COMPARISON OF CLEC-2 AND GPVI SIGNALLING IN PLATELETS: THE ROLE OF ADAPTOR PROTEINS

By

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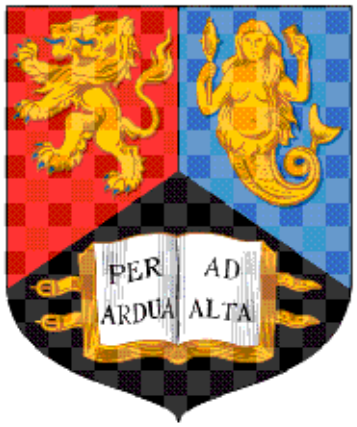

UNIVERSITY OF BIRMINGHAM

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Institute for Biomedical Research

College of Medical and Dental Science

The University of Birmingham

March 2010

Abstract

The platelet collagen receptor GPVI has been shown to activate platelets through an ITAM (immunoreceptor based activatory motif) signalling pathway mediated by sequential activation of Src and Syk tyrosine kinases leading to activation of PLCγ2. The recently discovered C-type lectin CLEC-2 has been shown to activate platelets using an ITAM-like sequence in its cytoplasmic tail that is also dependent on Src and Syk tyrosine kinases, but this shows a partial rather than an absolute dependence on adapter SLP-76 for activation of PLCγ2. The aim of this thesis is to understand some of the key differences in these two signalling pathways.

GPVI is found in a complex with FcRγ which contains the ITAM sequence consisting of two conserved tyrosine residues (Yxx(L/I)x_{6-12}Yxx(L/I)). These two tyrosines, when phosphorylated, provide a docking site for the tandem SH2 domains of the tyrosine kinase Syk, which plays a critical role in downstream signalling and platelet activation. In this thesis I show that CLEC-2 signalling through Syk is mediated by phosphorylation of the single CLEC-2 YxxL sequence and involves receptor dimerisation and cross-linking by the tandem SH2 domains in Syk. I also show that the differential requirement for the adaptor protein SLP-76 between GPVI and CLEC-2 is not mediated by the adaptor Gads, which is a constitutive SLP-76 binding partner and plays a similar, weak partial role in platelet activation by both receptors. Both signalling pathways also show a similar, partial dependency for the transmembrane adaptor protein LAT, although its role is more substantive that that of Gads. I also show that a novel membrane adapter protein, G6f, is not able to substitute for LAT in this signalling pathway and also exclude the LAT-family proteins PAG, LIME, LAX

and NTAL as potential LAT replacements in platelet activation by GPVI. Thus, these results extend our understanding of the mode of platelet activation by CLEC-2 in comparison to GPVI, with the major difference being that the former induces activation of Syk through a novel dimerisation pathway. The events that lie downstream of activation of Syk by CLEC-2 and GPVI on the other hand appear to largely similar.

Publications arising from this thesis

Hughes, C. E., A. Y. Pollitt, J. Mori, J. A. Eble, Michael G. Tomlinson, John H. Hartwig, Chris A. O'Callaghan, Klaus Fütterer and S. P. Watson (2010). "CLEC-2 activates Syk through dimerisation." Blood (***in press)***.

Hughes, C. E., J. M. Auger, J. McGlade, J. A. Eble, A. C. Pearce and S. P. Watson (2008). "Differential roles for the adapters Gads and LAT in platelet activation by GPVI and CLEC-2." J Thromb Haemost **6**(12): 2152-9.

Garcia, A., Y. A. Senis, R. Antrobus, **C. E. Hughes**, R. A. Dwek, S. P. Watson and N. Zitzmann (2006). "A global proteomics approach identifies novel phosphorylated signalling proteins in GPVI-activated platelets: involvement of G6f, a novel platelet Grb2-binding membrane adapter." Proteomics **6**(19): 5332-43.

"…all that is of the body is as coursing waters, all that is of the soul as dreams and vapours; life a warfare, a brief sojourning in an alien land; and after repute, oblivion. Where, then, can man find the power to guide and guard his steps? In one thing and one alone: the love of knowledge."

Marcus Aurelius

"We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the Universe. That makes us something very special."

Stephen Hawking

"When you are courting a nice girl an hour seems like a second. When you sit on a red-hot cinder a second seems like an hour. That's relativity."

Albert Einstein

Acknowledgments

First and foremost I would like to thank my supervisor Steve Watson who has been, and continues to be a constant source of encouragement and support. It has been thanks to his help that I feel like I can call myself a scientist. Thanks also to Mike Tomlinson, my second supervisor who has also been a source of great help and advice. Thanks to the University of Birmingham for our lab space and to the Wellcome Trust for funding.

Many thanks to our collaborators for reagents, mice and the use of their lab space and equipment. In particular Dr. Burkhart Schraven and Dr. Mauro Togni for allowing me to work in their lab in Magdeburg, Germany and for the use of many of their knock-out mice. Thanks to Dr. Jane McGlade for sending the Gads knock-out mice from Canada. Thanks to Dr. Chris O'Callaghan and his lab in Oxford for teaching me how to use their Biacore T-100 and thanks to Dr. Klaus Fütterer for his help with protein expression, ITC and tryptophan fluorescence in the School of Bioscience. Thanks also to John Herbert for his bioinformatical know how.

Thanks also to members of the Watson lab, both past and present; it wouldn't have been the same without you all. In no particular order, Andy for being a mentor and a great bloke. Gemma for being posh. Teresa for teaching me the geography of Europe, and for being a great friend. Simon aka Cpt. Starch Boy for consistently beating me at squash. Stevie T for consistently losing to me at squash, and for being my chauffer, and also being a great bloke. Yotis for making offers people cannot refuse. Mark and Jen for both being stalwart Pub Quizzers. Majd for being a computer whiz. Neil for making me feel like a cool, calm and collected individual. Jun for being funny, and for fitting underneath her desk. Sonia and Alex for teaching me about France. Dario for punching me, a lot, still to this day without any real explanation. Alice for being one of the nicest people to work with and for introducing me to studio cycling without ruining my butch image. Banjo for being a cross between a mother and a child. Jossy for laughing. Stuart for being the first Man Utd fan from Manchester I've met. Craig for being a great sport. Dean for the love. Ian aka Swerve for being an enigma. The Marmite Sandwiches for good times and the most fun I've had loosing at football, and last but definitely not least, Gayle for being easy to scare.

Also, thanks to Andy for being not only a great bloke but also a Best Man, and to both Andy and Gemma for countless evenings/nights/early mornings, for going on holiday with us, and for being Ben's Godparents. Birmingham just isn't the same without you two. To Kings Norton RFC for allowing me an outlet for my stress and making me feel like a sportsman.

To Mom, Dad, Emma and Brian, thanks for all your love and support. Hopefully Dad can now say "Thank you Doctor" without it being entirely erroneous. And finally to my loving wife Clare, and my son Benjamin. For your support and understanding and putting up with me through this from day one, you have made this thesis possible. I dedicate it to you.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BCR	B-cell receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
CRD	Carbohydrate recognition domain
CTLD	C-type lectin-like domain
CRP	Collagen related peptide
DAG	Diacylglycerol
DIC	Differential interference contrast
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
FAK	Focal adhesion kinase
FcR	Fc receptor
Gads	Grb2 adaptor downstream of Shc
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GEMs	Glycolipid enriched microdomains
GPVI	Glycoprotein VI
Grb2	Growth factor receptor bound protein-2
GST	Glutathione-S-transferase
GTP	Guanine triphosphate
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IP	Immunoprecipitation
IP_3	Inositol-1,4,5-trisphosphate
ITC	Isothermal titration calorimetry
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
ITSM	Immunoreceptor tyrosine based switch motif
kDa	Kilodalton
LAT	Linker for activation of T-cells
mAb	Monoclonal antibody
pAb	Polyclonal antibody
PAR	Protease activated receptor
PAS	Protein A sepharose
PBS	Phosphate buffered saline
PGI_2	Prostaglandin I_2
PGS	Protein G sepharose

PH	Pleckstrin homology
PI-3 kinase	Phosphatidyl inositol-3 kinase
PIP_2	Phosphatidyl inositol-4,5-bisphosphate
PIP_3	Phosphatidyl inositol-3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Phospholipase A
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PRP	Platelet rich plasma
PS	Phosphatidyl serine
PTB	Phosphotyrosine binding
PVDF	Polyvinyl difluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
SLP-76	SH2 containing leukocyte protein of 76 kDa
SPR	Surface plasmon resonance
Sulfo-EGS	Ethylene glycol bis (sulfosuccinimidylsuccinate)
TBS-T	Tris buffered saline-Tween
TCR	T-cell receptor
TPO	Thrombopoietin
TxA_2	Thromboxane A_2
VWF	Von Willebrand Factor
WT	Wild type

Contents

CHAPTER 1

GENERAL INTRODUCTION

1.1 Platelet physiology

1.1.1 Platelet overview

Platelets play a major haemostatic role in the body. They have a discoid shape and circulate at a relatively high concentration of $1.5 – 4x10^8$/ml in a quiescent state ready to form a haemostatic plug at sites of damage to the vasculature. Their shape, small size (1-3 μm) and the physics of laminar flow in blood vessels, whereby red blood cells cause the platelets to marginate to the vessel wall, means they are ideally placed to detect injury to the vessel wall. Activation is brought about by exposure to sub-endothelial matrix proteins, by positive feedback mechanisms from the platelets themselves and through the generation of thrombin (Gibbins, 2004). Pathologic platelet activation is found in areas of diseased endothelium and atherosclerotic plaque rupture causing thrombotic disorders such as myocardial infarction and stroke, two of the major causes of death in the Western world. Anti-platelet agents (e.g. aspirin and clopidogrel) are widely prescribed for individuals at high risk of arterial thrombosis. The following sections give an overview of platelet receptors, activation, and inhibition. For more extensive reviews see (Jackson, 2007; Jackson *et al*, 2009; Watson and Harrison, 2010).

1.1.2 Platelet genesis

Platelets have a life-span of approximately ten days (Fritz *et al*, 1986) before their removal by the reticuloendothelial system. Multipotent haematopoietic stem cells in the bone marrow give rise to all circulating blood cells (Figure 1.1). The cytokine, thrombopoietin (TPO), plays a critical role at the early stages of blood cell production

and in the maturation of megakaryocytes and subsequently, platelet production. Megakaryocytes are large polyploid cells that can grow up to 50 μm or more and which make up approximately 0.1% of bone marrow cells. They can each be viewed as factories that fragment, releasing 2,000–3,000 platelets into the blood stream (Hartwig and Italiano, 2003). They can also be used as a genetically tractable platelet model with the advent of RNA interference and viral expression technologies.

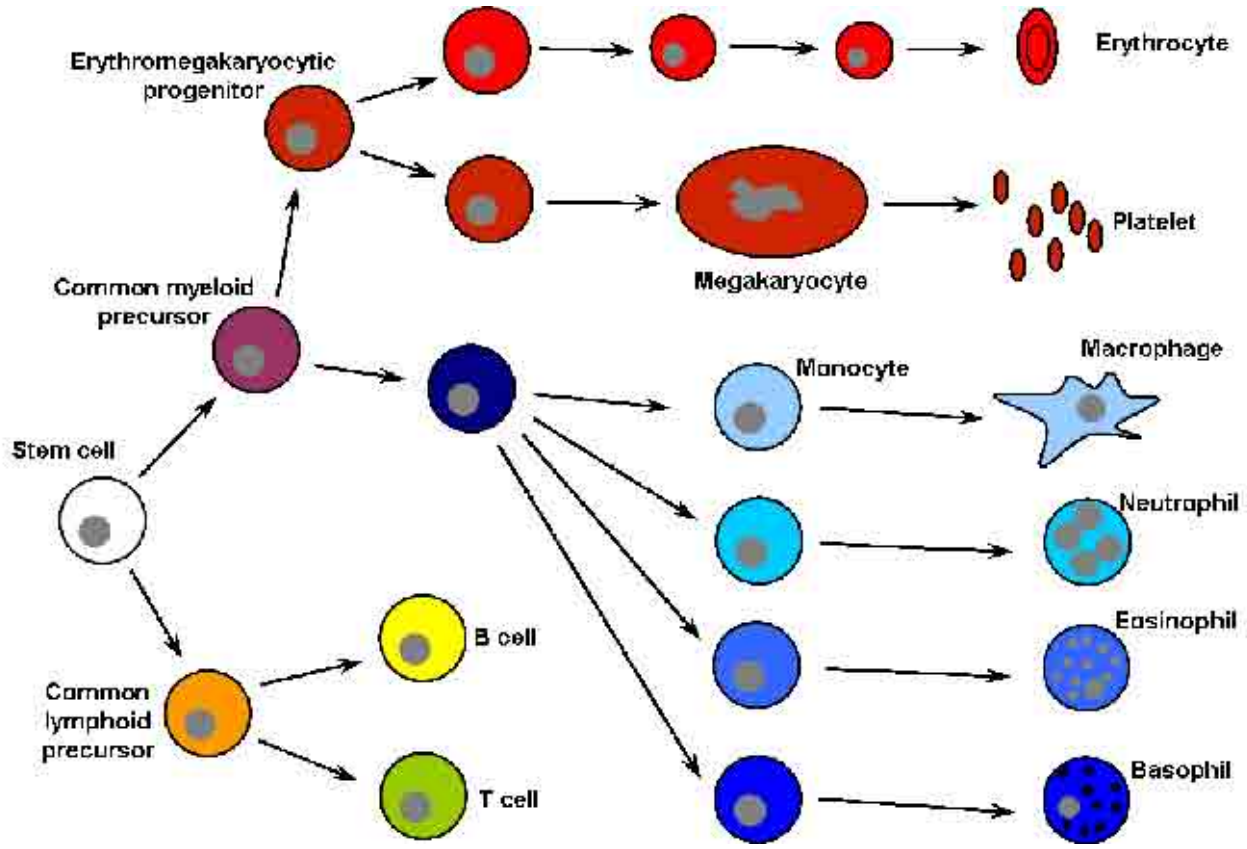

Figure 1.1 – Haematopoiesis. Based on (Kondo *et al*, 2003).

1.1.3 Platelet anatomy

Platelets are anucleate cells which have the capacity to undergo very limited *de novo* protein synthesis, although the physiological significance of this is unclear. The organelles, proteins and very low levels of mRNA are therefore synthesised in the parental megakaryocyte, although the platelet retains the ability to take up some proteins from the plasma, most notably fibrinogen. The plasma membrane is supported by an actin cytoskeleton and a ring of microtubules to maintain the resting discoid shape. The membrane contains a large network of invaginations known as the open canilicular system (OCS) which greatly increases the surface area of the platelet membrane thereby increasing the sites for release of granule content. The OCS also contains the dense tubular system which is a Ca^{2+} store and the site for cyclooxygenase-1, which is crucial in the production of thromboxane A_2 (TxA_2), a positive feedback platelet agonist. The plasma membrane contains a wide variety and diversity of glycoprotein receptors, many of which are expressed at high level such as integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa), which facilitate rapid platelet activation at sites of vascular damage. Phosphatidylserine (PS) is selectively concentrated on the inner envelope of the membrane and is exposed on activation to provide a pro-coagulant surface for thrombin generation which both further activates additional platelets and leads to fibrin generation as part of the coagulation cascade. Another clotting factor, Factor XII has also recently been shown to be activated by platelet-released polyphosphates (Muller *et al*, 2009).

The cytoplasm contains two distinct secretory vesicles which rapidly release their contents upon activation, namely α-granules and dense granules. α-granules contain a

wide variety of proteins which can be divided according to their function. These include adhesion proteins, such as fibrinogen and von Willebrand Factor (VWF), which play a critical role in aggregation and thrombus formation. They also contain a variety of chemokines, such as platelet factor 4 (PF4) and SDF-1 which attract leukocytes and stem cells to sites of vessel damage, and growth factors, including PDGF, EGF and VEGF which are involved in wound repair and angiogenesis. They also express a number of membrane proteins on their surface that support aggregation, including $\alpha_{IIb}\beta_3$, and support binding of leukocytes, namely P-selectin. Dense granules, so called due to their appearance in electron micrographs; contain the major feedback mediators adenosine diphosphate (ADP) and adenosine triphosphate (ATP), as well as Ca^{2+}, 5-HT (serotonin), and polyphosphates (Muller *et al*, 2009; Watson and Harrison, 2010).

1.1.4 Platelet function in haemostasis

Platelets circulate in healthy vessels in a quiescent state, continuously sampling the endothelium for sites of vascular damage. Ordinarily, platelets are unable to bind to the endothelial cells which release PGI_2 (prostacyclin) and nitric oxide and express the ectonucleoside triphosphate diphosphohydrolase (CD39/ENTPD1, which hydrolyses ATP and ADP to AMP) to prevent platelet activation (Kaczmarek *et al*, 1996). Damage to the endothelium leads to exposure of sub-endothelial matrix proteins which mediate powerful platelet adhesion and activation. Activated platelets will recruit other platelets and provide a surface to catalyse the coagulation cascade leading to formation of a thrombus and occlusion of the wound (Gibbins, 2004). The role of platelets in thrombus formation can be described in several stages (Figure 1.2) (Watson and Harrison, 2010).

(1) Tethering – Under low shear stress (i.e. venous flow) the collagen binding integrin $\alpha_2\beta_1$ can bind to exposed collagen fibres in the sub-endothelial matrix. This interaction has a slow rate of association, which under high shear stress (i.e. arterial flow), is unable to tether the platelets. However, the exposed collagen fibres become coated by soluble VWF which undergoes rapid binding to the GPIb-IX-V complex on the platelet surface as a result of a fast on-rate of association. This leads to capture (or tethering) of platelets but is unable to mediate stable adhesion due to a fast off-rate of dissociation. Thus, on a VWF coated surface, platelets role or slide in the direction of flow.

(2) Activation and stable adhesion – tethering to VWF brings the low affinity collagen receptor GPVI into close proximity with the exposed collagen fibres resulting in its activation and the conversion of integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ from an inactive to an active conformation. This in turn leads to stable adhesion through their interactions with VWF and collagen, respectively. The GPIb-IX-V complex also generates intracellular signals that, on their own, are insufficient to mediate rapid platelet activation but may act in synergy with those from GPVI to induce activation.

(3) Spreading – Activation-dependent actin polymerisation results in the sequential formation of filopodia and lamellipodia thereby increasing the surface area in contact with the exposed sub-endothelial matrix and strengthening attachment. This is in turn followed by the formation of actin stress fibres that further reinforce the growing aggregate (Calaminus *et al*, 2007).

(4) Secretion – Activated platelets rapidly synthesis thromboxane A_2 and secrete this along with the contents of their α- and dense granules in response to most activatory agonists. ADP is secreted from dense granules and acts in synergy with thromboxane A_2 to induce aggregation. Fibrinogen and VWF, which are stored in α-granules, support platelet aggregation through cross-linking and capture of circulating platelets, respectively (Kulkarni *et al*, 2000).

(5) Aggregation and thrombus growth – New, discoid shaped platelets are recruited to the growing thrombus by a combination of membrane tethers (Nesbitt *et al*, 2009) and binding to VWF. They are subsequently activated by ADP and TxA_2. Secretion of the adhesion proteins fibrinogen and VWF, and their recruitment from plasma, leads to the cross-linking of activated platelets (aggregation) through integrin $\alpha_{IIb}\beta_3$. As mentioned above, activated platelets also provide a pro-coagulant surface for the generation of thrombin through the coagulation cascade (thrombin is also generated by exposure of tissue factor). Thrombin activates further platelets and mediates conversion of fibrinogen to fibrin, resulting in a fibrin mesh which makes up the bulk of the growing thrombus and occludes the wound, preventing further blood loss.

(6) Late stage events in platelet activation – Several other transmembrane glycoprotein receptors have been shown to participate during the later stages of aggregation and thrombus formation by reinforcing (or consolidating) the growing aggregate, including ephrins and eph kinases (Prevost *et al*, 2005; Brass *et al*, 2008). There is also evidence that aggregation is further strengthened by sustained Src kinase activity and actin-

dependent contraction, independent of fibrin formation (Auger and Watson, 2008; Ono *et al*, 2008).

(7) Clot retraction – Further stabilisation of the thrombus is brought about by the active process of clot retraction which is mediated by contraction of the actin cytoskeleton via fibrin/fibrinogen-bound $\alpha_{IIb}\beta_3$.

Several roles for platelets in processes other than in haemostasis have also been described, including inflammatory processes through adhesion to activated endothelium and recruitment of leucocytes (Tull *et al*, 2006; Zarbock *et al*, 2006), cancer metastasis (Jain *et al*, 2007; Jain *et al*, 2009), vascular integrity (Aursnes and Pedersen, 1979; Kisucka *et al*, 2006) and recently, in the closure of the ductus arteriousus in newborn mice (Echtler *et al*, 2010). Other roles for platelets are reviewed by (Smyth *et al*, 2009).

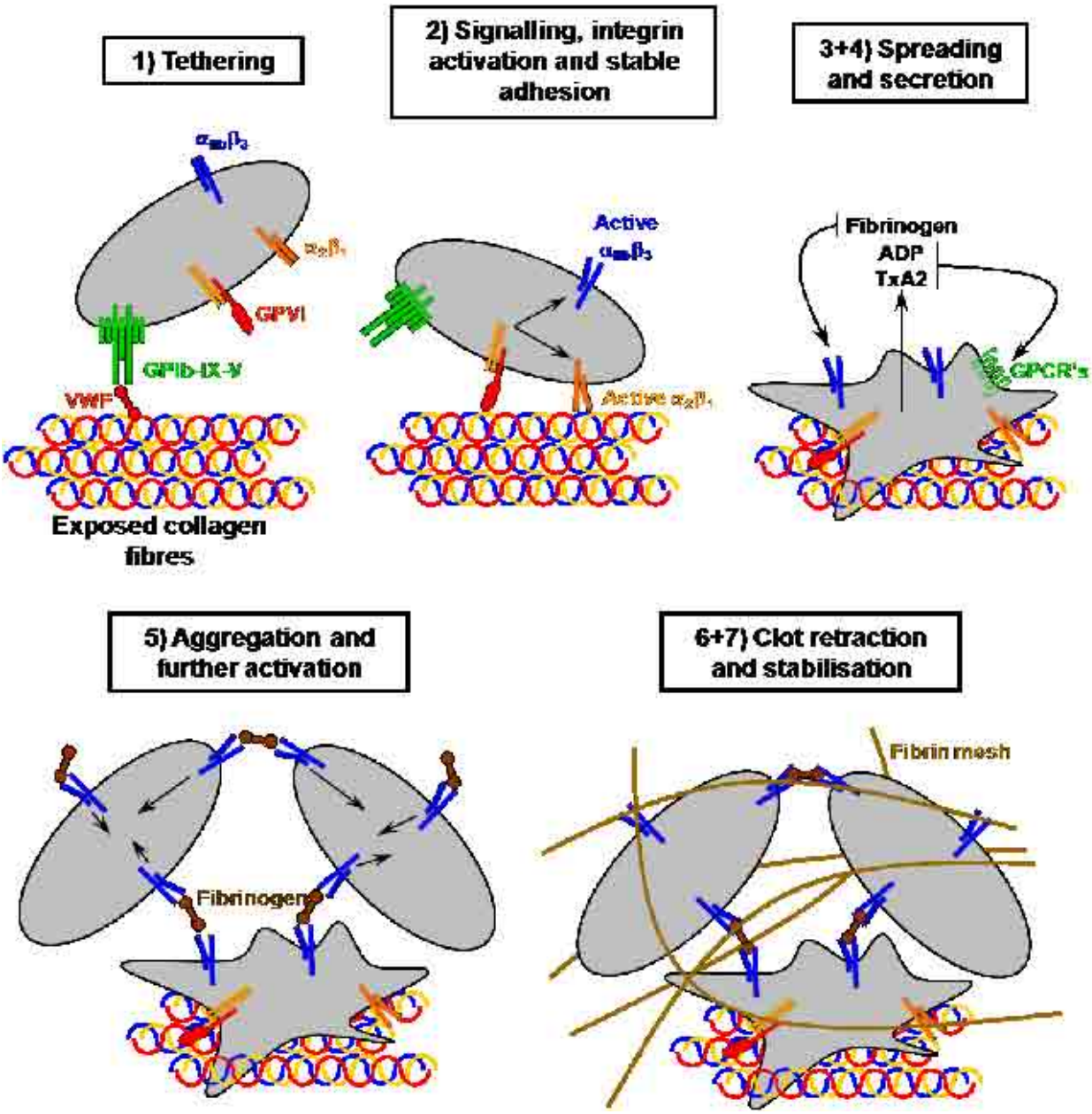

Figure 1.2 – Thrombus formation. Based on (Jackson *et al*, 2003; Sachs and Nieswandt, 2007; Varga-Szabo *et al*, 2008).

1.1.5 Platelet activation and inhibition

This section is a brief description of some of the major activatory and inhibitory agents and receptors, including, GPVI, which is of key importance in this thesis as it is used as a comparison to the novel activation receptor, CLEC-2.

Platelet activation – Collagen, a sub-endothelial matrix protein, is a powerful activator of platelets through GPVI and integrin $\alpha_2\beta_1$. Platelets become tethered to collagen via interactions with $\alpha_2\beta_1$ or GPIb-IX-V (via VWF) allowing for robust signalling through GPVI (Nieswandt and Watson, 2003). This is enhanced through inside-out activation of integrin $\alpha_2\beta_1$ which further facilitates binding to and activation of GPVI. Limited signalling by GPIb-IX-V and integrin $\alpha_2\beta_1$ has also been described but is of uncertain physiological significance (Inoue *et al*, 2003; Ozaki *et al*, 2005). The basement membrane protein laminin has also been shown to activate GPVI. Platelets can become tethered to laminin coated surfaces via interactions with $\alpha_6\beta_1$ or GPIb-IX-V (via VWF) (Inoue *et al*, 2006; Inoue *et al*, 2008) facilitating activation of GPVI. GPVI has also been shown to be activated by globular adiponectin, although the physiological relevance of this is unclear (Riba *et al*, 2008).

GPVI activates platelets through a tyrosine kinase-linked pathway (described in detail later) resulting in the activation of phospholipase C-γ (PLCγ) which produces the secondary mediators inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) which in turn mediate a cellular increase in both Ca^{2+} and protein kinase C (PKC)

activity. Ca^{2+} release from intracellular stores is mediated by IP_3. This Ca^{2+} release stimulates extracellular Ca^{2+} entry through the CRAC (Ca^{2+}-release-activated-Ca^{2+}) channel Orai-1by binding to STIM1which is localised to the intracellular stores (Varga-Szabo *et al*, 2009). PKCα has been shown to regulate secretion of both dense and α-granules in mouse platelets (Konopatskaya *et al*, 2009). There is also evidence for both activatory and inhibitory roles that are specific to other PKC isoforms (Hall *et al*, 2007; Pears *et al*, 2008). Additionally, DAG and Ca^{2+} together regulate CALDAG-GEF1, a Rap1 (aka Rap1b) GTP exchange factor. Rap1 plays a role in integrin $\alpha_{IIb}\beta_3$ activation (Chrzanowska-Wodnicka *et al*, 2005).

The major platelet integrin $\alpha_{IIb}\beta_3$ (making up 15% of surface protein) mediates platelet adhesion to VWF and platelet aggregation by cross-linking platelets through soluble VWF, fibrinogen and other adhesion proteins, including fibronectin and vitronectin. It is essential for aggregation as demonstrated using specific blocking reagents or platelets from patients that lack either subunit of the integrin, a condition known as Glanzmann's thrombasthaenia. Outside-in signalling through the integrin leads to further PLCγ activation reinforcing platelet activation, and activation of myosin light chain (MLC) which plays a role in shape change, actin stress fibre formation and clot retraction (Wonerow *et al*, 2003; Suzuki-Inoue *et al*, 2007; Coller and Shattil, 2008).

Activation of platelets is reinforced by release of TxA_2, which activates the TP thromboxane receptor, and by release of ADP, which signals through the $P2Y_1$ and $P2Y_{12}$ receptors. $P2Y_1$ and $P2Y_{12}$ couple to the $G\alpha_q$ and $G\alpha_i$ classes of G proteins,

respectively, while TP couples to $G\alpha_q$ and $G\alpha_{12/13}$. $G\alpha_q$ activates PLCβ, while $G\alpha_{12/13}$ activates p115Rho, a GTP exchange factor which activates Rho, which activates Rho kinase, which regulates Myosin light chain phosphatase, thereby playing a role in shape change and stress fibre formation. $G\alpha_i$ inhibits the formation of cAMP through adenylyl cyclase (Woulfe *et al*, 2002; Chrzanowska-Wodnicka *et al*, 2005) and also activates PI3-kinase which phosphorylates inositol-lipids in the membrane to provide binding sites for the pleckstrin homology domains of PLCβ and γ isoforms, and various other proteins. The $P2Y_{12}$ ADP receptor interacts synergistically with $G\alpha_q$ and $G\alpha_{12/13}$-coupled receptors to mediate powerful platelet activation. This synergy accounts for the powerful anti-platelet activation of the $P2Y_{12}$ antagonist clopidogrel which is used in the long term treatment of patients at risk of thrombosis. Platelets also express an ATP receptor, $P2X_1$, which is a Ca^{2+} ion channel. The physiological significance of $P2X_1$ is uncertain due to its low expression level.

The coagulation cascade generates thrombin (Factor IIa) which cleaves fibrinogen (Factor Ia) to form fibrin, forming a large part of the bulk of a thrombus. Thrombin also activates platelets through the protease-activated receptor (PAR) family of GPCRs which are linked to both $G\alpha_q$ and $G\alpha_{12/13}$. Thrombin cleaves the N-terminus of the receptors, exposing a tethered ligand. Human platelets express PAR-1 and PAR-4 isoforms of the receptor, while mouse platelets express PAR-4 and the PAR-3 isoform. The latter does not directly signal but facilitates the interaction between thrombin and PAR-4 (Kahn *et al*, 1998; Xu *et al*, 1998; Brass, 2003).

Table 1.1 Major platelet activation and adhesion receptors

Receptor	Agonists	Receptor Type	Signalling Pathway
GPVI	Collagen (physiological) Laminin (physiological) Globular Adiponectin (pathological?) CRP (collagen related peptide) (synthetic) Convulxin (snake toxin) JAQ1 (antibody)	Ig	Tyrosine kinase
$\alpha_2\beta_1$	Collagen (physiological)	Integrin	Tyrosine kinase (weak)
$\alpha_6\beta_1$	Laminin (physiological)	Integrin	
GPIb-XI-V	VWF (physiological) Ristocetin (snake toxin)	Leucine-rich	Tyrosine kinase
$\alpha_{IIb}\beta_3$	Fibrinogen (physiological) VWF (physiological)	Integrin	Tyrosine kinase
$P2Y_1$	ADP (physiological)	GPCR	$G\alpha_q$
$P2Y_{12}$	ADP (physiological)	GPCR	$G\alpha_i$
$P2X_1$	ATP (physiological)	Ca^{2+} channel	
TP	TxA_2 (physiological) U46619 (synthetic)	GPCR	$G\alpha_q$, $G\alpha_{12/13}$
PAR1	Thrombin (physiological) PAR1 peptide (synthetic)	GPCR	$G\alpha_q$, $G\alpha_{12/13}$
PAR4	Thrombin (physiological) PAR4 peptide (synthetic)	GPCR	$G\alpha_q$, $G\alpha_{12/13}$

Platelet inhibition – Healthy, intact endothelium has several mechanisms for inhibiting platelet activation. Probably the most powerful mechanism is through nitric oxide (NO) generation. Healthy endothelial cells continuously synthesise NO through nitric oxide synthase (NOS) (Jin *et al*, 2005; Naseem, 2005). NO is a membrane permeable gas with a short half life in the vasculature and so is concentrated close to the endothelial cell wall. NO activates guanylyl cyclase leading to formation of cyclic guanosine monophosphate (cGMP) which inhibits platelet activation. Endothelial cells release PGI_2 (prostaglandin I_2, prostacyclin) which also has short half life (Jin *et al*, 2005). PGI_2 acts on the platelet through the G protein-coupled PGI_2 receptor (IP) which is coupled to $G\alpha_s$. Activation of this receptor results in an accumulation of cyclic adenosine monophosphate (cAMP) and platelet inhibition. cAMP and cGMP activate protein kinase A (PKA) and protein kinase G (PKG) respectively, which phosphorylate several common target proteins in platelets, including Rap1b and vasodilator-stimulated phosphoprotein (VASP) (Munzel *et al*, 2003). In addition, PKG phosphorylates the IP_3 receptor-associated cGMP kinase substrate (IRAG), which is expressed in complex with PKG-1, and the type 1 IP_3 receptor (Antl *et al*, 2007). Phosphorylation of IRAG by PKG-1 inhibits IP_3-induced Ca^{2+} release. Another major mechanism of endothelial cells inhibiting platelets is by virtue of their expression of CD39. This enzyme will hydrolyse the platelet agonists ATP and ADP to AMP (Jin *et al*, 2005).

Platelets express several ITIM (immunoreceptor tyrosine based inhibitory motif)-containing receptors namely PECAM-1 (platelet endothelial adhesion molecule 1, CD31) (Falati *et al*, 2006; Dhanjal *et al*, 2007), CEACAM-1 (carcinoembryonic

antigen-related cell adhesion molecule 1, CD66a) (Wong *et al*, 2009), TLT-1 (TREM-like transcript 1) (Barrow *et al*, 2004; Washington *et al*, 2004) and G6b (Newland *et al*, 2007; Mori *et al*, 2008). ITIMs are binding sites for the inhibitory phosphatases SHP1 and 2 (SH2 containing tyrosine phosphatase), SHIP1 (SH2 containing inositol-5-phosphatase) and Csk (c-Src kinase). The physiological significance of ITIM receptors in platelets is unclear, although it is generally accepted that their inhibitory activation is relatively weak when compared to that of NO and PGI_2 (Dhanjal *et al*, 2007). It has been speculated that they serve to dampen down constitutive tyrosine kinase signalling under basal conditions (Mori *et al*, 2008).

Table 1.2 Major platelet inhibitory mechanisms

Receptor	Agonists	Receptor Type	Signalling Pathway
-	Nitric oxide	-	cGMP
IP	PGI_2	GPCR	$G\alpha_s$
CD39	ATP ADP	-	
G6b	Unknown	Ig	SHP1, SHP2, SHIP2
PECAM-1	PECAM-1	Ig	SHP1, SHP2, SHIP2
CEACAM-1	CEACAM-1	Ig	SHP1, SHP2, SHIP2
TLT-1	Unknown	Ig	Unknown

1.2 Platelet ITAM signalling

Since the GPVI signalling pathway is a major focus of this thesis, the following section describes this signalling pathway in detail and also the major ligands that are used to study GPVI function. The low affinity immune receptor, FcγRIIA, which signals via the same signalling motif and pathway to that of GPVI, is also described, as is the major platelet integrin $\alpha_{IIb}\beta_3$, which activates many of the same signalling proteins, although the proximal events in its signalling cascade are distinct.

1.2.1 The GPVI-Fc receptor γ-chain complex

GPVI is a member of the immunoglobulin (Ig) superfamily of receptors and contains two extracellular Ig domains, a single transmembrane domain and a short cytoplasmic tail (Clemetson *et al*, 1999). Its expression is restricted to platelets and megakaryocytes and has been estimated to be expressed on the cell surface at between 4,000 – 6,000 copies per platelet using a range of antibodies against GPVI (Furihata *et al*, 2001; Best *et al*, 2003; Samaha *et al*, 2004; Senis *et al*, 2009) although there are reports which differ from this generally accepted amount (Chen *et al*, 2002). It is constitutively associated with the FcRγ chain, which is present as a disulphide-linked homodimer and is required for surface expression of GPVI (Nieswandt *et al*, 2000).

Each FcRγ chain contains one copy of an immunoreceptor tyrosine-based activatory motif (ITAM) which is defined by the consensus $Yxx(L/I)x_{6-12}Yxx(L/I)$, which is essential for GPVI signalling. YxxL is the most frequent sequence, although YxxI does

appear in both B-cell receptor ITAMs and also in one of the T cell receptor ζ-chain ITAMs. ITAMs are also found in several of the major immunoreceptor complexes, including the T-cell and B-cell receptors and a number of Fc receptors, including FcεRI, FcγRI and FcγRIIA where they signal through Src and Syk tyrosine kinases resulting in cellular activation (Shaw *et al*, 1995; Barrow and Trowsdale, 2006; Underhill and Goodridge, 2007). They are also found on a number of virally encoded proteins which initiate signalling through the ITAM receptor cascade (Grande *et al*, 2007).

GPVI is the major signalling receptor for collagen on platelets. Collagen is comprised of three helical peptide chains, which are rich in prolines and hydroxyprolines, and with a glycine at every third residue. GPO (glycine-proline-hydroxyproline) is the specific sequence which activates GPVI (Knight *et al*, 1999). Several synthetic collagen-related peptides (CRP) have been developed based on repeats of the GPO sequence and shown to mimic collagen-induced platelet activation (Morton *et al*, 1995; Asselin *et al*, 1997). Depending on the number of GPO motifs and cross-linking, CRP has the potential to be a stronger agonist than collagen due to avidity caused by the repeated GPO sequence. Platelets also express a second collagen receptor, integrin α2β1, which binds sequences containing GER but not GPO (Zhang *et al*, 2003), and which has a greater affinity for collagen than GPVI. Thus, binding to integrin α2β1 can bring about a net increase in activation of GPVI by collagen. GPVI is also activated by monoclonal antibodies, such as JAQ1 (Nieswandt *et al*, 2000), and by snake venom toxins, such as convulxin (Jandrot-Perrus *et al*, 1997; Polgar *et al*, 1997). Convulxin, a snake C-type lectin protein produced by the South American rattlesnake (*Crotalus durissus terrificus*), was used in the original purification and cloning of GPVI (Clemetson *et al*, 1999).

Convulxin is a tetrameric protein consisting of α- and β-subunits and therefore able to cluster several GPVI molecules making it a powerful agonist.

Mice engineered to lack GPVI or FcRγ fail to undergo aggregation to collagen and exhibit defects in both adhesion and aggregation under flow (Poole *et al*, 1997; Best *et al*, 2003; Kato *et al*, 2003). FcRγ-deficient mice also have mildly delayed and decreased thrombus formation *in vivo* following mild but not severe laser injury model (Kalia *et al*, 2008; Senis *et al*, 2009). Furthermore, GPVI-immuno-depleted mice (i.e. mice treated with an α-GPVI antibody to deplete the protein from the platelet (Bergmeier *et al*, 2001)) have reduced thrombus formation in an *in vivo* ferric chloride injury model (Munnix *et al*, 2005) and or an *in vivo* laser injury model in response to a low but not a high level of laser injury (Hechler *et al*, 2009). Deficiency of either GPVI or FcRγ also results in a mild increase in tail bleeding in mice (Kato *et al*, 2003; Kalia *et al*, 2008) demonstrating that other compensatory mechanisms protect the animal from excessive blood loss.

GPVI is constitutively associated with FcRγ which contains one copy of an ITAM sequence which is responsible for the major component of signalling through the receptor. The FcRγ chain was identified in collagen stimulated platelets through its association with the tyrosine kinase Syk (Gibbins *et al*, 1996). Subsequently, GPVI in complex with FcRγ was identified as the collagen receptor on platelets via co-precipitation between GPVI and FcRγ, and by antibody cross-linking of GPVI to cause aggregation (Gibbins *et al*, 1997). More recently, definitive proof that GPVI and FcRγ

form a receptor for collagen was achieved through expression in a cell line model using a sensitive NFAT-report assay (Tomlinson *et al*, 2007). Experiments of this nature had been attempted previously by transfection of GPVI into the DAMI megakaryocyte cell line (Clemetson *et al*, 1999) and by expression in rat-Rbl cells (Zheng *et al*, 2001; Berlanga *et al*, 2002). However, the conclusions from these studies were unclear as convulxin was able to flux calcium in non-transfected DAMI cells, thereby demonstrating the presence of an endogenous receptor, while the Kahn group, although able to confer responsiveness to convulxin, were not able to show responsiveness to collagen. The Kahn group later suggested that this was a consequence of the level of receptor expression as they were able to reconstitute signalling in one out of 177 stable clones of GPVI expressed in Rbl cells (Chen *et al*, 2002). The success of the Tomlinson study in reconstituting a robust response to collagen was to use a highly sensitive reporter assay that had been previously used to characterise signalling by other ITAM receptors (Lin *et al*, 1999). Moreover, the Tomlinson study also demonstrated that there was no need for a critical level of GPVI to confer signalling to collagen.

1.2.2 The GPVI receptor signalling pathway

Following ligand binding, the conserved ITAM tyrosine residues are phosphorylated by Src family kinases (SFK). Fyn and Lyn are the major two Src kinases mediating FcRγ phosphorylation as shown using mutant mice and precipitation studies (Ezumi *et al*, 1998; Quek *et al*, 2000), although, significantly, a residual level of activation is seen in mice deficient in the two Src kinases indicating involvement of one or more other Src kinases (Quek *et al*, 2000). Both Fyn and Lyn have been shown to associate via their SH3 domains with a poly-proline region of the cytoplasmic tail of GPVI placing them

proximal to their substrate. Further, mutation of this region results in a partial inhibition of GPVI mediated signalling (Suzuki-Inoue *et al*, 2002; Bori-Sanz *et al*, 2003; Schmaier *et al*, 2009). The protein tyrosine phosphatase CD148 has also been shown to play a role in regulating Fyn and Lyn downstream of GPVI by dephosphorylating an inhibitory tyrosine residue which, due to intermolecular binding, holds the SFK in an inactive conformation (Senis *et al*, 2009). GPVI also contains a calmodulin (CaM) binding site which has shown to bind calmodulin in resting cells but which promotes dissociation upon activation. The functional significance of this site in mediating platelet activation is unclear (Andrews *et al*, 2002; Locke *et al*, 2003).

SFK-mediated phosphorylation of the two ITAM tyrosines provides a docking site for the tandem SH2 domains of the Syk family of tyrosine kinases, namely Syk, which is widely expressed in haematopoietic cells, and ZAP-70 (ζ-chain associated protein of 70 kDa), which is localised to T-cells and a sub-population of natural killer cells. Binding of Syk or ZAP-70 to a phosphorylated ITAM leads to activation through a combination of molecular re-organisation and phosphorylation by both SFKs and auto-phosphorylation (Futterer *et al*, 1998; Brdicka *et al*, 2005; Arias-Palomo *et al*, 2007; Deindl *et al*, 2007). Classically, SH2 domains have micromolar affinity for phosphotyrosine groups, but the interaction between the two ITAM tyrosines and the tandem SH2 domains of Syk has nanomolar affinity due to cooperativity (Grucza *et al*, 1999). Moreover, mutagenesis studies have shown that both of the conserved ITAM tyrosine residues and also both of the Syk SH2 domains are required for signalling thereby favouring a model in which Syk kinase binds to the dually phosphorylated ITAM (Kurosaki *et al*, 1995; Fruehling and Longnecker, 1997; Abtahian *et al*, 2006).

Mouse platelets which are deficient in Syk are unresponsive to collagen confirming the critical role of the tyrosine kinase in platelet activation by collagen (Poole *et al*, 1997).

GPVI-mediated activation of Syk has been shown to mediate phosphorylation of a number of downstream targets including the membrane scaffolding protein LAT (linker for activation of T-cells), which has nine conserved tyrosine residues (Pasquet *et al*, 1999; Judd *et al*, 2002) (Figure 1.3). When phosphorylated, LAT has been shown to bind to a range of SH2 domain-containing proteins thereby forming a 'signalosome' that plays a key role in mediating platelet activation by GPVI. Among the proteins that bind to LAT in platelets are the cytosolic adaptors proteins Grb2 (growth factor receptor bound protein 2) and Gads (Grb2 related adaptor protein downstream of Shc), and PLCγ2 (Asazuma *et al*, 2000). The adaptor protein SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) is also recruited to the signalosome through binding to Gads and PLCγ (Liu *et al*, 1999; Yablonski *et al*, 2001) and is critical for activation of PLCγ (Clements *et al*, 1999; Gross *et al*, 1999; Judd *et al*, 2002). LAT also binds to PI-3 kinase (phosphatidylinositol 3-kinase) in platelets (Gibbins *et al*, 1998). The product of PI 3-kinase, PIP_3, binds to the pleckstrin homology (PH) domain of PLCγ2 thereby supporting its localisation to the membrane. The LAT-Gads-SLP-76 signalling triad is the focus of the work in Chapter 4 and is discussed in further detail later (see Figure 1.3).

The recruitment of PLCγ2 to the signalosome allows it to become activated as a consequence of phosphorylation by the Tec family kinases, Btk (Bruton's tyrosine

kinase) and Tec, which also bind to PIP_3 in the membrane (Quek *et al*, 1998; Atkinson *et al*, 2003), and by binding to SLP-76 (Gross *et al*, 1999). A role for both Btk and Tec was shown using double-deficient mouse platelets which exhibited a much greater loss signalling through GPVI compared to the individual knock-out platelets (Atkinson *et al*, 2003). However, because of the interplay of proteins in the LAT signalosome, it is unclear whether additional kinases also mediate phosphorylation of PLCγ2, including Src family kinases.

The Vav family of guanine nucleotide exchange factors (GEF) have also been shown to be recruited to the LAT signalosome by binding to SLP-76, Btk/Tec and Syk, and to play a critical role in mediating PLCγ2 activation (Pearce *et al*, 2002; Pearce *et al*, 2004). In this pathway, Vav is believed to function as an adaptor rather than as a GEF for small G proteins, as mutation of the GEF domain does not inhibit TCR signalling (Kuhne *et al*, 2000). As with the SFKs and Btk/Tec kinases, there is redundancy between the Vav family members, as mice deficient in both Vav1 and Vav3 show defective GPVI signalling whereas mice deficient in Vav1, Vav2 or Vav3 alone, or a combination of both Vav1 and Vav2 show no obvious defect in GPVI signalling (Pearce *et al*, 2002; Pearce *et al*, 2004).

Activation of PLCγ2 leads to hydrolysis of its substrate, phosphatidylinositol-4,5-bisphosphate (PIP_2), to form the two second messengers, inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which release Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively. PLCγ2 knock-out mice have a severe

blockade in GPVI signalling, although this is not complete as mouse platelets also express a small amount of PLCγ1 (Suzuki-Inoue *et al*, 2003).

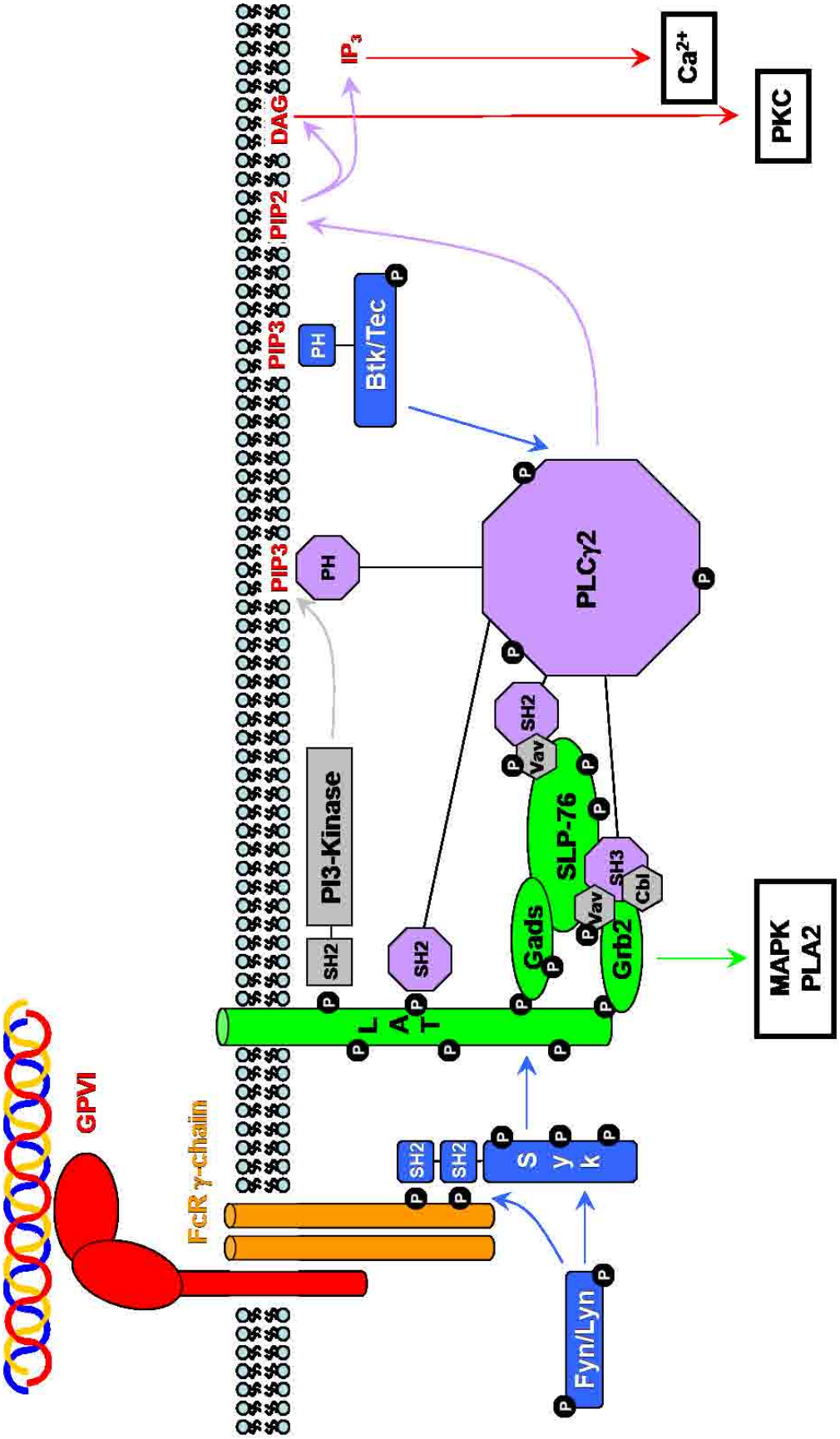

Figure 1.3 – The GPVI signalling pathway

1.2.3 FcγRIIA – A second platelet ITAM receptor

Human platelets express a second ITAM containing protein, FcγRIIA (CD32A), which is believed to use the same ITAM signalling pathway as GPVI, but because it is not found in the mouse genome (and therefore mouse platelets), the same repertoire of studies using mutant platelets have not been performed. FcγRIIA is a low affinity member of the Fcγ (IgG binding) family of Fc receptors and is activated by clustering as a result of binding to immune complexes. It has two extracellular Ig domains, a single transmembrane domain and a cytoplasmic tail, although unlike GPVI, this contains its own ITAM sequence. The ITAM sequence can be regarded as atypical in that it is the only ITAM in which the tandem YxxL sequences are separated by more than 8 amino acids (the number in FcγRIIA is 12). Nevertheless, it has been shown that this length of spacer is able to bind to Syk in platelets and B-cells (Chacko *et al*, 1994; Yanaga *et al*, 1995; Chacko *et al*, 1996). However, due to its more rigid tertiary structure, it is thought that an ITAM of this length would not be able to signal through Zap-70 (Brdicka *et al*, 2005; Deindl *et al*, 2007).

