

Structural Biology of IgG Fc Glycoforms

Kavitha Baruah

A thesis submitted for the degree of
Doctor of Philosophy

Lincoln College
Department of Biochemistry
University of Oxford

Hilary Term 2012

Table of Contents

Abstract.....	4
Acknowledgments	5
1. Introduction	7
1.1. Human IgG antibodies and functions- an overview	7
1.2. Structure of IgG Fc.....	12
1.3. Glycosylation of IgG Fc	16
1.4. Glyco-engineering of Antibodies.....	20
1.5. Therapeutic antibodies with modified effector functions	23
1.6. Deglycosylated antibodies and bacterial endoglycosidases in immune evasion.....	26
2. Materials and Methods	29
2.1. Cloning of Human IgG1 Fc.....	29
2.2. Large Scale DNA purification	30
2.3. Protein Expression and purification	30
2.4. Protein crystallisation.....	31
2.5. Structure determination	33
2.6. Structure refinement and validation.....	34
2.7. Cloning and expression of chimeric full length mouse collagen Type II antibody with human IgG1 Fc domain	35
2.8. Mass Spectrometric analysis of Fc linked N-glycans	36
2.9. Surface Plasmon Resonance (SPR) analysis	39
2.10. Differential scanning fluorimetry (DSF) assays for thermal unfolding of IgG Fc	40
2.11. ELISA	40
2.12. Structural analysis and illustrations.....	41
3. Intradomain plasticity of human IgG1 Fc	43
3.1. Summary	43
3.2. Cloning and Expression of FcWT	43
3.3. Crystallisation and Structure Determination of FcWT	44
3.4. Mass spectrometric analysis of FcWT glycans.....	47
3.5. Structural analysis of FcWT	48
3.6. Protein-glycan interactions of FcWT	51
3.7. Thermal stability of FcWT	54
3.8. Conclusions	54

4. Structure of a mutated human IgG1 Fc with reduced protein-carbohydrate interactions ..	57
4.1. Summary	57
4.2. Purification of Fc mutants with reduced protein-carbohydrate interactions.....	58
4.3. Crystallisation and structure determination of F241A Fc	60
4.4. Structural analysis of F241A Fc.....	62
4.5. Sugar protein interactions and Glycan processing in the Fc mutant	64
5. Structure and protein-carbohydrate interactions of biosynthetic intermediate IgG1 Fc glycoforms.....	69
5.1. Summary	69
5.2. Cloning and Expression of Fc Man ₉ GlcNAc ₂ and Fc Hybrid	71
5.3. Crystallisation and Structure Determination of Fc Man ₉ GlcNAc ₂	72
5.4. Mass spectrometric analysis of of Fc Man ₉ GlcNAc ₂ glycans	75
5.5. Structural Analysis of Fc Man ₉ GlcNAc ₂	77
5.6. Protein-glycan interactions of Fc Man ₉ GlcNAc ₂	80
5.7. FcγRIIIa binding and thermal stability of Fc Man ₉ GlcNAc ₂	80
5.8. Crystallisation and Structure Determination of Fc Hybrid	83
5.9. Mass spectrometric analysis of Fc Hybrid glycans	86
5.10. Structural analysis of Fc Hybrid.....	88
5.11. Protein-glycan interactions of Fc Hybrid.....	91
5.12. Thermal stability and FcγRIIIa binding of Fc Hybrid	94
5.13. Conclusions	94
6. Deglycosylated IgG: Structure and therapeutic applications.....	99
6.1. Summary	99
6.2. Purification, Crystallisation and Structure determination of Deglycosylated IgG Fc.	100
6.3. Structural analysis of deglycosylated Human IgGFc (Fc ^{GlcNAc})	103
6.4. Thermal stability of Fc ^{GlcNAc}	106
6.5. Carbohydrate selectivity of Endo S	106
6.6. Enhanced activity of oligomannose containing monoclonal antibodies by Endo S treatment of serum IgG	109
6.7. Conclusions	110
7. Final conclusions and perspectives.....	112
Appendix.....	119
Abbreviations	120
References.....	123

Structural Biology of IgG Fc Glycoforms

Kavitha Baruah

A thesis submitted for the degree of Doctor of Philosophy

Lincoln College, Department of Biochemistry

University of Oxford, Hilary Term 2012

Abstract

The conserved N-linked glycosylation site on the Fc domain of IgG1 antibodies is essential for maintaining a functionally active conformation of the antibody. Different glycoforms of the Fc exhibit widely different effector functions. Similarly, therapeutic antibodies, with engineered glycosylation, exhibit altered binding to cellular Fc receptors (FcRs). Here, X-ray crystallographic structures were obtained for biosynthetic intermediate glycoforms of human IgG1 Fc bearing: unprocessed oligomannose-type, intermediate hybrid-type, and mature complex-type glycans. The fully processed Fc protein crystallised in an “open” conformation with glycans forming canonical stabilising interactions on the protein surface. Analysis of the biosynthetic intermediates revealed that these stabilising hydrophobic protein-glycan interactions are formed only after processing by Golgi α -mannosidase II. Mutagenesis of hydrophobic residues on Fc disrupted crucial protein-glycan interactions resulting in the selective destabilization of the 3-arm of the glycan chain with the 6-arm closely matching that seen for the native structure. However, carbohydrate analysis of released glycans shows increased processing on both arms indicating a more accessible and flexible glycan in the mutant structure suggesting that the crystallographic structure of these antibody glycans represents a minor low-energy conformation. The importance of Fc glycosylation is highlighted by endoglycosidases which eliminate Fc effector function. The crystallographic structure of enzymatically deglycosylated IgG Fc revealed a significant collapse of the of C γ 2 domains resulting in a ‘closed’ quaternary conformation, incompatible with Fc receptor binding. This provides a structural explanation for immune deactivating properties of endoglycosidases including those under preclinical development for the treatment of antibody-mediated immune pathology. One such bacterial endoglycosidase, Endo S, was studied further and revealed a specificity for complex-type glycans of the type found on IgG but no hydrolytic activity towards an engineered IgG Fc with oligomannose-type glycans. Introduction of both the engineered monoclonal IgG and endoglycosidase in serum led to a dramatic increase in FcR binding as the competitive binding of serum IgG for FcRs was selectively eliminated. This approach is a general technique for boosting the effector signal of therapeutic antibodies.

Acknowledgments

The work described in this thesis was conducted in the Dr Chris Scanlan's laboratory and I am most grateful to him and Dr Max Crispin for all their guidance, supervision and encouragement and for giving me the opportunity to work on this project. It has been a most valuable learning experience for me.

All X-ray crystallographic experiments described in this thesis were conducted in close collaboration with Dr Tom Bowden at the Division of Structural Biology and Dr Matthew Higgins at the Laboratory of Molecular Biophysics. I am most grateful to both of them for their valuable guidance and tutorials on X-ray crystallography. I am especially thankful to Tom for his help in particular with the structural refinements of the Fc Hybrid and Fc^{GlcNAc} structures and for his advice and guidance on structural analysis. I would also like to thank Jonny Goodfellow and Professor Ben Davis for providing recombinant Endo S for the experiments described in Chapter 6. I am most grateful to Professor David Harvey for his collaboration and advice with all the mass spectrometry experiments.

My DPhil studies at Oxford have coincided with most tumultuous life events and I am very grateful for the help, support and encouragement that I received during this time from several people. I am especially grateful to Professor Raymond Dwek for the personal support and encouragement he gave me to continue my studies. I would also like to specially thank all my lab colleagues- Camille Bonomelli, Dan Perez, Fatma Mansab, Snezana Vasiljevic, Ben Yu and Martin Dalziel for being so helpful and supportive at various times during my DPhil.

Finally, to the most important, my god parents Nalin Nirula and Renoo Nirula and my family - I am most grateful for your support and love. I would have never had the strength and without you this DPhil was an impossible dream.

CHAPTER 1

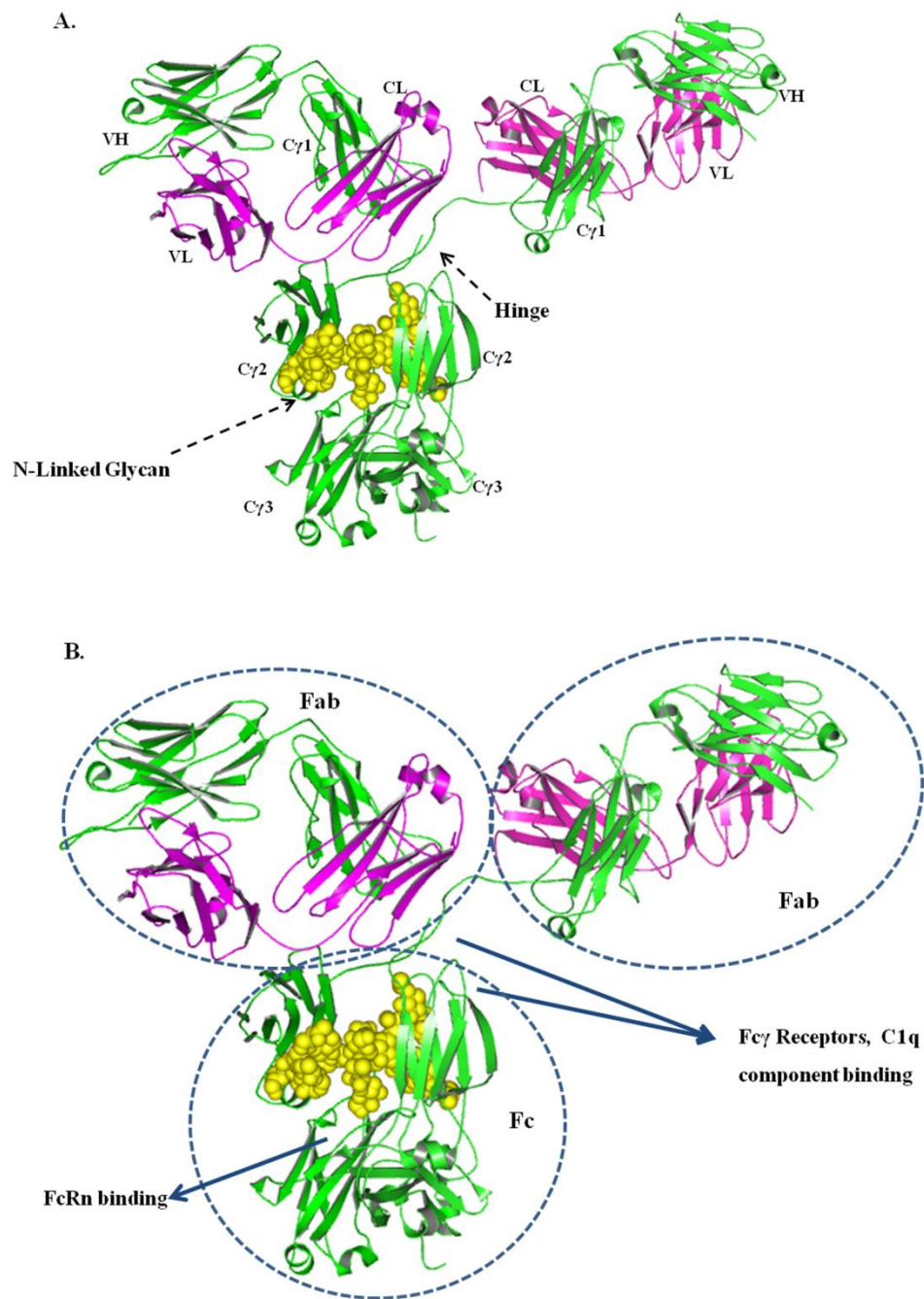
Introduction

1. Introduction

1.1. Human IgG antibodies and functions- an overview

Antibodies constitute the principal effector arm of the vertebrate adaptive immune system. The antibody molecule is classically described as a 'Y' shaped structure made up of two heavy chains and two light chains. In humans, there are two types of light chains (λ and κ) and five different types of heavy chains (μ , δ , γ , ϵ and α) and together they form five major classes of antibodies namely- IgM, IgD, IgG, IgE and IgA (1).

IgG antibodies form the largest subclass of antibodies in human serum. They comprise of two functional domains: the Fab or *fragment antigen binding* and the Fc or *fragment crystallisable* (Figure 1.1.1A) (2). While the Fab domain binds antigens through highly variable sequences of the variable domains, the Fc domain modulates effector functions through the engagement of soluble and cellular immune receptors. The Fab domain is made of the entire light chain (V_L and C_L) and the variable and first constant domains of the heavy chain (V_H and $C\gamma1$). The Fc domain on the other hand is made up entirely of the heavy chain constant sequence ($C\gamma2$ and $C\gamma3$ domains). Fab and Fc domains are connected by an extended peptide 'hinge' region which provides flexibility and which, through interchain disulphide bonds, provides further stability to the Fc dimer(3). The IgG Fc domain and hinge region form binding interfaces with Fc γ receptors, C1q component of complement system and the neonatal Fc receptor FcRn. Binding of Fc receptors and C1q component (4) activates complement dependent cellular cytotoxic responses while binding of FcRn modulates the serum half-life of antibodies and their transport across the placenta (Figure 1.1.1 B) (5-7). Amino acid residues of IgG Fc involved in binding Fc receptors and C1q have been identified by extensive mutational studies. Amino acid residues (Asp270, Lys322, Pro329, Pro331) of the B/C and F/G loops of IgG Fc form the binding interface with C1q, while the binding interface with Fc γ receptors is described in more detail in Section 1.3 (8-10). While X-ray crystallographic structures of IgG Fc, both free and in complex with Fc γ RIIIa are available, no structural studies on Fc-C1q binding have been reported to date(6). Residues of the Fc domain constituting the binding site of FcRn have been identified by mutational studies to be located in the $C\gamma2$ - $C\gamma3$ interface (11).



Human IgG antibodies are further divided into four subclasses based on their serum concentration. IgG1, 2, 3 and 4 differ from each other mainly in the length and composition of the hinge region (Figure 1.1.2). IgG1 and IgG3 exist mainly as a monomer *in vivo* while IgG2 is known to form covalent dimers with intermolecule disulphide bonds (13). IgG4 is known to alternate between a monospecific molecule with normal disulphide bonds, a half molecule with intrachain disulphide bonds and a bispecific form (14). IgG isotypes also differ in their affinity for various cellular Fc receptors and thus in their ability to elicit different effector functions (Table 1.1.1) (15).

Five classes of Fc receptors specific for IgG are known in humans: FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb (Figure 1.1.3) (16). FcγRI is the only high affinity receptor and comprises of three extracellular immunoglobulin like domains, a short membrane spanning domain and an intracellular immunoreceptor tyrosine based activation motif (ITAM) (17). The ITAM domain is common to all activatory Fcγ receptors- FcγRI, FcγRIIa, FcγRIIc and FcγRIIIa. Human FcγRIIIb exists with a glycosyl-phosphatidyl inositol membrane anchor in neutrophils only and does not contain a cytoplasmic activation motif. Activatory Fcγ receptors are found on most immune effector cells- monocytes, macrophages, NK cells, mast cells, eosinophils and platelets. Activatory Fcγ receptors are also present on dendritic cells but are absent from lymphoid cells (17). Binding of immune complexes to extracellular domains of activatory Fcγ receptors and subsequent cross linking of these domains leads to ITAM phosphorylation, triggering signal transduction pathways finally leading to phagocytosis, ADCC, degranulation and release of inflammatory mediators. In humans, FcγRIIb is the only inhibitory receptor and is distinguished by the presence of an immunoreceptor tyrosine based inhibitory motif (ITIM) in the cytoplasmic domain (18). This inhibitory receptor is expressed on B cells, where it is the only Fcγ receptor. It is also expressed in dendritic cells, macrophages, mast cells, eosinophils and platelets. FcγRIIb is absent from NK cells. Cross-linking of FcγRIIb and an ITAM containing activatory receptor by immune complexes blocks all inflammatory effects such as degranulation, phagocytosis and ADCC (19). FcγRIIb plays an important role in modulating B cell activation and proliferation or anergy and apoptosis depending on the involvement of the B cell receptor. In most immune effector cells both activatory and inhibitory Fcγ receptors are expressed and the ratio of their expression and activation determines the type and threshold for productive immune signalling (20-22).

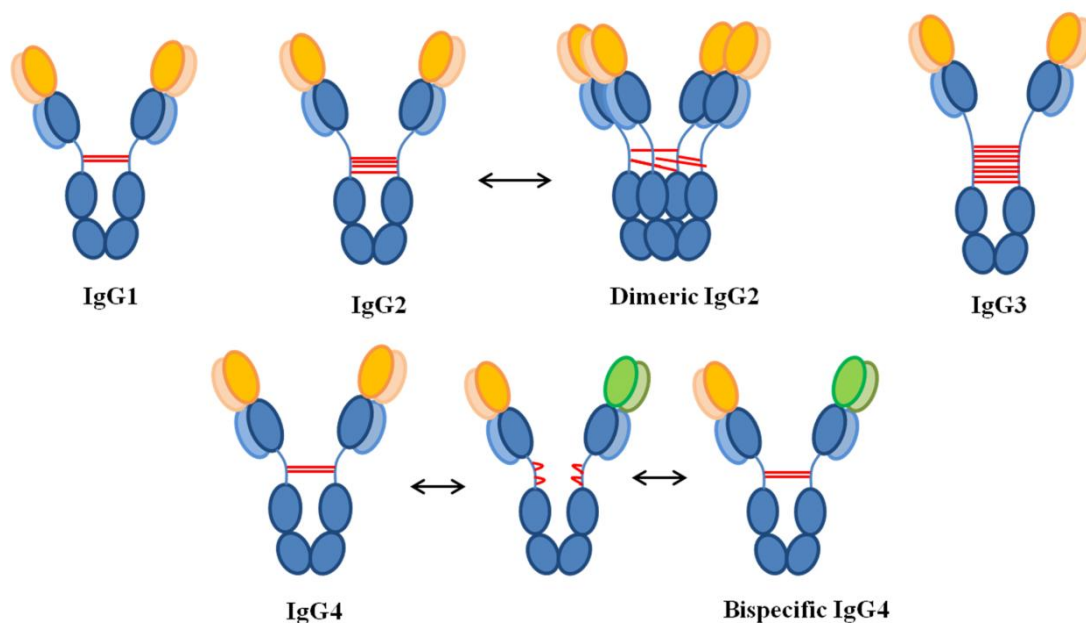


Figure 1.1.2. Human IgG subclasses. Human IgG1, IgG2, IgG3 and IgG4 are shown as cartoon representations with constant domains in blue, variable domains in yellow and hinge disulphide bonds shown in red. N-linked glycans have been omitted for clarity.

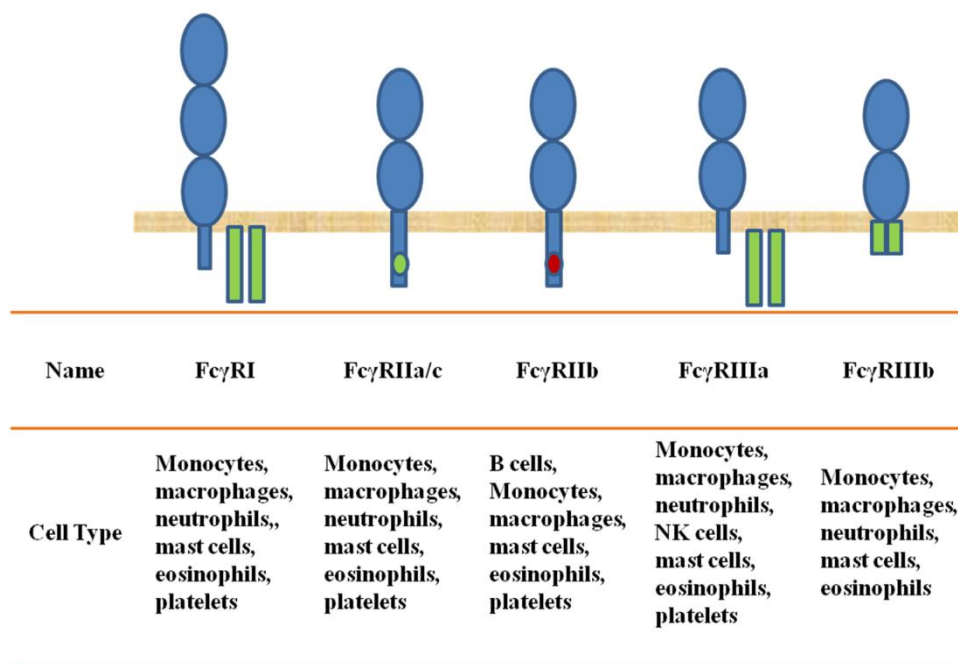


Figure 1.1.3. Human Fcγ receptors. Human activatory (green intracellular domains) and inhibitory (red intracellular domain) Fcγ receptors are shown along with their cellular distribution. All Fcγ receptors contain two extracellular immunoglobulin (Ig) like domains, except FcγRI which contains three Ig domains (blue). FcγRIIIb contains no intracellular signalling domain. Cell type distribution is based on references (17,23,24).

The ratio of activatory and inhibitory stimuli is further modulated by the different affinities of FcR binding for the various IgG subclasses (21). While IgG1 has the highest affinity for FcγRI, IgG2 shows no detectable binding. IgG3 has a higher affinity for FcγRIIIa than IgG1. All IgG subclasses bind to FcγRIIb with low affinity (25). IgG1 and IgG3 antibodies are therefore most pro-inflammatory.

Partly due to their ease of expression as monomers, IgG1 antibodies have been the major subclass of choice for therapeutic purposes, however the unique properties of other IgG isotypes are increasingly exploited in antibody engineering (26). Further variation in immune effector responses are also contributed by genetic polymorphisms of FcγRIIa (H131/R131), FcγRIIIa (V158/F158) and FcγRIIb (NA1 and NA2) (25). These varying affinities of Fcγ receptors for IgG subclasses were recently reported in detail by Bruhns *et al* (Table 1.1.1).

Table 1.1.1. Human Fcγ receptors and their affinities for various IgG subclasses. Data based on reference (25).

Fcγ Receptor	IgG1 (K _A M ⁻¹)	IgG2 (K _A M ⁻¹)	IgG3 (K _A M ⁻¹)	IgG4 (K _A M ⁻¹)
FcγRI	6.5×10 ⁷ +++++	NA	6.1×10 ⁷ +++++	3.4×10 ⁷ ++++
FcγRIIa (H131)	4×10 ⁶ ++++	4.5×10 ⁵ +++	1×10 ⁶ +++	2×10 ⁵ ++
FcγRIIa (R131)	4×10 ⁶ ++++	8×10 ⁴ ++	1×10 ⁶ +++	2×10 ⁵ ++
FcγRIIb	2×10 ⁵ ++	2.5×10 ⁴ +	1.7×10 ⁵ ++	2×10 ⁵ ++
FcγRIIc	2×10 ⁵ ++	2.5×10 ⁴ +	1.7×10 ⁵ ++	2×10 ⁵ ++
FcγRIIIa (V158)	1.5×10 ⁶ +++	8×10 ⁴ ++	5×10 ⁶ ++++	2×10 ⁵ ++
FcγRIIIa (F158)	1.5×10 ⁶ +++	2.5×10 ⁴ +	5×10 ⁶ ++++	2×10 ⁵ ++
FcγRIIIb (NA1)	2×10 ⁴ +	NA	1×10 ⁶ +++	NA
FcγRIIIb (NA2)	2×10 ⁴ +	NA	1×10 ⁶ +++	NA

1.2. Structure of IgG Fc

The earliest X-ray crystallographic structures of IgG and IgG Fc were reported by Huber and Deisenhofer in 1976 (27). IgG exists as Y shaped molecule with the two Fab domains forming the highly mobile upper arms and the Fc dimer forms the stem. A flexible hinge links the Fab and Fc domains and allows translation and rotation of the Fab arms (28). Electron density for the hinge polypeptide is rarely observed in X-ray crystal structures and no defined secondary structure has been attributed to this region most probably due to the large number of proline residues (28).

All Fab and Fc fragments of antibodies are made up of a basic tertiary structural unit called the immunoglobulin (Ig) domain (29). The Ig domain consists of either seven or nine β strands arranged in two β sheets, twisted to form a greek key β barrel domain. A conserved disulphide bond in the core of the barrel connects the two β sheets. The constant domains of IgG are comprised of seven stranded Ig fold domains, three strands in one β sheet and four in the other. They are numbered from A to G according to convention (Figure 1.2.1). The variable domain consists of nine stranded Ig domains with 5 and 4 stranded β sheets. The additional two β sheets in variable domains are labelled C' and C'' (30).

IgG Fc exists as a horse-shoe shaped dimer with the C γ 3 domains of the Fc dimer forming extensive protein-protein interactions (Figure 1.2.2). Disulphide linkages in the upper hinge effectively provide further stability the Fc dimer. There is no direct contact between the two C γ 2 domains; instead the N-linked glycan chain covalently attached to Asn297 occupies the interdomain space. Defined structures for Fc carbohydrate chains are observed regularly in crystal structures of IgG Fc. N-linked glycans of the Fc lie closely juxtaposed to the predominantly hydrophobic inner face of the C γ 2 domain and form several non-covalent interactions (6,28,31).

Most crystal structures of Fc deposited in the protein databank contain native complex bi-antennary type glycans. However some studies have reported modified glycans and sequential truncation of Fc glycan residues led to a progressive decrease in interdomain spacing and closing of the Fc dimer (32,33). This suggested a direct relationship between glycan size and Fc spacing. However more recent Fc structures, including those presented in this thesis, suggest a more dynamic model for interdomain spacing (Chapter 3).

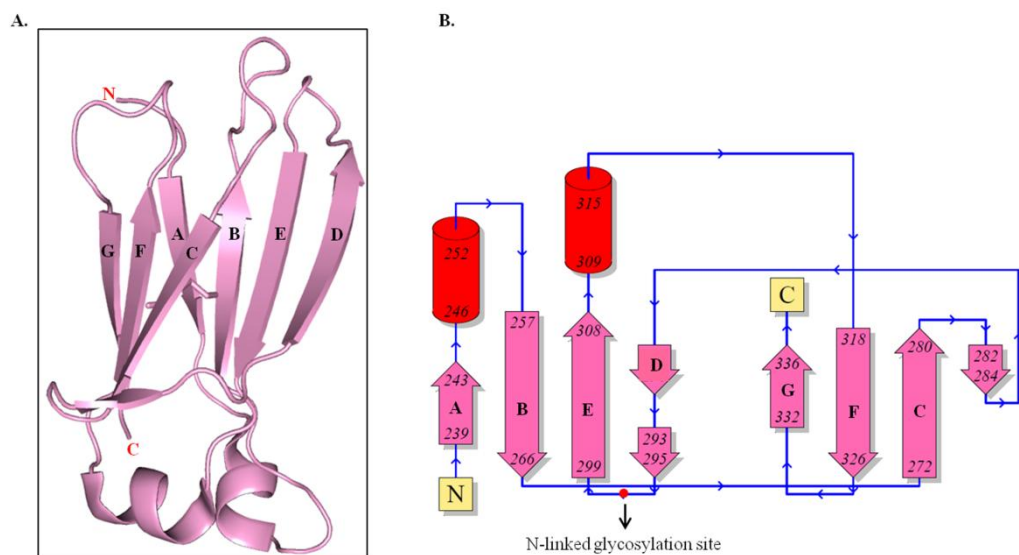


Figure 1.2.1. Structure and organisation of the immunoglobulin fold domain of IgG Fc. (A) The greek key β barrel motif of $C\gamma 2$ domain of IgG Fc. (B) Topology diagram of IgG Fc $C\gamma 2$ domain showing principal loops and position of N-linked glycosylation site was generated using PDB sum (34). All β strands are coloured in pink and α helices are shown in red. Loops are shown in blue.

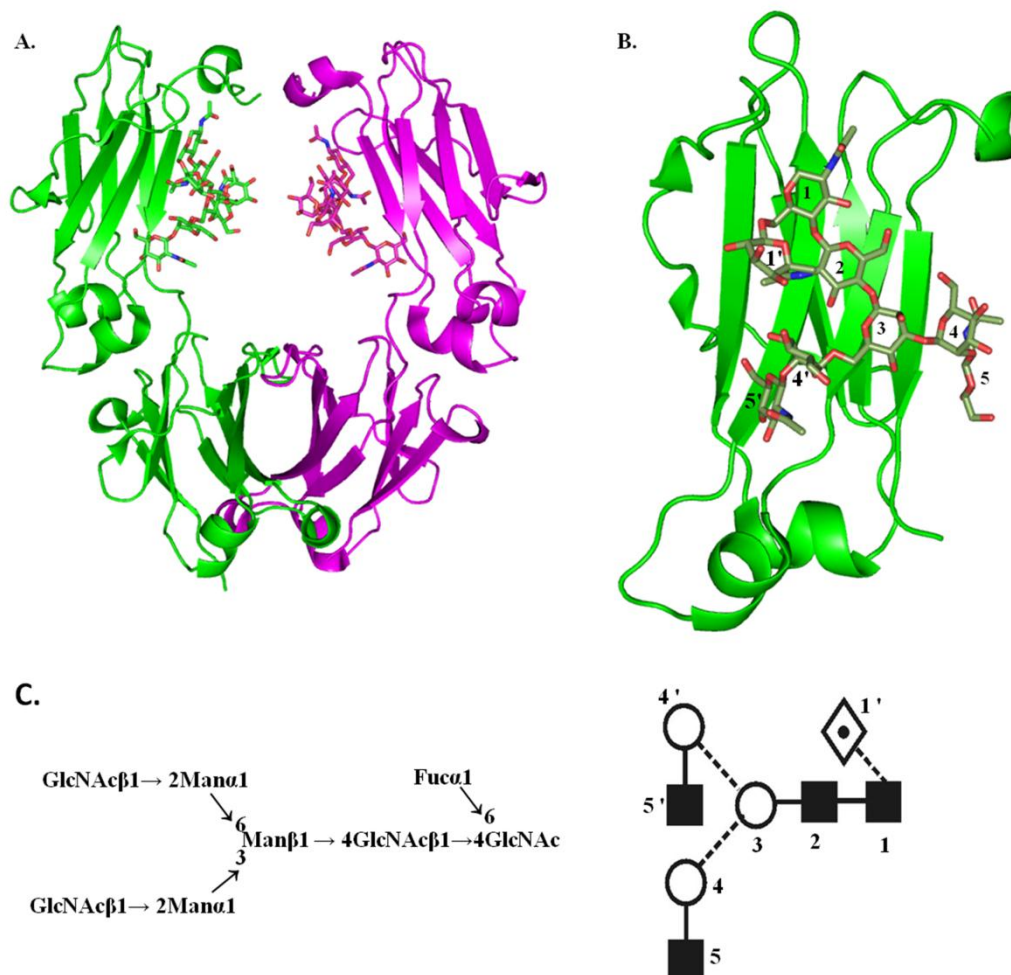


Figure 1.2.2. X-ray crystallographic structure of human IgG Fc (PDB ID 2DTQ). (A) Structure of IgG Fc with monomers in green and pink. N-linked glycan at Asn297 is shown in stick representation with nitrogen atoms coloured blue and oxygen atoms coloured red. (B) Magnified view of N-linked glycans with each carbohydrate residue numbered according to convention as described in Section 2.8. (C) Glycan composition and oligosaccharide nomenclature is according to Harvey *et al* (35) as described in Section 2.8.

The X-ray crystallographic structures of IgG Fc in complex with FcγRIIIa show asymmetric opening and 1:1 stoichiometry of binding to the Fc dimer. Analysis of the protein binding interface of Fc-FcγRIIIa complex shows that the Fc hinge (residues from Leu 234 to Ser 239), B/C, D/E and F/G loops make contacts with the receptor (Figure 1.2.1 and 1.2.3). Due to the asymmetric nature of Fc-FcγRIIIa binding, each chain of the Fc dimer makes different contacts with the Fc receptor. The structural stabilisation of these upper loops of the Cγ2 domain with receptor binding is discussed further in Section 3.5. Recent structural studies have also further revealed the crucial role of the $\alpha 1 \rightarrow 6$ linked core fucose residue in determining the affinity of Fc-FcγRIIIa binding interaction. This is discussed in further detail in Section 1.3 and Figure 1.3.3.

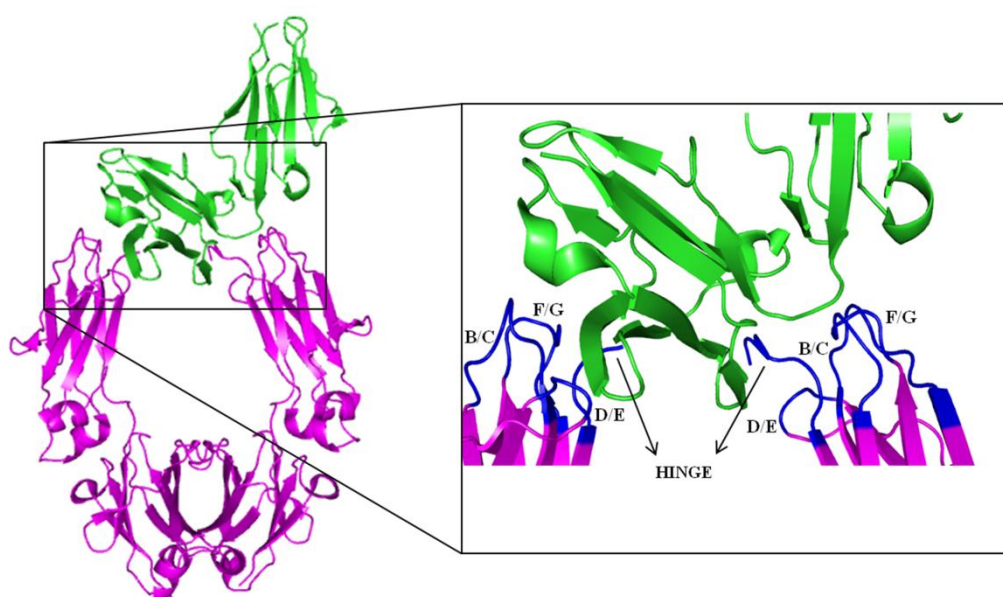


Figure 1.2.3. X ray crystallographic structure of human IgG1 Fc in complex with FcγRIIIa (PDB ID 3SGK). Magnified view of hinge, B/C, D/E and F/G loops of Fc Cγ2 domain in contact with FcγRIIIa are shown in blue.

1.3. Glycosylation of IgG Fc

All subclasses of human IgG contain a single conserved N-linked glycosylation site located on the Fc domain (Figure 1.2.1). Fc glycans are processed via the mammalian N-linked pathway (Figure 1.4.1) and the single N-linked carbohydrate chain has been reported to contain over 30 different glycoforms (36-38). However, the major IgG Fc glycoforms in human serum are based on a shared, biantennary core heptasaccharide glycan structure with variable additions of core fucose, bisecting GlcNAc and terminal extensions of galactose and sialic acid (Figure 1.3.1). Analysis of serum IgG Fc glycosylation shows that nearly 69% of serum IgG Fc contains fucosylated, biantennary glycans with terminal galactose residues (39). The remaining serum IgG Fc fraction is made up of defucosylated (21%) glycans. Around 11% of serum IgG Fc contains bisecting GlcNAc residues. Finally, less than 10% of serum IgG glycans are made up of terminal sialylated structures (40). While there are individual variations in percentages of different Fc glycoforms in serum between studies, the overall ratio of glycoforms remains constant with $G2F > G1F$ (galactose on the Man $\alpha 1 \rightarrow 6$ Man arm) $> G0F > G1F$ (galactose on the Man $\alpha 1 \rightarrow 3$ Man arm) $>$ monosialylated $G2F$ (3 arm) $\geq G0BF$. Structure of the canonical complex type N-glycan of IgG Fc and the system of nomenclature used throughout this thesis is described in Section 2.8, Figure 2.8.1.

Limited variability in glycosylation patterns between various IgG subclasses has been observed and all show a similar set of conserved biantennary glycans (41). The only differences observed have been in the preferential addition of galactose residues to the 6 arm of the chitobiose core in IgG1 and IgG4 while the 3 arm was preferred for galactosylation in IgG2. The preferential galactosylation of the 6 arm of the biantennary complex type Fc glycan has been attributed to preferential activity of Golgi galactosyltransferases (42). Similarly, there is a predominance of $\alpha 2 \rightarrow 6$ linked sialic acid residues in human IgG1 antibodies and further these residues are preferentially found on the 3 arm. This preferential sialylation has been linked to expression and substrate specificity of the $\alpha 2 \rightarrow 6$ sialyltransferase (ST6Gal) (43).

Comparison of glycosylation profiles of Fab and Fc fragments of IgG showed that Fab glycoforms are characterized by more galactosylated, sialylated and multi-antennary glycans (44). This comparatively limited diversity of Fc glycosylation indicates a role for the Fc structure in determining glycan processing. A crystallographic analysis of the

protein-carbohydrate interactions which modulate this “protein directed glycosylation” is presented in Chapter 4.

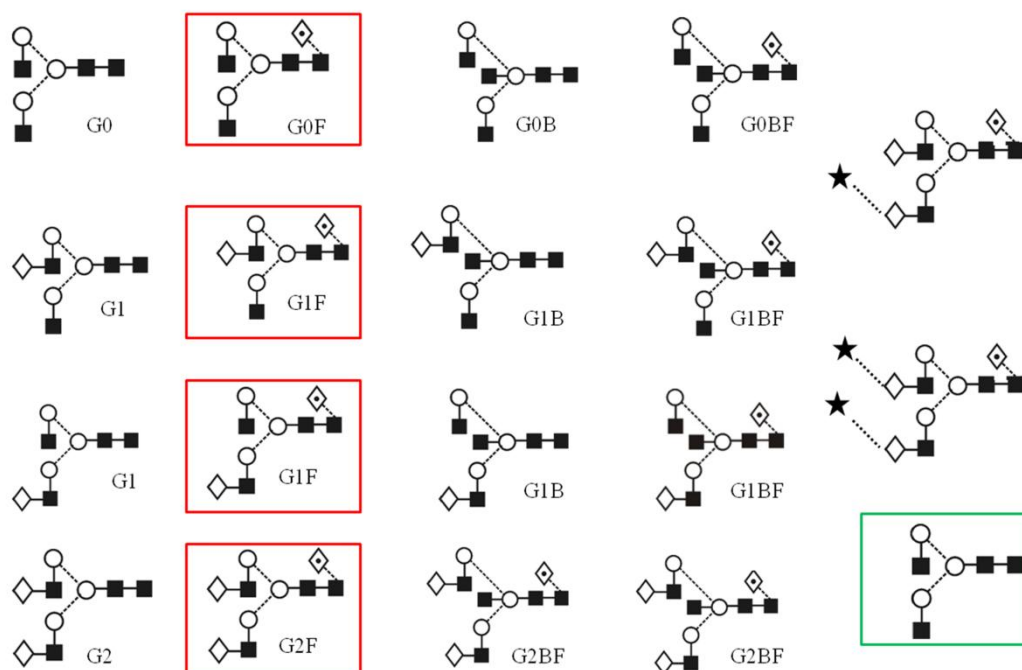


Figure 1.3.1. Principal IgG Fc glycoforms in human serum. Principal IgG Fc glycans observed in normal human serum are depicted according to nomenclature described in Section 1.8. The major glycan types found in serum consisting of galactosylated (labelled with prefix G), fucosylated (F) biantennary complex type glycans are shown in red boxes. The common biantennary heptasaccharide common to all IgG Fc glycoforms is shown in the green box.

Variations in the glycosylation profile of serum IgG have been correlated with age, pregnancy and several inflammatory diseases (45-47) (Figure 1.3.2). Levels of agalactosylated and bisected GlcNAc containing Fc glycans increase with age (36,44,46). Similarly, sialylation of IgG decreases with age (48). Elevated levels of agalactosylated Fc glycoforms have been observed in patients with rheumatoid arthritis (36). Early studies suggested that agalactosylated antibodies may play a causative role in inflammatory disease pathology (49) (50). However, reports of elevated levels of agalactosylated antibodies in several other inflammatory conditions have since been reported (44) and it now appears that agalactosylation may in fact be a consequence of inflammation. Terminal sialylation of IgG Fc has been shown to lead to potent anti-inflammatory effects (51). Sialylated IgG Fc has been shown to be the active anti-

inflammatory component of intravenous immunoglobulin (IVIG) therapy (52). Specifically, IgG Fc containing terminal $\alpha 2 \rightarrow 6$ linked sialic acid residues confer enhanced anti-inflammatory activity in both mouse models of thrombocytopenia and *in vitro* ADCC assays (52).

