INVESTIGATING THE ROLE OF SRC FAMILY KINASES IN αIIbβ3-MEDIATED PLATELET ACTIVATION

By

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Abstract

αIIbβ3 is the major integrin expressed in platelets and plays a critical role in platelet aggregation and cessation of bleeding. Signalling via this integrin is critically dependent on the Src family kinases of which there are eight members. There are several Src family kinases expressed in platelets, however the roles of the individual members in platelet signalling is not clear. Platelets also express G protein-coupled receptors whose signalling is classically thought to be dependent on their G proteins, however some evidence for dependence on both Src family kinases and other platelet receptors exists. Therefore, the aims of this thesis are to quantitate the level of Src family kinases expressed in human and mouse platelets and to characterise the roles of the individual members in functional responses downstream of αIIbβ3. The final aim of this thesis is to characterise the role of Src family kinases in G_i-coupled receptors.

Subsequent to work in our lab identifying Lyn, Fyn, Src and Fgr as the members of Src family kinases expressed in mouse platelets, I have demonstrated that there are differential levels of expression of SFKs in mouse and human platelets, with mouse platelets expressing Lyn at 10x the level of the other members and human platelets expressing 2x the level of Fyn in comparison to other SFKs. Further to this, utilising mutant mouse models, I demonstrate that Src plays a critical positive role in αIIbβ3-mediated spreading on immobilised fibrinogen, with Lyn playing a negative role. Interestingly, this negative role appears to be downstream of Src. In contrast to these results, individual Src family kinases do not appear to play a role in clot retraction or an *in vivo* tail bleeding assays, despite the Src family kinase inhibitor, Dasatinib having a significant effect. Finally, I demonstrate that adrenaline-mediated aggregation is dependent on signals from secondary mediators, particularly Thromboxane A_2

for full response. Remarkably, both G_i-coupled receptors in human platelets, i.e. α2A for adrenaline and $P2Y_{12}$ for ADP are critically dependent on Src family kinases and αIIbβ3 for signalling, with a partial dependence on PI 3-kinase isoforms. Interestingly, neither adrenaline nor ADP stimulate tyrosine phosphorylation of Src family kinases downstream of their G_i-coupled receptors in either platelets stimulated in buffer or plasma. This suggests a role for the basal phosphorylation of Src family kinases which may be dependent on αIIbβ3-mediated signalling.

In loving memory of

Percy Trevor Rowles

7 April 1929 – 23 March 2010

A wonderful Gramp and the man who first started my love of the natural world

Olive Mary Nash

26 June 1923 – 28 February 2012

A loving and caring Nan who will be forever missed

Publications arising from this thesis

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Severin S, Pollitt AY, Navarro-Nunez L, **Nash CA**, Mourao-Sa D, Eble JA, Senis YA, Watson SP. Syk-dependent Phosphorylation of CLEC-2 : A novel mechanism of Hem-Immunoreceptor tyrosine based activation motif signalling. J Biol Chem 2010; 289: 4107-4116

Severin S, **Nash CA**, Mori J, Zhao Y, Abram C, Lowell CA, Senis YA, Watson SP. Distinct functional roles of Src family kinases in mouse platelets. J Thromb Haemost 2012 (Under review)

“More gold has been mined from the thoughts of men than has been taken from the earth.”

Napoleon Hill

“Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science”

Jules Henri Poincaré

"The blood is the life!"

Bram Stoker, Dracula

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Abbreviations

5-HT 5-hydroxytryptamine (serotonin)
ACD Acid citrate dextrose
ADP Adenosine diphosphate
AMP Adenosine monophosphate
ATP Adenosine triphosphate
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
cGMP Cyclic guanosine monophosphate
CLEC-2 C-type lectin-like receptor 2
CRP Collagen related peptide
DAG Diacylglycerol
DIC Differential interference contrast
DMSO Dimethylsulfoxide
ECL Enhanced chemiluminescence
EDTA Ethylenediamine tetra-acetic acid
EGTA Ethylene glycol tetra-acetic acid
FAK Focal adhesion kinase
FcR Fc receptor
Gads Grb2 adaptor downstream of Shc
GDP Guanine diphosphate
GEF Guanine nucleotide exchange factor
GPCR G protein-coupled receptor
GPVI Glycoprotein VI
Grb2 Growth factor receptor bound protein-2
GST Glutathione-S-transferase
GTP Guanine triphosphate
HRP Horseradish peroxidase
Ig Immunoglobulin
IP Immunoprecipitation
IP_3 Inositol-1,4,5-trisphosphate
ITC Isothermal titration calorimetry
ITAM Immunoreceptor tyrosine based activation motif
ITIM Immunoreceptor tyrosine based inhibition motif
kDa Kilodalton
LAT Linker for activation of T-cells
mAb Monoclonal antibody
pAb Polyclonal antibody
PAR Protease activated receptor
PAS Protein A sepharose
PBS Phosphate buffered saline

PGI_2 Prostaglandin I_2
PGS Protein G sepharose
PH Pleckstrin homology
PI-3 kinase Phosphatidyl inositol-3 kinase
PIP_2 Phosphatidyl inositol-4,5-bisphosphate
PIP_3 Phosphatidyl inositol-3,4,5-trisphosphate
PKA Protein kinase A
PKC Protein kinase C
PKG Protein kinase G
PLA Phospholipase A
PLC Phospholipase C
PRP Platelet rich plasma
PS Phosphatidyl serine
PTB Phosphotyrosine binding
PVDF Polyvinyl difluoride
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFK Src family kinase
SH2 Src homology 2
SH3 Src homology 3
SLP-76 SH2 containing leukocyte protein of 76 kDa
TBS-T Tris buffered saline-Tween
TPO Thrombopoietin
TxA_2 Thromboxane A_2
VWF Von Willebrand Factor
WT Wild type

Contents

CHAPTER 1
GENERAL INTRODUCTION

1.1 Platelet overview

Platelets are discoid, anucleate cells which play a critical role in the processes of haemostasis and thrombosis. In the blood of healthy individuals, platelets circulate at levels of 150-400x10^9/L, however, individuals with levels as low as 50x10^9/L seldom display significant bleeding indicating considerable spare capacity.

Platelets circulate at the edge of blood vessels due to the presence of other, larger blood cells and the physics of blood as a laminar fluid. Platelets undergo rapid clumping following damage to the blood vessel wall or in diseased vessels leading to prevention of excessive blood loss or thrombosis, respectively. Arterial thrombotic disorders such as myocardial infarction and stroke are two of the major killers of man in the western world and individuals considered at risk of thrombosis are treated long term with low dose aspirin.

Platelet biogenesis

Platelets form by the budding from larger precursors, known as megakaryocytes. These cells extend long cytoskeletal protrusions, known as pro-platelets from which *de novo* platelets bud, generating 2000-3000 platelets each (Hartwig and Italiano, 2003). Megakaryocytes mature from a common stem cell progenitor, the haematopoetic stem cell. Under control of the cytokines, notably thrombopoietin, this progenitor matures to form the common myeloid progenitor (CMP), subsequently the erythromyeloid progenitor and finally the megakaryocyte (Kaushansky, 1995). Platelets have a life span of approximately 7-10 days before removal by the reticuloendothelial system.

Platelet structure

Platelets are discoid in shape whilst at rest, but change shape rapidly upon activation with the interchange regulated by a membrane cytoskeleton and microtubule network, consisting of three main components: an actin network, a spectrin membrane skeleton and a microtubule coil which forms a ring underneath the plasma membrane. These components also play a role in the movement of transmembrane receptors within the plasma membrane and between intracellular membranes.

The plasma membrane itself contains all of the receptors required for platelet activation and aggregation, including the platelet specific integrin, αIIbβ3, the most abundant platelet surface protein. In addition, phosphatidylserine is found within the inner leaflet of the plasma membrane and is exposed on platelet activation. This lipid is known to provide a procoagulant surface that supports the generation of thrombin.

Platelets are a major secretory cell which has four types of storage granule. These are α- and dense-granules, lysosomes and peroxisomes; the importance of the latter two is unknown at present. There are 5-9 dense granules per platelet containing high levels of the secondary mediator ADP as well as other components including 5-hydroxytryptamine (5-HT) which mediates vasoconstriction and polyphosphate, which activates factors of the coagulation cascade. There are ~80 α-granules per platelet, containing a wide range of proteins which play a variety of roles including provision of support for aggregation e.g. fibrinogen; vessel repair e.g. VEGF and PDGF; and attraction of leukocytes and circulating stem cells e.g. the chemokine SDF-1α (Watson and Harrison, 2010, White, 2006).

Within the platelet cytosol, there is a network of intracellular membranes designated the dense tubular system. This has many similarities with the endoplasmic reticulum of other cells and serves as a store for intracellular Ca^{2+}, which can be released in response to cellular stimuli. The dense tubular system is also the site for COX-1, the enzyme implicated in generation of thromboxane A_2 (TxA_2). The platelet surface also possesses an open canalicular system that acts to increase the surface area of the platelet and therefore allow for an increased surface area to facilitate rapid release of storage granules (Watson and Harrison, 2010, White, 2006).

1.2 Platelets haemostatic function

Platelets circulate in the blood in a resting state, maintained by the release of PGI_2 and nitric oxide (NO) from healthy endothelium. In addition, the ectonucleoside triphosphate diphosphohydrolase CD39 cleaves the platelet activator ADP to AMP (Kaczmarek et al., 1996). Endothelial damage exposes subendothelial matrix proteins, including collagen which is considered to be the most thrombogenic component in the vessel wall. Activation of platelets leads to occlusion of the breach and prevention of further blood loss. The stages of the process of aggregate/thrombus formation can be divided into 6 steps as shown in Figure 1.1 (Varga-Szabo et al., 2008, Watson and Harrison, 2010):-

1. *Platelet capture* – the receptors and mechanisms which mediate the process of capture and adhesion are mediated by the prevailing rheological conditions. Under low shear stress, i.e. in the venous system, the platelet integrin α2β1 can bind directly to collagen and tether the platelet to the surface. This interaction has a slow on-off rate, however, and therefore does not take place at higher rates of shear. At shear rates of greater than $1000s^{-1}$, the major receptor involved in tethering is the GPIb-IX-V complex. This receptor binds to the exposed collagen surface via von Willebrand factor, which has

unravelled and bound to the collagen surface. This interaction has a fast on-rate but also fast off-rate and mediates weak signalling which, on its own, does not mediate integrin activation and platelet adhesion.

2. *Platelet activation and stable adhesion* – in order for stable adhesion to occur, platelets integrins are regulated through a process known as inside-out activation. This is initiated by binding of the low affinity collagen receptor GPVI to collagen as a result of tethering of vWF to the GPIb-IX-V complex. This subsequently activates the integrins, αIIbβ3 and α2β1, which bind vWF and collagen, respectively.
3. *Platelet spreading* - platelets undergo a characteristic set of morphological changes following activation. They initially round up and then extend finger-like filopodia followed by lamellapodia. This increases the surface area in contact with the exposed subendothelial matrix and therefore strengthens adhesion.
4. *Secretion and aggregation* – the activated platelets secrete the contents of their α- and dense granules, and synthesise and release TxA_2. ADP and TxA_2 act in synergy to activate further platelets and thereby facilitate their capture from circulation, facilitated by the release of vWF and fibrinogen from platelet α-granules (Kulkarni et al., 2000). Platelets attach to each other through the cross-linking of αIIbβ3 via fibrinogen.
5. *Thrombin generation* –The procoagulant surface provided by phosphatidylserine supports the generation of thrombin which allows for further activation of platelets via protease activated receptors (PAR) and the generation of a fibrin mesh from soluble fibrinogen. This fibrin mesh acts to further occlude the breach in the endothelium.
6. *Further strengthening of the growing thrombus* – The fibrin rich clot is further strengthened by the process of clot retraction, which is mediated via binding of

integrin αIIbβ3 to the actin cytoskeleton through a pathway that is partially regulated by Src family kinases (Shattil et al., 1998, Suzuki-Inoue et al., 2007a). Several other membrane protein are also implicated in the late stage events underlying aggregation such as the binding of ephrins and eph kinases, although their overall functional significance is unclear (Prevost et al., 2005).

Other than this 'classical' role in haemostasis, platelets also play a role in various other physiological and pathological processes, such as angiogenesis (Sabrkhany et al., 2011), cancer metastasis (Jain et al., 2007, Jain et al., 2009), closure of the ductus arteriousus in mice (Echtler et al., 2010) and lymphatic development (Bertozzi et al., 2010a).

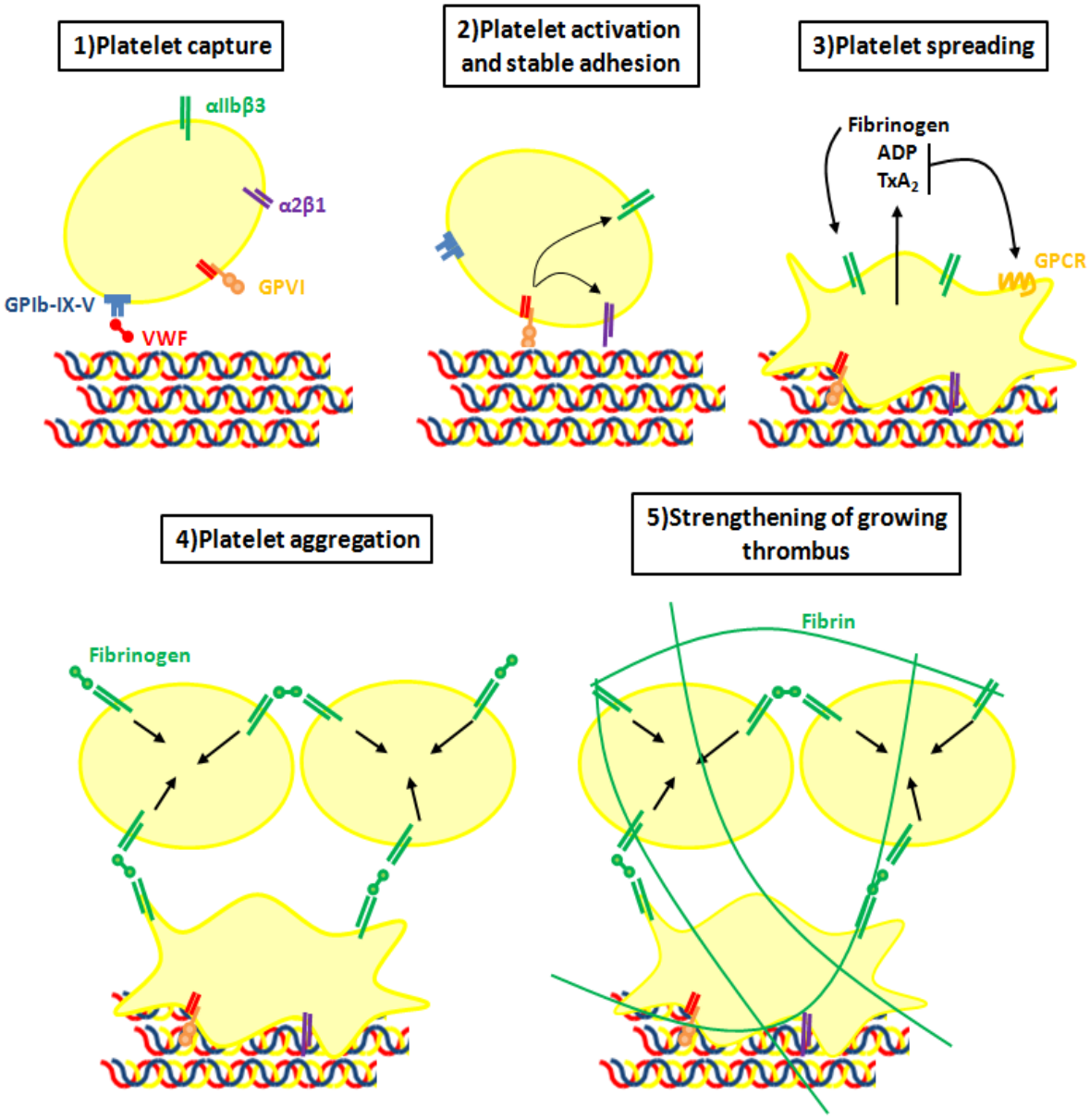


Figure 1.1 The process of thrombus formation. Based on (Varga-Szabo et al., 2008, Watson and Harrison, 2010)

1.3 Platelet signalling receptors

In order for platelets to respond to their environment, they must express a variety of transmembrane receptors, which transmit signals from the extracellular milieu to the inside of the cell. In platelets, these receptors fall into two major categories: single transmembrane receptors which signal via tyrosine kinases (notably via Src family kinases and Syk) mainly in response to immobilised matrix proteins or protein ligands on other cells, and seven transmembrane receptors which couple to G proteins and are activated by soluble mediators found within the plasma. In addition, platelets express a receptor-regulated ion channel receptor, $P2X_1$, of uncertain significance and several intracellular receptors, notably soluble guanylyl cyclase which is activated by NO.

1.3.1 Receptors which signal through Src family kinases and Syk.

Src family kinases

Src family kinases (SFKs) are a family of structurally related tyrosine kinases that are associated with the membrane through an N-terminal myristoyl group. There are eight members of the family expressed in the mammalian genome, namely Src, Lyn, Fyn, Fgr, Hck, Lck, Blk and Yes. These enzymes were first identified as genes which have transforming potential in viruses and later discovered as proto-oncogenes expressed in mammalian cells.

SFKs have been shown to be critical in many cellular processes, including immunity, antigen signalling, proliferation, differentiation, migration and adhesion (Boggon and Eck, 2004, Corey and Anderson, 1999, Thomas and Brugge, 1997). They also play a vital role in signalling downstream of many platelet receptors, including GPVI, CLEC-2 and αIIbβ3.

SFKs consist of an N-terminal (unique) SH4 domain, phosphotyrosine binding SH2 domain, PXXP binding SH3 domain and a kinase domain (also known as a SH1 domain). All SFKs have two conserved tyrosine residues which play a critical role in their regulation.

The activity of Src family kinases is tightly regulated through intramolecular interactions between conserved domains. In its basal state, the SH2 domain binds to a phosphorylated tyrosine residue within the C-terminal tail and the SH3 domain associates with a conserved PXXP motif within the SH2-kinase domain linker. These interactions must be disrupted to achieve activation. This can occur through dephosphorylation of the C-terminal tail, releasing the interaction with the SH2 domain, or by disruption of the interaction between the SH3 or SH2 domains and their binding partners. In the earlier scenario, the C-terminal tail is dephosphorylated by receptor tyrosine phosphatases, such as CD148 and PTPIb (Roskoski, 2005). In the latter scenario, dephosphorylation of the C-terminal tail is not required. This is illustrated by disruption of the SH3-PXXP interaction in Hck by the HIV protein Nef (Moarefi et al., 1997) or disruption of SH2-C-terminal tail interaction in the epidermal growth factor receptor (Luttrell et al., 1994). Disruption of the C-terminal tail tyrosine generates a kinase with significantly greater activity wild type protein as illustrated in the vSrc oncogene. vSrc is a viral oncogene expressed in the Rous Sarcoma virus that differs by a mutation removing the C-terminal regulatory tyrosine. (Cooper et al., 1986, Kmiecik and Shalloway, 1987). Full activation of Src family kinases requires phosphorylation of a second conserved tyrosine residue within the activation site (Su et al., 1999).

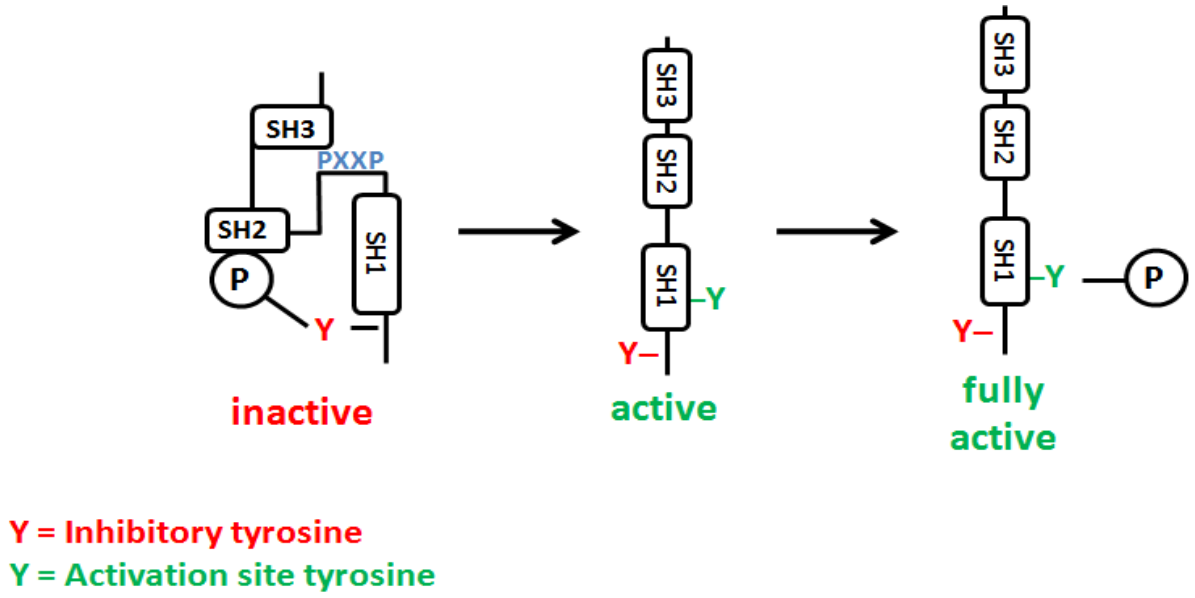


Figure 1.2 Activation of Src family kinases. Src family kinases are maintained in an inactivate state by intracellular interactions between their SH2 domain and intracellular tail, along with interaction between the SH3 domain and a conserved PXXP motif in the interdomain linker. Src family kinases are activated upon displacement of these interactions. However, for full activation to occur, a conserved tyrosine within the activation site must become phosphorylated.

SFKs are myristoylated at their N-terminus. This is necessary, but not sufficient to localise them to the membrane, with membrane localisation also requiring a polybasic region containing three alternating lysines (Silverman and Resh, 1992). Recent evidence has shown that defective myristoylation is associated with defects in signalling (Patwardhan and Resh, 2011). Six of the eight Src family kinases, namely Hck, Src, Lck, Fgr, Fyn and Lyn, contain a conserved Cys residue at the N-terminus (Paige et al., 1993, Shenoy-Scaria et al., 1993) which can undergo palmitoylation (Resh, 1994). The addition of palmitate allows the SFKs to be localised to lipid rafts. Lipid rafts are microdomains found within the plasma membrane enriched in cholesterol and glycosphingolipids as well as many receptors and signalling proteins.

Syk family kinases

The Syk family of tyrosine kinases consists of two members, Syk and ZAP-70. Syk has widespread distribution throughout the haematopoietic system, whereas ZAP-70 is restricted to T-cells and a subpopulation of natural killer cells (Bradshaw, 2010, Turner et al., 2000). Syk family kinases consist of dual SH2 domains followed by a kinase domain. Between these two regions is an interdomain region which contains several conserved tyrosines and is believed to play a role in substrate recruitment (Bradshaw, 2010).

Syk family kinases play a critical role in signalling downstream of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors. ITAMs consist of two YXXL sequences separated by 8-12 amino acids and were first identified by Reth in 1989 (Reth, 1989). When the tyrosine residues contained within the motif become phosphorylated, usually by SFKs, Syk family kinases are recruited through their dual SH2 domains which bind to the phosphotyrosine residues in a cooperative manner (Grucza et al., 1999). Mutation of one or both of the SH2 domains generates a mutant that is unable to signal downstream of ITAM receptors (Kurosaki et al., 1995). Recruitment allows for activation and removal of an inhibitory interaction between interdomain regions A and B and parts of the kinase domain of Syk. This event is then followed by autophosphorylation and phosphorylation by SFKs (Geahlen, 2009). Recently, a related motif consisting of a single YxxL has been identified in a subgroup of C-type lectin receptors, known as a hemITAM. Syk plays a critical role in signalling by this family of receptors through crosslinking of two phosphorylated hemITAMs. ITAM and hemITAM motifs are found in the GPVI-Fc γ-chain complex and CLEC-2, respectively, in platelets (Watson et al., 2010).

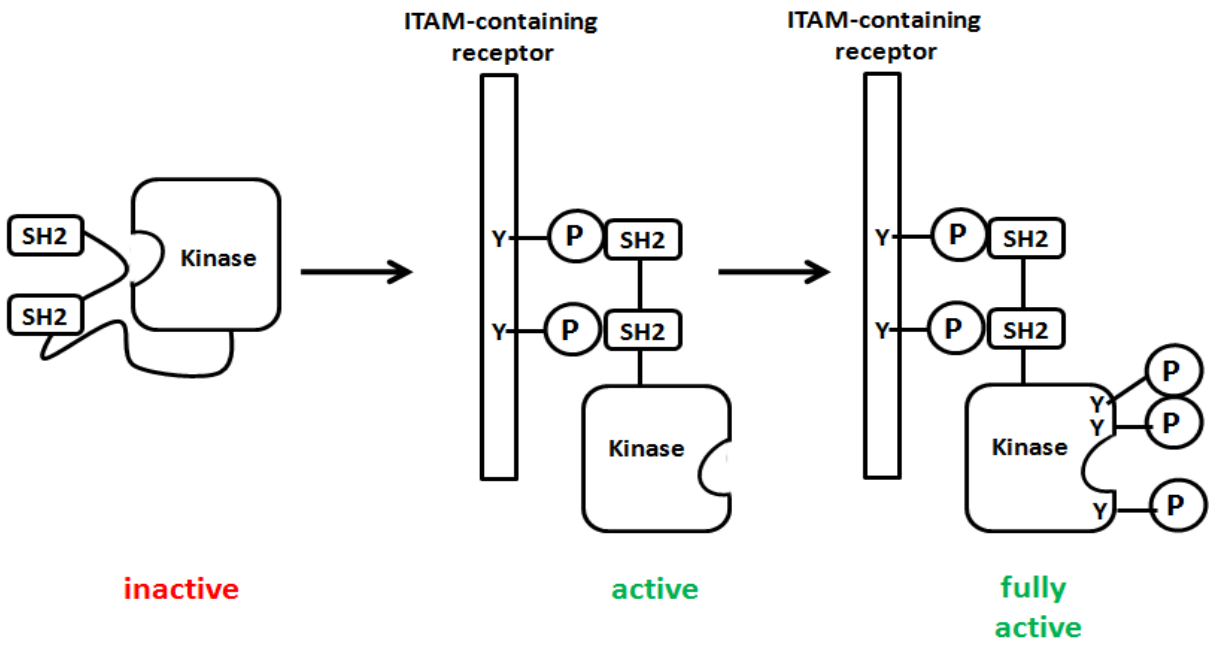


Figure 1.3 Activation of Syk by ITAM containing receptors. Syk is maintained in an inactive state by interaction between interdomain region B and the active site. Upon binding to a phosphorylated ITAM, this inhibition is removed and Syk becomes fully active. Following activation, Syk undergoes an autophosphorylation event and may also be phosphorylated by the Src family kinases, inducing full activity.

1.3.1.2 YXXL containing platelet receptors

GPVI

GPVI is an Ig-like receptor expressed at a level in the order of 2500-6000 per human platelet (Best et al., 2003, Samaha et al., 2004). It is the major signalling collagen receptor in the platelet and is known to bind to GPO motifs in fibrillar collagen. In the platelet membrane, it is non-covalently associated to the ITAM-containing Fc receptor (FcR) γ-chain. Mice deficient in either GPVI (Kato et al., 2003) or the FcR γ-chain (Kalia et al., 2008, Poole et al., 1997, Senis et al., 2009b) show defects in platelet aggregation to collagen in both stirring and flow conditions. The γ-chain has been shown to be essential for GPVI surface expression in both platelets and some cell lines (Nieswandt et al., 2000, Berlanga et al., 2002).

Upon ligation of GPVI, the conserved ITAM within the co-expressed FcR γ-chain is phosphorylated in a Src family kinase-dependent manner. This phosphorylation is mediated by both Lyn and Fyn (Ezumi et al., 1998, Quek et al., 2000). Lyn is thought to be responsible for initiation of GPVI stimulation as both aggregation and phosphorylation of the γ-chain are delayed in Lyn deficient platelets, followed by potentiation due to loss of feedback inhibitory signals. Lyn has also been demonstrated to play a key role in platelet adhesion to collagen under high shear conditions (Schmaier et al., 2009). Fyn deficient platelets, on the other hand, show a mild reduction in phosphorylation and aggregation but no delay in the onset of the response. Interestingly, another Src family kinase is also involved as a limited degree of signalling is seen in the combined absence of Lyn and Fyn (Quek et al., 2000).

The membrane tyrosine phosphatase, CD148, regulates the basal level of activity of all Src kinases in platelets through regulation of the inhibitory and stimulatory sites of phosphorylation (Ellison et al., 2010, Senis et al., 2009b). It dephosphorylates the C-terminal

tyrosine residue of all Src kinases, thereby increasing their activity (Senis et al., 2009b). It is unclear however whether GPVI signalling regulates the activity of CD148 or whether the tyrosine phosphatase is required to regulate the basal level of SFK activity. Following phosphorylation of the FcR γ-chain ITAM, Syk is recruited via it dual SH2 domains. Syk is then activated by phosphorylation by both Src family kinases and autophosphorylation (Futterer et al., 1998, Spalton et al., 2009, Geahlen, 2009).

The Src family kinases Fyn and Lyn have both been shown to be constituitively associated with GPVI through the binding of their SH3 domains to a conserved PXXP motif within the GPVI cytoplasmic tail (Bori-Sanz et al., 2003, Suzuki-Inoue et al., 2002, Schmaier et al., 2009). It has been proposed that this association holds the SFK in a 'primed' activation state to allow for rapid phosphorylation upon receptor ligation. However, deletion of the motif in GPVI reduces, but does not abrogate signalling in platelets and in transfected cell lines (Schmaier et al., 2009, Bori-Sanz et al., 2003).

Following Syk activation, LAT is phosphorylated on one or more of its nine tyrosine residues by Syk (Judd et al., 2002, Pasquet et al., 1999). This allows for the formation of a signalosome composed of the adapters LAT, SLP-76 and Gads, along with a number of effectors including phospholipase PLCγ2 (Asazuma et al., 2000). Deficiency of LAT and SLP-76 severely compromises or abolishes aggregation downstream of GPVI, respectively (Judd et al., 2002, Pasquet et al., 1999, Hughes et al., 2008). Deficiency of Gads, however, only generates a minor aggregation defect (Hughes et al., 2008). Other effector enzymes include the Vav family of GEFs (Pearce et al., 2004, Pearce et al., 2002), the Tec kinases, Btk and Tec (Atkinson et al., 2003) and PI 3-kinase α and β isoforms (Canobbio et al., 2009, Gilio et al., 2009).

Activation of PLCγ2 is critical in signalling downstream of GPVI (Suzuki-Inoue et al., 2003). PLCγ2 acts to hydrolyse its substrate phosphatidylinositol-4,5-bisphosphate to generate 1,2-diacylglycerol and 1,4,5-inositoltrisphosphate, which activate protein kinase C (PKC) and open intracellular Ca^{2+} channels, respectively.

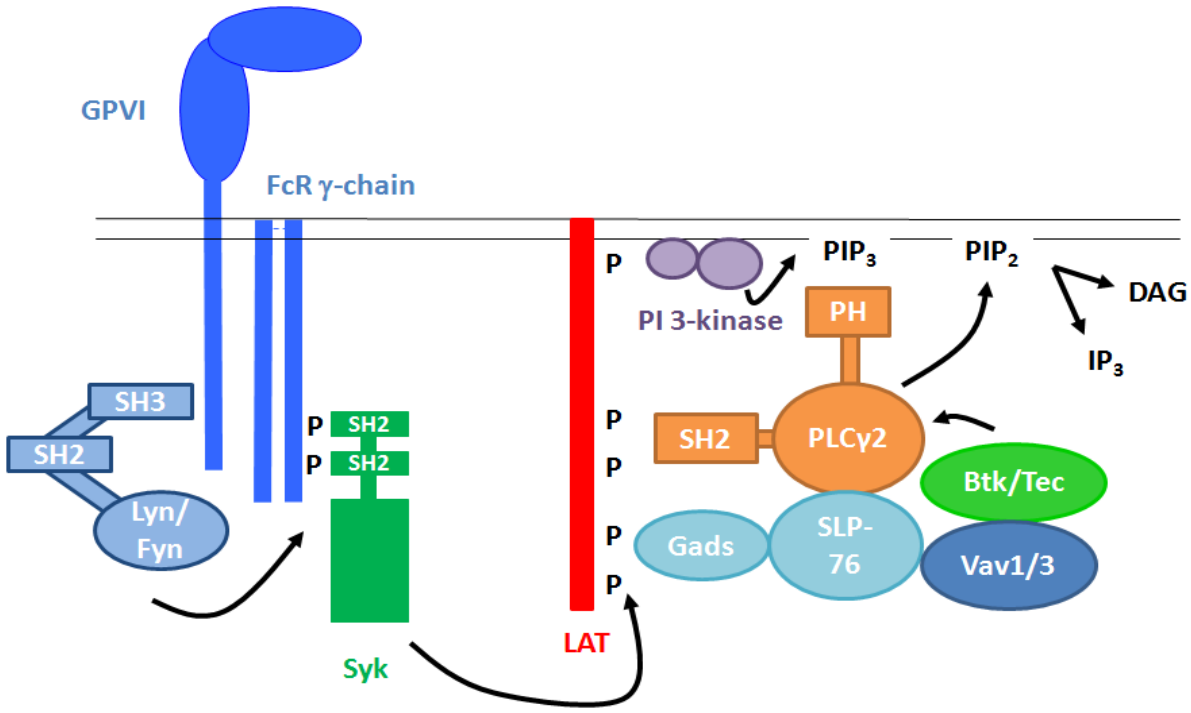


Figure 1.4 GPVI signalling cascade. Ligation of the collagen receptor GPVI activates Lyn and Fyn. Following this activation, Syk is recruited to the γ-chain and is activated. This activation induces phosphorylation of LAT and downstream recruitment of adapters such as Gads and SLP-76. Following this, PLCγ2 becomes activated and hydrolysis of PIP_3.

