CD148: a positive regulator of GPVI and αIIbβ3 proximal signalling in platelets

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Abstract

Platelets are small anucleate blood cells that plug holes in damage blood vessels. They do so by adhering to exposed extracellular matrix proteins at sites of injury and aggregating together. Platelet responsiveness to injury is controlled by a diverse repertoire of surface receptors that can be divided into two broad categories based on how they signal; the tyrosine kinased-linked receptors and the G protein-coupled receptors (GPCRs). There has been much work on elucidating the functions of tyrosine kinases in platelets, whereas protein tyrosine phosphatases (PTPs) have been under-investigated. To date, six non-transmembrane PTPs (NTPTPs), PTP-1B, Shp1, Shp2, MEG2-PTP, LMW-PTP and HePTP and a single receptor-like PTP (RPTP), CD148, have been identified in platelets. The main objective of this thesis was to determine the functional role of CD148 in platelets, which had never been studied in platelets. Using a mouse model, I demonstrate that CD148 is a critical positive regulator of signalling from the main collagen activation receptor GPVI and the fibrinogen integrin αIIbβ3, and also plays a minor role in regulating thrombin and thromboxane A_2 (TxA_2) mediated aggregation and secretion via the PAR-4 and TP receptors, respectively. The molecular mechanism of how CD148 regulates signalling from so many receptors is by maintaining a pool of active Src family kinases (SFKs) in platelets, which it does by dephosphorylating a tyrosine residue in the C-terminal of all SFKs. In an attempt to identify other PTPs that perform a similar function to CD148 in platelets, I analyzed platelets from PTP-1B- and TC-PTP-deficient mouse models for functional and phosphorylation defects. PTP-1B-deficient platelets exhibited minor aggregation/secretion and phosphorylation defects relative to CD148-deficient platelets; and TC-PTP, which I show to be expressed in human and mouse platelets for the first time, is involved in platelet development. My conclusion is that CD148, PTP-1B and TC-PTP have distinct functional roles in platelets.

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Abbreviations

AA	arachidonic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCR	B-cell receptor
CD148 TM-KO	CD148 transmembrane knockout mouse
CRP	collagen-related peptide
CSK	C-terminal Src kinase
COX1	cyclooxygenase-1
DAG	1,2-diacylglycerol
ECM	extracellular matrix
ER	endoplasmic reticulum
FNIII	fibronectin type 3
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HSC	haematopoietic stem cell
IP	immunoprecipitate
IP_3	inositol 1,4,5-trisphosphate
IRS-1	insulin receptor substrate-1
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
MAM	meprin/A4/μ domain
NO	nitric oxide
NTPTP	non transmembrane PTP
OCS	open canalicular system
PAS	protein-A sepharose
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I2
PGS	protein-G sepharose
PKA	protein kinase A
PS	phosphatidylserine
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTB	phosphotyrosine binding
RPTP	receptor-like PTP
SEM	sub-endothelial matrix
SFK	src family kinase
SH2	src homology 2 domain
SH3	src homology 3 domain
TCR	T-cell receptor
TKLR	tyrosine kinase-linked receptor
TM-KO	transmembrane knockout
TxA_2	thromboxane A2
VASP	vasodilator-stimulated phosphoprotein
VWF	Von Willebrand factor
WCL	whole cell lysates

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CHAPTER 1-

GENERAL INTRODUCTION

1.1 Platelet Overview

Platelets play a fundamental role in blood clotting and wound repair. In the event of vascular injury, the sub-endothelial extracellular matrix (ECM) becomes exposed. Exposure of circulating platelets to the ECM triggers their activation and subsequent formation of a haemostatic plug, preventing excessive blood loss. This process is crucial for normal haemostasis. However, unwanted platelet activation can have severe, adverse affects on human health. Uncontrolled thrombus formation in diseased vessels such as at the sites of ruptured atherosclerotic plaques, can lead to occlusion of the vessel resulting in myocardial infarction or stroke, which combined are the leading cause of death in the western world. Platelets are therefore a major target for current and new anti-thrombotic drugs.

1.2 Platelet Formation

Platelets are one of several types of blood cells that exist in the human body. All blood cells are related by a common ancestry, the haematopoietic stem cell (HSC), which resides in the bone marrow of adults. HSCs give rise to all the different types of blood cells and also have the capacity to self renew, so that there is always a pool of HSCs at sites of haematopoiesis. Blood cells are divided into two broad categories, the myeloid (macrophages, neutrophils, basophils, eosinophils, erythrocytes, platelets) and lymphoid (T and B cells) lineages (Figure 1.1). It is well established that platelets and erythrocytes (both of which are anucleate) are derived from a common progenitor, the colony forming unit-megakaryocyte/erythroid, which bifurcates into the megakaryocyte and erythroid lineages. Megakaryocytes are large polyploid cells that reside in the bone marrow and produce platelets, which enter the peripheral circulation. Immature megakaryocytes migrate along a SDF-1α gradient from the bone niche to the vascular niche in the bone marrow where they release platelets into the circulation (Avecilla et al. 2004). Megakaryocytes seldom if ever enter the circulation. The most well

established model of how platelets are formed is the proplatelet model (Italiano et al. 2003; Battinelli et al. 2007). At the vascular niche, megakaryocytes extend long, dynamic protrusions called 'proplatelets' into microvessels and platelets bud off of these protrusions (Figure 1.2) (Italiano et al. 2003; Battinelli et al. 2007). It is unclear how these bud-like structures that give rise to platelets are released from the proplatelet extensions. Platelet formation is regulated by the cytokine thrombopoietin, which is synthesized by the liver and kidneys and signals through the c-Mpl receptor on megakaryoctyes (Kaushansky 2009). It is estimated that each megakarycoyte produces between 5,000 – 10,000 platelets in its lifetime (Italiano et al. 2003). The physiological concentration range of platelets in the circulation of a healthy adult human is $1.5 – 4 \times 10^9$/litre, which is approximately 2,000-fold lower than the concentration of erythrocytes (A. Michelson, Platelets, 2nd Edition). The lifespan of a human platelet is 7 – 10 days. Old and defective platelets are removed from the circulation by resident phagocytes in the spleen and liver (A. Michelson, Platelets, 2nd Edition).

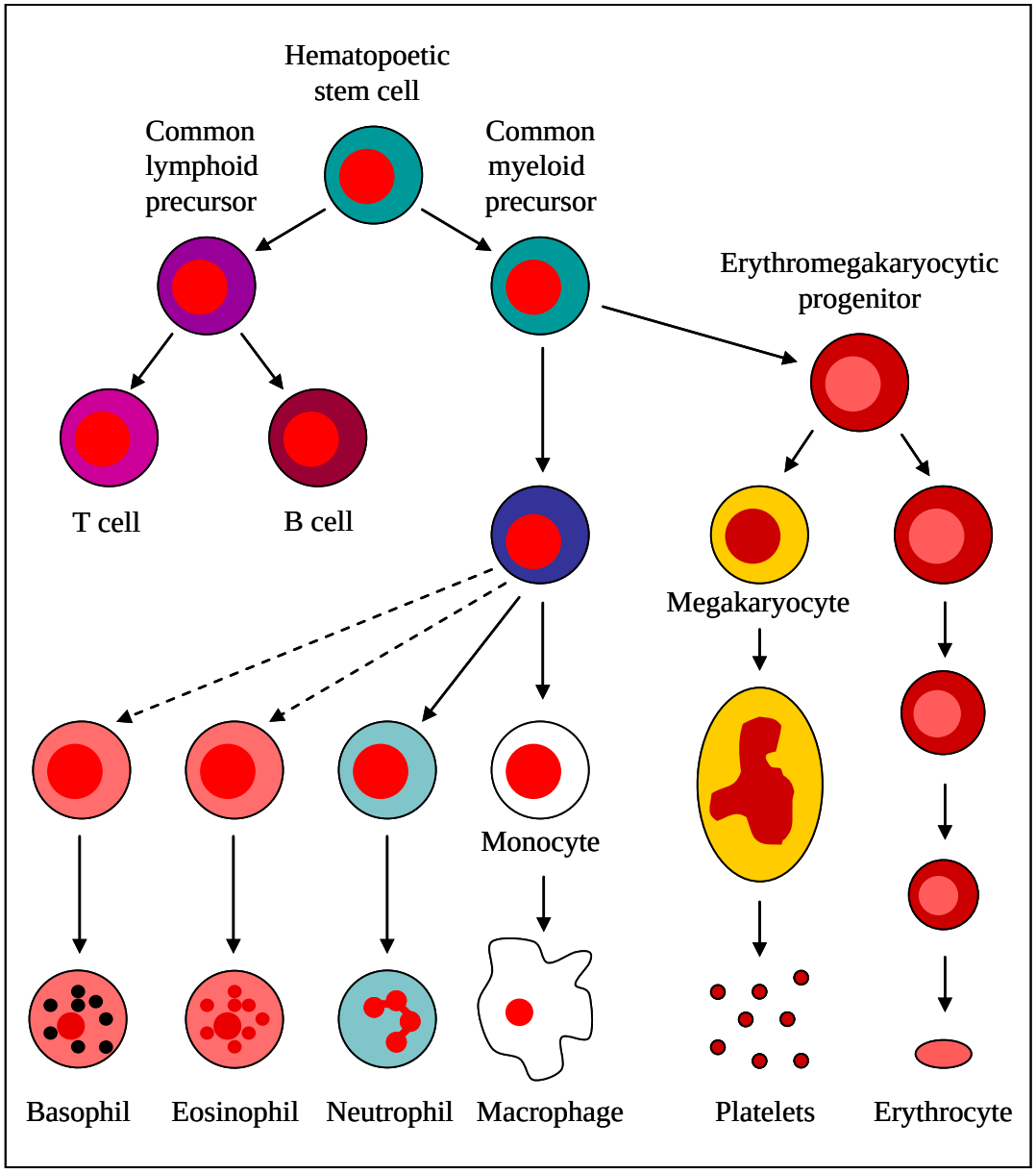


Figure 1.1: Haematopoiesis. A schematic representation of the various blood cell lineages in the human body including the pathway of platelet formation.

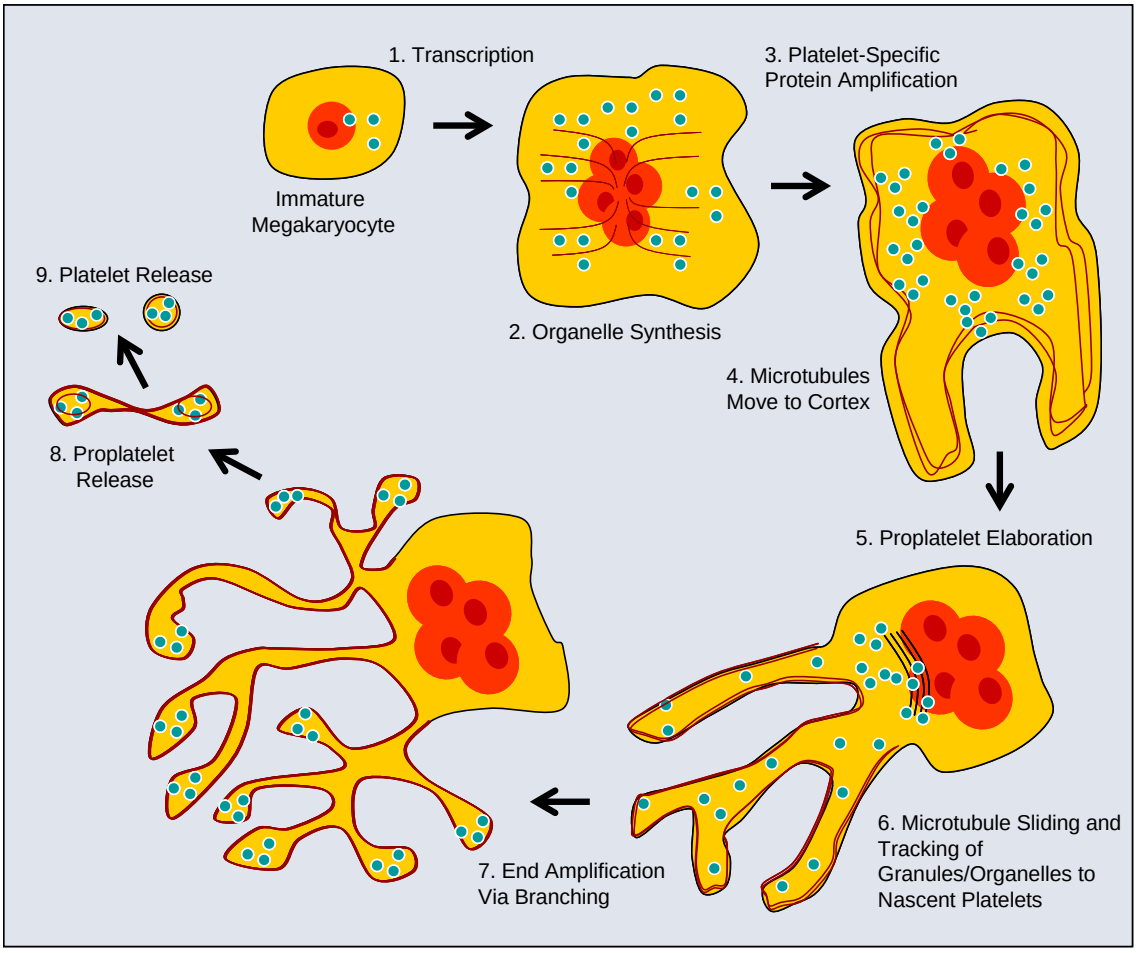


Figure 1.2: Proplatelet model of platelet formation from megakaryocytes. A number of events occur during the transition of an immature megakaryocyte to the release of platelets. Firstly the immature megakaryocyte undergoes endomitosis and transcriptional activation. Organelle synthesis and platelet-specific protein amplification then follows before the formation of microtubules within the cell, which move towards the cortex. The next stage is the initiation of proplatelet formation. Large pseudopodia elongate from the megakaryocyte and organelles are moved individually over the microtubules into the proplatelet ends, where nascent platelets assemble. The entire cytoplasm of the megakaryocyte is transformed into a mass of proplatelets, which bud off into the microcirculation where new platelets are released. (figure adapted from A. Michelson, Platelets, 2nd Edition)

1.3 Platelet Structure

Since platelets are sub-cellular fragments of megakaryocytes, they are composed of megakaryocyte membranes, cytoplasm, granules and organelles, but lack a nucleus. Platelets are the smallest of the blood cells and have a discoid shape when they are quiescent or 'resting'. The dimensions of a normal resting human platelet are 2 – 5 μm in diameter and approximately 0.5 μm in thickness (A. Michelson, Platelets, 2nd Edition). The small size and discoid shape enables platelets to be pushed towards the vessel edge by larger blood cells (primarily erythrocytes) in flowing blood, positioning them close to the endothelium, ideally placed to detect and rapidly respond to any vascular damage (Ruggeri 2009). The surface of the resting platelet appears featureless and lacks any protrusions. High-resolution scanning electron microscopy shows that the platelet plasma membrane is made up of many tiny folds that provides additional membrane needed when platelets spread on surfaces (Hartwig 2006). The plasma membrane also has a large network of invaginations called the open canalicular system (OCS) that provides a large surface area between the cytoplasm and blood to facilitate exchange of small molecules, but restricts entry of larger proteins such as antibodies (Hartwig 2006).

The cytoskeleton system of the platelet is essential for maintaining the discoid shape of the platelet as it encounters high fluid shear forces. The cytoskeleton also mediates the morphological changes that a platelet undergoes after it becomes activated or comes into contact with a site of vascular injury. The three layers of the platelet cytoskeletal system include: (1) a spectrin-based skeleton that is attached to the cytoplasmic surface of the plasma membrane; (2) a microtubule that is rolled into a coil and lines the circumference of the platelet; and (3) a rigid actin filament-based network that fills the cytoplasmic space of the cell (Italiano et al. 2003; Hartwig 2006). Actin is the most abundant platelet protein. Actin filaments are directly attached to the spectrin-based skeleton and are interconnected by filamin

and actinin (Italiano et al. 2003; Hartwig 2006). Of importance to the structural organization of the resting platelet is the interaction between filamin and one of the most abundant platelet surface glycoproteins GPIb-IX-V, which mediates tethering to sites of vascular injury. The microtubule coil beneath the plasma membrane is mainly composed of the β1 isoform of tubulin (Italiano et al. 2003; Hartwig 2006). Platelet activation initiates remodelling of the cytoskeleton that is required for formation of filopodia and lamellipodia. Actin-filament cleavage is mediated by gelsolin (Hartwig 2006). Following platelet aggregation, platelets must generate a contractile force that acts to stabilize the aggregate and plug the site of inujury. Platelets express non-muscle myosin IIA and B which are essential for generating the contractile force of platelets (Hartwig 2006).

Since platelets lack a nucleus they have a limited ability to express new proteins and as such are equipped with a wide range of pre-synthesized molecules needed to exert their physiological responses. The platelet cytoplasm contains typical cellular organelles such as mitochondria, lysosomes and endoplasmic reticulum as well as two types of platelet specific granules, α-granules and dense granules. α-granules are the largest and most abundant platelet granules (~80 α-granules/human platelet) with a size of approximately 0.2 - 0.4 μm in diameter (Hartwig 2006; Italiano et al. 2009). These granules store matrix adhesive proteins such as fibrinogen, fibronectin and VWF, and also have glycoprotein receptors embedded in their membranes, which promote adhesion between platelets and the ECM. P-selectin, which is not expressed on the surface of resting platelets is stored in the membranes of α-granules as well as a proportion of the major platelet adherence receptors, GPIb-IX-V and the integrin αIIbβ3. α-granules also contain coagulation factors, including prothrombin, factors V and XIII that promote thrombin generation and fibrin formation (Harrison et al. 1993).

There are approximately 10-fold fewer dense granules than α-granules in platelets. Dense granules are also smaller in size than the α-granule with a diameter of approximately

0.15 μm. Dense granules contain high concentrations of small molecules that play important roles in cell activation, including ADP, ATP, GDP, GTP, Ca^{2+} and the vasoconstrictor serotonin (Gunay-Aygun et al. 2004). ADP is necessary for the 'secondary' platelet activation and platelet recruitment to the growing thrombi. Dense and lysosomal granules also contain glycoproteins embedded in their membranes that are incorporated into the plasma membrane following granule fusion, including CD63, LAMP1, LAMP2 and CD68 (Nofer et al. 2004).

There are a number of additional features which enable the platelet to carry out its physiological functions. Platelets express high levels of glycoprotein receptors and signalling proteins in their plasma membrane that allow them to respond rapidly to vascular damage (described in detail below). In addition, the surface of the activated platelet becomes enriched with negatively charged phosphatidylserine (PS), following membrane flipping, which provides a platform for assembly of clotting factors, thereby initiating increased thrombin production at the site of injury and enhancing platelet activation (Zwaal et al. 2004).

1.4 Platelet Function

The main physiological function of platelets is to plug holes in damaged blood vessels, thus preventing excessive blood loss. There is a growing body of evidence that platelets are also involved in angiogenesis, inflammation and immunity. Platelets rapidly adhere to exposed ECM, become activated and recruit other platelets from the circulation to form a haemostatic plug at a site of vascular injury. The surface of activated platelets also accelerates thrombin generation at the site of injury that acts to consolidate the platelet plug. The role of platelets in thrombus formation can be broken down into several stages described on the next page and in Figure 1.3 (Ruggeri 2002; Auger et al. 2005; Bennett et al. 2009; Ruggeri 2009).

1) **Tethering, rolling and adhesion**. Under high shear conditions (>500/s), such as those found in small arteries, arterioles and stenotic diseased arteries, platelets initially tether and role along the site of injury. Tethering and rolling is mediated by the interaction between the high molecular weight plasma glycoprotein VWF that binds to exposed collagen at the site of injury, and the platelet surface receptor GPIb-IX-V. Platelet tethering and rolling on VWF occurs due to the rapid 'on/off' rates of association/dissociation and between GPIb-IX-V and immobilized VWF. This interaction also generates a weak activatory signal within the platelets that is believed to prime them for further activation. The VWF/GPIb-IX-V interaction is not required for platelet adhesion under low shear (<500/s) conditions, which are found in large arteries and veins. Under these conditions, platelets bind directly to collagen and other ECM proteins via the integrins $\alpha_2\beta_1$ (collagen), $\alpha5\beta1$ (fibronectin), $\alpha6\beta1$ (laminin) and $\alpha IIb\beta3$ (fibrinogen, fibronectin, VWF).

2) **Integrin activation and stable adhesion**. The initial tethering, rolling and non-firm adhesion of platelets with the ECM allows the low affinity collagen receptor GPVI to bind to collagen, cluster, and initiate a rapid, sustained activatory signal that triggers integrins ($\alpha IIb\beta3$ and $\alpha_2\beta_1$) to undergo a conformational change from a low affinity to a high affinity state. The platelets can then stably adhere to the exposed ECM and VWF.

3) **Spreading.** Platelet activation induces several dramatic morphological changes in the platelets mediated by cytoskeletal remodelling. Firmly adherent, activated platelets extent long finger-like projections call 'filopodia' that then fill in with 'lamellipodia'. Filopoida allow platelets to more firmly adhere to the damaged surface and also to trap

platelets flowing by in the bloodstream. Spreading allows the platelets to cover a larger surface area of the vascular lesion.

4) **Secretion.** Activated platelets rapidly secrete the contents of their α- and dense granules, and synthesize and release the potent platelet agonist thromboxane A_2 (TxA_2). ADP and TxA_2 released by activated platelets act synergistically to induce maximal platelet activation and also to recruit more platelets to the growing platelet plug. The release of adhesion proteins such as fibrinogen and VWF contribute to formation and growth of the thrombus. Released clotting factors (factors V, XIII and prothrombin) accelerate the localized generation of thrombin.

5) **Aggregation**. Plasma and platelet derived fibrinogen and VWF mediate platelet aggregation. High affinity interactions are formed between αIIbβ3 and fibrinogen, and αIIbβ3 and VWF, leading to rapid thrombus growth.

6) **Thrombus stabilisation**. PS exposed on the activated platelet surface provides a platform for assembly of components of the coagulation cascade (factors V, VIII, XI and X) leading to thrombin generation (Figure 1.4). Tissue factor exposed on damaged endothelial cells and smooth muscle cells in the vessel wall binds factor VIIa and initiates the extrinsic coagulation pathway, which also culminates in thrombin generation. A high localized concentration of thrombin enhances platelet activation and converts fibrinogen to fibrin, stabilizing the thrombus and making it more resistant to embolization due to the shear forces.

7) **Clot retraction**. The thrombus is stabilised by retraction of the platelet-fibrinogen/fibrin aggregate. Contractile forces are generated by non-muscule myosin

II pulling on the interlocking cytoskeletons of adjacent platelets. The interconnections between platelets are mediated by fibrinogen-αIIbβ3 interactions.

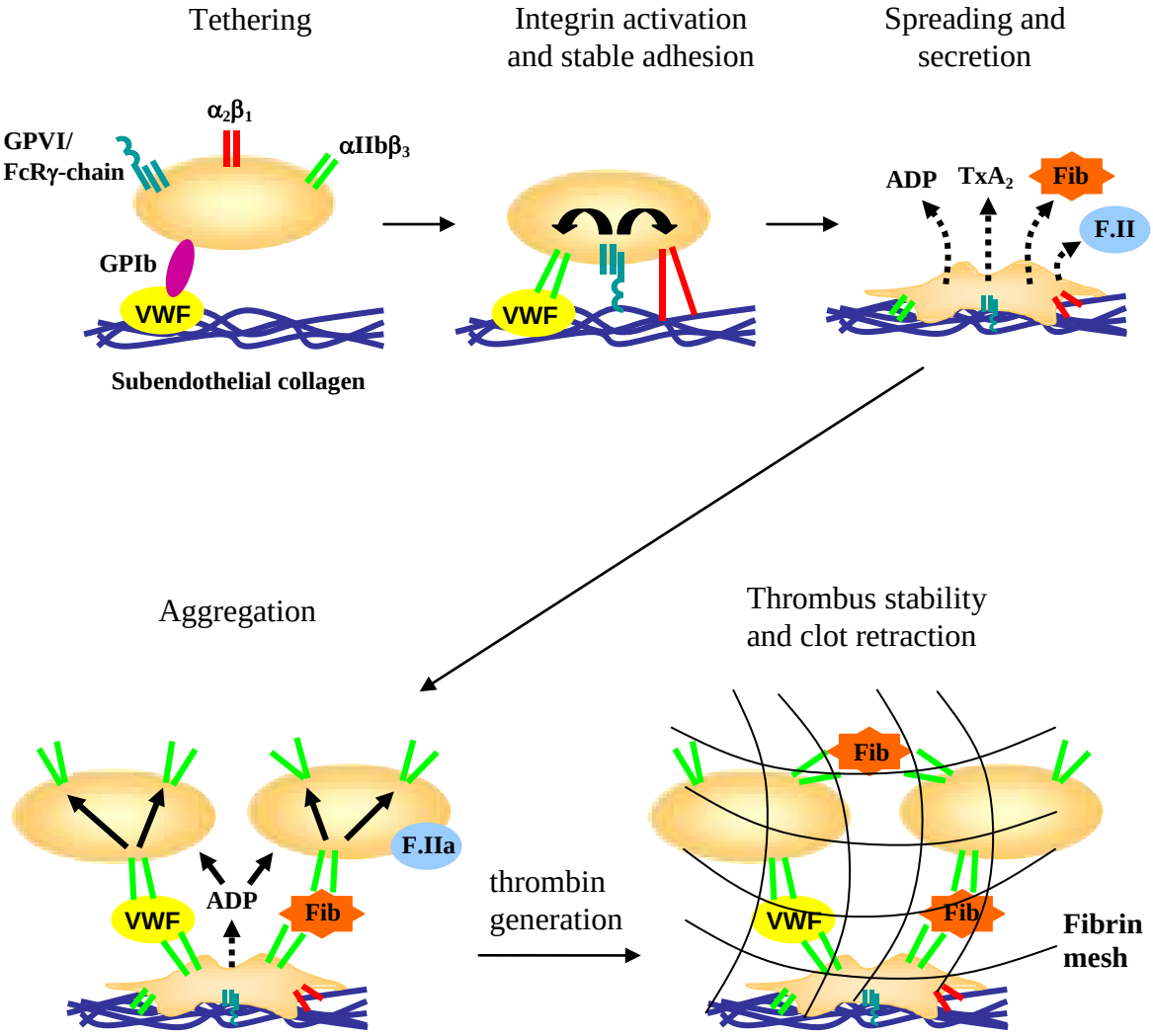


Figure 1.3: Platelet activation. A schematic representation of the various stages of platelet activation including: tethering, integrin activation and stable adhesion, spreading and secretion, aggregation and thrombus stability and clot retraction.

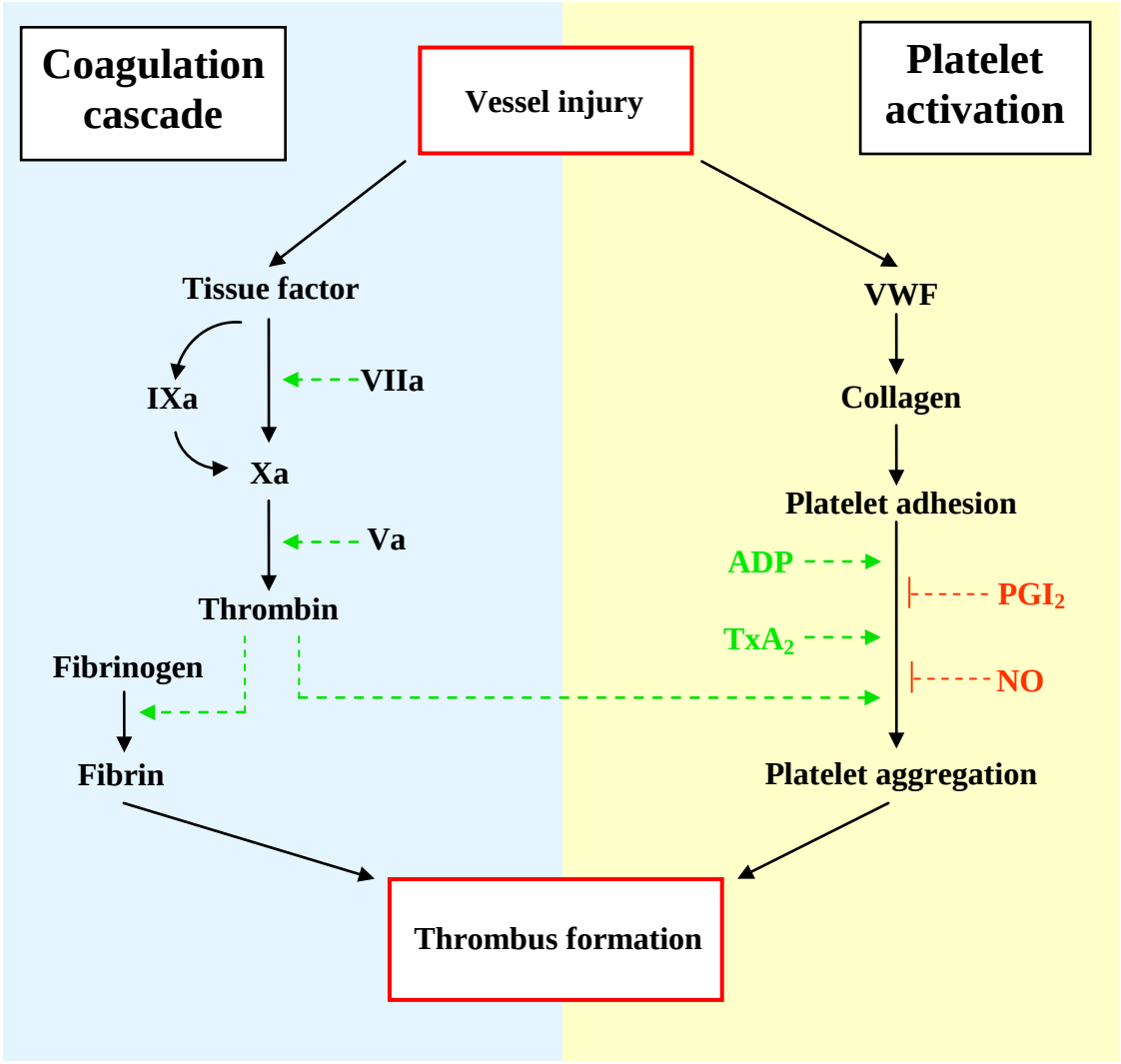


Figure 1.4: The coagulation cascade and platelet activation. Thrombin plays an essential role in both the coagulation cascade and platelet activation. Thrombin is generated on the surface of activated platelets. This greatly enhances the localised generation of thrombin. Surface thrombin is also protected from degradation and inhibition.

1.5 Platelet Receptors

Platelets express a wide variety of surface receptors that regulate all aspects of platelet function. They can be divided into two broad groups based on their signalling pathways: the tyrosine kinase-linked receptors and the G protein-coupled receptors (GPCRs). A large proportion of these receptors have been well characterized, however, novel receptors/receptor-like proteins continue to be discovered including CLEC-2, CD148 and CEACAM1 (Suzuki-Inoue et al. 2006; Senis et al. 2009; Wong et al. 2009). The functional roles and molecular functions of some of the most important and best characterized platelet receptors are described below. The focus is on tyrosine kinase-linked receptors as tyrosine phosphorylation/dephosphorylation is a major subject of investigation in this thesis, however, some of the main GPCRs will also be discussed.

1.5.1 Tyrosine kinase-linked receptors

Tyrosine kinase-linked receptors signal through a series of tyrosine phosphorylation events that culminate in specific cellular responses. Some of the most important and well characterized tyrosine kinase-linked receptors in platelets are the collagen activation receptor GPVI/FcR γ-chain complex; the collagen adhesion integrin $\alpha_2\beta_1$; the fibrinogen integrin αIIbβ3 and the VWF receptor GPIb-IX-V. The distinguishing features of these receptors and their signalling pathways are described in detail in the following sections.

1.5.1.1 Collagen receptors – GPVI and $\alpha_2\beta_1$

Collagen is the most abundant protein found in animals (Shoulders et al. 2009). This fibrous structural protein is the main component of the ECM, lining all vessel walls. Blood comes into contact with collagen following vessel injury. There are many different forms of

collagen, but the more thrombogenic types are collagen I and III found in the most luminal regions of the subendothelial matrix (Shoulders et al. 2009). Platelets express two receptors for collagen: the immunoreceptor GPVI and the integrin α2β1 (Nieswandt et al. 2003). GPVI and α2β1 bind to different regions of collagen fibrils, GPVI binding to the GPO repeat and α2β1 binding to the GFOGER repeat with low and high affinities, respectively. GPVI primarily mediates platelet activation through a rapid sustained signal and α2β1 primarily mediates firm adhesion (Nieswandt et al. 2003). The two receptors act in a cooperative manner to reinforce each other's activities, but also have distinct functions in regulating platelet adhesion and thrombus formation on collagen (Figure 1.6) (Holtkotter et al. 2002; Chen et al. 2003; Massberg et al. 2003; Auger et al. 2005; Sarratt et al. 2005).

1.5.1.1.1 GPVI/FcR γ-chain complex

GPVI is a 62 kDa type I transmembrane receptor composed of 2 IgG–like domains in its extracellular region, a mucin-like stalk, a transmembrane region, and a short 51-aa cytoplasmic tail that is expressed exclusively in platelets and megakaryocytes (Clemetson et al. 1999). Resting human platelets express approximately 2,000 – 4,500 copies of GPVI on their surface, which does not increase following platelet activation (Best et al. 2003; Samaha et al. 2004). However, it is shed from the surface of activated platelets as a means of regulating its signalling (Bergmeier et al. 2004; Gardiner et al. 2007). Within the transmembrane region of GPVI is a positively charged arginine which mediates a constitutive noncovalent interaction with the FcR γ-chain (Watson et al. 2005). The association of GPVI with the γ-chain is essential for expression of GPVI on the platelet surface and also critical for signalling (Gibbins et al. 1996; Poole et al. 1997). GPVI exists partly as a dimer on the surface of resting platelets and ligand mediated clustering triggers signalling, as described below (Herr 2009; Horii et al. 2009; Jung et al. 2009). Physiological ligands of GPVI include

collagen and laminin (Inoue et al. 2006). Non-physiological ligands commonly used for research purposes include: the GPVI-specific collagen-derived synthetic peptide collagen-related peptide (CRP) (Asselin et al. 1999); the snake toxin convulxin which also binds to GPIb-IX-V (Kanaji et al. 2003); and monoclonal antibodies which binds to the extracellular region of GPVI triggering signalling and shedding (Nieswandt et al. 2000; Nieswandt et al. 2001; Boylan et al. 2004).

The GPVI signalling pathway is essentially a hybrid of the T and B cell receptor signalling pathways sharing many of the same signalling molecules (Watson et al. 1998). A critical early GPVI signalling event is phosphorylation of the conserved immunoreceptor tyrosine-based activation motif (ITAM) (YXX[L/I]X_{6-12}YXX[L/I]) in the FcR γ-chain (Gibbins et al. 1996; Poole et al. 1997). This is mediated by Fyn and Lyn which are constitutively associated with a proline-rich region in the cytoplasmic tail of GPVI via their SH3 domains (Figure 1.5) (Ezumi et al. 1998; Briddon et al. 1999). Genetic evidence using Fyn and Lyn single and double-deficient mice demonstrated that Fyn positively regulates GPVI signalling; Lyn both positively and negatively regulates GPVI signalling; and other SFKs may compensate in the absence of Fyn and Lyn (Quek et al. 2000).

The tyrosine kinase Syk subsequently binds to the phosphorylated FcR γ-chain ITAM via its tandem SH2 domains and undergoes autophosphorylation and phosphorylation by SFKs, which renders it maximally activated and also mediates interactions with other signalling proteins (Figure 1.5) (Sada et al. 2001; Watson et al. 2005). Activated Syk, propagates the signal in lipid rafts, which involves the formation of the 'LAT signalosome' consisting of the adaptors LAT, SLP-76, Gads; Vav small GTPases; Tec family kinases; and effector proteins, including PI 3-kinase and PLCγ2 (Figure 1.5) (Watson et al. 2005). PLCγ2 induces formation of the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates PKC and IP_3 induces the release of Ca^{2+} from intracellular

stores and subsequent Ca^{2+} entry. GPVI signalling culminates in rapid integrin activation, secretion of α- and dense-granules, and the release of the secondary mediators ADP and TxA_2, which play an important role in amplifying primary stimulatory signals and thrombus growth (Nieswandt et al. 2003; Offermanns 2006).

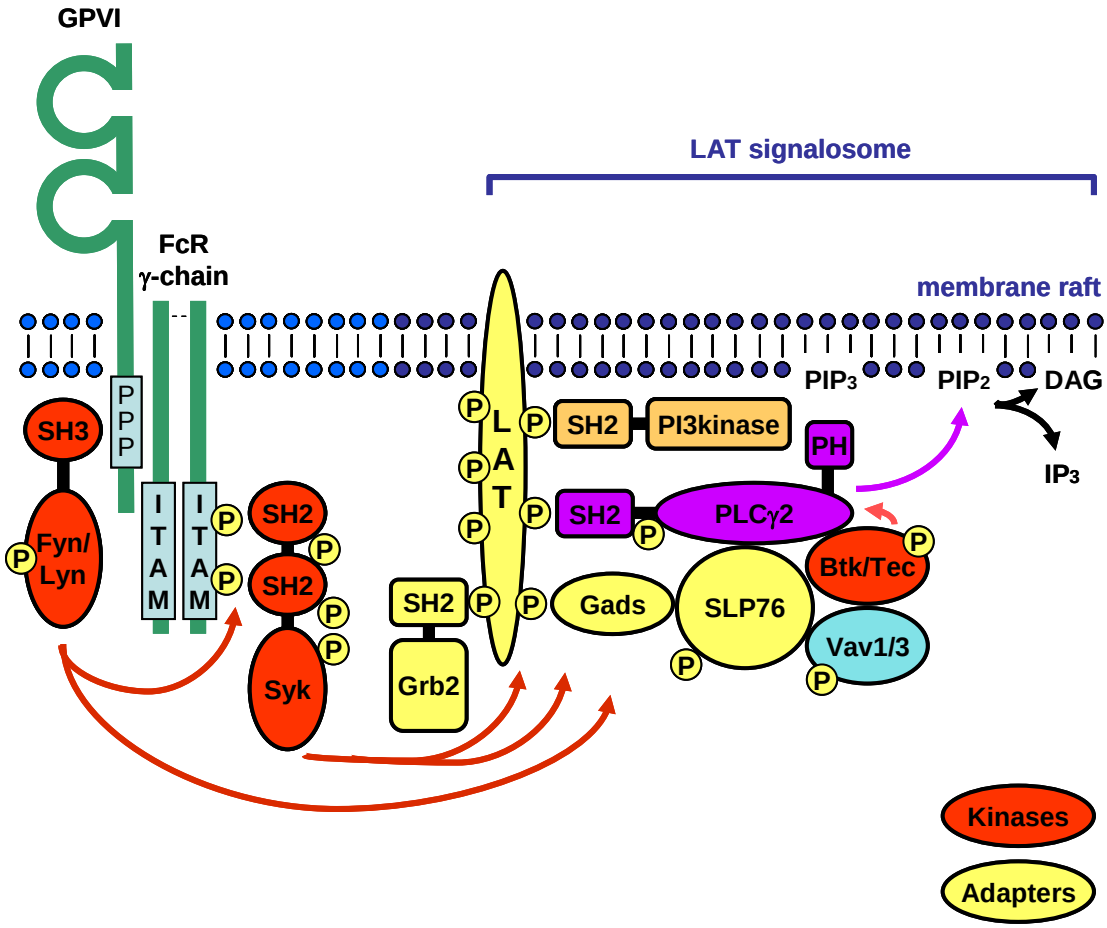


Figure 1.5: GPVI signalling cascade. Crosslinking of GPVI induces tyrosine phosphorylation of the FcRγ-chain ITAM by the Src family kinases, Fyn and Lyn, which are constitutively bound to the proline-rich region in the GPVI cytosolic tail. The phosphorylated ITAM recruits Syk and initiates a signalling cascade that leads to formation of the LAT signalosome and subsequent activation of PLCγ2. PLCγ2 associates directly with LAT, and indirectly via the adapters Gads and SLP-76. Members of the Tec and Vav families support activation of PLCγ2 (figure reproduced from Watson et al. 2005).

Patients with quantitative or qualitative defects in GPVI exhibit a mild bleeding tendency due to masking from various compensatory mechanisms. Several patients have been identified with reduced levels of GPVI surface expression due to auto-antibodies which induce GPVI shedding (Boylan et al. 2004). Two recent reports identified patients with genetic defects in GPVI resulting in mild bleeding diathesis (Dumont et al. 2009; Hermans et al. 2009). GPVI-deficient mice (genetically modified or GPVI-depleted) phenocopy the condition reported in GPVI deficient humans and confirm the essential role of this receptor in collagen-induced platelet activation, thrombosis and haemostasis (Konishi et al. 2002; Kato et al. 2003; Massberg et al. 2003; Konstantinides et al. 2006; Lockyer et al. 2006).

1.5.1.1.2 Integrin $\alpha_2\beta_1$

The integrin $\alpha_2\beta_1$ (also known as GPIa/IIa) was the first collagen receptor identified on platelets and serves mainly as an adhesion receptor (Figure 1.6) (Holtkotter et al. 2002; Nieswandt et al. 2003). It is widely expressed and binds with high affinity to collagen types I – V (Holtkotter et al. 2002; Leitinger et al. 2002). It is expressed at approximately 2,000 – 4,000 copies per resting human platelet, which varies considerably between individuals (Best et al. 2003; Samaha et al. 2004). Its level of expression does not change following platelet activation. The integrin $\alpha2\beta1$ is normally in a 'low-affinity' state on the surface of resting platelets and undergoes a conformation change to a 'high-affinity' state in response to agonist-induced 'inside-out' signalling (Jung et al. 2000; Lecut et al. 2004). In addition to mediating platelet adhesion, $\alpha_2\beta_1$ also reinforces the low affinity GPVI-collagen interaction and mediates weak 'outside-in' signalling (Figure 1.6) (Jung et al. 2000; Inoue et al. 2003; Nieswandt et al. 2003). Although the $\alpha2\beta1$ signalling pathway contains many of the same signalling molecules as the GPVI signalling pathway, including Src, Syk, SLP-76, and PLCγ2, $\alpha2\beta1$ signalling is much weaker than GPVI signalling, and likely plays little role in thrombus

formation (Jung et al. 2000; Inoue et al. 2003; Nieswandt et al. 2003). Genetic and pharmacological studies demonstrate that α2β1 plays a minor role in platelet aggregate formation on collagen under flow and has little or no protective effect in arterial thrombosis (Nieswandt et al. 2001; Holtkotter et al. 2002; He et al. 2003; Kuijpers et al. 2003; Auger et al. 2005; Kuijpers et al. 2007).

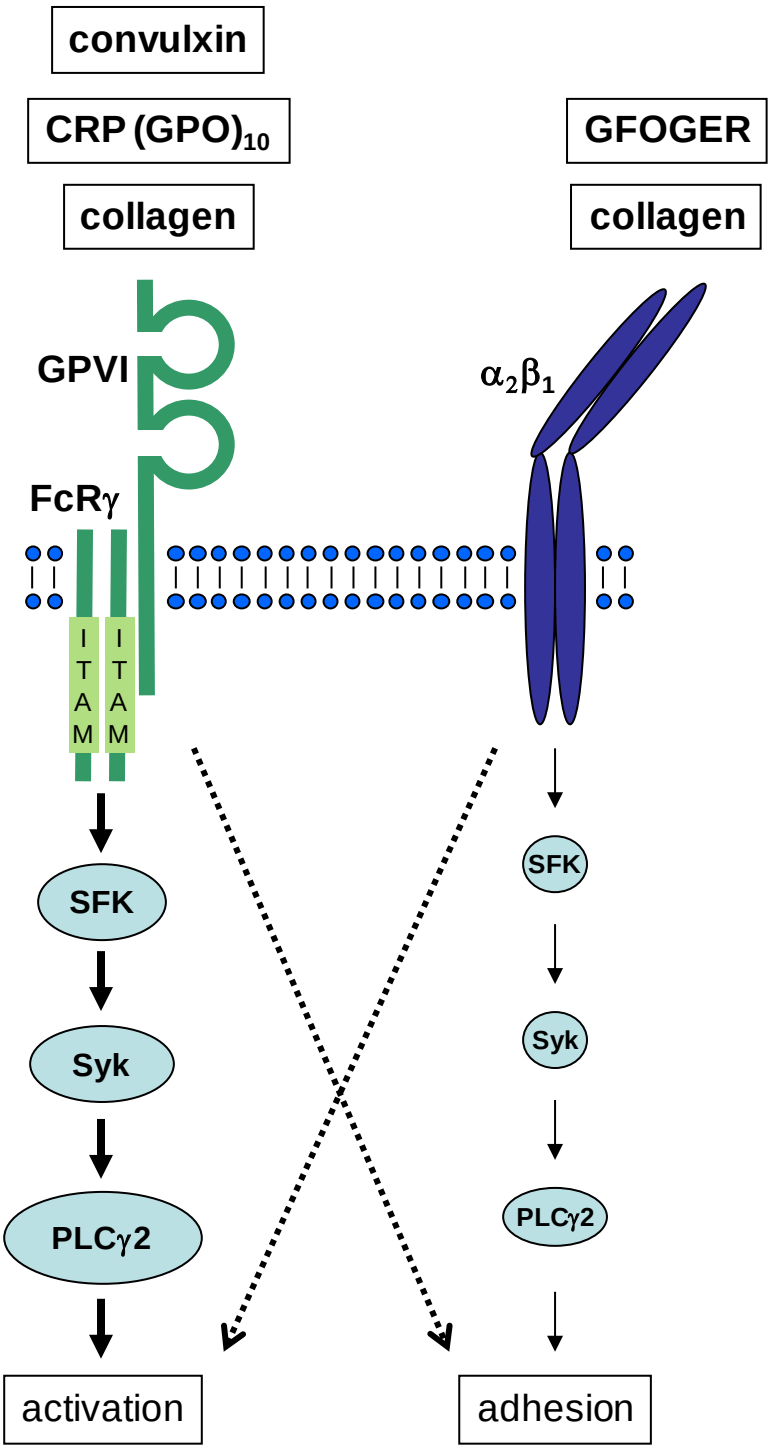


Figure 1.6: GPVI and $\alpha_2\beta_1$ act in a cooperative manner to reinforce each other's activity. Cross-linking of GPVI with the agonists collagen, collagen-related peptide (CRP $[GPO]_{10}$) and the snake toxin convulxin triggers platelet activation via a SFK-mediated signalling pathway ('outside-in' signalling) and also initiates activation of $\alpha_2\beta_1$ ('inside-out' signalling) to promote stable adhesion. Binding of collagen to $\alpha_2\beta_1$ reinforces collagen-GPVI interactions and also plays a minor role in platelet activation. The synthetic peptide GFOGER can also be used to initiate $\alpha_2\beta_1$ 'outside-in' signalling.

1.5.1.2 Fibrinogen receptor – integrin αIIbβ3

The integrin αIIbβ3 (also known as GPIIb/IIIa) is the most abundant glycoprotein expressed on the platelet surface (60,000 – 80,000 copies per resting human platelet). This increases by 30 – 50% following activation as αIIbβ3 stored in the α-granules translocates to the platelet surface (Shattil et al. 1998; Shattil et al. 2004). The αIIb subunit is expressed exclusively in the megakaryocyte/platelet lineage, whereas the β3 subunit is expressed in various other cell types where it interacts with other α subunits (Shattil et al. 2004). αIIbβ3 binds several ligands including fibrinogen, fibronectin and VWF. In resting platelets, αIIbβ3 exists primarily in a 'low-affinity' state and undergoes a conformational change to a 'high-affinity' state in response to agonist-induced inside-out signalling (Shattil et al. 2004). Ligand-mediated clustering of αIIbβ3 triggers outside-in signalling, filopodia and lamellipodia formation, secretion and clot retraction (Shattil et al. 1998). αIIbβ3 is essential for adhesion and aggregate formation at sites of vascular injury (Shattil et al. 2004; Coller et al. 2008).

Many similarities exist between the outside-in signalling events of αIIbβ3 and GPVI signalling (Watson et al. 2005; Kasirer-Friede et al. 2007). Fibrinogen-mediated clustering of αIIbβ3 leads to the sequential activation of SFKs, Syk family kinases, recruitment of adapter proteins such as SLP-76 and activation of downstream effector enzymes, including PLCγ2, PI 3-kinase and Vav small GTPases (Watson et al. 2005; Kasirer-Friede et al. 2007). Similar to the GPVI receptor, SFKs (Src and Fyn) are constitutively associated with the β3 subunit and Syk gets recruited to the intergrin in response to clustering. However, it is not clear whether Syk interacts directly with the β3 subunit, or whether an ITAM-containing protein mediates this interaction (Woodside et al. 2001; Abtahian et al. 2006; Mocsai et al. 2006). Two significant differences between αIIbβ3 and GPVI are that αIIbβ3 is not localized to lipid rafts,

whereas GPVI is; and the transmembrane adapter protein LAT is essential for GPVI signalling, whereas it is dispensable for αIIbβ3 signalling (Watson et al. 2005).

The molecular mechanism that initiates outside-in integrin αIIbβ3 signalling has been studied extensively over the past several years (Figure 1.7A). In resting platelets, integrin αIIbβ3-associated Src is maintained in an inactive conformation by Csk which phosphorylates an inhibitory tyrosine in the C-terminal tail of Src (Tyr-529) (Obergfell et al. 2002; Arias-Salgado et al. 2005). Upon integrin clustering, Csk dissociates from the αIIbβ3-Src complex and is replaced by the non-transmembrane protein tyrosine phosphatase, PTP-1B, which dephosphorylates the inhibitory tyrosine of Src and initiates the signalling pathway (Arias-Salgado et al. 2005). Syk is then recruited to the complex and becomes activated through phosphorylation by Src and autophosphorylated (Woodside et al. 2001; Abtahian et al. 2006; Mocsai et al. 2006). Other SFKs including Fyn, Lyn and Yes have been hypothesized or shown to play a role in initiating outside-in integrin αIIbβ3 signalling (Obergfell et al. 2002; Arias-Salgado et al. 2003; Reddy et al. 2008; Senis et al. 2009). Deletion of the two conserved tyrosines in the β3-tail prevents the interaction with Syk, as does tyrosine phosphorylation at these sites (Woodside et al. 2001).

Phosphorylation of the two conserved tyrosine residues in the β3 subunit has been shown to be functionally important (Law et al. 1999). The N-terminal tyrosine is found within a conserved NPXY motif that mediates binding to proteins with phosphotyrosine binding (PTB) domains, such as the Dok family of adapter proteins (Figure 1.7B) (Law et al. 1999; Calderwood et al. 2002; Arias-Salgado et al. 2003; Garcia et al. 2004). The second tyrosine lies within NXXY motif and has been shown to bind a distinct group of PTB domain containing proteins, including Shc (Phillips et al. 2001). Mutation of these tyrosines to phenylalanines results in increased bleeding and impairment in clot retraction in a knockin mutant mouse model (Law et al. 1999). The molecular basis of this defect is presently not

known, but has been proposed to be due to the loss of binding of myosin to the phosphorylated β3 tail (Figure 1.7B) (Jenkins et al. 1998; Phillips et al. 2001).

Lack or dysfunction of αIIbβ3 gives rise to Glanzmann thrombasthenia, a severe bleeding disorder associated with impaired adhesion and abolished aggregation of platelets (Nurden 2006). Similarly, mice lacking the β3 integrin subunit phenocopy Glanzmann thrombasthenia (Hodivala-Dilke et al. 1999). αIIb-deficient mice have also been generated and have been used to study haematopoiesis as well as platelet function (Tropel et al. 1997; Emambokus et al. 2003; Jacquelin et al. 2005). β3-deficient mice have markedly prolonged tail bleeding times and display spontaneous hemorrhage in all developmental stages. Intravital microscopy studies revealed that β3-deficient mice do not form thrombi at sites of vascular injury (Ni et al. 2000). The importance of αIIbβ3 in platelet adhesion and aggregation has made it an attractive pharmacological target for the prevention of ischemic cardiovascular events. Strategies to inhibit its function include antibodies (abciximab), peptides adapted from snake venom (eptifibitide), and analogues of an RGD peptide (lamifiban and tirofiban) that inhibit ligand binding (Coller et al. 2008). These inhibitors have been beneficial in a clinical setting but are not used extensively because of adverse bleeding side effects (Quinn et al. 2003).

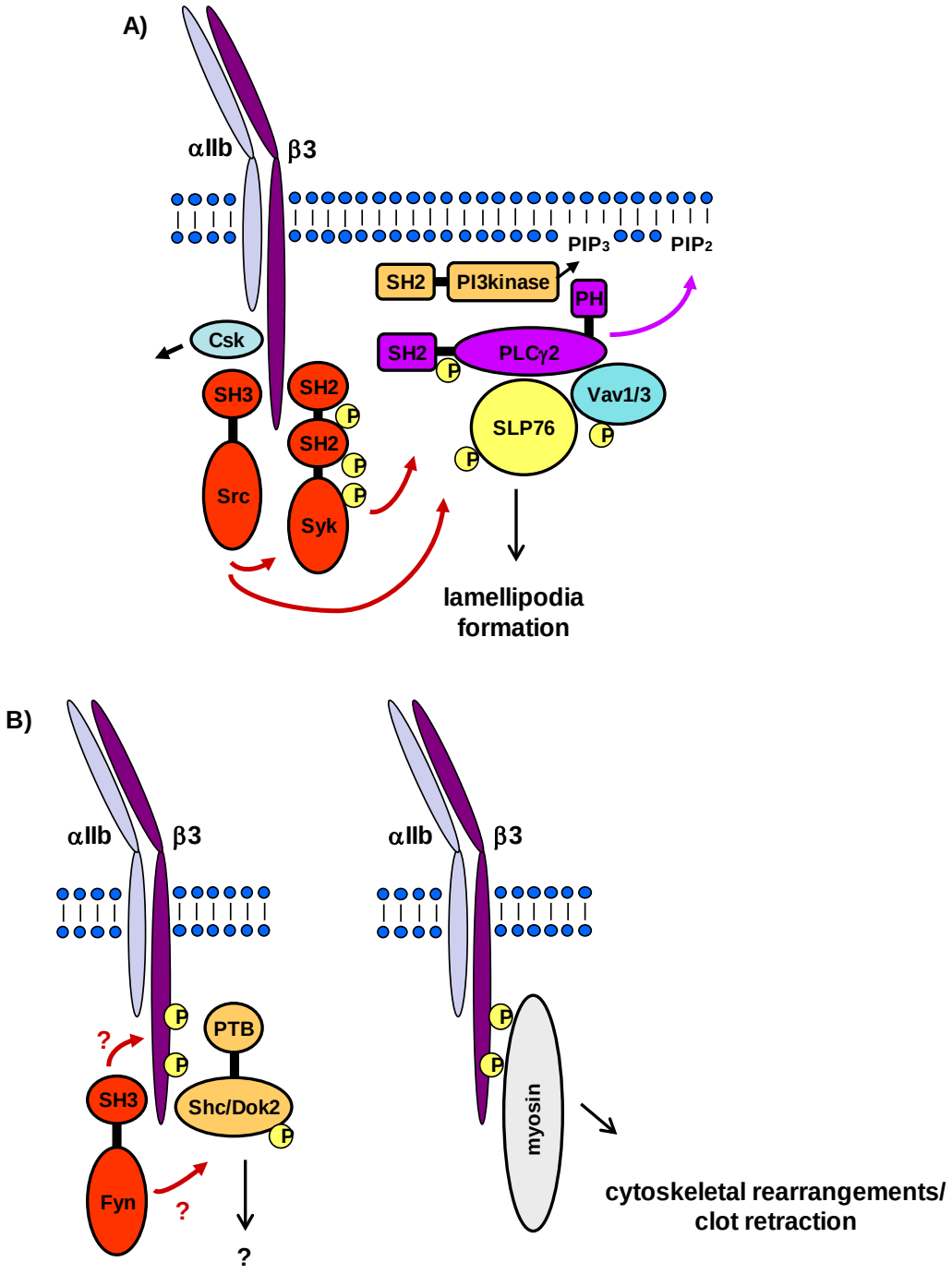


Figure 1.7: Integrin αIIbβ3 regulates Syk-dependent and -independent cascades. (A) Outside-in signalling through ligand engagement and clustering of integrin αIIbβ3 triggers a Syk-dependent intracellular signalling cascade. Syk is recruited to the β3-tail where it undergoes phosphorylation by Src. Activation of Syk leads to the activation of PLCγ2 through a pathway dependent on SLP-76 and Vav. (B) Tyrosine phosphorylation of two conserved tyrosines, possibly by Fyn, leads to binding and tyrosine phosphorylation of the adapters Shc and Dok2, and also recruitment of myosin.

1.5.1.3 VWF receptor – GPIb-IX-V complex

GPIb-IX-V is an abundant (25,000 copies per platelet) structurally unique receptor complex expressed exclusively in platelets and megakaryocytes (Berndt et al. 2001). Four different genes encode the receptor complex which consists of GPIb α- and β-subunits, GPIX, and GPV, all of which belong to the leucine-rich repeat protein superfamily (Berndt et al. 2001). Historically the receptor has been thought to be comprised of 2 GPIbα subunits, 2 GPIbβ subunits, 2 GPIX subunits and 1 GPV subunit (Berndt et al. 2001). More recent, Luo et. al. suggests a molar ratio of 2:4:2:1 of GPIbα, GPIbβ, GPIX, and GPV subunits, respectively (Luo et al. 2007).

The primary function of GPIb-IX-V is to recruit platelets to the site of injury by reducing their velocity to enable the interaction of other, higher affinity receptors with the damaged surface. At the high shear rates found in small arteries, arterioles, and stenosed arteries the interaction between GPIbα and VWF immobilized on collagen or on the surface of activated platelets is crucial for the initial tethering of the flowing platelets (Savage et al. 1998). This interaction on its own is insufficient to support stable adhesion. The activation of integrins is required to mediate stable adhesion.

The rapid on/off rate between GPIb-IX-V and VWF was first thought to be too short to allow sufficient time for a signal to be initiated (Goto et al. 1995; Savage et al. 1996). It was assumed that the GPIb-IX-V/VWF interaction only provides a physical interaction that tethers platelets to the sub-endothelial matrix (SEM), allowing sufficient time for collagen to associate with the collagen receptors to elicit intracellular activation signals. However, a growing body of evidence is now emerging that suggests GPIb-IX-V mediates intracellular signalling through Src family kinase and PLCγ2-related pathways, leading to platelet activation and αIIbβ3-mediated aggregate formation (Yap et al. 2002; Mangin et al. 2003). A hypothetical GPIb-IX-V signal transduction pathway was proposed to take place

predominantly in lipid rafts (Ozaki et al. 2005). In line with this model, GPIb-IX-V is constitutively associating with the p85 subunit of PI 3-kinase through 14-3-3ζ (Mangin et al. 2004; Mu et al. 2008). The interaction between GPIb-IX-V and VWF induces the binding between PI 3-kinase and Src, which then elicits downstream signals, leading to PLCγ2 activation.

Humans lacking or expressing dysfunctional GPIb-IX-V have a condition known as Bernard-Soulier syndrome (Lopez et al. 1998). This is a congenital bleeding disorder characterized by mild thrombocytopenia, giant platelets and the inability of platelets to aggregate in response to the antibiotic ristocetin (Lopez et al. 1998). Interestingly, GPIb-IX-V has also been shown to bind several coagulation factors including thrombin and factors XI, XII and VII, which may contribute to the severe bleeding defect exhibited by Bernard-Soulier syndrome patients (Dumas et al. 2003; Vanhoorelbeke et al. 2007).

1.5.2 G protein–coupled receptors

Platelets express a variety of GPCRs that play a central role in regulating platelet activation and inhibition (Woulfe 2005; Offermanns 2006). GPCRs signal in a different way to the tyrosine kinase-linked receptors described above. Some of the most important platelet activation receptors are: (1) the thrombin protease-activated receptors (PAR)-1 and PAR-4; (2) the ADP receptors $P2Y_1$ and $P2Y_{12}$; and (3) the TxA_2 receptor TP. Interestingly, several of these GPCRs also have a SFK signalling component. Below are brief descriptions of each of these receptors and their signalling pathways in platelets.

