Glycoprotein receptor mediated regulation of platelet morphology

by

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

Department of Physiology
University of Birmingham
Medical school
August 2007

THESIS CONTAINS CD/DVD

ABSTRACT

Spreading platelets sequentially form filopodia, lamellipodia, and stress fibres. This thesis demonstrates the formation of each actin structure in spread platelets, and in addition the formation of a novel actin structure, which I have termed an actin nodule. Actin nodules require Src kinase activation, and actin polymerisation, but are negatively correlated to ROCK and myosin-II activation.

This thesis has investigated the role of WAVE-1, Rho kinase (ROCK) and myosin-II in spreading and aggregate stability *in vitro* and *in vivo*. ROCK or myosin-II inhibition, prevents stress fibre formation leading to appearance of splits and holes (termed fenestrations) in spread platelets on collagen. In addition, ROCK or myosin-II inhibition compromises aggregate stability on collagen at arterial rates of flow. Lamellipodia formation is inhibited in WAVE-$1^{-/-}$ platelets spread on CRP, whilst shape change and aggregation downstream of GPVI is severely disrupted. However, GPCR agonists induce full lamellipodia formation on fibrinogen in WAVE-$1^{-/-}$. Aggregate formation on collagen under arterial rates of flow is unaffected further indicating WAVE-2 can compensate for WAVE-1. Thus, WAVE-1 maybe differentially regulated downstream of GPCR and glycoprotein signalling.

The actin regulatory proteins, Spin-90, β-Pix and Nck are tyrosine phosphorylated by multiple platelet agonists, but do not form a complex upon platelet adhesion. However, strikingly β-Pix is more heavily phosphorylated downstream of the collagen receptor integrin, $\alpha_2\beta_1$ in comparison to GPVI. I speculate β-Pix may play an important role in connecting PLCγ1 to Rac activation.

ACKNOWLEDGEMENTS

The biggest thank you must go to Professor Steve Watson, and Professor Laura Machesky for all their help and guidance throughout the last three years. It has been a privilege to work for both of you and I'm very grateful for everything you have done to help me. I must also thank the BHF for the three years of funding, without which I would not have been able to complete this thesis.

However, my PhD would also have been a much poorer place without the presence of many people and I hope I manage to remember to thank you all. Andy for putting up with many inane questions, Yotis for keeping us young, Owen for the running, Craig for being my Steve McClaren to my Sven, Jos for not killing me when sharing a fridge, Ban for being a second mum, Majd for being Majd, Mike for all the jokes, Dario for all the Italian food, Bori for being small, Gemma for being lovely, Caroline for her Gallic charm, Mark for the quiz knowledge, Ian 'the Swerve' for the goals, Gayle for being a bundle of fun, Neil ,Jun, Jen, Dean, Stuart for all the friendship, Kelly for all the enthusiasm, the marmites, for all the quality entertainment on the football pitch, the Machesky lab, especially Steve T, Simon, Hannah and Claire for all the help they have given me, and all the folks in the BMSU.

Finally a huge thank you to my loving wife, Emma, who has helped me through the ups and downs of the last three years, and without whom life would be a much poorer place. Also Mum, Dad, Rachel, Paul, Adam, Naomi, Ben and Jen for all the support over the years to get me this far, I love you all, and I dedicate this thesis to you.

PUBLICATIONS ARISING FROM THIS WORK INCLUDED WITHIN THIS THESIS

McCarty O.J.T, **Calaminus S.D.J,** Berndt M.C, Machesky L.M, Watson S.P. von Willebrand factor mediates platelet spreading through glycoprotein Ib and alpha(IIb)beta3 in the presence of botrocetin and ristocetin, respectively. *Journal of Thrombosis and Haemostasis* 2006 **4**:1367-78.

Calaminus S.D.J, McCarty O.J.T, Auger J.M, Pearce A.C, Insall R, Machesky L.M, Watson S.P. A major role for Scar/WAVE-1 downstream of GPVI in platelets. *Journal of Thrombosis and Haemostasis* 2006, **5**:535-541.

Calaminus S.D.J, Auger J.M, McCarty O.J.T, Wakelam, M.J.O, Machesky L.M, Watson S.P. MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability. *Journal of Thrombosis and Haemostasis*. 2007 Jul 23; [Epub ahead of print]

Calaminus S.D.J, Thomas S, Machesky L.M, Watson S.P. Platelet spreading on matrix proteins: identification of a novel, actin-rich structure. *Journal of Thrombosis and Haemostasis* submitted.

ABBREVIATIONS

Abi-2	Abl-kinase interactin protein-2
ACD	Acid/citric/dextrose
ADP	Adenosine diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
Arp2/3	Actin Related protein 2/3
ATP	Adenosine triphosphate
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N,N',N'-tetraacetic acid
BSA	Bovine Serum Albumin
Btk	Bruton`s tyrosine kinase
Ca^{2+}	Calcium
CDC42	Cell division cycle 42
CO_2	Carbon Dioxide
CRIB	CDC42 binding domain
CRP	Collagen related peptide
DAG	Diacylglycerol
DIC	Differential Interference Contrast
DMSO	Dimethylsulfoxide
DOCK2	Dedicator of Cytokinesis 2
ECM	Extracellular matrix
EGTA	Ethylenediamine tetra-acetic acid
EDTA	Ethylene-glycol-bis(b-aminoethylether)tetra-acetic acid
FAK	Focal adhesion kinase
FcRγ	Fc receptor γ chain
FITC	fluorescein isothiocyanate
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation Inhibitors
GDP	Guanine diphosphate
GEF	GDP exchange factors
GFOGER	Glycine-Phenylalanine-hydroxyproline-glycine-glutamic acid-arginine
GFP	Green fluorescent protein
GPCR	G protein coupled receptors
GPIb-IX-V	Glycoprotein-Ib-IX-V
GPO	Glycine-proline-hydroxyproline
GPVI	Glycoprotein VI
GTP	Guanine triphosphate
HSPC300	haematopoietic stem cell progenitor 300
IP_3	inositol-1,3,4-triphosphate
ITAM	Immunoreceptor Tyrosine Activating Motif
ITIM	Immunoreceptor Tyrosine Inhibiting Motif
LAT	Linker for Activation of T cells
MLC	Myosin Light Chain
MLCp	Myosin Light Chain phosphorylation
N-WASP	neural-Wiskott Aldrich Syndrome protein
NAP125	Nck associated protein
NO	Nitric oxide

PAK	p21-Activated Kinase
PAR	Protease activated receptor
PAS	Protein A sepharose
PBS	Phosphate Buffered Saline
PGI_2	Prostacyclin
PGS	Protein G sepharose
PH	Pleckstrin Homology
$cPLA_2$	Phospholipase A_2
PI 3-kinase	Phosphoinositol 3-kinase
PIP_2	Phosphatidylinositol-(4,5)-bisphosphate
PIP_3	Phosphatidylinositol-(3,4,5)-trisphosphate
PIR121	p53-inducible mRNA
PIX	PAK-interacting exchange factor
PKC	Protein Kinase C
PLCγ2	Phospholipase Cγ2
PPACK	D-phenyl-alanyl-1-prolyl-1 arginine chloromethyl ketone
PPP	Polyproline region
PRP	Platelet rich Plasma
PS	Phosphatidylserine
PVDF	Polyvinyldifluoride
RACK1	Receptor for activated C-kinase
RGD	arginine-glycine-aspartate
ROCK	Rho kinase
mRNA	messenger RiboseNucleicAcid
SCAR	suppressor of cyclic AMP repressor
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacylamide Gel Electrophoresis
SFLLRN	Serine-Phenylalanine-Leucine-Leucine-arginine-Asparagine
SH3	Src Homology 3
SH2	Src Homology 2
SLP-76	SH2 domain containing leukocyte protein of 76kDa
TP	Thromboxane receptor
TRAP	Thrombin receptor activating peptide
TXA_2	Thromboxanes
WASP	Wiskott Aldrich Syndrome family of proteins
WAVE	WASP-family verprolin-homologous protein
WRP	WAVE-associated RacGAP protein
VWF	von Willebrand Factor

CONTENTS

Chapter 2: Materials and Methods

Chapter 1

General Introduction

1.0 GENERAL INTRODUCTION

Platelets play a critical role in the prevention of excessive blood loss at sites of vessel injury. This is a highly important role as too little activation can cause a bleeding diathesis. However, too much platelet activation leads to formation of occlusive thrombi which can cause disorders such as myocardial infarction and stroke. Platelets are therefore a major target for anti-thrombotic drugs.

The platelet is also involved in several other physiological and pathological processes including inflammation (Bazzoni et al, 1991), removal of bacteria (Tang et al, 2002) and cancer metastasis (Jurasz et al, 2004). The multiple roles of the platelet may be due in part to its derivation from the same haematopoetic stem cell as the neutrophil and eosinophil (Fig.1.1) and its evolutionary origin as a haemocyte.

1.1 Platelet production and structure

The platelet is a small (0.5-3μm) discoid subcellular fragment, which is present at between 1.5-4.2x10^8/ml in whole blood. Platelets exist in the vasculature for between 7-10 days before removal from the blood by the spleen. Platelets are produced in the bone marrow from precursor cells, known as megakaryocytes. Megakaryocytes are multi-nucleated cells, which generate branch-like structures known as proplatelets that give rise to platelets within the vasculature (Becker et al, 1976). Proplatelets are elongated structures formed from the megakaryocyte cell body through rearrangement of both the microtubular and actin cytoskeletons. They are believed to protrude through the endothelial cells in the vascular niche of the bone marrow, from where proplatelets bud off into the vasculature. It is estimated that 3,000-4,000 platelets are generated from a single megakaryocyte and 10^{11} platelets are replenished in humans every day. The half-life of a platelet is 5 days for human and 2 days for mouse.

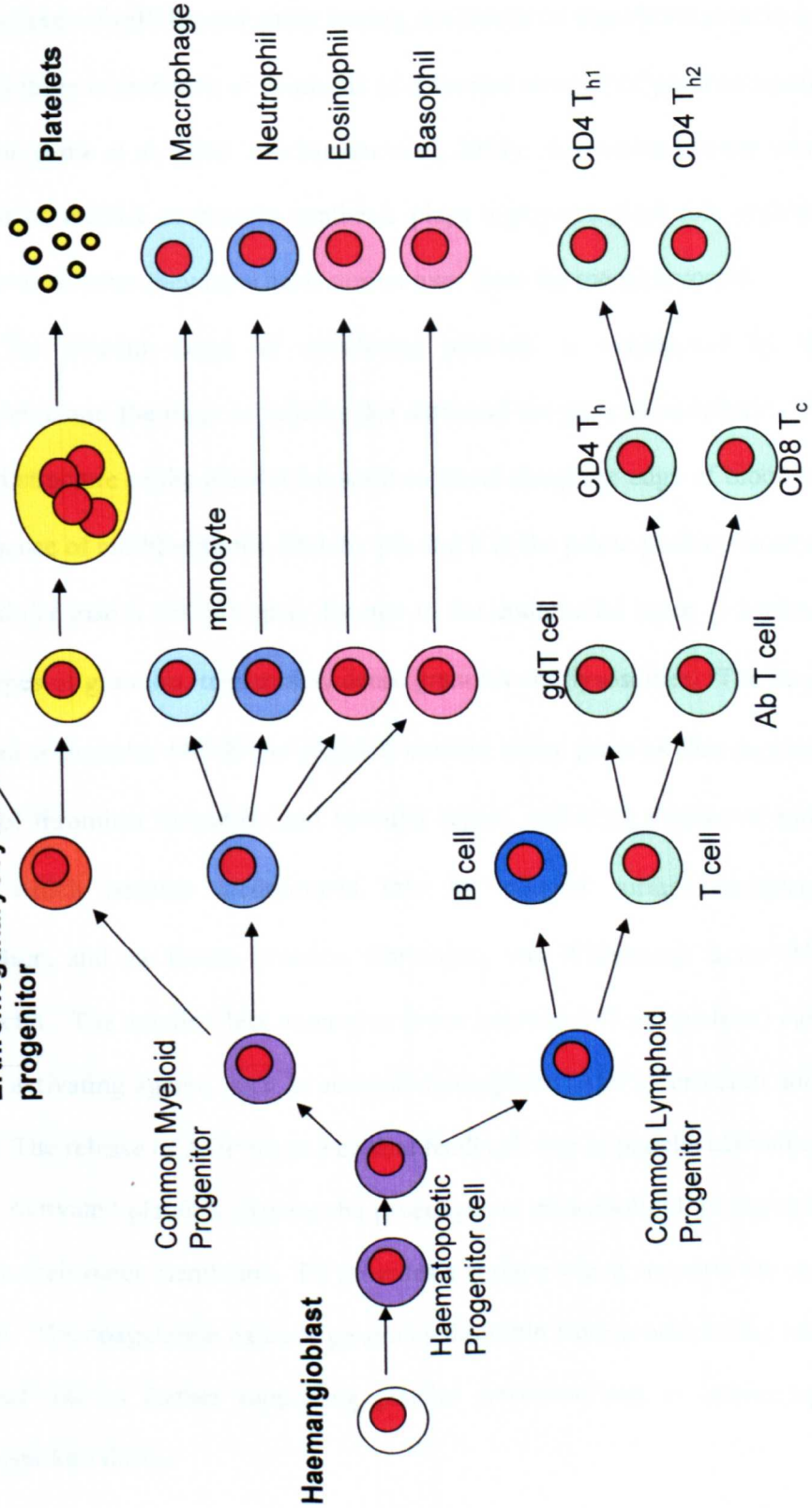

Figure 1.1: Common cell lineage of all haematopoeitic cells. Platelets are derived from haematopoetic stem cells. The multiple roles of the platelet stem from a common lineage to neutrophils, and eosinophils.

The platelet receives all its constituents, including granules and membranes from the megakaryocyte. The platelet does not possess a nucleus and, therefore, has a very low level of mRNA and under resting conditions no significant protein synthesis, although there is evidence of synthesis of a limited number of proteins upon platelet activation (Fink et al, 2003, Lindemann et al, 2001). It remains unclear whether the presence of mRNA or protein synthesis plays a physiological role within platelet function or whether they have been carried over from the megakaryocyte.

The discoid shape of circulating platelets is maintained by the actin cytoskeleton and the rings of tubulin that surround the platelet periphery. The small size and structure of the platelet causes it to travel along the edge of blood vessels in the presence of red blood cells, thereby placing it in the prime position to attach to the extracellular matrix (ECM) upon damage to the endothelial layer. Platelets contain three types of granules, α-granules, dense granules and lysosomes. The larger, more abundant α-granules (40-80 per platelet) contain many proteins that support various stages of thrombus formation and vascular repair, including P-selectin and integrin $\alpha_{IIb}\beta_3$, which become incorporated into the platelet surface membrane upon stimulation, and the matrix proteins, fibrinogen, von Willebrand factor (VWF) and fibronectin. The smaller, less numerous dense granules (~7 per platelet) carry small, soluble activating agents, such as adenine diphosphate (ADP), serotonin and calcium (Ca^{2+}). The release of ADP plays a critical feedback role in platelet activation.

Activated platelets express the procoagulant phospholipid, phosphatidylserine (PS), on their outer membrane. PS provides a surface which supports the coagulation cascade. The coagulation cascade generates thrombin from prothrombin, which plays a critical role in further supporting platelet activation and in converting plasma fibrinogen into fibrin.

1.1.2 The endothelial cell and extracellular matrix

The vasculature is lined with endothelial cells, which form a basement membrane that is a specialised form of the ECM. The endothelial cells continually monitor the vasculature and help prevent unwanted platelet activation through production of the inhibitors, prostacyclin and nitric oxide (NO). Disruption of their production can lead to platelet activation and is implicated in plaque formation (Shimokawa et al, 2007). Endothelial cells also produce heparin, a thrombin inhibitor, and provide a surface to support the fibrinolytic system.

The endothelial layer is usually nonthrombogenic, thereby preventing platelet attachment to the vessel wall. Damage to the endothelial layer leads to exposure of the basement membrane and exposure to collagen type IV and laminin which provide a surface for platelet adhesion and activation. Furthermore, deeper lesions lead to exposure to an increased number of matrix proteins, including collagens types I and III which also induce powerful platelet activation. The endothelial cells also produce VWF which plays a critical role in supporting platelet attachment and activation in the high shear rates found within arteries and arterioles (review, Ruggeri et al, 2007).

1.1.3 Thrombus formation

Thrombus formation is initiated via damage to the endothelial layer, leading to exposure of the ECM. Platelet activation then occurs via a defined sequence of events (Fig.1.2).

1) **Tethering**: Exposure to the ECM in a high shear environment leads to platelet tethering via binding of VWF, which has become immobilised on exposed collagen fibres, to the GPIb-IX-V complex. This interaction is transient due to

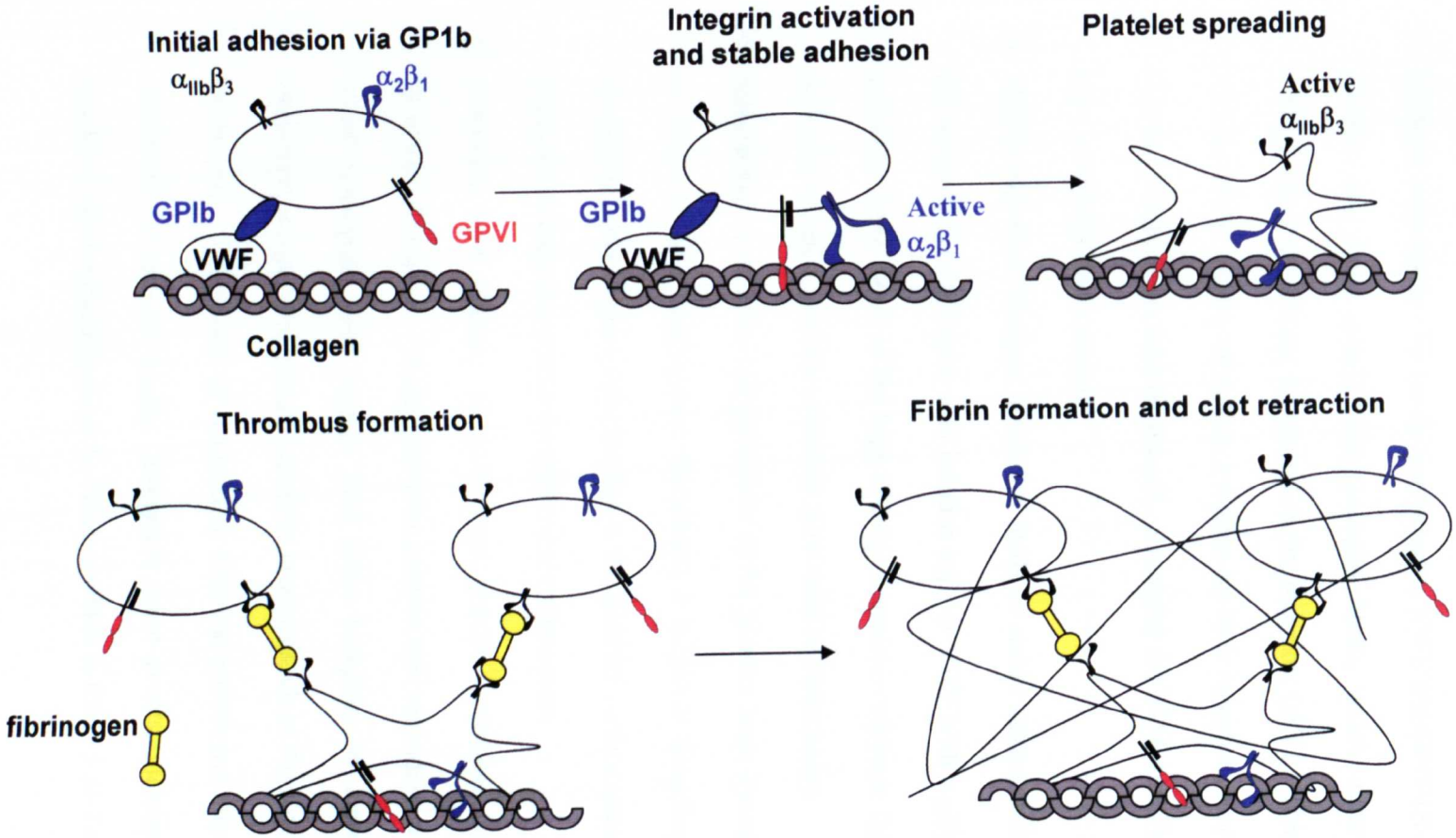

Figure 1.2: Platelet adhesion and activation at high shear. Platelets initially adhere via GPIb interaction with VWF bound to collagen. GPVI can then interact with collagen leading to stable adhesion via activated $\alpha_{IIb}\beta_3$ or $\alpha_2\beta_1$. Stable adhesion leads to platelet spreading, enabling the platelet to withstand the high shear environment. After platelet spreading, active $\alpha_{IIb}\beta_3$ binds fibrinogen, which acts as a crosslinker, leading to thrombus formation. During thrombus formation, thrombin cleaves fibrinogen to make fibrin, forming a meshwork of insoluble fibres.

a very high on-off rate of association/dissociation between VWF and GPIb-IX-V. The transient association causes platelets to roll along the exposed surface until further interactions lead to stable adhesion.

2) **Integrin activation:** The interaction between VWF and GPIb-IX-V brings the platelet into close contact with collagen fibres, which induce activation through the low affinity, immunoglobulin receptor, GPVI. This leads to an increase in the affinity of surface integrins for their receptors, a process known as integrin activation, including $\alpha_{IIb}\beta_3$, a receptor for VWF and fibrinogen, and $\alpha_2\beta_1$, a receptor for collagen.

3) **Stable adhesion**: Integrin activation leads to stable adhesion through either the interaction of collagen with integrin $\alpha_2\beta_1$ or VWF with $\alpha_{IIb}\beta_3$. The stable adhesion is the result of the high affinity interaction between the two ligands and their receptors and the relatively slow rates of dissociation.

4) **Spreading**: A massive reorganisation of the platelet actin cytoskeleton leads to filopodia and lamellipodia formation, a response described as platelet spreading. This dramatically increases the platelet surface area, and further strengthens adhesion within the high shear environment.

5) **Secretion and release**: α- and dense granule secretion further support thrombus formation. Dense granules contain the secondary mediator ADP which synergises with signals from other receptors to support thrombus formation. α-granules contain adhesive proteins such as fibrinogen, VWF and fibronectin. The fusion of α-granules with the membrane also leads to up-regulation of integrin $\alpha_{IIb}\beta_3$. Alongside these events, platelets release the feedback agonist thromboxane A_2 (TxA_2), which is formed *de novo* from

arachidonic acid liberated by activation of cytosolic phospholipase A_2 ($cPLA_2$).

6) **Aggregate formation**: Platelet aggregation is mediated by binding of circulating fibrinogen and VWF to activated integrin $\alpha_{IIb}\beta_3$. Platelet tethering to VWF and the subsequent activation of tethered platelets by ADP and TxA_2 plays a critical role in aggregation.
7) **Procoagulant activity:** Platelet activation leads to exposure of the membrane phospholipid, PS, which supports the coagulation cascade. Activation of the coagulation cascade drives thrombin generation, reinforcing platelet activation.
8) **Clot retraction**: Thrombin cleaves fibrinogen to form fibrin, which serves to further strengthen the thrombus through an active process known as clot retraction. Clot retraction is mediated by the action of fibrin and the actin cytoskeleton via integrin $\alpha_{IIb}\beta_3$.

Thrombus formation is thus mediated by the combination of activation of both platelets and the coagulation cascade.

1.1.4 Dependence of thrombus formation on shear

The process of thrombus formation is dependent on shear. The events described above govern thrombus formation at medium to high shear rates found within the arteriolar system. However, at the low shear rates found with the venous system, fibrinogen is the most important protein driving thrombus formation. It competes with VWF for binding to $\alpha_{IIb}\beta_3$, and probably due to its higher molar ratio in plasma, dominates binding to the integrin under these conditions. As the shear rate increases,

thrombus formation becomes increasingly dependent on VWF because of its critical role in the initial capture (or tether) of platelets.

Fibronectin is also implicated in thrombus formation as mice deficient in VWF and fibrinogen form thrombi upon damage to the endothelium (Ni et al, 2000). Fibronectin has been proposed as the protein driving this thrombus formation. Fibronectin-deficient mice (Ni et al, 2003) have deficient thrombus growth, initiation, and stability, implicating fibronectin as playing a role in each stage of thrombus formation. However, the role of fibronectin in supporting thrombus formation is not well defined.

1.2 PLATELET RECEPTORS

The platelet membrane contains many different types of receptors thereby enabling the platelet to respond to a large range of agonists. These receptors can be placed into two broad classes, G protein-coupled receptors (GPCRs) and tyrosine kinase receptors.

1.2.1 GPCR receptors

GPCRs are 7 transmembrane proteins that induce activation through a G protein, which is composed of α, β, and γ subunits. The G protein subtype takes its name from the α-subunit. Upon activation, GDP dissociates from the α-subunit and is replaced by GTP. This causes a conformational change that liberates the βγ complex. The α subunit is a GTPase and hydrolyses GTP to GDP, thereby switching off the G protein signal. Examples of platelet GPCRs include the two ADP receptors, $P2Y_1$ and $P2Y_{12}$, the thrombin receptors, Protease activated receptor (PAR)1 and PAR4, and the thromboxane receptor, TP (Murugappans et al, 2004, Coughlin, 2005). The $P2Y_{12}$ ADP receptor is coupled to $G_{i/z}$ family of G proteins, while the other four receptors are coupled to the G_q and G_{13} families. G_q signals via PLCβ leading to an increase in intracellular Ca^{2+} and protein kinase C (PKC) activation. $G_{12/13}$ signals via the RhoA/Rho kinase (ROCK) pathway leading to shape change, stress fibre formation and aggregation (Moers et al, 2004).

G protein signalling plays an important role within platelet activation. For example, platelets lacking $G\alpha_q$ or $G\alpha_{13}$ show a marked reduction in responsiveness to agonists such as thrombin and TxA_2. In addition these platelets will adhere normally to collagen, but cannot form thrombi. This implicates G protein signalling as playing

a fundamental role within platelet activation and thrombus formation (Moers et al, 2004).

1.2.2 Tyrosine kinase receptors

A distinct number of platelet glycoprotein receptors signal via tyrosine kinase receptors, including GPIb-IX-V, GPVI and integrins. These receptors signal through sequential activation of Src and Syk tyrosine kinases, leading to PLCγ2 activation, although each signalling pathway is distinct. All of these signalling cascades rely on adaptor proteins which serve as scaffolds integrating proteins into signalling complexes. These interactions are mediated through a number of protein and lipid binding domains including Src homology 2 (SH2), Src homology 3 (SH3) and pleckstrin homology (PH) domains. SH2 and SH3 protein binding domains interact with phosphotyrosine, and proline rich regions respectively (review Pawson et al, 2001, review Vidal et al, 2001). PH domains interact with lipid phosphoinositides in the membrane.

1.3 PLATELET AGONISTS AND THEIR RECEPTORS

The major platelet ligands and their receptors that support thrombus formation are discussed below.

1.3.1 Fibrinogen and integrin $\alpha_{IIb}\beta_3$

Fibrinogen, comprises of two sets of α, β and γ chains linked by both interchain and intrachain disulphide bonds (Doolittle et al, 1979). A sequence of 11 amino acids on the γ chain of fibrinogen is essential for integrin $\alpha_{IIb}\beta_3$ binding rather than the arginine-glycine-aspartate (RGD) sequence present on the α chains. Due to its structure, fibrinogen can mediate crosslinking of platelets through integrin $\alpha_{IIb}\beta_3$.

$\alpha_{IIb}\beta_3$ is the major integrin present on the platelet surface with over 80,000 copies on the plasma membrane and a further 40,000 copies on α-granules. It represents approximately 3% of the total protein within the cell (Niya et al, 1987, Phillips et al, 1988). $\alpha_{IIb}\beta_3$ is a heterodimer of two non-covalently bound subunits, α_{IIb} and $\beta 3$. In addition to binding fibrinogen, it also serves as a receptor for VWF, fibronectin, vitronectin and CD40 ligand. Patients with defects in expression or function of either subunit of the integrin have a major bleeding problem, known as Glanzmann's thrombasthenia (George et al, 1990).

$\alpha_{IIb}\beta_3$ is present on platelets in a low-affinity (or inactive) state, but can be induced to bind its ligands in response to 'inside-out signals' during platelet activation. This in turn leads to ligand binding and the generation of 'outside-in' signals (Fig.1.3) which serve to reinforce platelet activation. Clustering of $\alpha_{IIb}\beta_3$ leads to activation of the tyrosine kinases, Src and Syk. Src is constitutively associated with the β tail through its SH3 domain (Arias-Salgado et al, 2003), and is critical both for recruitment and activation of Syk (Obergfell et al, 2002). Src

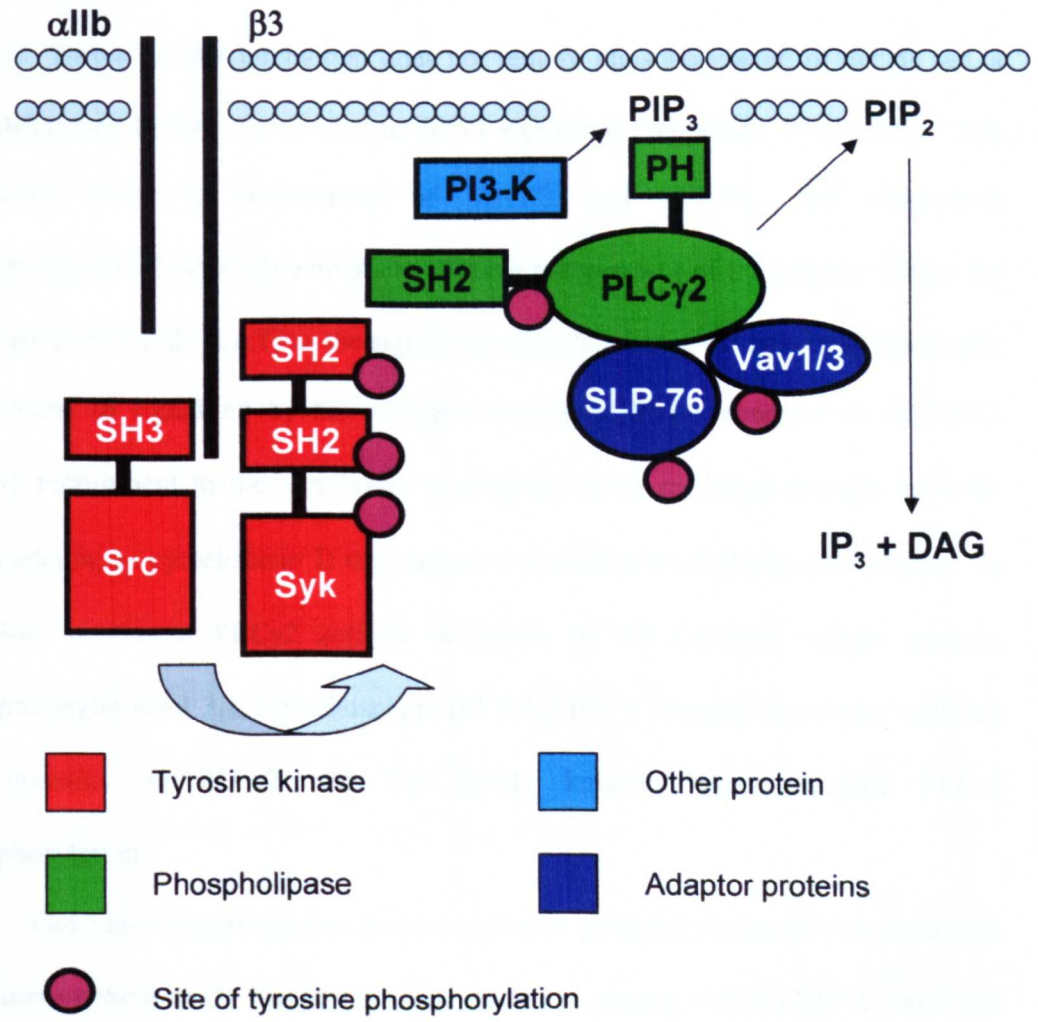

Figure 1.3: Syk dependent intracellular signalling induced by $\alpha_{IIb}\beta_3$ integrin activation after ligand binding. Ligand binding initiates the autophosphorylation of Src leading to the recruitment of Syk. Src mediated Syk phosphorylation leads to the recruitment of SLP-76, Vav1/3 and PLCγ2. PLCγ2 is then activated leading to the formation of diacylglycerol (DAG) and inositol phosphate (IP_3).

activation downstream of $\alpha_{IIb}\beta_3$ is mediated through autophosphorylation at position 418 (Arias-Salgado et al, 2003). This leads to Syk recruitment to the β3 tail through an interaction between its N-terminal SH2 domain and the last 28 amino acids of the β3 tail. However, this interaction does not require phosphorylation of the β3 tail, a key difference to Syk recruitment in GPVI signalling (Woodside et al, 2001). Syk activation leads to recruitment of Vav1/3 and SLP-76, and subsequent phospholipaseCγ2 (PLCγ2) activation. This is independent of the adapter Linker for Activation of T cells (LAT) as the signalling cascade takes place outside of lipid rafts, in contrast to signalling by the collagen receptor, GPVI (Wonerow et al, 2002). PLCγ2 recruitment to the membrane is proposed to be mediated through the actin cytoskeleton, as cytochalasin D can cause a decrease in PLCγ2 phosphorylation. In addition, maximum PLCγ2 activity is driven by PI 3-kinase, whose product, phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P3) is thought to interact with the PH domains on PLCγ2 and Tec family kinases, which mediate PLCγ2 phosphorylation.

Outside-in signalling also involves tyrosine phosphorylation of two conserved tyrosines on the β3 tail. The first tyrosine residue is present within a NPXY motif and upon phosphorylation associates with binding adaptor proteins such as DOK2. The second residue is present in a NXXY motif, and associates with adapter proteins such as Shc. Interestingly, the two tyrosine residues have been mutated to phenylalanine to make the diYF mouse (Law et al, 1999). This mouse has a significant increase in recurrent bleeding and impairment in clot retraction. This is associated with a defect in myosin binding to the β3 integrin tail (Jenkins et al, 1998). Both tyrosines are required for myosin binding. Tyrosine phosphorylation of the β3 tail also leads to formation of a complex between Receptor for activated C-kinase (RACK1) and

protein kinase C β (PKCβ), and an association with focal adhesion kinase (FAK) (Buensuceso et al, 2005).

1.3.2 VWF and GPIb-IX-V

VWF is a multimeric protein that is assembled into disulphide-linked multimers that consist of between 2-500 subunits and can have a molecular weight of several million. The major receptors for VWF are GPIb-IX-V and integrin $\alpha_{IIb}\beta_3$. There are approximately 25,000 copies of the GPIb-IX-V complex per platelet. This complex is a product of four genes: GPIbα, GPIbβ, GPXI, and GPV, each of which is characterised by a series of leucine-rich repeats. The binding of VWF to GPIb-IX-V complex takes place at intermediate to high rates of shear. However, this interaction can also be induced through use of the snake C-type lectin modulator, botrocetin, and the bacterial glycopeptide, ristocetin. The use of these reagents enables dissection of the VWF signalling cascades, as botrocetin induces signalling via GPIb-IX-V, and ristocetin via both GPIb-IX-V and $\alpha_{IIb}\beta_3$ (McCarty et al, 2006).

GPIb-IX-V is associated with the initial transient platelet adhesion that takes place under high shear conditions. In addition, there is evidence that GPIb-IX-V generates intracellular signals which are generally considered to be weak (Nesbitt et al, 2002, Jackson et al, 1994, and Asazuma et al, 1997), but under certain conditions can be strong (Canobbio et al, 2001, and Torti et al, 1999). The GPIb signalling cascade is similar to those used by GPVI and integrin $\alpha_{IIb}\beta_3$, but with several important differences. GPIb-IX-V signals via the non-tyrosine receptor kinases Fyn, Lyn, and Syk, and the adapters Shc and SLP-76 leading to PLCγ2 activation (Asazuma et al, 1997, Falati et al, 1999, Jackson et al, 1994, Marshall et al, 2002, Torti et al, 1999, and Wu et al, 2001). GPIb also physically associates with FcRγ-

chain and FcγRIIA (Cannobbio et al, 2001), although neither association is essential for signalling as demonstrated in mice deficient in the FcRγ-chain, while FcγRIIA is absent from the mouse genome.

The absence of GPIb-IX-V leads to Bernard Soulier syndrome, which is characterised by thrombocytopenia, enlarged platelets and a marked bleeding syndrome (Lopez et al, 1998, Tomer et al, 1994).

1.3.3 Collagen

Collagen is the most platelet reactive protein within the ECM. It supports platelet adhesion through direct and indirect (via VWF) mechanisms, and activates platelets leading to aggregation and formation of a pro-coagulant surface. Collagen-platelet interactions are most important at medium to high rates of shear (Nieswandt et al, 2003).

There are over 20 different forms of collagen within the human body, with 9 forms present within the vessel wall (Nieswandt et al, 2003). Fibrillar collagen type I and III are the major forms of collagen within the ECM. Collagens are made up of multiple repeats of glycine-X-Y, and are rich in prolines and hydroxyprolines. The sequence GPO (single amino acid code; O = hydroxyproline) is estimated to represent 10% of collagens types I and III. The collagen receptor GPVI binds selectively to this sequence to mediate platelet activation.

Collagen causes platelet activation through the immunoglobulin-like receptor GPVI and the integrin $\alpha_2\beta_1$. Collagen is proposed to activate platelets via the "two site-two step" model (Fig.1.4i). Here, the platelet initially interacts with collagen via GPVI, which generates intracellular signals that lead to activation of integrin $\alpha_2\beta_1$. Binding to integrin $\alpha_2\beta_1$ has the net effect of reinforcing the binding to GPVI and

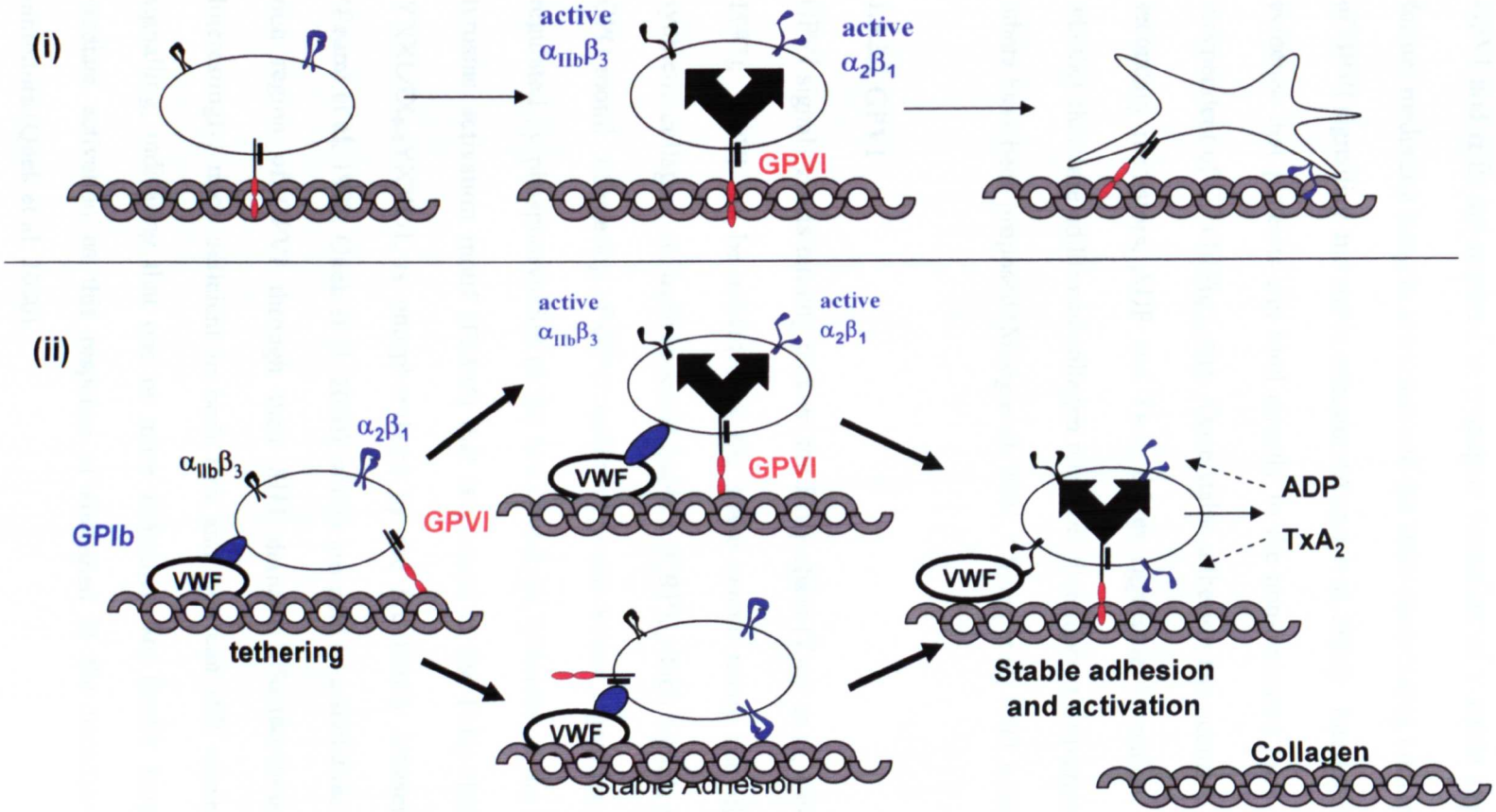

Figure 1.4: Collagen signalling in static and high shear conditions. (i) The Two site-Two step model. The platelet initially interacts with collagen via GPVI, generating intracellular signals leading to integrin $\alpha_2\beta_1$ activation. $\alpha_2\beta_1$ reinforces GPVI binding, increasing the net strength of signalling. **(ii)** Platelet adhesion to collagen at high shear. Initial platelet tethering via GPIb-VWF interaction. In the upper pathway, GPVI signals, activating the integrins $\alpha_2\beta_1$, and $\alpha_{IIb}\beta_3$, causing stable adhesion. In the lower pathway platelet adhesion is mediated via $\alpha_2\beta_1$, before GPVI mediated platelet activation. After stable adhesion, release of secondary mediators, aids thrombus formation. Redrawn from Auger et al, 2005.

thereby increasing the net strength of signalling. In addition, integrin $\alpha_2\beta_1$ generates weak intracellular signals of unknown physiological relevance. At high shear, both GPVI and $\alpha_2\beta_1$ are required to propagate formation of a stable aggregate, with the former mediating integrin activation and the latter contributing both to the net strength of GPVI signalling and stable adhesion (Auger et al, 2005). Interestingly, there is also evidence that platelets can bind directly to the non-activated form of integrin $\alpha_2\beta_1$ independent of GPVI (Fig.1.4ii). Once stable adhesion has occurred, secretion of the secondary mediators, ADP and TxA_2 drives aggregate formation. It is unknown whether there are additional collagen receptors that support activation, though several others have been proposed (Moog et al, 2001, Chiang et al, 1997 & 2002).

1.3.3.1 GPVI

GPVI signals via its binding partner, the FcRγ-chain (Tsuji et al, 1997; Gibbins et al, 1997). GPVI can be activated by the snake venom toxin, convulxin, and by the synthetic collagen, collagen repeated peptide (CRP), which signals through a repeat GPO motif. Clustering of GPVI initiates tyrosine kinase signalling (Fig.1.5) that is regulated by phosphorylation of the two conserved tyrosines in the immunoreceptor tyrosine activation motif (ITAM) that is present in the FcRγ-chain. This motif, $YXXL/IX_{6-8}YXXL/I$, is phosphorylated by the Src family kinases, Fyn and Lyn (Ezumi et al, 1998, Quek et al, 2000), which are held in association with the proline rich region of GPVI through their SH3 domains (Suzuki-Inoue et al, 2002). Interestingly, mice deficient in both Fyn and Lyn can still cause residual GPVI signalling, indicating that one or more additional Src family kinases are able to mediate activation as this response is abolished in the presence of Src kinase inhibitors (Quek et al, 2000).