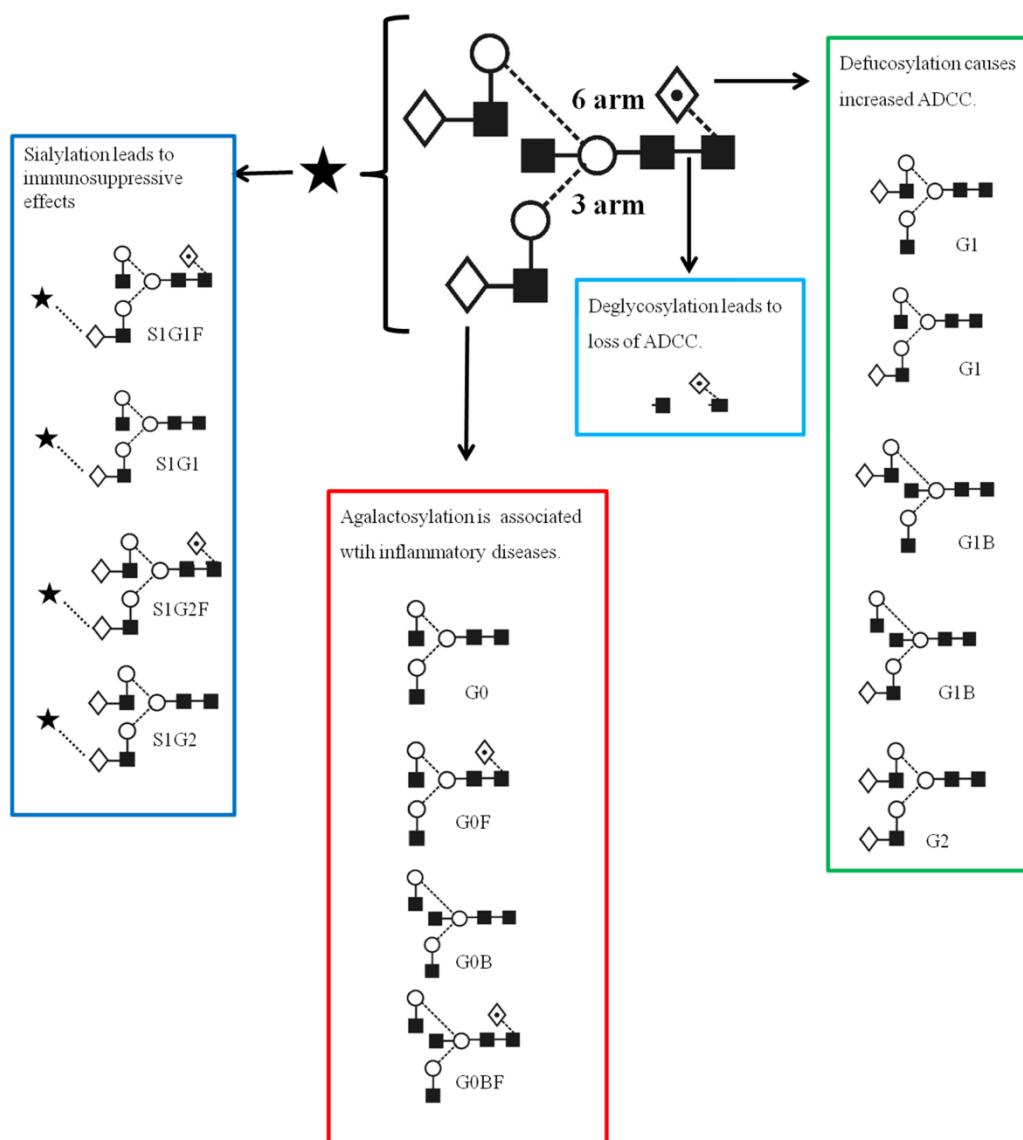


Figure 1.3.2. Human IgG Fc glycoforms and disease. Serum IgG Fc glycoforms associated with inflammatory and anti-inflammatory functions is depicted according to the nomenclature described in Section 2.8. Sialylated structures are labelled with prefix S followed by the number of terminal sialic acid residues. Galactosylated structures are labelled with prefix G followed by the number of terminal galactose residues.

While the exact mechanism for this activity is still not completely clear, recent evidence showed that mouse IgG Fc with sialylated Fc bind to SIGNR1, a mouse homologue of the human dendritic cell lectin, DCSIGN. Blockade of Fc-SIGNR1 binding or genetic deletion of SIGNR1 expression led to loss of anti-inflammatory properties of sialylated Fc (53).

Differential binding to cellular Fc γ receptors has been noted for some glycoforms of IgG. Sialylated Fc glycoforms have a lower affinity for Fc γ RIIIa (54) while the removal of N-linked glycans at Asn297 leads to loss of binding to all Fc γ receptors (55). Defucosylated IgG Fc exhibits higher affinity for Fc γ RIIIa and enhanced ADCC (56,57). A comparison of defucosylated and fucosylated Fc crystal structures revealed no significant differences in quaternary structures (58). However, further structural studies on Fc-Fc γ RIIIa complexes, revealed that high affinity binding of defucosylated Fc to Fc γ RIIIa requires glycosylation at a specific site (Asn162) on the receptor and the presence of a fucose on IgG Fc disrupts crucial stabilizing interactions between the Fc γ RIIIa carbohydrate and IgG Fc (59). Figure 1.3.3 depicts how defucosylated IgG Fc leads to the Asn162 linked glycan of Fc γ RIIIa to be oriented closer to the Fc protein surface in contrast to a more destabilised and protein-distal conformation for receptor glycans when in complex with fucosylated Fc. While, elevation in serum levels of fucosylated IgG were observed in association with fetal maternal allotype thrombocytopenia natural variations in the levels of fucosylated serum IgG in healthy or immune compromised conditions have not been reported to date (60).

1.4. Glyco-engineering of Antibodies

Given the importance of glycosylation for Fc function, the modification of N-linked glycosylation of IgG Fc has become a major focus for antibody engineering recently (61). The most common mammalian expressions systems used to produce recombinant antibodies are the CHO, NS0 and Sp2/0 cell lines. Glycosylation profiles of recombinant antibodies produced in these expression systems are characterised by predominantly fucosylated biantennary complex type glycans similar to serum IgG. However, all these cell lines show lower levels of galactosylated IgG Fc than in serum(62). NS0 and Sp2/0 cell lines are of murine origin and often contain glycans bearing α 1 $\rightarrow$ 3 linked galactose residues that are unusual in humans and have led to hypersensitivity reactions in patients(63).

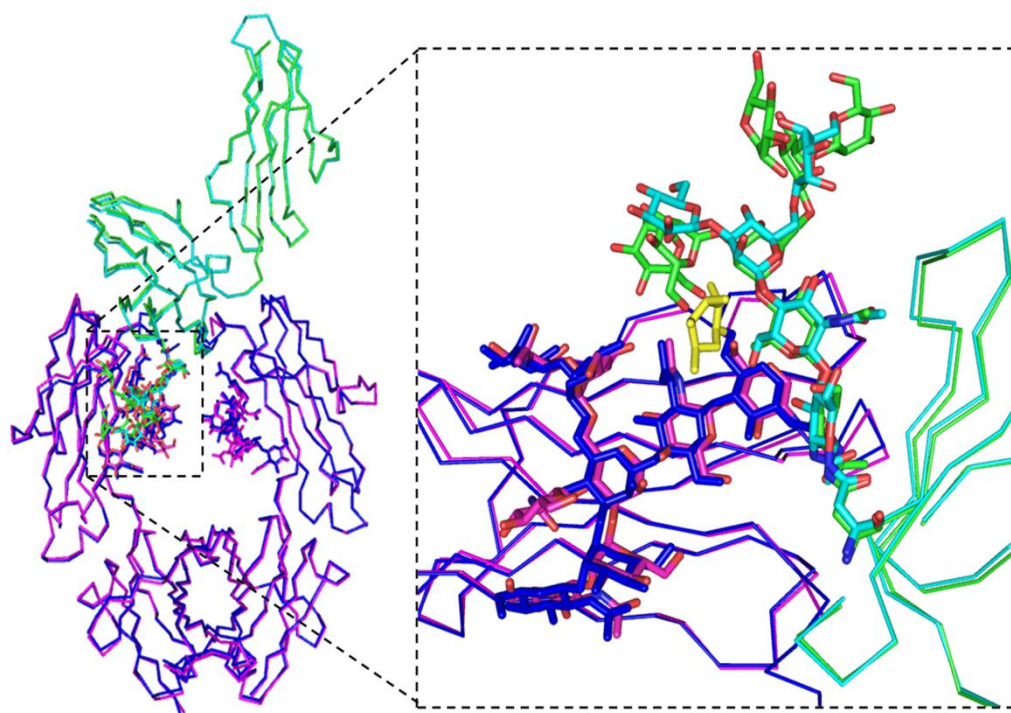


Figure 1.3.3. X-ray crystallographic structure of fucosylated and defucosylated IgG Fc in complex with FcγRIIIa. FcγRIIIa and its N-linked glycan at Asn162 (PDB ID 3SGJ) in complex with fucosylated IgG Fc are shown with the core fucose residue coloured yellow, receptor in cyan and Fc in blue. FcγRIIIa (PDB ID 3SGK) in complex with defucosylated IgG Fc is shown with the receptor coloured green and Fc in pink.

These differences in glycosylation profiles of CHO, NS0 and Sp2/0 cell lines and more recent studies on enhanced effector functions of defucosylated antibodies has led to more focus towards the production of recombinant antibodies with homogenous glycoforms.

Manipulations of the N-linked glycosylation pathway (Figure 1.4.1) can yield unnatural Fc glycoforms with modified effector functions. X-ray crystallographic structures of two such incompletely processed Fc glycoforms are reported in Chapter 5. Antibodies with oligomannose type, hybrid type and defucosylated complex type glycans have been shown to display differential binding to FcγRIIIa and varying levels of ADCC (64,65). Defucosylation of antibodies has been associated with the greatest increase in ADCC. Recently, a study comparing the ADCC effects of galactosylated antibodies reported that terminal galactosylation did not affect ADCC as potently as defucosylation (64). However, conflicting reports do exist on the role of Fc galactosylation in ADCC (56,66),

further experiments are needed to clarify this. To date, no correlation between core fucosylation and increase in CDC has been reported. However, differences in terminal glycan residues of IgG Fc have been associated with differences in CDC activity. While mapping of amino acid residues on IgG Fc crucial for C1q binding have been reported, there are currently no reported studies on the structural implications of IgG Fc glycosylation on Fc-C1q binding. Differences in ADCC and CDC activity of fucosylated and defucosylated IgG antibodies of all subclasses have again shown the potent effect of defucosylation on ADCC (57). These differences in IgG Fc effector functions with variations in Fc glycosylation are summarized in Table 1.4.1. Reduced binding affinity to Fc γ RIIIa for terminal sialylated IgG Fc has also been reported previously (51,54), however there no direct correlation between ADCC or CDC activity and Fc sialylation is known to date.

One of the first reported glycoengineered cell lines was generated to over express N-acetylglucosaminyltransferase III (GnT III), the Golgi enzyme responsible for the addition of a bisecting GlcNAc residue to the central β mannose of the Fc glycan. Recombinant antibodies produced in this cell line displayed enhanced ADCC (67). It was later discovered that the presence of bisecting GlcNAc residues inhibits core fucosylation and was responsible for the enhanced effector function (56). Ferrara et al further engineered GnT III for improved localization and along with over expression of Golgi α mannosidase II enzyme, produced recombinant antibodies with hybrid or complex type defucosylated glycans (68). This study led to the development of GA-101, a glycoengineered CD20 specific antibody that is now in Phase II clinical trials for non-Hodgkin's lymphoma (69). CHO cell lines lacking expression of α 1 $\rightarrow$ 6 fucosyltransferase (FT) are also being used to produce defucosylated recombinant antibodies with enhanced ADCC function (70). MEDI-563 is one such non-fucosylated antibody that has reached clinical testing (71). Besides mammalian expression systems, extensively glyco-engineered strains of *Pichia pastoris*, *Lemna*, *Nicotiana benthamiana* and moss have also been used to improve yields and lower cost of production of recombinant antibodies (40,64,72).

Table 1.4.1. Glycoforms of human IgG Fc and their associated effector functions and affinity for FcγRIIIa and C1q. Fucosylated and defucosylated Fc glycoforms are shown as +/- Fuc. Data not determined is shown as nd. Data based on references (56,57,64,73).

IgG Fc Glycoform	FcγRIIIa (V158)	C1q	FcRn	ADCC	CDC
Complex Type (+Fuc)	+++	+++	+++	+++	++++
Complex Type (-Fuc)	++++++	+++	+++	+++++	++++
Hybrid Type (+Fuc)	++	++	+++	+	++++
Hybrid Type (- Fuc)	++++	+++	+++	+++++	++++
Deagalactosylated (-Fuc)	++++	+++	nd	+++	nd
Deagalactosylated (+Fuc)	+++	nd	nd	+	nd
Galactosylated (-Fuc)	++++	nd	nd	+++	nd
Man ₅ GlcNAc ₂	++++	+	+	++++	+++
Man ₉ GlcNAc ₂	+++++	++	++++	++++	+++

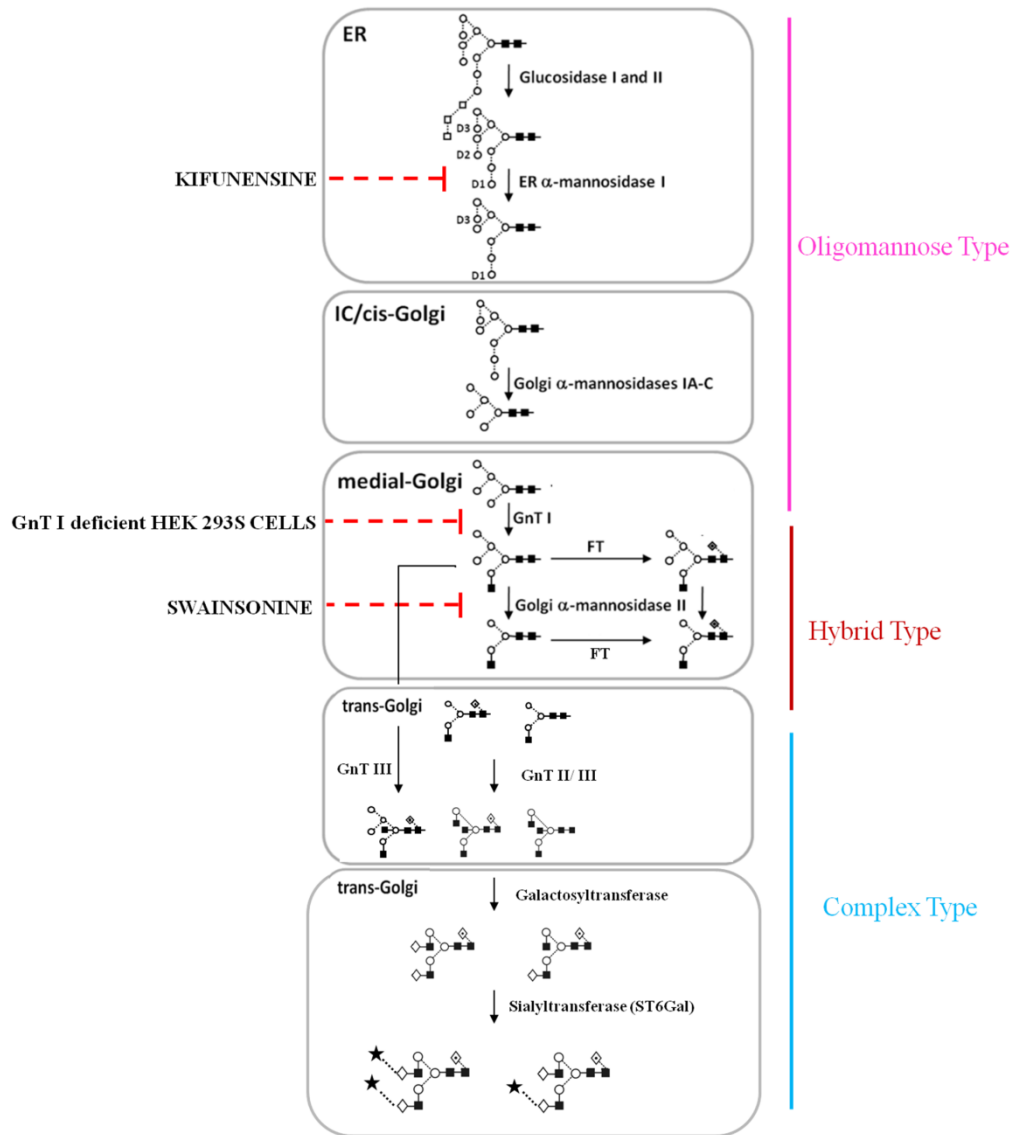


Figure 1.4.1. Mammalian N-Linked glycosylation pathway and Golgi glycan processing of IgG Fc. Glycan residues are labeled according to convention as described in Section 2.8. Inhibitors and mutant cell lines used to manipulate key steps of the glycan biosynthetic pathway are shown with red dotted lines.

1.5. Therapeutic antibodies with modified effector functions

The invention of hybridoma technology by Kohler and Milstein in 1975 followed by advances in humanisation of monoclonal antibodies signalled the beginning of therapeutic antibody research (74). There are now more than twenty five recombinant antibodies that are fully approved for clinical use and hundreds more are under clinical development (75).

Most therapeutic antibodies in clinical use today act by one or more combinations of the following: (1) High affinity binding to key ligand molecules blocking their binding to receptors. (2) High affinity binding to cellular receptors blocking downstream receptor functions. (3) Cytotoxicity mediated by ADCC and/or complement mediated lysis (CDC) (71).

Whilst research continues on novel formats for variable domains like bispecific antibody fragments, diabodies, single chain variable fragments (scFv), improvements in variable domain display technologies and monoclonal antibody engineering have largely eliminated the challenges of developing high affinity antigen binding domains (76). Therefore, in many cases, the downstream effector functions through the Fc domain are vital for antibody efficacy. Activation of ADCC by Fc γ R binding, complement dependent cytotoxicity by C1q binding and increased serum half-life by binding to FcRn are all mediated by binding of the Fc domain. Recently, many efforts have focused on the improvement of antibody effector functions by mutating the Fc protein backbone (77).

Extensive and detailed mutational analysis of the Fc domain and mapping of binding sites to Fc γ receptors was performed by Shields and co-workers and nearly 60 Fc variants with differential binding to Fc γ RI, IIa, IIb, IIIa were identified (10). This work was followed by several more studies using novel combinations of mutations (77). To date, several recombinant antibodies with mutated Fc domains showing enhanced ADCC and also modulation of complement mediated cytotoxicity have been reported (78). Design of these mutants was aided by the availability of X-ray crystallographic structures of the Fc-Fc γ RIIIa complex and took into account the asymmetric nature of Fc-Fc γ RIIIa binding (79). Fc engineered for minimal effector functions have made use of IgG2 and IgG4 Fc domains as these have lower affinities for Fc γ receptors (80,81). Mutants showing selective binding to the inhibitory Fc γ RIIb receptor are very few,

owing to its close similarity to the activatory FcγRIIa (82,83). Immunosilent Fc has also been generated by mutation of Asn297 linked glycosylation site resulting in the production of aglycosylated antibodies. IgG Fc mutants with prolonged serum half-life have been designed to bind FcRn with high affinity (84). Figure 1.5.1 depicts the key regions of the Fc Cγ2 and Cγ3 domains that have been successfully mutated for modified Fc effector functions.

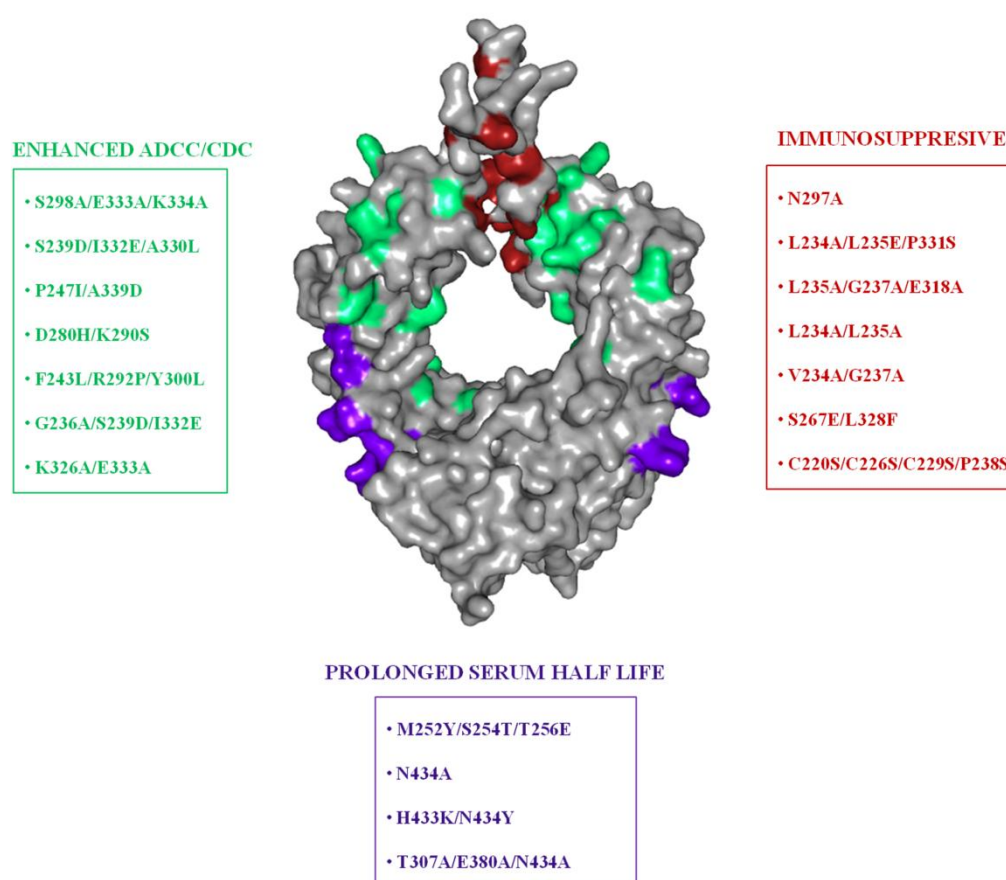


Figure 1.5.1. IgG1 Fc domain engineering for modified effector functions. Regions of human IgG Fc (PDB ID 1HZH) mutated for increased ADCC functions by binding to FcγRIIIa, FcγRIIa and CDC by binding to C1q (9,10,78,85-87) are colored green and listed in the green box on the left. Regions of IgG Fc mutated for increased immunosuppressive effector functions (81,82,88-92) are colored in red and listed in the red box on the right. Fc mutants with increased serum half-life through high affinity binding to FcRn (84,93-95) are shown in blue and listed in the blue box below. Relevant references are listed next to each mutation.

1.6. Deglycosylated antibodies and bacterial endoglycosidases in immune evasion

Removal of the N-linked glycan at Asn297 of the IgG Fc domains leads to loss of antibody effector functions and impaired complement activation (96). Recombinant antibodies expressed in bacteria or designed with mutations at the Asn297 N-linked glycosylation site at the Fc domain are aglycosylated and unable to trigger ADCC and inflammation. Such aglycosylated antibodies are particularly suited in a therapeutic setting where the mode of action of the recombinant antibody is through high affinity binding to target antigen and Fc mediated effector functions are unnecessary.

Deglycosylation of antibodies by bacterial endoglycosidases serves as an important strategy for immune evasion (97). Endoglycosidases are a class of enzymes that cleave the GlcNAc $\beta 1 \rightarrow 4$ GlcNAc linkage in the chitobiose core of N-linked glycans (Figure 1.6.1). Endo S is one such endoglycosidase secreted by the gram positive pathogenic bacteria- *Streptococcus pyogenes*. It exhibits a surprising specificity for native glycosylated IgG (98). This unique carbohydrate selectivity of Endo S is further investigated in Chapter 6.

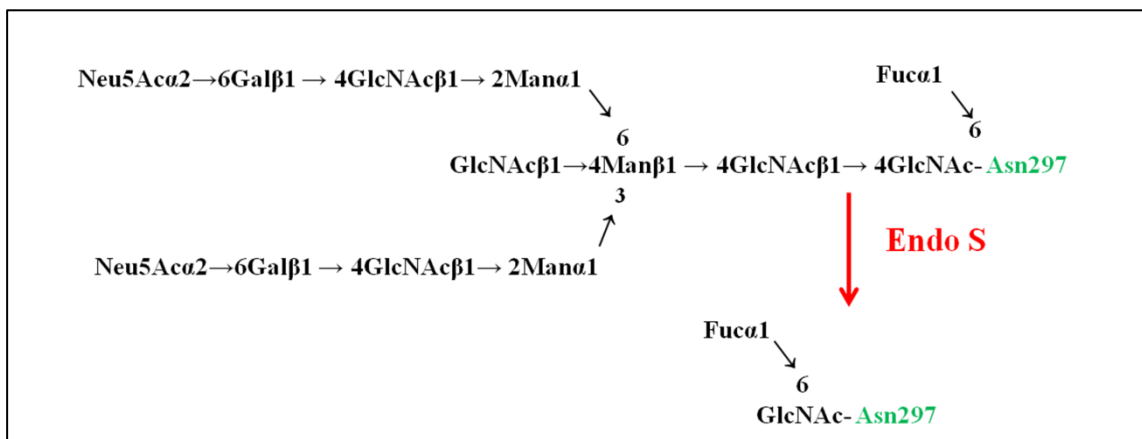


Figure 1.6.1. Endoglycosidase mediated cleavage of IgG Fc glycans. The canonical biantennary complex type N-glycan is shown here, however the finer carbohydrate specificity of Endo S is still under investigation.

Endo S can successfully hydrolyze N-linked glycans from all four subclasses of IgG and leads to loss of antibody effector functions and complement activation (99). This unique property has been further used to treat autoimmune disease phenotypes in animal models. Endo S treatment of mice models of systemic lupus erythematosus (SLE) led to significantly prolonged life and reduced disease (100). Further, treatment of healthy rabbits with low doses of Endo S led to no toxicity and minimal production of anti-Endo S antibodies. Endo S is currently being studied as a potential therapeutic for autoimmune diseases in humans (101). The structural basis for endoglycosidase-mediated Fc inactivation is also presented in Chapter 6.

In this thesis, X-ray crystallographic studies of five separate glycoforms of IgG1 Fc indicate a dynamic model for Fc domain conformations and reveal how Fc-glycan contacts develop during antibody biogenesis. It is also shown here that specific Fc glycoforms can be selectively modified by bacterial endoglycosidases providing a new approach to the enhancement of therapeutic antibody effector function.

CHAPTER 2

Materials and Methods

2. Materials and Methods

2.1. Cloning of Human IgG1 Fc

Human IgG1 Fc DNA coding for the full length hinge, C γ 2 and C γ 3 domains (Eu antibody numbering convention, residues 224-446 (102), GenBank accession no. J00228) was amplified by polymerase chain reaction (PCR) from the PhL-FcHis mammalian expression vector using specific primer sequences (Table 2.1.1) (103). PCR amplified DNA was visualised by 1% TAE (Tris acetate EDTA buffer) agarose gel electrophoresis. The amplified DNA fragment of 684 bp with 5' AgeI and 3' KpnI restriction sites was purified using QIAprep gel purification kits (Qiagen, Crawley, UK). Following gel purification, DNA was digested with AgeI and KpnI following using reaction mixtures and conditions as recommended by New England Biolabs (NEB, UK). Similarly, pHLsec vector DNA was digested with AgeI and KpnI restriction enzymes. Both vector and PCR amplified DNA inserts were separately purified by QIAprep gel purification as before. Enzyme digested pHLsec vector DNA was further treated with Antarctic phosphatase enzyme (NEB, UK) to prevent re-ligation.

Enzyme digested IgG Fc DNA inserts were ligated into pHLsec vector using the NEB quick ligation kit (NEB, UK). Briefly, 1X T4 DNA ligase buffer, 100ng IgG Fc DNA, 40ng pHLsec vector DNA, 1U NEB T4 ligase and milliQ distilled water were mixed together in a 10 μ l reaction mixture and incubated at room temperature for 30 minutes. 5 μ l of ligation mixture was added to 45 μ l of freshly thawed XL1 Blue competent *E.coli* cells (Agilent, UK) and incubated on ice for 30 minutes. This was followed by heat shock treatment at 42°C for 45 seconds after which competent cells were immediately placed on ice for 2 minutes. Pre-warmed NZY broth (450 μ l) was added to the heat-shocked cells and incubated on a rotary shaker at 37°C for 1 hour. Finally, transformed cells were spread onto LB-Agar plates containing 50 μ g ml⁻¹ carbenicillin and incubated overnight at 37°C for antibiotic selection of positive *E.coli* containing the pHLsec vector.

Single colonies grown on LB-carbenicillin plates were inoculated in 5ml of LB broth containing 50 μ g ml⁻¹ carbenicillin and grown overnight in a rotary shaker at 37°C. *E.coli* cultures were spun down and plasmid DNA was extracted using the Qiagen (UK) miniprep kit. Purified plasmid DNA was verified for the presence of full length IgG Fc DNA by DNA sequence analysis by Source Bioscience (Department of Biochemistry,

oxford, UK) sequencing facility. Sequencing primers pHLsecFor and pHLsec Rev are listed in Table 2.1.1.

Table 2.1.1. Primer design for IgG Fc cloning. Restriction enzyme sites are underlined.

Primer	Sequence (5' to 3')
Fc Hinge For	ATAC <u>ACCGGT</u> CACACATGCCCACCGTGC
Fc Hinge Rev	CGATGGT <u>ACC</u> ACCCGGAGACAGGGAGAGG
pHLsecFor	GCTGGTTGTTGTGCTGTCTCATC
pHLsecRev	CACCAGCCACCACCTTCTGATAG

F241A, F241A/F243A and F241A/F243A/V262E/V264E Fc mutants were cloned in pHLsec using the Quikchange site-directed mutagenesis kit (Agilent, UK) by Ben Yu. Transient transfection and protein purification of Fc mutants was performed as described below.

2.2. Large Scale DNA purification

Sequence verified clones were used for preparation of milligram quantities of endotoxin free DNA for transient expression of IgG Fc in HEK 293T cells. Overnight cultures in LB broth (0.5L) containing carbenicillin were set up from glycerol stocks. Plasmid DNA was isolated using the Endotoxin-free plasmid mega prep kit (Qiagen, UK) following manufacturer's instructions. Final yield and purity of DNA was assessed by measuring OD₂₆₀ and ensuring an OD₂₆₀/OD₂₈₀ ratio of greater than 1.30.

2.3. Protein Expression and purification

HEK 293T cells (0.5L) were used for all large scale recombinant expression of Fc glycoforms. Cells were cultured to 90% confluency in Dulbecco's modified eagles medium (DMEM, Sigma Aldrich, Manchester, UK) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen, Paisley, UK). Prior to transfection, endotoxin free DNA was mixed with Polyethyleneimine (PEI) at a DNA to PEI mass ratio of 1:1.5 in 30 mL of DMEM without FCS. Cells were transiently transfected with 2mg of DNA per 1L of 90% confluent cells in DMEM supplemented with 1% FCS and 1% penicillin/streptomycin. For production of Fc glycoforms with Man₉GlcNAc₂ glycans, 20μM Kifunensine (Toronto research chemicals, Ontario,

Canada) was added to the cell culture medium. For production of Fc Hybrid glycoforms, 20 μ M Swainsonine (Sigma Aldrich, UK) was added to the cell culture medium. Transfected HEK 293T cells were left undisturbed for 10-14 days at 37°C and 5% CO₂.

Harvested cell culture supernatant was filtered through 0.22 μ m pore sterile filters (Millipore, UK). Culture supernatant was diluted at 1:2 ratios with binding buffer consisting of PBS and 10 mM HEPES pH 8.0. Washed and pre-equilibrated chelating sepharose Fast Flow Ni²⁺ beads (GE healthcare, Buckinghamshire, UK) were then added to diluted cell culture supernatant and incubated at room temperature with gentle shaking for 2 hours. After incubation, beads were washed with twenty column volumes of PBS/HEPES binding buffer, followed by ten column volumes of PBS/Tris buffer with 5 mM imidazole. Finally, His tagged IgG Fc was eluted by washing the column with 500 mM imidazole buffer. Eluted protein was concentrated with 10kDa cut-off concentrators (Vivascience, Gloucestershire, UK). Protein yield and purity was first verified by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis using a 4-12% Bis-Tris agarose gel (Invitrogen, UK). Concentrated IgG Fc protein was finally purified by size exclusion chromatography using a superdex 16/60 200 pg gel filtration column (GE healthcare, UK) in 10mM HEPES pH 8.0, 150mM NaCl.

2.4. Protein crystallisation

IgG Fc protein preparations of high purity and average concentrations of 7mg/ml were used for crystallisation setup. The vapour diffusion sitting drop method was used for all crystallisation trials. A 1:1 mixture of protein and precipitant was equilibrated in a closed container with a reservoir of precipitant solution. Crystallisation experiments for all Fc glycoforms including FcWT and Fc^{GlcNAc} were carried out in collaboration with Dr Thomas Bowden at the Division of Structural Biology, University of Oxford. Crystallisation of F241A Fc was carried out in collaboration with Dr Matthew Higgins at the laboratory of molecular biophysics, University of Oxford and the Department of Biochemistry, protein crystallisation facility. Initial 96 well screens were set up using pipetting robotics. Here, 100 nL of protein solution was pipetted into crystallisation wells with 100 nL of precipitant. 96-well plates were sealed with Griener Bio One Viewseals (Stonehouse, UK). Precipitant screens were obtained from Hampton Research (Aliso Viejo, USA) and Molecular Dimensions (Suffolk, UK).

96-well plates were stored at room temperature and regularly checked under a microscope for the presence of crystals. Upon identification of a suitable precipitant condition for protein crystals, further optimisation was performed by varying pH, precipitant concentrations and protein to precipitant ratios. Optimisation experiments for FcWT, Fc Hybrid, Fc^{GlcNAc} and Fc Man₉GlcNAc₂ were carried out using robotic pipetting facilities. F241A Fc crystals were optimised by setting up 24-well plates using the hanging drop vapour diffusion method on inverted coverslips. Crystallographic conditions for all the proteins studied in this thesis, are listed in Table 2.4.1.

Table 2.4.1. Crystallographic conditions for various IgG Fc glycoforms.

Protein	Condition	Temperature (°C)	Cryoprotectant
FcWT	25% PEG 3350, 0.2M sodium chloride, 0.1M Bis-Tris pH 5.5	21	25% ethylene glycol
Fc Man ₉ GlcNAc ₂	20% w/v PEG monomethyl ether 5000, 0.1M Bis-Tris pH 6.5	21	25% ethylene glycol
Fc Hybrid	30% w/v PEG monomethyl ether 2000, 0.1M Sodium Acetate pH 4.6, 0.2M Ammonium sulphate	21	25% ethylene glycol
Fc ^{GlcNAc}	25% w/v PEG 1500, 0.1M SPG system pH 4.0	21	25% glycerol
F241A Fc	28% PEG monomethyl ether 2000, 0.1M Bis-Tris pH 6.5	21	30% polyethylene glycol 400

2.5. Structure determination

Fc protein crystals were immersed in suitable cryoprotectant (Table 2.1.1) to minimise radiation damage and immediately transferred to 100K gaseous nitrogen stream. X-ray diffraction data was collected by exposing protein crystals to high energy X-rays of defined wavelength and a charge-coupled device detector. Data for Fc crystals were collected at the Diamond light source synchrotron beamlines.

Initial screening for diffracting crystals was performed by short X-ray exposures of the crystal and the quality and resolution was checked. Fc crystals that gave satisfactory diffraction images were then further exposed to X-rays with a 0.2° oscillation of the crystal and a total of 900 images were recorded. Data indexing using HKL2000 or imosflm software (104) was used to calculate the space group and unit cell dimensions. Following data collection and indexing, integration and scaling of intensities of reflections from X-ray diffraction images was performed using imosflm and Scala programs (105). The overall quality of the dataset was then assessed by data completeness (%), $I/\sigma I$ (indicative of signal to noise ratio) and R_{merge} (%) values. Individual values for these statistics are presented for each Fc glycoform in the relevant chapters. Acceptable experimental datasets should contain greater than 95% completeness, $I/\sigma I$ greater than two and R_{merge} of less than 10% (106).

Finally, structure factors for each dataset are calculated from averaged scaled intensities using the CCP4 suite of programs (105). F_{hkl} , the structure factor describes the amplitude and phase for each given reflection. While the amplitude is directly measurable, the phase information is obtained using maximum likelihood algorithms for molecular replacement with previously reported Fc structures (106). Molecular replacement was performed using the program Phaser (107). Before each phaser run, water and carbohydrate molecules were removed from the search model to avoid any phase bias. The high resolution Fc structure with PDB ID 2DTQ was used as the model for molecular replacement for solving the structures of Fc glycoforms reported in this thesis. A further reason for choosing this structure as the model for molecular replacement runs was its error-free amino acid sequence. Previously reported structures of Fc including the very first structure reported by Deisenhofer et al, contained several errors in amide assignments.

2.6. Structure refinement and validation

The first electron density maps of the Fc structure obtained from molecular replacement solutions were subject to alternate rounds of real space model building using the software COOT (108) and model correction and global reciprocal space refinement using the program REFMAC (107). Restrained refinement runs were used where the model was refined to best fit the electron density whilst maintaining good geometry. Refinement using TLS (Translation-libration-screw) parameters were also performed to take into account the flexibility of rigid body movements in the structure (109). TLS groups for Fc were defined automatically by REFMAC and consisted of the C γ 2 and C γ 3 domains of each individual chain. Model quality and model bias during successive rounds of refinement was assessed by R_{work} and R_{free} values. R_{work} is a comparison of the observed (F_o) and calculated (F_c) structure factors. R_{free} value is calculated for 5% of the dataset that is set aside and not used at any stage of the structural refinement. R_{free} values not greater than 5% of the R_{work} values are generally considered acceptable for the final model.

Addition of carbohydrate residues was done after completing several initial rounds refinement of the protein structure. Initial atomic coordinates for carbohydrate moieties were taken from previously published Fc structures (PDB ID 2DTQ, 3SGK, 3SGJ). Carbohydrate residues were modelled into corresponding regions of electron density on COOT and the entire Fc model was subject to further rounds of refinement on REFMAC while monitoring the R_{work} and R_{free} values after each round of refinement. Terminal GlcNAc residues particularly on the 3 arm were challenging to model due to the absence of sufficient electron density. In cases where the electron density for terminal glycan residues did not improve with progressive refinement, these residues were omitted from the final model.

Final validation of the structural model and quality was verified by analysis of Ramachandran plots, rotamer conformations of side-chains and clashes between positions of hydrogen atoms using the MOLPROBITY server (110). Statistics from these analyses for each Fc structure is tabulated in the relevant chapters. There is currently no single defined application available for the automatic validation of carbohydrate structures. Therefore, all carbohydrate structures were validated manually by checking for the presence of correct $4C_1$ conformation, optimum distance of glycosidic bonds as reported in previous crystallographic structures of Fc. Further

validation of carbohydrate structures can also be obtained from measurement of glycosidic torsion angles and also ensuring the planarity of the NH-CO linkages in GlcNAc residues (111).

2.7. Cloning and expression of chimeric full length mouse collagen Type II antibody with human IgG1 Fc domain

CIIC1 hybridoma cells (8,112) were obtained from the Developmental Studies Hybridoma Bank, (University of Iowa, Department of Biology, Iowa City, IA 52242) and cultured in Iscove's modified Dulbeccos's medium (IMDM) supplemented with 10% FCS and 0.1% gentamycin sulphate (Calbiochem, Germany). Hybridoma cells were incubated at 37°C and 5% CO₂. Approximately 1×10^7 cells were used to prepare total RNA using the RNAspin Midi kit (GE healthcare, UK). This was immediately followed by isolation of mRNA using the Illustra mRNA purification kit (GE healthcare, UK). All RNA purification steps were carried out on freshly spun down hybridoma cells and precaution was taken to ensure all working areas and pipette tips and microcentrifuge tubes are free from RNase contamination (RNaseZap, Sigma Aldrich, UK). RNA and cDNA purification protocols were followed according to manufacturer's instructions for each kit.