The physiological role of FcγRIIA in platelets is unclear, although it has been proposed that FcγRIIA allows platelets to play a role in the innate immune system by binding to, and becoming activated by antibody bound pathogens, thus speeding their clearance. FcγRIIA is also important in heparin-induced thrombocytopenia, as consequence of platelet activation by antibody-mediated clustering.

1.2.4 Integrin $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ is a heterodimer of α and β subunits. It is highly expressed on the platelet surface at approximately 80,000 copies per platelet, with an estimated further 40,000 copies on platelet α-granules which become expressed upon platelet activation. It is activated by 'inside-out' signals which induce a conformational change and thereby increase its affinity for its endogenous ligands, including fibrinogen and VWF. In turn, ligand-induced clustering generates 'outside-in' signals which play a role in later stage processes such as thrombus stability and clot retraction as discussed above (for review see (Coller and Shattil, 2008)). $\alpha_{IIb}\beta_3$ outside-in signalling uses many of the same signalling proteins as used by ITAM receptors, thereby fuelling the debate as to whether it associates with one or more ITAM-containing proteins. Indeed, in human platelets, the group of Newman and colleagues have proposed FcγRIIA as the ITAM that facilitates outside-in signalling by the integrin (Boylan *et al*, 2008). Nevertheless, the absence of this protein from mouse platelets, and studies on transfected cell lines, shows that the integrin can signal independent of an ITAM sequence. Outside-in signalling by $\alpha_{IIb}\beta_3$ is described in more detail below.

The cytoplasmic tail of the β_3 subunit contains an RGD sequence at its C-terminus which allows for the constitutive association with SFKs, namely Src, Fyn and Yes (Arias-Salgado *et al*, 2003). There is also a separate binding site for Fyn (Reddy *et al*, 2008). Csk (C-Src kinase) is also associated with the tail under basal conditions (Obergfell *et al*, 2002). Csk regulates SFKs by phosphorylating the inhibitory tyrosine residue. Following ligand binding and receptor clustering, Csk dissociates, allowing the

SFKs to become active through dephosphorylation of the inhibitory tyrosine by the protein tyrosine phosphatases PTP1b (protein tyrosine phosphatase 1b) and CD148, and autophosphorylation of an activatory tyrosine residue (Arias-Salgado *et al*, 2005; Senis *et al*, 2009). Syk has been shown to associate with the β_3 tail, via an interaction between the integrin and the Syk N-terminal SH2 domain and inter-domain, which is independent of phosphorylation of the integrin (Woodside *et al*, 2001; Woodside *et al*, 2002). This brings Syk into the proximity of the SFKs and allows it to become activated. Following Syk activation, it can initiate a similar signalling cascade to the ITAM pathway with roles shown for SFKs, Syk, SLP-76, PLCγ2, PI 3-kinase, Tec, PKC and Vav family proteins (Judd *et al*, 2000; Obergfell *et al*, 2002; Goncalves *et al*, 2003; Wonerow *et al*, 2003; Pearce *et al*, 2007; Coller and Shattil, 2008). Interestingly, what has been termed late-stage activation of $\alpha_{IIb}\beta_3$ results in SFK mediated phosphorylation of the β_3 tail on two tyrosine residues, Y747 and Y759. These are not in an ITAM sequence and therefore do not allow for binding of Syk. In fact, their phosphorylation destabilises the interaction with Syk, causing dissociation (Woodside *et al*, 2001; Woodside *et al*, 2002). Y747 and Y759 have however been shown to bind to several other SH2 domain and PTB (phospho-tyrosine binding) domain containing proteins and also to myosin II, with evidence for a role in clot retraction (Law *et al*, 1999; Suzuki-Inoue *et al*, 2007).

There is evidence that $\alpha_{IIb}\beta_3$ couples to FcγRIIA in human platelets as discussed above. The Newman group have proposed that FcγRIIA is incorporated into $\alpha_{IIb}\beta_3$ clusters, bringing it into the proximity of active SFKs which then phosphorylate its ITAM sequence, providing a docking site for Syk (Boylan *et al*, 2008; Gao *et al*, 2009). The

mechanism and stoichiometry of the interaction of FcγRIIa and $\alpha_{IIb}\beta_3$ is not known. However, since FcγRIIa is expressed at approximately 5% of the level of $\alpha_{IIb}\beta_3$, it seems likely that it only associates with a fraction of the integrin. Interestingly, mouse platelets do not express FcγRIIa and this may explain why they exhibit much weaker signalling compared to human platelets on a fibrinogen coated surface.

1.2.5 The role of GEMs in platelet ITAM signalling

$\alpha_{IIb}\beta_3$ 'outside-in' signalling and GPVI signalling show distinct requirements on glycolipid-enriched microdomains (GEMs) that are also known as lipid rafts. GPVI signalling takes place in lipid rafts, with many of the signalling proteins required for GPVI signalling being found in these cholesterol rich membrane domains, including LAT which is considered a GEM marker and the Src kinases Fyn and Lyn (Wonerow *et al*, 2002). $\alpha_{IIb}\beta_3$ is found almost exclusively outside of GEMs and signals independent of LAT (Wonerow *et al*, 2002). The caveat to this is that approximately 2-5% of $\alpha_{IIb}\beta_3$ is found within rafts, which, due to the high expression of the integrin, equates to a level of protein comparable that of GPVI or FcγRIIA (Dorahy *et al*, 1996; Miao *et al*, 2001; Wonerow *et al*, 2002). Alternatively, this could be due to contamination of the GEM preparation.

The SFKs Fyn and Lyn are also able to localise to GEMs by virtue of palmitoylation. All SFKs have an N-terminal myristoylation which allows them to associate with the inner leaflet of the plasma membrane, however only some of them are palmitoylated.

Interestingly, Src lacks the residues for the critical palmitoylation, excluding it from GEMs. This suggests that Src could be the major SFK regulating signalling though the integrin (Paige *et al*, 1993; Koegl *et al*, 1994; Palacios and Weiss, 2004).

1.2.6 ITAM signalling in other haematopoietic cells

The signalling pathway downstream of GPVI follows the same pattern as that of other ITAM receptors; however, different members of the various protein families substitute for each other depending on the cell type. Thus, the GPVI pathway can be described as a hybrid of the T-cell and B-cell ITAM pathways (or vice versa) as shown in Figure 1.4. For example, GPVI and the B-cell receptor use Lyn and Syk, whereas the T-cell receptor uses Lck (leukocyte-specific kinase) and ZAP-70. On the other hand, GPVI and the T-cell receptor use the adaptors LAT-Gads-SLP-76, while the B-cell receptor uses BLNK (B-cell linker, SLP-65). BLNK is a cytoplasmic adaptor protein from the same family as SLP-76 and is capable of fulfilling the same role as the LAT-Gads-SLP-76 triad (Ishiai *et al*, 2000). The major effector protein of the ITAM pathway is PLCγ. The T-cell receptor uses the PLCγ1 isoform, whereas GPVI and the B-cell receptor use PLCγ2 (although, as mentioned previously, mouse platelets express a small amount of PLCγ1). The plasticity of these pathways has proved to be a useful tool for studying platelet GPVI signalling in the absence of a knock-out mouse model, by transfecting platelet receptors into wild type (WT) or mutant B- and T-cells (Tomlinson *et al*, 2007).

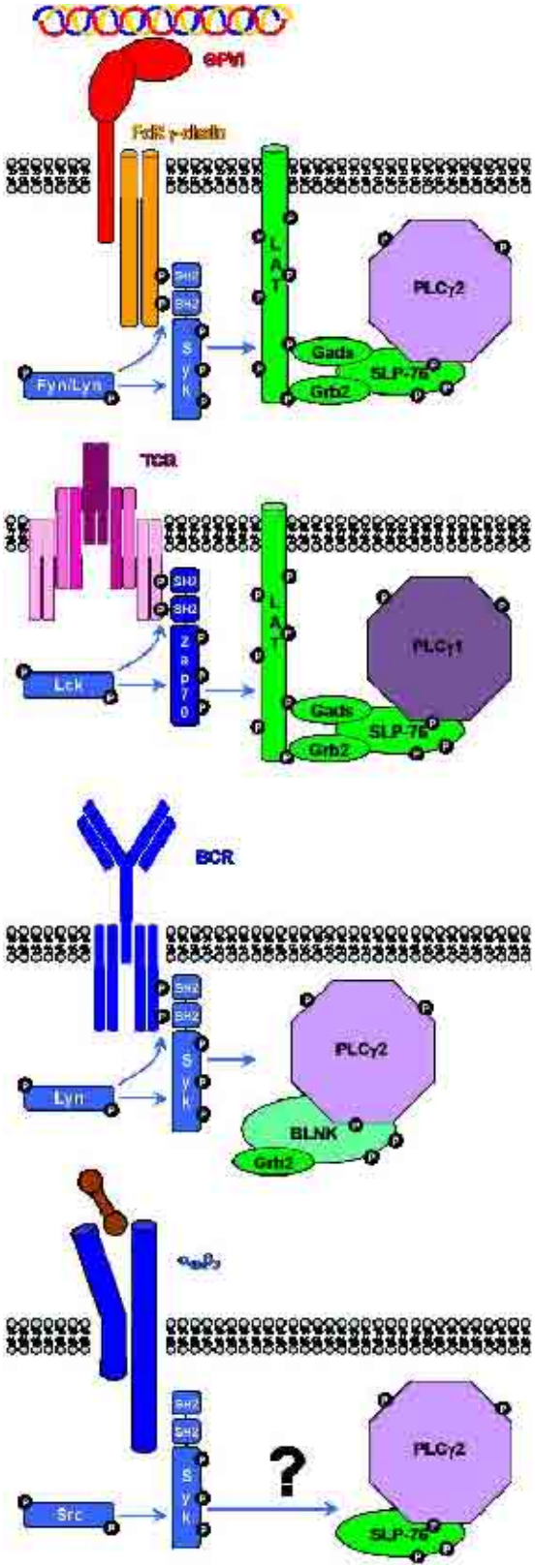

Figure 1.4 – Comparison of ITAM pathways. Platelet ITAM signalling is represented by the GPVI/FcRγ complex for comparison to T-cell receptor and B-cell receptor ITAMs. The integrin is shown using some of the signalling proteins independent of an ITAM.

1.3 The C-type lectin CLEC-2 in platelets

Determining the molecular basis of platelet activation by CLEC-2, which signals via a single YxxL sequence, is a major focus of this thesis. The following sections summarise the discovery of CLEC-2 and how it is currently known to mediate platelet activation. An endogenous ligand for CLEC-2, podoplanin, was reported in 2008 and this too is described along with two other C-type lectin receptors, which are structurally related to, and signal in a similar fashion to CLEC-2, namely Dectin-1 and CLEC9A.

1.3.1 CLEC-2

C-type lectin 2 (CLEC-2) was identified in platelets as a receptor for the snake venom toxin rhodocytin as described below (Suzuki-Inoue *et al*, 2006). CLEC-2 is a group V (non-classical) C-type lectin receptor that is expressed at high level on platelets and at low level in mouse but not human neutrophils, and also on rat liver sinusoidal endothelial cells (Chaipan *et al*, 2006; Kerrigan *et al*, 2009). mRNA for CLEC-2 (but not yet protein) has also been found in several other blood cells, including monocytes, dendritic cells and granulocytes (Colonna *et al*, 2000). CLEC-2 has an N-terminal cytoplasmic tail, a single transmembrane domain, a stalk region and a C-terminal carbohydrate-like recognition domain (CRD) that is referred to as a C-type lectin-like domain (CTLD) (Weis *et al*, 1998; Drickamer, 1999). In CLEC-2, the CTLD lacks the key residues for carbohydrate binding which indicates that ligands are likely to bind through a protein-protein interaction, as is the case for rhodocytin, which is not glycosylated.

The cytoplasmic tail of CLEC-2 has a single YxxL motif that is preceded by three acidic amino acids, but no other recognised signalling motif (Figure 3.1). The single YxxL sequence distinguishes CLEC-2 from ITAM-containing proteins, which have two YxxL sequences separated by 6 – 12 amino acids (Table 1.3). The CLEC-2 YxxL also lacks the conserved upstream amino acids that precede the single YxxL sequences of ITIM and ITSM (immunoreceptor tyrosine-based switch motif)-containing proteins that have been shown to mediate inhibition of ITAM signalling by recruitment of SH2 domain containing tyrosine phosphatases (SHP1 or SHP2) or inositol phosphatases (SHIP1 or SHIP2) (Table 1.3). Thus, the YxxL in CLEC-2 is distinct from that in other YxxL-containing proteins that are known to regulate intracellular signalling cascades.

Table 1.3 YxxL containing proteins

Type	Group	Name	Sequence
ITAM	**CONSENSUS**		**Y**xx(**L**/**I**)x_{6-12}**Y**xx(**L**/**I**)
	Fc receptor	FcRγ-chain	SDGV**Y**TG**L**-----STRNQET**Y**ET**L**
		FcRγRIIA	ADGG**Y**MT**L**NPRAPTDDDKNI**Y**LT**L**
	B-cell receptor	CD79a	DENL**Y**EG**L**-----NLDDCSM**Y**ED**I**
		CD79b	EDHT**Y**EG**L**-----DIDQTAT**Y**ED**I**
	T-cell receptor	ζ-chain 1	QNQL**Y**NE**L**-----NLGRREE**Y**DV**L**
		ζ-chain 2	QEGL**Y**NE**L**----QKDKMAEA**Y**SE**I**
		ζ-chain 3	HDGL**Y**QG**L**-----STARKDT**Y**DA**L**
		CD3γ	NDQL**Y**QP**L**-----KDREDDQ**Y**SH**L**
		CD3δ	NDQV**Y**QP**L**-----RDRDDAQ**Y**SH**L**
		CD3ε	PNPD**Y**EP**I**-----RKGQRDL**Y**SG**L**
	Intracellular	DAP12	TESP**Y**QE**L**-----QGQRSDV**Y**SD**L**
ITIM	**CONSENSUS**		(L/I/V)x**Y**xx(**L**/**V**)
		PECAM-1	VQ**Y**TE**V**
		CEACAM-1	VT**Y**ST**L**
			II**Y**SE**V**
		G6b	LL**Y**AD**L**
		TLT-1	VT**Y**AT**V**
ITSM	**CONSENSUS**		Tx**Y**xx(**L**/**V**)
		PECAM-1	TV**Y**SE**V**
		G6b	TI**Y**AV**V**
		TLT-1	TT**Y**TS**L**
ITAM-like (hemITAM)	**CONSENSUS**		(D/E)E(D/E)x**Y**xx**L**
	C-type lectin	CLEC-2	--------MQDEDG**Y**IT**L**
		Dectin-1	MEYHPDLENLDEDG**Y**TQ**L**
		CLEC9A	--------MHEEEI**Y**TS**L**

1.3.2 Rhodocytin

Rhodocytin (also known as aggretin) is a C-type lectin snake venom toxin that was purified from the Malayan Pit Viper *Calloselasma rhodostoma* more than ten years ago by two research groups (Huang *et al*, 1995; Shin and Morita, 1998). The ability of rhodocytin to stimulate powerful platelet activation was initially attributed to binding to integrin $\alpha_2\beta_1$ based on the use of a blocking antibody to the integrin (Huang *et al*, 1995; Suzuki-Inoue *et al*, 2001). This hypothesis was later refuted by the demonstration that the snake venom toxin was unable to bind to recombinant $\alpha_2\beta_1$ (Eble *et al*, 2001). At the same time, however, the Clemetson group reported that platelet activation by rhodocytin was inhibited by antibodies to both integrin $\alpha_2\beta_1$ and GPIbα (Navdaev *et al*, 2001), which was surprising given that a previous finding that cleavage of GPIbα had no effect on rhodocytin activation (Shin and Morita, 1998). Moreover, both of these receptors, and also the collagen receptor GPVI, were shown to be dispensable for platelet activation by rhodocytin snake toxin using mice deficient in the β_1–subunit of integrin $\alpha_2\beta_1$ which had been depleted of GPIbα and GPVI using O-sialoglycoprotein endopeptidase and *in vivo* treatment with GPVI monoclonal antibody, JAQ1, respectively (Bergmeier *et al*, 2001). Thus, rhodocytin stimulates powerful activation of platelets independent of the three receptors (Bergmeier *et al*, 2001).

The receptor underlying platelet activation by rhodocytin was identified as CLEC-2 based on a proteomic approach using rhodocytin affinity chromatography and mass spectrometry (Suzuki-Inoue *et al*, 2006). CLEC-2 was confirmed as a receptor for rhodocytin by expression studies in chicken DT40 cells using NFAT activation as a

reporter assay (Suzuki-Inoue *et al*, 2006). Furthermore, a polyclonal antibody to CLEC-2 was shown to mediate powerful activation of human platelets independent of the platelet low affinity Fc receptor, FcγRIIA, demonstrating that engagement of CLEC-2 was sufficient to mediate platelet activation (Suzuki-Inoue *et al*, 2006). The possibility that integrin $\alpha_2\beta_1$ may facilitate binding of rhodocytin to CLEC-2 was also refuted by the demonstration that azide-free preparations of $\alpha_2\beta_1$-blocking antibodies had no effect on platelet activation by the snake toxin (Fuller, 2006). Thus, these results demonstrate that CLEC-2 mediates platelet activation by rhodocytin.

1.3.3 The CLEC-2 signalling pathway

Rhodocytin stimulates Src kinase-dependent phosphorylation of the only tyrosine in the cytoplasmic tail of CLEC-2, located in the conserved YxxL sequence. Mutation of this tyrosine to phenylalanine abolishes NFAT activation in CLEC-2-transfected DT40 cells demonstrating the critical role of phosphorylation in mediating activation (Fuller *et al*, 2007). Rhodocytin stimulates a similar pattern of increase in tyrosine phosphorylation to that induced by GPVI in platelets, including phosphorylation of the tyrosine kinase Syk, thereby suggesting that it signals through an 'ITAM-like' pathway (Suzuki-Inoue *et al*, 2001; Suzuki-Inoue *et al*, 2006). Consistent with this, Syk has been shown to be essential for activation of mouse platelets and NFAT induction in transfected DT40 cells by rhodocytin (Suzuki-Inoue et al., 2006). In view of this similarity, the single YxxL sequence in CLEC-2 has been named a 'hemITAM' by the group of Reis e Sousa (Robinson *et al*, 2006) although the term 'ITAM-like' is used in this thesis.

The possible interaction between CLEC-2 and Syk has been further investigated through a series of pull-down and immunoprecipitation studies. A direct interaction between CLEC-2 and Syk is supported by the observation that Syk can be precipitated from platelets by a phosphorylated CLEC-2 peptide and that the tandem SH2 domains of Syk precipitate CLEC-2 from rhodocytin-stimulated platelets (Suzuki-Inoue *et al*, 2006). Furthermore, rhodocytin stimulated signalling is blocked in transfected DT40-cells by point mutations that disrupt phosphotyrosine binding of the N- and C-terminal SH2 domains of Syk (Fuller *et al*, 2007). On the other hand, neither the N- or C-terminal SH2 domains of Syk alone are able to precipitate CLEC-2 from rhodocytin-stimulated platelets suggesting that both SH2 domains are required for a stable interaction (Suzuki-Inoue *et al*, 2006). These results therefore provide evidence for a novel mode of Syk regulation in which two phosphorylated CLEC-2 receptors are cross-linked by its N- and C-terminal SH2 domains.

The use of mutant platelets have also highlighted critical roles for LAT, Vav1/3 and PLCγ2 in signalling by CLEC-2 as is the case for signalling by GPVI (Suzuki-Inoue *et al*, 2006). A role for SLP-76 has also been reported, but unlike GPVI signalling where SLP-76 is essential, the requirement for the adapter protein is overcome at high concentrations of rhodocytin (Suzuki-Inoue *et al*, 2006). Furthermore, the activation of platelets by CLEC-2 can also be distinguished from that of GPVI by its dependence upon the feedback mediators, ADP and TxA_2, and on actin polymerisation, which are essential for phosphorylation of the C-type lectin receptor and downstream phosphorylation events (Suzuki-Inoue *et al*, 2001; Pollitt *et al*, 2010). The molecular explanation for the differential requirement of CLEC-2 and GPVI on the adapter SLP-

76, secondary mediators and actin polymerisation is not known, but suggests fundamental differences in the proximal events underlying platelet activation by the two receptors. The study from Pollitt *et al* has also provided evidence that CLEC-2 signalling takes place in GEMs following activation of the receptor, which is not surprising given the critical role of LAT and the similarity to ITAM signalling. CLEC-2 phosphorylation takes place following translocation to GEMs (Pollitt *et al*, 2010).

1.3.4 Other platelet C-type lectin receptors

There are currently four other C-type lectins which are known to be expressed in platelets, namely DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin), P-selectin, CD23 and CD69. P-selectin (CD62P) has a well established role in platelets where it is present on α-granules and expressed at the cell surface upon platelet activation. It is widely used as a marker of platelet activation in flow cytometry assays. Its major role is in the recruitment of leukocytes during thrombus growth (Falati *et al*, 2003). The cytosolic tail of P-selectin does not have any recognised signalling motifs. Consistent with this, a study has shown that cross-linking P-selectin does not appear to generate regulatory signals in platelets (Sathish *et al*, 2004). P-selectin belongs to the selectin group of C-type lectins which are quite different to group V C-type lectins. The extracellular part of the selectins contain, along with the N-terminal CTLD, an EGF-like domain and a number of complement regulatory domain repeats. These domains are thought to be important for cellular interactions under flow conditions.

DC-SIGN (CD209) is highly expressed on dendritic cells where its major role is in the binding and internalisation of pathogens. Activation of DC-SIGN in dendritic cells has been shown to result in ERK and PI-3 kinase activation, PLCγ2 phosphorylation and Ca^{2+} mobilisation. It has also been shown to be associated with Lyn and Syk (Caparros *et al*, 2006) and to be linked to Raf-1 and possibly Ras, both part of the MAP (mitogen activated protein) kinase signalling pathway (Gringhuis *et al*, 2007; Gringhuis *et al*, 2010), although the role of MAP kinases in platelets is currently controversial as platelets do not have a nucleus and only limited functional roles have been described in platelets (Adam *et al*, 2008).

The cytoplasmic tail of DC-SIGN contains a YxxL sequence, however this is not preceded by the acidic DEDG sequence that is found in ITAM-like receptors (the DC-SIGN sequence is QTRG**Y**KS**L**). Furthermore, mutation of this YxxL sequence does not affect the ability of DC-SIGN to generate a weak signal upon cross-linking as measured using a NFAT reporter assay in DT40 cells (Fuller *et al*, 2007) . DC-SIGN is expressed at low levels on platelets where it has been shown to facilitate binding and internalisation of pathogens, in particular, viral particles (Boukour *et al*, 2006). DC-SIGN binds to several different viruses, including HIV (human immuno-deficiency virus), HCV (hepatitis C virus) and HPV (human papillomavirus) (Geijtenbeek *et al*, 2000; Pohlmann *et al*, 2003; Garcia-Pineres *et al*, 2006). The uptake of viral particles by platelets has been proposed to result in their degradation in the open canilicular system (Boukour *et al*, 2006). In some cases, however, the viruses remain infectious and the platelet can act as a means of viral dispersal throughout the body (Chaipan *et al*, 2006).

CD69 and CD23 are only expressed at low level on platelets and no function has, as yet, been attributed to their presence on platelets. CD69 does not contain any recognised signalling motifs in its cytoplasmic tail. There is however a single report that antibody cross-linking of CD69 on platelets promotes aggregation. This study was flawed however as FcγRIIA was not blocked thereby leaving the possibility that the Fc receptor mediated the aggregation response (Testi *et al*, 1990). CD23 is a low affinity IgE receptor and it has been shown to play an inhibitory role on IgE synthesis in B-cells through an unknown mechanism (Yu *et al*, 1994). A functional role for CD23 in platelets however has not been established. The cytoplasmic tail of CD23 contains a YxxI motif although there are no reports of its phosphorylation. Further, this sequence is preceded by EEGQ which does not correspond to the 'ITAM-like sequence' that is present in CLEC-2 and Dectin-1.

1.3.5 Endogenous CLEC-2 ligands

The first evidence for an endogenous ligand for CLEC-2 was shown in a study investigating binding of platelets to HIV-1 viral particles (Chaipan *et al*, 2006). This study reported that platelets could bind to and internalise the viral particles by a mechanism dependent on CLEC-2 and DC-SIGN (Chaipan *et al*, 2006). DC-SIGN was shown to be the major contributor through binding to the viral envelope protein Env via bound mannose sugars (Geijtenbeek *et al*, 2000). Interestingly, CLEC-2 binding to the viral particles was not dependant on the Env protein suggesting that it recognised a host protein derived from the cell used to make the virus. Blockade of both CLEC-2 and DC-SIGN severely inhibited binding to the viral particles suggesting that these two

proteins interacted together to aid the dissemination of the virus throughout the infected patient (Chaipan *et al*, 2006).

As the viral particles were grown in HEK-293T cells (human embryonic kidney), it was proposed that these cells expressed an endogenous ligand for CLEC-2. Consistent with this, addition of HEK-293T cells to platelets was shown to mediate aggregation, which was blocked by PD173952, an inhibitor of Src family kinases, and to stimulate NFAT production in DT40 cells transfected with CLEC-2 (Christou *et al*, 2008). At the time that the studies were performed, however, the nature of the activating ligand was not known.

Podoplanin was identified as a CLEC-2 ligand by Katsue Suzuki-Inoue, who had performed the original affinity purification studies that led to the identification of CLEC-2 (Suzuki-Inoue *et al*, 2007). Podoplanin is expressed on the leading edge of many tumour cells and underlies tumour cell-mediated platelet aggregation (Kato *et al*, 2003). It has been proposed that activation of platelets by tumour cells facilitates cancer metastasis by release of growth factors and metalloproteinases which facilitate tumour cell growth and invasion, respectively (Nash *et al*, 2002; Gupta and Massague, 2004). Activation of platelets by tumour cells occurs after a characteristic lag phase that is reminiscent of rhodocytin induced platelet aggregation (Kaneko *et al*, 2004; Kaneko *et al*, 2007). In view of this similarity, Suzuki-Inoue *et al* sought to identify the platelet receptor which was mediating platelet activation by podoplanin on tumour cells, hypothesising that CLEC-2 was the candidate.

Suzuki-Inoue *et al* demonstrated an interaction between the two proteins by flow cytometry using recombinant CLEC-2 binding to podoplanin expressing Chinese hamster ovary cells (CHO). This association was shown to be dependent on podoplanin glycosylation as it was lost when podoplanin was expressed in mutant CHO cells which lacked a sialic acid transporter and therefore are unable to fully glycosylate surface proteins (Suzuki-Inoue *et al*, 2007). Furthermore, the podoplanin transfected CHO cells were able to mediate platelet aggregation, whereas the mutant CHO cells could not. They confirmed that platelet aggregation was induced by podoplanin expressing tumour cells and showed that lymphatic endothelial cells (LECs), which endogenously express podoplanin at high level, could also mediate platelet aggregation. Furthermore, aggregation by podoplanin-expressing tumour cells or LECs was blocked by recombinant CLEC-2, and was dependent on SFKs (Suzuki-Inoue *et al*, 2007). Together, these data provided definitive evidence for podoplanin as a CLEC-2 ligand.

The above discovery enabled the demonstration that the ligand on HEK-293T cell was also podoplanin, a finding that was performed through a collaboration between the Birmingham Platelet Group and Chris O'Callaghan in Oxford (Christou *et al*, 2008). The association was confirmed using several methods, including flow cytometry using recombinant CLEC-2 and HEK-293T cells, and by precipitation studies from cell lysates, which pulled out a band of 36 kDa that was identified as podoplanin. Further, recombinant podoplanin and HEK-293T cells stimulated NFAT activity in CLEC-2 transfected DT40 cells and this was blocked with an α-podoplanin antibody (NZ-1). The affinity (K_D) of the interaction between the two proteins was calculated to be 24 μM using surface plasmon resonance (Christou *et al*, 2008).

Podoplanin (also known as aggrus, T1α, GP36, GP38) is a type I transmembrane sialoglycoprotein with a relatively large (206 amino acids), heavily glycosylated, extracellular domain, a single transmembrane domain and a short (9 amino acids) cytoplasmic tail (sequence – RKMSGRYSP). The tail contains two serine residues that are present in putative PKA and PKC phosphorylation sites and a single tyrosine residue, although there are no reports of a functional role for phosphorylation of any of these three residues. On the other hand, podoplanin has been proposed to interact with ERM (ezrin, radixin, moesin) family of proteins, with a direct interaction with ezrin and moesin being mediated by the three basic amino acids in the cytoplasmic tail (Martin-Villar *et al*, 2006). Expression of podoplanin in Madin-Darby canine kidney (MDCK) cells, an epithelial cell line, resulted in an up-regulation of RhoA and epithelial-mesenchymal transition (EMT). The EMT transition results in cells acquiring migratory features, a process required for many responses including tissue remodelling during development, as well as wound healing, inflammation and tumour invasion and metastasis (Bissell and Radisky, 2001; Thiery, 2002). Mutations based around the R/K group showed that the association of podoplanin with ERM family proteins was required for the increase in RhoA activity in the MDCK cells (Martin-Villar *et al*, 2006). These observations suggest that, in addition to being a ligand for CLEC-2, podoplanin may be a functional receptor in its own right.

Podoplanin has a wide expression profile and is expressed at particularly high levels on kidney podocytes (hence its name), type I alveolar cells, lymphatic endothelial cells and metastatic tumour cells. Two different podoplanin knock-out mice have been described as summarised in Table 1.4 (Ramirez *et al*, 2003; Schacht *et al*, 2003; Fu *et al*, 2008;

Mahtab *et al*, 2008; Uhrin *et al*, 2010). The two mice were made by deletion of the promoter and exon 1 (Mahtab *et al*, 2008) or by deletion of exons 2-5 (Ramirez *et al*, 2003) through insertion of a neomycin cassette, with neither mouse expressing podoplanin protein. Cardiac developmental problems were observed on a proportion of the first of these two knockouts, which is attributed to defective epithelial-mesenchymal transition (Mahtab *et al*, 2008). This knock-out shows 40% prenatal lethality. Of the null mice which survive to birth, 50% die within the first weeks after birth (the fate of the remaining 50% is not stated). Backcrossing from a 129 Sv x Swiss background to a C57Bl/6 background resulted in survival of approximately 20% of the null mice to adulthood and are fertile, but no prenatal lethality is described (Uhrin *et al*, 2010). Cardiac defects were not described for the second knockout, which was bred on a 129 Sv Ev background. The birth rate for this knockout was 75% of that expected from Mendelian genetics, although the authors stated that this figure was statistically insignificant and they did not report prenatal lethality (Ramirez *et al*, 2003). All of the mice died however within minutes to hours after birth due to defective lung development resulting in lungs that were unable to expand to perform gas exchange (Ramirez *et al*, 2003).

Both of the knock-out models show defective lymphatic separation during development, resulting in blood filled, dilated lymphatics and lymphoedema (Schacht *et al*, 2003; Fu *et al*, 2008; Uhrin *et al*, 2010). The most recent of these studies monitored development of the lymphatics and observed platelet aggregates in the separation zone between the cardinal vein and the newly forming lymph sacs in the wild type but not the knockout mice (Uhrin *et al*, 2010). This raises the possibility that platelets play a crucial role in

the separation of lymphatics through the interaction of CLEC-2 and podoplanin. Antibody blockade of podoplanin *in utero* resulted in the same phenotype as the knock-out (Uhrin *et al*, 2010).

A similar defect in lymphatic separation from the vasculature is seen in knock-out mice lacking Syk, SLP-76 or PLCγ2 (Abtahian *et al*, 2003; Sebzda *et al*, 2006; Ichise *et al*, 2009). The defect does not appear to be as severe, however, particularly in mice deficient in PLCγ2 which may be attributed to a low level of PLCγ1 (Suzuki-Inoue *et al*, 2003). Since these three proteins are required for CLEC-2 signalling, the results provide further evidence for a role for podoplanin-mediated CLEC-2 activation of platelets in development of the lymphatics. There are several explanations for the overlapping but distinct phenotypes of these three mice relative to those lacking podoplanin, including the possibility that podoplanin functions as a signalling receptor with CLEC-2 serving as a ligand.

Table 1.4 Podoplanin$^{-/-}$ summary

Reference	Defects
MOUSE USED – Generated by deletion of exons 2-5 by insertion of Neo cassette	
(Ramirez *et al*, 2003)	Mice born at close to Mendelian ratios. Die 3-10 min after birth due to respiratory failure. A few mice survived up to 4 hours. Lung morphology affected. Fewer and attenuated type I alveolar cells. Smaller airspaces with abundant surfactant. Lung defects attributed to disrupted epithelial-mesenchymal signalling.
(Schacht *et al*, 2003)	Defective lymphatic but not blood vessel pattern formation. Diminished lymphatic transport, congenital lymphoedema and dilated lymphatic vessels. Normal epidermal differentiation despite podoplanin expression in basal epidermis.
(Fu *et al*, 2008)	Blood-filled lymphatics. Similar phenotype seen in EHC T-Syn$^{-/-}$ (endothelial and haematopoietic specific knock-out of T-synthase) mice which have significantly reduced podoplanin due to loss of glycosylation. EHC T-Syn$^{-/-}$ mice survive and develop fatty liver disease due to misconnected lymphatics.
MOUSE USED – Generated by deletion of promoter and exon 1 by insertion of Neo cassette	
(Mahtab *et al*, 2008)	Increased embryonic and foetal lethality by 40%. 50% of neonates die within the first weeks of life. Cause of death unknown, but possibly cardiac in origin. Impaired cardiac development based on defective epicardial-myocardial interaction and reduced epithelial-mesenchymal transformation.
(Uhrin *et al*, 2010)	Mice were backcrossed onto a C57Bl/6 background. 55% of neonates die during the first week and are smaller than wild types. Some show petechia. 20% survive to adulthood achieving normal weights, life spans and are fertile. Blood filled, non-separated lymphatics. The lymph sacs bud off from the cardinal vein in development. This separation zone is podoplanin positive and platelet aggregates are found here. The knockout lacks platelet aggregates on lymphatic endothelium.

A recent publication has provided evidence for a further endogenous CLEC-2 ligand and for a critical role of CLEC-2 in haemostasis (May *et al*, 2009). In this study mice were treated with a CLEC-2 antibody which resulted in thrombocytopenia. Following recovery from this thrombocytopenia, the platelets were shown to be depleted of surface CLEC-2 for several days suggesting that the antibody had inhibited CLEC-2 expression on newly formed platelets. These CLEC-2-deficient platelets were unable to aggregate or express P-selectin in response to rhodocytin whereas the response to collagen, convulxin, thrombin, ADP, and U46619 was not altered (although only single doses of these agonists were tested therefore leaving the possibility that a dose-response curve may have highlighted a smaller defect). This result demonstrates that, in the low shear environment of an aggregation assay, CLEC-2 is only required for platelet activation by rhodocytin. However, when the CLEC-2-depleteted platelets were flowed in whole blood through collagen-coated capillaries at intermediate (1000 s^{-1}) and high (1700 s^{-1}) shear rates, there was a marked decrease in platelet aggregate formation. The initial attachment of the platelets was unaffected but the recruitment of further platelets and aggregate growth was inhibited in the absence of CLEC-2. In blood that was not anti-coagulated, and therefore thrombin generation allowed to occur, this defect was overcome. This suggests that CLEC-2 is playing a vital role in thrombus formation at arterial rates of shear. Moreover, using an *in vivo* ferric chloride injury model, the CLEC-2-depleted mice failed to fully occlude their vessels. Thrombi still formed at the injury site with similar kinetics to the control but due to increased embolisation and release of single platelets, the thrombi did not become occlusive. The CLEC-2 deficient mice also exhibited an increased bleeding time (by approximately 50%) as measured by a tail bleeding assay. As podoplanin expression is not found in blood cells, including platelets, this study suggests that CLEC-2 has another ligand which is either found in

plasma or is expressed by/secreted by platelets and which contributes to thrombus formation. It also highlights the potential of CLEC-2 as a drug target for anti-platelet therapy.

1.3.6 Dectin-1 and CLEC9A

CLEC-2 is a member of the group V family of C-type lectins which is closely related to the group II family having a single extracellular C-type lectin domain, a stalk region, a single transmembrane domain and a short cytoplasmic tail. Group II C-type lectins are considered the 'classical' C-type lectins, requiring Ca^{2+} for ligand binding, while group V are 'non-classical' C-type lectins as they do not require Ca^{2+} for binding (Drickamer, 1993). Many of the cytoplasmic tails of the group II and group V families of C-type lectins either contain recognised signalling motifs or associate with proteins with signalling motifs. Some examples are summarised in Table 1.5.

Table 1.5 Other C-type lectin signalling motifs

C-type lectin	Other names	References	Motif
CLEC12A	MICL (myeloid inhibitory C-type lectin)	(Marshall *et al*, 2004; Pyz *et al*, 2008)	ITIM
CLEC12B		(Hoffmann *et al*, 2007)	
CLEC5A	MDL-1 (myeloid DAP12 associating lectin)	(Bakker *et al*, 1999)	ITAM – associated with DAP12
CLEC6A	Dectin-2	(Sato *et al*, 2006)	ITAM – associated with FcRγ
CLEC4A	CD303, BDCA2 (blood dendritic cell antigen 2)	(Cao *et al*, 2007; Rock *et al*, 2007)	
CLEC4E	Mincle (macrophage-inducible C-type lectin)	(Wells *et al*, 2008; Yamasaki *et al*, 2008; Yamasaki *et al*, 2009)	

CLEC-2, Dectin-1 (CLEC7A) and CLEC9A appear to be unique within the group V family of C-type lectin receptors in that they have a single YxxL in their cytoplasmic tails which is preceded by three negatively charged amino acids and a glycine or isoleucine residue (Table 1.3). All three signal through sequential activation of Src and Syk tyrosine kinases downstream of phosphorylation of the conserved tyrosine in the YxxL sequence in their cytoplasmic tail (Fuller *et al*, 2007; Huysamen *et al*, 2008). The significance of the three acidic amino acid residues and glycine in the regulation of Syk by CLEC-2 has been investigated by expression of receptor mutants in DT40 cells (Fuller *et al*, 2007). Mutation of D3 and E4 to alanine had little effect on receptor signalling, while mutation of G6 to alanine abolished the response possibly due to steric hindrance. The significance of D5 could not be assessed as mutation to alanine prevented surface expression of CLEC-2. Thus, the overall significance of the acidic acid region in signalling by CLEC-2 is uncertain.

CLEC-2 and Dectin-1 share the same acidic amino acid sequence upstream of the single cytoplasmic YxxL motif, namely DEDG. In CLEC9A, this upstream sequence is EEEI, although the functional significance of this difference, if any, is not known. Unlike CLEC-2 and Dectin-1, CLEC9A is expressed as covalently-linked dimer on the cell surface. This dimerisation suggests a possible mechanism of regulation of Syk through binding of the two individual SH2 domains in the tyrosine kinase to separate, phosphorylated YxxLs in two molecules of CLEC9A, as illustrated in Figure 3.10. Moreover, a similar mechanism of regulation of Syk may also apply to CLEC-2 as activation is lost upon mutation of the phosphotyrosine binding motif in either of the Syk SH2 domains (Fuller *et al*, 2007). Because of the similarity in sequence, structure

and mode of signalling by Dectin-1 and CLEC9A to that for CLEC-2, these two C-type lectins are described in further detail below.

Dectin-1 – Dectin-1 is a group V C-type lectin receptor for β-glucan found primarily on dendritic cells (Brown *et al*, 2003), macrophages, monocytes, polymorphonuclear (PMN) cells but also on B-cells, eosinophils and mast cells (Willment *et al*, 2001; Olynych *et al*, 2006). β-glucans are immunostimulatory carbohydrate polymers found on in the cell wall of fungal, some plant and some bacterial cells (Brown and Gordon, 2001; Brown and Gordon, 2003). Dectin-1 also has a role in homeostasis as a currently unidentified endogenous ligand has been shown to be on T-cells (Ariizumi *et al*, 2000; Willment *et al*, 2001; Grunebach *et al*, 2002) and Dectin-1 has also been shown to be activated by apoptotic cells, again by an unidentified ligand (Weck *et al*, 2008). Activation of Dectin-1 results in dendritic cell maturation, phagocytosis, endocytosis and production and release of a variety of cytokines and chemokines (Brown, 2006; Reid *et al*, 2009)

Dectin-1 has two tyrosine motifs in its cytoplasmic tail, namely the ITAM-like motif, DEDGYxxL, which is described above, and a separate motif with the sequence YxxxL and YxxxI in human and mouse, respectively. The YxxxL sequence is dispensable for signalling by the C-type lectin receptor, whereas the ITAM-like sequence is essential for activation (Brown *et al*, 2003; Gantner *et al*, 2003; Herre *et al*, 2004; Rogers *et al*, 2005; Underhill *et al*, 2005; Fuller *et al*, 2007). Signalling by Dectin-1, as is the case for CLEC-2, is dependent on SFKs and Syk, as shown using selective inhibitors and

mutant mouse bone marrow-derived cells (Underhill *et al*, 2005). In addition, there is evidence that Dectin-1 can signal independently of Syk, again through the DEDGYxxL sequence, although the physiological relevance of this and the underlying mechanism is unknown (Herre *et al*, 2004; Rogers *et al*, 2005; Brown, 2006; Gringhuis *et al*, 2009).

CLEC9A – CLEC9A (DNGR-1 (DC, NK lectin group receptor-1)) is a group V C-type lectin receptor. It has been found in brain, thymus and spleen (Huysamen *et al*, 2008). CLEC9A is also expressed in subsets of haematopoietic cells, namely B cells, $BDCA3^{+}$ (thrombomodulin) dendritic cells (a rare subset of dendritic cells), $CD14^{+}CD16^{-}$ monocytes and $CD14^{-}CD11b^{-}CD64^{+}$ cells (Caminschi *et al*, 2008; Huysamen *et al*, 2008; Sancho *et al*, 2008).

The only functional role ascribed to CLEC9A to date is as a receptor for necrotic cells (Sancho *et al*, 2009). Sancho *et al* demonstrated that necrotic cells express a ligand for CLEC9A that becomes externalised during necrosis. However, they show that the functional role for CLEC9A is for efficient antigen-presentation by the dendritic cell, rather than for antigen uptake. Interestingly, the related C-type lectin receptor, Mincle, is also activated by cells undergoing apoptosis (Yamasaki *et al*, 2008).

It appears therefore that the major roles described for both Dectin-1 and CLEC9A are in the innate immune system and homeostasis through the activation of dendritic cells. These cells are the link between the innate and the adaptive immune system, expressing

a host of Toll-like receptors and C-type lectins which bind to and internalise invading pathogens (innate immunity). The internalised pathogens are processed, and fragments are attached to the major histocompatibility complex (MHC class II) which is in turn presented at the cell surface. The MHC and attached antigen is therefore presented for the activation of helper T-cells and killer T-cells (adaptive immunity). Because of this function, dendritic cells and macrophages are often referred to as antigen presenting cells (APC). B-cells can also perform this function but rather than binding to a pathogen directly, they bind to antibodies which have already recognised the pathogen, and subsequently internalise the complex.

Despite the similarities between the structure, sequence and signalling of CLEC-2, Dectin-1 and CLEC9A it is apparent that they play different roles and have different ligands. Dectin-1 has a well characterised exogenous ligand and is involved in the immune response to pathogens which express it. It also has unidentified endogenous ligands on T-cells and apoptotic cells. CLEC-2 has two or more endogenous ligands and appears to play roles in haemostasis and possibly development of the lymphatics and lungs. Whether it is possible that CLEC-2 can recognise further exogenous ligands (other than rhodocytin) and also plays a role in pattern recognition, or whether Dectin-1 has any role in development remain to be seen. CLEC9A also recognises an endogenous ligand on apoptotic cells with no other ligands as yet identified. It remains to be seen if CLEC9A has further endogenous or exogenous ligands and roles outside of the immune system.

1.4 Aims of the thesis

The proximal events underlying ITAM receptor signalling are reasonably well understood whereas, at the time that this work was begun, the mode of regulation of Syk by CLEC-2 and the related 'ITAM-like' receptors was uncertain. The aim of the work undertaken in this thesis was;

1. To investigate the mechanism of regulation of Syk by CLEC-2, with special focus on the hypothesis that activation is achieved through receptor dimerisation.

2. To compare the functional roles of the adapters Gads and LAT in platelet activation by GPVI and CLEC-2 given their differential requirement for SLP-76.

3. To investigate whether the LAT-independent component of platelet activation platelet by GPVI and CLEC-2 is due to the presence of one or more further LAT-like molecules in platelets. These studies were limited to platelet activation by GPVI in view of the similarity in the functional role of LAT in supporting platelet activation by the collagen receptor and CLEC-2.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies and reagents

The agonists and antagonists used in the course of this thesis are listed in Table 2.1 and 2.2 respectively. Table 2.3 lists the antibodies used. If unstated, materials used are from Sigma (Poole, UK) or from previously described sources (Atkinson *et al*, 2003; Bori-Sanz *et al*, 2003; Pearce *et al*, 2004)

Table 2.1 Agonists

Agonist	Target	Source
Collagen (HORM)	GPVI $\alpha_2\beta_1$	Nycomed (Munich, Germany)
CRP (YGKO$(GPO)_{10}$GKOG)	GPVI	Dr RW Farndale (Cambridge University, UK)
Rhodocytin	CLEC–2	Dr JA Eble (University of Frankfurt, Germany) (Eble *et al*, 2001)
Thrombin	PAR-1 PAR-3 PAR-4	Sigma (Poole, UK)
VWF	GPIb	Dr MC Berndt (Monash University, Australia)
Fibrinogen	$\alpha_{IIb}\beta_3$	Enzyme Research Laboratories (Swansea, UK)
ADP	$P2Y_1$ $P2Y_{12}$	Sigma (Poole, UK)
Phorbol Myristate Acetate (PMA)	PKC	Sigma (Poole, UK)
Ionomycin	Ca^{2+}	Sigma (Poole, UK)

Table 2.2 Antagonists

Inhibitors	Target	Source
Indomethacin	Cycloxygenase	Sigma (Poole, UK)
Apyrase	ADP	Sigma (Poole, UK)
EGTA	Ca^{2+}	Sigma (Poole, UK)
Lotrafiban	$\alpha_{IIb}\beta_3$	GlaxoSmithKline (USA)
Pervanadate	Tyrosine phosphatases	Sigma (Poole, UK)

Table 2.3 Antibodies

Antibody	Host species	Use*	Source
PRIMARY			
CLEC-2 (human)	Goat	IP: 2 μg/ml WB: 1 μg/ml	R+D Systems (Abingdon, UK)
G6f (human)	Rabbit	IP: 5 μl WB: 1/200	Invitrogen (UK)
G6f (mouse)	Rabbit	IP: 5 μl WB: 1/200	Invitrogen (UK)
Gads	Rabbit	IP: 2 μg/ml WB: 2 μg/ml	Millipore (Bucks, UK)
Goat IgG	-	-	R+D Systems (Abingdon, UK)
Grb2	Rabbit	IP: 2 μg/ml WB: 1/500	Santa Cruz Biotechnology (Heidelberg, Germany)
LAT	Rabbit	IP: 5 μg/ml WB: 1/500	Millipore (Bucks, UK)
Mouse IgG	-	-	R+D Systems (Abingdon, UK)
Myc (9B11)	Mouse	FC: 10 μg/ml WB: 1/1000	Cell Signalling Technology (Herts, UK)
Phosphotyrosine (4G10)	Mouse	IP: 2 μg/ml WB: 1/1000	Millipore (Bucks, UK)
PLCγ2	Rabbit	IP: 1/500 WB: 1/1000	Dr MG Tomlinson (Birmingham, UK)
P-Selectin FITC-conjugate (mouse)	Rat	FC: 1/100	BD Bioscience (Oxford, UK)
Rabbit IgG	-	-	Millipore (Bucks, UK)
Sheep IgG	-	-	Millipore (Bucks, UK)
SLP-76	Sheep	IP: 4 μg/ml WB: 1/500	Millipore (Bucks, UK)
Syk	Rabbit	IP: 1/500 WB: 1/1000	Dr MG Tomlinson (Birmingham, UK)
SECONDARY			
Goat IgG HRP-conjugate	Chicken	WB: 1/10000	R+D Systems (Abingdon, UK)
Mouse IgG FITC-conjugate	Sheep	FC: 1/100	Sigma (Poole, UK)
Mouse IgG HRP-conjugate	Sheep	WB: 1/10000	Amersham Bioscience (Bucks, UK)
Rabbit IgG HRP-conjugate	Donkey	WB: 1/10000	Amersham Bioscience (Bucks, UK)
Sheep IgG HRP-conjugate	Donkey	WB: 1/20000	R+D Systems (Abingdon, UK)

*IP: Immunoprecipitation, WB: Western Blot, FC: Flow cytometry

2.1.2 Plasmids and constructs

Human CLEC-2 sub-cloned into pEF6 vector with a C-terminal Myc tag (Invitrogen). A point mutation of tyrosine 7 to phenylalanine (Y7F) was generated and sub-cloned into pEF6 vector with an N-terminal FLAG tag. These were provided by Dr Gemma Fuller and Dr Andrew Pearce (University of Birmingham, UK). The CLEC-2 mutations shown in Table 2.4 were generated by a two-step PCR method (Higuchi *et al*, 1988) using the vector primers 5147 and 4150 in pEF6 with a C-terminal Myc tag using the restriction enzymes Kpn I (5') and Not I (3') (New England Biolabs, Herts, UK). Pfu Turbo DNA polymerase (Stratagene), Rapid ligation kit (Roche, East Sussex, UK), DH5α chemically competent *E.coli*, mini-prep and maxi-prep kits (Sigma, Poole, UK) were used for cloning according to manufacturers protocols. All cloning was verified by Plasmid to Profile sequencing. Human GPVI in pcDNA3 with a C-terminal Myc tag (Invitrogen) and untagged human FcRγ in pEF6 were provided by Dr Mike Tomlinson (University of Birmingham, UK). FcRγ with an N-terminal Myc tag was provided by Dr Jun Mori (University of Birmingham, UK) (Mori *et al*, 2008). The FcRγ mutations shown in Table 2.4 were generated by a one-step PCR method using a Quikchange II XL site-directed mutagenesis kit (Stratagene) into pEF6 with an N-terminal Myc tag. Human G6f was cloned into pEF6 vector with a C-terminal Myc tag (Invitrogen) using a two-stage, nested PCR approach, from K562 cDNA (human erythroleukaemic cell line) using the restriction enzymes Bgl II (5') and Eco RI (3') (New England Biolabs, Herts, UK). Mouse G6f was cloned into pEF6 vector with a C-terminal Myc tag (Invitrogen) using PCR from primary mouse megakaryocyte cDNA using the restriction enzymes Kpn I (5') and Eco RI (3') (New England Biolabs, Herts, UK). The nuclear factor of activated T-cells (NFAT)-luciferase reporter containing three copies of the

distal NFAT site from the IL-2 promoter (Shapiro *et al*, 1997) was provided by Prof Arthur Weiss (UCSF School of Medicine, USA). The pEF6-*lacZ* reporter was from Invitrogen. GST-fusion proteins of Syk N-SH2, C-SH2 and tSH2 domains were provided by Dr C Law (University of Washington, USA) (Law *et al*, 1996). A GST-fusion protein of the Syk tSH2 domains was also provided by Klaus Fütterer (University of Birmingham) (Futterer *et al*, 1998).