1.5.2.1 Thrombin receptors - Protease Activated Receptors (PARs)

Thrombin (also known as factor IIa) is a soluble plasma serine protease generated through a series of enzymatic reactions, commonly referred to as the ‘coagulation cascade’ that takes

place on the surface of activated platelets (Hoffman et al. 2001). The inactive zymogen prothrombin gets converted to active thrombin, which in turn cleaves soluble fibrinogen to form insoluble fibrin polymers (Figure 1.4). Fibrin forms a mesh like structure that stabilises the thrombus (Figure 1.3). Thrombin is also a powerful platelet agonist and triggers platelet activation by cleaving PAR-1 and PAR-4 to expose a short activatory peptide sequence known as a tethered ligand (Coughlin 2000). Human platelets express approximately 1,000 – 2,000 copies of PAR-1 (Brass et al. 1992; Ramstrom et al. 2008). PAR-1 and PAR-4 are coupled to heterotrimeric G proteins. Human platelets express PAR-1 and PAR-4 whilst mouse platelets express PAR-3 and PAR-4. PAR-1 and PAR-4 can couple to G_q, $G_{12/13}$ and G_i G protein α-subunits (Coughlin 2000). The G_q pathway is the main PAR-1 and PAR-4 activation pathway and leads to activation of PLCβ and generation of IP_3 and DAG, which in turn activate PKC and Ca^{2+} mobilization respectively; the $G_{12/13}$ pathway leads to activation of Rho kinase and cytoskeletal rearrangements; and G_i inhibits adenylyl cyclase, which promotes platelet activation (Coughlin 2000). Although G protein-coupled, there is also a minor Src kinase component to the signalling pathway downstream of the thrombin receptors (Coughlin 2000; Murugappan et al. 2005; Harper et al. 2006). PAR-3 does not signal, but acts as a cofactor that binds and localises thrombin in close proximity to PAR-4 receptor in mouse platelets (Coughlin 2000).

1.5.2.2 ADP receptors -$P2Y_1$ and $P2Y_{12}$

ADP is a platelet agonist that is released from the dense granules upon activation. ADP is also released from damaged endothelial cells at the sites of vascular injury (Gachet 2006). The physiological function of ADP is to enhance platelet activation via a positive feedback mechanism and is essential for recruitment of platelets to the growing thrombus (Offermanns 2006). Platelets express two ADP receptors, $P2Y_1$ and $P2Y_{12}$ (Gachet 2006). Human

platelets express very few copies of $P2Y_1$ (150 copies) and approximate ten-fold more of $P2Y_{12}$, however, together they induce rapid and strong platelet activation. $P2Y_1$ couples to G_q and $G_{12/13}$ heterotrimeric G protein α-subunits, whereas $P2Y_{12}$ couples to the G_i (Gachet 2006). PI 3-kinase also contributes to $P2Y_{12}$ signalling and Src plays a minor role. $P2Y_1$ receptor triggers Ca^{2+} mobilization, platelet shape change and weak, transient aggregation in response to ADP (Gachet 2006). $P2Y_{12}$ inhibits adenylyl cyclase, causing a decrease in intracellular cAMP levels, as well as activating PI3-kinase and Rap-1B (Gachet 2006). The functional outcome of $P2Y_{12}$ activation is sustained platelet aggregation and amplification of signals initiated by other agonists, including collagen, TxA_2 and the integrin αIIbβ3 (Gachet 2006). Interestingly, although $P2Y_1$ and $P2Y_{12}$ receptors signal through distinct mechanisms, Src kinase has been reported to be activated downstream of both the $P2Y_1$ and $P2Y_{12}$ receptors (Hardy et al. 2004; Shankar et al. 2006). Coactivation of $P2Y_1$ and $P2Y_{12}$ is necessary for normal ADP-induced platelet activation as inhibition of either receptor results in a marked decrease in platelet aggregation (Gachet 2006).

1.5.2.3 Thromboxane A_2 receptor - TP

TxA_2 is a short-lived lipid mediator that is synthesized by activated platelets to amplify activation signals and recruit additional platelets to the site of thrombus formation (FitzGerald 1991). TxA_2 also acts as a potent vasoconstrictor. In activated platelets, TxA_2 is generated from prostaglandin H_2 (PGH_2) by thromboxane-A synthase (Huang et al. 2004). Aspirin irreversibly inhibits platelet cyclooxygenase-1 (COX1) that converts arachidonic acid (AA) into prostaglandin H_2 (Huang et al. 2004). TxA_2 is lipid soluble and crosses the plasma membrane, exiting the platelet and binding to the TxA_2 receptor (TP) on the surface of the same platelet or other platelets in the vicinity in a autocrine and paracrine manner, respectively (Shen et al. 1998). Signalling through the TP receptor induces platelet activation

and secretion leading to platelet aggregation. In platelets, the activated TP receptor predominantly couples to G_q and $G_{12/13}$, similar to the thrombin receptors PAR-1 and PAR-4, and the $P2Y_1$ ADP receptor (Djellas et al. 1999; Klages et al. 1999). The TP receptor also has a minor Src signalling component (Huang et al. 2004).

The importance of TxA_2 as a positive feedback agonist is demonstrated by patients deficient in TxA_2 production, which display a mild bleeding disorder (Lagarde et al. 1978) and by the TP knockout mouse, which has a prolonged bleeding time (Thomas et al. 1998). TxA_2 works synergistically with released ADP to amplify platelet activation signals. Collagen in particular has a large dependency on the combined effects of TxA_2 and ADP to enhance the activation signal and promote more powerful aggregation (Figure 1.8).

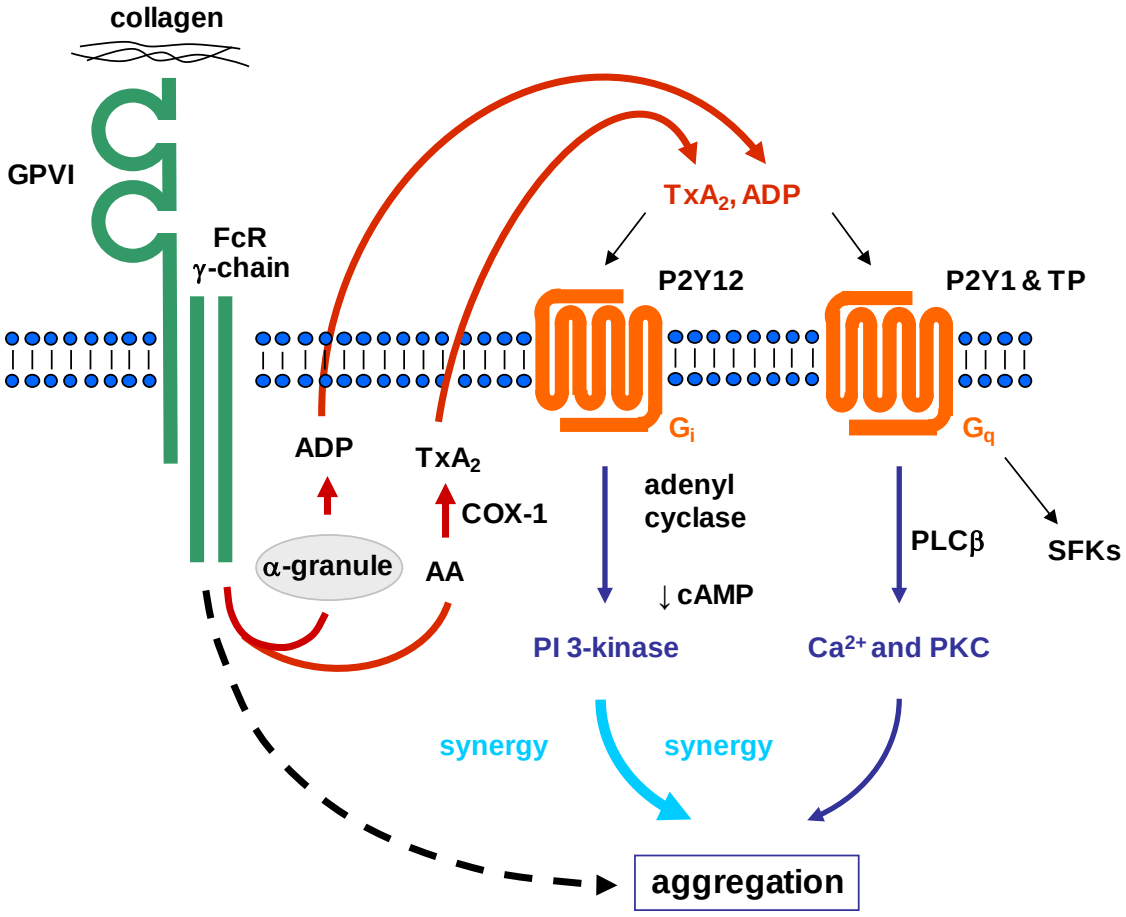


Figure 1.8: Aggregation by collagen is dependent on ADP and TxA_2. Released ADP and TxA_2 contribute to a positive feedback mechanism that enhances collagen signalling and platelet aggregation. TxA_2 signals through $P2Y_{12}$, whilst ADP signals through $P2Y_1$ and TP.

1.5.3 ITIM receptors

Platelets express several immunoreceptor tyrosine-base inhibition motif (ITIM)-containing receptors/receptor-like proteins including: PECAM-1 (CD31), TLT-1, G6b-B and CEACAM1. ITIMs are typically defined by the consensus sequence (L/I/V/S)-XY-X-X-(L/V) and are frequently found in pairs separated by 15 to 30 amino acid residues (Vivier et al, 1997; Ravetch et al, 2000). The SH2 domain containing non-transmembrane protein tyrosine phosphatases (PTPs) Shp1 and Shp2 interact with phosphorylated ITIMs. ITIM-

containing receptors were originally identified by their ability to inhibit signalling by ITAM receptors, as demonstrated by the selective inhibition of the B-cell receptor when cross-linked by surface IgG to FcγRIIb (Bijsterbosch et al, 1985; Daeron et al, 1995). However, this story was complicated by more recent evidence generated by studying platelets from PECAM-1-deficient mice, which demonstrated that ITIM-containing receptors can also inhibit activation by G protein-coupled receptors and that PECAM-1 positively regulates integrin-mediated responses (Cicmil et al, 2002; Dhanjal et al, 2007; Jones et al, 2001; Newman 2003; Patil et al, 2001).

PECAM-1 was the first ITIM-containing receptor identified in platelets and is the most well studied. It is highly expressed in platelets (~10,000 copies on the surface of resting platelets) and endothelial cells (~1,000,000 copies/endothelial cell) (Muller et al. 1992). Cross-linking PECAM-1 has been shown to inhibit signalling from the collagen ITAM-containing receptor GPVI-FcR γ-chain, the G protein-coupled receptor thrombin receptor PAR-4 and the VWF receptor GPIb-IX-V (Newman et al. 2003). The ligand for PECAM-1 is itself (homophilic interaction). It is hypothesised that the physiological function of PECAM-1 is to maintain platelets in an inactive state in healthy blood vessels and limit thrombus size at sites of injury. Work done using PECAM-1-deficient mice supports this model (Falati et al. 2006). However, the magnitude of the inhibitory function of PECAM-1 has been questioned by recent studies showing that PECAM-1 had only a minor inhibitory effect on platelet activation induced by ITAM signalling and that thrombus formation on collagen was not altered if PECAM-1 was absent (Dhanjal et al. 2007). Furthermore, Wee et al reported potentiation of aggregation in PECAM-1-deficient platelets in response to CRP, demonstrating the complexity of the functional role of PECAM-1 in platelets (Wee et al. 2005).

Work done on other ITIM-containing receptors in platelets supports their role as both activatory and inhibitory receptors and that they also regulate GPCR-mediated responses. TLT-1 which is stored in platelet α-granules and translocates to the surface upon platelet activation where it binds fibrinogen, positively regulates thrombosis (Washington et al. 2009); antibody-mediated cross-linking of G6b-B inhibits both GPVI- and ADP-mediated platelet aggregation (Newland et al. 2007); and work done using CEACAM1-deficient mice has shown that it negatively regulates most platelet responses mediated by tyroinse-kinase linked receptors (Wong et al. 2009).

1.6 Platelet Inhibition

Platelets must be maintained in a quiescent or 'resting' state in the peripheral circulation to prevent unwanted thrombus formation. Three mechanisms that maintain platelets in a resting state are: (1) certain ITIM receptors (PECAM-1 and CEACAM1) have been shown to reduce platelet activation (described above); (2) PGI_2 which is secreted from endothelial cells and (3) nitric oxide (NO), which is also released from endothelial cells. Below I briefly describe the later two mechanisms, which are better defined and have a much greater effect on inhibiting platelet activation than ITIM-mediated inhibition.

1.6.1 PGI_2 receptor

Prostaglandin I_2 (PGI_2) is a potent inhibitor of platelet activation, adhesion, aggregation and secretion. PGI_2 is synthesized in endothelial cells from PGH_2 by prostacyclin synthase and constitutively released into the circulation. PGI_2 is released at relatively low concentrations and has a short half-life, exerting its effects on platelets in close proximity to the vessel wall (Mitchell et al. 2008). PGI_2 binds to and signals through the G_s-coupled PGI_2 receptor. G_s activates adenylyl cyclase, which in turn generates cAMP, which activates the cAMP-

dependent Ser/Thr protein kinase A (PKA). PKA phosphorylates numerous proteins in platelets that may mediate its inhibitory effects. A major substrate of PKA in platelets is vasodilator-stimulated phosphoprotein (VASP), which interacts with various cytoskeletal proteins, focal adhesions, stress fibers and cell-cell contacts (Bundschu et al. 2006). Phosphorylation of VASP has been correlated with fibrinogen receptor inhibition. Phosphorylation of GPIb and the TxA_2 receptor may contribute to inhibition of platelet activation by PGI_2, whereas the effects of phosphorylation of the guanine nucleotide exchange factor Rap1B remain unknown.

1.6.2 Nitric oxide

NO is another potent inhibitor of platelet activation, adhesion, aggregation and secretion. NO is constitutively produced and release by endothelial cells and is important in maintaining platelets in a resting state. NO has a short half-life, having a high localised concentration near the vessel wall that drops off rapidly with increasing distance from the wall. NO freely crosses the platelet plasma membrane and enters the cytosol where it exerts its effect on platelet function. The inhibitory effects of NO are considered to be due to activation of soluble guanylyl cyclase resulting in production of cGMP. Increased intracellular levels of cGMP activate the Ser/Thr protein kinase G (PKG) as well as increase intracellular levels of cAMP which activates PKA (Walter et al. 2009). Both PKG and PKA phosphorylate VASP and inhibit platelet activation. The intracellular signalling effects of NO are not fully understood, but also involves inhibition of phospholipase C and IP_3-mediated mobilization of intracellular Ca^{2+}.

1.7 Platelet Kinases and Phosphatases

Protein phosphorylation on tyrosine residues is a fundamental cell-signalling mechanism, regulated by the combined activities of the protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The actions of both the PTKs and PTPs are under tight control as they regulate important cellular and physiological processes including cell growth and proliferation, differentiation, migration, metabolism and signal transduction (Tonks et al. 2001; Larsen et al. 2003). Disruption of the fine balance of tyrosine phosphorylation within the cell can trigger the onset of several human diseases, including, diabetes, autoimmunity and cancer (Blume-Jensen et al. 2001; Tonks et al. 2001; Zhang 2001). Therefore understanding the functional roles of these critically important enzymes has important implications for the development of novel therapies to treat disease.

The human and mouse genomes code for approximately the same number of catalytically active PTPs and PTKs (approximately 90 of each). However, PTPs in general are less well characterized and understood (Manning et al. 2002; Alonso et al. 2004). This is in part due to the first PTK being purified (Czernilofsky et al. 1980) and studied almost ten years prior to the first PTP (Charbonneau et al. 1989). In addition, PTPs were initially regarded as housekeeping enzymes that were constitutively active and dephosphorylated every substrate they encountered. In recent years however, a large body of evidence has emerged demonstrating that PTPs play very specific, precise roles in regulating tyrosine phosphorylation and in the regulation of many physiological processes (Fischer et al. 1991; Walton et al. 1993; Tonks et al. 1996; Mustelin et al. 2003; Stoker 2005).

1.7.1 Src family kinases

The most well studied family of PTKs is the Src family kinases (SFKs). Humans and mice express eight structurally-related SFKs (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src and Yes), all of

which are between 53 – 61 kDa in size and consist of an N-terminal SH3 domain, followed by an SH2 domain, a proline-rich linker region and a kinase domain (Roskoski 2004). The SH3 and SH2 domains mediate both intra- and inter-molecular interactions. SFKs also contain myristoylation and/or palmitoylation sequences in their N-terminus, which allow them to localize to the inner leaflet of the plasma membrane. They have been linked to a variety of cellular processes. One of their most well studied and important function is to initiate and propagate signals from surface receptors, including immunoreceptors, integrins and to a lesser extent G protein-couple receptors. SFKs have high catalytic activity, so they are tightly regulated. Aberrant SFK activity leads to various disease conditions including cancer and autoimmunity. Regulation of platelet SFKs is a major component of this thesis and is discussed below.

1.7.1.1 SFKs expressed in platelets

Human platelets express five of the eight SFKs, namely Src, Fyn, Lyn, Yes and Fgr (Golden et al. 1986; Rendu et al. 1989; Horak et al. 1990; Pestina et al. 1997; Quek et al. 2000; Obergfell et al. 2002). Src is the most abundant SFK in platelets, estimated to constitute ~0.2% of total platelet protein (Golden et al. 1986). Work done with cell lines, knockout mouse models and pharmacological inhibitors have clearly established SFKs as essential mediators of platelet activation, aggregation, adhesion and thrombosis (Ezumi et al. 1998; Gross et al. 1999; Quek et al. 2000; Obergfell et al. 2002). Src is essential for initiating integrin αIIbβ3 signalling, whereas Fyn and Lyn are essential for initiating GPVI signalling (Ezumi et al. 1998; Obergfell et al. 2002). The functions of Yes and Fgr in platelets are not clear. Although SFKs are known to play important roles downstream of many other platelet receptors, it is presently not known which specific SFKs act downstream of each receptor. It

is also not clear the extent of functional redundancy between the SFKs and unique functions performed by each.

1.7.1.2 Regulation of SFK activity

SFKs are tightly regulated by phosphorylation and intra-molecular interactions. SFKs are maintained in an inactive conformation by two important intra-molecular interactions, one of which is between the SH3 domain and the proline-rich linker sequence between the SH2 and kinase domains; and the second is between the SH2 domain and the phosphotyrosine residue in the C-terminal tail of the kinase (Tyr-529 in Src) (Figure 1.9) (Roskoski 2005). Both of these interactions must be disrupted in order for SFKs to be activated. This is achieved by either dephosphorylation of the C-terminal inhibitory tyrosine, which releases the interaction with the SH2 domain and has a knock-on effect that also disrupts the SH3 interaction, or by out-competing the SH3 and SH2 interactions through inter-molecular interactions (Roskoski 2005). In the latter scenario, the C-terminal inhibitory site does not have to be dephosphorylated. Dephosphorylation of the C-terminal inhibitory phosphotyrosine of SFKs 'primes' the kinase. Mutation of the C-terminal tyrosine residue of Src to a phenylalanine has been shown to increase its catalytic activity relative to wild-type Src (Cooper et al. 1986; Kmiecik et al. 1987). Primed SFKs become fully activated by *trans*-autophosphorylation of a tyrosine residue located within the activation loop of the kinase domain (Tyr-418 in Src) (Su et al. 1999). Phosphorylation of the activation loop maintains it in an 'open' confirmation, such that it does not obstruct the catalytic site of the enzyme (Xu et al. 1999).

SFKs are maintained in an inactive conformation by the structurally related tyrosine kinases Csk and Chk which phosphorylate the C-terminal inhibitory tyrosine of all SFKs (Okada et al. 1991; Zrihan-Licht et al. 1997; Cole et al. 2003). Csk is expressed in all mammalian cells, whereas Chk expression is restricted to hematopoietic cells, neurons, breast

and testes (Brown et al. 1996). Csk is maintained at the plasma membrane, in close proximity to the SFKs it regulates, by binding to phosphorylated Csk-binding protein also referred to as phosphoprotein associated with glycosphingolipid-enriched microdomains or paxillin (Roskoski 2004; Rathore et al. 2007).

Several PTPs have been shown to dephosphorylate the C-terminal inhibitory tyrosine residue of SFKs, including the RPTPs CD45, PTPα, PTPε and PTPλ and the NTPTPs PTP-1B, Shp1 and Shp2, whereas only CD45 and the PTP PTP-BAS have been shown to dephosphorylate the activation loop of SFKs (Roskoski 2005). SFK activity can also be reduced by dephosphorylation of the activation loop phosphotyrosine. CD45 and the NTPTP PTP-BL (PTP-BAS mouse homologue) are the only two PTPs to be shown to dephosphorylate this site to date. Interestingly, Palmer et al. demonstrated that PTP-BL specifically dephosphorylates Src at Tyr 418 but not Tyr-529; whereas CD45 dephosphorylate both the inhibitory and activation loop tyrosines of Lck (Tyr-507 and Tyr-396, respectively) downstream of the TCR (McNeill et al. 2007). CD45 has therefore been proposed to act as a switch modulating the sensitivity of cells to TCR signals by differentially regulating the two critical Lck tyrosine phosphorylation sites (McNeill et al. 2007).

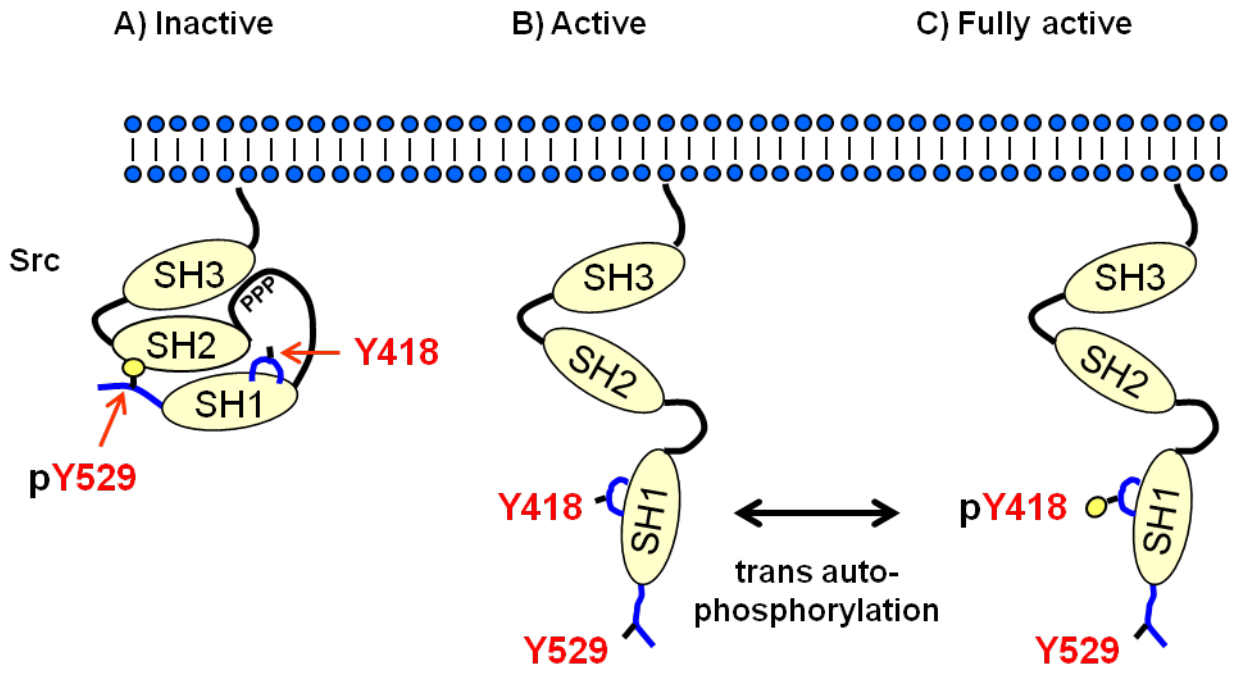


Figure 1.9: Regulation of Src kinase by tyrosine phosphorylation. (A) The inactive configuration of Src showing the SH2 domain interacting with the phosphorylated C-terminal tyrosine (pY529), the SH3 domain interacting with the polyproline SH2-linker region (PPP), and the dephosphorylated activation loop (Y418) folded back over the substrate binding site. (B) The active configuration of Src, showing SH2 and SH3 domains released from the intramolecular interactions and available for binding to substrates and regulatory molecules, the C-terminal tyrosine is dephosphorylated (Y529). (C) Src undergoes autophopshorylation of the activation loop (pY418) to become fully active.

1.7.2 Protein Tyrosine Phosphatases

1.7.2.1 Classification of PTPs

The defining feature of all PTPs is the presence of a PTP catalytic domain (~280 amino acids) containing an active-site signature motif HCX_5R, where the cysteine functions as a nucleophile and is essential for catalysis (Tonks 2006). Based on this definition, the human genome contains 107 genes that encode for PTPs (Alonso et al. 2004). Mouse orthologs exist for essentially all of these genes. The number of genes in the human genome that encode PTPs is surprisingly higher than the number of genes encoding PTKs (107 PTP genes compared with 90 PTK genes) (Manning et al. 2002; Alonso et al. 2004). However, this is somewhat misleading as of the 107 PTPs, 11 have no catalytic activity, 2 dephosphorylate mRNA and 13 dephosphorylate inositol phospholipids (Alonso et al. 2004). Consequently, only 81 of the PTPs are active protein phosphatases with the ability to dephosphorylate phosphotyrosines. Of the 90 PTK genes, 85 are thought to be catalytically active (Manning et al. 2002). Therefore, the number of active PTPs and PTKs are similar.

PTPs are classified into four separate groups (Classes I – IV) based on the amino acid sequences of their catalytic domains and their substrate specificity (Figure 1.10) (Alonso et al. 2004). The largest group is the Class I cysteine-based PTPs of which there are 99. Class I PTPs are divided into two broad categories, the ‘classical’ PTPs (38), which are strictly tyrosine-specific, and the ‘dual-specific’ PTPs (61), named for their broad substrate activity. The classical PTPs are further subdivided into the receptor-like PTPs (RPTPs) (21), and the non-transmembrane (NTPTPs) (17); and the dual-specific PTPs are subdivided into seven subgroups based on substrate specificity (Figure 1.10). Low molecular weight PTP (LMW-PTP) is the only Class II cysteine-based PTP, which appears to be more ancient than the Class I PTPs. The Class III cysteine-based PTP family contains only three members, all of which

are cell cycle regulators. In contrast to Class I – III cysteine-based PTPs, Class IV PTPs use a different catalytic mechanism, requiring a key aspartic acid and are cation-dependent.

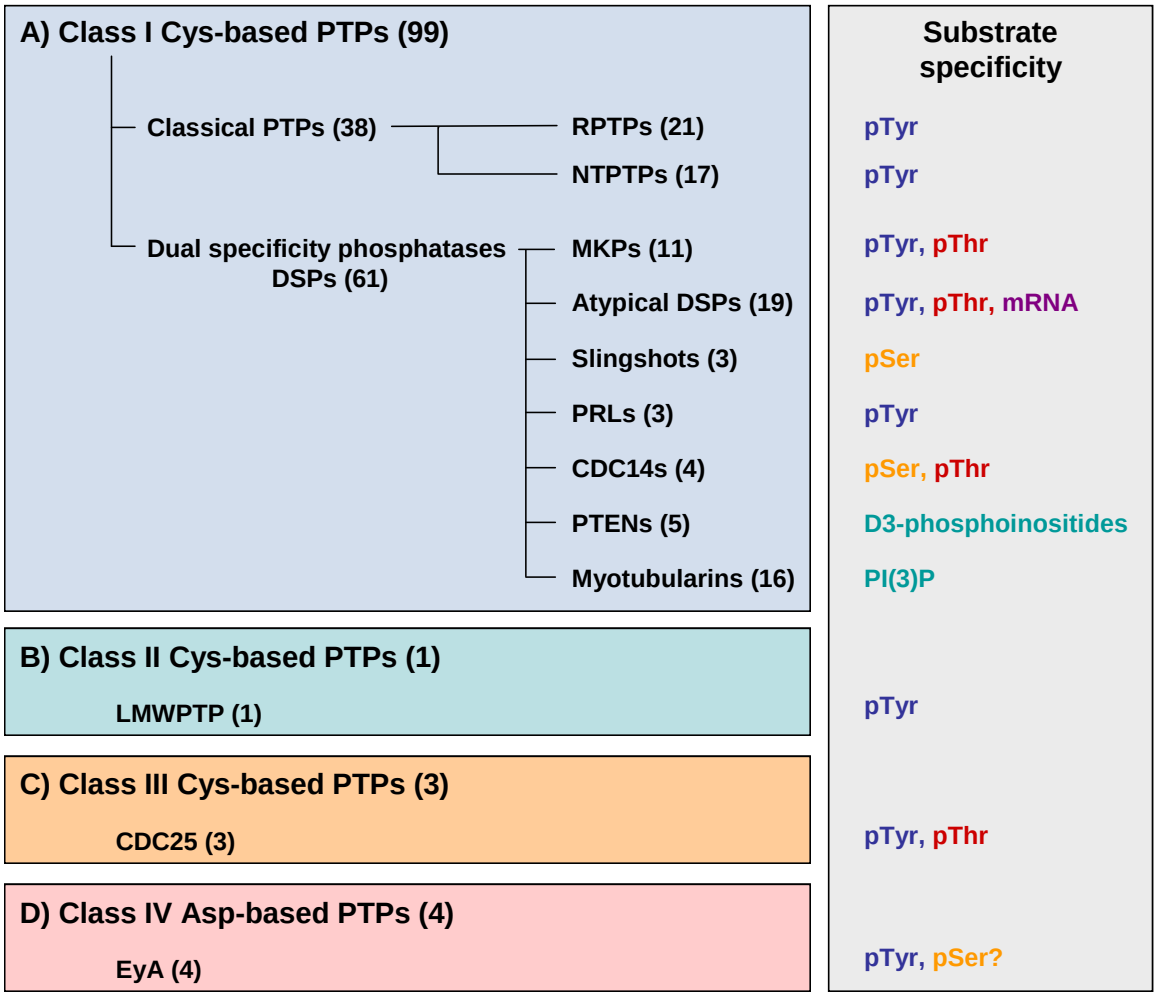


Figure 1.10: Classification and substrate specificity of the PTPs. Class I Cys-based PTPs (blue), class II Cys-based PTPs (green), class III Cys-based PTPs (orange), and Asp-based PTPs (pink). The substrate specificity of each class of PTP is listed (grey). Figure adapted from Alonso et al, 2004.

1.7.2.2 The classical PTPs

The classical PTPs, comprised of 21 RPTPs and 17 NTPTPs, are further divided into subgroups based on structural features and the extent of homology between their catalytic domains (Figure 1.11). All members of the classical PTP family have at least one PTP

domain with the majority of the RPTP subfamily containing a second pseudo-PTP domain within their cytoplasmic region that has little or no catalytic activity (Figure 1.11) (Tonks 2006). Nevertheless, the structural integrity of the pseudo-PTP domain is important for the activity, specificity and stability of the RPTP as a whole and may provide docking sites for substrates and regulatory proteins (Streuli et al. 1990; Felberg et al. 1998; Tonks et al. 2001). The pseudo-PTP domain is also important for protein-protein interactions that regulate dimerization.

The prototype and best studied RPTP is CD45. In general, the cytoplasmic domains of the RPTPs are relatively well conserved (~35% sequence identity), however their extracellular regions are structurally diverse (Figure 1.11). Extracellular regions of RPTPs are typically large (with the exception of PTPα and PTPε), highly glycosylated and contain domains present in receptors that regulate cell-cell and cell-ECM interactions, including fibronectin type III, immunoglobulin, MAM (Meprin/A4/μ domain) and carbonic anhydrase domains (Alonso et al. 2004). There is a growing body of evidence that RPTPs regulate cell-cell and cell-ECM adhesion.

The NTPTPs contain a wide variety of structural motifs flanking the catalytic domain that are important for regulating PTP activity, either directly by interaction with the active site, as shown for Shp2, or by controlling substrate specificity as demonstrated for PTP-PEST and $p130^{cas}$ (Figure 1.11) (Garton et al. 1997). These non-catalytic regions also target NTPTPs to subcellular locations within the cell, thereby indirectly regulating activity by controlling access to a particular subset of substrates. For example, Shp2 is targeted to the transmembrane protein PECAM-1 via its SH2 domain, following platelet activation and aggregation (Jackson et al. 1997).

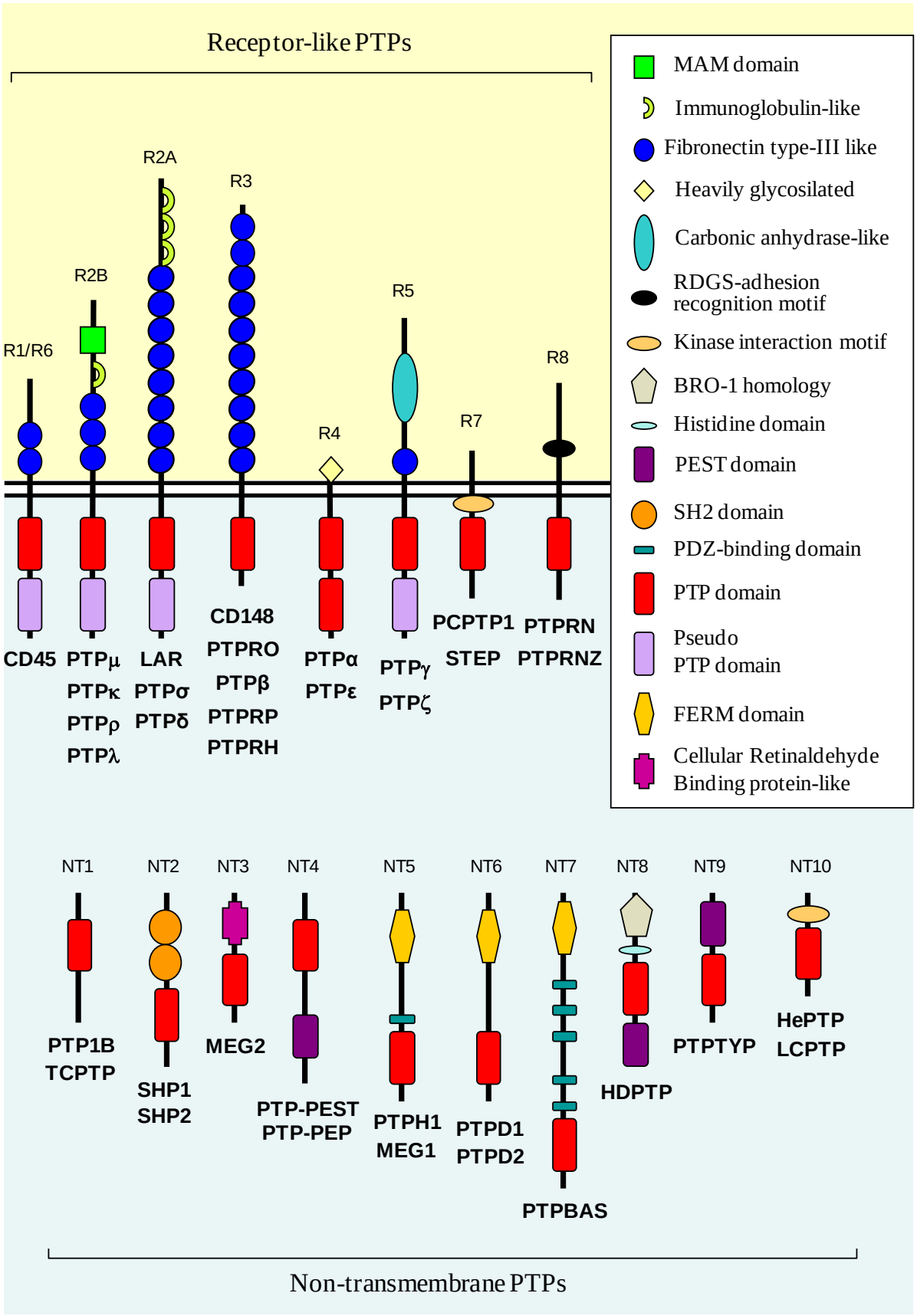


Figure 1.11: The Classical Protein Tyrosine Phosphatases. Schematic representation of the classical PTPs showing their varied structural domains.

1.7.2.3 Catalytic mechansim of PTPs

PTP-1B has been the prototype for understanding the reaction catalyzed by all PTPs. It was the first PTP identified and the first to have the crystal structure of its PTP domain solved (Tonks et al. 1988; Charbonneau et al. 1989; Barford et al. 1994). The crystal structures of the PTP-1B catalytic domain on its own and in complex with a peptide substrate were determined by the Tonks group (Barford et al. 1994). Sequence alignments of PTP catalytic domains revealed several conserved residues. The structure of PTP-1B illustrated that these residues were clustered in and around the active site and were demonstrated through site-directed mutagenesis to facilitate enzyme-substrate recognition and catalysis (Jia et al. 1995). Recently, work by Barr et. al. revealed the PTP domains of the classical PTPs have many structural variations which regulate activity and specificity (Barr et al. 2009). Other major findings from this study include: (1) a new atypical conformation for the WPD (Trp-Pro-Asp) catalytic loop; (2) a secondary substrate-binding pocket, initially reported for PTP-1B, can also be found in most other PTPs; and (3) a new 'head-to-toe' model to explain dimerization mediated inhibition of RPTP activity.

The molecular mechanism of all PTP catalyzed reactions is a nucleophilic attack of the substrate phosphate by the PTP. A schematic representation of the reaction catalyzed by PTP-1B and the key residues involved is shown in Figure 1.12 (Tonks 2006). Cys-215 in the PTP active site is fundamental for initiating catalysis (Figure 1.12A and B). Asp-181 located on a flexible WPD catalytic loop is another key residue of the reaction. Upon binding of substrate to the enzyme, the flexible WPD loop undergoes a conformational change, bringing Asp-181 into the active site, so that it protonates the tyrosyl leaving group of the substrate (Figure 1.12A and B). A phosphoryl-cysteine intermediate (PTP-Cys-PO_3) is subsequently formed (Figure 1.12C). The second part of the reaction involves the hydrolysis of the phospho-enzyme intermediate, which is stabilized within the catalytic cleft by Ser-222 (Figure 1.12D).

Hydrolysis is mediated by Asp-181, which now acts as a general base, and a Gln-262, which coordinates a water molecule in the active site (Figure 1.12D and E). The phosphate is released from the active site and the enzyme is ready to dephosphorylate another substrate molecule (Figure 1.12F). PTPs generally have a high catalytic activity that have been measured to be 3 orders of magnitude higher than PTKs (Zhang 2003).

1.7.2.4 Identification of PTP substrates

'Substrate-trapping mutants' have been important tools in the identification of physiological substrates and deciphering signalling pathways that PTPs regulate (Blanchetot et al. 2005; Tiganis et al. 2007). They were developed following the elucidation of the PTP catalytic mechanism. The basic principle of how they function is that they display impaired catalytic activity and bind substrates with higher affinity than their wild-type counter-parts (Garton et al. 1996; Flint et al. 1997). The most successful substrate-trapping mutants generated to date have either the signature motif cysteine replaced by a serine (C/S), or the aspartatic acid in the WPD loop mutated to alanine (D/A). The C/S mutation in the active site renders the PTP catalytically inactive, but still allows binding of the physiological substrate (S) leading to the formation of a stable enzyme-substrate interaction (PTP-Ser-PO_3-S) (Blanchetot et al. 2005). The D/A mutant is generally regarded as the best mutant to pulldown PTP substrates, as in the majority of cases it has greater affinity for substrates compared to the C/S mutant (Buist et al. 2000; Agazie et al. 2003). This is because the D/A mutant not only has impaired catalytic activity, but has the added benefit of the WPD loop flipping over and locking the substrate in the catalytic pocket (Blanchetot et al. 2005).

PTP substrate-trapping approaches have progressed to include double mutations as a means to improve trapping efficiency. Double mutants are effective when single mutants are slightly active and do not display sufficiently high binding affinities to trap substrates. Two

examples are the Shp2 C459S-D425A and PTP-1B D181A-Q262A double mutants, both of which proved to be much more effective in trapping substrates than either mutant alone (Xie et al. 2002; Agazie et al. 2003).

Although substrate-trapping mutants have been used with great effect to target substrates of PTPs, the binding of a phosphoprotein to a substrate-trapping mutant on its own is not sufficient to prove that a protein is a physiological substrate of a PTP. Therefore potential substrates are commonly validated by other means. Typically the criteria that needs to be fulfilled include: (i) the substrate must bind to the substrate trapping mutant via the catalytic site; (ii) dephosphorylation of the substrate should be visible *in vitro*; and (iii) an increase in tyrosine phosphorylation of the substrate should be detectable in a cell line or mouse model where the PTP has been knocked out or knocked down by RNA interference (Tiganis et al. 2007).

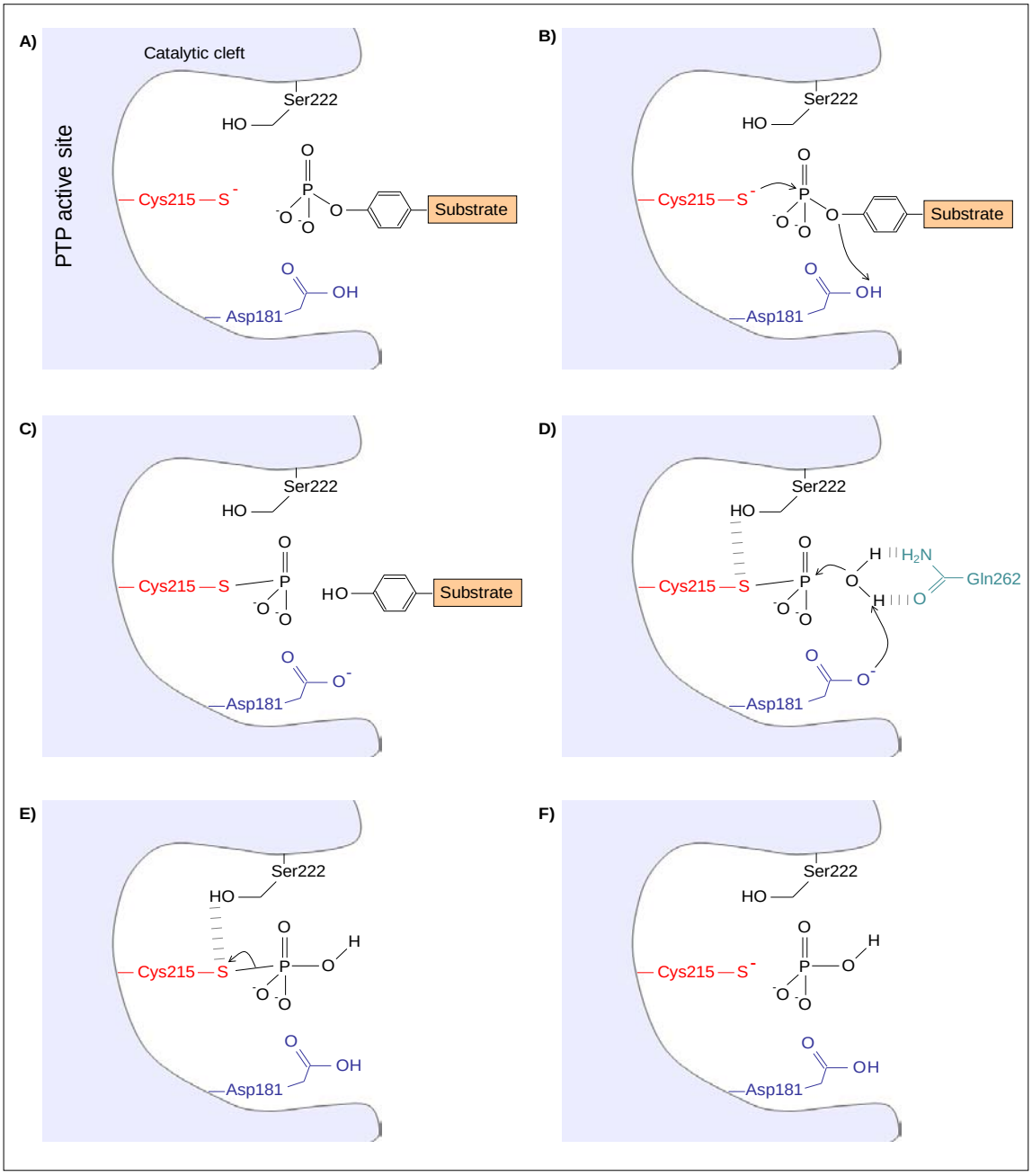


Figure 1.12: PTP-1B catalytic mechanism. (A) The tyrosine-phosphorylated substrate enters the PTP active site. (B) Nucleophilic attack occurs between the sulphur atom of the essential cysteine (Cys215) and the phosphate on the substrate, coupled with protonation of the tyrosyl leaving group by the conserved aspartic acid (Asp181), resulting in the formation of the phospho-enzyme intermediate (C). (D) Asp181 then acts as a general base to hydrolyze the phospho-enzyme intermediate, culminating in the release of phosphate (E and F).

1.7.2.5 Regulation of PTP activity

Disruption of the fine balance of tyrosine phosphorylation within a cell can severely effect functional responses of the cell. It is therefore essential that both PTKs and PTPs are under precise control. Below are outlined several ways in which PTPs are regulated (den Hertog et al. 2008):

1) Expression. The expression of PTPs is controlled at many different levels including: transcriptional regulation of mRNA, alternative mRNA splicing, mRNA stability, translation, post-translational modifications and protein stability. Some PTPs, such as PTP-1B and Shp2, are widely expressed in almost all cell types, whilst others are more selectively expressed, such as HePTP and CD45 which are expressed exclusively in hematopoietic cells (Pao et al. 2007). Some cell types appear to express many of the 38 classical PTP genes such as endothelial cells, whereas other cell types such as neurons and platelets appear to express only a select few (Kappert et al. 2005). The expression of certain PTPs can also be increased during development, such as PTPRO which is up-regulated during megakaryocyte differentiation (Taniguchi et al. 1999). Expression of CD148, LAR and RPTPμ are increased with increasing cell density (Longo et al. 1993; Ostman et al. 1994; Campan et al. 1996).

2) Subcellular localisation. The correct location within the cell is critical for a PTP to exert its physiological function. PTPs are targeted to their required subcellular locations by compartment-specific targeting domains and protein-protein interactions (Tonks 2006). Several RPTPs have been shown to regulate tyrosine phosporylation at the plasma membrane in response to a cell-cell or cell-ECM contact (Ostman et al. 2001; Burridge et al. 2006; Sallee et al. 2006). CD45 is expressed in the plasma membrane of lymphocytes where it regulates B- and T-cell receptor proximal signalling events (Hermiston et al. 2003). Some cytoplasmic

NTPTPs are recruited to the plasma membrane in response to cell stimulation, such as Shp1 and Shp2 which bind to phoshorylated ITIM receptors via their SH2 domains (Pao et al. 2007). PTP-1B is normally anchored to the cytosolic surface of the endoplasmic reticulum (ER) by a hydrophobic sequence in its C-terminus (Tonks 2003). Cleavage of this sequence by calpain releases PTP-1B from the ER allowing it to access substrates in other parts of the cell and also increases its specific activity (Frangioni et al. 1993).

3) Phosphorylation. Several PTPs are known to be phosphorylated on Ser and Tyr residues, including RPTPα, CD45, PTP-1B, PTP-PEST, Shp1 and Shp2, however, relatively little is known about how phosphorylation regulates PTPs. Phosphorylation of RPTPα at Ser-180 and Ser-204 increases catalytic activity, by a mechanism that is thought to inhibit dimer formation (den Hertog et al. 1995). Phosphorylation of RPTPα at Tyr-789 has a completely different function, mediating activation of Src and also recruitment of Grb-2, which antagonizes Src activation (Su et al. 1999). There is also evidence that PTPs can dephosphorylate other PTPs. For example, CD45 has been shown to dephosphorylate RPTPα at Tyr-789 *in vitro* and in T cells, implying that RPTPα is a direct substrate of CD45 (Maksumova et al. 2007). This suggests the possibility of PTP signalling cascades similar to the well documented, much studied kinase cascades.

4) Oxidation. It is well established that PTPs are reversibly inhibited by reactive oxygen species (ROS) that transiently oxidize the active site cysteine (Salmeen et al. 2005; Rhee 2006). Many PTPs have been shown to be oxidized following activation of tyrosine kinase-linked and G protein-coupled receptors, that elevate intracellular levels of ROS (Tonks 2005). Cell density, adhesion and migration also transiently affect levels of PTP oxidation (den Hertog et al. 2008). The major sources of ROS appear to be the mitochondria or NADPH

oxidases. The molecular mechanisms of PTP oxidation has been elucidated for some PTPs and involves formation of a covalent bond between the sulphur of the catalytic site cysteine and the nitrogen of the neighbouring serine (Salmeen et al. 2003; van Montfort et al. 2003; Yang et al. 2007). There is some variability in the sensitivity of PTPs to oxidation, which correlates with the conformation of the active site arginine (Groen et al. 2005; Ross et al. 2007). For example, Shp2 becomes oxidized following T cell stimulation with H_2O_2, whereas Shp1 does not (Kwon et al. 2005). Two commonly used general PTP inhibitors, vanadate and pervanadate, used throughout this thesis, work by oxidizing the active site cysteine of all classical PTPs (Huyer et al. 1997). Vanadate is a reversible inhibitor of PTPs whilst pervanadate is irreversible (Figure 1.13) (Huyer et al. 1997).

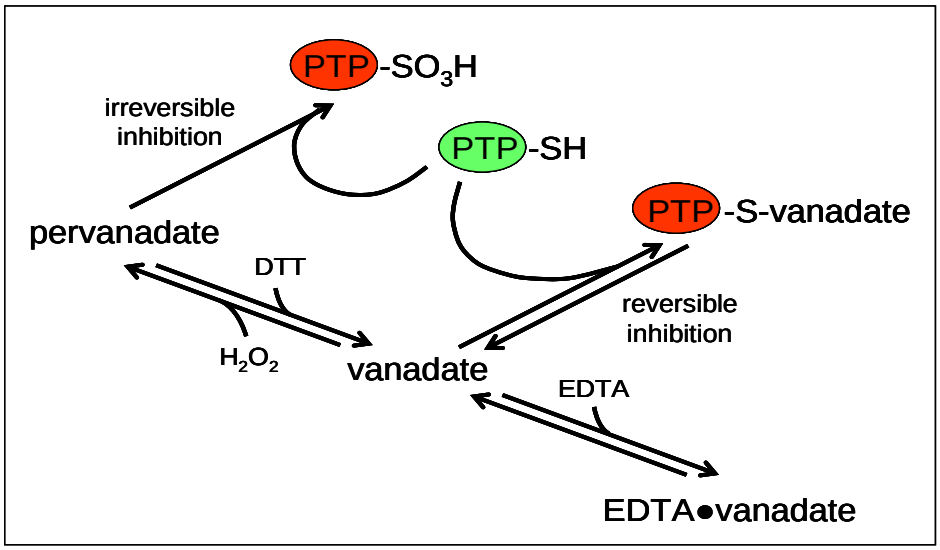


Figure 1.13. : Vanadate and pervanadate inhibition of PTPs. Figure adapted from Huyer et al. 1996. Inhibition by pervanadate is irreversible whilst inhibition by vanadate is reversible.

5) Ligands. The extracellular regions of RPTPs are typically large, highly glycosylated, structurally diverse and evolutionarily conserved, implying that they regulate PTP function. However, very few ligands of RPTPs have been identified to date. Table 1.1 summarizes our current knowledge of RPTP ligands. Interestingly, several RPTPs display homophilic interactions and/or interact with ECM proteins, suggesting that they regulate cell adhesion (den Hertog et al. 2008). The only RPTP extracellular region crystallized to date is that of RPTPμ, which was shown to form extended, rigid homophilic *trans* (anti-parallel) dimers through a 'spacer-clamp' mechanism (Aricescu et al. 2007). RPTPμ dimers are hypothesized to act as a distance gauge between adjacent cells and maintain the phosphatase in the appropriate functional location. RPTPζ is one of the best characterised RPTPs regulated by ligand binding. RPTPζ is inhibited following binding of the growth factor pleiotrophin (PTN), leading to an increase in tyrosine phosphorylation of proteins that regulate cell adhesion and cytoskeletal function (Meng et al. 2000). A size exclusion model predicts that some RPTPs are regulated by steric hinderance (Lin et al. 2003; Choudhuri et al. 2005). CD45 and CD148 are excluded from the tight immunological synapse between a lymphocyte and an antigen presenting cell due to the large size of their extracellular regions.

6) Dimerisation. Unlike receptor tyrosine kinases and tyrosine kinase-linked receptors which are activated by homodimerzation, several RPTPs have been shown to be inactivated following dimerzation. The long held, yet controversial 'inhibitory wedge' model proposes that an helix-turn-helix wedge domain in the membrane-proximal region of one RPTP obstructs the active site of the partner RPTP (Bilwes et al. 1996) (Figure 1.14). The prototype of this model is RPTPα. CD45 has also been proposed to be inactivated by the same mechanism (Majeti et al. 1998). However, recent work by Barr et. al. rejects the existence of an inhibitory wedge domain (Barr et al. 2009). The crystal structure of the complete

intracellular region of RPTPγ reveals a different mode of homodimer-mediated inhibition that involves the interaction between the D1 catalytic domain of one of the partners in the dimer with the D2 catalytic domain of the other partner. Barr et. al. recognize that the membrane-proximal region where the inhibitory wedge is thought to lie acts as a linker region that provides sufficient flexibility to allow the head-to-toe interaction to occur (Barr et al. 2009).

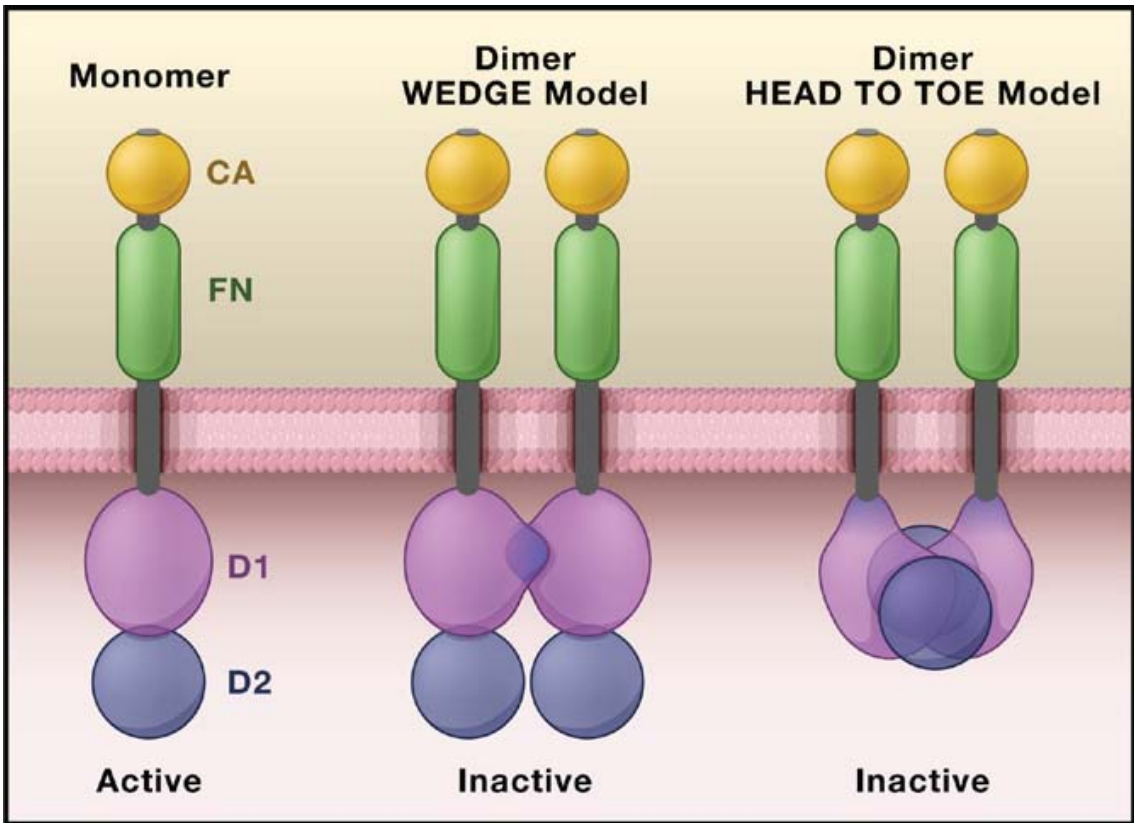


Figure 1.14. Models of homodimer-mediated inhibition of receptor-like protein tyrosine phosphatases. On the left is a schematic representation of a monomeric active R5 receptor-like protein tyrosine phosphatase (RPTP) (either RPTPγ or RPTPζ). The extracellular region consists of a carbonic anhydrase (CA) domain and a fibronectin type III (FN) domain. The 'wedge' model, based on studies of RPTPα and CD45, predicts that inhibition results from the wedge of one RPTP obstructing the catalytic domain (D1) of the partner RPTP. The 'head-to-toe' model, based on the crystal structure of the entire cytoplasmic region of RPTPγ (Barr et. al. *Cell* 2009), demonstrates that the D2 catalytic domain of one RPTP obstructs the D1 catalytic domain of the partner RPTP. (Figure from Tremblay et al, *Cell* 2009)

Table 1.1: Ligands of receptor-like protein tyrosine phosphatases (RPTPs)

RPTP	Class	Ligand(s)	Effect on activity	Comments	References
CD45	R1	- galectin-1	- inhibition	- interaction based on recognition of CD45 carbohydrates	(Walzel et al. 1999; Chen et al. 2007)
RPTPδ	R2A	- homophilic	- not known	- promotes adhesion and neurite outgrowth	(Wang et al. 1999; Gonzalez-Brito et al. 2006)
LAR	R2A	- LARFN5C - laminin-nidogen	- activation - not known	- homophilic interaction with LAR isoform - specific for LAR splice variant	(O'Grady et al. 1998; Yang et al. 2005)
dLAR	R2A	- syndecan - dallylike	- activation - inhibition	- dLAR ligand - competitive binding with snydecan	(Fox et al. 2005; Johnson et al. 2006)
LAR2	R2A	- homophilic	- not known	- induces repulsive responses in comb cells	(Johnson et al. 2006)
RPTPσ	R2A	- heparin sulphate proteoglycans (collagen XVIII, agrin) - nucleophilin - α-latrotoxin - unidentified component of developing muscle)	- not known	- ligand binding requires PTP dimerization	(Aricescu et al. 2002; Sajnani-Perez et al. 2003; Alete et al. 2006; Lee et al. 2007)
RPTPκ	R2B	- homophilic	- not known		(Sap et al. 1994)
RPTPμ	R2B	- homophilic	- not known	- 'spacer-clamp' mechanism in cell-cell adhesions; induces RPTPμ signalling in neurites	(Ensslen-Craig et al. 2005; Aricescu et al. 2007)
RPTPl	R2B	- homophilic	- not known		(Cheng et al. 1997)
CD148	R3	- components in matrigel	- activation	- identity of ligand(s) not known	(Sorby et al. 2001)
RPTPβ/ζ	R5	- pleiotrophin - tenascin - contactin - TAG-1/axonin-1	- inhibition - not known - not known - not known	- may activate several signalling pathways; not known whether inhibition occurs by induction of dimer formation	(Barnea et al. 1994; Peles et al. 1995; Milev et al. 1996)

dLAR, *Drosophila* LAR

1.7.2.6 PTPs in platelets

Although it has been known for many years that PTPs play important roles in regulating cell functions in both health and disease, they have been under-investigated in megakaryocytes, platelets and thrombosis. Significantly more emphasis has been placed on investigating PTKs in platelets. To date, five NTPTPs (PTP-1B, Shp1, Shp2, MEG-2 and LMW-PTP) and a single RPTP (CD148) have been conclusively demonstrated to be expressed in platelets (Table 1.2). However, this list is unlikely to be exhaustive as there are several PTPs which are widely expressed, such as PTP-PEST and TC-PTP. PTP-1B was the first PTP identified in platelets and is the most well studied. Far less is known about the other four NTPTPs in platelets. Prior to starting this thesis, there had been no studies investigating the functional role of any RPTP in platelets, including CD148. Below I provide a summary of each of the PTPs identified in platelets to date and what is known about their functions in platelets.

1.7.2.6.1 Receptor like PTPs expressed in platelets

1.7.2.6.1.1 CD148

CD148, also commonly referred to as DEP-1, PTPRJ or rPTPη (rat homologue), is a member of the R3 group of RPTPs (Figure 1.11). It is a large (180 – 250 kDa), surface glycoprotein consisting of an extracellular region containing 8 fibronectin type III (FNIII) repeats (~90 amino acids each), a single transmembrane domain and a cytoplasmic region containing a single PTP domain (~280 amino acids). It is expressed in hematopoietic cells, fibroblasts, endothelial cells, epithelial cells and smooth muscle cells (Borges et al. 1996; Autschbach et al. 1999). At the start of this thesis, only two studies had reported expression of CD148 in platelets, but neither had addressed its functional role (Borges et al. 1996; de la Fuente-Garcia et al. 1998). CD148 was also the only RPTP identified in platelets by multiple peptide hits using a proteomics-based approach (Senis et al. 2007; Lewandrowski et al. 2009).