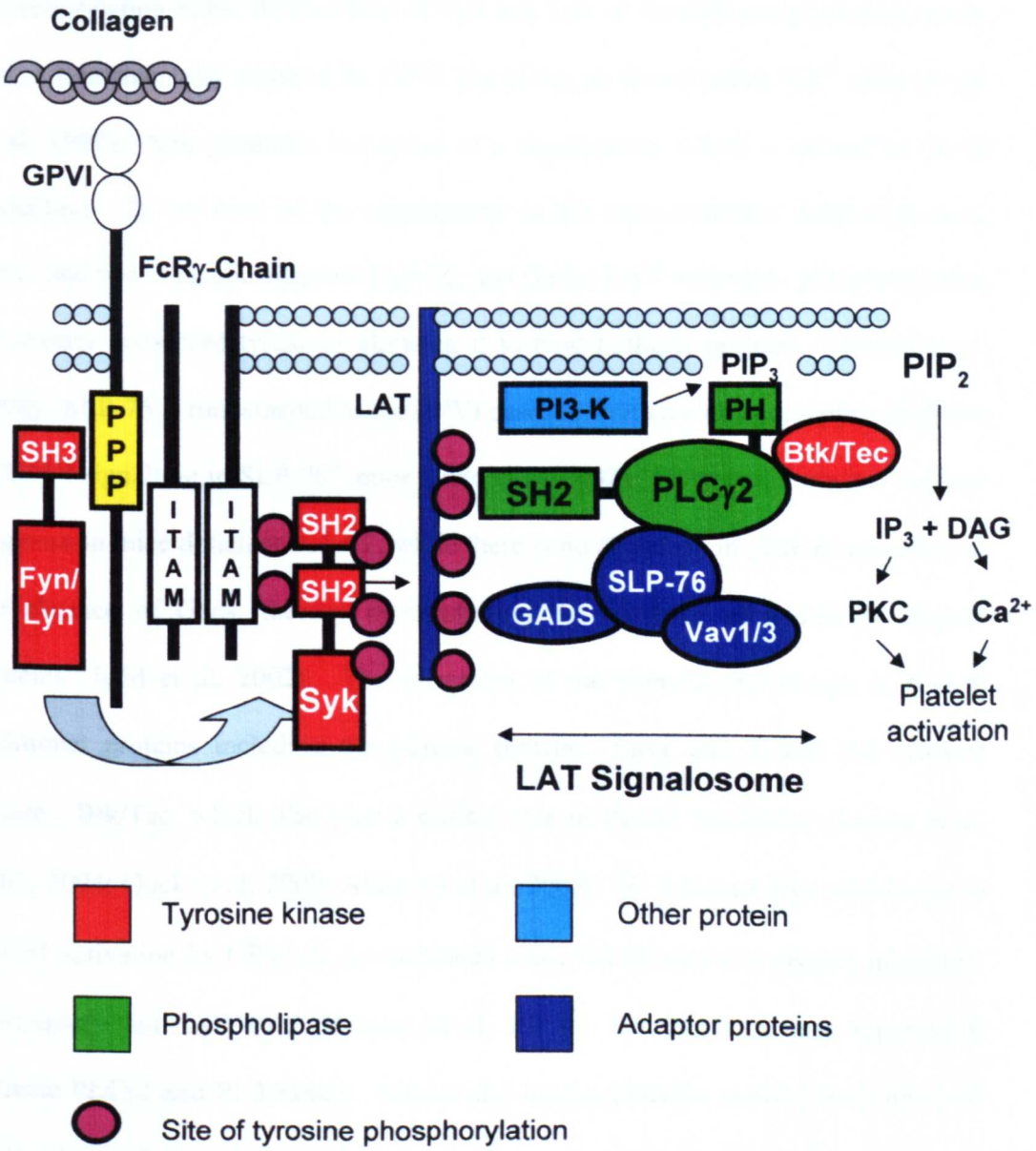

Figure 1.5: The GPVI signalling cascade. Collagen interaction with GPVI causes ITAM phosphorylation, by Fyn and Lyn. Syk is recruited, through interaction of the ITAMs and its tandem SH2 domains. Syk causes LAT phosphorylation, and the formation of the LAT signalosome, of SLP-76, Gads,Vav1/3, Btk, and PLCγ2. PLCγ2 is then activated leading to the formation of DAG and IP_3.

Syk recruitment to the FcRγ-chain ITAM via its two SH2 domains leads to its phosphorylation either downstream of Fyn and Lyn or through autophosphorylation. Syk is fundamentally required for GPVI signalling, as shown using $Syk^{-/-}$ mice (Poole et al, 1997). Syk generates formation of a signalosome which is central to GPVI signalling. At the core of the signalosome is the transmembrane adaptor protein, LAT, and two cytosolic adaptors SLP-76, and Gads. LAT undergoes phosphorylation on several conserved tyrosines allowing it to bind multiple proteins (Gibbins et al, 1998). SLP-76 is fundamental to the GPVI cascade with an almost complete abolition of GPVI signalling in $SLP\text{-}76^{-/-}$ mice (Judd et al, 2002). However, there is a residual response in mice deficient in LAT, while there is no alteration in platelet activation in the absence of Gads, thereby demonstrating the presence of additional adaptor proteins (Judd et al, 2002). The formation of the signalosome brings in several additional proteins including the adaptor proteins, Vav1 and 3, and the tyrosine kinases, Btk/Tec, which also play a critical role in PLCγ2 regulation (Pearce et al, 2002, 2004; Quek et al, 2000; Atkinson et al, 2003). PI 3-kinase also contributes to PLCγ2 activation by GPVI as demonstrated using the structurally distinct inhibitors, wortmannin and Ly294002 (Pasquet et al, 1999). Rac has also been reported to activate PLCγ2 and PI 3-kinase. Mouse and human platelets express both forms of PLCγ, 1 and 2, but the latter has the predominant role in mediating activation, as shown using $PLC\gamma2^{-/-}$ platelets (Wang et al, 2000). In addition, in human platelets, PLCγ1 does not undergo phosphorylation following GPVI activation.

As discussed above, GPVI plays an important role in platelet adhesion to collagen at high shear. However, although platelet adhesion to collagen, and thrombus formation is defective within GPVI deficient mice, there is only a minor bleeding phenotype associated with GPVI deficient mice and humans (review

Nieswandt et al, 2003), thereby demonstrating redundancy within the regulation of platelet activation.

1.3.3.2 Integrin $\alpha_2\beta_1$

$\alpha_2\beta_1$ mediates weak platelet activation as demonstrated by platelet spreading on an $\alpha_2\beta_1$-selective peptide, containing the sequence GFOGER, despite the peptide being unable to induce aggregation (Inoue et al, 2003, Suzuki-Inoue et al, 2001). Interestingly, integrin $\alpha_2\beta_1$ signals via a similar pathway to that used by integrin $\alpha_{IIb}\beta_3$, namely via Src kinase regulation of PLCγ2. It is possible that this signalling pathway helps to minimise the bleeding in mice deficient in GPVI (Nieswandt et al, 2001).

1.3.4 ADP and ATP, and the $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors

ADP signals via the two GPCRs, $P2Y_1$, and $P2Y_{12}$. $P2Y_1$ is expressed on multiple tissues, including blood vessels, platelets and heart. In platelets, it is coupled to G_q and G_{13}. $P2Y_1$ activation leads to shape change and weak, reversible aggregation (Leon et al, 1999). Mice deficient in $P2Y_1$ have impaired thrombus formation and an increase in bleeding time (Fabre et al, 1999), although the overall significance of the ADP receptor remains unclear since, as yet, no patients have been identified with a defect in the nucleotide receptor.

$P2Y_{12}$ is coupled to the $G\alpha_{i2}$, which inhibits adenyl cyclase and therefore potentiates platelet activation. In addition, $P2Y_{12}$ mediates $G\beta\gamma$ regulation of PI 3-kinase β and γ isoforms, which synergises with signals from Ca^{2+}-mobilising receptors to induce full platelet activation. In response to ADP, $P2Y_{12}^{-/-}$ platelets undergo transient aggregation through activation of $P2Y_1$ (Foster et al, 2001). $P2Y_{12}^{-/-}$

mice and $P2Y_{12}^{-/-}$ human patients have a marked increase in bleeding tendency thereby illustrating the critical role of this receptor in mediating activation (Foster et al, 2001, Hollopeter et al, 2001). $P2Y_{12}$ is the target for one of the two major classes of orally-available antiplatelet agents, clopidigrel (Bennet et al, 2001).

$P2X_1$ is a ligand-gated ion channel which serves as a receptor for ATP. Activation of $P2X_1$ leads to transient shape change but not aggregation (Takano et al, 1999, Rolf et al, 2001). Activation of $P2X_1$ potentiates granule release and aggregation to low-dose collagen, TXA_2 and thrombin receptors (Oury et al 2001, 2002). $P2X_1^{-/-}$ mice exhibit normal bleeding times, but show less tendency to form thrombi in both the ferric chloride and laser-induced model of vascular injury (Hechler et al, 2003). The overall significance of $P2X_1$, however, remains unclear as no patients with a bona fide mutation in the ATP receptor are reported.

1.3.5 Thromboxane A_2 and the TP receptor

TXA_2 is a lipid synthesised from arachidonic acid by the action of prostaglandin G/H synthase, and thromboxane synthase. It is a potent vasoconstriction agent, and signals in platelets through G_q and G_{13}, causing dense granule release and $\alpha_{IIb}\beta_3$ activation. Patients and mice deficient in TxA_2 formation have a mild bleeding phenotype (Thomas et al, 1998). The efficacy of aspirin as an anti-thrombotic agent is mediated by inhibition of TxA_2 formation. Aspirin acts by irreversibly inhibiting prostaglandin G/H synthase, and can be used at a low dose to selectively target platelets because they are unable to synthesise new protein.

1.3.6 Thrombin and the PAR receptors

Thrombin is a powerful platelet agonist produced by the cleavage of prothrombin to thrombin through the activity of Factor Xa and Va. Thrombin acts through PAR1 and PAR4 receptors in humans, and PAR3 and PAR4 in mouse. Thrombin does not bind directly to PAR1 and PAR4, but causes cleavage of their N-termini. This reveals a tethered ligand leading to intramolecular binding and intracellular signalling downstream of the two PAR receptors. In contrast, the role of PAR3 is to present thrombin to the PAR4 receptor, thereby increasing the net affinity of the interaction.

In platelets, PAR1 and PAR4 signal through G_q and G_{13}. In human platelets, PAR1 and PAR4 activation leads to aggregation and secretion. If PAR1 is blocked with antibodies, PAR4 will signal in response to high concentrations of thrombin (Kahn et al, 1999) demonstrating PAR1 and PAR4 respond to low and high concentrations of thrombin, respectively. $PAR4^{-/-}$ mice fail to undergo platelet shape change, mobilise Ca^{2+}, secrete ATP, or aggregate to thrombin, clearly demonstrating the requirement for PAR4 to mediate thrombin signalling (Sambrano et al, 2001). The action of PAR receptors is turned off due to internalization and degradation.

Thrombin plays a vital role in supporting thrombus formation and in mediating stabilisation as it cleaves fibrinogen to fibrin, a requirement for clot retraction. The prevention of thrombin activation through treatment of heparin is a major mechanism of prevention of deep vein thrombosis.

1.4 PLATELET ACTIN CYTOSKELETON

The actin cytoskeleton is important in maintaining platelet shape and integrity within the high shear environment of the vasculature. The platelet's discoid shape is maintained by a spectrin-based membrane skeleton, a tightly coiled region of microtubules, and a rigid network of cross-linked actin filaments. Following platelet adhesion to ECM proteins such as fibrinogen and collagen, the actin cytoskeleton undergoes a massive rearrangement leading to filopodia and lamellipodia formation and an overall 'fried-egg' appearance. This shape change is driven by the breakdown of the microtubular ring which maintains the initial discoid shape of the platelet and by actin polymerisation (Nachiamas et al, 1980).

1.4.1 The regulation of actin assembly

Actin is the most abundant platelet protein, with a cytoplasmic concentration of 0.55mM which corresponds to approximately two million copies of actin per cell (Nachmias et al, 1988). Of this, approximately 40% of actin is present in 2,000-5,000 filaments that make up a rigid network of cytoplasmic actin filaments (Hartwig et al, 1991). The filaments represent polymerised fibres of actin monomers. The remaining actin is present as monomers in the cytoplasm, most likely complexed with actin monomer binding proteins.

Actin filaments are polarised, with a fast growing (barbed) and a slow growing (pointed) end. The terms pointed and barbed originate from the arrow-like structure of actin filaments dotted with myosin in electron micrographs. The barbed end is oriented to the periphery of the cell toward the plasma membrane. Actin filaments are polymerised by the addition of ATP-actin to the barbed end (Fig.1.6). Upon polymerisation, ATP is quickly hydrolysed to ADP, but the phosphate group is not

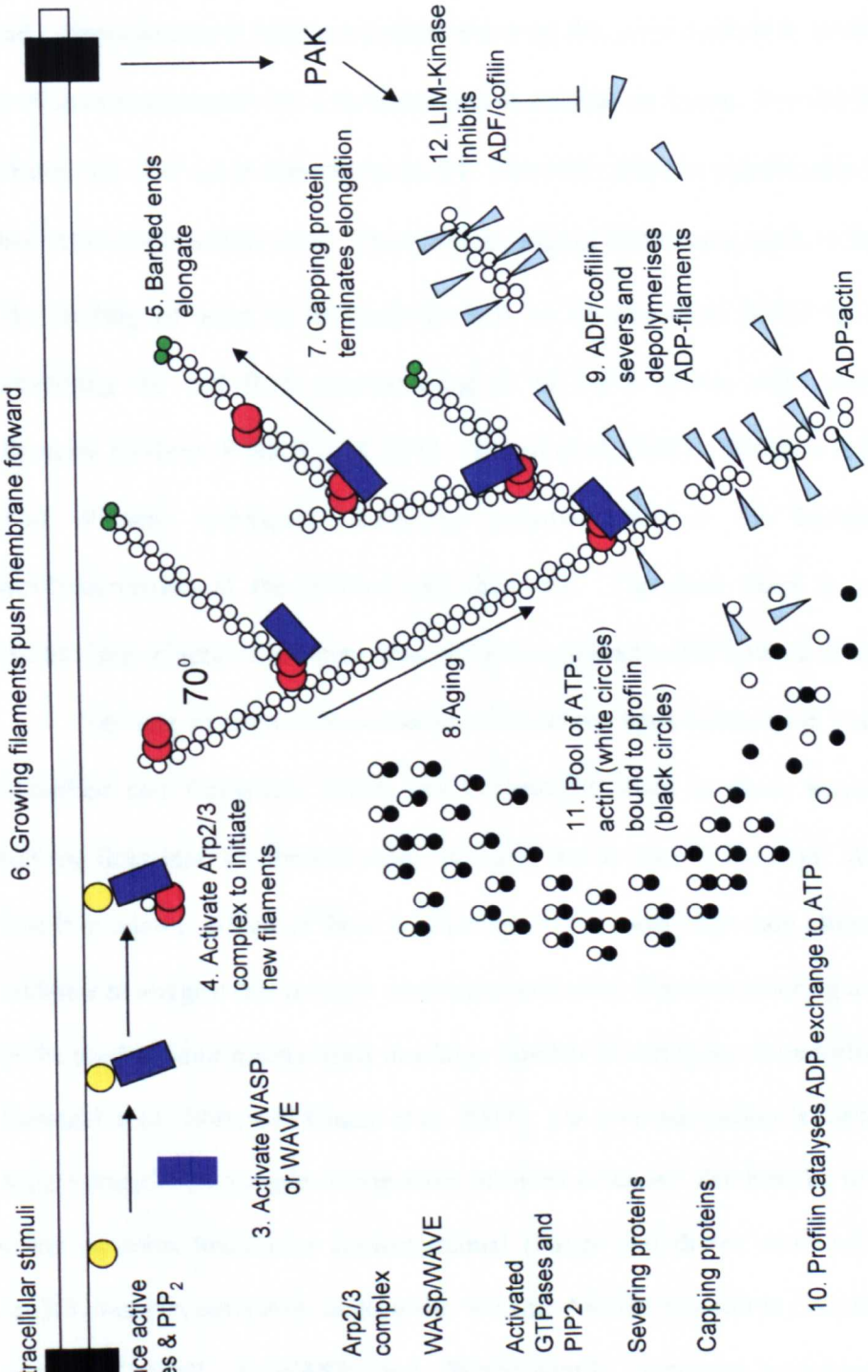

Figure 1.6: Model for actin polymerisation treadmilling at the leading edge. Stimulation, leads to RhoGTPase activation, and PIP_2 formation. WASP or WAVE are activated leading to activation of the Arp2/3 complex, and barbed end formation. Filament elongation is inhibited through capping proteins, and as filaments age, they are severed and depolymerised. ADP-actin binds profilin, which catalyses the exchange of ADP to ATP. ATP-actin can then be reincorporated into actin filaments. Redrawn from Pollard et al, 2003.

readily released. The half life for phosphate release is 6min. During this period the filament is stable. However, upon phosphate release, cofilin an actin severing and depolymerising protein, binds the filament causing depolymerisation at the pointed end. Depolymerised ADP-actin binds either to the actin nucleotide exchange factor profilin or to thymosin-β4, which prevents nucleotide exchange. Profilin has a greater affinity for ADP-actin than thymosin-β4. However, there is significantly less profilin than ADP-actin within cells. Therefore the excess ADP-actin binds to thymosin-β4. The binding of actin to thymosin-β4 acts as an important buffering mechanism, preventing the cell from incorporating all of the available actin monomers into filaments (reviews Pollard et al, 2003, Millard et al, 2005). Within a resting cell, an actin filament undergoes continuous polymerisation at the barbed end and depolymerisation at the pointed end (Fig.1.6). Therefore there is a very slow treadmilling of actin monomers from barbed to pointed end (Fujiwara et al, 2002).

The rate of actin polymerisation is increased upon stimulation via an increase in barbed end formation, which can be brought about in three ways: uncapping existing filaments, severing existing filaments and *de novo* nucleation. At the present time it is unclear which of these mechanisms is the most important although, there is evidence to suggest that *de novo* nucleation and actin filament severing are thought to be the predominant mechanisms in a large number of cell types (Condeelis et al, 1993, Zigmund et al, 1996, and Kiuchi et al, 2007). *De novo* nucleation is mediated via the Arp2/3 complex, a complex comprising of seven proteins. The binding of an activator to the complex leads to a conformational change and drives actin polymerisation. Arp2/3 complex activators include the Wiskott-Aldrich Syndrome (WASP) family of proteins, WASP, N-WASP and WASP-family verprolin-homologous protein (WAVE)-1-3 (also known as suppressor of cyclic AMP repressor (Scar)), and other

proteins such as cortactin, and Spin-90 (Kim et al, 2007, Bryce et al, 2005). The Arp2/3 complex mediates new barbed end formation by generating new actin filaments at 70° to the original filament, leading to formation of the 3D meshwork of actin filaments that is characteristic of lamellipodia. The role of actin severing in lamellipodia formation is also gaining recognition as this has been shown to be dependent on the production of new barbed ends by the action of cofilin, at least in MLTn3 mammary adenocarcinoma cells (Chan et al, 2000). However, in addition there is also strong evidence that cofilin regulates the actin cytoskeleton dynamics through regulation of actin monomer availability (Kiuchi et al, 2007).

Uncapping of filaments also plays an important role in increasing the number of barbed ends, and the correct localisation of actin polymerisation within a cell, i.e the periphery of the cell. In addition, capping also allows for the formation of short actin filaments that are of maximum strength to ensure maximum movement. There are multiple proteins that have been demonstrated to cap actin filaments including gelsolin, adducin, and capping protein (CP), a homologue of chicken CapZ (Cooper et al, 2000; Sun et al, 1999; Kuhlman et al, 1996). Within platelets gelsolin, CapZ, and α-adducin have all been identified (Falet et al, 2000; Gilligan et al, 1999; Barkalow et al, 1995). Barkalow et al (1995) identified the release of CP released barbed ends ready for polymerisation in permeabilised platelets stimulated by thrombin. This release was dependent on gelsolin. Falet et al (2000) reported the uncapping of gelsolin-capped filaments during actin polymerisation in platelets was required to mediate Arp2/3 complex mediated actin nucleation induced by CRP. Uncapping of barbed ends is mediated by membrane phosphoinositides downstream of Rac (Hartwig et al, 1995). In addition platelets also contain adducin, which in red blood cells has been identified as a capping protein (Kuhlman et al, 1996). Adducin is

associated with the resting platelet cytoskeleton, in conjunction with spectrin. Upon platelet activation PKC phosphorylates adducin leading to its disassociation from the cytoskeleton, and the movement of spectrin to the central region of the platelet. PKC is fundamental to the formation of lamellipodia within platelets. Due to the disassociation of adducin new barbed ends are formed ready for actin polymerisation (Barkalow et al, 2003).

1.4.2 The regulation of actin polymerisation by Rho family GTPases

The Rho GTPases, act as molecular switches and are involved in multiple signalling pathways leading to cell adhesion, migration, cytokinesis, proliferation and transformation (Nobes et al, 1995, Ridley et al, 1992a&b, reviewed Jaffe et al, 2005). Rho GTPases cycle between an active, GTP-bound and inactive, GDP-bound state. This cycling is tightly controlled by GDP exchange factors (GEFs), which stimulate replacement of GDP by GTP; GTPase-activating proteins (GAPs), which stimulate the innate GTPase activity of the small GTPases; and by guanine nucleotide dissociation inhibitors (GDIs), which act as negative regulators by blocking GDP-dissociation. The GEFs, GAPs and GDIs which regulate Rho family GTPases in platelets are unknown.

The most well known Rho GTPases are Cdc42, Rac, and RhoA. Their roles within cells have been demonstrated through microinjection of active forms (reviewed by Hall et al, 1998). This has led to the identification that these proteins play a fundamental role in filopodia, lamellipodia, and stress fibre formation respectively in a wide variety of cell types (Nobes et al, 1995).

1.4.3 The regulation of the actin cytoskeleton by WASP family proteins

In association with the RhoGTPases, the WASP family play a fundamental role within the regulation of the actin cytoskeleton. There are five members of the WASP family, WASP, N-WASP, WAVE-1, -2, and -3. WASP was first identified as a mutated gene in Wiskott-Aldrich syndrome, a rare X-linked chromosome disorder, associated with immmunological dysfunction and thrombocytopenia (Derry et al, 1994). WASP is only present in haematopoeitic cells, WAVE-1 and WAVE-3 are mainly neuronal in origin, but are also found in platelets, and N-WASP and WAVE-2 are ubiquitous (Miki et al, 1996, Suetsugu et al, 1999).

WASP and N-WASP are effectors of Cdc42. They have highly similar structures (Fig.1.7), with a C-terminal actin and Arp2/3 complex binding domain, and a N-terminal WASP homology domain. They are present within the cytoplasm in an autoinhibited state, but become activated by Cdc42 binding to the CRIB domain. Both WASP and N-WASP have been identified as potent activators of the Arp2/3 complex. However, their precise cellular role is not yet fully understood. Initial data indicated a role within filopodia formation (Miki et al, 1998) which is now disputed (Biyasheva et al, 2004). More recent data has identified a role for both WASP and N-WASP in vesicle trafficking (review Ridley et al, 2006, Benesch et al, 2002, and Snapper et al, 2001).

A lack of N-WASP is embryonically lethal at day 11 (Snapper et al, 2001). Defects were observed within the neural and cardiac tissue, which is similar to defects associated with WAVE-2$^{-/-}$ mice. WASP$^{-/-}$ mice however are viable, although a lack of WASP is associated with abnormalities of the immune system, including a reduction in lymphocytes, and thrombocytopenia (Zhang et al, 1999). The difference between the

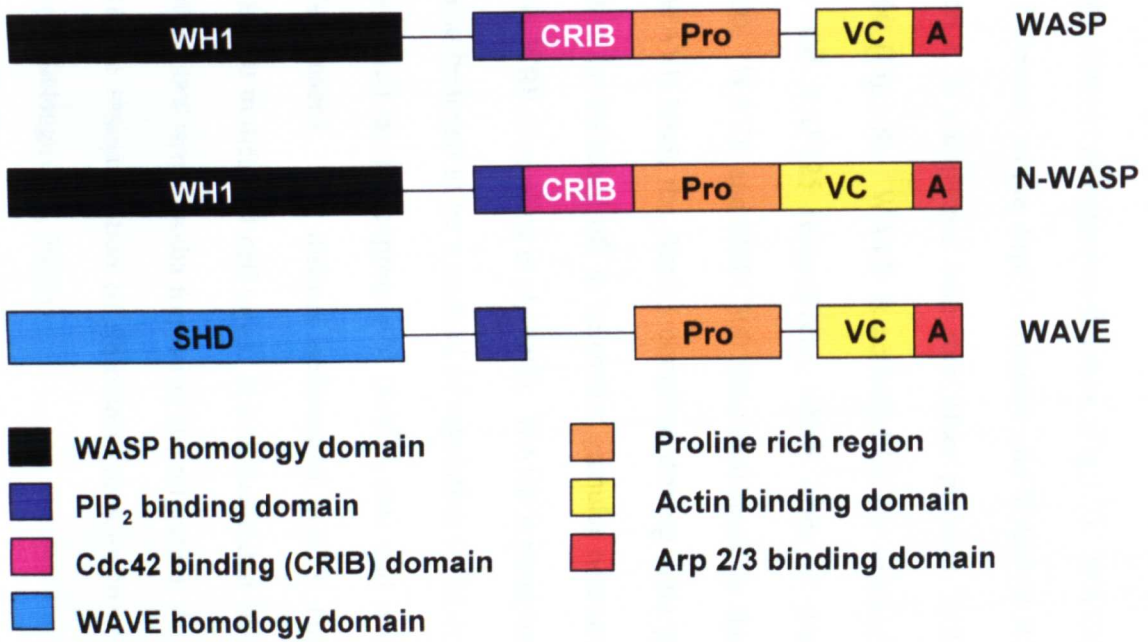

Figure 1.7: Structure of WASP family proteins. WASP family proteins contain an C-terminal VCA domain, which is responsible for Arp2/3 complex and actin binding, followed by a proline rich region. WASP and N-WASP contain a CRIB domain, which is fundamental in their activation via Cdc42. The PIP_2 binding domain potentiates Cdc42 binding. WASP and N-WASP have N-terminal WASP homology domain. WAVE contains a WAVE homology domain of unknown function.

two knockouts is consistent with the ubiquitous expression of N-WASP, in comparison to the haematopoietic-specific expression of WASP (Miki et al, 1996, Derry et al, 1994).

WAVE is a major target for the small GTPase Rac. WAVE is structurally similar to WASP and N-WASP, with a near identical C-terminal actin and Arp2/3 complex binding domain. However, the N-terminus of WAVE has a WAVE homology domain of unknown function (Fig.1.7). WAVE is constitutively active, a crucial difference to the Arp2/3 complex and WASP/N-WASP. To prevent WAVE activation it is complexed with four other proteins, PIR121, Nap125, Abi-2, and HSPC300 (Fig.1.8). WAVE is activated by the binding of GTP-Rac and Nck to PIR121 and NAP125 respectively, which leads to dissociation of WAVE and HSPC300. WAVE and HSPC300 then translocate to the leading edge of the cell, where WAVE binds the Arp2/3 complex, driving actin polymerisation. WAVE is reported to be switched off in cerebellar granular neurons through the activity of a Rac GEF, WRP (Soderling et al, 2003). WAVE is phosphorylated by Abl kinase, and this is also believed to be a method of regulation (Miki et al, 1999), although not in platelets which do not express Abl (SAGE data and western blotting unpublished Mike Tomlinson). The distinct isoforms of WAVE have been proposed to play different roles in different cell types. It is believed that WAVE-2 plays a fundamental role in fibroblast lamellipodia formation and migration. However, WAVE-1 has been implicated in transmigration of fibroblasts through the ECM but not lamellipodia formation (Suetsugu et al, 2002).

WAVE-$1^{-/-}$ mice have been produced by two different groups (Soderling et al, 2003; Dahl et al, 2003). Dahl et al, made WAVE-$1^{-/-}$ mice via gene trapping (Zambrowics et al, 1998). The Scar/WAVE-$1^{-/-}$ mice exhibited increased post-natal

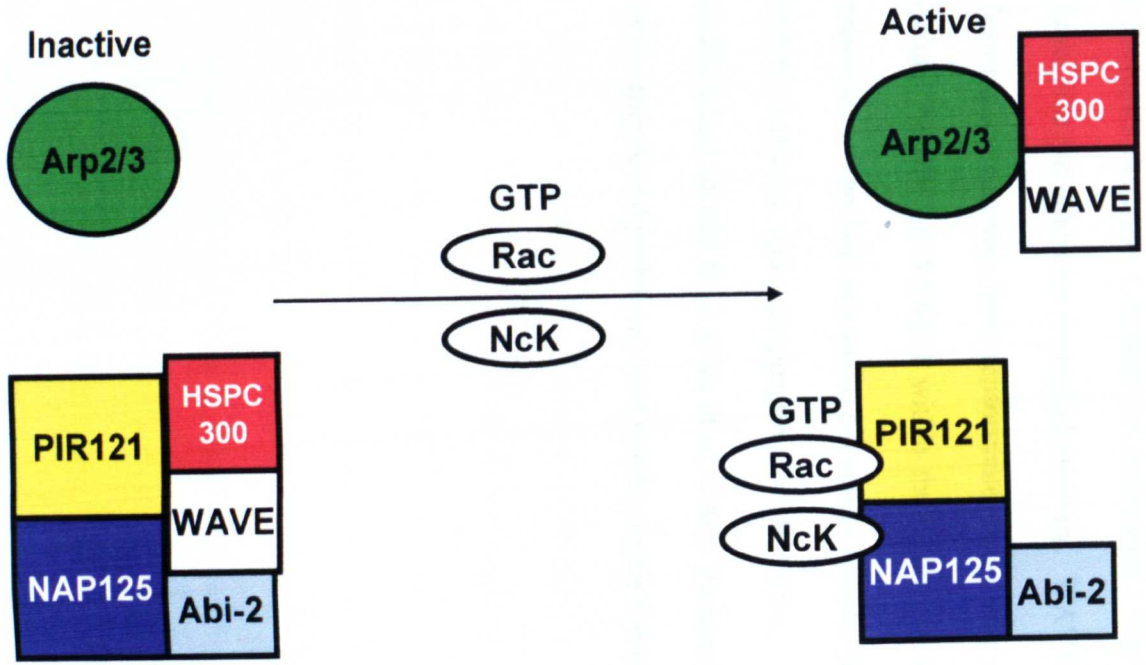

Figure 1.8: Mechanism of control of WAVE activation. WAVE is inactivated by incorporation into a complex of 5 proteins, PIR121, NAP125, HSPC300, and Abi-2. Upon Rac activation, Rac and NcK bind to PIR121, and NAP125 respectively. This causes WAVE and HSPC300 to leave the complex, and bind the Arp2/3 complex. The Arp2/3 complex is then activated leading to actin polymerisation. Redrawn from Millard et al, 2005.

mortality, living for only 3-4 weeks. This differs to Soderling et al, (2003) who produced WAVE-1$^{-/-}$ mice via WAVE-1 targeting vectors in ES cells. They identified a reduction in viability and a reduction in body mass, but by 8 weeks the WAVE-1$^{-/-}$ mice were comparable to wild-type. There was no increase in post-natal mortality. Although there are clear differences in the two studies, probably due to the differing targeting strategies there are a number of similarities. The mice are smaller, have reduced brain size but otherwise normal physiology. WAVE-1$^{-/-}$ mice show reduced anxiety, learning and memory, have sensorimotor retardation and a resting leg tremor. This illustrates the role of WAVE-1 within the central nervous system, correlating with its expression. The lack of a major phenotype outside of the brain indicates that other WAVE isoforms can compensate for WAVE-1. WAVE-2$^{-/-}$ mice are embryonically lethal at day 9-12 (Yan et al, 2003; Yamazaki et al, 2003) due to a number of developmental problems, most notably in the formation of the vasculature.

1.4.4 The regulation of filopodia formation

After stimulation, the actin cytoskeleton is initially reorganised into filopodia. Filopodia are highly dynamic rod-like extensions from the cell surface. They are made up of bundles of parallel actin filaments, which grow at the tip end (Oldenbourg et al, 2000), and are crosslinked with α-actinin, fascin and filamin. Their formation is independent of the Arp2/3 complex (Svitkina et al, 2003) and so they do not undergo branching, retaining their rod-like structure.

Filopodia were initially thought to be regulated downstream of Cdc42 through association of N-WASP or WASP with the Arp2/3 complex (Nobes et al, 1995; Stradal et al, 2004) (Fig 1.9). However, deletion of WASP (Biyasheva et al, 2004) or N-WASP (Lommel et al, 2001, Snapper et al, 2001) does not abolish Cdc42-induced filopodia formation. This is consistent with the presence of filopodia in both human and mouse $WASP^{-/-}$ platelets (Gross et al, 1999). Moreover, a recent study reported filopodia formation in $Cdc42^{-/-}$ fibroblastoid cells (Czuchra et al, 2005). However, it must be noted that another paper disputes this and demonstrates an important role for Cdc42 in filopodia formation in mouse embryo fibroblasts (Yang et al, 2006).

There is increasing evidence from several cell types that filopodia formation is dependent on the formin, mDia2 (Pellegrin et al, 2005; Schirenbeck et al, 2005). mDia2 has been identified at the tips of Cdc42 induced filopodia (review Faix et al, 2006). In addition, it can induce filopodia independently of Cdc42, but dependent on another RhoGTPase, Rif (Pellegrin et al, 2005; Ellis et al, 2000). Formins are prime candidates to drive filopodia extension as they drive linear actin polymerisation independent of the Arp2/3 complex (review Pollard et al, 2003). Allied to this, formins can uncap filaments, causing the presentation of barbed ends ready for polymerisation (Schirenbeck et al, 2005).

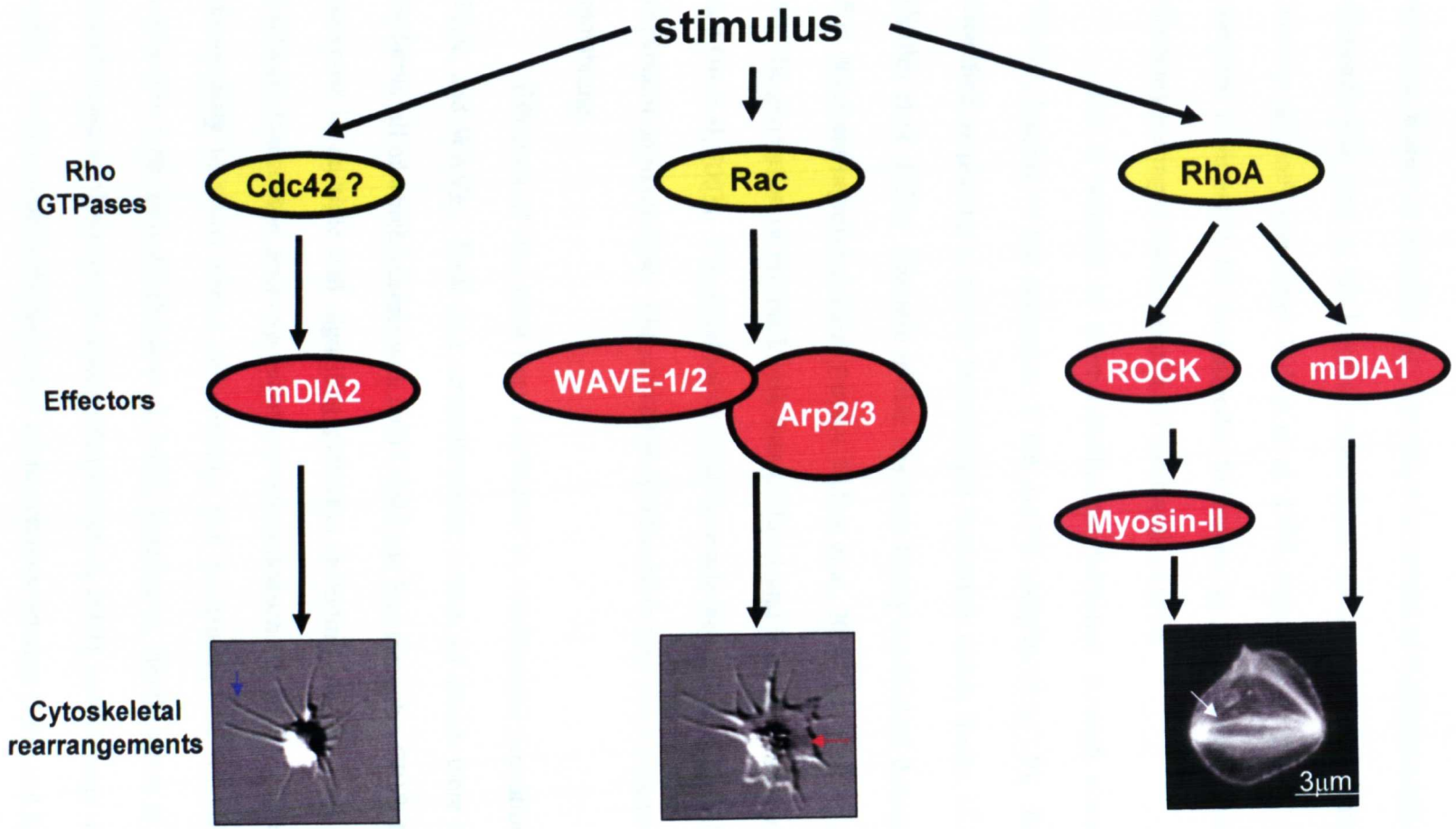

Figure 1.9: Cell signalling associated with actin cytoskeletal rearrangements after activation. After stimulation the cell undergoes three distinct actin rearrangements. The first is filopodia formation (blue arrows), driven by Cdc42, and its effector mDIA2. The second is lamellipodia formation (red arrows) driven by Rac activation, and subsequent activation of the Arp2/3 complex by WAVE. The third rearrangement is stress fibre formation (white arrows) driven by ROCK, and mDIA1.

1.4.5 The regulation of lamellipodia formation

After filopodia formation, the gaps between the filopodia are filled in by a sheet-like structure, known as lamellipodia. Lamellipodia consist of highly branched short actin filaments that form a 3D layer of cross-linked actin and are instrumental in cell motility and cell spreading (Ridley et al, 1995, Nobes et al, 1995). The Arp2/3 complex is essential for lamellipodia formation as it causes branching of actin filaments leading to formation of a 3D meshwork of actin.

Rac is believed to drive lamellipodia formation through association with WAVE, leading to the activation of the Arp2/3 complex (Fig 1.9). Rac was first identified as playing a role in lamellipodia formation within Swiss 3T3 fibroblasts (Ridley et al, 1992). The role of Rac has been firmly established through the use of Rac dominant-negative mutants (review Ridley et al, 2001, and Hartwig et al, 1995), and the generation of murine knock outs of Rac-1 and Rac-2 (Walmsley et al, 2003, and Gu et al, 2003). Three isoforms of Rac have now been identified, with expression dependent on tissue type. Rac activation is believed to take place beneath the plasma membrane.

Effectors of Rac have been implicated in lamellipodia formation, including PAK, and WAVE. PAK is a serine/threonine kinase, of which there are multiple isoforms, all of which interact with both Cdc42 and Rac through a CRIB domain. It is involved in multiple cell signalling pathways including the JNK and p38 kinase pathways that control gene expression (reviewed Bokoch 2003). PAK is implicated in disassembly of stress fibres, cell motility, and in lamellipodia formation through interaction with cortactin (Sells et al, 1997, Vidal et al, 2002), which is involved in lamellipodia stabilisation (reviewed Olazabal et al, 2001), and through activation of cofilin. PAK1 controls the formation of the complex between β-Pix and Rac-1, which

is vital for Rac-1 activation in HEK293 and MDCKII cells (Ten Klooster et al, 2006). Both PAK and β-Pix interact with the adaptor protein Nck, which is important in multiple tyrosine kinase signalling pathways, affecting adhesion and cytoskeletal reorganisation (Bokoch et al, 1996; Lim et al, 2003).

WAVE is vital for the activation of the Arp2/3 complex, as exemplified by the removal of WAVE-2 in both fibroblasts and endothelial cells leading to the abolition of lamellipodia. If WAVE-2 is returned to the cell, lamellipodia can be restored (Yamazaki et al, 2003).

In addition Spin-90, a Nck binding protein, with a SH3 domain, 3 proline rich regions, and a serine/threonine region (Lim et al, 2001 & 2003) is implicated within lamellipodia formation in HeLa cells. Spin-90 forms a complex with β-Pix, WASP and Nck upon adhesion within HeLa cells, and moves to lamellipodia upon cell spreading (Lim et al, 2003; Kim et al, 2006). In addition Spin-90 RNAi prevented lamellipodia formation (Kim et al, 2006). Furthermore, Spin-90 is able to bind actin and the Arp2/3 complex through its C-terminal region, and thereby drive actin polymerisation independent of the WASP family proteins (Kim et al, 2006 & 2007).

1.4.6 The regulation of stress fibre formation

After, or possibly during lamellipodia formation, the actin cytoskeleton undergoes further reorganisation as stress fibres are formed. Stress fibres are similar in arrangement to muscle cell sarcomeres, but the polarity is less regular. They contain the non-muscle counterparts to many of the muscle proteins, such as actin, myosin-II, tropomyosin and troponins. They are crosslinked with α-actinin, and can form dorsal or ventral stress fibres, and transverse arcs (Hotulainen et al, 2006). Ventral stress fibres are contractile assemblies that cross the cell, and are connected at either end to

focal adhesions. They are important in cell adhesion and contraction. Transverse arcs are curved actomyosin bundles not connected to focal adhesions. Dorsal stress fibres are connected to focal adhesion but rise vertically (Small et al, 1998). Stress fibres are important in cell adhesion and the maintenance of cell structure and integrity.

Stress fibre formation is mediated via RhoA activation of its effectors ROCK (Ridley et al, 1992) and the formin, mDIA1 (review Jaffe and Hall, 2005) (Fig.1.9). ROCK is a serine/threonine protein kinase. There are two different forms, ROCK I and II, both ubiquitously expressed within mouse, rat and human tissues (Yoneda et al, 2005). ROCK induces myosin light chain (MLC) phosphorylation, through inhibition of MLC phosphatase, leading to myosin-II activation. Non-muscle myosin-IIs are involved in multiple cellular responses, platelet shape change and stress fibre formation (Canobbio et al, 2005). They are made up of two heavy chains and four light chains, (two regulatory and two essential). Myosin-II interacts with the actin cytoskeleton, leading to contraction and thereby giving the cell structural integrity. At the same time as activating ROCK, RhoA also activates mDIA1 (review Jaffe and Hall, 2005). mDIA1 mediates stress fibre formation by driving actin polymerisation at focal adhesions (Hotulainen et al, 2006). ROCK also indirectly facilitates stress fibre formation via phosphorylation of LIMkinase. LIMKinase in turn phosphorylates cofilin, inhibiting its actin severing activity, thereby increasing actin filament stability.

1.4.7 Specialised actin structures associated with cell spreading

Within a spreading cell a number of additional actin structures have been both identified and characterized. These include podosomes, focal adhesions, dorsal ruffles and peripheral ruffles, yeast actin patches, and invadopodia. Each of these is discussed in detail below.