Table 2.4 Constructs

Construct	Primers
T9A CLEC-2	**FWD:** 5'-CAT GCA GGA TGA AGA TGG ATA CAT CGC CTT AAA TAT TAA AAC TCG-3' **REV:** 5'-CGA GTT TTA ATA TTT AAG GCG ATG TAT CCA TCT TCA TCC TGC ATG-3'
Δ21-28 CLEC-2	**FWD:** 5'-TAA AAC TCG GAA ACC AGC TCT CAT CTG GTG GCG TGT GAT GGC TTT GAT TC-3' **REV:** 5'-GAA TCA AAG CCA TCA CAC GCC ACC AGA TGA GAG CTG GTT TCC GAG TTT TA-3'
S21/27A CLEC-2	**FWD:** 5'-GAA ACC AGC TCT CAT CGC CGT TGG CTC TGC ATC CGC CTC CTG GTG GC-3' **REV:** 5'-GCC ACC AGG AGG CGG ATG CAG AGC CAA CGG CGA TGA GAG CTG GTT TC-3'
Y66F FcRγ	**FWD:** 5'- CAG ATG GTG TTT TCA CGG GCC TGA G-3' **REV:** 5'- CTC AGG CCC GTG AAA ACA CCA TCT G-3'
Y77F FcRγ	**FWD:** 5'- GGA ACC AGG AGA CTT TCG AGA CTC TGA AGC-3' **REV:** 5'- GCT TCA GAG TCT CGA AAG TCT CCT GGT TCC-3'
Human G6f (1st round)	**FWD:** 5'-TGG GGG AGA TCT ACC ATG GCA GTC TTA TTC CTC C-3' **REV:** 5'-TTT TTC ACC TGG GCT TGT GGG CA-3'
Human G6f (2nd round)	**FWD:** 5'-TGG GGG AGA TCT ACC ATG GCA GTC TTA TTC CTC CTC CTG-3' **REV:** 5'-TAG TAG GAA TTC CCT GGG CTT GTG GGC AGG TG-3'
Mouse G6f	**FWD:** 5'-TAG TAG GGT ACC ACC ATG GCA GTT GTA GTA TTC CTC CTG-3' **REV:** 5'-TAG TAG GAA TTC CCT GGT CTT GTG GTT AGG TGG G-3'

2.1.3 Mice

Gads deficient and LAT deficient mice were generated as previously described (Samelson *et al*, 1999; Zhang *et al*, 1999; Yoder *et al*, 2001). LAT mice were kindly provided by Dr Lawrence Samelson. Gads mice were kindly provided by Dr Jane McGlade (Hospital for Sick Children, Toronto, Canada). Mice were bred as heterozygotes allowing the use of litter matched wild-type control mice. LAT mice were provided on a C57Bl/6 background, Gads mice on a Balb-c background. Gads mice were subsequently back-crossed for nine generations onto a C57Bl/6 background. Housing and husbandry was in accordance with Home Office regulations under the Animals (Scientific Procedures) Act 1986. The mice used in chapter 5, deficient in PAG, LIME, NTAL, LAT, LAX, and the compound knock-out mice, NTAL/LAT deficient, NTAL/LAT/LAX deficient and PAG/LIME deficient were all provided by Dr Burkhart Schraven and were used in his labs in the Institute for Molecular and Clinical Immunology (Medical Faculty, Otto-Von-Guericke University, Magdeburg, Germany). These mice, and control mice, were all provided on a C57Bl/6 background.

2.2 Platelet preparation

2.2.1 Human washed platelet preparation

Blood was taken by venipuncture from healthy, drug free volunteers on the day of experiments into 10% sodium citrate. The blood was further anti-coagulated by addition of 10% acid citrate dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Platelet rich plasma (PRP) was retained following centrifugation for 20 minutes at 200 *g*. Prostacyclin (10 μg/ml) was added to inhibit platelet activation

prior to centrifugation for 10 minutes at 1000 *g*. The plasma was discarded and the platelet pellet washed in 25 ml modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH 7.3) and 3 ml ACD. Prostacyclin (10 μg/ml) was added prior to final centrifugation for 10 minutes at 1000 *g*. The platelet pellet was resuspended in Tyrode's-HEPES buffer at a concentration of 2 x 10^7/ml for static adhesion and spreading assays, 2 x 10^8/ml for aggregation assays and 5 x 10^8-1 x 10^9/ml for protein biochemistry. The platelets were allowed to rest for at least 30 minutes prior to experimentation.

2.2.2 Mouse washed platelet preparation

Mice were terminally CO_2-narcosed following isofluorane anaesthesia. A laparotomy was then performed and blood was taken from the descending aorta into 10% ACD. The blood was diluted further with addition of 200 μl of Tyrode's-HEPES buffer and centrifuged in a microcentrifuge at 2000 rpm for 5 minutes. The PRP and top third of erythrocytes were retained and centrifuged at 200 *g* for 6 minutes. PRP was retained and to maximise platelet recovery, a further 200 μl of Tyrode's-HEPES buffer was added to the remaining erythrocytes, mixed, and centrifuged again at 200 *g* for 6 minutes. The second collection of PRP was pooled with the first and following addition of 10 μg/ml prostacyclin, centrifuged at 1000 *g* for 6 minutes. The platelet pellet was resuspended in Tyrode's-HEPES buffer at a concentration of 2 x 10^7/ml for static adhesion and spreading assays, 2 x 10^8/ml for aggregation and ATP secretion assays and 5 x 10^8/ml for protein biochemistry. The platelets were allowed to rest for at least 30 minutes prior to experimentation.

2.3 Platelet functional studies

2.3.1 Aggregation and stimulation for biochemistry

Aggregation/stimulation was performed in a dual channel Born lumi-aggregometer (model 460VS; Chronolog, Labmedics, Manchester, UK) at 37˚C with stirring at 1200 rpm. The optical density of the platelet suspension was measured against a blank reading from Tyrode's-HEPES buffer and recorded in real time on a chart recorder (Chronolog, Labmedics, Manchester, UK).

Aggregation/stimulation experiments were carried out in silconised glass test tubes. The platelets were pre-incubated with stated inhibitors for 10 minutes before the experiment. Platelets were pre-incubated with EGTA (1 mM), indomethacin (10 μM) and apyrase (2 U/ml) to block aggregation, thromboxane A2 (TxA2) and ADP respectively. Platelets were then warmed to 37˚C for 1 minute followed by 1 minute with stirring followed by addition of the agonist. Aggregation was monitored for 5-10 minutes. Stimulations were allowed to proceed for the stated times and then the platelets were lysed by the addition of an equal volume of 2 x lysis buffer plus protease inhibitors (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 2% NP-40; pH 7.4 plus 2.5 mM Na_3VO_4, 100 μg/ml AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin).

2.3.2 ATP secretion

Secretion assays were carried out in the same way as aggregation studies. Platelets were pre-incubated for 2 minutes with Chrono-lume, a luciferin/luciferase reagent (Chronolog, Labmedics, Manchester, UK). After addition of agonist, light emission from the luciferase reaction, catalysed by secreted ATP was recorded on a chart recorder in real time.

2.3.3 Static adhesion and spreading

Glass coverslips were coated using either 100 μg/ml fibrinogen, 100 μg/ml collagen or 10 μg/ml VWF at 4˚C overnight followed by washing with three changes of PBS. The coverslips were then blocked with 5 mg/ml heat denatured BSA for 1 hour at room temperature followed by washing with three changes of PBS. VWF coated coverslips were then treated with 2 μg/ml botrocetin for 10 min at room at room temperature followed by washing with three changes of PBS. Washed platelets were then added to the coverslips and allowed to adhere and spread for 45 mins at 37˚C in the presence of 2 U/ml apyrase and 10 μM indomethacin. Non-adherent platelets were washed away with three changes of PBS. Adherent platelets were then fixed with 3.7% paraformaldehyde for 10 minutes at room temperature. The coverslips were then mounted onto glass slides using Hydromount (National Diagnostics, Atlanta, USA) and imaged by differential interference contrast (DIC) microscopy using a Zeiss Axiovert 200 M microscope. Platelet surface area was then analysed using ImageJ software (NIH, Bethesda, USA).

2.3.4 P-Selectin exposure

Washed mouse platelets were stimulated with stated agonists for 10 minutes at 37˚C in a volume of 50 μl in the presence of 1 mM EGTA to prevent aggregation. The platelets were stained with 100 μg/ml FITC-conjugated α-mouse P-selectin antibody for a further 10 minutes at 37˚C in the dark. The platelets were diluted by addition of 200 μl Tyrode's-HEPES buffer and analysed with a FACScalibur (BD Biosciences) and CellQuest software.

2.3.5 *In vitro* flow adhesion

Mouse blood was drawn as above, into sodium heparin (5 U/ml) and PPACK (40 μM). Glass capillary tubes (1 x 0.1 mm; Camlab, Cambridge, UK) were coated with 100 μg/ml Horm collagen for 1h at room temperature whilst rotating slowly. The capillaries were washed with PBS and blocked with 5 mg/ml heat-inactivated BSA for 1h at room temperature before being mounted on the stage of an inverted fluorescent microscope (DM IRB; Leica Microsystems Ltd, Milton Keynes, UK) equipped with a digital camera (CoolSnap ES, Photometrics, Huntington Beach, CA, USA). Anticoagulated whole blood was pre-incubated with 2 μM $DiOC_6$ for 15 minutes at 37˚C to fluorescently label the cells. The blood was then perfused through the capillary for 4 minutes at a wall shear rate of 1000 s^{-1} at 37˚C followed by washing for 3 minutes at the same shear rate with Tyrode's-HEPES buffer. Fluorescent images were recorded at approximately 2 second intervals for 2 minutes and the images linked to create a movie. Adherent cells were then fixed with 3.7% paraformaldehyde for 30 minutes and imaged using DIC microscopy on a Zeiss Axiovert 200 M microscope.

2.4 Cell culture

2.4.1 Cell culture

DT40 chicken B-cells were grown in RPMI supplemented with 10% foetal bovine serum (FBS), 1% chicken serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol and 20 mM GlutaMAX. Human endothelial kidney (HEK) 293T cells were grown in DMEM supplemented with 10% FBS 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM GlutaMAX. Jcam2 human Jurkat T-cells were grown in RPMI supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM GlutaMAX.

2.4.2 Transfection

DT40 cells and Jcam2 cells were transfected in a volume of 400 μl cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM HEPES, 0.04 mM ATP, 5 mM glutathione; pH 7.6) by electroporation using a GenePulser II (Biorad) set at 350 V and 500 μF. Cells were grown for 20 hours before use in experiments. HEK 293T cells were transfected by a calcium phosphate method: DNA was diluted in 500 μl water containing 252 mM $CaCl_2$ followed by drop wise addition of 500 μl of 2 x HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, 12 mM dextrose; pH 7.5). This was allowed to precipitate at room temperature for 10 minutes then added to cells and incubated for 24 hours after which time, the media was removed and replaced with fresh media for a further 24 hours after which time the cells were used for experiments.

2.5 Cell line reporter assay

2.5.1 NFAT-luciferase assay

DT40 cells or Jcam2 cells were transfected with stated amounts of stated receptors in addition to 20 μg of the luciferase reporter construct and also, 2 μg of pEF6-lacZ as a control for transfection efficiency. Twenty hours after transfection cells were counted in the presence of Trypan blue to exclude dead cells and the live cells were split for the luciferase assay, β-galactosidase assay for transfection efficiency and flow cytometry to assay surface expression of the receptor of interest. Stimulations were performed in triplicate in a 96-well plate using 10^5 cells per well in a final volume of 100 μl for 6 hours at 37˚C with stated agonists. PMA (50 ng/ml) with ionomycin (1 μM) were used as a positive control to show all cells were responsive. Following the 6 hour stimulation, cells were lysed with the addition of 11 μl harvest buffer (0.2 M potassium phosphate buffer; pH 7.8, 10% Triton X-100 and 1 mM DTT) for 5 minutes at room temperature. The lysate (100 μl) was added to an equal volume of assay buffer (0.2 M potassium phosphate buffer; pH 7.8, 20 mM $MgCl_2$, 10 mM ATP). Luciferase activity was measured with a Centro LB 960 microplate luminometer (Berthold Technologies, Germany) by addition of 50 μl of 1 mM luciferin (MP Biomedicals, UK). Data from the triplicates were averaged and normalised to β-galactosidase activity, then expressed as fold increase over basal.

2.5.2 β-galactosidase assay

From each transfection, live cells (10^5) were assayed in triplicate using the Galacto-Light chemiluminescent reporter assay, according to the manufacturer's instructions (Applied Biosystems, Bedford, Mass, USA). β-galactosidase activity was measured with a Centro LB 960 microplate luminometer.

2.5.3 Flow cytometry

From each transfection, live cells (10^5) were used to confirm surface expression of the receptor where possible. Cells were stained with primary antibody in a volume of 50 µl of PBS for 20 minutes on ice. Cells were then washed in 1 ml of PBS and stained with FITC- conjugated secondary antibody in a volume of 50 µl of PBS for 20 minutes on ice in the dark. Stained cells were then analysed with a FACScalibur (BD Biosciences) and CellQuest software.

2.6 Protein biochemistry

2.6.1 Immunoprecipitation and pull-downs

Lysates made as described above were pre-cleared with either protein A sepharose (PAS), or protein G sepharose (PGS) for immunoprecipitation (IP), or streptavidin-agarose for pull-downs. Lysates were pre-cleared for 60 minutes at 4˚C and then insoluble debris and the beads were pelleted by centrifugation at 18000 g in a microcentrifuge at 4˚C. Cleared lysates were transferred to a new tube and 50 µl was

removed and added to an equal volume of 2 x Laemmli sample buffer (Reducing: 20% glycerol, 10% β-mercaptoethanol, 4% SDS, 50 mM tris, trace Brilliant Blue R; Non-reducing: 20% glycerol, 4% SDS, 50 mM tris, trace Brilliant Blue R) for whole cell protein studies. For IP experiments, the remaining cleared lysate was incubated with stated antibody and PAS (rabbit antibodies) or PGS (mouse, sheep and goat antibodies) for 90 minutes at 4˚C. For pull-down experiments, the remaining cleared lysate was incubated with a biotinylated peptide and streptavidin-agarose for 90 minutes at 4˚C. The agarose or sepharose beads were subsequently washed with three changes of lysis buffer and associated proteins were eluted into 2 x Laemmli sample buffer and boiled for 5 minutes at 100˚C.

2.6.2 SDS-PAGE and Western Blotting

Denatured proteins were separated by electrophoresis through sodium dodecyl sulphate polyacrylamide gels (10% unless otherwise stated). Prestained molecular weight markers (Bio-Rad, Hemel Hempsted, UK) were run alongside samples. Separated proteins were then electro-transferred onto polyvinylidene difluoride (PVDF) membranes using a wet transfer method at 30 V for 90 minutes. Membranes were then blocked with blocking buffer (5% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) sodium azide in TBS-T (Tris-buffered saline (200 mM Tris, 1.37 M NaCl; pH 7.6 containing 0.1% Tween-100)) for 1 hour prior to Western Blotting.

The blocked membranes were then incubated with primary antibodies in blocking buffer for 1 hour followed by washing for 5 minutes at a time, with three changes of TBS-T.

The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBS-T for 1 hour followed by washing for 5 minutes at a time, with three changes of TBS-T. The membranes were then incubated with enhanced chemiluminescence (ECL, Amersham Bioscience, Buck, UK) reagent for 1 minute and then developed by exposure to autoradiographs. In the cases where the membranes were required for a second blotting the antibodies were removed by stripping. The membranes were incubated with stripping buffer (TBST-T containing 2% SDS) supplemented with 1% β-mercaptoethanol for 20 minutes at 80˚C followed by incubation with stripping buffer for a further 20 minutes at 80˚C. The membranes were then washed in several changes of TBS-T and blocked again in blocking buffer. The blotting process was then repeated.

2.6.3 Platelet surface protein cross-linking

Following platelet stimulation, Sulfo-EGS at a final concentration of 0.15 mM or 1.5 mM was added and allowed to incubate at room temperature for 30 minutes. The reaction was then quenched with the addition of Tris-HCl (pH 7.5) at a final concentration of 25 mM, and allowed to incubate for a further 20 minutes at room temperature. The platelets were then lysed with the addition of an equal volume of 2x ice cold NP-40 lysis buffer.

2.6.4 GST-fusion proteins

2.6.4.1 Expression

Constructs for the GST-tagged N-SH2, C-SH2 and tSH2 domains of Syk were transformed into DH5α *E.coli* cells and grown as a 100 ml overnight culture in Luria-Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per litre) plus ampicillin (100 μg/ml) at 37˚C with shaking. This was then split to inoculate four flasks of LB broth (1 litre) plus ampicillin (100 μg/ml) and grown at 37˚C with shaking until the optical density at 600 nm (OD_{600}) was approximately 0.75. The cultures were then allowed to cool to room temperature and induced to express the fusion proteins with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 mM). These were then incubated overnight with shaking at room temperature.

2.6.4.2 Purification

The cultures were centrifuged for 30 min at 4500 rpm (JLA 8.1000 rotor, BD) and 4˚C. The bacterial pellet was then resuspended in 200 ml of lysis buffer (PBS containing 1% Triton-X100, 1% aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 1 mM EDTA and 1 mM DTT) and sonicated on ice extensively. Cellular debris was then removed by centrifugation for 20 min at 15000 rpm (JA 25.50 rotor, BD) and 4˚C. The cleared lysate was then passed through a 10 ml column of glutathione-agarose and recycled five times. The column was then washed extensively with 500 ml PBS, then 300 ml Tris (10 mM Tris, 0.5 M NaCl and 1% NP40; pH 7) followed by 300 ml PBS. The bound GST-fusion proteins were then eluted from the column with 25 ml PBS containing 10 mM glutathione (pH7). For surface plasmon resonance studies the protein was used in this

form with the GST-tag still attached. Aggregated protein was removed using size-exclusion chromatography (HiLoad 16/60 Superdex-200 120 ml column, GE Healthcare, UK). For ITC and fluorescence titration experiments, the GST-tag was removed with the addition of 40 U of thrombin and incubated at room temperature for 6 hours. A further 40 U were added and left to incubate overnight at room temperature followed by a final addition of 25 U and incubated for 9 hours at room temperature. The free glutathione was then removed by extensive dialysis (three changes of buffer for 8 hrs at a time). The cleaved proteins were then passed through the regenerated glutathione-agarose column to deplete the free GST; recycling 10 times. Aggregated protein and remaining GST was removed using size-exclusion chromatography (HiLoad 16/60 Superdex-200 120 ml column, GE Healthcare, UK).

2.7 Surface Plasmon Resonance (SPR)

SPR experiments were performed using a Biacore 3000 machine (Biacore, GE Healthcare, UK). Biotin tagged CLEC-2 and FcRγ peptides were attached to CM5 research grade sensor chips (Biacore AB) following attachment of streptavidin to the chip using amine coupling. All experiments were performed at 25˚C in HBS-EP buffer (150 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.005% Surfactant P-20; pH 7.4). Different concentrations of N-SH2, C-SH2 and tSH2 domains were injected over all peptide surfaces to determine equilibrium dissociation constants (K_D). All injections were compared to a negative control flow cell containing no peptide.

2.8 Isothermal Titration Calorimetry (ITC)

ITC measurements were made in a VP-ITC isothermal titration calorimeter (MicroCal, GE Healthcare, UK). Both Syk tSH2 domains and CLEC-2 peptides were extensively dialysed into experimental buffer (50 mM HEPES, 150 mM NaCl, 150 mM glycine, 1 mM EDTA and 5 mM β-mercaptoethanol; pH 7.5) prior to experimentation. Protein and peptide concentrations were determined by spectrophotometry using a ND-1000 spectrophotometer (Nanodrop, Thermo Scientific, UK). The tSH2of Syk (8 μM) were loaded into the calorimeter cell (V = 1.42 ml) and the CLEC-2 peptide (2 mM) was loaded into the injection syringe. Titrations were computer controlled and performed at 25˚C. Negative control injections of peptide into buffer were performed under the same conditions.

2.9 Tryptophan fluorescence titration

Fluorescence measurements were performed in a PTI spectrofluorimeter (Photon Technology International Ltd. UK). Both Syk SH2 domains and CLEC-2 peptides were extensively dialysed into experimental buffer (50 mM HEPES, 150 mM NaCl, 150 mM glycine, 1 mM EDTA and 5 mM β-mercaptoethanol; pH 7.5) prior to experimentation. Protein and peptide concentrations were determined by spectrophotometry using a ND-1000 spectrophotometer (Nanodrop, Thermo Scientific, UK). The SH2 domains of Syk (3 ml of 250 nM) were loaded into a quartz cuvette and placed into the cell turret with constant stirring. The CLEC-2 peptide (2 mM) was titrated in and allowed to mix for 2 min before scanning. An excitation wavelength of 295 nm was used and emission spectra were collected over the range of 300-420 nm in 2.5 nm steps. Slit widths of

0.75 mm were used for both excitation and emission. The peak fluorescence of 340 nm was monitored for binding constant calculation.

2.10 Analysis of data

Data is shown from a single experiment which is representative of 3-5 experiments. Where data is expressed in chart form, results are shown as arithmetic mean ± standard error unless otherwise stated. NFAT-luciferase data is expressed as geometric mean ± standard error. Statistical analysis was carried out using unpaired Student's t-test. Significance was taken for $P < 0.05$. Where differences reached statistical significance, this is stated (with p values) on the corresponding figure and legend. Where statistical significance is not explicitly stated, any differences were not statistically significant.

CHAPTER 3

A CLEC-2 DIMER IS CAPABLE OF ITAM-LIKE SIGNALLING THROUGH SYK TYROSINE KINASE

3.1 Introduction

CLEC-2 has been shown to activate platelets using a signalling pathway that is similar to that which is used by GPVI, an ITAM receptor, despite having a single YxxL in its cytosolic tail (Suzuki-Inoue *et al*, 2006; Fuller *et al*, 2007). Critically, point mutations in the conserved tyrosine within the YxxL sequence in CLEC-2 or in either of the Syk SH2 domains, which destroying their phosphotyrosine binding capacity, cause complete ablation of CLEC-2 signalling (Fuller *et al*, 2007). Further, only a fusion protein encoding both Syk SH2 domains is able to precipitate CLEC-2 from rhodocytin-stimulated platelet lysates (Suzuki-Inoue *et al*, 2006; Fuller *et al*, 2007). These results demonstrate that both SH2 domains and the CLEC-2 YxxL sequence are critically required for signalling by the C-type lectin receptor.

Two models can be put forward to explain these results: (i) the presence of a second binding site in the CLEC-2 cytoplasmic tail that is required for binding, either directly or indirectly, to one of the Syk SH2 domains, or (ii) that CLEC-2 regulates Syk through dimerisation, thereby providing two YxxL sequences. The recent publication on CLEC9A, which is a covalent dimer, lends circumstantial evidence for this second model (Huysamen *et al*, 2008). Further, a CLEC-2 specific $F(ab')_2$ fragment, but not a F(ab) fragment, is able to mediate activation of mouse platelets providing further supporting this second model (May *et al*, 2009).

In regard to the first model, we hypothesised that a second binding site would be conserved between species and most likely with Dectin-1 and CLEC9A which are the

only other two known proteins that signal through a single YxxL motif. Sequence alignment of CLEC-2 from 9 species using ClustalW (www.ebi.ac.uk/clustalw/) highlighted the complete conservation of the MQDEDGYxxL sequence, and two serine residues at positions 21 and 27 (number from human CLEC-2). Several positively charged amino acids and a WR sequence at the juxtamembrane position were also conserved (Figure 3.1 a). Sequence alignment of Dectin-1 from 9 species also highlighted conservation of the DEDGYxxL sequence and of two serine residues in equivalent positions, but not the other conserved amino acids in CLEC-2 apart from the membrane WR sequence (Figure 3.1 b). The same relationship did not hold for the recently discovered third member of this family, CLEC9A (Figure 3.1c). The conserved DEDG sequence was replaced by the similarly charged EEEI sequence and just one of the serines was conserved.

Sequence alignment also highlighted the presence of a threonine residue at position 9 in CLEC-2, the Y+2 position in the YxxL. Dectin-1 had a conserved threonine at Y+1 of its YxxL and this was also seen in CLEC9A. Previous studies using mast cells or mouse platelets have shown that the presence of a threonine in position Y+1 in the FcRγ ITAM can be a target of PKC (δ or ε) phosphorylation, and that this phosphorylation allows for a tighter interaction with the Syk SH2 domains and maximal activation of Syk (Germano *et al*, 1994; Swann *et al*, 1999; Pears *et al*, 2008).

The aims of this chapter are to investigate the mechanism of regulation of Syk by CLEC-2 in order to distinguish between the two models proposed above. This will be

achieved by investigation of whether CLEC-2 signals as a monomer or as a dimer/oligomer; by mutation of the two conserved serine and threonine residues; and by co-expression of wild type and the Y7F mutant of CLEC-2 to investigate whether the latter inhibits signalling, as predicted by the dimerisation model. The presence of the conserved serine and threonine residues is of particular interest compared to other conserved sequences in CLEC-2 given their potential for phosphorylation. Alongside this, direct measurement of binding of the SH2 domains of Syk to peptides based on the cytosolic sequence of CLEC-2 using a variety of protein chemistry and biophysical techniques was performed.

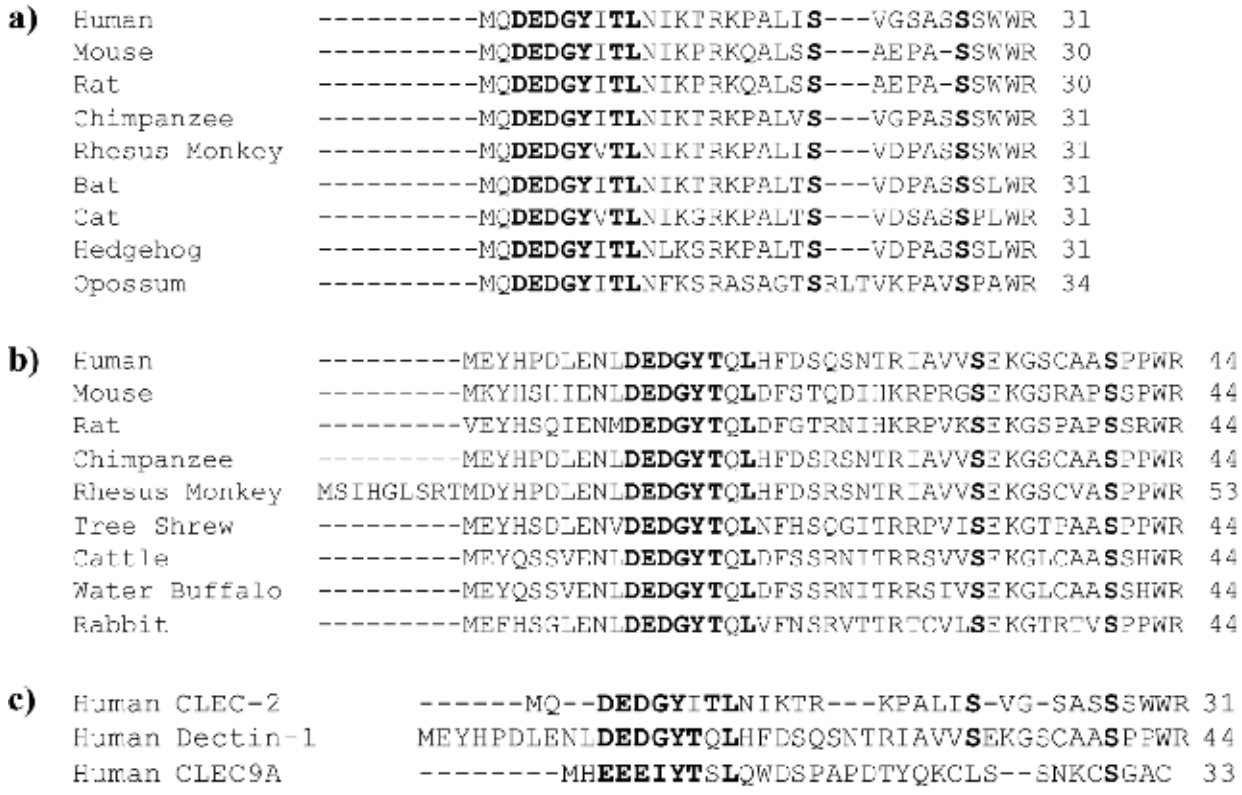

Figure 3.1 – Sequence alignment of CLEC-2 family proteins. a) 9 species of CLEC-2 were aligned using ClustalW web-based software, highlighting the conserved MQDEGDYxTL motif and serines 21 and 27. b) 9 species of Dectin-1 were aligned, highlighting the conserved DEDGYTxL motif and serine residues. c) Human CLEC-2, Dectin-1 and CLEC9A were aligned, highlighting the partially conserved DEDGYxxL motifs and serine residues.

3.2 Results

3.2.1 Structure-function relationships of the CLEC-2 cytoplasmic tail and Syk tandem SH2 domains

To investigate a potential role of conserved residues in the CLEC-2 tail, we used a combination of peptide pull-down studies, to ascertain if a CLEC-2 model peptide could associate with Syk from a platelet lysate, and a DT40 cell line assay using mutant forms of CLEC-2. Expression of WT CLEC-2 along with a NFAT-luciferase reporter gene can be used to monitor activation by rhodocytin in a DT40 cell line. This cell line has been used as a model system for GPVI-FcRγ and CLEC-2 signalling previously (Fuller *et al*, 2007; Tomlinson *et al*, 2007). In this model, CLEC-2 and GPVI-FcRγ use the ITAM signalling machinery of the B-cell receptor to activate PLCγ2, resulting in NFAT activation and subsequent expression of luciferase. In confirmation of the previously published result (Fuller *et al*, 2007) rhodocytin stimulated luciferase activity in DT40 cells transfected WT CLEC-2 but not Y7F mutant, in which the tyrosine of the single YxxL motif was mutated to phenylalanine (Figure 3.2 a). This confirms the critical role of the conserved tyrosine in signalling by CLEC-2.

In line with the above, Syk was precipitated from platelet lysates using a phosphorylated but not a non-phosphorylated peptide encoding the first 13 amino acids (aa) of the CLEC-2 tail (Figure 3.2 b). This region includes the conserved DEDGYxxL sequence. Furthermore, there was no observable increase in the amount of Syk pulled out when a tyrosine phosphorylated peptide encoding the full 31aa cytoplasmic tail of CLEC-2 was used (Figure 3.2 c) suggesting that the first 13aa, and therefore the YxxL

sequence, is sufficient for Syk association. It is important to note however that the interaction between the peptide and Syk in this experiment could be mediated through binding of two separate phosphorylated peptides to individual SH2 domains in Syk, bearing in mind that multiple phosphorylated peptides are attached to the beads used for precipitation. These observations therefore do not rule out a possible direct or indirect interaction between the Syk tandem SH2 domains and a second site in the CLEC-2 cytosolic tail which may take place in an intact cell.

Using a similar approach, the contribution of the two conserved serine residues was also investigated. Mutation of the serines to alanines, or deletion of the serine rich region 21-28, had no significant effect on the ability of the mutant CLEC-2 to induce NFAT activation relative to the WT receptor in transiently transfected DT40 cells (Figure 3.3 a). Likewise, a tyrosine phosphorylated 31aa peptide in which both S21 and S27 were replaced by alanine was able to associate with Syk to the same degree as the WT peptide (Figure 3.3 b). These results provide evidence against a functional role of the serine residues in mediating the association with Syk or signalling to PLCγ2 and subsequent NFAT activation.

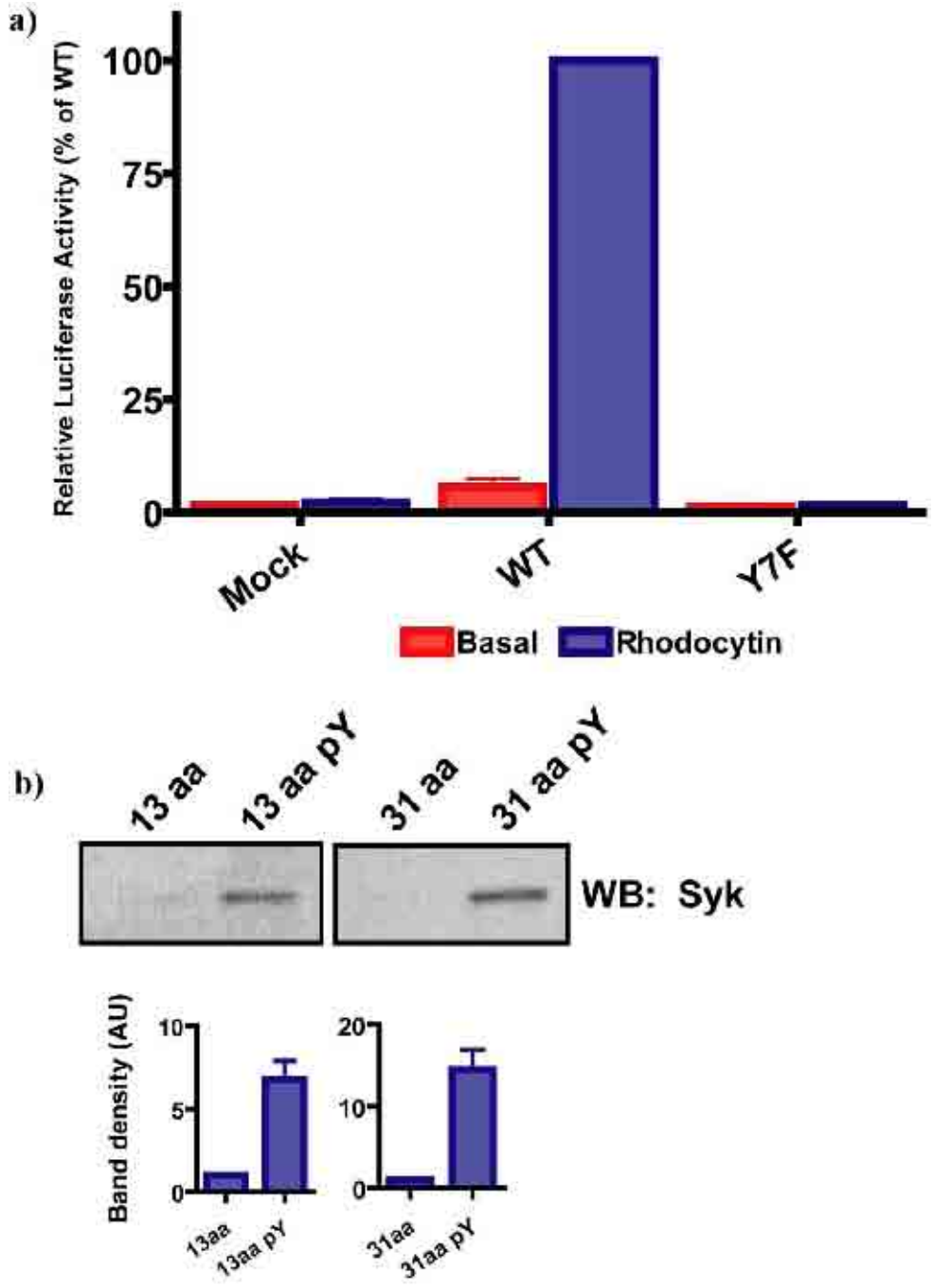

Figure 3.2 – YxxL is essential for Syk association and signalling through CLEC-2. a) DT40 cells were transfected with 10 μg/ml of the stated CLEC-2 construct and a NFAT-luciferase reporter plasmid. Transfected cells were stimulated with 50 nM rhodocytin for 6 hrs at 37˚C and then the luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the WT response. Error bars represent the geometric mean ± standard error of three to six separate experiments. b) Washed platelets (5 x 10^8/ml) were lysed with 2x NP40 lysis buffer, pre-cleared and interacting proteins precipitated with the addition of 10 μg of the relevant biotinylated CLEC-2 peptide. Precipitated proteins were separated by SDS-PAGE and western blotted for the presence of Syk. Densitometry is shown in the histograms. Result is representative of three experiments.

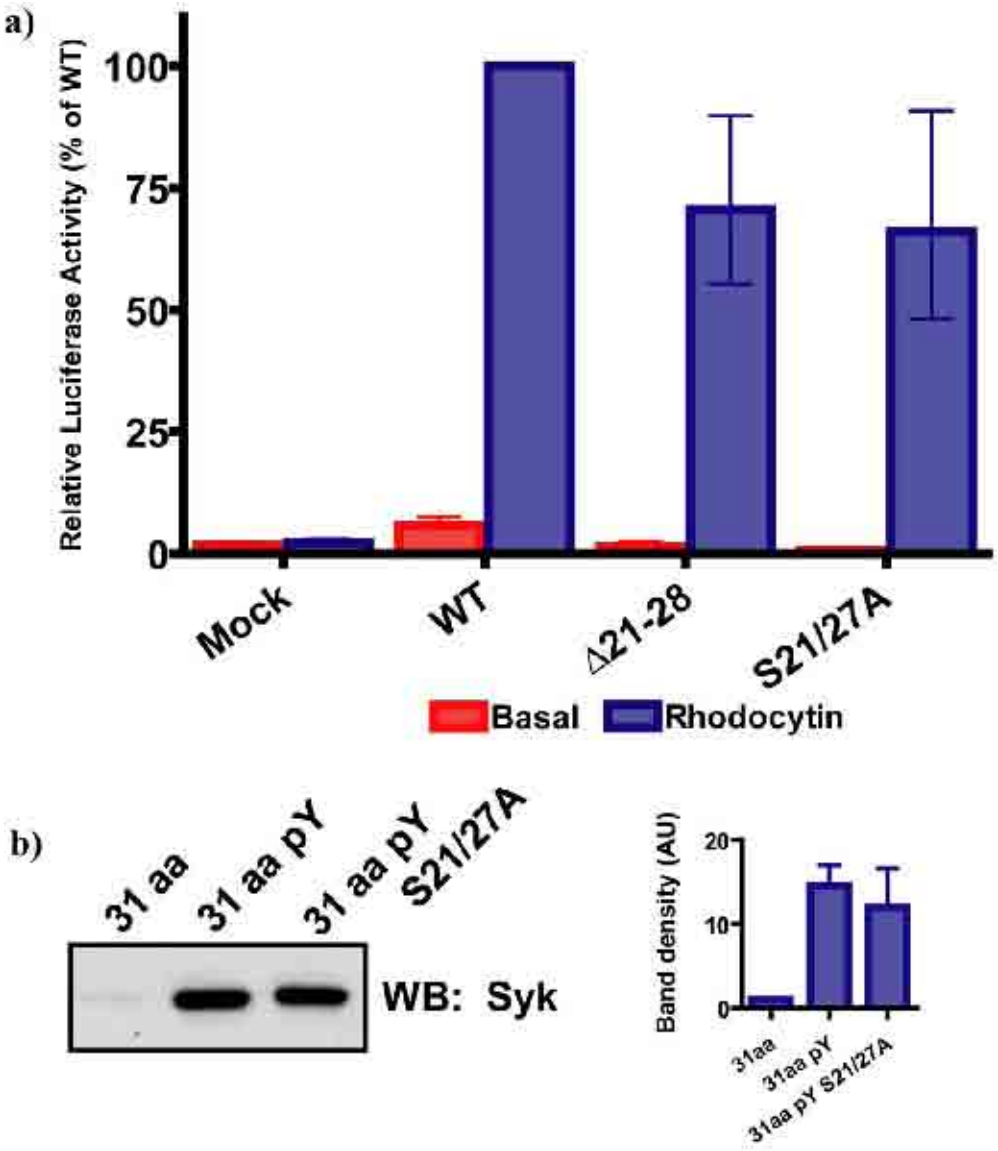

Figure 3.3 – Serines-21 and 27 are dispensable for Syk association and signalling through CLEC-2. a) DT40 cells were transfected with 10 μg/ml of the stated CLEC-2 construct and a NFAT-luciferase reporter plasmid. Transfected cells were stimulated with 50 nM rhodocytin for 6 hrs at 37˚C and then the luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the WT response. Error bars represent the geometric mean ± standard error of three to six separate experiments. b) Washed platelets (5 x 10^8/ml) were lysed with 2x NP40 lysis buffer, pre-cleared and interacting proteins precipitated with the addition of 10 μg of the relevant biotinylated CLEC-2 peptide. Precipitated proteins were separated by SDS-PAGE and western blotted for the presence of Syk. Densitometry is shown in the histograms. Result is representative of three experiments.

The same approach was also used to investigate the role of the conserved threonine in the Y+2 position in the CLEC-2 YxxL sequence. Mutation of the threonine to alanine had no significant effect on the ability of rhodocytin to stimulate NFAT activity in DT40 cells (Figure 3.4 a). Further, a 13aa peptide containing phosphotyrosine and phosphothreonine residues at position 7 and 9 was able to precipitate a similar level of Syk from a platelet lysate (Figure 3.4 b). This provides evidence against a functional role of the conserved threonine at position 9 in signalling by CLEC-2.

These results confirm a critical role for the phosphorylated YxxL sequence in binding to Syk and mediating signalling by CLEC-2, but demonstrate that the two conserved serines at positions 21 and 27 and the partially conserved threonine at position 9 are dispensable for these events. The functional role of the two conserved serines and threonine residues remains to be determined. The present results have therefore failed to provide evidence for a second binding site in the CLEC-2 tail that supports binding and activation of Syk and thereby indirectly favour a model in which Syk cross-links two molecules of CLEC-2 via its tandem SH2 domains, both of which are known to be essential for CLEC-2 signalling (Fuller *et al*, 2007).

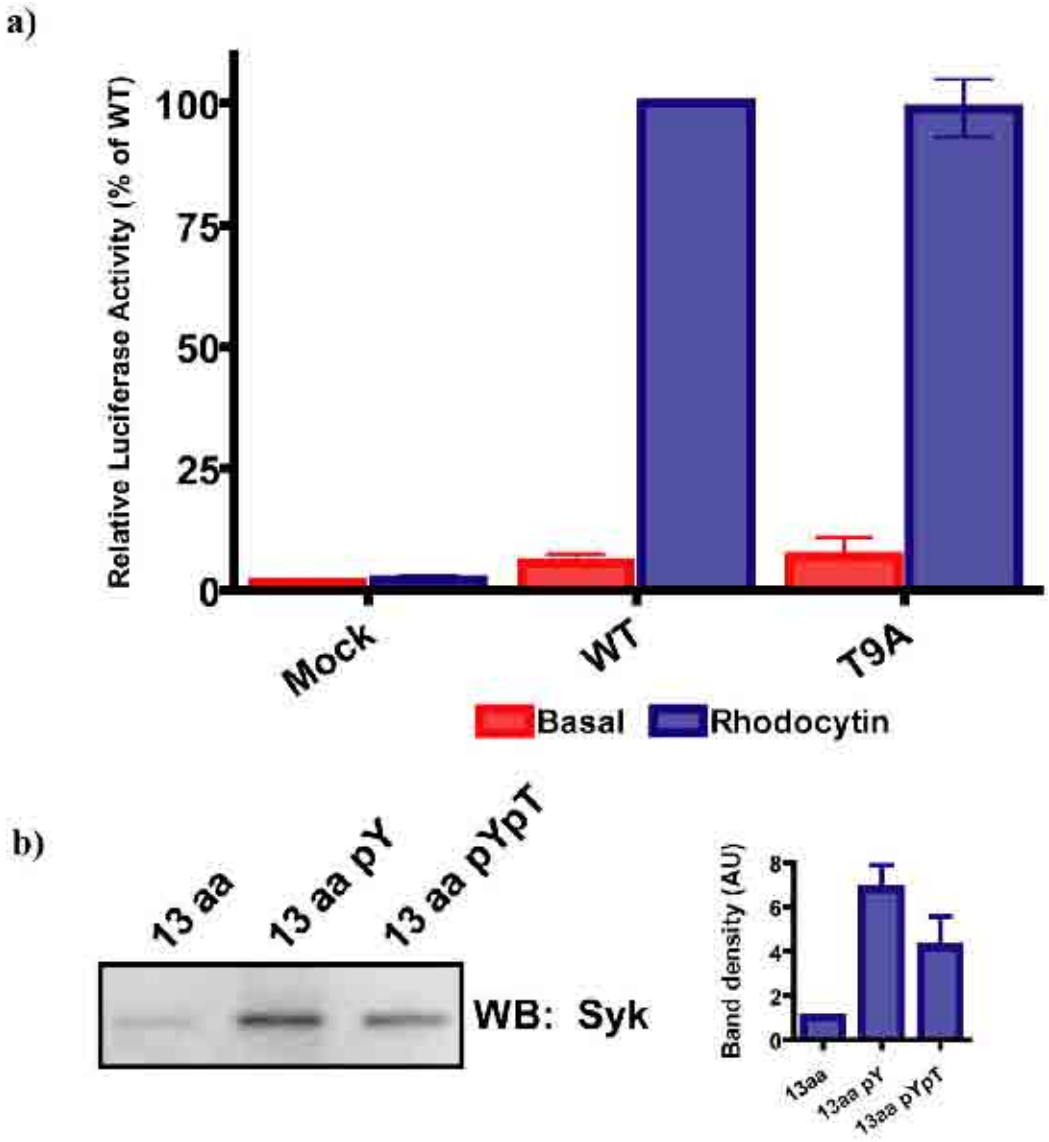

Figure 3.4 – Threonine-9 is dispensable for Syk association and signalling through CLEC-2. a) DT40 cells were transfected with 10 μg/ml of the stated CLEC-2 construct and a NFAT-luciferase reporter plasmid. Transfected cells were stimulated with 50 nM rhodocytin for 6 hrs at 37˚C and then the luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the WT response. Error bars represent the geometric mean ± standard error of three to six separate experiments. b) Washed platelets (5 x 10^8/ml) were lysed with 2x NP40 lysis buffer, pre-cleared and interacting proteins precipitated with the addition of 10 μg of the relevant biotinylated CLEC-2 peptide. Precipitated proteins were separated by SDS-PAGE and western blotted for the presence of Syk. Densitometry is shown in the histograms. Result is representative of three experiments.

3.2.2 Dominant negative effect of Y7F CLEC-2 on signalling by WT CLEC-2

In order to further investigate the mechanism of regulation of Syk by CLEC-2, I co-expressed wild type CLEC-2 with varying levels of the Y7F mutant of CLEC-2. The rationale for this experiment is that the Y7F mutant should have an inhibitory effect if the cross-linking/dimerisation model is correct whereas it will have no effect if Syk is activated by a single CLEC-2 receptor. The alternative possibility that the Y7F mutant is able to inhibit activation of Syk by a single CLEC-2 receptor seems unlikely. When expressed at a ratio of 3:1, Y7F to WT, NFAT activity was inhibited to approximately 25% that of the WT receptor. This response increases to approximately 40% when the ratio is decreased to 1:1 and is restored to approximately normal levels when the amount of Y7F CLEC-2 is titrated down below a ratio of 0.05:1 (Figure 3.5 a). Surface expression of WT CLEC-2 was measured by flow cytometry using an α-Myc antibody which only detected the WT CLEC-2 due to the presence of a Myc tag. The surface expression of WT CLEC-2 was not significantly affected by expression of the Y7F mutant (Figure 3.5 b). In contrast, the weak constitutive (agonist-independent) signal induced by expression of CLEC-2 that has been previously described (Mori *et al*, 2008) was not inhibited in the presence of the Y7F mutant suggesting that it may be mediated through a CLEC-2 monomer. These data provide evidence that ligand-activated CLEC-2 is unable to signal as a monomer in response to activation by rhodocytin and that expression of the Y7F inactive mutant has a 'dominant negative' effect as a result of forming dimeric or higher order structures with wild type receptors that are unable to signal.

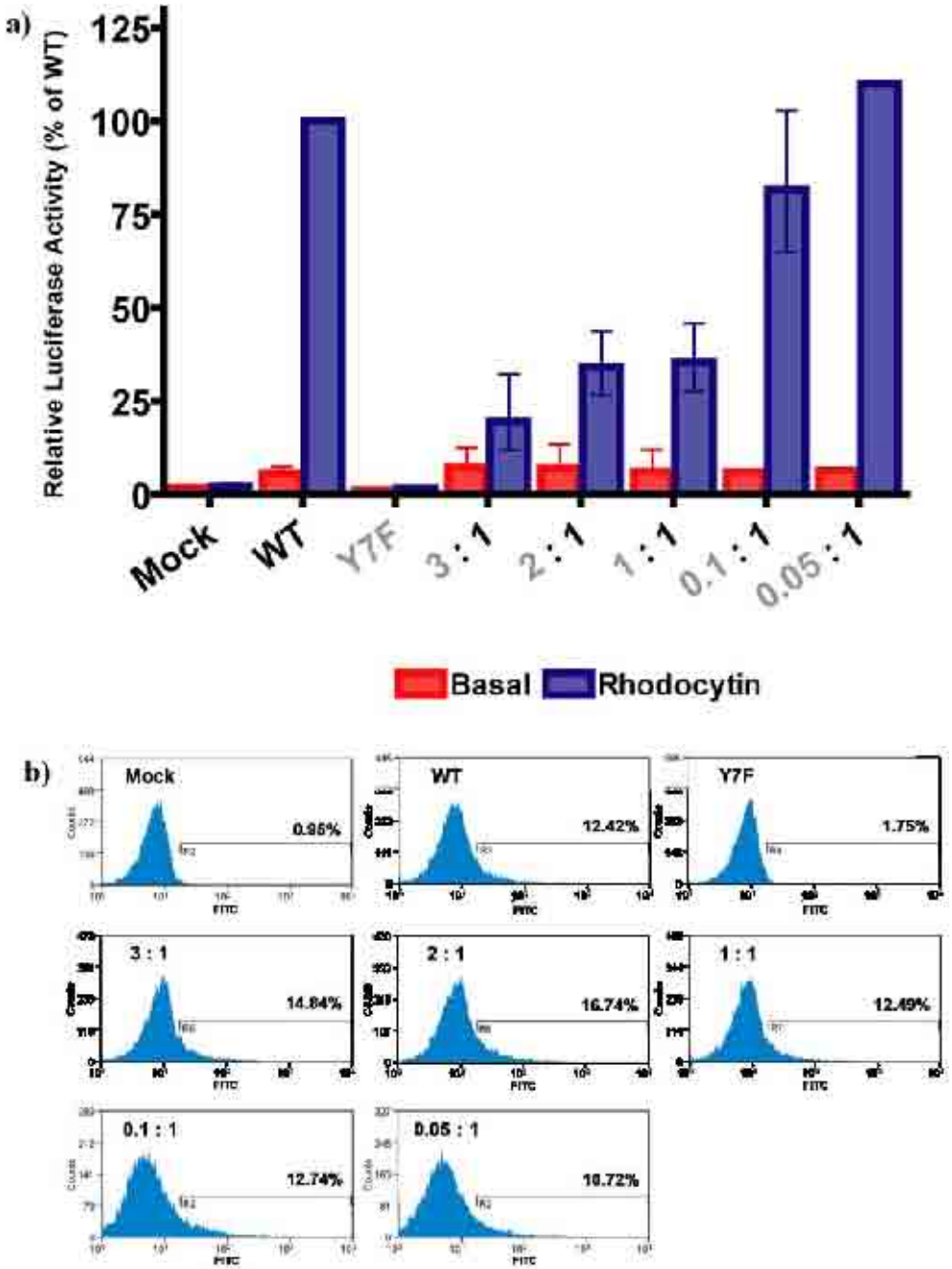

Figure 3.5 – The Y7F CLEC-2 mutant inhibits signalling by the wild type receptor. a) DT40 cells were transfected with 10 μg/ml of WT CLEC-2 (black text) and/or varying amounts of Y7F CLEC-2 (grey text) shown as a ratio, and a NFAT-luciferase reporter gene. Transfected cells were stimulated with 50 nM rhodocytin for 6 hrs at 37˚C after which time, the amount of luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the WT response. Error bars represent the geometric mean ± standard error of three to eight separate experiments. b) Transfected cells were analysed by flow cytometry for surface expression of Myc-tagged WT CLEC-2. The selected gate was drawn to exclude ~99% of mock transfected cells. No WT expression is seen in the Y7F transfection alone, and WT expression is similar when co-transfected with varying amounts of Y7F.