Table 1.2: Protein tyrosine phosphatases expressed in platelets

Category/ Name	Subcellular localization	Function	References
Non-transmembrane PTPs			
MEG2	binds to PIP2 and PIP3	modulates secretory vesicle genesis	Wang et. al. JEM 2005
PTP-1B	- tethered to the cytosolic surface of the ER - calpain-mediated shedding from the ER and translocation to the PM upon platelet activation	- first PTP identified in platelets - proposed to regulate the latter stages of platelet activation and aggregation - positive regulator of αIIbβ3 proximal signalling - dephosphorylates LAT and Src inhibitory tyrosine	Frangioni et. al. EMBO 1993 Ragab et. al. JBC 2003 Arias-Salgado et. al. Blood 2005 Kuchay et. al. MCB 2007
LMW-PTP	-cytosolic in resting platelets	- dephosphorylates the ITAM-containing FcγRIIA receptor *in vitro* and *in vivo*	Mancini et. al. Blood 2007
Shp1	- cytosolic/inactive in resting platelets - translocates to the PM and becomes activated via interaction with ITIM receptors upon platelet activation	- positive regulator of GPVI-mediated activation - interacts with actinin and Src - dephosphorylates Src inhibitory tyrosine	Somani et. al. JBC 1997 Hua et. al. JBC 1998 Pasquet et. al. JBC 2000 Lin et. al. JBC 2004 Senis et. al. MCP 2007
Shp2	- cytosolic/inactive in resting platelets - translocates to the PM and becomes activated via interaction with ITIM receptors upon platelet activation	implicated as positive and negative regulator of platelet activation	Hua et. al. JBC 1998 Cicmil et. al. Blood 2002 Barrow et. al. JI 2004 Washington et. al. Blood 2004 Wee et. al. Blood 2005 Dhanjal et. al. Platelets 2007
Receptor-like PTPs			
CD148	PM	- first RPTP identified in platelets - positive regulator of all SFKs in resting and activated platelets	Borgess et. al. Circ Res 1996 Fuente-Garcia et. al. Blood 1998 Senis et. al. MCP 2007 Senis et. al. Blood 2009

Studies on CD148 in non-haematopoietic cells have focused on its role as a negative regulator of cell growth and differentiation, as it is postulated to be a tumour suppressor (Ruivenkamp et al. 2002; Trapasso et al. 2004). More recently, studies have focused on the involvement of CD148 in regulating cell-cell and cell-ECM interactions. CD148 has been shown to inhibit PDGF-mediated cell migration and enhance cell-ECM adhesion of NIH3T3 mouse fibroblasts that inducibly express CD148 (Jandt et al. 2003). Similarly, rat fibroblasts stably expressing CD148 exhibit increased adhesion to substratum (Pera et al. 2005). In contrast, CD148 has also been shown to negatively regulate cell-ECM interactions, motility and chemoxtaxis of NIH3T3 inducibly expressing CD148 (Kellie et al. 2004). These contradictory findings suggest that CD148 can both positively and negatively regulate cell adhesion, depending on the cell type and level of expression.

There is a growing body of evidence that CD148 plays an important role in regulating immune receptor signalling in haematopoietic cells. Early studies suggested that CD148 was a negative regulator of signalling from the ITAM-containing T cell receptor (TCR) and the low affinity immune receptor FcγRIIA in lymphocytes and neutrophils, respectively (Hundt et al. 1997; de la Fuente-Garcia et al. 1998; Baker et al. 2001; Lin et al. 2003). These early studies were performed using transient and stable CD148 expressing cell lines, primary leukocytes and antibody-mediated cross-linking of CD148. A CD148 mutant mouse line in which the entire cytoplasmic region was replaced with green fluorescence protein led to embryonic lethality and severe defects in vascular development (Takahashi et al. 2003). The same group also demonstrated that antibody-mediated cross-linking of CD148 inhibited endothelial-cell growth and angiogenesis in mouse cornea *in vivo* (Takahashi et al. 2006). More recently two different CD148 knock-out and knock-in mouse models have been generated to determine the physiological functions of CD148. Trapasso and Fusco generated a CD148-deficient mouse model by deleting exons 3, 4 and 5 (Trapasso et al. 2006). In

contrast to the CD148:GFP knockin mutant mice described above, these mice were viable and healthy demonstrating that CD148 is dispensable for growth and development. However, this knockout mouse model was not studied in any detail for haematopoitic or immune system defects. A third mutant CD148 mouse model generated by Zhu et. al. lacking the transmembrane domain of CD148 (CD148 TM-KO) also did not exhibit any obvious defects in growth and development; however, detailed analysis of haematopoietic cells revealed a function of CD148 in the immune system (Zhu et al. 2008). It should be pointed out that the CD148 TM-KO mouse model expresses a truncated, soluble form of CD148.

Analysis of the T cell lineage, which express low levels of CD148, in CD148 TM-KO mice, did not reveal any defects in T cell development or function (Zhu et al. 2008). In contrast these mice exhibited almost an exact phenocopy of the block in B cell development observed in CD45-deficient mice. CD45:CD148 double deficient mice displayed substantial alterations in B and myeloid lineage development and defective immunoreceptor signalling (Zhu et al. 2008). B cell and macrophage functional responses were severally impaired, as were BCR- and Fc receptor-mediated signalling in CD45:CD148 double deficient B cells and macrophages, respectively. Biochemical analysis of CD45:CD148 double deficient B cells and macrophages revealed hyperphosphorylation of the C-terminal inhibitory tyrosine of SFKs, suggesting that the C-terminal inhibitory tyrosine of SFKs is a common substrate of both CD45 and CD148 and that decreased SFK activity is likely the cause of at least some of the phenotypes observed in mutant mice. These findings suggest a high level of redundancy between the two structurally distinct RPTPs in regulating SFKs (Zhu et al. 2008).

Several receptors and signalling proteins have been suggested to be substrates of CD148 (Table 1.3). The hepatocyte growth factor receptor MET, the adapter protein Gab1 and the junctional component p120 catenin were identified as potential substrates of CD148 in a study of human breast cancer cell lines (Palka et al. 2003). CD148 was also shown to

interact with p120 catenin in K562 erythroleukemia and A549 lung fibroblast cell lines (Holsinger et al. 2002). CD148 expression was concentrated at sites of cell-cell contact and was found to be co-localised with p120 catenin further reinforcing the theory that CD148 plays an important role in cell-cell contacts and adherens junctions. CD148 has also been shown to dephosphorylate the PDGFβ-receptor in a site-selective manner that acts to modulate rather than attenuate signalling; and to preferentially dephosphorylate the C-terminal inhibitory tyrosine of Src in transiently transfected rat fibroblasts (Kovalenko et al. 2000; Pera et al. 2005).

Table 1.3: Summary of substrates identified for CD148

Substrate	**Cell type**	**Reference**
MET	Human breast tumour cell lines	(Palka et al. 2003)
Gab1	Human breast tumour cell lines	(Palka et al. 2003)
p120cat	Human breast tumour cell lines, erythroleukemia and lung fibroblast cell lines	(Palka et al. 2003)
PDGFβ	Porcine aortic endothelial cells	(Kovalenko et al. 2000)
SFKs	Rat fibroblasts	(Pera et al. 2005)
PLCγ1	Jurkat T cell	(Baker et al. 2001)
LAT	Jurkat T cell	(Baker et al. 2001)

The presence of multiple FNIII domains in the extracellular region of CD148 suggests that it may be regulated by soluble ligands or adhesive molecules present on neighbouring cells or in the ECM, as FNIII domains are present in adhesion molecules and growth factor receptors (Patthy 1990). In support of this hypothesis, CD148 expressing X23 porcine aortic endothelial cells stimulated with Matrigel exhibited a >2-fold increase in CD148 specific activity suggesting that the ligand for CD148 may present in Matrigel (Sorby et al. 2001). It has also been suggested that CD148 localisation in the membrane may be regulated through a size exclusion model (Lin et al. 2003; Choudhuri et al. 2005).

The crystal structure of the CD148 PTP domain was recently determined by Barr et al (Barr et al. 2009). X-ray diffraction techniques and phosphatase activity assays were used to study the PTP domain structures and activities of a representative panel of classical PTPs. Interestingly, the CD148 WPD loop was found to be in a closed conformation and signifies a catalytically active state. In kinetics studies, recombinant CD148 PTP domain had one the highest enzymatic actives compared with a panel of 21 other recombinant PTP domains (Barr et al. 2009).

1.7.2.6.1.2 Other platelet RPTPs

Another RPTP that may be expressed in platelets is PTPRO (also referred to as GLEPP1 and PTP-U2), which belongs to the same subgroup and is structurally related to CD148 (Figure 1.11). PTPRO is primarily expressed in the brain and kidney, but is also present in hematopoietic stem cells, lungs, lymph nodes, spleen and placenta (Avraham et al. 1997; Aguiar et al. 1999; Beltran et al. 2003). It was recently shown to be expressed in human megakaryocytes and detected in a mouse megakaryocyte SAGE library (Taniguchi et al. 1999; Senis et al. 2007). It has been implicated in megkaryocytopoeisis and that its role is regulated by the SCF receptor, Kit. Importantly, knockdown of PTPRO expression in megakaryocytic

cell lines has been shown to significantly inhibit megakaryocyte progenitor proliferation. PTPRO knockout mice have defects in kidney function as a result of a change in podocyte structure, however, they have not been examined in detail for other physiological defects (Wharram et al. 2000).

The structurally distinct RPTP CD45, which is highly and exclusively expressed in all hematopoietic cells, except platelets and erythrocytes, was also recently detected in a large immature population of murine megakaryocytes, suggesting that it may be involved in megakaryocyte development and switched off during megakaryocyte maturation and platelet formation (Matsumura-Takeda et al. 2007).

1.7.2.6.2 Non-transmembrane PTPs

1.7.2.6.2.1 PTP-1B

PTP-1B is a small (50 kDa) NTPTP that is expressed in all tissues. Structurally, PTP-1B is comprised of an N-terminal PTP domain, followed by two tandem proline-rich motifs that may allow the interaction with SH3-domain containing proteins, and a short C-terminal hydrophobic stretch of amino acids that localize it to the cytoplasmic surface of the ER (Figure 1.11) (Frangioni et al. 1992). It is a major regulator of metabolism as demonstrated by two PTP-1B-deficient mouse models that are resistant to high fat-induced obesity and insulin hypersensitivity (Elchebly et al. 1999; Klaman et al. 2000). The obesity phenotype is due to its role in dephosphorylating Jak-2 downstream of the leptin receptor, and the insulin hypersensitivity is due to its role in dephosphorylating the insulin receptor and possibly insulin receptor substrate-1 (IRS-1). PTP-1B has also been linked to other diseases, including cancer and autoimmunity, through its role in regulating cytokine and growth factor receptor signalling in haematopoietic and lymphoid cells (Tonks 2006).

PTP-1B was the first PTP identified in platelets and is likely the most abundant, comprising ~0.2% of all soluble platelet proteins, comparable to that of Src kinase (Frangioni et al. 1993). Platelet activation accompanied by integrin αIIbβ3 engagement causes cleavage of the C-terminal hydrophobic tail of PTP-1B by calpain, releasing it from the ER and increasing its specific activity (Frangioni et al. 1993). Recent work using a PTP-1B:calpain-1 double deficient mouse model confirmed that calpain is essential for this cleavage (Kuchay et al. 2007). In support of PTP-1B being involved in outside-in αIIbβ3 integrin signalling, Ragab *et al* demonstrated that PTP-1B regulates FcγRIIA-mediated irreversible aggregation (Ragab et al. 2003).

Platelets from PTP-1B-deficient mice exhibit a number of functional defects attributed to defective outside-in integrin αIIbβ3 signalling (Arias-Salgado et al. 2005). These defects include: reduced spreading on fibrinogen, defective clot retraction, reduced aggregate formation on collagen under flow, and reduced thrombus formation *in vivo*. Interestingly, convulxin-, ADP- and PAR-4 peptide-induced responses were normal, suggesting that GPVI, $P2Y_1$, $P2Y_{12}$ and PAR-4 receptor signalling were normal. The molecular basis of the phenotype exhibited by PTP-1B-deficient platelets is a block in outside-in integrin αIIbβ3 proximal signalling, caused by hyperphosphorylation of the C-terminal inhibitory tyrosine (Tyr-529) of Src. Based on their findings, Arias-Salgado et. al. proposed a model whereby fibrinogen binding to αIIbβ3 triggers PTP-1B recruitment to the αIIbβ3-Src-Csk complex, dissociation of Csk, dephosphorylation of Src Tyr-529 resulting in Src activation and initiation of the signalling cascade (Arias-Salgado et al. 2005). PTP-1B therefore is an essential positive regulator of the initiation of outside-in integrin αIIbβ3 signalling in platelets. How PTP-1B gets activated downstream of the integrin αIIbβ3 remains to be answered. Further, why PTP-1B specifically activate Src downstream of αIIbβ3 and not other

SFKs downstream of other platelet surface receptors, such as Fyn and Lyn downstream of GPVI also needs to be addressed.

1.7.2.6.2.2 Shp1 and Shp2

Shp1 and Shp2 are a subfamily of NTPTPs characterized by the presence of two tandem SH2 domains, a classic PTP domain and a C-terminal tail containing two tyrosyl phosphorylation sites (Figure 1.11). Expression of Shp1 is restricted, with the highest levels found in haematopoietic cells, whereas Shp2 is widely expressed in a number of cell types (Neel et al. 2003). The SH2 domains of Shp1 and Shp2 play a crucial role in regulating PTP activity through intra- and inter-molecular binding that obstructs the catalytic cleft and mediate protein-protein interactions, primarily with ITIM proteins.

Shp1 is generally recognized as a negative regulator of tyrosine kinase-linked receptor signalling. For example, it has been proposed that Shp1 maintains the BCR in a non-signalling state by dephosphorylating the associated ITAMs and thereby controlling the threshold for receptor activation (Pani et al. 1995). Similarly, it has been suggested that Shp1 attenuates the earliest events in TCR signalling by dephosphorylating the SFK Lck and the tyrosine kinase ZAP-70 (Plas et al. 1996; Chiang et al. 2001). Shp1 has also been shown to inhibit integrin signalling in macrophages and neutrophils (Roach et al. 1998; Kruger et al. 2000; Griffiths et al. 2001; Peterson et al. 2001). Collectively, these findings are interesting from a platelet perspective as several candidate substrates of Shp1 downstream of the BCR and TCR are also involved in GPVI and/or integrin αIIbβ3 signalling (Kon-Kozlowski et al. 1996; Pani et al. 1996; Cuevas et al. 1999; Dustin et al. 1999; Mizuno et al. 2000; Stebbins et al. 2003; Mizuno et al. 2005). Two naturally occurring point mutations exist in the murine *Shp1* gene, resulting in no expression of Shp1 (*motheaten* allele) or expression of catalytically inactive Shp1 (*motheaten viable* allele). The phenotypes of the two mutations differ only in

severity, with *motheaten* mice dying earlier (2 – 3 weeks) than *motheaten viable* mice (9 – 12 weeks) due to severe inflammation and tissue damage (Van Vactor et al. 1998; Zhang et al. 2000; Neel et al. 2003). Mechanistically, most of the abnormalities of *motheaten* mice can be explained by the loss of negative regulatory pathways in the absence of Shp1. The functional role of Shp1 in platelets has been investigated using *motheaten viable* mice (Pasquet et al. 2000). This work demonstrated the surprising observation that Shp1 plays a positive role in supporting platelet activation through GPVI.

Shp2 is predominantly regarded as a positive regulator of the Ras-Erk pathway (Noguchi et al. 1994; Shi et al. 2000). It has also been implicated in regulating the PI-3 kinase and Rho pathways (Schoenwaelder et al. 2000; Zhang et al. 2002). The role of Shp2 in ITAM receptor signalling is less well understood than for Shp1. However, studies of TCR signalling and several inhibitory receptors implicate Shp2 as a negative regulator of immunoreceptor signalling pathways (Kwon et al. 2005). Another mechanism by which Shp2 inhibits TCR signalling may involve its association with inhibitory ITIM receptors, including CEACAM1 and PECAM-1 (Ilan et al. 2003; Gray-Owen et al. 2006). Shp2-deficient mice die between 8 and 10 weeks with a range of developmental defects (Van Vactor et al. 1998; Saxton et al. 2000). Interestingly, mice homozygous for the Shp2 D61G gain-of-function mutation die at mid-gestation, exhibiting a gross hemorrhagic phenotype, and heterozygotes develop a Noonan syndrome-like disorder with excessive bleeding (Araki et al. 2004). The dramatically different phenotypes exhibited by Shp1- and Shp2-deficient mutant mice demonstrate that they have different biological functions (Van Vactor et al. 1998).

It is presently not clear, whether Shp1 and Shp2 are predominantly negative or positive regulators of platelet activation. Although Shp1 and/or Shp2 have been shown to interact with each of the known platelet receptor-like ITIMs, namely PECAM-1, TLT-1, G6b-B and CEACAM1, the relative contributions of Shp1 and Shp2 to signalling by each of these

receptors is not known (Jackson et al. 1997; de Vet et al. 2001; Barrow et al. 2004; Newland et al. 2007; Senis et al. 2007; Wong et al. 2009).

1.7.2.6.2.3 MEG-2

MEG-2 is a NTPTP and is distinguished from other mammalian PTPs by the presence of an N-terminal lipid-binding domain. MEG-2 resides on internal membranes, including secretory vesicles and granules in neutrophils and lymphocytes, and has been shown to modulate murine development and platelet and lymphocyte activation through secretory vesicle function (Wang et al. 2005). Wang *et al* demonstrated that platelets from MEG-2-deficient mice did not aggregate when stimulated with a high dose of thrombin compared to wild-type platelets (Wang et al. 2005). In contrast, ADP-induced aggregation was only slightly impaired in the MEG-2 deficient platelets. The author suggests a link with defective release of α-granules however, the exact mechanism remains unclear.

1.7.2.6.2.4 LMW-PTP

LMW-PTP is a small (18 kDa) archaic PTP that consists of a single PTP domain containing the active-site signature motif HCX_5R and no other known domains or motifs (Alonso et al. 2004). It is not classified as a classical PTP, but rather is the only class II NTPTP based on its unique structural features. Although its physiological function is not known, it has been implicated in regulating cell adhesion and spreading (Chiarugi et al. 2000; Rigacci et al. 2002). One study reports the expression of LMW-PTP in platelets and DAMI human megakaryocytic cells (Mancini et al. 2007). Mancini *et. al*. demonstrate that human platelets express high levels of LMW-PTP (~0.05% of total protein) and that it dephosphorylates the ITAM-containing FcγRIIA receptor *in vitro* and in cell lines (Mancini et al. 2007). The

authors conclude that LMW-PTP plays an important role in regulating FcγRIIA receptor signalling in platelets.

1.8 Aims of this thesis

The main objective of this thesis was to determine the expression, biochemical properties and function of CD148 in platelets. The reason I focused on CD148 is because it was the only RPTP identified by multiple peptide hits in a global platelet membrane proteomics study conducted by my supervisor, Dr. Senis, and he hypothesized that platelets express a CD45-like RPTP that is critical for initiating signalling from the ITAM receptor GPVI. He also hypothesized that platelets express a PTPα-like RPTP that could initiate integrin αIIbβ3 signalling. These two hypotheses formed the basis of this thesis.

A third hypothesis which underpins the work on PTP-1B and TC-PTP in the final chapter of this thesis is that other PTPs compensate in the absence of CD148 in platelets. Since I was unable to conclusively demonstrate expression of another RPTP in platelets that could fulfil this functional redundancy, I turned my attention to PTP-1B and the structurally related TC-PTP. PTP-1B was the primary candidate as recent work by Arias-Salgado et. al. showed that PTP-1B was essential for activating integrin αIIbβ3-associated Src in platelets, by a similar mechanism to the one we had discovered for CD148. When we found that the aggregation, secretion and phosphorylation defects observed in PTP-1B-deficient platelets were much milder and complicated by strain variation compared with the defects observed in CD148-deficient platelets, we hypothesized that TC-PTP may have similar functional roles to CD148 in platelets, which is why I analyzed platelets from TC-PTP-deficient platelets for functional defects.

CHAPTER 2 -

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies and chemicals

All antibodies, agonists and inhibitors used in this thesis are detailed in Tables 2.1, 2.2 and 2.3, respectively. Alexa488-conjugated phalloidin was purchased from Molecular Probes. Bovine serum albumin (BSA) used for blocking western blots was purchased from First Link UK Ltd. Fatty acid free BSA used for blocking slides and coating plates was purchased from Sigma. Phosphopeptides corresponding to the activation loop and C-terminal inhibitory tail of SFKs were synthesized by GenScript (Piscataway, NJ, USA) (Table 2.5). All other chemicals were purchased from Sigma, unless otherwise indicated.

2.1.2 Molecular biology reagents

All expression constructs used in this thesis are detailed in Table 2.4. All cDNAs were sequenced at Alta Biosciences (University of Birmingham, UK), prior to use. TOP10 DH5α competent bacteria were purchased from Invitrogen. Mini-prep kits and maxi-prep kits were purchased from Sigma. HEK 293T cells and DT40 chicken B cells were kindly provided by Dr. Mike Tomlinson. RPMI, DMEM, foetal bovine serum, chicken serum, GlutaMAX, antibiotics and antimycotics were purchased from Gibco (Invitrogen).

2.1.3 Genetically modified mice

CD148 transmembrane knockout (CD148 TM-KO) mice on a C57BL/6 background were kindly provided by Professor Arthur Weiss (UCSF, California, USA) (Zhu et al. 2008). PTP-1B-deficient mice on a Balb/c and a C57BL/6 background were kindly provided by Professor Michel Tremblay (McGill University, Montreal, Canada) (Elchebly et al. 1999). All procedures were undertaken with United Kingdom Home Office approval in accordance with

the Animals (Scientific Procedures) Act of 1986 (Project License No: 40/2212, 40/2803, 40/2908 and 40/2749).

Table 2.1. Antibodies

Antigen (species)	**Species antibody raised in (clone or catalogue number)**	**Source**	**Working dilution**
αIIbβ3 (m, resting)	rat, PE-conjugated (Leo-H4)	Emfret Analytics	FC: 1/100
αIIbβ3 (m, high affinity)	rat, PE-conjugated (JON/A)	Emfret Analytics	FC: 1/100
actin (h, m)	mouse monoclonal (ac-40)	Sigma	WB: 1/1,000
CD148 (h, m)	mouse monoclonal (143-41)	Biosource	IP: 1/500 WB: 1/1,000
Csk (h)	rabbit polyclonal (sc-286)	Santa Cruz Biotechnology	IP: 1/1,000 WB: 1//1,000
FcR γ-chain (h,m)	rabbit polyclonal	Millipore	WB: 1/1,000
Fyn (h, m)	rabbit monoclonal (clone: 04-353)	Millipore	WB: 1/1,000
Fyn (h, m)	rabbit polyclonal (clone: BL90)	Dr. Joseph Bolen (DNAX)	IP: 1/1,000 WB: 1/1,000
Fyn p-Tyr 530 (h, m)	rabbit polyclonal (53690)	Abcam	WB: 1/1,000
goat IgG FITC-conjugate	chicken (HAF019)	R&D Systems	WB: 1/5,000
GPVI (m)	rat, FITC-conjugated (JAQ1)	Emfret Analytics	IP: 1/1,000 WB: 1//1,000
GPVI (h)	rabbit polyclonal	Professor Masaki Moroi (Japan)	FC: 1/100 WB: 1//1,000
LAT (h, m)	rabbit polyclonal (AB4093)	Millipore	WB: 1/500
Lyn (h, m)	rabbit polyclonal (sc-15)	Santa Cruz Biotechnology	WB: 1/1,000
Lyn p-Tyr 507 (h, m)	rabbit polyclonal (2731)	Cell Signaling	WB: 1/1,000
mouse IgG FITC-conjugate	sheep (26851)	Amersham	WB: 1/10,000
non-immune mouse IgG1	mouse (MOPC1)	Sigma	IP: 1/500
non-immune rabbit IgG	rabbit (12-370)	Millipore	IP: 1/500
PLCγ2 (h, m)	rabbit polyclonal (DN84)	Dr. Joseph Bolen (DNAX)	IP: 1/1,000 WB: 1/1,000

h, human; m, mouse; r, rat; p, phospho; FC, flow cytometry; IP: immunoprecipitation; WB: western blotting

Table 2.1 (continued)

Antigen (species)	**Species antibody raised in (clone or catalogue number)**	**Source**	**Working dilution**
P-selectin (h)	rat, FITC conjugated (AC1.2)	BD Biosciences	FC: 1/100
P-selectin (m)	rat, FITC conjugated (WUG.E4)	Emfret Analytics	FC: 1/100
p-Tyr (h, m)	mouse monoclonal (4G10)	Millipore	WB: 1/1,000
PTPRO (h, m)	goat polyclonal (sc-33415)	Santa Cruz Biotechnology	IP: 1/1,000 WB: 1/1,000
rabbit IgG FITC-conjugate	donkey (A50-201A)	Amersham	WB: 1/10,000
RPTPσ (h,m)	mouse monoclonal (17G7.2)	Dr. M.L. Tremblay (McGill University)	WB: 1/100
Src pan (h, m)	rabbit polyclonal (44656G)	Invitrogen	IP: 1/500 WB: 1/1,000
Src p-Tyr 418 (h, m)	rabbit polyclonal (44660G)	Invitrogen	WB: 1/1,000
Src p-Tyr 529 (h, m)	rabbit polyclonal (44-662G)	Biosource	WB: 1/1,000
Syk (h, m)	rabbit polyclonal (BR15)	Dr. Joseph Bolen (DNAX)	WB: 1/1,000
TC-PTP (h,m,r)	mouse monoclonal (252294)	R & D systems	WB: 1/1,000

h, human; m, mouse; r, rat; p, phospho; FC, flow cytometry; IP: immunoprecipitation; WB: western blotting

Table 2.2. Agonists

Agonist	Target receptor	Source
ADP	$P2Y_1$, $P2Y_{12}$	Sigma
collagen (HORM; equine; primarily types I and III)	GPVI, $\alpha 2\beta 1$	Nycomed
convulxin (snake toxin)	GPVI, GPIb-IX-V	Latoxan
collagen-related peptide (CRP; GCO-[GPO]10-GCOG-NH_2) chemically cross-linked as previously described (Morton et al, Biochem J, 1995; Asselin et al, Biochem J, 1999)	GPVI	Department of Biochemistry, Cambridge, UK
fibrinogen (human)	$\alpha IIb\beta 3$	Enzyme Research Laboratories
ionomycin	Ca^{2+} ionophore	Sigma
PMA	PKC	Sigma
thrombin (bovine)	PAR-1, PAR-4	Sigma
U46619	TP	Sigma
prostacyclin (PGI_2)	PGI_2 receptor	Camen Chemicals

Table 2.3. Inhibitors

Inhibitor	**Target**	**Final concentration**	**Source**
apyrase	ADP/ATP scavenger	2 U/ml	Sigma
indomethacin	cyclooxygenase	10 μM	Sigma
integrilin	αIIbβ3	9 μM	Sigma
EDTA	divalent cation chelator	1 μM	Sigma
EGTA	divalent cation chelator – primarily Ca^{2+}	1 μM	Sigma
pervanadate	protein tyrosine phosphatases	100 μM	Sigma
PP1	Src family kinases	10 μM	Calbiochem
PP2	Src family kinases	10 μM	Calbiochem
AEBSF	serine proteases	200 μg/ml	Sigma
leupeptin	serine proteases	10 μg/ml	Sigma
aprotinin	serine proteases	10 μg/ml	Sigma
pepstatin	serine proteases	10 μg/ml	Sigma
heparin	acts as a cofactor for anti-thrombin III, which inhibits coagulation factors, including thrombin	10 U/ml	Sigma

Table 2.5: Phosphopepetides used in *in vitro* phosphatase assay.

SFK peptide	peptide sequence
Lyn activation loop	VIEDNE**pY**$_{397}$TAREGA
Lyn inhibitory site	TATEGQ**pY**$_{508}$QQQP
Src activation loop	LIEDNE**pY**$_{419}$TARQGA
Src inhibitory site	TSTEPQ**pY**$_{530}$QPGENL
Fyn inhibitory site	TATEPQ**pY**$_{531}$QPGENL

Peptides custom manufactured by Genscript

2.2 Methods

2.2.1 Platelet preparation

2.2.1.1 Washed human platelets

Blood was drawn on the day of experiment from healthy, drug-free volunteers into 1:10 (v:v) sterile sodium citrate and 1:9 (v:v) acid citrate dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Platelet rich plasma (PRP) was obtained by centrifugation at 200 ×*g* for 20 minutes. Platelets were isolated from PRP by centrifugation at 1000 ×*g* in the presence of 10 μg/ml PGI_2. The platelet pellet was washed in 25 ml modified Tyrodes-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 followed by centrifugation at 1,000 ×*g* for 10 minutes in the presence of 10 μg/ml PGI_2. Platelets were counted using a Coulter Z_2 Particle Count and Size Analyzer (Beckman Coulter Ltd), and resuspended to a concentration of 2×10^8/ml for aggregation studies or 5×10^8/ml for protein studies in Tyrodes-HEPES buffer. Washed platelets were rested for at least 30 minutes prior to use to allow the effects of PGI_2 to wear off.

2.2.1.2 Washed mouse platelets

Blood samples were drawn from terminally CO_2-narcosed mice by cardiac puncture. Approximately 900 μl blood was collected from each mouse into 100 μl ACD. Subsequent to collection, anti-coagulated blood was further diluted with 200 μl modified Tyrodes-HEPES buffer. Blood was centrifuged at 180 ×*g* for 5 minutes and then the supernatant and the top third of the erythrocyte layer were removed and centrifuged at 200 ×*g* for 6 minutes. The PRP layer was transferred into a new tube and 300 μl of modified Tyrodes-HEPES buffer was added to the remaining erythrocyte layer. The diluted erythrocyte layer was centrifuged at 200 ×*g* for 6 minutes, the PRP layer was removed and pooled with the previously collected

PRP. Modified Tyrode's-HEPES buffer was added to the PRP to a final volume of 1 ml. Washed platelets were prepared by centrifugation of the diluted PRP at 1000 ×*g* for 6 minutes in the presence of 10 μg/ml PGI_2. The platelet pellet was resuspended in modified Tyrodes-HEPES buffer, platelets were counted using a Coulter Z_2 Particle Count and Size Analyzer, and platelets were diluted to 2×10^8/ml for aggregation studies or 5×10^8/ml for protein studies. All washed platelets were rested for at least 30 minutes prior to use to allow the effects of PGI_2 to wear off.

2.2.1.3 Preparation of ADP-sensitive mouse platelets

Blood samples were drawn from terminally CO_2-narcosed mice by cardiac puncture. Approximately 900 μl of blood was collected from each mouse into 100 μl 100 U/ml heparin diluted in modified Tyrodes-HEPES buffer. Anti-coagulated blood was further diluted with 200 μl of 10 U/ml heparin in modified Tyrodes-HEPES buffer. Blood was centrifuged at 180 ×*g* for 5 minutes and the platelet-rich plasma (PRP) was collected. Platelets were counted using a Coulter Z_2 Particle Count and Size Analyzer and diluted to a concentration of 2×10^8/ml with modified Tyrodes-HEPES buffer for aggregation studies.

2.2.1.4 Preparation of platelet poor plasma

Platelet poor plasma (PPP) was prepared by centrifuging the remaining erythrocyte layer following collection of PRP at 1000 ×*g* for 6 minutes. PPP was collected and diluted with Tyrodes-HEPES buffer to the same extent as PRP.

2.2.2 Platelet Functional Assays

2.2.2.1 Platelet Aggregation and ATP secretion

Platelet aggregation and ATP secretion were measured simultaneously, in real-time as a change in optical density and luciferase/luciferin generated luminescence, respectively, using a Born lumi-aggregometer (Chronolog, Labmedics). Samples (washed platelets or PRP at 2×10^8/ml) were maintained at 37°C with constant stirring at 1,200 rpm throughout the experiment. The optical density and luminescence of the each platelet suspension were recorded in real-time using a pen recorder over a 6 minute period (Chronolog, Labmedics). Modified Tyrodes-HEPES buffer and PPP were used as blanks for washed platelets and PRP, respectively. Glass tubes and stir bars in which samples were stimulated were siliconized. Samples were pre-warmed at 37°C for 2 minutes without stirring followed by 30 seconds at 37°C with continuous stirring at 1,200 rpm prior to addition of agonists. Inhibitors were added to platelets 5 – 10 minutes prior to addition of agonists.

2.2.2.2 Platelet spreading

Coverslips were coated with 200 μl of either 100 μg/ml fatty acid free BSA (control), 100 μg/ml collagen or 100 μg/ml fibrinogen and incubated overnight at 4°C. Coated coverslips were washed twice with phosphate buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) and blocked with 200 μl of denatured (10 minutes at 100°C) 5 mg/ml fatty acid free BSA for 60 minutes at room temperature. Coverslips were washed twice with PBS prior to addition of platelets (200 μl at 2×10^7/ml) pre-treated with 2 U/ml apyrase and 10 μM indomethacin. Platelets were incubated on protein-coated slides for 45 minutes at 37°C. Non-adherent platelets were washed away with modified Tyrode's-HEPEs buffer, platelets were fixed for 10 minutes with 3.7% paraformaldehyde (Sigma) then permeabilized with 0.2% Trition-X 100 for 5 minutes at

room temperature. Platelets were washed with 3 × 300 μl of PBS then actin filaments were stained with 300 μl of 0.5 μg/ml Alexa488-phalloidin in PBS at room temperature for 1 hr in the dark. Cover-slips were washed with 3 × 300 μl of PBS, mounted on microscope slides with Immuno Fluore Mounting Media (ICN Biomedicals, CA, USA) and platelets viewed using a Zeiss Axiovert 200M microscope with a 63 × oil immersion lens. Images were captured using a Hamamatsu Orca 285 cool digital camera (Cairn, UK). Images were analyzed using ImageJ software (NIH, Maryland, USA). Individual platelets (25 – 50 platelets per image; at least three images per slide) platelets were outlined and surface area quantified by determining the number of pixels within the outlined area. Imaging a graticule under the same conditions allowed the conversion of pixels to microns. For platelet adhesion analysis, the number of platelets per image were counted (at least three images per slide).

2.2.2.3 Flow cytometry

Washed human or mouse platelets (1×10^7/ml) in modified Tyrodes-HEPES buffer containing 1 mM EGTA were incubated with primary antibody (or isotype control antibody at the same concentration) for 20 minutes at room temperature in the presence and absence of 1 U/ml thrombin. Platelets were subsequently centrifuged at 1000 ×*g* for 6 minutes and resuspended in modified Tyrodes-HEPES buffer containing FITC-conjugated secondary antibody for 20 minutes at room temperature in the dark. The final volume was increased to 500 μl and platelets were analyzed using a FACScaliber instrument (Becton Dickinson) and Cell-Quest software. For P-selectin staining, washed platelets (1×10^7/ml) were stimulated with 1 U/ml thrombin (without stirring) for 10 minute at room temperature then stained with 100 μg/ml FITC-conjugated P-selectin antibody (or 100 μg/ml FITC-conjugated isotype control antibody) for 10 minutes. Two-hundred 200 μl of modified Tyrodes-HEPES buffer was added to samples prior to flow cytometric analysis. The same method was used to stain

active integrin αIIbβ3 PE-conjugated JON/A antibody, which only recognises the active, high affinity conformation of the integrin αIIbβ3. Total intergrin αIIbβ3 was stained using a PE-conjugated antibody that recognizes both active and inactive forms of the integrin.

2.2.3 Platelet biochemistry

2.2.3.1 Sample preparation of platelets in suspension

Washed platelets (5×10^8/ml) in modified Tyrodes-HEPES were pre-treated with: 100 μM EGTA or 9 μM integrillin, to inhibit outside-in integrin αIIbβ3 signalling; 10 μM indomethacin, to inhibit TxA_2 production; and 2 U/ml apyrase, to degrade released ADP. Platelets were stimulated with various agonists for various lengths of time (as indicated in the Results Chapters). Stimulations were performed in siliconized cuvettes at 37°C with constant stirring at 1,200 rpm. Following stimulation, platelets were lysed with an equal volume of 2 × lysis buffer containing protease and phosphatase inhibitors (300 mM NaCl, 20 mM Tris, 2mM EGTA, 2 mM EDTA, 2% NP-40 pH 7.4 with 2.5 mM Na_3VO_4, 100 μg/ml AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 0.5 μg/ml pepstatin).

2.2.3.2 Sample preparation of spread platelets

Ten centimetre diameter bacterial grade plastic plates were coated with 100 μg/ml fatty acid free BSA (control), 100 μg/ml collagen or 100 μg/ml fibrinogen and incubated overnight at 4°C. Plates were washed 2 × 5 ml PBS. Plates were subsequently blocked with 5 mg/ml denatured (10 minutes at 100°C) fatty acid free BSA for 1 hour at room temperature. Washed platelets (1.5 ml of 5×10^8/ml) in the presence of 10 μM indomethacin and 2 U/ml apyrase were placed on the various surfaces for 45 minutes at 37°C. Non-adherent platelets were removed from BSA-coated plates and lysed in an equal volume of ice cold 2× lysis buffer containing protease and phosphatase inhibitors (same as above). Adherent platelets were

washed from collagen- and fibrinogen-coated platelets with 2× 5 ml modified Tyrodes-HEPES buffer, and adherent platelets were lysed with 1× lysis buffer containing protease and phosphatase inhibitors. Plates were incubated on ice for 15 minutes following addition of lysis buffer to ensure complete lysis of platelets.

2.2.3.3 Immunoprecipitation

Platelet lysates generated as described above were pre-cleared by addition of 25 µl of a 50% slurry of protein-A or protein-G sepharose (PAS and PGS, respectively) hydrated in TBS-T (137 mM NaCl, 20 mM Tris, 0.1 % Tween-20, pH 7.6) for 30 minutes at 4°C with constant mixing. PAS, PGS and insoluble platelet debris were pelleted at 13,000 ×*g* for 10 minutes at 4°C. Supernatants were collected and protein concentrations determined using the BioRad-Detergent Compatible Protein Assay Kit according to the manufacturer's instructions. Whole cell lysates (WCLs) were prepared by adding 50 µl of 2× reducing Laemmli sample buffer (4 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol, 50 mM Tris, trace Brilliant Blue R) to 50 µl aliquots of the pre-cleared platelet lysate and heating to 100°C for 5 minutes. Specific proteins were immunoprecipited (IP'd) from the remaining WCLs by adding 1 – 5 µg of antibody, incubating for 30 minutes at 4°C with constant mixing then adding 20 µl of a 50% slurry of either PAS (to IP rabbit antibodies) or PGS (to IP mouse and goat antibodies) and incubating overnight at 4°C with constant mixing. The following day, beads were pelleted at 9,000 ×*g* for 30 seconds and washed 4 × 1 ml ice cold TBS-T. Proteins were eluted from beads by adding 2× Laemmli sample buffer under reducing conditions and heating to 100°C for 5 minutes.

2.2.3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Proteins were resolved on either 10% or pre-cast 4 – 12 % gradient SDS-PAGE gels (Invitrogen). Pre-stained molecular weight markers (Bio-Rad) were run on all 10% gels. Non-stained molecular weight markers (Bio-Rad) were run on all 4 – 12 % gradient gels. Proteins were transferred from SDS-PAGE gels to polyvinylidene diflouride membranes (PVDF) using a Semi-Dry Transfer apparatus (Bio-Rad). Gels and PVDF membranes were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS pH 9 containing 20% methanol) for 15 minutes prior to transferring at 110 mAmp per gel for 35 minutes. Membranes were blocked overnight at 4ºC in blocking buffer (TBS-T containing 5% [w/v] bovine serum albumin [BSA] and 0.1 % [w/v] sodium azide). Blocked membranes were immunoblotted with primary antibodies diluted in blocking buffer for 1 hour at room temperature or overnight at 4°C. Membranes were washed 3 times with 50 ml TBS-T for 15 minutes per wash and subsequently incubated for 1 hour with HRP-conjugated secondary antibodies in TBS-T. Membranes were washed 3 more times as described above. Bands were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Bioscience, Bucks, UK). Blots were stripped by incubating in stripping buffer (TBS-T, 2% SDS) containing 1% 2-mercaptoethanol for 20 minutes at 80ºC followed by 20 minutes in stripping buffer alone without 2-mercaptoethanol; re-blocked (TBS-T, 5% BSA, 0.1% azide) overnight; and re-blotted using the same protocol described above. For densitometry analysis, band intensities were calculated using Photoshop cs version 8.0.

2.2.4 Functional studies in DT40 cells

2.2.4.1 Cell culture

DT40 chicken B cells were cultured in RPMI supplemented with 10% foetal bovine serum, 1% chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM mercaptoethanol and 20 mM GlutaMAX.

2.2.4.2 DT40-NFAT luciferase assay

DT40 cells were transfected in a volume of 400 μl non-supplemented RPMI by electroporation using a GenePulser II (Bio-Rad) set at 350 V, 500 μF. DT40 cells were transfected with either 10 μg of CD148 wild-type or 10 μg CD148 catalytically inactive expression constructs and 2 μg GPVI and 2 μg FcR γ-chain constructs, in addition to 15 μg of the luciferase reporter construct and 2 μg of pEF6-*lac*Z to control for transfection efficiency. Twenty hours after transfection, live cells were counted by trypan blue exclusion, and samples were divided into three parts for luciferase assay, β-galactosidase assay and flow cytometry. Luciferase assays were performed in triplicate with 10^5 live cells in a total volume of 100 μl. Cells were stimulated for 6 hours with 10 μg/ml collagen, then lysates were prepared by adding 11 μl of harvest buffer (1 M potassium phosphate buffer pH 7.8 containing 12.5% Triton X-100 and 1 M DTT). 90 μl of lysate was added to 90 μl of assay buffer (1M potassium phosphate buffer pH 7.8 containing 0.1 M $MgCl_2$ and 0.1 M ATP). Following the addition of 50 μl of 1 mM luciferin (MP Biomedicals, UK), luciferase activity was measured with a Centro LB 960 microplate luminometer (Berthold Technologies, Germany). Data is expressed as either luminescence units normalised to β-galactosidase activity or as the fold increase in luminescence units over basal as indicated. All luciferase data is averaged from 3 readings and shown ± standard deviation. All experiments were performed 3 – 5 times.

2.2.4.3 β-galactosidase assay

β-galactosidase activity was measured in 5×10^5 cells using the Galacto-Light Chemiluminescent Reporter Assay according to the manufacturer's instructions (Applied Biosystems). Samples were measured in triplicate using a Centro LB960 Microplate luminometer Centro (Berthold Technologies, Wildbad, Germany). All luciferase measurements were normalised to β-galactosidase activity/5×10^5 cells.

2.2.4.4 Quantification of surface expression of receptors

Surface expression of GPVI and CD148 on transiently transfected DT40 cells was measured by flow cytometry. Half a million cells in 50 μl PBS were stained with 10 μg/ml of either anti-CD148 antibody or anti-GPVI antibody that recognize the extracellular regions of CD148 and GPVI, respectively, for 20 minutes. As negative controls, cells were stained with equal amounts of isotype control antibodies under the same conditions. Cells were washed and incubated for 20 minutes with 15 μg/ml FITC-conjugated secondary antibodies. Stained cells were analysed using the FACScalibur flow cytometer and CellQuest software.

2.2.5 *In vitro* substrate trapping

2.2.5.1 Bacterial transformation

Precast agar plates containing 100 μg/ml ampicillin and SOC medium (Invitrogen) were warmed at 37 °C. Competent DH5α bacteria (Invitrogen) were thawed on ice. Two microlitres of CD148 expression constructs (Table 2.4) were mixed with 50 μl of DH5α bacteria and incubated on ice for 30 minutes. Bacteria were heat shocked for 30 seconds at 42°C to induce take up of plasmids, then placed on ice for a further 5 minutes. Two-hundred and fifty microlitres of warm SOC medium was added to bacteria, which were incubated at 37°C for 30 minutes to allow expression of the ampicillin resistance gene. Bacteria were then

spread on the pre-warmed agar/ampicillin plates using sterile technique and grown overnight at 37°C. Colonies were picked the next day and expanded in 250 ml liquid broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0 containing 100 μg/ml ampicillin) overnight at 37°C. The following day 50% glycerol stocks of each construct culture were prepared and the remaining culture maxipreped using standard protocols.

2.2.5.2 Expression and purification of recombinant proteins

Pilot experiments were initially performed to determine optimal conditions for expression of MBP.CD148 fusion proteins, according to the vector manufacturer's protocol (pMAL Protein Fusion and Purification System, New England BioLabs). Five samples were collected at various stages of the expression/purification protocol to monitor expression levels of fusion proteins: (1) non-induced bacteria; (2) IPTG-induced bacteria; (3) total bacterial crude extract; (4) insoluble material from the crude extract; and (5) protein bound to amylose resin (Appendix - Figure 1).

Based on results from pilot experiments, the following protocol was devised for the expression and purification of the MBP.CD148 fusion proteins. Five millilitres of Liquid Broth (LB) containing 0.2 % glucose and 100 μg/ml ampicillin was inoculated with either MBP, MBP:CD148 wild-type, MBP:CD148 catalytically inactive or MBP:CD148 substrate trapping glycerol stocks and grown up overnight at 37°C with constant mixing. The following day, 1 ml of each culture was used to inoculate 250 ml LB containing glucose and ampicillin. Bacteria were cultured at 37°C until they reached an optical density of ~0.5 at an absorbance of 600 nm, corresponding to ~2×10^8 bacteria at the exponential growth phase. Expression of the fusion proteins was induced by the addition of 1 mM IPTG and incubation for 3 hours. Cultured bacteria were then pelleted at 4,500 ×*g* at 4 °C for 30 minutes, supernatant was discarded and pellets frozen at -20°C. Bacterial pellets were suspended in 20

ml ice cold column buffer containing protease inhibitors (20 mM Tris-HCl pH 7.4, 200 mM NaCl ,1 mM EDTA, 200 μg/ml AEBSF, 10 μg/ml leupeptin, 10 mM β-ME). Bacteria were lysed using a Vibra-Cell ultrasonic processor model VC130PB (Sonics & Materials, Inc., Newtown, CT, USA) for 15 second bursts for 2 minutes on ice. Samples were centrifuged at 9,000 ×*g* at 4°C for 30 minutes to remove insoluble bacterial debris. Crude supernatant was diluted 1:5 with column buffer.

Four 2.5 × 10 cm amylose resin columns were prepared. Columns were washed with 8 column volumes of column buffer prior to the addition of samples. Diluted crude extract was loaded on the columns and the flow rate adjusted to 10× (diameter of column in cm)^2 ml/hr (approximately 1 ml/minute for a 2.5 cm diameter column). Columns were then washed with 12 column volumes of column buffer. Fusion proteins were eluted from the columns with column buffer containing 10 mM maltose. Ten to twenty 3 ml fractions (1/5th column volume) were collected for each fusion protein. Fusion protein expression in fractions was monitored by measuring the absorbance at 280 nm of each fraction. Fractions with high fusion protein expression were pooled and concentrated to approximately 1 mg/ml using a Centriprep concentrator according to the manufacturer's protocol (Millipore).

2.2.5.3 Protein pull-down and elution

Substrate-trapping/pull-downs were performed using a similar protocol to that desribed by Palka *et al* (Palka et al, 2003). Washed human platelets (5 × 10^8/ml) in modified Tyrodes-HEPES buffer containing 1 mM EGTA, 10 μM indomethacin and 2 U/ml apyrase were stimulated with 100 μM pervanadate for 3 minutes at 37ºC with constant stirring. Platelets were washed with modified Tyrodes-HEPES buffer containing 1 mM EGTA then lysed with an equal volume of 2× lysis buffer containing protease and phosphatase inhibitors as described above (section 2.2.3.1), with the exception that 2.5 mM Na_3VO_4 was replaced with

2.5 mM iodoacetic acid. Following incubation on ice for 15 minutes dithiothreitol was added to a final concentration of 10 mM to inactivate any unreacted iodoacetic acid. Samples were pre-cleared with 1 µg recombinant MBP bound to amylose resin per 1 ml of platelet lysate, by incubating at 4°C for 30 minutes with constant stirring, then pelleting cell debris and beads at 13,000 $\times g$ for 15 minutes. Either MBP or MBP:CD148 fusion proteins bound to amylose resin (20 µl of a 50% slurry) was added to platelet lysates at a ratio of 1 µg of fusion protein to 500 µg of platelet lysate and incubated at 4°C for 2 hours. Resins were then washed 4 times with column buffer. Proteins were eluted from resins by boiling for 5 minutes in 2× Laemmli sample buffer under reducing conditions.

2.2.5.4 Sample preparation for mass spectrometry

Samples generated using the substrate-trapping/pull-down assay were resolved on 4 – 20% SDS-PAGE gradient gels (Invitrogen) and proteins stained with ProtoBlue Safe Colloidal Coomassie blue (Geneflow, Fradley, UK). Bands of interest were excised from gels using a clean scalpel blade in a laminar flow hood and prepared for mass spectrometry analysis according to the in-gel trypsinization protocol of Shevchenko et. al. (Shevchenko et al. 1996). Gel slices were put into 1.5 ml snap-cap tubes rinsed with 0.1% formic acid/50% acetonitrile; washed twice with 500 µl of 50% acetonitrile in 50 mM ammonium bicarbonate for 45 minutes/wash at 37°C with constant mixing; and dried using a SpeedVac Plus AR model SC110AR (Savant Instruments, Inc., Holbrook, NY, USA) connected to a Universal Vacuum System Plus model UVS400A (Long Island City, NY, USA). Gel slices were rehydrated in 50 µl of 50 mM DTT in 10% acetonitrile in 50 mM ammonium bicarbonate at 56°C for 1 hr. Any remaining supernatant was removed and 50 µl of 100 mM iodoacetamide in 10% acetonitrile in 50 mM ammonium bicarbonate was added and incubated at room temperature in the dark for 30 minutes. Any remaining supernatant was removed; gel slices were washed

three times with 10% acetonitrile in 40 mM ammonium bicarbonate for 15 minutes/wash at room temperature with constant mixing; and gel slices were dried using the SpeedVac (details above). Gel slices were rehydrated with 20 μl or 1.5× the gel volume if greater than 20 μl of 12.5 μg/ml sequence grade trypsin (Promega) in 10% acetonitrile in 40 mM ammonium bicarbonate for 1 hr at room temperature. A further 20 μl of 10% acetonitrile in 40 mM ammonium bicarbonate was added and incubated overnight at 37°C. The supernatant was collected, replaced with 30 μl of 3% formic acid and incubated for a further 1 hr at 37°C. This final step was repeated once more. Mass spectrometric analysis (peptide mass fingerprinting) and database searches were performed by Dr. Ángel García (University of Santiago de Compostela, Spain). Mass spectra of tryptic fragments were measured using a Bruker Autoflex Matrix-Assisted Laser Desorption/Ionization – Time-of-Flight (MALDI-TOF) mass spectrometer. Database searches were performed using the Mascot v2.1 search tool (Matrix Science, London, UK) screening SwissProt and MSDB databases.

2.2.6 *In vitro* dephosphorylation assays

2.2.6.1 Dephosphorylation of SFKs

WCLs were prepared of pervanadate (100 μM) stimulated human platelets as described above. Equal amounts (1 μg) of recombinant MBP, wild-type recombinant CD148 PTP domain and catalytically inactive MBP-CD148 cytoplasmic domain fusion protein were added to equal amounts (75 μg) of platelet lysates and incubated at 37°C for 30 minutes. Reactions were terminated by addition of an equal volume of 2× lysis buffer and boiling for 5 minutes at 100°C. Samples were resolved by SDS-PAGE and western blotted with phospho-specific antibodies.

2.2.6.2 Dephosphorylation of SFK-derived phosphopeptides

Rates of dephosphorylation of SFK-derived phosphopeptides (Table 2.5) by recombinant CD148 and PTP-1B PTP domains were measured using the EnzChek Phosphatase Assay Kit (Molecular Probes). The kit is based on the purine nucleoside phosphorylase (PNPase)-coupled assay developed by Webb (Webb 1992). Phosphopeptides were reconstituted to 3 mM in 10 mM HEPES buffer pH 7.5. Recombinant CD148 and PTP-1B PTP domains (kindly provided by Dr. Alistair Barr, Structural Genomics Consortium, Oxford) were diluted in gel filtration buffer (250 mM NaCl, 10 mM DTT in 10 mM HEPES pH 7.5). Reactions were performed in 384-well clear bottom plates at room temperature. Reactions consisted of: 125 μM substrate phosphopeptide and 20 ng of CD148 or PTP-1B PTP domain, in reaction buffer (100 mM NaCl, 1 mM DTT, 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside [MESG] and 1 U/ml purine nucleoside phosphorylase [PNPase]). MESG and PNPase concentrations were optimized to ensure that the phosphatase activity was rate-limiting. The plate was read in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance of 360 nm at 20 second intervals for 3 minutes.

2.2.8 Statistical analysis

Data shown throughout this thesis is representative of 2 – 5 separate experiments. Where experiments were performed in triplicate (e.g. DT40/NFAT luciferase assay) results were averaged for each experiment. Mean ± standard deviation or standard error of the mean are indicated. Statistical analysis was carried out using unpaired Student's t-test, with P values < 0.05 taken as significant. All graphs were generated using GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA).

Table 2.4 Expression constructs and uses

Expression construct	Backbone	Source	Protein expressed	Mutation	Use	Reference
CD148 catalytically inactive	pEF.BOS	AW	full length CD148	C1239S	DT40/NFAT luciferase assay	(Baker et al. 2001)
CD148 substrate trapping	pEF.BOS	AW	full length CD148	D1205A	DT40/NFAT luciferase assay	(Baker et al. 2001)
CD148 wild type	pEF.BOS	AW	full length CD148	none	DT40/NFAT luciferase assay	(Baker et al. 2001)
FcR γ-chain	pEF6	MT	FcR γ-chain	none	DT40/NFAT luciferase assay	(Tomlinson et al. 2007)
GPVI	pRC	MT	GPVI	none	DT40/NFAT luciferase assay	(Tomlinson et al. 2007)
LacZ	pEF6	Invitrogen	LacZ	none	DT40/NFAT luciferase assay	(Tomlinson et al. 2007)
MBP.CD148 catalytically inactive	pMAL.c2E	NT	cytoplasmic tail of CD148 as MBP fusion	C1239S	*in vitro* substrate trapping	(Palka et al. 2003)
MBP.CD148 substrate trapping	pMAL.c2E	NT	cytoplasmic tail of CD148 as MBP fusion	D1205A	*in vitro* substrate trapping	(Palka et al. 2003)
MBP.CD148 wild type	pMAL.c2E	NT	cytoplasmic tail of CD148 as MBP fusion	none	*in vitro* substrate trapping	(Palka et al. 2003)
NFAT reporter	pEF6	MT	NFAT reporter	none	DT40/NFAT luciferase assay	(Tomlinson et al. 2007)

NT, Professor Nicholas Tonks (Cold Spring Harbour Laboratory, NY, USA); AW, Professor Art Weiss (UCSF, CA, USA);
MT, Dr. Michael Tomlinson (University of Birmingham, UK)

Baker, J. E., et al. (2001). "Protein tyrosine phosphatase CD148-mediated inhibition of T-cell receptor signal transduction is associated with reduced LAT and phospholipase Cgamma1 phosphorylation." Mol Cell Biol **21**(7): 2393-403.

Palka, H. L., et al. (2003). "Hepatocyte growth factor receptor tyrosine kinase met is a substrate of the receptor protein-tyrosine phosphatase DEP-1." J Biol Chem **278**(8): 5728-35.

Tomlinson, M. G., et al. (2007). "Collagen promotes sustained glycoprotein VI signaling in platelets and cell lines." J Thromb Haemost **5**(11): 2274-83.

CHAPTER 3 –

EXPRESSION OF CD148 IN

HUMAN AND MOUSE PLATELETS

3.1 Aim

In this chapter, I checked for expression of CD148 in human and mouse platelets by western blotting and flow cytometry. CD148 was quantified in resting human platelets and changes in surface expression following platelet activation were investigated. Expression of PTPRO, RPTPσ and CD45 was also investigated in human and mouse platelets by either western blotting or flow cytometry.

3.2 Introduction

Pioneering work to elucidate the functional roles of PTPs in platelets relied heavily on the use of non-selective PTP inhibitors, such as pervanadate (Pumiglia et al. 1992). These studies demonstrated that PTPs were important for maintaining basal levels of phosphorylation in platelets (Pumiglia et al. 1992). PTPs were shown to regulate several important platelet functions including aggregation, clot retraction and microvesicle formation (Chiang 1992; Pumiglia et al. 1992; Pasquet et al. 1998; Osdoit et al. 2001; Ragab et al. 2003).

To date five non-transmembrane PTPs and a single RPTP have been identified in platelets. The non-transmembrane PTPs include: Shp1, Shp2, PTP-1B, PTP-MEG and the non-classical PTP, LMW-PTP (Frangioni et al. 1993; Somani et al. 1997; Hua et al. 1998; Wang et al. 2005; Xing et al. 2007). The lone RPTP is CD148 (Borges et al. 1996; de la Fuente-Garcia et al. 1998; Senis et al. 2007). Other RPTPs that may be expressed in platelets include PTPRO, RPTPσ and CD45. PTPRO was first identified in human megakaryocytes (Taniguchi et al. 1999). Forty-one SAGE tags of PTPRO were also identified in a mouse megakaryocyte SAGE library, suggesting that it is highly expressed (Senis et al. 2007). However, it has yet to be identified in human or mouse platelets. A single peptide from RPTPσ was recently identified in human platelets using a mass spectrometry approach, suggesting that it may be expressed at low levels (Senis et al. 2007). It is widely accepted that CD45 is not expressed in platelets, however it has been detected in megakaryocytes at a low level of expression (Stelzer et al. 1993; Qiao et al. 1996; Dahlke et al. 2004; Li et al. 2007). We therefore speculated that it may be expressed in platelets at a low level.

CD148 and PTPRO are structurally related classical PTPs that belong to the R3 subfamily of transmembrane PTPs (Tonks 2006). They consist of a large extracellular region composed of 8 fibronectin type III (FNIII) repeats, a single transmembrane segment and a single intracellular PTP domain. CD148 is expressed in all hematopoietic lineages,

endothelial cells, fibroblasts, epithelial cells and smooth muscle cells (Borges et al. 1996; Autschbach et al. 1999). The majority of research on CD148 has focused on its role in negatively regulating cell growth and differentiation. Recently, it has been demonstrated that CD148 negatively regulates cell-extracellular matrix contacts in fibroblasts by modulating Src and FAK activation (Jandt et al. 2003; Kellie et al. 2004; Pera et al. 2005). Knockin mice expressing a mutant CD148 that lacks the cytoplasmic phosphatase domain die at embryonic day 11.5 and have severe defects in vascular development (Takahashi et al. 2003). More recently, CD148 and the structurally distinct RPTP CD45 have been shown to have positively regulate B cell receptor signalling and Fcγ receptor signalling, in B cells and macrophages respectively, using single and double knockout mouse models (Zhu et al. 2008).

PTPRO is primarily expressed in the brain and kidney, but is also found in hematopoietic stem cells, lungs, lymph nodes, spleen and placenta (Avraham et al. 1997; Beltran et al. 2003). Alternatively spliced PTPRO transcripts are expressed in a tissue-specific manner (Aguiar et al. 1999). PTPRO has been studied primarily as a receptor involved in glomerulus function and axon guidance. The only paper describing its expression in megakaryocytes suggested that it is involved in megakaryocytopoeisis and that its role is regulated by the SCF receptor, c-Kit, which is a tyrosine kinase receptor (Taniguchi et al. 1999). PTPRO knockout mice have defects in kidney function as a result of a change in podocyte structure, however, they have not been examined in detail for other physiological defects (Wharram et al. 2000).

RPTPσ is structurally distinct from CD148 and PTPRO, belonging to the R2A subfamily of RPTPs. It consists of a large extracellular domain containing several immunoglobulin-like and FNIII repeats, a single transmembrane domain and a cytoplasmic tail containing a PTP catalytic domain and a pseudo-catalytic domain. It is predominantly expressed in the nervous system, with some expression also detected in the kidney and in

selected epithelial cells (Pulido et al. 1995). RPTPσ-deficient mice display severe neuroendocrine and neuronal defects, pituitary dysfunction, as well as central and peripheral nervous system abnormalities (Elchebly et al. 1999; Wallace et al. 1999; Batt et al. 2002).

CD45 is the prototypical RPTP belonging to the R1/R2 subfamily of RPTPs. The extracellular domain exists as multiple isoforms due to alternative splicing. It has a single transmembrane domain and a cytoplasmic region containing a PTP domain and a pseudo-PTP domain. CD45 is highly and specifically expressed in all nucleated haematopoietic cells. Different splice variants of CD45 are expressed in the different haematopoietic lineages. The lowest molecular weight form is termed CD45RO and the highest molecular weight form is CD45ABC. Naive T cells express primarily the RB isoform then switch to the RO isoform upon activation. B-cells primarily express the highest molecular weight isoform RABC, while the myeloid lineage cells generally express the RO isoform then switch to RA isoforms upon activation. There is a large body of research on the role of CD45 in immune cells, as it plays a central role in immune receptor signalling (Hermiston et al. 2003). It has redundant functions with CD148 in immune cells (Zhu et al. 2008). CD45 deficiency results in severe combined immunodeficiency phenotype in mouse and humans (Byth et al. 1996; Mee et al. 1999). More recently it has been implicated as a potential genetic modifier in autoimmune, infectious and malignant diseases (Tchilian et al. 2006).

