Protein Engineering of Human Properdin

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Hilary Term, 1995

Jonathan M.G. Higgins
Oriel College

MRC Immunochemistry Unit,
Department of Biochemistry,
University of Oxford

RADCLIFFE SCIENCE LIBRARY OXFORD
DEPOSITED
- 2 JUN 1995
THESIS

Acknowledgements

There are many people I must thank for making my time in the MRC Immunochemistry Unit such an enjoyable experience, and for helping with various aspects of the work. I am particularly grateful to Professor Ken Reid for being an excellent and encouraging supervisor, who always has time to talk. Alex Law and Bob Sim have also provided many reagents and lots of useful advice. I also thank Tony Gascoyne for providing the essential "purified plasma properdin", a phrase often repeated in this thesis, Tony Willis for protein sequencing and amino acid analysis, Beryl Moffat for her help in tissue culture, and Laura Le Gré for her work on sulphatide binding. There are too many people to name who have passed through the Unit and made it such a great place to work, but I thank Belen, Hans, Kazuo, Kath, Leo, Peter, Rajneesh, Uffe, Viv, Wallace, Christian, Steffen, Konrad, Jin-hua, Wang, Armin for their friendship, advice, and support through the last three or four years.

I wish to thank Drs Rupert Timpl and Hanna Wiedemann for the electron microscopy study, and to Fabianna Filippoussis and Dr John Lambris for the monoclonal antibody investigations (and hospitality on my short visit to Penn.).

I am extremely grateful to the Wellcome Trust for their generous studentship which has allowed me to carry out this project.

I particularly appreciate the patience of, and help provided by, Belen Carrillo, Peter Lawson and Viv Perkins over the last weeks and days while I was writing and printing this thesis.

I must thank those people who are not involved directly with work (since there are *some*!) for their friendship during my graduate years - they were just as good as the first four: Jon B., Dave, Kathryn, Kristina, Mike, and Tim, and Dan, Wils and Win have all been responsible for many entertaining hours in various parts of the country.

Finally, I thank my parents for putting up with my erratic telephone calls, and for their support and encouragement throughout my time in Oxford.

Abbreviations

bp	base pair(s)
C4bp	C4 binding protein
C	complement component
CCP	complement control protein
CS	circumsporozoite
cDNA	complementary DNA
CR	complement receptor
CTGF	connective tissue growth factor
CVF	cobra venom factor
DAF	decay accelerating factor
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EA	erythrocytes (E) sensitized with anti-sheep E antibody
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal cell growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
E_{hu}	human erythrocytes
ELISA	enzyme-linked immunosorbent assay
E_S	sheep erythrocytes
E_R	rabbit erythrocytes
Fab	antibody fragment - antigen binding
Fc	antibody fragment - crystallisation/constant
FGF	fibroblast growth factor
FPLC	fast protein liquid chromatography
Ig	immunoglobulin
K_a	association constant
kb	kilobase pair
kDa	kilo Dalton
LDL	low density lipoprotein
mAb	monoclonal antibody
MAC	membrane attack complex
MASP	MBP-associated serine protease
MBP	mannan binding protein
MCP	membrane cofactor protein
MES	2-(N-morpholino)ethanesulfonic acid

mRNA	messenger ribonucleic acid
MSX	L-methionine sulphoximine
nucl.	nucleotide
P	properdin
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNPP	4-nitrophenylphosphate disodium salt
RACE	rapid amplification of cDNA ends
RCA	regulators of complement activation
RNA	ribonucleic acid
RNase	ribonuclease
RP	reagent for measuring properdin
SDS	sodium dodecyl acid
SSP	sporozoite surface protein
TBS	tris buffered saline
TRAP	thrombospondin-related anonymous protein
Tris	tris(hydroxymethyl)-aminomethane
TSR	thrombospondin type I repeat
VWA	von Willebrand factor A domain
VWC	von Willebrand factor C domain

Abstract
Protein engineering of human properdin

Submitted for the degree of Doctor of Philosophy
Hilary Term, 1995
Jonathan M.G. Higgins
Oriel College

Properdin is a serum glycoprotein that upregulates the alternative pathway of complement by stabilizing the C3bBb complex. It also binds sulphated glycoconjugates, such as sulphatide, *in vitro*. Properdin is composed of cyclic dimers, trimers and tetramers of a 53 kDa monomeric subunit. The monomer contains an N-terminal region of no known homology and six thrombospondin type 1 repeats (TSRs) of approximately sixty amino acids. The sixth TSR of properdin contains an insertion of approximately 30 amino acids which corresponds to the position of an intron in the human properdin gene.

In order to identify the regions of properdin important for function, human properdin, and mutant forms each lacking a single TSR, were expressed in Chinese Hamster Ovary cells. In addition, limited tryptic digestion yielded "nicked" properdin by the cleavage of one peptide bond in TSR5. The structural and functional properties of the normal and altered forms of properdin were investigated. Wild type recombinant properdin is similar to properdin purified from plasma in size, immunoreactivity, N-terminal sequence, possession of N-linked sugar, oligomerization (as determined by electron microscopy and gel exclusion chromatography), and functional activity in an alternative pathway haemolytic assay, and in C3b and sulphatide binding assays.

Properdin "nicked" in TSR5 is unable to bind C3b, while retaining its overall structure and its ability to bind sulphatide. The removal of TSR5 prevents C3b and sulphatide binding. Properdin lacking TSR4 is unable to stabilize the C3bBb complex, but is able to bind C3b and sulphatide, and shows the presence of monomers and dimers in the electron microscope. Properdin without TSR3 is able to stabilize the C3bBb complex, to bind C3b and sulphatide, and forms dimers, trimers and tetramers. Properdin lacking TSR6 is unable to form oligomers. The N-linked carbohydrate of properdin is not required for oligomerization or stabilization of the C3bBb complex. Monoclonal antibodies which bind to the N-terminal region, TSR1, or TSR2 are able to inhibit properdin binding to C3b. A monoclonal antibody which binds TSR4 is able to inhibit properdin binding to sulphatide, but not to C3b.

The results confirm that TSRs are folded as independent units. The N-terminal end and TSR5 of properdin are implicated in C3b binding. The vertices of properdin oligomers may be important for interaction with C3b. TSR4 may also be involved in stabilization of the C3bBb complex. The sulphatide binding site is distinct from the C3b binding site, but TSR5, which contains many basic residues, may be important for both activities.

Contents

Acknowledgements
Abbreviations
Abstract
Contents i

Chapter 1
Introduction 1

1.1 An outline of the complement system 1
1.1.1 The classical pathway 1
1.1.2 The lectin pathway 2
1.1.3 Complement effector mechanisms 3
1.2 Properdin and the discovery of the alternative complement pathway 4
1.3 Isolation of the components of the alternative pathway 5
1.4 Activation and control of the alternative pathway 6
1.5 The role of properdin 7
1.6 Properdin structure: native and activated properdin 9
1.7 The function of native properdin 11
1.7.1 Stabilization of the C3bBb complex 11
1.7.2 Stabilisation of the C5 convertase 13
1.7.3 Protection against complement regulating proteins 14
1.7.4 Interaction with fluid phase complement components 14
1.7.5 The multivalency of properdin binding to C3b 15
1.7.6 Other ligands for properdin 15
1.8 Activators and non-activators of the alternative pathway 16
1.8.1 Activators circumvent the action of Factor H and Factor I 17
1.8.2 The role of sialic acid 17
1.8.3 The importance of other polyanions 19
1.8.4 Cell surface regulatory proteins 21
1.9 The structure of alternative pathway complement components 22
1.9.1 C3 (C4 and C5) 22
1.9.2 The serine proteases: Factors B, D and I (C2, C1r, C1s) 24
1.9.3 Regulators of complement activation: Factor H, CR1, DAF, MCP (C4bp) 24
1.10 Properdin: cDNA, gene and protein sequence 25
1.11 The function of the TSRs in properdin: C3b and sulphated glycoconjugate binding 27

1.12 Thrombospondin 29
1.12.1 Thrombospondin structure 29
1.12.2 The CSVTCG and RxR motifs in thrombospondin 30
1.12.3 The WSxWS motif in thrombospondin 31
1.12.4 Other ligands for the TSRs of thrombospondin 32
1.13 Proteins of the apicomplexan protozoa 32
1.13.1 The circumsporozoite protein 33
1.13.2 The thrombospondin-related anonymous protein and related molecules 35
1.14 Proteins involved in neuronal cell migration 36
1.14.1 F-spondin 36
1.14.2 Unc-5 36
1.15 Growth regulators related to connective tissue growth factor (CCN family) 37
1.16 Terminal components : C6, C7, C8 and C9 38
1.17 Properdin biosynthesis 39
1.18 Properdin deficiency 40
1.19 Aims of the project 42

Chapter 2

Materials and Methods **44**

2.1 Materials 44
2.2 Nucleic acid methods 45
2.2.1 Ethanol precipitation of DNA 45
2.2.2 Removal of protein by organic extraction 45
2.2.3 Oligonucleotides 45
2.2.4 Phosphorylation of oligonucleotides 46
2.2.5 Mini plasmid preparations 46
2.2.6 Maxi-preparation of plasmid DNA 47
2.2.7 Estimation of nucleic acid concentration 47
2.2.8 Denaturing polyacrylamide gel electrophoresis of DNA 48
2.2.9 Agarose gel electrophoresis of DNA 48
2.2.10 Capillary transfer of DNA from agarose gels to nylon membranes under alkaline conditions 48
2.2.11 Radiolabelling of oligonucleotides 48
2.2.12 Hybridization of radiolabelled oligonucleotide probes 49
2.2.13 Autoradiography 49
2.2.14 Isolation of DNA fragments from agarose gels 49
2.2.15 Restriction digestion 49
2.2.16 Dephosphorylation of cleaved vector DNA 49
2.2.17 Klenow fill reaction 50

2.2.18 Polymerase chain reaction 50
2.2.19 Ligations 50
2.2.20 Preparation of competent *E. coli* for transformations 50
2.2.21 Transformation of *E. coli* 50
2.2.22 Screening of transformants 51
2.2.23 Storage of Bacterial Strains 51
2.2.24 Double Stranded DNA Sequencing 52
2.2.25 Isolation of total U937 cell RNA 52
2.2.26 Agarose gel electrophoresis of RNA 52
2.2.27 Northern blot of RNA onto nitrocellulose membranes 53
2.2.28 Radiolabelling of cDNA fragment for use as a probe 53
2.2.29 Hybridisation with radiolabelled restriction fragments 53
2.2.30 First Strand cDNA synthesis 53
2.2.31 Primer Extension Analysis 54
2.2.32 Rapid Amplification of cDNA Ends (RACE PCR) 54
2.2.33 Construction of expression vector 55
2.2.34 Deletion mutagenesis 56
2.3 Cell culture and transfection techniques 57
2.3.1 Growth of U937 cells 57
2.3.2 Growth of CHO cells 57
2.3.3 Transfection of CHO cells 57
2.3.4 Amplification of properdin expression from CHO cells 57
2.3.5 Production of recombinant human properdin 57
2.4 Protein methods 58
2.4.1 SDS-Polyacrylamide gel electrophoresis 58
2.4.2 Coomassie Blue Staining of PAGE gels 58
2.4.3 Silver staining of PAGE gels 58
2.4.4 Dialysis and concentration of protein samples 58
2.4.5 Immunisation of rabbits 58
2.4.6 Preparation of rabbit immunoglobulin G 59
2.4.7 Determination of IgG concentration 59
2.4.8 Biotinylation of antibody 59
2.4.9 Polyclonal and monoclonal antibody antigen capture ELISA assays for human properdin 59
2.4.10 Western Blotting 60
2.4.11 Purification of recombinant properdin on monoclonal antibody affinity column 60
2.4.12 Purification of human plasma properdin 60
2.4.13 Purification of human plasma C3b 60

2.4.14 Preparation of polyclonal antibody affinity column 61
2.4.15 Purification of recombinant properdin on antigen-selected polyclonal antibody affinity column 61
2.4.16 Storage of properdin samples 61
2.4.17 Separation of properdin oligomers by size exclusion chromatography 61
2.4.18 Separation of properdin oligomers by cation exchange chromatography 62
2.4.19 Dissociation and reassociation of properdin oligomers 62
2.4.20 Removal of glycosylated proteins using lentil lectin 62
2.4.21 Enzymatic removal of oligosaccharide 62
2.4.22 Limited tryptic digest of properdin 62
2.4.23 Cross-linking of properdin oligomers 63
2.4.24 Preparation of erythrocytes for haemolytic assays 63
2.4.25 Haemolytic assay for human properdin 63
2.4.26 Preparation of properdin-depleted serum 63
2.4.27 Solid phase C3b binding assay 64
2.4.28 Solid phase lipid binding assay 64
2.4.29 Thin layer chromatography 64
2.4.30 Electron microscopy 65
2.4.31 N-terminal protein sequencing 65
2.4.32 Amino-acid and amino-sugar analysis 65
2.5 Sequence Analysis 66
2.5.1 Database searches 66
2.5.2 Definition of thrombospondin repeat boundaries. 66
2.5.3 Classification of thrombospondin repeats into groups by pairwise comparisons and dendrogram construction 66
2.5.4 Multiple alignment of thrombospondin repeats 67
2.5.5 Analysis of repetitive genomic elements 67
2.5.6 DNA sequence alignment 68

Chapter 3

Analysis of the genomic and protein sequence of properdin 69

3.1 Localisation of the transcription start site of the human properdin gene 69
3.1.1 Primer extension 70
3.1.2 Rapid amplification of cDNA ends (RACE-PCR) 70
3.2 Repetitive elements within the human properdin gene. 72
3.2.1 Alu repeats 73
3.2.2 MER20 74
3.2.3 MIR 74
3.3 Multiple Alignment and predicted structure of thrombospondin type I repeats 76

3.3.1 Reliability of the alignment 77
3.3.2 Sub-groups of TSRs 78
3.3.3 Properdin possesses six complete TSR modules 79
3.3.4 The sixth TSR of properdin contains are large insertion 79
3.4 Structural features of the TSR module 81
3.4.1 Prediction of disulphide bond patterns 81
3.4.2 The predicted secondary structure of the TSR module 82
3.5 The N-terminal region of properdin 83
3.6 The prediction of potentially important functional regions in properdin 84
3.6.1 Conservation of amino acid sequence between the different species in properdin 84
3.6.2 Insertions in the TSRs of properdin 85
3.7 Summary 86

Chapter 4
Expression of wild type recombinant human properdin in Chinese Hamster Ovary Cells **88**
4.1 Construction of the vector for expression of normal human properdin 89
4.2 Transfection and selection for stable transfectants 90
4.3 Preliminary characterisation of recombinant human properdin 90
4.4 Purification of recombinant properdin 91
4.5 SDS-PAGE of purified recombinant properdin 91
4.6 The N-terminal sequence of recombinant human properdin 92
4.7 Electron Microscopy of recombinant properdin 93
4.8 The oligomeric profile of recombinant properdin 93
4.9 The binding of recombinant properdin to a panel of monoclonal antibodies 96
4.10 Functional assays on recombinant human properdin 96
4.11 The N-linked carbohydrate of properdin 97
4.12 Summary 99

Chapter 5
Production of altered forms of human properdin **100**
5.1 Deletion of single thrombospondin repeats from the human properdin cDNA 100
5.2 Transfection of CHO cells and production of mutant forms of properdin 101
5.3 SDS-PAGE and immunoblotting of mutant forms of recombinant properdin 102
5.4 The oligomeric profile of the altered forms of properdin 103
5.4.1 Gel exclusion chromatography 103
5.4.2 Chemical crosslinking 104
5.5 Purification of the mutant forms of recombinant human properdin 105

5.6 Production of properdin "nicked" in TSR5 106
5.7 Oligomeric profile of purified altered forms of properdin 107
5.8 Electron microscopy of altered forms of properdin 108
5.9 Binding of the altered forms of properdin to anti-properdin monoclonal antibodies 109
5.10 Summary 111

Chapter 6
Stabilization of the C3bBb complex: the alternative pathway haemolytic assay 112
6.1 The alternative pathway haemolytic assay 112
6.2 Comparison of the specific activities of the oligomeric forms of properdin in haemolysis of rabbit and sheep erythrocytes 113
6.3 Binding of properdin to untreated erythrocytes 116
6.4 Haemolytic assay of the altered forms of human properdin 117
6.5 The effect of HYB3-3 mAb on stabilization of C3bBb 118
6.6 Summary 119
Figures for chapter 6

Chapter 7
The binding of altered forms of human properdin to C3b 120
7.1 The C3b binding assay 120
7.2 The binding of various oligomeric forms of properdin to C3b 121
7.3 Binding of altered forms of properdin to C3b 122
7.4 Inhibition of properdin binding to C3b by monoclonal antibodies 123
7.5 Summary 125
Figures for chapter 7

Chapter 8
The binding of altered forms of properdin to sulphatide 126
8.1 The sulphatide binding assay 126
8.2 Cation dependency of sulphatide binding 127
8.3 The specificity of properdin for sulphatide 127
8.4 The binding of properdin in normal human plasma to sulphatide 128
8.5 The binding of the various oligomeric forms of properdin to sulphatide 128
8.6 The binding of altered forms of properdin to sulphatide 129
8.7 Inhibition of sulphatide binding by the HYB3-3 monoclonal antibody 130
8.8 Summary 131
Figures for chapter 8

Chapter 9

Conclusions **132**

9.1 Preliminary sequence analysis: a model of the properdin monomer 132

9.2 Production of altered forms of human properdin 133

9.3 Characterization of wild type recombinant properdin and the mechanism of oligomerization 133

9.4 Characterization of altered forms of human properdin: identification of regions important for function 134

9.4.1 The N-linked oligosaccharide of properdin is not required for secretion, oligomerization, or C3bBb stabilization 135

9.4.2 The third TSR of properdin is not required for functional activity 135

9.4.3 The fourth TSR of properdin is not required for sulphatide or C3b binding, but may be required for C3bBb stabilization 135

9.4.4 The fifth TSR of properdin is involved in C3b and sulphatide binding 136

9.4.5 The sixth TSR of properdin is required for oligomerization 137

9.4.6 The N-terminal end of the properdin monomer may also be important for C3b binding 137

9.5 The vertices of properdin oligomers may be important for C3b binding 138

9.6 The sulphatide binding site of properdin is distinct from the C3bBb binding site(s) but may also involve TSR5 139

9.7 Possible roles *in vivo* of properdin binding to sulphated glycoconjugates 140

9.8 Future studies 141

References **142**

Chapter 1

Chapter 1
Introduction

1.1 An outline of the complement system

Complement is an important defence and clearance system in the circulation of vertebrates. Over thirty plasma and cell-surface molecules are involved in the activation and control of the system (for review see Law and Reid, 1988). The most abundant component is C3 (present in the plasma at 1.3 mg ml^{-1}). C3 is a large molecule (180 kDa) which can become covalently attached to material in the circulation (Law and Levine, 1977). The deposition of C3 onto foreign substances can be considered the central and most vital event during activation of the complement system since surface bound C3 can trigger a number of effector mechanisms, as outlined below. C3 contains an internal thioester bond (Tack *et al.*, 1980) (see section 1.9.1). Although this bond is potentially highly reactive and susceptible to hydrolysis, in whole C3 it is protected from the solvent. The covalent binding reaction of C3 usually requires the proteolytic cleavage of C3 to generate C3b, in which the thioester is exposed. While the bond will normally quickly be hydrolysed in the aqueous environment of the plasma, if the splitting of C3 occurs near a surface carrying suitable (hydroxyl or amino) chemical groups, then C3b can become covalently attached. The cleavage of C3 requires the formation of proteolytic enzymes which are specific for C3, known as C3 convertases. The activation of three major pathways can lead to generation of C3 convertases, and the subsequent deposition of C3 (see figure 1.1). The "classical" and "lectin" pathways are described in outline below. The "alternative" or properdin pathway is discussed in more detail later.

1.1.1 The classical pathway

The first pathway to be discovered was that activated by the binding of IgG or IgM antibodies, and now known as the classical pathway (see figure 1.1). Antibodies clustered on the surface of material in the circulation can be recognized by the C1 complex of complement (Reid, 1983). This complex contains three components. C1q is a large molecule comprising eighteen polypeptide chains of three types (A, B, and C) which associate to form trimers containing N-terminal collagenous regions and C-terminal globular heads. The disulphide cross-linking of six of these trimers forms the complete structure, which resembles a bunch of tulips. Associated with the collagenous regions of C1q are two related molecules, C1r and Cls, which form non-covalent, calcium dependent

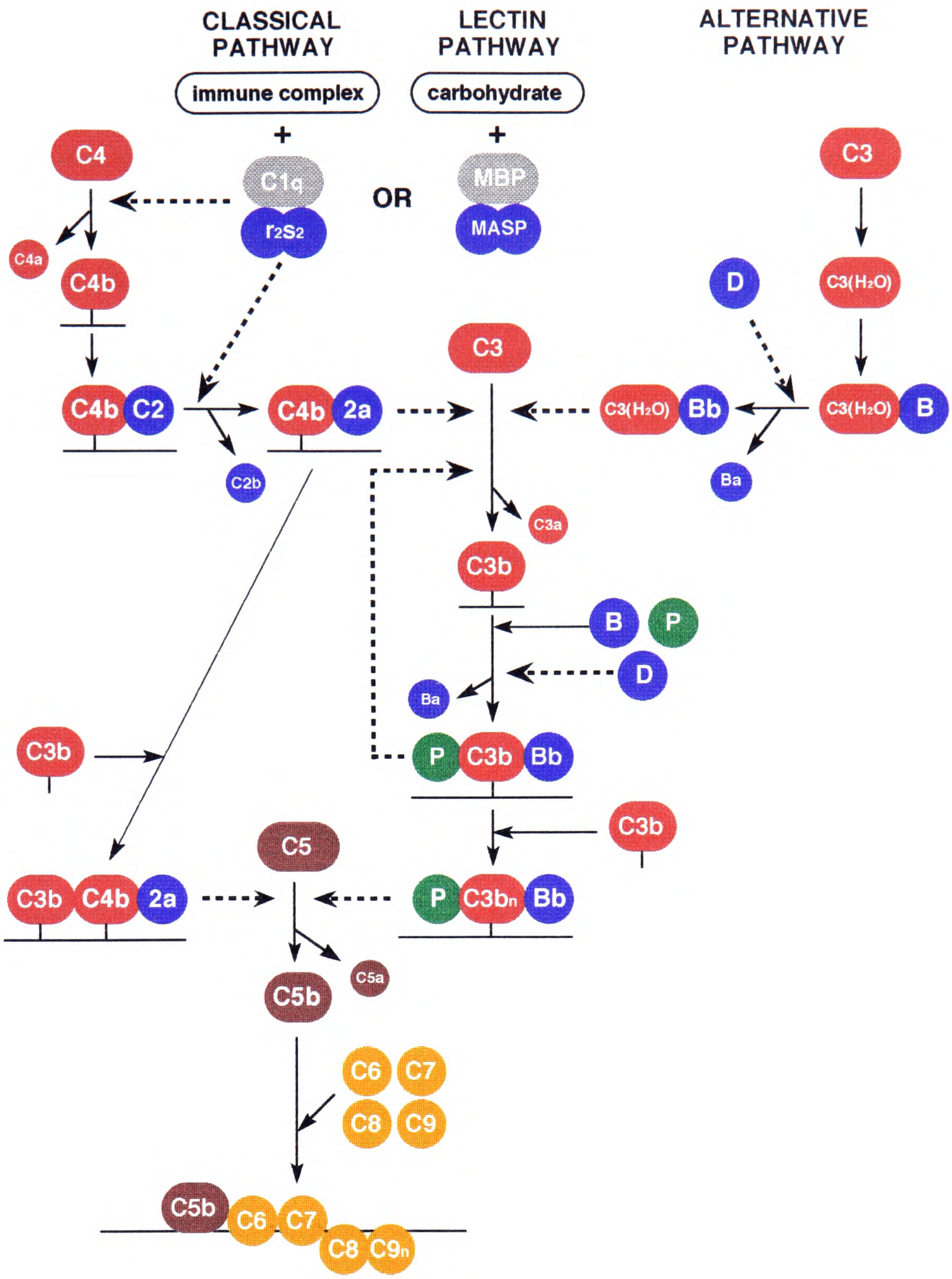


Figure 1.1
An outline of the complement system.
The classical pathway, left, is activated by the interaction of C1 with antibodies in immune complexes. The lectin pathway, centre, is activated by the binding of mannan-binding protein to carbohydrate.The alternative pathway, right, is continuously activated at a low level in the serum. Activation of all three pathways leads to amplification of C3b deposition by the properdin-stabilized C3 convertase, provided that the surface lacks complement controlling molecules. The thioester containing proteins, C3 and C4, are shown in red, while the related protein C5, which lacks a thioester, is in deep red. The serine proteases are shown in blue. The components of the membrane attack complex are shown in orange. C1q and MBP are shown in grey, and properdin in green. Enzymatic cleavages are indicated by dashed lines.

C1s-C1r-C1r-C1s tetramers in solution (Arlaud *et al.*, 1990). The globular heads of C1q possess binding sites for the Fc portions of immunoglobulins. Both C1r and C1s are pro-enzymes with potential serine protease activity. On association of at least two of the heads of C1q with bound Ig molecules, a change in the disposition of the stalks of C1q is thought to allow the autocatalytic activation of C1r (Burton, 1990). C1r is then able to cleave and activate C1s. The initial substrate for C1s is complement component C4. C4 is structurally and functionally related to C3, and also contains an internal thioester. The cleavage of C4 by C1s to generate C4b exposes this reactive internal bond, and C4b becomes attached onto the antibody coated surface. C4b has an altered conformation compared to C4, and is able to bind the C2 protein. C2 within the C4bC2 complex is also a substrate for activated C1s. The C4b2a complex thus generated is a C3 convertase enzyme, and is able to generate many molecules of C3b from C3.

The classical pathway is under the control of a number of regulatory proteins. The C1-inhibitor i) associates reversibly with the C1 complex, and prevents its activation in the fluid phase, and ii) forms covalent complexes with activated C1r and C1s, preventing their proteolytic activity (C1-inhibitor is a serpin). C4 binding protein (C4bp) encourages the dissociation of the classical pathway C3 convertase, and is a co-factor for the serine protease, Factor I, which is able to cleave and inactivate C4 (see later). A number of cell surface molecules which are similar in activity to C4bp also exist to prevent complement activation on host cells. These proteins are discussed in more detail later. These regulatory activities, in combination with the rapid hydrolysis of C3b and C4b in the aqueous serum, focus the deposition of C4b and C3b onto the activating surface.

1.1.2 The lectin pathway

Recently, it has been found that the formation of the classical pathway C3 convertase, C4b2a, can be triggered by a mechanism which is independent of antibodies and C1. Mannan binding protein (MBP) is a member of a group of proteins known as the "collectins" which are structurally related to C1q (Thiel and Reid, 1989; Holmskov *et al.*, 1994). Like C1q, MBP is present in the serum, has a bouquet-like shape and contains collagenous regions at the N-termini of its constituent chains. However, unlike C1q, MBP is made up of a single type of polypeptide which contains a C-type lectin domain at its C-terminus. The lectin module of MBP binds to carbohydrate in a calcium dependent manner. While the ligands recognized by MBP in physiological situations are not well defined (Hoppe and Reid, 1994), MBP is known to be able to bind to zymosan (yeast cell walls) and to mannan, a soluble glycan derived from the same source. The knowledge that MBP can initiate complement lysis of mannan-coated erythrocytes (Ikeda *et al.*, 1987) and can activate the $C1r_2$-$C1s_2$ complex in the absence of C1q (Lu *et al.*, 1990), led to the proposal that a third pathway of complement activation exists. The

discovery of another C1r/C1s-related serine protease which forms a complex with MBP (the MBP-associated serine protease, MASP) has lent further support to this hypothesis (Matsushita and Fujita, 1992). The binding of MBP to carbohydrate on the surface of bacteria or viruses is thought to lead to the activation of C1r/C1s or MASP, and the subsequent generation of a C3 convertase as in the classical pathway. The existence of this "lectin pathway" of complement activation may explain some earlier observations of non-classical complement activation (see for example, *Naff et al.*, 1980). However, since both the classical and lectin pathways are calcium dependent, and complement activation can occur in serum containing magnesium and ethylene glycol tetra-acetic acid (EGTA), an alternative pathway must exist.

1.1.3 Complement effector mechanisms

Once clusters of C3b have become attached to a foreign surface, a number of effector mechanisms can be called into action. For example, the addition of further C3b molecules to either the classical/lectin or alternative pathway C3 convertases leads to the generation of a C5 convertase. C5 is related to C3 and C4, but does not contain an internal thioester (see section 1.9.1). The cleavage of C5 generates C5b which binds non-covalently to C3b, and acts as the nucleus for formation of the membrane attack complex. This complex contains complement components C6, C7, C8, and multiple C9 molecules (see section 1.16), which are able to punch characteristic circular lesions into the membranes of bacteria and erythrocytes, leading to cytolysis.

In addition, there are a variety of host cell surface receptors for complement components. The best characterized of these are the receptors for fragments of C3b; CR1, CR2, CR3 and CR4. CR1 (a member of the RCA family, see section 1.9.3) and CR3 (an integrin) are both found on the surfaces of cells such as polymorphonuclear leukocytes and monocytes. These two molecules act in concert with Fc (antibody) receptors to mediate the ingestion of opsonized bacteria, viruses, and immune complexes by phagocytic cells (Law and Reid, 1988).

CR1 on erythrocyte cell surfaces is also important for the clearance of immune complexes from the circulation. In primates, C3b coated immune complexes are bound to red blood cells via CR1, and are carried by the cells to the liver where the immune complexes are removed by Kupffer cells (Cornacoff *et al.*, 1983). Also, the activation of the classical pathway is important for preventing the formation of large insoluble immune complexes *in vivo* (Ng and Schifferli, 1993).

Complement may also play a role in the regulation of the immune response. The primary immune response in guinea pigs which are deficient in C2 or C4 is reduced, and repeated immunization fails to produce the normal switch from IgM to IgG production (Böttger *et al.*, 1985). Signals transduced through the CR2 receptor on B cells may be

particularly important in modulation of the adaptive immune system by complement (Fearon, 1993).

Receptors for other components of the complement system are also likely to exist. For example, C1q or "collectin receptors" have been partially characterized on a variety of cell types. These receptors may mediate uptake of foreign particles with bound C1q, MBP or other collectin molecules (Malhotra *et al.*, 1990a, b).

Complement is also important in the inflammatory response. On cleavage of C3, C4 and C5, small C3a, C4a and C5a fragments are released (as well as the larger C3b, C4b and C5b fragments). These "anaphylotoxins" induce vasodilation, mast cell degranulation, and are chemotactic for phagocytic cells (C5a is the most potent). Thus, complement activation at a site of infection will lead to increased blood flow to the area, and immigration of macrophages and neutrophils from the circulation (Hugli, 1985).

1.2 Properdin and the discovery of the alternative complement pathway

The existence of an alternative pathway of complement activation, distinct from the classical and more recently discovered lectin pathways, was first suggested in the early nineteen-fifties by Louis Pillemer and his associates working at Western Reserve University, Cleveland, Ohio (Pillemer *et al.*, 1954). Pillemer observed that the incubation of zymosan (yeast cell walls) with human serum at 37°C lead to the selective inactivation of the third component of complement, without activation of the classical components C1, C2 and C4. Furthermore, Pillemer noted that incubation of zymosan with serum at 17°C caused little C3 inactivation, but prevented subsequent C3 inactivation in the serum by zymosan at 37°C. The factor that was removed from the serum by zymosan could be eluted from it, was found to be distinct from antibody, and was named properdin[1]. Serum treated with zymosan at 17°C was known as RP (reagent for measuring properdin, see chapter 6). The ability of RP to inactivate C3 could be reconstituted on the addition of zymosan and properdin. RP was also found to be deficient in bactericidal activity against gram-negative bacteria, and in anti-viral and haemolytic activity. These activities were returned to RP on the addition of properdin. However, properdin on its own was unable to bind zymosan, and had none of the activities described above. Pillemer suggested that properdin in conjunction with complement components (including a hydrazine sensitive "Factor A", a heat labile "Factor B" and magnesium ions) participated in a variety of innate immune responses.

Initially, these studies provoked much interest in the scientific community and excitement in the media (New York Times, 1954). However, work described by Robert

---

[1] from the Latin *perdere*, to lose or destroy.

Nelson (1958) suggested that the phenomenon described by Pillemer could be explained by classical antibody mediated complement activation. Nelson found evidence for anti-zymosan antibodies in normal human serum, and in partially purified preparations of properdin. In addition, the similar loss of C3 and C4 "reactive units" on incubation of zymosan with serum led Nelson to believe that the inactivation of C3 and C4 "attained a ratio approaching unity" (*i.e.* that there was not selective inactivation of C3).

For many years, the properdin system was ignored by most immunologists (Lepow, 1980). However, in the late sixties and early seventies, a number of reports highlighted systems in which inactivation of C3 occurred without the inactivation of C4. Gewurz *et al.* (1968) showed that the incubation of guinea pig serum with lipopolysaccharide or zymosan resulted in consumption of C3 to C9 without loss of C1, C2 or C4. Sandberg *et al.* (1970) found similar results with guinea pig IgG1 immune complexes. Both Sandberg *et al.* (1971) and Reid (1971) found that the $F(ab')_2$ of IgG would activate complement through C3 without C1, C2 or C4. In fact, Nelson had also demonstrated conditions in which the inactivation of C3 was much greater than C4. In this work, although the loss of C3 and C4 "reactive units" was similar, the definition of these units depends upon the assay system used for each protein. The *percentage* loss of C3 was much greater than C4, and the serum concentration of C3 is now known to be more than double that of C4. Furthermore, Pensky and co-workers (1968) had further purified properdin and shown that its function could be separated from contaminating immunoglobulin.

By 1971, the existence of the alternate complement pathway was accepted by many investigators. Marcus *et al.* (1971) speculated that the pathway was important in the absence of adequate amounts of antibody, but that it could amplify the classical response, and that it involved an enzyme activity similar but distinct from that of the classical C4b2a complex. They also noted that the pathway was probably related to the properdin pathway described by Pillemer.

1.3 Isolation of the components of the alternative pathway

The nineteen-seventies saw a better definition of the components of the alternative pathway. Gotze and Müller-Eberhard (1971) were working on a cobra venom protein (cobra venom factor, CVF) that was capable of forming a C3 inactivating enzyme in human serum. They discovered that a heat labile serum protein, which they named C3 pro-activator (C3PA), bound to CVF in the presence of metal ions and formed a C3 convertase. Incubation of human serum with zymosan or inulin lead to the cleavage of C3PA and the inactivation of C3. C3PA was inactive when purified, suggesting the need for another serum factor for the acquisition of C3 cleaving activity. This postulated factor

was named C3PA convertase (now known as Factor D). An outline of the alternative pathway was proposed in which an activating substance was involved in the activation of C3PA convertase, which then cleaved C3PA to form the C3 convertase activity. A relationship between C3PA and the heat labile Factor B of Pillemer was tentatively suggested, and their identity confirmed by the work of Goodkovsky and Lepow (1971). The term "Factor B" is now used for this protein.

The identity of Pillemer's hydrazine sensitive Factor A (or HSF) was unclear until the publication of another report from Müller-Eberhard and Gotze (1972). These workers treated serum with hydrazine and tested the ability of untreated serum fractions to reconstitute the ability of the treated serum to activate Factor B (and thus to cleave C3). Two factors were isolated. One was found to be native C3 itself, while the other factor, although not hydrazine sensitive, was the major cleavage product of C3, C3b. Thus, a role of C3 in its own cleavage was suggested and "the possibility [was] raised . . . that the C3 activator system is governed by a positive feedback mechanism." The availability of purified Factor A (C3/C3b) and Factor B (C3PA) allowed the screening of serum fractions for C3PA convertase activity. A reconstituted system of partially purified C3PA convertase (now known as Factor D), C3b, Factor B, and metal ions at 37°C was able to cleave C3 to C3b. Fearon *et al.* (1973) were also able to show that purified Factor B and Factor D would form a C3 convertase on the surface of sheep erythrocytes carrying C3b. Factor D was found to be a serine protease capable of cleaving Factor B (Fearon *et al.*, 1974a). These reports suggested that on an activating surface, C3b, Factor B, and Factor D would interact to form a multimeric haemolytically active intermediate of the alternative pathway.

1.4 Activation and control of the alternative pathway

Thus, by 1973, the essential components of the alternative pathway were known. However, a number of issues remained to be resolved. Firstly, the spontaneous activation of the system with purified components *in vitro* suggested, as Müller-Eberhard and Gotze (1972) recognised, that the alternative pathway must normally be controlled by a regulatory principle in serum. Secondly, the means of activation of the alternative pathway *in vivo* was unknown. Also, the role of properdin, the first component of the system to be identified, was unclear.

Müller-Eberhard and Gotze (1972) had suggested that a previously identified protein in serum, the C3b inactivator (Factor I) which cleaved cell surface C3b (Ruddy and Austen, 1971) might be important in down regulation of the alternative pathway. Nicol and Lachmann (1973) confirmed that immunochemical depletion of Factor I from human serum lead to the spontaneous activation of the alternative pathway (*i.e.* Factor B

and C3 cleavage). Furthermore, the status of the complement system after this *in vitro* removal of Factor I accurately mimicked that found *in vivo* in a single Factor I deficient patient (Abramson *et al.*, 1971). Later studies identified another serum protein, Factor H, which required for the action of Factor I (Whaley and Ruddy, 1976a; Pangburn *et al.*, 1977) and was also able to bind C3b directly, inhibiting Factor B binding, and also to encourage the dissociation of Bb, both leading to reduced C3 convertase activity (Whaley and Ruddy, 1976b; Weiler *et al.*, 1976). Nicol and Lachmann realised that, to explain the paradox that fixation of C3 by the alternative pathway required fixed C3, it might be "necessary to postulate that *in vivo* there is a minute degree of spontaneous C3 activation normally efficiently damped by inhibitors". They suggested that sufficient C3b may be generated by the action of serum or tissue proteases. However, the means of activation of the alternative pathway *in vivo* was still unclear. While properdin had been proposed as a possible activating principle, Nicol and Lachmann also suggested that "alternate pathway activators may act to stabilise the pathway normally spontaneously turning over," and reiterated the fact that the classical pathway can also activate the alternative pathway feedback mechanism. Later, Pangburn *et al.* (1981) suggested that C3 might be activated spontaneously in the fluid phase by hydrolysis of its internal thioester, leading to a conformational change to generate a C3b-like molecule (known as C3(H_2O)). This concept of alternative pathway activation is now widely accepted (Atkinson and Farries, 1987) although further work was required before differences between activators and non-activators of the alternative pathway became clear (see later).

1.5 The role of properdin

A spate of papers in the mid nineteen-seventies returned to the problem of the role of properdin in the alternative complement pathway. Initially, workers in this period tested the hypothesis that properdin might be involved in the triggering of Factor D by activators of the alternative pathway. Gotze and Müller-Eberhard (1974) purified properdin in a form which was able to initiate the alternative pathway in serum. The properdin was thus considered to be "activated". Activated properdin could not convert C3 or Factor B in isolation, but was necessary for efficient C3 and Factor B conversion in a system containing C3, Factor B, Factor D and Mg^{2+} at 37°C. It was proposed that activated properdin might interact with native C3, leading to the "expression of" Factor D activity. Fearon *et al.* (1974b) found that the incubation of purified activated properdin with Factor D conferred on the mixture the ability to form the C3bBb complex on sheep erythrocytes carrying C3b. However, the activation of Factor D by properdin was not demonstrated directly (Factor D is now thought not to require activation - see later).

The delineation of the role of properdin as a stabilizer of the alternative pathway C3 convertase began with studies of the dose-response and kinetics of alternative pathway activation (Fearon and Austen, 1975a). The sigmoidal dose-response and time courses of Factor B inactivation obtained using purified C3, Factor B, and Factor D suggested that two processes were occurring during alternative pathway activation. Firstly, C3, Factor B, and Factor D were able to interact to generate an initial C3 convertase which was able to cleave C3 to C3b resulting in the subsequent generation of the C3b-dependent C3 convertase. Properdin was found not to be essential for either process, but it was proposed that its action could be to stabilize the initial convertase and/or the C3b-dependent C3 convertase. Using purified components (C3, properdin, Factors B, D, H, and I) Schreiber *et al.* (1978) were able to reconstitute a system which behaved like the alternative pathway in serum. They demonstrated that the initial C3 convertase was formed in the fluid phase and confirmed that properdin was not essential for C3 consumption.

The first clear demonstration of the stabilizing role of properdin was reported by Fearon and Austen (1975b). Activated properdin was found to increase the haemolysis of sheep erythrocytes bearing C3b (E_sAC3b^2) in the presence of limited Factor B and excess Factor D. Properdin was effective whether previously bound to E_sAC3b or if added to the fluid phase with Factors B and D. The mechanism by which properdin augmented haemolysis became clear when the C3bBb complex was preformed on E_s and the washed cells were then incubated in the presence of various quantities of activated properdin. The half-life of the decaying C3bBb complex was found to be increased from 4 min in the absence of properdin to over 30 min in the presence of activated properdin. Since the number of C3 convertase sites present did not increase when properdin was added, the role of properdin was assigned to stabilization of the otherwise labile C3b-dependent C3 convertase. However, since properdin was not present during activation of the alternative pathway in these studies, the involvement of properdin in the formation of C3 convertases was not ruled out. Fearon and Austen (1975c) also found that in the presence of C3, Factor B, and limited Factor D, activated properdin was able to effect inactivation of C3 in the fluid phase. So, a role of properdin in stabilising the initial C3 convertase was also proposed.

---

[2] Sheep erythrocytes (E_s) are poor activators of the alternative pathway. C3b can be deposited on these cells through the classical pathway, triggered with anti-E_s antibody (A), giving E_sAC3b.

1.6 Properdin structure: native and activated properdin

A potential problem with the studies described above was the "activated" properdin used, which initiated the alternative pathway on addition to normal serum, could not be the form normally present in the blood. A form of properdin which was not "active" in this way had previously been recognised by various investigators (*e.g.* Fearon *et al.*, 1974b). Gotze, Medicus and Müller-Eberhard were able to partially purify "precursor" or "native" properdin from normal human serum using a procedure that omitted absorption onto zymosan (Medicus *et al.*, 1976a,b; Gotze *et al.*, 1977). Unlike activated properdin, this preparation of properdin did not activate the alternative pathway in serum. Minta (1976) had suggested that native properdin could be activated by limited proteolytic digestion. However, Müller-Eberhard and co-workers found no evidence for enzymatic conversion, and suggested that a conformational change could be responsible for the difference between the two forms.

The nature of the difference between native and activated properdin did not become clear until after studies of the structure of the properdin protein. Pensky *et al.* (1968) had originally found that purified properdin has a sedimentation coefficient of 5.2S and had calculated a molecular mass of approximately 220 kDa. Minta and Lepow (1974) found that properdin had a molecular mass of 184 kDa by sedimentation equilibrium, and a subunit mass of 49 kDa by SDS-PAGE (although this was later reported to be 54-59 kDa (Minta, 1976)). From these studies it was assumed that properdin was a tetramer of similar subunits. Protein sequencing of purified human properdin suggested that the four subunits were identical (Medicus *et al.*, 1980; Reid and Gagnon, 1981). DiScipio (1982) used a variety of methods (sedimentation ultracentrifugation and gel filtration) to calculate a molecular mass of 150 kDa for native properdin, and a subunit mass of 49 kDa. He concluded that properdin was a trimer of identical subunits.

That the discrepancy between the sizes of properdin oligomers reported might be due to the presence of a mixture of oligomeric forms was suggested by the multiple species of properdin found by gel exclusion chromatography on Sephacryl S300 (Reid, 1981). Smith *et al.* (1984a) confirmed the heterogeneity of purified human properdin on Sephacryl S300 and went on to subject the preparation to electron microscopy. The presence of "a polydisperse set of cyclic polymers constructed from a single highly asymmetric rod-like and flexible protomer, 26 nm in length and 2.5-3.0 nm in width" was described. Similar pictures were obtained by Farries *et al.* (1987) and in the work described in this thesis (chapters 4 and 5). Smith *et al.* (1991) carried out neutron and X-ray scattering studies on purified human properdin dimers and trimers. Simulations of the properdin structure in solution (from modelling based on the scattering curves obtained) agreed well with the images from electron microscopy. No monomeric properdin was observed by electron microscopy. Dimers comprised 30%, trimers 45%, tetramers

10% and higher forms 16% of the preparation. The results are in broad agreement with those of Pangburn (1989) and those presented in this thesis (chapter 4), which suggest a ratio of properdin dimers:trimers:tetramers in the serum of 26:54:20 (expressed as percentage by mass). The oligomers of properdin are extremely stable. For example, isolated trimers do not re-equilibrate to produce significant levels of the other oligomeric forms even after incubation at 37°C for 72 h (Smith *et al.*, 1984a; Pangburn, 1989) or at 4°C for several weeks (this thesis; Smith *et al.*, 1984a). The distribution of properdin oligomers is discussed in more detail in chapter 4.

Farries *et al.* (1987) found that native and activated properdin could be separated by gel exclusion chromatography on a Fractogel TSK HW-55(S) column. Activated properdin (which initiated C3 consumption in normal serum) was eluted close to the void volume and was found, by electron microscopy, to comprise large amorphous aggregates. Native properdin eluted later, as a heterogeneous peak which was found to contain dimers, trimers and tetramers by electron microscopy. Native properdin could be converted to activated properdin by freezing and thawing. Activated properdin could be converted to native properdin by treatment with mild denaturants. No aggregated properdin was found in fresh serum. Similar results were reported by Pangburn (1989). No difference was seen in the structure or function of native properdin after binding to zymosan in serum or after binding to E_SAC3bBb. Thus, it was concluded that "activated" properdin was simply an artefact of the purification procedure used, and that only native properdin was found *in vivo*. Any conformational change occurring during the interaction of properdin with components of the alternative pathway must be completely reversible.

A report from Whiteman *et al.* (1991) suggests that C3 is associated with high molecular mass "activated" properdin. A sandwich ELISA in which anti-properdin antibody was coated onto microtitre plates, and anti-C3 IgG was used as the detecting antibody (Mayes *et al.*, 1984) was used to detect such "P-C3 complexes". P-C3 was increased in normal human serum after incubation at 37°C for 1 h. While quantitation was not possible using this assay, it is clear that only minute quantities of the complexes were present in the samples tested. Since human serum is obtained from blood which is normally at 37°C, the relevance of the *in vitro* formation of such complexes to the activation of the alternative pathway *in vivo* is questionable. A further increase in formation P-C3 is found on the complete activation of the alternative pathway in serum with zymosan or CVF. This could reflect a low level of covalent binding of C3b to properdin, due to the proximity of C3bBb-bound properdin to sites of extensive C3b deposition.

1.7 The function of native properdin

Since the initial work on the function of properdin had been carried out using preparations containing activated (aggregated) properdin, it was important to repeat the experiments with preparations of native properdin which did not initiate C3 consumption in serum.

1.7.1 Stabilization of the C3bBb complex

Medicus *et al.* (1976a,b) found that native properdin, unlike activated properdin, was unable to bind E_sAC3b in the absence of Factors B and D unless low ionic strength conditions were used. However, native properdin was able to stabilize the C3b-dependent C3 convertase on E_sAC3b in the presence of Factors B and D. As in the experiments of Fearon and Austen using activated properdin (1975b), properdin had no effect on the number of convertase sites formed. The maximum uptake of properdin obtained in the presence of Factors B and D was approximately one molecule per thirty C3b molecules (although it is not clear if Factor B is present in sufficient excess to determine the maximum binding of properdin in these studies). This work confirmed that native properdin was able to stabilize the inherently labile C3bBb complex.

In a comprehensive study, DiScipio (1981) measured the avidities and stoichiometry of binding for various alternative pathway complement components for C3b bound to zymosan. Native properdin bound zymosan-C3b at physiological ionic strength with $K_a = 2.9 (\pm 0.4) \times 10^7 (M^{-1})$ and a ratio of P monomers to C3b molecules of 3-4 to 1. The results are in contrast to those of Medicus *et al.*, described above, who could not detect native properdin binding to C3b on the E_s cell surface at normal ionic strength. However, Farries *et al.* (1988a) reported similar binding studies using E_sAC3b and preparations of properdin from which the activated form had been removed by gel exclusion chromatography. Binding of native properdin to E_sAC3b could clearly be detected in the absence of Factor B at physiological ionic strength, although the avidity of binding was too low to measure. At half-physiological ionic strength, properdin bound to C3b with $K_a = 3 \times 10^7 (M^{-1})$, a value similar to that obtained by DiScipio for binding to zymosan-C3b at normal ionic strength. Daoudaki *et al.* (1988) also found that properdin in normal human serum was able to bind C3b at physiological ionic strength. In this case, C3b was not bound to the surface through its thioester by complement activation, as in the assay systems above, but was coated onto microtitre plate wells.

DiScipio found that in the presence of Factor B and 3 mM $MgCl_2$ (no Factor D) the avidity of properdin for zymosan-C3b increased to $K_a = 1.1 (\pm 0.1) \times 10^8 (M^{-1})$ with little change in stoichiometry. Furthermore, in the presence of properdin, the avidity of Factor B for zymosan-C3b increased from $K_a = 6.5 (\pm 0.5) \times 10^5 (M^{-1})$ to $K_a = 4.3 (\pm 0.5) \times 10^6$

(M^{-1}) (stoichiometry: B/C3b = 1.0). Farries *et al.* also reported an increase in properdin binding in the presence of Factor B on comparing the binding of properdin to E_sAC3b and E_sAC3bB. In the presence of Factor B, and 0.83 mM $MgCl_2$, at physiological ionic strength, the avidity constant for E_sAC3bB was 4×10^8 (M^{-1}). In agreement with DiScipio, the stoichiometry of this interaction was found to be 3-4 monomers of properdin per C3b molecule. Farries *et al.* also estimated the avidity constant for the binding of properdin to C3bBb. Although the experiment was conducted in non-equilibrium conditions (in the presence of a moderate excess of Factors B and D), the conversion of Factor B to Bb increased the avidity of properdin for the complex to at least 2×10^9 (M^{-1}).

The enhancement of properdin binding to C3b in the presence of Factor B, and the enhancement of Factor B binding to C3b in the presence of properdin suggests that properdin and Factor B interact directly in the C3bB complex. However, the results do not rule out the possibility that Factor B and properdin are able to induce conformational changes in C3b which indirectly enhance binding of the other molecule. Farries *et al.* (1988b) investigated further the possibility of a weak interaction between properdin and Factor B. Since the direct binding of the two components could not be demonstrated even at low ionic strength, Farries *et al.* used purified Factor B in an attempt to inhibit native properdin binding to E_sAC3b at low ionic strength. EDTA was also included to prevent the Mg^{2+} dependent binding of Factor B to C3b. At high concentrations, Factor B was able to partially compete with C3b for properdin binding. A direct interaction of properdin and Factor B was also indicated by the successful cross-linking of properdin to the Ba fragment of Factor B in a fluid phase C3iBP complex, using the homobifunctional reagent disuccinimidyl tartarate (DST). While the ability to detect properdin binding to a fluid phase C3iB complex at physiological ionic strength is surprising in the light of results discussed below (section 1.7.4), the use of relatively high properdin and Factor B concentrations compared to C3i, the use of radiolabelled proteins, and the use of an irreversible cross-linking agent may have allowed the detection of a very weak interaction between properdin and Factor B in the complex.

The studies described above also demonstrate that properdin is able to bind to C3b in the absence of Factor B at physiological ionic strength, although the avidity of interaction depends upon the system under investigation. The differences observed could reflect properties of the surface on which the C3 convertase is formed. E_s are poor activators of the alternative pathway, whereas zymosan is an efficient activator (Pillemer *et al.*, 1954; Pangburn *et al.*, 1983). E_s cells may have complement regulating molecules on the surface which could influence properdin binding to C3b (see chapter 6). Also, in the studies described here, each particle of zymosan carried far more molecules of C3b than the sheep erythrocytes (zymosan, 100 000 C3b per particle; E_s cells, 6000 C3b per cell). It is possible that the density of C3b on a surface might influence multivalent binding of properdin (see below) and thus explain some of the variation between the

reports described. Interestingly, Smith *et al.* (1984) were able to detect binding, at physiological ionic strength, of native properdin to E_sAC3b carrying 50 000 C3b per cell, and Farries *et al.* (1987) saw little binding of properdin to zymosan carrying C3b at approximately eight fold lower density than that used by DiScipio (1981).

Nevertheless, it is clear that properdin is able to bind, with increasing avidity, to C3b, C3bB, and C3bBb. On zymosan the avidity of properdin for C3b is forty fold higher than that of Factor B for C3b (DiScipio, 1981)[3]. The serum concentration of Factor B (2.2×10^{-6} M, 210 μg ml^{-1}) is approximately seventy fold that of properdin (3.0×10^{-8} M, 5 μg ml^{-1}). So, at the concentrations present in serum, it is possible that Factor B and properdin would occupy a similar proportion of the C3b sites available on zymosan (although *in vivo*, the situation would be complicated due to the binding kinetics of various complement components to C3b, *i.e.* non-equilibrium conditions). The possibility is raised that the role of properdin is not limited to stabilising preformed C3bBb complexes, but that it may also enhance formation of C3 convertases. This concept is supported by the work of Pangburn *et al.* (1983). The removal of properdin from normal human serum was found to increase the lag time observed before alternative pathway haemolysis of rabbit erythrocytes[4] (E_R) by two to five fold, and to reduce the maximum C3b deposition obtained by 50% (Sjöholm *et al.* (1988b) reported similar results when comparing the haemolysis of E_R in normal serum and in properdin-deficient serum from a patient with dysfunctional properdin (see section 1.18)). Also, the binding of properdin to E_R during alternative pathway activation in serum preceded the phases of rapid consumption and deposition of C3b, and the consumption of Factor B. These findings suggest that properdin is recruited early during formation of the C3bBb complex.

1.7.2 Stabilisation of the C5 convertase

The C5 convertase of the alternative pathway requires C3 for efficient cleavage of C5, like that of the classical pathway (Medicus *et al.*, 1976a; Daha *et al.*, 1976). It is thought that the C5 convertase requires two or more bound C3b molecules for its action. Properdin is able to stabilize this $C3b_nBb$ C5 convertase (Medicus *et al.*, 1976a).

---

[3] Note that the affinity of Factor B for C3b increases with increasing Mg^{2+} concentration (Kazatchkine *et al.*, 1979). The concentration of Mg^{2+} in the serum (0.75 mM - 1.1 mM) is less than that present in the assay used to determine the association constant utilised here (3 mM). Thus the affinity of Factor B for C3b *in vivo* may be less than that given.

[4] E_R, unlike E_S, activate the alternative pathway of complement without sensitization with C3b (Platts-Mills and Ishizaka, 1974).

1.7.3 Protection against complement regulating proteins

Medicus *et al.* (1976a) also found that native properdin was able to stabilize the C3bB complex on E_sA cells against the action of Factor I at physiological ionic strength. Activated properdin was able to prevent Factor I cleavage of C3b alone on the same cell type. They proposed that this might be an important function of properdin *in vivo.* Farries *et al.* (1988a) found that native properdin was able to protect E_sAC3b against the action of Factors H and I at low ionic strength. Since Factor I requires Factor H for activity, and Factor H is able to destabilize C3bBb complexes in the absence of Factor I (see earlier) it was not clear from these experiments alone how properdin was protecting the C3 convertase. However, both Medicus *et al.* (1976a) and Pangburn & Müller-Eberhard (1978) using E_sAC3b, and DiScipio (1981) using zymosan-C3b, found that there was no competition between native properdin and Factor H for binding to C3b. Farries *et al.* found that native properdin was able to prevent Factor I binding to E_sAC3b at low ionic strength. Thus, the protection of C3b from Factor I binding, but not directly from Factor H binding, may constitute another important function of properdin. Although Farries *et al.* and DiScipio found little change in the binding of properdin to C3b after treatment with Factors H and I, Medicus *et al.* (1976a) did note some release of properdin from E_sAC3bP after similar treatment.

1.7.4 Interaction with fluid phase complement components

The ability of activated properdin to trigger C3 consumption in the fluid phase had led to the suggestion that properdin might be able to stabilize the initial fluid phase C3 convertase of the alternative pathway (Fearon and Austen, 1975c). However, native properdin was unable to cause C3 consumption in the absence of an activating surface (Gotze *et al.*, 1977) Various workers later found that native properdin has very low affinity for fluid phase C3b, iC3b, or C3c. Daoudaki *et al.* (1988) found that fluid phase C3b inhibited properdin binding to microtitre plate bound C3b by 50% at 2.3×10^{-6} M at physiological ionic strength. The implied K_a ($= 4.3 \times 10^5$ (M^{-1})) is over sixty times less than that for zymosan-C3b (DiScipio, 1981). Farries *et al.*, (1988a) found that soluble C3b, iC3b and C3c all inhibited the binding of properdin to E_sAC3b at low ionic strength by 50% at a concentration of approximately 5×10^{-7} M. The implied K_a ($= 2 \times 10^6$ (M^{-1})) is fifteen times lower than that for the binding of properdin to E_sAC3b in the same conditions. No interaction of properdin with C3i could be detected. As expected, properdin had no impact on Factor I cleavage of *soluble* C3b in the presence of Factor H. It is unlikely that native properdin influences the fluid phase C3 convertase formed during the spontaneous tick-over of the alternative pathway. As Farries *et al.* recognized, properdin thus effectively focuses C3b deposition onto the activating surface, and away from the fluid phase.

1.7.5 The multivalency of properdin binding to C3b

The fact that properdin is oligomeric prompted DiScipio (1981) to test the hypothesis that properdin could bind multivalently to C3b. Properdin was allowed to bind to C3b-zymosan in the presence of Factor B and 3 mM $MgCl_2$. No further binding of fluid phase C3 or C3b to the C3bBP complexes could be detected, suggesting that properdin did not possess further unoccupied sites for C3b. However, later studies (as described above) showed that properdin has much lower avidity for fluid phase C3 or C3b than for bound C3b. Thus these studies do not rule out the multivalent binding of properdin to surface bound C3b.

Smith *et al.* (1984) tested the binding of various oligomers of properdin to E_sAC3b at physiological ionic strength. Farries *et al.* (1987), in a more detailed study, tested the binding of fractions of aggregated and native properdin separated by gel exclusion chromatography to E_sAC3b, E_sAC3bB, $E_sAC3bBb$, and zymosan-C3b. In both studies, the higher oligomeric forms of properdin displayed greater avidity for C3b than lower forms. Incubation of serum with zymosan led to the preferential removal of higher oligomers. The gradation in avidity of the oligomeric forms suggests that multivalent binding of properdin may occur. Such multivalent binding could be important during binding of properdin to C3b in the absence of Factor B (Farries *et al.*, 1988a).

Reid (1981) found that the specific activities of the three major forms of purified properdin eluted form Sephacryl S300 were similar in an alternative pathway haemolytic assay using E_R. However Pangburn (1989), using a similar assay but employing neuraminidase treated E_s[5], discovered that the ability of properdin dimers to reconstitute properdin-depleted serum was only one fifth that of tetramers and one third that of trimers on a mass or per subunit basis. Furthermore, during alternative pathway activation in normal serum containing ^{125}I-labelled properdin, tetramers and trimers bound to E_R more quickly than dimers, and to a higher percentage of the available properdin. The possible reasons for the discrepancies found between these assays are discussed in chapter 6. However, the results show that the difference in avidity for C3b of the various properdin oligomers is reflected in their activities during alternative pathway activation in some assay systems.

1.7.6 Other ligands for properdin

Properdin has been suggested to bind to erythrocytes and to a *Neisseria gonorrhoeae* protein. Konno *et al.* (1978) found that activated properdin was able to bind

---

[5] The removal of sialic acid from the surface of E_s renders them susceptible to the alternative pathway without sensitization with C3b.

to E_R and E_S in the absence of other complement components, and that these erythrocytes could subsequently be lysed by the alternative pathway in the absence of properdin. The binding of native properdin was not tested, and the identity of the surface structure(s) to which properdin bound was not determined.

Griffiss *et al.* (1991) found that absorption of normal human serum with serum sensitive strains of *Neisseria gonorrhoeae* at 0°C led to the loss of alternative pathway activity from the serum. Addition of properdin reconstituted the serum, suggesting that the loss of activity seen was due to the binding of properdin to the cells in the absence of marked complement activation. Preabsorption of serum containing magnesium and EGTA with E_R produced similar results. These findings are reminiscent of those of Pillemer *et al.* (1954) who found that properdin in serum was absorbed onto zymosan at 17°C. The means by which properdin binds to material such as zymosan, *N. gonorrhoeae*, or erythrocytes from serum is unclear. In the case of *N. gonorrhoeae*, Griffiss *et al.* found that properdin bound to a 39 kDa protein of a bacterial lysate on a Western blot, and suggested that the ability of the strain to bind properdin might determine its ability to activate the alternative pathway.

However, properdin does not bind to zymosan in the absence of other serum factors (Pensky *et al.*, 1968). A more general explanation for the binding of properdin in serum at low temperatures to various surfaces is suggested. It is possible that at low temperature, the incubation of zymosan with serum leads to a low level of activation of the alternative pathway and C3b deposition (fixation of C1, C2 and C4 is known to occur at 0°C, Pillemer *et al.*, 1954). As described in section 1.7.1, properdin could then bind to C3b on the zymosan (DiScipio, 1981; Pangburn, 1983). Since C3 is present at much higher concentration in the serum than properdin (7 μM C3 compared to 0.03 μM properdin), a high percentage of properdin binding could occur with a low percentage of C3 inactivation.

Properdin has also been found to bind sulphated glycoconjugates. This is discussed further in sections 1.8.3 and 1.11.

1.8 Activators and non-activators of the alternative pathway

The spontaneous low level formation of $C3(H_2O)$ and/or C3b in the fluid phase provides a mechanism for the triggering of the alternative pathway. However, the question remains as to how alternative pathway activators shift the system from fluid phase "tick over" to C3b amplification and deposition, while non-activators (in particular, host cells) escape. In the nineteen seventies, the action of Factors H and I as regulators of the alternative pathway in the fluid phase was clear. Since these were the only known inactivators, early work on the difference between activators and non-activators concentrated on these proteins.

1.8.1 Activators circumvent the action of Factor H and Factor I

Fearon and Austen (1977a) studied the action of Factors H and I on E_sAC4b3b (E_s cells are non-activators of the alternative pathway) and on zymosan-C3b (zymosan is an activator of the alternative pathway). Incubation of E_sAC4b3b with Factors H and I led to 95% inactivation of C3b, while a similar treatment of zymosan-C3b reduced C3b activity by only 35%. Factor H increased the rate of decay of the C3bBb complex on E_s cells but did not affect the decay of C3bBb on zymosan. The effect of adding zymosan to purified C3, properdin and Factors B, D, H, and I was similar to removing either Factor H or I from the system in the absence of zymosan. This led to the idea that activators of the alternative pathway were able to circumvent the normal regulatory mechanisms. The results suggested that C3b on the activating surface was somehow "protected" from the action of the complement regulatory proteins.

Similar results were obtained on comparison of E_s cells (which are not lysed on alternative pathway activation) with E_R cells (which are lysed) (Fearon and Austen, 1977b). The half-life of C3b on E_R was much longer than on E_s when incubated with Factors H and I, and the C3bBbP complex on E_s was more susceptible to dissociation by Factor H than on E_R. Again, complement on the surface of the alternative pathway activator appeared to be sheltered from the action of Factors H and I.

1.8.2 The role of sialic acid

Two groups of workers soon suggested a role for sialic acid in the regulation of the alternative pathway, but came to different conclusions about the mechanism involved. Fearon (1978) and Pangburn and Müller-Eberhard (1978) both found that the removal of sialic acid from the surface of E_s rendered them susceptible to alternative pathway mediated haemolysis. Fearon found that removal of sialic acid by neuraminidase treatment, or chemical modification of the polyhydroxyl tail of sialic acid using sodium periodate (followed by sodium borohydride) both caused a dose-dependent increase in the susceptibility of the cells to alternative pathway haemolysis. The increase in susceptibility also correlated with the concentration of Factor H required to dissociate a given proportion of Bb from membrane bound C3bBb. That is, the removal of 1% of the surface sialic acid led to a 1% decrease in the activity of Factor H. Because chemical modification of sialic acid had the same result as complete removal, it was supposed that the effect was not due to the exposure of previously masked sugars or due simply to a reduction in the negative charge on the membrane. It was proposed that membrane sialic acid facilitated the interaction between bound C3b and Factor H, preventing the cell from activating the alternative pathway. In a second report, the same workers looked at the effect of sialic acid on the competition between Factor B and Factor H for binding to C3b

on E_s cells and E_s cells treated with neuraminidase or sodium periodate (Kazatchine *et al.*, 1979). They found that the removal or modification of sialic acid had little effect on the affinity of Factor B or H for surface bound C3b. However, the number of high affinity sites for Factor H was reduced by four fifths after removal of 80% of the surface sialic acid, while the number of Factor B sites remained unchanged. A similar lack of high affinity Factor H binding sites, compared to Factor B binding sites, was found on zymosan-C3b. This work supported the concept that sialic acid directly enhanced the interaction of Factor H with C3b.

Pangburn and Müller-Eberhard (1978), on the other hand, compared the binding of alternative pathway components (Factors H, I, B, properdin) to C3b bound to E_R and E_s cells at similar multiplicities. Only the binding of Factor H (and consequently Factor I) was significantly different between the two cell types. The binding of Factor H to E_R was one tenth that to E_s. On treatment with neuraminidase, E_s cells became activators of the alternative pathway, and a pattern of binding similar to that found on E_R cells was reported. Further, it was known that E_R carried less surface sialic acid than E_s. Pangburn and Müller-Eberhard, in contrast to Fearon *et al.*, proposed that removal of sialic acid exposed Factor H "antagonists" which prevented the interaction of C3b and Factor H.

Okada *et al.* (1982) used liposomes sensitive to alternative pathway mediated lysis to investigate the ability of various incorporated glycolipids to influence activation of the alternative pathway. Of those tested, only sialylated lipids were found to prevent complement activation on the liposomes. Similar work by Michalek *et al.* (1988) demonstrated that the incorporation of sialylated lipids such as GM_1 into liposomes led to an increased number of Factor H binding sites. Using chemically modified lipids, these workers were able to define the structural requirements for the inhibition of the alternative pathway by sialic acid. In agreement with Fearon (1978), removal of the C9 carbon of the polyhydroxyl tail was sufficient to prevent the inhibitory action of sialic acid. Conversion of the carboxyl group to a hydroxyl group also abolished activity. These results support a direct role of sialic acid in the down regulation of complement, rather than the exposure of asialyloglycoconjugate Factor H antagonists.

A comparison of the ability of serum from twenty five mammalian species to lyse erythrocytes from different species allowed erythrocytes to be ranked in order of susceptibility to the alternative pathway. In general, the level of surface sialic acid (per mass protein) was found to be greatest on the least susceptible erythrocytes, supporting the role of sialic acid in protection against complement (Ish *et al.*, 1993). A few marked exceptions to this rule may be due to the presence of other regulatory molecules on the erythrocyte surface (see below). Tomlinson *et al.* (1992) have recently shown that resialation of E_s cells after neuraminidase treatment, using a recently characterized trans-sialidase, returns them to a non-activating state, further supporting the importance of sialic acid in protection against the alternative complement pathway.

1.8.3 The importance of other polyanions

Other anionic polysaccharides have also been found to inhibit the alternative pathway of complement. The sulphated glycosaminoglycan, heparin, has long been known to inhibit complement and coagulation. The effects of heparin on complement appear to be complex. Weiler *et al.* (1978) found that heparin prevented formation of the C3bBb complex on E_sAC4b3b cells, and proposed that it directly inhibited the interaction of C3b and Factor B. Heparin, at a higher concentration, was also found to increase the rate of decay of C3bBbP, but not C3bBb, suggesting a direct interaction with properdin. However, heparin was found to decrease the activity of Factor H, thus potentially upregulating complement. Blondin *et al.* (1993) reported similar findings for sulphated polysaccharides from brown seaweed (fucans). Fucans inhibited formation of the C3bB complex on E_sC3b cells. Also, fucan was more potent than heparin in decreasing the stability of C3bBbP, while neither compound affected the unstabilized complex. Wilson *et al.* (1984) found that chondroitin sulphate E from bone marrow derived mast cells or from squid was able to inhibit formation of C3bBb on E_sC3b in the presence of activated properdin and, to a lesser extent, native properdin. Activated properdin, but not native properdin, was inhibited from stabilizing the C3bBb complex in the presence of chondroitin sulphate E. Quigg (1992) found that "complement inhibitory chondroitin sulphate B proteoglycan" (GCRF) which is secreted by rat glomerular epithelial cells in culture could inhibit the action of C3bBbP on E_s cells, while not influencing the inherent decay of the complex. It was proposed that the GCRF was inhibitory by binding directly to the C3bBbP complex, although the site of binding was not established. The binding of properdin to sulphated sugars is discussed later.

Kazatchine *et al.* (1981) investigated the structural requirements for heparin to inhibit formation of the C3bBbP complex on EsAC4b3b cells. O-sulphation was required for activity. N-desulphation abolished activity, but either N-resulphation or N-acetylation returned it, suggesting that N-substitution was important. N-acetylated heparin had anti-complementary, but no anti-coagulant, activity, thus distinguishing the two functions. Weiler *et al.* (1992) confirmed these results *in vivo*. Heparin and N-acetylated heparin both inhibited CVF-induced activation of complement in guinea pigs.

Clearly, sulphated polysaccharides influence complement activity. However, the importance and primary mechanism of this action in physiological situations is unclear. The similarity between the effects of sulphated polysaccharides and sialic acid (and other polyanions) has been noted. Kazatchine *et al.* (1979) found that linking heparin to cyanogen bromide activated zymosan reduced the ability of zymosan to activate the alternative pathway in serum. The effect was not due to reduced C3bBb complex formation in the absence of Factors H and I. However, in a manner analogous to that of sialic acid, the presence of heparin on zymosan reduced the stability of C3bBb in the

presence of Factors H and I. It was proposed that sulphated polysaccharides, like sialic acid, might also regulate the activation of the alternative pathway on the surface of mammalian cells by promoting the interaction of C3b and Factor H. Carreno *et al.* (1989), using a chemically defined model system, proposed that C3b, Factor H and chemical groups on non-activating surfaces formed a ternary complex which would enhance the decay-accelerating activity of Factor H. Sephadex (α(1-6) cross-linked dextran) normally activates the alternative pathway. If the hydroxyl groups are modified to carboxymethyl groups, then the material becomes a non-activator, although the formation of C3bBb on the surface is not prevented in the absence of Factors H and I. Sephadex, carboxymethyl-Sephadex and Sephadex-C3b all have similar capacities for Factor H binding. However, carboxymethyl-Sephadex-C3b has increased affinity for Factor H. This suggested a requirement for C3b and surface carboxymethyl groups for the efficient interaction of C3b and Factor H in this system.

A possible basis for the formation of such ternary complexes was suggested by Meri and Pangburn (1990). Factor H was found to bind to heparin-agarose or fetuin-agarose (sialic acid containing) columns, suggesting the presence of a sialic acid/polyanion binding site on Factor H. Factor H was also found to be one of the major proteins binding to heparin from normal whole human plasma (Zammit *et al.*, 1993). Pangburn *et al.* (1991) localized the putative binding site within Factor H to a site 20 nm away from the C3b binding site (see section 1.9.3). However, a simple model in which surface polyanions encourage the localization of Factor H to surface bound C3b on non-activators was questioned. Meri and Pangburn (1990, 1994) found that *fluid phase* polyanions (such as dextran sulphate, heparin, chondroitin sulphate A and DNA) could enhance the binding of Factor H to C3b on small polysaccharides or zymosan. Furthermore, the fluid phase polyanions were able to enhance Factor H and I mediated cleavage of C3b bound to small soluble molecules, and thus were proposed to act as co-factors for Factor H. However, Koistinen (1993) found that while such fluid phase polyanions would increase Factor H binding to E_sC3b and Sepharose-C3b, they were unable to enhance Factor H co-factor or decay accelerating activity on these particles (indeed, high molecular mass dextran sulphate inhibited Factor H). While the mechanism of action remains unclear, Pangburn and Meri have proposed that molecules such as glycosaminoglycans and sialic acid may be important for restriction of complement activation on non-cellular surfaces such as basement membranes. However, since many complement components are found to bind to heparin (Sahu and Pangburn, 1993) including Factors B, D, and H, properdin, C1q, C2, C4, C4bp, C1-inhibitor, C6, C7, C8 and C9, it is clear that a multitude of other possible influences of these compounds on complement exist.

1.8.4 Cell surface regulatory proteins

It is now clear that on cellular surfaces, complement regulatory proteins other than Factors H and I are vital for preventing alternative pathway activation. Various phenomena originally suggested that Factors H and I could not explain all the properties of non-activators. For instance, although removal of sialic acid from E_s cells renders them susceptible to lysis by human serum, removal of sialic acid form human erythrocytes (E_{hu}) does not make them susceptible to autologous complement. Fearon (1979) used this observation to purify the first cell surface protein clearly shown to be a down-regulator of complement. Fractions of solubilized E_{hu} membranes were assayed for their capacity to decay-dissociate C3bBbP on E_s cells. Further purification of this activity revealed a protein, now known as CR1 (complement receptor 1, CD35), which enhanced the decay of C3bBbP on normal or desialated E_s cells, in contrast to Factor H which was relatively inactive on desialated cells. CR1 could also act as a co-factor for Factor I. It was proposed that such a membrane bound decay accelerating and Factor I co-factor activity could represent "one molecular basis for preventing inappropriate cell recognition". It is now clear that CR1 also functions as a receptor for C3b and C4b and has an important role in the removal of immune complexes from the serum on erythrocytes (Cornacoff *et al*, 1983). CR1 has a limited tissue distribution and is found mainly on peripheral blood cells.

Two other membrane proteins which regulate complement have been characterized. Nicholson-Weller *et al.* (1981, 1982) isolated a protein from erythrocyte stroma by butanol extraction which displayed decay accelerating activity for the classical C3 convertase, C4b2a. This glycoprotein was also able to accelerate decay of the alternative pathway C3bBbP complex. DAF (decay accelerating factor, CD55), as this glycoprotein is now known, is distinct from CR1. It does not have Factor I co-factor activity, and is linked to the membrane by a phosphatidyl inositol (GPI) anchor.

A third protein, membrane co-factor protein (MCP, CD46) was isolated from the monocytic U937 cell line. MCP is a highly efficient co-factor for Factor I cleavage of C3b, but does not have decay accelerating activity (Seya *et al.*, 1986). Thus, its properties are complementary to those of DAF. Both DAF and MCP are widely distributed on peripheral blood cells and tissues which are in contact with plasma complement components. It is now thought that these membrane proteins are of primary importance in the prevention of C3b amplification on host cells. While Factor H undoubtedly can contribute to control on cell surfaces, its major role is probably in limiting fluid phase tick-over of C3 (Atkinson and Farries, 1987).

Membrane bound proteins which regulate formation of the membrane attack complex also exist. CD59 and C8 binding protein/homologous restriction factor (C8bp/HRF) inhibit reactive lysis of erythrocytes independently of the earlier steps of complement

activation. CD59 is a GPI-anchored membrane protein that is thought to interfere with the association of the first C9 molecule with the C5b-8 complex (Lachmann, 1991). These proteins are also important in the prevention of lysis of self cells or "homologous restriction".

The disease paroxysmal nocturnal haemoglobinuria (PNH) is characterized by an acquired sensitivity of erythrocytes to alternative pathway haemolysis. The underlying defect is an inability of the cells to synthesize the GPI anchors which attach a variety of proteins to the cell surface. The sensitivity of these cells to the alternative pathway has been attributed to the lack of DAF, CD59 and C8bp/HRF on the cell surface. Patients of the Cromer null blood group are deficient in DAF, but do not show signs of increased haemolysis. In contrast, a single patient with inherited deficiency of CD59 has a mild PNH-like disease (Morgan and Walport, 1991). The absence of disease in the case of the DAF deficient subjects may reflect the presence of other C3bBb regulating proteins (MCP, CR1, and Factor H).

1.9 The structure of alternative pathway complement components

In the nineteen eighties, the cloning of the genes and/or cDNAs of many of the components was accomplished. This confirmed the expected structural relationships between some of the functionally similar components and revealed the modular nature of most of the complement proteins (figure 1.2). Many extracellular (and intracellular) proteins in higher eukaryotes are now known to be made up of "mosaics" of a relatively small number of protein "modules". Each type of module has characteristic conserved framework residues (often with good conservation of cysteine residues). Modules are independently folded structures within proteins, and are often encoded by discrete exons of similar phase. This allows them to be "shuffled" between different genes, and thus between different proteins (Patthy, 1987).

1.9.1 C3 (C4 and C5)

C3, C4 and C5 are all synthesised as single polypeptides which are processed before secretion to generate disulphide linked two or three chain molecules. C3 (190 kDa) is cleaved to form an α chain (115 kDa) and a β chain (75 kDa) held together in part by a disulphide bond. The amino acid sequences of C3, C4, C5 and also α2-macroglobulin have 20-30% identity along their lengths, and probably adopt similar three dimensional structures (Lambris *et al*, 1993). C3, C4 and C5 are the only complement components not composed of known modular units. However, recently a region of a modular *C. elegans* protein, Unc-6, has been found to contain a region similar to C3/C4/C5

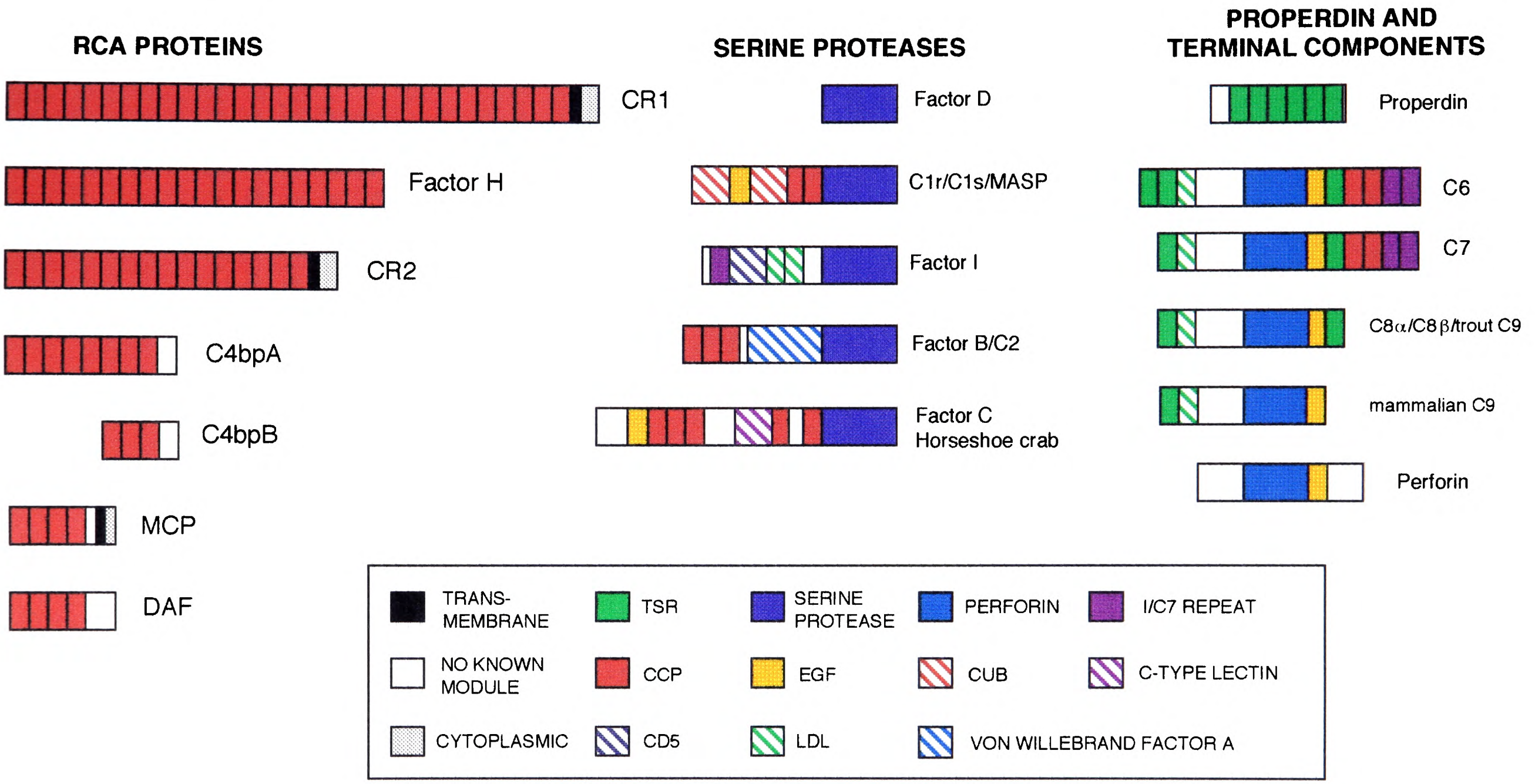


Figure 1.2
The modular construction of the components of the complement system.
Most of the proteins of the complement system are "mosaics" made up of a relatively small number of units containing conserved framework residues (see text).

(corresponding to the C-terminus of the α-chain of C3, see section 1.14.2). Other modules may yet be found in these molecules.

Within C3 and C4 an intramolecular thioester is formed between a cysteine and a glutamine residue in the peptide cys-gly-glu-gln. C5, which does not bind covalently, lacks the required cysteine and glutamine residues and does not have a thioester (Law and Reid, 1988). C3 and its major degradation products (C3(H_2O) which has a hydrolysed thioester, C3b formed by Bb cleavage, and the fragments iC3b and C3dg formed by Factor I cleavage) contain binding sites for a large number of other complement proteins including the serum components properdin and Factors B, H and I, and the membrane receptors CR1, CR2, CR3, CR4, MCP and DAF. Putative binding sites for a number of these proteins have been identified by protein fragmentation, peptide and competitive binding studies (Fishelson, 1991) (see figure 1.3). Lambris *et al.* localised the properdin binding site on human C3 by testing the binding of properdin to C3b, C3c, the α and β chains of C3, and fragments of the α and β chains. Properdin was found to bind to C3b, C3c and the α-chain immobilized on microtitre plate wells, but not to C3d or the β-chain. Proteolytic fragments of the α-chain were used to further localize the binding site to the C-terminal 40 kDa of the α-chain (Lambris *et al.*, 1984). Cyanogen bromide cleavage of the 40 kDa fragment produced a 17 kDa fragment (residues 1387-1540) which retained properdin binding activity. Alignment of the human C3 sequence with C3 from other species and with the related non-properdin binding proteins C4, C5 and α2-macroglobulin revealed a region within this fragment which was conserved in C3 but contained gaps in the other proteins (see figure 1.3). A 34 amino acid peptide encompassing this region (residues 1402-1435) was able to bind to human properdin but not to Factor H, and to inhibit the alternative pathway, while two other peptides from the α-chain were inactive (Daoudaki *et al.*, 1988). A peptide of nine amino acids within this region was later found also to bind properdin, and residues His-1431 and Ser-1432 were postulated to be important (Becherer *et al.*, 1989). This region is better conserved in rabbit, rat, guinea pig, mouse, *Xenopus*, cobra and trout C3 than the overall conservation of C3. Although it has been suggested that hagfish "C3" also retains this binding site (Ishiguro *et al.*, 1992) it is clear that both hagfish and lamprey "C3" have many features in common with C4. It is likely that these molecules are structurally and functionally similar to the common ancestor of C3 and C4 (Nonaka *et al.*, 1984; Ishiguro *et al.*, 1992). Certainly, within the putative properdin binding site these molecules possess features that are similar to mammalian C3, C4 and C5 (see figure 1.3). It is thus dangerous to propose the existence of properdin in these species from the sequence of "C3".

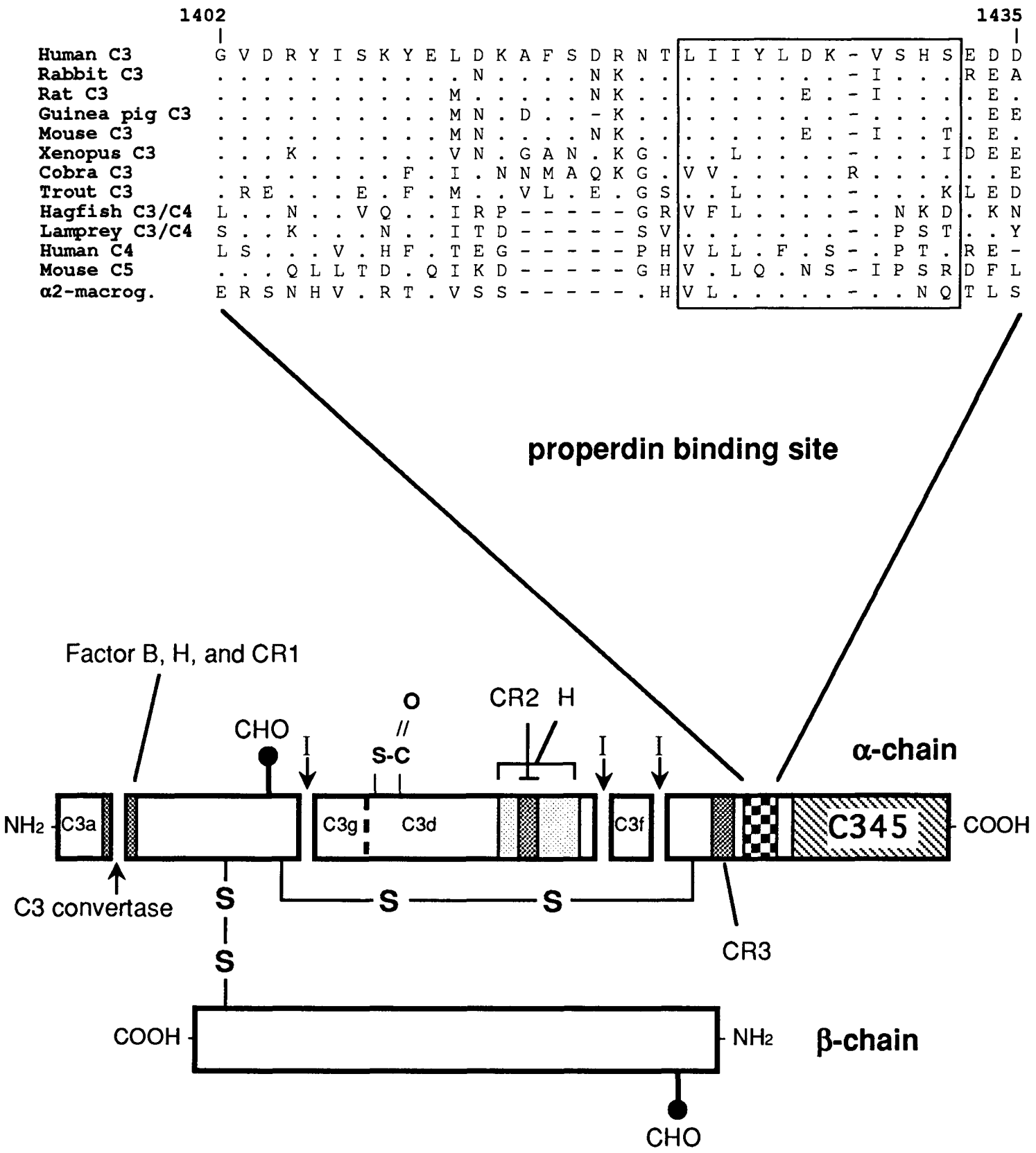


Figure 1.3
The structure of human C3, indicating the properdin binding site.
The upper portion of the figure shows the sequence of the 34 amino acid peptide from human C3 which binds properdin (Daoudaki *et al.*, 1988), compared to the sequences of C3, C4, C5, and α_2-macroglobulin from other species (Lambris *et al.*, 1993). The box shows the properdin binding region further localized by Becherer *et al.*, 1989. Residues identical to the human sequence are shown as dots, and gaps are shown as hyphens.
C3 is synthesized as a single polypeptide and cleaved before secretion to produce the alpha and beta chains. The sites for cleavage by the C3 convertase and Factor I are indicated by arrows. Binding sites for properdin, Factors B and H, and complement receptors CR1, CR2, and CR3 are also shown (Fishelson, 1991; Lambris *et al.*, 1993). CHO: N-linked carbohydrate, C345: module found in C3, C4, C5, Unc-6, netrins 1 and 2 (see text).

1.9.2 The serine proteases: Factors B, D and I (C2, C1r, C1s)

The major enzymes of the complement system are serine proteases, and contain the characteristic "catalytic triad" residues His-57, Asp-102 and Ser-195 (numbered as in chymotrypsin). The complement proteins are highly specific compared to other serine proteases such as trypsin and chymotrypsin, however. C1r, C1s, C2 and Factor B all circulate as pro-enzymes which require proteolytic cleavage before they are active. Inactive Factor B is cleaved by Factor D to form Ba, which is released from the C3bB complex, and Bb which remains to form the C3bBb convertase. Factor B and C2 which serve similar functions in the alternative and classical pathways (see figure 1.1) are also related in amino acid sequence outside the serine protease domain. The Ba fragment contains three CCP modules, which have been implicated in C3 and C4 binding (see section 1.9.3). This may support the proposed importance of Ba in the initial binding of Factor B to C3b and properdin (Pryzdial and Isenman, 1987; Farries *et al.*, 1988b). The Bb fragment contains a von Willebrand type A module and the enzymatic serine protease domain (see figure 1.2).

Factors D and I differ from the other complement serine proteases in that they do not circulate in a pro-enzyme form. However, both enzymes do require "co-factors" for activity. Factor I requires Factor H, CR1, MCP or C4bp (Pangburn *et al.*, 1977). Factor D only cleaves Factor B once it is associated with $C3(H_2O)$ or C3b. The recent determination of the structure of Factor D by crystallography has revealed that the catalytic triad is in an inactive configuration (Narayana *et al.*, 1993). It is thought that on binding, Factor B assumes a conformation which is able to bind Factor D. In turn, Factor D is only able to adopt the necessary active site conformation for enzymatic activity once it is bound to C3bB (or $C3(H_2O)B$). Factor D is thus functionally a zymogen.

1.9.3 Regulators of complement activation: Factor H, CR1, DAF, MCP (C4bp)

Sequencing of cDNAs encoding Factor H, CRI, DAF, MCP and C4bp has revealed that they are composed mainly of tandem repeats of an approximately sixty amino acid sequence which show between 20 and 40% identity within and between proteins (Reid & Day, 1989) (see figure 1.2). The structure of the genes encoding these proteins reveals that many of the repeats are encoded by discrete exons, although there are exceptions to this rule (Campbell *et al.*, 1988). The genes for CR1, CR2, Factor H, C4bp and DAF are all found within a 950 kb fragment on chromosome I in humans. The region has been named the "regulators of complement activation" or RCA cluster (Rodriguez de Corboba *et al.*, 1985; Law and Reid, 1988). These modules have been variously named "B-type modules" (they are found in Factor B - see above), "short consensus repeats" (SCRs), "complement control protein repeats" (CCPs) or "Sushi"

modules. The three dimensional structures of three such modules from Factor H have been solved by NMR (CCPs 5, 15 and 16). The structure is similar in each case, and reveals a fold dominated by five β-strands, with conserved hydrophobic residues forming the core, and the more variable regions forming exposed loops (Norman *et al.*, 1991; Barlow *et al.*, 1992). The solution structure of two adjacent modules (CCP 15 and 16) reveals relatively few contacts, and predicted flexibility, between the modules. Modelling of Factor H structure (20 CCPs) based on this information produces twisted "snake-like" structures reminiscent of electron micrographs of Factor H (Barlow *et al.*, 1993).

Factor H can be cleaved, using trypsin and mild reduction, into fragments representing CCPs 1-6, 6-12 and 12-15. By testing the activities of such fragments, the binding site for C3b with Factor H has been localised to the N-terminal six CCPs (Alsenz *et al.*, 1984) and Pangburn *et al.* (1991) have proposed the existence of a polyanion binding site within CCP 12-15. Photoaffinity labelled heparin could be cross-linked to CCPs 13-14, and examination of the Factor H amino acid sequence suggested that the highly cationic CCP 13 might represent the polyanion binding site (see earlier).

Since Factor H, CR1, CR2, DAF, MCP & C4bp all interact with C3b or C4b, it has been proposed that CCPs may be important in C3b/C4b/C5b binding in other complement proteins (Factor B, see above; C1r and C1s; terminal components, C6 and C7). However, CCPs are clearly found in other proteins unlikely to bind C3/C4/C5 (*e.g.* thyroid peroxidase) (Reid and Day, 1989).

1.10 Properdin: cDNA, gene and protein sequence

The amino acid sequence of approximately half of human properdin has been determined by protein sequencing (Reid and Gagnon, 1981). The analysis confirmed the unusual composition of properdin: glutamic acid, glutamine, proline, glycine and cysteine accounted for almost half of the total amino acids (Minta and Lepow, 1974). However, no evidence for short range repeats of the type found in collagen or C1q (Gly-X-Y) was found. Clones of cDNA representing all but the first four amino acids of mouse properdin (Goundis and Reid, 1988) and human properdin (Nolan *et al.*, 1991) have been sequenced. The derived amino acid sequence of human properdin contains a leader peptide and 442 amino acids encoding the mature form of plasma properdin and shows 76% identity to mouse properdin (with a single amino acid insertion). The sequence is in good agreement with the peptide fragments obtained by Reid and Gagnon. A single potential N-linked glycosylation site is located near the C-terminus. Properdin has previously been reported to contain 9.8% (w/w) carbohydrate (3.8% hexose, 1.5% hexosamine, 3.8% sialic acid, 0.8% fucose) (Minta and Lepow, 1974). The calculated mass of properdin from the derived amino acid sequence is 48 949 Da. Assuming 9.8%

(w/w) carbohydrate gives a total molecular mass of 53 300 Da for the properdin monomer - similar to that found for purified plasma properdin (Nolan and Reid,1990; Nolan, 1991). The predominance of basic amino acids in the derived sequence accounts for the high isoelectric point of over 9.5 observed for human properdin (Gotze and Müller-Eberhard, 1974) (see earlier). Smith *et al.* (1991) suggested that the high positive charge of the monomers might be responsible for maintaining the open cyclic structures adopted by properdin oligomers (see section 1.6), since the centres of the subunits would repel each other. The N- and C-terminal regions of properdin contain a large number of basic and acidic residues which could be involved in the stable predicted head-to-tail interactions of the monomers. The susceptibility of properdin oligomers to dissociation at low pH (Pangburn, 1989) fits in with the proposed ionic nature of the association (Nolan and Reid, 1993).

A closer examination of the derived amino acid sequence of mouse and human properdin revealed an N-terminal region of fifty amino acids with no homology to known proteins. However, the rest of the sequence was made up of five and a half or six repeating motifs of around sixty amino acids. Each repeat contains a framework of highly conserved residues with six cysteines, three tryptophans and a basic cluster featuring prominently. The structure of the human properdin gene (Nolan *et al.*, 1992) reflects this organization closely. The N-terminal region and the first five repeats are encoded by discrete exons. The remaining C-terminal region of the protein, including the possible sixth repeat, is encoded by two exons. The genomic structure of human properdin, and the existence of the sixth repeat are discussed further in chapter 3. Three repeats similar to those found in properdin have previously been identified in the cell adhesion molecule, thrombospondin, and have been named "thrombospondin type I repeats" or "properdin repeats". In this thesis they will be referred to as TSRs (thrombospondin type I repeats). Many other proteins containing TSRs have now been identified, including various protozoan parasite adhesive proteins (including the *Plasmodium* circumsporozoite (CS) protein), the terminal complement components, proteins of the rat, the frog, and *C. elegans* involved in neuronal cell guidance and a new family of vertebrate growth factors (see figure 1.4). These proteins are discussed in more detail below.

A consensus sequence for the TSRs of properdin and the closely related thrombospondin sequences is shown in figure 1.5. Multiple sequence alignments and a more detailed analysis of TSR module sequences are given in chapter 3 (see figures 3.9, 3.10b, and 3.14). Noteworthy features that will be discussed further in this introduction are the conserved WSxWS motif near the N-terminal end of the primary sequence of each TSR, the CSVTCG motif (which is highly conserved in the thrombopsondin and CS proteins, and partly conserved in properdin TSRs 1, 2, and 4), and the basic Rx(R/K) motif in the centre of the repeat (conserved in all the thrombospondin and CS TSRs and in all properdin TSRs except TSR4).

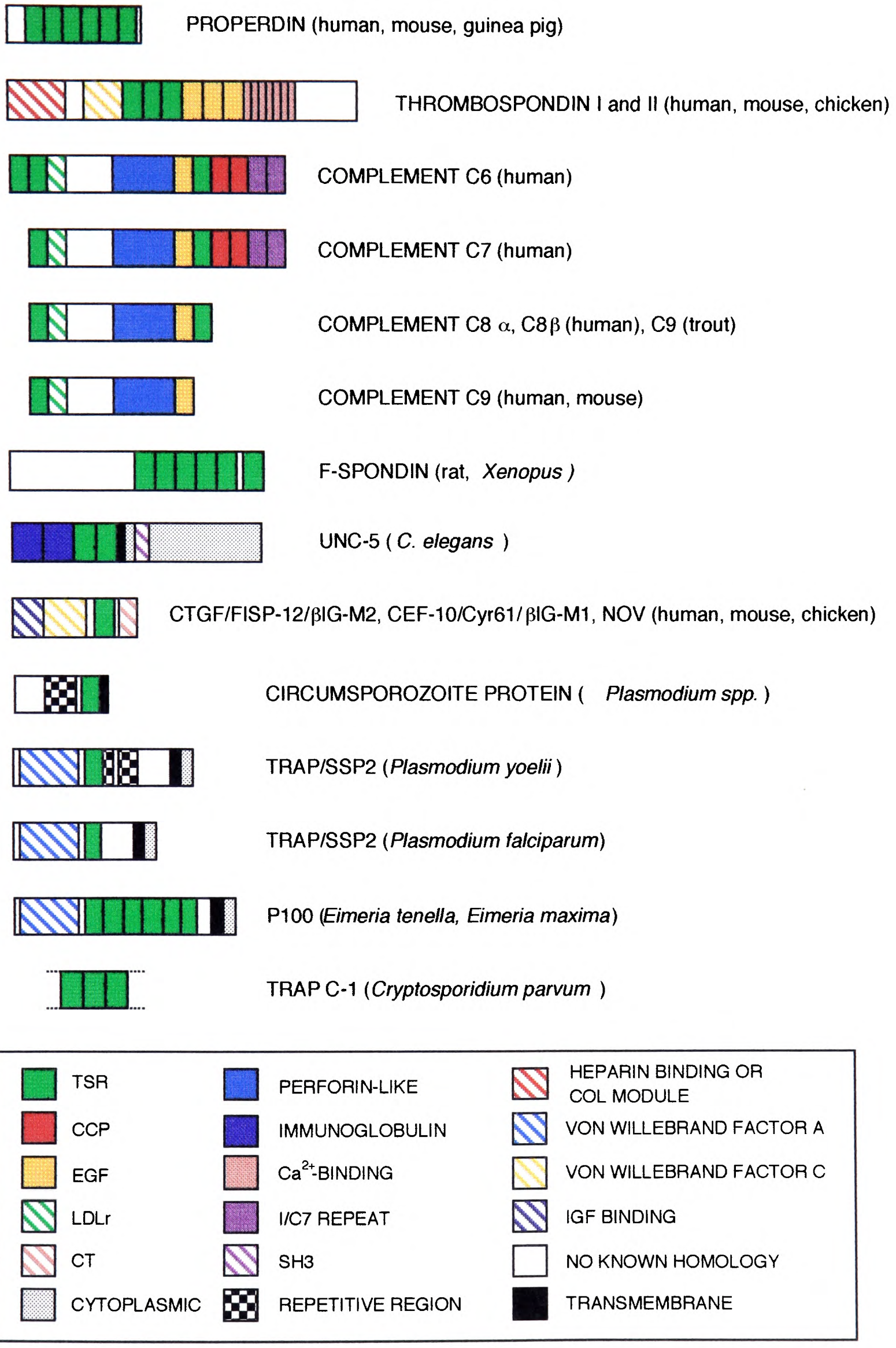


Figure 1.4
Proteins containing thrombospondin type I repeats (TSRs). See text for details.

xDGG**WS**x**WS**P**WS**x**CS**V**TCG**x**G**xQx**R**x**R**xCxxPxPQxx**G**xCxGxxx**E**/**Q** x**R**/**K** x**C**xxxx**C**P

xxIRxS**I**/**V**TxE**WS**P**CS**V**TCG**x**G**/**V**Rx**R**x**K**xxAx NKKPEELxL NDL**E**TEI**C**xMDK**C**S

Figure 1.5
Consensus sequences of the TSRs from properdin, thrombospondin and the malarial circumsporozoite proteins.
Top: consensus for the TSRs of properdin and thrombospondin.
Bottom: consensus for the TSRs of the *Plasmodium* circumsporozoite proteins.
Residues conserved in more than fifty percent of all TSRs are highlighted in **bold**. The WSxWS motif, the VTCG motif, and the basic cluster RxR motif discussed in the text are overlined. x represents non-conserved positions.
The consensus is taken from an alignment of all the known TSRs, described in more detail in chapter 3.

The three-dimensional structure of TSRs is unknown. Some predictions have been made, however. Fourier transform infrared spectroscopy was used to examine purified human properdin. The spectrum obtained suggested the presence of substantial quantities of β-sheet and β-turn structure, a result consistent with averaged secondary structure predictions made using the methods of Robson or Chou-Fasman, using the TSR sequences of properdin, thrombospondin and the terminal complement components (Perkins *et al.*, 1989). The far UV circular dichroism spectrum of purified properdin was found not to contain maxima typical of α-helix or β-structure, nor was it compatible with a conventional random coil assignment (Smith *et al.*, 1984). The presence of a large number of disulphide-linked cysteines in properdin may be the cause of the unusual results (Nolan and Reid, 1993). Estimates of TSR volume were made from the known composition of properdin, and a comparison with the known length of the properdin monomer from electron microscopy was made. This allowed the shape and size of a properdin TSR to be estimated. An elongated structure of length 3.3 - 4.3 nm and diameter of 1.7 nm was suggested (Perkins *et al.*, 1989; Smith *et al.*, 1991).

1.11 The function of the TSRs in properdin: C3b and sulphated glycoconjugate binding

Since properdin is composed almost entirely of TSRs, it is likely that one or more of these modules is important in properdin function. Properdin binds C3b and has been proposed to interact with both the Ba and Bb fragments of Factor B. The regions of properdin involved in these activities are unknown.

Knowledge of the protein sequence of properdin led to the prediction of another function for TSRs in properdin (and other proteins). Holt *et al.* (1989) were working on a salivary protein of the Mexican leech (*Haementeria officinalis*) which inhibits coagulation. This protein, antistasin, was found to bind to sulphatide (galactose(-3-sulphate)β1-1 ceramide) on thin layer chromatograms, but not to other anionic lipids such as sialylated gangliosides. The binding could be inhibited with dextran sulphate, fucoidan and heparin, but not with chondroitin sulphate A, B, or C, keratan sulphate or hyaluronic acid. Sequence comparisons of antistasin with other known sulphatide binding proteins (thrombospondin, von Willebrand Factor and β2-glycoprotein I) led Holt *et al.* to suggest a possible sulphatide binding motif (see figure 1.6). The sequence spans the conserved CSVTCG and basic RxR motifs of the thrombospondin TSRs. These workers also predicted that properdin and malarial circumsporozoite proteins might also bind to sulphated glycoconjugates. While this work highlighted the possible importance of these conserved regions in TSRs, it is clear that the similarity of the sequences of antistasin, von Willebrand Factor and the other proteins in this alignment is not significant. Antistasin

Protein	Sequence	Residues
Antistasin	R C R V H C P H G F Q - R S R W	32-46
	N C R K T C P N G L K - R D K L	87-101
Thrombospondin	S C S T S C G N G I Q Q R G R S	372-87
	S C S V T C G D G V I T R I R L	428-43
	I C S V T C G G G V Q K R S R L	485-500
von Willebrand factor	Q C H T V T C Q P D G Q T L L K S H R V	1135-54
β_2-Glycoprotein I	E I T Y S C K P G Y V S R G M G R K	27-44
Collagen Type IV:		
α1 chain	G Q P G A K G E P G G E F Y F D L R L K G	522-41
	A H G Q D L G T A G S C L R - K F	1482-97
α2 chain	A H N Q D L G L A G S C L A R F S T	1526-43
Glycoprotein E, *Herpes simplex* I	V C V V S C L A G T P K T S W R - R V	13-30
Circumsporozoite Coat Protein:		
P. falciparum	P C S V T C G N G I Q V R I K P	348-63
P. vivax	P C S V T C G V G V R V R S R V	315-30
P. knowlesi	P C S V T C G N G V R I R R K A	300-15
P. cyanomolgi	P C S V T C G K G V R M R R K V	315-30
P. yoelii	Q C S V T C G S G V R V R K R K	305-20
P. berghei	Q C N V T C G S G I R V R K R K	277-92
TRAP	P C S V T C G K G T R S R K R E	249-64
Properdin	P C S V T C S E G S Q L R H R R	61-76
	P C S V T C S K G T Q I R Q R V	120-35
	P C S V T C G L G Q T L E Q R T	241-56

Figure 1.6

Alignment of protein sequence homologies with antistasin (from Holt *et al.,* 1989).

Amino acids in common with the "sulphatide binding motif" proposed by Holt *et al.* (C.[S or T].V.[S or T].C.G.x.G.x.x.x.[R or K].x.[R or K]) are boxed. Gaps have been inserted (dashes), or residues inserted above the line to maximize alignment.

The significance of these alignments is discussed in the text.

The sequences from properdin shown correspond to TSRs 1, 2, and 4 of the mouse protein (see figure 3.10 for the corresponding regions of human and guinea pig properdin).

does not contain TSRs (see chapter 3) and the work of Cardin and co-workers has subsequently shown that, while the basic residues present in the antistasin motif suggested by Holt *et al.* (residues 87-101) contribute to sulphated sugar binding, the most active peptides derived from antistasin span residues 96-119, and contain a second cluster of basic amino acids. The peptide 87-101 is a poor inhibitor of antistasin binding to heparin (Blakenship *et al.*, 1990; Brankamp *et al.*, 1991, 1992). Nevertheless, the suggestion that many TSRs could be sulphated glycoconjugate binding modules stimulated a new area of research, described in more detail below.

In 1990, Holt *et al.* reported that properdin could bind specifically to sulphatide. Purified native properdin bound to sulphatide on thin layer chromatograms, but not to galactosylceramide[6], nor to other non-sulphated ceramides, nor sialylated gangliosides. A similar specificity of binding was observed on microtitre plates. "Activated" properdin bound more avidly to sulphatide than native properdin. The binding of native properdin could be inhibited by dextran sulphate (500 kDa) or fucoidan and in addition, the binding of activated properdin was inhibited by heparin (Holt *et al.*, 1990). Another group has also recently reported that properdin will bind to sulphatide, and with increased affinity to $S_{tri}1(III^3SO_3^{(-)}, II^3SO_3^{(-)}\text{-}Gg_3Cer)$ (a bisulphated ganglio-series glycosphingolipid found in the vascular membrane of rat glomerular capillaries) and to seminolipid ($I^3SO_3^{(-)}$-Gal-acylalkylglycerol,l-O-alkyl-2-O-acyl-3(β-3'-sulphogalactopyranosyl)-*sn*-glycerol) (Jennemann *et al.*, 1994). Although in these studies quantitative comparisons are difficult since the amount of the different glycolipids binding to the microtitre plates was not determined.

In contrast to these results, Stankowski *et al.* (1991) reported that although a peptide encompassing the "sulphatide binding motif" of Holt *et al.* from the second TSR of properdin (WSGWGWEP**CSVTC**SK**G**TRT**RR**A) bound to and aggregated liposomes containing sulphatide, the interaction was similar to that seen with another anionic lipid, palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphoglycerol (POPG). Similar results were obtained using intact purified human properdin. No significant difference was seen between liposomes or supported bilayers containing sulphatide or POPG, either in the presence of $CaCl_2$ or EDTA. It was concluded that properdin did not bind sulphatide specifically, and the interactions could be explained by electrostatic charge. The reason for the discrepancy between these assays and the lipid plating assays of Holt *et al.*, Jennemann *et al.*, and in this thesis (chapter 8) is not clear. The role of the sulphatide binding activity of properdin is not known, but it is interesting when considered with the effects of sulphated compounds on complement discussed in section 1.8.3. The phenomenon is discussed further in chapter 8.

---

[6] galactosylceramide (galactocerebroside) has the same structure as sulphatide, but lacks the sulphate group

1.12 Thrombospondin

Thrombospondin was first identified as a glycoprotein associated with the platelet membrane. It was found to be released from platelets on activation with thrombin - hence the name thrombospondin. As well as binding cell surfaces (through CD36, $\alpha_v\beta_3$ and sulphated polysaccharides), thrombospondin binds calcium ions, many extracellular matrix molecules (including heparan sulphate proteoglycan, fibronectin, laminin, collagen types I, IV, and V) and plasma proteins such as fibrinogen, plasminogen (for review see Frazier, 1991; Bornstein, 1992). Recently, thrombospondin has been found complexed with TGF-β on secretion from the α-granules of platelets (Schultz-Cherry *et al.*, 1994). Thrombospondin is synthesized by a wide variety of cells including fibroblasts, endothelial cells and smooth muscle cells. A role for thrombospondin in various processes during development and tissue repair has been proposed. Thrombospondin is incorporated into fibrin clots, is involved in platelet aggregation, promotes binding and spreading of many cell types, is a suitable substratum for neurite outgrowth, inhibits angiogenesis, and activates latent TGF-β (Lawler and Hynes, 1986; Frazier, 1991; Tolsma *et al.*, 1993; Schultz-Cherry *et al.*, 1994).