Podosomes were first identified within monocyte-derived cells such as osteoclasts (Marchisio et al, 1984). Podosomes consist of a dot-shaped F-actin enriched structure, which acts as a matrix contact point (Fig.1.10). Podosomes are usually found in large numbers and make up a rosette structure. They are 1-2μm in diameter, and between 200-400nm in height. Within the central core of a podosome, the actin filaments are surrounded by a ring consisting of vinculin, talin, α-actinin, Src, WASP, the Arp2/3 complex, gelsolin and tyrosine-phosphorylated proteins (Marchisio et al, 1988). The presence of actin polymerisation proteins such as the Arp2/3 complex and WASP differentiate podosomes from focal adhesions (Marchisio et al, 1984, 1988). Podosome formation in monocytes is PKC-dependent (Marchisio et al, 1988, and Giadano et al, 1990). The role of podosomes is little understood. A role in adhesion is proposed due to the similarity with focal adhesions, or due to the dynamic nature of podosomes; they might transfer membrane tension along the substrate, as the lamellipodia extends (review Buccione et al, 2004).

Focal adhesions connect stress fibres to the ECM. Focal adhesions are similar in shape to podosomes. Their structure is maintained by proteins such as FAK, vinculin, cortactin, and integrins. Neither the Arp2/3 complex nor WASP are present within focal adhesions, a defining difference to podosomes, although there is a report which indicates that the Arp2/3 complex can interact with focal adhesions through interactions with vinculin (DeMali et al, 2002). The production of a focal adhesion requires continual protein synthesis, and is thought to be a long process taking over 60min to complete (review Buccione et al, 2004).

Dorsal and peripheral ruffles are associated with lamellipodia formation. Peripheral ruffles occur at the leading edge of lamellipodia, and are thick areas of actin which are highly motile moving in a wave-like manner and are thought to play a

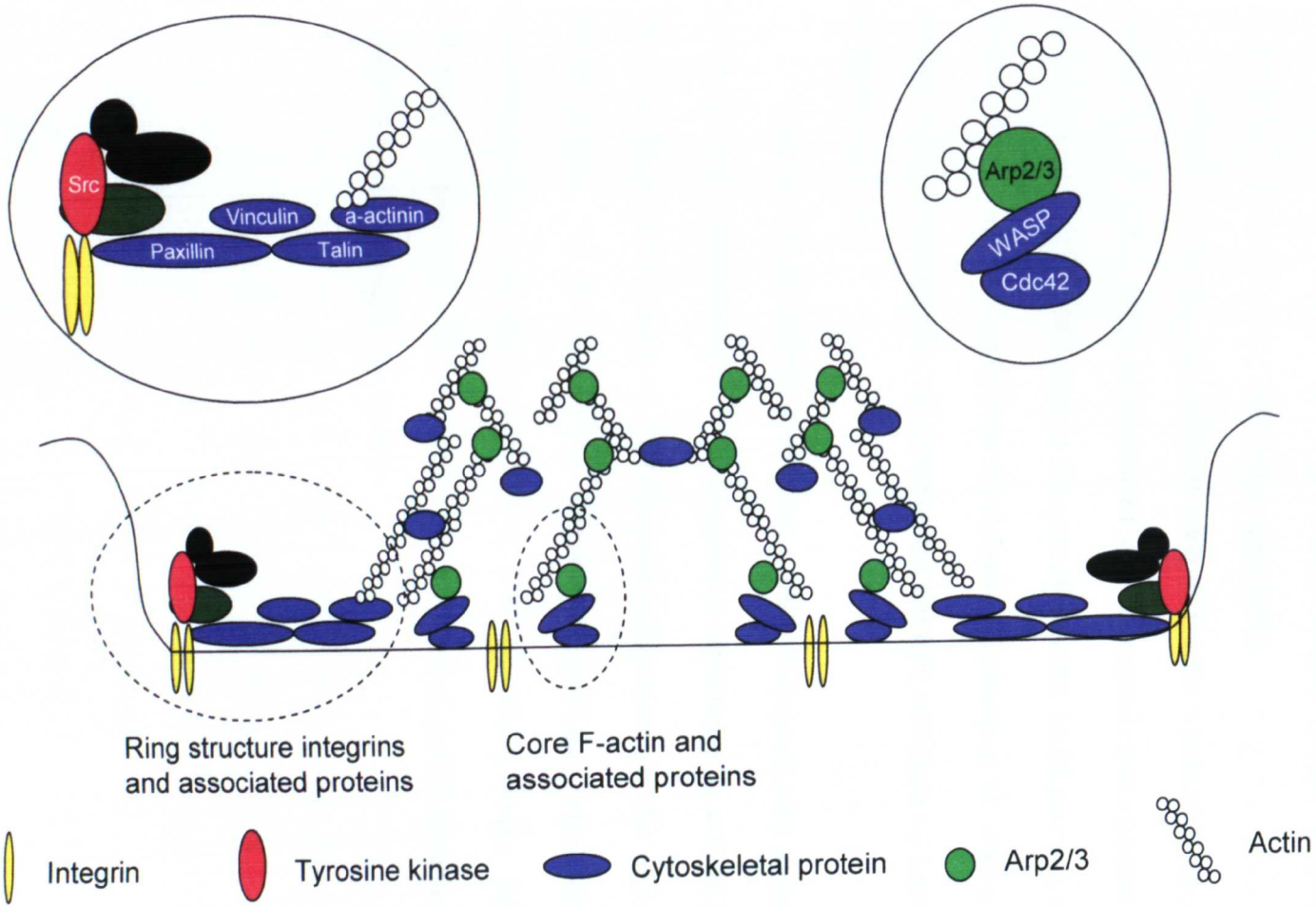

Figure 1.10: Structure of Podosomes. Podosome formation is mediated by multiple actin cytoskeletal proteins, of which the Arp2/3 complex, Src and WASP play fundamental roles. Podosomes are important in degradation of ECM aiding cellular invasion. Figure reproduced from Linder et al, 2005.

role in cell motility (Abercrombie et al, 1970). Dorsal ruffles are dynamic F-actin enriched waves, that occur behind the leading edge of the lamellipodia and extend in an upward direction (Legg et al, 2007). They are shortlived structures, which have a time course of 2-30min, and often form circular waves associated with macropinocytosis (Araki et al, 2000, Legg et al, 2007).

Yeast actin patches are enriched areas of F-actin within budding yeast cells (Adams et al, 1984). They are associated with endocytosis and exocytosis (review Moseley et al, 2006). They have a lifespan of between 10-20 seconds, are highly motile with a range of speed from 0.1-0.5μm/s (Carlsson et al, 2002). Motility requires actin polymerisation, as demonstrated through inhibition by Latrunculin A (Pelham et al, 2001).

Invadopodia are cellular protrusions usually associated with transformed or cancerous cells. Invadopodia are large structures *in vitro*, being on average 8μm wide and 2μm deep (Polishchuk et al, 2000), which approximates to the size of a fully spread platelet. Invadopodia secrete matrix metalloproteases which degrade the underlying matrix, allowing cellular invasion of the underlying matrix. Invadopodia formation is dependent on the activity of a N-WASP-Arp2/3-dynamin-cortactin complex (review Buccione et al, 2004).

1.4.8 Platelet filopodia formation

Within platelets it is not known whether Cdc42 drives filopodia formation. Cdc42 is present within platelets at an estimated cytosolic concentration of 0.21μM and moves to the cytoskeletal fraction upon platelet activation (Azim et al, 2000). It was previously believed that Cdc42 was responsible for filopodia formation through activation of WASP. However, filopodia formation has been reported on immobilised CRP in $WASP^{-/-}$ mouse platelets and human WASP patient platelets demonstrating a distinct pathway of regulation (Gross et al, 1999; Falet et al, 2002).

This is particularly interesting in the context of the recent discovery that the Cdc42 analagous Rho GTPase, Rif, is able to drive filopodia formation independent of Cdc42 (Pelligrin et al, 2005; Ellis et al, 2000) and that there is a high copy number of Rif in a SAGE library made from a highly purified preparation of megakaryocytes (Tomlinson unpublished data). However, at the present time there is no evidence for the role of Rif, or a Cdc42-independent mechanism of filopodia formation within platelets.

1.4.9 Platelet lamellipodia formation

The control of platelet lamellipodia formation is multifactorial. It has been identified that Rac plays a major role within lamellipodia formation, but that signalling molecules such as Src kinases, PLCγ2 and PI 3-kinase are also required for lamellipodia formation, most likely as they play a role within regulation of Rac.

Src kinase inhibition leads to removal of lamellipodia but maintenance of adhesion and filopodia on fibrinogen (Obergfell et al, 2002). This is due to the fundamental role of Src kinases within outside-in signalling of the integrin $\alpha_{IIb}\beta_3$. $PLC\gamma2^{-/-}$ platelets do not form lamellipodia when spread on fibrinogen (Wonerow et

al, 2003). PLCγ2 plays an important role in the regulation of both Ca^{2+} and PKC. Human platelets on fibrinogen treated with the PKC inhibitor Ro31-8220, or the Ca^{2+} inhibitor BAPTA-AM, do not form lamellipodia (Wonerow et al, 2003) although will form filopodia. In addition PKC induced phosphorylation of α-adducin is fundamental in the dissociation of adducin from the cytoskeleton, and the formation of new barbed ends ready for actin polymerisation, and lamellipodia formation (Barkalow et al, 2003). PI 3-kinase also plays an important role in lamellipodia formation. PI 3-kinase is involved in both $\alpha_{IIb}\beta_3$ and GPVI signalling. PI 3-kinase inhibition with either Ly294002, or wortmanin leads to inhibition of lamellipodia in human platelets spread on CRP and fibrinogen (Falet et al, 2000; Senis et al, 2005). The requirement for PI 3-kinase is likely to be due to the requirement for PIP_3 to recruit PLCγ2 to the membrane, and induce PKC activation and Ca^{2+} mobilization.

Rac activation is important for platelet lamellipodia formation. Platelets express only Rac-1, possibly -2, and do not express Rac-3. The presence of Rac-2 is controversial, but studies on Rac-$2^{-/-}$ platelets indicate that, even if it is present it does not play a functional role within platelet function. Rac is activated by multiple platelet agonists including TRAP (Azim et al, 2000), thrombin (Hartwig et al, 1995, Soulet et al, 2001), collagen (Soulet et al, 2001), ADP (Soulet et al, 2005), U46619 (Gratacap et al, 2001) and fibrinogen (Pearce et al, 2007). Rac-1 is responsible for lamellipodia formation upon platelet spreading on fibrinogen, laminin and collagen, with Rac-2 having little role or no role within platelets as demonstrated using mutant mouse platelets (McCarty et al, 2005). Rac is thought to play an important role alongside that of gelsolin in driving lamellipodia formation. Gelsolin drives the generation of new barbed ends through severing, whilst Rac activates the Arp2/3 complex driving actin polymerisation (Falet et al, 2002).

Rac targets play a role within platelet lamellipodia formation. PAK is required for lamellipodia formation induced by thrombin and to activate cortactin, a protein implicated in lamellipodia stabilisation (Vidal et al, 2002). Cofilin has been shown to play a role in actin reorganisation downstream of $\alpha_{IIb}\beta_3$ (Falet et al, 2005). Platelets express WAVE-1 and -2, and possibly WAVE-3 (Kashiwagi et al, 2005, Oda et al, 2005, Chapter 5).

1.4.10 Platelet Stress fibre formation

Platelet stress fibre formation is driven via the RhoA/ROCK pathway (Fig.1.11), and myosin-II activation, through MLC phosphorylation. RhoA is activated by multiple adhesive surfaces and by GPCR coupled receptors downstream of the $G_{12/13}$ pathway. Stress fibre formation on fibrinogen and collagen is inhibited by treatment with the ROCK inhibitors, C3 exoenzyme or Y27632 (Leng et al, 1998, Suzuki-Inoue et al, 2001). In addition to stress fibre inhibition, focal adhesion formation is also inhibited through the use of C3 exoenzyme (Leng et al, 1998).

Myosin-IIa is thought to be the major isoform present in platelets (Maupin et al, 1994). The role of myosin-IIa is demonstrated by the *MYH9* group of disorders, which include May-Hegglin, Fecthner, Epstein, and Sebastian syndromes. These syndromes are associated with thrombocytopenia and formation of large platelets (Seri et al, 2003). Upon platelet activation, the platelets show defective shape change, and spreading, though normal aggregation (Cannobio et al, 2005). Myosin-II also plays an important role within clot retraction (Suzuki-Inoue et al, 2007).

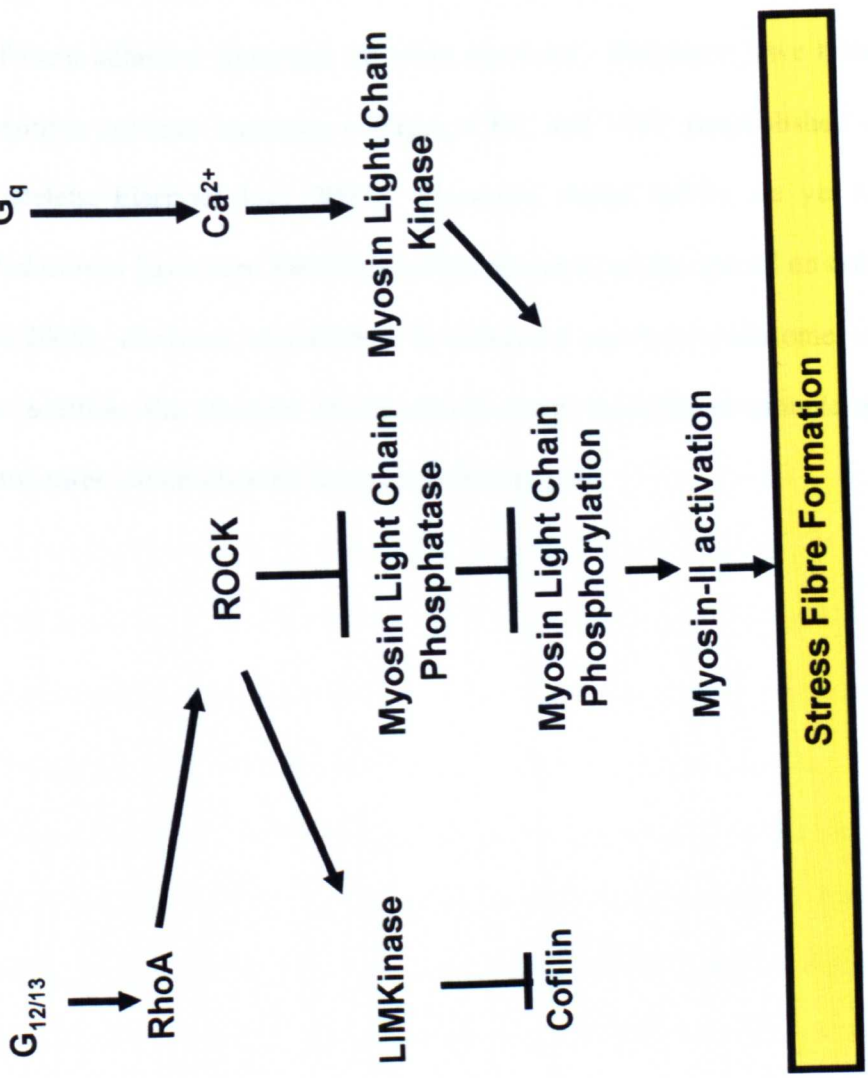

Figure 1.11: Cell signalling associated with stress fibre formation via $G_{12/13}$, and G_q. Upon platelet activation, stress fibres form after activation of either $G_{12/13}$ or G_q pathways. ROCK activation leads directly to inhibition of MLC phosphatase, increasing MLC phosphorylation, activating myosin-II and stress fibre formation. Simultaneously ROCK activates LIMKinase, inactivating cofilin, increasing actin filament stability. Gq activation increases MLC kinase activity increasing MLC phosphorylation, leading to stress fibre formation.

1.4.11 Other actin structures within platelets

Within platelets only a few other cytoskeletal structures other than filopodia, lamellipodia, and stress fibres have been clearly identified. Focal adhesions were identified by Leng et al, (1998) in 48% of spread platelets on fibrinogen, although interestingly only 2 or more focal adhesions were identified per platelet, and whether they are focal adhesions remains in doubt. However, there have been no other reports of focal adhesion formation on other matricies. Peripheral have been identified on multiple surfaces including collagen, CRP, and VWF (unpublished oberservations; Platelets, Elservier Inc., 2007). However, dorsal ruffles are yet to be reported. Podosomes have been identified within megakaryocytes spread on collagen (Sabri et al, 2006). However, to date there have been no reports of podosomes within platelets. In addition, the presence of invadopodia, and endocytic or granule associated actin structures within platelets have yet to be reported.

1.5 THE ROLE OF THE ACTIN CYTOSKELETON IN THROMBUS GROWTH

At the present time the role of the actin cytoskeleton within thrombus formation is unclear. This is because it has multiple roles within platelet activation, including shape change, clot retraction and GPVI signalling (Wilde et al, 2000; Wonerow et al, 2002; Suzuki-Inoue et al, 2007). However, it is possible to investigate the role of individual actin structures such as lamellipodia, with the use of mutant mice and specific inhibitors.

The role of actin polymerisation can be investigated using the inhibitors cytochalasin A-E or Latrunculin A. Cytochalasins prevent actin polymerisation by capping barbed ends inhibiting actin association and dissociation (Godette et al, 1986). Latrunculin A binds G-actin causing alterations within its structure preventing its incorporation into the actin cytoskeleton (Morton et al, 2000). These reagents partially inhibit signalling by glycoprotein receptors, including GPVI and integrin $\alpha_{IIb}\beta_3$, leading to a partial decrease in platelet aggregation to all agonists. The role of the cytoskeleton in secretion is unclear. There are conflicting reports with thrombin, calcium ionophore, ADP, and collagen, of having no effect, inhibiting, or augmenting secretion (Haslam et al, 1977; Cox et al, 1988; Hashimoto et al, 1986; Yatomi et al, 1986). However, recently Flaumenhaft et al, (2005), identified that dense and alpha granule secretion to the thrombin peptide (SFLLRN) was affected depending on the concentration of actin inhibitor used. Low concentrations of either Cytochalasin E or Latrunculin A accelerated alpha granule release, but did not affect dense granule release. However at higher concentrations alpha granule secretion was inhibited, but dense granule release was accelerated.

Inhibition of actin polymerisation does prevent platelet spreading but not platelet adhesion. At high shear both adhesion and initial thrombus formation is maintained, but the thrombi formed are highly unstable, and undergo embolisation, decreasing the overall height of the thrombi. This implicates the actin cytoskeleton in maintenance of thrombus stability.

The role of filopodia in thrombus assembly is unclear. A recent study has reported that inhibition of Cdc42, by secramine A leads to an inhibition of filopodia formation and aggregation to collagen (Pula et al, 2006). It is unclear however why secramine A, blocks both shape change and actin polymerisation, as similar effects are not seen with cytochalasin D. This indicates that secramine A maybe having additional effects. Studies on $Cdc42^{-/-}$ mice are required to address this.

The role of lamellipodia can be selectively investigated in $Rac\text{-}1^{-/-}$ mice. $Rac\text{-}1^{-/-}$ platelets exhibit normal aggregation and secretion to G protein receptor agonists, but have a mild impairment in signalling by the collagen receptor GPVI (McCarty et al, 2005). They fail to form lamellipodia on matrix proteins leading to the generation of thickened and elongated filopodia. The process of platelet adhesion and aggregation can still be observed on collagen at high shear, but is associated with increased embolisation and a decrease in overall thrombus growth (McCarty et al, 2005). Although, it is difficult to determine whether the mechanism behind the thrombus defect is due to impairment of GPVI signalling or lamellipodia formation, it is striking that a similar defect in aggregate assembly is not seen in mutant mice that cause a similar level of inhibition of GPVI signalling, and that aggregate formation is also impaired under flow conditions on a surface of thrombin and VWF (McCarty et al, 2005). Therefore, lamellipodia formation appears to be important for aggregate stability under flow conditions, rather than initial platelet adhesion and this

presumably contributes to the marked defect in thrombus formation observed in Rac-$1^{-/-}$ mice in vivo. In comparison, PLC$\gamma2^{-/-}$ platelets show a marked reduction in adhesion at high shear, and do not form thrombi, a clear difference to that seen with the Rac-$1^{-/-}$, with the difference being explained by loss of inside-out regulation of $\alpha_{IIb}\beta_3$ by GPVI (Suzuki-Inoue et al, 2003).

Several studies have investigated the role of stress fibre formation in adhesion and aggregate formation. Stress fibre formation mediated via the RhoA/ROCK pathway is activated by multiple adhesive surfaces and GPCR agonists in platelets (Leng et al, 1998; Suzuki-Inoue et al, 2001; Schoenwaelder et al, 2002; Jirouskova et al, 2007). RhoA or ROCK inhibition, using C3 exoenzyme and Y27632 respectively, has no effect on platelet aggregation to thrombin, collagen and ADP (Leng et al, 1998, Schoenwaelder et al, 2002) although a reduction in secondary aggregation to adrenaline has been reported (Leng et al, 1998). Similarly, RhoA or ROCK inhibition has a negligible or minor inhibitory effect on static adhesion to fibrinogen, VWF and collagen, or platelet capture by VWF under flow conditions (Leng et al, 1998; Suzuki-Inoue et al, 2001; Schoenwaelder et al, 2002; McCarty et al, 2006; Jirouskova et al, 2007). RhoA and ROCK have, however, been shown to be important in platelet tethering to VWF (Schoenwaelder et al, 2002). ROCK inhibition reduced shear dependent platelet adhesion on VWF and shear induced aggregation in suspension. However the role of RhoA/ROCK in high shear conditions on collagen has yet to be reported.

Inhibition of stress fibres with Y27632 or the myosin-II inhibitor, blebbistatin, inhibits clot retraction (Suzuki-Inoue et al, 2007). Clot retraction is important in consolidation of thrombus formation, restriction of thrombus size, and the production of an insoluble mesh to aid infiltration of other cell types into the damaged area.

1.6 Aims.

My goal was to investigate glycoprotein receptors regulation of morphological rearrangments associated with spreading and thrombus formation. This is important as this work could lead to the formation of new therapies to combat thrombosis and increase our understanding of bleeding disorders. This work can be broken down into the following aims:

1. Identification of key actin cytoskeletal rearrangements that occur when platelets spread and form thrombi.
2. Determination of the role of WAVE-1 in platelet adhesion and aggregate formation under static and flow conditions.
3. Determination of the role of RhoA and its target ROCK in stress fibre formation during platelet adhesion and aggregate formation under static and flow conditions.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 General materials: All general laboratory chemicals were obtained from Sigma-Aldrich (Dorset, UK). Glass capillary tubes were obtained from Camlab (Cambridge,UK). Chronolum reagent was obtained from Chronolog Corporation (Haverton, USA). Ketamine was obtained from Amersham Biosciences (Little Chalfont, UK) and Xylazine from Millpledge Pharmaceuticals (Scarborough, UK).

2.1.2 Blood anticoagulants: Sodium citrate (4%) was obtained from Sigma-Aldrich (Dorset, UK), P-PACK was purchased from Merck Biosciences (Nottingham, UK), and Heparin was obtained from CP Pharmaceuticals (Wrexham, UK).

2.1.3 Inhibitors: Y27632, blebbistatin, Latrunculin A, Ly294002 and AEBSF were obtained from Merck Biosciences (Nottingham, UK). Integrillin was obtained from GlaxoSmithKline (Durham, UK). Cytochalasin D, apyrase, indomethacin, leupeptin, aprotinin, pepstatin A, and BAPTA-AM were obtained from Sigma-Aldrich (Dorset, UK). Prostacyclin (PGI_2) was obtained from Alexis Biochemicals (Nottingham, UK). PD0173952 was a gift from Pfizer Global Research and Development (Ann Arbor, MI, USA and Sandwich, UK).

2.1.4 Platelet agonists: Thrombin, laminin, thrombin related activated peptide (TRAP) and ristocetin were obtained from Sigma-Aldrich (Dorset, UK). Fibrinogen and fibronectin were obtained from Enzyme Research (Swansea, UK). Fibrillar type 1 Horm collagen from equine tendon was obtained from Nycomed (Oxford, UK). VWF purified from human plasma was a gift from Dr MC Berndt (Monash

University, Melbourne, Australia. CRP was synthesised by Tana Laboratories (Houston, USA), and by Dr R Farndale (Cambridge, UK).

2.1.5 Fluorescent probes: FITC-phalloidin and rhodamine-phalloidin were obtained from Sigma-Aldrich (Dorset, UK). $DiOC_6$ and Alexa fluor 488 goat anti-rat IgG were obtained from Invitrogen (Paisley, UK). Purified anti-mouse CD41 was obtained from BD Pharminogen (Oxford, UK).

2.1.6 Fixative agents: Paraformaldehyde and glutaraldehyde were obtained from Sigma-Aldrich (Dorset, UK). Polyetheleneglycol (MW 40,000kDa) was obtained from Universal Biologicals (Cambridge, UK).

2.1.7 Mice: WAVE-$1^{-/-}$ mice were a kind gift of Seung Kwak at Wyeth Research, Princeton, NJ, USA. Mice were bred as heterozygotes and all experiments were performed on mice aged 14-20 days of age using litter-matched controls. The mice used at this age, as the absence of WAVE-1 leads to death at an early stage. Knockout mice were provisionally identified by the small size of the mice, and subsequently genotyped.

GFP-actin mice were a kind gift from Professor Andrew Matus at Friedrich Miescher Institute, Basel, Switzerland. Mice were bred as heterozygotes and all experiments were performed using mice at least 6 weeks old, using B57Bl6 wild-type controls (Charles River, Germany). Mice were genotyped at 1-3 days using uv light goggles by animal technicians within the BMSU.

2.1.8 Antibodies:

Antibody target	Details	Source	Experimental Concentrations*
α–$\alpha_{IIb}\beta_3$	Rabbit	Santa Cruz	IM:1/200
α-Arp2/3	Rabbit	Upstate Technology	IM:1/1000
α-β-Pix	Rabbit	Chemicon	WB:1/1000 IM:1/1000 IP:1/500
α-β_1 integrin	Mouse	Chemicon	IM:1/200
α-CD63	Human	Pharminogen	IM:1/100
α-Cortactin	Mouse	Upstate Technology	IM:1/600
α-Csk	Rabbit	Santa Cruz	IM:1/100
α-Fyn	Rabbit	Mike Tomlinson	IM:1/200
JonA	Rat	Emfret	FC:1/100
α-Myosin IIa	Rabbit	Covance	IM:1/1000
α-Nck	Rabbit	Chemicon	WB:1/1000 IM:1/5000 IP:1/1000
α-P-selectin	Rat	Pharminogen	IM:1/100 FC:1/100
α-tyrosine phosphotyrosine	Mouse (4G10)	Upstate Technology	WB:1/1000 IM:1/1000
α-mouse IgG	Rabbit, PE linked	Dako	IM:1/200
α-mouse IgG	Mouse	Sigma	IM:1/100
α-mouse IgG	Sheep, HRP-conjugated	Amersham	WB:1/10000
α-MLC	Rabbit	New England Biolabs	WB:1/1000
α-MLC phosphorylation	Rabbit	New England Biolabs	WB:1/1000
α-rabbit IgG	Alexa 660 conjugated	Molecular Probes	IM:1/100
α-rabbit IgG	Donkey, HRP conjugated	Amersham Pharmacia Biotech	WB:1/10000
α-Rac	Mouse	Upstate Technology	IM:1/200
α-WAVE-1	Mouse	Upstate Technology	WB:1/50
α-WAVE-2	Mouse	Upstate Technology	WB:1/50
α-Spin90	Mouse	Woo Keun Song	IM:1/1000 WB:1/1000 IP:1/1000
α-Src	Mouse	Upstate Technology	IM:1/100
α-Talin	Mouse	Sigma	IM:1/50
α-Vinculin	Mouse	Sigma	IM:1/100
α-Wasp	Rabbit	Upstate Technology	IM:1/100

***Key: IM:immunofluoresence, WB: western blotting, IP: immunoprecipitation, FC: flow cytometry**

2.2 METHODS

2.2.1 Genotyping: WAVE mouse genotyping was performed as described by Legg et al, (2007), and was performed by Hannah Morris, within Professor Laura Machesky`s lab.

2.2.2 Platelet preparation: Human venous blood was drawn by venipuncture into 4% sodium citrate. If plasma rich plasma (PRP) was to be used whole blood was centrifuged twice at 200g for 10min, and PRP removed. Platelet poor plasma was then prepared by centrifugation of whole blood at 1000g for 10min and the supernatant was removed. Platelets were left for 30min to rest before experimentation.

If washed platelets were to be prepared Acid/citrate/dextrose (ACD: 85mM sodium citrate, 67mM glucose, 42mM citric acid) was added to the blood to 10% of the blood volume. PRP was then prepared by centrifugation of whole blood at 200g for 20min. The platelets were isolated from PRP by centrifugation at 1000g for 10min in the presence of 0.1μg/ml prostacyclin (PGI_2) (Vargas et al, 1982). The pellet was gently resuspended in 25mls of modified HEPES-Tyrodes buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$; pH 7.3) and 3mls of ACD and 0.1μg/ml PGI_2 added. The platelets were then centrifuged at 1000g for 10min, before being resuspended in HEPES-Tyrodes buffer. 5μl platelets added to 10ml of isotonic fluid and the platelet number analysed using a Beckman Coulter counter. Platelet counts were taken twice, and averaged to obtain the platelet number. Platelets were left for 30min to rest before experimentation.

2.2.3 Mouse platelet preparation: Blood was withdrawn from anaesthetised, and terminally CO_2-narcosed mice via cardiac puncture into 10% ACD or 10unit heparin. PRP was prepared by centrifugation at 200g for 6min. PRP was removed and a further 200µl of HEPES-tyrodes was added to the remaining mixture to facilitate the maximisation of platelet recovery. Blood was recentrifuged at 200g for 6min and the PRP combined with that from the first centrifugation. For washed platelets, 0.1µg/ml PGI_2 was added to the PRP, and the PRP centrifuged at 1000g for 6min. Platelets were resuspended in 100µl modified HEPES-Tyrodes buffer. Platelet number was then counted as per human platelet samples. Platelets were resuspended to $5x10^8$/ml before being left to rest for 30min prior to experimentation (McCarty et al, 2005, Pearce et al, 2002, 2004, 2007).

2.2.4 Determination of haematological parameters of murine whole blood: A volume of 50µl of whole blood was taken up the ABX micros 60 (ABX Diagnostics, Montpelier, France). Levels of red blood cells, leucocytes, platelets, and platelet mean volume were measured.

2.2.5 Platelet aggregation and secretion: All experiments were performed in a Born-lumiaggregometer (Chronolog, Haverton, USA). A quantity of 300µl platelets ($2x10^8$/ml) was incubated at 37°C with the appropriate inhibitor for 60s. Chronolum reagent (20µl) was then added and platelets stirred for 60s at 1200rpm before addition of the appropriate agonist. Aggregations were allowed to proceed for 3min. The addition of an ATP standard (2nM) was used to measure ATP secretion. If shape was to be monitored, aggregation was blocked by the addition of the $\alpha_{IIb}\beta_3$ inhibitor, integrillin (9µM), which was added 2min prior to stimulation.

2.2.6 Monitoring of aggregation via single platelet counting: Prior to platelet aggregation a platelet count was taken as per section 2.2.2. A volume of 50µl of ($1.2x10^8$/ml) washed platelets were incubated at 37°C for 60s, and then for a further 60s whilst stirring, prior to aggregation to either 1µg/ml or 10µg/ml CRP, 0.02unit/ml or 0.1unit/ml thrombin, and 1µg/ml or 10µg/ml collagen. 2min post stimulation a platelet count was taken as per section 2.2.2. Analysis of pre/post counts identifies the percentage of platelet aggregation.

2.2.7 Immunoblotting: A volume of 300µl platelets ($1x10^9$/ml) were incubated with integrillin (9µM), and either DMSO (0.1%), Y27632 (3-50µM) or blebbistatin (100µM) for 2min at 37°C before stimulation with collagen (1-30µg/ml) for 1-15min. Platelets were lysed with a volume of 75µl 5xLaemmli sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 5% stacking buffer, and brilliant blue R) and boiled for 10min before centrifugation. Proteins were separated by SDS-PAGE (Bis-Tris 4-12%) at 180V for 50min. Samples were transferred to a PVDF membrane, 110mA for 35min, blocked with 5% BSA + 0.1% Sodium Azide for 60min at room temperature, and western blotted for MLC (1:1000) or phosphorylated MLC (1:1000), diluted in 5% BSA+0.1% Sodium Azide, at 4°C overnight. Membranes were washed in high salt (363mM NaCl) in TBS-Tween (0.1%) three times for 20min, before incubation with anti-rabbit secondary antibody (1:10000) in TBS-Tween (10ml, 0.1%) for 60min at room temperature. Blots were analysed using ECL western blotting kit and densitometry via Quantity One software (Bio-Rad, Hemel Hempstead, UK).

For immunoprecipitation samples, samples were run and transferred as above, but were blotted for SPIN-90, 4G10, β-Pix, or Nck overnight at 4°C, before washing,

and incubation with the appropriate secondary antibody. Blots were analysed using the ECL western blotting kit as above.

2.2.8 Immunoprecipitation: A volume of 500µl of platelets ($5x10^8$/ml) was incubated with integrillin (9µM) at 37°C for 2min, before stimulation with 30µg/ml collagen, 1unit/ml thrombin, and 0.3-10µg/ml CRP for 1-5min. 1-5min after stimulation 500µl of ice-cold 2x lysis buffer (300mM NaCl, 20mM Trizma base, 2mM EGTA, 2mM EDTA, 1mM AEBSF, 10µg/ml aprotinin, 10µg/ml Leupeptin, 0.7µg/ml pepstatin A, 2mM sodium orthovanadate, 2% NP-40, pH 7.3), 15µl of both PGS and PAS (50% w/v), and 1µl of mouse IgG was added to preclear samples. Samples were rotated for 60min at 4°C. Samples were pelleted by microcentrifugation, and the supernatent was removed. 100µl of the supernatent was added to 25µl of 5xLaemlli loading buffer and each sample was run as a whole cell lysate to verify stimulation. 1-3µl of the appropriate antibody was added to the remaining supernatent and rotated for 15min, before addition of 30µl of PGS or PAS depending on the antibody used (PAS for rabbit, PGS for mouse) and samples were rotated overnight at 4°C. Protein samples were pelleted by microcentrifugation and washed with 1ml 1xlysis buffer three times, before addition of 40µl of 2xLaemlli buffer. Protein samples were boiled for 10min, before microcentrifugation for 5min at 13,000rpm. Protein samples were run and transferred as per section 2.2.7.

2.2.9 Platelet morphology studies: Coverslips were coated with 100µg/ml fibrinogen, 100µg/ml collagen, 10µg/ml VWF+2µg/ml botrocetin, 10µg/ml VWF+2µg/ml ristocetin, 50µg/ml laminin, or 100µg/ml fibronectin overnight at 4°C.

BSA (5mg/ml) was boiled for 10min before being placed on ice, and filtered with a 0.45μm filter (Anachem, Luton, UK). Coverslips were washed twice with PBS, and were incubated with 300μl BSA (5mg/ml) for 60min at room temperature. Coverslips were washed twice with PBS prior to addition of platelets. All experiments were completed in the presence of apyrase (2unit/ml), and indomethacin (10μM). A volume of 200μl platelets ($2x10^7$/ml) were incubated for 2min with either DMSO (0.1%), Y27632 (50μM), blebbistatin (100μM), PD0173952 (20μM), Ly294022 (25μM), BAPTA-AM (40μM), or Latrunculin A (3μM) depending on the experiment (Wonerow et al, 2003, Senis et al, 2005, unpublished observations). After incubation platelets were then allowed to spread on the specific matrix for 45min at 37°C. For a separate set of experiments a volume of 200μl platelets ($2x10^7$/ml) were spread for 22.5min before addition of either DMSO (0.1%), PD0173952 (20μM), Ly294022 (25μM), BAPTA-AM (40μM), or Latrunculin A (3μM) depending on the experiment. Platelets were then allowed to spread for a further 22.5min. For both sets of experiments after 45min spreading, platelets were washed twice with PBS, and then fixed with a volume of 200μl paraformaldehyde (3.7%) for 10min at room temperature. Differential interference contrast (DIC) experiments required mounting with Hydromount (Fischer, Dublin, Ireland) and platelets were imaged using Köhler illuminated Nomarski DIC optics.

For immunohistochemistry samples, platelets were fixed as above, and then lysed with a volume of 200μl Triton X-100 (0.2%) for 5min at room temperature. Platelets were then washed twice with PBS. Platelets were incubated with FITC-phalloidin (2μM) or rhodamine phalloidin (2μM) and the appropriate antibody for 60min in the dark at room temperature. Platelets were washed twice with PBS, before

addition of 200µl of the appropriate secondary antibody and platelets incubated for 60min in the dark. Platelets were then mounted with Hydromount, and imaged using both fluorescent imaging with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope or DM IRE2 confocal microscope (Leica, Milton Keynes, UK), and DIC microscopy as above.

2.2.10 Realtime platelet morphological studies: All experiments were done in the presence of apyrase (2unit/ml) and indomethacin (10µM). A volume of 500µl platelets ($2x10^7$/ml) was incubated with the appropriate inhibitors for 2min before platelets were allowed to spread on the appropriate matrix at 37°C for 30min. For the appropriate experiments, platelets were allowed to spread for 15min before addition of either PBS or 0.03unit/ml thrombin. Platelets were allowed to spread for a further 15min. Platelet spreading was monitored using either Köhler illuminated Nomarski DIC optics, or with Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope, with an image taken every 5s.

2.2.11 Platelet spreading analysis: Surface area analysis was completed with a java plugin for ImageJ (NIH, USA). Platelets were individually outlined, and surface area quantified by determining the number of pixels within the outlined area. Imaging a graticule under the same conditions allowed the conversion of pixels to microns. For platelet adhesion analysis, the number of platelets per image were counted, and at least three images per slide were analysed. The adhesion results are presented as a percentage of the control.

2.2.12 F-actin assay: Filamentous actin was measured using a modified method from Machesky et al, (1997). A volume of 500µl platelets ($5x10^8$/ml) was incubated with apyrase (2unit/ml), indomethacin (10µM), and either DMSO (0.1%), Y27632 (50µM), or blebbistatin (100µM) for 2min, before being allowed to spread on collagen coated coverslips for 45min at 37°C. Collagen coated coverslips were prepared as per Section 2.2.9. A volume of 200µl was removed from the top platelet solution for each sample as a basal, and was fixed with 200µl of fixation buffer (20µM KH_2PO_4, 10µM pipes, 5µM EGTA, 2µM $MgCl_2$, 2% Triton X-100, 2µM FITC-phalloidin, 3.7% formaldehyde). Coverslips were washed twice with PBS before addition of 500µl of fixation buffer. Coverslips were placed on ice, and scraped three times to ensure removal of all platelets, and the sample was then rotated for 60min in the dark. Both basal and adhered platelet samples were pelleted by microcentrifugation for 2min, washed with 200µl saponin washing buffer (20µM KH_2PO_4, 10µM Pipes, 5µM EGTA, 2µM $MgCl_2$, 2% Triton X-100, 0.1% Saponin v/v), and were re-pelleted by microcentrifugation for 2min. FITC-phalloidin was extracted with 200µl methanol for at least 60min at room temperature under rotation, and measured at 520nm (emission wavelength), using 488nm (excitation wavelength) on a QuantaMaster spectrofluorimeter (Photon Technology International, West Sussex, UK). Alongside the F-actin assay, a second set of collagen coverslips was prepared, and platelets were allowed to spread as above. A basal sample was removed as above, and the spread platelets were lysed with ice cold 2xlysis buffer (300mM NaCl, 20mM Trizma base, 2mM EGTA, 2mM EDTA, 1mM AEBSF, 10µg/ml aprotinin, 10µg/ml Leupeptin, 0.7µg/ml pepstatin A, 2mM sodium orthovanadate, 2% NP-40, pH7.3). Protein levels of these samples were measured with Biorad Bradford assay kit to enable normalisation of F-actin results.

2.2.13 Flow Cytometry: Washed platelets ($2x10^7$/ml) in the presence of 2mM Ca^{2+}, were treated with 0.001-0.1unit/ml thrombin, 0.3-30μg/ml collagen, or 0.3-30μg/ml CRP in the presence of a FITC-conjugated anti-CD62P mAb (10μg/ml) and rhodamine-conjugated JonA for 15min at 37°C. In selected experiments, fluorescently labelled isotype-matched IgG mAbs were included for background fluorescence determination. Samples were diluted with PBS, and were analysed by flow cytometry on a Beckman Dickinson FACScalibur using Cell Quest software (Becton Dickinson, San Jose, CA). Platelets were identified by logarithmic signal amplification for forward and side scatter, and the geometric mean fluorescence of each specimen was recorded.

2.2.14 Flow adhesion studies: Glass capillary tubes were coated with 100μg/ml collagen and rotated for 60min at room temperature. BSA 5mg/ml was prepared as per Section 2.2.9. Capillary tubes were washed, blocked with BSA 5mg/ml, and rotated for 60min at room temperature. Washed platelets were prepared as per Section 2.2.1. Red blood cells were isolated by washing with HEPES-buffered saline (5mM Glucose, 140mM NaCl, 10mM HEPES) and three centrifugations at 2000g for 10min to remove plasma proteins. A volume of 150μl or 450μl washed platelets ($1x10^8$/ml) was incubated for 10min at 37°C with DMSO (0.1%), Y27632 (50μM) or blebbistatin (100μM). For real time analysis, both platelets and red blood cells were incubated with the fluorescent dye, $DiOC_6$ (2μM). Washed platelets and red blood cells were then reconstituted to a haematocrit of 50% and were flowed over collagen for 3min at $1000s^{-1}$ or at $3000s^{-1}$. Real-time image sequences were recorded using a CoolSnap Camera (Photometrics, Huntington Beach, CA, USA) on a DM IRB

microscope (Leica, Milton Keynes, UK) (McCarty et al, 2005; Auger et al, 2005). Platelet thrombi were washed with buffer for 5min, and fixed for 30min at room temperature with paraformaldehyde (3.7%). Platelets were then stained overnight with $DiOC_6$ (2μM) at room temperature. For determination of thrombus height, 0.5μm confocal slices of platelet thrombi were analysed. For flow analysis with murine whole blood, mouse blood was drawn into heparin (10IU/ml) and PPACK (40μM). Capillaries prepared as per Section 2.2.9. Anticoagulated whole blood was perfused for 4min at a wall shear rate of $1000s^{-1}$, followed by washing with modified Tyrode's for 3min, before imaging with phase-contrast microscopy. Image analysis was performed off-line using ImageJ. Platelet adhesion results are expressed as the percentage of surface area covered by platelets.

2.2.15 Intravital microscopy: This work was completed by Dr Jocelyn Auger, but analysed by Simon Calaminus. All procedures were undertaken with approval from the United Kingdom Home Office in accordance with the Animals (Scientific Procedures) Act of 1986. Male mice were anaesthetised via intraperitoneal injection with ketamine (100mg/kg Vetalar) and 2% xylazine (20mg/kg) before completion of tracheotomy and carotid artery cannulation. The cremaster muscle, a transparent muscle surrounding the testicle, was exteriorised and prepared through removal of connective tissue. The cremaster was superfused with bicarbonate-buffered saline (36°C) gassed with 5% CO_2, 95% N_2. Intravital experiments were performed as described by Falati et al, (2004). The endothelium of arterioles of 15-25μm in diameter was injured using a Coumann 440nM nitrogen laser at an appropriate level for the size of the vessel. To flourescently label platelets *in vivo*, a volume of 20μl of the secondary antibody Alexa Fluor 488 goat anti-rat IgG (2mg/ml) and a volume of

5μl of purified anti-mouse CD41 (0.5mg/ml) was added to a volume of 70μl of saline and infused via the carotid cannula. Thrombus formation was monitored with Olympus BX-61WI using both FITC and open channels. Images were captured with a high rate Senicam CCD camera (Cooke Corporation, Michigan, USA) coupled to a Gen III image intensifier (Videoscope Int Ltd, Virginia, USA). Several thrombi were induced and recorded for each mouse before intraperitoneal injection with Y27632 to give an estimated plasma concentration of 100μM, as described in the results. A further 5 thombi were induced in the presence of Y27632 between 5-30min following injection. All images were analysed using Slidebook 4.0 (Intelligent Imaging Innovations, Inc., Denver, USA). The background intensity of the free platelets was calculated and removed from the total intensity of the thrombus. The resulting intensity was identified for each time point, allowing for a kinetic analysis of thrombus formation and embolisation.