3.2.3 CLEC-2 oligomers are present on the platelet surface

The above results favour a dimerisation/oligomerisation model of signalling by CLEC-2. To further investigate this, we used the cross-linker Sulfo-EGS, which is known to cross-link surface proteins that lie within a distance of 16.1 Å (12 atoms) of each other (Browning and Ribolini, 1989; Dihazi and Sinz, 2003; Sinz, 2003). In non-stimulated or rhodocytin-stimulated platelets, CLEC-2 migrates on SDS-PAGE as a characteristic doublet due to differential glycosylation (Suzuki-Inoue *et al*, 2006; Watson *et al*, 2007). The molecular weights of the differentially glycosylated forms of CLEC-2 (30 and 40 kDa) suggest that both are present as a monomer and is consistent with the fact that they are reduced to a single band of just less than 30 kDa upon deglycosylation (Suzuki-Inoue *et al*, 2006). Following addition of a low or high concentration of the cross-linker Sulfo-EGS, the presence of several higher molecular weight bands of CLEC-2 can be seen together with a corresponding reduction in intensity of the 30 and 40 kDa bands (Figure 3.6 a). In the absence of stimulation, a new, broad band is observed at between 60 – 80 kDa which corresponds approximately to a doubling of the molecular weight of monomeric CLEC-2. With the higher concentration of cross-linker, there is the suggestion of higher order structures in non-stimulated platelets, with a corresponding reduction in both the monomeric and predicted dimeric forms of the receptor. In the presence of rhodocytin, Sulfo-EGS induces formation of the predicted CLEC-2 dimer along with higher order structures, most notably at the higher of the two concentrations, where there is almost no detectable monomeric CLEC-2. The broad nature and smearing of the bands could reflect a combination of higher oligomeric forms of CLEC-2 and possibly cross-linking to other proteins. Interestingly, western blotting studies failed to detect the presence of rhodocytin in the higher order structures suggesting that direct cross-linking to the toxin had not occurred (not shown).

A similar overall pattern of change in CLEC-2 dimerisation/oligomerisation was seen in whole cell lysates prepared from basal (not shown) and rhodocytin-activated platelets, as demonstrated by western blotting for CLEC-2 (Figure 3.7 b). In marked contrast, there was no apparent dimerisation/oligomerisation of the low affinity immune receptor, FcγRIIA, or the membrane-associated protein Src in the presence of the intermediate and high concentration of Sulfo-EGS in basal (not shown) or rhodocytin-activated platelets (Figure 3.7 b) as shown by the similar levels of the monomeric forms of both proteins and no higher molecular weight forms present as revealed by western blotting (Figure 3.7 b). This result demonstrates that the oligomerisation of CLEC-2 is not simply the result of a non-specific effect of cross-linking of platelet surface proteins.

Together, these results provide evidence for the presence of monomeric, dimeric and oligomeric forms of CLEC-2 on the platelet surface, although it should be noted that these structures may also be due to binding to one or more additional proteins. Nevertheless, the loss of the monomeric and the corresponding increase in the 60 – 80 kDa band is consistent with the proposal that CLEC-2 forms a dimer thereby providing adjacent binding sites for Syk through its phosphorylated YxxL domains.

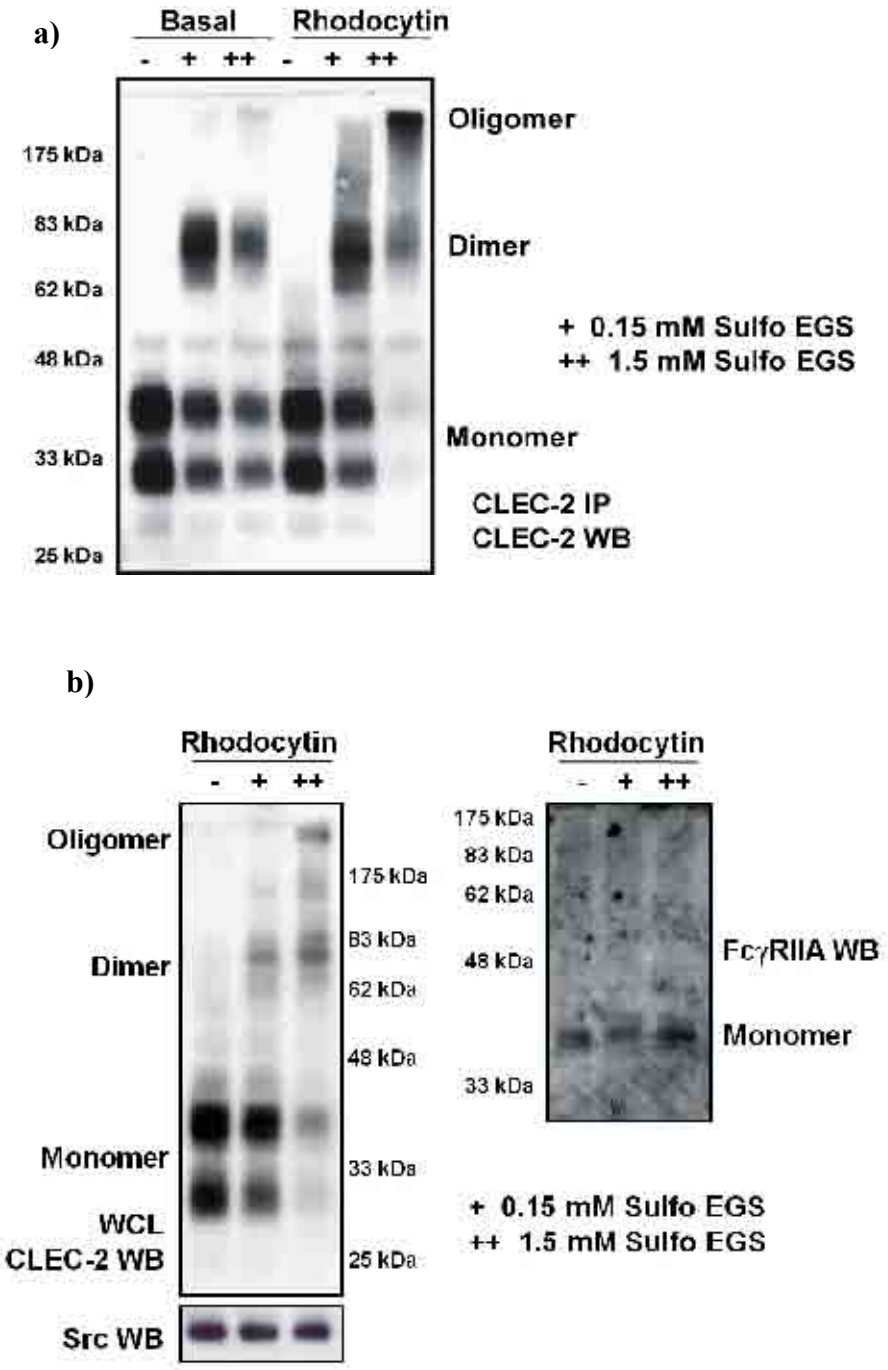

Figure 3.6 – CLEC-2 oligomers are present on the platelet surface. Washed platelets (5 x 10^8/ml) under basal or rhodocytin stimulated (100 nM) conditions had their surface proteins cross-linked with the addition of 0.15 mM or 1.5 mM Sulfo-EGS cross-linking reagent, with a linker length of 16 Å. The cross-linking reaction was subsequently blocked and then the platelets were lysed with 2x NP40 lysis buffer. a) The lysates were then pre-cleared with protein G Sepharose, then immunoprecipitated with α-CLEC-2 antibody and PGS. Precipitated proteins were separated by SDS-PAGE and western blotted for CLEC-2. b) Lysates were separated by reducing SDS-PAGE and western blotted for CLEC-2, FcγRIIA and Src. The results are representative of three experiments

3.2.4 Affinity measurements of Syk SH2 domains for CLEC-2

The above results support a model in which the tandem SH2 domains in Syk bind to the phosphorylated YxxL sequence in separate CLEC-2 monomers. This is consistent with our previous report that point mutants that disrupt binding of the individual SH2 domains to phosphotyrosine abrogate signalling by CLEC-2 (Fuller *et al*, 2007).

For this model to be correct, both SH2 domains of Syk must be able to bind to the phosphorylated YxxL sequence. This was investigated using surface plasmon resonance (SPR) to measure the equilibrium dissociation constants (K_D) of binding of the N-SH2, C-SH2 and the tandem SH2 (tSH2) domains of Syk to surfaces coated with the phosphorylated or non-phosphorylated 13aa CLEC-2 peptide (Figure 3.7 a). These proteins were expressed as GST-fusion proteins in DH5α *E.coli* cells and affinity purified on a glutathione-agarose column (Figure 3.7 b). No detectable binding was observed for any of the SH2 domains when flowed over the non-phosphorylated CLEC-2 peptide (not shown), confirming the requirement for phosphorylation of the conserved tyrosine residue. There was also no detectable binding observed for GST alone or in a reference flow cell (not shown). Specific binding was observed when the peptides were flowed over a tyrosine phosphorylated CLEC-2 peptide. From the resulting curves, we calculated a K_D of 10.5 μM (±2 μM) for the N-SH2 domain alone and 2.35 μM (±0.54 μM) for the C-SH2 domain alone demonstrating the ability of both SH2 domains to bind to phosphorylated CLEC-2 YxxL with similar affinity. Moreover, when the tSH2 domain protein was flowed over the tyrosine phosphorylated CLEC-2 peptide, a K_D of 256 nM (±59.9 nM) was calculated thereby demonstrating cooperativity in binding of the two SH2 domains, i.e. the divalent nature of the tSH2 domain protein binding to the

peptide surface under flow results in an increased affinity as a function of the affinities of the two SH2 domains and avidity of the surface. As a comparison, the individuals and tandem Syk SH2 domains were flowed over a dually phosphorylated peptide based on the FcRγ ITAM sequence. Cooperativity was again observed when the tSH2 domains were flowed over the peptide with a calculated K_D of 19.7 nM (±1.79 nM) compared to the individual K_Ds of 19.7 μM (±1.1 μM) and 1.71 μM (±0.36 μM), respectively, for the N- and C-SH2 terminal domains alone. These data therefore confirm that the Syk tandem SH2 domain protein binds with higher affinity than the single Syk SH2 domains to a surface containing multiple copies of a single or tandem phosphorylated YxxL-containing peptides. This has important implications for the binding of Syk to a dually phosphorylated ITAM protein or to the phosphorylated CLEC-2 tail when presented as a dimer. Consistent with this data, quantitative western blotting was used to show that twice the amount of a CLEC-2 peptide was able to associate with the tSH2 domains of Syk compared with the single SH2 domains alone providing further evidence of an interaction with a 2:1 stoichiometry of CLEC-2 to Syk (Figure 3.7 c).

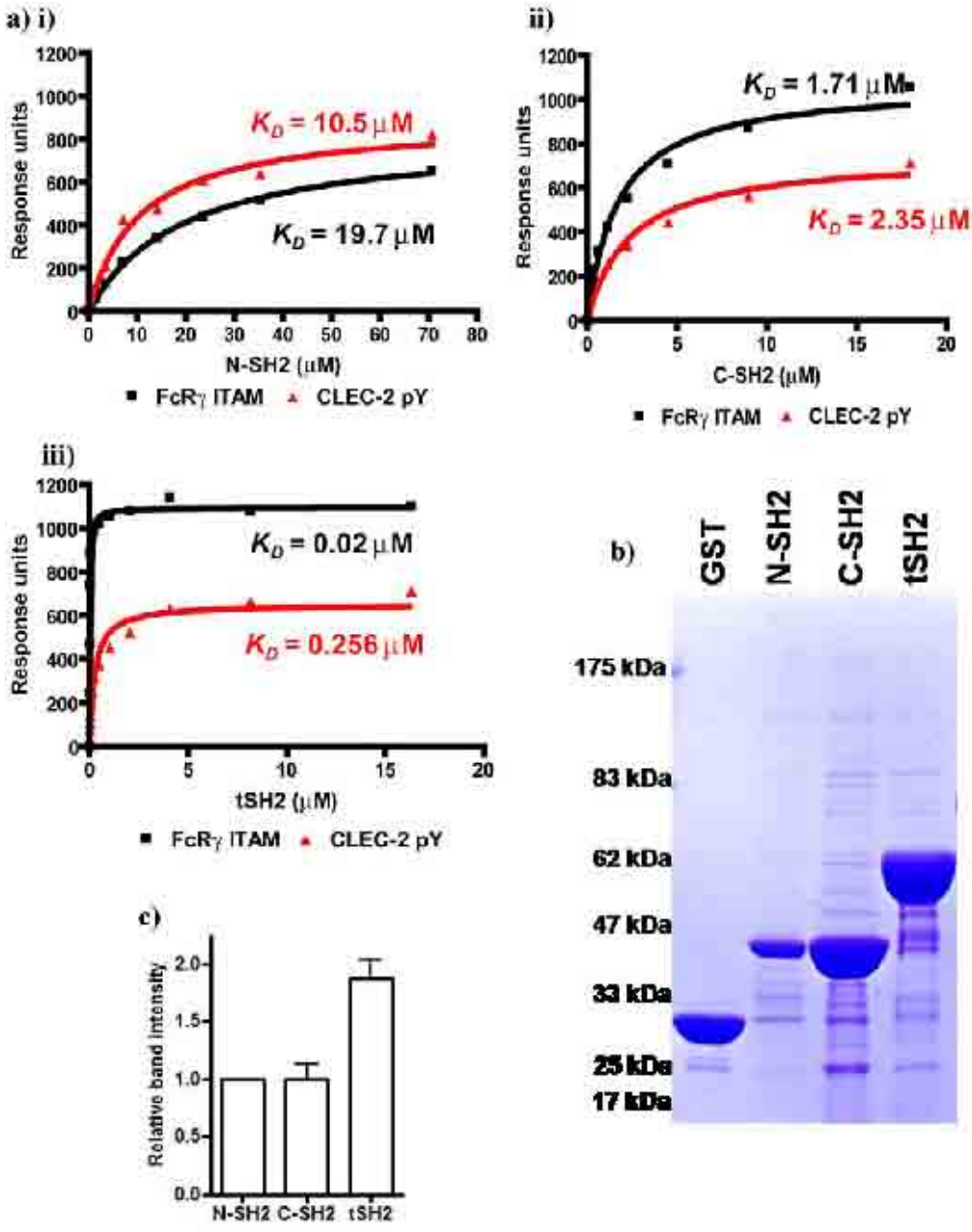

Figure 3.7 – Surface plasmon resonance measurements of the CLEC-2-Syk SH2 interaction. a) Biotinylated CLEC-2 and FcRγ peptides were bound to streptavidin-coated biosensor chips surfaces. The N-SH2 (ai), C-SH2 (aii) or the tSH2 (aiii) domains of Syk were flowed over the chip at a range of concentrations. Non-linear regression was used to analyse the data and calculate K_D values. The results are representative of three experiments. b) GST-fusion proteins were inducibly over-expressed in DH5α cells, and purified on a glutathione-agarose column. 20 μl samples were ran on SDS-PAGE and proteins visualised with Coomassie stain. c) GST-tagged Syk SH2 domain proteins were incubated with a 50-fold excess of biotinylated phospho-CLEC-2 peptide and precipitated with glutathione-agarose beads. The precipitated proteins were dot-blotted and the amount of associated CLEC-2 was measured using HRP-streptavidin and densitometric analysis. The result is representative of four experiments

The results from the SPR were particularly encouraging as the results for binding to the CLEC-2 peptide followed the same pattern of cooperativity as binding to the FcRγ ITAM peptide. However, to have greater confidence we wanted to use another technique to try to confirm the measured affinities. Firstly we chose to use isothermal titration calorimetry (ITC) due to its sensitivity, and because it is also possible to directly determine the stoichiometry of an interaction. ITC requires high concentrations of pure protein without any GST-tags attached. We therefore scaled up the production of the tSH2 protein, cleaved off the GST-tag with thrombin, and performed several purification steps with glutathione-agarose columns and size-exclusion chromatography (Figure 3.8 a). Titration of the phosphorylated CLEC-2 peptide caused a small heat exchange which was titratable down to only the heat exchanged due to the heat of dilution of buffer, thereby demonstrating an interaction (Figure 3.8 b). However, under the conditions required for this experiment (i.e. stirring conditions at room temperature for 3-4 hours), the tSH2 protein did not remain stable for long enough to collect high quality data. Without the GST-tag, the protein came out of solution after extended periods of time above 4˚C but these experiments could not be performed in the presence of the GST-tag due to the possibility of GST forming dimers and non-specific interactions with the peptide. Therefore, the data was insufficient to directly measure the stoichiometry of the interaction. However, it was possible to fit the collected data to a two-site but not to a single-site binding curve, consistent with the model in which the tandem Syk SH2 domains bind to two phosphorylated CLEC-2 peptides. The two calculated K_Ds from this fitting were 28 μM and 2.07 mM. These values do not correspond with the above results, most likely due to the instability of the proteins under the experimental conditions, thereby making the data unreliable. It may be possible to repeat these experiments in the future at 4ºC to bypass the instability of the SH2

domains however, the decreased temperature would mean that many of the measured thermodynamic parameters would not be comparable to those measured in assays at room temperature although the stoichiometry would presumably still be possible to calculated at the lower temperature.

Due to the failure of the ITC, we used a third approach to investigate binding of the tSH2 domains of Syk to a CLEC-2 peptide, namely tryptophan fluorescence titration. This technique is based on the principle that binding to the peptide causes a change in fluorescence of one or more tryptophan groups in the tandem Syk SH2 domains (Grucza *et al*, 1999; Kim *et al*, 2000). The N-terminal 13aa CLEC-2 peptide was used for these studies as this supports a similar level of binding of Syk to that of the 31aa phosphorylated peptide and does not contain any tryptophan residues. Addition of the peptide resulted in a saturatable decrease in peak tryptophan fluorescence of the tandem Syk SH2 domains at 340 nm, indicative of ligand binding (Figure 3.8 c). The resulting dissociation constant (K_D) of 5.72 μM (±2.14 μM) for the Syk tSH2:CLEC-2 interaction was of a similar order to the SPR derived K_D of the CLEC-2 peptide associating with either the N- or C-terminal Syk SH2 domains alone. In contrast, the fluorescence data recorded a 20-fold weaker binding to the tSH2 domains than was observed by SPR. This discrepancy may reflect the influence of avidity in the binding between the Syk tSH2 domains and a surface-immobilised peptide whereas the tryptophan fluorescence measurements were made in solution. In contrast, we were unable to detect binding of the CLEC-2 peptide to the single C-terminal SH2 domain of Syk over the same concentration range (not shown). The equivalent experiment was not valid for the N-terminal SH2 domain as this does not contain a tryptophan residue. It should also be

noted that there is also a single tryptophan residue in the SH2 linker region which is present only in the tSH2 domain protein. It is therefore possible that ligand binding is causing only the linker region tryptophan to change its fluorescence and this could explain the lack of fluorescence change with the C-terminal SH2 domain.

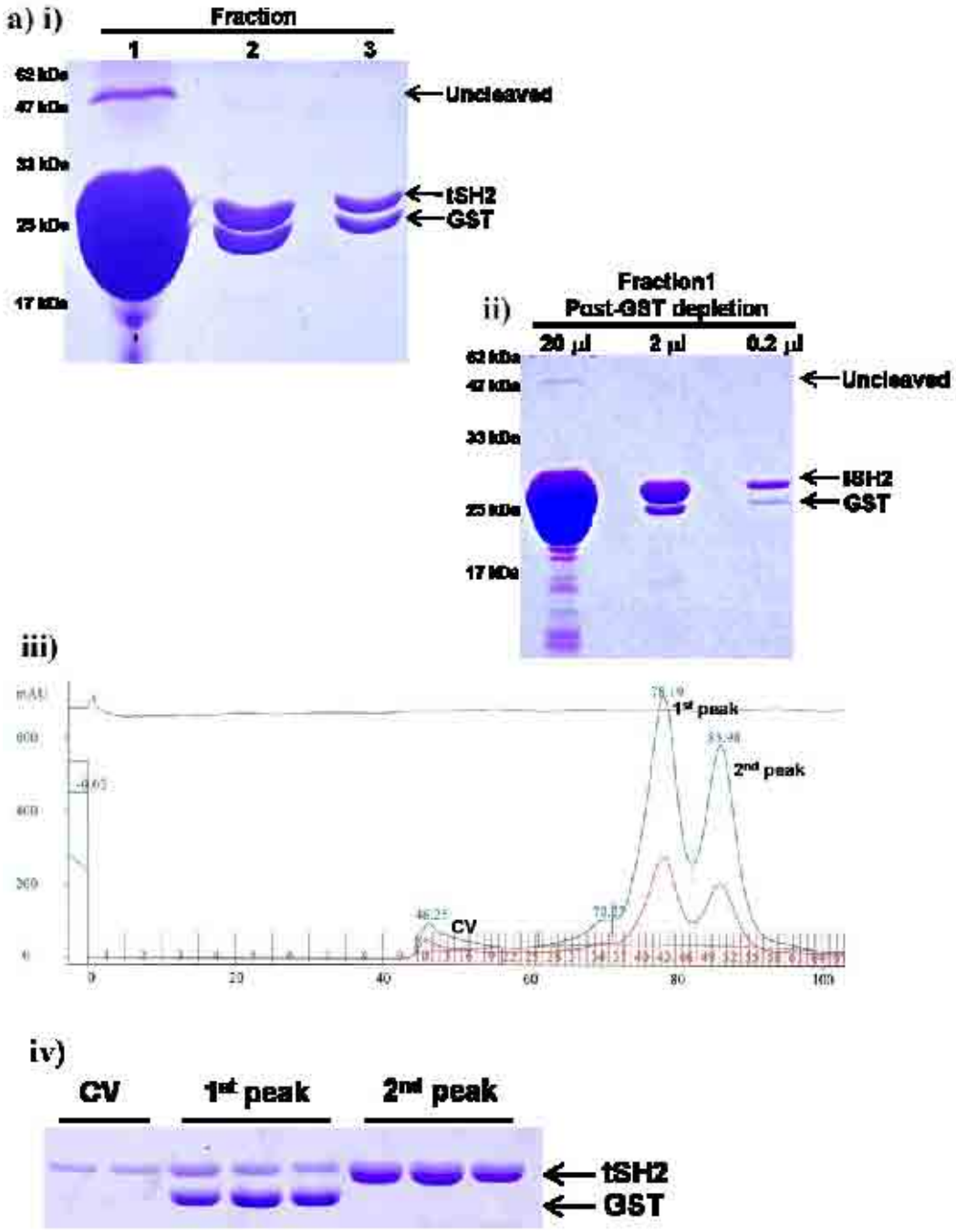

Figure 3.8 – SPR and tryptophan fluorescence measurements. a) GST-fusion tSH2 domain protein was inducibly over-expressed in DH5α cells, and purified on a glutathione-agarose column. Eluted fractions were treated with thrombin to cleave off the GST-tag. Samples were run on SDS-PAGE and proteins visualised with Coomassie stain (ai). Free GST was depleted using a glutathione agarose column. Samples were run on SDS-PAGE and proteins visualised with Coomassie stain (aii). Size exclusion chromatography was used to further purify the tSH2 domain protein (aiii). The blue line represents absorbance at 280 nm, the red line represents absorbance at 254 nm. Samples from CV (void column volume), and the two major peaks were run on SDS-PAGE and proteins visualised with Coomassie stain. The second peak contains pure tSH2 domain protein and was eluted off the column with a predicted MW of 29.65 kDa.

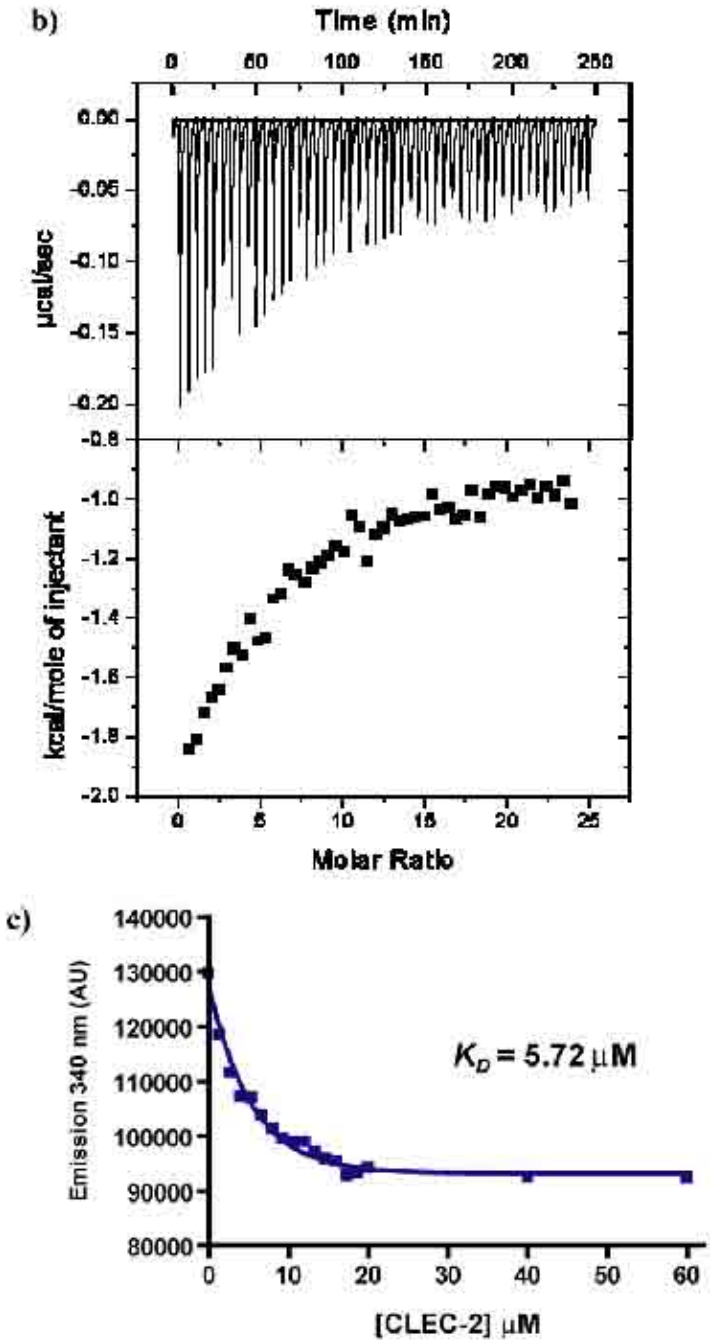

Figure 3.8 – continued. b) The purified tSH2 domains of Syk were loaded into the cell of an ITC instrument (MicroCal) and allowed to equilibrate to 25˚C. Computer controlled injections of a CLEC-2 peptide were performed and the power required for a reference cell to match the temperature in the experimental cell was plotted (upper panel), and integrated to calculated power per mole of peptide (lower panel). Origin Lab ITC software was used to fit the data to various binding models. The graph is representative of 2 experiments. c) The purified tSH2 domains of Syk were placed into a quartz cuvette in a PTI (Photon Technology International) spectrofluorimeter. Following excitation at 295 nm, emission at 340 nm was plotted during titration of a CLEC-2 peptide. Non-linear regression was used to analyse the data and calculate K_D values. The graph is representative of 3 experiments.

3.2.5 GPVI requires both FcRγ YxxL sequences to signal

The above observations that CLEC-2 is able to signal with only a single YxxL by forming a dimer, raises the question as to whether an ITAM receptor, containing tandem YxxL sequences, is able to activate Syk through cross-linking adjacent phosphorylated YxxLs or if binding to a doubly phosphorylated ITAM is essential for activation. This question was addressed through the generation of single and double point mutations (Y-F) of the conserved ITAM tyrosines in FcRγ which associates with GPVI. This work was performed in collaboration with Dr. Jun Mori. An N-terminal Myc-tagged version of FcRγ, which supports a similar level of signalling to that of the wild type protein (Mori *et al*, 2008), was used in these studies to enable measurement of the level of surface expression. As shown in Figure 3.9 a, mutation of the C-terminal ITAM tyrosine, or mutation of both ITAM tyrosines, abolished NFAT activation by collagen in DT40 cells transfected with GPVI and mutant FcRγ, whereas mutation of the N-terminal ITAM tyrosine alone suppressed the response by more than 95%. Expression of the various FcRγ mutants did not significantly affect the surface expression of GPVI as determined by flow cytometry using an α-GPVI antibody (Figure 3.9 b). Thus, these results demonstrate that both ITAM tyrosines are required for robust signalling by the GPVI-FcRγ complex and that FcRγ is unable to function efficiently through phosphorylation of a single YxxL.

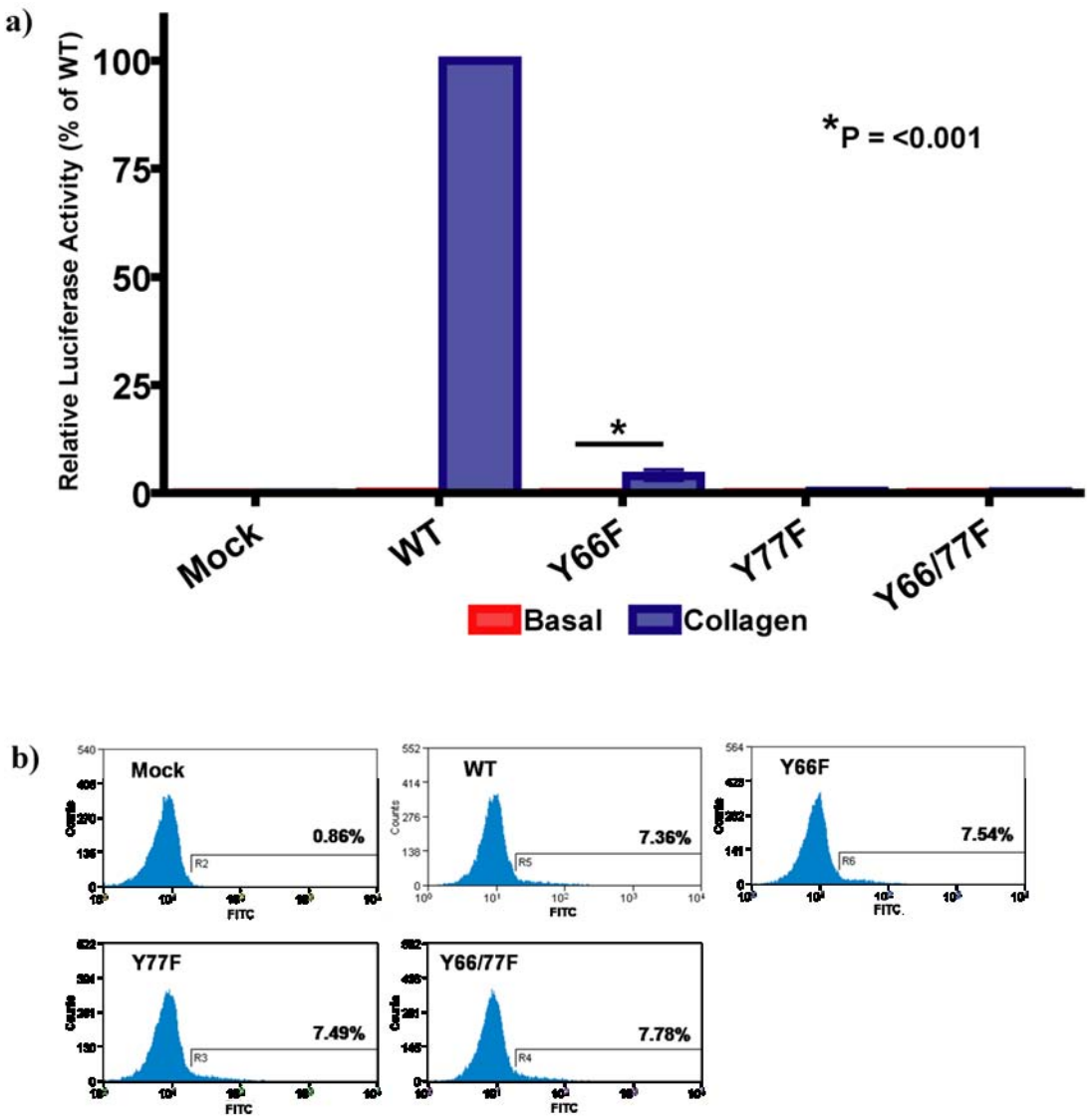

Figure 3.9 – GPVI signalling requires both ITAM tyrosines. a) DT40 cells were transfected with 2 μg/ml of stated FcRγ construct, 2 μg/ml of GPVI, along with a NFAT-luciferase reporter gene. Transfected cells were then stimulated with 10 μg/ml collagen for 6 hrs at 37˚C after which time, the amount of luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the WT response. Error bars represent the geometric mean ± standard error of three separate experiments. b) Transfected cells were analysed by flow cytometry for surface expression of GPVI. The selected gate was drawn to exclude ~99% of mock transfected cells. GPVI expression is similar when co-transfected with the various FcRγ point mutants.

3.3 Discussion

In this chapter I have considered two models for regulation of Syk by CLEC-2 via a single YxxL sequence, bearing in mind that it has previously been reported that activation requires the phosphotyrosine binding activity of both of the Syk SH2 domains (Fuller *et al*, 2007). The first model proposes the presence of a second (either direct or indirect) binding site for one of the Syk SH2 domains in the cytoplasmic tail of CLEC-2. This possibility was tested by mutational analysis of conserved residues using peptide pull-down assays and cell line reporter assays. Sequence alignment of multiple species of CLEC-2, and of the related family member Dectin-1, highlighted conservation of serines at position 21 and 27 (numbering from human CLEC-2). However, mutational analysis of these two residues failed to provide evidence for a functional role in mediating the association with Syk or for activation of NFAT activity in DT40 cells, which is critically dependent on engagement of Syk and PLCγ2 (Fuller *et al*, 2007). Likewise, the partially conserved threonine at the Y+2 position in the CLEC-2 YxxL sequence was shown to be dispensable for Syk association and NFAT activation. In fact, a short 13aa peptide was found to be sufficient to mediate the association with Syk. However, it may be possible that these residues are conserved throughout the family for some other function that is not required for the signalling pathway involving Syk.

The second model is through dimerisation, with CLEC-2 interacting with the tandem SH2 domains of Syk via phosphotyrosines found in adjacent CLEC-2 molecules (Figure 3.10). In support of this model, co-expression of functionally inactive Y7F CLEC-2

with the wild type receptor was found to inhibit signalling, with the response being restored by titrating down the amount of the Y7F mutant. Importantly, this effect of the Y7F mutant was not due to altered surface expression of the wild type receptor. This suggests that WT CLEC-2 is unable to signal when present as a complex with the Y7F mutant. This, taken with the dependence of both SH2 domains of Syk and the conserved tyrosine in the single YxxL of CLEC-2 (Fuller *et al*, 2007), suggests that the minimum signalling unit is a CLEC-2 dimer, recruiting a single molecule of Syk.

It is also interesting to consider that for Syk to be able to cross-link two CLEC-2 molecules, the linker region between the tandem SH2 domains would require a degree of flexibility. Various structural studies have shown that this linker region (interdomain A) interacts with the kinase domain helping to regulate its activity and that in Syk it is more flexible than in Zap-70 (Futterer *et al*, 1998; Zeitlmann *et al*, 1998; Grucza *et al*, 1999; Brdicka *et al*, 2005; Deindl *et al*, 2007). Therefore we can hypothesise that CLEC-2 signalling would be much less efficient through Zap-70 as it would be less likely to cross-link two CLEC-2 molecules and interact with the kinase domain correctly.

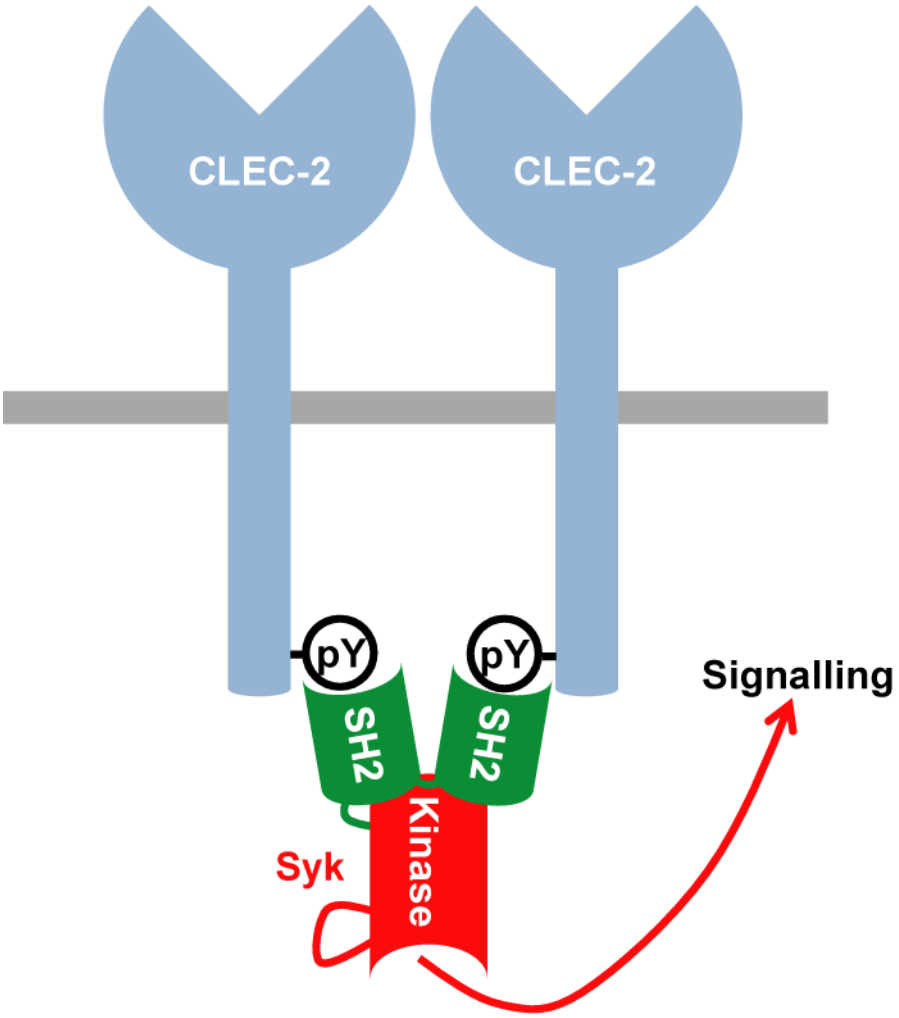

Figure 3.10 – Proposed model for Syk cross-linking a CLEC-2 dimer.

In further support of this model, cross-linking studies revealed the presence of dimeric and higher ordered complexes of CLEC-2. Moreover, the presence of these structures under basal conditions suggests that ligand binding is not required for the formation of CLEC-2 clusters. However, following rhodocytin stimulation, there was a shift towards higher molecular weight structures and a further lowering of the level of the monomeric form, suggesting that binding to rhodocytin induces or stabilises the formation of these higher, oligomeric forms. This is not surprising given the observation that rhodocytin is tetrameric and therefore potentially able to cluster up to eight molecules of CLEC-2 (Watson *et al*, 2008).

Using both SPR and tryptophan fluorescence we were able to measure the affinity of the interaction between the SH2 domains and CLEC-2 peptides. SPR showed an increase in affinity for the tSH2 domains compared to the single SH2 domains, thereby suggesting cooperative binding under the flow conditions of the assay. A similar result was found with an FcRγ peptide which is a known binding partner for the Syk tandem SH2 domains. This result was backed up with quantitative western blotting which showed that twice the amount of a CLEC-2 peptide was able to associate with the tSH2 protein compared to the single SH2 domains, giving further evidence that both of the SH2 domains are able to bind to a single CLEC-2 peptide. Tryptophan fluorescence was used to obtain a second measurement of the affinity of the interaction with the tandem SH2 domain peptide. The approximate one order of magnitude lower affinity is consistent with the affinity measured using SPR for binding to the individual Syk SH2 domains, reflecting the absence of cooperativity as the study was performed in solution

rather than on a monolayer. ITC measurements were unable to provide suitable data to directly measure the stoichiometry of the interaction.

This family of proteins may present a novel modification of ITAM signalling, which uses phosphorylation of the two conserved tyrosines in an ITAM sequence for optimal signalling. Given the dimerisation model, it is of interest to consider why FcRγ does not function in this way as shown in the NFAT studies by the abolition/dramatic inhibition of response following mutation of the individual FcRγ YxxL sequences. One possible explanation is that FcRγ, which is a covalent dimer, is expressed in a conformation that does not favour cross-linking of YxxL groups on separate chains, possibly to dampen or prevent signalling in the absence of agonist binding. It is also possible that it is a difference in agonists as rhodocytin is potentially able to cluster up to eight CLEC-2 molecules whereas, although collagen is able to cluster GPVI molecules, due to the spacing of the GPO repeats it may not bring the molecules into close enough proximity for the FcRγ molecules to be cross-linked by Syk. It may be that CRP would be able to induce a signal in the single point mutant of FcRγ by inducing tighter clustering of the receptor and being sufficient to initiate signalling through Syk.

On the other hand, the presence of two YxxL groups on the same chain facilitates binding to proteins with tandem SH2 domains without a need for receptor dimerisation/oligomerisation. This is illustrated by comparison of the K_Ds derived for binding of single and tandem SH2 domains of Syk to immobilised tyrosine

phosphorylated peptides in the surface plasmon resonance experiments described above. Dual YxxL groups that form an ITAM are found in separate exons (http://www.ensembl.org) suggesting that ITAM-like receptors preceded ITAM receptors and that the latter arose through gene duplication. The greater number of ITAM receptors over ITAM-like receptors may reflect an evolutionary advantage in having two YxxL groups on the same protein, although this may also reflect the fact that the majority of ITAM receptors are composed of multiple chains which may hamper dimerisation/oligomerisation.

CHAPTER 4

DIFFERENTIAL ROLES FOR THE ADAPTERS GADS AND LAT IN PLATELET ACTIVATION BY GPVI AND CLEC-2

4.1 Introduction

Although there are many parallels between the CLEC-2 and GPVI signalling pathways, one of the distinguishing features is in regard to the role of the adaptor protein, SLP-76, in the activation of PLCγ2. GPVI has been shown to have an absolute requirement for SLP-76 in platelet activation (Clements *et al*, 1999; Gross *et al*, 1999; Judd *et al*, 2000), whereas the response to low but not to high concentrations of rhodocytin are inhibited in the absence of the adapter (Suzuki-Inoue *et al*, 2006). Furthermore, this differential requirement is also seen between CLEC-2 and GPVI in DT40 cells using an NFAT reporter assay that is driven by activation of PLCγ2 (Fuller *et al*, 2007). SLP-76 has also been shown to play an essential role in signalling by other ITAM receptors, including the T-cell antigen receptor (Fang *et al*, 1996; Motto *et al*, 1996; Wardenburg *et al*, 1996) thereby indicating that the partial role in signalling by CLEC-2 may be a distinguishing feature between ITAM and ITAM-like receptors. It is therefore important to consider whether other proteins that regulate SLP-76 have a differential role in signalling by these two classes of receptor.

SLP-76 has been shown to be constitutively associated with the adaptor protein Gads (Grb2 adaptor downstream of Shc, also known as Mona/Grap2/GrpL/Grf40) and to form a complex with the membrane adapter LAT in activated platelets and T-cells (Liu *et al*, 1999). Gads has a central SH3 domain, flanked by two SH2 domains. The constitutive association with SLP-76 is by virtue of an atypical interaction between the SH3 domain of Gads and an RxxK motif on SLP-76 which is of very high affinity (~3 nM) relative to other SH3 interactions which fall within the micromolar range (Liu *et al*,

2003; Seet *et al*, 2007). Phosphorylation of the membrane bound adaptor protein LAT at position 191 (and to a lesser extent 171) (Zhang *et al*, 2000; Paz *et al*, 2001; Perez-Villar *et al*, 2002) by a Syk family kinase, leads to recruitment of Gads via an SH2-phosphotyrosine interaction, thereby recruiting SLP-76 to the membrane. The complex of LAT-Gads-SLP-76 recruits PLCγ2 and several other proteins to the membrane, including Vav and Tec family kinases, forming a LAT signalosome that is integral for activation of PLCγ.

Gads is a member of the Grb2 (Growth factor receptor bound protein) adaptor family which comprises Gads, Grb2 and Grap (Grb2 related adaptor protein). All three have the same domain organisation and moreover Grb2 has also been shown to bind to both LAT, via tyrosines Y171, Y191 and Y226 (Zhang *et al*, 2000; Paz *et al*, 2001; Perez-Villar *et al*, 2002), and to SLP-76, through a classical PxxP-SH3 interaction with a lower affinity (~3 μM) relative to the interaction between Gads and SLP-76 (Seet *et al*, 2007). Significantly, Grb2 has been shown to substitute for Gads in reconstitution of B-cell receptor signalling in chicken DT40 cells (Ishiai *et al*, 2000). This is a very artificial system, however, in which the B-cell equivalent of SLP-76 (BLNK/SLP-65) is removed by homologous recombination and cells transfected with various mutated forms of LAT and SLP-76 along with Gads or Grb2. Thus it is unclear whether Grb2 is able to substitute for Gads in more physiological systems.

As mentioned above, the ITAM containing T-cell receptor (TCR, and therefore the pre-TCR) also uses the LAT-Gads-SLP-76 signalosome for activation of PLCγ1. Mice

deficient in SLP-76 do not express any mature T-cells due to a blockade of signalling through the pre-TCR, which is analogous to blockade of GPVI signalling (Clements *et al*, 1998; Pivniouk *et al*, 1998; Clements *et al*, 1999). Mice deficient in LAT also do not express mature T-cells, again due to a block in pre-TCR signalling (Zhang *et al*, 1999), whereas GPVI signalling is able to bypass the requirement to LAT at high agonist concentrations (Pasquet *et al*, 1999). Similarly there is a loss of PLCγ1 activation by the TCR in either SLP-76 or LAT deficient Jurkat cells, which is a commonly used immortalised T lymphocyte cell line (Finco *et al*, 1998; Yablonski *et al*, 1998). Interestingly, mice deficient in Gads do have a limited degree of pre-TCR signalling which results in the production of mature T-cells, albeit in reduced numbers (Yoder *et al*, 2001). Consistent with this, disruption of the Gads-SLP-76 interaction impairs TCR signalling in Jurkat cells by approximately 50% (Yablonski *et al*, 2001; Jordan *et al*, 2007), confirming that Gads plays a partial role in TCR signalling.

The role of Gads in platelets has not been studied in detail. It has been shown to associate with SLP-76 (Liu *et al*, 1999; Asazuma *et al*, 2000) and, more recently, to undergo tyrosine phosphorylation downstream of GPVI, although the significance of this is unclear (Garcia *et al*, 2006). The only functional study using Gads-deficient mice reported that Gads was not required for platelet α-granule secretion following stimulation with a single, high concentration (20-30 nM) of the GPVI specific snake toxin, convulxin, whereas LAT was essential for activation by these concentrations of the toxin (Judd *et al*, 2002). Further, mutation of the Gads binding site on SLP-76 has been shown to impair platelet α-granule secretion by more than 50% in response to a

low dose (5 nM) of convulxin (Abtahian *et al*, 2006). The role of Gads in signalling by CLEC-2 is unclear.

The adapter SLP-76 has also been shown to shown to play a critical role in the regulation of PLCγ2 by integrin $\alpha_{IIb}\beta_3$ and the GPIb-IX-V complex (Judd *et al*, 2000; Judd *et al*, 2002; Liu *et al*, 2005), whereas the role of Gads in signalling by these two receptors is not established. In contrast, the membrane adapter LAT has been reported to have a differential role in signalling by integrin $\alpha_{IIb}\beta_3$ and the GPIb-IX-V. The membrane adaptor is localised to lipids rafts on the platelet surface, which are enriched in many signalling proteins including several Src family kinases. The integrin $\alpha_{IIb}\beta_3$ is excluded from these domains and, consistent with this, has been shown to regulate PLCγ2 independent of LAT (Wonerow and Watson, 2001; Wonerow *et al*, 2002). In contrast, the membrane adapter has been shown to play a partial role in the regulation of PLCγ2 by GPIb-IX-V, which has been shown to be recruited to rafts in activated platelets (Wu *et al*, 2001; Jin *et al*, 2007).

The aim of this chapter is to compare the role of Gads and LAT in platelet activation by the major membrane glycoprotein receptors using mutant mice, with special emphasis on activation by GPVI and CLEC-2 given their differential dependence on SLP-76.

