Expression and functional characterisation of the collagen receptor glycoprotein VI

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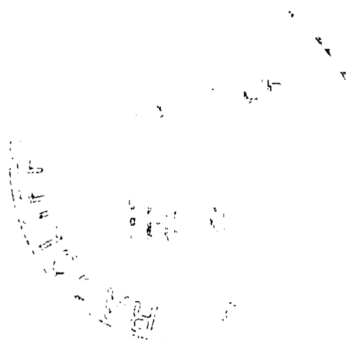

All of the work reported in this thesis was conducted by the author in the Department of Pharmacology. Except where acknowledgement is made, the work is his own and has not been submitted for any other degree in this or any other university or institute of learning.

A mi padre;

Por su amor, comprensión y sabiduría.

Porque siempre me dejó elegir.

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Expression and functional characterisation of the collagen receptor glycoprotein VI.

Abstract

This thesis is concerned with the study of the collagen receptor glycoprotein VI (GPVI). GPVI has been studied in a number of human haematopoietic cell lines and found to be expressed only in those showing mekaryocytic features. Moreover, differentiation of the human cell lines HEL and CMK to a megakaryocytic lineage using the phorbol ester PMA revealed upregulation of GPVI together with the associated FcR γ-chain. Further, primary cultures of mouse marrow cells demonstrated up-regulation of GPVI towards the end of differentiation, therefore confirming data obtained with cell lines and pointing to GPVI as a possible marker of megakaryocytic differentiation.

Structure/function studies of GPVI were carried out by means of transient and stable transfection of the receptor into COS-7 or K562 cells. These studies demonstrated the necessity of the transmembrane argine and the cytoplasmic tail of GPVI for association to the FcR γ-chain. Lack of association or absence of FcR γ-chain rendered GPVI unable to signal, despite binding to convulxin, a GPVI-specific ligand which causes activation of the receptor.

K562 cells expressing GPVI and the FcR γ-chain were able to reconstitute the proximal but not distal events in GPVI signaling. A detailed analysis demonstrated impairment in phosphorylation and translocation of SLP-76 to the membrane, despite the

presence and activation of others proteins known to be necessary for this phosphorylation/translocation to occur.

Stable expression of GPVI in K562 cells, which display low levels of expression of the collagen receptors $\alpha2\beta1$ and CD36, does not confer signaling properties in response to collagen. However, the cells respond to a collagen related peptide (CRP) which is specific for GPVI and to the snake venoms convulxin, alborhagin and alboaggregin-A, demonstrating that GPVI is one important component through which these venoms are acting.

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Abbreviations

Btk	Bruton's tyrosine kinase
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
CD	Cluster designation
CRP	Collagen-related peptide
DAG	Diacyl glycerol
DAPI	4,6-diamidino-2-phenylindole
DNA	deoxiribonucleic acid
ECL	Enhanced chemiluminescence
FACS	Fluorescence-activated cell sorting
FcαR	Receptor for Fc portion of IgAs
FcγR	Receptor for Fc portion of IgGs
FcεR	Receptor for Fc portion of IgEs
FcR	Fc receptor
FcR γ-chain	Fc receptor gamma-chain
FITC	Fluorescein isothiocyanate
FURA 2-AM	Pentapotassium salt
Gads	Grb2-related adapter protein downstream of Shc
GEM	Glycolipid-enriched microdomain
GFP	Green fluorescent protein
GP	Glycoprotein
GPVI	Glycoprotein VI
Grb2	Growth factor receptor binding protein 2
HSA	Human serum albumin
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kDa	Kilodalton
LAT	Linker for activation of T cells
NTP	Nucleotide tri-phosphate
dNTP	Deoxinucleotide tri-phosphate
PAS	Protein-A sepharose
PBS	Phosphate-buffered salinum
PCR	Polymerase chain reaction
PIR	Paired-immunoglobulin receptor
PLCγ	Phospholipase C-gamma
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid

RT-PCR	Retrotranscriptase-polimerase chain reaction
SDS	Sodium dodecyl-sulphate
SDS-PAGE	SDS-poly acrylamide gel electrophoresis
SH	Src homology
SLP-65	SH2 domain-containing leukocyte protein of 65 kDa
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
TBS-T	Tris-buffered salinum-Tween 20
Temed	N,N,N,N'-tetra-methyl-ethylendiamine
TM	Transmembrane

Chapter 1

Introduction

1.1 General introduction

Platelet formation is the function of the platelet precursor, the megakaryocyte. The megakaryocyte, meaning large nucleus, was named by Howell in 1890 and recognised by Wright in 1906 as the cell responsible for platelet manufacture (reviewed by Caen and Han, 1995). Megakaryocytes are large (25-50 μm), polyploid, lobular-nucleated cells that occur at their highest density in the bone marrow. Megakaryocytic development into platelets from haematopietic pluripotent stem cells involves the influence of cytokines and stromal cells (Caen and Han, 1995). Megakaryocytes produce the majority of proteins present in platelets meaning that drugs that influence megakaryocyte development can alter the produced platelets. The similarity in protein expression between the two cells allows utilisation of the megakaryocyte as a model of certain functional events that occur in the platelet.

Platelets are small (1-3 μm), abundant (2.5×10^8/ml whole blood), anuclear cells in the blood. The principal function of platelets is in the maintenance and repair of the vasculature, though they also participate in the immune response and particle clearing in the blood (Peerschke and Ghebrehiwet, 1998; Donald and Tennent, 1975). Exposure of platelets to subendothelial collagen at the site of tissue injury triggers activation, leading to secretion of platelet products which act to recruit additional platelets and stimulate vascular repair (reviewed in Watson et al., 2000). As activation of platelets constitutes the very earliest stage of a process which has potential for rapid amplification, this response must be finely regulated. A lack of platelets or failure to activate normally results in excessive bleeding. Conversely, over-activity of platelets is implicated in diseases such as myocardial infarction, artherosclerosis and stroke (reviewed in Flores and Sheridan, 1994; Caen and Han, 1997; Van der Loo and Martin, 1997; Van der Loo and Martin 1999). Platelet removal from the circulation is normally via the reticuloendothelial system.

1.1.1 Megakaryocyte development (megakaryocytopoiesis)

Megakaryocytes are primarily located in the bone marrow where they represent less than 0.1% of the cells. They arise from haematopietic cell precursors that develop into progenitor cells, BFU-MK (burst-forming units of megakaryocytes) and CFU-MK (colony-forming units of megakaryocytes), with proliferative capacity and a committed megakaryocytic lineage. The megakaryocyte then moves through four stages of morphologically discernible development with increasing size and cytoplasmic complexity (Levine, 1980). These stages of megakaryocyte development are termed (I) megakaryoblast, (II) promegakaryocyte, (III) granular megakaryocyte, and (IV) mature megakaryocyte (Fig. 1.1). Surface proteins act as markers of both megakaryocyte lineage and developmental stage. The platelet receptors GPIIb-IIIa (fibrinogen receptor, CD41/61) and GPIb-IX-V (von Willebrand factor receptor) are specific to the megakaryocyte lineage, whereas CD34 and CD4 are present on progenitors but progressively disappear during maturation. A charasteristic of the megakaryocyte is their polyploidy and ability to undergo endomitosis. In human, this can reach 64 n although for the majority of cells the ploidy is 16 n or 32 n (Paulus, 1970).

Mature megakaryocytes with their multilobular nuclei constitute the largest of the haematopietic cells. Cytoplasmic organelles with the aspect of platelet α-granules appear early during maturation, although their size and opacity are more heterogeneous than in platelets (Zucker-Franklin, 1970). A charasteristic of the megakaryocyte is the development of an internal membrane system, the demarcation membrane system (DMS) (Zucker-Franklin, 1970; Breton-Gorius et al., 1986). In young cells, the DMS is often concentrated in specific areas close to the plasma membrane. At the end of maturation, the DMS delimits territories resembling platelets, thus giving rise to the speculation that they represent pre-formed platelets inside the megakaryocyte.

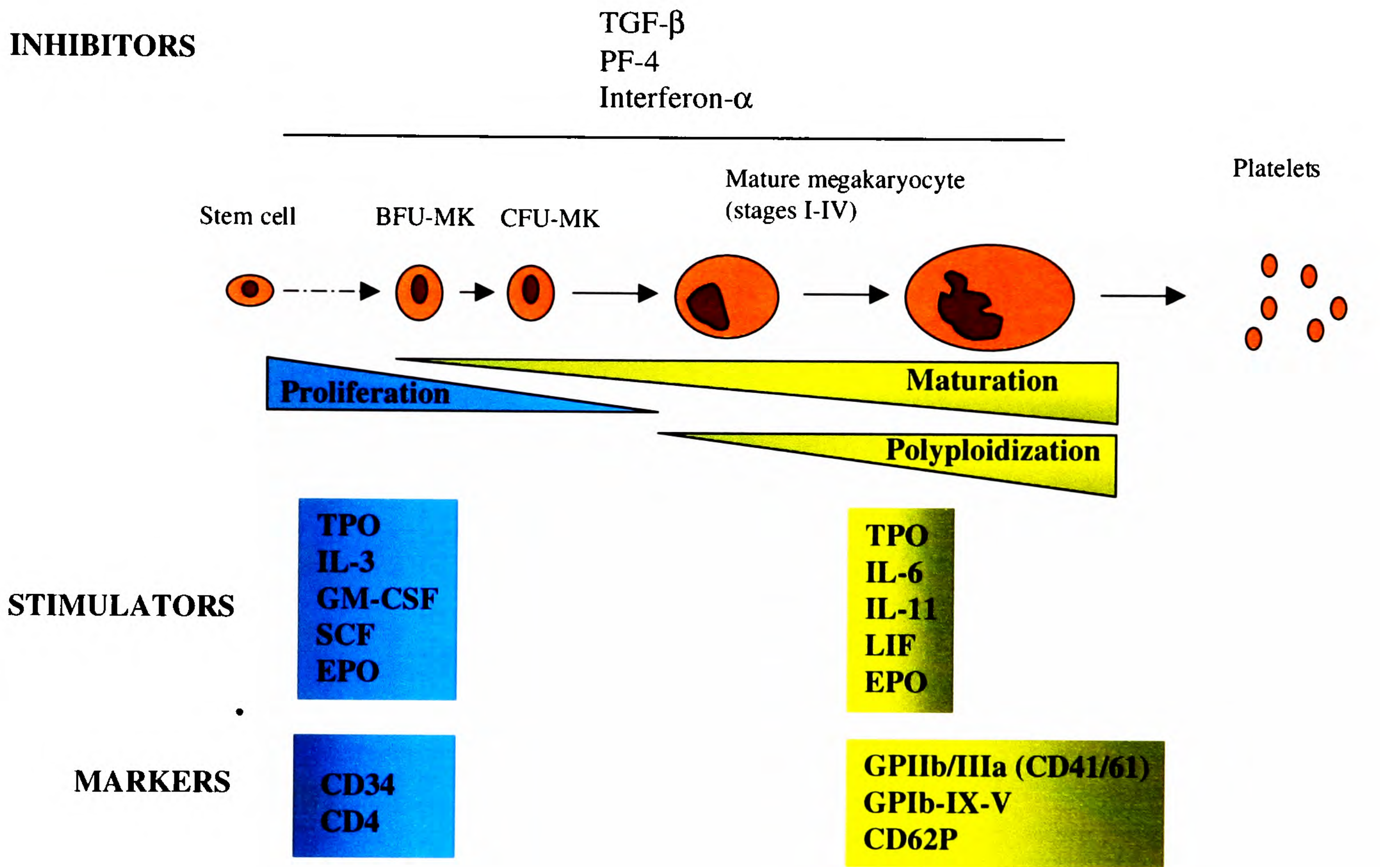


Figure 1.1. Schematic illustration of the cellular hierarchy and the regulation of megakaryocyte development.
BFU-MK, burst-forming unit-MK; CFU-MK, colony-forming unit-MK; EPO, erythropoietin; GM-CSF, granulocyte/ Macrophage colony-stimulating factor; IL, interleukin; LIF, leukaemia inhibitory factor; PF-4, platelet factor 4; SCF, stem cell factor, TGF-β, transforming growth factor-β, and TPO, thrombopoietin. Some markers defining different stages of differentiation are shown Below.

1.1.2 Regulation of megakaryocytopoiesis

The survival, proliferation, differentiation and function of normal haematopoietic cells are positively and negatively regulated by various cytokines (Fig. 1.1). Among the different haematopoietic lineages, megakaryocytopoiesis and platelet production represent complex processes whose regulation remains incompletely understood. The identification and cloning of thrombopoietin (TPO), the major humoral regulator of megakaryocyte development, represents a major advance in this area (Bartley et al., 1994; Lok et al., 1994; Kaushansky et al., 1995). Studies performed at a unicellular level and in serum-deprived medium demostrated that TPO directly promotes proliferation, growth and development of megakaryocytes from CD34+ human progenitor cells, polyploidization and terminal differentiation with proplatelet formation (Debili et al., 1995a; Debili et al., 1995b). When administrated to normal animals, the recombinant TPO molecule proved to be the most potent platelet-increasing factor of any cytokine yet described (Kaushansky, 1995). Nevertheless, TPO is not the sole regulator of platelet production and numerous pleiotropic cytokines act *in vivo* and *in vitro* on megakaryocytopoiesis. For instance, stem cell factor (SCF), the c-kit ligand, alone or in combination with the hematopoietic growth factors, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6, stimulates proliferation of megakaryocytic cell lines (Avraham et al., 1992; Debili et al., 1993). Moreover, SCF seems to have a role on inhibiting premature senescence by reducing TPO-induced apoptosis in megakaryocytes (Kie et al., 2002).

Another cytokine, IL-6, promotes megakaryocyte maturation by increasing the number, cell size, polyploidization and synthesis of platelet proteins and proplatelet formation (Ishibashi et al., 1989). In pre-clinical studies, administration of IL-6 accelerates platelet recovery in both normal and thrombocytopenic animals to a higher extent than IL-3. Because of its stimulatory action on platelet production, it was hypothesized that IL-6 was a cytokine

involved in platelet haemostasis. However, this conclusion is not supported by the generation of IL-6 knock-out mice which do not develop a thrombocytopenic state (Kishimoto et al., 1994). Other thrombocytopoietic growth factors include IL-11 and leukaemia inhibitory factor (LIF), which have overlapping biological properties with IL-6, and erythropoietin (EPO), which is able to promote megakaryocyte maturation.

It has become increasingly clear that normal megakaryocytopoiesis is the result of the balance between positive and negative regulators of proliferation and differentiation. These negative regulators include transforming growth factor β (TGFβ), platelet factor 4 (PF4), as well as interferon-α. Interestingly, most megakaryocytopoietic inhibitors are produced by megakaryocytes and platelets themselves, a method of regulation called negative feedback (Mitjavila et al., 1988; Caen and Han, 1995).

1.1.3 Formation of platelets from megakaryocytes

The final stage of megakaryocytopiesis, namely the fragmentation of mature megakaryocytes into proplatelets and ultimately platelets, is an area of controversy both in terms of the mechanism and location of this event. The use of electron microscopy and time-lapse cinematography gave rise to the so-called flow model (Radley and Haller, 1982) in which the production of platelets involves the extrusion across the vascular endothelium of megakaryocyte processes that subsequently break up in the vessel lumen. These processes, termed proplatelets, have been observed in *in vitro* megakaryocyte cultures. Studies of megakaryocytes *in vivo* showed similar beaded formations projecting into sinusoids with the presence of constrictions between individual segments. Rupture at these constrictions was proposed to be facilitated by shear stress (Radley and Haller, 1982). Groups of clustered megakaryocytes in the bone marrow are typically located close to a vascular sinusoid, along

the abluminal face of the sinus endothelium (Lichtman et al., 1978). Once the megakaryocytes are fully mature, they are thought to extend pseudopods through or between endothelial cells, which can enter into circulation. These long, cytoplasmic extensions or proplatelets remain attached to the megakaryocyte cell core and have been observed not only extruding into the lumen of the bone marrow sinusoids but orientating themselves in the direction of the blood flow (Poujol et al., 1998). However, megakaryocyte naked polyploid nuclei are rarely found in normal bone marrow biopsies, and images of proplatelet passage through endothelial cells are only seldom observed.

A second hypotheses, favoured by Martin and coworkers, is that migration of megakaryocytes into the peripheral circulation is followed by fragmentation in the pulmonary circulation (Martin et al., 1983; Slater et al., 1983; Trowbridge, 1988). In support of this, megakaryocytes are especially numerous in the human pulmonary circulation, and the lung has often been suggested as a site of pulmonary platelet production (Tingaard-Pedersen, 1978). The exact contribution of pulmonary megakaryocytes to total platelet production is the subject of debate and controversy, with estimates in the literature varying between 7% to 100% (Tingaard-Pedersen, 1978; Levine et al., 1993; Wilde et al., 1997)

1.2 Platelet regulation

Platelets are one of the major effectors of haemostasis, the others being cellular and acellular components of the vessel wall and clotting factors found in blood plasma. They initiate platelets responses such as shape change, secretion and aggregation.The interaction of all these components is required for efficient and regulated haemostasis.

1.2.1 General mechanism of platelet activation

The adhesive proteins von Willebrand factor and collagen, present in the subendothelium, become exposed upon injury of the vessel wall. Binding of platelets to these proteins results in platelet adhesion and exposure of ligand binding sites on the integrin $\alpha_{IIb}\beta3$ (Ruggeri, 1997). The signals leading to these responses are still largely unknown, although it is becoming clear that activation of isoforms of phospholipase C (PLC), in particular PLCγ2 is likely to play a key role (Wang et al., 2000). Shear stress-induced von Willebrand factor binding to the platelet glycoprotein Ib-V-IX complex results in hydrolisis of phosphoinositides, Ca^{2+} influx, synthesis of thromboxane A_2 (TxA_2), activation of protein kinase C (PKC), tyrosine phosphorylation of many proteins and platelet aggregation (Ikeda et al., 1993; Razdan et al.,1994; Kroll, 1994).

After initial activation of platelets, discoid platelets become spherical due to cytoskeletal reorganization, leading subsequently to formation of pseudopodia and filopodia. Meanwhile, dense alpha and lysosomal granules contents are secreted. Secretion of alpha granules leads to increased cell surface expression of adhesion molecules like P-selectin, and secretion of a spectrum of growth factors. Secreted contents also include fibrinogen fibronectin, clotting factors, von Willebrand factors, ADP, serotonin, TxA2, etc, all promoting secondary platelet activation and aggregation. Signalling components originating from these and other receptors upon platelet activation include PLC isoforms, phospholipase A_2 ($cPLA_2$), PKC, protein tyrosine kinases, etc. A review on platelet activation can be seen on Savage et al., 2001. A schematic representation showing different integrated signals leading to platelet activation are depicted in Figure 1.2

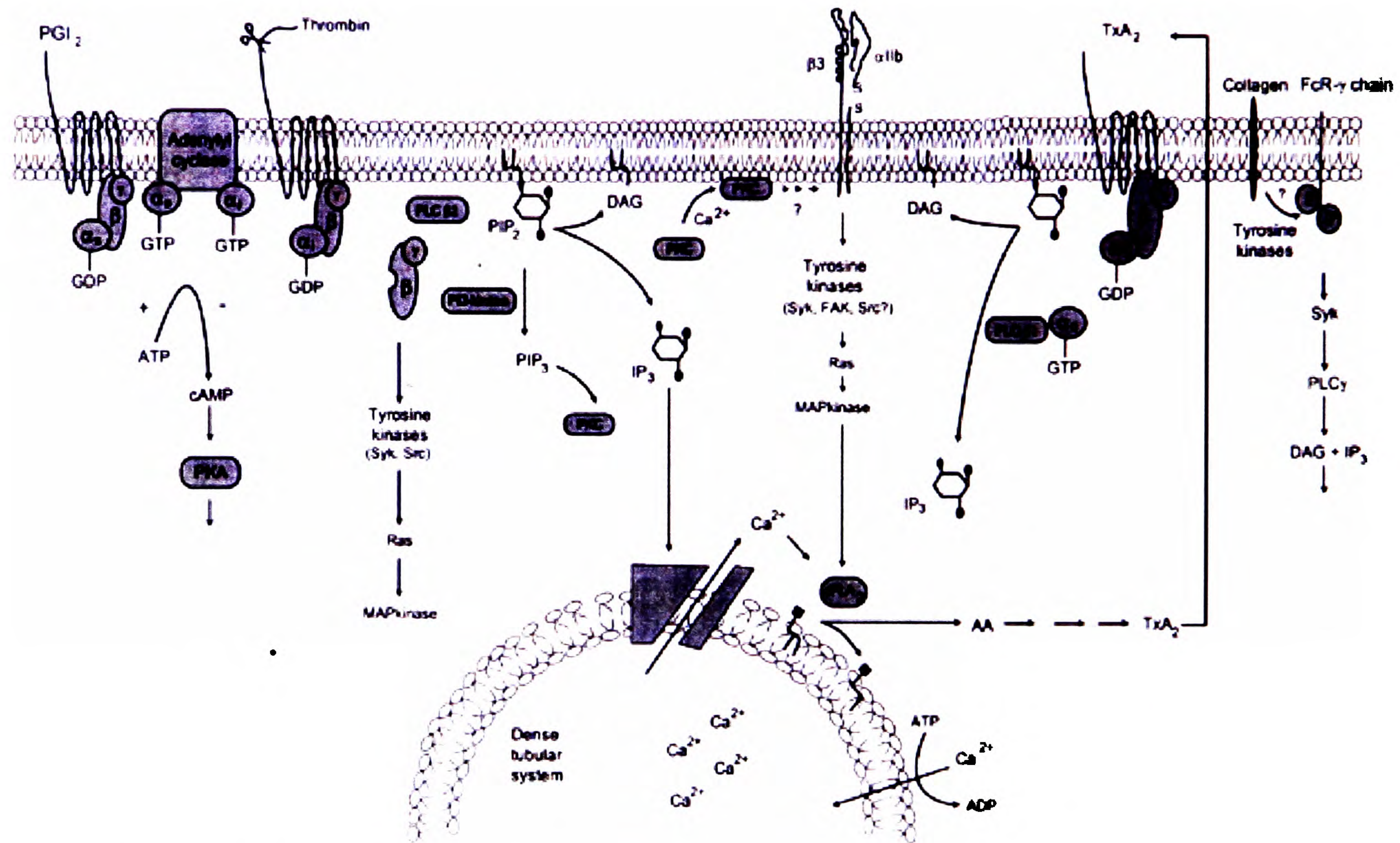


Figure 1.2. Schematic overview of signal transduction pathways in platelets. Abbreviations: AA, arachidonic acid; DAG, diacylglycerol; $cPLA_2$, cytosolic phospholipase A_2; IP_3, inositol-1,4,5-trisphosphate; MAP, mitogen-activated protein; PGI_2, prostaglandin I_2, PI 3-kinases, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; TxA_2, thromboxane A_2. Figure modified from Manning and Brass, 1991.

The soluble agonists that activate platelets include α-thrombin, platelet-activating factor (PAF), TxA_2, ADP, epinephrine, etc. (Kroll, 1994). Most of these agonists transduce signals via seven transmembrane domain receptors that are coupled to heterotrimeric GTP-binding proteins (G-proteins) (Manning and Brass, 1991). α-Thrombin, a serine protease, is formed as a result of activation of the coagulation cascade. A number of thrombin receptors has been described in platelets an megakaryocytes such as protease-activated receptor (PAR)-1 and PAR-3 and GPIb-V-IX (Ishihara et al., 1997). TxA_2 and ADP are involved in positive feedback pathways. ADP originates not only from platelets, but also from damaged red blood cells and endothelial cells. The mechanism by which ADP activates platelets is still unclear, although a rise in intracellular calcium is important. Platelets express at least two ADP receptors, P_{2X} and P_{2Y}; P_{2X} receptor is a ligand gated ion channel and responsible for the rapid Ca^{2+} influx seen with ADP (Mahaut-Smith et al., 1990), whereas P_{2Y} receptor is a G-protein-coupled receptor and is essential for ADP-induced platelet aggregation (Gachet et al., 1997).

Besides binding to G-proteins that activate platelets, the platelet receptors for α-thrombin, ADP and epinephrine are coupled to the inhibitory G-protein Gi. Activation of this G-protein results in inhibition of adenylate cyclase, the major inhibitory effector enzyme in platelets (Lerea et al., 1987; Hawiger et al., 1980).

Activation of platelets at sites of vascular injury occurs in response to exposed subendothelial matrix proteins. One of the most important of these matrix proteins is recognised to be collagen. Exposed collagen initiates two essential platelet functions, i.e. the adhesion of circulating platelets to the site of injury and subsequent activation, which

stimulates thrombus growth. The essential mechanism for platelet activation by collagen is explained below.

1.2.2 Receptors for collagen in platelets

Subendothelial fibrillar collagen is one of the major activators of blood platelets. Platelets are exposed to subendothelial collagen at sites of vascular injury. The activation of platelets by collagen is therefore a primary event in haemostasis. There are several putative receptors for collagen expressed on the platelet surface. This list includes the integrin $\alpha 2\beta 1$, glycoprotein IV (GPIV, CD36, GPIIIb), glycoprotein VI (GPVI), and a poorly characterised cloned cell surface protein p65 (Chiang et al., 1997). Fig. 1.3 shows a schematic representation of these collagen receptors.

Collagen-platelet interaction is a two-step process of adhesion and activation involving the sequential recognition of distinct receptors. $\alpha 2\beta 1$ has been proposed to play an essentially mechanistic role where it mediates the initial binding of platelets to collagen. In the two-site two-step model (Santoro et al., 1991), the binding of collagen fibres to $\alpha 2\beta 1$ provides the initial site of attachment for platelets travelling in vascular blood, causing platelets to appear to "roll" along the vascular wall, and promote further binding via a second receptor, such as GPVI (Fig. 1.4). GPVI is believed to be the collagen receptor responsible for signalling within the cell, promoting phosphorylation of several downstream proteins leading to platelet activation. Recently, however, it has been demonstrated that $\alpha 2\beta 1$ undergoes conformational changes to intermediate and high affinity states subsequent to platelet activation with a number of agonists (Jung and Moroi, 1998). The demonstration that $\alpha 2\beta 1$ exists in multiple conformations has important implications for the two-site two-

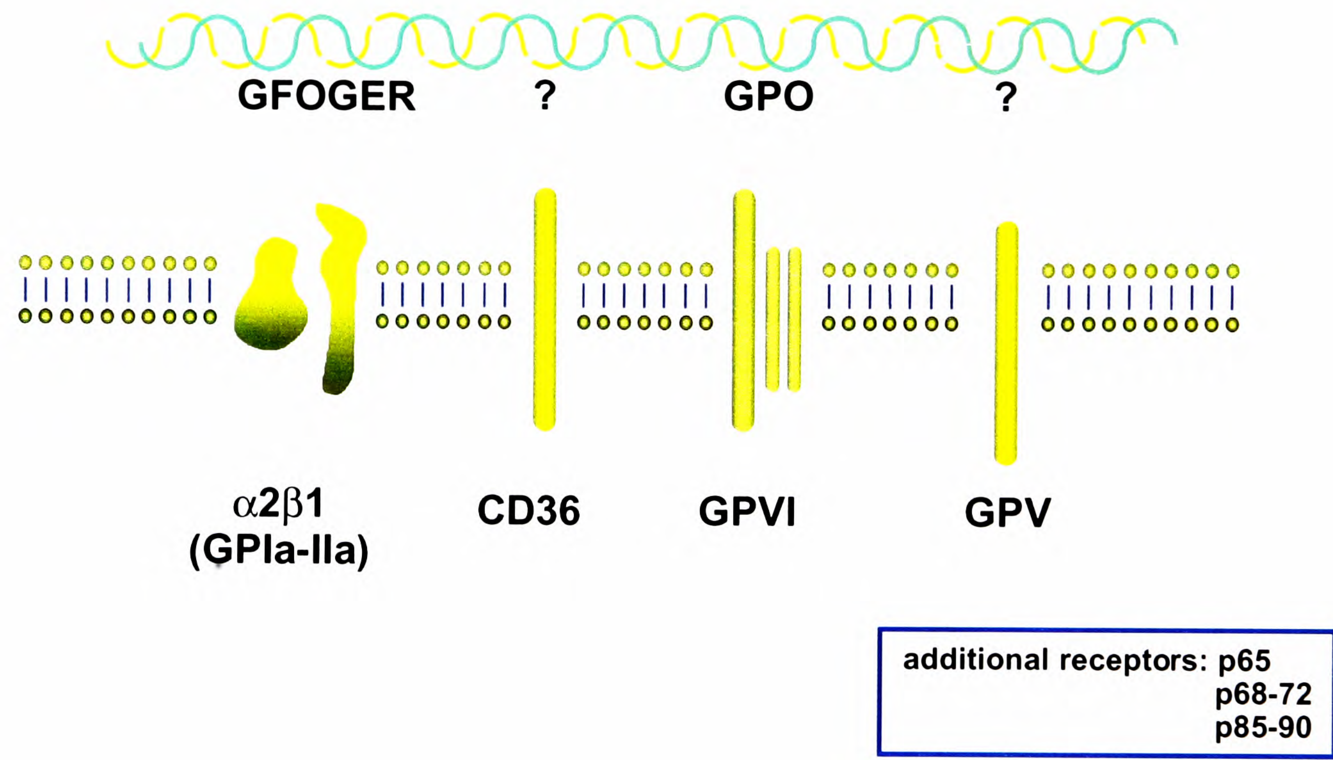


Figure1.3. Schematic representation of different collagen receptors present in platelets. Two major collagen Receptors have been described in platelets, the integrin a2b1 and GPVI. The peptide sequence within collagen responsible for binding to α2β1 and GPVI is shown (single letter code). Other collagen receptors in platelets include GPV (one of the components of the von Willebrand factor receptor GPIb-V-IX), CD36, p65, p68-72 and p85-90.

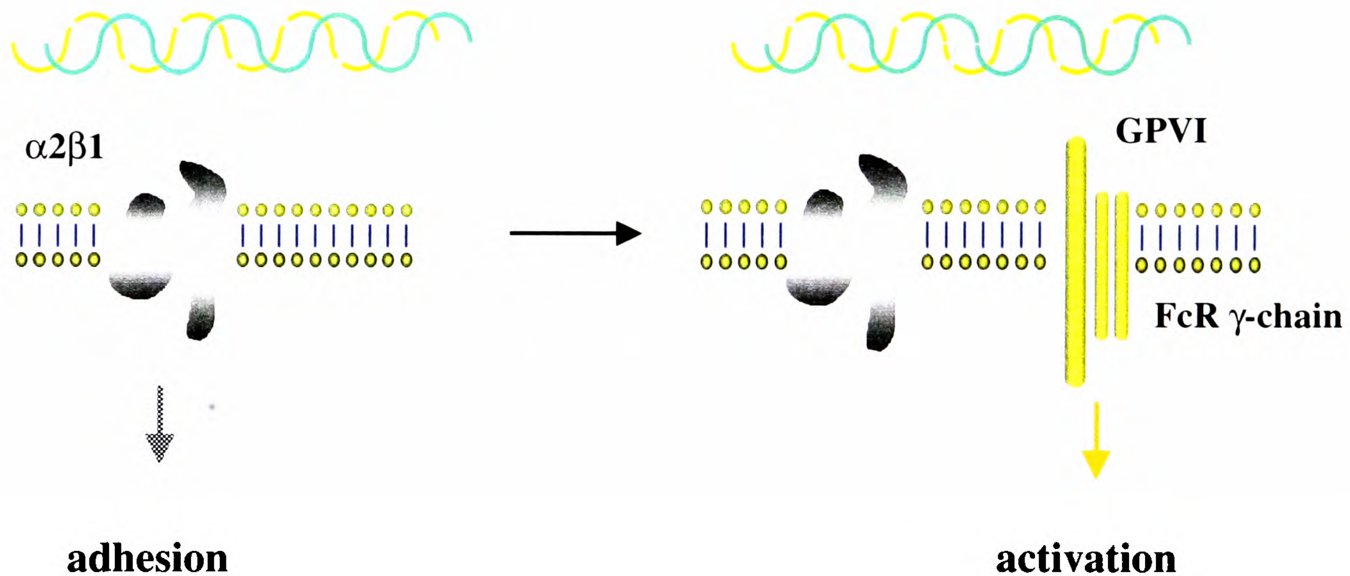


Figure 1.4. Two-site two-step model of platelet activation by collagen. This model postulates that collagen is binding to α2β1, which mediates initial adhesion of the platelet to the subendothelial collagen, and subsequently binds to GPVI, which strengthens adhesion of the platelet and is responsible to transmit the signal within the cell due to its associated FcR γ-chain.

steps model of the interaction of collagen with the platelet. In the original model it was proposed that collagen initially binds to the integrin $\alpha2\beta1$ and that this brings the adhesion molecule into the vicinity of a second receptor, now recognised as GPVI, mediating activation. It was assumed that crosslinking of $\alpha2\beta1$ itself does not generate intracellular signals. This model, however, does not take into account the existence of multiple affinity states of $\alpha2\beta1$ and the ability of the integrin to signal. Therefore, the two-site two-step model has been challenged and a modified version has been proposed which takes into account the different affinities states of $\alpha2\beta1$ (Watson et al, 2000). In this new model, collagen interacts with either $\alpha2\beta1$ or GPVI followed by rapid binding to the other receptor which serves to reinforce the adhesion. The generation of intracellular signals from GPVI or released agonists would convert $\alpha2\beta1$ to a high affinity state, increasing the strength of the adhesion. GPVI and possibly $\alpha2\beta1$ generate intracellular signals which contribute to platelet activation.

$\alpha2\beta1$

$\alpha2\beta1$ belongs to a large superfamily of cell surface proteins called integrins. These are composed of α and β subunits which are non-covalently associated as a heterodimer in the plasma membrane. To date, there are sixteen known isoforms of α subunit and eight of β subunit. Most α subunits can only associate with a single isotype of β subunit. Integrins are associated with cell-cell interactions and cell-extracellular matrix interactions.

The ability of $\alpha2\beta1$ to bind triple helical collagen is well documented. $\alpha2\beta1$ may play an essentially mechanistic role where it mediates initial binding of platelets to collagen in the so-called two-site two-step model, as explained above. Studies which have addressed whether signal transduction events occur downstream of $\alpha2\beta1$ remain controversial. It is notable that a $\alpha2\beta1$-selective peptide (Knight et al., 1999) or cross-linking of a range of antibodies to the $\alpha2$ or $\beta1$ subunits (Hers et al., 2000) do not stimulate tyrosine phosphorylation. This does not rule out the possibility that the integrin activates a tyrosine phosphorylation cascade in an "activated" conformation (Watson et al., 2000) or that it activates a different signalling pathway.

Glycoprotein VI

The existence of glycoprotein VI (GPVI) as a possible collagen receptor in platelets was first proposed when a platelet-activating antibody, anti-p62 IgG, was found in a patient with autoimmune thrombocytopenia and a selective deficiency in collagen-induced platelet aggregation (Sugiyama et al., 1987). The antibody immunoprecipitated a 62 kDa protein from normal platelets that was later identified as GPVI (Moroi et al., 1989). Five GPVI-deficient patients have been reported (Moroi et al., 1989; Ryo et al., 1992; Arai et al., 1995; Sugiyama et al., 1987) and all show defective platelet responses to collagen despite normal expression of the integrin $\alpha2\beta1$.

Human and mouse forms of GPVI were recently cloned and sequenced (Clemetson et al., 1999; Jandrot-Perrus et al., 2000), and found to belong to the immunoglobulin superfamily. GPVI is closely related to the Fc receptor for IgA (FcαR) and to natural killer cell inhibitory receptors. The cDNA for human GPVI contains an open reading frame of

1017 base pairs coding for a protein including leader sequence with 339 amino acids. The extracellular chain of GPVI has two Ig-like domains formed by disulphide bonds, a conserved N-linked glycosylation site at asparagine 93, a mucin-like stalk followed by the transmembrane domain, and a short cytoplasmic tail of 51 amino acids which shows little homology to the C-terminal part of other family members (Clemetson et al., 1999). Mouse GPVI shares 64% homology with the human protein, and contains a second potential glycosylation site at asparagine 244. The cytoplasmic tail is 24 amino acid shorter than in the human version. The expression of GPVI seems to be restricted to platelets and megakaryocytes (Jandrot-Perrus et al., 2000). A representation of GPVI, showing the different functional domains of the receptor, is displayed in figure 1.5.