Freshly isolated mRNA was used for first strand cDNA synthesis using the generic pdN6 primer with the Illustra first-strand cDNA synthesis kit (GE healthcare, UK). CIIC1 V_H and V_L sequences were amplified by PCR using specific primers (Table 2.7.1). Amplified variable domain DNA sequences were verified by PCR and ligated into TOPO pCRBlunt vector (Invitrogen, Paisley, UK) as before. *E.coli* colonies were picked and verified for carrying the correct plasmid by DNA sequencing analysis as described earlier. Blunt endligations were carried out according to manufacturer's instructions in the ZeroBlunt TOPO PCR cloning kit (Invitrogen, Paisley, UK). CIIC1 V_H and V_L DNA sequences were then first cloned into pHLsec vector as described in section 2.1. This was necessary for incorporating the secretion signal sequences from pHLsec vector. Finally, CIIC1 V_H and V_L DNA sequences were cloned into pFUSE-CHIg-hG1 vector (Invivogen, San Diego, California, and U.S.A) using the EcoRI and NheI sites for the heavy chain and BstEII and BsiWI restriction sites for the light chain. Primer sequences are shown below in Table 2.7.1. The pFUSE-CHIg-hG1 vector contains full length DNA sequences for human IgG1 C γ 1, C γ 2 and C γ 3 domains.

Table 2.7.1. Primer design for cloning of CIIC1 IgG in pFUSE-CHIg-hG1. Restriction sites are underlined.

Primer	Sequence (5' to 3')
CIIC1 pFuse VH For	ATACGAATTCGCCACCATGGGGATCCTTCCCAGCCCTG
CIIC1 pFuse VH Rev	CGATCGAGCTAGCTGCAGAGACAGTGACCAGAGT
CIIC1 pFuse VL For	ATACGGTCACCGCCACCATGGGGATCCTTCCCAGCCCTG
CIIC1 pFuse VL Rev	CGATCGTACGTTTTATTTCCAGCTTGGTCC

Intact CIIC1 antibody with complex and oligomannose-type glycans was transiently expressed in Human Embryonic Kidney cells as described in section 2.3. Successful expression of full length antibody was confirmed by SDS-PAGE analysis and also by ELISA assay for specific binding to mouse collagen Type II protein (Figure 2.7.1).

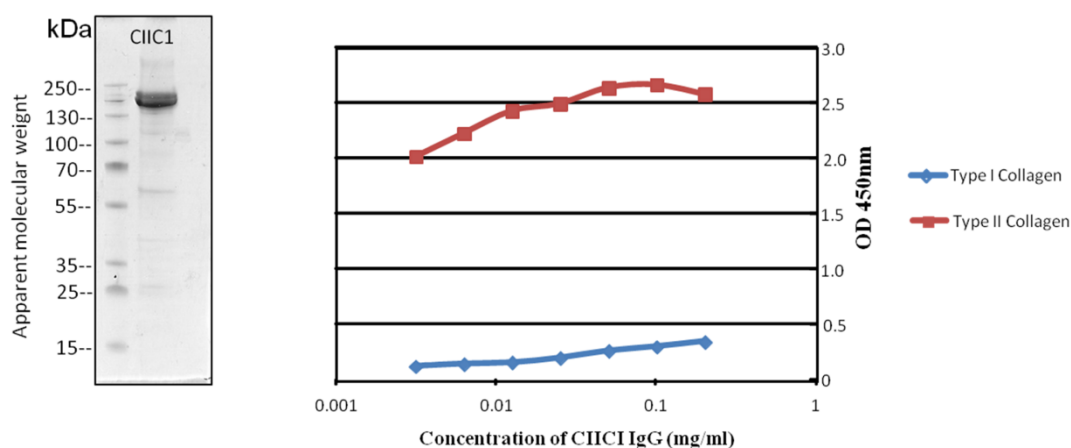


Figure 2.7.1. Expression of CIIC1 antibody. SDS-PAGE analysis of purified and concentrated CIIC1 antibody is shown on the right. Graph on the left shows ELISA data for binding to CIIC1 to mouse TypeI and Type II collagen.

2.8. Mass Spectrometric analysis of Fc linked N-glycans

Oligosaccharides were released from bands containing IgG Fc protein that were excised from Coomassie blue-stained reducing SDS-PAGE gels (113), washed with alternating water and acetonitrile and dried in a vacuum centrifuge, followed by rehydration with 100 Units/ml of PNGase F (New England Biolabs, MA, U.S.A.) and incubation for 12 hours at 37°C. PNGase F cleaves between the innermost N-Acetyl glucosamine residue (GlcNAc) and asparagines residue of N-linked glycoproteins (114).

The enzymatically released N-linked glycans were eluted with milliQ distilled water. Endoglycosidase digestion of glycans was performed by addition of 1µg of recombinant

Endo S (Provided by Professor Ben Davis, CRL, University of Oxford) or 1 µl of Endo H (500U/µl, New England Biolabs, MA, U.S.A.) and incubation for 12 hours at 37°C.

Aqueous solutions of the glycans were cleaned with a Nafion 117 membrane (115). Samples were prepared by adding 0.5 µL of an aqueous solution of the sample to the matrix solution (0.3 µL of a saturated solution of 2, 5-dihydroxybenzoic acid in acetonitrile) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then re-crystallized from ethanol. Positive ion MALDI-TOF mass spectra were recorded with a Shimadzu AXIMA TOF² MALDI TOF/TOF fitted with delayed extraction and a nitrogen laser (337 nm). The acceleration voltage was 20 kV; the pulse voltage was 3200 V; and the delay for the delayed extraction ion source was 500 ns. Negative ion, collision induced dissociation (CID) and fragmentation spectra for Fc glycans was obtained by electrospray ionisation mass spectrometry performed by Professor David Harvey (Glycobiology Institute, Department of Biochemistry, University of Oxford). Analysis of CID spectra and peak assignments were performed as described by Harvey *et al* (116-118).

Glycan structures corresponding to *m/z* peaks were assigned using calibration runs with dextran and by calculation of glycan composition based on the predicted *m/z* of 203.1 for N-Acetylglucosamine (GlcNAc), 162.1 for Hexoses (Mannose, Galactose residues) and 146.1 for Fucose residues. Symbolic representation of glycan structures follows that of Harvey *et al.* (37): ◇ = Galactose, ■ = GlcNAc, ○ = Mannose, ◆ = Fucose. The linkage position is shown by the angle of the lines linking the sugar residues (vertical line = 2-link, forward slash = 3-link, horizontal line = 4-link, back slash = 6-link). Anomerity is indicated by full lines for β-bonds and broken lines for α-bonds (Figure 2.8.1). Numbering of carbohydrate residues in all structural analysis follows the system by Berger and Vliegthart *et al* (119).

$\text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1$
 $\xrightarrow{6}$
 $\text{GlcNAc}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}-\text{Asn297}$
 $\xleftarrow{3}$
 $\text{Neu5Ac}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1$

Monosaccharide symbols

	Glc
	GlcNAc
	Gal
	GalNAc
	Fuc
	Man
	NeuNAc

β Linkage —————

α Linkage - - - - -

38

2.9. Surface Plasmon Resonance (SPR) analysis

Surface plasmon resonance (SPR) experiments were performed on a BIAcore T100 (GE Healthcare, Buckinghamshire, UK) at 25°C, to ascertain the affinity of binding of various Fc glycoforms to the Fc γ receptors- Fc γ RIIIa and Fc γ RIIb. Carboxymethyl 5 (CM5) chips (GE Healthcare, Buckinghamshire, UK) were activated and coupled with Fc γ RIIIa or Fc γ RIIb (R&D systems, Europe) upto 1000 response units (RU) using standard amine coupling methods. Fc glycoforms in HBS-N buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% v/v surfactant P20 at concentrations from 34.3 μ M, 17.15 μ M and 8.57 μ M were injected over immobilized Fc γ RIIIa at a flow rate of 30 μ l/min. After each binding cycle, chips were regenerated with Tris-glycine buffer (pH 1.0).

Non-specific binding was measured by keeping one flow cell without any coupled Fc receptor. Negligible non-specific binding was observed for all samples, and final sensograms were obtained after subtraction of background binding. Repeated low pH regeneration steps could also alter the binding of the coupled Fc receptor. To control for any loss of binding due to this, the first control FcWT sample was run again at the end to account for any attenuation in binding. No significant loss of binding signal was detectable after several regeneration steps. Another way of circumventing this problem would be to run internal controlled sample sets where FcWT control is repeated with each Fc glycoform. The sensograms shown in Figure A1 were all performed on the same CM5 chip.

Despite repeated attempts with different target levels of amine couple Fc γ RIIb, no significant binding could be detected for any of the Fc glycoforms. This could be due to loss of active conformation of the receptor during amine coupling. Further optimisation of Fc γ RIIb binding assays was not possible due to time constraints. Therefore, all binding data reported in this thesis are for the activatory Fc γ RIIIa. Analysis of binding sensograms and calculation of K_D values was performed with the Biacore Evaluation Software version 1.1 and affinity curves were fitted assuming a 1:1 Langmuir model(121). Despite obtaining measurable binding for each glycoform, it was challenging to fit data from repeat runs into a 1:1 Langmuir binding model to obtain standard errors for the reported K_D values.

2.10. Differential scanning fluorimetry (DSF) assays for thermal unfolding of IgG Fc

Thermal unfolding of Fc glycoforms was measured using differential scanning fluorimetry assays (122). Protein unfolding was measured in the presence of the fluorescent dye Sypro Orange (Invitrogen, Paisley, UK) which exhibits increased fluorescence in a non-polar and hydrophobic environment as opposed to an aqueous environment where the fluorescence is quenched. 1:1000 dilution of Sypro orange was mixed with 5 mg/ml of purified Fc protein in 10 mM HEPES buffer, pH 8.0. Temperature increments of 1.5°C ranging from 25°C to 90°C were set up and fluorescence measurements were performed in a Stratagene RT PCR 305 instrument. All sample and buffer controls were set up in triplicates. Thermal unfolding data were analysed and melting temperatures were calculated from first derivative curves of the thermograms on Excel. Final melting temperatures for Fc glycoforms are reported (Appendix, Figure A1) as average values from three independent experiments. Initial baseline readings for all Fc glycoforms (between temperatures 25°C to 50°C) were similar, ruling out any contributions due to non-specific binding of the Sypro orange dye to Fc glycoforms.

2.11. ELISA

Collagen binding ELISA assays for initial testing of full length CIIC1 antibodies were performed by coating mouse Type I or Type II collagen (Chondrex, Redmond, WA, USA) at 5µg/ml in collagen dilution buffer according to instructions on high-binding 96-well ELISA plates (Corning, USA). Overnight coating of antigen at 4°C was followed by blocking with 3% BSA/PBS for two hours at room temperature. Serial dilutions of CIIC1 antibody were added to antigen coated wells and binding was allowed to proceed for 2 hours. This was followed by 5-7 washes with PBS/0.05% Tween 20 and incubation with protein A-HRP (1:5000 dilution in PBS) for 1 hour at room temperature. Protein A-HRP binds to IgG Fc and binding was detected after 5-7 washes with PBS/Tween20 and incubation with TMB substrate (Thermo Scientific, USA) for colour development. Absorbance was measured at 450nm in a Spectramax 96 well plate reader.

FcγRIIIa (R&D systems, Minneapolis, U.S.A.) at 2.5µg/ml in PBS was coated on high-binding microtitre plates (3690, Corning, NY, U.S.A.) overnight at 4°C. Coated plates were washed with PBS containing 0.05% Tween 20 (Sigma-aldrich, U.S.A.) and

blocked for 2 hours at room temperature with 3% BSA in PBS. Serial dilutions of human serum (H4522, Sigma-Aldrich, U.S.A.) or recombinant human CIIC1 IgG1 glycoforms bearing Man₉GlcNAc₂ or Man₅GlcNAc₂ (starting concentration of 0.1mg/ml in PBS), was then added and allowed to bind for 2 hours at room temperature. Plates were washed five times with PBS containing 0.05% Tween and binding was detected using a HRP conjugated Fab fragment specific for murine IgG Fab (ab98659, Abcam, Cambridge, UK). TMB substrate (Thermo Scientific, Rockford, IL, U.S.A.) was used for colour development according to manufacturer's directions. Colour development was stopped by the addition of 2M H₂SO₄ and absorbance was measured at 450nm on a Spectramax M5 (Molecular Devices, California, U.S.A.) multiwall plate reader. For FcγRIIIa binding assays (Figure A1), serum was incubated overnight with 1μg/ml of Endo S or PBS at 37°C. For competition ELISA experiments, 1:5 dilution of serum was incubated with 1:100 dilution of Endo S (1mg/ml) or Endo H (500U/μl) overnight at 37°C. Control serum samples were mock treated with PBS and incubated overnight at 37°C. Data was processed and plotted using Prism (GraphPad software, California, U.S.A.). Apparent affinity was calculated as the concentration of oligomannose containing CIIC1 IgG corresponding to half-maximal binding on the ELISA binding curve.

2.12. Structural analysis and illustrations

All molecular superimpositions and root mean square deviation (r.m.s.d.) values were calculated using least square algorithms in COOT using FcWT or native Fc (PDB ID 2DTQ) as reference structure (108). Average B factor values for Fc Cγ2 domains were calculated using the temperature factor analysis in the CCP4 structural analysis suite of programs(105). PDB sum was used to generate LIGPLOT diagrams of carbohydrate-protein interactions for each Fc glycoform (34,123). All structural illustrations were prepared on PyMOL(124).

CHAPTER 3

Intradomain Plasticity of Human IgG1 Fc

3. Intradomain plasticity of human IgG1 Fc

3.1. Summary

The Fc domain of IgG antibodies plays a critical role in coupling antigen binding to cellular effector functions and is a focal point for interactions with several ligands. The 2.8Å X-ray crystallographic structure of IgG Fc was reported by J. Deisenhofer in 1981 (31). Variations in interdomain distances and symmetry of the Fc dimer in previous structural studies on both free and ligand bound IgG Fc and have been attributed to crystallographic conditions. There is currently no single definite model for the structure and conformations of IgG Fc domains. However a model whereby successive glycan trimming led to a progressively closed Fc structure has led to the suggestion that the “open” Fc conformation is favoured by Fc with larger glycans (32).

Therefore to investigate this further, the X-ray crystallographic structure of recombinant human IgG Fc with native complex type glycans was determined. Crystallographic studies were accompanied by MALDI-TOF analysis of the N-linked glycan composition. DSF analysis was performed to study the thermal stability of Fc γ 2 domains and functional binding to Fc γ RIIIa was measured by SPR.

3.2. Cloning and Expression of FcWT

Human IgG1 Fc domain including the upper, core and lower hinge sequences was cloned into the mammalian expression vector pHLsec and expressed transiently in HEK 293T cells as described in Section 2.1–2.3. Large scale expression in 1.5 L cell culture for 7–10 days was followed by purification of FcWT protein by immobilised metal affinity chromatography (IMAC) and SEC. Final yield of FcWT was 30 mg of pure protein per litre of cell culture.

FcWT eluted predominantly a dimer on SEC (Figure 3.2.1A) and the eluted fraction was verified as a 53kDa band on non-reducing SDS-PAGE and as 27kDa band on reducing SDS-PAGE (Figure 3.2.1B).

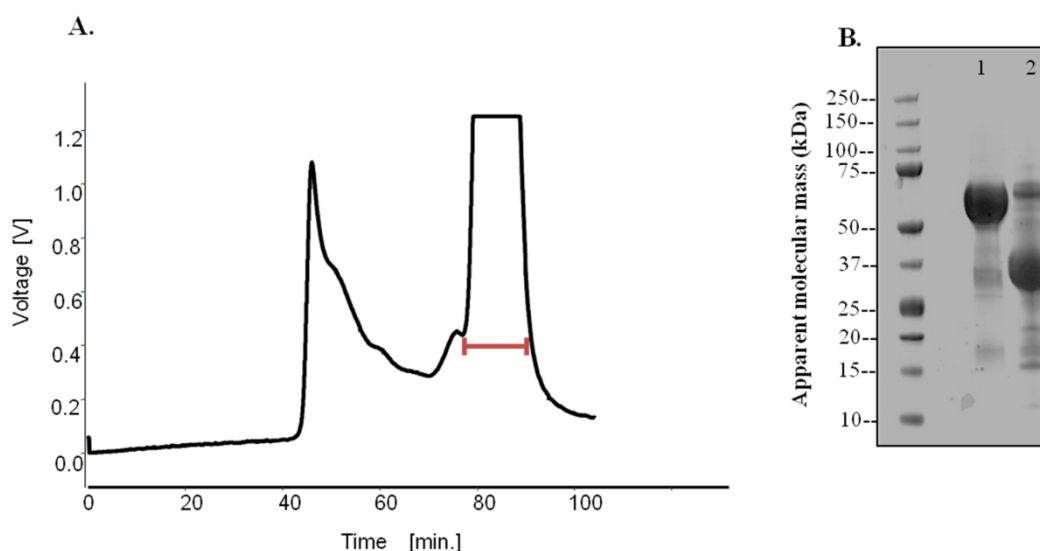


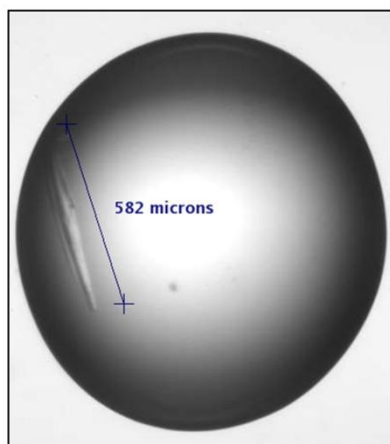
Figure 3.2.1. Purification of FcWT. (A) SEC curve of FcWT run on a Hiload 16/60 superdex 200 prepgrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peaks. (B) 4-12% gradient SDS-PAGE of SEC purified FcWT. Lane 1 is non-reduced and Lane 2 is reduced FcWT protein.

3.3. Crystallisation and Structure Determination of FcWT

Purified FcWT (10 mg/ml) was used for growing crystals by the sitting drop vapour diffusion method (Section 2.4). FcWT crystals grew at room temperature in 25% w/v PEG3350, 0.2 M Sodium Chloride and 0.1 M bis-Tris buffer, pH 5.5 after 3-6 days (Figure 3.2.2A). Crystals were immersed in cryoprotectant containing mother liquor in 25% ethylene glycol and transferred rapidly to gaseous nitrogen stream at 100K. X-ray diffraction datasets up to 2.6Å resolution were obtained at the Diamond I04 beamline.

Structure of FcWT was solved by molecular replacement with PHASER using native Fc structures (PDB ID 2DTQ) as search model (58). One Fc homodimer per asymmetric unit was identified, giving a solvent content of 50.07% (V_M of 2.46 Å³Da⁻¹). Model Building was performed with COOT (108) with several rounds of iterative refining with the program REFMAC5 (107) as described in Section 2.6. Molprobit was used for final validation of the model and for Ramachandran analysis (110). Ramachandran analysis showed that 98.8% of the residues were in the most favoured region with no residues in disallowed regions (Figure 3.3.1 B). Data collection and refinement statistics are presented in Table 3.3.1.

A.



B.

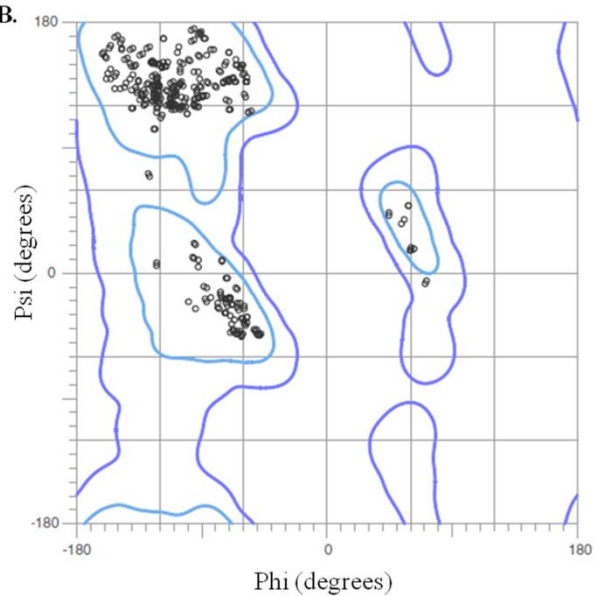


Figure 3.3.1. Crystallisation and structural analysis of FcWT.(A) FcWT crystals grew to a length of 582 μm . (B) Ramachandran plot generated with Molprobity(110) for the final refined FcWT structure showing 98.8% residues in the most favoured region.

Table 3.3.1. Data collection and refinement statistics for FcWT.

Data collection	
Beamline	Diamond I04
Resolution (Å) ^a	50.0-2.60 (2.69-2.60)
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	49.0, 74.6, 147.9
α, β, γ (°)	90.0, 90.0, 90.0
Wavelength (Å)	1.073
Unique reflections	31875 (3127)
Completeness (%)	98.6 (97.9)
<i>R</i> _{merge} (%) ^b	0.143 (0.760)
<i>I</i> /σ <i>I</i>	11.7 (1.9)
Average redundancy	6.9 (6.5)
Refinement	
Resolution range (Å)	41.0-2.60 (2.68-2.60)
Number of reflections	16116 (1147)
<i>R</i> _{work} (%) ^c	22.6
<i>R</i> _{free} (%) ^d	25.9
r.m.s.d.	
Bonds (Å)	0.010
Angles (°)	1.4
Main-chain bond (Å ²)	
Side-chain bond (Å ²)	
Molecules per a.s.u.	1
Atoms per a.s.u.	3327/85/184
(protein/carbohydrate/water)	
Average <i>B</i> -factors (Å ²)	
(protein/carbohydrate/water)	41.0/71.6/40.2
Model quality (Ramachandran plot)	
Most favored region (%)	98.8
Allowed region	1.2

^aNumbers in parentheses refer to the relevant outer resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I(\text{hkl}; i) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I(\text{hkl}; i)}$, where $I(\text{hkl}; i)$ is the intensity of an individual measurement and $\langle I(\text{hkl}) \rangle$ is the average intensity from multiple observations.(106)

^c $R_{\text{work}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$ (106)

^d *R*_{free} was calculated as *R*_{work} but after removal of 5% of data prior to refinement (Section 2.6).

3.4. Mass spectrometric analysis of FcWT glycans

N-linked glycans on FcWT were released by digestion with PNGaseF (Section 1.8) and subject to MALDI-TOF analysis to ascertain their structure and composition. Mass spectrometry revealed a major peak with m/z of 1485.1 corresponding to fucosylated biantennary complex type glycan (G0F, Figure 3.4.1). The other predominant peak with an m/z of 1647.1 corresponds to a monogalactosylated complex type glycoform (G1F). Further minor peaks corresponding to defucosylated biantennary complex type glycans (m/z of 1339.1, 1501.1) and a minor peak corresponding to digalactosylated biantennary glycan (m/z of 1809.1) were also observed.

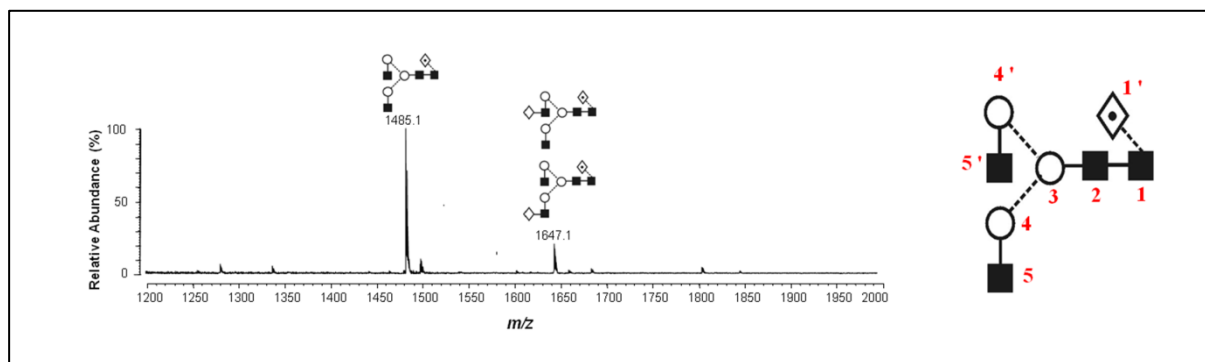


Figure 3.4.1. MALDI-TOF analysis of FcWT glycans. Composition and m/z of PNGase F released glycans from FcWT are shown. The schematic on the right shows the principal FcWT glycoform with individual glycan units numbered as described in Figure 2.8.1.

The glycan profile of FcWT is similar to that observed in serum and differs only with respect to the levels of galactosylated glycans. The principal glycoform observed in serum IgG contains digalactosylated biantennary complex type glycans (G1F, corresponding to m/z of 1647.1) while in recombinant FcWT agalactosylated glycoforms predominate(40). FcWT expressed recombinantly here, also showed no evidence of sialylated glycans again not largely variant from the low levels of serum IgG with sialylated termini (Section 1.3). All recombinant Fc glycoforms studied in this thesis, were expressed in HEK 293T cells to avoid variations in glycosylation due to cell culture conditions or expression protocols.

3.5. Structural analysis of FcWT

FcWT at 2.6Å resolution adopted a classical horseshoe shape common to IgG Fc domains. Electron density for both chains of the Fc dimer was clearly visible; however the hinge domain could not be traced (Figure 3.5.1). This is not unusual given the high mobility and flexibility of this region. Currently, only two Fc crystal structures have been reported with defined electron density for the hinge, of these, one Fc domain was part of full length IgG (PDB ID 1HZH) and the other was bound to FcγRIIIa (PDB ID 3SGJ) (28,125). Although, no defined secondary structure has been attributed to the hinge region, it forms a significant part of the Fc-FcγRIIIa binding interface and is crucial for productive binding and ADCC and complement mediated antibody activity (Figure 1.2.3) (28). Deletion of key residues of the lower hinge leads to complete abrogation of antibody effector functions (126,127).

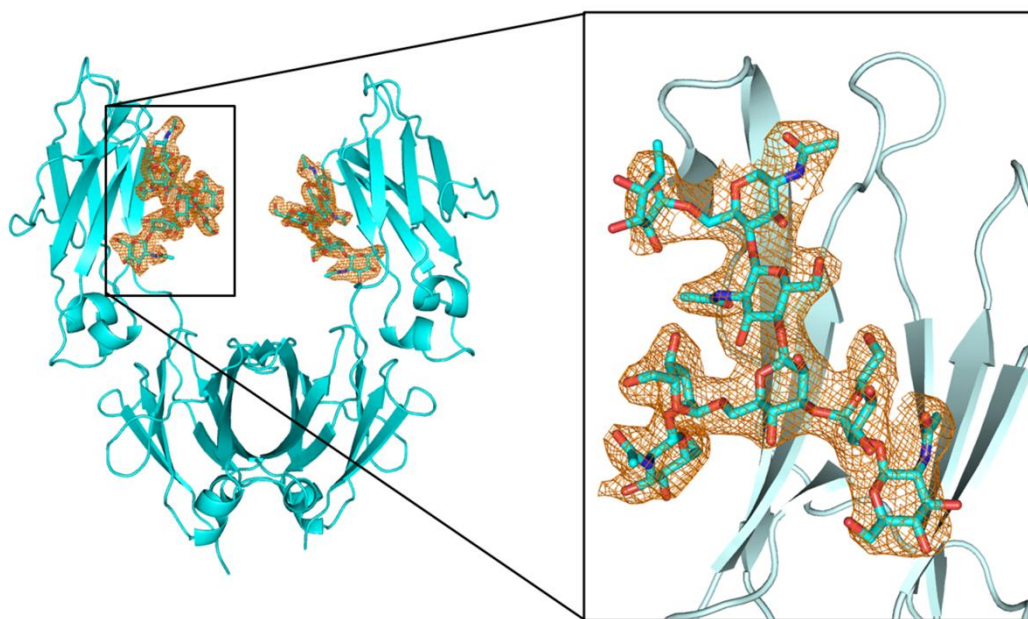


Figure 3.5.1. Structure of FcWT at 2.6 Å shown in cartoon representation. A maximum likelihood $2F_o - F_c$ map is displayed around the N-linked glycans, contoured to 1σ . Magnified view of the complex type biantennary glycan on Chain A is shown on the right.

Electron density for the principal agalactosylated biantennary glycan attached to Asn 297 was traceable on both Fc chains. All eight glycan residues were visible on chain A, while the terminal GlcNAc residue of the 3 arm on chain B could not be traced. Another observation from the FcWT structure is the asymmetric conformation of the two Fc monomers where chain B is rotated and tilted out and away from the central two-fold crystallographic axis.

B factor values of the C γ 2 domains of chain A and chain B were comparable ($B_{average}$ chain A is 52.713 Å² and $B_{average}$ chain B is 50.605 Å²) while upper loop regions both C γ 2 domains showed elevated *B* factor values indicating higher flexibility (Figure 3.5.2). The three upper loops (B/C, D/E and F/G) of the C γ 2 domain form part of the Fc-Fc γ RIIIa binding interface along with the lower hinge residues (Figure 1.2.3).

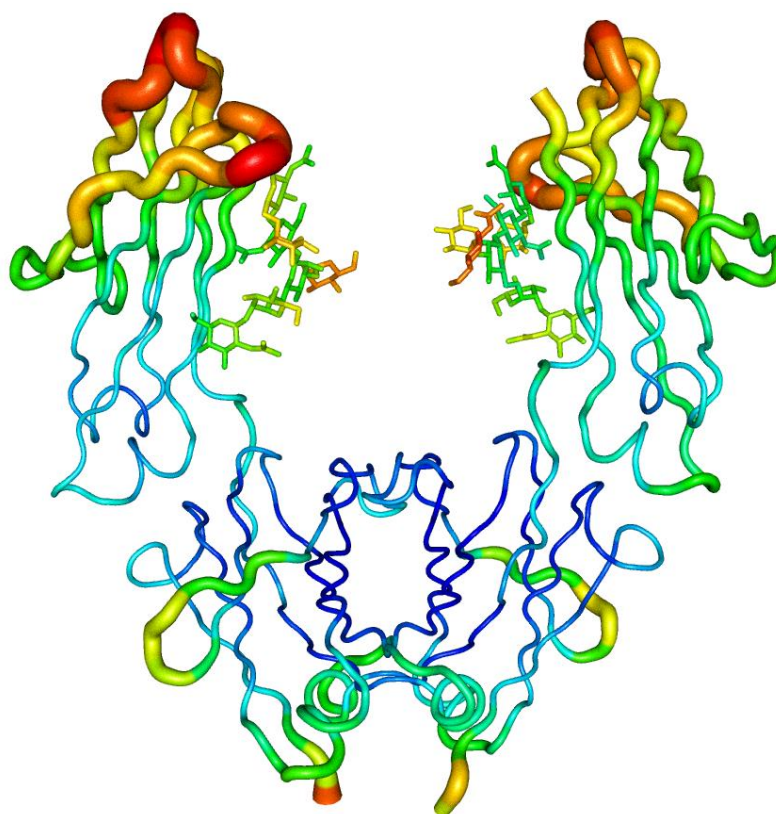


Figure 3.5.2. Conformational mobility of C γ 2 domains of FcWT. Protein backbone and attached glycans of FcWT are coloured according to their *B* factor values. Colours range from dark blue (lowest *B* factors) through to red (highest *B* factors).

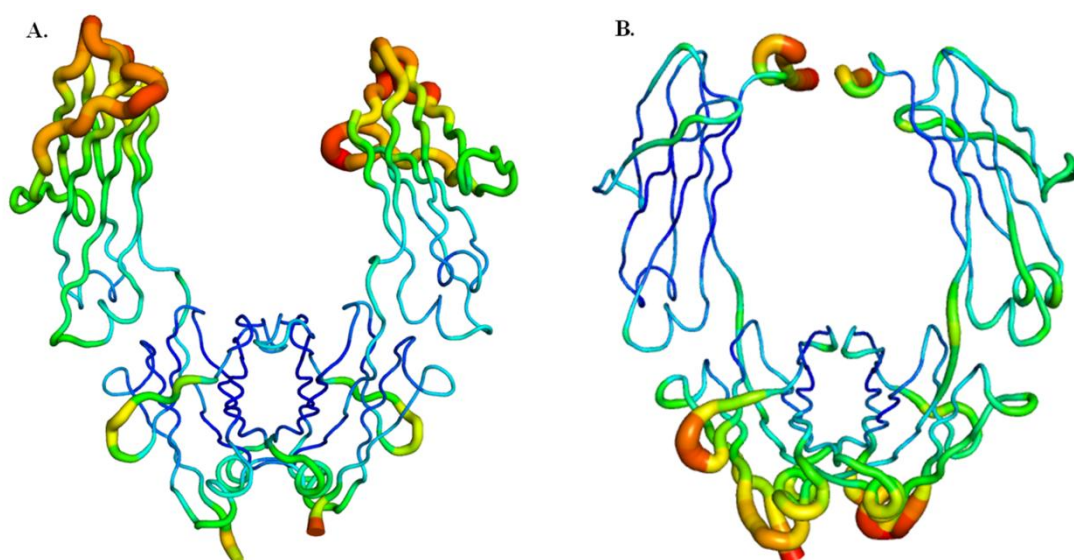


Figure 3.5.3. Comparison of *B* factor plots for free FcWT and Fc-Fc γ RIIIa complex. (A) Protein backbone of FcWT are coloured according to *B* factor values with higher *B* factor values coloured in red and progressing to blue for lowest *B* factor values. (B) Protein backbone of Fc domains of Fc-Fc γ RIIIa complex (PDB ID 3SGK) coloured according to *B* factor values.

These loop regions are consistently associated with high *B* factor values in Fc structures. However they were observed to be relatively stable when in complex with receptor (Figure 3.5.3). Stabilisation of the B/C, F/G and D/E loops of Fc in the Fc-Fc γ RIIIa complex could also be attributed to crystallographic contacts. However, a comparison of the *B* factor plots for all other Fc-Fc γ RIIIa structures revealed similar stabilisation of C γ 2 loops on engagement of Fc γ RIIIa receptor.

The FcWT structure reported here adopted an ‘open’ conformation with an interdomain spacing of 51.9Å (R291/R291). A comparison of the interdomain distances of all previously reported free native and receptor or ligand bound Fc domains reveals that IgG Fc can adopt a variety of ‘open’ conformations with interdomain distances varying from 38.0 Å to 50.6 Å (Figure 3.5.4, Appendix Table A1) and there is no one defined active, ‘open’ conformation for IgG Fc. The interdomain spacing observed here is larger than the equivalent interdomain distance of 48.1 Å observed for the Fc in complex with Fc γ RIIIa (PDB ID 3SGK). Asymmetric opening of the Fc dimer has been regularly noted in Fc-Fc γ RIIIa complexes (59,79). It is evident from the crystal structure of FcWT reported here that asymmetric ‘open’ conformations of free, unbound Fc are also feasible.

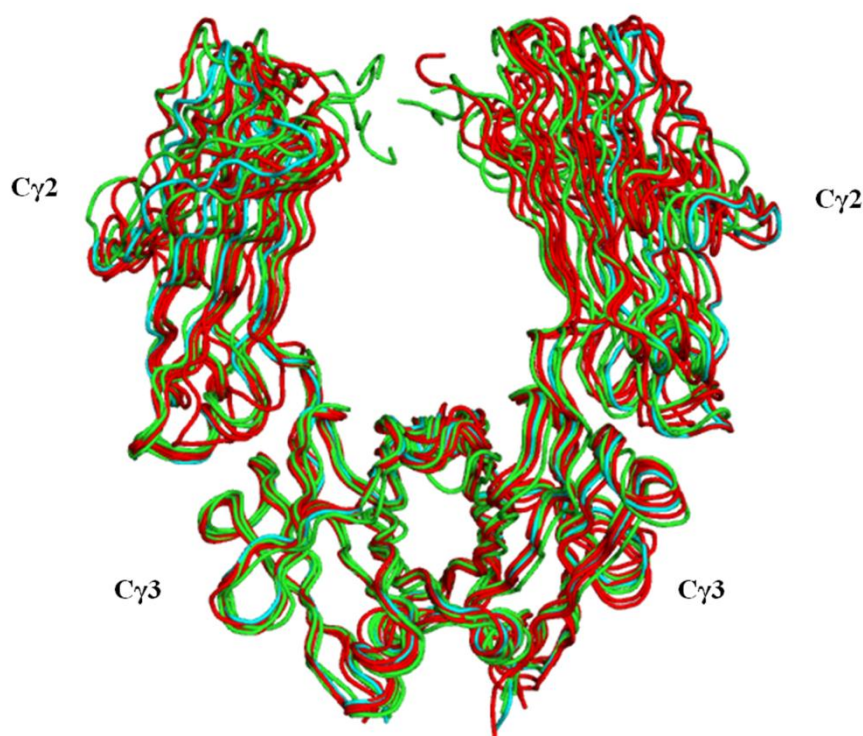


Figure 3.5.4. Conformational plasticity of FcWT C γ 2 domains. Protein backbone of all published Fc structures superimposed with FcWT (Cyan ribbon). Structures shown in red are free Fc structures and those depicted in green are from Fc γ RIIIa complexes. Structural superimpositions were performed as described in section 2.12 and interdomain distances are shown in Table 7.1.

3.6. Protein-glycan interactions of FcWT

Glycans attached to the single N-linked glycosylation site at Asn297 of IgG FcWT adopted a protein proximal conformation in the crystal structure, consistent with previous observations (31). GlcNAc 1, 2 and Man 3 residues lie close to the protein surface, with the 6 arm and 3 arm glycans following divergent paths on the protein surface (Figure 3.6.1). The 6 arm glycans make more contacts with amino acid residues of the C γ 2 domain and the terminal GlcNAc 5 residue lies adjacent to the previously described (PDB ID 1L6X) galactose pocket comprised of residues Pro244,Pro 245 and Glu258. GlcNAc 1 and GlcNAc 2 residues form hydrogen bonds with Asp265 and Arg301 respectively while the central β mannose residue along with the 6 arm Man 4' residue forms hydrophobic interactions with Phe241 (Figure 3.6.2).

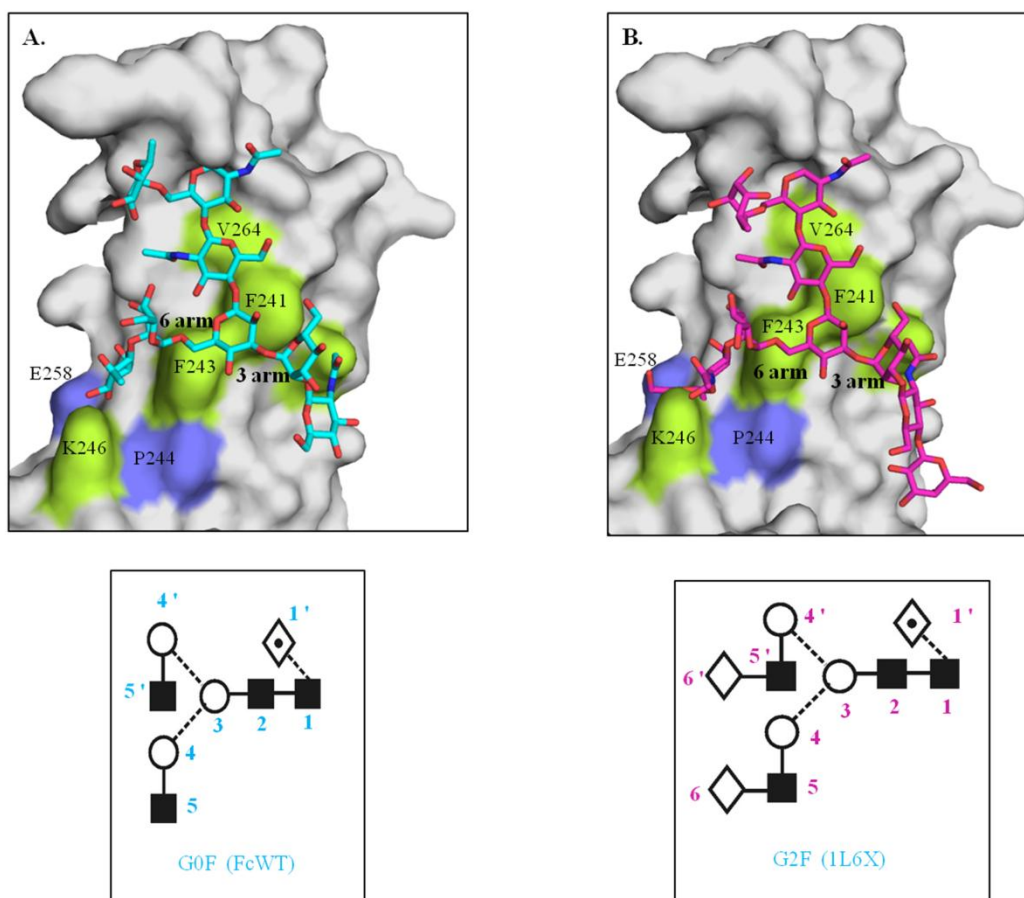


Figure 3.6.1. Structure and orientation of FcWT glycans. (A) Structure and trajectory of Asn-297 linked glycan on FcWT showing the 6 arm and 3 arm glycans on the protein surface. (B) Structure and trajectory of Asn-297 linked glycan on the high resolution FcWT structure (PDB ID 1L6X) showing the 6 arm and 3 arm glycans and terminal galactose residues on the protein surface.

