Effects of resistin on platelet function and its receptor, adenylyl cyclase associated protein 1

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ABSTRACT

Resistin is an adipokine which is found elevated in patients suffering from metabolic syndrome, affecting a large proportion of the population. It is characterised by obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD), which are the biggest contributors to morbidity and mortality in the modern world. Platelets play a major role in CVD, where their activation leads to formation of thrombi, which when uncontrolled, leads to the pathophysiological effects of CVD. The role of resistin in CVD progression and its mechanism of action on its target cells still remains debatable and surrounded by controversies. In a bid to assess this, the present study aims at illustrating the effect of resistin on platelet activation by deriving conclusions from platelet functional assays. This thesis makes novel observations that resistin blunts thrombin-mediated platelet aggregation and secretion and seemingly affects the platelet actin cytoskeleton. It also finds that resistin activates downstream effectors of phosphoinositide 3 kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways in platelets.

Adenylyl cyclase associated protein 1 (CAP1), a cytoskeletal protein, was recently identified as a receptor for resistin. When CAP1 was investigated in platelets, interesting observations were made, where CAP1 temporally translocated from the membrane fraction in response to thrombin mediated activation. Furthermore, in an attempt to improve our knowledge of platelet biology and to delineate future avenues for exploration, an observational proteomics investigation of cytoskeletal proteins and the change in abundance

upon thrombin activation was carried out, further highlighting their importance in platelet regulation. Additionally the subcellular localisation of specific cytoskeletal proteins (IQGAP1, Arp2/3, coronin1a, profilin, cofilin, villin, l-plastin, myosin IIa, and tropomyosin) that are either known or hypothesised to interact with CAP1 was also conducted. Further investigations with proteins IQGAP1 and Arp2/3 revealed that these proteins did not interact with CAP1.

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2D	Two-dimensional PAGE
5-HT	Serotonin or 5-hydroxytryptamine
ABP	Actin binding protein
AC	Adenylyl cyclase
AC-15	Antibody for monomeric actin
ADP	Adenosine diphosphate
AIP1	Actin interacting protein 1
ANOVA	Analysis of variance
APS	Ammonium persulphate
Apy	Apyrase
Arp2/3	Actin-related protein 2/3 complex
ATP	Adenosine triphosphate
BAPTA/AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)
BCA	Bicinchoninic Acid
BHF	British Heart Foundation
BMI	Body mass index
BSA	Bovine serum albumin
Bt	Bos taurus
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CAP1	Adenylyl cyclase associated protein 1
CD36/39/40L/148	Cluster of differentiation 36/39/40L/148
Cdc42	Cell division control protein 42

CEBPE	CCAAT/enhancer binding protein (C/EBP), epsilon
cGMP	Cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
Coll	Collagen
Cos7	Fibroblast-like tissue from monkey kidney tissue
COX1/2	Cycloxygenase 1/2
CRP	C-reactive protein
Csk	C-terminal Src kinase
c-Src	Cellular sarcoma
C-terminus	Carboxyl terminus
CVD	Cardiovascular disease
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTS	Dense tubular system
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase-1/2
F-actin	Filamentous actin
fAD	Full-length adiponectin
FAK	Focal adhesion kinase

FcRγ	Fc Receptor γ
FCS	Fetal calf serum
FERM	4.1 protein, ezrin, radixin and moesin
FITC	Fluorescein isothiocyanate
FIZZ 1/2/3	Found in inflammatory zone 1/2/3
G-actin	Globular actin
gAD	Globular adiponectin
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GLUT1/4	Glucose transporter 1/4
GP	Glycoprotein
GPCR	G-protein coupled receptor
GSK	Glycogen synthase kinase
GSNO	S-nitrosoglutathione
GTP	Guanosine triphosphate
HCI	Hydrochloric acid
HEK	Human embryonic kidney 293 cell line
HeLa	Carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	Helical fold domain
Hs	Homo sapiens
HT29	Human colorectal cancer cell line
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1 / CD54

Ig	Immunoglobulin
IL-1b/6/10	Interleukin 1b/6/10
IMAC beads	Phospho-protein enrichment beads
Indo	Indomethacin
IP_3	Inositol trisphosphate
IR	Insulin resistance
IRS-1	Insulin receptor substrate 1
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KCl	Potassium chloride
kDa	kilo Dalton
KO	Knock out strain
Lat A	Latrunculin A
Lat B	Latrunculin B
LC	Liquid chromatography
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein
MetS	Metabolic syndrome
min	Minutes
Mm	Mus musculus
MMP-2/9	Matrix metalloproteinase 2/9
MS	Mass spectrometry
MyD88	Myeloid differentiation primary response gene 88
Na_3VO_4	Sodium orthovanadate

NaCl	Sodium chloride
NO	Nitric oxide
N-terminus	Amino-terminus
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PAK	p21 activated kinases
PAR	Protease activated receptor
PBG	PBS-BSA-Gelatin solution
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PFA	Paraformaldehyde
PGI_2	Prostaglandin I_2
PI3K	Phosphoinositide 3-kinase
PIC	Protease Inhibitor Cocktail
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLCγ2	Phospholipase Cγ2
PMSF	Phenylmethanesulfonylfluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Phosphatidylserine
PTP-1B	Phosphotyrosine phosphatase-1B
PVDF	Polyvinylidene fluoride
RBP-4	Retinol binding protein 4
RELM	Resistin like molecule

Res	Resistin
RETN	Resistin gene
RNA	Ribonucleic acid
ROCK	Rho associated protein kinase
ROR1	Receptor tyrosine kinase like orphan receptor 1
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SFK	Src family kinase
sGC	Soluble guanylyl cyclase
SHP1	Src-homology 2 domain containing phosphatase-1
SIL	Stable isotope labelling
SN	Supernatant
SPR	Surface plasmon resonance
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
T2DM	Type-2 diabetes mellitus
TBS-T	Tris buffered saline -Tween 20®
TGF	Tumour growth factor
Thr	Thrombin
TLR-4	Toll-like receptor-4
TNF	Tumour necrosis factor
TP	TXA_2 receptor
t-PA	Tissue plasminogen activator
TPO	Thrombopoietin
TRITC	Tetramethylrhodamine isothiocyanate
TX-100	Triton X-100

TXA_2	Thromboxane A_2
TZD	Thiazolidinedione
VASP	Vasodilator-stimulated phosphoprotein
VCAM-1	Vascular cell adhesion protein 1 / CD106
vWF	Von Willebrand factor
WASP	Wiskott-Aldrich syndrome protein
WH2	WASP-homology domain 2
WHO	World Health Organisation

Amino acids

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acids	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

DEDICATIONS

To my mother

Who taught me that it is okay to have and pursue any dream

To my father

Who gives me the confidence in my ambitions

To my husband

Whose strength and support I always take for granted

To my son

The only reason I am…

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AUTHOR'S DECLARATION

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CHAPTER 1

INTRODUCTION

1.1 Platelets in metabolic syndrome: role of adipokines

The World Health Organisation (WHO) estimates that more than 1 billion adults, worldwide are overweight and of these, over 300 million are clinically obese (Body Mass Index [BMI] ≤ 30kg/m^2) suffering from the characteristic pathology of metabolic syndrome (MetS) (Miranda *et al.*, 2005; Santilli *et al.*, 2012). MetS as defined by the British Heart Foundation (BHF), is a combination of central obesity, impaired glucose metabolism, dyslipidemia, IR and arterial hypertension, making it a powerful and prevalent predictor of cardiovascular events and a leading cause of mortality in developed countries (Less, 2014). Obesity is an increasing public health problem constituting an independent cardiovascular risk factor. Abdominal obesity is directly related to IR, leading to increased blood pressure and alterations in the lipid profile and blood glucose levels (Anfossi, Russo and Trovati, 2009; Assumpção *et al.*, 2010). Clinical evidence supports the fact that IR plays a central role in metabolic syndrome, which is associated with around three times higher CVD mortality and approximately two times higher all-cause mortality in adults compared to healthy individuals (Assumpção *et al.*, 2010).

IR is a pathological condition whereby the target cells are unable to respond to normal levels of insulin. The outcome is the disruption of insulin-mediated glucose and lipid homeostasis, leading to increased requirement of insulin to

maintain normoglycaemia. The main characteristics of IR are disinhibited lipolysis in adipose tissue, impaired uptake of glucose by muscle and disinhibited gluconeogenesis, where compensatory obligate hyperinsulinemia is observed (Mlinar *et al.*, 2007).

Patients with MetS and T2DM have high risk of microcirculation complications and microangiopathies. An increase in thrombogenic risk is associated with platelet hyperaggregation, hypercoagulation, and hyperfibrinolysis (Alesso and Juhan-Vague, 2008).

Blood platelets are anucleate, discoid cells which are central to haemostasis. Haemostasis is a critical physiological process which prevents blood loss after vascular injury by formation of haemostatic plugs and helps regain blood fluidity thereby maintaining the integrity of the circulatory system. Blood components and vascular endothelium together form a regulatory system which aids in the process of haemostasis – the vascular endothelium which lines the circulatory system separates thrombogenic factors present in the vessel wall from the circulating blood (Versteeg *et al.*, 2013). Arterial blood flow generates shear stress pushing platelets into close proximity with the endothelial cell layer. Platelets remain quiescent during normal circulation under the influence of endothelial prostacyclin (PGI_2) and nitric oxide (NO) (Smolenski, 2012). Injury to the vascular endothelium breaks the physical barrier exposing subendothelial agonists to platelets which initiates their adhesion, activation and aggregation

and triggers the coagulation cascade. The coagulation cascade enables stable thrombus formation, thus regaining haemostasis (Heemskerk, Bevers and Lindhout, 2002).

Thrombus size is tightly regulated by neighbouring endothelial cells producing platelet inhibitors (PGI_2 and NO) which prevent occlusion of the artery by the growing thrombus in healthy individuals (Schwarz, Walter and Eigenthaler, 2001).

In addition to the most widely studied role of platelets in haemostasis, platelets also act as inflammatory effector cells and perform a broad spectrum of functions from acute inflammation to adaptive immunity (Rondina, Weyrich and Zimmerman, 2013). Thrombin and fibrinogen, which are generated during the formation of haemostatic plug function not only in the amplification of the platelet response in the coagulation cascade but also as key proinflammatory factors (Leger *et al*. 2006). Platelet surface immunoreceptors and platelet releasates containing proinflammatory chemokines enhance the inflammation at the site of injury. Platelets are therefore described as guardians of vascular integrity patrolling the circulation for vessel leakage in their quiescent, discoid shape and upon encountering molecular cues for vascular damage they change shape, undergoing dramatic cytoskeletal reorganisation (Aslan and Mccarty, 2013).

In pathological conditions like T2DM, obesity, CVD, and MetS, cumulative effects of dyslipidemia, hypertension and IR lead to increased risks of vascular harm. A key mechanism is involved, namely the progression of atherogenesis

(Alesso and Juhan-Vague, 2008). In atherosclerosis, blood flow within arteries is occluded due to platelet dysregulation leading to uncontrolled thrombosis and chronic inflammation increasing in severity over time. Endothelial barrier destabilisation, lipid deposition and plaque formation, angiogenesis and subsequent rupture cause thrombotic obstruction of blood circulation causing ischemia and infarctions in multitude of organs, often fatal (Badimon and Vilahur, 2014). Further aggravation of the MetS and T2DM disease occurs due to factors leading to platelet activation comprising IR, hyperglycaemia, non-enzymatic glycosylation, oxidative stress, and inflammation (Suslova *et al.*, 2014).

A number of platelet regulation mechanisms are affected in patients presenting MetS. Table 1.1 lists some of the effects of MetS on patients' platelets that have been correlated in clinical studies.

Table 1.1 Effects of metabolic syndrome (MetS) on patients' platelets

Effects of MetS on platelets	References
cGMP inhibitory pathway dysregulation	(Santilli *et al.*, 2012)
High platelet turnover	(Vaduganathan *et al.*, 2008; Santilli *et al.*, 2012)
Abnormal platelet morphology (high % of reticulated platelets)	(Vaduganathan *et al.*, 2008; Santilli *et al.*, 2012)
Increased ADP-induced P-selectin expression	(Vaduganathan *et al.*, 2008)
Increased platelet microparticles in circulation (increased CD40L, IL-1β, CRP, IL-6 and other inflammatory cytokines)	(Serebruany *et al.*, 2008; Santilli *et al.*, 2012; Gyulkhandanyan *et al.*, 2013)

It is discussed that platelets are sites of IR- thus causing the lack of the physiological action exerted by insulin on platelet function, such as reduction of the prothrombotic properties of agonists, including adenosine diphosphate (ADP), collagen, thrombin, epinephrine; activation of endothelial NO synthase, with increased NO formation and intra-platelet concentrations of cyclic adenosine monophosphate (cAMP); sensitization of platelets to the inhibitory actions of PGI_2 and NO on aggregation (Trovati *et al.*, 1997; Anfossi, Russo and Trovati, 2009; Gerrits *et al.*, 2012).

An understanding of the mechanisms and factors affecting platelet function are critical to assessing and improving treatments provided to patients suffering from MetS owing to the collateral complications that arise due to myriad of pathologies.

In a bid to isolate MetS effects on platelets further; in our study we investigate the role of an adipokine, resistin, and its effect on platelet activation.

1.2 Adipokines

Obesity and specifically the presence of excessive visceral adipose tissue has been correlated to IR and MetS in clinical investigations (Fantuzzi *et al.*, 2005; Rabe *et al.*, 2008; Assumpção *et al.*, 2010; Ayeser *et al.*, 2016). Adipose tissue as an endocrine organ in addition to energy storage organ has been extensively described. Adipose tissue secretes cytokines, termed adipokines, and components of the complement system (Table 1.2). It is therefore implicated in complex roles in vascular haemostasis, inflammation and metabolism (Bełtowski, 2003; Kershaw and Flier, 2004). Adipokines are primarily involved in the regulation of insulin sensitivity of target cells. Shearer *et al*. (2005) describe leptin, adiponectin, resistin, adipsin and visfatin as true adipokines, whereas additional chemokines secreted by the adipocytes are classified separately as they are secreted more abundantly by other cell types compared to adipocytes (Frühbeck and Salvador, 2004; Rabe *et al.*, 2008; Piya, McTernan and Kumar, 2013).

Table 1.2: List of adipokines: *Compilation of list of adipokines is derived from the following reviews - (Frühbeck and Salvador, 2004; Rabe et al., 2008; Piya, McTernan and Kumar, 2013)*

True adipokines	Other adipokines
Adiponectin	Tumor necrosis factor α (TNF-α)
Leptin	Interleukin 6 (IL-6)
Resistin	IL-10
Adispsin	Omentin
Visfatin	Retinol binding protein-4 (RBP-4)
Apelin	Monocyte-chemoattractant protein-1 (MCP-1)
	Transforming growth factor β (TGF-β)
	Ghrelin
	Chemerin
	RANTES (Regulated on activation normal T cell expressed and secreted)
	Plasminogen activator inhibitor 1 (PAI-1)
	C-reactive protein (CRP)

A differential role of adipokines in lean and obese individuals has been identified – white adipose tissue of lean individuals preferentially secreting anti-inflammatory cytokines like adiponectin, TGFβ, IL-10, apelin, whereas white adipose tissue in obese individuals have increased secretion of pro-inflammatory cytokines like resistin, leptin, TNF-α, IL-6, PAI-1, that subsequently affect the modulation of IR, and insulin signalling pathways (Piya, McTernan and Kumar, 2013).

In the following sections, true adipokines like leptin and adiponectin are further described. A more detailed account of resistin is presented in section 1.3, as it is the main protein of interest in this thesis. Other true adipokines like apelin, adipsin and visfatin are relatively newly identified and there is limited information available about their effects on platelet function and hence they are only described along with other adipokines.

1.2.1 Leptin

Leptin, which is a 16 kDa protein comprising of 167 amino acids, was one of the first adipokines described in literature and widely studied in the obese mouse model *ob/ob* (leptin deficient) and diabetic model *db/db* mice (leptin-receptor deficient) (Wang, Chandrasekera and Pippin, 2014). Leptin has been predominantly known for its role in regulation of food intake and body weight labelled as the hunger hormone. It elicits its function by activation of JAK-STAT signalling pathway in response to the leptin receptor activation. A dual

mechanism of action has been observed, as *db/db* mice, where leptin receptor activation and JAK-STAT is inhibited, glucose haemostasis was regulated by phosphatidylinositol-3 kinase (PI3K), suggesting that leptin activated PI3K and was required for maintaining normoglycemia in the wild-type and knockout mice (Stavros Konstantinides *et al.*, 2001). Additionally, leptin was found to inhibit insulin production in lean mice in response to induction of suppressor of cytokine signalling proteins 3 (SOCS-3) (Kershaw and Flier, 2004; Dellas *et al.*, 2008). Elevated levels of leptin in the plasma of patients suffering from T2DM has been described in HUVECs, where induction of tissue factor (TF) expression and upregulation of PAI and t-PA thus led to hypercoagulability in diabetic patients (Bobbert *et al.*, 2011). Platelets are thought to be the main target of leptin action owing to the presence of the leptin receptor on their surface. The long form of the receptor regulates platelet activity of downstream pathways involving JAK activation, PI3K, phospholipase Cγ2 (PLCγ2) and phospholipase A_2 (PLA_2) activation, which therefore alters cAMP hydrolysis, GPIIa/IIb expression and thromboxane A_2 (TXA_2) synthesis (Nakata *et al.*, 1999; Zhang and Colman, 2007; Santilli *et al.*, 2012). Clinical studies have shown platelets from obese donors are not resistant to the enhancing effects of leptin on ADP-induced platelet aggregation further corroborating leptin as an independent indicator of cardiovascular events due to platelet hyperactivity (Dellas *et al.*, 2008; Lukasik *et al.*, 2012).

1.2.2 Adiponectin

Adiponectin is a 244 amino acid protein that has two active forms – full-length and globular adiponectin with contrasting physiological functions. It is synthesised by adipocytes in response to increase in circulating pro-inflammatory factors (Kwon and Pessin, 2013). Full-length adiponectin (fAd) has anti-thrombotic properties and exerts its vasculoprotective effects through its direct actions in the vascular system, such as increasing endothelial NO production, inhibiting endothelial cell activation and endothelium-leucocyte interaction, enhancing phagocytosis, and suppressing macrophage activation, macrophage-to-foam cell transformation and platelet aggregation (Zhu *et al.*, 2008; Restituto *et al.*, 2010). fAD increases insulin sensitivity by regulating glucose metabolism via specific receptors on platelet surface, AdipoR1 and AdipoR2 that trigger p38 MAPK and PPARα (Kadowaki *et al.*, 2006; Santilli *et al.*, 2012)– PPARγ antagonists stimulate expression of adiponectin by adipocytes (Bełtowski, 2003; Kwon and Pessin, 2013). Enhanced platelet response to ADP and collagen was observed in adiponectin deficient mice, whereas preincubation with adiponectin in the platelet-rich plasma of patients suffering from MetS, prevented platelet aggregation when stimulated with ADP and epinephrine (Santilli *et al.*, 2012). Hypoadiponectinaemia is suggested to be involved IR and accelerated atherogenesis associated with obesity (Bełtowski, 2003; Lihn, Pedersen and Richelsen, 2005). Exogenous administration of adiponectin or overexpression in transgenic mice results in improved insulin sensitivity whereas adiponectin deficient mice develop high fat diet-induced

inflammation and IR suggesting that adiponectin plays a major role in prevention of atherosclerosis (Kato *et al.*, 2006).

The proteolytic cleavage of fAd by leukocyte elastase gives rise to a biologically active globular adiponectin (gAd) *in vitro*, but the *in vivo* production of gAd has yet to be characterised (Waki *et al.*, 2005). Most recent studies that explore the role of gAd in immune functions, reveals that gAd serves as a potent mobilizer of haematopoietic progenitors and leukocytes into the peripheral blood (Sung *et al.*, 2015). In cardiac fibroblasts and vascular endothelial cells, gAd plays a role in activation of inflammatory pathway mediated by NF-ƙB and proliferation by increased expression of angiotensin II (Goldstein *et al.*, 2007), although this has been challenged by Adya *et al*. (2012) and they propose that a synergistic, potentially protective mechanism of gAd occurs at the initiation site of the atherosclerotic plaque. gAd has been shown to trigger the GPVI-FcγR complex mediated activation pathway in platelets, where it triggers Src-family kinase, Syk and PLCγ2 (Riba *et al.*, 2008).

1.2.3 Other true adipokines

No data is available illustrating effects of adipokines like apelin, visfatin and adipsin on platelet function (Santilli *et al.*, 2012), but their mechanism of action in adipose tissue is documented. Apelin has been shown to be regulated by insulin overexpression and has been correlated with PI3K and MAPK stimulation (Boucher *et al.*, 2005). Visfatin is an adipokine which is responsible

for destabilisation of atherosclerotic plaques and enhanced expression of visfatin by macrophages was reported at the site of atherogenesis, thereby implicating it in the progression of acute cardiovascular outcomes (Romacho *et al.* 2013). Adipsin is an adipokine which is implicated in T2DM owing to its role in insulin regulation. Adipsin deficient mice lack glucose tolerance due to decreased levels of insulin whereas diabetic mice, when given exogenous adipsin have improved glucose metabolism and increased insulin expression in the β islets (Lo *et al.*, 2014).

1.2.4 Other adipokines

Several cytokines released from adipocytes are also secreted by other cell types. These affect insulin sensitivity and inflammatory responses in obese, MetS, T2DM patients. Ghrelin, which is mainly produced by the cells in gastrointestinal tract, is also secreted by adipose tissue where it regulates the adiposity of the tissue and Santilli *et al*. (2012) present it to have no effect on platelet aggregation (Tsubone *et al.*, 2005).

TNF-α, which is an inflammatory cytokine, modulates platelet function by activation of NF-ƙB pathway, MAPK pathway via ERK and p38, thus contributing towards the inflammatory role of platelets (Piya, McTernan and Kumar, 2013). Platelets express functional TNF receptors and engagement of TNF-α with its receptors elicits platelet activation, with enhanced TXA_2 biosynthesis and CD40L shedding (Santilli *et al.*, 2012).

A number of other cytokines like IL-6, IL-10, MCP-1, PAI-1, RBP-4, have activatory effects on platelets (Fantuzzi *et al.*, 2005; Santilli *et al.*, 2012), but they are not detailed as a part of this introduction as we restrict it to the products mainly contributed by adipocytes compared to other cell types.

1.3 Resistin

Resistin, also known as FIZZ3 (found in inflammatory zone 3) and ADSF (adipocyte-secreted factor), was initially identified in a screen for adipocyte-specific transcripts downregulated by treatment with antidiabetic thiazolidinedione (TZD) drugs (insulin sensitizers) (Schwartz and Lazar, 2011). It was first correlated to obesity in diabetic mice as the protein that conferred IR (Steppan *et al.*, 2001). In mice it is exclusively produced by white adipose tissue, while in humans high levels of resistin are expressed by macrophages, monocytes, leukocytes, spleen and bone marrow cells, followed by lung tissue, resting endothelial cells and placenta in addition to pre-adipocytes that are differentiating to adipocytes (Tarkowski *et al.*, 2010). High levels of circulating resistin are characteristic of obese mice and gender-based variations have been noted in mice and in humans, where plasma obtained from females has been shown to have higher levels of resistin (Steppan and Lazar, 2004). In rodents, resistin immunoneutralisation is reported to promote normoglycemia and improve IR in high-fat diet fed mice (Steppan *et al.*, 2001; Rajala *et al.*, 2004). The attempts at correlating resistin with obesity, MetS, T2DM and IR have been controversial owing to the differences in the techniques used to assess resistin expression -researchers correlated disease states to mRNA levels, circulating resistin in plasma or resistin concentrations detected using immunoblotting (Ukkola, 2002). An important difference which complicates extending resistin observations from mice to men is the fact that in humans, resistin is secreted by monocytes and macrophages, and negligible amounts produced by the adipose

tissue leading to physiological differences in its mechanism and target of action (Ukkola, 2002; Jamaluddin *et al.*, 2012).

1.3.1 Resistin structure

Human resistin is a 12.5kDa molecule that belongs to a cysteine-rich family of resistin-like molecule (RELMs) proteins - RELMα, RELMβ and resistin (FIZZ1, FIZZ2 and FIZZ3 respectively), where each member of the family has a unique tissue distribution pattern (Steppan *et al.*, 2001). Human and mouse resistin share 59% identity at the amino acid level. Its structural elements contain a variable N-terminal and a similar repeat sequence of cysteine residues found in both mouse and human isoforms (Al Hannan and Culligan, 2015). The two structurally distinct domains, N-terminal and C-terminal, are linked via a flexible neck domain. The C-terminal is a disulfide-rich beta-sandwich "head" domain and N-terminal alpha-helical "tail" segment. The alpha-helical segments associate to form three-stranded coiled coils, and surface-exposed interchain disulfide linkages (Cys6) mediate the formation of tail-to-tail hexamers, where the intertrimer formation is the key step in forming a functional resistin molecule. (Patel *et al.*, 2004). Figure 1.1 shows a homotrimer of resistin, where each colour represents a distinct molecule. However, the recombinant human resistin (PeproTech, USA) that was later used during experimentation was a 19.5kDa, disulfide-linked, homodimeric protein composed of two identical 92 amino acid

chains linked by a single disulfide bond. In order to address if the difference in oligomerisation caused any functional difference, control experiments were performed and can be found in sections 3.2.1.5 (Figure 3.6), and 3.2.3 (Figure 3.12).

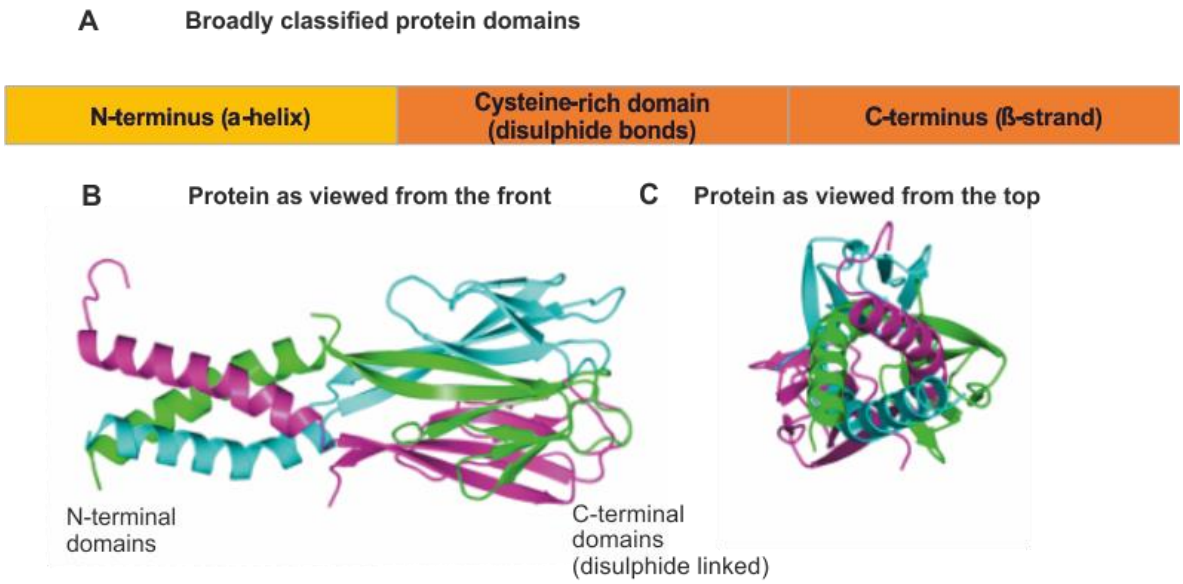


Figure 1.1 Resistin protein structure: Resistin exists as a homotrimer assembly of molecules. The N-terminal domains are found central to the assembly, while the C-terminal, cysteine-rich domains form the disulphide linkages that hold the trimer assembly together. (A) A broadly classified domain structure of the human resistin molecule of 108 a.a. is shown. (B) The α-helical domains at the N-terminal region and the β-strand sandwich at the C-terminal are clearly viewed in the front view of the protein trimer, where each colour represents one molecule of resistin (C) while the top view of the resistin protein trimer shows the central N-terminal domains towards the top and the C-terminal, disulphide linked domains towards the bottom. (Further details can be accessed at: http://www.ebi.ac.uk/pdbe/entry/pdb/1rgx)(Patel *et al.*, 2004).

1.3.2 Resistin gene expression

The human resistin gene, *RETN,* is located on chromosome 19p13.2 and was first identified as 10-cysteine containing protein (*XCP*) (Nagaev *et al.*, 2006). Resistin mRNA expresses 120 a.a. which contains a 20 a.a. signal peptide, and in its mature form it has 108 a.a. in humans and 94 a.a. in mice (Ukkola, 2002; Yang *et al.*, 2003). The expression of *RETN* is exclusively in myeloid cells and highest expression being in the bone marrow. Furthermore, expression of *RETN* is dependent on the myeloid-specific nuclear transcription factor, the CCAAT/enhancer binding protein epsilon (*CEBPE*). This transcription factor was found to be essential for and co-expressed with *RETN*. The disruption of *CEBPE* leads to silencing of *RETN* and its overexpression leads to expression of *RETN* even in non-myeloid cells (Chumakov *et al.*, 2004; Nagaev *et al.*, 2006). Chumakov *et al.* (2004) also showed that *RETN* played an important role in chemotaxis of bone-derived myeloid cells and specifically interacts with neutrophil α-defensins. Nagaev *et al.* (2006) observed that human and mouse resistin genes present with complications in understanding resistin's function as resistin gene lineages underwent various modifications during the evolution of different mammalian species. They also claim that resistin genes are not true homologues but the resulting proteins are functional analogues of each other, whereas Al Hannan & Culligan (2015) give the opposite view that resistin in mice and humans have distinct functional properties and expressed as a result of different signalling molecules.

In the studies related to resistin genetics, a polymorphism in the resistin gene (420 C>G) was reported in relation to the BMI of women, where women carrying the G-allele had lower BMI compared to C/C homozygote women (Feng and Zhang, 2011).

Resistin expression has been shown to be affected by insulin in adipocytes, where insulin-desensitiser (TZDs), PPARγ receptor agonists, caused an increase in resistin expression (Steppan *et al.*, 2001; Bełtowski, 2003). In a contradicting study stimulation of resistin expression in response to PPARγ agonists in obese rodent models was observed (Way *et al.*, 2001). Steppan & Lazar (2004) suggest that secretory profiles of resistin change, depending on which rodent model and/or study technique further contributing to the resistin conundrum.

1.3.3 Mechanism of action of resistin in endothelial and immune cell lines

Resistin exerts its influence over a number of cell types in a pleiotropic manner – via endocrine, paracrine and autocrine mechanisms. Implication of resistin in inflammation, MetS, T2DM, obesity, atherosclerosis and development of atherosclerosis, and cancer metastasis and the mechanisms involved therein are yet unclear owing to limited knowledge regarding resistin receptors (Codoner-Franch and Alonso-Iglesias, 2015). In addition to this, the role of resistin under healthy patients is as yet unclear. It is therefore, important to

identify a receptor for resistin and delineate both receptor-dependent and independent effects induced by this multifaceted hormone.

A number of putative resistin receptors have been described in the literature. An isoform of decorin (that lacks the glycation site) is reported as a resistin receptor in adipose progenitor cells, where it is reported to affect adipocyte differentiation, migration and proliferation (Daquinag *et al.*, 2011). Mouse resistin is also described to interact with tyrosine-kinase-like orphan receptor (ROR1) to mediate a decrease in glucose uptake and negatively affect adipogenesis in mouse adipocyte cell line (Sánchez-Solana, Laborda and Baladrón, 2012).

Toll-like receptor-4 (TLR-4), which is mainly known for its role in triggering inflammation responses in immune cells is also proposed as a receptor for resistin. Tarkowski *et al*. (2010) show that resistin binds to TLR-4 and induces cytokine production by initiation of NF-ƙB and MAPK signalling pathways, where it competes with a known TLR-4 ligand, LPS (lipopolysaccharide – present on the bacterial cell wall). Myeloid differentiation marker 88 (MyD88), which is essential for downstream signalling by TLRs also participates in the signalling cascade (Rondina, Weyrich and Zimmerman, 2013). Resistin-TLR-4 complex is also demonstrated to reduce insulin mediated phosphorylation of insulin receptor, upregulation of SOCS-3 and phosphotyrosine phosphatase-1B (PTP-1B), which are indicative of its role in IR (Benomar *et al.*, 2013). Most recently, resistin was shown to play a role in IR and hypertension in TLR-4 deficient mice by activation of the renin-angiotensin system (Jiang *et al.*, 2016) supporting the

view that resistin performs its inflammatory role by activating downstream signalling molecules of the TLR-4 pathway. A brief summary of resistin mediated signalling activated downstream of TLR4 are shown in Figure 1.2.

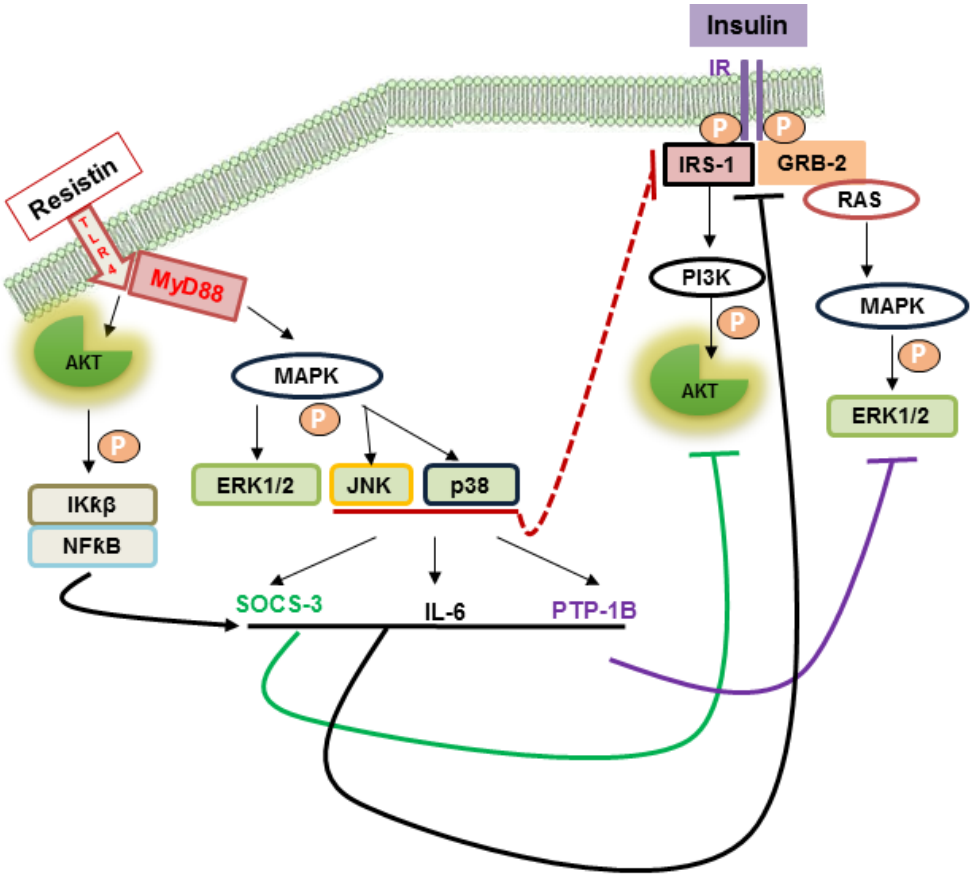


Figure 1.2 Resistin mediated activation of TLR4 signalling pathways and its influence on insulin signalling. Resistin mediated signalling occurs by binding TLR4 followed by MyD88 on the intracellular face triggering NF-ƙB and MAPK pathways. Phosphorylation of JNK and p38 downstream of MAPK activation and NF-ƙB phosphorylation leads to suppressor of cytokine signalling proteins 3 (SOCS-3), interleukin 6 (IL-6) and phosphotyrosine phosphatase-1B (PTP-1B) generation, which in turn inhibits insulin signalling in the cell, thus contributing to IR.

Another elaborate study claimed that adenylyl cyclase protein 1 (CAP1) was a bonafide receptor for resistin in a monocytic cell line. They presented evidence that interaction of resistin with CAP1 initiated adenylyl cyclase [or protein kinase A (PKA)] -mediated activation of NF-ƙB and the release of cytokines by the monocytes (Lee *et al.*, 2014). Lee *et al.* (2014) performed a detailed analysis of resistin mediated responses in cells where they silenced each of the four putative resistin receptors – CAP1, TLR-4, decorin and ROR1. They present evidence that cell migration, and cytokine expression upon resistin treatment was significantly reduced in cells lacking CAP1 compared to cell populations lacking TLR-4, decorin and ROR1, therefore claiming it as a confirmed receptor for resistin. As described in detail in section 1.6 of this chapter, CAP1 is mainly a cytosolic protein, that is associated with the membrane (Noegel *et al.*, 2004) but it has no transmembrane domains. It is therefore difficult to conceptualise the circumstances under which extracellularly released resistin might encounter cytosolic CAP1, as there is no evidence of resistin internalisation in the literature (Al Hannan and Culligan, 2015) and therefore, the resistin receptor conundrum remains unresolved.

Since we investigate the effects of resistin on platelets, a description of platelets and their cytoskeleton and regulation is given in the following sections and we investigate CAP1 in platelets subsequently.

1.3.4 Resistin function

The function of resistin in mice and men is controversial at best, as studies have not been able to clarify the precise role of resistin or its target cells under normal, healthy conditions. It has been difficult to assess whether elevated serum resistin is the cause of pathologies or if it is the effect of patients suffering from those pathologies like metabolic dysfunction in diabetes, obesity and IR that lead to the observed increase in resistin levels. Most recent observation regarding resistin function is that resistin may foster *in vivo* oxidative stress, thromboxane-dependent platelet activation and platelet-derived inflammatory proteins release in patients suffering from T2DM leading to atherothrombosis (Santilli *et al.*, 2016).

1.3.4.1 Resistin in MetS and associated CVD progression

The first functional attribute of resistin described in the literature is its role in IR in obese mice (Steppan *et al.*, 2001). Similar evidence was found in relation to increased expression of human resistin leading to IRin obese patients (McTernan *et al.*, 2002; Smith *et al.*, 2003) but simultaneously disputed due to inconsistency in the observations in other similar studies (Nagaev and Smith, 2001; Ukkola, 2002; Lee *et al.*, 2003). Several other studies provide positive correlation of elevated serum resistin in obesity and decreased insulin sensitivity and its role in progression of T2DM (Rajala *et al.*, 2004; Piya, McTernan and Kumar, 2013; Abate *et al.*, 2014; Nieva-Vazquez *et al.*, 2014). (Bobbert *et al.*, 2011) also provide evidence of resistin-mediated increase in procoagulability in

patients suffering from T2DM by causing an increase in expression of TF and disturbing the balance of fibrinolytic system by upregulation of PAI and t-PA expression, thus contributing towards increased CVD risk in patients suffering from T2DM. Resistin is described as a biomarker of systemic inflammation and atherosclerosis (Lehrke *et al.*, 2004; Reilly *et al.*, 2005). In mice, induction of hypertension and IR in response to administration of recombinant resistin was shown to be triggered via TLR4 pathway (Jiang *et al.*, 2016).

A number of controversies regarding the role of resistin in diabetes, obesity and IR have arisen over the years after it was first described in 2001 (Smith, 2002; Schwartz and Lazar, 2011). A unifying observation is that antidiabetic TZD drugs inhibit resistin gene expression in human macrophages as well as in mouse adipocytes suggesting that although resistin might be expressed in different tissues in humans and mice, it can potentially have similar function upon expression (Banerjee, 2004).

1.3.4.2 Resistin in inflammation: Effects on human monocytes and endothelial cells

Resistin is secreted from and affects monocyte behaviour, where it enhances monocyte-endothelial adhesion via p38 MAPK pathway, consequently increasing ICAM-1 and VCAM-1 expression (Hsu *et al.*, 2011), and enhances monocyte transmigration towards endothelial cell layer via TLR4-pathway (Pirvulescu *et al.*, 2014). The same research group had previously shown that

resistin caused increased expression of adhesion proteins (fractalkine) in human endothelial cells by activating p38 MAPK and NF-kB pathways, in comparable mechanisms to TNF-α (Manduteanu *et al.*, 2009) and that high glucose enhanced the effects of resistin mediated increase in fractalkine (Manduteanu *et al.*, 2010). These observations along with clinical examinations that recorded circulating serum resistin have shown that resistin plays a key role in inflammation and potential indicator of the degree of systemic inflammation (Fantuzzi *et al.*, 2005; Jamaluddin *et al.*, 2012; Kwon and Pessin, 2013; Piya, McTernan and Kumar, 2013; Al Hannan and Culligan, 2015).

1.3.4.3 Function of resistin in adipokine regulation in diabetic mouse models

Adiponectin, leptin and resistin each regulates the expression of others to maintain a balance of circulating adipokines. Direct reciprocal effect of adiponectin and resistin is seen in vascular endothelial cells where induction of ICAM-1 and VCAM-1 by resistin is inhibited by adiponectin (Kawanami *et al.*, 2004). In leptin deficient, obese mice, loss of resistin is shown to improve glucose homeostasis and thus contribute to its role in IRin diabetic mice (Qi *et al.*, 2006). As described earlier, leptin and resistin together contribute to increased CVD risk in T2DM patients (Bobbert *et al.*, 2011).

1.3.4.4 Function of resistin in glucose metabolism in rodent disease models and human endothelial cell lines

Dysregulation of glucose metabolism or glucose tolerance by resistin has been demonstrated in resistin deficient mice, where authors observed that blood glucose of fasting resistin deficient mice was lower than that of wild-type mice and this was recovered upon administration of recombinant resistin to the deficient mice (Banerjee, 2004). In humans also, hyperglycemia is always accompanied by high serum concentrations of resistin but with weak links to IR (Pagano *et al.*, 2006). Activation of SOCS proteins, that are inhibitors of insulin signalling, was observed in hyperresistinemic mice, leading to activation of PI3K and Akt phosphorylation, that was prevented in mice expressing dominant negative resistin with improvement in glucose tolerance and insulin sensitivity (Steppan *et al.*, 2005).

Glucose transporters, GLUT1 and GLUT4, are translocated to the membrane enabling glucose uptake by cells. Resistin reduces the uptake of glucose by rat skeletal muscle cells (Fan *et al.*, 2007), Further, resistin causes tyrosine phosphorylation of insulin-receptor substrate-like 1 (IRS-1) thus decreasing GLUT4 translocation inhibiting glucose permeability of cells (Palanivel *et al.*, 2006). Additionally, resistin downregulates the expression of GLUT1 in endothelial cells, thus impairing glucose permeability (Li *et al.*, 2016). Within the scope of disrupting metabolism, resistin disrupts glycogen synthesis under high insulin and high glucose levels by downregulating the levels of GSK3β,

integrating the observations that it causes SOCS-3 activation and Akt phosphorylation (Song *et al.*, 2013).

1.3.4.5 Upregulation of resistin in cancerous cells: Role in cell proliferation

Resistin promotes smooth muscle cell proliferation by activation of extracellular related kinase 1/2 (ERK1/2) and PI3K (Calabro *et al.*, 2004). Resistin mediated upregulation of matrix metalloproteinases, MMP-2 and MMP-9 was reported in breast cancer cell lines (Pan *et al.*, 2007). Progression of cancer, upon exogenous administration of recombinant resistin, in prostate cancer cell lines occurred via stimulation of PI3K/Akt signalling pathway (Housa *et al.*, 2008). In gastric cancer cell lines, resistin was shown to stimulate p38 MAPK thereby activating NF-ƙB pathway (Codoner-Franch and Alonso-Iglesias, 2015). In their review, Feng & Zhang (2011) describe evidence of studies that observe a correlation of elevated resistin levels in endometrial cancer, colorectal cancer and lung cancer patients.

1.4 Platelets

Platelets are anucleate cells, which are discoid when quiescent in normal circulation and undergo dramatic shape change to become flattened upon activation at site of injury, playing a major role in regulation of haemostasis and inflammation. They are present in the blood along with erythrocytes and leukocytes and under shear stresses of circulation they flow close to the endothelial cell lining the vessel walls. Platelets are derived from their progenitor cells, the megakaryocytes, in the bone marrow in response to the hormone thrombopoietin (TPO). Cytoskeletal changes in the mature megakaryocytes form protrusions called proplatelets or in state of inflammation undergo cytoplasmic fragmentation and microtubule reorganisation (Poulter and Thomas, 2015) determining their structural and functional attributes finally releasing platelets into circulation. The normal platelet count of blood ranges between 150 and 400 × 10^9/L and human platelets have a lifespan of 1-10 days, thus forming a heterogeneous population that varies in size and responsiveness to agonists (Bearer, Prakash and Li, 2002).

1.4.1 Platelet morphology

Platelets are about 3.0 by 0.5 µm in size in their resting state, containing a mean volume of 6-10 fL (Hartwig and Italiano, 2006). Platelets have characteristic morphological features including an unusual membrane system which comprises of an open canalicular system (OCS), which is a system of tunnelling invaginations of the plasma membrane that tracks through the platelet, and a

dense tubular system (DTS), which is thought to be derived from the endoplasmic reticulum of megakaryocytes and contains intracellular calcium stores (White, 1972). In the active platelet the OCS serves as a conduit system which enables fusion and release of granules together with accommodating the extra surface area of the spreading platelet (Hartwig and Michelson 2012). Additionally, platelets contain multiple organelles like mitochondria, lysosomes and granules including alpha and dense granules. Further details of the granular system are listed in Table 1.3.

Table 1.3 Details of the platelet granular system

Platelet granule	**Alpha-granules**	**Dense granules**	**Lysosomes**
# /platelet	50-80	5-6	Variable
Diameter	0.2-0.4µm	0.15µm	Variable
Contents	Adhesive proteins (Thrombospondin, P-selectin) Haemostatic factors (factor V, vWF, fibrinogen) Angiogenic factors (angiogenin, VEGF) Anti-angiogenic factors (angiostatin, PF4) Growth factors (PDGF, bFGF, SDF1α) Proteases (MMP-2, MMP-9) Necrotic factors (TNF-α, TNF-β) Cytokines	Serotonin ATP, ADP Polyphosphates (Calcium, Magnesium and Potassium)	Hydrolytic enzymes
References	(Harrison and Cramer, 1993; Whiteheart, 2011)	(McNicol and Israels, 1999)	(White, 2007)

The contents of granules are released in the process of platelet activation and their secretome has paracrine effects on activating neighbouring platelets and aiding the coagulation cascade (Fitch-Tewfik and Flaumenhaft, 2013). Platelet granules become centralised during activation. Interestingly, this centralisation is independent of platelet shape change and it has been proposed that depolymerisation of filamentous (F-) actin cytoskeleton is required for normal granule secretion (Flaumenhaft, 2003). Secretion of the granulomere initiates secondary activation in platelets, thus enhancing the platelet response at the haemostatic site.

The platelet cytoskeleton comprises of a peripheral band of microtubules, which gives it the discoid shape, and actin cytoskeleton and its associated proteins, which play an important role in cytoskeletal reorganisation of the activated platelet by formation of filopodia and lamellipodia finally merging to form a completely spread platelet with a centralised granulomere or hyalomere (Hartwig, Steffen and Cell, 1992; Blanchoin *et al.*, 2014).

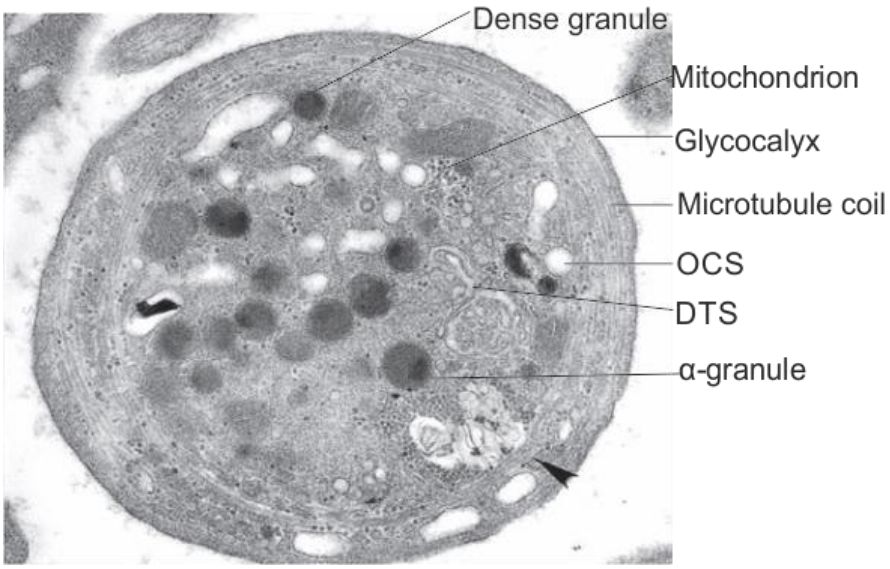


Figure 1.3 Ultrastructure of discoid, resting platelet. Platelet seen had been sectioned along the equatorial plane (magnification x 22,000). Glycocalyx, phospholipid bilayer forms the outermost layers of platelet. Open canalicular system (OCS) pits outline the surface of the platelet and the dense tubular system (DTS) within the platelet form a network of calcium rich zones. Underneath the outer layers lies the platelet cytoskeleton comprising of a microtubular coil and actin networks. A number of alpha and dense granules are observed along with other organelles like mitochondria. Figure modified from (White 2007).

1.4.2 Platelet plug formation

Platelets express a plethora of receptors on their surface that regulates platelet activity by responding to the variable microenvironments at the site of endothelial damage to enable their holistic role in haemostasis and inflammation. Platelet agonists like collagen, that initiate platelet adhesion and aggregation are present in the subendothelium (Roberts, McNicol and Bose, 2004). The subendothelial matrix is exposed upon vascular injury or rupture of an atherosclerotic plaque (Figure 1.4). Von Willebrand Factor (vWF), which is a plasma protein, interacts with collagen from the subendothelium altering the conformation of glycoprotein GP1b receptor complex, thus promoting transient tethering and rolling action of platelets on the exposed subendothelium (Jackson, 2011). Rolling platelets then adhere in response to binding of collagen to GPVI and $\alpha_2\beta_1$ receptors (Watson *et al.*, 2005). Soluble agonists are then generated at the site of injury which triggers activation of G-protein coupled receptors (GPCRs), followed by activation of integrin $\alpha_{IIb}\beta_3$, which results in aggregation and stable plug formation (Li *et al.*, 2010). Ligand binding at the membrane triggers signalling cascades that enable platelets to amplify their presence by secretion of their granulomere and the coagulation cascade ensues by formation of thrombin and thrombin-mediated conversion of fibrinogen to fibrin (Heemskerk, Bevers and Lindhout, 2002; Palta, Saroa and Palta, 2014). Platelet membrane expresses phosphatidylserine (PS) on the surface designating platelets with a procoagulant state within the thrombus. Fibrin strands ensure compaction and stability of the developing thrombus (Versteeg

et al., 2013). Thrombus consolidation occurs when endothelial cells surrounding the thrombus start generating platelet inhibitors like PGI_2 and NO (Smolenski, 2012).