The mouse gene for GPVI was mapped to chromosome 7, between the flanking markers D7Mit152 and D7Mit178 (Jandrot-Perrus et al., 2000). The human gene was mapped to the long arm of human chromosome 19, region1, band 3 (19q13) (Ezumi et al., 2000). This region is part of the leucocyte receptor complex (LCR) that contains various members of the Ig superfamily, to which GPVI is related (Ezumi et al., 2000).

The group of Takayama reported the cloning of human GPVI from PMA-differentiated CMK cells (Ezumi et al., 2000), and found the presence of three different isoforms of the receptor, referred to as GPVI-1, -2, and -3. GPVI-1 cDNA sequence was identical to the one published by the groups of Clementson and Jandrot-Perrus (Clemetson et al., 1999; Jandrot-Perrus et al., 2000), whereas GPVI-2 lacks an 18 amino acid sequence between the Ig-domains and the transmembrane domain without a frameshift. GPVI-3 has a 4-base insertion that causes a frame shift resulting in elongation of 361 amino acids with no apparent transmembrane domain.

GPVI is the receptor which underlies collagen-dependent immunereceptor-based

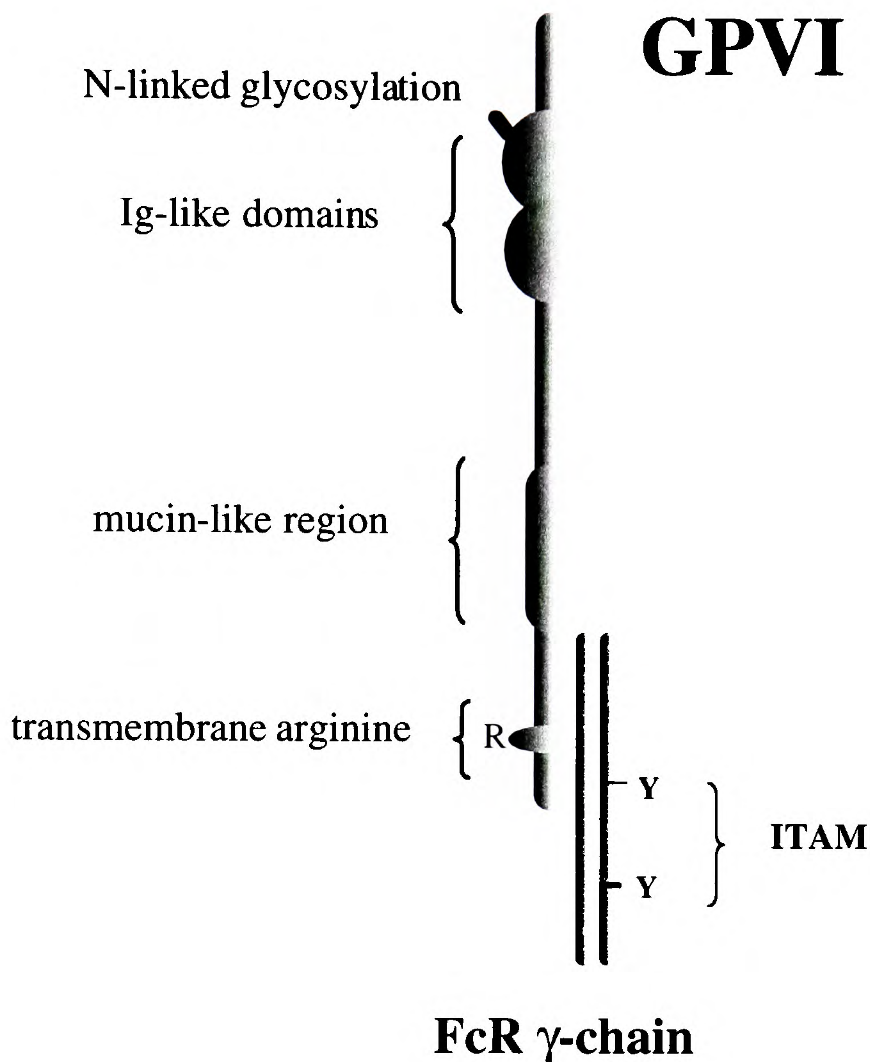


Figure 1.5. Schematic representation of GPVI. The receptor comprises two Ig-like domains with a potential site for N-glycosilation, an mucin-like domain, the transmembrane region containing an arginine (R) residue and a short cytoplasmic tail. The FcR γ-chain containing the ITAM motif is responsible to transmit the signal inside the cell.

tyrosine activatory motif (ITAM)-dependent signalling (Poole et al., 1997; Gibbins et al., 1996; Gibbins et al., 1997). GPVI transduces its signal in a similar fashion as immune receptors such as the receptors for IgG and IgE (FcγR and FcεR, respectively) and T and B cell antigen receptors (TCR and BCR respectively) in that it is closely associated with an ITAM-containing accessory molecule (reviewed in Watson and Gibbins, 1998), namely the γ chain of Fc receptors (FcR γ-chain) (Tsuji et al., 1997; Gibbins et al., 1997). A crucial arginine residue which resides in the transmembrane portion of GPVI is thought to be essential for association of GPVI with the FcR γ-chain (Clementson et al., 1999). Homology with other FcR γ-chain partners suggests that the association with the FcR γ-chain may be necessary not only for transducing the signal, but for stable expression of GPVI at the surface of the cell (Takai et al., 1994).

The study of GPVI-dependent signalling pathways has been aided by the availability of a synthetic collagen-like peptide known as collagen-related peptide (CRP) (Achison et al., 1996) and the snake venom toxin convulxin (Polgar et al., 1997) which are specific to GPVI and whose characteristics are explained below.

Other putative collagen receptors

Three other transmembrane proteins have been proposed as collagen receptors: GPIV, a 65 kDa protein (Chiang et al., 1997) and an uncharacterised 85/90 kDa protein. GPIV (also known as CD36 or GPIIIb) shares little homology with other platelet surface proteins. It is better known as the receptor for thrombospondin, a constituent of platelet granules. However, GPIV has also been shown to bind collagen although its role as a collagen receptor is unclear. Importantly, proximately 5% of the Japanese population lack expression

of GPIV but have no signs of haemostatic pathology, suggesting perhaps that this receptor does not play an essential role in regulating platelet activation. Huang et al. reported that GPIV is associated with Src family kinases Lyn, Fyn and Yes in resting platelets (Huang et al., 1991). However, the potencial signalling pathways downstream of GPIV remain unknown.

The 65 kDa collagen-binding cell surface protein has been cloned and characterised (Chiang et al., 1997), and the recombinant soluble receptor blocks binding of platelets to a collagen-coated surface, but not to a convulxin-coated surface (Francischetti et al., 2000). As yet, there have been no *in vivo* reports of possible functions of p65 in collagen-mediated regulation of platelet activation.

GPV, one of the components of the von Willebrand factor receptor, has been described to be a collagen receptor in platelets, and a recent report describes its capacity to bind collagen and participation in platelet adhesion and aggregation (Moog et al., 2001). As with the above receptors, however, further characterisation is required before a specific function can be assigned to this receptor for his role in platelet activation by collagen.

1.2.3 Collagen and collagen related peptide (CRP)

Collagen is a major component of extracellular matrix and a wide variety of types exist. Cells recognise collagen in different ways depending on sequence and structure. They can recognise predominantly primary sequence, they may require triple-helical structure or they can require fibrillar structures. Collagen binds directly to the platelet integrin receptor $\alpha2\beta1$, which binds to specific sequences in collagen in which the Gly-ER motif appears important. Platelet activation then follows from the recognition by the receptor GPVI of Gly-Pro-Hyp sequences in collagen. The different specific recognition motifs within the collagen

molecule are under intensive investigation in the search for possible methods to control platelet activation *in vivo*. This multimeric nature of collagen, however, hampers the study of the relative contribution of either receptor to the overall mechanism of platelet activation by collagen, and therefore the development of specific ligands for either receptor seemed essential.

A major advance in the field came with the development of simple collagen-like synthetic peptides (or collagen-related peptide, CRP), Gly-Lys-Hyp(Gly-Pro-Hyp)10-Gly-Lys-Hyp-Gly and Gly-Cys-Hyp(Gly-Pro-Hyp)10-Gly-Cys-Hyp-Gly which proved to be highly platelet-aggregatory and more active than collagen fibres (Morton et al., 1995). These peptides acted independently of the integrin $\alpha2\beta1$, suggesting the existence of a platelet receptor other than $\alpha2\beta1$ that underlies activation. However, CRP stimulation promoted tyrosine phosphorylation of the same pattern of proteins in platelets and megakaryocytes as collagen (Asselin et al., 1997; Melford et al., 1997; Mountford et al., 1999). Using platelets deficient for a number of receptors it has been shown that the receptor involved in platelet-activation by CRP was GPVI (Kehrel et al., 1998; Nieswandt et al., 2001). The peptides were cross-linked for experiments, and monomeric form was shown to be partial agonists at GPVI (Asselin et al., 1999). Several studies utilising CRP have proven this to be a very powerful ligand in the study of the signaling pathway generated by GPVI.

1.2.4 Snake venom C-type lectins: convulxin

Snake venoms contain a wide variety of biologically active peptides that enable the snake to paralyse, kill and digest its prey. Generally, poisonous snakes are divided into two major categories depending on whether their venom has predomonantly neurotoxic or vascular effects. Among those with vascular effects, the result can be either prolonged bleeding or

accelerated clotting, although the former is more common. The active components react with coagulation factors, platelets, endothelial cells or extracellular matrix. There are different major classes of molecules which interact with platelets, including metalloproteases, phospholipases, disintegrins, and snake class of C-type lectins.

C-type lectins were first described as carbohydrate-binding (hence lectin) and calcium dependent (hence C-type). The snake class of C-type lectins, however, do not bind sugars nor require calcium, but have other strong similarities with typical lectin domains (Lowe and Ward, 1997). Several different components from a number of snake venoms have been isolated and found to bind specifically to a given receptor on the platelet surface, promoting activation and a functional response (reviewed in Clemetson et al., 1998).

Convulxin is a C-type lectin which has been extensively used in this thesis. It is a potent platelet-aggregating glycoprotein from the venom of the South american rattlesnake *Crotalus durissus terrificus.* It consists of two subunits, α and β, joined by disulphide bridges in a hexameric structure. The binding of ^{125}I-convulxin to platelets has been analysed showing that the protein binds to platelet membrane, in a Ca^{2+}-independent manner at a small number of sites (1000 binding sites per cell) with very high affinity (K_d 30 pM) (Francischetti et al., 1997; Jandrot-Perrus et al., 1997). Binding is not inhibited by thrombin, fibrinogen, RGDS peptide, ADP, 5-hydroxytryptamine or adrenaline (Francischetti et al., 1997). There are several lines of evidence suggesting that convulxin binds to GPVI. Indirect evidence is provided by the observation that convulxin induces a similar pattern of increase in tyrosine phosphorylation to that induced by collagen, involving the time-dependent tyrosine phosphorylation of a number of identified proteins (Polgar et al., 1997; Asazuma et al., 2000). Convulxin binds to a glycosylated 62 kDa membrane component in platelet lysates, and to GPVI immunoprecipitated by human anti-GPVI antibodies. Moreover, convulxin

subunits inhibit both aggregation and tyrosine phosphorylation in respose to collagen (Polgar et al., 1997).

Convulxin has been cloned (Leduc and Bon, 1998), and widely used for studies on GPVI function on platelets and megakaryocytes.

1.3 Intracellular signalling events induced by collagen

Activation of platelets by collagen is associated with tyrosine phosphorylation of multiple proteins and subsequent increase in the levels of intracellular Ca^{2+}, leading to platelet shape change, secretion and aggregation. It is believed that GPVI is the receptor responsible for platelet activation by collagen and therefore a great effort has focussed in the study of signaling cascades emanating from this receptor. The initial signaling event following crosslinking of GPVI is the activation of Src family protein tyrosine kinases, namely Fyn and Lyn (Ezumi et al., 1998). These kinases phosphorylate the tyrosine-containing immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplsmic tail of the FcR γ-chain (Quek et al., 2000). A second tyrosine kinase, Syk, is recruited to the phosphorylated ITAM and subsequently become activated (Poole et al., 1997). One of the major, immediately downstream substrates of Syk in platelets is the adapter LAT (Asazuma et al., 2000), which is localised in characteristics microdomains within the plasma membrane known as GEMs (from glycolipid-enriched membrane microdomains) and which are explained in some detail below. Phosphorylated LAT represent a docking site for the SH2 domain of the adapter protein Gads (Zhang et al., 2000). This protein constitutively interacts via its SH3 domain with the adapter SLP-76 and, consequently, binding of LAT to phosphorylated LAT brings SLP-76 to the plasma membrane and more importantly, to the GEMs. Constitution of the LAT-Gads-SLP-76 trimer is followed by phosphorylation of

SLP-76 by Syk, thus providing the binding site for the SH2 domain of a member of the Tec family of PTKs, presumably Btk (Pasquet et al., 1999b; Oda et al., 2000). In parallel to Gads-SLP-76, PLCγ2 and the p85 regulatory subunit of PI3K bind to LAT in platelets (Gibbins et al., 1998; Asazuma et al., 2000). PI3K has been reported to positively modulate platelet activation upon GPVI stimulation (Pasquet et al., 1999a; Lagre et al., 1999) and is implicated in recruitment of PLCγ2 to the membrane in mouse megakaryocytes stimulated with CRP (Bobe et al., 2001). Activation of PLCγ2 results in cleavage of the inositol phospholipid, PI(4,5)P_2, and generates the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces calcium release whereas DAG activates protein kinase C. This tyrosine phosphorylation cascade and formation of second messengers leads to platelet shape change, secretion and aggregation. A schematic overview on those signals originated from GPVI leading to activation of PLCγ2 are depicted in figure 1.6.

Signals other than those leading to activation of PLCγ2 emanate from collagen-stimulated platelets. For instance, collagen promotes profound cytoskeletal reorganisation in platelets, which most dramatic phenotypic effect is reflected by the initial shape change that platelet suffer shortly after activation. Platelets deficient for GPVI show impairment in phosphorylation of vav, a guanosine exchange factor (GEF) for Rho family GTPases and which is implicated in cytoskeletal rearrangements (Ichinohe et al., 1997). On the other hand, it has recently been described the possibility of a cross-talk between GPVI and Gi-coupled receptors in collagen-stimulated platelets (Nieswandt et al., 2001a). These data suggest that signals additional to those implicated in PLCγ2 activation are important in GPVI signaling.

Within the plasma membrane of many cell types, including platelets, there are specialised regions enriched in sphingolipids and cholesterol that also contain a selective enrichment of proteins, including, particularly, proteins engaged in signal transduction (Schnitzer et al., 1995; Resh, 1994). These regions are referred to as GEMs (glycolipid-enriched membrane microdomains) and also known as rafts. In common with some other haematopoietic cells, platelets contain biochemically identifiable membrane microdomains (Dorahy et al., 1996). A variety of signalling molecules are concentrated in GEMs, including Src family tyrosine kinases, the adapter LAT and monomeric and heterotrimeric G proteins, as well as molecules involved in Ca^{2+} influx (reviewed in Simons and Ikonen, 1997; and Langlet et al., 2000). Other proteins, however, are recruited to GEMs upon stimulation of the cell. GEMs act as 'factories' for the generation of signals, where the proteins implicated in a given signalling pathway become concentrated in a close vicinity to each other, forming a so called signalosome. The importance of these regions in signalling is shown by studies in T and B cells where inhibition of protein recruitment to GEMs reduces the normal activation of the cell (Ishiai et al., 2000; Boerth et al., 2000).

There are no reports on studies of the presence of GPVI in GEMs, although many of the proteins implicated in GPVI signalling are important for signalling in B and T cells, where GEMs play a crucial role, and therefore it is predictable that the same applies for GPVI.

1.3.1 GPVI-FcR γ-chain complex and FcγRIIA: the ITAM motif

GPVI signals in a similar fashion to that of the low affinity receptor for immune complexes (FcγRIIA) present in platelets and megakaryocytes, with both receptors stimulating rapid and marked tyrosine phosphorylation of the tyrosine kinase Syk (Yanaga et

al., 1995). The Syk family of tyrosine kinases, of which there are two known members, Syk and ZAP-70, play a critical role in signalling by immune receptors as a consequence of the interaction with an immunoreceptor tyrosine-based activation motif (ITAM) present in one or more of the receptor chains. The ITAM contains two conserved tyrosine residues in a sequence, YxxLx6-12YxxL/Y (where X=any amino acid), both of which are capable of being phosphorylated following crosslinking of the immune receptor, and enabling binding to the tamdem SH2 domains in Syk and ZAP-70. Using a bacterially expressed GST fusion protein encoding the tandem SH2 domains of Syk, Gibbins et al. precipitated a 14 kDa protein from collagen-stimulated platelets under reduced conditions which was identified as the FcR γ-chain by immunoblotting (Gibbins et al., 1996). The FcR γ-chain contains one ITAM and was previously recognised for its role in signalling by a number of Fc receptors including FcγRI, FcγRII and FcγRIII. The importance of the FcR γ-chain and Syk in collagen receptor signalling was demonstrated by the complete inhibition of aggregation and dense granule secretion to collagen in mice platelets lacking either protein (Poole et al., 1997). This work demonstrated that collagen signals through the same fundamental pathway as that used by immune receptors with critical roles for the FcR γ-chain and Syk.

Several reports support a role for GPVI in the activation of the FcR γ-chain/Syk pathway by collagen. Crosslinking of GPVI using $F(ab')_2$ fragments of the antisera from the original GPVI-deficient patient stimulated rapid tyrosine phosphorylation of FcR γ-chain (Gibbins et al., 1997; Tsuji et al., 1997). Further, incubation of collagen-stimulated platelet lysates with the tandem SH2 domains of Syk precipitated a biotin-labelled surface protein which was identified as GPVI using the above antiserum (Gibbins et al., 1997). This suggested that the two proteins are present in a preformed complex as GPVI itself is not

tyrosine phosphorylated. The FcR γ-chain is absent in GPVI-deficient patients suggesting that the two proteins require each other for stable expression (Tsuji et al., 1997), consistent with results for a number of Fc receptors (Takai et al., 1994). More recently, using a monoclonal antibody raised to mouse GPVI, it was demonstrated that mice engineered to lack FcR γ-chain do not express detectable levels of GPVI in platelets nor megakaryocytes (Nieswandt et al., 2000).

Apart from the FcR γ-chain, there have been described two other proteins in platelets and megakaryocytes that bear an ITAM motif. One is the β chain of FcεRI, the high affinity receptor for IgE (Joseph et al., 1986; Joseph et al., 1997), and the other the low affinity receptor for IgGs, FcγRIIA (Rosenfeld et al., 1985). Little is known about the function of FcεRI in platelets and megakaryocytes. It has been shown to be expressed only in the cytoplasm of megakaryoblastic cell lines (Hasegawa et al., 1999). In platelets, it may be implicated in FcεRI-mediated cytotoxicity in response to certain infections by parasites (Joseph et al., 1997), although it level of expression seem low.

There are three FcγRII isoforms, A, B and C, which are highly homologous in the extracellular and transmembrane regions, but differ in their cytoplasmic tails. Of these, only FcγRIIA is expressed on platelets and megakaryocytes (Wu et al., 1996). FcγRIIA is a 40-45 kDa sialoglycoprotein which, unlike another Fc receptors, does not utilise accesory ITAM-containing subunits for signal transduction, since it contains an ITAM motif in its cytoplasmic tail. Clustering of FcγRIIA induces shape change, secretion and aggregation. FcγRIIA ligation induces tyrosine phosphorylation of multiple cellular proteins, including

the receptor itself (Huang et al., 1992), and displays a pattern of phosphorylation and functional responses similar to those of GPVI (Blake et al., 1994). On receptor engagement, the ITAM domain within the cytoplasmic tail is phosphorylated, allowing for recruitment of Syk, which is in turn phosphorylated and activated (Ravetch, 1994). Syk is responsible for transmission of the signal inside the cell (Bewarder et al., 1996), leading to activation of PLCγ2 and increase in intracellular calcium and culminating in secretion of the granule-contents and aggregation of the platelets. Phosphatidylinositol 3-kinase (PI3-K) and its product phosphatidylinositol 3,4,5-trisphosphate play a key role in the activation and adequate location of PLCγ2 induced by FcγRIIA crosslinking (Gratacap et al., 1998). More recently, it has been reported that co-activation of a Gi-dependent pathway is also required for efficient stimulation of PI3-kinase, $[Ca^{2+}]_i$ mobilisation and platelet aggregation (Gratacap et al., 2000) suggesting that platelet-activation after FcγRIIA crosslinking requires tyrosine phosphorylation-dependent and -independent signals.

Proteins containing SH2 domains transmit intracellular signals initiated by tyrosine kinase-linked receptors. Three-dimensional structures suggest mechanisms by which tandem SH2 domains confer higher specificity than individual SH2 domains. It has been shown that tandem SH2 domains bind ITAMs corresponding to its appropiate biological partner with highest affinity, illustrating that different ITAMs show greater specificity for one SH2 domain relate to another (Ottinger et al., 1998).

Figure 1.6. Schematic representation of PLCγ2 activation by GPVI-signalling. Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; LAT, linker for activation of T-cells; GPVI, glycoprotein VI; FcR γ-chain, Fc receptor gamma-chain; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; Gads, Grb2-related adapter downstream of Shc; Btk, Bruton's tyrosine kinase; PLCγ2, phospholipase C-gamma 2; PIP2. Phosphoinositide-4,5-bisphosphate; PIP3, phosphoinositide-3,4,5-trisphosphate; IP_3, inositol trisphosphate; DAG, diacylglycerol.

1.3.2 Cytoplasmic protein tyrosine kinases

Cytoplasmic protein tyrosine kinases (PTKs) have been grouped into distinct families which include the Syk/ZAP70 family, the Src-kinase family, the Tec kinase family, the JAK family and focal adhesion kinases.

Some tyrosine kinases such as p60src (Src) and p120fak (FAK) are associated with integrin and cytoskeletal signal transduction events (Clark and Brugge, 1993). JAKs are thought to play important roles in cytokine receptor signalling (Ihle, 1995). Others, like Syk, ZAP-70, the Src-like kinases Fyn, Lck and Lyn, and the Tec family kinases Bruton's tyrosine kinase (Btk) and IL-2 induced tyrosine kinase (Itk) are implicated in immune receptor signalling (Cheng et al., 1995; Rawlings and Witte, 1994; Takata et al., 1994). The emerging picture from several studies is one where similar patterns of signal transduction involving these groups of tyrosine kinases occur in different cell types. Many of these tyrosine kinases have been implicated in collagen receptor signalling in platelets. Description of different kinases involved in GPVI signalling is shown below.

1.3.3 Syk/ZAP-70 family

This tyrosine kinase family is characterised by the presence of tandem SH2 domains as well as a catalytic domain. The two SH2 domains bind to two phosphotyrosine residues on ITAM of tyrosine kinase-associated receptors. Syk is the major representative of this family of kinases in B cells, mast cells, macrophages and platelets, whereas ZAP-70 is primarily expressed in natural killer cells and T cells, including thymocytes (Chu et al., 1998). Syk is known to be associated with the tyrosine kinase phosphorylated ITAM of the B-cell receptor (BCR) (Takata et al., 1994), FcR γ-chain in mast cells (Kihara and Siraganian, 1994), and platelets (Gibbins et al., 1996). The association of Syk with

phosphorylated ITAM causes the kinase to autophosphorylate. Furthermore, Syk phosphorylates other substrates. Thus, Syk forms a vital link between phosphorylated ITAM and downstream substrates including tyrosine kinases and adapters leading to activation of PLCγ2 (Cheng et al., 1995; Poole et al., 1997).

The homology between Syk and ZAP-70 makes it possible for ZAP-70 to substitute for Syk in a reconstituted chicken B cell line depleted of Syk (Ishiai et al., 2000). In contrast to this, one major difference between Syk and ZAP-70 is the presence of a 'linker' region in Syk which is absent in ZAP-70, and which provides a critical function for the biological activity of Syk. An alternatively spliced form of Syk, termed SykB, has been identified which lacks a 23 amino acid sequence in the 'linker' domain. Syk B is inefficient at coupling stimulation of FcεRI on basophils or the antigen receptor on T cells. The functional defect in SykB correlates with its reduced ability to bind ITAMs in vitro and in vivo, demonstrating the importance of this region for immunoreceptor signalling (Latour et al., 1998).

Syk is not only phosphorylated and activated by GPVI or FcγRIIA crosslinking, but is also phosphorylated and activated by thrombin in platelets (Taniguchi et al., 1993; Sada et al., 1997). During the activation process, protein-tyrosine phosphorylation occurs in successive waves, and activation of Syk is regulated by initial integrin-independent and subsequent integrin-dependent mechanisms (Clark and Brugge, 1993; Clark et al., 1994). Since the thrombin receptor induces minimal phosphorylation of an ITAM-containing protein, the mechanism of thrombin-induced activation of Syk remains unclear. A representation of Syk and ZAP-70 is shown in figure 1.7.

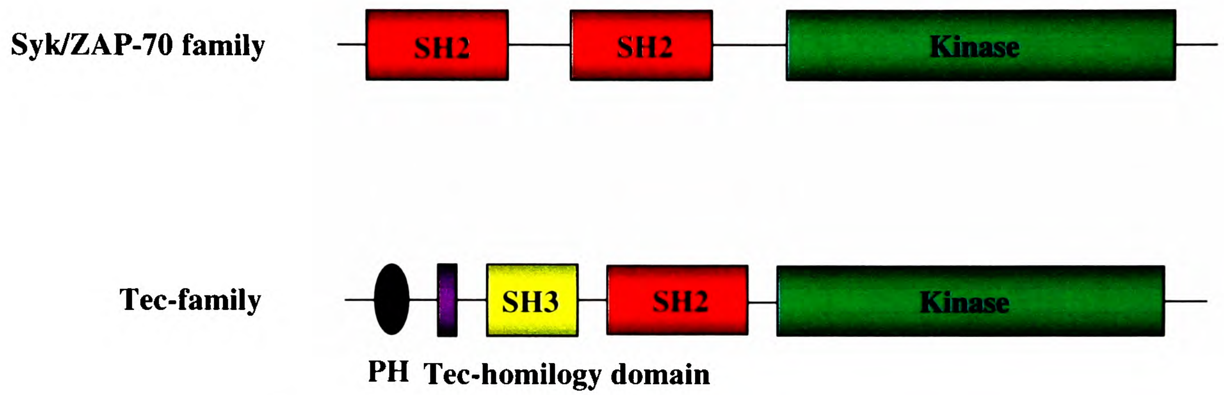


Figure 1.7. Tyrosine kinases in B-cells, T-cells and platelets. Linear representation of different regulatory domains of Tec-family and Syk/ZAP-70 family of protein tyrosine kinases.

1.3.4 Src-family tyrosine kinases

The Src family is the prototype for cytoplasmic tyrosine kinases. Src itself was discovered as the product of a viral oncogene v-src, found in Rous sarcoma virus, from whence it derived its name (sarcoma) (Strehlin et al., 1976). Apart from Src itself, Src-family kinases include Blk, Lyn, Lck, Fyn, Yes, Hck and Fgr. They are widely expressed in many cell types but much of the characterisation of these kinases has focused on haematopoietic cell lineages (reviewed in Chow and Veillette, 1995). The family is characterised by well conserved 'Src-homology' (SH) domains. Such homology domains have been found in a wide range of signalling proteins, including other protein tyrosine kinase (PTK) families, adaptor proteins, PI3-K and PLCγ isoforms. The SH1 domain is the catalytic kinase domain, situated at the C-terminus of the protein. Src family kinases also contain an SH2 and SH3 region, which interact specifically with phosphotyrosine sequences and proline-rich regions respectively (Pawson, 1995). Another characteristic of this family of kinases is the presence of lipid modifications in the N-terminus of the protein sometimes called the SH4 domain. Palmitoylation and myristoylation are responsible for the constitutive association of these enzymes with the intracellular surface of the plasma membrane, and may also play a role in mediating interactions of Src family kinases with cell surface receptors (Timson-Gauen et al., 1996).

Platelets and megakaryocytes express several Src-like kinases including Src, Lyn, Fyn, Yes, Hck and Fgr. It is thought that Src kinases are responsible for the tyrosine phosphorylation within the ITAM domain of the FcR γ-chain upon receptor engagement, enabling recruitment and activation of Syk, and subsequent transmission of the signalling cascade inside the cell (see Fig. 1.6). This hypothesis is supported by the observation that the selective Src family kinase inhibitor PP1 fully inhibits platelet activation by GPVI

agonists, but only marginally inhibits platelet responses to thrombin (Briddon and Watson, 1998; Ezumi et al., 1998). However, only Fyn and Lyn have been consistently shown to be associated with the complex GPVI-FcR γ-chain in human platelets, suggesting that these two Src family kinases are particularly important for GPVI signalling (Briddon and Watson, 1998; Ezumi et al., 1998). Murine platelets deficient in Lyn show a delay and then potentiation of the signal upon GPVI crosslinking, whereas Fyn-deficient platelets show a decrease in transmission. Lyn -/- Fyn -/- platelets show impairment of platelet aggregation in response to the GPVI-selective ligand CRP and a reduction in tyrosine phosphorylation relative to normal mice, reflecting the importance of these two Src family members in GPVI signalling (Quek et al., 2000).

1.3.5 Adapter proteins

Adapter proteins can be defined as proteins that mediate intermolecular interactions within a signal transduction pathway and that lack both intrinsic enzymatic and transcriptional activity. They express a variety of modular binding domains (e.g. SH2-, SH3- domains) or tyrosine based signaling motifs. These domains/motifs enable adapter proteins to mediate constitutive or inducible protein-protein or protein-lipid interactions with other signal transducing elements. The main function of adapter proteins is to integrate receptor mediated signals at the intracellular level and to couple signal transducing receptors complexes to intracellular effector systems by organising the dynamic ensemble of signaling scaffolds.

On the basis of their differential subcellular localization, adapter proteins can be classified as cytosolic or transmembrane adapter proteins. The former group includes not only molecules with well defined functions such as SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), SLP-65/BLNK (SH2 domain-containing leukocyte protein of

65 kDa/B-cell linker), Grb2 (growth factor receptor binding protein 2) and Gads, but also proteins whose functional role remains to be established (e.g. SKAP-55, SKAP-HOM, SLAP and SLAP-130/FYB). The group of transmembrane adapter proteins so far comprises four members: LAT (linker for activation of T cells), TRIM, SIT and PAG/Cbp. The main function of these adapter proteins seems to be to provide docking sites for the SH2-domains of intracellular signaling molecules (e.g. cytosolic adapter proteins) thereby targeting them to the inner leaflet of the plasma membrane. To facilitate this, the cytoplasmic domains of the four known transmembrane adapter proteins contain putative tyrosine based signaling motifs.

From a functional point of view, adapter proteins may be segregated into those exerting positive regulatory functions (e.g. SLP-76, LAT and Gads) and those possessing negative regulatory capabilities (e.g. PAG, SLAP). However, particular adapter proteins are probably capable of generating both negative and positive signals depending on cell type and subcellular localisation.

Crosslinking of GPVI and phosphorylation of the ITAM domain within the FcR γ-chain leads to a tyrosine phosphorylation-regulated cascade that involves Syk and PLCγ2, leading to platelet activation. A number of adapter proteins have been described to participate in this pathway, and examples are shown below and represented in figure 1.8.

LAT is a 36-38 kDa integral membrane adapter protein that was first identified in T cells (Zhang et al., 1998a), and shown to be present in NK cells, mast cells, megakaryocytes and platelets (Facchetti et al., 1999). LAT contains nine tyrosine residues in its cytoplasmic tail that are conserved between murine and human sequences, five of which are surrounded by the optimal binding sequence for binding to the SH2 domain of Grb2

(Zhang et al., 1998b). LAT plays a central role in T cell activation and is required for optimal GPVI-signaling in platelets. LAT becomes phosphorylated in platelets upon GPVI stimulation, thereby recruiting to the membrane a number of other proteins necessary for PLCγ2 activation (Asazuma et al., 2000). LAT tyrosine phosphorylation is abolished in CRP-stimulated, Syk-deficient mouse platelets, whereas Syk phosphorylation is maintained in LAT-deficient platelets, placing LAT downstream of the FcR γ-chain and Syk (Pasquet et al., 1999). Moreover, tyrosine phosphorylation of PLCγ2 is substantially reduced in LAT-deficient platelets but not completely inhibited (Pasquet et al., 1999).

SLP-76 is a cytoplasmic adapter protein that was initially identified as a tyrosine phosphorylated protein from stimulated T cells that associates with the SH3 domain of the Grb2 adapter *in vitro* (Zhang et al., 1998b). In platelets it was first identified on the basis of its association to a GST-Grb2 fusion protein upon FcγRIIA stimulation (Robinson et al., 1996). SLP-76 has a carboxyl-terminal SH2 domain that binds to tyrosine phosphorylated proteins, a central proline-rich region that associates with Grb2 and three N-terminal tyrosine residues. SLP-76 is believed to be a substrate for ZAP-70 in stimulated T cells and Syk in platelets (Fang and Koretzky, 1999; Pasquet et al., 1999). The importance of this adapter protein in platelet activation by collagen is illustrated by the fact that tyrosine phosphorylation of PLCγ2 upon collagen stimulation is reduced by greater than 90% in SLP-76-deficient platelets and there is a corresponding inhibition of platelet activation (Clements et al., 1999).

A third adapter protein, Gads (Grb2-related adapter downstream of Shc), also known as Grf40 or Grpl, is a Grb2-related protein originally identified on the basis of its interaction with the tyrosine phosphorylated form of the adapter Shc (Liu et al., 1999; Law

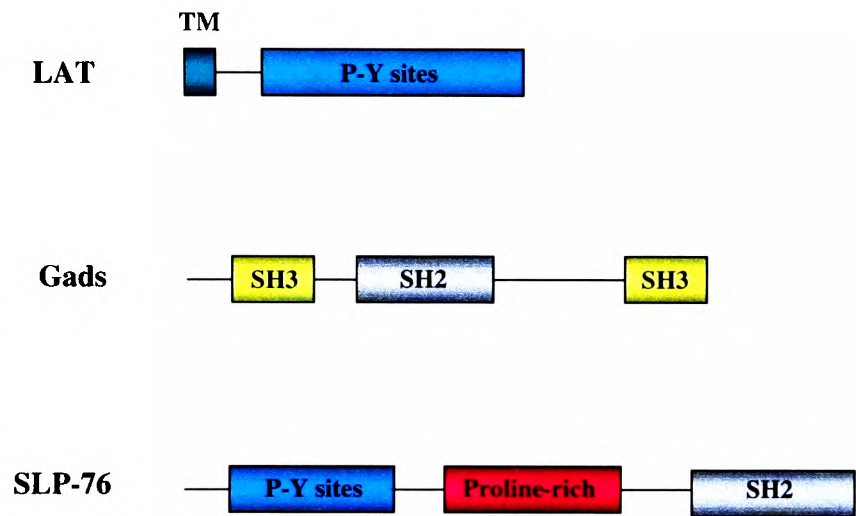


Figure 1.8. Adapter molecules in B-cells, T-cells and platelets. Linear structure of the adapter proteins LAT, Gads, and SLP-76, showing the different regulatory domains. TM: transmembrane domain. P-Y sites are regions containing tyrosine residues.

et al., 1999; Asada et al., 1999). It has one SH2 domain and two SH3 domains. Gads is constitutively associated to SLP-76 through the binding of the Gads SH3 domain in the cytoplasm of the platelet (Asazuma et al., 2000). Phosphorylation of PLCγ2 is severely compromised in T cells from mice-deficient for the adapter (Yoder et al., 2001). In platelets it has been described to associate to LAT upon GPVI stimulation (Asazuma et al., 2000).