FcγRIIa

FcγRIIa is a second ITAM-containing protein expressed at a level of 3000-5000 per human platelet. This receptor is thought to signal via a similar pathway to GPVI, although the investigation of this is held back by its absence from the mouse genome. FcγRIIa is a low affinity member of the IgG binding immune receptors and is activated by clustering in response to the Fc region of IgG isotype antibodies. Similarly to GPVI, FcγRIIa contains two extracellular Ig domains, a transmembrane region and an intracellular tail. However, in contrast to GPVI, FcγRIIa contains an ITAM sequence within the receptor itself. FcγRIIa has been shown to associate with Syk in platelets (Chacko et al., 1996, Chacko et al., 1994) in a Src family kinase dependent manner (Huang et al., 1992). This association induces phosphorylation of downstream targets such as LAT (Ragab et al., 2003) and PLCγ2 (Gratacap et al., 1998).

The C-type lectin receptor CLEC-2

C-type lectin 2 (CLEC-2) is a non-classical C-type lectin receptor expressed on platelets and at a low level on mouse neutrophils. CLEC-2 is also expressed on cells of the myeloid lineage and up-regulated upon LPS challenge (Chaipan et al., 2006, Mourao-Sa et al., 2011). It was first identified as a receptor for the snake venom toxin rhodocytin obtained from the Malayan pit viper *Calloselasma rhodostoma* (Suzuki-Inoue et al., 2006). It has subsequently been shown that CLEC-2 responds to the endogenous ligand podoplanin (Suzuki-Inoue et al., 2007b, Christou et al., 2008). This is expressed on the leading edge of many tumour cells as well as lung alveolar type I cells and kidney podocytes. It has been demonstrated that animals deficient in podoplanin (Fu et al., 2008, Schacht et al., 2003, Uhrin et al., 2010) and CLEC-2 show lymphatic separation defects (Bertozzi et al., 2010b, Suzuki-Inoue et al., 2010), suggesting that platelets and in particular CLEC-2 play a significant role in lymphatic development. It may be that this is the major role of CLEC-2 as platelets deficient in CLEC-2 do not demonstrate haemostatic defects (Hughes et al., 2010a), however the Nieswandt and Suzuki Inoue groups suggest that CLEC-2 does play a role in haemostasis (May et al., 2009, Suzuki-Inoue, 2011).

The structure of CLEC-2 consists of an N-terminal cytoplasmic domain, a single pass transmembrane region, a stalk region and a C-terminal carbohydrate-like recognition domain (CRD). This lacks the residues to bind carbohydrate but does mediate the interaction with protein ligands (Weis et al., 1998).

Within the N-terminal tail is a single YXXL motif preceded by a tri-acidic amino acid region. Mutation of the conserved tyrosine abolishes activation downstream of CLEC-2, suggesting that it plays a critical role in signalling downstream of the receptor (Fuller et al., 2007). Upon stimulation with rhodocytin, platelets show a similar increase in tyrosine phosphorylation to

that seen in the GPVI-Fc γ-chain pathway, including tyrosine phosphorylation of Syk. The use of both pharmacological inhibitors of Syk and Syk-deficient platelets has confirmed that the tyrosine kinase is critically important in signalling downstream of CLEC-2 (Severin et al., 2011, Spalton et al., 2009, Suzuki-Inoue et al., 2006). Due to the similarity with ITAM-mediated pathways, the YXXL has been named a hemITAM by the Reis e Sousa group (Mourao-Sa et al., 2011).

Evidence suggests that the interaction between Syk and CLEC-2 is direct, with a phosphorylated CLEC-2 peptide being able to precipitate Syk from a platelet lysate. (Suzuki-Inoue et al., 2006). CLEC-2, however, does not possess a second YXXL motif in order to bind the tandem SH2 domains of Syk, therefore the kinase must bind in a novel manner. In support of this hypothesis it has been shown by point mutation experiments that both SH2 domains of Syk are required for signalling downstream of CLEC-2 (Fuller et al., 2007) and that the receptor exists as a dimer on the surface of resting platelets (Hughes et al., 2010b). In addition to this, co-expression of CLEC-2 with a mutated tyrosine and wild type receptor abolishes signalling (Hughes et al., 2010b), most likely due to dimerisation of mutant and wild type receptors.

In addition to a requirement for Syk, it has also been demonstrated that signalling downstream of CLEC-2 requires the Src family kinases. In the presence of the pan-Src family kinase inhibitor PP2, platelet aggregation in response to CLEC-2 is abolished (Fuller et al., 2007, Suzuki-Inoue et al., 2006, Severin et al., 2011). Severin *et al* have recently demonstrated that in response to rhodocytin, phosphorylation of CLEC-2 and Syk is reduced but not completely abolished in the presence of PP2, as is the case for a CLEC-2 activating antibody. The phosphorylation and activation downstream of CLEC-2 has been demonstrated to be specifically downstream of the Src family kinase, Lyn, with platelets deficient in either Src or

Fyn showing no obvious defect over wild type control platelets. Mice doubly deficient in Lyn and either Src or Fyn show an increased defect compared to Lyn-deficient platelets downstream of CLEC-2 antibody but not rhodocytin, however, aggregation is not completely abolished. Taken with the results from PP2 treated cells, this demonstrates that the Src family kinases act in conjunction in order to activate pathways downstream of CLEC-2. In constrast to results seen with GPVI, CD148 is dispensable for signalling downstream of CLEC-2. These results, taken with the critical role for Syk in CLEC-2-mediated signalling, suggest that the role of the Src family kinases in this pathway is to phosphorylate Syk, rather than the receptor itself (Severin et al., 2011).

Downstream of Syk, CLEC-2 utilises a similar signalling cascade to that seen for GPVI. LAT, the Vav family of GEFs and PLCγ2 have all been shown to play critical roles in CLEC-2 signalling. SLP-76 has also been shown to play a role, however, the requirement for this can be overcome by high concentrations of rhodocytin (Suzuki-Inoue et al., 2006). In contrast to GPVI, however, CLEC-2 signalling is critically dependent on ADP and TxA_2. It also requires actin polymerisation and signalling via the Rac GTPase, which appears to be activated downstream of secondary mediators or actin in this pathway (Pollitt et al., 2010). These events have been demonstrated to be essential for CLEC-2 phosphorylation and downstream signalling.

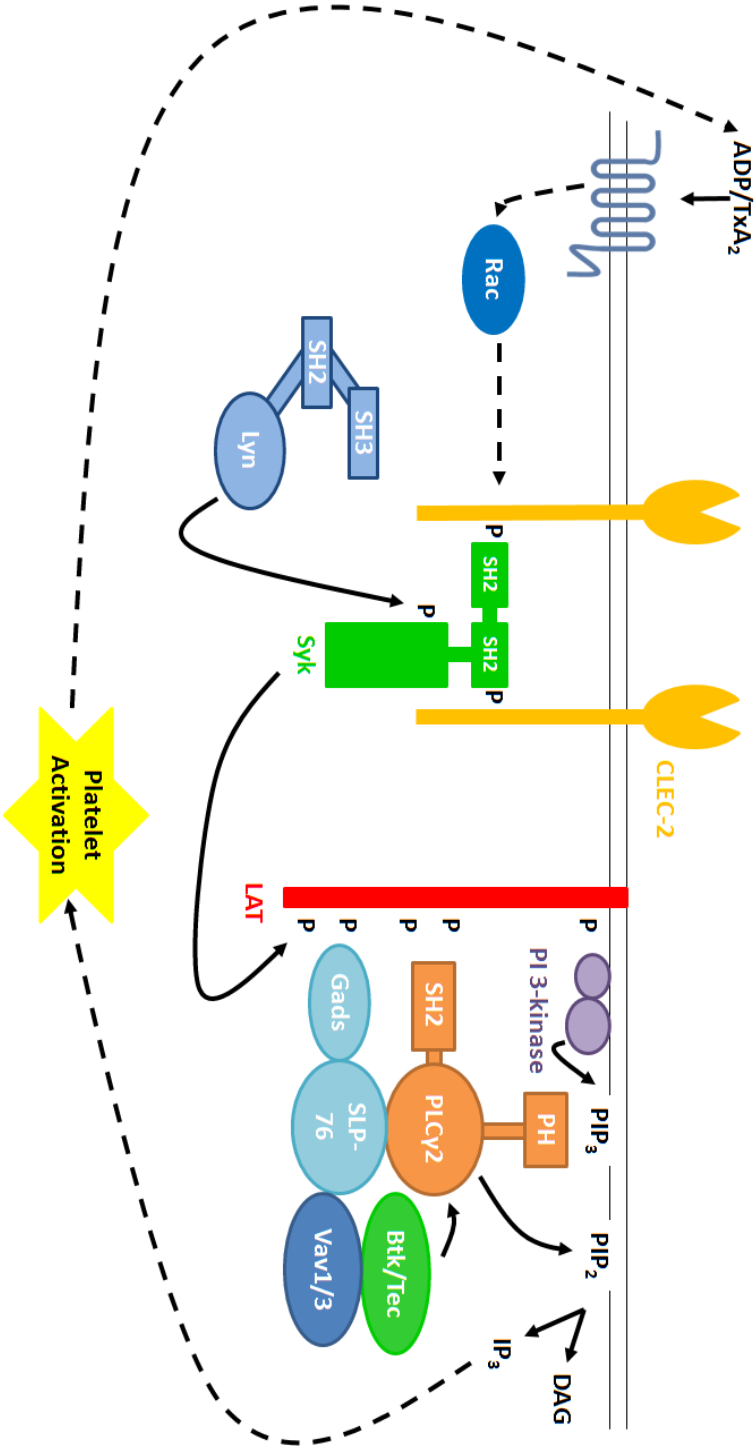


Figure 1.5. The CLEC-2 signalling cascade. Upon CLEC-2 ligation, Syk tyrosine kinase binds to the CLEC-2 dimer via one YXXL motif on each molecule. Syk is then activated in a SFK-dependent manner. Following activation, Syk phosphorylates LAT leading to recruitment of adapters such as SLP-76 and Gads. This leads to activation of PLCγ2. CLEC-2 dependent platelet aggregation is dependent on the release of secondary mediators from the platelet and activation of Rac.

1.3.1.2 Platelet integrins

αIIbβ3

αIIbβ3 heterodimer is the major receptor expressed on the platelet surface at a copy number of ~80,000 per resting human platelet (Wagner et al., 1996). This increases up to ~120,000 in activated platelets through docking of platelet α-granules in the membrane (Cramer et al., 1990). The αIIb-subunit is expressed specifically in the megakaryocyte/platelet lineage (Uzan et al., 1991), whereas β3 is ubiquitous. Mice and humans deficient in the integrin display strong platelet-based bleeding disorders due to their inability to aggregate (Nurden and Caen, 1974, Phillips et al., 1975, Hodivala-Dilke et al., 1999). αIIbβ3 also generates intracellular signals (known as outside-in signalling) upon ligand engagement which combine with other agonists to reinforce platelet activation.

The αIIb-subunit is composed of a disulphide-linked heavy and light chain, created by proteolytic processing of the protein. The αIIb-subunit has a 7 bladed β-propeller domain at the N-terminus which contains divalent cation binding motifs. The extracellular region of the protein also contains a 'thigh' region and 2 'calf' region. Between these two regions is a 'genu', a region with a knee-like bend which allows the molecule to maintain a compact structure. The transmembrane region consists of a single helix terminating in a bend (Vinogradova et al., 2000) and the intracellular region of human αIIb consists of 20 amino acids, six of the final eight of which are acidic and form a cation binding pocket (Haas and Plow, 1996, Vallar et al., 1999). The β3-integrin contains an A domain, which contains 3 cation binding sites, hybrid and PSI domains, all of which are implicated in integrin activation. The β3 extracellular domain also consists of a protease resistant domain and four endothelial growth factor (EGF)-like domains (Beglova et al., 2002) and a βTD domain (Haas

and Plow, 1997). The transmembrane helix extends into the 48 amino acid cytoplasmic tail that contains many of the motifs required for signalling (Ulmer et al., 2001). The description of this structure is based on the crystal structure of αvβ3 as shown in Figure 1.6 (Xiong et al., 2001) and NMR studies of αIIbβ3.

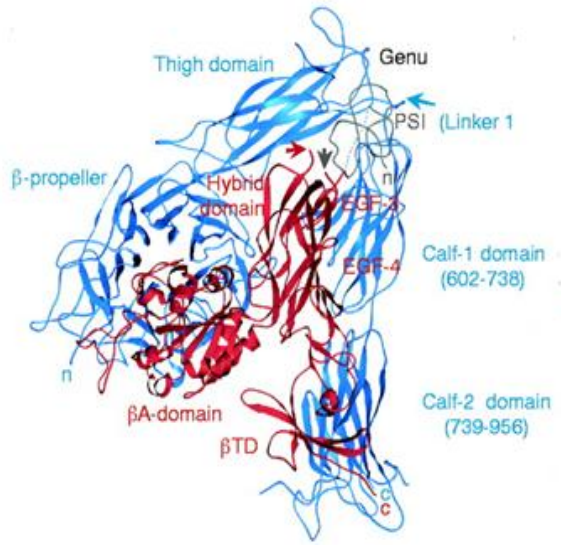


Figure 1.6. The crystal structure of the extracellular domain of αvβ3. The structure of αvβ3 has been used as a model to describe the 3D structure of αIIbβ3. αv is shown in blue and β3 in red. Taken from (Xiong et al., 2001).

Integrin αIIbβ3 recognises ligands which contain a fibrinogen γ-chain sequence or an RGD region (Plow et al., 1987). These two motifs bind to distinct allosteric sites (Cierniewski et al., 1999). Fibrinogen contains two RGD sequences and a single γ-chain, but only the latter mediates integrin binding (Farrell et al., 1992) whereas, for example, VWF binds through its RGD sequence.

Clustering of αIIbβ3 generates outside-in signals via Src family kinases, with Src being considered the principle player. Fibrinogen binding leads to the displacement of the negative regulatory of Src kinases, Csk, from the β3-subunit (Arias-Salgado et al., 2003, de Virgilio et

al., 2004, Obergfell et al., 2002, Ohmori et al., 2000) and subsequent dephosphorylation of the C-terminal inhibitory tyrosine residue by PTPIb (Arias-Salgado et al., 2005) or CD148 (Senis et al., 2009b). In turn, the Src family kinases induce phosphorylation and activation of the tyrosine kinase Syk as well as phosphorylation of two conserved tyrosines within the β3 tail which fall in a PTB binding domain; significantly, these are exclusive events with phosphorylation of the β3-tail inhibiting binding of Syk (Obergfell et al., 2002, Woodside et al., 2001, Woodside et al., 2002). Futher, mutation of the two conserved tyrosines within the β3 tail also prevents Syk binding (Woodside et al., 2002) and leads to mild bleeding and clot retraction defects in a mouse model (Law et al., 1999a).

Syk activation is critical for spreading on a fibrinogen-coated surface (Law et al., 1999b, Spalton et al., 2009, Hughan et al., 2007) through initiation of a signalling pathway that shares some homology with that of GPVI. The signalling proteins SLP-76, Vav and the Tec family kinases Btk and Tec have all been shown to play a role in signalling downstream of the integrin (Judd et al., 2000, Obergfell et al., 2001, Pearce et al., 2007, Atkinson et al., 2003). In addition, PLCγ2 is essential for spreading on a fibrinogen coated surface (Wonerow et al., 2003, Mangin et al., 2003). In contrast to GPVI, however, αIIbβ3-mediated PLCγ2 activation does not require LAT, which is localised to lipid rafts (Hughes et al., 2008, Wonerow et al., 2003). αIIbβ3 is found outside of lipid rafts and disruption of these microdomains does not affect αIIbβ3-mediated signalling (Wonerow et al., 2002). This observation corroborates the observation that Src, which is also localised outside of rafts, is critical for signalling downstream of the integrin.

Several other effector enzymes and adapters have been demonstrated to bind to the β3 tail tyrosines. The focal adhesion kinase family members p125FAK and Pyk2 have both been shown to interact with β3 tail in a phosphorylation-dependent manner (de Virgilio et al., 2004,

Ohmori et al., 2000). Further, deficiency of p125FAK in platelets has been demonstrated to cause diminished spreading on a fibrinogen coated surface (Hitchcock et al., 2008), however analysis of the interaction of Pyk2 knockout platelets with fibrinogen has not taken place.

The cytoskeletal protein talin binds to the β3 N-terminal NXXY motif via its PTB domain in a phosphorylation-independent manner (Tadokoro et al., 2003, Wegener et al., 2007). The association between the PTB domain of talin and αIIbβ3 has been shown to activate the integrin and allow ligand binding (Bouaouina et al., 2008, Calderwood et al., 1999). Talin consists of head and rod domains. Within the head domain is a FERM domain composed of three subdomains, F1, F2 and F3. The F3 domain is the PTB domain required for binding to integrins. The rod domain consists of a series of helical bundles which contains multiple binding sites for vinculin and a further integrin binding site. The C-terminus of the protein contains a THATCH domain, capable of directly binding actin (Moser et al., 2009).

Talin is essential for integrin activation (Tadokoro et al., 2003) and talin-deficient platelets fail to spread on fibrinogen or support aggregation under static and flow conditions (Nieswandt et al., 2007, Petrich et al., 2007b). Talin-deficient platelets have also been shown to have defective clot retraction, a process dependent on αIIbβ3 signalling to the cytoskeleton (Haling et al., 2011). Selective mutation of talin binding sites within the β3 integrin, i.e. Y747 and L746, also induces bleeding and defective inside-out integrin activation, even though outside-in signalling is intact in these platelets (Petrich et al., 2007a).

Kindlin family members show structural homology to talin in their FERM domain, containing F1, F2 and F3 domains along with a N-terminal domain. The main difference being that the F2 domain in the kindlin family is split by a plekstrin homology domain (Moser et al., 2009). Mutational studies in Chinese hamster ovary (CHO) cells and binding studies have shown that

kindlin proteins bind to the β3 tail via their PTB domain with a similar affinity to talin and promote integrin activation (Kloeker et al., 2004, Shi et al., 2007). It has also been demonstrated that the N-terminal domain in kindlins may aid integrin binding (Goult et al., 2009). In cell line studies, kindlin-2 has been shown to bind Y759, a distinct region to talin (Ma et al., 2008). Cells of haematopoetic origin express the kindlin-3 isoform (Moser et al., 2009) and platelets deficient in kindlin-3 also display significant defects in *in vivo* platelet aggregation and spreading on a fibrinogen coated surface (Moser et al., 2008). Taken together, the phenotypes of talin and kindlin-3 deficient platelets suggest that these proteins may act co-operatively to activate αIIbβ3 and cannot compensate for one another.

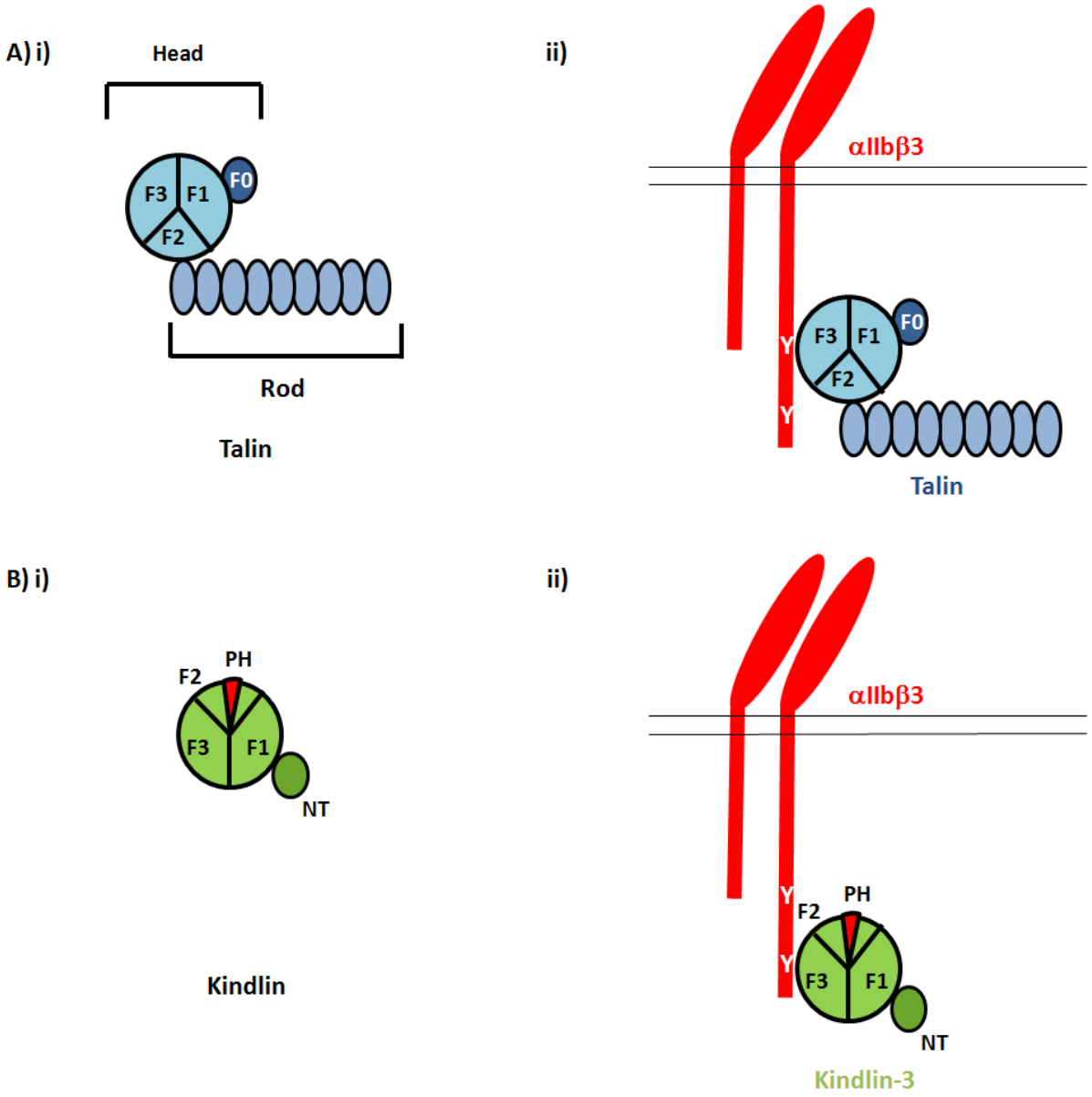


Figure 1.7 Differential structure and binding sites within αIIbβ3 of talin and kindlin-3. A) i) Talin consists of a head group contains the FERM domain, consisting of 3 subdomains, and a rod domain containing binding sites for the actin binding protein vinculin and a THATCH domain for directly binding actin. ii) Of the three subdomains of the FERM domain, F3 forms a PTB domain which is known to bind Y747 in the β3 tail of αIIbβ3. B) i) Kindlin family proteins consist of a FERM domain, of which the F2 subdomain is split by a plekstrin homology domain, and an N-terminal domain which is thought to play a role in integrin binding in an unknown manner. ii) In platelets, kindlin-3 binds the β3 tail of αIIbβ3 via the PTB domain formed by the F3 subdomain, however, this binds to Y759.

Members of the Dok family of adapter proteins have also been shown to associate with the phosphorylated β3 Y747 via their PTB domains. Dok family proteins associate with SHIP-1 and Grb2 downstream of the integrin and negatively regulate integrin activation (Calderwood et al., 2003, Hughan and Watson, 2007, Senis et al., 2009a). The differential dependence on tyrosine phosphorylation of talin and Dok binding demonstrates how phosphorylation at a given residue can act as a 'switch' converting a positive interaction into a negative one (Oxley et al., 2008) (See Figure 1.8).

Other proteins that interact with the αIIbβ3 integrin include myosin which associates to the β3 tail only when both conserved tyrosine residues are phosphorylated (Jenkins et al., 1998). Further, several actin-binding proteins are phosphorylated downstream of integrin engagement including α-actinin and cortactin (Senis et al., 2009a). α-actinin also associates with αIIbβ3 in platelets and modulate inside-out regulation in megakaryoblastic leukaemia CMK cells (Tadokoro et al., 2011).

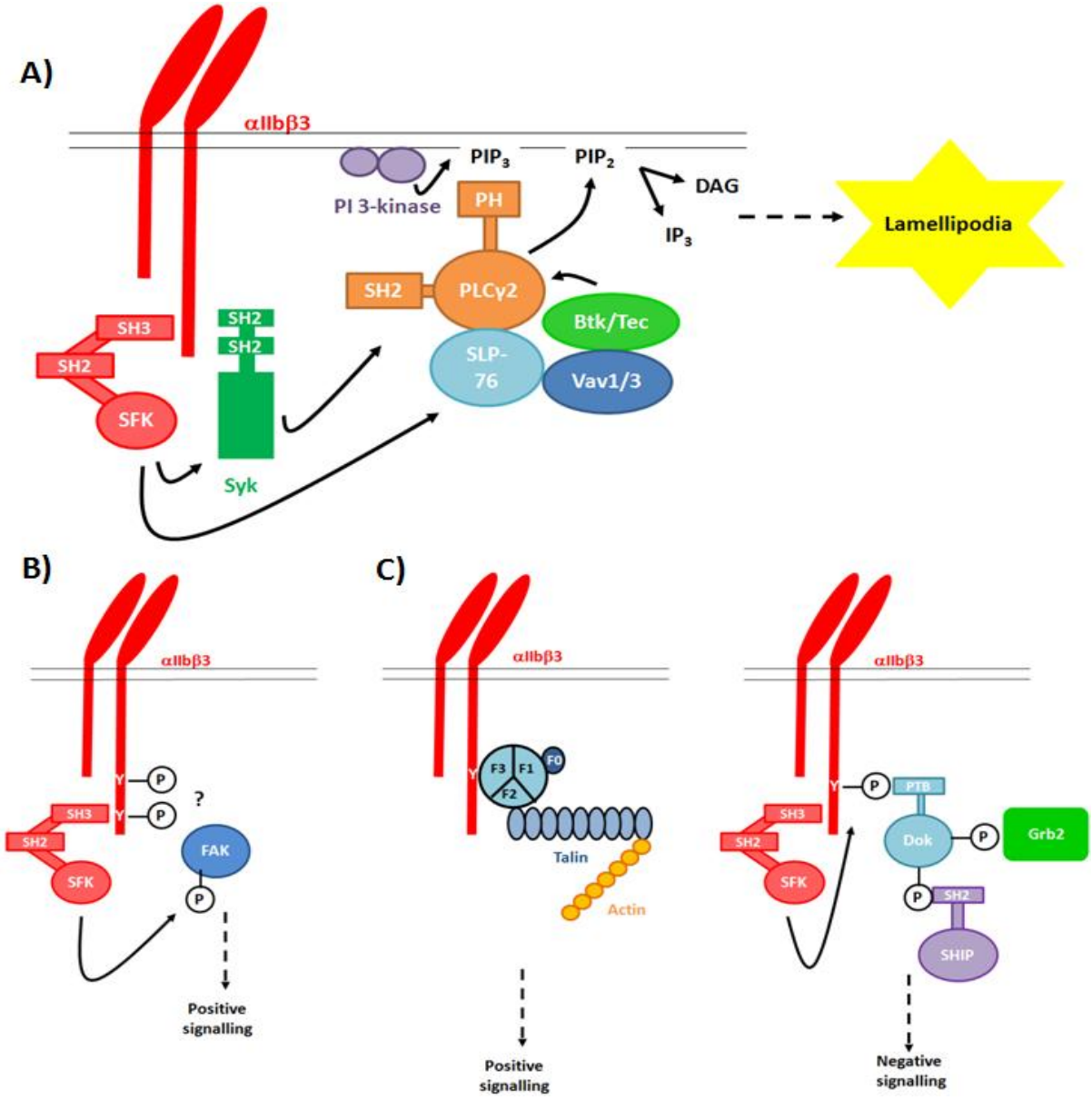


Figure 1.8 αIIbβ3 signalling pathways. A) Liagation of αIIbβ3 induces the activation of Src family kinases, leading to recruitment of Syk. Syk activation leads to the activation of PLCγ2 in a pathway which show similarity to ITAM signalling. B) Src family kinases downstream of αIIbβ3 can activate FAK, it is not known if this is dependent on β3 tail phosphorylation. C) Talin is recruited to the unphosphorylated N-terminal tyrosine residue, whereas phosphorylation of this residue allows for recruitment of Dok proteins and downstream effectors. This residue is thought to act as a 'switch' for αIIbβ3 mediated signalling.

It has recently been proposed that αIIbβ3 may also associate with and signal via an ITAM-containing receptor. Some evidence for a link between integrins and ITAM receptors has been described in neutrophils and mast cells, where deficiency of the ITAM containing Fcγ chain or DAP12 causes reduced integrin mediated signalling (Abram and Lowell, 2007). There are three activatory YXXL-containing proteins expressed in platelets, the GPVI-Fc γ chain complex, FcγRIIa and CLEC-2. It has recently been demonstrated that one of these, FcγRIIa, undergoes phosphorylation after platelet activation in an integrin-dependent manner and that blocking of this receptor with Fab fragments reduces spreading on a fibrinogen coated surface (Boylan et al., 2008). However, FcγRIIa is expressed at less than 10% the level of αIIbβ3 (Karas et al., 1982), suggesting that the Fc receptor can only associate with a fraction of the integrin. Moreover, mouse platelets do not express FcγRIIa and can still respond to fibrinogen, although not as strongly as human platelets. These two pieces of information, taken together, suggest that FcγRIIa is not essential for spreading on fibrinogen, however, it does appear to play some role. The likely reason for the ability of platelets to spread on fibrinogen in the absence of FcγRIIa is due to αIIbβ3 being able to activate essential signalling molecules, such as the Src and Syk family kinases, directly without the need for an ITAM.

Src family kinase members in αIIbβ3-mediated signalling

As described previously, the association of Src family kinases with αIIbβ3 is essential for downstream signalling processes. The association is mediated by interaction of the SH3 domain of the kinase with the β3 tail, although individual SFKs associate with different regions of the tail in that Src associates with the terminal 4 residues of the β3 tail, YRGT, (Arias-Salgado et al., 2003) and Fyn associates with residues 721-725, IHDRK (Reddy et al.,

2008). These two associations are atypical in that SH3 domains are typically thought to be associated with PXXP motifs.

Mutant mouse models have been used to elucidate the roles of Src family kinases in platelets downstream of αIIbβ3. Mice deficient in Fyn demonstrate an increase in re-bleeding and reduced spreading on a fibrinogen coated surface (Reddy et al., 2008). In contrast, platelets deficient in Lyn show increased spreading (Maxwell et al., 2004). Platelets from radiation chimera mice deficient in the four Src kinases, Fgr, Src, Hck and Lyn, but not platelets deficient in just Fgr, Hck and Lyn, have reduced spreading on a fibrinogen surface (Obergfell et al., 2002). From the latter result, Obergfell *et al* concluded that Src is the major Src family kinase required for spreading on fibrinogen. However, it is also possible that this result reflects net effect of loss of several Src kinases and not just Src itself. Therefore, the role of the individual members of Src family kinases involved in this process remains unclear.

Other platelet integrins

In addition to the major platelet integrin, αIIbβ3, platelets express several other integrins including α2β1 for collagen, αvβ3 for vitronectin and osteopontin, α5β1 for fibronectin and α6β1 for laminin.

Of these integrins, α2β1 is the most thoroughly studied. α2β1 is a relatively minor receptor on the platelet surface, expressed at a level of ~2000 copies on human platelets. (Best et al., 2003, Samaha et al., 2004) and is believed to reinforce the binding of the platelet collagen receptor, GPVI, to collagen fibres and induce firm collagen binding(Nieswandt et al., 2001, Kuijpers et al., 2003). It signals in a Src family kinase-dependent manner with PLCγ2 activation playing a key role in its signalling. However, α2β1 generates very weak signals and plays only a minor role in thrombus formation *in vivo*. This may be a consequence of the low

copy number of α2β1, at 40-60x lower than αIIbβ3 (Holtkotter et al., 2002, He et al., 2003, Kuijpers et al., 2007, Auger et al., 2005).

αvβ3 is expressed at only a few hundred copies per platelet but can be activated downstream of platelet agonists, including ADP (Paul et al., 2003). It appears that upon platelet activation, αvβ3 binds to osteopontin, which is found to be expressed in atherosclerotic plaques but not in healthy vessel walls (Bennett et al., 1997, Helluin et al., 2000).

α5β1 is the major receptor for fibronectin in platelets, although αIIbβ3 and αvβ3 can also bind this ligand. Binding of α5β1 to fibronectin can occur under static conditions without prior platelet activation (McCarty et al., 2004). Under shear conditions, α5β1 only contributes ~50% of the binding to fibronectin alongside αIIbβ3 (Beumer et al., 1994). Mice deficient in fibronectin display minor haemostatic defects due to loss of the αIIbβ3-fibronectin interaction (Ni et al., 2003).

α6β1 binds extracellular matrix laminins in a platelet activation-independent manner (Sonnenberg et al., 1988, Sonnenberg et al., 1991). Platelets adhered to laminins via α6β1 show filopodia formation with PI 3-kinase and cdc42 activities being increased above basal levels (Chang et al., 2005). In mice deficient in VWF and fibrinogen, α6β1, along with α5β1 has been hypothesised to play a role in thrombus formation after mesenteric artery injury (Ni et al., 2000).

1.3.1.3 Expression of Src family kinases in platelets

Seven of the eight Src family kinases have been shown to be expressed in human and mouse platelets in a variety of studies using several methods including gene expression analysis, proteomics and western blotting. Of all the Src family kinases, no study has shown Blk to be

expressed. On the other hand, it is widely accepted that Src, Fyn and Lyn are expressed in both human and mouse platelets (Golden et al., 1986, Horak et al., 1990, Quek et al., 2000, Corey and Anderson, 1999, Lannutti et al., 2003, Stenberg et al., 1997).