It has been known for many years that CD45 is essential for initiating B and T cell receptor signalling. It has also been known that structurally distinct RPTP PTPα plays an important role in initiating integrin signalling in fibroblasts. Based on these findings, we hypothesized that platelets express a CD45-like RPTP that is required to initiate signalling from the collagen receptor GPVI. The GPVI signalling pathway is essentially a hybrid of the B and T cell receptor signalling pathways. We also hypothesized that this platelet RPTP also

initiates integrin receptor signalling, similar to PTPα in fibroblasts. The primary candidate was CD148, followed by PTPRO, RPTPσ and CD45.

3.3 Results

3.3.1 Expression of CD148 in human platelets

I initially detected CD148 in human platelets by western blotting. Whole cell lysates prepared of washed human platelets were resolved by SDS-PAGE and western blotted with an anti-CD148 antibody. A single band was detected at approximately 220 kDa, which is the correct size for CD148 (Figure 3.1A). Flow cytometry was subsequently used to determine if CD148 was expressed on the surface of human platelets. Washed human platelets were stained with a monoclonal antibody to the extracellular region of CD148 followed by a FITC-conjugated anti-mouse secondary antibody prior to flow cytometric analysis (Figure 3.1B). Platelets were stained with an isotype control antibody instead of the primary antibody in parallel as a control for non-specific binding of the primary antibody. Platelets stained with the anti-CD148 monoclonal antibody showed a clear shift to the right in FITC fluorescence relative to platelets stained with the isotoype control antibody, demonstrating that CD148 was expressed on the platelet surface.

3.3.2 Quantification of surface expression of CD148 on resting human platelets

A commercially available flow cytometry-based assay was used to quantify surface expression of CD148 on resting human platelets. Washed human platelets from 6 healthy volunteers were stained with a monoclonal antibody to the extracellular region of CD148 followed by a FITC-conjugated anti-mouse secondary antibody prior to flow cytometric analysis. The expression level of CD148 on the platelet surface was quantified by plotting the geometric mean fluorescence intensities (GMFI) of immunostained platelets on a standard curve generated using calibration beads coated with a known number of binding sites for the secondary antibody (Figure 3.2A and B). The GMFI of platelets stained with the isotype control antibody was subtracted from the GMFI of platelets stained with the anti-CD148

antibody before the number of surface molecules per platelet was calculated. Human platelets were found to express 2,834 ± 90 (mean ± standard error of the mean) copies of CD148 on their surface using this method (Figure 3.2C). GPVI surface levels were quantified in parallel to CD148 levels on the same platelets as a positive control of the assay. GPVI surface expression was calculated to be 6,081 ± 252 copies per platelet, which is slightly higher than published levels of GPVI (3,500 – 5,000 copies/platelet) (Best et al. 2003). We suspect this discrepancy is related to a variety of reasons, which will be discussed later in this chapter. Based on these results, CD148 is expressed at approximately half the level of GPVI. This is an intermediate/low level of expression relative to other platelet surface glycoproteins. Interestingly, there was very little variability in surface levels of CD148 in the six individuals tested, suggesting that it may has an important functional role in platelets.

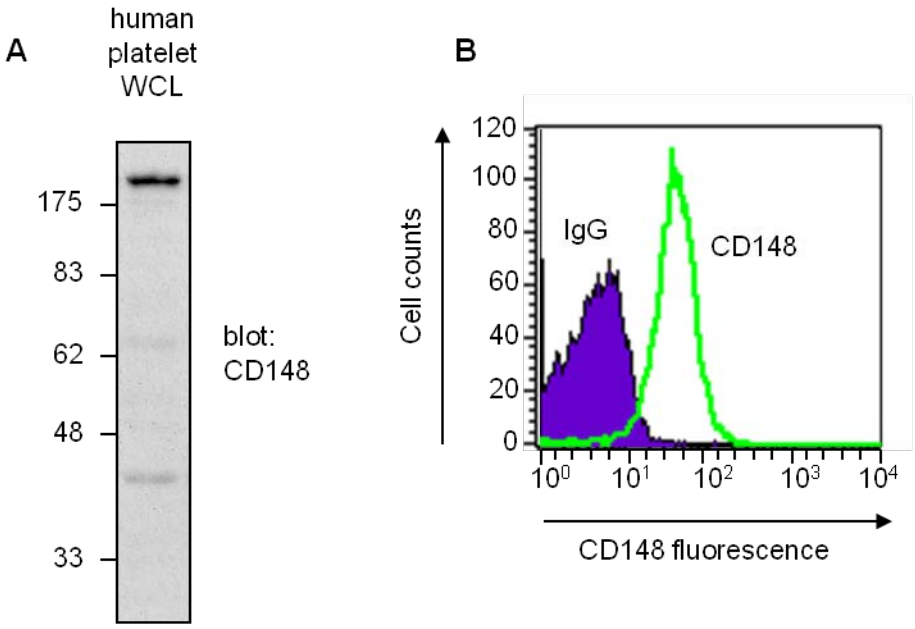


Figure 3.1: Expression of CD148 in human platelets. (A) Whole cell lysate (WCL) prepared from human platelets was western blotted for CD148 using a mouse anti-CD148 monoclonal antibody. A band at approximately 220 kDa was detected. (B) Resting human platelets were incubated with either anti-CD148 (green line) or an isotype control antibody (IgG, purple histogram) for 15 minutes, then stained for 15 minutes with a FITC-conjugated anti-mouse antibody before being analyzed by flow cytometry. n=6.

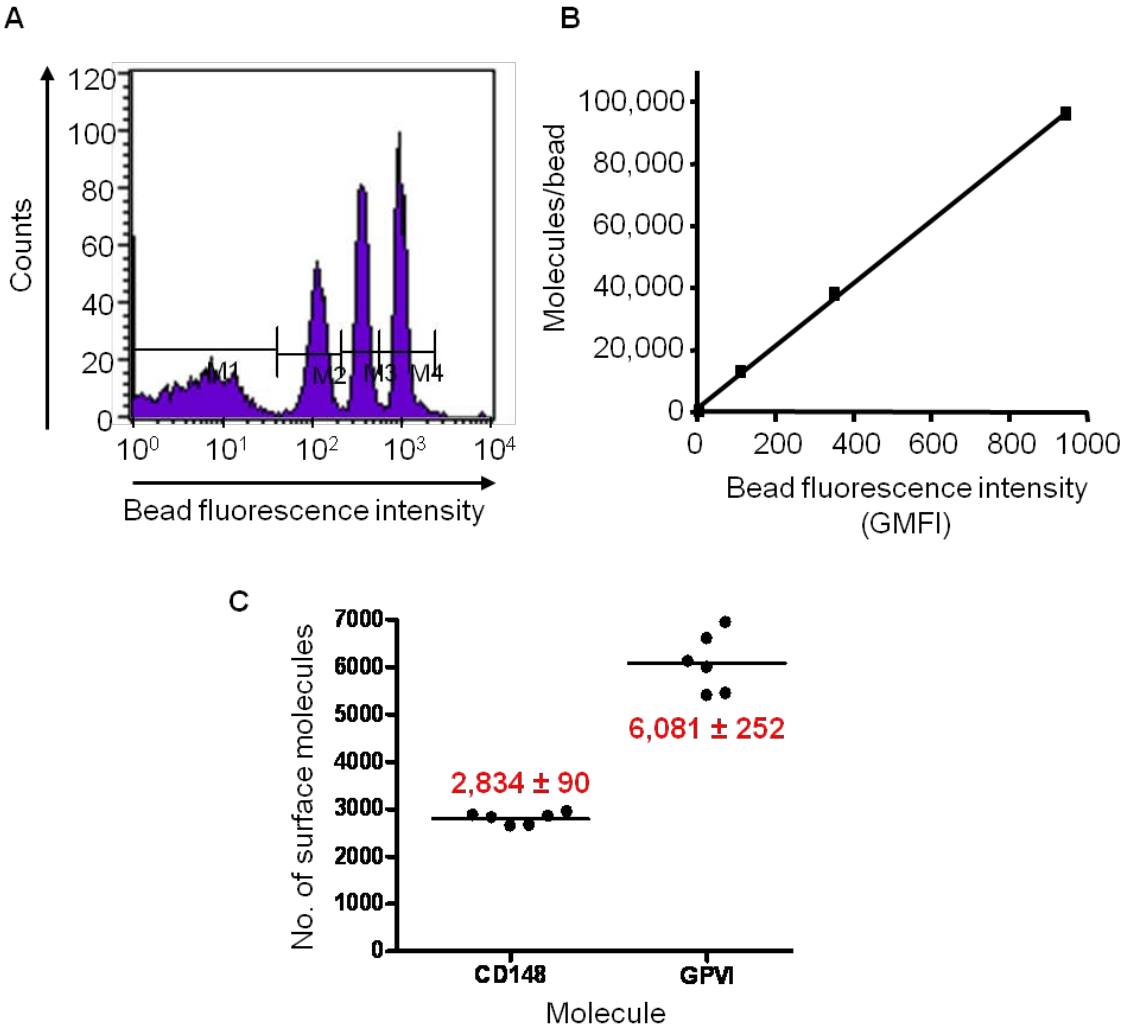


Figure 3.2: Expression level of CD148 on the surface of resting human platelets measured by quantitiative flow cytometry. (A) Calibration beads coated with a known number of antibody binding sites (490, 13,000, 38,000 and 96,000) were stained with FITC-conjugated anti-mouse antibody for 15 minutes and analysed by flow cytometry. (B) A calibration curve was obtained by plotting the geometric mean fluorescence intensity (GMFI) of the peaks in panel (A) against the known number of antibody binding sites for each peak. (C) The mean number of copies of CD148 on the surface of resting human platelets was calculated to be 2,834 ± 90 (± standard error of the mean). Each dot represents an individual and the horizontal line represents the mean. The number of copies of GPVI was also quantified.

3.3.3 Surface levels of CD148 in activated human platelets

Having established that CD148 is expressed on the surface of human platelets, I next wanted to investigate whether surface levels of CD148 are altered following platelet activation. There are several examples of platelet surface proteins that are up-regulated upon platelet activation including the integrin αIIbβ3, GPIb-IX-V, P-selectin and TLT-1 (Wagner et al. 1996; Berndt et al. 2001; Washington et al. 2004). Intracellular pools of these and other surface proteins are found in the open canalicular system and the platelet α-granules. Conversely, there are several examples of platelet surface glycoproteins that are shed from the surface of activated platelets, including GPVI and GPIb-IX-V (Bergmeier et al. 2004; Gardiner et al. 2007).

Surface levels of CD148 were compared between resting and thrombin activated platelets by flow cytometry. Platelets were stimulated with a high concentration (1U/ml) of the physiological agonist thrombin, which signals through PAR-1 and PAR-4 receptors in human platelets. A high dose of thrombin was used in order to induce maximal platelet activation and α-granule secretion. Under these conditions, surface expression of CD148 was not altered following thrombin stimulation (Figures 3.3A and B), suggesting the absence of any intracellular pool and that it does not get shed from the surface of platelets. P-selectin expression was used as a marker of α-granule secretion (Figures 3.3C and D).

3.3.4 Expression of CD148 in mouse platelets

I next checked for expression of CD148 in mouse platelets in order to establish the mouse as a model for studying the functional role of CD148 in platelets. CD148 had never before been shown to be expressed in mouse platelets. CD148 was detected on the surface of resting wild-type (WT) mouse platelets by flow cytometry. Washed mouse platelets were immunostained with a hamster anti-mouse CD148 monoclonal antibody to the extracellular

region of CD148 followed by a FITC-labelled anti-hamster secondary antibody prior to being analyzed by flow cytometry. The primary antibody was replaced with an isotype control antibody as a negative control. There was a clear shift to the right in FITC fluorescence of CD148 stained platelets relative to the isotype control antibody stained platelets, demonstrating that CD148 is expressed in mouse platelets (Figure 3.4A).

Western blotting was then used to confirm the expression of CD148 in wild-type mouse platelets. Platelets from CD148 transmembrane knockout (TM-KO) mice were used as a negative control. Platelet lysates were resolved by SDS-PAGE and immunoblotted with an anti-CD148 antibody. A band migrating at approximately 220 kDa was detected in the WT platelets, but not in platelets from CD148 TM-KO platelets, demonstrating that WT mouse platelets express CD148 and validating the CD148 TM-KO model (Figure 3.4B).

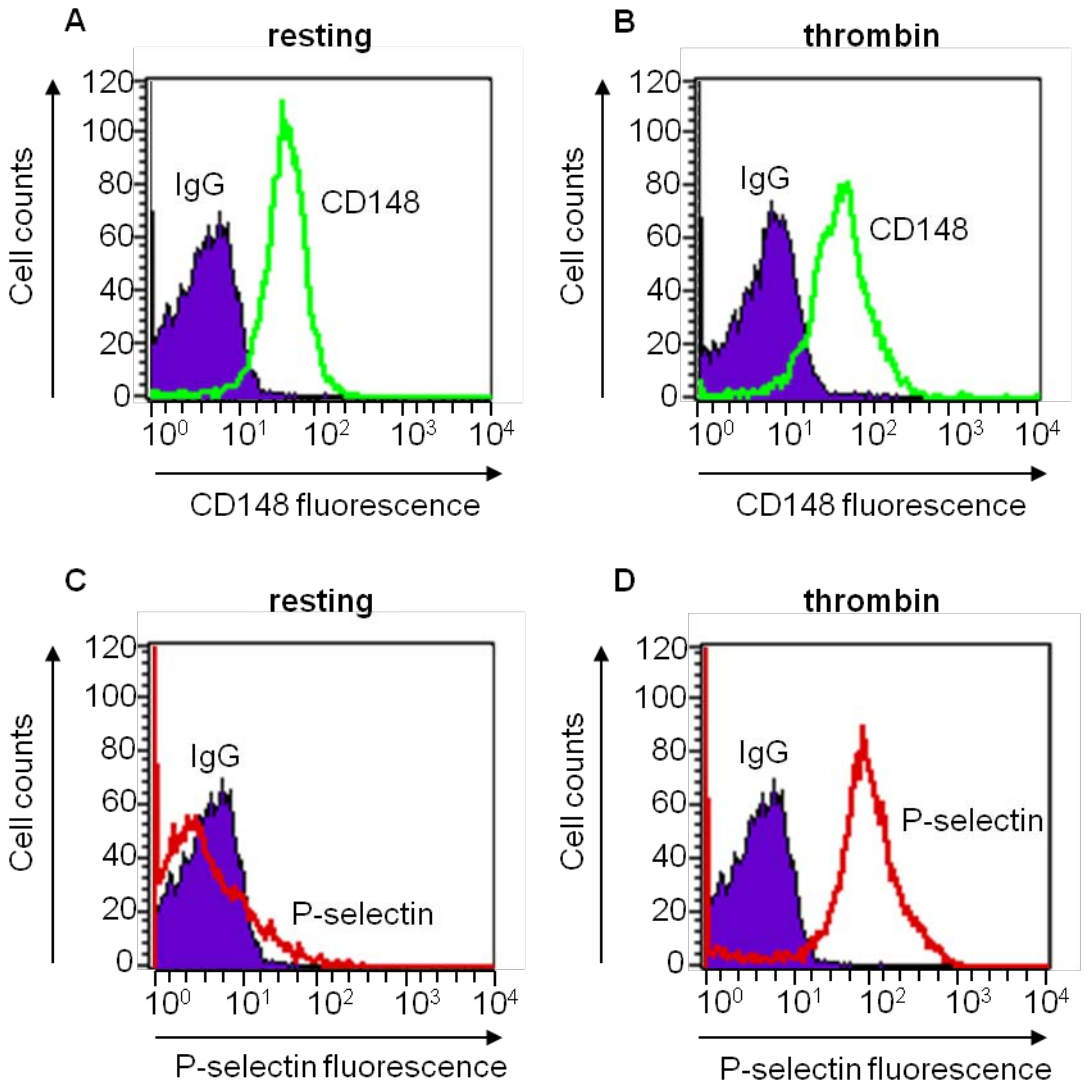


Figure 3.3: Subcellular localisation of CD148 in human platelets. Flow cytometry was used to investigate whether CD148 expression is restricted to the platelet surface. Resting (A) or thrombin stimulated human platelets (B) were incubated with 10 μg/ml of either anti-human CD148 monoclonal antibody (CD148, green peak), that recognises the extracellular region of CD148 or the same amount of isotype control antibody (IgG, purple histogram) for 15 minutes, then stained for 15 minutes with a FITC-conjugated anti-mouse antibody. As a positive marker for platelet activation and secretion, resting (C) and thrombin (D) stimulated human platelets were stained with 10 μg/ml anti-CD62P antibody (P-selectin, red peak) or the same amount of control antibody (IgG, purple histogram). n=3.

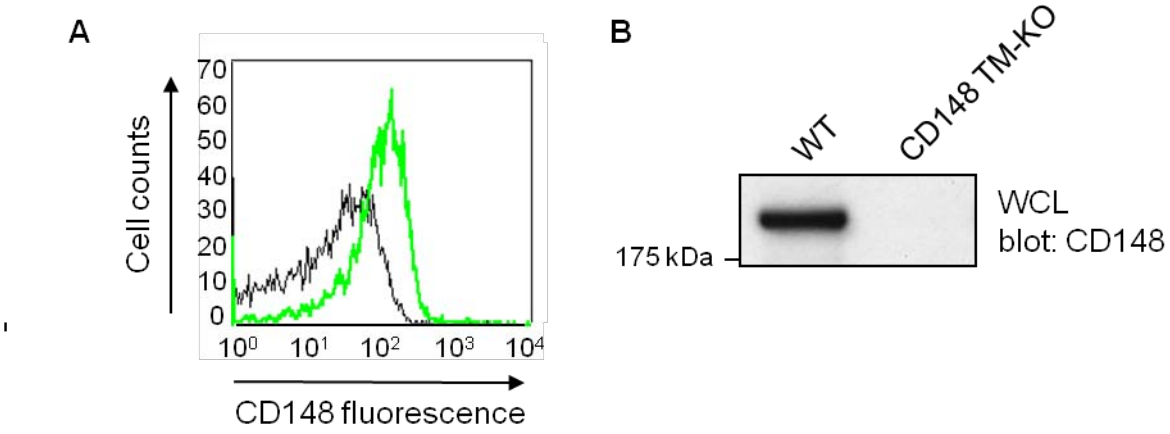


Figure 3.4: Expression of CD148 in mouse platelets. (A) Resting wild-type mouse platelets were incubated with either hamster anti-mouse CD148 (green line), that recognizes the extracellular region of mouse CD148, or an isotype control antibody (IgG, black line) for 15 minutes, then stained for 15 minutes with a FITC-conjugated anti-hamster antibody before being analyzed by flow cytometry. (B) Whole cell lysates (WCLs) prepared of wild-type (WT) and CD148 transmembrane-knockout (CD148 TM-KO) mouse platelets were western blotted for CD148 using the same monoclonal antibody used for flow cytometry in panel (A). A 220 kDa band was detected in the WT sample which was absent from the CD148 TM-KO sample. (Note: FACS data was initially generated by Mike Tomlinson and repeated by myself). n=3.

3.3.5 Expression of PTPRO in mouse platelets

Since PTPRO has been previously identified on the platelet progenitor cell, the megakaryocyte, I wanted to check for its expression on platelets (Taniguchi et al. 1999; Senis et al. 2007). Whole cell lysates of mouse platelets, mouse foetal liver-derived megakaryocytes, mouse splenocytes (positive control) and mouse brain (positive control) were resolved by SDS-PAGE and western blotted with a goat anti-PTPRO polyclonal antibody (Figure 3.5). PTPRO has been reported to migrate at 200 and 158 kDa, and a truncated splice variant, PTPROt, expressed in B cells migrates at 44 kDa (Aguiar et al. 1999). A prominent band at 158 kDa was detected in mouse brain, as expected (Figure 3.5). Similar size bands were also detected in the spleen and platelet WCLs, which could potentially be PTPRO. However, due to the large number of background bands present in all of the samples it is difficult to conclude which of these bands are PTPRO or its splice variants. A prominent band of the expected size for PTPRO was not detected in megakaryocytes. This is somewhat surprising as several tags for PTPRO were detected in a mouse megakaryocyte SAGE library, suggesting that they express high levels of the protein (Senis et al. 2007).

3.3.6 Expression of RPTPσ in human and mouse platelets

Whole cell lysates prepared from human platelets, mouse platelets, mouse foetal liver-derived megakaryocytes, mouse splenocytes and mouse brain were resolved by SDS-PAGE and western blotted with an anti-RPTPσ monoclonal antibody (Figure 3.6). RPTPσ is reported to migrate at 220 kDa and a splice variant at 75 kDa (Pulido et al. 1995). The prominent 75 kDa band detected in brain WCL is presumably RPTPσ. Although similar size band was also detected in the human platelet lysate, it resembled a background band rather than a

specifically labelled band. A prominent 220 kDa was also detected in the human platelet WCL that may represent RPTPσ.

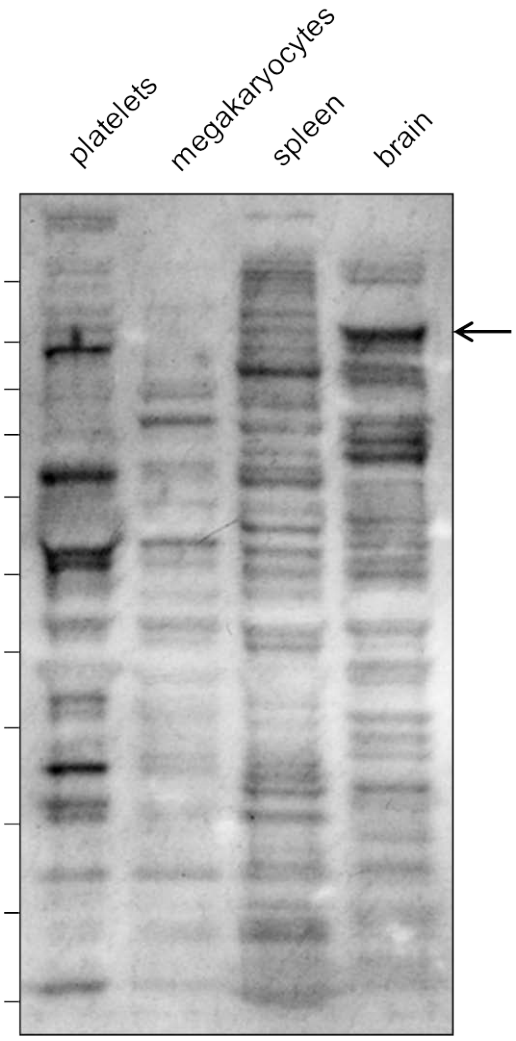


Figure 3.5: PTPRO may be expressed in mouse platelets. Whole cell lysates prepared of mouse platelets, mouse foetal liver-derived megakaryocytes, mouse splenocytes and mouse brain were western blotted with a goat anti-PTPRO polyclonal antibody. 10-20 μg of protein was loaded per lane. PTPRO has been reported to migrate at 200 and 158 kDa. A truncated splice variant, PTPROt, expressed in B cells migrates at 44 kDa. n=2.

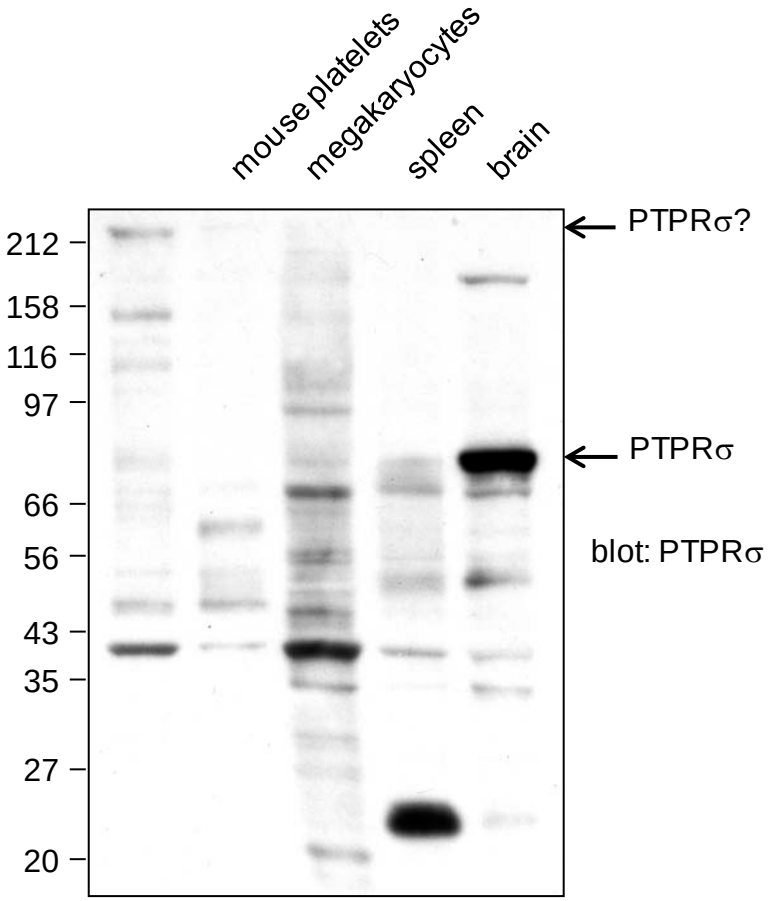


Figure 3.6: PTPRσ may be expressed in human platelets. Whole cell lysates prepared of human platelets, mouse platelets, mouse foetal liver-derived megakaryocytes, mouse splenocytes and mouse brain were western blotted with an anti-PTPRσ monoclonal antibody (courtesy of Professor Michel Tremblay, McGill University). 10-20 μg of protein was loaded per lane. PTPRσ has been reported to migrate at 220 and 75 kDa. A prominent band migrating at 75 kDa in brain lysate is PTPRσ. The high molecular band in the human platelet lysate may represent PTPRσ. n=2.

3.3.7 CD45 expression in human and mouse platelets

Although it is widely accepted that platelets do not express CD45, a recent study demonstrated that mouse megakaryocytes express low levels of CD45 (Dahlke et al, 2004). I therefore wanted to confirm whether CD45 is express in human and mouse platelets. Peripheral blood leukocytes which express high levels of CD45 were stained with a FITC-conjugated anti-CD45RA antibody as a positive control (Figure 3.7A). CD45RA is primarily found on cells of the myeloid lineage. Unstimulated or thrombin (1 U/ml) stimulated washed human platelets were stained with FITC-conjugated anti-CD45RA antibody prior to being analysed by flow cytometry (Figure 3.7B and C). Leukocytes, and resting and activated platelets were stained with an FITC-conjugated isotype antibody in parallel as a negative control. Leukocytes were strongly labelled for CD45 compared with the isotype control stained leukocytes, demonstrating that the antibody was working in this assay (Figure 3.7A). In contrast, both resting and thrombin stimulated platelets did not stain for CD45RA, demonstrating that platelets do not express this isoform of CD45 (Figures 3.7B and C respectively). The experiment was repeated with an antibody for CD45RO isoform, which is primarily expressed on T cells and myeloid cells, with the same result (data not shown).

The same experiment described above was also performed on mouse platelets. Mouse thymocytes were used as a positive control. Thymocytes showed a large shift to the right in CD45RA staining compared to control cells stained with the isotype control antibody, indicating very high levels of CD45 expression in thymocytes (Figure 3.8A). In contrast, unstimulated and thrombin stimulated mouse platelets exhibited comparable, relatively small shifts to the right relative to control stained platelets, suggesting mouse platelets may express very low levels of CD45RA (Figure 3.8B and C). Similar results were observed for CD45RO isoform (data not shown).

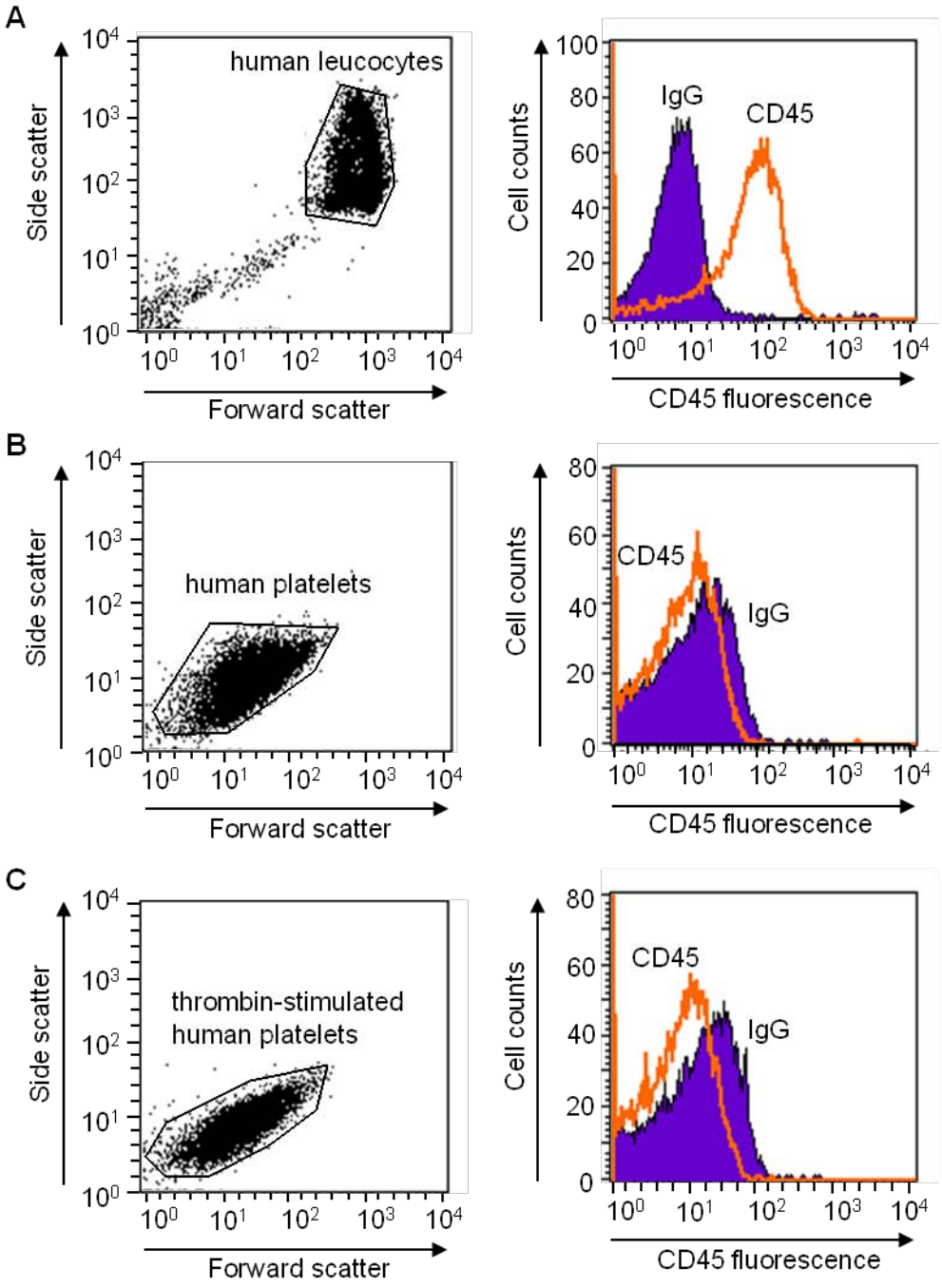


Figure 3.7: CD45 is not expressed on human platelets. Flow cytometry was used to investigate CD45 expression on human platelets. (A) As a positive control human leukocytes were stained with a FITC-conjugated anti-CD45 RA antibody (CD45, orange line) or an isotype matched IgG control antibody which was then detected with a secondary FITC antibody (IgG, purple peak). (B and C) Resting and thrombin stimulated human platelets were then analysed for CD45 expression using the same antibodies as in (A). n=2.

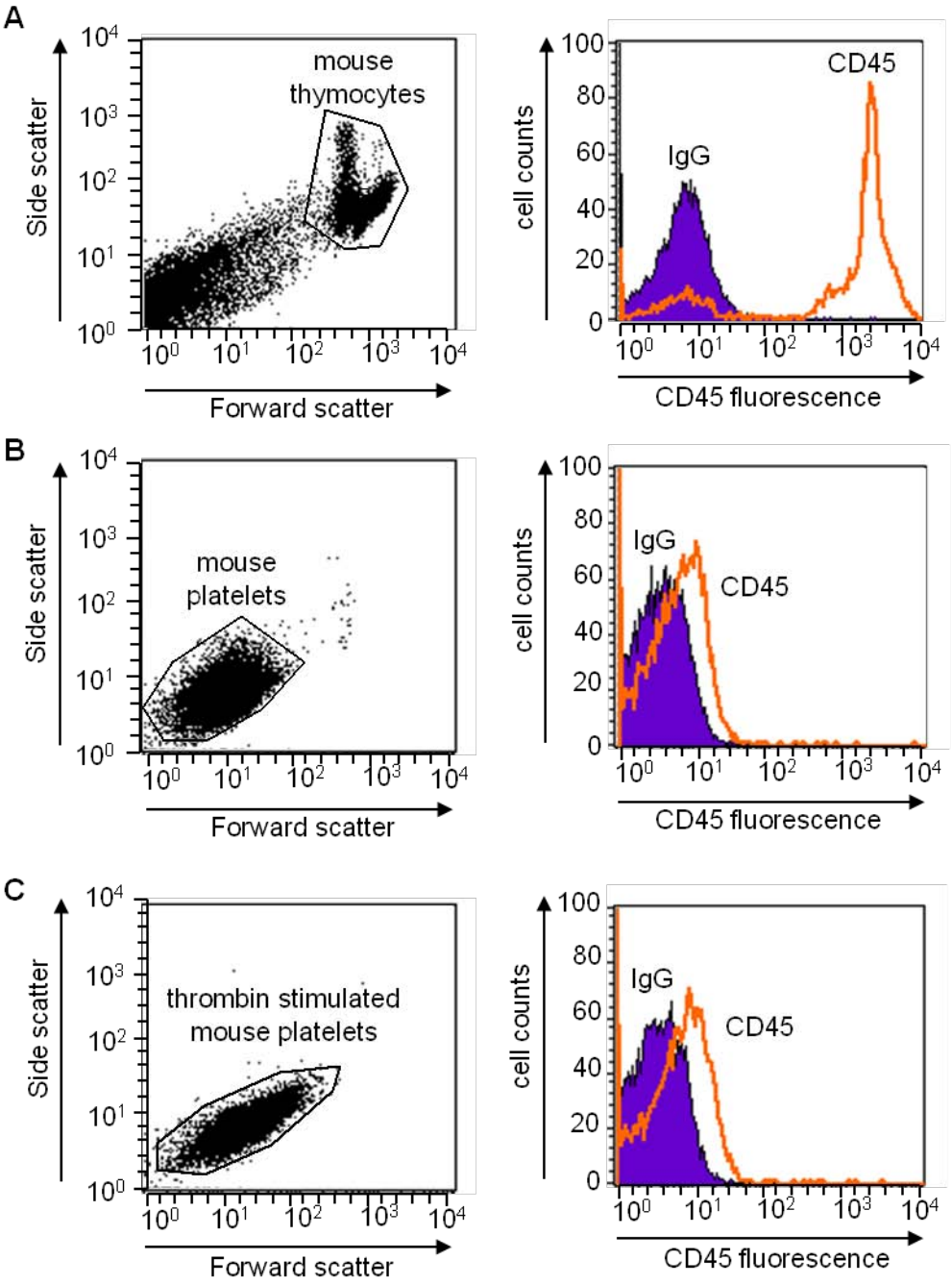


Figure 3.8: CD45 maybe expressed on mice platelets at low levels. Flow cytometry was used to investigate CD45 expression on mouse platelets. (A) As a positive control mouse thymocytes were stained with a FITC-conjugated anti-CD45 RA antibody (CD45, orange line) or an isotype matched IgG control antibody which was then detected with a secondary FITC antibody (IgG, purple peak). (B and C) Resting and thrombin stimulated mouse platelets were then analysed for CD45 expression using the same antibodies as in (A). n=2.

3.4 Discussion

To date, CD148 is the only RPTP identified in platelets by immunological-based techniques. It is a large glycoprotein that migrates at approximately 220 kDa by SDS-PAGE. Resting human platelets express approximately 2,800 copies on their surface. Surface levels are not altered upon platelet activation, demonstrating that platelets do not contain an intracellular pool of CD148 and that it does not get shed under the conditions tested. The very tight intra-individual surface levels on human platelets, suggests that it may play an important role in regulating platelet function. We suspect the discrepancy in the levels of GPVI I quantified on resting platelets (~6,000 copies) compared with published levels (3,500 – 5,000 copies) is due to a number of factors, including: different methods used to quantify GPVI levels; different batches of anti-GPVI antibody used to quantify; different numbers of secondary antibody binding sites on the calibration beads; different fluorescence intensities of the secondary antibodies; and sample size and variation.

The number of copies of CD148 on human platelets is intermediate/low relative to other surface glycoproteins and its expression is tightly regulated. The most abundant surface glycoprotein is the integrin αIIbβ3, which is present at approximately 80,000 copies on the surface of resting platelets (Wagner et al. 1996). Surface levels of other platelet glycoproteins are: 25,000 copies for GPIb-IX-V and 45,000 copies for CD9 (Hato et al. 1988); 10,000 copies for PECAM-1 (Newman et al. 1990); and 1,000 – 2,000 for the integrin $\alpha_2\beta_1$ (Bennett 1990). In contrast, the copy number of CD148 is approximately 20 fold greater than the ADP receptor, $P2Y_1$, which is amongst the least abundant platelet receptors expressed on the surface of resting human platelets at approximately 150 copies (Baurand et al. 2001).

To date no other RPTPs have been identified in human or mouse platelets by western blotting or flow cytometry. However, PTPRO and CD45 have been reported to be expressed in megakaryocytes, and a single peptide for RPTPσ was identified in human platelets by mass

spectrometry (Avraham et al. 1997; Tomer 2004; Senis et al. 2007). Presumably, PTPRO and CD45 are involved in regulating megakarycoyte function and/or development and their expression is turned off during differentiation and platelet formation. Based on findings presented in this chapter, I cannot conclude with any certainty whether PTPRO, RPTPσ or CD45 are expressed in human or mouse platelets. It may be that platelets express low levels or alternatively spliced isoforms of all three of these RPTPs. Further characterisation with better antibodies and positive control lysates are required in the case of PTPRO and RPTPσ. Although my findings suggest that mouse platelets may express low levels of CD45RA and RO, I believe that they probably do not. This is based on previously published findings and on a follow-up study performed by our collaborators in which they could not detect CD45 on mouse platelets (personal communication, Dr. Jing Zhu and Professor Arthur Weiss, UCSF, USA). Therefore, I suspect the minor shift in CD45RA and RO stained mouse platelets is likely due to lower fluorescence labelling of the isotyped control antibody relative to the anti-CD45 antibodies.

Collectively, these findings suggest that CD148 is the most abundant and possibly the only RPTP expressed on platelets. This is the opposite to other blood cells, with the exception of erythrocytes, which express high levels of CD45 and lower levels of CD148. The following chapters in this thesis focus on elucidating the biological and biochemical functions of CD148 in platelets.

CHAPTER 4 –

FUNCTIONAL CHARACTERIZATION OF CD148 IN MURINE PLATELETS

4.1 Aim

In this chapter, CD148 transmembrane knockout (CD148 TM-KO) mice, which lack cell surface expression of CD148, were utilized to investigate the functional role of CD148 in platelets. We hypothesized that CD148 plays a fundamental role in ITAM receptor signalling and integrin-mediated functional responses, as it has previously been shown to be involved in regulating ITAM and integrin receptor signalling in other cell types (Hundt et al. 1997; Tangye et al. 1998; Zhu et al. 2008).

4.2 Introduction

It has been known for many years that CD45 is essential for initiating ITAM receptor signalling in haematopoietic cells (Hermiston et al. 2003). In the case of the B and T cell receptors (BCR and TCR, respectively), it does so by modulating Lck downstream of the TCR and Lyn downstream of the BCR, leading to the recruitment and activation of Syk and PLCγ (McNeill et al. 2007; Zhu et al. 2008). Similarly, CD148 has been shown to play a fundamental role in regulating lymphocyte signal transduction, in particular CD148 is able to regulate BCR and TCR signalling pathways (Tangye et al. 1998; Baker et al. 2001). Recently, CD45 and CD148 have been shown to play redundant functions in activating SFKs downstream of BCRs and Fc receptors in B cells and macrophages, respectively (Zhu et al. 2008). Since the collagen activation receptor complex GPVI/FcR γ-chain signals in a similar way to the BCR and TCR, we hypothesized that CD148 plays a similar role in initiating GPVI signalling in platelets.

Work done in other cell types has shown that CD148 regulates cell adhesion and spreading (Jandt et al. 2003; Kellie et al. 2004; Pera et al. 2005; Dave et al. 2009). It has been hypothesized to do so by regulating key components of integrin signalling complexes, including SFKs, FAK and paxillin (Kellie et al. 2004; Pera et al. 2005). Similarly, the structurally distinct RPTP PTPα has been shown to positively regulate outside-in signalling from the fibronectin integrin α5β1 in fibroblasts (Chen et al. 2006). We therefore hypothesized that CD148 plays a similar function in regulating integrin signalling in platelets. Interestingly, GPVI and the fibrinogen integrin αIIbβ3 share the same backbone signalling pathway, consisting of the sequential activation of SFKs, Syk and PLCγ2 (Watson et al. 2005). SFKs have also been shown to play a minor role in signalling from various G protein-couple receptors (GPCRs), including the thrombin receptors PAR-1 and PAR-4, the thromboxane A_2 receptor TP and the ADP receptor $P2Y_1$ (Jarvis et al. 2000; Harper et al.

2006; Minuz et al. 2006). We therefore speculated that CD148 may also play a minor role in regulating signalling from various GPCRs in platelets.

The generation of a CD148 TM-KO mouse model that lacks any cell surface expression of CD148 has been an invaluable tool to study the functional role of CD148 in platelets (Zhu et al. 2008). In this chapter I utilised platelets from these mice to study the biological function of CD148 in platelets. I measured the ability of platelets from C148 TM-KO mice to aggregate together, secrete their contents and spread on collagen- and fibrinogen-coated surfaces. From these findings, I demonstrate that CD148 is an essential positive regulator of ITAM-, integrin- and to a lesser extent GPCR-mediated functional responses.

4.3 Results

4.3.1 CD148-deficient platelets exhibit impaired aggregation and secretion

The ability of platelets to aggregate and secrete was measured simultaneously, in real-time, using a lumi-aggregometer. Platelet aggregation was measured as a change in light transmission, through a platelet suspension (2×10^8/ml), following addition of an agonist. ATP secretion from platelet dense granules was measured as an increase in luminescence. This is mediated through luciferin, which is added to the platelet suspension, prior to stimulation.

Washed platelets from litter-matched WT and CD148 TM-KO mice were stimulated with low, intermediate and high doses of the GPVI-specific agonist, CRP (1, 3 or 10 μg/ml). CD148-deficient platelets exhibited marked impairment in aggregation and secretion responses to CRP compared with wild-type platelets (Figure 4.1A). Inhibition of platelet aggregation and secretion was observed even at the highest concentration of CRP tested (10 μg/ml), demonstrating that CD148 is an essential, positive regulator of GPVI-mediated aggregation and dense granule secretion. Aggregation and ATP secretion of CD148-deficient platelets were also inhibited in response to low and intermediate concentrations (1 and 3 μg/ml) of the physiological agonist collagen, which acts through both GPVI and the integrin α2β1 (Figure 4.1B). This effect was largely overcome at a higher concentration of collagen (10 μg/ml), which induced almost full aggregation and ATP secretion (Figure 4.1B). The selective recovery of response to high concentrations of collagen, but not CRP reflects the role of the integrin α2β1 in mediating activation to collagen (Chen et al. 2003).

Collagen-mediated aggregation and ATP secretion are heavily dependent on the release of the secondary mediators ADP and TxA_2 from platelets. The release of ADP from dense granules and synthesized TxA_2 amplifies the process of platelet activation by a positive feedback mechanism (FitzGerald 1991; Gachet 2006). To eliminate the contribution of these

secondary mediators to the collagen-induced aggregation and secretion responses, platelets were treated with the ADP scavenger, apyrase, and the cyclooxygenase inhibitory indomethacin prior to stimulation (Figure 4.1C). Under these conditions, CD148-deficient platelets exhibited impaired aggregation at all three concentrations of collagen tested (1, 3 and 10 μg/ml). The magnitude of the defects was enhanced in the presence of apyrase and indomethacin, demonstrating that ADP and indomethacin were masking defects in the collagen response. The greatest difference between WT and CD148-deficient platelet aggregation was observed at the highest dose of collagen (10 μg/ml). ATP secretion was effectively abolished in the presence of the inhibitors of secondary mediators. This data provides further evidence that CD148 is a positive regulator of GPVI- and α2β1-mediated signalling.

Aggregation and ATP secretion of CD148-deficient platelets were also tested in response to various GPCRs, which act through distinct receptors and signalling pathways. We initially investigated the ability of CD148-deficient platelets to aggregate and secrete ATP in response to the powerful GPCR agonist thrombin, which acts through the PAR-4 receptor in mouse platelets. Aggregation and ATP secretion were marginally reduced in response to low, intermediate and high doses of thrombin (0.03, 0.09 and 0.3 U/ml respectively) (Figure 4.1D), suggesting CD148 may play a minor role downstream of PAR-4. Platelets were then pre-treated with the Src kinase inhibitor PP1 before stimulation with thrombin to determine if SFKs were getting activated in mutant platelets. PP1 blocked the aggregation and secretion responses of CD148-deficient platelets to the same extent as WT platelets, demonstrating that SFKs are not completely inactive in mutant platelets. The only exception was at the intermediate does of thrombin (0.03 U/ml), where PP1 had more of an effect on the mutant platelets, suggesting that CD148 may regulate other components of the thrombin signalling pathway besides SFKs (Figure 4.1E).

We next investigated aggregation and ATP secretion in response to the TxA_2 analogue U46619, which signals through the TP receptor. CD148-deficient platelets exhibited a minor aggregation defect to all doses of U46619 (1, 3 and 10 μM) tested (Figure 4.1F). A minor delay in the secondary wave of aggregation and ATP secretion was also observed at 10 μM U46619 (Figure 4.1F). In contrast, CD148-deficient platelets exhibited normal aggregation and ATP secretion responses to ADP, which signals through the $P2Y_1$ and $P2Y_{12}$ receptors (Figure 4.1G). Together, these results demonstrate that CD148 is essential for tyrosine kinase-linked receptor-mediated aggregation and secretion responses, and also plays a minor role in selective GPCR-mediated responses.

Figure 4.1. CD148-deficient platelets exhibit impaired GPVI-mediated platelet aggregation and ATP secretion. Washed platelets (2×10^8/ml) prepared from littermatched wild-type (WT) and CD148 transmembrane-knockout ($CD148^{-/-}$) mice were stimulated with low, intermediate and high doses of: **A)** collagen- related peptide (CRP) (3, 10 and 30 μg/ml); **B)** collagen (1, 3 and 10 μg/ml); **C)** collagen (1,3 and 10 μg/ml) in the presence of secondary mediator inhibitors apyrase and indomethacin, **D)** thrombin (0.009, 0.03 and 0.09 U/ml) and **E)** thrombin in the presence of PP1. Platelet rich plasma prepared from litter-matched WT and CD148 TM-KO mice were stimulated with low, intermediate and high doses of: **F)** thromboxane A2 analogue U46619 (1, 3 and 10 μM) and **G)** ADP (1, 3 and 10 μM). Platelet aggregation was measured as a change in light transmission and ATP secretion was measured as luciferin/luciferase-mediated luminescence, using a lumi-aggregometer. Representative images are shown (n = 3 – 6 mice per condition).

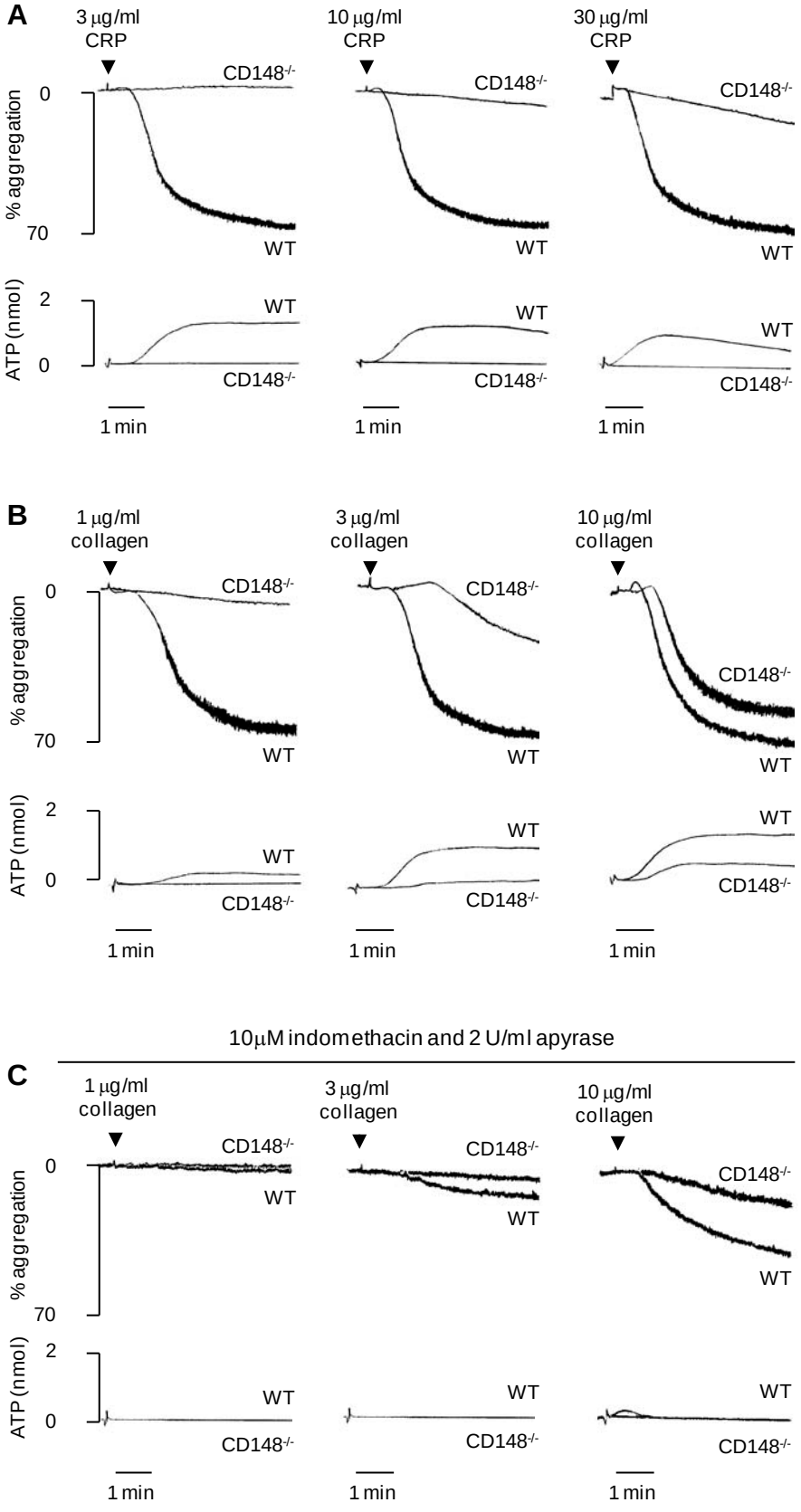

A
3 μg/ml CRP
10 μg/ml CRP
30 μg/ml CRP
CD148-/-
WT
% aggregation
0
70
ATP (nmol)
2
0
1 min
B
1 μg/ml collagen
3 μg/ml collagen
10 μg/ml collagen
10μM indomethacin and 2 U/ml apyrase
C

Figure 4.1.

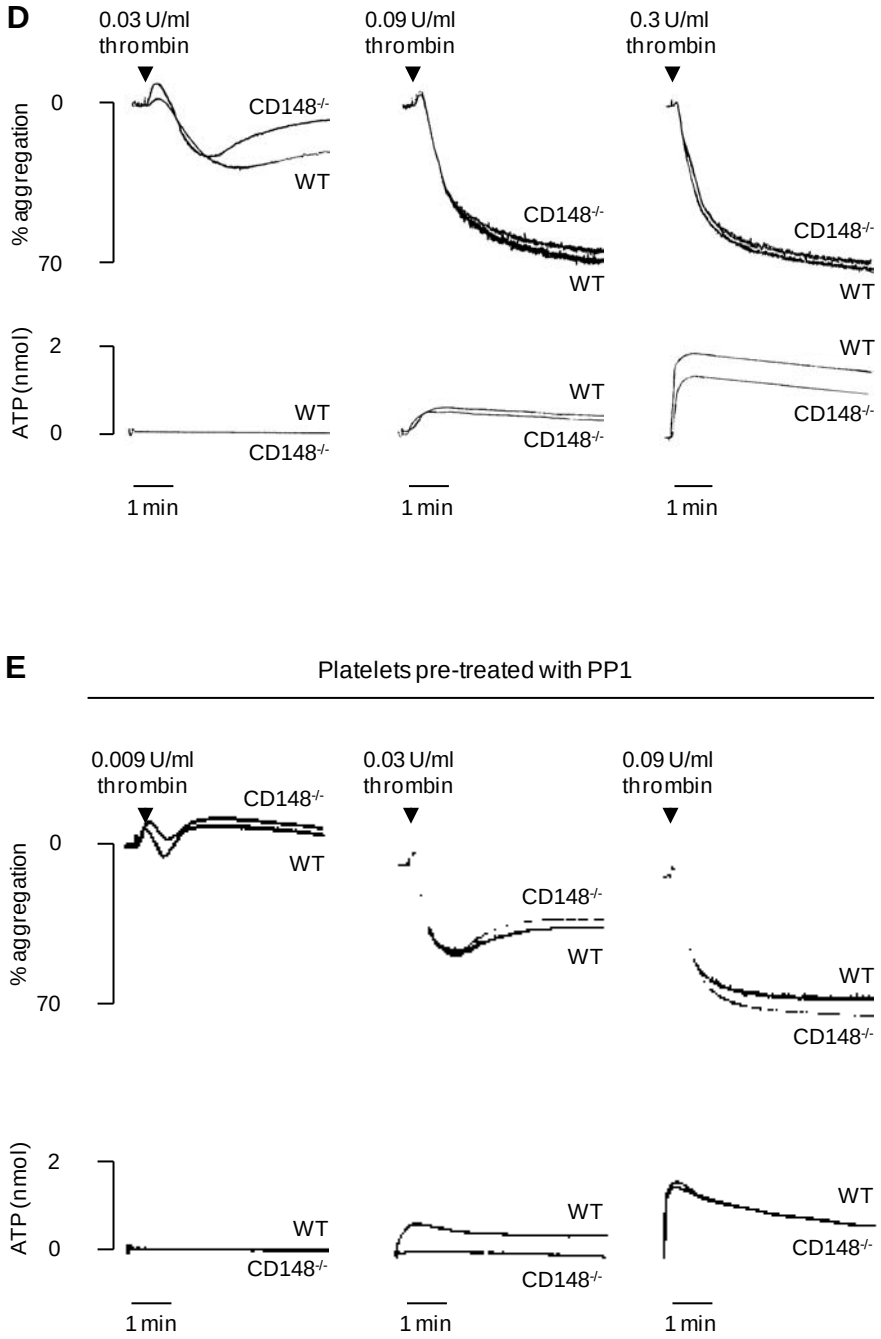


Figure 4.1 continued.

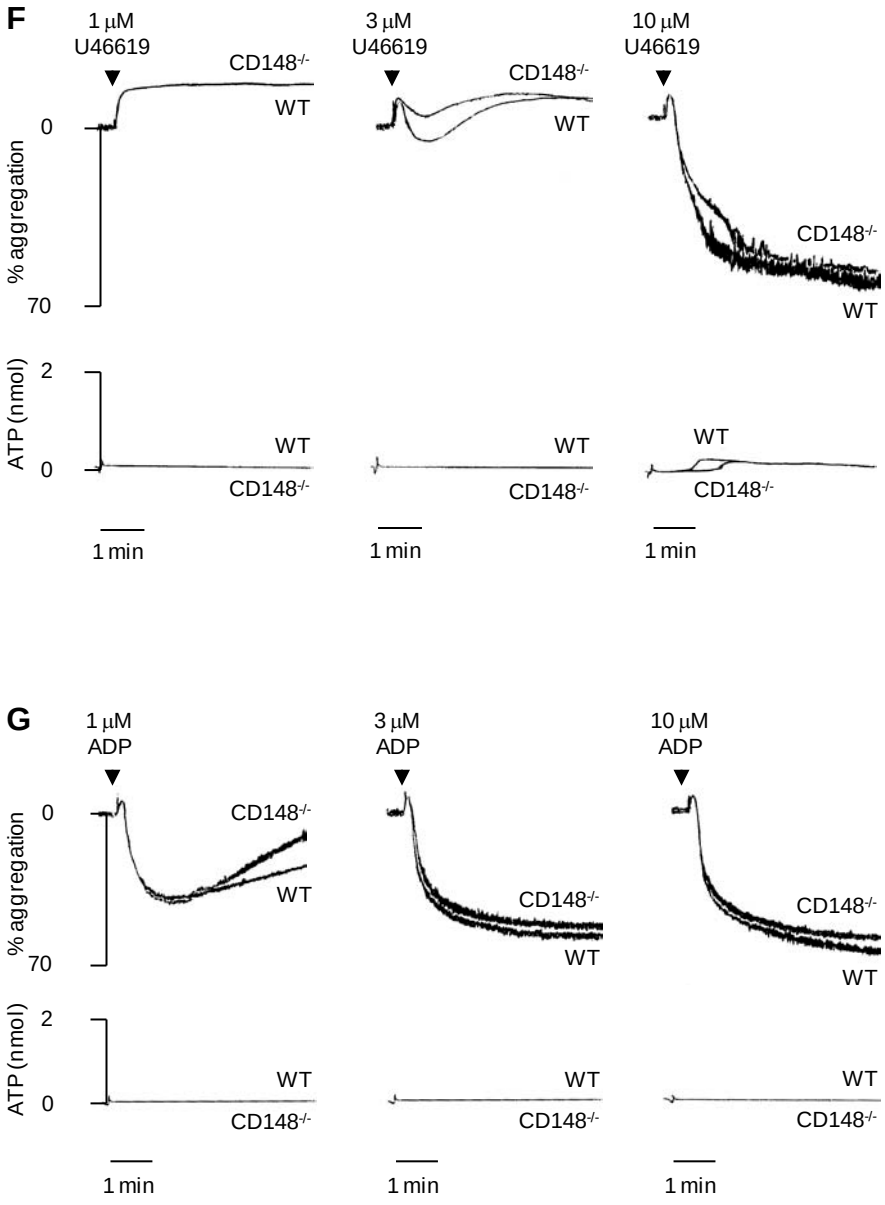


Figure 4.1 continued.

4.3.2 Impaired spreading of CD148-deficient platelets

We next investigated the ability of CD148-deficient platelets to adhere to and spread on a collagen-coated surface under static conditions. As with collagen-mediated aggregation, the spreading response is mediated through GPVI and the integrin $\alpha 2\beta 1$. Released ADP and TxA_2 enhance the response. WT and CD148-deficient platelets were placed on collagen-coated coverslips for 45 minutes. Adherent platelets were subsequently fixed, permeabilized and actin filaments stained with Alex 488-conjugated phalloidin. Although the same number of WT and CD148-deficient platelets adhered to the collagen-coated surface (data not shown), CD148-deficient platelets did not spread to the same extent as WT platelets (Figure 4.2A). There was approximately a 20% reduction in the surface area of collagen-adherent CD148-deficient platelets compared with WT control platelets (Figure 4.2B). Morphologically, mutant platelets looked the same as WT platelets, exhibiting filopodia and lamellipodia, except that they did not spread to the same extent as WT platelets. This demonstrated that CD148 positively regulates platelet spreading on collagen.

The same spreading experiment described above was also performed on fibrinogen-coated coverslips to investigate whether CD148 contributes to outside-in signalling by the integrin $\alpha IIb\beta 3$. The same number of CD148-deficient platelets adhered to the fibrinogen-coated surface however, mutant platelets exhibited a marked reduction in spreading on the fibrinogen-coated surface (Figure 4.3A). The fibrinogen spreading defect was more pronounced compared with the collagen spreading defect. CD148-deficent platelets exhibited a 30% reduction in spreading on fibrinogen compared with WT platelets under the same conditions (Figure 4.3B). Morphologically, mutant platelets had fewer filopodia and no lamellipodia, whereas all WT platelets had extended filopodia and several had formed lamellipodia. This data suggested that CD148 positively regulates $\alpha IIb\beta 3$ signalling in platelets.