1.12.1 Thrombospondin structure

Recently, it has become clear that there is a family of thrombospondin molecules, now known as thrombospondins 1 to 5, encoded by different genes. Alternative splicing may generate further variations of these proteins (Frazier, 1991; Adams and Lawler, 1993a). The original form of thrombospondin (now known as thrombospondin-1) is a trimeric 420 kDa glycoprotein. The amino acid sequence, derived from a cDNA clone, reveals its modular construction (Lawler and Hynes, 1986). In combination with information from electron microscopy and limited proteolysis of the molecule, an outline of the structure can be described. The globular N-terminal domain has no homology to proteins other than thrombospondin-2, can bind to heparin (Dixit et al., 1984) and is known as the heparin binding domain (HBD). Following this is a pair of conserved cysteines involved in trimerizing the molecule. A region with homology to procollagen type I and the von Willebrand C domain (VWC) is followed by three "thrombospondin type I repeats" (TSRs) of the type found in properdin, and three EGF-like repeats. This section forms the "stalk" region of thrombospondin which can be isolated as a 210 kDa trimer of a 70 kDa chymotryptic fragment (see later). The C-terminal region of the molecule contains seven calcium binding motifs related to those found in calmodulin, and a carboxy-terminal "cell binding domain" (CBD). The conformation of this region is highly sensitive to calcium binding. The homology of thrombospondin-1 with the other members of the family decreases from the N- to the C-terminus. Thrombospondin-2 is 86 to 97%

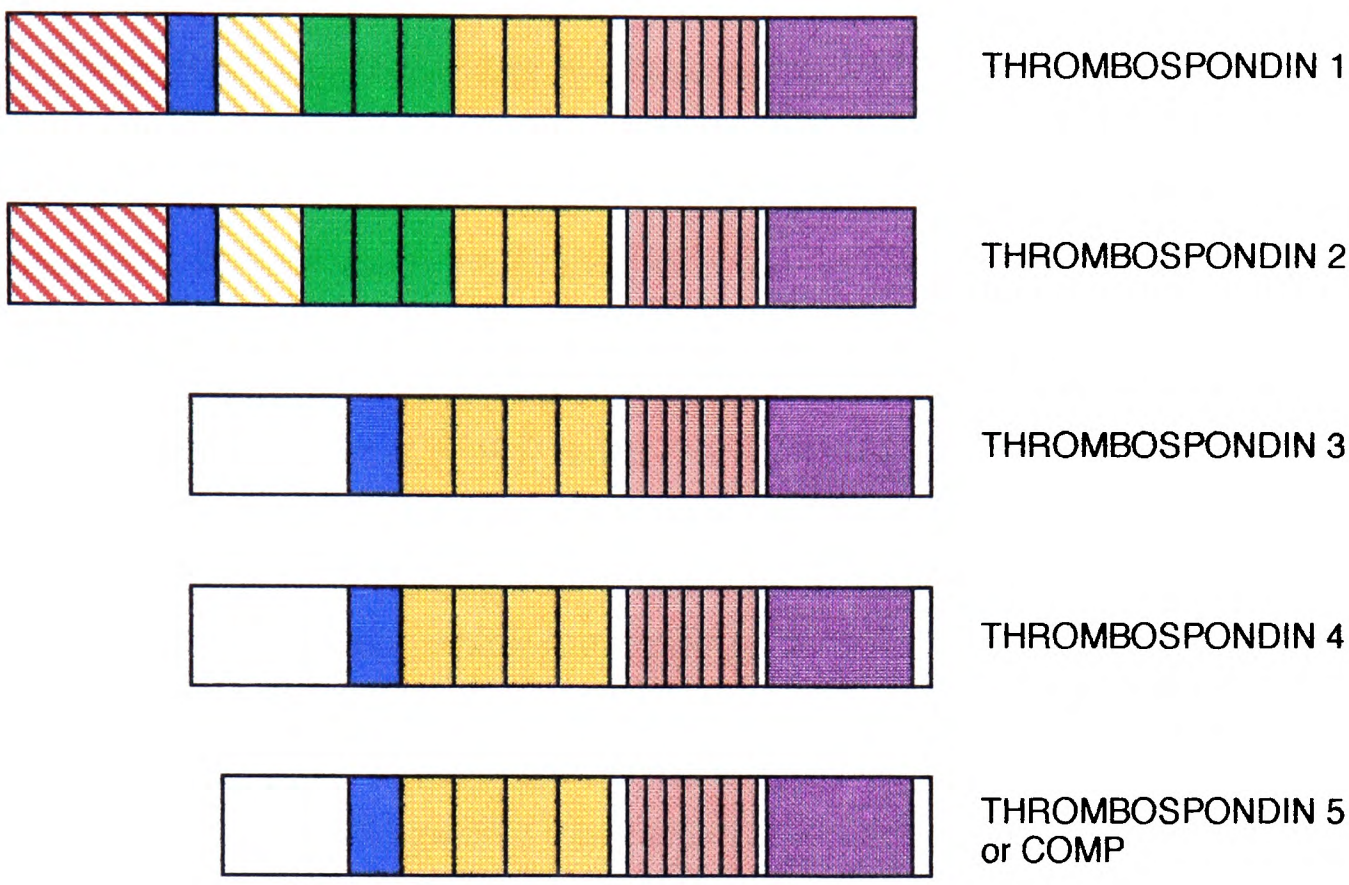


Figure 1.7
Outline structures of the members of the thrombospondin family.
COMP: cartilage oligomeric matrix protein.
See text for details (Adams and Lawler, 1993a; Bork, 1993)

similar to thrombospondin-1 from the HBD to the CBD. Thrombospondins-3,4 and 5 lack homology to the HBD, and although they retain the oligomerizing cysteines, they lack the three TSR modules and contain four rather than three EGF-like modules (see figure 1.7). In the genes of thrombospondin-1 and 2, the three TSRs are encoded by discrete exons.

1.12.2 The CSVTCG and RxR motifs in thrombospondin

The 70 kDa chymotryptic fragment of thrombospondin-1, and the three TSR modules within it have been implicated in a number of thrombospondin functions, although a considerable amount of controversy exists regarding the identity of the various cellular thrombospondin receptors, and their binding sites on thrombospondin. Dardik and Lahav (1991) and Prater *et al.* (1991) found that the 70 kDa fragment of thrombospondin would support adhesion of endothelial or melanoma cells. A monoclonal antibody to this region was able to inhibit the interaction. Heparin and other sulphated glycoconjugates were also found to partially inhibit the binding. Prater *et al.* synthesized peptides corresponding to the SxWxSCSxxCGxGxxxRxR region of each of the three TSRs. The peptides from TSRs II and III were also able to support cell attachment. It was proposed that the conserved CSVTCG motif of these two TSRs might be binding to a cell receptor, possibly with the involvement of glycosaminoglycan binding through the basic cluster at the C-terminus of the peptide. Tolsma *et al.* (1993) found that the same two peptides inhibited angiogenesis *in vivo* and *in vitro*.

Tuszynski *et al.* (1992) made shorter peptides from the same region of the thrombospondin TSRs, and found that the CSTSCG motif of TSR I, and the CSVTCG motif of TSRs II and III could increase the binding of various cell lines to plastic (controls: 0-17% binding compared to intact thrombospondin, TSR peptides: 22-49% binding). The CSVTCG peptide also inhibited ADP-induced platelet aggregation *in vitro* and reduced melanoma cell metastasis *in vivo*. These workers suggested that the CSVTCG motif is important in cell adhesion to thrombospondin. However, peptides containing arginine which are not found in thrombospondin (CSVTCR, CSTSCR, and CRVTCG), were better promoters of cell adhesion (to 79-104% binding compared to thrombospondin), and other different peptides have been found with similar activities (see later).

Tuszynski *et al.* (1993) have purified a possible receptor for the CSVTCG motif. Using a CSVTCG peptide affinity column, glycoproteins of 50 and 60 kDa were isolated from solubilized A549 human lung carcinoma cells. The so-called CSVTCG binding protein (CBP) bound purified thrombospondin *in vitro*, and the binding was inhibited by heparin. Antibodies raised to CBP prevented A549 cell spreading on thrombospondin. It was suggested that thrombospondin binding to CBP may be "mediated" by sulphated glycoconjugates on CBP (although the presence of such modifications was not

demonstrated). Thrombospondin is known to bind to sulphatide and heparan sulphate proteoglycan (Roberts *et al.*, 1985; Sun *et al.*, 1989) although the N-terminal HBD, which contains several basic amino acid clusters, has previously been proposed as the seat of this activity (see above). Thus, the possibility that there is a second heparin binding site in the TSRs of thrombospondin has been raised.

Other workers have proposed that the CSVTCG motif is required for binding to CD36, a glycoprotein receptor for thrombospondin found on platelets, monocytes and endothelial cells. Although the expression of CD36 in COS cells does not increase their capacity to bind thrombospondin (Oquendo *et al.*, 1989), expression of CD36 in Jurkat cells confers on them the ability to bind thrombospondin. Transfected, but not control, Jurkat cells could also bind a ^{125}I-YCSVTCG peptide, and this binding could be inhibited by an anti-CD36 mAb (Asch *et al.*, 1992). Catimel *et al.* (1992) were able to show that purified whole thrombospondin and the 70 kDa stalk fragment were able to bind purified CD36. CD36 was also able to bind CSVTCG peptide immobilized on microtitre plates. Thus, CSVTCG may be part of the CD36 binding site of thrombospondin. The difference seen between the COS and Jurkat cells above could indicate the requirement for other cell surface molecules for thrombospondin binding. More recently, the binding of thrombospondin to CD36 has been shown to be regulated by the phosphorylation of thr-92 in the extracellular thrombospondin binding site of CD36. Phosphorylation of thr-92 reduces thrombospondin binding (Asch *et al.*, 1993). So, differences in CD36 post-translational modifications may also influence the pattern of thrombospondin binding seen to different cell types.

1.12.3 The WSxWS motif in thrombospondin

A region other than the CSVTCG or RxR motifs of the thrombospondin TSRs has been proposed to be important for the binding of thrombospondin to heparin. Guo *et al.* (1992a) synthesized a variety of peptides based on the highly conserved N-terminal halves of TSRs I, II, and III, and tested their ability to inhibit the binding of thrombospondin, laminin, and apolipoprotein E to heparin-BSA or sulphatide on microtitre plate wells. A CSVTCG peptide was a good inhibitor of binding of both thrombospondin and laminin to heparin and sulphatide. However, a number of peptides based on the WSxWS motif of the TSRs were also found to inhibit the three proteins from binding. In a second report, Guo *et al.* (1992b) made small alterations to the WSxWS peptides to define the residues required for the inhibition of thrombospondin and laminin binding to heparin. The tryptophan, serine, and proline residues were all found to be important. The authors stress that if a single amino acid is added between the two tryptophans, then activity is lost. However, from their data it is clear that one or both of the amino acids between the tryptophans can be removed, producing a peptide quite unlike that found in

thrombospondin, and the activity is *not* altered. Although these workers clearly demonstrate that these peptides can bind heparin and/or sulphatide, the peptides inhibit the binding of all the proteins tested, whether or not they contain the WSxWS motif. It is far from clear that these regions are important for native thrombospondin binding to sulphated glycoconjugates. Stankowski *et al.* (1991), found that the fluorescence emission from the tryptophans in properdin was typical of a hydrophobic environment, shielded from the solvent. It is thus likely that these residues form part of the hydrophobic core of the TSR.

1.12.4 Other ligands for the TSRs of thrombospondin

Various other reports have implicated the thrombospondin TSRs in binding to other ligands. Takagi *et al.* (1993) found that a reduced 19 kDa fragment of bovine thrombospondin containing TSR I was able to bind collagen type V and heparin. Schultz-Cherry *et al.* (1994) found that a recombinant fragment of thrombospondin-1 containing only the three TSRs fused to glutathione-S-transferase was able to mimic whole thrombospondin in activating TGF-β. Thus this region may also bind TGF-β.

In summary, the TSRs of thrombospondin have been implicated in a variety of thrombospondin functions. However, a reliance on the use of peptides in an attempt to define motifs within the TSR region which are important has led to conflicting results of uncertain significance. The production of recombinant forms of thrombospondin, and the realisation that different cell types express different complements of thrombospondin receptors (Adams and Lawler, 1993b), should allow a better definition of the important amino acid residues in future.

1.13 Proteins of the apicomplexan protozoa

A number of proteins from apicomplexan protozoan parasites contain thrombospondin type I repeats. The similarity between the TSRs of thrombospondin and a conserved region ("region II") in the circumsporozoite (CS) proteins of the malarial parasites *Plasmodium knowlesi* (Ozaki *et al.*, 1983) and *P. falciparum* (Dame *et al.*, 1984) was first noted by Lawler and Hynes (1986). Subsequently, TSRs have also been found in the thrombospondin-related anonymous protein (TRAP or SSP2) of *P. falciparum* (Robson *et al.*, 1988; Rogers *et al.*, 1992a) and *P. yoelii* (Hedstrom *et al.*, 1990; Rogers *et al.*, 1992b), in the P100 proteins of *Eimeria tenella* and *E. maxima*, coccidiosis parasites of fowl (Tomley *et al.*, 1991; Pasamontes *et al.*, 1993) and in thrombospondin-related adhesion proteins (TRAP-C) of *Cryptosporidium parvum*, a parasite of the intestine (Spano *et al.*, 1994). See figure 1.4.

1.13.1 The circumsporozoite protein

The CS protein of *Plasmodium spp.* is the major surface protein of the liver-infecting sporozoite stage of the malarial parasite. Cloning and sequencing of the CS protein gene from a wide variety of species has confirmed that the region of homology to properdin and thrombospondin (region II) is highly conserved. This region is also less polymorphic than the rest of the CS protein. However, while workers in this field concentrate on this relatively short region (corresponding to the N-terminal half of a TSR), it is clear from multiple sequence alignments that the CS protein contains a complete TSR (see chapter 3). An outline of the CS protein structure is included in figure 1.4.

Region II within the TSR of the CS protein has been strongly implicated in the invasion of liver cells by malarial sporozoites. The first evidence that this region was important came from the work of Rich *et al.* (1990). These workers made a series of peptides corresponding to the sequence of region II. Peptides containing the highly conserved VTCG and Rx(R/K) motifs (EWTPCS**VTCG**VGV**R**V**R** and **VTCG**VGV**R**V**R**RR) were both found to support attachment of a variety of human haematopoietic cell lines when immobilized on plastic. Soluble peptides containing the VTCG motif were able to inhibit this binding (e.g. VTCGVGVRVR, 65% inhibition; VTCG, 45% inhibition, at the same dose). Recombinant *P. vivax* CS protein fragments containing region II were also partially inhibitory, but a fragment lacking the TSR was inactive.

Since region II was similar to motifs within properdin and thrombospondin which had been implicated in binding to sulphated glycoconjugates (Holt *et al.*, 1989, 1990; Prater *et al.*, 1991), Pancake *et al.* (1992) tested the ability of CS protein to bind to such compounds. Recombinant soluble CS protein of *P. yoelii* was able to bind to sulphated polysaccharides bound to Sepharose (dextran sulphate 500 kDa ≥ heparin > fucoidan > dextran sulphate 5 kDa >> chondroitin sulphate A or C). The same sulphated sugars inhibited CS protein binding to hepatocytes, and the infectivity of *P. bergei* sporozoites *in vitro* and *in vivo*. Whole *P. bergei* sporozoites were found to bind sulphatide, but not cholesterol-3-sulphate, GM_1, or phosphatidylserine immobilized on glass plates. Compared with the binding to normal Chinese Hamster Ovary (CHO) cells, the binding of CS protein to CHO cells deficient in heparan sulphate synthesis was much reduced. Together these findings suggested that the CS protein could be vital for the infection of host cells through binding cell surface glycosaminoglycans. Cerami *et al.* (1992a) obtained very similar results using recombinant fragments of *P. vivax* and *P. falciparum* CS proteins, and were able to narrow down the important sequence to region II. CS fragments containing region II were able to bind sulphatide and, to a lesser extent, cholesterol-3-sulphate on microtitre plates. CS fragments truncated before the TSR were inactive. The binding could be inhibited by a similar set of sulphated

glycosaminoglycans to those found by Pancake *et al.* Reduction and alkylation of the CS fragment was found to prevent binding. It was proposed that a disulphide bond within the CSVTCG motif was important. However, these cysteines are unlikely to be linked in the intact TSR (see chapter 3), and the requirement for non-reduced cysteines has since been attributed to the need for disulphide linked protein or peptide aggregates for sufficient binding avidity (see below).

Nussenzweig and co-workers have gone on to provide strong evidence for the importance of the TSR of CS protein in infection of the liver by malarial parasites. Immunohistochemical staining of sections of rat organs incubated with recombinant soluble CS protein from *P. falciparum* showed staining on the microvilli of hepatocytes in the Space of Disse of the liver. Recombinant CS protein lacking the central repeating region (see figure 1.4) bound to hepatocytes on sections or HepG2 cells, and a construct from which the TSR was removed was inactive (Cerami *et al.*, 1992b). Peptides of region II from *P. falciparum* or *P. bergei* could inhibit the binding. The presence of the Rx(R/K) motif was important. If RxK was changed to RxE, the inhibitory activity of the peptide was retained but reduced. Peptides containing ExE or DxD at this position were inactive. While an intact CxVTCG motif enhanced the activity of the peptides, it was not essential. Peptides containing only the CSVTCG motif or the basic cluster without the cysteine residues were inactive. The reason for the requirement for cysteines was found to be the necessity of the presence of disulphide linked peptide aggregates in order to attain sufficient binding avidity (Sinnis *et al.*, 1994). Cerami *et al.* (1994) showed that aggregates of recombinant CS proteins were also required for activity. When multimers of soluble ^{125}I-labelled recombinant CS protein from *P. falciparum* were injected into mice, the radiolabel was rapidly concentrated on hepatocyte membranes but not on endothelial or Kupffer cells or in other organs. Monomers of CS protein, or multimers of CS protein lacking the TSR were not specifically taken up by the liver. On the sporozoite surface, CS protein is naturally polymerized, and thus capable of avid binding to hepatocyte receptors.

Frevert *et al.* (1993) purified and partially characterized the CS binding receptors of HepG2 cells. Preliminary studies suggested that the receptors might be sulphated proteoglycans. Heparinitase (but not chondroitinase) treatment of liver sections or HepG2 cells prevented CS protein binding. HepG2 cells were biosynthetically labelled with $^{35}SO_4$, and surface proteins released by mild trypsin treatment. Immunoprecipitation of added soluble recombinant CS protein led to the co-precipitation of high molecular mass material that could be degraded with heparinitase. After further purification, amino acid and sugar analysis revealed a high content of glucosamine, serine and glycine. The CS binding material, although not completely characterized appears to be heparan sulphate proteoglycan (HSPG). Thus, it seems that malarial sporozoites

may gain entry to the host *via* the binding of the TSR module of surface CS protein to HSPG on the hepatocyte cell surface. Since the CSVTCG motif and basic cluster is found in host proteins, it may be an advantage for the malarial parasite to utilize this region for invasion, while avoiding an adaptive immune response. Indeed, Müller *et al.* (1993) found that it was difficult to raise antibodies against a peptide containing the CSVTCG and RxR motifs.

1.13.2 The thrombospondin-related anonymous protein and related molecules

The TRAP/SSP2 protein of *P. falciparum* contains a von Willebrand A domain (VWA, also found in Factor B and C2) and a single TSR (see figure 1.4). TRAP/SSP2 was first found to be expressed in the erythrocytic stage of the malarial life cycle. Thus a possible role for this protein in the well known cytoadherence of malaria-infected erythrocytes was proposed (Robson *et al.*, 1988). Interestingly, the thrombospondin receptor, CD36, is also known to be a cytoadherence receptor for Plasmodium-infected red blood cells in the absence of thrombospondin (for review see Berendt *et al.*, 1990). However, although the TSRs of thrombospondin are thought to contain the CD36 binding site (see above), there is no direct evidence for the binding of TSR-containing parasite-derived erythrocyte surface proteins to CD36.

Subsequently, TRAP/SSP2 of *P. falciparum* and *P. yoelii* has been found on the surface and in the micronemes[7] of sporozoites (Cowan *et al.*, 1992; Rogers *et al.*, 1992a). The related p100 protein of *Eimeria spp.*, which also contains a VWA domain, but has six TSRs[8], is also a major sporozoite microneme protein. The TRAP protein of *P. falciparum* has, like the CS protein, been implicated in hepatocyte binding *via* sulphated polysaccharide receptors. Müller *et al.* (1993) found that only recombinant TRAP constructs containing the WSPCSVTCGKGTRSRKR motif were able to bind to sulphatide and the surface of HepG2 cells. The interaction could be inhibited by sulphated glycoconjugates such as suramin (an anti-protozoan drug), pentosan polysulphate, and dextran sulphate. Suramin and anti-TRAP antibodies prevented the invasion of HepG2 cells by *P. falciparum* sporozoites *in vitro*. It was hypothesized that the apparent requirement for CS protein and TRAP for sporozoite infectivity may, assuming the two proteins have slightly different binding specificities, increase the specificity of sporozoite binding to liver cells *in vivo*. However, since TRAP is expressed at a low level on the sporozoite membrane, this has been questioned (Cerami *et al.*, 1994). It may be that TRAP is released from the micronemes during the invasion process

---

[7] the microneme is an intracellular apical organelle of apicomplexa, thought to be involved in trafficking or processing of cell surface receptors.

[8] although only five full TSRs were identified by Tomley *et al.* (1991), the "partial" sixth repeat also qualifies as a TSR (see chapter 3).

or at a different stage during the malarial life cycle. It should be noted that the basic cluster, but not the VTCG motif, is conserved in the *P. yoelii* homologue of TRAP (see chapter 3).

Two other partial gene sequences in the GenBank database from the apicomplexan parasite, *Cryptosporidium parvum*, contain TSR sequences (Spano *et al.*, 1994). TRAP C-1 is reported to be a micronemal adhesive protein, and the partial sequence contains three TSRs. Another partial sequence, TRAP C-2, contains a single TSR (see chapter 3).

1.14 Proteins involved in neuronal cell migration

Two proteins involved in neuronal cell guidance which contain TSRs have recently been characterized, and are described below.

1.14.1 F-spondin

F-spondin is produced by a gene expressed at high levels in the floor plate of the neural tube. The floor plate is thought to produce signals important for the control of cell identity and migration within the neural tube. F-spondin contains an N-terminal region of no known homology and six TSR repeats (with a small, highly charged, interruption of 30 amino acids between TSR5 and TSR6 which is not highly conserved between species) (see figure 1.4). Rat and *Xenopus* F-spondin show an overall sequence identity of 84% (Klar *et al.*, 1992; Ruiz I Altaba *et al.*, 1993). Expression of c-myc tagged F-spondin in COS cells revealed that F-spondin is a secreted protein, but that it bound to the cell surface. The expressed protein was also found to promote adhesion and outgrowth of dorsal root ganglion of dorsal spinal cord cells. This activity was inhibited by heparin, dextran sulphate and chondroitin sulphate, while cell adhesion to fibronectin was not so inhibited (Klar *et al.*, 1992). It was supposed that the TSR modules might be important by analogy with properdin, thrombospondin and the malarial proteins discussed above. Glycosaminoglycans are known to be important during neurite outgrowth (Fernaud-Espinosa *et al.*, 1994).

1.14.2 Unc-5

The Unc-5 gene is required for guiding dorsal migrating cells and axons along the body wall of *C. elegans*. The cloning and sequencing of the unc-5 gene revealed a predicted transmembrane protein with two membrane distal immunoglobulin domains, two membrane proximal TSRs, and a large cytoplasmic domain containing an SH3-like module

(see figure 1.4). Unc-5 lacks the VTCG motif in both TSRs, and the first TSR contains a fifteen amino acid insertion not found in other TSRs (see chapter 3). It is thought that Unc-5 may act as a navigational receptor expressed in motile cells and pioneering axons (Leung-Hagesteijn *et al.*, 1992). A probable receptor for Unc-5, Unc-6, has also been characterized. Unc-6 is required for dorsal and ventral cell and axon migration, and has strong homology to laminin, suggesting that it may be a basal lamina component. Intriguingly, Unc-6 also contains a C-terminal region ("C" domain) with some homology to C3, C4 and C5 of the complement system. The significance of this homology was questioned in the original report (Ishii *et al.*, 1992). However, several lines of evidence suggest that this region does constitute a new "module". Firstly, the sequence is also conserved in two recently cloned cDNAs from chicken which are homologous to Unc-6 (netrins 1 and 2 (Serafini *et al.*, 1994)). Also, in Unc-6 and the netrins, this region is bordered at the N-terminal end by a module found in laminin (V module) and at the C-terminus by the end of the polypeptide chain. Finally, precisely this C module is absent from the C-terminus of α2-macroglobulin when compared to C3, C4 and C5. The C module of C3 (residues 1480-1643) is found at the C-terminus of the α chain. This is on the same 17 kDa fragment of C3b which has been implicated in properdin binding and is close to the peptide (1402-1435) which has been shown to bind properdin (Daoudaki *et al.*, 1988). It is interesting to speculate that TSR:C domain interactions may be important in Unc-5:Unc-6 and in properdin:C3b binding.

1.15 Growth regulators related to connective tissue growth factor (CCN family)

A number of related proteins that comprise a new family of growth regulating molecules have recently been characterized. The presence of sequence similar to the thrombospondin type I repeat in these proteins was first noted by Brunner *et al.* (1991) and the mosaic modular structure was completely described later by Bork (1993) (see figure 1.4). In the cases where the gene structure is known, each module is encoded by a single exon (see chapter 2, section 2.5.2). The first member of the family described was CEF-10, an immediate early gene induced in chicken embryo fibroblasts by $pp60^{v\text{-}src}$ or serum growth factors (Simmons *et al.*, 1989). The mouse homologue, Cyr61 (O'Brien *et al.*, 1990) or βIG-M1 (Brunner *et al.*, 1991) is upregulated in mouse fibroblasts by PDGF, FGF or TGF-β1 and is expressed mainly in the lung. Cyr61 was shown to interact with the extracellular matrix, and fibroblast cell surfaces. Furthermore, Cyr61 was found to bind strongly to heparin (Yang and Lau, 1991). Although the presence of the TSR module was not recognized at the time, it is now clear that CEF-10/Cyr61/βIG-M1 is another example of a TSR-containing protein with affinity for sulphated polysaccharides. However, the WSxWS motif, the VTCG motif and the RxR motif are all poorly conserved

in this family of proteins. A cluster of basic amino acids is found towards the C-terminus of these TSRs (see chapter 3).

A second member of the family was identified as another serum stimulated gene of mouse fibroblasts and has been named βIG-M2 (Brunner *et al.*, 1991) or Fisp-12 (Ryseck *et al.*, 1991). Fisp-12 is a secreted protein also expressed predominantly in the lung. The human homologue of Fisp-12, connective tissue growth factor, CTGF (Bradham *et al.*, 1991) was purified as a protein related by immunoreactivity and activity to PDGF. CTGF is secreted by human umbilical vein endothelial cells in culture and is a chemoattractant and mitogen for fibroblasts. A third member of the family, Nov, was found to be overexpressed in myeloblastosis-associated virus type I induced nephroblastomas of chickens (Joliot *et al.*, 1992). Expression of a truncated form of the molecule was found to transform chicken embryo fibroblasts. The normal form is expressed in embryonic kidney, brain, and heart and in adult lung and brain. The human gene for Nov has been mapped, and is distinct from that of CTGF (Matinerie *et al.*, 1992). The possible interaction of the CTGF/Fisp-12 and c-Nov/h-Nov proteins with sulphated polysaccharides has not been examined.

1.16 Terminal components : C6, C7, C8 and C9

The components of the MAC; C6, C7 C8 (α and β chains) and C9 are a group of related modular proteins (Hobart *et al.*, 1993; Reid and Day, 1989) (see figure 1.4). All contain a cysteine-poor hydrophobic region that is related to a similar region in perforin. Since perforin is the pore-forming granule protein of cytotoxic T lymphocytes, it is thought that this portion of these proteins is involved in insertion into target membranes. C7, C8α, and C8β all contain two TSRs - one at the N- and one at the C-terminus (Patthy, 1988). While mammalian C9 lacks a C-terminal TSR, such a repeat is present in the C9 molecule of trout (Tomlinson *et al.*, 1993). C6 has an additional TSR at the N-terminus. The function of the TSRs in these molecules is unknown, although they are likely to be involved in the protein-protein interactions required to form the membrane attack complex. The terminal components also contain LDL receptor repeats and EGF repeats. In addition, C6 and C7 contain two CCPs each, and two copies of a repeat similar to a region of Factor I.

1.17 Properdin biosynthesis

Northern blot analysis of RNA from various tissues in the mouse using a mouse cDNA probe indicate that properdin mRNA is synthesized by activated macrophages, in the spleen, and at a low level in the lung. No message could be detected in the liver. The spleen is probably the major site of plasma properdin synthesis *in vivo*.

The ability of macrophages to synthesize properdin has been confirmed by studies showing the production of C3bBb stabilizing activity by human peripheral blood monocytes (Whaley, 1980), the secretion of properdin by U937 cells, a human monocytic cell line, (Minta and Pambrun, 1983; Minta, 1987; Goundis and Reid, 1987), the isolation of properdin cDNA clones from mouse macrophage and phorbol ester stimulated U937 cells (Goundis and Reid, 1988; Nolan *et al.*, 1991), and the detection of properdin mRNA in U937, Mono Mac 6 and freshly prepared human monocytes (Schwaeble *et al.*, 1994). The expression of properdin mRNA in U937 and Mono Mac 6 cells can be enhanced by phorbol esters, LPS or cytokine treatment (Nolan *et al.*, 1991; Schwaeble *et al.*, 1994). Properdin is also synthesized in small quantities by HL-60 cells after DMSO-stimulation (Farries and Atkinson, 1989). This has led to the suggestion that properdin might also be synthesized by neutrophils *in vivo*. While Maves and Weiler (1992) found no evidence for properdin mRNA in human neutrophils, and *de novo* synthesis of properdin in human peripheral neutrophils cannot be detected (Baggiolni *et al.*, personal communication), properdin is clearly detectable in the secondary granules of neutrophils by immunoblotting (Dr Paul Eggleton, personal communication), and is released from the granules on fMLP treatment (Baggiolni *et al.*, personal communication). Properdin mRNA has also been detected in human brain astrocytes by reverse transcription-PCR (Avery *et al.*, 1993) and Schwaeble *et al.* (1993) find properdin mRNA in peripheral blood $CD4^+$ and $CD8^+$ T cells, and confirm that T cell lines secrete functional properdin. Thus, peripheral monocytes, macrophages, neutrophils and possibly T cells, may provide another important source of properdin *in vivo*, and secretion of properdin by such cells could be important in the local upregulation of complement activation at sites of infection. Interestingly, neutrophil granules are known to be rich in sulphated glycoconjugates, for which properdin has demonstrable affinity (see section 1.11). The possible consequences of this remain to be explored.

Although many complement components are synthesized in the liver, properdin message was not detected in mouse liver or human HepG2 cells by Northern blot analysis (Goundis and Reid, 1988; Schwaeble *et al.*, 1994). However, properdin secretion by HepG2 cells has been reported (Minta, 1987), and properdin mRNA has been detected in HepG2 cells by reverse transcription-PCR (Maves and Weiler, 1993; Avery *et al.*, 1993). Thus synthesis of properdin may occur at a low level in the liver.

Farries and Atkinson (1989) carried out a more detailed study of properdin

biosynthesis in DMSO-stimulated HL-60 cells. An intracellular form of properdin was detected that was slightly smaller than the extracellular form, and was susceptible to endoglycosidase H digestion, suggesting that it represented an intermediate with high mannose N-linked carbohydrate before processing to more complex sugars. Growth of the cells in the presence of tunicamycin, an inhibitor of N-linked glycosylation (see chapter 4), or endoglycosidase F treatment yielded properdin 2-5 kDa smaller than the normal form, confirming the presence of at least one N-linked oligosaccharide. The intracellular form of properdin was capable of binding C3iBb-Sepharose and cross-linking with DST indicated that it was oligomeric. It was proposed that oligomerization is an early intracellular event, and perhaps carefully controlled to prevent anomalous aggregation.

1.18 Properdin deficiency

Inherited deficiencies of various complement components have provided insight into the importance of the different pathways of activation *in vivo*. For example, the lack of the classical pathway components C1, C2, or C4 leads to immune complex disorders such as systemic lupus erythematosus in up to 66% of individuals (Figueroa *et al.*, 1993). This confirms the importance of the classical pathway in dealing with immune complexes (see section 1.1.1). Classical pathway and lectin pathway (*i.e.* MBP) deficiencies are both associated with increased susceptibility to bacterial infection (Figueroa and Densen, 1991; Super *et al.*, 1989). Deficiencies of the late complement components (the constituents of the membrane attack complex) show a marked association with neisserial infections (particularly with *Neisseria meningitidis* which causes bacterial meningitis). Around fifty percent of C5, C6, C7, or C8 deficient individuals suffer recurrent neisserial infection, although the course of disease is often milder than found in the general population. This suggests that complement lysis is an important means of controlling such infection *in vivo*. But, this observation also indicates that bactericidal activity might contribute to pathogenesis in normal individuals, possibly through increased release of endotoxin from the lysed bacteria (Figueroa *et al.*, 1993), and that phagocytic clearance of *Neisseria* may also be important (Söderström *et al.*, 1991).

Around seventy cases of inherited properdin deficiency have been reported. Of these, around fifty percent have experienced meningococcal disease due to *Neisseria meningitidis* infection. In contrast to the deficiencies of late complement components mentioned above, properdin deficient individuals have been reported to suffer fulminant , non-recurrent infections, frequently leading to death (Sjöholm *et al.*, 1982; Densen *et al.*, 1987; Nielsen and Koch, 1987; Editorial, 1988). The basis of this difference is thought to be inefficient amplification of C3b deposition on the surface of bacteria in properdin deficient individuals due to a failure to form a stable C3bBb convertase. Such reduced

opsonization will result in decreased phagocytic clearance of *Neisseria* in addition to a reduction in complement lysis, and thus lead to more severe disease. Indeed, granulocyte-mediated opsonization and chemotactic activities against *N. meningitidis* are impaired in properdin deficient serum (Söderström *et al.*, 1991). Properdin deficient individuals can be immunized against neisserial infections since they retain a functional classical pathway (Densen *et al.*, 1987). The poor prognosis for properdin deficient individuals on infection with Neisseria has recently been questioned. Schlesinger *et al.* (1993) have reported three families of Tunisian Jews with properdin deficiency. Only mild meningococcal disease was found in these cases. A true picture of the clinical consequences of properdin deficiency may require analysis of a larger number of patients from a variety of ethnic groups.

The need for techniques for the detection of properdin deficiency carriers and for prenatal diagnosis is clear. Three types of properdin deficiency have been described on the basis of immunochemical and functional analyses. Type I deficiency was the first reported, and is characterized by protein levels below that detectable by ELISA or haemolytic assay (Sjöholm *et al.*, 1982; Densen *et al.*, 1987). Type II deficients have serum levels of properdin from 1 to 10% of normal (Nielsen and Koch, 1987; Sjöholm *et al.*, 1988a). In one case, the properdin present in such an individual was found to be functionally active (Sjöholm *et al.*, 1988a), although this may vary between pedigrees. In type III properdin deficiency, properdin is present when assayed immunochemically, but is dysfunctional (Sjöholm *et al.*, 1988b). In all cases, deficiency is found only in males, and is inherited in an X-linked manner (Goonewarda *et al.*, 1988). Thus, the diagnosis of properdin deficiency by immunochemical or functional means is complex and the firm detection of female carriers is impossible. The characterization of the human properdin gene (Nolan *et al.*, 1992) has led to the identification of a polymorphic dinucleotide "CA" repeat less than 15 kb downstream of the gene. This microsatellite marker can now be used for the unambiguous carrier detection of properdin deficiency (Kölble *et al.*, 1993). In addition, the availability of properdin DNA probes has allowed the properdin structural locus to be mapped to Xp11.3-Xp11.23 in humans (Goundis *et al.*, 1989; Coleman *et al.*, 1991) and to the syngenic region in mice (Evans *et al.*, 1990; Laval *et al.*, 1991).

Restriction and Southern blot analysis of genomic DNA from a Tunisian Jewish family with type I deficiency (Schlesinger *et al.*, 1990) failed to identify any gross structural changes in the properdin gene (Nolan, 1991). However, recently the defect underlying one case of type I deficiency has been identified as a point mutation causing a premature stop codon in the fifth exon of the gene (Fredrikson *et al.*, 1994).

1.19 Aims of the project

The aim of the project was to localize the regions of properdin molecule which are important for its activity. The best characterized role of properdin is to stabilize the C3bBb complex. Crude models of this complex can be constructed on the basis of electron microscopy and solution scattering studies (Smith *et al.*, 1982, 1984b, Sim and Perkins, 1990) (see figure 1.8) and a binding site for properdin on C3b has been suggested (see section 1.9.1). However, the regions of properdin which are important for stabilizing the complex and the configuration of C3bBbP are unknown.

Properdin has been reported to bind sulphated glycoconjugates, although the role of this activity *in vivo* is not known. Some of the TSRs in properdin contain amino acid motifs which have been implicated, by peptide studies, in cell and sulphated glycoconjugate binding in other proteins (see sections 1.12 and 1.13). However, whether these TSRs are required for the sulphatide binding activity of properdin is unknown.

Although it has been proposed that the N- and C-terminal regions of properdin are involved in forming intersubunit bonds, this has not been demonstrated directly, and the precise contact sites which form this unusually stable interaction are not known.

The modular nature of the properdin monomer suggested that removal of single TSRs, by protein engineering employing recombinant DNA techniques, would be a suitable initial approach for identifying important regions of the molecule. Although there was no direct experimental evidence that TSRs fold as independent units, this was considered likely in the light of the known structures of many similar modules (*e.g.* CCP and EGF-like repeats). TSRs are found next to a variety of different types of other module in different proteins, and in tandem arrays of different numbers (e.g. single TSRs are found in the terminal complement components and in the malarial adhesion proteins, TSR pairs in C6 and Unc-5, threes in thrombospondin, fives in F-spondin, and sixes in properdin and *Eimeria* proteins, see figure 1.2). These observations also suggest that TSRs are independently folded. The characterization of the human properdin gene revealed introns of the same phase at the junctions between exons encoding all of the TSR modules (see chapter 3). This added further support to the idea that the TSRs are structurally independent, since it has been proposed that such genes have evolved by shuffling of exons encoding discrete protein elements. Also, the knowledge of the intron/exon boundaries between the modules allowed the N- and C-termini of each properdin TSR to be defined, and the extent of the required deletions was thus clear. A similar approach has been used successfully to identify functional regions in other molecules. For example, the deletion of CCP modules from CR1 led to the identification of C3b and C4b binding sites (Klickstein *et al.*, 1988).

This thesis describes the completion of the characterization of the human properdin

gene and an analysis of the protein sequence of the TSRs of properdin and other proteins (chapter 3). The production and characterization of recombinant wild type human properdin is described in chapter 4, and the production of altered forms of properdin, including forms lacking single TSRs, is outlined in chapter 5. The ability of these proteins to stabilize the C3bBb complex, to bind directly to C3b, and to bind sulphatide is described in chapters 6, 7, and 8. The results are discussed as a whole in chapter 9.

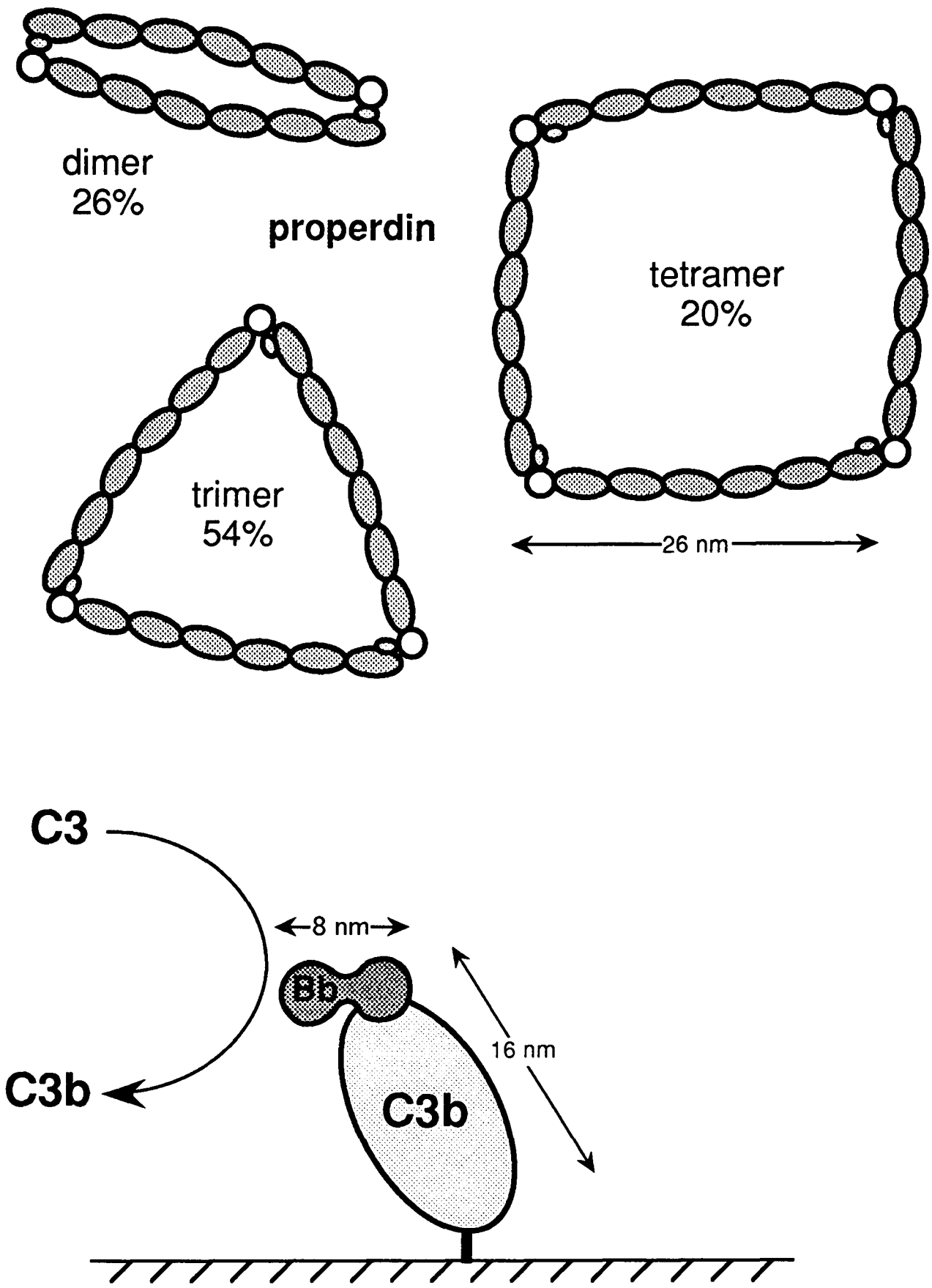


Figure 1.8
Models of the oligomers of properdin, and of the C3bBb complex. The proteins are shown roughly to scale, as judged by electron microscopy and solution scattering studies (Smith *et al.*, 1982, 1984a, 1984b, Sim and Perkins, 1990; Smith *et al.*, 1991). The manner in which properdin binds to the C3bBb complex is unknown. The sub-structure of each properdin monomer is discussed in more detail in chapter 3. The ratio of oligomeric forms of properdin on a weight basis is shown (Pangburn, 1989).

Chapter 2

Chapter 2
Materials and Methods

2.1 Materials

Chemicals were purchased from BDH Laboratory Supplies (Poole, UK), Sigma Chemical Co. (St Louis, USA) or Rose Chemicals Ltd (London, UK) unless otherwise stated.
Radiochemicals were purchased Amersham International plc (Amersham, UK).
DNA and RNA modifying enzymes were purchased from Amersham International plc (Amersham, UK), Promega Corporation (Madison, USA), and Boehringer-Mannheim GmbH (Lewes, UK).
Milli-Q purified water was used throughout.

Denhardt's reagent (x50): 1% (w/v) ficoll 400/1% (w/v) polyvinylpyrrolidine/1% (w/v) BSA
Phosphate-buffered saline (PBS): 10 mM Na_2HPO_4/1.8 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl, pH 7.4
Standard saline citrate (1x SSC): 150 mM NaCl/15 mM tri-sodium citrate, pH 7.0
Tris-borate-EDTA buffer (1x TBE) was 90 mM Tris/90 mM boric acid/2.5 mM EDTA, pH 8.3
Tris-buffered saline (TBS): 20 mM Tris(hydroxymethyl)aminomethane/150 mM NaCl, pH 7.4
Tris-EDTA buffer (TE): 10 mM Tris(hydroxymethyl)aminomethane/1 mM EDTA, pH 7.4

A monoclonal anti-human properdin IgG antibody (HYB 3-3, mouse IgG1, kappa) was kindly provided by Dr C. Koch, Statens Seruminstitut, Copenhagen, Denmark. An isotype matched control mouse plasmacytoma antibody (MOPC-21) was obtained from Sigma Chemical Co. (St Louis, USA).
E. coli for routine use: MC1061 *hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rosL thi.*
E. coli dam$^-$ strain: JM110 *dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔM15].*
Rabbit and sheep erythrocytes were purchased from TCS Biologicals Ltd, Buckingham, UK.

2.2 Nucleic acid methods

2.2.1 Ethanol precipitation of DNA

Samples containing DNA were made 0.3 M with respect to sodium acetate (by adding $^1/_{10}$ volume of 3 M sodium acetate, pH 5.2). After adding 2 volumes of ethanol, samples were mixed and incubated at -20° for approximately 15 min. After centrifugation at 13000 g for 10 min, the pellet was washed with 70% (v/v) ethanol, and resuspended in an appropriate volume of water.

2.2.2 Removal of protein by organic extraction

Before use, phenol was equilibrated with 1 M Tris, pH 8.0 by repeated mixing, centrifugation and removal of the aqueous phase until the pH of the phenol reached greater than 7.5. Phenol was subsequently stored under 0.1 M Tris, pH 8.0. Isoamyl alcohol was added to chloroform at a ratio of 1:24.

Extractions were carried out by adding an equal volume of organic solvent to the sample, vortexing, and centrifugation at 13000 g for 5 min. The upper aqueous phase was then removed without disturbing the interface between phases.

2.2.3 Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems DNA Synthesiser 381A by Mr T. Gascoyne of the MRC Immunochemistry Unit. Oligonucleotides were removed from the columns using 2 ml of concentrated ammonia solution (30%) and the β-cyanoethyl protecting groups removed by incubation at 55°C for approximately 15 h, before ethanol precipitation and resuspension in 600 µl water. Primers used were as shown below.

Primer name	Sequence (5' to 3')
J89/10	CATGGAACAACGGACGTGCAATC
K89/11	GACGGACATCGATGTGCCGG
L89/01	CAGCGCCTCTGCACACCCTT
C90/39	TCCCAGGTGCCACCTTTCCAG
C90/41	GTGGATGGGGAGTGGGACTCGTGGGGGGAG
E91/04	CTAGGAAGTTGCTGCACGCT
A92/06	CCCCCCAGATCTTAGAGTTCCTCTTCCTCAGG
A92/07	CCCCCCTCTAGATGATCACAGAGGGAGCGCAGGCCCCTCGATTG
B92/18	CCCCCCTCTAGAGCCGCCACCATGATCACAGAGGGAGCGC
A93/14	AGGGCAGGGCACAGCTGTGTTGCAGAT
A93/15	GGGGCAGACCTGCTGGGTGTCACAGGCCTC
A93/16	TTGAAAGGATCATGGTCAGAGTG
A93/17	GTGGCTGGGGGCTGGGGGCCTTGGGGCCCT
D93/10	CCCCCCAGATCTAGGGGCAGTGCTGGATGCTGTAGCA
trunc	CCCCCCAGATCTACTCACACCGTGGCAGCGGTC
TSR3 3'	TGGGCAGGGTGGCAGGCCGGT

2.2.4 Phosphorylation of oligonucleotides

If PCR products were to be cloned as blunt-ended fragments, the primers used were first phosphorylated as follows. Approximately 2.5 nmol of oligonucleotide was incubated at 37°C for 30 min with 10 U T4 polynucleotide kinase (Amersham) in 400 µM ATP/2 mM spermidine/10 mM $MgCl_2$/10 mM dithiothreitol/50 mM Tris-HCl, pH 7.4 (125 µl total volume). Phenol and chloroform extraction was then carried out to remove the kinase, and the resulting 20 µM primer solution used directly in PCR reactions.

2.2.5 Mini plasmid preparations

Colonies were incubated in 5 ml aliquots of 2x TY broth (80 µg ml^{-1} ampicillin) at 37°C overnight, with shaking. After centrifugation at 1400 g for 10 min, pellets were resuspended in 150 µl 50 mM glucose/10 mM EDTA/25 mM Tris-HCl, pH 8.0, incubated at room temperature for 10 min, then 400 µl 0.2 M NaOH/1% (w/v) SDS was added and the solution mixed. Incubation on ice for 5 min was followed by the addition of 300 µl 3 M NaOAc,pH 4.8, a further 10 min incubation on ice and centrifugation at 13000 g, 10 min.

After precipitation of the plasmid DNA in the supernatant with an equal volume of propan-2-ol and immediate centrifugation at 13000 g, the pellet was resuspended in 0.3 M NaOAc and extracted once with an equal volume of phenol and once with chloroform:isoamyl alcohol (24:1). After a second precipitation with propan-2-ol, the pellet was washed with 70% (v/v) ethanol, resuspended in water, and treated with RNase (1 ng boiled DNase-free pancreatic RNase A (Sigma); 37°C, 30 min). Alternatively, Magic Minipreps (Promega Corporation) were used, following the manufacturer's instructions.

2.2.6 Maxi-preparation of plasmid DNA

A colony of the required clone was incubated in a 5 ml aliquot of 2x TY broth (80 μg ml^{-1} ampicillin) at 37°C for a few hours, with shaking. One litre of L-broth with 100 μg ml^{-1} ampicillin was then inoculated with this 5 ml and incubated at 37°C overnight, shaking. After chilling at 4°C for 30 min, the bacteria were pelleted by centrifugation at 3000 g for 20 min. The pellet was resuspended in 40 ml of SET buffer (150 mM NaCl/5 mM EDTA/50 mM Tris-HCl, pH 8.0) and 80 ml of cold 0.2 M NaOH/1% (w/v) SDS. After incubation on ice for 5 min, 40 ml 3 M KOAc, pH 4.8 was added and the solution mixed by shaking. Cell debris was removed by centrifugation at 4000 g for 20 min and filtration of the supernatant through muslin. Nucleic acid was precipitated by adding an equal volume of propan-2-ol, leaving at -70°C for 30 min, and then centrifuging at 6000 g for 20 min. The pellet was washed with 70% (v/v) ethanol, dried in air, and resuspended in 6 ml of SET buffer (as above) containing 7 M CsCl and 0.017% (w/v) ethidium bromide. The solution was transferred to a 6 x 76 mm Beckman QuickSeal tube. The tube was then filled up with liquid paraffin, heat sealed and centrifuged at 55 000 rpm, 16 h, 18°C and then at 45 000 rpm, 1 h (no brake) in a Beckman L8-70M ultracentrifuge using a Ti70 rotor. This density gradient centrifugation separated the closed circular form of the plasmid from any RNA, nicked or linear DNA, and protein in the preparation. The plasmid was removed from the gradient using a syringe and hypodermic needle (Sambrook *et al.*, 1989). Ethidium bromide was then removed from the solution by repeated butan-1-ol extractions. After ethanol precipitation the pellet was washed in 70% ethanol and resuspended in 100-200 μl of water.

Alternatively, Qiagen maxi-prep column kits were used according to the manufacturer's instructions, with subsequent phenol and chloroform extractions.

2.2.7 Estimation of nucleic acid concentration

The optical density of an appropriately diluted solution was measured at 260 nm in a CECIL series 2 CE 292 Digital Ultraviolet Spectrophotometer. The nucleic acid

concentration was calculated assuming that an A_{260nm} of 1 corresponds to 50 mg ml^{-1} for double stranded DNA, 40 mg ml^{-1} for RNA or 34 mg ml^{-1} for oligonucleotides.

2.2.8 Denaturing polyacrylamide gel electrophoresis of DNA

Polyacrylamide/urea gels were 7 M urea/6 or 12% (w/v) acrylamide:bisacrylamide ("Accugel", National Diagnostics, Georgia, USA)/0.06% (w/v) ammonium persulphate in 1x TBE. N,N,N',N'-tetramethylethylenediamine (TEMED) was added to 0.1% (v/v) to initiate cross-linking. The gel running buffer was 1x TBE.

2.2.9 Agarose gel electrophoresis of DNA

Agarose at 0.6 to 3% (w/v) was dissolved in 1x TBE with 1 µg ml^{-1} ethidium bromide. The gel running buffer was 1x TBE. The gel loading buffer was $^1/_{10}$ sample volume of 0.25% (w/v) bromophenol blue/0.25% (w/v) xylene cyanol FF/30% (v/v) glycerol. For DNA fragment purification, NuSieve GTG or SeaPlaque GTG agarose (FMC BioProducts, ME, USA) was used.

2.2.10 Capillary transfer of DNA from agarose gels to nylon membranes under alkaline conditions (Reed and Mann, 1985)

Initially, agarose gels for DNA transfer were soaked in 0.25 M HCl for 15 minutes before rinsing in distilled water and then soaking twice in 0.5 M NaOH/1.5 M NaCl for 15 minutes. Two sponges (thin and dense) with two pieces of 3 MM Whatman paper on top were placed in a plastic tray and soaked in 0 25 M NaOH/1.5 M NaCl. The gel was positioned on top of the 3 MM Whatman paper and surrounded with Saran Wrap (Dow Chemical Company) so that the only route for the transfer buffer was through the gel. A piece of Hybond N (Amersham Life Science) nylon membrane was cut to size and laid on top of the gel followed by two pieces of 3 MM Whatman paper soaked in water and then a stack of paper towels. Finally, a glass plate and a small weight (≈300 g) were positioned on top of the paper towels. The transfer was carried out for approximately 6 h. Following transfer the membranes were dried in air.

2.2.11 Radiolabelling of oligonucleotides

End-labelling of 40 ng of oligonucleotide was carried out using 50 µCi [γ^{32}P]-dATP and 5 U T4 polynucleotide kinase (Amersham) in 1.7 mM spermidine/10 mM $MgCl_2$/10 mM dithiothreitol/50 mM Tris-HCl, pH 7.4 (total volume: 30 µl). The reaction was incubated at 37°C for 30 min.

2.2.12 Hybridization of radiolabelled oligonucleotide probes

Membranes were hybridised with 5 ml oligonucleotide hybridization buffer (5x Denhardt's reagent (Sambrook *et al.*, 1989), 6x SSC/0.1% (w/v) sodium pyrophosphate/0.1% (w/v) SDS/0.1% (w/v) boiled sheared salmon sperm DNA (Sambrook *et al.*, 1989)) with approximately 50 ng of the appropriate radiolabelled oligonucleotide probe (see section 2.2.11). Hybridizations were performed at 42°C overnight, in a rolling bottle incubator. The filters were washed twice at room temperature for 15 min and twice at 65°C for 15 min with 6x SSC/0.1% (w/v) SDS, shaking, before autoradiography.

2.2.13 Autoradiography

Kodak X-OMAT S film was exposed in radiographic cassettes. ^{35}S gels were exposed at room temperature. ^{32}P gels were exposed to pre-flashed film with intensifying screens (DuPont Cronex Lightening Plus) at -70°C. Films were developed using a Kodak X-OMAT ME-1 processor.

2.2.14 Isolation of DNA fragments from agarose gels (Heery *et al.*, 1990)

Gel slices containing the required fragment were excised and centrifuged at 6000 g for 15 min through a plug of siliconised glass wool. Any remaining agarose in the eluate was removed by phenol extraction before ethanol precipitation of the DNA and resuspension in water.

2.2.15 Restriction digestion

Restriction digests were carried out with 1-5 U enzyme per microgramme DNA in 1x restriction enzyme incubation buffer (A, B, L, M or H, from Boehringer-Mannheim) at 37°C for 30 min to 6 h.

2.2.16 Dephosphorylation of cleaved vector DNA

Vectors cleaved with only a single restriction enzyme were dephosphorylated before ligation reactions to prevent self-religation. Plasmid DNA (≈1 µg) was incubated at 37°C for 30 min with 1 U calf intestinal phosphatase (Boehringer-Mannheim) in 1x CIP buffer (Boehringer-Mannheim). The enzyme was then inactivated by heating to 65°C for 10 min.

2.2.17 Klenow fill reaction

To blunt-end PCR products for subsequent cloning, the Klenow fragment of DNA polymerase I was used as follows. In a total volume of 20 µl, 2 U Klenow fragment, the gel purified PCR product, and 0.25 mM dNTPs, 66 mM $MgCl_2$/0.5 M NaCl/10 mM dithiothreitol/66 mM Tris-HCl, pH 7.4 were incubated at 37°C for 30 min. After adding 80 µl of TE buffer, protein was removed by phenol and chloroform extractions.

2.2.18 Polymerase chain reaction

The polymerase chain reaction was carried out in a volume of 100 µl in 1x Taq buffer (Promega Corp.)/1 µM each primer/200 µM each of dATP, dCTP, dGTP and dTTP/2.5 U Taq ploymerase (Promega Corp.). The temperature cycle programs used varied according to the application, and are given in the appropriate sections.

2.2.19 Ligations

Various concentrations of insert were mixed (in excess) with 10-20 ng of cleaved vector DNA in ligase buffer (1 mM rATP (Boehringer-Mannheim)/20 mM dithiothreitol (Sigma)/10 mM $MgCl_2$/0.2 mg ml^{-1} BSA/5 mM Tris-HCl, pH 7.4). One to five units of T4 DNA ligase (Amersham) were added, followed by incubation at approximately 16°C for 5-16 h. Control ligations with cleaved vector and no insert were carried out.

2.2.20 Preparation of competent E. coli for transformations

A colony was picked from an overnight TYM-agar plate and incubated in 5 ml of 2x TY broth at 37°C for 2 h. These cells were then transferred to 100 ml of prewarmed TYM broth and incubated, with shaking, at 37°C until an A_{550} of 0.8-0.9 was reached (3-4 h). The cells were centrifuged at 2000 g, 10 min, the pellet resuspended in 40 ml TfbI (30 mM KOAc/50 mM $MnCl_2$/100 mM KCl/15% (v/v) glycerol, filter sterilised) and incubated on ice for 5 min. The cells were pelleted at 2000 g, 8 min, 4°C and resuspended in 4 ml TfbII (10 mM Na-MOPS/75 mM $CaCl_2$/10 mM KCl/15% glycerol, filter sterilised). Aliquots of 200 µl were stored at -70°C.

2.2.21 Transformation of E. coli

Competent *E. coli* (100 µl) were added to 10 µl of solution containing 10-20 ng plasmid DNA. The mixture was incubated on ice for 15 min and heat shocked at 37°C for 5 min before addition of 0.4 ml L-broth and incubation at 37°C for 45-60 min. Quantities

between 10 and 250 µl were plated out onto L-broth-agar plates containing 80 µg ml^{-1} ampicillin. Control transformations without plasmid and with uncut plasmid were usually included.

2.2.22 Screening of transformants

i) PCR amplification from whole cells.

Using a sterile pipette tip, each colony was picked into a 20-100 µl aliquot of L-broth-ampicillin. Then, in each case using the same tip, 1 µl samples of these stocks were transferred into aliquots of standard PCR mix (10 µl of 1x Taq buffer (Promega)/1 µM each primer/200 µM dNTPs). Primers were chosen which would yield bands revealing the presence of required inserts on an agarose gel. The following program was used: 20 cycles of 94°C, 1 min/55°C, 1 min/72°C, 1 min.

ii) Colony screening using radiolabelled oligonucleotide probes.

Transformants were plated on "master" Hybond-C filters (on L-broth-agar, 80 µg ml^{-1} ampicillin), and after allowing colonies to grow overnight at 37°C, replica filters were produced by pressing a second filter onto each master filter (and marking to allow subsequent alignment). Replicas were incubated on L-broth-agar (containing 80 µg ml^{-1} ampicillin) for 2-3 h at 37°C. The bacteria were lysed, and the DNA denatured, by treating the filters as follows: 5 min in 0.5 M NaOH, 5 min drying, 2 min in 1 M Tris-HCl pH 7.5, 2 min drying, 15 min in 0.5 M Tris-HCl/1.5 M NaCl, pH 7.5, 2 min drying. After wiping away cell debris, the filters were baked at 80°C for 2 h.

Hybridisation was carried out in 10 ml oligonucleotide hybridisation buffer (5x Denhardt's reagent (Sambrook *et al.*, 1989), 6x SSC/0.1% (w/v) SDS/0.1% (w/v) sheared salmon sperm DNA (Sambrook *et al.*, 1989)) with approximately 40 ng of the appropriate oligonucleotide probe (see section 2.2.11). Hybridisations were performed at 42°C overnight, shaking.

The filters were washed twice at room temperature for 15 min and twice at 65°C for 15 min with 6x SSC/0.1% (w/v) SDS, shaking. Positive colonies were identified using autoradiography and picked into overnight L-broth-ampicillin cultures.

2.2.23 Storage of Bacterial Strains

Glycerol stocks were prepared by resuspending pelleted cells from 1 ml of overnight bacterial culture in 500 µl Hogness buffer (3.6 mM K_2HPO_4/1.3 mM KH_2PO_4/2 mM sodium citrate/1 mM $MgSO_4$/4% (v/v) glycerol, in L-broth). The stocks were stored at either -20°C or -70 °C. Agar plates were stored at 4°C.

2.2.24 Double Stranded DNA Sequencing

This was performed using the Pharmacia T7 Sequencing Kit, according to the manufacturer's protocol, using approximately 2 μg of template DNA, 50 ng oligonucleotide primer and 10 μCi [α^{35}S]-dATP (Amersham). After heating at 80°C for 2 min and rapid cooling on ice, the fragments were separated on a 6% (w/v) polyacrylamide/7 M urea gels (as described in section 2.2.8) at 42 W for periods of 1.25 to 5 h. Gels were then washed in 10% (v/v) ethanoic acid, dried on Whatman 3M filter paper and visualised by autoradiography.

2.2.25 Isolation of total U937 cell RNA

Approximately $2x10^8$ U937 cells, which had been stimulated with 30 ng ml^{-1} PMA for three days, were washed with ice cold PBS (Oxoid) three times, recovering cells between washes by centrifuging at 1500 g for 10 min. The cells were then resuspended in 3 ml of freshly prepared 4 M guanidinium thiocyanate/1% (v/v) 2-mercaptoethanol. Three millilitres of 5.7 M CsCl/25 mM tri-sodium citrate/10 mM EDTA were added and mixed in by inversion. The suspension was layered onto a 3 ml cushion of 5.7 M CsCl/25 mM tri-sodium citrate/10 mM EDTA in a 16 x 76 mm Beckman QuickSeal tube. The tube was filled with liquid paraffin, heat sealed and centrifuged at 35 000 rpm for 16 h at 20°C in a Beckman L8-70M ultracentrifuge with a Ti70 rotor. Following centrifugation the top of the tube was removed using a hot pair of scissors and most of the supernatant removed using a sterile pipette. The remaining supernatant was tipped off and the RNA pellet washed twice with 70% (v/v) ethanol. After drying in air, the pellet was resuspended in 400 μl of 2.5 mM Tris-HCl/1 mM EDTA/0.2% (w/v) SDS/0.3 M sodium acetate, pH 6. After ethanol precipitation, the pellet was resuspended in 200 μl of the same buffer. The RNA was stored as a precipitate in ethanol at -20°C.

2.2.26 Agarose gel electrophoresis of RNA

Agarose was dissolved to 1% (w/v), by heating, in 1x MOPS buffer (20 mM 3-(N-morpholino)propane sulphonic acid/5 mM sodium acetate/1 mM EDTA, pH 7.0). After cooling, formaldehyde was added to 5% (v/v), before pouring the gel. The gel was prerun for 5 minutes in 1xMOPS buffer at 40 mA before loading. RNA samples were heated at 65°C for 15 min in 50% (v/v) deionized formamide/0.025% (w/v) bromophenol blue/0.025% (w/v) xylene cyanol FF/1x MOPS before cooling on ice and the addition of 1 μg ethidium bromide. The samples were then loaded and the electrophoresis carried out overnight at 40 mA.

2.2.27 Northern blot of RNA onto nitrocellulose membranes (Fourney *et al.*, 1988)

The gel was soaked for 10 minutes in 0.05 M NaOH/1x SSC and then twice in 10x SSC for 20 minutes. The transfer of RNA was essentially as described in section 2.2.10 for DNA transfer except that the transfer buffer in this case was 10x SSC and the membrane used was Hybond C Extra (Amersham Life Science), prewet in water and then 10x SSC before use. Transfer was carried out overnight. Following transfer the filter was baked at 80°C for 2 hours.

2.2.28 Radiolabelling of cDNA fragment for use as a probe

The Amersham Multiprime DNA labelling kit was used following the manufacturer's protocol. Both [α^{32}P]-dATP and [α^{32}P]-dCTP were incorporated to ensure high specific activity. The reaction was incubated at 37°C for 1 h or at room temperature for 5 h. Unincorporated nucleotides were removed using a Pharmacia Nick Column according to the manufacturer's recommendations.

2.2.29 Hybridisation with radiolabelled restriction fragments

Membranes were pre-soaked in 2x SSC for 5-10 min. Pre-hybridisation was carried out in a Hybaid bottle with 5 ml of hybridization buffer (50% (v/v) deionized formamide/50 mM Tris-HCl, pH 7.4/1 M NaCl/0.1% (w/v) sodium pyrophosphate /5x Denhardt's reagent (Sambrook *et al.*, 1989)/10% (w/v) dextran sulphate/ 0.1% (w/v) SDS/0.1% (w/v) boiled sheared salmon sperm DNA (Sambrook *et al.*, 1989) for 2 h at 42°C in a rolling bottle incubator. Probes were boiled for 5 min, and cooled rapidly on ice before addition to the hybridization buffer. Hybridization was allowed to proceed overnight. Subsequently, blots were washed twice for 15 min at room temperature in 2x SSC/0.1% (w/v) SDS, followed by washing twice for 15 min at 65°C in 1x SSC/0.1% (w/v) SDS, before exposure to autoradiography film.

2.2.30 First Strand cDNA synthesis

Synthesis of first strand bovine spleen cDNA was carried out using the Amersham cDNA Synthesis System Plus. The recommended reaction conditions were used but 10 µg total spleen RNA was used rather than 1 µg mRNA. The reaction was allowed to proceed for 45 min at 42°C before a further 20 U of reverse transcriptase was added. This was followed by another 45 min at 42°C and 20 min at 52°C. Oligo dT primers were used.

2.2.31 Primer Extension Analysis (Ausubel *et al.*, 1989)

Preparation of radiolabelled primer. Firstly, 100 ng of oligonucleotide was labelled with 0.2 µg [γ^{32}P]-dATP (200 mCi) using 5 U T4 polynucleotide kinase in 30 µl 10 mM $MgCl_2$/10 mM dithiothreitol/1.7 mM spermidine/50 mM Tris-HCl, pH 7.4. After incubation at 37°C for 1 h, followed by inactivation of the enzyme at 65°C for 5 min, the labelled primer was precipitated with ethanol and 20 µg tRNA as carrier. The pellet was resuspended in 30 µl formamide dye mix and, after incubation at 85°C for 5 min, the labelled oligonucleotide was purified by electrophoresis on a 12% polyacrylamide/7 M urea gel (as described in section 2.2.8) at 30 mA. The required band was identified by autoradiography and excised. The DNA was removed from the gel slice by incubation in 5 M ammonium acetate at 37°C, shaking, for approximately 16 h. Brief centrifugation allowed subsequent removal of the supernatant from the gel slice. After twice ethanol precipitating the DNA in a methylated spirit/dry ice bath, the pellet was resuspended in 50 µl 0.3 M sodium acetate.

Hybridisation and reverse transcription. Labelled primer ($5x10^5$ cpm) was ethanol precipitated with approximately 50 µg of total PMA-stimulated U937 cell RNA, and with 50 µg tRNA as a control, prior to resuspension in 30 µl 80% deionised formamide/40 mM PIPES (pH 6.4)/0.4 M NaCl/1 mM EDTA. After heating to 80°C for 5 min, hybridisation was carried out at 45°C for 3 h. The RNA with annealed primer was precipitated using ethanol. Reverse transcription was carried out in 25 µl RT mix (0.6 mM dNTPs/50 mM KCl/6 mM $MgCl_2$/50 mM Tris-HCl, pH 8.2, with 50 U RNasin (Promega) and 40 U AMV reverse transcriptase (Northumbria Biologicals Ltd, UK)) for 1 h at 42°C followed by 30 min at 50°C. The solution was made 20 mM EDTA to stop the extension reaction and the RNA then degraded by treatment with 1 µg RNase A at 37°C for 30 min. Addition of ammonium acetate to a concentration of 2 M was followed by phenol/chloroform extraction and ethanol precipitation with 20 µg tRNA carrier. The pellet was resuspended in a volume of formamide dye mix such that 3 µl contained approximately 10^5 cpm. After heating at 85°C for 5 min, this quantity of DNA was run on a 6% polyacrylamide/7 M urea gel against an M13 sequence ladder (produced using the Pharmacia T7 Sequencing kit) to determine the size of the product (which was visualised by autoradiography).

2.2.32 Rapid Amplification of cDNA Ends (RACE PCR) (Frohman *et al.*,1988)

A first strand cDNA template was synthesised from total PMA-stimulated U937 cell RNA (see section 2.2.25) as described in section 2.2.31 above, but using 20 ng non-labelled K89/10 primer ($^{5'}$AACTCTGTCGTCAGAAGGTGCGA$^{3'}$). The resulting cDNA was dissolved in 1 ml TE buffer, and centrifuged through a Centricon-100 ultrafiltration column (Amicon) at 1000 g for 5 min to remove unincorporated nucleotides and primers.

The cDNA in the retentate (≈120 µl) was precipitated with 0.8 volumes of 5 M ammonium acetate and 2 volumes of propan-2-ol for 1 h at -70°C. The pellet was resuspended in 11 µl water. A poly dA tail was then added to the cDNA by incubation at 37°C for 5 min in 250 mM dATP/1x tailing buffer (BRL) with 15 U terminal deoxynucleotide transferase (BRL). The enzyme was inactivated by incubation at 65°C for 5 min before diluting the tailed products to 500 µl in TE buffer.

The first round of PCR amplification was carried out using 10 µl of cDNA template in 1x PCR buffer (Promega)/200 µM dNTPs/0.2 µM dT_{17}-adaptor primer (K89/06: $^{5'}$GGTCGACTCTAGAGGATCC$T_{17}{}^{3'}$)/0.5 µM adapter primer (K89/05: $^{5'}$GGTCGACTCTAGAGGATCC$^{3'}$)/0.5 µM internal gene-specific primer (B91/08: $^{5'}$GCCGCCTCGTTGTTAGCTCCCCGGACGCG$^{3'}$)/2.5 U TAQ polymerase (Promega). The temperature cycling program used was as follows: 94°C 5 min, 55°C 5 min, 72°C 30 min, 40 cycles of [94°C 40 s, 60°C 1 min, 72°C 3 min], then 72°C 10 min.

A second round PCR amplification was then carried out using a second internal gene-specific primer and the adapter primer to increase the generation of specific products. Five microlitres of first round product was amplified in 1x PCR buffer (Promega)/200 µM dNTPs/0.5 µM phosphorylated adapter primer (K89/05)/0.5 µM phosphorylated gene-specific primer (K91/19: $^{5'}$CCAGAGGAAATGGAGCATAGG$^{3'}$)/2.5 U TAQ polymerase (Promega). The temperature cycling program used was as follows: 30 cycles of [94°C 40 s, 60°C 1 min, 72°C 3 min], then 72°C 10 min.

The RACE products were separated and purified from a NuSieve GTG agarose gel (see section 2.2.9). The Klenow fill reaction was used to blunt-end the isolated fragments (see section 2.2.17) and the products were then subcloned into *Hinc* II cleaved and phosphatased pBluescript SK(+) (Stratagene Ltd). Transformation of *E. coli* was carried out as described in section 2.2.21, and colonies screened for presence of inserts by PCR with T3 and M13 primers (complimentary to the pBluescript vector - see section 2.2.22). DNA was prepared by mini-prep from positive colonies (section 2.2.5), and the inserts sequenced using double stranded sequencing (see section 2.2.24).

2.2.33 Construction of expression vector

Two independent partial cDNA clones for human properdin had previously been isolated from a PMA-stimulated U937 cDNA library (Nolan *et al.,* 1991, Nolan *et al.,* 1992). One of these clones (pKNPCKS-1 (Nolan *et al.,* 1991)) bears a single base mutation in codon 430 (Nolan *et al.,* 1992), while the other clone (pKNPCKS-3) contains the correct sequence but is further truncated at the 5' end. Double stranded sequencing was used to verify the sequence of pKNPCKS-3 between the *Sph I* site and the 3' end, since this clone had not previously been fully sequenced. To generate the correct coding

sequence in pBluescript KS(-), the *Sph* I - *Xho* I fragment of pKNPCKS-1 was replaced with the *Sph* I - *Xho* I fragment of pKNPCKS-3 to create pKNPCKS-1/3.

This cDNA lacked an initiation codon and 10 nucleotides of leader sequence at the 5' end. To correct this, two rounds of PCR using primers with add-on sequences (i. A92/07 + C90/39 and ii. B92/18 + C90/39) were carried out to generate a fragment containing an *Xba* I site for subcloning, an ATG initiation codon in its optimal context (Kozak, 1991, Cavener and Ray, 1991) and the missing leader sequence. The fragment was digested with *Xba* I and *Bbs* I and ligated into *Xba* I + *Bbs* I cleaved pKNPCKS-1/3 to create pATGPCKS-1/3.

PCR was also used to generate a fragment from the 3' end of the coding sequence with a *Bgl* II site immediately after the stop codon (using primers L89/01 + A92/06). All PCR reactions were carried out with the following program: 20 cycles of 94°C 1 min, 60°C 1 min, 72°C 1 min. This fragment was digested with *Sph* I and ligated into *Sph* I + *Hinc* II cleaved pATGPCKS-1/3 to generate pBSproperdin.exp. The construct was checked by double stranded sequencing using the T7 sequencing kit (Pharmacia) according to the manufacturer's protocol. The full length coding region was excised with *Xba* I + *Bgl* II and subcloned into *Xba* I + *Bcl* I cleaved pEE6.HCMV.GS (Celltech) to create the final expression vector, pEE6properdin.

2.2.34 Deletion mutagenesis

Single TSRs were removed from pBSproperdin.exp using an inverse PCR method simplified from that of Hemsley *et al.* (1989). Oligonucleotides were phosphorylated using T4 polynucleotide kinase (Boerhinger-Mannheim). For each mutation, except the removal of TSR3, PCR on pBSproperdin.exp was carried out in 1x TAQ buffer (Promega)/200 µM dNTPs/2 µM each oligonucleotide/2.5 U TAQ polymerase (Promega) for 25 cycles of 94°C, 45 s/55°C, 45 s/72°C, 6 min. For the removal of TSR3, it was found necessary to reduce the concentration of primers to 0.2 µM, and to use a higher annealling temperature of 70°C in order to obtain the specific product. In each case, the 4 kb product was purified from a 0.8% SeaPlaque GTG agarose gel (FMC) (as described in section 2.2.9) and circularised by blunt-end ligation with T4 DNA ligase (Boerhinger-Mannheim) as described in section 2.2.19. The products were used to transform *E. coli* (section 2.2.21). Clones containing the required deletion were identified by double stranded sequencing (section 2.2.24). The region containing the deletion was then excised at unique flanking restriction sites and ligated into pBSproperdin.exp to reduce the amount of sequencing required to check the complete mutant construct for PCR errors. Mutant constructs were then transferred to the expression vector as described above. Plasmids containing the required insert were identified by restriction digestion with *Eco* RV.

2.3 Cell culture and transfection techniques

2.3.1 Growth of U937 cells

The human monocytic cell line U937 (American Type Culture Collection, 1991) was maintained in 10% heat inactivated foetal calf serum/RPMI-1640 (Gibco-BRL)/100 IU ml^{-1} penicillin/100 µg ml^{-1} streptomycin at 37°C in 5% CO_2. For phorbal ester stimulation, cells were treated with 10 ng ml^{-1} PMA for 3 days.

2.3.2 Growth of CHO cells

CHO-K1 (American Type Culture Collection, 1991) cells were maintained in Glasgow Minimal Essential Medium without glutamine (Advanced Protein Products) containing 5% (v/v) heat inactivated foetal calf serum (Gibco-BRL) at 37°C in 5% CO_2. For sub-culture, cells were washed with PBS, pH 7.4 and released using trypsin.

2.3.3 Transfection of CHO cells

Transfections were carried out using 20 µg plasmid DNA by the method of Chen and Okayama (1987) and the Stratagene mammalian transfection kit. Mock transfections with no DNA were also performed. After growth for 24 h in non-selective medium the cells were transferred to and incubated in selective medium (containing 15-30 µM L-methionine sulphoximine, MSX) (Bebbington and Hentschel, 1987). After fifteen days, wells containing resistant colonies were assayed for properdin by ELISA.

2.3.4 Amplification of properdin expression from CHO cells (Cockett *et. al.*, 1990)

CHO cell lines producing properdin were plated out in 96-well tissue culture plates (ICN Flow) and incubated in selective medium at 100-500 µM MSX until resistant colonies were visible.

2.3.5 Production of recombinant human properdin

Properdin secreting CHO-K1 cells and MSX-resistant mock transfectant control cells were grown beyond confluence in triple-layer 500 cm^2 flasks (Nunc) in 5% FCS/GMEM-S at the appropriate MSX concentration. The cells were then washed three times with PBS and incubated in 90 ml serum-free MSX/GMEM-S for 2 to 5 days. To produce non-glycosylated properdin, tunicamycin (Sigma) was included in the medium at 4 µg ml^{-1}. Leupeptin (Sigma) at a concentration of 50 µM was added during production of recombinant properdin lacking TSRs. Debris was removed from the medium by centrifugation and the supernatant stored at 4°C with 0.05% (w/v) sodium azide.

2.4 Protein methods

2.4.1 SDS-Polyacrylamide gel electrophoresis

SDS-PAGE on 7.5, 10, 12.5 or 15% (w/v) polyacrylamide gels (Protogel, National Diagnostics) was carried out as described by Laemmlli (1970). Sample loading buffer was $^1/_4$ volume of 60 mM Tris/8 M urea/3% (w/v) SDS/10% (v/v) glycerol/0.1% (w/v) bromophenol blue, pH 6.8. To reduce samples, 60 mM dithiothreitol was included. Samples were incubated at 100°C for 5 min prior to electrophoresis.

2.4.2 Coomassie Blue Staining of PAGE gels

Gels were stained by incubation with shaking in 0.1% (w/v) Coomassie Brilliant Blue R-250/40% methanol/10% ethanoic acid for several hours and destained in 35% methanol/15% ethanoic acid until a clear background was obtained.

2.4.3 Silver staining of PAGE gels

Gels were fixed by shaking in 50% methanol for 1 h before washing in three changes of deionized water for a total of 2 h. Gels were then incubated for 20 min in 3.3 µM dithiothreitol followed by 20 min in 6 mM silver nitrate. After a brief rinse in deionized water, colour development was carried out by the addition of 0.05% (v/v) formaldehyde/280 mM sodium carbonate. The reaction was stopped by the addition of citric acid to a final concentration of 0.2 M.

2.4.4 Dialysis and concentration of protein samples

Samples were dialysed using Spectra/Por 1 membrane (Spectrum Medical Industries Inc., CA, USA). Samples were concentrated using Microcon-3, Centricon-3, or Centricon-10 concentrators (Amicon Inc., MA, USA). Where necessary, samples were concentrated for SDS-PAGE by incubation with 10 µl Strataclean resin (Stratagene) for 15 min at room temperature. After removal of the supernatant, the resin was incubated at 100°C for 5 min in SDS-PAGE loading buffer, and the resin and buffer loaded onto the gel.

2.4.5 Immunisation of rabbits

Rabbits were immunised with an initial inoculation of 50 µg purified human properdin in 1 ml Freund's complete adjuvant, followed by a booster inoculation of the same quantity of antigen in Freund's incomplete adjuvant after one month.

2.4.6 Preparation of rabbit immunoglobulin G

Rabbit blood was allowed to clot overnight at 4°C. The serum was then dialysed into $^{2}/_{3}$ PBS, pH 7.4, and centrifuged at 3000 g to remove any precipitate. The solution was then made 18% (w/v) Na_2SO_4 and stirred at room temperature for 20 min. The precipitate was harvested after centrifugation at 3000 g and resuspended in $^{2}/_{5}$ the starting volume of $^{2}/_{3}$ PBS. The solution was then made 14% (w/v) Na_2SO_4 and stirred at room temperature for 20 min. After centrifugation, the precipitate was dissolved in $^{1}/_{5}$ the starting volume of $^{2}/_{3}$ PBS and reprecipitated at 14% (w/v) Na_2SO_4. After centrifugation, the IgG fraction was resuspended in $^{2}/_{3}$ PBS at a final concentration of 15 mg ml^{-1} and stored at -20°C.

2.4.7 Determination of IgG concentration

IgG concentrations were determined by measuring absorbance at 280 nm where, E_{280}^{1cm}=1.4 for 1 mg ml^{-1} IgG.

2.4.8 Biotinylation of antibody

Antibody at 6 mg ml^{-1} in 0.1 M $NaHCO_3$, pH 8.0 was incubated at room temperature for 3 h with 0.3 mg ml^{-1} NHS-biotin (Pierce Chemical Co.). The biotinylated antibody was subsequently dialysed into PBS, pH 7.4.

2.4.9 Polyclonal and monoclonal antibody antigen capture ELISA assays for human properdin (Kolble *et al.*, 1993)

The wells of a microtitre plate (Linbro, Flow Laboratories Inc, Virginia, USA), were coated with either 150 µl of 8 µg ml^{-1} rabbit polyclonal anti-human properdin IgG or with 150 µl of 0.5 µg ml^{-1} mouse monoclonal anti-human properdin IgG1 (HYB 3-3) by incubating at 37°C for 3 h. After washing the wells with TBS, non-specific binding sites were blocked using 200 µl 1% (w/v) BSA/TBS and incubating for 3 h at 37°C. The wells were then washed three times with TBS-Tween 20 (0.05% v/v polyoxyethylenesorbitan monolaurate in TBS). Serial dilutions of all samples and standards were carried out in 0.05% (w/v) BSA/TBS, at a final volume of 100 µl per well. The plates were incubated at 37°C for 2 h. The wells were then washed with TBS-Tween 20, and 150 µl 8 µg ml^{-1} biotinylated anti-human properdin IgG in TBS was added to each, and the plate incubated at 37°C for 1 h. After washing with TBS-Tween 20, 150 µl 0.4 µg ml^{-1} Extravidin-alkaline phosphatase conjugate (Sigma) in TBS was added. After incubation at 37°C for 1 h, the wells were washed with TBS-

Tween 20. Then, to each well, 50 µl 1 mg ml^{-1} phosphatase substrate (Sigma) in 100 mM NaCl/5 mM $MgCl_2$/Tris-HCl, pH 9.6 was added and the absorbance at 405 nm was measured after approximately 30 min.

2.4.10 Western Blotting

Western blotting was carried out as described in Sambrook (1989) using the BioRad minigel "Trans-blot" apparatus, using Hybond-C nitrocellulose membrane (Amersham). Western blots were blocked with 0.5% (w/v) BSA in PBS-Tween20. Blots were probed either with 30 µg ml^{-1} rabbit polyclonal anti-properdin IgG in PBS followed by goat anti-rabbit IgG-alkaline phosphatase secondary antibody (Sigma), or with 30 µg ml^{-1} anti-human properdin monoclonal antibody followed by sheep anti-mouse IgG-alkaline phosphatase (Sigma). Blots were developed using NBT and BCIP (Sigma) in 5 mM $MgCl_2$/TBS, pH 9.5 (Sambrook *et al.*, 1989).

2.4.11 Purification of recombinant properdin on monoclonal antibody affinity column

To prepare the affinity column, 24 mg anti- human properdin monoclonal antibody (HYB 3-3) were coupled to 6 g dry mass of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Cell culture supernatants were pre-incubated with 0.05 volumes of Sepharose 6B for 2 h at 4°C to reduce non-specific binding to the column. Samples were then applied to the mAb column, washed with 30 ml each of 3 M NaCl/PBS, pH 7.4 and PBS, pH 7.4 and then eluted with 30 ml 0.2 M glycine/0.5 M NaCl, pH 2.5. Eluted fractions were immediately neutralised with approximately $^1/_5$ volume of 2 M Tris, pH 7.4 and then dialysed into PBS, pH 7.4.

2.4.12 Purification of human plasma properdin

Human properdin was purified from serum by euglobulin precipitation, ion-exchange chromatography, and affinity chromatography on an anti-properdin monoclonal affinity column (method 2, as described by Nolan & Reid, 1993) and kindly provided by Mr Tony Gascoyne, MRC Immunochemistry Unit.

2.4.13 Purification of human plasma C3b

Human C3b, purified according to Dodds (1993), was kindly provided by Ms Vivienne Perkins, MRC Immunochemistry Unit.

2.4.14 Preparation of polyclonal antibody affinity column

To prepare the affinity column, 90 mg rabbit anti-human properdin polyclonal antibody were coupled to 2 g dry mass of CNBr-activated Sepharose 4B (Pharmacia LKB) according to the manufacturer's instructions. To allow the irreversible binding of properdin, 200 ml of human serum (dialysed into PBS, pH 7.4) were passed down the column.

2.4.15 Purification of recombinant properdin on antigen-selected polyclonal antibody affinity column

Firstly, antigen-selected polyclonal anti-properdin antibody was prepared. As an antigen column, the polyclonal antibody affinity column described above was used since after passing through human serum, approximately 1 mg properdin had become irreversibly bound. Approximately 30 mg rabbit anti-properdin polyclonal antibody in PBS, pH 7.4 were applied to the column. The column was then washed with PBS, pH 7.4 until the absorbance at 280 nm returned to baseline. The column was then washed with 15 ml 3M NaCl/PBS, pH 7.4, before antibody was eluted with 0.2 M glycine/0.5 M NaCl, pH 2.5. Approximately 0.5-1 mg of purified antigen-selected antibody was obtained. This fraction was coupled to 0.5 g dry mass of CNBr-activated Sepharose 4B (Pharmacia LKB) according to the manufacturer's instructions. The column was subsequently used as described for the monoclonal affinity column.

2.4.16 Storage of properdin samples

Samples of properdin were stored in 0.04% (w/v) sodium azide/PBS, pH 7.4 in glass tubes at 4°C. Purified properdin samples were not frozen.

2.4.17 Separation of properdin oligomers by size exclusion chromatography

Gel filtration was carried out on 24 ml Superose 6 or Superose 12 HR 10/30 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden) in PBS, pH 7.4, with a flow rate of 0.2 ml min^{-1} and a sample volume of 100 µl (unless otherwise stated). Eluted material was detected by absorbance at 214 or 280 nm. When separating low quantities of protein (≈10 µg), eluted fractions were collected into 0.1% (w/v) BSA/PBS, pH 7.4 in order to reduce losses through properdin binding to the tubes.

2.4.18 Separation of properdin oligomers by cation exchange chromatography (Pangburn, 1989)

Properdin oligomers were separated by cation exchange chromatography on a 1 ml Mono-S HR 5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was run in 50 mM sodium phosphate, pH 6.0, with a 40 ml gradient from 0.1 M NaCl to 0.3 M NaCl.

2.4.19 Dissociation and reassociation of properdin oligomers

Properdin oligomers were dissociated by dialysing samples into 0.1 M glycine/0.15 M NaCl, pH 2.5. Monomers were allowed to reassociate by neutralising with approximately $^{1}/_{10}$ volume of 2 M Tris, pH 7.4.

2.4.20 Removal of glycosylated proteins using lentil lectin

Serum free conditioned culture medium (4 ml) was adjusted to pH 7.0-7.5 and incubated, shaking, with 1 ml of lentil lectin coupled to Sepharose (kindly provided by Dr R. B. Sim) and 0.5 mM Pefabloc/0.5 mM IAA/10 μgml^{-1} SBTI/2 μgml^{-1} pepstatin A at 4°C overnight. The resin was removed afterwards by centrifugation.

2.4.21 Enzymatic removal of oligosaccharide

To remove N-linked glycosylation, properdin was incubated with recombinant N-glycosidase F (PNGase F, New England Biolabs) at a ratio of 100 U PNGase F to 1 µg properdin in PBS, pH 7.4 for 1-4 h at 37°C.

To remove sialic acid, properdin was incubated with recombinant neuraminidase (New England Biolabs) at a ratio of 0.25 mU enzyme to 0.5 µg properdin in MES, pH 6.0 for 16 h at 37°C.

To remove O-linked sugar, properdin was incubated with BSA-free O-glycosidase (Boehringer-Mannheim) at a ratio of 0.5 U enzyme to 0.5 µg properdin in MES, pH 6.0 for 16 h at 37°C.

2.4.22 Limited tryptic digest of properdin

Properdin was incubated with modified trypsin (Promega Corporation) at a ratio of 40:1 in PBS, pH 7.4 for 17 h at 28°C. The reaction was stopped by the addition of phenylmethylsulphonyl fluoride (PMSF, Sigma) to a final concentration of 0.5 mM.

2.4.23 Cross-linking of properdin oligomers

Bis(sulphosuccinimidyl) suberate (BS^3, Pierce Chemical Co.) was added to samples in PBS, pH 7.4 on ice to a final concentration of 1 mM. The reaction was allowed to proceed for 30 min at 37°C, before quenching with SDS-PAGE loading buffer. The stock solution of BS^3 was prepared immediately before use.

2.4.24 Preparation of erythrocytes for haemolytic assays (Liao *et al.*, 1973; Fearon, 1978; Gahmberg and Tolvanen, 1994)

Rabbit erythrocytes were prepared from whole blood in Alsever's solution by repeated centrifugation at 800 g and washing in PBS containing 5 mM $MgCl_2$ and 10 mM EGTA, pH 7.4 (PBS.Mg.EGTA).

Prior to periodate treatment, sheep erythrocytes were washed three times with 50 mM sodium acetate/150 mM sodium chloride, pH 6.0. The cells were diluted to 10^9 cell ml^{-1} in 1 to 3 mM sodium periodate/50 mM sodium acetate/150 mM sodium chloride, pH 6.0 (10^8 cell ml^{-1} has $A_{541nm} \approx 1.0$). The cells were then incubated on ice, in the dark, for 10 min. The cells were then washed five times with PBS.Mg.EGTA.

For neuraminidase treatment, sheep erythrocytes were washed three times with 50 mM sodium acetate/150 mM sodium chloride, pH 5.0. The cells were diluted to 10^9 cell ml^{-1} in 5 U ml^{-1} recombinant neuraminidase (New England Biolabs)/50 mM sodium acetate/150 mM sodium chloride, pH 5.0 and incubated for 2 h at 37°C. The cells were then washed five times with PBS.Mg.EGTA.

2.4.25 Haemolytic assay for human properdin (Platts-Mills and Ishizaka, 1974)

The concentration of erythrocytes was adjusted with PBS.Mg.EGTA so that full lysis of 25 µl of cells gave an A_{405nm} of 1.0 in the final assay volume (approximately 1.5×10^7 cells). Samples of 60 µl were assayed in duplicate by addition of 25 µl of dialysed properdin-deficient serum (see section 2.4.26) and 25 µl of rabbit or sensitised sheep erythrocytes in PBS.Mg.EGTA. After incubation at 37°C, for 15 min with rabbit cells or for 20 min to 35 min with sheep cells, 500 µl of ice cold PBS.Mg.EGTA were added and the assay tubes were centrifuged for 3 min at 6 000 g. The A_{405nm} of 250 µl of each supernatant was read in a microtitre plate.

2.4.26 Preparation of properdin-depleted serum

Dialysed properdin-depleted serum was prepared by passing normal human serum over an anti-human properdin monoclonal antibody affinity column. The eluate was subsequently dialysed into PBS/5 mM $MgCl_2$ /10 mM EGTA, pH 7.4.

2.4.27 Solid phase C3b binding assay (Lambris *et al.*, 1984)

The wells of a Maxisorb microtitre plate (Nunc) were coated with 150 µl 3.3 µg ml^{-1} C3b in 20% glycerol/PBS, pH 7.4 by incubation at 4°C overnight. The wells were then washed three times with PBS, pH 7.4. The plates were then blocked with 200 µl 1% BSA/PBS for 1 h at room temperature. After washing three times with PBS, serial dilutions of the sample were applied in 0.05% BSA/PBS, pH 7.4 and incubated for 1 h at room temperature. The wells were again washed with PBS, and the detection of bound properdin was carried out using biotinylated polyclonal rabbit anti-properdin IgG as described for the sulphatide binding assay above. In some assays, after the coating step, phosphate buffer at half physiological ionic strength (10 mM Na_2HPO_4/1.8 mM KH_2PO_4/69 mM NaCl/1.4 mM KCl, pH 7.4) was used in place of PBS, pH 7.4.

2.4.28 Solid phase lipid binding assay (Roberts, 1987; Holt *et al.*, 1990)

Sulphatides, galactocerebroside types I or II (all from bovine brain), cholesterol-3-sulphate (Sigma) or phosphatidylserine (Boehringer-Mannheim) in methanol were coated onto 96-well microtitre plates ("Polysorb", Nunc) by evaporation in air. The plates were then washed twice with PBS, pH 7.4, and then blocked with 200 µl 1% (w/v) BSA/PBS, pH 7.4 overnight at 4°C. After washing three times with PBS, pH 7.4, dilutions of samples were made in 1% (w/v) BSA/PBS, pH 7.4 at a final volume of 100 µl per well. Binding was allowed to occur overnight at 4°C. The wells were then washed with PBS, pH 7.4, and 150 µl 8 µgml^{-1} biotinylated rabbit anti-human properdin IgG in PBS, pH 7.4 was added to each, and the plate incubated at 37°C for 1 h. After washing three times with PBS, pH 7.4, 150 µl 0.4 µgml^{-1} Extravidin-alkaline phosphatase conjugate (Sigma) in PBS, pH 7.4 was added. After incubation at 37°C for 1 h, the wells were washed with PBS, pH 7.4. Then, to each well, 50 µl 1 mgml^{-1} phosphatase substrate (Sigma) in 100 mM NaCl/5 mM $MgCl_2$/Tris-HCl, pH 9.6 was added and the absorbance at 405 nm was measured after approximately 1 h.

2.4.29 Thin layer chromatography

Samples of lipids or steroids were dissolved in methanol, and run on silica gel thin layer chromatography plates in chloroform/methanol/water (60/35/7). After drying in air, non-nitrogenous sugars were detected using Winzler orcinol - sulphuric acid (1 volume 1.6% orcinol/7.5 volumes 50% sulphuric acid). Molybdenum blue was used to detect phospholipids. All components were detected by charring at > 100°C on a hot plate after spraying with sulphuric acid.

2.4.30 Electron microscopy

Samples were dialysed into 0.2 M ammonium bicarbonate and concentrated to approximately 50 μg ml^{-1} by Centricon as described in section 2.4.4. Rotary shadowing with carbon/platinum and electron microscopy were carried out by Drs Hanna Wiedemann and Rupert Timpl (Max-Planck Institut für Biochemie, Martisried, Munich, Germany) as described by Engel *et al.* (1981).

2.4.31 N-terminal protein sequencing (Matsudaira, 1987)

Samples were separated by SDS-PAGE as described in section 2.4.1, and electroblotted onto ProBlott membrane (Applied Biosystems, Warrington, UK) using the Mini "Trans-Blot " electrophoretic transfer apparatus (BioRad). Membranes were then stained with Coomassie Brilliant Blue R250 and the bands of interest excised. N-terminal sequencing was carried out on an Applied Biosystems 470A protein sequencer with an on line PTH analyser. Membrane samples (1 x 3 mm) were placed in an ABI "Blott" cartridge and sequenced using a modified programme for membrane-bound samples with optimised sample washing and extraction of the cleaved anilothiozolinone-amino acid. Data analysis was performed with Waters "Expert Ease" software (Millipore Ltd, Watford, UK). This procedure was kindly carried out by Mr A.C. Willis.

2.4.32 Amino-acid and amino-sugar analysis (Hendrikson & Meredith, 1984)

Samples for amino acid analysis were hydrolysed at either 110°C for 22 h or at 150°C for 75 min and, for amino-sugar analysis, at 110°C for 4 h. They were then applied to an ABI 420A derivatiser/analyser which utilises pre-column derivatisation with phenylisothiocyanate to form phenylthiocarbamyl amino acids. These are then automatically passed to a narrow-bore HPLC system for analysis. Data handling was performed by the ABI 920A Data Analysis System (Applied Biosystems, Warrington, UK). This procedure was kindly carried out by Mr A.C. Willis.

2.5 Sequence Analysis

2.5.1 Database searches

Searches of the non-redundant combined GenBank and Embl nucleotide sequence database, and of the SwissProt protein database were performed using BLAST at the National Centre for Biotechnology Information.

2.5.2 Definition of thrombospondin repeat boundaries.

Thrombospondin repeats were defined according to intron-exon boundaries where possible (human properdin TSRs 1-6, human thrombospondin I TSRs 1-3, mouse thrombospondins I and II TSRs 1-3, Complement C6 TSR3, mouse FISP-12, chicken and human Nov, N-terminus of Unc-5 TSR1, C-terminus of Eimeria TSR5 and N-terminus of TSR6(Nolan *et al.*, 1992; Wolf *et al.*, 1990; Lawler *et al.*, 1991; Bornstein *et al.*, 1991; Hobart *et al.*, 1993; Ryseck *et al.*, 1991; Joliot *et al.*, 1992; Matinerie *et al.*, 1992; Leung-Hagesteijn *et al.*, 1992; Tomley *et al.*, 1991)). The boundaries of other TSRs were defined by analogy to closely related proteins of known genomic structure, or by the presence of other identifiable structural features, such as known modules (see figure 1.4). Complement component TSR1s are at the extreme N-termini of these proteins. The circumsporozoite protein TSRs are limited at the N-terminal end by proline-rich repetitive sequence and at the C-terminal end by the presence of a hydrophobic transmembrane sequence. The TRAP/SSP2 TSRs are limited by the presence of a von Wilebrand A domain at the N-terminus, and a proline-rich stretch at the C-terminus. Eimeria parasite TSR1 is preceded by a von Wilebrand domain and TSR6 is bounded at the C-terminus by a glycine-rich element and a transmembrane region. Unc-5 TSR2 also has a transmembrane sequence immediately C-terminal. Of those proteins of known genomic structure, only the N-terminal TSRs of the terminal components of the complement pathway and the second TSR of Unc-5 do not coincide with intron/exon boundaries.The sources of the sequence data are given in the legend to figure 3.9.

2.5.3 Classification of thrombospondin repeats into groups by pairwise comparisons and dendrogram construction

All the TSRs were clustered by similarity to produce a dendrogram or tree representation of the clustering relationships. The PILEUP program of the GCG package was used to perform the pairwise comparisons (by the method of Needleman and Wunsch (1970)) and to determine the clustering relationships (the unweighted pair-group method using arithmetic averages (UPGMA), Sneath and Sokal (1973)). The symbol

comparison table used was a modified form of the Dayhoff PAM250 table normalised so that perfect matches are always preferred (Dayhoff *et al.*, 1979; Gribskov & Burgess, 1986). The FIGURE program of the same package was used to draw the resulting dendrogram (Deveureux *et al.*, 1984).

2.5.4 Multiple alignment of thrombospondin repeats

The alignment of TSRs was undertaken using the "Alignment of Multiple Protein Sequences" (AMPS Version D 1.0, 1988) package by G. Barton (Barton and Sternberg, 1987b). The Dayhoff PAM250 Mutation Data Matrix with a constant of 8 added to each score was used for all alignments. Initially, in order to create the basic alignment, all TSRs without large insertions (*i.e.* less than 65 amino acids) were aligned with each other using the pairwise algorithm of Needleman and Wunsch (1970) (with the program Multalign, mode=pairwise, gap_penalty=10). For each sequence pair, the sequences were shuffled and recompared one hundred times in order to find the expected distribution of scores that would be obtained if the sequences were unrelated but had the same length and composition. This allows a "significance score" to be assigned to each pairwise alignment. Subsequently, these significance scores were used to rank the TSRs in order of decreasing similarity (using the program Order). The TSRs were then aligned in this order to generate a single order alignment according to the method of Barton and Sternberg (1987b) (program: Multalign, mode=multiple, gap_penalty=10). Three iterative realignments of each sequence with the whole alignment were then carried out to produce the basic TSR multiple alignment. After this the TSRs over 65 amino acids in length (properdin TSRs 6, Eimeria TSRs 1 and TSRs 5 and Unc-5 TSR 1) were added to the existing alignment using the Multalign program (Multalign, mode=multiple, gap_penalty=10).

2.5.5 Analysis of repetitive genomic elements

Identification, classification and preliminary alignments of repetitive elements within the human properdin gene were carried out using the Pythia (version 2.0) E-mail server at the Argonne National Laboratory, Illinnois (pythia@anl.gov). The 8.3 kb properdin genomic sequence (embl:X70872:HSPROPG) was submitted to the "Rpts" server in order to identify potential repetitive elements as defined in the Reference Collection of Human Repetitive Elements (release 2.1, June 1994). Alu elements were subsequently aligned and classified by submitting sequences encompassing each element to the "Alu" server.

2.5.6 DNA sequence alignment

Pairwise alignment of nucleotide sequences was performed using the FASTA program of the GCG 7.0 package (Deveureux *et al.*, 1984).

Chapter 3

Chapter 3

Analysis of the genomic and protein sequence of properdin

A cosmid clone (COS4XP) containing the complete coding sequence of human properdin has been characterized by Dr Kathleen Nolan (Nolan *et al.*, 1992). The positions of introns within the coding sequence were identified by comparison with the partial cDNA sequence (Nolan *et al.*, 1991). In addition, the presence of a further intron, upstream of the translated region, was identified by reverse transcription-PCR of mRNA isolated from phorbol 12-myristate 13-acetate (PMA)-stimulated U937 cells. The identification of the transcription initiation site and a search of the genomic DNA sequence for the presence of repetitive elements are described in this chapter.

The structure of the human properdin gene reveals that the N-terminal region and the first five TSR modules are encoded by six discrete exons (figure 3.1). The remaining coding sequence at the C-terminal end (including the potential sixth TSR) is split into two exons. This information allowed the extent of properdin TSRs 1 to 5 to be clearly defined, and facilitated a multiple alignment of thrombospondin type I repeats from properdin and other proteins, as described below. This analysis led to the proposal of a new model for the structure of the C-terminal region of properdin, and allowed the identification of predicted structural features of properdin which could be important for its function.

3.1 Localisation of the transcription start site of the human properdin gene

Both primer extension analysis (Ausubel *et al.*, 1989) and rapid amplification of cDNA ends (RACE-PCR) (Frohman *et al.*, 1988) were used to define the start site for transcription of the properdin gene. Primer extension depends upon the hybridisation of a radiolabelled oligonucleotide primer to the mRNA of interest and its subsequent extension to the extreme 5' end of the mRNA using the enzyme reverse transcriptase. The size of the product formed reflects the distance between the primer and the 5' end of the mRNA, allowing the start site of transcription to be identified. RACE-PCR involves the addition of a homopolynucleotide tail to the 3' end of the primer extension product (*i.e.* to the 3' end of a "first strand" cDNA). This homopolymeric tail, together with a cDNA-specific sequence, can then be used as sites for complementary primers in the polymerase chain reaction. This process allows the 5' end of the mRNA to be amplified and sequenced in order to discover the transcription start site and, by comparison with genomic sequence, the position of any intron-exon boundaries.

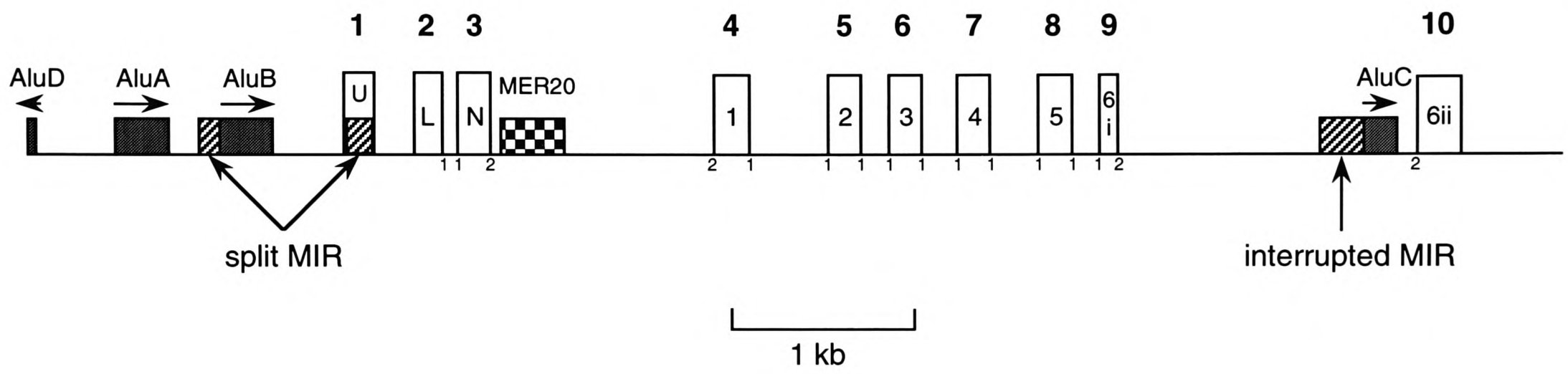


Figure 3.1
Outline structure of the 8.3 kb cosmid clone including the human properdin gene, showing the presence of repetitive elements. The positions of the ten exons of the human properdin gene are shown by large white boxes, numbered above **1** to **10**. The letters within the boxes represent: U, untranslated region; L, coding for the leader peptide; N, coding for the N-terminal region of mature properdin; 1 to 5, the coding for TSRs 1 to 5 of properdin; 6i and 6ii, the coding for the sixth TSR of properdin split over two exons. The exon phases are indicated below the boxes for each of the coding exons (Nolan *et al.*, 1992).
The positions of the repetitive elements described in this chapter are shown as shaded boxes.

The RNA used in these studies was prepared from phorbol 12-myristate 13-acetate (PMA)-stimulated U937 cells. This is a human monocyte-like cell line which assumes macrophage-like characteristics when stimulated with phorbol esters such as PMA and is the cell type from which the cDNA clones of human properdin were originally isolated (Nolan *et al.*, 1991). The presence of the properdin mRNA in the preparation was confirmed by probing a Northern blot of the total RNA preparation with radiolabelled human properdin cDNA (not shown).

3.1.1 Primer extension

Primer extension was carried out as described in chapter 2 (section 2.2.31) using the primer B91/08 which is complementary to the antisense strand of the properdin gene and corresponds to the cDNA sequence encoding the leader peptide of the protein (residues -22 to -13). RNA secondary structure can cause premature termination of reverse transcription. To avoid this problem, the extension reaction included an incubation step at 50°C. The major product produced was approximately 283 bp long. Another minor product of 286-287 bp was also visible (figure 3.2). These lengths correspond to a 5' untranslated region of approximately 238 bp or 241-242 bp. The predicted transcription start site in the properdin gene, assuming that only the single intron identified by Dr Nolan is present upstream of the primer B91/08, is shown in figure 3.3.

3.1.2 Rapid amplification of cDNA ends (RACE-PCR)

In order to confirm the results of the primer extension analysis, and to locate the positions of any introns at the extreme 5' end of the properdin gene (by comparison with the genomic sequence), RACE-PCR was carried out as described in chapter 2, section 2.2.32. Synthesis of the first strand cDNA was primed using the oligonucleotide K89/10, which is complementary to the antisense strand of the sequence encoding residues 25-32 of the mature properdin protein. As described above, the reverse transcription reaction included an incubation at 50°C. After homopolymeric tailing with ATP, the PCR was primed using the hybrid dT_{17}-adapter primer K89/06, the adapter primer K89/05 and the cDNA-specific primer B91/08 described above. Subsequently, a second round of PCR was carried out using K89/05 and K91/19. The K91/19 primer is complementary to the antisense strand of the gene and corresponds to nucleotides -66 to -86 of the 5' untranslated region of the mRNA (upstream of B91/08). This approach, using three nested cDNA-specific primers in successive steps, ensures the specificity of the products formed. The first round of RACE-PCR generated DNA of a range of sizes from 100 to 250 bp. After the second round, major products of approximately 200 bp were

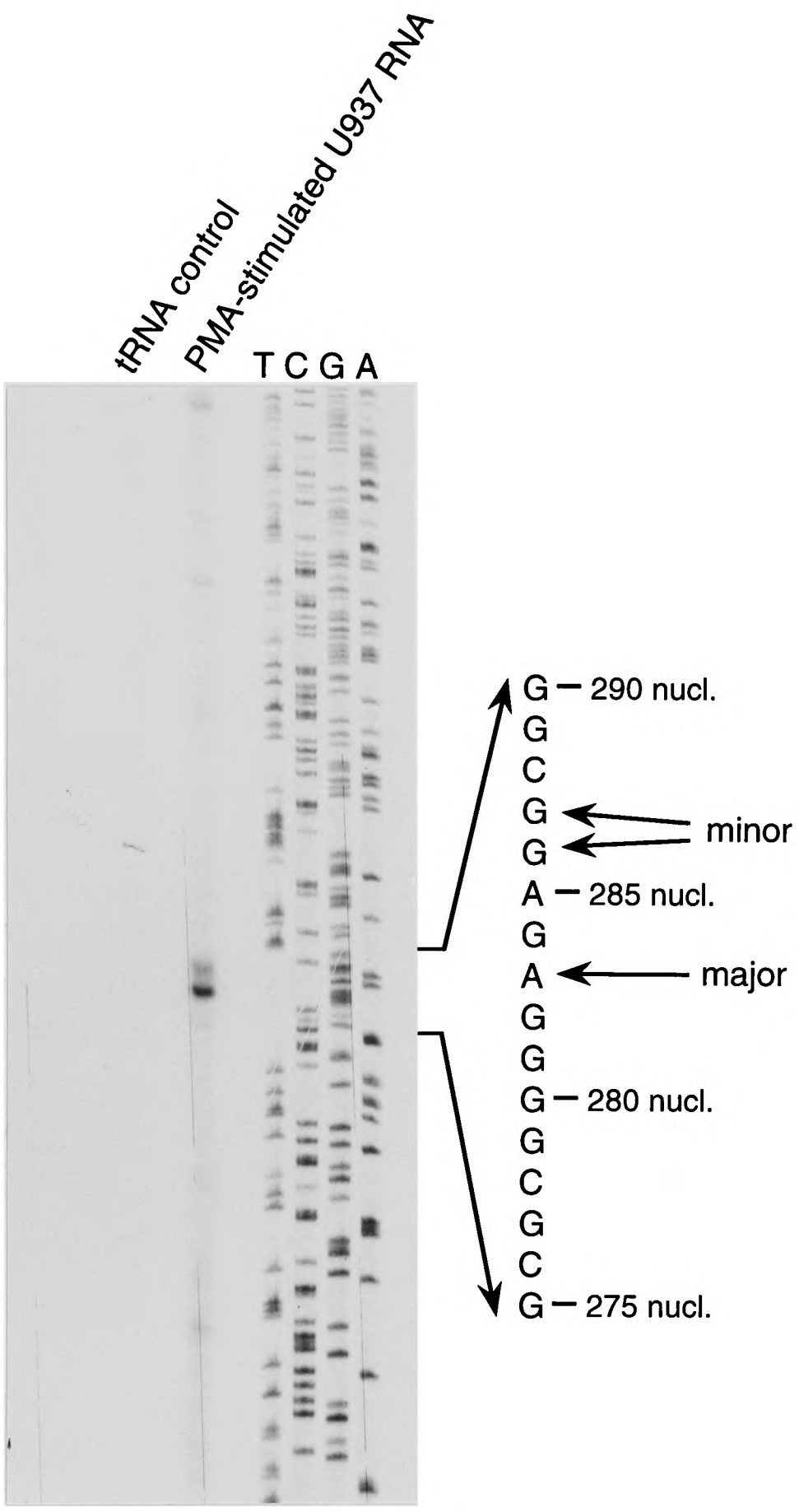


Figure 3.2
Primer extension analysis of the 5' end of the human properdin gene. Primer extension was carried out from the oligonucleotide B91/08 (bases 2221 to 2250 of the human properdin gene (numbering as in figure 3.3), corresponding to amino acids -22 to -13 of the leader peptide) on RNA isolated from PMA-stimulated U937 cells, with tRNA as a control (see materials and methods). The sizes of the products were determined by comparison with a sequence ladder generated from M13amp18 bacteriophage DNA using the "universal" primer (5'-GTAAAACGACGGCCAGT-3') separated on a 6% (w/v) acrylamide/7 M urea gel. The sizes of the major (283 nucleotides) and minor (286/287 nucleotides) products are indicated.

clear. To check that the DNA fragments obtained were indeed related to the 5' end of the properdin message, a Southern blot of the products was probed with radiolabelled oligonucleotide E91/04 (corresponding to nucleotides -87 to -106 of the 5' untranslated region). The oligonucleotide probe hybridised to the RACE products, confirming that the primers had specifically amplified the properdin cDNA (data not shown). The major products were then purified from an agarose gel (section 2.2.9), blunt-ended using Klenow enzyme (section 2.2.17), phosphorylated and cloned into *Hinc* II / pBluescript KS(+) plasmid (sections 2.2.19 to 2.2.21).

Four clones were sequenced and found to be identical to the 5' region of the human properdin gene. The sequences obtained from the clones were co-linear with the genomic sequence, indicating that no introns had been removed upstream of K91/19 to form the mRNA. The extreme 5' ends of the clones are indicated in figure 3.3. The 5' end of the longest clones is eight nucleotides short of the major primer extension product.