These adapters seem therefore critical for phosphorylation and activation of PLCγ2 in platelets. LAT serves as a linker molecule targeting key proteins to GEMs for regulated signalling in response to activation of GPVI, enabling an organised signalling cascade that leads to platelet activation (Asazuma et al., 2000). Despite its presence in the platelet, the function and regulation of adapter proteins requires further work to understand their role in collagen signalling. Although I have described those adapter proteins implicated in PLCγ2 activation in collagen-stimulated platelets, a number of other adapter proteins have been reported to be present in platelets, such as SKAP-HOM and SLAP-130 (Asazuma et al., 2000). However, these are probably not implicated in the regulation of PLCγ2.

1.3.6 PLCγ2 regulation

PLC isoforms form a central point upon which several signalling cascades converge. There are three subfamilies of PLC characterised so far: PLCβ, PLCδ and PLCγ. There are two known isoforms of PLCγ, 1 and 2. PLCγ1 is ubiquitously expressed whilst PLCγ2 has a distribution which is restricted to cells of haematopoietic lineages (Rhee and Bae, 1997). The structures of PLCγ1 and PLCγ2, based on cDNA sequences, are depicted in figure 1.9. All phosphoinositide-specific PLC isozymes share the catalytic subunit designated as X and

Y. Based on crystallographic structure of a PLCδ isoform shows that these two domains fold together to compose the catalytic sites (Essen et al., 1996). The region between X and Y domains is enlarged to contain SH2 and SH3 domains unique to the γ isozymes. Both of these motifs facilitate PLCγ association with other proteins. In addition to SH domains, PLCγ also contains a C2 domain and two PH domains, one of which is split. These domains are features shared with β and δ family members and likely to serve a general mechanism in the catalysis of $PI4,5P_2$. C2 domains mediate interaction with Ca^{2+}/phospholipids, while PH domains recognise polyphosphoinositides.

Homozygous disruption of PLCγ1 results in lethality at embrionic day (E) 9.0, demonstrating that the capacity of PLCγ1 to mobilise second messenger molecules is essential, and its absence is not compensated by other signaling pathways or other PLC isozymes (Ji et al., 1997). On the other hand, PLCγ2-deficient mice are viable, consistent with a more restricted expression relative to PLCγ1, but have decreased mature B cells. GPVI signaling is defective in PLCγ2-deficient platelets, resulting in a loss of collagen-induced platelet aggregation (Wang et al., 2000).

PLCγ2 is the main isoform in platelets (Blake et al., 1994). Collagen and CRP stimulate tyrosine phosphorylation of the PLCγ2 in platelets, which has been shown to be associated with a large signalling complex comprising of Syk, Lyn and LAT. The adaptor protein SLP76 is also thought to be involved in regulating PLCγ2 recruitment (Gross et al.,

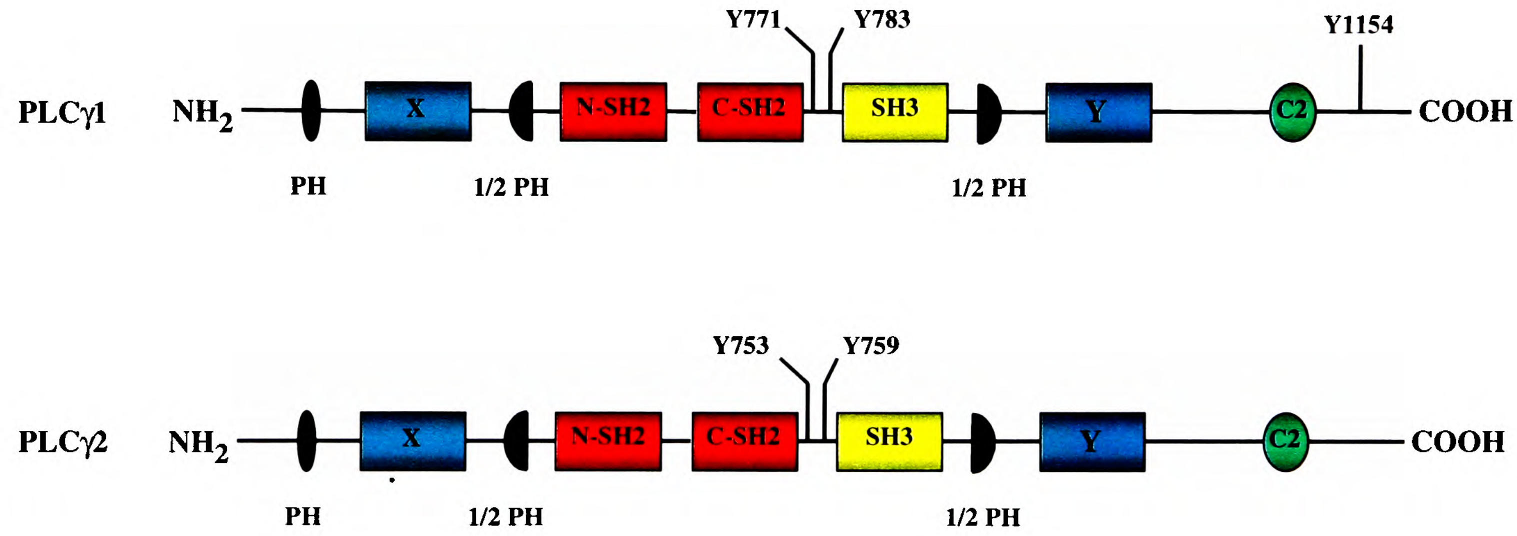


Figure 1.9. Organization of domains in PLC-γ isozymes. Based on primary sequence information, the linear arrangements of catalytic domains (X and Y) and regulatory domains (SH2, SH3, PH and C2) are depicted for PLCγ1 and PLCγ2.

1998). Knock-out mice for the FcR γ-chain, Syk, LAT and SLP-76 have proven these proteins to be important for activation of PLCγ2 in murine platelets (Poole et al., 1997; Clements et al., 1999; Pasquet et al., 1999).

3'phosphorylated phosphoinositides have also been shown to be necessary for full activation of PLCγ isoforms. The current model postulates that high affinity binding of the PH domain of PLCγ to $PI3,4,5P_3$ and its subsequent co-localisation with its substrate phosphoinositide 4,5-bisphosphate ($PI4,5P_2$) is necessary for PLC activity (Bae et al., 1998; Falasca et al., 1998; Gratacap et al., 1998; Rameh et al., 1998).

1.3.7 Negative regulation of collagen signalling in platelets: the ITIM motif

As shown above, GPVI plays an important signalling role in platelet activation. The activatory signal triggered from the ITAM motif must however be negatively regulated by inhibitory signals, in order to modulate the degree of activation of the platelet. This modulatory role is undertaken by immunoreceptor tyrosine-base inhibitory motif (ITIM) containing molecules (reviewed in Ravetch and Lanier, 2000). One ITIM molecule is known in platelets. CD31 (PECAM-1) is expressed in human and mouse platelets (Ohto et al., 1985). CD31 associates with the cytoplasmic phosphatases SHP-1 and SHP-2 and is phosphorylated upon platelet activation by collagen, thrombin and FcγRIIA crosslinking (Jackson et al., 1997; Cicmil et al., 2000; Noda et al., 2000). Mice deficient in CD31 show enhanced responses to GPVI agonists (Patil et al., 2000) demonstrating that CD31 is a negative regulator of platelet responses. The role of the ITIM inhibitory receptors in platelets is still obscure, and further work is necessary before we can start to understand the precise mechanisms of regulation.

Aims of the thesis

Within the last years, GPVI has emerged as crucial for platelet activation to exposed subendothelial collagen after blood vessel damage. However, the lack of a nucleus and small size makes it impossible to apply some of the modern cell and molecular biology techniques to the platelet. One difficulty in the field of platelet activation by collagen is the presence of several receptors at the surface of the cell, making it difficult to distinguish the contribution of one single receptor. Therefore, alternative systems seem necessary to further understand the biology of GPVI. It was one aim of this thesis to (i) screen a number of cell lines in order to find one expressing GPVI as a source of mRNA for the cloning of the receptor. Ectopic expression of GPVI in cell lines would permit to demonstrate which signals are attributable to GPVI without intervention of other collagen receptors. Moreover, genetic manipulation of those cells expressing GPVI could be useful to over-express, deplete or modify proteins implicated in the signaling pathway generated by GPVI. It was a further aim of this thesis to (ii) reconstitute the signaling pathway generated by GPVI leading to PLCγ2 activation. An additional aim was to (iii) use mutant receptors to make structure/function studies of the receptor in order to identify specific sites or domains necessary for its functionality.

The identification of new markers and their stage of expression during megakaryocytopoiesis are an important contribution in the field of megakaryocyte differentiation. As GPVI seems to be specific of the platelet, it was a further aim to (iv) detect its expression in the platelet precursor, the megakaryocyte, and to investigate the

relation between expression of GPVI and the stage of differentiation of the megakaryocyte.

Chapter 2

Materials and Methods

2. Material and Methods

2.1 Materials

Horm collagen was from Nycomed (Germany). Lyophilised convulxin was a generous gift of Dr. Mireille Leduc and Dr. Cassian Bon (Institute Pasteur, Paris, France). After resuspension in PBS its concentration was measured with a spectrophotometer at 280 nm (extinction coefficient=2.6) and its activity was assessed by bioassay on platelet aggregation (Jandrot -Perrus et al., 1998). Alboaggregin-a was supplied by Dr Robert Andrews (Baker Medical Research Institute, Melbourne, Australia). Alborhagin was from Sigma-Aldrich Ltd. (Poole, UK) or Venom Supplies (Tanunda, South Australia). Collagen-related peptide (CRP) in a crosslinked form (Morton et al., 1995) was supplied by Richard Farndale (Biochemistry department, University of Cambridge, UK).

RPMI-1640 medium, IMDM medium, foetal bovine serum, penicillin, streptomycin, hygromycin and neomycin (G418) antibiotics were from Gibco-RBL (Life Technologies Ltd, Paisley, UK). Recombinant murine IL-6, IL-11 and TPO were from R&D Systems (Oxfordshire, UK). Trypan blue was from Gibco-RBL. PMA and DMSO were from Sigma. Nucleotides tri-phosphate and deoxinucleotides tri-phosphate for primary cultures were from Pharmacia (Cambridge, UK).

All antibodies used are shown in annexe I. Prestained SDS-standards, Bradford reagent, acrylamide, temed and 0.2 μm PVDF membranes were from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). ECL reagents and Hyperfilm were from Amersham International (Amersham, Buckinghamshire, UK).

Trizol reagent was from Gibco-RBL. RNAgents, M-MLV reverse transcriptase and oligo$(dT)_{15}$-primer were from Promega (Southampton, UK). Qiagen Rneasy kit was from Quiagen Ltd (Crawley, UK). AmpliTaq Gold was from Perkin Elmer (Warrington, UK).

All other reagents were from Sigma-Aldrich Ltd.

2.2 Cell preparation

Platelet preparation

Human blood was taken from drug-free volunteers on the day of experiment using acidic citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min, and platelets were isolated by centrifugation at 1000 *g* for 10 min in the presence of prostacyclin (0.1 μg/ml). Platelets were resuspended in modified Tyrode's-Hepes buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM $Na_2HPO_4.12H_2O$, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3 at 37°C) in the presence of prostacyclin (0.1 μg/ml), recentrifuged at 1000 *g* for 10 min, and resuspended in the above buffer to a density of $5x10^8$ cells/ml.

Cell culture

HEL, K562, CMK, DAMI, MEG01 and Jurkat cells were grown in RPMI-1640 medium. UT-7 and COS-7 cells were grown in DMEM medium. All cell lines were supplemented with 1mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated FBS under 5% CO2 / 95% air in a humidified incubator. UT-7 cells stably

expressing the hTPO receptor c-mpl (UT-7/c-mpl) were a generous gift of Dr. Jorge Erusalimsky (University College London, The Rayne Institute, London, UK) (Hong et al., 1998). UT-7/c-mpl cells were grown in the presence of 5 ng/ml GM-CSF and 500 μg/ml neomycin. Cells were kept at exponential phase of growth.

To induce megakaryocytic differentiation, $2x10^5$ cells/ml were exposed to 10 nmol/L PMA or an equal volume of DMSO as control medium for the times indicated. To induce erythroid differentiation, $2x10^5$ cells were stimulated with 25 μM hemin for up to 3 days. Cell number and viability were determined by trypan blue dye exclusion method. Attached cells were harvested by adding phosphate-buffered saline (PBS) containing 2.5 mM EDTA at 37°C for 5 min. Cells were washed and resuspended in modified Tyrode's-Hepes buffer. 10^7 HEL cells/ml were stimulated with 20 nM convulxin at 37°C and reaction terminated by the addition of an equal volume of Laemmli sample buffer, then subjected to SDS-PAGE.

Bone marrow cell isolation and culture in vitro

Femurs and tibias from CD1 mice that were at least eight weeks old were taken, and bone marrow cells flushed out with cold Iscove's modified Dulbecco's medium (IMDM) using a $25G_{5/8}$ needle, centrifuged and resuspended for 5 min in lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) to remove red cells. After centrifugation at 250 g for 5 min, cells were resuspended in IMDM medium supplemented with 5 mg/ml BSA, 0.2 mg/ml transferrin, 10 μg/ml insulin, 50 μM β-mercaptoethanol, 40 μg/ml low density lipoprotein, 20 μM of each dNTP and NTP, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml IL-6, 10 ng/ml IL-11 and 50 ng/ml TPO. Cells were plated at a density of $1.5x10^6$

cells/ml and kept in culture for four days. Non-adherent cells were harvested for subsequent experiments. For acetylcholinesterase detection, cells were cytospined onto coverslips and incubated for 1-2 h in a solution of 100 mM sodium phosphate buffer pH 6 containing 0.66 mg/ml acetylthiocholine iodide, 5 mM sodium citrate, 3 mM copper sulphate and 0.5 mM potassium ferricyanide. Cells were washed with sodium phosphate buffer and fixed with 95% ethanol for 5 min. Cells were air-dried and incubated for 20 sec with Harris-Hematoxylin, washed and mounted on slides.

Transient and stable transfections

Two different methods of transfection were used, e.g. electroporation and calcium-phosphate.

Electroporation- $5x10^6$ cells were washed three times with cold serum-free medium and resuspended in a final volume of 800 μl in an electroporation cuvette already containing 10μg of plasmid DNA. After 5 min on ice, cells were electroporated under different conditions (see chapter 3) and placed on ice for another 5 min. Finally cells were diluted to a final volume of 5 ml with complete pre-warmed medium and placed in an incubator.

In chapter 5 a modified protocol was used. Cells were washed twice in cytomix buffer (120 mM KCl, 0.5 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, pH 7.6 adjusted with KOH) supplemented on the day of experiment with 5 mM glutathione and 2 mM ATP. Cells were resuspended in 400 μl of the above buffer and placed onto an electroporation cuvette already containing 10μg of DNA.

Cells were electroporated and incubated room temperature for 10 min, then placed on ice for another 10 min. Finally cells were added to 5-10 ml complete media and incubated for 48 h.

Calcium-phosphate- The calcium-phosphate method was employed with COS-7 cells. A mixture containing 500 μl sterile-distilled water, 186 μl of 1M $CaCl_2$ and 20 μg plasmid DNA was prepared. After 5 min, 750 μl of 2x HBS buffer (2x HBS is: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4 (anhydrous), 50 mM HEPES, 12 mM dextrose, pH 7.5) was added, drop by drop. After 1 min, 100 μl of this transfection mixture per ml of medium was added to the cells. After 8 h cells were washed 5-6 times with sterile PBS and then complete medium added.

For stable expression, plasmid DNA was linearised prior to transfections with a proper restriction enzyme, and the enzyme inactivated by heat. After 24 hours cells were split in 24-well plates and the antibiotic for selection added (500 μg/ml of G418 or 500 μg/ml of hygromycin, as required). Approximately 1 week after, individual clones of cells were selected and put in 96-well plates for expansion.

2.3 Cell analysis and functional assays

Flow cytometry studies

Cells and platelets were resuspended in Tyrodes-Hepes or PBS buffer containing 1% HSA and 0.02% sodium azide. For some experiments, cells were fixed with 3.7%

formaldehyde for 30 min and platelets with 1% formaldehyde during 1 hour, followed by 10 min incubation with 50 mM NH_4Cl, in order to neutralize aldehyde groups of the fixer. All incubation times were performed for 30 min unless otherwise indicated. For GPVI detection, cells and platelets were pre-incubated with either 10 μg/mL of anti human FcγRIIA receptor monoclonal antibody IV.3 or anti-mouse FcγRII-III, to avoid non-specific binding to the Fc receptor. Cells were incubated with 20 nmol/L convulxin, washed and incubated with 0.4 μg/mL anti-convulxin antibody, washed again and finally incubated with FITC-conjugated anti-rabbit IgG secondary antibody diluted 1:500. Incubation with convulxin was omitted to obtain background fluorescence. For GPIIIa detection, cells were incubated on ice with FITC-conjugated anti-GPIIIa antibody or its FITC-conjugated isotype control.

For GPIIb detection in primary cultures, cells were incubated for 30 min with anti-mouse GPIIb antibody or its isotype control (1:100), washed and incubated with FITC-conjugated anti rat IgG (1:500).

For ploidy analysis, cells were resuspended in Tyrodes-Hepes buffer or PBS and 50 μg/ml propidium iodide were added. Cells were permeabilised with NP-40 (0.1%) and analysed immediately using a FACScalibur (Becton Dickinson). Data were recorded and analysed using CellQuest software.

Measurement of intracellular Ca^{2+} concentration

HEL, K562 cells and mouse megakaryocytes, identified on the basis of size and morphology, were viewed on an inverted microscope. Single cell digital imaging of $[Ca^{2+}]_i$

was performed on an Axiovert 110 microscope (Zeiss, Germany) capable of fluorescent and brightfield imaging. Fluorescence video images were captured at excitation wavelenghts of 340 and 380 nm with emission at 510 nm. Calculation of $[Ca^{2+}]_i$ from the 340:380 ratio was performed by the use of a previously established calibration curve using Ca2+ standard solutions. Analysis was performed using Openlab software. Cells were stimulated with 20 nM convulxin. All experiments were performed at room temperature. Results are shown as the mean ±S.E.M. of at least three independent experiments. Statistical indications were made using Student's t-test.

Immunofluorescence

Attached cells grown onto coverslips were fixed for 30 min with PBS containing 3.7% methanol-free formaldehyde, washed and incubated for 10 min with PBS containing 50 mM NH_4Cl, in order to neutralize aldehide groups of the fixer. After washing, cells were blocked with 1% BSA or HSA at room temperature for 1 hour. Incubations with antibodies were carried out for 1 hour at 37°C. Before mounting on slides, cells were incubated for 10 min with DAPI for nuclear staining. Cells were visualized in a Zeiss microscope and images recorded with Openlab software.

2.4 Protein studies

Protein concentration measurement

A protein concentration curve was made using increasing concentrations of BSA mixed with Bradford reagent to a final volume of 1 ml, incubated at room temperature for 5 minutes and then read at 595 nm in a Ultrospec 2100 pro spectrophotometer. 2 µl of a

cell protein lysate was then mixed with Bradford reagent to a final volume of 1 ml and read at 595 nm. Absorbance values obtained were extrapolated in order to obtain protein concentration after multiplying by dilution factor (x500).

Immunoprecipitation

$2\text{-}5\times10^6$ cells were used per immunoprecipitation. Proteins were extracted by resuspending the cells in cold-lysis buffer (2X lysis buffer is: 300 mM NaCl, 20 mM Tris, 10 mM EDTA, pH 7.3, 2% (v/v) Triton X-100, and the protease inhibitors 2 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml pepstatin and leupeptin 10 μg/ml, pH 7.3). Samples were rotated at 4°C for 30 min, then centrifuged for 10 min and the supernatant taken for measuring protein concentration. Samples were pre-cleared for 1 hour with 30 μl protein A-sepharose (PAS) (50% vol/vol), centrifuged and supernatant used for immunoprecipitation. Incubations with specific antibodies were carried out for at least 4 hours, after which 30μl PAS were added and incubated for 1 hour. Samples were washed 4-6 times with cold-lysis buffer and resuspended in Laemmli sample buffer.

Affinity precipitation

Affinity precipitation is similar to immunoprecipitation except that a specific ligand (hence affinity), rather than an antibody (hence immuno), is used to pull down the protein of interest. For GPVI affinity precipitation, 5×10^8 platelet-protein extract or 500 μg of protein extract from the cell lines were incubated with 15 nM convulxin for 2 hours. 0.4 μg/ml anti-convulxin antibody were added and incubations carried out overnight, then 30 μl PAS were

added and incubated for 1 hour, and the samples washed 4-6 times with cold-lysis buffer and resuspended in Laemmli sample buffer.

Immunoblotting studies

30μg of whole protein extracts in Laemmli sample buffer per lane, or samples from immunoprecipitations, were loaded and separated by SDS-PAGE using 10% gels, or 12.5% gels for FcR γ-chain detection. For some experiments, 10-18% gradient slab gels were used. Gels were transferred to PVDF membranes and blocked with TBS-T (0.5M Tris, 1.5M NaCl,, 0.1% (v/v) Tween-20, pH 7.4) containing 10% (w/v) BSA for at least 1 hour. Primary and secondary antibodies were diluted in TBS-T containing 5% (w/v) BSA and incubated with western blots. All incubation times were 1 hour at room temperature. After washing, blots were developed using an enhanced chemiluminescence (ECL) detection system.

Ligand blotting

As above, a specific ligand was used to detect the protein of interest, For GPVI detection, membranes were blocked for 1 hour using TBS-T containing 5% skimmed milk. Membrane was then incubated with 10nM convulxin dissolved in TBS-T for 1 hour at room temperature, washed and incubated with anti-convulxin antibody for 1 hour. After washing again, membrane was incubated for 1 hour with secondary antibody and washed. All antibodies were dissolved in TBS-T.

2.5 Molecular biology

RNA preparation and RT-PCR

For RNA isolation, platelets were prepared as above but steps were taken to avoid contamination of another cells by taking only the uppermost 1/3 of platelet-rich-plasma (PRP) and filtered for leucocyte removal. Total RNA was extracted from platelets using RNAgents and from cultured cells using Trizol. 5μg RNA sample were reverse transcribed using M-MLV reverse transcriptase and oligo$(dT)_{15}$-primer according to the manufacturer's instructions. One fifth of the reverse transcribed RNA mixture was subjected to PCR amplification using AmpliTaq Gold. Oligonucleotides used for PCR reactions are described in annexe 2. Amplification products were electrophoresed on a 1.2%-agarose gel and visualised by ethidium bromide staining.

Plasmid constructs

cDNAs coding for wild type human GPVI (F1), GPVI mutated in the transmembrane arginine residue to alanine (R272A) (FA) or with the cytoplasmic tail deleted (A288STOP) (C1) were cloned into HindIII/XbaI sites of pRc plasmid (Invitrogen). All GPVI constructs were a generous gift of Dr. Masaaki Moroi (Kurume University, Kurume, Japan)

pMG plasmid (InvivoGen) is a bicistronic plasmid for simultaneous expression of two different proteins. The cDNA coding for human FcR γ-chain was subcloned into SmaI/XbaI sites of multi-cloning site 1.

pEGFP plasmid, coding for the green fluorescent protein (GFP), was supplied by Clontech. A chimeric protein GPVI-GFP was made by inserting the cDNA of GPVI into EcoRI site of EGFP plasmid.

pEF plasmid containing the cDNA for LAT-myc (Boerth et al., 2000), the cDNA for SLP-76 (Boerth et al., 2000) or the cDNA for the chimeric protein LAT/SLP-76 (Boerth et al., 2000), were generously provided by Gary Koretzky (University of Pennsylvania School of Medicine, Philadelphia, USA) .

Annexe 1. List of antibodies

Antibody	Modifications	Source	Species	Dilution*
Anty-phosphotyrosine 4G10		Upstate biotechnology	mouse/monoclonal (purified)	WB: 1:1000
Anty-human Syk N-19		Santa Cruz	rabbit/polyclonal (purified)	WB: 1:200
Anty-human Syk		Mike Tomlinson (USA)	rabbit/polyclonal (serum)	IP: 1:500
Anti-human PLCγ2 Q-20		Santa Cruz	rabbit/policlonal (purified)	WB: 1:500
Anti-human PLCγ2		Mike Tomlinson (USA)	rabbit/polyclonal (serum)	IP: 1:500
Anti-human LAT		Upstate biotechnology	rabbit/polyclonal (purified)	IP: 1:250; WB: 1:500
Anti-human SLP-76		Gary Koretzky (USA)	sheep/polyclonal (serum)	IP: 1:500; WB: 1:500
Anti-human Btk		Mike Tomlinson (USA)	rabbit/polyclonal (serum)	IP: 1:500; WB: 1:750
Anti-human Gads		Jon McGlade	rabbit/polyclonal (purified)	WB: 1:250
Anti-human FcR γ-chain		Upstate biotechnology	rabbit/polyclonal (purified)	WB: 1:500
Anti-human Lyn		Transduction laboratories	rabbit/polyclonal (purified)	IP:
Anti-mouse IgG	HRP-conjugated	Amersham	sheep/polyclonall (purified)	WB: 1:10000
Anti-rabbit IgG	HRP-conjugated	Amersham	donkey/polyclonal (purified)	WB: 1:10000

Antibody	Modifications	Source	Species	Dilution
Anti-human GPIIIa	FITC-conjugated	DAKO	mouse/monoclonal (purified)	F: 1:100
IgG1	FITC-conjugated	DAKO	mouse/monoclonal (purified)	F: 1:100
Anti-mouse GPIIb		Pharmingen	rat/monoclonal (purified)	F: 1:100
IgG2a		Pharmingen	rat/monoclonal (purified)	F: 1:100
Anti-convulxin		Mireille Leduc (Framce)	rabbit/polyclonal (purified)	WB, IP and F: 1:10000
Anti-rabbit IgG $F(ab')_2$	FITC-conjugated	Sigma	goat/polyclonal (purified)	F: 1:500
Anti-rabbit IgG $F(ab')_2$	R-phycoerythrin-conjugated	Sigma	goat/polyclonal (purified)	F and IH: 1:500
Anti-mouse FcγRII-III		Pharmingen		F: 10 µg/ml
Anti-human FcγRIIA (IV.3)			mouse/monoclonal (purified)	10 µg/ml
Anti-mouse IgG		Sigma	Rabbit/polyclonal (purified)	30 µg/ml

* WB: western blotting
IP: immunoprecipitation
F: flow cytometry
IH: immunohystochemistry

Annexe 2. Oligos

GPVI:

5'-AACCATGTCTCATCCCCGACC-3'

5'-CCGCTCGAGTGAACATAACCCGCG-3' (1042-bp fragment).

5'-AACCATGTCTCCATCCCC-3'

5'-TTCAGCGGTCATGAACATAA-3' (1034-bp fragment).

β-actin:

5'-TACCACTGGCATCGTGATGGACT-3'

5'-TCCTTCTGCATCCTGTCGGCAAT-3' (506-bp fragment).

Glycophoryn A

5'-AGCATCAAGTACCACTGGT-3'

5'-TTAAAGGCACGTCTCTGTC-3' (359-bp fragment).

GPIIIa

5'-AGATGCGAAAGCTCACCA-3'

5'-TGAGCTCACTATAGTTCTG-3' (553-bp fragment).

Hypoxanthine phosphoribosyltransferase (HPRT)

5'-AGTGATGATGAACCAGGT-3'

5'-GGCTTTGTATTTTGCTTTTC-3'. (620-bp fragment).

Chapter 3

GPVI expression in human and mouse megakaryocytes

3.1. Aim

The aim of this chapter was to establish whether (i) different human megakaryoblastic cell lines expressed GPVI, (ii) their stage of differentiation, and (iii) the possibility of transfection. Moreover, megakaryoblastic cell lines and mouse bone marrow cells were differentiated *in vitro* in order to (iv) establish the stage of differentiation at which GPVI is expressed. An additional aim was to study whether (v) GPVI was capable of generating functional responses in cell lines when cross-linked with the GPVI-specific ligand convulxin.

3.2. Introduction

GPVI is the collagen receptor underlying activation of platelets, and the signaling pathway generated by this receptor is a matter of intense research. Due to the lack of a nucleus and small size, many of the modern molecular and cell biology techniques cannot be applied to the platelet. Therefore an alternative system where genetic manipulation is possible is vital to further understand the mechanisms of regulation of GPVI. One logical option as an alternative system is the precursor of platelets, the megakaryocyte, because they share a variety of surface markers and functional responses. Megakaryocytes originate in the bone marrow from pluripotent stem cells through a differentiation process that involves stem-cell commitment, nuclear polyploidization and cytoplasmic maturation leading to the production of platelets. Due to their low number and fragility, their isolation and study is difficult, although techniques describing the isolation and *in vitro* megakaryocytic differentiation of bone marrow cells have developed, partly overcoming this problem (Debili et al., 1995a; Murphy and Leavitt, 1999). One other difficulty is access to human bone marrow samples on a regular basis.

A number of megakaryoblastic cell lines have been characterised. Although each has unique features which distinguish them from bone marrow megakaryocytes, they can be induced to undergo differentiation to varying degrees in the presence of cytokines and growth factors, and by phorbol esters such as PMA (Long et al., 1990, Roth et al., 1988, Papayannopoulou et al., 1987). Thus, PMA stimulates further megakaryocytic development, resulting in an inhibition of cell proliferation, nuclear polyploidization, and an increase in the expression of platelet/megakaryocyte proteins such as the integrin GPIIb/IIIa (CD41/CD61) (Long et al., 1988, Garcia et al, 1996, Tabilio et al., 1984). Human megakaryoblastic cell lines, derived from patients with leukaemia, have proven useful in the study of the biology, biochemistry and differentiation of megakaryocytes. To date, eighteen human cell lines with varying megakaryocytic properties have been reported (Caen and Han, 1997). Almost all megakaryocytic cell lines have been reported during the past 15 years. This is partly due to the fact that specific markers for a megakaryocytic lineage became established during this period of time.

GPVI is the collagen receptor underlying aggregation of platelets as shown by the selective impairment of response in GPVI-deficient patients (Arai et al., 1995, Ichinohe et al., 1997). Crosslinking of GPVI is associated with tyrosine phosphorylation of a number of proteins such as FcR γ-chain, Syk and PLCγ2, and subsequent increase in the levels of the messengers inositol 1,4,5-trisphosphate, 1,2-diacylglycerol, phosphatidylinositol 3,4,5-trisphosphate and intracellular Ca^{2+}, leading to platelet shape change and aggregation (Gibbins et al., 1996 and 1997, Poole et al., 1997, Tsuji et al., 1997, Polgar et al., 1997). GPVI can be selectively activated by the snake venom toxin convulxin, a C-type lectin isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus.*

Convulxin is comprised of two subunits, α and β, joined by disulphide bridges in a α3β3 structure (Leduc et al., 1998).

The data in this chapter demonstrate that GPVI is present at low levels in different megakaryoblastic cell lines, and that the levels of expression are higher in those cell lines exhibiting a more differentiated phenotype. In addition, this chapter identifies those cells that can be transfected using a plasmid encoding for GFP as a reporter protein. Two of these cell lines, namely HEL and CMK, were chosen due to their inmature megakaryocytic phenotype and the possibility to further differentiate them by means of phorbol ester stimulation. This chapter shows that expression of GPVI is up regulated during differentiation of HEL and CMK cells, leading to potentiation of functional responses. Further, primary cultures of mouse megakaryocytes differentiated *in vitro* from bone marrow cells were used to confirm the increase in expression and up regulation of activation with the stage of differentiation.

Results

3.3. Expression of GPIIIa and GPIIb in megakaryoblastic cell lines

Expression of the platelet/megakaryocyte markers GPIIIa and GPIIb was measured by flow cytometry using specific antibodies. Table 3.1 describes the presence of these proteins and their relative level of expression. GPIIIa was barely detectable in K562 and UT-7/c-mpl cells, while it was present at a low level in CMK cells. CHRF-288, HEL, MEG-01 and DAMI cells expressed a higher level of GPIIIa. On the other hand, GPIIb was absent in UT-7/c-mpl, K562 and CMK cells, and present at a low level in HEL and MEG-01. Only CHRF-288 expressed the protein at high levels. Among the cell lines, UT-7/c-mpl, CMK and K562 were the three cell lines with the lowest levels of GPIIIa and absence of

	GPIIIa	GPIIb
platelets	331.82	—
CMK	11.85	-0.36
CHRF-288	129.1	37.48
DAMI	66.33	8.89
HEL	52.79	7.74
K562	2.83	-0.15
MEG-01	22.23	5.32
UT-7	-3.3	1.49

GPIIIa

Platelets

K562

CHRF-288

Table 3.1. Relative expression of the megakaryocytic markers GPIIIa and GPIIb as measured by flow cytometry. Figures indicate median fluorescence values after subtracting basal fluorescence. GPIIb expression on platelets was not measured. An example of GPIIIa expression on platelets, K562 cells and CHRF-288 cells is shown on the right hand side, where shaded area represents fluorescence obtained with an anti-GPIIIa antibody, and non-shaded area is fluorescence obtained with an isotype control. Table shows one result representative of two independent experiments.

expression of GPIIb, whereas the other cell lines expressed both markers at various degrees. Bearing in mind that GPIIIa and GPIIb co-express on the surface, the difference in the absolute numbers obtained by flow cytometry may be due to differences in the antibodies used to detect either receptor.

3.4. PMA-induced endoreplication of megakaryoblastic cell lines

One characteristic feature of the megakaryocyte is its ability to endoreplicate, a process by which the cell increases its size and ploidy. Endoreplication can be induced *in vitro* by treatment with phorbol esters such as PMA, resulting in a cell resembling the megakaryocyte (Roth et al., 1988). Cell ploidy can be measured using propidium iodide, a reagent that binds to DNA and can be measured by flow cytometry (Garcia et al., 1996).

Megakaryocyte cell lines were induced to undergo megakaryocytic differentiation using 10 nM PMA and ploidy measured by flow cytometry (Table 3.2). PMA induced an increase in ploidy of CMK, HEL, DAMI, CHRF-288 and MEG-01 cells, which was evident after 48 hours and pronounced after 72 hours. HEL and DAMI cells reached ploidy values of 16n after 72 hours of PMA treatment, whereas CMK, MEG-01 and CHRF-288 reached a ploidy of 8n. Only a small population (3%) of K562 reached over 4n values, and UT-7/c-mpl cells died under these conditions.

Although the cell lines studied displayed expression at varies degrees of the megakaryocytic markers GPIIb and GPIIIa and were capable to endoreplicate with PMA, it is difficult to place them without doubt at an exact stage of differentiation. For instance, MEG-01 and CHRF-288 are the only cell lines in this study that are not expressing the erythroid marker glycophorin A (Ogura et al., 1988; Fugman et al., 1990), which could induce to believe that these two cell lines are more differentiated than the others. However,

	no PMA	72h PMA
CMK	2n 46% 4n 31.52% >4n 2.13%	2n 31.67% 4n 30.14% >4n 20.87%
CHRF-288	2n 67.1% 4n 26% >4n 3.3%	2n 41.6% 4n 31.3% >4n 20.22%
DAMI	2n 61.26% 4n 27.56% >4n 5%	2n 35.97% 4n 25.67% >4n 19.51%
HEL	2n 53.35% 4n 41.09% >4n 3.91%	2n 24.40% 4n 38.51% >4n 33.40%
K562	2n 62.23% 4n 34.97% >4n 1.91%	2n 36.72% 4n 38.43% >4n 4.79%
MEG-01	2n 53.02% 4n 17.25% >4n 4%	2n 36.57% 4n 19.88% >4n 20.55%
UT-7	2n 46.76% 4n 31.23% >4n 1.35%	2n 4n >4n

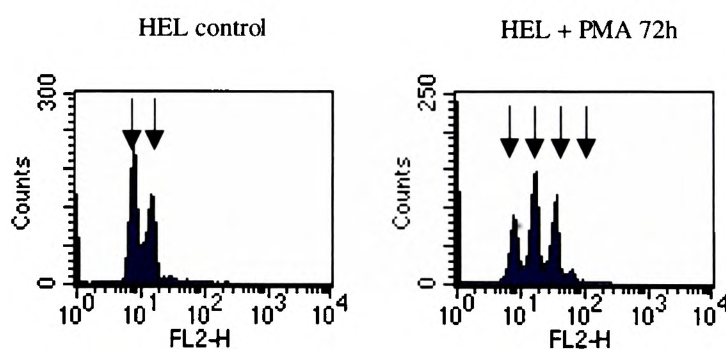


Table 3.2. Ploidy values of human megakaryoblastic cell lines upon differentiation with 10 nM PMA for 72 hours, as measured by flow cytometry using propidium iodide. Figures represent percentage of cells with a given ploidy value within the whole population. Under the conditions used UT-7/c-mpl cells died after PMA treatment and therefore no measures are shown. An example of the histograms obtained from HEL cells is shown below. Arrows indicate a given ploidy value, starting from 2n on the left. Table shows one result representative of two independent experiments.