Several reports have described the presence of members of the Src family kinases in platelets using a variety of methods, including SAGE analysis in megakaryocytes, western blotting, proteomics and microarray methods but all suffer from issues with reagents or contamination. Yes has been shown to be present in many mammalian tissues, including human platelets (Zhao et al., 1990). The presence of Lck and Fgr in human and rodent platelets has been shown by western blotting with kinase-specific antibodies (Pestina et al., 1997) (Stenberg et al., 1997). In contrast, Lanutti et al was unable to detect Lck in platelets by western blotting and microarray studies, although he demonstrated expression of Hck, Fgr, Yes, Src, Fyn and Lyn (Lannutti et al., 2003). However, in a recent studies performed in our lab, Sonia Severin and myself determined that only Src, Fyn, Lyn and Fgr were expressed in mouse platelets by western blot (unpublished: see Appendix and Chapter 3). Further, Mike Tomlinson demonstrated that only Src, Fyn and Lyn are expressed in mouse megakaryocytes as detected by SAGE analysis (Senis et al., 2007).

A summary of the major SFKs in platelets, along with the method and species in which the member was identified, is shown in Table 1.3. There are several explanations for the discrepancy between these studies, including the cross-reactivity between antibodies and contamination with other blood cell types. Haematopoetic cells express many of the Src family kinases and a small amount of contamination with these cell types would cause false positive results.

Table 1.3 Expression of Src family kinases in platelets and megakaryocytes. Platelets have been proposed to express seven of the eight Src family kinases in both human and mouse platelets. A variety of methods has been used to provide evidence for the presence of a Src family kinase and the publications in which these are used is outlined below.

Src family kinase	Species	Evidence	Reference
Src	Human and mouse	Western blot, SAGE, proteomics and microarray analysis	(Senis et al, 2007, Corey and Anderson, 1999, Golden et al., 1986, Lannutti et al., 2003(Senis et al., 2009a))
Lyn	Human and mouse	Western blot, SAGE, proteomics and microarray analysis, also functional analysis in gene deficient mice	(Senis et al, 2007, Corey and Anderson, 1999, Golden et al., 1986, Lannutti et al., 2003, Quek et al., 2000, Stenberg et al., 1997(Senis et al., 2009a))
Fyn	Human and mouse	Western blot, SAGE, proteomics and microarray analysis, also functional analysis in gene deficient mice	(Senis et al, 2007, Horak et al., 1990, Quek et al., 2000, Stenberg et al., 1997(Senis et al., 2009a))
Yes	Human and mouse	Western blot and microarray analysis	(Lannutti et al., 2003, Zhao et al., 1990)
Fgr	Human and mouse	Western blot and microarray analysis	(Severin, unpublished, Pestina et al., 1997, Stenberg et al., 1997)
Lck	Human	Western blot	(Pestina et al., 1997, Stenberg et al., 1997)
Hck	Human and mouse	Western blot and microarray analysis	(Lannutti et al., 2003)

1.3.2 G protein-coupled receptors

G protein-coupled, or seven transmembrane, receptors regulate platelet activation through heterotrimeric G proteins, although recently they have also been shown to signal via Src family kinases (See section 1.3.2.4).

1.3.2.1 Signalling via heterotrimeric G-proteins.

Heterotrimeric G proteins consist of α- and βγ-subunits. They are regulated by 7TM receptors which induce dissociation of the α- and βγ-subunits. Receptor activation leads to the release of GDP from the α-subunit and its exchange for GTP which is present at molar excess within the cell. In this process, the GPCR acts as guanine nucleotide exchange factor (GEF). The binding of GTP induces dissociation of the subunits enabling them to bind to their effector enzymes.

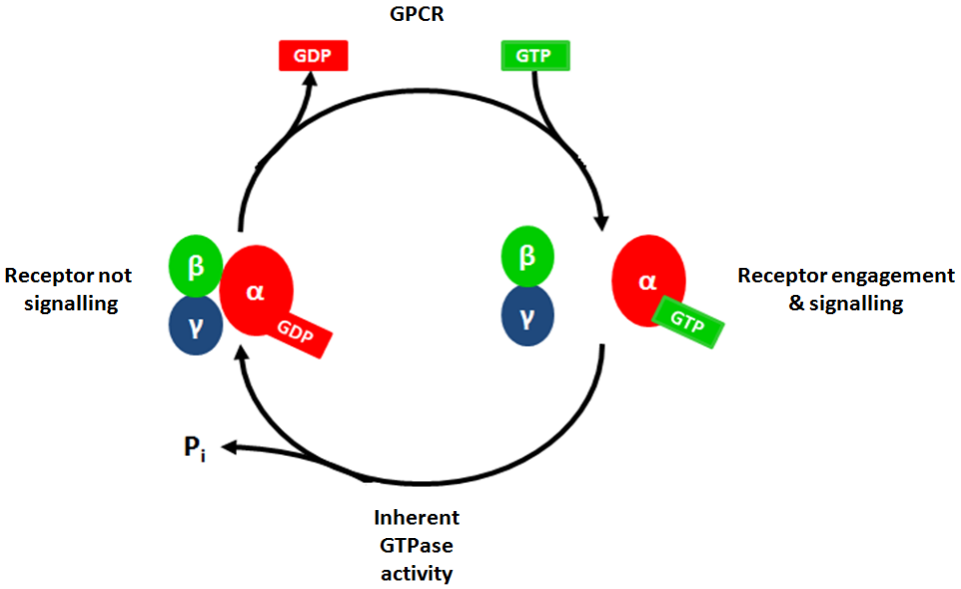


Figure 1.9 The G protein cycle. In their inactive state, heterotrimeric G proteins bind GDP and maintained in their inactive state. Upon exchange of GDP for GTP, catalysed by the G protein-coupled receptor, the α subunit dissociates from the βγ dimer, allowing for activation of downstream targets. Over time, the inherent GTPase activity of the α subunit converts GTP into GDP, allowing for reassociated of the three components of the heterotrimer.

There are 16 Gα genes within the mammalian genome which are subdivided into four major classes, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ (Simon et al., 1991). All of the α-subunits are either palmitoylated or myristoylated (Chen and Manning, 2001) and this enables their membrane association and interaction with 7TM receptors (Peitzsch and McLaughlin, 1993, Wedegaertner et al., 1995). Each class of α- subunit regulates a distinct group of effector enzymes. $G\alpha_s$ mediates activation of adenylyl cyclase enzyme, leading to an increase in cAMP (Simonds, 1999); $G\alpha_q$ regulates β- and ε-isoforms of phospholipase C, leading to formation of IP_3 and 1,2-diacylglycerol (Hubbard and Hepler, 2006); $G\alpha_{12/13}$ regulates Rho GTP exchange factors (GEFs) (Suzuki et al., 2009); and $G\alpha_i$ inhibits some forms of adenylyl cyclase (Simonds, 1999) and regulates Ca^{2+} and K^+ channels (Neves et al., 2002). The βγ-subunits also regulate effector enzymes with specificity being achieved through the release of different levels within the membrane rather than subtypes of β- and γ-subunits. Many of the actions of the βγ-subunits are attributed to the G_i-family of G proteins as these are usually expressed at a much greater level than the other G protein families (Smrcka, 2008).

All nine members of the adenylyl cyclase family identified to date are activated by $G\alpha_s$, however their sensitivity to the G protein does vary. Not all isoforms of this enzyme are inhibited by G_i (Simonds, 1999). cAMP produced by adenylyl cyclase is directly responsible for activation of protein kinase A. Protein kinase A consists of two enzyme catalytic units and two regulatory units. The consecutive binding of cAMP to each of the regulatory subunits induces activation of the enzyme. Once activated, the catalytic subunits are then free to serine/threonine phosphorylate targets containing a conserved RRXS/T sequence (Kemp and Pearson, 1990).

$G\alpha_q$ is well characterised as an activator of phospholipase C β-isoforms. An interaction between the C-terminal extension of PLCβ (Kim et al., 1996, Park et al., 1993) and a switch region within $G\alpha_q$ (Venkatakrishnan and Exton, 1996) induces the activation of PLCβ. The conformation of the switch region in $G\alpha_q$ alters upon exchange of GDP for GTP, allowing for the interaction to occur. The binding of $G\alpha_q$ to PLCβ increases the intrinsic GTPase activity of the Gα protein (Chidiac and Ross, 1999, Paulssen et al., 1996), allowing for reversible acitivation of the phospholipase enzyme. As with PLCγ, activation of PLCβ allows for the production of the second messengers, IP_3 and DAG.

$G\alpha_{12/13}$ family members activate RhoA family proteins indirectly via the activation of RhoGEFs (guanine nucleotide exchange factors). $G\alpha_{12/13}$ bind to RhoGEFs via conserved Rho homology (RH), pleckstrin homology (PH) and dbl homology (DH) domains in the GEF and a conserved helix within the G-protein, leading to activation of the RhoGEF (Hart et al., 1998, Wells et al., 2002). Once active, RhoA can bind many downstream targets, the best understood of these are the Rho kinases (ROCK1/2). Rho kinases phosphorylate targets such as FAK (Dubash et al., 2007, Iwanicki et al., 2008) and myosin light chain (MLC) phosphatase (Feng et al., 1999, Kimura et al., 1996), along with MLC itself (Amano et al., 1996, Narumiya et al., 1997, Totsukawa et al., 2000). The phosphorylation of FAK induces the formation of actin stress fibres whereas phosphorylation of MLC phosphatase inhibits its action, therefore along with the direct phosphorylation of MLC induces myosin driven cytoskeletal contraction.

In addition to the Gα subunits, there are also 5 Gβ and 12 Gγ subunits expressed in mammals (McCudden et al., 2005). Any combination of Gβ and Gγ subunits in a dimer is possible, except for the combination of $G\beta_2$ and $G\gamma_1$ which cannot pair together (Schmidt et al., 1992). In addition to the diversity of Gβγ dimer combinations possible, a dimer can also combine

with any member of the Gα family. This creates a huge diversity in signalling and cellular control.

βγ dimers were originally thought to be non-signalling units whose sole purpose was to slow the exchange of GDP for GTP in the Gα subunit of the heterotrimer and localise it to the 7 TM receptor. However, many studies have described a signalling role for the βγ dimer which has fairly diverse downstream targets. Among the first targets of the βγ dimer to be found were the G-protein inward-rectifier K^+ channels (GIRKs) (Logothetis et al., 1987). These channels are multimeric ion channels made up of of four GIRK subunits (GIRK 1-4) and βγ dimers have been shown to bind both the N- and C-terminus of all four subunit proteins and increase their activity (Doupnik et al., 1996, Huang et al., 1995, Kunkel and Peralta, 1995). PI 3-kinase γ is also known to be activated by direct binding of Gβγ dimers downstream of G_i-coupled receptors (Stephens et al., 1994, Stoyanov et al., 1995) (Leopoldt et al., 1998, Stephens et al., 1997, Suire et al., 2005). In addition to this, Gβγ dimers have also been implicated in the positive and negative regulation of adeylyl cyclase isoforms (Diel et al., 2006, Tang and Gilman, 1991) and the activation of PLCβ by direct binding (Boyer et al., 1992, Camps et al., 1992).

1.3.2.4 Interplay between G protein-coupled receptors and tyrosine kinase pathways

G-protein coupled receptors are classically thought to signal exclusively through their coupled G-proteins. However, there is much evidence to suggest that GPCR signalling can occur via Src family tyrosine kinase mediated pathways, either directly or through a cross-talk mechanism.

Src family kinases can be activated by several mechanisms downsteam of GPCRs. These include the direct binding of SFKs to the G protein-coupled receptor, binding of G proteins themselves to the kinase or mechanisms mediated by the arrestin proteins. In addition to this, Src family kinases may also be activated by indirect means, for example through signalling to integrin activation.

Some GPCRs have the ability to bind SFKs through conserved domain-binding motifs within their intracellular loops. Association of Src family kinases with $P2Y_2$ purinergic (Liu et al., 2004) and β3 adrenergic receptors (Cao et al., 2000) or conserved tyrosines within the β2-adrenergic receptor (Fan et al., 2001) prevents association of Src family kinases with the receptor via their SH3- and SH2-domains, respectively.

G proteins themselves also have the ability to activate Src family kinases. Direct interaction of GTP-bound $G\alpha_s$ or $G\alpha_i$ with Src family kinases results in activation of the kinase, however other members of the Gα family do not seem to have this effect (Ma et al., 2000). There is also evidence to suggest that Gβγ subunits can activate Src, with transient overexpression of these subunits in cell line generating increased tyrosine phosphorylation of Src at its

activation site, however this is not dependent on the direct association of the proteins (Luttrell et al., 1996, Ma et al., 2000).

G protein coupled receptor bound β-arrestins 1 and 2 can directly activate Src family kinases (Luttrell et al., 1999). The SFK-receptor interaction is mediated by proline-rich motifs in the β-arrestin and SH3 domains in Src family kinases and partly by the N-terminal region with the kinase domain (Miller et al., 2000). The recruitment of arrestins and Src to the GPCR appears to initiate a second wave of signalling and may be involved in MAPK activation in some cell types.

Src family kinases can also be activated indirectly by G-protein coupled receptors. The best characterised of these type of mechanisms is the activation of Src within focal adhesion complexes (Luttrell and Luttrell, 2004). G-protein coupled receptors can induce tyrosine phosphorylation of focal adhesion kinase on three sites conserved site (Hordijk et al., 1994, Rodriguez-Fernandez and Rozengurt, 1998, Sinnett-Smith et al., 1993, Shikata et al., 2003). One of these residues, Y-397 is a site for autophosphorylation and creates a docking site for SH2 domain containing proteins, including the SFKs (Schaller et al., 1994). Upon docking to FAK, SFKs are activated by displacement of the SH2 domain-C terminal tail interaction (Calalb et al., 1995) in a β integrin binding-dependent manner (Dikic et al., 1998).

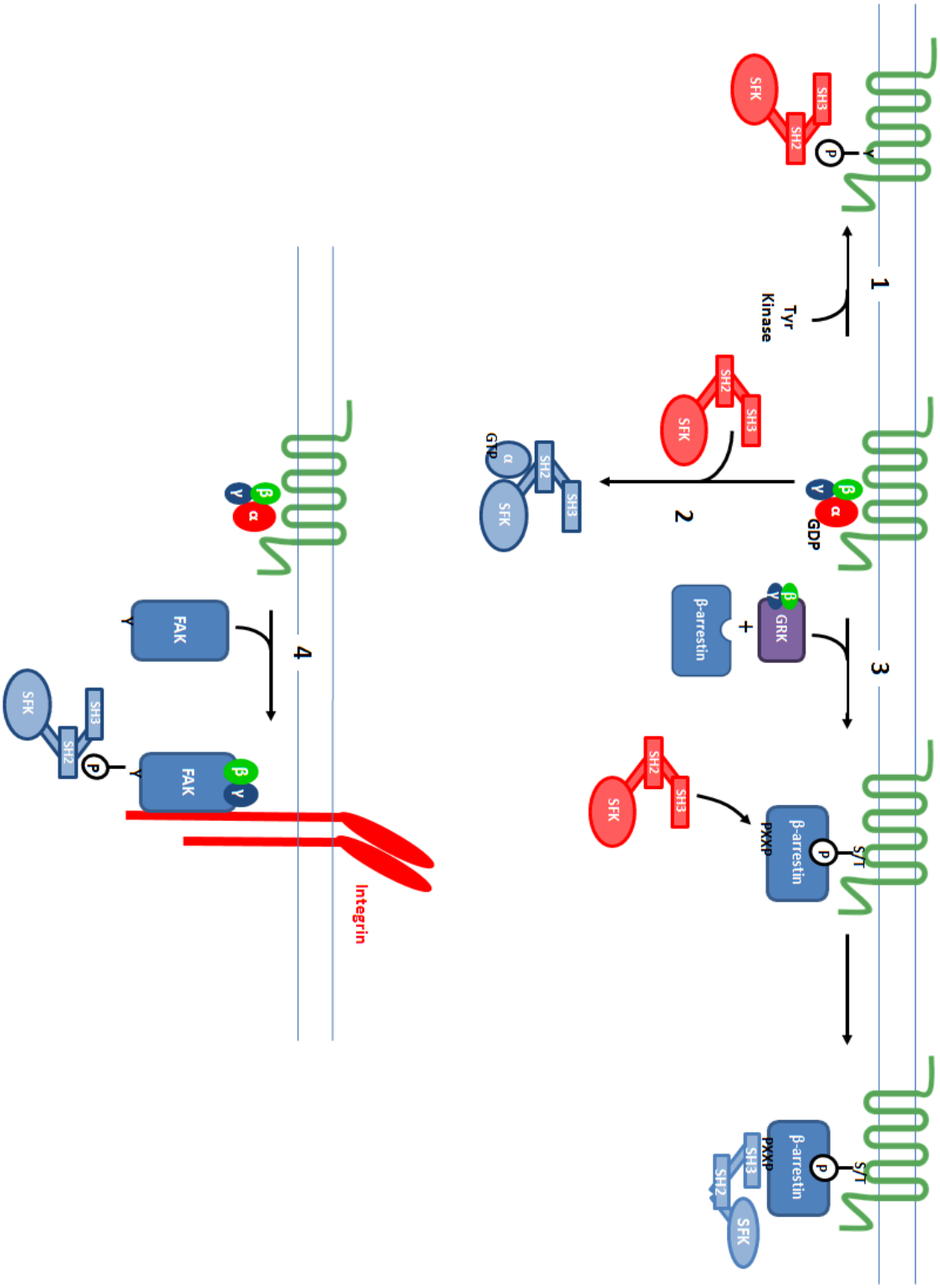


Figure 1.10 Mechanisms of SFK regulation by G protein coupled receptors. GPCRs can regulate SFKs by a variety of processes including 1) direct binding of kinase to GPCR, 2) activation via direct binding of G protein 3) activation by binding to β-arrestin 4) indirect activation via the activation of integrin-bound FAK.

Currently it is unclear which of these pathways is the major player in signalling via Src family kinases downstream of G-protein coupled receptors. It appears that different mechanisms apply to different receptors and no receptor mechanism can be applied to all GPCRs in general.

1.3.2.2 G protein-coupled receptor signalling in platelets

Platelets express a plethora of G protein-coupled receptors on their surface and members of this receptor type are required to respond to both activating and inhibitory signals.

Prostacyclin (PGI_2)

PGI_2 binds to the IP receptor expressed on the platelet surface and this GPCR is coupled to the $G\alpha_s$ family of G proteins (Dutta-Roy and Sinha, 1987). Subsequent activation of adenylyl cyclase and protein kinase A induces inhibitory serine/threonine phosphorylation of several targets (Cavallini et al., 1996, Quinton and Dean, 1992). IP receptor-deficient mice display reduced cAMP levels in their platelets and increased thrombus formation following challenge in an *in vivo* model (Murata et al., 1997, Yang et al., 2002). Interestingly, platelets from these mice do not display any *in vitro* defect. This suggests that low cAMP concentration is not sufficient to activate platelets alone.

Thrombin and the PAR receptors

Platelets respond to thrombin generated by the coagulation cascade. Thrombin activates PAR receptors by cleavage of a susceptible arginyl-X sequence in the N-terminal tail of the receptor (Ofosu, 2003, Vu et al., 1991). This neo-epitope, consisting of residues SFFRLN in PAR-1 (Furman et al., 1998) and GYPGKF in human PAR-4 (Xu et al., 1998), interacts with the ligand binding domain of the receptor mediating powerful activation of G_q and G_{13}

proteins. Interestingly, mouse platelets express PAR-3 rather than PAR-1 (Kahn et al., 1998), however, PAR-3 is not believed to have a direct signalling role, but acts as a co-factor to PAR-4 signalling (Nakanishi-Matsui et al., 2000, Kahn et al., 1998). Platelets deficient in G_q and G_{13} display platelet aggregation and shape change defects in response to thrombin (Moers et al., 2003, Offermanns et al., 1997).

Thromboxane A_2 and the TP receptor

Thromboxane A_2 (TxA_2) is generated by Thromboxane A_2 synthase from Prostaglandin H_2, synthesised by the cyclo-oxygenase (COX) enzyme. It has a very short half life in aqueous solution of approximately 30 seconds (Smith et al., 1976) and can therefore only act in the local environment. TxA_2 signals in platelets via the TP receptor (Saussy et al., 1986). There are two splice forms of this receptor in humans, α and β, which differ in their carboxyl terminus (Hirata et al., 1996, Raychowdhury et al., 1994). Although mRNA for both isoforms has been detected in the platelet, it is thought that only the α-variant exists in protein form (Habib et al., 1999). The TP α receptor couples to G_q (Shenker et al., 1991) and $G_{12/13}$ (Offermanns et al., 1994) G proteins. Mice deficient in G_q and G_{13} display defects in platelet aggregation and shape change to U46619 (Moers et al., 2003, Offermanns et al., 1997). Inhibition of the COX enzyme by drugs such as indomethacin and aspirin induces defects in aggregation to several platelet agonists, including collagen.

ADP

ADP signals via two receptors in platelets, $P2Y_1$ and $P2Y_{12}$ which signal via G_q and G_i-dependent mechanisms, respectively.

$P2Y_1$ is the major receptor involved in platelet shape change downstream of ADP. Activation of this receptor initiates a signalling cascade which leads to the activation of PLCβ via the receptors coupled G_q protein (Offermanns et al., 1997). Mouse platelets deficient in $P2Y_1$ do not display shape change in response to ADP, but can still aggregate at high concentrations of the agonist (Fabre et al., 1999, Leon et al., 1999). Interestingly, RhoA activation and MLC phosphorylation, events required for platelet shape change, do not occur in response to ADP in G_q-deficient platelets (Moers et al., 2004). This suggests that the shape change response downstream of ADP is not reliant on $G_{12/13}$ but requires Ca^{2+}-mediated signalling. These mice also show mildly increased tail bleeding times and some resistance to an ADP-induced thromboembolism model (Fabre et al., 1999, Leon et al., 1999).

PY_{12} is expressed at ~10x the level of $P2Y_1$ and signals via a Gi-dependent mechanism. Mice deficient in $P2Y_{12}$ show both a bleeding phenotype and a resistance to thromboemolism models (Andre et al., 2003, Foster et al., 2001). They also show a reduced aggregation to ADP, with shape change still intact (Andre et al., 2003). $P2Y_{12}$ is a major pharmaceutical target due to its major synergistic role with other platelet agonists with many drugs, such as prasugrel, clopidogrel and ticagrelor, having been developed to target this receptor. Human patients treated with these drugs or with defects in the receptor (Cattaneo et al., 2003) display mild platelet based bleeding.

Adrenaline and α_{2A}

Adrenaline is a hormone released from the adrenal glands. It is normally considered to play a role in the physiological 'fight or flight' response. Adrenaline has been shown to induce aggregation of human platelet rich plasma but not washed platelets. This has led many groups to define adrenaline as potentiating agonist (Lanza et al., 1988, Steen et al., 1993). Adrenaline induces a reduction in cAMP in platelets via a G_i-dependent pathway (Lenox et al., 1985), however it does not induce intracellular calcium release (Nieuwland et al., 1993).

The role of adrenaline in *in vivo* platelet aggregation is not entirely clear. It is possible that platelet dense granules contain small amounts of adrenaline that is released upon platelet activation, supporting aggregation as a feedback agonist. This hypothesis is supported by the observation of a tail rebleeding phenotype in mice deficient in α_{2A} (Pozgajova et al., 2006).

1.3.2.3 The molecular mechanism of activation by G_i-coupled receptors

As described previously, platelets express two major G_i-coupled receptors, the α_{2A}-adrenoreceptor and $P2Y_{12}$ ADP receptor, which are known to inhibit adenylyl cyclase and activate PI 3-kinases. Studies in mutant mice have shown that the two receptors couple to distinct members of the G_i family of G proteins, namely G_z (Yang et al., 2000, Kelleher et al., 2001) and $G\alpha_{i2}$ (Jantzen et al., 2001), respectively. Evidence for this is provided by the observation that these mice mirror the phenotype of that seen with the receptor knockout. The α_{2A}-adrenoreceptor and $P2Y_{12}$ ADP receptor also couple to a second member of the G_i family of G proteins in mice, most likely $G\alpha_{i1}$ (Patel et al., 2003, Yang et al., 2002). It is not known whether $P2Y_{12}$ and α_{2A} couple to the same G_i proteins in human platelets as they do in mice.

The $P2Y_{12}$ ADP receptor and the α_{2A}-adrenoreceptor undergo synergy with G_q- and $G_{12/13}$-regulated pathways, inducing powerful platelet activation (Dorsam et al., 2002, Nieswandt et al., 2002). They have also been shown to synergise together to induce platelet aggregation (Dorsam et al., 2005). On their own, however, they induce weak aggregation in plasma and no effect in 'washed' platelets. The molecular basis of activation in plasma and synergy with other agonists is not fully understood. It has been known since the 1970s that a reduction in cAMP is not sufficient to induce platelet aggregation, although it is unclear if this makes a contributory role (Daniel et al., 1999, Haslam, 1973, Yang et al., 2002). On the other hand, there is considerable evidence that activation of PI 3-kinases is critical for G_i-mediated platelet activation. The structurally distinct inhibitors of PI 3-kinases, wortmannin and LY294002, inhibit activation of integrin αIIbβ3 by ADP in $P2Y_1$-deficient mice platelets (Kauffenstein et al., 2001) and mice deficient in PI 3-kinase γ display a small reduction in aggregation to ADP (Hirsch et al., 2001). However, a much greater reduction in response is seen in the presence of the PI 3-kinase β inhibitor, TGX221 (Jackson et al., 2005, van der Meijden et al., 2008), and in PI 3-kinase β-deficient mice platelets (Martin et al., 2010, Canobbio et al., 2009) revealing this to be the predominant isoform downstream of $P2Y_{12}$. However, these observations have not yet been extended to the α_{2A} adrenoreceptor.

1.3.2.5 GPCR signalling via Src family kinases in platelets

In addition to other cell systems discussed previously, Src family kinases are also known to play a role in G protein-coupled receptor signalling in platelets.

The first platelet GPCR agonist demonstrated to induce tyrosine phosphorylation was thrombin. Under these conditions, the specific activity of Src increases in an aggregation dependent manner. Therefore, thrombin-mediated SFK stimulation is likely to be directly downstream PAR receptors. It has also been determined that PLCγ2 is phosphorylated downstream of thrombin (Tate and Rittenhouse, 1993). This is a well characterised signalling component activated downstream of SFKs and would not normally be activated by a G_q-mediated pathway. This provides further evidence for activation of SFKs in response to thrombin.

More recently, studies by the Kunapuli and Poole groups have described roles for Src family kinases in ADP receptor signalling. Hardy et al determined that ADP stimulates phosphorylation of Src on the conserved Y418 residue and that this response is dependent on signalling via the $P2Y_1$ receptor (Hardy et al., 2004). This study also demonstrates that blockade of SFKs inhibits the potentiation of $P2Y_1$-induced Ca^{2+} release by $P2Y_{12}$, providing evidence for a physiological role for SFKs downstream of these receptors (Hardy et al., 2004). In support of the physiological role for SFKs in ADP-mediated platelet responses, Jin et al demonstrate that aggregation to ADP is markedly reduced in the presence of the SFK antagonist PP2 (Jin et al., 2002). Taken together, these results demonstrate that Src family kinases are activated downstream of ADP receptors and this activation is important in ADP receptor-mediated functional responses.

Further studies have also demonstrated a role for Src family kinases in signalling by other platelet G-protein coupled receptors. Dorsam et al demonstrated that costimulation of G_i and G_z pathways led to platelet aggregation in a SFK-dependent manner (Dorsam et al., 2005). The authors also provide evidence for an increase in tyrosine phosphorylation at the Y418 site of SFKs downstream of G_i constimulation with both G_q and G_z (Dorsam et al., 2005). However, in this study, in contrast to previous studies by the same group, ADP-mediated aggregation was shown to be independent of SFK activation. A further study from this group also demonstrates that Src family kinase activity is required for MAPK activation downstream of ADP (Garcia et al., 2007).

The TxA_2 analogue U46619 has also been demonstrated to induce tyrosine phosphorylation of many proteins in human platelets, including Src family kinases at their activation site (Minuz et al., 2006). Minuz et al demonstrate that this phosphorylation occurs independently of αIIbβ3 activation, release of secondary mediators or a Rho kinase mechanism activated by $G_{12/13}$. This study also considers the potentiation of U46619-mediated signalling by adrenaline and finds that tyrosine phosphorylation is increased when adrenaline is used to stimulate platelets in conjunction with U46619, however adrenaline alone cannot stimulate phosphorylation. These results are in contrast to those seen by Dorsam et al who describe tyrosine phosphorylation of Src family kinases downstream of adrenaline alone (Dorsam et al., 2005).

These pieces of evidence suggest that a variety of G protein-coupled receptors in platelets can signal via Src family kinase-mediated mechanisms. What is not fully understood, however, are the exact mechanisms by which these platelet GPCRs stimulate Src activity, i.e. by direct receptor binding, G protein mediated effects or via β-arrestins and their functional

significance. Individual studies have shown evidence for mechanisms of activation by some platelet GPCRs. Torti et al demonstrate that G_i induces the association of G_i-family proteins with SFKs under conditions of adrenaline stimulation, however, it has not been demonstrated whether this has an effect on the kinase activity of SFKs (Torti et al., 1992). A recent study has demonstrated that Lyn can form stimulation-dependent complexes with arrestin-2 in response to thrombin and ADP in a manner that is PP2-senstive and –insensitive, respectively (Li et al., 2011). Although this study goes on further to demonstrate the phenotype of arrestin-2-deficient platelets, the effect of removing arrestin on SFK activation is not described. Therefore, it is unknown if the association of arrestin and Lyn is functionally relevant for its activity.

1.4 Aims of the thesis

The overall aim of this thesis is to investigate the role of SFKs in platelet activation by integrin αIIbβ3 and GPCRs. The specific goals are:

1. To quantitate the levels of Src family kinase expressed in human and mouse platelets.
2. To determine the role of the individual Src family kinases in αIIbβ3-mediated functional responses.
3. To address whether there is a role for αIIbβ3 integrin activation in adrenaline-mediated platelet aggregation and if this is mediated through Src family kinases.
4. Further to aim 3, to determine if stimulation of α_{2A} receptors induces tyrosine phosporylation in human platelets.

CHAPTER 2
MATERIALS & METHODS

2.1 Materials

2.1.1 Reagents and antibodies

The agonists and inhibitors used in this study are described in Table 2.1 and 2.2, respectively. Antibodies used are listed in Table 2.3 and recombinant proteins listed in Table 2.4. Materials used are from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Table 2.1 – Agonists

Agonist	Source	Target
Collagen (HORM)	Nycomed (Munich, Germany)	GPVI α2β1
CRP (YGKO(GPO)$_{10}$GKOG)	Prof R Farndale (University of Cambridge, UK) Cross-linked by Y.J Wang and Y. Senis (University of Birmingham, UK)	GPVI
Thrombin	Sigma-Aldrich (Poole, UK)	PAR-1 PAR-3 PAR-4
ADP	Sigma-Aldrich (Poole, UK)	$P2Y_1$ $P2Y_{12}$
Adrenaline	Sigma-Aldrich (Poole, UK)	α_{2A}
Fibrinogen	Enzyme Research Laboratories (Swansea, UK)	αIIbβ3
Thrombopoeitin	Sigma-Aldrich (Poole, UK)	cMpl

Table 2.2 Inhibitors

Inhibitor	Source	Target
Indomethacin	Sigma-Aldrich (Poole, UK)	Cycloxygenase
Apyrase	Sigma-Aldrich (Poole, UK)	ADP
Integrilin	University Hospital Birmingham Pharmacy (Birmingham, UK)	αIIbβ3
Recombinant cMpl	R&D Systems (Abingdon, UK)	TPO
Yohimbine	Prof N Barnes (University of Birmingham, UK)	α_{2A}
Dasatinib	LC Laboratories (Woburn, MA, USA)	SFKs Abl Ephrins cKit
Imatinib	LC Laboratories (Woburn, MA, USA)	Abl Ephrins cKit

Table 2.3 – Antibodies

Antibody	Host species	Use	Source
PRIMARY			
Src (pan Src)	Rabbit	WB: 1/1000 IP: 0.5µg	Invitrogen (Paisley, UK)
Fyn	Rabbit	WB: 1/200 IP: 0.5µg	Santa Cruz (Heidelberg, Germany)
Lyn	Rabbit	WB: 1/200 IP: 0.5µg	Santa Cruz (Heidelberg, Germany)
Fgr	Rabbit	WB: 1/200 IP: 0.5µg	Santa Cruz (Heidelberg, Germany)
SFK pY418	Rabbit	WB: 1/1000 FC: 1/50	Invitrogen (Paisley, UK)
Lyn pY507	Rabbit	WB: 1/1000	Cell Signalling (Hitchin, Herts, UK)
Fyn pY530	Rabbit	WB: 1/1000	Abcam (Cambridge, UK)
Src pY529	Rabbit	WB: 1/1000	Biosource (London, UK)
Phosphotyrosine (4G10)	Mouse	WB: 1/1000	Millipore (Bucks, UK)
Lck	Mouse	WB:1/1000	Dr M Tomlinson (Birmingham, UK)
P-selectin FITC-conjugate	Rat	FC: 1/100	BD Bioscience (Oxford, UK)
SECONDARY			
Mouse IgG HRP-conjugate	Sheep	WB:1/10000	Amersham Biosciences (Bucks, UK)
Rabbit IgG HRP-conjugate	Donkey	WB:1/10000	Amersham Biosciences (Bucks, UK)
Rabbit IgG FITC-conjugate	Goat	FC: 1/100	Sigma-Aldrich (Poole, UK)
Mouse IgG Dylight 800- conjugate	Goat	WB:1/10000	LiCor Biotechnology (Cambridge, UK)
Rabbit IgG Dylight 680-conjugate	Goat	WB:1/10000	LiCor Biotechnology (Cambridge, UK)

WB: Western blot, IP: Immunoprecipitation, FC: Flow cytometry,

Table 2.4 Recombinant proteins

Protein	Tag	Source
Human Src	GST	Enzo Life Sciences (Exeter, UK)
Human Lyn	GST	Enzo Life Sciences (Exeter, UK)
Human Fyn	His	Enzo Life Sciences (Exeter, UK)
Mouse Fgr (N-terminus)	GST	Generated as described in Section 2.2.3

2.1.2 Mice

Mice deficient in Lyn, Fyn, Src and Fgr were generated as described in publications outlined in Table 2.5. Lyn, Fyn and Src mice were obtained from Jackson Laboratories (Bar Harbor, USA). Fgr mice were a kind gift from Prof Clifford Lowell (UCSF, San Francisco, USA). Lyn/Fyn and Lyn/Fgr double deficient animals were generated by breeding of Lyn and Fyn or Lyn and Fgr single deficient animals, respectively. Fyn/Src and Lyn/Src were generated as radiation chimeras due to perinatal lethality of this genotype (See below). All mutant mice were maintained by heterozygote breeding on a C57/Bl6 background in order to allow for the use of litter-matched controls.