That the RACE clones were of cDNA origin, rather than from genomic contamination, is suggested by the presence of various lengths of homopolymeric adenine or thymine (from 18 to over 20 nucleotides) between the K89/05 and properdin sequence in the clones. This is the pattern expected for RACE products, since the hybrid dT_{17}-adapter primer contains seventeen thymine nucleotides, but may anneal at various positions on the homopolymeric tailed cDNA. In addition, there are very few thymine nucleotides in the genomic sequence upstream of the transcription start site, so that annealing of the hybrid dT_{17}-adapter primer to this region of a genomic contaminant is unlikely. Also, while some cDNA preparations showed the presence of genomic contamination on amplification with E91/04 and B91/08 (producing a band of 370 bp, rather than 150 bp, due to the presence of an intron), in parallel experiments the preparation used for the RACE did not (not shown).

The results of the primer extension analysis and the RACE experiment together with the results obtained using conventional PCR by Dr Kathleen Nolan define the structure of the 5' end of the properdin mRNA (Nolan *et al.*, 1992). The 5'-end of the gene contains a single intron, which is spliced out from the untranslated region of the properdin message 110 bp upstream of the initiation codon (figure 3.3). The primer extension experiment indicates that the predominant mRNA species extends to a total distance of 238 nucleotides upstream of the initiation codon. The sequence surrounding this primary site of transcription initiation (AGC<u>A</u>CCGCA) resembles the start site consensus sequence for higher eukaryotic genes reported by Corden *et al.* (1980), YYC<u>A</u>YYYYY (where Y represents a pyrimidine base, and <u>A</u> the initiation site). The secondary potential initiation site (ACT<u>GA</u>GCACC) shows little similarity to this consensus. It is possible that the slightly shorter length of the RACE products obtained indicates further heterogeneity in transcription initiation in the properdin gene.

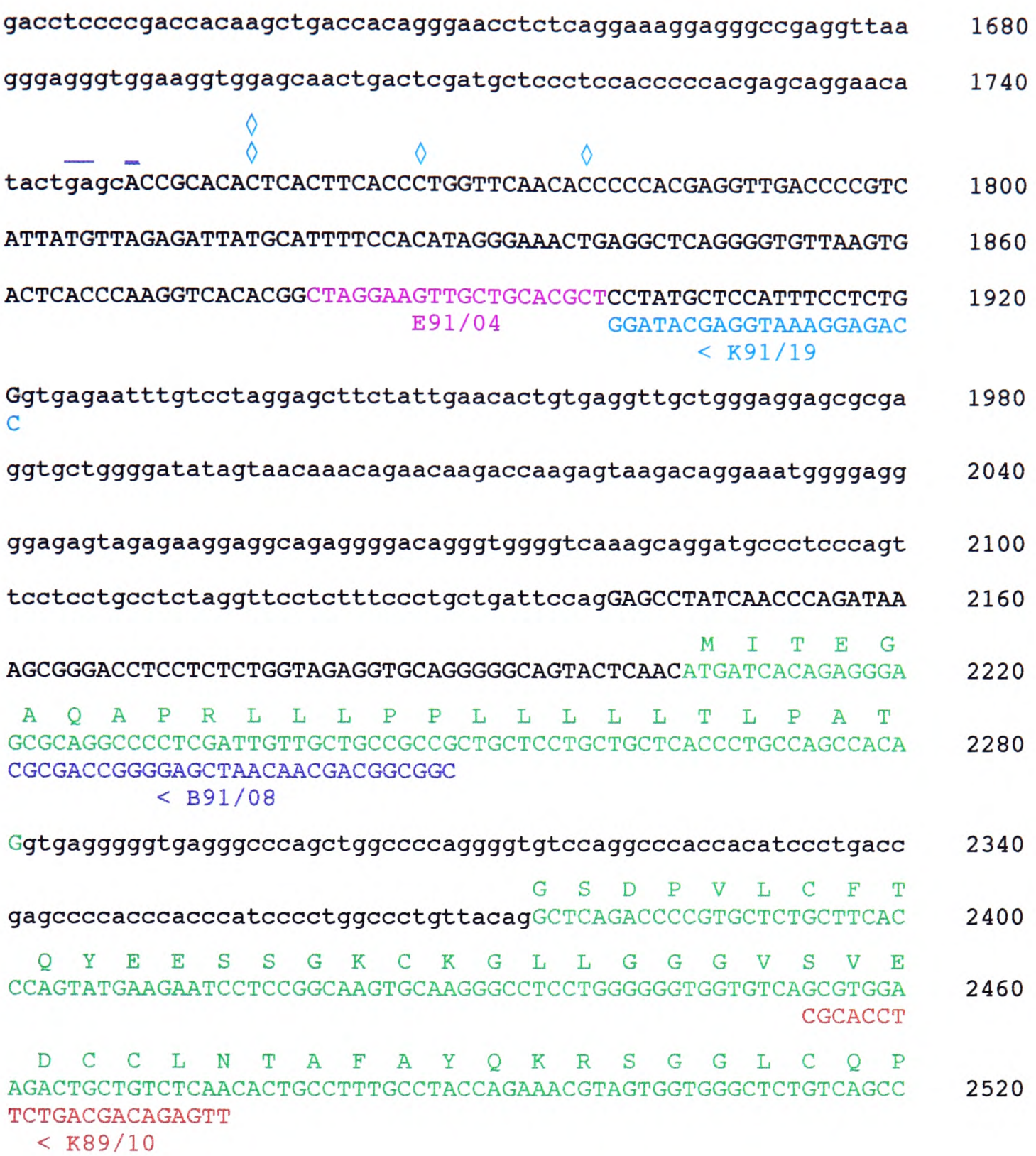


Figure 3.3

The transcription start site of the human properdin gene. Nucleotides 1621 to 2520 from the 5' end of the human properdin gene are shown (Nolan *et al.*, 1992).

DNA sequence present in the human properdin cDNA is shown in UPPER CASE, and intronic and upstream sequence in lower case. The coding region, and derived protein sequence, are shown in green. The oligonucleotide used for primer extension analysis is shown in blue. The 5' ends of the products obtained are shown by overlining (‾‾ : minor product, ‾ : major product). The primer used for cDNA synthesis during the RACE procedure is shown in red, the properdin-specific primer used in the first round of RACE PCR in blue, and that used in the final round of RACE PCR in cyan. The extreme 5' ends of the RACE clones obtained are also shown as ◊. The oligonucleotide used to check that the RACE products were related to properdin (by probing of a Sourthern blot, see text) is shown in magenta. Nucleotides 1749-1921 constitute exon 1, nucleotides 2141-2281 exon 2 and nucleotides 2376-2526 exon 3.

Transcription from multiple start sites is often a feature of genes which lack a TATA-box, a binding motif for the RNA polymerase II transcription factor, TFIID (Dynan, 1986). The human properdin promoter lacks a TATA-box (Nolan, 1992). However, the primer extension products obtained suggests that there is a major transcription initiation site, and a single secondary site. This pattern of initiation resembles that of another TATA-less gene, the human nerve growth factor receptor gene. An additional similarity between the two genes is the presence of several GGGCGG potential Sp1 binding motifs in the 5' upstream region (Nolan, 1992; Sehgal *et al.*, 1988). Sp1 may participate in the activation of transcription in the absence of a TATA-box (Pugh and Tjian, 1990). Inefficient cDNA synthesis prior to the RACE procedure may explain the slightly reduced length of the RACE products. The overall length of the properdin message calculated from this information and from sequencing of a human properdin cDNA clone (Nolan *et al.*, 1991) (1700 nucleotides without polyA tail) corresponds closely to that estimated from a Northern blot of PMA-stimulated U937 total RNA probed with a fragment of the human properdin gene (approximately 1.7 kb when compared to standard RNA markers (Gibco-BRL)(Nolan, 1991).

3.2 Repetitive elements within the human properdin gene.

The genomes of humans, as well as those of other eukaryotes, contain multiple copies of many types of repetitive sequence. Mammalian elements can be divided into the long interspersed repeats (LINEs) such as the LINE-1 or Kpn family (Fanning and Singer, 1987), the short interspersed repeats (SINEs)(Okada, 1991) including the Alu family, the mammalian interspersed repeat (MIR)(Donehower *et al.*, 1989; Jurka *et al.*, 1992), and various medium interspersed repeats (MERs)(Jurka, 1990; Kaplan et al., 1991), and also into mini- and microsatellites (Jeffreys, 1987; Beckmann & Weber, 1992). Some of these elements (*e.g.* the Alu family) are thought to have spread through the genome by retrotransposition. These events probably occurred through mRNA intermediates, since such elements often have polyA stretches at the 3' end. Alu repeats are also characterised by the presence of short direct repeats at each end. These short regions (5-20 nucleotides) are derived from the original genomic DNA sequence at the site of insertion of the transposon and are a result of the mechanism of integration. The MIR and many MER elements do not have clear polyA tracts or direct repeats. The means by which these elements became dispersed through the genome is not known.

Clearly, mobile DNA elements can directly alter the DNA sequence at a site of insertion. In addition, because repetitive elements create regions of homology at multiple sites within the genome, their presence can cause illegitimate recombinations, producing larger rearrangements of chromosomal DNA. These processes can lead to the

introduction of new transcription regulation elements, produce new splice sites and introduce new coding regions and thus can be important for the evolution of eukaryotic genes. Such instability is also known to lead to changes causing genetic disease (for example, in the C1 inhibitor gene (Stoppa-Lyonnet *et al.*, 1990)).

In order to identify repetitive elements in the human properdin gene, the properdin genomic sequence was submitted to the Pythia E-mail server (as described in chapter 2, section 2.2.5). This program identifies the occurrence of repetitive DNA elements from a database of prototypic sequences compiled by Jurka *et al.* (1992) and provides a preliminary alignment of the element with the target sequence.

3.2.1 Alu repeats

Alu repeats probably have their origins before the rodent-primate divergence (Quentin, 1994; Margalit *et al.*, 1994). However, the majority of Alu repeats found in the human genome are more modern and are thought to have colonised only primate genomes. The Alu elements so far sequenced can be classified into several groups. Each group is thought to have been generated by one wave of Alu repeats originating from one or a few progenitor sequence(s) (Britten *et al.*, 1988; Quentin, 1989). The evolution of the progenitor sequences can be reconstructed from a comparison of the Alu sub-families, allowing the period during which each Alu dispersal occurred to be estimated (Britten *et al.*, 1988; Jurka and Smith, 1988; Britten, 1994).

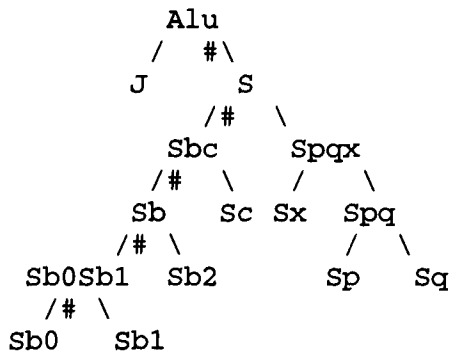
The Pythia package identified the Alu repeats previously known to be present in the human properdin gene (see figure 3.1). Pythia will also align known Alu elements with the human consensus sequence and classify each Alu into a sub-family based upon the identity of certain diagnostic residues within the Alu sequence (Jurka and Smith, 1988; Jurka and Milosavljevic, 1991; Jurka, 1993). The results are shown in figure 3.4. Following the notation of Nolan (Nolan, 1991); the dimeric "A" Alu sequence in the far 5' flanking region of the properdin gene is classified as an Alu-J and the dimeric "B" element (also in the 5' flanking region) as an Alu-Sx. The partial "D" Alu is found at the extreme 5' end of the genomic clone sequenced, and insufficient sequence is available for the repeat be classified unambiguously. The "D" element could represent either an Alu-J or an Alu-S.

The "C" element in the final intron of the gene superficially resembles a monomeric Alu repeat, a class of Alu which probably predates the rodent-primate divergence (Quentin, 1994). However, on closer examination, it appears that the first 70-80 bp of this element resemble the first 70-80 bp of the left half of the dimeric Alu-J family, while the remaining part more closely resembles the 3' end of the right-hand half of a dimeric Alu-J (in both the pattern of deletions, and in nucleotide sequence). Thus, it appears that this element is the result of the fusion of portions of more modern (*i.e.* primate-specific)

Figure 3.4

Alignment and classification of the ALU repeats of the human properdin gene using the Pythia program.

An Alu sequence is identified by performing a series of decisions as illustrated on the right. Each decision leads to the placement of the sequence in a more specific subfamily. As an example, the decisions leading to the identification of an Sb1 sequence are marked by '#'. (Sb0 denotes the members of Sb that are neither Sb1 nor Sb2.) Below, identities are marked "*", and transitions are marked ":".



PROPERDIN ALU "A" (bases 491-780)
score: 88

---

(1) Alignment against the Alu consensus (the consensus is on top):

```
top:  locus: CONSENSUS  beginning: 1  end: 289  length: 289
bottom:  locus: PROPA  beginning: 1  end: 290  length: 290
local_indels: 9  mismatches: 63  transitions: 46

         @10        @20        @30        @40        @50        @60
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGATCAC-CTGAG
**.*.**:*:*:*******:****************************:****:.**.:**..*: :***:
GGGCTGGTGTGATGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCTGAGGTTGGGAGAAAATATTGAA
         @10        @20        @30        @40        @50        @60        @70

@70        @80        @90        @100       @110       @120       @130
GTCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCC
*:********:*************.*****::::**:*****:***.***.:******.***.*****::
GCCAGGAGTTTGAGACCAGCCTGGGCAACACAACGAGACCCCATCTATACAGAAAATAAAAACATTAGTT
          @80        @90        @100       @110       @120       @130       @140

@140       @150       @160       @170       @180       @190       @200
GGGCGTGGTGGCGCGCGCCTGTAATCCCAGCTACTC-GGGAGGCTGAGGCAGGAGAATCGCTTGAACCCG
  *:********:*:*:********:************: ****.***:***::***:****:*****:***:
--GTGTGGTGGTGTGTGCCTGTAGTCCCAGCTACTTGGGGACGCTAAGGTGGGAAAATCACTTGAGCCCA
            @150       @160       @170       @180       @190       @200

 @210       @220       @230       @240       @250       @260       @270
GGAGGCGGAGGTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCCG
****.:.****:****:*****::.****:.:*****************::**:***  ***:******:*
GGAGTTTGAGGCTGCAATGAGCTATGATCAGACCACTGCACTCCAGCCCAGGTGAC--AGCAAGACTCTG
 @210       @220       @230       @240       @250       @260         @270

 @280
TCTC---AAAAAAA
****   ****.**
TCTCTTTAAAACAA
   @280       @290
```

---

(2) Identification of subfamily membership:

---

pos:	57	65	70	71	94	101	120	163	194	204	214	215	220	233	275	277
J:		*		*	*	*	*	*		*	*		*	*		*
S:			*						*						*	
weight:	5	18	5	9	20	2	5	12	22	20	7	1	7	6	6	8

locus contains an **Alu-J**

PROPERDIN ALU "B" (bases 1055-1342)
score: 192

---

(1) Alignment against the Alu consensus (the consensus is on top):

```
top:  locus: CONSENSUS  beginning: 1  end: 288  length: 288
bottom:  locus: PROPB  beginning: 1  end: 288  length: 288
local_indels: 0  mismatches: 31  transitions: 23

         @10       @20       @30       @40       @50       @60       @70
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGATCACCTGAGG
********:*****:****************:****************:****:**:****.********
GGCCGGGCACGGTGACTCACGCCTGTAATCCTAGCACTTTGGGAGGCCAAGGCAGGTGGATAACCTGAGG
         @10       @20       @30       @40       @50       @60       @70

         @80       @90       @100      @110      @120      @130      @140
TCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCCG
**********************.*****************:*****:**********************:*
TCAGGAGTTCGAGACCAGCCTGCCCAACATGGTGAAACCCTGTCTCCACTAAAAATACAAAAATTAGCTG
         @80       @90       @100      @110      @120      @130      @140

         @150      @160      @170      @180      @190      @200      @210
GGCGTGGTGGCGCGCGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGG
*:*:******::*:**.******************:*********************:*.*****.:****
GACATGGTGGTACACGGCTGTAATCCCAGCTACTCAGGAGGCTGAGGCAGGAGAATTGGTTGAAATCGGG
         @150      @160      @170      @180      @190      @200      @210

         @220      @230      @240      @250      @260      @270      @280
AGGCGGAGGTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCCGTC
****:************************:*.********.***************:***:********:**
AGGCAGAGGTTGCAGTGAGCCGAGATCGTGACACTGCACGCCAGCCTGGGCGACGGAGTGAGACTCCATC
         @220      @230      @240      @250      @260      @270      @280


TCAAAAAA
**:*.***
TCGACAAA
```

---

(2) Identification of subfamily membership:

---

pos:	57	65	70	71	94	101	120	163	194	204	214	215	220	233	275	277
J:																
S:		*	*	*	*	*	*	*	*	*	*		*	*	*	*
weight:	5	18	5	9	20	2	5	12	22	20	7	1	7	6	6	8

pos:	78	88	95	153	163	197	219
Sbc:							
Spqx:	*	*	*	*	*		*
weight:	36	28	5	3	13	2	1

pos:	155	244	262	272
Spq:				
Sx:	*	*	*	*
weight:	2	2	4	38

1ocus contains an **A1u-Sx**

PROPERDIN ALU "C" (complete element submitted, bases 7310-7484)
score: 10

---

(1) Alignment against the Alu consensus (the consensus is on top):

```
top:  locus: CONSENSUS  beginning: 129  end: 289  length: 161
bottom:  locus: PROPC  beginning: 1  end: 174  length: 174
local_indels: 13  mismatches: 28  transitions: 20

 @130                 @140       @150       @160       @170          @180
AAAAATTA----------GCCGGGCGTGGTGGCGCGCGCCTGTAATCCCAGCTAC---TCGGGAGGCTGA
*****.**          ***:***:::*****.*:****************.**    *:**********
AAAAAGTAGTGTTGGGAGGCCAGGCACAGTGGCTCACGCCTGTAATCCCAGCAACACTTTGGGAGGCTGA
         @10        @20        @30        @40        @50        @60        @70

    @190       @200       @210       @220       @230       @240       @250
GGCAGGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGC
********.***:*****:***:****::.****:**********::.***:*.***********.****
GGCAGGAGCATCACTTGAGCCCAGGAGATTGAGGCTGCAGTGAGCTATGATTGAGCCACTGCACTGCAGC
         @80        @90        @100       @110       @120       @130       @140

    @260       @270       @280
CTGGGCGACAGAGCGAGACTCCGTCTCAAAAAAA
******:******:*****:*:*:**********
CTGGGCAACAGAGTGAGACCCTGCCTCAAAAAAA
         @150       @160       @170
```

---

(2) Identification of subfamily membership:

---

pos:	57	65	70	71	94	101	120	163	194	204	214	215	220	233	275	277
J:										*	*		*	*	*	*
S:								*								
weight:	5	18	5	9	20	2	5	12	22	20	7	1	7	6	6	8

locus contains an **Alu-J**

PROPERDIN ALU "C" (first half of element submitted, bases 7329-7409)
score: -31

---

(1) Alignment against Alu consensus (consensus is on top):

```
top:  locus: CONSENSUS  beginning: 1  end: 79  length: 79
bottom:  locus: PROPC  beginning: 18  end: 99  length: 82
local_indels: 3  mismatches: 11  transitions: 7

         @10        @20        @30           @40        @50        @60
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAG---CACTTTGGGAGGCCGAGGCGGGCGGATCACCTG
****:***:*:**********************   *************:*****:**.*.*****:**
GGCCAGGCACAGTGGCTCACGCCTGTAATCCCAGCAACACTTTGGGAGGCTGAGGCAGGAGCATCACTTG
  @20        @30        @40        @50        @60        @70        @80

  @70
AGGTCAGGAGTT
**.:******.*
AGCCCAGGAGAT
  @90
```

---

(2) Alu subfamily identification:

---

pos:	57	65	70	71	94	101	120	163	194	204	214	215	220	233	275	277
J:	*	*	*	*												
S:																
weight:	5	18	5	9	20	2	5	12	22	20	7	1	7	6	6	8

locus contains an **Alu-J**

PROPERDIN ALU "D" (bases 86-27)
score: -37

---

(1) Alignment against Alu consensus (consensus is on top):

---

top: locus: CONSENSUS beginning: 1 end: 61 length: 61
bottom: locus: PROPD beginning: 1 end: 60 length: 60
local_indels: 1 mismatches: 9 transitions: 9

```
         @10        @20        @30        @40        @50        @60
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGAT
****:***::*** ********************:***********:::*****:**:****
GGCCAGGCATGGT-GCTCACGCCTGTAATCCCAACACTTTGGGAGATTGAGGCAGGTGGAT
         @10         @20        @30        @40        @50        @60
```

---

(2) Alu subfamily identification:

---

pos:	57	65	70	71	94	101	120	163	194	204	214	215	220	233	275	277
J:																
S:																
weight:	5	18	5	9	20	2	5	12	22	20	7	1	7	6	6	8

locus contains either an **Alu-J or an Alu-S**

---

dimeric elements. This assignment is supported by the alignment and classification provided by Pythia when the first 80 bp of "C" are submitted separately from the rest of the sequence (figure 3.4). Since the "C" element has a short direct repeat at its 5' and 3' ends (figure 3.7), it is likely that originally a single dimeric Alu-J was inserted at this point in the properdin gene. Subsequently the monomer-like element was probably formed by recombination within the Alu leading to loss of sequence from the middle of the dimer, rather than from recombination between two different dimeric elements.

3.2.2 MER20

In addition to the four Alu and partial Alu sequences previously identified, the Pythia search revealed a single MER20 repeat in the intron between exons 3 and 4, which encode the N-terminus and TSR1 of properdin respectively. This type of repetitive element was first identified by Kaplan *et al.* (1991) in an *Alu* I restriction fragment library of human genomic DNA by its similarity to a sequence in the first intron of the human gene for ribosomal protein S17. Hybridisation experiments gave an estimate of 200-400 copies per haploid genome. The MER20 DNA fragment also hybridises with bovine chromosomal DNA, indicating that the element may have been present early in the mammalian radiation. Pythia identified a region of approximately 190 bp between exons 3 and 4 which showed 66% homology with the central portion of the 323 bp MER20 sequence (GenBank accession number X59026). A lower degree of homology extends beyond this core region (see figure 3.5).

3.2.3 MIR

The Pythia search also identified a region in the final intron of properdin containing two overlapping potential repetitive elements known as DBR and MER24, and another region in the 5' flanking region with homology to MER24. The DBR and MER24 elements are ill-defined repeats which were first identified in the human prothrombin gene (Degan & Davie, 1987) and on human chromosome 17 (p11.2-p12), respectively (Donehower *et al.*, 1989; Jurka *et al.*, 1992). These two elements are now known to form part of the mammalian interspersed repeat, or MIR element. The DBR and MER24 elements constitute a region of lower conservation associated with the more highly conserved core region of the MIR element as defined by Donehower *et al.* (J. Jurka, personal communication). The DBR element may also be found independently and has been christened MIR2 (A. Smit and J. Jurka, personal communication). A preliminary extended MIR consensus of 265 bp was kindly provided by Dr J. Jurka of the Linus Pauling Institute of Science and Medicine, Paolo Alto, CA. A BLAST (Altschul *et al.*, 1990) search of the combined non-redundant GenBank and EMBL databases with this sequence, or

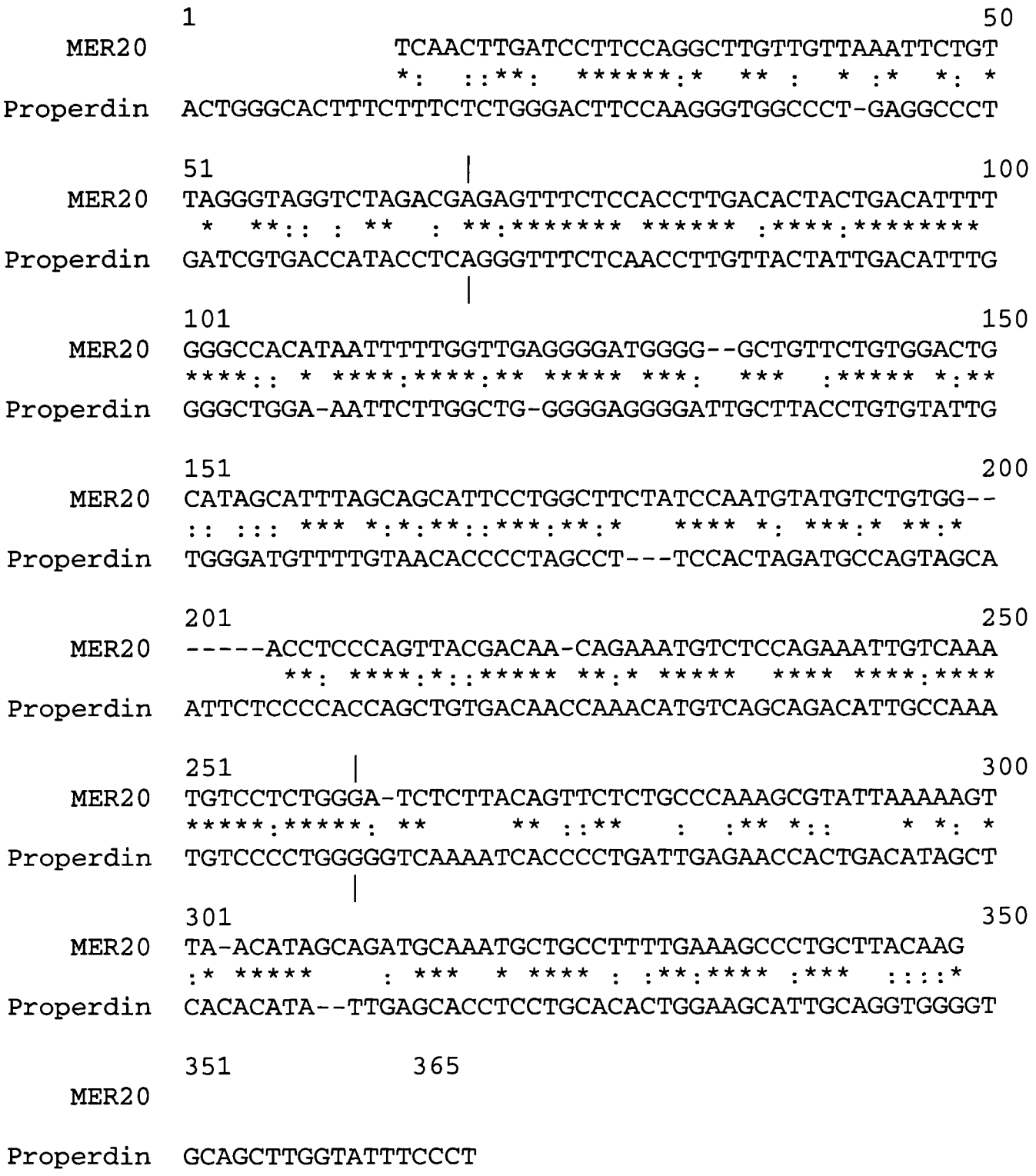


Figure 3.5

Alignment of the MER20 consensus element with sequence from the intron between the properdin gene exons 3 and 4 encoding the N-terminus and TSR1. The homology of this region of the properdin gene to the MER20 repeat was identified between bases 68 and 261 of the MER20 consensus by the Pythia program (66% identity over 194 bp, between the bars, "I", above). Identities are marked "*", and transitions ":". The region shown corresponds to bases 2591-2950 of the human properdin gene.

with a variant of it containing a dinucleotide insertion that is present in the core consensus of Donehower *et al.* (see figure 3.6) identified, among others, two MIR elements within the properdin gene.

The FASTA program of the GCG package (Deveureux *et al.*, 1984) was used to find the best match of the MIR consensus with the human properdin gene. The alignment revealed the presence of an MIR in the final intron of the properdin gene, in the same position located by the BLAST search, and as identified by Pythia as the DBR/MER24 homology (figure 3.7). This properdin MIR shows approximately 67% identity to the first 218 bp of the MIR consensus. The element is truncated at the 3' end by presence of the Alu-J element "C" described earlier (see figure 3.7). Furthermore, the short direct repeats of this Alu-J element, which are thought to be derived from the DNA at the site of Alu integration, appear to be derived from the MIR element. It is thus likely that a pre-existing MIR has been invaded by the Alu-J element. Moreover, Alu-J elements are thought to be the oldest class of dimeric Alu repeats. From an analysis of sequence divergence, the spread of Alu-J has been dated at about 55 million years ago, and certainly earlier than 30 million years ago (Britten *et al.*, 1988; Labuda and Striker, 1989; Britten, 1994). Thus the insertion event probably occurred soon after the rodent-primate divergence. Indeed, potential MIR elements have been found in primates, dogs, cows, sheep, pigs, rodents, lagomorphs and marsupials (in the BLAST search above; Donehower *et al.*, 1989; Jurka, personal communication) and thus they almost certainly arose early in mammalian evolution. This may have interesting implications for the reconstruction of the evolutionary history of the properdin gene (see below). The missing 3' portion of this MIR is not found immediately downstream of the "C" Alu element in the final intron, as might be predicted if a simple Alu repeat insertion had occurred. However, because of its short expected length (47 bp) and fairly low conservation (below 67%) it probably not possible to locate the fragment unambiguously.

The other MIR repeat identified by the BLAST search lies in the first exon of the human properdin gene, in the 5' untranslated region. Indeed, the MIR element constitutes most of the exon (see figure 3.8). The element showed 79% identity to the MIR consensus over 73 bp in the BLAST search, and 70% identity to the consensus over 102 bp in a FASTA alignment. This MIR lacks approximately the first 100 nucleotides of the consensus sequence. Interestingly, the partial MIR element identified as a MER24 repeat by Pythia in the 5' flanking region of the gene (70% identity over 52 bp) is related to the first 101 bp of the MIR consensus. This upstream region is also identified by a FASTA alignment of the MIR consensus with the 5' flanking region of the properdin gene (60% identity over 100 bp). Furthermore, the 3' end of the MIR homology in this upstream region coincides exactly with the 5' end of the "B" Alu element described above (see figure 3.8). Thus, this region is probably the missing 5' end of the partial MIR in the

```
  1 ATTCTTAGCA TGTGCCTAGT ATATAATAGC TAACACTTTT ATANAGCACT
  1 ATTCTTAGCA TGTGCCTAGT ATATAATAGC TAACACTTTT ATANAGCACT

 51 TACTATGTGC CAGGCACTAA GTNCTTTACA TNTATTAACT CATTTAATCC
 51 TACTATGTGC CAGGCACTAA GTNCTTTACA TNTATTAACT CATTTAATCC

101 TCACAACAAC CCTATGAATT GGTANTATTA TTATCCCCAT TTTACAGATG
101 TCACAACAAC CCTATGAATT GGTANTATTA TTATCCCCAT TTTACAGATG

151 AGGAAACTGA G--ACAGAGA AGTTAAATAA CTTGCCCAAG GTCACACAGC
151 AGGAAACTGA GGCACAGAGA AGTTAAATAA CTTGCCCAAG GTCACACAGC

201 TAGTAAGTGG TAGAGCNAGG ATTCAAACCC AGCAGTCTGT CTCCAGAGCC
201 TAGTAAGTGG TAGAGCNAGG ATTCAAACCC AGCAGTCTGT CTCCAGAGCC

251 ATGCTCTTNC ATCCTCC
251 ATGCTCTTNC ATCCTCC
```

Figure 3.6

Consensus MIR elements used to search the combined nucleotide sequence database. The central more highly conserved region identified by Donehower *et al.* is underlined. The top line shows the 265 bp preliminary consensus sequence supplied by Dr J. Jurka (personal communication). The bottom line shows a variant of this consensus containing a GC insertion in the central region which is present in the consensus of Donehower *et al.* (1989).

```
                    7071
properdin           GGTATATATTTCGGAGGTAAAAGAAAGTAGCTCATGCC-AACAAGTAACA
                                          :    **** ::**** *::* :***:*
MIR consensus                             ATTCTTAGCATGTGCCTAGTATATAATA

                    7120
properdin           TTCACCAGCATTTATTGAGCACTTGCTGTGTGCCAGGCACTGCTCTGAGC
                     ::* ** * ***** :*******:**:**************     :**:
MIR consensus       GCTAACA-CTTTTATANAGCACTTACTATGTGCCAGGCACT-----AAGT

                    7170
properdin           ACTTTACCTGAATTAACTCATTT-ATCCTCATTCAACCCCGTGAGGTGAG
                    :****** *: ************ *******  ****  * : *  ***
MIR consensus       NCTTTACATNTATTAACTCATTTAATCCTCA--CAACAACCCTA--TGA-

                    7219
properdin           TATTAATATTACTATTCCTACCATTCTCATTTTGCAGATGAGGGAAACCG
                     ***::**:** ****      **:*:******:******* ******:*
MIR consensus       -ATTGGTANTATTATT------ATCCCCATTTTACAGATGA-GGAAACTG

                    7269
properdin           AGGCACAGAGAAGCTAAAGAAATTGCCCAATATCCTATAGCTAAAAAGTA
                    ************:**** ** ******** :** :*:*****: ****:
MIR consensus       AGGCACAGAGAAGTTAAATAACTTGCCCAAGGTCACACAGCTAGTAAGTG

Alu J consensus     7319      GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAG---CACT
                              ****:***:*:************************   ****
properdin           GTGTTGGGAGGCCAGGCACAGTGGCTCACGCCTGTAATCCCAGCAACACT
                    **:  * :*
MIR consensus       GTAGAGCNAGGATTCAAACCCAGCAGTCTGTCTCCAGAGCCATGCTCTTN

Alu J consensus     TTGGGAGGCCGAGGCGGGAGGATCACTTGAGCCCAGGAGTT|GAGGCTGC
                    *********:*****:**** ****************** * ********
properdin           TTGGGAGGCTGAGGCAGGAGCATCACTTGAGCCCAGGAGATTGAGGCTGC
                                                                  7418
MIR consensus       CATCCTCC

Alu J consensus     AGTGAGCCGTGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGA
                    *******::****:* ********** **********:******:****
properdin           AGTGAGCTATGATTGAGCCACTGCACTGCAGCCTGGGCAACAGAGTGAGA
                                                                  7468

Alu J consensus     CCCTGTCTCAAAAAAA$_n$
                    *****:**********
properdin           CCCTGCCTCAAAAAAAAAAAAGGAAAAAAAAAAAAAAAAAAAGTAGCCCT
                                                                  7518

properdin           CGGCATATAGCAGGCACTCAATAAGAATTGAATGCATTCTTGCCTTCCCT
                                                                  7568
```

Figure 3.7
Alignment of properdin gene bases 7071 to 7568 from the final intron between exons 9 and 10 with the consensus sequences of the MIR element (red) and the Alu J repeat (blue, the position of the deletion in the Alu is marked in the consensus as "|", see text). The direct repeats of the Alu repeat are shown in magenta, identities are marked "*" and transitions are marked ":". The identity of the region of properdin shown with the first 218 bp of the MIR consensus is 67%. The presence of the MIR was detected by a BLAST search with the second consensus sequence shown in figure 3.5 (results: properdin nucleotides 7128-7160, 84% identity over 33 bp, $p=4.3\times10^{-5}$; properdin nucleotides 7261-7320, 80% identity over 60 bp, $p=2.9\times10^{-17}$)

a

```
                1                                                          58
MIR             attcttagcatgtgcctagtatataatagctaacacttttatanagcacttactatgt
                *  : :   **: :: * * *** ***   * * **    *:*:*****:******:*
Properdin       agtaggtgctcaataaatgttgaata-aatttatcaaac----acagagcacctactatat
                958                                                        1013

                59                                                102      119
MIR             gccaggcactaagtnctttacatntattaactcatttaatcctcacaacaaccctatgaat
                **********  **:**  * *::* ::**    ** * ****
Properdin       gccaggcact--gttctaaagactttccaaaagcttaactcctggccgggcacggtgactc
                1014                                              ********:*****:***
Alu                                                               ggccgggcgcggtggctc
                                                                  1
```

b

```
                41                                                         101
MIR             atanagcacttactatgtgccaggcactaagtnctttacatntattaactcatttaatcct
                 :   * :*  :       : **   *  *   :: : :   : * : :: *: :  :* :
Properdin       cccacgagcaggaacatactgagCACCGCACACTCACTTCACCCTGGTTCAACACCCCCAC
                1725                   1748                                1785

                102                                                        162
MIR             cacaacaaccctatgaattggtantattattatccccattttacagatgaggaaactgagg
                 * : ::****::* * *  ** : *  *****   ****** ** **::**********
Properdin       GAGGTTGACCCCGTCATTATGTTAGA-GATTAT--GCATTTTCCACATAGGGAAACTGAGG
                1786                                                       1843

                163                                                        223
MIR             cacagagaagttaaataacttgcccaaggtcacacagctagtaagtggtagagcnaggatt
                * ***:*: *****:*:***::**********:***** **** *: * :*::   *
Properdin       CTCAGGGGTGTTAAGTGACTCACCCAAGGTCACACGGCTAGGAAGTTGCTGCACGCTCCTA
                1844                                                       1904

                224                                               267
MIR             caaacccagcagtctgtctccagagccatgctcttncatcctcc
                ::  **   :  ****  *  ::* :::* **   :  *:** :
Properdin       TGCTCCATTTCCTCTGGgtgagaatttgtcctaggagcttctattgaacactgtgaggttg
                1905            1922                                       1965
```

Figure 3.8

Alignment of the MIR consensus element with two regions at the 5' end of the human properdin gene. The homology of the upstream element with the MIR consensus ends after 101 nucleotides. The "B" Alu element begins at nucleotide 102. The second MIR element lacks approximately the first 100 bp of the MIR consensus. Thus, the upstream region is the 5' end of a split MIR repeat.

a) Alignment of the first portion of the MIR consensus with bases 958-1072 of the properdin gene, a region upstream of the first exon identified by Pythia as Mer24 homology (70% identity over 52 bp) and by a FASTA alignment of the MIR consensus with the 5' end of the properdin gene (60% identity over 100 bp). The 5' end of the "B" Alu repeat is aligned with the Alu consensus sequence.

b) Alignment of bases 41-267 of the MIR consensus with bases 1725-1965 of the properdin gene. The partial MIR element was identified by a BLAST search (properdin nucleotides 1819-1891, 79% identity over 73 bp, $p=1.4 \times 10^{-9}$) and by a FASTA alignment (70% identity over 102 bp). Bases 1748 to 1921 correspond to the first exon of the properdin gene.

Identities are marked "*", and transitions are marked ":". Exonic properdin sequence is shown in UPPER CASE, and other sequence in lower case.

first exon of the gene (figure 3.8). Both MIR elements in the properdin gene have been disrupted by the insertion of Alu repeats (see figure 3.1).

As described by Donehower *et al.* (1989) and Armour *et al.* (1989), MIR elements show greater homology in the central region of 60-70 bp than in the surrounding sequence (figure 3.6). The MIRs in the properdin gene follow this pattern. Such a profile of sequence conservation is not found in relatively recently retrotransposed elements such as the Alu repeat, where the entire region shows a similar level of homology to the consensus sequence. This pattern of conservation may reflect partly the age of the MIR and also indicates that the central MIR sequence may have functional importance. The possibility that MIRs may bind a nuclear factor was suggested by preliminary footprinting experiments of Donehower *et al.* (1989) in which nuclear extracts of hepatoma cells altered the DNAse I hypersensitivity around the core region of the 17p11.2 to 17p12 MIR. Whether the MIRs within the properdin gene, or in other genes, have any role in regulation of transcription or DNA replication is unknown. The significance, if any, of the close correspondence of the first exon of the properdin gene to a region of MIR homology (including the conserved central region) is not clear.

No gross rearrangements within the properdin gene were found by restriction analysis of a properdin deficient kindred of Tunisian Sephardic origin (Nolan, 1991), and a point mutation resulting in a premature stop codon in exon five (TSR2) was found to be responsible for type I deficiency in another single individual (Fredrikson *et al.*, 1994). However, it remains possible that recombination between repetitive elements such as Alu, MIR or MER20 which are present in the properdin gene is responsible for at least some examples of properdin deficiency. It is clear that such events are responsible for genetic deficiency of C1 inhibitor in some families, for example (Stoppa-Lyonnet, 1990). It is also possible that such events were important during the evolution of the properdin gene (see later).

3.3 Multiple Alignment and predicted structure of thrombospondin type I repeats

All known "thrombospondin type I" or "properdin repeats" were aligned using the Alignment of Multiple Protein Sequences (AMPS) package of Barton (Barton & Sternberg, 1987b), as described in Materials and Methods. The alignment (figure 3.9) includes ninety-five TSRs, including those from analogous proteins in different species. These TSRs were identified from a search of the literature and of the annotations of the GenBank database. No further TSR containing proteins were identified by searches of the GenBank, EMBL, or SwissProt sequence databases with BLAST (Altschul, 1990) or

Figure 3.9

Alignment of thrombospondin repeat type I sequences generated using the AMPS package (Barton & Sternberg, 1987b).

Cysteine residues are shown in red. Residues which are conserved over fifty percent of all TSRs are shown in green. Residues which are conserved in fifty percent or more repeats within a subgroup are shown in cyan. Amino acid pairs which score two or over in the PAM250 Mutation Data Matrix of Dayhoff (Dayhoff *et al.*, 1979) are counted as being conserved (for example lysine and arginine). A secondary structure prediction is shown above each subgroup in blue (the positions of predicted beta-strands are represented by: e). A prediction of the disulphide bonding pattern is shown below the alignment. Note that the top loop of this prediction illustrates the possibility of two alternative bonding patterns in different classes of TSR (see text).

The classification of sequences is based upon a dendrogram produced by the PILEUP program of the GCG package (Deveureux *et al.*, 1984). The alignment procedure is described in detail in chapter 2. The secondary structure prediction was carried out using the PHDsec program (Rost & Sander, 1993, 1994). Note that sequences outside residues 1 to 111 are not aligned.

References, given in order in which the sequences appear in the alignment opposite: Nolan *et al.*, 1991; Goundis and Reid, 1988; Maves *et al.*, 1991; Lawler and Hynes, 1986; Lawler *et al.*, 1991; sp P35448 ; LaBell and Byers, 1993; Bornstein *et al.*, 1991; splP35440l; Leung-Hagesteijn *et al.*, 1992; DiScipio and Hugli, 1989; DiScipio *et al.*, 1988; Rao *et al.*, 1987; Haeflinger *et al.*, 1987; Tomlinson *et al.*, 1993; gp X05475 ; Klar *et al.*, 1992; Ruiz I Altaba *et al.*, 1993; Spano *et al.*, 1994; Tomley *et al.*, 1991; Pasamontes *et al.*, 1993; Robson *et al.*, 1988; Hedstrom *et al.*, 1990; Lal and Goldmen *et al.*, 1990; Dame *et al.*, 1984; Ozaki *et al.*, 1983; Arnot *et al.*, 1985; Galinski *et al.*, 1987; Lal *et al.*, 1990; Lal *et al.*, 1988; Eichinger *et al.*, 1986; Lal *et al.*, 1986; Simmons *et al.*, 1989; O'Brien *et al.*, 1990; Ryseck *et al.*, 1991; Bradham *et al.*, 1991; Joliot *et al.*, 1992.

Figure 3.9

Alignment of thrombospondin repeat type I sequences generated using the AMPS package (Barton & Sternberg, 1987b).

Cysteine residues are shown in red. Residues which are conserved over fifty percent of all TSRs are shown in green. Residues which are conserved in fifty percent or more repeats within a subgroup are shown in cyan. Amino acid pairs which score two or over in the PAM250 Mutation Data Matrix of Dayhoff (Dayhoff *et al.*, 1979) are counted as being conserved (for example lysine and arginine). A secondary structure prediction is shown above each subgroup in blue (the positions of predicted beta-strands are represented by: e). A prediction of the disulphide bonding pattern is shown below the alignment. Note that the top loop of this prediction illustrates the possibility of two alternative bonding patterns in different classes of TSR (see text).

The classification of sequences is based upon a dendrogram produced by the PILEUP program of the GCG package (Deveureux *et al.*, 1984). The alignment procedure is described in detail in chapter 2. The secondary structure prediction was carried out using the PHDsec program (Rost & Sander, 1993, 1994). Note that sequences outside residues 1 to 111 are not aligned.

References, given in order in which the sequences appear in the alignment opposite: Nolan *et al.*, 1991; Goundis and Reid, 1988; Maves *et al.*, 1991; Lawler and Hynes, 1986; Lawler *et al.*, 1991; sp P35448 ; LaBell and Byers, 1993; Bornstein *et al.*, 1991; spIP35440I; Leung-Hagesteijn *et al.*, 1992; DiScipio and Hugli, 1989; DiScipio *et al.*, 1988; Rao *et al.*, 1987; Haeflinger *et al.*, 1987; Tomlinson *et al.*, 1993; gp X05475 ; Klar *et al.*, 1992; Ruiz I Altaba *et al.*, 1993; Spano *et al.*, 1994; Tomley *et al.*, 1991; Pasamontes *et al.*, 1993; Robson *et al.*, 1988; Hedstrom *et al.*, 1990; Lal and Goldmen *et al.*, 1990; Dame *et al.*, 1984; Ozaki *et al.*, 1983; Arnot *et al.*, 1985; Galinski *et al.*, 1987; Lal *et al.*, 1990; Lal *et al.*, 1988; Eichinger *et al.*, 1986; Lal *et al.*, 1986; Simmons *et al.*, 1989; O'Brien *et al.*, 1990; Ryseck *et al.*, 1991; Bradham *et al.*, 1991; Joliot *et al.*, 1992.

```
                      50          60              70          80          90          100
TSR1        Human     SPRWSLWSTWAPCSVT    CSEGSQL    RYRRCVGWNGQCS  GKVAPGTLEWQLQA CEDQQCCP
            Mouse     SPQWSAWSLWGPCSVT    CSEGSQL    RHRRCVGRGGQCS  ENVAPGTLEWQLQA CEDQPCCP
            Guinea Pig SPRWSPWSSWAPCSVS   CSEGSQL    RHRRCIGWGGQCS  ENKAPGTLEWQLQA CEEQQCCP

                      110         120             130         140         150         160
TSR2        Human     EMGGWSGWGPWEPCSVT   CSKGTRT    RRRACNHPAPKC   GGHCPGQAQESEA  CDTQQVCP
            Mouse     EMGGWSEWGPWGPCSVT   CSKGTQI    RQRVCDNPAPKC   GGHCPGEAQQSQA  CDTQKTCP
            Guinea Pig EMGGWSNWEPWGPCTVT   CSKGTRI    RRRVCNNPAPKC   GGHCPGVAQESEA  CDTQQVCP

                         170         180          190         200         210         220
TSR3        Human     THGAWATWGPWTPCSAS   CHGGPHEPKETRSRKCSAPEPSQKPPGKPCPGLAYEQRR  CTGLPPCP
            Mouse     THGAWASWGPWSPRSGS   CLGGAQEPKETRSRSCSAPAPSHQPPGKPCSGPAYEHKA  CSGLPPCP
            Guinea Pig THGAWGPWGPWSSCLSS   CHGGPHKPVETRSRTCSAPEPSKNPPGNPCPGTAYEQQS  CAGLPPCP

                      230         240             250         260         270         280
TSR4        Human     VAGGWGPWGPVSPCPVT   CGLGQTM    EQRTCNHPVPQHG  GPFCAGDATRTHI  CNTAVPCP
            Mouse     VAGGWGPWSPLSPCSVT   CGLGQTL    EQRTCDHPAPRHG  GPFCAGDATRNQM  CNKAVPCP
            Guinea Pig VAGGWGPWGSVSPCSVT   CGLGQIL    EQRKCDNPVPQHG  GSFCTGDDTRAHI  CNTAVPCP

                      290         300         310         320         330         340         350
TSR5        Human     VDGEWDSWGEWSPCIRRNMKSISCQEIPGQQS  RGRTCRGRKFD   GHRCAGQQQDIRH  CYSIQHCP
            Mouse     VNGEWEAWGKWSDCSRLRM SINCEGTPGQQS  RSRSCGDRKFN   GKPCAGKLQDIRH  CYNIHNCI
            Guinea Pig VDGEWEPWGDWSTCTRPHLSAIRCKEIVGQQT  RVRICKGRKFN   GQRCPGKHQEIRH  CYNIQNCIFG

                      360         370         380         390         400         410
TSR6        Human     LKGSWSEWSTWGLCMPP      CGPNPTRA   RQRLCTPLLPKYPPTVSMVEGQGEKNVTFWGRPLPRCE
            Mouse     MKGSWSQWSTWSLCTPP      CSPNATRV   RQRLCTPLLPKYPPTGSMVEGQGEKNVTFWGTPRPLCE
            Guinea Pig EKGSWSQWTPWGLCTPP      CGANPTRV   RQRRCMASLPKFSPTVSVVEGQGEKNVTFWGKPLAQCE
```

Figure 3.10a
Manual alignment of the thrombospondin type I repeats of properdin from human and mouse, from Smith *et al.* (1991). The TSRs of guinea pig properdin have been added. Residues conserved in half or more of the properdin TSRs are shown in cyan. Cysteine residues are shown in red. Potential N-linked glycosylation sites are in blue. The numbering is that of the mature form of human properdin.

```
                        50          60          70          80          90          100
                        '           '           '           '           '           '
TSR1        Human       SPRWSLWSTWAPCSVT        CSEGSQL     RYRRCVGWNGQCS   GKVAPGTLEWQLQA  CEDQQCCP
            Mouse       SPQWSAWSLWGPCSVT        CSEGSQL     RHRRCVGRGGQCS   ENVAPGTLEWQLQA  CEDQPCCP
            Guinea Pig  SPRWSPWSSWAPCSVS        CSEGSQL     RHRRCIGWGGQCS   ENKAPGTLEWQLQA  CEEQQCCP

                        110         120         130         140         150         160
                        '           '           '           '           '           '
TSR2        Human       EMGGWSGWGPWEPCSVT       CSKGTRT     RRRACNHPAPKC    GGHCPGQAQESEA   CDTQQVCP
            Mouse       EMGGWSEWGPWGPCSVT       CSKGTQI     RQRVCDNPAPKC    GGHCPGEAQQSQA   CDTQKTCP
            Guinea Pig  EMGGWSNWEPWGPCTVT       CSKGTRI     RRRVCNNPAPKC    GGHCPGVAQESEA   CDTQQVCP

                            170         180             190         200         210         220
                            '           '               '           '           '           '
TSR3        Human       THGAWATWGPWTPCSAS           CHGGPHEPKETRSRKCSAPEPSQKPPGKPCPGLAYEQRR   CTGLPPCP
            Mouse       THGAWASWGPWSPRSGS           CLGGAQEPKETRSRSCSAPAPSHQPPGKPCSGPAYEHKA   CSGLPPCP
            Guinea Pig  THGAWGPWGPWSSCLSS           CHGGPHKPVETRSRTCSAPEPSKNPPGNPCPGTAYEQQS   CAGLPPCP

                        230         240         250         260         270         280
                        '           '           '           '           '           '
TSR4        Human       VAGGWGPWGPVSPCPVT       CGLGQTM     EQRTCNHPVPQHG   GPFCAGDATRTHI   CNTAVPCP
            Mouse       VAGGWGPWSPLSPCSVT       CGLGQTL     EQRTCDHPAPRHG   GPFCAGDATRNQM   CNKAVPCP
            Guinea Pig  VAGGWGPWGSVSPCSVT       CGLGQIL     EQRKCDNPVPQHG   GSFCTGDDTRAHI   CNTAVPCP

                        290         300         310         320         330         340         350
                        '           '           '           '           '           '           '
TSR5        Human       VDGEWDSWGEWSPCIRRNMKSISCQEIPGQQS    RGRTCRGRKFD     GHRCAGQQQDIRH   CYSIQHCP
            Mouse       VNGEWEAWGKWSDCSRLRM SINCEGTPGQQS    RSRSCGDRKFN     GKPCAGKLQDIRH   CYNIHNCI
            Guinea Pig  VDGEWEPWGDWSTCTRPHLSAIRCKEIVGQQT    RVRICKGRKFN     GQRCPGKHQEIRH   CYNIQNCIFG

                                360             370         380         390         400         410
                                '               '           '           '           '           '
TSR6        Human       LKGSWSEWSTWGLCMPP           CGPNPTRA    RQRLCTPLLPKYPPTVSMVEGQGEKNVTFWGRPLPRCE
            Mouse       MKGSWSQWSTWSLCTPP           CSPNATRV    RQRLCTPLLPKYPPTGSMVEGQGEKNVTFWGTPRPLCE
            Guinea Pig  EKGSWSQWTPWGLCTPP           CGANPTRV    RQRRCMASLPKFSPTVSVVEGQGEKNVTFWGKPLAQCE
```

Figure 3.10a
Manual alignment of the thrombospondin type I repeats of properdin from human and mouse, from Smith *et al.* (1991). The TSRs of guinea pig properdin have been added. Residues conserved in half or more of the properdin TSRs are shown in cyan. Cysteine residues are shown in red. Potential N-linked glycosylation sites are in blue. The numbering is that of the mature form of human properdin.

```
                    50        60                  70        80                  90        100
TSR1        Human   SPRWSLWSTWAPC   SVTCSEG   SQLRYRRCVGWNG   QCSGKVAPGT   LEWQLQACEDQQCCP
            Mouse   SPQWSAWSLWGPC   SVTCSEG   SQLRHRRCVGRGG   QCSENVAPGT   LEWQLQACEDQPCCP
        Guinea Pig  SPRWSPWSSWAPC   SVSCSEG   SQLRHRRCIGWGG   QCSENKAPGT   LEWQLQACEEQQCCP

                   110       120                 130       140                 150       160
TSR2        Human   EMGGWSGWGPWEPC   SVTCSKG   TRTRRRACNHPAP   KCGG HCPG   QAQESEACDTQQVCP
            Mouse   EMGGWSEWGPWGPC   SVTCSKG   TQIRQRVCDNPAP   KCGG HCPG   EAQQSQACDTQKTCP
        Guinea Pig  EMGGWSNWEPWGPC   TVTCSKG   TRIRRRVCNNPAP   KCGG HCPG   VAQESEACDTQQVCP

                       170          180       190       200          210       220
TSR3        Human   THGAWATWGPWTPC   SASCHGGPHEPKETRSRKCSAPEP   SQKPPGKPCPG   LAYEQRRCTGLPPCP
            Mouse   THGAWASWGPWSPR   SGSCLGGAQEPKETRSRSCSAPAP   SHQPPGKPCSG   PAYEHKACSGLPPCP
        Guinea Pig  THGAWGPWGPWSSC   LSSCHGGPHKPVETRSRTCSAPEP   SKNPPGNPCPG   TAYEQQSCAGLPPCP

                   230       240                 250       260                 270       280
TSR4        Human   VAGGWGPWGPVSPC   PVTCGLG   QTMEQRTCNHPVP   QHGGPFCAG   DATRTHICNTAVPCP
            Mouse   VAGGWGPWSPLSPC   SVTCGLG   QTLEQRTCDHPAP   RHGGPFCAG   DATRNQMCNKAVPCP
        Guinea Pig  VAGGWGPWGSVSPC   SVTCGLG   QILEQRKCDNPVP   QHGGSFCTG   DDTRAHICNTAVPCP

                   290       300       310       320                 330       340       350
TSR5        Human   VDGEWDSWGEWSPCIRRNMKSISCQEIP   GQQSRGRTCRGR   KFDGHRCAG   QQQDIRHCYSIQHCP
            Mouse   VNGEWEAWGKWSDCSRLRM SINCEGTP   GQQSRSRSCGDR   KFNGKPCAG   KLQDIRHCYNIHNCI
        Guinea Pig  VDGEWEPWGDWSTCTRPHLSAIRCKEIV   GQQTRVRICKGR   KFNGQRCPG   KHQEIRHCYNIQNCIFG

                           360       370       380       390       400       410       420       430       440
TSR6        Human   LKGSWSEWSTWGLC   MPPCGPNP   TRARQRLCTPLLPKYPPTVSMVEGQGEKNVTFWGRPLPRCEELQGQKLVVEEKRPCLHVPACKDPEEEEL
            Mouse   MKGSWSQWSTWSLC   TPPCSPNA   TRVRQRLCTPLLPKYPPTGSMVEGQGEKNVTFWGTPRPLCEALQGQKLVVEEKRSCLHVPVCKDPEEKKP
        Guinea Pig  EKGSWSQWTPWGLC   TPPCGANP   TRVRQRRCMASLPKFSPTVSVVEGQGEKNVTFWGKPLAQCEELQGQKVLLEEKRPCLHVPACKDPEEEEP
```

Figure 3.10b
Alignment of the thrombospondin type I repeats of properdin from human, mouse, and guinea pig, generated using the AMPS package of Barton (Barton and Sternberg, 1987b). Residues conserved in over fifty percent of all TSRs are shown in green. Those conserved in half or more of the properdin TSRs are shown in cyan. Cysteine residues are shown in red. Potential N-linked glycosylation sites are in blue. The numbering is that of the mature form of human properdin.

with PROFILESEARCH from the GCG package (Deveureux *et al.*, 1984). Sonnhammer and Kahn (1994) have divided the entire SwissProt database into putative "domains" using an automatic algorithm, initially employing BLASTP to locate homologous segment pairs. Consultation of the resulting ProDom 28.0 database (via the E-mail server prodom@toulouse.inra.fr) also failed to reveal any further TSR modules.

The extent of each TSR module was defined by various means. Originally, the TSR was defined by the alignment of protein sequences from thrombospondin (Lawler and Hynes, 1986). However, it is clear that in many cases protein modules are encoded by single exons (Patthy, 1987). In cases where the intron/exon boundaries of the gene were known, and the exons were found to coincide with TSR protein sequence, then the TSRs were defined by the extent of the exons. Such TSRs included those from properdin, thrombospondins I and II, and connective tissue growth factor (a more detailed list is given in materials and methods). The lengths of TSRs of other proteins were defined by analogy to these exon boundaries. Among those proteins of known genomic structure, only properdin TSR6, the N-terminal TSRs of the terminal complement components and the second TSR of Unc-5 are interrupted by introns. In some cases the lengths of TSRs could be defined by the presence of other predicted structural features in the proteins. For example, the TSRs of many of the malarial proteins are limited at the N-terminal end by the presence of short repetitive sequences or von Willebrand type A module, and at the C-terminal end by transmembrane or short repeating sequences (see chapter 1, figure 1.4 and chapter 2, section 2.5.2 for more details).

3.3.1 Reliability of the alignment

According to Barton and Sternberg (1987a, b) if the significance score for a pairwise alignment is over 5-6 S.D. then there is a high probability that the sequences will be correctly aligned over the regions which correspond to secondary structures, while a score over 15 S.D. suggests the alignment is correct over most of the length. The multiple alignment algorithm proceeds by the successive addition of sequences to a growing array, beginning with the most similar pair and progressing to less similar sequences. All the sequences added to the alignment (figure 3.9) score between 24.50 and 7.12 S.D. with a sequence already in the array (although not all possible pairwise comparisons score so highly). These scores indicate that all the sequences are related, and are likely to have a similar structure. Proteins such as antistasin (Nutt *et al.*, 1988; Holt *et al.*, 1989), which have been suggested to show limited similarity with thrombospondin and properdin (Prater *et al.*, 1991; Bornstein, 1992), do not contain TSRs when compared to this alignment. The maximum score obtained for antistasin when compared to the TSRs was 2.78 S.D. with human complement C6 TSR2 (most scores were below 1 S.D.). The region of homology within the circumsporozoite proteins

of *Plasmodium spp.* is often said to be limited to the N-terminal half of the TSR, to the so-called "region II" or "region II plus" (Dame *et al.*, 1984; Stankowski *et al.*, 1991; Cerami *et al.*, 1992a, b; Sinnis *et al.*, 1994). However, in this alignment, sequences from circumsporozoite proteins show homology to other TSRs extending beyond region II plus. In particular, the two highly conserved cysteine residues found at the C-terminus of over 90% of TSRs are maintained in the circumsporozoite proteins of all species known (the TSR of *P. reichenowi* has an align score of 5.91 S.D. with human thrombospondin I TSR2, and that of *P. bergei*, a score of 8.51 S.D. with the *E. maxima* p100 TSR3). Furthermore, the proposed TSR of the CS protein lies between short repetitive amino acid sequences at the N-terminus and a transmembrane region at the C-terminus (see figure 1.4). Also, although Tomley *et al.* (1991) and Pasamontes *et al.* (1993) classify the sixth repeats of the *Eimeria* P100 proteins as partial TSRs, these proteins probably contain a complete, though somewhat degenerate sixth TSR.

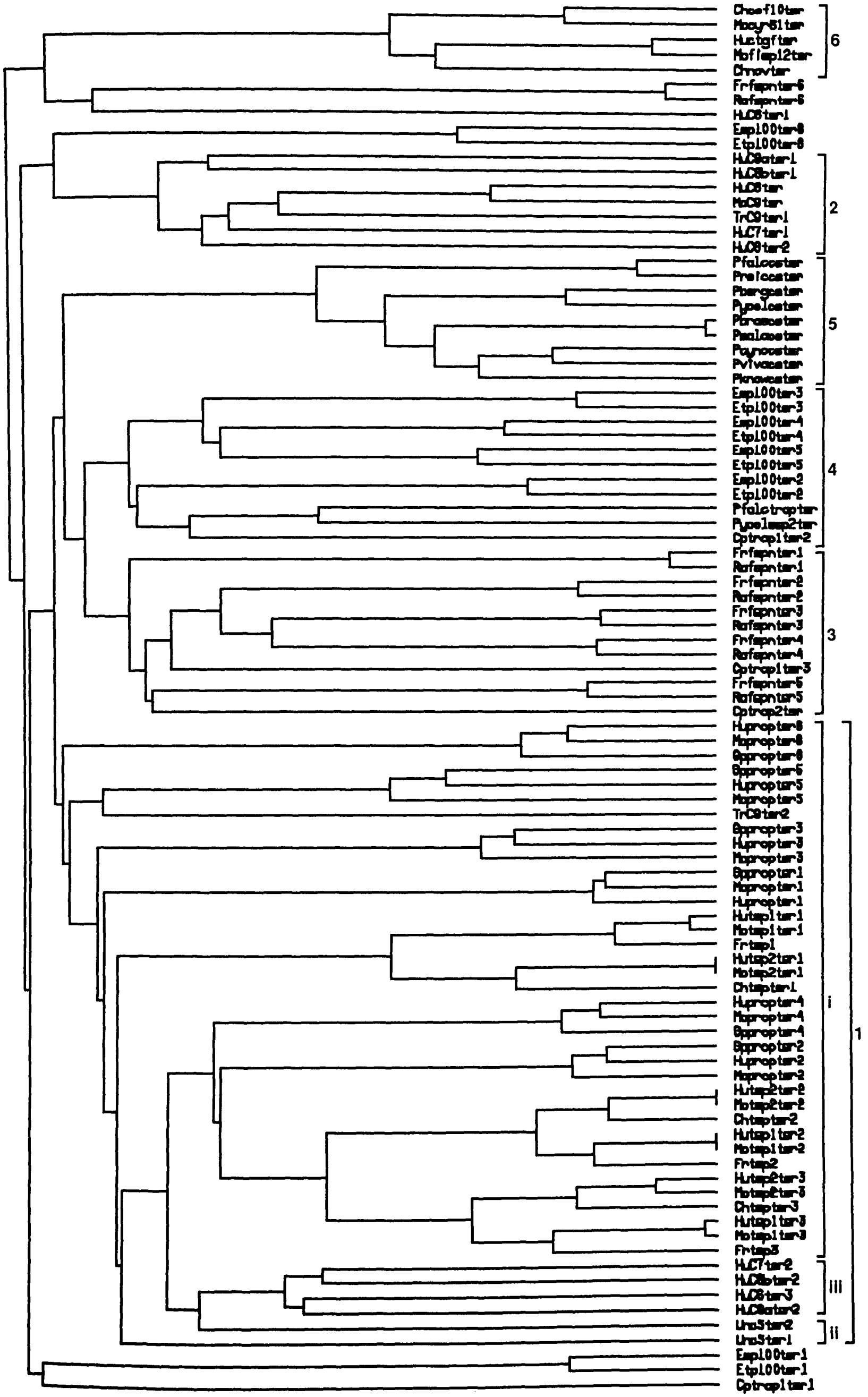
3.3.2 Sub-groups of TSRs

The sequences in the alignment can be clustered according to pairwise similarities using the methods of Needleman and Wunsch (1970) and Sneath and Sokal (1973), as implemented by the PILEUP program of the GCG package (see Materials and Methods). This process produces a tree which, while not showing phylogenetic relationships, does indicate subgroups of TSRs which have close overall sequence similarity (figure 3.11). In this way, the TSRs can be divided into six groups which are likely to be structurally similar, expanding the three group classification of Smith *et al.* (1991). Group 1 includes the properdin TSRs, thrombospondin TSRs and subgroups of (1.ii) the Unc-5 TSRs, (1.iii) the C-terminal TSRs of the terminal complement components. Group 2 comprises the N-terminal TSRs of the terminal complement components, group 3 the F-spondin and *Cryptosporidium* TRAP TSRs, and group 4 the TSRs of the *Eimeria* p100 proteins and of the *Plasmodium* TRAP/SSP2 proteins. Group 5 includes the circumsporozoite protein TSRs and group 6, the TSRs of the connective tissue growth factor-like proteins. The extra N-terminal TSR of complement component C6 does not fall simply into any of the groups, but is most similar to TSR6 of F-spondin, group 3. The pairwise significance scores used to build the single order AMPS alignment (using the method of Barton and Sternberg, see above) support this classification. Interestingly, while a functional relationship between the parasite protein TSRs and properdin or thrombospondin has been proposed, the parasite TSRs are more similar to the F-spondin sequences than to the other vertebrate sequences known.

Figure 3.11

Classification of TSRs into six families according to overall protein sequence similarity. The dendrogram was produced using the PILEUP program of the GCG package. Figure 3.9 shows a multiple alignment of TSR sequences grouped according to this classification.

Key: Hu, human; Mo, mouse; Ra, rat; Gp, guinea pig; Ch, chicken, Fr, frog; Tr, trout; Cp, *Cryptosporidium parvum*; Et, *Eimeria tenella*; Em, *E. maxima*; Pfalc, *Plasmodium falciparum*; Pyoel, *P. yoelli*; Preic, *P. reichenowi*; Pknow, *P. knowlesi*; Pviva, *P. vivax*; Pcyno, *P. cyanomolgi*; Pmala, *P. malariae*; Pbras, *P. brasilinum*; Pberg, *P. berghei*. prop, properdin; tsp, thrombospondin; fspn, F-spondin; trap, thrombospondin related anonymous protein; cs, circumsporozoite protein; ctgf, connective tissue growth factor.


Chaef10tar
Macyr61tar
Huctgftar
Moflap12tar
Chnovtar
Frfapntar6
Rafapntar6
HLC8tar1
Esp100tar8
Etp100tar8
HLC8atar1
HLC8btar1
HLC8tar
MaC9tar
TrC9tar1
HLC7tar1
HLC8tar2
Pfalcstar
Prelcstar
Pbergstar
Pypelcstar
Pbrascstar
Pmalcstar
Poynocstar
Pvlvacstar
Pknowcstar
Esp100tar3
Etp100tar3
Esp100tar4
Etp100tar4
Esp100tar5
Etp100tar5
Esp100tar2
Etp100tar2
Pfalctraptar
Pypelsap2tar
Cptrap1tar2
Frfapntar1
Rafapntar1
Frfapntar2
Rafapntar2
Frfapntar3
Rafapntar3
Frfapntar4
Rafapntar4
Cptrap1tar3
Frfapntar5
Rafapntar5
Cptrap2tar
Huproptar8
Maproptar8
Gppropta8
Gppropta5
Huproptar5
Maproptar5
TrC9tar2
Gppropta3
Huproptar3
Maproptar3
Gppropta1
Maproptar1
Huproptar1
Hutap1tar1
Matap1tar1
Frtap1
Hutap2tar1
Matap2tar1
Chtaptar1
Huproptar4
Maproptar4
Gppropta4
Gppropta2
Huproptar2
Maproptar2
Hutap2tar2
Matap2tar2
Chtaptar2
Hutap1tar2
Matap1tar2
Frtap2
Hutap2tar3
Matap2tar3
Chtaptar3
Hutap1tar3
Matap1tar3
Frtap3
HLC7tar2
HLC8btar2
HLC8tar3
HLC8atar2
Ura3tar2
Ura3tar1
Esp100tar1
Etp100tar1
Cptrap1tar1
6
2
5
4
3
i
1
III
II

3.3.3 Properdin possesses six complete TSR modules

The alignment indicates that properdin does possess six full TSR modules. While some reports have suggested that properdin has six repeats (Goundis & Reid, 1988; Nolan *et al.*, 1991; Smith *et al.*, 1991; Leung-Hagesteijn *et al.*, 1992), it is clear in these alignments that at the end of the sixth repeat the homology with other TSRs is very low, and that two conserved cysteines are absent, including one of the highly conserved C-terminal pair of cysteine residues (figure 3.10a) In one of the reports above, a multiple alignment of TSR sequences includes a sixth TSR of properdin that seems to have both of the conserved C-terminal cysteines of the other TSRs. However, this is due to an error in the properdin sequence used that substitutes a cysteine for a glycine residue (Leung-Hagesteijn *et al.*, 1992). It has been suggested that the C-terminal region of properdin has homology only to the first half of a full TSR (Nolan, 1991; Nolan *et al.,* 1992). However the analysis in this work included only the sequence after the end of TSR5 (residue 350) up to residue 413 of mature human properdin, a region chosen because of its similarity in length to other TSRs. While it is true that the homology to other TSRs extends only over the first half of this region, when the entire C-terminal end of properdin (from residue 351 to the final residue, 442) is included in the alignment procedure, it is clear that there is a further region of homology to the TSR close to the C-terminus of properdin (figure 3.9 and 3.10). A similar alignment of the sixth repeat of properdin is produced when the sequence is aligned either with the other TSRs from properdin, or when added to the extended TSR alignment (as described in materials and methods). Similar results are obtained for the sixth repeat of properdin from human, mouse and guinea pig.

3.3.4 The sixth TSR of properdin contains a large insertion

This new alignment of the sixth TSR of properdin allows some predictions to be made about the structure of the C-terminal end of the properdin monomer. The extra region of TSR homology identified in TSR6 includes both of the highly conserved cysteine residues found at the C-terminus of thrombospondin repeats. Thus it is likely that the sixth repeat is able to satisfy the normal disulphide bonding pattern of a TSR (see later). This was not possible for the previous alignments of the sixth repeat (Goundis & Reid, 1988; Nolan *et al.*, 1992; Smith *et al.*, 1991; Leung-Hagesteijn *et al.*, 1992), in which a cysteine present in 93 of the 95 other known TSRs was apparently missing in the sixth TSR (without the loss of a complementary cysteine elsewhere).

It is possible that the middle region of TSR6 which does not show homology to other TSRs forms a small sub-domain attached to a structure which is otherwise very similar to normal TSRs. This extra inserted sequence contains the conserved N-linked

glycosylation site of human, mouse and guinea pig properdin. A BLAST search of the combined GenEMBL database with the sequence of the insert region (residues 389-424, human numbering) did not reveal any significant overall matches. Intriguingly, a short sequence within this region was found to have homology to the C-terminal end of many CCP repeats, including modules from CR1, CR2, DAF, Factor H and P-selectin. While the CCP repeat is known to be involved in C3b binding in many of these proteins, the individual CCPs known to be required for this activity are not those that score highly in comparison to properdin TSR6. It is probably more likely that the homology detected reflects a conserved structural motif rather than a functional similarity. In the CCP, this region corresponds to the transition from a short β-strand, through a loop and into another β-strand.

The C-terminus of properdin extends for eight amino acids beyond the final cysteine of the TSR. Of these amino acids, six are charged lysine, aspartate or glutamate residues in all three species of properdin. The role of this "C-terminal charged tail" is unknown.

It is noticeable that the position of the insertion in TSR6 coincides with the position of the last intron of the human properdin gene. This intron interrupts the sequence encoding TSR6 within the codon for amino acid 388 (figure 3.10). The other five TSRs of properdin are encoded precisely by single exons. The introns between TSRs 1-6 are of phase 1-1, while the intron interrupting TSR6 is of phase 2-2. The similar phases of the introns between properdin TSRs 1 to 6 suggest that it is likely that the basic tandem TSR repeat structure of properdin evolved by exon shuffling, and that this was followed by the insertion of an intron of different phase into the last TSR6-encoding exon. It is possible that the coding for the extra amino acids in TSR6 was derived from this inserted DNA (perhaps due to the use of a new splice site in the intronic DNA, or by mutation of a splice site and use of a cryptic site). Alternatively, the presence of the intron may have allowed new coding sequence to be brought into the properdin gene by recombination. If such a process was responsible for the generation of the structure of properdin, then a final intron in the same position should also be present in the mouse and guinea pig properdin genes, since both have a similar highly conserved inserted amino acid sequence in TSR6. The structure of the properdin gene in these species is not known. However, the final intron of human properdin contains a MIR element that potentially could have been involved in such events, since MIR elements probably predate the rodent-primate divergence (see above). It would be interesting to examine the sequence of this region of the properdin gene in other species.

3.4 Structural features of the TSR module

3.4.1 Prediction of disulphide bond patterns

The multiple alignment allows a prediction of the possible disulphide bonding pattern of the TSR modules to be made. Cysteines 25 and 110 (numbering as in figure 3.9) are absent from the group 1.iii TSRs, while the group 5 TSRs have cysteines 15 and 104 in addition to 25 and 110. This suggests that cysteine 25 may link to cysteine 110, while cysteine 15 links to 104. This idea is supported by the absence of cysteines 15 and 104 from the second TSR of both *E. tenella* and *E. maxima* p100 proteins.

In group 1 TSRs, the prediction above suggests that cysteine 83 links to cysteine 51. However, in groups 2,3,4 and 6, while cysteine 83 is conserved, cysteine 51 is missing, and an alternative cysteine is present between positions 3 to 5. Thus, it is possible that these groups have an alternative disulphide linkage from cysteine 3-5 to cysteine 83. This is supported by the absence of cysteines 5 and 83 from TSR3 of the both *Eimeria spp.* p100 proteins. It seems that the conserved cysteine at position 83 may form disulphide bonds with cysteines in different positions in the primary sequence in different sub-groups of TSRs. A similar possibility is clear from alignments of the TNF-R/NGF-R module family (Mallet & Barclay, 1991). It is possible that such alternative disulphide bonds could be accommodated with little change in the overall fold if the alternative cysteines were near by in the tertiary structure.

The prediction agrees with that made for group 1 TSRs by Leung-Hagesteijn *et al.* (1992), but differs from that of Smith *et al.* (1991). In the latter case, the prediction relies on a manual alignment of relatively few TSRs. Furthermore, the prediction of the disulphide bonding pattern depends upon the supposed absence of cysteine 98 from properdin TSR6. However, as described above, TSR6 probably does possess the full complement of cysteine residues found in other TSRs. Although the pattern suggested here is consistent with the positions of the conserved cysteines in all the known TSRs, confirmation of the prediction requires disulphide mapping or 3-D structure determination of the modules involved. Such studies have not been reported for any TSRs.

Another feature clear from the alignment of the properdin TSRs, is the presence of an extra cysteine residue in both TSR1 and TSR2, and the apparent shift in position of the conserved cysteine 83 in TSR1 (numbering as in figure 3.9; residue 84 of the properdin protein). Since no free sulphydryl groups have been detected in human properdin (Nolan and Reid, 1993), it seems likely that there is a disulphide bond linking TSR1 to TSR2 in properdin (see figure 3.15). It is possible that this extra covalent bond between TSRs could restrict flexibility between these two modules. However, from electron microscopy, it seems that there may be flexible regions between most of the TSRs of properdin, since the elongated rods visible within the properdin oligomers do not

appear to be rigid (Smith *et al.*, 1984a). An insertion of two amino acids found between TSRs 5 and 6 of guinea pig properdin, compared to the other two species, suggests that there may be flexibility in this region.

3.4.2 The predicted secondary structure of the TSR module

Secondary structure predictions for the TSR module have previously been made using the methods of Garnier *et al.* (1978) and Chou and Fasman (1978). Both these methods are based on the secondary structure forming propensities of individual amino acids. These predictions indicate that TSRs are likely to contain predominantly β-turn and elements of β-sheet, although one of the methods suggests a region of α-helix. A predominance of β-turn and β-sheet over α-helix in the TSR is also suggested by FT-infrared studies of properdin and complement component C9 (Perkins et al., 1989; Perkins *et al.* 1993).

An E-mail server, PredictProtein@EMBL-Heidelberg.de, allows access to a secondary structure prediction algorithm which makes use of the extra information which can be derived from multiple sequence alignments. The prediction is produced by the profile network method, called PHDsec (Rost and Sander, 1993, 1994). In addition, the method has much improved β-strand prediction as a result of balanced training. The secondary structure prediction method is rated at over 72% average accuracy for water-soluble globular proteins, in the three states helix, strand, and loop. That is, over seventy percent of residues in 250 test proteins of known structure were correctly predicted to fall into one of the three secondary structure classes. For example, the program recently correctly predicted the position and class of seven of eight secondary structure regions in the src-homology SH3 domain (Rost and Sander, 1992). The program aligns submitted sequences with other similar sequences in the SwissProt database using the program MaxHom (Sander and Schneider, 1991), and then carries out the structural predictions on the alignment. Alternatively, user-defined multiple sequence alignments can be submitted.

Representatives of each of the six sub-groups of TSRs were submitted to the PredictProtein server, as well as the group 1.i multiple alignment discussed above. The results are shown in figure 3.12, and summarised together with the sequence alignment in figure 3.9. The predictions of secondary structure are similar for all of the TSR subgroups. Beta-strands are predicted for all sub-groups spanning the conserved arginine cluster in the centre of the TSR. In addition, β-strands are predicted for all the other conserved blocks of sequence in the multiple alignment in at least three of the sub-groups. The similarity of the secondary structure predictions for the different subgroups in regions which are diverse in primary sequence lends support to the hypothesis that all the sub-groups have similar folds.

In conclusion, the method predicts regions of β-strand coinciding with the conserved blocks of sequence in the multiple alignment, and loops outside these regions. The high proportion of loop structure predicted for the group 1 TSRs is due to the high proportion of proline, glycine and serine residues found in these modules. No α-helical regions are predicted. Such a structure, with conserved elements of secondary β-strand structure linked by less conserved loop regions is common in extracellular modules of known tertiary structure (e.g. the complement control module and the epidermal growth factor repeat (Norman *et al.*, 1991; Campbell and Bork, 1993)). Functional regions in such modules are often found in the loop regions, but the edges of conserved β-sheets can also be involved in interactions with other molecules.

3.5 The N-terminal region of properdin

The sequence of the N-terminus of properdin is known for human, mouse and guinea pig from cDNA sequence. In addition, the sequence of the first 38 amino acids of rabbit properdin have been reported from protein sequencing. The N-terminal region is not homologous to the TSRs which constitute the rest of properdin. A search of the SwissProt and combined translated GenEMBL databases using BLAST failed to identify regions in any other proteins which are related to this domain. The PHDsec algorithm was used to predict the secondary structure of the region from an alignment of the N-termini from the four species of properdin. The results are in marked contrast to those from a previous prediction based on the human and mouse sequences using the less sophisticated methods of Robson and Chou-Fasman (Perkins *et al.*, 1989; Smith *et al.*, 1991). Rather than the β-sheet structure previously suggested, the PHDsec algorithm strongly predicts the presence of two α-helical regions within the N-terminus, together with a shorter element of β-sheet (figure 3.13). This is consistent with the small proportion of α-helix predicted from analysis of the FT-infrared spectrum of properdin (Perkins *et al.*, 1989). It should be noted that because only four similar sequences were available to make this prediction, it may be less reliable than those for the TSRs. However, it is likely to be more accurate than the previous predictions.

Since there is an even number of cysteine residues in the N-terminal region of properdin, and no free sulphydryls have been detected in properdin (Nolan and Reid, 1993), it is likely that the six cysteines of the N-terminus are involved in intra-domain disulphide bonds.

Figure 3.12. PHDsec secondary structure prediction for the various groups of TSRs.
The program delivers:
`PHD sec`: prediction of secondary structure for all residues, with an expected average three-state accuracy of > 72% (helix (H), beta sheet (E) or loop (L));
`Rel sec`: reliability index for prediction (0 lowest, and 9 highest reliability);
`pr(H/E/L) sec`: score for each residue as helix, sheet or loop;
`SUB sec`: prediction of secondary structure for reliably scored residues only, with an expected three-state accuracy for these residues of > 82%;
`P_3 and PHD sec`: prediction of solvent accessibility for all residues with an expected average correlation to the experimentally observed values of 0.54 (three state prediction of buried (b) intermediate (i) or exposed (e) and ten state prediction between buried (0) and exposed (9));
`Rel acc`: reliability index for prediction (0 lowest, and 9 highest reliability);
`SUB acc`: prediction of solvent accessibility for reliably scored residues only, with an expected correlation between experimental observation and prediction of 0.69.

Group 1.i TSRs (AMPS group 1.i alignment, without propedin TSR6, submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,....6....,....7....,...
        AA      |....VNGQWGPWSPW.SAC......TVTCGGG....IRERSRLCNSPEP..QYGGKPCVG.DTKQHDMCNKR.DCPI.|
        PHD sec |                            EE          EEEEE                                  |
        Rel sec |997889997777888866764257611131499999753367642899988878888669987753212488989899|
detail:
        prH sec |000000001111100011000001111100000000000000000000010000000000111111101000000|
        prE sec |001000000000000001012321134454300000123677763100000011000110000013333210000000|
        prL sec |998889998778888877876577744434699988765321235899888888888779987765445678888899|
subset: SUB sec |LLLLLLLLLLLLLLLLLLL..LLL......LLLLLL..EEE..LLLLLLLLLLLLLLLLLLL.....LLLLLLLL|

ACCESSIBILITY
3st:    P_3 acc |eebebeeebbbbbebbbbbbbbbbebbbbeebbeebbbbbbbbbebeeeeeebeeebbebbbbbbbebbeebeebeee|
10st:   PHD acc |990909990000090000001010900009900990010001009099999919990090000000900990990999|
        Rel acc |991929993314495633983416938599942991435465899299999919992790313415959991996999|
subset: SUB acc |ee.e.eee...bbebb..bb.b.be.bbbeeb.ee.b.bbbbbbe.eeeeee.eee.be....b.bebbee.eebeee|
```

Group 1 TSRs (human properdin TSR4 submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,..
        AA      |VAGGWGPWGPVSPCPVTCGLGQTMEQRTCNHPVPQHGGPFCAGDATRTHCNTAVPCP|
        PHD sec |     E       EEEEE     EEEEE                              |
        Rel sec |999950318876725564899725775538999899998533589987888999999|
detail:
        prH sec |000001000000000000000000000000000000000000000000010000000|
        prE sec |000024341011156676100247887731000100000233200001000000000|
        prL sec |999974558887832223899752112268999899999875678888888899999|
subset: SUB sec |LLLLL...LLLLL.EEE.LLLL.EEEEE.LLLLLLLLLLL..LLLLLLLLLLLLLL|

ACCESSIBILITY
3st:    P_3 acc |eeeebbbbbebbbbbbbbeebeebbbbbbbeeebbeebbeebeeeeeeeeebeeeebe|
10st:   PHD acc |999900000900000000099099000000999009900990999999999909999909|
        Rel acc |999985377935075658990990025499993199039989999999909999949|
subset: SUB acc |eeeebb.bbe.b.bbbbbee.ee...bbbeee..ee..eebeeeeeeeee.eeeebe|
```

Group 1 TSRs (human thrombospondin 1 TSR2 submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,....
        AA      |FKQDGGWSHWSPWSSCSVTCGDGVITRIRLCNSPSPQMNGKPCEGEARETKACKKDACP|
        PHD sec |                          EEEEEE                          |
        Rel sec |988999713489998421125985038974489999999997767677753367999999|
detail:
        prH sec |000000000000000000000000000000000000000000010001111233100000|
        prE sec |000000143210000344432012458886310000000000111000000011000000|
        prL sec |998999745689998654457887431013689999999988878777866578999999|
subset: SUB sec |LLLLLLL...LLLLL.....LLLL..EEE..LLLLLLLLLLLLLLLLLL..LLLLLLL|

ACCESSIBILITY
3st:    P_3 acc |beeeeebbbbbebbebbbbbbebbbbbbbbbbeeebeeeeeeebebeeeebeebeeeeebe|
10st:   PHD acc |199999000009009000000900000000009909999999909099990990999909|
        Rel acc |099999625659779888792953148352799919999999809999909989999939|
subset: SUB acc |.eeeeeb.bbbebbebbbbb.eb..bb.b.beee.eeeeeeebe.eeee.eebeeee.e|
```

Group 2 TSRs (human complement C9 TSR submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,
        AA      |IDCRMSPWSEWSQCDPCLRQMFRSRSIEVFGQFNGKRCTDAVGDRRQCVPTEPCE|
        PHD sec |   E                  EEEEEEEE                         |
        Rel sec |992128998976788856851479964233777998674766873545799988 9|
detail:
        prH sec |000000000011100001000000000000000001102111013211000000 0|
        prE sec |004440000000010021024688876563111000111000000121100000 0|
        prL sec |995458998977788866875311023436878998786777875667788988 9|
subset: SUB sec |LL...LLLLLLLLLLLLLL..EEEE....LLLLLLLL.LLLLL.L.LLLLLLLL|

ACCESSIBILITY
3st:    P_3 acc |bebebbebbebbebbebbeebbbebbbebbbbbbebeebbeebeebeebeeeeeebe|
10st:   PHD acc |090900900900900900990009000900000090990099099099099999909|
        Rel acc |199987967965983966992659315901017929980994991997999992 9|
subset: SUB acc |.ebebbebbebbeb.ebbee.bbe..be....be.eeb.eebee.eebeeeee.e|
```

Group 3 TSRs (Rat F-spondin TSR2 submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,..
        AA       |STCTMSEWITWSPCSVSCGMGMRSRERYVKQFPEDGSVCMLPTEETEKCTVNEECSP|
        PHD sec  |  EE   EE     E EE    EEEEEEEE      EEEE       E         |
        Rel sec  |93262431222653432438644333335616999956664997633347887448 9|
 detail:
        prH sec  |00000000000000000000001333332100000000000000000000000000|
        prE sec  |03573335543223635631236566656742000077773001133631111320 0|
        prL sec  |96425664455776354268762100001157999921226998755368888668 9|
 subset: SUB sec |L..E.......LL......LL.......EE.LLLLLEEEE.LLLL....LLLL..LL|

 ACCESSIBILITY
 3st:    P_3 acc |eebbbbbbbebbbbbbbbbbbbbbebbbeebeeebebbbbeeeeeeebebeeeeee|
 10st:   PHD acc |99000000090000000000000090009909990900009999999090999999|
         Rel acc |99727328898819985943661689476994999292633999999989099999 9|
 subset: SUB acc |eeb.b..bbebb.bbbbbb.bb.bbebbbeebeee.e.b..eeeeeeebe.eeeeee|
```

Group 4 TSRs (*Eimeria tenella* p100 TSR4 submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,....6
        AA       |IDATCGEWTEYSACSRTCGGGTQERKREPWLDNAQHGGRTCMEQYPDGPISVRECNTQPC|
        PHD sec  |              EE          EEEEE           EE                 |
        Rel sec  |99988897898737613599874413442599989366732268999999998988899|
 detail:
        prH sec  |00000000000000000000000000000000000000001000000000000000 1000|
        prE sec  |00000001101137743200012245665200010311125510000000000000000 0000|
        prL sec  |99988898898861155699876643223799988577533788999999888888899|
 subset: SUB sec |LLLLLLLLLLL.EE..LLLLL.......LLLLLL.LLL...LLLLLLLLLLLLLLLLL|

 ACCESSIBILITY
 3st:    P_3 acc |eeeebbebbebbbbbbbbbebbbebebbbeeeeeebbbeebbeebeeeebeeeebbeeeb|
 10st:   PHD acc |99990090090000000090009090009999990009900990999909999009990|
         Rel acc |99994397697409778909781969246999999103999699699995999960999 0|
 subset: SUB acc |eeeeb.ebbebb.bbbbb.ebb.ebe.bbeeeeee...eebbeebeeeebeeeeb.eee.|
```

Group 4 TSRs (*Plasmodium falciparum* thrombospondin related anonymous protein TSR submitted)

```
                 ....,....1....,....2....,....3....,....4....,...
        AA       |KTASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSEIQEQCEEERCP|
        PHD sec  |  EE  E      EEEE    EEEEEEEE       E EEEEEE    |
        Rel sec  |91432122455991322399625677552467998611255312116 9|
 detail:
        prH sec  |00000000000000000000000000000000000000000000000000|
        prE sec  |04664454322005655300257787765221001244576555442 0|
        prL sec  |95235545677884333699742211223678998744422344557 9|
 subset: SUB sec |L........LLLL.....LLL.EEEEEE..LLLLLL...EE.....LL|

 ACCESSIBILITY
 3st:    P_3 acc |eebebebbbebbebbbbbbebbbbbeebbeeeeeeeebeeebebeebe|
 10st:   PHD acc |99090900090090000009000009900999999909990909909|
         Rel acc |99196966198798897929570469922999999919997919969|
 subset: SUB acc |ee.ebebb.ebbebbbbb.ebb.bbee..eeeeeeee.eeebe.eebe|
```

Group 5 TSRs (*Plasmodium falciparum* circumsporozoite protein TSR submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,
        AA       |KKIKNSISTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYENDIEKKICKMEKCS|
        PHD sec  |                     EEEEE                    EEEE   E  |
        Rel sec  |97424266324883134488528899169999996665545565111222121 69|
 detail:
        prH sec  |00000000000000000000000000010000000222212222233332110 00|
        prE sec  |01232321343113432310258898310000000001110000144443434 20|
        prL sec  |98656577656885556688741000479989997776666777422134454 79|
 subset: SUB sec |LL....LL...LL.....LLL.EEEE.LLLLLLLLLLLL.LLLL.........LL|

 ACCESSIBILITY
 3st:    P_3 acc |eebeeebbeebbebbbbbbebbbbbbeeeeeeeeeeeebebbeebeebbbebeebe|
 10st:   PHD acc |99099900990090000009000000999999999990900990990009099 09|
         Rel acc |99899970998699686939696772999999999996933997990799799 69|
 subset: SUB acc |eebeeeb.eebbebbbbb.ebbbbb.eeeeeeeeeeebe..eebee.bbebeebe|
```

Group 6 TSRs (human connective tissue growth factor TSR submitted)

```
                 ....,....1....,....2....,....3....,....4....,....5....,....6....,
        AA       |PTMIRANCLVQTTEWSACSKTCGMGISTRVTNDNASCRLEKQSRLCMVRPCEADLEENIKKGKKC|
        PHD sec  |       EEE                EEEE     EEEEEE  EEEE               |
        Rel sec  |97488661326873246556567971578759997354564225886489998660214569999|
 detail:
        prH sec  |00000100000000000001111000000000000000000000000000001224452220000|
        prE sec  |01210115552013322221211014778820001666676446887310000000000000000|
        prL sec  |98688774337876567667677884211179998322222552111689998774546779999|
 subset: SUB sec |LL.LLLL...LLL...LLLLLLLLL.EEEELLLLL.E.EE...EEEE.LLLLLLL....LLLLLL|

 ACCESSIBILITY
 3st:    P_3 acc |eeeeeeebbbbbbebbebbbbbbebbbbbbeeeeebbebbebbbbbbbbbbeeebeeeeeeeeee|
 10st:   PHD acc |99999990000009009000000900000099999009009000000010099909999999999|
         Rel acc |99999999723329869973492959256599999289629553791872899909999999999|
 subset: SUB acc |eeeeeeebb....ebbebb.bb.ebb.bbbeeeee.beb.ebb.bb.bb.beee.eeeeeeeeee|
```

```
Properdin N-terminus
                  ...,....1....,....2....,....3....,....4....,....
         AA       |DPVLCFTQYEESSGKCKGLLGGGVSVEDCCLNTAFAYQKRSGGLCQPCR|
         PHD sec  |  EEEEEE       HHHH     EE HHHHHHHHHHHHH   E     |
         Rel sec  |986998634798755132247992141456567999997239811457 9|
 detail:
         prH sec  |000000000000122455421000013667678999987530011000 0|
         prE sec  |017998763100001211221003562000000000000000044221 0|
         prL sec  |982001236788866323257995423222221000002458844668 9|
 subset: SUB sec  |LLEEEEE..LLLLLL.....LLL.....HHHHHHHHHHH..LL...LLL|