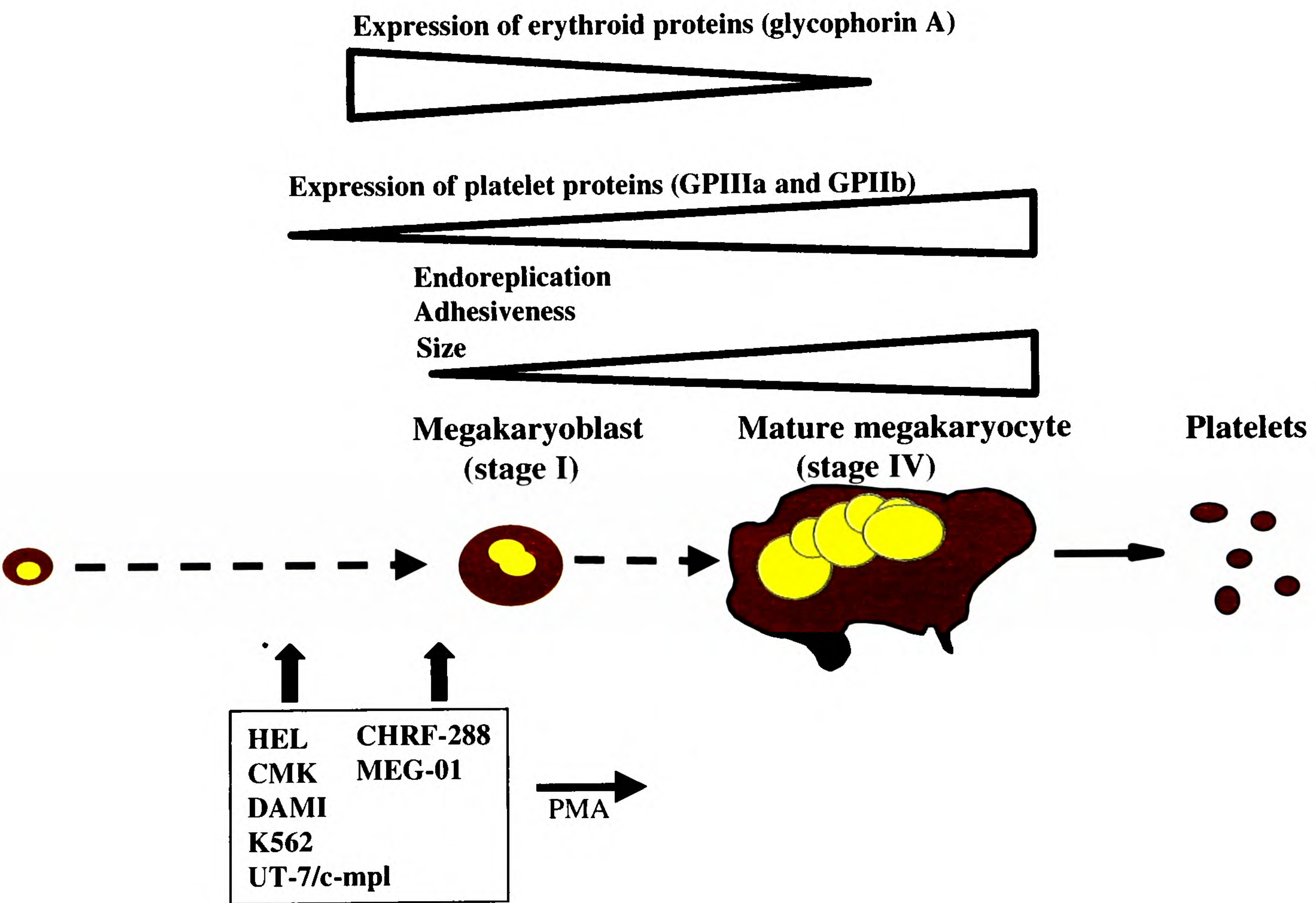


Figure 3.1. Representation of the relative position of human megakaryoblastic cell lines along the megakaryocytopoiesis. Cell lines expressing GPIIb and GPIIIa and are placed before the morphologically recognizable megakaryblastic cell. Stimulation of these cells with 10 nM PMA induces further megakaryocytic differentiation with increase in expression of megakaryocytic markers, decrease in expression of erythroid markers, and polyploidization.

MEG-01 cells express a lower level of GPIIIa than HEL, which express glycophorin A (Martin and Papayannopoulou, 1982) and endoreplicate further (Fig. 3.2). Moreover, other specific markers and morphological features can apply for their classification, such as GPIb expression (the von Willebrand factor receptor), adhesiveness to a plastic surface and increase in size. Although placing the different cell lines at a specific stage of differentiation seems therefore difficult, these can be broadly placed before the megakaryoblastic cell (stage I), with MEG-01 and CHRF-288 cells slightly further differentiated on the grounds of absence of glycophorin A and adhesive phenotype (Figure 3.1).

Once the relative stage of megakaryocytic differentiation of the cells was established, expression of GPVI was studied by flow cytometry and by RT-PCR.

3.5. GPVI is expressed in different megakaryoblastic cell lines

The expression of GPVI was detected by RT-PCR, and by flow cytometry using the GPVI-specific ligand convulxin and an antibody to convulxin. Total RNA from K562, HEL, CMK and CHRF-288 cells, which represent the different stages of differentiation, was obtained and reverse transcribed to obtain the cDNA. The same procedure was used with the T cell line Jurkat, the B cell line Daudi, and the promonocytic cell line U937, to check whether other haematopoietic cell lineages expressed mRNA for GPVI. Semiquantitative PCR using specific primers to GPVI revealed the presence of mRNA for GPVI in all of the megakaryoblastic cell lines, but not in the other haematopoietic lineages (Fig 3.2). The level of expression of mRNA in megakaryoblastic cells varied from low in HEL, K562 and CMK to high in CHRF-288. Although the conditions used to carry out the PCR reaction were not quantitative, this was repeated several times under exactly the same conditions, always obtaining a similar result as exemplified in figure 3.2.

Expression of GPVI in the different cell lines was further analyzed by flow cytometry using convulxin and an antibody to convulxin. GPVI was barely detectable in UT-7/c-mpl, K562 and CMK cells (Table 3.3), and expression was slightly higher in HEL, MEG-01, DAMI and CHRF-288 cells, although always the level of expression was low relative to platelets (Table 3.3).

3.6. GPVI and FcR γ-chain expression are up-regulated upon PMA-differentiation of HEL and CMK cells

To further characterise the expression of GPVI during megakaryocytic differentiation, HEL and CMK cells were stimulated with PMA. The majority of non-differentiated HEL and CMK cells had ploidy values of 2n (representative of cells in phase G1 of the cell cycle) and 4n (cells in G2/M). When exposed to PMA for 3 days, the ploidy values ranged from 2n to 16n confirming that differentiation had occurred (table 3.2). Expression of GPVI mRNA increased on differentiation as measured by RT-PCR. This was accompanied by an increase in mRNA encoding GPIIIa, a marker of megakaryocytic differentiation, and a decrease in mRNA encoding glycophoryn A, a marker of erythroid differentiation (Fig. 3.3). When the cells were induced to undergo erythroid differentiation using hemin (Hong et al., 1996), expression of mRNA for GPIIIa was down regulated and mRNA for GPVI was no longer detectable (Fig. 3.3). In contrast, the level of the "house-keeping" protein hypoxanthine phosphoribosyltransferase (HPRT) did not change significantly upon differentiation with PMA or hemin as measured by RT-PCR (Fig. 3.3). Flow cytometric studies demonstrated that surface expression of GPVI and GPIIIa was also increased in both cell lines during megakaryocytic differentiation (Fig. 3.4). In addition, ligand and western blotting studies in HEL cells revealed an up regulation of the glycoprotein receptor and its associated FcR γ-chain, whereas expression of Syk did not

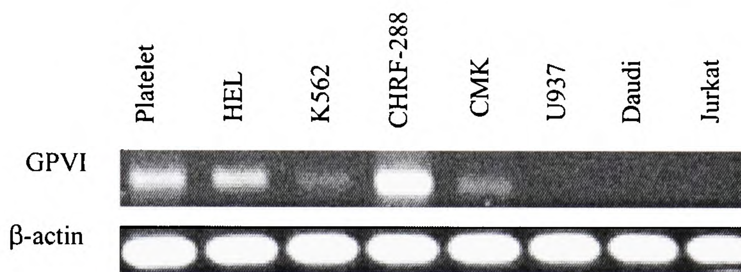
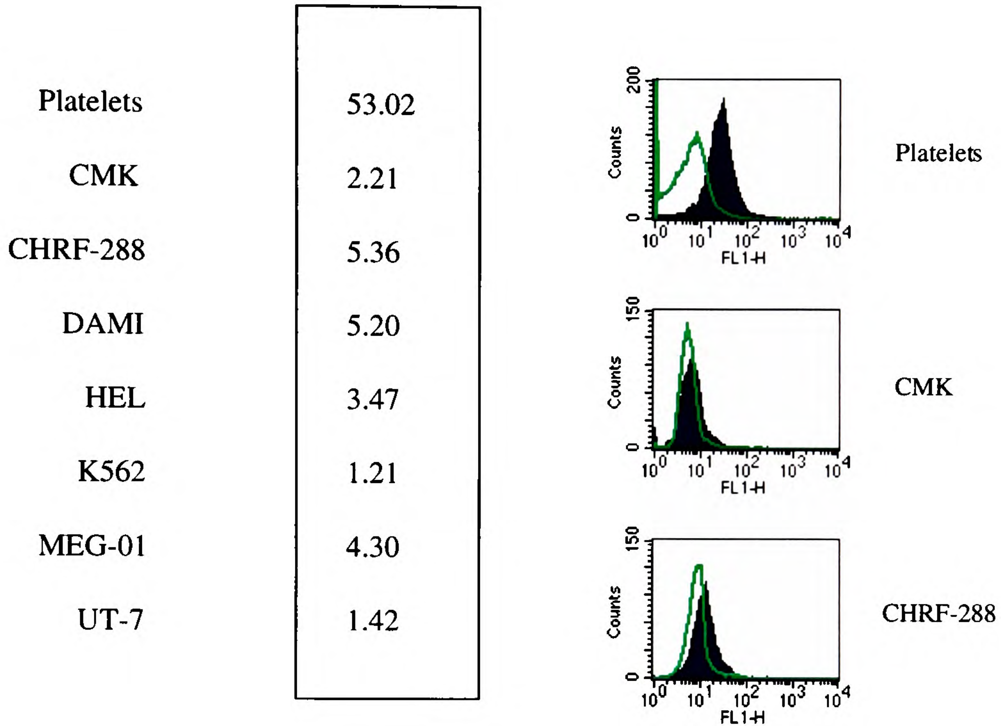


Figure 3.2. GPVI expression in haematopoietic cell lines and platelets. 2 μg RNA were reverse transcribed and 1/5 of the cDNA obtained used for 40 cycles PCR amplification. RT-PCR products were separated by agarose gels and stained with ethidium bromide to visualise GPVI and β-actin. mRNA for GPVI was not detected in non-megakaryoblastic cell lines. Among the megakaryoblastic cell lines GPVI was detected at a low level in HEL, K562 and CMK, and at a high level in CHRF-288. Figure shows one result representative of five independent PCRs.

GPVI



Cell type	GPVI
Platelets	53.02
CMK	2.21
CHRF-288	5.36
DAMI	5.20
HEL	3.47
K562	1.21
MEG-01	4.30
UT-7	1.42

Table 3.3. GPVI expression in human megakaryoblastic cell lines. Expression of GPVI was measured by flow cytometry using convulxin and an antibody to convulxin, then indirectly detected with a secondary FITC-conjugated anti-rabbit IgG. Background fluorescence was obtained in the absence of convulxin.·Figures represent median fluorescence value after substracting background fluorescence. Platelet GPVI-measurement is included for comparison. On the right hand side some examples of the histograms obtained by flow cytometry are shown, where shaded area represents fluorescence obtained with convulxin and non-shaded area is background fluorescence obtained in the absence of convulxin. Table shows one result representative of two independent experiments.

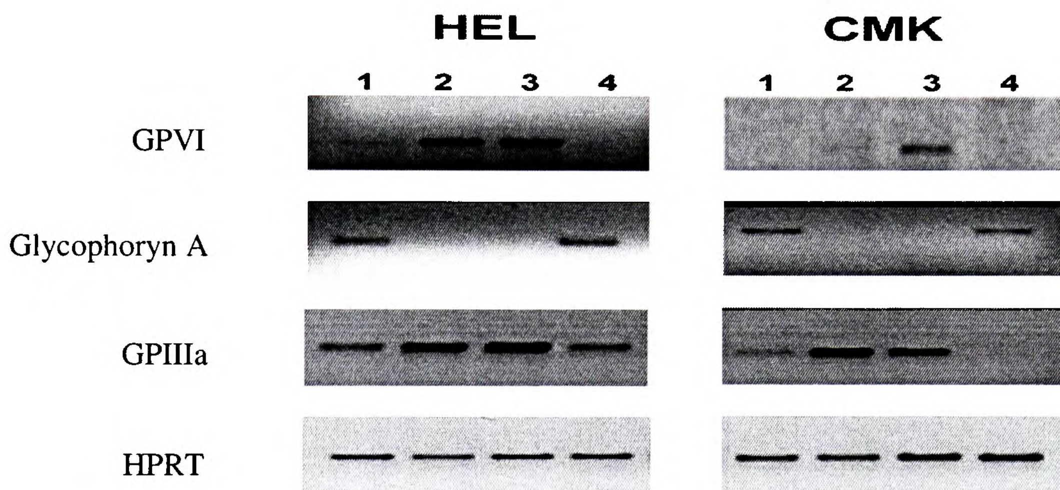


Figure 3.3. RT-PCR of GPVI in HEL and CMK cells upon PMA and hemin differentiation. Semi-quantitative RT-PCR of HEL and CMK cells showing expression of GPVI, glycophoryn A, GPIIIa (CD61) and HPRT before (lane 1) and after mekakaryocytic differentiation with PMA for 1 day (lane 2) and 3 days (lane 3) or erythroid differentiation using hemin for 3 days (lane 4).Figure shows one result representative of three independent experiments.

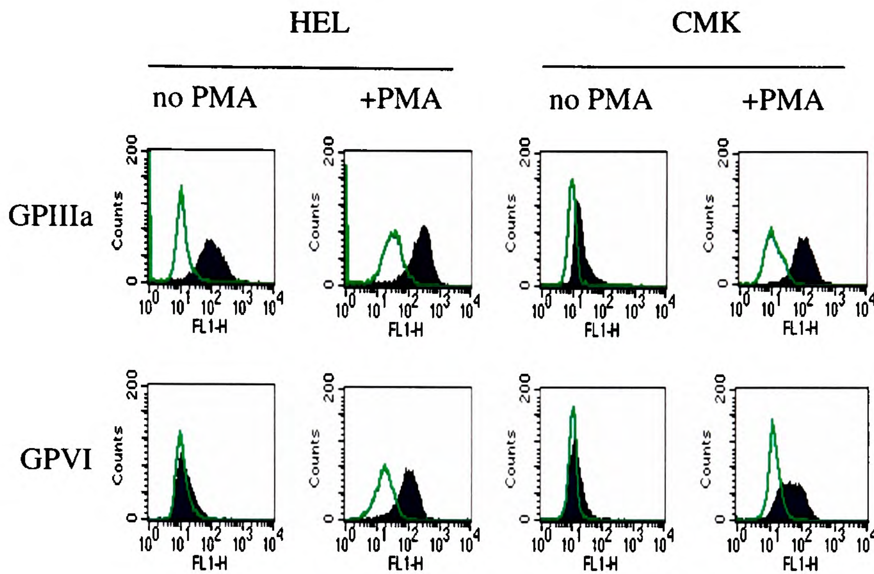


Figure 3.4. Surface expression of GPVI and GPIIIa (CD61) in HEL and CMK cells. HEL and CMK cells were differentiated with 10 nM PMA for 3 days and expression of GPVI detected by flow cytometry using convulxin, an antibody to convulxin and indirectly labelled with an anti-rabbit IgG conjugated with fluoresceine. GPIIIa expression was detected using a fluoresceine-conjugated anti-GPIIIa antibody. Figure shows one result representative of three independent experiments.

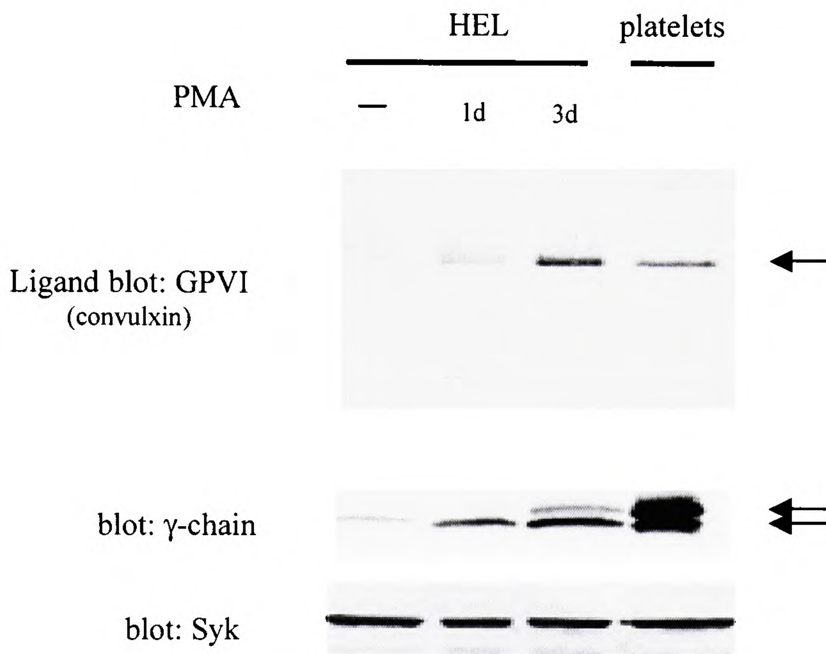


Figure 3.5. Ligand and western blotting of GPVI and FcR γ-chain in HEL cells. Cells were differentiated with10 nM PMA for 1 and 3 days (1d, 3d). 30 μg of HEL extract was loaded per lane and subjected to SDS-PAGE. GPVI was detected by ligand blotting using convulxin and an antibody to convulxin. FcR γ-chain expression was assessed by immunoblotting. Arrows indicate the relative position of GPVI (55 kDa) and FcR γ-chain (17-19 kDa). A platelet sample is included as a positive control; the platelet number used in either blot was different. The level of Syk measured by western blotting is also shown for comparison. Figure shows one result representative of two independent experiments.

change significantly (Fig. 3.5). Maximal increase in the expression of GPVI and FcR γ-chain was reached after 3 days of exposure to PMA. The correlation of expression of GPVI and FcR γ-chain corresponds to the observation made on platelets from patients deficient for GPVI (Tsuji et al., 1997).

3.7. GPVI stimulation with convulxin promotes increase in tyrosine phosphorylation in HEL cells

In order to investigate whether GPVI was functional on HEL cells, we measured tyrosine phosphorylation and $[Ca^{2+}]_i$ elevation in response to convulxin in differentiated and non-differentiated cells. In non-differentiated cells there was a slight increase in the overall level of tyrosine phosphorylation in response to convulxin, which was clearly seen after 90 sec of stimulation and maintained up to 270 sec (Fig. 3.6). The increase in tyrosine phosphorylation in differentiated cells was stronger and more rapid, being evident after 30 sec of convulxin stimulation and peaking at 90 sec. Major protein bands of 36, 72, 76, 80, 100, 130 and 148 kDa underwent increases in tyrosine phosphorylation on convulxin stimulation in cells differentiated with PMA, whereas in non-differentiated cells only weak increases in the phosphorylation of these bands was seen. Increases in phosphorylation of several other, more minor bands was also seen. This increase in tyrosine phosphorylation corresponds to the increase in expression of GPVI with differentiation. The identity of some of the proteins which underwent increases in tyrosine phosphorylation in response to convulxin stimulation was assessed following immunoprecipitation. The bands of 36, 72 and 148 kDa were shown to contain LAT, Syk and PLCγ2, respectively (Fig. 3.7). Additionally, FcR γ-chain was found to undergo tyrosine phosphorylation in response to

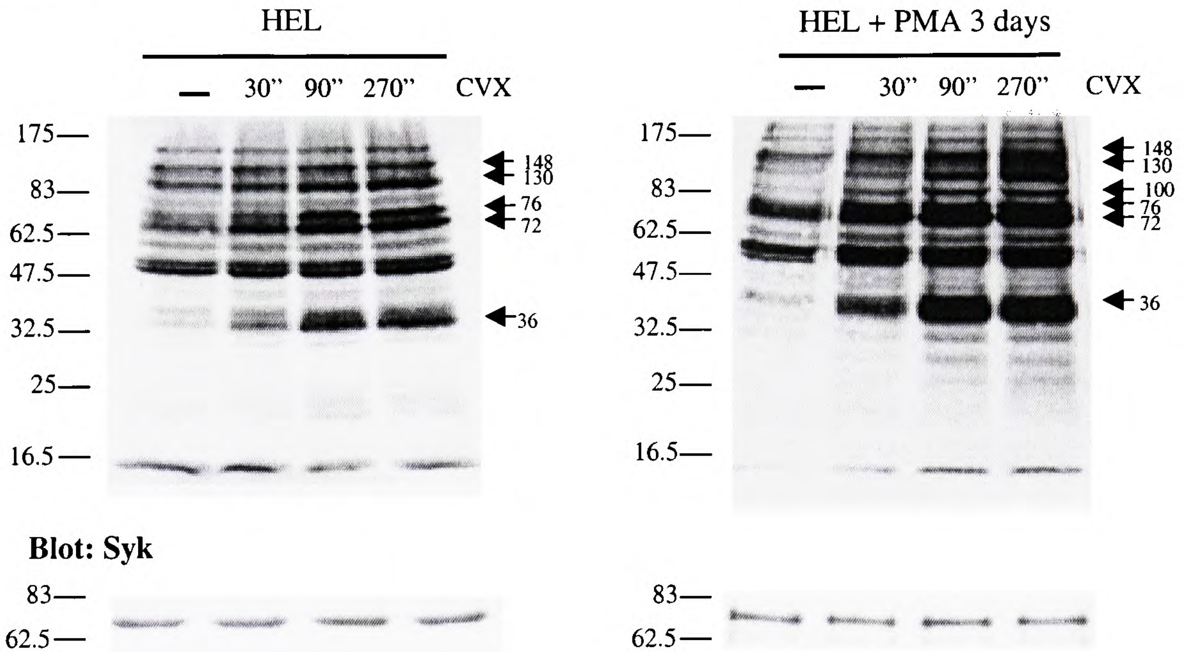


Figure 3.6. Stimulation of HEL cells with convulxin. Control and PMA-differentiated HEL cells for 3 days were stimulated with convulxin 20 nM at different times, lysed and subjected to SDS-PAGE, then blotted for tyrosine phosphorylation using mAb 4G10. Arrows indicate the major tyrosine phosphorylated bands.Membrane was stripped and re-blotted with anti-Syk antibody to ensure equal loading. Figure shows one result representative of three independent experiments.

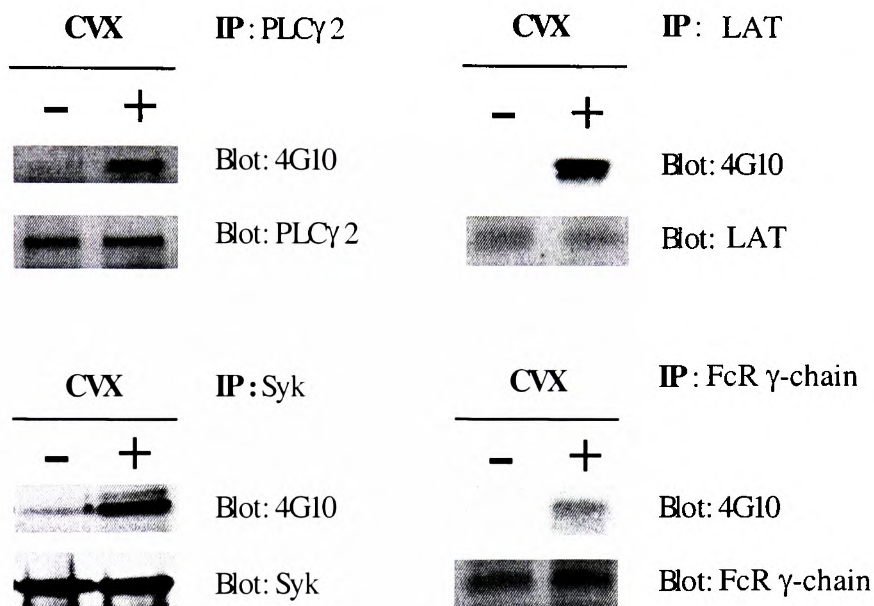
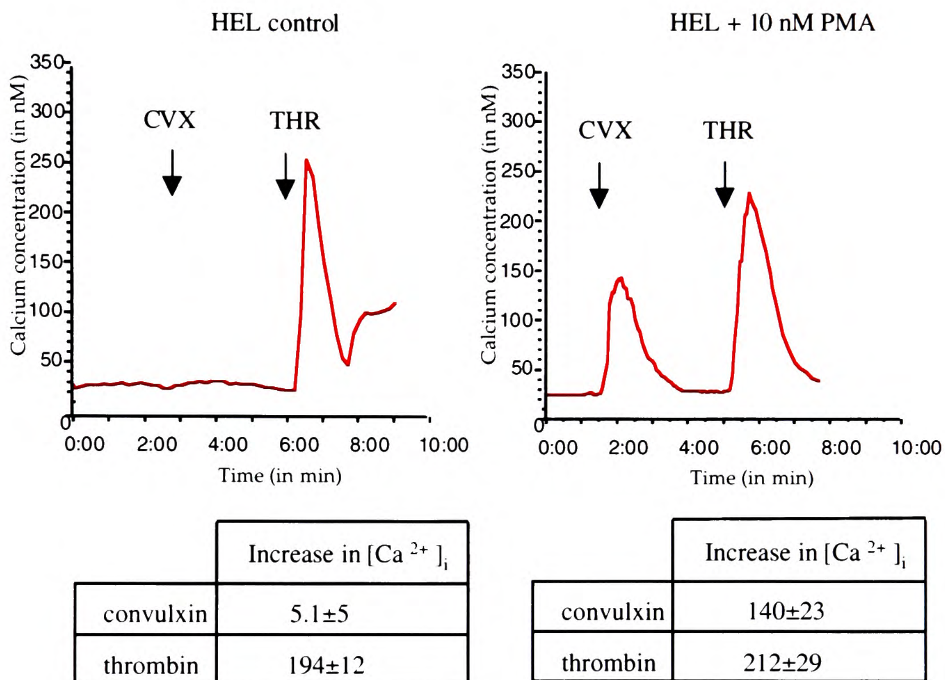


Figure 3.7. Phosphorylation of PLCγ2, LAT, Syk and FcR γ-chain in HEL cells. Differentiated HEL cells using 10 nM PMA for 3 days were stimulated with convulxin 20 nM for 90 sec and the indicated proteins immunoprecipitated with specific antibodies. Phosphorylation of immunoprecipitated proteins was detected by western blotting using mAb 4G10. Membranes were stripped and reprobed to check equal loading. Figure shows one result representative of two independent experiments.



	Increase in $[Ca^{2+}]_i$
convulxin	5.1±5
thrombin	194±12

	Increase in $[Ca^{2+}]_i$
convulxin	140±23
thrombin	212±29

Figure 3.8. Ca $^{2+}$ increase in convulxin-stimulated HEL cells. Control or PMA-differentiated HEL cells were stimulated with convulxin and thrombin, and $[Ca^{2+}]_i$ concentration measured using openlab software. Values indicate increase in $[Ca^{2+}]_i$± SEM (standard error). 20 cells were measured in each experiment. n=4.

convulxin stimulation (Fig. 3.7). This demonstrates that the major proteins undergoing phosphorylation in response to convulxin in HEL cells are the same as those in platelets.

3.8. Convulxin stimulation on PMA-differentiated HEL cells leads to an increase in $[Ca^{2+}]_i$

Cells were loaded with the reporter dye FURA-2, and $[Ca^{2+}]_i$ measured by single cell digital imaging. Cells less than 15 µm in diameter did not undergo a significant increase in $[Ca^{2+}]_i$ in response to convulxin (Fig. 3.8). Less than 0.1% of the cells in the culture were greater than 20 µm in diameter. Of these, the majority responded to convulxin with an elevation in $[Ca^{2+}]_i$. This population is thought to represent cells that have undergo differentiation. In contrast, both populations of small and large cells responded to thrombin with an elevation in $[Ca^{2+}]_i$ (Fig. 3.8). Upon differentiation with 10 nM PMA, approximately 99 % of cells responded to convulxin and thrombin with increases in $[Ca^{2+}]_i$ of 140±23 and 212±29 nM above basal, respectively (Fig. 3.8).

3.9. GPVI expression is up-regulated in mouse megakaryocytes

The expression of GPVI in mouse megakaryocytes derived from bone marrow cells was examined in order to determine if similar observations apply to primary cells. Cells were grown *in vitro* for up to four days in a medium designed to support megakaryocyte differentiation. Staining for acetylcholinesterase detection was used as a marker of megakaryocyte differentiation (Tanum-Jensen et al.,1981). Large, terminally differentiated bone marrow megakaryocytes make up less than 0.1% of total cell number at day 0, but undergo a significant expansion after 4 days *in vitro*, representing 2-5% of the cell population, as demonstrated by acetylcholinesterase staining, a specific marker of murine

megakaryocytes (Fig. 3.9). These *in vitro* grown cells were analysed for expression of GPVI and GPIIb by flow cytometry. Three different cell populations were gated based on their different size and complexity as previously reported (Shiraga et al., 1999). Of these, the population of larger cells, considered to be more mature megakaryocytes, was detectable only after 4 days of culture. A second population, containing cells of intermediate size, and a third population of smaller cells are thought to represent less differentiated megakaryocytes and other cell types (Fig. 3.10). Flow cytometry analysis making double stainings for GPIIb and GPVI demonstrated that after 4 days in culture, nearly 50% of the whole cell population were GPIIb-positive, and of these greater than 5% were GPVI-positive (Fig. 3.10). When cells of different sizes whithin the whole population were analysed, almost 100% of the large cell population were GPIIb-positive, whereas nearly 40% were positive for GPVI. In contrast, between 1-5% of the medium and small cells expressed GPVI (Table 3.4). This suggested a correlation in expression of both proteins, although GPVI was detected in fewer cells relative to GPIIb.

3.10. Convulxin stimulation increases $[Ca^{2+}]_i$ in mouse megakaryocytes

We examined the ability of convulxin to stimulate an increase in $[Ca^{2+}]_i$ in the primary megakaryocytes by single cell digital imaging. Only cells with a size greater than 20 μm were analysed (Fig. 3.11). The cells responded to different degrees, with about 50% of the cells responding to convulxin stimulation with a robust increase in $[Ca^{2+}]_i$, whereas all responded with a strong increase in response to thrombin. The percentage of cells responding to convulxin in this way correlates with the percentage of GPVI-positive megakaryocytes, as detected by flow cytometry.

3.12. GFP transfections in megakaryoblastic cell lines

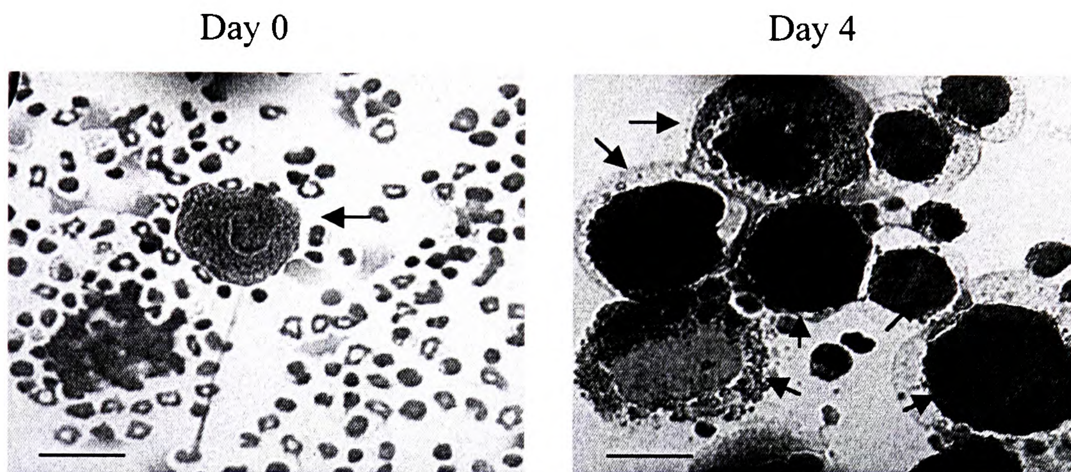


Figure 3.9. Acetylcholinesterase staining of mouse megakaryocytes differentiated *in vitro*. Bone marrow was harvested and cells induced to undergo megakaryocytic differentiation. Figure shows acetylcholinesterase staining before (left panel) and after differentiation (right panel). Arrows indicate mature megakaryocytes, easily distinguished from other cell types by its large size. Bar represents 20 μm. Figure shows one result representative of seven independent experiments.