4.2 Results

4.2.1 Comparison of the role of Gads and LAT in platelet aggregation and secretion

The role of Gads and LAT in supporting platelet aggregation by the GPVI-specific agonist CRP was compared using mice deficient in the two proteins. Platelet aggregation was monitored in a Born-lumi aggregometer which measures light transmission through the platelet suspension: as the platelets become activated and form larger but less numerous aggregates, the amount of light transmission increases. WT platelets responded to a range of CRP concentrations (0.3-10 μg/ml) with a characteristic shape change response (as reflected an initial decrease in light transmission) followed by full aggregation (increase in light transmission) (Figure 4.1). All of these concentrations of CRP were able to elicit a full aggregation response; however, the lower ones had a more pronounced shape change because of increase in the time to onset of aggregation.

In the absence of LAT, the dose-response curve for platelet aggregation to CRP was shifted such that a supra-maximal concentration of CRP (10 μg/ml) in washed platelets only induced a sub-maximal aggregation response. In contrast, full aggregation was achieved a concentration of 1 μg/ml in litter matched WT controls. Further, the onset of aggregation was also delayed in response to 10 μg/ml of CRP, whereas there was a much greater delay and reduction in aggregation with 3 μg/ml of CRP, while 1 μg/ml was unable to induce aggregation (Figure 4.1 a). In contrast, the dose-response curve was only slightly shifted in the absence of Gads. A CRP concentration of 1 μg/ml

which was ineffective with LAT-deficient platelets was able to induce full aggregation in the absence of Gads, although there was a notable delay in the onset of aggregation (Figure 4.1 a). Further, Gads-deficient platelets responded very weakly to 0.3 μg/ml CRP and with a large delay in onset. On the other hand, aggregation induced by a low concentration of the GPCR agonist thrombin (0.03 U/ml), was not altered in the absence of either LAT or Gads (Figure 4.1 b).

The aggregation response to rhodocytin in WT platelets is distinct from that of GPVI in that it has a characteristic delay which decreases with increasing concentration over the range 3-30 nM. At a threefold lower concentration of 1 nM prolonged shape change is seen which is eventually followed by weak aggregation. In the absence of LAT, there was a loss of aggregation to 3 nM rhodocytin which could be overcome by increasing the concentration to 30 nM, thereby restoring the response to the same level as WT platelets (Figure 4.1 c). This result is in agreement with previously published work (Suzuki-Inoue *et al*, 2006). In contrast, in the absence of Gads the response to 3 nM rhodocytin was comparable to the WT response. At the lower concentration of 1 nM, shape change was further prolonged relative to litter matched controls and aggregation was not seen (Figure 4.1 c).

These results demonstrate that the aggregation defect in the absence of Gads is very minor in comparison to the defect seen in the absence of LAT. However, the two mice were on different strain backgrounds thereby raising the possibility that this could account for the difference. To investigate this, the $Gads^{-/-}$ mice were backcrossed for

nine generations from the Balb-c background on which they were supplied onto a C57Bl/6 background to match the $LAT^{-/-}$ mice. With the $Gads^{-/-}$ mice on the equivalent background to the $LAT^{-/-}$ mice we were able to assess the possible effect of the background on the extent of the $Gads^{-/-}$ defect. Using the same concentrations of both CRP and Rhodocytin as used above, we found no apparent difference in the severity of the defect in the absence of Gads (Figure 4.1 d). There was again, slightly delayed but full aggregation to 1 μg/ml CRP and a partial response to 0.3 μg/ml CRP. The response to 3 nM rhodocytin was comparable to the WT response, while the lower concentration of 1 nM was unable to induce aggregation. However, the WT response is slightly delayed compared to the response in the Balb-c mice. This small difference may be due to agonist batch differences, as these experiments were performed several months after the initial Balb-c background experiments.

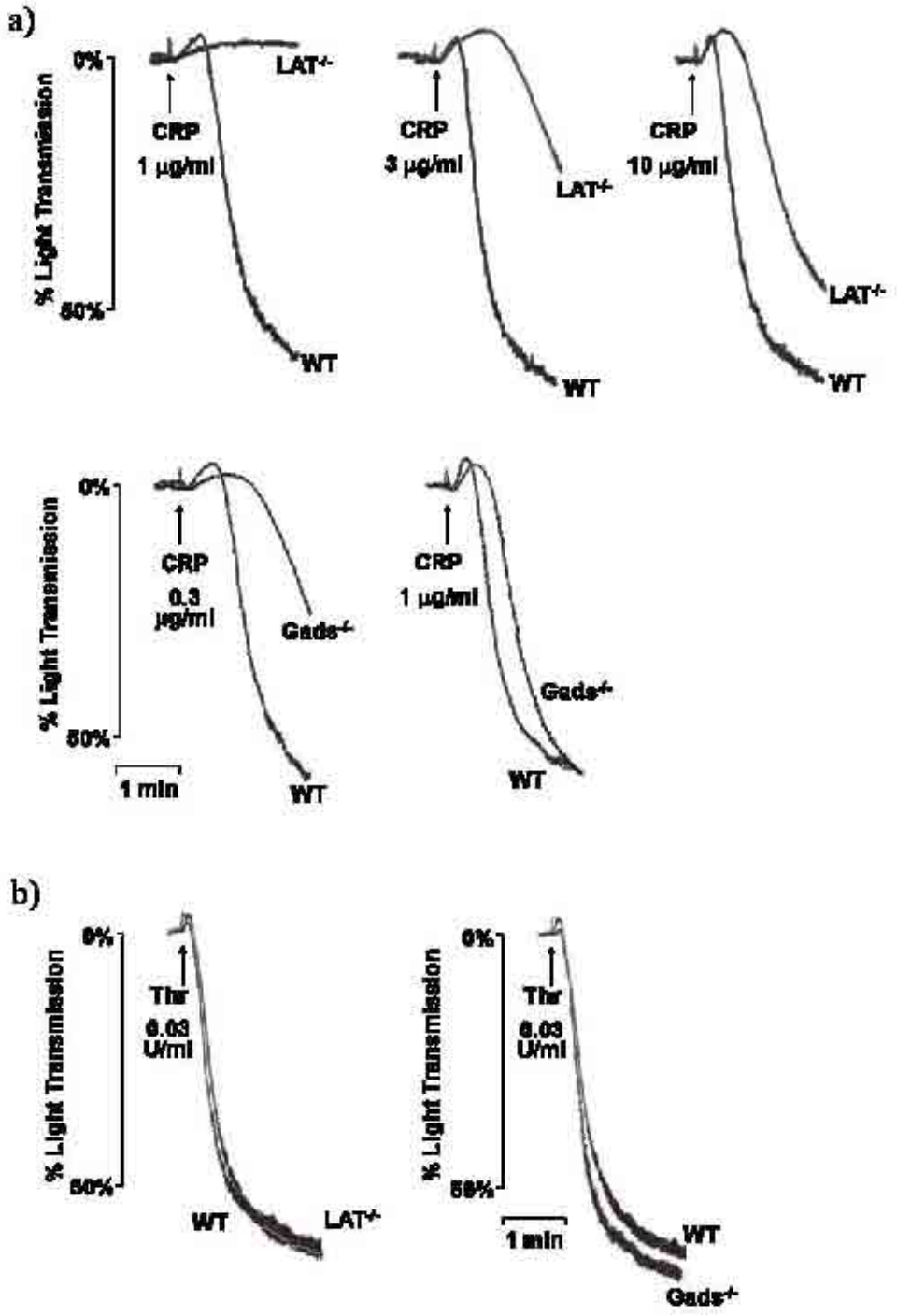

Figure 4.1 – Aggregation responses in Gads$^{-/-}$ and LAT$^{-/-}$ platelets.

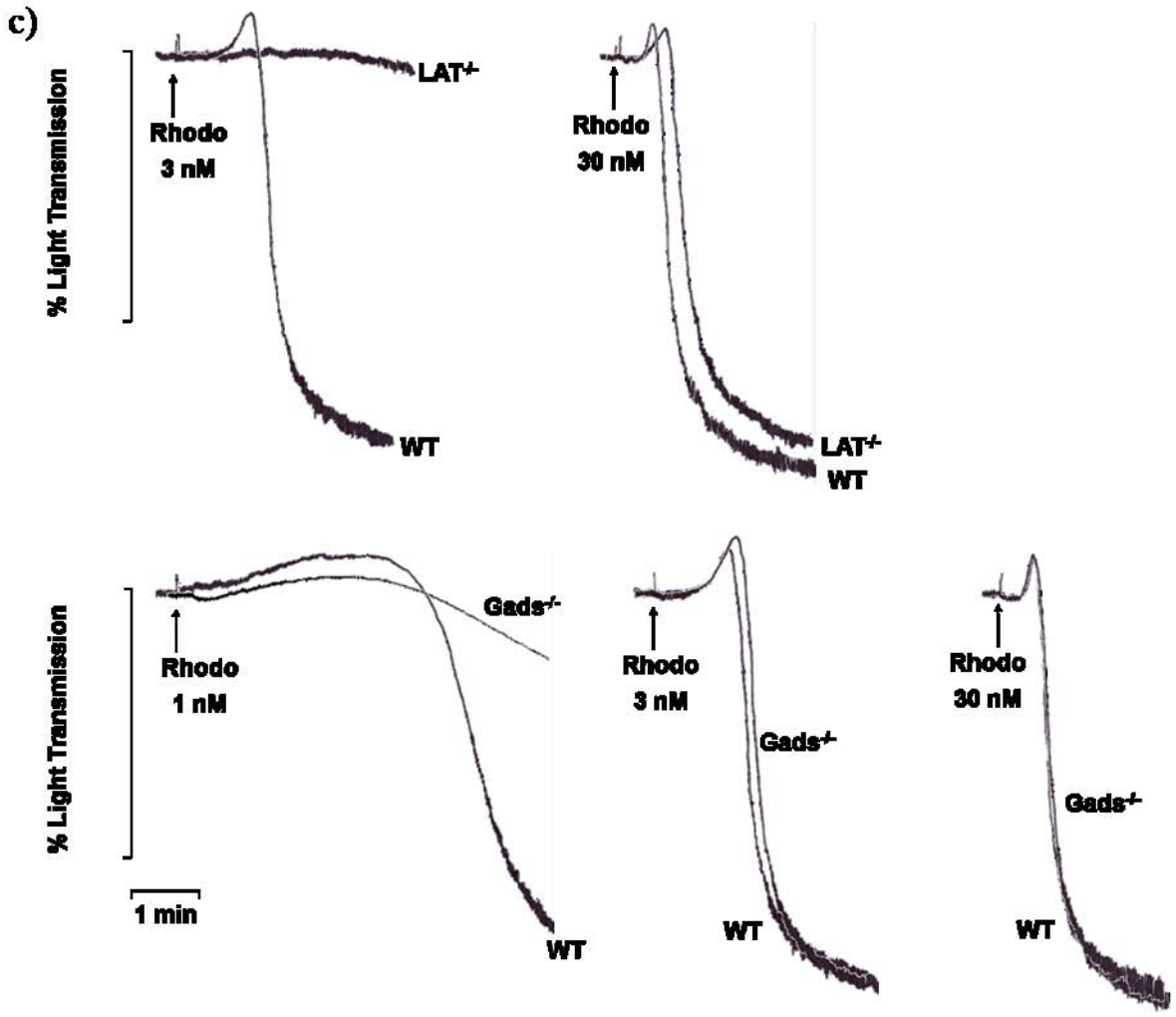

Figure 4.1 – continued. Washed platelets (2 x 10^8/ml) were stimulated in an aggregometer with CRP (a), thrombin (b) or rhodocytin (c) and allowed to aggregate. Percentage light transmission was calculated. (d) Washed platelets (2 x 10^8/ml) from Balb-c or backcrossed C57Bl/6 mice were stimulated in an aggregometer with CRP or rhodocytin and allowed to aggregate. Results are representative of between three and eight experiments.

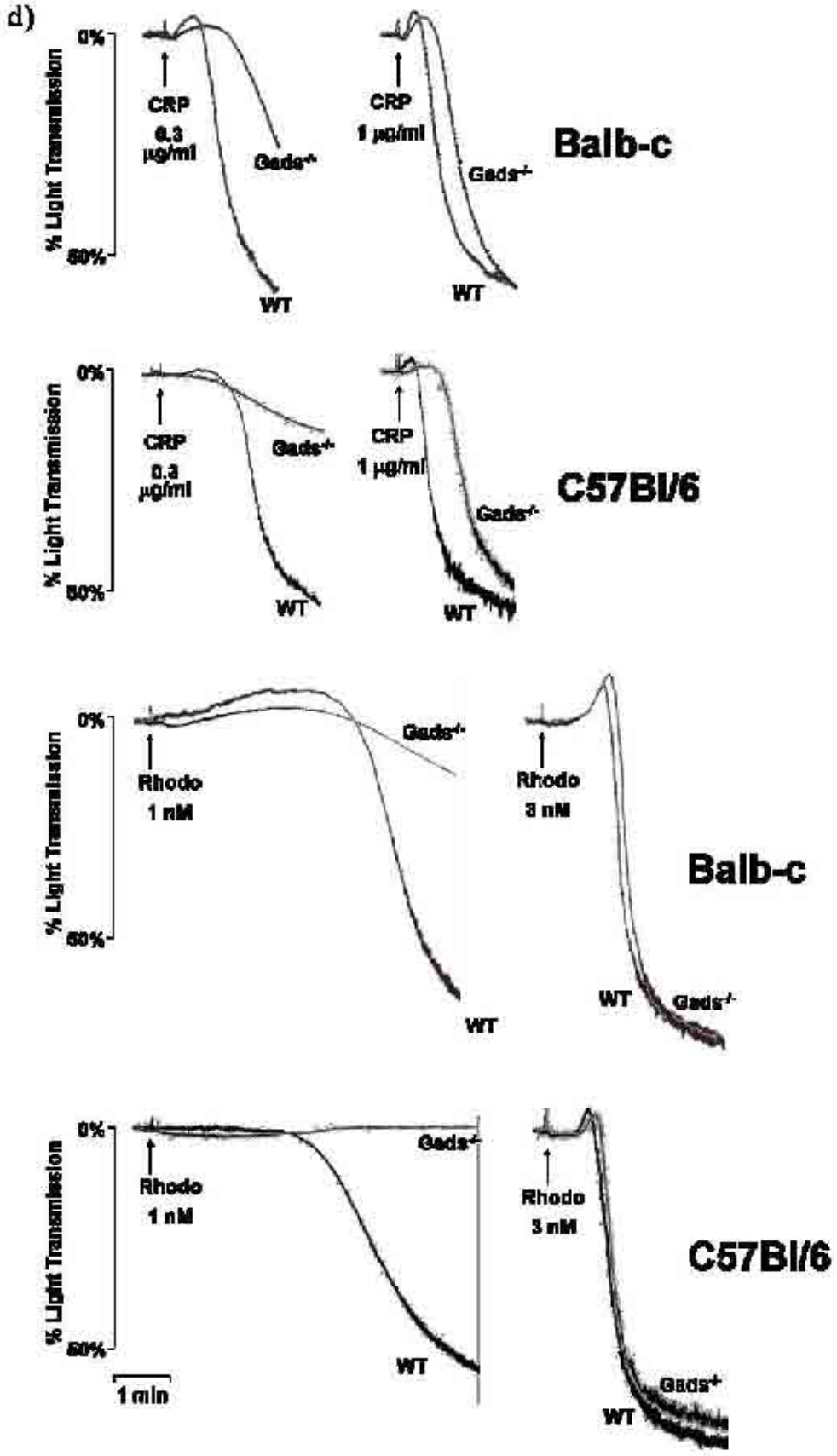

Figure 4.1 – continued

Dense granule secretion was monitored in real-time along with aggregation in a Born-lumi aggregometer. Secreted ATP was detected by virtue of its catalysis of a luciferin-luciferase light reaction. After the experiment was completed, the addition of a known amount of ATP enabled quantification. Both CRP and rhodocytin stimulate secretion of ATP in wild type platelets in an agonist-dependent manner. In both cases, there is a delay in secretion which coincides with the end of the shape change response (Figure 4.2).

Similar to aggregation, secretion was abolished in the absence of LAT in response to 1 μg/ml CRP but was partially restored at the higher concentration of 3 μg/ml. In comparison there was a marked delay and decrease in the rate of secretion in the absence of Gads in response to 1 μg/ml CRP. A lower concentration of 0.3 μg/ml shows a more pronounced defect in secretion (Figure 4.2 a). The overall pattern of the decrease in the Gads deficient mice is similar to that for aggregation although it appears to be more substantial.

There was also a reduction and delay in secretion induced by 1 nM rhodocytin in the absence of Gads, although there near full recovery at 3 nM. In comparison, the response to 3 nM rhodocytin was reduced by more than 90% in the absence of LAT, with partial recovery observed in response to 30 nM (Figure 4.2 b). These results therefore correspond to those for aggregation. In comparison, secretion to a low dose of thrombin was not altered in the absence of Gads or LAT (Figure 4.2 c). Secretion

experiments were performed on both backgrounds of $Gads^{-/-}$ mice but as with aggregation, not significant differences were seen (not shown).

These results demonstrate the relatively minor role of Gads in mediating platelet activation through both GPVI and CLEC-2, in contrast to the greater, yet dispensable role of LAT. Neither protein plays a significant role in platelet activation by thrombin.

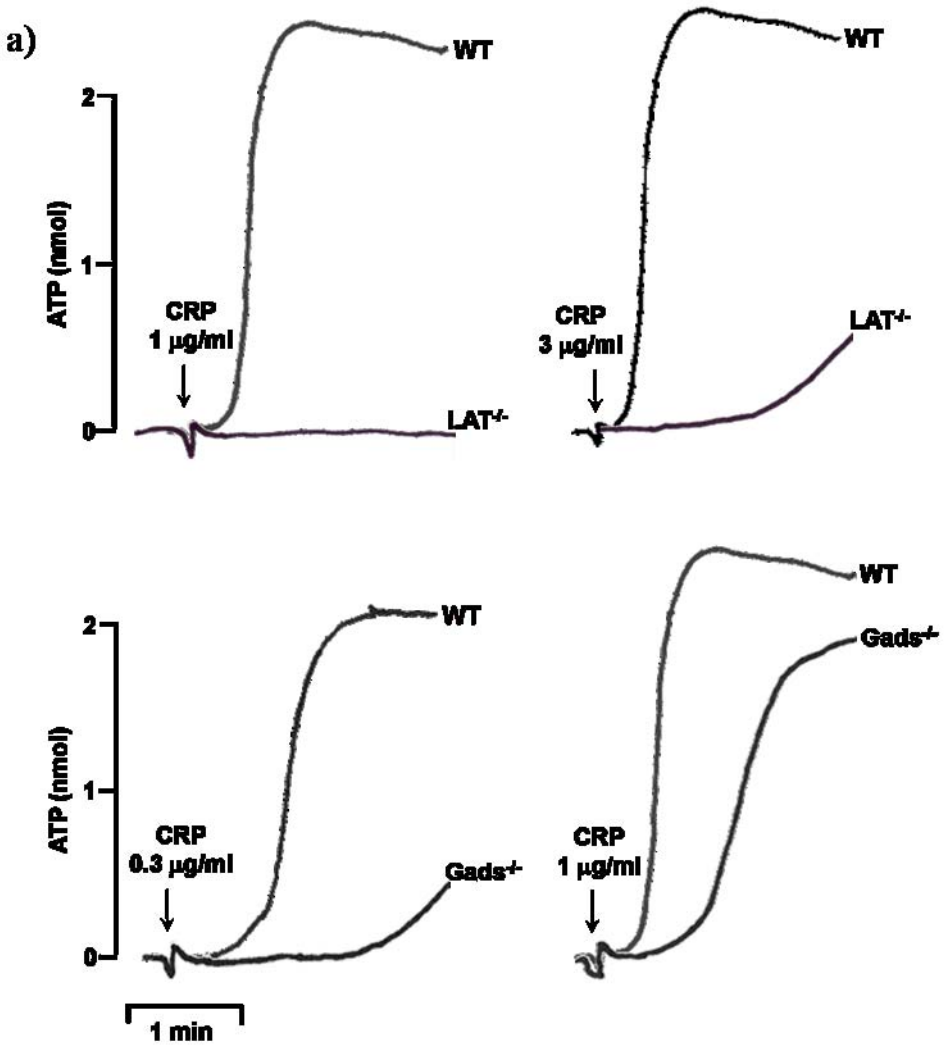

Figure 4.2 – ATP secretion in Gads$^{-/-}$ and LAT$^{-/-}$ platelets. Washed platelets (2 x 10^8/ml) were stimulated in an aggregometer with CRP (a), rhodocytin (b) or thrombin (c) and allowed to aggregate. ATP secretion was measured using light emission from luciferin/luciferase. Results are representative of between three and eight experiments.

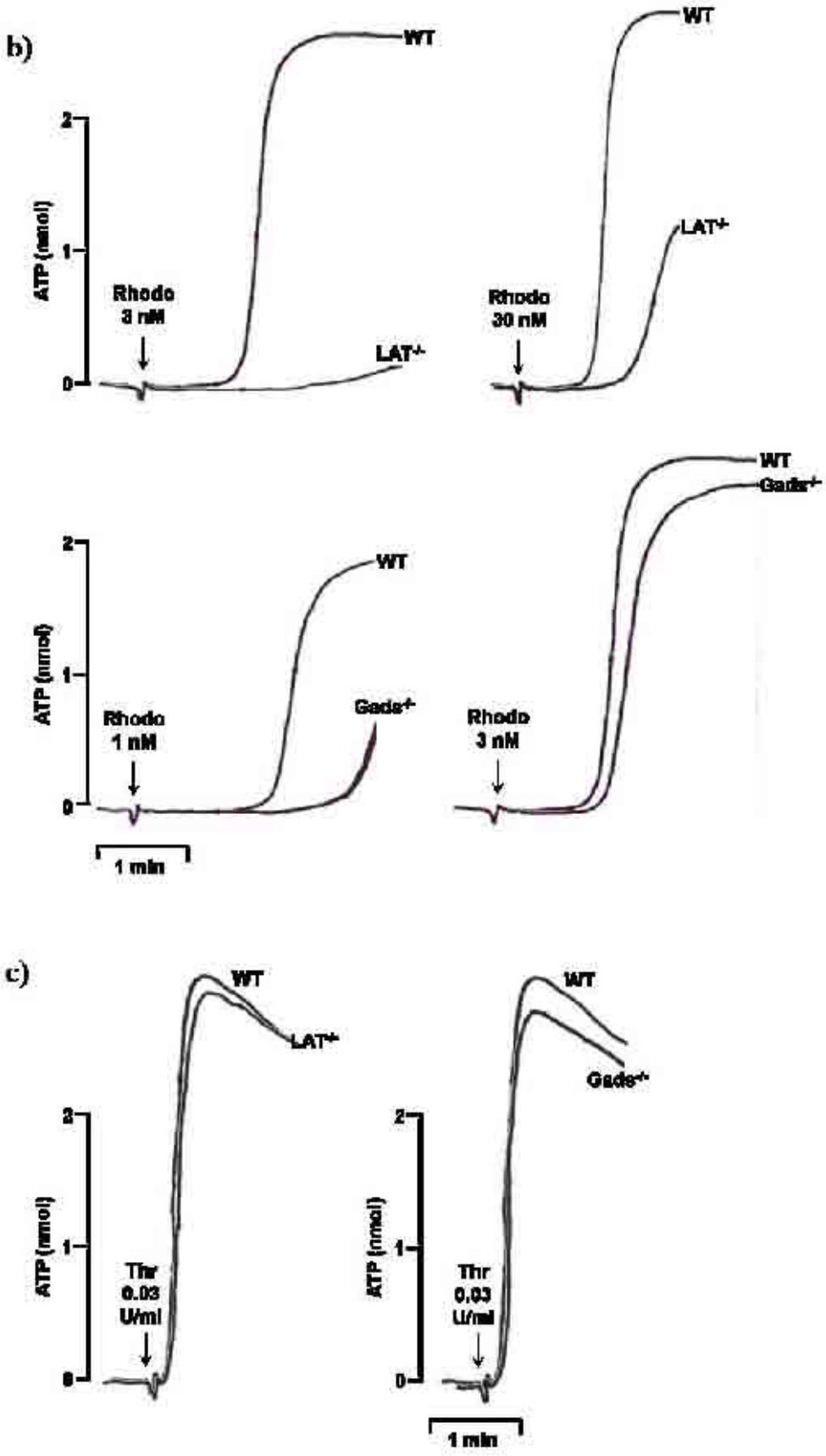

Figure 4.2 – continued

4.2.2 Platelet aggregation on collagen at arteriolar rates of flow

Platelet aggregometry takes place in a low shear environment in contrast to the high shear forces that exist within arterioles. Thus, it is possible that Gads and LAT may have a more significant role under flow conditions, where the speed of platelet integrin activation may be rate limiting in mediating platelet stable adhesion and aggregate growth. To address this, I monitored aggregate formation in heparinised whole blood over a collagen-coated surface at an intermediate arteriolar flow rate of 1000 s^{-1}. This assay allows monitoring of aggregate formation in real time using a monolayer of collagen which has been described as a highly thrombogenic component of the extracellular matrix.

When blood from WT mice was flowed at 1000 s^{-1} over the collagen surface, there was a rapid formation of a platelet monolayer followed by aggregate formation due to the capture and activation of further platelets. Fluorescent and DIC endpoint images show these large, elongated 3-dimensional aggregates present in abundance at 4 min (Figure 4.3). There was no detectable difference in the initial stages of adhesion and platelet aggregate growth using blood from $Gads^{-/-}$ mice compared to the WT (Figure 4.3 and not shown). In comparison, $LAT^{-/-}$ platelets were only able to form a monolayer on the collagen surface (Figure 4.3). This strongly suggests that the level of signalling through GPVI following adherence to collagen is sufficient to bypass the small defect caused by the absence of Gads but not the larger defect caused by the absence of LAT.

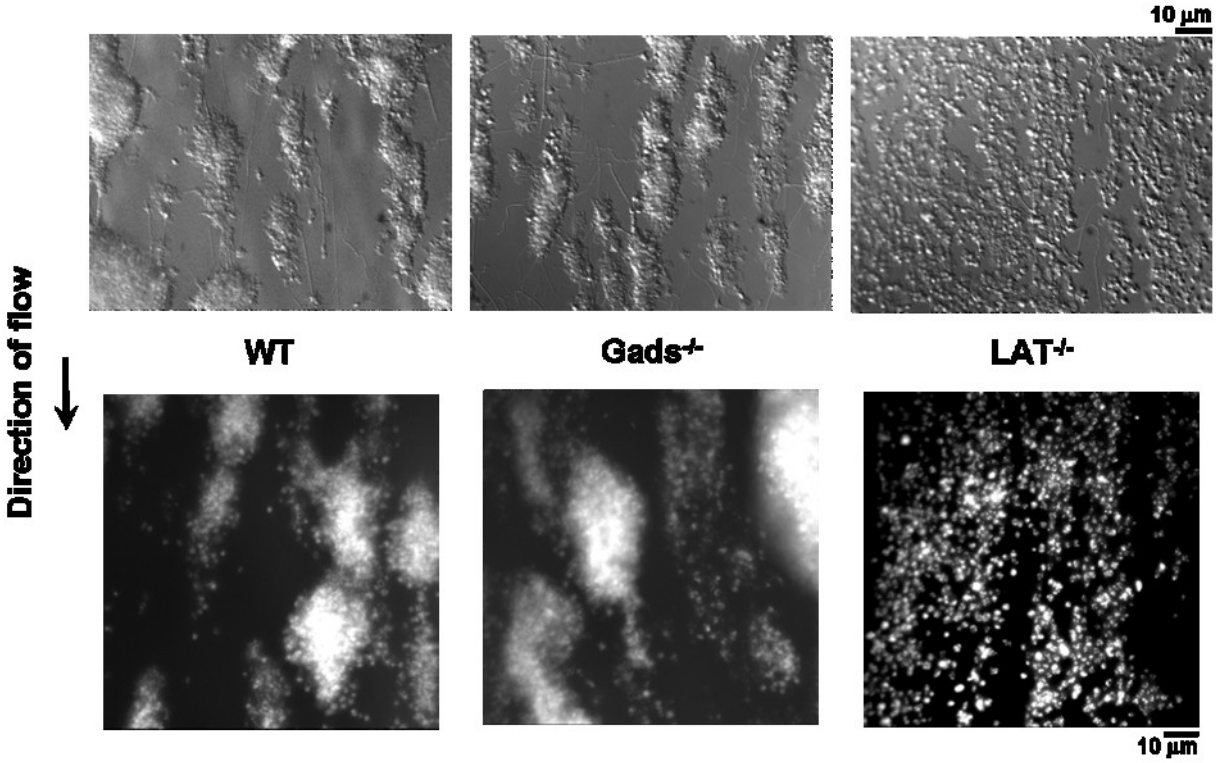

Figure 4.3 – Adhesion of Gads$^{-/-}$ and LAT$^{-/-}$ platelets on collagen under flow conditions. Heparinised whole mouse blood was fluorescently labelled with $DiOC_6$ and passed through collagen-coated glass capillaries at an intermediate shear rate of 1000 s^{-1}. Platelets were imaged with a fluorescent microscope (lower) and subsequently fixed and imaged by differential interference contrast (DIC) microscopy (upper). Results are representative of five experiments.

4.2.3 Measurement of protein tyrosine phosphorylation

The above results suggest a critical role for LAT in platelet function downstream of GPVI and CLEC-2 in contrast to the much more minor role of Gads. To investigate the molecular basis of this, we measured tyrosine phosphorylation of key signalling proteins in the two receptor cascades, Syk, SLP-76, and PLCγ2. Several concentrations of both CRP (0-3 μg/ml) and rhodocytin (0-300 nM) were used to stimulate platelets for up to one and two minutes respectively, before lysis. The tyrosine phosphorylation of these proteins was measured following immunoprecipitation and western blotting. Both agonists stimulated a dose-dependent increase in tyrosine phosphorylation of all three proteins (Figure 4.4). In the absence of Gads there was a small but consistent reduction in CRP-induced tyrosine phosphorylation of Syk, SLP-76 and PLCγ2 in comparison to wild type platelets (Figure 4.4 a), consistent with a supporting role for Gads in mediating activation of PLCγ2 through the LAT signalosome. There was also a minor reduction in phosphorylation of SLP-76 and PLCγ2 in $Gads^{-/-}$ platelets in response to low concentrations of rhodocytin, whereas Syk phosphorylation appeared unaltered (Figure 4.4 b). In comparison, there was a marked inhibition of phosphorylation of all three proteins in the absence of LAT in response to both CRP and rhodocytin, as previously reported (Pasquet *et al*, 1999; Suzuki-Inoue *et al*, 2006). The reduction in Syk phosphorylation in platelets deficient in LAT and, in the case of GPVI, Gads, indicates a role for the LAT signalosome in maintaining Syk phosphorylation. These results correspond to those for aggregation and secretion, with minor and major roles for Gads and LAT, respectively, in regulating tyrosine phosphorylation of PLCγ2 downstream of GPVI and CLEC-2.

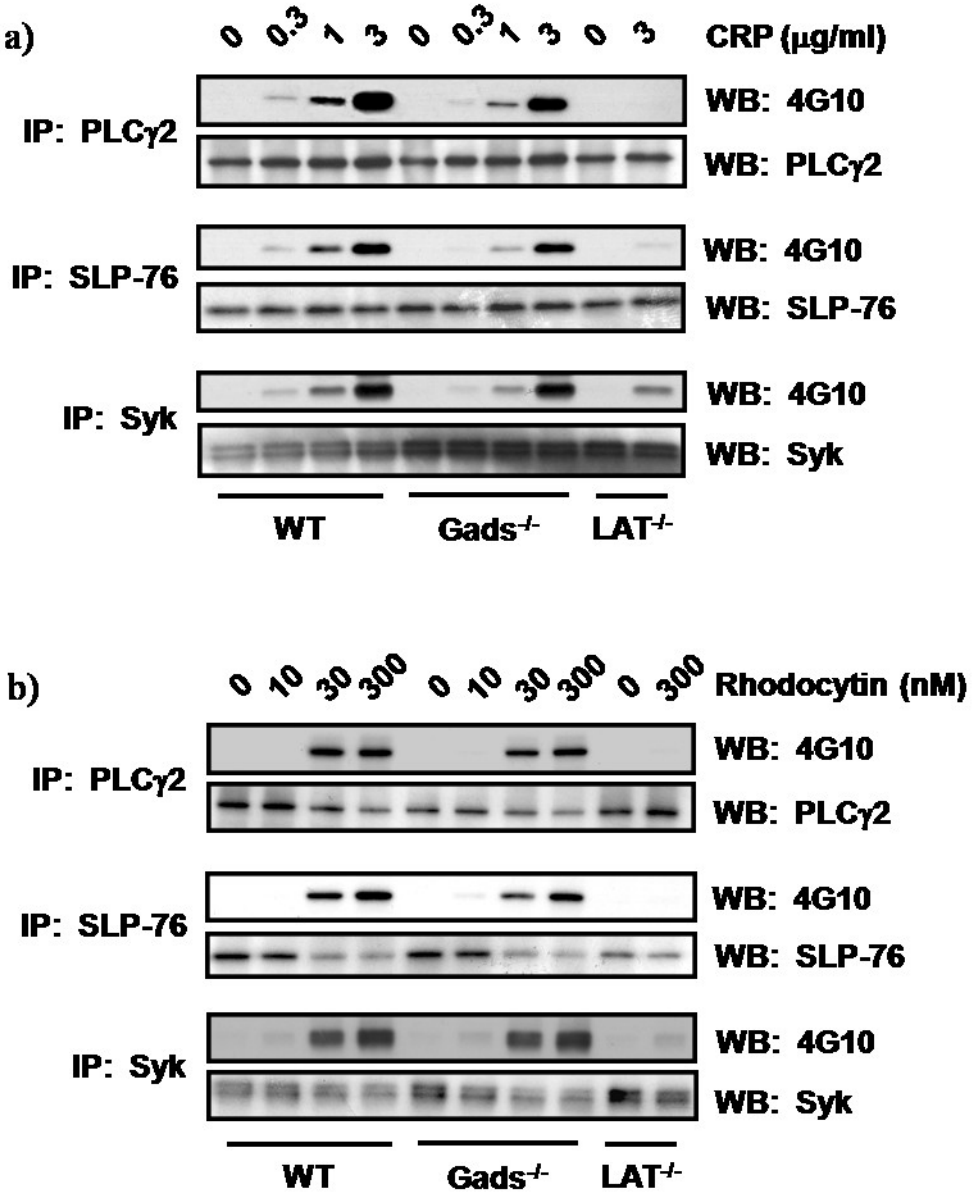

Figure 4.4 – Measurement of tyrosine phosphorylation in Gads$^{-/-}$ and LAT$^{-/-}$ platelets. Washed platelets (5x 10^8/ml) were stimulated with CRP for 60 sec (a) or rhodocytin for 120 sec (b) and subsequently lysed with NP-40 detergent. PLCγ2, SLP-76 and Syk were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for phosphotyrosine (4G10) and reprobed for PLCγ2, SLP-76 and Syk. Results are representative of between four and six experiments.

4.2.4 Grb2 association with the LAT-Gads-SLP-76 signalosome

The relatively minor role of Gads in mediating platelet activation by both CRP and rhodocytin raises the issue of whether there is a Gads-related protein that supports platelet activation downstream of GPVI and CLEC-2 through binding to LAT. Gads belongs to a family of three adaptors, of which only Gads and Grb2 are expressed in platelets. Grb2 has been shown to associate with both LAT and SLP-76 in T-cells and therefore has the potential to have a redundant role with Gads in platelets downstream of GPVI or CLEC-2. The data so far has shown no apparent difference in the contribution of Gads in signalling by GPVI and CLEC-2, with the adapter playing a very minor role in either pathway. Therefore, studies designed to compare the role of Gads and Grb2 focussed only on platelet activation by GPVI.

Gads and Grb2 were immunoprecipitated from basal and CRP (10 μg/ml) stimulated platelets and samples analysed for protein tyrosine phosphorylation (Figure 4.5). Tyrosine phosphorylated bands of 38 and 76 kDa, which co-migrate with LAT and SLP-76, respectively, were observed to immunoprecipitate with Gads, along with a band of 45 kDa that was detected after 90 seconds which co-migrates with a band that has previously been identified as Gads (Garcia *et al*, 2006). Two further unidentified tyrosine phosphorylated bands of 60 and 150 kDa were also present. In comparison, a major tyrosine phosphorylated band of 38 kDa that co-migrates with LAT is observed in the Grb2 immunoprecipitates, along with a weakly tyrosine phosphorylated band of 76 kDa that co-migrates with SLP-76. There is also a prominent tyrosine phosphorylated band of 125 kDa that has not been identified. Confirmation that the 38 and 76 kDa bands correspond to LAT and SLP-76 was achieved by immunoprecipitation of both

proteins and Western blotting (not shown), although it is possible that these bands contain additional proteins. These results indicate that Gads efficiently precipitates LAT and SLP-76 from platelets, which are presumably present as a complex. In comparison, Grb2 binds to other protein complexes, although the presence of a small amount of SLP-76 and a high level of LAT indicates that it may also form a complex with the two adapters.

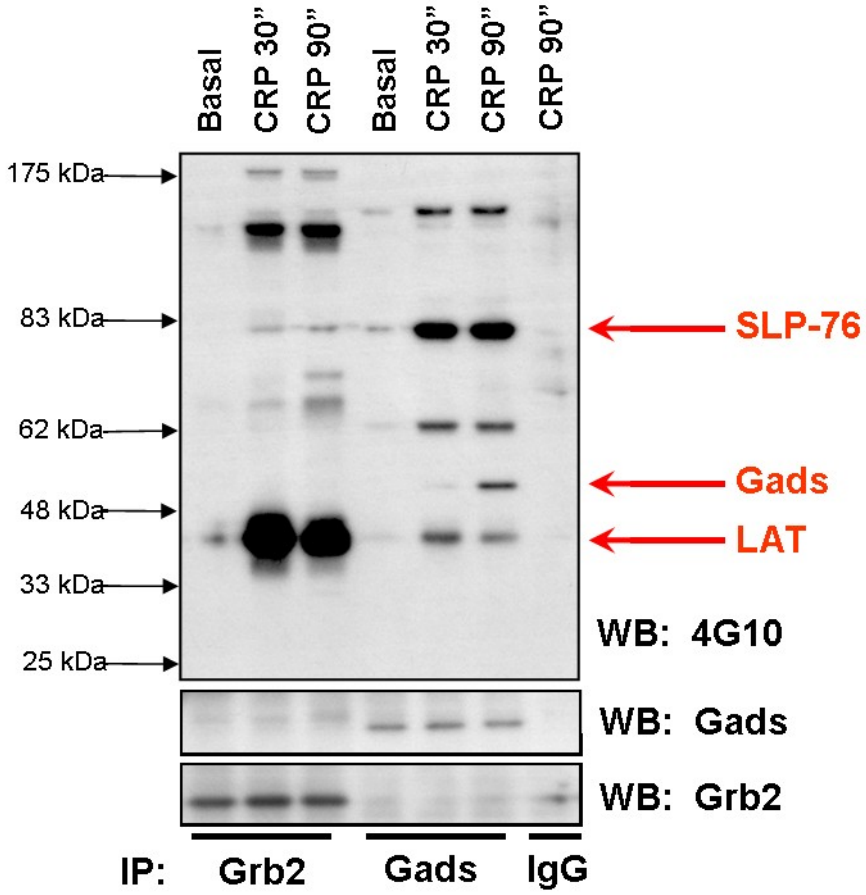

Figure 4.5 – Gads and Grb2 associate with LAT and SLP-76 in platelets. Washed platelets (5x 10^8/ml) were stimulated with 10 µg/ml CRP for 30 and 90 sec and subsequently lysed with NP-40 detergent. Gads and Grb2 were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for phosphotyrosine (4G10) and reprobed for Gads and Grb2. Results are representative of five experiments.

4.2.5 Spreading of platelets on collagen and other matrix proteins

As there is also a role for SLP-76 in signalling through $\alpha_{IIb}\beta_3$ and GPIb-IX-V, I also investigated a potential role for Gads and LAT in spreading of platelets on surfaces coated with fibrinogen which binds $\alpha_{IIb}\beta_3$ or VWF which can bind to both $\alpha_{IIb}\beta_3$ and GPIb-IX-V under the right conditions. Platelets were also spread on collagen coated surfaces as a comparison. As the assay is under static conditions, immobilised VWF was pre-treated with botrocetin to activate it. This pre-treatment confers specific binding to GPIb-IX-V but not to integrin $\alpha_{IIb}\beta_3$ which requires pre-treatment with ristocetin for specific binding (McCarty *et al*, 2006). Both botrocetin and ristocetin are snake venom toxins which act upon VWF causing conformational changes allowing binding to the above receptors. These changes are physiologically induced by the shear forces under flow. WT platelets adhere to the surface coated proteins and undergo spreading. Spreading occurs in stages, firstly with the formation of actin-rich finger-like protrusions (filopodia) followed by formation of actin-rich sheet-like structures (lamellipodia) which form between the filopodia. This is then followed by formation of stress fibres. This is referred to as full spreading, examples of which can be readily seen on collagen (Figure 4.6 a). In contrast to results for human platelets, WT mouse platelets generate only filopodia and limited lamellipodia on fibrinogen or VWF-botrocetin as illustrated in Figure 4.6 a and shown by others (Pearce *et al*, 2007; Calaminus *et al*, 2008).

In the absence of Gads or LAT there was no significant difference in the surface area or morphology of platelets spread on either fibrinogen or VWF-botrocetin compared to WT platelets after 45 minutes of spreading (Figure 4.6). This indicates that although

SLP-76 has been shown to be important in signalling downstream of $\alpha_{IIb}\beta_3$ and GPIb-IX-V, this role is independent of the LAT-Gads-SLP-76 signalosome. Surprisingly, there was no significant difference in the spreading of $LAT^{-/-}$ platelets on collagen compared to WT platelets, whereas there was a small but significant decrease in surface area in the absence of Gads (Figure 4.6 b). While this is consistent with a minor role for Gads in signalling by GPVI, the lack of effect in the absence of LAT argues against this interpretation. It is therefore possible that this reflects an unknown role for LAT in platelet development or in signalling by another receptor which contributes to platelet spreading.

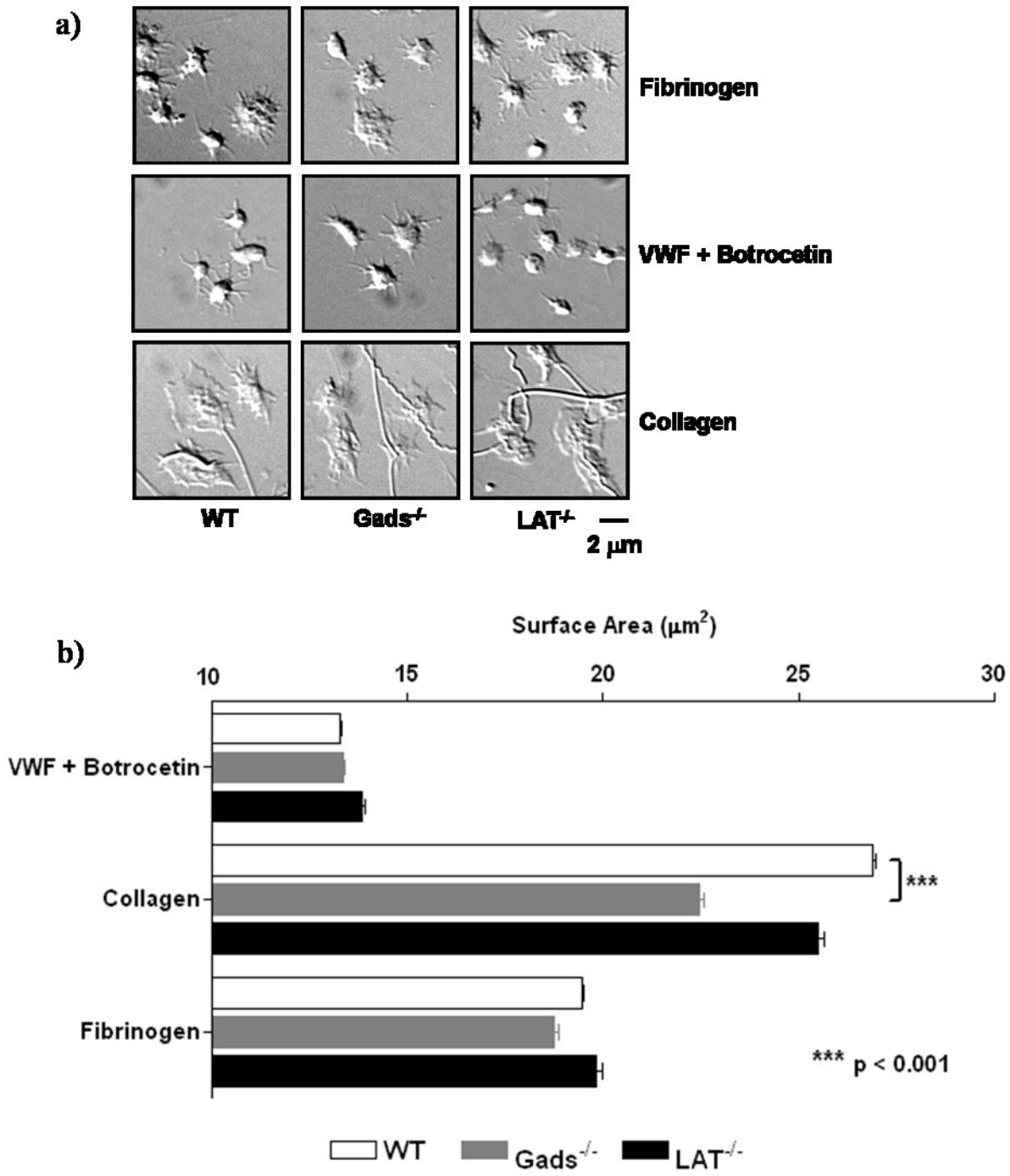

Figure 4.6 – Spreading of Gads$^{-/-}$ and LAT$^{-/-}$ platelets on matrix proteins. Washed platelets (2 x 10^7/ml) were allowed to spread on collagen, fibrinogen or botrocetin-treated VWF coated cover-slips for 45 min at 37˚C. Non-adherent platelets were subsequently washed away and adherent platelets were fixed and imaged by differential interference contrast (DIC) microscopy (a). Surface area of adherent platelets was calculated using ImageJ software (b). The results are representative of five fields of view from three mice ± 95% confidence interval. Statistical significance was calculated using a Student's *t*-test.

4.3 Discussion

The primary aim of the work in this Chapter was to compare the roles of Gads and LAT in the regulation of PLCγ2 by GPVI and CLEC-2 signalling in view of the differential role of SLP-76, which forms a signalosome with LAT and Gads, between the two receptors. SLP-76$^{-/-}$ mice have previously been shown to have a complete blockade of GPVI signalling and also lack any mature T-cells (Yablonski *et al*, 1998; Clements *et al*, 1999; Gross *et al*, 1999; Judd *et al*, 2000) whereas CLEC-2 signalling was able to overcome SLP-76-deficiency with sufficiently high agonist concentrations (Suzuki-Inoue *et al*, 2006). In agreement with previous studies, LAT was shown to have major role in GPVI-mediated aggregation and secretion, although high concentrations of CRP were able to induce aggregation and secretion in the absence of LAT. Furthermore, LAT$^{-/-}$ platelets formed a monolayer but were unable to support aggregation when flowed over collagen at an arteriolar rate of flow. This can be explained by the limited degree of platelet activation that occurs in the absence of LAT which is sufficient to support stable adhesion through integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, which bind to collagen and VWF, respectively, but being insufficient to mediate the robust secretion that is essential for capture of flowing platelets and aggregate growth (Auger *et al*, 2005; Sarratt *et al*, 2005). LAT also plays a critical role in aggregation and secretion induced by rhodocytin, although, as was the case for CRP, higher concentrations of rhodocytin are able to induce aggregation and secretion in the absence of LAT. In contrast, LAT was not required for adhesion or spreading induced by integrin $\alpha_{IIb}\beta_3$, GPIb-IX-V or collagen, which binds to both GPVI and integrin $\alpha_2\beta_1$. The lack of effect on collagen was surprising and suggests that LAT is either not essential or is not rate-limiting in the events that underlie aggregation and secretion. Because of the absence of a role in

signalling by collagen in this assay, it is not possible to draw conclusions surrounding the role of LAT in signalling by integrin $\alpha_{IIb}\beta_3$ and GPIb-IX-V.

In comparison, Gads was found to have a minor role in mediating platelet activation by both GPVI and CLEC-2, as illustrated by a slight delay in onset and reduction in the magnitude of aggregation and secretion to low but not intermediate or high concentrations of both CRP and rhodocytin. This minor effect did not translate into a defect in aggregate formation under flow on collagen suggesting that this effect has minor if any physiological significance. Gads is also not required for spreading induced by integrin $\alpha_{IIb}\beta_3$ or GPIb-IX-V although it was found to play a minor role in mediating spreading on collagen, although the molecular basis of this is unclear as no defect was observed in the absence of LAT as discussed above. We attribute the minor role of Gads in platelet activation by GPVI and CLEC-2 to supporting the recruitment of SLP-76 to the LAT signalosome, bearing in mind that it is able to efficiently precipitate both proteins in stimulated platelets. However, the relatively minor phenotype of the Gads-deficient platelets compared to that of platelets deficient in LAT or SLP-76 suggests the presence of one or more, more prominent pathways of regulation of SLP-76 by LAT. Furthermore, there must also be a LAT-independent pathway of regulation of SLP-76 in view of the fact that platelets deficient in this adapter protein have a more marked phenotype than those deficient in LAT, at least following activation by GPVI.

This minor role for Gads in supporting both GPVI and CLEC-2 signalling in platelets is in line with its more limited role in TCR signalling relative to that of LAT and SLP-76.

For example, mice deficient in Gads have a marked reduction in mature T-cells (Yoder *et al*, 2001) as a consequence of inhibited pre-TCR signalling, whereas mice deficient in LAT and SLP-76 have a blockade of pre-TCR signalling, resulting in no circulating T-cells. Further, mutation of the site of interaction of Gads with LAT reduces signalling through the T-cell receptor by approximately 50% (Zhang *et al*, 2000) in contrast to the full inhibition of T-cell signalling that is observed in the absence of LAT and SLP-76 (Finco *et al*, 1998; Yablonski *et al*, 1998). Thus, the relatively minor role of Gads in TCR, CLEC-2 and GPVI signalling in comparison with those of LAT and SLP-76, may be to facilitate the interaction of the two adapter proteins in response to threshold levels of receptor stimulation.