The 3 arm on the other hand, makes fewer contacts with the underlying protein surface. Despite of the weak nature of protein-carbohydrate interactions, structures and conformations of the 6 arm and 3 arm of FcWT glycans are fairly well conserved in all native Fc structures (31,58). Absence of terminal galactose residues on Fc glycans has been previously shown to contribute to increased mobility for the glycan chain (128). However, more recent NMR studies have suggested a more mobile and flexible state for the Fc glycans (129). These studies and a more detailed discussion on the mobility of Fc glycans is presented in Chapter 4, Section 4.8.

The protein-proximal position and close apposition of glycans in the central cavity of the Fc dimer suggests limited access to Golgi glycoprocessing enzymes and may explain the low levels of sialylated and multi-antennary complex type glycans in serum IgG Fc.

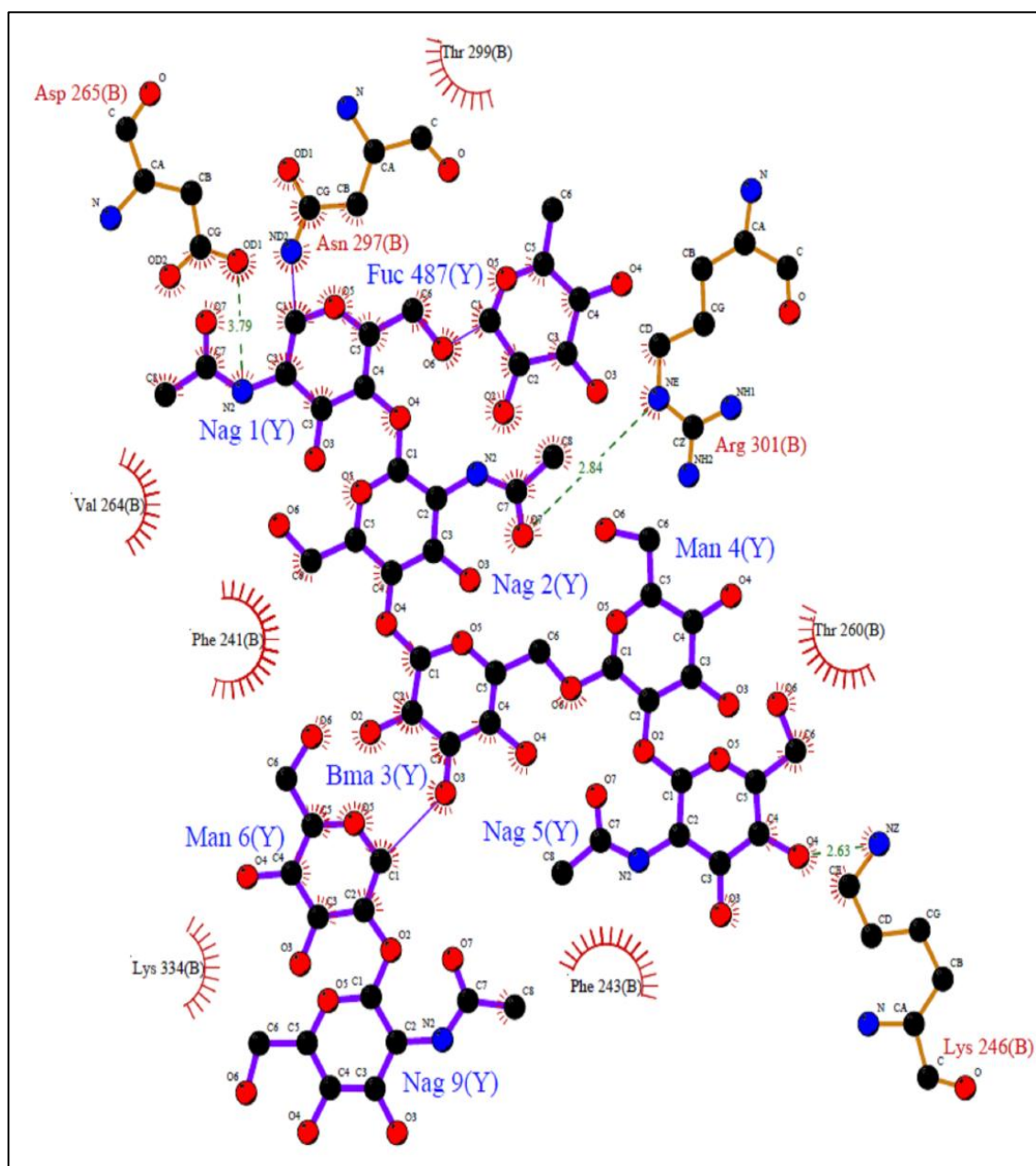


Figure 3.6.2. Protein-glycan interactions of glycans of FcWT. LIGPLOT(123) diagram showing hydrophobic interactions (red semicircles around residues) and hydrogen bonds (green dotted lines) formed by each carbohydrate residue of Fc Man₉GlcNAc₂ with amino residues on the Cy2 protein backbone.

3.7. Thermal stability of FcWT

It was evident from the structural analysis of FcWT that there is large amount of intradomain plasticity in the conformation and orientation of Fc C γ 2 domains. To ascertain if this 'open' conformation of FcWT altered its stability, DSF analysis was performed to measure the melting temperature of FcWT (Section 2.10). The thermogram of FcWT was characterised by a single thermal transition (Appendix, Figure A1) and the melting temperature of FcWT was calculated to be $69.87 \pm 0.48^\circ\text{C}$.

Previous studies on thermal unfolding of IgG1Fc using differential scanning calorimeter (DSC) have shown that the thermal unfolding of IgG1Fc over the temperature range of 5-100°C results in two characteristic thermal transitions (130). The first transition assigned to the C γ 2 domains was observed at $65.2 \pm 0.6^\circ\text{C}$ at pH 7.4. The second thermal transition assigned to the C γ 3 domains was observed at 81.9°C at pH 7.4. The second melting temperature resulting from the unfolding of the C γ 3 domains however remained glycoform independent. In the DSF experiments described in this thesis, the limit of sensitivity of Sypro orange is 75°C , therefore the second thermal transition for the C γ 3 domains were not measurable. The melting temperature of FcWT as measured by DSF is higher than that reported previously from DSC experiments. Due to unavailability of a working instrument, melting temperature of FcWT could not be calculated using DSC.

3.8. Conclusions

X-ray crystallographic structures of IgG Fc have been reported as part of several studies previously. The Fc portion of IgG has long been known to be amenable for crystallisation under various conditions and in fact derives its name for this propensity. There are currently 35 deposited structures of human IgG Fc domains in the protein data bank. Many of these are in complex with various other proteins and receptor molecules while a few have been modified by mutations. The majority of these structures contain the biantennary fucosylated complex type glycosylation common in serum IgG Fc.

Although the overall quaternary conformation of the Fc dimer is conserved in all reported structures, there are wide variations in interdomain distances between the two C γ 2 domains. The 2.6 Å crystal structure of human IgG1 Fc with native complex type glycosylation reported here adopted an 'open' conformation whilst maintaining

productive binding to Fc γ RIIIa. This IgG Fc has the largest interdomain spacing observed for native IgG Fc till date, with no change in thermal stability when compared to previously reported Fc conformations (Table 7.1).

These variations in Fc conformations along with the consistent disorder observed in C γ 2 loops point towards a more dynamic and flexible model for IgG Fc. This inherent plasticity of the C γ 2 domains may be crucial for the Fc domain to successfully engage a variety of binding partners in vivo. The intradomain plasticity of IgG Fc is discussed further in Chapter 7.

The second key observation from the FcWT structure was on the structure and orientation of the Asn297 linked glycan. Protein-carbohydrate interactions are generally known to be weak in nature and most glycoproteins contain highly mobile glycan structures, making them a hindrance to crystallisation and therefore very difficult structures to study. Fc glycans are unique in their comparatively rigid orientation and give well defined electron density in crystal structures. Comparison of the orientation of glycans on the FcWT C γ 2 protein surface with previously published structures showed the conserved nature of protein-glycan interactions. Indeed, comparison of mammalian IgG Fc sequences reveals that the Asn297 glycosylation site along with the key hydrophobic residues involved in glycan interactions are widely conserved across species (89). The FcWT structure reported here forms a template for comparison for all other modified Fc glycoforms reported in this thesis.

CHAPTER 4

Structure of a Mutated human IgG1 Fc with Reduced Protein-Carbohydrate Interactions

4. Structure of a mutated human IgG1 Fc with reduced protein-carbohydrate interactions

4.1. Summary

Structure of the complex type N-linked oligosaccharide on IgG Fc is widely conserved with the 6 arm of the glycan chain making several stable interactions with hydrophobic amino acid residues on the inner surface of the C γ 2 domain. Key amino acid residues that interact with the 6 arm glycans through hydrogen bonds and hydrophobic interactions include - Phe241, Phe243, Val264, Asp265, Lys246 and Arg301. Aromatic rings of Phe241 and Phe243 form CH- π interactions with GlcNAc 2 and GlcNAc 5' residues of the Fc glycan (131,132). The 3 arm on the other hand, makes fewer contacts with the protein backbone and hydrophobic interactions between Man 4 and Lys334 are the only observable protein-glycan interactions.

Influence of the hydrophobic protein backbone on IgG Fc glycosylation was first observed in mutational studies on mouse-human chimeric IgG3 antibodies (131). In this study, the authors observed that replacement of Phe241, Phe243 and Val264 with Ala resulted in elevated levels of mono and disialylated glycans and decreased binding to C1q and Fc γ RI activity. This suggested a relatively immobile and protein proximal conformation for Fc glycans with consequently reduced processing by Golgi galactosyltransferases and sialyltransferases.

Earlier NMR experiments compared the relaxation properties of galactosylated and agalactosylated Fc glycoforms and showed that lack of terminal galactose residues corresponds to increased mobility of the glycan (128). However, recent NMR spectroscopic studies show a much more dynamic and mobile role for the Fc glycan. The chemical shift patterns of isotopically labelled terminal galactose residues of Fc were studied at multiple fields and a range of temperatures. From these observations, the authors showed that the 6 arm of the galactosylated native Fc glycan alternates between protein-bound and unbound states in a temperature dependent manner (129).

Therefore, to test these alternate hypotheses and further clarify the role of protein-directed glycosylation in IgG Fc, the structure and glycan conformations of an abundantly sialylated Fc mutated at Phe241 was studied using X-ray crystallography.

4.2. Purification of Fc mutants with reduced protein-carbohydrate interactions

Human IgG1 Fc domain with F241A, F241A/F243A and F241A/F243A/V262E/V264E mutations cloned in the mammalian expression vector pHLsec, were transfected into HEK 293T cells as described in Section 2.1–2.3. Large scale (1.5 L) culture of transfected HEK 293T cells were allowed to grow at 37°C for 10 days following which cell culture supernatant was collected and mutant Fc was purified by immobilised metal affinity chromatography and SEC (Figure 4.2.1A, 4.2.2A, B). Purity of recombinant Fc protein was verified by SDS-PAGE where Fc was visualised as an approximately 53kDa band and as 27kDa band after reducing disulphide bonds (Figure 4.2.1B, 4.2.2C).

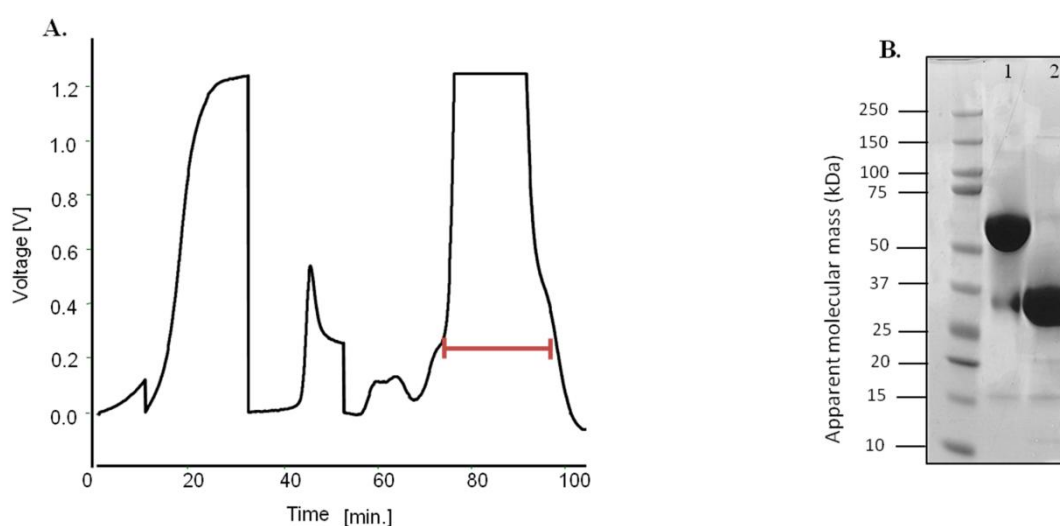


Figure 4.2.1. Purification of F241A Fc. (A) SEC curve of F241A Fc run on a Hiload 16/60 superdex 200 prepgrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peaks. (B) 4-12% gradient SDS-PAGE of concentrated F241A Fc after SEC purification. Lane1 contains non-reduced and Lane 2 contains reduced protein sample.

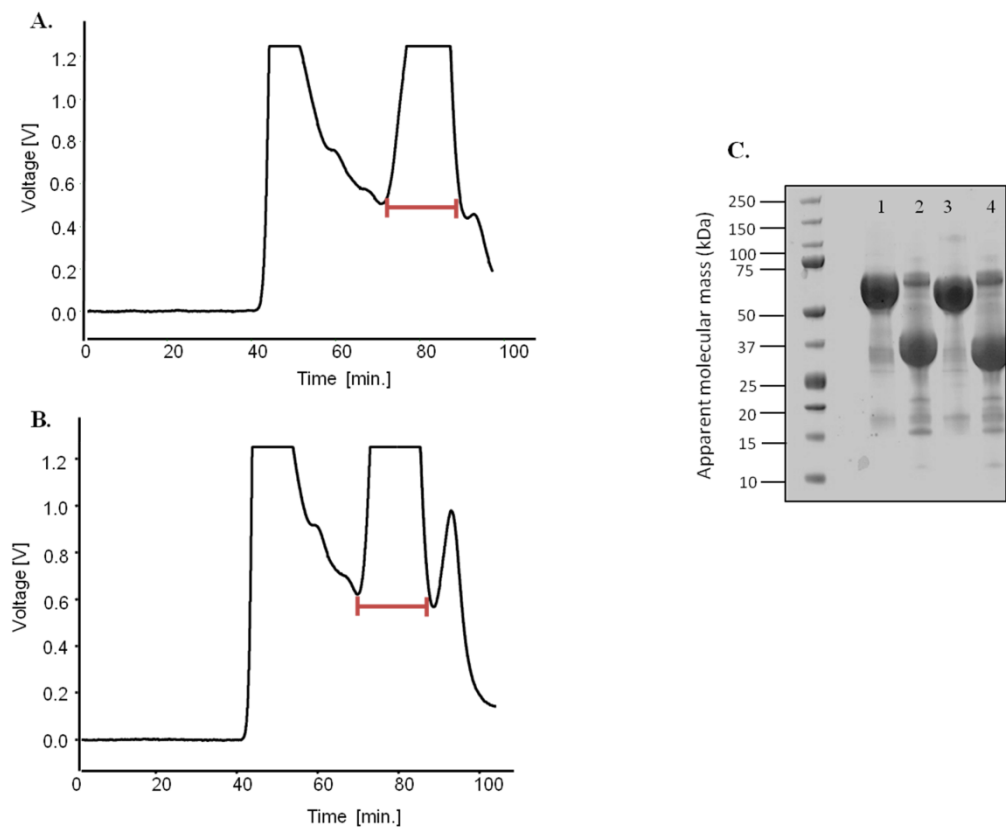


Figure 4.2.2. Purification of F241A/F243A and F241A/F243A/V262E/V264E Fc. SEC curve of (A) F241A/F243A Fc and (B) F241A/F243A/V262E/V264E Fc run on a Hiload 16/60 superdex 200 prepgrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peaks. 4-12% gradient SDS-PAGE of concentrated F241A/F243A Fc (Lanes 1 and 2) and F241A/F243A/V262A/V264A Fc (lanes 3 and 4) after SEC purification. Lanes 1 and 3 contained non-reduced and Lane 2 and 4 contained reduced protein sample.

4.3. Crystallisation and structure determination of F241A Fc

Recombinant mutant IgG1 Fc (F241A) was concentrated to 7.0 mg mL^{-1} and crystallised after 10 days using the hanging drop vapour diffusion method (Section 2.4). Crystals of F241A Fc grew at room temperature in a precipitant containing 28% polyethylene glycol monomethyl ether 2,000 in 0.1M BIS-TRIS buffer at pH 6.5. Crystals were flash frozen by immersion in a cryoprotectant containing the mother liquor diluted in 30% Polyethylene glycol and then rapidly transferred to a gaseous nitrogen stream. Crystallographic data were collected to a resolution of $1.93 \text{ \AA}$ at beamline I04 at the Diamond Light Source (Oxfordshire, UK). Data were processed and scaled using iMosflm (104) and Scala (133) and structure was solved using molecular replacement with the program PHASER (134) using native FcWT as search model. One Fc homodimer was identified in each asymmetric unit with a solvent content of 48.73% (V_M of $2.40 \text{ \AA}^3 \text{ Da}^{-1}$). Model building was performed with COOT (135) and iteratively refined using restrained refinement with TLS in the CCP4 supported program (136), REFMAC5 (137) as described in section 2.6. Model quality was validated with Molprobtity. Ramachandran analysis of the final structure showed 98.9% of residues in the most favoured region with no residues in disallowed regions. Data collection and refinement statistics for the F241A Fc structure are presented in Table 4.3.1.

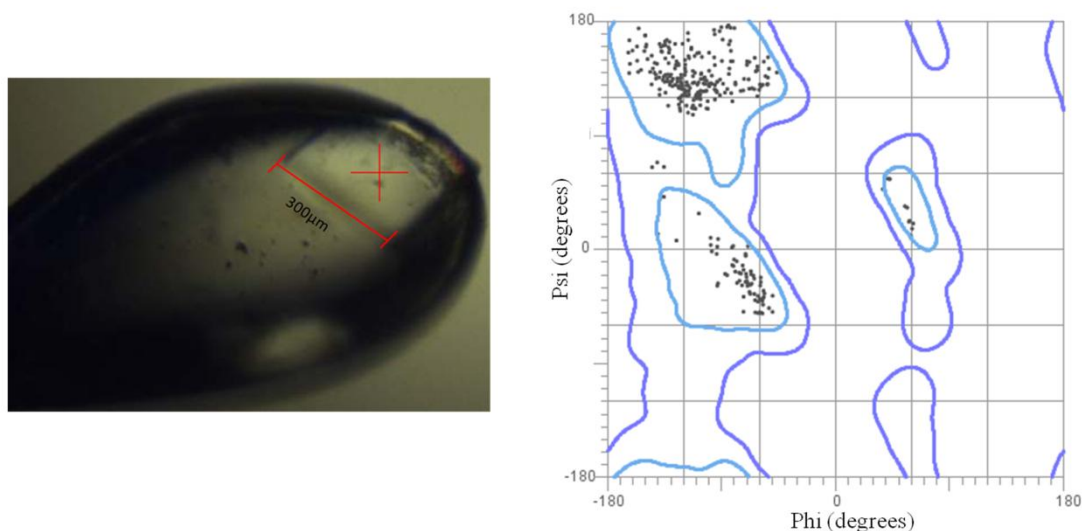


Figure 4.3.1. Crystallisation and structural analysis of F241A Fc. (A) F241A Fc crystals grew to a length of $300 \text{ }\mu\text{m}$. Red coloured cross indicates point of X-ray beam focus. (B) Ramachandran plot generated with Molprobtity(110) for the final refined F241A Fc structure showing 98.9% of residues in the most favoured regions.

Table 4.3.1. Data and refinement statistics for F241A-Fc

Data Collection			
Beamline	Diamond I04		
Resolution (Å)	36.72-1.93 (2.03-1.93) ^a		
Space group	P2 ₁ 2 ₁ 2 ₁		
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å) and angles (°)	48.90, 73.45, 135.47	90.00, 90.00,	90.00
Wavelength (Å)	0.917		
Unique reflections	37,174 (5,398)		
Completeness (%)	99.2 (99.9)		
<i>R</i> _{merge} (%) ^b	10.0(66.0)		
I/σI	10.8 (2.9)		
Refinement			
Resolution range (Å)	50.0-1.93 (1.98-1.93)		
Number of reflections	35,229 (2,598)		
<i>R</i> _{work} (%) ^c	22.1		
<i>R</i> _{free} (%) ^d	25.5		
r.m.s.d. ^e			
Bonds (Å)	0.010		
Angles (°)	1.2		
Average <i>B</i> -factors (Å ²)			
(protein/carbohydrate/water)	40.6/55.25/37.88		
Model quality (Ramachandran plot)			
Most favoured region (%)	98.9		
Allowed region	1.1		

^aNumbers in parentheses refer to the relevant outer resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I(\text{hkl};i) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I(\text{hkl};i)}$, where $I(\text{hkl};i)$ is the intensity of an individual measurement and $\langle I(\text{hkl}) \rangle$ is the average intensity from multiple observations.(106)

^c $R_{\text{work}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$ (106)

^d R_{free} was calculated as R_{work} but after removal of 5% of data prior to refinement(Section 2.6).

4.4. Structural analysis of F241A Fc

The crystal structure of F241A Fc was determined to a resolution of 1.93 Å. Electron density for the C γ 2 and C γ 3 domains for both protomers of the Fc dimer was observed. Electron density at amino acid position 241 revealed the loss of the aromatic ring and a clearly defined methyl group of Ala. All four immunoglobulin domains of the Fc were built, although only one chain (termed the A chain) had well defined electron density for the Asn297-linked carbohydrate and upper loops of the C γ 2 domain (Figure 4.4.1). These disordered regions from chain B were not included in the final crystallographic model. B factor values of C γ 2 domains of chain A and B ($B_{average}$ of chain A is 59.43 Å² and $B_{average}$ of chain B is 54.12 Å²) were comparable (Figure 4.4.2). This is indicative of a well defined conformation for the F241A Fc domain.

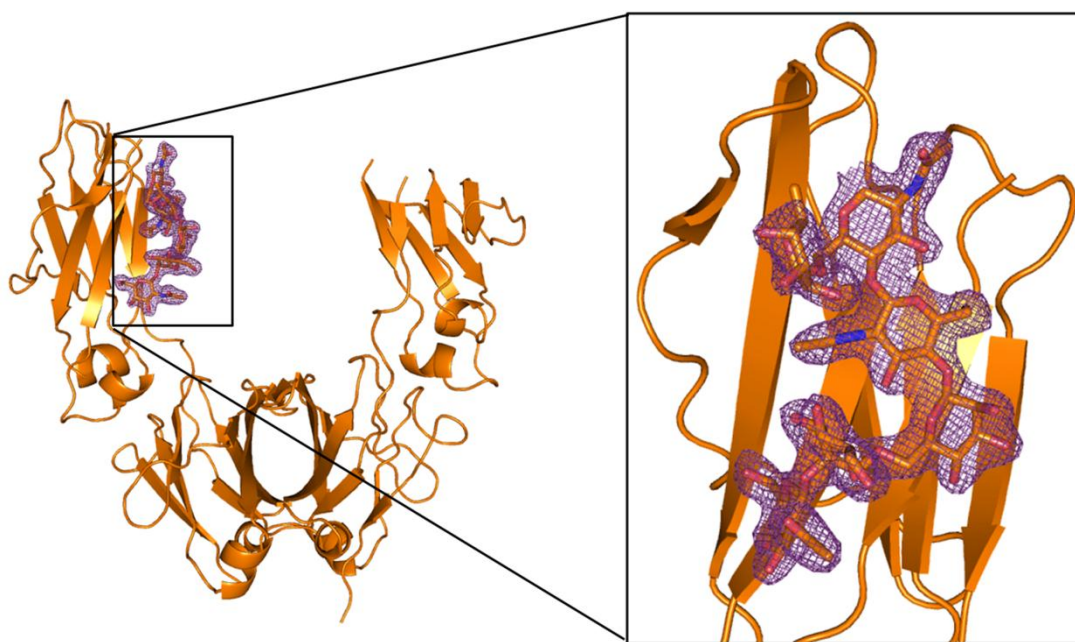


Figure 4.4.1. Structure of F241A Fc at 1.93 Å shown in cartoon representation. A maximum likelihood $2F_o - F_c$ map is displayed around the N-linked glycans, contoured to 1σ . Magnified view of the F241A Fc glycan on chain A is shown on the right.

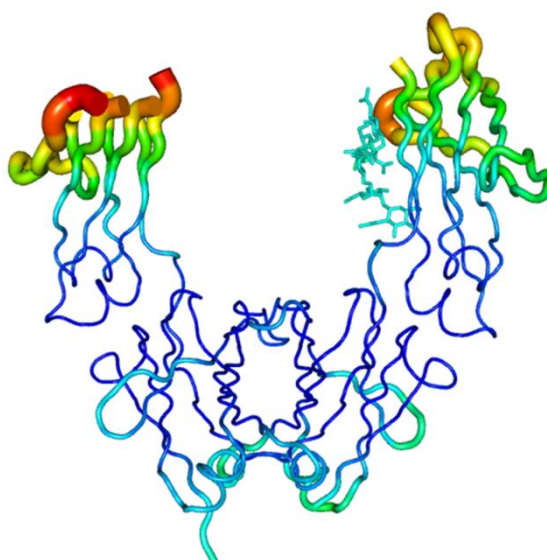


Figure 4.4.2. Conformational mobility of C γ 2 domains of F241A Fc. The protein backbone and attached glycans of F241A Fc are coloured according to their *B* factor values. Colours range from dark blue (lowest *B* factors) through to bright red (highest *B* factors).

F241A Fc adopts an ‘open’ conformation with asymmetric C γ 2 domains and an interdomain spacing of 60.1 Å (Table 7.1). A comparison of quaternary structures of F241A Fc with FcWT and Fc-Fc γ RIIIa complex showed significant similarity (r.m.s.d. of 0.4 over equivalent C α atoms of FcWT). However, the interdomain spacing of F241AFc is significantly larger than that reported for Fc- Fc γ RIIIa complexes (50.6 Å for 1E4K). Further, the significant disorder in the B/C, D/E and F/G loops of chain B of the Fc C γ 2 domain do not allow a definite interpretation for the quaternary conformation of the F241A Fc dimer.

Previous studies showed loss of binding to Fc γ RIIIa for a F241S Fc mutant (132) and F241A mutation in an IgG3 Fc resulted in loss of Fc γ RIa mediated activity (131). This mutation has also been associated with lowered thermal stability of the C γ 2 domains (132). These observations indicate that hydrophobic interactions between protein and glycan residues contribute to Fc stability and mediate productive binding to Fc γ RIIIa.

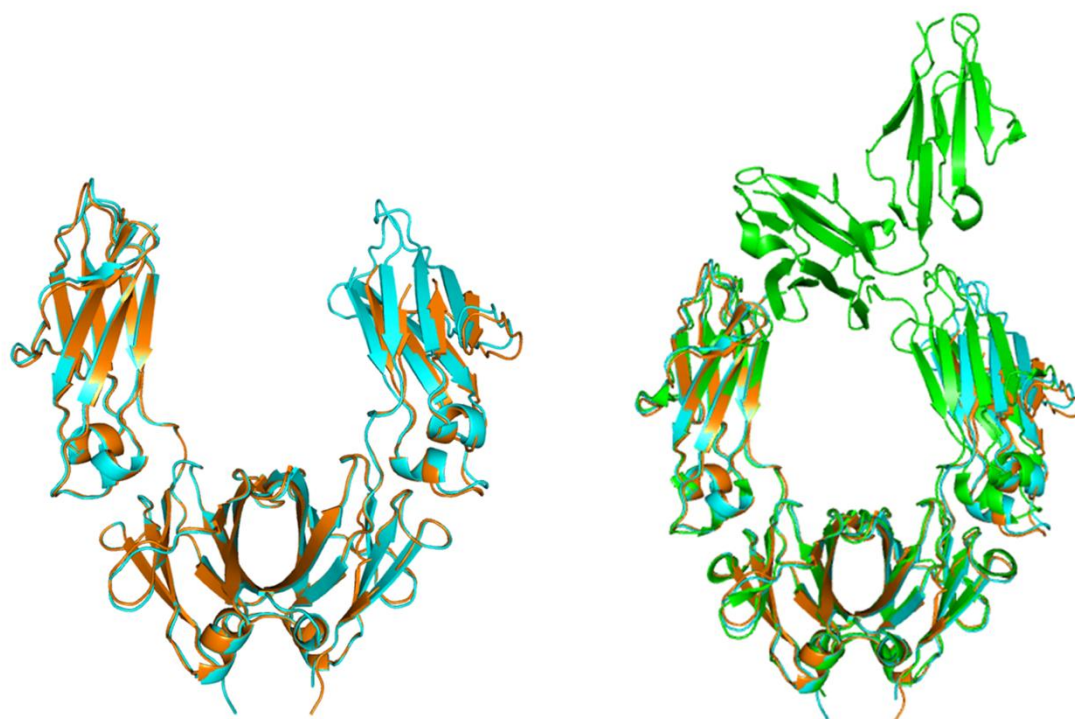


Figure 4.4.3. Structural analysis of F241A Fc. (A) Comparison of the quaternary structures of F241A Fc (orange) with FcWT (cyan). (B) Overlay of F241A Fc (orange) on FcWT (cyan) and Fc-FcγRIIIa complex structures (Green, PDB ID 3SGK).

4.5. Sugar protein interactions and Glycan processing in the Fc mutant

Analyses of glycans released from F241A Fc have previously shown elevated levels of galactosylation and sialylation (131,132). Electron density was visible for five out of the seven glycans constituting the chitobiose core of F241A Fc glycan (Figure 4.4.1). The 6 arm glycans were traceable upto GlcNAc5' while very thin density was visible for the 3 arm glycans. GlcNAc1 and GlcNAc2 residues on the reducing end and the core $\alpha 1 \rightarrow 3$ linked fucose followed a protein proximal path similar to that for FcWT. On the 6 arm, Man 4 and GlcNAc5' residues also adopted a protein proximal path making hydrophobic contacts with Phe243 and Thr260 and the C3 hydroxyl of GlcNAc5' forms hydrogen bonds with the amine of Lys246 (Figure 4.5.2). In the native Fc, Phe241 participates in well-characterized CH- π interactions with GlcNAc2 and Man3 residues (138,139). The F241A mutation eliminates this stabilizing interaction.

Given the increased processing of the glycan in the F241A mutant, it would be expected for the N-linked glycan to be released from the protein surface to provide increased access to Golgi-resident glycosyltransferases. However from the electron density map,

the Man3 residue collapses towards the C γ 2 domain β -strands and no electron density for the 3-arm is observable (Figure 4.5.1). This is indicative of increased mobility of the α 1 $\rightarrow$ 3 linked arm of the F241A Fc glycan in comparison to the corresponding arm in the FcWT structure. The average *B* factor values of the F241A Fc glycan chain (55.245 Å²) are lower in comparison to that of FcWT (70.982 Å²) while the *B* factor values for the C γ 2 domains remained comparable (52.71 Å² for FcWT and 59.428 Å² for F241A Fc). This infers an overall stable conformation for the chitobiose core and the attached 6 arm glycans of the F241A glycan chain. It is however important to also note that lower *B* factor values for F241A may also be contributed by stabilising crystal contacts.

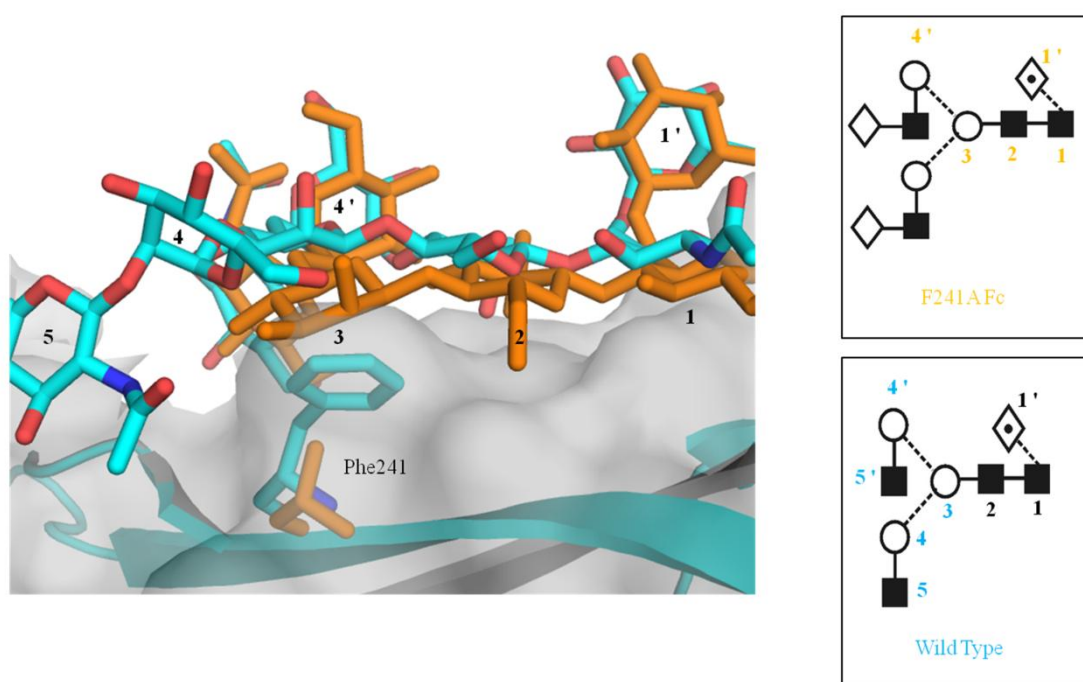


Figure 4.5.1. F241A glycan-protein interactions. Wild type (cyan) and F241A (orange) glycans interaction with Phe241 of the Fc and Ala241 of the F241A Fc mutant C γ 2 domain is shown with interacting carbohydrate residues labelled. The schematic on the right shows the glycan composition of the predominant glycoform.

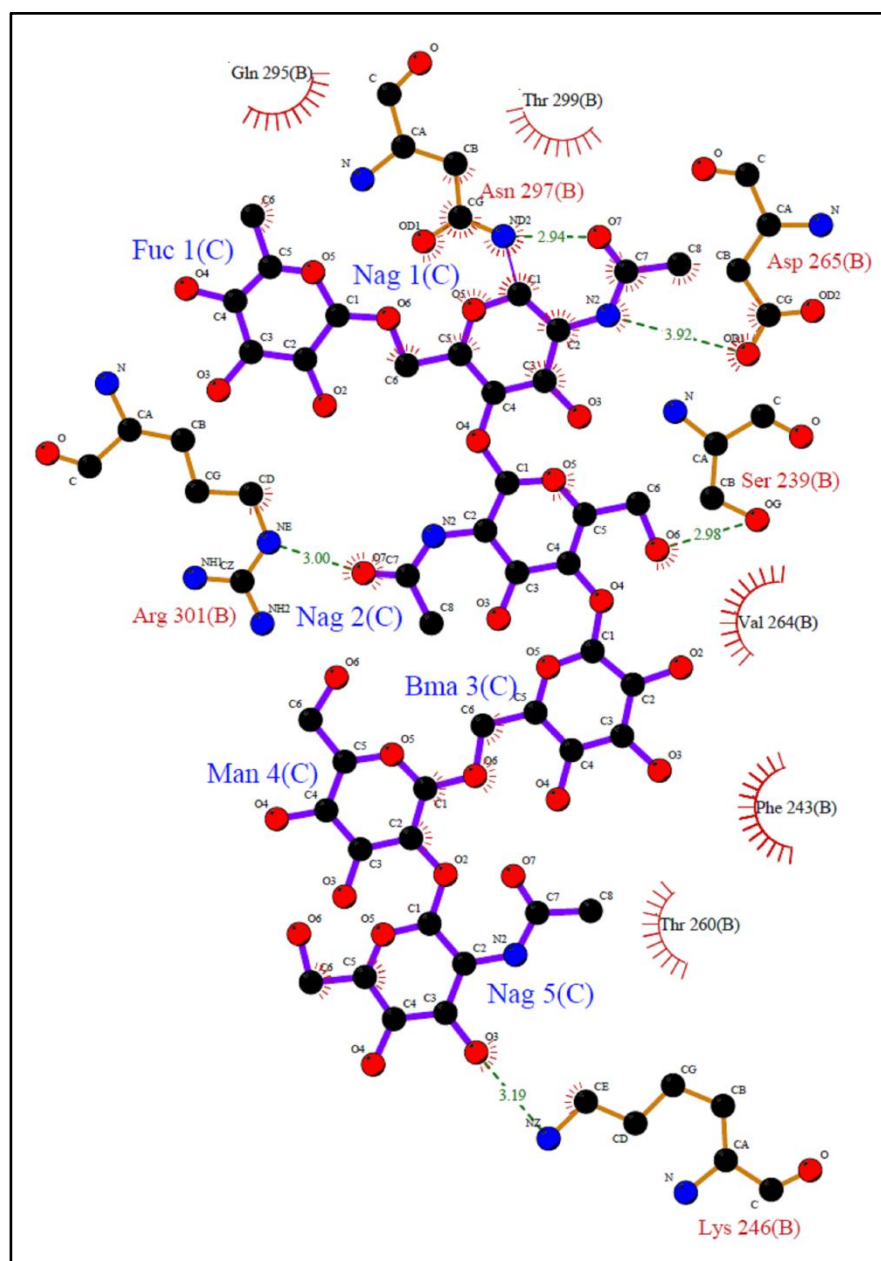


Figure 4.5.2. Protein-glycan interactions of the glycans of F241A Fc. LIGPLOT diagram showing hydrophobic interactions (red spokes around residues) and hydrogen bonds (green dotted lines) formed by each carbohydrate residue with amino residues on the C γ 2 protein backbone.

4.6. Conclusions

Comparison of glycosylation profiles of Fab and Fc domains in human serum reveals the presence of multi-antennary sialylated glycans on the Fab domain but surprisingly limited terminal processing of Fc glycans with the predominance of fucosylated biantennary complex type glycans (140,141). In mammalian recombinant expression cell lines such as HEK 293T, there is a predominance of agalactosylated IgG Fc glycans with low levels of monogalactosylated species. Similar to serum IgG glycosylations, sialylated glycans are rarely observed in IgG Fc expressed in HEK 293T cells.

The limited glycan diversity and close juxtaposition of glycans with hydrophobic amino acid residues of IgG Fc is supportive of a protein-directed glycosylation profile. The F241A Fc mutant showed increased sialylation of both arms of the biantennary glycan consistent with previous reports (131,132).