Table 2.5 Mouse models

Model	Obtained from	Original source	Initial publication
$Lyn^{-/-}$	Jackson Labs	Prof Clifford Lowell	Immunity, 7:69-81
$Fyn^{-/-}$	Jackson Labs	Prof Philippe Soriano	Cell, 70:741-50
$Src^{-/-}$	Jackson Labs	Prof Philippe Soriano	Cell, 64:693-702
$Fgr^{-/-}$	Prof Clifford Lowell	Prof Philippe Soriano	Genes Dev, 8:387-98
$Lyn^{-/-}Fgr^{-/-}$	$Lyn^{-/-}$ X $Fgr^{-/-}$ breeding pairs	N/A	N/A
$Lyn^{-/-}Src^{-/-}$	$Lyn^{-/-}$ X $Src^{+/-}$ breeding pairs	N/A	N/A
$Lyn^{-/-}Fyn^{-/-}$	$Lyn^{-/-}$ X $Fyn^{-/-}$ breeding pairs	N/A	N/A
$Fyn^{-/-}Src^{-/-}$	$Fyn^{-/-}$ X $Src^{+/-}$ breeding pairs	N/A	N/A

2.1.2.1 Genotyping of mutant mice

DNA was isolated from mouse ear clippings by isopropanol extraction. Briefly, ear clippings were incubated with tissue lysis buffer (100mM Tris-HCl; pH=8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) and 500µg/ml Proteinase K overnight at 55^{o}C. Following incubation, remaining tissue was vortexed before centrifugation at ~300xg in a microfuge. Supernatant was removed, added to an equal volume of 100% isopropanol and mixed. Following this, supernatant was discarded and the DNA pellet dried before being resuspended in 100µl autoclaved water.

Following isolation of DNA, samples were genotyped by the use of polymerase chain reaction (PCR), utilising the primers outlined in Table 2.6. Following PCR, samples were resolved on 1% agarose gel and imaged.

Table 2.6 Genotyping primers

Primer	Sequence
Lyn	
Wild Type	5’ - CGG CCT TGA TAT CCA TGA TTT CAC -3’
Common	5’ - CAG GTG GAG CAT ACC TGG CTG TTT -3’
Mutant	5’- CCT TGG GAA AAG CGC CTC CCC TAC - 3’
Fyn	
Wild Type FWD	5’ - TTA CCC TCT GAG CAT CTG AC – 3’
Wild Type REV	5’ - GCA AAA CAA CCC ACA CAG AG – 3’
Mutant FWD	5’ - CTT GGG TGG AGA GGC TAT TC - 3’
Mutant REV	5’ - AGG TGA GAT GAC AGG AGA TC – 3’
Src	
Wild Type	5’ - GTG ACG GTG TCC GAG GAG TTG AAG – 3’
Common	5’ - AGC AAC AAG AGC AAG CCC AAG GAC – 3’
Mutant	5’ - TCA TAG CCG AAT AGC CTC TCC AC – 3’
Fgr	
Wild Type	5’ - CAA GGC CGG ACT TCG TCC GTC TTT CC – 3’
Common	5’ - GAG AGC CTT ACT GGA ATC CCT CTT TAG C – 3’
Mutant	5’ - CAG TCA TAG CCG AAT AGC CTC – 3’

2.1.2.2 Generation of radiation chimera mice

Foetal liver cells were isolated from E12.5-14.5 embryos and stored in liquid nitrogen until required . Embryos were genotyped by PCR in order to confirm presence or absence of desired genes. One week prior to transplantation, 6-8 week old male wild type mice of C57Bl6 background were dosed with Baytril antibiotic in drinking water. Following this period, mice were irradiated with two doses of 0.5Gy raditation 2 hours apart. Mice were then given $1x10^6$ foetal liver cells via tail vein injection. Following transplantation, mice were allowed to recover for six weeks prior to experimentation.

2.2 Methods

2.2.1 Platelet preparation

2.2.1.1 Isolation of human platelet rich plasma

Blood was taken from healthy, drug-free volunteers via venipuncture from the antecubital vein into 1/10 volume of 3.8% sodium citrate. Following withdrawal, blood was centrifuged at 200x g for 20 min in a swing out bucket rotor centrifuge in order to obtain platelet rich plasma (PRP). The remaining blood was centrifuged again at 1000x g for 10 min to obtain platelet poor plasma (PPP) used as an optical standard for platelet aggregation. A Coulter Z3 particle counter, calibrated for platelet counting, was used to determine the concentration of platelets within the plasma. Where PRP was used for flow cytometry-based assays, it was first diluted to a platelet concentration of $1x10^7$/ml.

2.2.1.2 Preparation of washed human platelets

Blood from healthy, drug-free volunteers was taken into 1/10 volume 3.8% sodium citrate or 1/8 volume acid citrate dextrose (ACD: 120mM sodium citrate, 110mM glucose, 80mM citric acid) by antecubital venipuncture. Following this, 1/10 volume ACD was added to the blood taken into citrate before centrifugation at 200x g for 20 min to obtain PRP. 10µg prostaglandin I_2 (PGI_2) was then added in order to prevent spontaneous platelet activation. PRP was then centrifuged at 1000x g for 10 min to obtain a platelet pellet.

The pellet was then resuspended in 25ml modified Tyrode's buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$, 0.2U/ml apyrase; pH 7.3) and 3ml ACD, before adding 10µg PGI_2 and centrifuging for a

second time at 1000x g for 10 min. The pellet was then resuspended to the appropriate volume at a concentration of $2x10^8$/ml, $5x10^8$/ml and $1x10^7$/ml for aggregation, biochemistry and flow cytometry experiments, respectively. Platelets were left at room temperature for 30 min before experiments.

2.2.1.3 Preparation of washed mouse platelets

Blood was taken from terminally CO_2 narcosed mice from the descending vena cava into 10% acid citrate dextrose. The blood was then added to 200µl Modified HEPES-Tyrode's buffer. Following this, the whole blood was centrifuged at ~60x g for 5 min in a desktop microfuge in order to separate PRP and erythrocytes. Once complete, the PRP and ~30% of the erythrocytes were transferred to a clean eppendorf tube and centrifuged at 200x g for 6 min in a swing-out bucket rotor. The PRP and 'buffy coat' of the sample are then removed and retained. After the addition of 200µl HEPES Tyrodes buffer, the remaining erythrocytes were centrifuged again at 200x g in a swing-out bucket rotor. Platelet activation was inhibited by addition of 1µg prostacyclin (PGI_2) and then centrifuged at 1000x g for 6 min. The plasma (supernatant) was removed and the platelet pellet was then resuspended in 200µl HEPES-Tyrodes buffer. The resulting suspension was counted in a Coulter Z3 particle counter. The samples were then diluted to the desired concentration ($2x10^8$/ml, $2x10^7$/ml, and $1x10^7$/ml for platelet aggregation studies, spreading and FACs, respectively) and left to rest for at least 30 min to recover from PGI_2 treatment.

2.2.2 Platelet functional assays

2.2.2.1 Platelet aggregation and ATP secretion

Aggregation and secretion measurement in real time were performed in a Born-lumiaggregometer (Chronolog distributed by Lab Medics, Manchester, UK) by measuring light transmission and luminescence, respectively. $2x10^8$/ml platelets (400µl for human, 300µl for mouse), washed or in plasma, were warmed to 37^{o}C, without stirring for 1 min, at this stage 10µl (for washed platelets) or 40µl (for PRP) chrono-lume luciferin-luciferase reagent was added. Following this, platelets were warmed for a further 1 min with stirring (1200rpm). Samples were then stimulated by addition of the appropriate agonist. ATP secretion was calculated in comparison to a 2nmol ATP standard, added at the end of the aggregation experiment.

2.2.2.2 Stimulation for platelet biochemistry

$5x10^8$/ml washed platelets (500µl) were stimulated under stirring conditions in the presence of 9µM Integrilin (αIIbβ3), 10µM indomethacin (COX) and 2U/ml apyrase (ADP scavenger). For experiments where ADP was the agonist, apyrase was not used. Following stimulation, samples were either lysed with an equal volume of 2x lysis buffer (LB: 300mM NaCl, 200mM Tris, 2mM EDTA, 2mM EGTA, 2% (v/v) NP-40, 2mM AEBSF, 5mM Na_3VO_4, 10µg/ml aprotinin, 1µg/ml pepstatin and 10µg/ml leupeptin) or 2x sample buffer (SB: 20% glycerol, 10% stacking gel buffer (0.5M Tris-HCl; pH 6.8), 10% β-mercaptoethanol, 4% SDS, trace of Brilliant Blue R).

For monitoring protein phosphorylation in plasma, stimulations were carried out with PRP as described above for washed platelets.. Reactions were stopped by rapid cooling in ice. PRP

was then centrifuged at 1000x *g* in a microfuge for 2 min at 4^{o}C to obtain a platelet pellet. The pellet was then resuspended in 1ml ice cold PBS and centrifuged again for 2 min at 4^{o}C. This pellet was then lysed in an appropriate volume of 1 x sample buffer to resuspend platelets to a concentration equivalent to 5x10^{8}/ml.

2.2.2.3 Static adhesion and spreading

Glass coverslips were incubated with 100µg/ml fibrinogen at 4^{o}C overnight and washed three times with PBS. Coverslips were then blocked using 5mg/ml denatured BSA for 1 hr at room temperature. Washed platelets were added to the coverslips and incubated for 45min at 37^{o}C to allow them to adhere and spread. Non-adherent platelets were removed by washing three times with PBS. Adherent platelets were then fixed to coverslips using 3.7% paraformaldehyde for 10 mins at room temperature. Coverslips were mounted onto glass slides using Hydromount (National Diagnostics, Atlanta, USA) and imaged using differential intereference contrast (DIC) microscopy using a Zeiss Axiovert 200 M microscope. Platelet surface area was measured using ImageJ software (NIH, Bethesa, USA).

2.2.2.4 Clot retraction assays

PRP was isolated from mouse blood and diluted to a concentration of 2x10^{8}/ml. Immediately prior to the experiment, this PRP was supplemented with 2mg/ml fibrinogen and 2mM $CaCl_2$. 250µl PRP was added to an aggregometer tube containing a paper clip and clot retraction begun by the addition of 10U/ml thrombin. Clot retraction was allowed to occur for periods of 15, 30, 45 and 60 min. Percentage clot retraction was obtained by measuring the weight of liquid remaining at the time of interest in the experiment.

2.2.2.5 Flow cytometric analysis of mouse platelets

Washed mouse platelets were isolated and diluted to $1x10^7$/ml in Tyrode's buffer. For flow cytometric analysis of platelet receptor expression, platelets were then stained with 100µg/ml FITC-conjugated α-mouse antibodies to the receptor of interest (as indicated) or an IgG-FITC control antibody for 30min in the dark. Platelets were then diluted with 200µl Tyrode's buffer before analysis with a FACScalibur flow cytometer and CellQuest Software. Offline analysis was performed using Summit software in order to obtain median fluorescence values of each sample.

2.2.2.6 Flow cytometry of fixed and permeabilised human platelets

Platelet rich plasma (400µl) was stimulated with the indicated concentrations of agonists for 3 min at 37^{o}C. Following stimulation, an aliquot of cells was taken and added to an equal volume of 4% formalin and incubated for 10 min at room temperature. The platelets were then washed in PBS and the pellet produced resuspended in 0.1% saponin and incubated for 5 min at room temperature to permeabilise the platelets. Following this, platelets were resuspended in 0.1% saponin containing 100µg/ml FITC-conjugated anti-human or unconjugated anti-human antibodies, as indicated, an incubated for 30 min in the dark. For unconjugated antibodies, platelets were washed in 0.1% saponin before subsequent incubation with 0.1% saponin containing 30µg/ml of appropriate secondary antibody. Platelets were then analysed with a FACScalibur flow cytometer and CellQuest Software.

2.2.2.7 cAMP assay

Washed platelet ($6x10^7$/ml) were stimulated at 37^{o}C for 3 min with adrenaline, PGE_1 or both in conjunction. Stimulations were performed under non-stirring conditions to prevent platelet aggregation. Following stimulation and lysis, samples were analysed using the Parameter

cAMP ELISA kit (R&D systems, Abingdon, UK). Briefly, samples or standards were added to individual wells on a Goat anti-mouse antibody coated 96 well plate along with a calibrator reagent to determine non-specific binding. Following this, anti-cAMP primary antibody was added to all wells except non-specific binding. cAMP-HRP conjugate was also added and the plate incubated for 3 hours at room temperature with mixing. Following incubation, wells were aspirated and washed three times with wash solution. Following the final wash, substrate solution was added and the plate incubated for 30 min before the addition of stop solution. Following termination of the reaction, plates were analysed by reading at 450nm in a microplate reader. All reagents used were components of the Parameter cAMP ELISA kit.

2.2.2.8 Tail bleeding assays

Experiments were conducted on mice between 7 – 9 weeks of age, or 6 weeks after transplantation in deficient and litter-matched wild-type mice. Mice used post-transplantation were at an age of 12-14 weeks. For Dasatinib treated mice, animals were dosed by intraperitoneal injection of Dasatinib (5mg/kg) 2 hours prior to conducting the experiment. Mice were anesthetized with isofluorane, and buprenorphine was used as an analgesic. A 3mm portion of the tail tip was excised with a razor blade. Following excision of tail tip, any blood lost was collected in a clean eppendorf tube. Mice were allowed to bleed until they lost either 15% blood volume (assuming a blood volume of 70 µl/g) or for 30 min. Following termination of the experiment by cauterization of the tail, amount of blood lost was determined by weight of fluid within the tube.

2.2.2 Molecular biology and generation of recombinant protein.

2.2.2.1 Plasmids and constructs

Mouse Fgr cDNA was obtained from IMAGE consortium (Source Bioscience, Nottingham, UK). Fgr was cloned into pGEMTEasy vector (Promega, Southampton, UK). Fgr kinase insert was generated by a two-step PCR reaction using primers complementary to the 5' and 3' end of Fgr which contained EcoRI and NotI sites and mutational primers complementary to sequence within the Fgr cDNA sequence. The N-terminal (unique) domain of Fgr was from the above product using 5' primers as above and a primer complementary to a site 210bps into Fgr coding sequence. Inserts were subcloned into pGEX 4T2 (GE Life Sciences, Buckinghamshire, UK) vector by use of EcoRI and NotI restriction enzymes in order to generate an N-terminal Glutathione-S-Transferase (GST) tag. Hi-Fidelity Taq polymerase (Roche, East Sussex, UK), Rapid ligation kit (Roche, East Sussex, UK), chemically competent DH5α *E.coli* and mini-prep kits (Sigma-Aldrich, Poole, UK) were used according to manufacturer's instructions. All cloning was confirmed by Plasmid to Profile sequencing (School of Biosciences, University of Birmingham, UK).

Table 2.4 – Constructs

Construct	Primers
Full Length Fgr (1st round –5' section)	**FWD:** 5' – GAA TTC AAG GCT GTG TGT TCT GCA AGA A – 3' **REV:** 5' – GGG TCA GGG AAA TAG CGT TC – 3'
Full Length Fgr (1st round –3' section)	**FWD:** 5' – GAA CGC TAT TTC CCT GAC CC – 3' **REV:** 5' – GCG GCC GCG CTA TGT CTG GTC TCC AGG C – 3'
Full length Fgr (2nd round)	**FWD:** 5' – GAA TTC AAG GCT GTG TGT TCT GCA AGA A – 3' **REV:** 5' – GCG GCC GCG CTA TGT CTG GTC TCC AGG C – 3'
N-terminus of Fgr	**FWD:** 5' – GAA TTC AAG GCT GTG TGT TCT GCA AGA A – 3' **REV:** 5' – GCG GCC GCG AAT ATG GTC ACT CCG GTC C – 3'

2.2.2.2 GST-fusion proteins

Expression

Constructs for GST-tagged full length and N-terminus of Fgr were transformed into BL21 *E.coli* cells and grown as a 100ml overnight culture in Luria-Bertani broth (LB broth: 10g tryptone, 5g yeast extract, 10g NaCl per litre). This was then used to inoculate a flask of LB broth (1 litre) and ampicllin (100µg/ml) and was grown with shaking at 37^{o}C until an optical density at 600nm (OD_{600}) was between 0.4 and 0.6. Cultures were then induced to express fusion proteins with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG 100µM). This was incubated at room temperature overnight.

Purification

The cultures were centrifuged for 15 min at 2000x g (JLA 10.500 rotor, BD) at 4^{o}C. The bacterial pellet was resuspended in 40ml of lysis buffer (PBS containing 1% Triton-X100, sodium orthovanadate, 5µg/ml leupeptin, 1mM AEBSF, aprotinin and pepstatin-A) and sonicated repeatedly on ice. Debris was then removed by centrifugation at 16000x g for 30 min (JA 25.50 rotor, BD). The lysate was then passed through a 0.45µm filter to further remove any debris. The fully cleared lysate was then passed through a 4ml glutathione-agarose column and recycled 5 times. The column was then washed with 80ml lysis buffer followed by 80ml PBS. Bound GST-fusion proteins were then eluted from the column with 4ml PBS containing 10mM glutathione (pH=7).

2.2.3 Biochemical analysis

2.2.3.1 Western blotting

Electrophoresis was performed using 4-12% NuPAGE gradient gels (Invitrogen) or acrylamide gels of differing percentage, dependent on protein of interest. Proteins were then transferred to PVDF membrane by semi-dry transfer method and membranes blocked overnight using 5% bovine serum albumin (BSA) containing 0.1% sodium azide. Membranes were incubated for 1.5 hr at room temperature with the desired primary antibody (1:1000) diluted in 5% BSA containing 0.1% sodium azide. Membranes were then washed three times in high salt (0.7M NaCl) Tris-buffered-saline with 0.1% Tween (TBS-T) over a 30 min period. Following this, membranes were incubated with a horse-radish-peroxidase (HRP) conjugated secondary antibody (1:10000) or Alexa 488 conjugated secondary antibody (1:10000) diluted in TBS-T for 1 h. Membranes were subsequently washed in high salt TBS-T as above. For HRP-conjugated secondaries, membranes were then incubated for 1 min with an enhanced chemiluminescence (ECL) kit before being exposed to film. For Alexa 488-conjugated antibodies, images were taken using the Li-Cor Odyssey western blotting system.

2.2.3.2 Quantitation of Src family kinases in platelets

Recombinant protein for Src, Fyn and Lyn (Enzo Life Sciences, Exeter, UK) or the N-terminal portion of Fgr (generated as in Section 2.2.2) at final amounts of 30, 10, 3 and 1ng were resolved on 10% SDS-PAGE alongside whole cell lysates from five human donors or three wild type mice. Following transfer, blots were immunoblotted with appropriate primary and secondary antibodies. Proteins were detected and densitometry performed using the Odyssey infrared imaging system (Li-Cor, Cambridge, UK). Subsequently, densitometry data

from recombinant protein was used to generate a standard curve from which concentration of protein within the whole cell lysate could be determined.

CHAPTER 3
QUANTITATION OF THE SRC FAMILY KINASES IN MOUSE AND HUMAN PLATELETS

3.1 Introduction

Src family kinases are critically required for signalling downstream of a variety of platelet receptors, including GPVI and αIIbβ3 complex. As described previously, human and mouse platelets have been demonstrated to express seven of the eight Src family kinases that are found within the mammalian genome, these being Src, Fyn, Lyn, Yes, Fgr, Hck and Lck (Stenberg et al., 1997, Golden et al., 1986, Horak et al., 1990, Quek et al., 2000, Corey and Anderson, 1999, Lannutti et al., 2003). Studies by Dr Sonia Severin and myself have highlighted the presence of three Src family kinases in both human and mouse platelets, with differential expression of a fourth member. Human platelets have been shown to express Yes, whereas mouse platelets express Fgr (Appendix A).

Src family kinases consist of SH3, SH2 and SH1 domains, which display strong sequence conservation. However, the SH4 or unique domain has a divergent sequence between members of the family (Engen et al., 2008). This allows for antibodies to be raised to this region which can specifically recognise members of the family with little to no cross-reactivity. The SH4 domain is implicated in regulating the localisation of Src family kinases as illustrated by the interaction of Lck with CD4 and CD8 (Turner et al., 1990, Kim et al., 2003). This may explain why the SH4 domain is highly conserved between species (Figure 3.1). This conservation is the reason antibodies cross-react between human and mouse. One exception to this is Fgr which displays a divergent N-terminal region between human and mouse protein (Figure 3.2).

Figure 3.1 Alignment of mouse and human sequences of the major platelet expressed Src family kinases. Lyn, Fyn and Src display highly conserved sequences between mouse and human protein in the unique (SH4) domain. Region shown from human is also that to which polyclonal antibodies are raised *=absolutely conserved, :=highly conservative change .=conservative change. .

Figure 3.2 Alignment of mouse and human sequences of Fgr. Fgr shows significant differences between the mouse and human sequences in the unique (SH4) domain. Region shown from human is also that to which polyclonal antibodies are raised *=absolutely conserved, :=highly conservative change .=conservative change.

The Src family kinases show differential expression patterns which may reflect their importance in the regulation of cellular events in particular cells. Often deficiency of the most abundant SFK generates the greatest phenotype in mouse models. For example, Hong et al demonstrated that Lyn is the most expressed SFK in mast cells obtained from mouse bone marrow, with Fyn and Hck being expressed at lower levels (Hong et al., 2007). This is consistent with the major role of Lyn in positive and negative signalling in the mast cell (Xu

et al., 2005). The relative expression of Src family kinases in platelets is unknown, although it has been suggested that Src is the most abundant (Golden et al., 1986).

The regulation of the Src family kinases is critically dependent on phosphorylation as outlined in General Introduction. In order to interrogate phosphorylation of the Src family kinases, phospho-specific antibodies can be used. These antibodies are raised to either the C-terminal tail region or the active site region, which is highly conserved between members (Figure 3.3). Because of the latter, it is difficult to determine which members of the SFKs become phosphorylated downstream of various receptors, particularly as immunoprecipitation of SFKs is complicated by the presence of IgG heavy chain resolving at the same size as SFKs on SDS-PAGE gels.

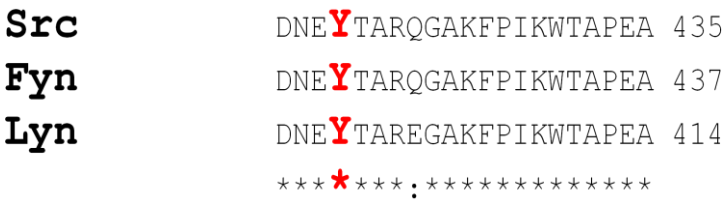


Figure 3.3 Conserved region in Src family kinase catalytic domain. Src family kinases are highly conserved in the region surrounding the important Tyr-418 (Src numbering) residue found in the catalytic domain. This activatory tyrosine residue is highlighted in red in the figure.

The aim of this chapter is to quantitate the level of all expressed SFKs in mouse platelets and and compare this to human platelets.

3.2 Results

3.2.1 Generation of recombinant Fgr protein in a bacterial system

In order to determine expression levels of the Src family kinases in human and mouse platelets, recombinant protein of the various SFKs was required. Recombinant protein for the majority of the human Src family kinases can be obtained commercially. This human protein can be used to quantitate the Src family kinases in both human and mouse platelets due to the highly conserved nature of the N-terminal region to which antibodies are raised, but with a caveat that it could lead to slight over or underestimates due to differences in affinity between the two species. The sequence to which antibodies are raised is as described in the Chapter Introduction. However, Fgr protein sequence at the N-terminus is divergent between the two species, therefore, mouse protein, which is not commercially available must be generated.

In order to generate recombinant protein, mouse Fgr was cloned into an expression vector. An IMAGE clone (ID: 5317449, Source Bioscience, Nottingham, UK) was obtained and primers generated to 5'- and 3'-end of the full length Fgr construct with EcoRI and NotI sites added to 5'- and 3'-ends, respectively. Following this, the construct was subcloned into pGEMTEasy (Promega, Southampton, UK) and subsequently subcloned into pGEX 4T2 (GE Life Sciences, Buckinghamshire, UK) using the restriction enzyme sites. Following this, BL21 *E.coli* were transformed with the construct and allowed to grow, followed by induction of expression with IPTG. However, due to the relatively low expression of the construct by bacteria, it proved difficult to generate sufficient purified protein to detect on a Coomassie stained gel (unpublished). The low expression of the construct may be due to the presence of the tyrosine kinase domain within the enzyme causing disregulated protein phosphorylation and therefore death of bacterial cells or secretion of protein.

In order to overcome this issue, a construct was generated which contained only the N-terminal unique region of mouse Fgr. This is the region recognised by Fgr-specific antibodies and lacks kinase activity. This construct was generated with primers complementary to the severe 5' of the Fgr cDNA and a 3' region 210 base pairs into the Fgr coding sequence. This construct was cloned into pGEX 4T2, expressed and purified as outlined above. Due to the much stronger expression of the Fgr N-terminus obtained than with the full length protein, a higher degree of purity was achieved upon column chromatography. Following chromatography, the concentration of GST-Fgr N-terminus was determined by generating a standard curve using known amounts of BSA (Figure 3.4).

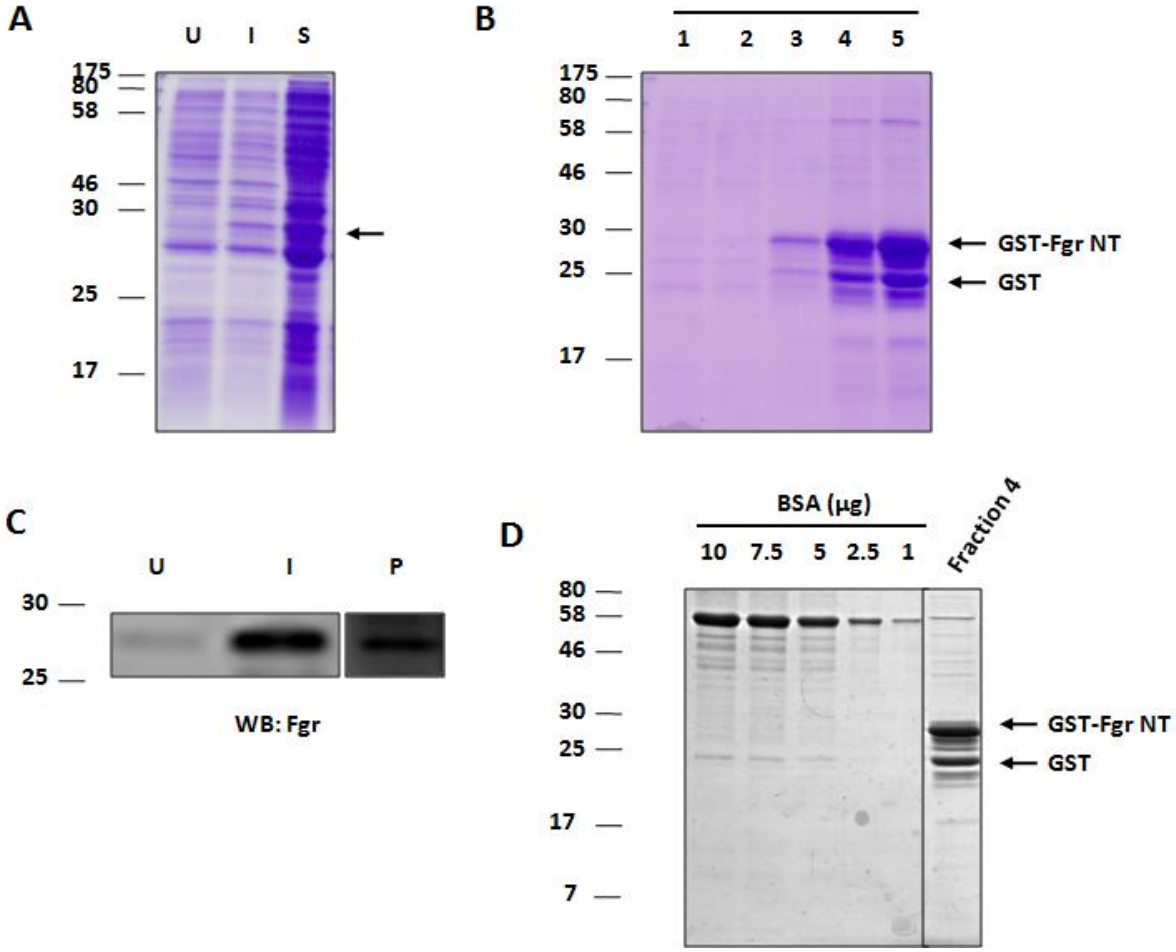


Figure 3.4 N-terminus of Fgr is expresses well in BL21 *E.coli*. A) Lysates from either uninduced (U), IPTG induced (I) or the soluble lysate (S) subsequent to triton lysis of BL21 *E.coli* transformed with GST-NT Fgr were run on 10% SDS-PAGE gels and stained with Coomassie blue. B) Five fractions obtained from glutathione column purification of *E.coli* transformed with GST-full length Fgr were run on 10% SDS-PAGE and subsequently stained with Coomassie blue. C) i) Bacterial lysates from uninduced (U) and IPTG induced bacteria (I) or glutathione column purified protein (P) were run on separate 10% SDS-PAGE gels before transfer and western blotting with indicated antibodies. D) A sample from fraction 4 (as shown in B) was run alongside the indicated amounts of BSA in order to generate a standard curve and therefore determine the concentration of protein in fraction 4.

3.2.2 Determination of levels of Src family kinases in mouse and human platelets.

The recombinant Fgr protein was used to determine both the concentration and copy number of Fgr expressed in mouse platelets. In addition to this, commercially purchased human Lyn, Fyn and Src protein (Enzo Life Sciences, Exeter, UK) were used to determine the copy number and concentration of their respective kinases in human and mouse platelets.

Known amounts (30, 10, 3 and 1µg) of purified recombinant protein were resolved alongside platelet lysates by SDS-PAGE and blotted with antibodies to the protein of interest. The recombinant protein was used to generate a standard curve from which the amount of protein in a platelet lysate was determined. As the equivalent number of cells in the lysate was known, along with the mass of a mol of the protein and volume of the platelet (9.5fl), this could be used to generate copy number and concentration from amount of protein using Equation 1 and Equation 2, respectively. As the bands seen for Lyn by western blot resolve as a doublet, the density of both bands were added to obtain the level of expression.

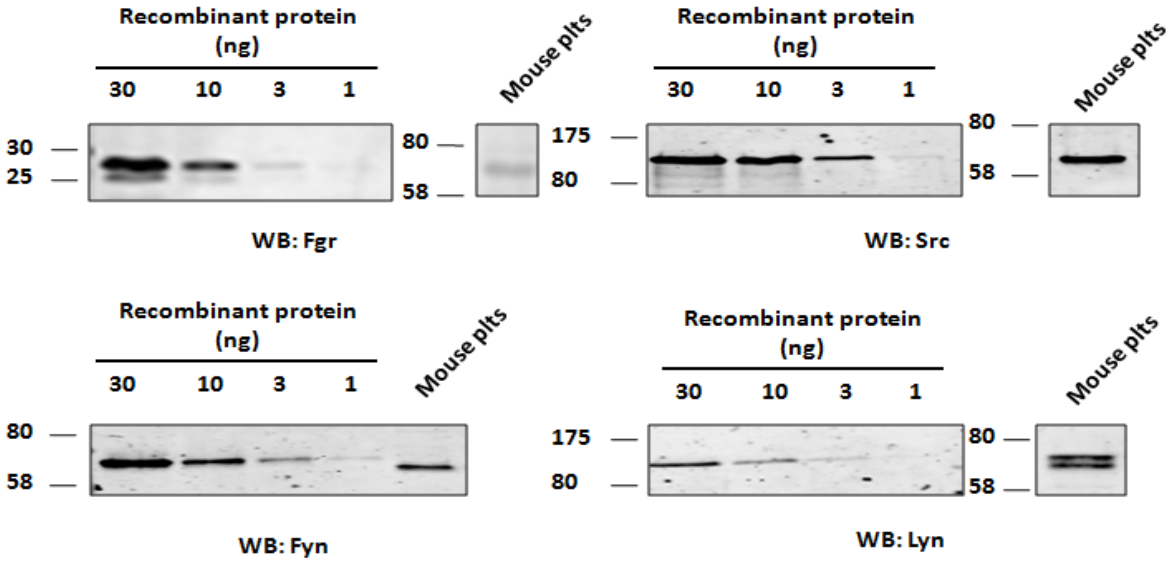
Equation 1

$$Copy\ number = \frac{\left(amount\ of\ protein \Big/ mass\ of\ mol\ of\ kinase \right)}{equivalent\ number\ of\ platelets\ in\ lane}$$

Equation 2

$$Concentration\ per\ platelet = \frac{\left(copy\ number \Big/ Avagadro's\ number \right)}{volume\ of\ a\ platelet}$$

Using these equations, the concentration of Lyn was determined to be the highest in mouse platelets, being expressed at approximately ten times that of the other Src kinases at 72 ± 3.2µM. Fyn and Src are expressed at very similar levels, 5.6 ± 0.70µM and 6.0 ± 2.4µM, respectively. Fgr is expressed at approximately one quarter to one fifth of the level of Fyn and Src (1.2 ± 0.04µM) (Figure 3.5).