Platelets express the Gads-related adapter, Grb2, which also binds to LAT and SLP-76. This raises the possibility that a role for Gads could be masked by the presence of Grb2, especially in light of studies in a DT40 cell model reporting that Gads and Grb2 are both able to couple LAT and SLP-76, albeit Gads does this more efficiently (Ishiai *et al*, 2000). Indeed, this is in line with the present result which demonstrates that Grb2 associates strongly with LAT but only weakly with SLP-76, whereas Gads is constitutively associated with SLP-76 (not shown). The strong binding of Grb2 to LAT is explained by the presence of three sites for association of Grb2 with LAT at phosphotyrosines Y171, Y191 and Y226 (Zhang *et al*, 2000; Paz *et al*, 2001; Perez-Villar *et al*, 2002), compared with a single, major site for Gads at phosphotyrosine Y191, and to a lesser extent Y171 (Zhang *et al*, 2000; Paz *et al*, 2001; Perez-Villar *et al*, 2002). On the other hand, the increased binding of Gads to SLP-76 is explained by the very high affinity of the association between the C-terminal SH3 domain of Gads and a

RxxK motif on SLP-76 of 3 nM (Liu *et al*, 1999; Seet *et al*, 2007). This is an atypical interaction, as the majority of SH3 domains bind to proline rich sequences with micromolar affinity, including the C-terminal SH3 domain of Grb2 which binds to a proline rich region in SLP-76 with an affinity of 3 μM (Seet *et al*, 2007). Grb2 knock-out mice are embryonically lethal due to its ubiquitous expression. For future work it would be interesting if a platelet specific Grb2 knock-out could be generated as this could be studied in regards to GPVI and CLEC-2 signalling and also crossed with the $Gads^{-/-}$ mouse to make platelets deficient in both proteins. This would make it possible to address the possible redundancy of these two proteins.

Overall, these observations, along with previously published data, emphasise that SLP-76 and LAT are the key adapters in bringing PLCγ2 to the membrane, with SLP-76 being essential for tyrosine phosphorylation and activation of PLCγ2. In the absence of LAT, a limited degree of tyrosine phosphorylation of PLCγ2 occurs which is sufficient to enable recovery of aggregation to high concentrations of CRP in the absence of shear. On the other hand, platelet aggregation on collagen at arteriolar shear is abolished in the absence of LAT resulting in increased tail-bleeding and impaired thrombus formation *in vivo* (Kalia *et al*, 2008). Interestingly, a reduction in tyrosine phosphorylation of Syk was also observed in the absence of LAT in platelets stimulated by CRP and rhodocytin. This reduction is possibly due to increased accessibility of tyrosine residues in Syk to protein tyrosine phosphatases in the absence of LAT, or due to the lack of signals that recruit Src kinases to the signalosome.

In comparison to LAT and SLP-76, Gads has a relatively minor role in recruiting SLP-76 to LAT and activation of PLCγ2, and does not contribute to platelet aggregation at arteriolar shear. Indeed, it may be that the evolution of a role for Gads in mediating the interaction between LAT and SLP-76 has occurred because of its role in facilitating weak signalling by the pre-TCR to ensure an optimal number of mature T-cells in the circulation rather than to facilitate TCR or platelet activation at higher agonist concentrations. Consistent with this theory, Gads can be bypassed in a LAT signalosome as PLCγ can be recruited to LAT through a direct interaction between LAT Y132 and its N-terminal SH2 domain (Zhang *et al*, 1998; Aguado *et al*, 2002; Sommers *et al*, 2002).

CHAPTER 5

INVESTIGATION OF CANDIDATE LAT-LIKE MOLECULES

5.1 Introduction

The previous chapter investigated the requirement for Gads in GPVI and CLEC-2 signalling using mice deficient in Gads, an adaptor protein which couples SLP-76 to LAT. SLP-76 is essential for activation of phospholipase C by GPVI whereas, in contrast, CLEC-2 is able to bypass the requirement for the adapter at high agonist concentrations. The membrane adapter LAT also plays a key role in platelet activation by GPVI and CLEC-2, although in both instances, high agonist concentrations bypass the requirement for the membrane adapter.

LAT belongs to a family of transmembrane adaptor proteins as described in detail in Appendix I. The partial requirement for LAT in GPVI and CLEC-2 signalling raises the possibility that one or more LAT-like molecules are present in platelets and exhibit redundancy with LAT. Furthermore, this could also explain why SLP-76 but not LAT is required in $\alpha_{IIb}\beta_3$-mediated outside-in signalling, especially given that a mutation in SLP-76 which destroys its ability to bind to LAT inhibits spreading on fibrinogen (Abtahian *et al*, 2006). Signalling by GPVI and CLEC-2 takes place in lipid rafts (also known as glycosphingolipid-enriched microdomains or GEMs), where LAT is localised, whereas $\alpha_{IIb}\beta_3$ signalling takes place outside of these domains (Wonerow *et al*, 2002; Pollitt *et al*, 2010). This may therefore explain why LAT does not play a role in signalling by the integrin.

Although structurally unrelated to the LAT family of adapter proteins, several candidate membrane adapter proteins/novel cell surface receptors have been identified which contain one or more cytoplasmic tyrosines that undergo tyrosine phosphorylation and mediate binding to SH2 domain-containing proteins. It is therefore possible that one or more of these could also explain the partial or lack of role of LAT in mediating activation of platelets by surface glycoprotein receptors. One such candidate is G6f which is found in the multi-histocompatibility complex class III gene region along with several other proteins that are expressed in platelets including the novel ITIM receptor, G6b-B (Ribas *et al*, 2001). G6f has a predicted molecular weight of 36 kDa, similar to that of LAT, and consists of a glycosylated extracellular Ig domain, a transmembrane domain and a cytoplasmic tail containing a single tyrosine residue at position 281 in the human sequence. Expression of wild type G6f but not the Y281F mutant in K562 cells and treatment with the tyrosine phosphatase inhibitor vanadate leads to association with Grb2 and Grb7, therefore establishing tyrosine 281 as a docking site for SH2 domain-containing proteins (De Vet *et al*, 2003). Furthermore, cross-linking G6f through an N-terminal T7 tag using a T7 antibody resulted in Erk phosphorylation in K562 cells, presumably as a result of recruitment of Grb2 and activation of Ras (De Vet *et al*, 2003). Importantly, we have identified the presence of G6f in platelets by analysing tyrosine phosphorylated proteins in CRP-stimulated human platelets (Garcia *et al*, 2006). Potentially, therefore, tyrosine phosphorylation of G6f in platelets could account for some of the redundancy with LAT bearing in mind that both proteins bind to the adapter Grb2.

The aim of this chapter is to investigate the presence of the various LAT family proteins and G6f in platelets and to investigate whether they are phosphorylated downstream of GPVI and CLEC-2. Furthermore, through collaboration with Dr Burkhart Schraven in Magdeburg, Germany, the functional roles of the LAT-like proteins have been investigated using mice deficient in one or more of the transmembrane family of adapters.

5.2 Results

5.2.1 Raising a G6f antibody

To confirm whether G6f is expressed in platelets, a rabbit polyclonal antibody was raised against a peptide based on the cytoplasmic tail (amino acids 259-274) of human G6f (Figure 5.1 a). The antibody was raised in rabbits using a commercial company, Invitrogen, and the bleeds tested against human G6f expressed in 293T HEK (human kidney epithelial) cells alongside mock transfected cells. To achieve this, I cloned human G6f into a pEF6 expression vector (Invitrogen) with a C-terminal Myc tag attached using a two-round, or 'nested' PCR approach. The cDNA was obtained from K562 cells, which were used in the original cloning of G6f (De Vet *et al*, 2003).

G6f was transfected into 293T HEK cells and the expressed protein immunoprecipitated using an antibody against the Myc tag. The immunoprecipitate was then used for testing of serum from two rabbits immunised against G6f. This work revealed the presence of a clear band of approximately 45 kDa with the serum from all three bleeds of both rabbits (not shown) The final bleed from the second rabbit had the strongest response as shown in Figure 5.1 b. Importantly, this band was not present in the mock transfected control cell lysates or in the pre-immune rabbit serum (not shown). This band was successfully reprobed for the presence of Myc, confirming that the antibody recognises G6f (Figure 5.1 b). There was also a minor band of approximately 85 kDa which was not visible when reprobed with the Myc antibody (not shown) indicating that it is not a dimer of G6f.

a)

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Human   253 SIVLWRQR-VRGAPGRDASIPQFKPEIQVYENIHLARLGPPAHK-- 294
Mouse   255 SIVLWRRRRAQGSRDREPSVPHFKPEVQVYENIHLARLSGSIPKGW 300
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b)

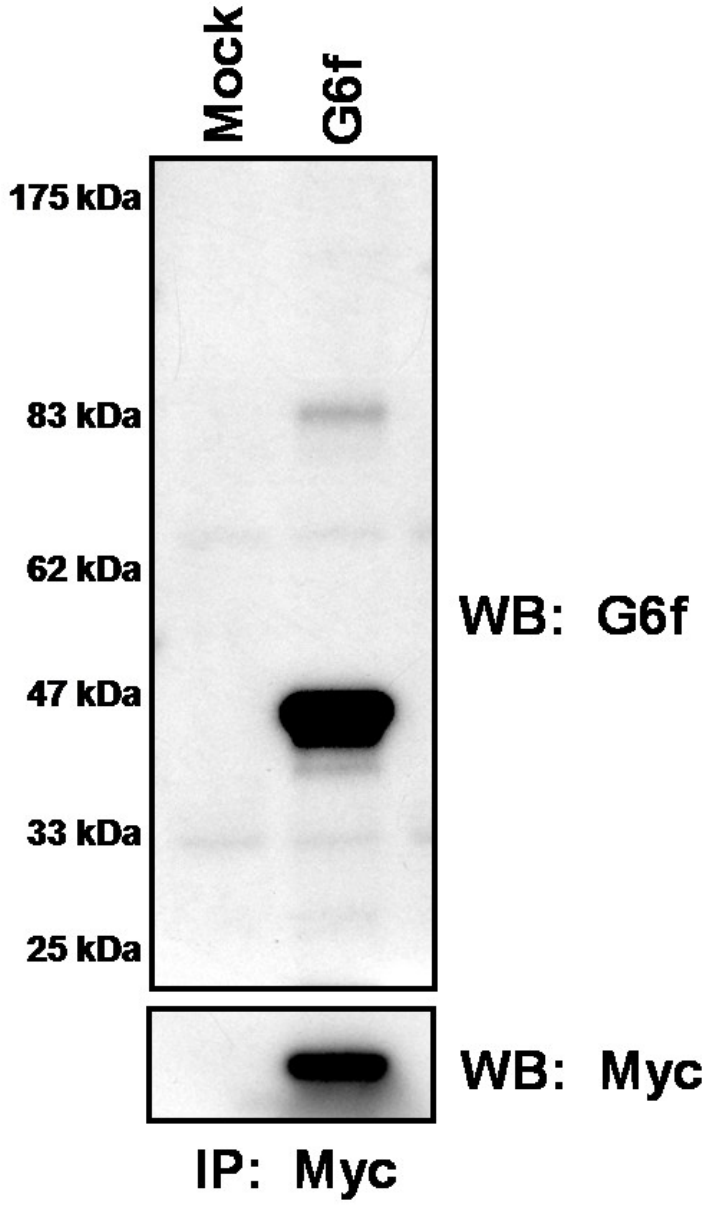

Figure 5.1 – A new antibody recognises transfected G6f. a) Human and mouse G6f were aligned using ClustalW web-based software highlighting the conserved YxN motif in bold, and the regions used as antigen are shown in red. b) 293T cells were transfected with G6f or an empty vector control and subsequently lysed with NP-40 detergent. Myc-tagged proteins were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting with the third bleed test serum and reprobed for Myc. Results are representative of three experiments. Similar results were seen with both first and second bleed serum samples, whereas the pre-immune serum was negative.

5.2.2 G6f is phosphorylated downstream of GPVI in platelets

G6f was identified by Dr Ángel García through analysis of tyrosine phosphorylated proteins in CRP-stimulated human platelets (Garcia *et al*, 2006). This approach identified several proteins that undergo tyrosine phosphorylation upon stimulation by CRP, including G6f. Confirmation of tyrosine phosphorylation of G6f was achieved using the novel antibody to the membrane adapter described above. Platelets were stimulated with CRP (10 μg/ml) for 30–300 sec and tyrosine phosphorylated proteins were immunoprecipitated using the monoclonal α-phosphotyrosine antibody, clone 4G10 and western blotted for G6f (Figure 5.2 a). A clear band for G6f was seen in the whole cell lysate lane, whereas there was a minor band in the immunoprecipitate from non-stimulated platelets. Following CRP stimulation, a marked increase in the amount of G6f was detected which peaked at 90 sec. These results confirm that G6f is present in platelets and strongly suggest that it undergoes tyrosine phosphorylation upon activation of GPVI. Interestingly, G6f runs as a smaller sized protein (~40 kDa) compared to over-expressed G6f in the HEK 293T cells, most likely due to differential glycosylation. Interestingly, a second band of approximately 70 kDa was also identified by western blotting using the novel antibody to G6f. This could represent a covalent dimer of the transmembrane protein or a non-specific band.

The G6f antibody was tested for its ability to immunoprecipitate G6f from platelets stimulated with CRP (10 μg/ml) in order to directly confirm that it undergoes tyrosine phosphorylation upon activation of the collagen receptor (Figure 5.2 b). Western blotting with the anti-phosphotyrosine antibody 4G10 revealed the presence of a major band of 40 kDa in CRP-stimulated platelets. Re-probing with the G6f serum identified

the same band thereby demonstrating that it is G6f. Several weakly phosphorylated bands were also observed on the 4G10 western blot, most notably at 50, 70 and 85 kDa, which are likely to represent co-precipitating proteins. In addition, G6f may interact with non-tyrosine phosphorylated proteins although these would not be seen using this experimental design.

The novel antibody was used to investigate the ability of other agonists to stimulate phosphorylation of G6f. These studies were performed in the absence or combined presence of apyrase and indomethacin in order to block the feedback effects of ADP and thromboxane A_2 and thereby establish whether phosphorylation was direct. Apyrase hydrolyses ATP and ADP to AMP and will therefore remove any ADP secreted by the activated platelets and indomethacin is a cycloxygenase (COX) 1 and 2 inhibitor which inhibits the production of thromboxanes from arachidonic acid in the platelet. CRP (10 μg/ml) stimulated robust phosphorylation of G6f which was not altered in the presence of apyrase and indomethacin (Figure 5.2 c). Collagen (10 μg/ml) also stimulated marked phosphorylation of G6f. In contrast, a high concentration of rhodocytin (300 nM) stimulated weak tyrosine phosphorylation of G6f which was completely blocked in the presence of apyrase and indomethacin suggesting that stimulation through CLEC-2 is critically dependent on the secondary mediators ADP and TxA_2. The G protein-coupled receptor agonist thrombin (1 U/ml) but not ADP (100 μM) also stimulated weak tyrosine phosphorylation of G6f. In addition, thrombin and rhodocytin stimulated weak tyrosine phosphorylation of a doublet of approximately 70 kDa which may be the same band as observed in CRP-stimulated platelets. A small increase in G6f phosphorylation was also seen when platelets were spread on a fibrinogen-coated

surface whereas there was no such increase on a BSA-coated surface (Figure 5.2 d). Reprobing the gel revealed the presence of a much higher amount of G6f in the fibrinogen-stimulated platelets. Despite this difference, the complete absence of a co-migrating tyrosine phosphorylated band in non-stimulated platelets supports the idea that integrin $\alpha_{IIb}\beta_3$ is able to induce weak phosphorylation of G6f.

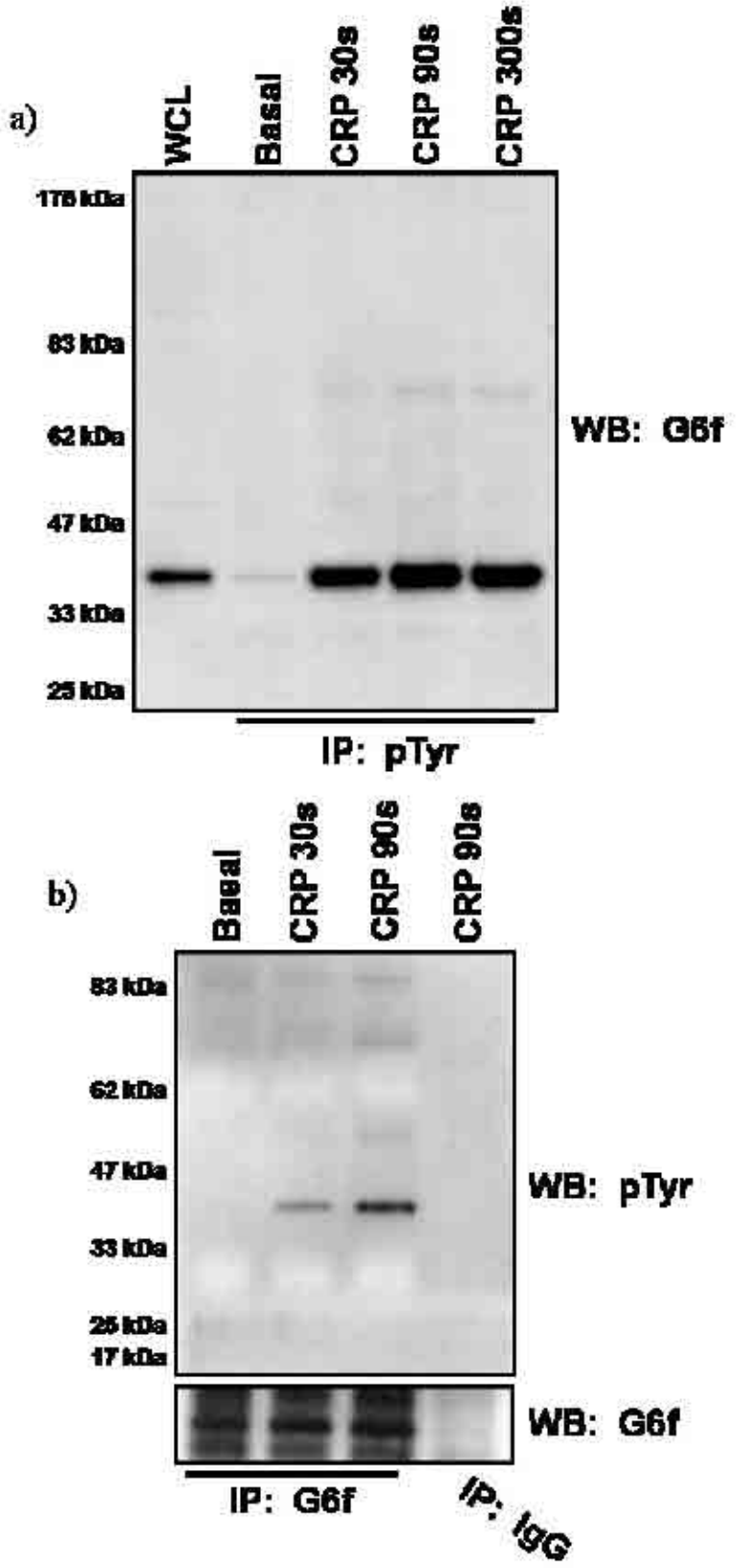

Figure 5.2 – G6f is undergoes tyrosine phosphorylation downstream of GPVI in platelets.

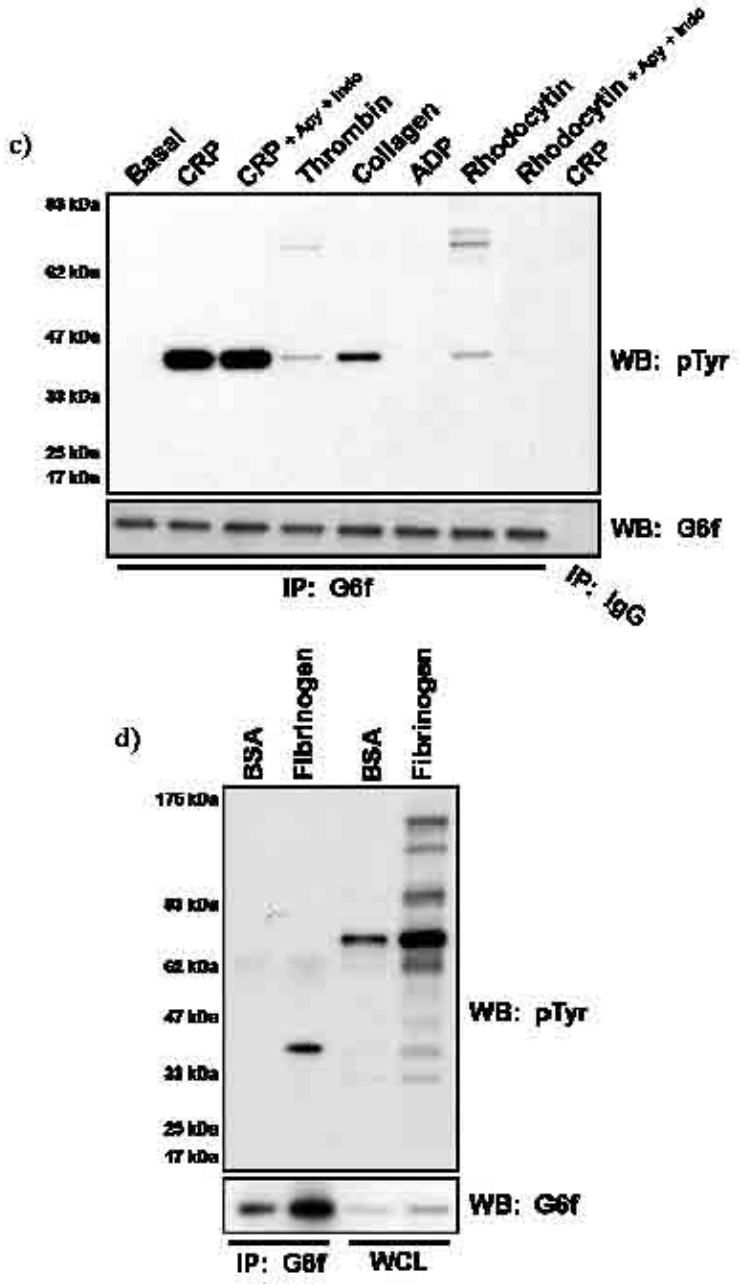

Figure 5.2 – continued. a) Washed platelets (5x 10^8/ml) were stimulated with 10 μg/ml CRP for up to 300 sec and subsequently lysed with NP-40 detergent. Tyrosine phosphorylated proteins were immunoprecipitated using 4G10. WCL = whole cell lysate. b) Washed platelets (5x 10^8/ml) were stimulated with 10 μg/ml CRP for up to 90 sec and subsequently lysed with NP-40 detergent. c) Washed platelets (5x 10^8/ml) were stimulated with 10 μg/ml CRP for 90 sec, 10 μg/ml collagen for 90 sec, 1 U/ml thrombin for 60 sec, 300 nM rhodocytin for 300 sec or 100 μM ADP for 60 sec, and subsequently lysed with NP-40 detergent. Apy = apyrase, Indo = indomethacin. d) Washed platelets (5x 10^8/ml) were spread on surfaces coated with 100 μg/ml fibrinogen or 5 mg/ml BSA for 45 mins at 37˚C. Adherent cells were lysed with NP-40 detergent. In b, c and d, G6f was immunoprecipitated using the newly raised antibody. Samples were all analysed by SDS-PAGE and western blotting for phosphotyrosine (4G10) and G6f. Results are representative of three experiments.

5.2.3 G6f is associated with Grb2 but not Gads

Grb2 was shown to associate with G6f in a phosphorylation dependent manner in K562 cells (De Vet *et al*, 2003). Co-immunoprecipitation studies where therefore performed to investigate whether Grb2 and the closely related adapter protein Gads associate with G6f in platelets. Platelets were stimulated with CRP (10 μg/ml) for 30 and 90 sec and lysed. Gads, Grb2 and G6f were immunoprecipitated from the lysates and the samples subjected to SDS-PAGE before western blotting for the presence of all three proteins (Figure 5.3). There was no detectable association between Gads with either Grb2 or G6f. Similarly, Grb2 was not detected following immunoprecipitation of G6f. On the other hand, G6f could be weakly detected in the Grb2 immunoprecipitate at 30 sec and robustly at 90 sec, confirming the phosphorylation-dependent association seen previously (De Vet *et al*, 2003). The absence of Grb2 in the G6f immunoprecipitate may be due to a steric effect as the peptide that was chosen for immunisation is only four amino acids upstream of Y281 (Figure 5.1 a).

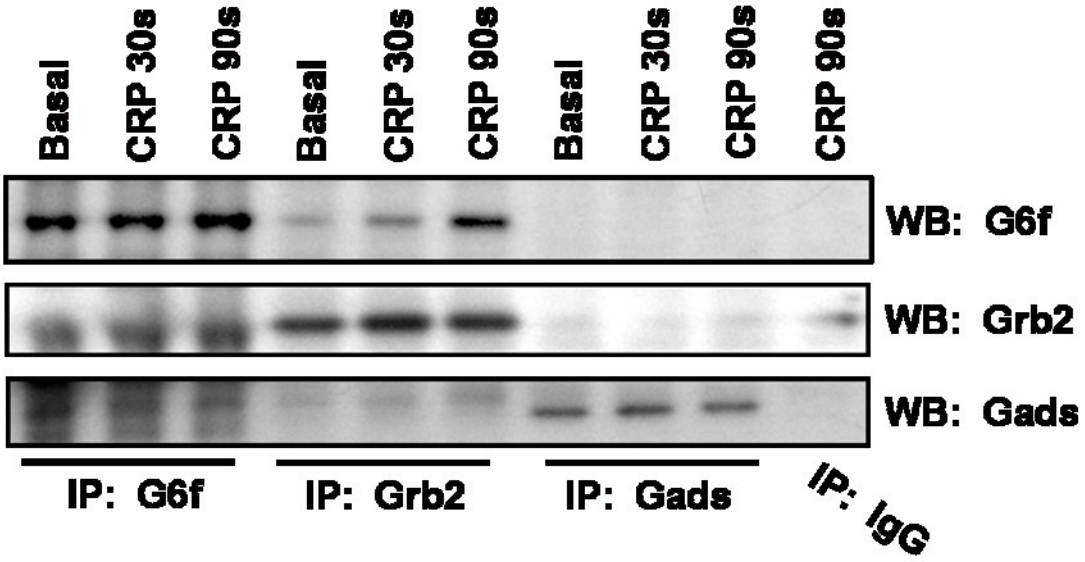

Figure 5.3 – G6f associates with Grb2 in a phosphorylation dependant manner. Washed platelets (5x 10^8/ml) were stimulated with 10 μg/ml CRP for up to 90 sec and subsequently lysed with NP-40 detergent. G6f, Gads and Grb2 were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for Gads, Grb2 and G6f. Results are representative of three experiments.

5.2.4 G6f cannot substitute for LAT in LAT-deficient cells

LAT-deficient Jurkat T-cells (Jcam2) were used to investigate whether G6f can act as a LAT-replacement molecule in the GPVI signalling pathway. The Jcam2 cells were generated by Dr Arthur Weiss (San Francisco) using a mutagenesis strategy and screening for the absence of LAT (Finco *et al*, 1998). The reintroduction of LAT into the cells served as a control to show that LAT is sufficient to restore activation bearing in mind that the mutagenesis strategy may have introduced other mutations. The Jcam2 cells were transfected with both GPVI and FcRγ and either LAT or G6f. Receptor activation was monitored using a NFAT-luciferase reporter construct as described in Chapter 3. Jcam2 cells transfected with GPVI and FcRγ were unresponsive to collagen unless co-transfected with LAT (Figure 5.4). Although it was noticeable that the response was weak compared to that in DT40 cells it was comparable to previous experiments in Jurkat cells (Fuller *et al*, 2007). In contrast, co-transfection of G6f was unable to restore the response to collagen suggesting that it is unable to mimic the loss of LAT in this cell line model.

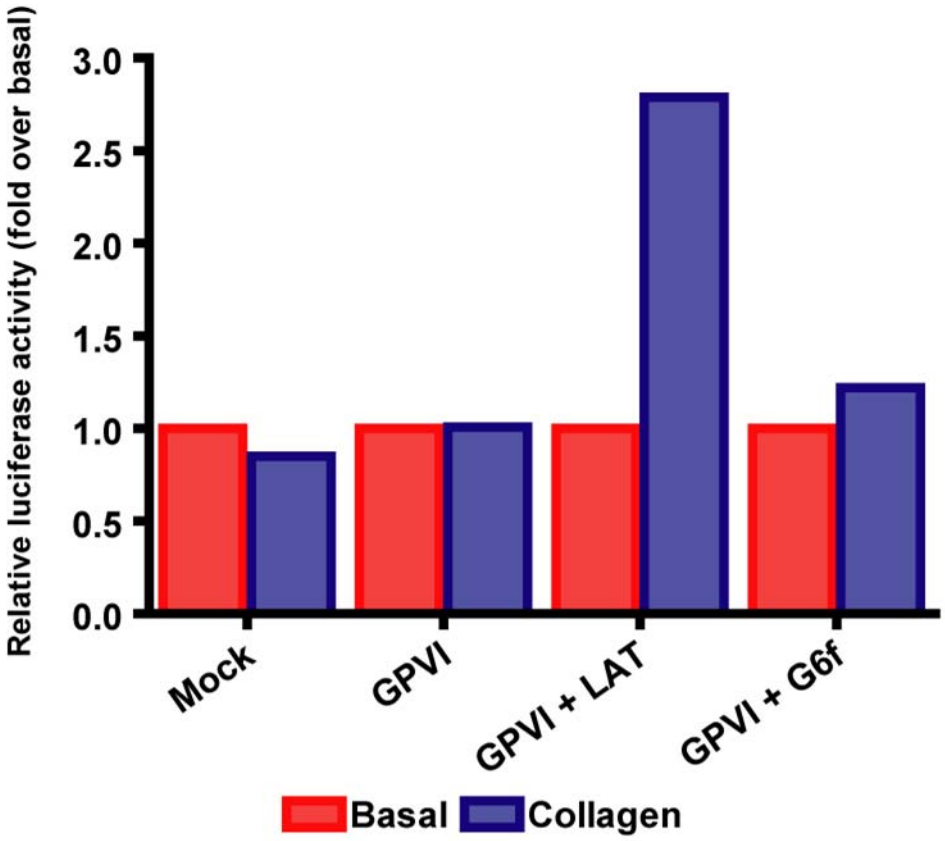

Figure 5.4 – G6f cannot substitute for LAT. Jcam2 cells were transfected with 2 μg/ml each of GPVI, FcRγ, G6f and LAT as specified, and a NFAT-luciferase reporter plasmid. Transfected cells were stimulated with 10 μM collagen for 6 hrs at 37˚C and then the luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as fold increase over basal. Result is representative of three experiments.

5.2.5 G6f is not a novel collagen receptor

G6f was identified in a proteomic analysis of CRP-stimulated platelets raising the possibility that it is a receptor for CRP or collagen and that it is able to confer activation of PLCγ2. To test this possibility, we monitored phosphorylation of G6f and NFAT activation in transfected DT40 cells stimulated by CRP or collagen. Cells transfected with GPVI/FcRγ exhibited a robust NFAT response to collagen and a smaller response to CRP (Figure 5.5 a). The difference between the two agonists in this model has been previously described and is attributed to weak but sustained signalling over the 6 hour incubation with collagen compared to powerful but short lived activation with CRP (Tomlinson *et al*, 2007). Transfection of G6f alone was unable to confer a response to collagen or CRP. Furthermore, the response to collagen or CRP in cells that had been co-transfected with G6f and GPVI/FcRγ was not significantly different to that in cells transfected with GPVI/FcRγ thereby demonstrating that G6f does not contribute to the activation of PLCγ2 in this cell line model.

As a second, indirect test, of whether G6f is a collagen receptor, I monitored tyrosine phosphorylation of the membrane adapter in G6f-transfected cells stimulated by CRP. The synthetic collagen was used in these studies since G6f was first identified as a tyrosine phosphorylated protein in CRP-stimulated platelets (Garcia *et al*, 2006). G6f is weakly tyrosine phosphorylated in basal, serum-starved DT40 cells with no significant increase in phosphorylation upon stimulation by CRP. In contrast, the tyrosine phosphatase inhibitor, pervanadate, induced a marked increase in tyrosine phosphorylation of G6f (Figure 5.5 b). The disparity between the level of tyrosine

phosphorylation of G6f in CRP and pervanadate treatment strongly suggests that G6f is not a receptor for CRP.

Higher agonist concentrations were not tested suggesting the possibility that G6f could show a signalling response or phosphorylation if stimulated with higher concentrations. However, these concentrations are considered to be maximal with regards to platelet stimulation and the DT40 NFAT assay and therefore it seems unlikely that any potential signalling to supra-high agonist concentrations would have any physiological relevance.

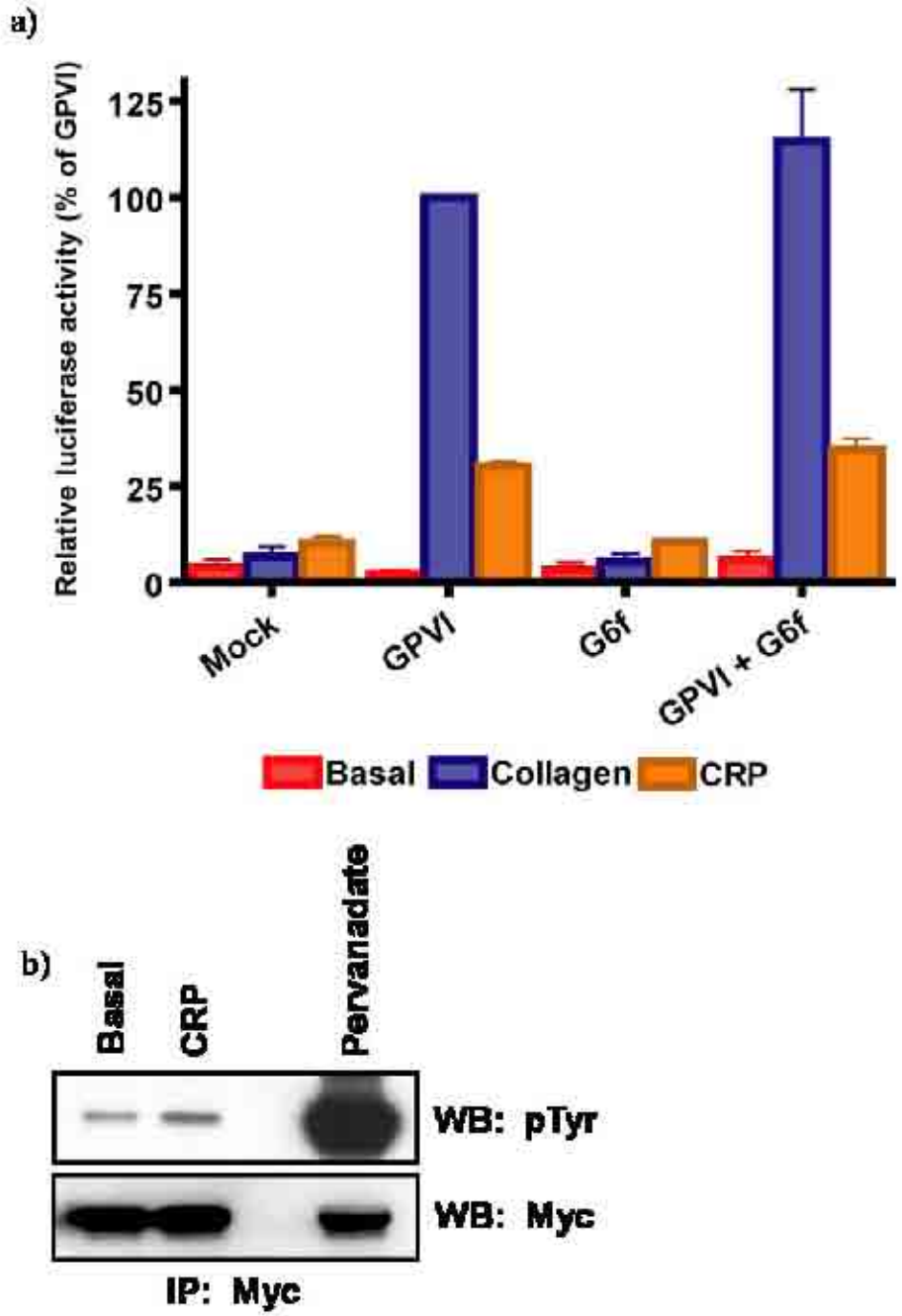

Figure 5.5 – G6f is not a CRP receptor. a) DT40 cells were transfected with 2 μg/ml of GPVI and FcRγ, G6f or a combination of both, and a NFAT-luciferase reporter plasmid. Transfected cells were stimulated with 10 μg/ml collagen or 10 μg/ml CRP for 6 hrs at 37˚C and then the luciferase activity was measured as a readout of signalling. Results were normalised for transfection efficiency and plotted as a percentage of the GPVI-collagen response. Error bars represent the geometric mean ± standard error of three experiments. b) DT40 cells were transfected with G6f. Following serum starvation, cells were stimulated with 10 μg/ml CRP or 0.1 mM pervanadate for 300 sec and subsequently lysed with NP-40 detergent. Myc-tagged proteins were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for G6f and Myc. Results are representative of three experiments.

5.2.6 Minimal expression of G6f in mouse platelets

To assess whether G6f is expressed in mouse platelets, I raised an antibody to the cytoplasmic tail of mouse G6f (amino acids 259-273, Figure 5.1 a shows a comparison of the mouse and human sequence), using the same commercial approach for the anti-human G6f antibody. The antibody was validated by cloning mouse G6f into a pEF6 expression vector (Invitrogen) with a C-terminal Myc tag attached. When this construct was expressed in 293T cells, mouse G6f was immunoprecipitated using the α-Myc antibody followed by western blotted using serum from the immunised rabbit. A clear band of approximately 45 kDa was observed which was absent in mock transfected cells. Reprobing the membrane for Myc confirmed that the band was mouse G6f (Figure 5.6 a). There was also the presence of a smaller sized protein (~40 kDa) in the transfected lysate which did not re-probe for Myc, suggesting either an unrelated protein or a break-down product of full length G6f protein.

Several attempts to demonstrate expression of G6f expression in whole cell lysates of mouse platelets by a combination of western blotting and immunoprecipitation were carried out but failed to provide definitive proof of expression. Only through the use of 4G10 to immunoprecipitate tyrosine phosphorylated proteins from basal and CRP stimulated mouse platelets was it possible to identify the presence of a weak band of approximately 55 kDa in GPVI stimulated platelets using the validated mouse G6f antibody (Figure 5.6 b). However, since this protein is much bigger than predicted (although this could be explained by glycosylation), and we were unable to immunoprecipitate G6f and detect its presence by western blotting, we suspect that the positive band is an unrelated protein that is phosphorylated upon stimulation by CRP.

Indeed, a serial analysis of gene expression (SAGE) on mouse megakaryocytes did not reveal any tags for mouse G6f (Senis *et al*, 2007). Several non-specific bands are also seen under both basal and stimulated conditions. We therefore conclude that G6f is absent or below the level of detection in mouse platelets.

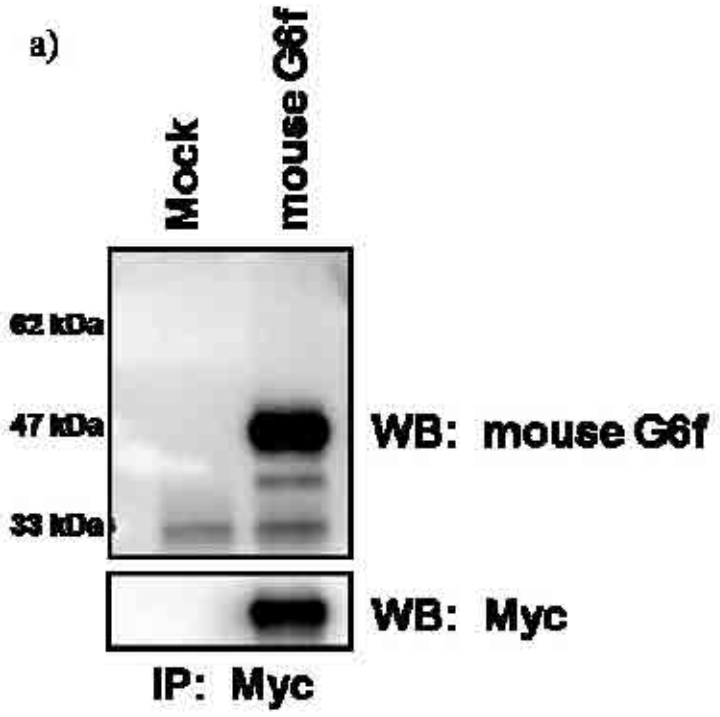

Figure 5.6 – G6f was not detected in mouse platelets.

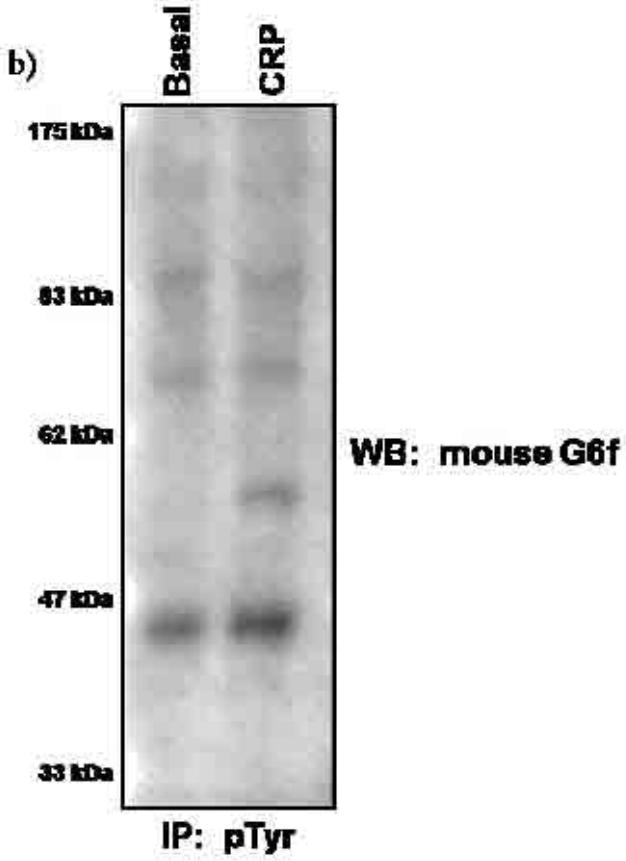

Figure 5.6 – continued. a) 293T cells were transfected with mouse G6f or an empty vector control and subsequently lysed with NP-40 detergent. Myc-tagged proteins were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting with the mouse G6f antibody and reprobed for Myc. Results are representative of three experiments. b) Washed mouse platelets (5x 10^8/ml) were stimulated with 10 μg/ml CRP for 90 sec and subsequently lysed with NP-40 detergent. Tyrosine phosphorylated proteins were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for mouse G6f. Results are representative of three experiments.

5.2.7 Studies on LAT-like proteins reveal the presence of PAG in platelets

To search for a LAT-like molecule that lies upstream of SLP-76 and PLCγ2 in platelets, we were given access to several knock-out mouse lines in Dr Burkhart Schraven's laboratory in Magdeburg, Germany. This approach has a number of benefits over that of western blotting of this family of adapters, as antibodies to this group of proteins are notoriously poor most likely due to their relatively low levels of expression. Thus, a more definitive guide to the functional presence of one or more LAT-like proteins was to investigate a possible phenotype using platelets deficient in one or more of the family of membrane adapters.

We were provided with access to platelets deficient in one LAT family protein, namely $LAT^{-/-}$, $NTAL^{-/-}$, $LAX^{-/-}$, $LIME^{-/-}$ and $PAG^{-/-}$ or compound knock-out mice, namely $LAT/NTAL^{-/-}$, $LAT/NTAL/LAX^{-/-}$ and $PAG/LIME^{-/-}$. Protein lysates were generated from all of these mice and used for western blotting. Platelet lysates from litter matched controls, primary T-cells and in the case of NTAL, primary B cells, were used to verify expression in platelets and to verify the efficacy of the antibodies. Using the specific antibodies, all of which were supplied by Dr Schraven, we were able to confirm expression of PAG in platelets which runs at approximately 70 kDa in both T-cells and in platelets, and which was absent in PAG knock-out platelets (Figure 5.7 a). On the other hand, we were unable to detect the presence of NTAL in platelets although we were able to confirm its expression in B cells where it runs as a 30 kDa protein (not shown). We were unable to detect the presence of LIME (Figure 5.7 b) and LAX (not shown) in platelets or in the T cell lysates, raising concern over the sensitivity of the available antibodies.

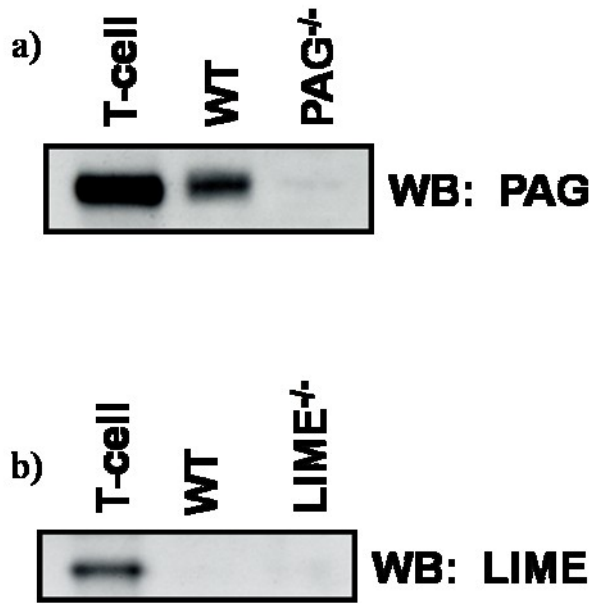

Figure 5.7 – PAG is expressed in mouse platelets. Washed platelets (1x 10^7) from wild type (WT) and knock-out mice lysed with SDS sample buffer. Platelet and T-cell lysates were analysed by SDS-PAGE and western blotting for a) PAG and b) LIME. Results are representative of two mice.

5.2.8 Contribution of LAT-family proteins to GPVI-mediated platelet activation

We used lysates from the various mutant mice platelets to investigate a possible role for the LAT-like adapter proteins in phosphorylation of PLCγ2 by GPVI. For these studies, washed platelets from WT, PAG$^{-/-}$, LIME$^{-/-}$ and PAG/LIME$^{-/-}$ mice were stimulated with collagen (10 μg/ml) or CRP (3 μg/ml CRP) for 90 sec. Syk and PLCγ2 were then immunoprecipitated and analysed for tyrosine phosphorylation by western blotting. There was no significant difference in tyrosine phosphorylation of Syk or PLCγ2 by CRP or collagen in the absence of any of the above LAT-like adapter proteins, with the exception that, in the absence of LIME alone, the CRP response appears slightly low in comparison to the other mice (Figure 5.8). On the other hand, phosphorylation of PLCγ2 was similar to that in the other lysates suggesting that the reduction in Syk may reflect loss of sample. Due to the limited availability of tissue, we were unable to test a full dose response curve to CRP or collagen, and to extend this to other agonists such as rhodocytin. We were also unable to monitor aggregation for the same reason.

LAT$^{-/-}$ platelets were also used as control (not shown) and results were similar to those seen in Figure 4.4. Similar experiments were also performed on LAX$^{-/-}$ and NTAL$^{-/-}$ platelets (not shown) which showed no obvious differences although we were unable to confirm or deny their expression in platelets making it difficult to draw any firm conclusions. Likewise, LAT/NTAL$^{-/-}$ and LAT/NTAL/LAX$^{-/-}$ platelets showed no obvious difference in comparison to LAT$^{-/-}$ platelets (not shown).

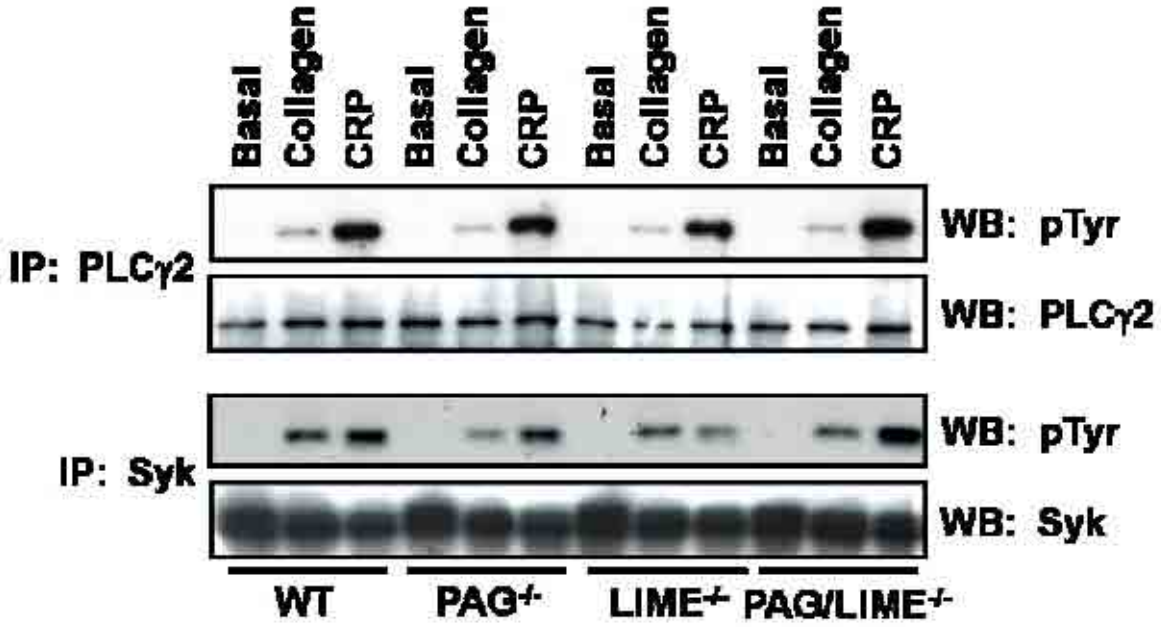

Figure 5.8 – Measurement of tyrosine phosphorylation in PAG$^{-/-}$, LIME$^{-/-}$ and PAG/LIME$^{-/-}$ platelets. Washed platelets (2x 10^8/ml) were stimulated with 3 μg/ml CRP for 60 sec or 10 μg/ml collagen for 90 sec and subsequently lysed with NP-40 detergent. PLCγ2 and Syk were immunoprecipitated and samples were analysed by SDS-PAGE and western blotting for phosphotyrosine (4G10) and reprobed for PLCγ2, and Syk. Results are representative of studies performed using platelets from two mice.

5.3 Discussion

The transmembrane adaptor protein LAT has previously been shown to be required for optimal GPVI and CLEC-2 signalling, but a residual response can be seen in its absence. This raises the possibility of expression of a LAT-like protein in platelets. The aim of this chapter was to test several candidate proteins which may have redundancy with LAT.