In the crystal structure of F241A Fc, electron density for the N-linked glycans could be traced on one Fc chain only and while the conformation of the reducing end of the 6 arm glycan chain was largely conserved, the 3 arm residues were significantly disordered. It is known that crystallographic studies of heterogeneous glycoproteins present an average density from distinct conformers. This particular conformation of F241A thus represents one of several stable conformations. Further, the selective destabilisation of glycan chains in F241A indicates greater conformational mobility and is supportive of a flexible and accessible model for Fc glycan structures. This is consistent with recent evidence from solution NMR studies that show that at 37°C, 70% of Fc glycan conformations exist in a protein uncoordinated state and lower temperatures are associated with larger proportion of protein-coordinated glycoforms (129). Given that most X-ray crystallographic measurements are made at extremely low temperatures (100K), observations from the F241A Fc structure may indicate a protein-coordinated minor Fc population. NMR studies of the F241A Fc at ambient temperatures would perhaps provide a more accurate picture of Fc glycan conformations. Finally, given these observations on the conformational mobility of Fc glycans and C γ 2 domains, analysis of Fc conformations under experimental conditions that more accurately represent *in vivo* temperatures are necessary for a more accurate model of IgG Fc structure.

CHAPTER 5

Structure and Protein-Carbohydrate Interactions of Biosynthetic Intermediate IgG1 Fc Glycoforms

5. Structure and protein-carbohydrate interactions of biosynthetic intermediate IgG1 Fc glycoforms

5.1. Summary

Mammalian N-linked glycosylation in the ER begins with the transfer of an oligosaccharide precursor from a lipid carrier to the nascent polypeptide at asparagine residues found in the sequence Asn-X-Ser/Thr where X can be any amino acid except proline (142). Presence of N-linked glycans at asparagines residues can also be determined by the presence of adjacent hydrophobic amino acids and on the non-classical NXC motif. The oligosaccharide lipid precursor comprises of nine mannose residues and three glucose residues all attached to the ER membrane bound lipid - Dolichol. After covalent linkage of this oligosaccharide moiety to the protein, the glucosylated high-mannose glycan is sequentially trimmed by the removal of the two terminal glucose residues by α -glucosidases I and II in the ER. The action of these glucosidases produces a monoglucosylated structure which can be bound by calnexin and calreticulin which promote protein folding. The final glucose is removed by a second activity of glucosidase II and the protein can either leave the folding cycle or, if unfolded, can be re-glucosylated by UGGT1 and re-bind to calnexin (143).

Oligomannose glycans that are released after glucosidase digestion undergo further processing in the ER and Golgi where, ER α -mannosidase I and Golgi α -mannosidases IA, IB and IC catalyze the removal of the terminal oligomannose glycans that are released as $\text{Man}_5\text{GlcNAc}_2$ bearing glycoprotein (Figure 1.4.1). The $\text{Man}_5\text{GlcNAc}_2$ glycan is an obligate intermediate prior to further diversification. The step in towards “complex-type” glycosylation requires GlcNAc transferase I which catalyses the addition of a single $\beta 1 \rightarrow 2$ linked GlcNAc to the 3 arm of the glycan chain to generate a “hybrid type glycan”. Core fucosylation of glycans is another commonly observed feature in IgG glycans and occurs after the GlcNAc transferase I step. The resulting $\text{Man}_5\text{GlcNAc}_3\text{Fuc}$ hybrid undergoes one final processing stage catalyzed by Golgi α -mannosidase II which removes the $\alpha 1 \rightarrow 3$ linked terminal mannose of the 6 arm. This truncated $\text{Man}_3\text{GlcNAc}_3\text{Fuc}$ glycan is then modified by the action of several GlcNAc transferases to yield biantennary and triantennary complex-type glycans with variable terminal galactose and sialic acid residues. This results in the different glycoforms of IgG observed in human serum (143).

Changes in the glycosylation state of IgG are known to modulate the effector functions of antibodies. Manipulation of N-linked glycosylation of antibodies has been used successfully to generate antibodies with enhanced effector functions (40). One such modification that is now undergoing clinical testing has been the production of IgG antibodies with oligomannose glycans (144). These unprocessed antibody glycoforms show enhanced binding to the activatory Fc γ RIIIa receptor and hence potent cytotoxic activity. Structural analysis of such glyco-engineered Fc domains could define novel protein-glycan interactions that may guide further engineering of antibodies for modified effector functions.

In this chapter, X-ray crystallographic analysis of human IgG Fc that have been arrested at different stages of glycan biogenesis using selective inhibitors of key steps in the mammalian N-linked glycosylation pathway is described (Figure 1.4.1). MALDI-TOF and negative ion ESI mass spectrometric assays were used to elucidate the composition, fine structure, and connectivity of the N-linked glycan chains of each Fc glycoform. SPR analysis for binding to Fc γ RIIIa and DSF assays for thermal unfolding were used to investigate the effect of glycan modifications on functional activity and C γ 2 domain stability.

5.2. Cloning and Expression of Fc Man₉GlcNAc₂ and Fc Hybrid

Human IgG1Fc domain was cloned into the mammalian expression vector pHLsec and expressed transiently in HEK 293T cells in the presence of 20 μ M Kifunensine or 20 μ M Swainsonine (Section 2.3). Kifunensine inhibits α -mannosidase class I enzyme in the endoplasmic reticulum resulting in the accumulation of immature oligomannose type glycans on secreted proteins (145). Swainsonine inhibits Golgi α -mannosidase II and results in the accumulation of proteins with Hybrid type glycoforms bearing a core Man₅GlcNAc₂Fuc with further processing of glycans of the 3 arm.

Large scale expression in 1.5 L cell culture for 7-10 days was followed by purification of Fc Man₉GlcNAc₂ and Fc Hybrid by IMAC and SEC and proteins were further concentrated to 7.7 mg/ml. SDS-PAGE analysis confirmed the purity and size of SEC purified Fc glycoforms (Figure 5.2.1 and 5.2.2). Purified Fc with oligomannose and hybrid type glycans, migrate as an approximately 53 kDa band on non-reducing SDS-PAGE and as a 27 kDa band on reducing SDS-PAGE

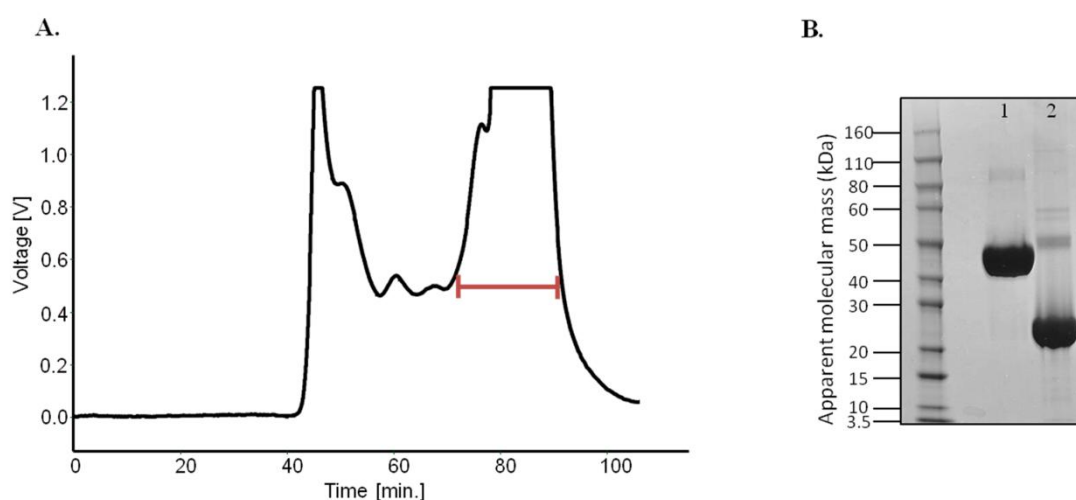


Figure 5.2.1. Purification of Fc Man₉GlcNAc₂ (A) SEC curve of Fc Man₉GlcNAc₂ run on a Hiload 16/60 superdex 200 prepgrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peaks. (B) 4-12% gradient SDS-PAGE of concentrated Fc Man₉GlcNAc₂ after SEC purification. Lane1 is non-reduced and Lane 2 is reduced Fc Man₉GlcNAc₂.

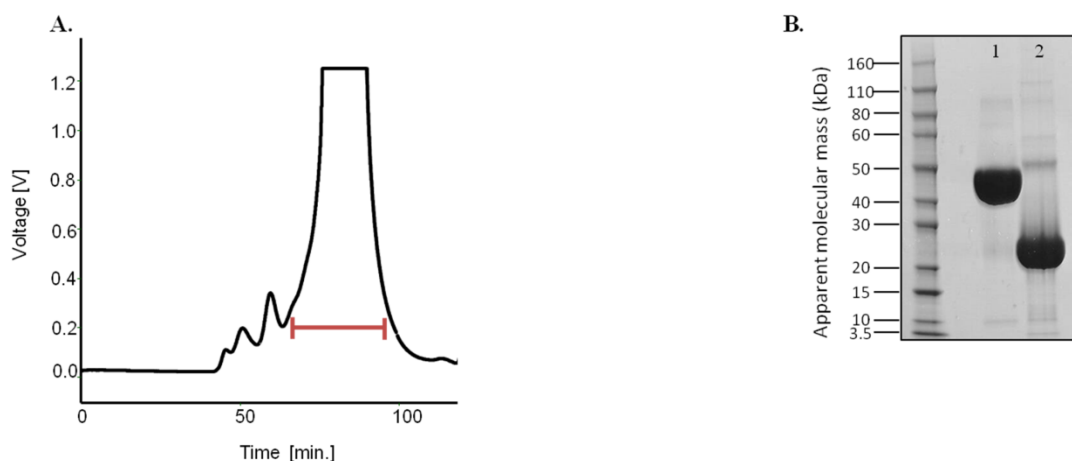


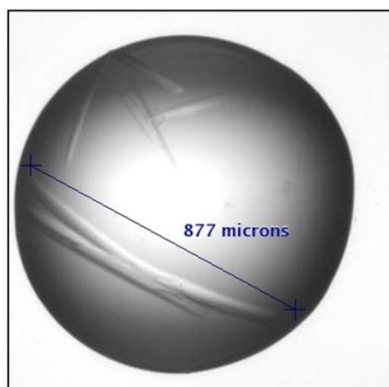
Figure 5.2.2. Purification of Fc Hybrid. (A) SEC curve of Fc Hybrid run on a Hiload 16/60 superdex 200 pregrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peaks. (B) 4-12% gradient SDS-PAGE of concentrated Fc Hybrid after SEC purification. Lane1 is non-reduced and Lane 2 is reduced Fc Hybrid.

5.3. Crystallisation and Structure Determination of Fc Man₉GlcNAc₂

Purified Fc Man₉GlcNAc₂ was used for growing crystals by the sitting drop vapour diffusion method (Section 2.4). Fc Man₉GlcNAc₂ crystals grew at room temperature in 20% w/v PEG monoethyl Ether 5000, 0.100M bis-Tris buffer, pH 6.5 after 3-6 days (Figure 5.3.1A). Crystals were immersed in cryoprotectant containing mother liquor in 25% ethylene glycol and transferred rapidly to gaseous nitrogen stream at 100 K. Data for Fc Man₉GlcNAc₂ crystals was collected at the I04 beamline at Diamond.

The FcMan₉GlcNAc₂ structure was solved by molecular replacement with Phaser using native Fc structures (PDB accession number 2DTQ) (58) as search model and was refined to a resolution of 2.24 Å. One Fc homodimer per asymmetric unit was identified, giving a solvent content of 55.26 % (V_M of 2.75 Å³ Da⁻¹). Model Building was performed with COOT along with several rounds of iterative refining with the program REFMAC5 as described in section 2.6. Molprobity was used for final validation of the model. Ramachandran analysis showed that 97.8% of the residues were in the most favoured region with no residues in disallowed regions (Figure 5.3.1B). Data collection and refinement statistics are presented in Table 5.3.1.

A.



B.

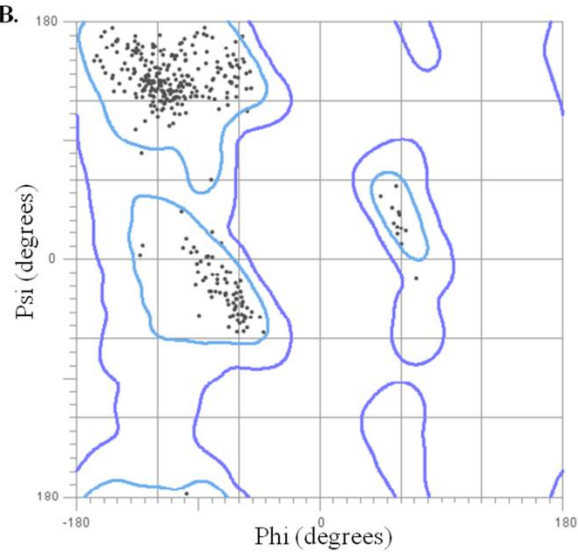


Figure 5.3.1 Crystallisation and structural analysis of Fc Man₉GlcNAc₂.(A) Fc Man₉GlcNAc₂ crystals grew to a length of 877 μm . (B) Ramachandran plot generated with Molprobity(110) for the final refined Fc Man₉GlcNAc₂ structure.

Table 5.3.1. Data collection and refinement statistics for Fc Man₉GlcNAc₂

Data collection	Fc Man ₉ GlcNAc ₂
Beamline	Diamond I02
Resolution (Å)	50.0-2.24 (2.32-2.24) ^a
Spacegroup	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions (Å) and angles (°)	<i>a</i> = 49.4, <i>b</i> = 75.2, <i>c</i> = 150.0, <i>a</i> = <i>b</i> = <i>γ</i> = 90
Wavelength (Å)	0.979
Unique reflections	27781 (2686)
Completeness (%)	99.7 (98.9)
<i>R</i> _{merge} (%) ^b	0.114 (0.828)
<i>I</i> / <i>σI</i>	20.0 (3.0)
Average redundancy	10.5 (9.4)
Refinement	
Resolution range (Å)	47.0-2.24 (2.29-2.24)
Number of reflections	26337 (1852)
<i>R</i> _{work} (%) ^c	20.9
<i>R</i> _{free} (%) ^d	24.9
r.m.s.d. bonds (Å)	0.011
angles (°)	1.3
Atoms per asymmetric unit (protein/water/glycan)	3299/219/151
Average <i>B</i> factors (protein/water/glycan) (Å ²)	45.6/40.5/80.9
Ramachandran Plot (Favored/allowed; %)	97.8/2.2

^aNumbers in parentheses refer to the relevant outer resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I(\text{hkl}; i) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I(\text{hkl}; i)}$, where $I(\text{hkl}; i)$ is the intensity of an individual measurement and $\langle I(\text{hkl}) \rangle$ is the average intensity from multiple observations.(106)

^c $R_{\text{work}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$

^d*R*_{free} was calculated as *R*_{work} but after removal of 5% of data prior to refinement(Section 2.6).

5.4. Mass spectrometric analysis of Fc Man₉GlcNAc₂ glycans

N-linked glycans of purified glycoproteins were released with PNGase F following SDS-PAGE analysis and examined by MALDI-MS (Figure 5.4.1). IgG1 Fc expressed in the presence of 20 μ M kifunensine resulted in a spectrum almost entirely dominated by m/z of 1905.6 corresponding to Man₉GlcNAc₂. A trace population of Man₈GlcNAc₂ glycans was observed at m/z 1743.5 indicating some residual Golgi α -mannosidase activity. Consistent with the inhibition of core fucosylation by terminal α 1 $\rightarrow$ 2-mannose residues (146,147) and the requirement for terminal 3-arm β 1 $\rightarrow$ 2 GlcNAc for full enzymatic activity (147,148), no α 1 $\rightarrow$ 6 fucosylation was observed.

Fragmentation of individual glycans from negative ion collision-induced dissociation spectra (CID) was analyzed as described in Section 2.8 (Figure 5.4.2). The principal A₆ ion with m/z of 1720.7 corresponds to the cleavage of the chitobiose core at the reducing terminus. The D and C_{3a} ions (m/z of 971.4 and 827.3 respectively) were diagnostic for the presence of the 6 arm and the branching Man $\mathbf{B}$ residue (Figure 5.4.2) and the D' and B₂ ions confirmed the presence of the terminal D1 and D3 mannose residues.

The composition, structure and connectivity of individual carbohydrate residues of Fc Man₉GlcNAc₂ glycans confirmed by MALDI-TOF analysis and ESI negative ion fragmentation analysis facilitated the accurate interpretation of electron density from carbohydrate residues in the Fc Man₉GlcNAc₂ structure.

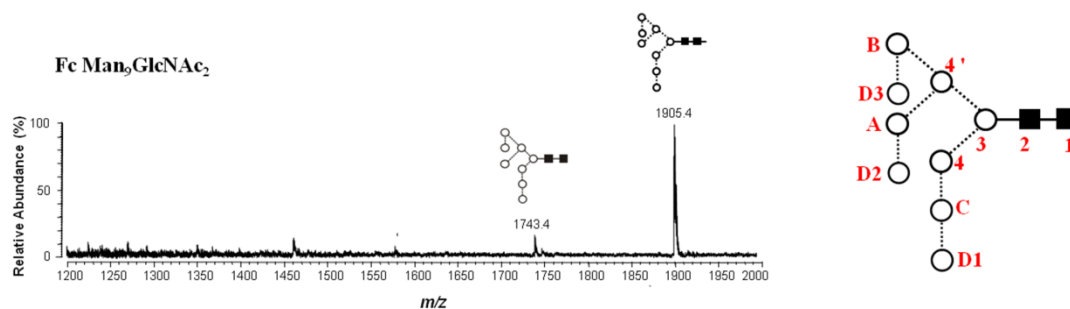


Figure 5.4.1. MALDI-TOF analysis of Fc Man₉GlcNAc₂ glycans. *m/z* and composition of all PNGase F released glycans from Fc Man₉GlcNAc₂ are shown. The glycan composition of each peak is shown. The schematic on the right shows the principal Fc Man₉GlcNAc₂ glycoform with individual glycan units numbered as described by Crispin *et al*(33).

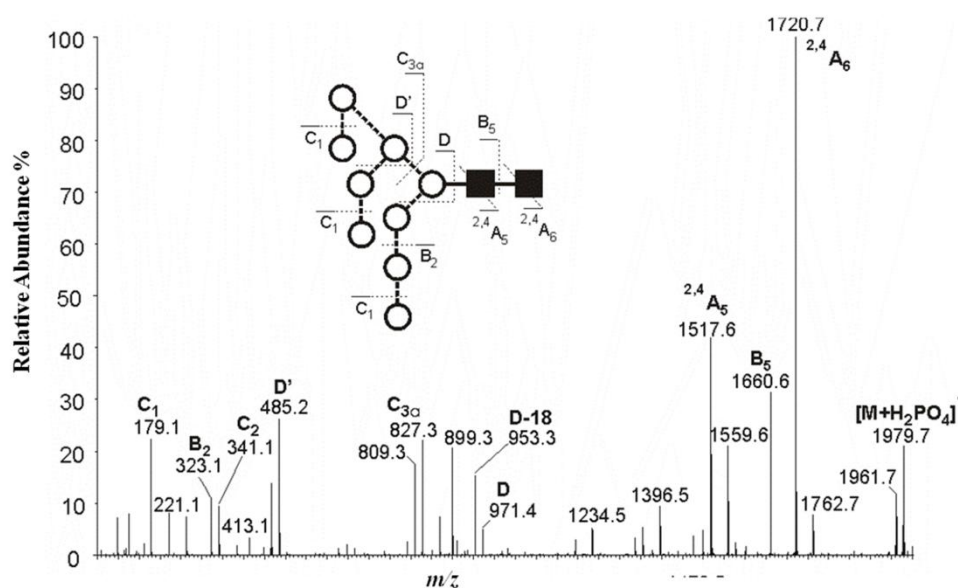


Figure 5.4.2. Negative ion ESI mass spectrometry of Fc glycans. ESI CID spectra of [M+H₂PO₄]⁻ ions of Fc Man₉GlcNAc₂. Fragmented ions are labelled according to scheme as devised by Domon and Costello (149).

5.5. Structural Analysis of Fc Man₉GlcNAc₂

Fc Man₉GlcNAc₂ retained the horseshoe shaped structure common to all wild type Fc structures and extensive branched electron density was observed at the Asn297 glycosylation site (Figure 5.5.1). On chain A, electron density was visible for all glycan residues comprising the Man₉GlcNAc₂ glycoform, barring the terminal α 1 $\rightarrow$ 2 linked mannose residues of the D1 and D2 arms of the oligomannose glycan. On chain B, electron density could only be traced for the first three glycan residues of the chitobiose core (Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc). All amino acids in both chains were traceable in electron density maps barring residues 272 to 274. A comparison of average *B* factor values for chain A and B (*B*_{average} Chain A of 53.613 Å² and *B*_{average} Chain B of 71.969 Å² for all main chain atoms of the C γ 2 domains) shows that chain B is more conformationally mobile (Figure 5.5.2). This is also reflected in the asymmetry of the Fc dimer with Chain B further “tilted” out resulting in an increase in interdomain spacing.

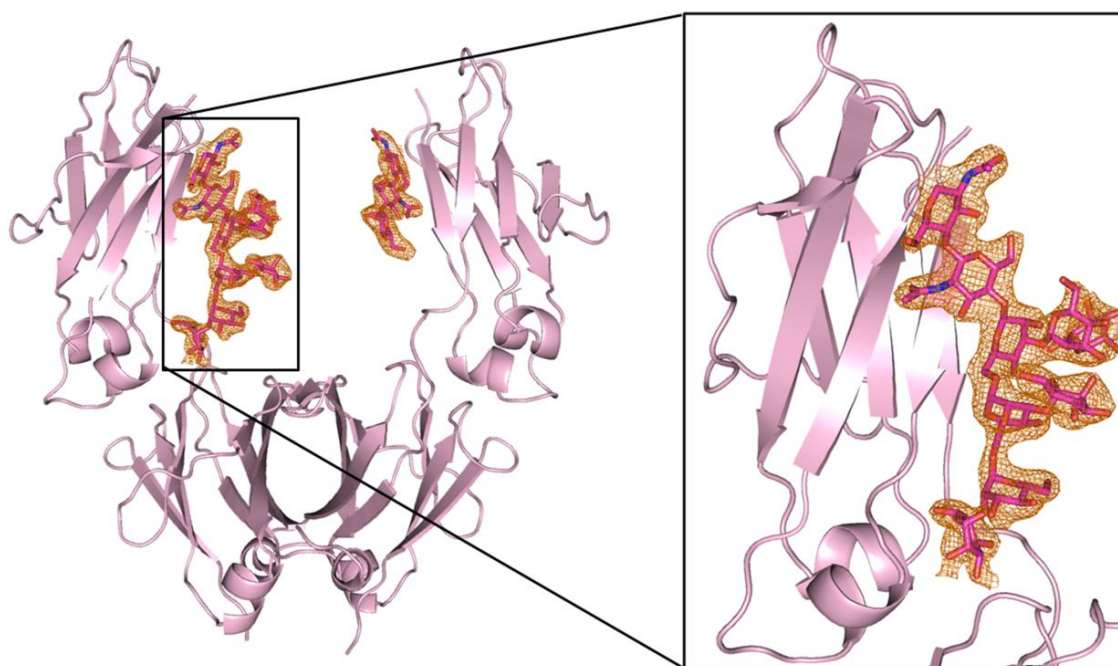


Figure 5.5.1. Structure of Fc Man₉GlcNAc₂ at 2.24 Å shown in cartoon representation. (A) A maximum likelihood $2F_o-F_c$ map is displayed around the N-linked glycans, contoured to 1σ . (B) Magnified view of the Man₉GlcNAc₂ glycans on chain A.

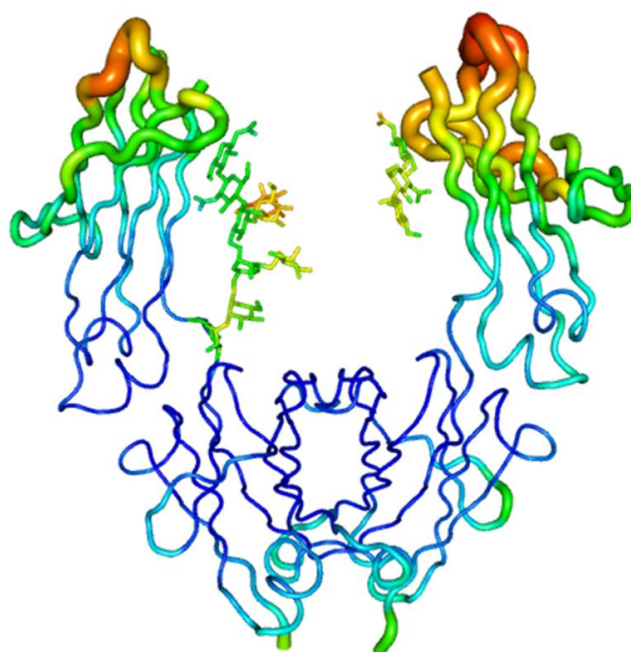


Figure 5.5.2. Conformational mobility of C γ 2 domains of Fc Man₉GlcNAc₂. Protein backbone and attached glycans of Fc Man₉GlcNAc₂ are coloured according to their *B* factor values. Colours range from dark blue (lowest *B* factors) through to bright red (highest *B* factors).

Fc Man₉GlcNAc₂ structure was similar to the FcWT structure (r.m.s.d. of 0.4211 Å over equivalent C α atoms) in its ‘open’ conformation and also had similar interdomain distances (Figure 5.5.3, Table 7.1).

X-ray crystallographic structure of Fc bearing oligomannose type glycans has been previously reported (PDB ID 2WAH) (33). This structure was lacking a full length hinge sequence. Comparison of the Fc Man₉GlcNAc₂ structure with 2WAH revealed no significant differences between the two structures (r.m.s. deviation of 0.25 Å over equivalent C α atoms) thus excluding a role of the hinge in determining interdomain spacing. A more detailed analysis of the orientation and packing of oligomannose glycans revealed that a “closed” conformation was not feasible owing to numerous steric clashes between the oligomannose glycans of both chains of the Fc dimer.

Here, both FcWT and Fc Man₉GlcNAc₂ structures adopt very similar “open” conformations (r.m.s.d. of 0.42 Å over equivalents C α atoms). This is further evidence of the conformational plasticity of the C γ 2 domains and indicates that “open” conformations are not necessarily restricted to oligomannose type Fc glycans.

Fc Man₉GlcNAc₂ was highly similar to the Fc domains of the recently published high affinity defucosylated Fc in complex with the activatory FcγRIIIa receptor (PDB ID 3SGK, r.m.s.d. of 0.65 Å over equivalent Cα atoms). In the fucosylated Fc-FcγRIIIa complex, steric inhibition caused by the α1→6 linked core fucose of the Fc significantly weakens the binding of FcγRIIIa receptor (Figure 1.3.4) (59). In the absence of this core fucose, several hydrogen bonds are formed between the two core GlcNAc residues of the reducing terminal of the glycan at Asn162 of the FcγRIIIa and the GlcNAc1 of the Fc contributing to the higher affinity between the two molecules. This also explains the high affinity of Fc Man₉GlcNAc₂ for FcγRIIIa.

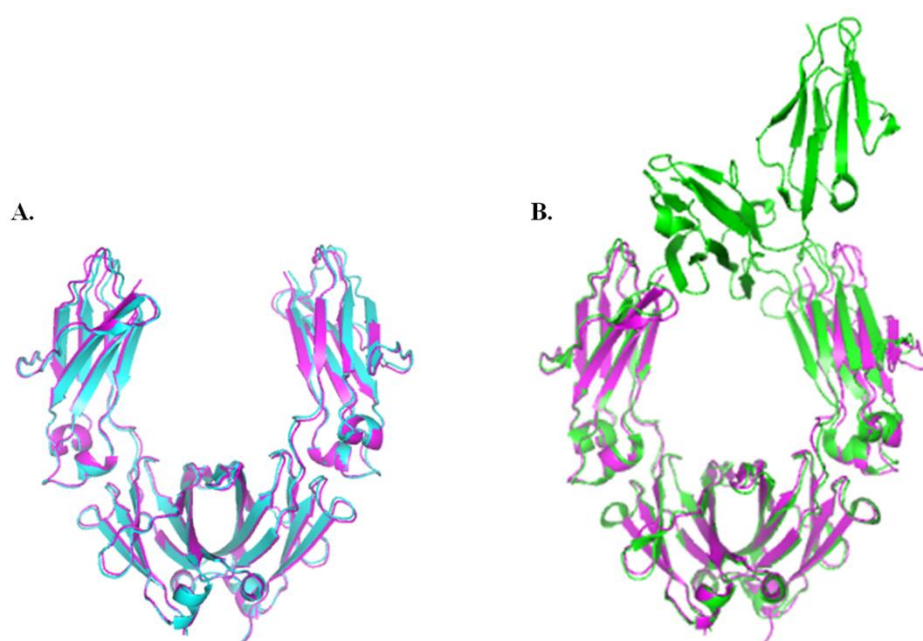


Figure 5.5.3. Structural analysis of Fc Man₉GlcNAc₂. (A) Fc Man₉GlcNAc₂ (pink ribbon) overlayed with FcWT structure (cyan ribbon) (B) Overlay of Fc Man₉GlcNAc₂ (pink ribbon) with Fc-FcγRIIIa complex (PDB ID. 3SGK, green ribbon).

5.6. Protein-glycan interactions of Fc Man₉GlcNAc₂

The glycan chains attached to Asn297 of Fc Man₉GlcNAc₂ were largely ordered on chain A in contrast to those in chain B, where only the core GlcNAc 1, 2 and β 1 $\rightarrow$ 4 linked Man3 residues were visible. Electron density on chain A for 9 of the carbohydrate moieties of the total 11 that comprise the Man₉GlcNAc₂ glycan was traced.

Hydrophobic interactions and hydrogen bonds between Asp265, Val264, Arg301 and Phe241 and GlcNAc1 and 2 of the reducing terminal of the glycan chain were maintained as previously observed in the FcWT structure (Figure 5.6.1). Similarly, trajectory of the 3 arm glycans was similar to that observed in the FcWT structure as they extend towards the interdomain space away from the hydrophobic protein backbone.

The 6 arm glycans of Fc Man₉GlcNAc₂ however, adopt a significantly altered path in comparison to FcWT (Figure 5.6.2). The terminal GlcNAc 5' residue on FcWT forms conserved hydrophobic interactions with Phe243 and truncation of this GlcNAc residue has been shown to lead to destabilization of the Fc domain(150). However, in Fc Man₉GlcNAc₂, the equivalent ManA residue is projected away from the protein backbone. ManB forms hydrophobic interactions with Pro244 and ManD3 is immobilised at the C γ 2–C γ 3 interface by hydrogen bonds formed by the C3 and C4 hydroxyls with Asp376. The loss of stabilising interactions with Phe243 may indicate lowered stability for Fc Man₉GlcNAc₂ protein.

5.7. Fc γ RIIIa binding and thermal stability of Fc Man₉GlcNAc₂

Fc Man₉GlcNAc₂ was assessed for activity by measuring the binding to the activatory Fc receptor, Fc γ RIIIa. Recombinant Fc γ RIIIa (R&D systems, Minneapolis, U.S.A.) was immobilised on carboxymethyl CM5 sensor chips to 1000 RU using standard amine coupling protocols as described in Section 2.9. Fc Man₉GlcNAc₂ and FcWT at 34.3 μ M, 17.15 μ M and 8.57 μ M were used as analytes and binding was measured as relative response units (RU). The Fc Man₉GlcNAc₂ glycoform binds Fc γ RIIIa with higher affinity (K_D of 0.884 μ M) in comparison to FcWT (K_D of 2.8 μ M) (Appendix, Figure A1).

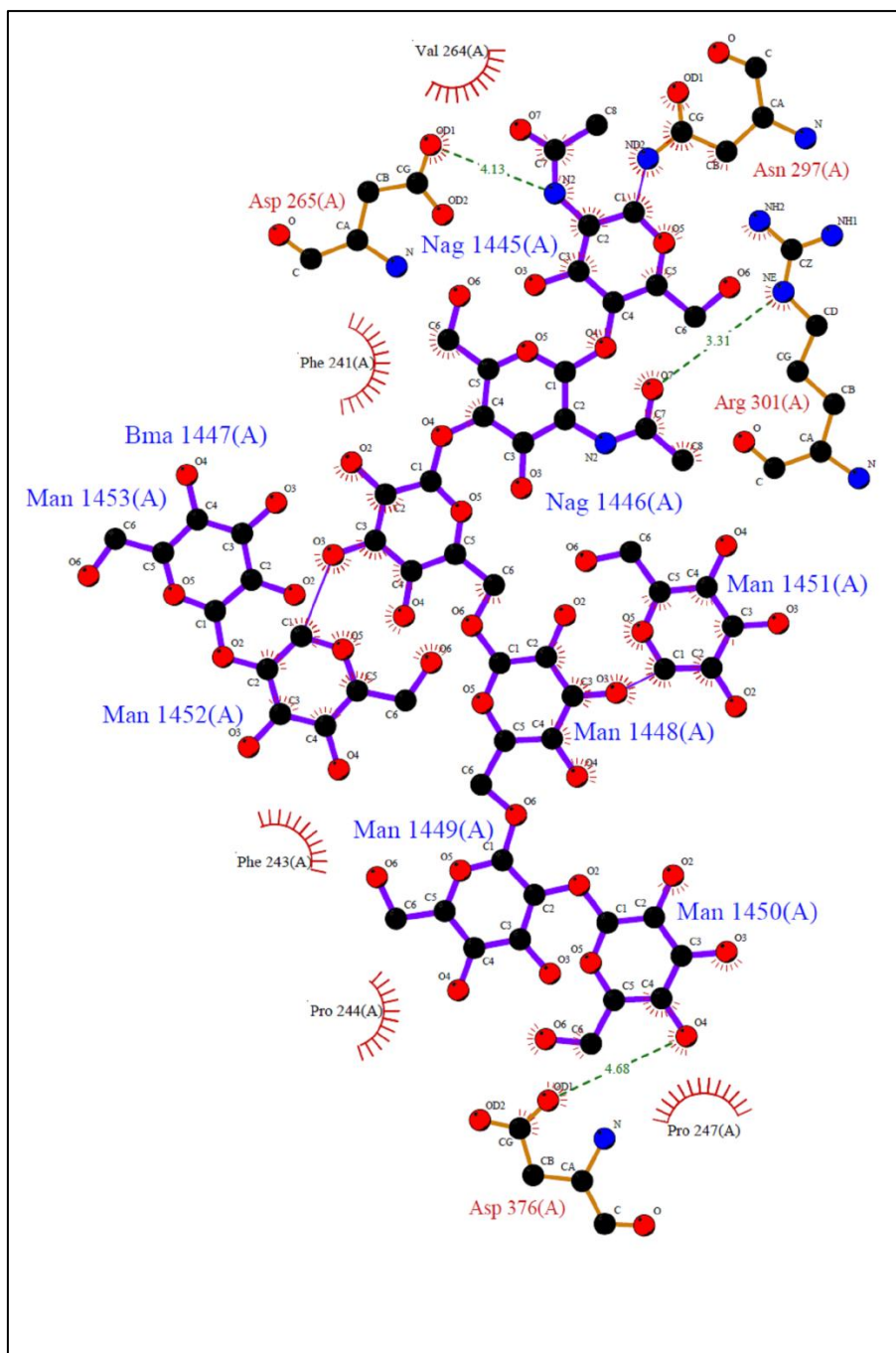


Figure 5.6.1. Protein-glycan interactions of the oligomannose glycans of Fc Man₉GlcNAc₂. LIGPLOT diagram showing hydrophobic interactions (red spokes around residues) and hydrogen bonds (green dotted lines) formed by each carbohydrate residue of Fc Man₉GlcNAc₂ with amino residues on the C γ 2 protein backbone.

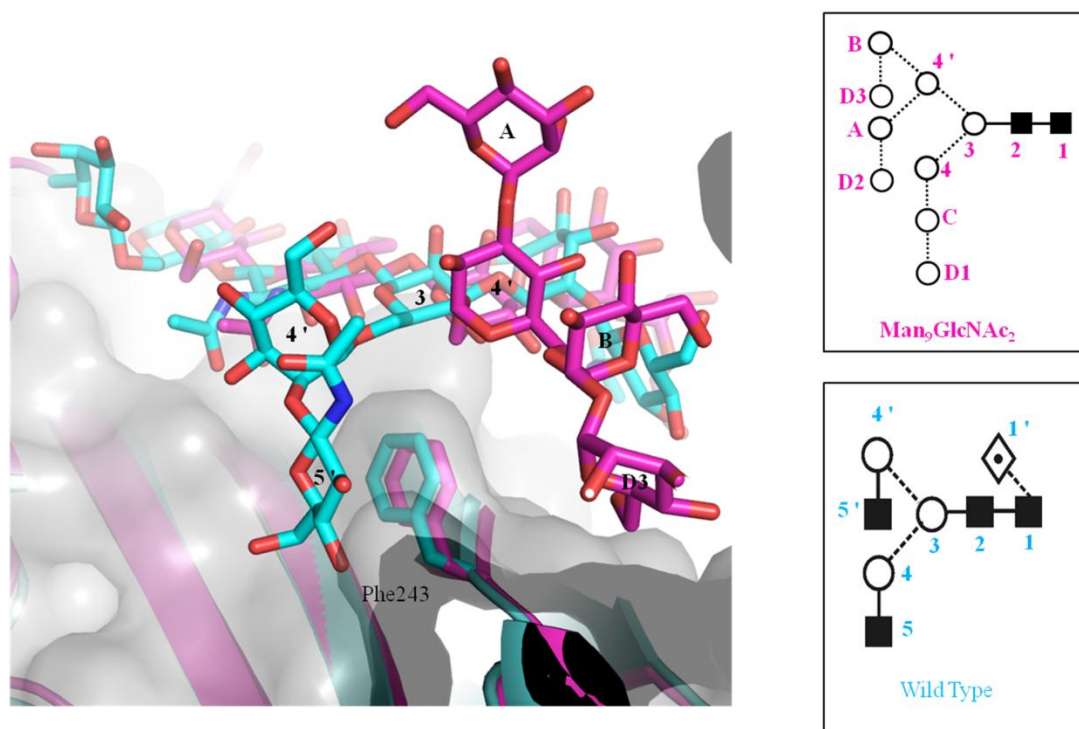


Figure 5.6.2. FcWT and Fc Man₉GlcNAc₂ glycan-protein interactions. Wild type (cyan) and oligomannose (pink) glycans interaction with Phe243 of the Fc Cy2 domain is shown with interacting carbohydrate residues labelled. The schematic on the right shows the glycan composition of each glycoform.

These SPR results taken together with the observed “open” conformation of Fc Man₉GlcNAc₂ in X-ray crystallographic studies would initially appear to suggest that this quaternary conformation was contributory to the high affinity binding to FcγRIIIa. However, the FcWT structure also crystallised in an “open” conformation whilst maintaining low affinity binding to the receptor. This is consistent with previous observations of Fc bearing oligomannose glycans and defucosylated antibodies binding to FcγRIIIa with similar affinities (65). These SPR data also confirm that the crystallographic structure of recombinant expressed Fc Man₉GlcNAc₂ was a functionally active conformation.

Thermal unfolding assays were performed to study the effects of Fc glycosylation on the stability of Cγ2 domains. Thermal stability assays were performed using differential scanning fluorimetry (DSF) as described in Section 2.10. Unfolding of Cγ2 domains of Fc Man₉GlcNAc₂ was measured in the presence of fluorescent Sypro orange dye which exhibits increased fluorescence with progressive exposure of hydrophobic residues as a protein progressively unfolds with increasing temperature (122).

Fc Man₉GlcNAc₂ followed the same pattern of thermal unfolding as that of FcWT and resulted in a single thermal transition (Appendix, Figure A1). The melting temperature of Fc Man₉GlcNAc₂ was lower ($66.74 \pm 0.54^{\circ}\text{C}$) than that of FcWT ($69.87 \pm 0.48^{\circ}\text{C}$). Structural analysis of Fc Man₉GlcNAc₂ indicated that the significantly altered trajectory of the 6 arm of the oligomannose glycans leads to loss of the conserved interaction of GlcNAc5' with Phe243 whilst the terminal ManD3 residue was anchored by hydrogen bonds with Asp376. This decrease in melting temperature of the Fc Man₉GlcNAc₂ is consistent with the destabilization due to the absence of GlcNAc5'.