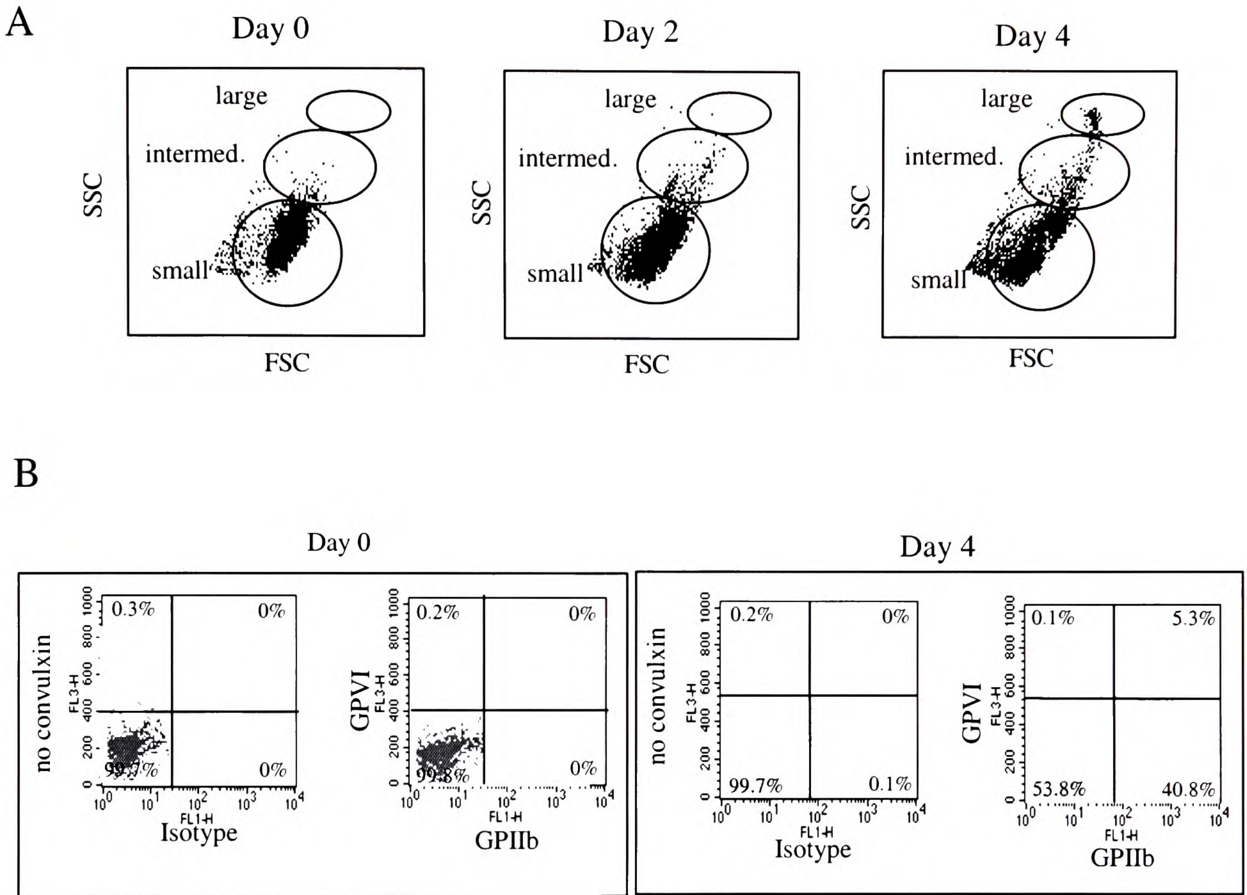
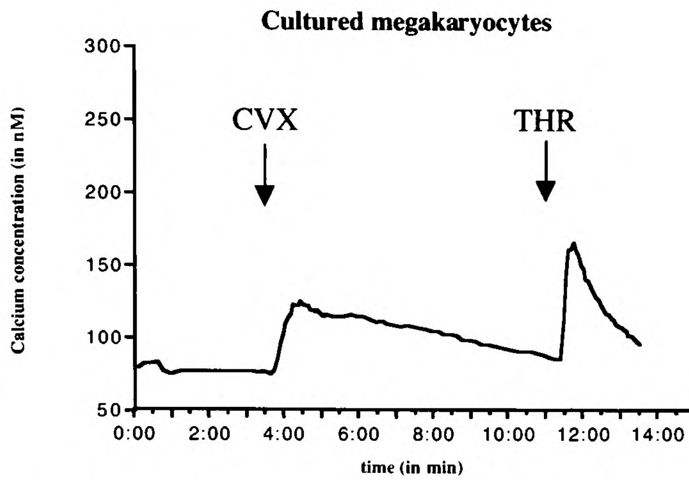


Figure 3.10. FACS analysis of mouse differentiated bone marrow cells. **A**. Dot plots for forward (FSC) and side (SSC) light scatter profiles of 10000 cells. In each blot, three arbitrary analysis gates have been drawn. The gate at the lower includes small cells, the middle gate intermediate-size cells, and the gate at the upper large cells. Note a progressive increase in the proportion of intermediate-size and large cells. **B**. Dot plots from day 0 and 4 cultures showing double staining for GPIIb, using a specific antibody and indirectly labelled with a anti-rat antibody conjugated to FITC, and for GPVI using convulxin, anti-convulxin and indirectly labelled with an anti-rabbit antibody conjugated to R-phycoerythrin. Control plots were obtained using and isotype control antibody to GPIIb or in the absence of convulxin for GPVI. Each dot plot has been divided into 4 different areas, with low-left corner representing double-negative cells, and top-right corner double-positive cells. Note an increase in GPIIb and GPVI expression with time. Figure shows one result representative of five independent experiments.

Table 3.4. Expression of GPIIb and GPVI during *ex vivo* expansion of megakaryocytes

	Day 0	Day 2		Day 4		
	small	small	intermed.	small	intermed.	large
GPIIb	0.5%*	0.25%	75.0%	6.8%	75.0%	96.5%
GPVI	0.1%	0.2%	5.0%	3.1%	4.2%	40.3%

*Values represent the percentage of cells expressing a given surface protein and are the average of three independent experiments



	Increase in $[Ca^{2+}]_i$
convulxin	84±18
thrombin	99±18

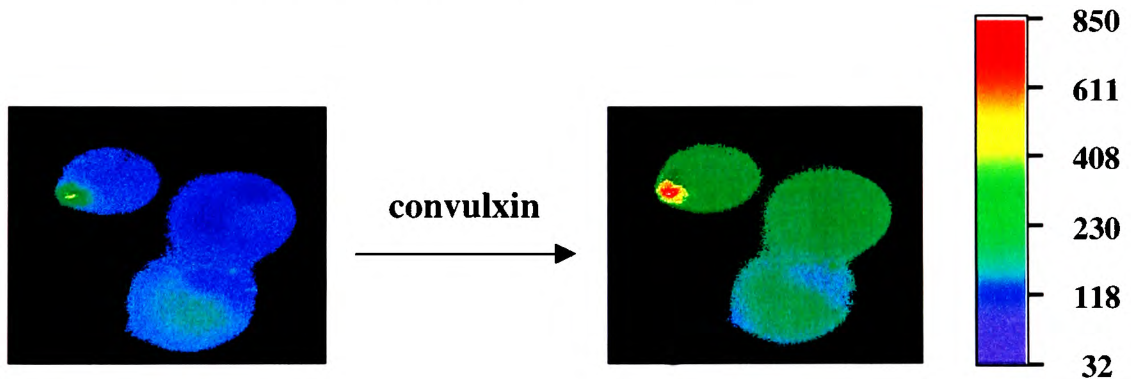


Figure 3.11. Calcium increase in mouse megakaryocytes. *In vitro* differentiated mouse megakaryocytes were analysed for calcium increase by single cell digital imaging. Top panel shows elevation of $[Ca^{2+}]i$ on convulxin and thrombin stimulation, and table indicates the increase above basal level of calcium. A representation of the single cell digital image obtained in the microscope is shown, with a color scale on the right indicating the gradient from basal (blue) to activated cells (green-red). n=5.

Cell line	Condition	Transfection efficiency	Number surviving cells ($x10^6$)
CMK	1	24%	2.5
	2	17%	4.5
	3	0%	0.8
CHRF-288	1	0%	0.2
	2	0%	1.0
	3		
DAMI	1	0%	1.0
	2	0%	1.6
	3	0%	1.0
HEL	1	34%	2.0
	2	16%	5.5
	3	0%	0.35
K562	1	21%	2.5
	2	16%	4.5
	3		
MEG-01	1	21%	2.0
	2	16%	4.0
	3	0%	0.5
UT-7	1	17%	0.6
	2	7%	2.5
	3		

Table 3.5. GFP transfection into human megakaryoblastic cells. $5x10^6$ cells were transfected with 5 μg plasmid DNA encoding for the GFP, and efficiency of transfection and viability measured by flow cytometry and cell counting, respectively, after 48 hours from transfection. Conditions used were as follows; 1: 960μF-300v; 2; 960μF-250v; 3: 25μF-2000v.

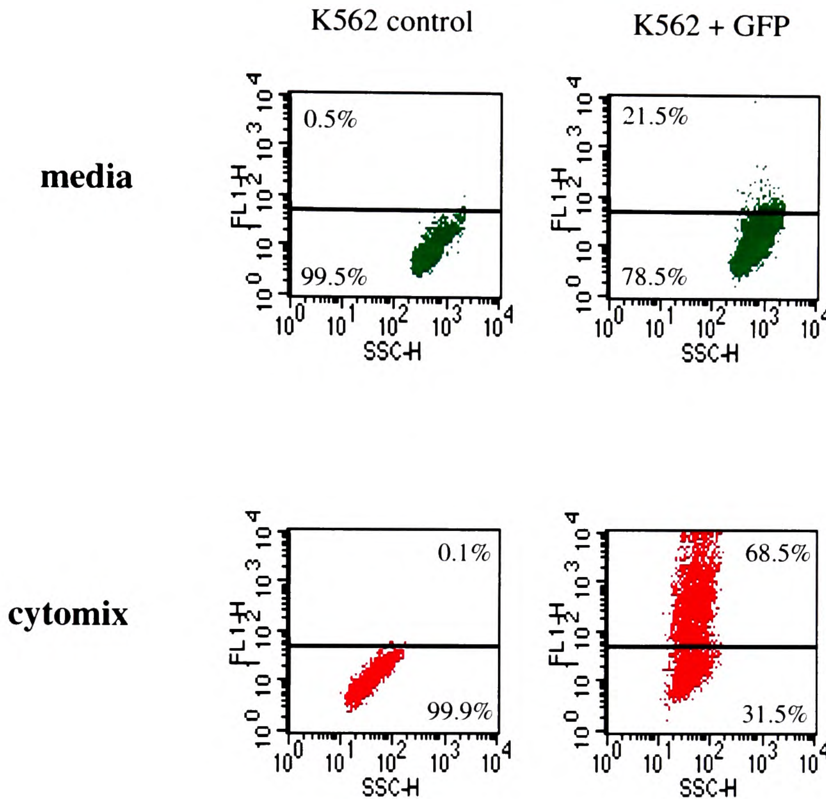


Figure 3.12. Efficiency of transfection in K562 cells. K562 cells were transient transfected by electroporation with the reporter gene for GFP using cells suspended either in serum-free media (top panels) or cytomix bufffer (bottom panels). After 48 hours in culture cells were harvested and efficiency measured by flow cytometry. Panels on the left hand side represent basal fluorescence from non-transfected cells, whereas panels on the right show dot plots obtained from transfected cells. A line has been drawn across each panel to separate cells with background fluorescence at the bottom from cells expressing the reporter gene at the top. Numbers indicate the percentage of cells above or below the line. Figure shows one result representative of 5 independent experiments.

It was important to establish whether the different cell lines were suitable for transfection in consideration of future studies. GFP is a well known reporter protein which can be easily detected by flow cytometry, and can therefore be used to measure the percentage of cells transfected. Although many different transfection methods exist, electroporation is the one preferred by most groups working with these cells (Garcia et al., 1996), and was therefore the method chosen in this thesis.

$5x10^6$ cells were electroporated under three different conditions, i.e. 950 μF-250 v, 950 μF-300 v, and 25 μF-2000 v. Fourty eight hours after transfection cells were counted and analyzed by flow cytometry. In all cases dead cells were removed from the analysis by means of propidium iodide staining. Table 3.5 reflects the efficiency of transfection in each cell line, as well as the capacity of recovery and/or proliferation of the cells after transfection. The maximum efficiency was achieved with HEL cells, with 33% of the cells transfected, whereas MEG-01, CMK, UT-7 and K562 varied between 15-25% efficiency. It was not possible to transfect CHRF-288 and DAMI cells under these conditions. Maximum efficiency was obtained using 950 μF-300 v, although this was also the more severe condition as either less of the cells survive or begin to restart proliferation.

A modified protocol in which cells were resuspended in cytomix buffer prior to electroporation was assessed, and found to increase the efficiency of transfection by more than two, probably due to a greater cell survival (van den Hoff et al., 1992). Figure 3.12 shows a graphic comparison between K562 cells transfected with GFP under either condition.

3.13. Discussion

The results in this chapter demonstrate the presence of GPVI in a number of megakaryoblastic cell lines and the suitability of these cells for transfection. Moreover, expression of GPVI has been shown to be up-regulated towards the end of megakaryocytic differentiation. The expressed receptor is functional as demonstrated with the use of the GPVI-specific agonist convulxin.

Detection of the megakaryocyte/platelet markers GPIIIa and GPIIb, along with determination of ploidy value after megakaryocytic differentiation with PMA, allowed the characterisation of the cells and comparison with previous studies. Although expression of different megakaryocytic markers in the cells have been described, some studies show discrepancies regarding expression of these markers and cell behavior when differentiated with phorbol esters (Leary et al.,1987; Ogura et al.,1998; Molla et al., 1995). These differences are partly due to the particular growth and experimental conditions employed. For instance, depending on the antibody used for detection it has been shown that expression of GPIIIa and GPIIb in HEL cells varies from 34% of the cells with P2 antibody, to 1% with the AC7 antibody (Molla et al.,1995). Accordingly, although GPIIb has been detected in some of the cell lines used in this study, it must be pointed out that the antibody used did not seem to work as expected in comparison with other reports, and therefore the results cannot be taken as conclusive. Rather, it can be said that most cells express GPIIb, although no correlation can be made between the different cell lines screened.

This chapter shows that the cells studied display different phenotypic features, which account for their classification at different stages of megakaryocytic differentiation. Those cells with low or undetectable levels of megakaryocytic markers are considered as more immature, whereas increased expression of megakaryocytic markers and absence of

erythroid markers is considered to be a sign of cells more differentiated. However, each cell line has a unique property with a combination of various erythroid, myeloid and megakaryocytic markers. Thus, it is quite difficult to divide the different cell lines into a specific group without exception. Each of these cell lines is quite possibly biologically different from normal megakaryocytes (Hoffman, 1989). One typical example of the abnormal properties of these cell lines is that they all carry the Philadelphia chromosome, the result of a translocation between chromosomes 9 and 22, leading to the generation of the oncogenic fusion protein BCR-ABL present in chronic myeloid leukaemia patients.

Using flow cytometry, GPVI was detected at various levels among the cell lines studied.However, expression of GPVI was low in all cases. RT-PCR revealed that GPVI was present only in cell lines with some megakaryocytic feature, but was not present in cell lines of other hematopoietic lineages. The possibility arises that GPVI is a specific marker of megakaryocytic differentiation. It has previously been described that GPVI is not present in a number of megakaryoblastic cell lines, namely HEL and MEG-01 (Mountford et al., 1999). There are a number of differences between that study and the present one. In the work by Mountford et al., an antibody to GPVI was used to detect the protein by western blotting, whereas in the present study convulxin has been used to detect GPVI by flow cytometry, in a protocol consisting of three steps of amplification, achieving a considerable increase in the sensitivity of the technique. Keeping in mind the low levels of expression of GPVI in these cell lines, the differences in the respective experimental procedures apply for the differences observed in the results.

In this chapter it has been observed an increase in expression of GPVI in HEL and CMK cells differentiated with PMA. Differentiated HEL cells showed an increase in the level of the FcR γ-chain, indicating that both proteins are up-regulated during the process of differentiation, consistent with their co-expression.

Non-differentiated HEL cells, which express a low level of GPVI, respond with a weak increase in the overall level of tyrosine phosphorylation to convulxin, with only larger cells (<0.1% of the total cell population) exhibiting an increase in $[Ca^{2+}]_i$. In contrast, PMA-differentiated HEL cells respond to convulxin with a powerful increase in tyrosine phosphorylation with nearly all undergoing increases in $[Ca^{2+}]_i$. It is possible that the increase in tyrosine phosphorylation observed in the non-differentiated megakaryocytes is primarily taking place at the sub-population of larger cells which exhibit an increase in $[Ca^{2+}]_i$, and which may represent cells which have undergo differentiation. A previous study on the megakaryocytic cell line DAMI also described an elevation of $[Ca^{2+}]_i$ in response to convulxin, which was increased when GPVI was introduced by transfection (Clemetson et al., 1999). Studies in mouse megakaryocytes grown *in vitro* also demonstrate that expression of GPVI increases upon differentiation, accompanied by an increase in $[Ca^{2+}]_i$ elevation to convulxin.

GPVI is suggested to be a novel marker of megakaryocyte differentiation. It has been reported that in human megakaryocytes, GPVI acts as an early marker of differentiation, co-expressing with GPIIb and GPIa, although its level of expression at this early stage is very weak (Lagrue-Lak-Hal et al., 2001). In the work presented here there seems to be a correlation between GPIIb and GPVI expression in mouse megakaryocytes. Both proteins are mainly detected in intermediate-size and large cells, indicating up-regulation with differentiation. Although GPVI is detected only in 5% and 40 % of the intermediate and large cells, respectively, this may be due to the poor affinity of convulxin for murine GPVI relative to human GPVI (unpublished results) in combination with the low levels of expression at early stages of the differentiation as reported above.

It has previously been reported that non-differentiated human megakaryoblastic cell lines did not respond to collagen or collagen-related peptide (CRP) with an increase in tyrosine phosphorylation or elevation of $[Ca^{2+}]_i$ (Mountford et al., 1999). The major difference between this study and the present one is the use of convulxin. The trimeric convulxin is a much more powerful ligand than collagen or CRP, causing a much greater increase in response in platelets (Asazuma et al., 2000). A similar observation was made by Clemetson et al, who reported that the megakaryocytic cell line DAMI responded to convulxin, but not collagen, through an increase in $[Ca^{2+}]_i$ (Clemetson et al., 1999).

The cell lines were tested for transfection with the reporter protein GFP as it was important to determine the efficiency achieved under the experimental conditions used in this study. Maximum efficiency was detected with HEL cells, where up to 33% of the cells can be transfected, while all the others cell lines rendered efficiency values of no more than 25%. However, the efficiency can be increased by means of cytomix buffer, which rendered a considerable increase in the efficiency of transfection. Only two of the cell lines tested, namely CHRF-288 and Dami, were not possible to be transfected under these conditions.

In conclusion, this chapter shows evidence that GPVI is present in some megakaryoblastic cell lines but not in others, and that the presence of GPVI seems to correlate with the level of expression of other megakaryocytic markers. In addition, all the cell lines tested except two can be transfected and therefore suitable for genetic manipulation. This chapter presents evidence that GPVI is expressed in platelets and megakaryocytes, and that expression increases towards the end of megakaryocyte differentiation. Whilst further work is required to confirm that GPVI is exclusively expressed on platelets and megakaryocytes, the results indicate that GPVI is likely to be a novel marker of megakaryocytopoiesis. The question arises as to why GPVI expression increases with late-stage megakaryocyte differentiation. One possible reason for this is to prevent activation of

the immature, developing megakaryocyte through exposure to surrounding collagen. It is also possible that GPVI plays a role in the end-stage megakaryocyte differentiation/platelet formation. However, GPVI-deficient individuals have normal levels of platelets and show impairment in response only to collagen (Arai et al., 1995, Ichinoche et al., 1997), indicating that the role of GPVI is primarily-linked to the control of platelet function. Engineering of GPVI-deficient mice would enable a detailed investigation of this.

Chapter 4

Studies on GPVI translocation to the membrane and association to the FcR γ-chain

4.1. Aim

The aim of this chapter was to establish the nature of the interaction between GPVI and the FcR γ-chain, and the ability of the receptor complex to signal in a reconstituted system in which different mutant versions of GPVI alone or in combination with the FcR γ-chain have been expressed.

4.2. Introduction

GPVI is the major signaling collagen receptor present in platelets and megakaryocytes. It belongs to the superfamily of immunoblobulin receptors and is closely related to Fcα receptor (FcαR) and natural killer (NK) receptor (Clemetson et al., 1999). GPVI forms a complex with the Fc receptor γ-chain (FcR γ-chain), which is responsible for signalling through GPVI (Tsuji et al., 1997, Gibbins et al., 1997, Poole et al., 1997). Previous reports on two receptors sharing homology with GPVI, FcαR and PIR-A, revealed that they translocate to the membrane independently of the FcR γ-chain in cell lines (Craig Morton et al., 1995; Ono et al., 1999) but that co-expression with FcR γ-chain increases the level of expression of the receptor at the surface (Craig Morton et al., 1995; Hayami et al., 1997). The interaction between the FcαR or PIR-A and the FcR γ-chain occurs in the transmembrane region due to oppositely charged amino acid residues (Taylor and McVicar, 1999; Ono et al., 1999). The ITAM domain within the cytoplasmic tail of the

FcR γ-chain is responsible for signaling after engagement of the receptor complex (Maeda et al., 1998).

Results in this chapter demonstrate translocation of GPVI independently of the FcR γ-chain in COS-7 and K562 cells, and that both the transmembrane arginine and cytoplasmic domain of GPVI are necessary for association with the FcR γ-chain. Moreover, the FcR γ-chain is necessary and sufficient to initiate the signaling events after GPVI engagement as demonstrated in a reconstituted system where both GPVI and FcR γ-chain have been stably expressed.

Results

4.3. GPVI translocates to the membrane independently of the γ-chain in COS-7 and K562 cells

COS-7 cells were transiently transfected with wild type GPVI (COS/F1) or a number of mutant constructs of the receptor. Expression of GPVI was detected by flow cytometry using the GPVI-specific ligand convulxin and an antibody to convulxin (Fig. 4.1.1). COS/F1 or mutant versions in which the transmembrane arginine was mutated to alanine (R272A) (COS/FA) or the cytoplasmic tail depleted (A288STOP) (COS/C1) were expressed on the surface of COS-7 cells with similar efficiency, despite low percentage of transfection (Fig. 4.1.1). Relative amount of each receptor version was similar, as detected by ligand blotting (Fig. 4.1.2). Since GPVI is non-covalently and constitutively associated with FcR γ-chain in platelets (Tsuji et al. 1997), COS-7 cells were co-transfected with the different

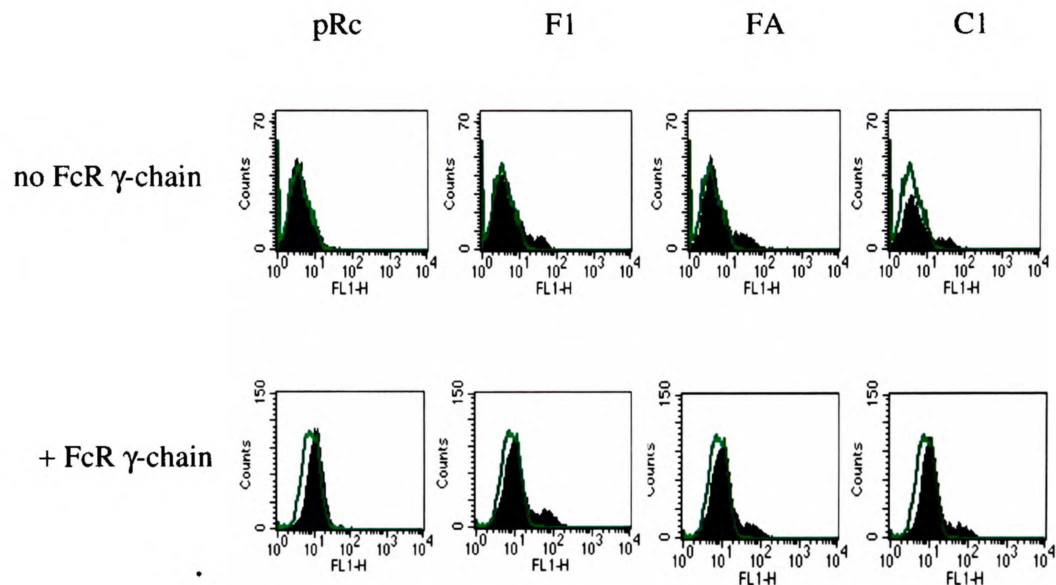


Figure 4.1.1. GPVI translocates to the membrane independently of the FcR γ-chain in COS-7 cells. Cells were transiently transfected with type GPVI (F1), GPVI mutated in a transmembrane arginine (R272A)(FA), or depleted of the cytoplasmic tail (A288STOP)(C1), together (bottom panels) or not (upper panels) the FcR γ-chain. Control cells were transfected with empty plasmid (pRc). GPVI was detected by flow cytometry in the surface of the cells by means of convulxin, an antibody to convulxin and indirectly labeled with a FITC-conjugated anti-rabbit IgG (purple hystograms). Background fluorescence was obtained in the absence of convulxin (green line) Figure shows one result representative of three independent experiments.

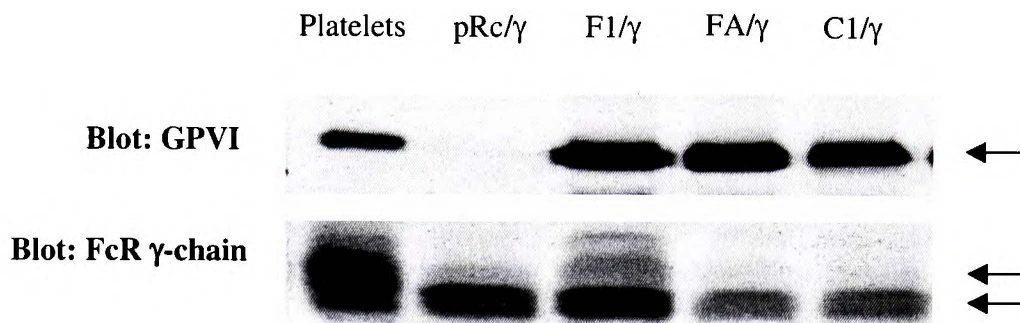


Figure 4.1.2. GPVI and FcR γ-chain transfection in COS-7 cells. Cells were transient transfected with FcR γ-chain together with wild type GPVI (F1/γ), GPVI mutated in the transmembrane arginine (FA/γ) or depleted of the cytoplasmic tail (C1/γ). Control cells were transfected with FcR γ-chain and empty plasmid (pRc/γ). Cells were lysed and 30 μg protein concentration per lane separated by SDS-PAGE in 12.5% gels. After transferring, membranes were blotted to detect the indicated proteins using convulxin and an antibody to convulxin for GPVI-detection, and an specific antibody to FcR γ-chain. A platelet sample was included as a positive control. Figure shows one result representative of two independent experiments.

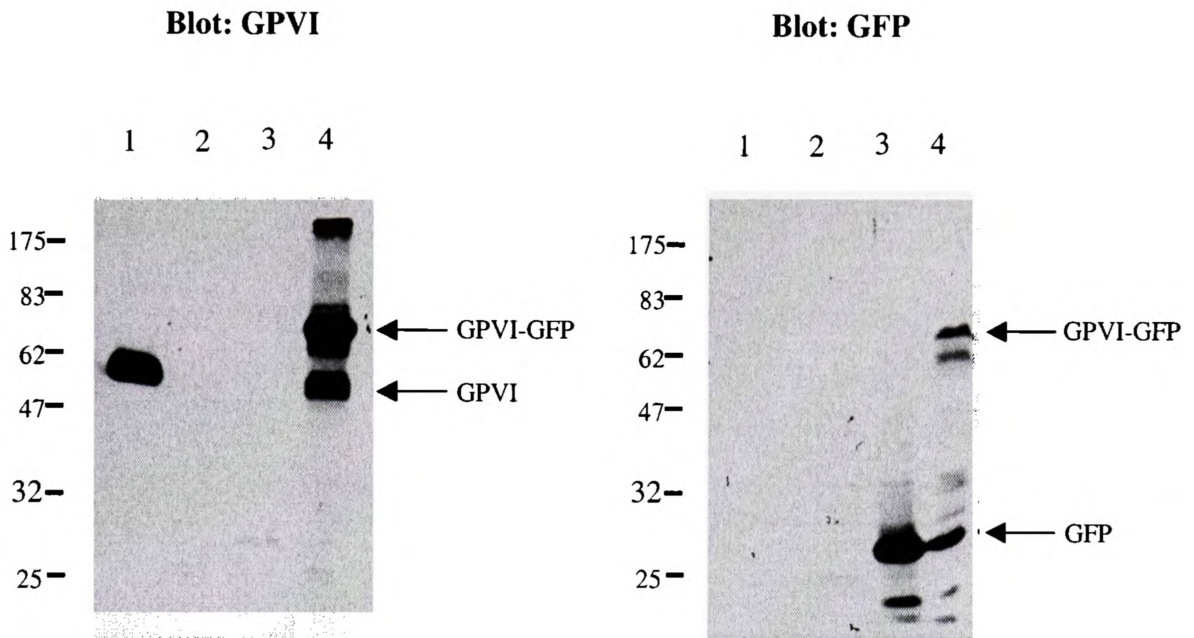


Figure 4.1.3. Expression of a chimeric protein GPVI-GFP in COS-7 cells. COS-7 cells transient transfected with either GFP (lane 3) or a chimeric protein GPVI-GFP (lane 4) were lysed and 30 μg protein separated by SDS-PAGE. Transferred proteins were detected using convulxin and an antibody to convulxin (left hand side blot) or anti-GFP antibody (right hand side blot). A whole cell lysate from human platelets (lane1) and COS-7 cells (lane 2) were included as controls. Arrows indicate the position of the corresponding proteins. Figure shows one result representative of three independent experiments.

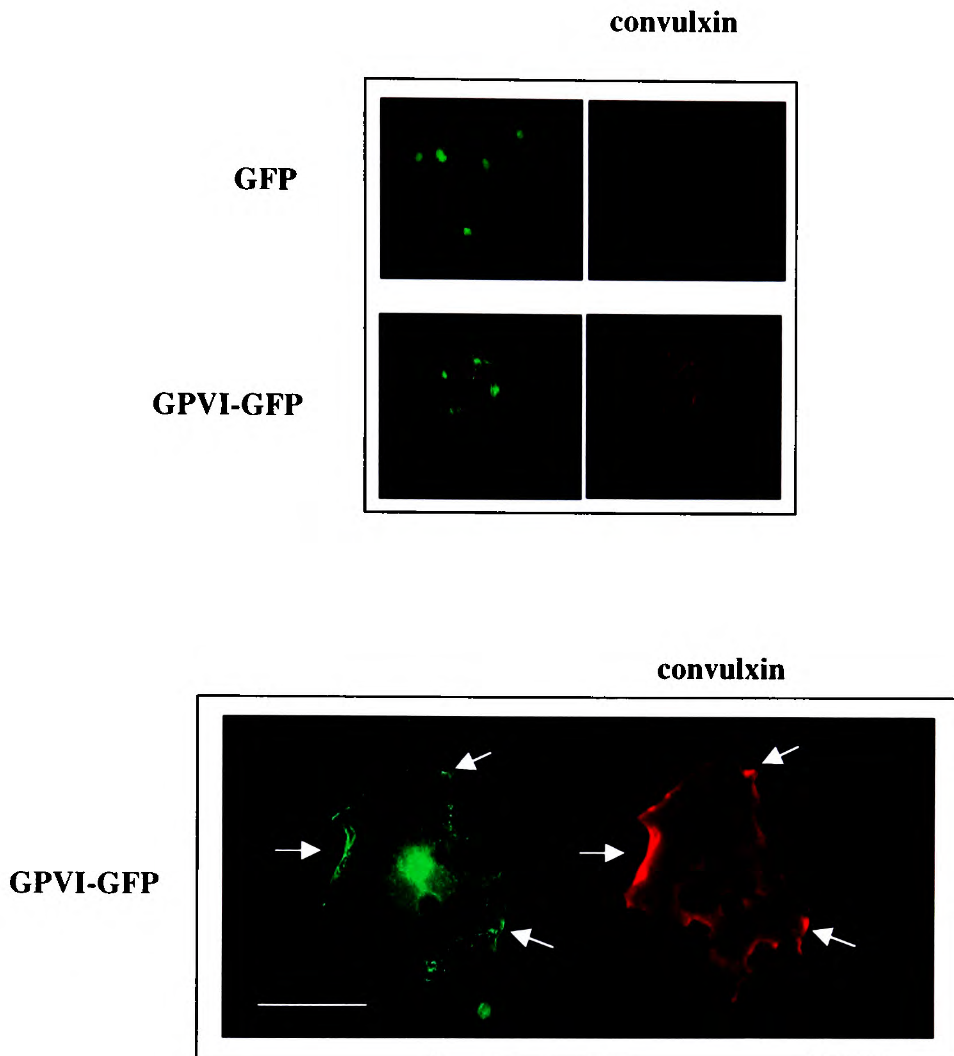


Figure 4.1.4. Convulxin colocalizes at the membrane with a chimeric protein GPVI-GFP in the absence of FcR γ-chain in COS-7 cells. Cells growing on coverslips were transient transfected with a plasmid encoding for either GFP or GPVI-GFP. After 48 h cells were fixed. GPVI-GFP was visualised by incubation with convulxin, an antibody to convulxin, and indirectly labelled with a R-Phycoerythin-conjugated anti-rabbit IgG. Fluorescent GFP is directly visualised in the microscope. Upper panel shows cytoplasmic localization of GFP (top-left), and absence of binding of convulxin to these cells (top right). GPVI-GFP localizes to the membrane (low left) and is recognized by convulxin (low right). Magnification x20. Bottom panel is a magnification of cells transfected with GPVI-GFP and incubated with convulxin. Arrows indicate spots of co-localization between GPVI-GFP and convulxin. Bar indicates 30 μm. n=3.

constructs of GPVI together with FcR γ-chain. Co-transfection of FcR γ-chain did not affect the surface expression level of the different GPVI constructs, judging from flow cytometry studies (Fig. 4.1.1).

Translocation of GPVI to the membrane independently of FcR γ-chain was further analysed by means of transient transfection in COS-7 cells of a chimeric protein GPVI-GFP. The chimeric protein was detected by immuno or ligand blotting using an anti-GFP antibody or convulxin, respectively (Fig. 4.1.3). A band with a relative molecular weight of 75 kDa, belonging to the chimeric protein, was detected in both cases. Surprisingly, additional bands appeared, probably as a result of degradation. The presence of strong bands with the same molecular weight as GPVI, as detected with convulxin, and GFP as detected with an anti-GFP antibody, suggested that degradation was taking place mainly, but not only, at the site of junction of both proteins in the chimeric form. Cellular localization of GPVI-GFP in COS-7 cells was analysed by immunohistochemistry. Cells transfected with GFP or with GPVI-GFP were incubated with convulxin, anti-convulxin and indirectly labeled with an R-Phycoerythrin-conjugated anti-rabbit IgG (Fig. 4.1.4). GFP localized in the cytoplasm of the cell, whereas GPVI-GFP was detectable at the surface. Moreover, convulxin did not bind to COS-7 cells transfected with GFP, whereas it did bind to all the cells expressing GPVI-GFP. A magnification of the latter revealed co-localization between GPVI-GFP and convulxin, therefore further demonstrating translocation to the surface of GPVI independently of FcR γ-chain.

The results reported above demonstrated translocation of GPVI to the membrane independently of the FcR γ-chain when transiently expressed, but the possibility remained that FcR γ-chain was necessary for stable surface expression of the receptor. To address this

point, GPVI was stably transfected into the erythroleukemic cell line K562, which lack expression of FcR γ-chain (Ernst et al. 1993 and Fig 4.3). Mock transfected cells (K562/pRc) do not express GPVI as detected by flow cytometry and ligand blotting (Fig. 4.2.1 and 4.3, respectively), whereas cells transfected with full length GPVI (K562/F1) or a cytoplasmic deleted GPVI (A288STOP) (K562/C1) expressed the receptor at the surface (Fig. 4.2.1). When FcR γ-chain was co-transfected, there was no major change in expression of GPVI (Fig. 4.2.2 and 4.3, respectively), demonstrating that FcR γ-chain was not necessary for expression of GPVI in these cells.

The level of expression of GPVI and FcR γ-chain was increased after treatment of the cells with the phorbol ester PMA (Fig. 4.2.1, Fig. 4.2.2, and Fig. 4.3). This was an increase of transfected proteins, as mock-transfected cells did not show detectable endogenous GPVI or FcR γ-chain after PMA-treatment, as assessed by flow cytometry (Fig. 4.2.1) and western or ligand blotting (Fig. 4.3). It was unclear whether the increase in expression with PMA was a cellular effect due to differentiation of the cells or whether it was due to stimulation of the CMV promoter of the plasmid. The latter seems more likely, as demonstrated in other cell types (Stein et al., 1993). It is noteworthy that those cells transfected with only FcR γ-chain expressed the protein at an undetectable level before treatment with PMA, and to a low level after differentiation with the phorbol ester for 3 days (Fig. 4.3). However, when GPVI was co-transfected, expression of FcR γ-chain was up-regulated (Fig. 4.3), suggesting a regulatory mechanism of co-expression between these two proteins.