2.2.16 Electron microscopy: Washed platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips, in the presence of apyrase (2unit/ml), indomethacin (10μM), and either DMSO (0.1%), Y27632 (50μM), or blebbistatin (100μM) for 45min at 37°C. Platelets were then rinsed twice with PBS before addition of an extraction solution (50μM imidazole, pH 6.8, 50μM KCl, 0.5μM MgCl, 0.1μM EDTA, 1μM EGTA, 1% Triton X-100, 4% PEG (MW 40,000)) for 3-5min at room temperature. Platelets were rinsed with cytoskeleton buffer (50μM imidazole, pH 6.8, 50μM KCl, 0.5μM MgCl, 0.1μM EDTA, 1μM EGTA) 2-3 times, before fixation overnight at 4°C, in a volume of 400μl of 2% glutaraldehyde (EM grade) in 0.1M sodium cacodylate, pH 7.3. Before processing specimens were brought back to room temperature. Glutaraldehyde solution was removed, and a volume of 300μl 0.1%

aqueous tannic acid (Mallinckrodt, Inc, Kentucky, USA) was added for 20min at room temperature. Platelets were then rinsed in distilled water twice, before incubation in distilled water for 5min. 0.1-0.2% uranyl acetate solution was then added for 20min at room temperature before, rinsing with distilled water. To undergo critical point drying, samples were dehydrated by transferring the platelets through graded ethanols (10%, 30%, 50%, 70%, and 100% (twice)), with a 5min incubation at each grade. Platelets were then placed in 0.1-0.2% uranyl acetate in 100% ethanol for 20min. Platelets were incubated twice for 5min in 100% ethanol, followed by two incubations for 5min in 100% ethanol dried over molecular sieves. Samples were placed within the critical point dryer and were submerged within 100% ethanol dried with molecular sieves. The critical point dryer was cooled to 5-10°C and CO_2 added. CO_2 changed every 5 min with a maximum of 10 changes. After critical point drying the coverslips were coated with carbon to achieve a 3D definition of the cytoskeleton. Images were processed using a Philips XL-30 FEG Environmental scanning electron microscope (Oxford, UK).

2.2.17 Analysis of results: Where applicable, results are shown as mean ± SEM. Statistical significance of differences between means was determined by student's t-test or one-tailed ANOVA. If means were shown to be significantly different with an ANOVA, multiple comparisons were performed using a Tukey test. Probability values of $P < 0.05$ were selected to be statistically significant. In addition where appropriate, a frequency distribution of the data was obtained, from a combination of three separate experiments. In addition where the data obtained was not normally distributed, geometric means were obtained, before statistical analysis as above.

Chapter 3

Platelet spreading on matrix proteins: identification of a novel, actin-rich structure

3 GENERAL INTRODUCTION

3.0 Summary

During platelet spreading, the actin cytoskeleton undergoes a massive reorganisation, which leads to formation of multiple actin rich structures, including filopodia, lamellipodia, and stress fibres. The aim of this Chapter was to provide insight into the mechanisms of the actin dynamics and structures formed when platelets adhere and spread on various matricies. This work has led to the unexpected identification of a novel actin structure in platelets, which we have termed an actin nodule. In this Chapter I provide a detailed analysis of its kinetics, composition and dependence on adhesion.

3.1 Introduction

Filopodia, lamellipodia, and stress fibres are among the most well known actin structures formed during cell spreading, whose regulation is mediated by the Rho GTPases, Cdc42, Rac1, and RhoA, respectively (Nobes et al, 1995). In addition, several additional types of actin structures have been described in other cell types, although very little is known as to whether they contribute to platelet spreading. These include focal adhesions (Abercrombie et al, 1971), focal complexes (reviewed by Zamir et al 2001), invadopodia (review Buccione et al, 2004), podosomes (review Linder et al 2005), yeast actin patches (review Moseley et al, 2006), and dorsal and peripheral ruffles (Abercrombie et al, 1970 & 1971). However, at the present time only focal adhesions (Leng et al, 1998) and peripheral ruffles (unpublished observations; Platelets, Elservier Inc, 2007) have been identified within platelets.

I have used staining of fixed platelets with the actin-binding protein FITC-phalloidin and platelets from mice expressing a transgene encoding GFP-actin to monitor actin polymerisation during platelet spreading on various matrix proteins. This work has led to identification of punctate areas of actin staining that are formed early on during platelet spreading which I have termed actin nodules. The actin nodule is most readily seen when stress fibre formation is inhibited or under certain conditions of weak platelet activation. The actin nodule can also be labelled with antibodies to several actin-binding proteins, including the Arp2/3 complex, talin and cortactin. I propose that the actin nodules are formed during the early stages in actin polymerisation and that they could represent an important contact site between platelet and matrix.

3.2 RESULTS

3.2.1 Monitoring actin polymerisation during platelet spreading.

Human platelets were spread on fibrinogen coated coverslips in the presence of inhibitors of the major secondary agonists, apyrase (2unit/ml) and indomethacin (10μM), which remove ADP and inhibit TxA_2 formation, respectively. Spreading was monitored in real-time using DIC microscopy and at specific time points by fixation and staining with FITC-phalloidin (2μM). After initial adhesion to fibrinogen, actin-rich fingerlike protrusions termed filopodia extend from the platelet body (Fig.3.1i). This is followed by the formation of the sheet like extension, lamellipodia (Fig.3.1ii) leading to the characteristic fried egg shape of a fully spread platelet. Within the fully spread platelet, stress fibres can be identified by staining the actin cytoskeleton with FITC-phalloidin (Fig.3.1iii). The timescale for formation of these actin structures is demonstrated by Fig.3.2. Strikingly, even after full platelet spreading, the outer membrane of the platelet undergoes continuous movement (supplementary video 1), most likely reflecting peripheral ruffle formation.

3.2.2 Identification of a new actin structure termed actin nodules

The above analysis identified the presence of prominent, highly-stained actin structures which could also be detected via DIC microscopy (Fig.3.3). These structures have been termed actin nodules. Using real-time DIC microscopy, the nodules can be shown to move continuously throughout the various stages of platelet spreading, continuing after lamellipodia formation is complete (Fig.3.2 and supplementary video 1). The presence of nodules in resting platelets is difficult to identify. However, this reflects either their absence or that the platelets have not yet

Figure 3.1: Demonstration of filopodia, lamellipodia, and stress fibres. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10µM) for 45min. Platelets were fixed with paraformaldehyde (3.7%). **(i)** After initial attachment, filopodia form (white arrow). **(ii)** This is followed by lamellipodia formation (white arrow). **(iii)** After the platelet has fully spread, in order to visualise stress fibres, platelets were lysed with Triton X-100 (0.2%), and stained with FITC-phalloidin (2µM). Stress fibres are identified by the white arrow. Images are representative of at least three experiments.

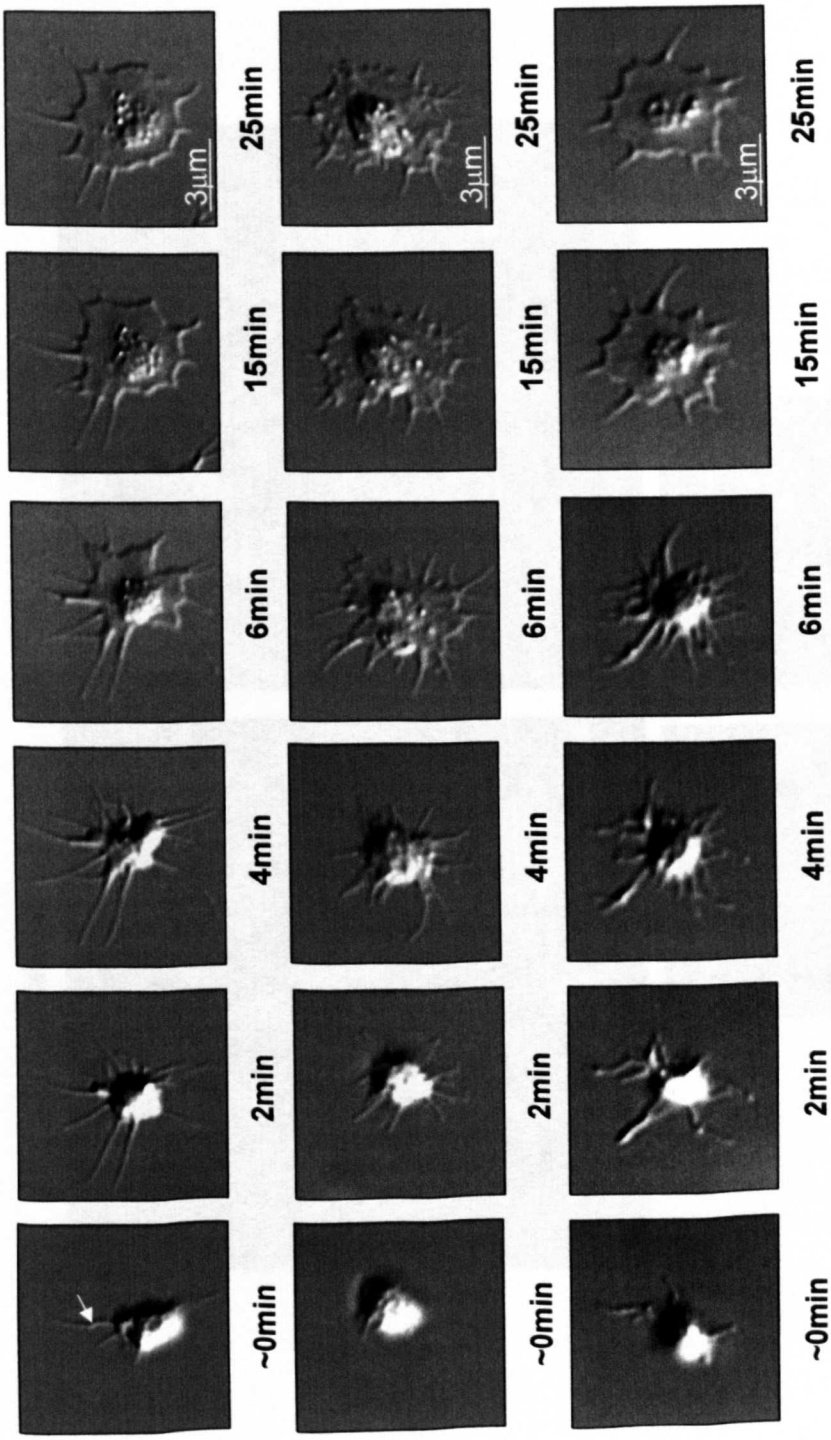

Figure 3.2: Time course of platelet spreading on fibrinogen. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) for 30min. Platelet spreading was monitored using real-time DIC microscopy, with an image taken every 5s. Videos were analysed using ImageJ software. After initial attachment, filopodia form, followed by lamellipodia. After 25min the platelet has fully spread forming its typical fried egg appearance. Images are three representative platelets of al least three experiments.

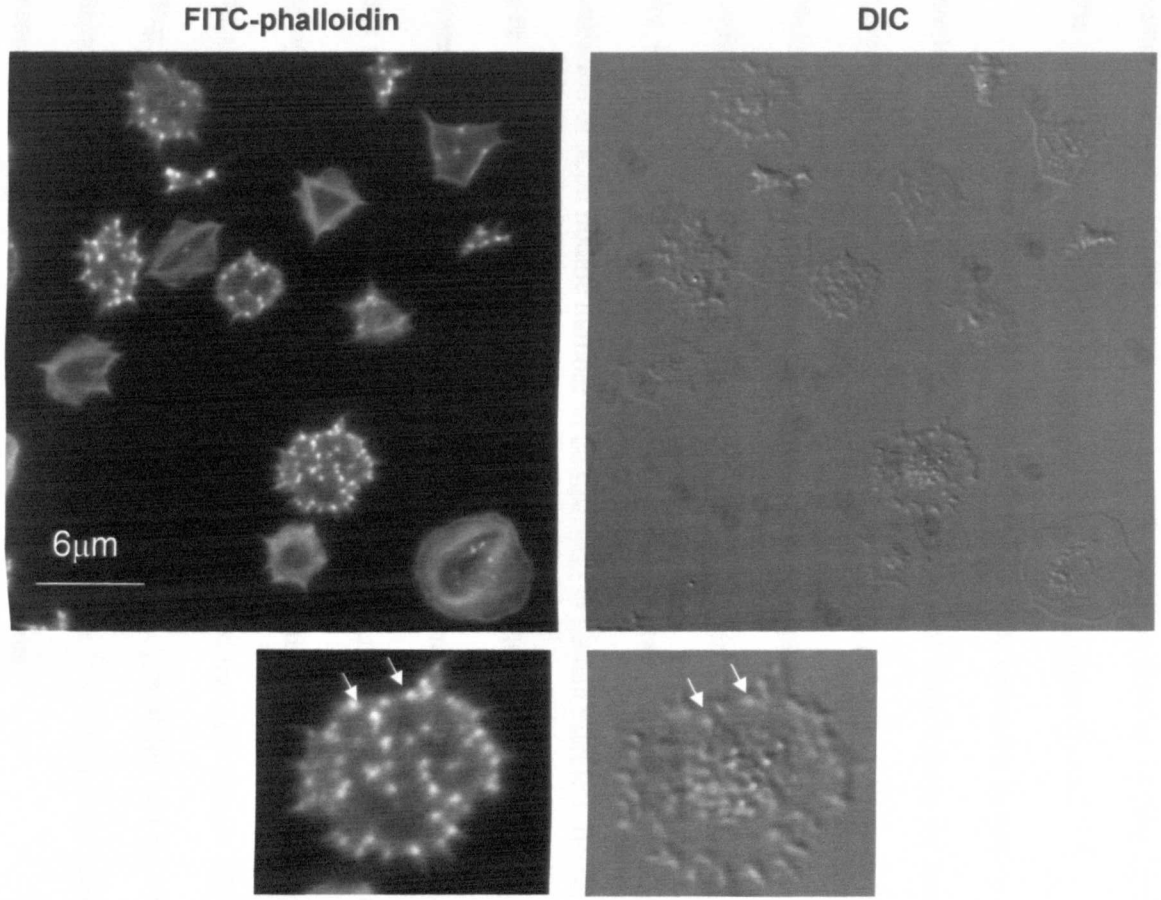

Figure 3.3: **Identification of actin nodules in platelets spread on fibrinogen.** Human platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) for 45min. Platelets were then fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with FITC-phalloidin (2μM). White arrows indicate punctate stains within FITC-phalloidin images and raised circular spots within DIC images. Images are representative of more than three experiments.

settled onto the surface and so actin nodules cannot be resolved. However, actin nodules are rapidly detected upon contact with the matrix protein (Fig.3.3). Within a population of human spread platelets that had been allowed to spread on fibrinogen for 45min, 35.4±3.7% of immobilised platelets contain nodules (data not shown). Within the platelets containing actin nodules, there is great variation within the number of actin nodules, with a mode of 5, and a median of 14 (Fig.3.4). Strikingly, the nodules were absent in platelets that had formed stress fibres.

The above studies with human platelets were extended to mouse platelets. In contrast to human platelets, mouse platelets do not form extensive lamellipodia on fibrinogen unless stimulated by an agonist such as thrombin (Thornber et al, 2006). Nevertheless, as is the case for human platelets, prominent actin-rich structures or nodules can be seen in mouse platelets that have been allowed to spread on fibrinogen for 45min (Fig.3.5i). Murine platelets which contain actin nodules, have a similar variation of actin nodule number in comparison to human platelets, despite the smaller size of the mouse platelets (data not shown). However, there was a significant increase in the number of platelets containing actin nodules within mouse platelets that had spread on fibrinogen, with 80.3±2.7% of platelets containing actin nodules in comparison to 39.5±2.7% of human platelets containing actin nodules. Addition of thrombin (0.1unit/ml), caused full platelet spreading and stress fibre formation (Fig.3.5ii), with only 6.3±1.7% of platelets containing nodules and 93.7% containing stress fibres when measured at 45min (Fig.3.5iii). Therefore in agreement with human platelets, actin nodules form before stress fibres.

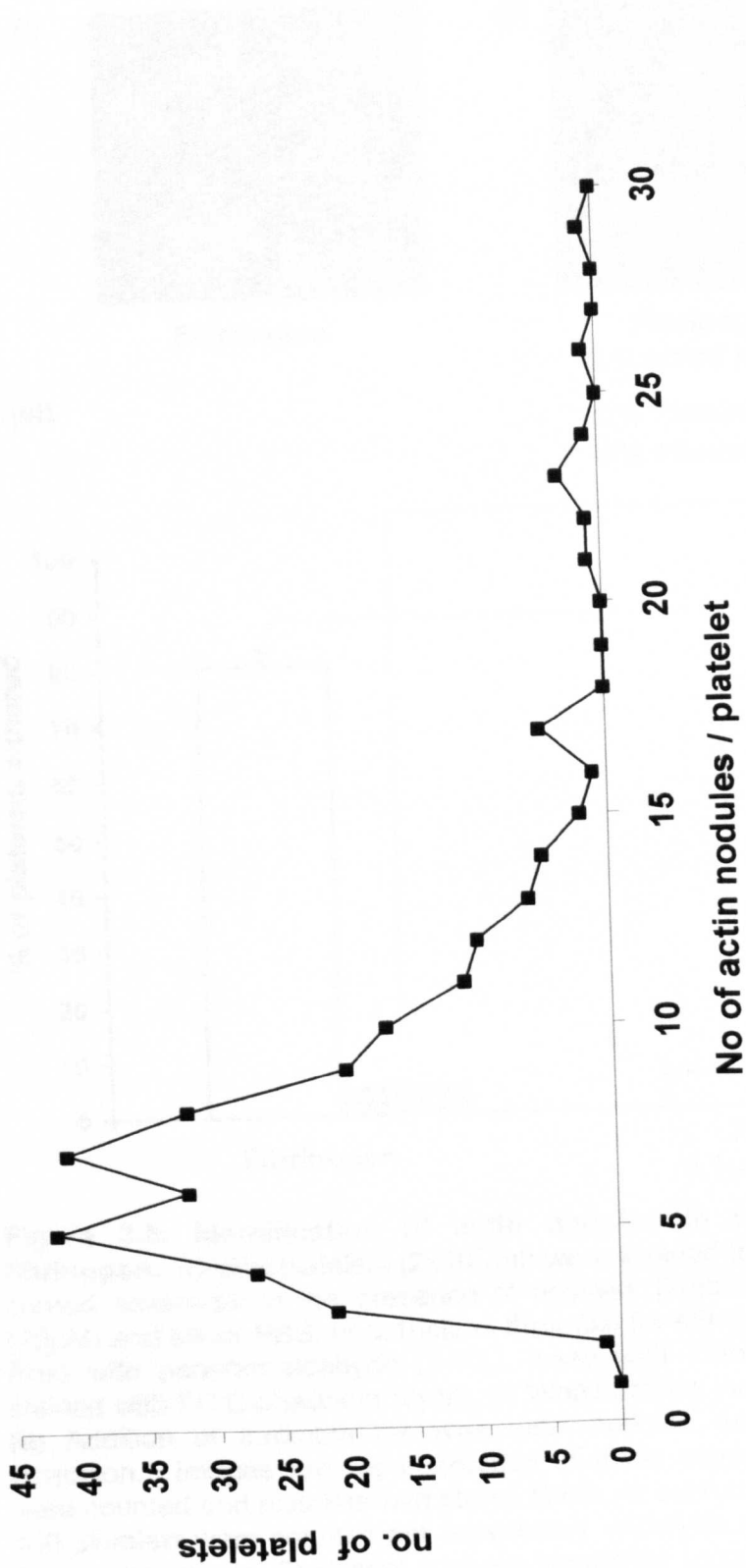

Figure 3.4: **Identification of the number of actin nodules per platelet within human platelets.** Platelets were allowed to spread as per Fig.3.3. Platelets that contained actin nodules were identified, and the number of actin nodules per platelet was counted. At least 50 platelets were counted per experiment. Three experiments were analysed and numbers grouped to obtain a frequency distribution of the number of actin nodules within a platelet.

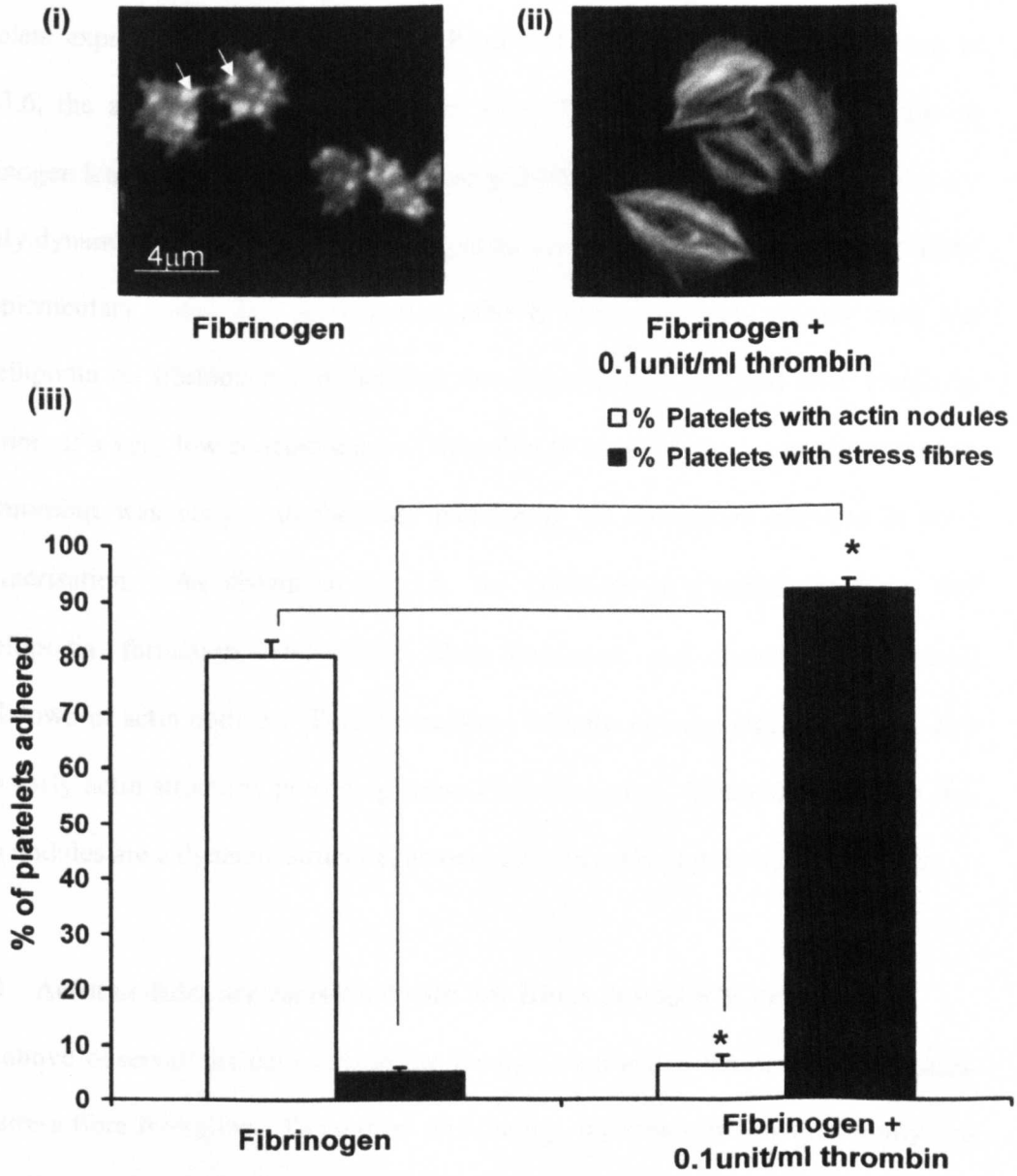

Figure 3.5: Identification of actin nodules in murine platelets on fibrinogen. Murine platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) and either PBS, or 0.1unit/ml thrombin for 45min. Platelets were then fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with FITC-phalloidin (2μM). **(i)** White arrows indicate punctate stains. **(ii)** Addition of thrombin removes actin nodules and drives stress fibre formation. Images are representative of three experiments. **(iii)** Platelets were counted and platelets with stress fibres, or actin nodules were identified. 100 platelets were counted per experiment. Analysis is an average±SEM of three experiments. Statistical analysis completed using a paired student`s t-test. * $p<0.05$ relative to fibrinogen

Confirmation that the nodules are actin structures was achieved using mouse platelets expressing a transgene for GFP-actin (Ludin et al, 1996). As shown in Fig.3.6, the actin cytoskeleton undergoes a rapid rearrangement upon adhesion to fibrinogen leading to actin nodule formation and filopodia. Moreover, the nodules are highly dynamic structures and undergo rapid movement throughout the spread platelet (supplementary video 2). As discussed above, mouse platelets do not form full lamellipodia on fibrinogen and therefore lamellipodia were induced after 15min by addition of a very low concentration of thrombin (0.03unit/ml). A low concentration of thrombin was chosen to facilitate monitoring of subsequent changes in actin polymerisation. As shown in Fig.3.6, the addition of thrombin leads to full lamellipodia formation, and stress fibre formation and therefore subsequent breakdown of actin nodules. This is consistent with the concept that the actin nodule is an early actin structure, preceding stress fibre formation. These data confirm that actin nodules are a dynamic structure, associated with early platelet actin dynamics.

3.2.3 Actin nodules are correlated with low RhoA and ROCK activity

The above observations demonstrate the inverse relationship between actin nodules and stress fibre formation. To confirm this finding, the effect of two structurally and mechanistically distinct inhibitors of stress fibre formation on actin nodule formation was investigated in platelet spreading of human platelets on fibrinogen. These studies were completed in the presence of inhibitors of the secondary mediators apyrase and indomethacin, the ROCK inhibitor Y27632 and the myosin-II inhibitor, blebbistatin. Platelets were fixed, and stained with FITC-phalloidin (2μM) before imaging using fluorescent microscopy as described above. As expected, treatment with Y27632 and blebbistatin inhibited stress fibre formation (Fig.3.7), although neither platelet

Figure 3.6: Time course of actin nodule formation in GFP-actin mice. Murine platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) for 30min. Platelet spreading was monitored using real-time fluorescent microscopy, with an image taken every 5s. The white arrows indicate the presence of actin nodules. Upon full spreading (30min) and stress fibre formation actin nodules breakdown. Images are two representative platelets of three separate experiments.

adhesion nor platelet surface area was affected (Fig.3.8). Strikingly, inhibition of ROCK or myosin-II caused a significant increase in the number of platelets containing actin nodules from 39.5±2.4% to 72.0±1.1%, and 80.0±2.0% respectively (Fig.3.9i). As the numbers of platelets containing actin nodules is increased the number containing stress fibres decreased. Interestingly, the range of actin nodules present within spread platelets is not affected by treatment with either Y27632, or blebbistatin (Fig.3.9ii). These data demonstrate that actin nodule formation is inversely related with ROCK and myosin-II activity and stress fibre formation, and suggest that they represent a novel, early actin-rich structure.

3.2.4 Actin nodule formation requires actin polymerisation and Src kinase activity.

A variety of pharmacological inhibitors were used to investigate whether resting platelets contain actin nodules. These include latrunculin A, which prevents actin polymerisation by binding G-actin causing alterations within its structure preventing its incorporation into the actin cytoskeleton (Morton et al, 2000), PD0173952, which blocks Src kinases, Ly294002, which inhibits PI 3-kinases and BAPTA-(AM), which blocks Ca^{2+} elevation. These inhibitors were chosen as they have all been shown to disrupt spreading on fibrinogen. Inhibition of Src kinases, PI 3-kinase and Ca^{2+} elevation blocks lamellipodia formation, although filopodia are retained (Wonerow et al, 2003, Senis et al, 2005). In comparison, latrunculin A completely blocks platelet spreading.

Platelets were pre-incubated with apyrase, indomethacin and the above inhibitors before being allowed to spread on fibrinogen for 45min. They were then fixed, lysed and stained with FITC-phalloidin (2μM). In agreement with previous

Figure 3.7: Stress fibre inhibition increases the number of platelets containing actin nodules. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml), indomethacin (10μM), and either DMSO (0.1%), Y27632 (50μM) or blebbistatin (100μM) for 45min. Platelets were then fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with FITC-phalloidin (2μM). Platelets were analysed using fluorescent microscopy. White arrows indicate stress fibres. Images are representative of at least three experiments.

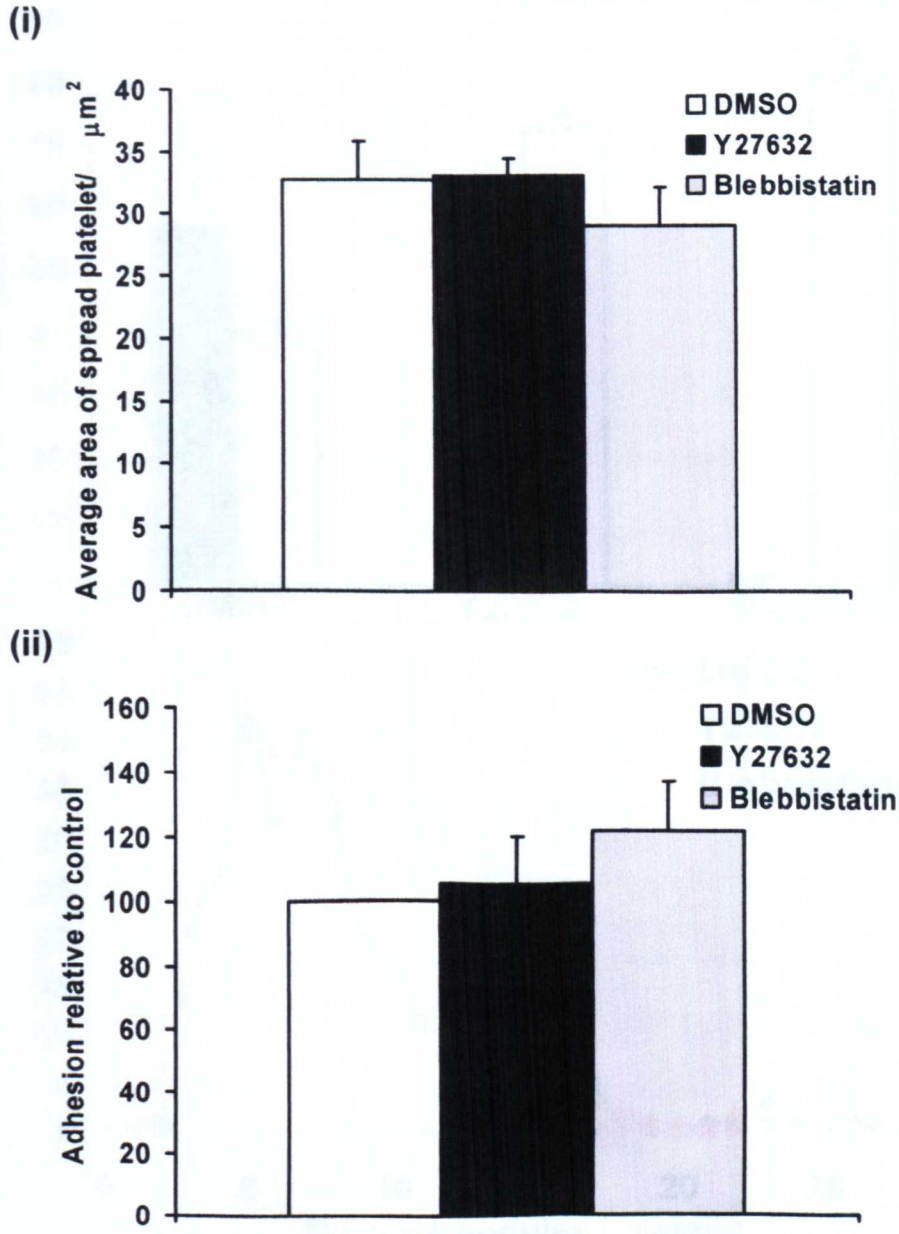

Figure 3.8: **ROCK and myosin-II inhibition does not inhibit human platelet spreading or adhesion on fibrinogen.** Platelets were allowed to spread as per Fig.3.7. **(i)** Average surface area was analysed using ImageJ. 100 platelets were analysed per experiment, and results are representative of one experiment. **(ii)** The number of platelets adhered were counted per experiment. Results are an average of three experiments±SEM. Statistical analysis was completed using ANOVA, with no significant result identified.

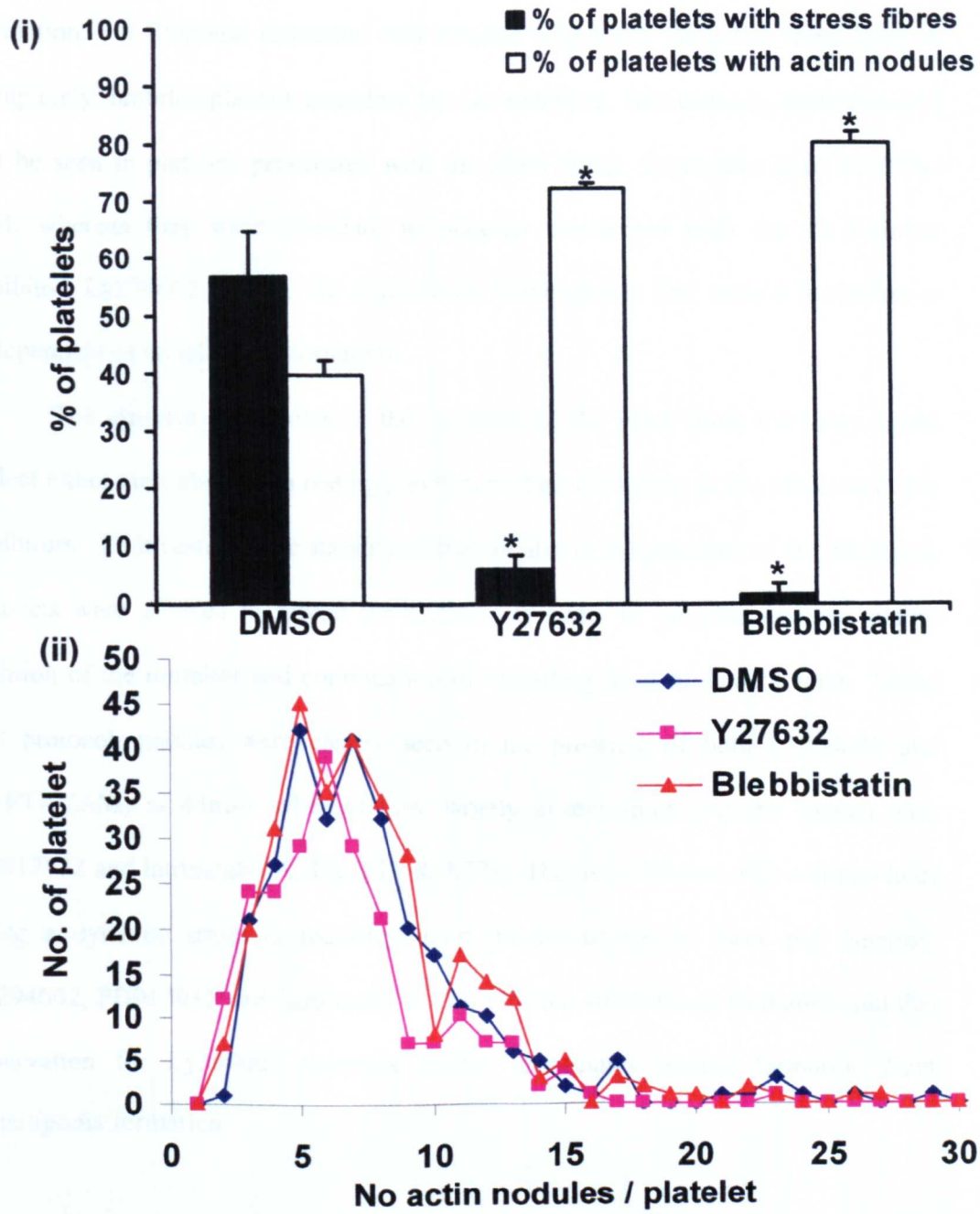

Figure 3.9: Actin nodule formation is negatively correlated with ROCK and Myosin-II activity, but does not affect actin nodule number within platelets. Platelets were allowed to spread as per Fig 3.7. **(i)** Platelet`s containing actin nodules were identified and counted. Platelets were counted from three separate experiments. Results analysed with an ANOVA, followed by a Tukey test. *$p<0.05$ is relative to DMSO control **(ii)** Platelets that contained actin nodules were identified, and the number of actin nodules per platelet was counted. At least 50 platelets were counted per experiment. Three experiments were analysed and a frequency distribution obtained.

observations, PD0173952, Ly294002 and BAPTA-(AM) blocked lamellipodia formation, but filopodia formation was retained (Fig.3.10). However, latrunculin A completely inhibited platelet spreading but not adhesion. Interestingly, nodules could not be seen in platelets pre-treated with the PD0173952, latrunculin A or BAPTA-AM, whereas they were abundant in platelets pre-treated with the PI 3-kinase inhibitor, Ly294002. Thus, the latter result demonstrates that nodule formation is independent of lamellipodia formation.

The absence of nodules in the presence of the other three inhibitors could reflect either their absence in resting platelets or their disruption in the presence of the inhibitors. To investigate the stability of the nodules in the presence of the inhibitors, platelets were allowed to spread for 22.5min, in order to generate nodules, before addition of the inhibitor and continuation of spreading for a further 22.5min. Using this protocol, nodules were clearly seen in the presence of both Ly294022 and BAPTA-(AM) at 45min, whereas they largely absent from platelets treated with PD017952 and latrunculin A (Fig.3.11 & 3.12). This is consistent with actin nodules being a dynamic structure requiring actin polymerisation to form and function. Ly294002, PD017952 and latruncalin A also blocked stress fibres formation and this observation for Ly294002 therefore further dissociates nodule formation from lamellipodia formation.

3.2.5 Actin nodules are large structures, extending through the platelet height.

Confocal and scanning electron microscopy was used to identify the structure of the actin nodules. Confocal microscopy was used to measure the height of the actin nodules by taking 0.25μm slices through the platelet. The average height of the nodules was 1.20±0.12μm after spreading for 45min and this was unaltered in the

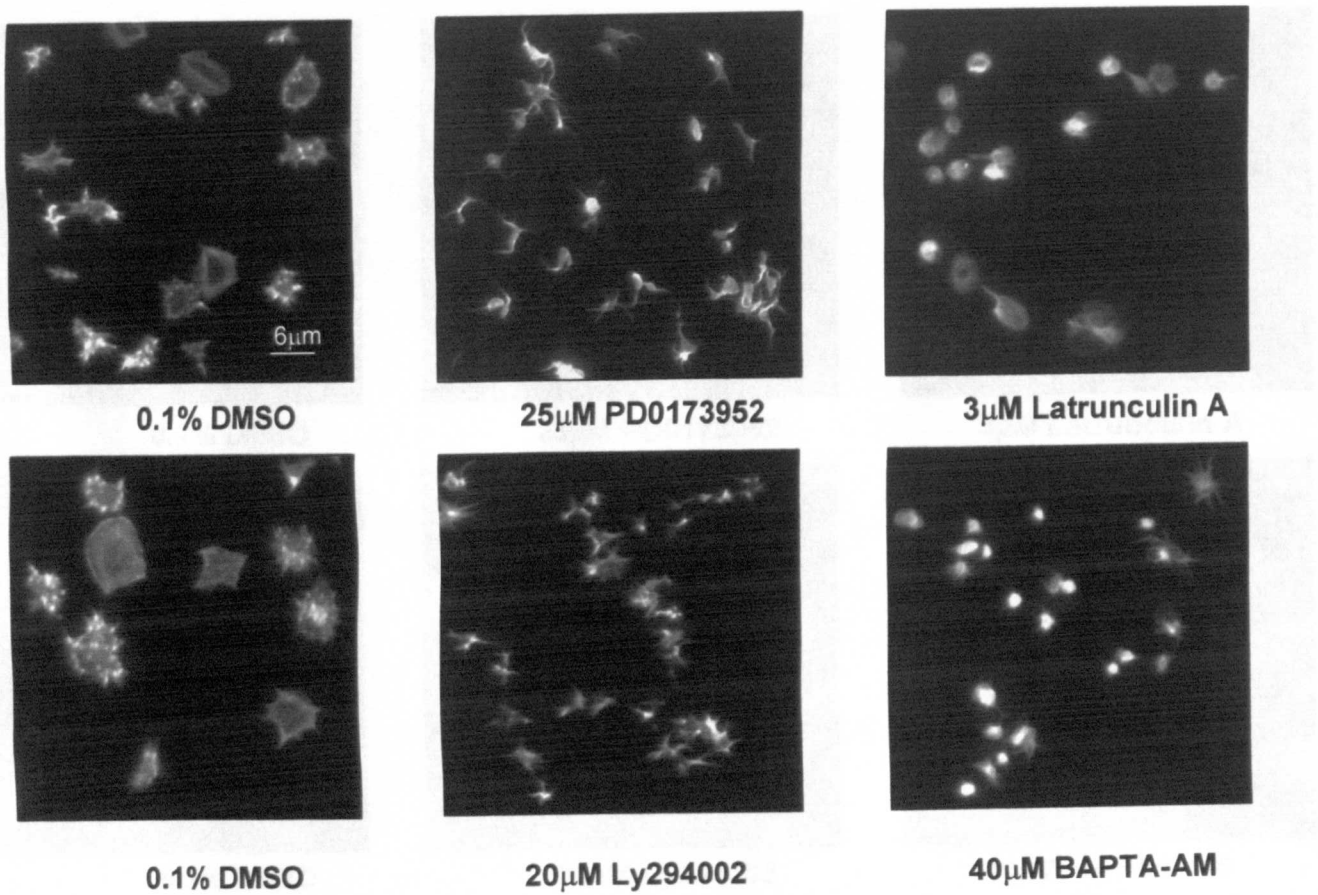

Figure 3.10: Actin nodule formation is independent of lamellipodia formation. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) and either DMSO (0.1%), PD0173952 (25μM), Ly294002 (20μM), BAPTA-AM (40μM) or Latrunculin A (3μM) for 45min. Platelets were fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and then stained with FITC-phalloidin (2μM). Images are representative of at least three experiments.