 ACCESSIBILITY
 3st:    P_3 acc  |eebbbbbbbeeeeeebeebbeeebbbbbbbbbbbbbeeeeeebbbebe|
 10st:   PHD acc  |990000009999990990099900000000000009999990000909|
         Rel acc  |997999238999999899769997073299932463699999939297 9|
 subset: SUB acc  |eebbbb..beeeeeebeebbeeeb.b..bbb..bb.beeeeee.b.ebe|
```

Figure 3.13

Secondary structure prediction by PHDsec for properdin N-terminal region.

For details see legend of figure 3.12.

3.6 The prediction of potentially important functional regions in properdin

3.6.1 Conservation of amino acid sequence between the different species in properdin

The conservation of amino acid residues in the same protein of different species can indicate regions that are important for function. For example, a candidate properdin binding site in C3b was first identified through a comparison of the sequences of C3 from various species, and then confirmed by peptide binding studies (Daoudaki *et al.*, 1988). The region is conserved in the species sequenced to date which clearly have a C3 molecule distinct from C4 (see introduction and figure 1.3) (including trout, Xenopus and mammalian C3 (Lambris *et al.*, 1993)). The protein sequences of human, mouse and guinea pig properdin were compared (figure 3.10b), and pairwise and three-way percentage identities were calculated for each module. In addition, the pairwise significance scores from the AMPS alignment were averaged for each module, to give an indication of the overall homology between species for each TSR, and for the N-terminus (table 3.1). On the basis of the three-way percentage identity and the average pairwise identities, the first and second TSRs of properdin are the most conserved between the three species, and the fifth TSR is the least conserved. However, on the basis of the significance scores derived from the Mutation Data Matrix of Dayhoff (which scores residues based on their conservation in analogous proteins during evolution), the sixth TSR is the most highly conserved. It is clear that comparisons of the properdin TSRs at this level can provide little clue as to the regions important for function. It may be that conservation of smaller motifs, or a few crucial residues distant in the primary sequence but close in the tertiary fold, are important.

An analysis of the conservation of charged, polar and non-polar residues in properdin was also carried out in order to find potentially interesting features (figure 3.14). The N-terminus, and particularly the C-terminus of TSR6 contain many conserved acidic and basic residues. It is possible that some of these residues are involved in the strong non-covalent head-to-tail association of the properdin monomers. The finding that the oligomers of properdin are dissociated at pH 2.5 (see chapter 4 and Pangburn, 1989) supports the contention that ionic interactions are important for oligomerization. All the TSRs in the multiple alignment, including the properdin TSRs, have a region containing conserved arginine residues in the centre of the primary sequence. Such strong conservation may be indicative of a structural role for these amino acids. Indeed, a recent report highlights the presence of arginine residues in the core of many proteins (Borders *et al.*, 1994). This RxR motif has been implicated in the binding of TSR modules of some proteins to sulphated glycoconjugates (see introduction), suggesting that it may be exposed. This region of the TSR is predicted to form a β-strand in all of the sub-classes discussed above.

Table 3.1

Conservation of properdin protein sequence between human, mouse and guinea pig.

region	three-way % identity[1]	average % pairwise identity[2]	average significance score[3]
N-terminus	59	74	16.7
TSR1	76	83	17.2
TSR2	74	81	17.8
TSR3	67	71	20.1
TSR4	69	79	21.8
TSR5	50	61	17.9
TSR6	67	77	23.5

[1]Percentage of residues conserved in properdin of all three species

[2]Average of the three pairwise percentage identities

[3]Average of the three pairwise significance scores from the AMPS multiple alignment, calculated using the Dayhoff Mutation Data Matrix (PAM250).

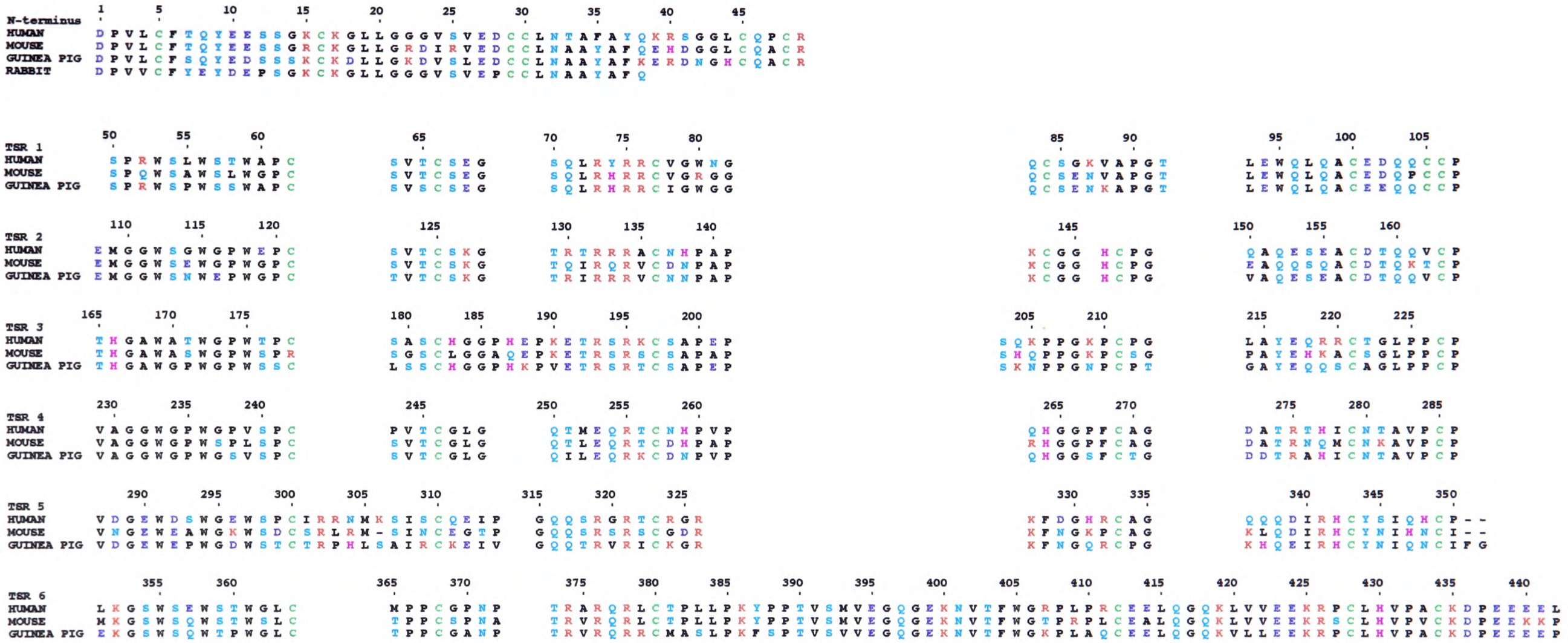


Figure 3.14

The conservation of charged, polar and non-polar amino acids in the protein sequences of human, mouse, guinea pig, and rabbit properdin. Acidic residues are shown in blue, basic residues in red, histidines in magenta, polar residues in cyan, and cysteines in green. The thrombospondin repeat alignment was generated using the AMPS package (see text).

Studies on a variety of glycosaminoglycan binding proteins have revealed an essential role for cationic amino acids (Lander, 1994). These sulphated sugar binding sites are often formed by the juxtaposition of amino acids that are distant in the primary structure (for example in anti-thrombin III (Carrell *et al.*, 1994)). The second TSR of properdin is noteworthy for an extended basic region around the RxR motif in the primary sequence. Also, the fifth TSR is highly charged, with a preponderance of acidic residues near the N-terminal end, and a larger number of basic residues throughout the module. The positions of these residues in the tertiary structure is, of course, unknown.

3.6.2 Insertions in the TSRs of properdin

The multiple alignment and secondary structure prediction highlight regions of properdin which are likely to form loops not found in other TSRs and which may be important in function. For example, the third TSR in all three species contains an short insert region immediately N-terminal of the conserved RxR motif. This sequence contains both acidic and basic residues, but is not highly conserved in the three species of properdin known. The long insertion present in TSR6 has been discussed above. Much of this region is highly conserved in the three species, possibly reflecting an important role (perhaps in oligomerization).

The fifth TSR of properdin also contains an insertion of five to six amino acids which is not typical of thrombospondin type I repeats. This insertion is in the same region which often contains the CSVTCG motif in other TSRs. It is likely that this region forms an exposed loop since mild trypsin digestion of human properdin results in specific cleavage of the polypeptide chain within this sequence (Reid and Gagnon, 1981, and chapter 4). Hubbard *et al.* (1994) suggest that loops susceptible to trypsin in this way are normally at least nine residues in length. In human properdin, the tryptic cleavage site lies within a sequence of nine amino acids between two cysteine residues. This suggests that despite the proximity of the cysteines, which are likely to be involved in core disulphide bonds with other cysteines, the loop must be exposed to solvent and highly extended. The loop is not highly conserved in the three species of properdin sequenced to date. Indeed, in the mouse sequence the insertion in TSR5 is one amino acid shorter than in the other species. The protease susceptibility of this region in the fifth repeat of properdin suggests that the CSVTCG motif found at this position in other TSRs also may be at the surface of the protein, and available for interaction with other molecules (as discussed in the introduction). This CSVTCG motif is highly conserved in TSRs of thrombospondin, F-spondin, and the TRAP and circumsporozoite parasite proteins. The motif is absent from the terminal complement components and from Unc-5, and poorly conserved in Eimeria p100 proteins and in the connective tissue growth factor-like proteins. Thus it is possible that this sequence may be important in function

(as discussed in the introduction) rather than being a core structural motif. In properdin, TSR1 and TSR2 have the completely conserved sequence PCSVTCS in all three species, while the sequence PCSVTCG is conserved in mouse and guinea pig TSR4. In human TSR4 the analogous sequence is PCPVTCG. The remaining TSRs of properdin clearly do not possess this motif.

3.7 Summary

Multiple sequence alignment of the thrombospondin type I repeats from properdin and other proteins allowed classification of the sequences into six sub-families, and the identification of conserved and unusual features (figure 3.9). The alignment indicates that, contrary to some previous reports, properdin does contain six complete TSRs. The sixth repeat contains an insertion of around thirty amino acids which is peculiar to this module. A new model for the structure of the properdin monomer is proposed (figure 3.15). The alignment also highlights an insertion of six amino acids in properdin TSR5 which, while not highly conserved in sequence between species, is found in properdin of all known species and not in any other TSR. This region is highly sensitive to trypsin digestion and thus is almost certainly at the surface of the protein. Furthermore, this loop is located in the same position within the primary sequence of the TSR as the CSVTCG motif which has been implicated in the function of TSRs from thrombospondin and *Plasmodium* surface proteins. The multiple alignment also allowed the prediction of disulphide bonding patterns for the various TSR sub-families which are consistent with the positions of the cysteine residues in all the known TSRs. Secondary structure predictions suggest that TSRs are composed predominantly of beta-strands, connected by loops of variable length. The N-terminal region of properdin is predicted to contain at least one alpha-helical region. These predictions are consistent with the results of FT-infra red spectroscopy (Perkins *et al.*, 1989).

Primer extension analysis and RACE-PCR have allowed the transcription initiation site at the 5' end of the human properdin gene to be defined. These results, together with those of Nolan (1991), make clear the genomic structure of human properdin (see figure 3.1). The gene has ten exons. The first exon is untranslated, while the second exon includes sixty-five untranslated nucleotides and coding sequence for all but two residues of the protein leader peptide. Exon three encodes the N-terminal region and exons four to eight encode precisely each of the first five TSR modules of properdin. The coding for the sixth TSR is split over exons nine and ten. The final intron (between exons nine and ten) interrupts TSR6 at the 5' end of the inserted sequence. It is possible that the extra coding region found in TSR6 originates from the intron. A number of repetitive elements are found in and around the human properdin gene (see figure 3.1).

In addition to the Alu repeats previously identified (Nolan, 1991), a MER20 element is located between exons 3 and 4. Two MIR elements, both interrupted by Alu repeats, are also found in the properdin gene. It is possible that recombination between such repetitive elements was important during the evolution of the properdin gene, and that similar events may be responsible for some cases of properdin deficiency.

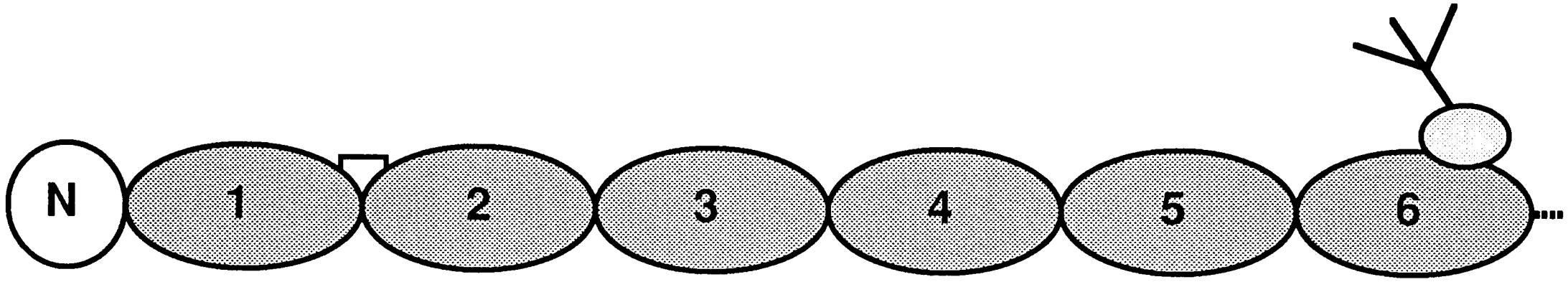


Figure 3.15
A new model for the structure of the properdin monomer (see text for details).

Chapter 4

Chapter 4

Expression of wild type recombinant human properdin in Chinese Hamster Ovary Cells

Human properdin was expressed in Chinese Hamster Ovary (CHO) cells. This decision was made for several reasons. A mammalian cell line was chosen in order to maximize the likelihood that the oligomers of properdin would be synthesized and secreted in their native conformation. It was considered more probable that a mammalian cell would have the machinery necessary for the synthesis of a complex molecule such as properdin rather than a bacterial, or yeast, cell. Mammalian cells are more likely to have the chaperone molecules needed to ensure the correct folding and formation of oligomers in the complex environment of the endoplasmic reticulum. Also, properdin contains a high number of cysteine residues which are involved in the twenty-three disulphide bonds estimated to be present in each monomer. Problems are often encountered with expression of such proteins in yeast and bacterial cells. Indeed, attempts to express single TSRs from properdin in these cell types in this laboratory have yielded only covalently linked aggregates (Dr Armin Sepp, personal communication). Furthermore, mammalian cells add complex carbohydrate chains to expressed proteins. This is not the case in bacterial or yeast cells, and varies in insect cell expression systems depending on factors such as the period of culture (Davidson & Castellino, 1991). A preliminary ELISA for human properdin showed no immunoreactivity in conditioned 10% serum-containing medium from CHO cells, suggesting that these cells would be appropriate for subsequent purification of recombinant properdin.

The glutamine synthetase expression system has been used with success with Chinese Hamster Ovary (CHO) cells for the production of a number of secreted proteins, including the tissue inhibitor of metalloproteinases (Cockett *et al.*, 1990), complement C4 (Sepp *et al.*, 1993), and a secreted form of rat CD4 (Davis *et al.*, 1990). In this system, the glutamine synthetase gene (GS) is used as a dominant selectable marker in cells that already contain active GS genes. CHO-K1 cells are able to grow in medium without glutamine provided that glutamate and ammonia are provided as substrates for GS. This essential endogenous GS activity can be inhibited with methionine L-sulphoximine, leading to the death of normal CHO cells. However, if intracellular GS levels are increased by the provision of exogenous GS on a plasmid, then transfected cells can overcome the lethal metabolic block. This allows the selection of cells which have taken up the plasmid DNA (Bebbington and Hentschel, 1987).

If a gene encoding a protein of interest is included on the GS plasmid, downstream of a strong promoter, then the system can be used for expression of a

foreign protein. The vector pEE6.HCMV.GS contains the a GS minigene under the SV40 late promoter, the human cytomegalovirus promoter upstream of a cloning site, an SV40 early polyA signal downstream of this site, a bacterial origin of replication and an ampicillin resistance gene (figure 4.1). This vector can be maintained in *E. coli*, simplifying manipulation of the vector, and insertion of the foreign coding region into the cloning site. After transfection of CHO cells, those cells which have taken up the plasmid can be selected by growth in medium without glutamine and containing MSX. In some cases, the plasmid will become integrated into the chromosomal DNA of the host cell so that the exogenous coding sequences are retained as the cell divides. Such stable transfectants are useful for the expression of high levels of the foreign protein.

A further advantage of this expression system is that GS is an amplifiable marker. That is, if cell-lines containing the GS gene are grown in the presence of increasing concentrations of the selective agent (MSX), then variant clones will emerge that are more resistant to the drug. This occurs through an increase in, or "amplification" of, the number of copies of the GS gene. These extra copies of the GS gene reside either within the chromosome or as smaller extrachromosal regions of DNA. Because the region of DNA that is amplified in such cases is often much larger than the marker gene itself, this process also leads to amplification of the foreign gene and to increased expression of the foreign protein (Cockett *et al.*, 1990).

4.1 Construction of the vector for expression of normal human properdin

Before producing mutant forms of properdin, it was important to check that wild type recombinant human properdin was identical, in structure and function, to properdin purified from normal human serum. To this end, a pEE6.HCMV.GS expression vector containing the coding sequence for human properdin was constructed, as described in Materials and Methods. An almost full-length coding sequence was generated in pBluescript KS(-) by the fusion of two partial human properdin cDNAs cloned and sequenced by Dr Kathleen Nolan (to create the vector pKNPCKS-1/3, see figure 4.2). A PCR fragment containing coding for the leader sequence was produced by amplification of the 5' end of the properdin cDNA using a 5' primer with the appropriate additional nucleotides, according to the genomic sequence. In addition, a further round of PCR was used to create a DNA fragment with an efficient translation start site (GCCGCCACCAUG (Kozak, 1991; Cavener and Ray, 1991) and an *Xba* I restriction site at the beginning of the leader sequence. This fragment was cloned into the pKNPCKS-1/3 at the 5' end of the properdin cDNA. PCR was also used to create a fragment with a *Bgl* II restriction site at the extreme 3' end of the cDNA. This fragment was cloned into pKNPCKS-1/3 at the 3' end of the properdin cDNA to create

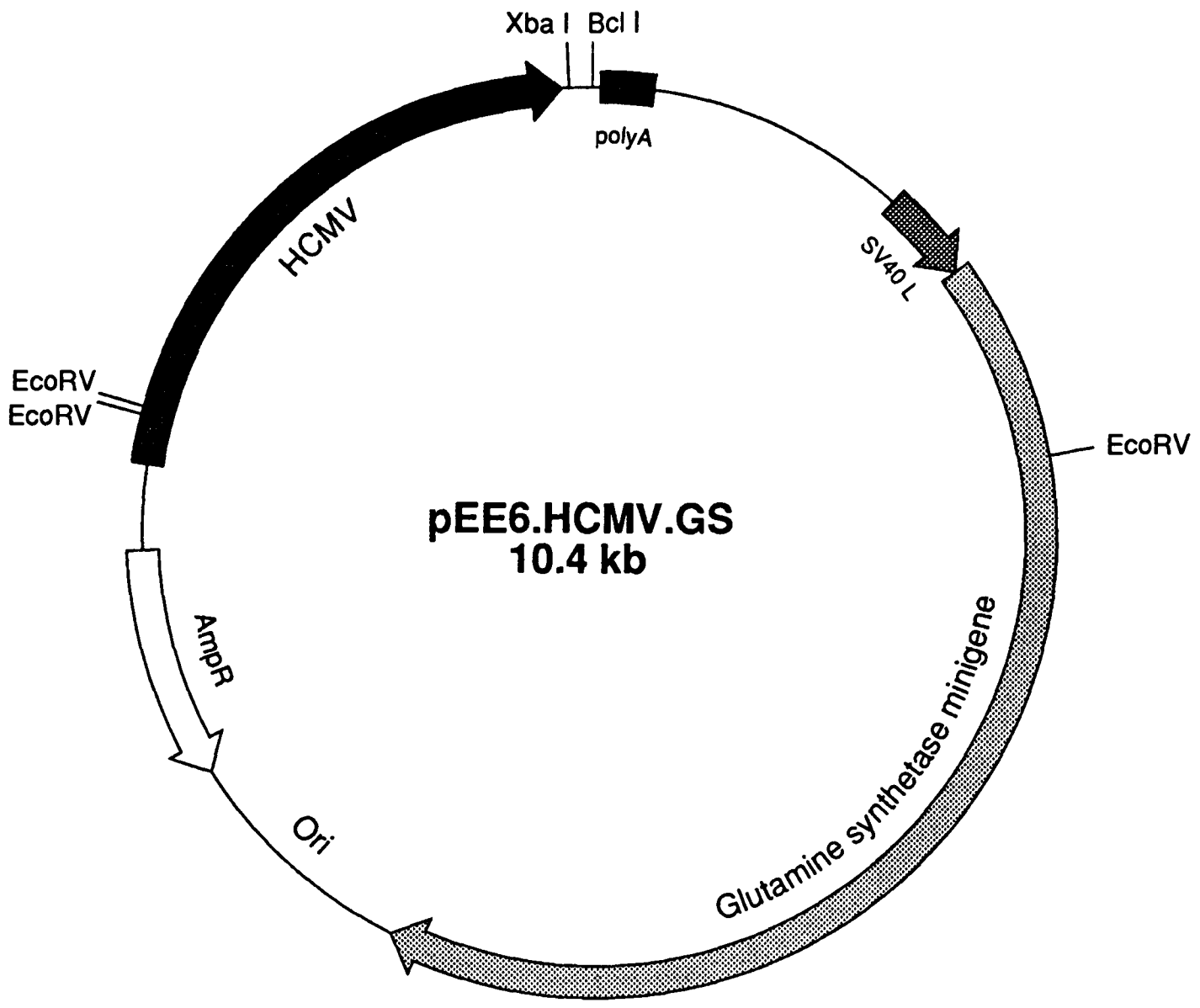
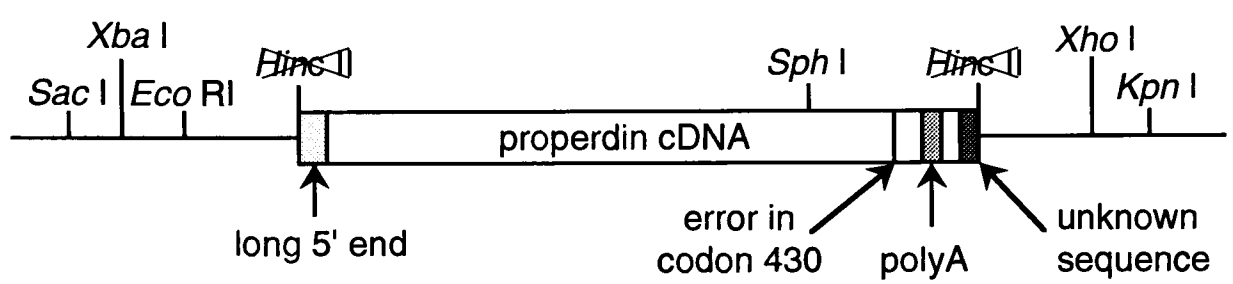


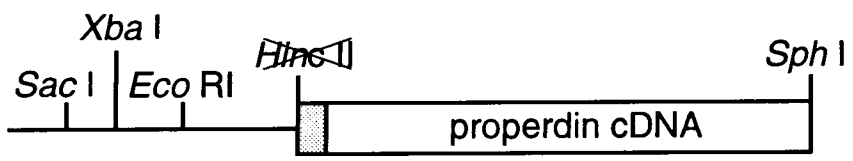
Figure 4.1
The expression vector used for production of recombinant human properdin in Chinese Hamster ovary cells, pEE6.HCMV.GS (Stephens & Cockett, 1989). The vector contains the human cytomegalovirus major immediate early promoter (HCMV) to drive expression of the foreign gene, and the Sendai Virus 40 early polyadenylation signal sequence (polyA). Between these two elements is the multiple cloning site. Properdin constructs were inserted uni-directionally into *Xba* I - *Bcl* I cut vector. Cleavage with *Eco* RV was used to confirm the presence of inserts.
The vector also carries a glutamine synthetase gene under the Sendai Virus 40 late promoter (SV40L) for selection in mammalian cells, and a bacterial origin of replication (Ori) and an ampicillin resistance gene (AmpR) for selection in *E. coli*.

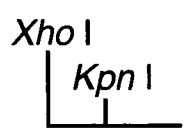
clone pKNPCKS-1



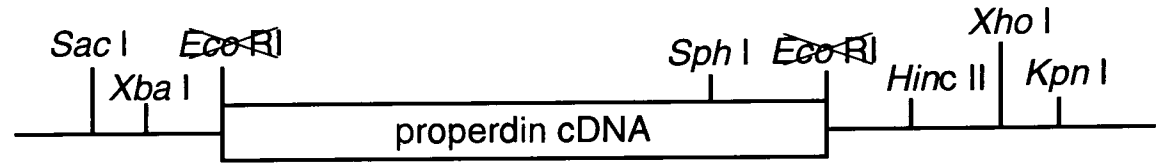
Sph I + *Xho* I

isolate cleaved vector



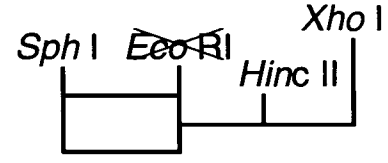


clone pKNPCSK-3



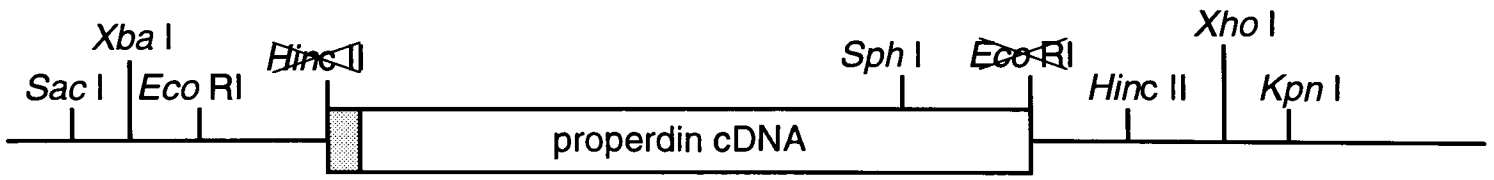
Sph I + *Xho* I

isolate fragment

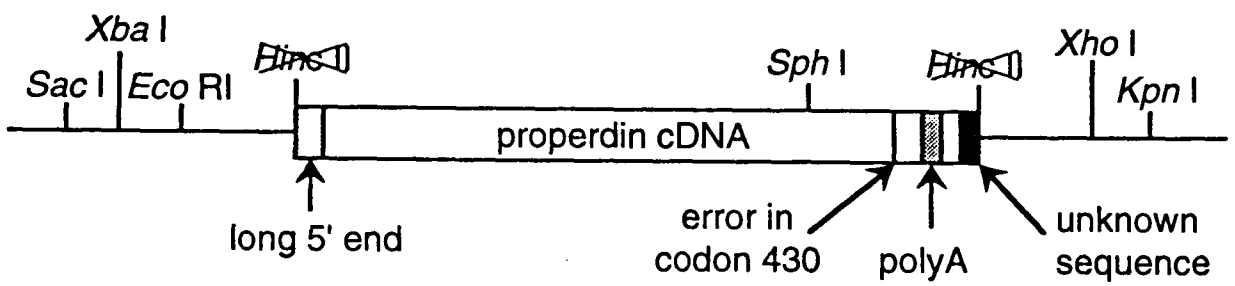


Ligation

clone pKNPCKS-1/3

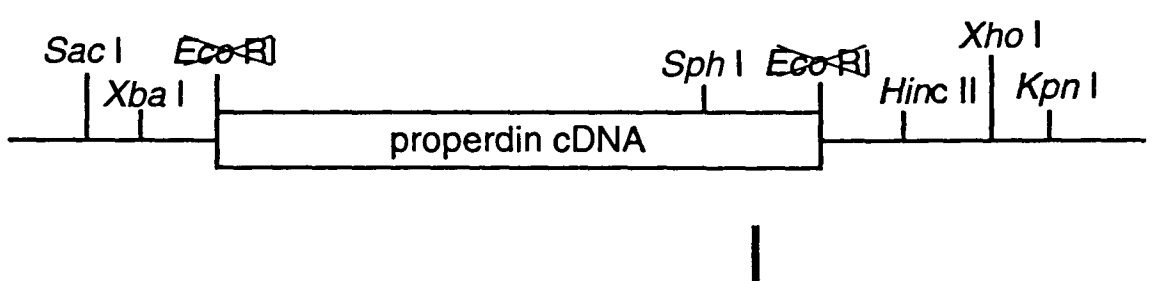


clone pKNPCKS-1

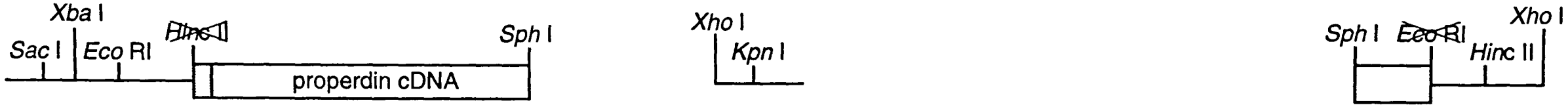


Sph I + *Xho* I — isolate cleaved vector

clone pKNPCSK-3



Sph I + *Xho* I — isolate fragment



Ligation

clone pKNPCKS-1/3

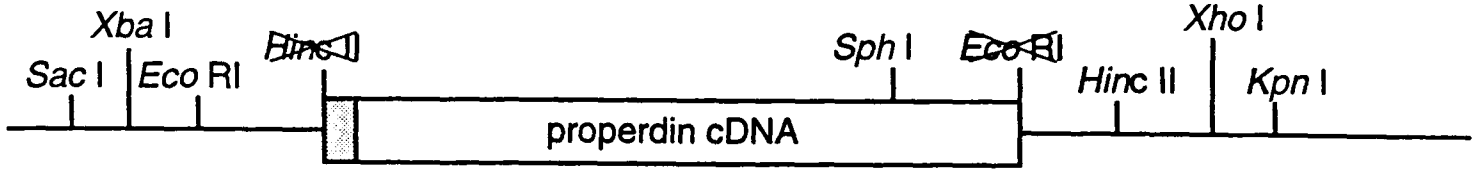


Figure 4.2
Construction of an almost full length coding sequence for human properdin in pBluescript KS(-) (not to scale).
The plasmid, pKNPCKS-1, comprises pBluescript KS(-) with a human properdin cDNA clone (at the *Hinc* II site) that contains the complete 3' untranslated region, but has a single base error in codon 430 which changes a histidine to an arginine residue. This clone lacks the 5' untranslated region and the first five codons of the leader sequence (Nolan *et al.*, 1991, 1992).
The plasmid, pKNPCSK-3, comprises pBluescript SK(-) with a human properdin cDNA clone (at the *Eco* RI site) that lacks the polyadenylated region and thirteen codons of the leader sequence, but has the correct coding for histidine at codon 430 (Nolan, 1991).
In order to generate an almost full length coding region for human properdin, the *Sph* I - *Xho* I fragment of pKNPCSK-3 was ligated into *Sph* I + *Xho* I cleaved pKNPCKS-1 to generate pKNPCKS-1/3.

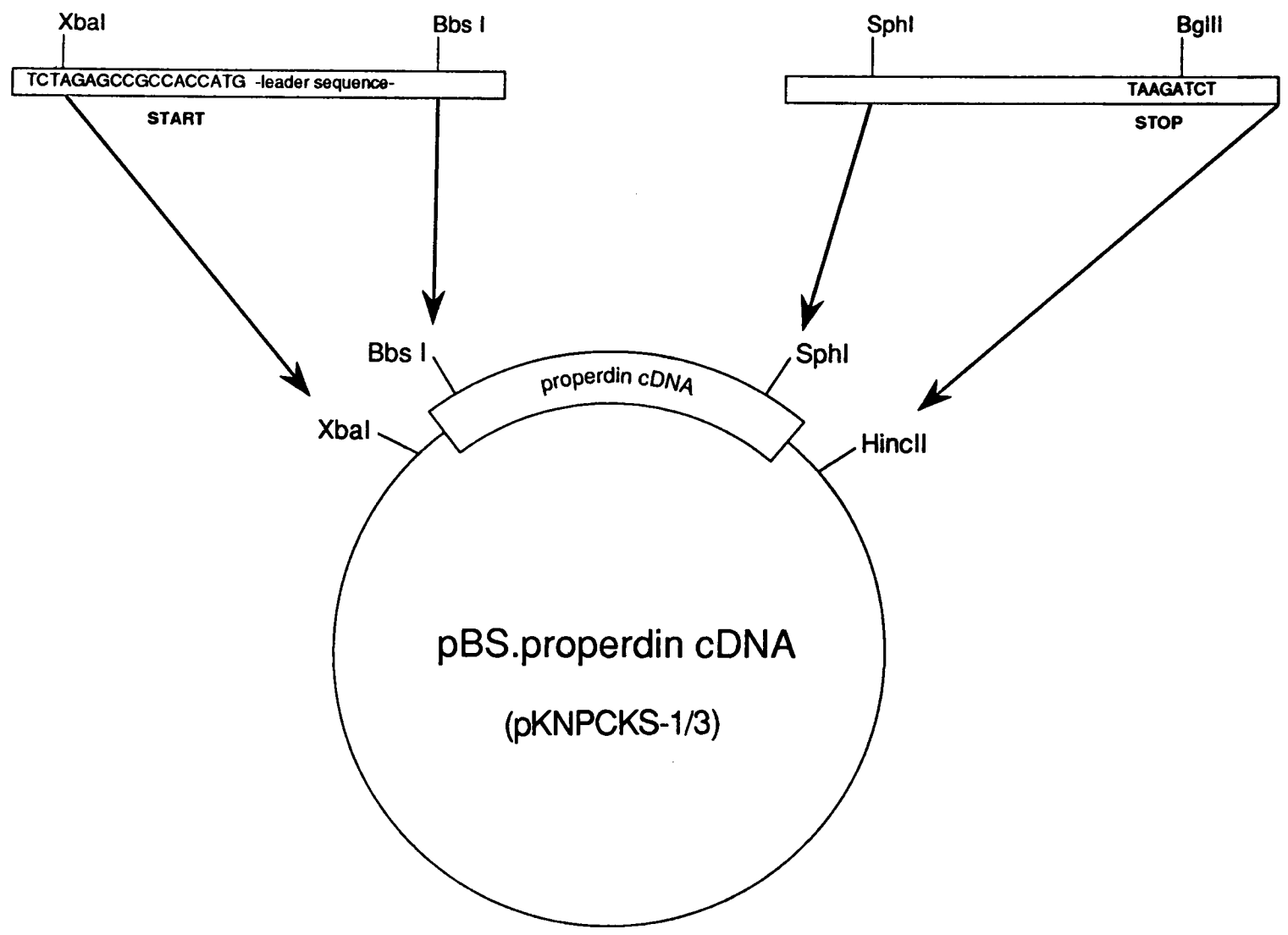


Figure 4.3
Engineering of the cDNA construct for expression of properdin.
Summary of the replacement of the 5' and 3' ends of the properdin cDNA in pBluescript with PCR generated fragments, in order to complete the coding region and add unique restriction sites for subsequent cloning into the expression vector. The 5' fragment contains a *Xba* I site for sub-cloning, a strong translation start site, and coding for the missing amino acids of the leader peptide. The 3' fragment contains a *Bgl* II site for sub-cloning immediately after the stop codon. The final plasmid was named pBS.properdin.exp.
See section 4.1, and Materials and Methods for more details.

pBS.properdin.exp. (see figure 4.3). Although the pEE6 vector contains *Xba* I and *Bcl* I restriction sites for incorporation of the foreign sequence, the properdin construct was produced with *Xba* I and *Bgl* II restriction sites at the ends. This was done because of the presence of an internal *Bcl* I site in the properdin leader sequence. However, the overhanging ends produced by *Bgl* II and *Bcl* I are compatible in subsequent ligation, allowing the full length properdin cDNA construct to be inserted into the pEE6.HCMV.GS expression vector. The entire coding sequence of the properdin construct was checked by double stranded sequencing, to ensure the absence of PCR-generated errors and inaccurate ligation joints.

4.2 Transfection and selection for stable transfectants.

CHO-K1 cells were transfected with the pEE6.HCMV.GS.properdin expression vector using the modified calcium phosphate precipitation method of Chen and Okayama (1987). Assuming a plating efficiency of 100%, a transfection efficiency of approximately 2×10^{-4} was obtained using either 15 μg or 25 μg of plasmid DNA. Selection at increasing concentrations of MSX from 15 μM to 30 μM yielded decreasing numbers of resistant colonies in the transfection plates, but also decreased the number of spontaneously resistant colonies obtained among the mock transfectants. Overall, no significant difference in the number of resistant colonies due to incorporation of the pEE6 plasmid was seen between the transfections at these concentrations of DNA or MSX.

An ELISA assay (described in Materials and Methods) was used to screen the resistant colonies for secretion of properdin. Medium from mock transfected cells did not show ELISA reactivity. However, approximately 75% of the resistant transfected colonies appeared to secrete properdin. These colonies were maintained in selective medium, and transferred to progressively larger flasks. Rescreening indicated that ninety percent of the clones continued to secrete properdin after a further week of sub-culture. The level of secretion varied from clone to clone, and showed no relationship to the concentration of MSX used. Clones secreting the highest levels of properdin were selected for further characterisation. Aliquots of these clones were frozen and stored in liquid nitrogen.

4.3 Preliminary characterisation of recombinant human properdin

SDS-PAGE of the conditioned medium in reducing conditions revealed the presence of a protein of the same molecular mass as plasma purified properdin which was not present in mock transfectant medium (see figure 5.4, in which medium from wild

type transfectants is compared with that from cells expressing mutant forms). A Western blot probed with rabbit anti-human IgG confirmed the presence of an immunoreactive band of the same size as plasma purified properdin, and displaying the multiple bands under non-reducing conditions typical of properdin. No immunoreactivity was detected in mock transfectant medium (figure 4.4). Preliminary alternative pathway haemolytic assays confirmed that the conditioned medium from transfected cells could substitute for plasma properdin in properdin-depleted serum, indicating that the recombinant material was functionally active.

4.4 Purification of recombinant properdin

Since the preliminary results indicated that the recombinant properdin was structurally and functionally similar to plasma purified properdin, the expressed material was further purified. Properdin-secreting CHO cells were grown to confluence in foetal calf serum-containing medium with the appropriate concentration of MSX (30 μM for the cell line selected). After washing the cells three times with PBS to remove traces of serum, serum-free medium containing 30 μM MSX was added. The cells were then cultured for approximately one week, until they began to detach from the vessel surface, before the medium was harvested. Using this procedure, yields of up to 8 μg ml^{-1} of properdin were obtained without the need for further GS gene amplification.

The medium was removed from the cells and the recombinant properdin purified from it using an anti-human properdin monoclonal IgG affinity column (as described in Materials and Methods). Serum-free medium was used during this procedure, since properdin is a serum protein and it was necessary to avoid contamination of the preparation with bovine properdin. This was particularly important since bovine properdin also binds the anti-human monoclonal antibody used for the affinity column (Vivienne Perkins, personal communication).

4.5 SDS-PAGE of purified recombinant properdin

This purification procedure yields a single protein detectable on Coomassie Blue or silver stained reducing SDS-PAGE gels (figure 4.5). The protein is similar in size to plasma purified properdin. However, a small but reproducible difference in the size of recombinant and plasma properdin is detectable. It is thought that this difference may be due a small difference in the N-linked glycosylation pattern of the two molecules (see section 4.11).

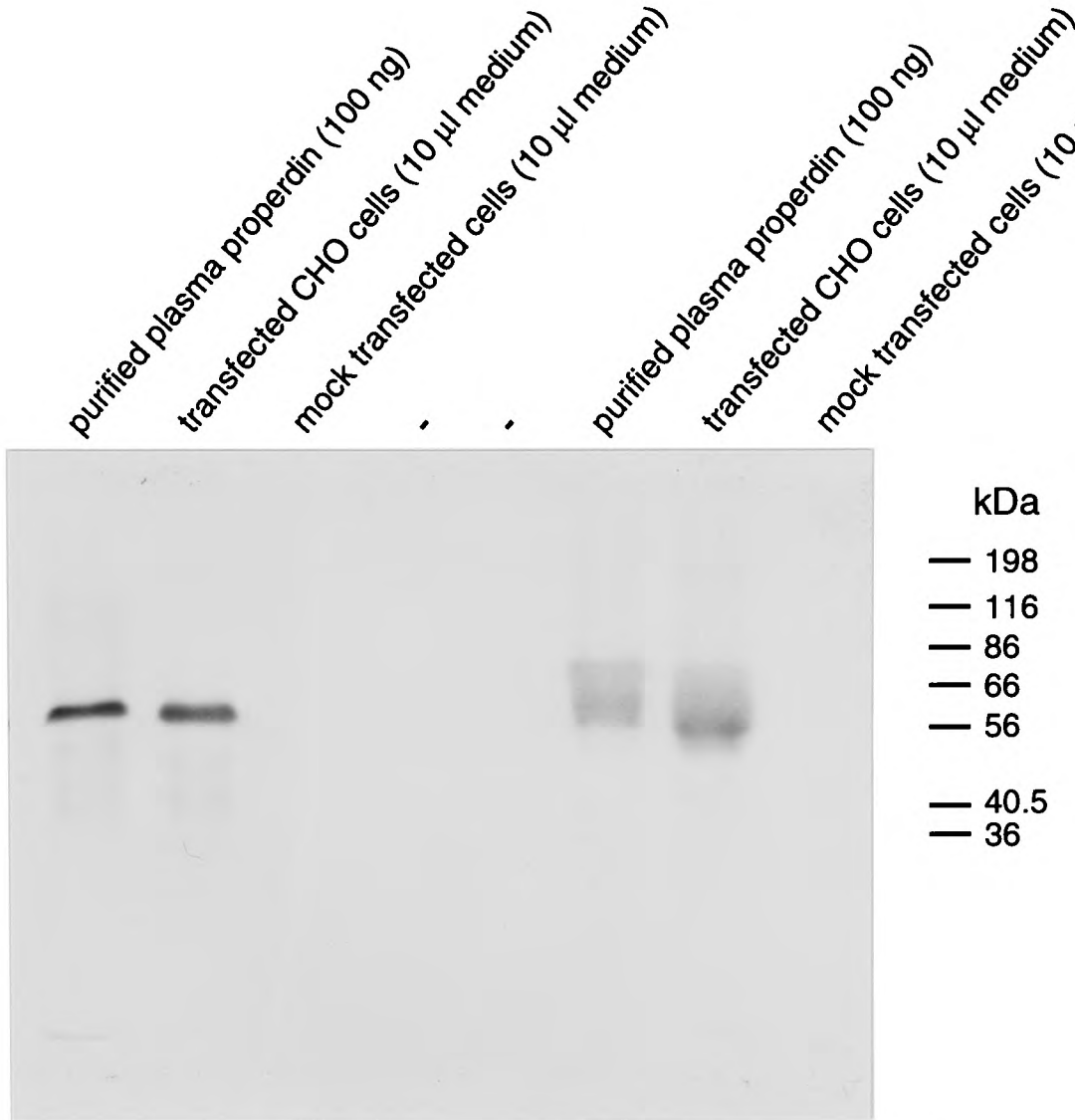
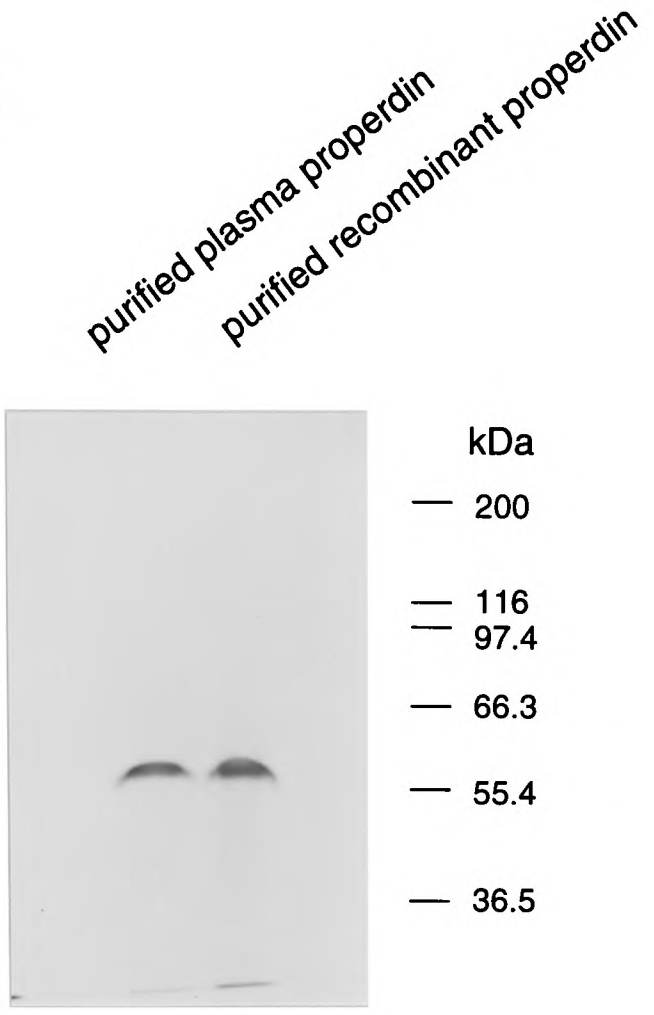


Figure 4.4
Western blot of serum-free medium from transfected and mock-transfected CHO cells probed with anti-properdin antibody. Samples were separated by 10% SDS-PAGE in reducing and non-reducing conditions (as indicated). The proteins were then transferred to nitrocellulose, and properdin detected with rabbit polyclonal anti-properdin IgG and standard procedures described in materials and methods.

a) Reducing conditions



b) Non-reducing conditions

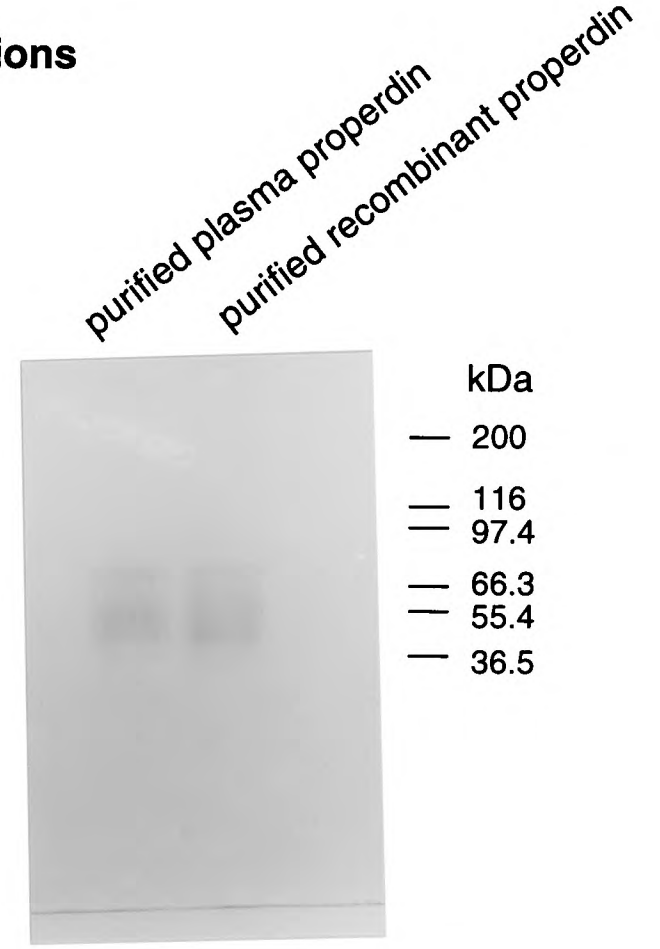


Figure 4.5

SDS-PAGE of purified recombinant properdin compared with properdin purified from normal human plasma (2.5 μg each sample).

a) Reducing conditions, 7.5% SDS-PAGE, Coomassie blue stained.

b) Non-reducing conditions, 10% SDS-PAGE, Coomassie blue stained

Under non-reducing conditions, plasma purified properdin runs as a characteristic multiple band pattern of at least three major bands (figure 4.5). The monoclonal anti-human properdin antibody binds to all the bands equally well, indicating that the pattern is not due to contaminating proteins (not shown). The pattern is also typical of rabbit properdin (Nakano *et al.*, 1986), bovine and porcine properdin (Vivienne Perkins, personal communication). The same pattern is found for recombinant human properdin. The origin of this heterogeneity is unclear. The different bands can be excised individually, and will remigrate in the same position on subsequent electrophoresis (Farries & Atkinson, 1989), suggesting that a post-translational modification of properdin is responsible, rather than an artefact of the electrophoresis. Properdin lacking N-linked glycosylation shows the same pattern of bands, ruling out differences in N-linked glycosylation as the cause (Farries & Atkinson, 1989). The various properdin oligomers, after separation by Mono-S ion exchange chromatography, also show the same pattern in each case (not shown). Treatment of human properdin with sialidase and O-glycosidase also fails to alter either the apparent size of properdin on SDS-PAGE (see figure 4.6) or the appearance of the multiple band pattern (while a control digest of fetuin results in a clear reduction in molecular mass, not shown). Furthermore, an amino sugar analysis of hydrolysed properdin (kindly carried out by Mr Anthony Willis, MRC Immunochemistry Unit) did not reveal the presence of any N-acetyl galactosamine, indicative of a lack of classical O-linked sugar (not shown). No heterogeneity was detected in the N-terminal sequence of plasma or recombinant properdin. The reason for the multiple band pattern thus remains a mystery. However, the fact that recombinant properdin retains the property suggests that it is intrinsic to the single polypeptide chain encoded by the cloned cDNA and that, if it is due to a post-translational modification, that CHO cells are capable of carrying it out.

4.6 The N-terminal sequence of recombinant human properdin

The N-terminal sequence of the recombinant molecule was determined as described in Materials and Methods. The sequence was identical to that of human properdin at all positions. The sequence is different from that of bovine properdin (Vivienne Perkins, personal communication). This confirms that the recombinant properdin is correctly processed to remove the leader peptide during its synthesis and secretion, and that the protein isolated is not contaminating bovine properdin.

Plasma purified human properdin (from protein and cDNA sequencing):	DPVLCFTQYE
Recombinant human properdin(from protein sequencing):	DPVL-FTQYE

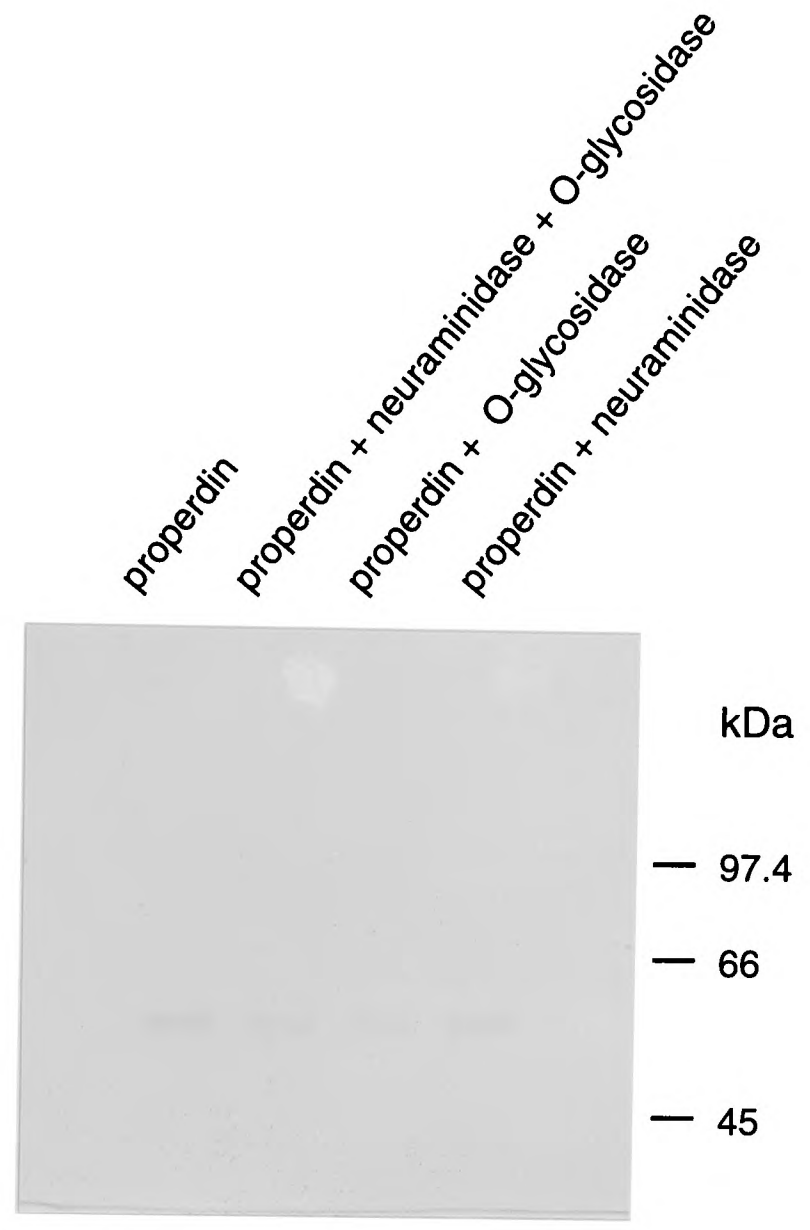


Figure 4.6

Treatment of plasma purified properdin with O-glycosidase and neuraminidase.

Properdin purified from plasma (0.5 μg) in MES, pH6.0 was incubated with 0.25 mU recombinant neuraminidase (New England Biolabs) or 0.5 U O-glycosidase (BSA-free, Boehringer-Mannheim) at 37°C for 16 h. In one case, neuraminidase treatment was carried out before O-glycosidase treatment.

7.5% SDS-PAGE, reducing conditions, Coomassie Blue stained.

4.7 Electron Microscopy of recombinant properdin

Recombinant properdin was compared with plasma properdin by electron microscopy (kindly carried out by Drs Hanna Wiedemann and Rupert Timpl, Max-Planck Institut für Biochemie, Martinsried, Munich, Germany). The recombinant protein was indistinguishable from plasma properdin (see chapter 5, figure 5.14). Both preparations contained characteristic trimers and tetramers, and a few pentamers, as previously reported (Smith *et al.*, 1984a). Trimers dominated in both samples. Dimers were not easily identified due to the large size of the grains used to stain the oligomers. The length of the monomers within the oligomers of both preparations is similar to the 26 nm previously reported (Smith *et al.*, 1984a). Covalent linking of the oligomeric forms using a homobifunctional cross-linking reagent confirmed the presence of dimers, trimers, tetramers in recombinant properdin (see chapter 5).

4.8 The oligomeric profile of recombinant properdin

The various oligomeric forms of properdin can be separated by cation exchange chromatography on a Mono-S column (Pangburn, 1989). A comparison of the elution profile of purified plasma properdin detected by absorbance at 280 nm with that obtained by ELISA of the eluted fractions showed that the ELISA provides an accurate estimate of the number of properdin monomers present in the sample. This was also confirmed by comparison of the A_{280nm} and ELISA estimations of the concentrations of properdin present in separate preparations of dimers, trimers and tetramers purified by size exclusion chromatography. That is, the ELISA results are proportional to the mass of properdin present in the sample, rather than to the molarity of oligomers. The two methods estimated the same ratio of oligomers to be present in the purified preparations (within 5%).

If the constituents of whole normal human serum or conditioned CHO cell medium are separated in this way, then the ELISA assay can be used to determine the proportions of oligomers present. Figure 4.7 shows that normal human serum contain the polydisperse distribution of oligomers reported by Pangburn (1989). Furthermore, medium from transfected CHO cells also shows a similar pattern, confirming that the recombinant molecule is secreted as oligomers. Medium from mock transfected cells did not contain any detectable properdin in this assay. The ratio of oligomers in plasma reported by Pangburn is 26:53:21. In this study, the ratio found was 25:52:23. It is encouraging that the results obtained are similar, since the results of the monoclonal antibody competitive RIA assay used by Pangburn were found to be proportional to the molar concentrations of oligomer, rather than to the mass of properdin present, in contrast

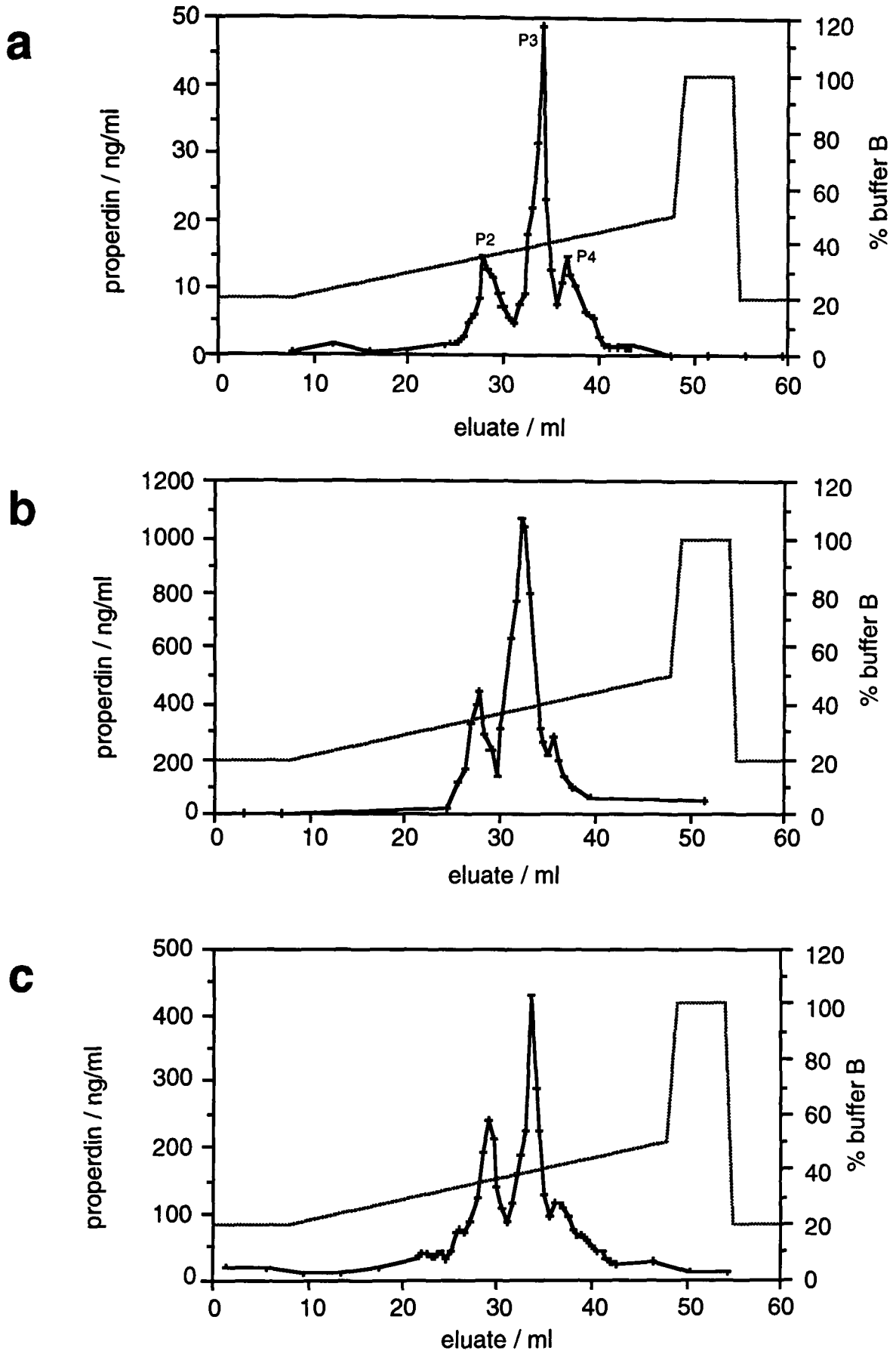


Figure 4.7
Elution profiles of properdin samples from Mono-S ion exchange chromatography. The properdin concentration in each fraction was determined by ELISA of duplicate samples (see text). Buffer A was 50 mM sodium phosphate, pH6. Buffer B was 0.5 M NaCl/50 mM sodium phosphate, pH6. P2,P3,P4 indicate the dimer, trimer and tetramer forms of properdin.
a) Normal human serum (30 µl).
b) Purified plasma properdin (12 µg).
c) Recombinant properdin in serum-free medium (1.3 µg).

to this assay. The ratio of recombinant properdin oligomers found was 33:49:18. This apparent difference in the ratio of oligomers present is discussed below.

The oligomeric forms of *purified* recombinant properdin were also separated by Mono-S cation exchange chromatography (figure 4.8) or by size exclusion chromatography on Superose 6 (see chapter 5). These experiments confirmed the presence of the various oligomeric forms. However, the ratio in which the oligomers were found varied in different preparations of recombinant properdin. Since there were a number of variables between the methods used during these purifications, a closer examination of the phenomenon was undertaken.

The purification procedure includes an elution step from the mAb affinity column in 0.2 M glycine, pH 2.5. These conditions are known to dissociate properdin oligomers (Pangburn, 1989). This raised the possibility that the ratio of oligomers present in purified preparations was dependent upon the concentration of monomers available during neutralization of pH. This was investigated by dissociating a sample of purified plasma properdin (at 1 mg ml^{-1}) into its component monomers by dialysis into 0.2 M glycine/0.15 M NaCl, pH 2.5. The sample was then diluted to different extents in the same buffer over a one thousand fold range of concentration (*i.e.* 1 µg ml^{-1}, 10 µg ml^{-1}, 0.1 mg ml^{-1}, 1 mg ml^{-1}). Each sample was then quickly neutralized by the addition of 2 M Tris, pH 7.4. The ratios of the oligomeric forms in each sample was determined by separation on Superose 6 size exclusion chromatography, and detection of the eluted properdin by absorbance at 280 nm or 214 nm and ELISA of the eluted fractions.

The results are shown in table 4.1 and figure 4.9. It is clear that the higher the concentration of properdin monomers present during reassociation, the greater the proportion of the higher oligomers that are formed. An explanation for this may be as follows. As an oligomer is formed by the association of additional monomers, there are two possible fates for the growing structure. Either i) another subunit can be added, forming a higher oligomer, or ii) the free ends of the structure can come together (ring closure) and prevent further association of monomers. If the concentration of monomers is high, there is an increased chance that another monomer will associate with the growing oligomer rather than of the ring closing, leading to the formation of a greater proportion of higher oligomers (see figure 4.10). The differences seen are not due to a concentration-dependent shift in equilibrium association of oligomers, since the bonds between monomers are extremely stable. Little or no re-equilibration of purified dimers, trimers, or tetramers was seen after several weeks at 4°C (not shown). Similar findings have been reported by Smith *et al.* (1984a) and Pangburn (1989).

Table 4.1

The influence of concentration on the oligomerization of human properdin

properdin sample	Ratio of oligomers P_2	P_3	P_{4+}
before dissociation	30	51	19
reassociation at 0.001 mg ml^{-1}	46	46	8
reassociation at 0.01 mg ml^{-1}	41	46	13
reassociation at 0.1 mg ml^{-1}	40	47	14
reassociation at 1 mg ml^{-1}	34	50	16

These findings suggested that the rate of synthesis of properdin in the cell may also be able to influence the ratio of oligomers produced, since a higher rate of synthesis could lead to a higher local concentration of properdin monomers in the endoplasmic reticulum during oligomerization. It had previously been observed that the presence or absence of MSX in the culture medium of transfected CHO cells influenced both the rate of cell growth and the amount of recombinant properdin produced. If MSX was absent, the cells grew and divided more quickly, but the yield of properdin per cell was decreased. The ratios of oligomers produced from cells grown in the presence or absence of MSX were compared. The oligomers present in conditioned medium were separated directly by Mono-S cation exchange chromatography, and the oligomeric ratio determined by ELISA of the eluted fractions. It was found that the cells synthesising properdin at the higher rate (*i.e.* those grown in the presence of MSX) produced more of the higher oligomeric forms of properdin than the cells producing properdin more slowly (see figure 4.11). These results are consistent with the proposal that the ratio of oligomers formed depends upon the concentration of monomers present during their formation.

The results above provide a likely explanation for the differences seen in the ratio of oligomers found in preparations of recombinant properdin and plasma properdin and between different preparations of recombinant properdin. The ratio of recombinant oligomers produced by transfected CHO cells depends upon the rate of synthesis by the cells. Furthermore, in purified preparations, the ratio of oligomers found depends upon the concentration of properdin present during neutralization after affinity chromatography. Samples of recombinant properdin were always at lower concentrations than samples of plasma properdin during this step and so it is not surprising that pure recombinant properdin often contains a greater proportion of dimers than purified plasma properdin.

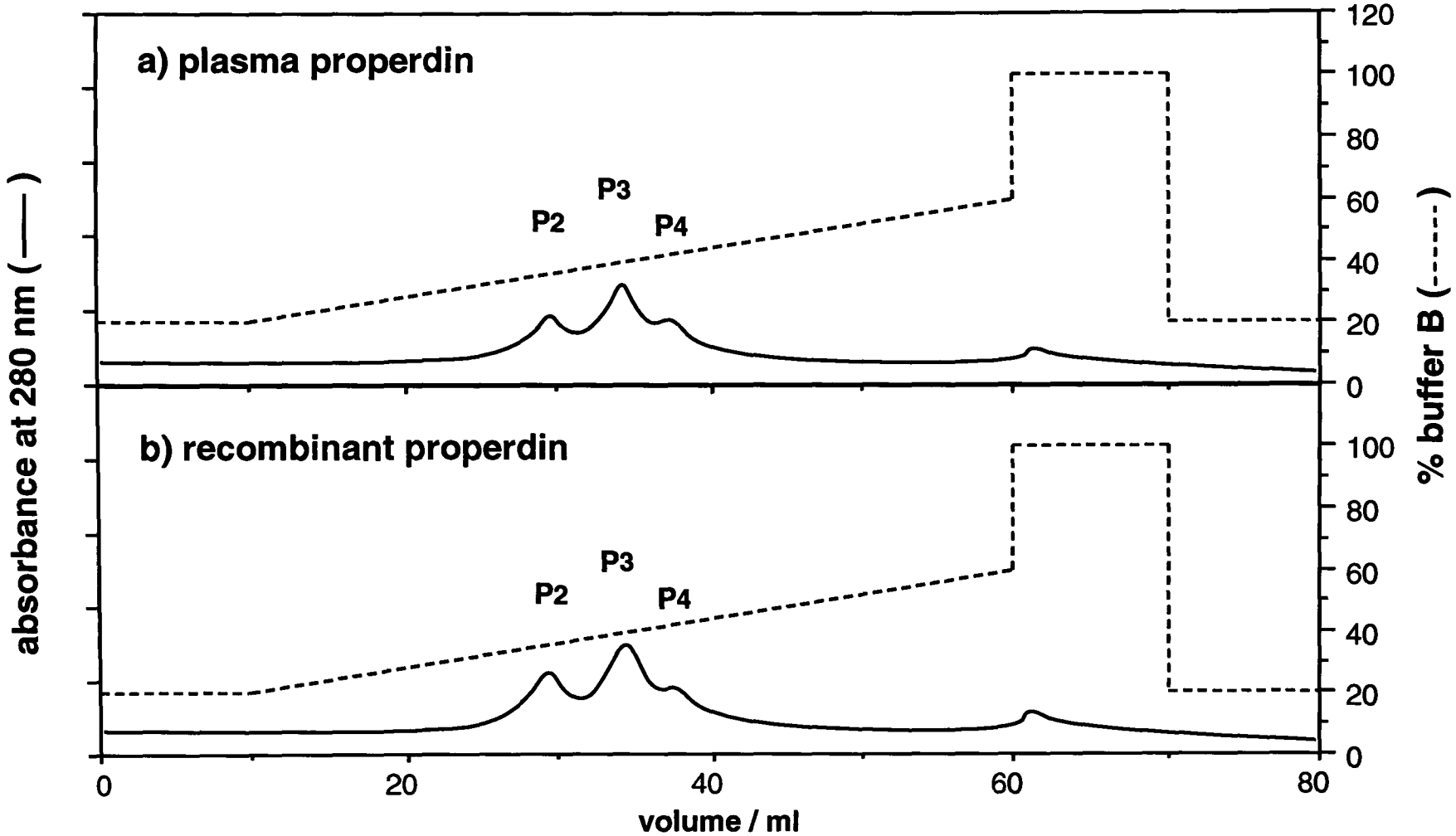


Figure 4.8
Comparison of approximately 10 µg of purified (a) human plasma properdin and (b) recombinant normal human properdin on Mono-S cation exchange chromatography. Buffer A was 50 mM sodium phosphate, pH 6. Buffer B was 0.5 M NaCl/50 mM sodium phosphate, pH 6. Eluted properdin was detected by absorbance at 280 nm. P2, P3, and P4 indicate the dimer, trimer and tetramer forms of properdin.

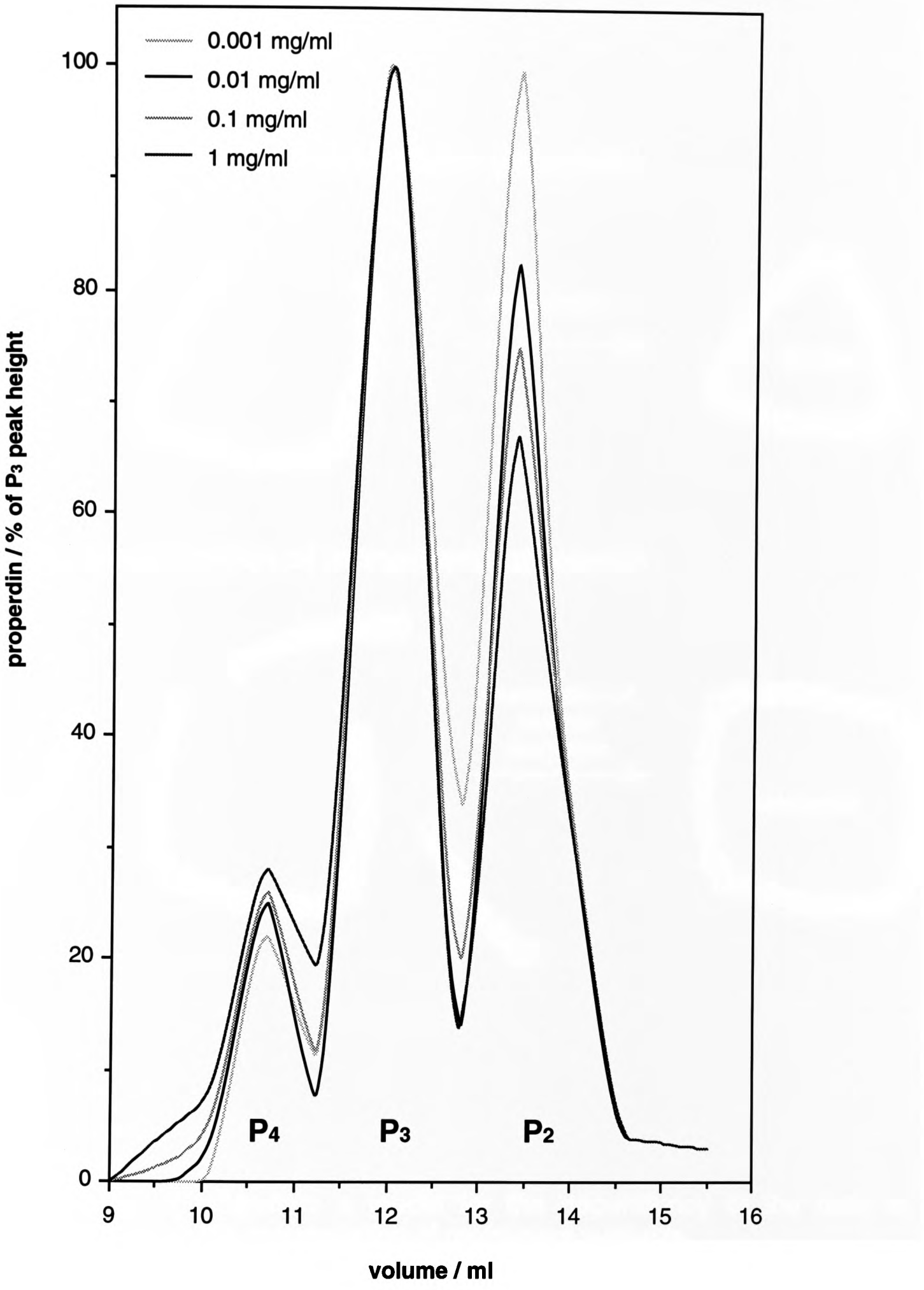


Figure 4.9

The ratio of oligomers of properdin formed depends upon the concentration of monomers present during reassociation.

Properdin was dissociated at pH2.5, and reassociated at the concentrations shown by rapid adjustment to pH7.4. The oligomers formed were separated by gel exclusion chromatography on Superose 6, and detected by absorbance at 280 nm or 214 nm, and by ELISA of eluted fractions. The results are adjusted in each case to percentage of the trimer peak height.

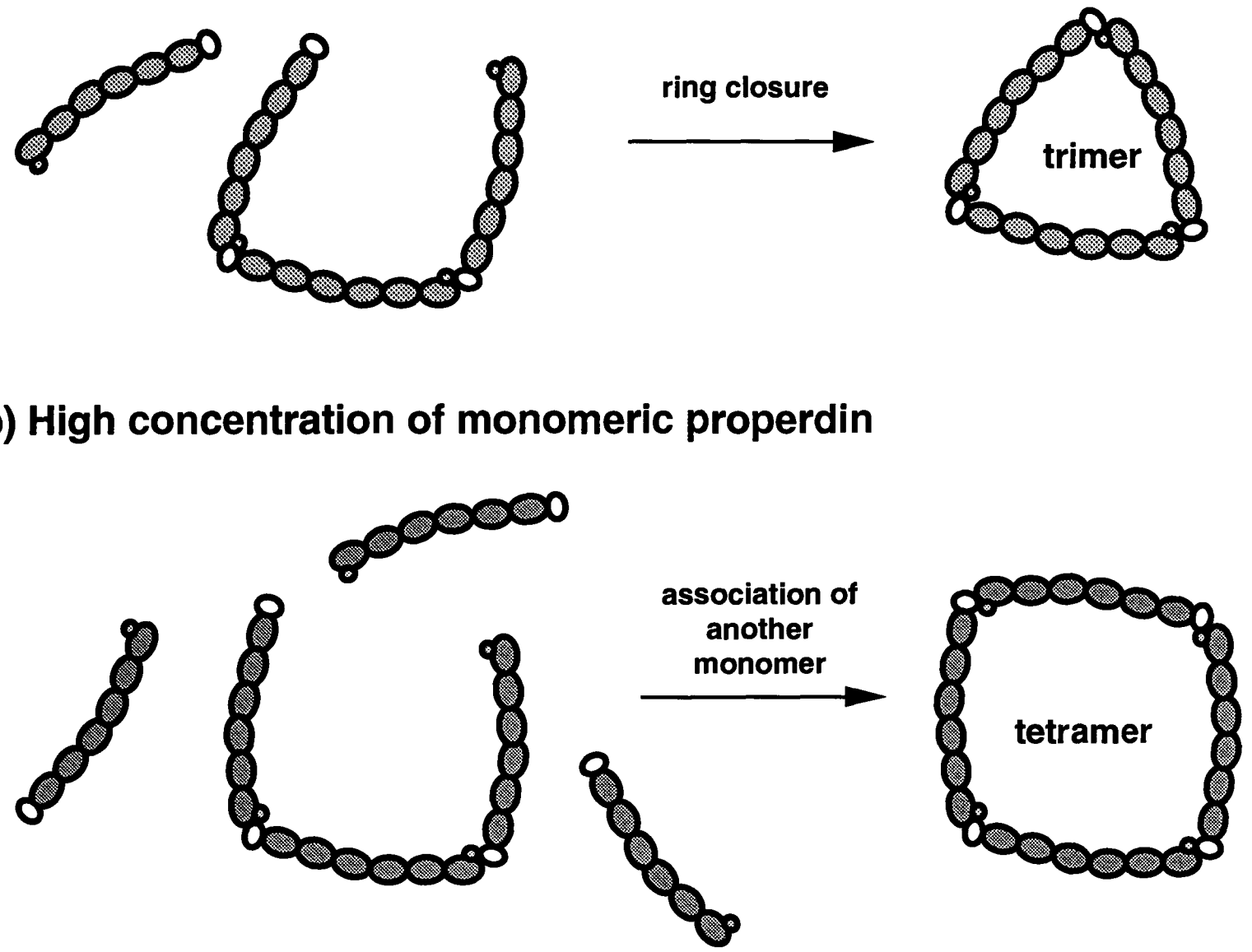


Figure 4.10
Oligomerization of properdin: a model for the dependency of the ratio of oligomers formed on the concentration of monomers available.
a) At low concentrations of monomeric properdin, the likelihood of another subunit associating with a growing oligomer is low, and ring closure is favoured. Thus, a high proportion of small oligomers are formed.
b) At higher concentrations of monomeric properdin, the chance of another subunit associating with the growing oligomer is higher, and more larger oligomers are formed.

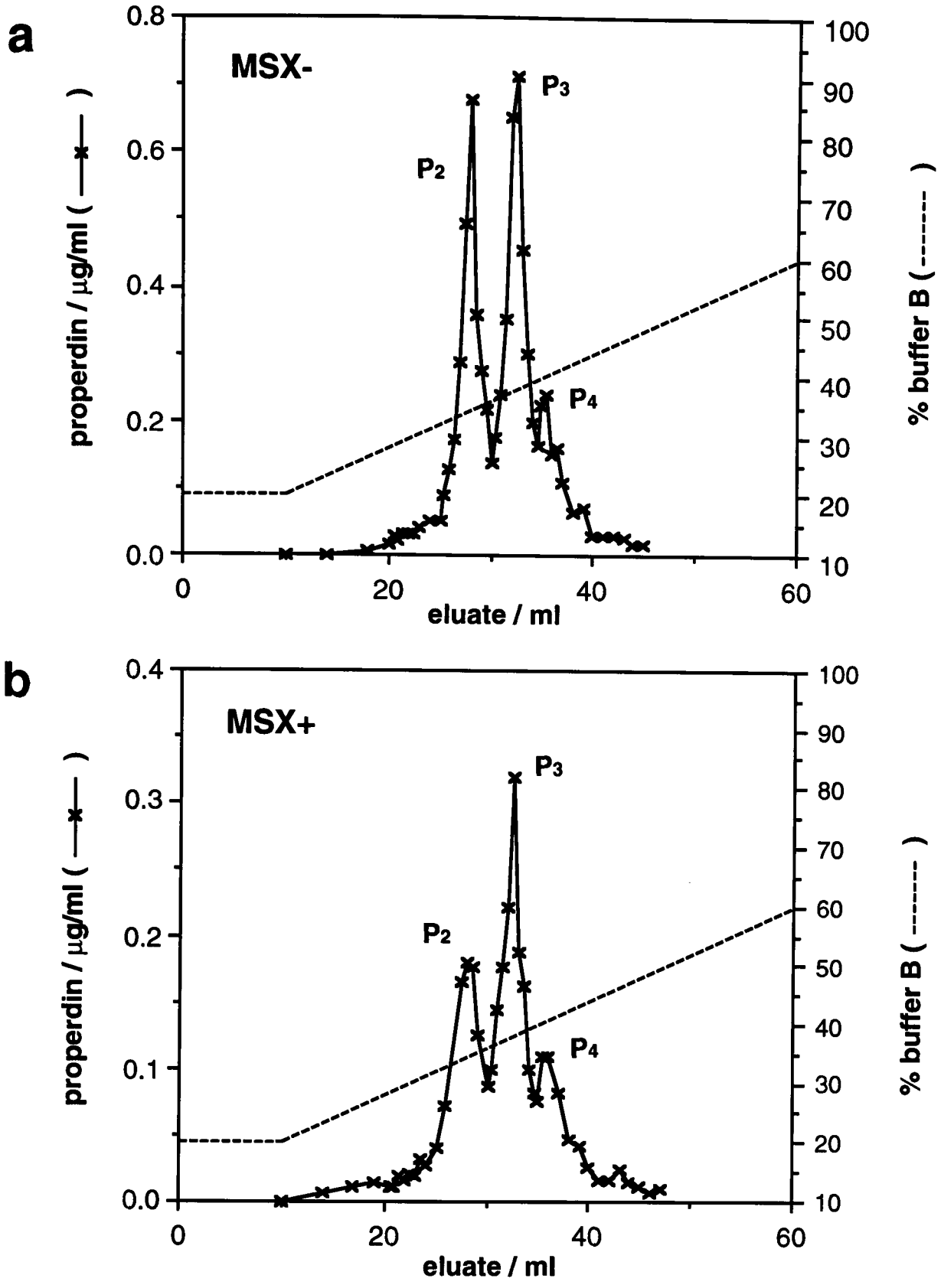


Figure 4.11

Oligomer profiles of properdin produced by transfected cells grown under different conditions. Two flasks were seeded with 3 x 10^6 transfected CHO cells. One culture was grown in the presence of 30 µM MSX and the other without MSX. After harvesting of the medium, the quantity of properdin produced by each was determined by ELISA, and the number of cells present was counted by haemocytometry. These values were used to estimate the synthesis rate of properdin by each culture. The ratio of oligomers formed was determined after separation of the medium on Mono-S ion exchange chromatography. The properdin concentration in each fraction was determined by ELISA (see text). Buffer A was 50 mM sodium phosphate, pH6. Buffer B was 0.5M NaCl/50 mM sodium phosphate, pH6.

a) Recombinant properdin produced from CHO cells in the absence of MSX. The estimated rate of synthesis was ≈0.1 pg/day/cell. The ratio of oligomers produced is approximately 38:47:15 (P2:P3:P4).

b) Recombinant properdin produced from CHO cells in the presence of MSX. The estimated rate of synthesis was ≈0.4 pg/day/cell. The ratio of oligomers produced is approximately 32:50:20 (P2:P3:P4).

While the quantities of recombinant properdin obtained were inadequate for a careful study of the dependence of oligomer ratio on concentration during association, the ratios obtained were those expected given the concentration of monomers present. It will be important to bear these findings in mind when measuring the proportions of properdin oligomers found *in vivo*. Such studies will be required to test the hypothesis that the higher oligomeric forms of properdin are utilised first during alternative pathway activation on infection (Pangburn, 1989). Clearly the analysis should be carried out on whole plasma (or serum) or after a purification procedure that does not involve dissociation of the oligomers.

4.9 The binding of recombinant properdin to a panel of monoclonal antibodies

Recombinant properdin binds to the HYB 3-3 monoclonal antibody used during the purification procedure with the same affinity as plasma properdin when tested by ELISA assay or Western blot. The monoclonal antibody binds to non-reduced properdin with much higher affinity than to reduced properdin, indicating that the presence of the epitope is dependent on the conformation of the molecule, and thus that the recombinant molecule is folded correctly in this region. The binding to recombinant properdin of a panel of monoclonal antibodies raised against human plasma properdin was kindly tested by Ms Fabianna Filippoussis and Dr John D. Lambris (University of Pennsylvania, Philadelphia, USA). All eleven of the antibodies tested bound to the recombinant protein, providing further confirmation that the molecules are antigenically similar (see chapter 5, section 5.9).

4.10 Functional assays on recombinant human properdin

The ability of recombinant properdin to reconstitute properdin-depleted human serum in a rabbit erythrocyte haemolytic assay was tested. The assay was carried out in then presence of magnesium ions and the calcium chelator, EGTA, so that only the alternative pathway of complement can operate (see chapter 6). The dose-response and time course of haemolytic activity are indistinguishable for purified plasma and recombinant properdin (see figure 4.12). In addition, recombinant properdin bound to C3b with the same affinity as plasma properdin in a solid phase C3b binding assay, as shown in figure 4.13 (see chapter 7). Binding of recombinant properdin to sulphatides in a microtitre plate assay also showed no significant difference from the binding of plasma properdin, as shown in figure 4.13 (see chapter 8).

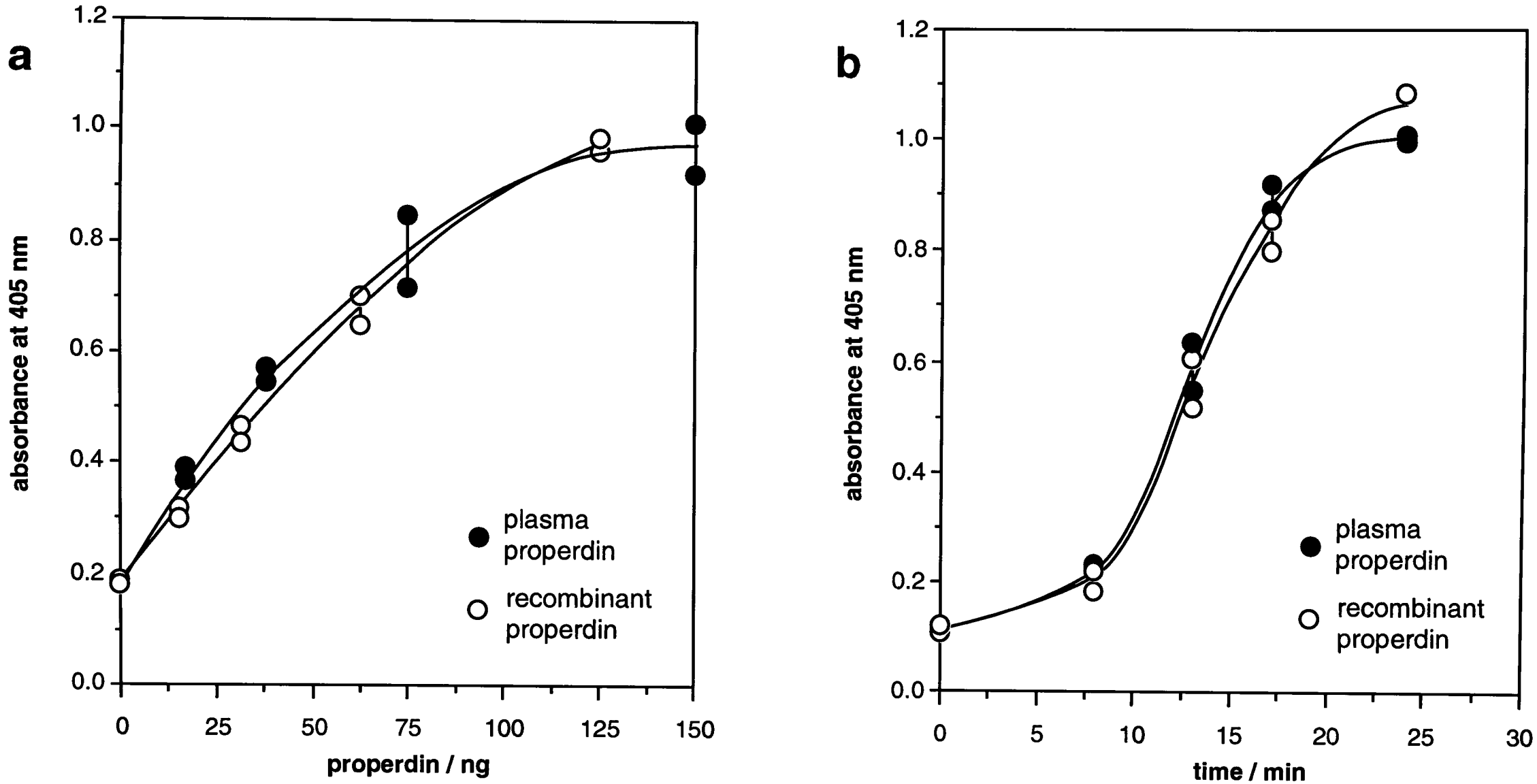


Figure 4.12
Comparison of the activity of plasma and recombinant purified properdin in the alternative pathway haemolytic assay (see text for details). The results of duplicate experiments are shown.
a) The dependency of haemolysis on the dose of plasma or recombinant properdin.
b) The time course of haemolysis with 100 ng of plasma or recombinant properdin.

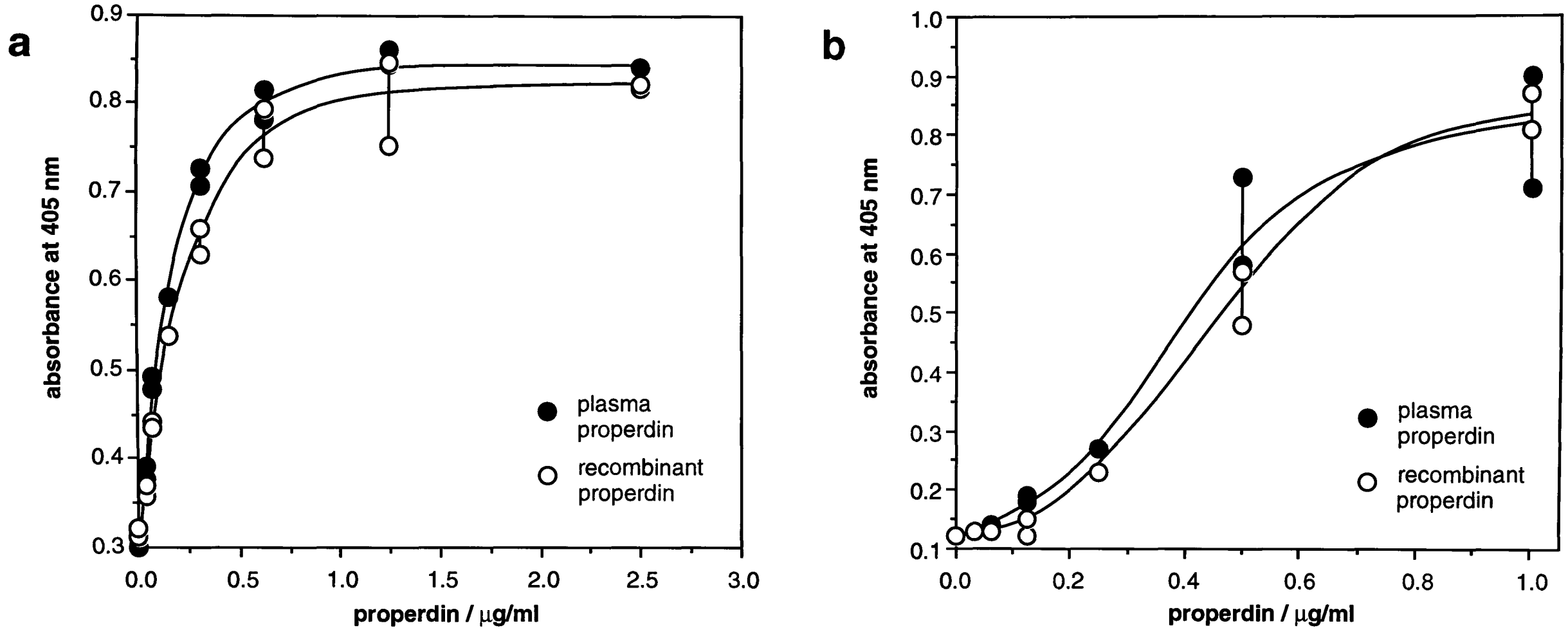


Figure 4.13
Direct microtitre plate C3b and sulphatide binding assays (see Materials and Methods).
a) Comparison of purified plasma properdin and recombinant properdin in C3b binding assay. The results of duplicate experiments are shown.
b) Comparison of purified plasma properdin and recombinant properdin in sulphatide binding assay. The results of duplicate experiments are shown.

4.11 The N-linked carbohydrate of properdin

Chemical analysis shows that properdin contains approximately ten percent carbohydrate (Minta and Lepow, 1974). Limited trypsin digestion of properdin at 28°C for 17 h cleaves a single peptide bond in the fifth TSR, and generates two protein fragments visible after SDS-PAGE in reducing conditions (see chapter 5). The larger N-terminal fragment is designated T_1, and the smaller C-terminal fragment, T_2. Two early reports suggest that the carbohydrate in properdin resides mainly on the T_1 fragment. Minta and Kunar (1976) found that periodic-Schiff's reagent stained only the T_1 fragment, and Reid and Gagnon (1981) deduced that the carbohydrate was linked to this fragment from the apparent molecular masses of cyanogen bromide peptides derived from human properdin. However, the cDNA sequence of human properdin reveals that the only putative N-linked carbohydrate site is on the T_2 fragment (Nolan *et al.*, 1991). In order to resolve this discrepancy, human plasma properdin was treated with PNGase F to remove N-linked oligosaccharide, and subsequently digested under mild conditions with trypsin to produce the T_1 and T_2 fragments. As seen in figure 4.14, only the T_2 fragment was reduced in size, showing that the N-linked carbohydrate is on the C-terminal fragment, in accordance with the prediction from the cDNA sequence. From a comparison with standard proteins, the size of the N-linked sugar was estimated as 5 kDa. This agrees well with the quantity found by chemical analysis (9.8%, or approximately 5 kDa).

A similar approach was used to determine if the small size discrepancy between recombinant and plasma properdin was due to a difference in the N-linked carbohydrate. Treatment with PNGase F appeared to reduce both the recombinant and plasma proteins to an equal size. After, limited trypsin digestion, it is the T_2 fragment which appears to be different in size between the two proteins (not shown). So, it seems that the small size difference is due to an altered glycosylation pattern generated by the CHO cell, when compared with the natural source of human properdin. It is known that CHO cells can glycosylate proteins differently from human cells. For example, CHO cell glycoproteins can contain terminal α(1-3) linked galactose (Ashford *et al.*, 1993). The enzyme responsible for this modification is absent in humans.

Recombinant properdin lacking N-linked sugar was also produced by growing transfected CHO cells in the presence of tunicamycin. Tunicamycin blocks the transfer of N-acetyl glucosamine-1-phosphate to dolichol monophosphate, and thereby inhibits N-linked glycosylation of proteins (Tkacz and Lampen, 1975). Since the tunicamycin was dissolved in DMSO before addition to the culture medium, control cultures were also set up containing DMSO alone. Treatment with DMSO only had no visible effect on the growth of the cells. A Western blot of the resulting conditioned medium (figure 4.15), when probed with anti-human properdin IgG, revealed the

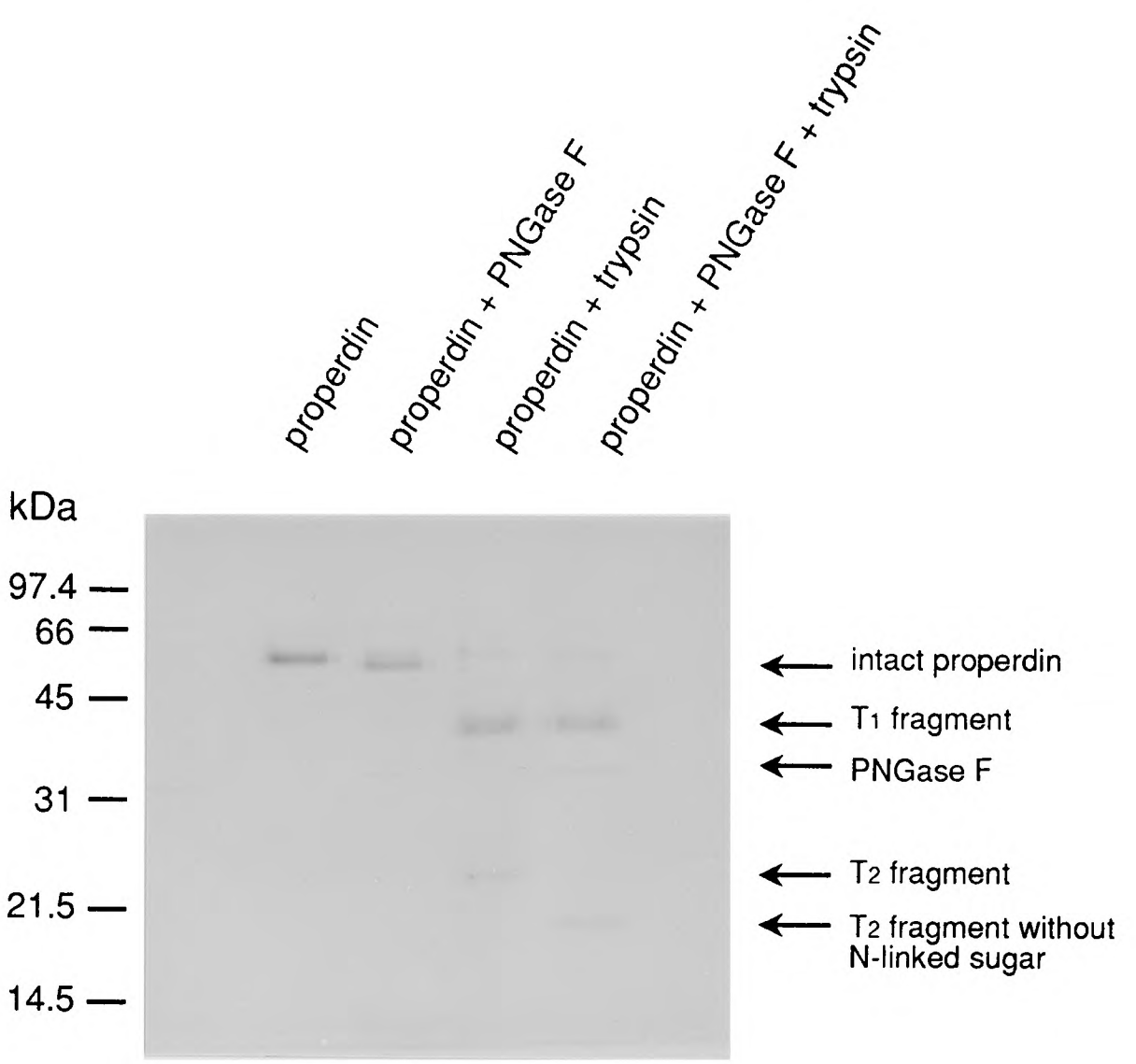


Figure 4.14

The N-linked glycosylation of human properdin resides on the C-terminal T_2 tryptic fragment.

The N-linked oligosaccharide of purified plasma properdin (0.5 µg) was removed by incubation with recombinant PNGase F (New England Biolabs) by incubation at 37°C for 8 h. Limited tryptic digestion of properdin to yield the N-terminal T_1 fragment, and the C-terminal T_2 fragment was carried out with a properdin:trypsin ratio of 40:1 at 28°C for 17 h.

12.5% SDS-PAGE, reducing conditions, Coomassie Blue stained.

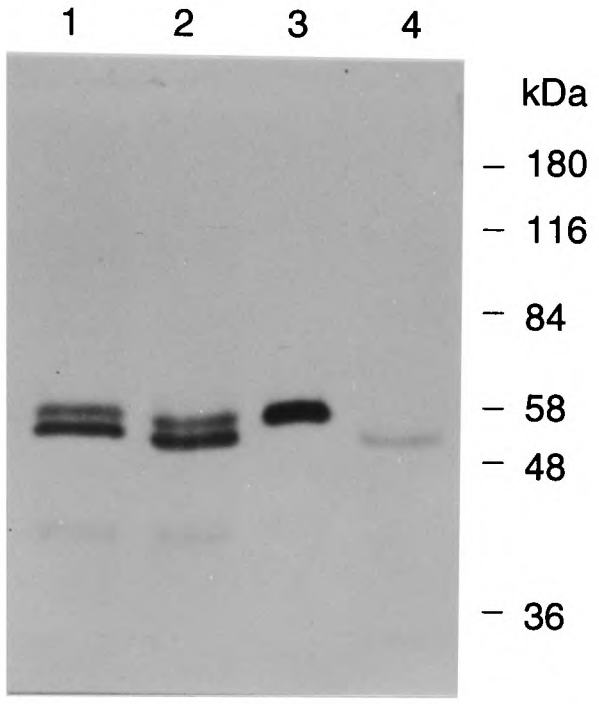


Figure 4.15

Production of recombinant human properdin lacking N-linked oligosaccharide. Western blot of serum-free samples of recombinant properdin probed with anti-human properdin IgG. 10% SDS-PAGE was carried out in reducing conditions. Lanes contain serum-free culture supernatants of:

1) Recombinant properdin produced in the presence of 4 μg/ml tunicamycin
2) Recombinant properdin produced in the presence of 10 μg/ml tunicamycin
3) Normal recombinant properdin
4) Lentil lectin-treated recombinant properdin produced in the presence of tunicamycin

presence of two immunoreactive species from cells treated with tunicamycin. The predominant species was approximately 5 kDa smaller than normal recombinant properdin. The less abundant upper band was the same size as normal recombinant properdin and could be selectively removed from the medium using immobilized lentil lectin. Together these results indicate that the smaller species is properdin lacking N-linked carbohydrate. The fact that recombinant properdin binds to lentil lectin suggests that its N-linked sugar is a bi- or tri-antennary-type oligosaccharide containing a core fucose (figure 4.16) (Cummings, 1994). This is consistent with the composition reported for normal plasma properdin: 3.8% hexose, 3.8% sialic acid, 1.5% hexosamine, 0.7% fucose (Minta and Lepow, 1974), but the binding of normal human plasma properdin to lentil lectin was not tested.

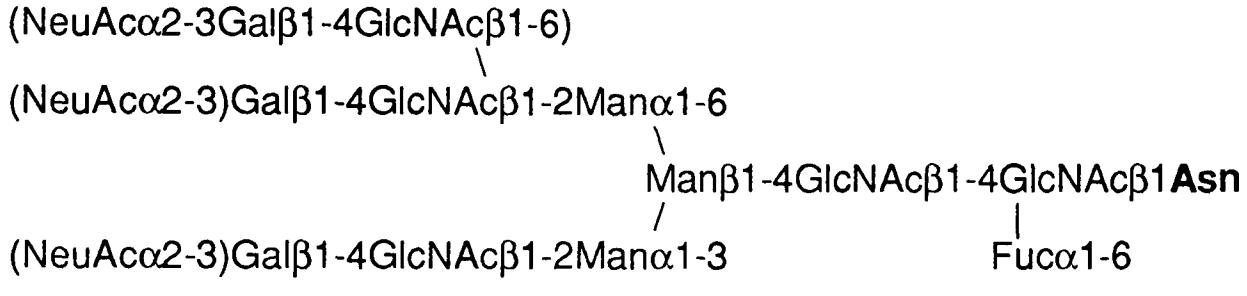
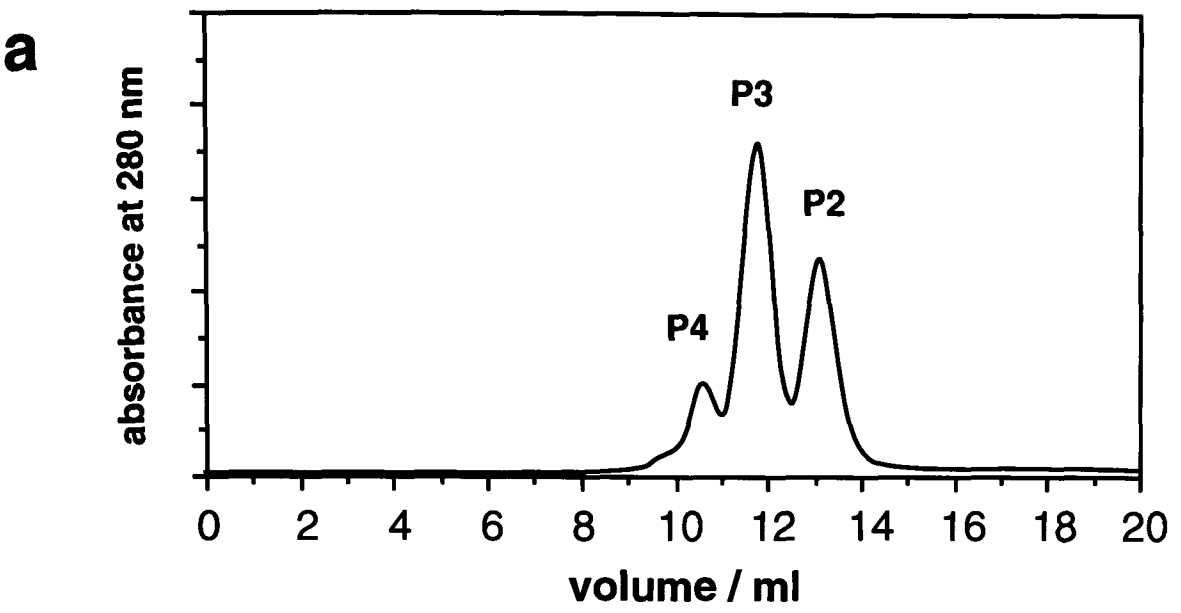
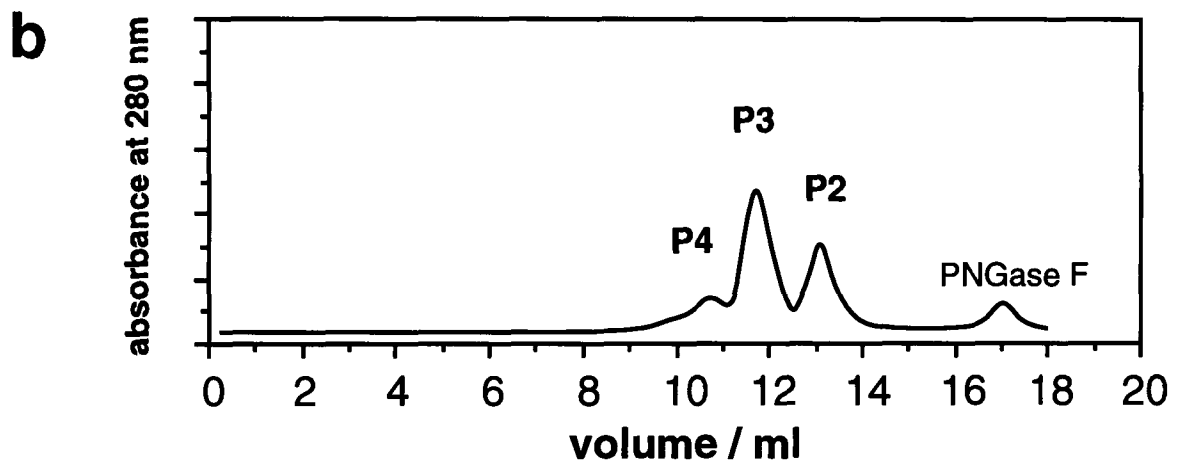


Figure 4.16 Summary of the N-linked sugars to which Lentil Lectin binds
(Residues in brackets can vary or be absent)

The finding that properdin can be secreted in the absence of N-linked sugar confirms the results of Farries and Atkinson (1989) who used tunicamycin to inhibit the glycosylation of properdin secreted from the human pro-myelotic cell line, HL-60 after DMSO stimulation. Properdin without N-linked sugar showed the presence of oligomers whether the sugar was removed enzymatically, or its addition during synthesis was prevented (see figure 4.17). Thus, the N-linked sugar is vital neither in the formation nor in the maintenance of properdin oligomers. The ease with which the N-linked sugar was removed from properdin using PNGase F (complete removal was obtained in under one hour in non-denaturing conditions, figure 4.18) also suggests that the sugar moiety is relatively exposed. Farries and Atkinson reported that properdin without N-linked carbohydrate was still able to bind C3iBb-sepharose. In addition the recombinant properdin produced by tunicamycin-treated CHO cells, and the enzymatically deglycosylated plasma properdin from the present study were both found to be fully active in the alternative pathway haemolytic assay described in chapter 6. N-linked sugar is not required for the stabilization of the C3bBb complex by properdin (figure 4.19).





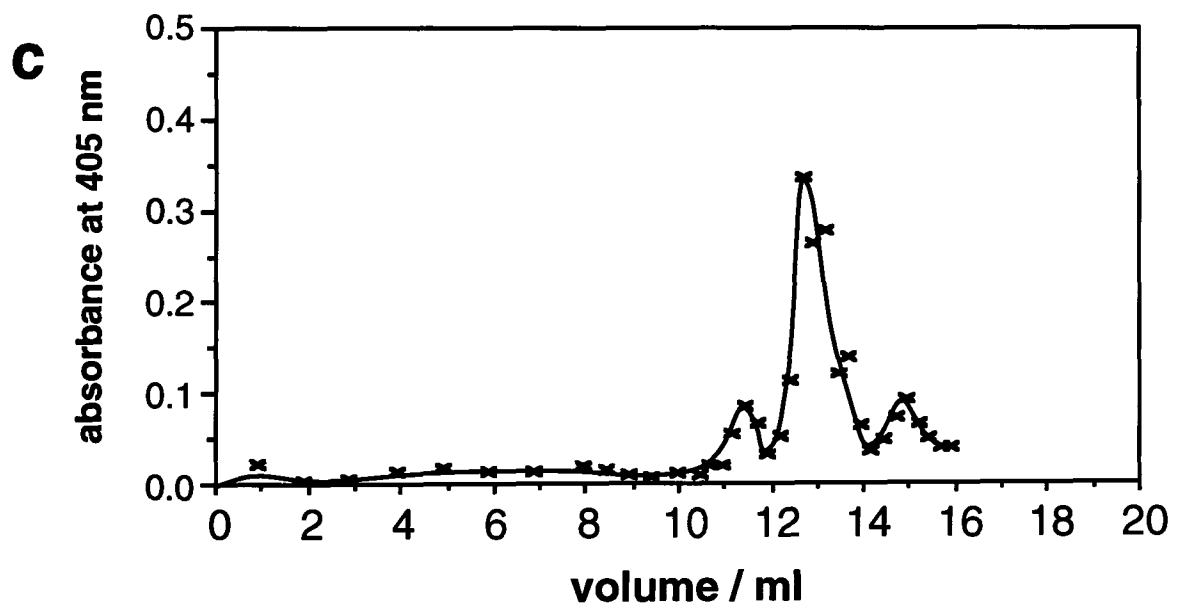


Figure 4.17

Gel exclusion chromatography comparison of the oligomeric profiles of
a) purified plasma properdin,
b) plasma properdin treated with PNGase F, and
c) recombinant properdin produced in the presence of tunicamycin.
[(a) and (b) determined by absorbance at 280 nm, (c) determined by triplicate ELISA of eluted fractions.

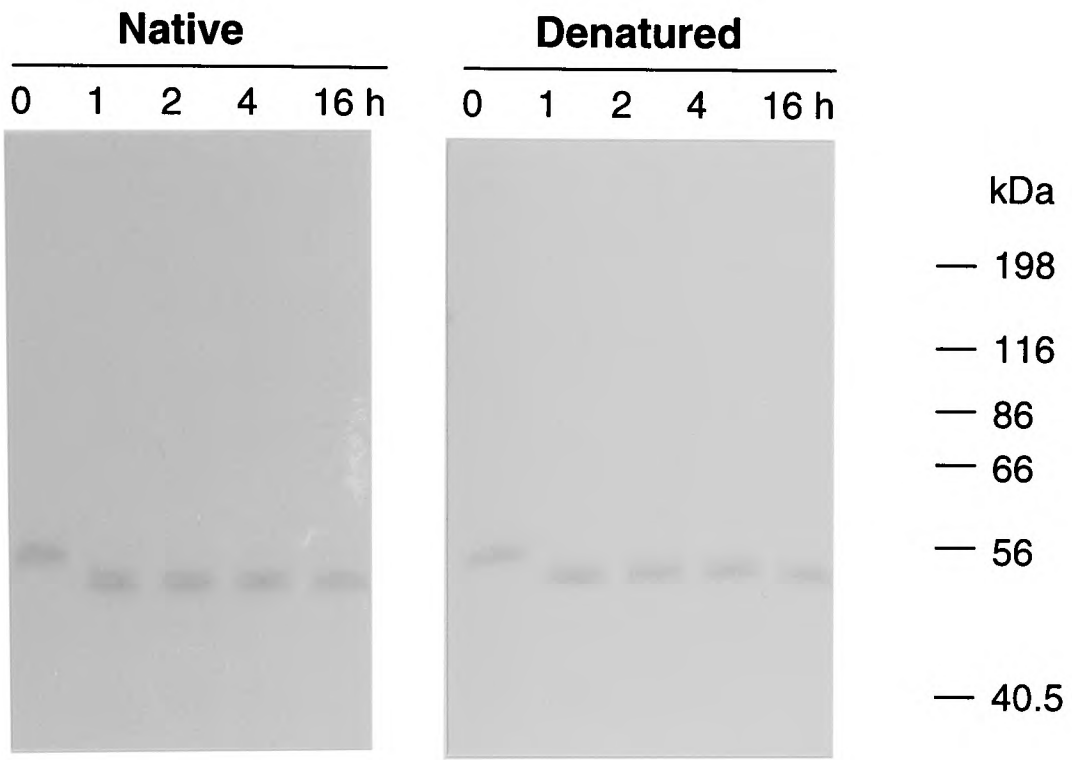


Figure 4.18

Time course of deglycosylation of purified plasma properdin with PNGase F (New England Biolabs).

Removal of N-linked sugar was carried out as recommended by New England Biolabs. Properdin (10 µg in 100 µl) was incubated with 2000 U PNGase F in either a) native conditions or b) with properdin denatured with 0.5% SDS/1% 2-mercaptoethanol prior to addition of the enzyme and NP-40 to a final concentration of 1%. Reactions were incubated at 37°C, and samples withdrawn for analysis at the times indicated.

10% SDS-PAGE, reducing conditions, Coomassie Blue stained.

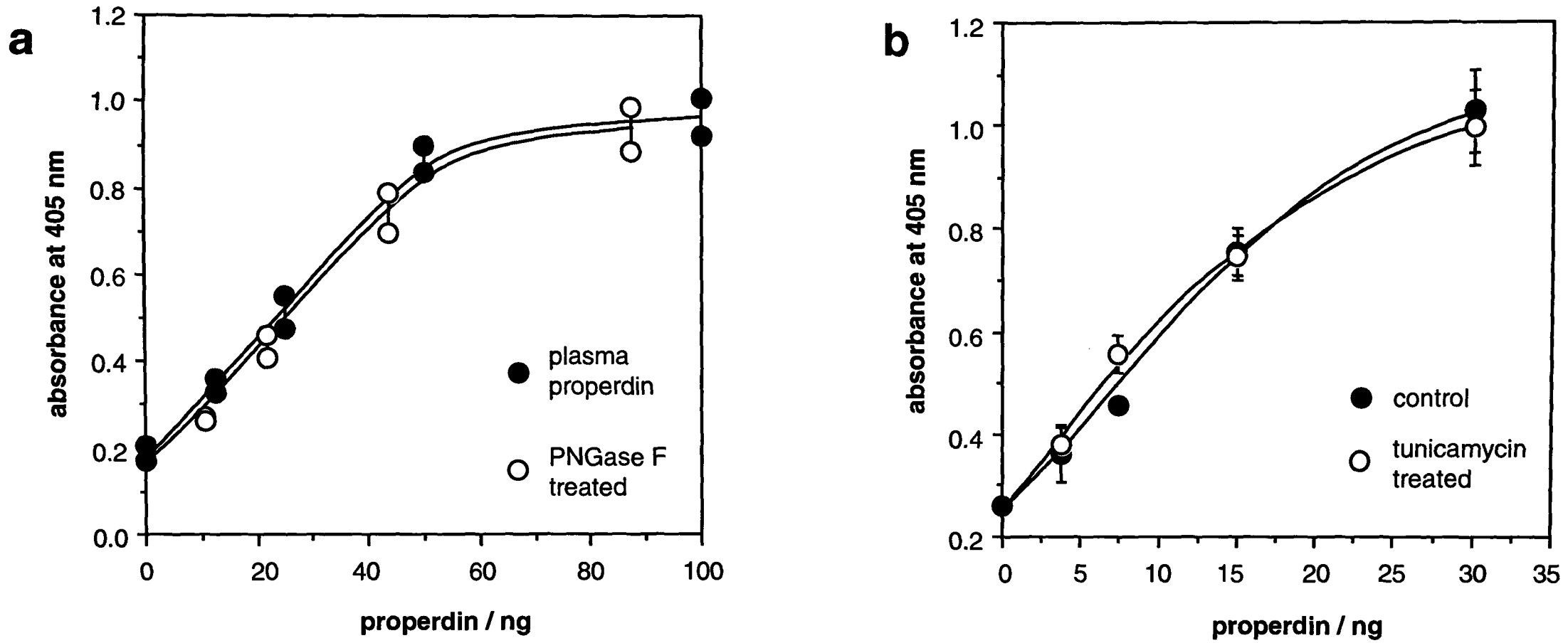


Figure 4.19
The haemolytic activity of properdin lacking N-linked oligosaccharide.
a) Comparison of plasma properdin before and after deglycosylation with PNGase F. The results of duplicate experiments are shown.
b) Comparison of recombinant properdin produced in the presence and absence of tunicamycin (control cells were grown in the presence of DMSO only, treated cells were grown in the presence of tunicamycin and DMSO). The means ± standard deviation are shown, n=4.

Some evidence of limited degradation of recombinant properdin was seen when transfected CHO cells were grown in the presence of tunicamycin (and DMSO) but not with DMSO alone. This suggests that the N-linked carbohydrate of properdin may protect the molecule against proteolysis. Such a role for carbohydrate has been suggested for a number of other proteins (Lis and Sharon, 1993). In addition, the oligosaccharide is also probably important in the removal of molecules such as properdin from the serum, through the removal of terminal sialic acid residues and binding to receptors such as the asialoglycoprotein receptor (Ashwell and Morell, 1984).

4.12 Summary

Recombinant wild type human properdin was produced in Chinese Hamster Ovary cells. The recombinant protein is similar to the plasma protein in size, immunoreactivity, N-terminal sequence, and appearance under the electron microscope. The expressed material formed oligomers in a similar ratio to that found in plasma. However, the precise distribution of oligomeric forms was found to depend on the concentration of monomers present during oligomerization. The recombinant properdin was also indistinguishable from plasma properdin in an alternative pathway haemolytic assay, and in C3b and sulphatide binding.

Recombinant properdin had a similar level of N-glycosylation to plasma properdin, but a small difference in the size of the oligosaccharide may exist. However, the N-linked sugar is not required for oligomerization, secretion, or function of properdin in the alternative pathway. It was confirmed that the N-linked oligosaccharide is attached to the C-terminal 20 kDa of properdin. The CHO cell system for expression of properdin was thus considered suitable for the expression of mutant forms of properdin.

Chapter 5

Chapter 5
Production of altered forms of human properdin

As discussed in the previous chapter, it was found that expression of wild type human properdin in Chinese Hamster ovary cells led to the secretion of a protein with structural and functional properties essentially identical to that of properdin isolated from human plasma. Having established this, mutant forms of properdin lacking single thrombospondin repeats were produced using the same system. In addition, normal plasma properdin was treated with trypsin to produce a form of properdin with a "nick" in the polypeptide backbone in TSR5. The production of properdin without N-linked glycosylation was described in chapter 4. A summary of the various altered forms that were made is shown in figure 5.1.

5.1 Deletion of single thrombospondin repeats from the human properdin cDNA

Four mutant constructs were produced each lacking one of TSR3, TSR4, TSR5, or TSR6. Also, a fifth construct with a deletion of the last twenty-nine amino acids was made. The extent of the amino acid sequence of each TSR was defined by the exon structure of the human gene. However, the deletions were made in phase 0 (between codons), rather than according to the precise phase of the properdin exons, in order to maintain the reading frame (see figure 5.2). TSRs three, four and five were removed from the human properdin cDNA (in pBSproperdin.exp) using an inverse PCR method simplified from that of Hemsley *et al.* (1989). This method involves the PCR amplification of a plasmid using phosphorylated primers which flank the region to be deleted (see figure 5.3). The resulting large PCR products (approximately 4 kb) are then circularised by blunt-end ligation. After transformation of *E. coli,* clones containing the required deletion can be identified by double stranded sequencing. The PCR is likely to create errors during amplification. So, in each case, the region containing the deletion was excised at unique flanking restriction sites and ligated into fresh pBSproperdin.exp (see below and figure 5.2). It was then only necessary to sequence through the restriction sites and the insert to verify the sequence of the mutant construct.

TSR3 was removed by PCR using primers *A93/15* (corresponding to amino acids 164-155) and *A93/17* (amino acids 229-238). In this case, sequencing revealed the presence of a single base substitution in the cleavage sequence of the restriction

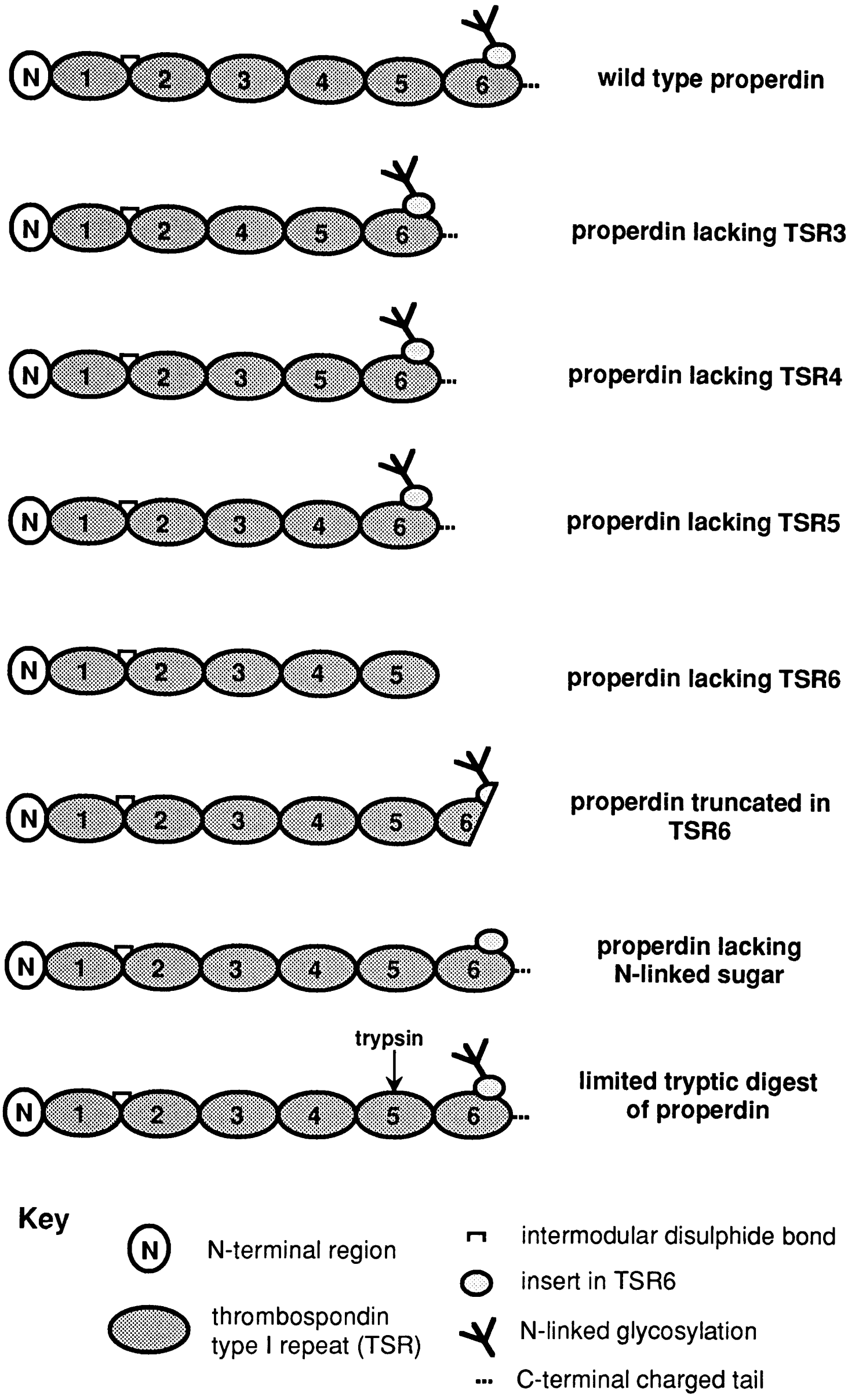


Figure 5.1
Outline structures of the monomers of the various altered forms of properdin produced in this study.