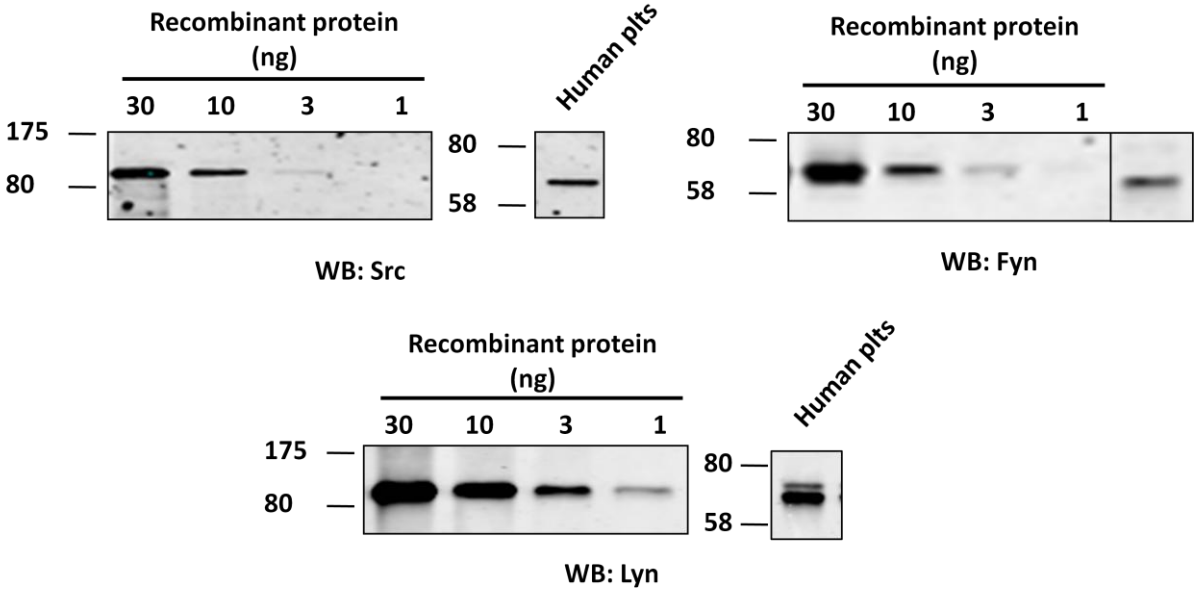


	Copy no per platelet	Concentration (μM)
Fgr	8900 ± 90	1.2 ± 0.04
Src	33000 ± 15000	6.0 ± 2.4
Fyn	32000 ± 4100	5.6 ± 0.70
Lyn	420000 ± 18000	72 ± 3.2

Figure 3.5 Determination of mass levels of SFKs in mouse platelets. Recombinant protein for SFKs, as indicated, of 30, 10, 3 and 1ng in amount were resolved alongside mouse platelet lysates and immunoblotted with indicated antibodies. Recombinant band intensity was then used to determine amount of protein in mouse platelets. Recombinant proteins resolve differently to platelet proteins due to the presence of epitope tags. Fgr, Src and Lyn proteins all contain GST tags (+~25kDa), whereas Fyn recombinant protein contains a His tag which does not significantly alter molecular weight. Gels are representative of 3 separate experiments with 3 different mice, with numbers representing the arithmetic mean ± SEM.

To ascertain the concentration of the three major members of the Src family expressed in human platelets, known concentrations of commercially purchased recombinant protein were resolved alongside human platelet lysates on SDS-PAGE and western blotted with an antibody specific to the protein of interest. The recombinant protein was then used to determine the copy number and concentration by generation of a standard curve and utilising equation 1 and 2, as outlined above. Src, Fyn and Lyn mass levels were determined in this study. Other Src family kinase members were not studied due to lack of availability of recombinant protein from Yes or non detection of Lck, Fgr, Hck, or Blk protein in platelet lysates.

Lyn and Src were expressed in human platelets at 6.0 ± 2.4μM and 4.6 ± 1.8μM, respectively. Interestingly, Fyn is expressed at twice the level of the other two members of the family at 12 ± 2.4μM. This result is very different from mouse platelets where Lyn is expressed at a significantly higher level than the other three members (Figure 3.6).



	Copy no per platelet	Concentration (μM)
Src	26000 ± 10000	4.6 ± 1.8
Fyn	68000 ± 13000	12 ± 2.4
Lyn	34000 ± 6800	6.0 ± 2.4

Figure 3.6. Fyn is expressed at twice the level of Lyn and Src in human platelets. Recombinant protein for either Src, Fyn and Lyn, as indicated, of 30, 10, 3 and 1ng in amount were resolved alongside human platelet lysates and immunoblotted with indicated antibodies. Recombinant band intensity was then used to determine amount of protein in human platelets. Recombinant proteins resolve differently to platelet proteins due to the presence of epitope tags. Fgr, Src and Lyn proteins all contain GST tags (+~25kDa), whereas Fyn recombinant protein contains a His tag which does not significantly alter molecular weight.Gels are representative of 3 separate experiments using 5 different donors, with numbers in table representing the arithmetic mean ± SEM.

3.2.3 Identification of Src family kinase bands in a phosphotyrosine blot

Analysis of mouse platelet whole cell lysates by western blotting with an anti-pY418 Src family kinase antibody reveals three distinct bands in the region at which the Src family kinases should resolve (~60kDa). SFK deficient platelets were used to determine which of these bands corresponds to a specific member of the Src family kinases.

The two lower bands on a phosphotyrosine blot were absent in Lyn-deficient platelets, suggesting that they correspond to the two splice variants of Lyn (p53 and p56) (Figure 3.7, panel 4). The remaining upper band migrates with Src, Fyn and Fgr. In Src- or Fyn-deficient platelets, the intensity of the upper band detected using the anti-pY418 Src antibody was reduced by approximately 50% (Figure 3.7, panels 2 and 3), consistent with the similar levels of Fyn and Src. No difference was observed in the Fgr-deficient platelets (Figure 3.3, panel 5), possibly because it is present at a much lower level.

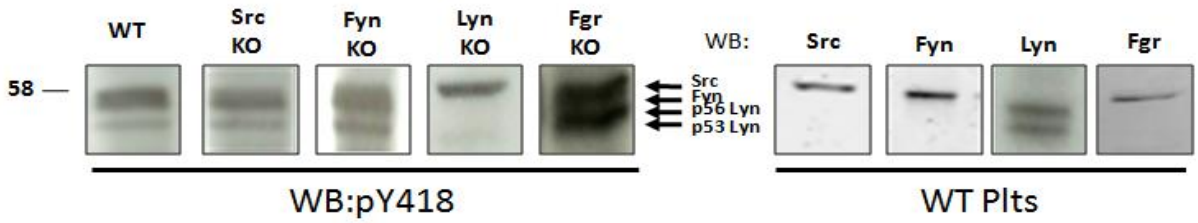


Figure 3.7 Identification of bands shown in region of SFKs in phosphotyrosine blots. Lysates from WT and appropriate knockout platelets were resolved on 10% SDS-PAGE gels before being transferred and blotted with either pY418 SFK or specific Src family kinase antibodies. Gels were then aligned in order to determine the indentity of the band of interest.

3.3 Discussion

The results of this chapter demonstrate that Lyn is expressed at approximately 10x the level of Src and Fyn and 100x the level of Fgr in mouse platelets. In contrast, Fyn has the highest expression in human platelets and the individual Src family kinases are expressed at very different concentrations to those in mice. This may reflect differing roles for the individual Src kinases in the two species, although the absence of specific inhibitors prevents this being tested in human platelets.

Lyn co-migrates with the lower two bands of the triplet three bands seen in anti-pY418 SFK blots of mouse platelets. Deficiency of Lyn in mouse platelets removes these two bands from the blot, whereas deficiency of either Src or Fyn simply reduces the upper band. In contrast, deficiency of Fgr in mouse platelets does not alter pY418 SFK blots. This data is consistent with the quantitation data in this study and taken together both suggest that Lyn is the predominantly expressed member of the Src family kinases in mouse platelets.

The high level of expression of Lyn in mouse platelets suggests that it plays the predominant role in signalling downstream of platelet activation. This is consistent with the observation that aggregation under arterial flow over collagen and in platelet aggregation in a Born-aggregometer show a strong dependence on Lyn (Schmaier et al., 2009, Quek et al., 2000). Lyn-deficient platelets also have increased megakaryopoeisis (Lannutti et al., 2006). This suggests that, as well as its critical role in mouse platelet signalling, Lyn also plays a negative role in the generation of stem cell progenitors of platelets.

The present data is in contrast to reports that Src is the most highly expressed of the SFKs in human platelets, representing 0.2-0.4% of total platelet protein (Golden et al., 1986). Although this may be due to the difference in methods used between the two studies, with

Golden et al using an immunoprecipitation approach to concentrate Src, it could also be due to limitations in the selectivity of antibodies in the mid 1980s. A SAGE library made from mouse megakaryocytes reveals that Lyn and Src each have 9 expression tags, Fyn has 3 tags and Fgr has none (Senis et al., 2009a). SAGE analysis is a method by which small mRNA sections from a cell of interest are studied. The similar number of tags for Lyn and Src may reflect the use of megakaryocytes over platelets. Rowley et al (2011) also reported that Lyn has the high highest level in mouse platelets by an RNA-Seq method, with Src and Fyn being at a lower level (Rowley et al., 2011). RNA-Seq is a method used to assess the whole transcriptome of a cell. It involves purification of coding mRNA through use of polyT magnetic beads through the virtue of the polyA tail on the coding RNA. Following this, samples are analysed by next generation DNA sequencing.

In conclusion, Src family kinases are expressed at differing levels in mouse and human platelets with Lyn being expressed at the highest level in mouse and Fyn in human. This data is corroborated by that from other studies.

CHAPTER 4
INVESTIGATING THE ROLE OF SRC FAMILY KINASES IN αIIbβ3-MEDIATED PLATELET FUNCTION

4.1 Introduction

The major platelet integrin, αIIbβ3, is expressed at ~80,000 copies per human platelet. αIIbβ3 is critical for platelet aggregation as revealed by the increase in bleeding in patients and mice deficient in either subunit (Nurden and Caen, 1974, Phillips et al., 1975, Hodivala-Dilke et al., 1999). Fibrinogen is the major physiological ligand underlying aggregation, although the binding of the integrin to several other matrix proteins including VWF and fibronectin also supports this process.

Ligation of αIIbβ3 by fibrinogen regulates intracellular signalling cascades, including a SFK-Syk-PLCγ2 pathway (see Introduction). These signalling pathways combine with each other and with signals from other receptors to regulate actin polymerisation and platelet spreading. Studies on genetically deficient mice and specific inhibitors have revealed a key role for Src family kinases in platelet spreading, although the precise role of individual members is unclear (Reddy et al., 2008, Maxwell et al., 2004, Obergfell et al., 2002). Src has been described as the major Src family kinase mediating spreading on fibrinogen, however, the evidence provided for this uses a quadruple knockout mouse of Src/Hck/Lyn/Fgr (Obergfell et al., 2002). Both Lyn and Fgr are found in mouse platelets, whereas Hck is not. Therefore, it is necessary to understand the role of these individual Src family kinases in spreading on fibrinogen.

Clot retraction is a process mediated by the bridging of the actin cytoskeleton with αIIbβ3 (Morgenstern et al., 2001, Osdoit and Rosa, 2001, Hodivala-Dilke et al., 1999). The process of clot retraction has been shown to be dependent on the conserved tyrosines within the β3 tail and partially dependent on the Src family kinases (Law et al., 1999a, Suzuki-Inoue et al.,

2007a). However, no investigation has occurred into which of the individual SFKs play a role in this assay.

A role for the Src family kinases in haemostasis has been highlighted utilising the Src family kinase inhibitor Dasatinib (Gratacap et al., 2009), although it is not known to what extent this reflects the role of the SFKs in integrin signalling alongside that in GPVI and GPCR signalling. Further, the individual roles of the individual SFKs in haemostasis are not known.

The aims of this chapter therefore are to identify the role of individual Src family kinases in αIIbβ3-mediated platelet signalling using mice deficient in one or two Src family kinases. Clot retraction and spreading on fibrinogen were chosen so as to compare the roles of the individual Src family kinases in assays which are partially and fully SFK-dependent, respectively.

4.2 Results

4.2.1 Deficiency of one or more Src family kinases does not affect receptor expression or platelet count

Transgenic mice were used to investigate the role of Src family kinases in αIIbβ3-mediated platelet activation. All of the single-deficient Src family kinases animals used in this study, namely Fgr, Fyn, Lyn and Src, were born at Mendelian frequencies, although many of the Src deficient animals had a lifespan of just a few weeks due to the lack of teeth and onset of malnutrition despite the use of liquid agar diet. Platelet counts were not affected up to 12 weeks of age in any of the single deficient Src family kinase mice (Table 4.1), although at later time, the Lyn-deficient mice became severely thrombocytopenic in agreement with previous findings (Harder et al., 2004). Thus, mice aged approximately 6 – 10 weeks were used for subsequent studies.

Animals double deficient for Fyn/Lyn and Fgr/Lyn were also born at Mendelian frequencies. Mice deficient in Fyn and Lyn had normal platelet counts but an increase in mean platelet volume compared to their wild type littermates (Table 4.1). In contrast, mice deficient in Fgr and Lyn however had a significantly reduced platelet counts compared to wild type animals. This suggests that Fgr and Lyn may play a role in thrombopoeisis or in regulating platelet removal. However, deficiency of Lyn and either Fgr or Fyn does not cause a change in the expression of any of the major receptors or receptor subunits (Table 4.1).

Mice deficient in Fyn/Src and Lyn/Src showed a high level of perinatal lethality. Therefore, in order to perform platelet experiments, radiation chimera animals were generated as described in Materials & Methods. Radiation chimera animals deficient in Fyn and Src do not display significantly altered platelet counts or mean platelet volume compared to wild type

counterparts. In contrast, radiation chimera animals deficient in Lyn and Src show significantly reduced platelet counts, but no alteration in mean platelet volume (Table 4.2). Deficiency of Fyn and Src causes no alteration of receptor expression in mouse platelets, however, deficiency of Lyn together with Src causes a significant decrease in GPIb (Table 4.2)

Table 4.1 Expression of platelet receptors in conventional knockout platelets. Platelets obtained from wild type and indicated kinase deficient mice were analysed by flow cytometry to determine receptor expression using antibodies for indicated proteins. Results are presented as geometric mean ± SEM from median fluorescence readings. Platelet counts and mean platelet volume values were obtained using a whole blood cell analyser (ABX Pentra 60) calibrated for mouse blood. Readings were obtained from 3 or more mice and statistically analysed by two-way ANOVA followed by Bonferroni post-test. **:$p<0.01$. PC = platelet count, MPV = mean platelet volume.

	Control	***src*-/-**	***fgr*-/-**	***lyn*-/-*fyn*-/-**	***lyn*-/-*fgr*-/-**
PC (10^3/μl)	866 ± 42	822 ± 52	843 ± 45	823 ± 47	267 ± 49**
MPV (fl)	5.5 ± 0.1	5.0 ± 0.6	5.2 ± 0.2	6.2 ± 0.2**	6.3 ± 0.2**
GPVI	16.7 ± 1.0	20.3 ± 2.2	17.2 ± 1.1	22.2 ± 1.6	15.9 ± 0.5
GPIb	50.4 ± 1.0	42.8 ± 5.3	43.0 ± 4.3	50.8 ± 4.9	37.9 ± 3.5*
αIIb	89.5 ± 12.8	102 ± 25	128 ± 14	108.9 ± 15.6	104 ± 23
α2	3.6 ± 0.5	4.5 ± 0.5	2.82 ± 0.4	3.3 ± 0.4	3.7 ± 1.0

Table 4.2 Expression of platelet receptors in radiation chimera knockout platelets. Platelets obtained from wild type and indicated kinase deficient mice were analysed by flow cytometry to determine receptor expression using antibodies for indicated proteins. Results are presented as geometric mean ± SEM from median fluorescence readings. Platelet counts and mean platelet volume values were obtained using a whole blood cell analyser (ABX Pentra 60) calibrated for mouse blood. Readings were obtained from 3 or more mice and statistically analysed by two-way ANOVA followed by Bonferroni post-test. *:$p<0.05$, **:$p<0.01$. PC = platelet count, MPV = mean platelet volume.

	Chimeric control	***fyn*-/-*src*-/-**	***lyn*-/-*src*-/-**
PC (10^3/µl)	744 ± 52	813 ± 32	481 ± 49**
MPV (fl)	5.4 ± 0.1	5.2 ± 0.1	5.7 ± 0.1
GPVI	19.9 ± 2.4	18.5 ± 2.5	16.7 ± 0.4
GPIb	41.2 ± 2.5	44.5 ± 3.3	36.2 ± 1.9*
αIIb	103.5 ± 17.0	118.3 ± 12.3	89.6 ± 7.9
α2	3.4 ± 0.3	3.6 ± 0.1	3.1 ± 0.4

4.2.2 The role of Src and Lyn in spreading on a fibrinogen-coated surface.

In order to determine the roles for the individual members in functional responses by αIIbβ3, platelets from mice deficient in either Src, Fyn, Lyn or Fgr were allowed to spread on fibrinogen-coated coverslips and imaged by DIC microscopy. Results from these mice were compared with wild type controls and controls treated with the Src family kinase inhibitor, PP2 (20μM).

Wild type mouse platelets adhere and spread on fibrinogen coated surfaces, sending out filopodia to give a 'spiky' appearance to the spread platelet (Figure 4.1, upper left panel). Wild type controls were taken for all experiments and pooled due to no significant difference between wild types from different mice colonies. In contrast to wild type platelets, PP2-treated platelets do not spread and produce a rounded appearance on fibrinogen (Figure 4.1, lower right panel). Platelets obtained from knockouts of Fyn or Fgr knockout mice display no significant difference in spread surface area, adhesion and appearance when compared to their wild type controls (Figure 4.1, upper middle and left panels). Interestingly, platelets obtained from Src knockout animals display a significantly lower degree of spreading, demonstrated by the reduction (over 70%) in spread surface area on fibrinogen (Figure 4.1, lower middle panel). In contrast to this, platelets from Lyn deficient animals display increased spreading on a fibrinogen coated surface, to a level of ~200% (Figure 4.1, lower left panel). These results, taken together, suggest a positive role for Src in αIIbβ3-mediated signalling and a negative role for Lyn.

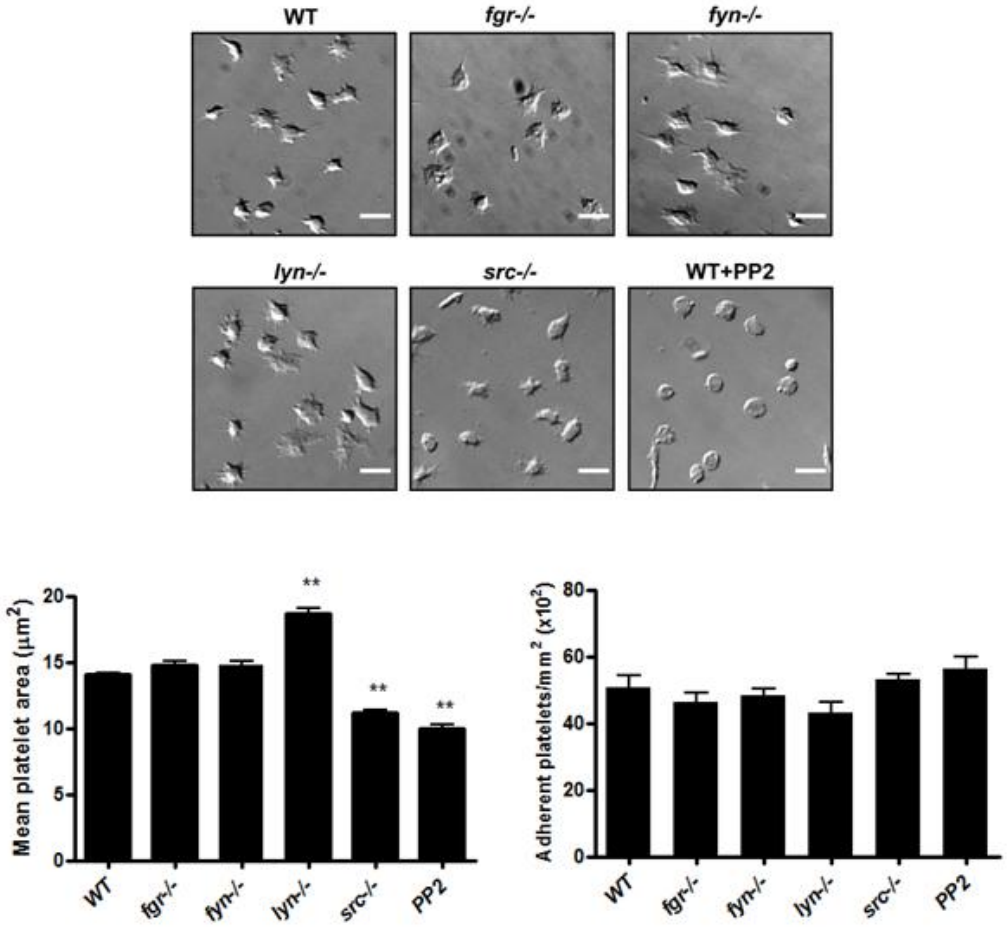


Figure 4.1 Spreading on fibrinogen is dependent on Src. Lyn also plays a negative role. Washed mouse platelets ($2x10^7$/ml) were spread on fibrinogen coated coverslips for 45 min, subsequently fixed and imaged by DIC microscopy. Analysis of spreading and adhesion was performed offline using Image J software. All images are representative of at least 3 experiments. Results were statistically analysed by two way ANOVA followed by Bonferoni post-test.

4.2.3 Double knockout platelets of Src in conjuction with either Lyn or Fyn display similar phenotypes to that seen with the Src knockout.

As described previously, there are four members of the Src family kinases expressed in mouse platelets (General Introduction and Appendix). The Src family kinases are well known to display compensatory mechanisms, i.e. when one is knocked out in a system, one or more of the other members of the family will fulfil the role of the missing member. In order to test if these compensatory mechanisms exist in mouse platelets spreading on fibrinogen, double knockouts of the Src family kinases were generated. As Lyn is the major Src family kinase expressed in mouse platelets (Chapter 3) and deficiency of the kinase shows a significant potentiation on spreading on fibrinogen, mice deficient in Lyn/Fyn and Lyn/Fgr were studied in this static adhesion assay. Also, as Src deficient platelets show a significant reduction in spreading of fibrinogen, mice deficient in Lyn/Src and Src/Fyn were generated, particularly given the previously published role of Fyn in platelet spreading, along with the constitutive association of Src and Fyn with the β3 tail (Arias-Salgado et al., 2003, Reddy et al., 2008).

Fyn/Lyn doubly deficient platelets display a phenotype which was very similar to that observed with the Lyn deficient platelets, in that there is a potentiation of spreading on a fibrinogen coated surface of 135% when compared to wild type controls. Interestingly, platelets deficient in Lyn/Src show a reduction similar to that seen in PP2 treated platelets (Figure 4.2, lower right panel). However, Fyn/Src-deficient platelets show a significant reduction in spreading, ~50% of that seen in wild type platelets. Platelets doubly deficient in Fgr and Lyn do not show a reduction in platelet spread area when compared to wild type controls, however, there is a significant reduction in the number of platelets with filopodia in animals deficient in these two kinases (41.3 % ± 1.9 % versus 73.4 % ± 3.4 % of Fgr/Lyn-deficient platelets relative to wild-type platelets).

Taken together, these results suggest that Src plays a major role in spreading on fibrinogen, with the potentiation seen in the absence of Lyn requiring Src activity. In contrast to previously published results (Reddy et al, 2008) we do not observe a role for Fyn in spreading in our hands. Interestingly, the potentiating role of Lyn observed in single deficient platelets is overcome in the absence of Fgr.

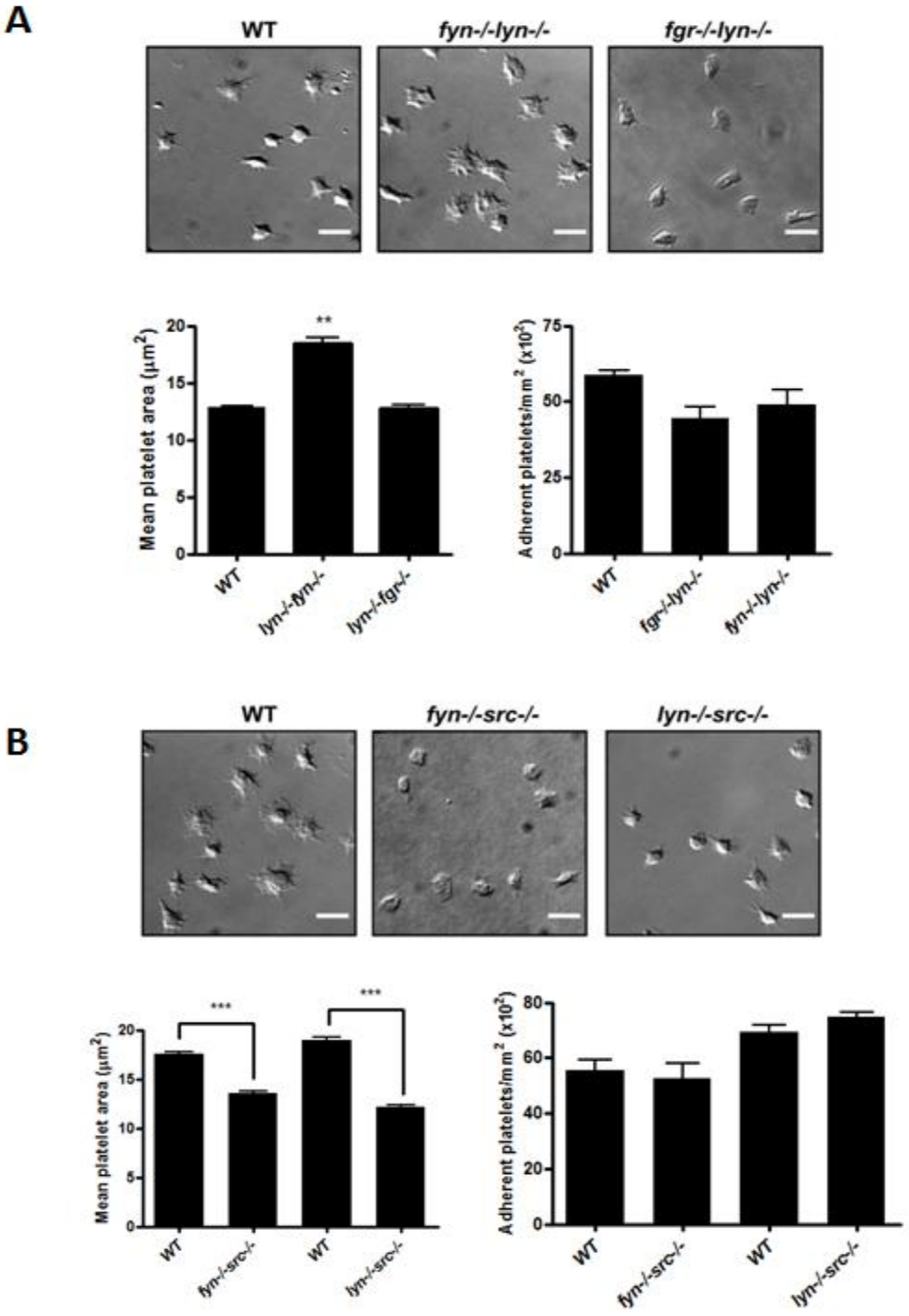


Figure 4.2 Spreading of platelets doubly deficient in Lyn/Fyn, Lyn/Src and Fyn/Src on fibrinogen. Mouse platelets from animals constituitively doubly deficient (A) or doubly deficient radiation chimera animals (B) for the indicated kinases were spread on fibrinogen-coated coverslips for 45 mins before being fixed and imaged. Offline analysis of spreading and adhesion was performed using Image J. All images are representative of at least 3 experiments. Results were statistically analysed by two way ANOVA followed by Bonferoni post-test.

4.2.4 Clot retraction is a time and Src family kinase dependent event.

Clot retraction is a physiological event which allows for the reduction of thrombus size. This event is known to be both αIIbβ3 and actin cytoskeleton dependent, and partially dependent on Src kinases.

The *in vitro* clot retraction assay used within our lab was characterised in order to determine whether the event of clot retraction was dependent on SFK signalling in mouse platelets and therefore could be inhibited by the inhibitor Dasatinib. Another aim of these experiments was determine the most useful time points at which Src family kinase deficient platelets could be studied. In order to perform this experiment, mouse platelet rich plasma was isolated and diluted to an appropriate concentration ($2x10^8$/ml) with Tyrodes buffer. Platelet rich plasma was supplemented with fibrinogen (2mg/ml) and Ca^{2+} (2mM) due to dilution of fibrinogen in plasma and the presence of the Ca^{2+} chelator sodium citrate, respectively. Retraction was initiated with 10U/ml thrombin. Using this method, it was found clot retraction was approximately 50% of the maximal at 10 min and almost complete (90%) by 60 min (Figure 4.3A).

Following this, the time points of 10 and 60 min were used to determine the effect of Src family kinase inhibition on clot retraction. At 10 and 60 min, clot retraction was reduced by approximately 25% in the presence of Dasatinib (Figure 4.3B). This suggests that Src family kinases play a small, but significant role in clot retraction and confirms the results seen by Suzuki-Inuoue et al (Suzuki-Inoue et al., 2007a).

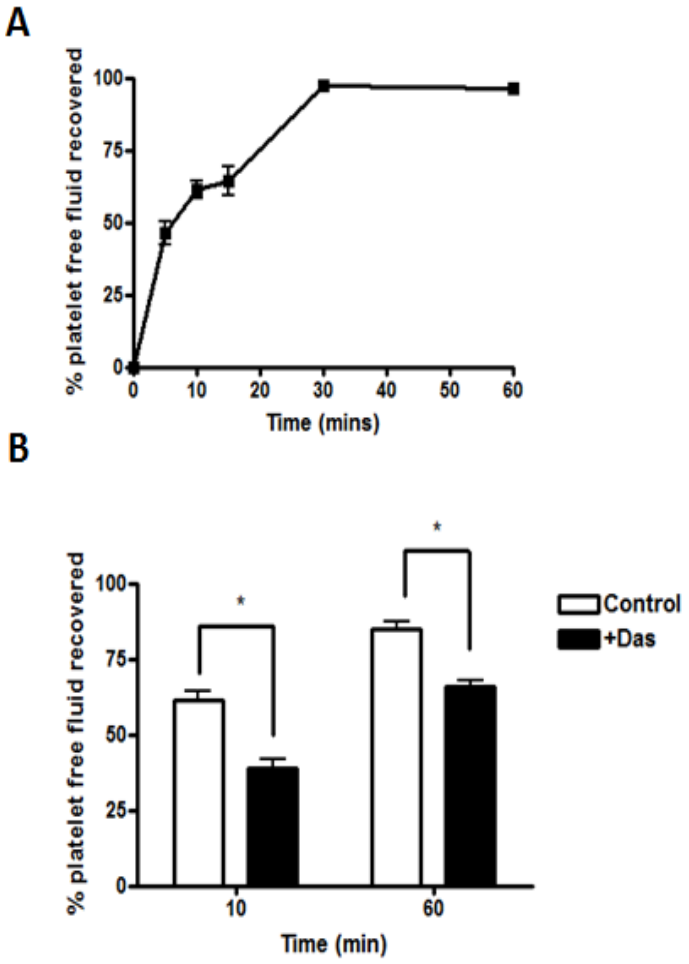


Figure 4.3 Clot retraction is time and Src family kinase dependent. Mouse platelet rich plasma (supplemented with fibrinogen (2mg/ml) and Ca^{2+} (2mM)) were stimulated with thrombin (10U/ml). A) Samples were allowed to clot retract for times indicated before measurement of percentage retraction by weight. B) Mouse PRP was allowed to clot retract for either 10 or 60 min in the presence or absence of dasatinib (20µM). n=5. Results in B were analysed by Students t-test. *:$p<0.05$.

4.2.5 Clot retraction is not affected by deficiency of individual members of the Src family kinases.

In order to determine if individual members of the Src family kinases play a role in clot retraction, SFK-deficient platelet rich plasma was used in a clot retraction assay. This assay was performed as in Section 4.2.4.

Clot retraction proceeded normally in wild type controls from these mice, reaching approximately 50% after 10 min and 90% after 60 min. Interestingly, however, no individual knockouts of the Src family kinases displayed a phenotype of reduced or potentiated clot retraction. Results with Dasatinib suggest that, although some role is played by the Src family kinases in the signalling to clot retraction machinery, the use of Src family kinase platelets suggest that no particular role is played by an individual member of the Src family kinases.

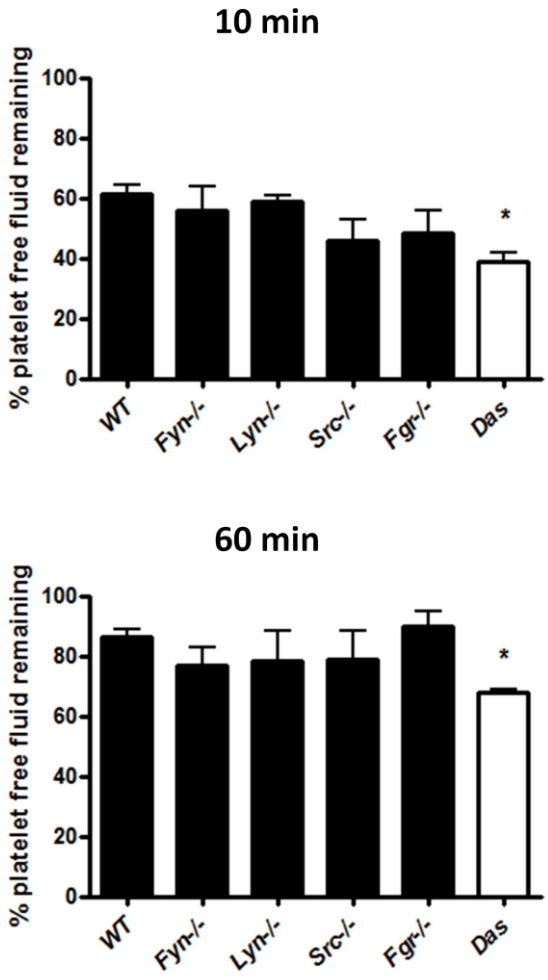


Figure 4.4 Clot retraction is not dependent on a particular member of the Src family kinases. Mouse platelet rich plasma (supplemented with fibrinogen (2mg/ml) and Ca^{2+} (2mM)) from either knockout animals or wild type controls were stimulated with thrombin (10U/ml). Samples were allowed to clot retract for either 10 or 60 min, as indicated, before measurement of percentage retraction by weight. Data is from 3 or more experiments. Results were analysed by two way ANOVA followed by Bonferoni post-test.