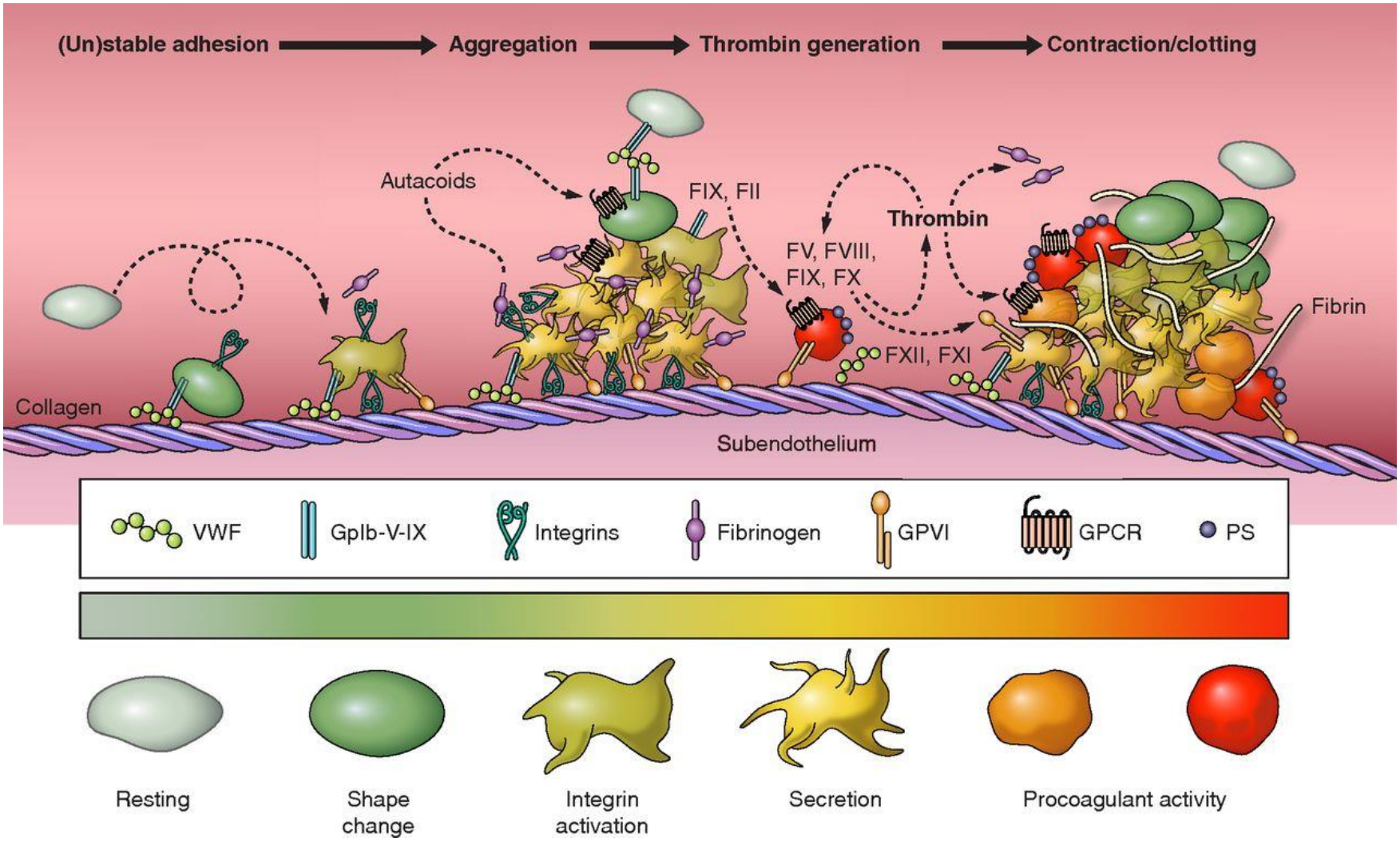

(Un)stable adhesion
Aggregation
Thrombin generation
Contraction/clotting
Autacoids
FIX, FII
Thrombin
FV, FVIII,
FIX, FX
FXII, FXI
Fibrin
Collagen
Subendothelium
VWF
GpIb-V-IX
Integrins
Fibrinogen
GPVI
GPCR
PS
Resting
Shape change
Integrin activation
Secretion
Procoagulant activity

Figure 1.4 Platelet plug formation. At the site of endothelial interruption, platelets begin to roll inducing transient platelet adhesion by binding to plasma protein vWF. The GP1b-IX-V complex then mediates binding to collagen in the subendothelial matrix completing platelet adhesion. As platelets adhere, they begin to change shape from discoid to form more flattened structures. Outside-in signalling triggers activation of integrins and therefore initiate platelet aggregation. Platelet granulomere is released from its cytosol providing additional platelet agonists, like ADP, serotonin and coagulating factors. Presence of factor V, VIII lead to conversion of prothrombin into active thrombin protease. As the coagulation cascade begins, thrombin converts fibrinogen into fibrin, enabling the stabilisation of the growing thrombus. Thrombin, ADP and TXA_2 also activate G-protein coupled receptors amplifying platelet activation and recruiting more platelets to the site of injury and enabling platelet procoagulant activity. Thrombus consolidation mediated by platelet inhibitory pathways triggered by NO and PGI_2 production by neighbouring endothelial cells prevents the thrombus from occluding the vessel. Figure from Versteeg *et al*. 2013.

1.4.2.1 Platelet adhesion: vWF/GP1b complex mediated platelet activation

At the site of injury, shear rates within the vasculature promote transient binding of vWF in the subendothelium to platelet receptor complex GP1b-IX-V. This then enables binding collagen and therefore, the initial platelet adhesion, leading to integrin activation and integrin-dependent stable platelet adhesion and aggregation (Li *et al.*, 2010). Binding of the receptor complex to collagen induces an early increase in intracellular calcium (Ca^{2+}) due to interaction with Src-family kinases (SFK) and PI3K. An increase in intracellular cGMP (cyclic guanosine monophosphate) levels is also observed when GP1b-IX-V complex is activated in response to stimulation of protein kinase G (PKG) (Riba *et al.* 2006).

1.4.2.2 Platelet adhesion: Collagen/GPVI mediated platelet activation

At the site of vascular rupture, integrin $\alpha_2\beta_1$ receptors enable platelet adhesion to collagen thereby enabling GPVI mediated activation. This binding of immunoglobulin receptor GPVI to collagen then causes tyrosine phosphorylation of FcγRIIA. This then causes recruitment of Syk at the platelet membrane (Janmey and Matsudaira, 1988). Next, phosphorylation of Syk activates PLCγ2, which then leads to generation of diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 is freely soluble in the cytosol and translocates and binds DTS, initiating the release of intracellular stores of Ca^{2+} in response to IP_3 receptor. Intracellular calcium levels are further elevated upon opening of the calcium channels in the plasma membrane, whereas DAG activates protein kinase C

(PKC). Ca^{2+}, as a secondary messenger, together with DAG mediate characteristic platelet activation leading to shape change, granule secretion and aggregation (Roberts, McNicol and Bose, 2004). Granule secretion enhances platelet activation when platelets are presented with low concentrations of collagen.

1.4.2.3 GPCR mediated platelet activation

Platelets adhere at the site of injury initially forming a monolayer which serves as a base for platelet aggregation. This process is dependent on binding of bivalent fibrinogen to integrin $\alpha_{IIb}\beta_3$ (Parise, 1999). Aggregation is mediated by a process involving capture of platelets by vWF bound to $\alpha_{IIb}\beta_3$ and activation of $\alpha_{IIb}\beta_3$ by the secondary mediators, ADP and TXA_2, and by the product of the coagulation cascade, thrombin (Watson *et al.*, 2005). Soluble platelet agonists (that are not bound to subendothelium surface) are released from cellular damage and produced as a result of coagulation or platelet exocytosis, activate platelets via G-protein coupled receptors (GPCR) (Li *et al.*, 2010).

GPCRs are 7 transmembrane domain containing proteins coupled to G proteins. G-proteins consist of α, β, and γ subunits. The extracellular N-terminus of G-proteins provides a connection to the intracellular C-terminus (Ma *et al.*, 1998). The α-subunits bind GDP in inactive state and GTP in their active form. There are four categories of the α-subunits – G_q, $G_i/G_o/G_z$, $G_{12/13}$, and G_s. G_q, $G_i/G_o/G_z$, $G_{12/13}$ are responsible for activation of platelets, whereas G_s plays a role in

platelet inhibition. The β- and γ- subunits are regulated by acylation and prenylation, respectively, in close association with each other and are responsible for G-protein association to the membrane (Offermanns, 2006; Li *et al.*, 2010).

In platelets, there is one $G\alpha_s$ subunit mediating platelet inhibition, four $G\alpha_i$, which inhibit adenylyl cyclases thereby activating platelets, three $G\alpha_q$ subunits, which activate PLCβ thereby enabling phosphotidylinositol 4,5 bisphosphate (PIP_2) to form IP_3 (leading to elevation of intracellular Ca^{2+}) and diacylglycerol (DAG) (leading to PKC activation), and $G\alpha_{12}$ and $G\alpha_{12/13}$ subunits, which activate Rho A, and thus regulate actin cytoskeletal reorganisation. The inhibition and consolidation of thrombus formation by α_s is described in section 1.4.2.6.

G_q and $G_{12/13}$ deficient mice have been reported to be unresponsive to thrombin, TXA_2 and that G_i stimulation or only the βγ subunits is not efficient in platelet stimulation (Simon *et al.*, 1997; Moers *et al.*, 2003). These mice also have severely impaired thrombosis and haemostasis, as evidence from these mice shows that collagen-induced activation of mouse platelets resulting in platelet aggregation and thrombus formation is completely dependent on intact G protein-mediated signalling pathways (Moers *et al.*, 2004). These observations emphasise the importance of G-protein signalling in comprehensive platelet activation under shear rate.

GPCR signalling pathways in platelets are represented in Figure 1.5 and details of agonist specific responses are given in the following sections.

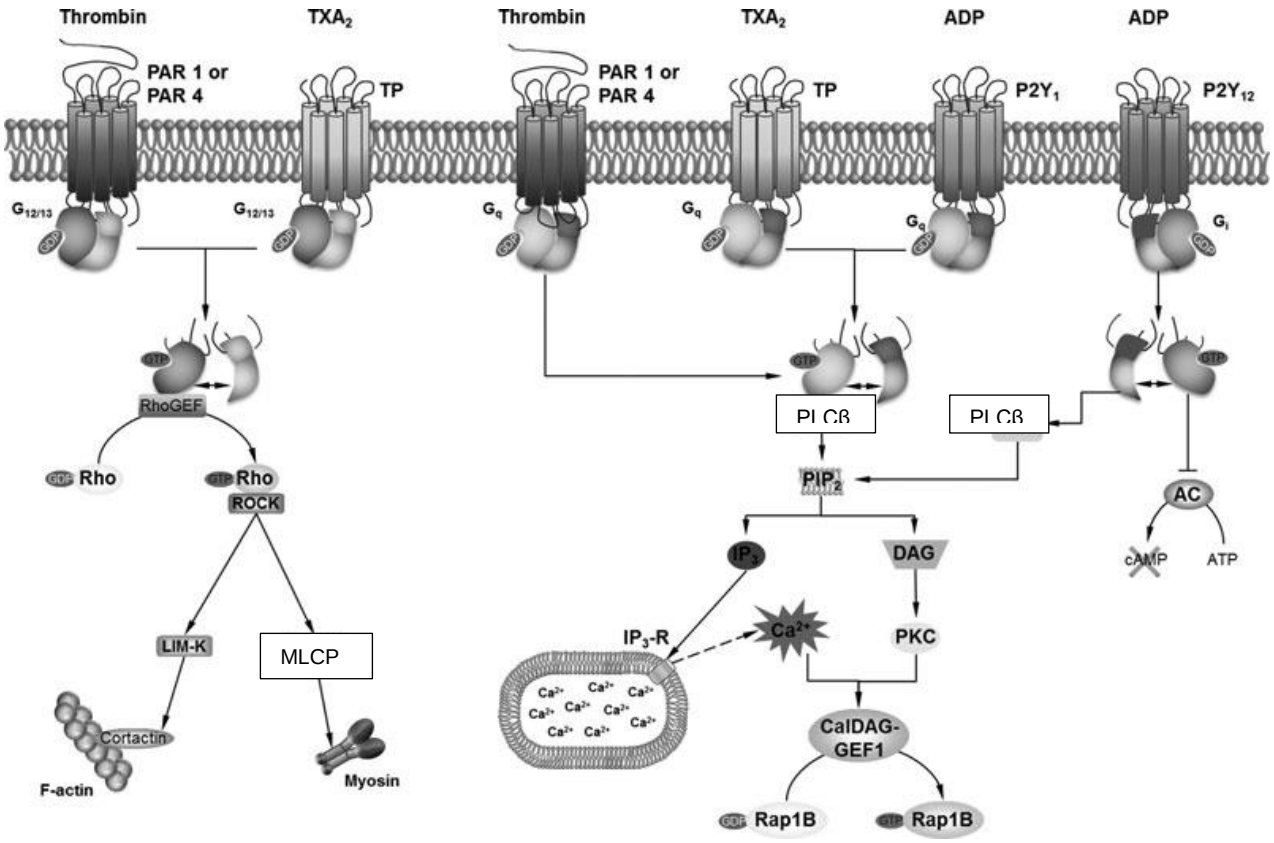


Figure 1.5 GPCR signalling pathways in platelet activation. The key events that occur downstream of GPCR activation by agonists like thrombin, thromboxane and ADP include cytoskeletal reorganisation following Rho signalling (ROCK-LIMK mediated regulation of the actomyosin complexes), and phospholipase mediated calcium mobilisation via PIP_2 and PKC activation**.** From (Brass, 2003; Goggs and Poole, 2012) with modifications.

1.4.2.3.1 Platelet activation pathways triggered by thrombin

Thrombin causes activation of G_q, $G_{12/13}$ via protease activated receptor, PAR1/PAR4 activation (Coughlin, 2000). PAR1 and PAR4 differentially regulate the actin cytoskeleton and the granule secretion (Flaumenhaft *et al.*, 2005). Coupling of PARs to G_q subunits initiates PLC activation, followed by production of IP_3 and DAG from PIP_2 and subsequent release of Ca^{2+} from the DTS and PKC activation, whereas $G_{12/13}$ subunits allows interactions with Rho GEFs (Rho guanosine exchange factor) such as p115RhoGEF and causes activation of the Rho family of small GTPases. Rho GTPases mediate rearrangement of the platelet actin cytoskeleton and subsequent generation of filopodia and lamellipodia (Brass, 2003; Leger, Covic and Kuliopulos, 2006; Soh *et al.*, 2010).

1.4.2.3.2 Platelet activation pathways triggered by ADP

ADP binds purinergic receptors like $P2Y_1$ and $P2Y_{12}$ via G_i and activates the PI3K pathway aiding in amplification of platelet activity (Kim, Jin and Kunapuli, 2003; Damman *et al.*, 2012). The ADP-mediated coupling of $P2Y_1$ to G_q induces a transient and reversible aggregation mediated by increase in intracellular Ca^{2+} levels in response to PLCβ and is amplified further by activation of $P2Y_{12}$. The coupling of $P2Y_1$ to G_q additionally induces Rac1 mediated granule secretion and formation lamellipodia, and coupling to $G_{12/13}$ leads to ROCK activation and is responsible for platelet shape change (Dorsam and Kunapuli, 2004; Hardy *et al.*, 2005). ADP stimulation of platelets also leads to the coupling of $P2Y_{12}$ to the

$G_{\beta\gamma}$ subunits which then associate with G_i, to initiate signalling events involved in integrin $\alpha_{IIb}\beta_3$ activation (Goggs and Poole, 2012).

1.4.2.3.3 Platelet activation pathways triggered by thromboxane A_2, serotonin and epinephrine

TXA_2 coupling with prostaglandin receptor, TXA_2 receptor (TP)activates G_q, which then initiates PKC activation and stimulation of PLCβ. TP coupling with $G_{12/13}$ causes shape change by activation of ROCK (Goggs *et al.*, 2015). Serotonin (5-HT - 5 hydroxytryptamine) couples with G_q leading to platelet activation via PLCγ2 activation of PKC (Versteeg *et al.*, 2013). Epinephrine couples with G_i and G_z, and blocks PKA by PI3K activation (Goggs and Poole, 2012).

1.4.2.3.4 GPCR ligand desensitisation

Interestingly, the ligand binding to receptors mediated by GPCR undergo desensitisation after rapid signal transduction. PAR receptors are concentrated in OCS pits. Once activated by agonist-mediated cleavage, they become internalised and are degraded in the platelet lysosomes or shed in platelet derived microparticles, although differences in PAR1 and PAR4 internalisation are yet unexplored (Molino *et al.*, 1997; Duvernay *et al.*, 2013). Since platelets contain a limited pool of PAR, restricted within the OCS, no other PARs are available at the membrane after initial activation, signal transduction, and

internalisation (Goggs and Poole, 2012). Therefore, platelets are able to respond to thrombin only once. Similarly, P2 receptors undergo desensitisation when stimulated by ADP. In case of P2 receptors, they are transiently endocytosed and this internalisation is reversible (Hardy *et al.*, 2005). Whether the processing of P2 receptors is clathrin-dependent or independent is still unclear (Goggs and Poole, 2012).

1.4.2.4 Amplification of platelet activation: Granule secretion

Activation of agonist receptors triggers platelet exocytosis. Once activated, α and dense granules release a number of autocrine and paracrine stimulators, which activate and recruit platelets to the growing thrombus. Centralisation of platelet granules is believed to be required for fusion of granules with each other as well as with the OCS and the plasma membrane (Offermanns, 2006). The contents of α and dense granules are discussed in section 1.4.1. The key characterisation of α granule secretion involves assessment of expression of all or a combination of responses of P-selectin, thrombospondin, and epinephrine release (Harrison and Cramer, 1993; Furie, Furie and Flaumenhaft, 2001; Blair and Flaumenhaft, 2009). Dense granule release is mainly characterised by the measurement of the release of ADP and adenosine triphosphate (ATP) (McNicol and Israels, 1999; Murugappan *et al.*, 2004). A mutual coordination and regulation of platelet secretion exists as ADP-induced TXA_2 generation in human platelets requires coordinated signalling through integrin activation and P2 receptors (Jin *et al.*, 2002). Thus, TXA_2 is generated *de novo* when platelets are activated by $P2Y_{12}$. Phospholipase A_2 (PLA_2) enables the release of arachidonic acid from the plasma membrane, in response to elevated Ca^{2+} levels post-stimulation in a PI3K-dependent manner. Arachidonic acid is then converted to TXA_2 by serial actions of cyclooxygenases and thromboxane synthase. TXA_2 augments platelet activation by binding to TP receptor and coupling G-proteins at the membrane, therefore initiating a greater platelet response by potentiating Ca^{2+} (Bye, Unsworth and Gibbins, 2016). Influence of

secondary platelet agonists like ADP and TXA_2 are analysed by using apyrase, which is an ADP degrading enzyme and indomethacin, aspirin that block the generation of TXA_2 by inhibiting cyclooxygenases.

Platelet degranulation involves activation of PLC and subsequent cleavage of PIP_2 into DAG and IP_3. DAG directly activates PKC, and IP_3 mobilizes intracellular Ca^{2+}, which synergistically contributes to release of the platelet secretome (Golebiewska and Poole, 2015). This is supported by evidence that platelet secretion in G_q- or PLCβ-deficient mice and humans is weakened significantly (Gabbeta *et al.*, 1997; Simon *et al.*, 1997; Golebiewska and Poole, 2015).

1.4.2.5 Integrin $\alpha_{IIb}\beta_3$ activation as a central event in platelet activation

Integrin activation marks a central point in platelet activation signalling pathways. Signalling mechanisms that affect the cytoplasmic tail of the $\alpha_{IIb}\beta_3$ initiate 'inside-out signalling' response without which platelet fail to adhere or aggregate. These interactions at the cytoplasmic tail of $\alpha_{IIb}\beta_3$ are responsible for changing it from low affinity 'bent' conformation to enable 'open' conformation having a high affinity towards fibrinogen, vWF, thrombospondin-1 and fibronectin (Shattil and Newman, 2004). $\alpha_{IIb}\beta_3$ binds divalent fibrinogen and multivalent vWF facilitating platelet-platelet interaction, forming a thrombus. *In vivo* it is likely that pathways triggered by activation of GP1b-IX-V complex, GPVI, by soluble agonists like thrombin, ADP, TXA_2, are all activated during the

course of platelet responses to vascular injury and act synergistically to efficiently activate $\alpha_{IIb}\beta_3$ and ensure redundancy for an important signalling event (Goggs and Poole, 2012). Key events in the signalling mechanisms leading to $\alpha_{IIb}\beta_3$ activation include DAG and IP_3 formation by PLCβ activation. The IP_3–related increase in cytosolic Ca^{2+} is required for $\alpha_{IIb}\beta_3$ activation, but it is insufficient for inside-out signalling. DAG-related PKC activation by phorbol esters also leads to $\alpha_{IIb}\beta_3$ activation (Offermanns, 2006). Mice deficient in G_q, G_{13}, and G_i are reported to have aggregation defects (Moers *et al.*, 2003; Woulfe *et al.*, 2004a; Offermanns, 2006).

$\alpha_{IIb}\beta_3$ assembles in a complex with actin binding proteins like talins and kindlins that support activation of the actin cytoskeleton in response to stimulation (Bennett *et al.*, 1999). Additional information about the actin binding proteins is described in section 1.5.2.3. The small GTPase Rap1b is an important regulator of integrin $\alpha_{IIb}\beta_3$ activation (Goggs and Poole, 2012; Goggs *et al.*, 2015).

Activation of PI3K is another central event in sustained platelet activation, and this modulates Rap1b. PI3K is known to act by inhibiting the GTPase-activating protein RASA3, which negatively regulates Rap1b activation (Offermanns, 2006; Bye, Unsworth and Gibbins, 2016). A role of PI3K in the $\alpha_{IIb}\beta_3$ activation is also supported by observations in PI3K-deficient mice, which show reduced aggregation responses to ADP, as well as by studies using specific inhibitors of PI3K, which suggests that this enzyme plays an important role in sustaining platelet aggregation in response to low concentrations of platelet activators. Consistent with these observations, platelets lacking the downstream effectors

of PI3K, Akt1, and/or Akt2 show reduced aggregation (Woulfe *et al.*, 2004a; O'Brien *et al.*, 2011).

Outside-in signalling pathways are triggered by ligand binding to $\alpha_{IIb}\beta_3$, and therefore induce cytoskeletal rearrangement, platelet aggregate formation, granule secretion, procoagulant surface expression, and clot retraction (Goggs and Poole, 2012). Integrin clustering is a characteristic of this phase of activation (Campbell and Humphries, 2011). Kinase c-Src (cellular-sarcoma) and other Src-family kinases (SFK) are bound to the β_3-tails and remain in an autoinhibited form by phosphorylation of tyrosine residues by kinase, Csk (C-terminal src kinase), until $\alpha_{IIb}\beta_3$ activation. The receptor-like PTP-CD148 maintains a pool of active SFKs at the plasma membrane, thus contributing towards outside-in signalling. $\alpha_{IIb}\beta_3$ activation by binding fibrinogen potentiates the recruitment of protein phosphatases like PTP-1b to replace Csk. This dissociation of Csk, frees c-Src to phosphorylate and activate several downstream effector proteins like SHP1 (Src-homology 2 domain containing phosphatase-1), Syk, PLCγ2 and PI3K, thus enhancing Ca^{2+} mobilisation (Senis, Mazharian and Mori, 2014).

1.4.2.6 Regulation of platelet activation

Although thrombus formation at the site of injury is important for haemostasis, regulation of the size of thrombus becomes vital to prevent occlusion of blood vessel and ensure continuous blood flow. In the absence of inhibitory

mechanisms, spontaneous microangiopathies can lead to morbid outcomes when thrombi remain unregulated. Under haemostatic conditions, platelet activation by vascular ADP is prevented by continuous degradation by CD39, an endothelial ectonucleotidase (NTPDase-1), which ensures that platelets circulate in their resting state under physiological conditions (Michelson, 2012). Adenosine released as a result of cellular damage or degradation of ADP by CD39, activates G_s-coupled, A_{2A} receptor to inhibit platelet function (Offermanns, 2006).

As discussed previously, vWF, which is a multimeric protein synthesised by endothelial cells plays an essential role in the adhesion of platelets to collagen under the high-flow conditions found in arteries. It is secreted in a high molecular weight form, that requires cleavage by the metalloprotease, ADAMTS13, thus preventing it from binding to platelets spontaneously and causing thrombus formation (Brass, 2003).

Endothelial cell-mediated platelet inhibition is being characterised and the key mechanisms that are investigated involve PGI_2 and NO, which elevate intracellular secondary messengers – cAMP and cGMP, respectively. Both cAMP and cGMP pathways involve a number of mechanisms including the inhibition of intracellular calcium elevation, cytoskeletal reorganisation, granule secretion and integrin activation required for suppressing platelet activation (Schwarz, Walter and Eigenthaler, 2001; Smolenski, 2012)

PGI_2 is generated as result of serial enzymatic actions including conversion of prostaglandin H_2 by PGI_2 synthase. Inhibition of platelets occurs as a result of PGI_2 receptor IP, activating G_s. This activation of G_s activates adenylyl cyclases (AC) which converts ATP to cAMP. cAMP influences activation of PKA and inhibits shape change (Aburima *et al.*, 2013). PKA activation phosphorylates VASP at serine 157, thereby influencing the actin cytoskeleton (Bearer, Prakash and Li, 2002; Raslan and Naseem, 2014). Platelet activators such as ADP and thrombin block AC function through inhibitory G_i proteins, resulting in a drop in cAMP levels during platelet activation. PKA thus provides an inhibitory function during thrombus formation (Brass, 2003).

NO is a gaseous free-radical, generated as a result of enzymatic reactions between NO synthase (NOS) and L-arginine. NO permeates into the platelet plasma membrane to stimulate soluble guanylyl cyclases (sGC) which convert GTP to cGMP, which acts a secondary messenger. cGMP then activates PKG, which in turn phosphorylates a number of activation inhibitory proteins, including VASP at serine 239 (Aburima, Riba and Naseem, 2010).

Recent investigations have revealed a high degree of interconnection between activating platelets and cAMP/cGMP-dependent inhibitory signalling pathways at multiple levels, including cAMP/cGMP synthesis and breakdown, and PKA/PKG-mediated substrate phosphorylation (Smolenski, 2012).

cAMP and cGMP pathways strongly inhibit the elevation of cytosolic Ca^{2+} concentrations in platelets by suppressing the IP_3- receptor on DTS, required to

release intracellular Ca^{2+}, in response to stimulation by thrombin or collagen (Bilodeau and Hamm, 2007).

Additional regulation of PKA and PKG activation is carried out by phosphodiesterases (PDE) that hydrolyse cAMP and cGMP. Several isoforms of PDEs have been identified in platelets including PDE2, PDE3 and PDE5 (Schwarz, Walter and Eigenthaler, 2001). PDE2 and PDE5 are stimulated by cGMP whereas PDE3 is inhibited by cGMP but stimulated by PKA. PDE3A acts on cAMP, PDE2A acts on both but is not abundant, whereas PDE5 preferentially acts upon cGMP (Smolenski, 2012).

VASP phosphorylation by PKA and PKG activation inhibits actin polymerisation and actin bundling, thus preventing cytoskeletal remodelling post-stimulation (Schwarz, Walter and Eigenthaler, 2001). VASP deficient mice showed a suppression in cAMP and cGMP-mediated platelet inhibition, fibrinogen binding was enhanced, which reiterated the importance of VASP in integrin regulation and actin cytoskeletal reorganisation (Aszódi *et al.*, 1999).

1.5 The platelet cytoskeleton

Platelet cytoskeleton is a series of molecular scaffolds that attribute platelets their characteristic discoid shape and comprises of microtubules, submembranous membrane skeleton and interconnected cytoplasmic actin cytoskeletal framework (Figure 1.6). The platelet cytoskeleton also binds and activates signalling molecules. In resting platelets, secondary messengers and signalling molecules are localised in compartments within the cytoplasm connected to the membrane cytoskeleton where they can be recruited to and activated in response to platelet stimulation. Complexes of receptors and signalling molecules therefore transmit signalling cascades enabling rapid cytoskeletal reorganisation. The cytoskeletal reorganisation then facilitates dynamic changes in platelets allowing them to aggregate, contract and spread at the site of injury (Fox, 2001).

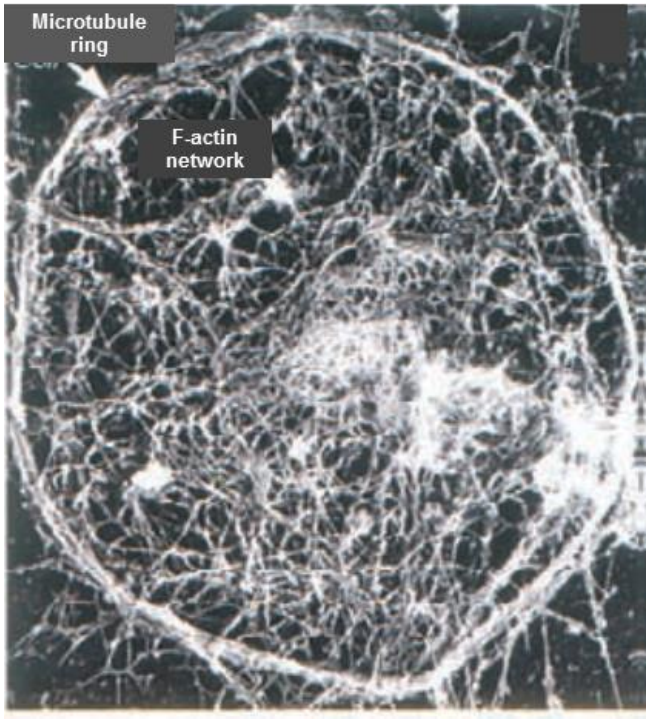


Figure 1.6 Microtubule coil and actin cytoskeleton in the resting platelet. A single microtubule is coiled 8 to 12 times. The actin cytoskeleton is a three-dimensional network of approximately 1μm long filaments (Michelson, 2012).

1.5.1 Cytoskeleton of the resting platelet

In resting platelets, situated underneath the plasma membrane is a marginal circumferential band of 8-12 filamentous rings of microtubules, as polymers of αβ-tubulin dimers (Silver, 1966; Behnke, 1967). This microtubule coil is responsible for maintaining the discoid shape under the high shear forces that platelets experience when flowing in close proximity to the endothelium during circulation (White and Rao, 1998). Limited information is available with respect to the mechanisms involved in maintaining the microtubule coil in resting platelets or about depolymerisation and repolymerisation of microtubules during platelet activation (Patel-Hett *et al.*, 2008). Evidence from cultured fibroblasts suggests that microtubules play a role in inducing integrin-based signalling events (Danowski, 1989; Kaverina, Rottner and Small, 1998). Transgenic mice lacking tubulin1 possess non-discoid platelets with defective marginal bands containing only 2 to 3 microtubule coils. β1-tubulin–deficient mice experience thrombocytopenia (platelet counts 50% of wild-type) and prolonged bleeding times and it has been suggested that microtubules might be responsible for spatial interconnections and activation of signalling molecules in platelets (Fox, 2001; Patel-Hett *et al.*, 2008).

A spectrin-based membrane skeleton lies underneath the plasma membrane which is responsible for binding membrane glycoproteins like GPIb (Fox, 1985). Short actin filaments of 14 monomers (37nm length) are found connecting spectrin, actin-binding protein (ABP) and the plasma membrane. The ultrastructural investigations of membrane skeleton reveal that actin filament

ends that bind spectrin, arrive at the plasma membrane originating from filaments in the cytoplasm, therefore linking the cytoplasmic actin networks to the receptors present in the plasma membrane (Hartwig and Michelson 2012). Inspection of platelets by transmission electron microscopy shows a cytoplasmic microfilamentous network in a mesh-like pattern in resting platelets (Bearer, 2005).

The 42 kDa protein actin is present at 0.55mM concentration and accounts for a significant part of the platelet protein content constituting approximately 15-20% of total platelet proteins (Hartwig *et al.*, 1999). The actin cytoskeleton exists in two distinct pools within platelets characterised by the difference in their sedimentation properties – the membrane-bound actin skeleton that mediates transmembrane signalling and the cytoplasmic actin network that provides the contractile component of the platelet (Fox *et al.*, 1988).

A number of key features of actin sequestration, nucleation, and assembly-disassembly regulate the platelet cytoskeleton and changes upon stimulation. Actin filaments are polarised structures, where the barbed-end is the site of polymerisation and the pointed end is the site of depolymerisation. Actin exists in a state of dynamic equilibrium as filamentous, F-actin and globular, G-actin by a mechanism known as actin turnover, where depolymerised G-actin from the pointed-end of the aging actin filament is substituted by another molecule from the G-actin pool at the barbed end. Actin turnover is discussed is further detail in section 1.5.2 ahead. It is estimated that about 40% of actin in resting platelets is in its F-actin form. The actin molecule maintains its tertiary structure by retaining

in specific pockets, either ATP or ADP and divalent cations, Mg^{2+} or Ca^{2+}. The nucleotide within the actin pockets defines its status in actin turnover, thus determining the rate of actin polymerisation. ATP-bound G-actin has affinity for the barbed-end, whereas the ADP-bound G-actin is depolymerised from the pointed end. Actin molecule has four functional sites – three sites interact with other actin monomers in a filament and one site which interact with actin binding proteins, which are responsible for nucleation of new filaments, bundling and stabilising filaments as well the depolymerisation and polymerisation of actin filaments required in actin turnover (Bugyi & Carlier 2010; Blanchoin *et al*. 2012; Bearer *et al*. 2002).

In platelets, F-actin and G-actin are maintained in their respective states with the help of several actin associated proteins. Thymosin-β4 sequesters monomeric actin, specifically, making it unavailable for polymerisation (Xue and Robinson, 2013). Profilin, also a monomer sequestering protein, transfers the G-actin from thymosin-β4 and acts as an ATP exchange factor for actin (Goldschmidt-Clermont *et al.*, 1991). αγ-adducins and Capping protein/CapZ, cap the F-actin filaments at the membrane and prevents depolymerisation (Hartwig *et al.*, 1999). Arp2/3, which is known as an actin-nucleator; in platelets, it also caps F-actin filaments (Falet *et al.*, 2002). Additionally, vasoactive-stimulated phosphoprotein (VASP) bundles actin filaments preventing the severing actions of gelsolin in resting platelets (Smolenski, 2012). Actin filaments are stabilised by filamin, tropomyosin and spectrin. Filamin connects cytoplasmic actin to the GP1b-IX-V complex at the platelet surface in

preparation of activation (Hartwig and Michelson 2012). Together, actin associated proteins and actin networks, help platelets maintain their discoid shape, prevent shape change by regulating the availability of G-actin and barbed-ends on F-actin (Bearer *et al*. 2002). Multivariate, synchronised choreography of myriad actin regulating proteins therefore controls the dynamics of all cellular actin. A number of proteins that affect actin dynamics are discussed in Chapter 5.

1.5.2 Actin rearrangement in stimulated platelets: A CAP1 perspective

Platelet actin assembly following the addition of agonists begins as the globular actin (G-actin) pool from the cytoplasm polymerises into dynamic filamentous actin (F-actin). Aging F-actin strands undergo disassembly by recycling actin from the cytoplasm pool controlled by multiple actin associated proteins. This process of actin turnover or treadmilling is regulated by ATP hydrolysis releasing inorganic phosphate (P_i) to generate G-actin subunits that are bound to ADP molecules. ADP-bound actin subunits are recognised by a number of proteins involved in accelerating the F-actin disassembly process. The binding of cofilin to actin filaments destabilises them, releasing G-actin subunits. Cofilin exists in a phosphorylated, inactive form in resting platelets (Mcgrath *et al.*, 2000). Further, it has been shown that cofilin becomes active upon dephosphorylation in stimulated platelets and can remain dephosphorylated for extended periods of time if integrins become ligated to it (Aslan *et al.*, 2013).

Evidence from nucleated cells has shown that cofilin, which is inactive at neutral or acidic pH, is rescued by adenylyl cyclase-associated protein 1 (CAP1) which accelerates its ability of severing actin (Normoyle and Brieher, 2012). CAP1 is discussed in further detail in section 1.6. CAP1 is a key protein that affects the turnover of actin in spreading or motile cells in cooperation with cofilin and profilin and promotes the nucleotide exchange on the actin subunits (Goldschmidt-Clermont and Janmey, 1991; Zhou, Zhang and Field, 2014). As seen in figure 1.7, cofilin in conjunction with CAP1 severs F-actin from the pointed end to release ADP-bound G-actin complex. Upon severing the actin filament, cofilin dissociates from the complex followed by nucleotide exchange promoted by formation of a complex between CAP1 and profilin to efficiently recycle actin into ATP-bound G-actin which is then free to polymerise at the barbed end of the growing actin filament (Balcer *et al.*, 2003). The N-terminus of CAP1 in yeast and mouse, forms hexamers that accelerate binding and recycling of cofilin during actin disassembly (Chaudhry *et al.*, 2013; Jansen *et al.*, 2014). Further, profilin-dependent nucleotide exchange is enhanced by the C-terminus of CAP1 thus aiding in elongating the actin filament (Balcer *et al.*, 2003).

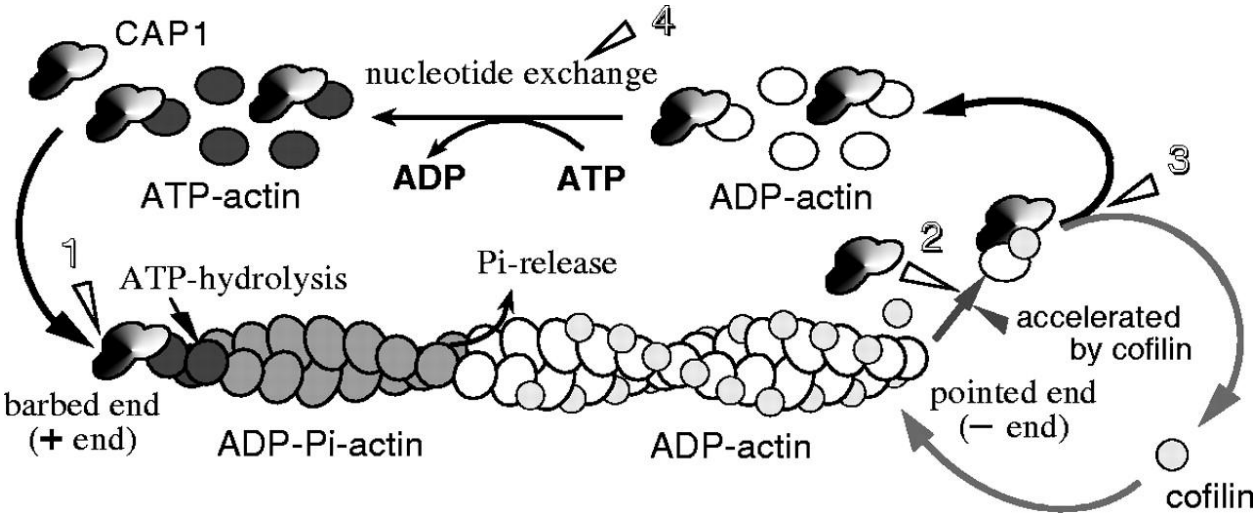


Figure 1.7: CAP1-mediated actin turnover. During actin polymerisation, G-actin subunits are added at the barbed end (1). CAP1 accelerates cofilin-dependent severing of the actin filament at the pointed end, releasing ADP-G-actin (2-3). CAP1 also enhances profilin-mediated nucleotide exchange on the actin subunit, which is then released as ATP-bound G-actin (4). ATP-actin is then free to bind to the leading edge (barbed end) of the actin filament (1). Taken from (Moriyama and Yahara, 2002).

1.5.3 Cytoskeleton of the activated platelet: Formation of actin structures

In their primary role, platelets attach, change shape, spread, release their secretome and retract over the damaged vascular area to create a haemostatic plug. Reproducible temporal changes in the platelet cytoskeleton have been recorded mediating the formation of the haemostatic plug and are dominated by the cytoplasmic dynamics of actin polymers, their availability and assembly-disassembly (Bearer, 2005).

All platelet agonists initiate change in platelet shape from discoid into a rounded sphere owing to depolymerisation of cytoplasmic actin networks, consequently generating increased levels of ATP-bound G-actin and simultaneously creating multiple barbed ends that are available for nucleation of new actin filaments. Filopodia or finger-like projections begin emanating from the cell periphery and lamellipodial extensions are formed as the F-actin doubles compared to the content in the resting platelet. Actin nodules are formed that are thought to drive the actin filament assembly in the lamellipodia and bundling of filaments to form stress fibres, which then define the shape of a completely spread platelet (Calaminus *et al.*, 2008).

The platelet flattens as granules fuse with the OCS and they are squeezed with other organelles towards the centre of the platelet. It must be noted that secretion of granules is not dependent on the actin cytoskeleton, but their transport towards the centre is a function of actin depolymerisation (Flaumenhaft *et al.*, 2005; Blair and Flaumenhaft, 2009; Fitch-Tewfik and

Flaumenhaft, 2013). Microtubule fragmentation and reassembly is thought to occur at this stage but evidence for it is still preliminary (Patel-Hett *et al.*, 2008).

A dynamic phase of membrane motility then proceeds with digestion of spectrin-based membrane skeleton leading to formation of membrane ruffles which cause platelets to retract inwards. This is thought to be mediated by forces generated from actin-myosin interplay (Bearer, *et al*. 2002; Hartwig and Michelson 2012). Doubling of F-actin is achieved as cortical networks are derived from new actin filament polymerisation as blunt filopodia extend from the cell and are rotated around the periphery.

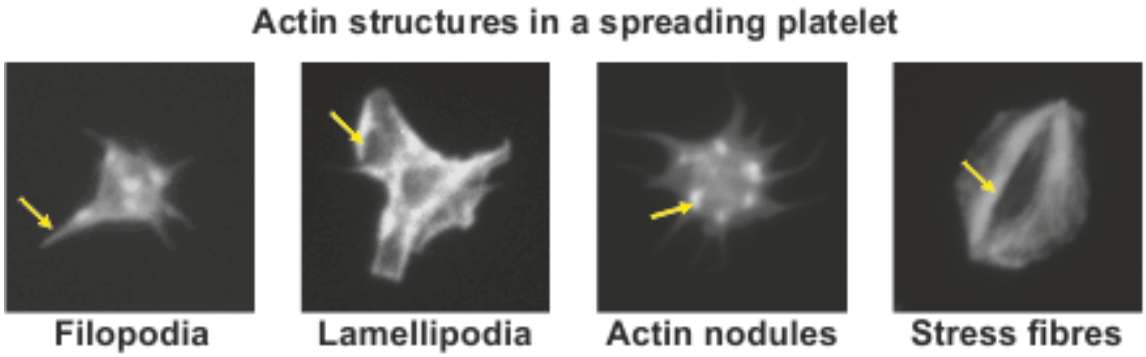


Figure 1.8 Actin structures found developing in a spreading platelet. Upon activation of platelets with agonists, filopodia or finger-like projections begin emanating from the cell periphery and lamellipodial extensions are formed as the F-actin doubles compared to the content in the resting platelet. Actin nodules, adhesive actin structures, are formed that are thought to drive the actin filament assembly in the lamellipodia and bundling of filaments to form stress fibres, which then define the shape of a completely spread platelet.

1.5.2.1 Filopodia formation in platelets

Filopodia are used by cells for accurately gauging extracellular matrix cues and for the identification of adhesion receptor proteins that bind to them. Filopodia are formed in activated platelets in response to signalling leading to actin polymerisation (Figure 1.8). Spatially restricted and temporally controlled actin filament nucleation is critical for generating membrane protrusions (Bearer, Prakash and Li, 2002; Yang *et al.*, 2007). These actin filament-rich structures are formed, where barbed-end of filaments is observed at the protruding end of the filopodia. This actin polymerisation event is thought to occur via RhoGTPase, Cdc42, that regulates the formation of filpodia by interacting with WASP and Arp2/3 (Nobes and Hall, 1995; Li, Kim and Bearer, 2002). Further, the formation of parallel actin filaments in filpodia are bundled by proteins like formins that give filopodia their characteristic shape (Yang *et al.*, 2007).

1.5.2.2 Lamellipodia formation in platelets

Lamellipodia are extensions of the cytoplasmic front and present as thin-sheet like actin-rich structures (Figure 1.8). Lamellipodia are thought to be formed to bridge gaps generated by filopodia. Activated platelets undergo membrane ruffling which acts as a precursor to formation of lamellipodia. The formation of lamellipodia is governed by small Rho GTPase, Rac1. Rac1 regulates the downstream signalling mechanisms via WAVE/Arp2/3 and PAK/Cofilin mediated actin polymerisation. Rac1 activated WAVE and Arp2/3 are responsible for actin nucleation and branching of existing actin filaments, whereas, PAK/Cofilin are

responsible for depolymerisation of F-actin fragments, thus ensuring availability of actin for the expanding actin network in the lamellipodia (Aslan and Mccarty, 2013).

1.5.2.3 Actin nodules in platelets

Actin nodules are F-actin rich structures which were first identified and described in 2008 by Calaminus *et al*. Actin nodules are described as transient actin structures that disappear on the formation of stress fibres in a spreading platelet. Since then, actin nodules have been further characterised as podosome-like adhesive actin structures in a spreading platelet (Figure 1.8). The formation of actin nodules is driven by WASp mediated action of Arp2/3. Actin nodules were found to be enriched in Arp2/3 and other Tyr-phosphorylated proteins. Further, actin nodules were characterised as adhesive structures based on co-localisation of proteins around the site of the actin nodules – adhesion-related proteins talin and vinculin in addition to integrins formed a ring-like structure around the actin nodule (Poulter *et al.*, 2015).

1.5.2.4 Stress fibres in platelets

In platelets, stress fibres are a characteristic of completely spread platelet (Figure 1.8). In non-muscle cells, stress fibres are used as contractile structures. Stress fibres provide contractility by the action of the actomyosin complex and are regulated by Rho GTPase, Rho A. Rho A mediates actin polymerisation by

regulating its kinases like ROCK, LIMK and their downstream effectors like myosin II and cofilin, respectively (Ridley and Hall, 1992; Aburima *et al.*, 2013). Actin bundles of 20-30 parallel actin filaments are cross-linked by actin-binding proteins and provide structural stability to the spread platelet. Stress fibres act as the focal adhesions anchoring the platelet at the site of activation (Aslan and Mccarty, 2013; Goggs *et al.*, 2015).

1.5.3 Brief overview of signalling related to actin cytoskeletal reorganisation

The changes in the cytoskeleton described are complemented by cognate biochemical signalling pathway activation. Phosphoinositides serve to initiate actin filaments assembly directly and perform the critical function of targeting this assembly to the membrane-cytoskeleton interface. This is especially important as all filament assembly begins underneath the plasma membrane (Janmey 1995; Hartwig and Michelson 2012).

Barbed-end capping proteins are inactive when bound to membrane phospholipids. Receptor ligation upon activation induces transient elevation in cytosolic Ca^{2+} levels, where platelets lose their discoid shape and become rounded. The increase in cytosolic Ca^{2+} is mediated by activation of PLC. PLC hydrolyses membrane bound phospholipid, PIP_2 from its precursor phosphatidylinositol 4 phosphate (PIP) at the surface of plasma membrane and is regulated by profilin (Goldschmidt-Clermont *et al.*, 1991). Profilin is thought to inhibit PLC and dissociate from the membrane upon hydrolysis giving rise to profilin-actin complexes that are able to polymerise on to barbed end of actin filaments.

A major role in cytoskeletal regulation is played by phosphoinositide signalling which subsequently leads to elevation of Ca^{2+} levels is necessary for efficient interaction of actin binding proteins with actin monomers and filaments. A number of actin regulating proteins like gelsolin, require readily available Ca^{2+}

for severing actin and freeing it for polymerisation at alternative sites (Janmey and Matsudaira, 1988).

Inhibition of phosphoinositides has been shown to impair production of barbed-ends thereby decreasing the rate of actin assembly. In contrast, overproduction of phosphoinositides in neutrophils has revealed increased actin assembly due to increased barbed end exposure in addition to increase in membrane ruffling (Bezanilla *et al.*, 2015). PI3K is stimulated directly or indirectly by phospholipases downstream of G-protein activation by cognate agonists like ADP, thrombin, epinephrine, TXA_2. PI3K phosphorylates the D3-position of inositol lipids. Upon receptor ligation, dramatic increase in the D3-containing phosphoinositides has been shown to promote filpodia formation as a result of actin polymerisation (Guinebault *et al.*, 1995; Hartwig *et al.*, 1996). Actin polymerisation occurs at the newly created barbed-ends by activation of Arp2/3 complex aided by actin sequestering proteins like profilin and thymosin β4. Polymerisation of actin filaments to form filopodia and lamellipodia therefore provides the protrusive force required in a spreading platelet.

A brief overview of platelet agonists and pathways they trigger that lead to cytoskeletal reorganisation is found in Figure 1.9.

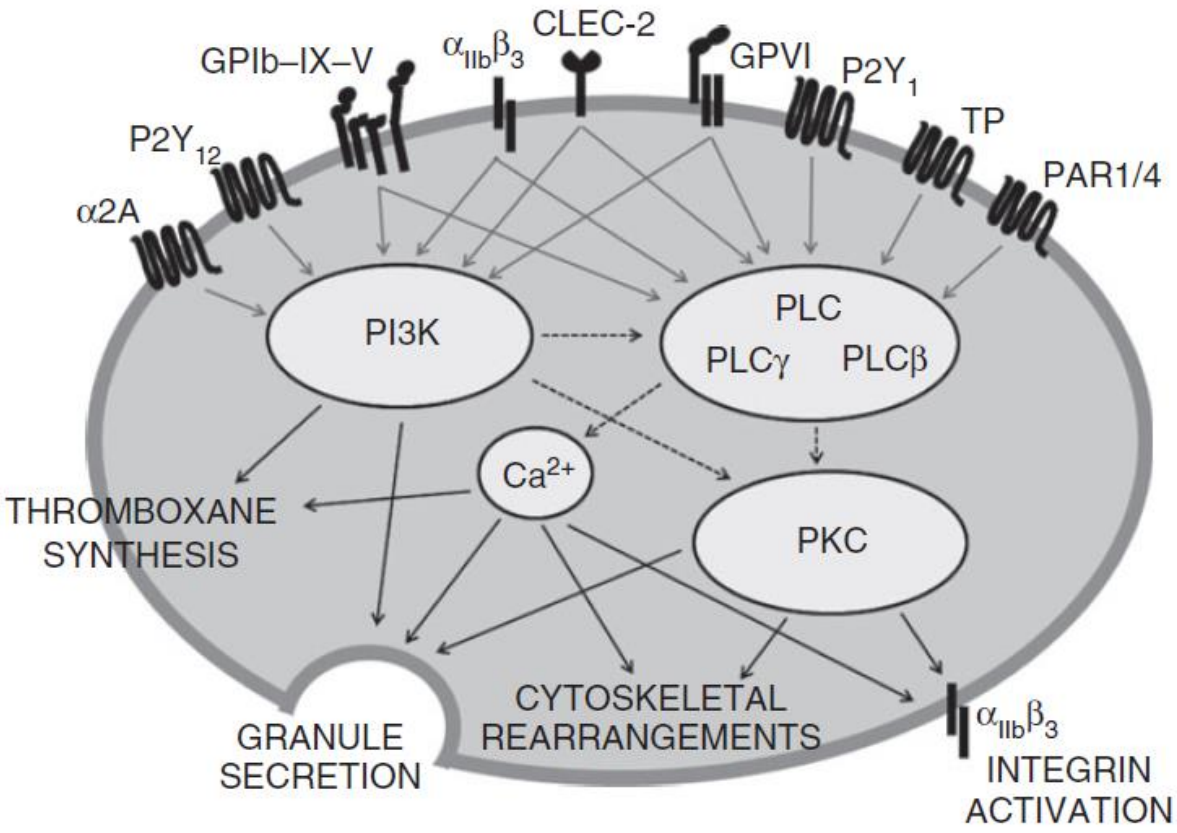


Figure 1.9 Biochemical signals affecting cytoskeletal reorganisation in platelets. A number of platelet agonists bind to receptors (α_2A, P_2Y_{12}, GP-1b-IX-V, $\alpha_{IIb}\beta_3$, CLEC-2, GPVI, P_2Y_1, TP, PAR1/4) and activate platelets leading to activation of phospholipase C (PLC) and activation of phosphatidylinositol 3 kinase (PI3K) thereby elevating intracellular calcium levels, PKC activation and secondary agonist release, together contributing to cytoskeletal reorganisation in platelets (Bye, Unsworth and Gibbins, 2016).

In unstimulated platelets, actin and actin associated proteins such as talin, kindlin and α-actinin restrict $\alpha_{IIb}\beta_3$ by binding its cytoplasmic tail intracellularly, thereby preventing its association with fibrinogen. Talin-1 is an abundant cytoplasmic protein able to interact with other cytoskeletal proteins including actin and vinculin, thereby connecting the integrins with the actin cytoskeleton. These binding sites involved in actin-binding proteins and those for integrin β tails are buried in an auto-inhibited conformation and are only exposed during platelet activation. Talin-1 contains a FERM (band 4.1, ezrin, radixin, moesin) domain, approximately 150 amino acids long, that is involved in localising it to the plasma membrane (Goggs and Poole, 2012). When platelet agonists initiate actin turnover in the membrane skeleton by activation of actin-severing proteins like gelsolin and cofilin, they relieve the constraints on $\alpha_{IIb}\beta_3$ allowing it assume a high affinity conformation required for binding fibrinogen and subsequent platelet aggregation (Bennett *et al.*, 1999).