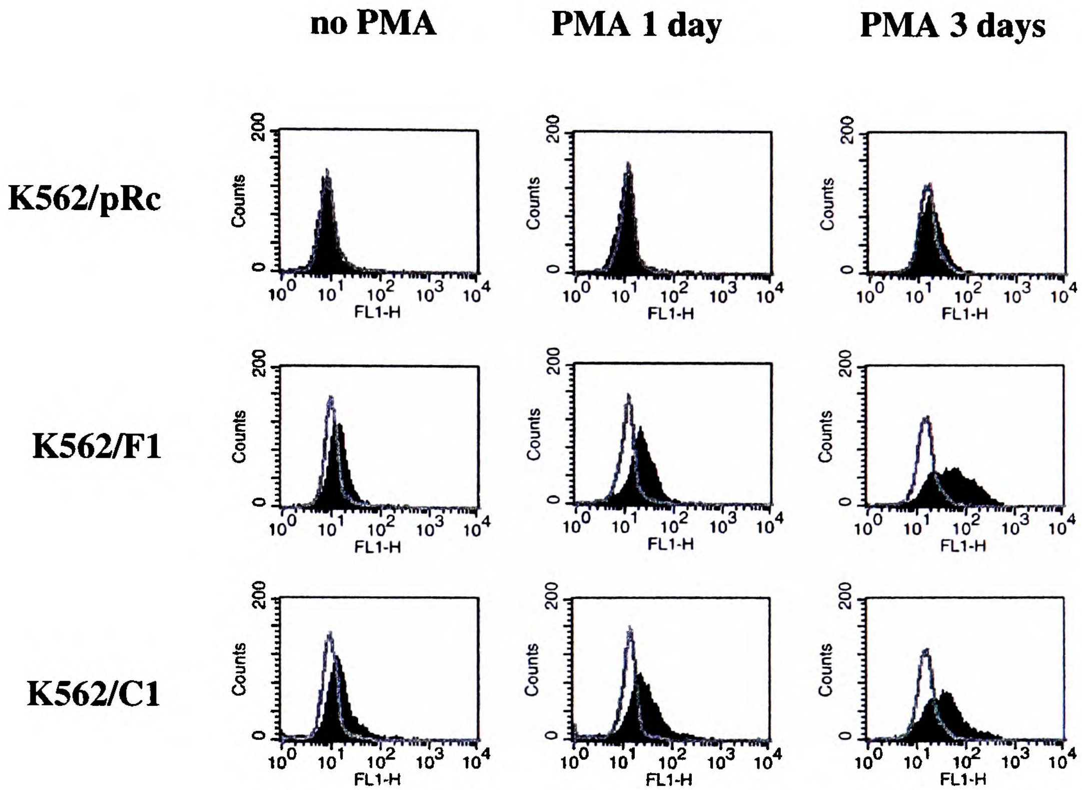


Figure 4.2.1. Surface expression of GPVI in stably-transfected K562 cells. K562 cells mock-transfected (K562/pRc), transfected with wild type GPVI (K562/F1) or with a cytoplasmic tail-deleted mutant (K562/C1), were differentiated with 10 nM PMA for 1 and 3 days, and expression of GPVI detected by flow cytometry using the GPVI-specific ligand convulxin and an antibody to convulxin (shaded area). Non-shaded are represents background fluorescence in the absence of convulxin. Figure shows one result representative of three independent experiments.

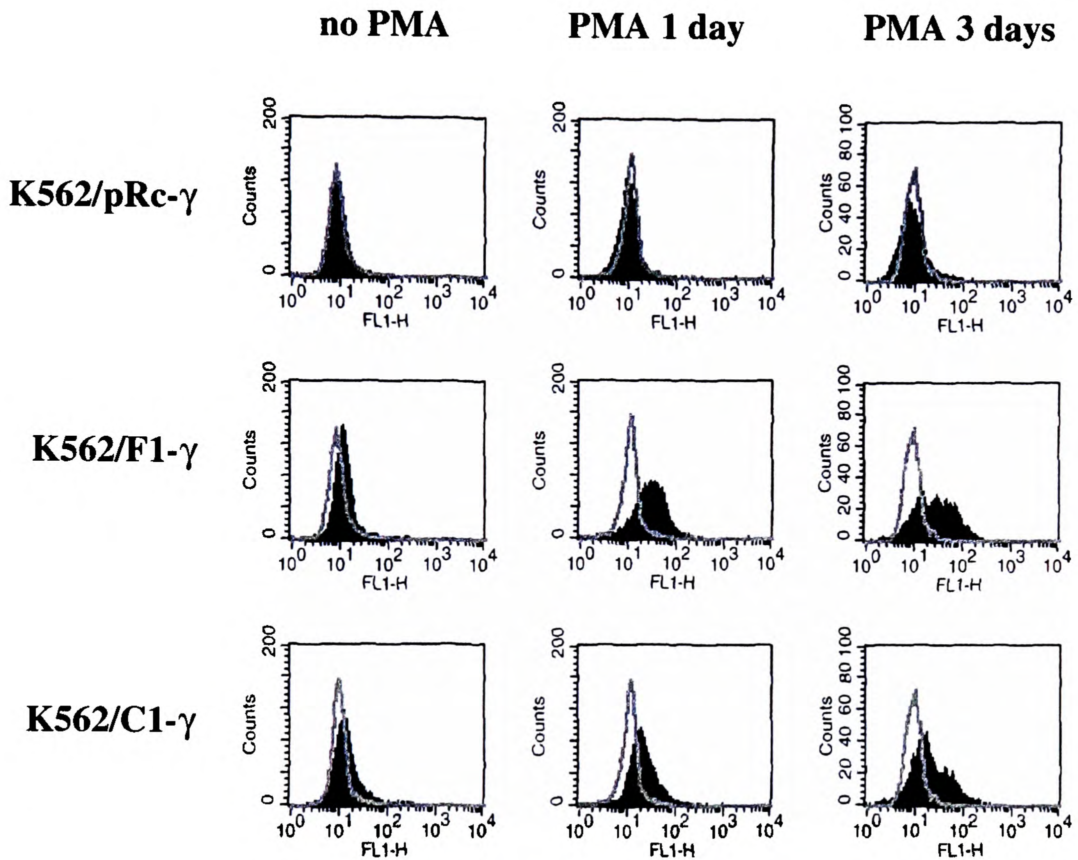


Figure 4.2.2. Surface expression of GPVI in FcR γ-chain expressing K562 cells.
K562 cells stably transfected with FcR γ-chain and co-transfected with either empty plasmid (K562/pRc-γ), wild type GPVI (K562/F1-γ) or with a cytoplasmic tail-deleted mutant GPVI (K562/C1-γ) were differentiated with PMA for 1 and 3 days, and expression of GPVI detected by flow cytometry using the GPVI-specific ligand convulxin (shaded area). Non-shaded area represents background fluorescence in the absence of convulxin. n=3.

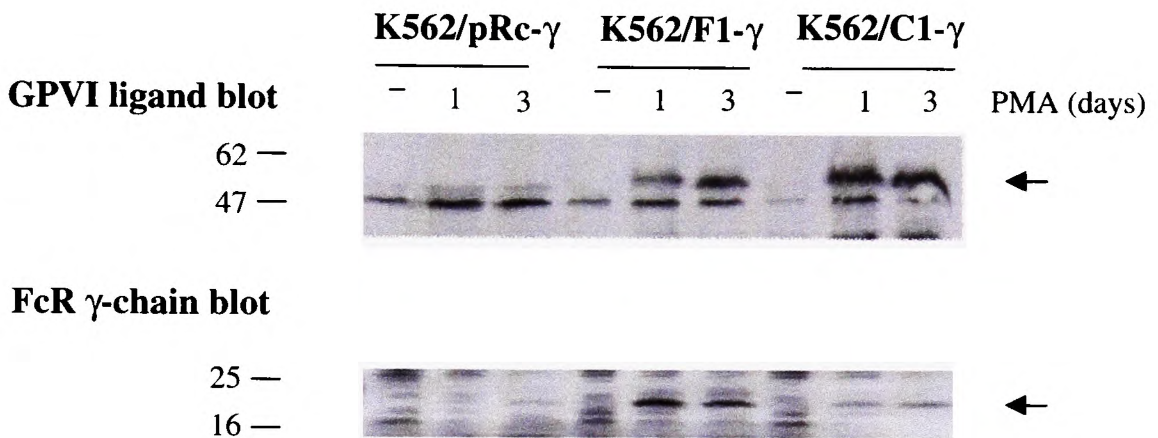


Figure 4.3. GPVI and FcR γ-chain expression in K562 cells. K562 cells were stably transfected with FcR γ-chain together with pRc vector alone (K562/pRc-γ) or coding for wild type GPVI (K562/F1-γ) or a cytoplasmic depleted GPVI (K562/C1-γ). Cells were stimulated with PMA for the times indicated and protein extracts separated by SDS-PAGE under non-reducing conditions. Membranes were blotted for GPVI or FcR γ-chain detection. Arrows indicate the position of GPVI or the FcR γ-chain. n=2.

Affinity precipitation: GST- Syk-SH2

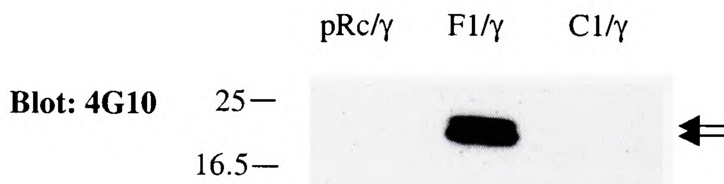


Figure 4.4. FcR γ-chain is phosphorylated in response to convulxin. K562 cells stably expressing FcR γ-chain (pRc/γ), or co-transfected with either wild type GPVI and the FcR γ-chain (F1/γ) or a cytoplasmic tail-deleted GPVI and FcR γ-chain (C1/γ) were stimulated for 90 seconds with 20 nM convulxin. Protein concentration was measured by Bio-Rad assay and equalized in each condition. Samples were affinity precipitated using GST-conjugated SH2 domain of Syk, and precipitated proteins subjected to SDS-PAGE under non-reducing conditions, then blotted to detect tyrosine phosphorylated proteins. Arrows indicate a characteristic doublet representing the phosphorylated forms of FcR γ-chain. Figure shows one result representative of three independent experiments.

IP: Syk

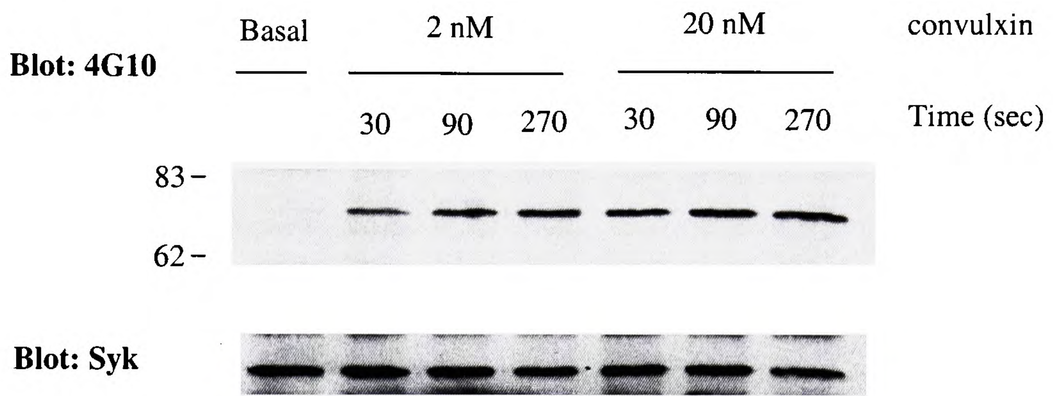


Figure 4.5. Dose response and time course of phosphorylation of Syk in GPVI-expressing K562 cells. K562 cells stable co-transfected with GPVI and FcR γ-chain And differentiated with 1o nM PMA for 24 hours were stimulated with convulxin at the concentration and times indicated. Protein lysates were immunoprecipitated using an anti-Syk antibody and subjected to SDS-PAGE, then blotted to detect tyrosine phosphorylated bands using mAb 4G10.Membrane was stripped and re-probed for Syk. Figure shows one result representative of two independent experiments.

4.4. FcR γ-chain is necessary for GPVI-signalling upon convulxin stimulation

In order to investigate whether GPVI was functional we measured tyrosine phosphorylation of the FcR γ-chain and the tyrosine kinase Syk, which is tyrosine phosphorylated and activated early after GPVI engagement. Phosphorylation of FcR γ-chain was assessed in K562 cells expressing wild type GPVI (K562/F1) or a mutant receptor lacking the cytoplasmic tail (K562/C1), along with FcR γ-chain. Cells were stimulated with 20 nM convulxin for 90 sec, and FcR γ-chain pulled down using a fusion protein consisting of GST conjugated with the SH2 domain of Syk, which recognizes the phosphorylated form of FcR γ-chain (Poole et al., 1997). Figure 4.4 shows tyrosine phosphorylation of FcR γ-chain in cells expressing both wild type GPVI and FcR γ-chain (K562/F1-γ), but not in those cells expressing only the FcR γ-chain (K562/pRc-γ), demonstrating that stimulation with convulxin phosphorylates the FcR γ-chain only when this is co-expressed with GPVI. Interestingly, GPVI lacking the cytoplasmic tail was unable to phosphorylate the FcR γ-chain under the same conditions.

Next, we performed a dose response and time course phosphorylation of Syk in K562/F1-γ cells expressing high levels of the receptor complex GPVI/ FcR γ-chain. Stimulation of these cells with 2 and 20 nM convulxin led to increase in tyrosine phosphorylation of Syk (Fig 4.5). The increase was evident after 30 sec of stimulation and maximum after 90 sec, remaining phosphorylated for up to 270 sec. Stimulation with 20 nM

convulxin after 30 sec promoted a stronger phosphorylation relative to 2 nM for the same time, although longer times of stimulation with both concentrations of convulxin rendered a similar intensity of the phosphorylated band (Fig 4.5). When K562/F1-γ cells expressing low levels of the receptor complex GPVI/ FcR γ-chain were stimulated with 20 nM convulxin for 90 sec, Syk became phosphorylated too, although to a lower degree relative to K562 cells expressing high levels of GPVI/ FcR γ-chain (Fig 4.6). The use of PMA to increase the level of expression of the receptor complex GPVI/FcR γ-chain was necessary to generate a more powerful signal. Bearing in mind that in the absence of PMA the signal was also generated, it can be concluded that the magnification of this signal in PMA-treated cells was probably due to the increase in the number of receptors at the surface of the cells, and not to up-regulation or activation of another proteins. A similar set of experiments were carried out with K562 cells which had been stably transfected with the cytoplasmic tail-deleted form of GPVI together with FcR γ-chain (K562/C1-γ). No increase in phosphorylation of Syk was detected in these cells upon convulxin stimulation (Fig. 4.6), demonstrating that deletion of the cytoplasmic tail makes the receptor unable to signal to the tyrosine kinases. Further, K562 cells stably transfected with only GPVI, and therefore lacking FcR γ-chain (K562/F1), did not show any detectable increase in tyrosine phosphorylation of Syk after 20 nM convulxin stimulation for 90 sec, even when expressing higher levels of GPVI after PMA-treatment (Fig. 4.7).

Altogether, our data indicate that in K562 cells, co-transfection of GPVI and the FcR γ-chain leads to its association in a receptor complex which can be activated with the GPVI-specific ligand convulxin, and that this activation brings about the phosphorylation of the protein kinase Syk, therefore reproducing the proximal events of GPVI signaling in

platelets. Moreover, the FcR γ-chain was shown to be crucial for the generation of this signal, as cross-linking of GPVI in the absence of FcR γ-chain was unable to promote any increase in phosphorylation of Syk.

4.5. Transmembrane arginine^{272} residue and the cytoplasmic tail of GPVI are necessary for its association to FcR γ-chain

We next performed experiments to determine whether there was a physical association between GPVI and FcR γ-chain as described on platelets, and the role that the transmembrane arginine and cytoplasmic tail of GPVI play in this association. COS-7 cells were co-transfected with the different GPVI constructs along with FcR γ-chain. Affinity precipitation of GPVI using convulxin and an antibody to convulxin, and subsequent immunoblotting to detect FcR γ-chain, showed an association between the latter and wild type GPVI, but not with any of the mutant GPVI receptors, demonstrating that the transmembrane arginine and the cytoplasmic tail of GPVI are essential for its association to the FcR γ-chain (Fig. 4.8.1).

A similar set of experiments were carried out using K562 cells stably expressing either wild type GPVI or with the cytoplasmic tail-deleted mutant receptor, together with FcR γ-chain. The cells were stimulated with PMA for 24 h prior to experiment to increase the expression of both proteins and facilitate its detection. As observed in COS-7 cells, only wild type GPVI was able to bind FcR γ-chain (Fig. 4.8.2), showing a requirement of the cytoplasmic tail for association with FcR γ-chain. The lack of association between the FcR

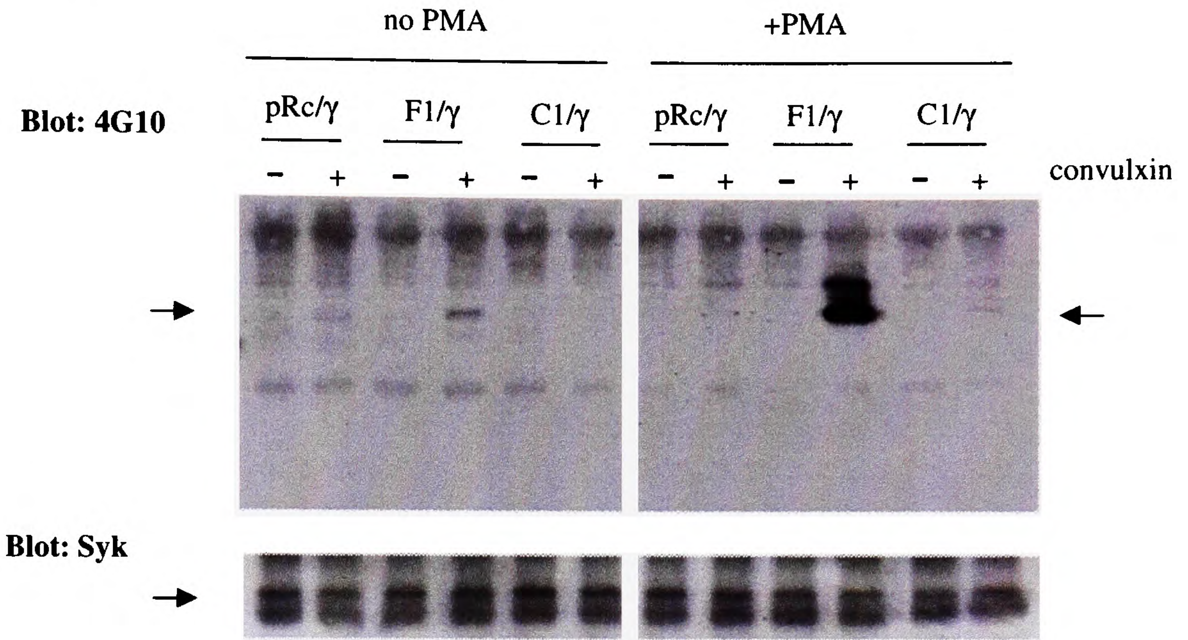


Figure 4.6. Syk phosphorylation in GPVI and FcR γ-chain expressing cells.
K562 cells stably transfected with FcR γ-chain and co-transfected with vector alone (pRc/γ) or wild type GPVI (F1/γ) or a cytoplasmic tail-depleted GPVI (C1/γ) were treated or not with 10 nM PMA for 24 h, then stimulated with 20 nM convulxin for 90 sec and protein lysates subjected to Syk immunoprecipitation. Immunoprecipitated proteins were separated by SDS-PAGE and transfer to a PVDF membrane. After blocking with BSA, phosphorylated proteins were detected using the monoclonal antibody 4G10. Membrane was stripped and re-blotted for Syk. Arrows indicate the relative position of Syk. Figure shows one result representative of three independent experiments.

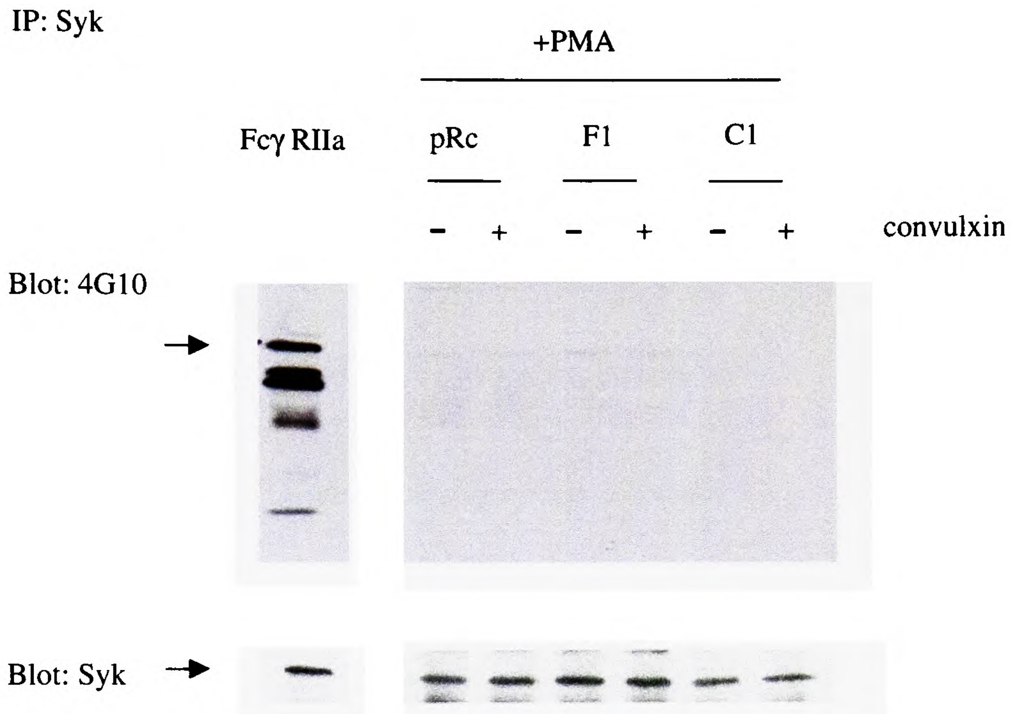


Figure 4.7. Syk phosphorylation in GPVI-transfected K562 cells. K562 cells Mock-transfected (pRc) or stably transfected with wild type GPVI (F1) or a cytoplasmic tail-depleted GPVI (C1) were treated with 10 nM PMA for 24 h, then stimulated with convulxin for 90 sec. Syk was immunoprecipitated using a specific antibody, and subjected to SDS-PAGE. After transfer, membranes were blotted to detect tyrosine phosphorylated bands using the monoclonal antibody 4G10. Membrane was stripped and reblotted for Syk detection, to ensure equal loading. A sample from FcγRIIA-stimulated cells was included as a positive control.Figure shows one result representative of two independent experiments.

1- WCL platelets
2- COS7/pRc-γ
3- COS7/F1-γ
4- COS7/FA-γ
5- COS7/C1-γ

Affinity precipitation: GPVI

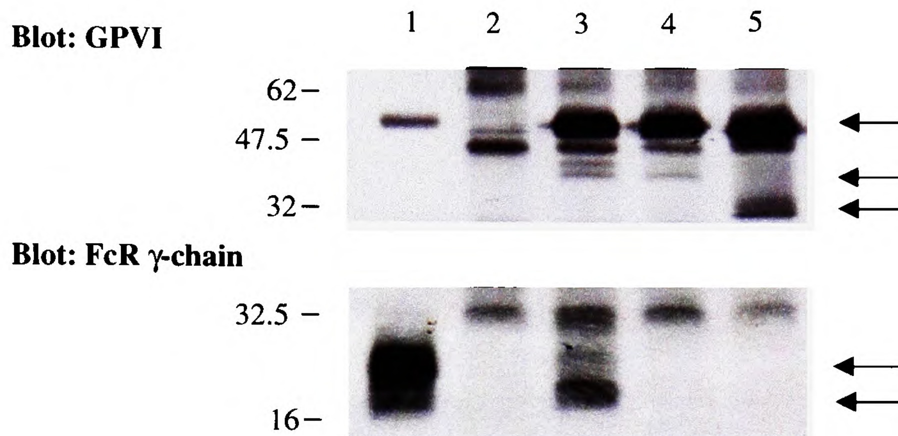


Figure 4.8.1. GPVI association to the FcR γ-chain in COS7 cells. COS7 cells were transient transfected with wild type or different mutant forms of GPVI along FcR γ-chain. GPVI was affinity precipitated using convulxin, and samples subjected to SDS-PAGE under non-reducing conditions in a 12.5% gel. Top part of the membrane was blotted for GPVI detection using convulxin and an antibody to convulxin, whereas bottom part was blotted for FcR γ-chain detection. A whole cell lysate (WCL) from platelets was included as a positive control (lane 1). GPVI was detected in top blot using convulxin and an antibody to convulxin as a band with a relative molecular weight of 55 kDa, whereas additional bands of around 40 kDa and 30 kDa were detected too in lanes 3-5, possibly representing degraded or non-glycosylated forms of GPVI. Bottom blot shows co-precipitated FcR γ-chain, which associates with wild type GPVI (F1) (lane 3) but not with GPVI mutated at the transmembrane arginine (R272A) (FA) (lane 4) or lacking the cytoplasmic tail (A288STOP)(C1) (lane 5). Lane 2 are COS7 cells co-transfected with FcR γ-chain and pRc plasmid. Arrows on top blot indicate GPVI, and on bottom blot a doublet representing differently phosphorylated forms of FcR γ-chain. Figure shows one result representative of two independent experiments.

Affinity precipitation: GPVI

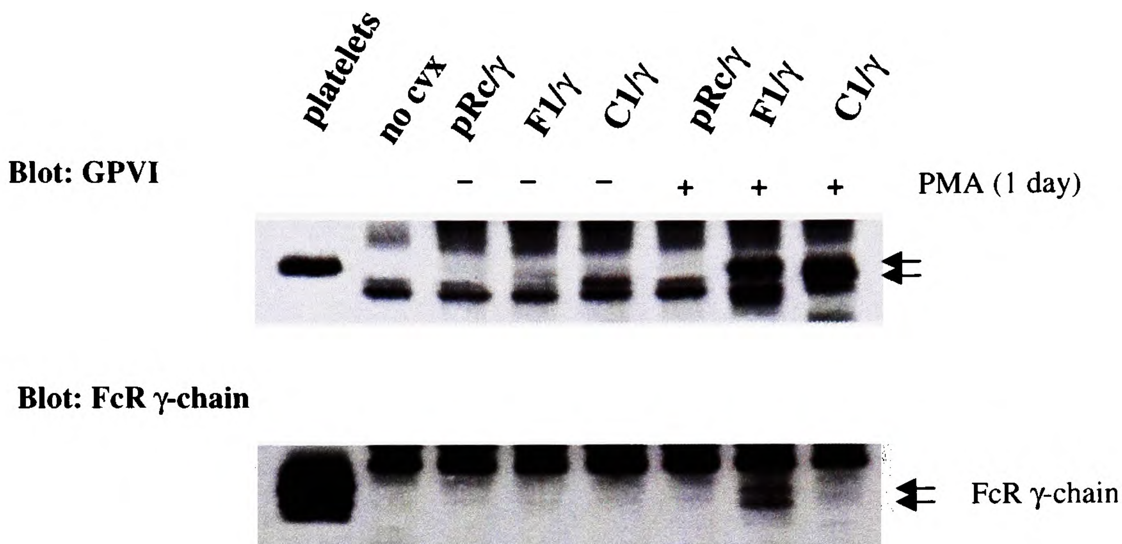


Figure 4.8.2. Association between GPVI and FcR γ-chain in K562 cells. K562 cells were stably transfected with FcR γ-chain and co-transfected with either empty plasmid (pRc/γ) or plasmid containing wild type GPVI (F1/γ) or a cytoplasmic tail-deleted mutant (C1/γ). Cells were differentiated or not with 10 nM PMA for 1 day to increase expression of transfected GPVI and FcR γ-chain. GPVI was affinity precipitated using convulxin, and detected by ligand blotting. The associated FcR γ-chain was detected by western blotting using a specific antibody. A sample of pRc-F1/γ stimulated with PMA Was used for precipitation in the absence of convulxin as a control. A platelet sample is included as a positive control. Arrows indicate the position of GPVI or the FcR γ-chain. Figure shows one result representative of two independent experiments.

γ-chain and a mutant GPVI deleted of its cytoplasmic tail explains the failure of the mutant receptor in promoting a functional response when stimulated with convulxin, as shown above.

4.6. Discussion

The recent cloning of GPVI allows for the generation of mutant receptors to further understand its biology and function. GPVI/FcR γ-chain is a unique receptor complex when compared with another FcR γ-chain partners, as it has been reported to function as a collagen receptor with no apparent function in the immune response, despite sharing similar structure and signaling features with another FcRs (Gibbins et al., 1997, Arai et al., 1995). This difference in function may be explained on the grounds that GPVI seems to be specific to the platelet and megakaryocyte, which are involved in functions distinct to those of other haematopietic cells. However, the similarity between GPVI and different FcRs provides a starting point in the study of the structure-function relationship of GPVI and the signaling pathways. FcRs and their associated β and γ-chains bind to each other through the transmembrane (TM) domain resulting in a receptor complex which is able to signal inside the cell (Taylor and McVicar, 1999; Ono et al., 1999). The nature of this binding is dependent on oppositely charged amino acid residues within the TM domain of each subunit (reviewed in Harrison, 1996). Point mutations of these amino acids have revealed the importance of the arginine residue in the TM domain of the immunoglobulin receptor (Taylor and McVicar, 1999; Craig-Morton et al., 1995). However, the cytoplasmic domain of these Ig receptors is not essential for the binding to the FcR γ-chain. Results in this chapter show that GPVI depends on both the transmembrane arginine and the cytoplasmic tail for its

association to the FcR γ-chain, although it is unclear as to how the cytoplasmic tail supports this association. It is possible that the cytoplasmic tail enables GPVI to adopt a conformation in which the association with the FcR γ-chain is no longer possible.

The cytoplasmic tail of different FcRs do not contain any recognizable tyrosine phosphorylation-dependent signaling motif, but they seem to alter the signaling properties of the receptors to which they bind (Ono et al., 1999, Edberg et al., 1999). There is also no tyrosine phosphorylation-dependent recognisable signalling motif within the cytoplasmic tail of GPVI. Although the role of this cytoplasmic tail is still obscure, it has recently been reported that a specific serine residue within the cytoplasmic tail of FcαR mediates cytokine-induced inside-out signaling of the receptor (Bracke et al., 2001). As there are different serine residues within the cytoplasmic tail of GPVI, it is possible that these serine and/or other residues are somehow modulating the function of the receptor.

In this chapter it has been shown by means of transient and stable transfections of GPVI into COS7 and K562 cells, respectively, that GPVI translocates to the cell surface independently of the FcR γ-chain. This is a property that has previously been described for the GPVI-related receptors PIR-A and FcαR (Ono et al., 1999, Craig Morton et al., 1995) and for the FcR γ-chain partner FcγRI (Takai et al., 1994) when transfected into cell lines. However, experiments using transgenic and knock-out mice demonstrated that *in vivo*, the FcR γ-chain was necessary for translocation to the membrane of FcαR and FcγRI (Van Egmond et al., 1999; Takai et al., 1994; Van Vugt et al., 1996). Moreover, FcR γ-chain seemed to be necessary to prevent degradation of its partners *in vivo* (Weissman et al.,

1989; Kurosaki et al., 1991). Consistent with this, mice depleted of the FcR γ-chain did not express detectable GPVI (Nieswandt et al., 2000), and the possibility remains that *in vivo*, unlike cell lines, GPVI may be degraded in the absence of the FcR γ-chain.

The co-transfection of FcR γ-chain with GPVI did not increase the level of surface expression of the latter relative to transfection of GPVI alone, either on transient or stable transfections. The same observation has been reported by the group of Takayama on transient transfections of GPVI into COS7 cells (Ezumi et al., 2000). This is a difference with the related PIR-A and Fcα-receptor (Maeda et al., 1998; Craig Morton et al., 1995), and suggests that in cell lines FcR γ-chain is acting as a signaling and stabilizing subunit for some receptors, such as the Fcα-receptor, but only as a signaling subunit for others, namely GPVI.

It is well known that the FcR γ-chain is essential for signalling by those receptors to which associates (Craig Morton et al., 1995; van Egmond et al., 1999, Maeda et al., 1998), and therefore it has been suggested that the same accounts for GPVI. Mice engineered to lack FcR γ-chain do not express detectable GPVI (Nieswandt et al., 2000), making it impossible to determine whether GPVI signals in the absence of FcR γ-chain *in vivo*. K562 cells do not express detectable levels of endogenous GPVI or FcR γ-chain, as assessed by flow cytometry and ligand and western blotting, even after differentiation with PMA. This fact makes K562 cells a suitable system where studying the signaling events triggered by GPVI in the presence or absence of FcR γ-chain following transfection of either protein. It

has been shown here that GPVI requires the FcR γ-chain to generate a signal, but the latter is not required for the recognition of the ligand, in this case convulxin. This illustrates the dual mechanism of the complex, with one subunit, namely GPVI, responsible for binding to the ligand, and the other subunit, FcR γ-chain, responsible for the transmission of the signal within the cell.

Chapter 5

GPVI signaling in human megakaryocytes

5.1. Aim

The aim of this chapter was to reconstitute the signaling pathway generated by GPVI which leads to phosphorylation of PLCγ2 and subsequent $[Ca^{2+}]_i$ increase, and comparison to those signals from the low affinity receptor for immunoglobulins G, Fc gamma RIIA (FcγRIIA), in K562 cells stably expressing GPVI and the FcR γ-chain.

5.2. Introduction

Stimulation of GPVI triggers a tyrosine kinase-dependent mechanism resulting in activation of the platelet. Tyrosine phosphorylation of the FcR γ-chain and the tyrosine kinase Syk are early events in this pathway, eventually leading to activation of PLCγ2 and elevation of intracellular calcium. Phosphorylation of FcR γ-chain and Syk serve as a docking site for the recruitment and activation of several downstream proteins. Among these, adapter proteins play an important role by acting as a scaffold between different proteins. Similarly to T-cell receptor (TCR) signaling, it is thought that proteins implicated in GPVI-signaling concentrate in glycolipid-enriched membrane microdomains (GEMs) upon receptor activation. These are compartments known to be critical for concentrating signaling molecules after TCR and probably GPVI engagement. Recruitment and activation of proteins to GEMs gives rise to activation of the platelet signalosome, a term coined in 1998 by the group of Dubiel to define protein complexes implicated in signal transduction (Seeger et al., 1998). Although much is known about both the proximal phosphorylation events as well as the downstream signaling pathways required for GPVI

signaling, the mechanisms by which these steps are integrated after GPVI ligation remain less clear. Several studies have shown that the early phosphorylation events are required for the creation of multimolecular protein complexes that coordinate the various signals important for successful GPVI signaling. These complexes are nucleated by adapter molecules, proteins that contain modular domains responsible for mediating interactions with other molecules within the cell. Two such adapter proteins, SLP-76 and LAT, have been shown to be important for GPVI signaling in platelets (Clements et al., 1999; Pasquet et al., 1999b).

SLP-76 is a cytosolic adapter protein consisting of an NH2-terminal acidic region containing several tyrosines, a central proline-rich region which enable for constitutive association with the adapter Gads, and a COOH-terminal region containing an SH2 domain (Musci et al., 1997). SLP-76 becomes phosphorylated and associates to LAT early after GPVI crosslinking (Asazuma et al., 2000).

Due to posttranslational fatty acid modifications, the adapter LAT is targeted to GEMs and is essential for the recruitment of downstream proteins in T cells (Lin et al., 1999; Zhang et al., 1998b). In platelets LAT seems important for collagen signaling as demonstrated by the impaired response to collagen in LAT deficient platelets (Pasquet et al., 1999b). LAT and SLP-76 associate in GEMs upon GPVI stimulation (Asazuma et al., 2000; Wonerow and Watson, unpublished results), therefore transmitting the signal further within the cell.