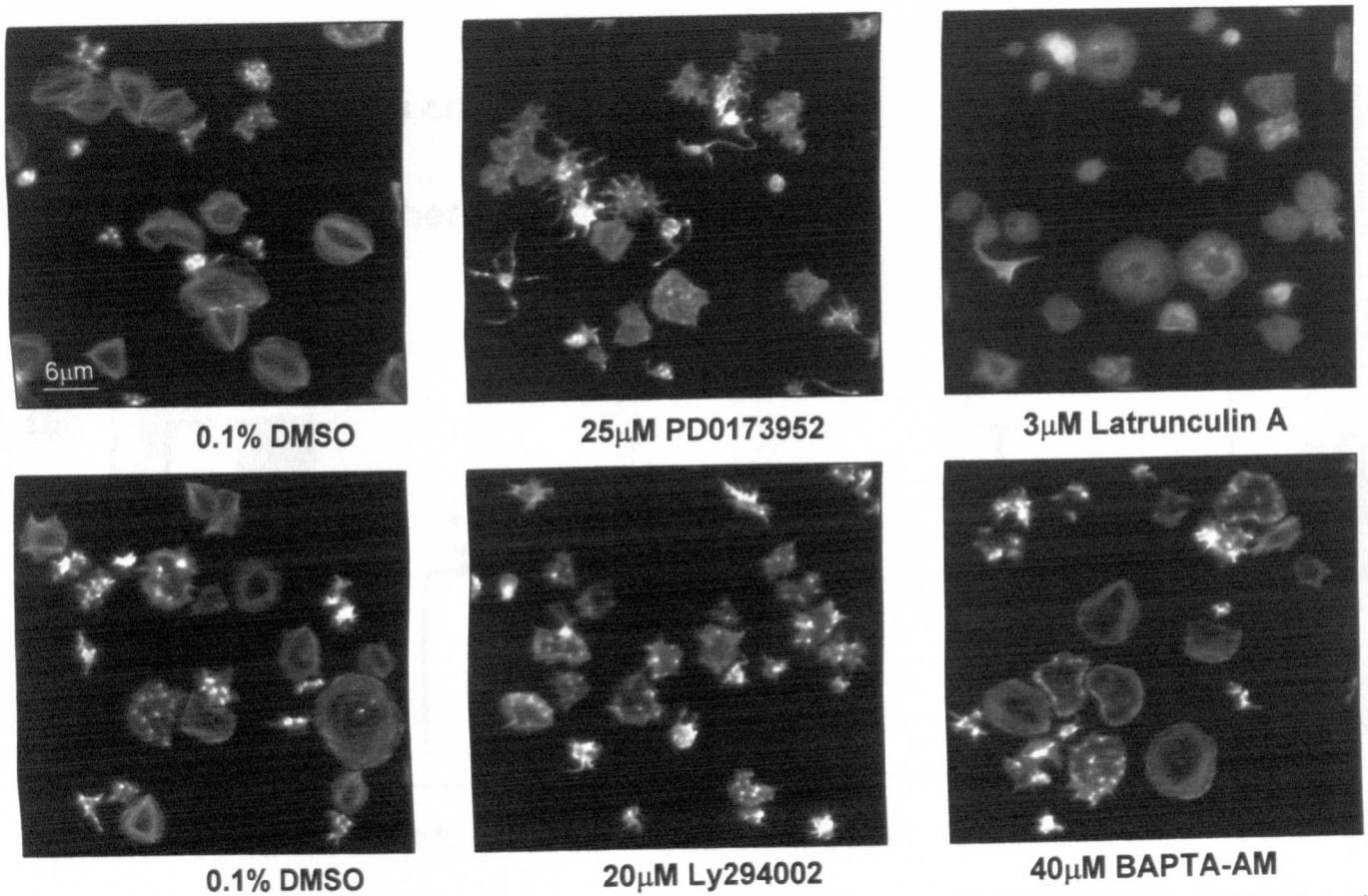

Figure 3.11: Actin nodule formation requires actin polymerisation and Src kinase activity. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml) and indomethacin (10μM) for 22.5min before addition of either DMSO (0.1%), PD0173952 (25μM), Ly294002 (20μM), BAPTA-AM (40μM) or Latrunculin A (3μM) and were allowed to spread for a further 22.5min. Platelets were fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%) and then stained with FITC-phalloidin (2μM). Images are representative of at least three experiments

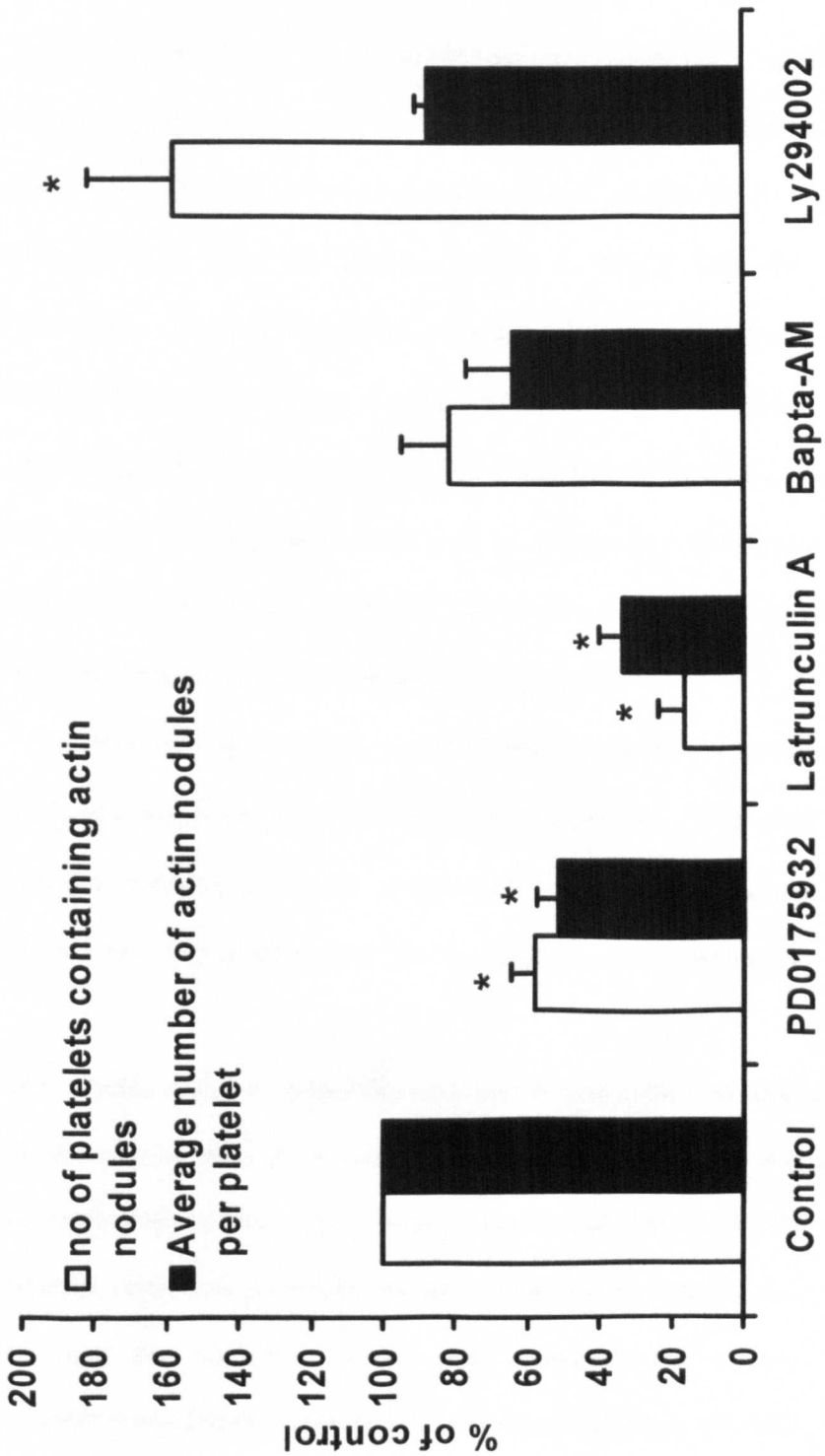

Figure 3.12: Src kinases and actin polymerisation are required for actin nodule formation. Platelets were allowed to spread as per Fig.3.11. Images were analysed and the number of actin nodules per platelet and the number of platelets containing actin nodules were counted. Averages were then normalised against the control. Normalised average±SEM are representative of at least three experiments. Statistical analysis was completed using an ANOVA followed by a Tukey test. * $p<0.05$ to the control.

presence of Y27632, or blebbistatin. The actin nodules were further investigated using scanning electron microscopy, after spreading for 45min. Analysis clearly identified filopodia (white arrows), stress fibres (red arrows), and actin nodules (yellow arrows) (Fig.3.13). Filopodia are thin bundles of actin filaments extending from the platelet body. In comparison stress fibres are thick bundles of actin, linear in nature, and define the edge of the spread platelet. Actin nodules could also clearly be identified and were substantially greater in height than the rest of the actin cytoskeleton. They represent dense areas of actin from which actin cables extend, as if the nodule is the central point upon which actin polymerisation is being driven. The actin nodules are also frequently found at the base of filopodia, though whether they have a role in filopodia formation is unknown. Consistent with the above observations, inhibition of ROCK or myosin-II inhibited stress fibres, whereas filopodia and actin nodules were present (Fig.3.13).

This data demonstrate that the actin nodule is a substantial actin structure, which extends throughout the height of the platelet. The actin nodule has actin filaments radiating from it in all directions. Overall these results indicate the actin nodule could play an important role within platelet actin dynamics.

3.2.6 Actin nodules co-localise with significant actin regulatory proteins.

The association of actin-binding proteins with actin nodules was investigated using immunohistochemistry. The results reveal that Rac, Fyn, the Arp2/3 complex, cortactin, talin, Csk, β1 and β3 integrin all colocalise with the actin nodules (Fig.3.14 and data not shown). These actin nodules also contain heavily tyrosine phosphorylated proteins, although the identity of these is unknown. Proteins that are not associated with actin nodules include WASP, and Src, both of which play

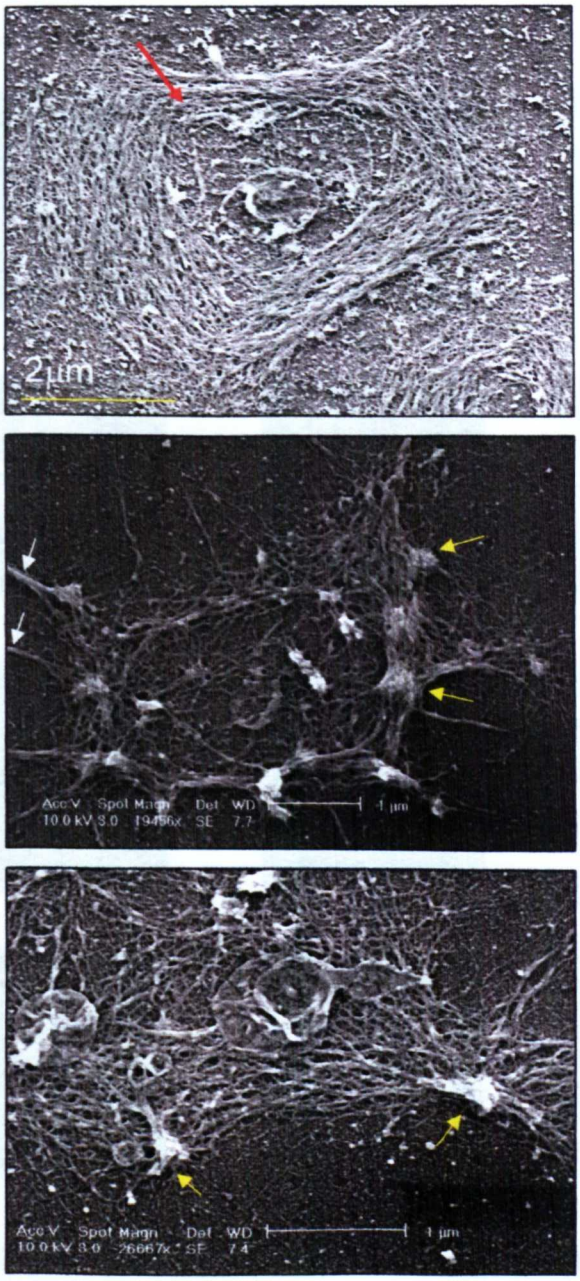

Figure 3.13: Actin nodules are distinct circular actin structures. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml), indomethacin (10µM) and either DMSO (0.1%), Y27632 (50µM), or blebbistatin (100µM) for 45min. Platelets were fixed with phalloidin and 4% PEG for 10min, and overnight using 2% glutaraldehyde (EM grade). Samples were dried with graded alcohols, critical point dried, and coated with carbon. Samples were analysed using scanning elecronmicroscopy. Red arrows indicate stress fibre formation, and yellow arrows identified the formation of actin nodules. Images are representative of two experiments.

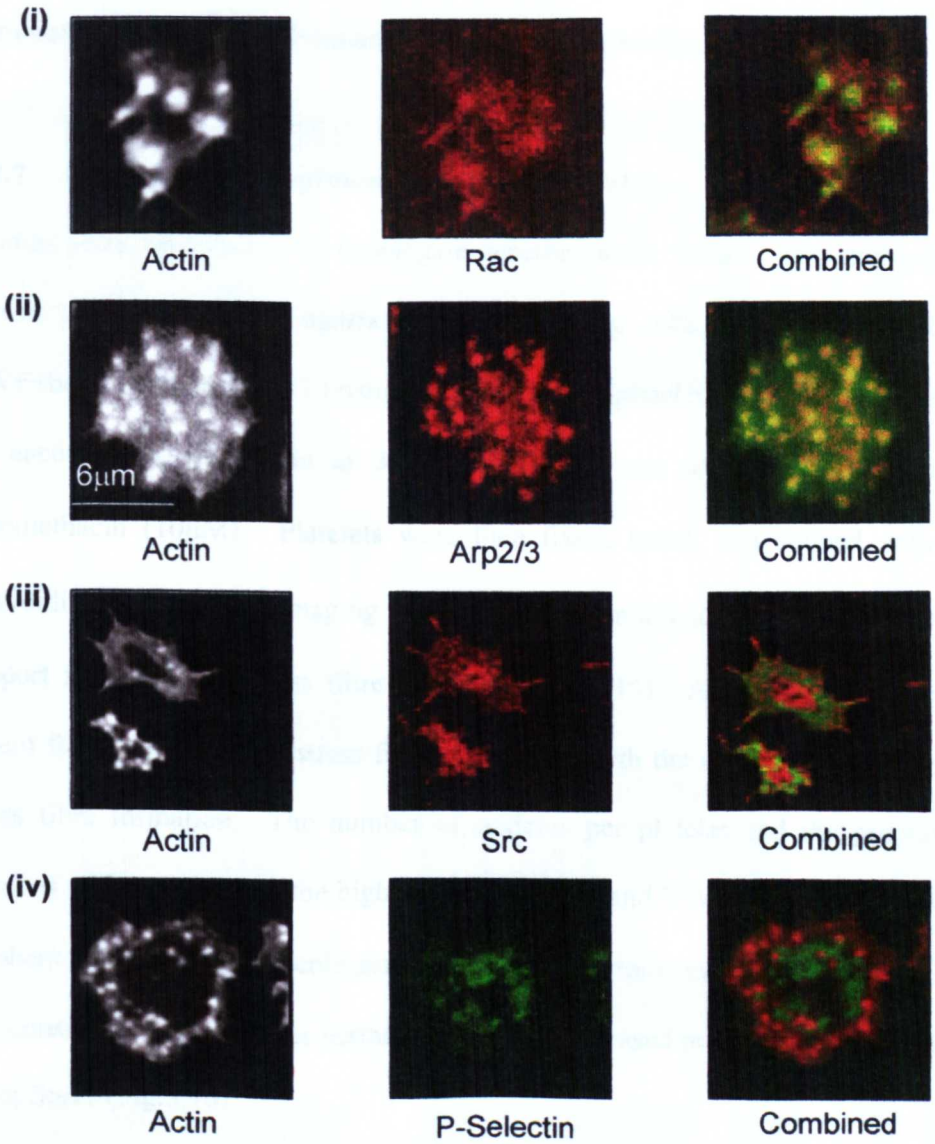

Figure 3.14: Proteins associated with actin nodules present on fibrinogen. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips in the presence of apyrase (2unit/ml), and indomethacin (10μM) for 45min. Platelets were then fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with **(i-iii)** FITC-phalloidin (2μM) or **(iv)** rhodamine-phalloidin (2μM) and the appropriate antibody. Platelets washed with PBS, before the addition of the appropriate secondary antibody. Platelets were analysed by confocal microscopy. Images are representative of three experiments.

important roles in the formation and control of a number of other actin structures such as podosomes. P-selectin and CD63 did not colocalise with the actin nodules, demonstrating that the nodules are not α- or dense granules.

3.2.7 Actin nodules are present on multiple matrices.

Studies were undertaken to investigate whether actin nodules are also present in spread platelets on other matrix proteins including collagen, fibronectin, laminin, VWF+botrocetin, and VWF+ristocetin. Therefore platelets ($2x10^7$/ml) were spread on each matrix for 45min at 37°C, in the presence of apyrase (2unit/ml) and indomethacin (10μM). Platelets were then fixed, lysed, and stained with FITC-phalloidin (2μM), before imaging with fluorescence microscopy. All of the matricies support filopodia and stress fibre formation (Fig.3.15). Again, actin nodules were absent from platelets with stress fibres consistent with the concept that they precede stress fibre formation. The number of nodules per platelet and the proportion of platelets with nodules was the highest on fibrinogen and VWF+botrocetin, while both numbers were reduced on collagen, fibronectin, laminin and VWF+ristocetin. This was consistent with the latter surfaces having an increased proportion of platelets with stress fibres (Fig.3.16).

These results demonstrate that actin nodules are present on all surfaces and again emphasise that they are absent in platelets with stress fibres suggesting they may precede their formation.

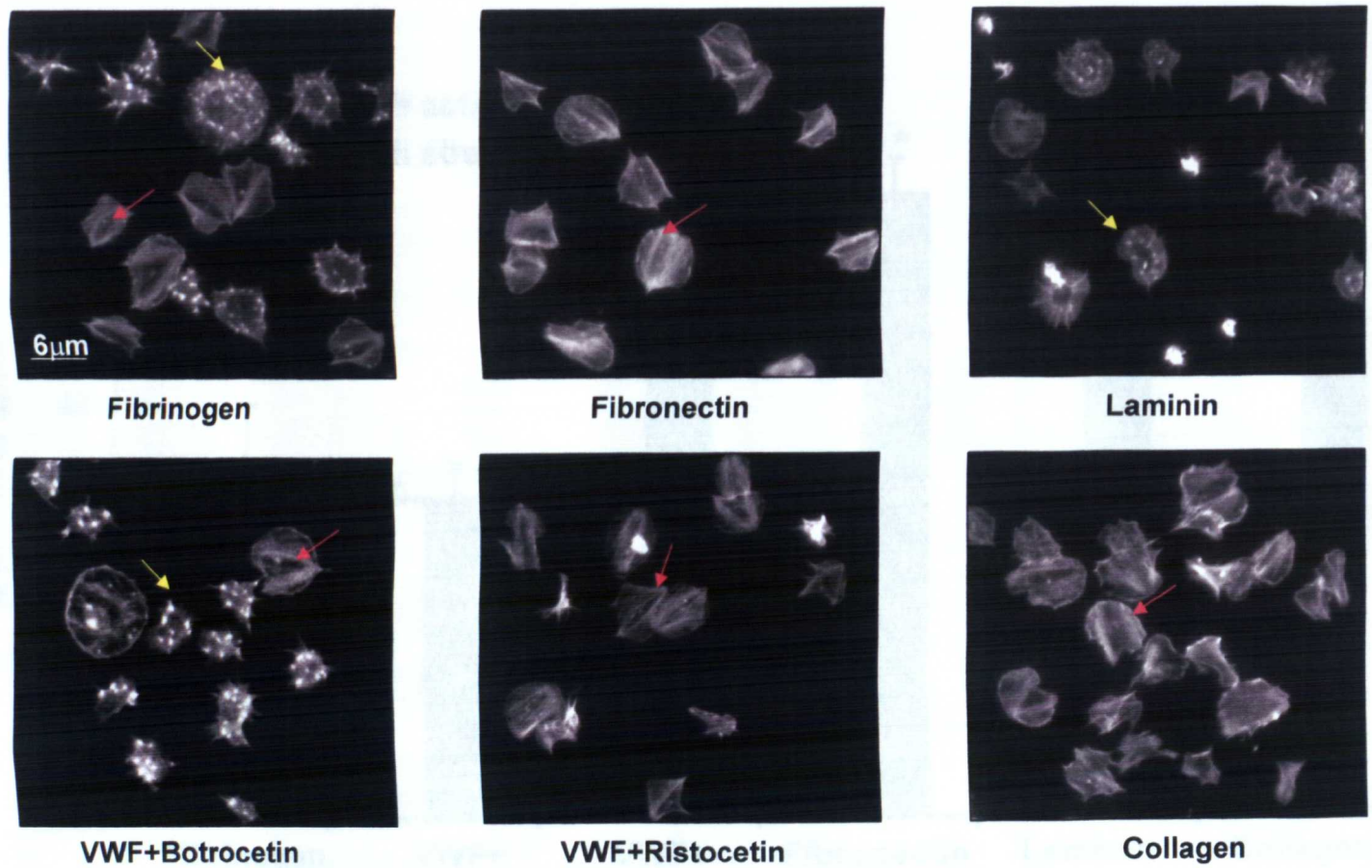

Figure 3.15: Identification of actin nodules on a number of different matrices. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen, VWF+botrocetin, VWF+ristocetin, laminin, collagen, or fibronectin coated coverslips, in the presence of apyrase (2unit/ml) and indomethacin (10μM) for 45min. Platelets were then fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%) and stained with FITC-phalloidin (2μM). Yellow arrows indicate actin nodules, and red arrows indicate stress fibres. Images are representative of three experiments.

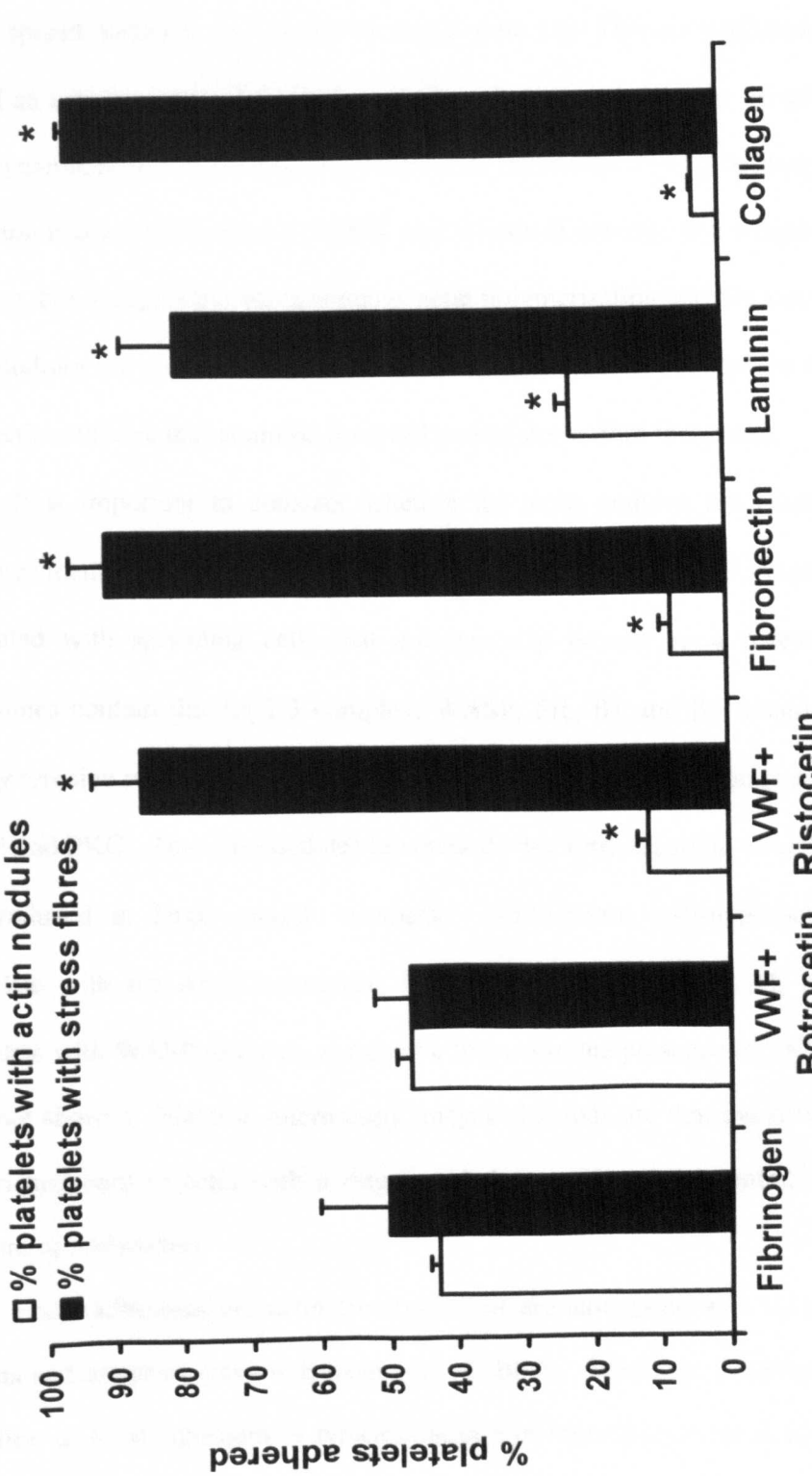

Figure 3.16: Identification of actin nodules on a number of different matrices. Platelets were allowed to spread as per Fig.3.15. Images were analysed and platelets with stress fibres, or actin nodules were counted. A minimum of 100 platelets were counted per experiment. Analysis is an average±SEM of at least three experiments. Statistical analysis was completed using an ANOVA followed by a Tukey test. *$p<0.05$ relative to fibrinogen.

3.3 DISCUSSION

The results presented in this chapter identify the formation of a novel actin structure within spread platelets on a variety of matrix proteins. This actin structure has been termed an actin nodule. The nodules are identified at an early stage of spreading and are a dynamic structure, undergoing continuous movement throughout the cell. Their formation is inversely related to ROCK and myosin-II activity, and independent of PI 3-kinase, but is dependent on continuous actin polymerisation and Src kinase activity. Actin nodules are absent in platelets with stress fibres. Actin nodules are therefore an early actin structure that could be involved within stress fibre formation.

It is important to consider whether the actin nodules are related to actin structures identified in other cells. Podosomes are F-actin enriched, circular structures associated with spreading cells that are typically present as a rosette structure. Podosomes contain the Arp2/3 complex, WASP, Src, β1 and β3 integrins, and are heavily tyrosine phosphorylated (Linder et al, 2005). Their formation is dependent on WASP and PKC. The actin nodules however do not form a rosette structure, and are not produced in large enough numbers. Furthermore, although actin nodules colocalise with the Arp2/3 complex, Rac, and integrins β1 and β3, they do not colocalise with WASP or c-Src, and can be formed in the presence of PKC inhibition (data not shown). Electron microscopy images also indicate that the structure is not an enriched core of actin with a ring round the outside of the centre, such as the structure of podosomes.

Focal adhesions are actin structures that are associated with phosphorylated proteins and integrins (review Buccione et al, 2004). However, the time course for formation of focal adhesions is typically 60min or more (Buccione et al, 2004) and

our actin nodules are rapidly formed short-lived structures. Additionally, focal adhesions formation is promoted by stress fibre formation.

The actin nodules are also unlikely to be invadopodia, peripheral ruffles or dorsal ruffles. Invadopodia are large structures of up to 8 μm in size and are present in protrusions from the cell to facilitate degradation of the ECM. Peripheral ruffles are composed of branched networks of actin found at the periphery of the platelet and are associated with the continual wave like motion of the periphery upon full spreading. Dorsal ruffles are short-lived, dynamic F-actin enriched waves, which occur at the top of the cell. They have a time course of 2-30min and they often form circular waves accompanied by macropinocytosis (Araki et al, 2000). However, each of these structures is not reminiscent of the structure of actin nodules.

The above discussion strongly suggests that the actin structures identified in other cell types do not correspond to the actin nodules observed within spreading platelets. Furthermore, the actin nodules do not appear to correspond to known platelet structures, including α- and dense granules. However, it seems very likely that the actin nodules have been observed in previous studies in platelets, although there was no specific comment on their presence (e.g. Chang et al 2005, Falet et al 2002, McCarty et al 2006, Wonerow et al 2003, Atkinson et al 2003, Jirouskova et al 2007).

The critical question is the function of the nodules. At present, this is subject to speculation, but their early presence and absence in platelets with stress fibres gives rise to the possibility that they may represent novel sites of actin nucleation, possibly related to stress fibre formation. In this context, it is noteworthy that the actin nodule is enriched in many platelet regulatory proteins, including Src kinases, interestingly not Src but Fyn, Rac, Talin, Csk, β1 and β3 integrins, and the Arp 2/3 complex, and

that there is a high proportion of tyrosine phosphorylated proteins within the nodules. The presence of such important actin and signalling proteins indicates an important role for the nodules within platelet spreading. Further investigation of the nodules necessitates identification of the interplay between different actin structures within platelet spreading. It is necessary to disrupt actin nodule formation dynamically, through the use of inhibitors, to monitor how their disruption affects platelet spreading and function. Therefore platelets should be pre-incubated, and incubated with inhibitors after 15min of spreading to monitor if actin nodules can initially form, and if they are affected by the addition of the inhibitor once formed.

However, in addition further work must be completed to ensure that the actin nodule is an actin structure, and is not associated with vesicle movement, or secretion of granules. The inhibition of dynamin, should prevent endo and exo-cytosis and so if used within the GFP-actin mice, will demonstrate if the nodules are vesicles. In addition the use of Hermansky-Pudlack, patients who do not have dense granules, and staining with antibodies to fibrinogen, should help to eliminate the possibility of the actin nodules being alpha and dense granules.

In conclusion this Chapter demonstrates the formation of a novel actin structure, which is illustrated to form prior to stress fibre formation. It requires both actin polymerisation and Src kinase activity, whilst being negatively correlated to ROCK and myosin-II activity. It is present on multiple matricies and has all the proteins required for a major role within actin dynamics.

Chapter 4

Myosin-II contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability

4 GENERAL INTRODUCTION

4.0 Summary

During platelet spreading the actin cytoskeleton undergoes multiple rearrangements the last being stress fibre formation. Stress fibre formation is mediated by ROCK and myosin-II activation. The aim of the Chapter was to investigate the functional significance of myosin-II in platelet spreading and thrombus formation on collagen using inhibitors of ROCK (Y27632) and myosin-II (blebbistatin). This work led to the identification of fenestrations within spread platelets, and thrombus instability both *in vivo* and *in vitro* on collagen at high shear in the presence of both inhibitors.

4.1 Introduction

In response to an appropriate stimulus, the platelet actin cytoskeleton generates finger-like protrusions (filopodia), actin-rich sheet-like protrusions (lamellipodia) and bundles of actin and myosin filaments (stress fibres). This actin polymerisation is regulated by Rho GTPases, with specific family members regulating distinct structures. For example, Cdc42, Rac and RhoA generate filopodia, lamellipodia and stress fibres, respectively, in a wide variety of cell types (Ridley et al, 1992a; Ridley et al, 1992b).

Platelets sequentially generate filopodia, lamellipodia and stress fibres as they spread on a variety of adhesive surfaces, including collagen and fibrinogen (Leng et al, 1998; Suzuki-Inoue et al, 2001). Cdc42 is activated during platelet spreading on immobilised collagen (Suzuki-Inoue et al, 2001) and laminin (Chang et al, 2005), but at present there are no reports of a direct link between Cdc42 activation and filopodia formation in platelets. Rac is activated by a wide range of receptor agonists in

platelets, including those for ADP, collagen, thrombin and TxA_2 (Azim et al, 2000; Pearce et al, 2002; Soulet et al, 2001; Soulet et al, 2005; Gratacap et al, 2001; Hartwig et al, 1995). In addition, constitutively active Rac drives actin polymerisation in permeabilised platelets (Hartwig et al, 1995) and studies using mutant murine platelets have shown that Rac-1 is essential for lamellipodia formation on fibrinogen, collagen, laminin and von Willebrand factor (VWF) both in the absence or presence of GPCR agonists (McCarty et al, 2005).

The small GTPase RhoA is activated by a wide variety of adhesive surfaces and GPCR agonists in platelets, with activation being mediated downstream of the $G_{12/13}$ family of heterotrimeric G proteins (Gratacap et al, 2001). Activation of ROCK by RhoA in platelets is associated with myosin light chains (MLC) phosphorylation and subsequent stress fibre formation and 'shape change' as measured in an aggregometer (Suzuki-Inoue et al, 2001; Bauer et al, 1999; Wilde et al, 2000). In addition, stress fibre formation and shape change are regulated through elevation of Ca^{2+} which activates MLCkinase leading to MLC phosphorylation (Bauer et al, 1999). On the other hand, RhoA or ROCK inhibition, using C3 exoenzyme and Y27632, respectively, has no effect on platelet aggregation to thrombin, collagen and ADP (Leng et al, 1998), although a reduction in secondary aggregation to adrenaline is reported (Leng et al, 1998). Similarly, RhoA or ROCK inhibition has a negligible or minor inhibitory effect on static adhesion to fibrinogen, VWF and collagen, or platelet capture by VWF under flow conditions (Leng et al, 1998; Suzuki-Inoue et al, 2001; Schoenwaelder et al, 2002). RhoA and ROCK, however, do contribute to stable platelet adhesion on VWF under arterial rates of shear (Schoenwaelder et al, 2002).

Myosin-IIs are involved in a wide variety of cellular responses, including cytokinesis, phagocytosis and cell motility (Canobbio et al, 2005). Myosin-IIs consist

of two heavy chains and four light chains. Myosin-II activation is mediated via MLC phosphorylation, either through an increase in MLCkinase activity through Ca^{2+} elevation or by inactivation of myosin phosphatase downstream of ROCK. The importance of these two pathways in the regulation of MLC phosphorylation can be identified using the pharmacological inhibitors Y27632 for ROCK and BAPTA-AM for Ca^{2+}. In addition, it is possible to block the activity of myosin-II using blebbistatin. This inhibitor locks myosin-II in a low affinity state, even after its phosphorylation by MLCkinase, thereby preventing its interaction with actin (Allingham et al, 2005; Kovacs et al, 2004).

The major isoform of myosin-II in platelets is myosin-IIa (Maupin et al, 1994), although the presence of a low level of myosin-IIb is reported (Bodie et al, 2001). Mutations of myosin-IIa lead to the group of *MHY9* disorders, which include May-Hegglin anomaly, Sebastian, Fechtner and Epstein syndromes (Seri et al, 2003). This group of disorders are caused by an autosomal dominant mutation in the gene encoding non-muscle myosin heavy chain IIa (NMMHC-IIA) and are associated with thrombocytopenia, giant platelets and a mild bleeding disorder, as well as different combinations of additional clinical features. Platelets from *MHY9* patients exhibit a defect in cytoskeletal reorganisation in response to activation, leading to impairment in shape change and spreading. However, platelet aggregation is maintained (Canobbio et al, 2005).

In the present Chapter I have demonstrated that ROCK and myosin-II are required for stress fibre formation, and that stress fibre inhibition causes the formation of fenestrations in spread platelets on collagen. In addition, myosin-II activation is required for thrombus formation at high shear both *in vitro* and *in vivo*. Although this is not mediated by inhibition of platelet aggregation, or dense granule secretion.

Therefore I speculate that stress fibre formation contributes to maintenance of platelet morphology and thrombus stability.

4.2 RESULTS

4.2.1 Collagen regulates myosin light chain phosphorylation through ROCK

Collagen (30μg/ml) stimulates marked MLC phosphorylation in a suspension of human platelets which peaks at 1min and is reduced at 5min and 15min (Fig.4.1(i) and data not shown). Phosphorylation is inhibited in a concentration dependent manner by the ROCK inhibitor Y27632, with maximal inhibition observed at 30-50μM (Fig.4.1(ii)). Y27632 induced a much greater inhibition of MLC phosphorylation following stimulation by collagen for 5min (86±6% inhibition) than after 1min (44±8%), consistent with previous reports that Ca^{2+} elevation induces rapid phosphorylation of MLC and that ROCK mediates sustained MLC phosphorylation (Bauer et al, 1999, Paul et al, 1999). Similar observations were made with 1 and 10μg/ml collagen (data not shown). In contrast in the presence of blebbistatin, used at a concentration that completely inhibits platelet stress fibre formation, MLC phosphorylation induced by collagen (1-30μg/ml) over a time course of 1-15min, was unaffected (Fig.4.1(i) and data not shown). This observation is consistent with previous reports that blebbistatin traps myosin-II in a low affinity state for actin but does not inhibit MLC phosphorylation (Allingham et al, 2005; Kovacs et al, 2004).

4.2.2 Myosin-II is required for full aggregation and secretion to collagen

A series of experiments were designed to investigate the effect of Y27632, blebbistatin, and the actin polymerisation inhibitor, cytochalasin D, on aggregation and secretion induced by collagen (1 and 10μg/ml). Collagen (1μg/ml) stimulated partial aggregation and weak ATP secretion with a characteristic delay (Fig.4.2). At a ten fold higher concentration, collagen stimulated full aggregation and a more marked

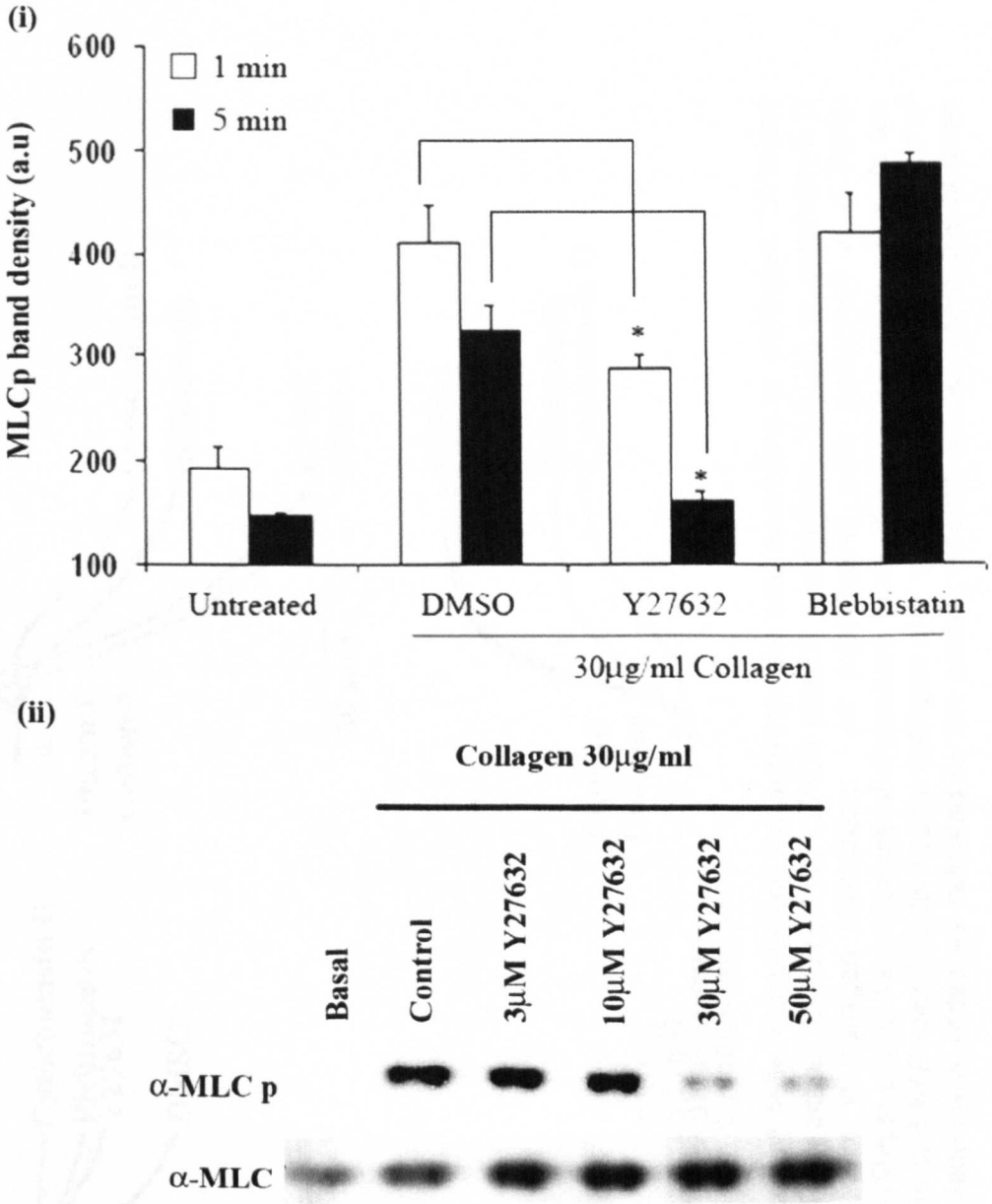

Figure 4.1: Collagen stimulates phosphorylation of MLC through a ROCK-dependent pathway. Platelets were treated with either DMSO (0.1%), Y27632 (50µM) or blebbistatin (100µM) for 2min prior to stimulation with 30µg/ml collagen for 1min, or 5min at 37°C, and then lysed. Samples were run on SDS-PAGE gels, transferred to PVDF and western blotted for MLC using anti-pan MLC and anti-phospho MLC (MLCp) antibodies. **(i)** The intensity of MLCp western blots were quantified by densitometry, and the results are shown as the mean±s.e.m of three experiments. * $p<0.05$ relative to DMSO control. Statistical analysis completed with a ANOVA. **(ii)** Example western blot of MLCp and MLC in the presence of Y27632 (3-50µM) at 5min. Images are representative of three experiments.

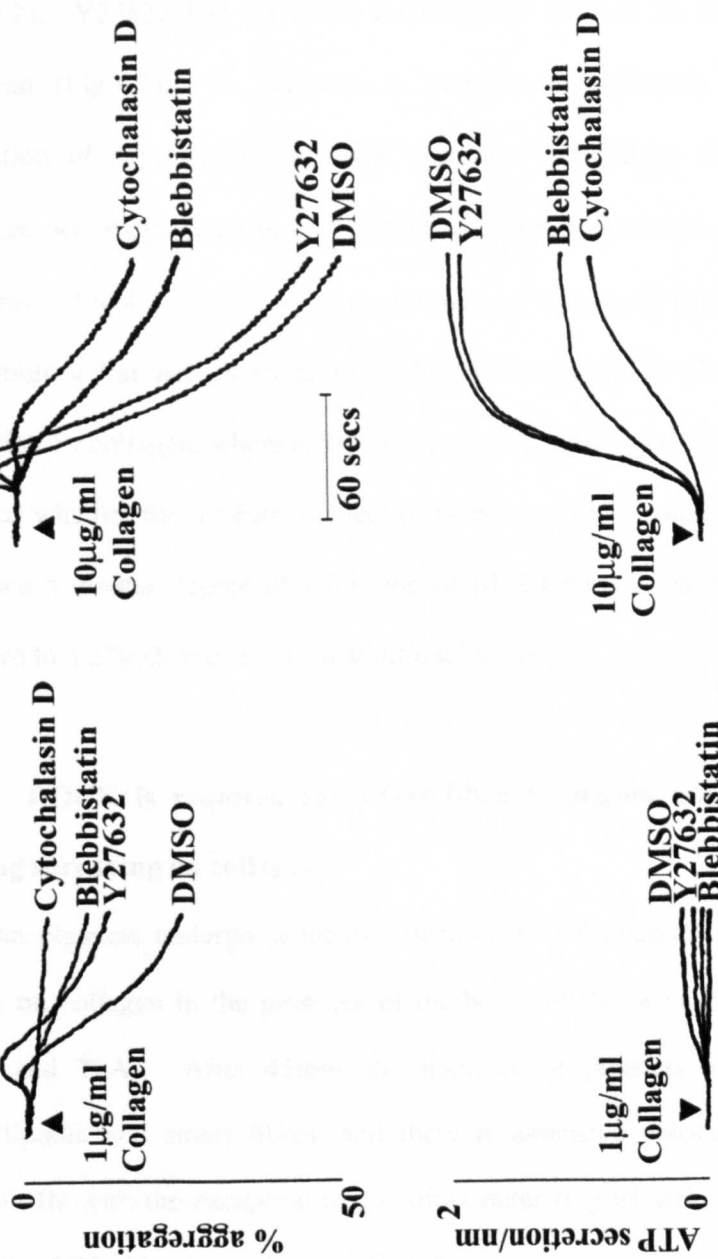

Figure 4.2: The effect of inhibitors of actin remodelling on aggregation and secretion to collagen. Washed platelets ($2x10^8$/ml) were incubated with either DMSO (0.1%), Y27632 (50µM), blebbistatin (100µM) or cytochalasin D (10µM) for a minimum of 2min prior to collagen stimulation. All experiments were performed in a Born-lumiaggregometer with stirring at 1200rpm and in the presence of chronolum to monitor ATP secretion. Results are representative traces of aggregation and ATP secretion to 1µg/ml collagen and 10µg/ml collagen in the presence of inhibitors of actin remodelling. Collagen was added as indicated by the arrow. Results are representative of three experiments

ATP secretion (Fig.4.2). In both cases, aggregation was preceded by shape change. Strikingly, treatment with Y27632 (50μM), caused a more pronounced shape change response to both concentrations of collagen, although the rate and final extent of aggregation was unaffected when the increase in shape change is taken into account (Fig.4.2). Y27632 had no effect on secretion induced by either concentration of collagen (Fig.4.2). In comparison, blebbistatin (100μM) caused a significant inhibition of aggregation and ATP secretion induced by both concentrations of collagen, while cytochalasin D (10μM) had a slightly greater inhibitory effect on both responses (Fig.4.2 & 4.3). The results with cytochalasin D provide evidence for a contributory but non-essential role of actin assembly in platelet aggregation and secretion by collagen, whereas ROCK activity is not required for either response. It is unclear whether the inhibitory effect of blebbistatin is a consequence of its ability to produce a greater degree of inhibition of MLCkinase at early times of stimulation relative to Y27632 or through an additional action.