4.2.6 Single and double knockouts of Src family kinases do not display a phenotype in an *in vivo* tail bleeding assay

As both single and doubly deficient Src family kinase mice were observed to have significant defects in αIIbβ3-mediated spreading, it was of interest to determine if this signalling defect could also be observed in an *in vivo* setting.

In order to test this, both single and doubly deficient mice, generated as outlined previously, were subjected to an *in vivo* tail bleeding assay. In contrast to that observed in *in vitro* αIIbβ3-dependent assays, no individual or double knockout of the Src family kinases displayed a significant *in vivo* tail bleeding phenotype. Interestingly, however, animals treated with the Src family kinase inhibitor, Dasatinib, displayed significant bleeding above saline treated controls, suggesting some mechanism of compensation between Src family kinases in this assay.

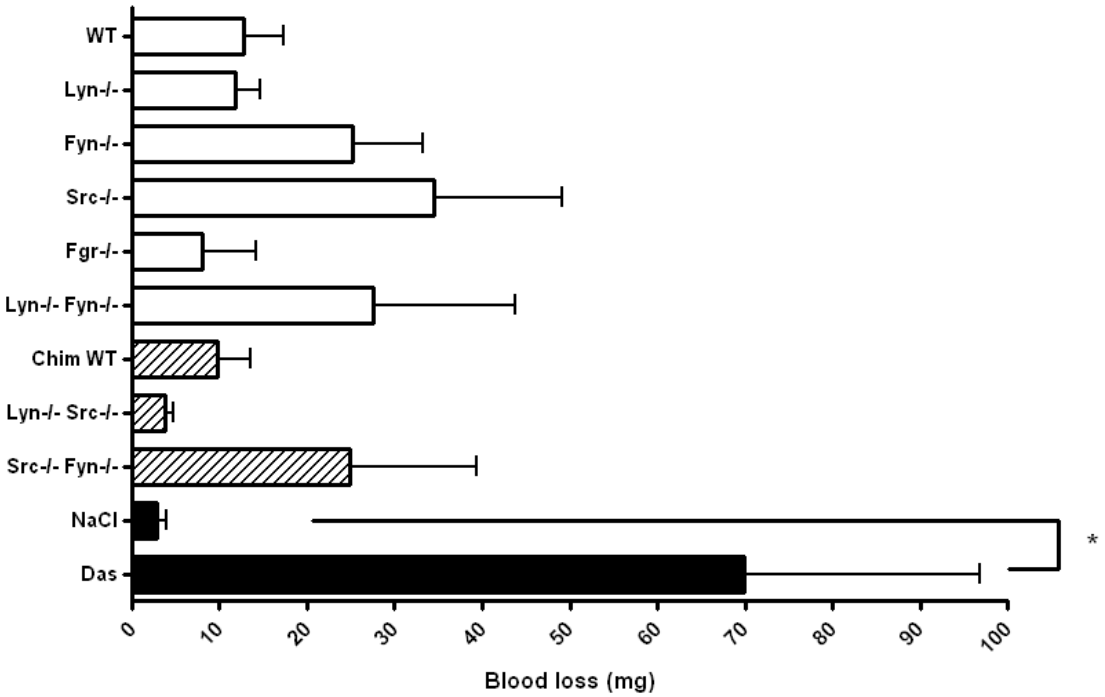


Figure 4.5 *In vivo* tail bleeding is not defective in Src family kinase-deficient animals. Conventional knockout (white bars), Radiation chimera (hatched bars) and drug treated (black bars) animals had a 3mm portion of tail removed and were allowed to bleed for up to 30min or until 15% blood volume had been lost. Wild type data is pooled from mice from several colonies. Drug treated animals were given a single dose of indicated drug 2hr prior to experiment. n=10 for all groups other than drug treated, where n=5. Results were statistically analysed using Kruksal-Wallace test. *:$p<0.05$

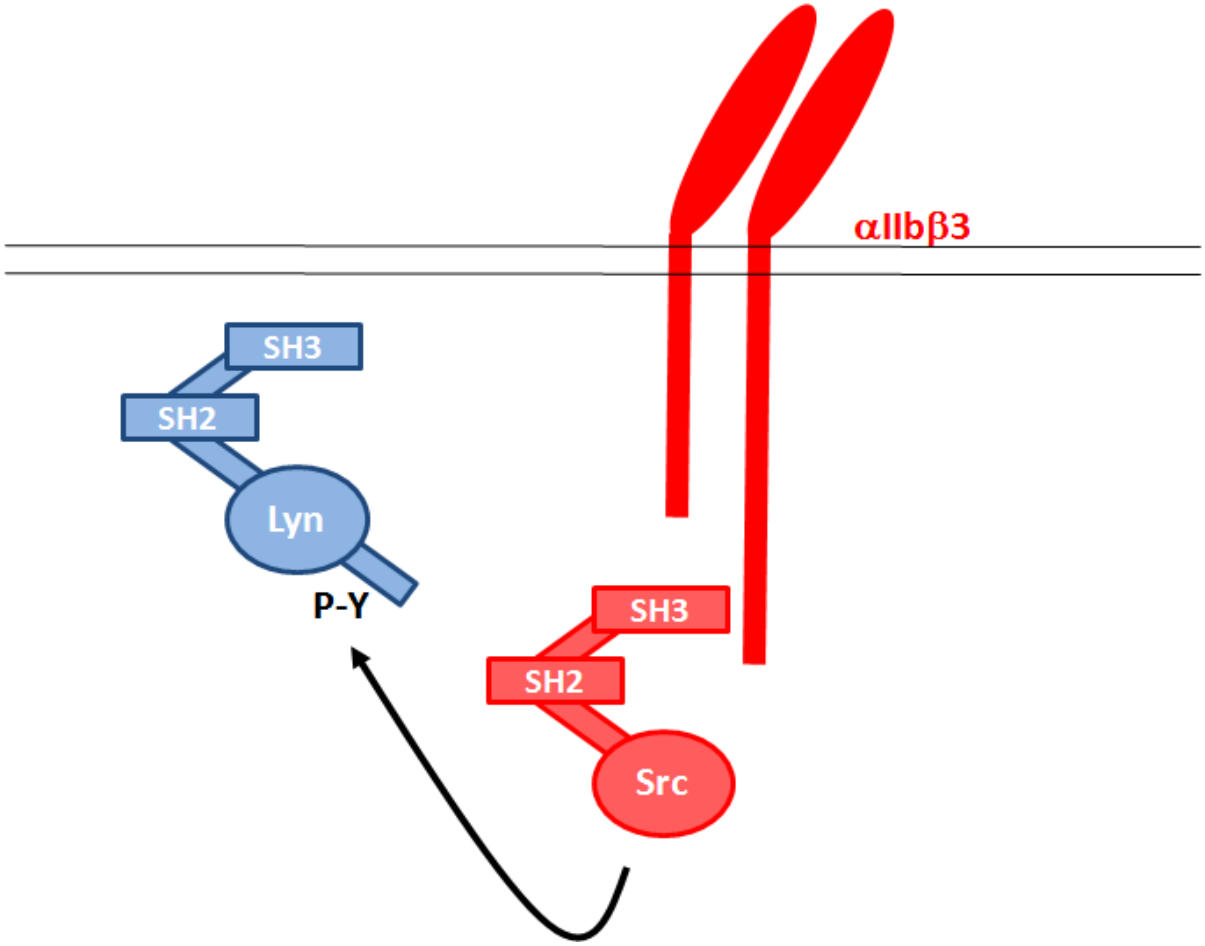
4.3 Discussion

In this chapter, I have demonstrated that the Src family kinases play a critical role in the spreading of platelets on immobilised fibrinogen. I have also demonstrated that Src family kinases a minor yet significant role in another αIIbβ3-mediated event, clot retraction but that this is not mediated by a particular member of the SFKs. In addition to this, it has also been demonstrated that total Src family kinase inhibition in mice leads in an increase in tail bleeding. Importantly, this study demonstrates that Src is the major SFK downstream of αIIbβ3, at least for spreading, and that deficiency of Src in addition to another member of the family does not produce a more severe phenotype. Significantly, knockout of either individual members, or two members in conjunction, does not lead to a significant tail bleeding phenotype.

Interestingly, although αIIbβ3-mediated spreading on fibrinogen is dependent on Src, with potentiation occurring in the absence of Lyn, clot retraction assays do not display any dependence on these individual members. This may suggest a difference in mechanism between clot retraction and spreading on immobilised fibrinogen, however, this may also demonstrate a difference in sensitivity for Src family kinases between the two assays. Some evidence for this is provided by Suzuki-Inuoue et al (2005) who demonstrated that clot retraction has partial dependence on PLCγ2 (a reduction of 10%) but a larger dependence on Src family kinases (inhibition causes a reduction of 20%). In the assay as performed, PAR-3 and PAR-4 thrombin receptors may also play a role as signalling from these receptors will also increase intracellular calcium and induce signalling to the cytoskeleton along with signalling via secondary mediators such as ADP and TxA_2 subsequent to their release. In conclusion, many factors play a role in the clot retraction process and this may mask any significant role for a member of the Src family kinases.

The phenotype of the Src/Lyn double knockout mouse for fibrinogen spreading is no more severe than the Src phenotype, however the potentiation observed with Lyn deficient platelets is no longer present. This suggests that Src may be upstream of the negative signalling of Lyn in the αIIbβ3 pathway. A mechanism which may explain this phenomenon is that Src may act as a negative regulator of Lyn, as well as a positive regulator of β3 tail phosphorylation (Putative model 4.1). Therefore, when Src is activated by receptor ligation, Lyn becomes phosphorylated on its inhibitory tyrosine residue and spreading is allowed to occur due to the removal of negative signals, such as that to SHIP (Maxwell et al., 2004). However, in the absence of Src alone, the negative signal from Lyn dominates and a spreading defect is observed. This would account for the phenotype seen in Lyn deficient platelets, whereby the positive signal from Src dominates and the phenotype seen in the compound knockout as there are no positive or negative signals present, therefore no spreading occurs. This mechanism is similar to that described by Hong et al for Hck and Lyn in mast cells (Hong et al., 2007), whereby it is demonstrated that Hck can phosphorylate the inhibitory site of Lyn.

Putative model 4.1 Src inactivates Lyn via the phosphorylation of the C-terminal tail. Src may potentially inhibit the activity of Lyn downstream of αIIbβ3 by phosphorylating the inhibitory tyrosine found within the C-terminal tail of Lyn. This would remove the negative signal initiated by Lyn and allow the positive Src signal to predominate.



Interestingly, deficiency of Fyn in conjunction with Lyn retains the potentiating phenotype seen in Lyn deficient platelets. Taken with observations from the Fyn singly deficient animals, this suggests that in our assay, Fyn plays very little positive role in αIIbβ3-mediated function. This data is in contrast to that published where Fyn is observed to play a major role in αIIbβ3-mediated spreading on fibrinogen (Reddy et al., 2008). Some reasons for the difference between these observations include a difference in background of the mice and slight differences in the use of the assay, including the use of differing time periods for

spreading and concentrations of fibrinogen. Further to the phenotype of Fyn and Lyn double deficient animals, the phenotype of Fgr and Lyn double deficient animals is also unexpected.

Fgr/Lyn deficient platelets spread to the same degree as wild type platelets, however display fewer filopodia. This may however be a consequence of the platelets having some haematopoetic defect displayed as fewer platelets circulating in the blood of Fgr/Lyn doubly deficient animals. This reduction is also the case for Src/Lyn deficient radiation chimeras. Lyn deficient mice have been demonstrated to develop mild myeloproliferative disease (MPD) after 8 weeks of age and this is associated with increased myeloid progenitors and decrease in B-cells (Harder et al., 2001, Harder et al., 2004). This deficiency also leads to decreased numbers of platelets with increasing age, suggesting a defect at the haematopoetic stem cell (HSC) stage. Loss of Lyn with other members of the Src family kinases induces other phenotypes associated with defective haematopoeisis, including marked fibrosis and lung invasion by immune cells in Lyn/Hck animals (Xiao et al., 2008). In contrast to this however, Lyn/Hck/Fgr mice display no overt signs of altered haematopoesis (Meng and Lowell, 1997). The defects in Lyn/Src and Fgr/Lyn mice in this study may be due to altered HSC function and the beginning of a mild MPD.

Interestingly, although individual Src family kinases play a significant role in both signalling from GPVI and αIIbβ3, there appears to be no role for the individual members in *in vivo* tail bleeding. The reasons for this may be that there are many receptors involved in the process of haemostasis that signal via different mechanisms and as more than one of these is required, defiency in signalling of just one or two receptor may not induce a strong defect in a whole animal. It is also possible that due to multiple members of Src family kinases, i.e. more than two, being expressed in platelets that removal of a single or two members can be compensated for by other members. Finally, a whole animal bleeding phenotype does not rely on just

platelets, with coagulation and other mechanisms of stemming blood loss playing a role, such as vasoconstriction. It is highly unlikely that these will be affected by the removal of a single intracellular kinase, particularly in radiation chimeras where the kinases are only removed in the haematopoetic system. However, due to the variability of this assay it is difficult to make absolute conclusions.

In conclusion, the Src family kinases Src and Lyn play positive and negative roles in spreading on a fibrinogen matrix, respectively. They do not, however, appear to play a role in clot retraction or *in vivo* most likely due to redundancy with other members of the Src family kinases. The phenotype of Src/Lyn double deficient platelets may indicate a potential role for Src as a negative regulator of Lyn downstream of αIIbβ3.

CHAPTER 5
INVESTIGATING THE ROLE OF SRC FAMILY KINASES AND αIIbβ3 IN G_i-MEDIATED PLATELET AGGREGATION

5.1 Introduction

Platelets express two G_i family-coupled receptors, $P2Y_{12}$ and α_{2A}-adrenoreceptor. These two receptors are able to initiate platelet aggregation in human platelet rich plasma, but cannot do so in washed platelets. Both receptors, however, are able to potentiate platelet aggregation in washed platelets (Steen et al., 1993, Lanza et al., 1988, Andre et al., 2003, Foster et al., 2001). Further, the release of ADP from dense granules and subsequent activation of the $P2Y_{12}$ receptor plays a critical role in reinforcing platelet activation to most agonists (Andre et al., 2003, Foster et al., 2001).

Studies from the Kunapuli group and others suggest that G_i-coupled receptors synergise with Ca^{2+}-releasing receptors to activate integrin αIIbβ3 and to induce secretion. Full aggregation to ADP requires concomitant signals from G_q and G_i as shown using mutant mice models (Andre et al., 2003, Foster et al., 2001, Jantzen et al., 2001). Further, the absence of G_q or the $P2Y_1$ ADP receptor also abolishes signalling to adrenaline, demonstrating that a Ca^{2+}-releasing receptor is essential for signalling by the G_i-coupled receptor (Fabre et al., 1999, Leon et al., 1999).

Two separate studies in 2002 also reported platelet aggregation via a synergy between G_{13}- and G_i-coupled receptors. Dorsam and colleagues reported that YFLLRNP peptide, which stimulates $G_{12/13}$ via the PAR-1 thrombin receptor, and adrenaline, which signal via G_i, synergise to induce platelet aggregation (Dorsam et al., 2002). Further, aggregation to U46619, which activates the TxA_2 receptor which is can signal via both G_{13} and G_q, is blocked in G_q-deficient platelets but is restored in the presence of adrenaline (Nieswandt et al., 2002).

Receptors that couple to different members of the G_i-family can also undergo synergy and induce full aggregation. For example, in the presence of the $P2Y_1$ antagonist, MRS2179, adrenaline and ADP together stimulate full aggregation in washed platelets, although neither agonist can stimulate aggregation alone (Dorsam et al., 2005). Further, this synergy is associated with Ca^{2+} mobilisation whereas neither receptor can induce a detectable increase in cytosolic Ca^{2+} (Dorsam et al., 2005). Taken together, these data support a model in which G_i-coupled receptors require elevation of cytosolic Ca^{2+} to induce platelet activation.

The ability of adrenaline to induce aggregation in plasma but not in Tyrode's buffer could reflect the presence of a plasma component that induces a low level of Ca^{2+}-release that is subthreshold for platelet aggregation, but which is sufficient for synergy with the G_i-coupled receptor. Plasma contains several candidates that synergise with a range of platelet agonists even though they are unable to induce activation on their own, including the cytokine thrombopoetin (TPO). This includes ADP, in the presence of the $P2Y_1$ receptor antagonist, MRS2179, and adrenaline (Campus et al., 2005). Receptors which signal via Src and Syk family kinases are also candidates for supporting the synergy with G_i-coupled receptors. Tyrosine phosphorylation of a number of effector proteins including PLCγ2 can be detected in platelets in the absence of known agonists and which is reduced in the presence of selective inhibitors of Src and Syk kinases (Mori et al., 2008). It is highly likely that this constitutive activation of Src and Syk kinases is dependent on several surface receptors which regulate these two kinases, including major platelet integrin, αIIbβ3, and the GPI-IX-V complex, but further research is required to confirm this. Further, this mechanism alone does not explain the synergy in plasma as it can also be observed in platelets resuspended in Tyrode's buffer.

The aim of this chapter is to investigate molecular basis of adrenaline-mediated platelet aggregation in platelet rich plasma, with a special focus on the role of Src family kinase –

regulated signalling pathways and the major platelet integrin αIIbβ3. This work will therefore potentially address a further role for Src family kinases in integrin signalling, namely a potential role in synergising with the G_i-coupled α_{2A}-adrenoceptor.

5.2 Results

5.2.1 Adrenaline mediated aggregation and secretion is dependent on $P2Y_1$, $P2Y_{12}$ and TxA_2

Adrenaline-mediated aggregation proceeds in a characteristic biphasic manner, with secondary aggregation occurring concomitantly with secretion. To investigate the mediators of second phase aggregation, pharmacological inhibitors were used to determine the relative effects of $P2Y_1$, $P2Y_{12}$ and TxA_2.

ADP activates platelets through the Gq-coupled $P2Y_1$ receptor and the Gi-coupled $P2Y_{12}$ receptor and acts as an important secondary mediator of platelet activation. The second wave of aggregation to adrenaline was partially reduced in the presence of the $P2Y_1$ antagonist MRS2179 and secretion was markedly reduced (Figure 5.1, left panels). The $P2Y_{12}$ antagonist Cangrelor also reduced second phase aggregation and secretion to adrenaline (Figure 5.1, left panels). In contrast, the second phase of aggregation was abolished in the presence of the cyclooxygenase inhibitor indomethacin with the primary phase unaltered (Figure 5.1, left panels). This raises the possibility that the role of the $P2Y_1$ and $P2Y_{12}$ ADP receptors in reinforcing platelet activation to adrenaline is mediated downstream of TxA_2 formation. A similar profile of results was observed throughout the adrenaline concentration response curve (Figure 5.1, upper and lower right panels).

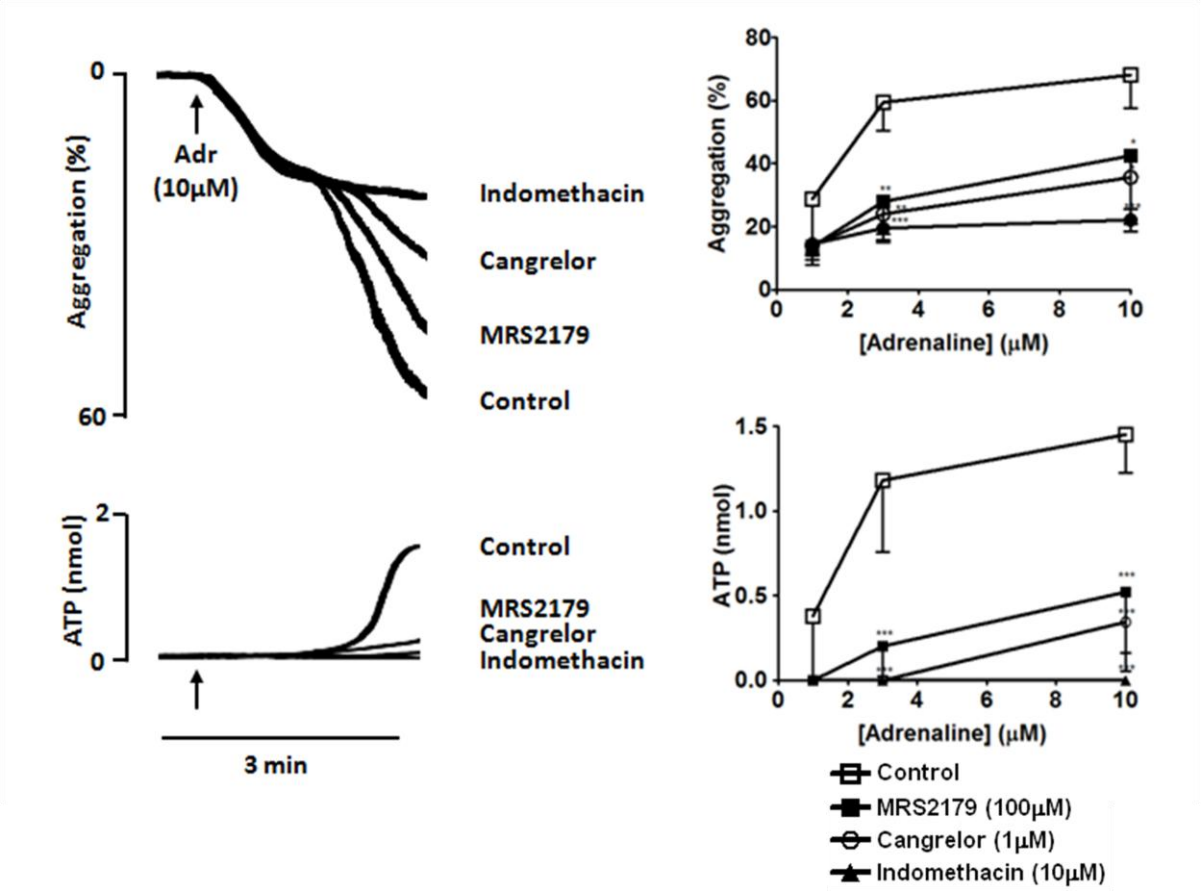


Figure 5.1 Adrenaline-mediated aggregation and secretion is dependent on ADP and TxA_2-mediated signalling. Platelet rich plasma was stimulated with adrenaline (10μM) in the presence of vehicle control or either MRS2179 (100μM), Cangrelor (1μM) or indomethacin (10μM). Data is from 3 experiments and graphs show arithmetic mean ± SEM. Data was analysed by 2-way ANOVA with Bonferoni post test. *p<0.05, **p<0.01, ***p<0.001

5.2.2 Secretion downstream of adrenaline is dependent on αIIbβ3

Under certain conditions, the generation of TxA_2 and secretion of platelet granules to weak agonists has been shown to be dependent on integrin αIIbβ3 (Prevost et al., 2009, Jin et al., 2002). The role of αIIbβ3 in adrenaline-mediated platelet activation was therefore investigated using the αIIbβ3 receptor antagonist Integrilin.

As expected, Integrilin blocked primary and secondary phase aggregation to adrenaline (Figure 5.2, upper left panel). In addition, secretion was also abolished (Figure 5.2, lower left panel) thereby demonstrating that aggregation is critical for adrenaline-induced secretion.

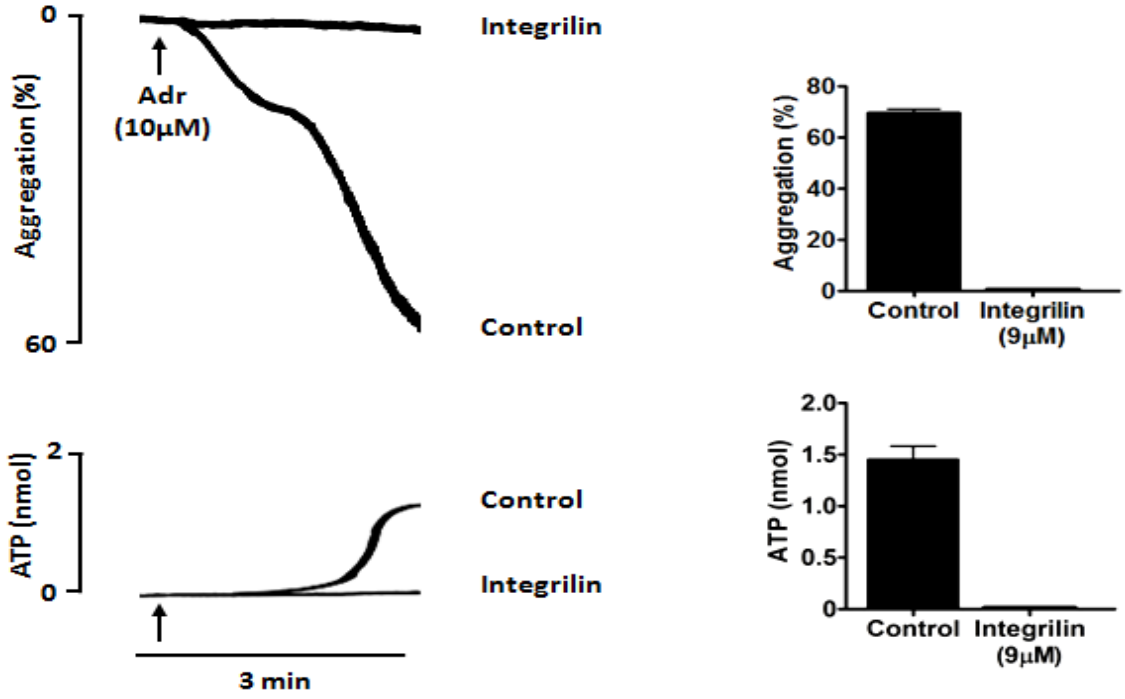


Figure 5.2 Adrenaline-mediated secretion is dependent on αIIbβ3. Platelet rich plasma was stimulated with adrenaline (10μM) in the presence or absence of Integrilin (9μM). Data is from 3 experiments and graphs show arithmetic mean ± SEM.

5.2.3 The effect of Src family kinase inhibitor, Dasatinib on adrenaline-induced platelet activation

The role of αIIbβ3 in adrenaline induced secretion could be mediated downstream of regulation of Src family kinases by the integrin. The absence of availability of inhibitors of Src kinases that are biologically available in plasma has until recently hampered investigation of this potential mechanism. Dasatinib is a dual inhibitor of both Src and Bcr-Abl family kinases which is available in plasma and used in the clinic to treat Imatinib-resistant chronic myeloid leukaemia. Both inhibitors block Abl kinases and so the additional efficacy of Dasatinib is believed to be due to inhibition of Src family kinases. The use of both inhibitors therefore provides a mechanism for investigation of the role of Src kinases in platelet secretion induced by adrenaline.

Experiments were initially performed to establish the concentration range for inhibition of Src family kinase by Dasatinib in plasma. This was achieved by monitoring the response to the GPVI-specific agonist, CRP. Activation by GPVI is critically dependent on Src family kinases. Aggregation to CRP was inhibited by Dasatinib in plasma in a concentration-dependent manner with complete blockade at Dasatinib (3μM) (Figure 5.3A). The specificity of Dasatinib in inhibiting Src kinases was shown using washed platelets by the complete abolition of tyrosine phosphorylation in basal and CRP-stimulated platelets (Figure 5.3B) Washed platelets were used in these studies because of problems in measuring tyrosine phosphorylation in plasma due to the high level of albumin and other proteins (see Chapter 6). Interestingly, Dasatinib inhibited phosphorylation of all proteins including those which migrated in the region of Src kinases (Figure 5.3B). Inhibition of both the activation site of SFKs (pY418 SFK) and inhibitory site of Src (pY529) is also observed. This is in contrast to pattern of inhibition observed with the well characterised SFK inhibitor, PP2 (Figure 5.3B),

and suggests that although Dasatinib is an effective SFK inhibitor, it must also inhibit Csk which inhibits all Src kinases though phosphorylation of a conserved inhibitory tyrosine.

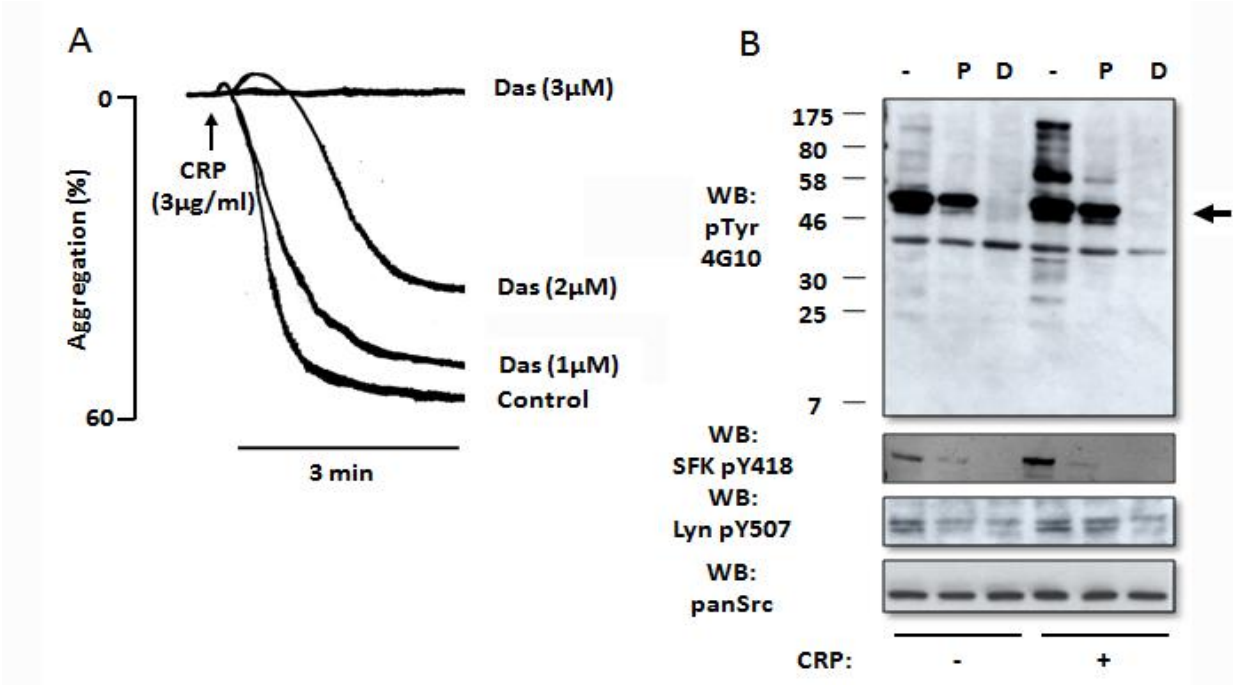


Figure 5.3. Platelet aggregation and phosphorylation is inhibited by Dasatinib. A) Platelet rich plasma stimulated with CRP (3µg/ml) in the presence or absence of Dasatinib at indicated doses. Aggregation was allowed to continue for 3 mins. B) Washed platelets ($5x10^8$/ml) were stimulated with CRP (10µg/ml) in the presence of PP1 (P:10µM), Dasatinib (D: 1µM) or vehicle control and subsequently lysed. Lysates were resolved on 10% SDS-PAGE gel, transferred and western blotted with indicated antibodies. Arrow indicates region in which Src family kinases migrate on SDS-PAGE.

Dasatinib was used to determine if Src family kinases are required for aggregation and secretion to adrenaline in platelet rich plasma. However, because of concerns over the specificity of Dasatinib, these studies were performed alongside those with Imatinib, which is structurally similar but does not inhibit Src family kinases, and a second, structurally distinct inhibitor of Src kinases, PD0173952, which has also been shown to be bioavailable in plasma but to have uncertain specificity (Auger et al., 2005).

In the presence of a vehicle control, aggregation to adrenaline induces biphasic activation with a secretion dependent secondary phase. In contrast, in the presence of Dasatinib or PD0173952 (Figure 5.4A), aggregation and secretion are completely abolished whereas they are not altered in the presence of Imatinib (Figure 5.4B). These results therefore provide evidence for a critical role for Src family kinases in adrenaline-mediated aggregation.