Cross-linking of the monoclonal antibody IV.3 to selectively activate the low affinity receptor for IgG, FcγRIIA induces a robust increase in tyrosine phosphorylation in K562 cells (Mountford et al., 1999). As GPVI signals in a similar fashion to that of the FcγRIIA (Yanaga et al., 1995; Chacko et al., 1994), it is reasonable to think that the GPVI signaling machinery necessary for the activation of PLCγ2 and subsequent $[Ca^{2+}]_i$ increase is functional in K562 cells.

Results in the previous chapter report reconstitution of the early events of the GPVI signaling in K562 cells stably expressing GPVI and the FcR γ-chain. This response is characterised by increase in phosphorylation of the FcR γ-chain and Syk when stimulated with convulxin. This chapter further analyses the signaling cascade triggered by GPVI that leads to PLCγ2 activation, in PMA-differentiated K562 cells stably expressing wild type GPVI and the FcR γ-chain (F1/γ), and compares it to that one generated by FcγRIIA.

Results

5.3. Phosphorylation of Syk and PLCγ2 after GPVI or FcγRIIA engagement

GPVI was cross-linked in K562 cells stably expressing GPVI and FcR γ-chain (F1/γ) using 20 nM convulxin for 90 sec. FcγRIIA was engaged using 10 μg/ml mAb IV.3 antibody for 60 sec, then cross-linked with 30 μg/ml of an anti-mouse IgG for 60 sec. Cells were lysed after stimulation and Syk immunoprecipitated using a specific antibody. Syk was tyrosine phosphorylated to a similar level after stimulation of either receptor (Fig.

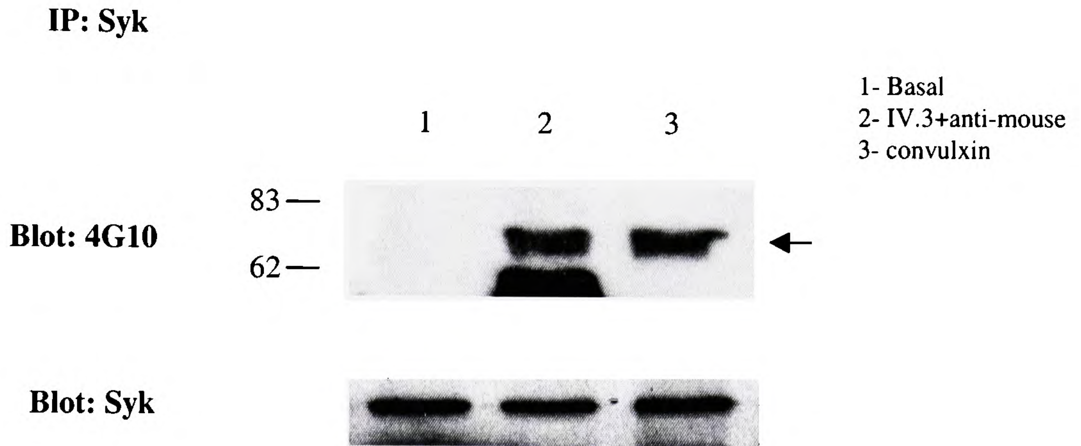


Figure 5.1- Syk is phosphorylated in K562 F1/γ cells in response to convulxin and FcγRIIA cross-linking. Cells were differentiated with 10 nM PMA for 24 hours and stimulated by cross-linking either FcγRIIA receptor using mAb IV.3 or GPVI using convulxin, and Syk immunoprecipitated using a specific antibody. After SDS-PAGE membrane was blotted to detect tyrosine phosphorylation using mAb 4G10. Arrow indicates tyrosine phosphorylated Syk. A band below the phosphorylated Syk in lane 2 belongs to IV.3 antibody used for the stimulation. Membrane was stripped and re-blotted for Syk to assure equal loading. Figure shows one result representative of five independent experiments.

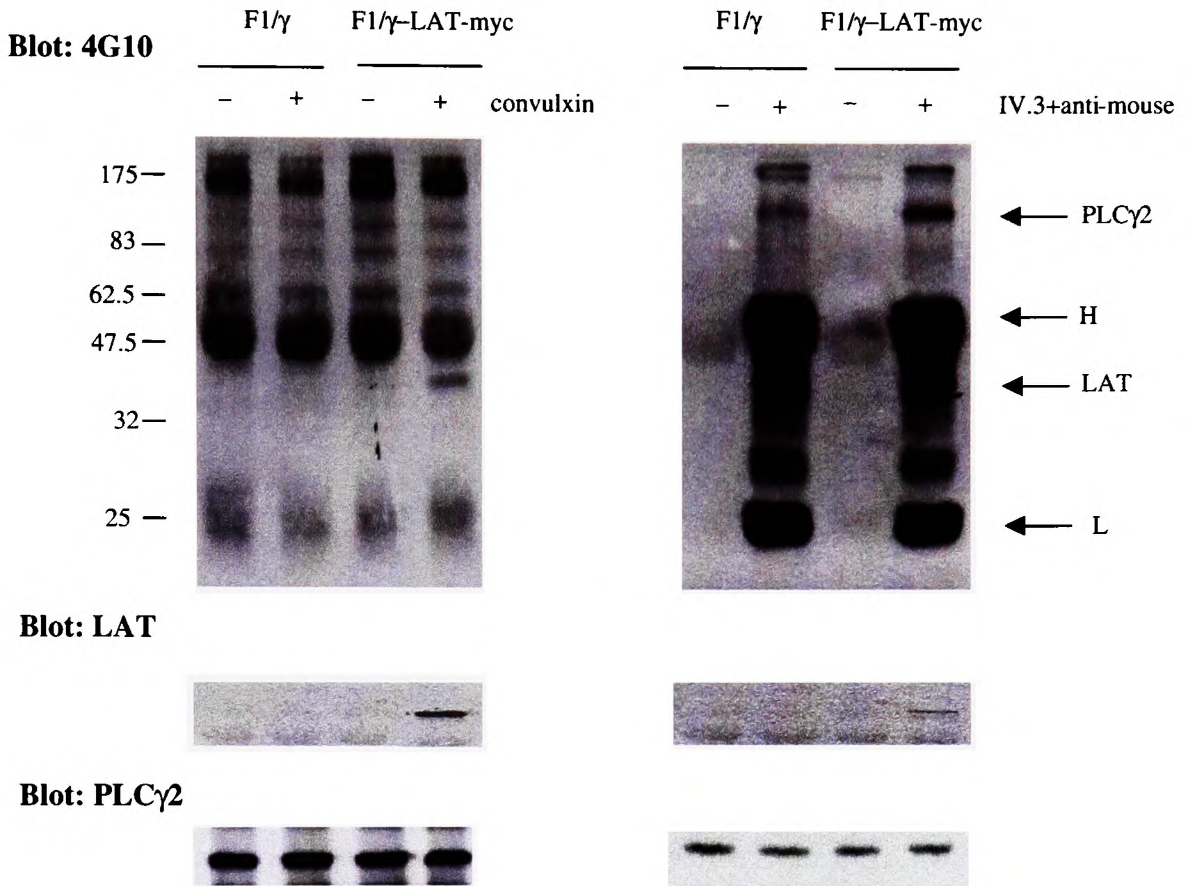


Figure 5.2.1. PLCγ2 is phosphorylated by FcγRIIA but not GPVI cross-linking in F1/γ cells. Cells transient-transfected with LAT (F1/γ-LAT-myc) or not (F1/γ) were Differentiated with 10 nM PMA for 24 hours and stimulated with either convulxin (left panel), or with IV.3 antibody followed by coss-linking with anti mouse IgG (right panel). PLCγ2 was immunoprecipitated and immuno complexes subjected to SDS-PAGE followed by western blotting with mAb 4G10 to detect tyrosine phosphorylated proteins. A band with a relative molecular weightof 150 kDa corresponding to PLCγ2 was tyrosine phosphorylated upon activation of FcγRIIA but not GPVI. A co-precipitated phosphorylated band of around 40 kDa was detected in convulxin-stimulated cells expressing LAT. This band was shown to contain LAT (middle panel). A phosphorylated band with a similar molecular weight, probably corresponding to the phosphorylated Fc receptor, was detected in cells stimulated with IV.3 antibody. Arrows on the right hand side indicate the heavy (H) and light (L) chains corresponding to the antibodies used for the stimulations and/or the immunoprecipitation. Membrane was re-probed for PLCγ2 to ensure equal loading.Figure shows one result representative of four independent experiments.

5.1). As phosphorylation of Syk by these receptors leads to phosphorylation and activation of downstream proteins in platelets, including PLCγ2, we next examined whether F1/γ cells were able to reconstitute the downstream events after Syk phosphorylation. GPVI and FcγRIIA were stimulated with convulxin or IV.3 antibody as above, and PLCγ2 immunoprecipitated. After SDS-PAGE, membranes were blotted for tyrosine phosphorylation. Surprisingly, PLCγ2 became phosphorylated upon FcγRIIA but not after GPVI cross-linking (Fig. 5.2.1). As GPVI and FcγRIIA cross-linking led to a similar increase in phosphorylation of Syk (Fig. 5.1), suggesting that the strength of the signal was likely to be similar, it would appear that the two receptors regulate PLCγ2 through distinct pathways downstream of Syk.

5.4. Ectopic-expressed LAT is phosphorylated in response to convulxin stimulation in F1/γ cells

In order to identify whether any of the proteins described to be important for PLCγ2 phosphorylation in GPVI signaling was missing in K562 cells, immunoprecipitation studies of different proteins using specific antibodies were carried out. Among the different proteins investigated, the adaptors SLP-76 and Gads were present and constitutively associated as previously described (Liu et al., 1999) (Fig. 5.3). The src-kinase Lyn and the tyrosine kinase Btk were also present, but the adapter molecule LAT was absent in these cells (Fig. 5.3). LAT has been shown to interact with multiple adapter proteins in GPVI-activated platelets (Asazuma et al., 2000), and platelets deficient for LAT show a substantial reduction in phosphorylation of PLCγ2 upon GPVI stimulation

(Pasquet et al., 1999). Therefore, absence of LAT can apply for the lack of phosphorylation of PLCγ2 in F1/γ cells stimulated with convulxin.

A myc-tagged construct of LAT (LAT-myc) was transient transfected in F1/γ cells by means of electroporation using cytomix buffer, achieving efficiencies of transfection in the order of 60-70% as explained in chapter 3. LAT-myc was detected by western blotting and compared with wild type LAT from platelets, and shown to migrate slightly different due to the myc tag (Fig. 5.3). GPVI and FcγRIIA were stimulated in LAT-myc- expressing F1/γ cells, and PLCγ2 immunoprecipitated. Western blot analysis revealed that in the presence of LAT-myc, GPVI activation was still unable to promote an increase in phosphorylation of PLCγ2 (Fig. 5.2.1). However, a phosphorylated band of 38-40 kDa, corresponding to the transfected LAT-myc, co-precipitated with PLCγ2 (Fig. 5.2.2), indicating that stimulation of GPVI promotes the phosphorylation of LAT and subsequent association with PLCγ2, independently of phosphorylation of the latter. Moreover, cross-linking of FcγRIIA is able to induce an increase in phosphorylation of PLCγ2 in the absence of LAT-myc, and there is a potentiation of this phosphorylation when LAT-myc is present (Fig. 5.2.1). Due to phosphorylation of the FcγRIIA receptor itself, which migrates to the same region than the transfected LAT-myc, it was not possible to distinguish whether phosphorylated LAT-myc co-precipitated with PLCγ2 in FcγRIIA-activated cells (Fig. 5.2.1).

5.5. Impairment in calcium increase in F1/γ cells stimulated with convulxin or IV.3 antibody

Phosphorylation and activation of PLCγ2 leads to formation of the second messengers inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), leading to an increase in the concentration of intracellular calcium. F1/γ cells were loaded with the calcium reporter dye FURA-2 and intracellular calcium measured after cross-linking of GPVI or FcγRIIA. Neither receptor, when cross-linked, was able to induce an increase in the level of intracellular calcium (Fig. 5.4), regardless the presence of LAT-myc. The lack of phosphorylation of PLCγ2 with convulxin was consistent with the inability of the receptor to induce elevation of $[Ca^{2+}]_i$. The data also demonstrated that although PLCγ2 was phosphorylated after cross-linking of FcγRIIA, subsequent functional responses did not occur.

5.6. SLP-76 is not phosphorylated in F1/γ cells stimulated with convulxin or IV.3 antibody

Impairment in phosphorylation/activation of PLCγ2 in GPVI- or FcγRIIA-stimulated FI/γ cells was further analysed in order to establish the site of lesion in the pathway. Among the proteins implicated in PLCγ2 phosphorylation in platelets, Btk and SLP-76 play an important role (Quek et al., 1998; Clements et al., 1999). Platelets made deficient for SLP-76 show complete impairment in PLCγ2 phosphorylation and functional responses in response to collagen (Clements et al., 1999). Furthermore, transient over-expression of SLP-76 in a T-cell line potentiates transcriptional activation after T-cell receptor ligation, while loss of SLP-76 expression abrogates several T-cell

IP: LAT

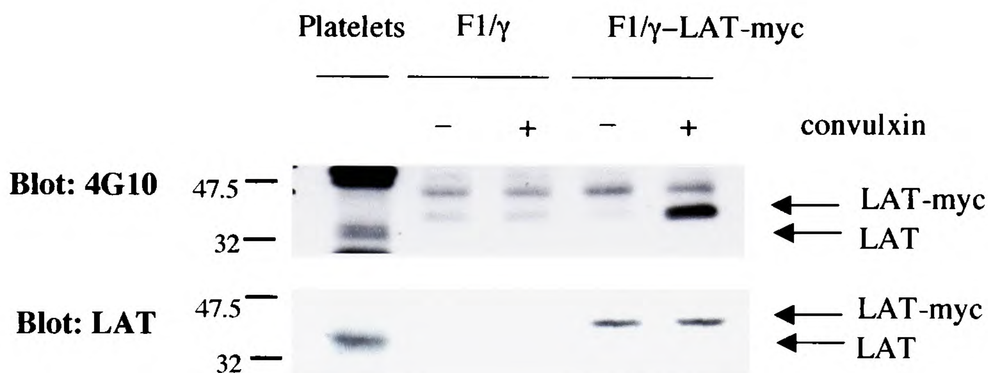


Figure 5.2.2. LAT is phosphorylated upon GPVI cross-linking. F1/γ cells were transient transfected with a construct encoding for LAT-myc (F1/γ-LAT-myc), differentiated with 10 nM PMA for 24 hours and stimulated with 20 nM convulxin for 90 sec. LAT was immunoprecipitated and separated by SDS-PAGE. After transferring membranes were blotted with mAb 4G10 to detect tyrosine phosphorylation. Transfected LAT-myc was found to be phosphorylated after activation of GPVI using convulxin. A platelet whole cell lysate is included as a positive control. Arrow indicate the position of the phosphorylated LAT-myc. Membrane was stripped and re-blotted to ensure equal loading. Note that transfected LAT-myc migrates slightly different relative to endogenous LAT from platelets due to the myc tag. Figure shows one result representative of three independent experiments.

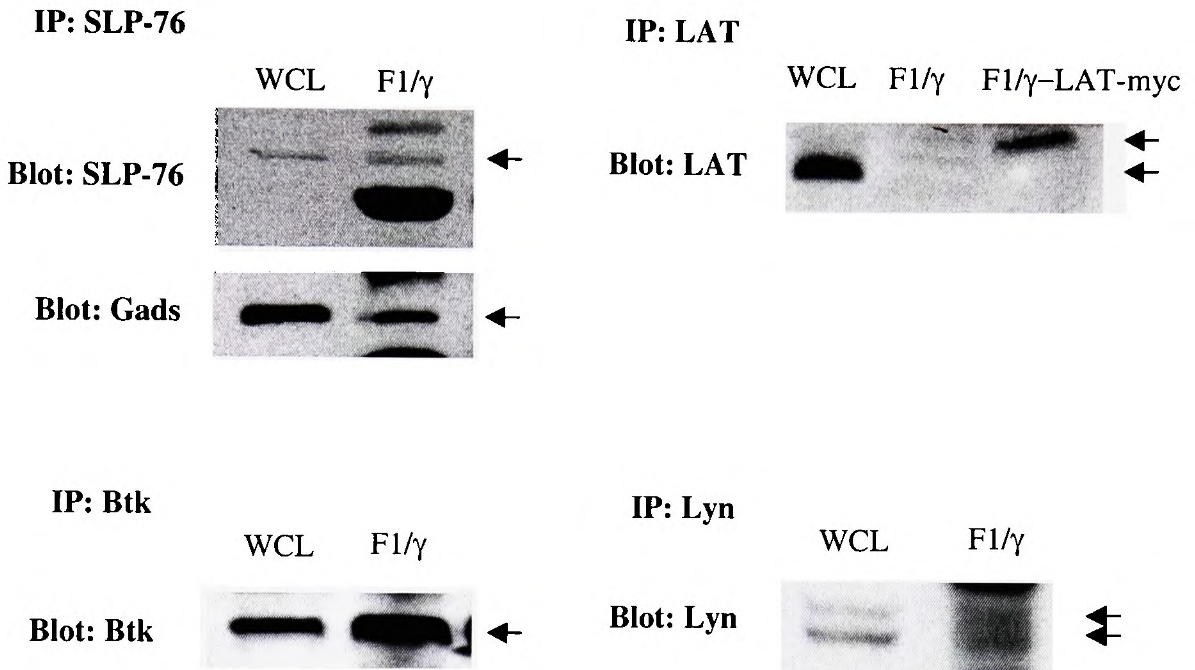


Figure 5.3. SLP-76, Gads, Lyn and Btk but not LAT are expressed in K562 cells. F1/γ cells were lysed and the indicated proteins immunoprecipitated using specific antibodies. Immuno complexes were separated by SDS-PAGE and immunoblotted to detect the corresponding protein. Gads was found to be constitutively associated to the adaptor SLP-76 as both proteins co-precipitated in SLP-76 pull downs. LAT was not detected in F1/γ cells, but a LAT-myc construct ectopic expressed was detected with a retardation in migration due to its higher molecualr weight. Arrows indicate the position of the blotted proteins. A whole cell lysate (WCL) from human platelets was included in all cases as a positive control in the western blots. n=2.

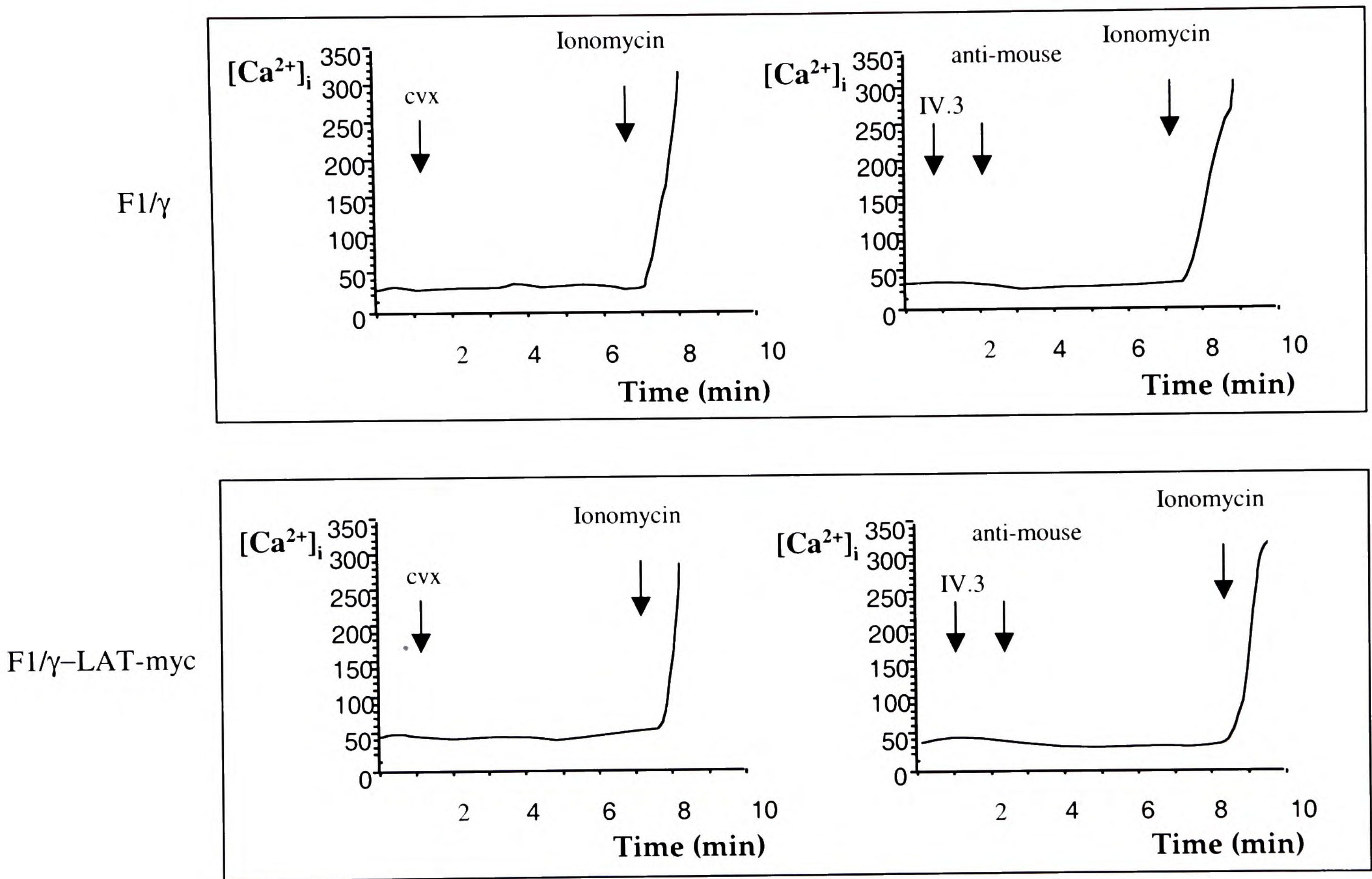


Figure 5.4. Calcium increase in F1/γ cells. Cells transfected (F1/γ-LAT-myc) or not (F1/γ) with LAT were loaded with the calcium reporter dye Fura-2. Cells were stimulated with 20 nM convulxin or with 10 mg/ml IV.3 antibody followed by cross-linking with anti-mouse IgG. After 7-8 min cells were stimulated with ionomicyn as a positive control.Changes in calcium concentration were visualised in a Zeiss microscope and analysed with Openlab software. n=5.

receptor–dependent signaling pathways (Musci et al., 1997). On the other hand, aggregation, dense granule secretion and calcium mobilisation are significantly diminished in platelets from XLA patients, who lack functional expression of Btk, in response to GPVI stimulation (Quek et al., 1998). In order to investigate whether SLP-76 and Btk were phosphorylated in F1/γ cells, GPVI and FcγRIIA were activated and Btk and SLP-76 immunoprecipitated. Immuno complexes were analysed by western blotting to detect tyrosine phosphorylation. Btk was found to be phosphorylated after stimulation of either receptor, even in the absence of LAT-myc (Fig.5.5). However, neither GPVI nor FcγRIIA induced a detectable increase in tyrosine phosphorylation of SLP-76 (Fig. 5.6). Transient co-overexpression of wild type SLP-76 and LAT-myc in F1/γ cells did not induce PLCγ2 phosphorylation upon GPVI cross-linking (data not shown), indicating that the failure was not due to a lesion in endogenous SLP-76. The data suggested that the failure to phosphorylate or activate PLCγ2 upon GPVI or FcγRIIA cross-linking might be due to an inability to phosphorylate SLP-76.

Studies in B and T cells give us a model of the proximal events in the signaling by immune receptors. For SLP-76 to transmit the signal downstream in the cell, its translocation to the membrane and phosphorylation are required (Clements et al., 1998; Pivniouk et al., 1998; Fang et al., 1996; Boerth et al., 2000; Ishiai et al., 2000). Translocation is dependent on its association with Gads, which binds to the phosphorylated LAT already located in the membrane (Zhang et al., 2000; Asada et al., 1999). Once SLP-76 is in the membrane, it is phosphorylated by Syk/ZAP-70 (Noraz et al., 2000; Wardenburg et al., 1996). On the other hand, the Tec-family member Btk has

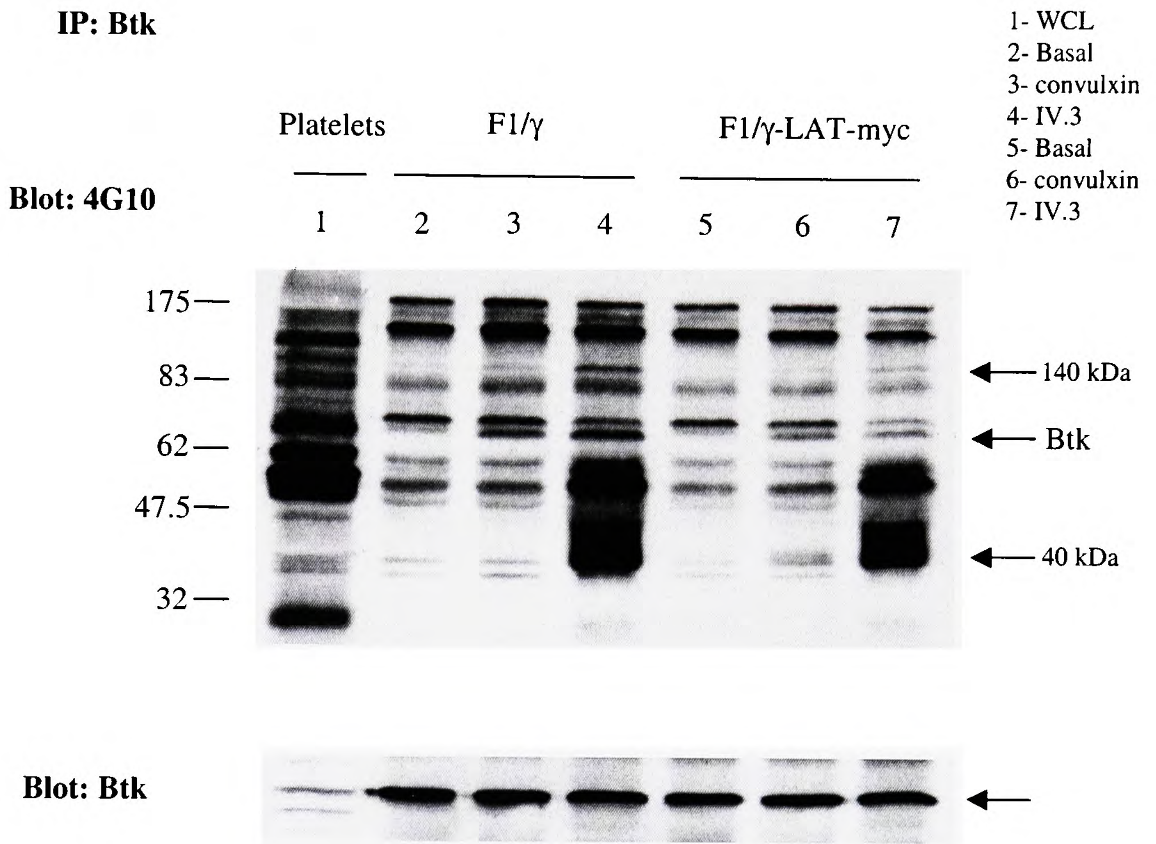


Figure 5.5. Btk is phosphorylated in F1/γ cells following GPVI and FcγRIIA stimulation. F1/γ cells expressing (lanes 5-7) or not (lanes 2-4) LAT were differentiated with 10 nM PMA for 24 hours and stimulated with either convulxin or with mAb IV.3 followed by anti-mouse IgG. Lysates were subjected to immunoprecipitation using an anti-Btk antibody, and immunocomplexes separated by SDS-PAGE. Membranes were blotted with mAb 4G10 to detect tyrosine phosphorylated proteins. Btk migrates approximately at 72 kDa as indicated, and was found to be phosphorylated after stimulation of either receptor. An unidentified tyrosine phosphorylated band of about 140 kDa co-precipitated with Btk in stimulated cells. The transfected LAT-myc (40 kDa) was co-precipitated with Btk in cells stimulated with convulxin (lane 6). A strongly phosphorylated band with a similar molecular weight co-precipitated with Btk following stimulation of FcγRIIA. This band corresponds to the phosphorylated receptor itself (lanes 4 and 7). Membrane was stripped and re-blotted to check loading. A whole cell lysate from human platelets was included as a positive control for western blotting (lane 1).Figure shows one result representative of two independent experiments.

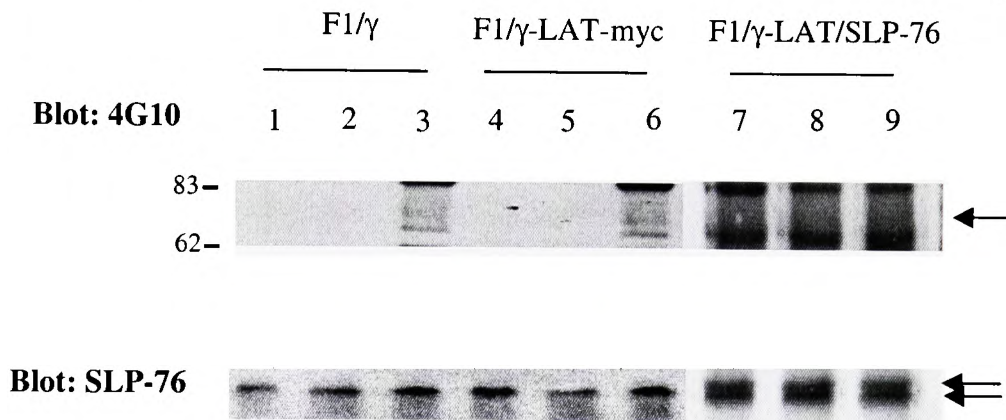


Figure 5.6. SLP-76 phosphorylation upon GPVI and FcγRIIA activation in F1/γ cells. F1/γ cells transient transfected (lanes 4-6) or not (lanes 1-3) with LAT, or transient transfected with a chimeric protein LAT/SLP-76 (lanes 7-9), were differentiated with 10 nM PMA for 24 hours and stimulated with convulxin, or with mAb IV.3 followed by anti-mouse IgG. Lysates were subjected to immunoprecipitation using an anti-SLP-76 antibody, and immunocomplexes separated by SDS-PAGE. Membranes were blotted with mAb 4G10 to detect tyrosine phosphorylated proteins. Endogenous SLP-76 migrates approximately at 76 kDa, and chimeric LAT/SLP-76 at 78 kDa. Membrane was stripped and re-blotted to check loading.Figure shows one result representative of three independent experiments.

been shown to play a role in translocation to the membrane of SLP-76 or its homologue SLP-65 in T and B cells, respectively (Su et al., 1999). Altogether, we have a picture where the complex SLP-76-Gads binds to the phosphorylated LAT at the membrane. SLP-76 is then phosphorylated by Syk, and binds to the SH2 domain of Btk. PLCγ2 is then translocated to the membrane trough its association to LAT and then phosphorylated and activated, leading subsequently to an elevation in the concentration of intracellular calcium.

The failure of SLP-76 to be phosphorylated in Fl/γ cells after GPVI or FcγRIIA activation might therefore be due to the inability of the protein to translocate to the membrane. Consistent with this, immunoprecipitation of LAT followed by western blotting to detect co-precipitated SLP-76 revealed a lack of association between both proteins (Fig. 5.7.1). Moreover, immunoprecipitation of SLP-76 and subsequent western blot to detect co-precipitated Btk showed some basal level of association between both proteins which was not increased upon stimulation (Fig. 5.7.2). It can be concluded from these results that SLP-76 does not form a complex with LAT-Gads.

The question raised whether translocation of SLP-76 to the membrane would suffice to induce PLCγ2 phosphorylation upon stimulation of GPVI or FcγRIIA in these cells. It has recently been reported that expression of a chimeric protein LAT/SLP-76 in T cells lacking LAT is sufficient to restore responses upon TCR ligation, indicating that targeting SLP-76 to GEMs through its association with LAT is a vital step in TCR signaling (Boerth et al., 2000). The LAT/SLP-76 chimeric protein was transient

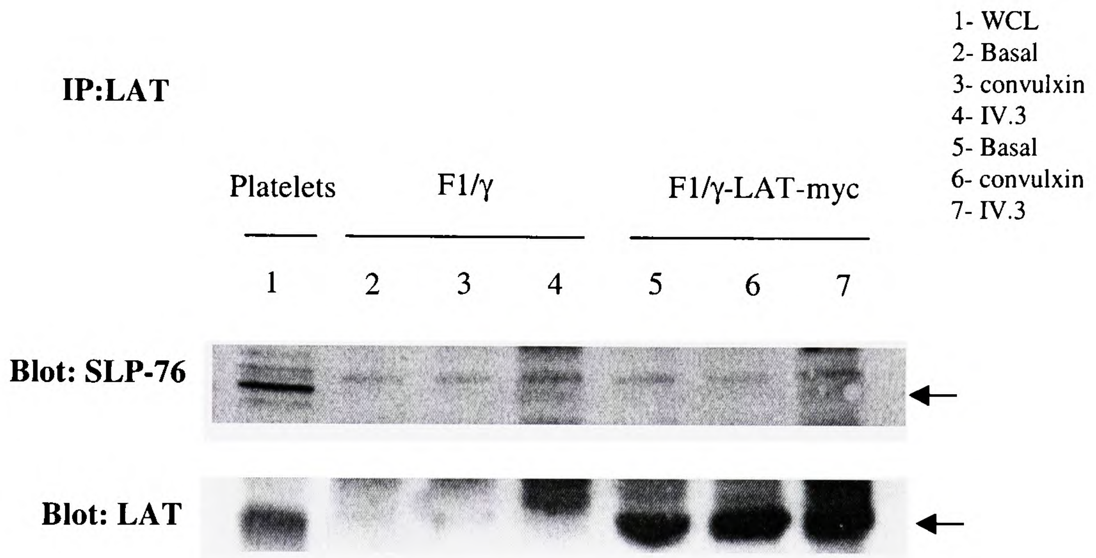


Figure 5.7.1. LAT-myc does not associate to SLP-76 upon GPVI or FcγRIIA cross-linking. F1/γ cells transient transfected (F1/γ-LAT-myc) or not (F1/γ) with LAT were differentiated with 10 nM PMA for 24 hours and stimulatedconvulxin (lanes 3 and 6), or with IV.3 antibody followed by cross-linking with anti-mouse IgG (lanes 4 and 7), and LAT immunoprecipitated using a specific antibody. Immuno complexes were separated by SDS-PAGE and blotted for either LAT (bottom blot) or SLP-76 (upper blot). A band corresponding to LAT-myc was detected in cells transfected with the protein, whereas SLP-76 was not detected in association with LAT-myc. A platelet whole cell lysate was included as a positive control for western blotting. Arrows indicate the position of the blotted proteins. Figure shows one result representative of two independent experiments.