4.2.3 ROCK is required for stress fibre formation and structural integrity during spreading on collagen

Human platelets undergo sequential formation of filopodia, lamellipodia and stress fibres on collagen in the presence of inhibitors of the secondary feedback agonists, ADP and TxA_2. After 45min, the majority of platelets have formed extensive lamellipodia and stress fibres, and there is extensive colocalisation of actin with myosin-IIa with the exception of a distinct outer ring of actin (Fig.4.4). The ROCK inhibitor Y27632 inhibited stress fibre formation on collagen and markedly reduced the co-localisation of actin and myosin-IIa (Fig.4.4). However, there was no significant change in platelet surface area, at either 22.5 or 45min, in the presence of

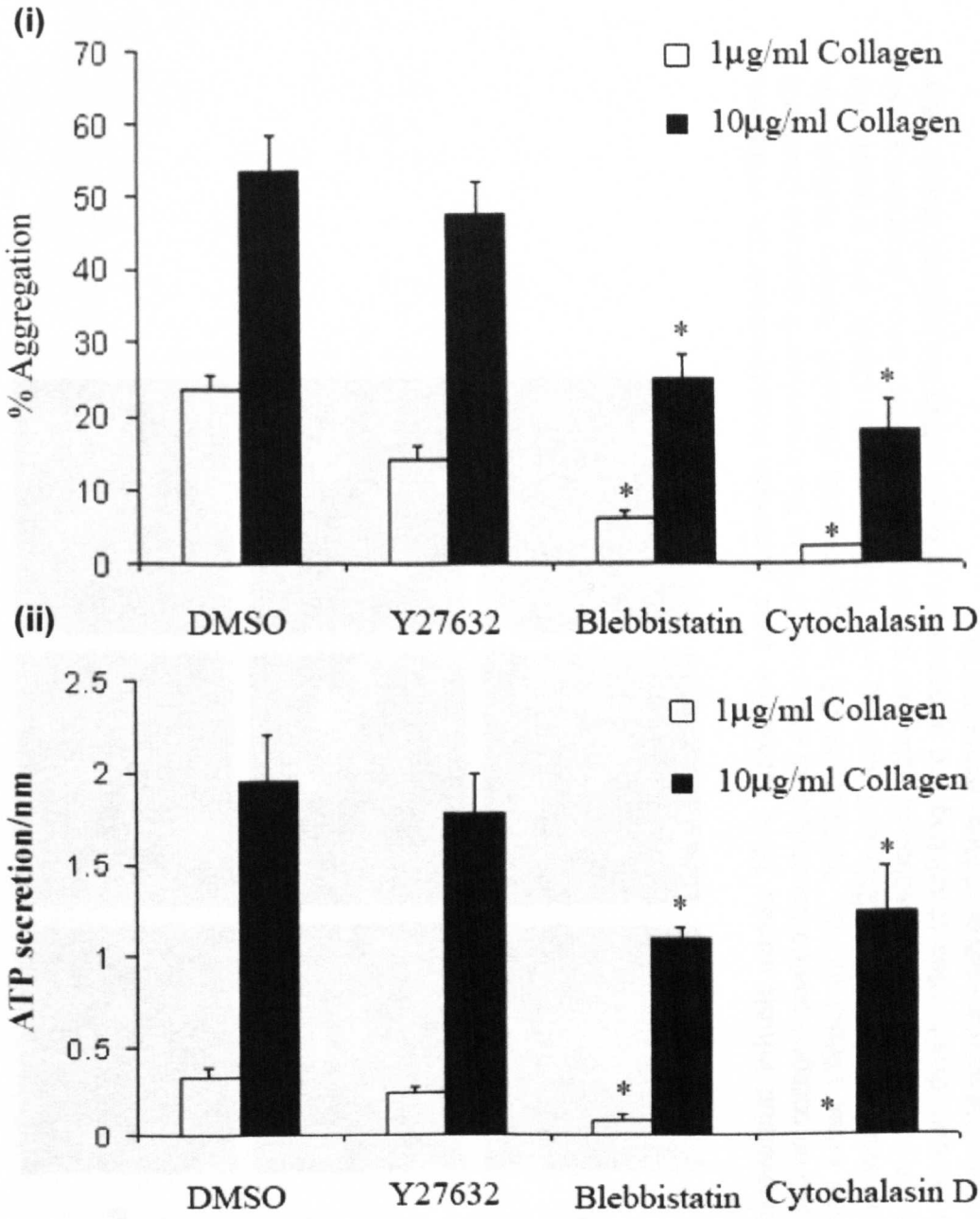

Figure 4.3: The effect of inhibitors of actin remodelling on aggregation and secretion to collagen. Maximum aggregation **(i)** and ATP secretion **(ii)** for experiments described in Fig.4.2. Results are mean±s.e.m of three experiments. *p<0.05 relative to the control. Statistical analysis completed with an ANOVA, followed by a Tukey test.

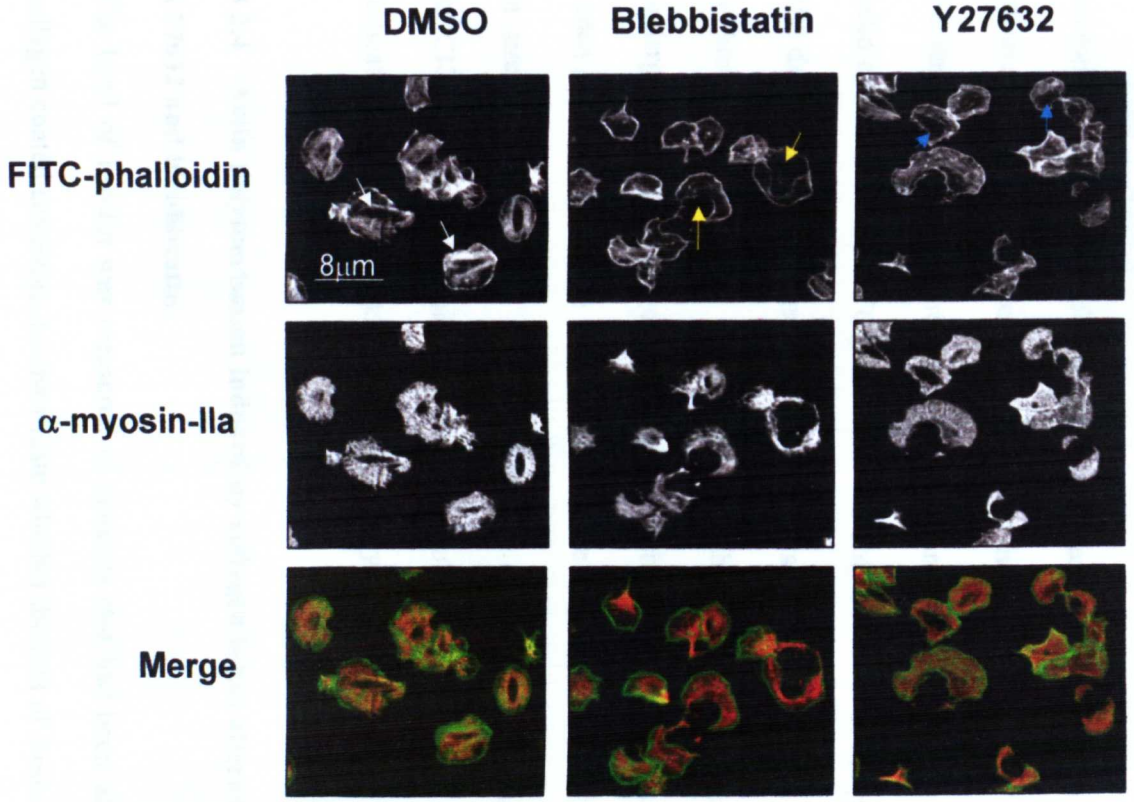

Figure 4.4: Y27632 and blebbistatin inhibit stress fibre formation and generate fenestrations on collagen. Platelets were allowed to spread on collagen coated coverslips for 45min at 37°C in the presence of apyrase (2unit/ml) and indomethacin (10μM), and either DMSO (0.1%), Y27632 (50μM) or blebbistatin (100μM), before fixation with paraformaldehyde (3.7%), lysis with Triton X-100 (0.2%) and staining with FITC-phalloidin (2μM), and a myosin-IIa antibody. The overlay shows the comparison of localisation of actin and myosin-IIa. The white arrow illustrates stress fibres, and the yellow and blue arrows identify sites of splitting and appearance of holes within the platelet, respectively (collectively fenestrations). Images are representative of three experiments.

Y27632 and blebbistatin in comparison to the DMSO control (Fig.4.5 and data not shown), demonstrating that lamellipodia formation was unaltered. In addition platelet adhesion in the presence of either Y27632 or blebbistatin is unaffected (data not shown). Strikingly, the presence of internal holes and splits could be seen in 10-15% of the platelets that had spread on collagen in the presence of Y27632 and blebbistatin, whereas similar structures were present in <0.5% of control cells (Fig.4.4 & 4.5). These structures have been termed fenestrations (i.e. windows). To investigate whether the fenestrations are due to an increase in permeability of the plasma membrane, platelets were fixed and incubated with FITC-phalloidin (2μM) in the absence or presence of Triton X-100 (to induce permeabilisation). Staining of the actin cytoskeleton by FITC-phalloidin was only observed in the presence of Triton X-100 demonstrating that membrane integrity is maintained in the presence of Y27632 or blebbistatin (Fig.4.6). Consistent with this, the fenestrations were observed to undergo continuous movement throughout the recording (Fig.4.7 & supplementary video 3). Together, these studies demonstrate a critical role for ROCK and myosin-II in stress fibre formation in spread platelets. Furthermore, following inhibition of ROCK or myosin-II, a significant number of platelets exhibit fenestrations, which indicates a structural weakening of the actin cytoskeleton.

4.2.4 Actin polymerisation induced by collagen is not altered in the presence of Y27632 and blebbistatin.

The level of F-actin was measured in platelets that had been allowed to spread on collagen coated coverslips to investigate whether the loss of stress fibre formation and appearance of fenestrations was associated with a change in the level of actin polymerisation. There was no significant difference in the level of F-actin in spread

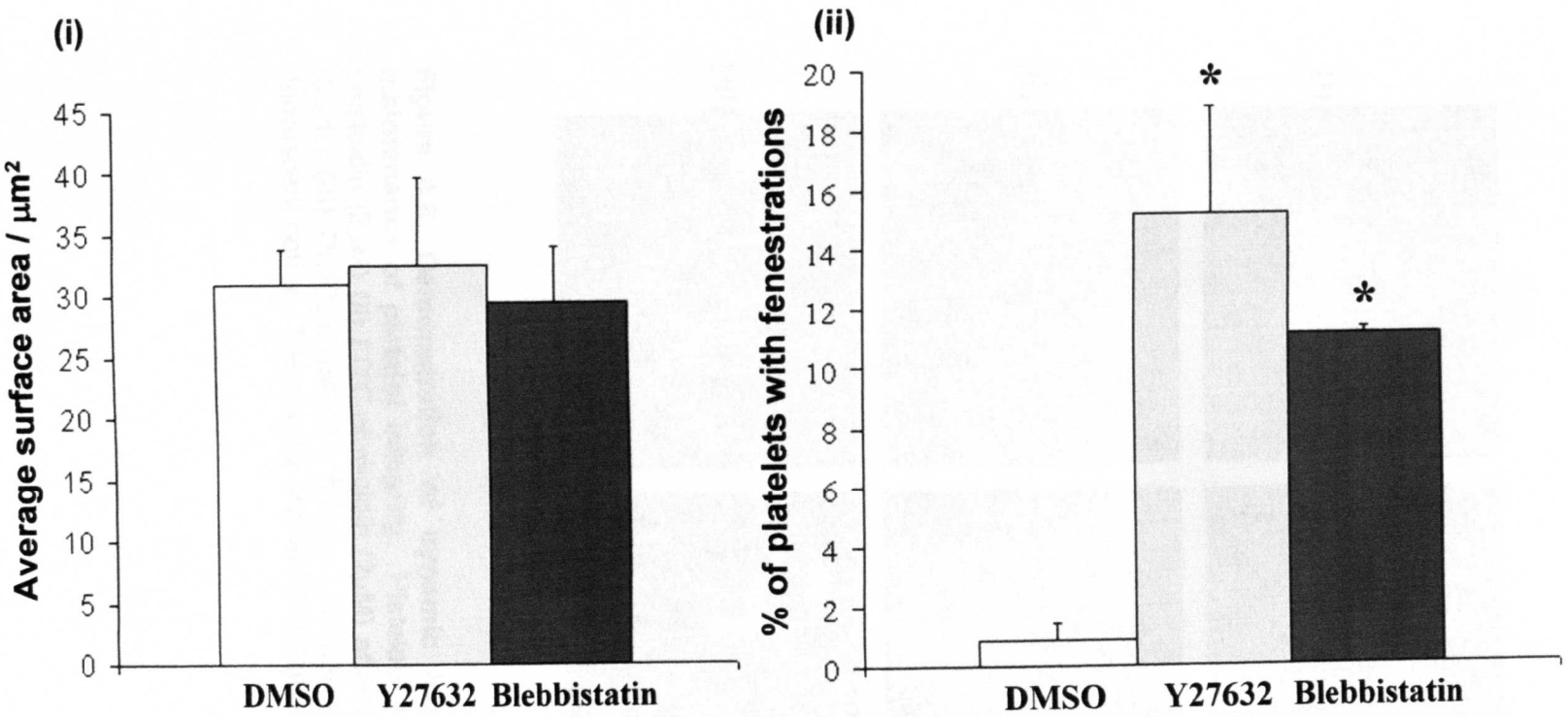

Figure 4.5: Normal lamellipodia formation, but an increase in fenestration formation in the presence of Y27632 and Blebbistatin. Platelets were allowed to spread for 45min on collagen coated coverslips in the presence of apyrase (2 unit/ml) and indomethacin (10μM), and either 0.1% DMSO (white) Y27632 (50μM); grey, or blebbistatin (100μM); black. Platelets were then fixed with paraformaldehye (3.7%) for 30min. **(i)** DIC microscope images were recorded and platelet surface area was quantified. Results represent the mean±s.e.m of a minimum of 100 platelets from one experiment that is representative of three. **(ii)** Platelets were lysed with Triton X-100 (0.2%) and stained with FITC phalloidin (2μM). The percentage of platelets with fenestrations was counted (mean±s.e.m). A minimum of 100 platelets were analysed per experiment and n=3. * $p<0.05$ relative to the control. Statistical analysis completed with ANOVA followed by a Tukey test.

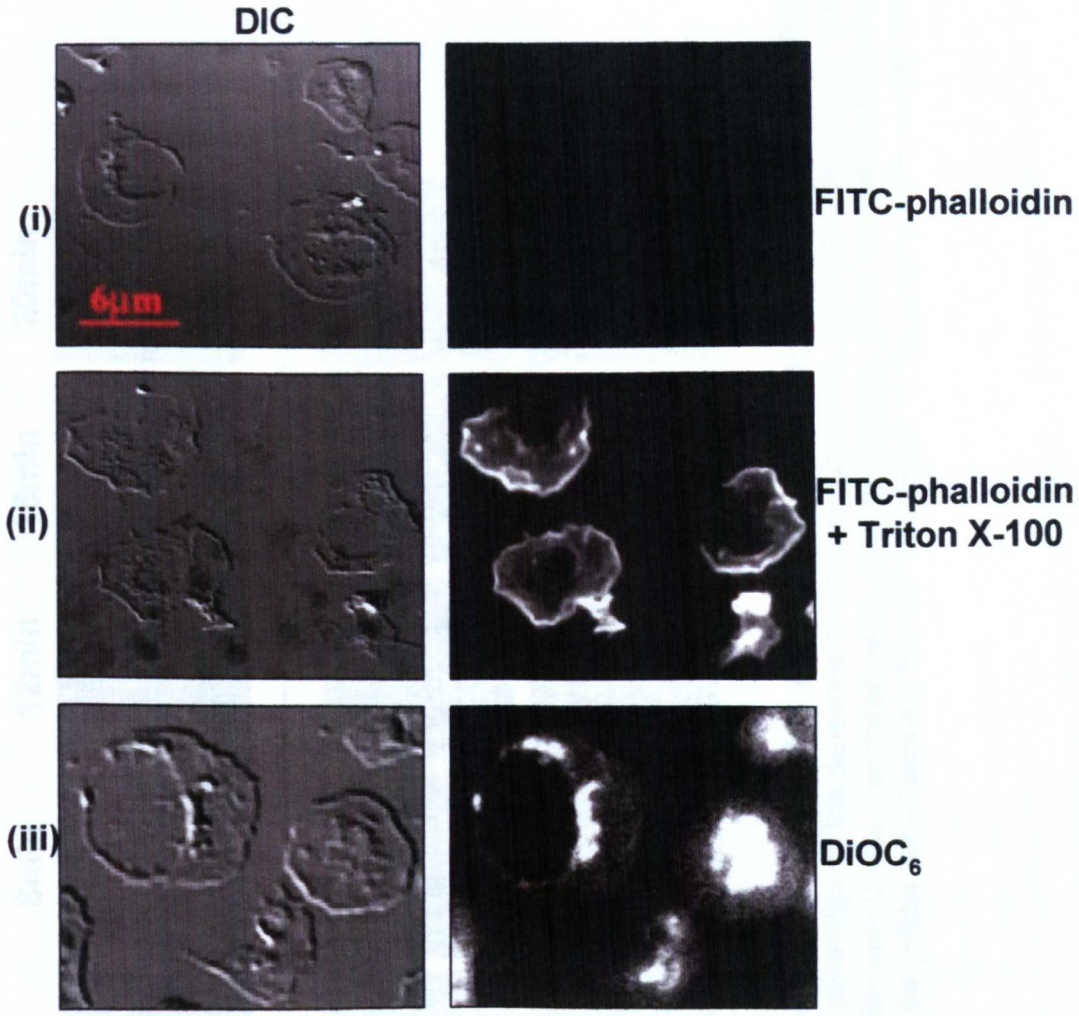

Figure 4.6: Demonstration of dynamic fenestration formation and maintenance of platelet integrity. Platelets were stained with **(i)** FITC-phalloidin (2µM) **(ii)** FITC-phalloidin (2µM) after treatment with Triton X-100 (0.2%) **(iii)** $DiOC_6$ (2µM), for 60min. Images were taken using DIC and fluorescent optics. The results are representative of three experiments.

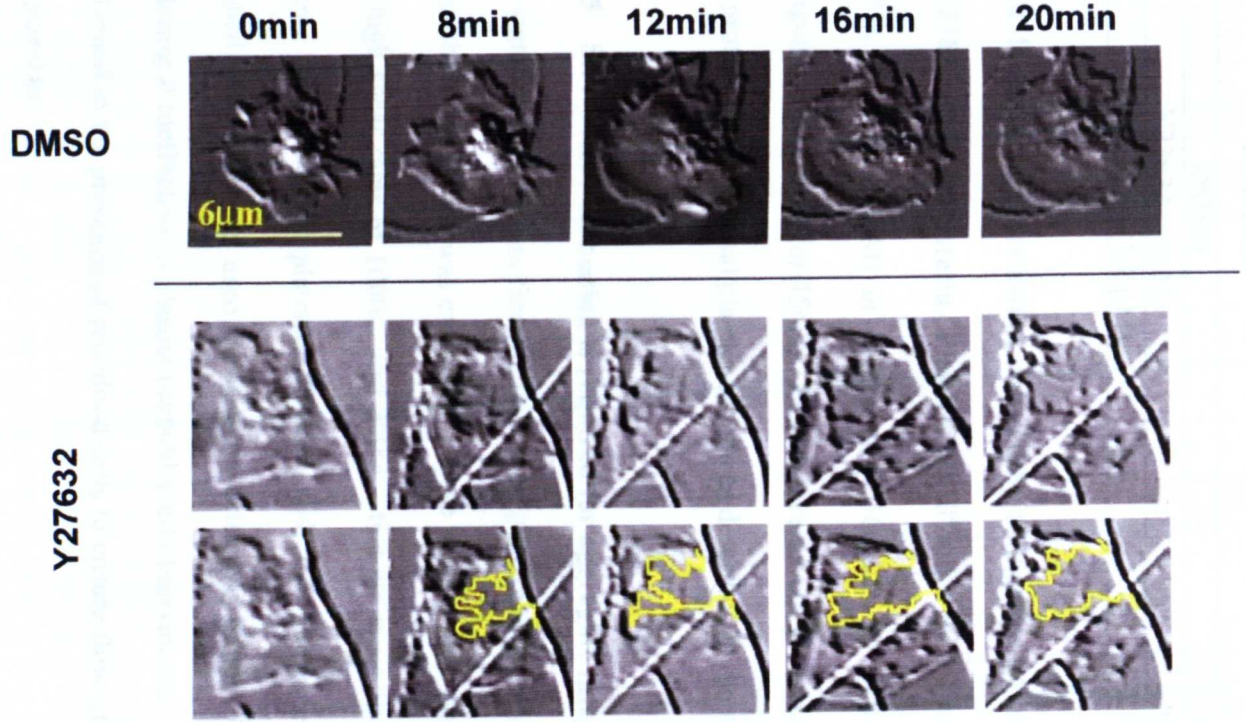

Figure 4.7: Demonstration of dynamic fenestration formation and maintenance of platelet integrity. Platelets were allowed to spread on collagen coated coverslips in the presence of apyrase (2unit/ml), indomethacin (10μM) and either DMSO (0.1%), or Y27632 (50μM). Spreading was monitored using DIC microscopy. The images illustrate the dynamic movement of the membrane, with the yellow lines following the cell membrane of the platelet. The results are illustrative of three experiments

platelets treated with either Y27632 or blebbistatin in comparison to the DMSO control (Table 1).

Treatment	Fold increase in F-actin in comparison to basal
DMSO	1.81±0.03
Y27632 (50μM)	2.04±0.14
Blebbistatin (100μM)	2.04±0.36

Table 1: Actin polymerisation induced by collagen is not altered in the presence of Y27632 and blebbistatin. Platelets ($2x10^8$/ml) were incubated with either DMSO (0.1%), Y27632 (50μM) or blebbistatin (100μM) for 2min before spreading on collagen-coated plates for 45min. F-actin levels were measured and compared to that of a protein matched basal platelet sample as described in the methods.

4.2.5 Stress fibre formation is required for aggregate integrity under flow

The significance of stress fibre inhibition on platelet aggregate formation on collagen under shear conditions was examined. These studies were performed at intermediate and high shear rates of $1000s^{-1}$ and $3000s^{-1}$ that are typically found in the arteriolar system, using washed platelets labelled with the fluorescent dye $DiOC_6$ (2μM). Washed platelets were used because of extensive protein binding of blebbistatin rendering it ineffective in plasma (unpublished observations). The experiments were performed in the presence of red blood cells to ensure flow of platelets at the edge of the chamber.

Inhibition of ROCK and myosin-II caused a significant reduction in mean aggregate height at both shear rates, although the effect was more pronounced at $3000s^{-1}$ (Fig.4.8i&ii). The relative small nature of the inhibition at $1000s^{-1}$ indicates

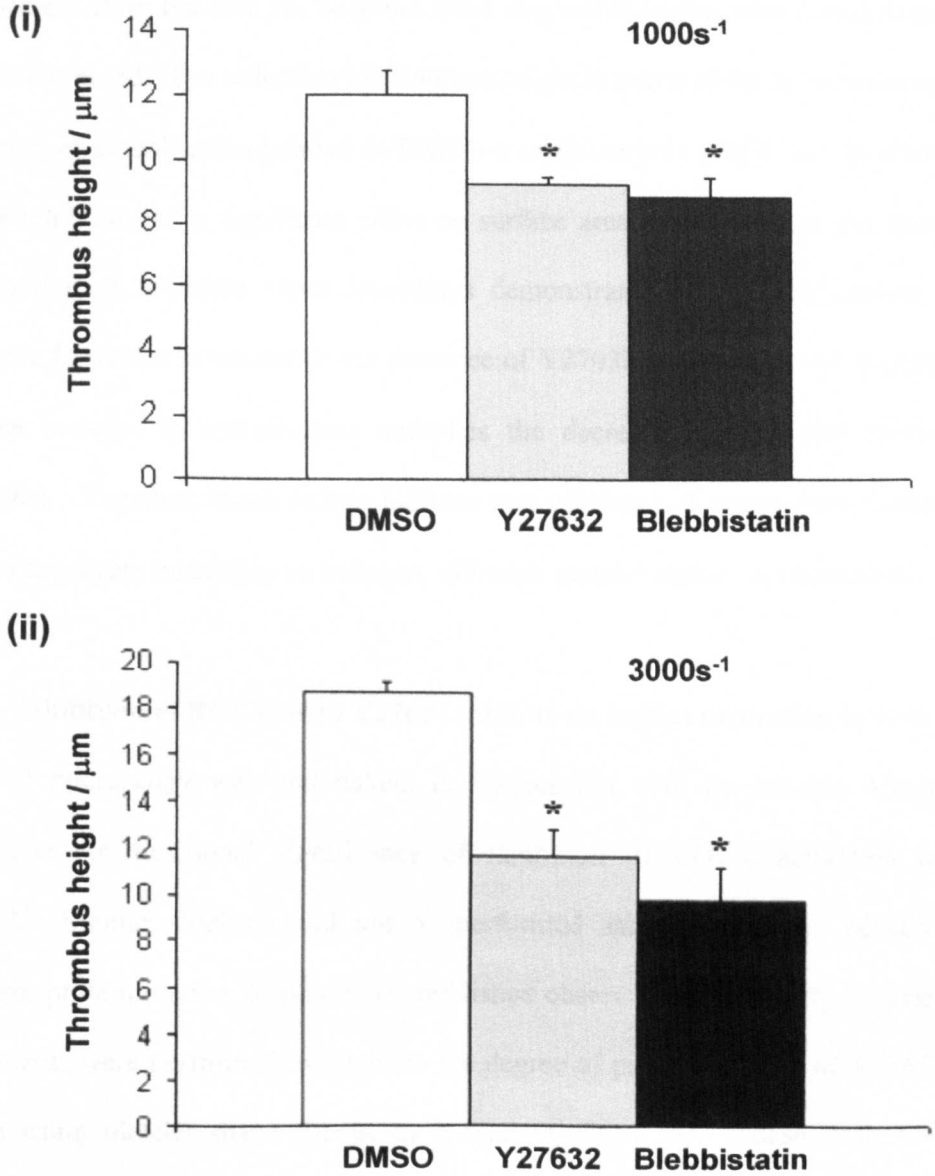

Figure 4.8: Y27632 and blebbistatin inhibit platelet aggregate formation on collagen. Washed platelets were reconstituted with red blood cells and incubated with either DMSO (0.1%), Y27632 (50μM), or blebbistatin (100μM) for 10min at 37°C. Platelets were then flowed over collagen for 3min at either $1000s^{-1}$ or $3000s^{-1}$, before washing with Tyrode`s buffer for 5min. Thrombi were fixed using paraformaldehyde (3.7%) for 30min and incubated with $DiOC_6$ (2μM) overnight at room temperature. Thrombus height at **(i)** $1000s^{-1}$ **(ii)** $3000s^{-1}$ was calculated using confocal microscopy. Statistical analysis completed with an ANOVA, followed by a Tukey test.

that aggregate formation is largely independent of ROCK and myosin-II at intermediate shear but that this becomes more dependent on the actin cytoskeleton as shear is increased. The reduction in thrombus height is mirrored by an increase in the frequency of embolisation relative to DMSO-treated controls (Fig.4.9i). In contrast, neither inhibitor had a significant effect on surface area coverage (data not shown). Examination of real-time video recordings demonstrates that platelet capture and aggregate formation is similar in the presence of Y27632 and blebbistatin suggesting that the increase in embolisation underlies the decrease in aggregate formation (Fig.4.9ii). Together, these results indicate that blockage of stress fibre formation leads to aggregate instability on collagen, although platelet capture is maintained.

4.2.6 Inhibition of ROCK with Y27632 inhibits thrombus formation in vivo

Intravital microscopy was undertaken, in conjunction with Dr Jocelyn Auger, to investigate the functional significance of inhibition of ROCK activation using Y27632. Similar studies could not be performed using blebbistatin because of extensive protein binding in plasma (unpublished observations). Initially, a series of experiments were performed to establish the degree of protein binding of Y27632 in plasma using platelet shape change as a readout (Fig.4.10). These studies were performed in BAPTA-AM loaded platelets to prevent MLCkinase activation by Ca^{2+} elevation. Y27632 (10μM&100μM) caused partial and complete block of shape change to the PAR-4 specific peptide, TRAP, respectively (Fig.4.10i). Similar results were obtained in washed platelets (Fig.4.10ii), demonstrating that Y27632 is minimally protein bound in plasma. This observation was used to estimate the dose of Y27632 to induce inhibition of ROCK following intraperitoneal injection in mice, on the assumption that Y27632 distributes freely in the aqueous compartments in the

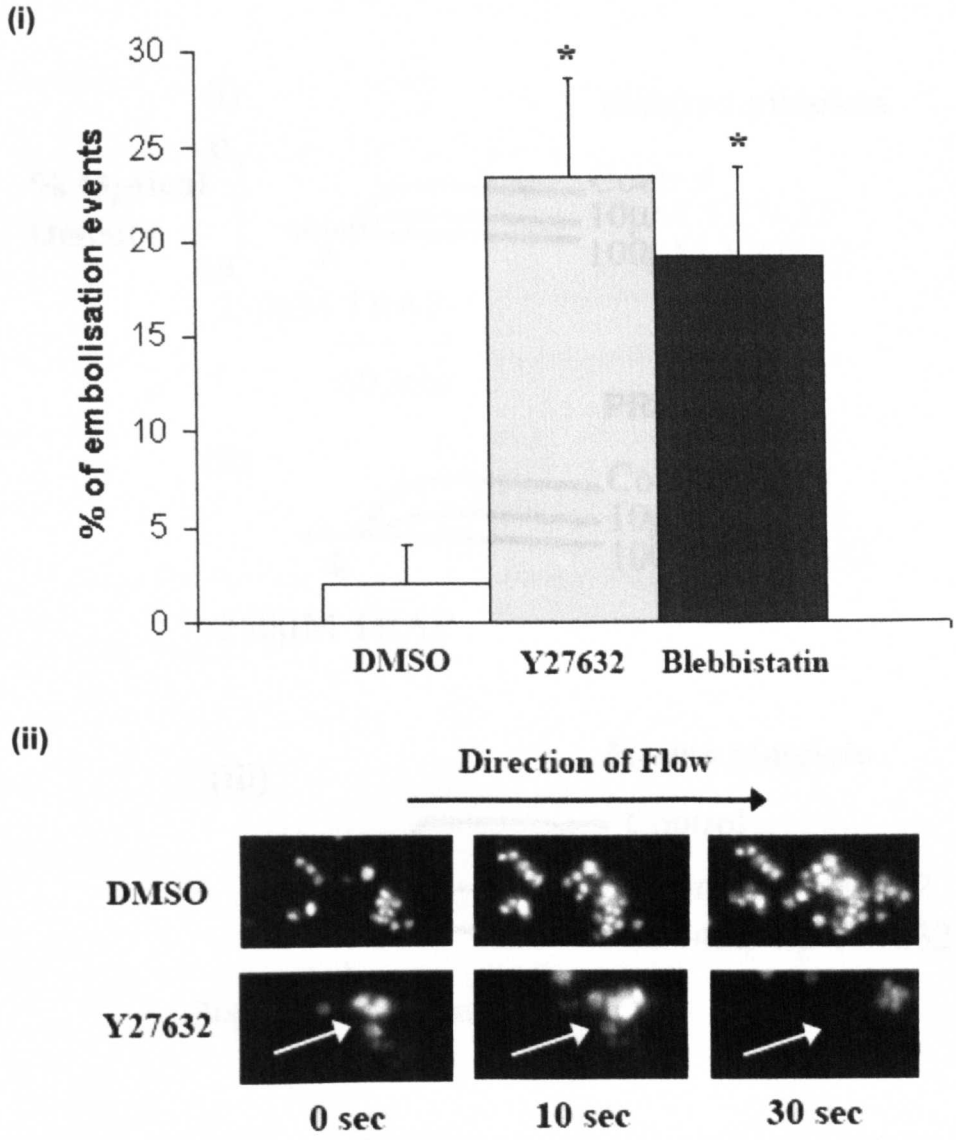

Figure 4.9: Y27632 and blebbistatin inhibit platelet aggregate formation on collagen. Thrombi were formed and imaged as per Fig.4.8. **(i)** The rate of embolisation was measured by analysis of realtime time courses. Embolisation was defined as movement of platelets from original point of contact. Results are shown as mean±s.e.m of three experiments. Statistical analysis completed with an ANOVA, followed by a Tukey test. * $p<0.05$ relative to the control. **(ii)** An example recording illustrating thrombus embolisation in the presence of Y27632 (50μM). The arrow at 0sec illustrates the build up of a small thrombus, which then embolises in the direction of flow. Images are representative of three experiments.

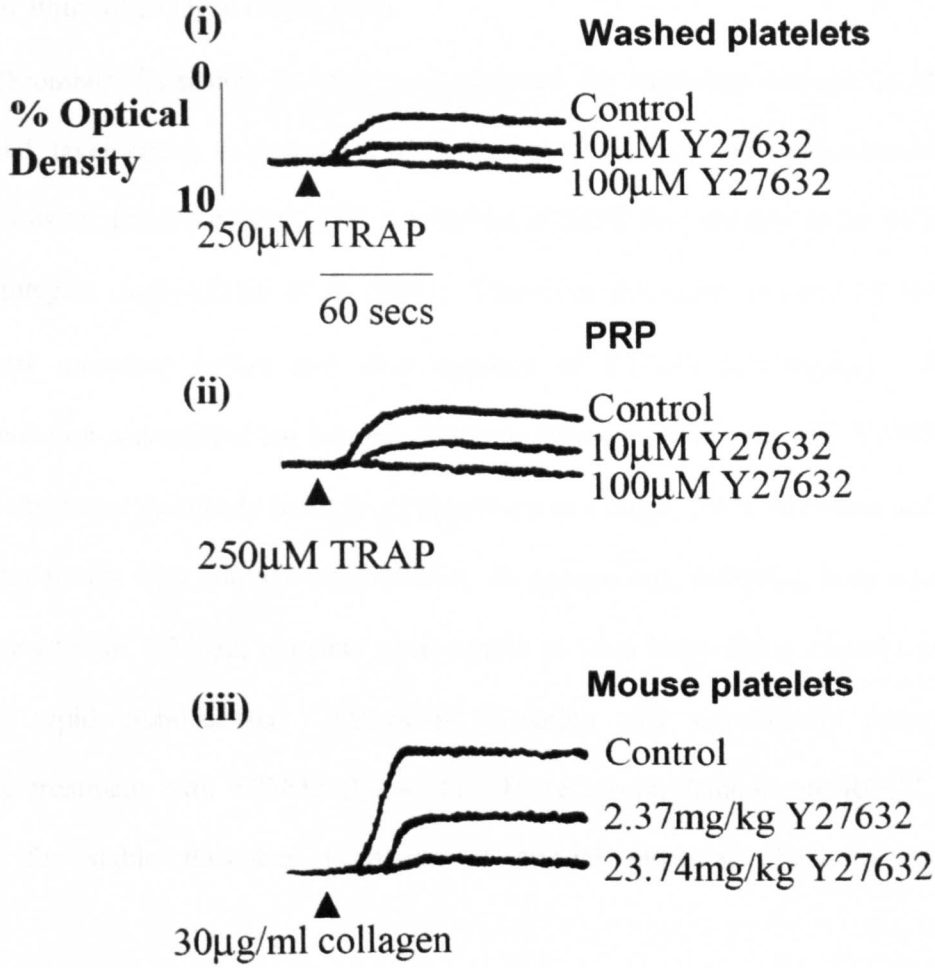

Figure 4.10: Inhibition of platelet shape change after administration of Y27632 to washed platelets and platelet-rich plasma. **(i)** Washed platelets were treated with BAPTA-AM (40μM) for 30min to block the regulation of shape change downstream of Ca^{2+}, and Y27632 (10-50μM) for 2min, before stimulation by TRAP (250μM) (indicated by the arrow). Aggregation was inhibited with αIIbβ3 blocker, integrillin (9μM). **(ii)** Platelets were stimulated in platelet-rich-plasma by TRAP (250μM) (indicated by black arrowhead) under the same conditions as for **(i)**. **(iii)** Mice injected with saline, 2.37, or 23.74mgs/kgs Y27632 for 30min before exsanguination. Platelet rich plasma was prepared and loaded with BAPTA-AM for 30min before stimulation with 30μg/ml collagen (indicated by arrow). All traces are representative of three experiments.

cell. *Ex vivo* analysis of platelet shape change in BAPTA-AM loaded platelets revealed that 2.37mg/kg, and 23.7mg/kg induce partial and full blockade of shape change to 30μg/ml collagen (Fig.4.10iii).

Thrombus formation *in vivo* was analysed by inducing damage to the endothelial layer using a nitrogen dye laser and monitoring realtime thrombus formation using a non-blocking FITC-conjugated antibody that recognises the major platelet integrin $\alpha_{IIb}\beta_3$ (Falati et al, 2004). Thrombus formation induced by laser injury was measured before and after injection of Y27632 (23.7mg/kg). All experimentation was carried out between 5-30min following treatment with Y27632. Fig.4.11 illustrates the steady build up of platelets into a large, stable thrombus under control conditions with minimal embolisation. In comparison, following laser injury in the presence of Y27632, platelets were unable to form large stable thrombi and exhibited rapid embolisation. Thrombus formation was significantly reduced following treatment with Y27632 (Fig.4.11). These results demonstrate ROCK is required for stable thrombus formation at arterial rates of shear *in vivo*.

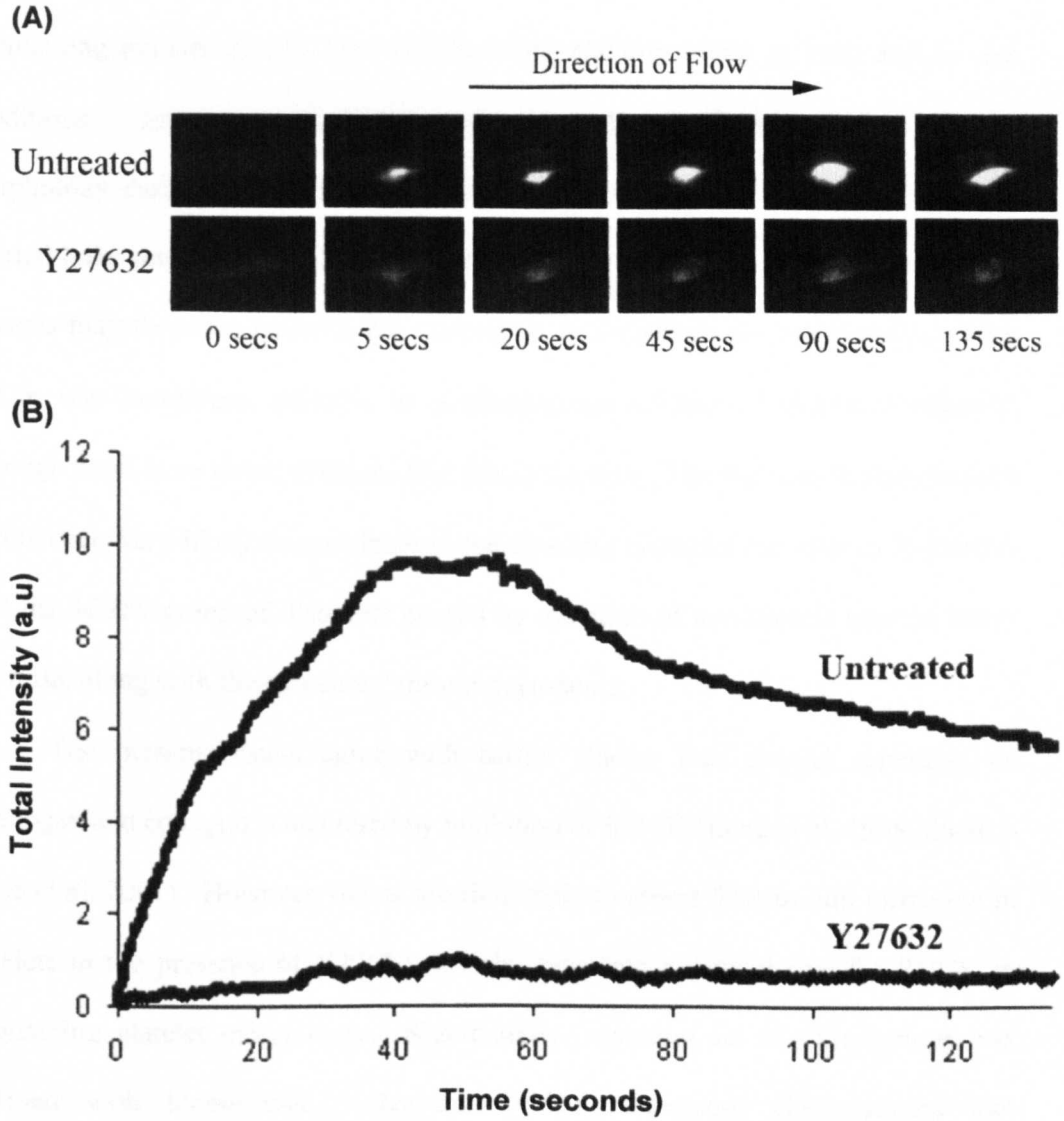

Figure 4.11: Decreased thrombus formation in Y27632 treated mice after laser induced injury. The effect of Y27632 (23.74mgs/kg) on thrombus formation was monitored within the same animal, with an average of five thrombi produced both before and after administration of Y27632 (with recordings between 5–30min following Y27632 injection). **(A)** An example recording illustrating thrombus embolisation in control and Y27632 treated animals (100μM). **(B)** Mean integrated fluoresence of five thrombi pre and post Y27632 injection. The data is representative of experiments on three animals.

4.3 DISCUSSION

The present study demonstrates ROCK and myosin-II play critical roles in maintaining platelet morphology and thrombus stability under *in vitro* and *in vivo* conditions. Inhibition of ROCK and myosin-II significantly affects platelet morphology during platelet spreading on collagen, as demonstrated by formation of internal holes and splits (fenestrations) and blockade of stress fibre formation. These changes may therefore underlie the increase in embolisation observed under *in vitro* and *in vivo* conditions, possibly as a consequence of loss of structural integrity, although there is no direct evidence that this is the case. The increase in embolisation is, however, very likely to contribute to the bleeding diathesis that is seen in patients with the *MHY9* group of disorders caused by mutation of non-muscle myosin heavy chain IIa, along with the associated thrombocytopenia.