```
TGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCAC      -30    pBS

                                m  i  t  e  g  a  q  a  p  r      -18    leader
CGCGGTGGCGGCCGCTCTAGAGCCGCCACCATGATCACAGAGGGAGCGCAGGCCCCTCGA       30
                |                |
              XbaI             BclI

 l  l  l  p  p  l  l  l  l  l  t  l  p  a  t  g  s  D  P  V        3
TTGTTGCTGCCGCCGCTGCTCCTGCTGCTCACCCTGCCAGCCACAGGCTCAGACCCCGTG      90

 L  C  F  T  Q  Y  E  E  S  S  G  K  C  K  G  L  L  G  G  G       23
CTCTGCTTCACCCAGTATGAAGAATCCTCCGGCAAGTGCAAGGGCCTCCTGGGGGGTGGT     150    N-terminus

 V  S  V  E  D  C  C  L  N  T  A  F  A  Y  Q  K  R  S  G  G       43
GTCAGCGTGGAAGACTGCTGTCTCAACACTGCCTTTGCCTACCAGAAACGTAGTGGTGGG     210
         |
       BbsI

 L  C  Q  P  C  R  S  P  R  W  S  L  W  S  T  W  A  P  C  S       63
CTCTGTCAGCCTTGCAGGTCCCCACGATGGTCCCTGTGGTCCACATGGGCCCCCTGTTCG     270

 V  T  C  S  E  G  S  Q  L  R  Y  R  R  C  V  G  W  N  G  Q       83
GTGACGTGCTCTGAGGGCTCCCAGCTGCGGTACCGGCGCTGTGTGGGCTGGAATGGGCAG     330    TSR1

 C  S  G  K  V  A  P  G  T  L  E  W  Q  L  Q  A  C  E  D  Q      103
TGCTCTGGAAAGGTGGCACCTGGGACCCTGGAGTGGCAGCTCCAGGCCTGTGAGGACCAG     390

 Q  C  C  P  E  M  G  G  W  S  G  W  G  P  W  E  P  C  S  V      123
CAGTGCTGTCCTGAGATGGGCGGCTGGTCTGGCTGGGGGCCCTGGGAGCCTTGCTCTGTC     450

 T  C  S  K  G  T  R  T  R  R  R  A  C  N  H  P  A  P  K  C      143
ACCTGCTCCAAAGGGACCCGGACCCGCAGGCGAGCCTGTAATCACCCTGCTCCCAAGTGT     510    TSR2

 G  G  H  C  P  G  Q  A  Q  E  S  E  A  C  D  T  Q  Q  V  C      163
GGGGGCCACTGCCCAGGACAGGCACAGGAATCAGAGGCCTGTGACACCCAGCAGGTCTGC     570
                                  CTCCGGACACTGTGGGTCGTCCAGACG
                                                < A93/15

 P  T  H  G  A  W  A  T  W  G  P  W  T  P  C  S  A  S  C  H      183
CCCACACACGGGGCCTGGGCCACCTGGGGCCCCTGGACCCCCTGCTCAGCCTCCTGCCAC     630
GGG

 G  G  P  H  E  P  K  E  T  R  S  R  K  C  S  A  P  E  P  S      203
GGTGGACCCCACGAACCTAAGGAGACACGAAGCCGCAAGTGTTCTGCACCTGAGCCCTCC     690    TSR3

 Q  K  P  P  G  K  P  C  P  G  L  A  Y  E  Q  R  R  C  T  G      223
CAGAAACCTCCTGGGAAGCCCTGCCCGGGGCTAGCCTACGAGCAGCGGAGGTGCACCGGC     750
                                  |                TGGCCG
                                NheI

 L  P  P  C  P  V  A  G  G  W  G  P  W  G  P  V  S  P  C  P      243
CTGCCACCCTGCCCAGTGGCTGGGGGCTGGGGGCCTTGGGGCCCTGTGAGCCCCTGCCCT     810
GACGGTGGGACGGGT              A93/17 >
 < TSR3.3'

 V  T  C  G  L  G  Q  T  M  E  Q  R  T  C  N  H  P  V  P  Q      263
GTGACCTGTGGCCTGGGCCAGACCATGGAACAACGGACGTGCAATCACCCTGTGCCCCAG     870    TSR4
                              J89/10 >

 H  G  G  P  F  C  A  G  D  A  T  R  T  H  I  C  N  T  A  V      283
CATGGGGGCCCCTTCTGTGCTGGCGATGCCACCCGGACCCACATCTGCAACACAGCTGTG     930
                                  TAGACGTTGTGTCGACAC
                                      < A93/14

 P  C  P  V  D  G  E  W  D  S  W  G  E  W  S  P  C  I  R  R      303
CCCTGCCCTGTGGATGGGGAGTGGGACTCGTGGGGGGAGTGGAGCCCCTGTATCCGACGG     990
GGGACGGGA            C90/41 >
```

```
TGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCAC      -30    pBS

                                m  i  t  e  g  a  q  a  p  r      -18    leader
CGCGGTGGCGGCCGCTCTAGAGCCGCCACCATGATCACAGAGGGAGCGCAGGCCCCTCGA       30
                |                |
               XbaI             BclI

 l  l  l  p  p  l  l  l  l  l  t  l  p  a  t  g  s  D  P  V        3
TTGTTGCTGCCGCCGCTGCTCCTGCTGCTCACCCTGCCAGCCACAGGCTCAGACCCCGTG      90

 L  C  F  T  Q  Y  E  E  S  S  G  K  C  K  G  L  L  G  G  G       23
CTCTGCTTCACCCAGTATGAAGAATCCTCCGGCAAGTGCAAGGGCCTCCTGGGGGGTGGT     150    N-terminus

 V  S  V  E  D  C  C  L  N  T  A  F  A  Y  Q  K  R  S  G  G       43
GTCAGCGTGGAAGACTGCTGTCTCAACACTGCCTTTGCCTACCAGAAACGTAGTGGTGGG     210
         |
        BbsI

 L  C  Q  P  C  R  S  P  R  W  S  L  W  S  T  W  A  P  C  S       63
CTCTGTCAGCCTTGCAGGTCCCCACGATGGTCCCTGTGGTCCACATGGGCCCCCTGTTCG     270

 V  T  C  S  E  G  S  Q  L  R  Y  R  R  C  V  G  W  N  G  Q       83
GTGACGTGCTCTGAGGGCTCCCAGCTGCGGTACCGGCGCTGTGTGGGCTGGAATGGGCAG     330    TSR1

 C  S  G  K  V  A  P  G  T  L  E  W  Q  L  Q  A  C  E  D  Q      103
TGCTCTGGAAAGGTGGCACCTGGGACCCTGGAGTGGCAGCTCCAGGCCTGTGAGGACCAG     390

 Q  C  C  P  E  M  G  G  W  S  G  W  G  P  W  E  P  C  S  V      123
CAGTGCTGTCCTGAGATGGGCGGCTGGTCTGGCTGGGGGCCCTGGGAGCCTTGCTCTGTC     450

 T  C  S  K  G  T  R  T  R  R  R  A  C  N  H  P  A  P  K  C      143
ACCTGCTCCAAAGGGACCCGGACCCGCAGGCGAGCCTGTAATCACCCTGCTCCCAAGTGT     510    TSR2

 G  G  H  C  P  G  Q  A  Q  E  S  E  A  C  D  T  Q  Q  V  C      163
GGGGGCCACTGCCCAGGACAGGCACAGGAATCAGAGGCCTGTGACACCCAGCAGGTCTGC     570
                                        CTCCGGACACTGTGGGTCGTCCAGACG
                                                  < A93/15

 P  T  H  G  A  W  A  T  W  G  P  W  T  P  C  S  A  S  C  H      183
CCCACACACGGGGCCTGGGCCACCTGGGGCCCCTGGACCCCCTGCTCAGCCTCCTGCCAC     630
GGG

 G  G  P  H  E  P  K  E  T  R  S  R  K  C  S  A  P  E  P  S      203
GGTGGACCCCACGAACCTAAGGAGACACGAAGCCGCAAGTGTTCTGCACCTGAGCCCTCC     690    TSR3

 Q  K  P  P  G  K  P  C  P  G  L  A  Y  E  Q  R  R  C  T  G      223
CAGAAACCTCCTGGGAAGCCCTGCCCGGGGCTAGCCTACGAGCAGCGGAGGTGCACCGGC     750
                                  |                     TGGCCG
                                 NheI

 L  P  P  C  P  V  A  G  G  W  G  P  W  G  P  V  S  P  C  P      243
CTGCCACCCTGCCCAGTGGCTGGGGGCTGGGGGCCTTGGGGCCCTGTGAGCCCCTGCCCT     810
GACGGTGGGACGGGT              A93/17 >
 < TSR3.3'

 V  T  C  G  L  G  Q  T  M  E  Q  R  T  C  N  H  P  V  P  Q      263
GTGACCTGTGGCCTGGGCCAGACCATGGAACAACGGACGTGCAATCACCCTGTGCCCCAG     870    TSR4
                              J89/10 >

 H  G  G  P  F  C  A  G  D  A  T  R  T  H  I  C  N  T  A  V      283
CATGGGGGCCCCTTCTGTGCTGGCGATGCCACCCGGACCCACATCTGCAACACAGCTGTG     930
                                              TAGACGTTGTGTCGACAC
                                                    < A93/14

 P  C  P  V  D  G  E  W  D  S  W  G  E  W  S  P  C  I  R  R      303
CCCTGCCCTGTGGATGGGGAGTGGGACTCGTGGGGGGAGTGGAGCCCCTGTATCCGACGG     990
GGGACGGGA          C90/41 >
```

```
TGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCAC      -30    pBS

                                m  i  t  e  g  a  q  a  p  r      -18    leader
CGCGGTGGCGGCCGCTCTAGAGCCGCCACCATGATCACAGAGGGAGCGCAGGCCCCTCGA       30
                |                |
              XbaI             BclI

 l  l  l  p  p  l  l  l  l  l  t  l  p  a  t  g  s  D  P  V        3
TTGTTGCTGCCGCCGCTGCTCCTGCTGCTCACCCTGCCAGCCACAGGCTCAGACCCCGTG      90

 L  C  F  T  Q  Y  E  E  S  S  G  K  C  K  G  L  L  G  G  G       23
CTCTGCTTCACCCAGTATGAAGAATCCTCCGGCAAGTGCAAGGGCCTCCTGGGGGGTGGT     150    N-terminus

 V  S  V  E  D  C  C  L  N  T  A  F  A  Y  Q  K  R  S  G  G       43
GTCAGCGTGGAAGACTGCTGTCTCAACACTGCCTTTGCCTACCAGAAACGTAGTGGTGGG     210
         |
       BbsI

 L  C  Q  P  C  R  S  P  R  W  S  L  W  S  T  W  A  P  C  S       63
CTCTGTCAGCCTTGCAGGTCCCCACGATGGTCCCTGTGGTCCACATGGGCCCCCTGTTCG     270

 V  T  C  S  E  G  S  Q  L  R  Y  R  R  C  V  G  W  N  G  Q       83
GTGACGTGCTCTGAGGGCTCCCAGCTGCGGTACCGGCGCTGTGTGGGCTGGAATGGGCAG     330    TSR1

 C  S  G  K  V  A  P  G  T  L  E  W  Q  L  Q  A  C  E  D  Q      103
TGCTCTGGAAAGGTGGCACCTGGGACCCTGGAGTGGCAGCTCCAGGCCTGTGAGGACCAG     390

 Q  C  C  P  E  M  G  G  W  S  G  W  G  P  W  E  P  C  S  V      123
CAGTGCTGTCCTGAGATGGGCGGCTGGTCTGGCTGGGGGCCCTGGGAGCCTTGCTCTGTC     450

 T  C  S  K  G  T  R  T  R  R  R  A  C  N  H  P  A  P  K  C      143
ACCTGCTCCAAAGGGACCCGGACCCGCAGGCGAGCCTGTAATCACCCTGCTCCCAAGTGT     510    TSR2

 G  G  H  C  P  G  Q  A  Q  E  S  E  A  C  D  T  Q  Q  V  C      163
GGGGGCCACTGCCCAGGACAGGCACAGGAATCAGAGGCCTGTGACACCCAGCAGGTCTGC     570
                                CTCCGGACACTGTGGGTCGTCCAGACG
                                                < A93/15

 P  T  H  G  A  W  A  T  W  G  P  W  T  P  C  S  A  S  C  H      183
CCCACACACGGGGCCTGGGCCACCTGGGGCCCCTGGACCCCCTGCTCAGCCTCCTGCCAC     630
GGG

 G  G  P  H  E  P  K  E  T  R  S  R  K  C  S  A  P  E  P  S      203
GGTGGACCCCACGAACCTAAGGAGACACGAAGCCGCAAGTGTTCTGCACCTGAGCCCTCC     690    TSR3

 Q  K  P  P  G  K  P  C  P  G  L  A  Y  E  Q  R  R  C  T  G      223
CAGAAACCTCCTGGGAAGCCCTGCCCGGGGCTAGCCTACGAGCAGCGGAGGTGCACCGGC     750
                                |                TGGCCG
                              NheI

 L  P  P  C  P  V  A  G  G  W  G  P  W  G  P  V  S  P  C  P      243
CTGCCACCCTGCCCAGTGGCTGGGGGCTGGGGGCCTTGGGGCCCTGTGAGCCCCTGCCCT     810
GACGGTGGGACGGGT                A93/17 >
 < TSR3.3'

 V  T  C  G  L  G  Q  T  M  E  Q  R  T  C  N  H  P  V  P  Q      263
GTGACCTGTGGCCTGGGCCAGACCATGGAACAACGGACGTGCAATCACCCTGTGCCCCAG     870    TSR4
                                J89/10 >

 H  G  G  P  F  C  A  G  D  A  T  R  T  H  I  C  N  T  A  V      283
CATGGGGGCCCCTTCTGTGCTGGCGATGCCACCCGGACCCACATCTGCAACACAGCTGTG     930
                                TAGACGTTGTGTCGACAC
                                    < A93/14