A potential candidate was highlighted by the identification of G6f using proteomics (Garcia *et al*, 2006). G6f is a potential orphan receptor with a single transmembrane domain and a cytoplasmic tail containing one tyrosine residue (Ribas *et al*, 2001). After raising and validating an antibody against human G6f, we were able to confirm its presence in human platelets and demonstrate that it undergoes tyrosine phosphorylation in response to CRP. G6f was also robustly phosphorylated by collagen, whereas it was only weakly phosphorylated in response to fibrinogen, thrombin and rhodocytin. The weak response to rhodocytin was dependent on the formation of the secondary mediators, ADP and thromboxanes, as is the case for phosphorylation of CLEC-2 itself and downstream proteins (Pollitt *et al*, 2010). These results show that GPVI is the major receptor mediating G6f phosphorylation, although other receptors can also mediate weak G6f phosphorylation

The single cytoplasmic tyrosine residue, Y281 is in a consensus sequence for Grb2 binding via its SH2 domain, namely YxN. In agreement with this, and also with the initial study on G6f (De Vet *et al*, 2003), we were able to confirm a phosphorylation-

dependent association with Grb2 using CRP-stimulated platelets. In contrast, no association with the Grb2 family member Gads was observed. The functional contribution of the G6f-Grb2 association, if any, to platelet activation by GPVI is unclear. However, the observation that G6f was unable to restore the response to collagen in GPVI-FcRγ-transfected LAT-deficient Jurkat T-cells argues against a role in the regulation of PLCγ2. As a caveat to this experiment, it should be noted that we were unable to confirm expression of G6f at the cell surface by flow cytometry due to the absence of a suitable antibody. Following CRP, thrombin and rhodocytin stimulation, several larger, unidentified tyrosine phosphorylated proteins were seen to co-precipitate with G6f, but their overall level of tyrosine phosphorylation was weak compared to the level of G6f phosphorylation. Identifying these proteins is an interesting line of future work on G6f as it is unclear if they are constitutively associated proteins or if they associate with G6f following its phosphorylation, and whether this is via Grb2 or an independent association.

G6f contains an extracellular Ig domain, thus raising the possibility that it may be a novel collagen receptor by analogy to GPVI. Transfection of DT40 cells with G6f however failed to result in a NFAT response to collagen or CRP and nor were we able to detect significant tyrosine phosphorylation downstream of GPVI. Therefore it seems likely that G6f is phosphorylated downstream of GPVI in platelets rather than serving as a collagen receptor. The observation that collagen and CRP stimulate robust phosphorylation of G6f argues for a potentially important role in mediating platelet activation. However, a functional role for G6f seems less likely following our failure to find conclusive evidence for expression of the mouse homologue in mouse platelets,

either using a validated antibody or by a SAGE analysis (Senis *et al*, 2007). Caution is necessary in regard to this conclusion however as the major receptor for thrombin in human platelets, PAR-1 is not expressed in mouse platelets, where PAR-4 plays the major role. It is possible that a similar scenario may exist for a putative G6f-like protein. Also, rather than a role as a receptor it may be that G6f acts as an adaptor protein for GPVI signalling, however ligand hunting for G6f would be an interesting line of future work as it may have a novel ligand for platelet activation.

I also investigated the possible expression of several other LAT-like molecules in mouse platelets, thereby able to demonstrate expression of PAG. I was unable to detect the presence of LIME, NTAL or LAX, although concerns remain in regard to the efficacy of available antibodies for the latter two adapters. PAG-deficiency had no significant effect on CRP- or collagen-mediated Syk and PLCγ2 phosphorylation. In T-cells, PAG acts as a membrane anchor for the Src family kinase regulatory protein, Csk (Brdicka *et al*, 2000; Kawabuchi *et al*, 2000). Csk mediates phosphorylation of Src kinases at their inhibitory tyrosines. The lack of an effect of the absence of PAG on platelet activation by GPVI therefore argues against a major role for the membrane adapter in regulating Csk in platelets downstream of collagen. However, extending future studies with this knock-out mouse to look at integrin $\alpha_{IIb}\beta_3$ signalling would be particularly interesting as Csk has been shown to mediate Src family kinases downstream of the integrin (Obergfell *et al*, 2002).

The hypothesis that formed much of the basis of the work in this chapter, namely that the LAT-independent component of platelet activation by GPVI and CLEC-2 reflects the presence of a LAT-like molecule that overlaps with LAT in terms of function, is still valid as there are other LAT-like proteins whose expression has yet to be confirmed, as well as other potential candidates. However, the alternative possibility that there is no such protein should also be considered, especially given our inability to identify robust expression of one or more LAT-like proteins other than that of PAG. In such a scenario, it may be that the role of LAT is to amplify signalling by GPVI but that a residual level of activation of PLCγ2 can occur in its absence. In a similar argument, the role of Gads may be to amplify GPVI signalling but again the pathway can function independent of Gads or a related family protein. Studies on mice deficient in Gads and Grb2 are required to investigate this. Neither LAT nor Gads are required for signalling downstream of $\alpha_{IIb}\beta_3$ consistent with the localisation of LAT and the integrin to lipids rafts and non-raft regions, respectively. Again, it remains unclear if platelets express one or more LAT- and Gads-like proteins that facilitate signalling by the integrin. Nevertheless, the much greater level of expression of integrin $\alpha_{IIb}\beta_3$ (80,000 – 120,000 copies in human platelets) relative to GPVI (4,000 – 6,000), and the fact that the latter is the more powerful signalling receptor, provides an indirect argument against the presence of a LAT-like protein downstream of activation of Syk by the integrin.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of results

In this thesis I have provided evidence for a unique mode of signalling through Syk and a single YxxL sequence via cross-linking of CLEC-2 molecules. This *trans* binding is a novel mechanism through Syk which until now was thought to activate either through *cis* binding to the dual tyrosines of an ITAM or through a phosphotyrosine independent mechanism through integrin $\alpha_{IIb}\beta_3$. I have also shown a minimal role for the adaptor protein Gads in both GPVI and CLEC-2 signalling pathways. It appears that Gads is as dispensable for GPVI and CLEC-2 signalling as LAT is, whereas CLEC-2 is capable of a small degree of signalling independently of its constitutive binding partner, SLP-76. I have also investigated the possible role of a number of other LAT-like molecules in the ITAM signalling pathway, but failed to identify any potential candidates. In this, the final chapter, I will discuss some of the wider aspects of the work.

6.2 The evolution of ITAM receptors and C-type lectins

After performing numerous database searches with the help of John Herbert we have found that orthologs for a number of ITAM proteins can be found as far back as zebrafish (*Danio rerio*). Due to the size of and divergence of the C-type lectin family, only one (CLEC14) was found to have an ortholog in zebrafish. Other zebrafish C-type lectins were too divergent in sequence to be directly linked to a human C-type lectin. We were however, able to find a single zebrafish protein which had both a C-type lectin domain and a putative ITAM-like sequence (Figure 6.1). Both Syk and Zap-70 can also be found in the zebrafish.

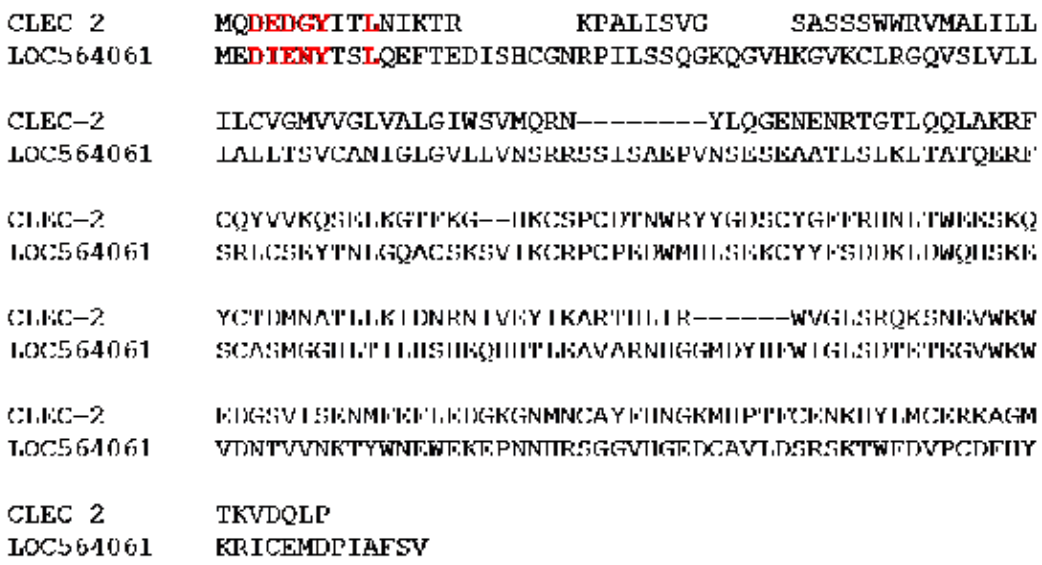

Figure 6.1 – Sequence alignment of human CLEC-2 and a hypothetical zebrafish C-type lectin LOC564061. Sequences were aligned using ClustalW web-based software.

As discussed in Chapter 3, we have found that a number of the ITAM-containing proteins have their ITAM sequence split over two exons. We therefore hypothesised that the possibility of gene duplication of the ITAM-like sequence resulted in a protein which was able to signal more efficiently due to a higher affinity for Syk. As the known ITAM-like C-type lectins have multiple roles, in haemostasis, lymphatic development and in the innate immune system, it is possible that at one time this family of proteins mediated many of these responses in a thrombocyte forerunner. A review by Pancer and Cooper discusses the evolution of the adaptive immune system and they conclude that this appeared along with the rearrangement of IgG domain receptors in jawed fish (Pancer and Cooper, 2006). The jawless fish have an immune system that utilises Leucine-rich-repeat receptors. This step in evolution from innate towards adaptive immunity could be linked to the appearance of the variable IgG receptors and possibly the presence of an ITAM. Before this ITAM-like molecules may have been the less efficient mode of signalling used.

The opposite hypothesis to this would be that the ITAM-like molecules may have evolved through convergent evolution with ITAM proteins and use a common signalling pathway. This is perhaps the more likely scenario given that ITAMs are found mostly on IgG superfamily proteins, whereas the only ITAM-like proteins we know of so far are C-type lectins. Also, the ITAM-like proteins are type II proteins whereas the ITAM proteins are type I and are therefore quite different structurally. However, the structure and activity of Syk versus Zap-70 is also of interest when thinking about the evolution and role of ITAMs and ITAM-like receptors although it is

of note that Syk is also able to be activated by a phosphotyrosine independent mechanism through integrin $\alpha_{IIb}\beta_3$.

Syk is expressed in a number of tissues whereas the expression of Zap-70 is restricted to haematopoietic cells, in particular, T-cells and natural killers (NK) cells where its role is in ITAM signalling. In B-cells, platelets, granulocytes and macrophages Syk plays this role although Syk is also expressed in thymocytes (a T-cell precursor), but then lost following T-cell maturation. The sequence of Syk and Zap-70 reveals an N-terminal SH2 domain, a short inter-domain linker (interdomain A), a C-terminal SH2 domain, a SH2-kinase linker (interdomain B), and a kinase domain (Figure 6.2). A structural analysis of inactive Zap-70 shows that these domains are arranged such that the tandem SH2 domains present their phosphotyrosine binding pockets in line facing in one direction, and the kinase domain presents the active site in the opposite direction (Deindl *et al*, 2007). This arrangement allows for tight binding to the ITAM sequence and therefore, membrane localisation of the kinase domain such that it is orientated to phosphorylate its targets.

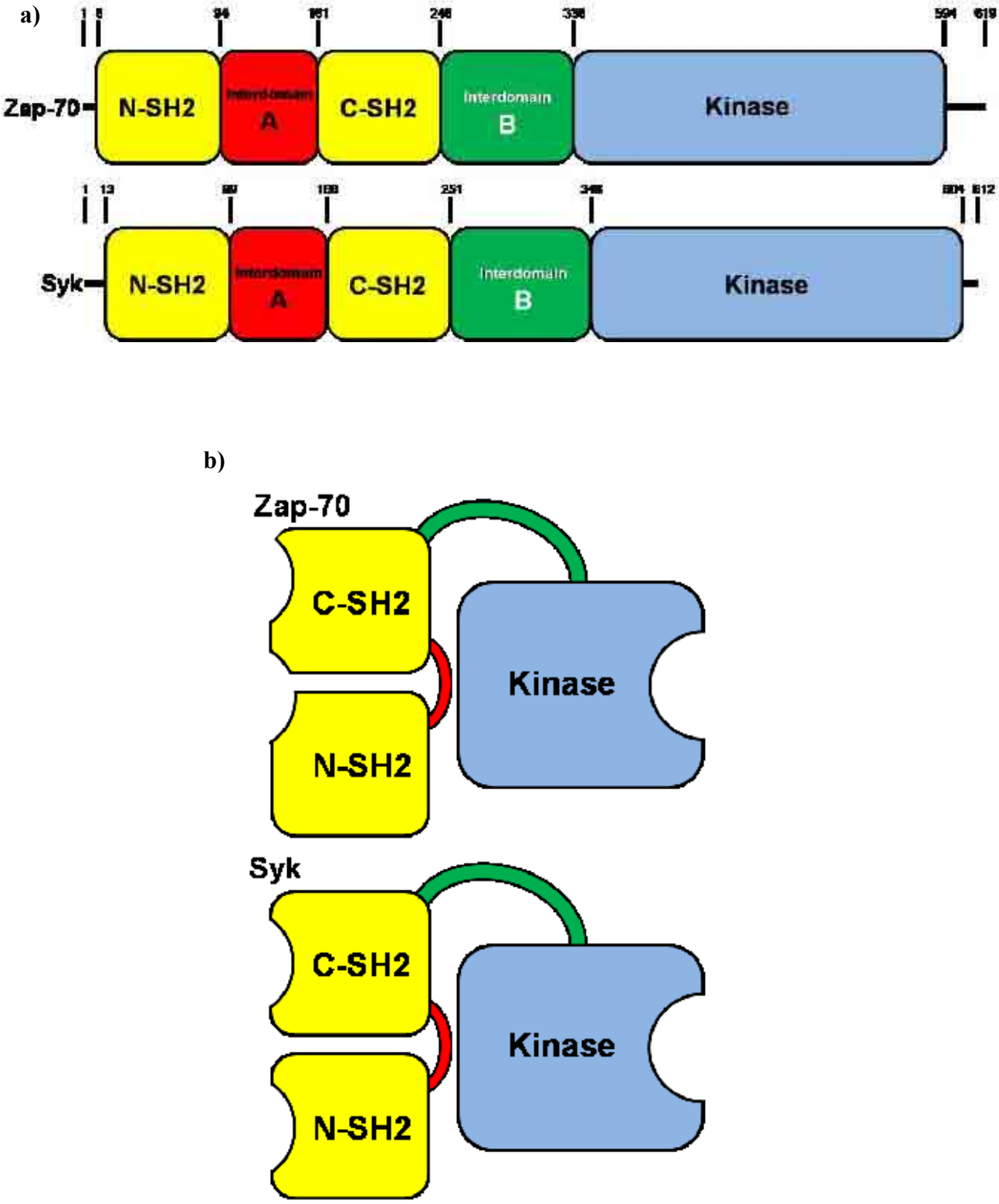

Figure 6.2 – Domain arrangement of Syk and Zap-70. a) Linear representation of the domains and their sizes (in number of amino acid residues) of Syk and Zap-70. b) Colour-coded cartoon representing the 3D arrangement of the domains of Syk and Zap-70 including the phosphotyrosine binding pockets of the SH2 domains.

Interestingly, in Syk, the two phosphotyrosine binding pockets are complete, one per SH2 domain. However, Zap-70 is slightly different with one complete binding pocket present in the C-SH2 domain, and the second binding pocket is partially formed by both SH2 domains. Therefore, the N-SH2 domain of Zap-70 alone should not be able to bind to phosphotyrosine and for Zap-70 to bind to an ITAM, both SH2 domains have to align correctly. In comparison, we have presented data in Chapter 3 confirming that both of the single SH2 domains of Syk are capable of phosphotyrosine binding. A further difference between the two kinases is in their kinase domains, with the kinase domain of Syk being shown as up 100 times more active than that of Zap-70 in an *in vitro* kinase assay (Latour *et al*, 1996). Furthermore, interdomain A in Syk has been shown to be more flexible than in Zap-70 suggesting it would be able to accommodate binding between two chains much more efficiently than Zap-70.

Therefore, we can hypothesise that ITAM-like molecules came first in evolution, and that they were able to mediate a number of functions (haemostasis, innate immunity, development) in a generic blood cell. This is mediated through cross-linking by Syk which is made possible due to the ability of both SH2 domains to bind to phosphotyrosine residues and the high intrinsic activity of its kinase domain. The appearance of an ITAM could result in a higher affinity interaction with Syk and more efficient signalling. The high activity of Syk would be advantageous in a platelet-like cell which is required to activate fully and rapidly in times of vascular damage, however, immune cells which evolved for the adaptive immune system, like T-cells, have to undergo positive and negative selection and the high activity of Syk could possibly affect these processes. Therefore the use of Zap-70 with its lower intrinsic

kinase activity and requirement for binding to an ITAM and not an ITAM-like molecule could be a way that evolution refined the ITAM signalling pathway.

Future work on the structure-activity relationship between CLEC-2 and Syk will be carried out to investigate these hypotheses by comparing the ability of CLEC-2 to signal through Syk compared to Zap-70 using the NFAT-luciferase cell line reporter assay and cells deficient in either Syk or Zap-70. Interestingly, it has previously been published that CLEC-2 can signal in a Jurkat T-cell line (Zap-70 expressing) using the NFAT-luciferase reporter assay, although the increase over basal in these cells is much lower than the increases seen in the DT40 B-cell line (Fuller *et al*, 2007). Chimeric versions of Syk and Zap-70 would also be interesting for further experiments. Constructs with the SH2 domains of Zap-70 and the kinase domain of Syk, and *vice versa* would allow us to investigate if it is the less active kinase domain or the unusual SH2 domains of Zap-70 which were the key to any differences found. Furthermore, the Zap-70 kinase domain may become more active when paired with the Syk SH2 domains due to intramolecular interactions; however *in vitro* kinase assays can be used to study this also.

Various CLEC-2 mutants can also be investigated looking at the importance of the DEDG sequence preceding the YxxL and also CLEC-2/ITAM chimeras to investigate if CLEC-2 can signal with two YxxL sequences in its cytoplasmic tail, and would this also be possible through Zap-70?

It would also be interesting to extend studies into the zebrafish and investigate the ITAM-like C-type lectin we found to be expressed there. Use of *in situ* hybridisation could be used to look at the expression of this protein. If it were present in thrombocytes, cells which play an analogous role to platelets found in birds and fish, it would be particularly provocative as it may play a CLEC-2-like role. Further, the use of morpholino knock-down technology could be used to look at the development of the fish in the absence of the protein, and also the use of a laser injury model to investigate a haemostatic role for the protein. DT40 cell line studies could also be performed following cloning of the zebrafish protein to see if it can signal in a similar fashion to CLEC-2.

6.3 Final thoughts

During the course of this thesis, many new developments for a physiological role of CLEC-2 have been published. While this does not directly influence the work in this thesis which has focussed on the downstream signalling pathway it does validate the importance of working on the receptor. Unlike GPVI which has a well defined haemostatic role though its interaction with collagen, it seems that the major physiological role of CLEC-2 is in development through its interaction with podoplanin. This field of work is showing the importance of platelets not only as a means of preventing blood loss, but as a crucial cell required for the development of, and continued maintenance of the lymphatic system (Carramolino *et al*, 2010; Uhrin *et al*, 2010). Unpublished, ongoing work in our lab with a recently generated CLEC-2 knock-out mouse has shown the mutation to be lethal, highlighting the importance of this molecule. Furthermore, radiation chimeric mice have blood filled lymph providing

evidence for a continued role of CLEC-2 in the maintenance of the lymphatics in adult mice (Hughes, Pollitt, Finney and Watson, Unpublished).

REFERENCES

ABTAHIAN, F., N. BEZMAN, R. CLEMENS, E. SEBZDA, L. CHENG, S. J. SHATTIL, M. L. KAHN and G. A. KORETZKY (2006). "Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells." Mol Cell Biol **26**(18): 6936-49.

ABTAHIAN, F., A. GUERRIERO, E. SEBZDA, M. M. LU, R. ZHOU, A. MOCSAI, E. E. MYERS, B. HUANG, D. G. JACKSON, V. A. FERRARI, V. TYBULEWICZ, C. A. LOWELL, J. J. LEPORE, G. A. KORETZKY and M. L. KAHN (2003). "Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk." Science **299**(5604): 247-51.

ADAM, F., A. KAUSKOT, J. P. ROSA and M. BRYCKAERT (2008). "Mitogen-activated protein kinases in hemostasis and thrombosis." J Thromb Haemost **6**(12): 2007-16.

AGUADO, E., S. RICHELME, S. NUNEZ-CRUZ, A. MIAZEK, A. M. MURA, M. RICHELME, X. J. GUO, D. SAINTY, H. T. HE, B. MALISSEN and M. MALISSEN (2002). "Induction of T helper type 2 immunity by a point mutation in the LAT adaptor." Science **296**(5575): 2036-40.

ANDREWS, R. K., K. SUZUKI-INOUE, Y. SHEN, D. TULASNE, S. P. WATSON and M. C. BERNDT (2002). "Interaction of calmodulin with the cytoplasmic domain of platelet glycoprotein VI." Blood **99**(11): 4219-21.

ANTL, M., M. L. VON BRUHL, C. EIGLSPERGER, M. WERNER, I. KONRAD, T. KOCHER, M. WILM, F. HOFMANN, S. MASSBERG and J. SCHLOSSMANN (2007). "IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation." Blood **109**(2): 552-9.

ARIAS-PALOMO, E., M. A. RECUERO-CHECA, X. R. BUSTELO and O. LLORCA (2007). "3D structure of Syk kinase determined by single-particle electron microscopy." Biochim Biophys Acta **1774**(12): 1493-9.

ARIAS-SALGADO, E. G., F. HAJ, C. DUBOIS, B. MORAN, A. KASIRER-FRIEDE, B. C. FURIE, B. FURIE, B. G. NEEL and S. J. SHATTIL (2005). "PTP-1B is an essential positive regulator of platelet integrin signaling." J Cell Biol **170**(5): 837-45.

ARIAS-SALGADO, E. G., S. LIZANO, S. SARKAR, J. S. BRUGGE, M. H. GINSBERG and S. J. SHATTIL (2003). "Src kinase activation by direct interaction with the integrin beta cytoplasmic domain." Proc Natl Acad Sci U S A **100**(23): 13298-302.

ARIIZUMI, K., G. L. SHEN, S. SHIKANO, S. XU, R. RITTER, 3RD, T. KUMAMOTO, D. EDELBAUM, A. MORITA, P. R. BERGSTRESSER and A. TAKASHIMA (2000). "Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning." J Biol Chem **275**(26): 20157-67.

ASAZUMA, N., J. I. WILDE, O. BERLANGA, M. LEDUC, A. LEO, E. SCHWEIGHOFFER, V. TYBULEWICZ, C. BON, S. K. LIU, C. J. MCGLADE, B. SCHRAVEN and S. P. WATSON (2000). "Interaction of linker for activation of T cells with multiple adapter proteins in platelets activated by the glycoprotein VI-selective ligand, convulxin." J Biol Chem **275**(43): 33427-34.

ASSELIN, J., J. M. GIBBINS, M. ACHISON, Y. H. LEE, L. F. MORTON, R. W. FARNDALE, M. J. BARNES and S. P. WATSON (1997). "A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1." Blood **89**(4): 1235-42.

ATKINSON, B. T., W. ELLMEIER and S. P. WATSON (2003). "Tec regulates platelet activation by GPVI in the absence of Btk." Blood **102**(10): 3592-9.

AUGER, J. M., M. J. KUIJPERS, Y. A. SENIS, S. P. WATSON and J. W. HEEMSKERK (2005). "Adhesion of human and mouse platelets to collagen under shear: a unifying model." Faseb J **19**(7): 825-7.

AUGER, J. M. and S. P. WATSON (2008). "Dynamic tyrosine kinase-regulated signaling and actin polymerisation mediate aggregate stability under shear." Arterioscler Thromb Vasc Biol **28**(8): 1499-504.

AURSNES, I. and O. O. PEDERSEN (1979). "Petechial hemorrhage in the ciliary processes of thrombocytopenic rabbits. An electron microscopic study." Microvasc Res **17**(1): 12-21.

BAKKER, A. B., E. BAKER, G. R. SUTHERLAND, J. H. PHILLIPS and L. L. LANIER (1999). "Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells." Proc Natl Acad Sci U S A **96**(17): 9792-6.

BARROW, A. D., E. ASTOUL, A. FLOTO, G. BROOKE, I. A. RELOU, N. S. JENNINGS, K. G. SMITH, W. OUWEHAND, R. W. FARNDALE, D. R. ALEXANDER and J. TROWSDALE (2004). "Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2." J Immunol **172**(10): 5838-42.

BARROW, A. D. and J. TROWSDALE (2006). "You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling." Eur J Immunol **36**(7): 1646-53.

BERGMEIER, W., D. BOUVARD, J. A. EBLE, R. MOKHTARI-NEJAD, V. SCHULTE, H. ZIRNGIBL, C. BRAKEBUSCH, R. FASSLER and B. NIESWANDT (2001). "Rhodocytin (aggretin) activates platelets lacking alpha(2)beta(1) integrin, glycoprotein VI,

and the ligand-binding domain of glycoprotein Ibalpha." J Biol Chem **276**(27): 25121-6.

BERLANGA, O., D. TULASNE, T. BORI, D. C. SNELL, Y. MIURA, S. JUNG, M. MOROI, J. FRAMPTON and S. P. WATSON (2002). "The Fc receptor gamma-chain is necessary and sufficient to initiate signalling through glycoprotein VI in transfected cells by the snake C-type lectin, convulxin." Eur J Biochem **269**(12): 2951-60.

BEST, D., Y. A. SENIS, G. E. JARVIS, H. J. EAGLETON, D. J. ROBERTS, T. SAITO, S. M. JUNG, M. MOROI, P. HARRISON, F. R. GREEN and S. P. WATSON (2003). "GPVI levels in platelets: relationship to platelet function at high shear." Blood **102**(8): 2811-8.

BISSELL, M. J. and D. RADISKY (2001). "Putting tumours in context." Nat Rev Cancer **1**(1): 46-54.

BORI-SANZ, T., K. S. INOUE, M. C. BERNDT, S. P. WATSON and D. TULASNE (2003). "Delineation of the region in the glycoprotein VI tail required for association with the Fc receptor gamma-chain." J Biol Chem **278**(38): 35914-22.

BOUKOUR, S., J. M. MASSE, L. BENIT, A. DUBART-KUPPERSCHMITT and E. M. CRAMER (2006). "Lentivirus degradation and DC-SIGN expression by human platelets and megakaryocytes." J Thromb Haemost **4**(2): 426-35.

BOYLAN, B., C. GAO, V. RATHORE, J. C. GILL, D. K. NEWMAN and P. J. NEWMAN (2008). "Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets." Blood **112**(7): 2780-6.

BRASS, L. F. (2003). "Thrombin and platelet activation." Chest **124**(3 Suppl): 18S-25S.

BRASS, L. F., L. ZHU and T. J. STALKER (2008). "Novel therapeutic targets at the platelet vascular interface." Arterioscler Thromb Vasc Biol **28**(3): s43-50.

BRDICKA, T., M. IMRICH, P. ANGELISOVA, N. BRDICKOVA, O. HORVATH, J. SPICKA, I. HILGERT, P. LUSKOVA, P. DRABER, P. NOVAK, N. ENGELS, J. WIENANDS, L. SIMEONI, J. OSTERREICHER, E. AGUADO, M. MALISSEN, B. SCHRAVEN and V. HOREJSI (2002). "Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling." J Exp Med **196**(12): 1617-26.

BRDICKA, T., T. A. KADLECEK, J. P. ROOSE, A. W. PASTUSZAK and A. WEISS (2005). "Intramolecular regulatory switch in ZAP-70: analogy with receptor tyrosine kinases." Mol Cell Biol **25**(12): 4924-33.

BRDICKA, T., D. PAVLISTOVA, A. LEO, E. BRUYNS, V. KORINEK, P. ANGELISOVA, J. SCHERER, A. SHEVCHENKO, I. HILGERT, J. CERNY, K. DRBAL, Y. KURAMITSU, B. KORNACKER, V. HOREJSI and B. SCHRAVEN (2000). "Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation." J Exp Med **191**(9): 1591-604.

BRDICKOVA, N., T. BRDICKA, P. ANGELISOVA, O. HORVATH, J. SPICKA, I. HILGERT, J. PACES, L. SIMEONI, S. KLICHE, C. MERTEN, B. SCHRAVEN and V. HOREJSI (2003). "LIME: a new membrane Raft-associated adaptor protein involved in CD4 and CD8 coreceptor signaling." J Exp Med **198**(10): 1453-62.

BROWN, G. D. (2006). "Dectin-1: a signalling non-TLR pattern-recognition receptor." Nat Rev Immunol **6**(1): 33-43.

BROWN, G. D. and S. GORDON (2001). "Immune recognition. A new receptor for beta-glucans." Nature **413**(6851): 36-7.

BROWN, G. D. and S. GORDON (2003). "Fungal beta-glucans and mammalian immunity." Immunity **19**(3): 311-5.

BROWN, G. D., J. HERRE, D. L. WILLIAMS, J. A. WILLMENT, A. S. MARSHALL and S. GORDON (2003). "Dectin-1 mediates the biological effects of beta-glucans." J Exp Med **197**(9): 1119-24.

BROWNING, J. and A. RIBOLINI (1989). "Studies on the differing effects of tumor necrosis factor and lymphotoxin on the growth of several human tumor lines." J Immunol **143**(6): 1859-67.

BRUYNS, E., A. MARIE-CARDINE, H. KIRCHGESSNER, K. SAGOLLA, A. SHEVCHENKO, M. MANN, F. AUTSCHBACH, A. BENSUSSAN, S. MEUER and B. SCHRAVEN (1998). "T cell receptor (TCR) interacting molecule (TRIM), a novel disulfide-linked dimer associated with the TCR-CD3-zeta complex, recruits intracellular signaling proteins to the plasma membrane." J Exp Med **188**(3): 561-75.

CALAMINUS, S. D., J. M. AUGER, O. J. MCCARTY, M. J. WAKELAM, L. M. MACHESKY and S. P. WATSON (2007). "MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability." J Thromb Haemost **5**(10): 2136-45.

CALAMINUS, S. D., S. THOMAS, O. J. MCCARTY, L. M. MACHESKY and S. P. WATSON (2008). "Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading." J Thromb Haemost **6**(11): 1944-52.

CAMINSCHI, I., A. I. PROIETTO, F. AHMET, S. KITSOULIS, J. SHIN TEH, J. C. LO, A. RIZZITELLI, L. WU, D. VREMEC, S. L. VAN DOMMELEN, I. K. CAMPBELL, E. MARASKOVSKY, H. BRALEY, G. M. DAVEY, P. MOTTRAM, N. VAN DE VELDE, K. JENSEN, A. M. LEW, M. D. WRIGHT, W. R. HEATH, K. SHORTMAN and M. H.

LAHOUD (2008). "The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement." Blood **112**(8): 3264-73.

CAO, W., L. ZHANG, D. B. ROSEN, L. BOVER, G. WATANABE, M. BAO, L. L. LANIER and Y. J. LIU (2007). "BDCA2/Fc epsilon RI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells." PLoS Biol **5**(10): e248.

CAPARROS, E., P. MUNOZ, E. SIERRA-FILARDI, D. SERRANO-GOMEZ, A. PUIG-KROGER, J. L. RODRIGUEZ-FERNANDEZ, M. MELLADO, J. SANCHO, M. ZUBIAUR and A. L. CORBI (2006). "DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production." Blood **107**(10): 3950-8.

CARRAMOLINO, L., J. FUENTES, C. GARCIA-ANDRES, V. AZCOITIA, D. RIETHMACHER and M. TORRES (2010). "Platelets Play an Essential Role in Separating the Blood and Lymphatic Vasculatures During Embryonic Angiogenesis." Circ Res.

CHACKO, G. W., J. T. BRANDT, K. M. COGGESHALL and C. L. ANDERSON (1996). "Phosphoinositide 3-kinase and p72syk noncovalently associate with the low affinity Fc gamma receptor on human platelets through an immunoreceptor tyrosine-based activation motif. Reconstitution with synthetic phosphopeptides." J Biol Chem **271**(18): 10775-81.

CHACKO, G. W., A. M. DUCHEMIN, K. M. COGGESHALL, J. M. OSBORNE, J. T. BRANDT and C. L. ANDERSON (1994). "Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase p72syk." J Biol Chem **269**(51): 32435-40.

CHAIPAN, C., E. J. SOILLEUX, P. SIMPSON, H. HOFMANN, T. GRAMBERG, A. MARZI, M. GEIER, E. A. STEWART, J. EISEMANN, A. STEINKASSERER, K. SUZUKI-INOUE, G. L. FULLER, A. C. PEARCE, S. P. WATSON, J. A. HOXIE, F. BARIBAUD and S.

POHLMANN (2006). "DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets." J Virol **80**(18): 8951-60.

CHEN, H., D. LOCKE, Y. LIU, C. LIU and M. L. KAHN (2002). "The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion." J Biol Chem **277**(4): 3011-9.

CHRISTOU, C. M., A. C. PEARCE, A. A. WATSON, A. R. MISTRY, A. Y. POLLITT, A. E. FENTON-MAY, L. A. JOHNSON, D. G. JACKSON, S. P. WATSON and C. A. O'CALLAGHAN (2008). "Renal cells activate the platelet receptor CLEC-2 through podoplanin." Biochem J **411**(1): 133-40.

CHRZANOWSKA-WODNICKA, M., S. S. SMYTH, S. M. SCHOENWAELDER, T. H. FISCHER and G. C. WHITE, 2ND (2005). "Rap1b is required for normal platelet function and hemostasis in mice." J Clin Invest **115**(3): 680-7.

CLEMENTS, J. L., J. R. LEE, B. GROSS, B. YANG, J. D. OLSON, A. SANDRA, S. P. WATSON, S. R. LENTZ and G. A. KORETZKY (1999). "Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice." J Clin Invest **103**(1): 19-25.

CLEMENTS, J. L., B. YANG, S. E. ROSS-BARTA, S. L. ELIASON, R. F. HRSTKA, R. A. WILLIAMSON and G. A. KORETZKY (1998). "Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development." Science **281**(5375): 416-9.

CLEMETSON, J. M., J. POLGAR, E. MAGNENAT, T. N. WELLS and K. J. CLEMETSON (1999). "The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors." J Biol Chem **274**(41): 29019-24.

COLLER, B. S. and S. J. SHATTIL (2008). "The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend." Blood **112**(8): 3011-25.

COLONNA, M., J. SAMARIDIS and L. ANGMAN (2000). "Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells." Eur J Immunol **30**(2): 697-704.

DE VET, E. C., B. AGUADO and R. D. CAMPBELL (2003). "Adaptor signalling proteins Grb2 and Grb7 are recruited by human G6f, a novel member of the immunoglobulin superfamily encoded in the MHC." Biochem J **375**(Pt 1): 207-13.

DEINDL, S., T. A. KADLECEK, T. BRDICKA, X. CAO, A. WEISS and J. KURIYAN (2007). "Structural basis for the inhibition of tyrosine kinase activity of ZAP-70." Cell **129**(4): 735-46.

DHANJAL, T. S., E. A. ROSS, J. M. AUGER, O. J. MCCARTY, C. E. HUGHES, Y. A. SENIS, C. D. BUCKLEY and S. P. WATSON (2007). "Minimal regulation of platelet activity by PECAM-1." Platelets **18**(1): 56-67.

DIHAZI, G. H. and A. SINZ (2003). "Mapping low-resolution three-dimensional protein structures using chemical cross-linking and Fourier transform ion-cyclotron resonance mass spectrometry." Rapid Commun Mass Spectrom **17**(17): 2005-14.

DORAHY, D. J., L. F. LINCZ, C. J. MELDRUM and G. F. BURNS (1996). "Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36." Biochem J **319 (Pt 1)**: 67-72.

DRICKAMER, K. (1993). "Evolution of Ca(2+)-dependent animal lectins." Prog Nucleic Acid Res Mol Biol **45**: 207-32.

DRICKAMER, K. (1999). "C-type lectin-like domains." Curr Opin Struct Biol **9**(5): 585-90.

EBLE, J. A., B. BEERMANN, H. J. HINZ and A. SCHMIDT-HEDERICH (2001). "alpha 2beta 1 integrin is not recognized by rhodocytin but is the specific, high affinity target of rhodocetin, an RGD-independent disintegrin and potent inhibitor of cell adhesion to collagen." J Biol Chem **276**(15): 12274-84.

ECHTLER, K., K. STARK, M. LORENZ, S. KERSTAN, A. WALCH, L. JENNEN, M. RUDELIUS, S. SEIDL, E. KREMMER, N. R. EMAMBOKUS, M. L. VON BRUEHL, J. FRAMPTON, B. ISERMANN, O. GENZEL-BOROVICZENY, C. SCHREIBER, J. MEHILLI, A. KASTRATI, M. SCHWAIGER, R. A. SHIVDASANI and S. MASSBERG (2010). "Platelets contribute to postnatal occlusion of the ductus arteriosus." Nat Med **16**(1): 75-82.

EZUMI, Y., K. SHINDOH, M. TSUJI and H. TAKAYAMA (1998). "Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets." J Exp Med **188**(2): 267-76.

FALATI, S., Q. LIU, P. GROSS, G. MERRILL-SKOLOFF, J. CHOU, E. VANDENDRIES, A. CELI, K. CROCE, B. C. FURIE and B. FURIE (2003). "Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin." J Exp Med **197**(11): 1585-98.

FALATI, S., S. PATIL, P. L. GROSS, M. STAPLETON, G. MERRILL-SKOLOFF, N. E. BARRETT, K. L. PIXTON, H. WEILER, B. COOLEY, D. K. NEWMAN, P. J. NEWMAN, B. C. FURIE, B. FURIE and J. M. GIBBINS (2006). "Platelet PECAM-1 inhibits thrombus formation in vivo." Blood **107**(2): 535-41.

FANG, N., D. G. MOTTO, S. E. ROSS and G. A. KORETZKY (1996). "Tyrosines 113, 128, and 145 of SLP-76 are required for optimal augmentation of NFAT promoter activity." J Immunol **157**(9): 3769-73.

FINCO, T. S., T. KADLECEK, W. ZHANG, L. E. SAMELSON and A. WEISS (1998). "LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway." Immunity **9**(5): 617-26.

FRITZ, E., H. LUDWIG, W. SCHEITHAUER and H. SINZINGER (1986). "Shortened platelet half-life in multiple myeloma." Blood **68**(2): 514-20.

FRUEHLING, S. and R. LONGNECKER (1997). "The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction." Virology **235**(2): 241-51.

FU, J., H. GERHARDT, J. M. MCDANIEL, B. XIA, X. LIU, L. IVANCIU, A. NY, K. HERMANS, R. SILASI-MANSAT, S. MCGEE, E. NYE, T. JU, M. I. RAMIREZ, P. CARMELIET, R. D. CUMMINGS, F. LUPU and L. XIA (2008). "Endothelial cell O-glycan deficiency causes blood/lymphatic misconnections and consequent fatty liver disease in mice." J Clin Invest **118**(11): 3725-37.

FULLER, G. L. (2006). Identification of the platelet rhodocytin receptor. The Medical School. Birmingham, The University of Birmingham. **PhD:** 231.

FULLER, G. L., J. A. WILLIAMS, M. G. TOMLINSON, J. A. EBLE, S. L. HANNA, S. POHLMANN, K. SUZUKI-INOUE, Y. OZAKI, S. P. WATSON and A. C. PEARCE (2007). "The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade." J Biol Chem **282**(17): 12397-409.

FURIHATA, K., K. J. CLEMETSON, H. DEGUCHI and T. J. KUNICKI (2001). "Variation in human platelet glycoprotein VI content modulates glycoprotein VI-specific prothrombinase activity." Arterioscler Thromb Vasc Biol **21**(11): 1857-63.

FUTTERER, K., J. WONG, R. A. GRUCZA, A. C. CHAN and G. WAKSMAN (1998). "Structural basis for Syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide." J Mol Biol **281**(3): 523-37.

GANTNER, B. N., R. M. SIMMONS, S. J. CANAVERA, S. AKIRA and D. M. UNDERHILL (2003). "Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2." J Exp Med **197**(9): 1107-17.

GAO, C., B. BOYLAN, D. BOUGIE, J. C. GILL, J. BIRENBAUM, D. K. NEWMAN, R. H. ASTER and P. J. NEWMAN (2009). "Eptifibatide-induced thrombocytopenia and thrombosis in humans require FcgammaRIIa and the integrin beta3 cytoplasmic domain." J Clin Invest **119**(3): 504-11.

GARCIA-PINERES, A. J., A. HILDESHEIM, M. TRIVETT, M. WILLIAMS, L. WU, V. N. KEWALRAMANI and L. A. PINTO (2006). "Role of DC-SIGN in the activation of dendritic cells by HPV-16 L1 virus-like particle vaccine." Eur J Immunol **36**(2): 437-45.

GARCIA, A., Y. A. SENIS, R. ANTROBUS, C. E. HUGHES, R. A. DWEK, S. P. WATSON and N. ZITZMANN (2006). "A global proteomics approach identifies novel phosphorylated signaling proteins in GPVI-activated platelets: involvement of G6f, a novel platelet Grb2-binding membrane adapter." Proteomics **6**(19): 5332-43.

GEIJTENBEEK, T. B., D. S. KWON, R. TORENSMA, S. J. VAN VLIET, G. C. VAN DUIJNHOVEN, J. MIDDEL, I. L. CORNELISSEN, H. S. NOTTET, V. N.

KEWALRAMANI, D. R. LITTMAN, C. G. FIGDOR and Y. VAN KOOYK (2000). "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells." Cell **100**(5): 587-97.

GERMANO, P., J. GOMEZ, M. G. KAZANIETZ, P. M. BLUMBERG and J. RIVERA (1994). "Phosphorylation of the gamma chain of the high affinity receptor for immunoglobulin E by receptor-associated protein kinase C-delta." J Biol Chem **269**(37): 23102-7.

GIBBINS, J., J. ASSELIN, R. FARNDALE, M. BARNES, C. L. LAW and S. P. WATSON (1996). "Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets." J Biol Chem **271**(30): 18095-9.

GIBBINS, J. M. (2004). "Platelet adhesion signalling and the regulation of thrombus formation." J Cell Sci **117**(Pt 16): 3415-25.

GIBBINS, J. M., S. BRIDDON, A. SHUTES, M. J. VAN VUGT, J. G. VAN DE WINKEL, T. SAITO and S. P. WATSON (1998). "The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin." J Biol Chem **273**(51): 34437-43.

GIBBINS, J. M., M. OKUMA, R. FARNDALE, M. BARNES and S. P. WATSON (1997). "Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain." FEBS Lett **413**(2): 255-9.

GONCALVES, I., S. C. HUGHAN, S. M. SCHOENWAELDER, C. L. YAP, Y. YUAN and S. P. JACKSON (2003). "Integrin alpha IIb beta 3-dependent calcium signals regulate platelet-fibrinogen interactions under flow. Involvement of phospholipase C gamma 2." J Biol Chem **278**(37): 34812-22.

GRANDE, S. M., G. BANNISH, E. M. FUENTES-PANANA, E. KATZ and J. G. MONROE (2007). "Tonic B-cell and viral ITAM signaling: context is everything." Immunol Rev **218**: 214-34.

GRINGHUIS, S. I., J. DEN DUNNEN, M. LITJENS, M. VAN DER VLIST, B. WEVERS, S. C. BRUIJNS and T. B. GEIJTENBEEK (2009). "Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk." Nat Immunol **10**(2): 203-13.

GRINGHUIS, S. I., J. DEN DUNNEN, M. LITJENS, B. VAN HET HOF, Y. VAN KOOYK and T. B. GEIJTENBEEK (2007). "C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB." Immunity **26**(5): 605-16.

GRINGHUIS, S. I., M. VAN DER VLIST, L. M. VAN DEN BERG, J. DEN DUNNEN, M. LITJENS and T. B. GEIJTENBEEK (2010). "HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells." Nat Immunol **11**(5): 419-26.

GROSS, B. S., J. R. LEE, J. L. CLEMENTS, M. TURNER, V. L. TYBULEWICZ, P. R. FINDELL, G. A. KORETZKY and S. P. WATSON (1999). "Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets." J Biol Chem **274**(9): 5963-71.

GROSS, B. S., S. K. MELFORD and S. P. WATSON (1999). "Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets." Eur J Biochem **263**(3): 612-23.

GRUCZA, R. A., K. FUTTERER, A. C. CHAN and G. WAKSMAN (1999). "Thermodynamic study of the binding of the tandem-SH2 domain of the Syk kinase to a dually

phosphorylated ITAM peptide: evidence for two conformers." Biochemistry **38**(16): 5024-33.

GRUNEBACH, F., M. M. WECK, J. REICHERT and P. BROSSART (2002). "Molecular and functional characterization of human Dectin-1." Exp Hematol **30**(11): 1309-15.

GUPTA, G. P. and J. MASSAGUE (2004). "Platelets and metastasis revisited: a novel fatty link." J Clin Invest **114**(12): 1691-3.

HALL, K. J., M. L. JONES and A. W. POOLE (2007). "Coincident regulation of PKCdelta in human platelets by phosphorylation of Tyr311 and Tyr565 and phospholipase C signalling." Biochem J **406**(3): 501-9.

HARTWIG, J. and J. ITALIANO, JR. (2003). "The birth of the platelet." J Thromb Haemost **1**(7): 1580-6.

HAYASHI, K., R. NITTONO, N. OKAMOTO, S. TSUJI, Y. HARA, R. GOITSUKA and D. KITAMURA (2000). "The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells." Proc Natl Acad Sci U S A **97**(6): 2755-60.

HECHLER, B., C. NONNE, A. ECKLY, S. MAGNENAT, J. Y. RINCKEL, C. V. DENIS, M. FREUND, J. P. CAZENAVE, F. LANZA and C. GACHET (2009). "Arterial thrombosis: relevance of a model with two levels of severity assessed by histological, ultrastructural and functional characterization." J Thromb Haemost.

HERRE, J., A. S. MARSHALL, E. CARON, A. D. EDWARDS, D. L. WILLIAMS, E. SCHWEIGHOFFER, V. TYBULEWICZ, C. REIS E SOUSA, S. GORDON and G. D. BROWN (2004). "Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages." Blood **104**(13): 4038-45.

HIGUCHI, R., B. KRUMMEL and R. K. SAIKI (1988). "A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions." Nucleic Acids Res **16**(15): 7351-67.

HOFFMANN, S. C., C. SCHELLACK, S. TEXTOR, S. KONOLD, D. SCHMITZ, A. CERWENKA, S. PFLANZ and C. WATZL (2007). "Identification of CLEC12B, an inhibitory receptor on myeloid cells." J Biol Chem **282**(31): 22370-5.

HOREJSI, V., W. ZHANG and B. SCHRAVEN (2004). "Transmembrane adaptor proteins: organizers of immunoreceptor signalling." Nat Rev Immunol **4**(8): 603-16.

HUANG, T. F., C. Z. LIU and S. H. YANG (1995). "Aggretin, a novel platelet-aggregation inducer from snake (Calloselasma rhodostoma) venom, activates phospholipase C by acting as a glycoprotein Ia/IIa agonist." Biochem J **309 (Pt 3)**: 1021-7.

HUR, E. M., M. SON, O. H. LEE, Y. B. CHOI, C. PARK, H. LEE and Y. YUN (2003). "LIME, a novel transmembrane adaptor protein, associates with p56lck and mediates T cell activation." J Exp Med **198**(10): 1463-73.

HUYSAMEN, C., J. A. WILLMENT, K. M. DENNEHY and G. D. BROWN (2008). "CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes." J Biol Chem.

ICHISE, H., T. ICHISE, O. OHTANI and N. YOSHIDA (2009). "Phospholipase Cgamma2 is necessary for separation of blood and lymphatic vasculature in mice." Development **136**(2): 191-5.

INOUE, O., K. SUZUKI-INOUE, W. L. DEAN, J. FRAMPTON and S. P. WATSON (2003). "Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2." J Cell Biol **160**(5): 769-80.

INOUE, O., K. SUZUKI-INOUE, O. J. MCCARTY, M. MOROI, Z. M. RUGGERI, T. J. KUNICKI, Y. OZAKI and S. P. WATSON (2006). "Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI." Blood **107**(4): 1405-12.

INOUE, O., K. SUZUKI-INOUE and Y. OZAKI (2008). "Redundant mechanism of platelet adhesion to laminin and collagen under flow: involvement of von Willebrand factor and glycoprotein Ib-IX-V." J Biol Chem **283**(24): 16279-82.

ISHIAI, M., M. KUROSAKI, K. INABE, A. C. CHAN, K. SUGAMURA and T. KUROSAKI (2000). "Involvement of LAT, Gads, and Grb2 in compartmentation of SLP-76 to the plasma membrane." J Exp Med **192**(6): 847-56.

JACKSON, S. P. (2007). "The growing complexity of platelet aggregation." Blood **109**(12): 5087-95.

JACKSON, S. P., W. S. NESBITT and S. KULKARNI (2003). "Signaling events underlying thrombus formation." J Thromb Haemost **1**(7): 1602-12.

JACKSON, S. P., W. S. NESBITT and E. WESTEIN (2009). "Dynamics of platelet thrombus formation." J Thromb Haemost **7 Suppl 1**: 17-20.

JAIN, S., S. RUSSELL and J. WARE (2009). "Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models." J Thromb Haemost **7**(10): 1713-7.

JAIN, S., M. ZUKA, J. LIU, S. RUSSELL, J. DENT, J. A. GUERRERO, J. FORSYTH, B. MARUSZAK, T. K. GARTNER, B. FELDING-HABERMANN and J. WARE (2007). "Platelet glycoprotein Ib alpha supports experimental lung metastasis." Proc Natl Acad Sci U S A **104**(21): 9024-8.

JANDROT-PERRUS, M., A. H. LAGRUE, M. OKUMA and C. BON (1997). "Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha2beta1." J Biol Chem **272**(43): 27035-41.

JANSSEN, E., M. ZHU, W. ZHANG, S. KOONPAEW and W. ZHANG (2003). "LAB: a new membrane-associated adaptor molecule in B cell activation." Nat Immunol **4**(2): 117-23.

JIN, R. C., B. VOETSCH and J. LOSCALZO (2005). "Endogenous mechanisms of inhibition of platelet function." Microcirculation **12**(3): 247-58.

JIN, W., O. INOUE, N. TAMURA, K. SUZUKI-INOUE, K. SATOH, M. C. BERNDT, M. HANDA, S. GOTO and Y. OZAKI (2007). "A role for glycosphingolipid-enriched microdomains in platelet glycoprotein Ib-mediated platelet activation." J Thromb Haemost **5**(5): 1034-40.