Receptor binding to extracellular matrix ligands initiates inside-out signalling, where integrins associate with the actin cytoskeleton through several molecular linkages and undergo conformational changes to become “active” (Shattil and Newman, 2004). Followed by a reorganisation in the actin cytoskeleton, outside-in signalling occurs in a discrete pattern whereby ligand binding initiates integrin clustering and assembly of a nascent signalling complex proximal to the cytoplasmic tails of $\alpha_{IIb}\beta_3$, followed by the growth of a larger actin-based signalling complex (Shattil *et al.*, 1996). F-actin is polymerised at adhesion sites or the integrin clusters, where actin binding proteins like profilin and VASP are

recruited. Additionally, recruitment of WASP and Arp2/3 at these sites lead to further actin polymerisation and reorganisation (Calderwood, Shattil and Ginsberg, 2000). Increase in Ca^{2+} towards the later stages, activates calpain, which is calcium-dependent protease, which cleaves the β_3 cytoplasmic domain and initiates clot retraction (Moscardó *et al.*, 2013).

1.6 Adenylyl cyclase-associated protein 1 (CAP1)

Adenylyl cyclase associated protein 1 (CAP1) is an actin-monomer binding protein that plays a role in dynamic actin reorganisation, briefly introduced in section 1.5.2 (Ono, 2013; Xue and Robinson, 2013; S. Lee *et al.*, 2014). It was first described in yeast, as suppressor of hyperactive RAS2(V19) (*Srv2*) (Field *et al.*, 1988), a RAS and adenylyl cyclase pathway effector protein (Field *et al.*, 1990). Porcine CAP1 was found to have identical sequence to protein, ASP-56 which was previously isolated from porcine platelets, , and described as an actin-monomer binding protein (Gieselmann and Karlheinz, 1992). The multifunctional role of CAP1 in the regulation of yeast cytoskeleton was identified much later (Freeman *et al.*, 1996). A number of cytoskeleton regulatory functions for CAP1 have been described in mammalian cells (Ono, 2013), without any role in adenylyl cyclase regulation and details can be found in sections, 1.6.1.1 and 1.6.2.

1.6.1 CAP1 structure

CAP1 is a 56 kDa protein containing 450-550 amino acids found in various mammalian cell-types, and it is 70 kDa (Srv2/CAP) in *Saccharomyces cerevisiae* (Field *et al.*, 1990; Ono, 2013). Vertebrates have two isoforms of CAP – CAP1 and CAP2, which share 64% sequence homology and yet, have distinct, non-redundant functions (Peche *et al.*, 2007, 2013). The expression profiles of the two CAP proteins also vary significantly, where CAP1 is widely expressed in a number of tissues, except skeletal muscle, and CAP2 is

expressed predominantly in brain, heart, skeletal muscle and testis (Bertling *et al.*, 2004; Peche *et al.*, 2007). While CAP1 and CAP2 both bind G-actin, CAP2 was additionally found to regulate sarcomere assembly in heart and skeletal striated muscles (Yu, Swiston and Young, 1994; Bertling *et al.*, 2004; Peche *et al.*, 2007). In a CAP2 KO mouse model, CAP2 deficiency caused dilated cardiac myopathies (Peche *et al.*, 2013) and impeded wound healing in human skin (Kosmas *et al.*, 2015). CAP1, on the other hand determined cell polarity, motility and receptor mediated endocytosis along with mitochondrial shuttling of actin during apoptosis (Noegel *et al.*, 2004; Sultana *et al.*, 2005; Wang *et al.*, 2008). There are no biochemical studies comparing CAP1 and CAP2, and differences, if any, are not documented (Peche *et al.*, 2013). Although CAPs have distinct functions, they have similar domain structures as seen in figure 1.10.

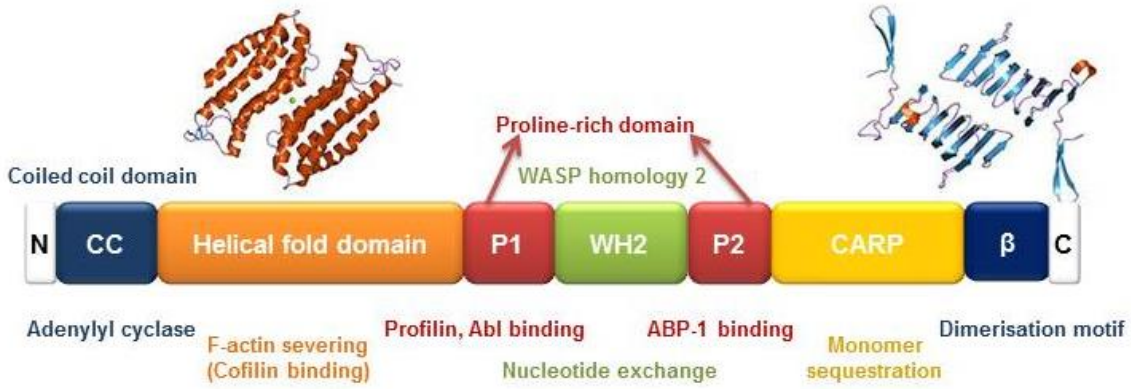


Figure 1.10 Domain structure of CAP: The N-terminus contains a coiled coil domain, responsible for binding to adenylyl cyclase, followed by a helical fold domain (HFD) (crystal structure shown above the domain), which binds to cofilin. A WH2 domain responsible for nucleotide exchange during actin turnover is flanked by two proline-rich (P1, P2) domains, which bind other actin binding proteins like profilin, Abl and ABP-1. The C-terminal CARP domain binds ADP-G-actin, followed by a dimerisation motif comprising of β-strands (dimerised crystal structure shown above it). [Figure adapted from (Ono, 2013)]

The N-terminus of CAP is comprised of a coiled-coil domain, which is responsible for binding adenylyl cyclase and stimulating cAMP activity and self-oligomerisation of Srv2/CAP (Quintero-Monzon *et al.*, 2009). The formation of coiled-coil domain is yet to be described in a crystal structure (Ono, 2013). The remainder of N-terminal CAP comprises of a helical fold domain (HFD) containing a stable bundle of six anti-parallel helices, which associate laterally with helices from another CAP molecule forming dimers (Yusof *et al.*, 2005). The HFD is responsible for binding actin-depolymerising factor (ADF)/cofilin (Moriyama and Yahara, 2002) and the interaction between cofilin and CAP1 enhances the severing of F-actin (Chaudhry *et al.*, 2013).

The central region of CAP1 contains two proline-rich domains – P1 and P2, where P1 is conserved across all eukaryotes and P2 is more variable (Freeman *et al.*, 1996; Yu *et al.*, 1999). The P1 domain of CAP1 binds Src-homology domain (SH3) of tyrosine-kinase Abl (Freeman *et al.*, 1996), and profilin (Makkonen *et al.*, 2013). Actin-binding protein (ABP-1) binds to the P2 domain of Srv2/CAP (Freeman *et al.*, 1996). The Wiskott-Aldrich Syndrome protein homology domain 2 (WH2) is also located centrally and is responsible for CAP interaction with G-actin playing a key role in nucleotide exchange/ recharging of actin monomers during actin turnover (Chaudhry *et al.*, 2010).

The C-terminus of CAP comprises of β-strands which are arranged into six coils of right-handed β-helices, which enable dimerization of CAP molecule (Dodatko *et al.*, 2004). The C-terminus also contains a sequence motif called the CARP

domain (Ono, 2013), that binds to G-actin, independent of the WH2 domain (Chaudhry *et al.*, 2010; Nomura and Ono, 2013).

CAPs have been identified in most representative eukaryotic species, where, during evolution, the domain structure of CAP has been conserved to a great extent among species - fungi, protists and invertebrates have one CAP gene, whereas multiple isoforms of CAP are found in vertebrates, nematodes and plants (Ono, 2013). The function of CAP as regulators of the cytoskeleton is also conserved among most eukaryotic species, with only a few species have it is as a component of the Ras-cAMP signalling pathway (Gerst *et al.*, 1991; Hubberstey and Mottillo, 2002; Ono, 2013).

Oligomerisation of CAP1 is an essential mechanism by which CAP1 carries out the modulation of actin turnover (Section 1.5.2, Figure 1.7). A high molecular mass complex of 600 kDa containing six molecules each of CAP1 and actin (CAP1-actin complex) is a conserved feature in multiple organisms (Balcer *et al.*, 2003; Quintero-Monzon *et al.*, 2009). Self-association of CAP1 is mediated by the N-terminal coiled-coil domain (Yu *et al.*, 1999; Quintero-Monzon *et al.*, 2009), by the HFD (Yusof *et al.*, 2005), and the C-terminal dimerisation motif (Dodatko *et al.*, 2004; Iwase and Ono, 2016). Chaudhry *et al.* (2013) have elucidated that, *in vitro*, the C-terminal dimerises and helps organise the HFD oligomerisation to form a cluster of hexameric, symmetrical protrusions (shurikens-like structure) and enhances the CAP1-actin association. The observation that multimer formation enhances its function of regulating actin was reiterated by Iwase & Ono (2016) in relation to the C-terminal dimerisation

in relation to CAP isoform of *C. elegans*. They show that the evolutionarily conserved dimerisation motif of CAP is essential for its C-terminal region to exert the actin monomer-specific regulatory function. In summary, N-terminal CAP1 can bind N and C-terminal of a second CAP1 molecule for oligomerisation, while the C-terminal dimerisation motif is key for forming a complex with actin and thus hexamer formation via the HFD domain (Hubberstey *et al.*, 1996; Zelicof *et al.*, 1996; Chaudhry *et al.*, 2013; Iwase and Ono, 2016). Observations in *Dictyostelium* suggests that several configurations of CAP dimer are possible but the actual arrangement of the molecules *in vivo* is currently unknown (Ono, 2013).

1.6.2 CAP1 function

Although a number of CAP1 effector functions related to actin regulation and adenylyl cyclase association are known, the regulation of CAP1 itself and the underlying signalling mechanisms and pathways remain poorly investigated. The phosphorylation of mouse CAP1 reported by Zhou *et al.* (2014) suggests a kinase signalling pathway that regulates CAP1 localisation and function, where phosphorylation lead to inactivation of CAP1. Further, phospho-CAP1 was found diffused throughout the cytosol and CAP1 was found at the periphery. Additionally, CAP1 as an intracellular substrate of matrix metalloproteinase-9 (MMP-9), suggests that levels of cellular CAP1 may be regulated by proteolysis (Cauwe *et al.*, 2008; Ono, 2013; Xie *et al.*, 2014).

1.6.2.1 Adenylyl cyclase activity

The original discovery in *Saccharomyces cerevisiae* assigned the role of adenylyl cyclase regulation to CAP (Field *et al.*, 1990). In *Saccharomyces cerevisiae*, Ras and cyclic AMP (cAMP) signalling coordinates cell growth and proliferation with nutritional sensing (Suzuki *et al.*, 1993; Wang *et al.*, 1993). Similar role for CAP linking cell-growth and cAMP activity were also observed in *Candida albicans* and *Magnaporthe oryzae* (Bahn and Sundstrom, 2001; Zhou *et al.*, 2012). The generation of cAMP is catalysed by conversion of ATP by adenylyl cyclase, and one of the stimulation mechanisms involves binding of GTP-bound Ras and Srv2/CAP proteins (Gerst *et al.*, 1991; Freeman *et al.*, 1996). Elevation of cAMP levels leads to dissociation of the PKA regulator

proteins to yield active A kinases which affect processes such as cell cycle progression and cell migration in yeast, although direct cross-talk between adenylyl cyclase association and actin regulation are only conjectured in *Dictyostelium* (Noegel *et al.*, 1999, 2004; Sultana *et al.*, 2012). Further downstream effects of CAP-adenylyl cyclase interaction include reactive oxygen species (ROS) production as a stress response effect of cAMP elevation leading to apoptosis (Gourlay and Ayscough, 2006).

Later, CAP1, as a mediator of similar adenylyl cyclase activity, was noted as a function linked to cell polarity determination and starvation-related aggregation in *Dictyostelium* cells, but there was no evidence of direct binding between CAP and adenylyl cyclases (Noegel *et al.*, 2004; Sultana *et al.*, 2012).

1.6.2.2 Role of CAP1 in actin regulation

The significance of CAP1 in binding actin monomers can be established by the number of domains present on CAP1 molecule that bind G-actin - the WH2-domain, the CARP domain and HFD which binds to cofilin-G-actin complex (Chaudhry *et al.*, 2010; Makkonen *et al.*, 2013; Jansen *et al.*, 2014; Iwase and Ono, 2016).

CAP1 binds to G-actin in 1:1 ratio to inhibit spontaneous polymerisation *in vitro* and to F-actin at sub-stoichiometric ratios (Hubberstey and Mottillo, 2002). CAP1-actin binding does not affect filament formation but rather accelerates cofilin-mediated F-actin depolymerisation (Zhang *et al.*, 2013). The mechanistic

role of CAP1 in actin turnover is mainly described in reference to its association to cofilin (Moriyama and Yahara, 2002). CAP1 acts as the fourth component of F-actin severing machinery which includes, AIP1, coronin and cofilin. AIP1 prevents severed G-actin from reannealing to F-actin, coronin increases cofilin loading on F-actin and improves cofilin-mediated severing, while CAP1 is speculated to destabilise F-actin making it more susceptible to severing in addition to exchanging nucleotide and releasing cofilin thus acting as a cofilin recycling factor (Blanchoin *et al.*, 2012; Normoyle and Brieher, 2012; Zhang *et al.*, 2013). Additionally, at neutral and acidic pH where cofilin is rendered inactive, CAP1 rescues cofilin and increases severing frequency of cofilin by seven-folds (Normoyle and Brieher, 2012). Studies in CAP1 knockdown cell-lines have revealed that CAP1 affects the phosphorylation state of cofilin, but the precise mechanism is yet unconfirmed (Zhang *et al.*, 2013).

Nucleotide exchange is the key to recycling actin monomers during actin turnover, where ADP-G-actin is severed from the pointed-end and ATP-G-actin then polymerises onto the barbed or elongating-end of F-actin. CAP1 catalyses nucleotide exchange in an evolutionarily conserved manner (Makkonen *et al.*, 2013). Traditionally, profilin, which can overcome the inhibitory effects of cofilin (Xue and Robinson, 2013), was thought to catalyse this reaction (Goldschmidt-Clermont *et al.*, 1991), but it was later demonstrated that profilin only weakly promotes nucleotide exchange on its physiological substrate, the cofilin-bound ADP-G-actin (Balcer *et al.*, 2003). Instead now, CAP1 having similar affinities to ADP-G-actin and ATP-G-actin, has been shown to promote the nucleotide

exchange via its WH2 domain (Chaudhry *et al.*, 2010; Nomura and Ono, 2013). The functional relation between CAP1 and profilin in mammalian cells is unclear, although CAP1 has binding site for profilin (Ono, 2013). It has been speculated that profilin and CAP1 catalyse nucleotide exchange independently and that CAP1-profilin interaction potentially enhances filament elongation, where profilin-actin monomer complex can dissociate from CAP1 and be used for formin- or Ena/VASP-catalysed filament polymerisation (Bugyi and Carlier, 2010; Makkonen *et al.*, 2013; Blanchoin *et al.*, 2014).

All scenarios described above provide evidence of a central role for CAP1 in modulating actin cytoskeleton. CAP1 knockdown in cell-lines have shown that cells show alterations in cell motility due to increase in lamellipodia and stress fibres (Zhou, Zhang and Field, 2014). CAP1 knockdown in HeLa cells presented a role for CAP1 in cell adhesion. In the CAP1 knockdown cells, focal adhesion kinase (FAK), an important regulator of cell adhesion, is activated by phosphorylation of its Tyr397 residue thus promoting its association with Src and affecting cell motility. Additionally, FAK and talin, which is a key component of focal adhesions that provides link between integrin activation and actin cytoskeleton, were identified in co-IP of cells overexpressing CAP1, which emphasises the role of CAP1 in cell adhesion and motility (Zhang *et al.*, 2013).

1.6.2.2.1 **In vivo *effects of CAP1-mediated actin regulation***

Several CAP1 binding partners have been identified that suggest involvement of CAP1 in plethora of physiological functions – CAP1 in chemotaxis, cell motility, cytokinesis and membrane trafficking in *Dictyostelium* (Noegel *et al.*, 1999, 2004; Sultana *et al.*, 2005), endocytosis and neuronal development in *Drosophila* (Stevenson *et al.*, 2000; Hubberstey and Mottillo, 2002) and neuronal growth cone formation in rats (Lu *et al.*, 2011), Arp2/3-mediated actin regulation in plant development in *Arabidopsis* (Deeks *et al.*, 2007), and extracellular function as it is implicated as an autoantigen in rheumatoid arthritis (Kinloch *et al.*, 2005; Ono, 2013), along with a role in tumourigenesis.

In yeast and *Dictyostelium*, CAP1 localises to the cortical regions (Yu, Swiston and Young, 1994; Noegel *et al.*, 1999). In mammalian cells, it was found in dynamic actin structures like membrane ruffles, lamellipodia (Moriyama and Yahara, 2002; Bertling *et al.*, 2004) and also in stress fibres in fibroblasts (Freeman and Field, 2000) (Figure 1.11). CAP1 promotes actin filament dynamics, thus playing a key role in cell migration, adhesion and cytokinesis. Overexpression of CAP1 is evident in several cancer phenotypes like pancreatic cancer (Yamazaki *et al.*, 2009), breast cancer (Liu X *et al.*, 2014), hepatocellular carcinoma (Liu Y *et al.*, 2014), ovarian cancer (Hua *et al.*, 2015), lung cancer (Tan *et al.*, 2013) by affecting the invasiveness of the cancer cells contributing towards tumourigenesis (Ono, 2013; Zhou, Zhang and Field, 2014).

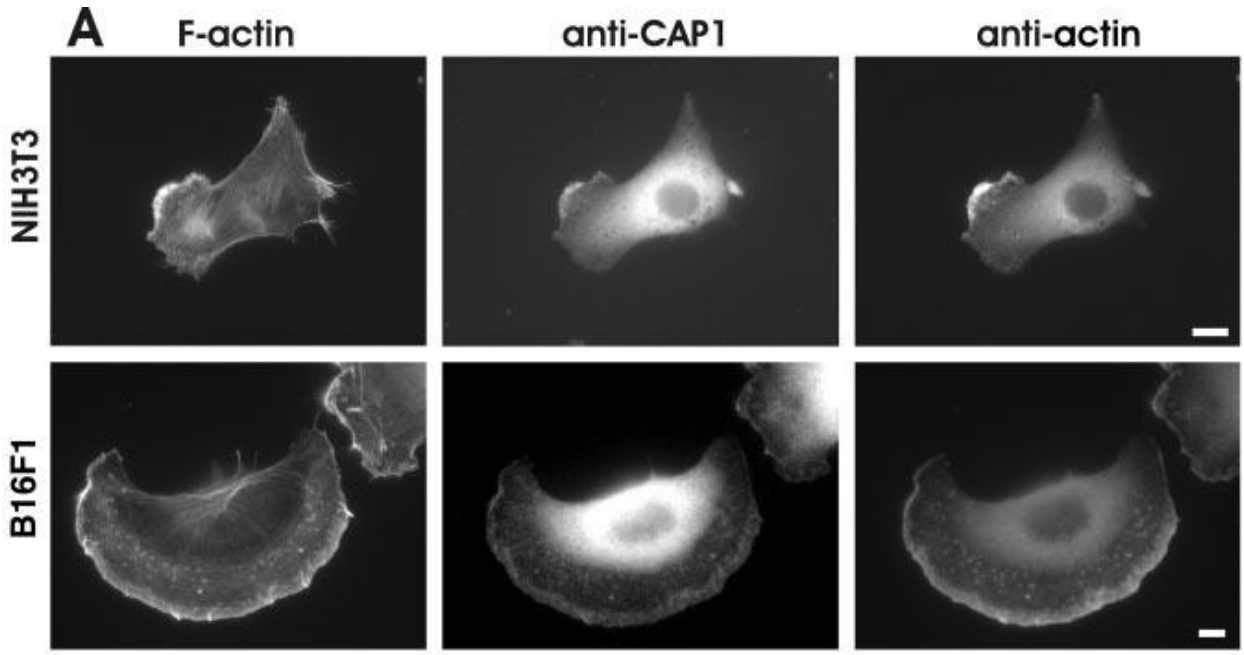


Figure 1.11 CAP1 localisation in mammalian cells. F-actin is observed by phalloidin staining, CAP1 localisation is identified using isoform specific antibody and AC-15 antibody is used to stain monomeric actin. CAP1 is diffused in the cytoplasm and localises at the leading edge of the cortical region in both cell types – NIH3T3 fibroblast cells and B16F1 melanoma cell lines. CAP1 is also seen to be abundant in the regions with higher proportions of monomeric actin. Scale bar = 10µm [Image from *Bertling et al.*(2004)].

Recent evidence has suggested a pro-apoptotic role for CAP1 in mammalian cells (Wang *et al.*, 2008). In the paper, they show that CAP1 translocates to mitochondria in response to endogenous apoptosis inducers independent of its role in actin depolymerisation, but instead acts in a shuttling mechanism with cofilin transporting actin to mitochondria (Wang *et al.*, 2008). Further, an interesting observation was that the translocation of CAP1 appeared as distinct structures, similar to CAP1-mediated actin-aggregate translocation to mitochondria seen during apoptosis in yeast (Gourlay and Ayscough, 2006). Characteristic apoptosis markers like release of cytochrome c and ROS accumulation were found to be reduced in CAP1 knockdown mammalian cells, where the deficiency of CAP1 protected cells from apoptosis (Wang *et al.*, 2008). Interestingly, yeast CAP, links actin cytoskeleton and Ras-cAMP pathway. Actin stabilisation (actin-aggregates) signal to activate Ras, which subsequently leads to ROS production, suggesting that the function of CAP in apoptosis is conserved but are governed by distinct mechanisms in yeast and mammalian cells respectively (Gourlay and Ayscough, 2006; Wang *et al.*, 2008). These observations that propose that CAP1 is pro-apoptotic further corroborate the role of elevated CAP1 in cancer progression and invasion.

A recent investigation revealed CAP1 as a receptor for adipokine, resistin, where it activates adenylyl cyclase pathway, triggering NF-ĸB pathway mediated cytokine expression in monocytes (Lee *et al.*, 2014). This study provides preliminary evidence for a potentially novel mechanism for CAP1 in mediating adenylyl cyclase activity in mammalian cells independent or partially dependent

on its known role in regulation of actin dynamics (Ono, 2013; Blanchoin *et al.*, 2014; Al Hannan and Culligan, 2015). A number of questions remain unanswered following this study in respect to the circumstances of CAP1-resistin interaction and its relation to the actin cytoskeletal dynamics in mammalian cells, thus making it difficult to establish their mode of action in disease states like metabolic syndrome, and regulation of haemostasis and glucose homeostasis.

AIMS AND OBJECTIVES

Obese, diabetic patients (300 million adults worldwide in 2012) suffering from MetS have increased levels of resistin in their circulation. Elevated levels of resistin have since been established as a contributor to IR and correlated to the progression of CVD. Platelets play a key role in CVD and yet little is known about the effects of elevated resistin levels on their physiological function and if this affects the severity of CVD in patients suffering from MetS. We therefore address this by characterising the effects of increased resistin in platelet function. Additionally, the mechanism of action of resistin in its target cells is unclear including identification of a receptor mediating resistin signalling, which is still under considerable debate. Recently, Lee *et al* (2014) reported CAP1 to be a 'bonafide' receptor for resistin. They suggested that CAP1 performed its role as a resistin-receptor by triggering its adenylyl cyclase function, a role which is not commonly reported in mammalian cells. Since CAP1 is predominantly known as a cytoskeletal protein, a preliminary investigation into the localisation of it and other cytoskeletal components within platelets was carried out which would aid in further delineating the role of platelet cytoskeletal proteins.

The key focus of this thesis is investigating how resistin affects human platelets, a cell model presenting dynamic cytoskeletal changes when stimulated with agonists, and establish and characterise CAP1 in platelets.

Objectives achieved in this thesis are outlined here:

1. Understanding the effect of resistin on platelet activation by assessing platelet functional assays including aggregation, secretion and spreading.

2. Characterising the cytoskeletal protein, CAP1, by investigating its localisation within platelets and its translocation during stimulation of platelet regulatory pathways – activation by thrombin and collagen and inhibition by prostacyclin.

3. Establishing the significance of cytoskeletal proteins in platelets using proteomics based data mining and establishing the localisation of selected cytoskeletal proteins in platelets by investigating their distribution pattern in spread platelets and in the platelet subcellular fractions of resting platelets.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of human blood platelets

2.1.1 Isolation of human blood platelets

Human blood was taken from drug-free volunteers by clean venepuncture using acid citrate dextrose (ACD) (29.9 mM of sodium citrate, 113.8 mM of glucose, 72.6 mM of sodium chloride, and 2.9 mM of citric acid (pH 6.4) as anticoagulant. Platelet-rich plasma was obtained by centrifugation of whole blood at 200xg at 20°C for 20 minutes. Platelet-rich plasma was treated with prostacyclin (50 nM) or citric acid (6mM) and was centrifuged at 800xg for 12 minutes. Platelets were washed in modified Tyrode's buffer (150 mM NaCl, 5 mM HEPES, 0.55 mM NaH_2PO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, and 5.6 mM D-glucose) containing 50nM prostacyclin, when isolated using prostacyclin or washed using wash buffer (0.036 mM citric acid, 0.01 mM EDTA, 0.005 mM D-glucose, 0.05 mM KCl, 0.09 mM NaCl) and centrifuged at 800xg for 12 minutes. Sedimented platelets were finally resuspended at required concentration in modified Tyrode's buffer and incubated at 37°C for 45 minutes prior to use for experiments (Aburima *et al.*, 2013).

2.1.2 Determination of platelet count

Washed platelets (5 µL) were suspended in 10 mL of IsotonII Diluent (Coulter) and platelet count per millilitre was determined using Beckman Coulter Z1 D counter.

2.2 Turbidimetric analysis of platelet aggregation

Washed platelet count was adjusted to 2.5 $x10^8$ platelets/mL and platelet suspension was used in aggregometer cuvettes for measurement using aggregometer (Aggrolink, Chrono-log/490-D) and output was recorded using AggroLink software. The turbidimetric assay is based on the principal of light transmission through platelet suspension which is detected by a photocell. Suspended platelets scatter light and inhibit it from reaching the photocell. When an agonist is introduced in the suspension, platelets start aggregating causing more light to pass through to the photocell and this transmission of light is directly proportional to the degree of platelet aggregation. The aggregometer was calibrated, where washed platelet suspension indicated 0% aggregation and Tyrode's buffer indicated 100% aggregation (Born and Cross, 1963). All agonists were diluted to required concentration in modified Tyrode's buffer.

2.3 Measurement of platelet dense granule secretion

Platelet dense granule secretion is quantified as being proportional to the release of ATP from the granules. ATP-dependent luciferin-luciferase assay

determines the ATP-release. Chrono-Log lumi-aggregometer was used to measure ATP release. Platelets (2.5 x10^8 platelets/mL) were pre-incubated with Chronolume (luciferin-luciferase reagent #395) for 1 minute prior to stimulation in the lumi-aggregometer. As described in section 3.2.1.6, stimulation was carried out using varying doses of thrombin (Sigma) for 2 minutes to record secretion and aggregation in real-time. ATP standard was added at the end of stimulation, which serves as an internal standard for each experiment. The lumi-aggregometer was calibrated, where washed platelet suspension indicated 0% aggregation and Tyrode's buffer indicated 100% aggregation; whereas the baseline for secretion was obtained after one minute pre-incubation with Chronolume and maximum was determined by addition of the ATP standard at the end of stimulation (White and Jennings, 1999). Secretion from dense granules was then determined by the comparison of test to the ATP standard using following formula.

$$Calculation\ of\ ATP\ release = \frac{Gain\ in\ sample}{Gain\ in\ ATP\ standard} \times 2nM$$

2.4 Platelet visualisation

2.4.1 Spreading on fibrinogen or collagen

The platelet count was adjusted before seeding at 2x10^7 platelets/mL. Platelet suspension (200 µL) was applied to 12-mm glass coverslips that were pre-

coated with fibrinogen [100 µg/mL in PBS - phosphate buffered saline (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4. pH of 7.4)] (Human fibrinogen plasminogen - Enzyme Research Swansaea, UK) or collagen (10 µg/mL in SKF solution) (collagen type IV and SKF solution form Horm-Kollagenreagens, Takeda) in 24-well cell culture plates (Corning) overnight at 4˚C. Platelets were allowed to adhere and spread for 30-45 minutes (in a humid chamber at 37°C) (variations in experiments have been noted in the figure legends) (Gibbins and Mahaut-Smith, 2004). In experiments that assess effects of resistin (Peprotech, USA), platelets were pre-incubated with resistin prior to spreading on fibrinogen. In experiments requiring further activation, spreading on fibrinogen was followed by stimulation with 0.1 U/mL thrombin (Sigma) for designated time periods (ranging from 0 to 180 seconds) at room temperature without shaking. The platelets were fixed at the end of stimulation period using ice-cold 4% paraformaldehyde (PFA) (Sigma) in PBSfor 10 minutes. Any unbound platelets were removed by aspiration and washed with PBS prior to blocking with PBG (0.5% BSA, 0.05% Fish gelatin in PBS).

2.4.2 Platelet stimulation in suspension

Washed platelets ($5x10^8$ platelets/mL) were stimulated with 0.1U/mL thrombin in suspension (shaking) and fixed at the end of stimulation using 1:1 ice-cold 4% PFA. Platelet suspension was then applied to poly-L-lysine (Sigma) (0.01% in PBS) pre-coated 12-mm glass coverslips and centrifuged at 3,440xg for 10 minutes to allow fixed platelets to attach to the matrix. Any unbound platelets

were removed by aspiration and washed with PBS prior to blocking with PBG (For ingredients see 2.4.1).

2.4.3 Immunostaining platelets

Human platelets that have previously adhered and/ or spread on coverslips (see sections 2.4.1 and 2.4.2) were permeabilised using 0.3% Triton-X 100 solution (Roth, UK) in PBS before immunostaining with primary (see Appendix 2) and corresponding fluorescent-tagged secondary antibodies [Alexa 488 (Molecular Probes) or Alexa 568 (Molecular Probes) – (1:2000 in PBS)]. When staining for actin, FITC-phalloidin (Sigma) or TRITC-phalloidin (Sigma) was used. Intermediate washing steps were carried out using PBG (For ingredients see section 2.4.1).

2.4.4 Imaging platelets

Platelets were imaged using a fluorescence microscope (Olympus XL-100) at 100x magnification (Chapter-4), using Zeiss Axio VertA.1 at 100x magnification (Chapter-3), and Zeiss ApoTome.2 (Chapter-5) and images were captured by Micropublisher 3.3RTV camera or XM10 CCD camera or AxioCam 506, respectively. All images were processed using PhotoShop 5.5 (Adobe) and CorelDRAW X6 (Corel).

2.4.5 Analysis of imaged platelets

Analysis of unprocessed images (see section 2.4.4), saved in TIFF format, was carried out using ImageJ software. Signal intensities, cell size, and cell numbers were measured using ImageJ and were recorded in MS Excel. When comparisons were carried out, values were normalised to respective controls. For classification of platelets according to the actin structures, following criteria were used – cells having actin nodules, and cells having stress fibres. Platelets having both actin nodules and stress fibres were classified twice. Platelets showing a bright spots were not characterised as they are not spread platelets. Figure 2.1 shows a representative classification of platelets.

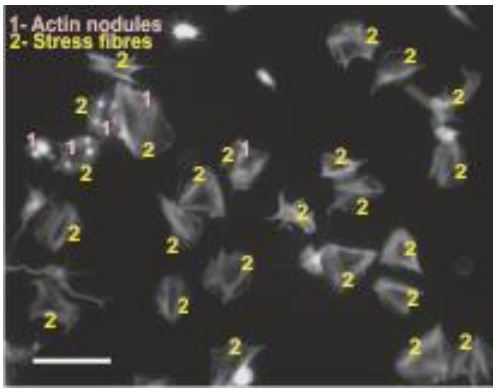


Figure 2.1 Classification of platelets for characterisation of actin structures in platelets. All platelets labelled (1) are classified as having actin nodules and (2) represent all platelets having stress fibres. Platelets showing a bright spot are not characterised as they are not spread platelets. Scale bar - 10µm.

2.5 Preparation of whole cell lysate

Washed platelets were adjusted to 5 x 10^8 platelets/mL and treated with platelet agonists at required concentration under stirring condition. Reaction between platelets and agonist was stopped using 1:1, ice-cold lysis buffer [1% TX-100, 10 mM Tris-HCl, 10 mM EGTA, 1:100 (v/v) protease inhibitor cocktail, 1:200 (v/v) phenylmethanesulfonylfluoride (PMSF)]. 20µL aliquot was stored at 4°C to measure protein concentrations (see section 2.6) . 2X SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue, 4% β-mercaptoethanol] was added to remainder of reaction mixture and boiled at 95°C for 5 minutes.

2.6 BCA (Bicinchoninic acid) Protein determination assay

Protein concentrations of platelet lysates (see section 2.5) and mammalian cell lysates (see section 2.10.4) were measured with Pierce® BCA Protein Assay Kit according to manufacturer's protocol. Absorption was measured at 563 nm using a TECAN 2000 plate reader.

2.7 Subcellular fractionation of platelets

Human blood platelets, 3 x 10^8, were lysed in an isotonic sucrose solution [100 mM Sucrose, 4 mM HEPES, 0.5 mM Na_3VO_4, 1:100(v/v) Protease inhibitor cocktail, 1:200 (v/v) PMSF] by 5-6 cycles of rapid freeze thaw in liquid nitrogen. Cell debris and unlysed platelets were removed by centrifugation of lysates at

1,000xg at 4˚C for 3 minutes. Supernatant lysates were then spun at 100,000xg for 1 hour using Beckman Coulter Optima MAX-XP ultracentrifuge (Castle, 2004). Pellet contained the membrane fraction which was resuspended in 2X SDS sample buffer (1:1 (v/v)) and boiled at 95°C for 5 minutes. Supernatant contained the cytosolic fraction and prepared by adding 2X SDS buffer (1:1) followed by a boiling step at 95°C for 5 minutes.

2.8 Isolation of the detergent insoluble pellet

Human blood platelets, 3 x 10^8 ,were lysed in TX-100 containing lysis buffer [2% TX-100, 10 mM Tris-HCl, 10 mM EGTA, 1:100 (v/v) protease inhibitor cocktail, 1:200 (v/v) PMSF]. Lysates were spun at 15,600xg (low speed) or 100,000xg (high speed) as indicated (Fox, 1985). Pellet containing the detergent insoluble proteins were resuspended in 2X SDS sample buffer (see section 2.7) and supernatant was prepared by adding 2X SDS buffer. Pellet and supernatant were boiled at 95°C for 5 minutes and samples were resolved using Western blotting (see section 2.9).

2.9 Immunoblotting

Specific proteins of interest were identified from lysates containing complex mixture of proteins using Western blotting. Western blotting constitutes protein separation based on their molecular weight under denaturing conditions of SDS-containing polyacrylamide gel. Separated proteins are then electrophoretically transferred to adhesive membranes to enable protein detection using protein specific antibodies. Unspecific binding sites on the membranes are then blocked using casein or serum albumin followed by incubation with antibodies generated against the proteins or peptides of interest. Fluorescently or HRP-tagged secondary antibodies then help to generate a signal equivalent to the concentration of protein that is transferred onto the membrane.

2.9.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight under denaturing conditions of SDS-PAGE. Samples resuspended in (1:1 2xSDS buffer) were resolved on gels and electrophoresis was done using MiniPROTEAN® (BioRad, UK) system. 10% or 12% separating gels were prepared according to the following proportions (Table 2.1). The gels were placed in the gel chambers containing 1X SDS running buffer (25 mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.3). The samples and molecular weight ladder (EZ-Run TM Prestained Rec Protein Marker, Fisher Scientific, UK) were loaded into wells and run at 80-150V until the bromophenol dye containing loading front ran out the bottom of the gel.

Table 2.1: Composition of SDS-PAGE

Chemicals	12% (Separating gel)	10% (Separating gel)	4% (Stacking gel)
Acrylamide:Bisacrylamide (37.5:1) (Rotiphorese 30%) (CarlRoth, Germany)	12 mL	10 mL	4.08 mL
1.5 M Tris-HCl pH 8.8 ((Sigma, UK)	7.5 mL	7.5 mL	--
10% SDS (Melrose, UK)	300 µL	300 µL	300 µL
Distilled water	9.9 mL	12.9 mL	17.16 mL
10% Ammonium persulphate (APS) (Sigma, UK)	300 µL	300 µL	225 µL
TEMED (Sigma, UK)	30 µL	30 µL	150 µL
0.5 M Tris-HCl pH 6.8 (Sigma, UK)	--	--	8.4 mL

2.9.2 Blotting of proteins onto PVDF or nitrocellulose membranes

Gels were equilibrated in ice-cold blotting buffer (25 mM Tris-base, 193 mM Glycine, 20% Methanol) for 2-3 minutes. PVDF membranes were activated by soaking in methanol for one minute. Nitrocellulose membranes were activated by soaking in distilled water. Blotting paper soaked in blotting buffer was used

on either side to form a cassette. Wet-blot transfer was carried at 100V for 1.5 hours in a MiniPROTEAN® gel chamber (Biorad, UK) under constant stirring at 4˚C.

2.9.3 Immuno-detecting proteins bound to membranes

PVDF or nitrocellulose membrane carrying the blotted protein was first equilibrated in TBS-T buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1 % Tween-20] before blocking any unspecific binding sites using 5% BSA prepared in TBS-T for 45 minutes. Membrane was washed and incubated with primary antibodies at appropriate dilutions overnight. Membrane was washed again before incubating it with appropriate peroxidase-coupled or fluorescently-tagged secondary antibodies. The chemiluminescence reaction for detecting peroxidase-coupled antibodies was carried out using peroxide containing ECL buffer 1, and luminol containing ECL buffer 2 (See Table 2.2). The membrane was exposed on Amersham HyperFilm ECL (GE Healthcare, UK) and developed using developer and fixer solutions (Kodak, UK). Developed films were scanned and densitometric analysis was carried out using ImageJ software(version 1.50b). Fluorescently-tagged secondary antibodies were detected using LiCor Odyssey CLx Imaging System and densitometric analysis was carried out using ImageStudio software (Image Studio lite version 5.2).

Table 2.2: Composition of enhanced chemiluminescence (ECL) buffers

Chemical	Volumes	
	ECL1	**ECL2**
10% Tris base (Sigma, UK)	100 mM	100 mM
Hydrogen peroxide (Sigma, UK)	--	30% (v/v)
Luminol (Sigma, UK)	250 mM	--
Coumaric acid (Sigma, UK)	90 mM	--

2.9.4 Processing of the densitometric values for graphical representation

After densitometric analysis was carried out using ImageStudio software (Image Studio lite version 5.2), the values were exported to MS Excel for further processing. The densitometric values of protein of interest were first normalised to the loading control (GAPDH, CD36, Integrinβ3 or beta-actin, as relevant and is specified in the respective experiments and the figure legends). In situations where treatment conditions were compared to the basal or untreated, resting platelets, the values that were normalised to the loading control were further normalised to the basal to allow clear comparison of changes post-treatments, if any. Graphical representations were generated in MS Excel, while statistical analysis was carried out as specified in section 2.11.

2.10 Cell culture and transfection

2.10.1 Culture of mammalian cells

293T HEK cells (Human Embryonic Kidney cells) and HeLa (carcinoma cell line) were retrieved from frozen stock vial (5 x 10^6 cells/mL) and warmed to 37°C and resuspended in DMEM. The cells were centrifuged at 200xg for 5 min and resuspended in fresh medium for seeding on a 10 cm culture dish (Corning, UK). Cells were incubated at 37°C in a humidified incubator supplied with 5% CO_2 in appropriate culture medium (Supplemented DMEM – see below). The medium was changed the following day and cells were allowed to proliferate until confluent before passaging.

Supplemented DMEM:

DMEM high glucose (Sigma, UK)

10% heat-inactivated FCS (Life Technologies, UK)

2 mM glutamine (Sigma, UK)

100 U/mL penicillin, 100 µg/mL streptomycin (PAA, UK)

1 mM sodium pyruvate (Sigma, UK)

2.10.2 Transfection of mammalian cells

293T HEK cells and later HeLa were transiently transfected with either IQGAP1WW-GFP plasmid, or IQGAP2-WW-GFP plasmid, in addition to myc-CAP1 plasmid using Lipofectamine 2000 (Invitrogen, Life Technologies Ltd.) according to manufacturer's recommendations (GFP-CAP1 was used to

generate a control sample). All plasmids were generated previously in the lab of Dr Rivero and their details can be found in Table 2.3.

Table 2.3 List of plasmid constructs

Plasmid name	Vector	Insert
pRK5-myc-HsCAP1-C (242-475)	pRK5 myc	HsCAP1 C terminus (242-475)
pRK5-myc-HsCAP1-N (1-324)	pRK5 myc	HsCAP1 N terminus (1-324)
pGEM-Teasy/HsCAP1	pGEM-Teasy	Hs CAP1
HsIQGAP2-WW/pEGFP-C2	pEGFP-C2	HsIQGAP2 WW-region (542-686)
HsIQGAP1-WW/pEGFP-C3	pEGFP-C3	HsIQGAP1 WW-region (626-767)

Transfection was confirmed using fluorescence microscope using GFP expression as marker for successful transfection. Transfection efficiency is shown in Figure 2.2.

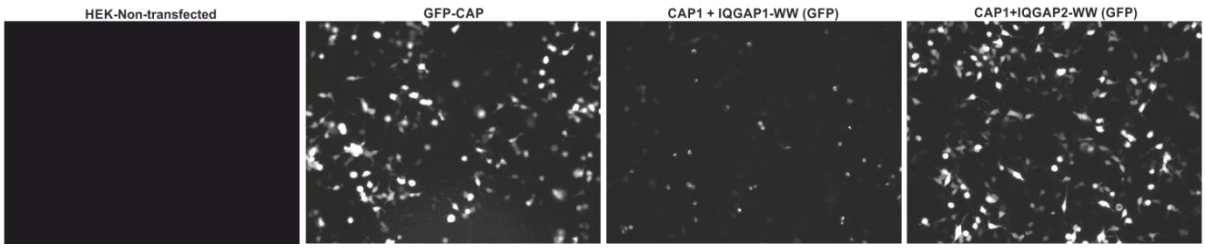


Figure 2.2 Transfection efficiency. Plasmids were transfected transiently in HEK cells and efficiency was assessed by viewing under a fluorescence microscope. GFP-CAP1 and IQGAP2-WW (GFP) transfection was highly efficient. IQGAP1-WW (GFP) transfection was minimal.

2.10.3 Cryopreservation of mammalian cells

A confluent monolayer of 293T HEK cells on a 10 cm culture plate was trypsinised using Trypsin/EDTA (Life Technologies, UK) for 5 min at 37˚C and resuspended in cold DMEM (without FCS). The cells were centrifuged for 10 min at 200xg at 4°C. The pellet was resuspended in 1 mL of freezing medium (see below) and transferred to cryotubes. The cryotubes were placed in pre-cooled cryorack Nalgene® Mr Frosty™ Cryo (soaked in isopropanol) and left at -80°C overnight before transferring to liquid nitrogen until further use. For reusing the frozen cells method is as described in section 2.10.1.

Freezing medium:

70% DMEM High glucose (Sigma, UK)

20% FCS (Life Technologies, UK)

10% DMSO (Sigma, UK)

2.10.4 Lysis of mammalian cells

A confluent layer of mammalian cells was washed with PBS and lysed using a lysis buffer (see below). Lysates were then collected and centrifuged at 2000xg for 10 min at 4°C and stored at -20°C until further use.

Lysis buffer:

150 mM NaCl (Merck, UK)

1% Triton-X 100 (Roth, Germany)

50 mM Tris-HCl (pH 8.0) (Sigma, UK)

0.5 mM PMSF (Sigma, UK), 1:100 Protease Inhibitor Cocktail (Sigma, UK)

2.10.5 Immunoprecipitation: Transfected mammalian cells

Cell debris was removed from lysed, transfected mammalian cells by centrifugation at 10,000xg at 4°C for 10 minutes and epitope tagged proteins were immunoprecipitated using a μMACS epitope tag protein isolation kit with magnetic beads conjugated with anti-Myc antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol.

2.11 Statistical analysis

All data was primarily computed in MS Excel and used to produce graphs. Statistical analysis was carried out using IBM SPSS 23 software. Student's t-test (two-tailed, unpaired) was carried out when comparing two individual sample sets. One-way ANOVA was carried out when data of more than two sets was compared to a control or basal sample set. LSD (Fisher's least significant difference) post-hoc correction was applied for smaller data sets (For example, control is compared to two other variable treatment conditions) and Bonferroni correction was applied for larger and/ or multiple data sets (including n numbers greater than 4). P-values are presented in the figure legends. Parametric tests were undertaken assuming normal distribution of data, owing to random selection of blood donors from the population, thus ensuring a more robust statistical analysis.

2.12 Source of chemicals and reagents

All solvents (methanol, ethanol, isopropanol) were bought from Fischer Scientific, UK and unless otherwise stated, remainder of the chemicals and reagents were bought from Sigma, UK. A list of antibodies used and their test conditions can be found in Appendix 2.

CHAPTER 3

EFFECT OF ELEVATED RESISTIN LEVELS ON PLATELET FUNCTION

High levels of circulating resistin are present in patients with MetS and associated conditions. Several studies correlate elevated levels of resistin to obesity, T2DM and CVDs suggesting that resistin might play a major role in aetiology of MetS and therefore contribute towards CVD development and progression leading to complications (Miranda *et al.*, 2005; Vykoukal and Davies, 2011). Resistin promotes atherogenesis by virtue of its pro-inflammatory effects on vascular endothelial and smooth muscle cells *(*Jung *et al.,* 2006; Pirvulescu *et al.*, 2014). In atherosclerotic lesions elevated resistin levels were found associated with macrophages and, when present at high levels locally in the arterial vessel wall, resistin significantly contributed to an enhanced accumulation of macrophages by increasing monocyte chemotaxis (Manduteanu *et al.*, 2010; Cho *et al.*, 2011). Also, evidence from endothelial cell lines suggests that resistin increases procoagulability in T2DM by upregulation of TF, which is required for thrombin generation, in addition to upregulation of PAI and t-PA, which are required for fibrinolysis – together leading to the procoagulatory state (Bobbert *et al.*, 2011).

The imminent question in understanding the correlation between the hormone and disease is to find out if and how far these elevated levels of resistin affect thrombotic events. Platelets, as first responders to the site of vascular injury, are exposed to dynamic thrombotic microenvironments during the process of haemostasis, presenting as an interesting model cell to investigate the effect of resistin on their function. Although there is evidence in the literature that a strong link exists between elevated resistin levels and MetS-associated CVD (Bełtowski, 2003; Reilly *et al.*, 2005; Anfossi, Russo and Trovati, 2009; Piya, McTernan and Kumar, 2013; Huang and Yang, 2015), there are no thorough reports outlining the effects of resistin on platelet function. Additionally, there are discrepancies surrounding research on resistin (Lee *et al.*, 2003) – resistin secretory cells vary between mice and humans as do the target cells and the effects they cause. In mice, resistin is secreted by adipocytes and acts in an autoregulatory manner (Steppan *et al.*, 2001) with supplementary endocrine effects like causing IR in obese, diabetic mice (Steppan *et al.*, 2005). In humans, however, monocytes and macrophages (Patel *et al.*, 2003; S. Lee *et al.*, 2014) are the main cells responsible for resistin secretion and its effects on IR and glucose metabolism are keenly debated and evidence for and against it exits depending on the experimental designs of various investigators (Cho *et al.*, 2001; Zhang *et al.*, 2003; Banerjee, 2004; Manduteanu *et al.*, 2010; Piya, McTernan and Kumar, 2013; Huang and Yang, 2015).

This chapter therefore narrows the scope of study and aims to broadly investigate how recombinant human resistin affects platelet activation in functional studies involving platelet aggregation, secretion and adhesion assays. Additionally, biochemical signalling pathways involving activation of integrin $\alpha_{IIb}\beta_3$ and its downstream effector proteins in PI3K and MAPK pathways are briefly examined.

3.1 Aims and objectives

The aim of this chapter is to examine the effects of resistin on various platelet activation processes using agonists that stimulate platelets through distinct pathways and carry out a preliminary evaluation of potential biochemical signalling pathways involved therein.

The objectives are:

1. Investigating the effect of resistin on platelet aggregation:
    - In GPCR mediated platelet activation using thrombin
    - In tyrosine kinase mediated platelet activation using collagen
    - Evaluating the time and dose response to resistin pre-treatment of platelets

2. Examining the effect of resistin on secondary platelet response by
    - Inhibiting ADP and TXA_2 and assessing effect on platelet aggregation
    - Assessing dense granule secretion/ATP release using luciferase assay in platelet aggregation

3. Investigating the effect of resistin on integrin-mediated platelet spreading by
    - Evaluation of time and dose response of resistin on immobilised fibrinogen
    - Understanding the effect of resistin on secondary response during spreading

4. Assessing the effect of resistin on potential biochemical signalling pathways by
    - Evaluating phosphorylation by AGC kinases
    - Assessing phosphorylation of PI3K/Akt and MAPK, ERK1/2, in response to resistin stimulation.

3.2 Results

3.2.1. Effects of resistin in suspended platelets

3.2.1.1 Effects of resistin on platelet aggregation

In order to study the effects of resistin on platelets, a preliminary functional study was carried out to assess if resistin caused platelets to aggregate in a dose dependent manner. In figure 3.1, it was observed that treatment of platelets with various doses of resistin alone did not have any effect on platelet aggregation, similar to basal platelets left stirring in the aggregometer with no agonist stimulation.

The doses were chosen in the high physiological range to severe disease conditions (50ng/mL to 1µg/mL) (Qiu *et al.*, 2014). In order to account for the competency of platelets post-preparation, a positive control test was carried out using 0.1U/mL of thrombin.

Therefore, resistin does not appear to act as a platelet agonist.

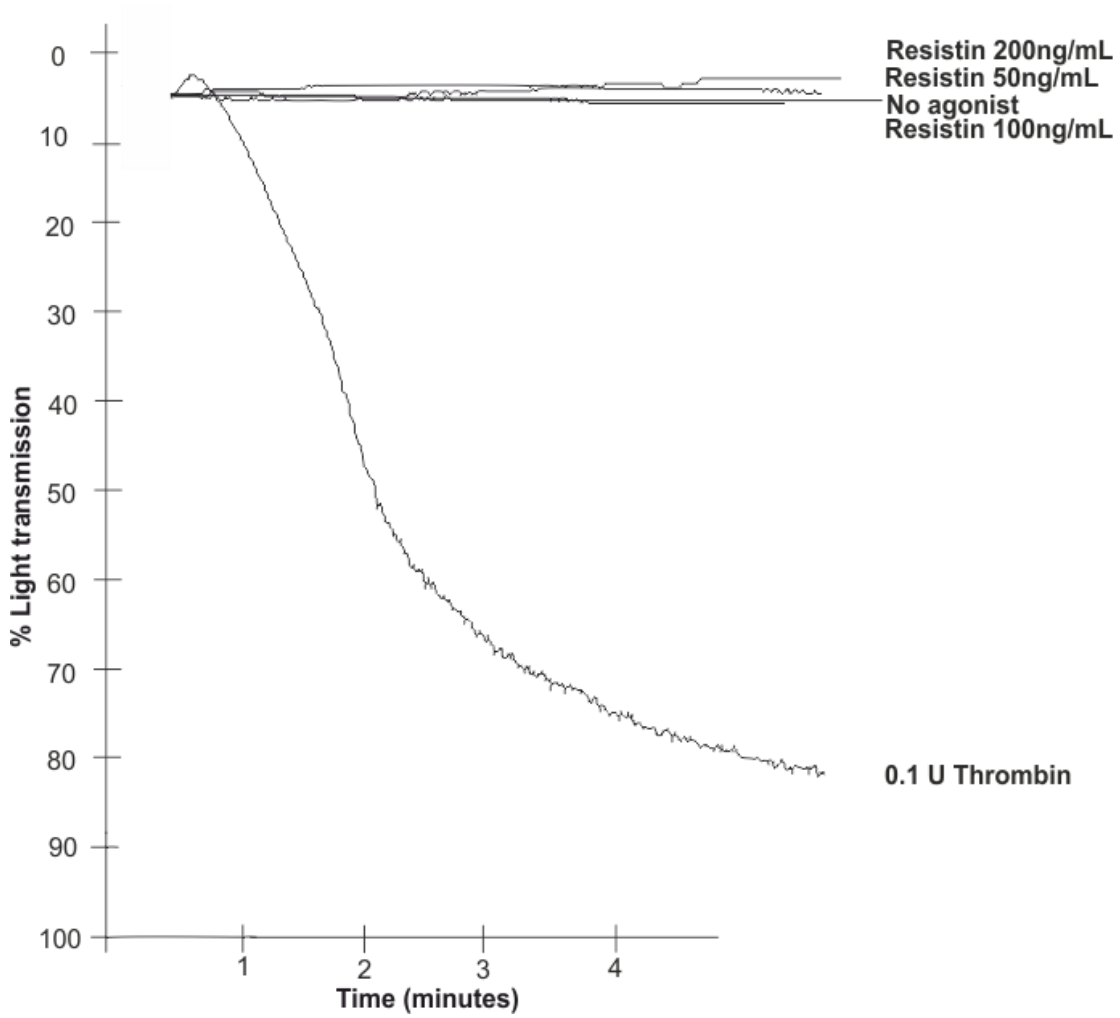


Figure 3.1: Effect of resistin on platelet aggregation: Multiple doses of resistin (Res) were tested to assess effect on aggregation response of 2 x 10^8 /mL blood platelets in aggregometer. 0.1U/mL thrombin is used as a positive control to assess platelet function and basal was the negative control which received no agonist. Aggregation traces are representative of four independent experiments.