 P  C  P  V  D  G  E  W  D  S  W  G  E  W  S  P  C  I  R  R      303
CCCTGCCCTGTGGATGGGGAGTGGGACTCGTGGGGGGAGTGGAGCCCCTGTATCCGACGG     990
GGGACGGGA              C90/41 >
```

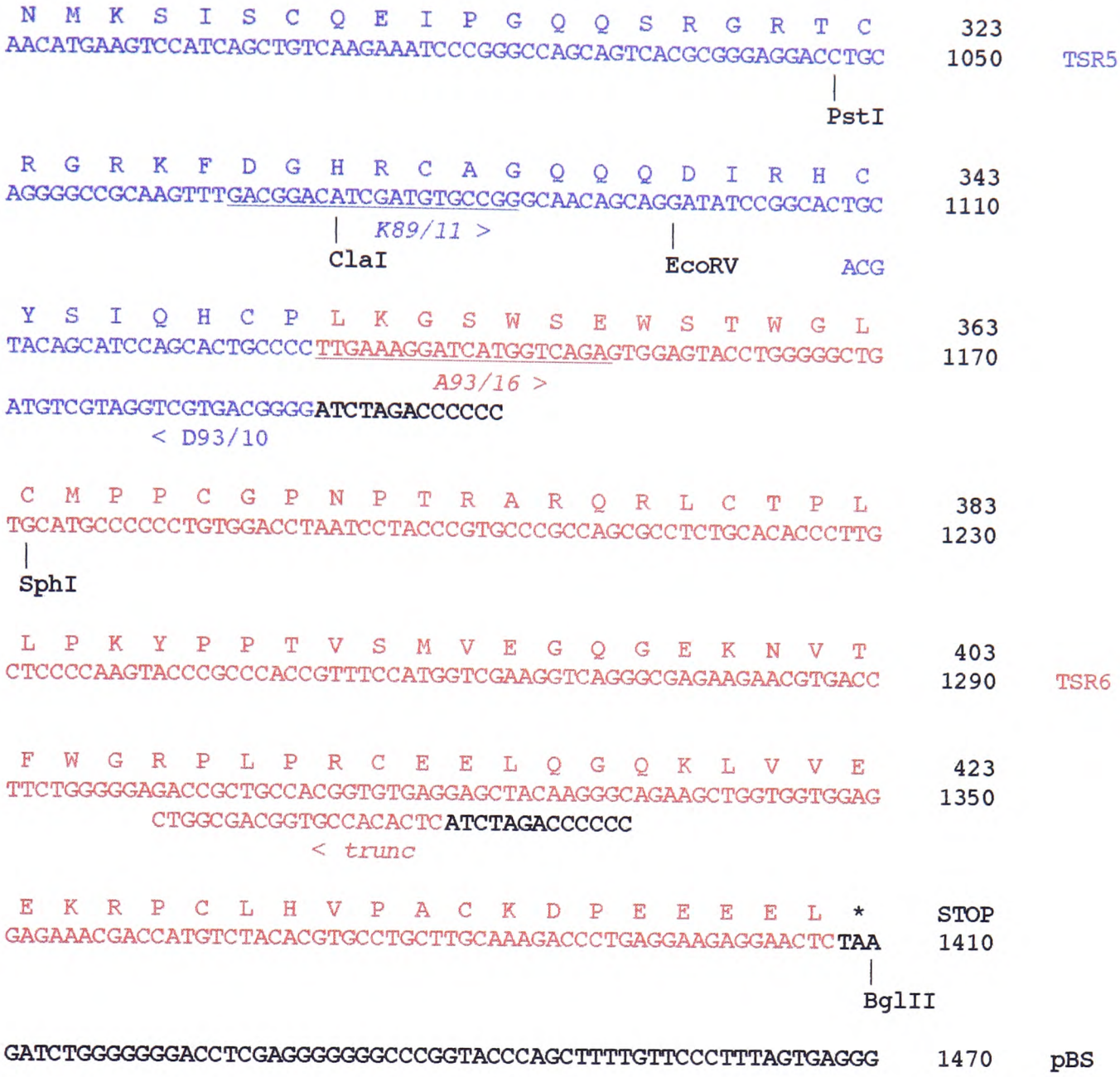


Figure 5.2

The nucleotide sequence of the human properdin expression cassette in Bluescript (pBS) and the derived amino acid sequence, showing the position of primers and restriction sites used during deletion mutagenesis.

The DNA sequence is numbered from the first nucleotide of the translation initiation codon, ATG. The protein sequence is numbered from the first amino acid of the mature form of human properdin (the leader sequence is shown in lower case). The sequence is divided into the leader peptide, the N-terminal region and the six TSRs by colour differences. Unique restriction sites used in cloning procedures are shown below the sequence. The primers used are also shown (from 5' to 3' in the direction of the >).

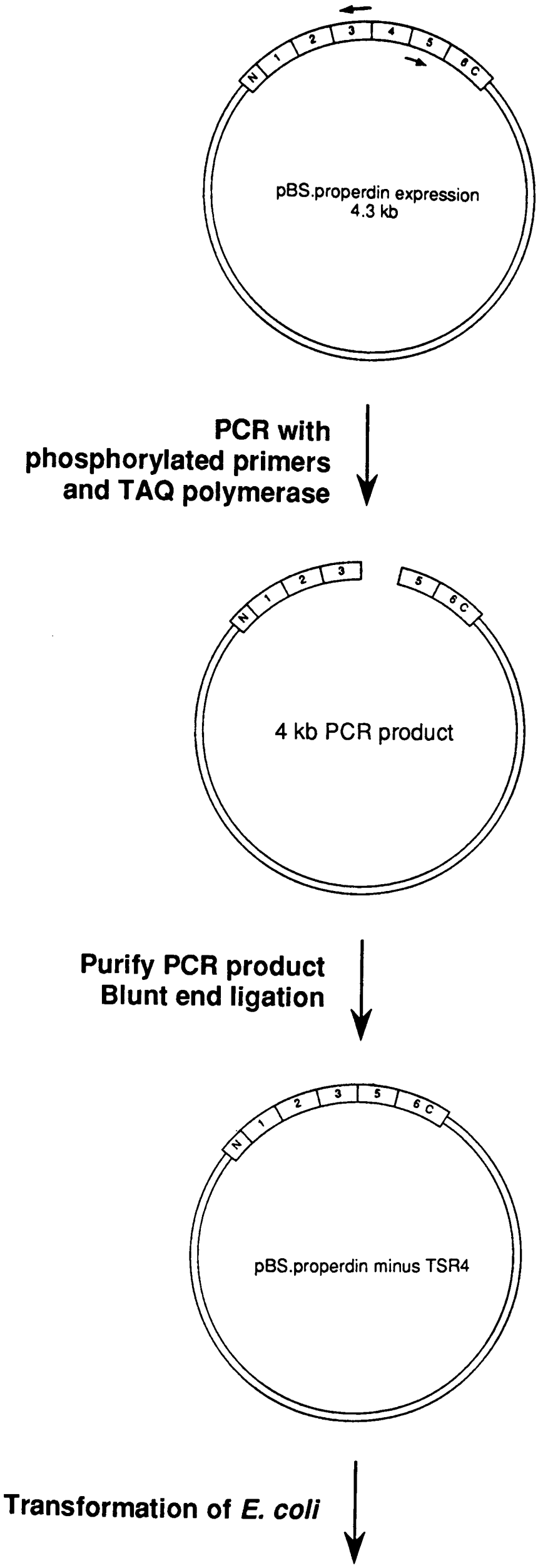


Figure 5.3
Outline of the method for deletion of single TSR modules from the human properdin cDNA in pBluescript. The technique is a simplified version of that of Hemsley *et al.* (1989). The removal of the coding for TSR4 is shown as an example. Mutant plasmids lacking the coding for TSR3 and TSR5 were obtained by the same method.
Oligonucleotides flanking the region to be deleted were phosphorylated and used to amplify the plasmid by PCR. The resulting PCR product, lacking the coding for TSR4, was purified and circularised by blunt end ligation. The resulting plasmid was used to transform *E. coli*, and clones containing the required deletion were identified by double stranded sequencing. See Materials and Methods for details.

enzyme *Bbs* I. However, the region containing the deletion was excised[1] using *Bbs* I and *Cla* I and after ligation into fresh pBSproperdin.exp (cleaved with *Bbs* I and *Cla* I) and transformation of *E. coli*, five out of six clones contained the correct sequence due to *in vivo* repair of the plasmid. TSR4 was removed by a similar procedure in which the primers *TSR3.3'* (corresponding to amino acids 228-222) and *C90/41* (amino acids 287-296) were used in the PCR and, after sequencing, the *Nhe* I - *Cla* I fragment was ligated into *Nhe* I + *Cla* I cleaved pBSproperdin.exp. TSR5 was removed by PCR with the primers *A93/14* (corresponding to amino acids 286-278) and *A93/16* (amino acids 351-357) and, after sequencing, the *Nhe* I - *Sph* I fragment was ligated into *Nhe* I + *Sph* I cleaved pBSproperdin.exp.

To produce a construct lacking TSR6 (and the C-terminal charged tail), the PCR was used to amplify a DNA fragment from TSR5 which contained a stop codon and a *Bgl* II site at the 3' end of TSR5. The primers used were *J89/10* (corresponding to amino acids 252-258) and *D93/10* (corresponding to amino acids 350-343 with additional stop codon and *Bgl* II site). This fragment was cleaved with *Pst* I and *Bgl* II and then ligated into *Pst* I + *Bgl* II cleaved pBSproperdin.exp. A construct known as "trunc" was also produced using a similar procedure. This form has a deletion of the last twenty-nine amino acids of properdin (those amino acids beyond the region previously defined as TSR6 by the alignment of Smith *et al.* (1991), see chapter 3, figure 3.10a). The PCR was carried out using the primers *K89/11* (corresponding to amino acids 329-335) and *trunc* (amino acids 413-407 with additional stop codon and *Bgl* II site). This fragment was cleaved with *Sph* I and *Bgl* II and then ligated into *Sph* I + *Bgl* II cleaved pBSproperdin.exp.

Full length coding regions containing deletions of single TSRs were then excised with *Xba* I +*Bgl* II and subcloned into *Xba* I + *Bcl* I cleaved pEE6.HCMV.GS to create the final expression vectors.

5.2 Transfection of CHO cells and production of mutant forms of properdin

CHO cells were transfected and stable transfectants isolated as described for the wild type protein, using 25 or 30 μM MSX (see chapter 4). The polyclonal anti-properdin IgG sandwich ELISA was again used to screen culture supernatants for secreted properdin. Clones were isolated which secreted immunoreactive material in each case. However the yields obtained were much lower than for the wild type protein (typical yields in serum-free medium: properdin lacking TSR3, 0.1 μg ml^{-1}; lacking TSR4, 1.5 μg ml^{-1}; lacking TSR5, 0.02 μg ml^{-1}; lacking TSR6, 0.1 μg ml^{-1}; trunc, 0.1 μg ml^{-1}).

[1] The recognition sequence of *Bbs* I is distinct from the cleavage site, so excision was still possible

However amplification of the glutamine synthetase gene by selection for clones resistant to higher concentrations of MSX (100 or 500 μM) led to the derivation of cell populations secreting higher levels of the mutant proteins. Typical yields in serum-free medium after amplification were as follows: properdin lacking TSR3, 0.6 μg ml^{-1}; lacking TSR4, 1.8 μg ml^{-1}; lacking TSR5, 0.6 μg ml^{-1}; lacking TSR6, 0.5 μg ml^{-1}; trunc, 0.2 μg ml^{-1}. Such yields were adequate for the production and purification of the mutant proteins for functional assays.

The estimates of the concentrations of the mutant samples were made using the polyclonal anti-properdin ELISA. In this thesis the concentrations of the mutant proteins are expressed in microgrammes per millilitre as determined by ELISA in comparison with normal plasma properdin. These estimates were later confirmed by amino acid analyses of the purified proteins, but no attempt was made to precisely quantitate the molar concentrations of the various oligomers present in these complex mixtures.

5.3 SDS-PAGE and immunoblotting of mutant forms of recombinant properdin

SDS-PAGE in reducing conditions of serum-free medium from cultures of cells secreting each of the mutant forms of properdin failed to show clear bands of the expected size, due to the low yields obtained (figure 5.4). However, a Western blot probed with rabbit anti-properdin IgG confirmed the presence of immunoreactive species in each case which were not found in mock transfectant cell supernatants (figure 5.5).

Transfection with the construct lacking TSR3 produced a single main immunoreactive protein with the expected size of 46 kDa in reducing conditions. Under non-reducing conditions multiple bands similar in appearance to those found for wild type properdin were visible.

Transfection with the plasmid containing a deletion of TSR4 produced a protein of the expected size of 47 kDa, but the predominant bands seen were a doublet at around 30 kDa. A single minor band of approximately 20 kDa was also seen in some preparations (in reducing conditions, see figure 5.6). Under non-reducing conditions the intensity of the lower bands was reduced, and the largest protein appeared as closely spaced multiple bands. Since it was considered likely that the smaller bands represented degradation products of the required protein (see section 5.6), the conditions of cell culture were altered in an attempt to reduce the extent of proteolysis. The cysteine and serine protease inhibitor leupeptin (which is not toxic for mammalian cells) was included in the culture medium, and the period of cell culture was limited. As shown in figure 5.6, the yield of intact properdin lacking TSR4 could be increased by reducing the period of cell culture to two days and by the inclusion of leupeptin in the culture medium.

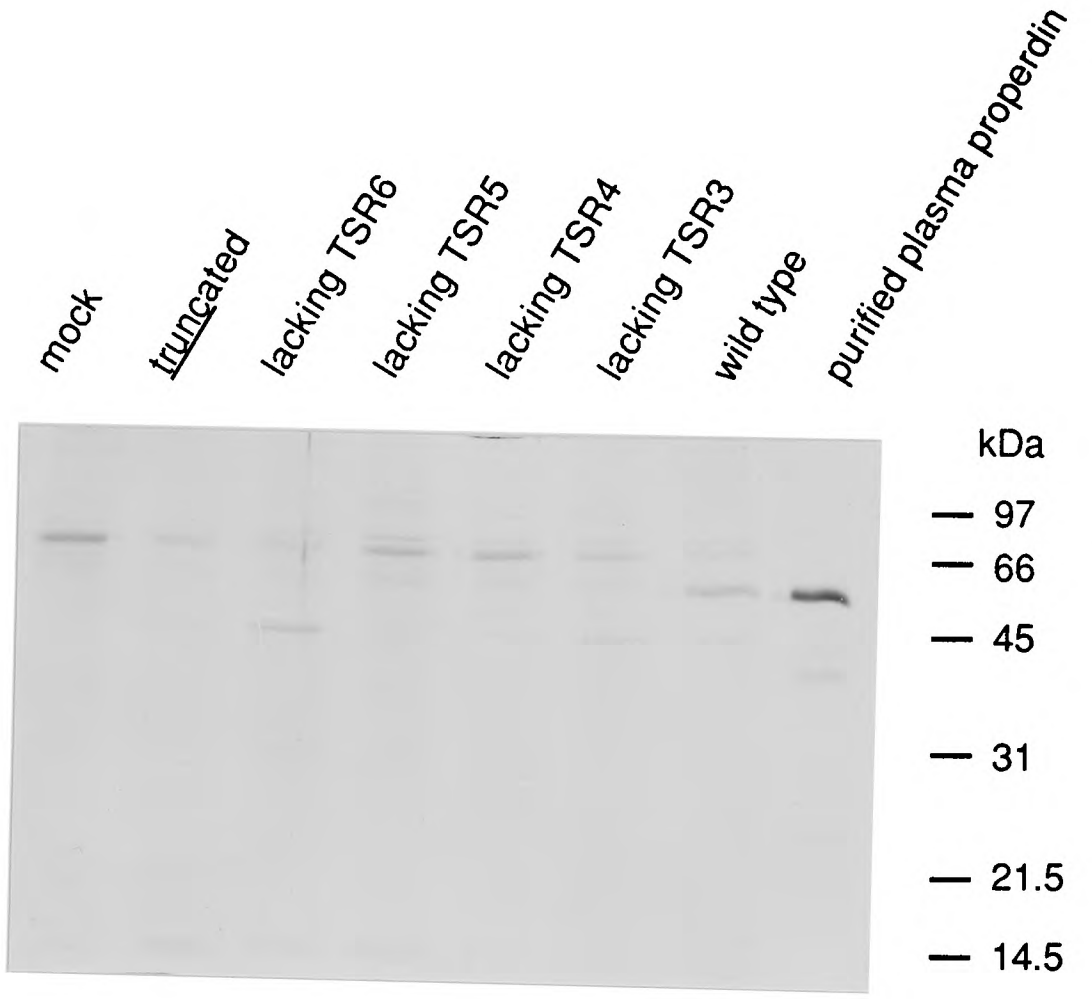


Figure 5.4

Serum-free conditioned medium from cells transfected with expression constructs for the various mutant forms of properdin, as indicated.

1.3 µg of purified plasma properdin were applied as a standard.

50 µl samples of each culture supernatant were loaded.

12.5% SDS-PAGE, reducing conditions, Coomassie Blue stained.

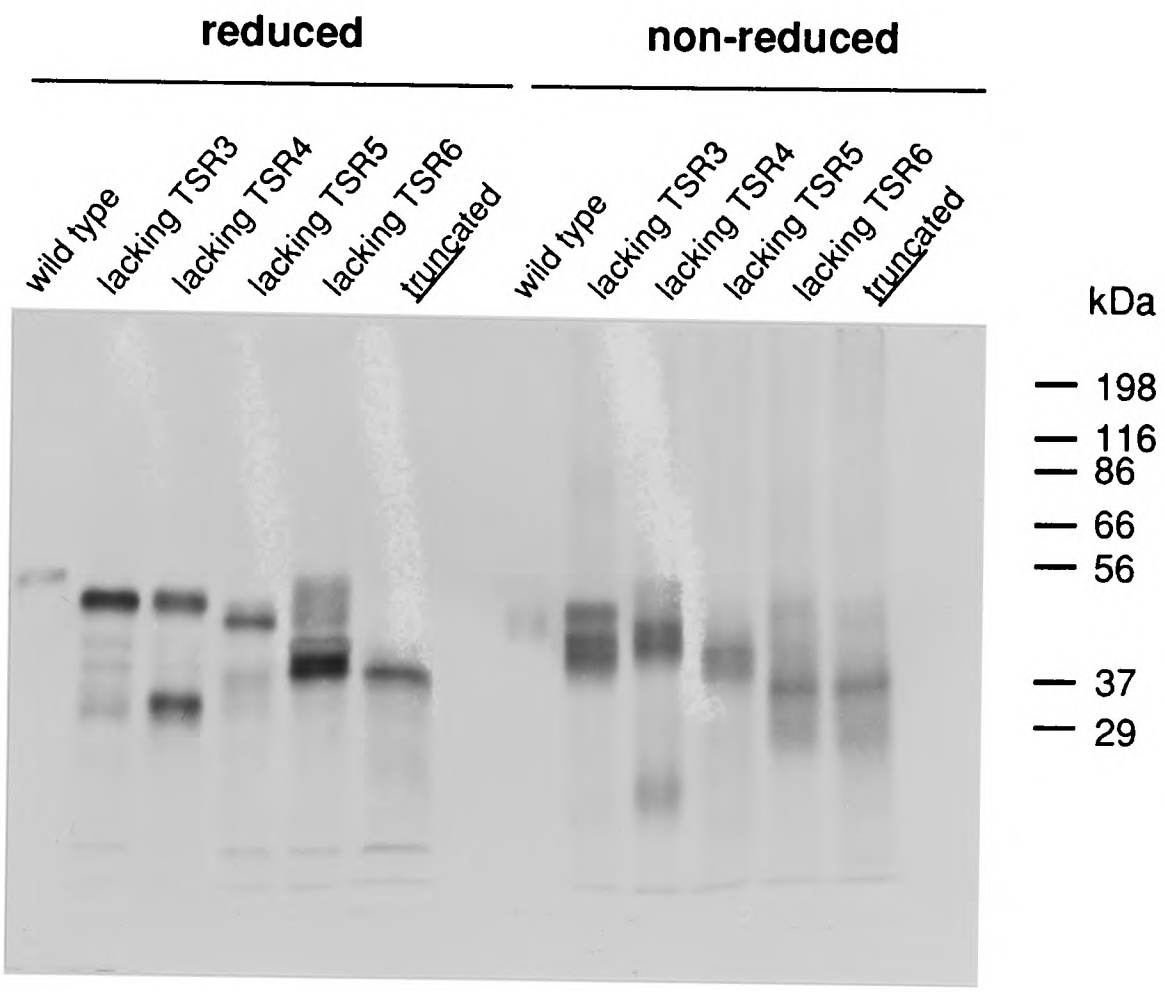


Figure 5.5

Western blot of serum-free medium from transfected CHO cells expressing the various mutant forms of recombinant properdin, probed with anti-properdin antibody.

Samples were separated by 10% SDS-PAGE in reducing and non-reducing conditions (as indicated). The proteins were then transferred to nitrocellulose and properdin detected with rabbit polyclonal anti-properdin IgG and standard procedures (as described in materials and methods).

Medium samples containing approximately 50 ng of properdin (as determined by ELISA) were loaded, except for wild type recombinant properdin (6 ng).

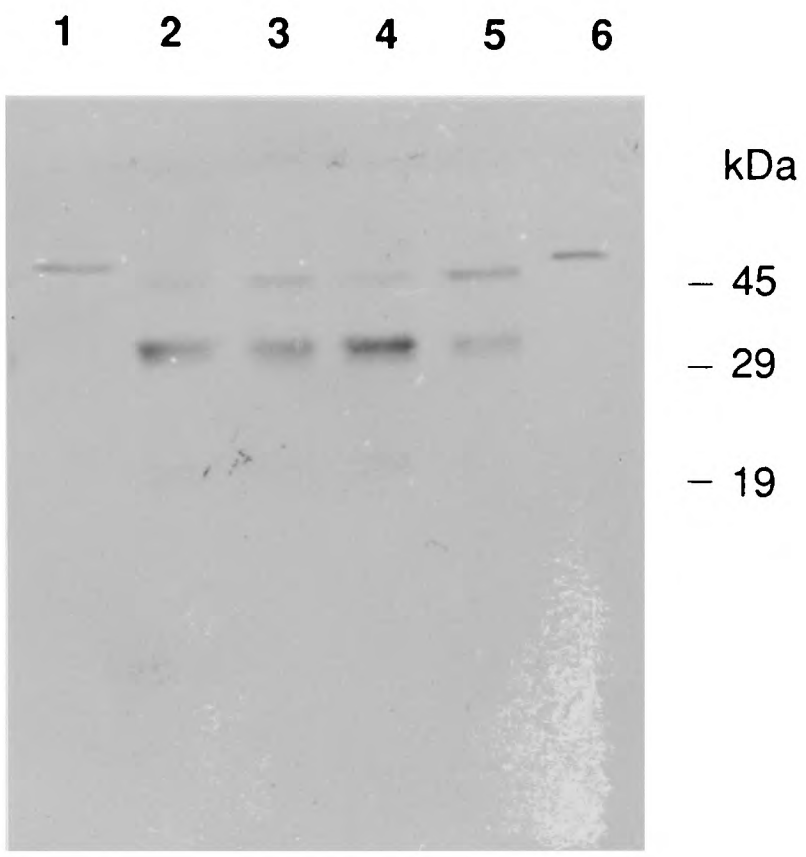


Figure 5.6

Production of recombinant properdin lacking TSR4.

CHO cells transfected with a construct bearing a deletion of the TSR4 were grown under various conditions in serum-free medium. After 12.5% SDS-PAGE in reducing conditions and electroblotting, the blot was probed with anti-properdin IgG. Lanes contain serum-free culture supernatants of:

1. Recombinant normal properdin (40 ng)
2. Recombinant properdin without TSR4, 5 day culture, no leupeptin
3. Recombinant properdin without TSR4, 2 day culture, no leupeptin
4. Recombinant properdin without TSR4, 5 day culture, with leupeptin (100 μM)
5. Recombinant properdin without TSR4, 2 day culture, with leupeptin (100 μM)
6. Plasma purified properdin (40 ng).

Transfection with the construct lacking TSR5 produced a protein of the expected size of 46 kDa and a lower band at around 43-44 kDa under reducing conditions. Again, the extent of degradation could be reduced by the inclusion of leupeptin in the culture medium and with short culture periods. However, it was not possible to prevent the degradation to better than approximately fifty percent of the total immunoreactive protein. In non-reducing conditions a more diffuse staining was found at 35-40 kDa.

Cells transfected with the TSR6 lacking construct secreted an immunoreactive protein of the predicted size (38 kDa) and a less abundant band of approximately 35 kDa which is likely to represent a degradation product (see also figure 5.12, and section 5.6). In addition, a higher molecular mass immunoreactive smear was observed between 43 and 53 kDa in reducing conditions. The origin of this material is unclear. In non-reducing conditions very diffuse staining from below 20 to over 40 kDa was observed.

Transfection with the trunc construct led to the production of a protein of the expected size of 50 kDa provided that leupeptin was present during a short period of cell culture. In the absence of leupeptin or if the period of culture was extended beyond two days, the protein was almost entirely degraded to around 34 kDa.

5.4 The oligomeric profile of the altered forms of properdin

5.4.1 Gel exclusion chromatography

Initially, to determine the size of oligomers formed by each of the mutant forms, whole conditioned serum-free medium was fractionated by gel exclusion chromatography on Superose 6, and properdin was detected by polyclonal ELISA of the fractions. The results are shown in figure 5.7. Wild type recombinant properdin showed the expected three main peaks representing dimer, trimer and tetramer fractions, all of which elute before IgG (150 kDa) (properdin behaves as if it were a larger molecule than predicted on the basis of its mass, almost certainly because of its elongated shape). The recombinant protein lacking TSR3 also elutes as multiple peaks, the smallest of which co-elutes with ovalbumin (44 kDa) and thus probably represents monomeric protein. The larger peaks are consistent with the expected migration positions of dimers, trimers and tetramers lacking TSR3. The mutants lacking TSR4 or TSR5 show similar patterns to each other. Again, a peak is observed which co-elutes with ovalbumin and may be monomeric protein, and a second peak is found eluting at or before the position of IgG which could represent dimeric (and possibly trimeric) molecules. Both properdin lacking TSR6 and the trunc mutant elute as single peaks between ovalbumin (44 kDa) and BSA (66 kDa), and thus appear not to form oligomers.

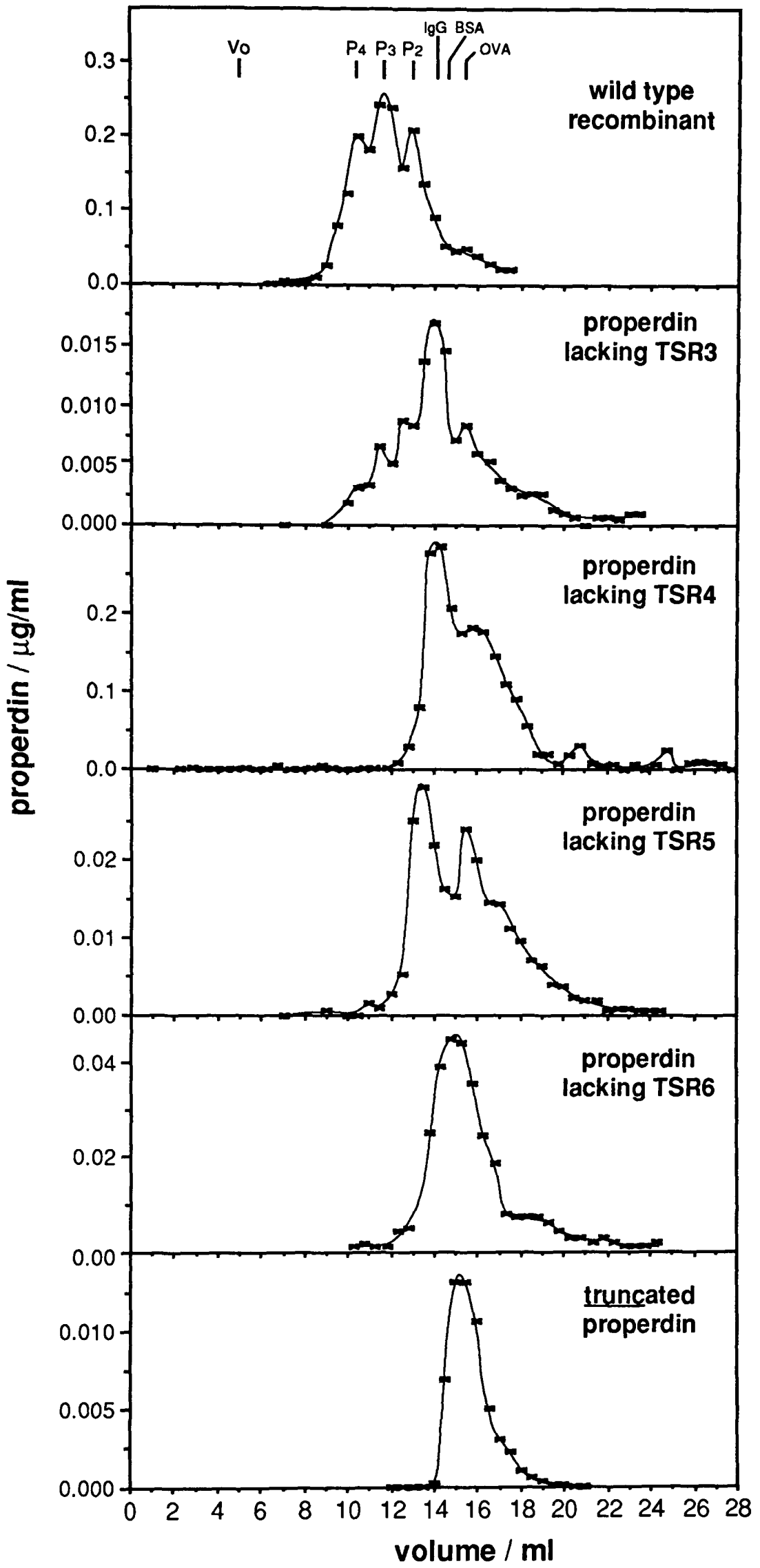


Figure 5.7

Elution profiles of recombinant forms of properdin from gel exclusion chromatography. Samples of serum-free conditioned media from transfected cell lines were dialysed into PBS, pH 7.4 and concentrated to between 4 and 10 µg/ml with Centricon-3 spin concentrators. The samples were loaded onto a Superose 6 column using a 100 µl loop, except for the truncated form, where a 200 µl loop was used. Properdin in the eluted fractions was detected by polyclonal ELISA. The void volume (Vo), and the elution positions of plasma properdin (P4, P3, P2) and of IgG (150 kDa), BSA (66 kDa) and OVA (44 kDa) are shown.

5.4.2 Chemical crosslinking

Properdin migrates anomalously on gel exclusion chromatography. This makes it difficult to determine unequivocally which oligomeric forms of the (smaller) mutant proteins are present. An independent means of visualising the oligomers was sought. The method employed was that of chemical cross-linking. A number of homo-bifunctional chemical cross-linking agents are available. These reagents contain moieties which will react with and covalently link to lysine residues on polypeptides. If two lysine residues are found at an appropriate distance apart (for instance, on two subunits within an oligomer), they can be cross-linked with bifunctional reagents. Two such cross-linkers were tried in preliminary experiments with purified plasma properdin. BS^3 (Bis(sulphosuccinimidyl)suberate) is 1.14 nm in length, is water soluble, has a molecular mass of 572 Da and is non-reducible. DTSSP (3,3'-dithiobis(sulphosuccinimidyl) propionate) is 1.2 nm in length, water soluble, has a molecular mass of 608 Da and is reducible. Cross-linking was carried out at different temperatures (22 or 37°C), for different times (30 or 60 min) and at various concentrations of crosslinker (0.1 to 10 mM) in order to determine the optimal conditions. After cross-linking 0.5 µg properdin with 1 mM BS^3 for 30 min at 37°C, monomers, dimers, trimers, tetramers and some higher forms of properdin could be clearly visualised by reducing SDS-PAGE and silver staining (not shown). The treatment also had the effect of broadening and slightly increasing the size of the monomeric properdin band. This is likely to be due to the attachment of reagent to some of the many lysines present in properdin (eighteen per monomer) without forming a cross-link (since hydrolysis of the reagent is a major competing reaction and not all lysines will be in the proximity of another lysine).

It is possible in experiments with purified proteins that under efficient cross-linking conditions, molecules which are not normally associated with each other will be cross-linked. In subsequent experiments, the cross-linking was carried out directly in serum-free medium from transfected cells, and the crosslinked products were detected by SDS-PAGE followed by Western blotting and detection with anti-properdin polyclonal antibody. In these samples properdin is not the major species present (see figure 5.4), and thus non-specific cross-linking would be expected to produce smears due to properdin becoming cross-linked to various other proteins. This was not observed. However, in these experiments, a shift in size due to properdin specifically binding to another protein, though unlikely, cannot be excluded formally.

The result of these experiments is shown in figure 5.8. After cross-linking, wild type recombinant properdin clearly shows bands of the size expected for monomers, dimers, trimers, tetramers and higher forms. Mutant properdin lacking TSR3 shows a similar pattern shifted to lower molecular mass, again suggesting the presence of dimers, trimers, tetramers, and possibly higher forms. Both the mutants lacking TSR4 or TSR5

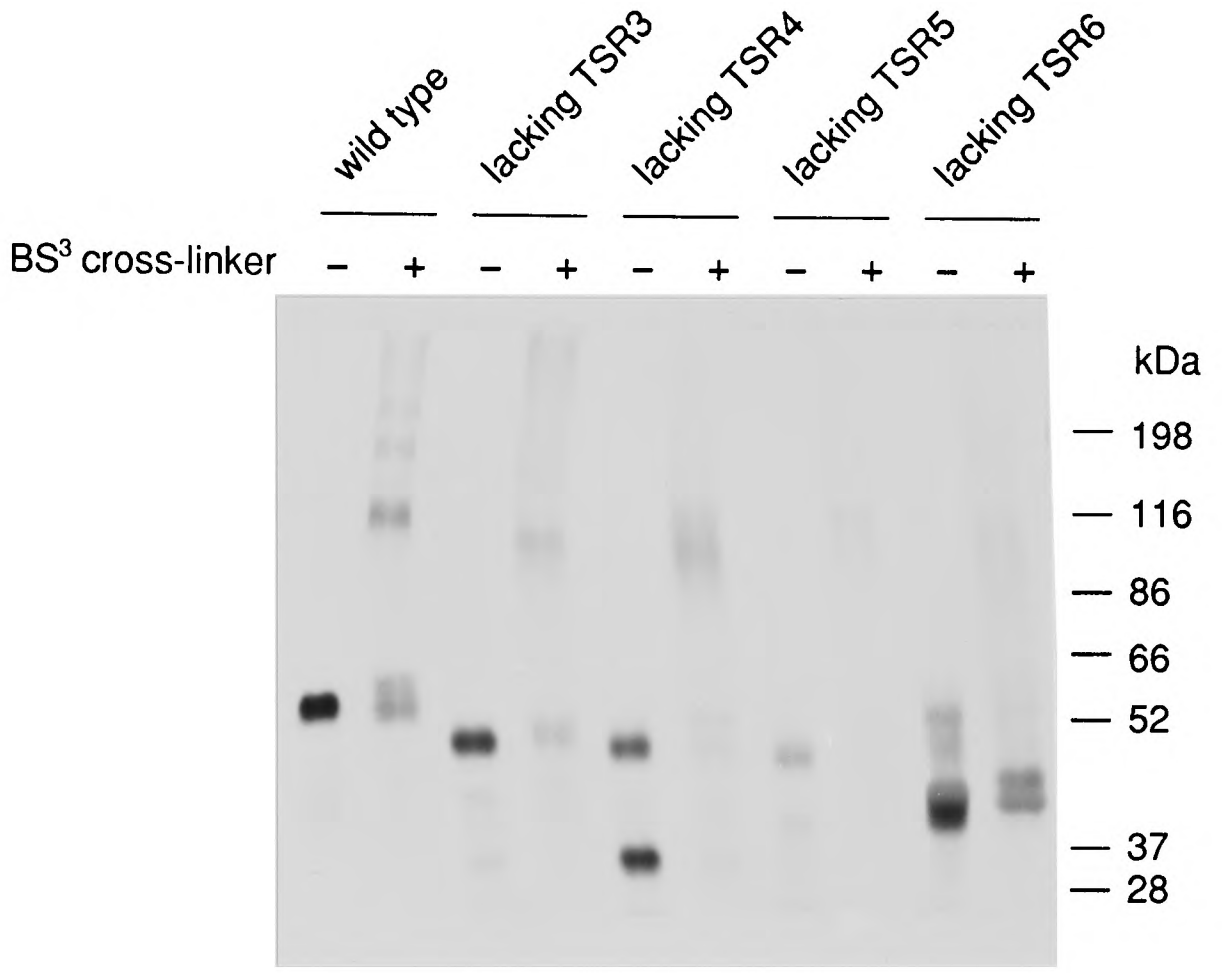


Figure 5.8

Cross-linking of oligomers of the various mutant forms of properdin in serum-free conditioned medium from CHO cell transfectants.

Serum-free supernatants were dialysed into PBS, pH 7.4, and concentrated to 10 μg/ml using Centricon-3 microconcentrators. Samples containing 100 ng properdin were crosslinked with 1 mM BS^3 in a final volume of 11 μl for 30 min at 37°C. After 7.5% SDS-PAGE in reducing conditions, proteins were transferred to nitrocellulose, and the blot was probed with polyclonal rabbit anti-properdin IgG as described in materials and methods.

give a band at around the expected size for dimers after cross-linking. It is possible that some of the size heterogeneity of the TSR4 lacking properdin dimeric band can be attributed to the limited proteolysis of this form previously observed (see above).

The results of the experiment with the TSR6 lacking mutant are more difficult to interpret. For instance, the oligomerization status of the unidentified immunoreactive material of between 43 and 53 kDa is not known. However, despite the presence of more TSR6 lacking mutant in this experiment than of the other forms, there is no clear band corresponding to the size expected for a dimeric molecule (76 kDa). The staining visible on the blot corresponds to that seen on silver staining of the cross-linked sample after SDS-PAGE. Thus, in combination with the gel exclusion chromatography results above, there is no clear evidence for oligomerization of properdin lacking TSR6.

5.5 Purification of the mutant forms of recombinant human properdin

The TSR3 lacking mutant was purified on the anti-properdin monoclonal antibody affinity column as for wild type properdin (see chapter 4). However, the mutants lacking TSR5 and TSR6 bound only with low affinity to this mAb, while binding of the form lacking TSR4 was undetectable (see below). Therefore, a polyclonal anti-properdin IgG affinity column was prepared (see materials and methods). Initially, high affinity binding sites on the column were saturated with approximately 1.5 mg of properdin in over 200 ml of serum (dialysed into PBS, pH 7.4). However, the percentage yields obtained from this column with either normal serum properdin or with the TSR4 lacking recombinant form were very low, and elution occurred in a very broad peak (washing of the 7 ml column with approximately 1 litre of elution buffer failed to reduce the levels of properdin below that detectable by silver staining or ELISA). Therefore, this column was considered inappropriate for purification of the low levels of recombinant properdin obtained from the CHO cell transfectants.

To circumvent this problem it was decided to prepare an "antigen-selected" fraction of the polyclonal antibody which could be used to make a second anti-properdin antibody affinity column. This was done by purifying a fraction of the polyclonal anti-properdin IgG on a properdin column. Since the first polyclonal anti-properdin column had up to 1 mg of human properdin irreversibly attached to it (see figure 5.9), this was used as the properdin column. Thirty milligrammes of anti-properdin IgG were passed down the properdin column and, after washing, elution was carried out under the same conditions as for the antibody affinity columns (see materials and methods). This process produced between 0.5 and 1 mg of antigen-selected anti-properdin IgG (see figure 5.9) which were then attached to CNBr-activated Sepharose to make an antigen-selected anti-properdin affinity column. This column behaved like the monoclonal anti-

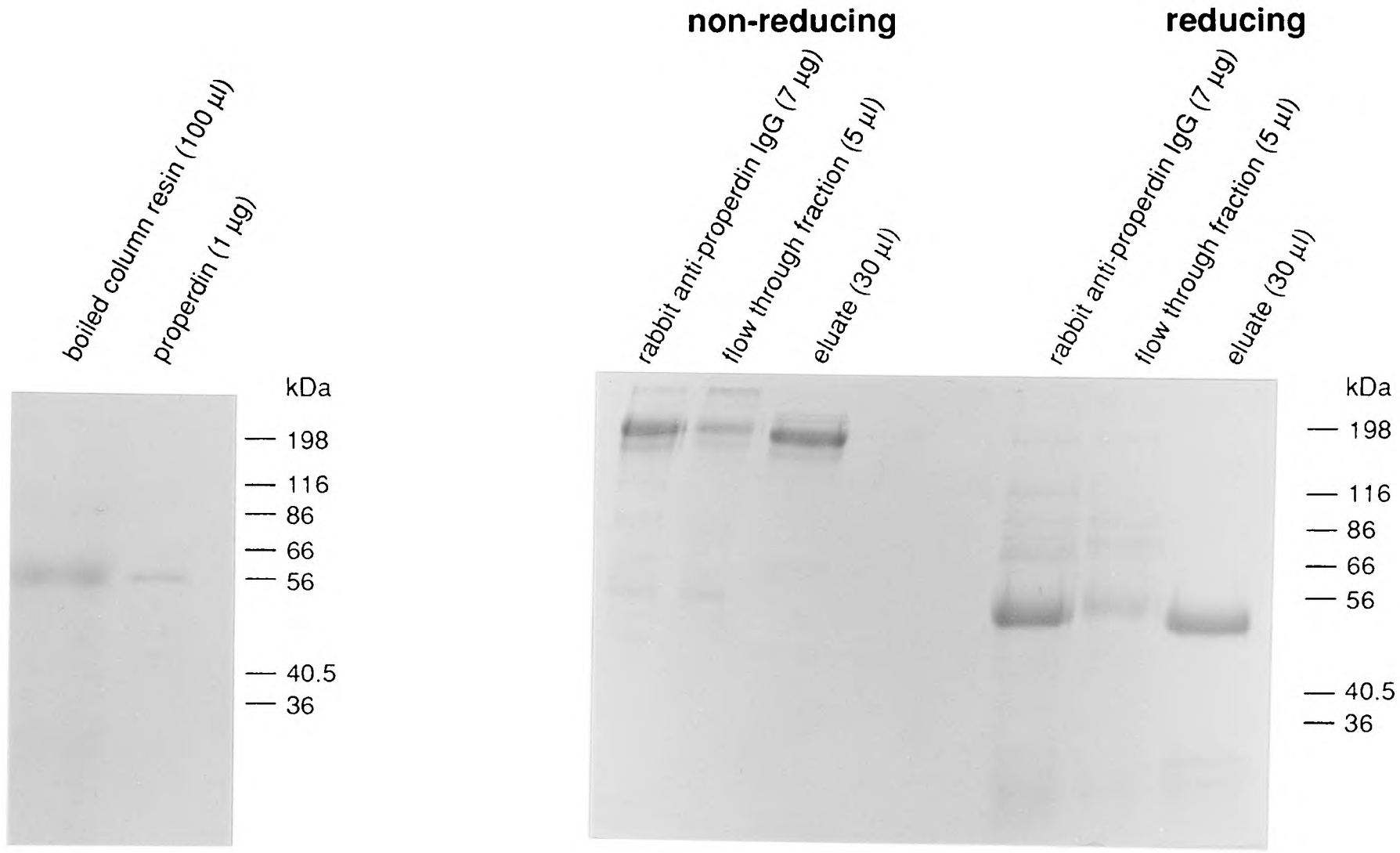


Figure 5.9
Purification of antigen-selected rabbit anti-properdin IgG.
a) Demonstration that human properdin is attached to the polyclonal anti-properdin column. 100 µl of resin from the column was boiled in SDS-PAGE sample buffer for 5 min, and analysed on 10% SDS-PAGE in reducing conditions. The multiple band pattern typical of properdin was observed in non-reducing conditions (not shown).
b) Purification of an antigen-selected fraction of rabbit anti-properdin IgG. Whole rabbit anti-properdin IgG (30 mg) was applied to the column in PBS. After washing with 3 M NaCl/PBS, bound antibody was eluted with 0.2 M glycine/0.5 M NaCl, pH 2.5. The starting material, a flow through fraction, and the eluate were analysed by 10% SDS-PAGE in reducing and non-reducing conditions as indicated. The gels were stained with Coomassie Blue.

properdin antibody column in its elution characteristics and in the percentage yields obtained.

Recombinant properdins lacking TSR4, or TSR5, or TSR6 were purified using the antigen-selected antibody column. In each case only those bands visible in Western blots of medium samples were observed by silver staining of SDS-PAGE gels, confirming the efficacy of the purification procedure (see figure 5.10). The mutants produced showed the characteristic multiple band pattern observed for wild type properdin in non-reducing conditions (figure 5.10). In the case of properdin lacking TSR6 bands of the size of the higher molecular mass immunoreactive material co-purified with the band of the expected size. Attempts to derive N-terminal sequence data from this protein were unsuccessful, while the N-terminal sequence of the smaller band at around 38 kDa was identical to human properdin over the ten amino acids checked (DPVL-FTQYE). Also, efforts to purify the protein of the expected size from the contaminating protein using Mono-S ion exchange chromatography were unsuccessful. The production of larger quantities of mutant properdin lacking TSR6 will be required for an improved purification of this protein.

5.6 Production of properdin "nicked" in TSR5

A further altered form of properdin was produced by a limited tryptic digest of purified plasma properdin. This treatment leads to the production of two fragments of 33 kDa (T_1) and 20 kDa (T_2) visible on reducing SDS-PAGE (figure 5.11). This is due to the cleavage of a peptide bond in the sequence IRRNMK↓SIS found between the first two cysteines of TSR5, as described by Reid & Gagnon (1981) (see chapter 3, section 3.6.2). However, due to the presence of intramodular disulphide bonds, this "nicked" form of properdin remains intact on non-reducing SDS-PAGE (figure 5.11).

It is possible that some of the degradation seen during production of recombinant properdin lacking TSR4 or TSR6 is also due to cleavage in this sequence. Both these deletions could potentially make the trypsin-sensitive loop in TSR5 more accessible to proteolytic enzymes. The size of the major degradation product of TSR6 lacking properdin is similar to the T_1 fragment (figure 5.12). The larger degradation product of properdin lacking TSR4 is similar to that expected for a T_1 fragment lacking TSR4. Indeed, N-terminal sequence analysis of this fragment confirmed that it had an N-terminus identical to T_1 and intact properdin. Insufficient protein was available for sequencing of the smaller degradation product of properdin without TSR4, but its size is similar to that of the T_2 fragment (figure 5.6).

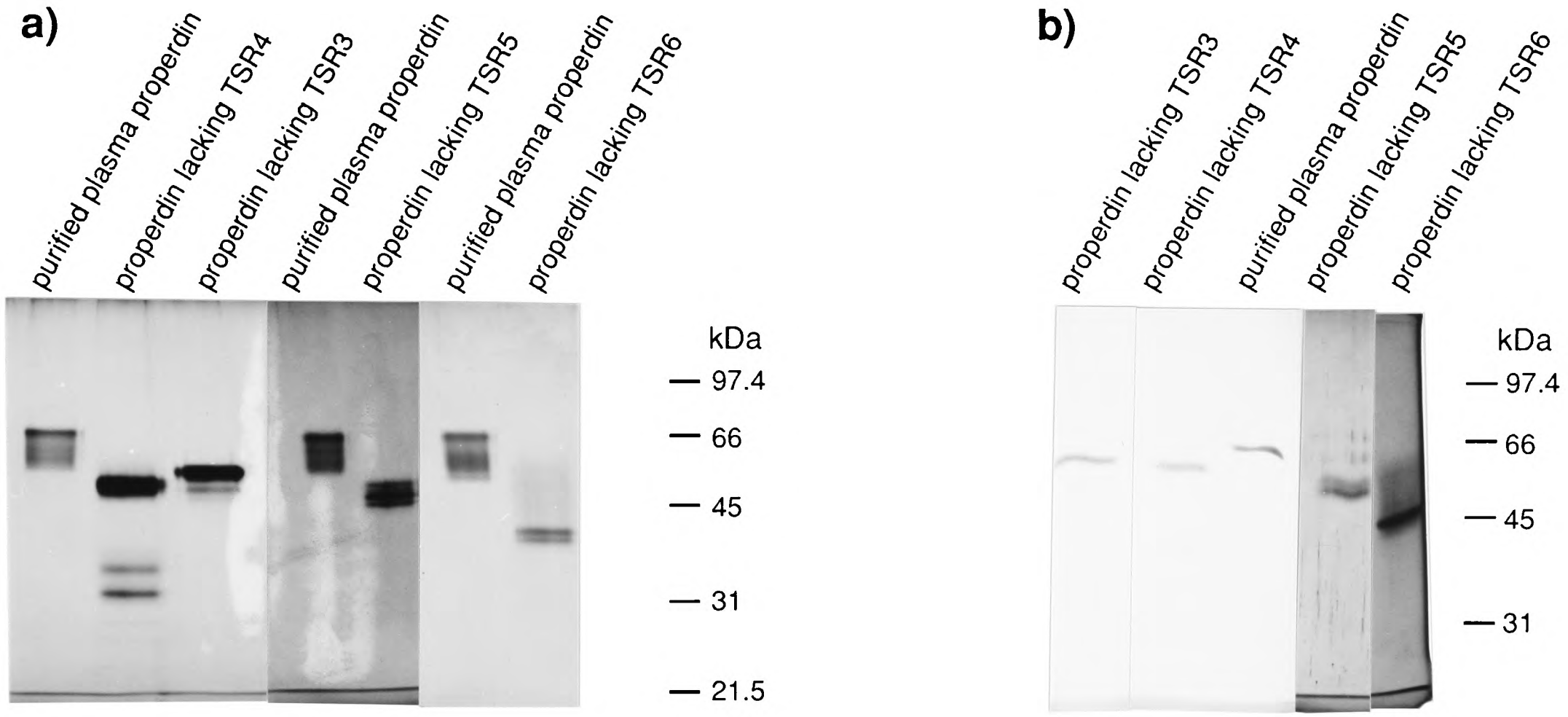


Figure 5.10
SDS-PAGE of purified mutant recombinant properdin forms compared with normal human properdin purified from plasma (200 ng). a) Non-reducing conditions, and b) reducing conditions. Both 10% SDS-PAGE, silver stained.

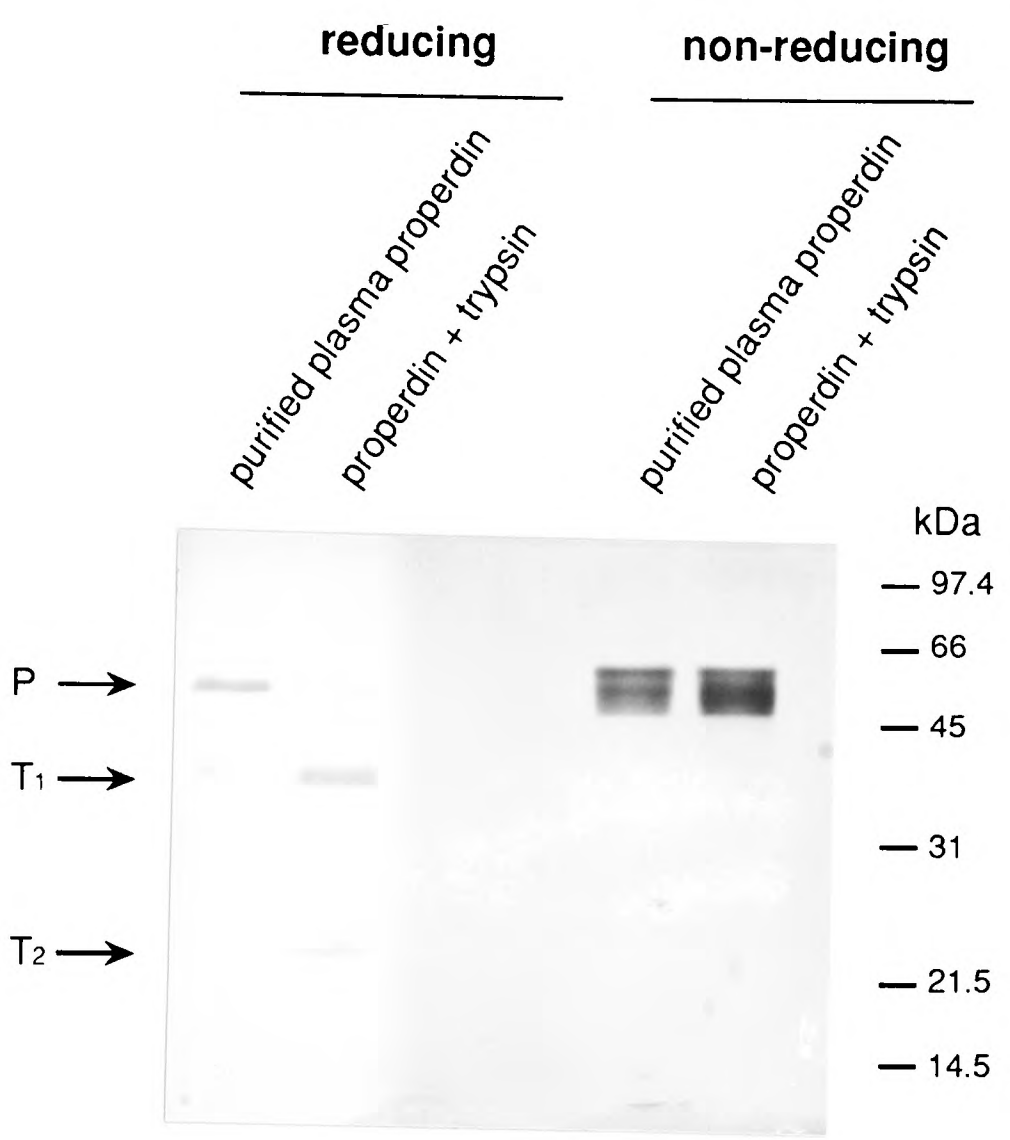


Figure 5.11

Limited tryptic digestion of properdin

Properdin (90 µg/ml in PBS) was digested with trypsin (at a ratio of 40:1) for 17 h at 28°C.

The products were analysed by 12.5% SDS-PAGE in reducing and non-reducing conditions, as indicated. Proteins were visualised by silver staining (see matarials and methods).

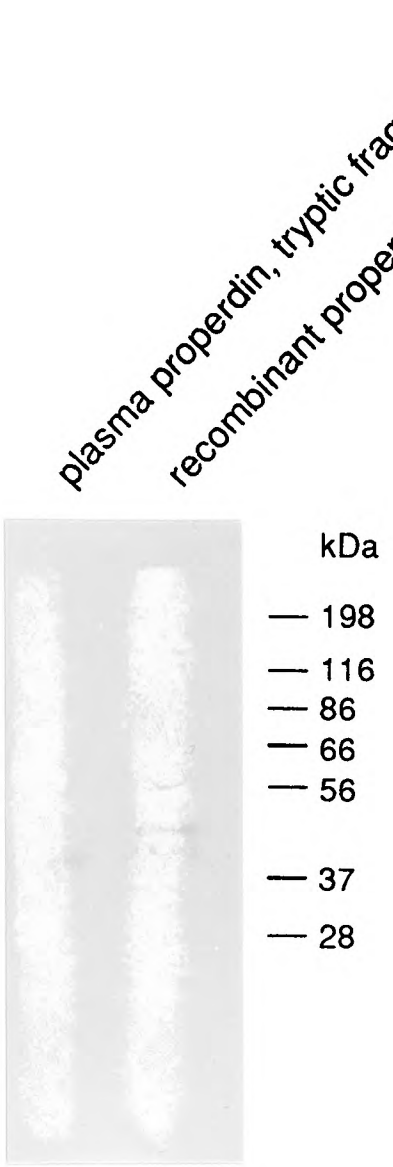


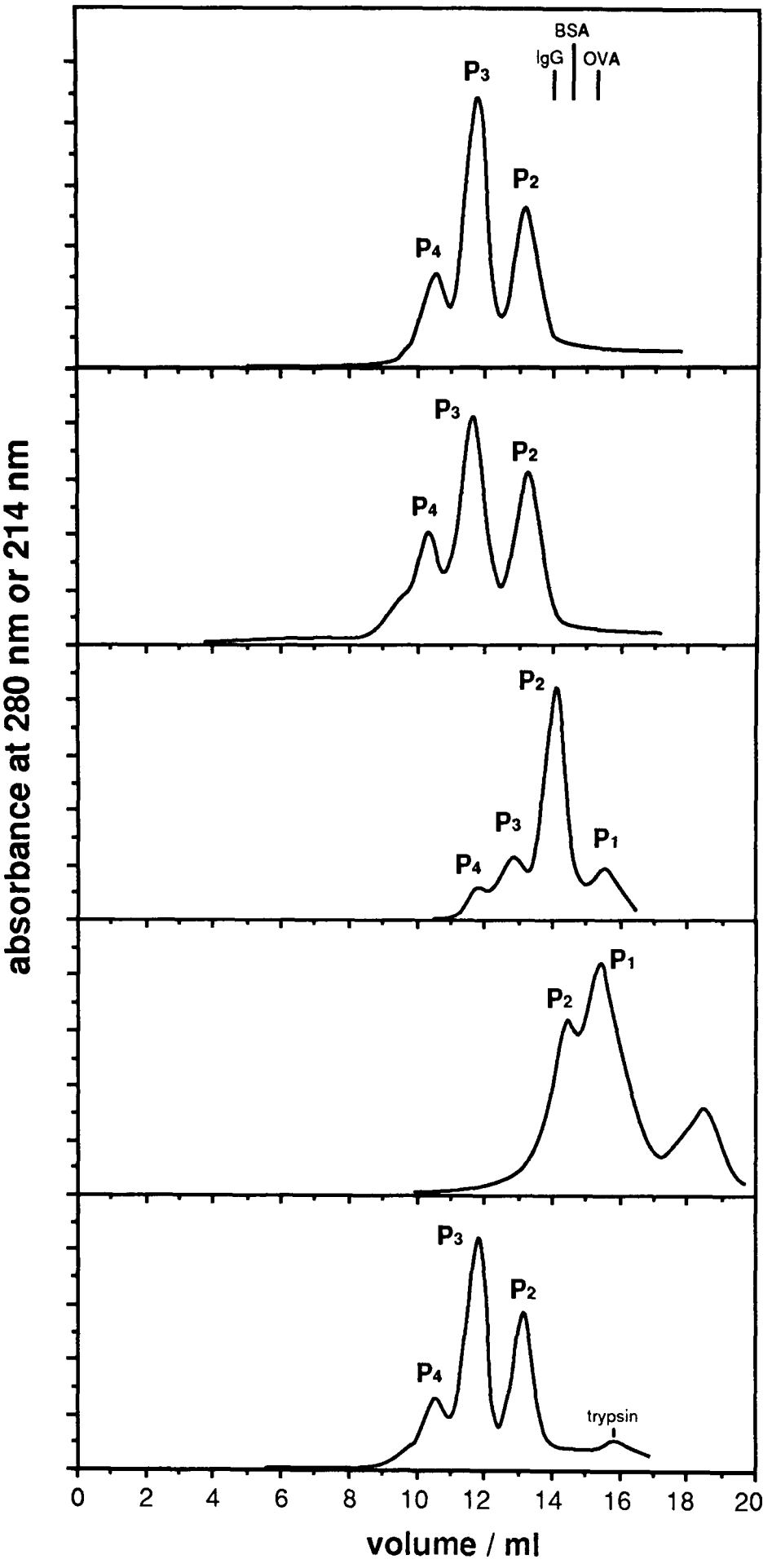
Figure 5.12
Comparison of the size of recombinant properdin lacking TSR6 with the N-terminal T1 trypsin fragment of plasma properdin.
Conditioned serum-free medium (45 µl) from CHO cells producing properdin without TSR6 and a limited tryptic digest of purified properdin were subjected to 10% SDS-PAGE in reducing conditions. After transfer to nitrocellulose, the blot was probed with rabbit anti-properdin IgG and developed using standard procedures (see materials and methods).

5.7 Oligomeric profile of purified altered forms of properdin

Since the purification procedure for the isolation of the recombinant mutant forms of properdin involved an elution step at pH 2.5 which is known to dissociate properdin oligomers (see chapter 4), it was important to confirm the state of oligomerization of the mutant proteins after purification. In addition it was necessary to determine the effect of limited tryptic digestion on the oligomerization of normal plasma properdin. This was done using gel exclusion chromatography as described above. The results are shown in figure 5.13.

The mutant lacking TSR3 clearly forms a distribution of oligomers. The profile indicates the presence of tetramers, trimers, dimers and monomers as found before purification. The distribution of oligomers is shifted towards smaller forms when compared to normal properdin. This marked change in distribution cannot be explained simply in terms of the concentration of monomers present during oligomerization (see chapter 4, section 4.8 and figure 4.9). The difference may be due to the smaller overall length of the monomer lacking TSR3. This reduction in the distance between N and C-terminal regions may favour ring closure during the assembly of the cyclic oligomers, and thus the formation of smaller oligomers. The forms lacking TSR4 and TSR5 behave similarly to each other and formed both monomers and dimers. The ratio of dimers:monomers appeared to be reduced after purification compared to that found in cell culture medium. This could be due to the denaturation or cleavage of the samples during purification, or due to inefficient reassociation of the dimers after the dissociation at pH 2.5. The presence of monomers in these three preparations is in some respects surprising considering the extremely strong association observed between subunits and the lack of monomers in preparations of normal properdin. The reason for the presence of monomers is unclear. It is possible the "monomer" peak represents a proportion of the preparation that is denatured or degraded in some way. Alternatively, the removal of TSRs from properdin may introduce steric constraints on oligomerization or lead to a change in the conformation of the regions of the protein involved in oligomerization so that the formation of monomers is favoured.

After purification of properdin lacking TSR6, the major peak seen on gel exclusion chromatography elutes at the position of IgG. However, anti-properdin ELISA of the eluted fractions indicates that this peak is not the major immunoreactive protein. The main immunoreactive peak eluted between ovalbumin and BSA as before purification, and thus this probably represents monomeric properdin lacking TSR6. The identity of the approximately 150 kDa protein is not known but it is likely to correspond to the material of around 50 kDa found on reducing or non-reducing SDS-PAGE.



Plasma properdin

Wild type recombinant

Recombinant properdin lacking TSR3

Recombinant properdin lacking TSR4

Properdin "nicked" by limited tryptic digestion

Figure 5.13a

The oligomeric profiles of purified altered forms of properdin as determined by gel exclusion chromatography on Superose 6.

Purified samples of properdin in PBS, pH 7.4 were concentrated to approximately 100 µg/ml with Centricon-3 spin concentrators, and 100 µl of each loaded onto a Superose 6 column in PBS, pH 7.4. The eluted protein was detected by absorbance at 280 nm or, for properdin lacking TSR4, at 214 nm.

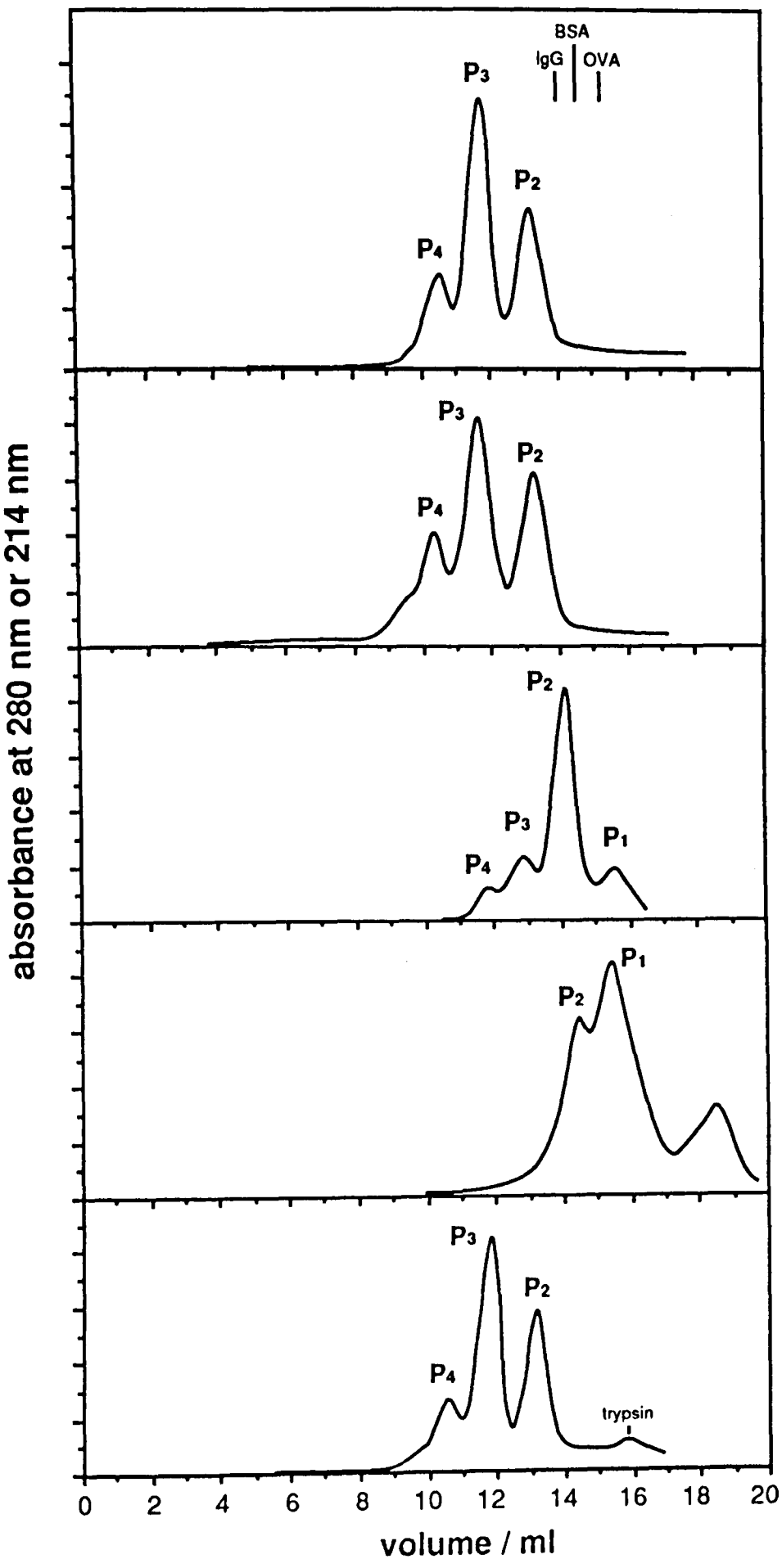


Figure 5.13a
The oligomeric profiles of purified altered forms of properdin as determined by gel exclusion chromatography on Superose 6.
Purified samples of properdin in PBS, pH 7.4 were concentrated to approximately 100 µg/ml with Centricon-3 spin concentrators, and 100 µl of each loaded onto a Superose 6 column in PBS, pH 7.4. The eluted protein was detected by absorbance at 280 nm or, for properdin lacking TSR4, at 214 nm.

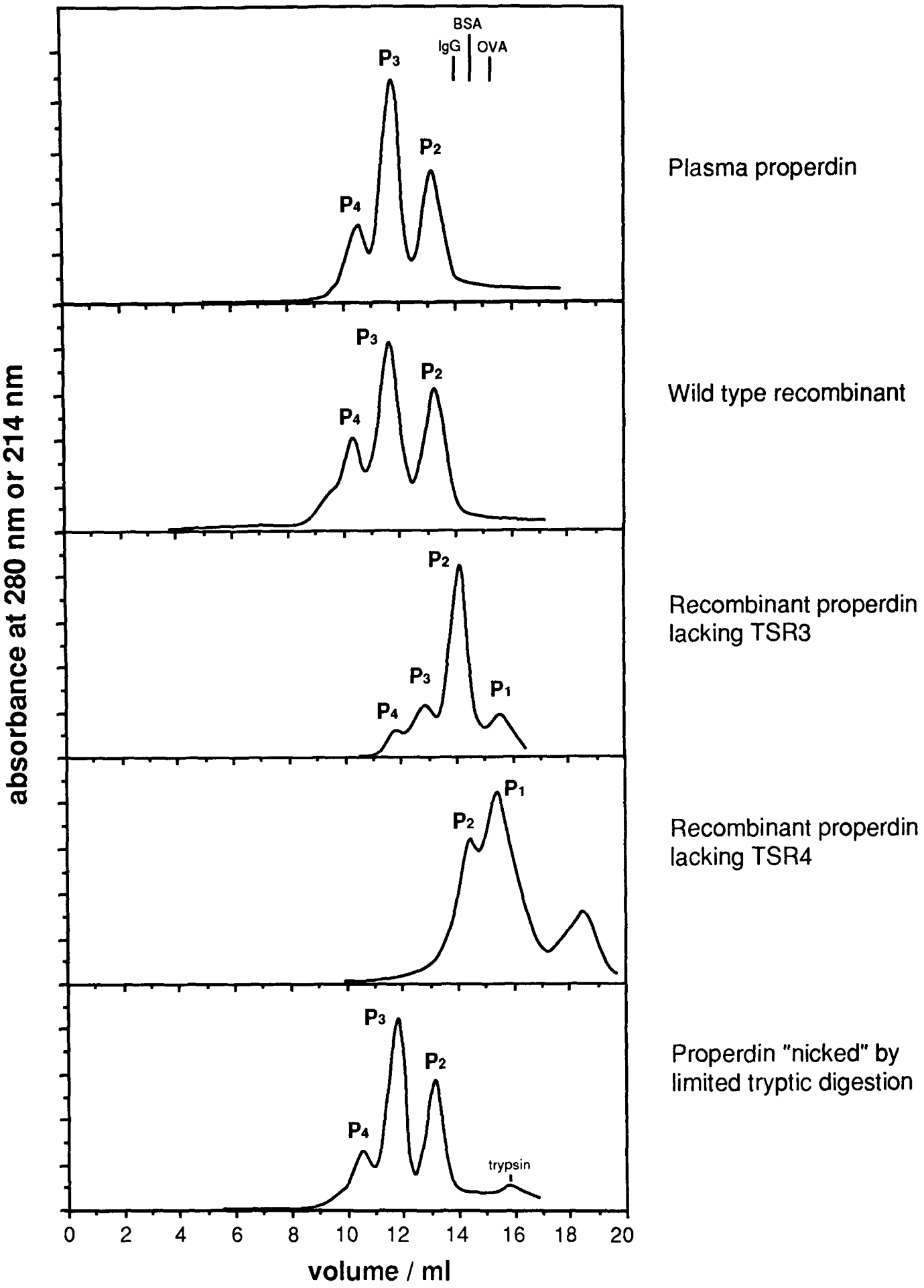


Figure 5.13a
The oligomeric profiles of purified altered forms of properdin as determined by gel exclusion chromatography on Superose 6.
Purified samples of properdin in PBS, pH 7.4 were concentrated to approximately 100 µg/ml with Centricon-3 spin concentrators, and 100 µl of each loaded onto a Superose 6 column in PBS, pH 7.4. The eluted protein was detected by absorbance at 280 nm or, for properdin lacking TSR4, at 214 nm.

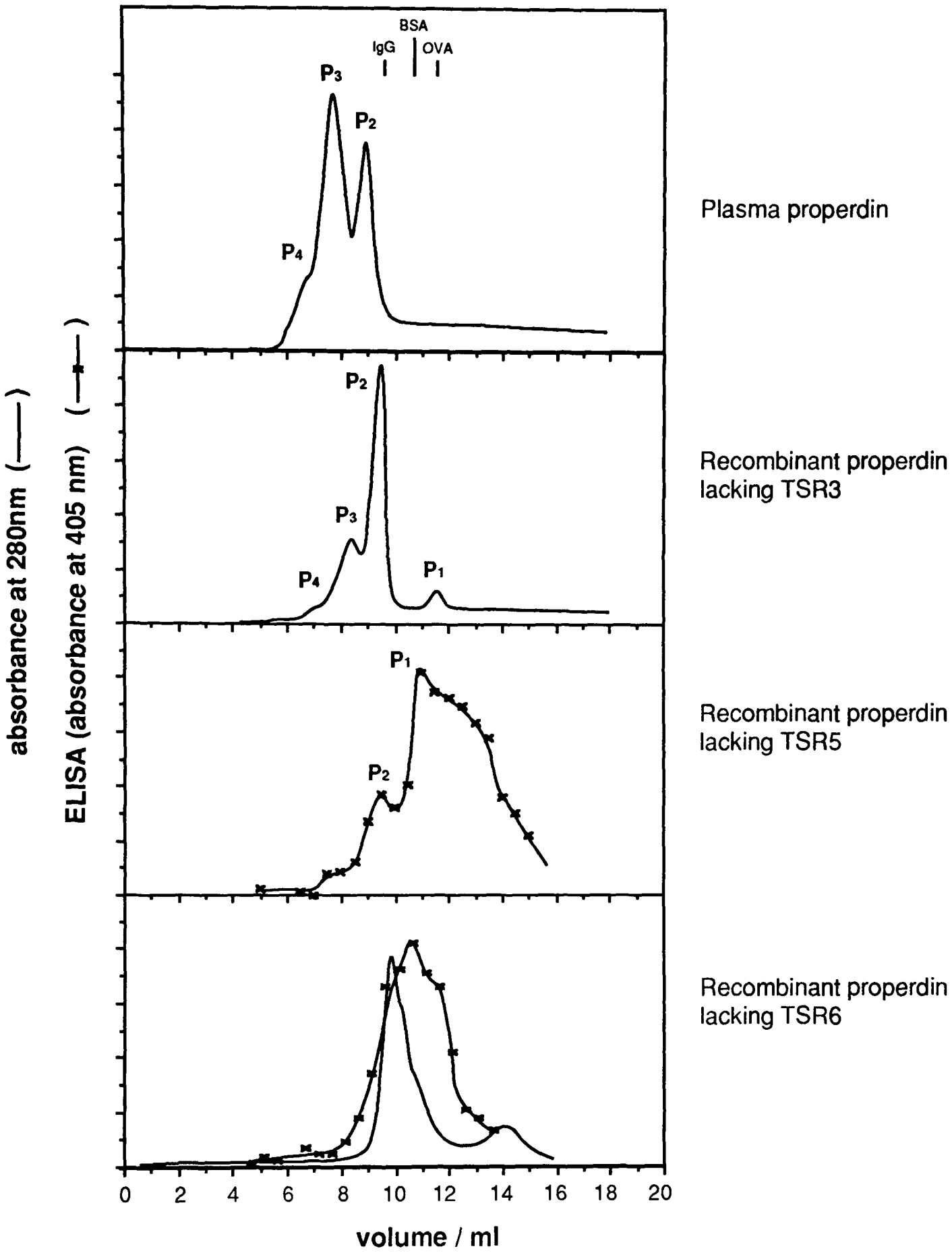


Figure 5.13b
The oligomeric profiles of purified altered forms of properdin as determined by gel exclusion chromatography on Superose 12.
Purified samples of properdin in PBS, pH 7.4 were concentrated to approximately 100 µg/ml (or 20 µg/ml for properdin lacking TSR5) with Centricon-3 spin concentrators, and 100 µl of each loaded onto a Superose 12 column in PBS, pH 7.4. The eluted protein was detected by absorbance at 280 nm or, where the quantity of protein was small, by duplicate ELISA of the collected fractions.

After limited tryptic digestion, plasma properdin gives exactly the same profile as untreated properdin on gel exclusion chromatography. It is striking that nicking of properdin in TSR5 does not affect the oligomerization of the molecule.

5.8 Electron microscopy of altered forms of properdin

Electron microscopy, carried out by Drs Hanna Wiedemann and Rupert Timpl (Max-Planck Institut für Biochemie, Martinsried, Munich, Germany), was used to examine the gross structure of properdin lacking TSR3 or TSR4 and plasma properdin after limited tryptic digestion (figure 5.14). The preparation of properdin lacking TSR3 contains cyclic oligomers similar to those seen for normal properdin, but the length of the subunits is reduced to approximately seventy percent of that found for normal properdin (to around 20 nm). The oligomers show thickening at the vertices typical of normal properdin. Also, the preparation contains predominantly dimers, with fewer trimers and tetramers than observed for normal properdin. These observations confirm the conclusions drawn from the gel exclusion chromatography and cross-linking results. Properdin lacking TSR3 is able to form a distribution of cyclic oligomers which is shifted towards smaller forms when compared to normal properdin.

The preparation of properdin lacking TSR4 contains a large number of monomeric subunits, many showing a thickening at one end. Again, the length of the monomeric subunit is reduced to around seventy percent of that seen for normal human properdin. In addition to the monomeric subunits, dimers are also visible. While some of these dimers are cyclic (and similar in appearance to those found for properdin lacking TSR3), some appear as "open" dimers, with intersubunit contacts at only one end. As suggested above, it may be that removal of TSR4 introduces some strain into the cyclic dimer, or reduces the affinity of subunits for each other so that open dimers and monomers are favoured. It should be noted that "open" oligomers were observed, though rarely, in the normal properdin preparation examined by Smith and co-workers (1984). Alternatively, these structures may be artefacts due to partial proteolysis of the molecule.

The electron micrographs of properdin "nicked" in TSR5 show predominantly cyclic dimers, trimers and tetramers indistinguishable from untreated properdin. Thus, this treatment has no effect on oligomerization of properdin, as predicted from gel exclusion chromatography. In addition, the treatment has no effect on the overall structure of the molecule.

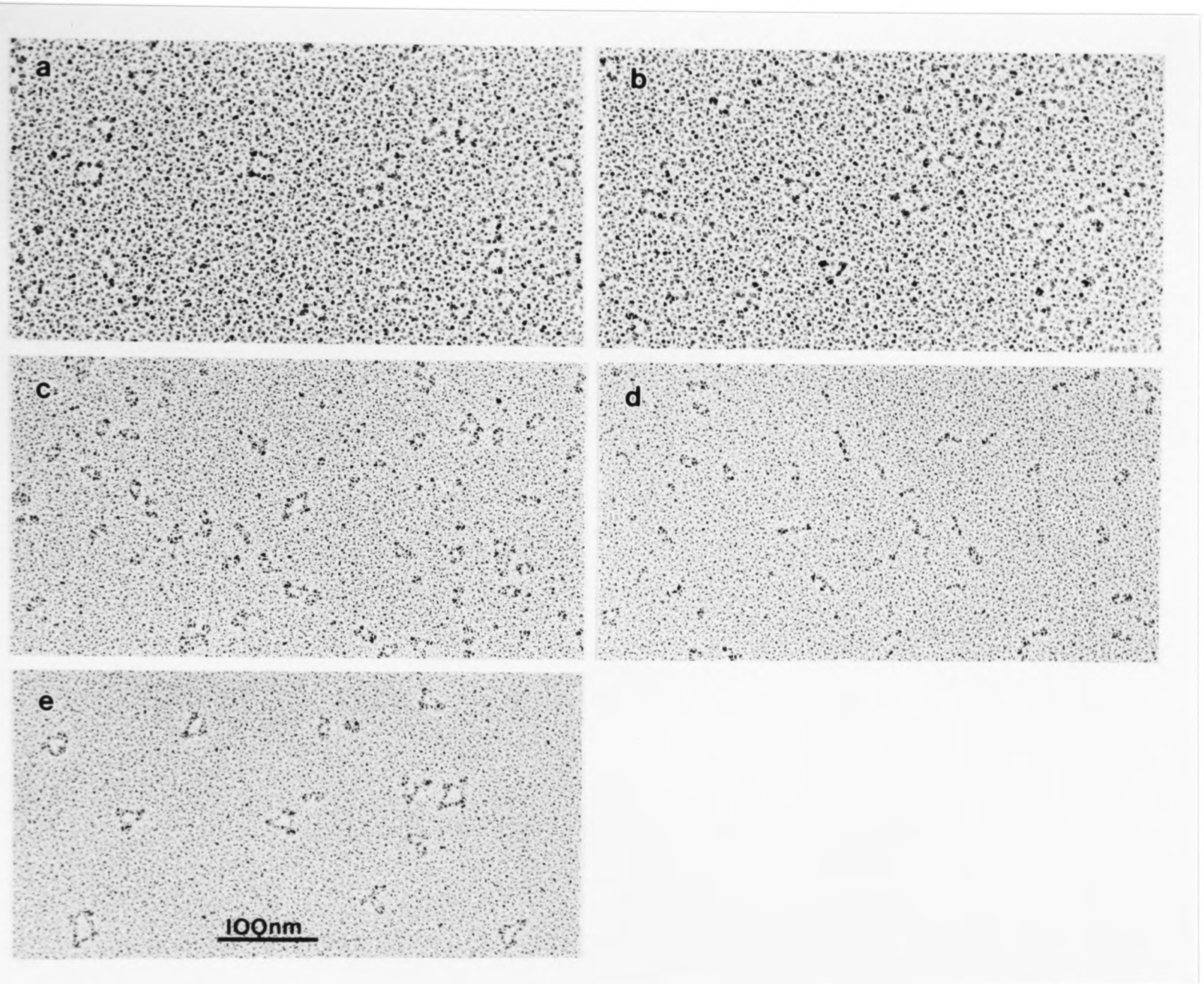


Figure 5.14

Electron microsopy of various altered froms of properdin.

Rotary shadowing with carbon/platinum and electron microscopy were carried out by Drs Hanna Wiedemann and Rupert Timpl, Max-Planck Institut für Biochemie, Martinsried, Munich, Germany.

The samples shown (approximately 50 μg ml^{-1} in 0.2 M ammonium bicarbonate) are as follows:

a) Purified properdin from normal human plasma

b) Purified recombinant human properdin

c) Purified recombinant properdin lacking TSR3

d) Purified recombinant properdin lacking TSR4

e) Limited tryptic digest of purified plasma properdin

5.9 Binding of the altered forms of properdin to anti-properdin monoclonal antibodies

The binding of the various altered forms properdin to the HYB3-3 anti-human properdin monoclonal antibody (kindly provided by Dr C. Koch, Statens Seruminstitut, Copenhagen, Denmark) was analysed. The binding assay used was a sandwich ELISA in which the monoclonal antibody was coated onto the plate. Since it had previously been confirmed that all the mutant forms reacted with the polyclonal anti-properdin antibody, biotinylated polyclonal anti-properdin IgG was used for detection of properdin which bound to the monoclonal antibody. The results are shown in figure 5.15. Recombinant wild type properdin is indistinguishable from plasma properdin in its affinity for the monoclonal antibody. The mutant recombinant molecule lacking TSR3 and purified plasma properdin "nicked" in TSR5 are also similar in their affinities for the mAb. However, mutants lacking TSR5 or TSR6 bind to HYB3-3 with at least ten-fold lower affinity than normal properdin. Furthermore, no binding of the mutant form of properdin lacking TSR4 can be detected over the range of concentration used (figure 5.15). Similar results were obtained using samples of the recombinant proteins in serum-free medium prior to purification (not shown). These results suggest that the epitope for HYB3-3 lies in TSR4 of properdin. However, the possibility that removal of TSR4 causes a conformational change that prevents the antibody binding elsewhere in the molecule cannot be excluded formally.

The binding of a panel of other monoclonal antibodies against human properdin to the various mutant forms of properdin was tested by Ms Fabianna Filippoussis and Dr J. D. Lambris of the University of Pennsylvania. The samples provided were serum-free culture media dialysed into PBS/0.04% (w/v) sodium azide, pH 7.4. The concentration of recombinant protein in each sample was estimated by polyclonal ELISA and was as given in section 5.2 (after amplification). The assay used was similar to the monoclonal ELISA described above and in Materials and Methods, except that the monoclonal antibodies were coated at 0.15 $\mu g\ well^{-1}$ on microtitre plates, blocking was carried out with 2% milk powder, and goat polyclonal anti-properdin and peroxidase conjugated rabbit anti-goat antibodies were used for detection. Examples of the results obtained are shown in figure 5.16 and a summary is shown in table 5.1.

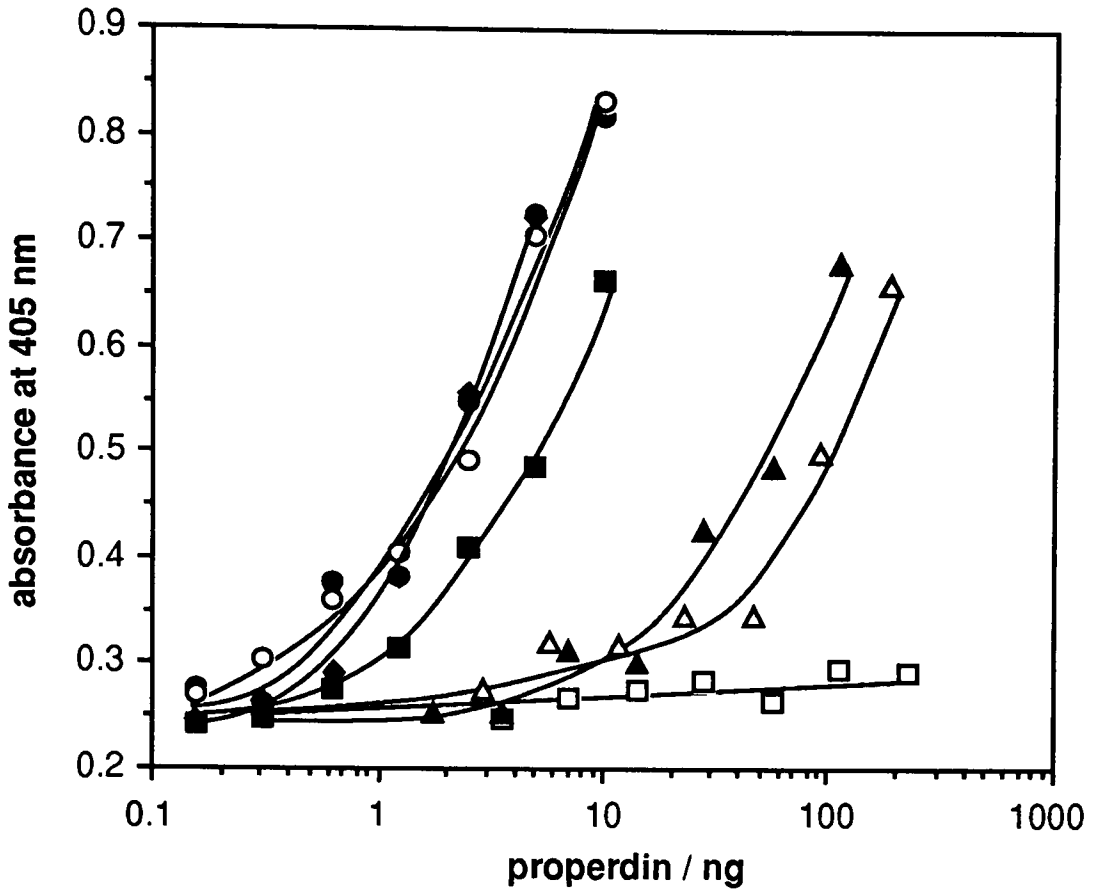
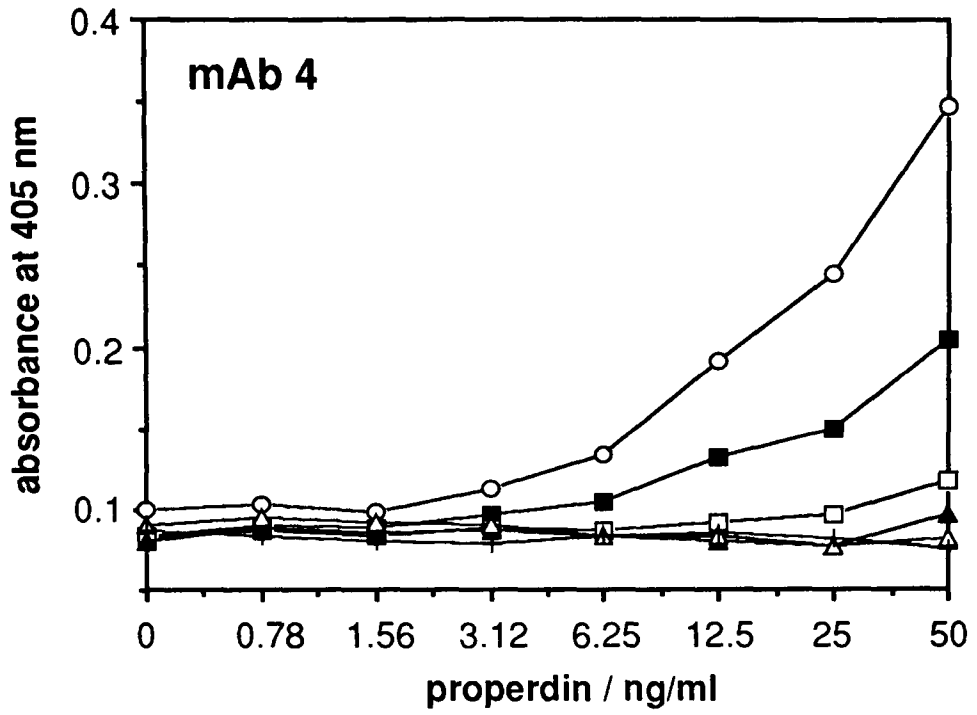
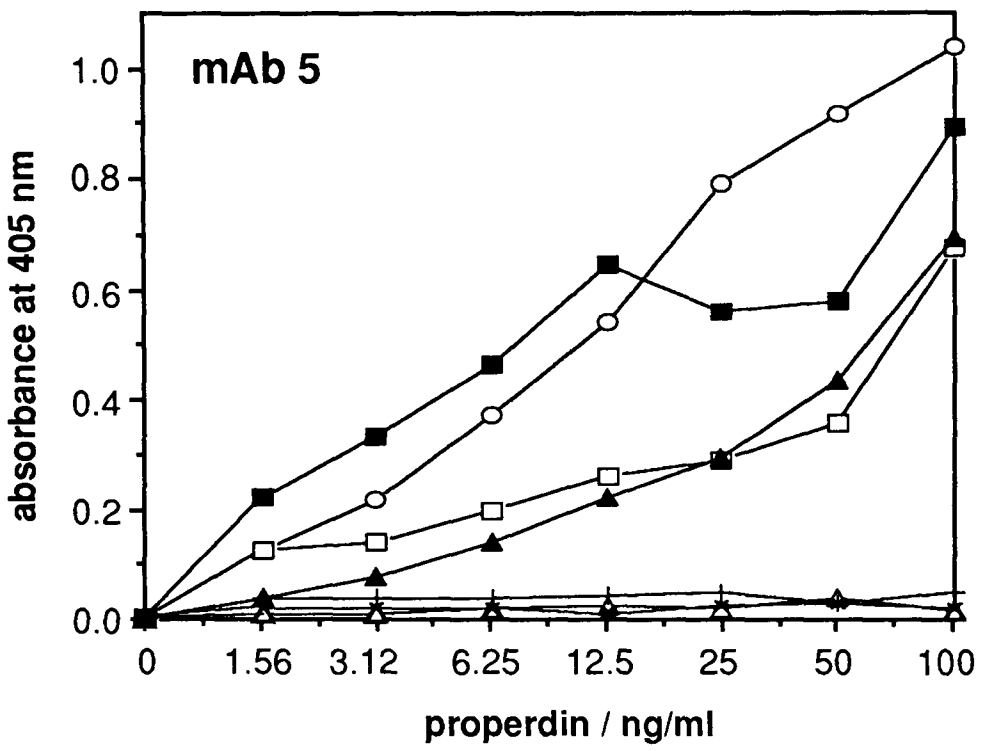
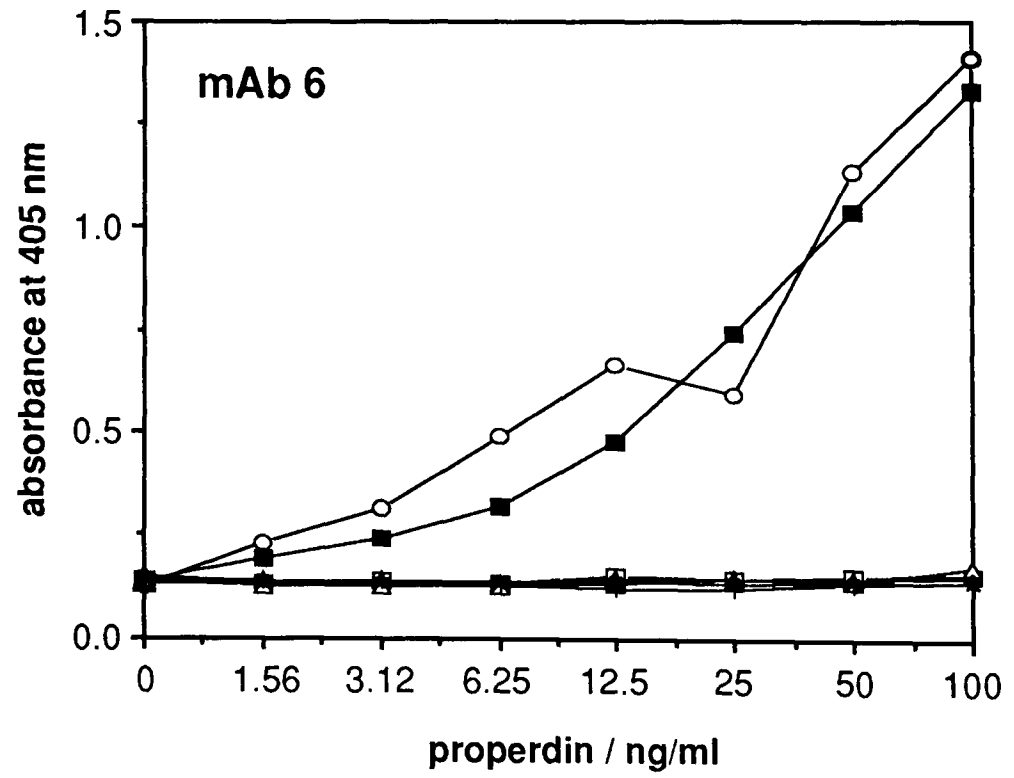
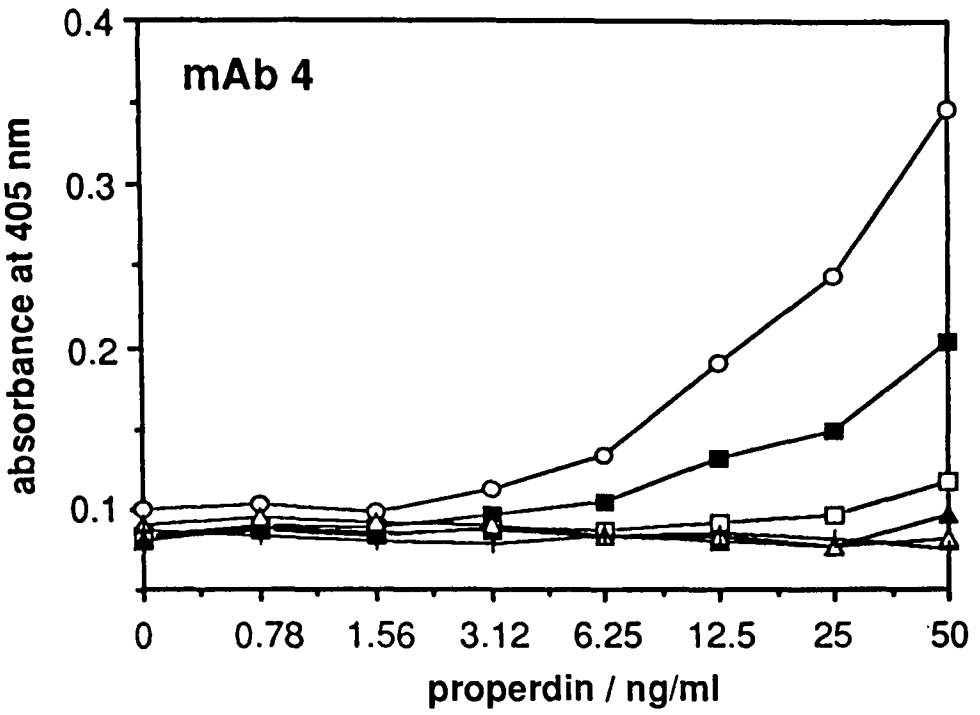


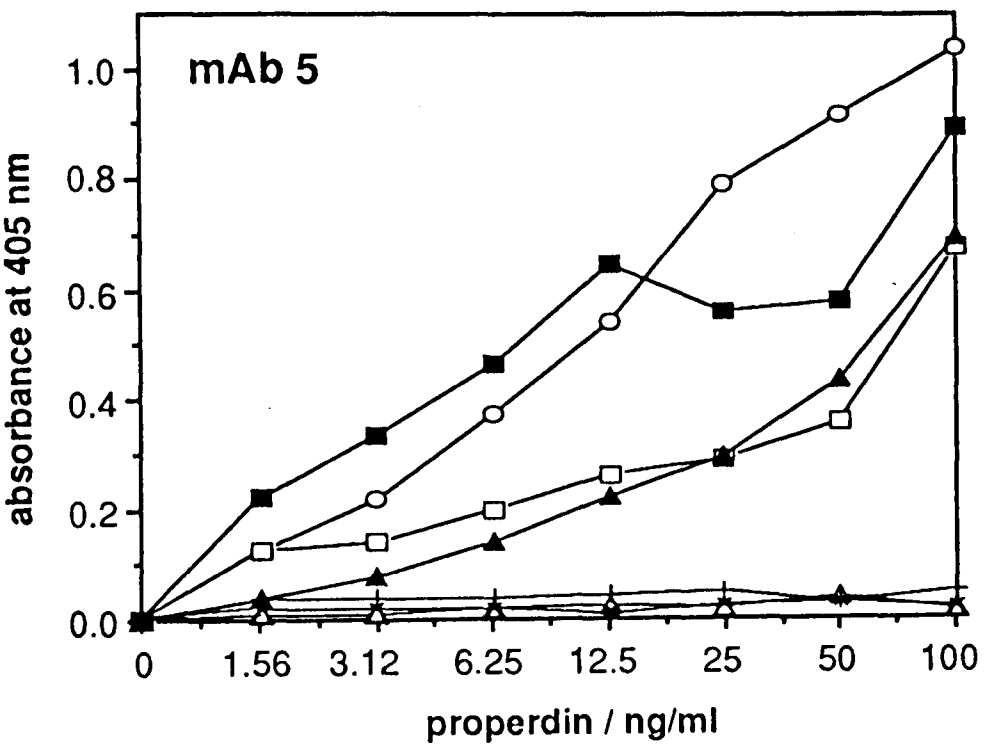
Figure 5.15
Binding of the various altered forms of properdin to the HYB3-3 anti-human properdin monoclonal antibody (kindly provided by Dr C. Koch, Statens Seruminstitut, Copenhagen, DK). Briefly, the mAb was coated onto microtitre plate wells. After blocking, serial dilutions of purified preparations of plasma properdin, a limited tryptic digest of properdin, and the various forms of recombinant properdin, were applied to the plate. After washing, bound properdin was detected with biotinylated polyclonal anti-properdin IgG using standard procedures (see text). The averages of duplicate experiments are shown.

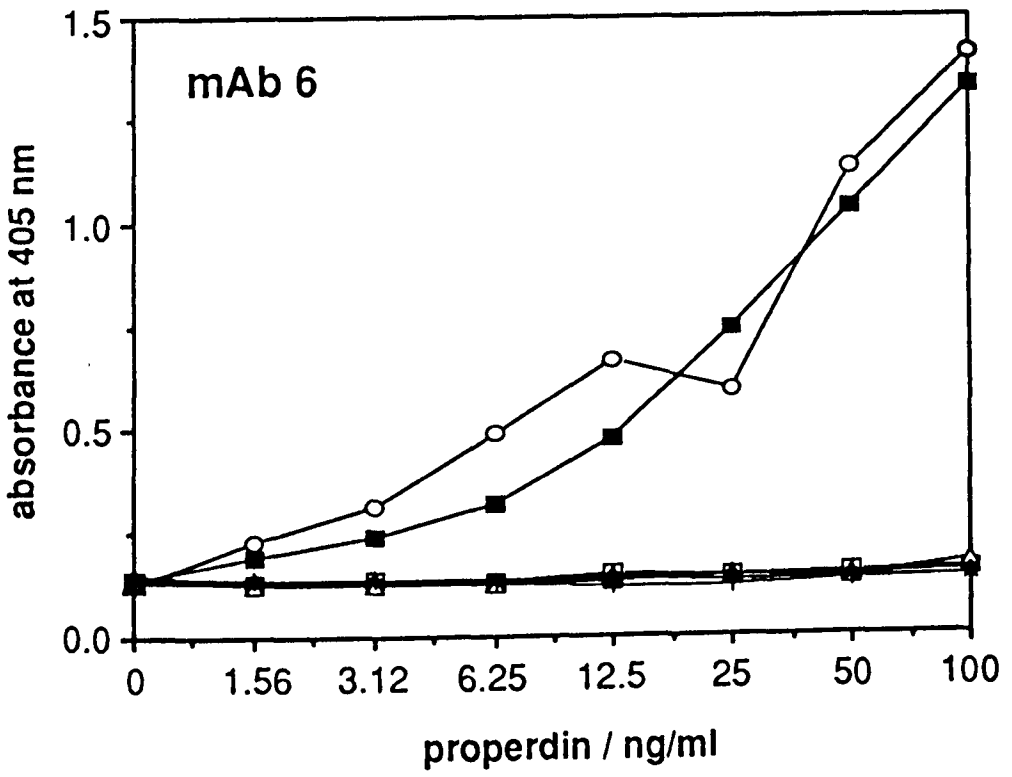

mAb 4
absorbance at 405 nm
0.4
0.3
0.2
0.1
0
0.78
1.56
3.12
6.25
12.5
25
50
properdin / ng/ml

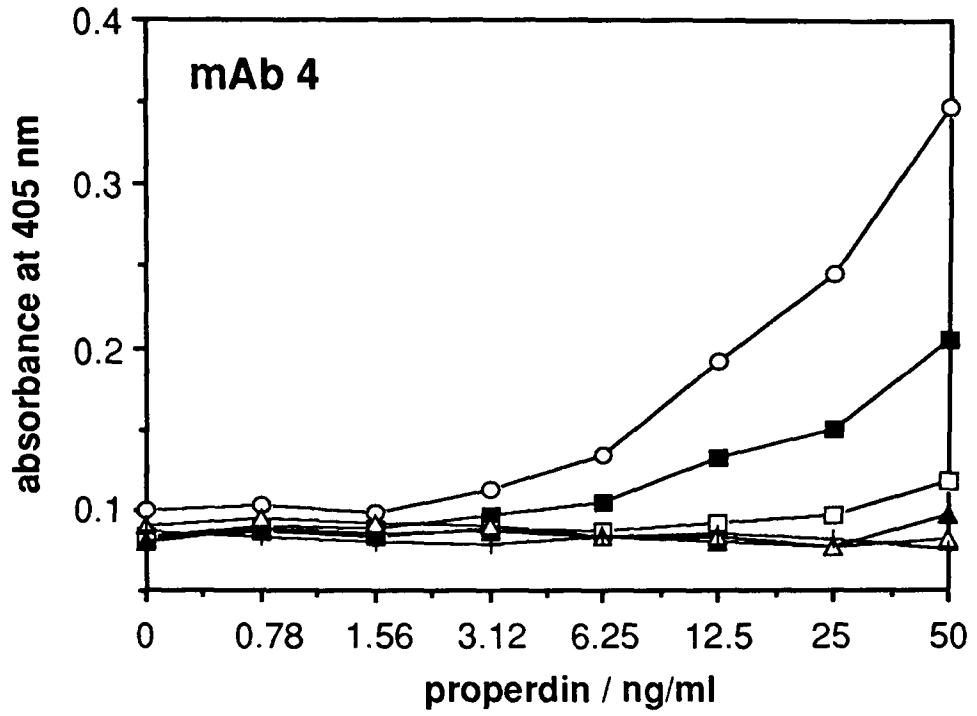

mAb 5
absorbance at 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml

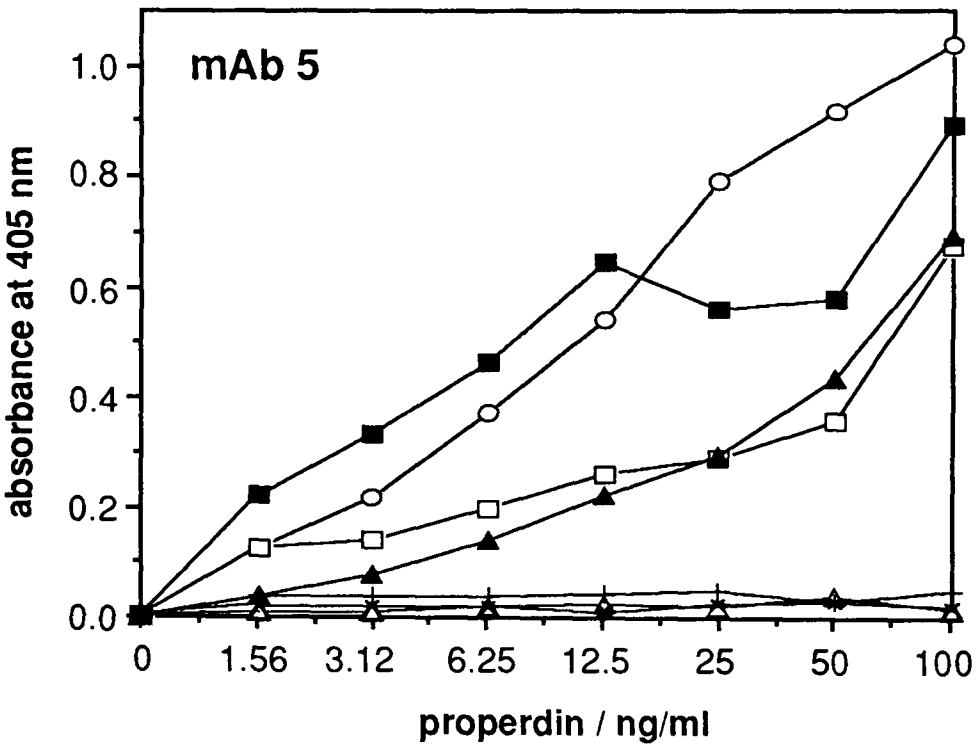

mAb 6
absorbance at 405 nm
1.5
1.0
0.5
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml

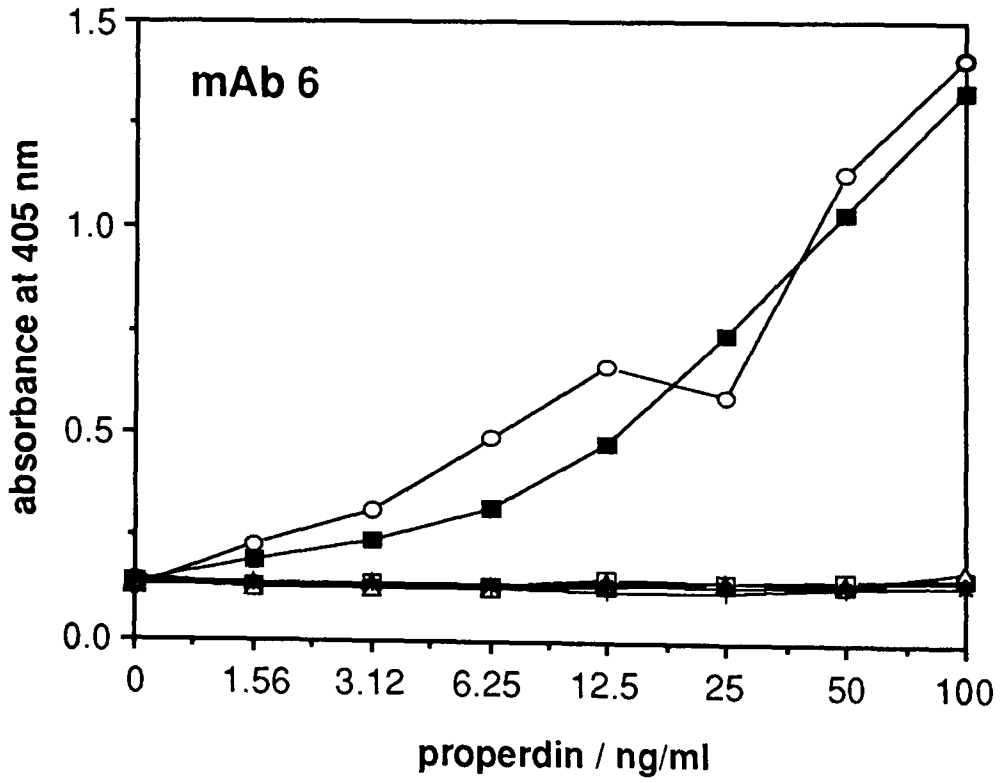

mAb 4
absorbance at 405 nm
0.4
0.3
0.2
0.1
0
0.78
1.56
3.12
6.25
12.5
25
50
properdin / ng/ml

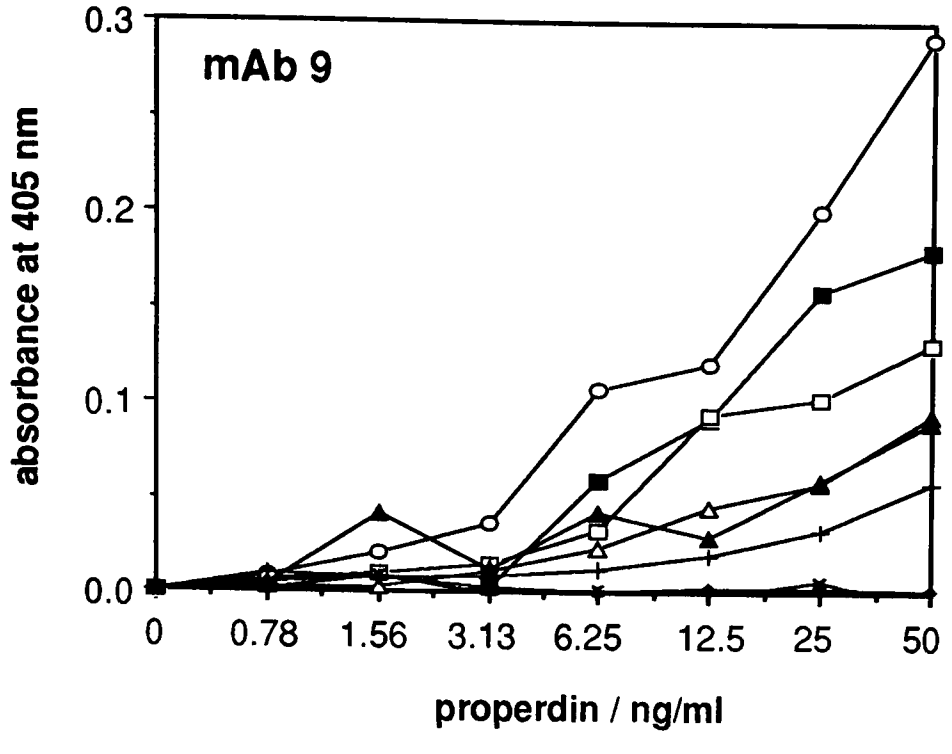

mAb 5
absorbance at 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml

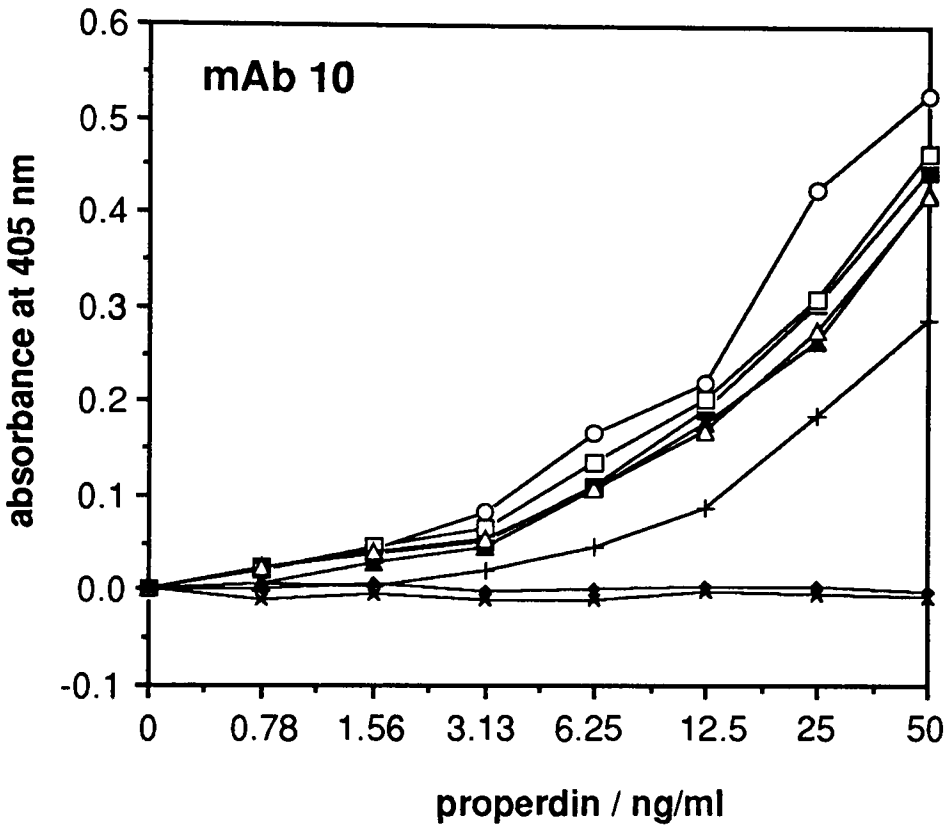

mAb 6
absorbance at 405 nm
1.5
1.0
0.5
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml


mAb 4
absorbance at 405 nm
0.4
0.3
0.2
0.1
0
0.78
1.56
3.12
6.25
12.5
25
50
properdin / ng/ml


mAb 5
absorbance at 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml


mAb 6
absorbance at 405 nm
1.5
1.0
0.5
0.0
0
1.56
3.12
6.25
12.5
25
50
100
properdin / ng/ml





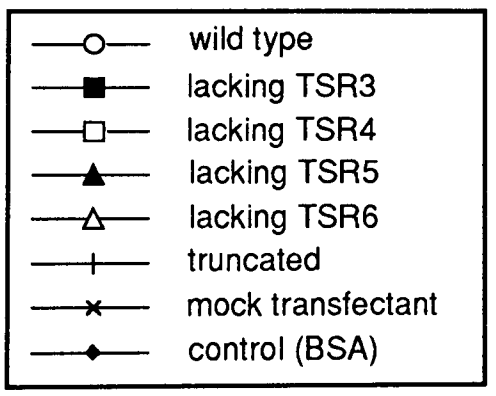


Figure 5.16
Binding of the various recombinant mutant forms of properdin to anti-properdin mAbs. Briefly, mAbs were coated onto microtitre plate weels. After blocking, serial dilutions of serum-free medium samples containing the recombinant proteins were applied to the plate. After washing, bound properdin was detected with goat polyclonal anti-properdin and peroxidase-conjugated anti-goat IgG. Assay carried out by Ms Fabianna Filippoussis and Dr John D. Lambris, University of Pennsylvania.

Table 5.1

The binding of mutant forms of recombinant properdin to a panel of monoclonal antibodies

form of properdin	monoclonal antibody number									
	1*	4	5	6	7	9	10	11	12	13
plasma properdin	++	++	++	++	++	++	++	++	++	++
recombinant properdin	++	++	++	++	++	++	++	++	++	++
properdin lacking TSR3	++	++	++	++	++	++	++	++	+	+
properdin lacking TSR4	-	+	++	-	++	++	++	++	-	-
properdin lacking TSR5	+	-	++	-	++	++	++	++	-	-
properdin lacking TSR6	+	-	-	-	-	++	++	++	-	-
truncated properdin	nd	-	-	-	-	+	++	+	-	-
control medium	-	-	-	-	-	-	-	-	-	-

* mAb 1 is HYB3-3

++ indicates clear immunoreactivity

\+ indicates low immunoreactivity

\- indicates no detectable immunoreactivity

nd indicates reactivity not determined

The results allow the epitopes of the monoclonal antibodies to be localised to some extent. The binding sites for monoclonals 9, 10, and 11 cannot reside in TSRs 3, 4, 5 or 6, and so must be within the N-terminus, TSR1 or TSR2. Monoclonals 5 and 7 do not bind to TSR3, 4 or 5, and thus bind to the N-terminus, TSR1, 2 or 6. The finding that these antibodies do not bind to properdin lacking TSR6 suggests that they may bind to this repeat. However, it remains possible that removal of TSR6 leads to the loss of an epitope elsewhere in the molecule, particularly since TSR6 is likely to be involved in oligomerization. Monoclonals 6, 12 and 13 may bind to the N-terminus, TSRs1, 2, 4, 5 or 6 since they bind to properdin lacking TSR3. Monoclonal 4 binds to the N-terminus, TSRs1, 2, 5 or 6.

Further mutants lacking the N-terminus or TSRs 1 plus 2 will allow us to further localize the binding sites of these antibodies (these constructs are in preparation). The inhibitory activities of these antibodies are compared with their likely epitopes in subsequent chapters (chapters 6, 7 and 8).

5.10 Summary

A number of mutant forms of recombinant human properdin were produced in CHO cells. After selection for clones that had undergone gene amplification at elevated concentrations of MSX, yields of 0.5 - 2 $\mu g\ ml^{-1}$ were obtained in serum-free medium for several mutant forms of properdin lacking single TSRs (TSR3, TSR4, TSR5 or TSR6). Another mutant form of properdin (trunc), lacking the C-terminal twenty-nine amino acids, was also produced in lower quantities. The inclusion of the protease inhibitor, leupeptin, and restricted periods of cell culture were required to obtain good yields of non-degraded properdin without TSR4, TSR5, or TSR6, or the truncated form of properdin. The mutant lacking TSR3 was stable for extended periods of culture. Prior to purification, properdin lacking TSR3, TSR4, or TSR5 formed oligomers as determined by ELISA of fractions from gel exclusion chromatography and covalent cross-linking with homo-bifunctional reagents. The forms lacking TSR6 or the C-terminal twenty-nine amino acids (trunc) showed no evidence of oligomerization. The mutant proteins were purified on a monoclonal antibody affinity column (properdin lacking TSR3) or an antigen-selected polyclonal antibody column (properdin lacking TSR4, TSR5, or TSR6). In each case, proteins of the expected size were obtained. The preparation of properdin lacking TSR6 also contained immunoreactive material that was larger than expected (on SDS-PAGE and gel exclusion chromatography). The identity of this material remains unclear. The purified mutant lacking TSR3 formed a range of oligomers as judged by gel exclusion chromatography. The mutants lacking TSR4 or TSR5 formed monomers and dimers. Electron microscopy confirmed the presence of oligomers of the mutants lacking either TSR3 or TSR4. Tryptic cleavage of a bond in an exposed loop within TSR5 of purified plasma properdin did not alter the oligomerization or overall structure of the protein as determined by gel exclusion chromatography and electron microscopy. The availability of mutant forms of properdin carrying known deletions allowed the epitopes of a number of monoclonal antibodies to be mapped.

Chapter 6

Chapter 6

Stabilization of the C3bBb complex: the alternative pathway haemolytic assay

A haemolytic assay was used to investigate the ability of the various altered forms of properdin to stabilize the alternative pathway C3 convertase, the C3bBb complex. Initially, the properties of normal plasma properdin in the assay were assessed.

6.1 The alternative pathway haemolytic assay

Activation of the complement system leads to the formation of membrane attack complexes on permissive cells. These structures disrupt the plasma membrane and can cause cell lysis. If erythrocytes are used as the target cell *in vitro*, then the release of haemoglobin can be used to quantitate the extent of lysis, and thus of complement activation. The classical complement pathway requires calcium ions for activity (the association of the $C1r_2C1s_2$ complex is Ca^{2+}-dependent), while the association of C3b and Bb in the alternative pathway requires magnesium ions. So, complement activation can be limited to the alternative pathway *in vitro* in the presence of EGTA and $MgCl_2$ (EGTA chelates calcium much more efficiently than magnesium ions). The release of haemoglobin from erythrocytes lysed by complement in the presence of EGTA and magnesium ions can thus be used as an assay of alternative pathway activity.

The sensitivity of erythrocytes to complement lysis depends upon the species from which the cells are obtained and on the species from which the serum is derived. This may be due to some extent to the presence of downregulatory proteins on the erythrocyte surface, and the compatibility of these proteins with the serum complement proteins. However, in a large survey of the lysis of many different types of red blood cell by complement from many sources, the extent of lysis observed correlated closely with the quantity of sialic acid on the surface of the erythrocyte (Ish *et al.*, 1993). Desialylation of sheep red blood cells (E_S) renders them susceptible to attack by human complement (Fearon, 1978; Pangburn and Müller-Eberhard, 1978), while resialylation again confers protection (Tomlinson *et al.*, 1992). These findings are thought to be due to the binding of the down-regulatory complement molecule factor H to sialic acid on the erythrocyte surface. The loss of sialic acid from the cell surface correlates closely with the binding of factor H to the cells (Fearon, 1978). This phenomenon is discussed in more detail in the introduction. On the other hand, rabbit red blood cells (E_R) have very low

levels of surface sialic acid and are susceptible to human complement without prior treatment.

The ability of properdin to stabilize the alternative pathway C3 convertase, C3bBb, can be judged using a modification of the haemolytic assay described above. The assay measures the ability of a sample to reconstitute the haemolytic activity of serum depleted of properdin. Originally, properdin was removed from serum by incubation with zymosan at 17°C. The same can now be accomplished more specifically using an anti-properdin monoclonal antibody column. The depleted serum (RP) is dialysed into buffer containing magnesium and EGTA before use. Either rabbit red blood cells or neuraminidase treated sheep red blood cells can be used as targets for lysis.

6.2 Comparison of the specific activities of the oligomeric forms of properdin in haemolysis of rabbit and sheep erythrocytes

Initially the properties of normal human properdin in the rabbit erythrocyte alternative pathway assay were investigated. Properdin-depleted human serum reconstituted with purified human properdin shows haemolytic activity in a dose and time dependent manner (see chapter 4, figure 4.12). The various oligomeric forms of properdin were separated by gel exclusion chromatography and assayed for haemolytic activity (see figure 6.1). Dimers, trimers and tetramers of properdin had the same specific activity. The results confirm those of Reid (1981) using the same assay procedure. However, these results are in contrast to those of Pangburn (1989). Using neuraminidase treated sheep erythrocytes as the target cell, Pangburn found that, on a per monomer basis, properdin tetramers were five fold more active than dimers, and trimers were three fold more active than dimers. Pangburn also investigated the rate at which the various species of properdin were utilised during activation of the alternative pathway. In this experiment, radiolabelled properdin (a mixture of dimers, trimers, and tetramers) was added to normal human serum containing magnesium and EGTA, and *rabbit* erythrocytes were used as the target. The disappearance of properdin from the supernatant (due to attachment to the cells) was determined. At each time point, dimers, trimers, and tetramers were separated by gel exclusion chromatography, and the percentage of each oligomer remaining was measured. Tetramers bound to the cells first, with trimers lagging only slightly. Dimers were utilised more slowly, and less than 20% of this species ever bound, compared to around 70% of tetramers and trimers. These results are consistent with the increased avidity of higher properdin oligomers for C3b (see chapter 1, section 1.7.5).

In order to examine these discrepancies, the haemolytic assay was repeated with E_R or neuraminidase treated E_S using the same buffers, the same RP reagent, and the

same preparations of purified properdin dimers, trimers and tetramers. Other than the different cell type used, the only difference between the assays was that it was found to be necessary to extend the incubation time with the treated sheep erythrocytes from 15 min to 35 min in order to obtain the same degree of haemolysis. That is, even after neuraminidase treatment under the conditions used, the sheep erythrocytes were more resistant to complement than the rabbit cells. Treatment of sheep cells in the same conditions but without neuraminidase did not cause sensitization (figure 6.2).

The results of the comparison of the effect of properdin oligomers on lysis of the two cell types are shown in figure 6.3. In agreement with the work of Pangburn, differences in the specific activities of the various oligomeric forms of properdin were found in the treated sheep erythrocyte system. By comparing the areas of the dimer, trimer and tetramer peaks found by absorbance at 280 nm or ELISA and by sheep erythrocyte haemolytic assay, tetramers were found to be three fold more active than dimers, and trimers 2.5-fold more active than dimers on a per monomer basis. These results are comparable with those of Pangburn, and clearly different from those obtained using rabbit cells as the target (figures 6.1 and 6.3).

Sheep erythrocytes can also be made sensitive to lysis by the alternative pathway of human complement by periodate treatment. When the reaction is performed under mild conditions (low temperature, short period), periodate is not transported into the cells, and only sialic acid on the erythrocyte surface is oxidized (Liao *et al.*, 1973). The treatment specifically oxidizes adjacent hydroxyl groups on the C7, C8 and C9 carbons of sialic acid, forming C7 and C8 aldehydes. The sensitization of sheep erythrocytes by periodate treatment was dose-dependent (figure 6.4). Treatment with 1.2 mM sodium periodate for 10 min at 4°C (as described in chapter 2) sensitized the cells to a similar extent to the neuraminidase treatment (35 min was required for the same extent of lysis as rabbit cells after 15 min). Treatment with 3 mM periodate made the cells almost as sensitive to complement attack as rabbit cells (20 min required for the same degree of lysis). Similar treatment of E_S cells in the absence of periodate caused little increase in sensitivity (not shown).

The effectiveness of different properdin oligomers in enhancing the lysis of periodate treated cells was also investigated. The results are shown in figure 6.3. Sheep cells treated with 3 mM periodate showed a pattern similar to that with rabbit erythrocytes, where dimers, trimers and tetramers were equally active on a per monomer basis. Treatment of E_S with a lower concentration of periodate (1.2 mM) produced cells which behaved more like the neuraminidase treated cells (*i.e.* properdin tetramers were more effective than dimers in enhancing alternative pathway haemolysis), although the differences seen were not statistically significant, and the assays were not repeated (not shown).

The reason for the difference observed between the erythrocyte cell types is not known. Although the quantity of sialic acid removed or modified by these procedures was not measured directly, the results indicate that the difference seen in the activity of the properdin oligomers becomes less pronounced as more sialic acid is removed. It is clear that E_S remain relatively poor activators of the alternative pathway after neuraminidase treatment (these results, and Pangburn *et al.*, 1983; Fearon, 1978; Pangburn and Müller-Eberhard, 1978)). Neuramindase treated E_S cells bind less C3b at saturation than E_R cells (Pangburn *et al.*, 1983), although E_S cells are actually more sensitive to the terminal lytic pathway than E_R at similar multiplicities of C3b binding (Fearon and Austen, 1977b). It may be that in conditions in which activation of the alternative pathway is restricted to some extent (*i.e.* on poor activators), any difference in the activities of the properdin oligomers is more pronounced. The reason why neuraminidase treated E_S cells are less efficient activators of the alternative pathway than E_R is not clear. It may be that neuraminidase treatment does not remove all of the sialic acid from the cell surface. The greater sensitivity of periodate treated cells to haemolysis may be due to more efficient modification of sialic acid residues. However, it cannot be discounted that the periodate treatment also modifies other molecules on the erythrocyte surface, or that the potentially reactive aldehyde groups formed by periodate treatment influence the results obtained (in these experiments, the periodate treated cells were not subsequently treated with a reducing reagent such as sodium borohydride).

It is also possible that once sialic acid is removed from the erythrocyte surface, then other complement regulating molecules may influence alternative pathway activation. Interestingly, although both E_R and E_S have been shown to possess decay accelerating and/or Factor I co-factor activity (Horstmann and Müller-Eberhard, 1986; Ezzell and Parker, 1992), protein(s) on the rabbit cells are unable to accelerate decay of the human C3bBb complex, nor to act as a co-factor for human Factor I (while both activities are observed when rabbit C3b and Bb are used). A molecule on the surface of E_S cells does act as a co-factor for human Factor I. If the different oligomeric forms of properdin have varying abilities to inhibit Factor I cleavage of C3b (or the binding of a membrane bound RCA protein to C3b), then a difference will be seen in the ability of properdin oligomers to stabilize the C3bBb complex on neuraminidase-treated E_S cells, but not on E_R cells (assuming that Factor H action is inefficient in these cases).

Another possibility is that, if properdin is multivalent for C3b binding (see chapter 1, section 1.7.5), then the density of clustered C3b on a surface may influence the relative binding avidities of the different oligomers of properdin. For example, it is possible that a high density of C3b might be required in order to observe high avidity binding of tetrameric properdin. This possibility is assessed in chapter 7. It is conceivable that the pattern and density of C3b deposition may vary on different erythrocyte surfaces.

An outstanding discrepancy that remains is that while Pangburn demonstrated that the different species of properdin bind to E_R to different extents and with different kinetics (see above), the various oligomers of properdin were equally active when haemolysis of E_R was measured (Reid, 1981; and this thesis). At least two factors may contribute to the difference observed. Firstly, Pangburn measured the binding of properdin oligomers to E_R, and not the ability of bound properdin to enhance haemolysis. Secondly, in Pangburn's experiment, the various oligomeric forms of properdin were present at the same time, and thus were competing with each other for C3bBb binding. In the assays of Reid, and those reported here, the oligomers were assayed separately. It is possible that the observed differences in the activities of the various species would be more pronounced in a mixture of oligomers. It may be interesting to investigate further the ability of the different oligomers of properdin to bind C3b, to stabilize the C3bBb complex, and to inhibit the action of complement regulating molecules.

6.3 Binding of properdin to untreated erythrocytes

Konno *et al.* (1978) reported that "activated" (*i.e.* aggregated) properdin bound to E_R (or E_S) cells in the absence of other complement components to form an intermediate, "E aP", which could then be lysed efficiently by the action of the alternative pathway in the absence of properdin. The identity of the properdin binding molecule(s), and the ability of native properdin to bind E were not investigated. Since this was proposed to be "a new function of activated properdin", and because such binding might influence the differences seen in the ability of properdin oligomers to enhance lysis of erythrocytes, an attempt was made to repeat these experiments using native properdin.

E_R (1.5×10^7 cells) were incubated for 15 min at 37°C in PBS with various quantities of purified properdin. After a single wash with PBS, the cells were resuspended in PBS.Mg.EGTA containing properdin-depleted serum (RP) and incubated at 37°C for 15 min before the extent of haemolysis was measured. Control cells were incubated in PBS for 15 min at 37°C in the absence of properdin, washed and then incubated with PBS.Mg.EGTA, RP, and various concentrations of properdin for 15 min at 37°C for haemolysis to occur. The details of the experiment, and the results are shown in figure 6.5. Although only one washing step was included between the pre-incubation with properdin and the addition of RP, no significant lysis of E_R was found (over 1000-fold more properdin is required to obtain the same extent of lysis if it is only present during the pre-incubation step). In contrast, using "activated" properdin, Konno *et al.* reported 66% lysis of E_R after preincubation with properdin, compared to 100% lysis when the same quantity of properdin was present during alternative pathway activation.

Thus no evidence was found for the direct binding of native properdin to E_R in a manner which will allow subsequent enhancement of the alternative pathway.

Very similar experiments have been reported in abstract form by Jarvis and Griffiss (1992). These workers suggest that native properdin can function as an initiator of the alternative pathway by binding directly to membrane surfaces. However, the quantity of properdin used was greater than utilized in the experiment reported here. It seems likely that the response observed is due to residual non-specific binding of properdin to the E_R surface. In addition, Medicus *et al.* (1976a) and Farries *et al.* (1988) found that native properdin had no measurable affinity for E_S cells, even in the presence of bound C3b.

6.4 Haemolytic assay of the altered forms of human properdin

Recombinant wild type properdin was indistinguishable from plasma properdin in the rabbit erythrocyte haemolytic assay (see chapter 4). The activities of the various purified altered forms of properdin were assayed in a similar way. The rabbit erythrocyte assay was used since in this assay, each oligomeric form of properdin had similar activity, and the preparations of the mutant proteins contained different proportions of various oligomers.

Properdin lacking TSR3 showed little difference from normal properdin in its ability to stabilize the C3bBb complex in both dose-dependence and time course experiments (figure 6.6). To investigate the activity of the various forms of properdin lacking TSR3, the oligomers were separated by gel exclusion chromatography on Superose 6. Fractions were assayed for properdin by ELISA and by rabbit erythrocyte haemolytic assay (figure 6.7). The tetramer, trimer and dimer peaks all contained properdin haemolytic activity. Within the error of the experiment, the activity of each oligomeric form is similar on a per monomer basis as found for normal properdin. However, the fractions containing "monomeric" properdin lacking TSR3 are not haemolytically active. While this may suggest that oligomerization of properdin is necessary for C3bBb stabilization, it cannot be ruled out that these fractions contain denatured properdin. It is striking that removal of an entire TSR module from the centre of the properdin monomer has no effect on the ability of the molecule to bind and stabilize the C3bBb complex. Thus, TSR3 is not directly involved in binding to C3b or to Bb, nor is it required to maintain a proper disposition of binding sites in other regions of the molecule.

Properdin lacking TSR4 showed no activity in the haemolytic assay, even at concentrations five fold greater than that required to give 100% lysis with normal properdin (figure 6.8). Since in the rabbit red blood cell haemolytic assay the different

oligomers of properdin are equally active, this lack of activity is not due to the absence of trimers or tetramers in the preparation. Furthermore, after separation of the dimers and monomers of properdin without TSR4 (as described above for properdin lacking TSR3), neither the dimer nor monomer fractions displayed haemolytic activity (figure 6.9). ELISA of the same fractions confirmed the presence of properdin. So, removal of TSR4 prevents properdin from stabilizing the C3bBb complex.

Properdin lacking TSR5 was also inactive in the haemolytic assay (figure 6.10). Furthermore, plasma properdin "nicked" in TSR5, as described in chapter 5, also lost its ability to stabilize the C3bBb complex (figure 6.10). Nicked properdin retains its overall structure and remains oligomerized as determined by electron microscopy and gel exclusion chromatography (see chapter 5). These results implicate TSR5 in the stabilization of the C3bBb complex.

Partially purified properdin lacking TSR6 (and the C-terminal tail) did not enhance haemolysis of rabbit erythrocytes (figure 6.11). However, we cannot determine from these results if this is due to the failure of this form of properdin to oligomerize, or due to the removal of important functional residues. The absence of N-linked oligosaccharide (which is normally linked to TSR6) is not responsible for the lack of activity, since properdin lacking N-linked carbohydrate is able to stabilize the alternative pathway C3 convertase in this assay (see chapter 4).

6.5 The effect of HYB3-3 mAb on stabilization of C3bBb

The effect of the anti-properdin monoclonal antibody, HYB3-3, on the haemolysis of rabbit erythrocytes in the alternative pathway haemolytic assay was tested. Purified properdin (40 ng), and various quantities of the antibody were mixed, and 25 µl RP, and 1.5×10^7 E_R added to a final volume of 110 µl in PBS.Mg.EGTA. The extent of haemolysis was measured by the absorbance of the supernatant at 405 nm. The results are shown in figure 6.12. The antibody had no effect on haemolysis up to approximately 20-fold molar excess. Larger quantities of antibody enhanced the observed haemolysis. Thus this mAb, which is thought to bind to TSR4 of properdin (see chapter 5), does not inhibit stabilization of the C3bBb complex by properdin. The enhanced lysis seen at high doses of HYB3-3 may be due to the ability of antibody Fab regions to activate the alternative pathway (Sandberg *et al.*, 1971; Reid, 1971) (see chapter 1, section 1.2). The HYB3-3 antibody produced very similar results when included in a similar assay of properdin lacking TSR3. That is, HYB3-3 was unable to inhibit the ability of properdin lacking TSR3 to stabilize the C3bBb complex.

6.6 Summary

The ability of properdin to stabilize the C3bBb complex of the alternative pathway can be measured using a haemolytic assay. Dimers, trimers and tetramers of purified human plasma properdin were equally active in such an assay when rabbit erythrocytes were used as the target cells. However, when neuraminidase treated sheep erythrocytes were utilised in the same assay, higher oligomers of properdin were found to be more effective than the lower forms. The reason for this difference is not known. Purified properdin was unable to bind to rabbit erythrocytes in a form which could subsequently enhance haemolysis.

The altered forms of properdin described in chapter 5 were tested for their ability to stabilize the C3bBb complex using the rabbit erythrocyte haemolytic assay. Strikingly, removal of TSR3 has no effect on the proficiency with which properdin stabilized the C3bBb complex. Removal of TSR4 abolishes the ability of the protein to function in this assay. However, the HYB3-3 monoclonal antibody to properdin, which is thought to bind TSR4, does not inhibit haemolysis of rabbit erythrocytes *via* the alternative pathway. Removal of TSR5, or tryptic "nicking" of a single peptide bond within an exposed loop in TSR5, also prevents the molecule from stabilizing the C3 convertase. Properdin with a deletion of TSR6 (including the C-terminal charged tail) is also inactive, although removal of the N-linked sugar which is attached to this module does not, in itself, alter the activity.

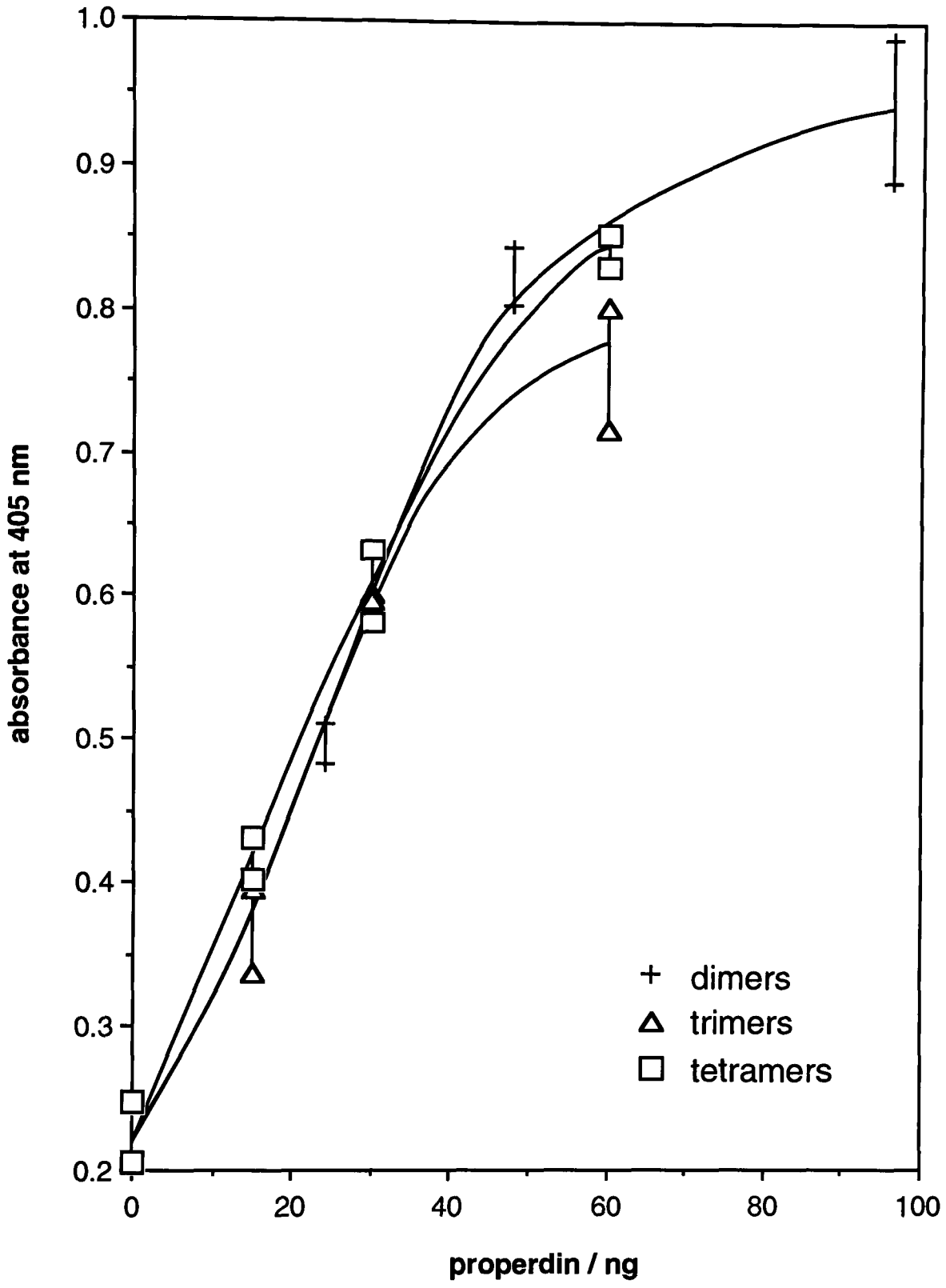


Figure 6.1
Comparison of the activity of the various oligomeric forms of human properdin in the rabbit erythrocyte haemolytic assay.
Purified plasma properdin was separated by gel exclusion chromatography on Superose 6. The three peak fractions, corresponding to dimers, trimers and tetramers, were used in the activity assay. The quantity of properdin in each fraction was determined by ELISA.

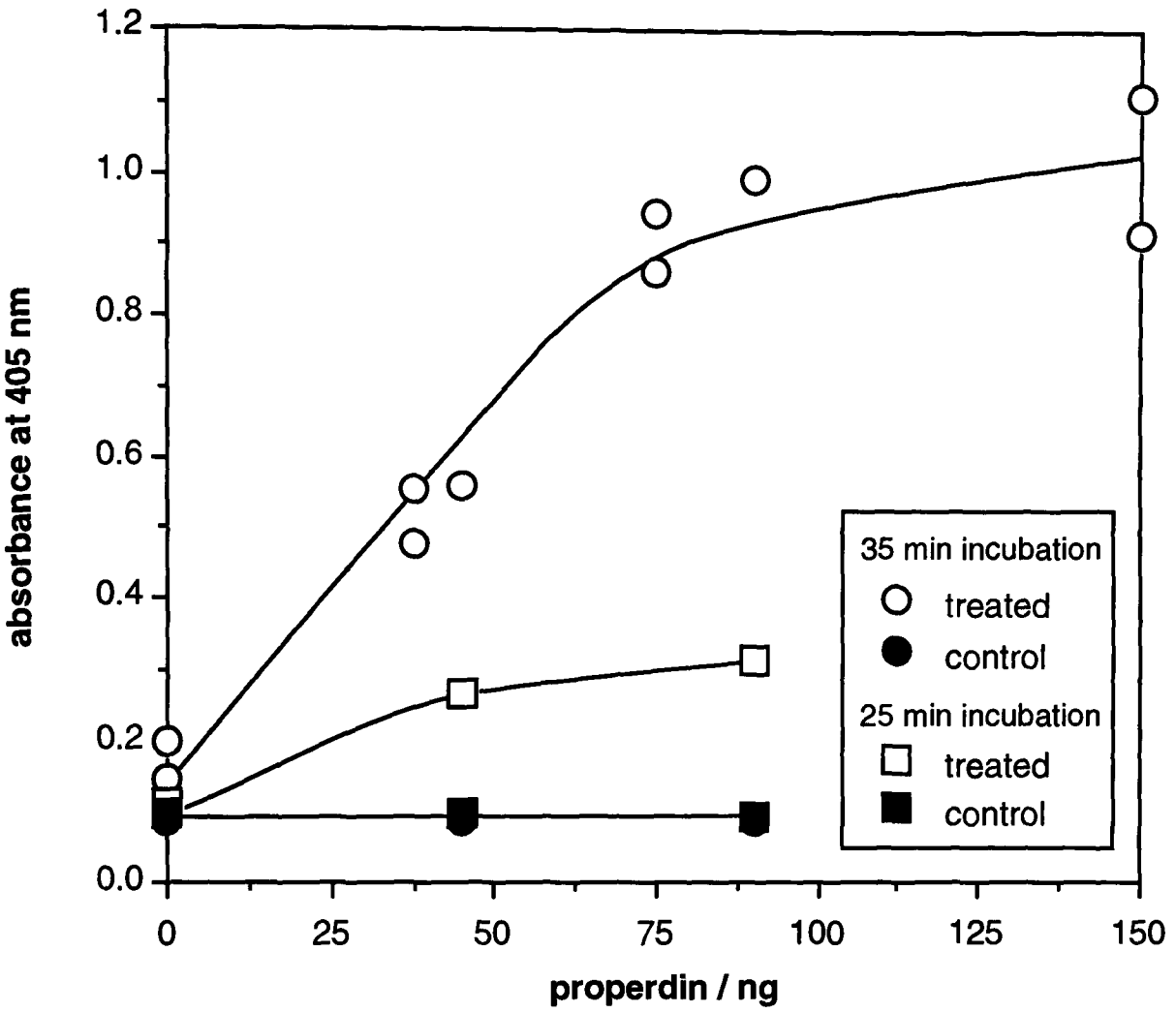
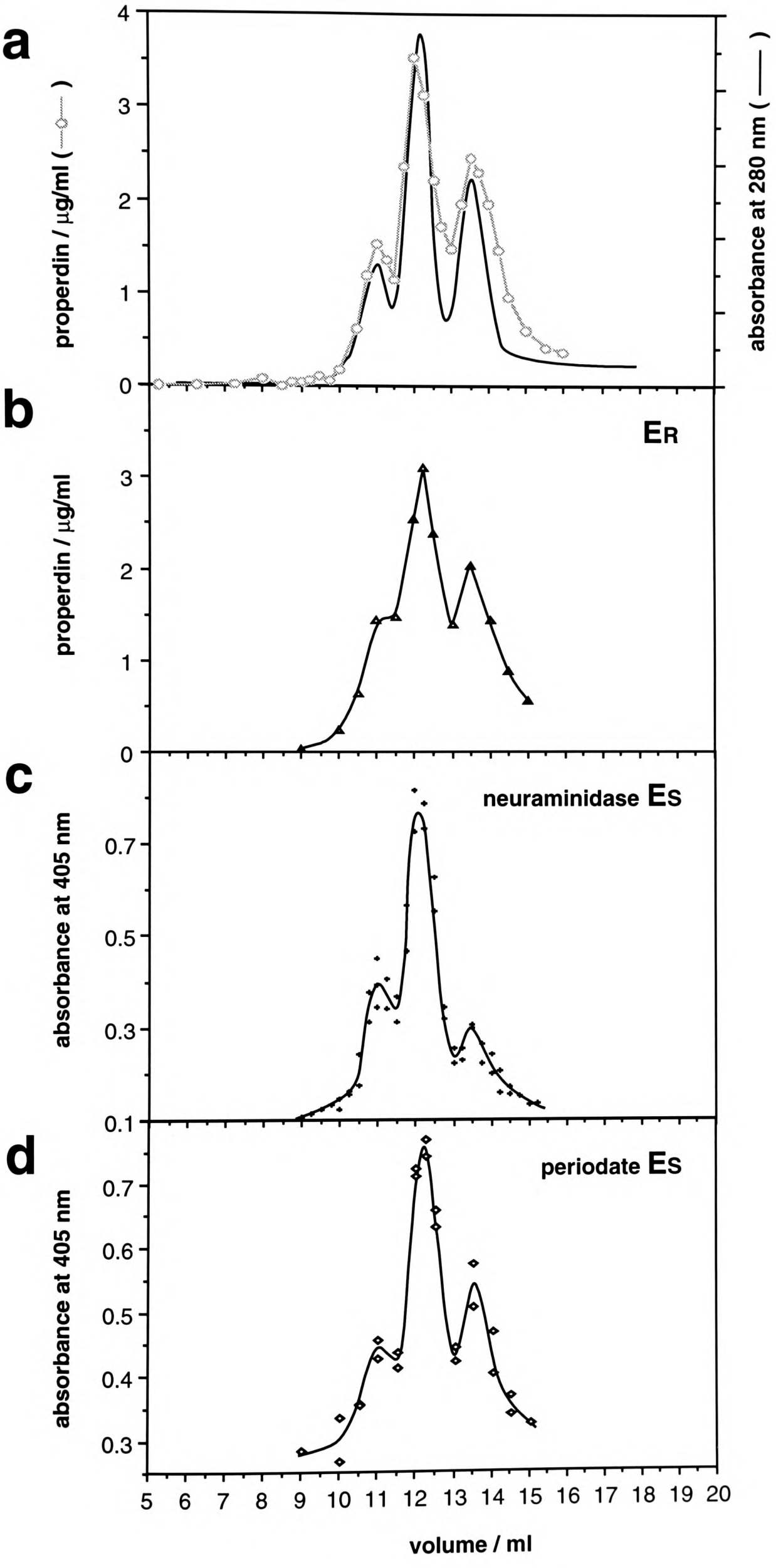


Figure 6.2
Haemolysis of neuraminidase treated sheep erythrocytes.
Sheep red blood cells were treated with neuraminidase at pH 5 for 2 h at 37°C ("treated"), or incubated in the same conditions without neuraminidase ("control"). The cells were then incubated with properdin-depleted serum/PBS.Mg.EGTA with various concentrations of plasma properdin for 25 or 35 min at 37°C, and haemolysis determined by absorbance of the supernatant at 405 nm.

Figure 6.3
Comparison of the activities of the various oligomers of properdin in the rabbit and treated sheep erythrocyte haemolytic assays.
a) The elution profile of purified plasma properdin from gel exclusion chromatography on Superose 6. Properdin was detected by absorbance at 280 nm and by ELISA. The concentration in each fraction was determined by ELISA in comparison with known concentrations of properdin.
b) The activity of fractions determined by rabbit eyrthrocyte haemolytic assay. The results, which are representative of three independent experiments, are expressed as properdin concentration calculated by comparison with properdin standards of known concentration.
c) The activity of fractions determined by neuraminidase-treated sheep eyrthrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (see figure 6.2).
d) The activity of fractions determined by periodate-treated sheep erythrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (not shown).

Figure 6.3
Comparison of the activities of the various oligomers of properdin in the rabbit and treated sheep erythrocyte haemolytic assays.
a) The elution profile of purified plasma properdin from gel exclusion chromatography on Superose 6. Properdin was detected by absorbance at 280 nm and by ELISA. The concentration in each fraction was determined by ELISA in comparison with known concentrations of properdin.
b) The activity of fractions determined by rabbit eyrthrocyte haemolytic assay. The results, which are representative of three independent experiments, are expressed as properdin concentration calculated by comparison with properdin standards of known concentration.