5.8. Crystallisation and Structure Determination of Fc Hybrid

SEC purified Fc Hybrid was crystallised by the sitting drop vapour diffusion method as described in section 2.4. Crystals of Fc Hybrid grew after 5-10 days in 30% w/v PEG Monomethyl Ether 2000, 0.100 M Sodium Acetate buffer at pH 4.6 and 0.200 M Ammonium Sulphate (Figure 5.8.1A). Crystals were immersed in cryoprotectant containing mother liquor in 25% ethylene glycol and transferred rapidly to gaseous nitrogen stream at 100 K. Data for Fc Man₅GlcNAc₃Fuc crystals were collected at the ESRF BM14 beamline.

Fc Hybrid structure was solved by molecular replacement with the software Phaser using native Fc structures (PDB 2DTQ) as search models. The final structure was refined to a resolution of 2.25 Å. One Fc homodimer per asymmetric unit was identified with a solvent content of 45.95% (V_M of 2.27 Å³ Da⁻¹). Model building with COOT was accompanied by iterative round of refinements with the program REFMAC5. Molprobit was used to validate the final model. Ramachandran analysis showed that 99.5% of residues were in the most favoured region with no residues in the disallowed regions (Figure 5.8.1B).

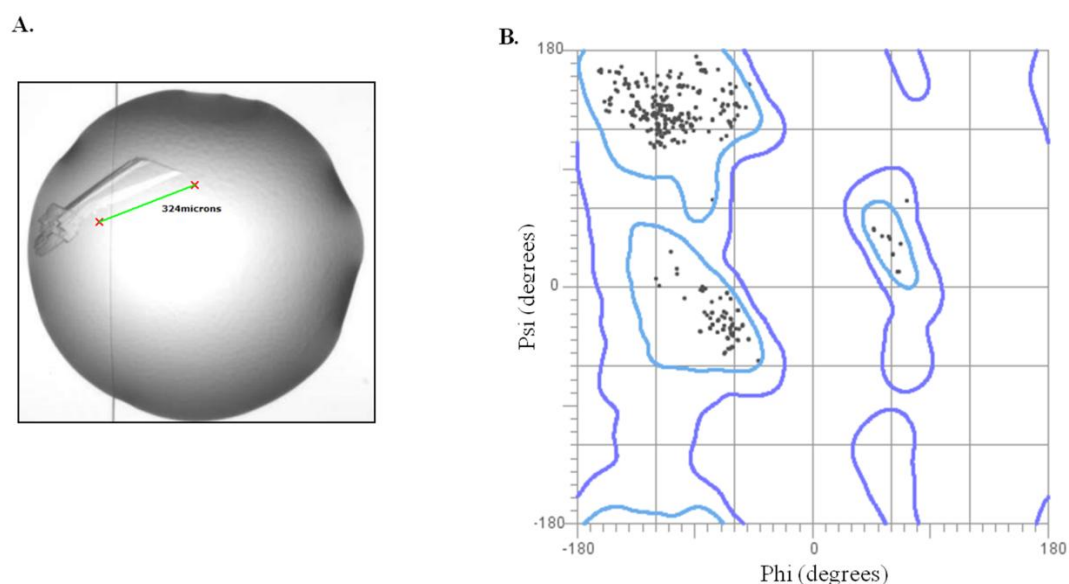


Figure 5.8.1. Crystallisation and structural analysis of Fc Hybrid. (A) Fc Hybrid crystals grew to a length of 324 µm. (B) Ramachandran plot generated with Molprobit(110) for the final refined Fc Hybrid structure.

Table 5.8.1. Data collection and refinement statistics for Fc Hybrid

Data collection	Hybrid
Beamline	ESRF BM14
Resolution (Å)	50-2.25 (2.30-2.25) ^a
Spacegroup	<i>P2₁2₁2₁</i>
Cell dimensions (Å) and angles (°)	<i>a</i> = 48.7, <i>b</i> = 72.8, <i>c</i> = 137.8, <i>a</i> = <i>b</i> = <i>γ</i> = 90
Wavelength (Å)	0.977
Unique reflections	23410 (1501)
Completeness (%)	99.29 (95.7)
<i>R</i> _{merge} (%) ^b	0.094 (0.649)
<i>I</i> / <i>σI</i>	10.9 (2.1)
Average redundancy	4.9 (4.9)
Refinement	
Resolution range (Å)	29.28-2.25(2.35-2.25)
Number of reflections	24634 (2778)
<i>R</i> _{work} (%) ^c	19.19
<i>R</i> _{free} (%) ^d	24.9
r.m.s.d. bonds (Å)	0.011
angles (°)	1.16
Atoms per asymmetric unit (protein/water/glycan)	3495/316/80
Average <i>B</i> factors (protein/water/glycan) (Å ²)	33.20/35.25/78.08
Ramachandran Plot (Favoured/allowed; %)	99.5/0.5

^aNumbers in parentheses refer to the relevant outer resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I(\text{hkl}; i) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I(\text{hkl}; i)}$, where $I(\text{hkl}; i)$ is the intensity of an individual measurement and $\langle I(\text{hkl}) \rangle$ is the average intensity from multiple observations.(106)

^c $R_{\text{work}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$

^d *R*_{free} was calculated as *R*_{work} but after removal of 5% of data prior to refinement(Section 2.6).

5.9. Mass spectrometric analysis of Fc Hybrid glycans

Glycan composition of Fc Hybrid was analysed by PNGase F mediated in-gel release of N-linked glycans as described before (Section 2.8). The glycan profile of this Fc glycoform comprised of hybrid type glycans, all with a core Man₅GlcNAc₂Fuc and varying levels of processing in the 3 arm (Figure 5.9.1A). The principal glycoform observed at *m/z* 1606.5 corresponded to Man₅GlcNAc₂Fuc. The second predominant peak with *m/z* of 1768.5 corresponds to the addition of a single β 1 $\rightarrow$ 4 linked Gal6 residue on the 3 arm of the core Man₅GlcNAc₂Fuc. Other glycoforms observed were the defucosylated Man₅GlcNAc₂ structure with *m/z* of 1257.4 representing the immature oligomannose structure. Some residual activity of Golgi α -mannosidase II was observed as evident from the glycoform observed at *m/z* of 1444.4 which corresponds to a core Man₃GlcNAc₂Fuc structure with additional GlcNAc and Gal on the 3 arm. Further, peaks with *m/z* at 1809.6 and 1971.6 were observed and these contain a bisecting β 1 $\rightarrow$ 4 linked GlcNAc in addition to the previously observed Man₅GlcNAc₃Fuc core structure.

Peak with *m/z* of 2035.6 was unique in that it had not been observed previously in the mass spectrometric profiles of native or oligomannose bearing Fc glycans. An *m/z* of 2035.6 corresponds to a sialylated glycoform containing a sialic acid residue in the 3 arm of the Fc Man₅GlcNAc₃Fuc glycan chain.

To further confirm the presence of sialylated glycans in Fc Hybrid we repeated MALDI-TOF analysis of the PNGase F released glycans after desialylation. Desialylation was performed by addition of 1% Acetic acid followed by incubation at 90°C for 1 hour. MALDI-TOF spectra of desialylated glycans from Fc Man₅GlcNAc₃Fuc, resembled that of the original spectra, however no peak with *m/z* 2035.6 was observed (Figure 5.9.1B). The fine structure and connectivity of various glycan residues of the sialylated peak with an *m/z* of 2035.6 was finally confirmed using negative ion ESI mass spectrometry (as described in section 2.8, Figure 5.9.2). The CID spectra showed the presence of terminal sialic acid residue indicated by the B₁ ion at *m/z* 290. Absence of an ion at *m/z* 306 indicated that this ion is α 2 $\rightarrow$ 3 linked (151).

Mass spectrometric analysis of Fc Hybrid confirmed the inhibition of Golgi α mannosidase II by Swainsonine (Figure 1.4.1). The glycan profile of IgG Fc glycans after swainsonine treatment has been previously reported (65), however the presence of sialylated glycans was not noted. Other proteins expressed in the same HEK293T

expression system in the presence of swainsonine shows similar increase in $\alpha 2 \rightarrow 3$ linked sialic acid bearing glycans (152). This suggests that elevated sialylation levels observed here could be protein independent and more likely determined by the preferential activity of $\alpha 2 \rightarrow 3$ sialyltransferase enzyme (ST3Gal) towards hybrid type glycans. Terminal sialic acid residues with $\alpha 2 \rightarrow 3$ linkages in IgGFc are uncommon in human serum, as $\alpha 2 \rightarrow 6$ linkages predominate (39).

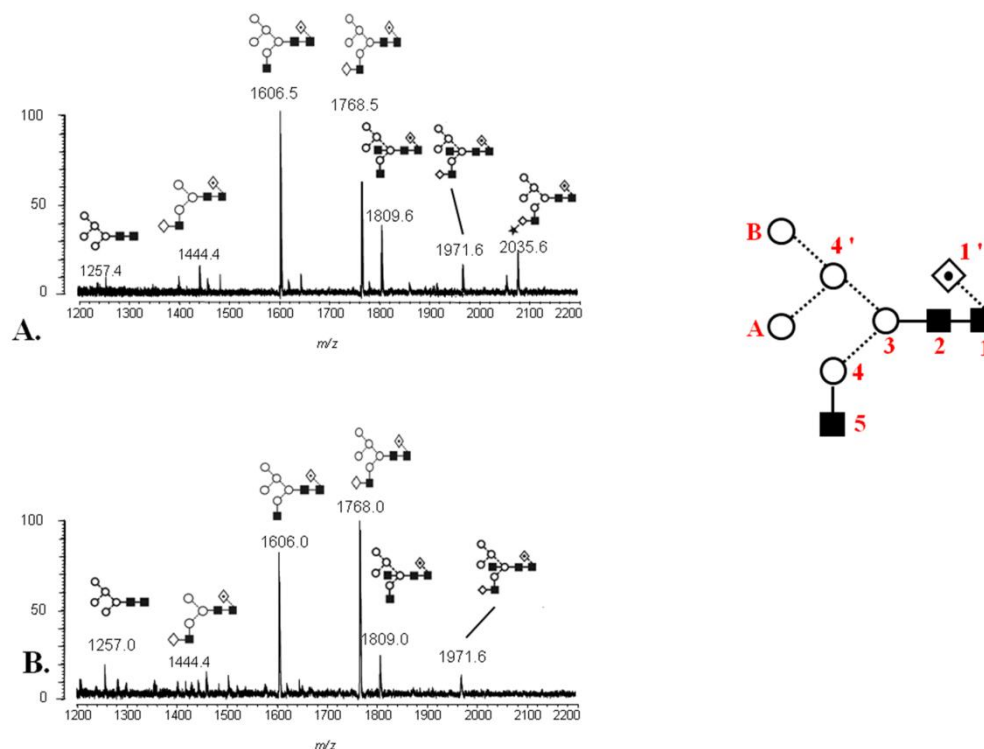


Figure 5.9.1 MALDI TOF analysis of Fc Hybrid glycans. (A) MALDI TOF spectra of PNGase F release glycans from Fc Hybrid. (B) MALDI TOF spectra of Fc Hybrid glycans after desialylation. Y axis of both spectra represents the percentage abundance (%). The glycan composition of each peak is shown. The schematic on the right shows the principal Fc Hybrid glycoform with individual glycan units numbered according to convention.

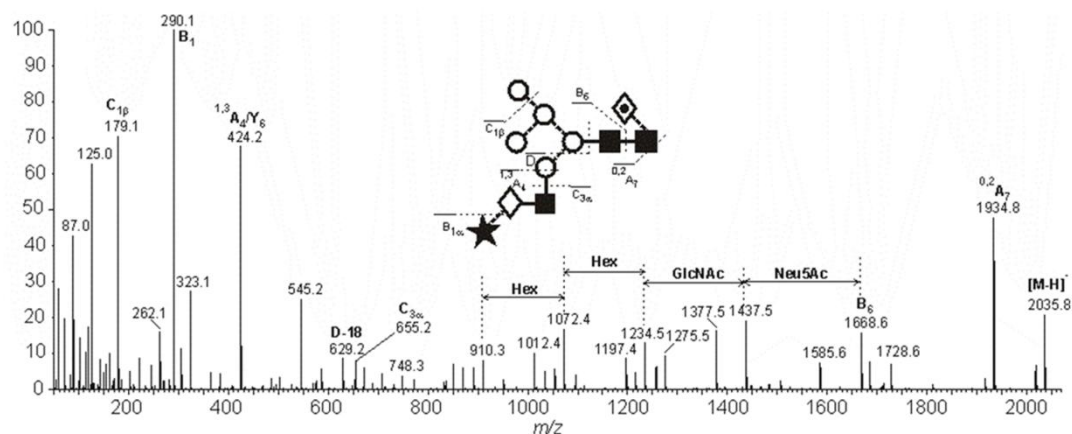


Figure 5.9.2. Negative ion fragmentation of sialylated Fc Hybrid glycan. Fragmented ions are labelled according to scheme as devised by Domon and Costello(149). Percentage abundance of glycan species is shown on the Y axis and corresponding m/z is shown on the X axis.

5.10. Structural analysis of Fc Hybrid

X-ray crystallographic structure of Fc Hybrid at 2.25 Å was characterised by a horseshoe shaped dimer and was most similar to the FcWT structure (0.45 Å r.m.s.d. in Cas over equivalent residues, Figure 5.10.1A). This glycoform adopted an “open” conformation similar to Fc Man₉GlcNAc₂ (r.m.s.d. of 1.02 Å over equivalent Cas). Asymmetry of Cγ2 domains was most pronounced with chain B significantly “tilted” out and away from the central two fold axis of symmetry. The interdomain distance was 56.5 Å (Table 7.1), higher than that observed for FcWT (51.9Å) and Fc Man₉GlcNAc₂ (50.9Å). Comparison with FcγRIIIa bound fucosylated Fc domain (PDB ID 3SGJ, r.m.s. deviation of 0.923 over equivalent Cα residues) showed that the receptor bound Fc was more “closed” (interdomain distance of 46.0 Å) than Fc Hybrid (figure 5.10.1B).

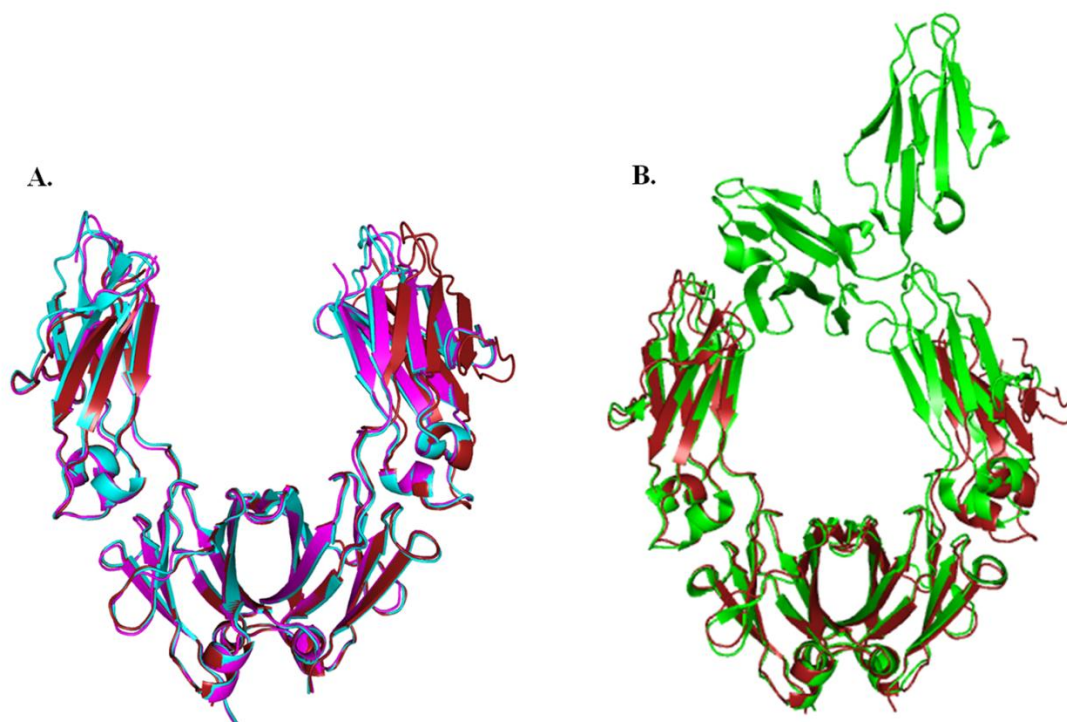


Figure 5.10.1. Structural analysis of Fc Hybrid. (A) Comparison of the quaternary structures of Fc Hybrid (Red) with FcWT (cyan) and Fc Man9GlcNAc2 (pink). (B) Overlay of Fc Hybrid (red) structure of Fc-FcγRIIIa complex (Green, PDB ID 3SGK).

Electron density for 8 of the 9 carbohydrate moieties of the principal $\text{Man}_5\text{GlcNAc}_3\text{Fuc}$ glycan was visible on chain A (Figure 5.10.2). There was no visible electron density for the glycans on chain B. This was accompanied by absence of electron density at Asn297 and residues 294 to 296 of the D/E loop on chain B. This increased disorder was evident in the average B factor values for Cγ2 domains of chain A and B. Chain A showed lower B factor values in comparison to chain B (B_{average} chain A of $27.02 \text{ \AA}^2$ and B_{average} chain B $45.82 \text{ \AA}^2$, Figure 5.10.3). Residues spanning from Val266 to Asp270 of the B/C loop and Val323 to Leu328 of the F/G loop of the Cγ2 domains of chain B were omitted from the final model due to absence of detectable electron density. These disordered upper loops of Fc Cγ2 domain constitute the binding interface with FcγRIIIa (Figure 1.3.3) and have been a common feature of all the Fc glycoforms studied here (153).

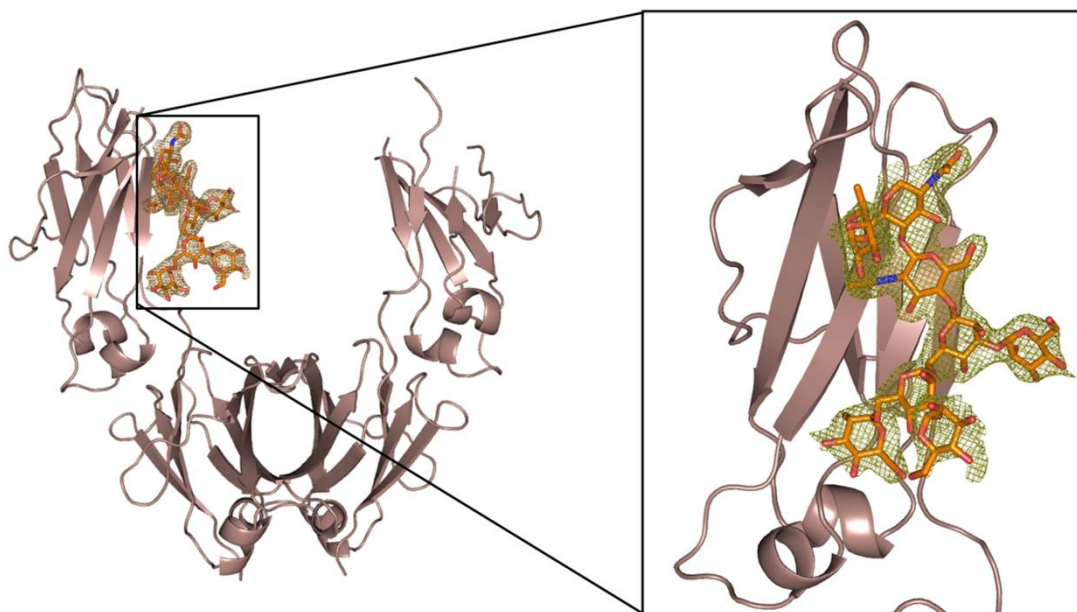


Figure 5.10.2. Structure of Fc Hybrid at 2.25 Å shown in cartoon representation. (A) A maximum likelihood $2F_o - F_c$ map is displayed around the N-linked glycans, contoured to 1σ . (B) Magnified view of the $\text{Man}_5\text{GlcNAc}_2\text{Fuc}$ glycans on chain A.

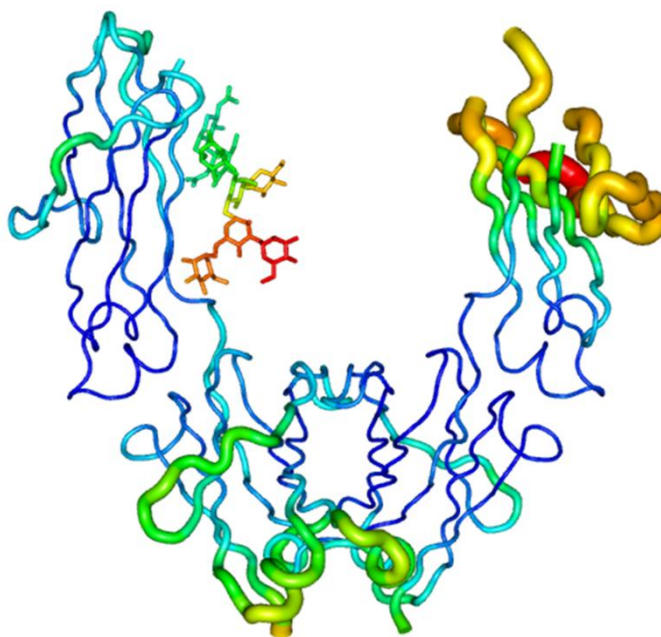


Figure 5.10.3. Conformational mobility of $\text{C}\gamma_2$ domains of Fc glycoforms. The protein backbone and attached glycans of Fc Hybrid are coloured according to their B factor values. Colours range from dark blue (lowest B factors) through to bright red (highest B factors).

5.11. Protein-glycan interactions of Fc Hybrid

The Man₅GlcNAc₂Fuc glycan traced on chain A of Fc Hybrid structure begins with the close apposition of the reducing terminal GlcNAc residues to the C γ 2 protein backbone. GlcNAc1 and the α 1 $\rightarrow$ 6 linked core fucose residue form several hydrophobic interactions with Val264, Gln295 and Thr299 (Figure 5.11.1). Hydrogen bonds between GlcNAc1 and Asp265 and GlcNAc2 and Arg301 also further stabilise this region of the glycan chain. These interactions are similar to those observed in FcWT and Fc Man₉GlcNAc₂ glycan chains. The central β mannose (Man3) lies near the protein surface forming hydrophobic interactions with Phe241. On the 3 arm of the glycan chain, the α 1 $\rightarrow$ 3 linked Man 4 residue projects towards the interdomain space, tracing a path similar to that adopted by the 3 arm glycans of FcWT and forming hydrophobic interactions with Lys334.

Elevated sialylation on the 3 arm of the glycan chain was evident from mass spectrometric analyses. However, no electron density for the terminal GlcNAc, galactose and sialic acid residues on the 3 arm was observed, indicating higher conformational mobility for these residues. Glycans of the 6 arm comprising of Man4', Man A and Man B adopted a significantly different conformation than equivalent residues in FcWT and Fc Man₉GlcNAc₂ (Figure 5.11.2). In the Fc Hybrid structure, Man 4' is oriented away from the underlying protein backbone, perpendicular to the equivalent Man 4' residue of FcWT free of hydrophobic interactions with Phe243 and Thr260. While the terminal Man A residue is oriented towards the interdomain space, similar in orientation to the equivalent residue in Fc Man₉GlcNAc₂, Man B is oriented along the protein surface and forms hydrophobic interactions with Lys246. This pronounced “flip” in the conformation of Man 4' residue of Fc Hybrid leads to loss of stabilizing protein-glycan interactions and orients the glycan chain away from the hydrophobic C γ 2 protein backbone.

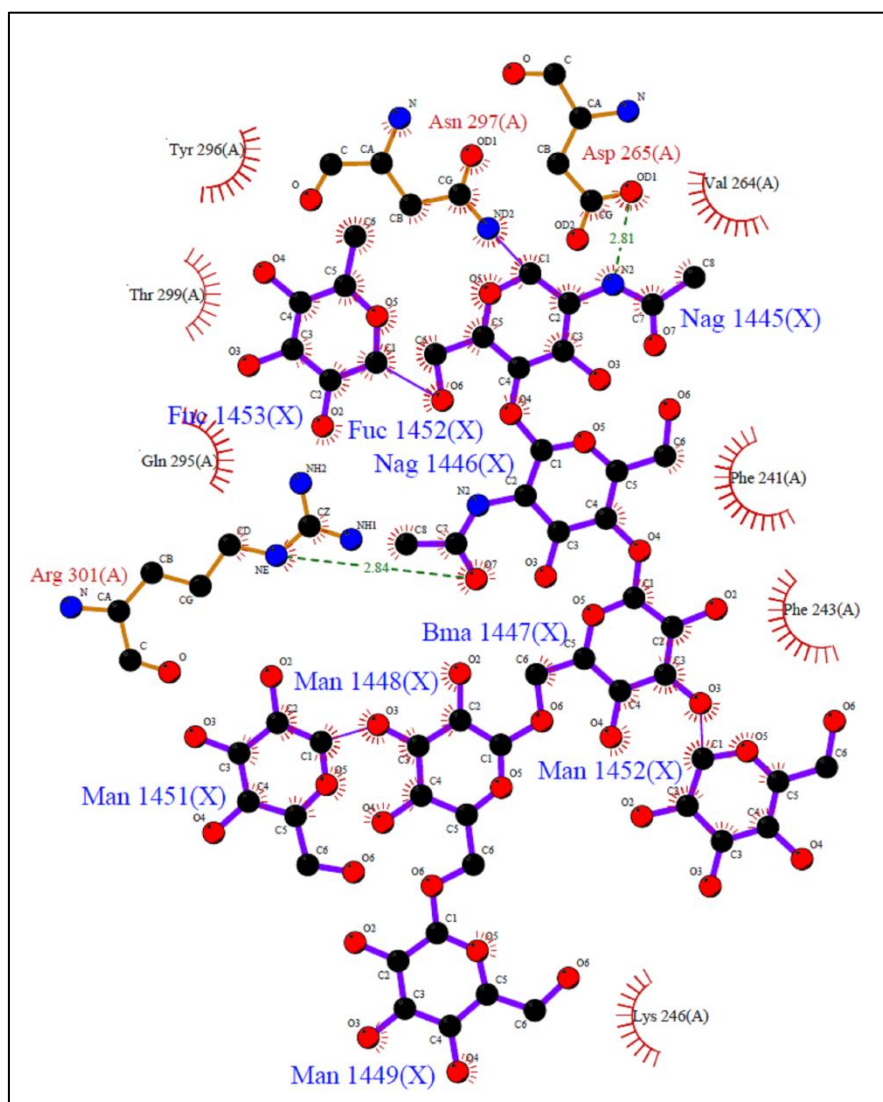


Figure 5.11.1. Protein-glycan interactions of the oligomannose glycans of Fc Hybrid. LIGPLOT diagram showing hydrophobic interactions (red spokes around residues) and hydrogen bonds (green dotted lines) formed by each carbohydrate residue of Fc Man₉GlcNAc₂ with amino residues on the Cγ2 protein backbone

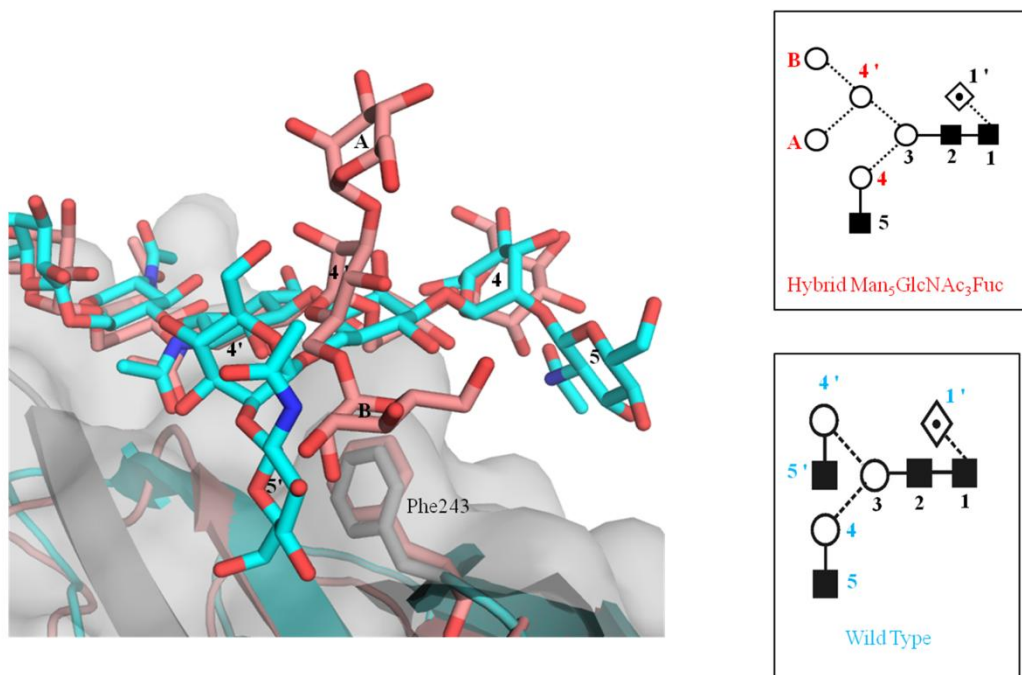


Figure 5.11.2. FcWT and Fc Hybrid glycan-protein interactions. Wild type (cyan) and hybrid (red) glycans interact differently with Phe243 of the Fc C γ 2 domain. Interacting carbohydrate residues are labelled and a schematic on the right shows the glycan composition of each glycoform.

5.12. Thermal stability and FcγRIIIa binding of Fc Hybrid

SPR experiments to measure the activity of Fc Hybrid were performed with immobilised FcγRIIIa as described in section 2.9. Fc Hybrid at 34.3μM, 17.15μM and 8.575 μM was used as the analyte. Representative SPR sensograms for Fc Hybrid, FcWT and Fc Man₉GlcNAc₂ are shown in Appendix, Figure A1. Fc Hybrid showed diminished binding to FcγRIIIa (K_D of 3.6 μM) in comparison to FcWT (K_D of 2.8 μM) and Fc Man₉GlcNAc₂ (K_D of 0.88 μM). This has been noted in previous studies where modified glycoforms of anti-CD20 therapeutic antibodies with hybrid type N-linked glycans showed lower affinities for FcγRIIIa compared to antibodies with complex type and oligomannose glycans (65). The sialylation status of hybrid type IgG1 antibodies used in these binding studies was not known. The presence of α2→3 linked terminal sialic acid residues on Fc Hybrid could be contributory to the low affinity for FcγRIIIa (54).

Thermal stability of Fc Hybrid was measured using DSF as described before (Section 2.10). With a T_m of 65.57 ± 0.2 °C, Fc hybrid was less thermostable than FcWT (4.3 °C lower than T_m of FcWT, Appendix Figure A1). It was however not significantly different from Fc Man₉GlcNAc₂, with the T_m of both glycoforms differing by only 1.2°C. The limit of sensitivity of DSF restricts thermal stability assays to 75°C, thus no measurements of the unfolding of Cγ3 domains could be made. Decreased thermal stability of IgG1Fc domain with hybrid type glycans correlates with the altered conformation and loss of glycan-protein interactions for the Man₅GlcNAc₂Fuc glycan observed in the crystallographic structure.

5.13. Conclusions

Folding of IgG Fc domains in the mammalian endoplasmic reticulum proceeds co-translationally and at a rapid pace (154). N-linked glycosylation occurs on nascent polypeptide chains and the immature glucosylated high mannose bearing Fc Cγ2 domains undergo processing by ER glucosidases (Figure 1.4.1). Activity of ER glucosidases and the folding of Cγ1 domain are crucial events in the quality control of immunoglobulin folding (155). Dimerization of IgG Fc begins with the formation of Cγ3 dimers and is further stabilised by disulphide bonds in the hinge region (155). Cγ2 domains interact with each other through their N-linked glycan chains. Further processing of the N-linked glycan chain by ER and Golgi enzymes results in the biantennary complex type glycosylation that is characteristic of wild type serum IgG Fc.

While crystallographic structures of native Fc with fully processed glycans have been reported before, a systematic study of unnatural and incompletely processed glycoforms of Fc has not been reported till date. Crystallographic structures of Fc Man₉GlcNAc₂ and Fc Hybrid represent such glycoforms trapped at two key biosynthetic steps of the mammalian N-linked glycan processing pathway. Both glycoforms were secreted as full-length dimers with patent hinge disulphide bonds indicating that the inhibition of glycan processing by ER α -mannosidase I and Golgi α -mannosidase II did not disrupt the stable secretion and folding of full length IgG Fc.

Two immediate observations from the crystallographic structures of Fc Man₉GlcNAc₂ and Fc Hybrid were the “open” conformation and pronounced asymmetry of the Fc dimer. Productive binding of Fc to Fc γ receptors has been attributed to “open” and asymmetric Fc conformations. This was evident in the crystallographic structures of Fc-Fc γ RIIIa complex. Previously, IgG Fc structures with sequentially truncated complex type glycans caused progressive “closing” of the Fc dimer and diminished Fc γ RI and complement binding (32). This led to the conclusion that N-linked glycans of C γ 2 domains determine the degree of opening of the Fc dimer (32). Comparison of previously reported structures of FcWT in the previous chapter showed that C γ 2 domains bearing complex type glycans could adopt different “open” conformations with varying interdomain distances. The quaternary structures of both Fc glycoforms described here were characterised by “open” conformations further confirming that the composition of the glycan chain does not always predict the conformational movement of C γ 2 domains (Table 7.1).

Structure and orientation of biantennary complex type glycans in wild type IgG Fc is well studied and high resolution crystallographic structures have been reported for these. Protein-glycan interactions in Fc contribute to the stability of C γ 2 domains. While the composition of N-linked glycans in Fc does not affect the relative opening of the Fc dimer, loss of protein-glycan interactions by truncation of glycan residues, does lead to closing of the Fc dimer and lowered thermal stability (150,156). Fully mature complex type glycans of FcWT interact closely with hydrophobic residues in the protein backbone. Comparison of the structure and interactions of the distinct glycan chains of Fc Man₉GlcNAc₂, Fc Hybrid and FcWT (Figure 5.13.1) show that stabilising hydrophobic protein-glycan interactions are formed only after processing by Golgi α -mannosidase II. Lack of protein-glycan contacts in the 6 arm residues of Fc

Man₉GlcNAc₂ and Fc Hybrid correlate with lower protein thermal stability. These observations are also consistent with NMR studies on Fc glycoforms which showed conformational changes in the lower hinge, F/G and D/E loops due to loss of GlcNAc5' and GlcNAc5 residues (156).

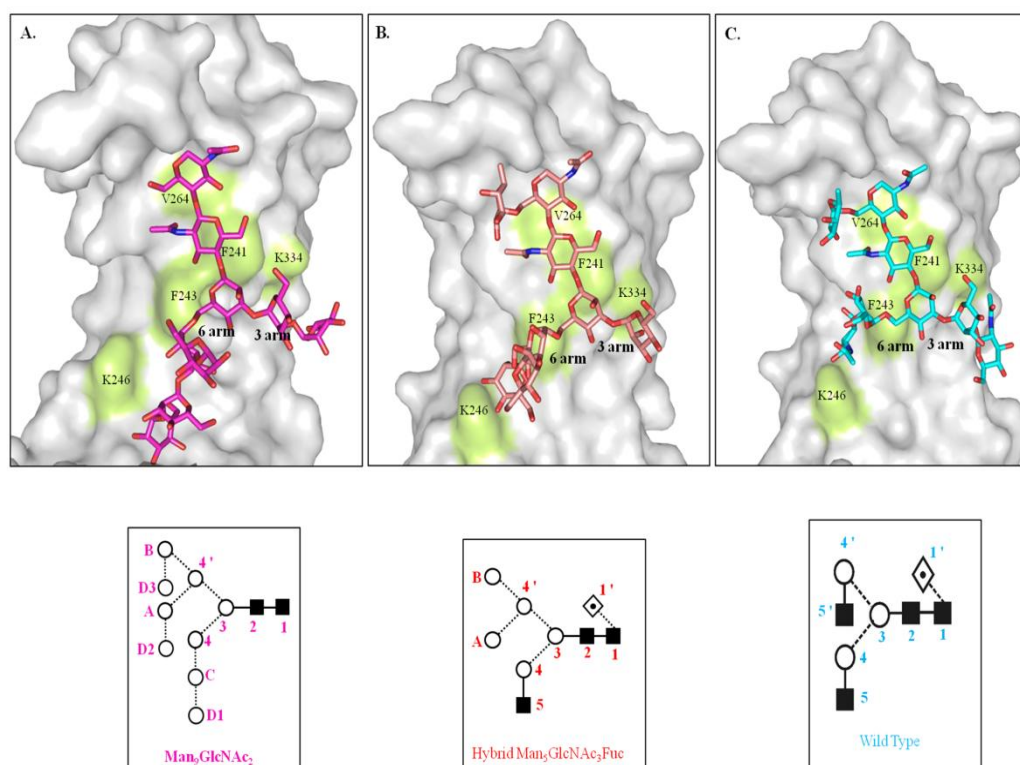


Figure 5.13.1. Stabilisation of protein-glycan interactions on Fc after Golgi α -mannosidase processing. Orientation of glycans attached to Asn297 on IgG Fc protein surface before processing by ER α -mannosidase I (A) and Golgi α -mannosidase II (B). The final processed complex type biantennary glycan of FcWT (C) is shown for comparison. Composition and structure of each glycoform is shown in box below. The N-glycan processing pathway indicating these enzymatic steps is shown in Figure 1.4.1.

While the quaternary structures of Fc Man₉GlcNAc₂ and Fc Hybrid were similar to Fc domains in complex with FcγRIIIa, both glycoforms showed significantly different affinities for binding to FcγRIIIa. The core α1→6 linked fucose residue of Fc glycans significantly attenuates Fc-FcγRIIIa binding. In the case of Fc Man₉GlcNAc₂, the absence of this core fucose residue may contribute to its higher affinity for FcγRIIIa (65). Similarly the low affinity of Fc Hybrid for FcγRIIIa is consistent with previous reports and could be a consequence of increased terminal sialylation. The importance of glycans in Fc structure is further highlighted by the structural consequences of their removal by endoglycosidases- as described in chapter 6.

CHAPTER 6

Deglycosylated IgG:

Structure and Therapeutic Applications

6. Deglycosylated IgG: Structure and therapeutic applications

6.1. Summary

The importance of glycosylation for antibody structure is well established through *in vitro* experiments (55,96). However, the critical role played by Fc glycans is perhaps most clearly evidenced by the evolution of bacterial enzymes which cleave the Fc glycan as an immune evasion strategy. Endoglycosidase S (Endo S), secreted by the bacterial pathogen *Streptococcus pyogenes*, is an active immunomodulatory molecule which prolongs bacterial infections by virtue of its ability to specifically cleave the GlcNAc β 1 $\rightarrow$ 4GlcNAc linkage of human IgG Fc glycans (Section 1.6). Experiments *in vitro* and in whole blood have revealed that Endo S treated antibodies fail to bind leukocytes, activate complement or elicit phagocytosis (157). While evidence for the specificity of Endo S for human IgG is available, the finer carbohydrate specificity of this enzyme is not known (97,158). Similarly the structural basis for the inactivation of the Fc by endoglycosidases has not been established for human Fc.

A murine Fc (lacking a hinge domain) where the Fc glycan was entirely removed using a N-glycosidase F (PNGase F) showed a “closed” conformation with the closest known distance between the C γ 2 domain (34.6 Å). Given the ability of Fc structures to sample a range of quaternary arrangements (Chapter 3) and the importance of the hinge domain in FcR binding, a detailed investigation of the structural basis for endoglycosidase inactivation of human IgG Fc was undertaken.