3.2.1.2 Effects of resistin on platelet aggregation driven by agonists

Having shown that resistin doesn't act as an agonist itself, we asked whether it may modulate the response to well characterised agonists.

Physiologically platelets are exposed to resistin in circulating blood before they encounter thrombotic conditions in the vascular system (Vykoukal and Davies, 2011). In order to simulate that, washed platelets were exposed to resistin prior to agonist stimulation.

3.2.1.2.1 Effect of resistin on thrombin-stimulated aggregation

Thrombin is a strong platelet agonist. *In vivo* it is generated by adhered platelets to enhance thrombus formation by causing recruitment and activation of quiescent platelets from circulation, further helps demonstration of a thrombotic event. Thrombin causes platelets to generate an inside-out signal through the G-protein (G_q) coupled receptors PAR1 and PAR4 (in humans) leading to activation of integrin $\alpha_{IIb}\beta_3$ required for platelet aggregation (Coughlin, 2000; Brass, 2003).

Blood platelets were treated with 200ng/mL resistin for 5 minutes prior to stimulating them with thrombin in a dose dependent manner, where untreated platelets were used as positive control for each dose of thrombin. Figure 3.2 shows percent aggregation of platelets upon thrombin stimulation. It was observed that resistin pre-incubation caused a small reduction in aggregation of platelets across multiple doses of thrombin stimulation. The most significant

reduction in aggregation of ~30% ($p=0.006$, one-way ANOVA) was observed when resistin pre-treated platelets received 0.025U/mL thrombin dose.

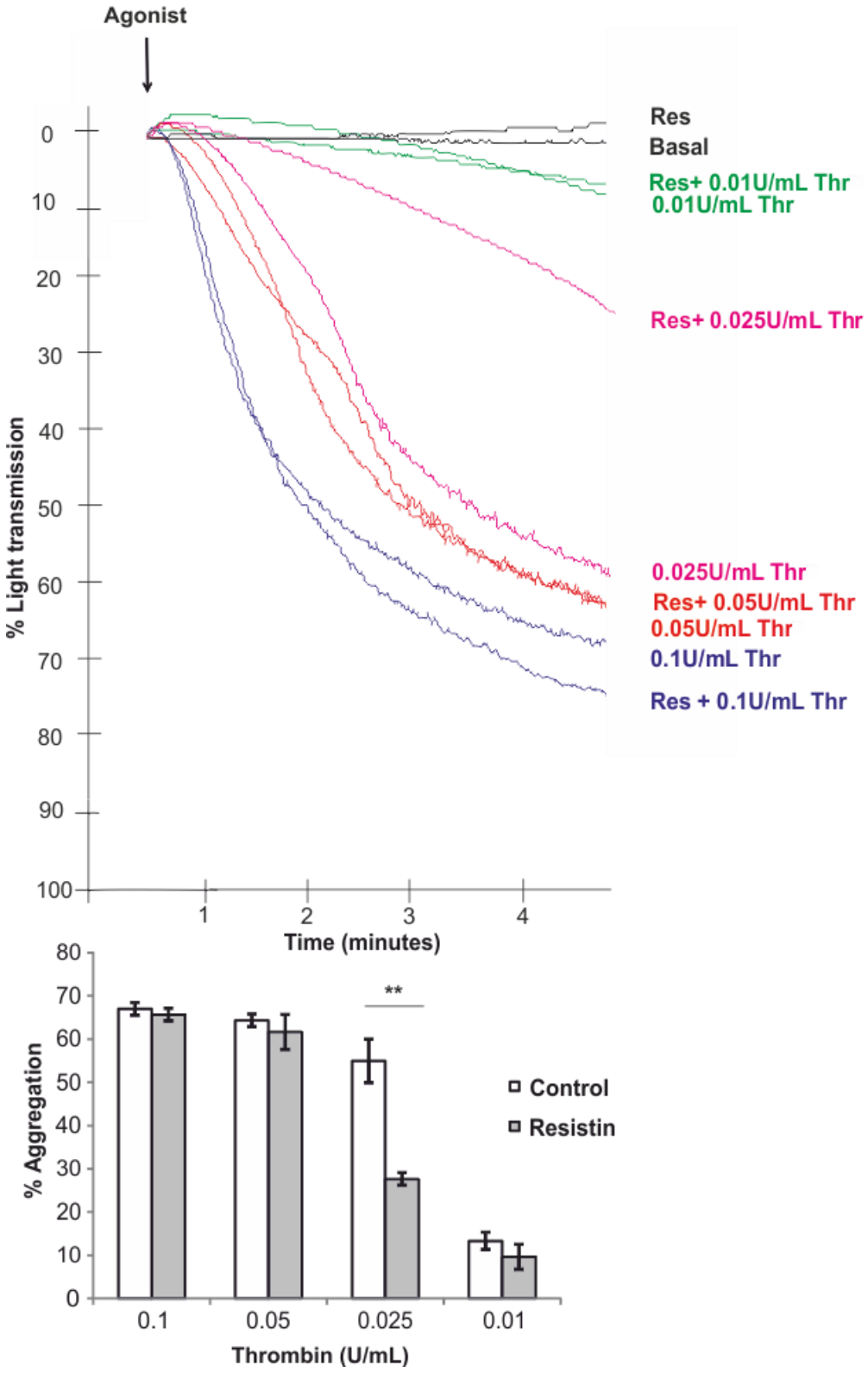

Agonist
0
10
20
30
40
50
60
70
80
90
100
% Light transmission
1
2
3
4
Time (minutes)
Res
Basal
Res+ 0.01U/mL Thr
0.01U/mL Thr
Res+ 0.025U/mL Thr
0.025U/mL Thr
Res+ 0.05U/mL Thr
0.05U/mL Thr
0.1U/mL Thr
Res + 0.1U/mL Thr
80
70
60
50
40
30
20
10
0
% Aggregation
**
Control
Resistin
0.1
0.05
0.025
0.01
Thrombin (U/mL)

Figure 3.2: Effect of resistin pre-incubation on platelet aggregation with thrombin: Human blood platelets (2 x 10^8 /mL)were incubated with 200ng/mL resistin for 5 minutes before assessing the effect on thrombin stimulation. Untreated platelets were used as a control against resistin treatment for each dose of thrombin agonist tested in the aggregometer. Basal indicates platelets with no agonist stimulation, while “Res” indicates 200ng/mL resistin with no thrombin agonist. Data are represented as mean ± standard error of mean of three independent experiments. Statistical significance calculated using one-way ANOVA (**p = 0.006).

3.2.1.2.2 Effect of resistin on collagen-stimulated aggregation

The inhibition of aggregation upon resistin treatment of platelets after thrombin stimulation prompted an assessment to identify if this effect was thrombin pathway specific. Collagen, another strong platelet agonist, upon vascular injury, acts as the most thrombogenic component of the endothelium initiating platelet activation. Similar to thrombin dose response (section 3.2.1.2.1), collagen agonist was examined inresistin pre-treated platelets. Since collagen acts via binding glycoprotein receptors distinct from thrombin receptors, it was considered as an alternative agonist to assess changes in platelet aggregation post resistin treatment. Collagen binds tyrosine kinase-linked receptors GPVI-FcR-γ activating PLCγ2, leading to inside-out signal causing integrin $\alpha_{IIb}\beta_3$ activation required for platelet aggregation (Watson *et al.*, 2005).

The experimental set-up was similar to that of section 3.2.1.2.1. Platelets were pre-incubated with 200ng/mL resistin for 5 minutes before stimulation with collagen in an aggregometer. Aggregation of untreated platelets with respective collagen doses were used as controls for resistin treatment. Figure 3.3 shows that platelet aggregation of resistin-treated platelets was slightly less than control at all collagen doses, but this reduction was not statistically significant (ANOVA). Like thrombin (Figure 3.2), figure 3.3 also showed a trend towards collagen responding to resistin by causing reduction in aggregation at lower doses greater than that at higher doses.

As the effects of resistin in collagen-stimulated platelet aggregation were relatively mild compared to thrombin stimulation, further investigation of the effects of resistin on platelet function were only restricted to GPCR-related stimulation using thrombin and tyrosine-kinase related stimulation by collagen was not investigated more in this chapter.

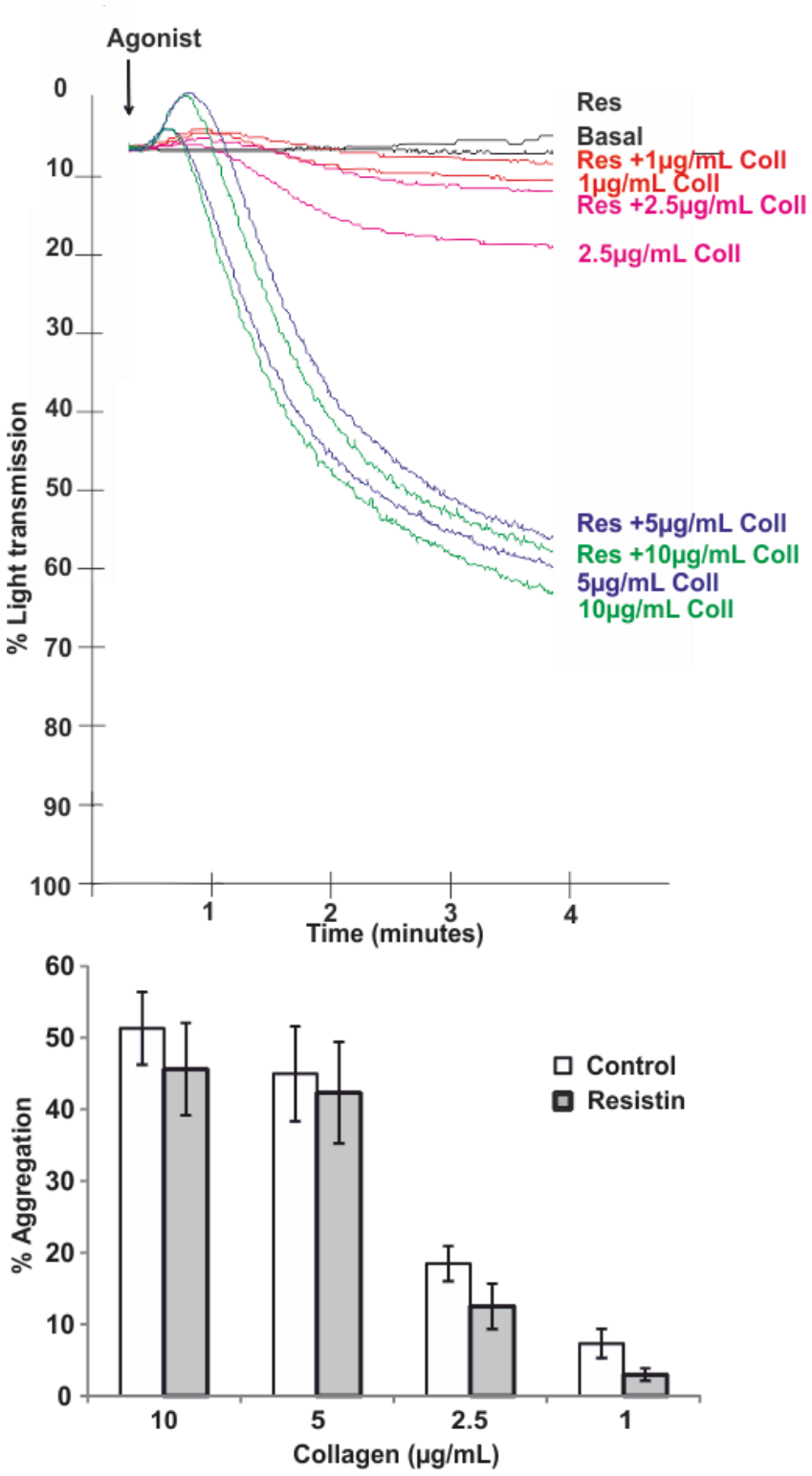


Figure 3.3: Effect of resistin pre-incubation on collagen stimulated platelet aggregation: Human blood platelets (2 x 10^8 /mL) were incubated with

200ng/mL resistin (Res) for 5 minutes before assessing the effect on aggregation with collagen (Coll). Untreated platelets were used as a control against resistin treatment for each dose of the agonist stimulation. Data are represented as mean ± standard error of mean of three independent experiments. Statistical significance calculated using one-way ANOVA, but no significance within each collagen dose was observed.

3.2.1.3 Characterisation of resistin pre-incubation on thrombin stimulation of platelets

A preliminary hypothesis emerged that resistin inhibits platelets when stimulated with thrombin. It was important to assess if it was the time of resistin pre-incubation that had an effect on the outcome or if it was the dose of resistin used for the treatment of washed platelets. Hence, it was important to investigate time and dose dependence of the effect of resistin on aggregation when stimulated with a fixed dose of thrombin.

3.2.1.3.1 Time dependence of resistin pre-incubation on thrombin related platelet aggregation

Washed platelets were incubated with 200ng/mL resistin for varying times to assess effects on aggregation (Figure 3.4). Resistin and thrombin were administered together, which was considered first time point at zero minutes and showed minimal change in aggregation compared to control which only received 0.025U/mL thrombin and no resistin. Following time points at 1, 5, 15, 30 minutes pre-incubation show decline in percent aggregation compared to control. This might have indicated when changes were triggered in platelets that lead to dampening of platelet activation, useful for further investigation. Statistically significant ($p \leq 0.001$, one-way ANOVA) reduction was observed when washed platelets were incubated with resistin for 15 and 30 minutes compared to percent aggregation of control samples. Multiple control samples were run on the aggregometer for the later incubation times to account for loss

of platelet activity, if any, during the long incubation in buffer between tests, but only one representative control aggregation trace is shown in figure 3.4.

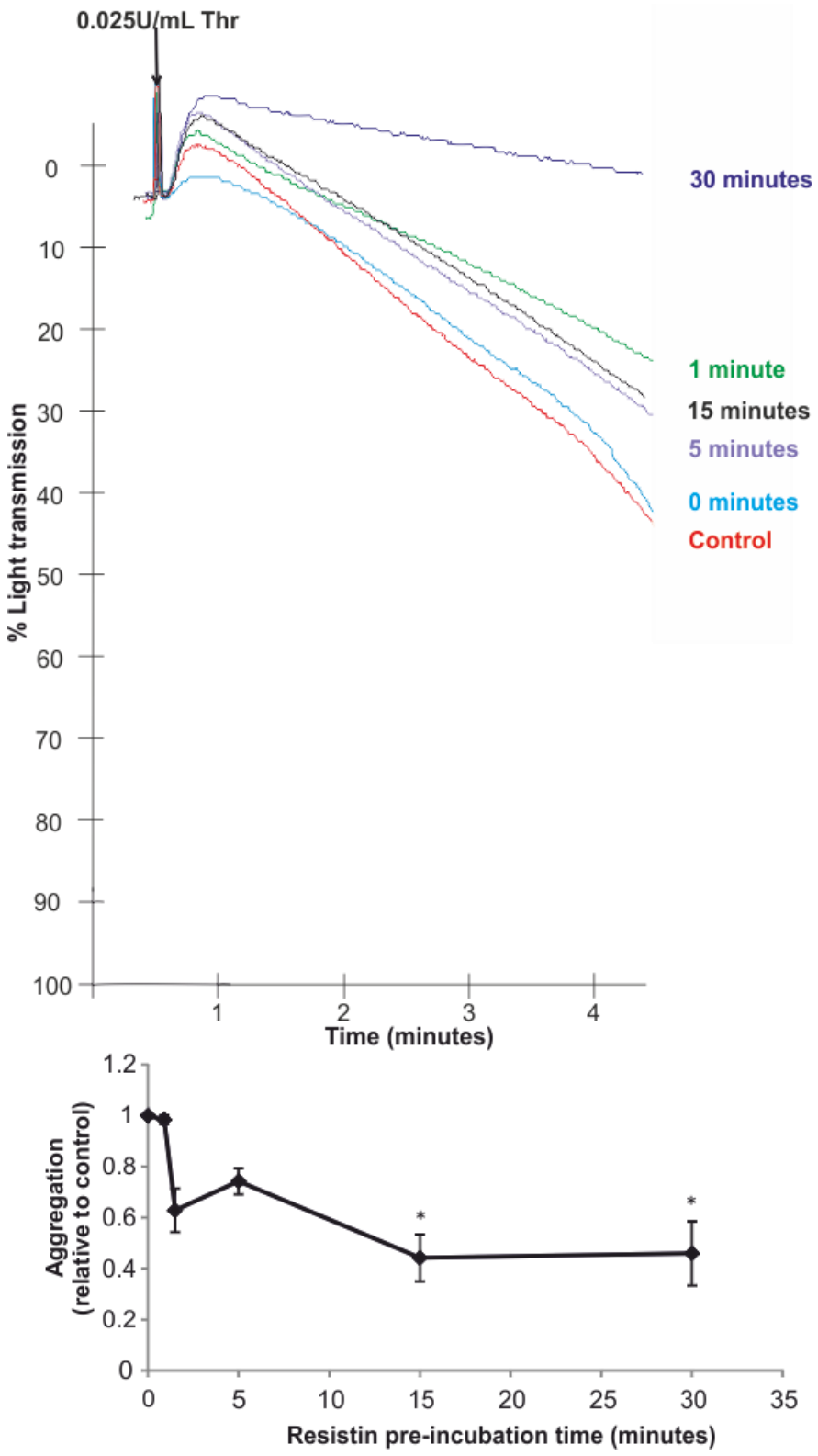


Figure 3.4 Time response of resistin pre-incubation on thrombin related platelet aggregation: Human blood platelets (2 x 10^8 /mL) were incubated for

increasing length of time with 200ng/mL resistin before assessing the effect on aggregation with 0.025U/mL thrombin. Aggregation of platelets when stimulated with 0.025U/mL thrombin was used as control. Fold change in aggregation was calculated with respect to aggregation of control test in order to normalise data from multiple experiments. Data are represented as mean ± standard error of mean of four independent experiments. Statistical significance is calculated using one-way ANOVA (*$p \leq 0.001$).

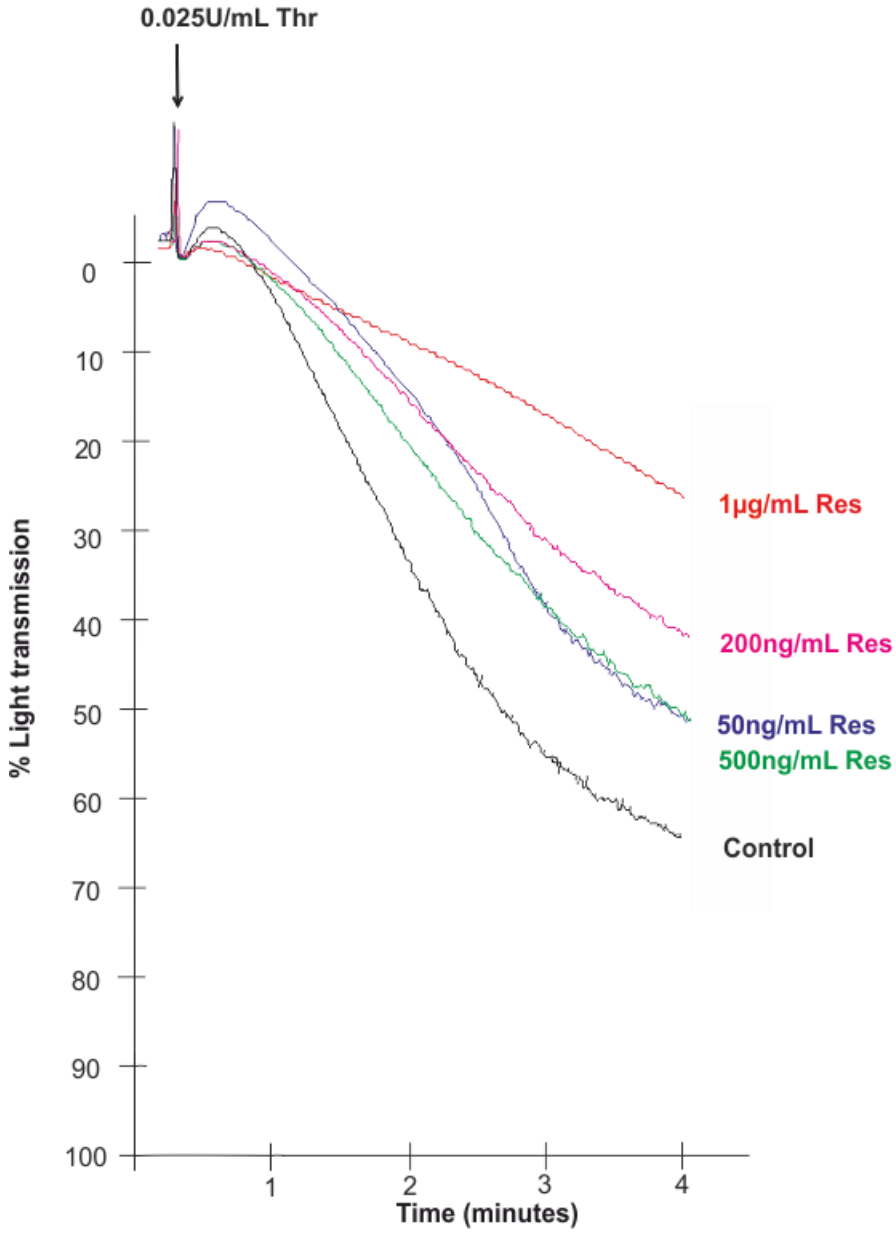
3.2.1.3.2 Dose dependence of resistin pre-incubation on thrombin related platelet aggregation

The conclusion which emerged from Figure 3.2 and 3.4 was that 30 minutes of resistin incubation caused maximum inhibition of platelets in aggregation when stimulated with 0.025U/mL thrombin. It was important to identify if the appropriate dose of resistin was used in the pre-incubation and that the effect sustained or varied when the dose of resistin was altered. Hence, resistin doses which range from early disease on-set to chronic hyperresistinemia were chosen - 50ng/mL to 1µg/mL, respectively, were tested in aggregation. As previously shown, in figure 3.1, these doses alone do not initiate aggregation of platelets.

As seen in figure 3.3, 100ng/mL and 200ng/mL dose of resistin during platelet incubation caused statistically significant ($p \leq 0.05$) reduction in percent aggregation compared to control (which only received 0.025U/mL thrombin with no resistin). Further doses did not result in an increased reduction in platelet aggregation.

500ng/mL resistin dose led to similar reduction in aggregation as 50ng/mL resistin. Maximum effect was achieved around 200ng/mL and higher doses did not produce any further effect, hence the following experimental analyses were carried out using 200ng/mL resistin pre-incubation of washed platelets for 30 minutes before further testing.

The dose and time dependent reduction in aggregation upon stimulation with thrombin also suggested that platelets were likely not inhibited, but rather pre-activated. An assessment of these emerging hypotheses is carried out in the forthcoming sections.


0.025U/mL Thr
% Light transmission
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
Time (minutes)
1µg/mL Res
200ng/mL Res
50ng/mL Res
500ng/mL Res
Control

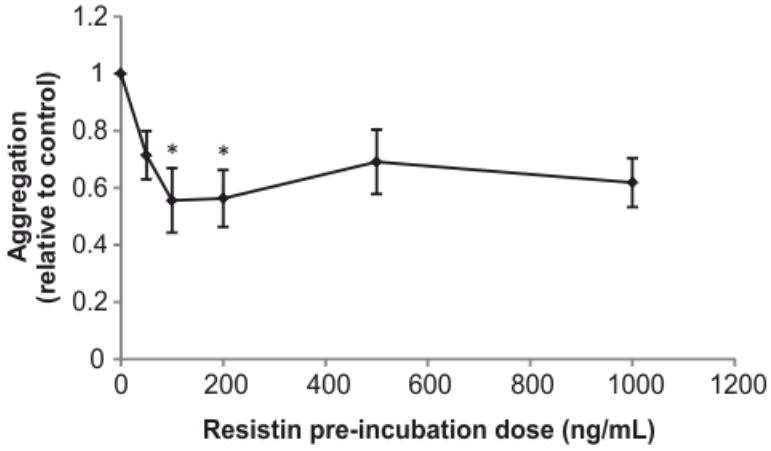

Aggregation
(relative to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
200
400
600
800
1000
1200
Resistin pre-incubation dose (ng/mL)

Figure 3.5 Dose response of resistin pre-incubation on thrombin related platelet aggregation: Human blood platelets, 2 x 10^8 /mL, were incubated with various doses of resistin for 30 minutes before assessing the effect on stimulation with 0.025U/mL thrombin. Aggregation of platelets in response to 0.025U/mL thrombin is used as control. Fold change in aggregation upon resistin pre-incubation was calculated with respect to aggregation control test in order to normalise data. Data are represented as mean ± standard error of mean of four independent experiments. Statistical significance is calculated using one-way ANOVA (*$p \leq 0.05$).

3.2.1.4. Validating recombinant human resistin activity

Recombinant human resistin was used in all experiments and it was important to validate the following listed assumptions that were made before investigating the role of resistin further. The assumptions were: (1) Recombinant human resistin was the only active compound that was received from the supplier and contained no other substances which might inhibit platelets; (2) Resistin itself did not react with thrombin or was not a substrate for thrombin protease activity. These assumptions were corroborated by (A) heat-inactivating recombinant human resistin at 95˚C for 5 minutes before incubating with washed platelets for 30 minutes at 37˚C and (B) by creating a pre-mix of thrombin and resistin, incubating for 10 minutes on ice and testing in aggregation with untreated platelets.

Doses of 0.025U/mL thrombin and 200ng/mL resistin that were recorded as significant in previous experiments were used in this validation experiment. The outcome, as seen in figure 3.6, confirms all the assumptions of the previous investigations, as there was no difference in aggregation when comparing the test samples to the control, whereas platelets that were treated for 30 minutes with active recombinant human resistin (200ng/mL) showed expected decrease in platelet aggregation when stimulated with 0.025U/mL thrombin.

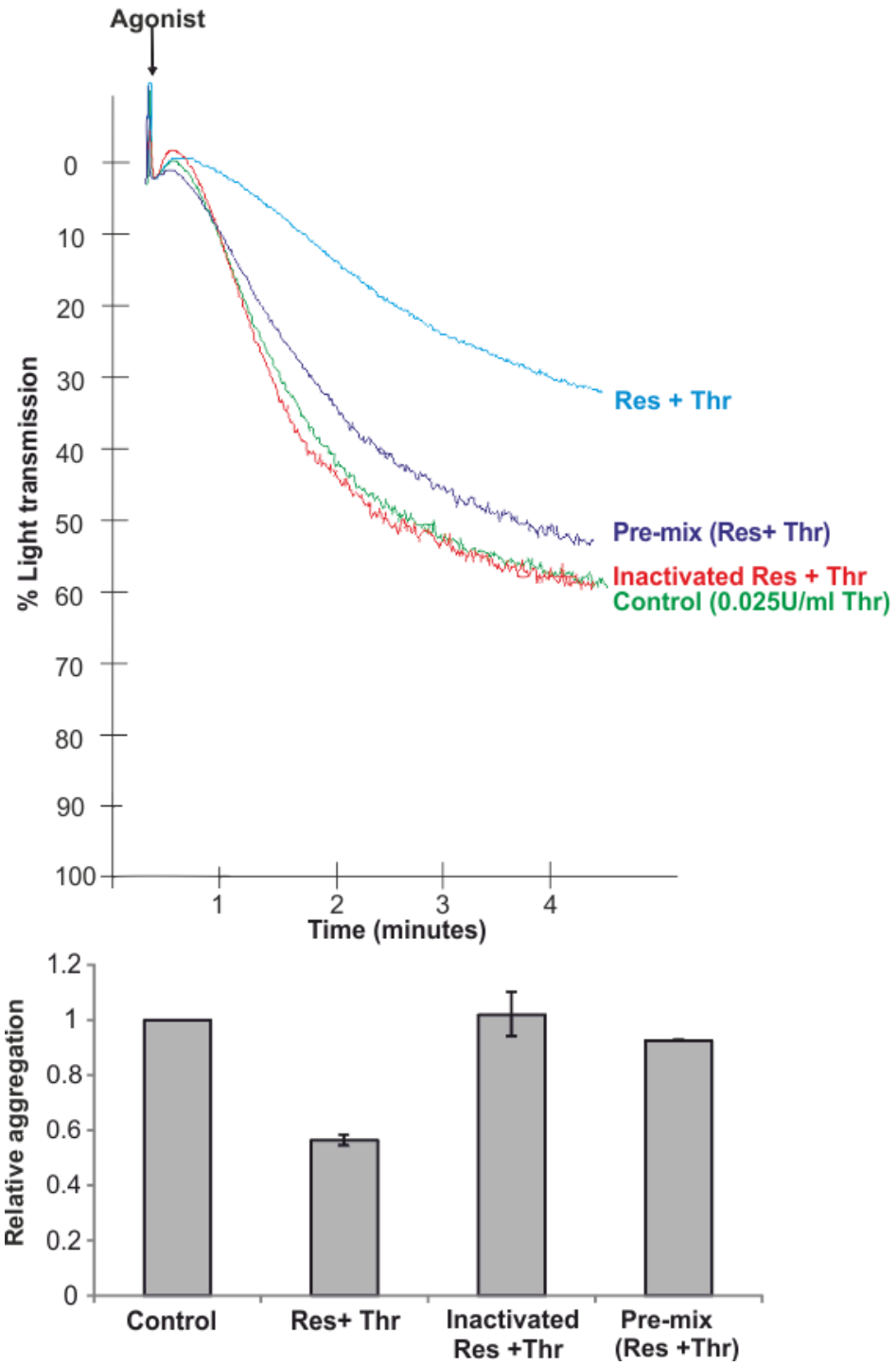


Figure 3.6 Effect of resistin inactivation and pre-mixing resistin and thrombin in platelet aggregation: Platelets were pre-incubated with resistin or heat-inactivated resistin (200ng/mL) for 30 minutes before assessing the effect on thrombin stimulation in aggregation. 0.025U/mL thrombin was incubated with

200ng/mL resistin for 10 minutes and the pre-mix was tested in untreated platelets. Aggregation of 0.025U/mL thrombin in untreated platelets was used as a control. Aggregation traces are representative of two independent experiments and data are represented as mean ± standard deviation of the mean of the same.

3.2.1.5 Effect of resistin on aggregation of indomethacin and apyrase inhibited platelets

In order to isolate effects of primary and secondary aggregation, platelets were inhibited using indomethacin and apyrase. These inhibitors prevent secondary aggregation by blocking two potent agonists secreted by platelet granules upon stimulation of primary aggregation: TXA_2 and ADP. Indomethacin inhibits cyclooxygenases, preventing the conversion of arachidonic acid to TXA_2 (Ruggiero and Lapetinat, 1986; Y. Qiu *et al.*, 2014), whereas apyrase degrades ADP (Damman *et al.*, 2012; Y. Qiu *et al.*, 2014).

Washed platelets were inhibited by treating them with 10µM indomethacin and 1U/mL apyrase for 10 minutes followed by incubation in the presence or absence of 200ng/mL resistin for another 30 minutes before examining the effect on aggregation with 0.1U/mL thrombin. Since platelets were inhibited using strong, irreversible inhibitors, 0.025U/mL thrombin showed no effect on aggregation (data not shown), hence a stronger dose of 0.1U/mL thrombin had to be used to reliably measure percent aggregation upon stimulation. In figure 3.7, similar to the observation from figure 3.2, there was no significant reduction in resistin-treated platelets when compared to control. Additionally, aggregation of indomethacin and apyrase inhibited platelets in response to thrombin did not vary significantly when compared to their resistin-treated equivalent samples. The only valid statistical significance, as would be expected, was seen between control and indomethacin and apyrase inhibited platelets ($p=0.059$, one-way ANOVA).

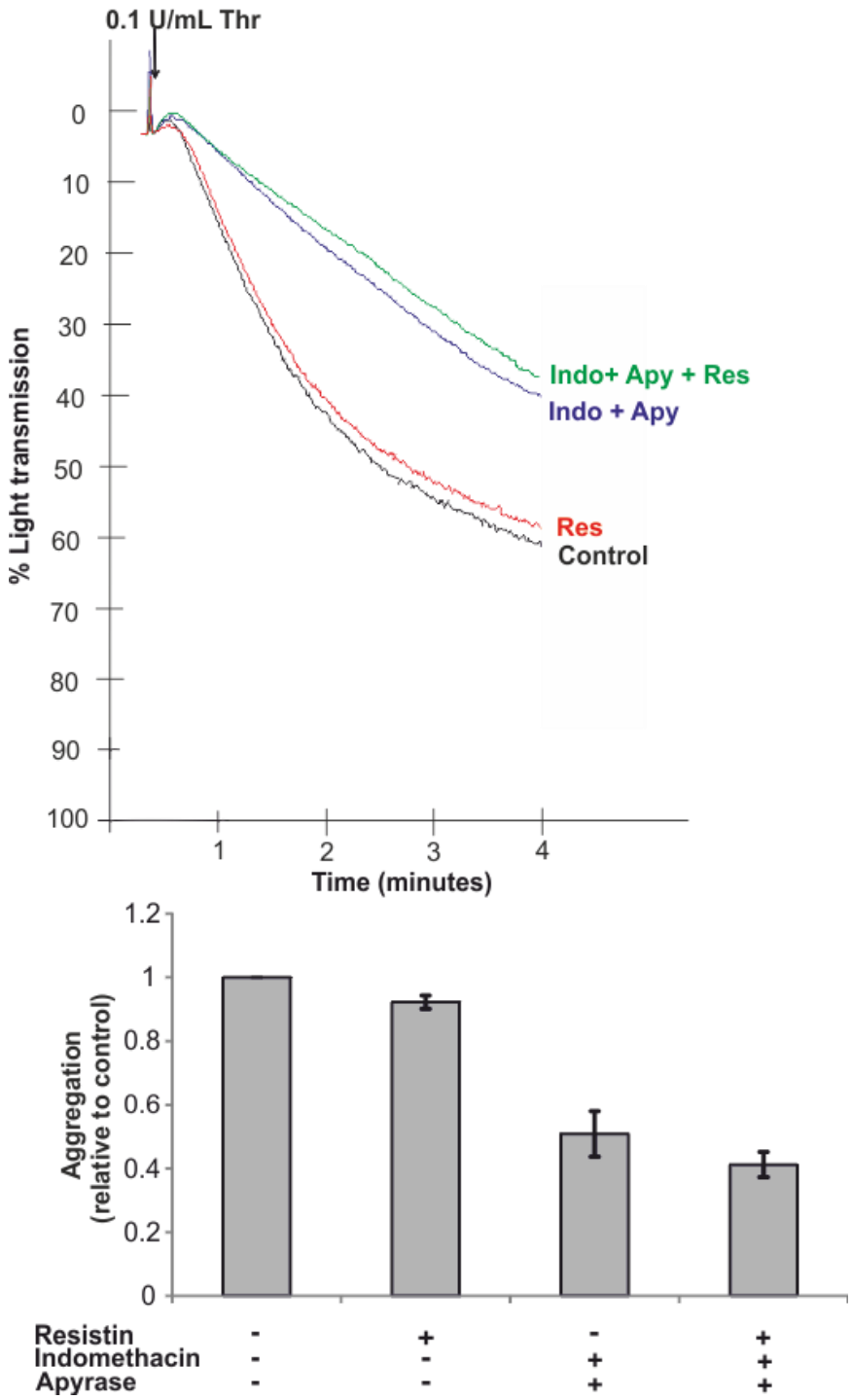


Figure 3.7 Effect of resistin on aggregation in indomethacin and apyrase inhibited platelets: Platelets were pre-incubated with indomethacin (10μM) and apyrase (1U/mL) in the presence or absence of 200ng/mL resistin for 30 minutes before assessing the effect on thrombin stimulation. 0.1U/mL thrombin

stimulation of untreated platelets was used as a control. Percent aggregation of tests were normalised to the percent aggregation of the control test in order to generate data representing a fold change compared to control. Aggregation traces are representative of three independent experiments and data are represented as mean ± standard error of the mean of the same. Statistical significance is calculated using one-way ANOVA but no significance was observed between control and resistin-treated groups.

In conclusion, there were no statistically significant differences between control and resistin-treated platelet aggregation and between inhibited control and inhibited, resistin-treated platelet aggregation. Forthcoming sections in this chapter will help shed more light on the underlying mechanisms of resistin effects.

3.2.1.6 Effect of resistin on granule secretion upon thrombin stimulation

The effect of resistin on reduction of platelet aggregation when stimulated with thrombin together with blunting of that effect upon apyrase treatment suggested that resistin might have an effect on granule secretion. When platelets undergo activation, they release their secretome, which is contained within its alpha (α) and dense granules. While dense granules release small signalling molecules like serotonin, ATP, ADP and polyphosphates (McNicol and Israels, 1999; Murugappan *et al.*, 2004), α-granules release the bulk of the secretome which includes hemostatic factors, cytokines, angiogenic and anti-angiogenic factors, proteases, and necrotic factors, which aid in augmenting the platelet response (Furie, Furie and Flaumenhaft, 2001).

An established method of assessing platelet secretion is by using lumiaggregometry where ATP release is measured along with aggregation (Flaumenhaft, 2003; Woulfe *et al.*, 2004a). In order to substantiate the hypothesis that resistin affects granule secretion, ATP release from 200ng/mL resistin-treated platelets was compared to untreated platelets after stimulation with thrombin. In figure 3.8, panel A shows representative secretion traces of ATP release during thrombin stimulation, where green traces correspond to resistin treatment and black traces are their respective controls. An internal control (ATP standard) to account for loss of luciferase-luciferin substrate activity was used for all samples. Panel B shows ATP release values that were normalised to the maximum dose of thrombin (0.1U/mL) to eliminate donor variations in total secretion. A statistically significant decrease in ATP release

was observed when comparing resistin treatment to untreated platelets. Since, ATP is released from dense granules, it can be concluded that dense granule secretion is negatively affected by treatment of platelets with resistin.

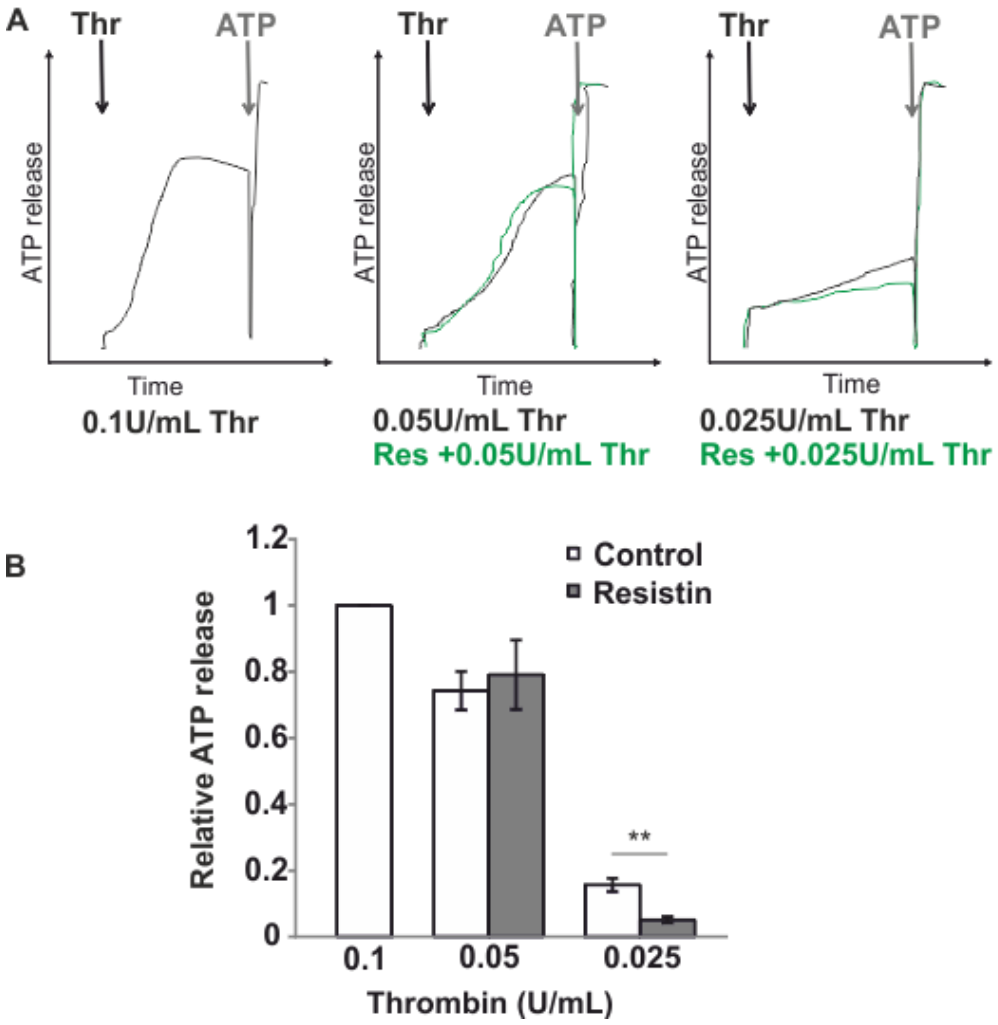


Figure 3.8 ATP release in resistin treated platelets: ATP release by 2.5 x 10^8/mL platelets upon stimulation with thrombin was tested in untreated and 200ng/mL resistin treated platelets. **(A)** Representative secretion traces – **control** and **resistin** are shown and arrows indicate the addition of thrombin and ATP standard (2nM). Traces were normalised to the effect of adding ATP to the control **(B)** ATP release was calculated using in gain in sample compared to gain in ATP standard and normalised to maximum thrombin dose (0.1U/mL). Values of ATP release used are relative to control and represent mean ± standard error of the mean of three independent experiments. Statistical significance is calculated using one way-ANOVA, where resistin treated samples were compared to the control (**p=0.009).

3.2.2. Effect of resistin on platelet spreading

Upon vascular injury, platelets are exposed to agonists in the subendothelial matrix which cause activation leading to shape change and adhesion to the subendothelium and to each other, forming a thrombus to regain haemostasis. Further evaluation of resistin effects on platelet function was therefore carried out using adhesion assays where fibrinogen was used as the activating matrix protein. Immobilised fibrinogen helped to investigate integrin $\alpha_{IIb}\beta_3$ activation more specifically (Parise, 1999; Payrastre, Missy and Trumel, 2000; Shattil and Newman, 2004).

3.2.2.1 Time dependence of effect of resistin on fibrinogen spreading

When clot formation begins, platelets are exposed to fibrin matrices and are activated by binding integrin $\alpha_{IIb}\beta_3$ (Qiu *et al.*, 2014). Adhesion of platelets to immobilised fibrinogen and the average surface area of the spread platelets have been used as parameters for assessment of integrin activation (Schachtner *et al.*, 2013). The effect of resistin pre-treatment of platelets was assessed by allowing them to spread on fibrinogen coated coverslips for variable lengths of time. Platelets were treated with 200ng/mL resistin for 30 minutes, as in previous experiments, followed by incubation on 100µg/mL fibrinogen-coated coverslips for 10, 20 and 45 minutes at 37˚C; all time points had respective controls without resistin pre-treatment. Time points indicate the length of time platelets were incubated before fixing with 4% PFA.

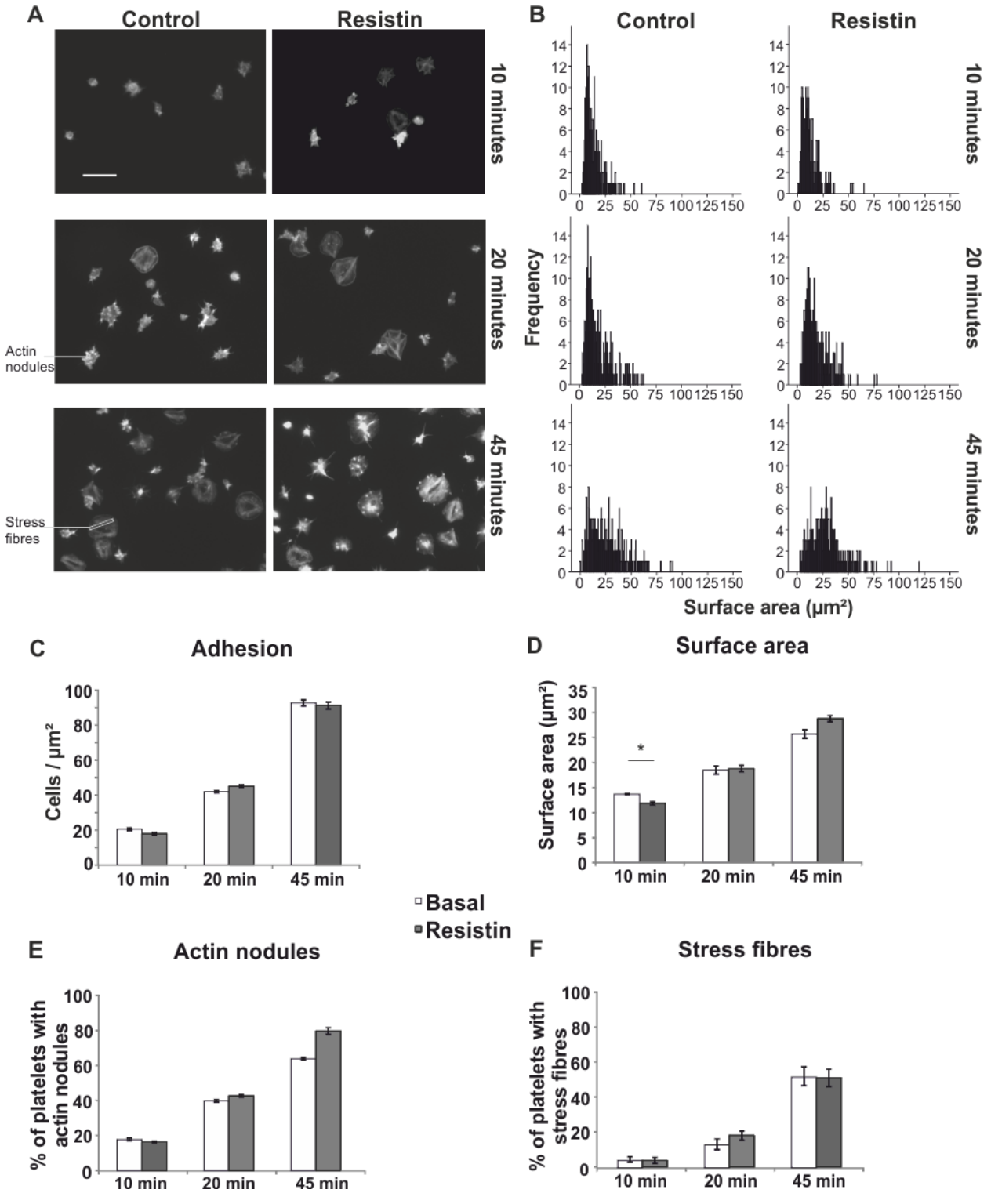


Figure legend on following page.

Figure 3.9 Time dependence of resistin treatment on platelet spreading: (Figure on previous page) Platelets were pre-treated for 30 minutes with 200ng/mL resistin and allowed to spread for 10, 20 and 45 minutes on 100µg/mL fibrinogen and stopped using paraformaldehyde. Untreated platelets allowed to spread under similar conditions were used as control for each time point. FITC-phalloidin was used for highlighting actin structures in platelets (actin nodules/stress fibres) using fluorescence imaging techniques. All comparisons between basal and resistin-treated outcomes were analysed using one-way ANOVA and LSD post-hoc correction. (**A**) Images are representative of three independent experiments.Actin nodules and stress fibres used for classification for platelets in (E) and (F) are labelled adjacent to the panel for control images for 20 minutes and 45 minutes, respectively. Scale bar =10µm. (**B**) Surface area (µm²) of spread platelets is represented as frequency (with 0.5µm² interval) of approximately 300 platelets per experimental condition. **(C)** Number of cells adhering to the fibrinogen matrix was recorded. Data represent mean ± standard error of the mean of three independent experiments across 5 fields of view (12,406µm²) per experiment and represented as cells observed per unit area (µm²) (**D**) Surface area of platelets is represented as mean ± standard error of the mean of 300 individual platelets across three independent experiments. (**E**) Spread platelets showing presence of actin nodules are represented as percent of total platelets adhered per image. Classification criterion for spread platelets was based as either platelets showing presence or absence of actin nodules. (**F**) Spread platelets showing presence of stress fibres are represented as percent of total platelets adhered per image. Classification criterion for spread platelets was based as either platelets showing presence or absence of stress fibres.

As seen in figure 3.9 (A), representative images of fixed platelets show an expected outcome – there is an increase in number of platelets attaching to the fibrinogen surface as the length of incubation on the matrix increases. No differences, however, were observed in the numbers adhering (per field of view) when platelets were pre-treated with resistin compared to controls (Figure 3.9 C). Similarly, surface area measurement, indicated that at 10 minutes the average platelet surface area was moderately low compared to the control (Figure 3.9 D). Statistical significance between surface areas of spread platelets was not observed at later time points.

During measurement of surface area it became apparent that although the average surface area of spread platelets was not different, platelets appeared to spread better, where the curve of surface distribution shifts to higher values, when pre-treated with resistin compared to their respective untreated controls. In order to quantitate this observation, a frequency distribution graph was generated using 0.5μm^2 as the interval width – number of platelets with their surface area within 0.5μm^2 range was considered as one group. Figure 3.9 (B) shows that at 20 minutes and 45 minutes, more number of platelets with surface area of 25μm^2 and over were present in the resistin-treated groups compared to their respective controls. Additionally, at 20 minutes, most platelets in the control group were smaller than 25μm^2, while the resistin treated showed the maximum surface area of spread platelets of more than 75μm^2.

The above observation meant that although the average surface area and number of cells adhering to fibrinogen was not altered, a resistin effect was

clear in the way platelets spread. In order to understand if there were any morphological differences upon resistin treatment, adhered platelets were further analysed and classified based on the presence of different cytoskeletal features – actin nodules and stress fibres (Calaminus, Thomas, McCarty, Machesky, & Watson, 2008; Schachtner et al., 2013).

Figure 3.9 E and F, show that there were no statistical differences in the proportion of platelets showing actin nodules or stress fibres. In Figure 3.9 E though, after 45 minutes of incubation on fibrinogen, a trend is observed, where more resistin treated platelets had actin nodules compared to their control– this was consistent with visual observation that platelets with large surface area, which normally would only display stress fibres, showed a high number of actin nodules. This observation was difficult to quantify but a representation can be observed in Figure 3.9 A.

In conclusion, resistin treatment had an apparently mild effect on platelet cytoskeleton as seen from the surface area distribution (Figure 3.9 B) and actin nodule data (Figure 3.9 E), but there was no effect on overall adhesion of platelets on the immobilised fibrinogen.

3.2.2.2 Dose dependence of effect of resistin on platelet spreading on fibrinogen

In order to assess the effect of elevated levels of circulating resistin on platelets it was important to identify if higher doses of resistin treatment augmented or altered the behaviour of platelets spreading on fibrinogen. Also, a trend showing that there were differences in number of actin nodules observed in resistin treated platelets after spreading on fibrinogen for 45 minutes, meant the investigation could reveal if resistin concentration affected their adhesive properties.

Platelets were incubated with 100ng/mL, 200ng/mL, 500ng/mL and 1µg/mL resistin for 30 minutes and were incubated on 100µg/mL fibrinogen coated coverslips for 45 minutes and compared to untreated platelets incubated under similar conditions. As in section 3.2.2.1, adhesion of platelets per field of view and surface area were measured as relevant parameters for integrin $\alpha_{IIb}\beta_3$ activation on fibrinogen.

In Figure 3.10 A and C, it can be observed that there was no statistically significant difference between the adhesion numbers of any dose of resistin treatment or when compared to untreated control. Also, no differences were observed in the surface area of spread platelets under all the different resistin treatments (Figure 3.10 D).