c) The activity of fractions determined by neuraminidase-treated sheep eyrthrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (see figure 6.2).
d) The activity of fractions determined by periodate-treated sheep erythrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (not shown).

Figure 6.3
Comparison of the activities of the various oligomers of properdin in the rabbit and treated sheep erythrocyte haemolytic assays.
a) The elution profile of purified plasma properdin from gel exclusion chromatography on Superose 6. Properdin was detected by absorbance at 280 nm and by ELISA. The concentration in each fraction was determined by ELISA in comparison with known concentrations of properdin.
b) The activity of fractions determined by rabbit eyrthrocyte haemolytic assay. The results, which are representative of three independent experiments, are expressed as properdin concentration calculated by comparison with properdin standards of known concentration.
c) The activity of fractions determined by neuraminidase-treated sheep eyrthrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (see figure 6.2).
d) The activity of fractions determined by periodate-treated sheep erythrocyte haemolytic assay. The results of duplicate experiments are shown. All values lie on the linear portion of a dose-response curve with unfractionated plasma properdin in the same assay (not shown).


a
properdin / µg/ml (----○----)
absorbance at 280 nm (——)
4
3
2
1
0
b
properdin / µg/ml
ER
3
2
1
0
c
absorbance at 405 nm
neuraminidase ES
0.7
0.5
0.3
0.1
d
absorbance at 405 nm
periodate ES
0.7
0.6
0.5
0.4
0.3
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
volume / ml

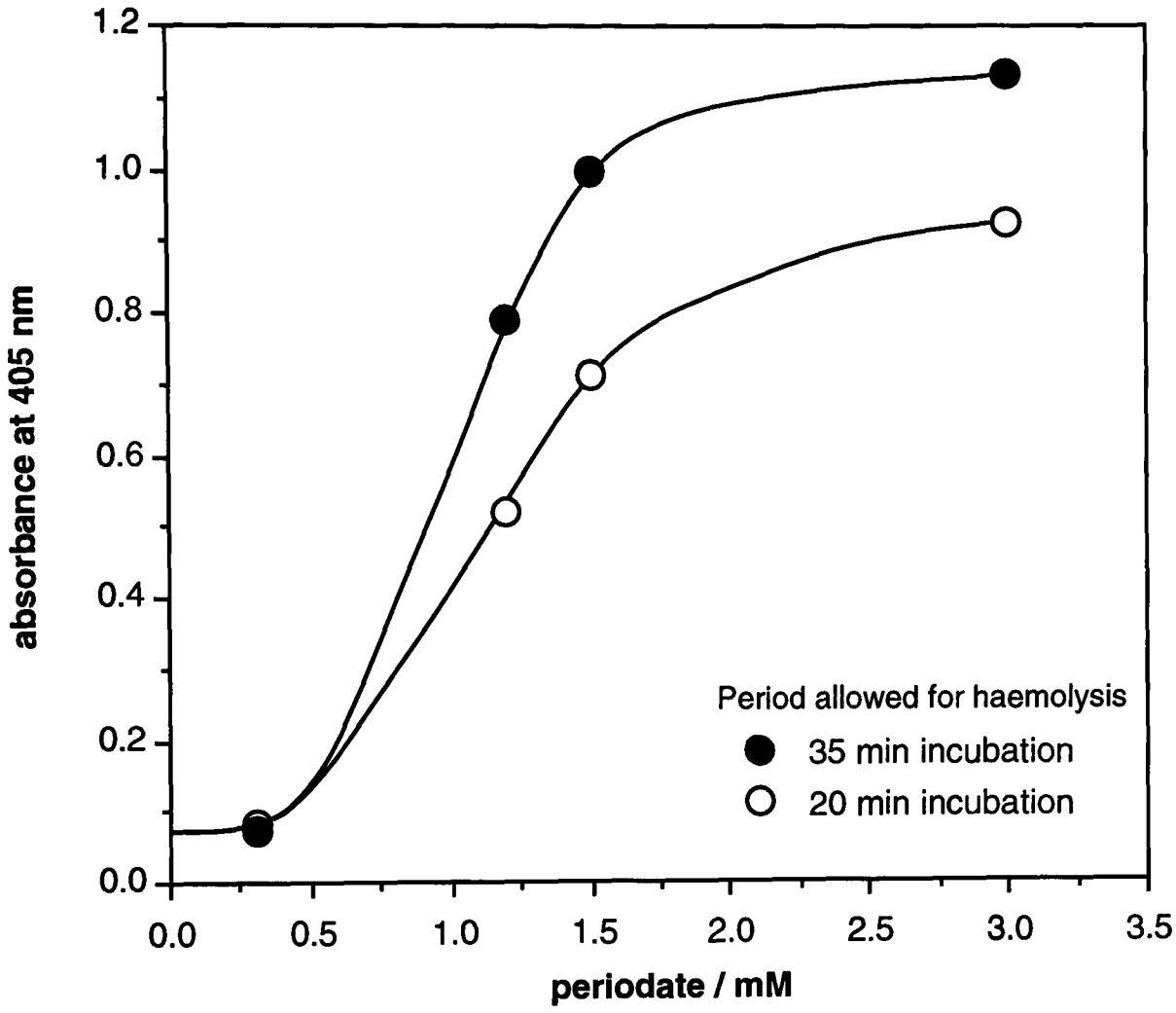


Figure 6.4
Effect of sodium periodate on the sensitivity of sheep erythrocytes to alternative pathway haemolysis.
The quantity of sodium periodate used to treat sheep red blood cells (4°C, 10 min) was titrated, and the effect on the sensitivity of the cells was tested by incubation with 100 ng plasma properdin in properdin-depleted serum/PBS.Mg.EGTA for 20 or 35 min at 37°C.

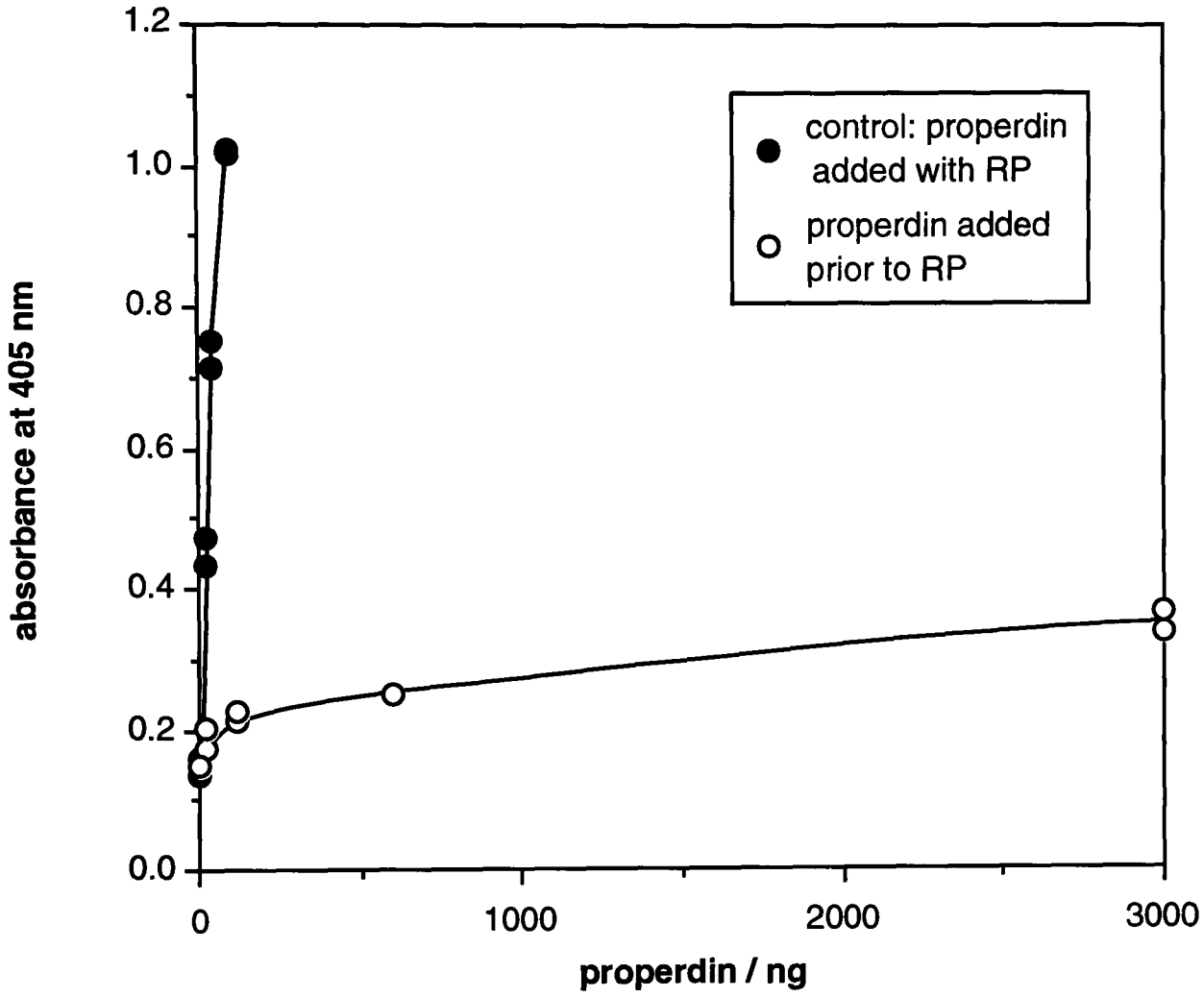
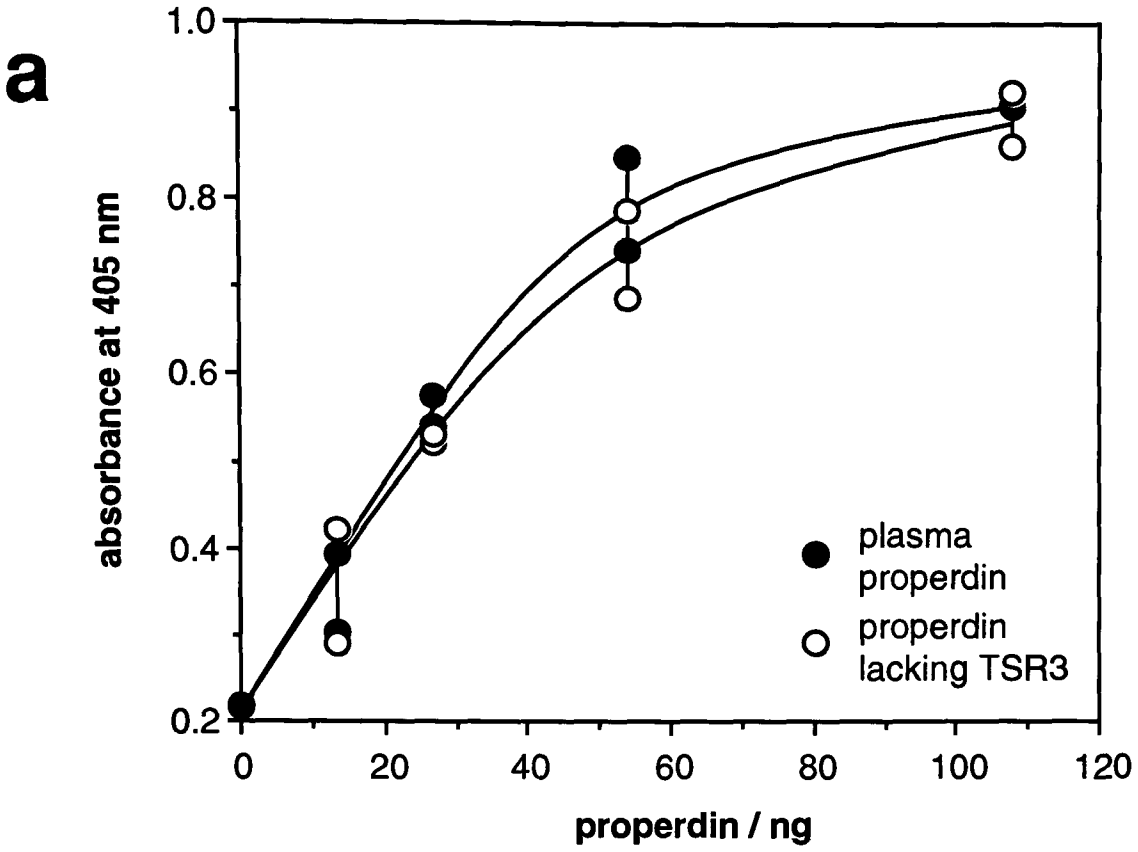


Figure 6.5
Haemolysis of rabbit erythrocytes in properdin-depleted serum after pre-incubation with human properdin.
Rabbit erythrocytes (1.5×10^7 cells) were incubated for 15 min at 37°C with the indicated quantities of human properdin in a total volume of 85 µl PBS. After a single wash with 150 µl PBS, the cells were incubated with 25 µl RP in a total volume of 110 µl PBS.Mg.EGTA. As a control, cells were incubated for 15 min at 37°C in the absence of properdin, and after washing, incubated for 15 min at 37°C with the indicated quantities of properdin with 25 µl RP in a total volume of 110 µl PBS.Mg.EGTA.



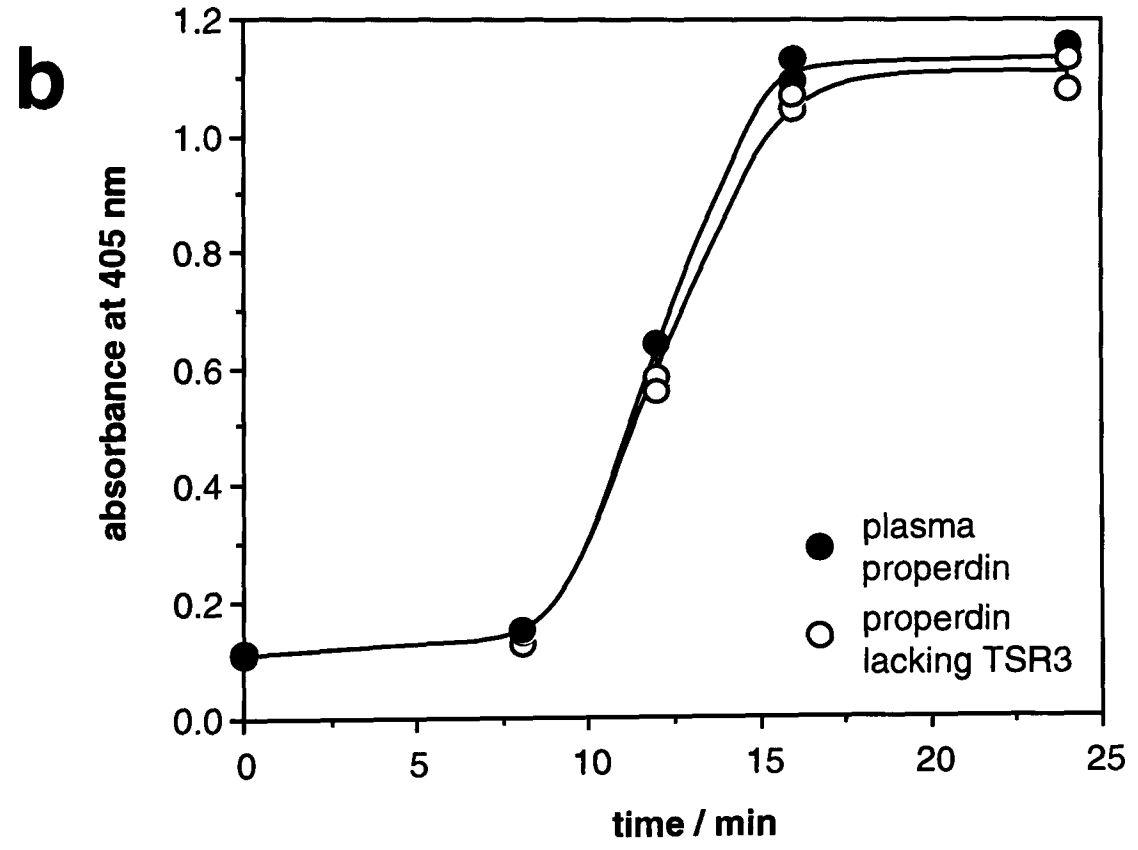
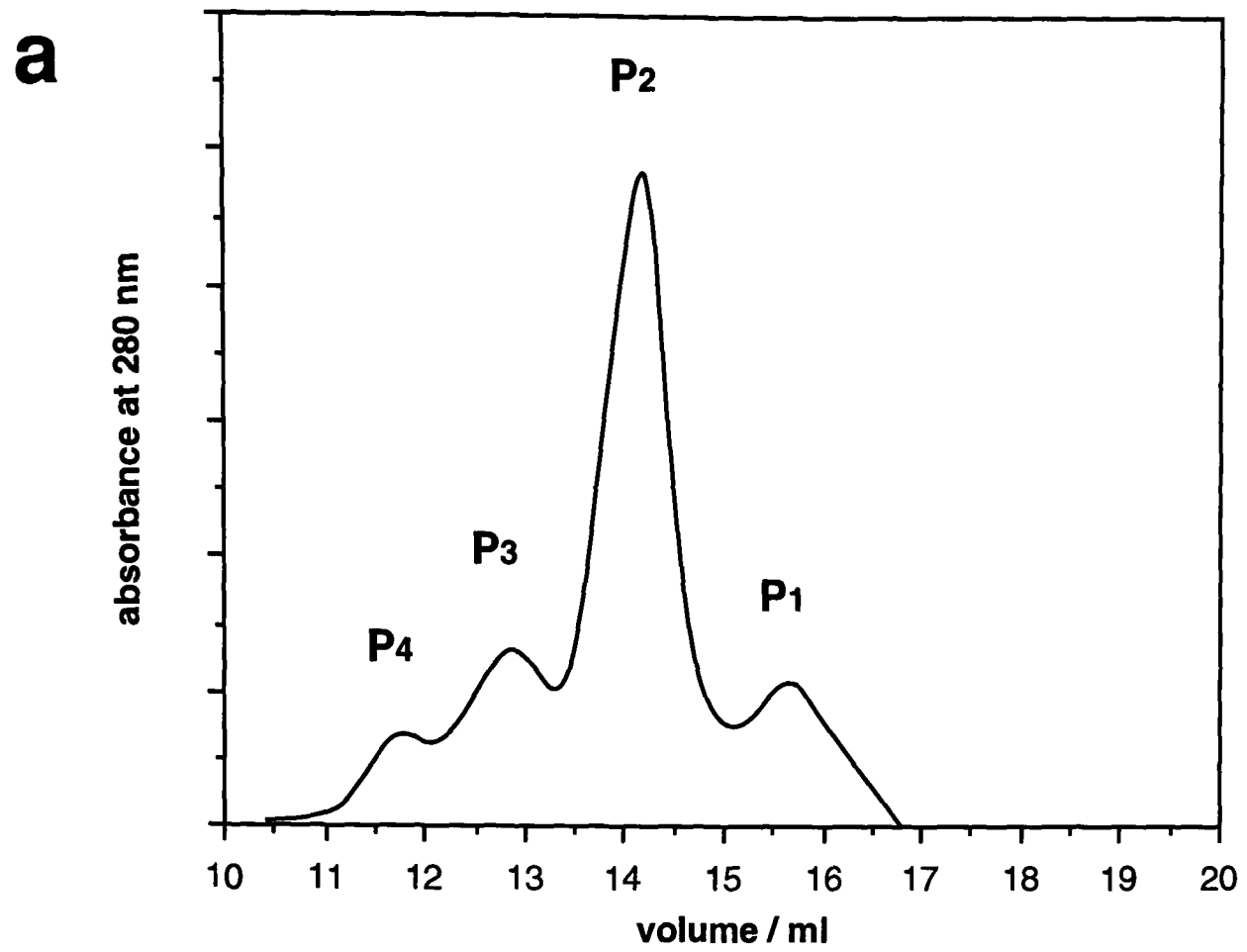


Figure 6.6
Comparison of properdin lacking TSR3 with plasma properdin in the rabbit erythrocyte haemolytic assay.
a) Dependence of haemolysis on dose of properdin
b) Time course of haemolysis (with 90 ng each form of properdin)
The results of duplicate experiments are shown.



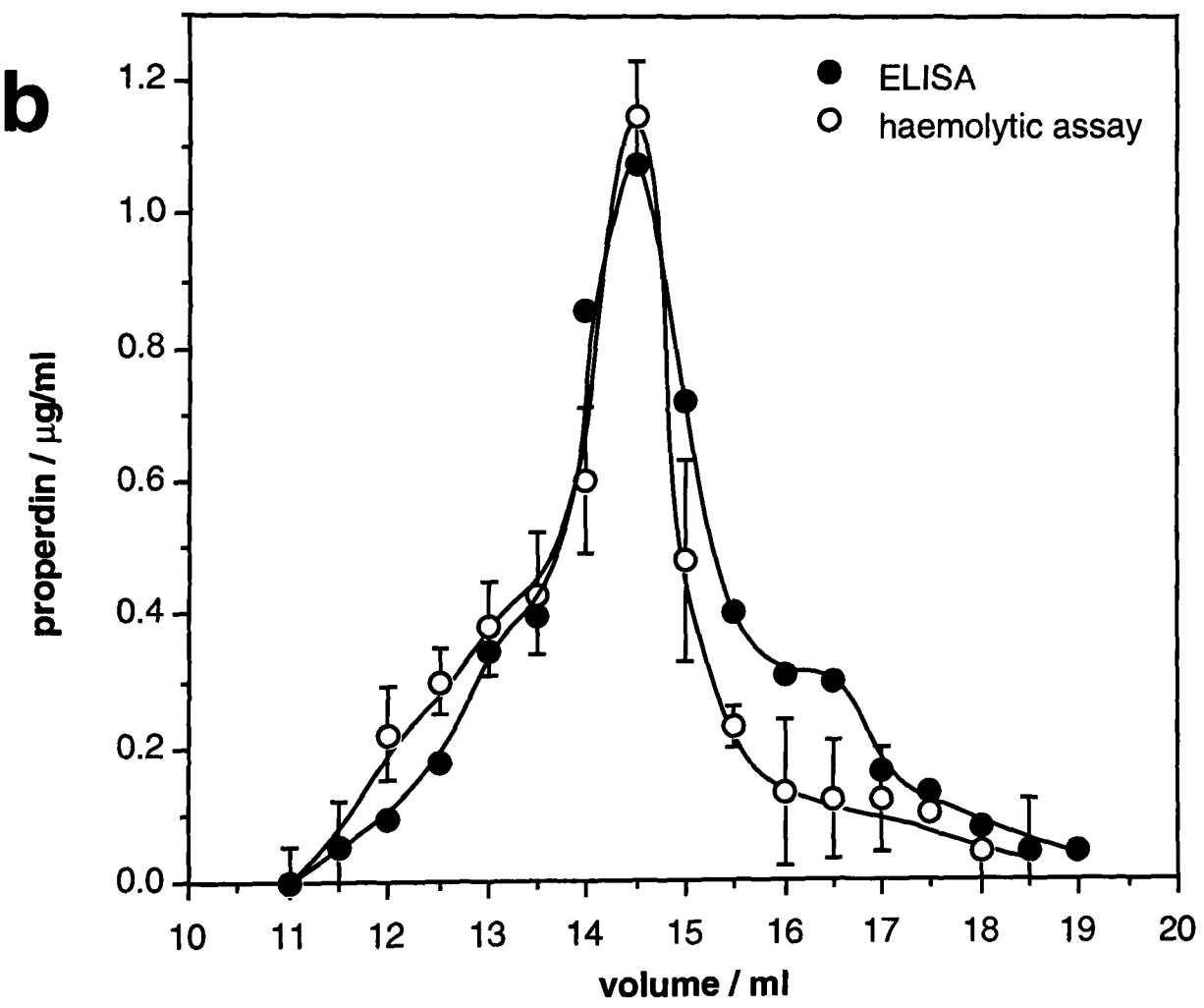


Figure 6.7
Haemolytic assay of properdin lacking TSR3 after separation of oligomers on Superose 6 gel exclusion chromatography. The position of the peaks suggested to correspond to monomers, dimers, trimers and tetramers are marked P1, P2, P3, and P4 (see chapter 5).
a) Elution profile determined by absorbance at 280 nm.
b) Comparison of the elution profiles as determined by ELISA and rabbit erythrocyte haemolytic assay. Average error in the ELISA is ± 0.05 µg/ml. The haemolytic assay results are shown as mean ± standard deviation, n=5.

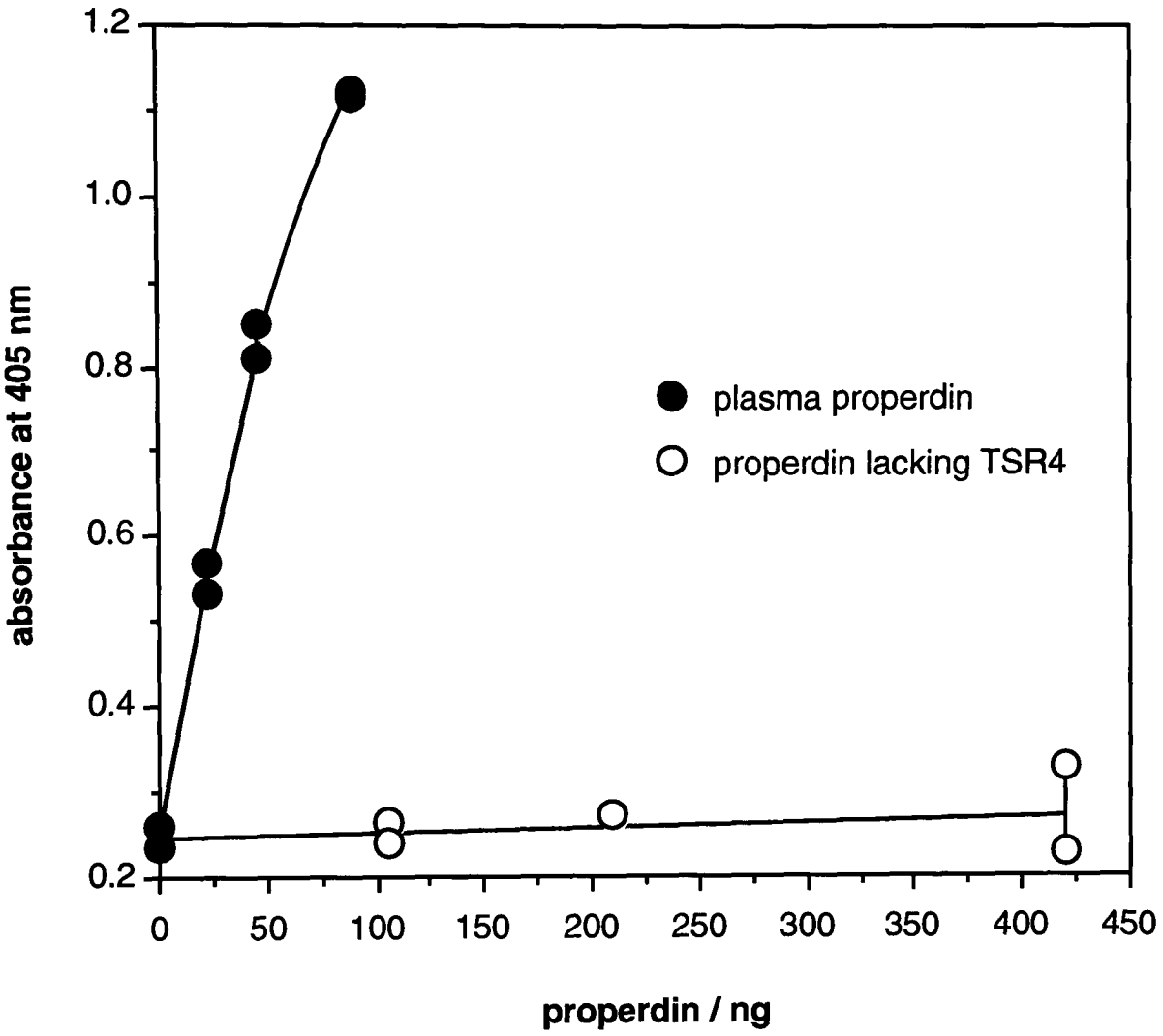
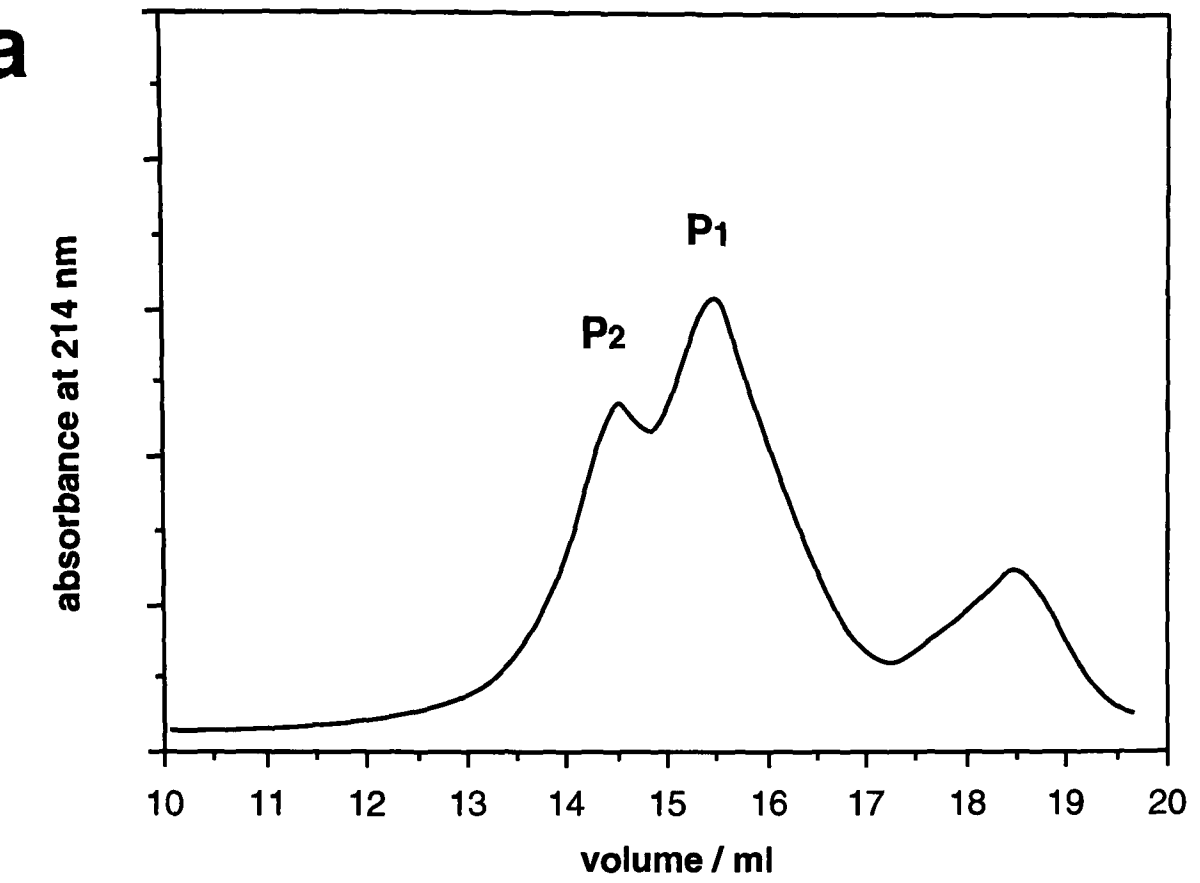


Figure 6.8
Comparison of recombinant properdin lacking TSR4 and plasma properdin in the rabbit erythrocyte haemolytic assay. The results of duplicate experiments are shown.



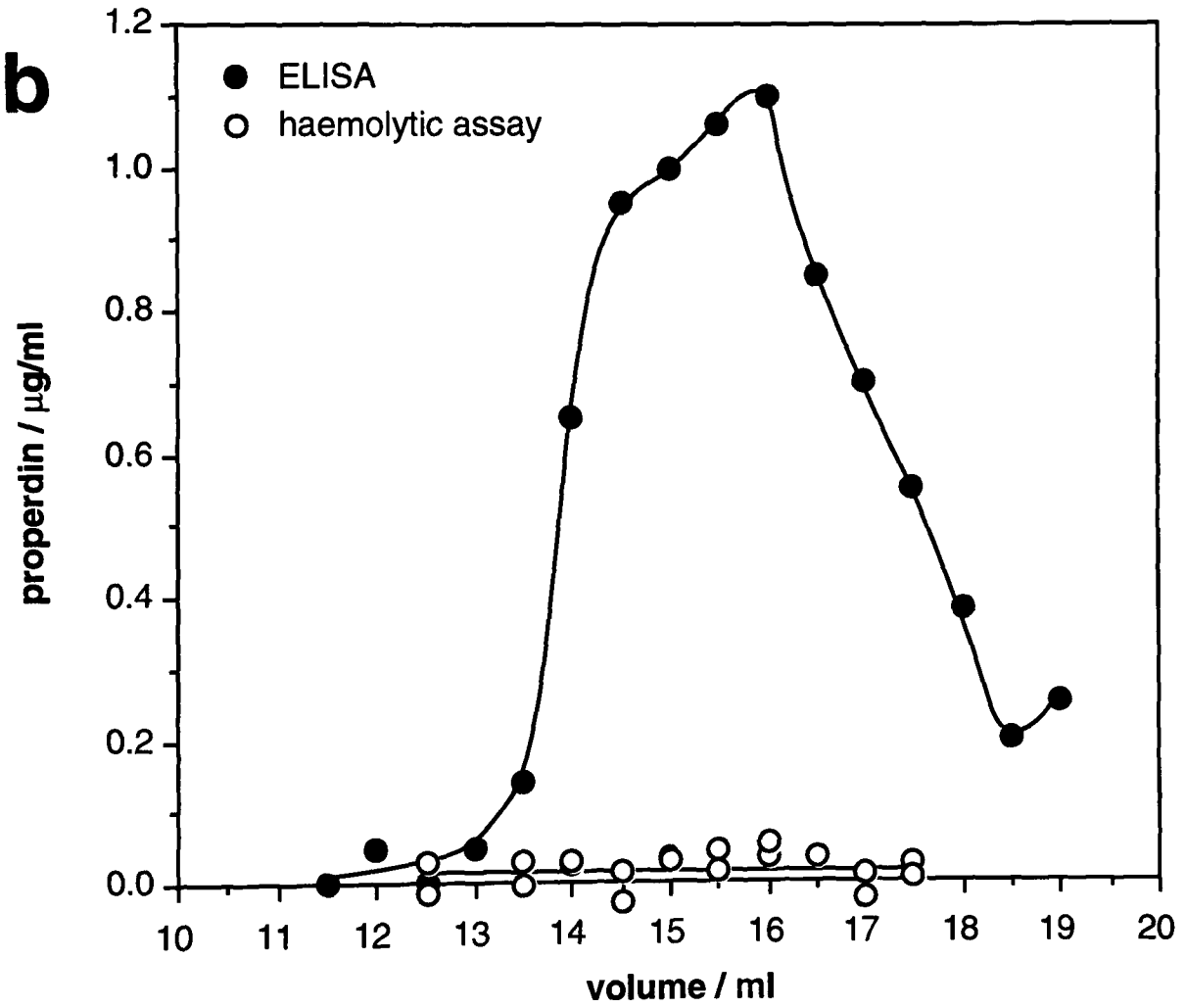


Figure 6.9
Haemolytic aasay of properdin lacking TSR4 after separation of oligomers on Superose 6 gel exclusion chromatography. The positions of peaks suggested to correspond to dimers and monomers are marked P2 and P1.
a) Elution profile determined by absorbance at 214 nm.
b) Comparison of the elution profiles as determined by ELISA and rabbit erythrocyte haemolytic assay. Average error in the ELISA is ±0.05 µg/ml. The results of duplicate experiments are shown for the haemolytic assay.

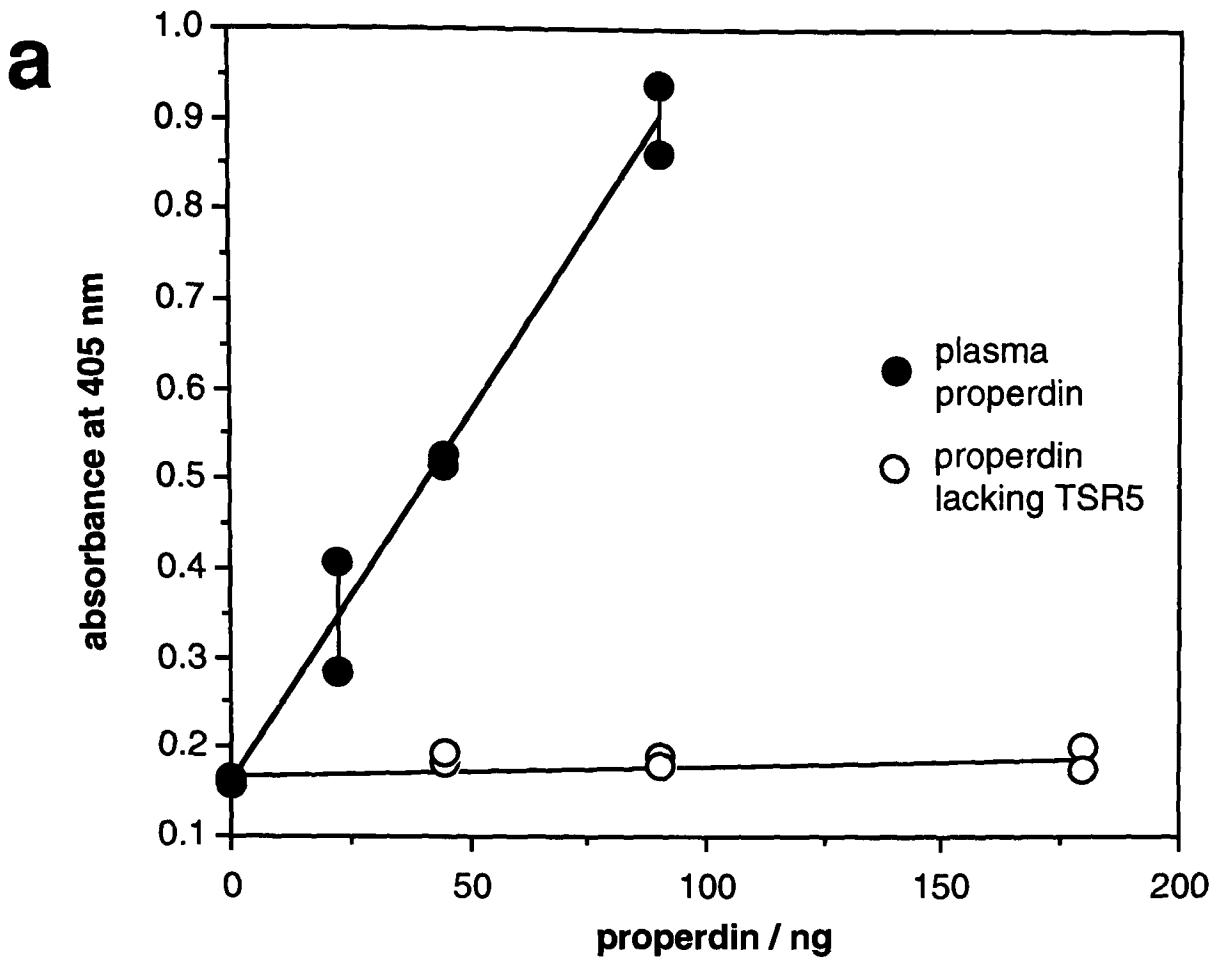


Figure 6.10
Comparison of properdin lacking TSR5 or properdin "nicked" in TSR5 with plasma properdin, using the rabbit erythrocyte haemolytic assay.
a) Comparison of plasma properdin with recombinant properdin lacking TSR5.
b) Comparison of plasma properdin with properdin "nicked" in TSR5 by limited tryptic digestion.
The results of duplicate experiments are shown.

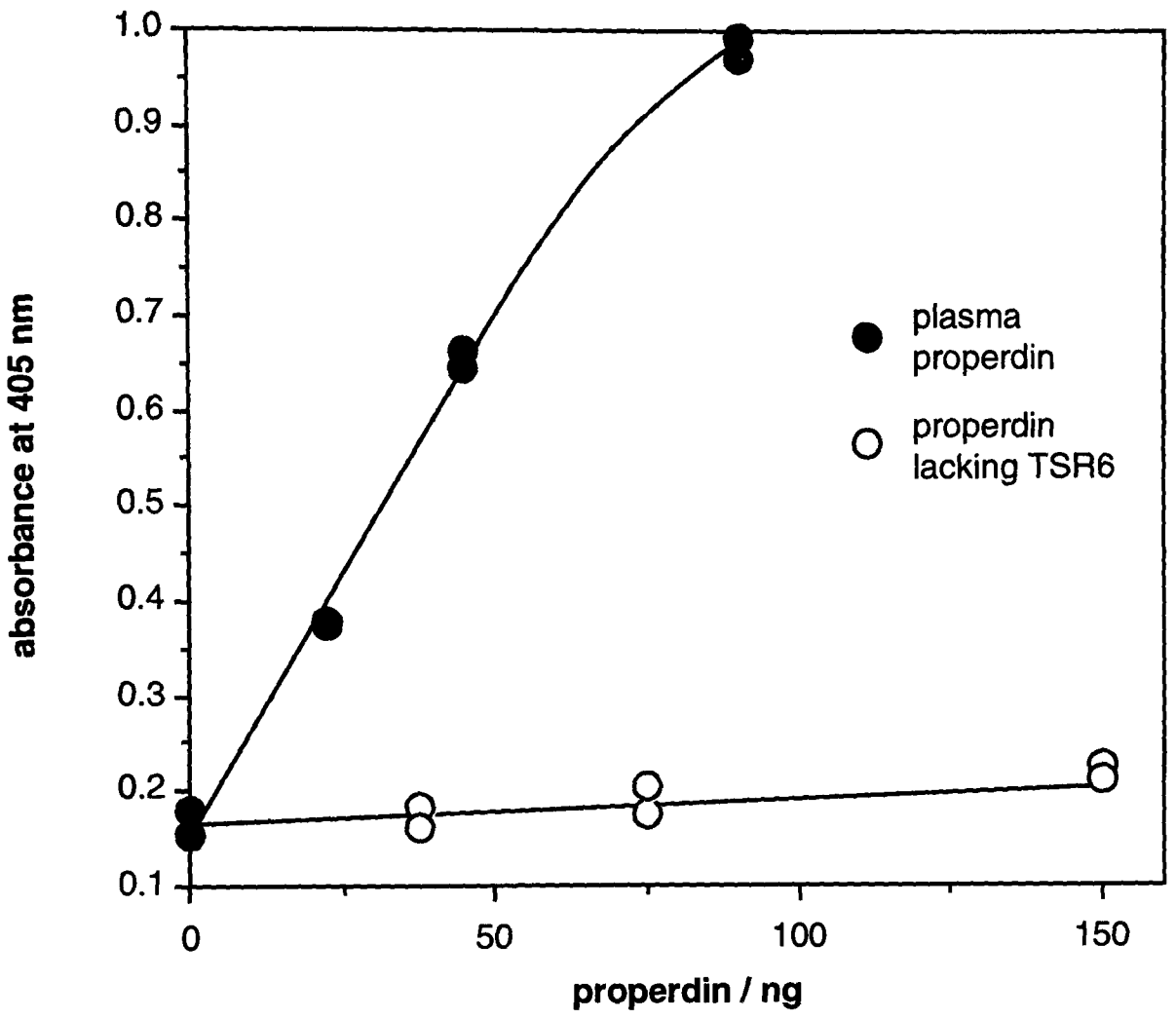


Figure 6.11
Comparison of the activity of recombinant properdin lacking TSR6 (and the C-terminal charged tail) and plasma properdin in the rabbit erythrocyte haemolytic assay.
The results of duplicate experiments are shown.

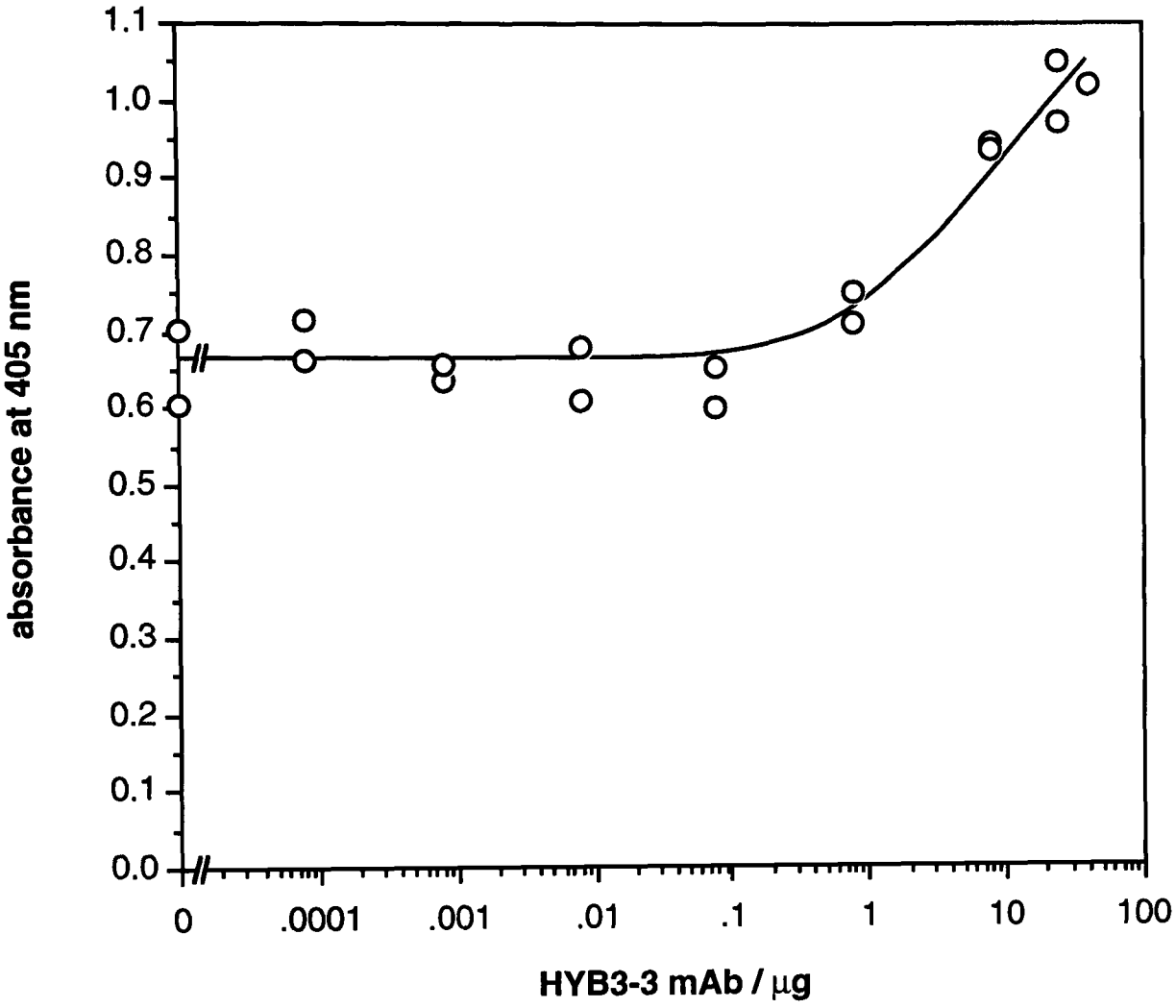


Figure 6.12
The effect of HYB3-3 anti-properdin monoclonal antibody on haemolysis of rabbit erythrocytes *via* the alternative pathway. The quantity of mAb indicated was mixed with 40 ng of purified properdin in PBS, before addition of RP and rabbit erythrocytes in PBS.Mg.EGTA. The extent of haemolysis was measured by absorbance at 405 nm after incubation at 37°C for 15 min. The results of duplicate experiments are shown.

Chapter 7

Chapter 7

The binding of altered forms of human properdin to C3b

Binding of properdin to C3b is an important interaction in the stabilization of the C3 convertase of the alternative pathway of complement (see chapter 1, section 1.7.1). However, the failure of an altered form of properdin to stabilize the C3bBb complex does not rule out the possibility that the molecule is able to bind C3b. The altered forms of properdin described in chapter 5 were tested for their ability to bind to C3b using a microtitre plate binding assay.

7.1 The C3b binding assay

The binding of native properdin to C3b can be detected in the absence of factor B at physiological ionic strength and pH (see section 1.7.1 of the introduction). For example, the binding of radiolabelled properdin to C3b on zymosan or sheep erythrocytes has been reported (DiScipio, 1981; Smith *et al.*, 1984; Farries *et al.*, 1988a). For the work described in this thesis, a microtitre plate binding assay similar to that developed by Dr J. D. Lambris and co-workers was used (Lambris *et al.*, 1984). Purified C3b (kindly provided by Viv Perkins, MRC Immunochemistry Unit) was coated onto microtitre plates in 20% glycerol/PBS, pH 7.4. After blocking with BSA, samples were applied and incubated at room temperature for 1 h. Bound properdin was detected using biotinylated polyclonal anti-properdin IgG and standard procedures (described in materials and methods).

Initially the binding characteristics of normal plasma properdin were examined at different concentrations of coating C3b and at different ionic strengths (figure 7.1). No binding of properdin to the plates was observed in the absence of coated C3b. However, as the quantity of coated C3b increased, clear dose-dependent binding of properdin was seen. This binding was enhanced at low ionic strength, in agreement with the results of DiScipio (1981) and Farries *et al.* (1987, 1988a). Since it was important to detect potentially weak binding of mutant forms of properdin, subsequent assays were conducted at half-physiological salt strength.

7.2 The binding of various oligomeric forms of properdin to C3b

Some of the mutant forms of properdin did not form oligomers other than dimers. Therefore, it was important to determine the binding characteristics of the various oligomeric forms of normal plasma properdin so that comparisons with the altered forms could be made. Dimeric, trimeric and tetrameric properdin fractions were obtained by gel exclusion chromatography of whole purified plasma properdin on Superose 6. The amount of properdin in each fraction was determined by ELISA and by absorbance at 280 nm. The results of the C3b binding assay on the isolated oligomers are shown in figures 7.2 and 7.3. At saturation, the binding of higher oligomers results in greater detected absorbance than the binding of smaller oligomers. However, care must be taken in interpreting the differences seen in this assay since the binding of a properdin tetramer, for example, will result in the immobilization of more properdin subunits onto the plate than the binding of a dimer, even if the affinity of each oligomer for C3b is the same. The binding of one tetramer is likely to lead to a higher detected absorbance than the binding of a single dimer. In the assay used here, this problem is further complicated because different oligomeric forms of properdin are not detected directly, but by using a polyclonal anti-properdin antibody. While in the sandwich ELISA using this detection method, the different forms of properdin are clearly detected equally according to the number of monomers present (see chapter 4), this is not necessarily the case in the C3b binding assay. Thus it is not possible to determine the number of properdin oligomers binding to C3b in this assay, and it is unwise to draw any conclusions about the number of binding sites available for each oligomer from the apparent differences seen in the absorbance measured at saturation.

The data suggest that the various oligomeric forms do not have very different avidities for C3b, although the quantity of tetramers needed for saturation of C3b binding appears lower than for the other forms of properdin, and thus tetramers may have higher avidity for C3b than the other oligomers. Higher oligomeric forms of properdin bind to C3b coated sheep erythrocytes or zymosan with higher avidity than lower forms in direct binding assays using radiolabelled properdin (Smith *et al.*, 1984; Farries *et al.*, 1987). In these experiments, the percentage of the radiolabelled protein which bound to C3b was determined. So, the differences seen between the binding avidities of the oligomers cannot be attributed simply to improved detection of the higher forms.

If properdin is multivalent for C3b binding, then the difference seen in the activities of the various oligomers may depend on the density of C3b available for binding (see chapter 6). To investigate this, C3b was coated on to microtitre plates at concentrations between 0.1 and 5.0 $\mu g\ well^{-1}$, and the profile of C3b binding activity of properdin oligomers fractionated by gel exclusion chromatography on Superose 6 was determined (as in the experiment shown in figure 7.3, at half-physiological ionic strength).

However, the density of C3b had no marked effect on the relative binding avidities of the various oligomeric forms of properdin (not shown). It is possible that the range of C3b density examined was insufficient to observe any change in the ability of the different properdin species to bind C3b.

The lack of a striking difference in the avidities of the various oligomeric forms in the assay used here may be due to the use of C3b which is not oriented on the surface through its thioester bond. C3b immobilized on plastic as in this microtitre plate assay may be bound in various orientations, leading to a reduction in multivalent properdin - C3b interactions.

Although it is difficult to interpret the differences between the oligomeric forms of properdin seen in this assay, the procedure was considered suitable for analysis of the various altered species of properdin if similar oligomeric forms were compared. Furthermore, since only microgramme quantities of the recombinant forms of properdin were available, direct labelling of the proteins would have been difficult.

7.3 Binding of altered forms of properdin to C3b

The binding of purified preparations of the various forms properdin to C3b was investigated using the assay described above. C3b was coated onto microtitre plate wells at 3.3 $\mu g\ ml^{-1}$ (0.5 $\mu g\ well^{-1}$), and properdin binding was carried out in half-physiological salt strength phosphate buffer. The binding of recombinant wild type properdin to C3b in this assay was identical to that of purified plasma properdin (see chapter 4).

As expected from the ability of the mutant form of properdin lacking TSR3 to stabilize the C3bBb complex (chapter 6), this form of properdin binds to C3b (see figures 7.4). Considering the high proportion of dimers present in this preparation compared to normal properdin, it is surprising that the binding curves for properdin without TSR3 and normal properdin are so similar. It may be that properdin lacking TSR3 actually has increased C3b binding activity. Careful quantitative studies will be required to check this possibility. The profile of C3b binding activity among the oligomeric forms of properdin lacking TSR3 after separation by gel exclusion chromatography (figure 7.5) is similar to that obtained for normal properdin. The likely monomeric fraction of the preparation does not show detectable binding to C3b. The inability of these molecules to stabilize the C3 convertase complex (see chapter 6) is probably due to their inability to bind C3b. It remains possible that the assay procedure is unable to detect the binding of monomeric properdin, although this seems unlikely since polyclonal anti-properdin was employed for detection.

Properdin lacking TSR4 is unable to stabilize the C3bBb complex (see chapter 5). However, this form of properdin retains C3b binding activity (see figure 7.6). The preparation of properdin lacking TSR4 contains only monomers and dimers. When compared to the binding of the various oligomeric forms of plasma properdin, the binding seen for the form lacking TSR4 is similar to that of normal dimeric properdin. So, the apparent reduced binding of properdin lacking TSR4 compared with that normal properdin can be ascribed to the absence of higher oligomers in the preparation. Removal of TSR4 from properdin does not diminish the ability of dimers to bind C3b. TSR4 is likely not to play a direct role in C3b binding in normal properdin. However, since properdin lacking TSR4 is unable to stabilize the C3bBb complex (see chapter 6), it is possible that TSR4 may be involved in Factor B binding, or in causing a conformational change in C3b that increases the stability of the complex. The mechanism through which properdin stabilizes the C3bBb complex is not known (for a discussion of this see chapter 1, section 1.7.1).

Purified recombinant properdin lacking TSR5 does not show detectable binding to C3b, despite forming a similar distribution of oligomers to that found for properdin without TSR4 (figure 7.7a). Furthermore, properdin "nicked" in TSR5 by limited tryptic digestion also loses its ability to bind C3b (figure 7.7b). The low level of binding seen for "nicked" properdin is probably due to the residual intact properdin which remains after trypsin treatment (see figure 5.13). Limited tryptic digestion of properdin has no effect on the overall structure of the oligomers as determined by gel exclusion chromatography and electron microscopy (see chapter 5). So, together these results strongly suggest that TSR5 is required for the binding of properdin to C3b.

Partially purified properdin lacking the sixth TSR does not bind to C3b in this assay. This form of properdin does not form oligomers. It is clear that the response seen in the assay used is dependent upon the oligomerization of properdin. The probable monomeric form of properdin lacking TSR3 does not show detectable binding to C3b (figure 7.5). Thus, from these results it cannot be determined if the lack of binding of properdin without TSR6 is due to the removal of important binding residues or to the inability of monomeric properdin to bind C3b (or to an inability to detect the binding of monomers using this assay procedure).

7.4 Inhibition of properdin binding to C3b by monoclonal antibodies

In chapter 5, the use of the recombinant mutant forms of properdin to localize the epitopes of a panel of anti-human properdin monoclonal antibodies was described. The ability of these antibodies to inhibit properdin binding to C3b was also assessed by Ms Fabianna Filippoussis and Dr John D. Lambris of the University of Pennsylvania,

Philadelphia, PA. The assay used was similar to that described by Lambris *et al.* (1984). Briefly, normal human plasma was pre-incubated with the monoclonal antibody under examination before the samples were applied to microtitre plates coated with C3b (as described in materials and methods). Bound properdin was detected using goat anti-human properdin IgG, with peroxidase conjugated rabbit anti-goat IgG as the second antibody. Results are shown in figure 7.9 and summarised in table 7.1.

Table 7.1

The binding of mutant forms of recombinant properdin to a panel of monoclonal antibodies, and the ability of the mAbs to inhibit properdin binding to C3b.

form of properdin	monoclonal antibody number										
	1*	4	5	6	7	8	9	10	11	12	13
plasma properdin	++	++	++	++	++	++	++	++	++	++	++
recombinant properdin	++	++	++	++	++	nd	++	++	++	++	++
properdin lacking TSR3	++	++	++	++	++	nd	++	++	++	+	+
properdin lacking TSR4	-	+	++	-	++	nd	++	++	++	-	-
properdin lacking TSR5	+	-	++	-	++	nd	++	++	++	-	-
properdin lacking TSR6	+	-	-	-	-	nd	++	++	++	-	-
truncated properdin	nd	-	-	-	-	nd	+	++	+	-	-
control medium	-	-	-	-	-	-	-	-	-	-	-
inhibition of P binding	-	-	o	oo	o	-	o	oo	o	-	-

* mAb 1 is HYB3-3

++ indicates clear immunoreactivity

\+ indicates low immunoreactivity

\- indicates no detectable immunoreactivity

oo indicates strong inhibition of properdin binding

o indicates clear inhibition of properdin binding

\- indicates no inhibition of properdin binding

nd indicates reactivity not determined

Since the TSR containing the epitope for most of the antibodies could not be unambiguously defined (see chapter 5), many of the results do not provide a clear indication of the importance of individual modules. Nevertheless, some information can be obtained from the data.

Monoclonal antibodies 9, 10 and 11 bind to the N-terminal end of the properdin monomer, within the N-terminal region, TSR1 or TSR2. Pre-incubation of human plasma with any of these antibodies prevents properdin within the plasma from binding to C3b. Thus the involvement of the N-terminal-TSR1-TSR2 portion of the properdin monomer is implicated in C3b binding.

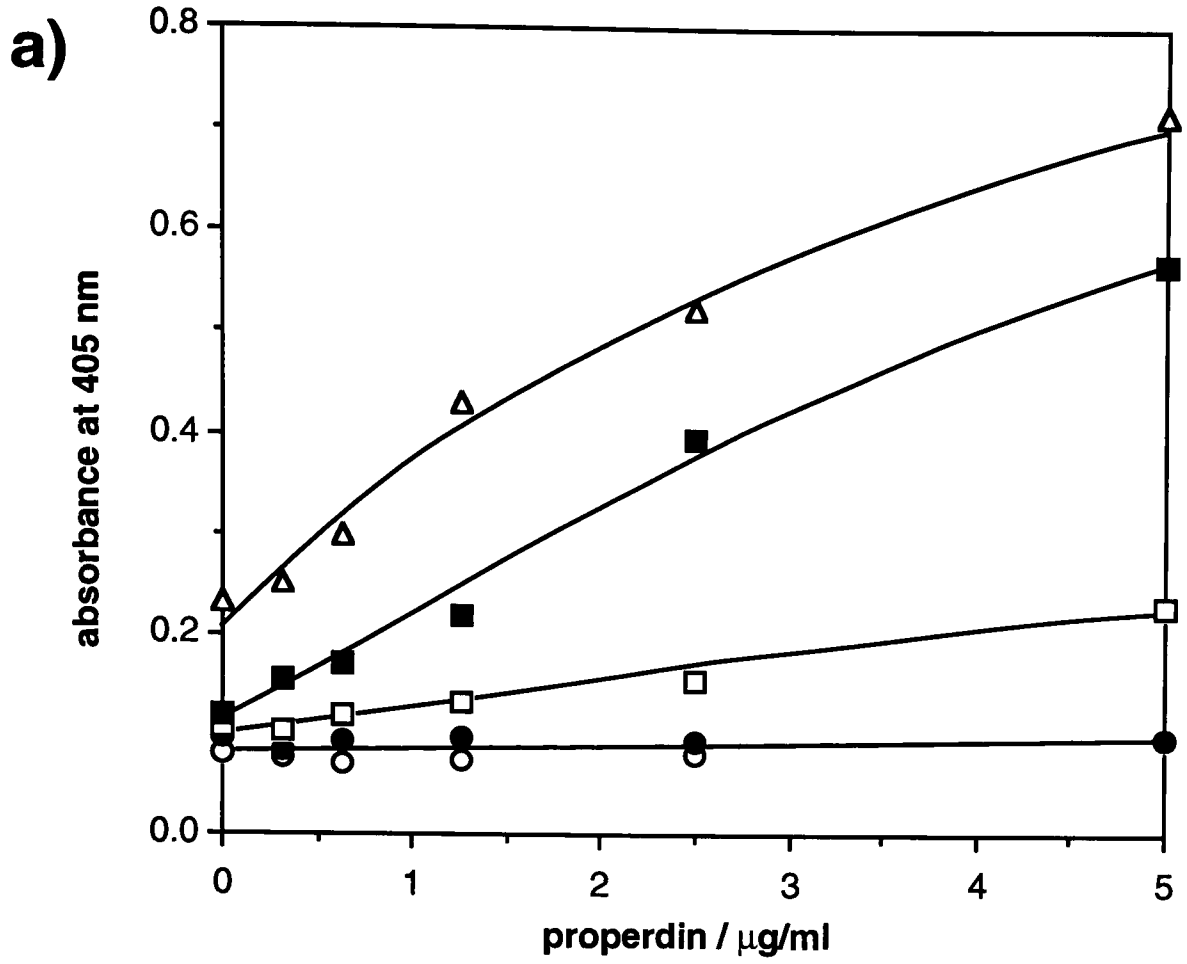
Monoclonal antibodies 5 and 7 inhibit properdin binding to C3b. These antibodies do not bind to TSRs 3,4 or 5 and, although the data suggest that they may bind to TSR6, it can not be excluded formally that they bind to the N-terminus, TSR1 or TSR2 (see chapter 5). Antibody number 6 also strongly inhibits properdin binding to C3b. However, the epitope of this mAb could not be localised further than it being outside TSR3. As expected from direct experiments with the recombinant forms of properdin, none of the inhibitory antibodies bind to TSR3. HYB3-3 (mAb 1) does not inhibit properdin binding to C3b, consistent with its inability to reduce lysis of rabbit erythrocytes *via* the alternative pathway (chapter 6).

7.5 Summary

A solid phase binding assay was used to investigate the binding of normal and mutant forms of properdin to C3b. Binding of normal human properdin to C3b could be detected at physiological ionic strength, and the binding was enhanced at half-physiological salt.

Recombinant wild type properdin was identical to plasma properdin in its ability to bind C3b. In low ionic strength conditions, properdin lacking either TSR3 or TSR4 was able to bind to C3b. Thus it is unlikely that these modules are directly involved in C3b binding. Removal of TSR5 or "nicking" of a loop within TSR5 both abolish the interaction of properdin with C3b, implicating this module in direct interaction with C3b. Properdin lacking TSR6 is unable to bind to C3b in this assay, but it is unclear from these results whether this is due to the removal of important residues, the lack of oligomers, or the inability to detect monomer binding using this assay.

The use of monoclonal antibodies demonstrated that binding of antibody to the N-terminal-TSR1-TSR2 portion of properdin was able to inhibit the interaction of properdin with C3b. Antibodies with epitopes somewhere within the N-terminus, TSR1, TSR2, or TSR6 are also able to prevent properdin binding to C3b.



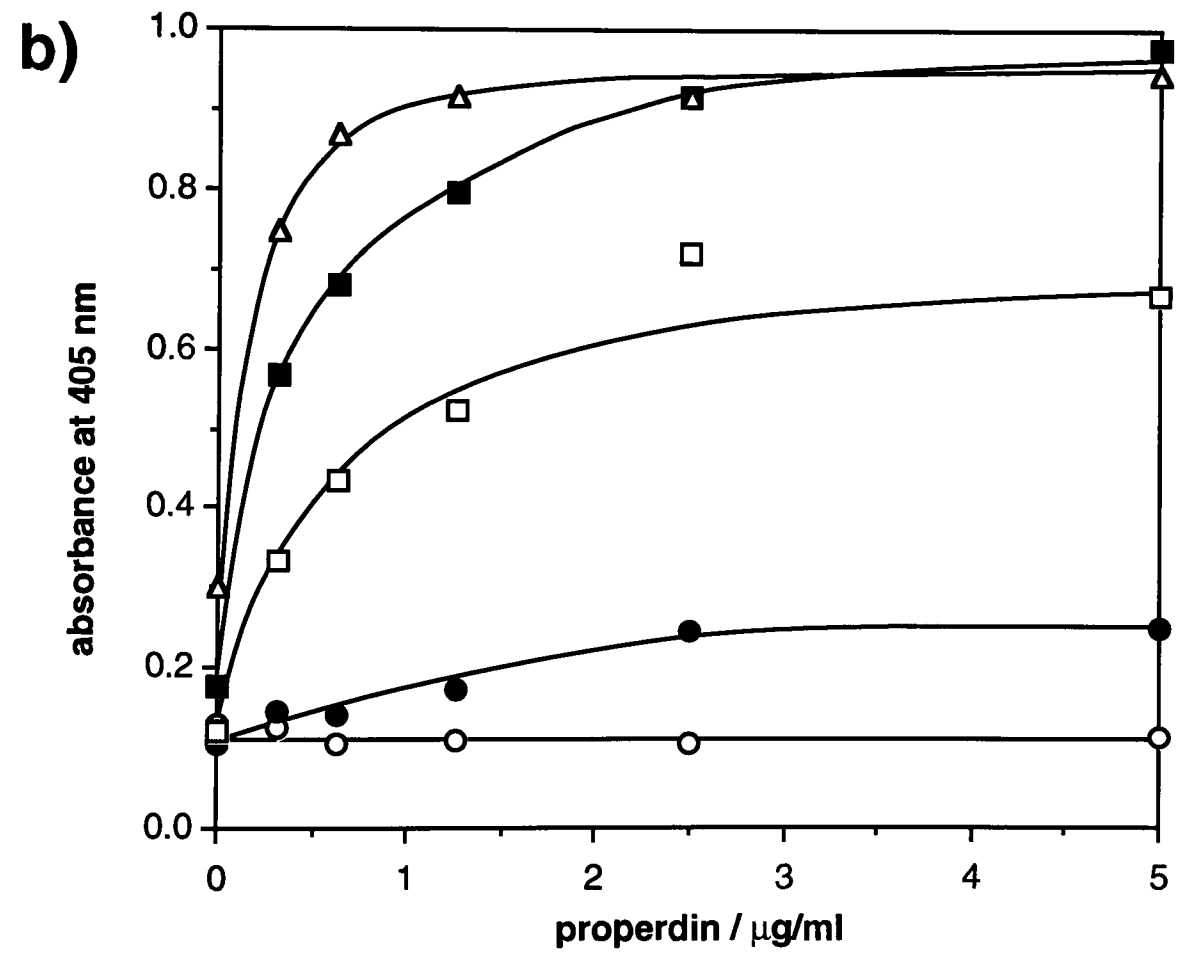
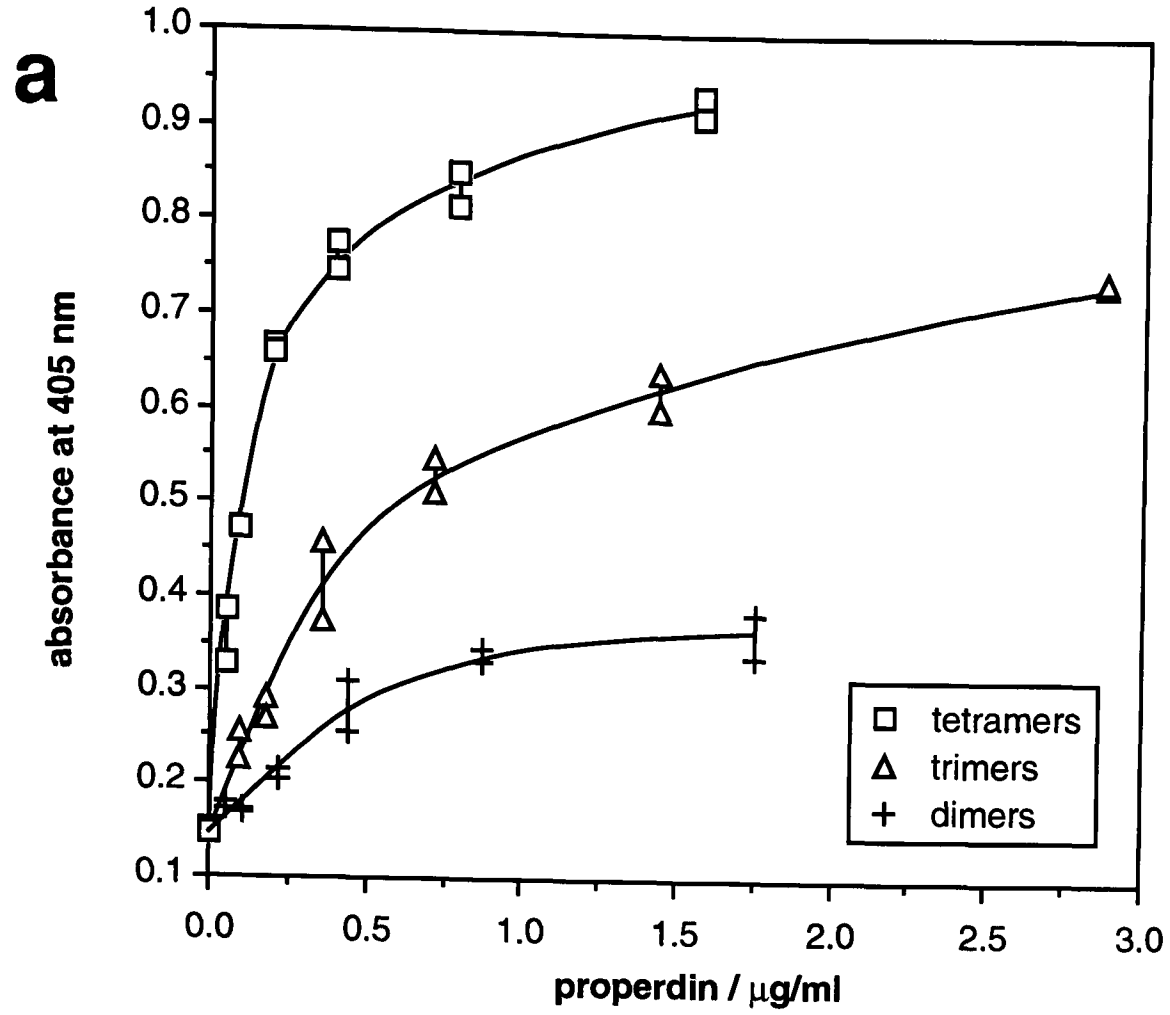


Figure 7.1
Binding of normal human properdin to C3b immobilized on microtitre plate wells.
a) Purified human plasma properdin binding to human C3b at physiological salt strength (150 mM NaCl).
b) Purified human plasma properdin binding to human C3b at half physiological salt strength (75 mM NaCl).
C3b was coated onto the plates at various concentrations:
○ 0.0 µg/well, ● 0.13 µg/well, □ 0.25 µg/well, ■ 0.5 µg/well, △ 2.0 µg/well.



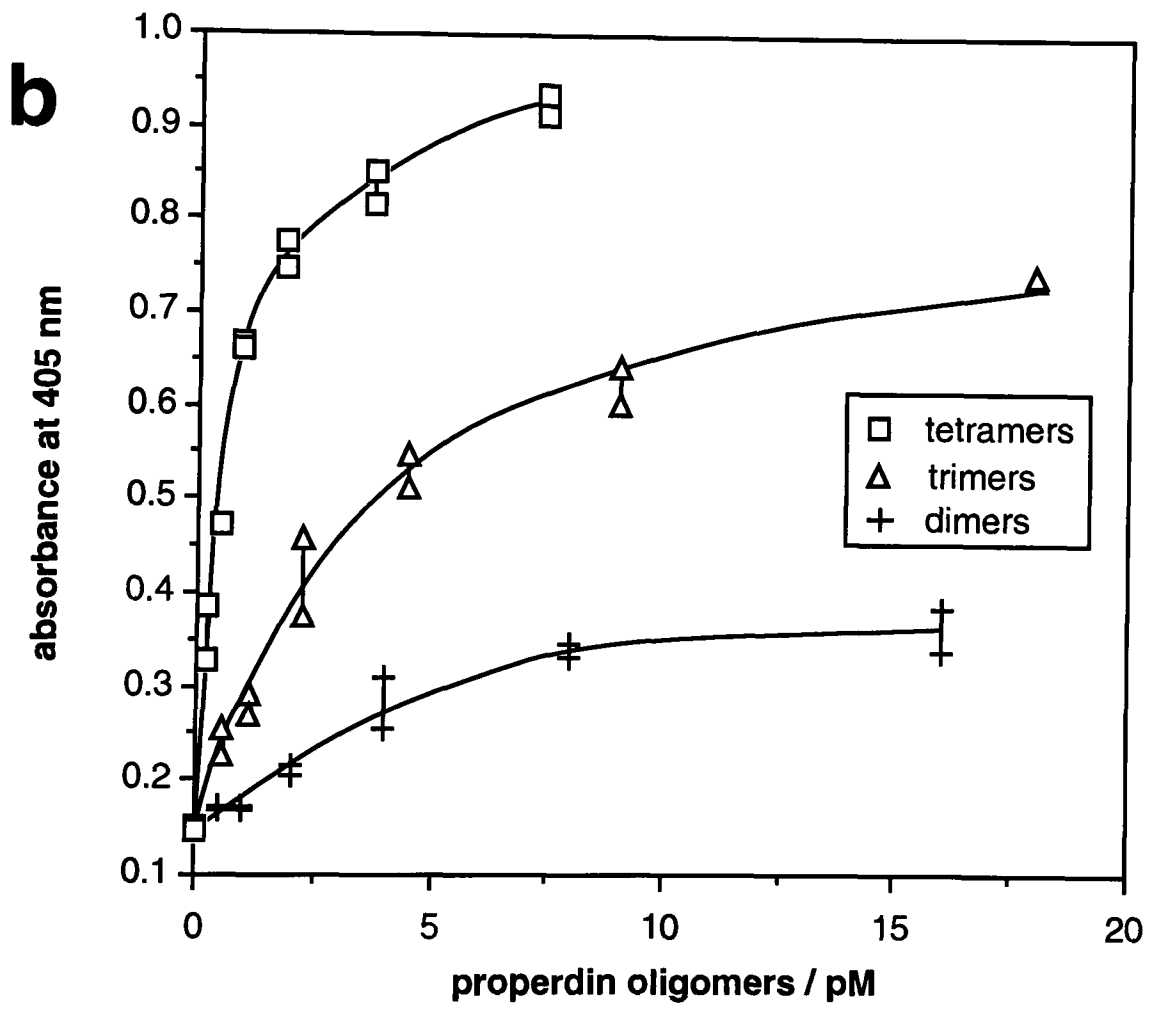


Figure 7.2
Binding of the various oligomeric forms of human properdin to human C3b immobilized on microtitre plate wells. The assay was carried out in half physiological salt strength as described in the text. The results of duplicate experiments are shown.
a) Dose dependency of properdin dimers, trimers and tetramers binding to C3b.
b) Data as in (a), but adjusted for the concentration of each type of oligomer added. (Molecular masses were assumed to be as follows: dimer, 106.6 kDa; trimer, 159.9 kDa; tetramer, 213.2 kDa).

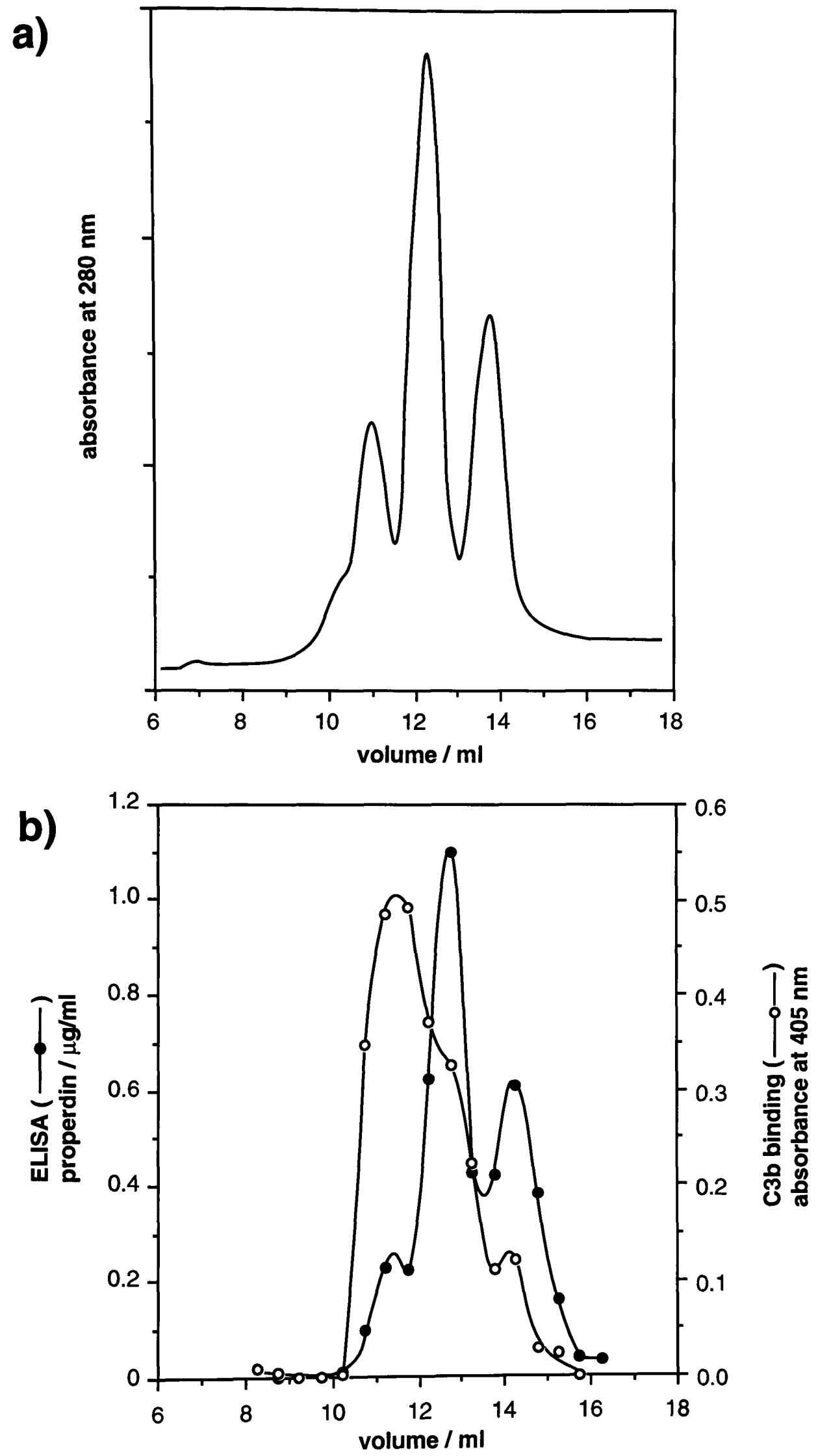


Figure 7.3
C3b binding activity of the various forms of plasma properdin separated by gel exclusion chromatography on Superose 6.
a) The absorbance at 280 nm (arbitrary units).
b) Properdin in the eluted fractions was detected by polyclonal anti-properdin sandwich ELISA. The values shown are the concentrations of properdin present in the C3b binding assay. The C3b binding activity of each fraction was also determined using the microtitre plate C3b binding assay. In both cases, the average of duplicate experiments is shown.

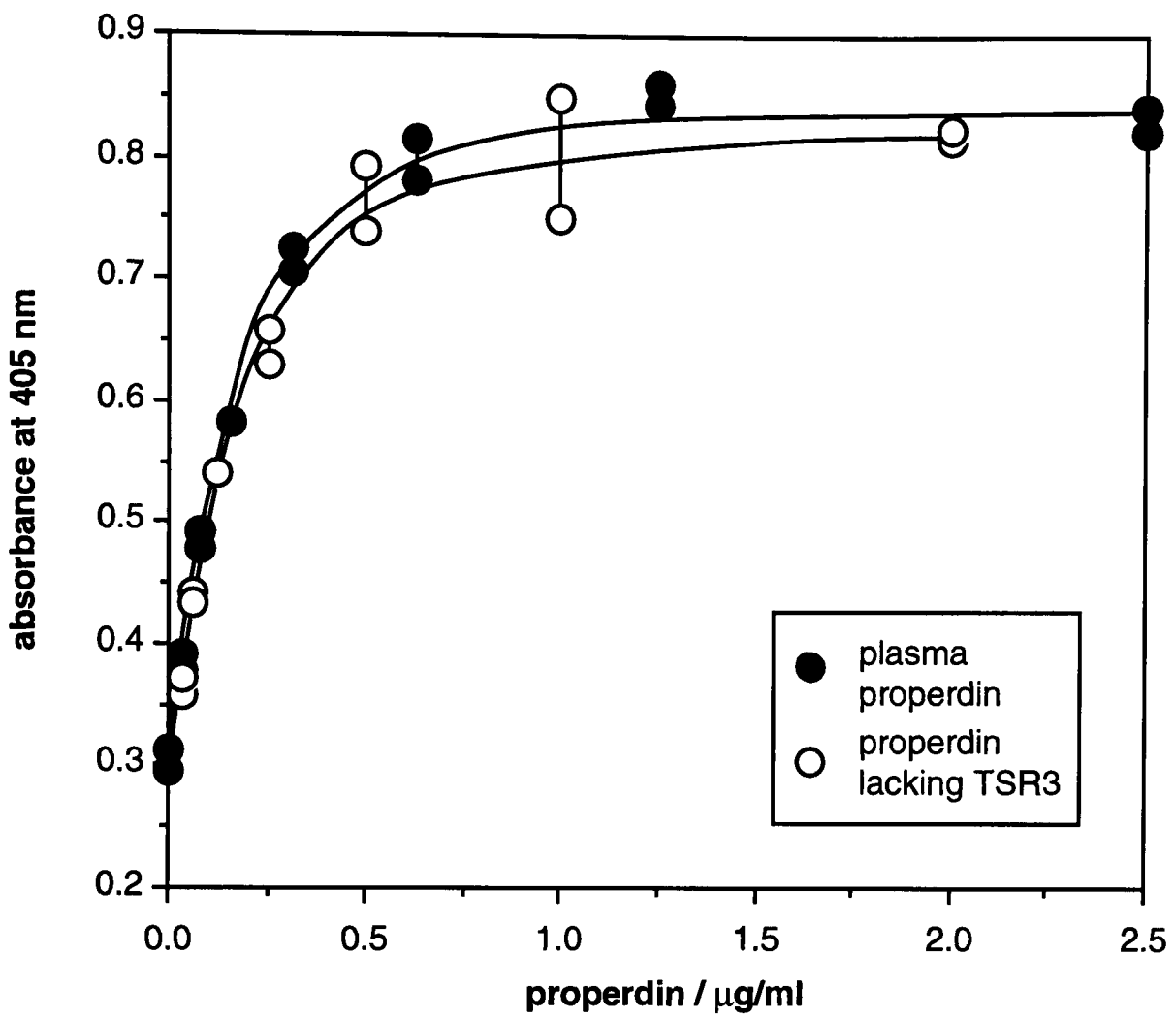
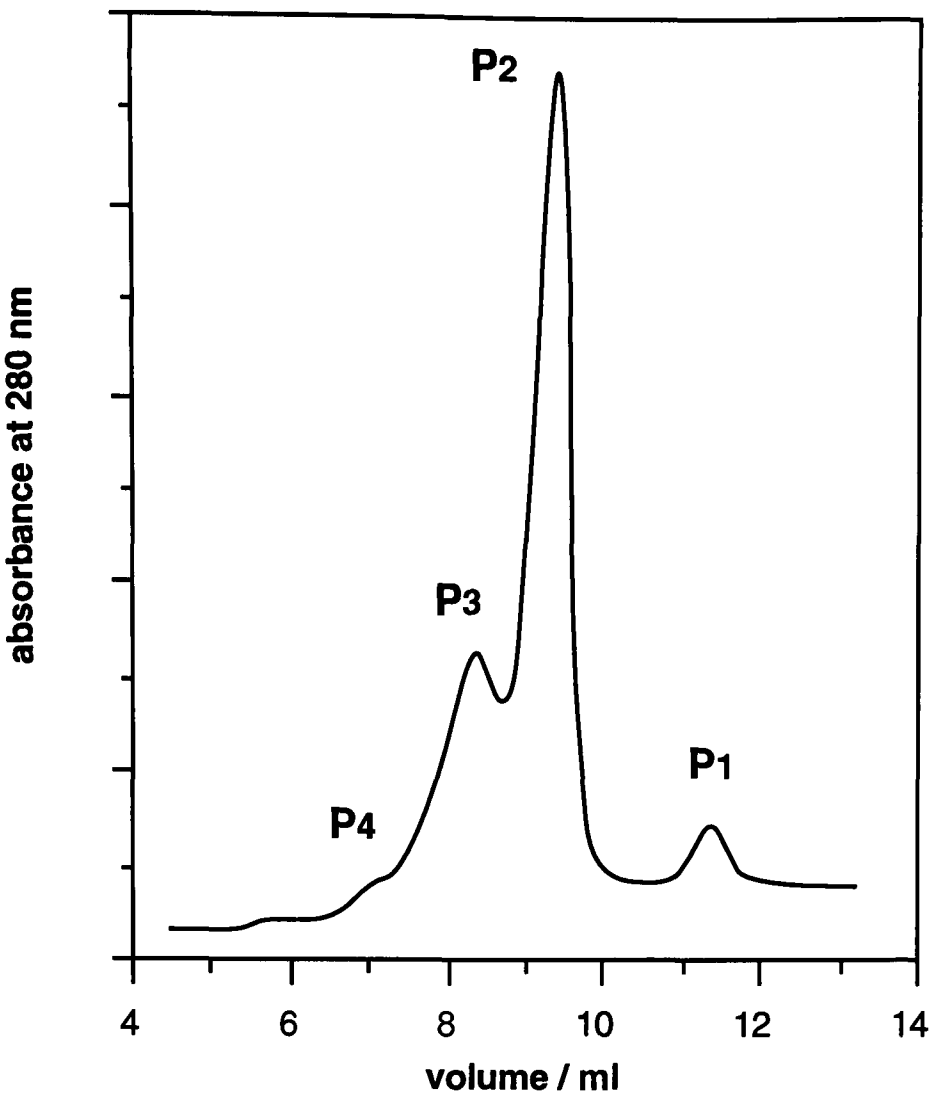


Figure 7.4
Binding of recombinant human properdin lacking TSR3 to human C3b immobilized on microtitre plate wells. The binding of normal human plasma properdin is also shown. The assay was carried out in half physiological salt strength, as described in the text. The results of duplicate experiments are shown.



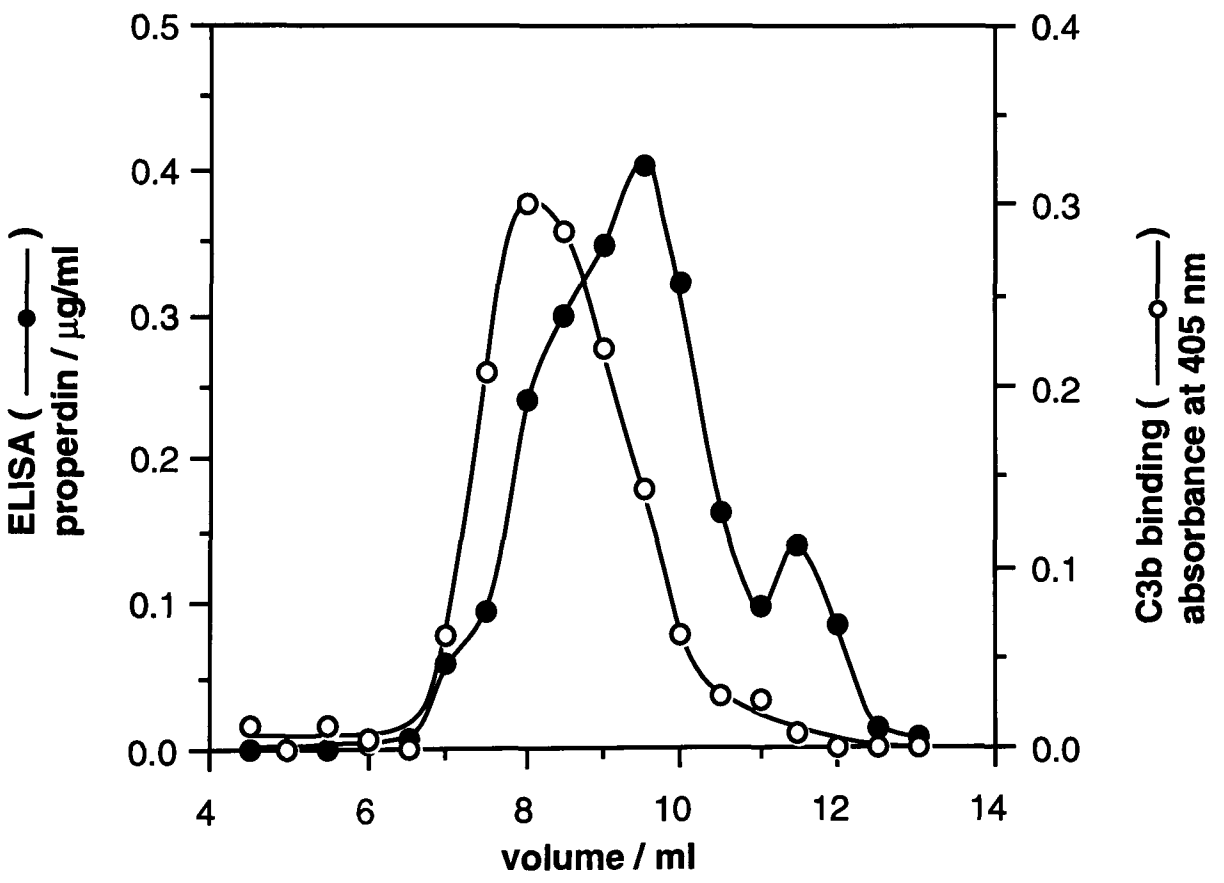


Figure 7.5
C3b binding activity of the various oligomeric forms of recombinant properdin lacking TSR3 separated by gel exclusion chromatography on Superose 12.
a) The absorbance profile at 280 nm (arbitrary units).
b) Properdin in the eluted fractions was detected by polyclonal anti-properdin sandwich ELISA. The values shown are the concentrations of properdin present in the C3b binding assay. The C3b binding activity of each fraction was also determined using the microtitre plate C3b binding assay. In both cases, the average of duplicate experiments is shown.

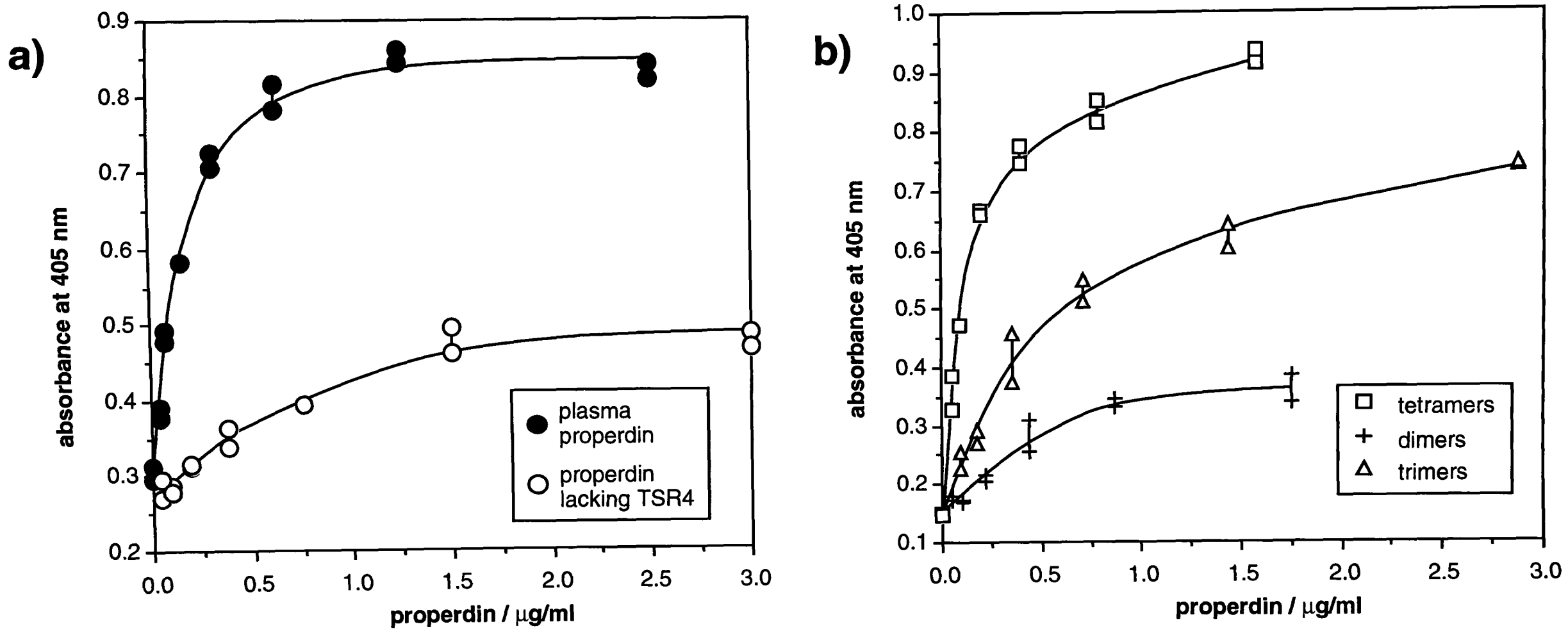


Figure 7.6
Binding of recombinant human properdin lacking TSR4 to human C3b immobilized on microtitre plates.
The binding of properdin lacking TSR4 is compared to that for (a) whole plasma properdin, (b) the various oligomeric forms of plasma properdin. The preparation of properdin lacking TSR4 contains only dimers and monomers. The binding to C3b seen is similar to that found for normal properdin dimers. The results of duplicate experiments are shown.

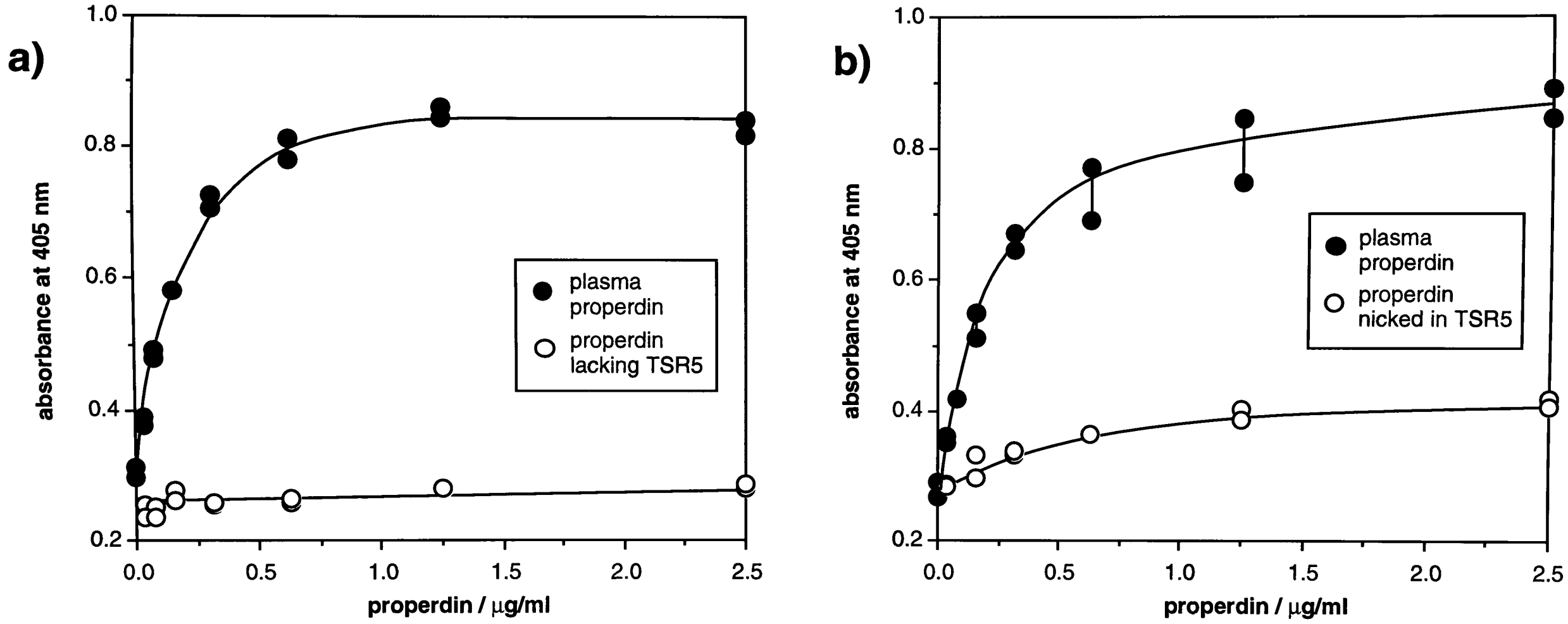


Figure 7.7

Binding of recombinant properdin lacking TSR5, and properdin "nicked" in TSR5, to C3b immobililized to microtitre plate wells. The results of duplicate experiments, carried out at half-physiological ionic strength, are shown.

a) The binding of recombinant human properdin lacking TSR5 compared to normal human plasma properdin.

b) The binding of human plasma properdin "nicked" in TSR5 by trypsin treatment, compared to normal human properdin.

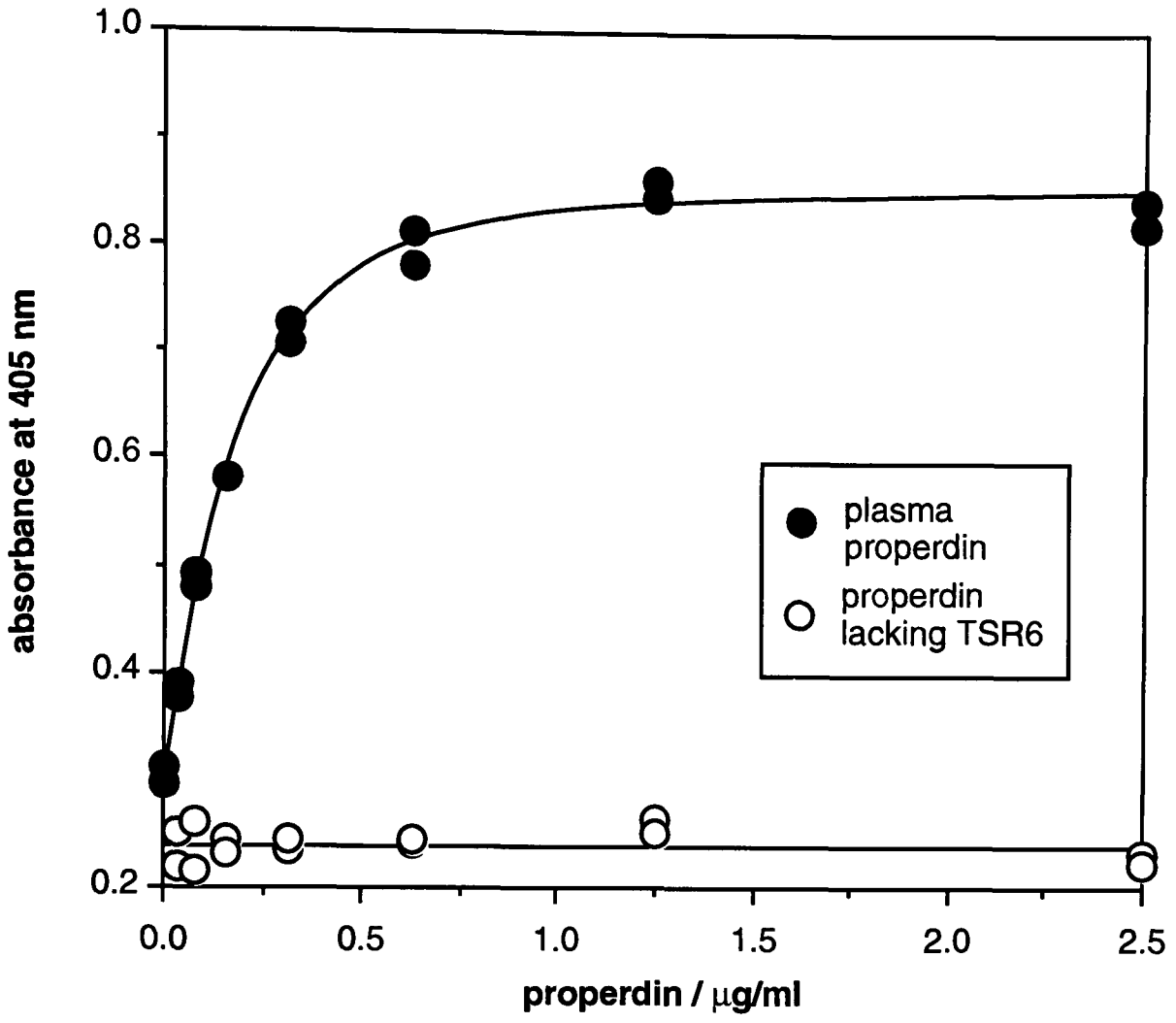
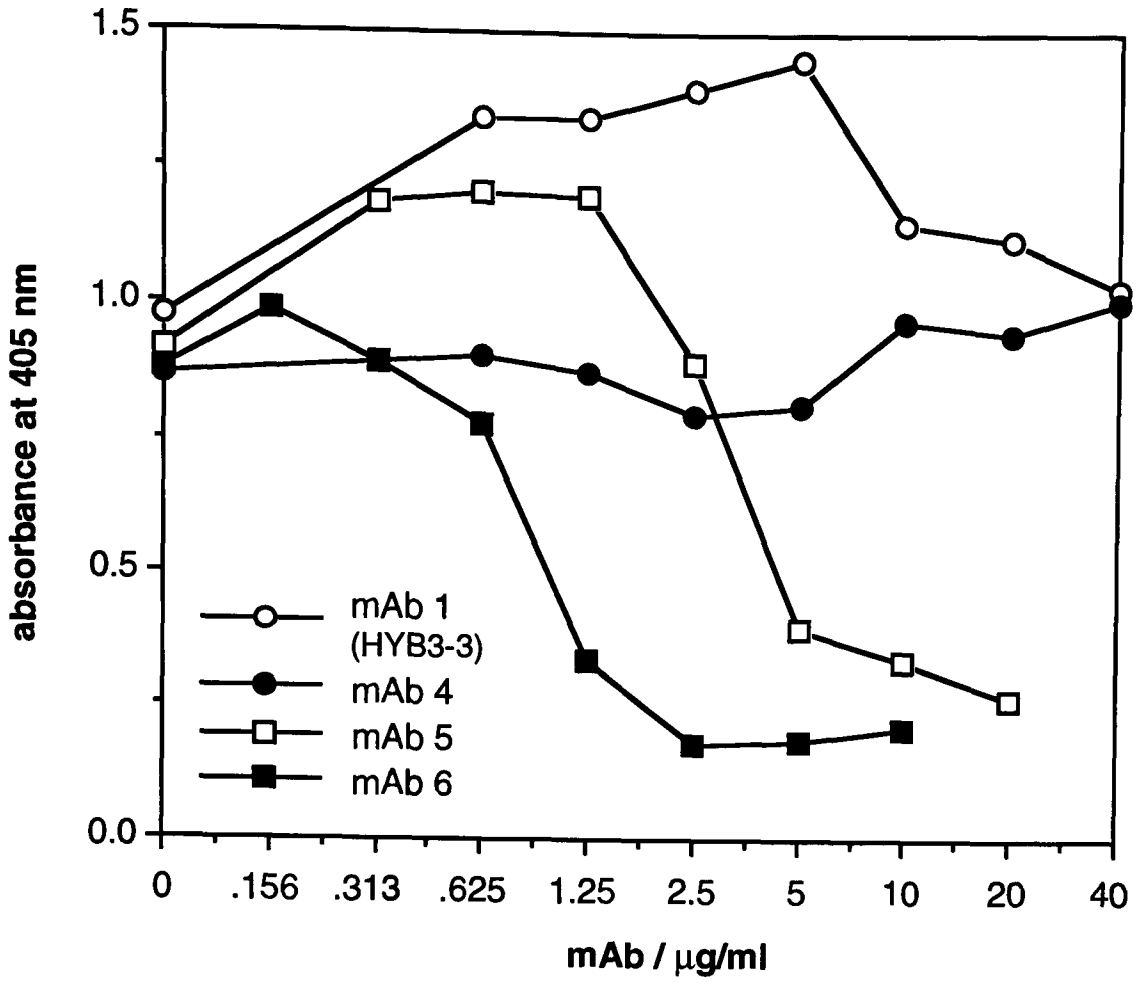


Figure 7.8
The binding of recombinant human properdin lacking TSR6 to human C3b immobilized on microtitre plates wells, compared to that for purified normal human plasma properdin. The results of duplicate experiments are shown.



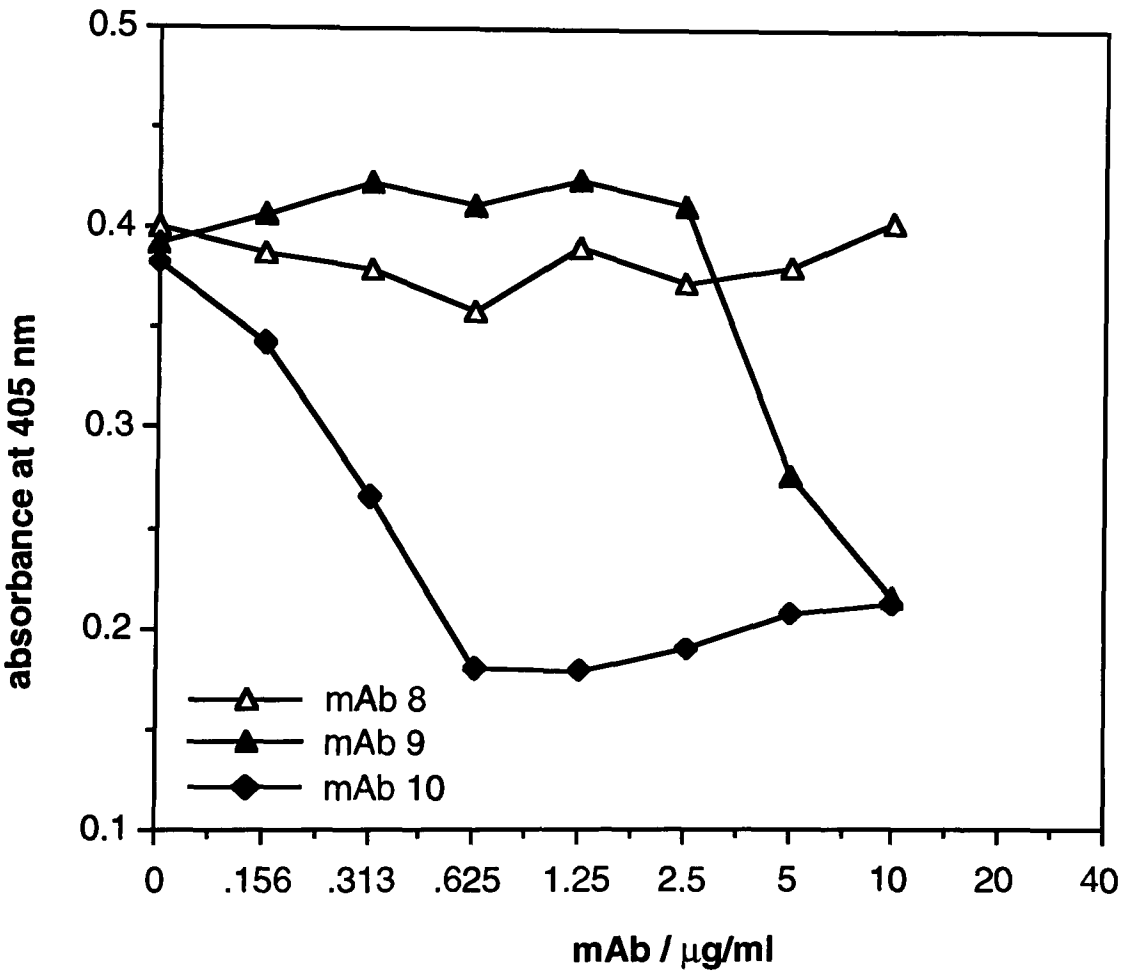


Figure 7.9
Inhibition of properdin binding to C3b by anti-properdin monoclonal antibodies.
Microtitre plates were coated with C3 at 0.5 µg/well, and then blocked with 2% milk powder. Serial dilutions of the various mAbs which had been pre-incubated with a 1/40 dilution of normal human plasma were then applied. Bound properdin was detected with goat anti-properdin polyclonal antibody, and peroxidase conjugated rabbit anti-goat antibody. The assays were carried out by Ms Fabianna Filippoussis and Dr John D. Lambris, University of Pennsylvania.

Chapter 8

Chapter 8

The binding of altered forms of properdin to sulphatide

Properdin has been reported to bind to sulphated glycoconjugates (Wilson *et al.*, 1984; Holt *et al.*, 1990; Jennemann *et al.*, 1994), although the role of this activity *in vivo* is unknown (see chapter 1, sections 1.8.3 and 1.11). The TSRs of properdin contain motifs which have been implicated in sulphated polysaccharide binding in the related molecules thrombospondin and circumsporozoite protein (see chapter 1, sections 1.12 and 1.13.1). The various altered forms of properdin were tested for their ability to bind sulphatide (galactose(-3-sulphate)β1-1 ceramide) in a microtitre plate binding assay. Some of the preliminary assays described in this chapter were carried out by Ms Laura Le Gré, an undergraduate working in the MRC Immunochemistry Unit. These experiments are acknowledged where appropriate.

8.1 The sulphatide binding assay

Properdin has been reported to bind specifically to the sulphated glycolipid, sulphatide, on microtitre plates. These experiments were repeated to determine if the assay could be used to examine the sulphated glycoconjugate binding capacity of the altered forms of properdin.

The microtitre plate assay was carried out essentially as described by Roberts (1987) and Holt *et al.* (1990). Bovine brain sulphatide was obtained from Sigma. Briefly, sulphatide in methanol was coated onto the wells of microtitre plates by evaporation. After blocking with 1% BSA/PBS, samples were applied to the wells in the presence of 1% BSA. Bound properdin was detected using biotinylated rabbit anti-properdin polyclonal IgG, and alkaline phosphatase conjugated extravidin (see materials and methods).

The dose dependency of properdin binding to various quantities of sulphatide was tested (figure 8.1). In the absence of sulphatide, no binding of properdin was detected. At increasing concentrations of coated sulphatide, clear dose dependent and saturable binding of properdin at physiological ionic strength was seen. No increase in the properdin binding was observed above a coating concentration of 30 $\mu g\ ml^{-1}$ sulphatide (i.e. 3.8 $\mu g\ well^{-1}$), suggesting that coating with this quantity of lipid saturated the well. This agrees well with the maximum quantity of sulphatide suggested for microtitre plate well coating by Roberts of approximately 20 $\mu g\ ml^{-1}$ (Roberts, 1987). In subsequent assays, coating of lipids was carried out at 30 $\mu g\ ml^{-1}$ (4 $\mu g\ well^{-1}$) unless otherwise stated.

8.2 Cation dependency of sulphatide binding

The properdin binding assays of Holt *et al.* (1990) were carried out in buffer containing 1 mM $CaCl_2$, although the cation dependency of the interaction was not discussed. In the experiment described in section 1.1, properdin binding to sulphatide could clearly be detected in the absence of added calcium. The binding assay was repeated in the presence of 5 mM EDTA (figure 8.2). Only a small decrease in binding was observed.

When 1 mM $CaCl_2$ was included throughout the assay procedure, the apparent binding of properdin increased approximately two fold. However, when calcium was absent only during the properdin binding step, but present during the subsequent washing and detection steps, no difference was seen from the binding observed when calcium was present throughout (assays carried out by Laura Le Gré). Thus the apparent increase in properdin binding in the presence of calcium could be attributed to effects on other steps of the assay. Properdin binding to sulphatide is calcium-independent. Jennemann *et al.* (1994) have also recently reported that the absence of calcium in a similar assay "resulted in only marginal alterations of properdin binding".

8.3 The specificity of properdin for sulphatide

The specificity of properdin for binding of sulphatide was checked by coating other lipids or steroids on microtitre plates by the same method (see materials and methods). Purified plasma properdin or recombinant wild type properdin did not bind to galactocerebroside[1] type I or type II, or to phosphatidyl serine (figure 8.3). In some assays, a very low level of binding to cholesterol-3-sulphate was observed (see figure 8.3b).

However, since essentially no binding of properdin to these lipids could be detected, it was important to determine if the lipids were indeed immobilized on the plates. To check this, the lipids (10 μg $well^{-1}$) were evaporated onto microtitre plate wells using the normal procedure, and subsequently blocked with 1% BSA/PBS before a mock binding assay was performed. This involved the same incubation and washing steps as the usual assay, but without the addition of properdin or antibodies. After the last wash, the wells were dried in air, and any lipid remaining in the well was eluted with methanol. These samples were then analysed by thin layer chromatography in chloroform/methanol/water (60/35/7) and compared to standard quantities of the appropriate lipid run on the same chromatogram (see materials and methods). This

[1] galactocerebrosides from bovine brain were obtained from Sigma. Type I contains 98% α-hydroxy fatty acids. Type II contains 98% non-hydroxy fatty acids.

procedure was carried out by Laura Le Gré. All the lipids migrated as single bands, suggesting that the preparations were pure. In all cases, material that co-migrated with the standard could be eluted from the plate (not shown). The order of binding efficiency of the lipids to the wells was found to be galactocerebroside type II > galactocerebroside type I > cholesterol-3-sulphate > phosphatidyl serine > sulphatide. Since all the lipids tested bound to the microtitre plates to a greater extent than sulphatide, the specificity of properdin for sulphatide was confirmed. Properdin fails to bind to galactocerebroside, which has the same structure as sulphatide, but lacks the sulphate group, suggesting that this sulphate group is important for binding. Also, properdin does not bind to the anionic compounds cholesterol-3-sulphate and phosphatidyl serine, indicating that the binding is not simply a non-specific electrostatic interaction. These results confirm and extend the findings of Holt *et al.* (1990).

8.4 The binding of properdin in normal human plasma to sulphatide

The ability of properdin within normal human plasma to bind sulphatide was tested. Serial dilutions of plasma were applied to sulphatide coated microtitre plate wells, and bound properdin was detected as previously described (assay conducted by Laura Le Gré). Clear dose dependent binding of properdin is observed (figure 8.4). This is unlikely to be due to complement activation since the presence of EDTA will prevent the classical, lectin and alternative pathways from depositing C3b onto the surface. Although the presence of EDTA may also inhibit competing cation-dependent sulphatide binding proteins in the plasma, the result indicates that the binding of properdin to sulphated glycoconjugates could occur in physiological situations.

8.5 The binding of the various oligomeric forms of properdin to sulphatide

The binding of the various oligomeric forms of properdin to sulphatide has not previously been examined. As with the C3b binding assay described in chapter 7, it was important to determine the responses of the different species of properdin in the assay so that comparisons with preparations of the mutant proteins could be made. Dimers, trimers, and tetramers of normal plasma properdin were separated by gel exclusion chromatography on Superose 6 or Superose 12. The binding of these different species to sulphatide is shown in figures 8.5 and 8.6. The results obtained are similar to those obtained for C3b binding (chapter 7). As discussed in chapter 7, the interpretation of the differences between the binding of the various oligomers is complex. However, saturation of binding occurs at a lower molar concentration of properdin tetramers than of

trimers or dimers (figure 8.5), suggesting that higher oligomeric forms have greater avidity for sulphatide than the lower forms in this assay. A necessity for multivalency for high avidity binding to sulphatide has previously been reported for the related circumsporozoite protein of *Plasmodium*. Only disulphide-linked multimers of recombinant CS protein or covalently coupled peptides derived from the TSR of the same protein will bind to sulphatide, or to the sulphated polysaccharides on liver cells (Cerami *et al.*, 1992a, b; Sinnis *et al.*, 1994) (see chapter 1, section 1.13.1).

8.6 The binding of altered forms of properdin to sulphatide

The binding of purified preparations of recombinant forms of properdin to sulphatide was investigated. The binding of recombinant wild type properdin was identical to that of purified plasma properdin (see chapter 4). Recombinant properdin also bound specifically to sulphatide (figure 8.3).

Properdin lacking TSR3 retained sulphatide binding activity (figure 8.7). Indeed, this mutant appears to have increased affinity for sulphatide compared to normal properdin, although the interpretation of such differences is difficult due to the altered distribution of oligomers found in this preparation. The binding of the various oligomers of properdin without TSR3, separated by gel exclusion chromatography, shows the shift in apparent binding activity to higher oligomeric forms typical of normal properdin (*cf.* figures 8.6 and 8.8). All oligomeric fractions of properdin lacking TSR3 in this assay show higher specific binding to sulphatide than similar fractions of normal properdin (*cf.* figures 8.6 and 8.8). However, careful quantitative direct binding assays will be required to confirm this observation. The probable monomeric fraction of properdin lacking TSR3 does not bind to sulphatide. Properdin without TSR3 retains its specificity for sulphatide binding. The mutant does not bind to galactocerebroside, cholesterol-3-sulphate, phosphatidyl serine, or uncoated wells (figure 8.9).

Properdin lacking TSR4 was also able to bind to sulphatide, although the apparent binding observed was less than that for normal properdin (figure 8.10a). However, since the preparation of properdin lacking TSR4 contained only monomers and dimers, it was important to compare the binding observed with that of dimers, trimers, and tetramers of normal properdin (figure 8.10b). It is clear that the response obtained for the mutant protein is similar to that expected for a preparation containing predominantly dimeric properdin. Thus it was concluded that dimeric properdin lacking TSR4 did not show diminished sulphatide binding activity compared with normal properdin dimers. The binding of properdin without TSR4 was also specific for sulphatide. No binding was found to galactocerebroside, cholesterol-3-sulphate, phosphatidyl serine, or uncoated wells (figure 8.11).

Purified recombinant properdin lacking TSR5 did not bind to sulphatide, despite an oligomerization profile similar to that of properdin lacking TSR4 (figure 8.12a). Similarly, no binding of this mutant in serum-free medium prior to purification was detectable (not shown). However, the tryptic "nicking" of a bond within TSR5 did not alter the ability of properdin to bind sulphatide (figure 8.12b). This is in contrast to the results obtained for C3b binding, where both the deletion of TSR5, and "nicking" of the exposed loop within TSR5, caused the loss of activity. So, it is likely that, although TSR5 may be important for both C3b and sulphatide binding, the binding sites are not identical.

Partially purified properdin lacking TSR6 and the C-terminal charged tail (figure 8.13), or the same protein in serum-free medium before purification (not shown) did not bind to sulphatide. It is not clear from these results if this is due to the removal of residues directly important for sulphatide binding, or due to the failure of this form of properdin to oligomerize.

8.7 Inhibition of sulphatide binding by the HYB3-3 monoclonal antibody

The ability of the HYB3-3 anti-properdin monoclonal antibody to inhibit properdin binding to sulphatide was tested. HYB3-3 is a mouse IgG1, kappa antibody. An isotype matched control mAb (mouse myeloma protein MOPC21) was obtained from Sigma. The similar concentration and purity of the two antibodies was confirmed by SDS-PAGE (not shown).

Serial dilutions of each antibody were included during the binding of 0.5 $\mu g\ ml^{-1}$ purified plasma properdin to sulphatide in 1% BSA/PBS. Dose dependent inhibition of binding was seen with the HYB3-3 mAb, but not with the control antibody (figure 8.14a). Fifty percent inhibition was obtained with approximately ten-fold molar excess of HYB3-3 over properdin. Similar results were seen when the binding of properdin lacking TSR3 was examined (figure 8.14b). HYB3-3 is thought to bind to TSR4 (chapter 5). As expected, HYB3-3 was no more effective than the control mAb in inhibiting the binding of properdin lacking TSR4 to sulphatide (not shown). Roberts and others have observed low affinity non-specific binding of several mouse IgG and IgM monoclonal antibodies to sulphatide and some phospholipids (Roberts, 1987). This may explain the marginal inhibition of sulphatide binding obtained with high doses of the control mAb in these studies.

The HYB3-3 antibody does not inhibit the binding of properdin to C3b (chapter 7) nor the stabilization of the C3bBb complex in an alternative pathway haemolytic assay (chapter 6). Thus, this monoclonal antibody distinguishes between the C3b/Factor B and sulphatide binding activities of properdin.

8.8 Summary

Clear dose dependent and saturable binding of properdin to sulphatide on microtitre plate wells was observed. The binding is specific, since similar interactions were not observed with galactocerebroside, cholesterol-3-sulphate or phosphatidyl serine, despite the presence of greater quantities of these compounds. Properdin binding to sulphatide is calcium-independent, and can be observed in whole human plasma. Tetramers of properdin have higher avidity for sulphatide than lower oligomers. The HYB3-3 anti-properdin monoclonal antibody inhibits the binding of properdin to sulphatide.

Recombinant forms of properdin lacking TSR3 or TSR4 retain sulphatide binding activity. "Nicking" of a single exposed loop within TSR5 also has no effect on sulphatide binding. However, properdin lacking TSR5 altogether is unable to bind sulphatide. Properdin without TSR6 does not bind sulphatide. It is not known if this is due to the lack of oligomers of this form of properdin, or due to a direct requirement for residues within TSR6 for binding.

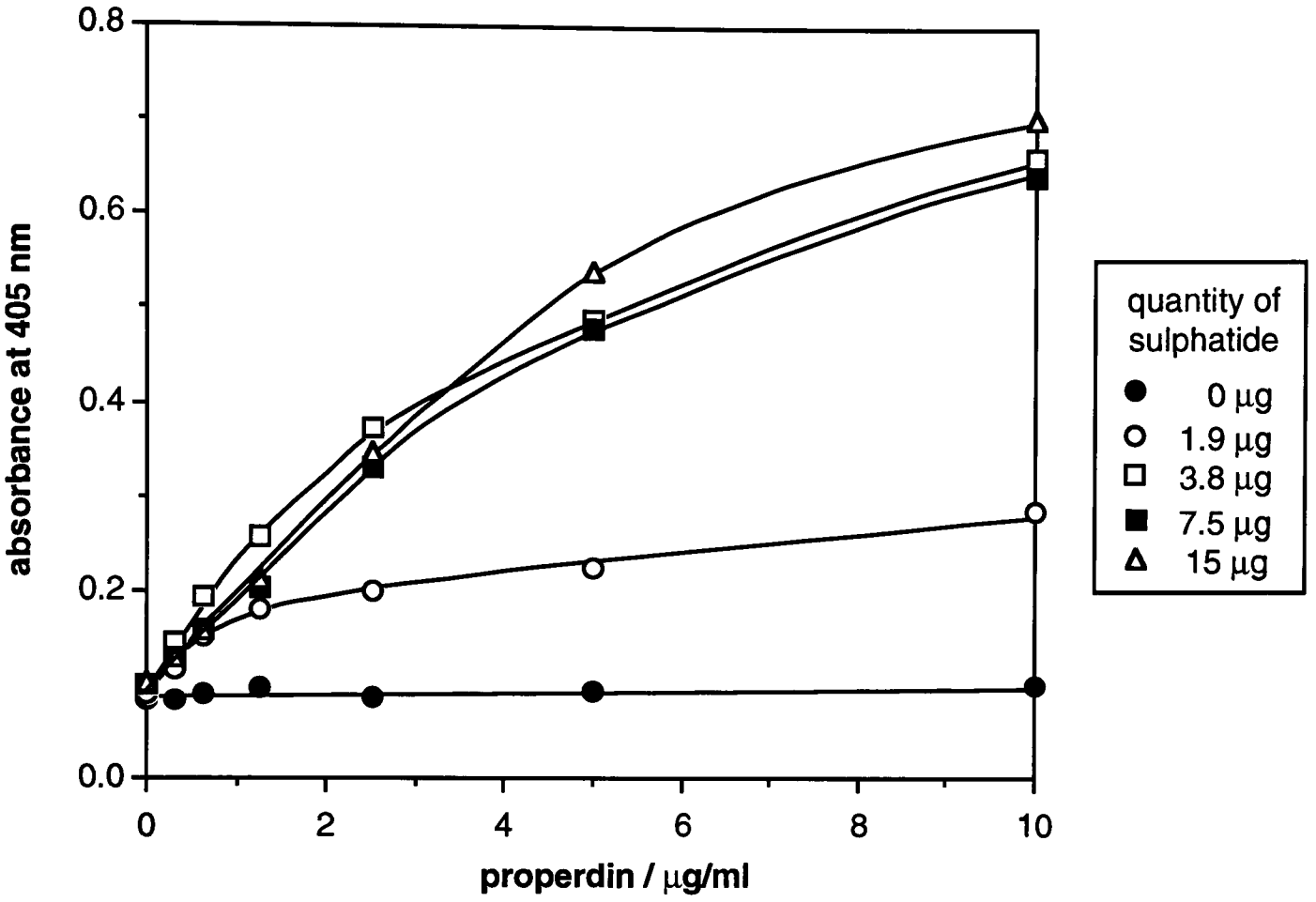


Figure 8.1

Binding of purified human plasma properdin to different quantities of sulphatide.

Microtitre plate wells were coated, by evaporation, with sulphatide dissolved in 150 µl methanol. The assay was carried out as described in materials and methods. The averages of duplicate or triplicate experiments are shown. The maximum range of duplicates was 0.03 absorbance units.

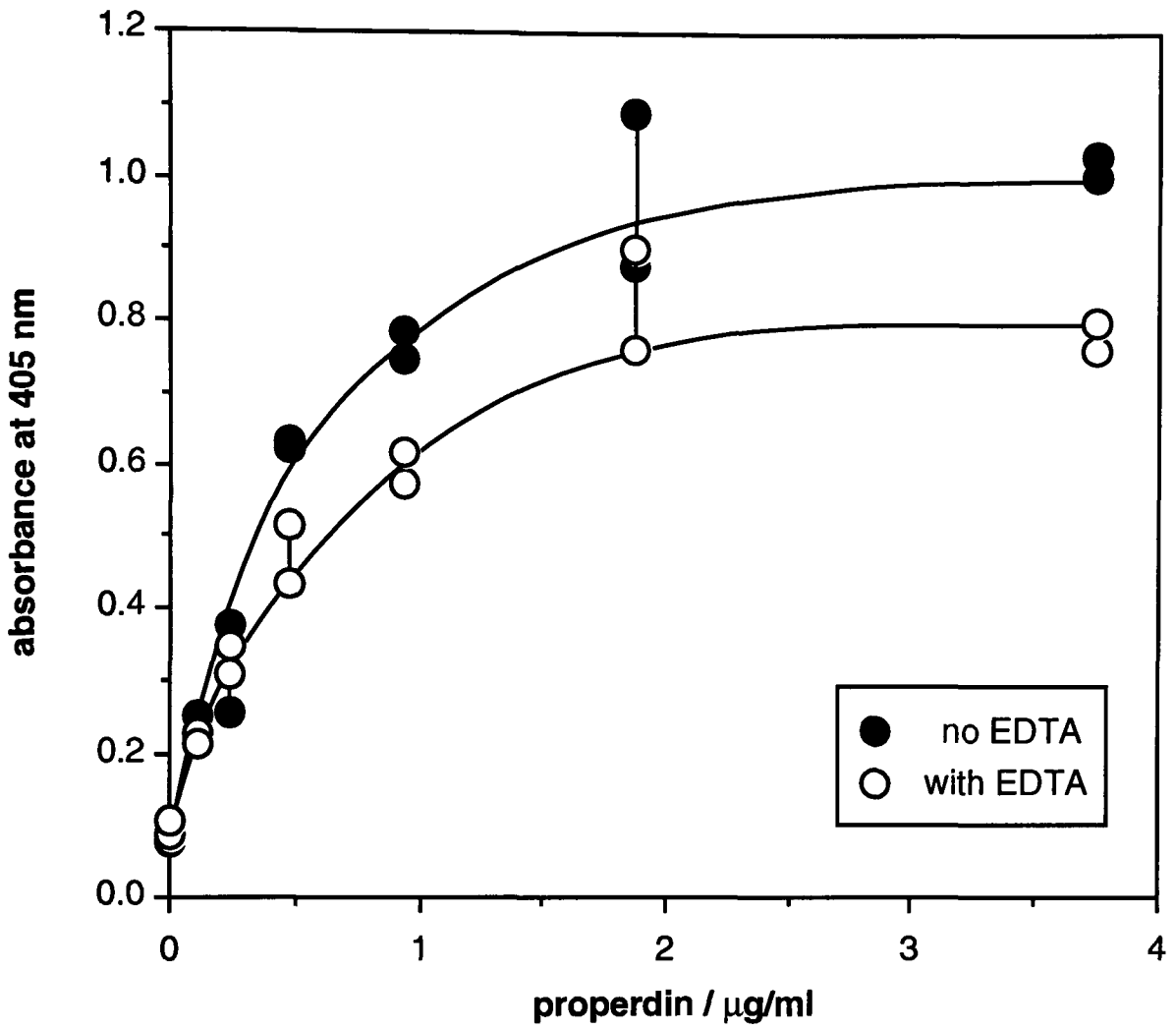
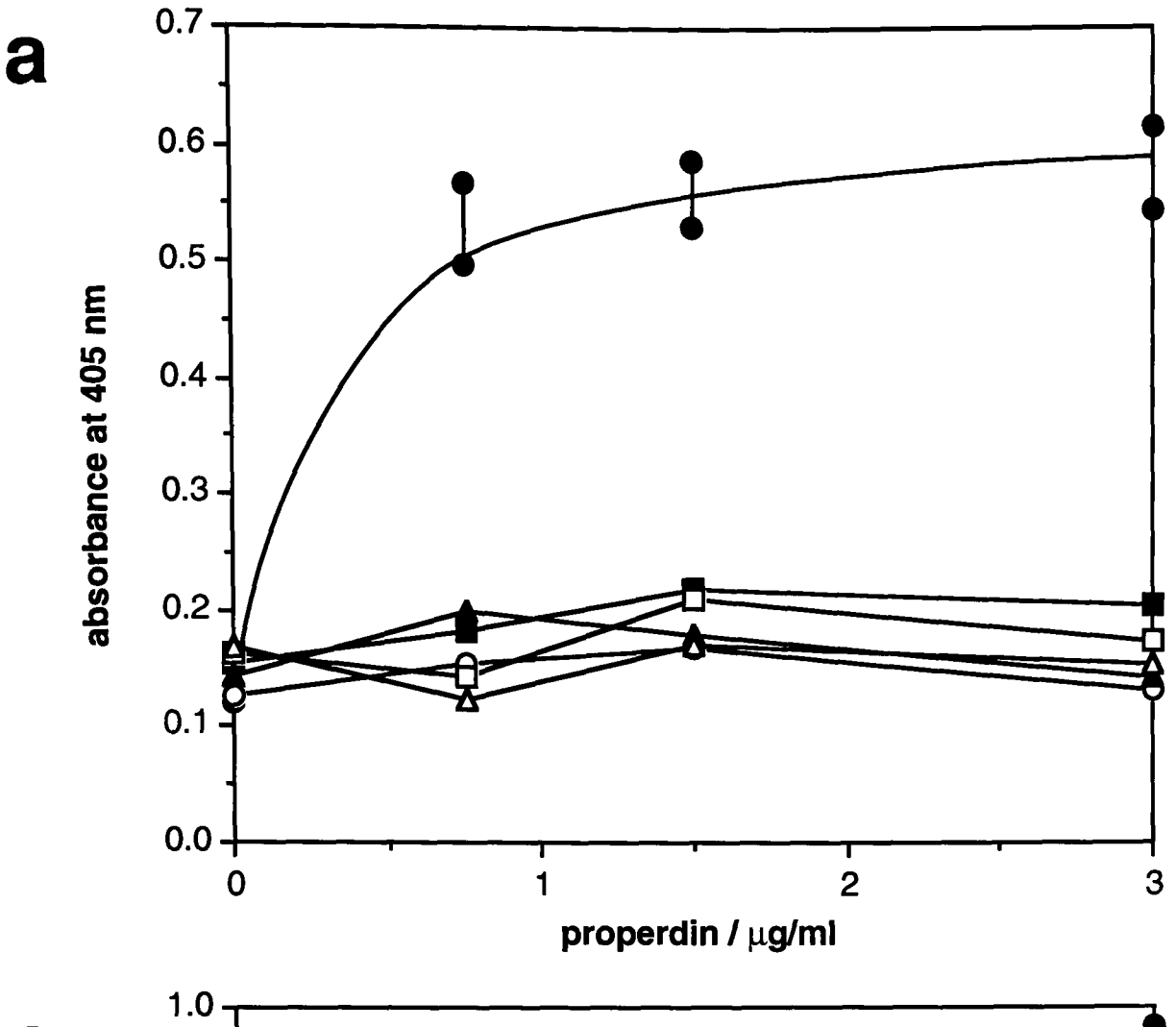


Figure 8.2
Binding of purified human plasma properdin to sulphatide in the presence and absence of 5 mM EDTA.
Microtitre plate wells were coated, by evaporation, with 4 µg/well sulphatide dissolved in methanol. The results of duplicate experiments are shown.



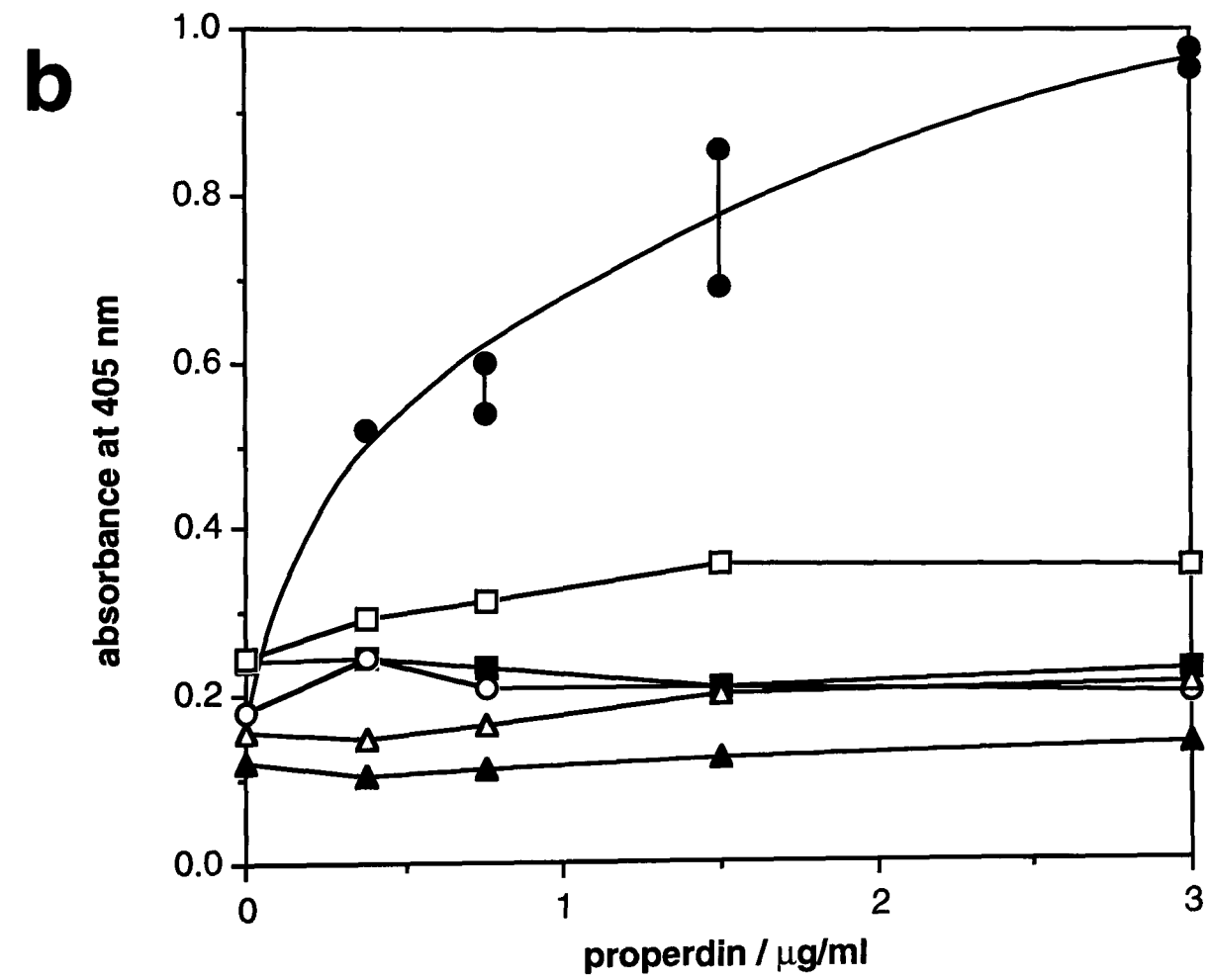


Figure 8.3
Binding of properdin to various lipids coated on microtitre plate wells. Lipids were dissolved in methanol at 30 μg/ml. Wells were coated by evaporation using 150 μl of each lipid solution. For binding to sulphatide, the results of duplicate experiments are shown. For clarity, the averages of duplicate experiments are shown for binding to the other lipids.
a) Binding of purified plasma properdin.
b) Binding of purified normal recombinant properdin.

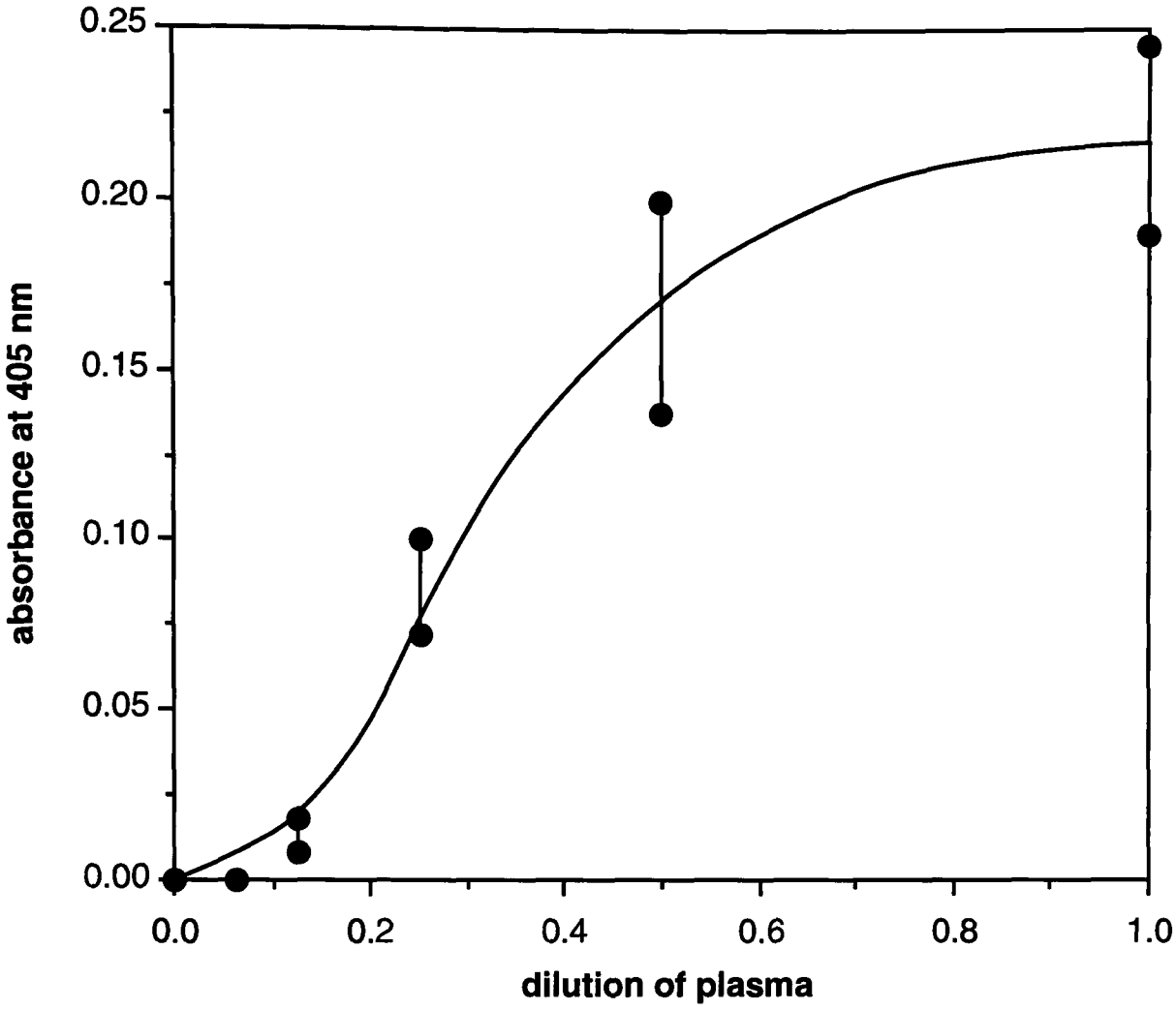
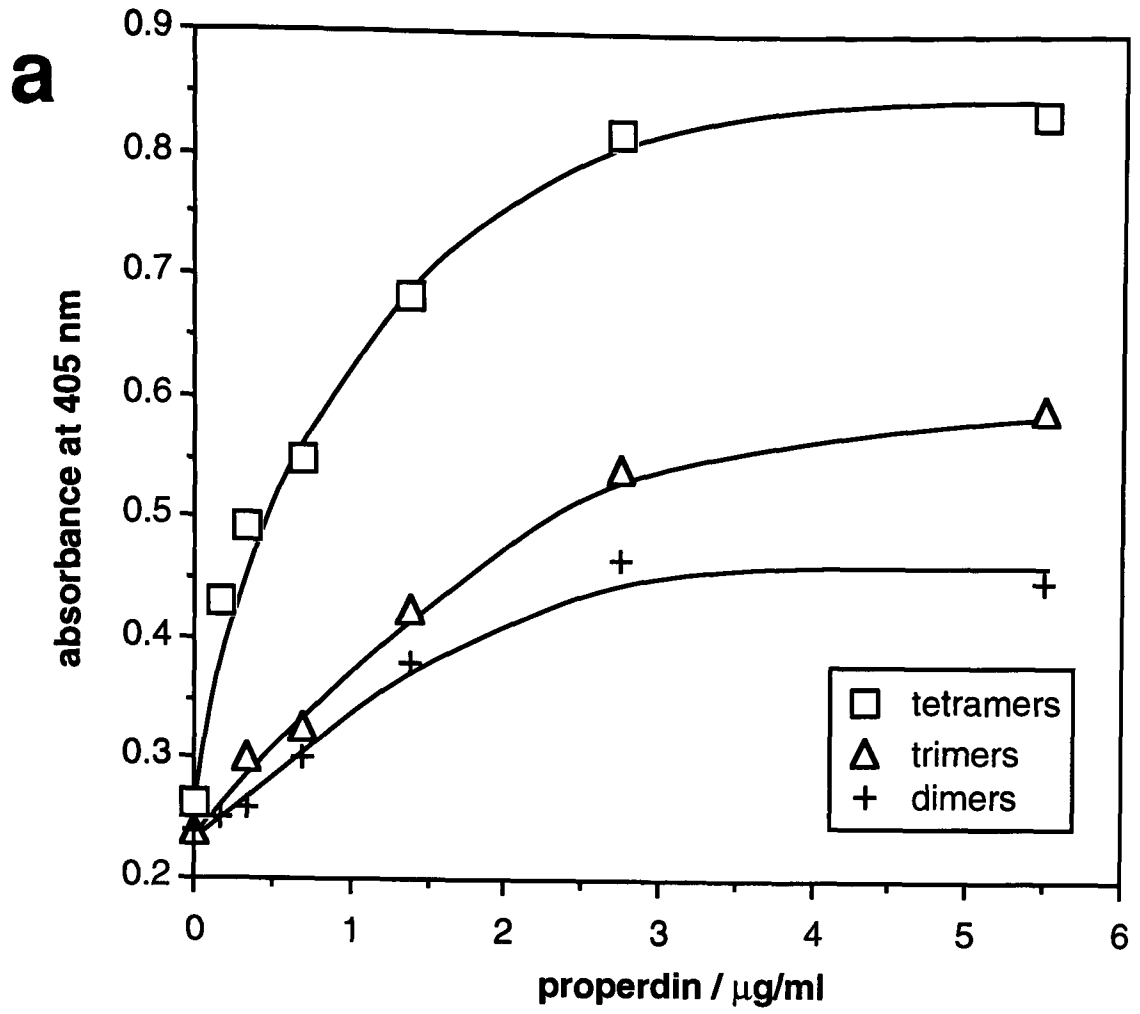


Figure 8.4
Binding of properdin in normal human plasma to sulphatide coated on microtitre plate wells.
Sulphatides in methanol were coated at 10 µg/well by evaporation. The assay was carried out as described in materials and methods. Dilutions of human plasma in PBS/10 mM EDTA were used as the source of properdin (100 µl final volume). The results of duplicate experiments are shown, after subtraction of binding in the absence of sulphatide. The assay was carried out by Laura Le Gré.



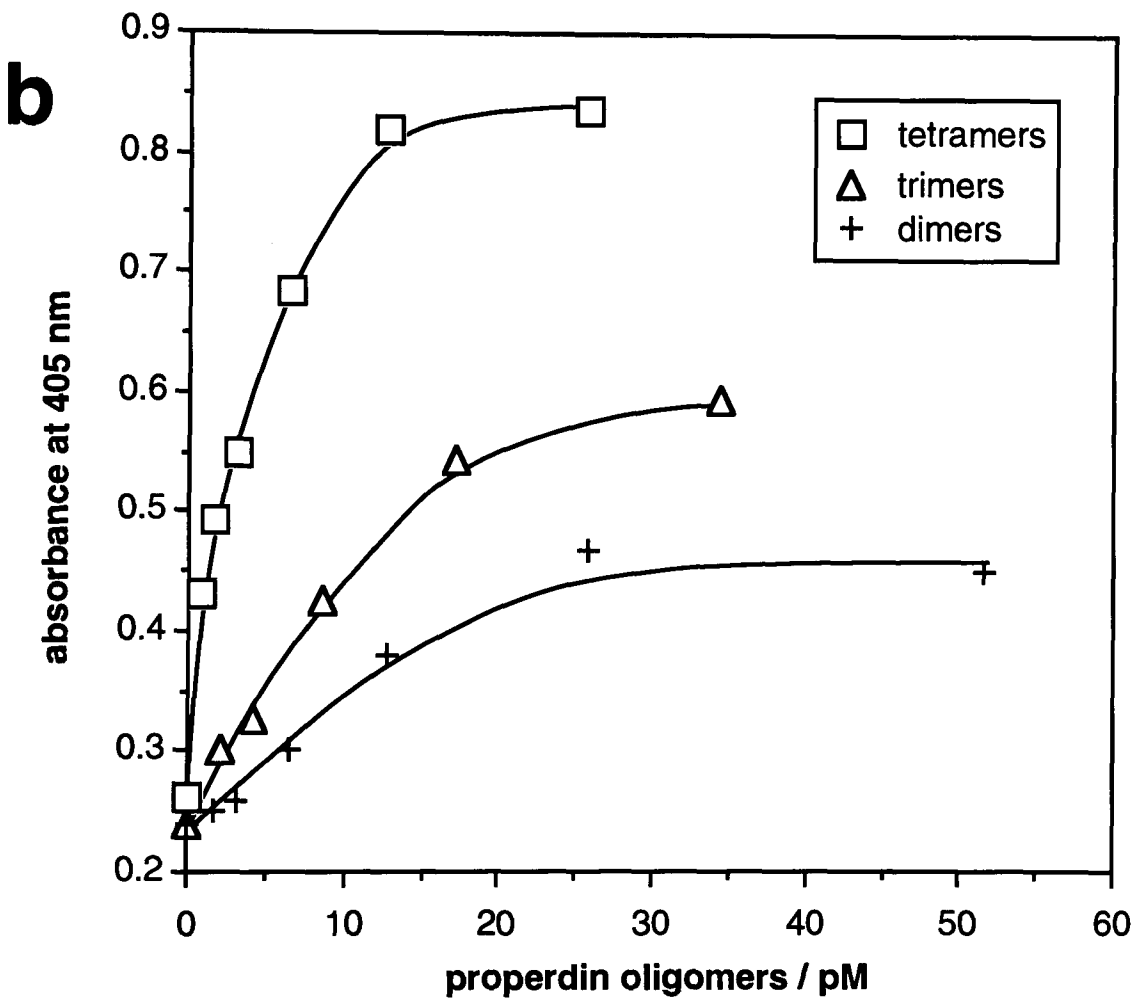


Figure 8.5
Binding of the various oligomeric forms of human properdin to sulphatide.
Sulphatide was coated onto microtitre plate wells at 4 µg/well in methanol by evaporation. Properdin oligomers were separated by gel exclusion chromatography on Superose 6. The averages of duplicate experiments are shown. The average range of the duplicates was 0.08 absorbance units. Similar results were obtained in a second independent experiment.
a) Dose dependency of properdin dimers, trimers and tetramers binding to sulphatide.
b) Data as above, but adjusted for the molar concentration of each oligomer added. (Molecular masses assumed: dimer, 106.6 kDa; trimer, 159.9 kDa; tetramer, 213.2 kDa).

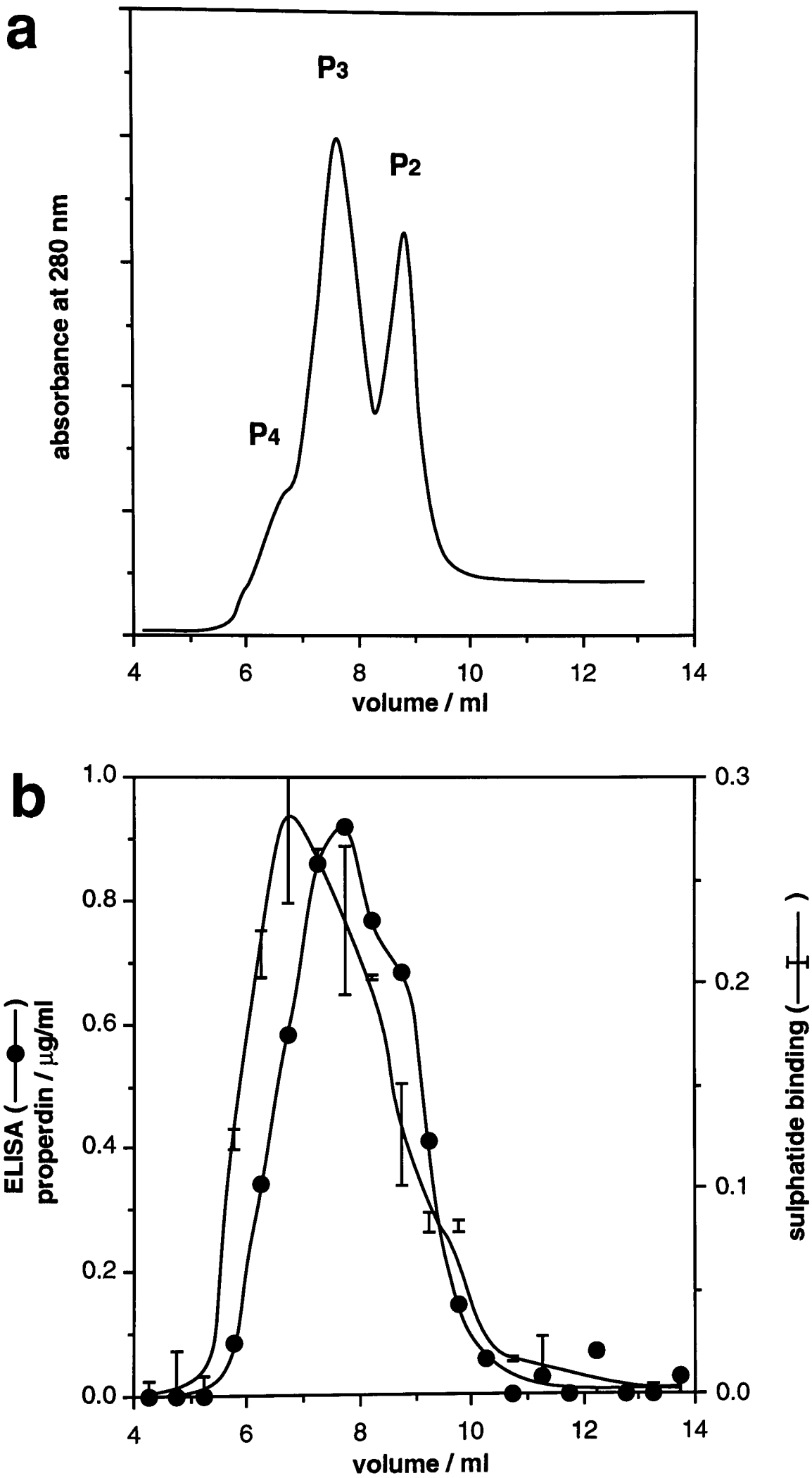


Figure 8.6

Sulphatide binding activity of the various forms of plasma properdin separated by gel exclusion chromatography on Superose 12.

a) The absorbance at 280 nm (arbitrary units)

b) Properdin in the eluted fractions was detected by polyclonal sandwich ELISA. The averages of duplicate experiments are shown. The sulphatide binding activity of each fraction also was determined using the microtitre plate binding assay. The ranges of duplicate experiments are shown.

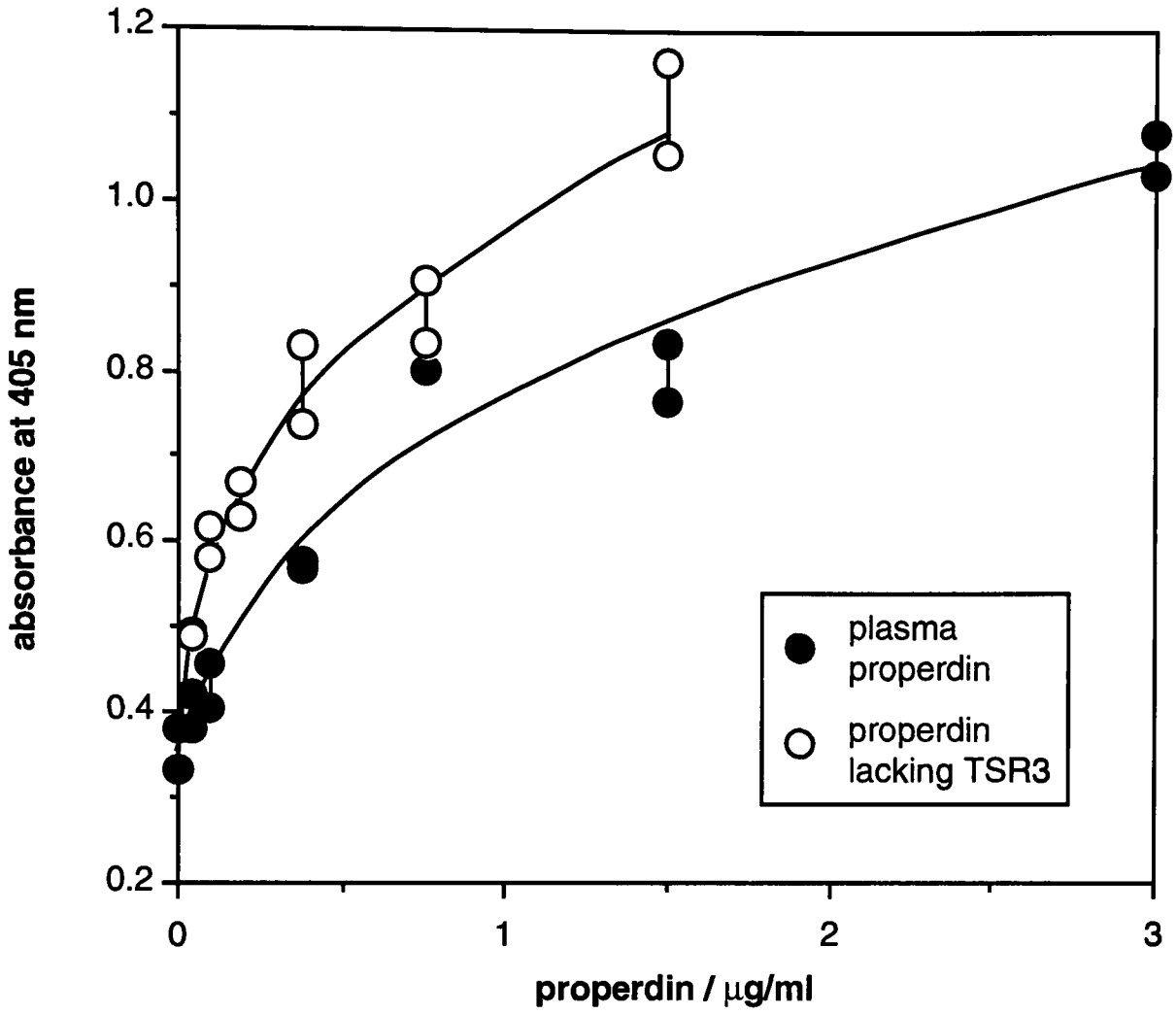
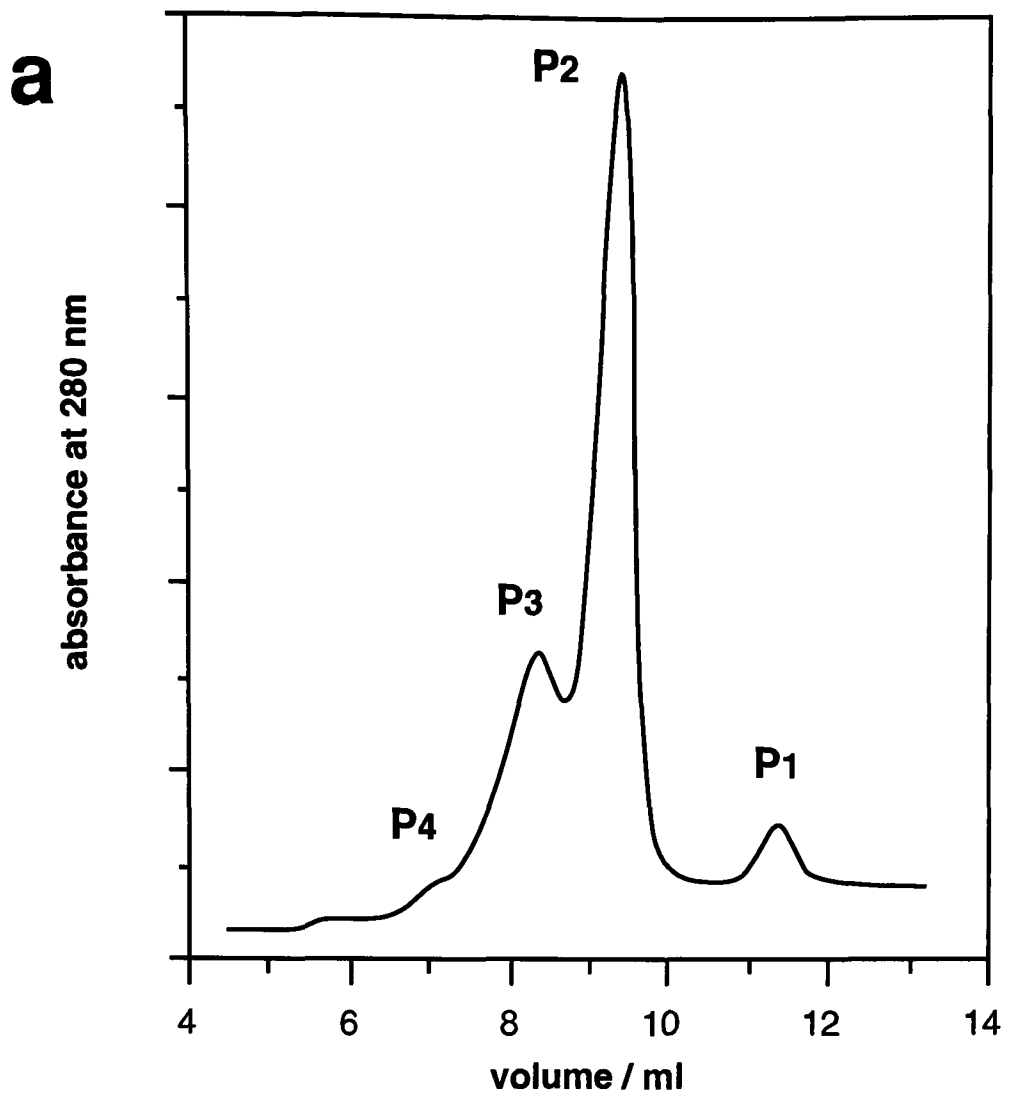


Figure 8.7
Binding of purified recombinant properdin lacking TSR3 to sulphatide. The binding of purified human plasma properdin is shown for comparison. Sulphatides were coated at 4 µg per microtitre plate well. The results of duplicate experiments are shown.



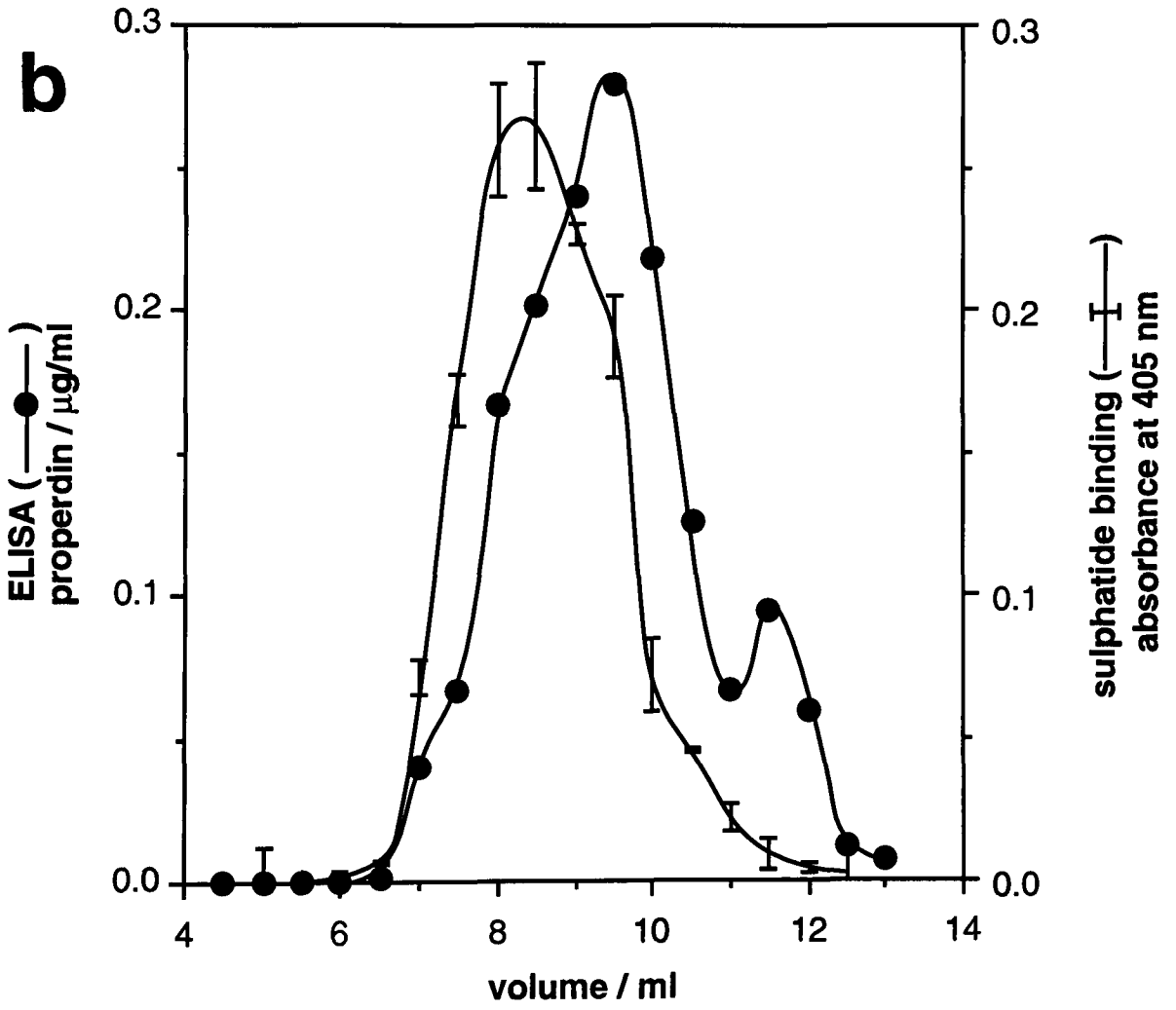


Figure 8.8
Sulphatide binding assay of the various oligomeric forms of recombinant properdin lacking TSR3 separated by gel exclusion chromatography on Superose 12.
a) The absorbance profile at 280 nm (arbitrary units).
b) Properdin in the eluted fractions was detected using the polyclonal anti-properdin sandwich ELISA. The values shown are the concentrations present in the sulphatide binding assay (averages of duplicate experiments). The sulphatide binding activity of each fraction was also determined using the microtitre plate binding assay. The ranges of duplicate experiments are shown.

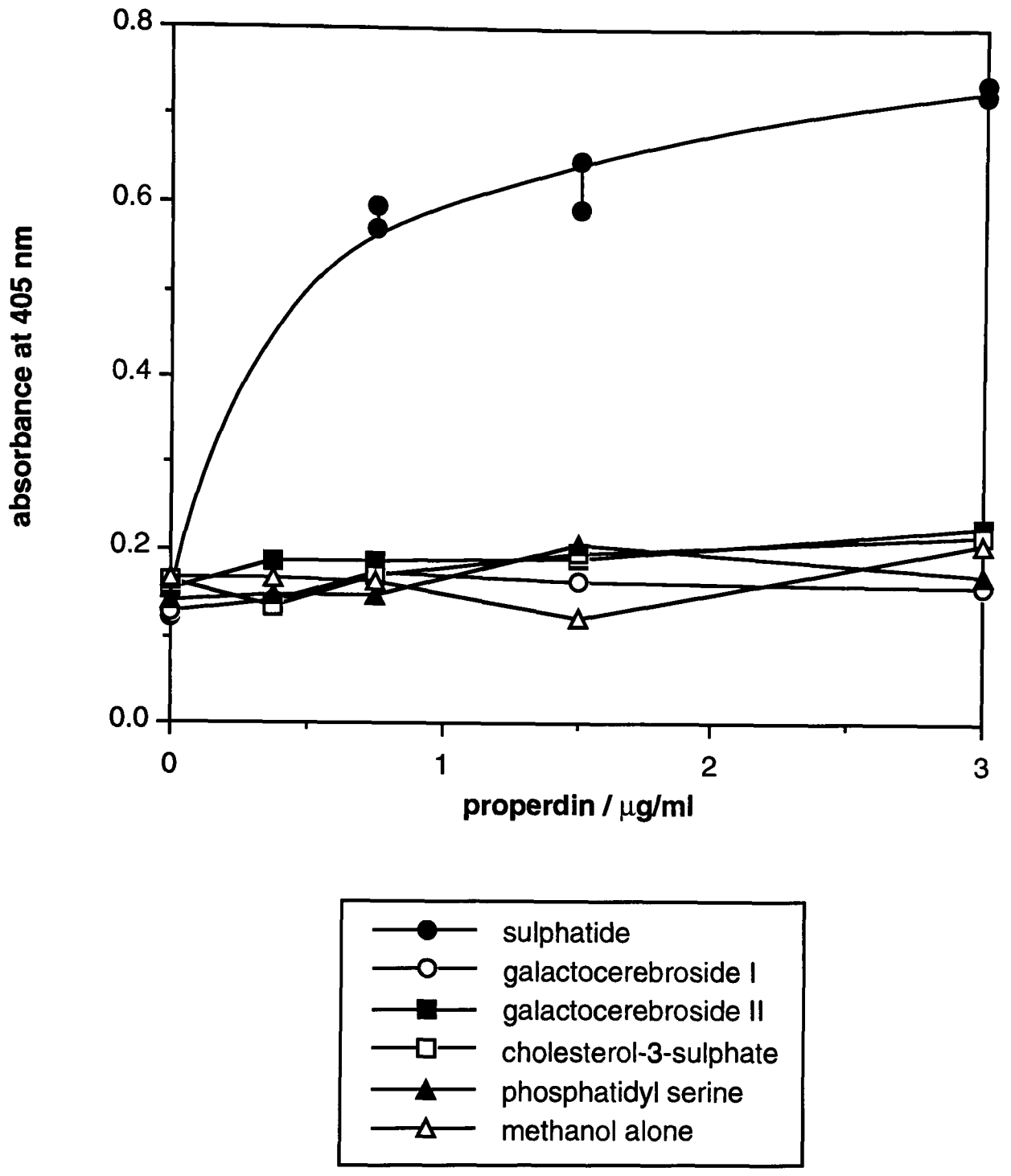


Figure 8.9

The specificity of recombinant properdin lacking TSR3 for sulphatide.

Lipids were dissolved in methanol at 30 µg/ml. Wells were coated by evaporation using 150 µl of each lipid solution. For binding to sulphatide, the results of duplicate experiments are shown. The averages of duplicate experiments are shown for binding of the other lipids, for clarity.

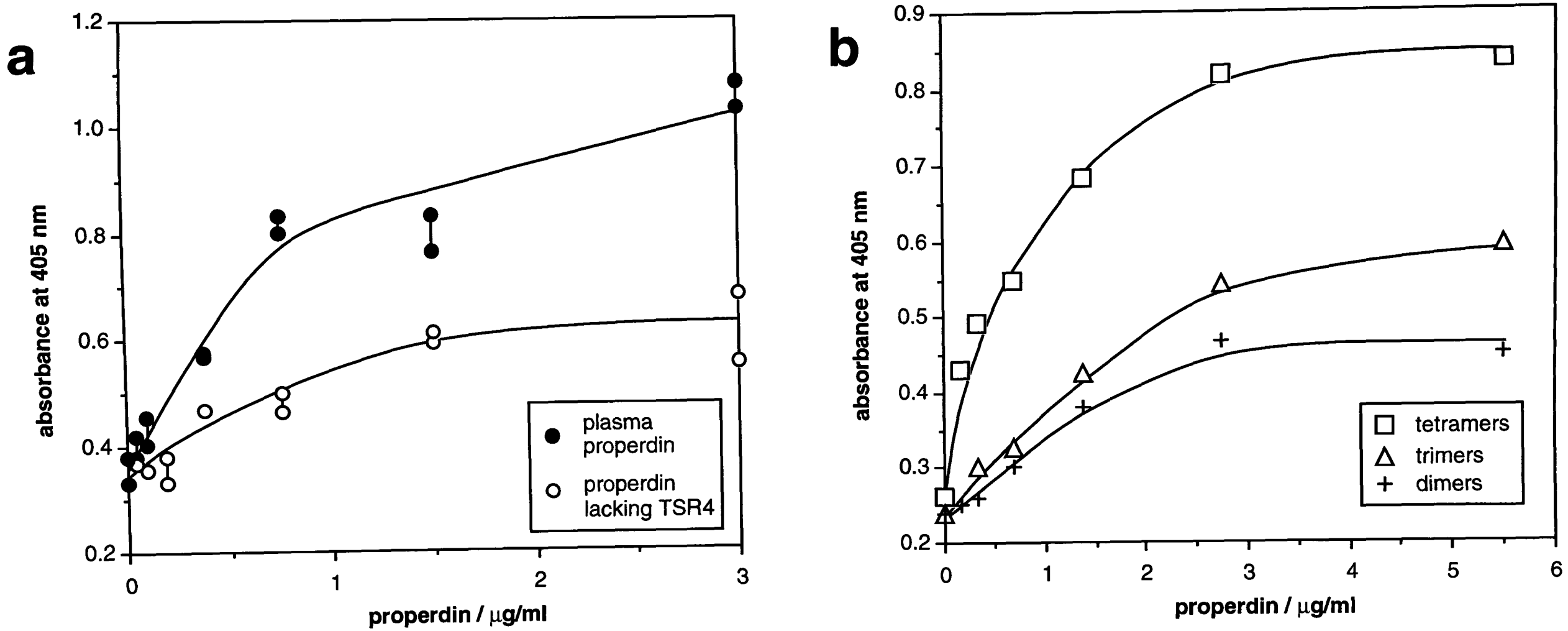


Figure 8.10
Binding of recombinant human properdin lacking TSR4 to sulphatide.
The binding of properdin lacking TSR4 is compared to that for (a) whole plasma properdin, (b) the various oligomeric forms of plasma properdin. The preparation of properdin lacking TSR4 contains only dimers and monomers. The binding to sulphatide seen is similar to that found for normal properdin dimers. The results of duplicate experiments are shown in (a). The results in (b) are as figure 8.5. The assay was carried out as described in the legend of figure 8.6.

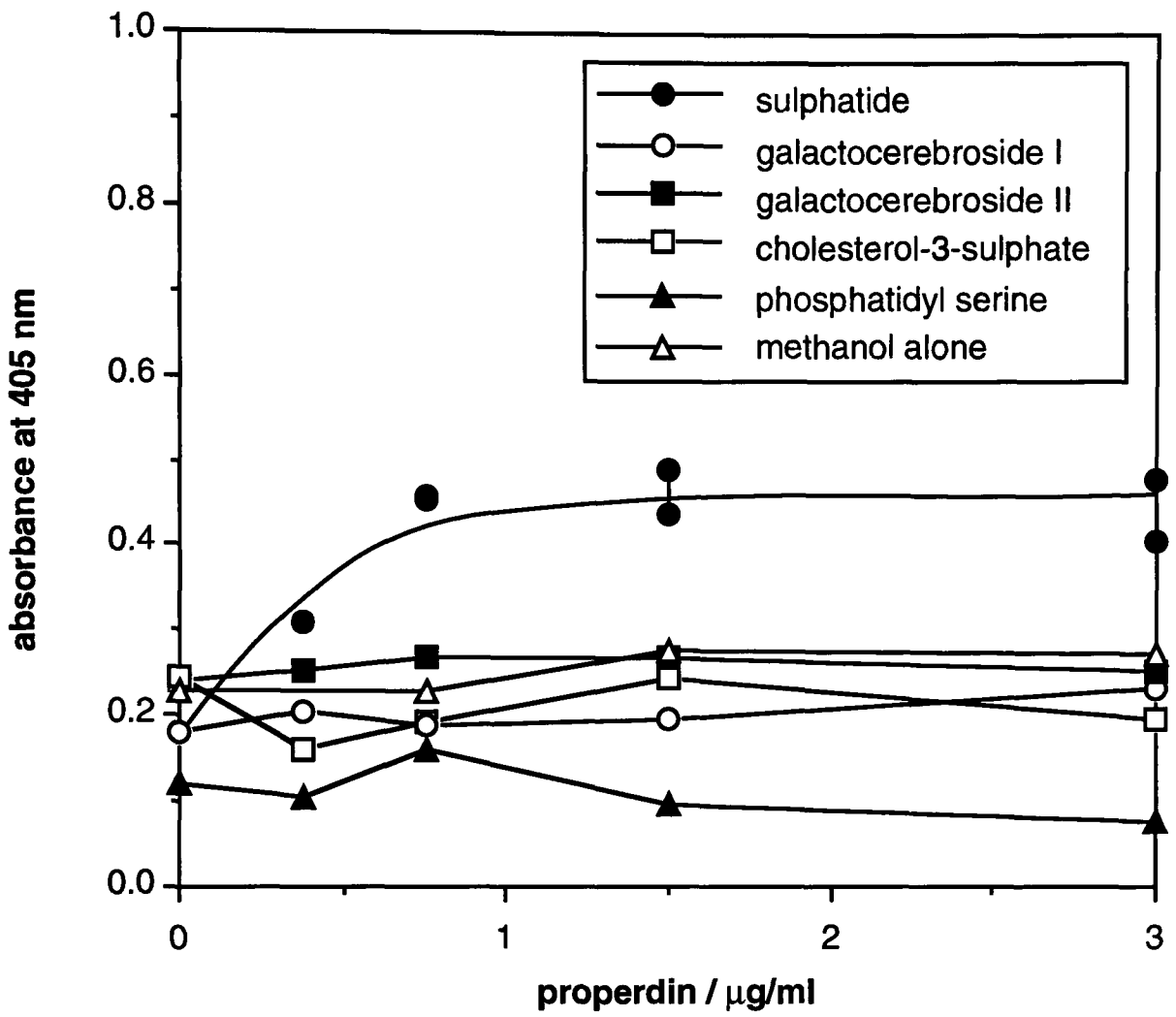


Figure 8.11

The specificity of recombinant properdin lacking TSR4 for sulphatide.

Lipids were dissolved in methanol at 30 µg/ml. Wells were coated by evaporation using 150 µl of each lipid solution. For binding to sulphatide, the results of duplicate experiments are shown. The averages of duplicate experiments are shown for the binding to the other lipids, for clarity.

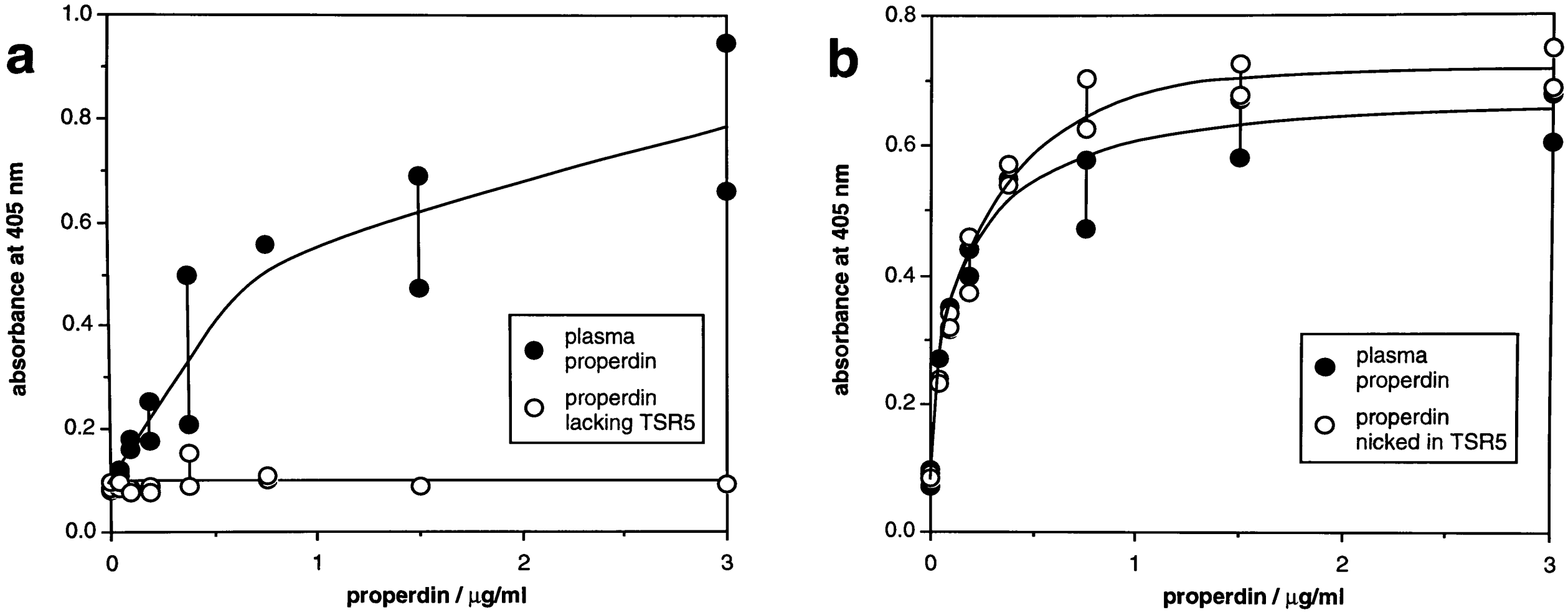


Figure 8.12
Binding of recombinant properdin lacking TSR5, and properdin "nicked" in TSR5, to sulphatide.
Microtitre plate wells were coated, by evaporation, with 4 µg/well sulphatide in methanol. The results of duplicate experiments are shown.
a) The binding of recombinant human properdin lacking TSR5 compared to normal human plasma properdin.
b) The binding of human plasma properdin "nicked" in TSR5 by trypsin treatment, compared to untreated human plasma properdin.

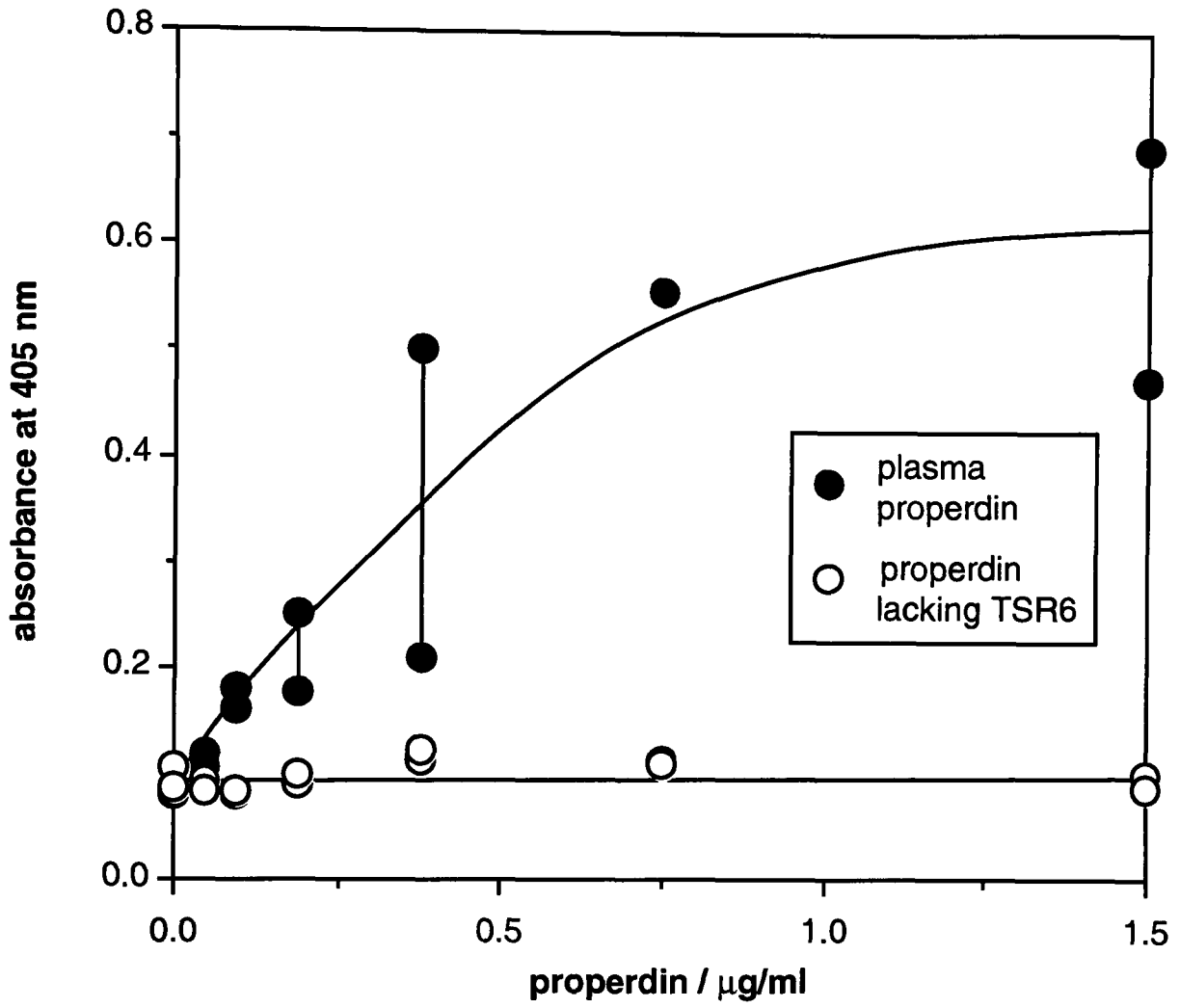
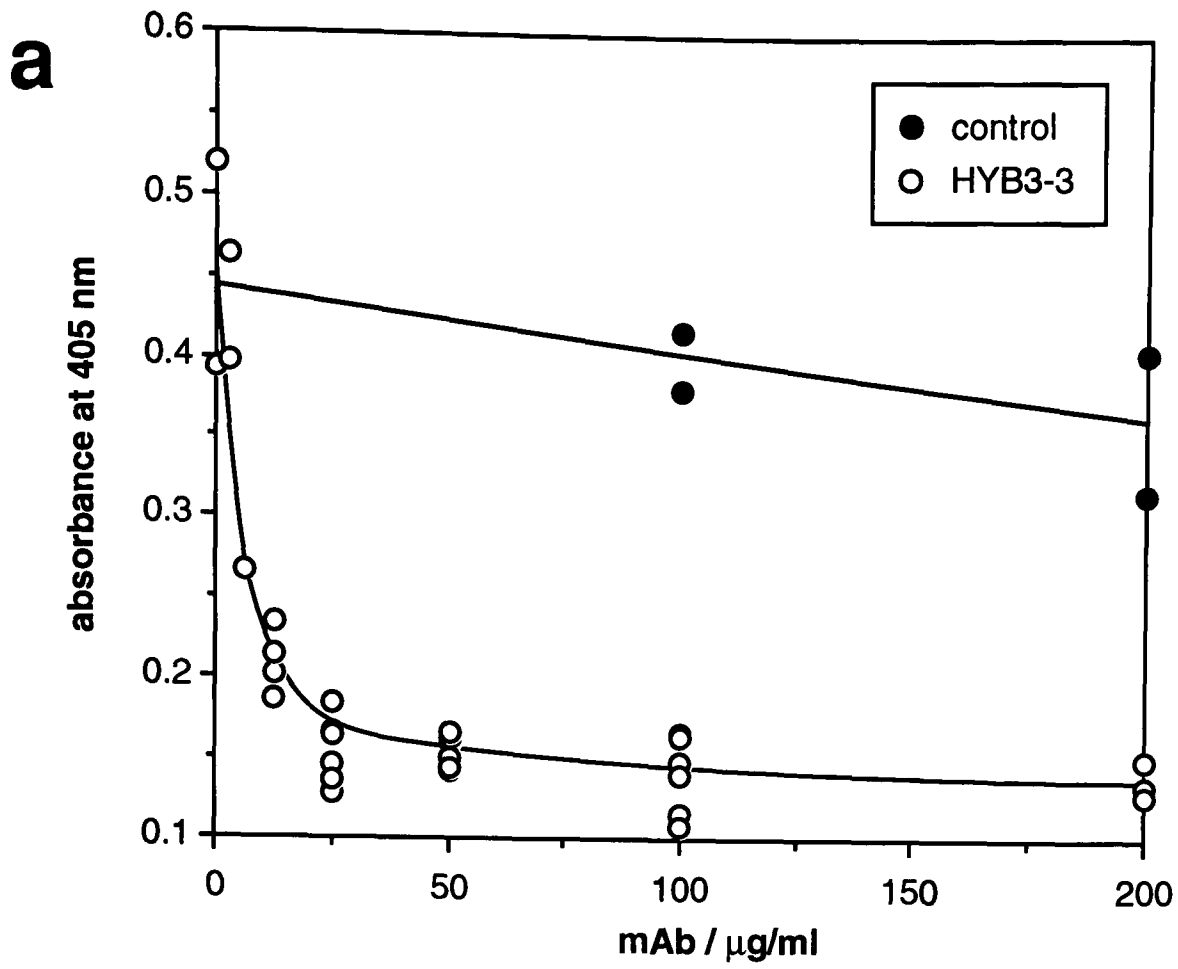


Figure 8.13
Binding of recombinant properdin lacking TSR6 to sulphatide. The binding of purified human plasma properdin is shown for comparison. Sulphatides were coated at 4 μg per microtitre plate well. The results of duplicate experiments are shown.



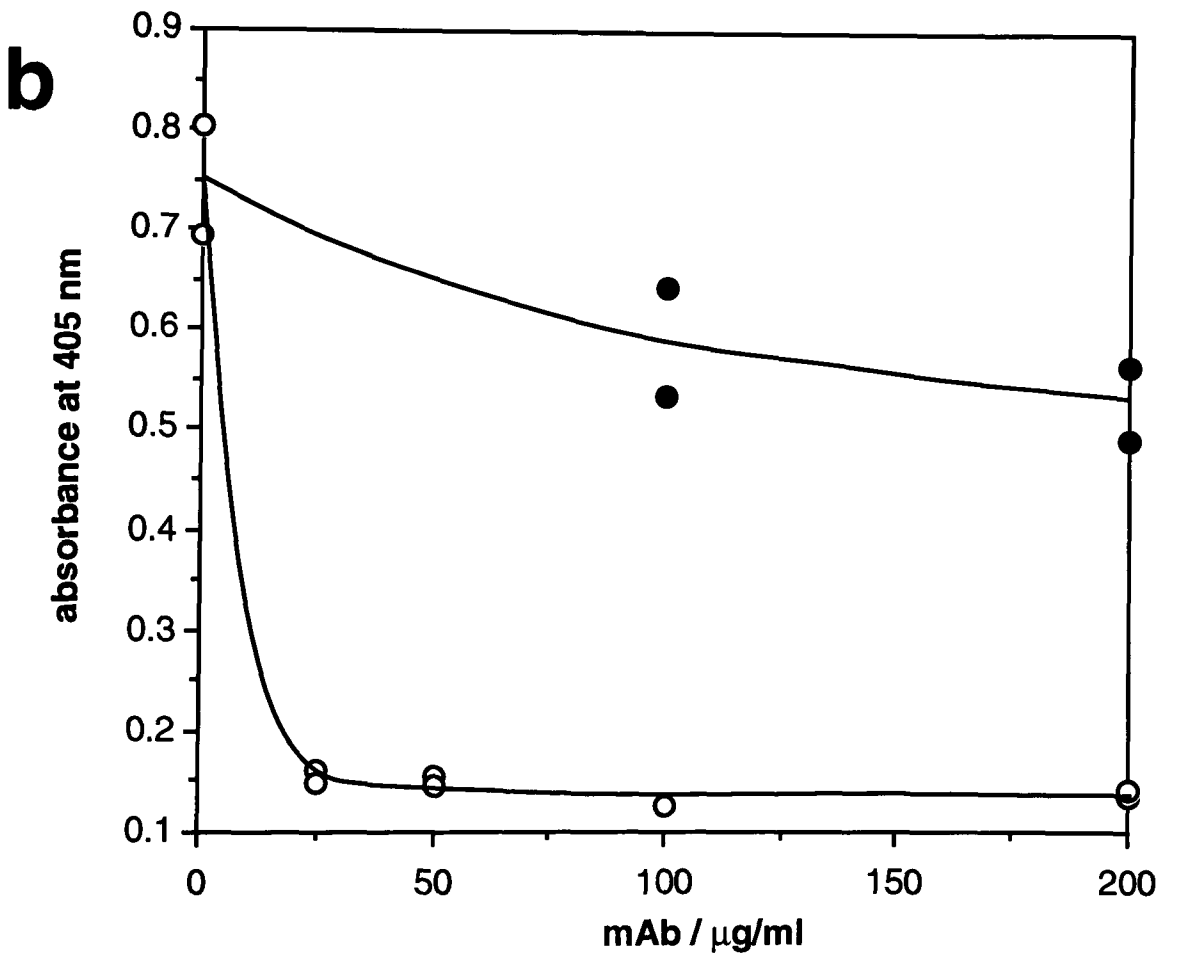


Figure 8.14
Inhibition of properdin binding to sulphatide by HYB3-3 anti-properdin monoclonal antibody.
Sulphatide was coated at 4 µg/well. Properdin samples (0.5 µg/ml) were incubated with serial dilutions of the HYB3-3 mAb, or an isotype matched control mAb, MOPC21 (mouse IgG1, kappa).
a) Purified plasma properdin.
b) Recombinant properdin lacking TSR3.

Chapter 9

Chapter 9
Conclusions

9.1 Preliminary sequence analysis: a model of the properdin monomer

The aim of the work described in this thesis was to localize the regions of human properdin important for functional activity. The amino acid sequence of properdin derived from mouse and human cDNA clones (Goundis and Reid, 1988; Nolan *et al.*, 1991) had previously shown that each subunit of properdin contains a number of repeats of a type first identified in thrombospondin. Such thrombospondin type I repeats (TSRs) are now known to be present in a wide variety of proteins from many species. There has been some debate about whether properdin contains five and a half or six copies of the TSR consensus sequence. The multiple sequence alignment of all known TSRs presented in chapter 3 suggests that properdin has six complete TSRs. A new model for the structure of properdin is proposed (see figure 3.15). Properdin contains an N-terminal region of no known homology and five TSRs which are each encoded by single exons, and a sixth TSR encoded by two exons. Each TSR is expected to be folded as an independent unit or "module" with similar three dimensional structure. The sixth repeat contains all the cysteine residues required to satisfy the normal disulphide bonding pattern of a TSR. The inserted region may form a sub-domain attached to a module with tertiary structure similar to the other TSRs. This inserted region carries the N-linked carbohydrate of human properdin. The position of the amino acid insertion in TSR6 corresponds closely to the position of the final intron of the human properdin gene. It is possible that an intron insertion event was responsible for introducing the extra coding into this region. Following the sixth TSR is a short eight amino acid sequence containing six charged residues in properdin from the three species known. This is referred to as the "C-terminal charged tail".

The multiple alignment also highlights a short insertion in TSR5 of properdin which is not found in any other TSR. A bond within this region can be specifically "nicked" by limited tryptic digestion of properdin (Reid and Gagnon, 1981), suggesting that it forms an exposed loop in the TSR structure. Interestingly, it is precisely this region that contains the CSVTCG motif implicated as a receptor binding site in the TSRs of thrombospondin and malarial proteins (see chapter 1).

9.2 Production of altered forms of human properdin

Based on the model structure of properdin, a number of mutant forms of the molecule were produced in a mammalian cell expression system. Normal recombinant human properdin, properdin lacking single TSRs (*i.e.* TSR3, TSR4, or TSR5), properdin lacking TSR6 and the C-terminal charged tail, and properdin lacking N-linked sugar were expressed and purified for functional studies. The properties of purified plasma properdin after "nicking" in TSR5, or removal of N-linked sugar by PNGase F treatment were also investigated.

9.3 Characterization of wild type recombinant properdin and the mechanism of oligomerization

Initially, wild type recombinant properdin was expressed in Chinese Hamster Ovary (CHO) cells. The recombinant properdin is essentially identical to properdin purified from normal human plasma in size, immunoreactivity, N-terminal sequence, possession of N-linked sugar, appearance under electron microscopy, and function in haemolytic, C3b binding and sulphatide binding assays. The recombinant protein forms oligomers in a similar distribution to that found in serum. It has been proposed that the polymerization of properdin may be carefully controlled (see section 1.17) (Farries and Atkinson, 1989). However, since properdin can be efficiently synthesized in hamster ovary cells (which are unlikely to produce properdin *in vivo*), it is unlikely that specific control processes are required for the assembly of oligomers. This suggestion is also supported by the finding that after denaturation and renaturation *in vitro,* properdin again forms a similar distribution of oligomers (Pangburn, 1989). These findings do not rule out a role for less specific chaperone molecules in properdin assembly *in vivo*.

The precise distribution of oligomers secreted by transfected CHO cells was found to be dependent on the conditions of cell culture. Cells synthesizing properdin at high rates produced ratios of oligomers shifted towards higher forms. Dissociation (at low pH) and reassociation of purified oligomers *in vitro* revealed that the distribution formed depends upon the concentration of monomers present during oligomerization. At high concentrations, a greater proportion of tetrameric properdin is formed. It is possible to speculate that when properdin synthesis is upregulated, for example by cytokine action on macrophages (Schwaeble *et al.*, 1994), then the ratio of oligomers secreted may be shifted towards higher forms. This could help to compensate for the relatively rapid depletion of the higher oligomers of properdin that may occur during alternative pathway activation (Pangburn, 1989). The design of experiments to test these hypotheses must

avoid the use of low pH during properdin purification since this will cause a redistribution of oligomers and mask any differences in the ratio of oligomers initially present.

9.4 Characterization of altered forms of human properdin: identification of regions important for function

Since recombinant properdin was functionally identical to plasma properdin, the same expression system was considered suitable for the production of mutant forms of properdin. The results of functional assays on these proteins are presented together in figure 9.1, and the properties of the proteins are summarized in table 9.1.

Table 9.1

Summary of the structural and functional characteristics of normal and altered forms of human properdin.

form of properdin	oligomerization[a]	C3bBb stabilization[b]	C3b binding[c]	Sulphatide binding[d]
plasma properdin	P_4,P_3,P_2	+++	+++	+++
recombinant properdin	P_4,P_3,P_2	+++	+++	+++
properdin lacking TSR3	P_4,P_3,P_2,P_1	+++	+++	+++
properdin lacking TSR4	P_2,P_1	-	++	++
properdin lacking TSR5	P_2,P_1	-	-	-
properdin nicked in TSR5	P_4,P_3,P_2	-	(+)[e]	+++
properdin lacking TSR6	P_1	-	-	-
truncated properdin	P_1	nd	nd	nd
properdin lacking N-linked sugar	P_4,P_3,P_2	+++	nd	nd

+++ indicates activity close to that of normal properdin

++ indicates clear activity

+ indicates low activity

- indicates no activity

nd indicates activity not determined

[a] predominant oligomeric forms present, see chapter 5 for details

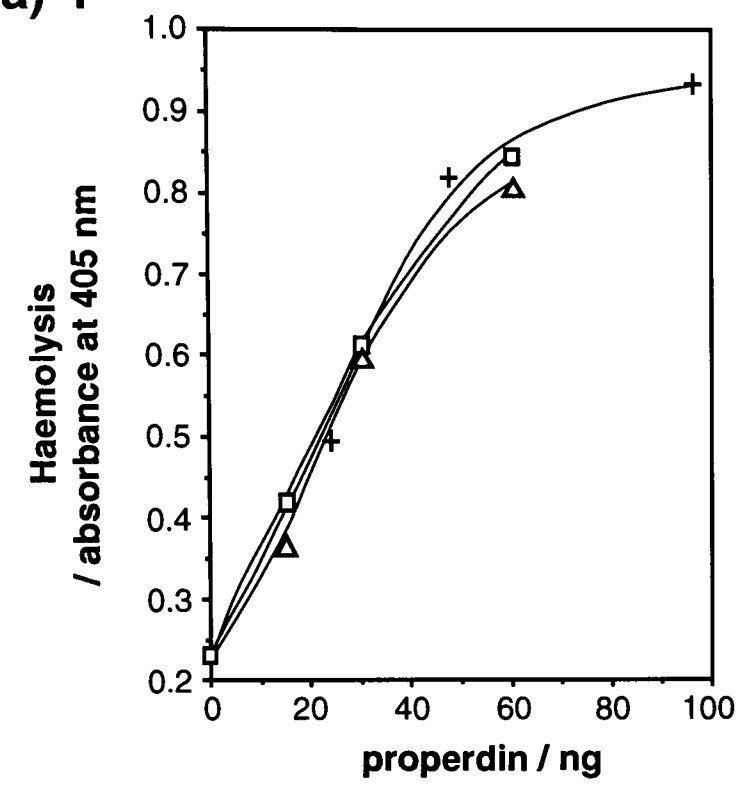
[b] assessed by haemolytic assay, see chapter 6 for details

[c] by direct binding ELISA in half-physiological salt, see chapter 7 for details

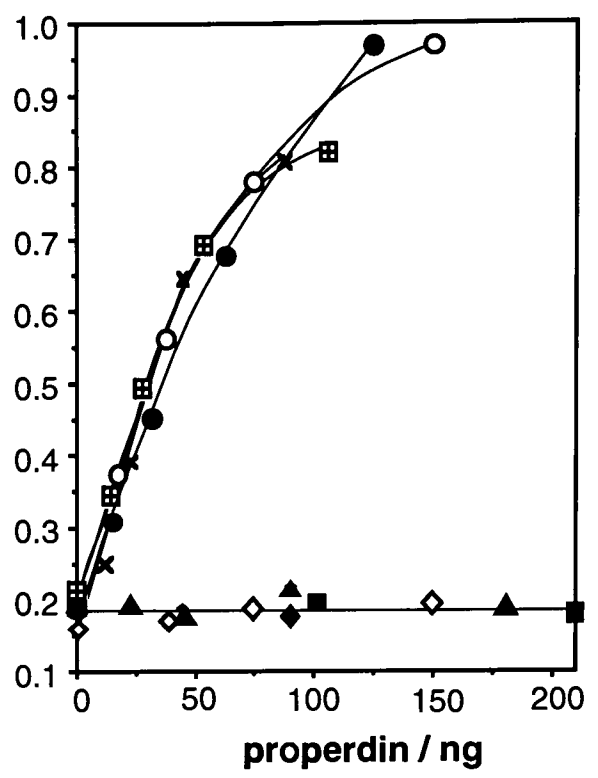
[d] by direct binding ELISA in PBS, see chapter 8 for details

[e] probably activity due to the presence of residual non-nicked properdin

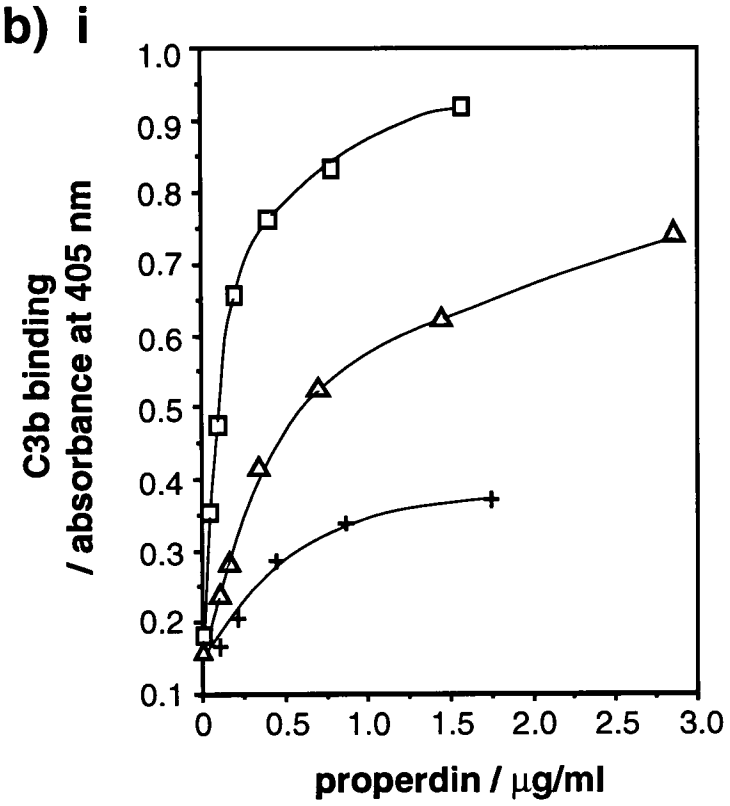
a) i



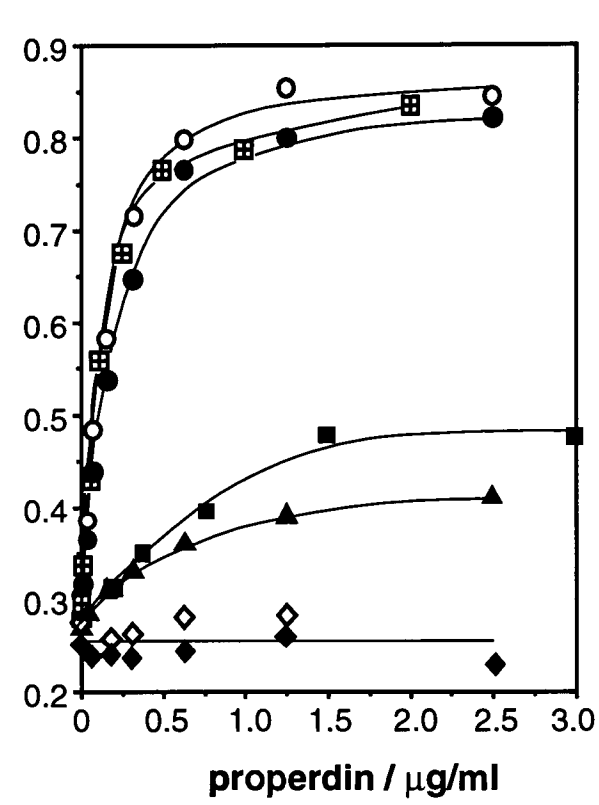
ii



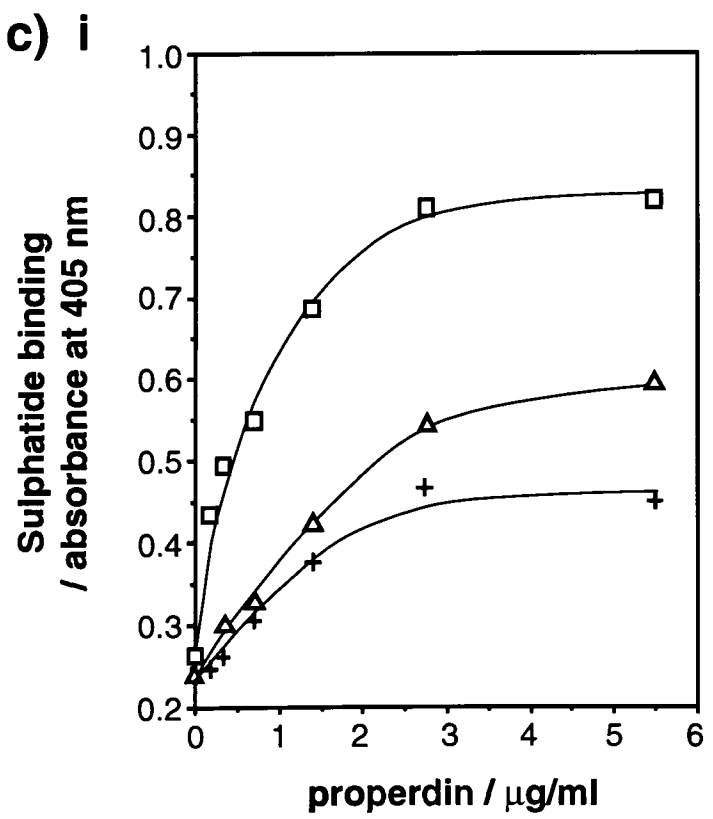
b) i



ii



c) i



ii

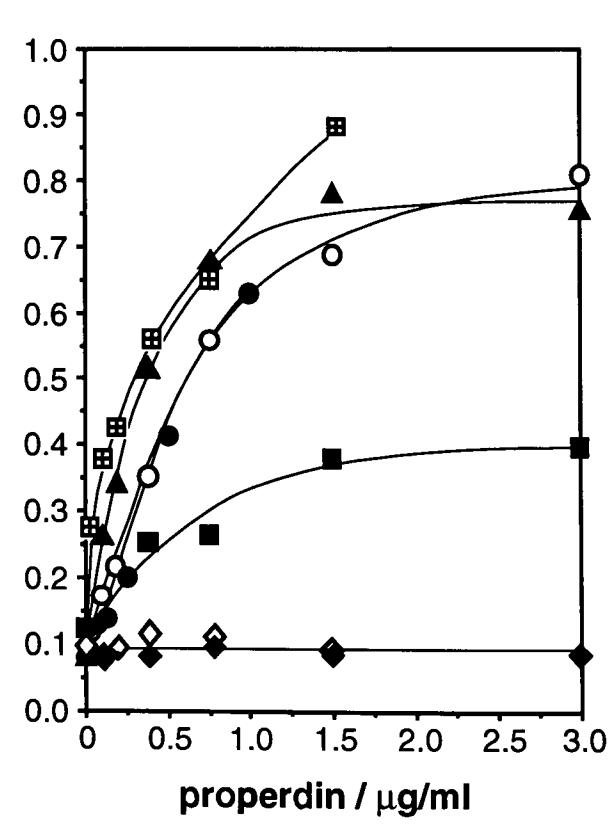


Figure 9.1

Summary comparison of the functional activities of normal and altered forms of properdin. The data presented in the previous chapters were normalised to the standard curve obtained using purified plasma properdin in each assay. The averages of duplicate experiments are shown.

a) Alternative pathway haemolytic assay. i) Comparison of the activity of purified preparations of normal plasma properdin dimers, trimers and tetramers. ii) Comparison of the activities of normal and mutant forms of properdin.

b) C3b binding assay. i) and ii) as for (a).

c) Sulphatide binding assay. i) and ii) as for (a).

Key: Plasma properdin dimers (+), plasma properdin trimers (△), plasma properdin tetramers (□), whole plasma properdin (○), wild type recombinant properdin (●), properdin lacking TSR3 (⊞), properdin lacking TSR4 (■), properdin lacking TSR5 (◆), properdin nicked in TSR5 (▲), properdin lacking TSR6 (◇), properdin lacking N-linked sugar after PNGase F treatment (✕).

9.4.1 The N-linked oligosaccharide of properdin is not required for secretion, oligomerization, or C3bBb stabilization

Recombinant properdin produced in the presence of tunicamycin lacks N-linked carbohydrate. This form of properdin is approximately 5 kDa smaller than normal recombinant properdin. A similar shift in size is seen on treatment of plasma properdin with PNGase-F (see chapter 4). Thus, recombinant properdin carries a similar level of N-glycosylation to that of plasma properdin. The results confirm that N-glycosylation is not required for secretion of properdin, nor for the formation of oligomers. Similar results have previously been obtained for properdin produced by DMSO-stimulated HL-60 cells in the presence of tunicamycin (Farries and Atkinson, 1989). The results also demonstrate that properdin lacking N-linked sugar is fully functional in an alternative pathway haemolytic assay, consistent with the previous report that endoglycosidase-F treated properdin is able to bind C3iBb-Sepharose (Farries and Atkinson, 1989).

9.4.2 The third TSR of properdin is not required for functional activity

Properdin lacking the third TSR is able to form a distribution of oligomeric forms including tetramers, trimers, dimers and monomers as judged by electron microscopy, gel exclusion chromatography and cross-linking. The shift in the distribution towards smaller forms, compared to normal properdin, may be due to the shorter overall length of the monomer lacking TSR3. Under the electron microscope, the length of a subunit of properdin lacking TSR3 is about 70% that seen for full length properdin. This reduction in the distance between N and C-terminal regions may favour ring closure during the assembly of the cyclic oligomers. Strikingly, properdin lacking TSR3 is essentially unaltered in its ability to bind C3b and to stabilize C3bBb in the haemolytic assay. Also, TSR3 is not required for binding to sulphatide. The discovery that TSR3 can be removed from properdin without loss of oligomerization or function is the first clear demonstration that thrombospondin type I repeats form independently folded modules. The result also shows that an alteration in the length of the properdin monomer does not, in itself, diminish the ability of the protein to stabilize the C3 convertase.

9.4.3 The fourth TSR of properdin is not required for sulphatide or C3b binding, but may be required for C3bBb stabilization

Properdin lacking the fourth TSR forms at least dimers as judged by gel exclusion chromatography, electron microscopy, and cross-linking. This form of properdin is unable to stabilize the alternative pathway C3 convertase in the haemolytic assay. However, properdin lacking TSR4 is able to bind to C3b in half-physiological ionic strength buffer.

The pattern of binding is similar to that found for a preparation of plasma properdin containing only dimers. So, removal of TSR4 prevents functional interaction of properdin with C3bBb, but does not prevent the molecule from binding to C3b. A monoclonal antibody (HYB3-3) which is thought to bind to TSR4 does not inhibit C3bBb stabilization or C3b binding. This is consistent with the ability of properdin without TSR4 to bind to C3b. The inability of HYB3-3 to inhibit the alternative pathway may suggest that properdin TSR4 is not directly involved in stabilizing the complex, or simply that the epitope of this antibody is distinct from the functional region. Binding of properdin to Bb is thought to be weak, but has been inferred from the chemical cross-linking of properdin to Bb (Farries *et al.*, 1988b). Further studies will be required to determine if TSR4 interacts directly with Bb.

The binding of properdin lacking TSR4 to sulphatide is similar to that seen with a preparation of normal properdin dimers. Thus, although this module of properdin contains a motif (**C**P**VTCG**L**G**QTMEQ**R**) similar to a sequence (**CSVTCG**x**G**xxx**R**x**R**) which has been implicated in binding to sulphated glycoconjugates in other TSR containing proteins (see introduction), TSR4 is not required for the binding of properdin to sulphatide. However, the HYB3-3 monoclonal antibody which binds to TSR4 does inhibit properdin binding to sulphatide. It is possible that this antibody sterically inhibits the interaction of residues in the adjacent TSR5 with sulphated sugar (see below). Clearly, this antibody distinguishes the C3b/Factor B and sulphated glycoconjugate binding sites of properdin.

9.4.4 The fifth TSR of properdin is involved in C3b and sulphatide binding

Properdin lacking the fifth TSR shows a pattern of oligomerization very similar to that lacking TSR4. However, properdin without TSR5 is not active in the haemolytic assay, and is unable to bind to C3b in the ELISA system used. Furthermore, properdin containing a tryptic "nick" in TSR5 also loses its ability to bind to C3b and to stabilize the C3 convertase, despite being unaltered in overall structure (as seen by electron microscopy). Together these results strongly implicate TSR5 in the stabilization of the alternative pathway C3 convertase, and perhaps directly in binding to C3b. Trypsin cleaves TSR5 in a small inserted sequence of amino acids between the first two cysteines of the module, a region not found in any other TSR known. Interestingly, this sequence in TSR5 is in the same position as the CSVTCG motif that is found in many other TSRs. This region almost certainly forms an exposed loop in the TSR structure. This putative loop is present in the derived amino acid sequences of human, mouse, and guinea pig properdin, and in a partial cDNA sequence of bovine properdin (not shown). Rabbit, bovine and porcine properdin (Nakano *et al.*, 1986; Viv Perkins, personal communication) can all be cleaved into similar T_1 and T_2 fragments by trypsin treatment, suggesting that the loop is also present in these molecules. However, the sequence of

the loop is not conserved between species. If the loop is directly involved in C3b binding then corresponding amino acid changes must be present in C3. The putative properdin binding site in C3 is highly conserved between these three species (Lambris *et al.*, 1993) (see figure 1.3). It may be that the loop in TSR5 is not directly involved in the interaction, but that it is important in maintaining the conformation of another part of the module that is necessary for C3b binding.

Properdin lacking TSR5 is unable to bind sulphatide in the assay used, while limited tryptic digestion has no effect on binding. These results suggest that residues in TSR5 are required for binding to sulphated glycoconjugates, but that the region that is important is distinct from that involved in C3bBb binding.

9.4.5 The sixth TSR of properdin is required for oligomerization

Properdin lacking the sixth TSR and the short C-terminal charged tail is unable to form oligomers. This is consistent with the head-to-tail association model proposed by Smith *et al.* from inspection of electron micrographs of purified properdin (Smith *et al.*, 1984a). It is likely that residues in the highly charged N-terminal and TSR6 modules (or in the C-terminal charged tail) are involved in the association of monomers to form oligomers. The dissociation of properdin oligomers observed at low pH is consistent with this prediction. Properdin lacking TSR6 is unable to stabilize C3bBb, or to bind C3b or sulphatide in the assay conditions used. From our results, we cannot determine whether this is due to the prevention of oligomerization *per se* or due to the removal of important functional residues. Since properdin without N-linked carbohydrate is active in the haemolytic assay, the loss of activity of the TSR6 lacking mutant is not due to the lack of N-linked sugar.

9.4.6 The N-terminal end of the properdin monomer may also be important for C3b binding

Experiments with a panel of monoclonal antibodies show that those which bind to the N-terminus, TSR1 or TSR2 are able to inhibit properdin binding to C3b. Thus the N-terminal portion of properdin is also implicated in C3b binding. Antibodies which may bind to TSR6[1] also inhibit properdin binding to C3b.

---

[1] Although it has not been definitively excluded that these mAbs also bind N-TSR1-TSR2 (see chapter 5).

9.5 The vertices of properdin oligomers may be important for C3b binding

The results summarized above implicate TSR5 (and possibly TSR6) and the N-terminal-TSR1-TSR2 region in binding to C3b. TSR3 and TSR4 are not required for this activity. These findings might suggest that two C3b binding sites on properdin are required for high affinity interaction (N/TSR1/TSR2 and TSR5/TSR6) and that the distance between them is not crucial (since removal of TSR3 or TSR4 does not prevent C3b binding). Alternatively, the implication that two different regions are important can be reconciled when one considers that the N-terminal and C-terminal regions of properdin are brought into close proximity by oligomerization. It is thus possible that the vertices of properdin oligomers constitute the C3b binding sites (figure 9.2). This is consistent with the lack of monomeric properdin in plasma, and the inactivity of monomeric properdin lacking TSR3 or TSR6. However, this region might be predicted to adopt rather different conformations in the different oligomers of properdin, since the intersubunit angles are widely different in each form (see figure 1.8). It is perhaps surprising, therefore, that such a region should form the C3b binding site. However, various investigators have reported that the higher forms of properdin have higher apparent affinity for C3b than lower forms, and are more active in stabilizing the C3bBb complex (Smith *et al.*, 1984a; Farries *et al.*, 1987). While this has been attributed to increased avidity of higher oligomers due to multivalent binding, the stoichiometry of properdin:C3b interaction has been reported to be close to one oligomer:one C3b molecule (DiScipio, 1981). The possibility is raised that the differences in activity of the oligomers may also be explained, in part, by differences in the affinity of the C3b binding site, due to varying geometry at the oligomer vertices.