The present results agree with earlier studies that platelet spreading on fibrinogen and collagen is unaltered by inhibition of ROCK (Leng et al, 1998; Suzuki-Inoue et al, 2001). However, this is the first study to report fenestration formation in platelets in the presence of Y27632, thereby revealing a critical role for ROCK in maintaining platelet morphology. Significantly, a similar set of observations was obtained with blebbistatin. Real-time video microscopy demonstrated that fenestration formation in the presence of Y27632 and blebbistatin was not simply due to a loss of platelet integrity, as the change in morphology was continuous, and moreover actin fibres could not be stained with the membrane-impermeable toxin, FITC-phalloidin, without prior permeabilisation. Fenestration formation was not due to a reduction in F-actin production. The present results are similar to the effect of RhoA inhibition by C3 toxin in Swiss 3T3 cells (Rottner et al, 1999). In this study,

the presence of fenestrations is readily apparent in C3 toxin-treated but not in control fibroblasts that have been allowed to undergo spreading.

The possibility that the reduction in thrombus stability is due to the loss of structural integrity is consistent with the proposal by Canobbio et al (2005) that GTPase incorporation into the cytoskeleton is vital for late events of thrombus formation. On the other hand, thrombus formation is regulated by multiple factors and it is possible that additional actions of ROCK and myosin-II, rather than fenestration formation or stress fibre inhibition, contribute to this instability. For example, ROCK inhibition by Y27632 is reported to reduce aggregate formation in a cone-and-plate visocometer and adhesion of platelets to VWF within a high shear environment (Schoenwaelder et al, 2002). In addition, it is possible that the inhibitors have other inhibitory actions that have contributed to the reduction in thrombus stability. For example, blebbistatin causes a partial reduction in secretion and aggregation but this cannot explain the reduction in thrombus stability as Y27632 had a negligible effect on these two responses. On the other, ROCK has been implicated in a number of other pathways that could have potentially contributed to the thrombus stability that was seen *in vivo*, including expression of tissue factor and pro-coagulant activity (Kunedia et al, 2003; Rolfe et al, 2005). Pro-coagulant activity is known to play an important role in the laser-induced injury model alongside that of the thrombin receptor, PAR4, and the collagen receptor, GPVI (Mangin et al, 2006; Dubois et al, 2006). Therefore the contribution of coagulation to thrombus formation after laser induced injury may be reduced in the presence of Y27632. It is also possible that the contribution of ROCK to contraction of vascular smooth muscle, reduction in blood pressure, nitric oxide synthesis (eNOS), and maintenance of vascular tone effects thrombus formation *in vivo* (Budzyn et al, 2006) has contributed

to the decrease in thrombus formation. Importantly, however, inhibition of these responses is unable to account for the increase in embolisation that is seen under *in vitro* flow conditions that are seen in the presence of Y27632.

The effect of Y27632 *in vivo*, is further complicated by Y27632 affecting other cells in the bloodstream, including endothelial cells. ROCK inhibition is associated with an increase in nitric oxide synthesis from endothelial cells, which could drive vasodilation and reduce platelet activation (Wolfrum et al, 2004). ROCK inhibition also leads to a reduction in migration, cell adhesion, and cell permeability.

The present results can be compared with the recent observations of McCarty et al (2005) who demonstrated increased platelet embolisation on immobilised collagen or immobilised VWF/thrombin in Rac-$1^{-/-}$ platelets. Rac-$1^{-/-}$ platelets cannot form lamellipodia and therefore also have altered stress fibre formation which could have contributed to the thrombus instability observed in the absence of the small GTPase. Therefore, our results are consistent with stable thrombus formation requiring both lamellipodia formation and stress fibre generation and that Rac and Rho co-ordinate with each other to mediate stable thrombus formation *in vivo* under flow.

In conclusion I have demonstrated a requirement for ROCK and myosin-II in maintaining the platelet cytoskeleton and thrombus stability on collagen at arterial rates of flow. These studies further underscore the role of the cytoskeleton in contributing to thrombus formation *in vivo*. Additionally, this study emphasises the potential of a ROCK inhibitor in the treatment of hypertension and its associated thrombotic complications, as the inhibitor would have both an antihypertensive and antihrombotic action.

Chapter 5

A major role for Scar/WAVE-1 downstream of GPVI in platelets

5 GENERAL INTRODUCTION

5.0 Summary

WASP family proteins are implicated within platelet lamellipodia formation. The aim of this chapter was to identify the role of WAVE-1 within platelet activation and thrombus formation at high shear. This work led to identification that WAVE-1 plays a major role in lamellipodia, and platelet activation downstream of GPVI. GPCR signalling pathways were unaffected in WAVE-1$^{-/-}$ platelets, indicating either WAVE-1 was not required or that WAVE-2 could compensate for the lack of WAVE-1.

5.1 Introduction

Actin reorganisation is a consequence of platelet activation. F-actin levels almost double after platelet activation as the platelet changes from a discoid shape to a sphere, and then forms filopodia and lamellipodia. These distinct actin reorganisations are mediated via multiple different signalling pathways.

The Rho GTPase family plays a fundamental role, with Cdc42, Rac, and RhoA implicated in the formation of filopodia, lamellipodia, and stress fibres respectively (Nobes et al, 1995). Rac-1 was recently identified as fundamental for lamellipodia formation and F-actin production in platelets. In addition Rac-1$^{-/-}$ cannot form stable thrombi, implicating lamellipodia formation in thrombus formation (McCarty et al, 2005).

WAVE is an important Rac target. WAVE is important in lamellipodia formation, as it is activated by Rac, leading to WAVE binding the Arp2/3 complex. Removal of WAVE leads to the removal of lamellipodia (Yamazaki et al, 2003). There are three isoforms of WAVE, of which WAVE-1 and -3 are mainly neural, and

WAVE-2 is ubiquitous. Due the redundancy in the WAVE structures, it is possible that the isoforms could compensate for each other in vivo. This is demonstrated by the viability of the WAVE-1$^{-/-}$ mouse.

Platelets express WAVE-1, -2, and possibly -3 (Kashiwagi et al, 2005; Oda et al, 2005). WAVE-1 may play a role in megakaryocytes, as WAVE-1 is upregulated by five fold during megakaryocyte differentiation. WAVE-2 expression is maintained at a constant level (Kashiwagi et al, 2005). WAVE-1 is also implicated within migration through the ECM (Suetsugu et al, 2002) a process that requires metalloprotease activity. This process is required by megakaryocytes to allow platelet budding into the vasculature.

The aim of this Chapter was to identify the role of WAVE-1 within platelet activation, spreading and aggregate formation. I demonstrate that WAVE-1 is important within the GPVI signaling cascade, but plays a much smaller role within GPCR signaling. In addition WAVE-1 does not play a role within aggregate formation *in vitro*.

5.2 RESULTS

5.2.1 Confirmation of expression of WAVE isoforms present within human and murine platelets.

In order to identify the role of WAVE within platelets, it was necessary to identify the WAVE isoforms present within platelets. Therefore wild-type and WAVE-1$^{-/-}$ platelet lysates were Western blotted with WAVE specific antibodies, alongside human lysates. It is clear that human and murine platelets contain both WAVE-1 and -2 (Fig.5.1). WAVE-3 could not be identified in human or murine samples using available antibodies. WAVE-1$^{-/-}$ platelets however, contain just WAVE-2 (Fig.5.1C&D). Importantly, the expression of WAVE-2 is not increased within WAVE-1$^{-/-}$ platelets, indicating WAVE-2 is not upregulated to compensate for the lack of WAVE-1.

5.2.2 Platelet production is maintained within WAVE-1$^{-/-}$ mice

The mean platelet volume and platelet number of the WAVE-1$^{-/-}$ mice was investigated in view of the defect in platelet production that has been described in the absence of WASP (Ochs et al, 1980). Table.1 demonstrates that both platelet number and volume is not significantly different within the WAVE-1$^{-/-}$, to a wild-type control.

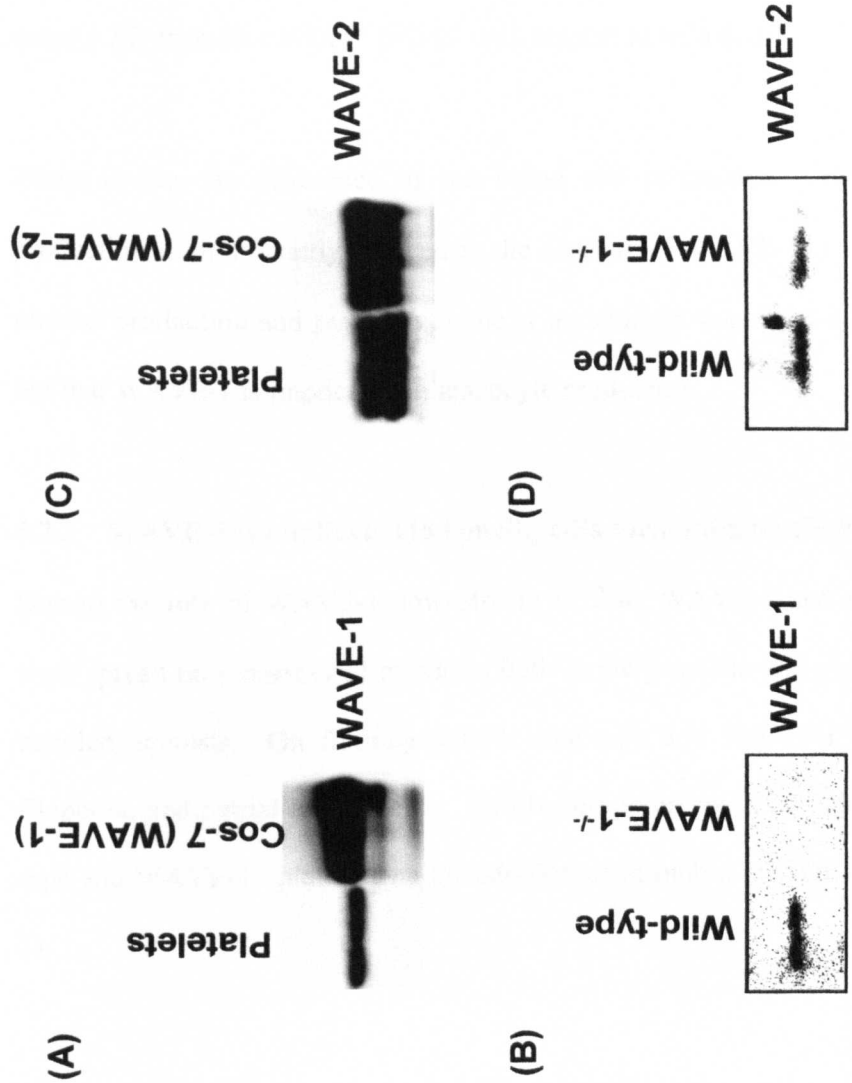

Figure 5.1: WAVE isoforms are expressed in human and murine platelets. Equal amounts of human **(A&C)** and murine wild-type and WAVE-1$^{-/-}$ **(B&D)** platelet lysates were analyzed for WAVE expression using WAVE-1 and WAVE-2 antibodies. Lysates from Cos-7 cells transfected with a vector containing **(A)** WAVE-1 or **(C)** WAVE-2 were included as a positive control.

Parameter	Wild-Type	WAVE-1
Leucocytes 10^6/ml	3.39±0.67	1.09±0.21*
Erythrocytes 10^6/ml	3.54±0.14	3.39±0.57
Platelets, 10^6/ml	329.2±18.9	260.4±47.8
mean Platelet volume, fL	5.24±0.07	5.28±0.16

Table 1: Haematological parameters of wild-type and WAVE-1$^{-/-}$ mice. Samples of whole blood (50µl) were analysed using an ABX Micros 60. Data are presented as mean ± SD from five mice. * $p<0.01$ with respect to wild-type.

There is also no difference in red blood cell production. However, leucocyte production is significantly reduced in the absence of WAVE-1$^{-/-}$. This suggests that platelet production and platelet volume is maintained as normal in WAVE-1$^{-/-}$ mice, but that WAVE-1 is implicated in leucocyte production.

5.2.3 WAVE-1 is implicated in lamellipodia formation on CRP and laminin

Due to the role of WAVE-1 downstream of Rac, WAVE-1 and wild-type platelets were spread on a number of matricies both in the presence and absence of G-protein coupled agonists. On fibrinogen both wild-type and WAVE-1$^{-/-}$ platelets formed filopodia, and partial lamellipodia. Full lamellipodia could be induced both in wild-type and WAVE-1$^{-/-}$ platelets by the addition of thrombin or ADP (Fig.5.2 and Table 2).

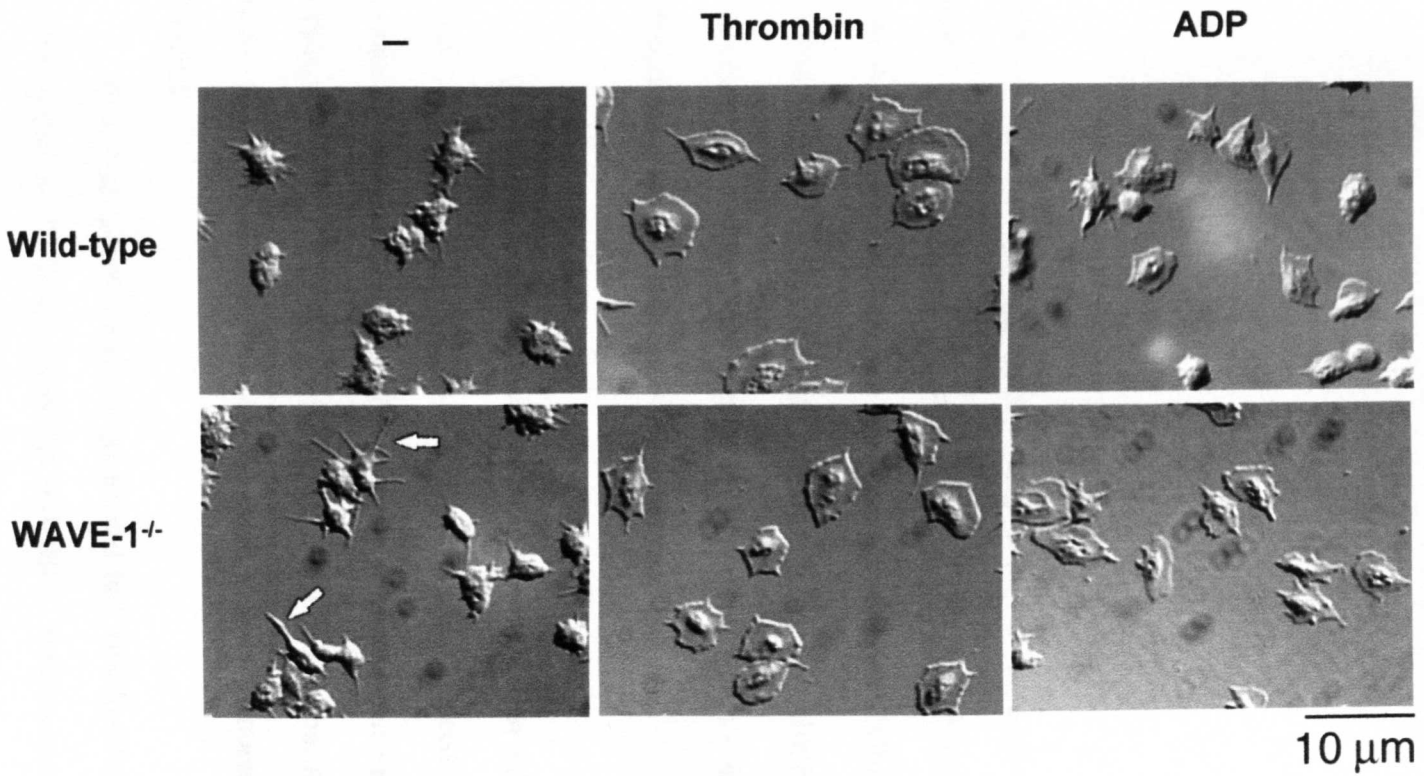

Figure 5.2: Role of WAVE-1 in $\alpha_{IIb}\beta_3$-mediated murine platelet spreading. Purified wild-type and WAVE-1$^{-/-}$ murine platelets ($2x10^7$/ml) were placed on coverslips coated with fibrinogen with or without 1unit/ml thrombin for 45min at 37°C, fixed with paraformaldehyde (3.7%), and imaged using DIC microscopy. In separate experiments, platelets were treated with 10μM ADP. Note the elongated filopodia extended by a portion of the WAVE-1$^{-/-}$ platelets on fibrinogen under non-stimulated conditions as indicated by the arrows. Results are representative of at least three experiments.

Surface	Treatment	Wild-type	WAVE-1$^{-/-}$
Fibrinogen	-	14.7±0.12	14.2±0.28
Fibrinogen	ADP	18.4±0.18	18.1±0.16
Fibrinogen	Thrombin	24.9±0.13	24.6±0.22
Collagen	-	18.6±0.22	18.0±0.08
Collagen	ADP	18.5±0.2	18.7±0.11
Collagen	Thrombin	18.8±0.17	18.2±0.15
Laminin	-	24.8±0.21	15.5±0.18*
Laminin	ADP	25.2±0.11	23.8±0.1
Laminin	Thrombin	25.7±0.19	24.0±0.12
CRP	-	21.9±0.18	15.8±0.21*
CRP	ADP	23.8±0.21	24.3±0.09
CRP	Thrombin	24.6±0.19	25.1±0.18

Table 2: Average platelet surface area in wild-type and WAVE-1$^{-/-}$. Purified human and wild-type and WAVE-1$^{-/-}$ murine platelets ($2x10^7$/ml) were placed on coverslips coated with fibrinogen, collagen, laminin, and CRP for 45min at 37°C. In selected experiments, platelets were treated with thrombin (1unit/ml) or ADP (10μM). Values are reported as follows: platelet surface area (μm^2) = mean±SEM of at least 200 platelets. * $p<0.05$ with respect to wild-type.

Surface area analysis indicated no difference between wild-type and WAVE-1$^{-/-}$ mice. Interestingly, 26.2±0.3% WAVE-1$^{-/-}$ platelets induced elongated filopodia in the absence of thrombin and ADP, which are at least as twice as long as seen within wild-type platelets. This is a similar result to that seen within Rac1$^{-/-}$ (McCarty et al, 2005), and is presumably due to increased F-actin monomer incorporation into filopodia.

Wild-type platelets form filopodia and full lamellipodia on collagen, laminin or CRP (Fig.5.3). Strikingly, although WAVE-1$^{-/-}$ platelets spread fully on collagen, they do not form lamellipodia on laminin (which signals via α6β1, and GPVI) (Inoue

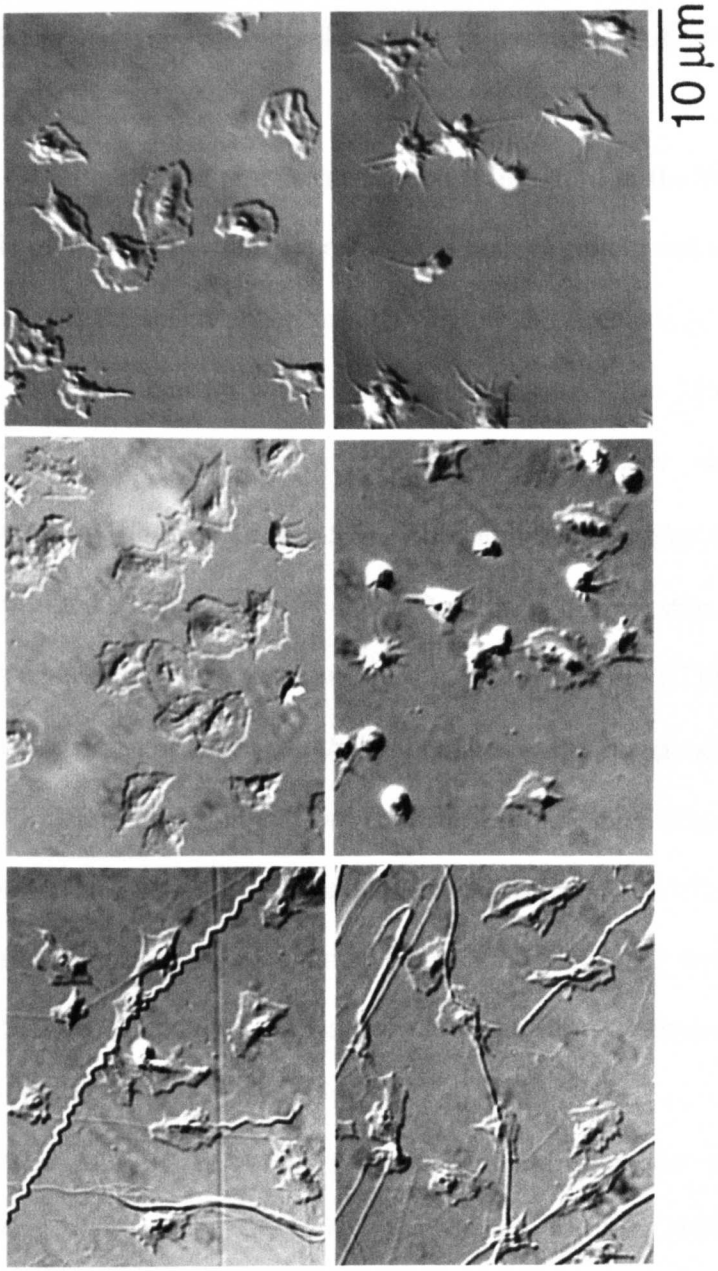

Figure 5.3: Role of WAVE-1 in GPVI-murine platelet spreading. Purified wild-type and WAVE-1$^{-/-}$ murine platelets ($2x10^7$/ml) were placed on collagen, laminin or CRP coverslips coated for 45min at 37°C, fixed with paraformaldehyde (3.7%), and were imaged using DIC microscopy. Images are representative of at least three experiments.

et al, 2005) and CRP (which signals via GPVI). Interestingly, lamellipodia formation can be induced in the presence of thrombin or ADP. However, WAVE-1$^{-/-}$ filopodia formation is unchanged on all surfaces. Therefore, these results suggest a role for WAVE-1 in mediating lamellipodia formation downstream of GPVI but not GPCRs.

5.2.4 GPVI mediated platelet activation is impaired in the WAVE-1$^{-/-}$ mice

The role of WAVE-1 within aggregation to both G protein and tyrosine kinase based receptors was measured either through single platelet counting, or by measuring the change in optical density with a Born aggregometer. The latter demonstrated that WAVE-1$^{-/-}$ platelets showed a marked delay of shape change and impaired aggregation to 1μg/ml CRP (Fig.5.5). The inhibition of aggregation to 1μg/ml CRP, was confirmed by single platelet counts 2min after agonist stimulation (75.1%±5.8% wild-type platelets within aggregates v. 42.3±5.2% WAVE-1$^{-/-}$ platelets). However, a high concentration of CRP (10μg/ml) stimulated shape change and full aggregation of WAVE-1 platelets, a result that was confirmed by single platelet counting (91.9±3.0% wild-type platelets within aggregates v. 92.5±4.2% WAVE-1$^{-/-}$ platelets within aggregates). In contrast stimulation of WAVE-1$^{-/-}$ platelets with either low or high concentrations of collagen did not induce a significant difference between wildtype and WAVE-1$^{-/-}$ mice responses (Fig.5.5).

The role of WAVE-1 in alpha granule secretion was investigated by monitoring of P-selectin exposure. Surprisingly analysis indicated that P-selectin exposure was decreased by between 30-50% for CRP, thrombin and collagen, although the reduction in P-selectin exposure to CRP was greater than that to thrombin (Fig.5.6). Therefore, this raises the possibility that the WAVE-1$^{-/-}$ mice had reduced P-selectin expression, or there could be a secretion defect associated with the

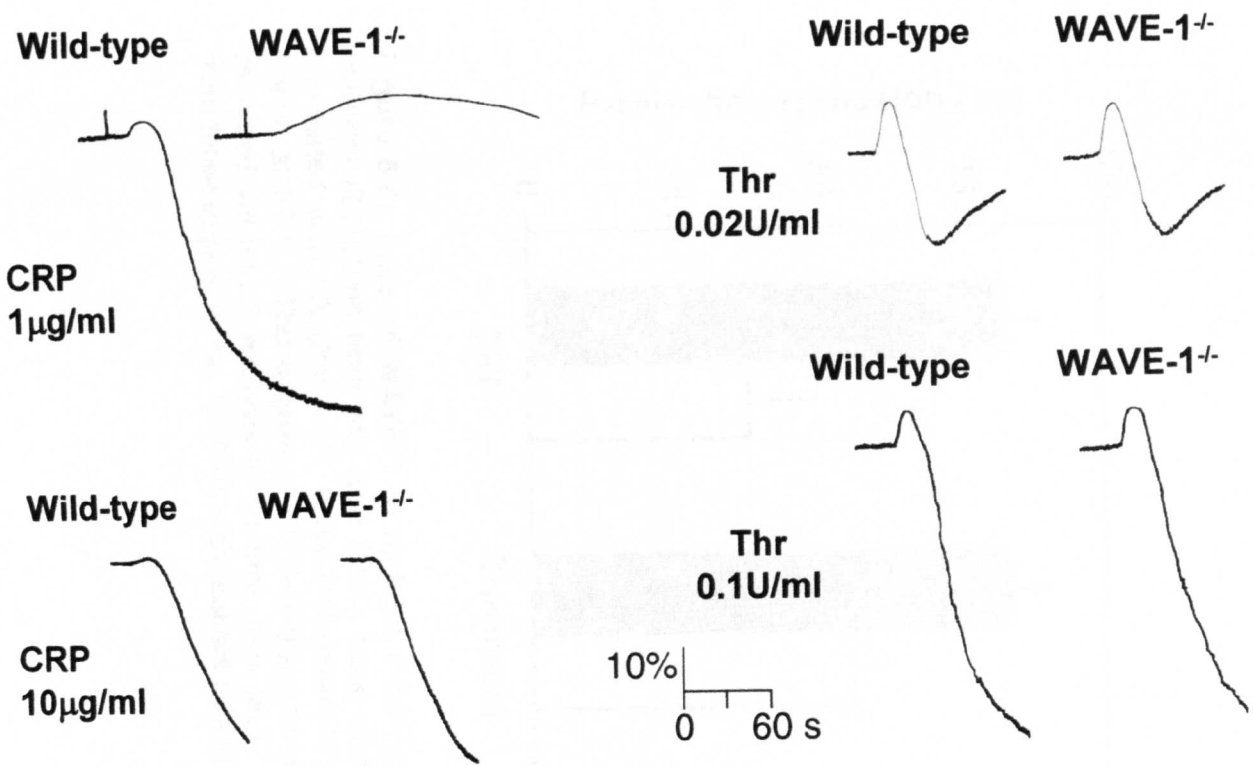

Figure 5.4. Role of WAVE-1 in platelet aggregation to GPVI and GPCR agonists. Washed platelets ($2x10^8$/ml) from wild-type (WT) and WAVE-$1^{-/-}$ mice were stimulated with **(A)** 1-10μg/ml CRP or **(B)** 0.02-0.1unit/ml thrombin (thr), and the change in optical density indicative of aggregation recorded. Images representative of one experiment of three.

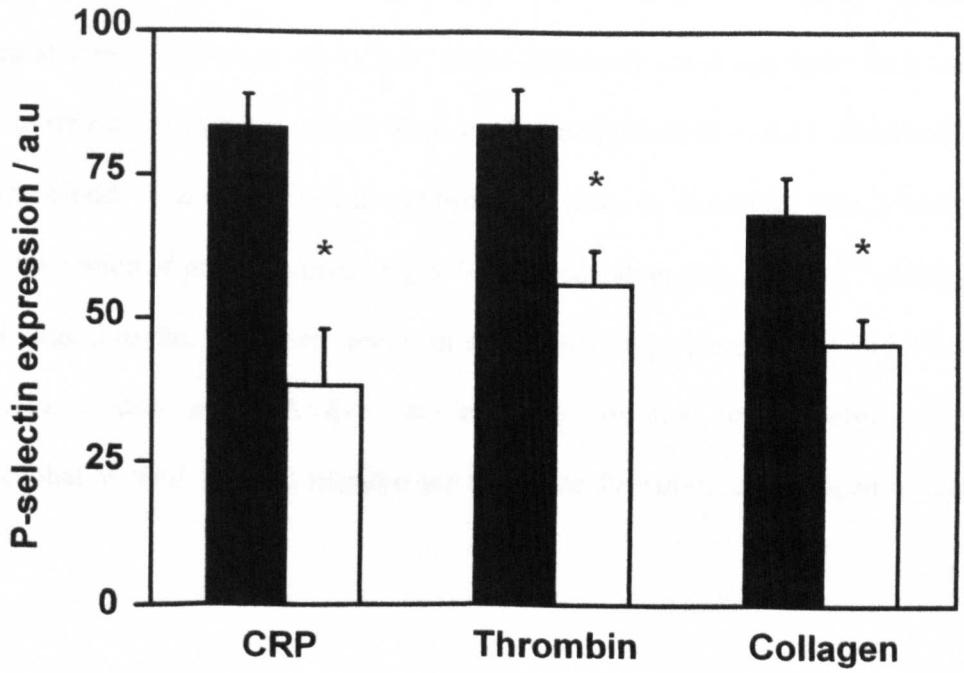

Figure 5.5:. Role of WAVE-1 in platelet P-Selectin exposure. Washed platelets ($2x10^7$/ml) from wild-type (black bars) and WAVE-$1^{-/-}$ (white bars) stimulated with 10μg/ml CRP, 0.1unit/ml thrombin or 30μg/ml collagen for 15min at 37°C. The degree of FITC-conjugated anti P-Selectin mAb was recorded via FACS analysis in arbitary units (au). Values are mean±SEM from three experiments. *$p<0.01$ with respect to wild-type.

WAVE-1$^{-/-}$mice. Unfortunately the low yield of platelets from the WAVE-1$^{-/-}$ mice did not allow for the further investigation of this effect.

5.2.5 The role of WAVE-1 in aggregate formation under high shear.

The functional role of WAVE-1 platelets within thrombus formation was investigated using an *in vitro* flow based assay. Aggregate formation on collagen could be monitored using fluorescent microscopy at high shear. Whole blood was flowed over collagen at 1000s^{-1} for 4min. WAVE-1$^{-/-}$ blood generated stable aggregate formation with densely packed platelets, which were similar in appearance to those observed in wild-type blood. The similarity was confirmed by analysis of surface area coverage and quantification of protein lysates (Fig.5.7A&B) indicating that WAVE-1$^{-/-}$ platelets formed large thrombi, that were similar in size to wild-type platelets (51.7±5.7% v. 60.0±5.7%, control and WAVE-1$^{-/-}$ surface area coverage respectively). This indicates that WAVE-1 is not required for aggregate formation on collagen at high shear.

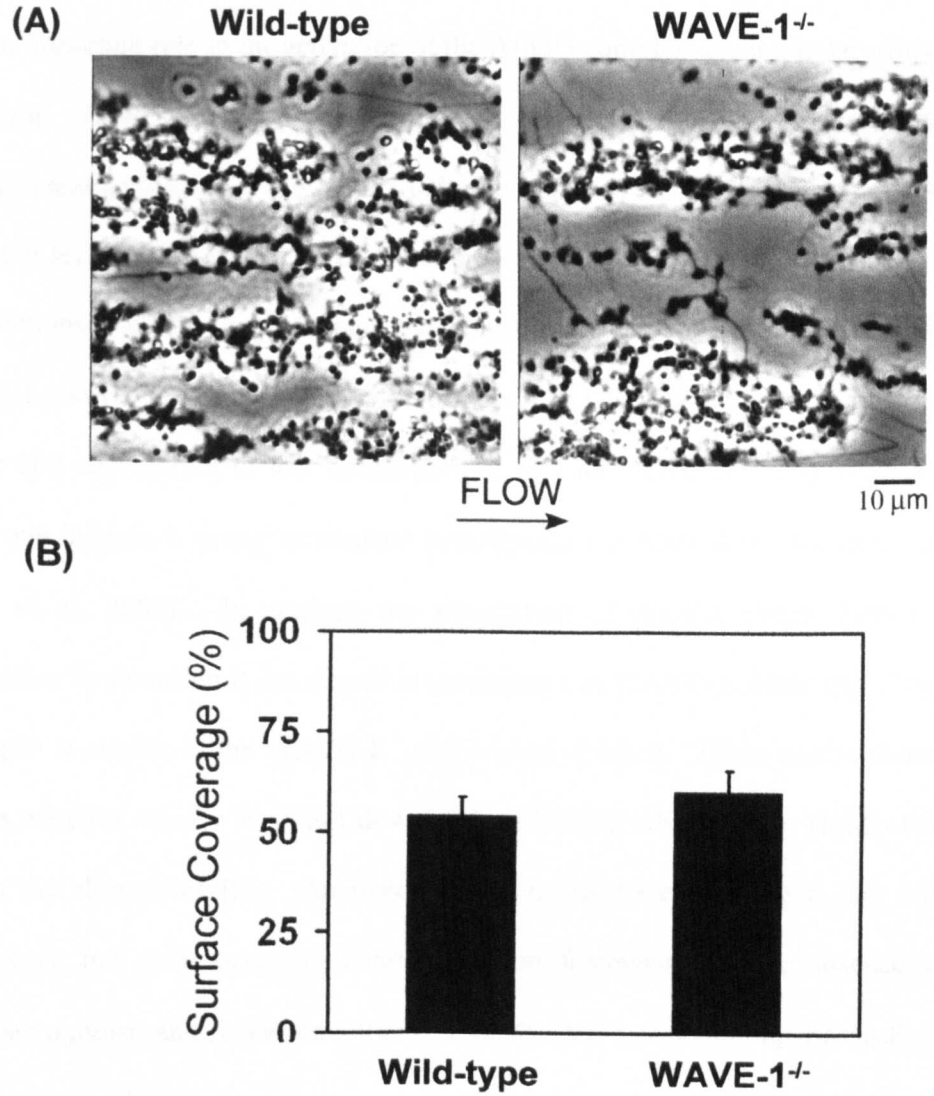

Figure 5.6: Role of WAVE-1 in platelet adhesion and aggregate stability on collagen under flow. Mouse blood anticoagulated with P-Pack and heparin was perfused through collagen coated microslide at a shear rate of 1000s-1 for 4min follwed by modified Tyrode`s buffer for 3min to remove non-adherent platelets. Slides were then visualised using phase-contrast microscopy. **(A)** Representative images of platelet adhesion from wild-type (left panel) and WAVE-1$^{-/-}$ (right panel) mice are shown. Images are representative of three experiments. **(B)** Platelet adhesion results are expressed as mean±SEM of surface area covered by platelets. Work completed by Dr.J.Auger.

5.3 DISCUSSION

WASP family proteins have been implicated as downstream effectors of Rac, and thus have an important role in the activation of the Arp2/3 complex leading to lamellipodia formation. However, WASP, is reported to play little role within platelet actin dynamics and activation of the Arp2/3 complex (Falet et al, 2002, Gross et al, 1999) while the levels of N-WASP in platelets are extremely low. Within this chapter I have demonstrated that both human and mouse platelets contain WAVE-1 and -2. WAVE-1 was required for lamellipodia formation on CRP and laminin, and for shape change and aggregation to low concentrations of CRP. CRP is highly specific for GPVI, and laminin is shown to mediate lamellipodia formation downstream of GPVI (Inoue et al, 2005). In contrast the stimulation of platelet shape change and aggregation by thrombin is not altered in the absence of WAVE-1, while spreading on fibrinogen is similar in the WAVE-$1^{-/-}$ and control platelets. These results therefore imply a selective role for WAVE-1 downstream of GPVI, which maybe similar to that seen in the absence of Rac. An important caveat to the conclusion is that mouse platelets do not form extensive lamellipodia on fibrinogen (in the absence of a secondary agonist) and so a more minor role for WAVE-1 in signalling via $\alpha_{IIb}\beta_3$ may have been missed.

The inability of WAVE-$1^{-/-}$ platelets to form lamellipodia on CRP, or laminin, and the defect identified within shape change and aggregation to low concentrations of CRP indicates a GPVI signalling defect. However, this is at odds to the normal spreading identified on collagen. This may be due to collagen signalling via $\alpha_2\beta_1$, or that binding to collagen causes a net increase in the affinity for GPVI (Watson et al, 2005), enabling collagen to act as a more powerful agonist thereby masking the role of WAVE-1. This argument may not apply to laminin, which requires binding to

$\alpha_6\beta_1$, in order to enable its interaction with GPVI, as laminin has a tenfold lower affinity for GPVI, or $\alpha_6\beta_1$ may induce a smaller signal than $\alpha_2\beta_1$.

These results indicate that GPVI may induce lamellipodia through WAVE-1, and GPCR agonists via WAVE-2. If this is the case then this suggests that WAVE-1 is involved in tyrosine phosphorylation cascades. Tyrosine phosphorylation of WAVE has been identified in other cell types (Miki et al, 1999), but platelets lack the Abl kinases involved within tyrosine phosphorylation of WAVE and I was not able to demonstrate tyrosine phosphorylation of WAVE downstream of CRP (unpublished data). This indicates that the regulation of WAVE may differ depending on the cell type. Interestingly the observation that platelets undergo full spreading on collagen indicates in the absence of WAVE-1, collagen can also regulate WAVE-2.

The removal of WAVE did not prevent either filopodia or lamellipodia formation on fibrinogen. Indeed a proportion of platelets had abnormally long filopodia, as was also seen with Rac-$1^{-/-}$ platelets on fibrinogen (McCarty et al, 2005). This could indicate a shift in the incorporation of actin filaments into filopodia from lamellipodia, as previously reported in gelsolin$^{-/-}$ platelets (Falet et al, 2000). Gelsolin is thought to be important in the generation of the new barbed ends that are required for Arp2/3 complex induced lamellipodia formation (Falet et al, 2005).

It has been recently reported Rac-1 is critical for lamellipodia formation in platelets. In addition Rac-$1^{-/-}$ platelets do not form stable thrombi on collagen at high shear (McCarty et al, 2005). However, this chapter identifies that WAVE-$1^{-/-}$ have normal aggregate formation on collagen, in agreement with the normal WAVE-$1^{-/-}$ spreading on collagen. In addition this difference could be due to the secondary mediators, ADP and TxA_2, driving lamellipodia formation through WAVE-2 (Yan et al, 2003, Yamazaki et al, 2003). The production of either a WAVE-$2^{-/-}$ or a WAVE-

$1/2^{-/-}$, would investigate this hypothesis. However, in order to identify the role of WAVE-2 within platelets a conditional knock out must be made as a traditional knock out is embryonically lethal (Yan et al 2003; Yamazaki et al, 2003; Dahl et al, 2003, Soderling et al, 2003). However, the recent description of a PF4-Cre mouse that confers megakaryocyte/platelet specific knockdowns is a welcome contribution to the field (Tiedt et al, 2007). Interestingly there is already some precedent for a difference in role between WAVE-1 and WAVE-2 with mouse embryonic fibroblasts, where WAVE-$1^{-/-}$ has little or no effect on lamellipodia formation whereas WAVE-2 has a major effect on lamellipodia formation (Yan et al 2003; Yamazaki et al, 2003).

At the moment it is unclear whether WAVE-1 is critical for cytoskeletal rearrangements downstream of CRP, or whether WAVE-1 plays an additional important role within the GPVI signalling cascade. The reduction in aggregation and elongation in shape change to CRP, is very similar to that seen with the Rac-$1^{-/-}$ to low doses of CRP. In both cases the aggregation can be recovered with the use of high doses of CRP. The similarity of phenotype may be to be expected as WAVE is a Rac target. Interestingly recent data within Rac-$1^{-/-}$ mice, indicates a reduction in PLCγ2 and Syk phosphorylation at low doses of CRP (Pearce unpublished data). Unfortunately it was not possible to extend this experiment to WAVE-$1^{-/-}$ platelets, due to the limited amount of material.

WAVE-$1^{-/-}$ mice did not have altered red blood cells, platelet number or mean platelet number. This is in agreement with the Rac-$1^{-/-}$ mouse data (McCarty et al, 2005). Rac-1 deficient fibroblasts have been identified as having normal chemotaxis and migration (Vidali et al, 2006). This suggests that the Rac/WAVE pathway is not required for movement of megakaryocytes from the osteoblastic to the vascular niche (Avercilla et al, 2004). This is markedly different to the WASP$^{-/-}$ mouse that has

defective platelet production (Sabri et al, 2006). Interestingly leucocyte production is significantly affected within the WAVE-$1^{-/-}$ mice, clearly indicating a role for WAVE-1 within leucocyte production. Further experiments are required to establish the basis of this defect.

This chapter indicates that WAVE-1 plays an important role within GPVI signalling, relative to GPCR signalling, raising the possibility that WAVE-1 is specifically regulated by tyrosine kinase linked receptors.

Chapter 6

Studies on the cytoskeleton-regulatory proteins β-Pix, Nck and Spin-90

6 GENERAL INTRODUCTION

6.0 Summary

Cell activation is associated with cytoskeletal rearrangements that are regulated downstream of multiple signalling pathways. The role of many cytoskeletal-associated proteins in the regulation of actin assembly is unclear. The aim of this Chapter is to characterise two actin-binding proteins that have been recently identified in platelets using a proteomics-based approach (Garcia et al, 2006), namely the Arp2/3 complex-binding protein Spin-90 and the Rac GTP exchange factor β-Pix. This work has confirmed that each of these proteins is tyrosine phosphorylated in platelets downstream of GPCR and tyrosine kinase-linked receptors, but has demonstrated that in contrast to observations in other cells, these proteins do not form a complex with each other or the adapter Nck upon platelet activation either in suspension or following adhesion to a surface.

6.1 Introduction

In the majority of cells, cytoskeletal dynamics is controlled by the Rho GTPases Cdc42, Rac, and RhoA leading to filopodia, lamellipodia, and stress fibre formation, respectively. The mechanism of regulation of these RhoGTPases within platelets and many other cell types, however, is not well understood. Recently, two novel actin-regulatory proteins, Spin-90 and β-Pix, were reported to be tyrosine phosphorylated downstream of the collagen receptor GPVI in platelets using a novel proteomics-based approach (Garcia et al, 2006). Although this was the first report of their expression in platelets, they had been previously been implicated in the regulation of the cytoskeleton in several other cell types (Lim et al, 2003, Ten Klooster et al, 2006).

Interestingly, in cardiac myocytes, Spin-90, β-Pix, and WASP form a complex in suspension, which upon adhesion to a surface recruits Nck (Lim et al, 2003). Tyrosine phosphorylation of Spin-90 was responsible for recruitment of Nck, and the complex of all four proteins was implicated in the stabilisation of adhesion. Furthermore, Spin-90 has been shown to localise to lamellipodia where it binds to the Arp2/3 complex through its C-terminus, stimulating actin polymerisation (Kim et al, 2006 & 2007). These observations clearly implicate Spin-90 as an important regulator of actin dynamics and lamellipodia formation in cardiac myocytes (Kim et al, 2006, Lim et al, 2003). Importantly, β-Pix has been shown to lie upstream of activation of the Rho GTPase Rac in HEK293 and MDCKIII cells (Ten Klooster et al, 2006), thereby suggesting that it is also involved in lamellipodia formation. In addition β-Pix and GIT1, an adaptor protein, can bind PLCγ1, leading to the activation of both Cdc42 and Rac, within multiple cell types and so is a requirement for cell motility and spreading (Jones et al, 2007).

In the present Chapter, I have set out to confirm expression and to investigate the regulation and function of Spin-90, β-Pix, and Nck following activation of platelets in suspension and upon adhesion by GPCR and tyrosine kinase-linked receptors.

6.2 RESULTS

6.2.1 Characterisation of Spin-90, β-Pix, and Nck antibodies

The minimum amount of the Spin-90 antibody that could be used in immunoprecipitation studies was determined prior to experimentation in view of a limited supply, since the antibody was kindly donated by Professor Woo Keun Song. The β-Pix and Nck antibodies were also characterised simultaneously. Platelets ($5x10^8$/ml) were lysed with 2xlysis buffer and proteins immunoprecipitated with either 1, 2 or 3μl of each antibody, or the relevant IgG control. Immunoprecipitation and western blotting were performed as described in the methods. Fig.6.1A&B identifies that 3μl each of the β-Pix, and Nck antibodies generated a robust band and so were used in further immunoprecipitations. Fig.6.1C identifies that 2μl of Spin-90 generated the strongest band, but due to limited supply of the antibody, 1μl of antibody was chosen for experimentation. IgG controls identified that there were no overlapping bands associated with the relevant bands for each antibody.