Although mean surface area of platelets across all treatment groups was not different, visual observation that all resistin doses of platelet treatment had more

surface area than control was quantified using a frequency distribution graph (where the interval width was 0.5μm^2 (Figure 3.10 B). Larger surface area (more than 75μm^2) of platelets was observed in all resistin treated samples compared to control – where maximum number of platelets with large surface area was seen at 200ng/mL resistin pre-treatment dose.

Adhered platelets were further characterised to identify cytoskeletal differences, as in section 3.2.2.1 and classified into cells showing actin nodules and/or stress fibres. No statistical differences in the proportion of cells displaying actin nodules or stress fibres were observed across all treatment groups (Figure 3.10 E and F). Although statistically not significant, a higher proportion of resistin treated platelets had stress fibres compared to control (Figure 3.10 F).

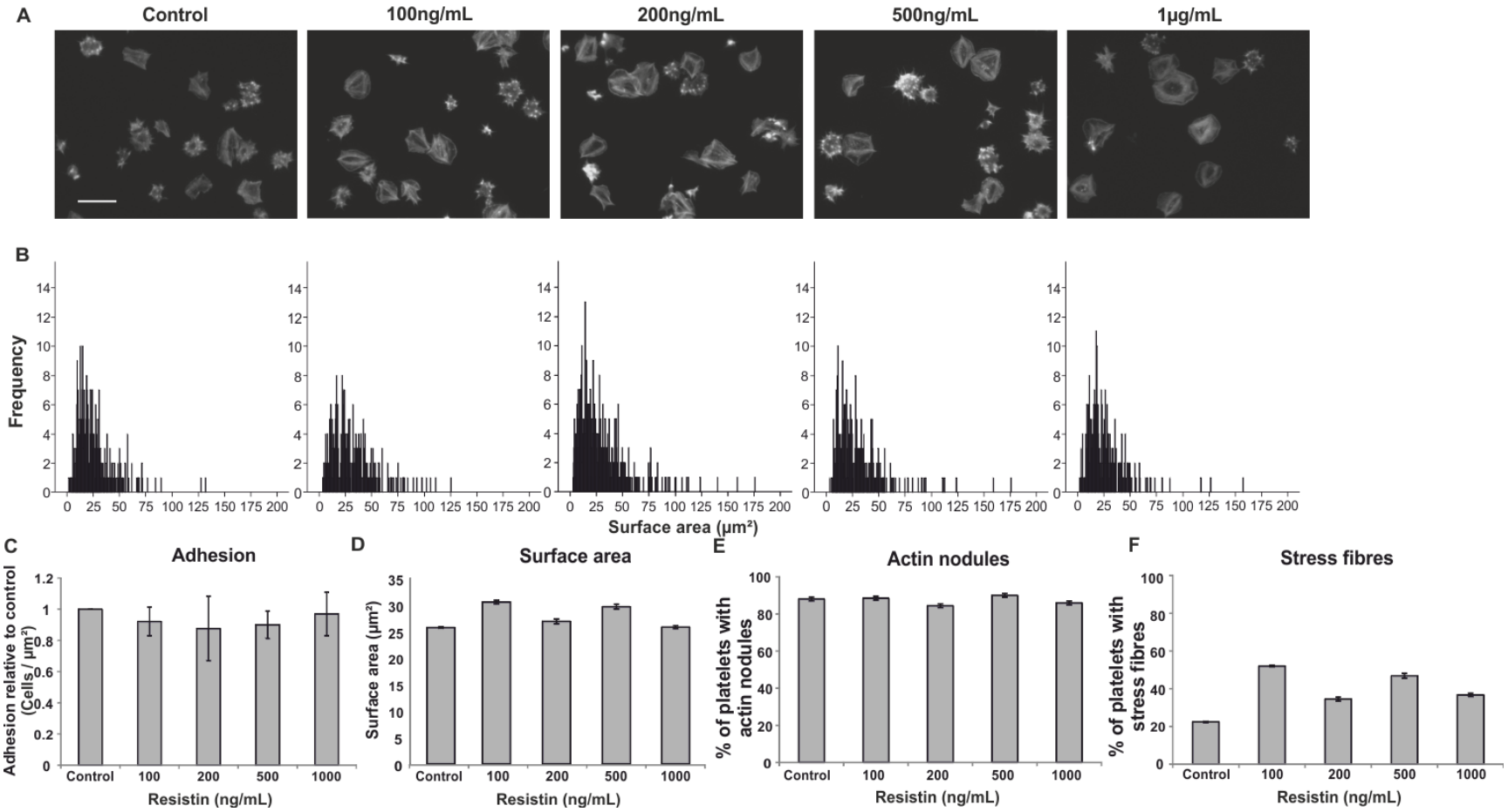

A
Control
100ng/mL
200ng/mL
500ng/mL
1µg/mL
B
Frequency
Surface area (µm²)
C
Adhesion
Adhesion relative to control (Cells / µm²)
Resistin (ng/mL)
D
Surface area
Surface area (µm²)
Resistin (ng/mL)
E
Actin nodules
% of platelets with actin nodules
Resistin (ng/mL)
F
Stress fibres
% of platelets with stress fibres
Resistin (ng/mL)

Figure 3.10 Dose dependence of resistin treatment on platelet spreading: Platelets were pre-treated for 30 minutes with various doses of resistin and allowed to spread for 45 minutes on 100µg/mL fibrinogen-coated coverslips and stopped using paraformaldehyde. Untreated platelets allowed to spread under similar conditions were used as control. FITC-phalloidin was used for highlighting actin structures in platelets (actin nodules/stress fibres) using fluorescence imaging techniques.(**A**) Images are representative of three independent experiments. Scale bar =10µm. (**B**) Surface area (µm²) of spread platelets is represented as frequency (with 0.5µm² interval width) of approximately 300 platelets per experimental condition. (**C**) Number of cells adhering to the fibrinogen matrix per unit area (µm²) is recorded relative to control, as different microscope and image-recorder was used for one set of data. This was performed in order to normalise all values. Data represent mean ± standard error of the mean of three independent experiments across 5 images per experiment. (**D**) Surface area of platelets is represented as mean ± standard error of the mean of 300 individual platelets across three independent experiments. (**E**) Spread platelets showing presence of actin nodules are represented as percent of total platelets adhered per image. Classification criterion for spread platelets was based as either platelets showing presence or absence of actin nodules. (**F**) Spread platelets showing presence of stress fibres are represented as percent of total platelets adhered per image. Classification criterion for spread platelets was based as either platelets showing presence or absence of stress fibres. Statistical significance between and within doses of resistin was calculated using one-way ANOVA, but no significance was observed.

3.2.2.3 Effect of resistin treatment on platelet spreading using inhibitors

Platelet activation upon adhesion onto immobilised fibrinogen can cause secretion of agonists such as TXA_2 and ADP, which in turn can lead to paracrine activation of neighbouring platelets (Qiu *et al.*, 2014). In order to narrow the reason for the observed differences of surface area distribution between resistin treated and untreated groups, was an effect of agonist secretion or resistin pre-treatment, platelets were treated with inhibitors like indomethacin and apyrase to block TXA_2 synthesis and degrade ADP, respectively.

The presence of inhibitors caused less total adhesion of platelets to immobilised fibrinogen compared to control but no statistical difference in the number of adherent cells was observed between inhibitor treated group and inhibitor + resistin treated group (Figure 3.11 A and D). When surface area of spread platelets was analysed, the inhibitor treated groups, in the presence or absence of resistin, did not show any significant variation (Figure 3.11 C). The surface area frequency distribution, on the other hand, showed that the most platelets in the resistin treated group were around the 25 μm^2 range (Figure 3.11 B), whereas the untreated (inhibitor) group included platelets that covered larger surface area.

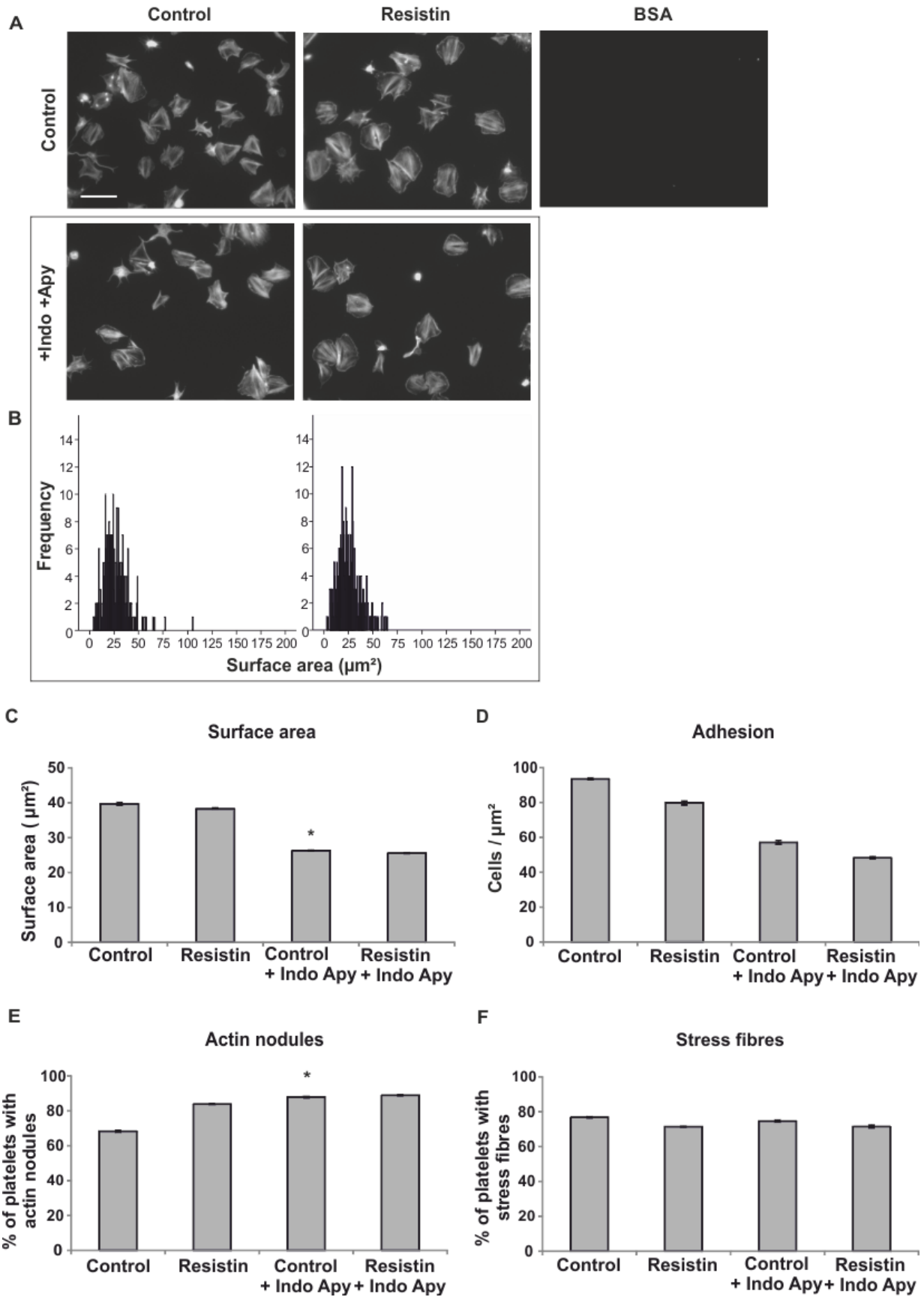

A
Control
Resistin
BSA
Control
+Indo +Apy
B
Frequency
Surface area (µm²)
C
Surface area
Surface area (µm²)
Control
Resistin
Control + Indo Apy
Resistin + Indo Apy
D
Adhesion
Cells / µm²
E
Actin nodules
% of platelets with actin nodules
F
Stress fibres
% of platelets with stress fibres

Figure 3.11 Effect of resistin treatment on platelet spreading using inhibitors: Platelets were pre-treated for 30 minutes with indomethacin (10µM), apyrase (2U/mL) in presence and absence of 200ng/mL resistin and allowed to spread for 45 minutes on 100µg/mL fibrinogen-coated coverslips and stopped using paraformaldehyde. Untreated platelets allowed to spread under similar conditions were used as control. FITC-phalloidin was used for highlighting actin structures in platelets (actin nodules/stress fibres) using fluorescence imaging techniques**. (A)** Images are representative of three independent experiments. Scale bar =10µm. **(B)** Surface area (µm²) of spread platelets is represented as frequency (with 0.5µm² interval width) of approximately 300 platelets per experimental condition. **(C)** Surface area of platelets is represented as mean ± standard error of the mean of 300 individual platelets across three independent experiments. **(D)** Number of cells adhering to the fibrinogen matrix is recorded relative to control, as different microscope and image-recorder was used for one set of data. This was performed in order to normalise all values. Data represent mean ± standard error of the mean of three independent experiments across 5 images per experiment. **(E)** Spread platelets showing presence of actin nodules are represented as percent of total platelets adhered per image. Classification criterion for spread platelets was based as either platelets showing presence or absence of actin nodules. **(F)** Spread platelets showing presence of stress fibres are represented as percent of total platelets adhered per image. . Classification criterion for spread platelets was based as either platelets showing presence or absence of stress fibres. Statistical significance was calculated using one-way ANOVA; significance of $p<0.05$ was observed when comparing means of control with indomethacin and apyrase treatment when measuring surface area and the percent of platelets with actin nodules, rest of the comparisons show no significance.

3.2.3. Effect of resistin on platelet signalling pathways

Resistin has been investigated in monocytic cell lines, endothelial cells, smooth muscle cells and adipocytes in some detail for its role in causing IR (Nagaev *et al.*, 2006; Manduteanu *et al.*, 2009; Cho *et al.*, 2011). While there is still much speculation about a specific receptor that binds resistin (Tarkowski *et al.*, 2010; Lee *et al.*, 2014; W. Qiu *et al.*, 2014), downstream events are better characterised. Evidence from smooth muscle cells and endothelial cells indicates that resistin induces adhesion between the cells via an ERK1/2 MAPK dependent pathway (Calabro *et al.*, 2004). Evidence from adipocytes indicated that resistin play a role in SOCS3 activation (inhibitor of insulin signalling) by phosphorylation of insulin receptor and substrate, activation of PI3K, which subsequently phosphorylates and activates Akt (which is required for stimulation of glucose transport) (Steppan *et al.*, 2005).

Recombinant human resistin was tested in human umbilical vein endothelial cells (HUVEC) to reiterate evidence from literature and to assess if resistin potentiated similar effects under present laboratory conditions. It can be seen from Figure 3.12 that resistin caused phosphorylation of PI3K/Akt and MAPK, ERK1/2.

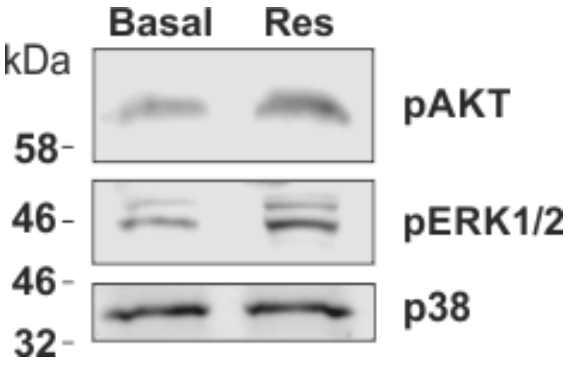


Figure 3.12 Effect of recombinant resistin in HUVEC lysates: Human umbilical vein endothelial cells were treated with 50ng/mL resistin for 15 minutes and phosphorylation of Akt and ERK1/2 was compared to untreated basal. Lysates of the above conditions were prepared using RIPA buffer and equal amounts of protein were loaded on 12% SDS-PAGE prior to immunoblotting using the indicated antibodies. p38 is the loading control. Image is representative of one duplicate of the experiment.

With confidence that recombinant human resistin affected signalling in human cells, it was further tested in human blood platelets to understand if there were similarities in signalling effects of resistin. Phosphorylation of PKC substrates in relation to signal transduction events like adhesion, secretion and cell migration upon external stimulation has been previously noted in literature (Newton, 1995; Murugappan *et al.*, 2004; Cimmino and Golino, 2013) and from our observations can be extended to stimulation by resistin.

In order to assess resistin effect in platelets, platelets were treated with 200ng/mL resistin for 30 minutes. A positive marker for PKC activation is phosphorylation of its substrates at Ser residues surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position on PKC substrates. PKC substrate phosphorylation levels upon resistin treatment were compared to basal levels in platelets. Interestingly, three independent experiments showed an increase in total PKC substrate phosphorylation in response to resistin stimulation. Figure 3.13 represents an array of proteins that were phosphorylated at Ser residues on PKC substrates more than in the basal platelet lysates.

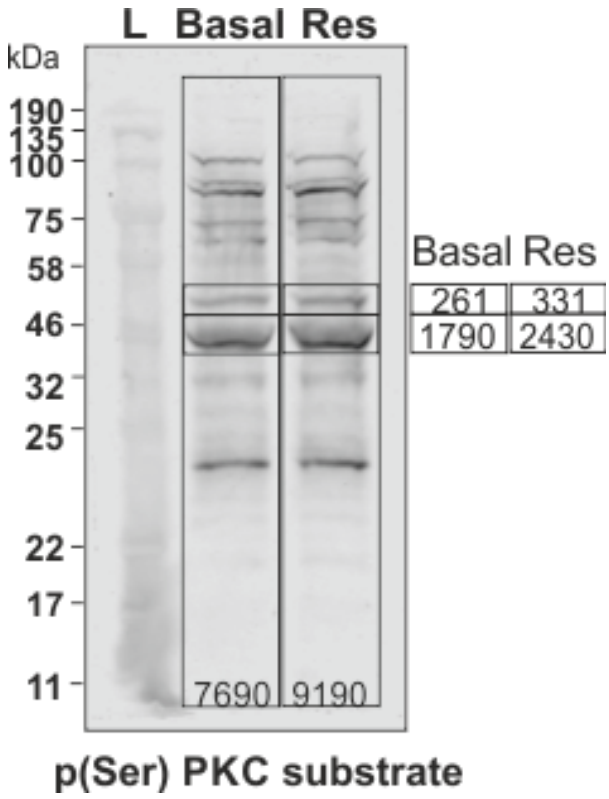


Figure 3.13 Phosphorylation of PKC substrates: PKC dependent serine phosphorylation in basal and resistin treated (200ng/mL - 30 minutes) platelet whole cell lysate (8 x10^8 /mL) was identified by immunoblotting using a phosphor-serine PKC substrate antibody. Ladder is indicated by (L) in the image. Densitometric measurements were carried out to observe change in phosphorylation. Values for the whole lane are indicated at the bottom of the respective lanes. The values in the adjacent boxes indicate respective locations of the rectangles identified in the lanes –these are specific proteins that would potentially be of interest as they show maximum change in phosphorylation upon resistin treatment.

Further, involvement of resistin in PKA and PKG activation was evaluated to underscore the effect of resistin on thrombin induced aggregation. Increased PKA/PKG activity due to resistin treatment would support the argument in favour of platelet inhibition as opposed to platelet pre-activation.

Phosphorylation of VASP at two distinct serine residues 157 and 239 were used as markers of PKA and PKG activation respectively. Known PKA and PKG activators, PGI_2 and S-nitrosoglutathione (GSNO), respectively, were used as positive control to assess relative platelet activation. Basal and 200ng/mL resistin treated platelets were compared using one-way ANOVA. The phosphorylation of VASP at both serine residues 157 and 239 are statistically significant ($p<0.05$) when comparing basal to PGI2. All other comparisons are not significant. As the aggregation traces and relevant protein kinase inhibitors indicating these compounds were functionally competent are lacking, the tentative conclusion drawn from the graphs in Figure 3.14, is that resistin most likely does not have an effect on PKA or PKG activation.

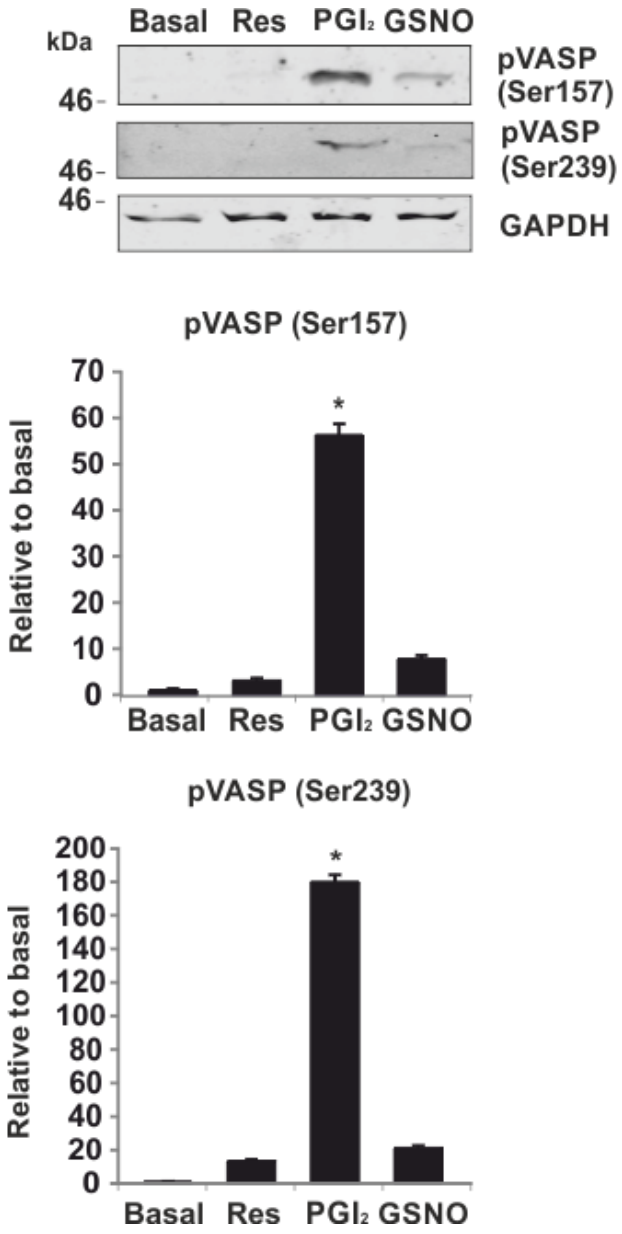


Figure 3.14 Effect of resistin treatment on PKA and PKG activation of platelets: Whole cell lysates of 8 x 10^8 /mL platelets stimulated using 200ng/mL resistin (30minutes) (Res), 100µM prostaglandin I_2 (5 minutes) (PGI_2) and 20µM S-Nitrosoglutathione (20 minutes) (GSNO) were tested using immunoblotting for phosphorylation of VASP Serine 157 and Serine 239. Lysates containing equal total protein were loaded on 12% SDS-PAGE before immunoblotting using the indicated antibodies. Densitometry data are normalised first, to loading control (GAPDH) and then calculated relative to basal (untreated) whole cell lysates. Data represent mean ± standard error of the mean of three independent

experiments. Statistical significance is calculated using one-way ANOVA (*$p<0.05$).

As it was noted in smooth muscle cells and endothelial cells (Calabro *et al.*, 2004) that resistin stimulation caused phosphorylation of PI3K/Akt and MAPK, a similar test was carried out using platelets. Phosphorylation of Akt, as a marker for PI3K activation (Steppan *et al.*, 2005), and phosphorylation of ERK1/2, as indicator for MAPK activation, were investigated in platelets treated with in 200ng/mL resistin for 30 minutes. As seen in Figure 3.15, phosphorylation of Akt at Ser473 was significantly increased ($p<0.001$). Phosphorylation of ERK1/2 was also significantly increased ($p<0.01$) at only one site of phosphorylation. Unfortunately, the site of phosphorylation could not be positively identified as being either threonine 202 or tyrosine 204 in this preliminary assessment. Despite this, it can be convincingly deduced that downstream effectors of PI3K and MAPK are involved in stimulation of platelets in response to resistin. As resistin itself did not stimulate or cause aggregation of platelets, it can be implicated to have an effect on PI3K and MAPK pathways.

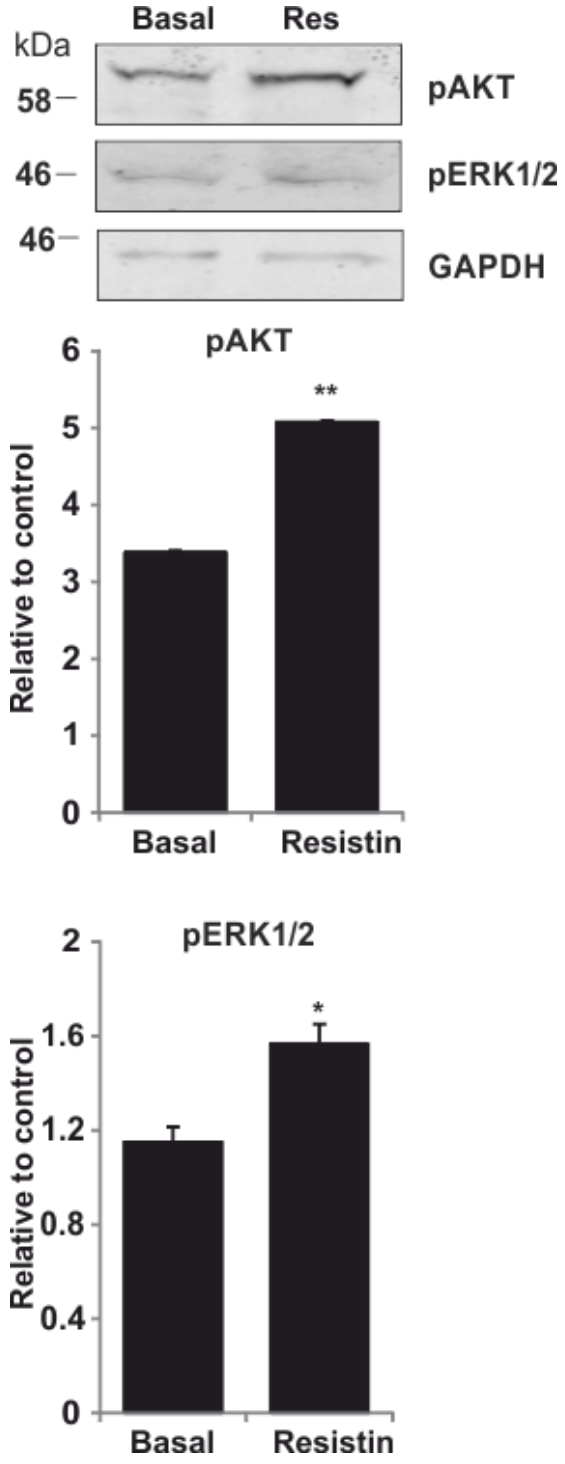


Figure 3.15 Activation of PI3K and MAPK: Phosphorylation of AKT and ERK1/2 in basal and resistin treated (200ng/mL - 30 minutes) platelet whole cell lysate (8 x10^8 /mL) was measured by loading equal proteins (loading control GAPDH) on 12% SDS-PAGE and tested using immunoblotting for the indicated

antibodies. Densitometry data are normalised to loading control and represented as mean of three independent experiments ± standard error of the mean. Statistical significance was calculated using one-way ANOVA (*p≤0.01 and **p≤0.001).

3.3 Discussion

Resistin, like other adipokines including leptin and adiponectin, increases the risk of thrombosis in patients with T2DM (Bełtowski, 2003; Kato *et al.*, 2006). Adiponectin stimulates fatty acids oxidation, decreases plasma triglycerides, and improves glucose metabolism by increasing insulin sensitivity. In platelets, adiponectin has a dual role; globular adiponectin induces platelet aggregation via GPVI-FcRγ interaction but not the full-length adiponectin (Riba *et al.*, 2008), whereas full-length adiponectin inhibits platelet aggregation and activates eNOS to attenuate its response to oxidative stress (Wang *et al.*, 2011). Leptin, another adipokine, described to play an important role in atherogenesis in obese patients with T2DM, has been revealed to elevate platelet aggregation responses via interactions with specific leptin receptor found in platelets (Konstantinides *et al.*, 2001).

In non-platelet cell models like monocytes and macrophages, leptin and resistin induce procoagulability in T2DM by upregulating PAI and t-PA, disturbing the balance of the fibrinolytic system (Bobbert *et al.*, 2011). Additionally, full-length adiponectin inhibits resistin mediated induction of ICAM-1 and VCAM-1 in the vascular endothelial cell model (Kawanami *et al.*, 2004). Together, above evidence indicates a potential role for adipokines in regulating each other *in vivo* or in disease states.

3.3.1 Resistin treatment causes a thrombin dependent reduction in platelet aggregation

A prothrombotic role for resistin has been hypothesised owing to elevated levels found in circulation of patients suffering from metabolic syndrome (Jamaluddin *et al.*, 2012; Lee *et al.*, 2014; Huang and Yang, 2015). Interestingly, we observed that resistin pre-treatment caused attenuation of platelet aggregation when stimulated via the GPCR targeting agonist thrombin, but not significantly when stimulated with the tyrosine kinase-activating agonist collagen. This inhibitory effect of almost 30% of the untreated control was most pronounced when stimulation was carried out with 0.025U/mL dose of thrombin (Figure 3.2). The results suggested that resistin might affect the G-protein coupled receptor pathway that leads to integrin $\alpha_{IIb}\beta_3$ activation and PLC-related activation of PKC leading to $\alpha_{IIb}\beta_3$ activation (Li *et al.*, 2010). The observation that endothelial cells and smooth muscle cells signal via TLR-4 and $G_{i/o}$ pathway in response to resistin helps substantiate the claim that a GPCR pathway is affected in platelets (Pirvulescu *et al.*, 2014).

We report that as dose and time of resistin pre-treatment increased, the platelet aggregation reduced significantly (Figure 3.4 and 3.5). The experiments helped to identify 30 minutes and 200ng/mL resistin doses as ideal for all experimental purposes. Under physiological conditions, 0.1U/mL-10U/mL thrombin concentrations are present at the site of a haemostatic plug (Angiolillo et al. 2010), whereas lower doses seem to make platelets prone to activation without causing aggregatory effects. *In vitro* however, since platelets are devoid of

plasma containing clotting factors and they are not subjected to circulation where agonists undergo continuous dilution, 0.025U/mL thrombin is sufficient to cause significant aggregation even in untreated platelets, as seen in Figure 3.2. Hence, it makes for a very interesting observation that only 0.025U/mL thrombin stimulation caused reduction of aggregation of resistin pre-treated platelets whereas higher and lower doses of thrombin caused minimal inhibition.

These observations therefore led to the preliminary conclusion that platelets were inhibited by resistin and that the inhibition levels increased with higher doses and longer incubation times. On prolonged resistin exposure platelets were basally inhibited compared to untreated control. This inhibition, therefore, caused them to aggregate less and not reach similar aggregation levels as untreated controls post thrombin stimulation.

3.3.1.1 Resistin negatively affects platelet dense granule secretion.

Since a higher dose of thrombin, 0.1U/mL instead of 0.025U/mL had to be used to assess detectable difference in platelet aggregation with the secondary aggregation inhibitors, it wasn't sufficient to make conclusions about the effect of resistin on secondary platelet responses. Further analysis of dense granule secretion was carried out to clarify the effect of resistin.

It was observed that 200ng/mL resistin caused a significant decrease in ATP release when stimulated with 0.025U/mL thrombin, which suggested that dense granule secretion was attenuated by resistin treatment. Although not

investigated in this chapter, α-granule secretion by platelets, using P-selectin (released from α-granules on platelet activation) was carried out by Qiu *et al*., (2014). They show a concentration dependent increase in P-selectin expression after resistin treatment of platelets. The P-selectin expression was further enhanced upon resistin treatment of platelets with subsequent thrombin stimulation. Their study lacks key controls making it difficult to make definitive conclusions. If their conclusions are assumed to be true and correct, then it would suggest that that resistin might differentially affect alpha and dense granule secretion. The suggestion that granular systems are uniquely affected by chemical agents has been previously shown in literature with regards to the actin cytoskeletal inhibitors like cytochalasin E (Flaumenhaft *et al.*, 2005).

The inhibitory effect of resistin was further substantiated by examining the results in presence of secondary aggregation mediators like TXA_2 and ADP (Flaumenhaft, 2003) as discussed ahead. The caveat here is that a higher dose of thrombin was used, thus making an unbiased conclusion difficult (see section 3.2.2). In order to circumvent the issue of incomparable data, owing to difference in agonist concentration, another experimental set up could be used to give more definitive conclusions. An investigation into P-selectin expression in response to thrombin receptor agonists, which is a platelet marker for procoagulability, could be assessed using FACS. Experimental set up would include positive controls for the thrombin receptor agonists, in addition to independent controls for each inhibitor (resistin, indomethacin, apyrase), before using a combination of the inhibitors in tests. The increase or decrease in P-

selectin exposure in platelets in response to inhibitors would allow for unbiased conclusion in understanding if platelets are truly inhibited in the presence of resistin. This would also shed light on the effect resistin has on secondary activation mediated by α-granule secretion in platelets, either supporting or rejecting the data presented by Qiu *et al* (2014) and the hypothesis of differential regulation of α- and dense granule secretion by resistin (Flaumenhaft *et al.*, 2005).

3.3.3 Resistin affects integrin $\alpha_{IIb}\beta_3$-mediated surface area distribution of platelets

When investigating the effects of resistin on platelet adhesion on immobilised fibrinogen, it was observed that there were no apparent differences between resistin treated and control platelets. Similarly, there were no significant differences in the average surface area of platelets between resistin pre-treatments and control. Remarkably though, when frequency distribution of surface area was plotted, more resistin-treated platelets had larger surface area when spread on immobilised fibrinogen compared to their respective controls, suggesting that resistin affected platelet cytoskeletal proteins. There was lack of any obvious difference between the experimental groups in their platelet morphology markers- number of platelets displaying actin nodules and/or stress fibres. Nevertheless, visual observation indicated that resistin-treated platelets contained more number of actin nodules than their respective controls. It was an observation that was very difficult to quantify owing to the fact that there were

large number of actin nodules present per platelet. Additionally,these actin nodules lay in different planes and not always clearly visualised, thus introducing error due to the lack of accuracy in identifying spots as actin nodules from background noise.

Previous research by Calabro *et al*. (2004), has shown that human aortic smooth muscle cell proliferation is promoted by resistin via activation of ERK1/2 and PI3K pathways, which suggests that the cytoskeletal differences in resistin-treated platelets could be owing to activation of similar pathways in platelets. The reason for the derivation being that cell proliferation pathways coincide with cytoskeletal pathways (Parise, 1999; Barry *et al.*, 2003; Woulfe *et al.*, 2004a; Li *et al.*, 2010; Senis, Mazharian and Mori, 2014).

Further, in the experiment where secondary aggregation was blocked using inhibitors like indomethacin and apyrase, the frequency distribution of surface area highlighted relatively large size of platelets when compared to similarly inhibited platelets that were also treated with resistin. Pre-treatment with inhibitors obstructed the granule secretion and therefore explains the lack of higher surface area platelets in the resistin treated groups. This corroborates the previous observation from aggregation and secretion data (section 3.2.1.6) that resistin affects platelet granule secretion, which consequently causes platelets to spread more and have larger surface area in the resistin treated groups when compared to control, as seen in the sections, 3.2.2.1 and 3.2.2.2.

Platelet cytoskeleton is responsible for transporting granules to the centre of platelet during stimulation, where the secretome is released. As granule secretion is tightly regulated by the platelet cytoskeleton (Flaumenhaft, 2003; Murugappan *et al.*, 2004; Woulfe *et al.*, 2004a; Flaumenhaft *et al.*, 2005; Zelen, 2012), it was important to understand if it was the cytoskeleton that was affected by resistin treatment. CAP1, which is an actin binding protein (Moriyama and Yahara, 2002; Jansen *et al.*, 2014), was recently shown to be a receptor for resistin (Lee *et al.*, 2014) and its presence and function in platelets will be investigated in the following chapter 4.

3.3.5 Resistin affects downstream effectors of PI3K and MAPK pathways

PKC substrate activation occurs downstream in platelets undergoing GPCR-related stimulation (Parise, 1999; Murugappan *et al.*, 2004; Watson *et al.*, 2005; Cimmino and Golino, 2013). Evidence of elevated PKC substrate phosphorylation at serine residues was observed in resistin-treated whole cell lysates compared to basal platelet lysates thus introducing a contradiction to the observation that resistin inhibits platelets. Interestingly, a study investigating a downstream effector of PKC, PKD2, showed a similar inhibition pattern of differential granule secretion in its gene knock-in mouse model. They show that dense granule release and secretion is affected but not α-granule secretion (Konopatskaya *et al.*, 2011). It may be that resistin alters phosphorylation sites downstream of PKC activation as an inhibitory mechanism in platelets, as yet undefined.

Additionally, the minimal phosphorylation upon PKA and PKG activators (PGI_2 and GSNO, respectively) further substantiates the lack of activation of the traditional platelet inhibition pathways, suggesting that alternative inhibitory mechanisms in platelets were activated in response to resistin exposure of platelets.

Due to constraints in the availability of inhibitors of the respective AGC kinases, confirmation of resistin activity was sought by investigating its known activators - PI3K effector protein Akt and MAPK effector protein, ERK1/2 (Calabro *et al.*, 2004). Statistically significant phosphorylation of Akt and ERK1/2 in resistin treated whole cell lysates was observed on comparison with control. This meant that the mechanism of action of resistin clearly involved activation of PI3K and MAPK in platelets. Although enhanced phosphorylation of ERK1/2 was observed in smooth muscle cells (Calabro *et al.*, 2004) contradicting data was shown in platelets (Qiu *et al.*, 2014), which suggested that p38 MAPK was phosphorylated in response to resistin and not ERK1/2 by assessing P-selectin expression using their respective inhibitors. The lack of immunoblotting data supporting the observation by Qiu *et al.*, (2014), that resistin treatment of platelets caused selective phosphorylation of p38 instead of ERK1/2 meant that firm conclusions cannot be sought in the literature for identifying which or if multiple MAPK proteins' phosphorylation affected resistin activity in platelets. Evidence by Flevaris *et al.*, (2009) show that phosphorylation of p38 and ERK1/2 in platelets is transient in nature owing to its negative regulation by integrin outside-in signalling. Put together, these observations conflicts with the

results from Qiu *et al*. (2014) where they only assessed platelets for P-selectin expression.

Phosphorylation of Akt is mediated by PI3K and evidence only adds strength to the evidence from literature relating to resistin activity (Steppan *et al.*, 2005). Another interesting observation from platelets isolated from Akt2 deficient mice was that they present an agonist-specific defect (G_q and G_i receptor binding–like thrombin) in platelet aggregation, fibrinogen binding and secretion (Woulfe *et al.*, 2004a), all of which were overcome at higher agonist concentrations, similar to observations from resistin-treated platelet aggregation (Figure 3.2). Further, another research group showed that Akt2 deficient mice present T2DM like IR and impaired glucose homeostasis (Cho *et al.*, 2001).

All the evidence together with observations from resistin-treated platelets suggests that resistin causes attenuation of platelet aggregation and dense granule secretion response. Prolonged resistin exposure also affects the platelet cytoskeleton by causing them to spread more than control platelets. Biochemical signalling data however, contradicts the inhibition hypothesis in response to resistin. Although resistin inhibits platelets, it can be inferred that it likely occurs downstream of GPCR-related agonist activation, PKC activation, and is mediated by Akt/PI3K and tentatively by ERK1/2 MAPK. This also suggests that alternative, as yet undefined platelet inhibitory pathway must be activated by resistin exposure, thus dampening the platelet aggregation and secretion responses to thrombin.

3.3.6 Extension of the study of resistin effect on platelet function

Resistin levels in circulation vary greatly between individuals. Further, the observations made in this chapter that prolonged exposure to high doses of resistin affects platelet function makes it necessary to step back and look at the circulating resistin levels in the healthy blood donors prior to blood donation. In order to facilitate the analysis and normalise the overall resistin exposure *in vitro*, an estimation of serum resistin levels using ELISA kits that are commercially available, prior to experimentations would have improved the credibility of the results. Once this estimation was carried out, it would provide ideal conditions to study effect of thrombin in resistin treated plasma using thrombin receptor agonists, PAR1 or PAR4 to isolate the potential downstream pathways that are triggered.

Insulin is known as a platelet inhibitor (Trovati *et al.*, 1997; Santilli *et al.*, 2016). Since resistin is believed to work antagonistically to insulin, a direct comparison of the two (insulin and resistin) in platelet aggregation would improve the conclusions of the experiments, thus providing a clearer context to resistin effects on platelet activity.

Limitations of this chapter in elucidating the complete mechanism of action of resistin on platelets are owing to lack to negative controls to give more confidence in the data obtained. Rosiglitazone, a TZD and a generic PPARγ inhibitor, known to sensitise cells to insulin, has been previously used as a resistin inhibitor by Steppan *et al.* (2001), in experiments to aid more robust conclusions, although, admittedly, it is not a resistin-specific inhibitor.

Since resistin plays a key role in glucose metabolism (Nogueiras *et al.*, 2009), glucose enriched media, glucose analogues, or use of sugars not recognised by insulin receptors, to study aggregation, adhesion, and secretion in platelets in the presence and absence of resistin would have enabled a better understanding of its role in glucose homeostasis with reference to thrombosis.

The observation that platelet cytoskeleton was affected when platelets were exposed to resistin meant that interesting findings can be sought by depolymerising actin using various cytoskeleton disrupting agents like latrunculins and cytochalasins to assess if resistin effects were altered in any way.

CHAPTER 4

CHARACTERISATION OF CAP1 IN THE PLATELET CYTOSKELETON

In chapter 3, when platelets were exposed to resistin, a cytoskeletal effect was observed owing to mild activation of platelets. Actin rearrangement drives platelet activation by receptor mediated signalling mechanisms. Platelets change shape when they adhere to blood vessel wall and spread by flattening at the site of injury. In this process, platelets are required to reorganise their cytoskeleton to accommodate for the shape change. Membranes of activated platelets undergo changes in lateral distribution of glycoprotein and integrin receptors to achieve directional spreading (Li *et al.*, 2010). Actin assembly/disassembly and proteins involved therein during this reorganisation elicit a number of physiological responses of platelets like formation of filopodia and lamellipodia and granule release including signal transduction pathways with active participation of actin associated proteins (Hartwig, Steffen and Cell, 1992). This process of receptor redistribution then triggers inside-out signalling pathways which change the conformation of major platelet integrins like $\alpha_{IIb}\beta_3$, all of which act in a paracrine manner to help recruit more platelets to the site of repair (Shattil and Newman, 2004).

One of the major functions of CAP1 lies in actin rearrangement and turnover, details of which can be found in section 1.5 of the introduction (Figure 1.7).

In a recent study, CAP1 was identified as a receptor for resistin (Lee S *et al.*, 2014). In CAP1 knockdown and overexpression monocyte cell models, the authors showed that the effects of resistin - NF-ƙB mediated cytokine expression and PKA activation were modulated by CAP1. They also showed that the SH3-binding domain (P1 domain) (Figure 1.8) of CAP1 was responsible for binding resistin. The main criticism of this publication is that CAP1 is not a transmembrane protein and is therefore unlikely to bind resistin under physiological conditions as resistin binds receptors on the plasma membrane. Alternatively, internalisation of resistin by cells has not been reported. Most importantly, a possible role of CAP1 in activation of adenylyl cyclases in mammalian cells has not been elucidated yet (Ono, 2013; Zhou, Zhang and Field, 2014).

In the light of our evidence that resistin affects the platelet cytoskeleton, in addition to the potential link between resistin and CAP1, as a resistin receptor, along with CAP1 playing a major role in actin rearrangement, we aim to investigate the role of CAP1 in platelet biology. In this chapter, we characterise CAP1 subcellular localisation in resting platelets and in platelets stimulated by activation of distinct signalling pathways, as an initial step in establishing the role of CAP1 in platelet actin dynamics and how it might be affected in the presence of resistin.

4.1 Aims and objectives

CAP1 is an important actin regulatory protein and yet there are no reports investigating CAP1 in human platelets (Gieselmann and Karlheinz, 1992). A lack of information on the presence and distribution pattern of CAP1 in human platelets therefore prompted us to initially investigate these aspects. The current chapter therefore aims to establish the presence of CAP1 in human blood platelets and examine the effect of agonist stimulation on the subcellular localisation of CAP1.

The objectives are:

1. Characterise the localisation and distribution of CAP1 in human platelets using immunofluorescence and multiple subcellular fractionation approaches

2. Investigate the dynamics and actin dependence of CAP1 localisation upon thrombin and collagen stimulation

3. Investigate the dynamics of CAP1 localisation upon prostacyclin treatment and assess how it affects the changes elicited by subsequent thrombin stimulation

4. Preliminary assessment of the effect of resistin on CAP1 distribution

4.2 Results

4.2.1. CAP1 in resting platelets

4.2.1.1 Characterisation of CAP1 antibodies

Prior to collecting data using the anti-CAP1 (rabbit monoclonal -ab133655, Abcam, UK) antibody it was important to characterise it for its specificity towards CAP1 in human platelets.

In order to test if the antibody could identify the presence of CAP1 in human platelets and be reliably used to quantitate a range of CAP1 concentrations, varying amounts of whole platelet lysates of known total protein concentration were immunoblotted for CAP1. CAP1 protein is ~55-60 kDa and a clear band at 56 kDa is observed in the blot presented in Figure 4.1 A indicating that the antibody was highly specific to CAP1. Additionally, it was observed that an increase in protein concentration was accompanied by a proportional increase in band density.

GAPDH, which is a cytosolic marker, was used as a loading control and proportional variations were observed in the control as well, indicating that there was no loading error. We conclude that anti-CAP1 antibody was a reliable tool to study CAP1 in human platelets.

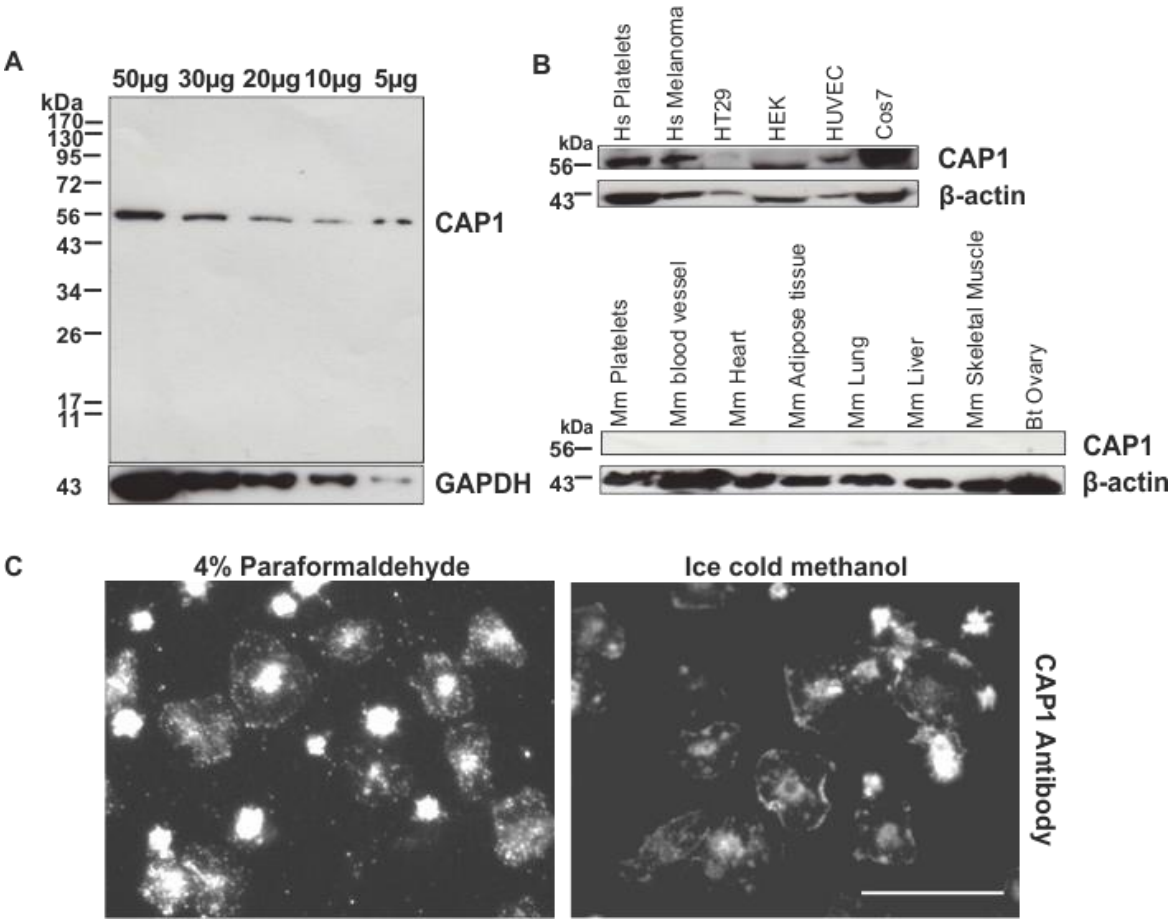


Figure 4.1 Specificity of anti-CAP1 antibody: (A) Variable protein concentrations of human blood platelets. Whole cell lysate of human platelets (3 x10^8 /mL platelets) was loaded (between 50µg - 5µg total protein), resolved on 12% SDS-PAGE, blotted onto PVDF membrane and probed with antibodies for the indicated proteins, where GAPDH is a loading control. **(B) Various animal tissue and cell line lysates (30µg total protein)** were resolved on a 12% SDS gel, blotted onto PVDF membrane and probed with antibodies for CAP1. The blots were probed for beta-actin as a loading control. Human platelets (Hs platelets), human melanoma (Hs melanoma), human colorectal cancer cell line (HT29), human embryonic kidney 293 cell line (HEK), human umbilical vein endothelial cells (HUVEC), fibroblast-like tissue from monkey kidney tissue (Cos 7), murine (Mm) and bovine (Bt) tissue lysates were prepared from freshly collected tissue from culled animals or from cultured cells.

(C) Investigating the localisation of CAP1 in platelets using different fixation methods. Human platelets (2 x10^7 platelets/mL) were allowed to spread on fibrinogen-coated (100µg/ml) coverslips for 30 minutes and were fixed using either ice-cold methanol or 4% paraformaldehyde for 20 min. The platelets were then permeabilised and immunostained using anti-CAP1 antibody and Alexa 568-coupled secondary antibody. Images were captured using a fluorescence microscope. Scale bar = 10µm

Various animal tissue lysates were obtained and samples were resolved and blotted to investigate the specificity of the anti-CAP1 (rabbit monoclonal ab133655) antibody. Figure 4.1 B shows that only human and monkey cells/cell lines gave positive results for presence of CAP1.

Human platelets were chosen as they were the main tissue type of interest followed by human melanoma cells from a patient and HT29, a colorectal cancer cell line, as altered CAP1 expression in cancer cells has been reported previously (Li *et al.*, 2013; X. Liu *et al.*, 2014). Human embryonic cell lines like HEK and HUVEC were also investigated. Most importantly we validate the presence of CAP1 in human blood platelets.

The anti-CAP1 antibody (ab133655) did not identify the CAP1 protein in murine or bovine tissue lysates. However, unpublished work from our lab using an alternate CAP1 antibody has detected CAP1 in murine platelets. Previous research in CAP1 has illustrated that most murine tissues including heart, lungs and liver expressed CAP1 in abundance and skeletal muscle expressed the least CAP1 (Peche *et al.*, 2007; Kosmas *et al.*, 2015). The β-actin control shows that there indeed was protein transferred after a Western blot and that it was not a loading error. It can therefore be concluded that the ab133655 antibody specifically recognises human CAP1 and cannot be used to investigate CAP1 expression in murine and bovine samples.

A suitable fixation method for spread human blood platelets needed to be identified for use of anti-CAP1 in immunofluorescence experiments to assess CAP1 localisation. Two common fixatives, ice-cold methanol and 4% paraformaldehyde (PFA) were tested for their effectiveness on platelets spread on fibrinogen (Figure 4.1 C). Reassuringly, the localisation of CAP1 was similar using both fixatives but the appearance of CAP1 varied - PFA fixation led to more punctate staining of CAP1, whereas methanol fixation led to more continuous staining along the cortical regions. Overall, ice-cold methanol fixation gave clearer and more defined localisation signals for CAP1 than 4% PFA. Phalloidin, which specifically stains F-actin, cannot be used in conjunction with methanol fixation as methanol disrupts actin during fixation, thus leading to inaccurate representation of actin when stained with phalloidin (Small *et al.*, 1999). Therefore, 4% PFA was deemed the preferred method of fixation as it provided relatively better conditions for immunostaining for CAP1 and allowed visualising actin filaments simultaneously without compromising on the quality of staining.