Although TSR4 is not required for properdin binding to C3b, properdin lacking TSR4 is unable to stabilize the C3bBb complex in an alternative pathway haemolytic assay. The means by which properdin stabilizes the C3bBb complex is unclear, although a direct weak interaction of properdin and Factor B has been proposed (see introduction 1.7.1) (Farries *et al.*, 1988b). It is tempting to speculate that TSR4 may be involved in Factor B binding. If this is the case, and the distance between the C3b and Factor B binding sites is crucial for activity, then the region of properdin which bridges the two sites must span the junction between monomers, since removal of TSR3 has no effect on the ability of properdin to stabilize the C3bBb complex (see figure 9.2).

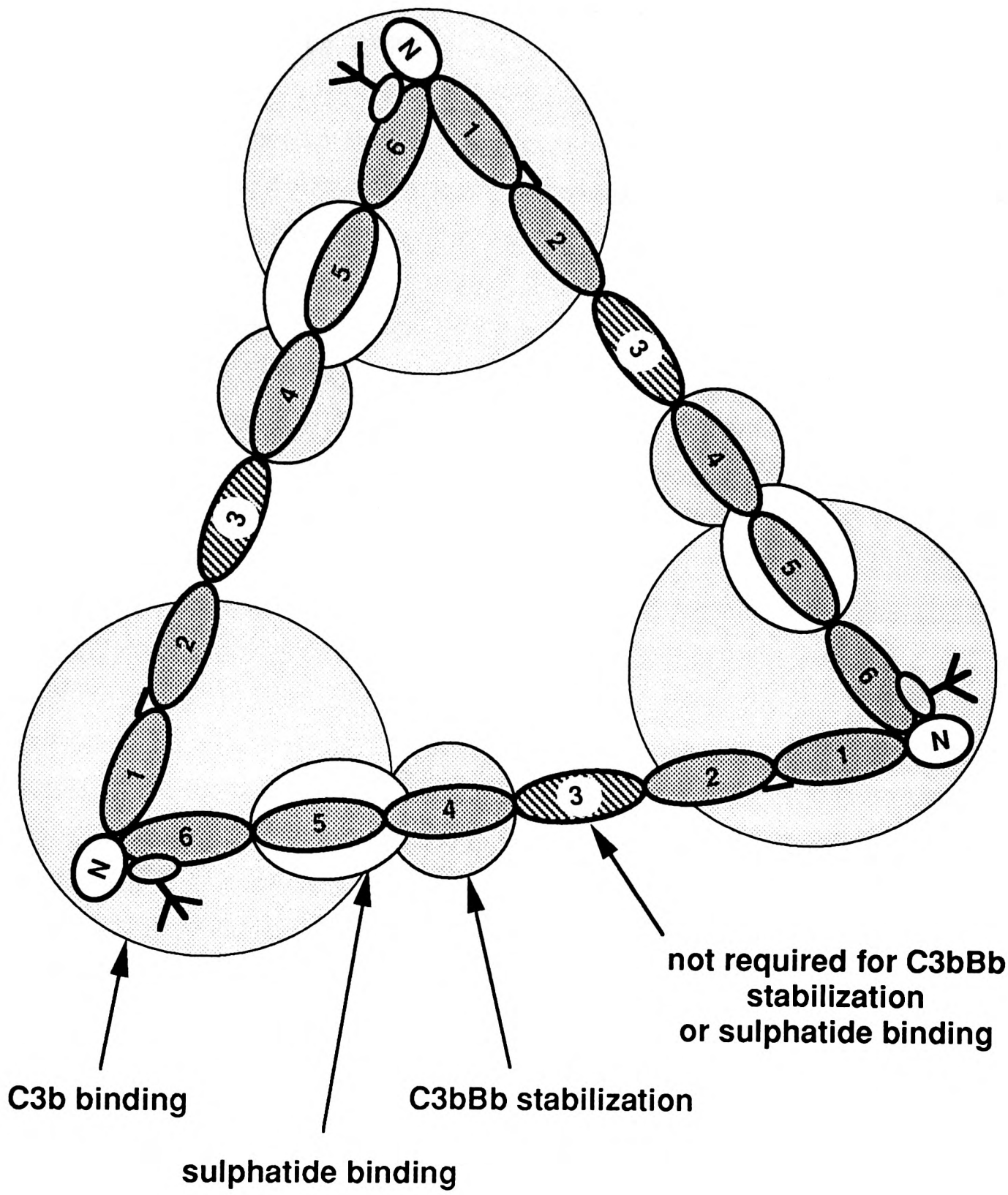


Figure 9.2
A model of a human properdin trimer showing potentially important functional regions.
The substructure of each monomer is described in figure 3.15. The circled regions represent regions of properdin which have been implicated in the activities shown by protein engineering, trypsin digestion and monoclonal antibody inhibition studies (explained in the text). The details of the interactions between subunits at the head-to-tail junctions are not clear.

9.6 The sulphatide binding site of properdin is distinct from the C3bBb binding site(s) but may also involve TSR5

"Nicking" of properdin in TSR5 prevents C3b binding but does not influence sulphatide binding. The HYB3-3 monoclonal antibody which binds to TSR4 will inhibit sulphatide binding, but not stabilization of the C3bBb complex. Thus, these two activities of properdin (C3bBb stabilization and sulphatide binding) probably involve distinct sites. However, sulphatide binding, like C3b binding, may require TSR5. Properdin lacking TSR3 or TSR4 retains the ability to bind to sulphatide, but removal of TSR5 abolishes this activity.

TSR5 contains a sequence related to the WSxWS motif implicated by Guo *et al.* (1992a,b) in heparin binding by thrombospondin (properdin TSR5 sequence: WDSWGE**WS**). However, as discussed in chapter 1, section 1.12.3, it seems likely that these tryptophan residues are buried within the TSR structure. The WSxWS motif is highly conserved in TSRs of groups 1 to 4 (as defined in chapter 3), whether or not they have been implicated in sulphated sugar binding. Furthermore, the malarial CS proteins, for which the strongest evidence of TSR-mediated sulphated sugar binding exists (and also Cyr-61 which binds heparin), do not possess the WSxWS motif (T($^{E}/_{T}$)EWS is the consensus in this region of group 5 and 6 TSRs). TSR5 completely lacks the CSVTCG motif which has been implicated in sulphated glycoconjugate binding by some workers (Holt *et al.*, 1989, 1990; Prater *et al*, 1991; Cerami *et al.*, 1992a; Tusynski *et al.*, 1993). Indeed, tryptic cleavage of TSR5 in the loop which contains this motif in other TSRs does not prevent sulphatide binding. The requirement for the CSVTCG motif in peptides which bind heparin has since been suggested to be due to the need for disulphide linked oligomers for high affinity binding (Sinnis *et al.*, 1994). The CSVTCG motif may be important for binding to CD36 (Asch *et al.*, 1992) or other glycoprotein receptors (Tuszynski *et al.*, 1993).

Studies on a variety of glycosaminoglycan binding proteins have revealed an essential role for cationic amino acids (reviewed in Jackson *et al.*, 1991; Lander, 1994). This is the case, for example, in anti-thrombin III (Carrell *et al.*, 1994). Such binding sites can be formed by the juxtaposition of amino acids that are distant in the primary structure. TSR5 of properdin contains a large number of lysine, arginine and histidine residues which could contribute to sulphated sugar binding (see figure 3.14). The basic cluster in peptides derived from the TSRs of CS protein and thrombospondin has been implicated in sulphated glycoconjugate binding in a number of studies (Prater *et al.*, 1991; Guo *et al.*, 1992a; Sinnis *et al*, 1994).

9.7 Possible roles *in vivo* of properdin binding to sulphated glycoconjugates

Although properdin, like many other proteins, binds to sulphatide *in vitro*, this may not be the natural ligand *in vivo* (Roberts *et al.*, 1987). Sulphated sugar binding proteins are known to possess high affinity for specific patterns of sulphation on particular sugar residues (San Antonio *et al.*, 1993). The sulphated ligand of properdin, and the role of such binding *in vivo* is unknown. An interaction might be proposed with either i) glycosaminoglycans of the host (on cell surfaces, in granulocyte granules, or in the extracellular matrix) or ii) with foreign material (particularly bacteria).

In the first case, interaction of properdin with host sulphated proteoglycans might provide a means of regulating complement activation at sites of tissue injury and infection (changes in glycosaminoglycan synthesis, secretion, and shedding are known to occur in these situations (Klein *et al.*, 1992)). The proximity of the sulphatide binding site and the C3bBb binding region suggested by the work in this thesis (figure 9.2) may explain how sulphated glycoconjugates are able to inhibit properdin function (see introduction) (Weiler *et al.*, 1978; Wilson *et al.*, 1984). Immobilization on sulphated proteoglycans on cell surfaces or in the extracellular matrix is now of recognized importance in modulating the local concentration of various cytokines, and thus the adhesion of leukocytes (Tanaka *et al.*, 1993). It is also possible that binding to sulphated glycoconjugates might be important in the interaction of properdin with cell surface receptors. Heparan sulphate is required for basic-FGF binding to cellular receptors and its mitogenic action (Rapraeger *et al.*, 1991), and cell-associated proteoheparan sulphate mediates binding and endocytosis of thrombospondin by vascular endothelial cells (Schön *et al.*, 1992). A potential properdin receptor has been identified on U937 cells (Goundis, 1988). Thus properdin could be involved in the uptake of complement opsonized material by phagocytes. Reduced granulocyte chemotaxis and phagocytic uptake of *Neisseria meningitidis* has indeed been observed in properdin-deficient serum (Söderström *et al.*, 1991). However, it is difficult to divorce the action of properdin in increasing C3b deposition on the bacterial surface from a direct interaction with cellular receptors in these studies.

In the second case, properdin could interact directly with molecules on the surface of bacterial cells. A role for properdin in initiating the alternative pathway has been proposed by some workers (Griffiss *et al.*, 1991; Griffiss and Jarvis, 1992). It has recently been suggested that the immobilization of cytokines on bacterial cells might promote their killing by leukocytes (George, 1994). Bacteria can express glycosaminoglycan-like surface molecules. The K5 capsule of *E. coli* has a polysaccharide with backbone structure similar to that of heparin, although it is not sulphated (Vann *et al.*, 1981). Sulphated glycoconjugates have been reported on the surface of *Halobacteria* (Wieland *et al.*, 1980) and *Chlamydia* (Zang and Stephens,

1992). *Mycobacteria* possess sulphatide-like glycolipids (Goren *et al.*, 1976). Thus binding of properdin to such structures on bacterial surfaces could enhance activation of the alternative complement pathway. However, sulphated sugars have not been reported, to my knowledge, on the surface of *Neisseria* (Verheul *et al.*, 1993), the main cause of infection in properdin deficient patients. The sulphated sugar binding capacity of properdin may represent an interesting future direction of properdin research.

9.8 Future studies

Future work will include the deletion of TSRs1 and 2. These modules will be removed together since they are likely to be linked by an intermodular disulphide bond (see chapter 3). A mutant lacking the N-terminal region should also be generated. These constructs may provide some insight into the important modules for oligomerization and C3b binding within the N-TSR1-TSR2 region. Furthermore, they should allow the epitopes of the inhibitory monoclonal antibodies against properdin to be mapped more precisely.

Smaller deletions and point mutations within the TSRs of properdin will provide information on the sequence motifs which are important for the various activities. Mutagenesis of basic residues in TSR5 might reveal those which are required to form the sulphated sugar binding site. Mutations within the exposed loop of TSR5 will help to determine the function of this region in C3b binding. It will be interesting to see if point mutations in TSR4 are able to prevent properdin from stabilizing the C3bBb complex without altering C3b binding activity. In the longer term it might be possible to identify regions within properdin with complement-modulatory activity for therapeutic use.

Since TSR3 can be removed from properdin with little alteration in the structure or function of the molecule, it is very likely that this region forms an independently folded unit. TSR3 of properdin may be a good choice of thrombospondin type I repeat for expression in bacterial or yeast systems, in order to obtain sufficient quantities for solution structure studies by 2D-NMR. This information would be useful since similar modules are found in a wide variety of proteins, and the structure is, at present, unknown.

References

References

Abramson, N., Alper, C.A., Lachmann, P.J., Rosen, F.S., and Jandl, J.H. (1971) Deficiency of C3 inactivator in man. J. Immunol. 107, 19

Adams, J.C. and Lawler, J. (1993a) The thrombospondin family. Curr. Biol. 3, 188-190.

Adams, J.C. and Lawler, J. (1993b) Diverse mechanisms for cell attachment to platelet thrombospondin. J.Cell Sci. 104, 1061-1071.

Alsenz, J., Lambris, J.D., Schulz, T.F. and Dierichi, M.P. (1984) Localization of the complement-component-C3b-binding site and the cofactor activity for factor I in the 38kDa tryptic fragment of factor H. Biochem. J. 224, 389-398.

Altschul, S.F., Gish, W., Miller, W. Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

American Type Culture Collection, (1991) *Catalogue of Cell Lines and Hybridomas.*

Arlaud, G.J., Thielens, N.M. and Illy, C. (1990) Arrangement of the C1 complex of complement. Biochem. Soc. Trans. 18, 1148-1151.

Armour, J.A.L., Wong, Z., Wilson, V., Royle, N.J. and Jeffreys, A.J. (1989) Sequences flanking the repeat arrays of human minisatellites: association with tandem and dispersed repeat elements. Nucl. Acid. Res. U, 4925-4935.

Arnot, D.E., Barnwell, J.W., Tam, J.P., Nussenzweig, V., Nussenzweig, R.S. and Enea, V. (1985) Circumsporozoite protein of *Plasmodium vivax*: Gene cloning and characterisation of the immunodominant epitope. Science 230, 815-818.

Asch, A.S., Liu, I., Briccetti, F.M., Barnwell, J.W., Kwakye-Berko, F., Dokun, A., Goldberger, J. and Pernambuco, M. (1993) Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. Science 262, 1436-1440.

Asch, A.S., Silbiger, S., Heimer, E. and Nachman, R.L. (1992) Thrombospondin sequence motif (CSVTCG) is responsible for CD36 binding. Biochem.Biophys.Acta. 182, 1208-1217.

Ashford, D.A., Alafi, C.D., Gamble, V.M., Mackay, D.J.G., Rademacher, T.W., Williams, P.J., Dwek, R.A., Barclay, A.N., Davis, S.J., Somoza, C., Ward, H.A., and Williams, A.F. (1993) Site specific glycosylation of recombinant rat and human CD4 variants in Chinese Hamster Ovary cells. J. Biol. Chem. 268, 3260-3267.

Atkinson, J.P. and Farries, T. (1987) Separation of self from non-self in the complement system. Immunology Today 8, 212-215.

Ausubel, F.M., Brent, R., Kingston, R.E., et al. *Short protocols in molecular biology*, Greene Publishing Associates with Wiley-Interscience, 1989.

Avery, V.M., Adrian, D.L. and Gordon, D.L. (1993) Detection of mosaic protein mRNA in human astrocytes. Immunol.Cell Biol. 71, 215-219.

Barlow, P.N., Norman, D.G., Steinkasserer, A., Horne, T.J., Pearce, J., Driscoll, P.C., Sim, R.B. and Campbell, I.D. (1992) Solution structure of the fifth repeat of factor H: a second example of the complement control protein module. Biochemistry 31, 3626-3634.

Barlow, P.N., Steinkasserer, A., Norman, D.G., Kieffer, B., Wiles, A.P., Sim, R.B., Campbell, I.D. (1993) Solution structure of a pair of complement modules by nuclear magnetic resonance. J. Mol. Biol. 232, 268-284.

Barton, G.J. and Sternberg, M.J.E. (1987a) Evolution and improvements in the automatic alignment of protein sequences. Protein Engineering 1, 89-94.

Barton, G.J. and Sternberg, M.J.E. (1987b) A strategy for the rapid multiple alignment of protein sequences: confidence levels from tertiary structure comparisons. J. Mol. Biol. 198, 327-337.

Bebbington, C.R. and C.C.G. Hentschel (1987) The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells. In *DNA cloning, a practical approach, volume III,* D.M. Glover, ed. IRL Press, Oxford, p.163-188.

Becherer, J.D., Alsenz, J. and Lambris, J.D. (1989) Molecular aspects of C3 interactions and structural/functional analysis of C3 from different species. Curr.Top.Microbiol.Immunol. 153, 45-72.

Beckmann, J.S. and Weber, J.L. (1992) Survey of human and rat microsatellites. Genomics 12, 627-631.

Berendt, A.R., Ferguson, D.J.P. and Newbold, C.I. (1990) Sequestration in Plasmodium falciparum. Malaria: sticky cells and sticky problems. Parasitology Today. 6, 247-254.

Borders, C.L., Broadwater, J.A., Bekeny, Salmon, J.E., Lee, A.S., Eldridge, A.M. and Pett, V.B. (1994) A structural role for arginine in proteins: multiple hydrogen bonds to backbone carbonyl oxygens. Protein Sci. 3, 541-548

Bork, P. (1993) The molecular architecture of a new family of growth related to connective tissue growth factor. FEBS Letts. 327, 125-130.

Bornstein, P. (1992) Thrombospondins: structure and regulation of expression. FASEB J. 6, 3290-3299.

Bornstein, P., Alfi, D., Devarayalu, S., Framson, P. and Li, P. (1990) Characterization of the mouse thrombospondin gene and evaluation of the role of the first intron in human gene expression. J.Biol.Chem. 265, 16691-16698.

Bornstein, P., Devarayalu, S., Li, P., Disteche, C.M. and Framson, P. (1991) A second thrombospondin gene in the mouse is similar in organisation to thrombospondin 1 but does not respond to serum. Proc.Natl.Acad.Sci.USA 88, 8636-8640.

Böttger, E.C., Hoffmann, T., Hadding, U. and Bitter-Suermann, D. (1985) Influence of genetically inherited complement deficiency on humoral response in guinea pigs. J. Immunol. 135, 4100-4107.

Bradham, D.M., Igarashi, A., Potter, R.L. and Grotendorst, G.R. (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J. Cell Biol. 114, 1285-1294.

Brankamp, R.G., Manley, G.D., Owen, T.J., Krstenansky, J.L., Smith, P.L. and Cardin, A.D. (1991) Demonstration that [$A^{103,106,108}$] antistasin 93-119 inhibits the specific binding of antistasin to sulphatide [Gal(3-SO_4)β1-1Cer). Biochem. Biophys. Res. Commun. 181, 246-251.

Brankamp, R.G., Manley, G.D., Owen, T.J., Krstenansky, J.L. and Cardin, A.D. (1992) Specific inhibition of binding of antistasin and [$A^{103,106,108}$] antistasin 93-119 to sulfatide (Gal(3-SO_4)β1-1Cer) by glycosaminoglycans. FEBS Letts. 296, 145-147.

Britten, R.J. (1994) Evidence that most human Alu sequences were inserted in a process that ceased about 30 million years ago. Proc. Natl. Acad. Sci. 91, 6148-6150.

Britten ,R.J., Baron, W.F., Stout, D.B. and Davidson, E.H. (1988) Sources and evolution of human Alu repeated sequences. Proc. Natl. Acad. Sci. 85, 4770-4774.

Brunner, A., Chinn, J., Neubauer, M. and Purchio, A.F. (1991) Identification of a gene family regulated by transforming growth factor-b. DNA Cell Biol. 10, 293-300.

Burton, D.R. (1990) Antibody: the flexible adaptor molecule. Trends Biochem. Sci. 15, 64-69.

Campbell, I.D. and Bork, P. (1993) Epidermal growth factor-like modules. Curr. Op. Struct. Biol. 3, 385-392.

Campbell, R.D., Law, S.K.A., Reid, K.B.M. and Sim, R.B. (1988) Structure, organization and regulation of the complement genes. Annu.Rev.Immunol. 6, 161-195.

Carrell, R.W., Stein, P.E., Fermi, G. and Wardell, M.R. (1994) Biological implication of a 3 Å structure of dimeric antithrombin. Structural 2, 257-270.

Carreno, M.P., Labarre, D., Maillet, F., Jozefowicz, M. and Kazatchkine, M.D. (1989) Regulation of the alternative complement pathway: formation of a ternary complex between factor H, surface-bound C3b and the chemical groups on nonactivating surfaces. Eur. J. Immunol. 19, 2145-2150.

Catimel, B., Leung, L., Ghissasi, H.E, Mercier, N. and McGregor, J. (1992) Human platelet glycoprotein IIIb binds to thrombospondin fragments bearing the C-terminal region, and/or the type I repeats (CSVTCG motif), but not to the N-terminal heparin-binding region. Biochem.J. 284, 231-236.

Cavener, D.R. and Ray, S.C. (1991) Eukaryotic start and stop translation sites. Nucleic Acids Research 19, 3185-3192.

Cerami, C., Kwakye-Berko, F. and Nussenzweig, V. (1992a) Binding of malarial circumsporozoite protein to sulfatides [Gal(3-SO_4)β1-Cer] and cholesterol-3-sulfate and its dependence on disulfide bond formation between cysteines in region II. Mol.Biochem Parasitol. 54, 1-12.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M.J. and Nussenzweig, V. (1992b) The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell 70, 1021-1033.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B. and Nussenzweig, V. (1994) Rapid clearance of malaria circumsporozoite protein (CS) by hepatocytes. J.Exp.Med. 179, 695-701.

Chen, C. and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol. 7, 2745-2752.

Chou, P.Y. and Fasman, G.D. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45-148.

Cockett, M.I., Bebbington, C.R., and Yarrington, G.T. (1990) High level expression of tissue inhibitor of metalloproteinases in Chinese Hamster ovary cells using glutamine synthetase gene amplification. Biotechnology 8, 662-667

Coleman, M.P., Murray, J.C., Willard, H.F., Nolan, K.F., Reid, K.B.M., Blake, D.J., Lindsay, S., Bhattacharaya, S.S., Wright, A. and Davies, K.E. (1991) Genetic and physical mapping around the properdin P gene. Genomics 11, 991-996.

Corden, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C. and Chambon, P. (1980) Promoter sequences of eukaryotic protein-coding genes. Science 209, 1406-1414.

Cornacoff, J.B., Hebert, L.A., Smead, W.L., Vanaman, M.E., Birmingham, D.J. and Waxman, F.J. (1983) Primate erythrocyte - immune complex-clearing mechanism. J. Clin. Invest. 71, 236.

Cowan, G., Krishnan, S., Crisanti, A. and Robson, K. (1992) Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. The Lancet 339, 1412-1413.

Cummings, R.D. (1994) Use of lectins in analysis of glycoconjugates. Methods in Enzymol. 230, 66-90.

Daha, M.R., Fearon, D.T., Austen, K.F. (1976) C3 requirements for formation of the alternative pathway C5 convertase. J. Immunol. 117, 630-634.

Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wirtz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.D., Schneider, I., Roberts, D., Sanders, G.S., Reddy, E.P., Diggs, C.L. and Miller, L.H. (1984) Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science 225, 593-599.

Daoudaki, M.E., Becherer, J.D. and Lambris, J.D. (1988) A 34-amino acid peptide of the third component of complement mediates properdin binding. J.Immunol. 140, 1577-1580.

Dardik, R. and Lahav, J. (1991) Cell binding of endothelial cell thrombospondin: localization to the 70 kDa core fragment and determination of binding characteristics. Biochemistry 30, 9378-9386.

Davidson, D.J. and Castellino, F.J. (1991) Asparagine-linked oligosaccharide processing in lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected spodoptera frugiperda (IPLB-SF-21AE) cells. Biochemistry 30, 6167-6174.

Davis, S.J., Ward, H.A., Puklavec, M.J., Willis, A.C., Williams, A.F., and Barclay, A.N. (1990) High level expression in Chinese Hamster Ovary cells of soluble forms of CD4 T lymphocyte glycoprotein including glycosylation variants. J. Biol. Chem. 265, 10410-10418.

Dayhoff, M.O., Schwarz, R.M., and Orcutt, B.C. (1979) A model of evolutionary change in proteins", in Atlas of Protein Sequence and Structure, vol. 5, suppl. 3, pp. 345-362 (M.O. Dayhoff, ed.). National Biomedical Research Foundation, Washington D.C., 1979.

Degan, S.J.F. and Davie, E.W. (1987) Nucleotide sequence for human prothrombin gene. Biochemistry 26, 6165-6167.

Densen, P., Weiler, J.M., Griffiss, J.M. and Hoffmann, L.G. (1987) Familial properdin deficiency and fatal meningococcemia. Correction of the bactericidal defect by vaccination. N.Engl.J.Med. 316, 922-926.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387-395.

DiScipio, R.D. and Hugli, T.E. (1989) The molecular architecture of human complement component C6. J.Biol.Chem. 264, 16197-16206.

DiScipio, R.G. (1981) The binding of human complement proteins C5, factor B, β_1H and properdin to complement fragment C3b on zymosan. Biochem.J. 199, 485-496.

DiScipio, R.G. (1982) Properdin is a trimer. Mol.Immunol. 19, 631-635.

DiScipio, R.G., Chakravarti, D.N., Müller-Eberhard, H.J. and Fey, G.H. (1988) The structure of human complement component C7 and C5b-7 complex. J.Biol.Chem. 263, 549-560.

DiScipio, R.G., Gehring, M.R., Podack, E.R., Kan, C.C., Hugli, T.E. and Fey, G.F. (1984) Nucleotide sequence of cDNA and derived amino acid sequence of human complement component C9. Proc.Natl.Acad.Sci.USA 81, 7298-7302.

Dixit, V.M., Grant, G.A., Santoro, S. and Frazier, W.A. (1983) Isolation and characterization of a heparin-binding domain from the amino terminus of platelet thrombospondin. J. Biol. Chem. 259, 10100-10105.

Dodds, A.W. (1993) Small scale preparation of complement components C3 and C4. Methods Enzymol. 223, 46-61.

Donehower, L.A., Slagle, B.L., Wilde, M., Darlington, G. and Butel, J.S. (1989) Identification of a conserved sequence in the non-coding regions of many human genes. Nucl. Acid. Res. 17, 699-710.

Dynan, W.S. (1986) Promoters for housekeeping genes. Trend. Genet. 2, 196-197.

Editorial (1988) Properdin deficiency. Lancet i, 95-96

Eichinger, D.J., Arnot, D.E., Tam, J.P., Nussenzweig, V. and Enea, V. (1986) Circumsporozoite protein of *Plasmodium berghei*: gene cloning and identification of the immunodominant epitopes. Mol.Cell.Biol. 6, 3965-3972.

Engel, Z., E. Odermatt, A. Engel, Z.A. Madri, H. Furthmayr, H. Rohde and R. Timpl (1981) Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. J. Mol. Biol. 150, 97-.

Evans, E.P., Burtenshaw, M.D., Laval, S.H., Goundis, D., Reid, K.B.M. and Boyd, Y. (1990) Localization of the properdin factor complement locus Pfc to band A3 on the mouse X chromosome. Genet.Res.,Camb. 56, 153-155.

Ezzell, J.L. and Parker, C.J. (1992) Cell-surface regulation of the human alternative pathway of complement. Sheep but not rabbit erythrocytes express factor I-dependent cofactor activity. Scand.J.Immunol. 36, 79-87.

Fanning, T.G. and Singer, M.F. (1987) LINE-1: a mammalian transposable repeat. Biochim. Biophys. Acta 910, 203-212.

Farries, T.C. and Atkinson, J.P. (1989) Biosynthesis of properdin. J.Immunol. 142, 842-847.

Farries, T.C., Finch, J.T., Lachmann, P.J. and Harrison, R.A. (1987) Resolution and analysis of "native" and "activated" properdin. Biochem.J. 243, 507-517.

Farries, T.C., Lachmann, P.J. and Harrison, R.A. (1988a) Analysis of the interactions between properdin, the third component of complement (C3), and its physiological activation products. Biochem.J. 252, 47-54.

Farries, T.C., Lachmann, P.J. and Harrison, R.A. (1988b) Analysis of the interaction between properdin and factor B, components of the alternative-pathway C3 convertase of complement. Biochem.J. 253, 667-675.

Fearon, D.T. (1978) Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Natl. Acad. Sci. USA. 75, 1971-1975.

Fearon, D.T. (1979) Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc. Natl. Acad. Sci. USA. 76, 5867-5871.

Fearon, D.T. (1993) The CD19-CR2-TAPA-1 complex, CD45 and signaling by the antigen receptor of B lymphocytes. Curr. Op. Immunol. 5, 341-348.

Fearon, D.T., Austen, K.F. and Ruddy, S. (1973) Formation of a hemolytically active cellular intermediate by the interaction between properdin factors B and D and the activated third component of complement. J.Exp.Med. 138, 1305-1313.

Fearon, D.T., Austen, K.F. and Ruddy, S. (1974a) Properdin factor D: characterisation of its active site and isolation of the precursor form. J.Exp.Med. 139, 355-366.

Fearon, D.T., Austen, K.F. and Ruddy, S. (1974b) Properdin factor D. II Activation to D by properdin. J. Exp. Med. 140, 426-436.

Fearon, D.T. and Austen, K.F. (1975a) Initiation of C3 cleavage in the alternative complement pathway. J. Immunol. 6, 1357-1361.

Fearon, D.T. and Austen, K.F. (1975b) Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. J.Exp.Med. 142, 856-863.

Fearon, D.T. and Austen, K.F. (1975c) Properdin: initiation of the alternate complement pathway. Proc. Natl. Acad. Sci. 72, 3220-3224.

Fearon, D.T. and Austen, K.F. (1977a) Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. Proc.Natl.Acad.Sci.USA 74, 1683-1687.

Fearon, D.T. and Austen, K.F. (1977b) Activation of the alternative complement pathway with rabbit erythrocytes by circumvention of the regulatory action of endogenous control proteins. J. Exp. Med. 146, 22-33.

Fernaud-Espinosa, I., Nieto-Sampedro and Bovolenta, P. (1994) Differential effects of glycosaminoglycans on neurite outgrowth from hippocampal and thalamic neurones. J. Cell Sci. 107, 1437-1448.

Figueroa, J., Andreoni, J. and Densen, P. (1993) Complement deficiency states and meningococal disease. Immunol. Res. 12, 295-311.

Figueroa, J.E. and Densen, P. (1991) Infectious diseases associated with complement deficiencies. Clinical Microbiology Reviews 4, 359-395.

Fishelson, Z. (1991) Complement C3: a molecular mosaic of binding sites. Molecular Immunology 28, 545-552.

Fourney, R.M., Miyakoshi, J., Day, R.S. and Patterson, M.C. (1988) Northern blotting: Efficient RNA staining and transfer. Bethesda Research Laboratories Focus 10 , 5-7.

Frazier, W.A. (1991) Thrombospondins. Current Opinion in Cell Biology 3, 792-799.

Fredrikson, G.N., Westberg, J., Uhlen, M., Sjoholm, A.G., and Truedsson, L. (1994) Type I properdin deficiency: a point mutation in exon five generates a stop codon. Clin. Exp. Immunol. 97 (suppl. 2), 37 (abstr.).

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B. and Nussenzweig, V. (1993) Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. J.Exp.Med. 177, 1287-1298.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc.Natl.Acad.Sci.USA 85, 8998-9002.

Galinski, M.R., Arnot, D.E., Cochrane, A.H., Barnwell, J.W., Nussenzwelg, R.S. and Enea, V. (1987) The circumsporozoite gene of the *Plasmodium cynomolgi* complex. Cell 48, 311-319.

Gahmberg, C.G. and Tolvanen, M. (1994) Nonmetabolic radiolabeling and tagging of glycoconjugates. Methods of Enzymology. 230, 33-37.

Garnier, J., Osguthorpe, D.J., and Robson, B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97-120.

George, A.J.T. (1994) Surface bound cytokines - a possible effector mechanism in bacterial immunity? Immunol. Today, 15, 88-89.

Gewurz, H., Shin, H.S., Mergenhagen, S.E. (1968) Interactions of the complement system with endotoxic lipopolysccharide: consumption of each of the six terminal components. J. Exp. Med. 128, 1049-1057.

Goodkofsky, I. and Lepow, I.H. (1971) Functional relationship of factor B in the properdin system to C3 proactivator of human serum. J.Immunol. 107, 1200-1204.

Goonewardena, P., Sjöholm, A.G., Nilsson, L-Å. and Petterson, U. (1988) Linkage analysis of the properdin deficiency gene: Suggestion of a locus in the proximal part of the short arm of the X chromosome. Genomics 2, 115-118.

Goren, M.B., Brokl, O., Roller, P., Fales, H.M., and Das, B.C. (1976) Sulfatides of Mycobacterium tuberculosis: the structureof the principal sulfatide (SL-1). Biochemistry, 15, 2728-2735.

Gotze, O., Medicus, R.G. and Müller-Eberhard, H.J. (1977) Alternative pathway of complement: nonenzymatic, reversible transition of precursor to active properdin. J.Immunol. 118, 525-528.

Gotze, O. and Müller-Eberhard, H.J. (1971) The C3-Activator system: an alternate pathway of complement activation. J. Exp. Med. 134, 90-108.

Gotze, O. and Müller-Eberhard, H.J. (1974) The role of properdin in the alternate pathway of complement activation. J.Exp.Med. 139, 44-57.

Goundis, D. (1988) *Structure, biosynthesis and cDNA cloning studies of properdin of the serum complement system*, DPhil. Thesis, University of Oxford.

Goundis, D., Holt, S.M., Boyd, Y. and Reid, K.B.M. (1989) Localization of the properdin structural locus to Xp11.23-Xp21.1. Genomics 5, 56-60.

Goundis, D. and Reid, K.B.M. (1987) Biosynthesis of complement proteins by the U937 cell line. Biochem. Soc. Trans. 15, 650-651.

Goundis, D. and Reid, K.B.M. (1988) Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. Nature 335, 82-85.

Gribskov, M. and Burgess, R.R. (1986) Nucl. Acids Res. 14, 6745-6763.

Griffiss, J.M., Jarvis, G.A., O'Brien, J.P., Eads, M.M. and Schneider, H. (1991) Lysis of *Neisseria gonorrhoeae* initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain specific, properdin-binding-dependant alternative complement pathway activation. J.Immunol. 147, 298-305.

Guo, N.H., Krutzsch, H.C., Nègre, E., Vogel, T., Blake, D.A. and Roberts, D.D. (1992a) Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. Proc.Natl.Acad.Sci.USA 89, 3040-3044.

Guo, N.H., Krutzsch, H.C., Nègre, E., Zabrenetcky, V.S. and Roberts, D.D. (1992b) Heparin binding peptides from the type I repeats of thrombospondin. J. Biol. Chem. 267, 19349-19355.

Haefliger, J.A., Tschopp, J., Naedelli, D., Wahli, W., Kocher, H.P., Tosi, M. and Stanley, K.K. (1987) Complementary DNA cloning of complement C8β and its sequence homology to C9. Biochemistry 26, 3551-3556.

Hedstrom, R.C., Campbell, J.R., Leef, M.L., Charoenvit, Y., Carter, M., Sedegah, M., Beaudoin, R.L. and Hoffman, S.L. (1990) A malaria sporozoite surface antigen distinct from the circumsporozoite protein. WHO Bulletin OMS 68 (supplement), 152-157.

Heery, D.M., Gannon, F. and Powell, R. (1990) A simple method for subcloning DNA fragments from gel slices. TIG 6, 173.

Hemsley, A., N. Arnheim, M.D. Toney, G. Cortopassi and D.J. Galas. 1989. A simple method for site directed mutagenesis using the polymerase chain reaction. Nuc. Acid Res. 17, 6545-6551.

Hendrikson, R.L. and Meredith, S.L. (1984) Amino acid analysis by reverse phase high performance liquid chromatography: precolumn derivitization with phenylisothiocyanate. Anal. Biochem. 136, 65-74.

Hobart, M.J., Fernie, B. and DiScipio, R.G. (1993) Structure of the human C6 gene. Biochemistry. 32, 6198-6205.

Holmskov, U., Malhotra, R., Sim, R.B. and Jensenius, J.C. (1994) Collectins: collagenous C-type lectins of the innate immune defense system. Immunology Today. 15, 67-73.

Holt, G.D., Krivan, H.C., Gasic, G.J. and Ginsburg, V. (1989) Antistatin, an inhibitor of coagulation and metastasis, binds to sulfatide (Gal(3-SO_4)β1-1Cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates. J.Biol.Chem. 264, 12138-12140.

Holt, G.D., Pangburn, M.K. and Ginsburg, V. (1990) Properdin binds to sulfatide [Gal(3-SO_4)β1-1Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. J.Biol.Chem. 265, 2825-2855.

Hoppe, H-J. and Reid, K.B.M. (1994) Collectins - soluble proteins containing collagenous regions and lectin domains - and their roles in innate immunity. Protein Sci. 3, 1143-1158.

Hubbard, S.J., Eisenmenger, F. and Thornton, J.M. (1994) Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. Prot. Sci. 3, 757-768.

Hugli, T.E. (1985). In Müller-Eberhard, H-J and Miescher, P.A. (eds), Complement. Springer-Verlag, Berlin, p.73.

Horstmann, R.D. and Müller-Eberhard, H.J. (1986) Demonstration of C3b receptor-like activity and of decay-accelerating factor-like activity on rabbit erythrocytes. Eur. J. Immunol. 16, 1069-1073.

Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T. and Yamashina, I. (1987) Serum lectin with known structure activates complement through the classical pathway. J. Biol. Chem. 262, 483-489.

Ish, C., Ong, G.L., Desai, N. and Mattes, M.J. (1993) The specificity of alternative complement pathway-mediated lysis of erythrocytes: a survey of complement and target cells from 25 species. Scand.J.Immunol. 38, 113-122.

Ishiguro, H., Kobayashi, K., Suzuki, M., Titani, K., Tomonaga, S. and Kurosawa, Y. (1992) Isolation of a hagfish gene that encodes a complement component. EMBO J. 11, 829-837.

Ishii, N., Wadsworth, W.G., Stern, B.D., Culotti, J.G. and Hedgecock, E.M. (1992) UNC-6, a laminin-related protein, guides cell and pioner axon migrations in C.elegans. Neuron. 9, 873-881.

Jackson, R.L., Busch, S.J. and Cardin, A.D. (1991) Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. Physiol. Rev. 71, 481-539.

Jarvis, G.A. and Griffiss, J.M. (1992) Characterization of properdin as an initiator of the alternative complement pathway activation. 8th International Congress of Immunology Abtstracts. Springer-Verlag, Budapest, p.177 (abstr.)

Jeffreys, A.J. (1987) Highly variable minisatellites and DNA fingerprints. Biochem. Soc. Trans. 15, 309-317.

Jennemann, R., Schulze, M., Bauer, B.L., Kurtz, C. and Wiegandt, H. (1994) Serum immunoglobulins in Heymann's experimental nephritis modulate binding of properdin and factor-H to sulpho-glycosphingolipids $II^3SO3^{(-)}$-Gg_3Cer and $III^3SO_3^{(-)}$-,$II^3SO^{(-)}$-Gg_3Cer. J.Biochem. 116, 450-456.

Joliot, V., Martinerie, C., Dambrine, G., Plassiart, G., Brisac, M., Crochet, J. and Perbal, B. (1992) Proviral rearrengements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. Mol. Cell. Biol. 12, 10-21.

Jurka, J. (1990) Novel families of interspersed repetitive elements from the human genome. Nucl. Acid. Res. 18, 137-141.

Jurka, J. (1993) A new subfamily of recently transposed human Alu repeats. Nucl. Acid. Res. 21, 2252.

Jurka, J. and Milosavljevic, A. (1991) Reconstruction and analysis of human Alu genes. J. Mol. Evol. 32, 105-121.

Jurka, J. and Smith, T. (1988) A fundamental division in the *Alu* family of repeated sequences. Proc.Natl.Acad.Sci.USA 85, 4775-4778.

Jurka, J. Walichiewicz, J. and Milosavljevic, A. (1992) Prototypic sequences for human repetitive DNA. J. Mol. Evol. 35, 286-291.

Kaplan, D.J., Jurka, J., Solus, J.F. and Duncan, C.H. (1991) Medium reiteration frequency repetitive sequences in the human genome. Nucl. Acid. Res. 19, 4731-4738.

Kazatchkine, M.D., Fearon, D.T. and Austen, K.F. (1979) Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β1H for cell bound C3b. J.Immunol. 122, 75-81.

Kazatchkine, M.D., Fearon, D.T., Silbert, J.E. and Austen, K.F. (1979) Surface-associated heparin inhibits zymosan-induced activation of the human alternative complement pathway by augmenting the regulatory action of the control proteins on particle-bound C3b. J.Exp.Med. 150, 1202-1215.

Kazatchkine, M.D., Fearon, D.T., Metcalfe, D.D., Rosenberg, R.D. and Austen, K.F. (1981) Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. J. Clin. Invest. 67, 223-228.

Klar, A., Baldassare, M. and Jessel, T.M. (1992) F-Spondin: A gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. Cell. 69, 95-110.

Klein, N.J., Shennan, G.I., Heyderman, R.S. and Levin, M. (1992) Alteration in glycosaminoglycan metabolism and surface charge on human umbilical vein endothelial cells induced by cytokines, endotoxin and neutrophils. J. Cell Sci. 102, 821-832.

Klickstein, L.B., Bartow, T.J., Miletic, V., Rabson, L.D., Smith, J.A. and Fearon, D.T. (1988) Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. J.Exp.Med. 168, 1699-1717.

Koistinen, V. (1993) Effects of sulphated polyanions on functions of complement factor H. Mol.Immunol. 30, 113-118.

Kölble, K., A.J. Cant, A.C. Fay, K. Whaley, M. Schlesinger and K.B.M. Reid. (1993) Carrier detection in families with properdin deficiency by microsatellite haplotyping. J. Clin. Invest. 91, 99-102.

Konno, T., Hirai, H. and Tamura, N. (1978) Binding of activated properdin to untreated erythrocytes. A new function of activated properdin. Immunology 34, 207.

Kozak, M. (1991) An analysis of vertebrate mRNA sequences: Intimations of translation control. J.Cell.Biol. 115, 887-903.

LaBell, T.L. and Byers, P.H. (1993) Sequence and characterization of the complete human thrombospondin 2 cDNA: potential regulatory role for the 3' untranslated region. Genomics 17, 225-229.

Labuda, D. and Striker, G. (1989) Sequence conservation in Alu evolution. Nucl. Acid. Res. 17, 2477-2491.

Lachmann, P.J. (1991) The control of homologous lysis. Immunology Today 12, 312-315.

Laemmlli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lal, A.A., La Cruz, V.F., Campbell, G.H., Procell, P.M., Collins, W.E., and McCutchan, T.F. (1988) Circumsporozoite protein gene from *Plasmodium malariae*. Mol. Biochem. Parasitol. 30, 291-294.

Lal, A.A. and Goldman, I.F. (1991) Circumsporozoite protein gene from *Plasmodium reichenowi*, a chimpanzee malaria parasite evolutionarily related to the human malaria parasite *Plasmodium falciparum*. J.Biol.Chem. 266, 6686-6689.

Lal, A.A., de la Cruz, V.F., Collins, W.E., Campbell, G.H., Procell, P.M. and McCutchan, T.F. (1988) Circumsporozoite protein from *Plasmodium brasilianum*. Animal reservoirs for human malaria parasites? J.Biol.Chem. 263, 5495-5498.

Lal, A.A., de la Cruz, V.F., Welsh, J.A., Charoenvit, Y., Maloy, W.L. and McCutchan, T.F. (1987) Structure of the gene encoding the circumsporozoite protein of *Plasmodium yoelii.* A rodent model for examining antimalarial sporozoite vaccines. J.Biol.Chem. 262, 2937-2940.

Lambris, J.D., J. Alsenz, T.F. Schulz and M.P. Dierich (1984) Mapping of the properdin-binding site of in the third component of complement. Biochem J. 217, 323-.

Lambris, J.D., Z. Lao, J. Pang and J. Alsenz (1993) Third component of trout complement. cDNA cloning and conservation of functional sites. J. Immunol. 151, 6123-.

Lander, A.D. (1994) Targeting the glycosaminoglycan binding sites on proteins. *Chemistry and Biology 1:73.*

Laval, S.H., Chen, Z.-Y. and Boyd, Y. (1991) The properdin structural locus (Pfc) lies close to the locus for tissue inhibitor of metallothionine protease (Timp) on the mouse X chromosome. Genomics 10, 1030-1034.

Law, S.K.A., and Levine, R.P. (1977) Interaction between the third component of complement and cell surface macromolecules. Proc. Natl. Acad. Sci. 74, 2701-2705.

Law, S.K.A. and Reid, K.B.M. (1988) Complement. In: *"In Focus" series,* edited by Male, D. Oxford, UK: IRL Press, 1988.

Lawler, J., Duquette, M. and Ferro, P. (1991) Cloning and sequencing of chicken thrombospondin. J.Biol.Chem. 266, 8039-8043.

Lawler, J., Duquette, M., Ferro, P., Copeland, N.G., Gilbert, D.J. and Jenkins, N.A. (1991) Characterization of the murine thrombospondin gene . Genomics 11, 587-600.

Lawler, J. and Hynes, R.O. (1986) The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. J.Cell.Biol. 103, 1635-1648.

Lepow, I.H. (1980) Presidential address to American Association of Immunologists in Anaheim, California, April 16, 1980: Louis Pillemer, properdin and scientific controversy. J.Immunol. 125, 471-478.

Leung-Hagesteijn, C., Spence, A.M., Stern, B.D., Zhou, Y., Su, M., Hedgecock, E.M. and Culotti, J.G. (1992) UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. Cell. 71, 289-293.

Liao, T., Gallop, P.M. and Blumenfeld, O.O. (1973) Modification of sialyl residues of sialoglycoprotein(s) of the human erythrocyte surface. J. Biol. Chem. 248, 8247-8253.

Lis, H. and Sharon, N. (1993) Protein glycosylation: structural and functional aspects. Eur. J. Biochem. 218, 1-27.

Lu, J., Thiel, S., Wiedemann, H., Timpl, R. and Reid, K.B.M. (1990) Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2$ $C1s_2$ complex, of the classical pathway of complement, without involvement of C1q. J.Immunol. 144, 2287-2294.

Malhotra, R., Thiel, S., Reid, K.B.M. and Sim, R.B. (1990a) Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J.Exp.Med. 172, 955-959.

Malhotra, R., Sim, R.B. and Reid, K.B.M. (1990b) Interaction of C1q, and other proteins containing collagen-like domains, with the C1q receptor. Biochem. Soc. Trans. 18, 1145-1148.

Marcus, R.L., Shin, H.S., Mayer, M.M. (1971) An alternate complement pathway: C3 cleaving activity, not due to C4b2a on endotoxic lipopolysaccharide after treatment with guinea pig serum; relation to properdin. Proc. Natl. Acad. Sci. 68, 1351-1354.

Margalit, H., Nadir, E. and Ben-Sasson, S.A. (1994) A complete Alu element within the coding sequence of a central gene. Cell 78, 173-174.

Martinerie, C., Viegas-Pequignot, E., Guenard, I., Dutrillaux, B., Nguyen, V.C., Bernheim, A. and Perball, B. (1992) Physical mapping of human loci homologous to the chicken nov proto-oncogene. Oncogene. 7, 2529-2534.

Mallet, S. and Barclay, A.N. (1991) A new superfamily of cell surface proteins related to the nerve growth factor receptor. Immunol. Today 12, 220-223.

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262, 10035-10038.

Matsushita, M. and Fujita, T. (1992) Activation of the classical complement pathway by mannose binding protein and a novel C1s-like serine protease. J. Exp. Med. 176, 1497-1502.

Maves, K.K., Moser, D.R., Densen, P. and Weiler, J.M. (1991) Comparison of properdin sequences in three species shows conservation of thrombospondin repeats. Complement and Inflammation 8, 191-192. (Abstract)

Maves, K.K. and Weiler, J.M. (1992) Detection of properdin mRNA in human peripheral blood monocytes and spleen. J. Lab. Clin. Med. 120, 762-766.

Maves, K.K. and Weiler, J.M. (1993) Hep G2 cells produce functional properdin. Clinical Res. 41, No.3 (abstr.)

Mayes, J.T., Schreiber, R.D., and Cooper, N.R. (1984) Development and application of an ELISA for quantitation of aternative complement pathway activation in human serum. J. Clin. Invest. 73, 160.

Medicus, R.G., Esser, A.F., Fernandez, H.N. and Müller-Eberhard, H.J. (1980) Native and activated properdin: interconvertibility and identity of amino- and carboxy-terminal sequences. J.Immunol. 124, 602-606.

Medicus, R.G., Gotze, O. and Müller-Eberhard, H.J. (1976) Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway. J.Exp.Med. 144, 1076-1093.

Medicus, R.G., Schreiber, R.D., Götze, O. and Müller-Eberhard, H.J. (1976) A molecular concept of the properdin pathway. Proc.Natl.Acad.Sci.USA 73, 612-616.

Meri, S. and Pangburn, M.K. (1990) Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. Proc.Natl.Acad.Sci.USA 87, 3982-3986.

Meri, S. and Pangburn, M.K. (1994) Regulation of alternative pathway complement activation by glycosaminoglycans: specificity of the polyanion binding site on factor H. Biochem.Biophys.Res.Commun. 198, 52-59.

Michalek, M.T., Bremer, E.G. and Mold, C. (1988) Effect of gangliosides on activation of the alternative pathway of human complement. J. Immunol. 140, 1581-1587.

Minta, J.O. (1976) Purification of native properdin by reversed affinity chromatography and its activation by proteolytic enzymes. J. Immunol. 117, 405-412.

Minta, J.O. (1987) Biosynthesis of properdin by U-937 cells and Hep-G2 cells. Fed.Proc. 46, 771. (abstr.)

Minta, J.O. and Lepow, I.H. (1974) Studies on the sub-unit structure of human properdin. Immunochemistry 11, 361-368.

Minta, J.O. and Kunar, E.S. (1976) Effect of proteolytic digestion on the structure and function of human properdin. J.Immunol. 116, 1099-1104.

Minta, J.O. and Pambrun, L. (1985) Biosynthesis of complement proteins by U-937 cells. Fed.Proc. 44, 991. (abstr.)

Morgan, B.P. and Walport, M.J. (1991) Complement deficiency and disease. Immunology Today 12, 301-306.

Müller, H-M., Reckmann, I., Hollingdale, M.R., Bujard, H., Robson, K.J.H. and Crisanti, A. (1993) Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. EMBO J. 12, 2881-2889.

Müller-Eberhard, H.J. and Gotze, O. (1972) C3 proactivator convertase and its mode of action. J.Exp.Med. 135, 1003-1008.

Naff, G.B., Hedrick, S.W., Ratnoff, W.D., Novak, M.A., Smith, M.C., Steiner, C. and Todd, E.W. (1980) The rabbit properdin system. J. Immunol. 124, 2625-2631.

Nakano, Y., Matsuda, T., Sakamoto, T. and Tomita, M. (1986) Isolation and charaterization of rabbit properdin of the alternative complement pathway. J.Immunol.Meth. 90, 77-83.

Narayana, S.V.L., Moore, D., DeLucas, L.J., Kilpatrick, J.M., Yamauchi, Y., Macon, K.J., and Volanakis, J.E. (1993) Unique structural features of human Factor D and its zymogen form. Mol. Immunol. 30 (suppl. 1), 37 (abstr.)

Needleman, S.B. and Wunsch, C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. 48, 443-453.

Nelson, R.A.,Jr. (1958) An alternative mechanism for the properdin system. J.Exp.Med. 108, 515.

New York Times (1954) Key to innate immunity to disease believed found in blood protein. August 25, p.29.

Ng, Y.C. and Schifferli (1993) Complement, immune complexes and immune complex disease. In Complement in Health and Disease. Whaley, K., Loos, M and Weiler, J.M. (eds) Kluwer Academic Publishers, Dordrecht, The Netherlands. p.199-228.

Nicol, P.A.E. and Lachmann, P.J. (1973) The alternative pathway of complement activation. Immunology. 24, 259-275.

Nicholson-Weller, A., Bunge, J. and Austen, K.F. (1981) Purification from guinea pig erythrocyte stroma of a decay accelerating factor for the classical C3 convertase, C4b2a. J. Immunol. 127, 2035

Nicholson-Weller, A., Bunge, J., Fearon, D.T., Weller, P.F. and Austen, K.F. (1982) Isolation of a human erythrocyte membrane glycoprotein with decay accelerating activity for C3 convertases of the complement system. J. Immunol. 129, 184.

Nielsen, H.E. and Koch, C. (1987) Congenital properdin deficiency and meningococcal infection. Clinical Immunology and Immunopathology 44, 134-139.

Nolan, K.F. (1991) *Studies at the DNA level of human properdin.* DPhil. Thesis, University of Oxford.

Nolan, K.F. and Reid, K.B.M. (1990) Complete primary structure of human properdin: a positive regulator of the alternative pathway of the serum complement system. Biochem.Soc.Trans. 18, 1161-1162.

Nolan, K.F., Schwaeble, W., Kaluz, S., Dierich, M.P. and Reid, K.B.M. (1991) Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. Eur.J.Immunol. 21, 771-776.

Nolan, K.F., Kaluz, S., Higgins, J.M.G., Goundis, D. and Reid, K.B.M. (1992) Characterization of the human properdin gene. Biochem.J. 287, 291-297.

Nolan, K.F. and Reid, K.B.M. (1993) Properdin. Methods Enzymol. 223, 35-47.

Nonaka, M., Fujii, T., Kaidoh, T., Natsuume-Sakai, S., Yamaguchi, N. and Takahashi, M. (1984) Purification of a lamprey component protein homologous to the third component of mammalian complement system. J.Immunol. 133, 3242-3249.

Norman, D.G., Barlow, P.N., Baron, M., Day, A.J., Sim, R.B. and Campbell, I.D. (1991) Three-dimensional structure of a complement control protein module in solution. J.Mol.Biol. 219, 717-725.

Nutt, E., Gasic, T., Rodkey, J., Gasic, G.J., Jacobs, J.W., Friedman, P.A. and Simpson, E. (1988) The amino acid sequence of antistasin. J. Biol. Chem. 263, 10162-10167.

O'Brien, T.P., Yang, G.P., Sanders, L. and Lau, L.F. (1990) Expression of cyr61, a growth factor-inducible immediate-early gene. Mol. Cel. Biol. 10, 3569-3577.

Okada, N. (1991) SINEs. Curr. Opin. Genet. Dev. 1, 498-504.

Okada, N., Yasuda, T. and Okada, H. (1982) Restriction of alternative complement pathway activation by sialosyl glycolipids. Nature 299, 261-263.

Oquendo, P., Hundt, E., Lawler, J., and Seed, B. (1989) CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. Cell 58, 95-101.

Ozaki, L.S., Svec, P., Nussenzweig, R.S., Nussenzweig, V. and Godson, G.N. (1983) Structure of the *Plasmodium knowlesi* gene coding for the circumsporozoite protein. Cell 34, 815-822.

Pangburn, M.K. (1989) Analysis of the natural polymeric forms of human properdin and their functions in complement activation. J.Immunol. 142, 202-207.

Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1977) Humancomplement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protease β1H for cleavage of C3b and C4b in solution.

Pangburn, M.K. and Müller-Eberhard, H.J. (1978) Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. Proc. Natl. Acad. Sci. USA. 75, 2416-2420.

Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1981) Formation of the initial C3 convertase of the alternative complement pathway. J. Exp Med. 154, 856-867.

Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1983) C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. J. Immunol. 131, 1930-1935.

Pangburn, M.K., Atkinson, M.A.L. and Meri, S. (1991) Localization of the heparin-binding site on complement factor H. J.Biol.Chem. 266, 16847-16853.

Pancake, S.J., Holt, G.D., Mellouk, S. and Hoffman, S.L. (1992) Malaria sporozoites and circumsporozoite proteins bind specifically to sulfated glycoconjugates. J. Cell Biol. 117, 1351-1357.

Pasamontes, L., Hug, D., Humbelin, M. and Weber, G. (1993) Sequence of a major Eimeeria maxima antigen homologous to the Eimeria tenella microneme protein Etp100. Mol. Biochem. Parasitol. 57, 171-174.

Patthy, L. (1987) Intron-dependent evolution: preferred types of exons and introns. FEBS Letts. 214, 1-7.

Patthy, L. (1988) Detecting distant homologies of mosaic proteins. J.Mol.Biol. 202, 689-696.

Pensky, J., Hinz, C.F., Todd, E.W., Wedgwood, R.J., Boyer, J.T. and Lepow, I.H. (1968) Properties of highly purified human properdin. J.Immunol. 100, 142-158.

Perkins, S.J., Nealis, A.S., Haris, P.I., Chapman, D., Goundis, D. and Reid, K.B.M. (1989) Secondary structure in properdin of the complement cascade and related proteins: A study by Fourier transform infrared spectroscopy. Biochemistry 28, 7176-7182.

Perkins, S.J., Smith, K.F., Haris, P.I., Chapman, D., Harrison, R.A. and Morgan, B.P. (1993) Analysis of the domains in C6, C7, C8 and C9 by FT-IR spectroscopy, scattering and molecular graphics. Mol. Immunol. 30 (Suppl. 1), 43 (abstr.).

Pillemer, L., Blum, L., Lepow, I.H., Ross, O.A., Todd, E.W. and Wardlaw, A.C. (1954) The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science 120, 279-285.

Platts-Mills, T.A.E. and Ishizaka, K. (1974) Activation of the alternate pathway of human complement by rabbit cells. J. Immunol. 113, 348-358.

Prater, C.A., Plotkin, J., Jaye, D. and Frazier, W.A. (1991) The properdin-like type I repeats of human thrombospondin contain a cell attachment site. J.Cell Biol. 112, 1031-1040.

Pryzdial, E.L.G. and Isenman, D.E. (1987) Alternative complement pathway activation fragment Ba binds to C3b. Evidence that formation of the factor B-C3b complex involves two discrete points of contact. J.Biol.Chem. 262, 1519-1525.

Pugh, B.F. and Tjian, R. (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell 61, 1187-1197.

Quentin, Y. (1989) Successive waves of fixation of B1 variants in rodent lineage history. J. Mol. Evol. 28, 299-305.

Quentin, Y. (1994) A master sequence related to a free left Alu monomer (FLAM) at the origin of the B1 family in rodent genomes. Nucl. Acid. Res. 22, 2222-2227.

Quigg, R.J. (1992) Inhibiton of the alternative pathway of complement by glomerular chondroitin sulphate proteoglycan. Immunology 76, 373-377.

Rao, A.G., Howard, O.M.Z., Ng, S.C., Whitehead, A.S., Colten, H.R. and Sodetz, J.M. (1987) Complementary DNA and derived amino acid sequence for the α subunit

of human complement protein C8: evidence for the existence of a separate α subunit mRNA. Biochemistry 26, 3556-3564.

Rapraeger, A.C., Krufka, A. and Olwin, B.B. (1991) Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science 252, 1705-1708.

Reed, K.C. and Mann, D.A. (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Research 13, 7207-7221.

Reid, K.B.M. (1971) Complement fixation by the $F(ab')_2$-fragment of pepsin-treated rabbit antibody. Immunology 20, 649-658.

Reid, K.B.M. (1981) Preparation of human properdin. Methods in Enzymology, 80, 143-150.

Reid, K.B.M. (1983) Proteins involved in the activation and control of the two pathways of human complement. Biochem. Soc. Trans. 11, 1-12.

Reid, K.B.M. and Gagnon, J. (1981) Amino acid sequence studies of human properdin-N-terminal sequence analysis and alignment of the fragments produced by limited proteolysis with trypsin and the peptides produced by cyanogen bromide treatment. Mol.Immunol. 18, 949-959.

Reid, K.B.M. and Day, A.J. (1989) Structure-function relationships of the complement components. Immunology Today 10, 177-180.

Rich, K.A., George IV, F.W., Law, J.L. and Martin, W.J. (1990) Cell-adhesive motif in region II of malarial circumsporozoite protein. Science 249, 1574-1577.

Roberts, D.D. (1987) Sulfatide-binding proteins. Methods in Enzymology. 138, 473-483.

Roberts, D.D., Haverstick, D.M., Dixit, V.M., Frazier, W.A., Santoro, S.A. and Ginsburg, V. (1985) The platelet glycoprotein thrombospondin bind specifically to sulfated glycolipids. J. Biol. Chem. 260, 9405-9411.

Robson, K.J.H., Hall, J.R.S., Jennings, M.W., Harris, T.J.R., Marsh, K., Newbold, C.I., Tate, V.E. and Weatherall, D.J. (1988) A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature 335, 79-82.

Rodriguez de Cordoba, S., Lublin, D.M., Rubinstein, P. and Atkinson, J. P. (1985) Human genes for three complements that regulate the activation of C3 are tightly linked. J. Exp. Med. 161, 1189-1195.

Rogers, W.O., Malik, A., Mellouk, S., Nakamura, K., Rogers, M.D., Szarfman, A., Gordon, D.M., Nussler, A.K., Aikawa, M. and Hoffman, S.L. (1992a) Characterization of *Plasmodium falciparum* sporozoite surface protein 2. Proc.Natl.Acad.Sci.USA 89, 9176-9180.

Rogers, W.O., Rogers, M.D., Hedstrom, R.C. and Hoffman, S.L. (1992b) Characterization of the gene encoding sporozoite surface protein 2, a protective Plasmodium yoelli sporozoite antigen. Mol. Biochem. Parasitol. 53, 45-52.

Rost, B. and Sander, C. (1992) Jury returns on structure prediction. Nature 360, 540.

Rost, B. and Sander, C. (1993) Prediction of protein structure at better than 70% accuracy. J. Mol. Biol. 232, 584-599.

Rost, B. and Sander, C. (1994) Combining evolutionary information and neural networks to predict protein secondary structures. Proteins 19, 55-72.

Ruddy, S. and Austen, K.F. (1971) C3b inactivator in man. II. Fragments produced by C3b inactivator cleavage of cell bound or fluid phase C3b. J. Immunol. 107, 742-750.

Ruiz I Altaba, A., Cox, C., Jessel, T.M. and Klar, A. (1993) Ectopic neutral expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. Proc. Natl. Acad. Sci. USA. 90, 8268-8272.

Ryseck, R., Macdonald-Bravo, H., Mattei, M. and Bravo, R. (1991) Structure, mapping, and expression of fisp-12, a grow factor-inducible gene encoding a secreted cysteine-rich protein. Cell Growth and Differentiation. 2, 225-233.

Sahu, A. and Pangburn, M.K. (1993) Identification of multiple sites of interaction between heparin and the complement system. Mol. Immunol. 30, 679-684.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor, N.Y.:Cold Spring Harbor, Laboratory Press, 1989.

San Antonio, J.D., Slover, J., Lawler, J., Karnovsky, M.J., Lander, A.D. (1993) Specificity in the interactions of extracellular matrix proteins with subpopulations of the glycosaminoglycan heparin. Biochemistry 32, 4746-4755.

Sandberg, A.L., Osler, A.G., Shin, H.S. and Oliveira, B. (1970) The biologic activities of guinea pig antibodies. II. Modes of complement interaction with γ1 and γ2 immunoglobulins. J. Immunol. 104, 329-334.

Sandberg, A.L., Oliveira, B. and Osler, A.G. (1971) Two complement interaction sites in guinea pig immunoglobulins. J. Immunol. 106, 282-285.

Sander, C and Schneider, R. (1991) Database of homology derived structures and the structural meaning of sequence alignment. Proteins 9, 56-68.

Schön, P., Vischer, P., Volker, W., Schmidt, A. and Faber, V. (1992) Cell-associated proteoheparan sulphatemediates binding and uptake of thrombospondin in cultured porcine vascular endothelial cells. Eur. J. Cell Biol. 59, 329-339.

Schlesinger, M., Mashal, U., Levy, J. and Fishelson, Z. (1993) Hereditary properdin deficiency in three families of Tunisian Jews. Acta Paediatr. 82, 744-777.

Schlesinger, M., Nave, Z., Levy, Y., Slater, P.E. and Fishelson, Z. (1990) Prevalence of hereditary properdin, C7 and C8 deficiencies in patients with meningococcal infections. Clin.Exp.Immunol. 81, 423-427.

Schreiber, R.D., Pangburn, M.K., Lesavre, P.H. and Müller-Eberhard, H.J. (1978) Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. Proc.Natl.Acad.Sci.USA 75, 3948-3952.

Schultz-Cherry, S., Lawler, J. and Murphy-Ullrich, J.E. (1994) The type 1 repeats of thrombosopndin 1 activate latent transforming growth factor-β. J.Biol.Chem. 269, 26783-26788.

Schwaeble, W., Dippold, W.G., Schafer, M.K.H., Pohla, H., Jonas, D., Luttig, B., Huemer, H.P., Dierich, M.P., Reid, K.B.M. (1993) Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells. J. Immunol. 151, 2521-2528.

Schwaeble, W., Huemer, H.P., Möst, J., Dierich, M.P., Ströbel, M., Claus, C., Reid, K.B.M., Ziegler-Heitbrock, H.W.L. (1994) Expression of properdin in human monocytes. Eur. J. Biochem. 219, 759-764.

Sehgal, A., Patil, N. and Chao, M. (1988) A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol.Cell.Biol. 8, 3160-3167.

Sepp, A., Dodds, A.W., Anderson, M.J., Campbell, R.D., Willis, A.C. and Law, S.K.A. (1993) Covalent binding properties of human complement protein C4 and hydrolysis rate of the internal thioester upon activation. Prot. Sci. 2, 706-716.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessel, T.M. and Tessier-Lavigne, M. (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell. 78, 409-424.

Seya, T., Turner, J. and Atkinson, J.P. (1986) Purification and characterization of a membrane protein (gp 45-70) that is a cofactor for cleavage of C3b and C4b. J.Exp.Med. 163, 837-855.

Sim, R.B. and Perkins, S.J. (1990) Molecular Modelling of C3 and its ligands. Curr. Top. Microbiol. 153, 209-222.

Simmons, D.L., Levy, D.B., Yannoni, Y. and Erikson, R.L. (1989) Identification of a phorbol ester-repressible v-src-inducible gene. Proc. Natl. Acad. Sci USA. 86, 1178-1182.

Sinnis, P., Clavijo, P., Fenyö, D., Chait, B.T., Cerami, C. and Nussenzweig, V. (1994) Structural and functional properties of region II-plus of the malaria circumsporozoite protein. J.Exp.Med. 180, 297-306.

Sjöholm, A.G., Braconier, J.-H. and Söderström, C. (1982) Properdin deficiency in a family with fulminant meningococcal infections. Clin.Exp.Immunol. 50, 291-297.

Sjöholm, A.G., Söderström, C. and Nilsson, L.-Å. (1988a) A second varient of properdin deficiency: the detection of properdin at low concentrations in affected males. Complement 5, 130-140.

Sjöholm, A.G., Kuijper, E.J., Tijssen, C.C., Jansz, A., Bol, P., Spanjaard, L. and Zanen, H.C. (1988b) Dysfunctional properdin in a Dutch family with meningococcal disease. New Eng.J.Med. 319, 33-37.

Smith, C.A., Vogel, C.W. and Müller-Eberhard, H.J. (1982) Ultrastructure of cobra venom factor-dependent C3/C5 convertase and its zymogen, factor B of human complement. J.Biol.Chem. 257, 9879-9882.

Smith, C.A., Pangburn, M.K., Vogel, C.W. and Müller-Eberhard, H.J. (1984a) Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. J.Biol.Chem. 259, 4582-4588.

Smith, C.A., Vogel, C.W. and Müller-Eberhard, H.J. (1984b) MHC class III products: an electron microscopy study of the C3 convertases of human complement. J. Exp. Med. 159, 324-329.

Smith, K.F., Nolan, K.F., Reid, K.B.M. and Perkins, S.J. (1991) Neutron and X-ray scattering studies on the human complement protein properdin provide an analysis of the thrombospondin repeat. Biochemistry 30, 8000-8008.

Sneath, P.H.A. and Sokal, R.R. (1973). In Numerical Taxonomy, W.H. Freeman & Co. San Fransisco, CA, pp. 230-234.

Söderström, C., Braconier, J.H., Sjöholm, A.G. and Thuresson, B. (1991) Granulocyte functions and Neisseria meningitidis: influence of properdin-deficient serum. APMIS. 99, 965-971.

Sonnhammer, E.L.L. and Kahn, D. (1994) Modular arrangement of proteins as inferred from analysis of homology. Prot. Sci. 3, 482-492.

Spano, F.S., L.R. Ranucci, F.C. Catteruccia, S.S. Saccheo & A.C. Crisanti. (1994). Thrombospondin related protein in *Cryptosporidium*. Adhesive protein with conserved amino acid motif involved in *Cryptosporidium* host cell interaction. *GenBank acc. nos X77586 & 77587.*

Stankowski, S., Wey, J., Kalb, E. and Goundis, D. (1991) Membrane interaction of 'peptide P' derived from the repeating motif of properdin. Biochim.Biophys.Acta 1068, 61-67.

Stephens, P.E. and Cockett, M.I. (1989) The construction of a highly efficient and versatile set of mammalian expression vectors. Nucleic Acids Research. 17, 7110.

Stoppa-Lyonnet, D., Carter, P.E., Meo, T. and Tosi, M. (1990) Clusters of intragenic Alu repeats predispose the human C1 inhibitor locus to deleterious rearrangements. Proc. Natl. Acad. Sci. 87, 1551-1555.

Sun, X., Mosher, D.F. and Rapraeger, A. (1988) Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin. J. Biol. Chem. 5, 2885-2889.

Super, M., Lu, J., Thiel, S., Levinsky, R.J. and Turner, M.W. (1989) Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet. 25, ii, 1236-1239.

Tack, B.F., Harrison, R.A., Janatova, J., Thomas, M.L. and Prahl, J.W. (1980) Evidence for presence of an internal thioester bond in the third component of complement. Proc.Natl.Acad.Sci.USA 77, 5764-5768.

Takagi, J., Fujisawa, T., Usui, T., Aoyama, T. and Saito, Y. (1993) A single chain 19-kDa fragment from bovine thrombospondin binds to type-V collagen and heparin. J.Biol.Chem. 268, 15544-15549.

Tanaka, Y., Abams, D.H. and Shaw, S. (1993) Proteoglycans on endothelial cells present adhesion inducing cytokines to leukocytes. Immunol. Today 14, 111-115.

Thiel, S. and Reid, K.B.M. (1989) Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS Letts. 250, 78-84.

Tkacz, J.S., and Lampen, J.O. (1975) Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. Biochem. Biophys. Res. Commun. 65, 248-257.

Tolsma, S.S., Volpert, O.V., Good, D.J., Frazier, W.A., Polverini, P.J. and Bouck, N. (1993) Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. J.Cell Biol. 122, 497-511.

Tomley, F.M., Clarke, L.E., Kawazoe, U., Dijkema, R. and Kok, J.J. (1991) Sequence of the gene encoding an immunodominant microneme protein of Eimeria tenella. Molecular and Biochemical Parasitology. 49, 277-288.

Tomlinson, S., Pontes de Carvalho, L., Vandekerckhove, F. and Nussenzweig, V. (1992) Resialylation of sialidase-treated sheep and human erythrocytes by *Trypanosoma cruzi* trans-sialidase: restoration of complement resistance of desialylated sheep erythrocytes. Glycobiology 2, 549-551.

Tomlinson, S., Stanley, K.K. and Esser, A.F. (1993) Domain structure, functional activity, and polyerization of trout complement protein C9. Dev.Comp.Immunol. 17, 67-76.

Tuszynski, G.P., Rothman, V.L., Deutch, A.H., Hamilton, B.K. and Eyal, J. (1992) Biological activities of peptides and peptide analogues derived from common sequences present in thrombospondin, properdin, and malarial proteins. J.Cell Biol. 116, 209-217.

Tuszynski, G.P., Rothman, V.L., Papale, M., Hamilton, B.K. and Eyal, J. (1993) Identification and characterization of a tumor cell receptor for CSVTCG, a thrombospondin adhesive domain. J.Cell Biol. 120, 513-521.

Vann, W.F., Schmidt, M.A., Jann, B., and Jann, K. (1981) The structure of the capsular polysaccharide (K5 antigen) of urinary tract infective Escherichia coli O10:K5:H4: a polymer similar to desulphoheparin. Eur. J. Biochem. 116, 359-364.

Verheul, A.F.M., Snippe, H., and Poolman, J.T. (1993) Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol. Rev. 57, 34-49.

Weiler, J.M., Daha, M.R., Austen, K.F., and Fearon, D.T. (1976) Control of the amplification convertase of complement by the plasma protein β1H. Proc. Natl. Acad. Sci. 73, 3268-3272.

Weiler, J.M., Yurt, R.W., Fearon, D.T., Austen, K.F. (1978) Modulation of the formation of the amplification convertase of complement, C3bBb, by native and commercial heparin. J. Exp. Med. 147, 409-421.

Weiler, J.M., Edens, R.E., Linhardt, R.J. and Kapelanski, D.P. (1992) Heparin and modified heparin inhibit complement activation in vivo. J.Immunol. 148, 3210-3215.

Whaley, K. (1980) Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. J.Exp.Med. 151, 501-516.

Whaley, K. and Ruddy, S. (1976a) C3b INA accelerator: a serum cofactor for C3b inactivator necessary for the full expression of its activity. J. Immunol., 116, 1754 (abstr.)

Whaley, K. and Ruddy, S. (1976b) Modulation of C3b haemolytic activity by a plasma protein distinct from C3b-inactivator. Science 193, 1011-1013.

Whiteman, L.Y., Purkall, D.B. and Ruddy, S. (1991) Association of activated properdin with complexes of properdin with C3. J.Immunol. 147, 1344-1351.

Wieland, F. Dompert, W., Bernhardt, G. and Sumper, M. (1980) Halobacterial glycoprotein saccharides contain covalently linked sulphate. FEBS Letts. 120, 110-114.

Wilson, J.G., Fearon, D.T., Stevens, R.L., Seno, N. and Austen, K.F. (1984) Inhibition of the function of activated properdin by squid chondroitin sulfate E glycosaminoglycan and murine bone marrow derived mast cell chondroitin sulfate E proteoglycan. J.Immunol. 132, 3058-3063.

Wolf, F.W, Eddy, R.L., Shows, T.B. and Dixit, V.M. (1990) Structure and chromosomal localization of the human thrombospondin gene. Genomics 6, 685-691.

Yang, G.P. and Lau, L.F. (1991) Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. Cell Growth and Differentiation. 2, 351-357.

Zammit, A., Pepper, D.S., and Dawes, J. (1993) Interaction of immobilized unfractionated and LMW heparins with proteins in whole human plasma. Thromb. Haemost. 70, 951-958.

Zang, J.P. and Stephens, R.S. (1992) Mechanism of *C. trachomatis* attachment to eukaryotic host cells. Cell 69, 861-869.