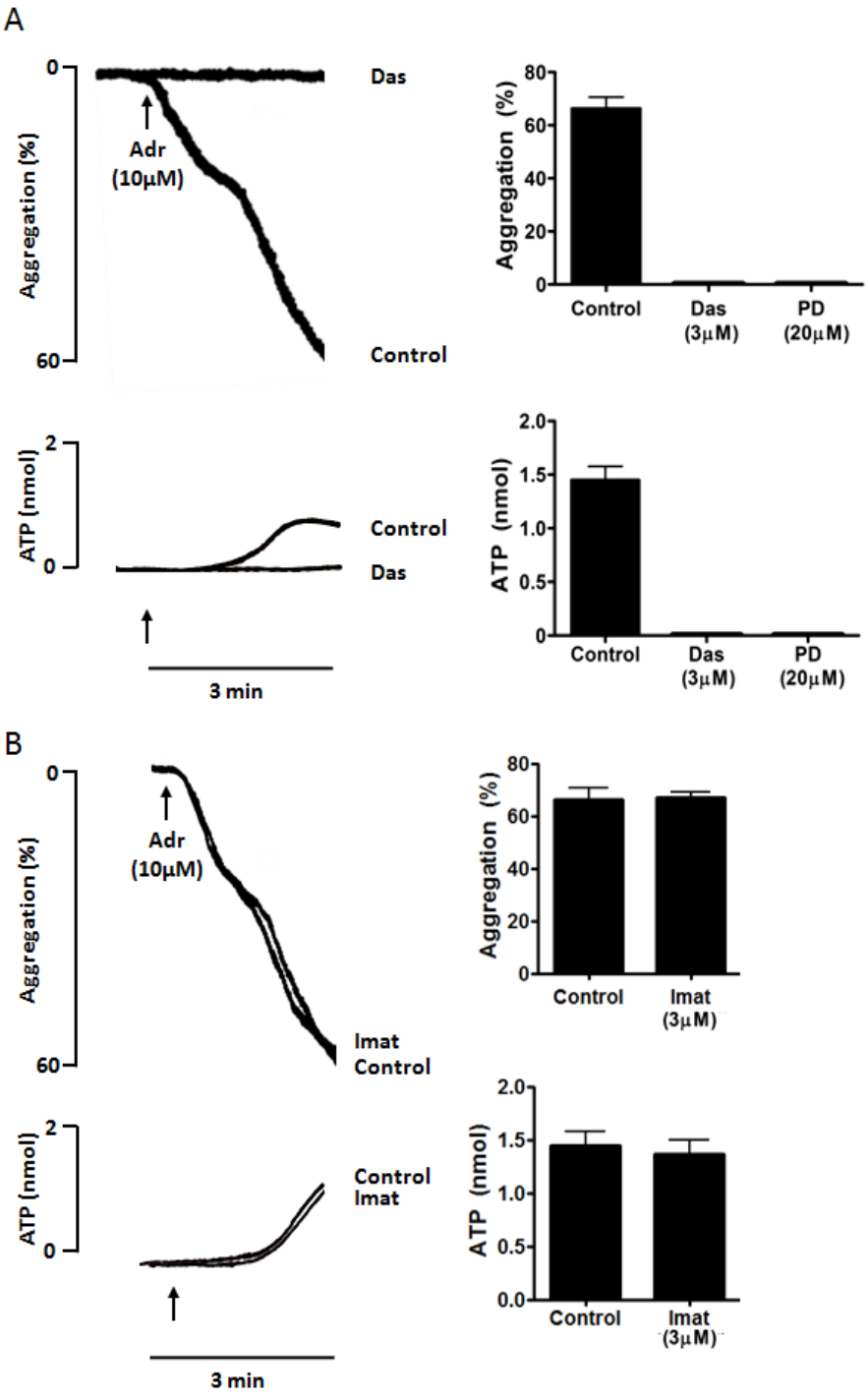


Figure 5.4 Adrenaline-mediated aggregation and secretion is dependent on Src family kinases. A) Platelet rich plasma was stimulated with adrenaline (10µM) in the presence of either Dasatinib (3µM) or PD0173952 (20µM). B) Platelet rich plasma was stimulated with adrenaline (10µM) in the presence of Imatinib (0.6µM). Data is from five independent experiments, mean ± SEM.

5.2.5 Src family kinases are not required for inhibition of cAMP by adrenaline.

An ELISA-based assay was used to determine if the blockade of aggregation and secretion by the Src family kinase inhibitors is due to an effect on adenylate cyclase. Adrenaline induces a small decrease in the basal level of cAMP (Figure 5.5). PGE_1 stimulates cAMP formation which is reduced in the presence of adrenaline (Figure 5.5). Interestingly, neither Src family kinase inhibitor had a significant effect on the ability of adrenaline to reduce cAMP in platelets at concentrations that block adrenaline-induced aggregation and secretion.

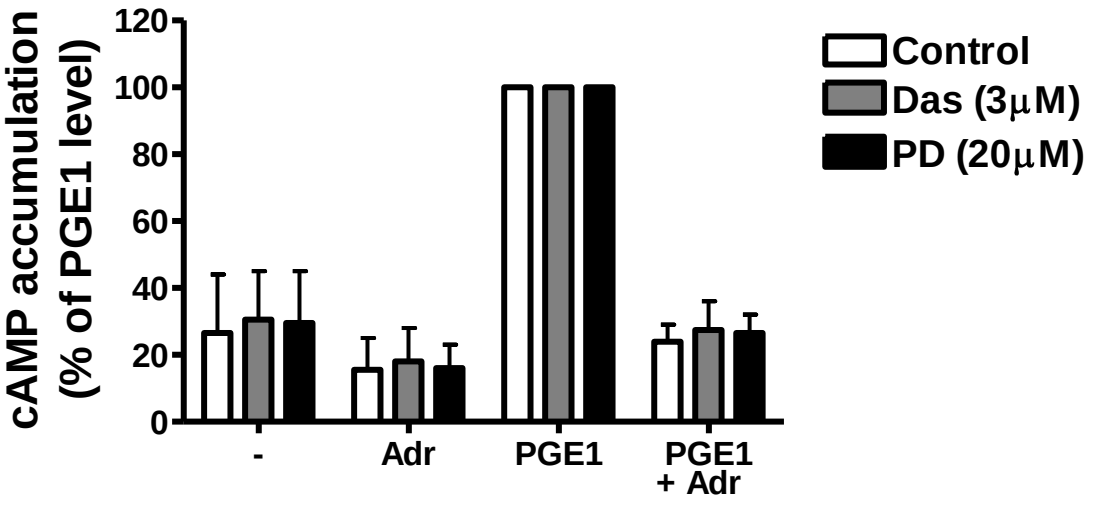


Figure 5.5 Adrenaline-mediated cAMP reduction is not dependent on Src family kinase signalling. Washed platelets ($5x10^7$/ml) were stimulated with either PGE_1, adrenaline (10μM) or both in conjunction in the presence of either vehicle control (0.1% DMSO), Dasatinib (3μM) or PD013752 (20μM). Cells were then lysed and ELISA performed according to manufacturers instructions. Results are from 3 replicates and arithmetic mean ± SEM Statistical analysis performed using two way ANOVA and Bonferroni post test, with all results compared to control.

5.2.6 Aggregation to adrenaline is not dependent on Syk activity.

Src family kinase-mediated signalling is known to lie upstream of Syk mediated signalling in many signalling pathways, such as that from GPVI. As adrenaline-mediated signalling is critically dependent on Src family kinase activity, it is possible that these kinases lie upstream of Syk.

In order to test this hypothesis, the Syk inhibitor PRT318 (Portola Pharmaceuticals, San Francisco, USA) was used in a preliminary experiment. Platelet rich plasma was preincubated with PRT318 (25μM), a dose sufficient to inhibit aggregation to the GPVI agonist CRP (not shown), and stimulated with 10μM adrenaline. Aggregation to adrenaline was unaffected (Figure 5.7). This suggests that aggregation to adrenaline, although dependent on Src family kinases, is not dependent on Syk.

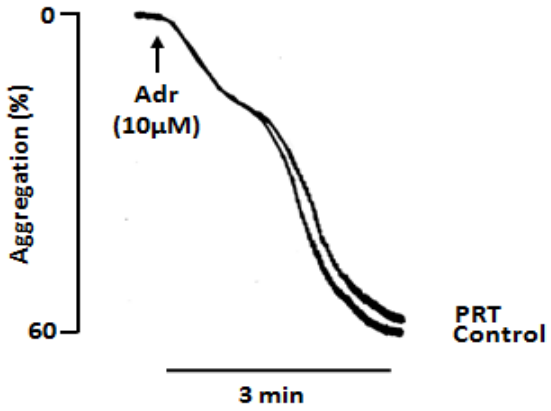


Figure 5.6 Aggregation to adrenaline is not dependent on Syk in a preliminary experiment. A) Human platelet rich plasma was stimulated with adrenaline (10µM) in the presence or absence of the Syk kinase inhibitor, PRT318 (25µM) n=1.

5.2.6 PI3K is required for signalling downstream of adrenaline

In order to determine if the phosphoinositide 3-kinase (PI3K) family of enzyme were also required for signalling downstream of the G_z-coupled α_{2A} receptor, platelet rich plasma was stimulated with adrenaline in the presence of the PI3K inhibitor, LY294002 (50μM). LY294002 was used at a concentration known to inhibit platelet activation by $P2Y_{12}$ (Kauffenstein et al., 2001). In the presence of the PI 3-kinase inhibitor, secondary aggregation and secretion to adrenaline were abolished, and primary aggregation to adrenaline was reduced (Figure 5.7). This demonstrates a critical role for PI 3-kinase in platelet activation by adrenaline.

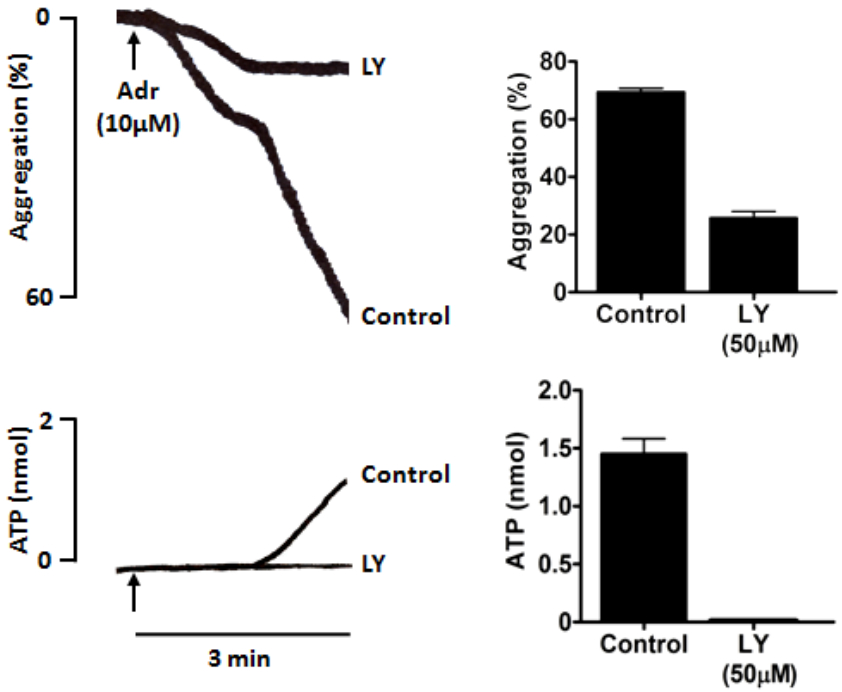


Figure 5.7 Adrenaline-mediated aggregation is partially dependent and secretion is fully dependent on PI 3-kinase. Platelet rich plasma was stimulated with adrenaline (10μM) in the presence of either LY294002 (50μM). Data is from three independent experiments, mean ± SEM.

5.2.7 $P2Y_{12}$-mediated platelet aggregation is also dependent on Src family kinases.

In order to determine if the results seen with adrenaline could be expanded to the G_i-coupled $P2Y_{12}$ receptor, platelets were stimulated with ADP in the presence of the $P2Y_1$ antagonist MRS2179. These experiments are of particular interest due to the physiological role of $P2Y_{12}$ in reinforcing platelet aggregation.

ADP (10μM) stimulates full aggregation with shape change and concomitant secretion. In the presence of the $P2Y_1$ receptor antagonist, MRS2179, there is no secondary phase aggregation and shape change (Figure 5.8A). Under these conditions, the primary phase of aggregation is completely inhibited by the presence of Dasatinib (3μM) (Figure 5.8, upper left panel). Further, platelets treated with Dasatinib in the absence of MRS2179 have reversible aggregation with no secretion (Figure 5.8A, lower left panel). In contrast to this, Dasatinib does not have a significant effect on aggregation to thrombin (0.1U/ml), an agonist which signals primarily through the G_q protein (Figure 5.8B). These data suggest that Src family kinases also play a critical role in the signalling downstream of $P2Y_{12}$, with the reduction in aggregation seen when platelets are stimulated with ADP in the presence of Dasatinib alone being caused by the removal of signalling by $P2Y_{12}$.

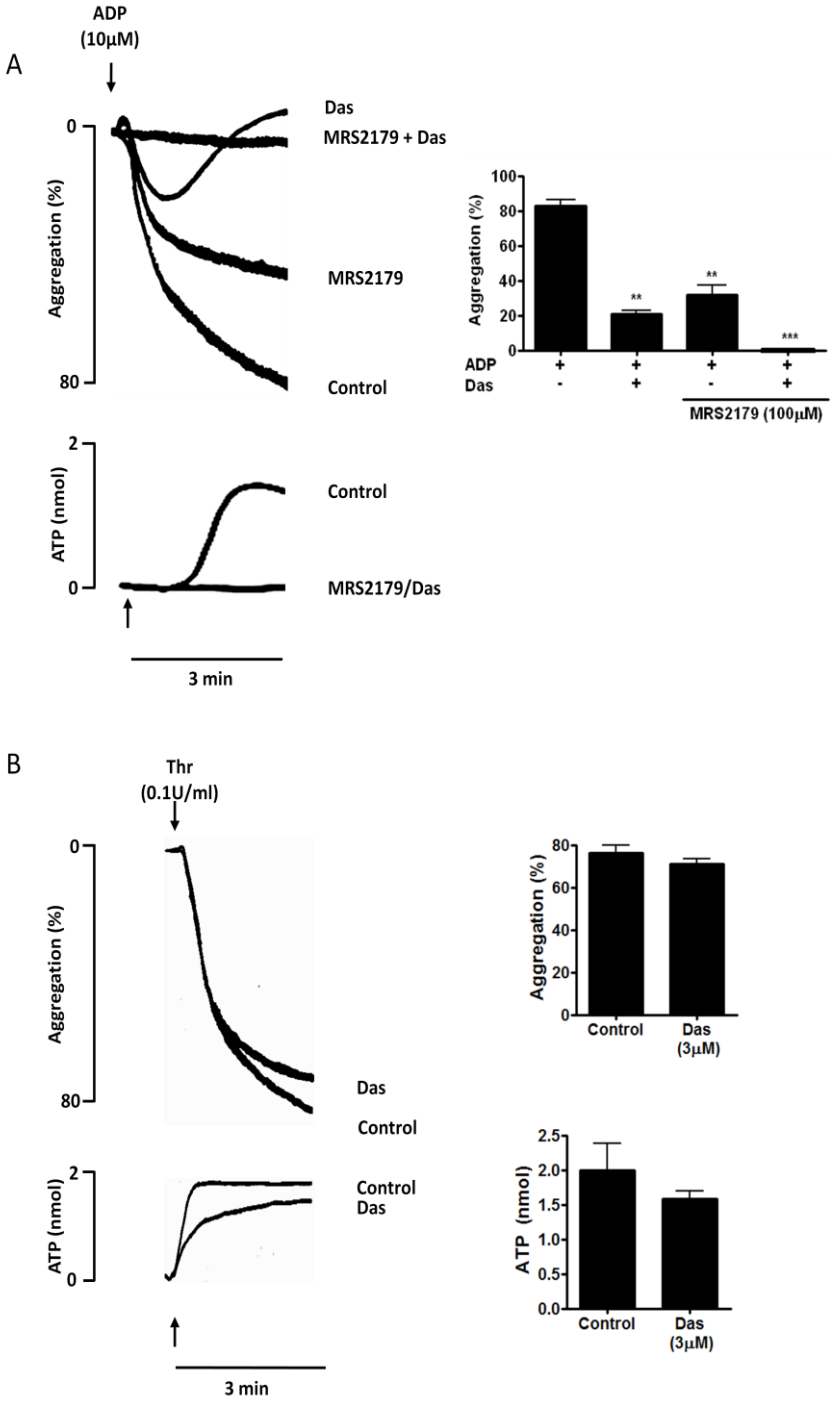


Figure 5.8 P2Y12-mediated aggregation is dependent on Src family kinases. A)Platelet rich plasma from healthy human donors was stimulated with ADP (10µM) in the presence or absence of MRS2179 (100µM). In addition, PRP was also preincubated with either vehicle control or Dasatinib (3µM). Data was analysed by two-way ANOVA and subsequently by Bonferoni post test comparing to control. B) Washed platelets were stimulated with thrombin (0.1U/ml) in the presence or absence of Dasatinib (3µM). Results are from 3 experiments ± SEM. Data was analysed by Students t-test.

5.2.8 Addition or inhibition of signalling by plasma components in washed platelets does not alter aggregation to adrenaline.

Washed platelets do not aggregate upon the addition of exogenous adrenaline, despite the catecholamine causing aggregation in platelet rich plasma and potentiating signalling to other platelet agonists (Figure 5.9A). This suggests that there is some component of plasma, removed by the washing process, which adrenaline can synergise with to produce full aggregation. There are several potential candidates for this, including thrombopoetin (TPO), fibrinogen and 5-HT.

In order to test if TPO or fibrinogen act as a co-stimulus to adrenaline, physiological levels of TPO (50ng/ml) and fibrinogen (2mg/ml) were added to washed platelets 1 min prior to activation with adrenaline. This concentration has previously been shown to potentiate aggregation and secretion to agonists in washed platelets (Campus et al., 2005). The addition of TPO and fibrinogen, separately or both in conjunction, had no effect on the ability of washed platelets to aggregate to adrenaline. (Figure 5.9A). Another potential co-stimultory agonist present in plasma is 5-hydroxytryptamine (serotonin), however, the addition of Ritanserin, a 5-HT_{2A} antagonist, to PRP also showed no effect (Figure 5.9B). The dose of ristanserin used was above that which fully inhibited 5-HT-mediated potentiation of aggregation to ADP (McBride et al., 1990).

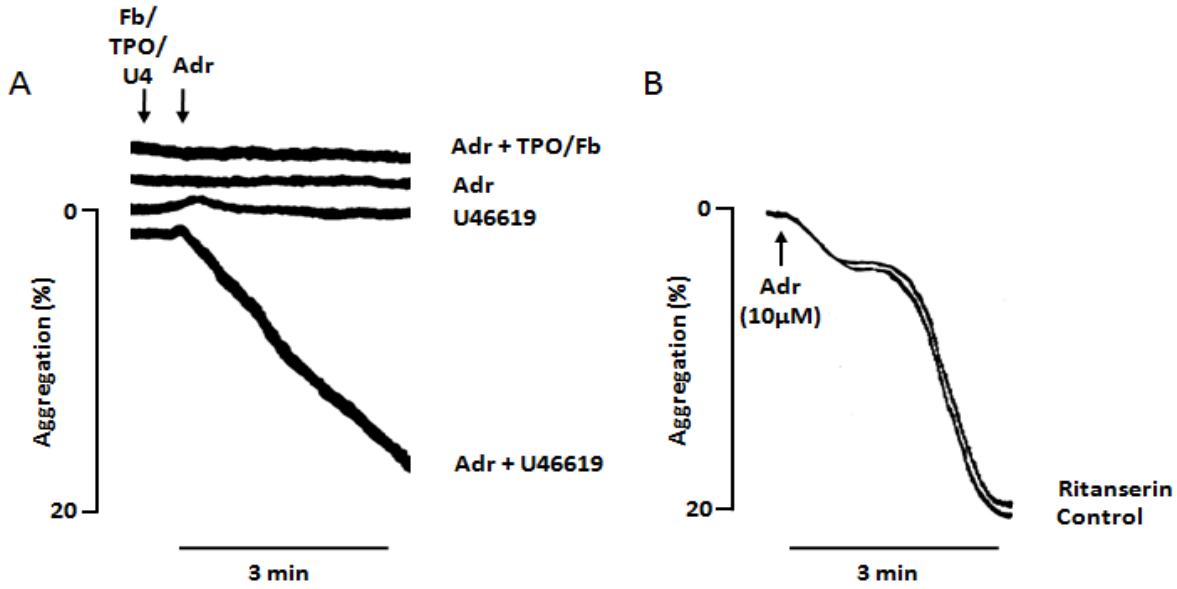


Figure 5.9 Aggregation to adrenaline is not dependent on Fb, TPO or 5-HT_{2A}. A) Human washed platelets were stimulated with adrenaline (Adr: 10µM), U46619 (U4: 20nM) in the presence or absence of TPO (50ng/ml) or fibrinogen (Fb: 2mg/ml). B) Human platelet rich plasma was stimulated with adrenaline (10µM) in the presence or absence of Ritanserin (1µM). Results are representative of three different experiments.

5.3 Discussion

The results in this chapter demonstrate that adrenaline-mediated secondary aggregation and secretion is dependent on $P2Y_1$, $P2Y_{12}$, TxA_2 and PI 3-kinase to varying degrees, with PI3 kinases and TxA_2 being critical for secondary aggregation. The main observation of this chapter, however, is that primary signalling to both adrenaline and ADP downstream of $P2Y_{12}$ are critically dependent on the Src family kinases, even though the receptors couple to distinct G_i-family members (Yang et al., 2000, Jantzen et al., 2001, Kelleher et al., 2001). This observation is not due to the inhibition of adenylyl cyclase by the G_i-protein. The results therefore demonstrate a previously unappreciated role for the Src kinases in signalling downstream of G_i-mediated receptors. However, it is also possible that the Src family kinase inhibitors, used at these concentrations, may have some off-target effects on targets such as Abl, Ephrins and cKit, although this seems unlikely due to the structurally distinct nature of the two inhibitors and the known role of the Src family kinases in G-protein coupled receptors. Also, the use of Imatinib, which inhibits similar targets to Dasatinib except the Src family kinases, shows no effect on aggregation to adrenaline.

Although Src family kinase signalling is critical for adrenaline-mediated aggregation, it is not known which Src kinases mediate the response to α_{2A} and $P2Y_{12}$. The nature of these family members cannot be investigated using mouse platelets, as adrenaline does not induce aggregation in mouse PRP (not shown), as also shown by others (Pozgajova et al., 2006), and $P2Y_{12}$-mediated aggregation in mouse platelets is not tyrosine kinase-dependent (Kauffenstein et al., 2001). Furthermore, there are no SFK member-selective inhibitors for use in human platelets.

The requirement for Src family kinases in aggregation to G_i-coupled receptors suggests that these enzymes are activated downstream of the receptor in some way. Activation of SFKs can be monitored by alteration in the phosphorylation of their inhibitory and activatory sites. Alternatively, it is possible that Src family kinases can play a role without a change in phosphorylation due to a low level of constitutive activity. It has been demonstrated that unstimulated platelets show a basal level of tyrosine phosphorylation that is ablated by SFK inhibitors (Mori et al., 2008). This is further investigated in Chapter 6.

Aggregation of adrenaline occurs in PRP but not in washed platelets and this is likely due to a component of the plasma being removed during the washing process. Plasma components such as TPO or insulin-like growth factor which, although do not induce aggregation alone, can potentiate aggregation induced by other agonists (Campus et al., 2005, Kim et al., 2007). However, the addition of these agonists to washed platelets does not support aggregation to adrenaline. It is also possible that plasma fibrinogen is responsible for the aggregation in PRP. This explanation is consistent with the observation that secretion to adrenaline does not occur in the presence of the αIIbβ3 antagonist, Integrilin. However, the addition of physiological concentrations of fibrinogen to washed platelets does not rescue aggregation to adrenaline. One possibility is that a combination of agonists found in plasma may be required for aggregation to adrenaline.

Interestingly, secretion downstream of adrenaline is dependent on αIIbβ3, demonstrating that this process is aggregation-dependent. Several previous studies have demonstrated that signalling from αIIbβ3 is required for release of TxA_2 and this could provide a molecular basis for this through synergy with the α_{2A}-adrenoceptor (Jin et al., 2002, Liu et al., 2005). This is supported by the abolition of secretion and second wave aggregation in the presence of a cyclooxygenase inhibitor.

The present results also demonstrate a partial role for PI3-kinase in adrenaline induced primary aggregation and secretion. Src family kinases lie in a signalling cascade upstream of PI3-kinase activation and this is also likely to be the case for G_i-coupled receptors. One potential mechanism for this is tyrosine phosphorylation leading to recruitment of PI3-kinases by virtue of it either or both of the SH2 domains in the catalytic subunit. They also demonstrate that G_i-coupled receptors do not signal via a classical 'ITAM-like' signalling pathway via Src kinases and Syk, as addition of a Syk inhibitor, at a concentration that blocks signalling by collagen, does not cause a defect in adrenaline-mediated aggregation. These observations provide evidence for a signalling pathway downstream of G_i-coupled receptors in which both Src kinases and PI3-kinases play a role in the inside-out activation of αIIbβ3 and platelet aggregation.

CHAPTER 6
DETERMINING TYROSINE PHOSPHORYLATION DOWNSTREAM OF G_i-COUPLED RECEPTORS

6.1 Introduction

As outlined in Chapter 5, the Src family kinases are critical for aggregation to adrenaline. Src family kinases are critically required for signalling and functional responses downstream of many platelet receptors, including the ITAM receptors, GPVI, FcγRIIA and CLEC-2, and the major glycoproteins αIIbβ3 and GPIb-IX-V (see Introduction). Activation of Src family kinases by these receptors is associated with dephosphorylation of the inhibitory site and increased phosphorylation of the activation site as monitored using phophospecific antibodies (Su et al., 1999, Xu et al., 1999, Senis et al., 2009b).

G-protein coupled receptors have also been demonstrated to activate Src family kinases in a variety of cell types, including platelets (see Introduction). This includes the ADP $P2Y_1$ and PAR-1 receptors which have been reported to increase phosphorylation of the activation site in human platelets (Hardy et al., 2004, Murugappan et al., 2005). On the other hand, phosphorylation of Src kinases in washed human platelets is not altered in the presence of adrenaline or downstream of $P2Y_{12}$, however, only adrenaline has been shown to potentiate tyrosine phosphorylation in response to the TxA_2 analogue U46619 (Dorsam et al., 2002, Dorsam et al., 2005, Minuz et al., 2006, Hardy et al., 2004).

As reported in Chapter 5 and by others, adrenaline is unable to activate platelets following isolation from platelet rich plasma (so-called 'washed platelets'). On the other hand, the effect of adrenaline on Src family kinases in platelet rich plasma has not been investigated because of the problems in studying phosphorylation in the presence of high levels of plasma proteins, including albumin. The monitoring of protein phosphorylation in plasma requires the development of specialist conditions to remove the plasma proteins whilst retaining phosphorylation. The aim of this chapter therefore is to develop methodology to determine

tyrosine phosphorylation in plasma and to monitor the regulation of Src family kinases by adrenaline. This work will establish whether adrenaline directly regulates Src kinases in platelets in the presence of plasma proteins.

6.2 Results

6.2.1 Measurement of protein phosphorylation in platelet rich plasma

The challenge to overcome in monitoring protein phosphorylation in plasma is to remove the high levels of the plasma proteins, which interfere with western blotting, while preserving phosphorylation. For example, the usual methods for separation of platelets from plasma such as centrifugation or gel chromatography take time and stress the platelets and so lead to a change in phosphorylation. To overcome this, we developed a method in which platelets were immersed in ice at the end of the period of stimulation to inhibit both kinase and phosphatase activity. Further, the platelet sample was diluted with 25 x excess of ice-cold PBS before centrifugation at 4^oC and resuspension of the pellet in lysis buffer.

Lysates from non-stimulated platelets prepared from washed and PRP platelet suspensions were resolved by SDS-PAGE and blotted as indicated below. In a side-by-side comparison, the lysates from the PRP samples had a markedly (75%) reduced level of tyrosine phosphorylation at the Src inhibitory site (Src pY529) than in washed platelets, despite having similar levels of protein as indicated by the panSrc western blot (Figure 6.1). This suggests either that dephosphorylation occurs during the washing procedure despite the use of ice-cold medium or that isolation of washed platelets using the standard procedures leads to an increase in phosphorylation. Despite this difference however longer exposure of samples from PRP showed clear tyrosine phosphorylated bands thereby enabling investigation of the effect of platelet agonists on phosphorylation of Src family kinases and other substrates (Figure 6.1).

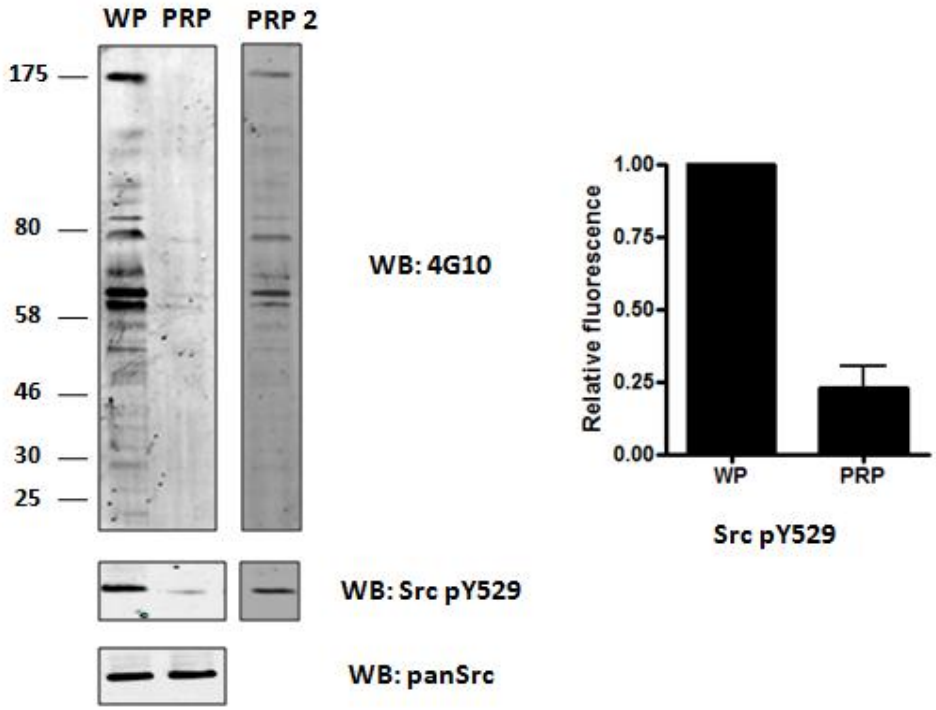


Figure 6.1 Comparison of basal platelet lysates obtained from platelet rich plasma and washed platelets. Basal platelet lysates were obtained from both PRP and washed platelets, as described in methods. Lysates were subsequently analysed by SDS-PAGE and western blot, with indicated antibodies utilising the Odyssey western blotting system. PRP-2 indicates a stronger exposure of PRP lane. WP: washed platelets, PRP: platelet rich plasma, WB: western blot. Data representative of three independent experiments.

6.2.2 Neither adrenaline nor $P2Y_{12}$ stimulate tyrosine phosphorylation.

In view of the critical role of Src family kinases in mediating platelet stimulation downstream of both α_{2A}- and $P2Y_{12}$ receptor stimulation (Chapter 5), an assessment of tyrosine phosphorylation in both washed and platelet rich plasma was made. Studies in plasma required rapid cooling of platelet samples post-stimulation to allow separation of platelets from plasma proteins as described above.

In these experiments, platelets were stimulated in the presence of Integrilin in order to prevent aggregation and where $P2Y_{12}$ was studied, platelets were stimulated with ADP in the presence of MRS2179, a $P2Y_1$ antagonist. Under these conditions, neither α_{2A}- nor $P2Y_{12}$ receptor stimulated tyrosine phosphorylation as measured using the pan-antiphosphotyrosine antibody 4G10 in washed platelets (Figure 6.2A) or platelet rich plasma, although interestingly, adrenaline induced dephosphorylation of an uncharacterised band at 50kDa in washed platelets (Figure 6.3A), however this could not be observed in PRP (Figure 6.3B) . In contrast, a dramatic increase in tyrosine phosphorylation was seen upon stimulation with the GPVI ligand CRP in washed platelets (Figure 6.3A) and in platelet rich plasma (Figure 6.3B). This increase is abrogated in the presence of the Src family kinase inhibitor, Dasatinib in washed platelets (Figure 6.2A).

Studies using a whole cell phospho-tyrosine antibody such as 4G10 may mask changes in the phosphorylation of individual proteins. Src family kinases are regulated by the phosphorylation of inhibitory and activatory tyrosine residues. Phosphorylation of the conserved activation loop tyrosine in all Src family kinases can be assessed by western blotting with a phosphospecific antipeptide antibody while phosphorylation of the inhibitory site can be studied by Src family kinase-specific phosphoantibodies. Neither adrenaline nor

ADP acting upon $P2Y_{12}$ induced a detectable change in tyrosine phosphorylation of activation (SFK Y418) or inhibitory tyrosine residues in Fyn (Y530), Lyn (Y507) or Src (Y529) (Figure 6.2B and Figure 6.3B) of the Src family kinases. This suggests that G_i-coupled receptors do not activate Src family kinases or if they do, activation falls below detectable levels.