4.2.1.2 Subcellular localisation of CAP1 in resting platelets

Platelets are highly specialised cell fragments which made it very important to verify if the localisation of CAP1 is similar to that observed in other cell types. It is known from several other cell types and various organisms that some CAP1 localises at the cell cortex and most of it is cytosolic (Peche *et al.*, 2007; Lee *et al.*, 2014).

4.2.1.2.1 Visualisation of CAP1 in spread platelets

In order to visualise the CAP1 distribution platelets were spread on fibrinogen-coated or on collagen-coated coverslips, fixed, permeabilised, immunostained using anti-CAP1 antibody and counterstained with FITC-phalloidin for actin. It was observed (Figure 4.2 A and B) that CAP1 is mostly cytosolic with some peripheral localisation. CAP1 co-localises with actin in the cortical regions but is missing near the cytosolic regions presenting stress fibres. CAP1-actin co-localisation is also observed centrally, where the platelet granulomere is expected.

4.2.1.2.2 Subcellular distribution of CAP1

In order to quantify CAP1 distribution we carried out a simple subcellular fractionation. Resting platelets were resuspended in an isotonic sucrose solution and lysed using rapid freeze-thaw in liquid nitrogen before spinning them at 100,000xg to separate cytosol and membrane fractions. As seen in

Figure 4.2C, most (77%) of CAP1 is cytosolic and the rest associates with the membrane fraction. CD36, a platelet membrane marker and Syk, a cytosolic marker in resting platelets confirmed that membrane and cytosol fractions were free from cross-contamination.

Since CAP1 is an actin-binding protein, further investigations were made to identify if this membrane association is actin mediated. Resting platelets were treated with Latrunculin B (LatB, 20μM) to depolymerise F-actin prior to subcellular fractionation.

There was no statistically significant difference in CAP1 association to the membrane in the absence (23%) or presence (27%) of LatB using Student's t-test and therefore it can be inferred that the association of CAP1 to platelet membranes is independent of its association with F-actin.

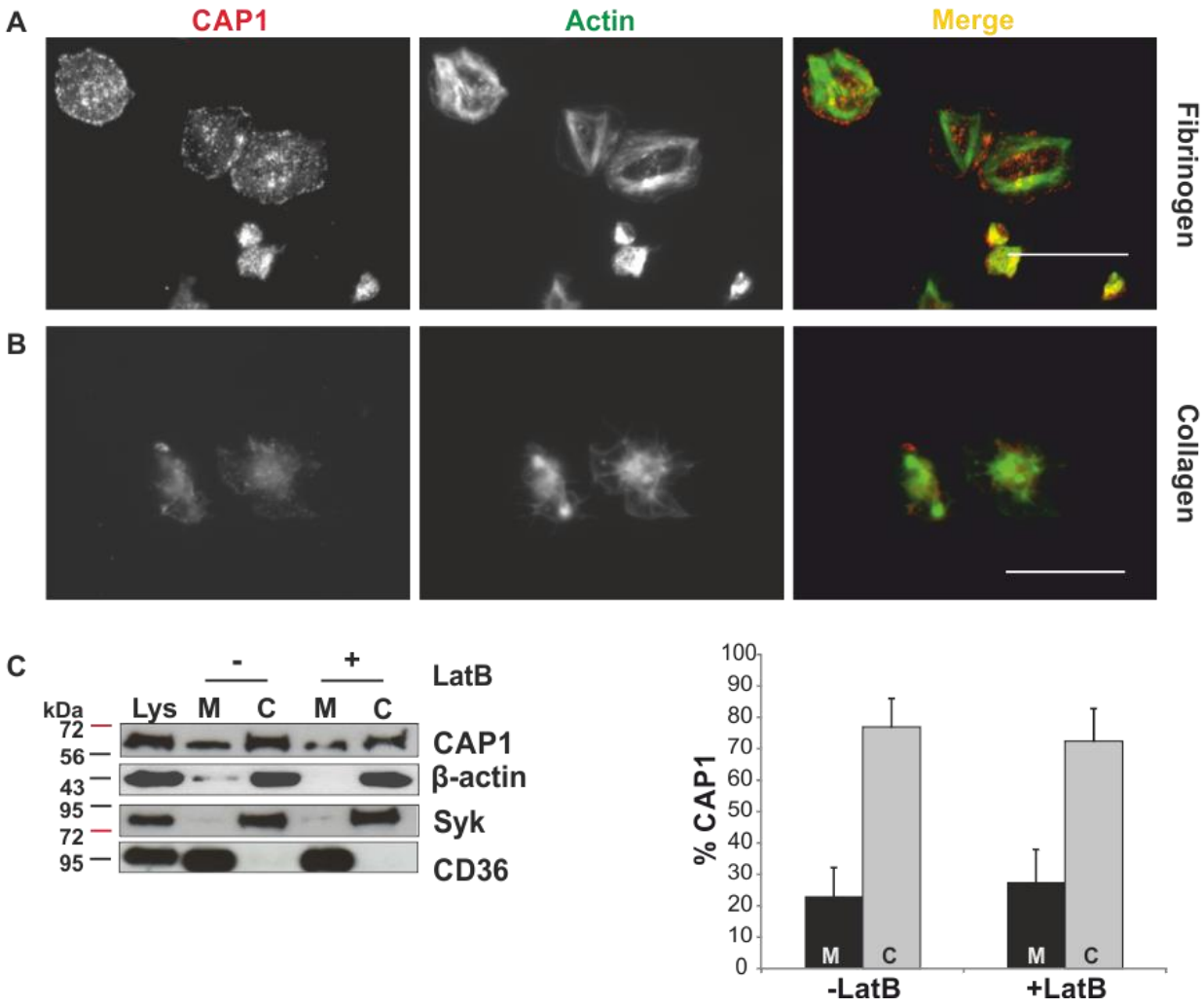


Figure 4.2 Subcellular localisation and distribution of CAP1. Platelet spreading: Human blood platelets (2 x 10^7 platelets) were allowed to spread on (**A**) fibrinogen coated coverslips or (**B**) collagen coated coverslips. Platelets were fixed, immunostained for CAP1 (Alexa 568, secondary antibody, red) and counterstained for actin filaments (FITC-phalloidin, green). Scale bar = 10μm. (**C**) **Subcellular fractionation**: Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume and resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with antibodies for the indicated proteins. CD36 was used as membrane marker and Syk as a cytosolic marker in resting platelets. Latrunculin B (LatB; 20μM; 20

min) was used to depolymerise F-actin prior to lysis. CAP1 distribution was quantified by densitometry and expressed as percent relative to the total CAP1 in the lysate. Data are presented as mean ± standard deviation of two independent experiments.

4.2.1.3 Association of CAP1 to the detergent insoluble pellet

The most described role of CAP1 in mammalian cells is in coordinating actin turnover. In order to assess this role in platelets, detergent insoluble fractions of platelets were isolated. The rationale behind isolating this pellet lies in the fact that most proteins are soluble in detergents, but actin filaments and associated proteins remain insoluble. The contents of the insoluble fractions depend on the extent of crosslinking of the actin filament networks and can be sedimented at various g-forces from the detergent lysates. Most of the actin filaments are cross-linked into networks that can be sedimented at low speed (15,600xg) (Fox, 2001). These are thought to represent the filaments that course throughout the body of the platelet. The filaments that are crosslinked to the plasma membrane and which form part of the membrane skeleton are sedimented at high speed (100,000xg). The proteins that are sedimented at this speed are associated with the lipid bilayer in the membrane (Fox, 1985; Fox *et al.*, 1988). In order to characterise the association of CAP1 to actin in platelets, resting platelets were lysed in a buffer containing the detergent, Triton X-100, and separated into soluble and insoluble fractions by centrifugation at low and high speeds. Detergent soluble fraction is the supernatant and the insoluble fraction is the pellet containing F-actin and associated proteins. Supernatant comprises of mostly G-actin and its associated proteins. Experiments were carried out in the presence and absence of LatB, which was used to depolymerise F-actin.

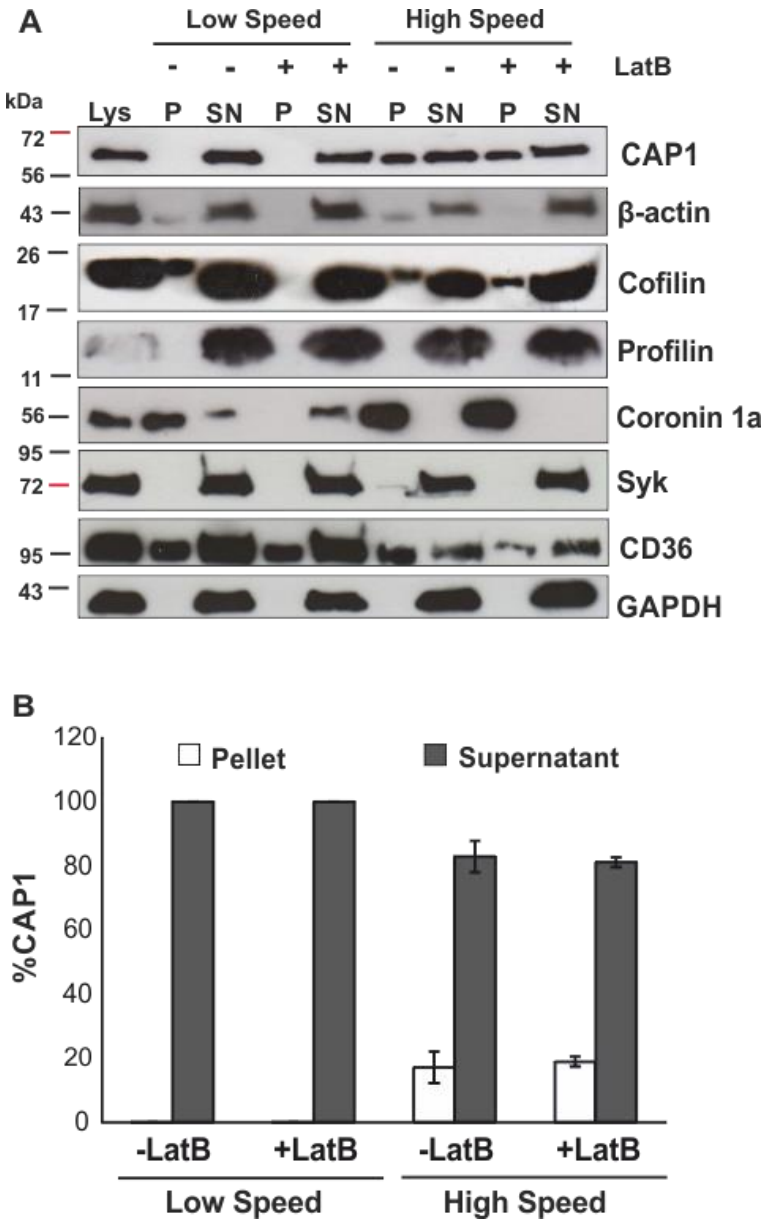


Figure 4.3 Association of CAP1 to actin in detergent insoluble pellet. Human blood platelets (8 x 10^8/mL platelets) were lysed in the presence of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. (**A**) The supernatant (SN) and pellet (P) were normalised by volume and resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with antibodies for the indicated proteins. Latrunculin B (LatB; 20µM, 20 min) was used to depolymerize F-actin prior to lysis. (**B**) CAP1 concentration in pellet and supernatant were quantified by densitometry as

percent of total (pellet + supernatant). Data are presented as mean ± standard deviation of three independent experiments.

In Figure 4.3 it was observed that CAP1 was not present in the low speed pellet where only long actin filaments and associated proteins are sedimented but strong bands for CAP1 appear in the high speed pellet, where membrane-associated or lipid bilayer complexed cytoskeletal proteins are present. 17% of CAP1 was found in the high speed detergent insoluble pellet of resting platelets. Notably, no significant difference in CAP1 was observed in the LatB treated samples (19%). An important conclusion is that the presence of CAP1 in the high speed detergent insoluble pellet is independent of its association with actin, similar to the observations from the previous fractionation experiments (Figure 4.2, Section 4.2.1.2.2) indicating that the membrane association was independent of actin.

CD36, which is a membrane protein, is mainly observed in the supernatant, but significant amount remains in both the low and high speed detergent insoluble pellet, probably attached to lipid rafts.

Syk and GAPDH, which are cytosolic proteins in resting platelets, were only observed in the supernatant fraction across the two isolation speeds, which confirmed the absence of cytosolic proteins in the pellets.

Further, cofilin and profilin, which are known to interact with actin in co-ordination with CAP1 in actin turnover were also characterised in the samples to see if all results were consistent (Balcer *et al.*, 2003; Zhang H *et al.*, 2013). Interestingly, profilin is only observed in the supernatant, owing to its association to only monomeric actin. On the other hand, cofilin, which interacts with F-actin in addition to G-actin, is observed in high and low speed detergent insoluble pellets, but not in the low speed pellet of LatB treated platelets. Coronin1a, which specifically binds F-actin is observed in the low speed detergent insoluble pellet, but is absent when platelets were treated with LatB. Further discussion about these proteins can be found in the following chapter 5.

In the following experiments, the behaviour of CAP1 will be examined only in the high speed detergent insoluble pellet.

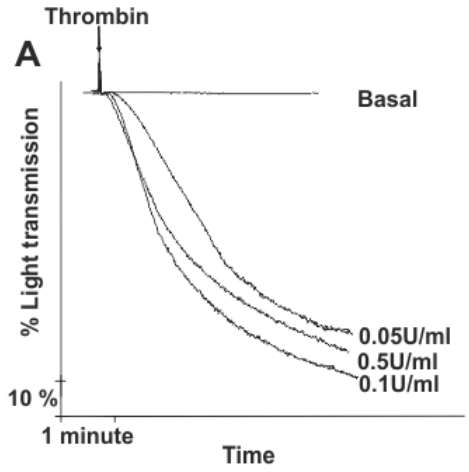
4.2.2. Dynamics of CAP1 upon thrombin stimulation

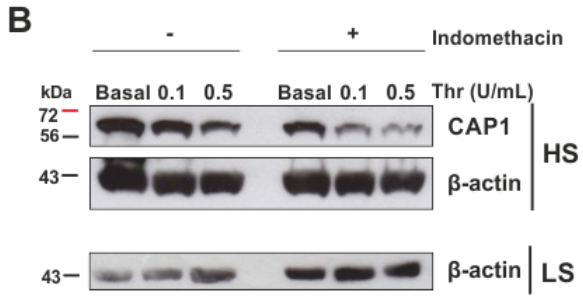
Thrombin is a potent platelet activator and causes cytoskeletal changes through G-protein coupled receptors PAR1 and PAR4, leading to shape change, platelet aggregation and spreading (Kahn *et al.*, 1999; Coughlin, 2000) and subsequently in high speed detergent insoluble pellet to assess any potential effects on the localisation of CAP1.

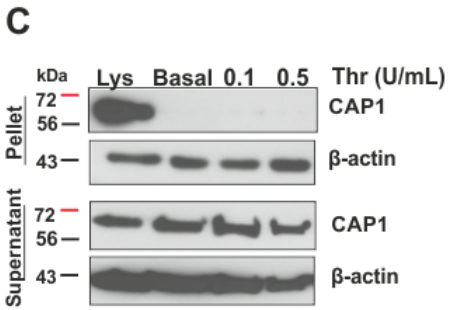
4.2.2.1 CAP1 moves away from the detergent insoluble pellet in response to thrombin stimulation

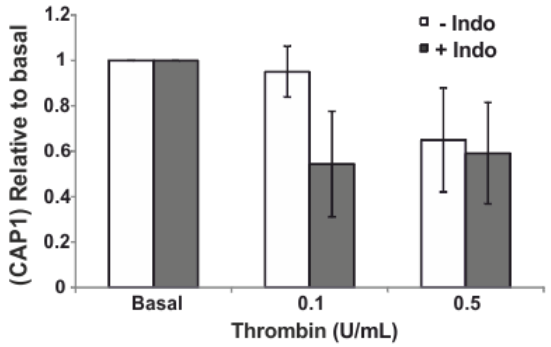
To assess which concentration of thrombin would give a significant cytoskeletal change in platelets, different doses were tested in aggregation and subsequently in high speed detergent insoluble pellet. As seen in Figure 4.4 A, 0.1U/mL thrombin caused the greatest response in terms of aggregation. In this particular case, less aggregation was observed for 0.5U/mL dose of thrombin compared to 0.1U/mL. Work in our and other labs show maximum aggregation response to thrombin at 0.1U/mL.

CAP1 localisation was assessed in the presence and absence of the TXA_2 synthesis-blocker, indomethacin prior to thrombin stimulation to prevent paracrine activation of platelets. Platelets were treated with indomethacin (10µM – 20 minutes), followed by thrombin stimulation for one minute and lysis in buffer containing 1% Triton X-100 before centrifugation at high speed (100,000xg).


Thrombin
A
Basal
% Light transmission
0.05U/ml
0.5U/ml
0.1U/ml
10 %
1 minute
Time


B
-
+
Indomethacin
kDa
Basal 0.1 0.5
Basal 0.1 0.5
Thr (U/mL)
72
56
43
CAP1
β-actin
HS
β-actin
LS


C
kDa
Lys
Basal 0.1 0.5
Thr (U/mL)
Pellet
72
56
43
CAP1
β-actin
Supernatant
72
56
43
CAP1
β-actin


- Indo
+ Indo
(CAP1) Relative to basal
1.2
1
0.8
0.6
0.4
0.2
0
Basal
0.1
0.5
Thrombin (U/mL)

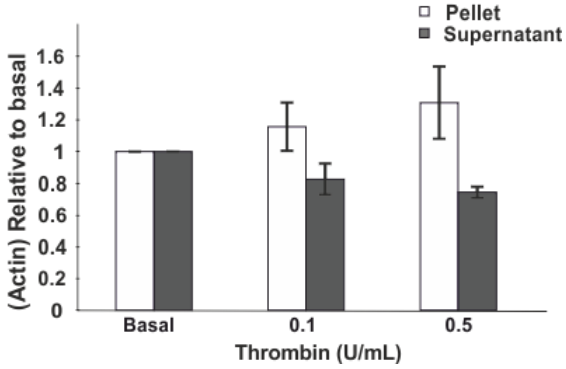

Pellet
Supernatant
(Actin) Relative to basal
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Basal
0.1
0.5
Thrombin (U/mL)

Figure 4.4 (from previous page) **Response of CAP1 in detergent insoluble pellet upon thrombin stimulation: (A)** Representative aggregation traces upon thrombin stimulation are shown. The X-axis shows the time post-stimulation with agonist, while the Y-axis shows percent light transmitted through the platelet suspension. **(B)** Detergent insoluble high speed (HS) pellet upon stimulation of resting platelets with the indicated thrombin doses for 1 minute followed by lysis in Tritin-X-100 based buffer. The experiment was performed in the presence or absence of indomethacin (10µM). In all experiments, pellet and supernatant were normalised by volume and resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with antibodies for the indicated proteins. Beta-actin was used as loading control. In the graph, CAP1 was normalised to the actin control and represented relative to the corresponding basal pellet samples. Data are represented as mean ± standard deviation of three independent experiments. Statistical significance calculated compared to basal using one-way ANOVA. **(C)** Actin concentration in pellet and supernatant of low speed samples (LS) were quantified by densitometry and expressed as percentage of platelet fraction. This percentage is represented relative to basal to enable clear visualisation of any changes. Data are presented as mean ± standard deviation of three independent experiments.

Interestingly, CAP1 in the high speed pellet decreased with increasing dose of thrombin. This decrease in CAP1 from the detergent insoluble pellet upon thrombin stimulation was more noticeable in the indomethacin pre-treated group (Figure 4.4 B). Although statistical analysis did not reveal any significant differences, the loss of CAP1 from the pellet after stimulation with thrombin is clear from the immunoblots. This observation will be investigated further to assess if the response is time dependent in the following sections of this chapter (4.2.2.4).

In the high speed detergent insoluble pellet, 0.1U/mL and 0.5U/mL of thrombin both caused a significant increase in actin in the pellet fractions (data not shown, only representative blot in Figure 4.5 B). The change in actin concentration in the pellet was more apparent in the low speed pellets (Figure 4.4 C) compared to high speed pellets, where smaller fragments of F-actin are sedimented skewing the results for total actin polymerisation.

The expected actin response to thrombin stimulation is a significant increase in F-actin to aid platelet spreading; this was not clear in the high speed detergent insoluble pellet. As a confirmation of the anticipated actin response to thrombin stimulation of platelets, detergent insoluble pellets were obtained at low speed and actin concentration was evaluated upon thrombin stimulation in Figure 4.4 C. The concentration of actin in the low speed pellet increased with increasing doses of thrombin and decreased in the supernatant thus providing a control measure for high speed detergent insoluble pellet experiments.

4.2.2.2 Time course of CAP1 translocation upon thrombin stimulation

When platelets experienced dose-dependent thrombin stimulation, there was a shift in CAP1 concentration in the detergent insoluble pellet. We assessed this change in association of CAP1 further in a time course experiment. Platelets were stimulated with 0.1U/mL thrombin and the reaction was stopped with lysis buffer at time points from 15 seconds to 5 minutes after stimulation. High speed detergent insoluble pellets were then generated to assess any time-dependent changes in association of CAP1 to the detergent insoluble fraction.

CAP1 concentration in the high speed detergent insoluble pellet shows a decrease of over 40% upon thrombin stimulation, becoming statistically significant after 3 minutes (80% reduction).

In Figure 4.5, actin concentrations in the low speed detergent insoluble pellet shows a statistically significant increase compared to basal within 30 seconds, reaches a maximum (1.8 fold increase) around 60 seconds and decreases further to reach a plateau after that. This experiment helps to illustrate that F-actin in the cytoskeleton increases after thrombin stimulation but a comparable increase cannot be noted in the high speed detergent insoluble pellet due to limitations that have been described previously (Section 4.2.1.3). Assessing the changes in actin in the high speed detergent insoluble pellet therefore does not give an accurate representation of the dynamic changes in F-actin concentration occurring after thrombin stimulation.

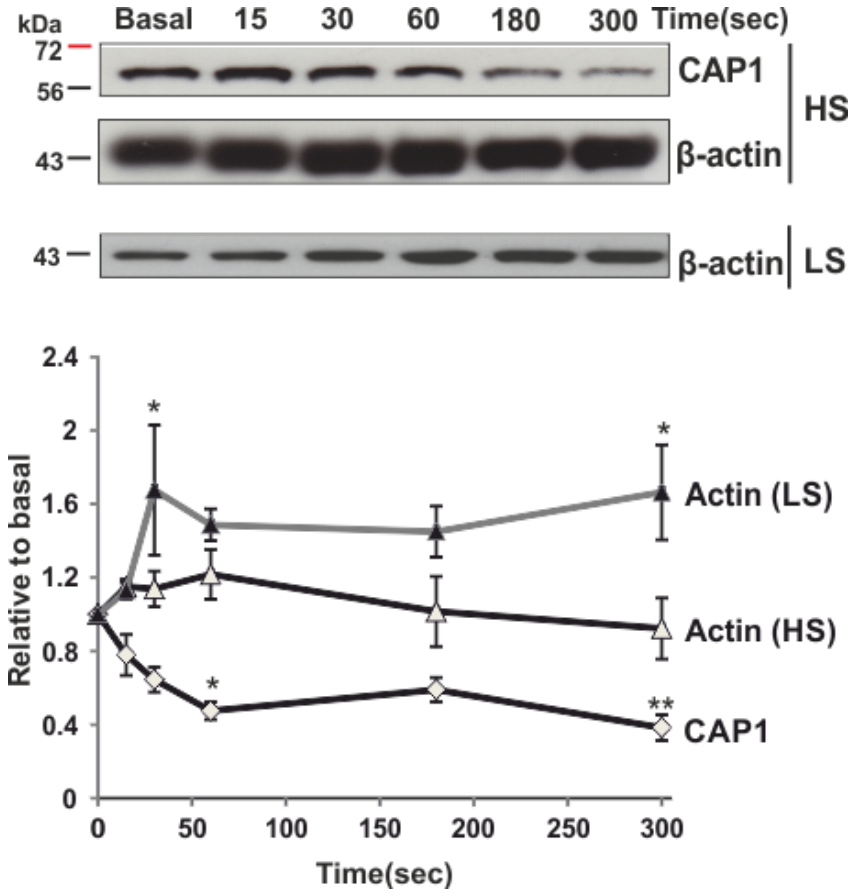


Figure 4.5 CAP1 in the high speed detergent insoluble pellet of thrombin stimulated platelets decreases with time. Human blood platelets (8 x 10^8/mL platelets) were stimulated with 0.1U/mL thrombin prior to lysis in Triton X-100 based buffer and spun at high speed (100,000xg) for 1 hour. Only pellet samples were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with antibodies for the indicated proteins. CAP1 concentration in pellet was quantified by densitometry and expressed as values relative to the basal pellet fraction. The experiment was repeated with centrifugation at low speed (15,600xg) to indicate increase in actin in pellet. Data is represented as mean ± standard error of the mean of four independent experiments. Statistical significance relative to basal is calculated using one-way ANOVA, *$p \leq 0.05$, **$p \leq 0.005$.

4.2.2.3 CAP1 translocates from membrane into cytosol upon thrombin stimulation

The decrease in CAP1 in the high speed detergent insoluble pellet after thrombin stimulation suggested that there was CAP1 movement that occurred after activation. In order to further investigate this we observed changes in CAP1 in different platelet fractions upon thrombin stimulation. Platelet fractions were generated upon increasing duration of thrombin stimulation, from 15 seconds to 3 minutes.

Platelets were stimulated with 0.1U/mL thrombin and were subsequently fractionated into membrane and cytosol as previously described (section 4.2.1.2). A clear reduction of CAP1 in the membrane fraction is observed with increasing time post-thrombin stimulation (Figure 4.6). A corresponding increase, as would be expected after thrombin stimulation, is not observed in actin, perhaps due to the fact that fractionation of platelets into membrane and cytosol is not based on the F-actin content. All CAP1 concentration values were therefore normalised to actin before normalising to basal values for this reason. CD36, which is a platelet membrane marker, shows no change in band intensities reinforcing that the lack of change in actin concentration is not a loading error. Further cytosolic control, GAPDH, confirms that all membrane fractions were devoid of cytosolic contamination.

Consistent with observations in spread platelets, a statistically significant ($p \leq 0.01$, ANOVA) reduction of 50% membrane bound CAP1 is observed around 30-60 seconds. Statistical significance in reduction of CAP1 in membrane

fractions further increases around 3 minutes ($p \leq 0.001$, ANOVA) post-stimulation.

This is mostly consistent with the decrease in CAP1 concentrations in the detergent insoluble fraction as time after thrombin stimulation increases which suggests that the membrane association of CAP1 reduces and therefore less CAP1 associates with the detergent insoluble pellet which contains lipid raft associated proteins. Figure 4.2 has shown that membrane association of CAP1 is actin independent, and together with observations from Figure 4.5, leads to the inference that CAP1 translocation occurs independent of its association with actin and most likely due to its association with lipid bilayer associated proteins in the platelet membrane.

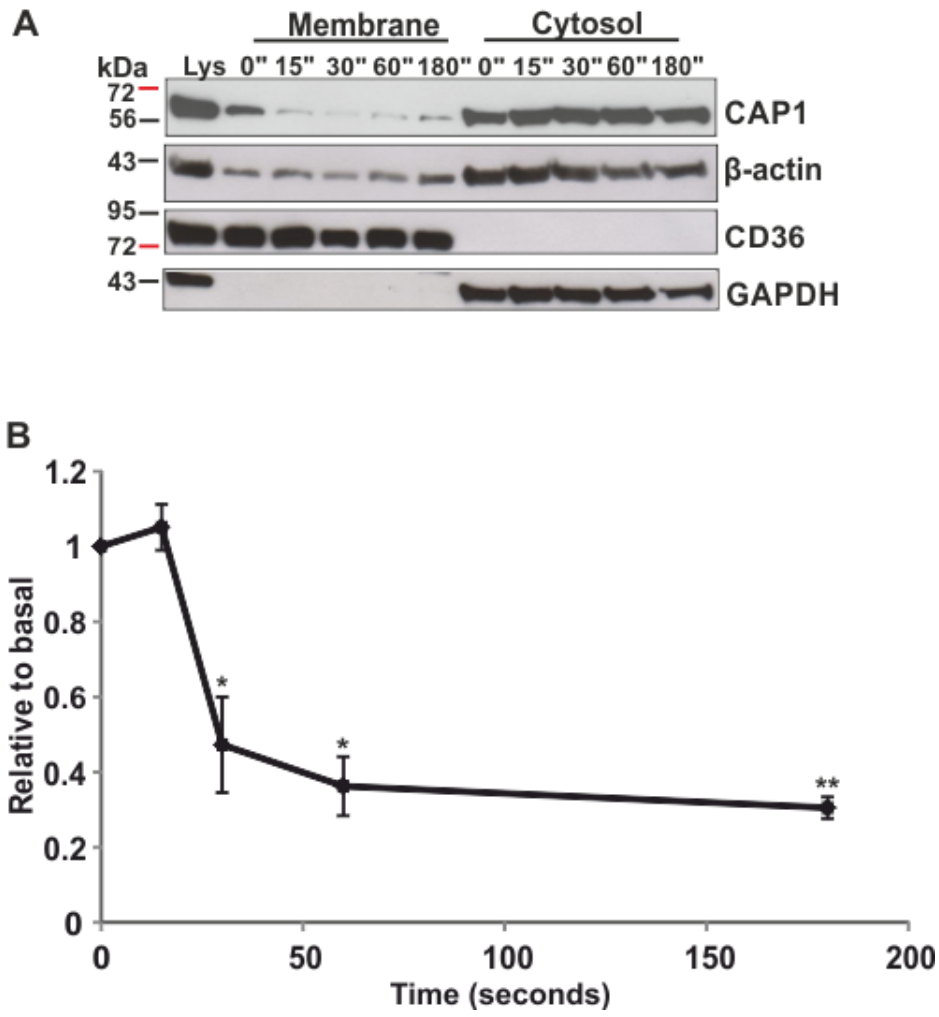


Figure 4.6 Reduced membrane-associated CAP1 upon thrombin stimulation. (A) Human blood platelets (8 x 10^8/mL platelets) were treated with 0.1U/mL thrombin for indicated times prior to lysis by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions and resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with antibodies for the indicated proteins – CD36, membrane marker; GAPDH, cytosolic marker. **(B)** CAP1 was quantified by densitometry and expressed relative to the CAP1 in the basal (0") membrane fraction. CAP1 was normalized to actin in the membrane fraction. Data are represented as mean ± standard error of the mean of four independent experiments. Statistical significance is calculated using one-way ANOVA, *p ≤ 0.01, **p ≤ 0.001 relative to 0".

4.2.2.4 Visualisation of the effect of thrombin stimulation on CAP1 localisation

4.2.2.4.1 Visualisation on fibrinogen

In order to visualise the CAP1 localisation upon thrombin stimulation, platelets were spread on immobilised fibrinogen (100µg/mL) for 30 minutes followed by stimulation with 0.1U/mL thrombin for increasing durations before fixing with PFA.

Confirming the observations from Figure 4.2, most CAP1 is mainly cytosolic and some outlines the peripheral membrane in platelets spread on fibrinogen without additional stimulation. Upon stimulation of spread platelets with thrombin (Figure 4.7), peripheral CAP1 seems to disperse into the cytosol with increasing length of thrombin stimulation. At 60 seconds, all CAP1 is cytosolic and negligible amounts localise near the periphery of platelets. After 3 minutes of thrombin stimulation, CAP1 seems more punctate and overall reduced but localisation appears more cortical than observed after one minute.

A profile of CAP1 distribution was generated from the processed images which helps elucidate the movement of CAP1 from the membrane into cytosol in a time dependent manner upon stimulation with thrombin (Figure 4.8). It can be seen from the control image that peaks for presence of CAP1 at the membrane reduce dramatically after stimulation with thrombin and the loss from the membrane is the greatest around 60 seconds post-stimulation.

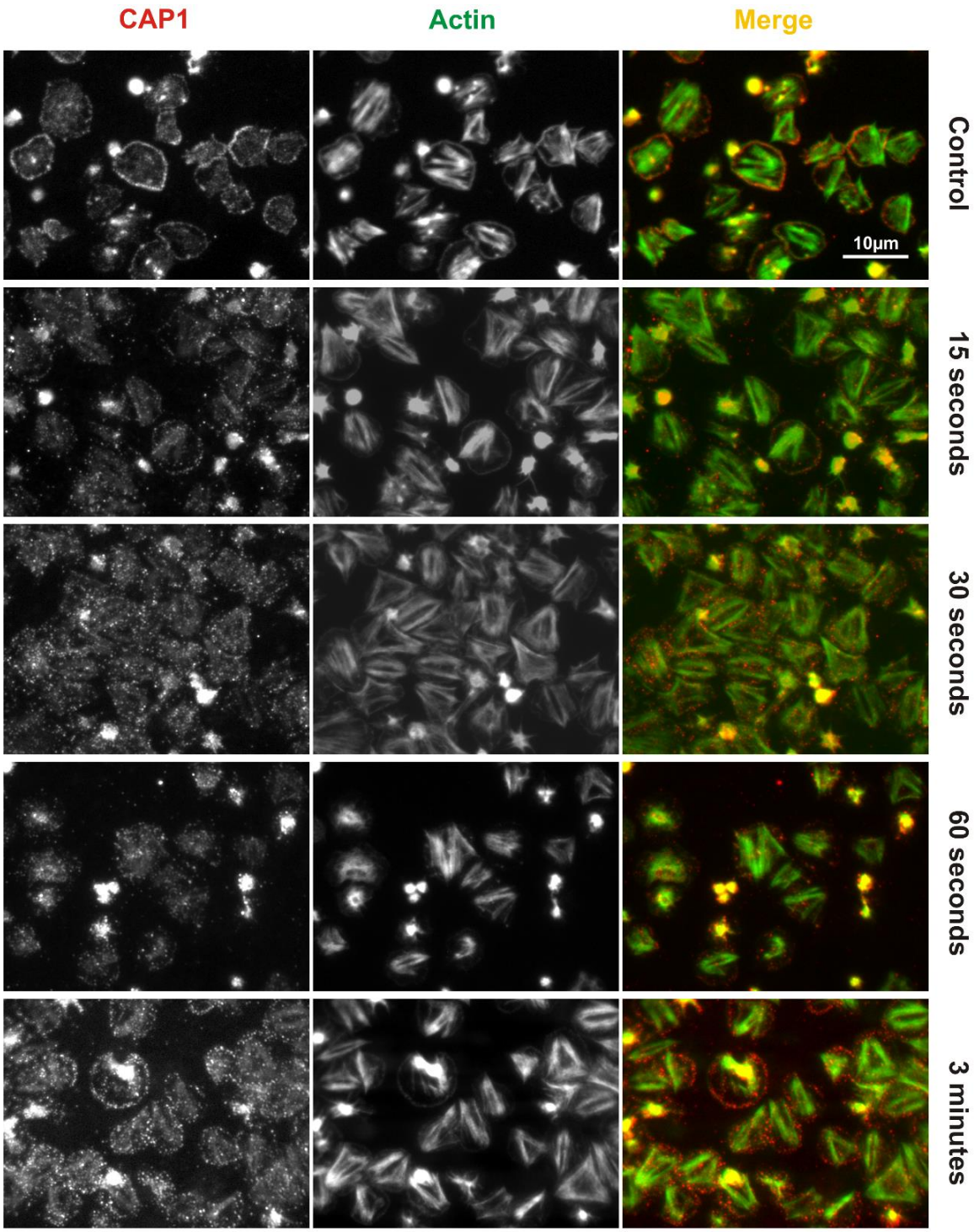


Figure 4.7 CAP1 re-localisation in spread platelets stimulated with thrombin. Human platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen-coated coverslips for 30 minutes. This was the "control" condition.

Platelets were then stimulated with 0.1U/mL thrombin for the indicated times, fixed with 2% PFA and immunostained for CAP1 (red). Actin filaments were visualized by FITC phalloidin staining (green). Images were captured using a fluorescent microscope. Scale bar = 10μm

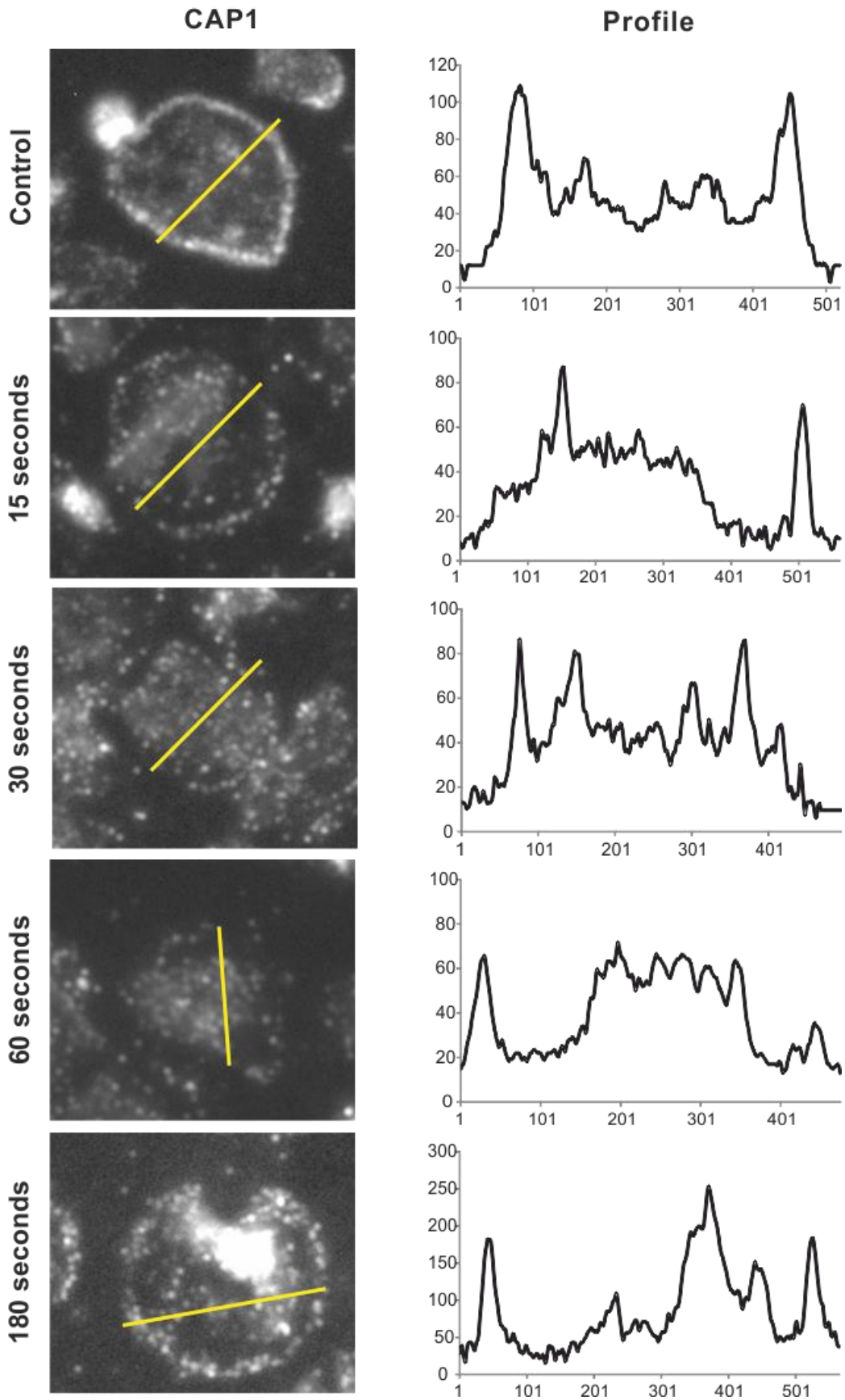

CAP1
Profile
Control
15 seconds
30 seconds
60 seconds
180 seconds
120
100
80
60
40
20
0
1
101
201
301
401
501
300
250
200
150
50

Figure 4.8 (previous page**) CAP1 translocation profile upon thrombin stimulation of spread platelets**. Human platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen-coated coverslips for 30 minutes. This was the "control" condition. Platelets were then stimulated with 0.1U/mL thrombin for the indicated times, fixed with 2% PFA and immunostained for CAP1. Images obtained in the previous figure (Figure 4.8) were further analysed by plotting a profile of CAP1 distribution across a spread platelet for each condition. Each yellow line in the left panel is represented as the cross-sectional profile of that platelet in the right panel. Using ImageJ, the signal intensity of CAP1 across the cross-section of a single platelet (Y-axis) was plotted against the arbitrary units of distance (X-axis). The changes in distribution pattern of CAP1 are evident with increasing stimulation with thrombin.

4.2.2.4.2 Visualisation on poly-L-lysine

It was important to investigate if CAP1 translocation effect was evident only in spread platelets that have already undergone activation when adhering to fibrinogen matrix, or if this can already be visualised in platelets that were stimulated with thrombin in suspension. In order to achieve this, platelets were stimulated with 0.1U/mL thrombin in suspension, fixed and then adhered onto poly-L-lysine coated coverslips. The outcomes in a time course experiment were inconclusive as the platelets stimulated in suspension did not dramatically increase in size as observed on fibrinogen but maintained their discoid shape and some filopodia were observed in platelets stimulated with thrombin for over one minute (Figure 4.9). As the platelets remained in their smaller, discoid shape, visualisation of CAP1 changes in distribution was rendered impossible under available microscopy capabilities.

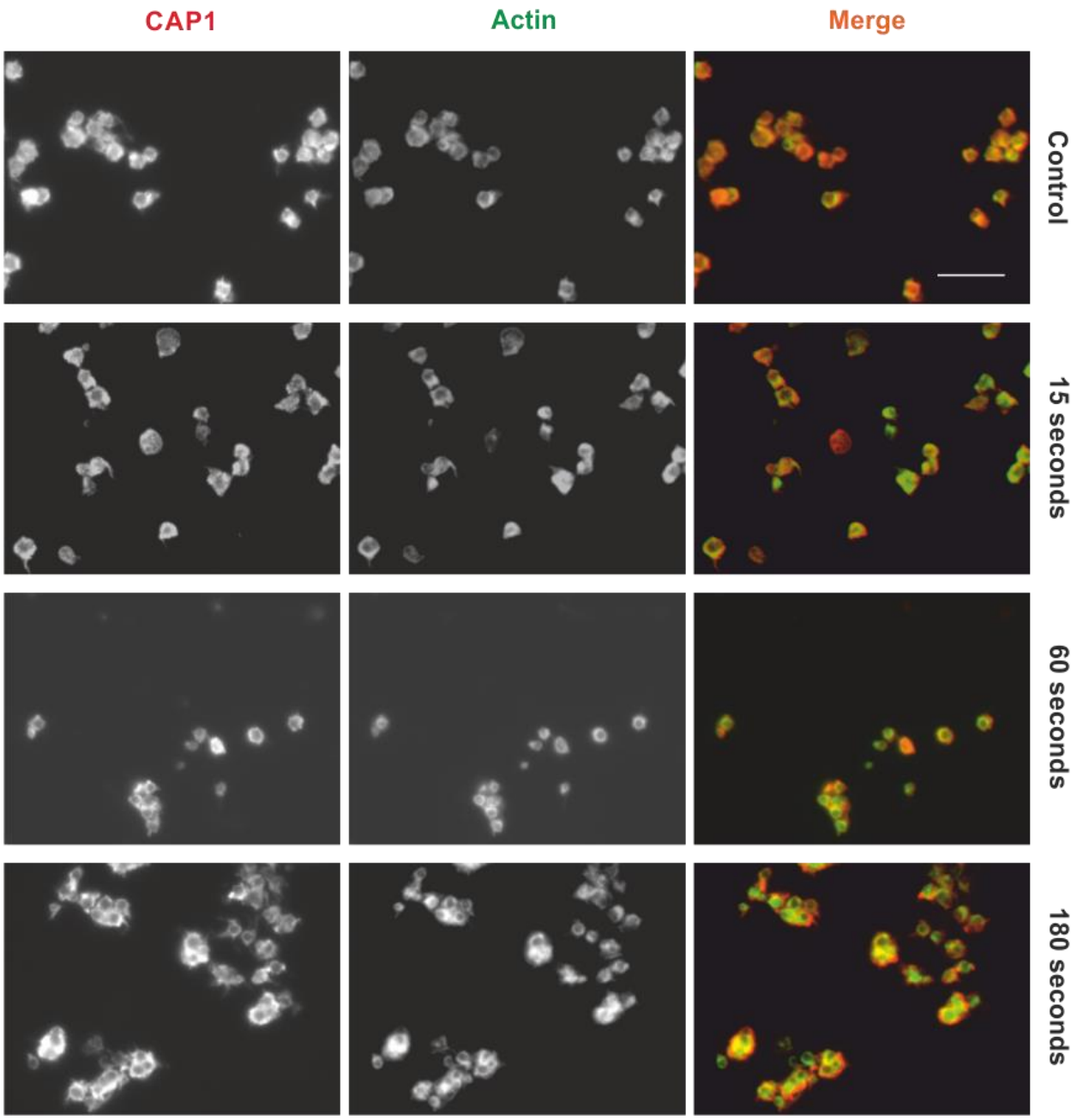


Figure 4.9 CAP1 visualisation of thrombin stimulation of platelets in suspension. Human platelets (2 x 10^7 platelets) were stimulated with 0.1U/mL thrombin in suspension in a time dependent manner, fixed with 2% PFA in suspension, followed by centrifugation at 4000xg to adhere to poly-L-lysine coated coverslips. Platelets were immunostained for CAP1 (red). Actin filaments were stained green with FITC-phalloidin. Scale bar = 10µm

4.2.2.5 Assessing involvement of thrombin receptor agonists in CAP1 translocation from membrane to cytosol

Thrombin stimulates platelets by cleavage of G-protein coupled receptor. Specifically, thrombin cleaves protease-activated receptors (PAR) and exposes tethered ligands to induce self-activation. Human platelets express two PARs – PAR1 and PAR4. It has been established that PAR1 and PAR4 differentially regulate downstream signalling in platelets (Coughlin, 2000). PAR1 is known to be activated at lower concentrations of thrombin compared to PAR4 (Kahn *et al.*, 1999), and PAR4 leads to elevated thrombin production by mediating secretion and microparticle generation (Duvernay *et al.*, 2013), and increased calcium mobilisation (Holinstat, 2006) compared to PAR1. The following experiment using PAR1-activating peptide – SFLLRN and PAR4-activating peptide AYPGKF to specifically target their respective PARs will enable us to evaluate their involvement during CAP1 translocation.

PAR1-activating peptide did not alter CAP1 localisation in the membrane at higher doses but at very low concentration, it was comparable to response of 0.1U/mL thrombin, with no statistical significance observed. PAR4-activating peptide on the other hand caused a moderate decrease in CAP1 concentration in the membrane at low concentration but indicated a dose-dependent increase to reach basal levels and a dramatic loss of CAP1 from the membrane at the highest tested dose of 100µM. It might be inferred from Figure 4.10, although insufficiently, that PAR4-activating peptide caused a greater translocation of CAP1 from the membrane to the cytosol compared to PAR1-activating peptide.

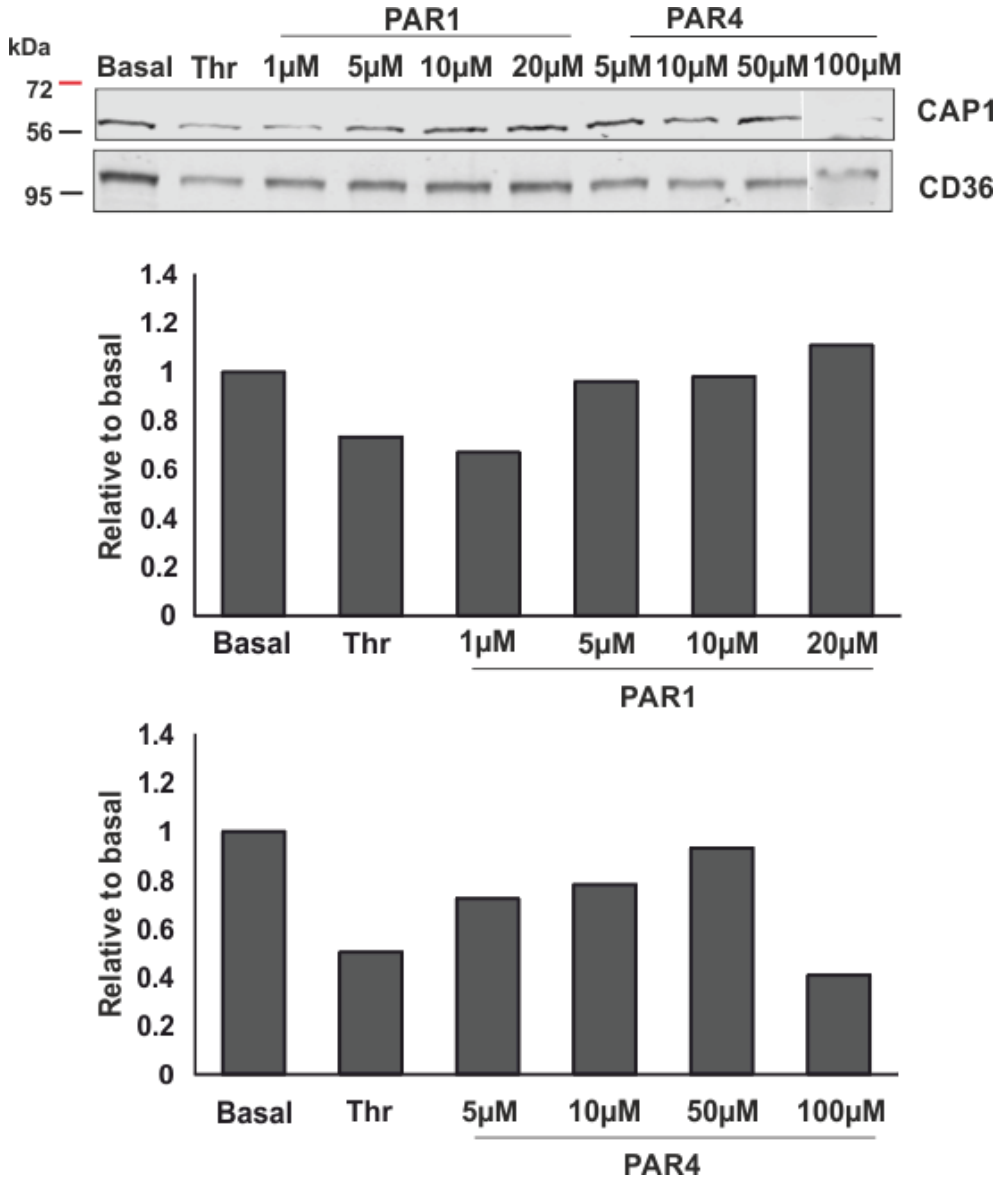


Figure 4.10 Subcellular localisation of CAP1 upon stimulation with thrombin receptor agonists: Dose response to thrombin receptor agonists PAR1 (SFLLRN) and PAR4 (AYPGKF) was compared to basal using 0.1U/mL thrombin stimulation as standard from previous experiments. Platelets were stimulation was for 3 minutes for each condition prior to subcellular fractionation. Membrane fractions were resolved on 12% SDS-PAGE and probed for the

presence of CAP1. CD36 was used as a loading control and marker for membrane fraction. Data represent mean of two independent experiments.

4.2.3. Dynamics of CAP1 distribution in collagen-stimulated platelets

Collagen is one of the key platelet agonists and acts via activation pathways distinct to thrombin. It was therefore important to understand if the CAP1 translocation event observed upon thrombin stimulation of platelets was agonist-specific. Collagen concentration of 10µg/mL which resulted in maximal aggregation (as observed in figure 3.5) was used for stimulation to assess CAP1 translocation. Although collagen mechanistically acts in a different manner to thrombin, stimulation times used was three minutes to provide easy comparison between the two agonists.

CAP1 localisation was simultaneously compared by fractionating platelets into membrane and cytosol and in high speed detergent insoluble pellet upon collagen stimulation. It was observed in Figure 4.11 that collagen stimulation did not cause any alterations in levels of CAP1 at the membrane or its cross-linking to the membrane based on the observations from the high speed detergent pellet.