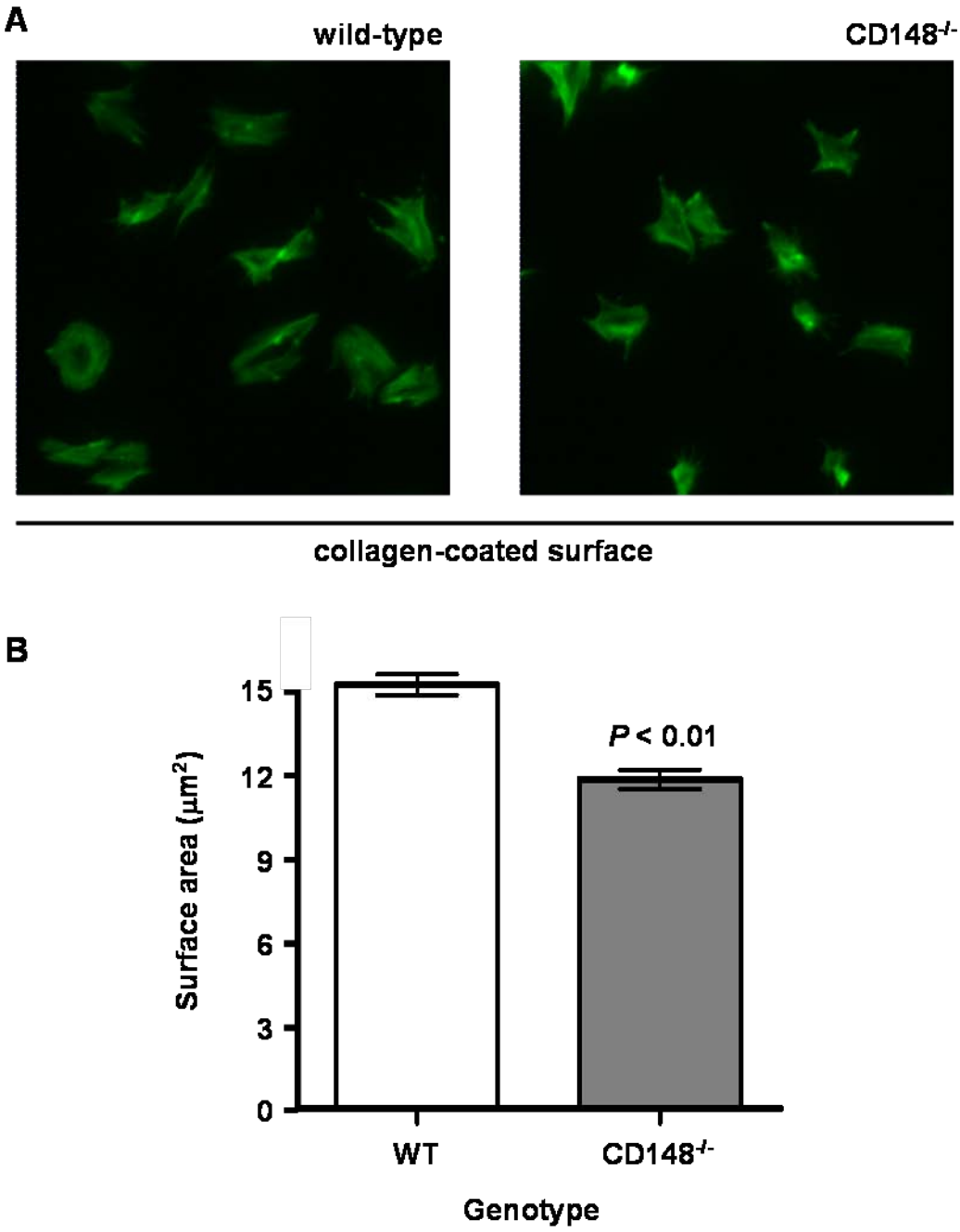


Figure 4.2: CD148-deficient mouse platelets have a spreading defect on collagen. Washed platelets (2×10^7/ml) were placed on collagen-coated cover slips for 45 mins at 37°C. Adherent platelets were permeabilized with 0.2% Triton-X 100 and actin filaments stained with Alex 488-conjugated phalloidin. (A) Representative images of wild-type and CD148-deficient platelets spread on collagen. (B) The surface area of 50 - 100 platelets from 3 wild-type and 2 $CD148^{-/-}$ mice was quantified using ImageJ software (mean ± SEM).

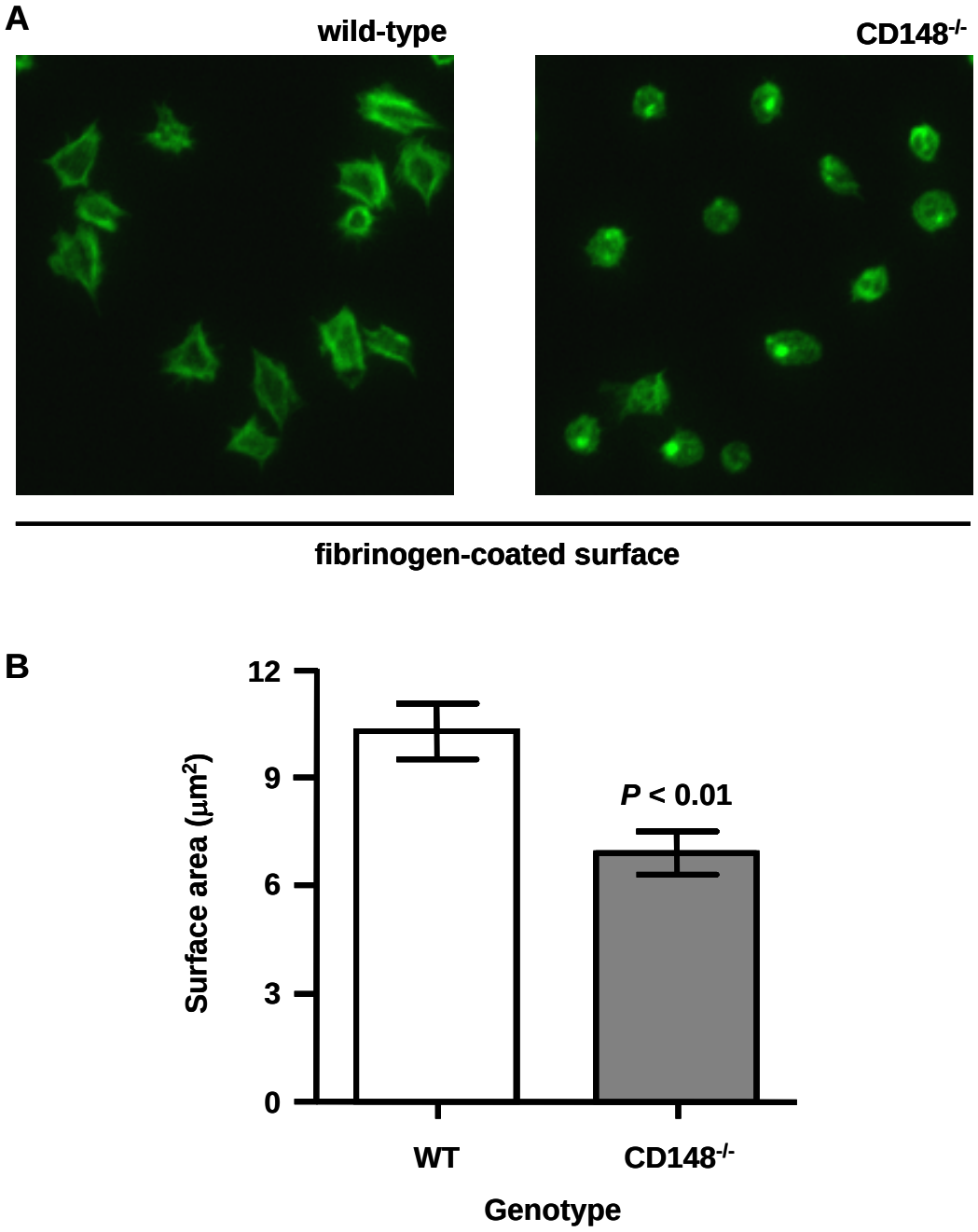


Figure 4.3: CD148 deficient mouse platelets do not spread properly on fibrinogen. Washed platelets (2×10^7/ml) were placed on fibrinogen-coated cover slips for 45 mins at 37°C. Adherent platelets were permeabilized with 0.2% Triton-X 100 and actin filaments stained with Alex 488-conjugated phalloidin. (A) Representative images of wild-type and CD148 deficient platelets spread on fibrinogen. (B) The surface area of 50 - 100 platelets from 3 wild-type and 2 $CD148^{-/-}$ mice was quantified using ImageJ software (mean ± SEM).

4.4 Discussion

Results from this Chapter demonstrate for the first time that CD148 is an essential positive regulator of platelet function. GPVI- and αIIbβ3-mediated functional responses were severely inhibited in CD148-deficient platelets. In contrast, thrombin- and TxA_2-mediated responses were minor, and ADP-mediated responses were normal. Impaired responsiveness of CD148-deficient platelets to collagen was less apparent at high concentrations of collagen. Conversely, it was enhanced in the presence of inhibitors of the secondary mediators ADP and TxA_2, demonstrating that these agonists were getting released and masking any defects in GPVI and α2β1 signalling. The more severe spreading defect exhibited by CD148-deficient platelets on a fibrinogen- rather than a collagen-coated surface is also probably due to masking by released ADP and TxA_2. The spreading defects suggest defects in outside-in integrin αIIbβ3 and α2β1 signalling. This is the first time that a RPTP has been shown to inhibit such a broad range of functional response to a variety of ITAM, integrin and GPCR agonists.

A potential explanation for the CRP- and collagen-mediated functional defects of CD148-deficient platelets is a reduction in GPVI and/or α2β1 integrin levels. GPVI levels were in fact reduced by 42 ± 1.5% (mean ± standard error; n = 9) of controls, whereas integrin α2β1 and αIIbβ3 levels were normal on platelets from CD148-deficient mice (appendix: Senis et al. 2009). However, despite this almost 60% reduction in GPVI levels on the surface of CD148-deficient platelets, CD148-deficient platelets exhibited more severe aggregation and secretion defects compared with FcR γ-chain heterozygous-deficient (γ-chain$^{+/-}$) platelets, which express comparable surface levels of GPVI (Snell et al. 2002; Senis et al. 2009).

The reduction in ATP release from CD148-deficient platelets may be due to reduced dense granule secretion as thrombin-mediated ATP secretion was not as severely reduced. A

similar reduction in P-selectin expression on the surface of CRP- and thrombin-stimulated CD148-deficient platelets, suggesting a concomitant reduction in α-granule secretion from mutant platelets (appendix: Senis et al. 2009). Integrin αIIbβ3 activation on the surface of CRP-stimulated CD148-deficient platelets was also reduced in mutant platelets. Collectively, these findings provide further evidence suggesting that CD148 positively regulates GPVI signalling.

Platelet adhesion and spreading are complex processes that require bi-directional integrin signalling. Platelets undergo a series of characteristic morphological changes during adhesion and spreading on collagen- and fibrinogen-coated surfaces. These include initially extending filopodia that then firmly adhere to the surface and subsequently fill in with lamellipodia. Failure of CD148-deficient platelets to spread properly on these surfaces suggested defective ITAM and integrin receptor signalling. Spreading on collagen is mediated by GPVI, which triggers inside-out signalling to the integrin α2β1 causing it to undergo a conformational change to a high affinity state. GPVI and α2β1 recognize different amino acid sequences in collagen fibrils (GPO repeats and GFOGER repeats, respectively). GPVI also triggers release of the secondary mediators ADP and TxA_2, which act in a paracrine and autocrine manner to enhance platelet activation. Spreading on fibrinogen is mediated primarily by the integrin αIIbβ3, without the need of an activation receptor and less of a contribution from secondary mediators. The less severe spreading defect exhibited by CD148-deficient platelets on a collagen-coated surface is likely due to synergy between GPVI and α2β1 signalling and amplification by released ADP and TxA_2. The pronounced spreading defect exhibited by mutant platelets on a fibrinogen-coated surface suggests CD148 also plays an important role in regulating outside-in integrin αIIbβ3 signalling. Interestingly, real-time imaging demonstrated that CD148-deficient platelets take longer and extend fewer filopodia than WT control platelets on fibrinogen (appendix: Senis et al. 2009). Filopodia

extended by mutant platelets did not remain firmly attached to the fibrinogen surface, but retract with time (appendix: Senis et al. 2009). Taken together, these findings demonstrate an essential, positive regulatory role of CD148 in ITAM and integrin receptor signalling.

Minor aggregation and secretion responses of CD148-deficient platelets were also detected in response to thrombin and TxA_2, which signal through the GPCRs PAR-4 and TP receptors in mouse platelets, respectively. This is the first time that a RPTP has been shown to regulate GPCR-mediated functional responses in any cell type, to our knowledge. In contrast, ADP receptor responses were normal in CD148-deficient platelets, demonstrating that platelet responses were differentially affected by deletion of CD148. We hypothesize this is due to a minor SFK component that is required for optimal thrombin- and TxA_2-mediated responses, but not for ADP responsiveness. This will be discussed in further detail in the General Discussion.

The physiological relevance of the above functional defects have been characterized by several members of the Watson and Senis groups and were recently published in *Blood* (appendix: Senis et al. 2009). CD148-deficient mice had a minor but significant prolongation of tail bleeding time and exhibited reduced thrombus formation and stability using two separate models of arterial thrombosis, namely the ferric chloride- and laser-induced injury models (appendix: Senis et al. 2009).

CHAPTER 5 –

ITAM AND INTEGRIN RECEPTOR SIGNALLING DEFECTS IN CD148-DEFICIENT PLATELETS

5.1 Aim

In the previous chapter, I demonstrated that CD148 positively regulates ITAM and integrin receptor mediated platelet responses. The aim of this chapter was to investigate the molecular mechanism underlying these defects. Experiments were designed to investigate differences in collagen- and fibrinogen-mediated signalling between wild-type and CD148-deficient platelets. Previous reports have demonstrated that CD148 regulates ITAM receptor signalling and SFK phosphorylation (Pera et al. 2005; Zhu et al. 2008). Our hypothesis was that CD148 would regulate proximal ITAM and integrin receptor signalling events, similar to CD45 and PTPα in lymphocytes and fibroblasts, respectively (Zeng et al. 2003; McNeill et al. 2007). I therefore focussed my attention on investigating the phosphorylation status of three of the main platelet SFKs, Fyn, Lyn and Src, as well as other key signalling proteins downstream of GPVI and αIIbβ3.

5.2 Introduction

SFKs are critical for initiating and propagating signalling from most platelet tyrosine kinase-linked receptors. They have also been shown to play a minor role in propagating signalling from some platelet GPCRs, including the thrombin receptors PAR-1 and PAR-4, and the TxA_2 receptor TP. To date, platelets have been demonstrated to express five of the eight SFKs, namely Fyn, Lyn, Src, Fgr and Yes with Src being the most abundant (Huang et al. 1991; Pestina et al. 1997).

The earliest identified GPVI signalling event is the activation of SFKs. Previous studies using mutant mouse models and transfected cell lines have shown that the SFKs Fyn and Lyn are constitutively associated with the proline-rich region of GPVI via their SH3 domains (Ezumi et al. 1998; Suzuki-Inoue et al. 2002). Similarly, Src is constitutively associated with the carboxy-terminal tail of the β3 integrin cytoplasmic tail (Obergfell et al. 2002; Arias-Salgado et al. 2003; Watson et al. 2005). It is thought that by having constitutively associated SFKs, GPVI and αIIbβ3 can signal more rapidly in response to ligand mediated cross-linking.

The activity of SFKs is tightly regulated by tyrosine phosphorylation and intra-molecular interactions, as described in the General Introduction. Activation of SFKs requires disruption of the intra-molecular SH2 and SH3 interactions, allowing substrates to access the catalytic site. Phosphorylation of the C-terminal tail inhibitory tyrosine residue by Csk and the related kinase Ctk/Chk maintains the molecule in an inactive conformation, whereas dephosphorylation of this site allows the SFK to adopt an open, active conformation (Okada et al. 1991; Murphy et al. 1993). SFKs can also be activated by out-competing the SH2 and SH3 interactions through inter-molecular interactions (Xu et al. 1999). Maximal SFK activation also requires *trans*-autophosphorylation of the activation loop tyrosine residue, which locks it in an open conformation (Xu et al. 1999).

Several PTPs have been shown to dephosphorylate the C-terminal inhibitory tyrosine residue of SFKs, including the RPTPs CD45, PTPα, PTPε and PTPλ and the non-transmembrane PTPs PTP-1B, Shp1 and Shp2. In the case of the B and T cell receptors this action is primarily carried out by the RPTP CD45, with the structurally distinct CD148 also playing a minor role in B cells (Lin et al. 2003; McNeill et al. 2007; Zhu et al. 2008). To date, only CD45 and the non-transmembrane PTP PTP-BAS have been shown to dephosphorylate the activation loop of SFKs (Roskoski 2005). Recently, CD148 was also shown to interact directly with Src and to dephosphorylate both of its regulatory phosphorylation sites *in vitro* (Pera et al. 2005). The interaction was also observed in transfected cells, however, CD148 only dephosphosphorylated the inhibitory site and not the activation site in transfected cells (Pera et al. 2005).

5.3 Results

This section is divided in the following three sub-sections: (1) Regulation of collagen-mediated signalling by CD148; (2) Regulation of fibrinogen-mediated signalling by CD148; and (3) Tyrosine phosphorylation of CD148 in human platelets.

5.3.1 Regulation of collagen-mediated signalling by CD148

5.3.1.1 CD148 positively regulates GPVI proximal signalling in mouse platelets

In the previous chapter, CD148-deficient mouse platelets were shown to have severely reduced responses to CRP. Western blotting was used as a way of identifying signalling defects in resting and CRP-stimulated CD148-deficient platelets. Washed platelets in suspension from wild-type and CD148-deficient mice were stimulated with a high concentration (10 μg/ml) of CRP, in the presence of inhibitors of the secondary mediators TxA_2 and ADP, and the integrin αIIbβ3, in order to focus specifically on GPVI signalling. There was a striking inhibition of the increase in tyrosine phosphorylation of most proteins to CRP in the CD148-deficient platelets throughout a 300 second time course (Figure 5.1). This included a loss of tyrosine phosphorylation of the 12 kDa doublet that has been previously identified as FcR γ-chain, thereby establishing a proximal signalling defect in the GPVI pathway (Gibbins et al. 1996; Poole et al. 1997). Consistent with this observation, there was a marked reduction in inducible tyrosine phosphorylation of important downstream signalling molecules, such as Syk and PLCγ2 (Figure 5.2A and B).

Since FcR γ-chain phosphorylation was decreased, we turned our attention to the GPVI-associated SFKs, Lyn and Fyn, which directly phosphorylate the FcR γ-chain (Ezumi et al. 1998; Quek et al. 2000). Lyn exhibited increased phosphorylation at its C-terminal inhibitory tyrosine residue (Tyr-507) in resting and CRP-stimulated CD148-deficient platelets, suggesting that higher proportions of Lyn were in an inactive conformation in

resting and activated CD148-deficient platelets (Figure 5.3A). Fyn and Src also showed a slight increase in phosphorylation at their C-terminal inhibitory tyrosine residues (Tyr-530 and Tyr-529 respectively) which was fully reproducible, suggesting they too are in an inactive conformation in resting and activated CD148-deficient platelets (Figure 5.3B and C). In line with this, SFKs were hypo-phosphorylated at their respective activation loop tyrosine residues in CD148-deficient platelets (Figure 5.3C). Since this is a *trans*-autophosphorylation event, it indicates a general reduction in SFK activity in the absence of CD148. Together, these results demonstrate that SFKs are in a state of reduced activation in both resting and CRP-stimulated CD148-deficient platelets.

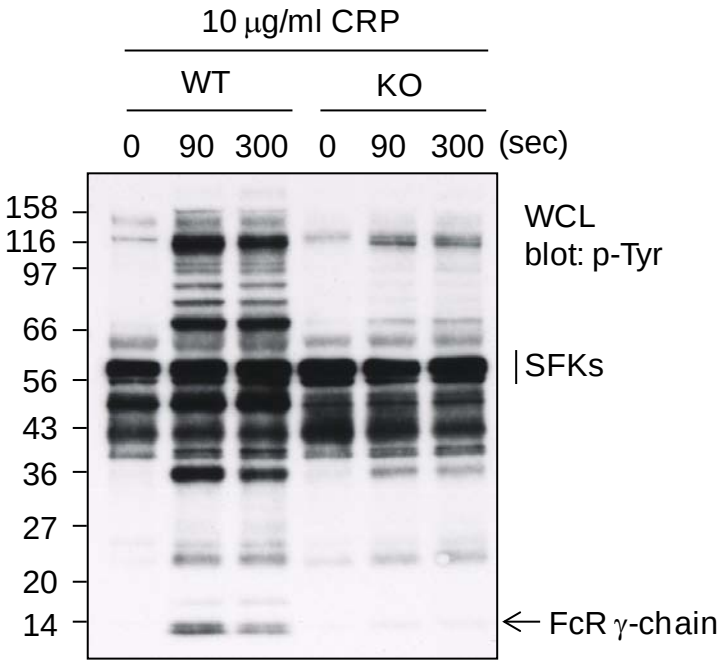


Figure 5.1. Reduced whole cell phosphorylation in CD148-deficient platelets. Whole cell lysates (WCL) of resting and collagen-related peptide (CRP) activated platelets from wild-type (WT) and CD148 transmembrane-knockout (KO) mice were prepared and western blotted with an anti-phosphotyrosine antibody (p-Tyr). Platelets were stimulated with 10 μg/ml CRP for 90 and 300 seconds (sec). Bands corresponding to Src family kinases (SFKs) and FcR γ-chain are indicated. Blots are representative of 4 – 6 experiments.

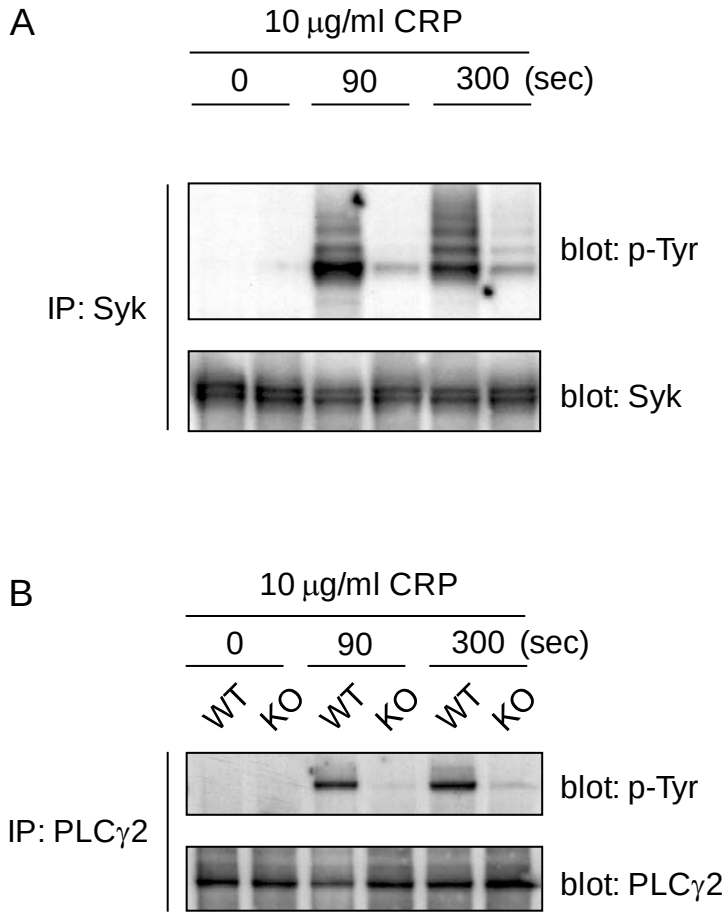


Figure 5.2. Reduced Syk and PLCγ2 tyrosine phosphorylation in CRP-stimulated CD148-deficient platelets. (A) Syk and (B) PLCγ2 were immunoprecipitated (IP) from equal amounts of whole cell lysates prepared from both wild-type (WT) and CD148 transmembrane-knockout (KO) mouse platelets. Platelets were stimulated with 10 μg/ml collagen-related peptide (CRP) for 90 and 300 seconds (sec). Membranes were blotted with an anti-phosphotyrosine (p-Tyr) antibody, and subsequently stripped and re-blotted with anti-Syk (Syk) and anti-PLCγ2 (PLCγ2) antibodies. Blots are representative of 4 – 6 experiments.

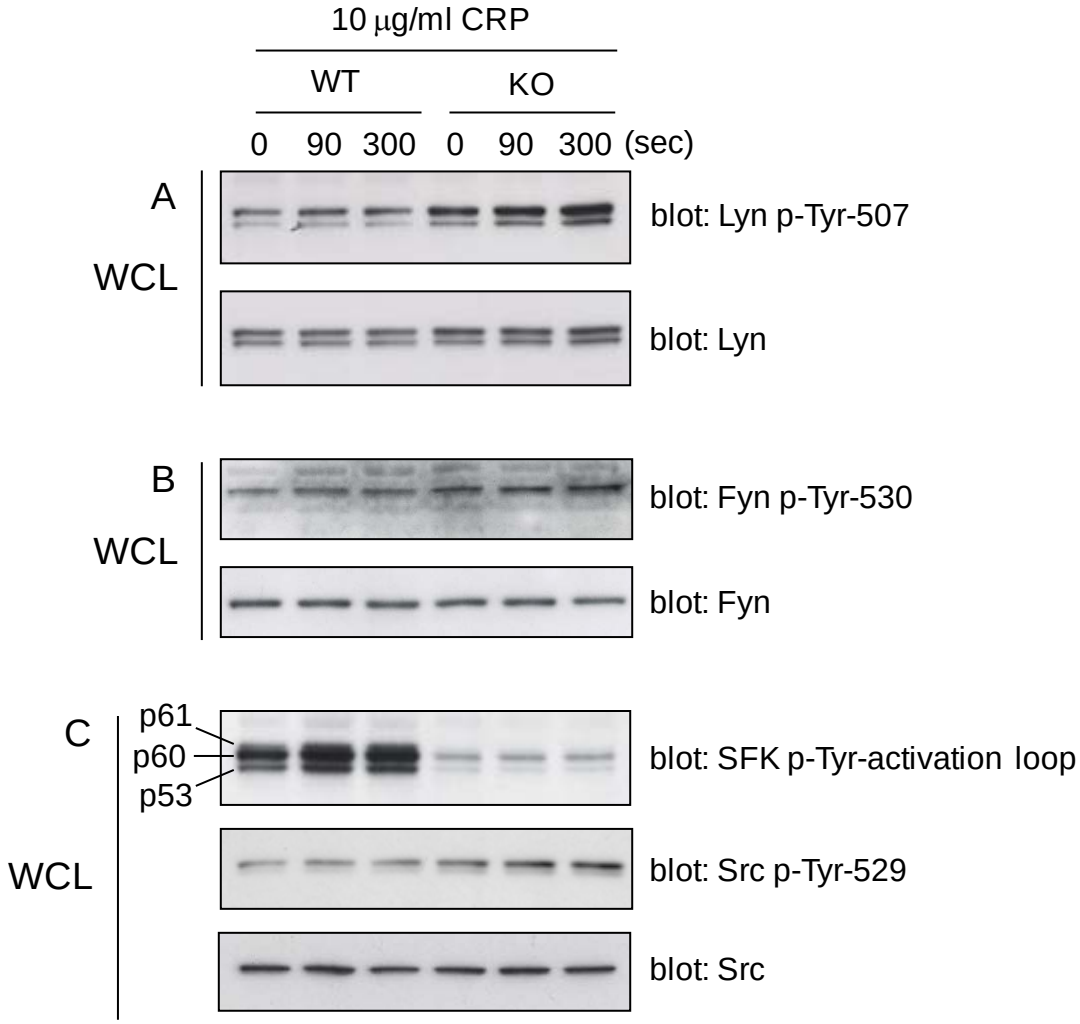


Figure 5.3. Reduced Src family kinase (SFK) activation in resting and CRP-stimulated CD148-deficient platelets. Whole cell lysates (WCLs) prepared from resting and collagen-related peptide (CRP)-stimulated wild-type (WT) and CD148 transmembrane-knockout (KO) platelets were western blotted with phosphospecific anti-SFK antibodies. Platelets were stimulated with 10 μg/ml CRP for 90 and 300 seconds. Membranes were blotted with: (A) anti-Lyn p-Tyr-507 and anti-Lyn-pan antibodies; (B) anti-Fyn p-Tyr-530 and anti-Fyn-pan antibodies; and (C) anti-SFK activation loop p-Tyr, anti-Src p-Tyr-529 and anti-Src-pan antibodies. Blots are representative of 4 – 6 experiments.

5.3.1.2 CD148 positively regulates collagen-mediated signalling in mouse platelets

In the previous chapter I demonstrated that defects in collagen-mediate responses of CD148-deficient platelets could be partially overcome at high concentrations of collagen (Chapter 4). Here I checked for signalling defects in collagen-stimulated CD148-deficient platelets. Unlike CRP, which is GPVI-specific, collagen binds to and signals through both GPVI and the integrin α2β1 simultaneously (Jarvis et al. 2002; Snell et al. 2002). Further, collagen-mediated responses are heavily dependent on positive feedback from released secondary mediators, ADP and TxA_2 (Atkinson et al. 2003). Initially, I checked whole cell phosphorylation in collagen-stimulated CD148-mutant platelets in the presence of an integrin αIIbβ3 antagonist to prevent platelet aggregation and outside-in integrin αIIbβ3 signalling. A dramatic reduction in the number and intensity of most bands was observed in CD148-deficient platelets compared with wild-type platelets, suggesting a block in GPVI and integrin α2β1 proximal signalling (Figure 5.4). Phosphorylation of the FcR γ-chain was marginally reduced in mutant platelets under these conditions (Figure 5.4).

SFKs were hypo-phosphorylated at their activation loop tyrosine in resting and collagen-stimulated CD148-deficient platelets relative to control platelets (Figure 5.5). Collagen-stimulated mutant platelets did however show a slight increase in phosphorylation of the activation loop of SFKs with time, suggesting a marginal increase in activity (Figure 5.5, top panel). Lyn, Fyn and Src were concomitantly hyper-phosphorylated at their C-terminal inhibitory tyrosines Tyr-507, Tyr-530 and Tyr-529, respectively, suggesting that these may be substrates of CD148 (Figure 5.5). Interestingly, Lyn was more highly phosphorylated at its inhibitory site than Fyn or Src, suggesting that CD148 may preferentially dephosphorylate the Lyn inhibitory tyrosine.

Experiments similar to those described above were performed in the presence of ADP and TxA_2 antagonists. Platelets were initially stimulated with 10 μg/ml collagen, but this was

subsequently increased to 100 μg/ml in order to better visualize weak staining bands, such as the FcR γ-chain (Figure 5.6). A dramatic reduction in tyrosine phosphorylation of most proteins was observed in CD148-deficient platelets stimulated with 10 μg/ml collagen compared with wild-type platelets throughout a 300 second time course (Figure 5.6A). Reduced whole cell phosphorylation in both mutant and control platelets the presence of ADP and TxA_2 antagonists (Figures 5.4 and 5.6A), demonstrated that released ADP and TxA_2 enhance collagen-mediated signalling. Interestingly, stimulation of mutant platelets with a 10-fold higher dose of collagen (100 μg/ml) induced more phosphorylation in mutant platelets compared with the lower dose (10 μg/ml collagen) (Figures 5.6A and B), indicating a partial block in collagen signalling in mutant platelets. This was supported by a marginal enhancement of tyrosine phosphorylation of the SFK activation loop (middle bands) over time in the CD148-deficient platelets, suggesting a very slight increase in SFK activity (Figure 5.7B). Although slight, this marginal increase in phosphorylation was fully reproducible, however to support this conclusion gel scanning should be performed. This observation correlated with increased phosphorylation of the FcR γ-chain with time (Figure 5.6A and B).

A critical early event in GPVI signalling is the recruitment and activation of Syk tyrosine kinase to the phosphorylated FcR γ-chain (Watson et al. 2005). This interaction is mediated by the tandem SH2 domains of Syk and the ITAM of the FcR γ-chain. Syk activation is also important for $\alpha_2\beta_1$ signalling (Keely et al. 1996). Using a phospho-specific antibody, Syk was found to be hypo-phosphorylated at Tyr-352 in collagen-stimulated CD148-deficient platelets. This was clearly observed at 100 μg/ml collagen (Figure 5.7B). Phosphorylation of Syk Tyr-352 is a SFK-mediated event that enhances activation of PLCγ (Law et al. 1994).

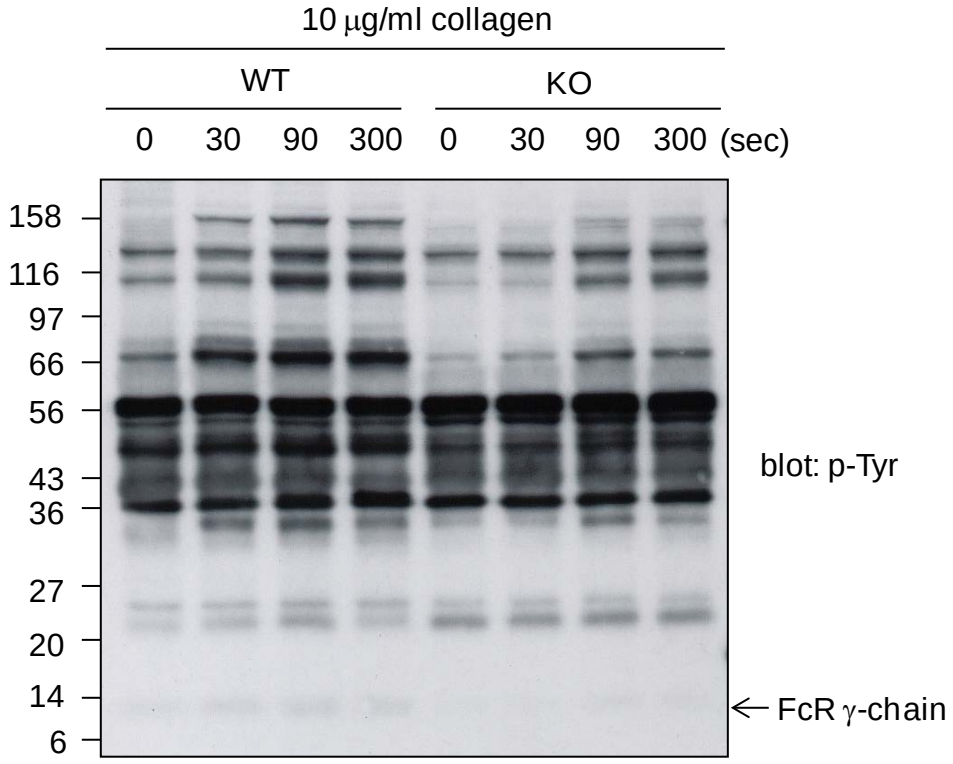


Figure 5.4. Reduced whole cell phosphorylation in CD148-deficient platelets. Whole cell lysates (WCL) prepared from resting and collagen-stimulated platelets from wild-type (WT) and CD148 transmembrane-knockout (KO) mice were western blotted with an anti-phosphotyrosine antibody (p-Tyr). Platelets were stimulated with 10 μg/ml collagen in the presence of 9 μM integrillin for 30, 90 and 300 seconds (sec). Blots are representative of 3 experiments.

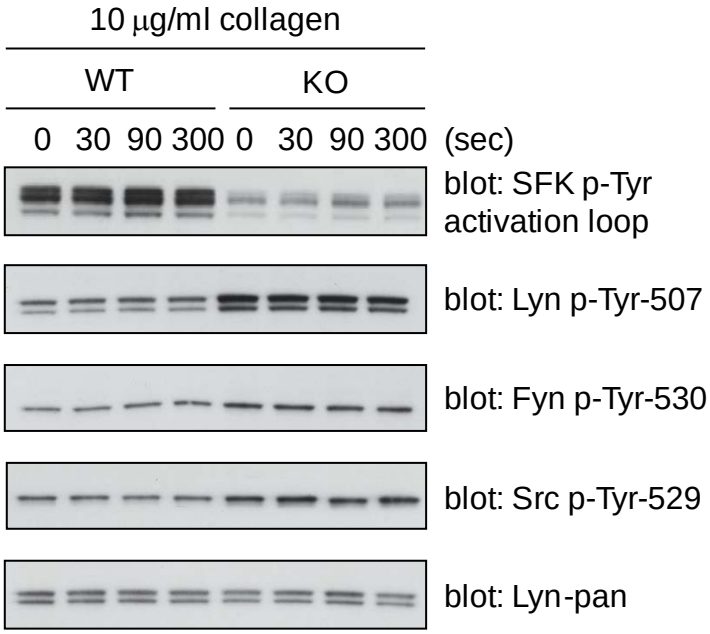


Figure 5.5. Impaired collagen-mediated Src family kinase activation in CD148-deficient platelets. (A) Platelet whole cell lysates (WCLs) prepared from wild-type (WT) and CD148 transmembrane knockout (KO) mice stimulated with 10 μg/ml collagen for 30, 90 and 300 seconds in the presence of 9 μM integrillin. WCLs were western blotted with phosphotyrosine-specific anti-Src family kinase (SFK), -Lyn, -Fyn, -Src antibodies, and anti-Lyn-pan antibody. Blots are representative of 3 experiments.

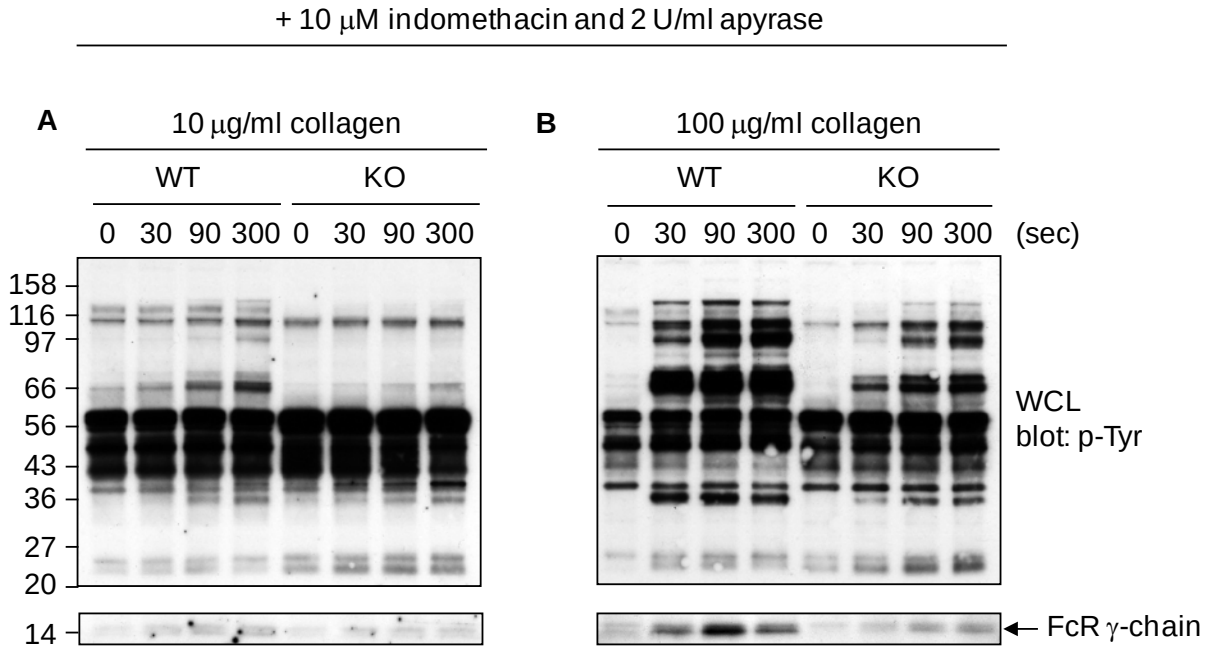


Figure 5.6. Reduced whole cell phosphorylation in CD148-deficient platelets. Whole cell lysates (WCL) prepared from resting and collagen-stimulated platelets from wild-type (WT) and CD148 transmembrane-knockout (KO) mice were western blotted with an anti-phosphotyrosine antibody (p-Tyr). Platelets were stimulated with either (A) 10 μg/ml or (B) 100 μg/ml collagen in the presence of 9 μM integrillin for 30, 90 and 300 seconds (sec). The bands corresponding to the FcR γ-chain are shown in the lower panels. Blots are representative of 3 experiments.

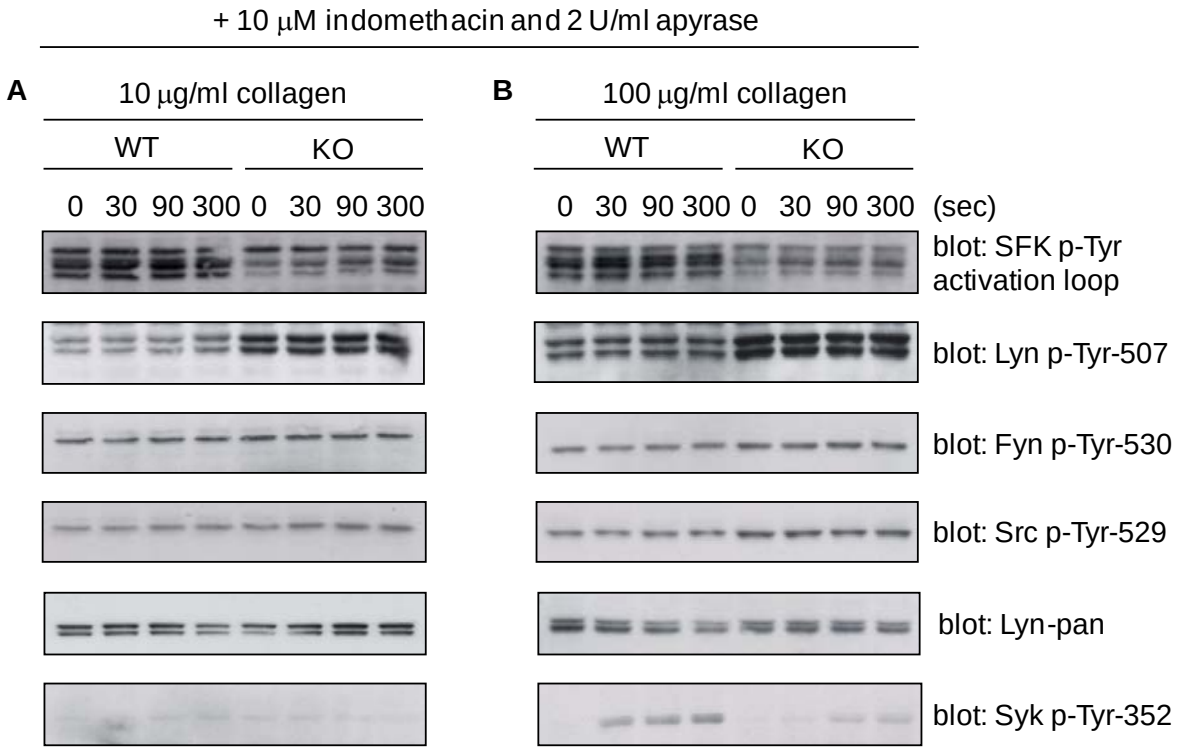


Figure 5.7. Impaired collagen-mediated Src family kinase and Syk kinase activation in CD148-deficient platelets. Platelet whole cell lysates (WCLs) prepared from wild-type (WT) and CD148 transmembrane knockout (KO) mice stimulated with 10 μg/ml collagen for 30, 90 and 300 seconds. Platelets were treated with 10 μM indomethacin (cyclooxygenase inhibitor) and 2 U/ml apyrase (ADP scavenger) prior to being stimulated with either (A) 10 μg/ml or (B) 100 μg/ml collagen. WCLs were western blotted with phosphotyrosine specific anti-Src family kinase (SFK), -Lyn, -Fyn, -Src antibodies, anti-Lyn-pan antibody. and phosphotyrosine specific anti-Syk antibody. Blots are representative of 3 experiments.

5.3.2 Regulation of fibrinogen-mediated signalling by CD148

5.3.2.1 CD148 positively regulates αIIbβ3 proximal signalling in mouse platelets

We next investigated the molecular mechanism underlying the fibrinogen spreading defect exhibited by CD148-deficient platelets described in Chapter 4. We hypothesized that this was due to a signalling defect as platelets from mutant mice expressed normal levels of integrin αIIbβ3 (Senis et al. 2009). Consistent with this theory, western blotting of whole cell lysates prepared of fibrinogen-spread WT and mutant platelets revealed a significant reduction in phosphorylation of most proteins in CD148-deficient platelets, including Syk and PLCγ2, both of which are essential components of the αIIbβ3 signalling cascade (Figure 5.8A – C). Both Src and Lyn were hyper-phosphorylated at their C-terminal inhibitory sites (Tyr-529 and Tyr-507, respectively) in fibrinogen-spread CD148-deficient platelets relative to control platelets (Figure 5.8D). In line with the result GPVI signalling results, SFKs were concomitantly hypo-phosphorylated at their activiaton loops relative to controls in BSA non-adherent and fibrinogen-spread CD148-deficient platelets (Figure 5.8D). Together, these results demonstrate that CD148 positively regulates SFKs downstream of αIIbβ3, providing a molecular explanation for the fibrinogen spreading defect exhibited by CD148-deficient platelets.

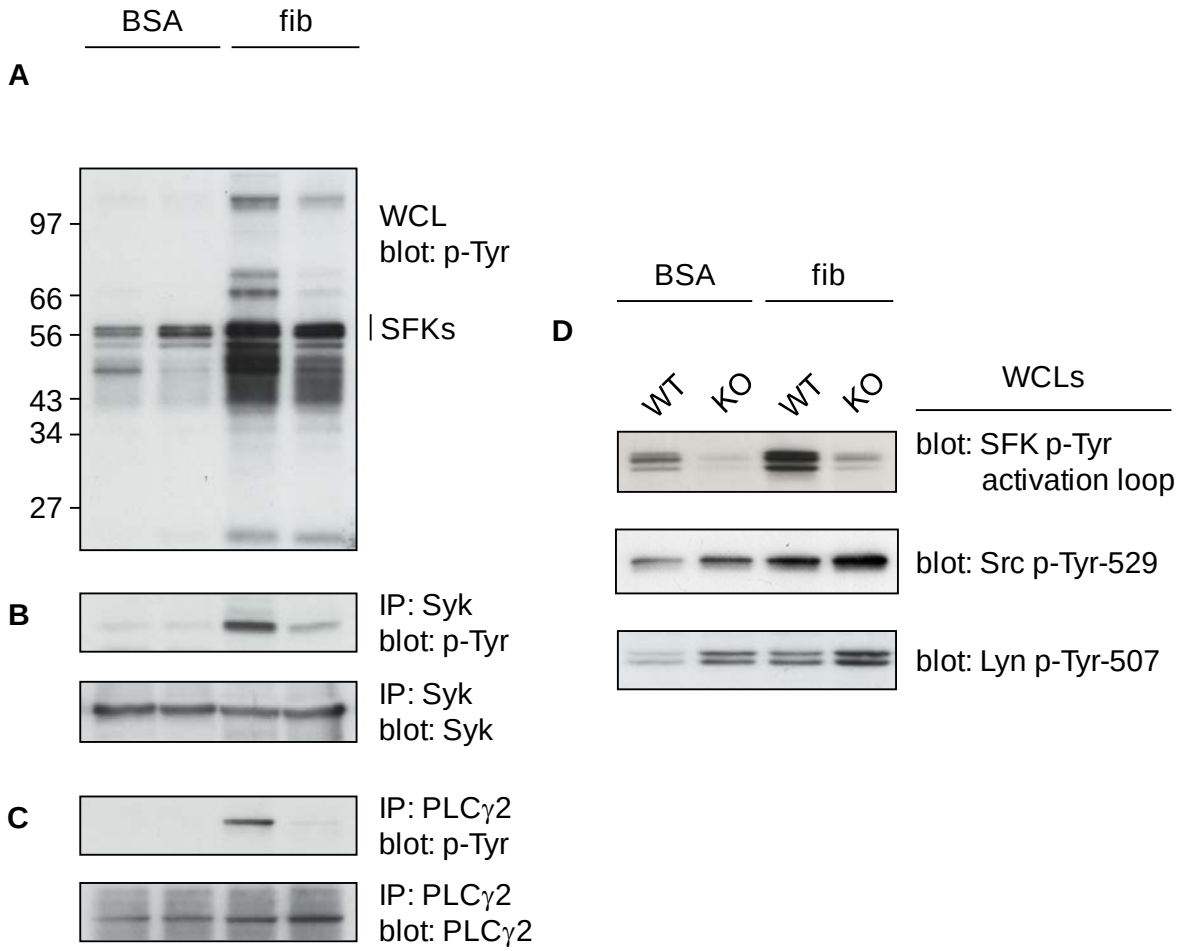


Figure 5.8. Defective αIIbβ3 signalling in CD148-deficient platelets. Wild-type (WT) and CD148-deficient (KO) platelets were plated on BSA- and fibrinogen-coated surfaces for 45 minutes at 37°C. Whole cell lysates (WCLs) were prepared from BSA (BSA) non-adherent and fibrinogen (fib) adherent platelets. (A) Equal amounts of total protein were resolved by SDS-PAGE and western blotted with an anti-phosphotyrosine antibody. (B and C) Syk and PLCγ2 were immunoprecipitated from equal amounts of WCLs and blotted with an anti-phosphotyrosine antibody. Membranes were subsequently stripped and re-blotted with anti-Syk and anti-PLCγ2 antibodies. (D) WCLs were western blotted with an anti-Src family kinase (SFK) activation loop p-Tyr antibody, an anti-Src p-Tyr-529 antibody and an anti-Lyn p-Tyr-507 antibody. Results are representative of three experiments.

5.3.3 Tyrosine phosphorylation of CD148

Tyrsoine phosphorylation has been shown to regulate the activity of some RPTPs, including PTPα and PTPε (Chen et al. 2006; Berman-Golan et al. 2007). In this sub-section, I investigated tyrosine phosphorylation of CD148 in resting and activated platelets, the rationale being that this information may provide some insight into the regulation of CD148 in platelets.

5.3.3.1 Tyrosine phosphorylation of CD148 in suspended platelets

Immunoprecipitation and western blotting was used to check the phosphorylation status of CD148 in resting and activated human platelets in suspension. CD148 was found to be constitutively phosphorylated in resting platelets and this did not change in response to stimulation with 10 μg/ml CRP and 5 U/ml thrombin for 3 minutes (Figure 5.9Ai and ii). A significant increase in tyrosine phosphorylation of CD148 was however observed in platelets treated with 100 μM of the general PTP inhibitor pervanadate, demonstrating that it was submaximally phosphorylated in resting, CRP and thrombin activated platelets (Figure 5.9Ai and ii).

Pre-treatment of platelets with the general Src kinase inhibitor PP2, prior to stimulation with CRP and thrombin, abolished tyrosine phosphorylation of CD148 in resting, CRP and thrombin activated platelets, demonstrating that this is SFK-dependent event (Figure 5.9Ai). PP2 significantly blocked, but did not completely inhibit pervandate-induced tyrosine phophorylation of CD148, demonstrating that CD148 can also be phosphorylated by other kinases besides SFKs (Figure 5.9Ai).

5.3.3.2 Tyrosine phosphorylation of CD148 in spread platelets

Tyrosine phosphorylation of CD148 was also investigated in fibrinogen-spread platelets, in order to determine whether outside-in integrin αIIbβ3 signalling alters its phosphorylation. Washed human platelets were placed on either a BSA- or fibrinogen-coated surface for 45 minutes. BSA non-adherent (control) or fibrinogen adherent platelets were subsequently lysed, CD148 was immunoprecipitated and western blotted. CD148 was constitutively phosphorylated to a similar extent in BSA non-adherent and fibrinogen adherent platelets (Figure 5.9B).

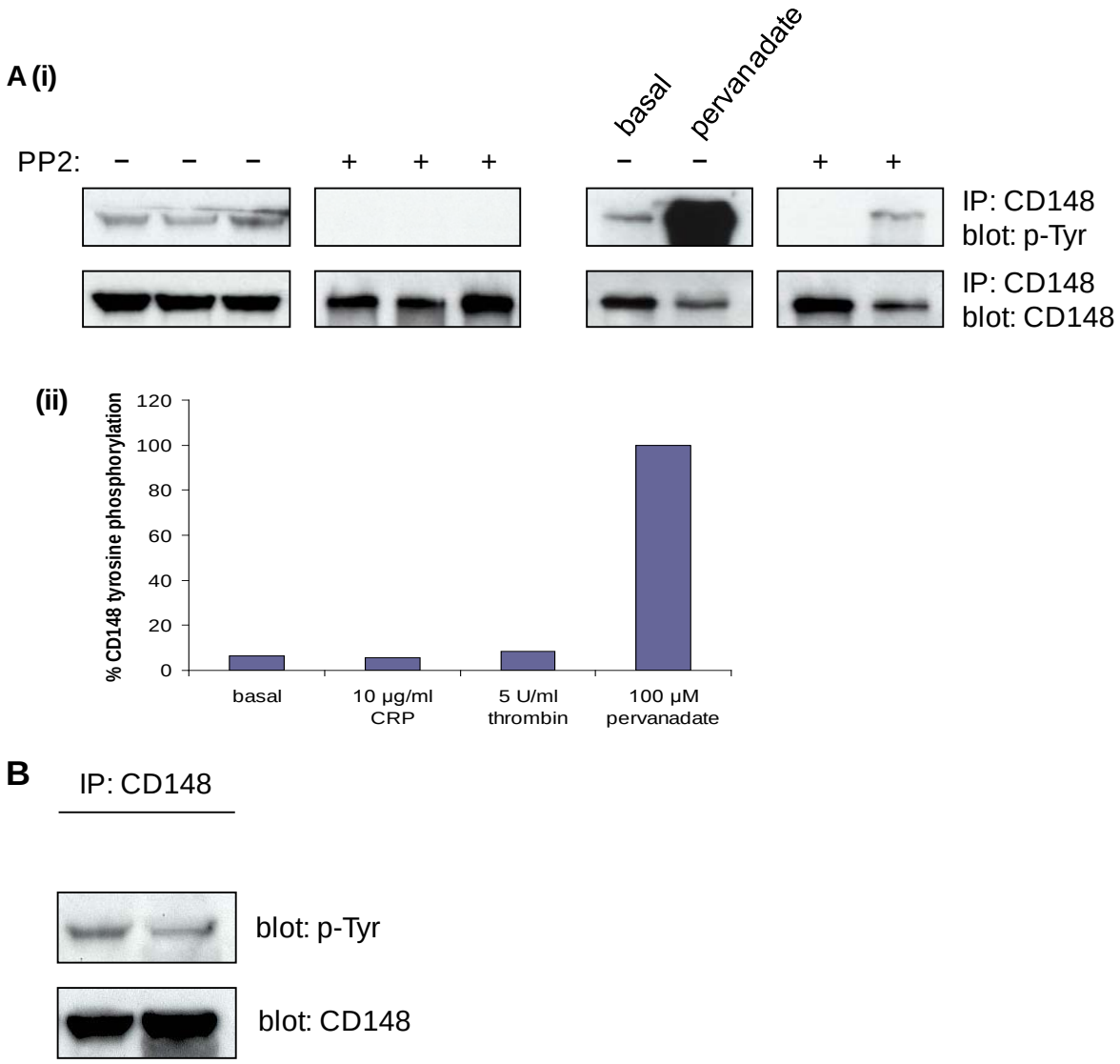


Figure 5.9. CD148 is constitutively tyrosine phosphorylated by Src kinases. (Ai) CD148 was immunoprecipitated (IP) from lysates prepared of washed human platelets under the following conditions: unstimulated (basal), stimulated with 10 µg/ml CRP, 5 U/ml thrombin and 100 µM pervanadate for 3 minutes. IP's were western blotted with an anti-phosphotyrosine antibody (p-Tyr). Membranes were subsequently stripped and re-blotted with an anti-CD148 antibody (CD148). Pre-treatment of platelets with 20 µM PP2 (Src kinase inhibitor) inhibited tyrosine phosphorylation of CD148 under all conditions tested. (Aii) Band intensities were quantified. (B) CD148 was IP'd from lysates prepared of BSA non-adherent (BSA) and fibrinogen adherent (fib) washed human plateles. IP's were western blotted with an anti-phosphotyrosine antibody (p-Tyr). Membranes were subsequently stripped and re-blotted with an anti-CD148 antibody (CD148). n=2.

5.4 Discussion

In this chapter I demonstrate proximal signalling defects downstream of both the collagen receptor GPVI and the integrin αIIbβ3 in CD148-deficient platelets. In both cases this appears to be due to a global reduction in SFK activity in resting and activated platelets. The C-terminal inhibitory sites of Lyn, Fyn and Src appear to be substrates of CD148, as all three proteins were hyper-phosphorylated at their respective inhibitory sites in CD148-deficient platelets. Without this pool of active SFKs in resting CD148-deficient platelets, ligand-mediated cross-linking of GPVI and αIIbβ3 does not elicit optimal signalling.

Until now, the earliest characterized signalling event downstream of GPVI was the activation of constitutively associated SFKs Lyn and Fyn (Ezumi et al. 1998; Quek et al. 2000; Suzuki-Inoue et al. 2002). However, the molecular mechanism of how SFKs are activated in platelets had yet to be addressed. Biochemical data presented in this chapter demonstrated a GPVI proximal signalling defect in the absence of CD148 as tyrosine phosphorylation of the FcR γ-chain, Syk and PLCγ2 were all substantially reduced in response to CRP. Additionally, SFKs exhibited markedly reduced phosphorylation of their activation loop tyrosines in parallel with increased phosphorylation of their C-terminal inhibitory tyrosines in the absence of CD148. Similar findings were observed in collagen-stimulated platelets, however these defects were less severe than those observed with CRP and could be partially overcome with high dose collagen. These differences can be explained by the contributions of α2β1, ADP and TxA_2 to collagen signalling (Inoue et al. 2003; Nieswandt et al. 2003). SFK activation-loop phosphorylation did not appear to change significantly following stimulation of wild type platelets with collagen. A potential reason for this is the contribution of α2β1 to collagen signalling. A higher dose of collagen maybe required to replicate the increase in SFK activation-loop phosphorylation observed with the GPVI specific agonist CRP. Collectively, these findings demonstrate that CD148 plays a

critical role in activating SFKs in resting platelets and downstream of GPVI. CD148 would therefore appear to play a similar role to that of the structurally distinct RPTP CD45 in B and T cell receptor signalling (Hermiston et al. 2003; Zhu et al. 2008).

Defective spreading of CD148-deficient platelets on a fibrinogen-coated surface can also be explained by altered tyrosine phosphorylation of SFKs, resulting in reduced SFK activity and signalling downstream of the integrin αIIbβ3. The Shattil group recently demonstrated a critical role for the non-transmembrane tyrosine phosphatase, PTP-1B, in activating Src specifically downstream of the integrin αIIbβ3. Interestingly, their work suggests that PTP-1B acts specifically downstream of the integrin and is not involved in regulating collagen-, thrombin- or ADP-mediated responses (Arias-Salgado et al. 2005). Therefore, CD148 appears to be a global regulator of SFKs in platelets, whereas PTP-1B acts specifically on αIIbβ3-associated Src.

The biochemical explanation why CD148 is constitutively tyrosine phosphorylated in platelets is presently not known. This is a SFK-dependent event and does not change in response to various platelet agonists, including CRP, thrombin and fibrinogen. Tyrosine phosphorylation may alter activity, protein-protein interactions or the half-life of CD148. Tyrosine phosphorylation has previously been shown to regulate the activity of other RPTPs, such as PTPα and PTPε, however, this does not seem to be a general mechanism that applies to all RPTPs (Chen et al. 2006; Berman-Golan et al. 2007).

The major finding of this chapter is that global SFK activity is significantly reduced in resting and activated CD148-deficient platelets compared with wild-type platelets. This appears to be due to hyperphosphorylation of SFKs at their C-terminal inhibitory tyrosine residues, so they are in an inactive conformation. A pool of active SFKs is therefore essential for initiating a rapid and optimal response when platelets come in contact with the ECM at sites of vascular injury.

CHAPTER 6 –

IDENTIFICATION OF CD148

SUBSTRATES IN PLATELETS

6.1 Aim

The three objectives of this chapter were: (1) to confirm that CD148 is a positive regulator of GPVI using a cell line model; (2) to confirm that Lyn, Fyn and Src are substrates of CD148; and (3) to identify physiological substrates of CD148 in platelets using an *in vitro* substrate-trapping approach.

6.2 Introduction

Results from the previous two chapters demonstrate that CD148 is a critical positive regulator of platelet function, and that it does so by maintaining a pool of active SFKs in platelets by dephosphorylation their C-terminal inhibitory tyrosines. However, several important questions arise from these findings and conclusions I have made thus far, including: (1) Are the functional defects observed in CD148-deficient mouse platelets due to developmental or signalling defects?; (2) Does CD148 have other substrates in platelets besides SFKs?; and (3) Are SFKs direct substrates of CD148?