JORDAN, M. S., J. S. MALTZMAN, S. KLICHE, J. SHABASON, J. E. SMITH, A. OBSTFELD, B. SCHRAVEN and G. A. KORETZKY (2007). "In vivo disruption of T cell development by expression of a dominant-negative polypeptide designed to abolish the SLP-76/Gads interaction." Eur J Immunol **37**(10): 2961-72.

JUDD, B. A., P. S. MYUNG, L. LENG, A. OBERGFELL, W. S. PEAR, S. J. SHATTIL and G. A. KORETZKY (2000). "Hematopoietic reconstitution of SLP-76 corrects hemostasis and platelet signaling through alpha IIb beta 3 and collagen receptors." Proc Natl Acad Sci U S A **97**(22): 12056-61.

JUDD, B. A., P. S. MYUNG, A. OBERGFELL, E. E. MYERS, A. M. CHENG, S. P. WATSON, W. S. PEAR, D. ALLMAN, S. J. SHATTIL and G. A. KORETZKY (2002). "Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling." J Exp Med **195**(6): 705-17.

JUMAA, H., B. WOLLSCHEID, M. MITTERER, J. WIENANDS, M. RETH and P. J. NIELSEN (1999). "Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65." Immunity **11**(5): 547-54.

KACZMAREK, E., K. KOZIAK, J. SEVIGNY, J. B. SIEGEL, J. ANRATHER, A. R. BEAUDOIN, F. H. BACH and S. C. ROBSON (1996). "Identification and characterization of CD39/vascular ATP diphosphohydrolase." J Biol Chem **271**(51): 33116-22.

KAHN, M. L., Y. W. ZHENG, W. HUANG, V. BIGORNIA, D. ZENG, S. MOFF, R. V. FARESE, JR., C. TAM and S. R. COUGHLIN (1998). "A dual thrombin receptor system for platelet activation." Nature **394**(6694): 690-4.

KALIA, N., J. M. AUGER, B. ATKINSON and S. P. WATSON (2008). "Critical Role of FcR gamma-Chain, LAT, PLCgamma2 and Thrombin in Arteriolar Thrombus Formation upon Mild, Laser-Induced Endothelial Injury In Vivo." Microcirculation **15**(4): 325-35.

KANEKO, M., Y. KATO, A. KUNITA, N. FUJITA, T. TSURUO and M. OSAWA (2004). "Functional sialylated O-glycan to platelet aggregation on Aggrus (T1alpha/Podoplanin) molecules expressed in Chinese hamster ovary cells." J Biol Chem **279**(37): 38838-43.

KANEKO, M. K., Y. KATO, A. KAMEYAMA, H. ITO, A. KUNO, J. HIRABAYASHI, T. KUBOTA, K. AMANO, Y. CHIBA, Y. HASEGAWA, I. SASAGAWA, K. MISHIMA and H. NARIMATSU (2007). "Functional glycosylation of human podoplanin: glycan structure of platelet aggregation-inducing factor." FEBS Lett **581**(2): 331-6.

KATO, K., T. KANAJI, S. RUSSELL, T. J. KUNICKI, K. FURIHATA, S. KANAJI, P. MARCHESE, A. REININGER, Z. M. RUGGERI and J. WARE (2003). "The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion." Blood **102**(5): 1701-7.

KATO, Y., N. FUJITA, A. KUNITA, S. SATO, M. KANEKO, M. OSAWA and T. TSURUO (2003). "Molecular identification of Aggrus/T1alpha as a platelet aggregation-inducing factor expressed in colorectal tumors." J Biol Chem **278**(51): 51599-605.

KAWABUCHI, M., Y. SATOMI, T. TAKAO, Y. SHIMONISHI, S. NADA, K. NAGAI, A. TARAKHOVSKY and M. OKADA (2000). "Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases." Nature **404**(6781): 999-1003.

KERRIGAN, A. M., K. M. DENNEHY, D. MOURAO-SA, I. FARO-TRINDADE, J. A. WILLMENT, P. R. TAYLOR, J. A. EBLE, C. REIS E SOUSA and G. D. BROWN (2009). "CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils." J Immunol **182**(7): 4150-7.

KIM, A. S., L. T. KAKALIS, N. ABDUL-MANAN, G. A. LIU and M. K. ROSEN (2000). "Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein." Nature **404**(6774): 151-8.

KIRCHGESSNER, H., J. DIETRICH, J. SCHERER, P. ISOMAKI, V. KORINEK, I. HILGERT, E. BRUYNS, A. LEO, A. P. COPE and B. SCHRAVEN (2001). "The transmembrane adaptor protein TRIM regulates T cell receptor (TCR) expression and TCR-mediated signaling via an association with the TCR zeta chain." J Exp Med **193**(11): 1269-84.

KISUCKA, J., C. E. BUTTERFIELD, D. G. DUDA, S. C. EICHENBERGER, S. SAFFARIPOUR, J. WARE, Z. M. RUGGERI, R. K. JAIN, J. FOLKMAN and D. D. WAGNER (2006). "Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage." Proc Natl Acad Sci U S A **103**(4): 855-60.

KNIGHT, C. G., L. F. MORTON, D. J. ONLEY, A. R. PEACHEY, T. ICHINOHE, M. OKUMA, R. W. FARNDALE and M. J. BARNES (1999). "Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen." Cardiovasc Res **41**(2): 450-7.

KOEGL, M., P. ZLATKINE, S. C. LEY, S. A. COURTNEIDGE and A. I. MAGEE (1994). "Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif." Biochem J **303 (Pt 3)**: 749-53.

KONDO, M., A. J. WAGERS, M. G. MANZ, S. S. PROHASKA, D. C. SCHERER, G. F. BEILHACK, J. A. SHIZURU and I. L. WEISSMAN (2003). "Biology of hematopoietic stem cells and progenitors: implications for clinical application." Annu Rev Immunol **21**: 759-806.

KONOPATSKAYA, O., K. GILIO, M. T. HARPER, Y. ZHAO, J. M. COSEMANS, Z. A. KARIM, S. W. WHITEHEART, J. D. MOLKENTIN, P. VERKADE, S. P. WATSON, J. W. HEEMSKERK and A. W. POOLE (2009). "PKCalpha regulates platelet granule secretion and thrombus formation in mice." J Clin Invest **119**(2): 399-407.

KUHNE, M. R., G. KU and A. WEISS (2000). "A guanine nucleotide exchange factor-independent function of Vav1 in transcriptional activation." J Biol Chem **275**(3): 2185-90.

KULKARNI, S., S. M. DOPHEIDE, C. L. YAP, C. RAVANAT, M. FREUND, P. MANGIN, K. A. HEEL, A. STREET, I. S. HARPER, F. LANZA and S. P. JACKSON (2000). "A revised model of platelet aggregation." J Clin Invest **105**(6): 783-91.

KUROSAKI, T., S. A. JOHNSON, L. PAO, K. SADA, H. YAMAMURA and J. C. CAMBIER (1995). "Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling." J Exp Med **182**(6): 1815-23.

LATOUR, S., L. M. CHOW and A. VEILLETTE (1996). "Differential intrinsic enzymatic activity of Syk and Zap-70 protein-tyrosine kinases." J Biol Chem **271**(37): 22782-90.

LAW, C. L., S. P. SIDORENKO, K. A. CHANDRAN, Z. ZHAO, S. H. SHEN, E. H. FISCHER and E. A. CLARK (1996). "CD22 associates with protein tyrosine phosphatase 1C, Syk, and phospholipase C-gamma(1) upon B cell activation." J Exp Med **183**(2): 547-60.

LAW, D. A., F. R. DEGUZMAN, P. HEISER, K. MINISTRI-MADRID, N. KILLEEN and D. R. PHILLIPS (1999). "Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function." Nature **401**(6755): 808-11.

LIN, J., A. WEISS and T. S. FINCO (1999). "Localization of LAT in glycolipid-enriched microdomains is required for T cell activation." J Biol Chem **274**(41): 28861-4.

LIU, J., C. W. JACKSON, R. A. GRUPPO, L. K. JENNINGS and T. K. GARTNER (2005). "The {beta}3 subunit of the integrin {alpha}IIb{beta}3 regulates {alpha}IIb-mediated outside-in signaling." Blood.

LIU, Q., D. BERRY, P. NASH, T. PAWSON, C. J. MCGLADE and S. S. LI (2003). "Structural basis for specific binding of the Gads SH3 domain to an RxxK motif-containing SLP-76 peptide: a novel mode of peptide recognition." Mol Cell **11**(2): 471-81.

LIU, S. K., N. FANG, G. A. KORETZKY and C. J. MCGLADE (1999). "The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors." Curr Biol **9**(2): 67-75.

LOCKE, D., C. LIU, X. PENG, H. CHEN and M. L. KAHN (2003). "Fc Rgamma - independent signaling by the platelet collagen receptor glycoprotein VI." J Biol Chem **278**(17): 15441-8.

MAHTAB, E. A., M. C. WIJFFELS, N. M. VAN DEN AKKER, N. D. HAHURIJ, H. LIE-VENEMA, L. J. WISSE, M. C. DERUITER, P. UHRIN, J. ZAUJEC, B. R. BINDER, M. J. SCHALIJ, R. E. POELMANN and A. C. GITTENBERGER-DE GROOT (2008). "Cardiac malformations and myocardial abnormalities in podoplanin knockout mouse embryos: Correlation with abnormal epicardial development." Dev Dyn **237**(3): 847-57.

MARIE-CARDINE, A., H. KIRCHGESSNER, E. BRUYNS, A. SHEVCHENKO, M. MANN, F. AUTSCHBACH, S. RATNOFSKY, S. MEUER and B. SCHRAVEN (1999). "SHP2-interacting transmembrane adaptor protein (SIT), a novel disulfide-linked dimer regulating human T cell activation." J Exp Med **189**(8): 1181-94.

MARSHALL, A. S., J. A. WILLMENT, H. H. LIN, D. L. WILLIAMS, S. GORDON and G. D. BROWN (2004). "Identification and characterization of a novel human myeloid inhibitory C-type lectin-like receptor (MICL) that is predominantly expressed on granulocytes and monocytes." J Biol Chem **279**(15): 14792-802.

MARTIN-VILLAR, E., D. MEGIAS, S. CASTEL, M. M. YURRITA, S. VILARO and M. QUINTANILLA (2006). "Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition." J Cell Sci **119**(Pt 21): 4541-53.

MAY, F., I. HAGEDORN, I. PLEINES, M. BENDER, T. VOGTLE, J. EBLE, M. ELVERS and B. NIESWANDT (2009). "CLEC-2 is an essential platelet activating receptor in hemostasis and thrombosis." Blood.

MCCARTY, O. J., S. D. CALAMINUS, M. C. BERNDT, L. M. MACHESKY and S. P. WATSON (2006). "von Willebrand factor mediates platelet spreading through glycoprotein Ib and alpha(IIb)beta3 in the presence of botrocetin and ristocetin, respectively." J Thromb Haemost **4**(6): 1367-78.

MIAO, W. M., E. VASILE, W. S. LANE and J. LAWLER (2001). "CD36 associates with CD9 and integrins on human blood platelets." Blood **97**(6): 1689-96.

MINEGISHI, Y., J. ROHRER, E. COUSTAN-SMITH, H. M. LEDERMAN, R. PAPPU, D. CAMPANA, A. C. CHAN and M. E. CONLEY (1999). "An essential role for BLNK in human B cell development." Science **286**(5446): 1954-7.

MORI, J., A. C. PEARCE, J. C. SPALTON, B. GRYGIELSKA, J. A. EBLE, M. G. TOMLINSON, Y. A. SENIS and S. P. WATSON (2008). "G6b-B inhibits constitutive and agonist-induced signaling by glycoprotein VI and CLEC-2." J Biol Chem **283**(51): 35419-27.

MORTON, L. F., P. G. HARGREAVES, R. W. FARNDALE, R. D. YOUNG and M. J. BARNES (1995). "Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity." Biochem J **306 (Pt 2)**: 337-44.

MOTTO, D. G., S. E. ROSS, J. WU, L. R. HENDRICKS-TAYLOR and G. A. KORETZKY (1996). "Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production." J Exp Med **183**(4): 1937-43.

MULLER, F., N. J. MUTCH, W. A. SCHENK, S. A. SMITH, L. ESTERL, H. M. SPRONK, S. SCHMIDBAUER, W. A. GAHL, J. H. MORRISSEY and T. RENNE (2009). "Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo." Cell **139**(6): 1143-56.

MUNNIX, I. C., A. STREHL, M. J. KUIJPERS, J. M. AUGER, P. E. VAN DER MEIJDEN, M. A. VAN ZANDVOORT, M. G. OUDE EGBRINK, B. NIESWANDT and J. W. HEEMSKERK (2005). "The glycoprotein VI-phospholipase Cgamma2 signaling pathway

controls thrombus formation induced by collagen and tissue factor in vitro and in vivo." Arterioscler Thromb Vasc Biol **25**(12): 2673-8.

MUNZEL, T., R. FEIL, A. MULSCH, S. M. LOHMANN, F. HOFMANN and U. WALTER (2003). "Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase [corrected]." Circulation **108**(18): 2172-83.

NASEEM, K. M. (2005). "The role of nitric oxide in cardiovascular diseases." Mol Aspects Med **26**(1-2): 33-65.

NASH, G. F., L. F. TURNER, M. F. SCULLY and A. K. KAKKAR (2002). "Platelets and cancer." Lancet Oncol **3**(7): 425-30.

NAVDAEV, A., J. M. CLEMETSON, J. POLGAR, B. E. KEHREL, M. GLAUNER, E. MAGNENAT, T. N. WELLS and K. J. CLEMETSON (2001). "Aggretin, a heterodimeric C-type lectin from Calloselasma rhodostoma (malayan pit viper), stimulates platelets by binding to alpha 2beta 1 integrin and glycoprotein Ib, activating Syk and phospholipase Cgamma 2, but does not involve the glycoprotein VI/Fc receptor gamma chain collagen receptor." J Biol Chem **276**(24): 20882-9.

NESBITT, W. S., E. WESTEIN, F. J. TOVAR-LOPEZ, E. TOLOUEI, A. MITCHELL, J. FU, J. CARBERRY, A. FOURAS and S. P. JACKSON (2009). "A shear gradient-dependent platelet aggregation mechanism drives thrombus formation." Nat Med **15**(6): 665-73.

NEWLAND, S. A., I. C. MACAULAY, A. R. FLOTO, E. C. DE VET, W. H. OUWEHAND, N. A. WATKINS, P. A. LYONS and D. R. CAMPBELL (2007). "The novel inhibitory receptor G6B is expressed on the surface of platelets and attenuates platelet function in vitro." Blood **109**(11): 4806-9.

NIESWANDT, B., W. BERGMEIER, V. SCHULTE, K. RACKEBRANDT, J. E. GESSNER and H. ZIRNGIBL (2000). "Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain." J Biol Chem **275**(31): 23998-4002.

NIESWANDT, B. and S. P. WATSON (2003). "Platelet-collagen interaction: is GPVI the central receptor?" Blood **102**(2): 449-61.

OBERGFELL, A., K. ETO, A. MOCSAI, C. BUENSUCESO, S. L. MOORES, J. S. BRUGGE, C. A. LOWELL and S. J. SHATTIL (2002). "Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton." J Cell Biol **157**(2): 265-75.

OLYNYCH, T. J., D. L. JAKEMAN and J. S. MARSHALL (2006). "Fungal zymosan induces leukotriene production by human mast cells through a dectin-1-dependent mechanism." J Allergy Clin Immunol **118**(4): 837-43.

ONO, A., E. WESTEIN, S. HSIAO, W. S. NESBITT, J. R. HAMILTON, S. M. SCHOENWAELDER and S. P. JACKSON (2008). "Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth." Blood **112**(1): 90-9.

OYA, K., J. WANG, Y. WATANABE, R. KOGA and T. WATANABE (2003). "Appearance of the LAT protein at an early stage of B-cell development and its possible role." Immunology **109**(3): 351-9.

OZAKI, Y., N. ASAZUMA, K. SUZUKI-INOUE and M. C. BERNDT (2005). "Platelet GPIb-IX-V-dependent signaling." J Thromb Haemost **3**(8): 1745-51.

PAIGE, L. A., M. J. NADLER, M. L. HARRISON, J. M. CASSADY and R. L. GEAHLEN (1993). "Reversible palmitoylation of the protein-tyrosine kinase p56lck." J Biol Chem **268**(12): 8669-74.

PALACIOS, E. H. and A. WEISS (2004). "Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation." Oncogene **23**(48): 7990-8000.

PANCER, Z. and M. D. COOPER (2006). "The evolution of adaptive immunity." Annu Rev Immunol **24**: 497-518.

PAPPU, R., A. M. CHENG, B. LI, Q. GONG, C. CHIU, N. GRIFFIN, M. WHITE, B. P. SLECKMAN and A. C. CHAN (1999). "Requirement for B cell linker protein (BLNK) in B cell development." Science **286**(5446): 1949-54.

PASQUET, J. M., B. GROSS, L. QUEK, N. ASAZUMA, W. ZHANG, C. L. SOMMERS, E. SCHWEIGHOFFER, V. TYBULEWICZ, B. JUDD, J. R. LEE, G. KORETZKY, P. E. LOVE, L. E. SAMELSON and S. P. WATSON (1999). "LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI." Mol Cell Biol **19**(12): 8326-34.

PAZ, P. E., S. WANG, H. CLARKE, X. LU, D. STOKOE and A. ABO (2001). "Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells." Biochem J **356**(Pt 2): 461-71.

PEARCE, A. C., O. J. MCCARTY, S. D. CALAMINUS, E. VIGORITO, M. TURNER and S. P. WATSON (2007). "Vav family proteins are required for optimal regulation of PLCgamma2 by integrin alphaIIbbeta3." Biochem J **401**(3): 753-61.

PEARCE, A. C., Y. A. SENIS, D. D. BILLADEAU, M. TURNER, S. P. WATSON and E. VIGORITO (2004). "Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen." J Biol Chem **279**(52): 53955-62.

PEARCE, A. C., J. I. WILDE, G. M. DOODY, D. BEST, O. INOUE, E. VIGORITO, V. L. TYBULEWICZ, M. TURNER and S. P. WATSON (2002). "Vav1, but not Vav2,

contributes to platelet aggregation by CRP and thrombin, but neither is required for regulation of phospholipase C." Blood **100**(10): 3561-9.

PEARCE, A. C., P. WONEROW, S. J. MARSHALL, J. FRAMPTON, T. K. GARTNER and S. P. WATSON (2004). "The heptapeptide LSARLAF mediates platelet activation through phospholipase Cgamma2 independently of glycoprotein IIb-IIIa." Biochem J **378**(Pt 1): 193-9.

PEARS, C. J., K. THORNBER, J. M. AUGER, C. E. HUGHES, B. GRYGIELSKA, M. B. PROTTY, A. C. PEARCE and S. P. WATSON (2008). "Differential roles of the PKC novel isoforms, PKCdelta and PKCepsilon, in mouse and human platelets." PLoS One **3**(11): e3793.

PEREZ-VILLAR, J. J., G. S. WHITNEY, M. T. SITNICK, R. J. DUNN, S. VENKATESAN, K. O'DAY, G. L. SCHIEVEN, T. A. LIN and S. B. KANNER (2002). "Phosphorylation of the linker for activation of T-cells by Itk promotes recruitment of Vav." Biochemistry **41**(34): 10732-40.

PFREPPER, K. I., A. MARIE-CARDINE, L. SIMEONI, Y. KURAMITSU, A. LEO, J. SPICKA, I. HILGERT, J. SCHERER and B. SCHRAVEN (2001). "Structural and functional dissection of the cytoplasmic domain of the transmembrane adaptor protein SIT (SHP2-interacting transmembrane adaptor protein)." Eur J Immunol **31**(6): 1825-36.

PIVNIOUK, V., E. TSITSIKOV, P. SWINTON, G. RATHBUN, F. W. ALT and R. S. GEHA (1998). "Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76." Cell **94**(2): 229-38.

POHLMANN, S., J. ZHANG, F. BARIBAUD, Z. CHEN, G. J. LESLIE, G. LIN, A. GRANELLI-PIPERNO, R. W. DOMS, C. M. RICE and J. A. MCKEATING (2003). "Hepatitis C

virus glycoproteins interact with DC-SIGN and DC-SIGNR." J Virol **77**(7): 4070-80.

POLGAR, J., J. M. CLEMETSON, B. E. KEHREL, M. WIEDEMANN, E. M. MAGNENAT, T. N. WELLS and K. J. CLEMETSON (1997). "Platelet activation and signal transduction by convulxin, a C-type lectin from Crotalus durissus terrificus (tropical rattlesnake) venom via the p62/GPVI collagen receptor." J Biol Chem **272**(21): 13576-83.

POLLITT, A. Y., B. GRYGIELSKA, B. LEBLOND, L. DESIRE, J. A. EBLE and S. P. WATSON (2010). "Phosphorylation of CLEC-2 is dependent on lipid rafts, actin polymerisation, secondary mediators and Rac." Blood: blood-2009-12-257212.

POOLE, A., J. M. GIBBINS, M. TURNER, M. J. VAN VUGT, J. G. VAN DE WINKEL, T. SAITO, V. L. TYBULEWICZ and S. P. WATSON (1997). "The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen." Embo J **16**(9): 2333-41.

PREVOST, N., D. S. WOULFE, H. JIANG, T. J. STALKER, P. MARCHESE, Z. M. RUGGERI and L. F. BRASS (2005). "Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets." Proc Natl Acad Sci U S A **102**(28): 9820-5.

PYZ, E., C. HUYSAMEN, A. S. MARSHALL, S. GORDON, P. R. TAYLOR and G. D. BROWN (2008). "Characterisation of murine MICL (CLEC12A) and evidence for an endogenous ligand." Eur J Immunol **38**(4): 1157-63.

QUEK, L. S., J. BOLEN and S. P. WATSON (1998). "A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen." Curr Biol **8**(20): 1137-40.

QUEK, L. S., J. M. PASQUET, I. HERS, R. CORNALL, G. KNIGHT, M. BARNES, M. L. HIBBS, A. R. DUNN, C. A. LOWELL and S. P. WATSON (2000). "Fyn and Lyn

phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway." Blood **96**(13): 4246-53.

RAMIREZ, M. I., G. MILLIEN, A. HINDS, Y. CAO, D. C. SELDIN and M. C. WILLIAMS (2003). "T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth." Dev Biol **256**(1): 61-72.

REDDY, K. B., D. M. SMITH and E. F. PLOW (2008). "Analysis of Fyn function in hemostasis and alphaIIbbeta3-integrin signaling." J Cell Sci **121**(Pt 10): 1641-8.

REID, D. M., N. A. GOW and G. D. BROWN (2009). "Pattern recognition: recent insights from Dectin-1." Curr Opin Immunol **21**(1): 30-7.

RIBA, R., C. E. HUGHES, A. GRAHAM, S. P. WATSON and K. M. NASEEM (2008). "Globular adiponectin induces platelet activation through the collagen receptor GPVI-Fc receptor gamma chain complex." J Thromb Haemost **6**(6): 1012-20.

RIBAS, G., M. J. NEVILLE and R. D. CAMPBELL (2001). "Single-nucleotide polymorphism detection by denaturing high-performance liquid chromatography and direct sequencing in genes in the MHC class III region encoding novel cell surface molecules." Immunogenetics **53**(5): 369-81.

ROBINSON, M. J., D. SANCHO, E. C. SLACK, S. LEIBUNDGUT-LANDMANN and C. REIS E SOUSA (2006). "Myeloid C-type lectins in innate immunity." Nat Immunol **7**(12): 1258-65.

ROCK, J., E. SCHNEIDER, J. R. GRUN, A. GRUTZKAU, R. KUPPERS, J. SCHMITZ and G. WINKELS (2007). "CD303 (BDCA-2) signals in plasmacytoid dendritic cells via a BCR-like signalosome involving Syk, Slp65 and PLCgamma2." Eur J Immunol **37**(12): 3564-75.

ROGERS, N. C., E. C. SLACK, A. D. EDWARDS, M. A. NOLTE, O. SCHULZ, E. SCHWEIGHOFFER, D. L. WILLIAMS, S. GORDON, V. L. TYBULEWICZ, G. D. BROWN and C. REIS E SOUSA (2005). "Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins." Immunity **22**(4): 507-17.

SACHS, U. J. and B. NIESWANDT (2007). "In vivo thrombus formation in murine models." Circ Res **100**(7): 979-91.

SAMAHA, F. F., C. HIBBARD, J. SACKS, H. CHEN, M. A. VARELLO, T. GEORGE and M. L. KAHN (2004). "Measurement of platelet collagen receptor density in human subjects." Arterioscler Thromb Vasc Biol **24**(11): e181-2.

SAMELSON, L. E., S. C. BUNNELL, R. P. TRIBLE, T. YAMAZAKI and W. ZHANG (1999). "Studies on the adapter molecule LAT." Cold Spring Harb Symp Quant Biol **64**: 259-63.

SANCHO, D., O. P. JOFFRE, A. M. KELLER, N. C. ROGERS, D. MARTINEZ, P. HERNANZ-FALCON, I. ROSEWELL and C. REIS E SOUSA (2009). "Identification of a dendritic cell receptor that couples sensing of necrosis to immunity." Nature **458**(7240): 899-903.

SANCHO, D., D. MOURAO-SA, O. P. JOFFRE, O. SCHULZ, N. C. ROGERS, D. J. PENNINGTON, J. R. CARLYLE and C. REIS E SOUSA (2008). "Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin." J Clin Invest **118**(6): 2098-110.

SARRATT, K. L., H. CHEN, M. M. ZUTTER, S. A. SANTORO, D. A. HAMMER and M. L. KAHN (2005). "GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow." Blood **106**(4): 1268-77.

SATHISH, J. G., S. FALATI, K. CROCE, C. CRUMP, B. C. FURIE, B. FURIE and A. W. POOLE (2004). "Antibody cross-linking of human platelet P-selectin induces calcium entry by a mechanism dependent upon Fcgamma receptor IIA." Thromb Haemost **92**(3): 598-605.

SATO, K., X. L. YANG, T. YUDATE, J. S. CHUNG, J. WU, K. LUBY-PHELPS, R. P. KIMBERLY, D. UNDERHILL, P. D. CRUZ, JR. and K. ARIIZUMI (2006). "Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses." J Biol Chem **281**(50): 38854-66.

SCHACHT, V., M. I. RAMIREZ, Y. K. HONG, S. HIRAKAWA, D. FENG, N. HARVEY, M. WILLIAMS, A. M. DVORAK, H. F. DVORAK, G. OLIVER and M. DETMAR (2003). "T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema." Embo J **22**(14): 3546-56.

SCHMAIER, A. A., Z. ZOU, A. KAZLAUSKAS, L. EMERT-SEDLAK, K. P. FONG, K. B. NEEVES, S. F. MALONEY, S. L. DIAMOND, S. P. KUNAPULI, J. WARE, L. F. BRASS, T. E. SMITHGALL, K. SAKSELA and M. L. KAHN (2009). "Molecular priming of Lyn by GPVI enables an immune receptor to adopt a hemostatic role." Proc Natl Acad Sci U S A **106**(50): 21167-72.

SEBZDA, E., C. HIBBARD, S. SWEENEY, F. ABTAHIAN, N. BEZMAN, G. CLEMENS, J. S. MALTZMAN, L. CHENG, F. LIU, M. TURNER, V. TYBULEWICZ, G. A. KORETZKY and M. L. KAHN (2006). "Syk and Slp-76 mutant mice reveal a cell-autonomous hematopoietic cell contribution to vascular development." Dev Cell **11**(3): 349-61.

SEET, B. T., D. M. BERRY, J. S. MALTZMAN, J. SHABASON, M. RAINA, G. A. KORETZKY, C. J. MCGLADE and T. PAWSON (2007). "Efficient T-cell receptor signaling

requires a high-affinity interaction between the Gads C-SH3 domain and the SLP-76 RxxK motif." Embo J **26**(3): 678-89.

SENIS, Y. A., M. G. TOMLINSON, S. ELLISON, A. MAZHARIAN, J. LIM, Y. ZHAO, K. N. KORNERUP, J. M. AUGER, S. G. THOMAS, T. DHANJAL, N. KALIA, J. W. ZHU, A. WEISS and S. P. WATSON (2009). "The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis." Blood **113**(20): 4942-54.

SENIS, Y. A., M. G. TOMLINSON, A. GARCIA, S. DUMON, V. L. HEATH, J. HERBERT, S. P. COBBOLD, J. C. SPALTON, S. AYMAN, R. ANTROBUS, N. ZITZMANN, R. BICKNELL, J. FRAMPTON, K. S. AUTHI, A. MARTIN, M. J. WAKELAM and S. P. WATSON (2007). "A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel immunoreceptor tyrosine-based inhibitory motif protein." Mol Cell Proteomics **6**(3): 548-64.

SHAPIRO, V. S., K. E. TRUITT, J. B. IMBODEN and A. WEISS (1997). "CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites." Mol Cell Biol **17**(7): 4051-8.

SHAW, A. S., L. K. GAUEN and Y. ZHU (1995). "Interactions of TCR tyrosine based activation motifs with tyrosine kinases." Semin Immunol **7**(1): 13-20.

SHIN, Y. and T. MORITA (1998). "Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib." Biochem Biophys Res Commun **245**(3): 741-5.

SINZ, A. (2003). "Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes." J Mass Spectrom **38**(12): 1225-37.

SMYTH, S. S., R. P. MCEVER, A. S. WEYRICH, C. N. MORRELL, M. R. HOFFMAN, G. M. AREPALLY, P. A. FRENCH, H. L. DAUERMAN and R. C. BECKER (2009). "Platelet functions beyond haemostasis." J Thromb Haemost.

SOMMERS, C. L., C. S. PARK, J. LEE, C. FENG, C. L. FULLER, A. GRINBERG, J. A. HILDEBRAND, E. LACANA, R. K. MENON, E. W. SHORES, L. E. SAMELSON and P. E. LOVE (2002). "A LAT mutation that inhibits T cell development yet induces lymphoproliferation." Science **296**(5575): 2040-3.

SU, Y. W. and H. JUMAA (2003). "LAT links the pre-BCR to calcium signaling." Immunity **19**(2): 295-305.

SUZUKI-INOUE, K., G. L. FULLER, A. GARCIA, J. A. EBLE, S. POHLMANN, O. INOUE, T. K. GARTNER, S. C. HUGHAN, A. C. PEARCE, G. D. LAING, R. D. THEAKSTON, E. SCHWEIGHOFFER, N. ZITZMANN, T. MORITA, V. L. TYBULEWICZ, Y. OZAKI and S. P. WATSON (2006). "A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2." Blood **107**(2): 542-9.

SUZUKI-INOUE, K., C. E. HUGHES, O. INOUE, M. KANEKO, O. CUYUN-LIRA, T. TAKAFUTA, S. P. WATSON and Y. OZAKI (2007). "Involvement of Src kinases and PLCgamma2 in clot retraction." Thromb Res **120**(2): 251-8.

SUZUKI-INOUE, K., O. INOUE, J. FRAMPTON and S. P. WATSON (2003). "Murine GPVI stimulates weak integrin activation in PLCgamma2-/- platelets: involvement of PLCgamma1 and PI3-kinase." Blood **102**(4): 1367-73.

SUZUKI-INOUE, K., Y. KATO, O. INOUE, M. K. KANEKO, K. MISHIMA, Y. YATOMI, Y. YAMAZAKI, H. NARIMATSU and Y. OZAKI (2007). "Involvement of the snake

toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells." J Biol Chem **282**(36): 25993-6001.

SUZUKI-INOUE, K., Y. OZAKI, M. KAINOH, Y. SHIN, Y. WU, Y. YATOMI, T. OHMORI, T. TANAKA, K. SATOH and T. MORITA (2001). "Rhodocytin induces platelet aggregation by interacting with glycoprotein Ia/IIa (GPIa/IIa, Integrin alpha 2beta 1). Involvement of GPIa/IIa-associated src and protein tyrosine phosphorylation." J Biol Chem **276**(2): 1643-52.

SUZUKI-INOUE, K., D. TULASNE, Y. SHEN, T. BORI-SANZ, O. INOUE, S. M. JUNG, M. MOROI, R. K. ANDREWS, M. C. BERNDT and S. P. WATSON (2002). "Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling." J Biol Chem **277**(24): 21561-6.

SWANN, P. G., S. ODOM, Y. J. ZHOU, Z. SZALLASI, P. M. BLUMBERG, P. DRABER and J. RIVERA (1999). "Requirement for a negative charge at threonine 60 of the FcRgamma for complete activation of Syk." J Biol Chem **274**(33): 23068-77.

TESTI, R., F. PULCINELLI, L. FRATI, P. P. GAZZANIGA and A. SANTONI (1990). "CD69 is expressed on platelets and mediates platelet activation and aggregation." J Exp Med **172**(3): 701-7.

THIERY, J. P. (2002). "Epithelial-mesenchymal transitions in tumour progression." Nat Rev Cancer **2**(6): 442-54.

TOMLINSON, M. G., S. D. CALAMINUS, O. BERLANGA, J. M. AUGER, T. BORI-SANZ, L. MEYAARD and S. P. WATSON (2007). "Collagen promotes sustained glycoprotein VI signaling in platelets and cell lines." J Thromb Haemost **5**(11): 2274-83.

TULL, S. P., S. I. ANDERSON, S. C. HUGHAN, S. P. WATSON, G. B. NASH and G. E. RAINGER (2006). "Cellular pathology of atherosclerosis: smooth muscle cells

promote adhesion of platelets to cocultured endothelial cells." Circ Res **98**(1): 98-104.

UHRIN, P., J. ZAUJEC, J. M. BREUSS, D. OLCAYDU, P. CHRENEK, H. STOCKINGER, E. FUERTBAUER, M. MOSER, P. HAIKO, R. FASSLER, K. ALITALO, B. R. BINDER and D. KERJASCHKI (2010). "Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation." Blood.

UNDERHILL, D. M. and H. S. GOODRIDGE (2007). "The many faces of ITAMs." Trends Immunol **28**(2): 66-73.

UNDERHILL, D. M., E. ROSSNAGLE, C. A. LOWELL and R. M. SIMMONS (2005). "Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production." Blood **106**(7): 2543-50.

VARGA-SZABO, D., A. BRAUN and B. NIESWANDT (2009). "Calcium signaling in platelets." J Thromb Haemost **7**(7): 1057-66.

VARGA-SZABO, D., I. PLEINES and B. NIESWANDT (2008). "Cell adhesion mechanisms in platelets." Arterioscler Thromb Vasc Biol **28**(3): 403-12.

WARDENBURG, J. B., C. FU, J. K. JACKMAN, H. FLOTOW, S. E. WILKINSON, D. H. WILLIAMS, R. JOHNSON, G. KONG, A. C. CHAN and P. R. FINDELL (1996). "Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function." J Biol Chem **271**(33): 19641-4.

WASHINGTON, A. V., R. L. SCHUBERT, L. QUIGLEY, T. DISIPIO, R. FELTZ, E. H. CHO and D. W. MCVICAR (2004). "A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets." Blood **104**(4): 1042-7.

WATSON, A. A., J. BROWN, K. HARLOS, J. A. EBLE, T. S. WALTER and C. A. O'CALLAGHAN (2007). "The crystal structure and mutational binding analysis of

the extracellular domain of the platelet-activating receptor CLEC-2." J Biol Chem **282**(5): 3165-72.

WATSON, A. A., J. A. EBLE and C. A. O'CALLAGHAN (2008). "Crystal structure of rhodocytin, a ligand for the platelet-activating receptor CLEC-2." Protein Sci **17**(9): 1611-6.

WATSON, S. P. and P. HARRISON (2010). The Vascular Function of Platelets. Postgraduate Haematology (Sixth Edition). A. V. HOFFBRAND, D. CATOVSKY and E. G. D. TUDDENHAM.

WECK, M. M., S. APPEL, D. WERTH, C. SINZGER, A. BRINGMANN, F. GRUNEBACH and P. BROSSART (2008). "hDectin-1 is involved in uptake and cross-presentation of cellular antigens." Blood **111**(8): 4264-72.

WEIS, W. I., M. E. TAYLOR and K. DRICKAMER (1998). "The C-type lectin superfamily in the immune system." Immunol Rev **163**: 19-34.

WELLS, C. A., J. A. SALVAGE-JONES, X. LI, K. HITCHENS, S. BUTCHER, R. Z. MURRAY, A. G. BECKHOUSE, Y. L. LO, S. MANZANERO, C. COBBOLD, K. SCHRODER, B. MA, S. ORR, L. STEWART, D. LEBUS, P. SOBIESZCZUK, D. A. HUME, J. STOW, H. BLANCHARD and R. B. ASHMAN (2008). "The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans." J Immunol **180**(11): 7404-13.

WILLMENT, J. A., S. GORDON and G. D. BROWN (2001). "Characterization of the human beta -glucan receptor and its alternatively spliced isoforms." J Biol Chem **276**(47): 43818-23.

WONEROW, P., A. OBERGFELL, J. I. WILDE, R. BOBE, N. ASAZUMA, T. BRDICKA, A. LEO, B. SCHRAVEN, V. HOREJSI, S. J. SHATTIL and S. P. WATSON (2002). "Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-

mediated phospholipase Cgamma2 regulation in platelets." Biochem J **364**(Pt 3): 755-65.

WONEROW, P., A. C. PEARCE, D. J. VAUX and S. P. WATSON (2003). "A critical role for phospholipase Cgamma2 in alphaIIbbeta3-mediated platelet spreading." J Biol Chem **278**(39): 37520-9.

WONEROW, P. and S. P. WATSON (2001). "The transmembrane adapter LAT plays a central role in immune receptor signalling." Oncogene **20**(44): 6273-83.

WONG, C., Y. LIU, J. YIP, R. CHAND, J. L. WEE, L. OATES, B. NIESWANDT, A. REHEMAN, H. NI, N. BEAUCHEMIN and D. E. JACKSON (2009). "CEACAM1 negatively regulates platelet-collagen interactions and thrombus growth in vitro and in vivo." Blood **113**(8): 1818-28.

WOODSIDE, D. G., A. OBERGFELL, L. LENG, J. L. WILSBACHER, C. K. MIRANTI, J. S. BRUGGE, S. J. SHATTIL and M. H. GINSBERG (2001). "Activation of Syk protein tyrosine kinase through interaction with integrin beta cytoplasmic domains." Curr Biol **11**(22): 1799-804.

WOODSIDE, D. G., A. OBERGFELL, A. TALAPATRA, D. A. CALDERWOOD, S. J. SHATTIL and M. H. GINSBERG (2002). "The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin beta cytoplasmic domains." J Biol Chem **277**(42): 39401-8.

WOULFE, D., H. JIANG, R. MORTENSEN, J. YANG and L. F. BRASS (2002). "Activation of Rap1B by G(i) family members in platelets." J Biol Chem **277**(26): 23382-90.

WU, Y., K. SUZUKI-INOUE, K. SATOH, N. ASAZUMA, Y. YATOMI, M. C. BERNDT and Y. OZAKI (2001). "Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling." Blood **97**(12): 3836-45.

XU, W. F., H. ANDERSEN, T. E. WHITMORE, S. R. PRESNELL, D. P. YEE, A. CHING, T. GILBERT, E. W. DAVIE and D. C. FOSTER (1998). "Cloning and characterization of human protease-activated receptor 4." Proc Natl Acad Sci U S A **95**(12): 6642-6.

YABLONSKI, D., T. KADLECEK and A. WEISS (2001). "Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT." Mol Cell Biol **21**(13): 4208-18.

YABLONSKI, D., M. R. KUHNE, T. KADLECEK and A. WEISS (1998). "Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell." Science **281**(5375): 413-6.

YAMASAKI, S., E. ISHIKAWA, M. SAKUMA, H. HARA, K. OGATA and T. SAITO (2008). "Mincle is an ITAM-coupled activating receptor that senses damaged cells." Nat Immunol **9**(10): 1179-88.

YAMASAKI, S., M. MATSUMOTO, O. TAKEUCHI, T. MATSUZAWA, E. ISHIKAWA, M. SAKUMA, H. TATENO, J. UNO, J. HIRABAYASHI, Y. MIKAMI, K. TAKEDA, S. AKIRA and T. SAITO (2009). "C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia." Proc Natl Acad Sci U S A **106**(6): 1897-902.

YANAGA, F., A. POOLE, J. ASSELIN, R. BLAKE, G. L. SCHIEVEN, E. A. CLARK, C. L. LAW and S. P. WATSON (1995). "Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor." Biochem J **311 (Pt 2)**: 471-8.

YODER, J., C. PHAM, Y. M. IIZUKA, O. KANAGAWA, S. K. LIU, J. MCGLADE and A. M. CHENG (2001). "Requirement for the SLP-76 adaptor GADS in T cell development." Science **291**(5510): 1987-91.

YU, P., M. KOSCO-VILBOIS, M. RICHARDS, G. KOHLER and M. C. LAMERS (1994). "Negative feedback regulation of IgE synthesis by murine CD23." Nature **369**(6483): 753-6.

ZARBOCK, A., K. SINGBARTL and K. LEY (2006). "Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation." J Clin Invest **116**(12): 3211-9.

ZEITLMANN, L., T. KNORR, M. KNOLL, C. ROMEO, P. SIRIM and W. KOLANUS (1998). "T cell activation induced by novel gain-of-function mutants of Syk and ZAP-70." J Biol Chem **273**(25): 15445-52.

ZHANG, W., J. SLOAN-LANCASTER, J. KITCHEN, R. P. TRIBLE and L. E. SAMELSON (1998). "LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation." Cell **92**(1): 83-92.

ZHANG, W., C. L. SOMMERS, D. N. BURSHTYN, C. C. STEBBINS, J. B. DEJARNETTE, R. P. TRIBLE, A. GRINBERG, H. C. TSAY, H. M. JACOBS, C. M. KESSLER, E. O. LONG, P. E. LOVE and L. E. SAMELSON (1999). "Essential role of LAT in T cell development." Immunity **10**(3): 323-32.

ZHANG, W., R. P. TRIBLE, M. ZHU, S. K. LIU, C. J. MCGLADE and L. E. SAMELSON (2000). "Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling." J Biol Chem **275**(30): 23355-61.

ZHANG, W. M., J. KAPYLA, J. S. PURANEN, C. G. KNIGHT, C. F. TIGER, O. T. PENTIKAINEN, M. S. JOHNSON, R. W. FARNDALE, J. HEINO and D. GULLBERG (2003). "alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens." J Biol Chem **278**(9): 7270-7.

ZHENG, Y. M., C. LIU, H. CHEN, D. LOCKE, J. C. RYAN and M. L. KAHN (2001). "Expression of the platelet receptor GPVI confers signaling via the Fc receptor gamma -chain in response to the snake venom convulxin but not to collagen." J Biol Chem **276**(16): 12999-3006.

ZHU, M., E. JANSSEN, K. LEUNG and W. ZHANG (2002). "Molecular cloning of a novel gene encoding a membrane-associated adaptor protein (LAX) in lymphocyte signaling." J Biol Chem **277**(48): 46151-8.

APPENDIX I

I.1 The LAT-family of adaptor proteins

The members of the family of LAT-like transmembrane adaptor proteins (TRAPs) are NTAL, LAX, PAG, LIME, TRIM and SIT (Figure I.1, adapted from (Horejsi *et al*, 2004). All of these family members are structurally related to LAT in that they have a short or limited extracellular domain, a transmembrane domain and a tyrosine-rich cytoplasmic tail. LAT has nine tyrosine residues in its cytoplasmic tail which, when phosphorylated, enable LAT to act as a protein scaffold to several SH2 domain-containing proteins including Gads, Grb2 and PLCγ2. LAT also contains an inner membrane proximal CxxC motif which undergoes palmitoylation and thereby allows LAT to integrate into GEMs (glycolipid enriched microdomains, aka lipid rafts). PAG, NTAL and LIME also have a CxxC motif proximal to the membrane which mediates their association with GEMs. SIT, TRIM and LAX are not found associated with GEMs and lack this CxxC motif.

Although LAT was first identified in T-cells, it is also found in platelets, NK cells and in B-lineage, but not mature B-cells, playing a role downstream of the pre-BCR (Jumaa *et al*, 1999; Minegishi *et al*, 1999; Pappu *et al*, 1999; Hayashi *et al*, 2000; Oya *et al*, 2003; Su and Jumaa, 2003). NTAL (Non-T-cell Activation Linker, also known as LAB, Linker for Activation of B-cells) is expressed in B-cells, NK and myeloid cells including mast cells. It contains eight cytoplasmic tyrosine residues which allow association with Grb2 but not PLCγ or SLP-65 and SLP-76 downstream of the BCR or Fc receptor (Brdicka *et al*, 2002; Janssen *et al*, 2003). PAG (Protein Associated with GEMs, also known as CBP, (CSK Binding Protein)), appears to be ubiquitously

expressed and contains nine cytoplasmic tyrosine residues, although the only clearly defined binding partner is Csk (c-Src Kinase). It has been proposed that PAG is a membrane anchor for Csk, allowing it to phosphorylate and thereby inhibit Src family kinases (Brdicka *et al*, 2000; Kawabuchi *et al*, 2000). LIME (Lck-Interacting Membrane Protein) is expressed in T-cells and contains five cytoplasmic tyrosine residues. It has been shown to associate with Lck, Fyn and Csk. Paradoxically, despite the association with Csk, Lck has been demonstrated to have increased activity when associated with LIME suggesting it is present in an active conformation (Brdickova *et al*, 2003; Hur *et al*, 2003).

LAX (Linker for Activation of X cells (where X is an unidentified cell)) has been shown to be expressed in T- and B-lymphocytes. It contains eight cytoplasmic tyrosine residues which are able to bind to Gads, Grb2 and PI-3kinase although it is unable to reconstitute T-cell receptor signalling in LAT-deficient Jurkat T-cells (Zhu *et al*, 2002). TRIM (T-cell Receptor Interacting Molecule) is expressed in T-cells where it forms a disulphide-linked homodimer. Each monomer contains three cytoplasmic tyrosines and the only binding partner described so far is PI-3 kinase. Over-expression of TRIM in Jurkat T-cells was shown to increase, by approximately two-fold, the levels of expression of the T-cell receptor on the cell surface and therefore the magnitude of TCR-mediated signalling (Bruyns *et al*, 1998; Kirchgessner *et al*, 2001). SIT (SHP2-Interacting TRAP) is expressed in T-cells where it forms a disulphide-linked homodimer. Each monomer contains five cytoplasmic tyrosines which, following TCR mediated activation, allow binding of Grb2, SHP2 and Csk. The association with SHP2 and Csk suggests an inhibitory role and indeed this has been shown experimentally by

over-expression in Jurkat T-cells (Marie-Cardine *et al*, 1999; Pfrepper *et al*, 2001). Interestingly, SIT is the only member of this family with an N-linked glycosylation group, which is found in the small extracellular domain. It has been proposed that this may be a binding site for an unidentified ligand although there is no direct evidence for this (Marie-Cardine *et al*, 1999).

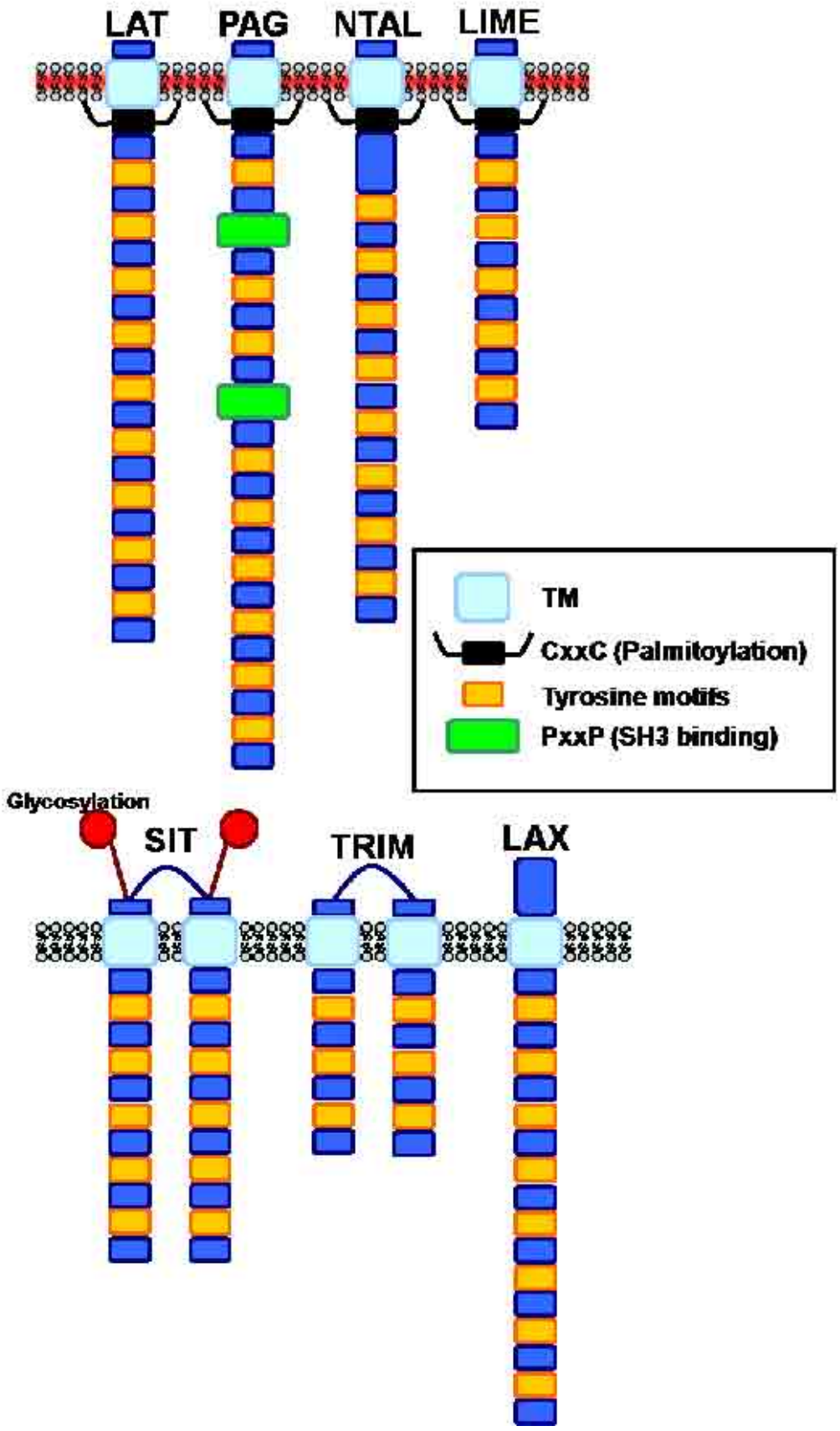

Figure I.1 – Transmembrane adaptor proteins. Adapted from (Horejsi *et al*, 2004).