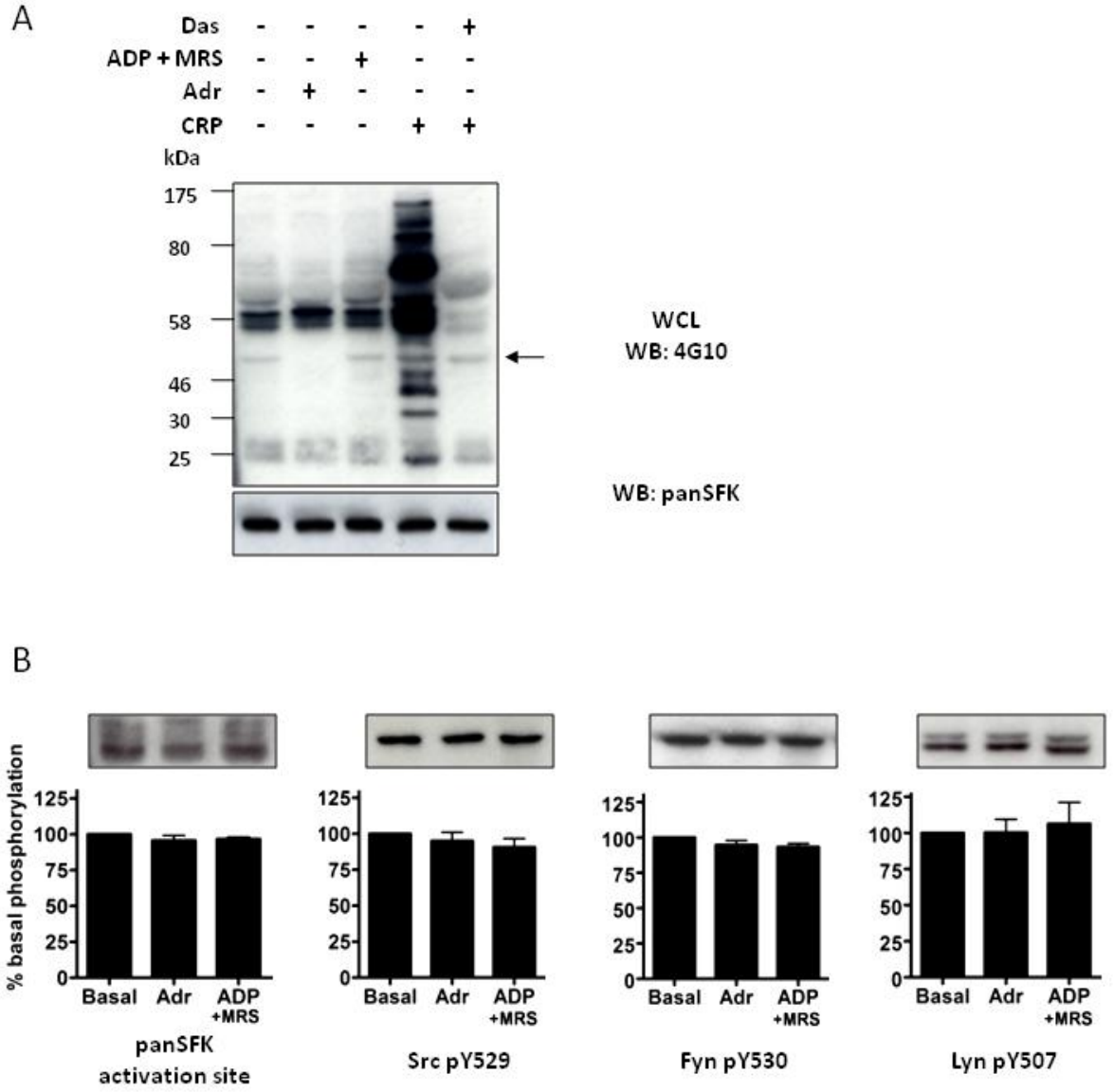


Fig. 6.2 Adrenaline (Adr) or $P2Y_{12}$ ADP receptor activation does not stimulate tyrosine phosphorylation in washed platelets. Human platelet rich plasma was stimulated with adrenaline (1 mM), ADP (10 µM) in the presence of MRS2179 (MRS) (100 µM) or collagen-related peptide (CRP) (10 µg/mL) in the presence or absence of dasatinib (Das) (1 µM), and subsequently lysed. Samples were immunoblotted with pan-pTyr monoclonal antibody 4G10 or phosphospecific antibodies, as shown. Mean data are representative of three independent experiments ± standard errors of the mean.

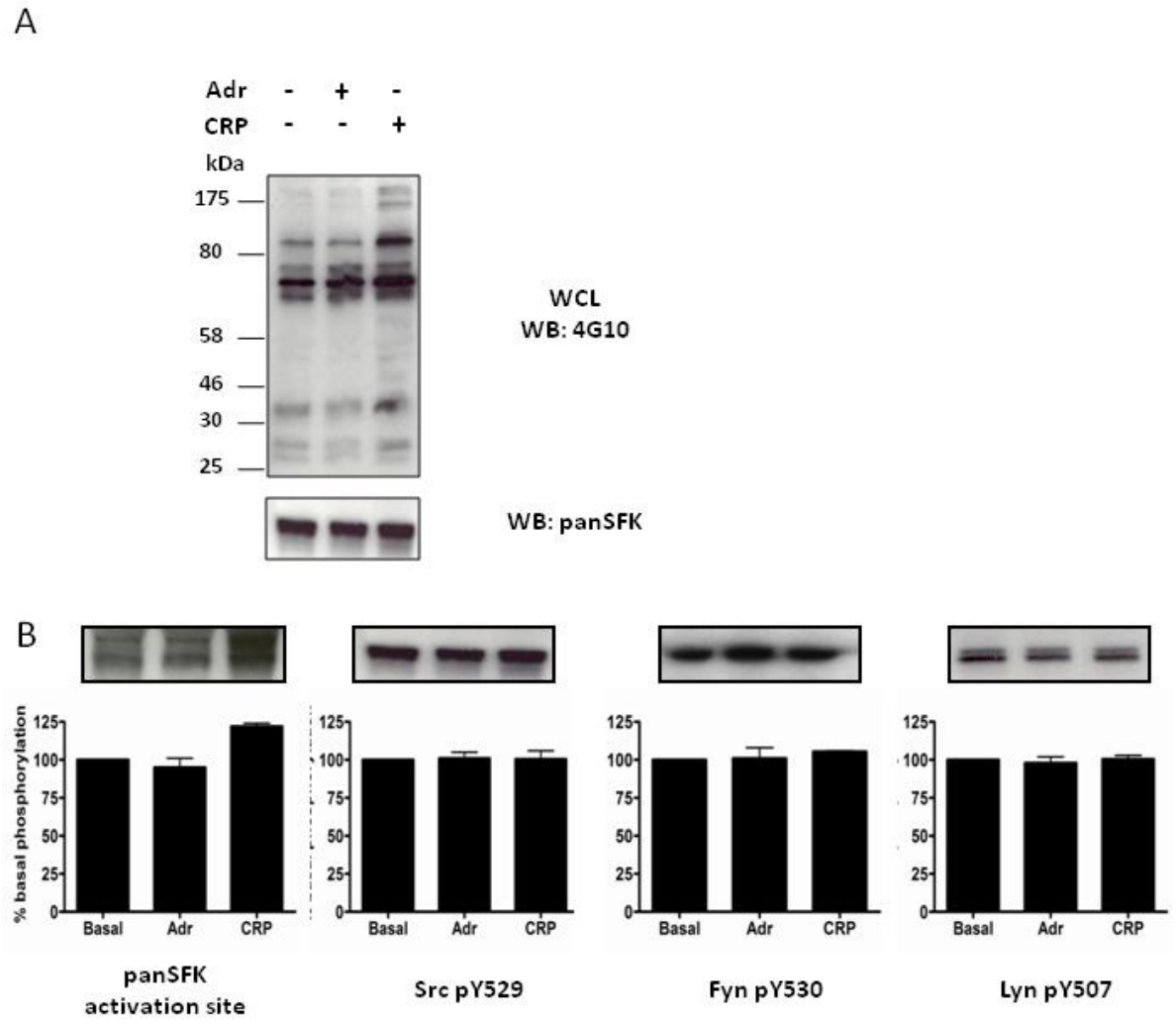


Fig. 6.3 Adrenaline (Adr) does not stimulate tyrosine phosphorylation in platelet rich plasma. Human washed platelets were stimulated with adrenaline (1 mM) or collagen-related peptide (CRP) (10 µg/mL) and subsequently lysed. Samples were immunoblotted with pan-pTyr monoclonal antibody 4G10 or phosphospecific antibodies, as shown. Mean data are representative of three independent experiments ± standard errors of the mean.

6.2.3 Study of Src family kinase phosphorylation in permeabilised cells by flow cytometry.

As observed in Figure 6.1, phosphorylation in platelets stimulated in PRP is much lower than that observed in washed platelets, despite matched levels of protein. The reason for this is not known but could be due to a delayed inhibition of phosphatase activity relative to kinase activity during the freezing process. For this reason, another method to study phosphorylation in platelets stimulated in PRP was required that involved an immediate fixing of the platelets. To achieve this, we investigated phosphorylation in permeabilised platelets by flow cytometry following fixation.

Platelets were stimulated in platelet rich plasma with either CRP (10μg/ml) or adrenaline (10μM) for 3 mins under non-stirring conditions as this prevents platelet aggregation Following stimulation, platelets were fixed with an equal volume of 4% formalin and permeabilised with saponin. Platelets were then incubated with either a directly labelled anti-P-selectin FITC-conjugated antibody or anti SFK pTyr418 and a FITC-conjugated secondary antibody.

P-selectin is expressed on the surface of activated platelets as shown following stimulation with CRP in PRP (Figure 6.4, upper left panel). Further, P-selectin can also be used to demonstrate successful permeabilisation of platelet. Platelets stimulated with CRP also show a significant increase in fluorescence with pSFK Y418 antibody relative to unstimulated controls (Figure 6.4, lower left panel). On the other hand, adrenaline stimulation does not lead to an increase in binding of this antibody to permeabilised platelets (Figure 6.4, lower right panel). This further demonstrates that activation of platelets with adrenaline does not induce tyrosine phosphorylation of Src family kinases.

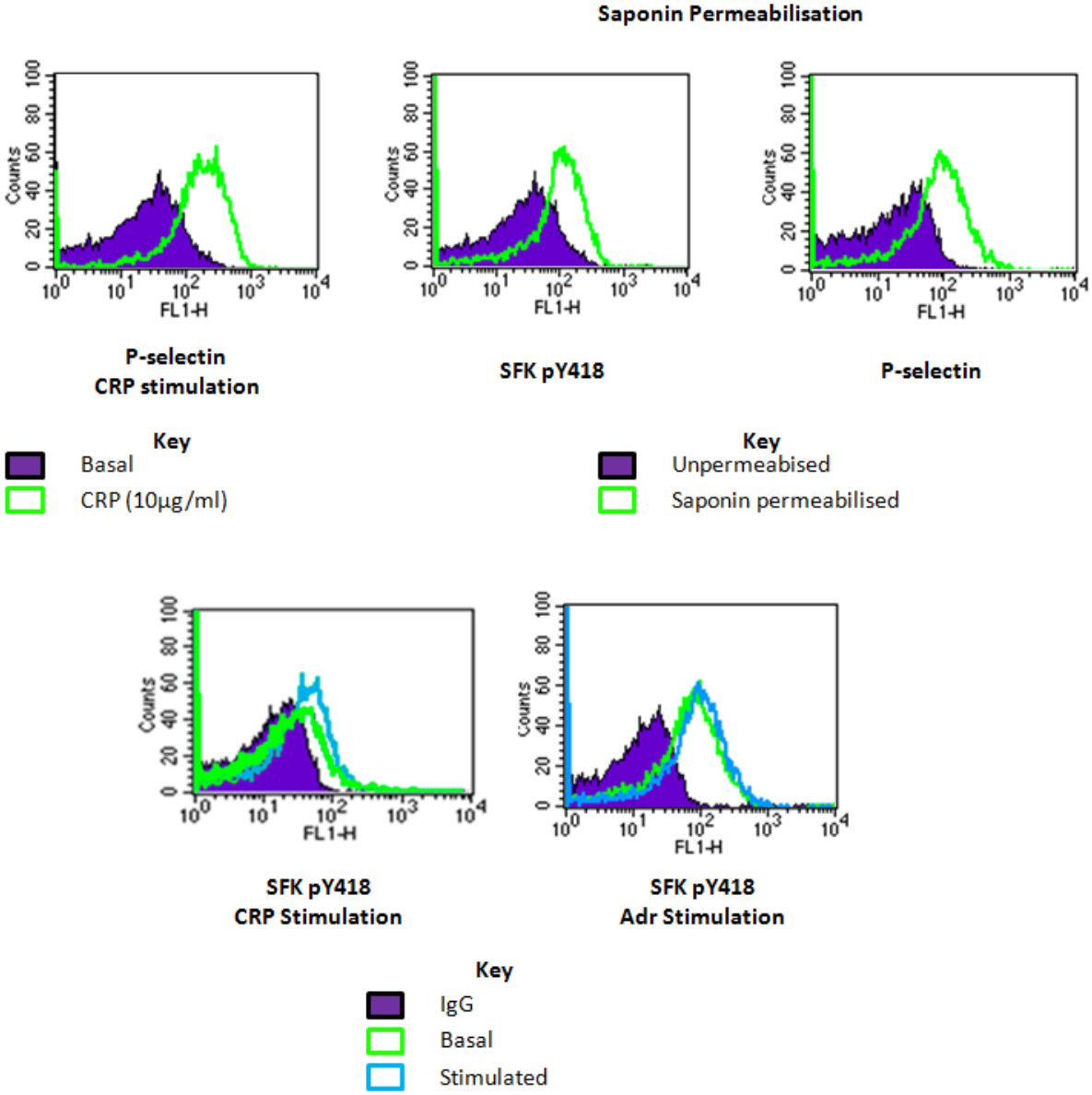


Fig. 6.4 Src family kinase phosphorylation in platelet rich plasma can be detected by FACS but adrenaline does not stimulate phosphorlyation. Platelet rich plasma was stimulated with either CRP (10µg/ml), Adr (1mM) or left unstimulated, as indicated. PRP was then permeabilised where indicated and incubated with the appropriate antibody before analysed by FACS. Data representative of three independent experiments.

6.3 Discussion

In this chapter, two methods were developed in order to study the phosphorylation of platelet proteins by adrenaline and $P2Y_{12}$ in platelet rich plasma. These assays were used, together with a conventional approach to studying phosphorylation in washed platelets, to investigate whether adrenaline or $P2Y_{12}$ regulate Src family kinases. Results from this chapter, taken together with the results from Chapter 5, demonstrate that, although required for G_i-mediated aggregation of platelets, Src family kinase phosphorylation is not altered downstream of these receptors.

The development of an assay for detecting phosphorylation in plasma raised some key issues which had to be overcome during the course of this study. Platelets stimulated in plasma appear to undergo a process of tyrosine dephosphorylation, likely due to the absence of any phosphatase inhibitors subsequent to termination of the experiment. This is potentially due to the longer time frame of inhibition of tyrosine phosphatases of that they may even retain activity at 4^oC. As an altenative method to overcome this problem, it was necessary to stimulate platelets and determine phosphorylation under conditions where the experiment could be stopped at a given timepoint. Thus a second flow cytometry-based method was developed.

The results in this chapter are similar to those seen by the Kunapuli group and other publications, whereby neither α_{2A} or $P2Y_{12}$ were unable to regulate tyrosine phosphorylation of the Src family kinases alone, however, they may do so in conjunction with other receptors (Dorsam et al., 2002, Dorsam et al., 2005, Nieswandt et al., 2002, Hardy et al., 2004). These results, together with those from Chapter 5, suggest a role for the basal phosphorylation of Src family kinases in α_{2A}- and $P2Y_{12}$- mediated platelet aggregation.

It is possible that the amount of basal activity present in human platelets is sufficient to synergise with those signals provided by the G_i-coupled receptor and induce platelet aggregation. It may also be that this basal phosphorylation is elevated in plasma than in washed platelets due to the presence of further platelet agonists within the plasma.

In conclusion, this chapter, along with the results from Chapter 5, demonstrate a critical role for the Src family kinases in the stimulation of aggregation by G_i-coupled receptors in platelets. Interestingly, neither adrenaline nor ADP signalling through α_{2A} or $P2Y_{12}$, respectively regulate Src family kinase phosphorylation, therefore suggesting a potential role for the basal levels of Src family kinase activity in platelet aggregation induced by adrenaline.

CHAPTER 7
GENERAL DISCUSSION

7.1 Summary of results

In this thesis, I have presented evidence for a differential level of expression of the Src family kinases in mouse platelets, with Lyn being expressed at 10x the level of Src and Fyn and 30x the level of Fgr. I have also demonstrated that in human platelets, Fyn is expressed at a higher concentration than other members, but the magnitude of difference is less than that seen in mouse platelets. The data in mouse platelets has been confirmed using Src family kinase-deficient platelet lysates and phospho-specific antibodies. I have gone on to demonstrate that Src plays a critical positive role in spreading of mouse platelets on fibrinogen, with Lyn playing a negative role. This negative role appears to require prior signalling from Src to occur and I hypothesise that this could be due to direct regulation of Lyn by Src. In contrast to these observations, clot retraction and tail bleeding assays do not show a significant requirement for individual members of the Src family kinases. Further to this, I have demonstrated that both the Src family kinases and αIIbβ3 play a critical role in aggregation and secretion to adrenaline. The induction of aggregation to adrenaline requires the presence of plasma, although the active component has not been identified. Platelet aggregation in response to adreanline does not induce phosphorylation of Src family kinases, suggesting a critical role for the basal activity of Src family kinases in this phenomenon. In this final chapter, I will discuss the wider implications for this work.

7.2 The role of Src family kinase expression level in platelets

The high expression of Lyn in mouse platelets is consistent with its major role in regulating both stimulatory and inhibitory pathways in platelets (Senis et al., 2007, Rowley et al., 2011, Maxwell et al., 2004, Quek et al., 2000). Although the positive regulatory role of Lyn in GPVI signalling is clear, i.e. phosphorylation of the FcR γ-chain, its negative role in both

GPVI- and αIIbβ3-mediated signalling is less clear. In GPVI signalling pathways, Lyn and PECAM-1, an ITIM containing receptor, have been shown to be interdependent regulators of GPVI. The authors of this paper suggest that Lyn is solely responsible for the phosphorylation of PECAM-1 under GPVI stimulated conditions and that this is the basis of both PECAM-1 and Lyn's inhibitory action on platelets (Ming et al., 2011). It is also possible that other ITIMs in platelets, however, such as G6b-B, also play a role. In αIIbβ3-mediated signalling, Lyn mediates phosphorylation of SHIP1 (Maxwell et al., 2004), a lipid phosphatase involved in the negative regulation of platelet activation through hydrolysis of the second messenger phosphatidylinositol 3,4,5-bisphosphate (PIP_3). It is also possible, however, that phosphorylation of ITIM receptors has a critical role in this negative regulation.

7.3 Compensation between Src family kinases

Mice deficient in Lyn display defects in GPVI signalling and potentiation of αIIbβ3-mediated signals. Src deficient mice, on the other hand display significant defects in αIIbβ3-mediated signals. This demonstrates that there are differential roles for Src family kinase members in platelets, potentially mediated by the differential localisation and/or expression level of the family members. Interestingly, upon deletion of Src or Lyn in conjunction with other SFKs, or with each other, more pronounced phenotypes are seen in both GPVI- (Severin, unpublished) and αIIbβ3-mediated signalling (Chapter 4). This suggests that there is some level of compensation between the members of this family. However, deletion of members of the SFKs does not affect the level of other members (Severin et al., 2011, Quek et al., 2000), suggesting that this compensation is not simply due to a compensatory increase in kinase levels.

7.4 Src family kinases *in vivo* – a potential thrombotic role

Haemostasis and thrombosis are distinct processes with different initiating stimuli, however many of the molecular players are the same. Binding of GPIb-IX-V to vWF, followed by activation of GPVI and α2β1 by collagen and subsequent inside-out activation and cross-linking of αIIbβ3 via fibrinogen are involved in the binding of platelets to injured vessels in haemostasis (See Introduction). These receptors are also required for binding to the surface of a ruptured atherosclerotic plaque during the process of thrombosis. Subsequent release of secondary mediators and fibrin generation by the coagulation cascade are also critical processes in the progress of thrombosis and haemostasis. A major difference between these two processes is that arterial thrombi form exclusively in the vascular lumen acting to occlude the blood vessel, whereas haemostatic plugs form at the vessel wall to stem loss of blood (Figure 7.1). This induces distinct blood flow conditions at the site of the thrombi and haemostatic plugs that can further influence the developing platelet aggregates (Jackson, 2011).

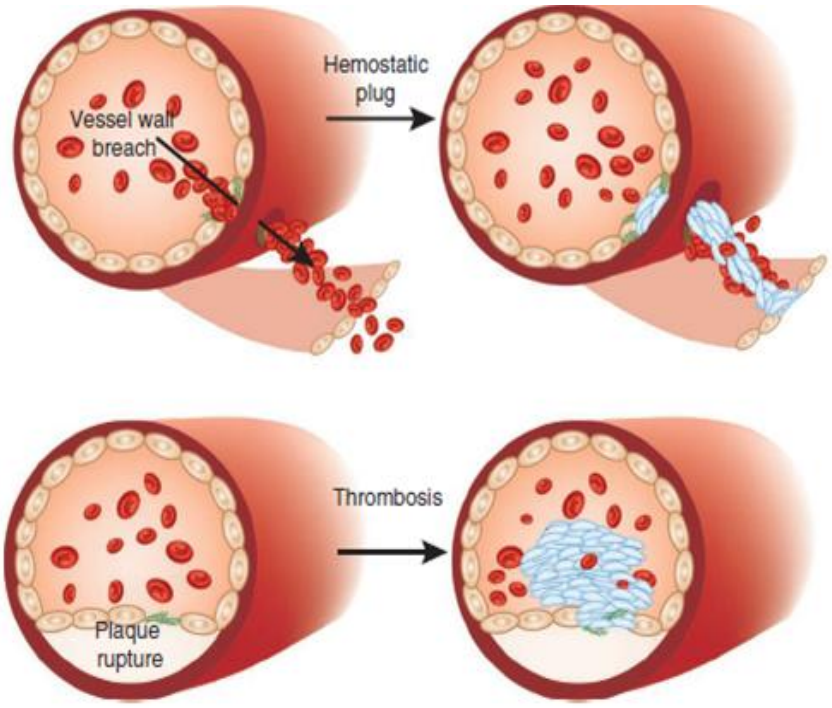


Figure 7.1 The differences between haemostasis and thrombosis. As indicated in this figure, haemostatic plugs form purely within the vessel wall of an injured artery, whereas thrombus forms within the lumen of the blood vessel acting to occlude the vessel. Image taken from (Jackson, 2011)

Several factors determine the propensity of platelets to induce thrombosis. An important factor is the thrombogenicity of atherosclerotic plaques, particularly the presence of tissue factor and fibrillar collagen. Deficiency of either of the platelet collagen receptors GPVI or α2β1 does not induce bleeding, but may potentially provide protection from thrombosis highlighting them as targets for development of a new class of antithrombotic (Massberg et al., 2003, Moroi et al., 1989, Rekhter, 1999, Toschi et al., 1997). A deficiency in the release of the inhibitory mediators of platelet activation, namely PGI_2 and NO, or the ecto-ADPase CD39, as a consequence of the build up of an atherosclerotic plaque is also likely to contribute to thrombus formation (Cheng et al., 2002, Enjyoji et al., 1999, Gkaliagkousi and Ferro, 2011, Marcus et al., 1997). Heightened platelet reactivity is also implicated in the

initiation of thrombosis. For example, diabetic patients have platelets which are more sensitive to agonist stimulation and display greater resistance to antiplatelet therapies and increased thrombotic events (Lincoff, 2003). Disturbed blood flow has also been demonstrated to play a role in the initiation of thrombosis, possibly through alteration of shear forces affecting deposition of platelets on thrombotic surfaces. Together, these results highlight a critical role for the strict regulation of platelet signalling especially as some of these steps such as shear induced activation are not affected by classic antithrombotic drugs such as aspirin (Jackson, 2011, Nesbitt et al., 2009). This, along with major bleeding defects seen on some antiplatelet drugs, highlights the requirement for further, novel anti-platelet drugs.

The Src family kinases Lyn and Src play key roles in signalling downstream of GPVI and αIIbβ3, respectively, but animals deficient in these kinases do not show bleeding phenotypes *in vivo*. As both of these receptors are also implicated in thrombosis, this suggests that they may be novel targets as an antithrombotics. Pharmacological inhibition of Syk also has no effect on bleeding in mice suggesting that it may also be a novel target (Reilly et al., 2011). Indeed two recent reports highlight Syk as a target in inhibiting thrombosis and also atherosclerotic plaque development (Reilly et al., 2011, Hilgendorf et al., 2011). As Syk is downstream of Src family kinases, this together with the data within this thesis provides evidence for a potential role of SFKs in thrombosis.

It can therefore be proposed that individual members of the SFKs show promise as drug targets for the treatment of thrombosis. These would provide strong drug targets due to their inhibition causing reduction in the initial stages of platelet activation without a minimal bleeding, although it should be borne in mind that bleeding is a multifactorial disorder and that Src kinase inhibition could synergise with a clinically unrecognised defect in platelet

activation to mediate bleeding. Further, there are difficulties with development of drugs of this type due to the high level of sequence homology in the kinase domain between members of the Src family.

An aid to the development of these drugs could be provided by novel mouse models generated by Taconic Artemis and were initially developed by Shokat et al (Bishop et al., 2000). These animals are known as KinaseSwitch animals and are available with Src family kinases mutated at the ATP binding pocket. This allows the binding of drugs that inhibit a specific kinase family member selectively above others of the same family. This technology works by mutation of the ATP binding pocket within the kinase in manner that does not affect ATP binding but allows the binding of a drug specific to this mutated kinase (Figure 7.2) The use of these animals would allow determination of effects of a kinase-specific drug and overcome any issues caused by the compensation of the kinase function by other members of the family, potentially found in mutant mouse models. This is important as genetically deficient animals are deficient in kinase activity throughout their life, whereas administration of drugs can occur over an acute time frame and this may cause different effects *in vivo*. Further to this, these animals may aid with the further development of drugs which are specific to the individual wild type kinase by aiding further understanding of critical residues within the catalytic region. Once developed, these drugs could be used in a similar strategy to asprin, i.e. a low dose of an irreversible inhibitor. This would be a good strategy for targeting platelets due to their low potential for synthesising new protein, therefore allowing specific inhibition of platelets over their lifespan. Cells with higher transcriptional and translational activity would be relatively unaffected due to their much faster turnover of protein.

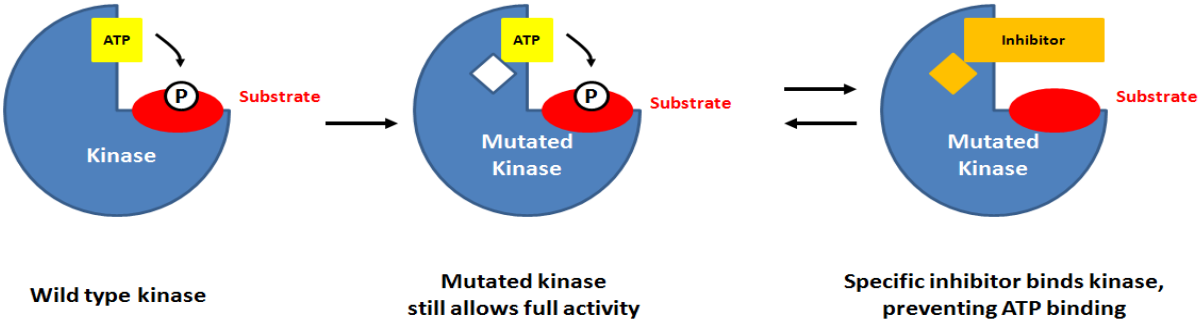


Figure 7.2 Principle of KinaseSwitch technology. Mutated kinase phosphorylates substrates normally in the absence of an inhibitor, however, in the presence of a kinase-specific inhibitor, kinase activity is prevented.

7.5 A mechanism for G_i-mediated platelet aggregation

As described in the above summary and Chapters 5 and 6, Src family kinases are critically required for platelet aggregation in response to G_i-coupled receptor stimulation, however phosphorylation of these kinases is not induced by these receptors. These findings allow for the development of a model for G_i-mediated platelet aggregation.

Upon stimulation of a G_i-coupled receptor in platelets, members of the G_i family act to inhibit adenylyl cyclase, thereby reducing cAMP, although this does not appear to play a major role in mediating platelet activation. It appears that G_i coupled receptors synergise with a constitutive, low level activity of Src family kinases in unstimulated platelets. These Src family kinases may be activated by association tyrosine kinase-mediated receptors, including αIIbβ3 and GPIb-IX-V complex or, alternatively, their activity may be regulated by the balance of kinase and phosphatases at the platelet surface. Several pathways could be regulated by the synergy between Gi-coupled receptors and SFKs including the regulation of PI 3-kinase isoforms.

Adrenaline initially induces primary wave aggregation through inside-out regulation of αIIbβ3 thereby leading to engagement of its endogenous ligand fibrinogen. Upon clustering, outside-in signalling from αIIbβ3 induce activaton of $cPLA_2$ and the generation of TxA_2 and subsequent dense granule secretion as a consequence of synergy with the G_i-mediated signals. The release of TxA_2 and secretion of mediators such as ADP from the dense granules support secondary aggregation. This process is summarised in Figure 7.3.

In this model, the synergy of outside in signals from αIIbβ3 with those from a G_i-coupled receptor is critical for secretion and second wave aggregation. However, because of the interplay and synergy between the various receptors, it is likely that each component is required to convert the weak signal from adrenaline to full aggregation and secretion.

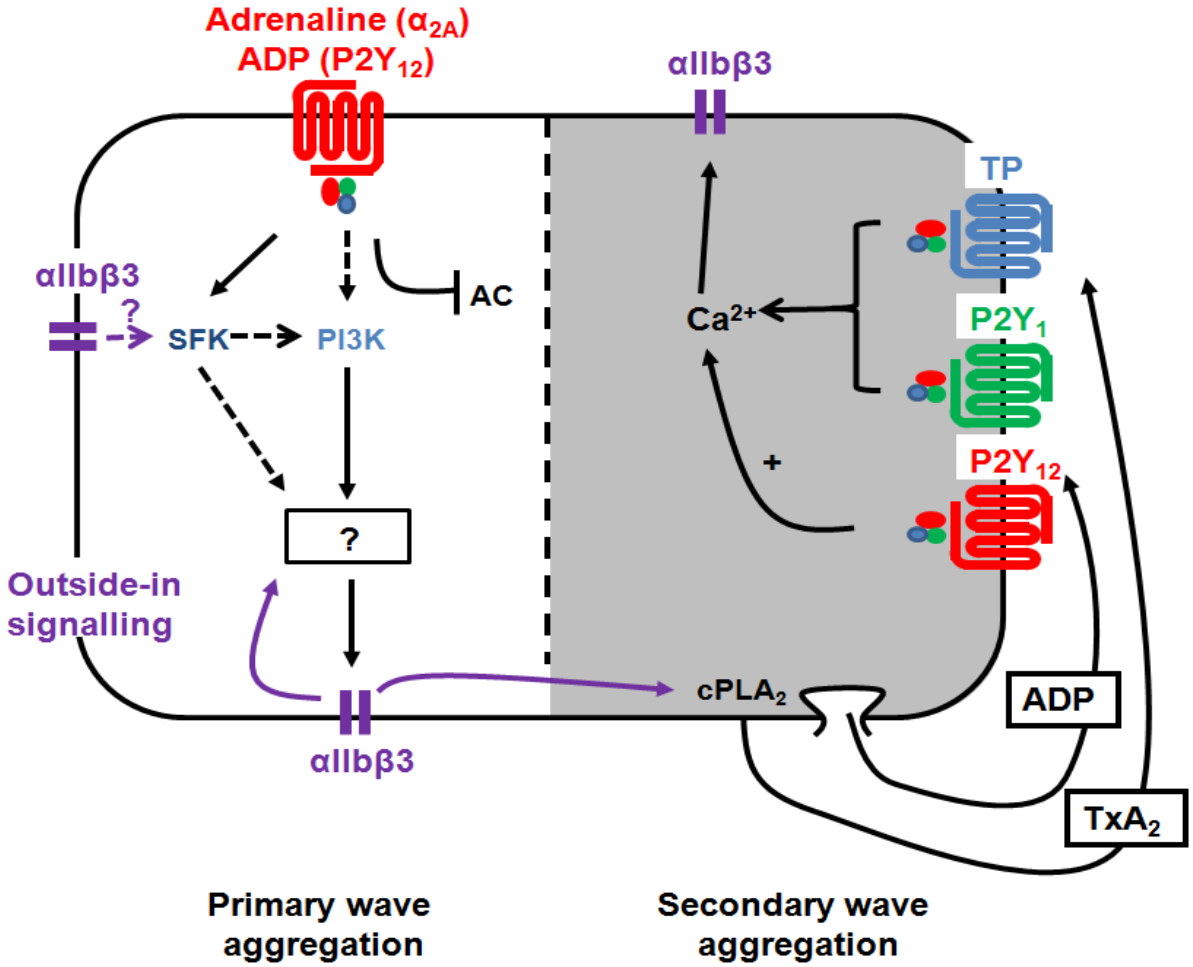


Figure 7.3 Putative model for signalling by G_i-coupled receptors in platelets. Upon stimulation with a G_i-coupled receptor agonist, platelets signal via a Src family kinase dependent mechanism to induce aggregation. These Src family kinases may be stimulated downstream of αIIbβ3. Following αIIbβ3, secretion is induced allowing for further secondary aggregation to occur.

7.6 Final thoughts

During this thesis, I have demonstrated that Src family kinases play a critical role in a variety of signalling pathways, some of which were well appreciated, such as for αIIbβ3, some which were previously unappreciated, i.e. for G_i-coupled receptors. However, the precise role of Src family kinases in G_i-coupled signalling is not known and further research is required to gain a greater understanding of how Src family kinases play this critical role in signalling

downstream of these GPCRs and what precise role αIIbβ3 plays in aggregation from these receptors.

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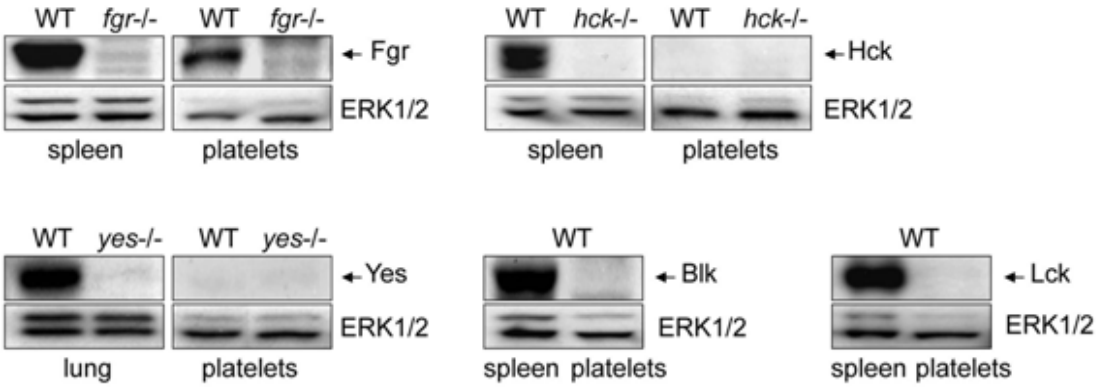
Appendix



Expression of Src family kinases in mouse platelets. Whole cell lysates were prepared from spleen, lung or washed platelets of control and different members of Src family kinases (Yes, Hck and Fgr)-deficient mouse. Expression of the different Src family kinase members (Yes, Hck, Fgr, Blk and Lck) were detected by western blot using specific antibodies. Data are representative of at least 3 independents experiments. This work was performed by Dr Sonia Severin.