6.2.2 Spin-90 is tyrosine phosphorylated downstream of GPVI

A series of experiments were designed to confirm that Spin-90 undergoes tyrosine phosphorylation upon platelet activation. Platelets ($5x10^8$/ml) were stimulated with a range of concentrations of CRP and for differing times as shown in Fig.6.2. The synthetic collagen stimulates marked tyrosine phosphorylation of Spin-90 at 60s which increases slightly by 300s (Fig.6.2A). However, there was no significant increase in phosphorylation at 20s, even though CRP induces a marked increase in tyrosine phosphorylation of several proteins in the whole cell lysate (Fig.6.2B).

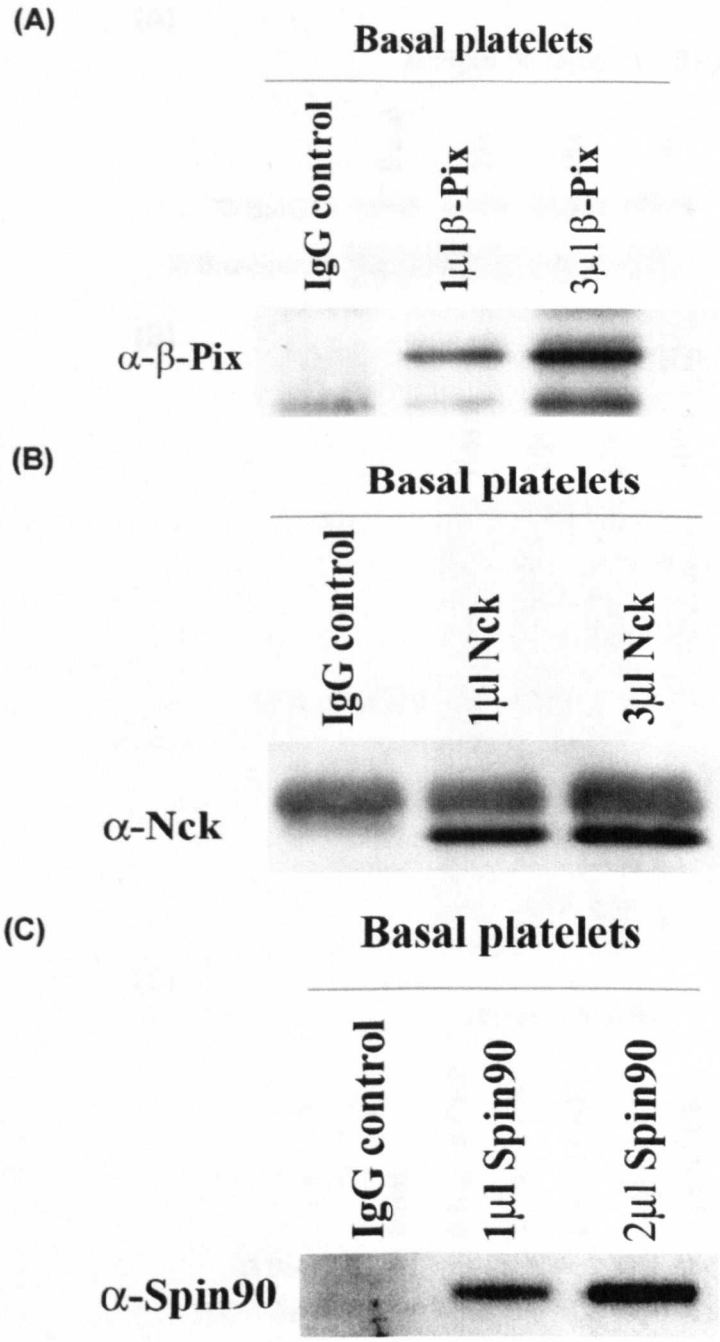

Figure 6.1: Characterisation of Spin-90, β-Pix and Nck for immunoprecipitation. Platelets ($5x10^8$/ml) were lysed without stimulation with 2xlysis buffer and immunoprecipitated with **(A)** 1 or 3μl of β-Pix **(B)** 1μl and 3μl of Nck **(C)** 1, or 2μl of Spin-90. In addition the relevant IgG control was immunoprecipitated. Images are representative of two experiments.

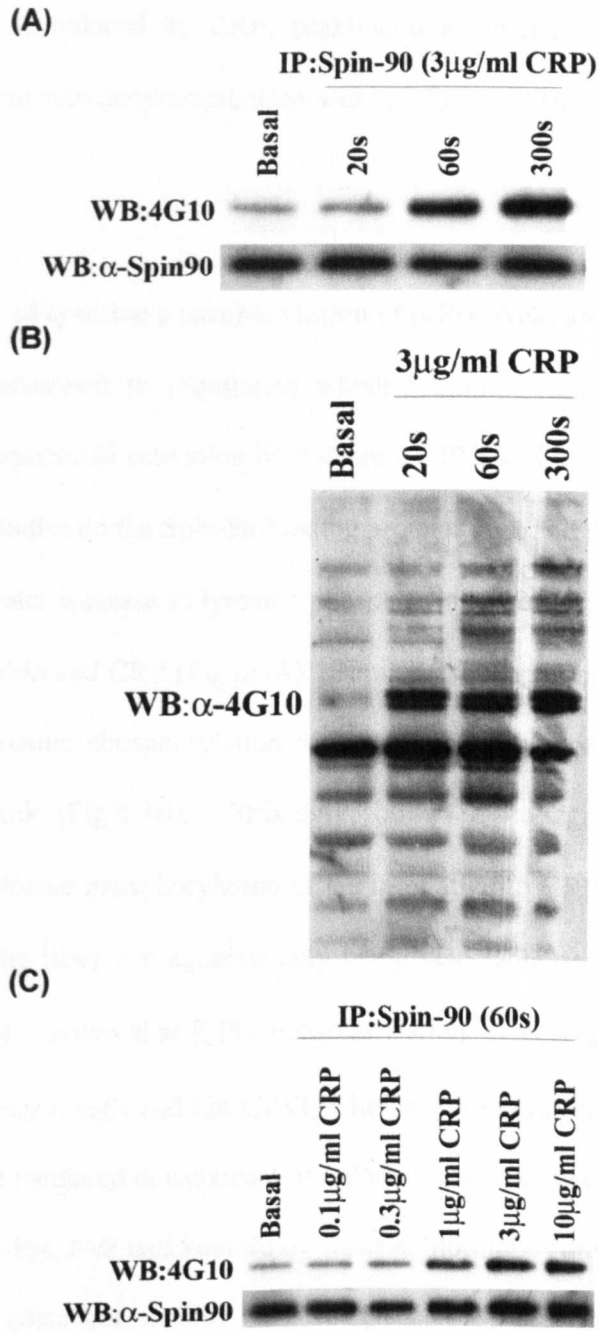

Figure 6.2: Spin-90 is tyrosine phosphorylated downstream of GPVI. Platelets ($5x10^8$/ml) were stimulated with 0.3-10μg/ml CRP, for 0-300s, before lysis with 2xlysis buffer. **(A)** A timecourse of immunoprecipitated Spin-90 **(B)** Whole cell lysates of timecourse samples **(C)** Dose response curve of immunoprecipitated Spin-90 to CRP were western blotted for tyrosine phosphorylation. For both **A&C** Spin-90 was reprobed for Spin-90 to ensure equal loading. Image are representative of two experiments.

Fig.6.2C demonstrates the concentration-dependent nature of the increase in tyrosine phosphorylation that is induced by CRP, peaking at a concentration of 3μg/ml. Therefore all remaining immunoprecipitations were performed using 60s stimulation and 3μg/ml CRP.

6.2.3 Measurement of tyrosine phosphorylation of β-Pix, Nck, and Spin-90

Experiments were performed to investigate whether Spin-90 undergoes tyrosine phosphorylation in response to activation by collagen, GPVI and the GPCR agonist, thrombin, alongside studies on the Spin-90-binding proteins Nck and β-Pix. Collagen induced a slightly greater increase in tyrosine phosphorylation of Spin-90 relative to that induced by thrombin and CRP (Fig.6.3A). Similarly, collagen also stimulated a greater increase in tyrosine phosphorylation of Nck, with the response to thrombin being particularly weak (Fig.6.3B). Strikingly, however, collagen stimulates a marked increase in tyrosine phosphorylation of β-Pix relative to CRP and thrombin, with the response to the latter two agonists only being detectable on long exposures (Fig.6.3C). This result implies that β-Pix phosphorylation by collagen is mediated downstream of the integrin $\alpha_2\beta_1$ and not GPVI, whereas phosphorylation of Spin-90 and Nck is likely to be mediated downstream of GPVI. I was unable to find evidence for co-association of β-Pix, Nck and Spin-90 by western blotting of the corresponding immunoprecipitations (data not shown) suggesting that they are not present as a complex in platelets, which is consistent with distinct modes of regulation.

A further series of studies were performed to investigate whether β-Pix, Nck and Spin-90 undergo tyrosine phosphorylation and form a complex following adhesion to fibrinogen. For these studies, platelets ($5x10^8$/ml) were allowed to spread on a fibrinogen-coated petri dish for 45min. Prior to lysis, a 500μl aliquot of platelets

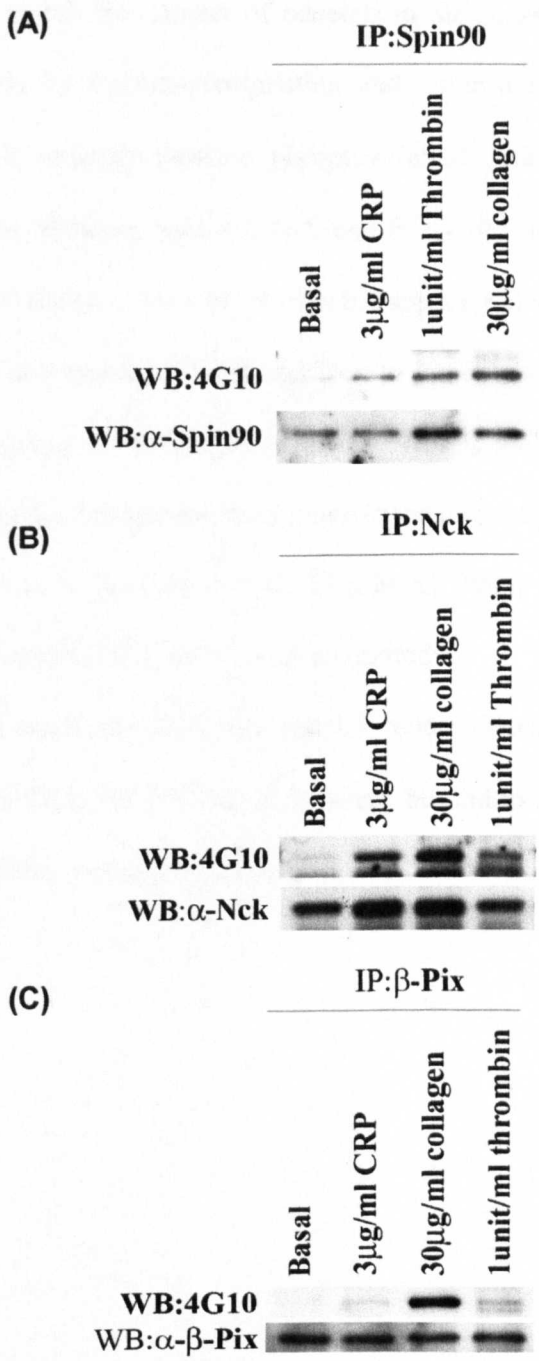

Figure 6.3: β-Pix, Nck, and Spin-90 are tyrosine phosphorylated by both GPCR and tyrosine kinase signalling pathways. Platelets ($5x10^8$/ml) were stimulated with 3μg/ml CRP, 30μg/ml collagen, or 1unit/ml thrombin for 60s before lysis with 2xlysis buffer. Basal platelets were also lysed, before immunoprecipitation with **(A)** Spin-90 **(B)** β-Pix, **(C)** Nck. Samples were western blotted for β-Pix, 4G10 (tyrosine phosphorylation), Spin-90, and Nck for each immunoprecipitation. Images are representative of two experiments.

was removed from the suspension and used as a basal sample. A protein assay was performed in order to match the number of platelets in the suspension and adhered samples before analysis by immunoprecipitation and western blotting. Strikingly, neither β-Pix nor Nck undergo tyrosine phosphorylation following adhesion to fibrinogen (Fig.6.4). In addition, Spin-90, Nck and β-Pix did not form a complex upon adhesion (data not shown). Consistent with the apparent absence of regulation, there was no apparent movement of β-Pix and Nck to lamellipodia in platelets that have been allowed to spread on fibrinogen (Fig.6.5). This is in contrast to Spin-90, which, although it stains the cytosplasm, does move to the platelet periphery and stain the lamellipodia. This is in agreement with Kim et al, 2006, who identified the presence of Spin-90 at lamellipodia, with the same antibody.

Thus, the above results reveal agonist-specific modes of regulation of tyrosine phosphorylation of Spin-90, β-Pix and Nck in platelets, but fail to reveal formation of a complex of the three actin-regulatory proteins.

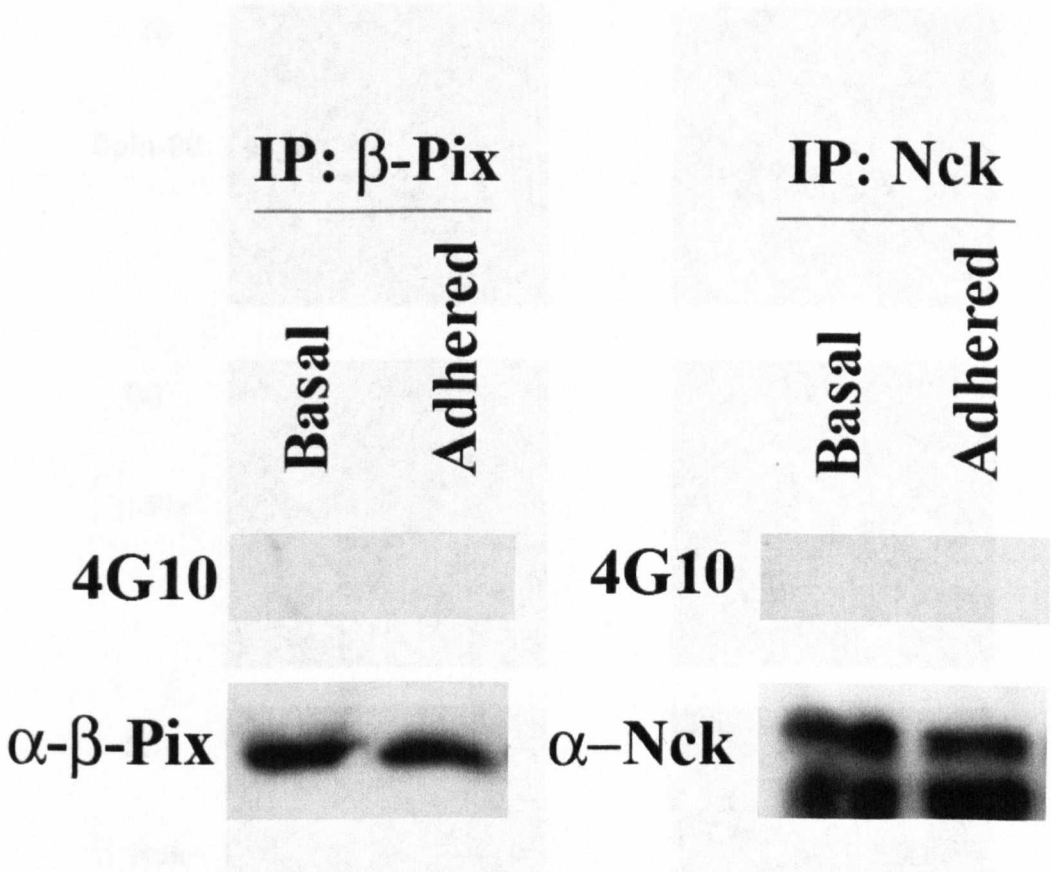

Figure 6.4: β-Pix and Nck are not tyrosine phosphorylated upon adhesion to fibrinogen. Platelets ($5x10^8$/ml) were allowed to spread on fibrinogen coated petri dishes before lysis with 2xlysis buffer. Prior to lysis, 500μl of platelets were removed from the supernatent, lysed with 2xlysis buffer and used as a basal sample. Immunoprecipitations were completed with both β-Pix and Nck, followed by western blotting for Spin-90, 4G10, β-Pix, and Nck. This experiment was performed once.

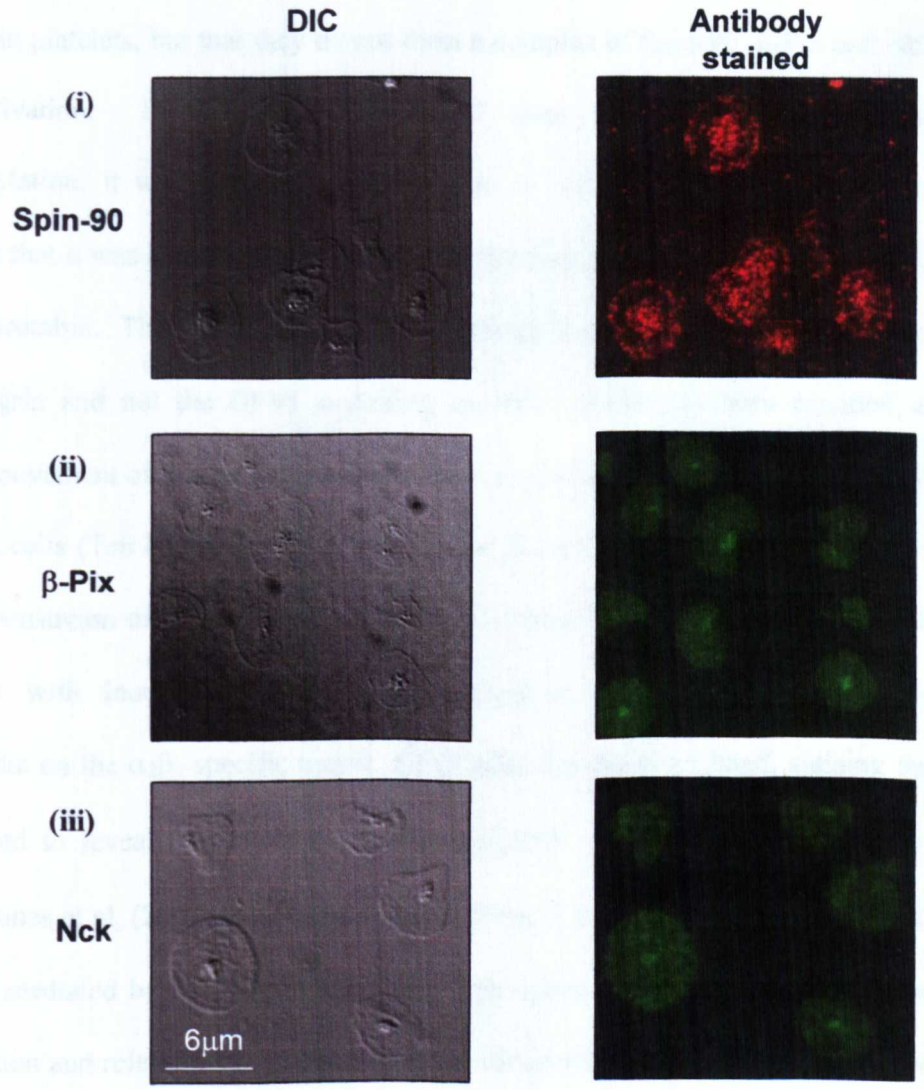

Figure 6.5: Spin-90 moves to the platelet periphery upon adhesion. Platelets ($2x10^7$/ml) were allowed to spread on fibrinogen coated coverslips for 45min at 37°C. Platelets were fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with **(i)** Spin-90, **(ii)** β-Pix and **(iii)** Nck before addition of the appropriate secondary antibody for a further 60min. Platelets were imaged using fluorescent microscopy. Images are representative of two experiments.

6.3 DISCUSSION

The results in this Chapter demonstrate that Spin-90, β-Pix, and Nck undergo tyrosine phosphorylation downstream of both GPCR and tyrosine kinase signalling cascades in platelets, but that they do not form a complex of Spin-90, β-Pix and Nck upon activation. Furthermore, although all three proteins undergo tyrosine phosphorylation, it was notable that the profile of phosphorylation of β-Pix was distinct in that it was heavily phosphorylated in response to collagen, but not to either CRP or thrombin. This indicates that β-Pix is primarily regulated downstream of the $\alpha_2\beta_1$ integrin and not the GPVI signalling cascade. β-Pix has been reported to promote movement of Rac to membrane ruffles and lamellipodia within HEK293 and MDCKIII cells (Ten Klooster et al, 2006) raising the possibility that a similar set of events downstream of $\alpha_2\beta_1$ promotes lamellipodia formation in platelets. This is in agreement with Inoue et al, (2001), who reported that platelets could form lamellipodia on the $\alpha_2\beta_1$ specific matrix, GFOGER. On the other hand, staining for β-Pix failed to reveal movement to platelet periphery upon platelet adhesion. In addition Jones et al, (2007) have identified that β-Pix is required for cell motility and spreading mediated by β1 integrin signalling. Therefore, a possible mechanism for the activation and role of β-Pix in platelets is identified within Fig.6.6. $\alpha_2\beta_1$ activates PLCγ1, leading to the recruitment of β-Pix and possibly the adaptor protein GIT1 (recently identified within platelets (Sato et al, 2007)). This leads to the recruitment of Rac, WAVE, and the Arp2/3 complex, causing actin reorganisation. This potentially explains the missing link between PLCγ1 and Rac activation within platelets.

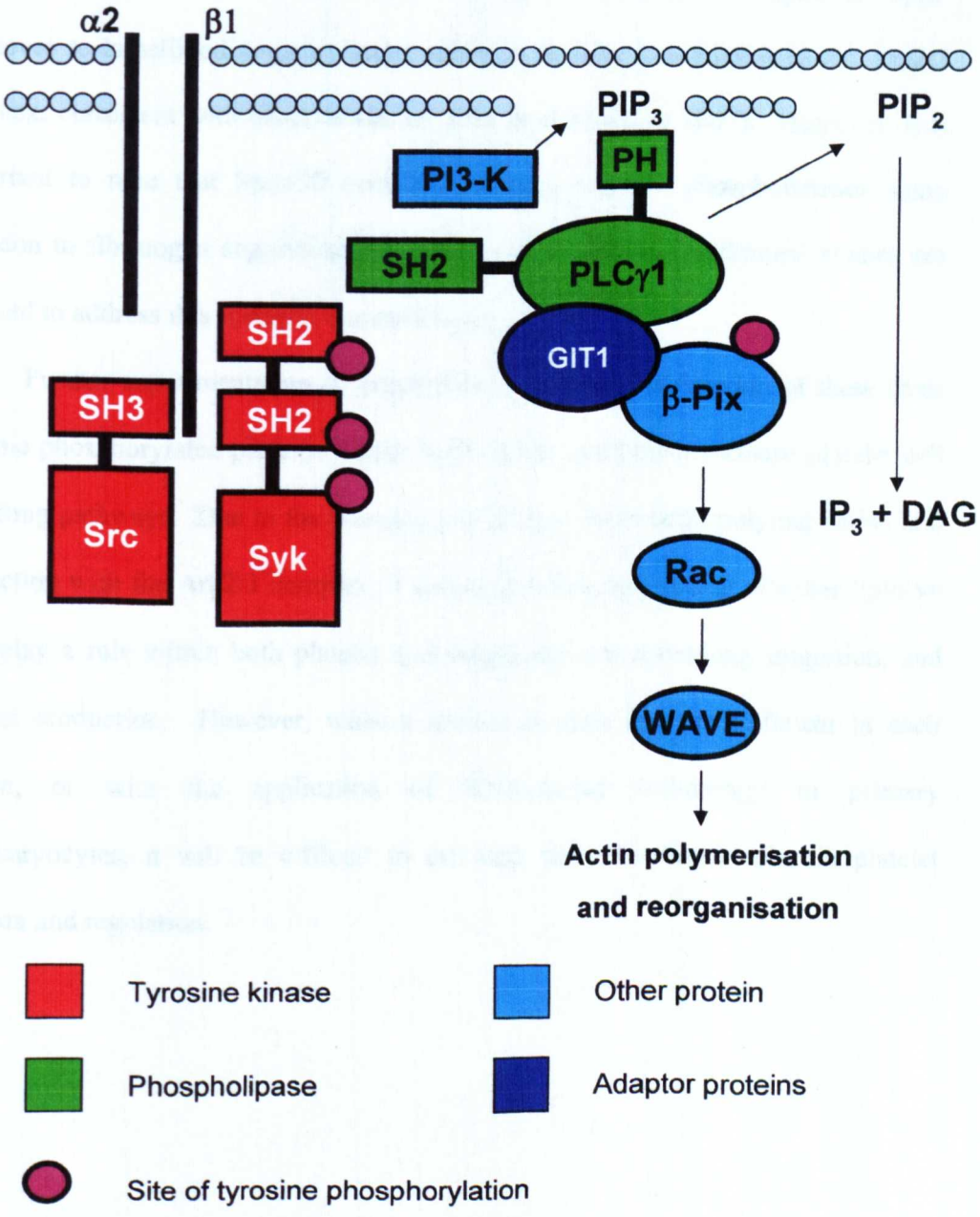

Figure 6.6: Actin reorganisation downstream of $\alpha_2\beta_1$ is dependent on PLCγ1 and β-Pix. α2β1 activates a signalling pathway dependent on Src kinase activation of PLCγ1. Downstream of PLCγ1 GIT1 and β-Pix, are activated, leading to Rac activation, and then actin reorganisation

The lack of formation of a complex between β-Pix, Spin-90, and Nck in suspended and adhered platelets indicates a difference in cell signalling in comparison to cardiac myocytes. However, there are similarities between the two systems. Spin-90 moves to lamellipodia upon platelet adhesion thereby localising it to the Arp2/3 complex, consistent with observations by Kim et al (2006 & 2007). However, it is important to note that Spin-90 does not undergo tyrosine phosphorylation upon adhesion to fibrinogen suggesting that it may not be active. Additional studies are required to address this apparent contradiction.

Further experimentation is required to understand the function of these three tyrosine phosphorylated proteins within both GPCR and tyrosine kinase platelet cell signalling pathways. Due to the possible role of Spin-90 in actin polymerisation and interaction with the Arp2/3 complex, it would be interesting to test whether Spin-90 does play a role within both platelet and megakaryocyte spreading, migration, and platelet production. However, without access to mice that are deficient in each protein, or with the application of RNAi-based technology in primary megakaryocytes, it will be difficult to establish their functional roles in platelet function and regulation.

Chapter 7

General Discussion

7.0 Summary

This thesis is concerned with the actin structures associated with platelet spreading and thrombus formation downstream of glycoprotein receptors. It demonstrates the importance of WAVE-1, the RhoA/ROCK signalling pathway, and myosin-II within lamellipodia and stress fibre formation and the formation of a novel actin structure with unknown function. In addition, the present results further emphasise the role of the actin cytoskeleton within thrombus formation, both *in vitro* and *in vivo*.

7.1 Actin morphological rearrangements associated with platelet activation downstream of glycoprotein receptors

Chapters 3-5 focus on the actin structures associated with platelet spreading on multiple matricies. Each Chapter builds on previous knowledge, furthering the investigation of the roles of actin morphological changes within platelet activation and function.

One of the initial actin structures that is associated with platelet activation is filopodia. However, the observations in Chapter 3 also identify an actin structure that we have termed an actin nodule. Actin nodule formation on collagen and fibrinogen is dependent on Src kinases. This is not surprising as Src kinases play a fundamental role in cell signalling downstream of both $\alpha_{IIb}\beta_3$ and GPVI. The actin regulatory proteins Rac and the Arp2/3 complex are present in the actin nodules, further emphasising their likely importance in actin assembly. However, to date the function and physiological role of actin nodule formation is unknown.

After the formation of actin nodules and filopodia, lamellipodia are formed. Rac-1 is fundamental for lamellipodia formation in platelets (McCarty et al, 2005). Chapter 5 builds on this observation by demonstration of a specific role for WAVE-1

in lamellipodia formation mediated downstream of GPVI. Furthermore, as is also the case for Rac-1, WAVE-1 is required for aggregation and secretion to low concentrations of GPVI-specific agonists. Nevertheless, WAVE-1 is not required for thrombus growth and stability, presumably because of the presence of WAVE-2 within platelets. Thus, there appear to be agonist-specific pathways of regulation of WAVE-1, downstream of GPVI, and WAVE-2, downstream of GPCR agonists.

The final actin rearrangement, stress fibre formation, requires activation of the RhoA/ROCK signalling pathway and myosin-II. Inhibition of either ROCK or myosin-II inhibits formation of stress fibres, causes fenestrations, and thrombus instability, both *in vitro* and *in vivo*. However, interestingly, neither ROCK nor myosin-II play a significant role in aggregation and secretion. I therefore speculate that the thrombus instability is due to inhibition of stress fibre inhibition. However, as yet, there is no direct evidence linking thrombus instability with cytoskeletal defects.

A series of studies were also undertaken to investigate the role of the actin binding adapter proteins, Spin-90 and Nck, and also the GDP/GTP exchange factor, β-Pix, downstream of glycoprotein receptors. All three of these proteins have been reported to form a complex within cardiac myocytes upon adhesion (Lim et al, 2001 & 2003). Moreover, Spin-90 and β-Pix were identified as novel tyrosine phosphorylated proteins in GPVI-activated platelets using a proteomics approach (Garcia et al, 2006). However, these three proteins do not form a complex in platelets either when stimulated in suspension or upon adhesion even though all three undergo tyrosine phosphorylation in suspension. Interestingly, however, collagen stimulates a marked increase in tyrosine phosphorylation of β-Pix relative to that induced by CRP, thereby indicating that phosphorylation maybe downstream of integrin $\alpha_2\beta_1$. In

addition the activation of β-Pix downstream of $\alpha_2\beta_1$ could be the missing link leading to Rac activation, as indicated by Fig.6.6 and Jones et al (2007).

7.2 General Discussion

7.2.1 The role of the cytoskeleton in intracellular signalling cascades

The present thesis, and earlier observations, demonstrate an important role for the cytoskeleton in intracellular signalling downstream of GPVI. This is illustrated by treatment of platelets with the actin polymerisation inhibitor, cytochalasin D, which causes a partial inhibition of GPVI-mediated tyrosine phosphorylation of PLCγ2, Syk and FAK (Obergell et al, 2002; Wonerow et al, 2002). Importantly, the reduction in phosphorylation of Syk argues that inhibition occurs at proximal stage in the GPVI signalling pathway, possibly being mediated by a defect in GPVI clustering. Strikingly though, cytochalasin D-treated platelets can still adhere, secrete and undergo aggregation to a number of agonists even though they cannot form stable thrombi under high shear (Chapter 5, Auger thesis 2005). However, it is unclear whether the loss of thrombus stability is due to a defect in signalling or a defect in thrombus formation.

There is also a reduction in GPVI signalling within the Rac-1$^{-/-}$ platelets, although this defect is smaller than that seen with cytochalasin D treatment demonstrating that the effect of the latter is not solely due to blockade of lamellipodia. Furthermore, other mutant platelet lines, for example LAT$^{-/-}$ platelets, have a similar reduction in PLCγ2 phosphorylation, but show little inhibition of lamellipodia. Therefore it is likely the lamellipodia defect associated with the Rac-1$^{-/-}$ is cytoskeletal rather than due to a cell signalling defect, associated with loss of Rac.

Although cytochalasin D-treated platelets will secrete α- and dense-granules, their secretion is differentially regulated. For example, α-granule release is inhibited by blockade of actin polymerisation, whilst dense granule release is unaffected (Flaumenhaft et al, 2005). However, it is unclear if cytochalasin D prevented the signalling events that lead to release or whether it prevents the actin rearrangements that are needed for release.

ROCK and myosin-II inhibition cause a novel cytoskeletal phenotype that is associated with inhibition of stress fibres formation, unstable thrombi and the appearance of fenestrations. On the other hand, ROCK inhibition does not affect platelet aggregation or dense-granule secretion. Myosin-II inhibited platelets, in contrast to ROCK inhibited platelets, have a small degree of platelet inhibition that is similar to that seen with cytochalasin D, although high concentrations of agonists can overcome the inhibitory effect. In addition it is also possible that this inhibitory effect is due to an additional action of blebbistatin. Allied to this, and bearing in mind that platelet activation in the presence of a ROCK inhibitor is normal, the thrombus instability is likely to be cytoskeletal in origin. This is further reinforced as the higher the shear stress the greater the reduction in thrombus height, indicating a greater role for the cytoskeleton with increasing shear stress.

Although the majority of the evidence demonstrates that the cytoskeleton plays an important but relatively minor role in platelet activation, it should be noted in other cell types the cytoskeleton is required for processes such as vesicle trafficking, and correct localisation of signalling proteins. Therefore, in other cell types the cytoskeleton may play a more major role, especially in motile cells, where movement and cell signalling have to be intricately linked in order to function appropriately.

7.2.2 Does platelet spreading play a role in thrombus formation *in vivo?*

Under *in vitro* conditions, platelets undergo a characteristic pattern of actin reorganisation that is associated with filopodia, actin nodules, lamellipodia, and stress fibres formation as demonstrated in Chapter 3-6. However, although it is possible to assign a role for each structure *in vitro*, the role of these specific actin structures *in vivo*, including whether they are formed, is unclear.

The major role of the platelet *in vivo* is prevention of blood loss through thrombus formation. Within thrombi whilst it is possible to identify the formation of filopodia (Maxwell et al, 2006, Remijn et al, 2001), the formation of lamellipodia, and therefore stress fibres is less well defined. *In vitro* it is reported that platelets will spread and form lamellipodia in aggregates, and that fibrinogen is a requirement for this spreading, and for formation of densely packed thrombi (Remijn et al, 2001). This is in agreement with *in vitro* flow data, which indicates that lamellipodia and fully spread platelets (and therefore it is presumed stress fibres) are clearly identified on the bottom layer of a thrombus (Fig.7.1; McCarty et al, 2005). However, confocal analysis of aggregates *in vitro* (Fig.7.1) indicates that platelets not in contact with collagen do not spread, and so the upper levels of thrombi consist of rounded platelets, not spread platelets. Interestingly however, Maxwell et al (2007) identify that within thrombi *in vivo* platelets seem to lose their spherical shape, and become elongated, possibly indicating platelet spreading.

Although platelet spreading in vivo is not clearly demonstrated it is likely that *in vivo* thrombus formation will mimic *in vitro* in terms of initial platelet adhesion to the damaged endothelium. Therefore, the first layer of platelets should spread, aiding the prevention of blood loss, due to the increase in surface area coverage of a spread platelet. However if the bottom layer of the thrombus spreads, and recruits further

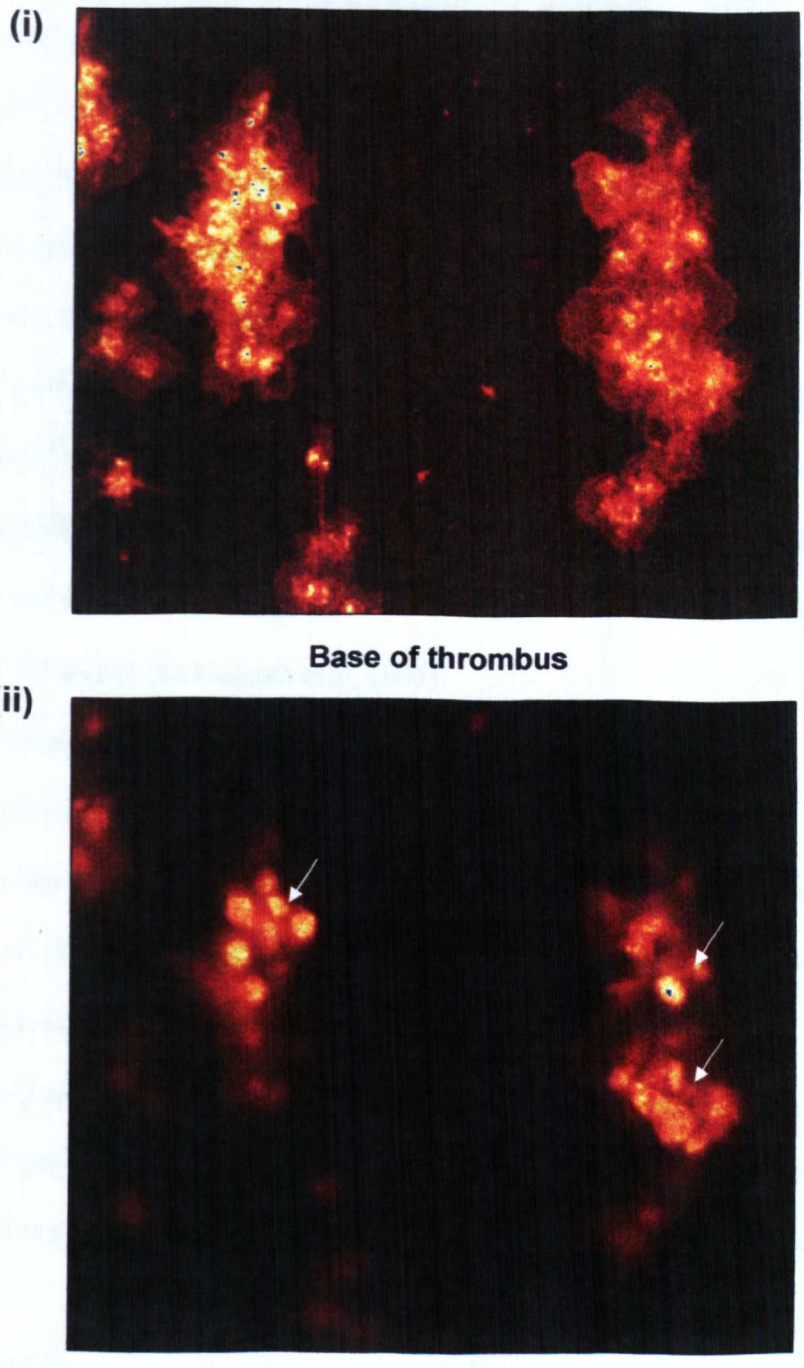

Figure 7.1: Identification of rounded platelets within thrombi. Whole blood was flowed across collagen at high shear before fixation with paraformaldehyde (3.7%), and staining with $DiOC_6$ (2μM). Images taken by 0.5μm slices through thrombi using confocal microsocopy **(i)** Thrombi imaged at their base and spread platelets are clearly identified. **(ii)** White arrows identify rounded platelets within the upper regions of thrombi. Images are representative of 9 experiments.

platelets to the thrombus, it is important to consider if these also spread. This is an important question and illustrates an important limitation of focussing on the events that take place on the surface i.e. within a 2D environment. However, the platelets that lie above the monolayer are interacting in a 3D environment. This is likely to change the actin rearrangements that take place because (i) a change in the agonist that mediates activation, and (ii) the matrix is no longer uniform, nor fixed in place. There is precedent in other cell types that a 3D environment is very different to that of a 2D environment. Fibroblasts alter their morphologies by developing a more starlike and elongated shape within a 3D matrix. Interestingly, differences in phosphorylation downstream of integrin signalling have been detected within fibroblasts within a 3D relative to a 2D matrix (Cukierman et al, 2001).

In addition to platelets responding differently to a 3D environment, the shear stress may also affect cytoskeletal rearrangements. Maxwell et al, (2006) identified that at low shear stress ($600s^{-1}$) platelets extended both tethers and filopodia. At higher shear ($1,800\text{-}5,000s^{-1}$) platelets adopted a spherical morphology with numerous surface projections. At very high shear ($10,000\text{-}20,000s^{-1}$) filopodia were retracted and the platelets developed a smooth ball like appearance. The reasons for the changes in shape are unclear but the changes were controlled by both microtubules, and the cytoskeleton indicating that morphology could play a role in thrombus formation.

Several lines of evidence suggest that it is important for platelets to reorganise their actin within a thrombus. For example, Rac-$1^{-/-}$ platelets, or treatment with cytochalasin D, form thrombi that readily embolise. Moreover, although these platelets have a defect in GPVI signalling, a similar defect in thrombus stability is seen on a monolayer of thrombin and VWF (McCarty et al, 2005). Interestingly,

however, under these conditions, the monolayer that lies at the base of the thrombus usually does not embolise, demonstrating that the defect is associated with the upper regions of the thrombus. This is similar to the situation observed for the ROCK- and myosin-II-inhibited platelets. Interestingly, analysis of the thrombus using confocal microscopy, reveals that the upper layers contain circular platelets (Fig.7.1). This clearly illustrates that the morphological rearrangements identified within a 2D system, may not be the same as those within a 3D system.

7.2.3 The role of the cytoskeleton as a platelet drug target

This thesis indicates that the cytoskeleton plays a minor role within platelet cell signalling, but that it plays an important role in platelet spreading and thrombus stability. Therefore, the question of whether the cytoskeleton a viable drug target for thrombosis is worthy of consideration.

The actin cytoskeleton is implicated in multiple disease states, including cancer metastasis, plaque formation, hypertension (Mallat et al, 2003, review Shimokawa et al, 2007, Jurasz et al, 2004), through activity of RhoGTPases. This is clearly demonstrated by the use of the ROCK inhibitor, fasudil as an anti-hypertensive drug, which reduces blood pressure and plaque formation (Higashi et al, 2003).

In the context of targeting thrombosis, the observation that the cytoskeleton plays a minor role in cell signalling is significant, as platelet activation would be largely unaffected. Therefore, the observation that interference in platelet spreading, through disruption of lamellipodia, or stress fibres, or actin polymerisation, leads to thrombus instability offers hope as a new means of preventing excessive thrombus formation (McCarty et al, 2005, Chapter 5, Auger et al unpublished data). On the

other hand, ROCK and Rac are both present within all cells and so such targets could give rise to many side effects.

A further consideration is whether inhibition of actin polymerisation would cause a bleeding phenotype. The *in vivo* data indicates that thrombus formation is heavily affected by interference of the cytoskeleton, but that there is still partial thrombus formation.

7.4 Future Directions

The work in this thesis has focused on the regulation and role of lamellipodia and stress fibre formation in thrombus formation. Similar studies are warranted for filopodia, which are believed to be regulated downstream of Cdc42, but with no reports to confirm this. Further to this, it would be interesting to investigate whether inhibition of filopodia also leads to a reduction in thrombus stability.

In addition to the above, further work is needed to completely understand the function and physiological relevance of the actin nodules. It is surprising that a such structure does not appear to have been identified in other cell types. However, it may that the platelet's small size has enabled its identification.

Finally the role of the actin cytoskeleton within megakaryocytes is a growing field. Much is known about the role of microtubules within megakaryocytopoesis, and platelet production (Italiano et al, 1999), but little about the role of the cytoskeleton. Clearly the cytoskeleton is vital for migration, allowing the megakaryocytes to move from the osteoblastic niche, to the vascular niche, but its role within platelet production is unclear. At the present time, both the RhoA/ROCK signalling pathway and myosin-II is reported to play a negative role in proplatelet formation (Chang et al, 2007). However, interestingly, the Rac-1, Rac-2, Rac-1/2$^{-/-}$,

and Scar-1$^{-/-}$ all have normal platelet numbers indicating no role for these important actin regulatory proteins within proplatelet formation. However, Cdc42/Rac double knockout has a major defect in platelet production, which may possibly be mediated at earlier stage in development, clearly indicating that there is a role for the cytoskeleton within platelet production (Pleines et al, 2007).

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APPENDIX

Supplementary Video 1: Human platelet spreading on fibrinogen

Supplementary Video 2: Murine GFP-spreading on fibrinogen

Supplementary Video 3: Human platelet spreading on collagen in the presence of Y27632

These can be found in the CD in the back sleeve.