In this chapter, the X-ray crystallographic structure of enzymatically deglycosylated human IgG1 Fc is described and the MALDI-TOF analysis of the carbohydrate selectivity of Endo S is reported. These studies provide a detailed structural picture for endoglycosidase-mediated immunosuppression. Finally, knowledge of the carbohydrate specificity of Endo S permits the selective modulation of the Fc function of serum and monoclonal antibodies providing a new route to significantly enhanced FcR binding and effector function under physiological conditions.

6.2. Purification, Crystallisation and Structure determination of Deglycosylated IgG Fc

To elucidate the structural basis for immune deactivation by endoglycosidases, deglycosylated human IgG Fc ($\text{Fc}^{\text{GlcNAc}}$) was prepared by treatment of oligomannose glycan containing Fc with Endoglycosidase H (Section 2.8). $\text{Fc}^{\text{GlcNAc}}$ was purified by SEC to a final concentration of 7 mg/ml (Figure 6.2.1A). SDS-PAGE analysis confirmed the reduced size of the $\text{Fc}^{\text{GlcNAc}}$ after deglycosylation (Figure 6.2.1B).

Crystals of $\text{Fc}^{\text{GlcNAc}}$ grew after 15-30 days at room temperature by the vapour diffusion method from sitting drops of 100 nl of protein and 100 nl of precipitant (Table 2.4.1. , Figure 6.2.2). Crystals were immersed in cryoprotectant comprising of 25% ethylene glycol in mother liquor and were stored in a steady stream of gaseous nitrogen until data collection. Data was collected at the Diamond IO3 beamline.

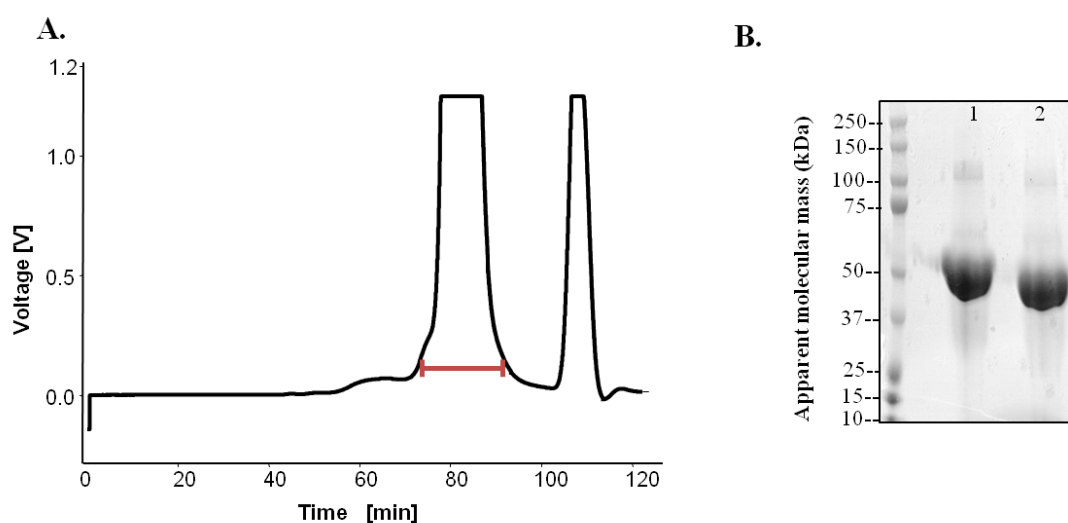


Figure 6.2.1. Purification of $\text{Fc}^{\text{GlcNAc}}$. (A) SEC curve of $\text{Fc}^{\text{GlcNAc}}$ run on a Hiloal 16/60 superdex 200 pregrade column. Red bar indicates the peak corresponding to the 53kDa Fc protein that was collected for crystallisation. Calibration standards were used to assign peak. (B) 4-12% gradient SDS-PAGE of concentrated $\text{Fc}^{\text{GlcNAc}}$ after SEC purification. Lane1 is FcWT and Lane 2 is $\text{Fc}^{\text{GlcNAc}}$.

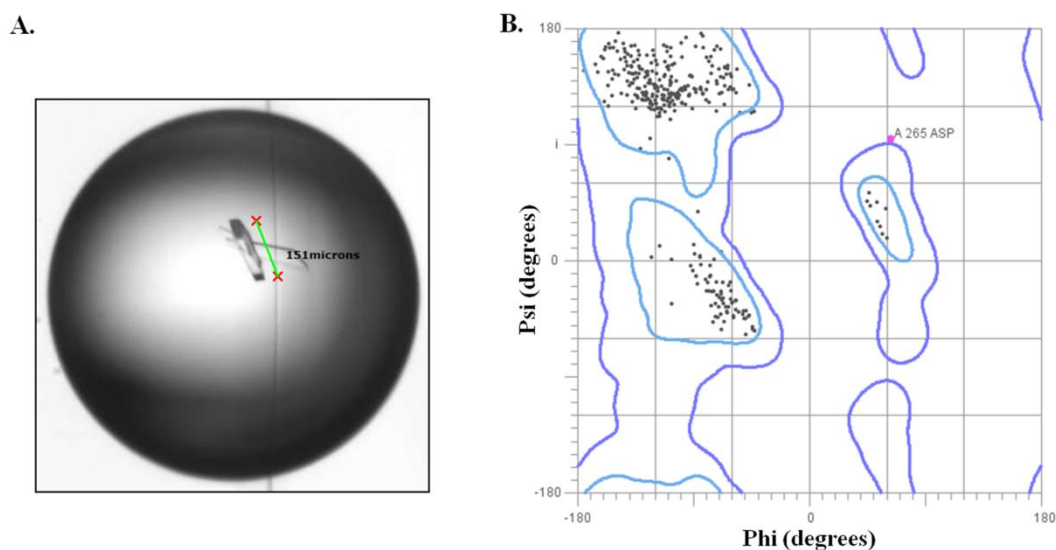


Figure 6.2.2. Crystallisation and model validation of Fc^{GlcNAc}. (A) Fc^{GlcNAc} crystals grew to a size of 1.51 μ m. (B) Ramachandran plot generated with Molprobit (110) for the final refined Fc^{GlcNAc} structure.

Structure of Fc^{GlcNAc} was solved by molecular replacement using PHASER and refined to a resolution of 2.5 Å. The previously reported, FcWT structure (PDB ID 2DTQ) was used as model for molecular replacement. One Fc^{GlcNAc} dimer was identified in each asymmetric unit with a solvent content of 56.1% and a Matthew's coefficient of 2.8 Å³/Da. Iterative structural refinements were performed with REFMAC and COOT (Section 2.6). The final model was validated with MOLPROBITY (110). Ramachandran analysis of phi and psi angles for the final Fc^{GlcNAc} structural model shows 97.7% residues in the most favoured regions (Figure 6.2.2B). Data collection and refinement statistics for the Fc^{GlcNAc} structure are summarised in Table 6.2.1.

Table 6.2.1. Crystallographic Data and Refinement Statistics for IgG1 Fc^{GlcNAc}.

Data collection Fc^{GlcNAc}	
Beamline	Diamond I03
Resolution (Å)	50.0-2.49 (2.59-2.49) ^a
Spacegroup	C2
Cell dimensions (Å)	<i>a</i> = 69.3 <i>b</i> = 110.9 <i>c</i> = 77.5
and angles (°)	β = 107.9, α = γ = 90
Wavelength (Å)	0.976
Unique reflections	19399 (1923)
Completeness (%)	99.9 (99.9)
<i>R</i> _{merge} (%) ^b	0.108 (0.666)
<i>I</i> /σ <i>I</i>	19.5 (2.2)
Average redundancy	8.4 (3.7)
Refinement	
Resolution range (Å)	34.3-2.49 (2.56-2.49)
Number of reflections	18408 (1348)
<i>R</i> _{work} (%) ^c	19.5
<i>R</i> _{free} (%) ^d	23.4
r.m.s.d. bonds (Å)	0.008
angles (°)	1.2
Atoms per asymmetric unit	
(protein/water/glycan)	3266/25/14
Average <i>B</i> factors (protein/water/glycan)	77.3/60.9/104.0
(Å ²)	
Ramachandran plot (Favored/allowed; %)	97.7/ 2.3

^aNumbers in parentheses refer to the relevant outer resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I(\text{hkl}; i) - \langle I(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I(\text{hkl}; i)}$, where $I(\text{hkl}; i)$ is the intensity of an individual measurement and $\langle I(\text{hkl}) \rangle$ is the average intensity from multiple observations.

^c $R_{\text{work}} = \frac{\sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{\text{hkl}} |F_{\text{obs}}|}$

^d R_{free} was calculated as R_{work} but after removal of 5% of data prior to refinement (Section 2.6).

6.3. Structural analysis of deglycosylated Human IgGFc (Fc^{GlcNAc})

Fc^{GlcNAc} retained the classic homodimeric horseshoe shape of IgG and electron density for the single GlcNAc residue on chain B was observed (Figure 6.3.1.). No visible electron density for Asn297 and the single GlcNAc residue attached to chain A was visible. Minimal electron density for the B/C, D/E and F/G loops on chain A were indicative of increased mobility. These regions were omitted from the final crystallographic model. Increased disorder of the C γ 2 domains of Fc^{GlcNAc} were reflected in the significantly elevated B factor values (Figure 6.3.2, Table A.1).

A comparison of the Fc^{GlcNAc} structure with the FcWT structure revealed a significant deviation in the quaternary conformation of the C γ 2 domains (r.m.s.d of 1.96 Å for Ca atoms of residues 238 to 444 of chain B). Both chains of the Fc^{GlcNAc} dimer move inwards with chain B showing a significant rotation and translation about the central axis (Figure 6.3.3). Comparison of interdomain distances with other glycosylated Fc structures in the protein data bank confirmed that Fc^{GlcNAc} had the shortest interdomain distance (34.6 Å). This was consistent with previously reported X-ray crystal structure of murine deglycosylated Fc fragment which also shows a “closed” C γ 2 conformation (PDB codes 3HKF) (159).

Comparison of the Fc^{GlcNAc} structure with the receptor bound Fc structure shows that the “closed” conformation of Fc^{GlcNAc} is largely incompatible with asymmetric ‘open’ Fc bound to Fc γ RIIIa (59,79). The D/E loop containing Asn297 linked glycosylation site is particularly significant as disorder and increased mobility of this loop has been associated with decreased stability of C γ 2 domains. Sequential truncation of glycans attached to Asn297 has been shown to increase mobility of the D/E loop. In Fc^{GlcNAc}, the D/E loop of chain A was too disordered to show electron density, while on chain B, the D/E loop is significantly tilted inwards, making engagement with the receptor very difficult (Figure 6.3.4). Further significance of the stability of this loop was shown in mutational studies, where mutations of the D/E loop (S298G, K290E, T299A) restored receptor binding of aglycosylated Fc (89).

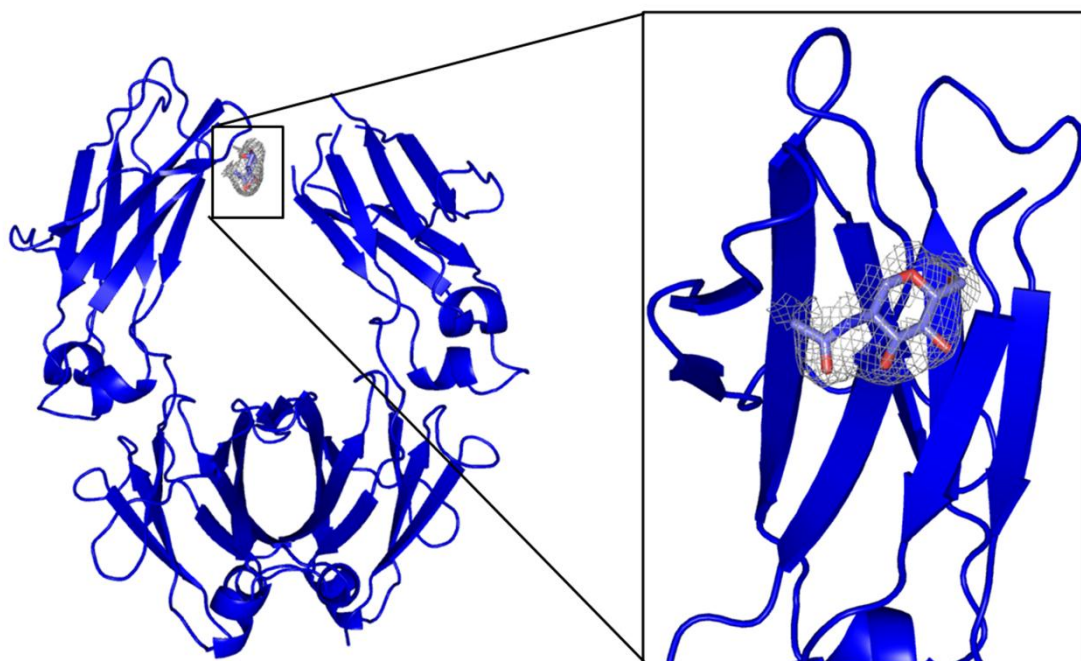


Figure 6.3.1. Structure of Fc^{GlcNAc} at 1.93 Å shown in cartoon representation. A maximum likelihood $2F_o - F_c$ map is displayed around the N-linked glycan, contoured to 1σ . Magnified view of the Fc^{GlcNAc} glycan on chain A is shown on the right.

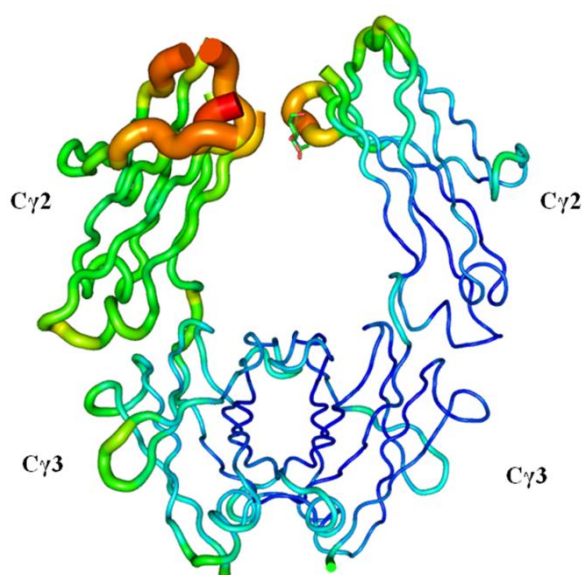


Figure 6.3.2. Conformational mobility of C γ 2 domains of Fc^{GlcNAc}. The protein backbone and attached glycan of Fc^{GlcNAc} (on chain A) are coloured according to their **B** factor values. Colours range from dark blue (lowest **B** factor values) through to bright red (highest **B** factor values).

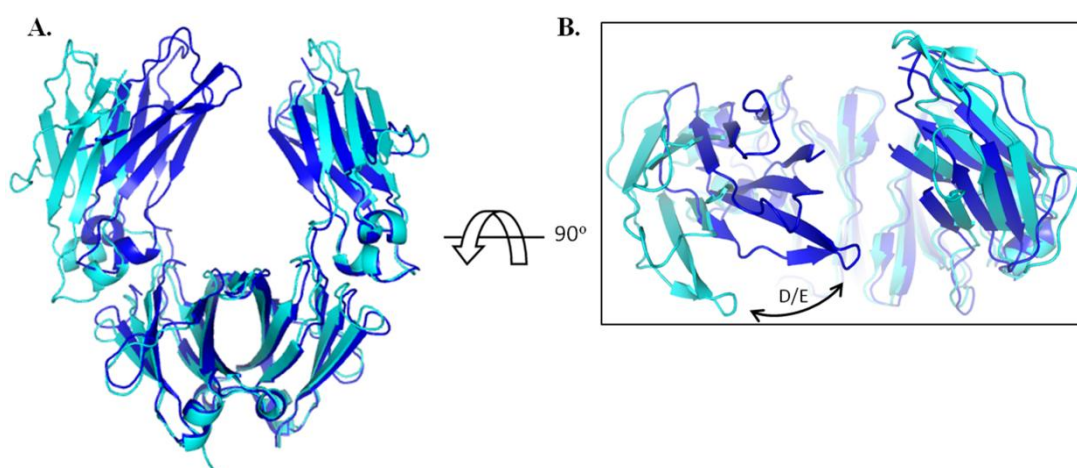


Figure 6.3.3. Structural analysis of Fc^{GlcNAc}. (A) Fc^{GlcNAc} (blue) overlaid with FcWT (Cyan) structure. (B) Closer view of D/E loops of Fc^{GlcNAc} and FcWT.

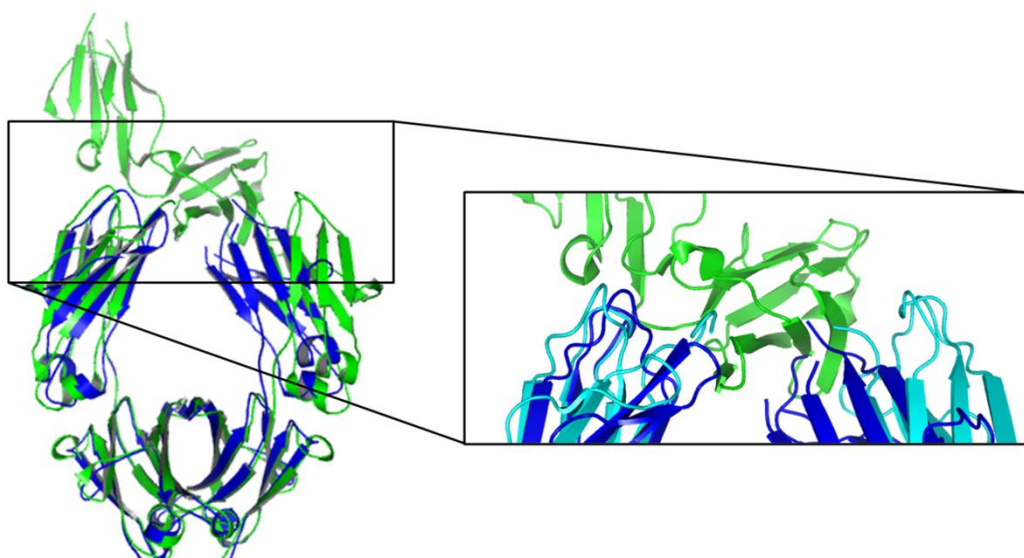


Figure 6.3.4. Structural comparison of Fc^{GlcNAc} with Fc-FcγRIIIa complex. IgG Fc in complex with FcγRIIIa complex (cyan and green, PDB ID 3SGK) is overlaid with Fc^{GlcNAc} (blue) and a magnified view of the Cγ2 loops (D/E, B/C and F/G) of Fc are shown.

6.4. Thermal stability of Fc^{GlcNAc}

DSF analysis of thermal unfolding of Fc GlcNAc showed a single thermal transition corresponding to the Cγ2 domain (Appendix, Figure A.1). Melting temperature of Fc^{GlcNAc} (64.16°C) was significantly lower than that of FcWT (69.8°C). Lowered stability of Fc^{GlcNAc} was consistent with high B factor values and disordered loop regions observed in the crystal structure. Previous studies on IgG1Fc showed decrease in melting temperature with sequential truncation of glycans (150). These observations confirm the important role of Fc glycans in the stability of Cγ2 domain and are consistent with the structural observations on the instability of the D/E loop of Fc^{GlcNAc}.

6.5. Carbohydrate selectivity of Endo S

The bacterial endoglycosidase, Endo S exhibits unique specificity towards IgG glycans. We therefore hypothesised that an Fc with non-natural glycans would be resistant to Endo S mediated hydrolysis. To test this hypothesis, N-linked glycans released by PNGaseF digestion from FcWT and Fc Man₉GlcNAc₂ (containing non-natural, oligomannose type N-linked glycans, as described in Section 5.4) were incubated with recombinant Endo S (1μg) or mock treated with PBS, overnight at 37°C. Following

this, glycans were purified by filtration through a MultiScreen 96-Well Filtration Plate (Millipore, Billerica, MA, U.S.A.) and dried down and subject to MALDI-TOF analysis (as described in Section 2.8).

Endo S completely hydrolysed all complex type N-linked glycans on FcWT; however oligomannose glycans from Fc Man₉GlcNAc₂ remained undigested. The MALDI-TOF spectra for control and EndoS hydrolysed glycans are shown in Figure 6.5.1. This carbohydrate specificity was further characterised using another well characterised endoglycosidase- Endo H. Endo H is known to be highly specific for oligomannose type N-linked glycans (160,161). Analysis of Endo H digested N-linked glycans from FcWT and Fc Man₉GlcNAc₂ showed complete hydrolysis of the oligomannose glycans from Fc Man₉GlcNAc₂ while leaving the complex glycans from FcWT unperturbed. Comparison of the carbohydrate specificity of Endo S with other known endoglycosidases revealed most similarity to Endo D which also preferentially hydrolyses complex type N-linked glycans (162).

One important difference between Endo S and other endoglycosidases is that most carbohydrate specificity studies for these enzymes were performed on free N-glycans or with denatured glycoprotein, while it has been reported that Endo S activity specifically requires the presence of native IgG (98,162). To investigate the ability of Endo S to digest N-linked glycans on whole Fc Man₉GlcNAc₂ and FcWT proteins, we incubated 3mg/ml of purified Fc Man₉GlcNAc₂ and FcWT proteins overnight with 1µg/ml of recombinant Endo S. Control samples of Fc Man₉GlcNAc₂ and FcWT were mock treated with PBS overnight at 37°C. Endo S digested Fc Man₉GlcNAc₂ and FcWT proteins were then tested for their ability to bind to the activatory FcγRIIIa by SPR analysis as described in Section 2.9. Consistent with previous, Endo S treated FcWT protein showed significantly reduced binding to FcγRIIIa in contrast to the oligomannose glycan containing Fc Man₉GlcNAc₂ protein that retained high affinity binding to the receptor even after overnight incubation with Endo S (55).

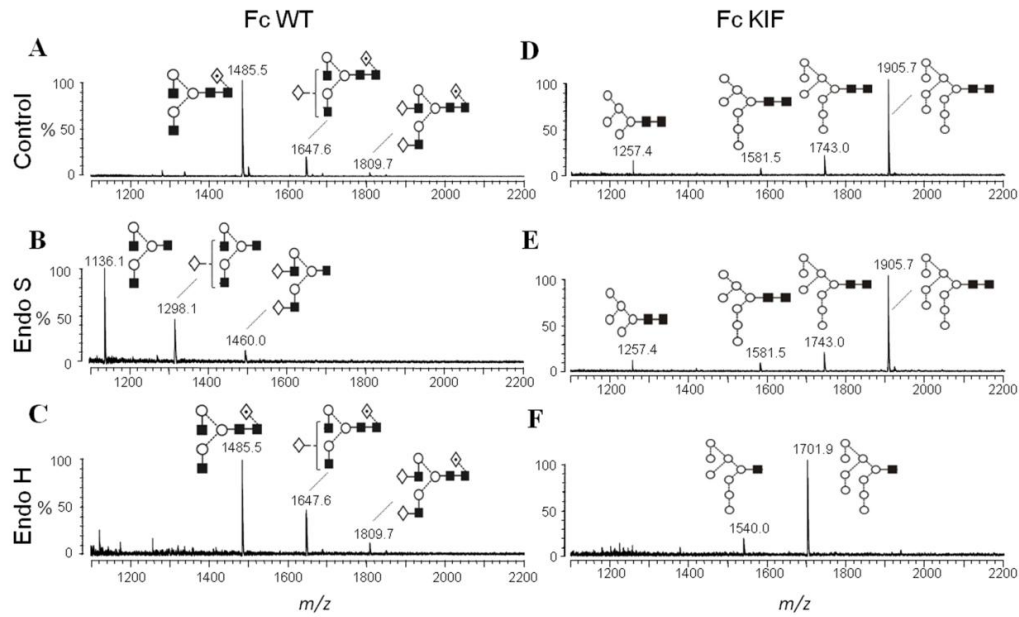


Figure 6.5.1. MALDI-TOF analysis of Endo S and Endo H digested glycans from FcWT (complex type N-linked glycans) and Fc Man₉GlcNAc₂ (Oligomannose type glycans). The cleavage of the core GlcNAc β 1 $\rightarrow$ 4GlcNAc bond results in the removal of a single GlcNAc and a Fucose residue from complex glycans (predicted $\Delta m/z = 349.1$), and a GlcNAc from oligomannose-type glycans (predicted $\Delta m/z = 203.1$).

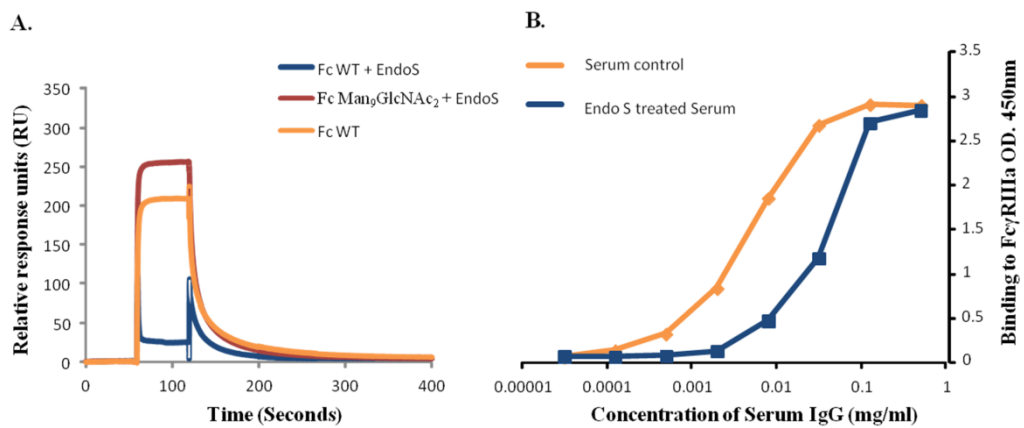


Figure 6.5.2. Fc γ RIIIa binding for deglycosylated FcWT and serum IgG. (A) Background subtracted SPR sensorgrams for binding of Endo S treated recombinant FcWT (1mg/ml) and Fc Man₉GlcNAc₂ (1mg/ml) to Fc γ RIIIa. (B) ELISA binding of control or Endo S treated human sera to Fc γ RIIIa.

These results show selective activity of Endo S towards IgG with complex type glycosylations versus that for monoclonal antibodies with oligomannose type glycans. This carbohydrate selectivity of Endo S may have potential therapeutic applications. One consideration is to use this unique specificity of Endo S to overcome the role of natural IgG in blocking monoclonal FcR activity. Preliminary experiments to test this hypothesis are described below.

6.6. Enhanced activity of oligomannose containing monoclonal antibodies by Endo S treatment of serum IgG

Serum IgG has been shown to competitively inhibit the activity of therapeutic antibodies(163). Therefore, we hypothesised that the selective deactivation of serum IgG by Endo S would enhance the therapeutic activity of monoclonal antibodies with oligomannose type glycosylation. To test this hypothesis, a competitive ELISA experiment was designed to reflect the binding of mAb to the activatory Fc receptor FcγRIIIa, in the presence of serum. For this a recombinant monoclonal antibody (mAb CIIC1) bearing human IgG1 Fc domains and murine Fab fragments specific for mouse collagen type II was cloned and expressed with oligomannose type glycans as described in section 2.7. This unnaturally glycosylated mAb along with human serum was incubated with recombinant Endo S at 37°C overnight and the next day a competition ELISA was performed to measure the binding of CIIC1 mAb to FcγRIIIa in the presence of Endo S treated and untreated serum IgG (Section 2.11). The results of this competition ELISA are shown in Figure 6.6.1.

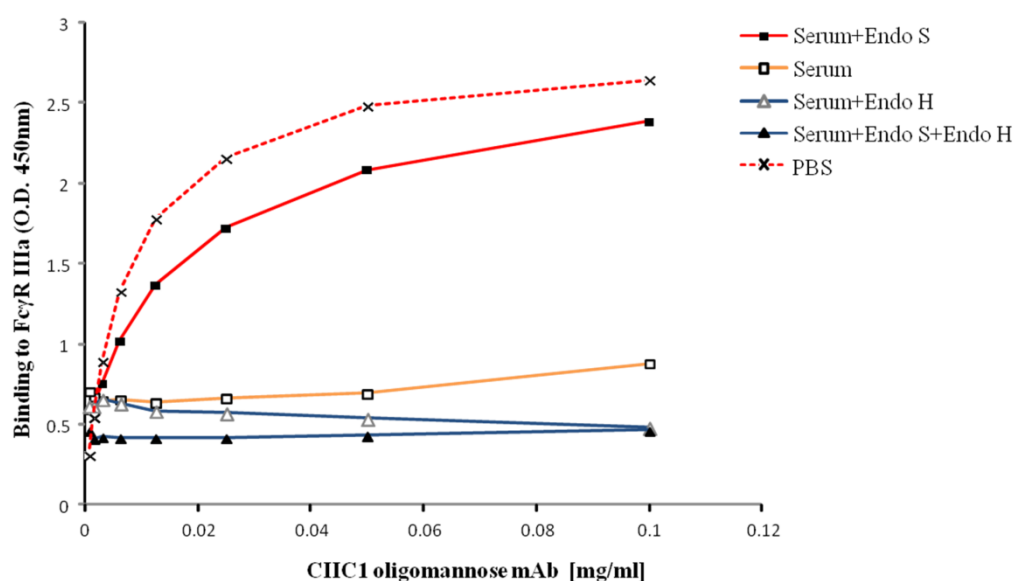


Figure 6.6.1 Binding of oligomannose bearing CH1C1 mAb to FcγRIIIa in the presence of Endo S treated serum IgG. Competition ELISA with Endo S and Endo H treated serum and serial dilutions of CH1C1 antibody bearing oligomannose glycans.

Endo S treatment of serum significantly enhanced the binding of oligomannose bearing mAb to FcγRIIIa. In the absence of serum, IgG bearing oligomannose type glycans exhibit higher affinity for FcγRIIIa in comparison to native glycosylated antibodies (65) (Appendix, Figure A.1). The competitive ELISA experiment described here in the presence of Endo S treated serum shows that the affinity of oligomannose bearing IgG for FcγRIIIa was restored to a level approaching that of serum-free mAb.

6.7. Conclusions

A significant drawback of therapeutic antibodies whose mode of action is through engagement of cellular receptors is the necessity for very high therapeutic doses. Serum trough levels of the well characterised, anti-cancer therapeutic antibody (Trastuzumab) are nearly 1000 fold higher than effective concentrations needed for the same activity *in vitro* (164). This requirement for high therapeutic doses is due to the inhibitory effect of endogenous serum IgG which competes with monoclonal antibody for receptor binding (163).

Pathogenic bacteria regularly evade the human immune system by secreting several proteases and endoglycosidases that selectively target and hydrolyse antibodies (126).

Endo S is one such highly evolved bacterial enzyme that specifically hydrolyses native human IgG. Endo S mediated digestion of IgG Fc fragments results in the cleavage of the core GlcNAc β 1 $\rightarrow$ 4GlcNAc bond, leaving a single GlcNAc and fucose residue attached to the Asn297 residue. This deglycosylation of antibody Fc domains abrogates binding to Fc receptors leading to the deactivation of downstream cellular immune responses. The structural basis for this was evident from the 2.5 Å resolution crystal structure of Fc^{GlcNAc} which showed a significant inward collapse of C γ 2 domains incompatible with Fc γ receptor binding.

Effector functions of therapeutic antibodies are directly dependent on two key factors. First, is the affinity of the therapeutic antibody for cellular Fc receptors and second is the density of target antigen. Serum inhibition of therapeutic antibody activity is more pronounced under low target antigen densities (163). Hence, engineering therapeutic antibodies with higher affinity for cellular receptors may overcome serum inhibition only in the presence of high target antigen density. Endo S mediated deactivation of serum IgG could enhance therapeutic antibody effector functions independent of target antigen density. The combination of Endo S and monoclonal antibodies with oligomannose type glycans led to significant enhancement in binding of monoclonal antibodies to Fc γ RIIIa, while selectively eliminating serum IgG inhibition. This new approach for refocusing antibody receptors to specifically tailored monoclonal antibodies may be used to significantly reduce the effective doses of therapeutic antibodies and also bring previously inaccessible target antigens within the therapeutic window.

CHAPTER 7

Final Conclusions and Perspectives

7. Final conclusions and perspectives

Crystallographic structures of IgG Fc glycoforms reported in this thesis show distinct glycan conformations accommodated within a relatively limited variation in the overall quaternary structure of the Fc dimer. All Fc glycoforms studied here contained identical amino acid sequences and adopted an ‘open’ conformation under different crystallographic conditions (Table 2.4.1.) and glycosylation profiles. The most ‘open’ conformation was adopted by F241A Fc with an interdomain distance of 60.1Å (Table 7.2), while the most ‘closed’ conformation was that of Fc^{GlcNAc} with an interdomain distance of 34.6 Å. All other Fc glycoforms (Fc Man₉GlcNAc₂, Fc Hybrid and FcWT) adopted varying ‘open’ conformations which were also larger than the interdomain distance of IgG Fc in Fc-FcγRIIIa complexes. A systematic comparison of interdomain spacing with all previously reported human IgG Fc structures showed that a range of ‘open’ conformations are possible for the Fc dimer (Figure 3.5.4 , Figure 7.1).

While the presence of hinge disulphides was verified for each Fc glycoform by SDS-PAGE under reducing conditions and size-exclusion chromatography, selective crystallisation of the Fc dimers without hinge disulphide bonds may also be a possibility. Direct SDS-PAGE and size-exclusion chromatography of protein samples from crystal loops used in the X-ray diffraction experiments, would allow a more accurate determination of the presence of disulphide linked hinge domains in each Fc structure. Previously, variations in the interdomain spacing of Fc dimers prepared under matching conditions and containing similar complex type glycosylation (PDB ID 1H3V, 1H3Y) were attributed to differences in crystallographic contacts (32). Similarly, ‘open’ conformations of Fc dimers have been attributed to point mutations (165) and to differences in glycosylation. An initial comparison of crystal contacts of Fc glycoforms revealed no immediate correlations with interdomain spacing.

Table 7.1. Interdomain distances of all reported human IgG Fc structures in the protein data bank.

Human IgG1 Fc (PDB ID)	State	Glycoform	R291/R291 Å
1H3U	Free Fc	Man ₃ GlcNAc ₂ Fuc	46.5
1H3V	Free Fc	G2F	45.7
1H3W	Free Fc	G2F (desialylated)	50.0
1H3X	Free Fc	G0F	46.2
1H3Y	Free Fc	G2F	38.0
1H3T	Free Fc	Man ₁ GlcNAc ₂ Fuc	45.6
1FC1	Free Fc	G1F	46.8
2DTQ	Free Fc	G0F	41.6
2DTS	Free Fc	G0	41.7
2WAH	Free Fc	Man ₉ GlcNAc ₂	56.2
Fc WT	Free Fc	G0F	51.9
Fc KIF	Free Fc	Man ₉ GlcNAc ₂	50.9
Fc Hybrid	Free Fc	Man ₅ GlcNAc ₂ Fuc	56.5
Fc GlcNAc	Free Fc	GlcNAc	34.6
Fc F241A	Free Fc	Man ₂ GlcNAc ₃ Fuc	60.1
1T83	FcγRIIIb bound	G1F/G0F	44.7
1T89	FcγRIIIb bound	G1F	49.1
3SGJ	FcγRIIIa bound	G0/G0F	46.0
3SGK	FcγRIIIa bound	G0	48.1
3RY6	FcγRIIa bound	A2G2F/G0F	47.9
1E4K	FcγRIIIa Bound	G1F	50.6
1HZH	Full length IgG	G1F/G1	42.8
1MCO	Full length IgG	G2F	38.3
1L6X	Fc-Protein A	G2F	43.7
2GJ7	Fc-gE-gI	G1F	46.6
2J6E	Fc-RF61	G1F	42.1
1FC2	Fc-Protein A		46.2
1OQO	Fc-Protein A	G0F	47.4
1OQX	Fc-Protein A	G0F	41.5
1FCC	Fc-Protein G	G1F	48.3
1DN2	Fc- Protein A	G1F	45.7
2IWG	Fc-TRIM	G1F	44.0
2QL1	Mutant with enhanced ADCC	G1F	47.6
3FJT	Mutant with extended half -life	G0F	41.0
3C2S	Mutant lacking effector functions	G1F	45.7

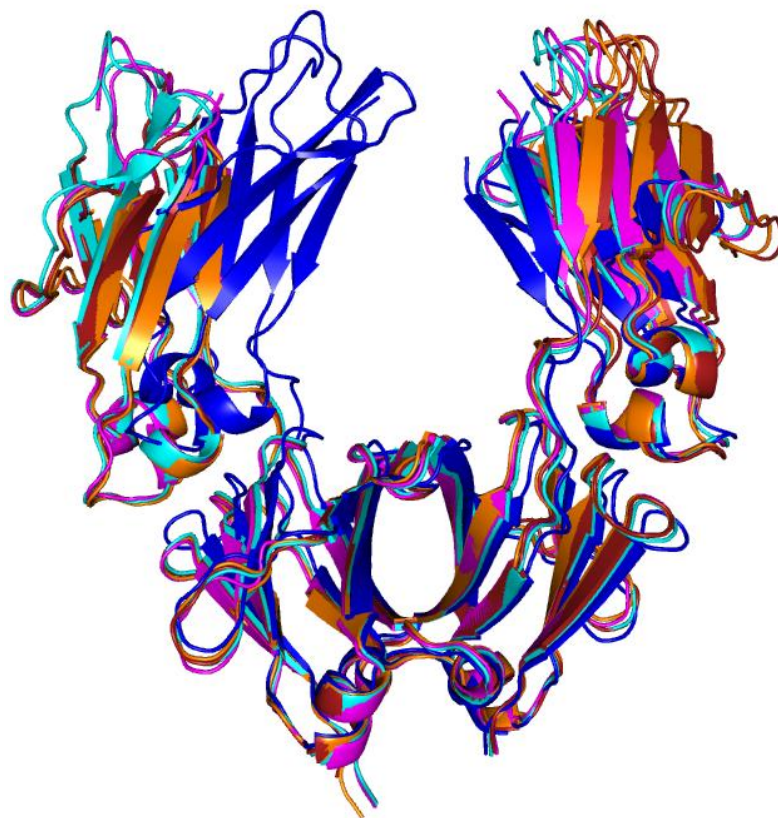


Figure 7.2. Structural comparison of Fc glycoforms. Overlay of FcWT(Cyan), Fc Man₉GlcNAc₂(Pink), Fc Hybrid(Red), Fc^{GlcNAc} (Blue) and F241A Fc (Orange) shown in cartoon representation.

Another common feature of all Fc structures was the disorder and mobility of the B/C, D/E and F/G loops of the C γ 2 domains which form part of the binding interface with Fc γ RIIIa. This was also reflected in the high B factor values of C γ 2 domains of Fc glycoforms in comparison to C γ 3 domains (3). Neighbouring crystal contacts can also significantly affect the stability of C γ 2 loops and a more thorough study of crystal contacts for each Fc glycoform is required. Despite differences in crystallographic conditions these disordered regions are a common feature of IgG Fc (7) and may in fact be a prerequisite for functionally active IgG Fc capable of engaging many different ligands (166) (132).

Table 7.3. *B* factor values of C γ 2 and C γ 3 domains of Fc glycoforms. (A) $B_{average}$ values for C γ 2 domains of chain A and chain B for each Fc glycoform. (B) $B_{average}$ values for C γ 3 domains of chain A and chain B for each Fc glycoform. *B* factor values were calculated using the temperature factor analysis software in the CCP4 suite (105).

A.

Fc Glycoform	$B_{average}$ (C γ 2)/ chain A ($\text{\AA}^2$)	$B_{average}$ (C γ 2)/ chain B($\text{\AA}^2$)
Fc WT	52.713	50.605
Fc Man ₉ GlcNAc ₂	53.613	71.969
Fc Hybrid	27.024	45.823
F241A Fc	59.428	54.125
Fc ^{GlcNAc}	73.079	108.440

B.