Although genetically modified mice are the model of choice for studying protein function in platelets, one of their limitations is developmental defects masking the functional role of a protein. It is therefore important to validate functions observed in platelets from mouse models in a cell-based system. To address this point, I used the DT40 chicken B cell/NFAT luciferase assay to measure the effects of CD148 on GPVI signalling. This assay has been used extensively by immunologists to dissect the B cell receptor signalling pathway (Yasuda et al. 2004). More recently this assay has been adapted by the Watson group for studying GPVI, CLEC-2 and G6b-B signalling (Fuller et al. 2007; Tomlinson et al. 2007; Mori et al. 2008). One of the main strengths of this assay is that it is particularly good for measuring weak, sustained signals.

I also wanted to confirm and build on the finding that SFKs are substrates of CD148 in platelets; and identify other substrates of CD148 in platelets. Several phosphatases have been demonstrated to have a broad range of substrate specificity *in vitro* and *in vivo* (Blanchetot et al. 2005). Comparing the GPVI and the integrin αIIbβ3 signalling pathways provides potential CD148 targets. Some of the signalling molecules common to both receptors are: (1) the tyrosine kinases Btk, Tec, and Syk; (2) the lipid kinase PI 3-kinase; (3) the phospholipase PLCγ2; and (4) the small GTPases Vav1, Vav3, and Rac1 (Watson et al. 2005). There are

also some important differences between the GPVI and αIIbβ3 signalling pathways, including the adaptor protein LAT, which nucleates formation of an essential signalling complex downstream of GPVI, but is not required for αIIbβ3 signalling (Watson et al. 2001; Wonerow et al. 2002).

Potential physiological substrates of CD148 identified in other cell systems including: (1) the tyrosine kinase-linked receptors Met and PDGFβ; (2) the adapter proteins LAT and Gab1; (3) the adherens junction protein p120catenin; (4) PLCγ1; and (5) more recently the p85 subunit of PI 3-kinase (Kovalenko et al. 2000; Baker et al. 2001; Holsinger et al. 2002; Palka et al. 2003; Tsuboi et al. 2008). Interestingly, LAT, PLCγ1 and PI 3-kinase all lie downstream GPVI signalling cascade, raising the possibility that CD148 regulates multiple points in the same signalling cascade (Pasquet et al. 1999; Suzuki-Inoue et al. 2003; Watanabe et al. 2003).

In this chapter I confirmed that CD148 plays a positive regulatory role in GPVI signalling using the DT40/NFAT luciferase reporter assay and showed that its catalytic activity is essential for this function. In addition, using *in vitro* biochemical assays, I demonstrate that the SFKs, Src, Lyn and Fyn interact with and are dephosphorylated on their activation and inhibitory sites by CD148. Finally, using a mass spectrometry-based approach, I identified myosin heavy chain 9 (MYH9 protein) as a potential novel substrate of CD148.

6.3 Results

6.3.1 CD148 positively regulates GPVI signalling in a cell line model

In order to determine whether CD148 catalytic activity is required to enhance GPVI signalling I used a nuclear factor of activated T-cells (NFAT)–luciferase transcriptional reporter assay, which provides a highly sensitive readout and is widely used to study signalling through the ITAM-containing B-cell and T-cell receptors. The NFAT reporter contains three copies of a combined NFAT– activator protein-1 (AP-1) element from the human interleukin-2 (IL-2) gene promoter, and is maximally activated by mutual Ca^{2+} elevation and RAS/mitogen-activated protein kinase (MAPK) signalling, which activate NFAT and AP-1, respectively. NFAT-driven luciferase generation over a period of six hours provides a direct readout for sustained ITAM signalling (Tomlinson et al. 2007).

DT40 chicken B cells were transiently transfected with the GPVI/FcR γ-chain complex on its own or in combination with either wild-type or catalytically inactive CD148, and the NFAT-luciferase reporter. Cells were grown overnight and stimulated the following day with 1 μg/ml collagen for 6 hrs. Luciferase activity was measured in lysates and used as a direct readout of GPVI signalling. Co-expression of wild-type CD148 with the GPVI/FcR γ-chain complex enhanced collagen-induced NFAT reporter activity by approximately 2.5-fold compared with the GPVI/FcR γ-chain complex alone (Figure 6.1). Interestingly, basal GPVI signalling was also marginally enhanced in the presence of catalytically active CD148. In contrast, catalytically inactive CD148 had no effect on GPVI signalling in the same assay (Figure 6.2). These findings correlated with the platelet results from Chapters 4 and 5, demonstrating that catalytically active CD148 enhances GPVI signalling.

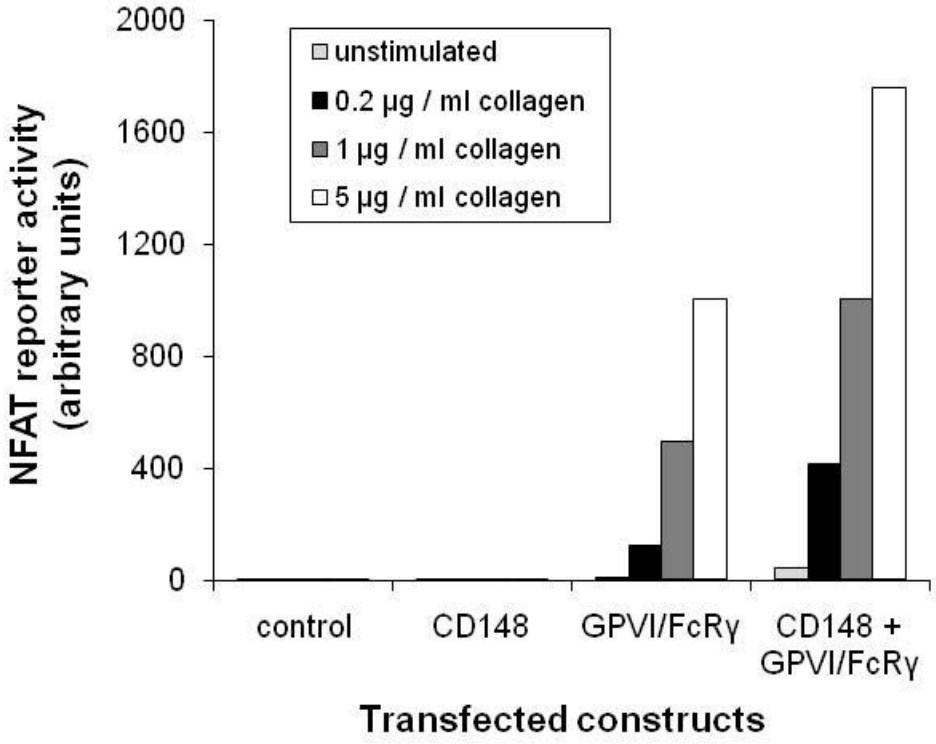


Figure 6.1: CD148 positively regulates GPVI in transiently transfected DT40 cells. An NFAT luciferase reporter assay was used to investigate whether CD148 regulates GPVI signalling. DT40 B cells were transiently transfected with wild-type CD148 (CD148) on its own, the GPVI-FcR γ-chain complex (GPVI/FcRγ) on its own, or CD148 and GPVI/FcRγ together. The NFAT luciferase reporter gene was co-transfected in all cases. Transfected cells were stimulated with either 0.2, 1 or 5 µg/ml collagen for 6 hrs at 37°C and subsequently lysed. Luciferase activity was measured in a luminometer by addition of luciferin. Luminenscence is directly proportional to the amount of GPVI signalling. The assay demonstrated that CD148 positively regulates GPVI signalling. Data shown is representative of 3 separate experiments.

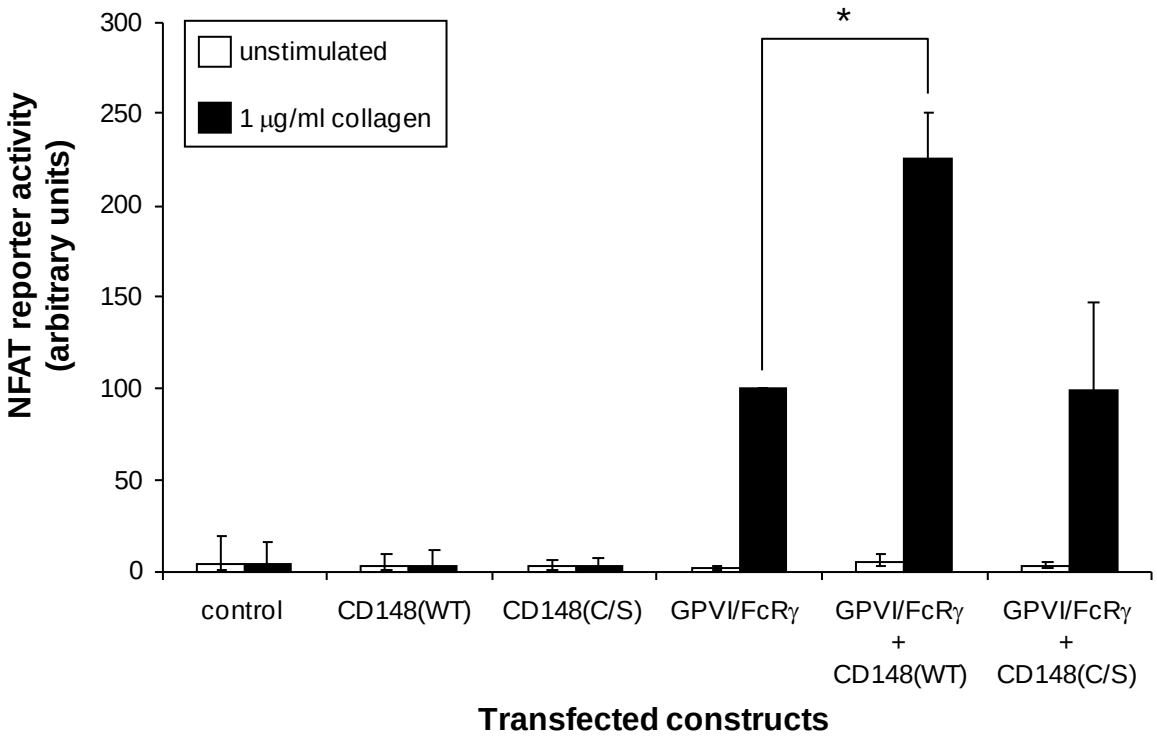


Figure 6.2. CD148 catalytic activity is essential for regulating GPVI signalling in transiently transfected DT40 cells. DT40 B cells were transiently transfected with various combinations of the following expression plasmids: wild-type CD148 (CD148[WT]), catalytically inactive CD148 (CD148[C/S]), GPVI and FcR γ-chain (GPVI/FcRγ), and the NFAT luciferase reporter. Transfected cells were stimulated with 1 μg/ml collagen for 6 hrs at 37°C and subsequently lysed. Luciferin was added to lysates and luciferase activity was measured in a luminometer. Luminenscence is directly proportional to the amount of GPVI signalling (mean ± standard error of the mean, n = 4).

6.3.2 Identification of CD148 substrates in platelets

Since CD148 enzyme activity is essential to positively regulate GPVI signalling, I next wanted to identify substrates of CD148 in platelets. To do this, I used an *in vitro* substrate-trapping pulldown assay. Variations of this assay have been used to identify substrates of various PTPs including CD148 (Palka et al. 2003). Wild-type and mutant forms of the cytoplasmic tail of CD148 were N-terminally tagged with maltose-binding protein (MBP) and used to pull down potential substrates and interacting proteins from pervanadate stimulated platelet lysates. A mutant form of CD148 (D1205A) was used to trap substrates (MBP-CD148[D/A]) (Figure 6.3). This mutation within the catalytic pocket of CD148 blocks catalysis and results in a more stable enzyme and substrate complex (Flint et al. 1997; Palka et al. 2003; Blanchetot et al. 2005). Wild-type (MBP-CD148[WT]) and catalytically inactive (C1239S; MBP-CD148[C/S]) were also used as controls to distinguish between substrates and interacting proteins (Figure 6.3). Bacterially expressed recombinant proteins were purified and coupled to amylose-coated beads, which were then used to pulldown CD148 interacting proteins (Figure 6.4). Expression and purification of mutant proteins can be found in the appendix.

Platelets were treated with the general PTP inhibitor pervanadate, so that intracellular proteins would be maximally phosphorylated. Optimal conditions for pervanadate stimulation were determined by performing a dose response and time course of pervanadate stimulation of platelets. Washed human platelets were stimulated with either, 1, 3, 10, 30 or 100 μM pervanadate for 3 minutes (Figure 6.5A), or with 100 μM pervanadate for 0, 30, 60, 90 or 180 seconds (Figure 6.5B). Platelets were subsequently lysed, proteins were resolved by SDS-PAGE and western blotted with an anti-phosphotyrosine antibody. The optimal concentration and time for pervanadate stimulation was determined to be 100 μM pervanadate for 3 minutes, and was used for all subsequent substrate-trapping experiments.

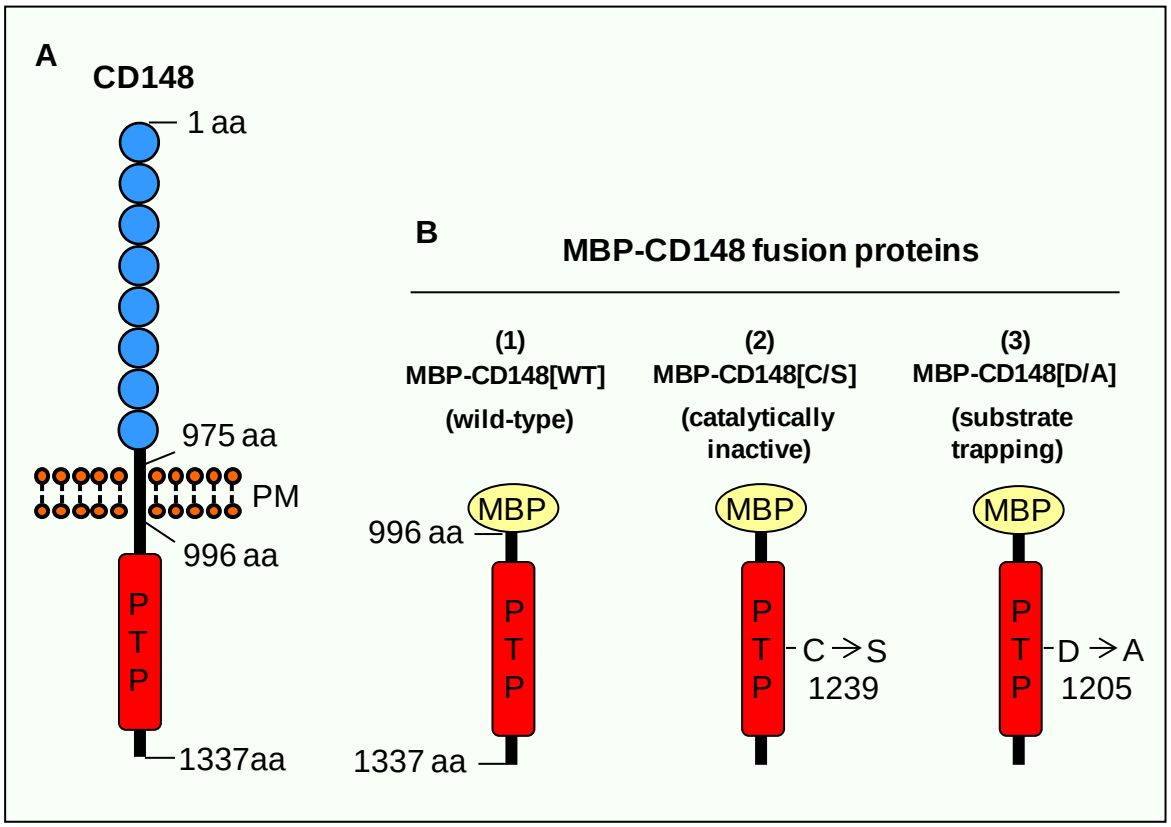


Figure 6.3: Recombinant MBP-tagged versions of CD148 cytoplasmic tail used in *in vitro* substrate-trapping pull down assay. (A) Full length wild-type CD148 protein consists of a 975 amino acid (aa) extracellular region, a 21 aa transmembrane domain and a 341 aa cytoplasmic region. The cytoplasmic tail consists of a 20 aa juxtamembrane region, followed by a 284 aa catalytic PTP domain (red box labelled 'PTP') and a 16 aa cytoplasmic tail. (B) Three recombinant maltose-binding protein (MBP)-tagged versions of the entire CD148 cytoplasmic region were used in the substrate-trapping pulldown assay: (1) wild-type (MBP-CD148[WT]); (2) catalytically inactive (MBP-CD148[C/S]), in which cysteine 1239 is replaced with a serine; and (3) substrate trapping mutant (MBP-CD148[D/A]), in which aspartic acid 1205 is replaced with an alanine. Expression plasmids for all recombinant proteins were kindly provided by Professor Nicholas Tonks (Palka et al. 2003) .

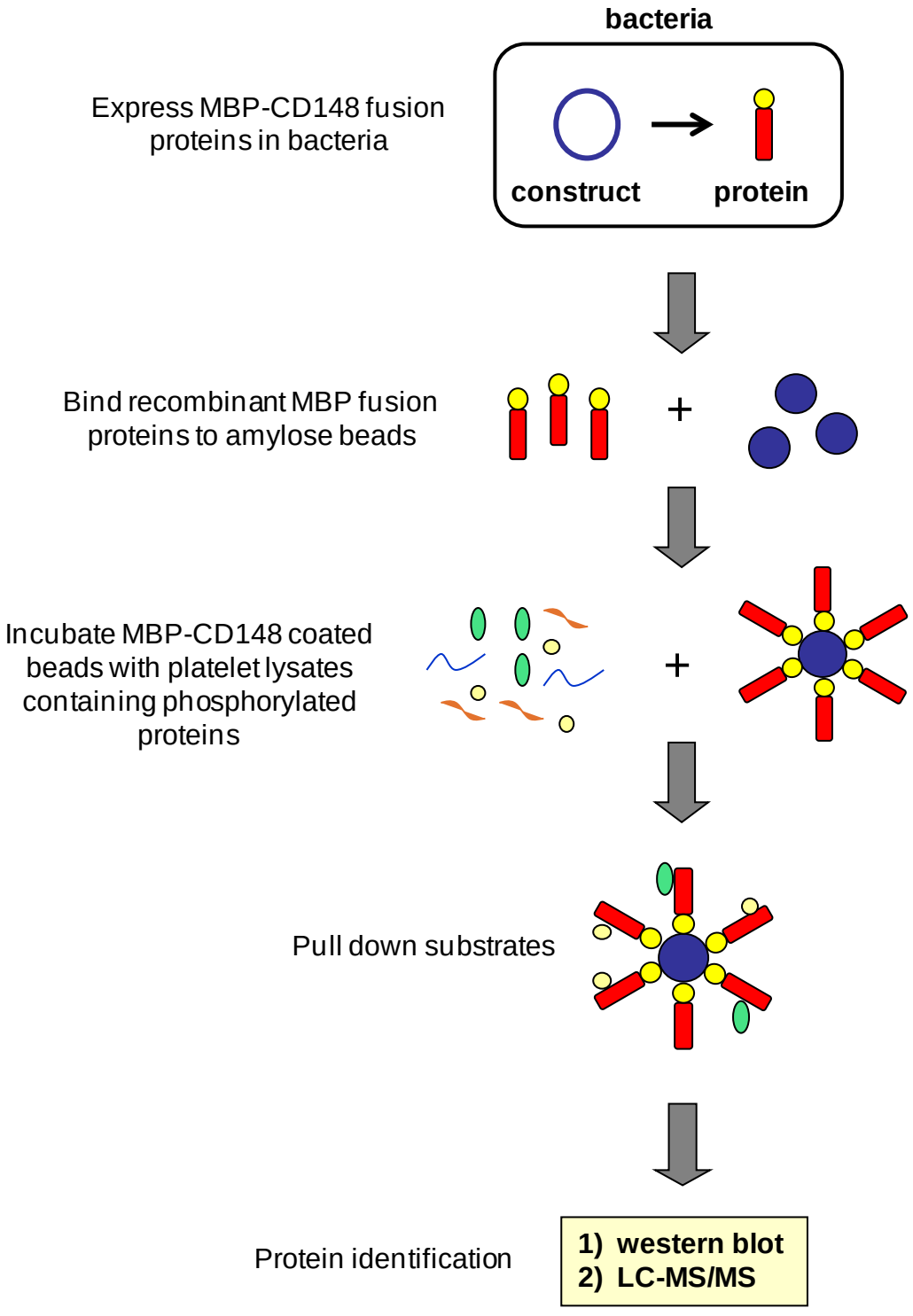


Figure 6.4: Flow diagram of substrate-trapping pulldown assay used to identify substrates and interacting proteins of CD148 in platelets.

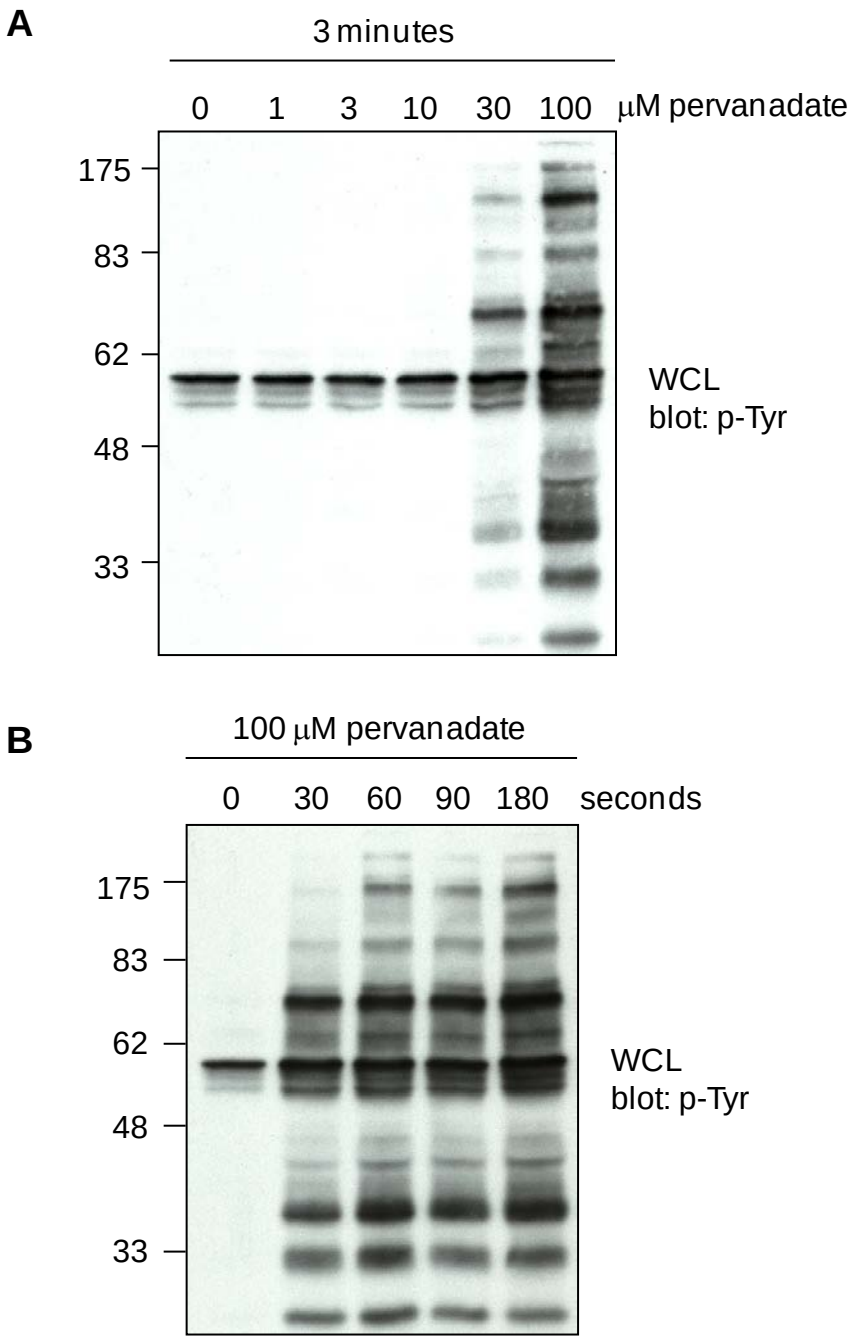


Figure 6.5. Pervanadate dose response and time course. (A) Washed human platelets (5×10^8/ml) were stimulated with either 1, 3, 10, 30 or 100 μM pervanadate for 3 minutes, or (B) 100 μM pervanadate for 0, 30, 60, 90 or 180 seconds. Platelets were subsequently lysed with 1% NP-40. Whole cell lysates (WCL) were resolved by SDS-PAGE and western blotted with an anti-phosphotyrosine antibody (p-Tyr). Blots are representative of 3 experiments.

6.3.3 SFKs interact with CD148 in platelets

An initial screen to identify interacting proteins of CD148 in platelets using the above assay was implemented. Recombinant bacterially expressed MBP-tagged CD148 fusion proteins were not purified from bacterial lysates prior to coupling to amylose-resin. Rather, an equal amount of amylose-resin was incubated with equal amounts of crude bacterial lysate expressing the different wild-type and mutant version of CD148 cytoplasmic region. Using this approach, Lyn and Src were found to preferentially interact with the substrate-trapping form of CD148 by western blotting, suggested that they may be substrates of CD148 (Figure 6.6). Interestingly, Csk interacted equally well with all three versions of the cytoplasmic region of CD148, suggesting that it may be constitutively associated with CD148 (Figure 6.6).

We next wanted to confirm these interactions using a cleaner, more well-defined assay. This was accomplished by purifying recombinant MBP-tagged fusion proteins from bacterial lysates by affinity chromatography, quantifying and coupling equal amounts of recombinant proteins to equal amounts of amylose-resin. Using this refined approach, we could now only detect interactions with phosphoproteins using the substrate-trapping recombinant protein (Figure 6.7). Prominent bands migrating at 90, 56, 43 and 40 kDa were specifically pulled down with MBP-CD148[D/A] (Figure 6.7). Several minor bands were also identified at 160, 130 and 100 kDa (Figure 6.7). Bands at 90 and 56 kDa co-migrated with MBP-CD148[D/A] and SFKs, respectively. Lyn, Fyn and Src were now only detected with the CD148 substrate-trapping mutant by western blotting (Figure 6.7). Re-blotting the membranes for MBP demonstrated equal amounts of each MBP-tagged recombinant protein in each lane (Figure 6.7). These results supported the hypothesis that Lyn, Fyn and Src are substrates of CD148.

Pulldown samples shown in Figure 6.7 were also immunoblotted for other signalling proteins that we hypothesized may be substrates of CD148. LAT, PLCγ2, as well as other

components of the GPVI signalling pathway, including Syk, SLP-76 and the FcR γ-chain were not detected, suggesting they are not substrates of CD148.

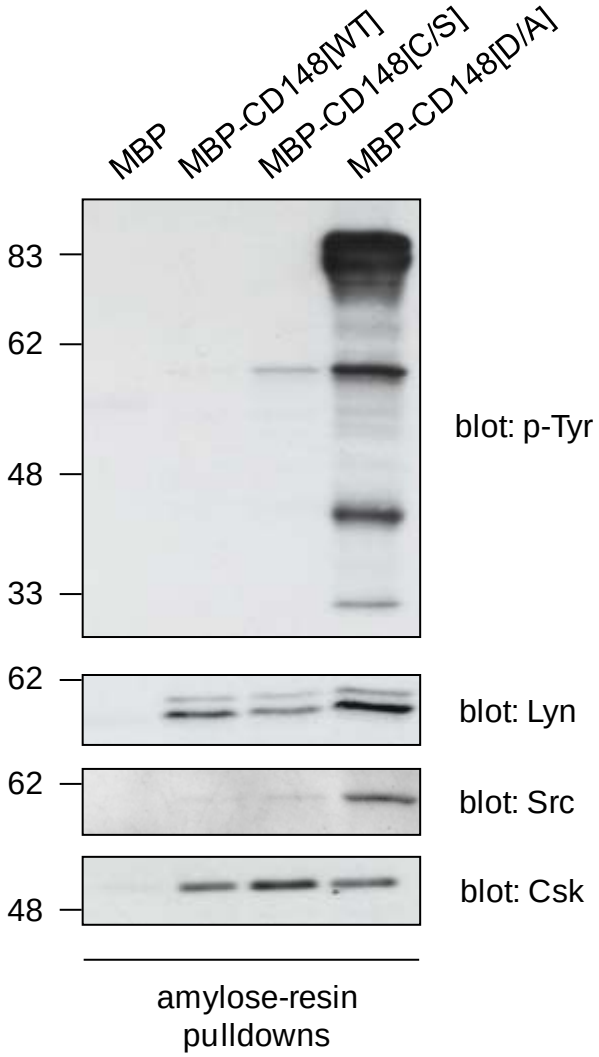


Figure 6.6. Crude screening approach reveals SFKs and Csk interactions with CD148 cytoplasmic region. Pulldowns were performed from pervandate stimulated platelets lysates (100 μM pervanadate for 3 minutes) using amylose-resin coated with equal amounts of crude lysates prepared of bacteria expressing MBP-tagged wild-type, catalytically inactive or substrate-trapping versions of the cytoplasmic region of CD148 (MBP-CD148[WT], MBP-CD148[C/S] or MBP-CD148[D/A], respectively). Non-specifically bound proteins were removed by washing with column buffer. Specifically bound proteins were eluted from resin and western blotted with: anti-phosphotyrosine (p-Tyr), anti-Lyn (Lyn), anti-Src (Src) and anti-Csk (Csk) antibodies. Blots are representative of 3 experiments.

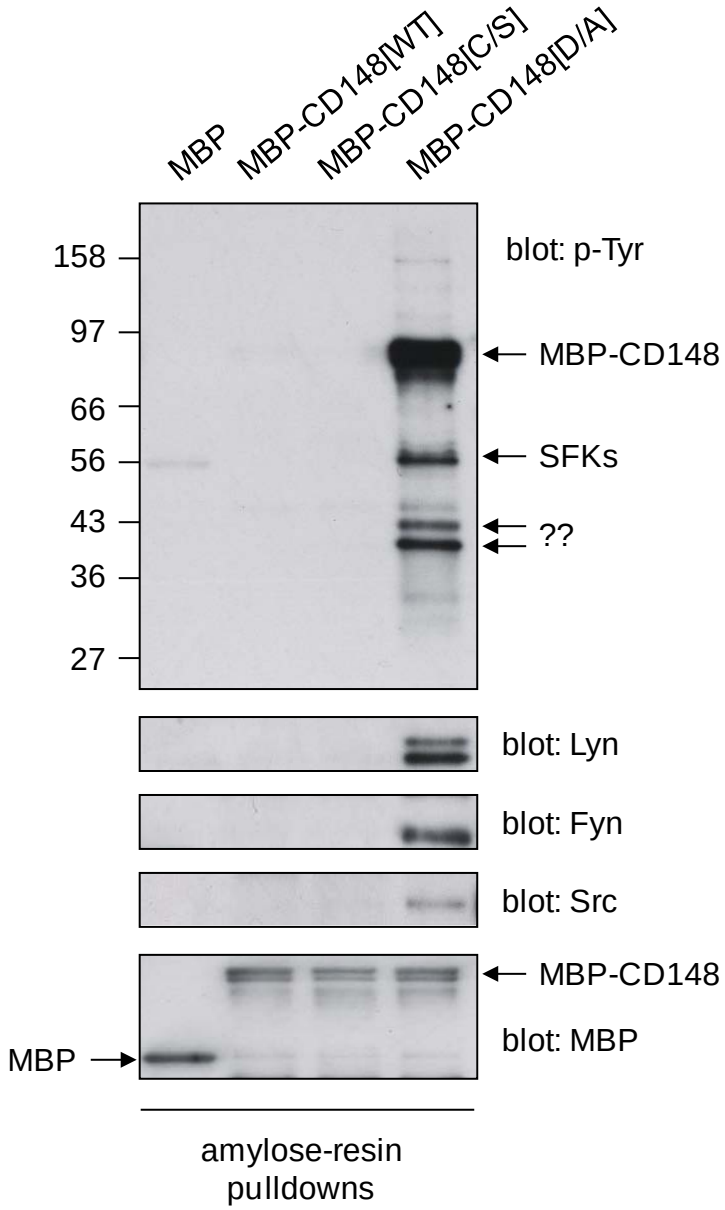


Figure 6.7. SFKs interact specifically with CD148 substrate-trapping mutant *in vitro*. Equal amounts of purified recombinant MBP-tagged fusion proteins of the cytoplasmic tail of wild-type, catalytically inactive and substrate-trapping forms of CD148 (MBP-CD148[WT], MBP-CD148[C/S] and MBP-CD148[D/A]) were coupled to amylose-resin and used to pulldown interacting proteins from pervanadate stimulated human platelet lysates. Specifically bound proteins were western blotted with: anti-phosphotyrosine (p-Tyr), anti-Lyn (Lyn), anti-Fyn (Fyn), anti-Src (Src) and anti-MBP (MBP) antibodies. Major phosphotyrosine bands were only observed with the MBP-CD148[D/A] substrate-trapping mutant. Prominent bands at 90 and 56 kDa likely correspond to MBP-CD148[D/A] and SFKs, respectively. The identity of the prominent doublet below 43 kDa is not known (?). Lyn, Fyn and Src were only pulled down with MBP-CD148[D/A]. Blots are representative of 5 experiments.

6.3.4 Src, Lyn and Fyn bind to the active site of CD148

A vanadate competition assay was used to confirm that Lyn, Fyn and Src interact with the catalytic site of CD148. Recombinant MBP-CD148[D/A] was treated with 2 mM vanadate before being incubated with pervanadate treated platelet lysate. Vanadate binds to the catalytic site of all PTPs and blocks any interactions with substrates. In the absence of vanadate the SFKs Lyn, Fyn and Src were pulled down from pervanadate-treated platelet lysates. However, in the presence of 2 mM vanadate, Src, Lyn and Fyn were not pulled down, demonstrating that these SFKs interact directly with the active site of CD148 and are likely substrates (Figure 6.8).

6.3.5 Recombinant CD148 dephosphorylates platelet-derived SFKs *in vitro*

I next investigated whether recombinant CD148 PTP domain could dephosphorylate Src, Lyn and Fyn *in vitro*. Lysates were prepared in the same way as for the substrate-trapping assay before being incubated with either purified recombinant MBP, MBP-CD148[WT] or catalytically inactive MBP-CD148[C/S]. Lysates were subsequently western blotted with phospho-specific antibodies that recognize either the activation loop tyrosine residues of all SFKs or the C-terminal inhibitory tyrosine residues of Src, Lyn and Fyn (Tyr-529, Lyn-507 and Fyn-530, respectively) (Figure 6.9). These findings demonstrate that CD148 can dephosphorylate both the activation loop tyrosines of SFKs and the inhibitory tyrosines of Lyn, Fyn and Src *in vitro*. Catalytically inactive CD148 did not dephosphorylate any of these sites. These findings suggest that CD148 preferentially dephosphorylates the activation loop tyrosine of SFKs and differentially dephosphorylates the C-terminal inhibitory tyrosines of SFKs, preferring the Lyn inhibitory tyrosine over those of Src and Fyn.

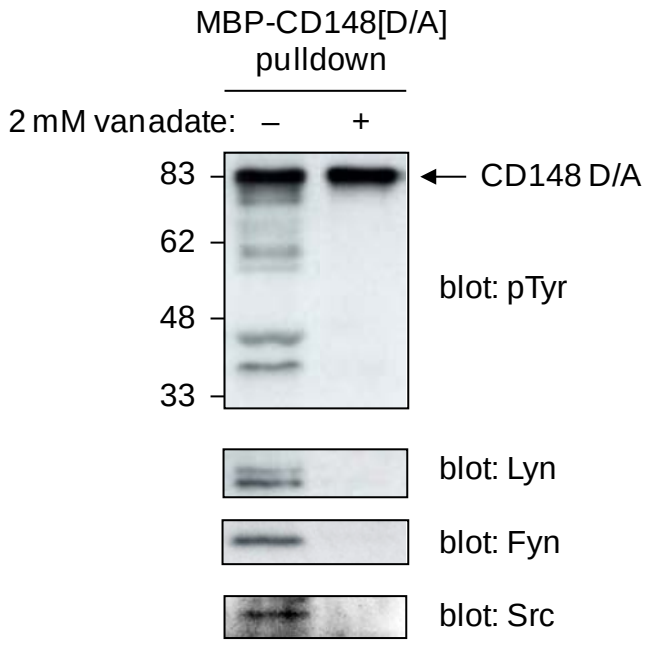


Figure 6.8: Vanadate out-competes interaction of CD148 substrate-trapping mutant with Lyn, Fyn and Src. Washed human platelets (5×10^8/ml) were treated with 100 μM pervanadate and lysed with 1% NP-40 lysis buffer. MBP-tagged CD148 substrate trapping fusion protein (MBP-CD148[D/A]) was pre-incubated with (+) or without (−) 2 mM vanadate prior to being incubated with the platelet lysates. Proteins were pulled down using amylose-resin and eluted off of the beads by boiling in 2× Laemmeli buffer. Samples were western blotted with either anti-phosphotyrosine antibody (p-Tyr), anti-Lyn (Lyn), anti-Fyn (Fyn) or anti-Src (Src) antibodies. Blots are representative of 3 experiments.

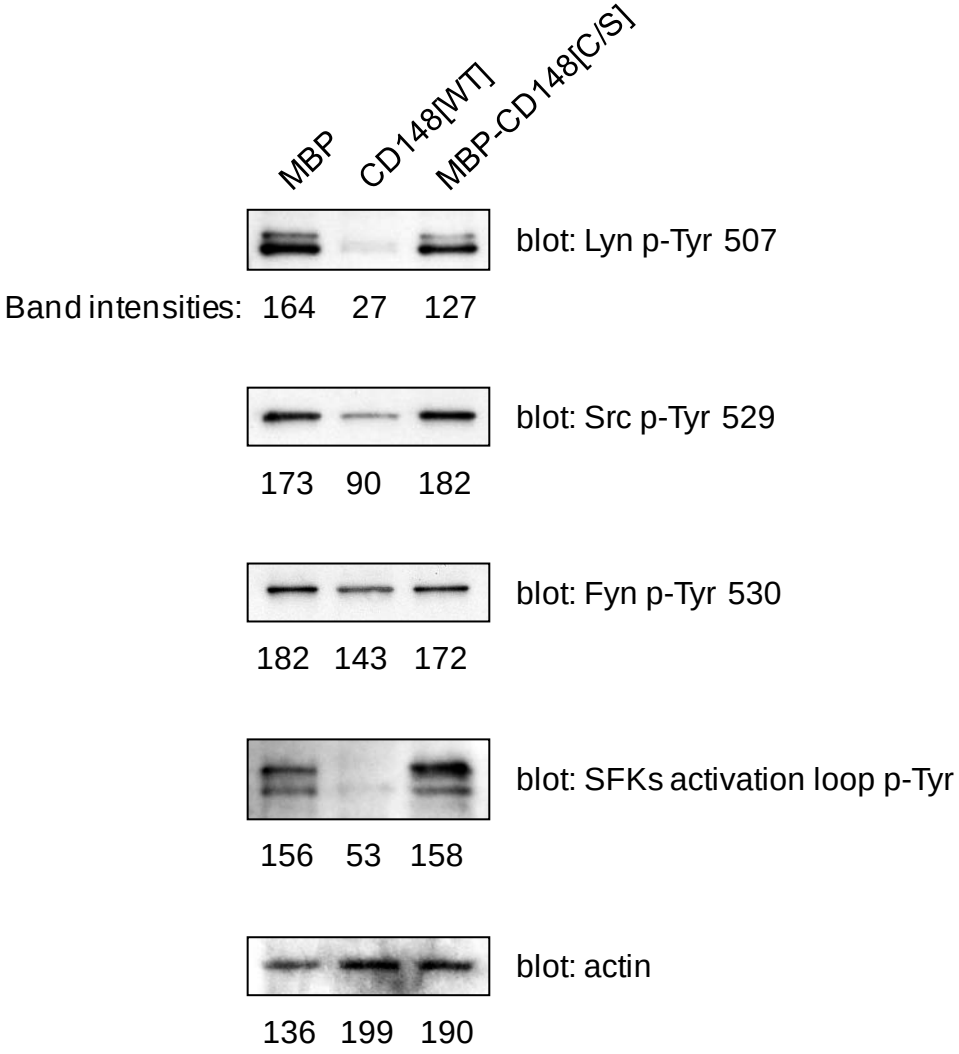


Figure 6.9. CD148 dephosphorylates both the activation loop and inhibitory site tyrosines of SFKs *in vitro*. Washed human platelets were treated with 100 μM pervanadate with stirring for 3 minutes prior to lysis. Lysates were incubated with MBP, wild-type CD148 PTP catalytic domain (CD148[WT]) or catalytically inactive MBP-CD148 (MBP-CD148[CS]) fusion proteins for 30 minutes at room temperature. Lysates were subsequently western blotted with phosphospecific antibodies that recognized either the Lyn inhibitory site tyrosine (Lyn p-Tyr 507), Src inhibitory site tyrosine (Src p-Tyr 529), Fyn inhibitory site tyrosine (Fyn p-Tyr 530) or the activation loop tyrosines of all SFKs (SFKs activation loop p-Tyr). Band intensities (arbitrary units) indicated below each panel were quantified using Adobe Photoshop cs version 8.0. Representative data from 2 experiments.

6.3.6 Kinetics of dehosphorylation of SFK-derived phopho-peptides

The kinetics of dephosphorylation of a panel of synthetic SFK-derived phospho-peptides by recombinant CD148 PTP domain was measured in real-time using the EnzCheck spetrophotometric assay. This work was done in collaboration with Dr. Alastair Barr (Structural Genomics Consortium Oxford). Phosphopeptides are shown in Table 2.5. The assay is based on a method originally described by Webb for measuring inorganic phosphate (Pi) released from enzymatic reactions (Webb 1992). In the presence of Pi, the substrate 2-amino-6-mercapto-7-methylpurine-riboside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. Enzymatic conversion of MESG results in a spectrophotometric shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product. Absorbances were measured continuously at 360 nm using a Spectramax plate reader and initial reaction rates were calculated over a 5 min period. Using this assay, recombinant CD148 PTP domain was found to dephosphorylate phosphopeptides corresponding the activation loop and inhibitory site of SFKs in the following order: Lyn activation loop = Src activation loop > Lyn inhibitory site > Src inhibitory site = Fyn inhibitory site (Figure 6.10A). These findings correlated with what was observed in the *in vitro* dephosphorylation assay (Figure 6.9).

PTP-1B catalytic domain was also tested in this assay as PTP-1B is highly expressed in platelets and has been shown to be an essential positive regulator of αIIbβ3-associated Src in platelets (Figure 6.10B). Interestingly, the PTP-1B catalytic domain had the opposite specificity to that of CD148, preferentially dephosphorylated SFK C-terminal inhibitory peptides rather than activation loop peptides (Figure 6Aii). These findings suggest that CD148 and PTP-1B differentially regulate SFK activity.

The calculated *Vmax* (absorbance [Abs] 360 nm/sec) of dephosphorylation of Lyn phospho-peptides by CD148 PTP domain was comparable for the activation loop and

inhibitory sites (5.6 ± 0.5 Abs 360 nm/sec versus 5.3 ± 0.7 Abs 360 nm/sec, respectively) (Figure 6.11). However, the *Km* for the Lyn activation loop phospho-peptide was half of that for the Lyn inhibitory site phospho-peptide (301 ± 56 μM versus 621 ± 144 μM, respectively), demonstrating a two-fold higher affinity for the activation loop phospho-peptide compared with the inhibitory site phospho-peptide (Figure 6.11).

Together these results suggest that CD148 has a marginal preference for dephosphorylating the activation loop of SFKs rather than the inhibitory site and that Lyn is a preferred substrate over Src and Fyn.

Table 6.1: Alignment of the activation loop and C-terminal inhibitory tyrosine motifs of SFKs

Subfamily	Src family kinase	Length (AA)	Predicted molecular weight (Da)	Activation loop tyrosine motif	C-terminal inhibitory tyrosine motif
Lyn	Lyn	512	58,574	ARVIEDNE**Y**$_{397}$TAREGAKFP	YTATEGQ**Y**$_{508}$QQQP
	Hck	526	59,600	ARVIEDNE**Y**$_{411}$TAREGAKFP	YTATESQ**Y**$_{522}$QQQP
	Lck	509	58,001	ARLIEDNE**Y**$_{394}$TAREGAKFP	FTATEGQ**Y**$_{505}$QPQP
	Blk	505	57,706	LARIIDSE**Y**$_{389}$TAQEGAKFP	YTATEGQ**Y**$_{501}$ELQP
Src	Src	536	59,835	ARLIEDNE**Y**$_{419}$TARQGAKFP	FTSTEPQ**Y**$_{530}$QPGENL
	Yes	543	60,801	ARLIEDNE**Y**$_{426}$TARQGAKFP	FTATEPQ**Y**$_{537}$QPGENL
	Fyn	537	60,762	ARLIEDNE**Y**$_{420}$TARQGAKFP	FTATEPQ**Y**$_{531}$QPGENL
	Fgr	529	59,479	ARLIKDDE**Y**$_{412}$NPCQGSKFP	FTSTEPQ**Y**$_{523}$QPGDQT
SFK-related	Brk	451	51,834	ARLIKEDV**Y**$_{342}$LSHDHNIPY	RLSSFTS**Y**$_{447}$ENPT
	Frk	505	58,254	FKVDNEDI**Y**$_{387}$ESRHEIKLP	YFETDSS**Y**$_{497}$SDANNFIR
	Srm	488	54,507	ARLLKDDI**Y**$_{380}$SPSSSSKIP	FATLREKLHAIHRCHP

Information obtained from Swiss-Prot database
Note: the Fyn activation loop sequence is the same as the Src activation loop sequence

Green high-lighted peptides: phosphopepetides used in *in vitro* phosphatase assay (Table 2.5).

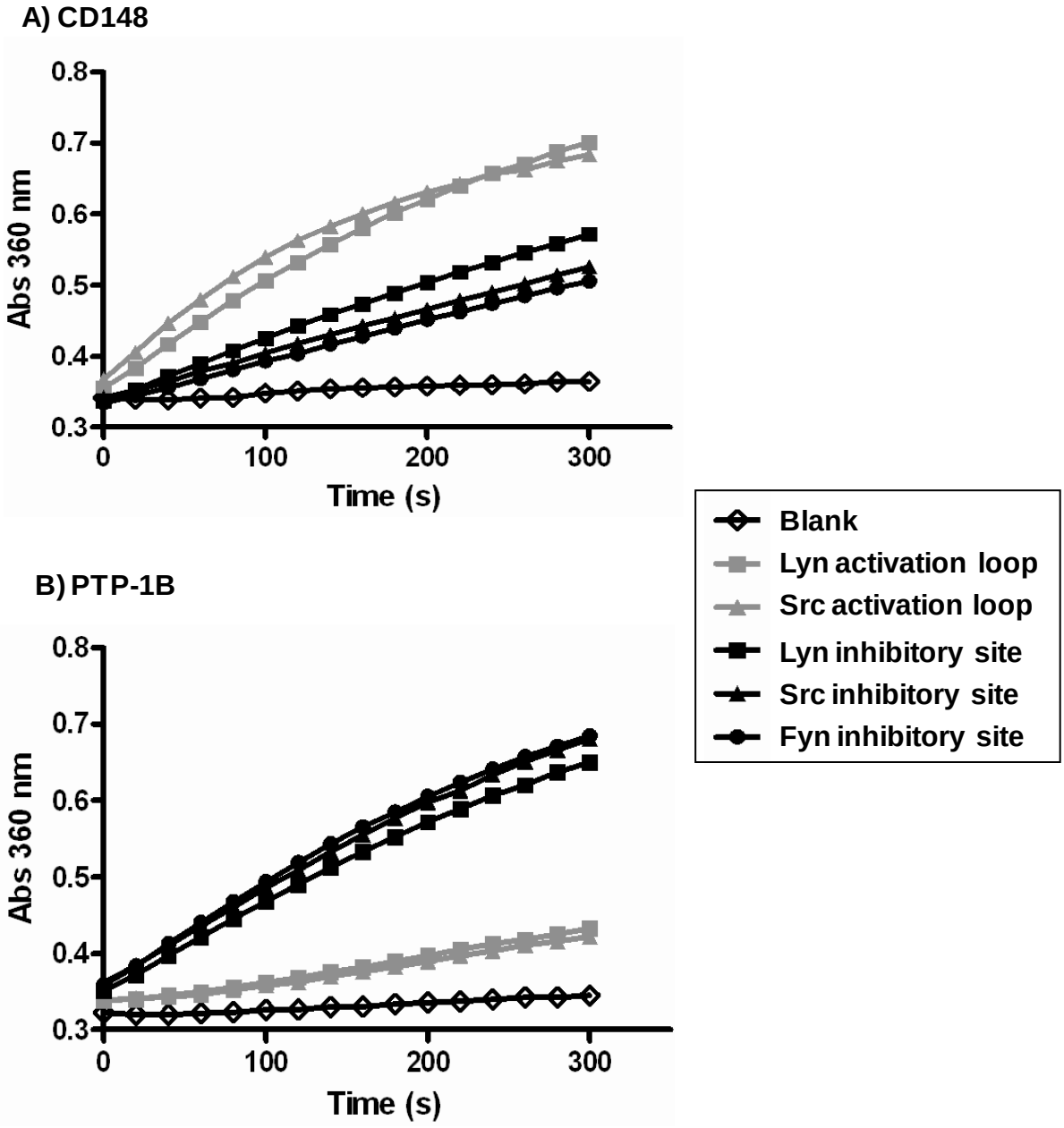
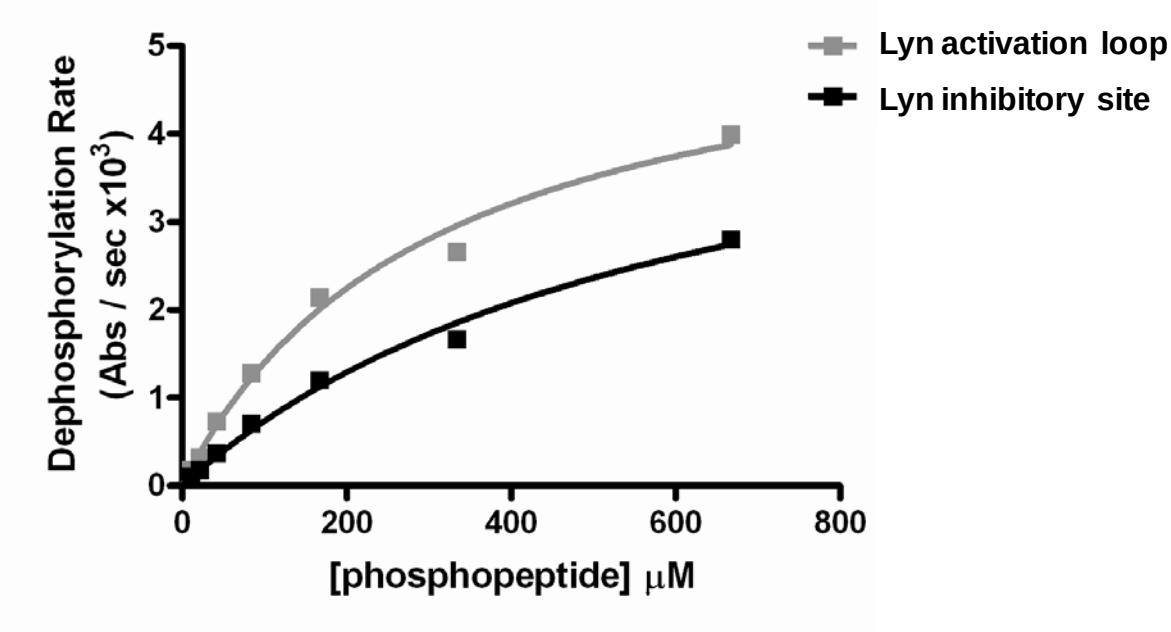


Figure 6.10. CD148 differentially dephosphorylates SFK phosphorylation sites *in vitro*. The kinetics of dephosphorylation of phosphopeptides, corresponding to the activation loop and inhibitory site of Src family kinases by recombinant CD148 and PTP-1B PTP domains, were measured using the EnzCheck spectrophotometric continuous assay. The reaction contained: 2-amino-6-mercapto-7-methylpurine-riboside (MESG), purine nucleoside phosphorylase (PNP), phosphopeptides and either: (A) recombinant CD148 PTP domain or (B) PTP-1B PTP domain. Inorganic phosphate released from the phosphopeptide was measured as an increase in absorbance at 360 nm using a Spectramax plate reader over 5 minutes. Data generated in collaboration with Dr.Alistair Barr, SGC, Oxford.



Enzyme	Phosphopeptide	*Km*	*Vmax*
CD148	Lyn activation loop	301±56	5.6±0.5
CD148	Lyn inhibitory site	621±144	5.3±0.7

Figure 6.11. CD148 PTP domain has a higher affinity for the Lyn activation loop phosphopeptide compared with the inhibitory site phosphopeptide *in vitro*. The initial rates of dephosphorylation of phosphopeptides corresponding to the activation loop and inhibitory site of Lyn were measured using the EnzCheck spectrophotometric continuous assay. The reaction contained: 2-amino-6-mercapto-7-methylpurine-riboside (MESG), purine nucleoside phosphorylase (PNP), SFK phosphopeptide and recombinant CD148 PTP domain. Inorganic phosphate released from the phosphopeptide was measured as an increase in absorbance at 360 nm using a Spectramax plate reader over 5 minutes. Calculated *Vmax* (Abs 360 nm/sec) and *Km* (μM) for each peptide are shown. Data generated in collaboration with Dr.Alistair Barr, SGC, Oxford.

6.3.7 Identification of CD148 substrates in platelets

I next wanted to identify other substrates of CD148 in platelets besides SFKs. The same substrate-trapping/pulldown assay described above was used for this purpose. Samples were resolved by SDS-PAGE and stained with colloidal Coomassie blue to visualize the proteins (Figure 6.12). Several proteins were pulled down specifically with the MBP-CD148(D/A) mutant, suggesting these may be substrates. The most prominent bands migrated at approximately 160, 90, 60 and 43 kDa (Figure 6.12). The diffuse band at 60 kDa may be SFKs. The 160, 90 and 43 kDa bands were excised and protein identification was attempted by MALDI-TOF. CD148 was identified in the 90 kDa band. This probably represents the fusion protein. Presumably other highly phosphorylated co-migrating substrates are also present in this region. Unfortunately, the 43 kDa protein could not be identified due to sample degradation.

The protein corresponding to the 160 kDa band was found to be non-muscle myosin heavy chain IIa (Swiss-Prot: P35579), also referred to a myosin heavy chain 9 or MYH9 protein (Figure 6.13). Twenty-nine unique peptides were identified, giving 22% coverage of the protein. There are two isoforms of non-muscle myosin heavy chain IIa. Isoform 1 is 1,960 amino acids and has a predicted molecular mass of 226,532 Da, and isoform 2 is 1,382 amino acids and has a predicted molecular weight of 159,864 Da. Non-muscle myosin heavy chain IIa was recently shown to be phosphorylated at Tyr-754 and Tyr-1408 (Rikova et al. 2007).

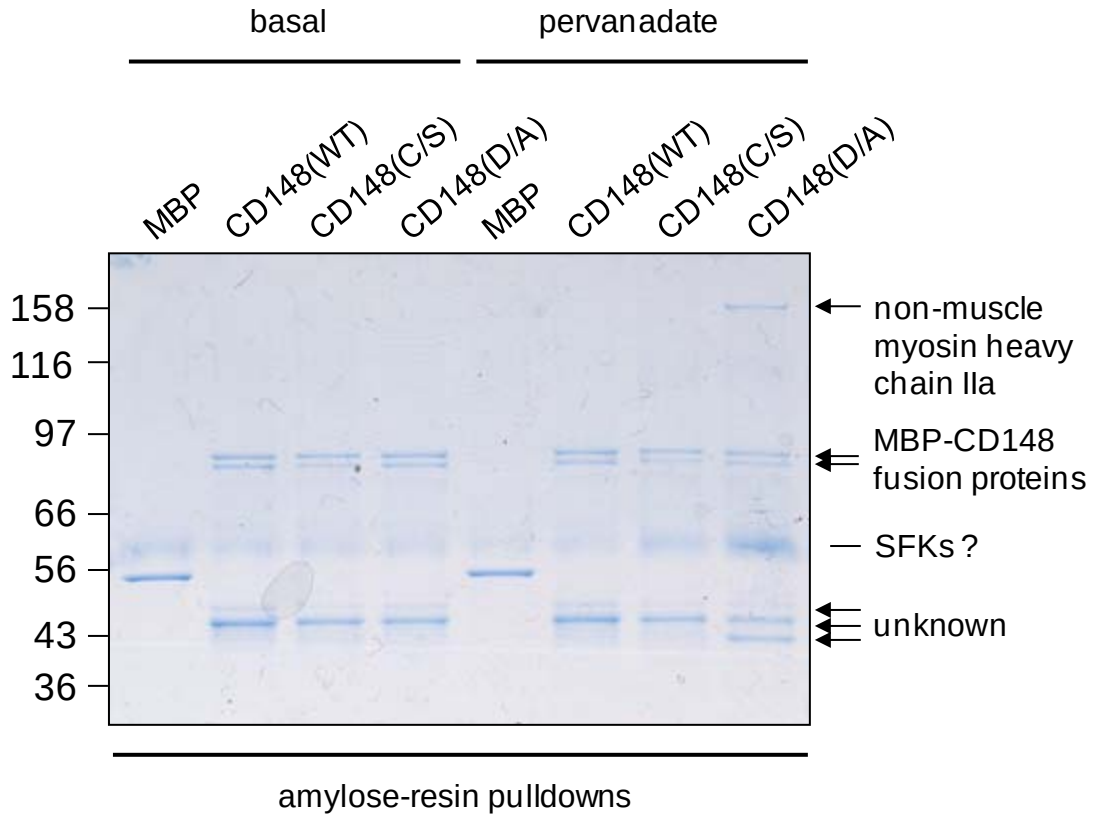


Figure 6.12: Nonmuscle myosin heavy chain IIa interacts with a substrate-trapping form of CD148 *in vitro*. Potential substrates of CD148 were pulled down from pervandate-stimulated platelet samples as in Figure 6.9. Samples were resolved by SDS-PAGE and proteins stained with Colloidal Coommassie blue. Bands were subsequently excised and proteins sent for analysis by electronstray ionization-liquid chromatography-tandem mass spectromety. Non-muscle myosin heavy chain IIa (Swiss-Prot no: P35579) was identified in the high molecular weight band, indicated by the arrow. CD148 was identified in the doublet where the MBP-CD148 fusion protein migrates. Proteins have yet to be identified in the other bands. n=2.

```
   1 AQQAADKYLY VDKNFINNPL AQADWAAKKL VWVPSDKSGF EPASLKEEVG
  51 EEAIVELVEN GKKVKVNKDD IQKMNPPKFS KVEDMAELTC LNEASVLHNL
 101 KERYYSGLIY TYSGLFCVVI NPYKNLPIYS EEIVEMYKGK KRHEMPPHIY
 151 AITDTAYRSM MQDREDQSIL CTGESGAGKT ENTKKVIQYL AYVASSHKSK
 201 KDQGELERQL LQANPILEAF GNAKTVKNDN SSRFGKFIRI NFDVNGYIVG
 251 ANIETYLLEK SRAIRQAKEE RTFHIFYYLL SGAGEHLKTD LLLEPYNKYR
 301 FLSNGHVTIP GQQDKDMFQE TMEAMRIMGI PEEEQMGLLR VISGVLQLGN
 351 IVFKKERNTD QASMPDNTAA QKVSHLLGIN VTDFTRGILT PRIKVGRDYV
 401 QKAQTKEQAD FAIEALAKAT YERMFRWLVL RINKALDKTK RQGASFIGIL
 451 DIAGFEIFDL NSFEQLCINY TNEKLQQLFN HTMFILEQEE YQREGIEWNF
 501 IDFGLDLQPC IDLIEKPAGP PGILALLDEE CWFPKATDKS FVEKVMQEQG
 551 THPKFQKPKQ LKDKADFCII HYAGKVDYKA DEWLMKNMDP LNDNIATLLH
 601 QSSDKFVSEL WKDVDRIIGL DQVAGMSETA LPGAFKTRKG MFRTVGQLYK
 651 EQLAKLMATL RNTNPNFVRC IIPNHEKKAG KLDPHLVLDQ LRCNGVLEGI
 701 RICRQGFPNR VVFQEFRQRY EILTPNSIPK GFMDGKQACV LMIKALELDS
 751 NLYRIGQSKV FFRAGVLAHL EEERDLKITD VIIGFQACCR GYLARKAFAK
 801 RQQQLTAMKV LQRNCAAYLK LRNWQWWRLF TKVKPLLQVS RQEEEMMAKE
 851 EELVKVREKQ LAAENRLTEM ETLQSQLMAE KLQLQEQLQA ETELCAEAEE
 901 LRARLTAKKQ ELEEICHDLE ARVEEEEERC QHLQAEKKKM QQNIQELEEQ
 951 LEEEESARQK LQLEKVTTEA KLKKLEEEQI ILEDQNCKLA KEKKLLEDRI
1001 AEFTTNLTEE EEKSKSLAKL KNKHEAMITD LEERLRREEK QRQELEKTRR
1051 KLEGDSTDLS DQIAELQAQI AELKMQLAKK EEELQAALAR VEEEAAQKNM
1101 ALKKIRELES QISELQEDLE SERASRNKAE KQKRDLGEEL EALKTELEDT
1151 LDSTAAQQEL RSKREQEVNI LKKTLEEEAK THEAQIQEMR QKHSQAVEEL
1201 AEQLEQTKRV KANLEKAKQT LENERGELAN EVKVLLQGKG DSEHKRKKVE
1251 AQLQELQVKF NEGERVRTEL ADKVTKLQVE LDNVTGLLSQ SDSKSSKLTK
1301 DFSALESQLQ DTQELLQEEN RQKLSLSTKL KQVEDEKNSF REQLEEEEEA
1351 KHNLEKQIAT LHAQVADMKK KMEDSVGCLE TAEEVKRKLQ KDLEGLSQRH
1401 EEKVAAYDKL EKTKTRLQQE LDDLLVDLDH QRQSACNLEK KQKKFDQLLA
1451 EEKTISAKYA EERDRAEAEA REKETKALSL ARALEEAMEQ KAELERLNKQ
1501 FRTEMEDLMS SKDDVGKSVH ELEKSKRALE QQVEEMKTQL EELEDELQAT
1551 EDAKLRLEVN LQAMKAQFER DLQGRDEQSE EKKKQLVRQV REMEAELEDE
1601 RKQRSMAVAA RKKLEMDLKD LEAHIDSANK NRDEAIKQLR KLQAQMKDCM
1651 RELDDTRASR EEILAQAKEN EKKLKSMEAE MIQLQEELAA AERAKRQAQQ
1701 ERDELADEIA NSSGKGALAL EEKRRLEARI AQLEEELEEE QGNTELINDR
1751 LKKANLQIDQ INTDLNLERS HAQKNENARQ QLERQNKELK VKLQEMEGTV
1801 KSKYKASITA LEAKIAQLEE QLDNETKERQ AACKQVRRTE KKLKDVLLQV
1851 DDERRNAEQY KDQADKASTR LKQLKRQLEE AEEEAQRANA SRRKLQRELE
1901 DATETADAMN REVSSLKNKL RRGDLPFVVP RRMARKGAGD GSDEEVDGKA
1951 DGAEAKPAE
```

Figure 6.13. Tryptic fragments of non-muscle myosin heavy chain IIa identified by mass spectrometry. The entire amino acid sequence of non-muscle myosin heavy chain IIa (Swiss-Prot no: P35579) is shown. Tryptic fragments identified by mass spectrometry are highlighted in bold and red. Tyrosine phosphorylation sites at positions Tyr-754 and Tyr-1,408 are highlighted in bold, blue and underlined.