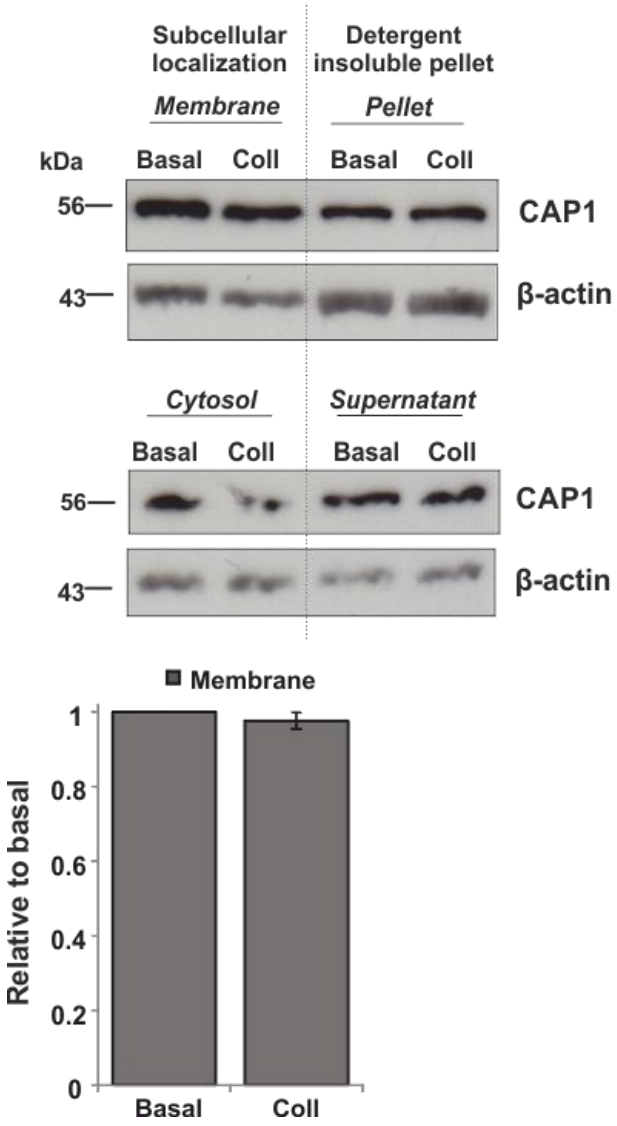


Figure 4.11 Distribution of CAP1 in collagen stimulated platelets. Subcellular localization: Platelets were stimulated with 10µg/mL collagen and subcellular localization was compared to basal, untreated platelets. Detergent insoluble pellet: Platelets were lysed using a Triton-X 100 based lysis buffer and centrifuged at high speed (100,000xg) for one hour to separate pellet and supernatant. Cytosol and supernatant were 6 times more diluted than the corresponding membrane and pellet samples. All samples were resolved on 12% SDS-PAGE and probed for the indicated proteins. Blots are representative of three independent experiments of subcellular fractionation and one detergent

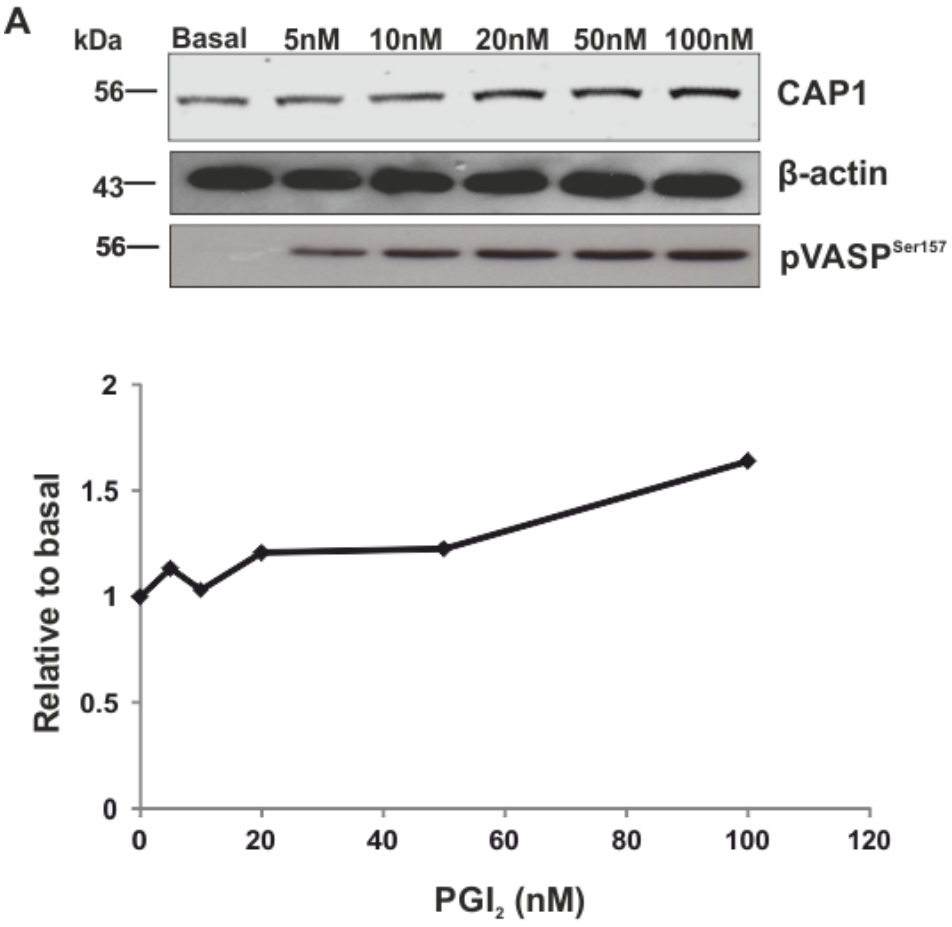
insoluble pellet experiment. Data in bar graph are presented as mean ± standard error of the mean of three independent experiments.

4.2.4. Dynamics of CAP1 in PGI_2 treated platelets

In a recent study Lee *et al*. (2014) put forth their finding that CAP1 is a receptor to adipokine, resistin, which has been described in chapter 3. They present an increase in PKA activation recorded as a response to elevated secondary messenger (cAMP) upon exposure to resistin in a monocytic cell line overexpressing CAP1. A role for CAP1 in cAMP elevation and activation of adenylyl cyclases has been described in *Saccharomyces* but no such evidence has been documented in mammalian cells yet (Hubberstey and Mottillo, 2002; Ono, 2013).

In platelets, PGI_2 is known to activate PKA via adenylyl cyclase activation, thus inhibiting platelets (Aszódi *et al.*, 1999). As an initial step in considering a role for CAP1 in PKA activation in highly specialised cells like platelets, an alteration in CAP1 localisation in response to PGI_2 treatment was investigated.

Figure 4.12 A clearly shows a dose dependent elevation of up to 50% in CAP1 in the membrane fraction in response to 3 minute PGI_2 treatment. Statistically significant increase in CAP1 level at the membrane was observed when platelets were treated with 100nM dose of PGI_2. Phosphorylation of VASP at Ser157 is used as a positive control for PGI_2 treatment.


A
kDa
Basal
5nM
10nM
20nM
50nM
100nM
56
CAP1
43
β-actin
56
pVASP Ser157
Relative to basal
2
1.5
1
0.5
0
0
20
40
60
80
100
120
PGI2 (nM)

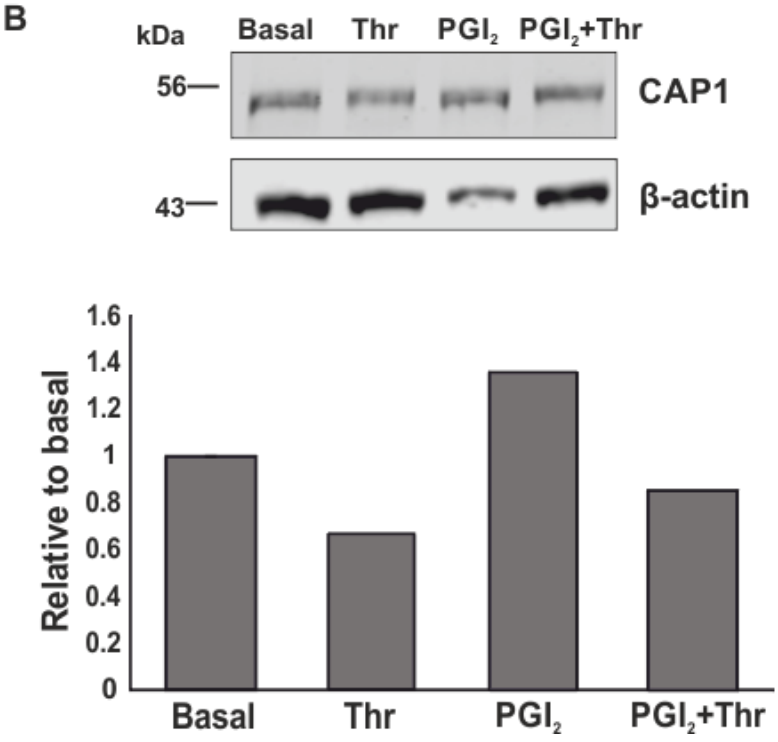

B
kDa
Basal
Thr
PGI2
PGI2+Thr
56
CAP1
43
β-actin
Relative to basal
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Basal
Thr
PGI2
PGI2+Thr

Figure 4.12 Subcellular localisation of CAP1 upon stimulation with prostacyclin: (A) CAP1 localisation was assessed as dose response to prostacyclin (PGI_2) and compared to basal, resting platelets. Platelets were treated with various doses of PGI_2 for 3 minutes prior to subcellular fractionation. Only membrane fractions were resolved on 12% SDS-PAGE and probed for the presence of CAP1. Beta-actin was used as a loading control and pVASP as a treatment control. Data represent mean of two independent experiments. **(B) Effect on CAP1 localisation upon PGI_2 treatment followed by thrombin stimulation.** Platelets were treated with 100nM PGI_2 for 3 minutes followed by 0.1U/mL thrombin for further 3 minutes prior to subcellular fractionation. Membrane fractions were resolved on 12% SDS-PAGE and probed for the presence of CAP1. Beta-actin was used as a loading control. Data represent mean of two independent experiments.

4.2.4.1 CAP1 localisation upon PGI_2 pre-treatment followed by thrombin stimulation

PGI_2, which is a known PKA activator, led to increased CAP1 association to the membrane. Thrombin, which is known to negatively regulate PKA activation, led to decrease in CAP1 association to the membrane. Although, PKA activation is not directly assessed in our experiments, a speculation can be made suggesting it might play a role in CAP1 translocation and initial investigations are made in this section.

The logical step was to identify differences in CAP1 translocation from the membrane when platelets are pre-treated with PGI_2 followed by stimulation with thrombin. As expected, 100nM PGI_2 pre-treatment for 3 minutes followed by 0.1U/mL thrombin stimulation for 3 minutes caused CAP1 levels in membrane to be higher when compared to thrombin stimulation alone and lower when compared to PGI_2 treatment alone (Figure 4.12 B). When compared to basal, the difference in CAP1 localisation was not found to be statistically significant in any of the cases, but a trend is observed which would require substantially more data to corroborate it.

4.2.5. Dynamics of CAP1 in resistin-treated platelets

In the study published by Lee *et al.* (2014) CAP1 is identified as a receptor for resistin mediating its role in inflammation. In the previous chapter (Chapter 3) we note that resistin pre-treatment caused an attenuation of the thrombin response in platelet aggregation. In the present chapter we show that CAP1 translocated from membrane into cytosol upon thrombin stimulation. The above observations together prompted an investigation to establish CAP1 localisation upon resistin treatment.

Platelets were treated with 0.1U/mL thrombin or 200ng/mL resistin and then fractionated into membrane and cytosol. As established before in Figure 3.5, 24% CAP1 is membrane associated under basal conditions and the membrane association reduces upon thrombin stimulation to 15%. Interestingly, when platelets were treated with resistin, the membrane association of CAP1 greatly diminished compared to basal, at 11% (Figure 4.13).

Clear conclusions cannot be derived from the observations as only one experiment was performed to test CAP1 localisation upon resistin treatment, but the definite reduction in CAP1 levels are promising and resistin pre-treatment in addition to thrombin stimulation would have added value to the observations.

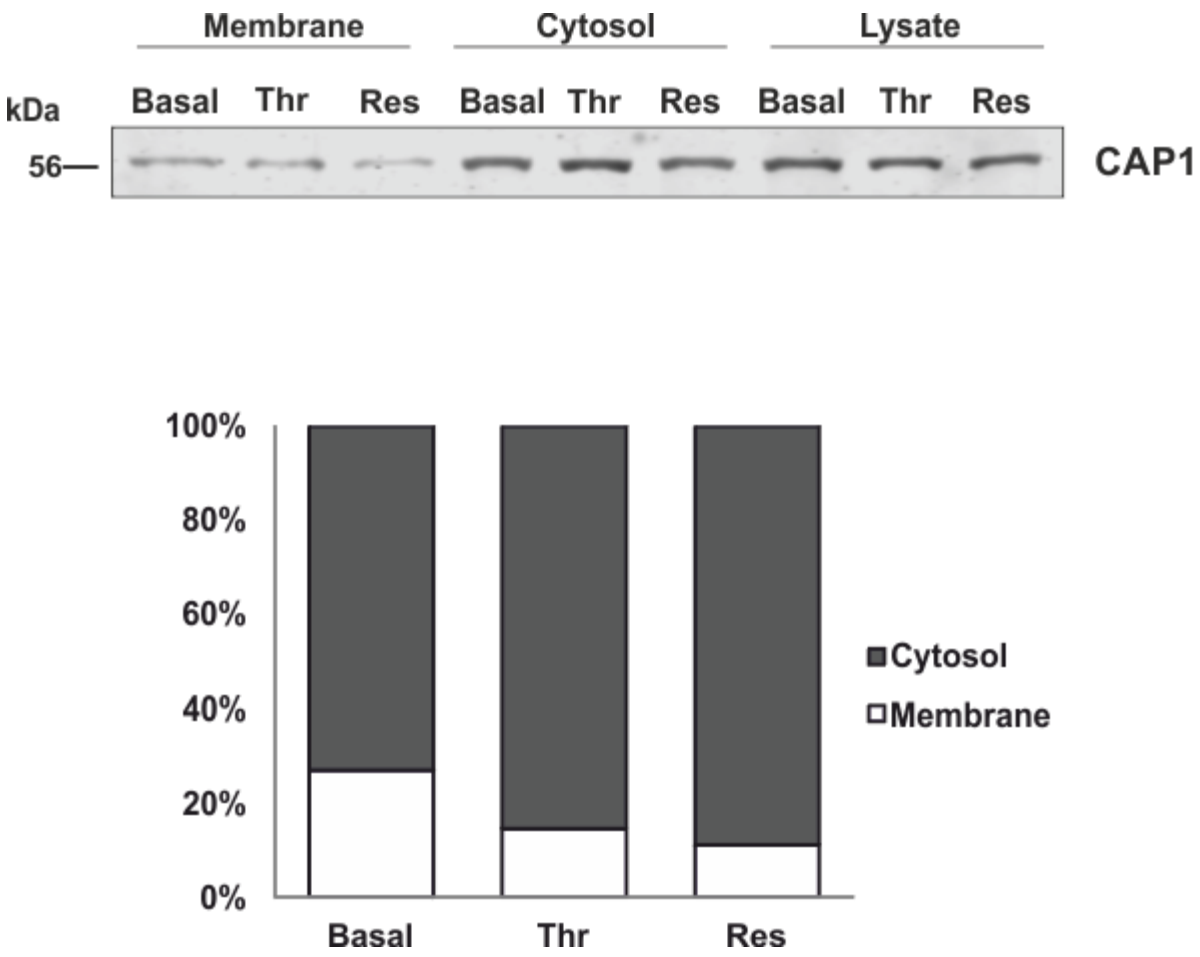


Figure 4.13 Subcellular localisation of CAP1 upon resistin treatment: Platelets were treated with 0.1U/mL thrombin for 3 minutes or with 200ng/mL resistin for 15 minutes before fractionating them into membrane and cytosolic fractions. Samples were resolved on 12% SDS-PAGE and probed with anti-CAP1 antibody. CAP1 concentrations were normalised to the whole cell lysates (assuming that whole cell lysate = membrane + cytosol) and values depicted in the graph are relative to the basal, untreated platelets from one experiment.

4.3 Discussion

4.3.1 Membrane association of CAP1 is independent of its association to actin

CAP1 which is a cytoskeletal, actin-binding protein, was interesting to study in human blood platelets owing to their rapid cytoskeletal reorganisation upon stimulation with agonists. It was reaffirming to find out that highly specialised cell fragments like platelets expressed CAP1 (Figure 4.1) and that the distribution in membrane (~20%) and cytosol (~80%) in resting platelets (Figure 4.2). The localisation of CAP1 in the cortical regions of cells was consistent with observations made in other cell types like *Dictyostelium* (Noegel *et al.*, 1999), *Arabidopsis* (Deeks *et al.*, 2007), fungi like *Magnaporthe* (Zhou *et al.*, 2012), mitochondrial membrane association in mammalian cell lines (Wang *et al.*, 2008), and THP-1 monocytic cell line (Wakeel, Kuriakose and McBride, 2009; Lee S *et al.*, 2014).

Interestingly, depolymerisation of F-actin with LatB did not result in significant changes in the levels of CAP1 observed in the membrane fraction, which suggested that membrane association of CAP1 was independent of actin in resting platelets. Additionally, observations using the high speed detergent insoluble fraction of platelets (Figure 4.3), which contains F-actin and its associated proteins, confirmed that CAP1 behaved independently of actin. The presence of CD36, a membrane protein in the detergent insoluble fractions indicated that membranous proteins, most likely associated to lipid rafts, were also sedimented with actin-associated proteins. The observations together with

the cortical localisation of CAP1 signify high probability of CAP1 association to lipid rafts or other membrane associated proteins. Another speculation is derived that CAP1 might play a role at the membrane of platelets as a receptor or a protein closely bound to a transmembrane receptor, evidence for which has been recently shown by Lee *et al*., 2014, where they propose CAP1 to be a mediator of inflammatory responses by interacting with the cytokine resistin in monocytic cells. Further investigations into the platelet response to resistin and its effect on CAP1 affecting the underlying mechanisms of CAP1 at the membrane of platelets are discussed ahead.

4.3.2 CAP1 translocates from membrane to cytosol upon thrombin stimulation

Platelets regulate their dynamic shape change upon stimulation through a number of mechanisms. As an initial step in investigating a role for CAP1 in response to external stimuli, a potent platelet agonist which leads to cytoskeletal rearrangement, thrombin was tested in established experimental systems. Both in membrane and in detergent insoluble pellet, CAP1 levels diminished in a time dependent manner upon thrombin stimulation, making it a novel finding in platelet biology. The only reference to CAP1 translocation was described by Wang *et al*. (2008), where CAP1 shuttled actin to mitochondria in apoptotic mammalian cell line. Our observations suggested that although the membrane bound CAP1 does not interact with F-actin under resting state, it could likely be interacting with actin as it translocates from membrane into cytosol owing to the

fact that there is quantifiable increase in F-actin as it polymerises during shape change in platelets.

This observation becomes more interesting by the fact that F-actin disassembly is required for normal granule secretion (Flaumenhaft *et al.*, 2005) and that one of the key known functions of CAP1 is F-actin depolymerisation by binding cofilin (Zhang H *et al.*, 2013; Makkonen *et al.*, 2013). Further experiments using LatB to depolymerise F-actin would have shed light on the actin dependence of the CAP1 translocation event. If CAP1 translocation is prevented or reduced after depolymerising F-actin, then it would prove that CAP1 translocation occurs because of its interaction with actin. If CAP1 translocation was dependent on actin, cytochalasins, which prevent actin polymerisation, would add to the understanding of the specific role CAP1 might play in stimulated platelets, where G-actin from cytoplasm polymerises to F-actin as platelet changes shape. Additional experiments using indomethacin (to block TXA_2 signalling) and apyrase (to prevent ADP signalling) to block the platelet secretome release would clarify involvement of CAP1 in granule release as co-localisation of CAP1 and actin was observed in fibrinogen spread platelets (Figure 4.8).

In spread platelets (on fibrinogen), time dependence of translocation of CAP1 from membrane to cytosol after thrombin stimulation was observed, suggesting that CAP1 might be involved in either aiding in actin turnover or might be involved in an actin independent event considering the platelets are already completely spread on fibrinogen having undergone prior stimulation and release of its secretome. Platelet secretome inhibition will give more information about

CAP1. The lack of evidence from thrombin-stimulated platelets in suspension makes further analysis of visualisation of CAP1 translocation controversial.

It was recently documented that CAP1 provides a connection between actin cytoskeleton to mitochondria by shuttling actin in response to apoptosis-signalling pathways (Wang *et al.*, 2008). The authors also showed that CAP1 translocates to mitochondria to promote apoptosis, an event which they prove is independent of actin binding. Considering subcellular fractionated platelet membrane fractions include all cellular and organelle membranes, this decrease might not be limited to peripheral membrane but an event occurring at organelle membranes, including mitochondrial membrane. These findings, therefore, add value to our CAP1 movement related observations so far and it would be interesting to investigate if CAP1 translocates to mitochondria in platelets as well. In light of the finding that resistin also causes similar translocation of CAP1 from membrane into cytosol (Figure 4.6) together with the fact that resistin is intricately involved in mitochondria-regulated metabolism (Jamaluddin *et al.*, 2012) a potential biochemical link begins to emerge between CAP1 and resistin.

4.3.3 PAR4 mediated PI3K pathway may be responsible for translocation of CAP1

PAR1 and PAR4 differentially regulate platelet response upon thrombin stimulation (Coughlin, 2000; Duvernay *et al.*, 2013). Observations that PAR4 receptor agonist lead to CAP1 translocation equivalent to or greater than

thrombin indicated that PAR4 receptor was most likely involved in engaging with CAP1 directly or through other proteins that interact with CAP1 and aid in the translocation of CAP1 from the membrane.

Evidence from inhibiting TXA_2 signalling in figure 4.4, where CAP1 translocation is greater in indomethacin treated platelets compared to resting platelets suggesting a trend that secondary platelet mediators inhibit the movement of CAP1. This means that CAP1 might, at the membrane, potentially interact or bind GPCRs that are engaged when the platelet secretome is released after activation. This also helps explain the increase in CAP1 at the membrane at lower doses of PAR4 agonist but likely surpasses the interaction at higher doses.

Evidence in the literature is that tethering of PAR4 at the membrane initiates activation of SFK which lie upstream of PI3K and work in conjunction with PKC to propagate PAR4 signalling to induce calcium mobilisation (Holinstat, 2006; Senis, Mazharian and Mori, 2014), by extension to the observations from CAP1, provide justification for investigation into PI3K dependent protein Akt. Also, a recent study in CAP1 signalling has demonstrated that a PI3K inhibitor caused modest stimulation in phosphorylation of CAP1 (Zhou *et al.*, 2014). The activation of PI3K and MAPK have been previously reviewed under stimulation by thrombin in platelets (Li *et al.*, 2010).

A broad hypothesis can be formulated from figures 4.8, 4.9 and 4.10 that the decrease in membrane bound CAP1 upon thrombin stimulation follows a

comparable time pattern of increase in phosphorylation of the PI3K-dependent protein Akt and that a trend is observed, where PAR4 may be responsible for triggering it.

It is further noted that the translocation of CAP1 is agonist dependent. The stable nature of membrane bound CAP1 in collagen stimulated platelets indicates that collagen has little effect on receptors or proteins interacting with CAP1 that enable its migration. From the lack of CAP1 translocation upon collagen stimulation, which is not dependent on GPCRs, together with evidence from thrombin and PGI_2 stimulation, which are dependent on GPCRs, it can be deduced that CAP1 localisation is dependent on binding GPCR.

4.3.4 Membrane bound CAP1 increases in response to prostacyclin

CAP1 studies in *Saccharomyces cerevisiae* have revealed a Ras-mediated elevation in cAMP levels owing to interaction of adenylyl cyclases and CAP1 under nutritional deprivation. Hubberstey & Mottillo (2002) further suggest two independent roles for CAP1; one in modulating the PKA pathway by generation of secondary messengers (cAMP) and a second pathway independent of PKA in regulation of the actin cytoskeleton. Although mammalian CAP1 has not been shown to be involved in Ras signalling directly, the results from Lee *et al.* (2014) have shown CAP1-dependent changes in cAMP in a knockdown and overexpression monocytic cell model in response to resistin. They infer that this CAP1 dependent change in PKA activation is responsible for resistin-induced IR in monocytic cells, thus mimicking an equivalent event of nutritional stress.

Hubberstey & Mottillo (2002) propose that CAP1 might be indirectly participating in PKA pathway via interacting with one or several of the Ras effector proteins, including PI3K and members of the Rho family of G-proteins that remodel the actin cytoskeleton. They propose this interaction might present links to a potential role for CAP1 in Ras signalling in mammalian cells. Further, there is scientific evidence that has shown decreased CAP1 phosphorylation in presence of a PKA activator (Zhou *et al.*, 2014).

PGI_2, a physiological platelet inhibitor, is a known activator of the PKA pathway and activates, via GPCR, adenylyl cyclases (Goggs and Poole, 2012). PGI_2 treatment of platelets provided an ideal set up to assess CAP1 localisation during PKA activation. A dose dependent increase in membrane bound CAP1

was observed in PGI_2 treated platelets. This indicated an increased association of CAP1 to the membrane in signal transduction pathway during PKA activation.

Although actin dependence of this association was not validated, an explanation for the increased presence of CAP1 in the membrane could be attributed to (a) direct association with the a membrane receptor that was active during PGI_2 treatment, or (b) an interaction with other membrane-associated proteins linked to Ras signalling pathways, like scaffold protein IQGAP1, which is known to regulate PI3K (White, Erdemir and Sacks, 2012) and which is discussed in detail in the chapter 5.

4.3.5 Extension of the study on CAP1 translocation in platelets

CAP1 knock-out mice or inhibitors to block the function of CAP1 are unavailable and silencing proteins is not established in platelets due to their obvious lack of nucleus and their limited ability to translate proteins, which makes it difficult to attribute a precise role for CAP1 in platelets. Extensive experimentation is required to completely justify the CAP1 translocation, including selective inhibition of PARs to critically assign a role in CAP1 translocation. Additionally, it may be useful to assess changes in membrane bound CAP1 in response to PKC-inhibitors and calcium chelators to help identify which stage in the signalling pathway is actually responsible for the translocation effect, or which step requires CAP1 translocation to occur beforehand. In a study investigating phosphoproteome of platelets, Serine 307 on CAP1 was identified as a phosphorylation-site (Zahedi *et al.*, 2008). It is also known than mouse CAP1 is phosphorylated by GSK3β at Ser309 (GSK3β dependent phosphorylation of CAP1 is dependent on primary phosphorylation by other kinases at Thr314). They suggest that phosphorylation of CAP1 renders it inactive by indirectly decreasing its association with cofilin (Zhou *et al.*, 2014) and it would provide great insight in how this might be regulated in human platelets by using a combination of signalling pathway activators and inhibitors – for example investigating the phosphorylation profile of CAP1 in thrombin stimulated platelets by initial treatments using GSK3 inhibitors (LiCl, 6-BIO, SB216763), inhibitors of PI3K (LY294002), ERK inhibitor (U0126) and activators of PKA

(Forskolin) and PKC (PMA) to shed light on the role in biochemical signalling pathways.

A study involving platelets of Akt knock-out mice, where secretion and aggregation defects in platelets are already described (Woulfe *et al.*, 2004a) would enable establish a role for CAP1, if any, in platelet functional over biochemical aspects. In order to extend observations from thrombin stimulation, other GPCRs can be investigated to understand if the effect is truly linked to G-proteins or if other protein complexes that bind G-proteins are responsible for binding CAP1 at the membrane. In addition, immunoprecipitation with potential protein candidates from the Ras effector proteins that might interact with CAP1 would help identify if CAP1 plays a distinct role in PKA activation, independent of its role in actin remodelling.

4.3.6 Model of CAP1 localisation in platelets in response to agonists

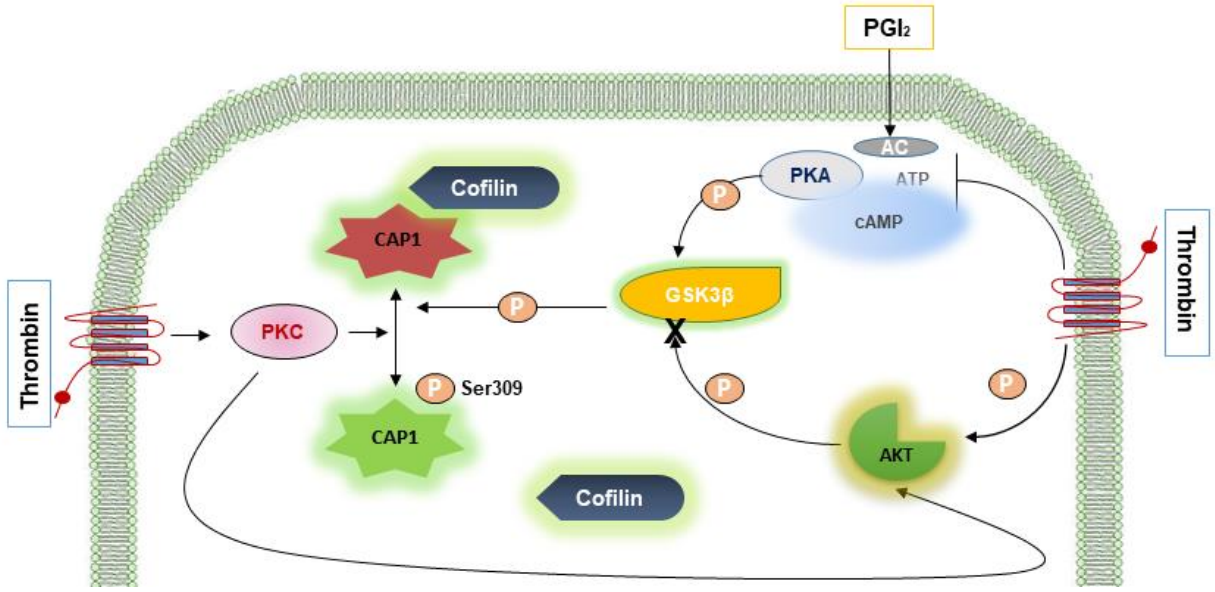


Figure 4.14 Model for CAP1 translocation in response to agonists in platelets: The speculative model is derived from evidence from endothelial and embryonic cell systems. CAP1 (red) represents the inactive form, whereas, CAP1 (green) represents its active form. Phosphorylation of CAP1, Akt and GSK3β substrates are indicated by (P). CAP1 phosphorylation occurs in response to thrombin stimulation and initiates its translocation from membrane; Phospho-CAP1 (phosphorylation by GSK3β at Ser 309) localises to the cytosol in its inactive form. In order for GSK3β to potentially phosphorylate CAP1 at Ser 309, CAP1 first is primed by phosphorylation by kinases like PKC at Thr 314. Therefore, CAP1 is rendered inactive by phosphorylation upon platelet activation. CAP1 dephosphorylation is enhanced by PI3K/Akt-dependent phosphorylation of GSK3β (inhibiting its enzymatic ability). Dephosphorylated CAP1 shows enhanced cofilin-binding and localises more cortically. Adenylyl cyclases activated in response to PGI_2 mediate phosphorylation of GSK3β, this in turn leads to dephosphorylation of CAP1 increasing its association to the peripheral membrane and cofilin. Therefore, platelet inhibitory mechanisms lead to CAP1 dephosphorylation.

When all the data is compiled and analysed against evidence from other cell types, a model of CAP1 translocation begins to emerge (Figure 4.14). Established scientific evidence suggests that (a) GSK3β causes CAP1 phosphorylation (Zhou *et al.*, 2014), (b) phosphorylation of GSK3β, which inhibits its enzymatic capability and increases sensitivity of platelets to agonist-induced aggregation, itself is mediated by PI3K dependent phosphorylation of Akt (Li, August and Woulfe, 2008), (c) Akt phosphorylation increases with thrombin stimulation (Woulfe, 2010), (d) thrombin negatively regulates PKA activation (Zhang and Colman, 2007), (e) PKA enhances phosphorylation of substrates by GSK3β (Zhang *et al.*, 2003), (f) GSK3β phosphorylation mediates cell survival (Jacobs *et al.*, 2012), and (g) CAP1 is pro-apoptotic (Wang *et al.*, 2008). These observations complement the data presented in this chapter, where thrombin causes translocation of CAP1 from membrane into cytosol and PKA activation via PGI_2 treatment and enhances its association with the membrane. Hypothetically, therefore, CAP1 dephosphorylation would cause it to localise to the membrane and phosphorylation would cause it to translocate to the cytosol, where the phosphorylation is regulated by GSK3β in response to PI3K pathway. Phosphorylation of CAP1 most likely renders it inactive in its role in actin filament turnover (as it indirectly undergoes a loss in its association with cofilin), a phenomenon similar to its binding partner cofilin (Paavilainen *et al.*, 2004; Zhou *et al.*, 2014).

Further, it can be deduced that thrombin stimulation most likely causes platelets to undergo apoptosis-like signalling (Leytin, 2012), where CAP1 would feature in its pro-apoptotic role acting as link between actin cytoskeleton and mitochondrial membrane where additional cell death pathways are triggered. The schematic depicted in Figure 4.14 helps in illustrating the CAP1 localisation occurring upon agonist stimulation.

CHAPTER 5
INVESTIGATIONS ON THE ACTIN CYTOSKELETON OF PLATELETS

The cytoskeletal protein, CAP1, was investigated in the chapter 4, owing to evidence that it may play roles as a receptor for resistin. CAP1 is responsible for actin remodelling, an event that it co-ordinates in association with several other actin binding proteins like cofilin and profilin. Our investigations revealed a change in localisation pattern of CAP1 in stimulated platelets compared to resting platelets. Also, changes in the phosphorylation patterns of cytoskeletal proteins in activated versus resting platelets are known (Zelen, 2012; Burkhart *et al.*, 2014) and are briefly summarised in a proteomics meta-analysis below (section 5.2.1).

The investigations into the platelet cytoskeleton have traditionally used approaches that include protein inhibitors, signalling pathway inhibitors, specific stimulators or mouse knock out models and the role of these proteins are reviewed in several key publications (Hartwig *et al.*, 1999; Fox, 2001; Bearer, Prakash and Li, 2002; Cerecedo, 2013; Goggs *et al.*, 2015). Additionally, platelets being anucleate, enforces that they cannot be cultured in great abundance or manipulated using common recombinant DNA technologies (Senis and García, 2012). As a result, platelet research has lagged behind that of nucleated cells. During the last decade proteomics allowed the discovery of many platelet receptors and additional insights into signalling proteins, some of which are being studied as antithrombotic drug targets (Vélez and García,

2015). Proteomics, using mass spectrometry approaches, is ideally suited for identifying low-abundance proteins, protein-protein interactions, and post-translational modifications in complex protein mixtures (Senis and García, 2012; Burkhart *et al.*, 2014; Vélez and García, 2015), and a general schematic is shown in figure 5.1.

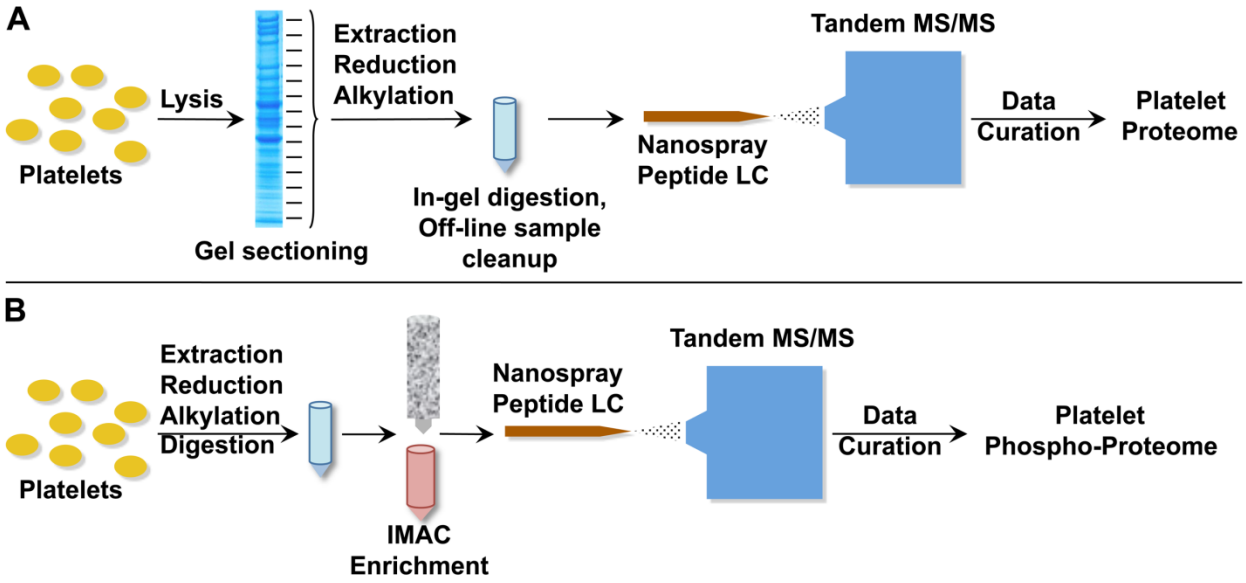


Figure 5.1 Schematic representation of platelet proteome analysis. Platelets isolated from human samples are subjected to lysis followed by gel-sectioning using SDS-PAGE, reduction/alkylation of extracted proteins. The proteins then undergo in-gel digestion and clean-up before being separated using light chromatography (LC). When generating the phosphoproteome, extracted proteins are incubated with IMAC beads to help enrich the phosphorylated proteins. Nanospray allows fine spraying of proteins and these are then detected using a mass spectrometer (MS). Data curation softwares then identify the proteins based on databases to generate the platelet proteome. Taken from (Qureshi *et al.*, 2009)

An analysis of platelet subproteomes (secretomes, exosomes, and microvesicles) has been most popular strategy for investigations into platelet biochemistry leading to higher sensitivity in identification of critical protein complexes (Vélez and García, 2015). The main drawback though is that this strategy ignores a number of other crucial proteins that do not necessarily fall into the subproteome categories.

The key proteins of interest to our lab are platelet cytoskeletal proteins. The crucial role that the cytoskeleton plays in platelet function is highlighted evermore when assessing the outcomes from studies carried out in the platelet proteome. Figure 5.2 shows that half of all platelet proteins are cytoskeletal proteins, where surprisingly, 37% are actin-myosin cytoskeleton proteins (Burkhart *et al.*, 2014). Although cytoskeletal proteins are identified as a result of proteomics analysis, we found that these studies lacked critical assessment of these proteins from a proteomics standpoint, including a lack of focus on the changes in these proteins upon thrombin stimulation. Phosphorylation of proteins is a key mechanism for proteins to become active or inactive and their phosphorylation determines the role they play in regulating multiple signalling pathways (Johnson and Barford, 1993).

Our proteomics meta-analysis indicated vast phosphorylation changes in actin and its associated proteins upon thrombin stimulation. We therefore pursued them in our investigations to include IQGAP, a scaffold protein interacting with multiple actin binding proteins; Arp2/3 complex, which nucleates and branches actin filaments, coronin 1a, which interacts with the Arp2/3 complex and is an F-

actin binding protein; villin, which is normally found in epithelial cells to perform the function of severing and capping of actin filaments, l-plastin, which is an actin filament bundling protein; myosin and tropomyosin, which form part of the actomyosin complex that drives actin filament dynamics. In order to further understand the localisation of these proteins that interact in a concerted manner in remodelling actin and in a bid to identify how other cytoskeletal proteins behave in resting platelets, we investigated above mentioned proteins that have distinct, yet related roles in numerous aspects of actin dynamics, including filament nucleation, branching, elongation, bundling, stabilisation, severing and its coordination as an actomyosin complex that provides forces within cells.

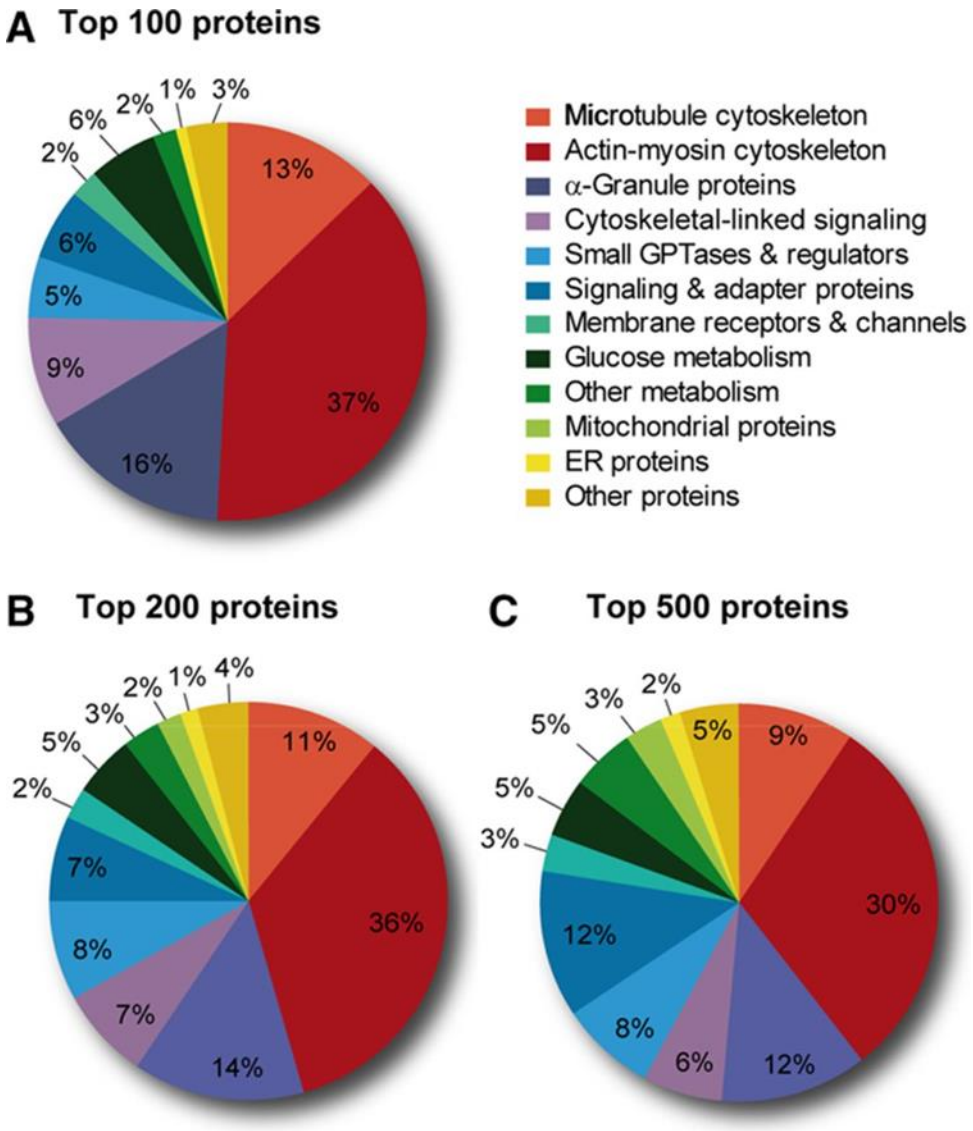


Figure 5.2 Distribution of proteins within the platelet proteome. Top 100, 200, 500 most abundant proteins are classified into functional categories. Top-100 proteins have estimated >35,000 copies per platelet, the top-200 have estimated >16,000 copies per platelet and the top-500 have estimated >6,700 copies per platelet. ER - endoplasmic reticulum. Taken from Burkhart *et al*. (2014).

5.1 Aims and objectives

A number of aspects of the platelet cytoskeleton remain unexplored due to the improbability of using recombinant DNA technology. Investigations using the platelet proteome have enabled identification of critical cytoskeletal components (Gevaert *et al.*, 2000). Further, the importance of cytoskeleton dynamics in the functional capability of platelets along with the complexity and cross-interactions in platelet cytoskeletal proteins make it extremely interesting to understand these proteins. Investigations carried out in the current chapter aim to gather preliminary information about actin associated proteins in platelets.

The main aims are:

- Perform a proteomics meta-analysis to emphasize the role of the platelet cytoskeleton

- Investigate a possible interaction between IQGAP proteins and CAP1

- Preliminary investigation of possible interaction between Arp2/3 and CAP1

- Investigate the localisation of cytoskeletal proteins (coronin1a, villin, l-plastin, myosin IIa, and tropomyosin) involved in the remodelling of actin based on their interactions with CAP1 and cross-interactions with each other.

5.2 Results

5.2.1 Proteomics meta-analysis of platelet cytoskeletal proteins

The cytoskeleton determines the shape of the platelet: discoid in resting platelets in circulation and more dynamic with flattened shape with presence of filopodia and lamellipodia during platelet adhesion and activation (Cerecedo, 2013). The cytoskeletal changes in platelets encourage thrombus formation and subsequently provide stability to sustain the thrombus under high shear forces within the arterial vessel wall (Versteeg *et al.*, 2013). Cytoskeletal changes also mediate microparticle release from the platelet secretome (Jurk *et al.*, 2005).

Platelet proteomics and transcriptomics related literature including over 87 papers and reviews, that included research in human platelet proteomics and transcriptomics in subproteomes of platelets, in proteomes of agonist-induced platelets, organelle specific proteomes and specific-protein deficient platelets or platelet proteomes of patients with platelet-related diseases were screened and further restrictions were applied to acquire information specific to thrombin induced phospho-proteome related variations in the whole platelet proteome. It was further narrowed down to include only the platelet cytoskeletal proteome which comprises around 40% of the platelet proteome (Figure 5.2). A meta-analysis of proteomics publications that review and investigate variation in the cytoskeletal proteins (based only on the functional aspect and not localisation of the respective proteins), by comparing resting platelet proteome and thrombin-induced proteome was undertaken (Gevaert *et al.*, 2000; Marcus, Moebius and Meyer, 2003; Moebius *et al.*, 2005; Macaulay *et al.*, 2005; Gnatenko and Bahou,

2006; Gnatenko, Perrotta and Bahou, 2006; Senis *et al.*, 2007; Esteso *et al.*, 2008; Zahedi *et al.*, 2008; Qureshi *et al.*, 2009; Senzel, Gnatenko and Bahou, 2009; Yu *et al.*, 2010; Májek *et al.*, 2010; Rowley *et al.*, 2011; Burkhart *et al.*, 2012, 2014; Randriamboavonjy *et al.*, 2012; Senis and García, 2012; Zufferey *et al.*, 2013; Londin *et al.*, 2014; Osman *et al.*, 2015; Vélez and García, 2015).

In this chapter we restricted the meta-analysis to the publications assessing whole platelet proteomics including thrombin induced variations in whole platelet proteomes and excluded several publications. The literature that was excluded from the analysis included proteomic variations induced by other (non-thrombin) platelet agonists, disease-state platelet proteomics or literature which investigated only a specific proteome subset; only exception to the rule was the inclusion of Gevaert *et al*. (2000), where they specifically investigate proteins translocating to the cytoskeleton. A list of publications analysed with a brief overview and methodology they used can be found in Table 5.1.

Table 5.1: Publications included in the proteomics meta-analysis detailing the methodology used and a brief overview of the investigations.

Publication	Type of study	Overview of publication	Methodology
(Gnatenko *et al.* 2006)	Transcriptomics	Human platelet mRNA transcriptome analysis	RNA-seq, Microarray, SAGE
(Rowley *et al.* 2011)	Transcriptomics	Comparison of transcriptomes of rat versus human platelets	RNA-seq, Microarray, SAGE
(Londin *et al.* 2014)	Transcriptomics	Correlation between transcriptomics versus proteomics in human platelets	RNA-seq, Microarray, SAGE
(Osman *et al.* 2015)	Transcriptomics	Correlation between transcriptomics versus proteomics in pathogen-reduced versus platelet concentrates (human platelets)	RNA-seq, Microarray, SAGE
(Marcus *et al.* 2003)	Proteomics	Phosphorylated proteins in resting versus thrombin stimulation in human platelets	2D-PAGE, MALDI-TOF MS, Nano LC-MS/MS
(Moebius *et al.* 2005)	Proteomics	Membrane proteome of human platelets in resting versus thrombin stimulation	2D-PAGE, LC-MS/MS
(Zahedi *et al*. 2008)	Proteomics	Phosphoproteome of human platelets in resting versus thrombin stimulation	IMAC-beads, SCX - protein enrichment, LC-MS
(Randriambo avonjy *et al*. 2012)	Proteomics	Comparison of proteomes human platelets of control versus diabetic patients	2D-PAGE, LC-MS/MS, Western blotting
(Gevaert *et al.* 2000)	Proteomics	Comparison of human platelet proteins that translocate to the	TX-100 insoluble pellet, 2D-PAGE, MALDI-TOF MS

		cytoskeleton upon thrombin stimulation	
(Esteso et al. 2008)	Proteomics	Porcine platelet proteome and alterations induced by thrombin	2D-PAGE, MALDI-TOF MS, Nano LC-ESI/MS
(Yu et al. 2010)	Proteomics	Global analysis of rat and human platelet proteome in thrombin induced platelets	2D-PAGE, IEF, LC-MS/MS, iTraq labelling, 2DLC
(Burkhart *et al.*, 2012)	Proteomics	Comprehensive proteomics analysis of human platelets	RNA-seq, IEF, iTraq labelling, TiO2 enrichment, SCX-protein enrichment, LC-MS/MS
(Qureshi *et al.*, 2009)	Proteomics	Proteome of human platelets in resting versus thrombin stimulation	2D-PAGE, MALDI-TOF MS, Nano LC-ESI/MS

Table 5.1: (Continued from previous page) ***Publications included in the proteomics meta-analysis detailing the methodology used and a brief overview of the investigations.***

Transcriptomics studies included non-coding RNA in addition to ribosomal and messenger RNA from megakaryocytes, which would have skewed our analysis and clear functional relevance of cytoskeletal protein transcripts could not be derived from the available information, and were therefore omitted. Analysis was further narrowed to include only proteomics publications that investigated the whole platelet proteome and assessed thrombin induced phosphorylation changes (Marcus, Moebius and Meyer, 2003; Zahedi *et al.*, 2008; Qureshi *et al.*, 2009; Burkhart *et al.*, 2012), as they presented the most comprehensive outlook towards changes in the cytoskeletal proteins.

A key theme that we validated in our analysis was that the cytoskeletal machinery is the most abundant protein category and also the most variable in platelet proteome when undergoing thrombin-mediated activation *in vitro*. This machinery included cytoskeletal proteins mainly associated with actin and tubulin along with signalling proteins that modulated the cytoskeletal proteins (Figure 5.1 and 5.2).

A list of cytoskeletal proteins and the changes in their phosphorylation status within the proteome of platelets, or lack thereof, upon thrombin stimulation has been compiled using UniProt accession number of the proteins in the Appendix 1. Tables were retrieved and compiled from proteomics publications (Marcus, Moebius and Meyer, 2003; Zahedi *et al.*, 2008; Qureshi *et al.*, 2009; Burkhart *et al.*, 2012) and proteins were manually segregated into several groups - actin and associated proteins, tubulin and associated proteins, intermediate filament proteins, myosins and other motor proteins. The classification of proteins was

based on their cytoskeletal function previously assigned in other publications (Janmey, 1995; Bearer, Prakash and Li, 2002; Bearer, 2005; Uribe and Jay, 2009; Blanchoin *et al.*, 2012, 2014; Xue and Robinson, 2013; Bezanilla *et al.*, 2015). Additionally, only heavy chains of motor proteins were included as they associate with actin or tubulin and light chains were omitted from the Appendix.

A mathematical model was generated in IBM SPSS Statistics 23, where each protein category was coded and the number of proteins under each classification was tallied. These were then converted to percentages based on total number of cytoskeletal proteins analysed and shown as a doughnut in Figure 5.3 using MS Excel.