Fc Glycoform	$B_{average}$ (C γ 3)/ chain A ($\text{\AA}^2$)	$B_{average}$ (C γ 3)/ chain B($\text{\AA}^2$)
Fc WT	30.043	31.333
Fc Man ₉ GlcNAc ₂	34.102	34.185
Fc Hybrid	34.973	26.925
F241A Fc	25.258	29.094
Fc ^{GlcNAc}	59.312	73.370

Removal of N-linked glycans by endoglycosidase treatment led to the most pronounced change in Fc structure and function (Figure 7.2, Section 6.3). The similarity of this structure to an N-glycosidase treated murine Fc suggests that while “open” and “closed” conformations of glycosylated Fc may represent a dynamic continuum of structures, the deglycosylated set of Fc structures form a distinct group of “collapsed” quaternary structures. Effect of Fc glycans on the conformation and stability of the D/E loop was evident from the Fc^{GlcNAc} structure (Figure 6.3.3) and were consistent with observations on mutations of residues on this loop, contributing to functional activity (89).

The importance of N-linked glycosylation of IgG Fc is evident from the highly conserved nature of this site in mammalian IgG protein sequences (89). Hydrophobic amino acid residues involved in interactions with the N-linked glycan and residues involved in binding to Fc γ receptors are also conserved across species and IgG subtypes. Increased sialylation and galactosylation of Fc mutated at these hydrophobic residues has been reported previously and is often cited as evidence for the protein-directed

glycosylation of Fc (131). The F241A Fc crystal structure revealed increased destabilisation and mobility of the N linked glycan in comparison to previous reported Fc structures. However, significant protein-proximal conformation of the N-linked glycan residues remained visible in the F241A Fc electron density maps despite the significant increase in terminal processing observed for this glycan.

These data indicate the crystallographic isolation of low energy stable glycan conformers and is consistent with recent NMR studies which led the authors to propose that Fc glycans exist in both protein bound and unbound conformations (129). Changes in the minimum energy conformation leads to significant increase in processing– this would be interesting to further asses by NMR studies of the F241A mutant Fc. Taken together, these results support a highly dynamic and flexible model for the IgG Fc and Fc glycans. While crystallographic studies provide snapshots of Fc and Fc glycan conformations, it would be most instructive to study these Fc glycoforms in solution and in more ambient temperatures. Analytical ultracentrifugation and small angle X-ray diffraction studies in combination with fluorescence resonance energy transfer (FRET) experiments could provide more accurate information on the interdomain spacing of IgG Fc under physiological conditions.

Finally, manipulation of the cellular glycan processing pathways to produce homogenous glycoforms of IgG Fc was used in combination with the knowledge of the fine glycan specificity of endoglycosidases as a potential therapeutic strategy for selectively recruiting the cellular immune system to single defined IgG glycoforms (Section 6.6). Preliminary experiments showed significant enhancement in the receptor binding of a monoclonal antibody with oligomannose glycans in the presence of endoglycosidase treated serum IgG. Further experiments on the detailed carbohydrate specificity of Endo S are ongoing.

Studies on inhibition of antibody effector functions by serum IgG have till date only focused on the activity of FcγRIIIa (167-169). However, effector functions of therapeutic antibodies *in vivo* are also determined by the presence of other activating and inhibitory Fc receptors (FcγRIIa, FcγRIIb), density of target antigen and density of Fc receptors on effector cells. Avidity of large antigen-antibody aggregates can also overcome serum inhibition and compensate for the low affinity and fast kinetics of Fc-

FcR binding interaction. These factors will determine the effectiveness of Endo S mediated removal of inhibitory serum IgG *in vivo*.

Finally, in a clinical setting, the efficacy and serum trough levels of therapeutic antibodies are also determined by their biological half-lives and biodistribution properties. This is especially relevant in treatments targeting tumour antigens where large and frequent doses of therapeutic antibody are required for effective distribution of therapeutic antibodies in target sites. Another crucial factor determining the feasibility of using Endo S for *in vivo* applications will be its antigenicity. While studies in healthy rabbits injected with recombinant Endo S did not show significant toxic effects (170), there are no reports from clinical studies in human volunteers.

Fc mutants designed to selectively engage specific Fc receptors with high affinity and Fc–Endo S conjugates may help overcome some of the problems listed above. There are to date, no reports of clinical trials using intravenous Endo S treatment. Further cell based assays to test the effectiveness of Endo S mediated deglycosylation of serum in an *ex vivo* setting are ongoing in our laboratory.

Appendix

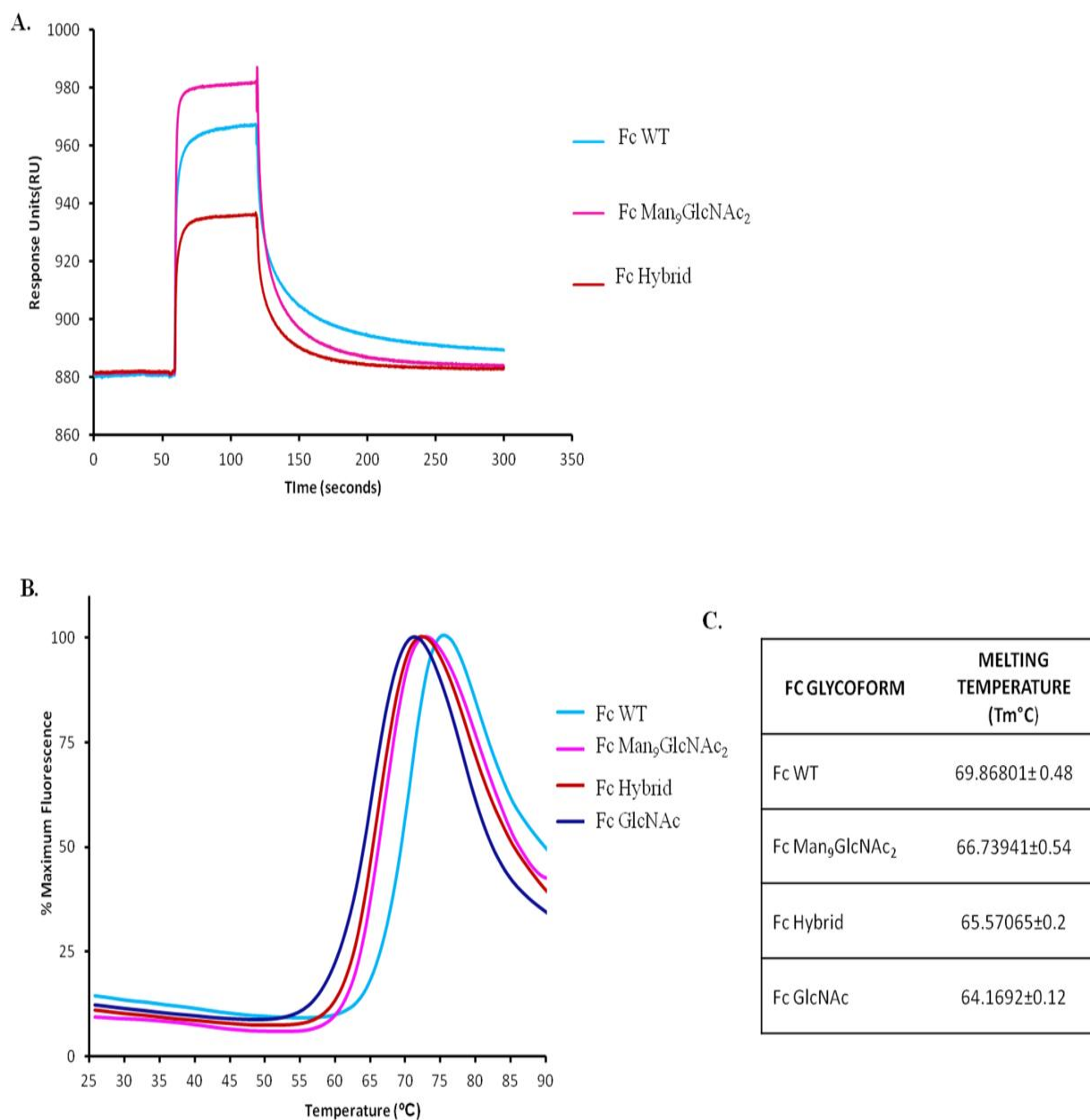


Figure A1. FcγRIIIa binding and Thermal stability of Fc glycoforms. (A) SPR binding of Fc glycoforms to immobilised FcγRIIIa. Background subtracted sensograms are shown. (B) Differential scanning fluorimetry (DSF) to measure thermal unfolding of Fc glycoforms. Blank subtracted fluorescence at each temperature is shown. (C) Melting temperatures of Fc glycoforms calculated from first derivative curves of DSF thermograms. Each T_m is the average of three independent experiments and is listed ± SD.

Abbreviations

µg	Microgram
µl	Microlitre
µM	Micromolar
Å	Angstrom
a.s.u	Asymmetric Unit
ADCC	Antibody Dependent Cellular Cytotoxicity
bp	Base Pair
BSA	Bovine Serum Albumin
CCP4	Collaborative Computational Project 4
CDC	Complement Dependent Cytotoxicity
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary
CID	Collision Induced Dissociation
CM5	Carboxymethyl 5
COOT	Crystallographic Object Oriented Toolkit
DCSIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSF	Differential Scanning Fluorimetry
ELISA	Enzyme Limited Immunosorbent Assay
Endo H	Endoglycosidase H
Endo S	Endoglycosidase S
ER	Endoplasmic Reticulum
ESI	Electrospray Ionisation
Fab	Fragment Antigen Binding
Fc	Fragment Crystallisable
FcR	Fc Receptor
FcRn	Neonatal Fc Receptor
FCS	Fetal Calf Serum

FRET	Fluorescence Resonance Energy Transfer
FT	$\alpha 1 \rightarrow 6$ Fucosyltransferase
Gal	Galactose
GlcNAc	N-Acetylglucosamine
GnTIII	GlcNAc Transferase III
H₂SO₄	Sulphuric Acid
HBS-N	10mm HEPES pH 7.4, 150mm Sodium Chloride, 3mm EDTA, 0.005% Surfactant P20
HEK 293T	Human Embryonic Kidney Cells
HEPES	4-(2-Hydroxyethyl)Piperazine-1-Ethanesulfonic Acid Hemisodium Salt
HRP	Horseradish Peroxidase
Ig	Immunoglobulin Fold
IgG	Immunoglobulin G
IMAC	Immobilised Metal Affinity Chromatography
IMDM	Iscoe's Modified Dulbecco Medium
ITAM	Immune Receptor Tyrosine Activation Motif
ITIM	Immune Receptor Tyrosin Inhibition Motif
IVIG	Intravenous Immunoglobulin
K	Kelvin
K_D	Equilibrium Dissociation Constant
L	Litre
LB	Luria-Bertani
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry
Man	Mannose
mAb	Monoclonal antibody
mg	Milligram
mM	Millimolar
ng	Nanogram
NK cells	Natural Killer Cells
NMR	Nuclear Magnetic Resonance
NZY	5g/L Sodium Chloride, 10g/L NZ Amine, 5g/L Bacto-Yeast Extract, 2g/L Magnesium Sulphate pH 7.4

OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene Glycol
PEI	Polyethylenimine
PNGase F	N-Glycosidase F
r.m.s.d.	Root Mean Square Deviation
RNA	Ribonucleic Acid
RU	Response Units
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SIGNR1	Specific ICAM3 Grabbing Non-Integrin Related 1
SLE	Systemic Lupus Erythematosis
SPR	Surface Plasmon Resonance
ST6Gal	$\alpha 2 \rightarrow 6$ Sialyltransferase
ST3Gal	$\alpha 2 \rightarrow 3$ Sialyltransferase
TAE	Tris Acetate EDTA
TMB	3,3',5,5'-Tetramethylbenzidine
U	Unit
UGGT1	UDP-Glucose:Glycoprotein Glucosyltransferase 1
VH	Variable Heavy
VL	Variable Light
WT	Wild Type

References

1. Kindt T, Osborne B, Goldsby R. 2006. *Kuby Immunology*. W. H. Freeman
2. Porter RR. 1967. The structure of immunoglobulins. *Essays in biochemistry* 3:1-24
3. Coloma MJ, Trinh KR, Wims LA, Morrison SL. 1997. The hinge as a spacer contributes to covalent assembly and is required for function of IgG. *J Immunol* 158:733-40
4. Duncan AR, Winter G. 1988. The binding site for C1q on IgG. *Nature* 332:738-40
5. Nezlin R, Ghetie V. 2004. Interactions of immunoglobulins outside the antigen-combining site. *Advances in immunology* 82:155-215
6. Woof JM, Burton DR. 2004. Human antibody-Fc receptor interactions illuminated by crystal structures. *Nature Reviews Immunology* 4:89-99
7. Jefferis R, Lund J, Pound JD. 1998. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunological reviews* 163:59-76
8. Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, et al. 2000. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164:4178-84
9. Idusogie EE, Wong PY, Presta LG, Gazzano-Santoro H, Totpal K, et al. 2001. Engineered antibodies with increased activity to recruit complement. *J Immunol* 166:2571-5
10. Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, et al. 2001. High Resolution Mapping of the Binding Site on Human IgG1 for Fc γ RI, Fc γ RII, Fc γ RIII, and FcRn and Design of IgG1 Variants with Improved Binding to the Fc γ RI. *Journal of Biological Chemistry* 276:6591-604
11. Ghetie V, Ward ES. 2000. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. *Annu Rev Immunol* 18:739-66
12. Saphire EO, Parren PWHI, Pantophlet R, Zwick MB, Morris GM, et al. 2001. Crystal Structure of a Neutralizing Human IgG Against HIV-1: A Template for Vaccine Design. *Science* 293:1155-9
13. Yoo EM, Wims LA, Chan LA, Morrison SL. 2003. Human IgG2 can form covalent dimers. *J Immunol* 170:3134-8
14. Aalberse RC, Schuurman J. 2002. IgG4 breaking the rules. *Immunology* 105:9-19
15. Jefferis R, Lund J. 2002. Interaction sites on human IgG-Fc for Fc γ R: current models. *Immunology Letters* 82:57-65
16. Ravetch JV. 2003. *Fundamental Immunology*. pp 685-700.
17. Ravetch JV, Kinet JP. 1991. Fc receptors. *Annu Rev Immunol* 9:457-92
18. Bolland S, Ravetch JV. 1999. Inhibitory pathways triggered by ITIM-containing receptors. *Advances in immunology* 72:149-77
19. Daeron M. 1997. Fc receptor biology. *Annu Rev Immunol* 15:203-34
20. Nimmerjahn F, Ravetch JV. 2008. Fc γ receptors as regulators of immune responses. *Nat Rev Immunol* 8:34-47
21. Ravetch JV, Bolland S. 2001. IgG Fc receptors. *Annu Rev Immunol* 19:275-90
22. Ravetch JV. 2002. A full complement of receptors in immune complex diseases. *J Clin Invest* 110:1759-61

23. Ravetch JV, Bolland S. 2001. IgG Fc receptors. In *Annual Review of Immunology*, pp. 275-90
24. van de Winkel JGJ, Capel PJA. 1993. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunology Today* 14:215-21
25. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, et al. 2009. Specificity and affinity of human Fc γ receptors and their polymorphic variants for human IgG subclasses. *Blood* 113:3716-25
26. Salfeld JG. 2007. Isotype selection in antibody engineering. *Nat Biotech* 25:1369-72
27. Huber R, Deisenhofer J, Colman PM. 1976. Crystallographic structure studies of an IgG molecule and an Fc fragment. *Nature* 264:415-20
28. Saphire EO, Stanfield RL, Max Crispin MD, Parren PWHI, Rudd PM, et al. 2002. Contrasting IgG structures reveal extreme asymmetry and flexibility. *Journal of Molecular Biology* 319:9-18
29. Halaby DM, Poupon A, Mornon J. 1999. The immunoglobulin fold family: sequence analysis and 3D structure comparisons. *Protein engineering* 12:563-71
30. Stanfield RL, I.A. 2009. Antibody molecular structure. In *Therapeutic monoclonal antibodies from bench to clinic*, ed. Z An, pp. 51-63. Hoboken, New Jersey: John Wiley & Sons, Inc.
31. Deisenhofer J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å... resolution. *Biochemistry* 20:2361-70
32. Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P. 2003. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *Journal of Molecular Biology* 325:979-89
33. Crispin M, Bowden TA, Coles CH, Harlos K, Aricescu AR, et al. 2009. Carbohydrate and Domain Architecture of an Immature Antibody Glycoform Exhibiting Enhanced Effector Functions. *Journal of Molecular Biology* 387:1061-6
34. Laskowski RA. 2009. PDBsum new things. *Nucleic acids research* 37:D355-9
35. Harvey DJ, Royle L, Radcliffe CM, Rudd PM, Dwek RA. 2008. Structural and quantitative analysis of N-linked glycans by matrix-assisted laser desorption ionization and negative ion nanospray mass spectrometry. *Anal Biochem* 376:44-60
36. Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, et al. 1985. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 316:452-7
37. Pučić M, Knežević A, Vidič J, Adamczyk B, Novokmet M, et al. 2011. High Throughput Isolation and Glycosylation Analysis of IgG—Variability and Heritability of the IgG Glycome in Three Isolated Human Populations. *Molecular & Cellular Proteomics* 10
38. Takegawa Y, Deguchi K, Nakagawa H, Nishimura S-I. 2005. Structural Analysis of an N-Glycan with “ β 1–4 Bisecting Branch” from Human Serum IgG by Negative-Ion MSn Spectral Matching and Exoglycosidase Digestion. *Analytical Chemistry* 77:6062-8
39. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. 2007. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* 25:21-50
40. Jefferis R. 2009. Glycosylation as a strategy to improve antibody-based therapeutics. *Nat Rev Drug Discov* 8:226-34

41. Lund J, Takahashi N, Nakagawa H, Goodall M, Bentley T, et al. 1993. Control of IgG/Fc glycosylation: A comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs. *Molecular immunology* 30:741-8
42. Fujii S, Nishiura T, Nishikawa A, Miura R, Taniguchi N. 1990. Structural heterogeneity of sugar chains in immunoglobulin G. Conformation of immunoglobulin G molecule and substrate specificities of glycosyltransferases. *J Biol Chem* 265:6009-18
43. Barb AW, Brady EK, Prestegard JH. 2009. Branch-specific sialylation of IgG-Fc glycans by ST6Gal-I. *Biochemistry* 48:9705-7
44. Holland M, Yagi H, Takahashi N, Kato K, Savage CO, et al. 2006. Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochimica et biophysica acta* 1760:669-77
45. Rook GA, Steele J, Brealey R, Whyte A, Isenberg D, et al. 1991. Changes in IgG glycoform levels are associated with remission of arthritis during pregnancy. *Journal of autoimmunity* 4:779-94
46. Parekh R, Roitt I, Isenberg D, Dwek R, Rademacher T. 1988. Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. *J Exp Med* 167:1731-6
47. van de Geijn FE, Wuhler M, Selman MH, Willemsen SP, de Man YA, et al. 2009. Immunoglobulin G galactosylation and sialylation are associated with pregnancy-induced improvement of rheumatoid arthritis and the postpartum flare: results from a large prospective cohort study. *Arthritis Res Ther* 11:R193
48. Knezevic A, Gornik O, Polasek O, Pucic M, Redzic I, et al. 2010. Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. *Glycobiology* 20:959-69
49. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nature medicine* 1:237-43
50. Garred P, Madsen HO, Marquart H, Hansen TM, Sorensen SF, et al. 2000. Two edged role of mannose binding lectin in rheumatoid arthritis: a cross sectional study. *The Journal of rheumatology* 27:26-34
51. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. *Science* 313:670-3
52. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. 2008. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320:373-6
53. Anthony RM, Wermeling F, Karlsson MC, Ravetch JV. 2008. Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci U S A* 105:19571-8
54. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. 2007. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. *Molecular immunology* 44:1524-34
55. Lund J, Tanaka T, Takahashi N, Sarmay G, Arata Y, Jefferis R. 1990. A protein structural change in aglycosylated IgG3 correlates with loss of huFc gamma R1 and huFc gamma R111 binding and/or activation. *Molecular immunology* 27:1145-53
56. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, et al. 2003. The Absence of Fucose but Not the Presence of Galactose or Bisecting N-

- Acetylglucosamine of Human IgG1 Complex-type Oligosaccharides Shows the Critical Role of Enhancing Antibody-dependent Cellular Cytotoxicity. *Journal of Biological Chemistry* 278:3466-73
57. Niwa R, Natsume A, Uehara A, Wakitani M, Iida S, et al. 2005. IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides. *J Immunol Methods* 306:151-60
 58. Matsumiya S, Yamaguchi Y, Saito J, Nagano M, Sasakawa H, et al. 2007. Structural comparison of fucosylated and nonfucosylated Fc fragments of human immunoglobulin G1. *J Mol Biol* 368:767-79
 59. Ferrara C, Grau S, Jäger C, Sondermann P, Brünker P, et al. 2011. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcγRIII and antibodies lacking core fucose. *Proceedings of the National Academy of Sciences* 108:12669-74
 60. Wuhler M, Porcelijn L, Kapur R, Koeleman CAM, Deelder AM, et al. 2009. Regulated glycosylation patterns of IgG during alloimmune responses against human platelet antigens. *Journal of Proteome Research* 8:450-6
 61. Jefferis R. 2009. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends in Pharmacological Sciences* 30:356-62
 62. Birch JR, Racher AJ. 2006. Antibody production. *Advanced drug delivery reviews* 58:671-85
 63. Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, et al. 2008. Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose. *The New England journal of medicine* 358:1109-17
 64. Forthal DN, Gach JS, Landucci G, Jez J, Strasser R, et al. 2010. Fc-Glycosylation Influences Fcγ Receptor Binding and Cell-Mediated Anti-HIV Activity of Monoclonal Antibody 2G12. *The Journal of Immunology* 185:6876-82
 65. Kanda Y, Yamada T, Mori K, Okazaki A, Inoue M, et al. 2007. Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. *Glycobiology* 17:104-18
 66. Kumpel BM, Rademacher TW, Rook GAW, Williams PJ, Wilson IBH. 1994. Galactosylation of human IgG monoclonal anti-D produced by EBV-transformed B-lymphoblastoid cell lines is dependent on culture method and affects Fc receptor-mediated functional activity. *Human Antibodies and Hybridomas* 5:143-51
 67. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotech* 17:176-80
 68. Ferrara C, Brünker P, Suter T, Moser S, Püntener U, Umaña P. 2006. Modulation of therapeutic antibody effector functions by glycosylation engineering: Influence of Golgi enzyme localization domain and co-expression of heterologous β 1, 4-N-acetylglucosaminyltransferase III and Golgi α -mannosidase II. *Biotechnology and Bioengineering* 93:851-61
 69. Mössner E, Brünker P, Moser S, Püntener U, Schmidt C, et al. 2010. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood* 115:4393-402
 70. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, Kusunoki M, Iida S, et al. 2004. Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal

- host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol Bioeng* 87:614-22
71. Chan AC, Carter PJ. 2010. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* 10:301-16
 72. Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, et al. 2006. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nat Biotech* 24:210-5
 73. Kanda Y, Yamane-Ohnuki N, Sakai N, Yamano K, Nakano R, et al. 2006. Comparison of cell lines for stable production of fucose-negative antibodies with enhanced ADCC. *Biotechnol Bioeng* 94:680-8
 74. Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495-7
 75. Reichert JM. 2010. Antibodies to watch in 2010. *mAbs* 2:84-100
 76. Carter PJ. 2006. Potent antibody therapeutics by design. *Nat Rev Immunol* 6:343-57
 77. Strohl WR. 2009. Optimization of Fc-mediated effector functions of monoclonal antibodies. *Current Opinion in Biotechnology* 20:685-91
 78. Lazar G, Dang W, Karki S, Vafa O, Peng J, et al. 2006. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci USA* 103:4005 - 10
 79. Sondermann P, Huber R, Oosthuizen V, Jacob U. 2000. The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. *Nature* 406:267-73
 80. Reddy MP, Kinney CA, Chaikin MA, Payne A, Fishman-Lobell J, et al. 2000. Elimination of Fc receptor-dependent effector functions of a modified IgG4 monoclonal antibody to human CD4. *J Immunol* 164:1925-33
 81. Cole MS, Stellrecht KE, Shi JD, Homola M, Hsu DH, et al. 1999. HuM291, a humanized anti-CD3 antibody, is immunosuppressive to T cells while exhibiting reduced mitogenicity in vitro. *Transplantation* 68:563-71
 82. Chu SY, Vostiar I, Karki S, Moore GL, Lazar GA, et al. 2008. Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and Fc gammaRIIb with Fc-engineered antibodies. *Molecular immunology* 45:3926-33
 83. Horton HM, Chu SY, Ortiz EC, Pong E, Cemerski S, et al. 2011. Antibody-Mediated Coengagement of Fc gammaRIIb and B Cell Receptor Complex Suppresses Humoral Immunity in Systemic Lupus Erythematosus. *The Journal of Immunology* 186:4223-33
 84. Dall'Acqua WF, Kiener PA, Wu H. 2006. Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). *J Biol Chem* 281:23514-24
 85. Allan B, Watkins J D. 2006.
 86. Stavenhagen JB, Gorlatov S, Tuaillon N, Rankin CT, Li H, et al. 2007. Fc optimization of therapeutic antibodies enhances their ability to kill tumor cells in vitro and controls tumor expansion in vivo via low-affinity activating Fc gamma receptors. *Cancer Res* 67:8882-90
 87. Richards JO, Karki S, Lazar GA, Chen H, Dang W, Desjarlais JR. 2008. Optimization of antibody binding to Fc gammaRIIa enhances macrophage phagocytosis of tumor cells. *Molecular cancer therapeutics* 7:2517-27
 88. Bolt S, Routledge E, Lloyd I, Chatenoud L, Pope H, et al. 1993. The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties. *Eur J Immunol* 23:403-11

89. Sazinsky SL, Ott RG, Silver NW, Tidor B, Ravetch JV, Wittrup KD. 2008. Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. *Proc Natl Acad Sci U S A* 105:20167-72
90. Hutchins JT, Kull FC, Jr., Bynum J, Knick VC, Thurmond LM, Ray P. 1995. Improved biodistribution, tumor targeting, and reduced immunogenicity in mice with a gamma 4 variant of Campath-1H. *Proc Natl Acad Sci U S A* 92:11980-4
91. Xu D, Alegre ML, Varga SS, Rothermel AL, Collins AM, et al. 2000. In vitro characterization of five humanized OKT3 effector function variant antibodies. *Cellular immunology* 200:16-26
92. Davis PM, Abraham R, Xu L, Nadler SG, Suchard SJ. 2007. Abatacept binds to the Fc receptor CD64 but does not mediate complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity. *The Journal of rheumatology* 34:2204-10
93. Yeung YA, Leabman MK, Marvin JS, Qiu J, Adams CW, et al. 2009. Engineering human IgG1 affinity to human neonatal Fc receptor: impact of affinity improvement on pharmacokinetics in primates. *J Immunol* 182:7663-71
94. Ward ES. 2006.
95. Petkova SB, Akilesh S, Sproule TJ, Christianson GJ, Al Khabbaz H, et al. 2006. Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease. *International immunology* 18:1759-69
96. Walker MR, Lund J, Thompson KM, Jefferis R. 1989. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors. *The Biochemical journal* 259:347-53
97. Collin M, Olsen A. 2001. EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *The EMBO journal* 20:3046-55
98. Collin M, Olsen A. 2001. Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect Immun* 69:7187-9
99. Allhorn M, Olin AI, Nimmerjahn F, Colin M. 2008. Human IgC/FcγR interactions are modulated by streptococcal IgG glycan hydrolysis. *PloS one* 3
100. Albert H, Collin M, Dudziak D, Ravetch JV, Nimmerjahn F. 2008. In vivo enzymatic modulation of IgG glycosylation inhibits autoimmune disease in an IgG subclass-dependent manner. *Proc Natl Acad Sci U S A* 105:15005-9
101. Collin M, Shannon O, Bjorck L. 2008. IgG glycan hydrolysis by a bacterial enzyme as a therapy against autoimmune conditions. *Proc Natl Acad Sci U S A* 105:4265-70
102. Edelman GM, Cunningham BA, Gall WE, Gottlieb PD, Rutishauser U, Waxdal MJ. 1969. The covalent structure of an entire gammaG immunoglobulin molecule. *Proceedings of the National Academy of Sciences of the United States of America* 63:78-85
103. Aricescu AR, Lu W, Jones EY. 2006. A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta crystallographica. Section D, Biological crystallography* 62:1243-50
104. Battye TGG, Kontogiannis L, Johnson O, Powell HR, Leslie AGW. 2011. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallographica Section D* 67:271-81
105. Collaborative Computational Project N, . 1994. The CCP4 Suite: Programs for Protein Crystallography. *Acta Cryst* D50:760-3

106. Rupp B. 2009. *Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology*. New York: Garland Science. 850 pp.
107. Murshudov GN, Vagin AA, Dodson EJ. 1997. Refinement of macromolecular structures by the maximum-likelihood method. *Acta crystallographica. Section D, Biological crystallography* 53:240-55
108. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography* 60:2126-32
109. Winn MD, Murshudov GN, Papiz MZ. 2003. Macromolecular TLS refinement in REFMAC at moderate resolutions. *Methods in enzymology* 374:300-21
110. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, et al. 2007. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic acids research* 35:W375-83
111. Lutteke T. 2009. Analysis and validation of carbohydrate three-dimensional structures. *Acta crystallographica. Section D, Biological crystallography* 65:156-68
112. Nandakumar KS, Andrén M, Martinsson P, Bajtner E, Hellström S, et al. 2003. Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcγRIIB. *European Journal of Immunology* 33:2269-77
113. Küster B, Wheeler SF, Hunter AP, Dwek RA, Harvey DJ. 1997. Sequencing of N-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal. Biochem.* 250:82-101
114. Moore GL, Chen H, Karki S, Lazar GA. 2010. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *mAbs* 2:181-9
115. Börnsen KO, Mohr MD, Widmer HM. 1995. Ion exchange and purification of carbohydrates on a Nafion(R) membrane as a new sample pretreatment for matrix-assisted laser desorption-ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 9:1031-4
116. Harvey DJ. 2005. Fragmentation of negative ions from carbohydrates: part 3. Fragmentation of hybrid and complex N-linked glycans. *Journal of the American Society for Mass Spectrometry* 16:647-59
117. Harvey DJ. 2005. Fragmentation of negative ions from carbohydrates: part 2. Fragmentation of high-mannose N-linked glycans. *Journal of the American Society for Mass Spectrometry* 16:631-46
118. Harvey DJ. 2005. Fragmentation of negative ions from carbohydrates: part 1. Use of nitrate and other anionic adducts for the production of negative ion electrospray spectra from N-linked carbohydrates. *Journal of the American Society for Mass Spectrometry* 16:622-30
119. Berger EG, Buddecke E, Kamerling JP, Kobata A, Paulson JC, Vliegenthart JF. 1982. Structure, biosynthesis and functions of glycoprotein glycans. *Experientia* 38:1129-62
120. Harvey DJ, Merry AH, Royle L, Campbell MP, Rudd PM. 2011. Symbol nomenclature for representing glycan structures: Extension to cover different carbohydrate types. *Proteomics* 11:4291-5
121. Merwe PAvd. 2001. Surface Plasmon Resonance. In *Protein-Ligand interactions: hydrodynamics and calorimetry*, ed. SHaPZ Chowdhry:137-70: Oxford University Press. Number of 137-70 pp.

122. Niesen FH, Berglund H, Vedadi M. 2007. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat. Protocols* 2:2212-21
123. Wallace AC, Laskowski RA, Thornton JM. 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein engineering* 8:127-34
124. Jung ST, Kang TH, Kelton W, Georgiou G. 2011. Bypassing glycosylation: engineering aglycosylated full-length IgG antibodies for human therapy. *Current Opinion in Biotechnology* 22:858-67
125. Radaev S, Sun PD. 2001. Recognition of IgG by Fcγ receptor. The role of Fc glycosylation and the binding of peptide inhibitors. *J Biol Chem* 276:16478-83
126. Brezski RJ, Jordan RE. 2010. Cleavage of IgGs by proteases associated with invasive diseases: an evasion tactic against host immunity? *mAbs* 2:212-20
127. Hindley SA, Gao Y, Nash PH, Sautes C, Lund J, et al. 1993. The interaction of IgG with Fc gamma RII: involvement of the lower hinge binding site as probed by NMR. *Biochemical Society transactions* 21:337S
128. Wormald MR, Rudd PM, Harvey DJ, Chang SC, Scragg IG, Dwek RA. 1997. Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* 36:1370-80
129. Barb AW, Prestegard JH. 2011. NMR analysis demonstrates immunoglobulin G N-glycans are accessible and dynamic. *Nat Chem Biol* 7:147-53
130. Ghirlando R, Lund J, Goodall M, Jefferis R. 1999. Glycosylation of human IgG-Fc: influences on structure revealed by differential scanning micro-calorimetry. *Immunol Lett* 68:47-52
131. Lund J, Takahashi N, Pound JD, Goodall M, Jefferis R. 1996. Multiple Interactions of IgG with Its Core Oligosaccharide Can Modulate Recognition by Complement and Human Fcγ Receptor I and Influence the Synthesis of Its Oligosaccharide Chains. *Journal of Immunology* 157:4963-9
132. Voynov V, Chennamsetty N, Kayser V, Helk B, Forrer K, et al. 2009. Dynamic fluctuations of protein-carbohydrate interactions promote protein aggregation. *PloS one* 4:e8425
133. 1994. The CCP4 suite: programs for protein crystallography. *Acta crystallographica. Section D, Biological crystallography* 50:760-3
134. McCoy AJ, Gross-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007. Phaser crystallographic software. *J. Appl. Cryst.* 40:658-74
135. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60:2126-32
136. 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 50:760-3
137. Winn MD, Murshudov GN, Papiz MZ. 2003. Macromolecular TLS refinement in REFMAC at moderate resolutions. *Methods Enzymol.* 374:300-21
138. Deisenhofer J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* 20:2361-70
139. Lund J, Takahashi N, Pound JD, Goodall M, Jefferis R. 1996. Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fc gamma receptor I and influence the synthesis of its oligosaccharide chains. *J Immunol* 157:4963-9

140. Mimura Y, Ashton PR, Takahashi N, Harvey DJ, Jefferis R. 2007. Contrasting glycosylation profiles between Fab and Fc of a human IgG protein studied by electrospray ionization mass spectrometry. *J Immunol Methods* 326:116-26
141. Takahashi N, Ishii I, Ishihara H, Mori M, Tejima S, et al. 1987. Comparative structural study of the N-linked oligosaccharides of human normal and pathological immunoglobulin G. *Biochemistry* 26:1137-44
142. Marth JD. 1999. N-Glycans. In *Essentials of Glycobiology.*, ed. CR Varki A, Esko J, et al.: Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Number of.
143. Stanley P, Schachter H, Taniguchi N. 2009. N-Glycans. In *Essentials of Glycobiology*, ed. A Varki, RD Cummings, JD Esko, HH Freeze, P Stanley, et al. Cold Spring Harbor (NY). Number of.
144. Zhou Q, Shankara S, Roy A, Qiu H, Estes S, et al. 2008. Development of a simple and rapid method for producing non-fucosylated oligomannose containing antibodies with increased effector function. *Biotechnol Bioeng* 99:652-65
145. Elbein AD, Tropea JE, Mitchell M, Kaushal GP. 1990. Kifunensine, a potent inhibitor of the glycoprotein processing mannosidase I. *Journal of Biological Chemistry* 265:15599-605
146. Crispin M, Aricescu AR, Chang VT, Jones EY, Stuart DI, et al. 2007. Disruption of alpha-mannosidase processing induces non-canonical hybrid-type glycosylation. *FEBS Lett.* 581:1963-8
147. Crispin M, Harvey DJ, Chang VT, Yu C, Aricescu AR, et al. 2006. Inhibition of hybrid and complex-type glycosylation reveals the presence of the GlcNAc transferase I-independent fucosylation pathway. *Glycobiology* 16:748-56
148. Reeves PJ, Callewaert N, Contreras R, Khorana HG. 2002. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc. Natl Acad. Sci. USA* 99:13419-24
149. Domon B, Costello CE. 1988. Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass spectrometry. *Biochemistry* 27:1534-43
150. Mimura Y, Church S, Ghirlando R, Ashton PR, Dong S, et al. 2000. The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Molecular immunology* 37:697-706
151. Wheeler SF, Harvey DJ. 2000. Negative ion mass spectrometry of sialylated carbohydrates: discrimination of N-acetylneuraminic acid linkages by MALDI-TOF and ESI-TOF mass spectrometry. *Anal Chem* 72:5027-39
152. Crispin M, Harvey DJ, Chang VT, Yu C, Aricescu AR, et al. 2006. Inhibition of hybrid- and complex-type glycosylation reveals the presence of the GlcNAc transferase I-independent fucosylation pathway. *Glycobiology* 16:748-56
153. Sondermann P, Huber R, Oosthulzen V, Jacob U. 2000. The 3.2-Å... crystal structure of the human IgG1 Fc fragment-FcγRIII complex. *Nature* 406:267-73
154. Bergman LW, Kuehl WM. 1978. Temporal relationship of translation and glycosylation of immunoglobulin heavy and light chains. *Biochemistry* 17:5174-80
155. Feige MJ, Hendershot LM, Buchner J. 2010. How antibodies fold. *Trends in Biochemical Sciences* 35:189-98

156. Yamaguchi Y, Nishimura M, Nagano M, Yagi H, Sasakawa H, et al. 2006. Glycoform-dependent conformational alteration of the Fc region of human immunoglobulin G1 as revealed by NMR spectroscopy. *Biochimica et biophysica acta* 1760:693-700
157. Allhorn M, Briceno JG, Baudino L, Lood C, Olsson ML, et al. 2010. The IgG-specific endoglycosidase EndoS inhibits both cellular and complement-mediated autoimmune hemolysis. *Blood* 115:5080-8
158. Allhorn M, Collin M. 2009. Sugar-free antibodies - The bacterial solution to autoimmunity? , ed. Y Shoenfeld, M Gershwin, pp. 664-9
159. Feige MJ, Nath S, Catharino SR, Weinfurter D, Steinbacher S, Buchner J. 2009. Structure of the Murine Unglycosylated IgG1 Fc Fragment. *Journal of Molecular Biology* 391:599-608
160. Robbins PW, Trimble RB, Wirth DF, Hering C, Maley F, et al. 1984. Primary structure of the Streptomyces enzyme endo-beta-N-acetylglucosaminidase H. *J Biol Chem* 259:7577-83
161. Yamamoto K. 1994. Microbial Endoglycosidases for Analyses of Oligosaccharide Chains in Glycoproteins. *Journal of Biochemistry* 116:229-35
162. Garbe J, Collin M. 2012. Bacterial Hydrolysis of Host Glycoproteins - Powerful Protein Modification and Efficient Nutrient Acquisition. *Journal of innate immunity*
163. Preithner S, Elm S, Lippold S, Locher M, Wolf A, et al. 2006. High concentrations of therapeutic IgG1 antibodies are needed to compensate for inhibition of antibody-dependent cellular cytotoxicity by excess endogenous immunoglobulin G. *Molecular immunology* 43:1183-93
164. Baselga J, Albanell J. 2001. Mechanism of action of anti-HER2 monoclonal antibodies. *Ann Oncol* 12 Suppl 1:S35-41
165. Oganessian V, Damschroder MM, Leach W, Wu H, Dall'Acqua WF. 2008. Structural characterization of a mutated, ADCC-enhanced human Fc fragment. *Molecular immunology* 45:1872-82
166. Dunker AK, Obradovic Z. 2001. The protein trinity--linking function and disorder. *Nat Biotechnol* 19:805-6
167. Iida S, Kuni-Kamochi R, Mori K, Misaka H, Inoue M, et al. 2009. Two mechanisms of the enhanced antibody-dependent cellular cytotoxicity (ADCC) efficacy of non-fucosylated therapeutic antibodies in human blood. *BMC Cancer* 9
168. Iida S, Misaka H, Inoue M, Shibata M, Nakano R, et al. 2006. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcγRIIIa. *Clin Cancer Res* 12:2879-87
169. Nechansky A, Schuster M, Jost W, Siegl P, Wiederkum S, et al. 2007. Compensation of endogenous IgG mediated inhibition of antibody-dependent cellular cytotoxicity by glyco-engineering of therapeutic antibodies. *Molecular immunology* 44:1826-8
170. Collin M, Shannon O, Björck L. 2008. IgG glycan hydrolysis by a bacterial enzyme as a therapy against autoimmune conditions. *Proceedings of the National Academy of Sciences of the United States of America* 105:4265-70