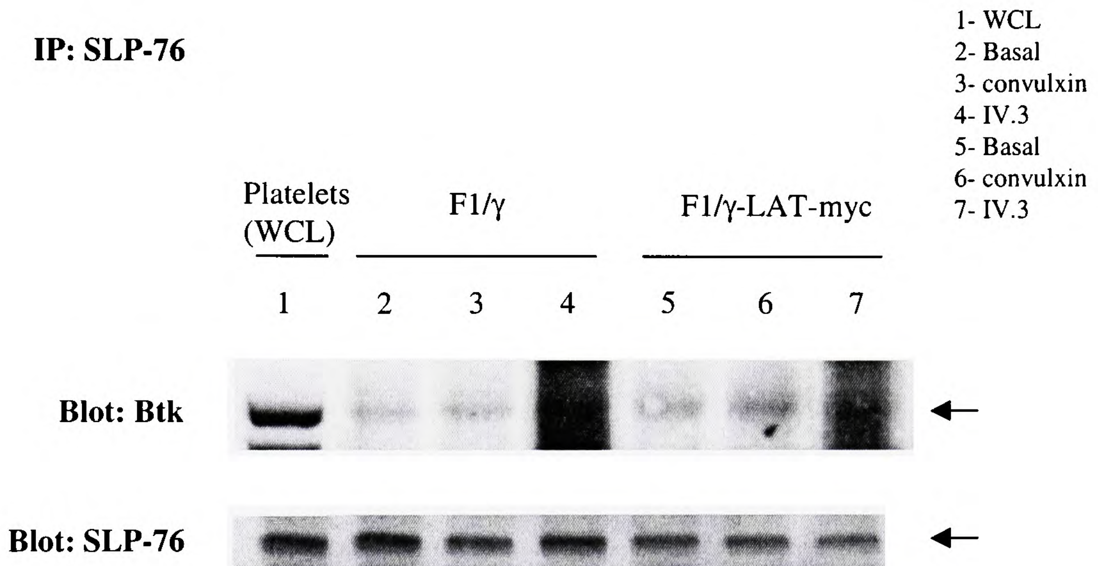


Figure 5.7.2. SLP-76 and Btk co-associate in F1/γ cells. F1/γ cells transient-transfected (F1/γ-LAT-myc) or not (F1/γ) with LAT were differentiated with 10 nM PMA for 24 hours and stimulated with convulxin (lanes 3 and 6), or with IV.3 antibody followed by cross-linking with anti-mouse IgG (lanes 4 and 7), and SLP-76 immunoprecipitated using a specific antibody. Immuno complexes were separated by SDS-PAGE and blotted for either SLP-76 (bottom blot) or Btk (upper blot). Btk was found to co-precipitate with SLP-76 in all cases, independently of stimulation. A platelet whole cell lysate was included as a positive control for western blotting. Arrows indicate the position of the blotted proteins. Figure shows one result representative of three independent experiments.

overexpressed in F1/γ cells and PLCγ2 phosphorylation assessed by western blotting after immunoprecipitation of the protein (Fig. 5.6). The results show that enforced localisation of SLP-76 to GEMs was not sufficient to promote phosphorylation of PLCγ2. Moreover, SLP-76 itself was not phosphorylated, suggesting that a previously undetected mechanism of regulation of SLP-76 is missing or defective in these cells.

5.7. Discussion

Results in this chapter showed a differential regulation of PLCγ2 phosphorylation after engagement of either receptor. Despite a similar initiation in the signaling events from both receptors, PLCγ2 was phosphorylated after FcγRIIA cross-linking but not upon GPVI stimulation. The similar levels of phosphorylation of Syk suggested that the absence and/or deficiency of a component in the cascade was the most likely explanation for the defect. The adapter LAT was demonstrated to be absent in these cells, but ectopic expression of a LAT-myc construct was not able to restore the phosphorylation of PLCγ2 by convulxin, despite phosphorylation of LAT-myc itself and its subsequent association to PLCγ2. The association between PLCγ2 and LAT was probably due to a consensus motif in the cytoplasmic tail of LAT for the binding to the SH2 domain of PLCγ2 (Songyang et al., 1993; Zhang et al., 1998a). Phosphorylation of PLCγ2 in response to FcγRIIA cross-linking was potentiated in the presence of LAT-myc, consistent with previous reports demonstrating potentiation in the presence of LAT upon cross-linking of Fcγ receptors in myeloid cells (Tridandapani et al., 2000). However in neither case mobilisation of calcium

was restored, suggesting that FcγRIIA can regulate phosphorylation of PLCγ2 by a novel phosphorylation way, but not one in which is of functional relevance.

LAT has previously been proven to be constitutively present in GEMs in T cells (Zhang et al., 1998a; Lin et al., 1999). Immunoprecipitation of LAT-myc in GPVI-stimulated cells showed increase in phosphorylation of the transfected protein. As shown above, upon stimulation with convulxin phosphorylated LAT was capable to recruit PLCγ2 to the membrane, and this suggested that GPVI was able to transmit the signal to GEMs in K562 cells.

It has been reported that phosphorylation of PLCγ2 is necessary but not sufficient to induce an elevation in calcium, as different domains in the protein function as regulators (reviewed in Carpenter and Ji., 1999). Moreover, PLCγ2 must be phosphorylated at different tyrosine residues to achieve full activation of the protein (Kim et al., 1991). Several proteins have been implicated in the regulation of PLCγ2 phosphorylation and calcium increase in B and T cells and in platelets. Among these the adapter LAT, which is constitutively targeted to GEMs in T cells (Zhang et al., 1998a; Lin et al., 1999), serves as a docking site for the recruitment and concentration of signaling proteins. In this report, phosphorylation of LAT upon receptor engagement suggested that both GPVI and FcγRIIA were capable to signal to GEMs, and therefore that the impairment in the signaling cascade was not due to a failure for either receptor to communicate with GEMs. SLP-76 in T cells and platelets and its homologue SLP-65 in B cells appear to be crucial.

In T cells, PLCγ1 is recruited to the phosphorylated LAT even in the absence of SLP-76, but complete TCR-mediated PLCγ1 phosphorylation and activation require phosphorylation of SLP-76 (Fang et al., 1996; Yablonski et al., 1998). Consistent with this, phosphorylation of PLCγ2 and subsequent activation events are abolished in platelets deficient for SLP-76 upon collagen stimulation (Clements et al., 1999). In this report it has been shown that SLP-76 was not phosphorylated upon GPVI or FcγRIIA cross-linking. Ectopic expressed SLP-76 was neither phosphorylated, indicating that the failure was not due to a defective endogenous SLP-76. The lack of phosphorylation of SLP-76 was likely the reason for the impairment in calcium elevation, despite phosphorylation of PLCγ2 upon FcγRIIA activation.

SLP-76 facilitates the assembly of macromolecular signaling complexes at the membrane which are necessary for the transmission of the signal (Boerth et al., 2000; Ishiai et al., 2000). Several studies have suggested that LAT and SLP-76 function coordinately to promote downstream signaling. SLP-76 must be recruited to the membrane and phosphorylated. This recruitment takes place trough the SH2 domain of the SLP-76-associated adaptor Gads (Yoder et al., 2001), which binds to the phosphorylated LAT present in GEMs. Consistent with this, enforced localisation of SLP-76 to GEMs replaces the requirement for LAT and Gads in T cell receptor signaling, demonstrating that translocation to the membrane of SLP-76 is primarily a function of LAT and Gads in T cells (Ishiai et al., 2000; Boerth et al., 2000). On the other hand, phosphorylated SLP-76 binds through its SH2 domain to the phosphorylated Btk located at the membrane (Su et al., 1999). Platelets from XLA patients, which develop B cell deficiency due to lack of

expression of functional Btk, show defects in the platelet response to collagen, which are associated with a reduction in tyrosine phosphorylation of PLCγ2 (Quek et al., 1999). It has been reported in this chapter that SLP-76 does not associate to LAT or to Btk upon GPVI or FcγRIIA cross-linking in F1/γ cells, despite phosphorylation of LAT and Btk itself and that SLP-76 forms a complex with Gads. Moreover, ectopic expression of a chimeric protein LAT/SLP-76 which constitutively localises to GEMs (Boerth et al., 2000) was unable to become phosphorylated or restore the response upon stimulation of GPVI or FcγRIIA. This strongly suggested that some unknown component important in SLP-76 regulation was missing in K562 cells, therefore blocking the transmission of the signal after engagement of either receptor.

Chapter 6

CRP but not collagen stimulate GPVI in K562 cells

6.1. Aim

The aim of this chapter was to use GPVI-expressing K562 cells in order to demonstrate whether collagen, CRP and a number of snake toxins can signal through GPVI.

6.2. Introduction

Collagens are a major component of extracellular matrix. Cells recognise collagen in different ways depending on sequence and structure. They can recognise predominantly primary sequence, they may require triple-helical structure or they can require fibrillar structures. Since collagens are major constituents of the subendothelium that determine the thrombogenicity of the injured or pathological vessel wall, a major role is induction of platelet activation and aggregation as the start of repair process. Platelets have at least two direct (the integrin $\alpha2\beta1$ and GPVI) and one indirect (via von Willebrand factor) receptor for collagen, and collagen has specific recognition motifs for these receptors. These receptors and recognition motifs are under intensive investigation in the search for possible methods to control platelet activation *in vivo*.

The interaction between platelets and collagen involves first adhesion and subsequently, activation leading to second phase adhesion, secretion and ultimately aggregation (Morton et al., 1989; Santoro et al., 1991). It is accepted that the integrin $\alpha2\beta1$ is the major receptor supporting initial platelet adhesion to collagen, whereas GPVI mediates activation (Kehrel et al., 1998; Gibbins et al., 1997). However recent reports

have challenged this idea, proposing instead that GPVI is a major collagen receptor for adhesion (Nieswandt et al., 2001c). It is likely that both receptors are important for adhesion and probably activation of platelets (Watson et al., 2000).

Due to the multimeric nature of collagen, the development of specific ligands to either receptor is essential for the understanding of the relative contribution of one receptor to the overall mechanism of platelet activation by collagen. Among these, collagen-related peptide (CRP) is thought to signal specifically through GPVI as demonstrated by the lack of response to the peptide in GPVI-deficient platelets (Kehrel et al., 1998). On the other hand, convulxin has been an invaluable tool for the understanding of GPVI signaling. However, snake toxins other than convulxin that recognise alternative domains within GPVI may be useful for the development of anti-thrombotic drugs. Alboaggregin-A is a 50 kDa tetrameric C-type lectin consisting of 2 α and 2 β subunits, isolated from *Trimeresurus albolabris*. Alboaggregin-A has been reported to bind specifically to the platelet glycoprotein (GP)-Ibα (Fujimura et al., 1996). In a recent study, alboaggregin-A was observed to promote tyrosine phosphorylation of the FcR γ-chain leading to phosphorylation and activation of Syk, events that are critical in signaling by the collagen receptor GPVI ((Falati et al., 1999; Gibbins et al., 1996). Partly on the basis of these observations, it was proposed that GPIbα signals through a GPVI-like pathway.

Targeted disruption of one receptor, or expression of one receptor in the absence of the others, in combination with the use of specific ligands, seem essential approaches to unravel the function of each receptor. This chapter combines some of these techniques in

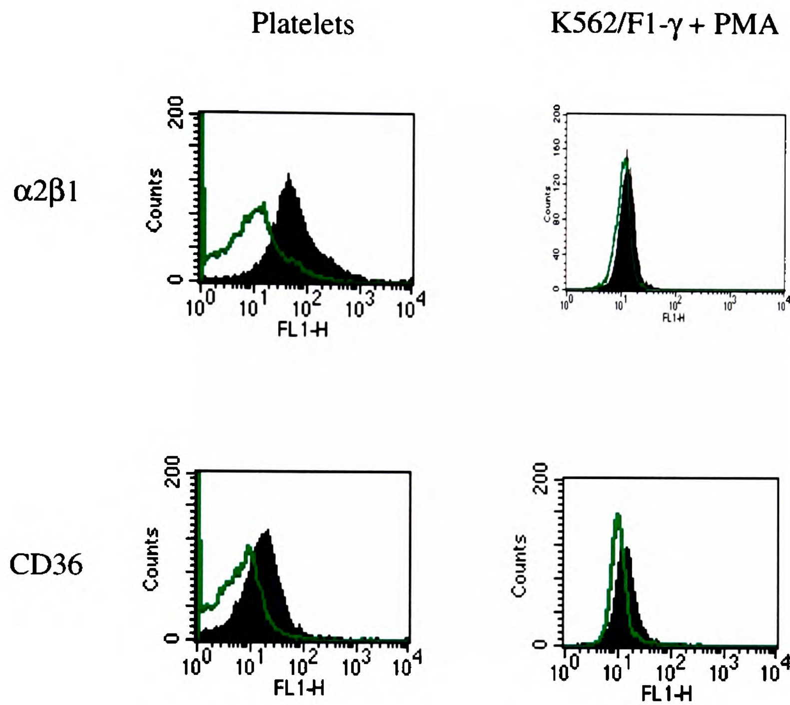


Figure 6.1. α2β1 and CD36 expression in K562/F1-γ cells. K562 cells stably expressing GPVI and the FcR γ-chain (K562/F1-γ) were differentiated with 10 nMPMA for 24 h. Expression of the α2-subunit of the integrin α2β1, and CD36, was assessed by flow cytometry using specific antibodies to either receptor (purple area). Background fluorescence was obtained with an isotype control antibody (green line). Expression of either receptor in platelets is shown as a positive control.Figure shows one result representative of two independent experiments.

order to establish a number of ligands that signal through GPVI. This will provide a model system to test the effect of inhibition of specific collagen-platelet receptor interactions for effectiveness and should provide assay systems for the development of small molecule inhibitors.

Results

6.3. K562 cells display low levels of expression of α2β1 and CD36

K562 cells were analysed to detect expression of the integrin α2β1 and CD36, in order to determine whether collagen receptors other than GPVI were present in these cells. Flow cytometry studies demonstrated low levels of expression of both receptors when compared to platelets (Fig. 6.1). The studies were performed in K562 cells differentiated with PMA for 24 hours and stably expressing GPVI and the FcR γ-chain (K562/F1-γ), since it was important for subsequent functional studies to determine the level of expression of the receptors in PMA-differentiated GPVI-expressing K562 cells.

6.4. CRP but not collagen stimulate Syk phosphorylation in GPVI-expressing K562 cells

PMA-differentiated K562 cells stably expressing FcR γ-chain alone (K562/pRc-γ) or together with GPVI (K562/F1-γ) were stimulated with collagen, CRP and convulxin for 90 sec. and Syk immunoprecipitated. After transfer, membranes were blotted to detect

tyrosine phosphorylation (Fig. 6.2). 20 nM convulxin induced a robust increase in phosphorylation of Syk, whereas CRP induced a comparably weak increase in phosphorylation albeit at similar concentration to those that induce platelet activation. However, collagen was unable to promote an increase in phosphorylation of Syk, even when used at high concentrations of 100 µg/ml. When stimulated under the same conditions in the absence of GPVI, the cells did not display an increase in phosphorylation of Syk. This demonstrates that GPVI expression was sufficient to reproduce the phosphorylation of Syk to convulxin and CRP, but not to collagen. It must be pointed out that collagen and CRP are already obtained in a crosslinked form (see Morton et al., 1995), and although the molecular weight of collagen is estimated to be 300000 Da, this is certainly very heterogeneous. As calculation of concentration in molarity results therefore impossible, the concentrations used for this experiment are those within a range which are causing maximum activation on platelets (Watson et al., 2000).

It has been reported that endogenous and transfected GPVI from cell lines migrates further on SDS-PAGE relative to native GPVI from human platelets, suggesting incomplete glycosilation (Jandrot-Perrus et al., 2000; Zheng et al., 2001). To assess whether the same applied to GPVI-transfected K562 cells, protein lysates from these cells and platelets were separated by SDS-PAGE and transferred. Membranes were blotted with convulxin to detect GPVI. Consistent with previous reports in different cell lines, GPVI from transfected K562 cells migrate further that GPVI from human platelets (Fig. 6.3). Moreover, GPVI from the human T cell line Jurkat stably transfected with the receptor

migrate further that GPVI from transfected K562 cells (Fig. 6.3), suggesting that different cells express differentialy glycosylated forms of the receptor.

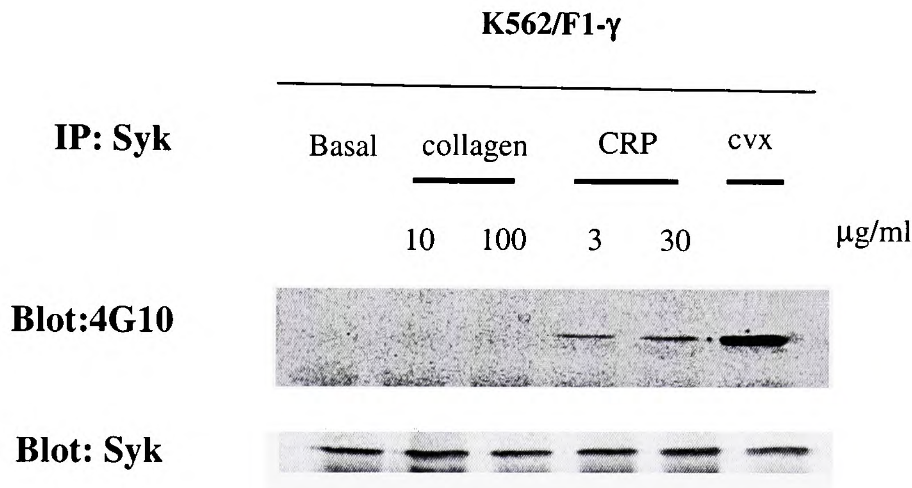


Figure 6.2. Syk phosphorylation upon collagen and CRP stimulation. K562 cells stably expressing GPVI and the FcR γ-chain (K562/F1-γ)were stimulated with the indicated concentrations of collagen and CRP. Syk was immunoprecipitated and immuno complexes separated by SDS-PAGE. Membranes were blotted with 4G10 antibody to detect tyrosine phosphorylated bands. Upper panel shows phosphorylation of Syk. Membrane was stripped and reprobed to check equal loading. A positive control using 20 nM convulxin for 90 sec is included for comparison of phosphorylation levels. Figure shows one result representative of three independent experiments.

Ligand blot: GPVI

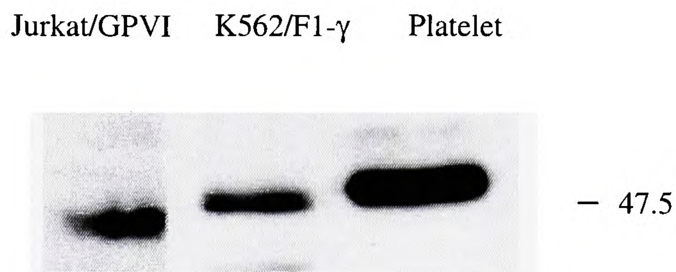
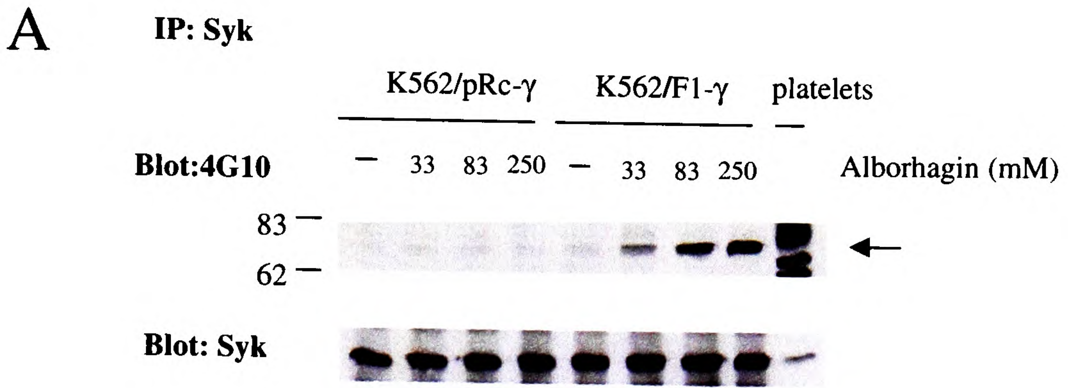


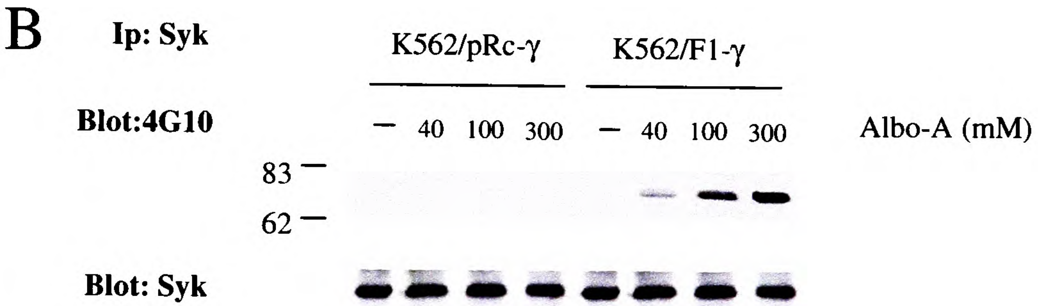
Figure 6.3. Differential GPVI migration on SDS-PAGE. A protein lysate from human platelets, Jurkat cells stably transfected with GPVI (Jurkat/GPVI) or K562 cells stably transfected with GPVI and the FcR γ-chain (K562/F1-γ) were subjected to SDS-PAGE and transferred to a PVDF membrane. Membranes were blotted with convulxin and an antibody to convulxin to detect GPVI. Notice a slight difference in migration of GPVI from Jurkat/GPVI, K562/F1-γ cells or from platelets. Figure shows one result representative of three independent experiments.

6.5. The snake venoms alboaggregin-A and alboraghin are agonists at the collagen receptor GPVI

The ability of the snake venoms alboaggregin-A and alborhagin to bind GPVI was studied using PMA-differentiated K562 cells expressing or not GPVI. Alboaggregin-A has been proposed to specifically recognise GPIbα and signal through a GPVI-like pattern. Stimulation of K562/F1-γ cells with alboaggregin-A promoted an increase in phosphorylation of Syk which was dose dependent, whereas in the absence of GPVI the increase in phosphorylation of Syk was no longer detectable (Fig. 6.4). Since these cells do not express GPIbα, as assessed by flow cytometry (Fig. 6.4), the increase in phosphorylation of Syk demonstrated that alboaggregin-A was binding to GPVI. A novel viper venom metalloproteinase, alborhagin, isolated from white-lipped tree viper (*Trimeresurus albolabris*) venom, has been shown to cause Syk phosphorylation in association with platelet activation (Andrews et al., 2001). Stimulation of K562/F1-γ cells with alborhagin caused an increase in phosphorylation of Syk which was dose dependent (Fig. 6.4).

Binding of convulxin (20 nM) to GPVI-expressing K562 cells was partially inhibited by alborhagin in a dose dependent manner. There was ~40% inhibition at a final concentration of 10 μg/ml alborhagin, with no increased inhibition up to 100 μg/ml (Fig. 6.5). On the other hand, binding of convulxin was partially inhibited by alboagreggin-A in a dose dependent manner. There was maximum inhibition of ~20% at 100 μg/ml





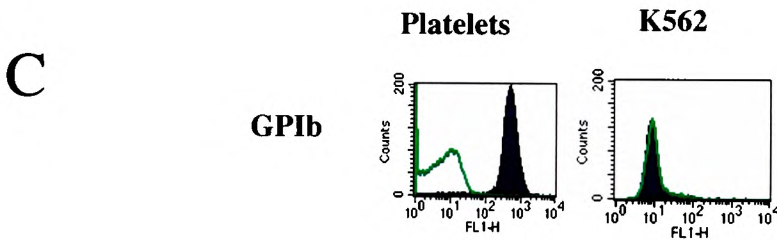


Figure 6.4. Alboaggregin-A and alborhagin stimulate Syk phosphorylation in K562/F1-γ cells. K562 cells stably expressing the FcR γ-chain alone (K562/pRc-γ) or together with GPVI (K562/F1-γ) were diferentiated with 10 nM PMA for 24 hours, then stimulated with different concentrations of alborhagin (**A**) or alboaggregin-A (Albo-A) (**B**), and Syk immunoprecipitated and blotted to detect tyrosine phosphorylation. Membranes were stripped and re-blotted to ensure equal loading. Expression of GPIb was assessed by flow cytometry in K562 cells differentiated with 10 nM PMA for 24 hours and compared to platelets (**C**). Purple area represents specific binding whereas green line represents background fluorescence obtained with an isotype control antibody. Figure shows one result representative of two independent experiments.

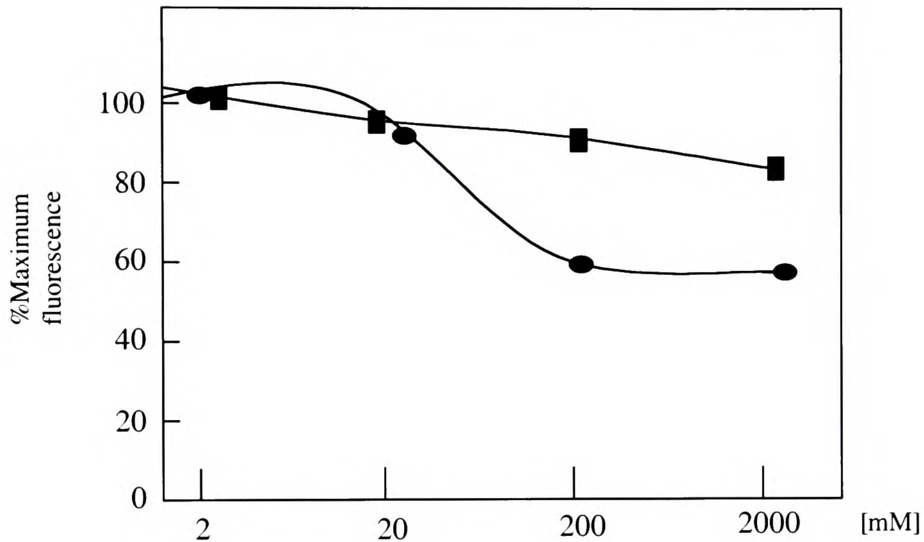


Figure 6.5. Alborhagin and alboaggregin-A binding to GPVI in competition with convulxin. Effect of different concentrations of alborhagin (filled circles) and alboaggregin-A (filled squares) on binding of convulxin (20 nM) to K562/F1-γ cells. Convulxin binding was assessed using FITC-conjugated anti-convulxin IgG, and results are expressed relative to maximum binding measured in the absence of alborhagin or alboaggregin-A. Figure shows one result representative of 3 independent experiments.

alboaggregin-A, which decreased up to 3% inhibition with 0.1 μg/ml alboaggregin-A (Fig. 6.5). This suggested that the binding site for the two toxins are partially overlapping.

6.6. Discussion

Recent studies of human platelets that are unresponsive to collagen (Moroi et al., 1989), mouse knockouts (Nieswandt et al., 2000), and platelet signaling have generated the hypothesis that the platelet surface protein GPVI mediates collagen signaling. Transient expression of GPVI has been demonstrated to confer a slight calcium signal to collagen in the DAMI megakaryocytic cell line (Clemetson et al., 1999), but these cells express endogenous GPVI, α2β1 (Strouse et al., 1996) and perhaps other collagen receptors. Stable expression of GPVI in RBL-H3 cells, a rat basophilic leukemia cell line that expresses FcR γ-chain but not GPVI or other collagen receptors, show intracellular signal when stimulated with convulxin, but not to collagen, and a small response to CRP when used 50 times in excess relative to platelets (Zheng et al., 2001). Results in this chapter using K562 cells show a lack of response to collagen but unlike RBL-2H3 cells, K562 cells responded to normal concentrations of CRP. The lack of response to collagen may suggest the necessity of other collagen receptors such as α2β1, which is up-regulated during megakaryocytic differentiation in parallel to GPVI (Lagrue-Lak-Hal, 2001), or it might reflect the requirement for a certain receptor-density at the membrane. The difference in response to the GPVI-specific ligand CRP between RBL-2H3 cells and K562 cells suggests, however, a qualitative difference at the expressed GPVI. It could be that binding of human GPVI to the endogenous rat FcR γ-chain in RBL-2H3 cells is affecting

to the conformation of GPVI, which now binds to CRP with a lower efficiency relative to K562. In addition, it could reflect a differential posttranslational modification of GPVI which is affecting the binding of the ligand. In support of the latter, it has been shown that GPVI from megakaryocytes migrates slightly different to GPVI from platelets (Jandrot-Perrus et al., 2000), and that transfection of GPVI into different cell lines, i.e. K562 and Jurkat, affects to the size of the receptor on SDS-PAGE. Although formal evidence is necessary, the data strongly suggest that different cells express a qualitatively different receptor, which could explain some of the differences observed between K562 and RBL-2H3 cells regarding the response to CRP.

Snake venoms provide a powerful tool in the study of platelet receptors, as many of them bind and activate specifically to one receptor. It was important to establish whether venoms other than convulxin targeted GPVI. Alborhagin has been reported to induce a tyrosine phosphorylation profile similar to that induced by convulxin (Ichinohe et al., 1997; Hers et al., 2000). In this chapter it is shown that alborhagin, like convulxin, induces GPVI-dependent phosphorylation of Syk in FcR γ-chain-expressing K562 cell cotransfected with GPVI but not in untransfected cells. The same observation has been made with the snake toxin alboaggregin-A, which was thought to bind specifically to the von Willebrand factor receptor GPIbα (Fujimura et al., 1996). Interestingly, although both alborhagin and alboaggregin-A appeared to target GPVI, they partially inhibited convulxin binding with different efficiencies in GPVI-expressing K562 cells. Alborhagin was capable to block by 40% convulxin binding, whereas the same concentration of

alboaggregin-A inhibited convulxin binding by 15%. This partial inhibition suggests that both venoms recognise GPVI at different sites to that recognised by convulxin.

Responses to alboaggregin-A through GPVI demonstrated that this venom is binding to GPVI, in addition to the reported binding to GPIbα (Fujimura et al., 1996), and therefore cannot be used to identify signaling pathways linked to GPIbα in cells that express GPVI. This is in agreement with Dörmann et al., who recently reported that alboaggregin-A binds to GPVI (Dörmann et al., 2001).

Chapter 7

General discussion

General Discussion

Summary of results

In this thesis I make a study of the collagen receptor GPVI in megakaryocytes. In chapter 3 I undertake the characterisation of a number of human megakaryoblastic cell lines and primary cultures of mouse megakaryocytes to demonstrate up-regulation of GPVI with megakaryocytic differentiation. Moreover, the receptor is functional and leads to functional responses when stimulated with convulxin, a specific ligand at GPVI. In chapter 4 I make stable transfections of GPVI and the associated FcR γ-chain in K562 cells to demonstrate the necessity of the latter for transmission of the signal. Moreover, by co-precipitation studies I demonstrate that the transmembrane arginine and cytoplasmic tail of GPVI are necessary for association to the FcR γ-chain. The transfected receptor is able to reconstitute the proximal events in GPVI signaling as demonstrated by phosphorylation of Syk. In chapter 5 it is shown that the cells are incapable to signal further. An analysis of different proteins implicated in this cascade demonstrates impairment in phosphorylation of the adapter SLP-76. Finally, chapter 6 demonstrates that the GPVI-specific ligand CRP but not collagen is capable to signal in GPVI-expressing K562 cells, and that the snake venoms alborhagin and alboaggregin-A are active at GPVI.

Discussion

Before commencing this work, GPVI was a protein known to be present in human and mouse platelets. Studies from patients with defective expression for GPVI and suffering from mild bleeding disorders pointed at this protein as a crucial receptor for collagen in platelet activation. This was confirmed using several different approaches including the

effect of collagen-related peptides (CRP) in GPVI-deficient platelets, or by the generation of FcR γ-chain knock-out mice whose platelets lack expression of GPVI and are unresponsive to collagen. However, the presence of different collagen receptors at the platelet surface made it necessary to develop specific ligands to GPVI capable to bind and activate the receptor without prior activation of another collagen receptors. The use of these specific ligands, namely the snake venom convulxin and the synthetic peptide CRP, allowed confirmation of the similarity between the signaling pathway generated by GPVI and that from TCR, BCR and other immune receptors. Advances in the understanding of collagen signaling in platelets have been hampered by the impossibility of genetically manipulate these cells, as they are anucleated. However, cell lines can be manipulated to alter expression or structure of a given protein, and offer the theoretical possibility to be used as an alternative model system to study certain aspects of the platelet biology.

Within the last three years, the major contribution to the study of GPVI came from the cloning of the receptor by three different groups. Not surprisingly was found to share homology with immune receptors, consistent with their in-common signaling features. Cloning of GPVI has opened new possibilities in the study of the receptor. It has been expressed in different cell types in order to elucidate the binding to GPVI of different ligands in the absence of another collagen receptors. In this thesis, expression of recombinant GPVI in K562 cells has proven important to demonstrate binding to and activation of the receptor by different unrelated snake toxins and CRP, but not collagen itself. Other groups have reported similar results, raising the possibility that ectopic-

expressed GPVI on its own is unable to bind collagen and suggesting that other collagen-receptors are required.

Cloning of GPVI has allowed also for the generation of mutant receptors which have been transfected into different cell lines. In this thesis it has been demonstrated that the FcR γ-chain is essential for the transmission of the signal within the cell after GPVI stimulation. In addition, particular sites within GPVI, namely the transmembrane arginine and the cytoplasmic tail, have proven essential for association with the FcR γ-chain and therefore for functional expression of GPVI.

Historically, the study of the signaling pathway generated by GPVI has been mainly studied in human platelets or in platelets from normal mice or mice deficient for one or more proteins. Studies with platelets from mice deficient for a given protein have been essential to unravel the network of proteins implicated in GPVI signaling which lead to PLCγ2 activation and elevation of $[Ca^{2+}]_i$. Moreover, they have greatly contributed to know the relative position of a certain protein within the whole cascade. However, further genetic manipulation of these platelets is not possible. This can be partly overcome by the use of the platelet precursor, the megakaryocyte. There are few reports on GPVI signaling in megakaryocytes. This is mainly due to the difficulty to obtain fresh megakaryoctes from bone marrow, where they represent less than 0.1% of the total cell number. This thesis reports studies with different human megakaryoblastic cell lines and with primary cultures of mouse bone marrow cells grown in a medium which supports megakaryocyte differentiation. Cell lines and megakaryocytes cultured *in vitro* are reported here to

undergo increase of GPVI expression with differentiation. The receptor is functional as demonstrated in experiments to detect $[Ca^{2+}]_i$ elevation upon convulxin stimulation. However, stable expression of GPVI and its associated FcR γ-chain in K562 cells, which show some megakaryocytic features but very low levels of GPVI, resulted in a cell capable to initiate the signaling events by the receptor, but failed to transmit the signal further to induce calcium mobilization. Analysis of key proteins necessary for the transmission of the signal by GPVI demonstrated lack of expression of the adapter LAT, which is crucial for $[Ca^{2+}]_i$ elevation upon GPVI stimulation. However, ectopic expression of LAT did not restore the response despite phosphorylation of LAT itself. Further analysis demonstrated impairment in phosphorylation of the adapter SLP-76. Although the exact nature of the failure has not been determined yet, it all points to a new mechanism of regulation for SLP-76 phosphorylation.

Studies from this thesis and from another groups suggest that GPVI may be a specific marker of megakaryocytic differentiation. It is important for the study of megakaryocytopoiesis to establish the exact moment during differentiation at which different proteins are expressed. Although GPVI seems to be an early marker of megakaryocytic differentiation, further work is necessary to conclusively establish the exact moment of expression of GPVI duringmegakaryocytopoiesis, and whether it is specific of the megakaryocyte or it is expressed somewhere else.

Overall, this thesis presents data demonstrating the presence of GPVI in megakaryocytes and up-regulation of the protein with differentiation. Structure function

studies of GPVI have shown the absolute requirement of the FcR γ-chain for the transmission of the signal within the cell. Moreover, the transmembrane arginine and cytoplasmic tail of GPVI have proven necessary for the association to the FcR γ-chain. Both GPVI and the FcR γ-chain have successfully been stably transfected within a megakaryoblastic cell line, allowing for reconstitution of the proximal signaling events upon GPVI engagement. This GPVI-expressing cell line has been useful for the characterisation of two different snake toxins which are agonists at GPVI.

Future work will involve further characterisation of K562 and another cell types stably transfected with GPVI, in order to reconstitute the complete signaling pathway leading to elevation of intracellular calcium. Moreover, the cells shall be co-transfected with different collagen receptors in combination with GPVI in order to achieve reconstitution of the response to collagen. Finally, generation of a GPVI knock-out will answer many questions regarding the importance of this receptor to the overall mechanism of platelet activation by collagen.

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