6.4 Discussion

The main findings of this chapter were: (1) CD148 positively regulates GPVI signalling in DT40 cells; (2) SFKs are direct substrates of CD148; (3) CD148 differentially dephosphorylates the activation loop and inhibitory tyrosine residues of SFKs; and (4) Non-muscle myosin heavy chain IIa may be a novel substrate of CD148 in platelets. These findings provide additional information of the molecular mechanism of how CD148 regulates GPVI-mediated platelet activation.

Here I build on data presented in the previous chapter that CD148 is a positive regulator of GPVI signalling, by demonstrating that CD148 positively regulates GPVI signalling in transiently transfected DT40 cells. I show that CD148 up-regulates basal and collagen-induced GPVI signalling by approximately 2 – 3 fold. I also demonstrate that CD148 catalytic activity is essential for mediating this effect. These findings also establish this DT40 NFAT-luciferase report assay as a good tool for studying the role of CD148 in GPVI signalling. The DT40 chicken B cell line has been used extensively for studying B cell receptor signalling (Yasuda et al. 2004). There a large panel of mutant DT40 cells that have been generated which lack expression of specific signalling molecules, including Shp1, Shp2, SHIP-1 and Dok-3, making it possible to investigate the importance of particular molecules downstream of specific receptors (Ono et al. 1997; Maeda et al. 1998; Stork et al. 2007). It can also be used for structure function analysis of CD148.

In this chapter I demonstrate that Lyn, Fyn and Src interact directly with the active site of CD148 and undergo dephosphorylation by recombinant CD148 *in vitro*, providing further evidence that these SFKs are physiological substrates of CD148 in platelets. I also show that recombinant CD148 is able to dephosphorylate both the inhibitory and activation loop tyrosines of Lyn, Fyn and Src. Furthermore, our data indicates that CD148: (1) has a preference for dephosphorylation of the activation loop tyrosine over the inhibitory site *in*

vitro, and (2) favours dephosphorylation of Lyn over Fyn and Src. In a recent study of T cell receptor signalling, the RPTP, CD45, was proposed to act as a rheostat, modulating the sensitivity of cells to TCR signals by differentially regulating the two critical Lck inhibitory and activation loop tyrosine phosphorylation sites (McNeill et al. 2007). We propose a similar mechanism may exist for the regulation of SFKs by CD148 in platelets, whereby CD148 firstly triggers SFK activation by dephosphorylation of the inhibitory tyrosine, following GPVI or αIIbβ3 stimulation, and then prevents prolonged signalling by dephosphorylation of the activation loop tyrosine.

Several phosphoproteins besides SFKs were specifically pulled down with the substrate trapping mutant form of CD148. Their sizes ranged from 160 – 43 kDa. Attempts to identify these proteins by western blotting were not successful. Based on the literature and sizes of the bands we immunblotted for PLCγ2, Syk, Csk, LAT, SLP-76 and the FcR γ-chain. Csk appeared to be constitutively associated with CD148 cytoplasmic tail in a crude version of the pull down assay. However, subsequent attempts to pull down Csk from pervanadate treated platelets using purified recombinant CD148 substrate trapping mutant were unsuccessful. Attempts to co-immunprecipitated Csk with anti-CD148 antibody from resting and CRP-activated platelets were inconclusive. These negative results may be due to the lack of an interaction or sub-optimal conditions for the interaction to occur. Interestingly, Csk has been previously shown to interact with the non-transmembrane PTP Lyp using a yeast 2-hybrid system (Cloutier et al. 1996). The same group also demonstrated that the association was highly specific and speculated that Lyp may be an effector and/or regulator of Csk in T cells and other hematopoietic cells.

Non-muscle myosin heavy chain IIa isoform 2 was identified as a potential novel substrate of CD148 in platelets using an *in vitro* substrate-trapping/pulldown assay and mass spectrometry. Human platelets exclusively express myosin IIa, which is an important

component of the intracellular cytoskeleton (Maupin et al. 1994; Sellers 2000). In resting platelets, the cytoskeleton is mainly comprised of polymerized actin and actin cross-linking proteins. Upon stimulation with a variety of agonists, actin undergoes further polymerization, and interacts with myosin and other contractile proteins to promote platelet shape change and granule secretion (Blockmans et al. 1995). Outside-in integrin αIIbβ3 signalling promotes the association of the actin-myosin filaments with membrane glycoproteins, including αIIbβ3 itself, and other signal transduction components such as tyrosine kinases, lipid metabolizing enzymes, and small GTP-binding proteins (Grondin et al. 1991; Jackson et al. 1994; Torti et al. 1999). Tyrosine phosphorylation of myosin heavy chain IIa has been implicated as an early event in human platelet activation following thrombin stimulation (Harney et al. 2003). Interestingly, Tyr-1408 is within a consensus immunoreceptor tyrosine-based inhibitory motif (ITIM), which has been shown to bind Shp1 (Baba et al. 2003). Mutations in the gene encoding non-muscle heavy chaing IIa, *MYH9*, cause May-Hegglin anomaly, characterized by thrombocytopenia, giant platelets and leukocyte inclusions (Kelley et al. 2000; Seri et al. 2000; Heath et al. 2001). The interaction between CD148 and mysosin IIa may provide further insight into how CD148 regulates platelet function. However, further work is required to validate the interaction.

Unfortunately, the identities of the phosphoproteins comprising the most prominent bands (90 and 40 kDa doublet) eluded identification. The broad band at 90 kDa appears to be composed of multiple overlapping bands representing several highly phosphorylated proteins that maybe substrates of CD148. Two attempts at identifying the constituents of this broad band by mass spectrometry only revealed CD148, likely representing the MBP-CD148 substrate trapping fusion protein, which co-migrates at this size. This finding does however raise the possibility that CD148 dephosphorylates itself. Another explanation is that it is hyperphosphorylated CD148 trapping mutant. In previous chapters I demonstrated that SFKs

phosphorylate CD148. Perhaps the CD148 trapping mutant is getting hyperphosphorylated by trapped SFKs?

CHAPTER 7 –

REGULATION OF GPVI AND INTEGRIN αIIbβ3 SIGNALLING BY PTP-1B AND TC-PTP

7.1 Aims

The non-transmembrane PTP, PTP-1B, has previously been shown to be an essential positive regulator of outside-in integrin αIIbβ3 signalling in platelets (Arias-Salgado et al. 2005). It does so by dephosphorylating the C-terminal inhibitory tyrosine of αIIbβ3-associated Src, at position Tyr-529, activating the kinase, initiating integrin signalling (Arias-Salgado et al. 2003). However, its role in GPVI signalling has not been thoroughly investigated. In Chapters 5 and 6 I demonstrated that the RPTP, CD148 is a global regulator of SFK activity in platelets and that it positively regulates both GPVI and αIIbβ3 signalling in mouse platelets through a mechanism that involves activating SFKs by dephosphorylating the C-terminal inhibitory tyrosine of SFKs. Therefore, CD148 and PTP-1B positively regulate SFK activity by an apparently similar mechanism. The main aim of this chapter was to investigate the role of PTP-1B in GPVI and integrin αIIbβ3 signalling and to start to address the question of possible functional redundancy between CD148 and PTP-1B in platelets. Since I found that PTP-1B-deficient platelets had minor functional and phosphorylation defects that were influenced by strain variation, we asked the question whether the structurally related NTPTP, TC-PTP, was playing a similar function in platelets and could possibly compensate in its absence (Figure 1.11). I checked for expression of TC-PTP in human and mouse platelets and tested platelets from TC-PTP-deficient mice for functional defects.

7.2 Introduction

PTP-1B is a ubiquitously expressed non-transmembrane PTP that is localized to the outer surface of the endoplasmic reticulum (Frangioni et al. 1992). Being the prototypic PTP, PTP-1B has been extensively studied and is best known for its role in insulin signalling (Elchebly et al. 1999). PTP-1B has been implicated as a negative regulator of insulin action and as an important mediator in the pathogenesis of insulin-resistance and non-insulin dependent diabetes mellitus (Byon et al. 1998; Shi et al. 2004; Bodula et al. 2005). Other roles for PTP-1B include down regulation of cytokine receptor signalling and as a negative regulator of macrophage development through CSF-1 signalling (Cheng et al. 2002; Gu et al. 2003; Heinonen et al. 2006).

In platelets, PTP-1B has been shown to get cleaved from the ER surface by the calcium-dependent protease calpain, following agonist-induced platelet activation (Frangioni et al. 1993; Ragab et al. 2003; Kuchay et al. 2007). This results in translocation of PTP-1B from the ER membrane to the cytosol. Recent work by Arias-Salgado et. al. demonstrated that fibrinogen-binding to αIIbβ3 triggers PTP-1B recruitment to the αIIbβ3-Src-Csk complex. Platelets lacking PTP-1B expression exhibited reduced integrin αIIbβ3-mediated signalling (Arias-Salgado et al. 2003; Arias-Salgado et al. 2005). The molecular basis of this defect was shown to be a lack of dissociation of Csk from the αIIbβ3-Src-Csk complex, and concomitant hyper-phosphorylation and inactivation of Src at its inhibitory tyrosine (Arias-Salgado et al. 2005). The functional consequences included reduced spreading on fibrinogen, clot retraction and calcium signalling. PTP-1B-deficient mice also exhibited reduced thrombus formation *in vivo*. Interestingly, GPVI signalling was reported to be normal in PTP-1B-deficient platelets. This was somewhat surprising, as the SFKs Fyn and Lyn, are essential for initiating GPVI signalling. This suggests that PTP-1B specifically regulates Src in platelets, perhaps through enzyme specificity or compartmentalization. However, one

limitation of the GPVI signalling data in the Arias-Salgado et. al. study was that GPVI signalling was being initiated by a high concentration of the snake toxin convulxin, which is not GPVI-specific, but also signals through the receptor for VWF, GPIb-IX-V (Andrews et al. 2000; Andrews et al. 2003).

Two PTP-1B knockout (KO) mouse models have been generated by two separate groups in order to determine the physiological function of PTP-1B. One was generated in the Tremblay laboratory and the other in the Neel laboratory by homologous recombination using slightly different strategies (Elchebly et al. 1999; Klaman et al. 2000). The PTP-1B KO mouse model generated in the Tremblay laboratory was made by deleting a portion of the gene that includes exon 5 and the tyrosine phosphatase active site in exon 6 (Elchebly et al. 1999). This mouse model (PTP-1B Ex5/6 KO) was bred onto a Balb/c background. The PTP-1B KO mouse model generated in the Neel laboratory was made by deleting exon 1 of the gene. This mouse model (PTP-1B Ex1 KO) was bred onto a mixed C57BL/6 × 129SvJ background. Both knockout strategies generated mice that lacked any expression of PTP-1B protein. Despite the strain variations, both knockout mouse models exhibited similar phenotypes, including: resistance to weight gain, insulin sensitivity and glucose tolerance (Elchebly et al. 1999; Klaman et al. 2000). Both models exhibited increased phosphorylation of the insulin receptor and the adaptor protein IRS-1, demonstrating that PTP-1B negatively regulates insulin receptor signalling. They also exhibited enhanced basal metabolic rate and total energy expenditure (Klaman et al. 2000).

The PTP-1B mouse model used by Arias-Salgado et. al. to show that PTP-1B is an essential positive regulator of platelet integrin αIIbβ3 signalling was the PTP-1B Ex1 KO. Whereas the PTP-1B Ex5/6 KO backcrossed onto a C57BL/6 background were used to generate compound PTP-1B/calpain-1 double-deficient mice and demonstrate that PTP-1B is a physiological substrate of calpain in platelets (Kuchay et al. 2007). PTP-1B activity was

significantly increased in calpain-1-deficient mouse platelets and correlated with reduced tyrosine phosphorylation of platelet proteins, platelet aggregation, and impaired clot retraction (Kuchay et al. 2007). Interestingly, these defects were rescued in the PTP-1B/calpain-1 double knockout mice (Kuchay et al. 2007).

TC-PTP belongs to the same subfamily as PTP-1B. It is ubiquitously expressed, with high levels in hematopoietic cells, however, it has not previously been shown to be expressed in platelets. Although structurally similar, PTP-1B and TC-PTP are not functionally redundant, regulating both common and distinct signalling pathways. A common pathway both proteins are involved in regulating is the insulin signalling pathway. TC-PTP has been mainly implicated in negatively regulating the Jak/STAT signalling pathway downstream of cytokine and growth factor receptors (Simoncic et al. 2002; Shields et al. 2008). TC-PTP-deficient mice are of normal size and health following birth, however, after approximately 2 weeks begin to display signs of runting, and by 3 to 5 weeks of age exhibit closed eyes, hunching, decreased mobility and diarrhoea followed by death (You-ten et al. 1997). Low hematocrit levels in these mice indicate they also suffer from severe anaemia contributing to their early death (You-Ten et al. 1997).

In this chapter, I test two hypotheses through the use of mouse models: (1) PTP-1B regulates GPVI and integrin αIIbβ3 signalling in platelets; and (2) TC-PTP regulates GPVI signalling in platelets. Findings presented in this chapter demonstrate that PTP-1B plays a minor role in GPVI and integrin αIIbβ3 signalling, that is influenced by strain variation or modifier loci. This demonstrates that CD148 and PTP-1B have fundamentally different functions in platelets. Finally, I show that TC-PTP is expressed in human and mouse platelets and that it plays a role in platelet development.

7.3 Results

7.3.1 PTP-1B

7.3.1.1 PTP-1B-deficient platelets on a Balb/c background exhibit minor aggregation and secretion defects to low concentration CRP

It has previously been reported that PTP-1B is a selective positive regulator of αIIbβ3-associated Src kinase, with no role downstream of the collagen activation receptor GPVI (Arias-Salgado et al. 2005). However, we question this conclusion as it was based on work done using a high concentration of the snake toxin convulxin, which is a non-specific GPVI agonist (Andrews et al. 2000). To test our hypothesis that PTP-1B positively regulates GPVI signalling, we measured the ability of platelets from PTP-1B Ex5/6 KO mice on a Balb/c background to aggregate to the GPVI-specific agonist, CRP. PTP-1B-deficient platelets were stimulated with either low (0.3 μg/ml) or intermediate (1 μg/ml) concentrations of CRP and aggregation was measured as a change in light transmission using an optical aggregometer. Platelets from PTP-1B-deficient mice did not aggregate in response to low concentration CRP (Figure 7.1Ai). This block in aggregation was partly overcome at a higher concentration of CRP (1 μg/ml) (Figure 7.1Aii). PTP-1B-deficient platelets responded normally to an intermediate concentration of thrombin (0.06 U/ml), demonstrating that this was a GPVI-specific aggregation defect (Figure 7.1B). This was the first evidence that that PTP-1B has a positive regulatory role downstream of GPVI signalling.

7.3.1.2 PTP-1B-deficient platelets on a Balb/c background have impaired P-selectin expression and αIIbβ3 activation in response to CRP

Flow cytometry studies were carried out to investigate the role of PTP-1B in α-granule secretion and integrin αIIbβ3 activation. P-selectin expression on the surface of platelets was used as a measure of α-granule secretion and inside-out activation of αIIbβ3 was detected

using JON/A antibody binding, which only recognizes the active, high-affinity conformation of the integrin. Platelets were stimulated with either 10 μg/ml CRP or 1 U/ml thrombin for 10 minutes then stained with either FITC-conjugated anti-P-selectin or JON/A antibodies. Samples were subsequently analyzed by flow cytometry. CRP-mediated P-selectin expression and integrin αIIbβ3 activation were reduced in PTP-1B-deficient platelets compared with wild-type (WT) control platelets (Figure 7.2A and B), whereas thrombin-mediated responses were normal (Figure 7.2C and D). These findings provided further evidence of a positive regulatory role of PTP-1B downstream of GPVI.

Surface expression of GPVI and αIIbβ3 in resting PTP-1B deficient and WT platelets were also analysed by flow cytometry. GPVI levels were reduced by approximately 33% (Figure 7.3A and B), whereas integrin αIIbβ3 levels were normal (Figure 7.3C and D). This reduction in GPVI expression may partially explain the functional defects, as platelets expressing 50% of GPVI levels were previously reported to exhibit a two-fold shift in the dose response curve in aggregation to collagen, convulxin and the anti-GPVI antibody JAQ1 (Snell et al. 2002).

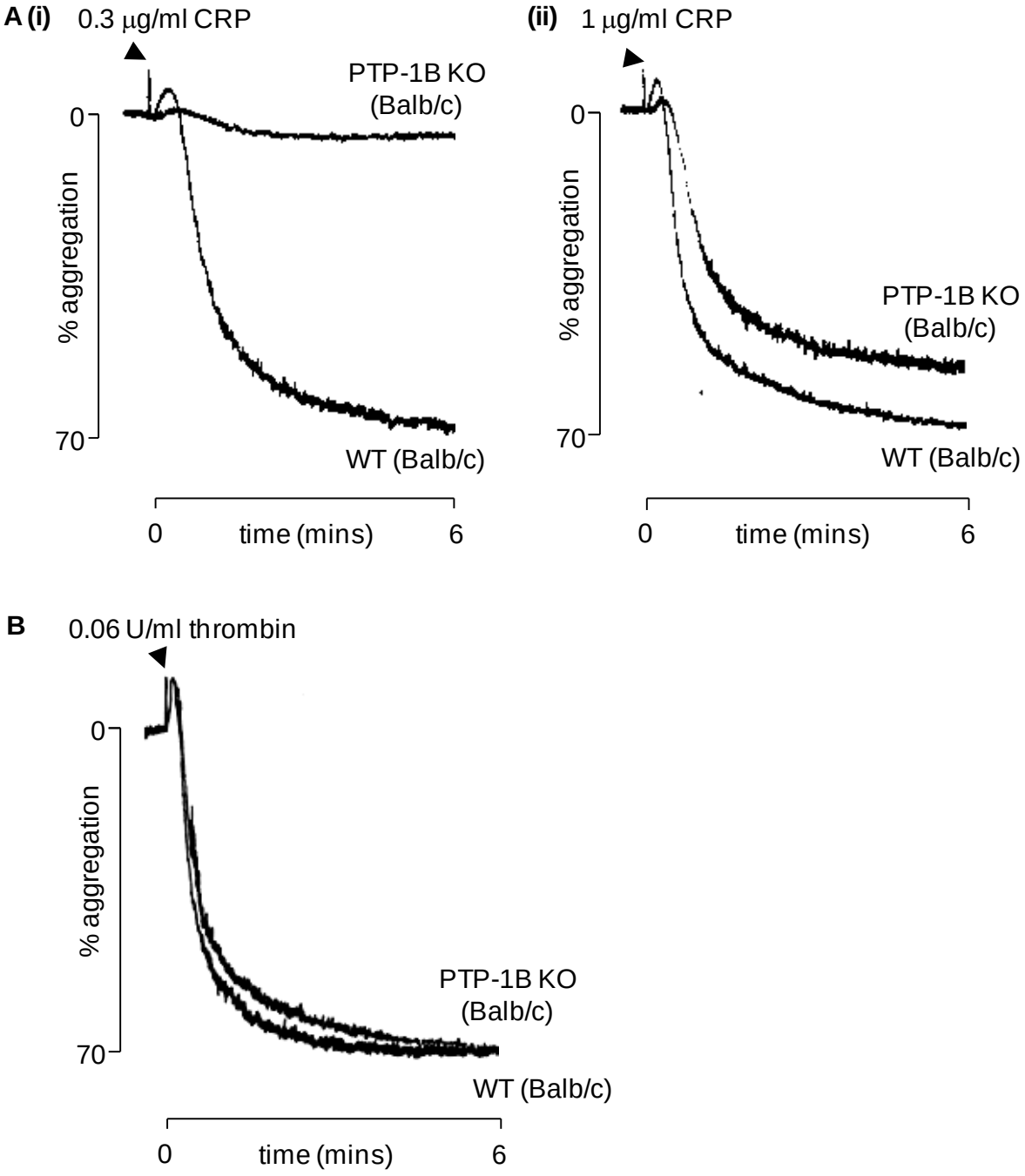


Figure 7.1: Platelets from PTP-1B-deficient mice (Balb/c background) exhibit impaired GPVI-mediated platelet aggregation. Washed platelets (2×10^8/ml) prepared from litter-matched wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a Balb/c background were stimulated with either (Ai) 0.3 μg/ml CRP, (Aii) 1 μg/ml CRP or (B) 0.06 U/ml thrombin. Platelet aggregation was measured as a change in light transmission using a lumi-aggregometer. Representative images are shown (n = 2-3 mice per condition).

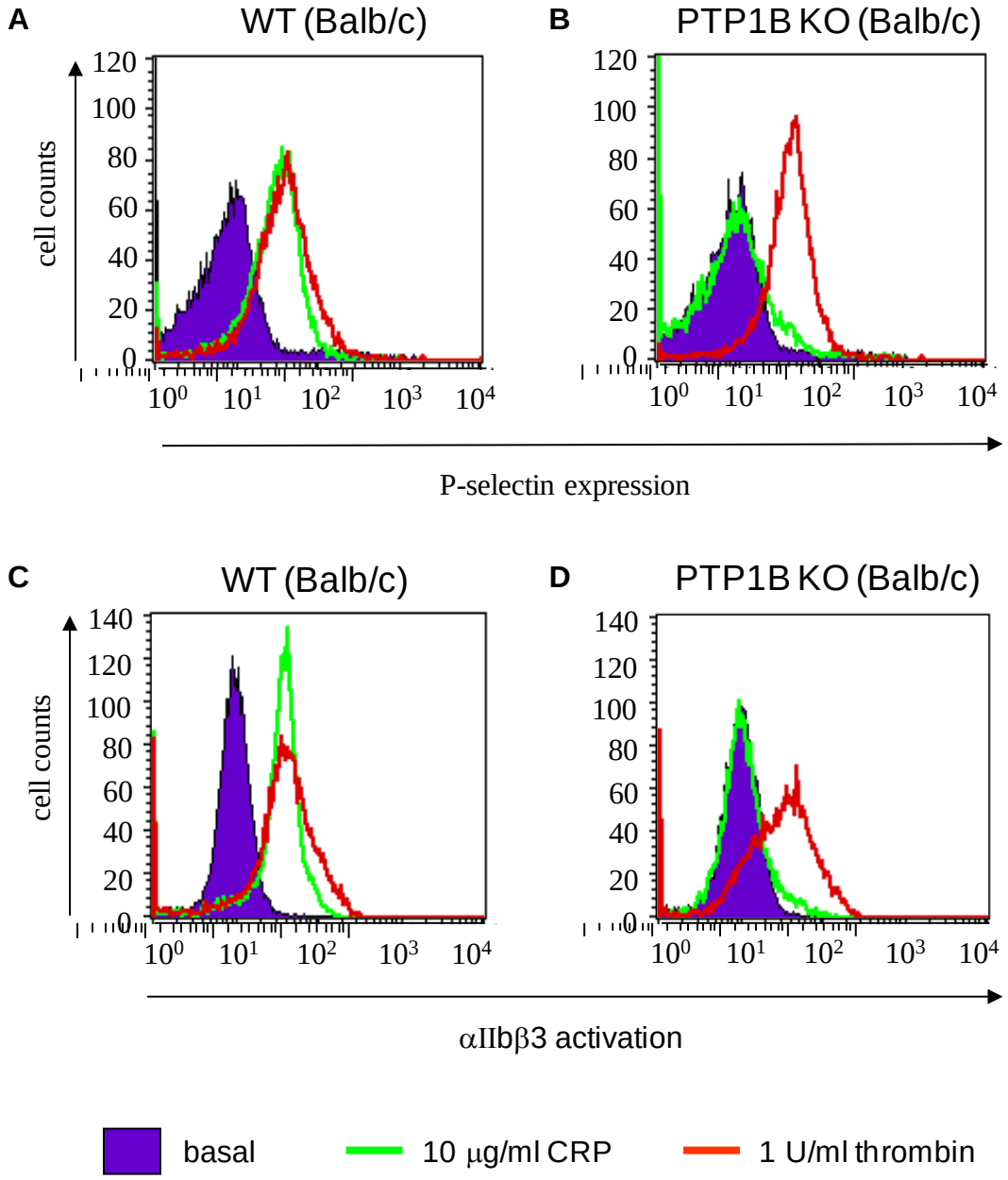


Figure 7.2: GPVI-mediated P-selectin expression and integrin αIIbβ3 activation are impaired in PTP-1B-deficient platelets (Balb/c background). Platelets from wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a Balb/c background were unstimulated (purple histograms), or stimulated with 10 μg/ml collagen-related peptide (CRP) or 0.06 U/ml thrombin (green and red lines respectively), stained with a FITC-conjugated P-selectin antibody (A and B) or JON/A antibody (C and D) and analysed by flow cytometry. Results are representative of three mice per genotype.

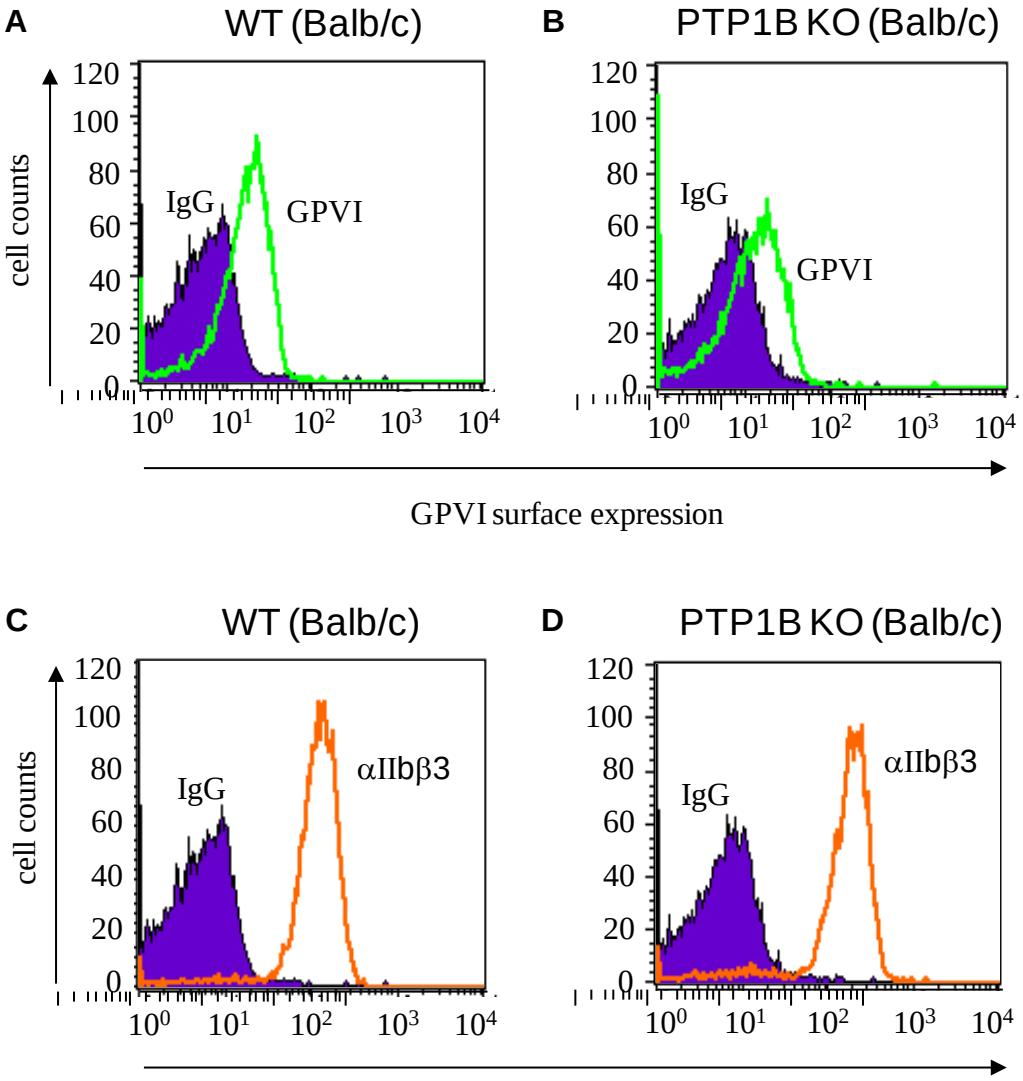


Figure 7.3: Reduced GPVI and normal integrin αIIbβ3 expression on PTP-1B-deficient platelets (Balb/c background). Wild-type (WT) and PTP-1B-deficient platelets (PTP-1B KO) on a Balb/c background were immunostained with either a FITC-conjugated GPVI (green lines-panels A and B) and αIIbβ3 antibodies (orange lines-panels C and D). Purple histograms represent platelets stained with FITC-conjugated IgG isotype control antibody.

7.3.1.3 GPVI signalling is normal in PTP-1B-deficient platelets on a Balb/c background

Although less severe than the GPVI-mediated functional defects reported in CD148-deficient platelets (Senis et al. 2009), the above observations demonstrated that PTP-1B positively regulates GPVI-mediated functional responses. We therefore wanted to investigate the molecular basis of this functional defect. Since PTP-1B has been shown to be a positive regulator of Src tyrosine kinase in platelets (Arias-Salgado et al. 2005), we hypothesized that it also regulates Fyn and Lyn SFKs, which are essential for initiating and propagating GPVI signalling (Watson et al. 2005). We started by analysing protein tyrosine phosphorylation in resting and CRP-stimulated platelets. The pattern of whole cell phosphorylation was near identical between WT and PTP-1B-deficient platelets stimulated with 10 μg/ml CRP for 0, 90 and 300 seconds (Figure 7.4). This was in stark contrast to the overall decrease in protein tyrosine phosphorylation observed in CRP-simulated CD148-deficient platelets (Chapter 5, Figure 5.1). Tyrosine phosphorylation of the FcR γ-chain was normal in CRP-stimulated PTP-1B-deficient platelets, suggesting that Fyn and Lyn were getting properly activated following GPVI engagement and cross-linking. This was confirmed by immunoblotting with phospho-specific antibodies for the SFK activatory and inhibitory tyrosine phosporylation sites. Fyn, Lyn and Src were all found to be phosphorylated to the same extent at both their activatory and inhibitory sites in resting and CRP-stimulated mutant platelets compared with WT control platelets (Figure 7.5). These findings demonstrated that PTP-1B is not involved in regulating these SFKs in resting or CRP-stimulated platelets. This was in complete contrast to the observation in CD148-deficient platelets, demonstrating that CD148 and PTP-1B have fundamentally different functions in platelets (Senis et al. 2009).

We next wanted to determine if there was a signalling defect further downstream in the GPVI signalling pathway. We investigated tyrosine phosphorylation of Syk and PLCγ2 as

they are essential components of the GPVI signalling pathway and lie downstream of the FcR γ-chain (Watson et al. 2005). Syk and PLCγ2 were immunoprecipitated from CRP-stimulated wild-type and PTP-1B-deficient platelets, resolved by SDS-PAGE and western blotted with an anti-phosphotyrosine antibody. Consistent with the whole cell lysate data, there was no reduction in CRP-induced tyrosine phosphorylation of Syk and PLCγ2 in PTP-1B-deficient platelets (Figure 7.6). This result suggested the defect may lie further downstream of PLCγ2 or in a different biochemical process.

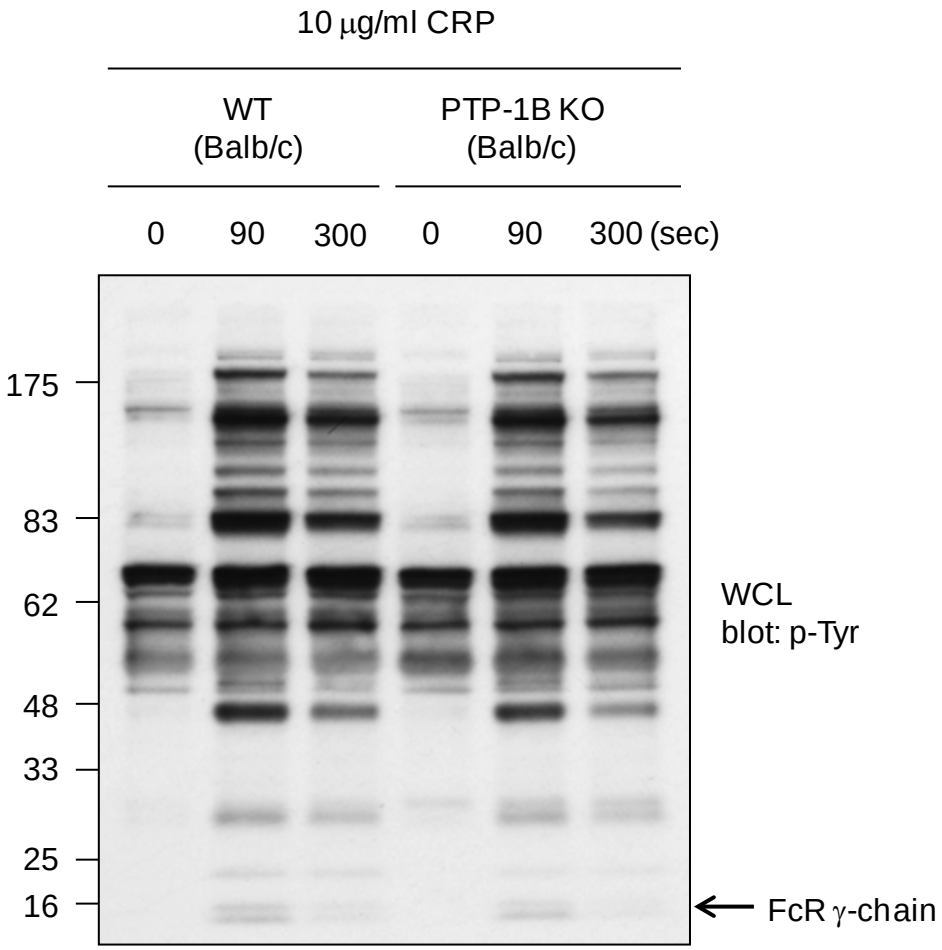


Figure 7.4: Normal GPVI signalling in PTP-1B-deficient platelets (Balb/c background). Washed platelets (5×10^8/ml) from wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a Balb/c background were stimulated with 10 μg/ml CRP for 0, 90 and 300 seconds. Whole cell lysates (WCLs) were resolved on a 10% SDS-PAGE gel then western blotted with an anti-phosphotyrosine antibody (p-Tyr). The doublet corresponding to the FcR γ-chain is indicated at the bottom of the gel. Blot representative of 3 experiments.

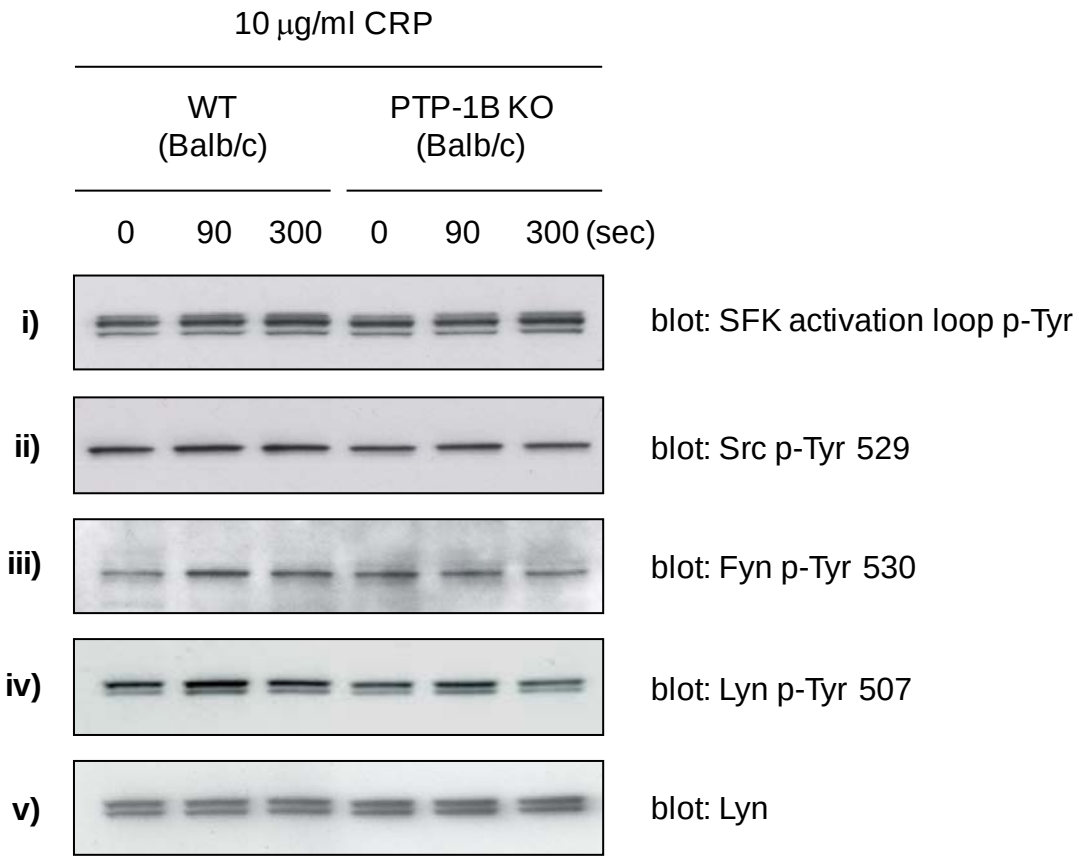


Figure 7.5: Normal Src family kinase phosphorylation in resting and CRP-activated PTP-1B-deficient platelets (Balb/c background). Washed platelets (5×10^8/ml) from wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a Balb/c background were stimulated with 10 μg/ml CRP for 0, 90 and 300 seconds. Whole cell lysates (WCLs) were resolved on a 10% SDS-PAGE gel then western blotted with: (i) an anti-Src family kinase (SFK) activation loop p-Tyr antibody; (ii) an anti-Src p-Tyr 529 antibody; (iii) an anti-Fyn p-Tyr 530 antibody; (iv) an anti-Lyn p-Tyr 507 antibody and (v) an anti-Lyn antibody as a control for protein loading. Blot representative of 3 experiments.

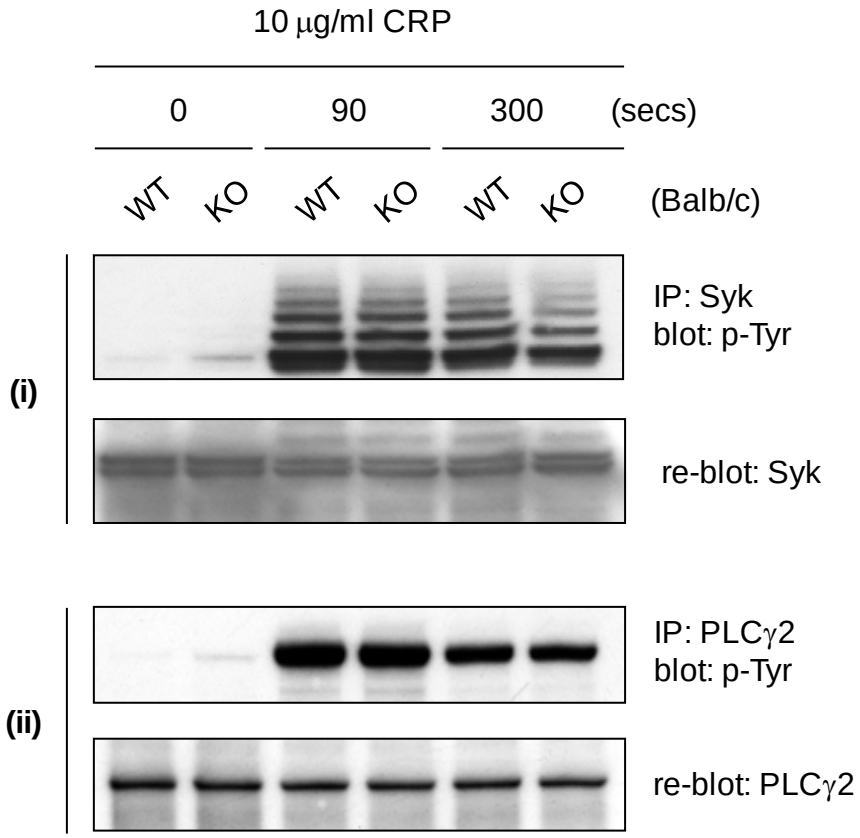


Figure 7.6: Normal Syk and PLCγ2 phosphorylation in resting and CRP-activated PTP-1B-deficient platelets (Balb/c background). Washed platelets (5×10^8/ml) from wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a Balb/c background were stimulated with 10 μg/ml CRP for 0, 90 and 300 seconds. (i) Syk and (ii) PLCγ2 were immunoprecipitated from whole cell lysates and western blotted with an anti-phosphotyrosine antibody, then stripped and re-blotted with anti-Syk and anti-PLCγ2 antibodies, respectively. Blot representative of 2 experiments.

7.3.1.4 PTP-1B-deficient platelets on a C57BL/6 background aggregate normally in response to low concentration CRP

One possible explanation for the discrepancy in our findings with those of Arias-Salgado et. al. regarding GPVI-mediated functional defects in PTP-1B-deficient platelets is that the mouse models that were analyzed are different. These differences may be due to strain variation or modifier loci. In our study, we used PTP-1B Ex5/6 KO mice on a Balb/c background, whereas Arias-Salgado et. al. used PTP-1B Ex1 KO mice on a mixed C57BL/6-129SvJ background. Neither mouse model generated a truncated version of PTP-1B. To test the possibility that strain variation can alter the phenotype, PTP-1B Ex5/6 KO mice backcrossed on a C57BL/6 background (>12 generations) were analyzed for functional and biochemical defects.

Unlike PTP-1B-deficent platelets on a Balb/c background which exhibit aggregation and secretion defects to low concentration (0.3 and 1 μg/ml) CRP, PTP-1B-deficient platelets on a C57BL/6 background exhibited normal aggregation and a reduction in ATP secretion to the same concentrations of CRP (Figures 7.1 and 7.7). This finding suggests that modifier loci in different strains of mice can alter the GPVI-mediated aggregation/secretion phenotype. It should be noted that PTP-1B-deficient platelets on a C57BL/6 background aggregated/secreted normally to low dose thrombin stimulation (data not shown).

7.3.1.5 GPVI signalling is normal in PTP-1B-deficient platelets on a C57BL/6 background

PTP-1B-deficient platelets on a C57BL/6 background were next examined for GPVI signalling defects. Platelets were stimulated with an intermediate concentration of CRP (5 μg/ml) for 0, 30, 60, 120 and 240 seconds in the presence of apyrase (ADP scavenger), indomethacin (cyclooxygenase inhibitor) and lotrafiban (αIIbβ3 antagonist). Whole cell

phosphorylation was almost identical between WT and mutant platelets pre- and post-CRP stimulation (Figure 7.8i). Some bands, including a prominent band at approximately 25 kDa, were marginally hyperphosphorylated in mutant platelets, suggesting they may be substrates of PTP-1B (Figure 7.8i). Phosphorylation of the FcR γ-chain was normal in resting and activated mutant platelets, as was phosphorylation of Syk at its activation loop (Tyr525/526) (Figure 7.8ii and iv), demonstrating normal GPVI proximal signalling in PTP-1B-deficient platelets on a C57BL/6 background.

The same experiments described above were repeated in the absence of lotrafiban to allow platelets to aggregate following CRP stimulation. The purpose of these experiments was to test whether PTP-1B was involved in aggregation-dependent signalling via the integrin αIIbβ3. Whole cell phosphorylation was normal in mutant platelets under these conditions (Figure 7.9i). Further, phosphorylation of the FcR γ-chain and the activation loop of Syk were also normal, demonstrating that PTP-1B is not involved in aggregation-dependent signalling.

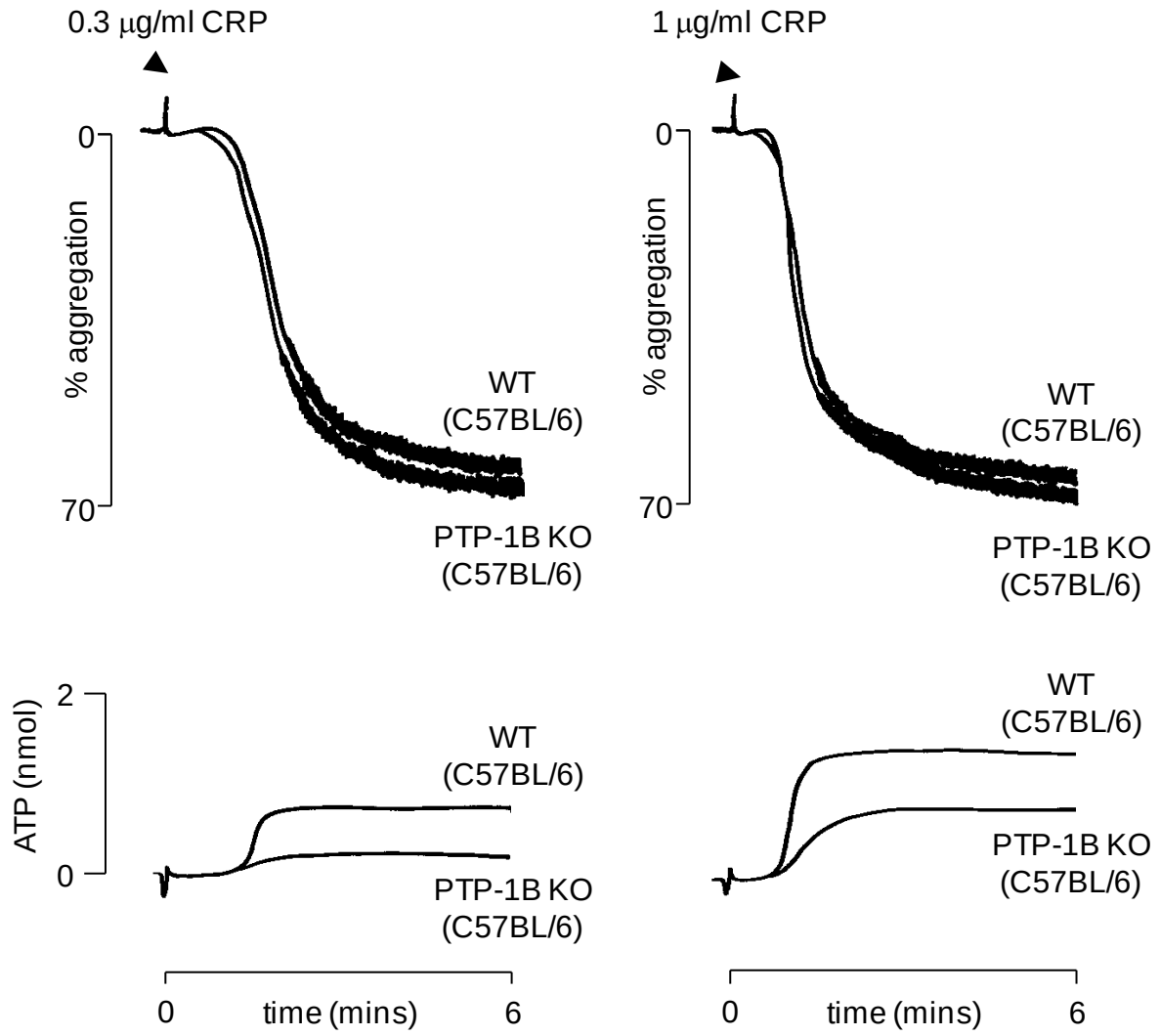


Figure 7.7: Platelets from PTP-1B-deficient mice (C57BL/6 background) aggregate normally and have an ATP secretion defect to CRP. Washed platelets (2×10^8/ml) prepared from litter-matched wild-type (WT) and PTP-1B-deficient (PTP-1B KO) mice on a C57BL/6 were stimulated with 0.3 and 1 μg/ml CRP. Platelet aggregation was measured as a change in light transmission using a lumi-aggregometer. Representative images are shown (n = 2 mice per condition).

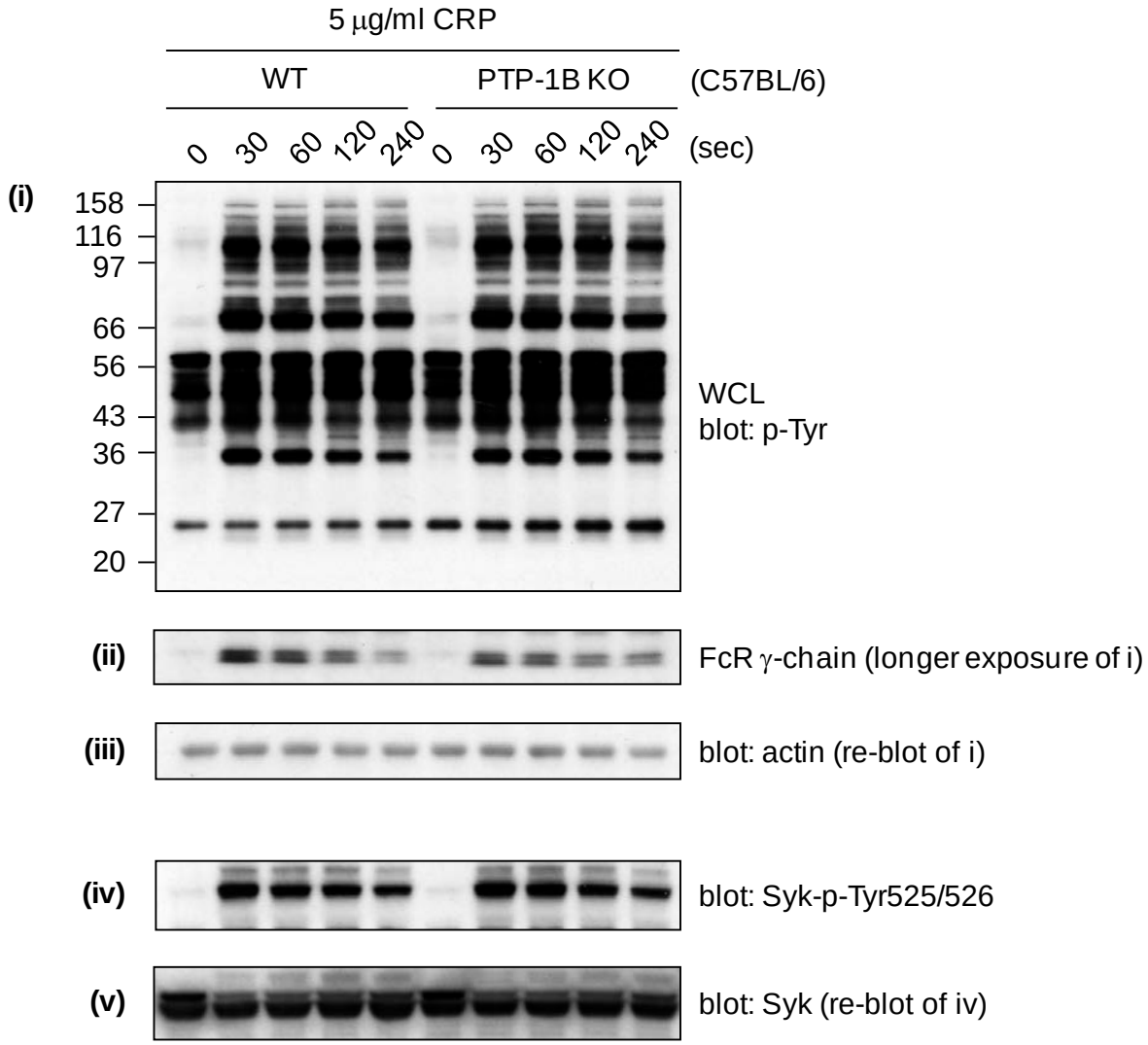


Figure 7.8: Normal GPVI signalling in PTP-1B-deficient platelets (C57BL/6 background) in suspension. Washed platelets (2×10^8/ml) pooled from three wild-type (WT) and three PTP-1B-deficient (PTP-1B KO) mice on a C57BL/6 background were stimulated with 5 μg/ml collagen-related peptide (CRP) in the presence of 2 U/ml apyrase, 10 μM indomethacin and 10 μM lotrafiban for 30, 60, 120 and 240 seconds (sec). Whole cell lysates (WCL) prepared at the indicated times were resolved on 4 – 12% SDS-PAGE gels and western blotted with: (i) anti-phosphotyrosine (p-Tyr); (iv) anti-Syk-phosphotyrosine 525/526 (Syk-p-Tyr525/526) antibodies. (ii) Longer exposure of the region of membrane (i) where FcR γ-chain migrates (~12 kDa). (iii) Membrane (i) was re-blotted with an anti-actin antibody. (v) Membrane (iv) was re-blotted with an anti-Syk antibody. Representative images are shown (n = 2 separate experiments).

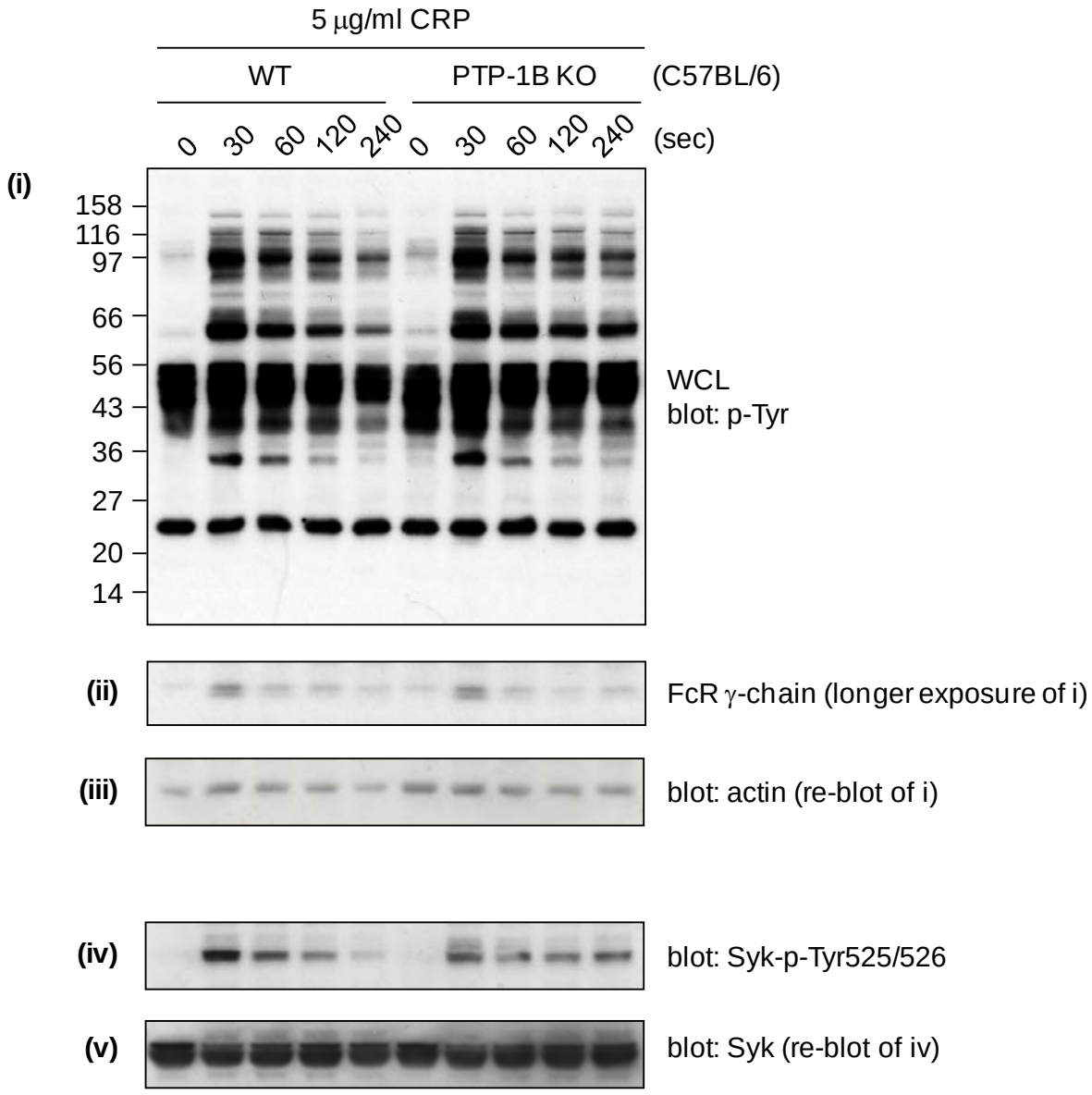


Figure 7.9: Normal GPVI signalling in aggregated PTP-1B-deficient platelets (C57BL/6 background). Washed platelets (2×10^8/ml) pooled from three wild-type (WT) and three PTP-1B-deficient (PTP-1B KO) mice on a C57BL/6 background were stimulated with 5 μg/ml collagen-related peptide (CRP) in the presence of 2 U/ml apyrase and 10 μM indomethacin for 30, 60, 120 and 240 seconds (sec). Whole cell lysates (WCL) prepared at the indicated times were resolved on 4 – 12% SDS-PAGE gels and western blotted with: (i) anti-phosphotyrosine (p-Tyr); (iv) anti-Syk-phosphotyrosine 525/526 (Syk-p-Tyr525/526) antibodies. (ii) Longer exposure of the region of membrane (i) where FcR γ-chain migrates (~12 kDa). (iii) Membrane (i) was re-blotted with an anti-actin antibody. (v) Membrane (iv) was re-blotted with an anti-Syk antibody. Representative images are shown (n = 2 separate experiments).

7.3.1.6 Integrin αIIbβ3 signalling in PTP-1B-deficient platelets on a C57BL/6 background

The main finding of the study by Arias-Salgado et. al. is that PTP-1B is essential for initiating outside-in integrin αIIbβ3 signalling by dephosphorylating the inhibitory site of αIIbβ3-associated Src, which activates the kinase. These findings are based on observations made using PTP-1B-deficient platelets (Ex 1 deleted on a mixed C57BL/6-129SvJ background) (Arias-Salgado et al. 2005). To test this finding, we investigated outside-in integrin αIIbβ3 signalling in fibrinogen spread PTP-1B-deficient platelets (Ex 5/6 deleted on a C57BL/6 background). Whole cell phosphorylation was found to be virtually identical in BSA non-adherent and fibrinogen-adherent WT and mutant platelets (Figure 7.10). Further, the SFKs Src, Fyn and Lyn were all found to be phosphorylated to the same extent at their activation and inhibitory sites in non-adherent and adherent WT and mutant platelets (Figure 7.11). Contrary to the findings reported by Arias-Salgado et. al., our findings suggest that PTP-1B is not required for initiating outside-in integrin αIIbβ3 signalling. This contradiction may reflect differences in the mouse models used by the two groups or differences in the experimental protocols.

The only potential biochemical difference in integrin signalling identified between WT and mutant platelets was in the phosphorylation and interacting proteins associated with the signalling molecules Syk and PLCγ2. Both Syk and PLCγ2 lie downstream of Src in the αIIbβ3 signalling pathway, and both were found to be marginally hyper-phosphorylated in fibrinogen adherent mutant platelets compared with WT platelets (Figures 7.12 and 7.13). In addition, differences were observed in both Syk and PLCγ2 co-associated phosphoproteins before and/or after adhesion to fibrinogen.

Taken together, these findings demonstrate that PTP-1B is not essential for initiating integrin αIIbβ3 signalling, but it may be involved in regulating signalling further downstream.

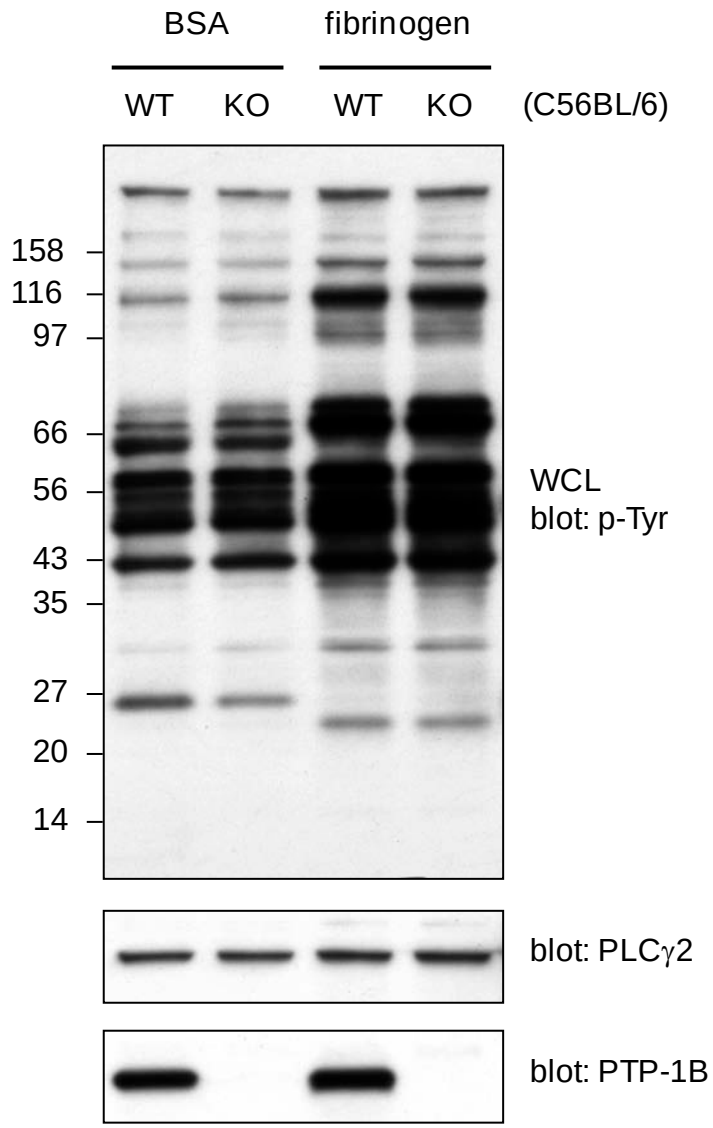


Figure 7.10: Normal integrin αIIbβ3 signalling in fibrinogen-adherent PTP-1B-deficient platelets (C57BL/6 background). Washed platelets (5×10^8/ml) pooled from five wild-type and five PTP-1B-deficient (KO) platelets on a C57BL/6 background were placed on BSA- and fibrinogen-coated surfaces for 45 minutes at 37°C. (i) Whole cell lysates (WCLs) prepared of BSA-non-adherent and fibrinogen-adherent platelets were resolved on 4 – 12% SDS-PAGE gels and western blotted with an anti-phosphotyrosine antibody (p-Tyr). (ii and iii) Membrane (i) was stripped and re-blotted with an anti-PLCγ2 antibody, then stripped and re-blotted a second time with an anti-PTP-1B antibody. Results are representative of 2 separate experiments.

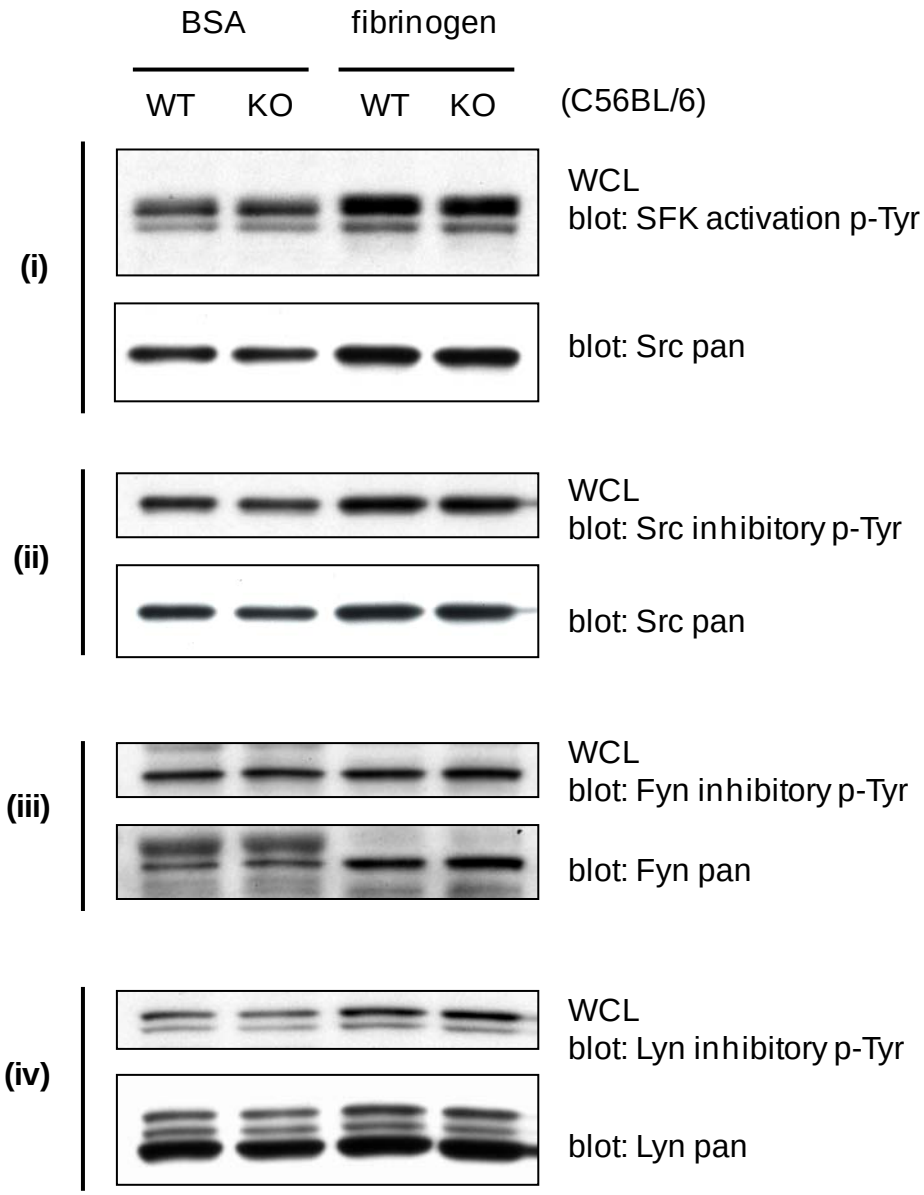


Figure 7.11: Normal Src family kinase (SFK) phosphorylation in BSA non-adherent and fibrinogen adherent PTP-1B-deficient platelets (C57BL/6 background). Washed platelets (5×10^8/ml) pooled from five wild-type and five PTP-1B-deficient (KO) platelets on a C57BL/6 background were placed on BSA- and fibrinogen-coated surfaces for 45 minutes at 37°C. Whole cell lysates (WCLs) prepared of BSA non-adherent and fibrinogen adherent platelets were resolved on 4 – 12% SDS-PAGE gels and western blotted with: (i) an anti-SFK activation loop phosphotyrosine antibody (SFK activation p-Tyr); (ii) an anti-Src inhibitory p-Tyr antibody; (iii) an anti-Fyn inhibitory p-Tyr antibody; and (iv) an anti-Lyn inhibitory p-Tyr antibody. Membranes (i – iv) were stripped and re-blotted with an anti-Src pan, and-Fyn pan and anti-Lyn pan antibodies, respectively. Results are representative of 2 separate experiments.

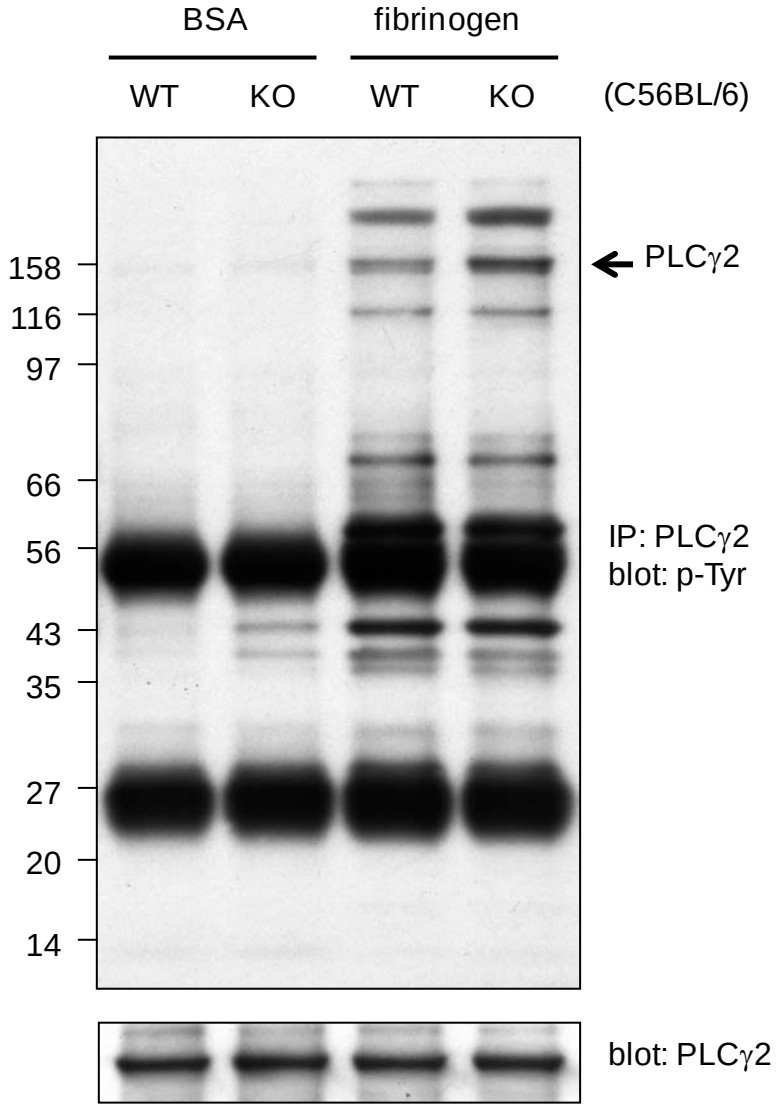


Figure 7.12: PLCγ2 phosphorylation and interacting proteins in BSA non-adherent and fibrinogen adherent PTP-1B-deficient platelets (C57BL/6 background). Washed platelets (5×10^8/ml) pooled from five wild-type and five PTP-1B-deficient (KO) platelets on a C57BL/6 background were placed on BSA- and fibrinogen-coated surfaces for 45 minutes at 37°C. PLCγ2 was immunoprecipitated (IP) from whole cell lysates (WCLs) prepared of BSA non-adherent and fibrinogen adherent platelets. IPs were resolved on 4 – 12% SDS-PAGE gels and western blotted with an anti-phosphotyrosine antibody (p-Tyr) then stripped and re-blotted with an anti-PLCγ2 antibody. Blot representative of 2 experiments.

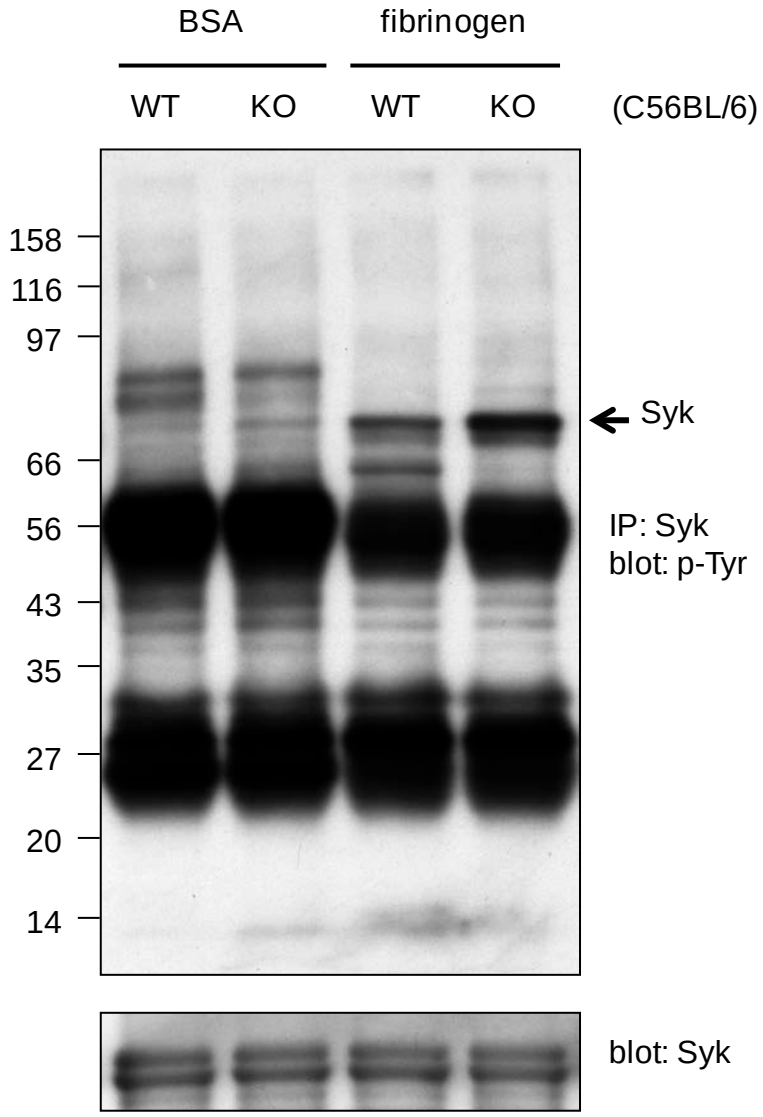


Figure 7.13: Syk phosphorylation and interacting proteins in BSA non-adherent and fibrinogen adherent PTP-1B-deficient platelets (C57BL/6 background). Washed platelets (5×10^8/ml) pooled from five wild-type and five PTP-1B-deficient (KO) platelets on a C57BL/6 background were placed on BSA- and fibrinogen-coated surfaces for 45 minutes at 37°C. Syk was immunoprecipitated (IP) from whole cell lysates (WCLs) prepared of BSA non-adherent and fibrinogen adherent platelets. IPs were resolved on 4 – 12% SDS-PAGE gels and western blotted with an anti-phosphotyrosine antibody (p-Tyr) then stripped and re-blotted with an anti-Syk antibody.

7.3.2 TC-PTP expression and functional characterization in platelets

TC-PTP and PTP-1B are structurally similar PTPs, sharing 74 % sequence homology in their catalytic domains (Romsicki et al. 2003; Doody et al. 2009). They also display similar enzyme kinetics (Iversen et al. 2002; Romsicki et al. 2003). We therefore hypothesized that TC-PTP may compensate in the absence of PTP-1B. In this section I confirmed expression of TC-PTP in mouse and human platelets, and analyzed GPVI-mediated functional responses in TC-PTP-deficient platelets.

7.3.2.1 TC-PTP is expressed in human and mouse platelets

Whole cell lysates prepared from human and WT mouse platelets were immunoblotted with an anti-TC-PTP antibody. Jurkat T cells and mouse thymocytes, which express high levels of TC-PTP, were used as positive controls. Faint bands migrating at the correct size for TC-PTP (45 kDa), were observed in both the human and mouse platelet (Figure 7.14A). Two C-terminal end splice variants of TC-PTP (45 and 48 kDa) are known to be expressed in various cell types. Each has distinctly different subcellular localizations and substrate preferences. The 45 kDa variant of TC-PTP tends to associate with proteins at the plasma membrane whereas the 48 kDa variant is localized to the endoplasmic reticulum (Tiganis et al. 1998; Tiganis et al. 1999). Our data suggests the 45 kDa splice variant is expressed in human and mouse platelets.

7.3.2.2 TC-PTP-deficient platelets have impaired P-selectin expression in response to CRP and thrombin

I next investigated the functional responses of TC-PTP-deficient platelets. TC-PTP-deficient mice exhibit an assortment of developmental defects and die within weeks of birth, making it difficult to collect sufficient amounts of blood for aggregation studies. Due to the poor

platelet recovery from mutant mice, flow cytometry was used to measure P-selectin expression on the surface of CRP- and thrombin-activated TC-PTP-deficient platelets. TC-PTP-deficient mice exhibited severely reduced levels of P-selectin on their surface in response to high doses of both CRP and thrombin (10 μg/ml and 1 U/ml, respectively) compared with litter-matched wild-type platelets (Figure 7.14B). Surface expression of GPVI was also severely reduced in resting TC-PTP-deficient platelets, whereas expression of the integrin αIIbβ3 was normal (Figure 7.15)

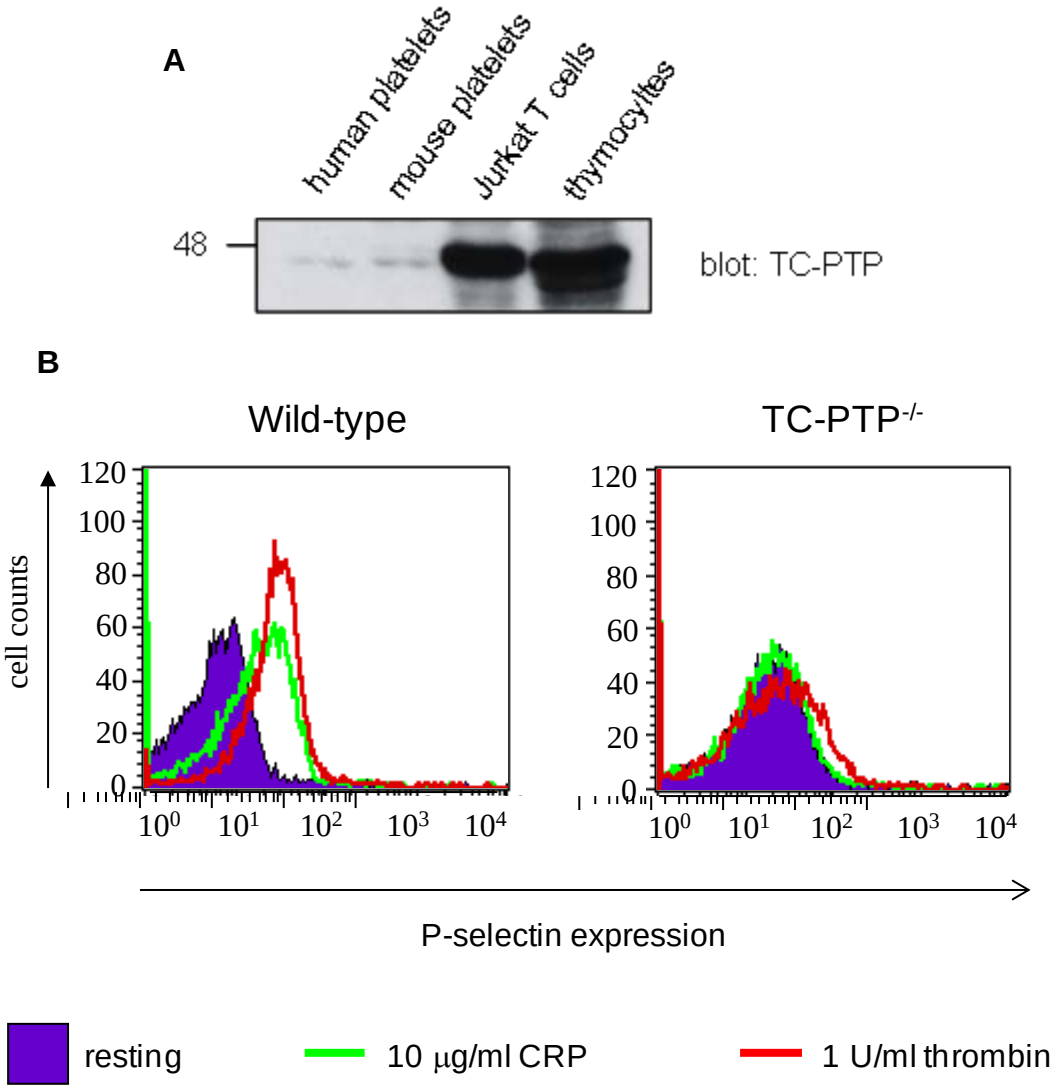


Figure 7.14. TC-PTP-deficient platelets exhibit decreased P-selectin expression in response to synthetic collagen peptide and thrombin. (A) Whole cell lysates prepared of washed human and mouse platelets, Jurkat T cells and mouse thymocytes were western blotted with an anti-TC-PTP antibody. (B) Washed platelets obtained from wild-type (WT) and TC-PTP-deficient (TC-PTP$^{-/-}$) mice were stimulated with 10 μg/ml of the GPVI-specific agonist collagen-related peptide (CRP) or 1 U/ml of thrombin. Surface expression of P-selectin was measured using FITC-conjugated anti-mouse P-selectin antibody and is directly proportional to the amount of α-granule secretion. Results are representative of 2 mice of each genotype.

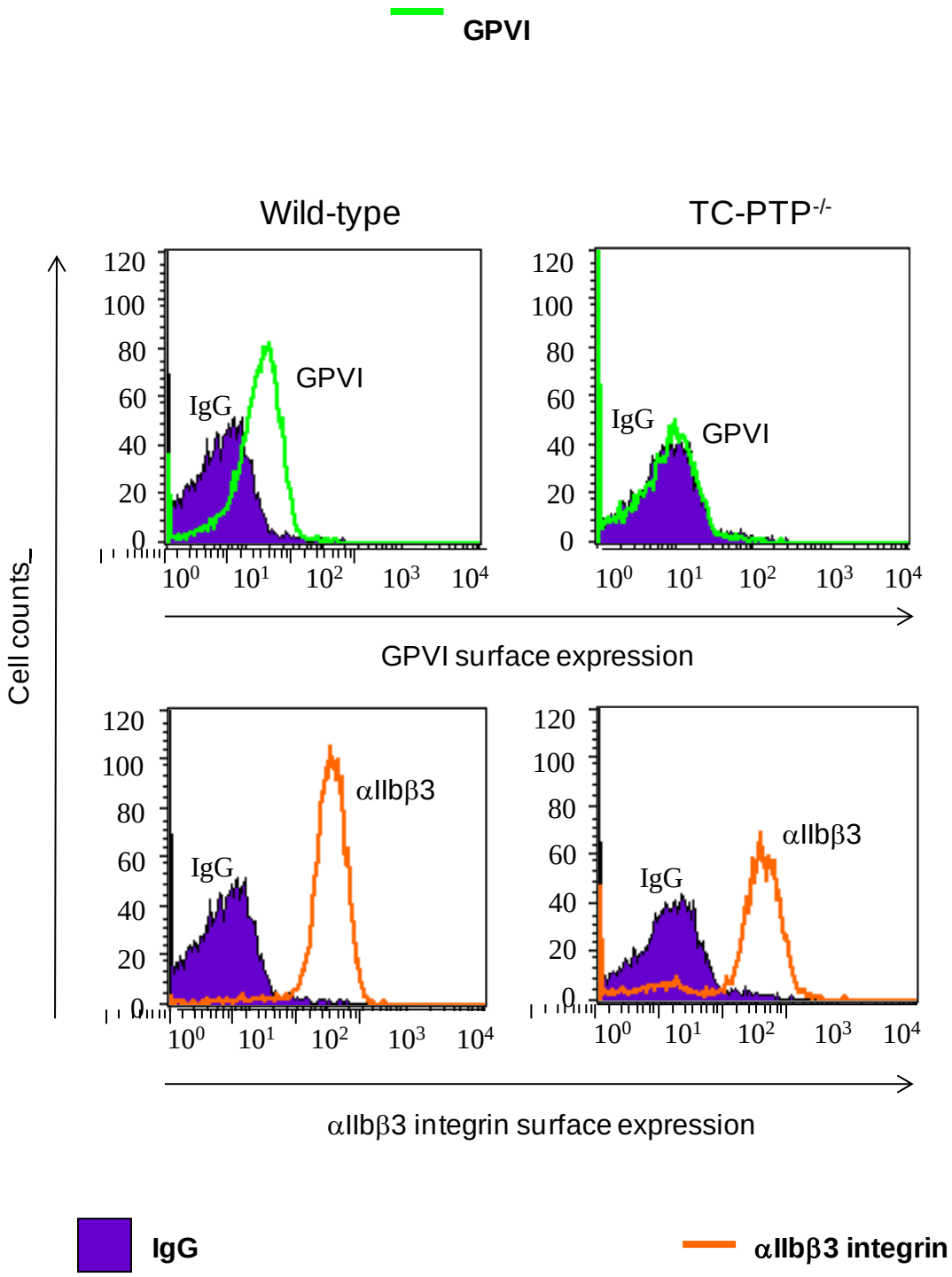


Figure 7.15. TC-PTP-deficient platelets do not express GPVI on their surface, but express normal levels of the integrin αIIbβ3. Resting, washed platelets from wild-type (WT) and TC-PTP-deficient (TC-PTP$^{-/-}$) mice were stained with either FITC-conjugated anti-mouse GPVI antibody or FITC-conjugated anti-mouse αIIbβ3 antibody. Results are representative of 3 mice of each genotype.

7.4 Discussion

In this chapter I demonstrate that PTP-1B plays little or no role in regulating GPVI or integrin αIIbβ3 signalling in platelets. However, this conclusion is complicated by strain variation which alters the platelet phenotype of PTP-1B-deficient mouse models. Platelets from PTP-1B-deficient mice on a Balb/c background exhibit minor CRP-mediated aggregation and secretion defects, whereas platelets from PTP-1B-deficient mice on a C57BL/6 background aggregated normally to CRP with a reduction in secretion. Neither PTP-1B mouse model exhibited any overt phosphorylation defects to CRP or fibrinogen, demonstrating that CD148 and PTP-1B have fundamentally different functions in platelets. I also demonstrate for the first time that the structurally related PTP, TC-PTP, is expressed in human and mouse platelets at relatively low levels and that platelets from TC-PTP-deficient mice have severely reduced P-selectin expression in response to CRP and thrombin stimulation. The cause of the CRP-mediated defect is likely the almost complete absence of GPVI on the surface of TC-PTP-deficient platelets. TC-PTP-deficient mice also appear to be severely thrombocytopenic, suggesting that TC-PTP is involved in platelet formation.

We chose to investigate the functional role of PTP-1B in platelets in parallel with CD148 because we hypothesized that these two structurally distinct phosphatases have redundant functions in platelets. This is based on the fact that the functional and biochemical defects exhibited by CD148-deficient platelets were partial and could be overcome with high doses of agonists (Chapters 4 and 5). CD45 is the main RPTP that regulates immune receptor function in B and T cells, however they also express CD148 which has been shown to have some redundant functions with CD45 (Zhu et al. 2008). Since we could not conclusively identify another RPTP in platelets, we speculated that a non-transmembrane PTP could perform this function. The most obvious candidate was PTP-1B because it was recently shown to be an important positive regulator of Src downstream of the integrin αIIbβ3 (Arias-

Salgado et al. 2005). Interestingly, PTP-1B selectively regulates Src downstream of αIIbβ3, but not Fyn and Lyn downstream of GPVI, which was different to what we were observing in CD148-deficient platelets. We speculated that this may be due to masking resulting from use of high concentration of the powerful GPVI agonist convulxin (Arias-Salgado et al. 2005). Further, convulxin is not GPVI-specific, but also binds to and activates GPIb-IX-V on platelets (Kanaji et al. 2003).

Collectively, results presented in this chapter demonstrate that PTP-1B and CD148 have fundamentally different roles in platelets. Functional defects observed in both PTP-1B-deficient mouse models were minor compared to those observed in CD148-deficient platelets (Chapter 4). Moreover, there were no major phosphorylation defects in PTP-1B-deficient platelets, whereas dramatic defects were observed in CD148-deficient platelets (Chapter 5). It should be pointed out that both the PTP-1B- and CD148-deficient mouse models were on a C57BL/6 background, eliminating the possibility of strain variation between the two models.

One of the most interesting findings of this chapter that demonstrates the different biochemical functions of CD148 and PTP-1B in platelets was that SFK activity was normal in resting PTP-1B-deficient platelets, whereas it was significantly reduced in CD148-deficient platelets. SFK activity was indirectly measured as phosphorylation of the SFK activation loop, which is a *trans*-autophosphorylation event, and phosphorylation of the FcR γ-chain, which is also mediated by SFKs. These findings demonstrate that CD148 is critical for maintaining a pool of active SFKs in platelets, which keeps platelets primed, so that they can respond rapidly when they come into contact with ECM proteins at sites of vascular injury.

We suspect the reason for the discrepancy between our findings and those previously reported by Arias-Salgado et. al. on the functional role of PTP-1B in platelets reflects strain variation and the effects of modifier loci on the PTP-1B-deficient mouse models studied by the two groups. Strain variation may be compounded by differences in experimental

conditions. We studied PTP-1B Ex5/6 KO mice on either a Balb/c or a C57BL/6 background, whereas Arias-Salgado et. al. studied PTP-1B Ex1 KO mice on a mixed C57BL/6-129SvJ mice. Neither the PTP-1B Ex5/6 KO mouse nor the PTP-1B Ex1 KO mouse express truncated forms of PTP-1B and the metabolism phenotypes previously reported in these mice were very similar. Therefore, it seems unlikely that either mouse model accidentally acquired other mutations besides ablation of the PTP-1B gene during generation. However, the possibility exists that the models drifted apart with time due to naturally occurring mutations. Another possibility for the different findings between ours and the Arias-Salgado groups is differences in experimental protocols.

Better understanding the functional role of PTP-1B in platelets will require identifying its substrates and interacting proteins. Some of the known substrates expressed in platelets are Src and the adaptor protein Dok-1, which is also involved in regulating outside-in integrin αIIbβ3 signalling (Calderwood et al. 2003). Another important question that needs to be addressed is how PTP-1B translocates from the outer surface of the ER to the plasma membrane following caplain-mediated cleavage. Further, how does PTP-1B dock to or near GPVI and αIIbβ3? One hypothesis is that Dok-1 and -2 may mediate docking of PTP-1B to the β3-subunit (Arias-Salgado et al. 2005). Recent work has demonstrated that PTP-1B is targeted to newly formed cell-matrix adhesion complexes (Hernandez et al. 2006). Live cell imaging has also been used to investigate trafficking of PTP-1B to the plasma membrane (Yudushkin et al. 2007).

Although structurally similar, TC-PTP and PTP-1B have distinct physiological functions, as knockout mouse models exhibit dramatically different phenotypes (You-Ten et al. 1997; Elchebly et al. 1999; Doody et al. 2009). PTP-1B-deficient mice live to adulthood, but have dramatic glucose and fat metabolism defects, whereas TC-PTP-deficient mice die 3 – 5 weeks after birth of erythropoietic and lymphopoietic deficits, indicating a critical role for

TC-PTP in bone marrow maturation (You-Ten et al. 1997). The two phosphatases have also been implicated in regulating different components of the insulin signalling pathway (Dube et al. 2005).

Our hypothesis that TC-PTP compensates for the lack of PTP-1B or CD148 in platelets does not appear to be correct. Preliminary findings presented in this chapter suggest that TC-PTP plays a role in platelet formation, which correlates with other haematopoietic defects previously reported in TC-PTP-deficient mice (Doody et al. 2009). Our flow cytometry data demonstrated that platelets isolated from these mice were unresponsive to CRP and thrombin agonists. The reduced responsiveness to CRP is likely due to the very low levels of GPVI expressed in TC-PTP-deficient platelets. Interestingly, αIIbβ3 levels were normal in mutant platelets, demonstrating that TC-PTP does not regulate expression of all surface proteins. The lack of GPVI expression may be due to a defective in development, trafficking or shedding.

In summary, PTP-1B plays little role GPVI or integrin αIIbβ3 signalling in platelets, that is influenced by modifier loci. PTP-1B has little or no role in regulating SFK activity in resting or activated platelets, whereas CD148 is a global regulator of SFK activity in platelets. TC-PTP and PTP-1B also appear to have fundamentally different roles in platelets. One of the main functions of TC-PTP appears to be in platelet development. Interestingly, CD148, PTP-1B and TC-PTP all positively regulate surface expression of GPVI on platelets however, we suspect this is through different mechanisms.

CHAPTER 8 –

GENERAL DISCUSSION

8.1 Summary of findings

The main objective of this thesis was to investigate the functional role of CD148 in platelets. Findings from this thesis establish CD148 as a key regulator of platelet responsiveness to collagen and fibrinogen. I also demonstrate that CD148 plays a minor role in regulating platelet responsiveness to thrombin and TxA_2. We believe the molecular mechanism underlying how CD148 regulates platelet responsiveness to such a diverse array of agonists is through regulation of SFK activity. We demonstrate that CD148 is a global regulator of SFKs in platelets, maintaining a pool of primed/active SFKs in resting platelets. Subsequent ligand mediated cross-linking of the collagen activation receptor GPVI and the fibrinogen receptor αIIbβ3 triggers receptor signalling. Although no other RPTPs were identified in platelets, I identified the non-transmembrane TC-PTP in platelets for the first time and work was initiated to investigate functional redundancy between CD148, PTP-1B and TC-PTP in platelets. I focused on PTP-1B as recent work by the Shattil group demonstrated that that PTP-1B is essential for activating Src kinase specifically downstream of the integrin αIIbβ3 (Obergfell et al. 2002; Arias-Salgado et al. 2003; Arias-Salgado et al. 2005). However, my results demonstrate that unlike CD148, PTP-1B does not maintain a pool of active SFKs in platelets and it is not involved in initiating GPVI or integrin αIIbβ3 signalling in platelets. PTP-1B plays a minor role relative to CD148 in regulating GPVI-mediated platelet aggregation and secretion, and αIIbβ3 signalling, however these functions are strain-dependent, suggesting that modifier loci influence its activity. We speculate that the discrepancies between our findings and those of the Shattil group are due to strain variation of PTP-1B-deficient mouse models. TC-PTP appears to play a developmental role in platelets. Therefore, I conclude that CD148, PTP-1B and TC-PTP have distinct functional roles in platelets.

8.2 Functional roles of CD148 in platelets

In this thesis, I demonstrated that CD148 positively regulates several important platelet functional responses, including aggregation, secretion and spreading. The most dramatic defects observed in CD148-deficient platelets were downstream of GPVI and the integrin αIIbβ3, both of which are tyrosine kinase-linked receptors. Minor defects were also detected to thrombin and TxA_2, which signal through the G protein-coupled receptors PAR-4 and TP in mouse platelets, respectively. ADP-mediated responses were however normal.

Platelet aggregation and ATP secretion of CD148-deficient platelets were abolished to the GPVI-specific agonist CRP at the concentrations tested. Platelet spreading on collagen was also attenuated, as was platelet adhesion to collagen under physiological flow conditions (Senis et al. 2009). These defects cannot be explained by reduced GPVI expression alone as FcR γ-chain heterozygous-deficient platelets, which express comparable levels of GPVI to CD148-deficient platelets, respond better to CRP and collagen (Senis et al. 2009). This result provided strong evidence that CD148-deficient platelets had a concomitant signalling defect. The reduction in surface levels of GPVI may be due to a reduction in expression, reduced trafficking or enhanced shedding from the platelet surface. This change in surface expression appeared to be specific to GPVI, as α2β1 and αIIbβ3 levels were normal (Senis et al. 2009).

Platelet adhesion and spreading on fibrinogen is mediate by the integrin αIIbβ3. The molecular mechanism involves αIIbβ3 clustering, leading to outside-in integrin signalling (Shattil et al. 2004). Platelets placed on a fibrinogen-coated surface initially extend filopodia, which become firmly attached to the fibrinogen. The spaces between filopodia subsequently fill in with lamellipodia. CD148-deficient platelets took longer and extended fewer filopodia than WT platelets (Senis et al. 2009). Moreover, filopodia did not remain firmly attached to the surface, compared with WT platelets. As a result, CD148-deficient platelets could not maintain filopodia extensions, and therefore did not form lamellipodia that WT platelets

eventually partially developed on fibrinogen (Senis et al. 2009). Since CD148-deficient platelets expressed normal levels of αIIbβ3 on their surface, these findings suggested that outside-in integrin αIIbβ3 signalling was abrogated.

The defects described above in CD148-deficient platelets contribute to a mild bleeding tendency, delayed thrombus formation and thrombus instability *in vivo* (Senis et al. 2009). These findings demonstrate a novel and vital role of CD148 in mediating haemostasis and thrombus formation *in vivo* (Senis et al. 2009). However, it should be noted that because CD148 is also expressed in endothelial cells, these defects may be partly due to defective endothelial cell function. The lack of evidence for a severe bleeding disorder in CD148 mutant mice makes it a potentially promising anti-thrombotic drug target. Structural and functional features of CD148 also lend it to drug targeting, including its large extracellular domain that could be targeted by small molecule inhibitors without the need to cross the plasma membrane.

8.3 CD148 regulates global SFK activity in platelets

SFKs are essential for initiating and propagating signalling from several major platelet tyrosine kinase-linked receptors. They also contribute to signalling downstream of a number of stimulatory GPCRs (Harper et al. 2006; Minuz et al. 2006). SFK activity is tightly regulated by tyrosine phosphorylation and two intramolecular interactions, that maintain the kinases in an inactive conformation (Xu et al. 1997; Xu et al. 1999). These weak interactions can be disrupted through inter-molecular interactions or by dephosphorylation of the inhibitory site of SFKs. One of the main findings of this thesis was that global SFK activity, as detected using phospho-specific antibodies, was significantly reduced in both resting and activated CD148-deficient platelets compared with WT platelets. We hypothesize that the pool of active SFKs in resting WT platelets is required to initiate a rapid and maximal

response following contact with ECM proteins at a site of vessel injury. Without this pool of active SFKs, clustering of GPVI and αIIbβ3 does not trigger sufficiently rapid or strong activatory signals (Figure 8.1). This model would predict that there is a small amount of basal signalling from GPVI and αIIbβ3 receptors in resting WT platelets. Recent work demonstrates that this is the case for GPVI signalling (Mori et al. 2008). Residual signalling from GPVI and αIIbβ3 in CD148-deficient platelets may be due to other PTPs compensating in the absence of CD148 or to activation of SFKs through inter-molecular interactions with proline-rich and phosphotyrosine-containing proteins.

We believe the global reduction in SFK activity in CD148-deficient platelets also explains the reduced responsiveness of CD148-deficient platelets to thrombin and TxA_2. This is because both the PAR-4 and TP receptors have a minor Src signalling component (Offermanns 2006). We speculate the reason for the normal ADP response of CD148-deficient platelets is because ADP signals through two receptors one of which has no SFK component. The G_q-coupled $P2Y_1$ receptor, is responsible for shape change and initiation of aggregation and has a small SFK component; whereas the G_i-coupled $P2Y_{12}$ receptor, which is required for sustain platelet aggregation does not signal through SFKs (Gachet 2006). I also demonstrate that the normal ADP response partially masks the reduced collagen responsiveness of CD148-deficient platelets.

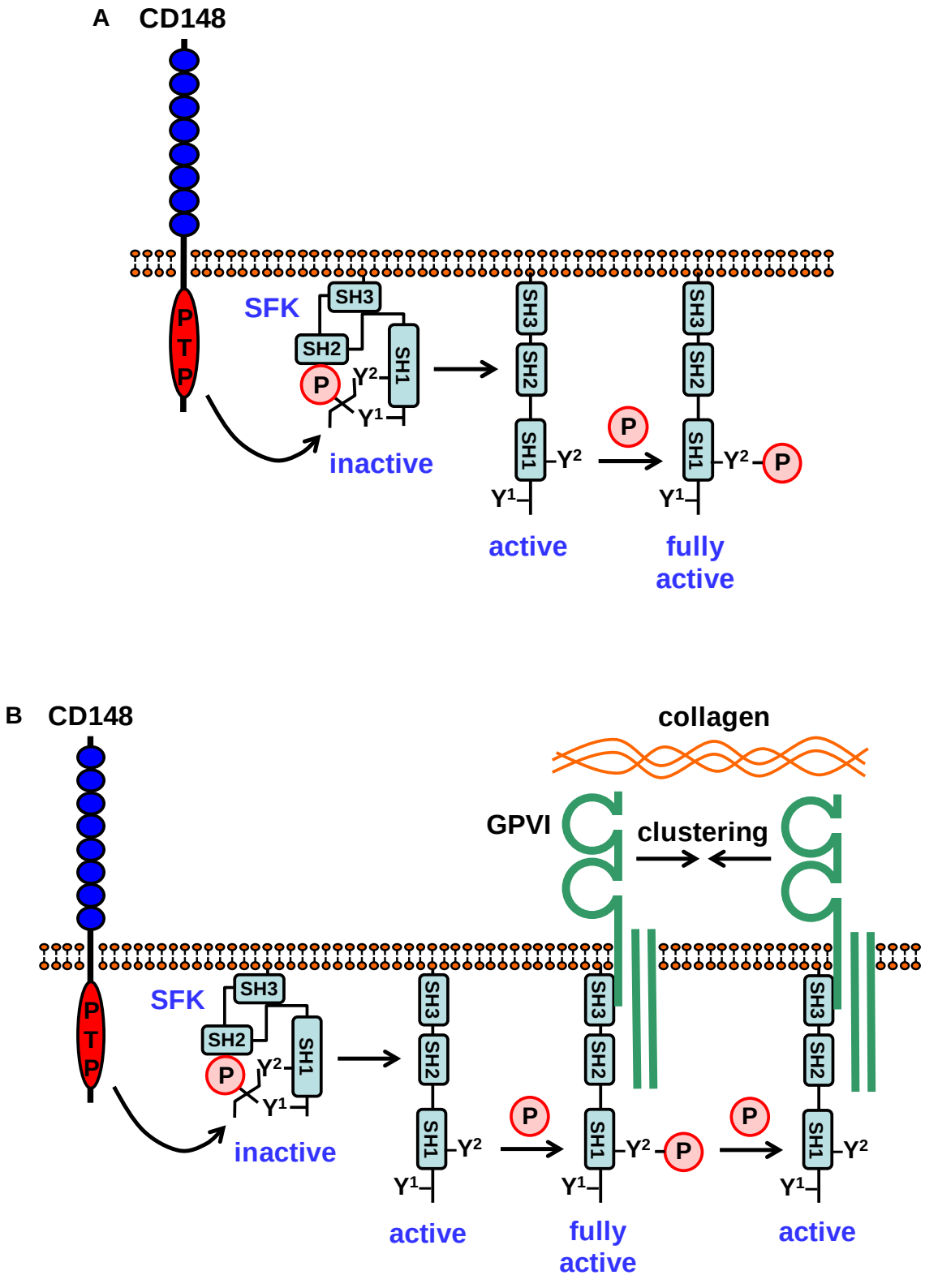


Figure 8.1: CD148 maintains a pool of active SFKs in platelets that are essential for initiating GPVI signalling. (A) SFKs are maintained in a primed/active state by dephosphorylation of their inhibitory C-terminal tyrosine (Y^1) by CD148. SFKs then become fully active by trans-autophosphorylation of the tyrosine residue in the active loop (Y^2). (B) The pool of activated SFKs is required to initiate a strong activatory signal following GPVI clustering. This model also holds true for αIIbβ3 signalling.

Findings from this study support the hypothesis that SFKs are direct substrates of CD148 (Senis et al. 2009). Here I show that CD148 interacts with and dephosphorylates both the activation and inhibitory sites of Fyn, Lyn and Src. Interestingly, the *in vitro* data demonstrates that recombinant CD148 marginally favours dephosphorylating the activation sites of Fyn, Lyn and Src rather than their inhibitory sites. Of the three inhibitory site phospho-peptides tested, CD148 had a marginal preference for the Lyn-derived phospho-peptide. These *in vitro* findings appear paradoxical to our platelet findings as the net effect of deleting CD148 from platelets reduces responsiveness to collagen and fibrinogen. To unify these findings, we hypothesize that CD148 initially activates SFKs, by dephosphorylating their C-terminal inhibitory tyrosine and subsequently attenuates their activity by dephosphorylating their activation loop tyrosine. In this way, CD148 both positively and negatively regulates SFK activity. Co-ordinated increased phosphorylation of the inhibitory sites of SFK, by Csk or its related kinase Ctk/Chk, effectively inhibit prolonged SFK-mediated signalling. The proposed model is illustrated in Figure 8.2. CD45 is thought to regulate Lck and Fyn downstream of the TCR via a similar mechanism (Hermiston et al. 2003). Therefore, the activation status of SFKs is tightly regulated by the opposing activities of CD148 and Csk.

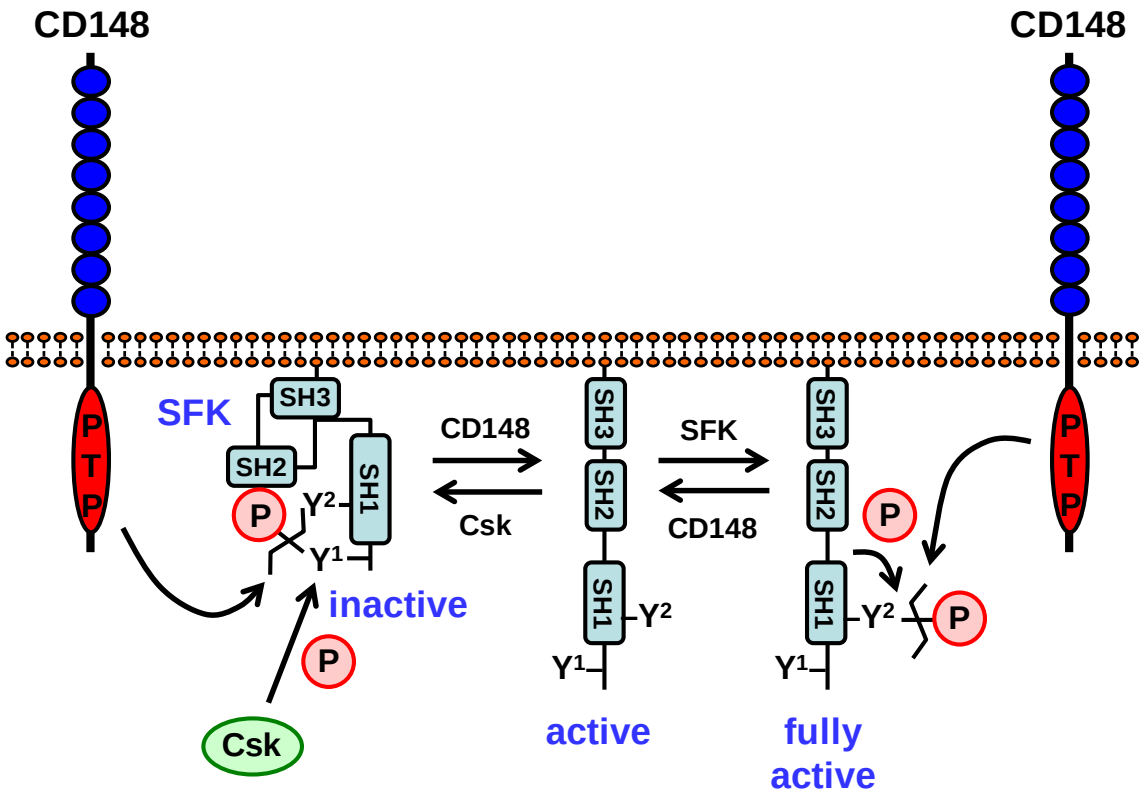


Figure 8.2: Model of how CD148 modulates SFK activity. CD148 initially activates SFKs, by dephosphorylating their C-terminal inhibitory tyrosine (Y^1) (left of figure) and subsequently attenuates their activity by dephosphorylating their activation loop tyrosine (Y^2) (right of figure). Co-ordinated increased phosphorylation of the inhibitory sites of SFK, by Csk or its related kinase Ctk/Chk, effectively inhibit prolonged SFK-mediated signalling .

8.4 Physiological substrates of CD148 in platelets

Other potential physiological substrates of CD148 include: the tyrosine kinase-linked receptors Met and PDGFβ, the adapter proteins LAT and Gab1, the adherens junction protein p120catenin, PLCγ1 and more recently the p85 subunit of PI 3-kinase, although the latter is not tyrosine phosphorylated in platelets (Gibbins et al. 1998; Kovalenko et al. 2000; Baker et al. 2001; Holsinger et al. 2002; Palka et al. 2003; Tsuboi et al. 2008). Interestingly, LAT, PLCγ1 and PI 3-kinase all lie downstream of SFKs in the GPVI signalling cascade, therefore CD148 may be regulating multiple points of the GPVI signalling pathway (Pasquet et al. 1999; Pasquet et al. 1999; Suzuki-Inoue et al. 2003; Watanabe et al. 2003).

Using a substrate trapping-pull down approach in conjunction with mass spectrometry, protein bands with molecular weights of 160, 90, 60 and 43 (doublet) kDa were isolated from pervanadate stimulated platelet lysates. Identification of proteins in the broad band at 90 kDa was complicated by the co-migrating recombinant MBP-CD148 fusion protein. The intensity and large size of this band may be due to several, highly phosphorylated, co-migrating proteins. It may also be due to the MBP-CD148 trapping mutant being hyper-phosphorylated by trapped SFKs. We suspect the 60 kDa band may be SFKs. The 43 kDa band was particularly prominent and warrants further investigation.

The identity of the 160 kDa protein pulled down from platelet lysate with the MBP-CD148 trapping mutant was determined to be non-muscle myosin heavy chain IIa (Swiss-Prot no: P35579), whereas the 60 and 43 kDa bands remain unknown. Non-muscle myosin heavy chain IIa is a novel and unexpected candidate substrate that requires further validation by other means. It was recently shown to be phosphorylated at Tyr-754 and Tyr-1,408, however, the functional consequences of phosphorylating these sites is not known (Rikova et al. 2007). Interestingly, the latter of these tyrosine residues is within a consensus ITIM, what has been shown to bind Shp1 (Baba et al. 2003). Indentifying non-muscle myosin heavy chain IIa as a

potential substrate of CD148 is an interesting finding as defects in the gene encoding this protein, namely *MYH9,* cause May-Hegglin anomaly, characterized by thrombocytopenia, giant platelets (Chen et al. 2007; Althaus et al. 2009).

8.5 Regulation of CD148 activity

Regulation of CD148 activity and localization in the platelet plasma membrane are now critical to understanding how CD148 regulates platelet function. A question that arises from our proposed mechanism is how ~2,800 copies of CD148 molecules can maintain the entire platelet pool of SFKs in an active conformation. We believe the explanation partly lies in the high catalytic activity of PTPs, which have *kcat* values up to three orders of magnitude greater than those of protein tyrosine kinases (Zhang 2003). We also hypothesize that expression of low levels of CD148 relative to SFKs may serve to have more of an activatory role rather than an inhibitory role. This hypothesis is largely based on recent work by McNeill et. al. investigating the effects of expressing various amounts of CD45 in transgenic mice (McNeill et al. 2007). They showed that expression of a low amount of CD45 (3 – 10%) is required to dephosphorylate the inhibitory site of Lck (Tyr-505) and allow T cell receptor signalling to occur. Conversely, expression of higher levels of CD45, as seen in wild-type T cells are required to attenuate TCR signalling by dephosphorylating the activation loop tyrosine of Lck (Tyr-394). It has also been suggested that net activation of an SFK by CD45 and other RPTPs requires a degree of separation between the RPTP and the SFK to promote dephosphorylation of the C-terminal tail whilst simultaneously allowing autophosphorylation of the activation loop tyrosine (Roach et al. 1997; Thomas et al. 1999; Johnson et al. 2000). Conversely, a high amount of a RPTP or close proximity to a SFK will have a net inhibitory effect on SFK activity by dephosphorylating the activation loop tyrosine. The fact that CD148 is membrane

localised and has a relatively low copy number in platelets may explain why it has a positive regulatory role on SFKs rather than an inhibitory one.

The ligand for CD148 is presently not known. Very few ligands for RPTPs have been identified to date. The most well established ligands for RPTPs are quite diverse and include: homophilic interactions of RPTPμ, pleitrophin for PTPλ and heparin sulphate and collagen for RPTPσ (Maeda et al. 1998; Aricescu et al. 2002; Sajnani-Perez et al. 2003; Ulbricht et al. 2003; Fox et al. 2005). The first study of CD148 demonstrated that cell-cell contact causes an increase in its surface expression and activity, suggesting that its ligand may be a counter receptor on an adjacent cell (Ostman et al. 1994). Another report suggested that its ligand may be an extracellular matrix protein. Sorby et. al. demonstrated that cells ectopically expressing CD148 exhibited increased CD148 catalytic activity when placed on Matrigel (Sorby et al. 2001). This hypothesis is supported by the fact that the extracellular domain of CD148 contains eight fibronectin type III repeats found in a variety of receptors and extracellular matrix proteins. An Arg-Gly-Asp (RGD) module within the FNIII domain conveys cell adhesion properties to proteins (Main et al. 1992). A recent biophysical study by Matozo et. al. suggests that dimerization of CD148 catalytic domains would auto-inhibit, according to the inhibitory wedge model defined in PTPα (Matozo et al. 2006). However, a recent large-scale structural study of all classical PTP catalytic domains argues strongly against the long-held inhibitory wedge model (Barr et al. 2009). Another possibility is that CD148 does not have a ligand, but rather moves freely about the cell surface due to electrostatic forces or steric hindrance according to the size-exclusion model (Lin et al. 2003; Choudhuri et al. 2005). This can be tested in a cell line model using mutant forms of CD148 lacking specific regions of the extracellular domain or containing point mutations disrupting glycosylation sites.

8.6 Redundancy between CD148 and other platelet PTPs

Residual GPVI and αIIbβ3 signalling in CD148-deficient platelets raises the possibility that one or more PTPs partially compensating in its absence. Attempts to identify other RPTPs expressed in platelets were either negative or inconclusive. Recent evidence suggests that megakaryocytes express low levels of CD45; PTPRO is expressed in human and mouse megakaryocytes; and RPTPσ may be expressed in human platelets (Taniguchi et al. 1999; Matsumura-Takeda et al. 2007; Senis et al. 2007). However, I could neither detect CD45 in human and mouse platelets nor conclusively demonstrate expression of PTPRO and RPTPσ on human and mouse platelets. This may be due to low levels of expression of these RPTPs in platelets, as well as questionable quality of the antibodies used for detection.

Since I did not conclusively detect another RPTP in platelets, as is the case in most other haematopoietic cell lines that express CD45 and CD148 (with the exception of erythrocytes), we speculated that perhaps a non-transmembrane PTP may have redundant functions with CD148 in platelets. The primary candidate was PTP-1B as recent evidence showed that PTP-1B dephosphorylates and activates Src downstream of αIIbβ3 (Obergfell et al. 2002; Arias-Salgado et al. 2003; Arias-Salgado et al. 2005). Other candidates include the SH2 domain-containing PTPs, Shp1 and Shp2, as they have been previously shown to regulate proximal ITAM receptor signalling events in immune cells (Shi et al. 2000; Zhang et al. 2000). Shp1 has also been shown to interact with Src in platelets and to positively regulate Src activation by preferentially dephosphorylating inhibitory Tyr-529 (Somani et al. 1997).

Findings presented in this thesis demonstrate that CD148 and PTP-1B have distinct functions in platelets. CD148 is essential for maintaining a pool of active SFKs in platelets, whereas PTP-1B plays little or no role in activating SFKs in resting or activated platelets. This may be due to redundancy with another PTP. We suspect the reason for the discrepancy between our findings and those of the Shattil group, regarding the function of PTP-1B in

GPVI and αIIbβ3 signalling, is strain variation of the mouse models. Another possibility is that another phosphatase compensates in the absence of PTP-1B, which is why we investigated TC-PTP in platelets. Although structurally similar, TC-PTP and PTP-1B appear to have distinct functional roles in platelets. Severely reduced platelet counts and GPVI expression in platelets from TC-PTP-deficient mice suggest that TC-PTP may be involved in platelet development (You-Ten et al. 1997). Collectively, these findings demonstrate that PTPs have distinct functional roles in platelets.

Appendix

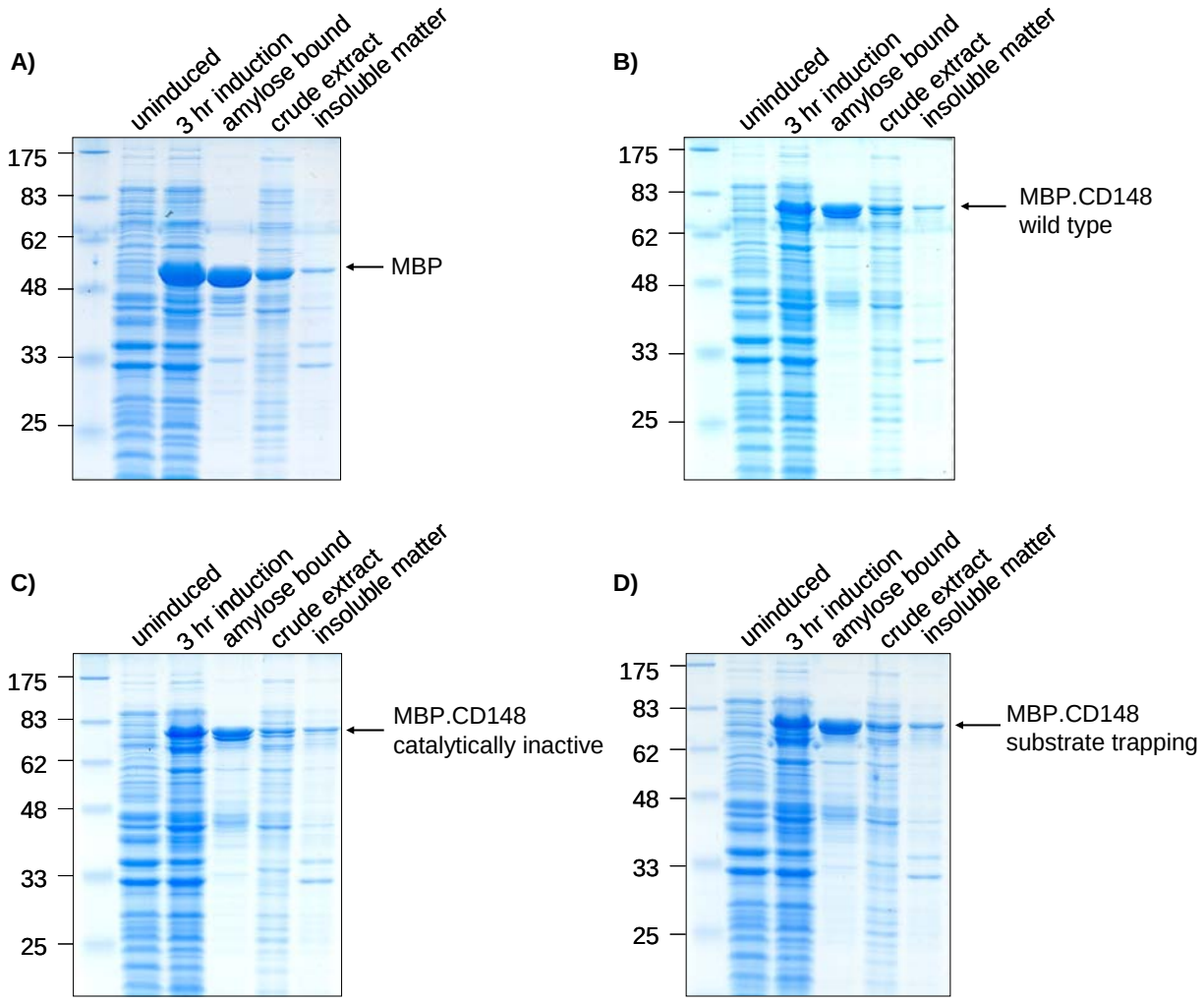


Figure 1: Confirmation of MBP.CD148 fusion protein expression

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