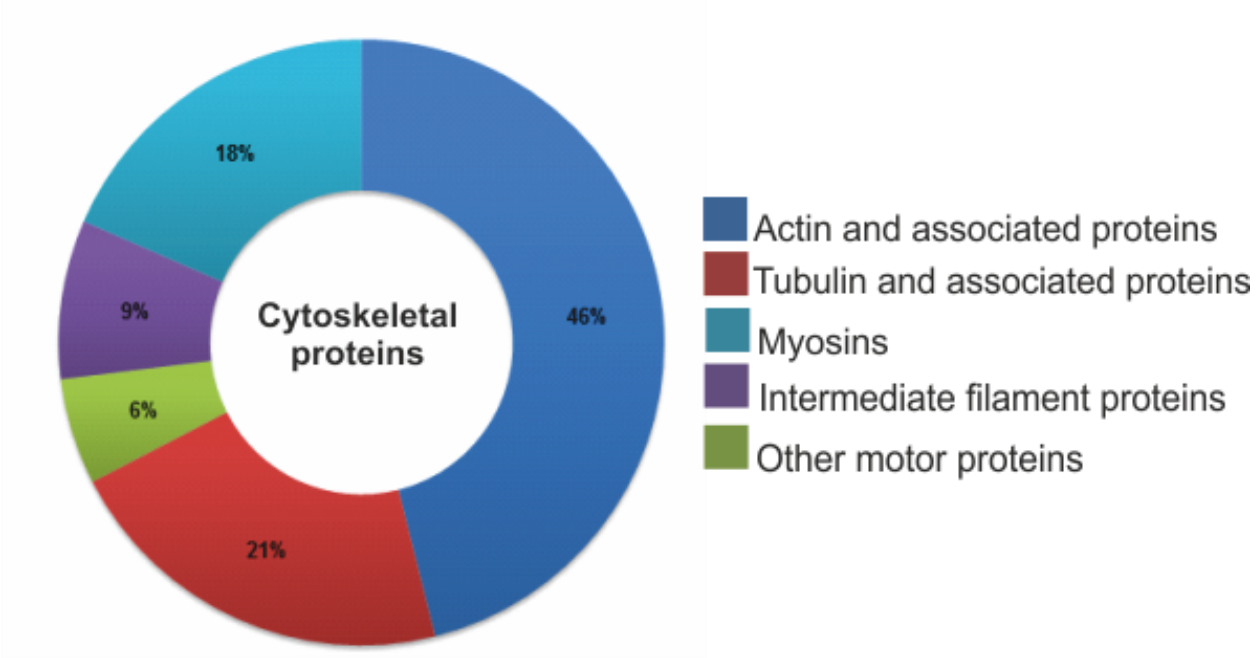


Figure 5.3: Proportions of number of cytoskeletal proteins in resting platelets. Number of cytoskeletal proteins and their distribution within the platelet proteome (from Appendix 1) is shown in the central doughnut. The key to the distribution charts is shown next to the doughnut.

Figure 5.3 shows the proportions of cytoskeletal proteins within the platelet proteome based on the total number of proteins. The distribution based on copy numbers (Figure 5.4) of the distinct types of cytoskeletal proteins was carried out next, providing a more global outlook based on the abundance of proteins. Similar to analysis described in reference to figure 5.3, a mathematical model in IBM SPSS Statistics 23 was generated which tallied copy numbers under a given category of cytoskeletal proteins (actin and associated proteins, tubulin and associated proteins, intermediate filament proteins, myosins and other motor proteins) and represented as a percentage of total copy number of cytoskeletal proteins within the platelet proteome as a mean of copy numbers from Burkhart *et al*. (2012); Marcus *et al.* (2003); Zahedi *et al.* (2008); Qureshi *et al.* (2009) and shown as a doughnut using MS Excel. A change in phosphorylation status upon thrombin stimulation – increase, decrease or no change in dot intensity on 2D-PAGE and auto-radiography was used for further classification of the cytoskeletal protein categories. An additional category included proteins where no information was available within the publications we scrutinised (Marcus, Moebius and Meyer, 2003; Zahedi *et al.*, 2008; Qureshi *et al.*, 2009; Burkhart *et al.*, 2012). These 4 sub-categories were coded accordingly in IBM SPSS Statistics 23 to generate numerical data showing variations in copy numbers. The results were converted into percentage of total copy numbers of proteins within each category and depicted as pie charts in figure 5.4 using MS Excel.

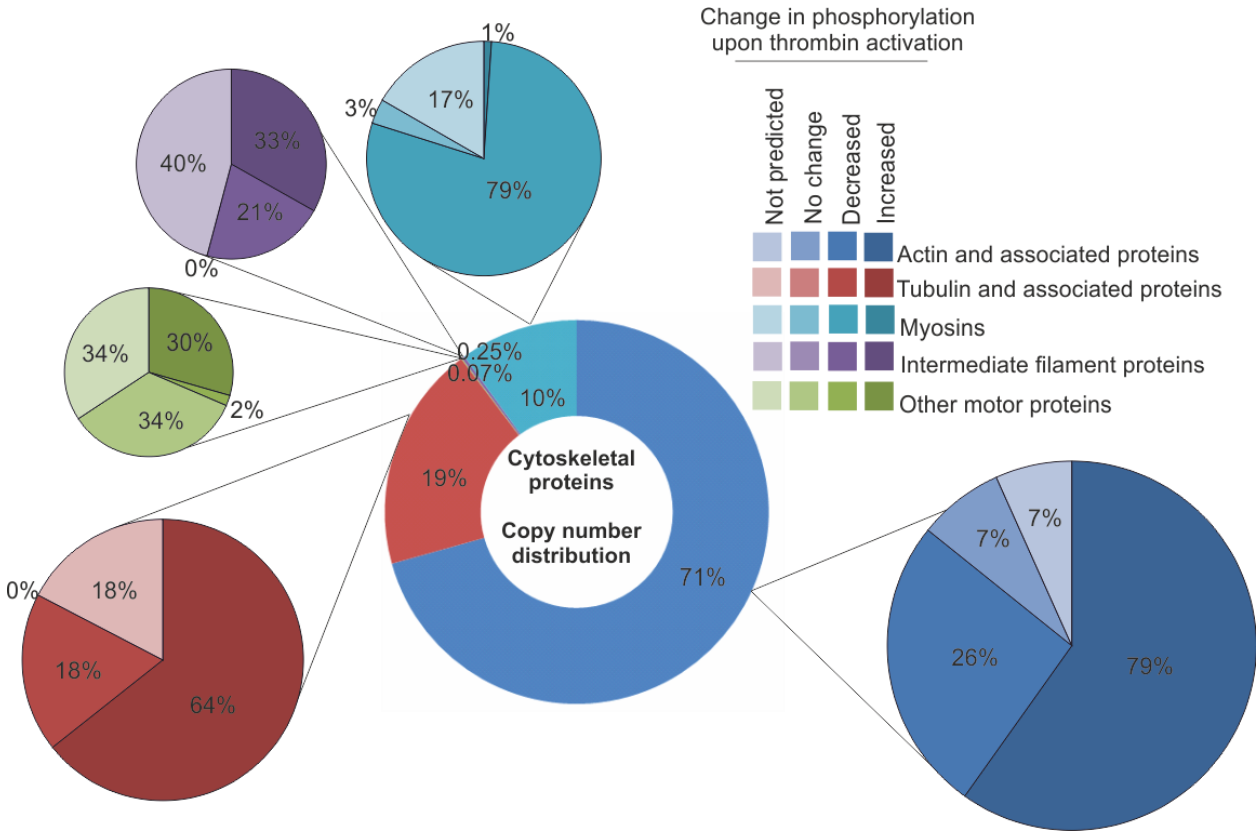


Figure 5.4 Copy numbers (abundance of protein) of cytoskeletal proteins within the resting state and those undergo change in phosphorylation status in thrombin-stimulated platelet proteome. Abundance of cytoskeletal proteins and their distribution based on copy numbers within the platelet proteome (from Appendix 1) is shown in the central doughnut. The surrounding pie-charts represent the different cytoskeletal protein groups that undergo change in their phosphorylation status upon thrombin stimulation. The key to the distribution charts is shown in the top left corner of the figure.

It is evident from both the figures 5.3 and 5.4 that the actin and actin associated proteins form the largest group of proteins forming 71% of all the platelet cytoskeletal proteins, with regards the copy numbers, and 46% of the total number of cytoskeletal proteins. Actin and associated proteins also form a significant proportion of the proteins undergoing an increase in phosphorylation upon thrombin stimulation of 79% of total actin and associated proteins, based on the abundance in copy numbers. This helps emphasize the significant role the actin cytoskeleton plays during platelet stimulation by thrombin. Tubulin and associated proteins form the second largest group comprising about 19%, in terms of the platelet cytoskeletal proteome, of which, 64% undergoes an increase in protein phosphorylation upon thrombin stimulation. The abundance of intermediate filaments, motor proteins and myosins is less compared to the tubulin and actin associated proteins, with a cumulative proportion of around 10% of total cytoskeletal proteins (Figure 5.4).

Since actin and associated proteins were the largest proportion of cytoskeletal proteins that were undergoing phosphorylation upon thrombin stimulation, we sought to identify antibodies that were well-characterised and available in our laboratory. A preliminary assessment of actin associated proteins that included, Arp2/3, IQGAP1, coronin1a, villin, l-plastin, myosin IIa and tropomyosin with respect to their localisation within platelets was carried out and detailed in the rest of the chapter, but investigation into the changes in phosphorylation and subsequent translocations, as observed in chapter 4, was not possible due to time constraints.

5.2.2 Is IQGAP an interaction partner of CAP1?

CAP1, as described in (section 1.6), is an actin-associated protein responsible in mediating actin turnover. When assessing the protein architecture, the central region of CAP1, more specifically the proline-rich region (Figure 1.8), is well conserved across species and therefore interesting for investigation in its role as a potential signalling hub. Six well defined proline recognition domains have been described (Li, 2005), namely the SH3 (Src-homology domain 3), WW, EVH1, GYF, UEV domains. CAP1 is also known to interact with small protein like profilin, which also contains the SH3 domain. Amongst these potential CAP1 partners, , only the interaction of CAP with profilin is well established (Goldschmidt-Clermont and Janmey, 1991). In order to generate a more comprehensive list of SH3 domain containing proteins that might potentially interact with CAP1, a process of elimination based on presence or absence of motifs was used. Interaction of CAP1 with EVH1 domain-containing proteins like Ena, VASP, WASP, N-WASP and Homer was deemed unlikely, as these require PPxxF (Homer) or FPPPP (Ena/VASP) motifs that are not present in any of the human CAPs. A similar argument was applied to UEV (PTAP motif) and GYF [(R/K/G)XXPPGX(R/K) motif]. The focus was then shifted to the SH3 domain containing a minimal PXXP consensus recognition sequence present in both CAP1 and CAP2 (although non-canonical motifs exist) (Peche *et al.*, 2007). Various SH3 proteins bind to the yeast CAP homolog, including Abp1, Sla3 and Rvs167 (Drees *et al.*, 2001), Abl *in vitro* (Freeman *et al.*, 1996). There are over 500 SH3-domains in proteins encoded in the human genome, very often

signalling proteins (Mayer, 2001). A screening of a library containing 296 human SH3 domains in M13-derived phagemid vectors with CAP1 as bait, but thatyielded no candidates (Kärkkäinen *et al.*, 2006).

The WW domain was therefore investigated. It is a 35-40 residues stretch with two conserved tryptophan residues approximately 20 residues apart that bind a variety of P-rich peptide motifs and are found in less than 100 domains in proteins encoded in the human genome (Kay, Williamson and Sudol, 2000; Ingham *et al.*, 2005). Hu *et al*, (2004) carried out the first comprehensive protein-peptide interaction map for a human modular domain using individually expressed WW domains and genome predicted PPxY-containing synthetic peptides. Their study revealed four candidate proteins that may bind to the P-rich region of CAP (the first three also investigated by Ingham *et al* (2005)): GAS7 (Growth arrest-specific 7 isoform b), a protein involved in neuronal development; Formin-binding protein 21 / WBP-4, involved in pre-mRNA splicing; FE65-like 2, an amyloid betaA4 precursor protein-binding protein; and IQGAP2. The IQGAP family of scaffold proteins that play important roles in actin remodeling was chosen for further investigation (Brandt and Grosse, 2007). The family consists of three members with the same architecture, of which IQGAP1 (1000 copies per platelet) and IQGAP2 (81,000 copies per platelet) are present in platelets (Burkhart *et al.*, 2012) (Appendix 1).

IQGAPs are known to scaffold multiple MAPK proteins and regulate focal adhesion formation and MAPK signaling from endosomes. IQGAP1 is also known to localize at the leading edge of migrating cells (Rac1, Cdc42-

dependent manner) (Smith, Hedman and Sacks, 2015), a pattern of localisation similar to CAP1 (Bertling *et al.*, 2004; Ono, 2013). In platelets, IQGAP2 associates with cdc42-F-actin cytoskeletal complex and rac1-complex that scaffolds Arp2/3 in a cytoplasmic complex in a GTP-dependent manner, thus functioning as a distinctive thrombin-regulated scaffolding protein in platelet activation. Further, studies in IQGAP1 null platelets implicated it in a shear-restricted procoagulant pathway modulating α-granule secretion (Schmidt *et al.*, 2003; Bahou *et al.*, 2004) making it an exciting protein to investigate within the platelet cytoskeleton.

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5.2.2.1 Co-immunoprecipitation of CAP1 and IQGAP proteins

In order to investigate a potential interaction of CAP1 with the WW domain of IQGAP a co-immunoprecipitation approach was used. 293FT human embryonic kidney (HEK) cells were transfected with Myc-tagged CAP1and GFP-tagged WW domains of IQGAP1 or IQGAP2. CAP1 was immunoprecipitated and the immune complexes were analysed by Western blotting for the presence of IQGAP-WW. Under these conditions, CAP1 is apparently able to co-immunoprecipitate IQGAP1-WW rather than IQGAP2-WW (Figure 5.5 B). Next, an attempt was made to verify that this interaction requires the proline-rich region of CAP1. Two truncated overlapping CAP1 constructs were already available in the lab. CAP1-N spans residues 1-324 and contains the proline-rich region. CAP1-C spans residues 242-475. 293FT HEK cells were transfected with Myc-tagged CAP1 constructs and GFP-tagged IQGAP1-WW. IQGAP1-WW

was immunoprecipitated and the immune complexes were analysed by Western blotting for the presence of CAP1 fragments. As shown in Figure 5.5 C, no clear co-immunoprecipitation of IQGAP1-WW with any of the CAP1 fragments was apparent. Furthermore, an attempt by Dr Wei Ji in our lab at co-immunoprecipitating endogenous CAP1 and IQGAP1 from human platelet lysates was also negative (Figure 5.5 D).

IQGAP1 and IQGAP2 play a role in integrating calcium signaling and cAMP mediated signaling, where modulation of calcium ions was found to affect IQGAP-related cell motility (Smith, Hedman and Sacks, 2015). We therefore performed this experiment in the presence or absence of calcium ions but did not observe variations in the outcome (Figure 5.5 D).

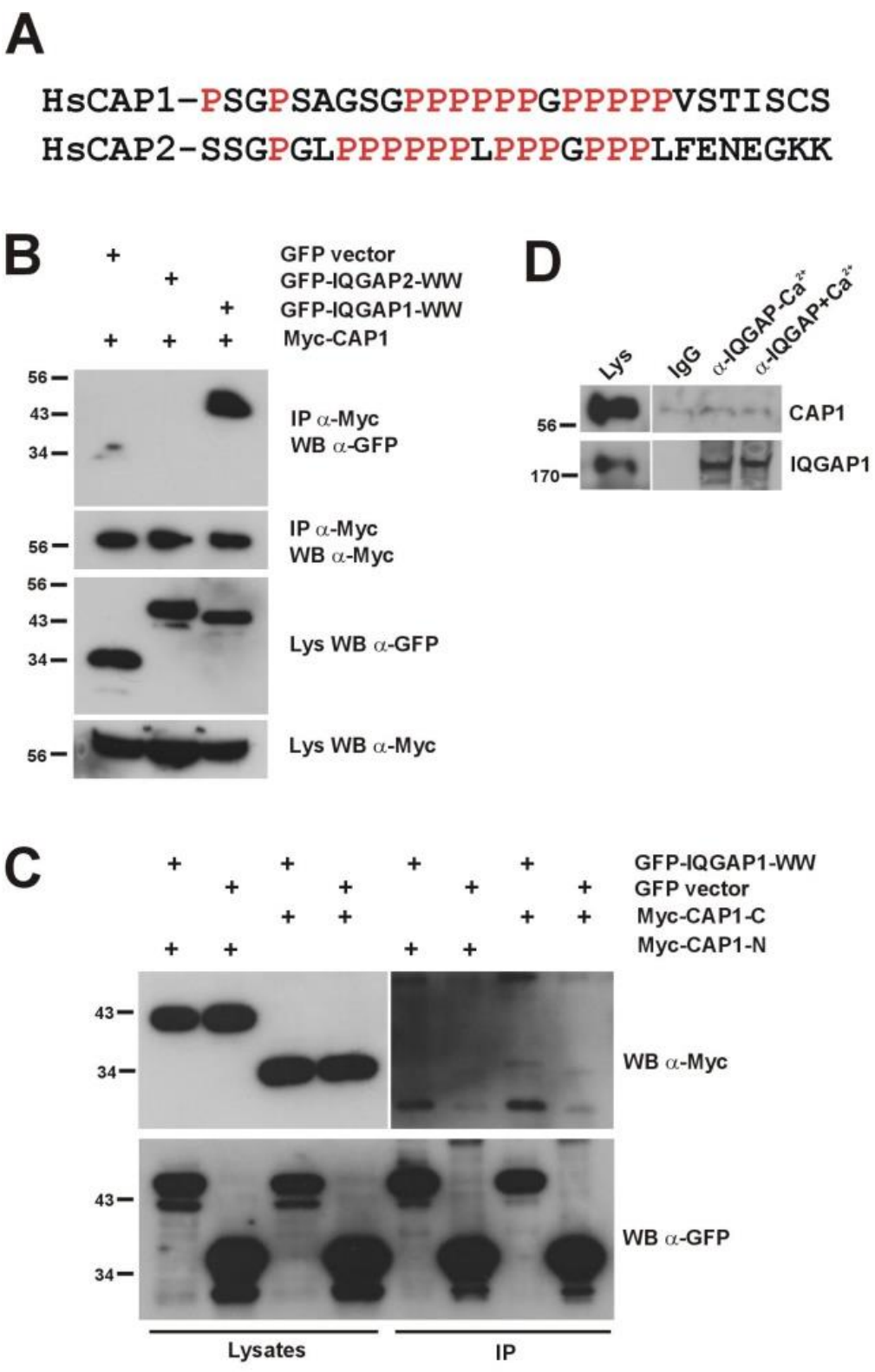
A
HsCAP1-PSGPSAGSGPPPPPPGPPPPPVSTISCS
HsCAP2-SSGPGLPPPPPPLPPPGPPPLFENEGKK
B
GFP vector
GFP-IQGAP2-WW
GFP-IQGAP1-WW
Myc-CAP1
IP α-Myc
WB α-GFP
IP α-Myc
WB α-Myc
Lys WB α-GFP
Lys WB α-Myc
56
43
34
D
Lys
IgG
α-IQGAP-Ca2+
α-IQGAP+Ca2+
CAP1
IQGAP1
170
C
GFP-IQGAP1-WW
GFP vector
Myc-CAP1-C
Myc-CAP1-N
WB α-Myc
WB α-GFP
Lysates
IP

Figure 5.5 No apparent interaction of CAP1 with IQGAP1. (A) Proline rich regions of human CAP1 and CAP2. (B) CAP1 apparently co-immunoprecipitates the WW region of IQGAP1. HEK293T cells were transfected with the indicated Myc or GFP-tagged constructs. Protein complexes were immunoprecipitated with anti-Myc antibody coupled to magnetic beads and along with the lysates (Lys) examined by Western blot for the presence of tagged proteins. **(C) No apparent interaction of IQGAP1-WW with truncated CAP1.** HEK293T cells were transfected with the indicated Myc or GFP-tagged constructs and processed as in B but using anti-GFP antibody coupled to magnetic beads. CAP-N encompasses a. a. 1-324 and includes the proline-rich region; CAP-C encompasses a. a. 242-475. The IP blot probed with anti-Myc antibody shows a longer exposure than the lysates blot. **(D) Immunoprecipitation of endogenous CAP1 and IQGAP1 in human platelets.** Platelet lysates were incubated with anti-IQGAP1 antibody or rabbit immunoglobulin (IgG) in the presence 1 mM EGTA ($-Ca^{2+}$) or 5 mM calcium ($+Ca^{2+}$). Immunocomplexes with protein A Sepharose and examined by Western blot along with the lysates (Lys). No apparent co-immunoprecipitation of CAP1 and IQGAP1 above unspecific binding. Data in panel C and D is generated with the kind help of Dr Wei Ji.

5.2.2.2 Immuno-localisation of CAP1 and IQGAP proteins

As a preliminary, visual indicator of interaction between IQGAP proteins and CAP1, immunofluorescence based co-localisation was assessed in platelets spread on fibrinogen. Platelets were allowed to spread on fibrinogen matrix for 30 minutes, fixed, permeabilised and immunostained for IQGAP1 and CAP1. Figure 5.6 A indicates that there is little co-localisation of the proteins in the cortical regions of platelets and is predominantly in different planes of analysis.

In order to further clarify if the observed co-immunoprecipitation was due to the interaction between WW domain of IQGAP1 and CAP1, HeLa cells were transfected with GFP-tagged IQGAP1 and IQGAP1-WW constructs (Images captured by Dr Wei Ji). If WW domain had a conspicuous localisation near CAP1-rich zones, that would support the probability of protein interaction. Although overlayed images show that there is some co-localisation of IQGAP1 and CAP1, there was no apparent interaction between the WW-domain of IQGAP1 and CAP1 as only GFP transfection alone has a similar distribution pattern. Although confocal microscopy is insufficient to identify interactions, proximity ligation assay would improve visualisation of the interaction between the two proteins, if any.

On the basis of observations made using immunoprecipitation and immunostaining techniques, we could not find robust interaction between CAP1 and WW domain of IQGAP1 (and probably also IQGAP2) and assume it to be unlikely under the current experimental conditions.

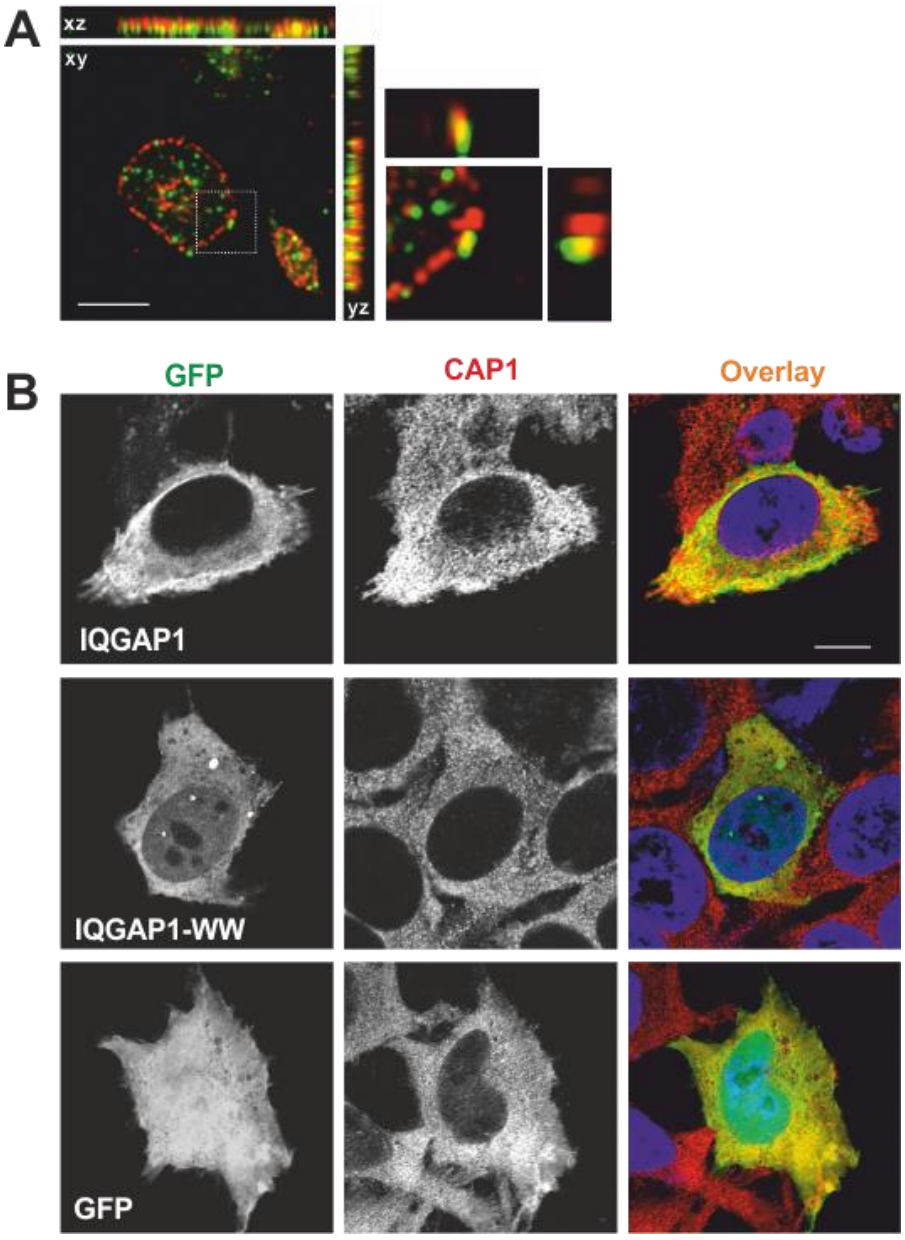

Figure 5.6 (A) Co-immunostaining of CAP1 (red) and IQGAP1 (green) in spread platelets. Platelets were allowed to spread on fibrinogen, fixed and immunostained with mouse anti-CAP1 and rabbit anti-IQGAP antibodies followed by Alexa568-labeled anti-mouse and Alexa488-labeled anti-rabbit antibodies. Optical sections were acquired with a fluorescence microscope equipped with a structured illumination attachment. Sections were 230 nm apart.

Shown is a maximum projection image in 3 planes after deconvolution and single planes of the region indicated with a square. CAP1 is shown in red, IGGAP1 in green. Scale bar-5µm. **(B) Co-staining of CAP1 (red) and IQGAP1 (green) in HeLa cells.** Cells were transfected with GFP-tagged IQGAP1 constructs fixed and immunostained with anti-CAP1 antibody. Nuclei were visualized with DAPI (blue). Images were acquired as in (A) and deconvolved single planes overlaid. Scale bar-10 µm. Images were captured in collaboration with Dr Wei Ji.

5.2.3 A possible interaction between Arp2/3 and CAP1: preliminary approach

The Arp2/3 complex is known to nucleate elongating actin filaments and is well characterised for its role in actin dynamics in platelets (Bearer, Prakash and Li, 2002; Li *et al.*, 2002). Arp2 and Arp 3 proteins with 5 additional associated proteins (p16, p20, p21, p34 and p40) form the Arp2/3 complex. When assessing their crystal structures, Arp2 and Arp3 resemble actin monomers. *In vitro* nucleation models of actin filaments have shown that "daughter" actin filaments start elongating at a 70˚ angle from the "mother" filament (Dyche Mullins, Heuser and Pollard, 1998). Further, it has been shown that different activators of the Arp2/3 complex, like Neuronal-Wiskott-Aldrich Syndrome Protein (N-WASP) and *Scar-1* (suppressor of cAMP receptor 1) spatially regulate nucleation and branching of actin filaments in 3D (Remedios *et al.*, 2003).

Li *et a*l. (2002) have shown that the Arp2/3 complex localises at the platelet cortex in response to agonist stimulation in the early stages of filopodia formation. They show that actin polymerisation rather than actin reorganisation activates Arp2/3 complex as it binds to the sides of existing filaments. N-WASP is a more potent activator of Arp2/3 compared to WASP (which is more abundantly present in platelets).

CAP1 and Arp2/3 have been shown to be active at similar times during actin filament elongation in actin turnover models (Bugyi and Carlier, 2010). Profilin, which is a known interacting partner of CAP1 (Goldschmidt-Clermont and

Janmey, 1991) is also known to bind Arp2 of the Arp2/3 complex (Remedios *et al.*, 2003). In *Arabidopsis*, CAP1-null in addition to mutation in Arp2/3 complex synergistically enhanced morphological defects, which suggested that CAP1 and Arp2/3 either regulates actin in co-ordination or that they perform related functions in similar actin regulatory pathway (Deeks *et al.*, 2007). In order to investigate the distribution of Arp2/3 in resting platelets and to evaluate its localisation in spread platelets with respect to CAP1, we used anti-Arp2/3 (Millipore p34-Arc/ARPC2) to immunostain platelets and in Western blot of fractionated resting platelet samples.

Localisation of Arp2/3 towards the cortical regions of platelets when spread on fibrinogen and collagen is consistent with observations made by Li *et al*. (2002) where they first described it in platelets spread on glass. Although, in our observations (Figure 5.7 A), CAP1 and Arp2/3 were both present in the platelet cortical regions, the punctate staining did not show any co-localisation in the fibrinogen-spread platelets and irregular co-localisation patterns were observed in collagen-spread platelets, which made it difficult to ascertain co-localisation, if any.

The distribution of Arp2/3 in resting platelets was assessed by fractionation into membrane and cytosol as described in (section 2.7) and samples that previously characterised (Figure 4.3) as devoid of contamination from either membrane or cytosolic fractions were used. Latrunculin B was used to depolymerise F-actin to assess actin-dependence of Arp2/3 to the membrane or cytosolic fractions. It was observed that 26% of Arp2/3 was found in membrane

and 74% was present in the cytosol. This did not vary significantly when F-actin was depolymerised – 28% was found to be membrane associated compared to 72% present in the cytosol (Figure 5.7 B).

The association of Arp2/3 to the detergent insoluble pellet was carried out in samples generated and characterised previously (Figure 4.4). The presence of Arp2/3 pellets and supernatants isolated at low and high speeds suggests that not all Arp2/3 is associated to the detergent insoluble pellet when platelets are in their quiescent state. When actin filaments are depolymerised using LatB prior to lysis of resting platelets, it becomes clear that the association of Arp2/3 to the detergent insoluble pellet depends entirely on binding actin, evident from its absence in LatB treated pellet samples, at both low and high speeds (Figure 5.7 C).

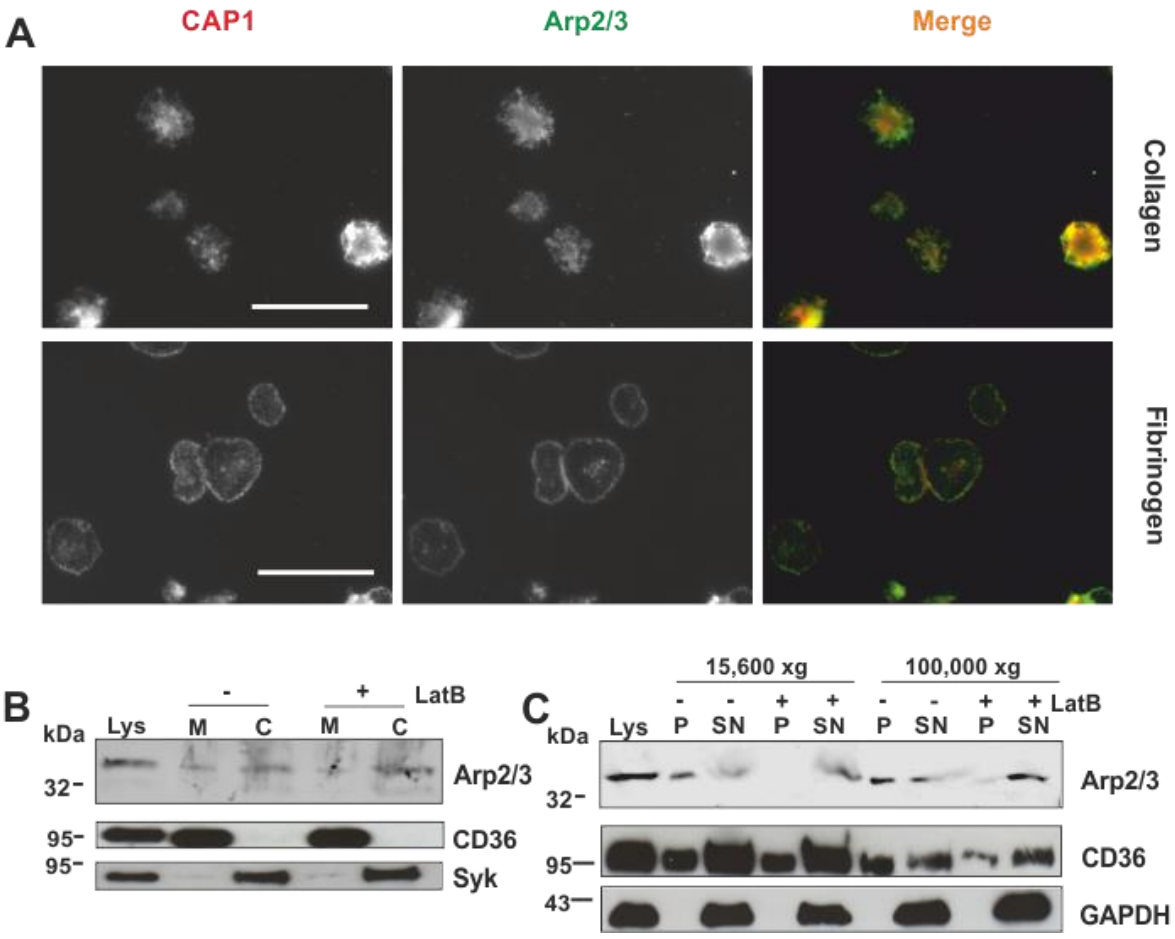

Figure 5.7 Subcellular localisation and distribution of Arp2/3. (A) Arp2/3 localisation with respect to CAP1: Human blood platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen coated coverslips or collagen coated coverslips. Platelets were fixed, immunostained for Arp2/3 (Alexa 568, secondary antibody, red) and co-stained for CAP1 (Alexa 488, secondary antibody, green) for visualisation using a fluorescent microscope. Scale bar = 10µm. (**B**) **Subcellular distribution of Arp2/3 by fractionation**: Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume and analysed using Western blotting. CD36 was used as a membrane marker, whereas as Syk was used as a cytosolic marker in resting platelets. **(C) Association of Arp2/3 to the detergent insoluble pellet:** Human

blood platelets (8 x 10^8/mL platelets) were lysed in the presence of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. The supernatant (SN) and pellet (P) were normalised by volume for Western blotting. CD36 was used as a pellet marker, whereas GAPDH was used as a marker for the supernatant.

All samples were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-Arp2/3 (anti-p34). Latrunculin B (LatB; 20µM; 20 min) was used to depolymerise F-actin prior to lysis.

5.2.4 Distribution of coronin 1a in resting and spread platelets

Coronins are F-actin binding proteins that play a role in remodelling the actin cytoskeleton. Coronin 1a is mainly expressed in hematopoietic tissues and cells and to a lesser extent in neuronal tissues. Structurally, it has 7-bladed β-propellers which contain WD-40 repeats. Interestingly, coronins also contain an acidic domain towards the C-terminal, which resembles Scar proteins, which are known to activate actin nucleating proteins like Arp2/3 (Uetrecht and Bear, 2006). Research evidence confirms a role for coronin in recruiting Arp2/3 to create nucleation sites on growing actin filaments. Additionally, coronin seems to modulate cofilin interaction with F-actin – at the barbed end, it protects the filament from cofilin-mediated disassembly whereas at the pointed end, it enhances the function of cofilin causing an increase in F-actin disassembly. Overall, coronins increase the plasticity of the actin cytoskeleton by differential modulation of other actin binding proteins (Gandhi and Goode, 2013).

Coronin 1a is required for phagocytosis, cell adhesion and spreading as shown in neutrophils and it is known to localise at the F-actin rich membrane protrusions in T-lymphocytes. In macrophages, it is proposed that coronin 1a function is mediated by phosphorylation by PI3K and is responsible for binding Arp2/3 (Gandhi and Goode, 2013; Jayachandran and Pieters, 2015). Coronin 1a depletion has also been show to protect endothelial cells from TNF-α-induced apoptosis via the modulation of p38β expression and signalling (Kim *et al.*, 2015).

In Figure 5.8 A and B, it can be seen that coronin co-localises with F-actin on fibrinogen and collagen matrices, an observation similar to those made in other haematopoietic cells (Jayachandran and Pieters, 2015). When assessing the subcellular distribution, it was observed that most coronin 1a was cytosolic (66%) and 34% was membrane associated. When F-actin was depolymerised prior to fractionation using LatB, coronin 1a association to the membrane was reduced to 24%, indicating that coronin 1a at the membrane is not entirely F-actin associated and that it may be responsible for binding other platelet membrane proteins.

When assessing its association to the detergent insoluble platelet fraction, as described previously in (Figure 4.4), it was observed that almost all coronin 1a was recovered in the F-actin rich detergent insoluble pellet at low speed of isolation and that this was reversed when F-actin was depolymerised. These observations further reinforce the strong interaction between F-actin and coronin 1a that is observed in other mammalian cell types (Uetrecht and Bear, 2006).

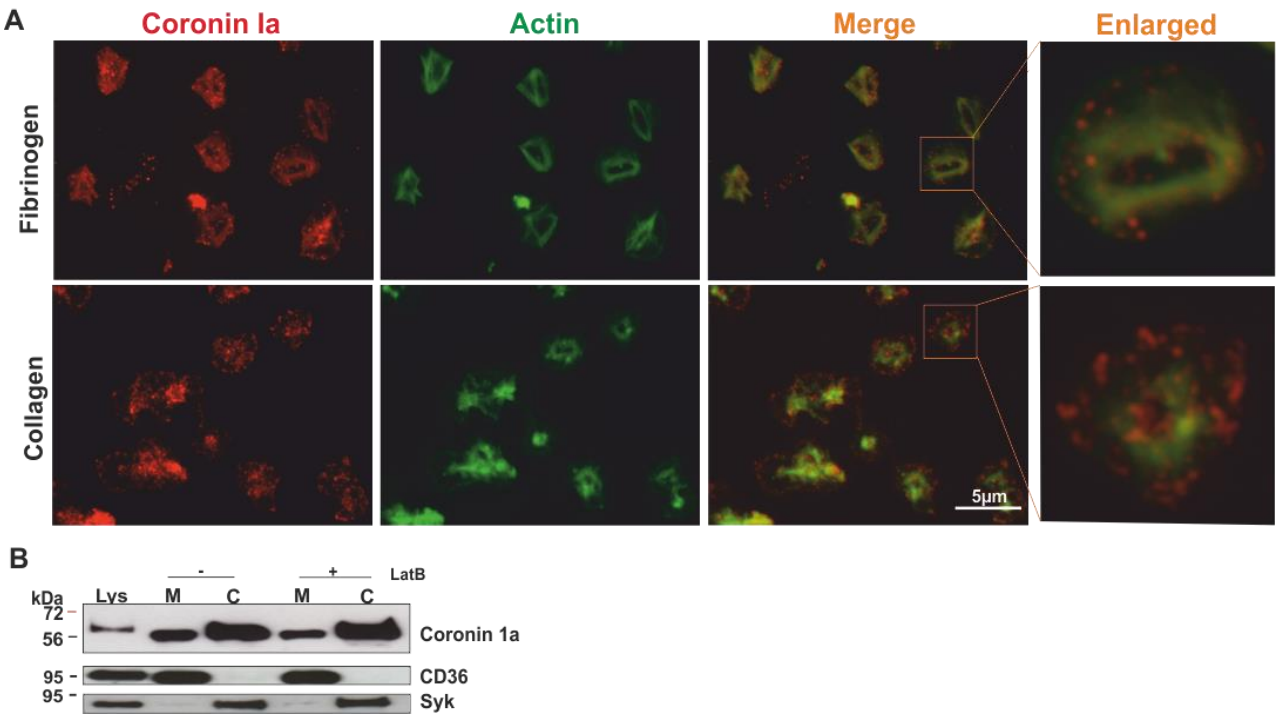

Figure 5.8 Subcellular localisation and distribution of coronin 1A. (A) Coronin1A localisation on different matrices: Human blood platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen coated coverslips or collagen coated coverslips. Platelets were fixed, immunostained for coronin 1A (Alexa 568, secondary antibody, red) and counterstained with FITC-phalloidin (green) for visualisation using a fluorescent microscope. Scale bar = 10µm. (**B**) **Subcellular distribution of coronin 1A by fractionation**: Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume for Western blotting. CD36 was used as a membrane marker, whereas Syk was used as a cytosolic marker for resting platelets. All samples were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-coronin1A. Latrunculin B (LatB; 20µM; 20 min) was used to depolymerise F-actin prior to lysis.

5.2.5 Distribution of villin in resting and spread platelets

Villin is 92.5 kDa that belongs to the gelsolin superfamily of actin binding proteins and is predominantly found in microvilli of epithelial cells. Villin contains six gelsolin repeats which form the core of the protein and a headpiece that contains the carboxyl-terminal domain which is similar to that of actin cross-linking proteins like dematin, supervillin and advillin. Villin has been described as actin-severing, -capping, -nucleating, and –bundling protein (Remedios *et al.*, 2003).

Actin binding functions of villin have been shown to vary according to the calcium concentrations – capping actin filaments at 10-30 nM and severing at 100-200 µM. Another regulation of villin occurs by tyrosine phosphorylation which reduces its affinity for F-actin. Tyrosine phosphorylated villin can sever actin at nanomolar concentration of calcium. Actin-nucleating is inhibited by tyrosine phosphorylation and high concentration of calcium is required. Actin-bundling function of villin is enhanced by its interaction with PIP_2 and inhibited by tyrosine phosphorylation. Actin-capping function on the other hand, is reduced as a result of PIP_2 interaction and is enhanced by calcium (Khurana and George, 2008). Other actin binding proteins like tropomyosin (discussed in section 5.2.8) can inhibit villin-actin interaction. The function of villin described in epithelial cells suggests a role in communicating between cytoskeleton and signal transduction pathways owing to its interaction with secondary messengers like calcium and PIP_2.

Interestingly, a recent study of the platelet proteome of resting platelets shows that 1,600 copies of villin (P09327) and 620 copies of supervillin (O95425) are present per platelet making it a novel target of investigation in platelets.

Anti-villin antibody generated in mouse host (10_2C_3) was used for the investigations. Antibody was previously characterised and used in our laboratory (Grimm-Gunter *et al.*, 2009). Platelets were spread on fibrinogen to allow visualisation of villin in platelets. Figure 5.9 A shows that villin is distributed across the platelet and seems concentrated towards the central region of the spread platelet. Interestingly, actin and villin co-localisation was observed in central region, site of granule release, but no co-localisation with F-actin-rich stress fibres was evident.

When resting platelets were fractionated into membrane and cytosol, as previously described (Figure 4.3), villin was found to be predominantly cytosolic and minimal presence was observed in the membrane fraction (Figure 5.9 B). LatB treatment to depolymerise F-actin in resting platelets did not alter the distribution of villin, as expected.

The presence of villin in the pellet and supernatant at both low and high speeds is indicative that all villin is bound to the detergent insoluble fraction (Figure 5.9 C; for controls see Figure 4.4). Further, depolymerisation of actin in resting platelets using LatB shows a lack of villin in the detergent insoluble pellets at both isolation speeds. This proves that the association of villin to the detergent

insoluble pellet in resting platelets is entirely dependent on its association with F-actin.

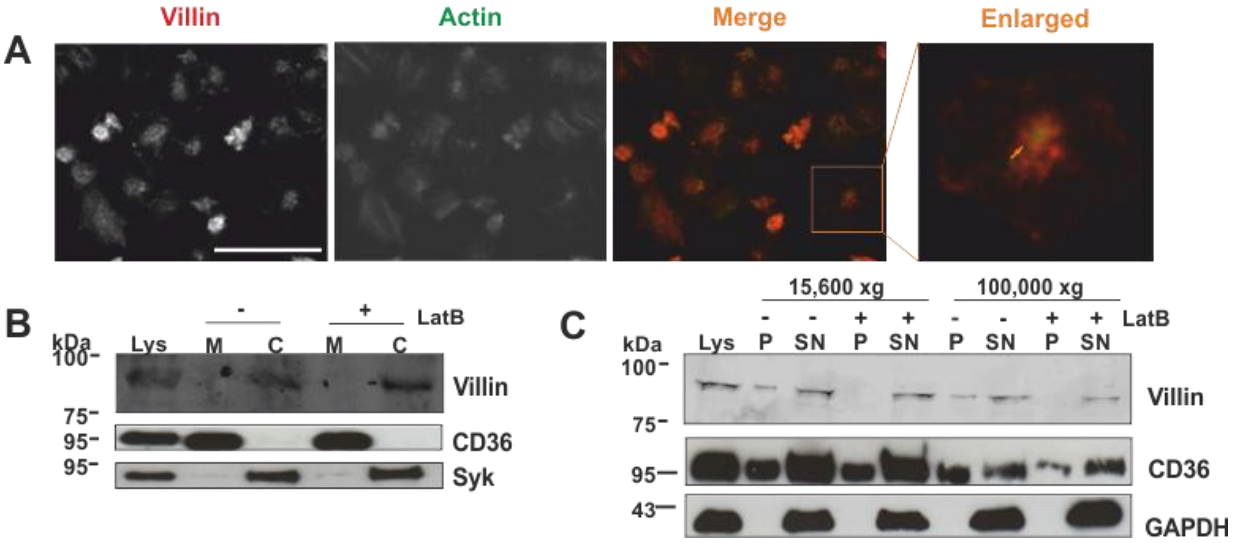

Figure 5.9 Subcellular localisation and distribution of villin. (A) Subcellular localisation of villin: Human blood platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen coated coverslips. Platelets were fixed, immunostained for villin (Alexa 568, secondary antibody, red) and counterstained for actin filaments (FITC-phalloidin, green) for visualisation using a fluorescent microscope. Scale bar = 10μm. **(B) Subcellular distribution of villin**: Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume for Western blotting. CD36 was used as a membrane marker, whereas as Syk was used as a cytosolic marker in resting platelets **(C) Association of villin in detergent insoluble pellet.** Human blood platelets (8 x 10^8/mL platelets) were lysed in the presence of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. (**A**) The supernatant (SN) and pellet (P) were normalised by volume for Western blotting.

CD36 was used as a pellet marker, whereas GAPDH was used as a marker for the supernatant.

All samples from (B and C) were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-villin. Latrunculin B (LatB; 20µM, 20 min) was used to depolymerise F-actin prior to lysis.

5.2.6 Distribution of L-plastin in resting and spread platelets

Plastins are actin-bundling proteins which belong to the α-actinin superfamily. There are three isoforms of plastin in mammals – L-plastin, T-plastin and I-plastin, all of which are structurally similar and differ only in their terminal sequences (Shinomiya, 2012). L-plastin is characteristically found in haematopoietic cells, T-plastin has a more diverse expression profile including immune cells, fibroblasts, endothelial cells, and epithelial cells and I-plastin is found in the small intestine, colon, and kidney and stereocilia of inner ear (Chen *et al.*, 2003; Grimm-Gunter *et al.*, 2009; Morley, 2012; Shinomiya, 2012).

The key features of plastin structure that differentiates it from other proteins in the α-actinin superfamily are the two tandem actin binding domains which allow simultaneous binding of two actin filaments via the calponin-homology domains (CH-domain) thus cross-linking and bundling the filaments (Shinomiya, 2012).

The actin-bundling function is initiated in response to adhesion signals generated in the immune cells - L-plastin-rich podosomal structures have been identified in migrating macrophages and osteoclasts (Morley, 2012; Shinomiya, 2012). L-plastin binds cytoplasmic domains of β_1 and β_2 integrins and it has been suggested that it might regulate inside-out signalling in leukocytes (Chen *et al.*, 2003; Le Goff *et al.*, 2010). Interestingly though, LPL deficient mice do not show any alteration in integrin-linked adhesion of neutrophils but they were found deficient in Syk activation as the function of LPL has been characterised to be downstream of FcγR activation (Morley, 2012).

L-plastin is regulated by serine phosphorylation and secondary messengers like calcium. Phosphorylation of L-plastin increased its affinity to binding F-actin and caused a consequential increase in its presence in the focal adhesions sites. In the investigations carried out in neutrophils, the phosphorylation of LPL occurred independently of its translocation to podosomes or calcium flux and actin polymerisation and a clear understanding of how it affects F-actin bundling is yet unclear (Morley, 2012). Proteomics studies in resting platelets have identified three isoforms – 1,600 copies of I-plastin (Plastin1 -P14651), 2,000 copies of L-plastin (Plastin2 -P13796), and 4,500 copies of T-plastin (Plastin3 -P13797) per platelet (Burkhart *et al.*, 2012).

In this section we characterise L-plastin in platelets using anti-L-plastin. Antibody was previously characterised and used in our laboratory (Grimm-Gunter *et al.*, 2009). In order to visualise the localisation of L-plastin in platelets, they were allowed to spread on fibrinogen for 30 minutes and immunostained for L-plastin and counterstained for actin using phalloidin. Consistent with the observations in neutrophils, L-plastin in platelets was predominantly found to be cytosolic and it co-localised with F-actin in the central region of the spread platelets (Figure 5.10 A).

When resting platelets were fractionated as previously described (section 2.7), it was confirmed that L-plastin was only present in the cytosol and this did not alter when F-actin was depolymerised using LatB (Figure 5.10 B). Although the blots presented in Figure 5.10 B and C do not give a clear picture for quantitation owing to technical issues in the blotting process, protein bands are

evident and thus we only make preliminary inferences regarding protein localisation.

The association of L-plastin to the detergent insoluble pellet in both low and high speed isolations suggests that L-plastin is not only bound to the detergent insoluble fraction but also present in the soluble fraction (Figure 5.10 C). When F-actin in platelets was depolymerised before isolation of detergent insoluble pellet, there were no differences when compared to untreated controls, which suggested that although L-plastin is associated with the detergent insoluble fraction, it is independent of its association to actin and that other it might be bound to other detergent insoluble proteins or protein complexes in the platelet cytosol.

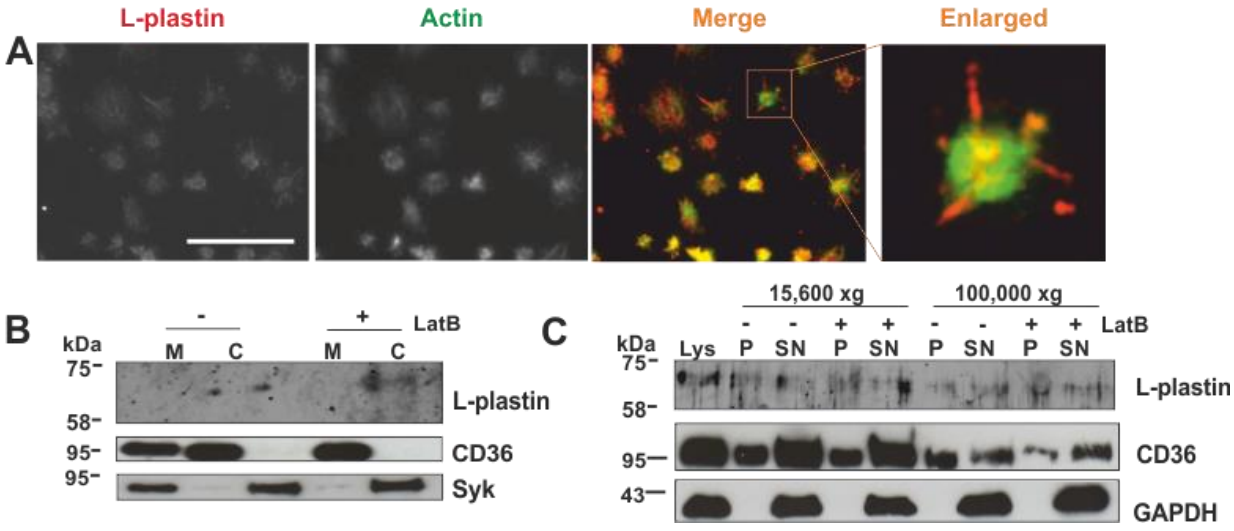

Figure 5.10 Subcellular localisation and distribution of L-plastin. (A) Subcellular visualisation of L-plastin: Human blood platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen coated coverslips. Platelets were fixed, immunostained for villin (Alexa 568, secondary antibody, red) and counterstained for actin filaments (FITC-phalloidin, green) for visualisation using a fluorescent microscope. Scale bar = 10µm. **(B) Subcellular distribution of L-plastin:** Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume for CD36 was used as a membrane marker, whereas Syk was used as a cytosolic marker for resting platelets **(C) Association of L-plastin in detergent insoluble pellet.** Human blood platelets (8 x 10^8/mL platelets) were lysed in the presence of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. (A) The supernatant (SN) and pellet (P) were normalised by volume for Western blotting. CD36 was used as marker for pellet fraction, whereas, GAPDH was used as marker for the supernatant fraction.

All samples from (B and C) were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-L-plastin. Latrunculin B (LatB; 20µM, 20 min) was used to depolymerise F-actin prior to lysis.

5.2.7 Distribution of myosin IIA in resting and spread platelets

The regulation of the cytoskeleton requires ATP-driven motor proteins like myosin IIA that regulate cell motility, cell division, migration, adhesion and polarity. In embryonic stem cell fibroblasts, myosin IIA has been shown to restrain random migration of cells and promote microtubule dynamics – thereby mediating cell contractility/motility and providing coupling between microtubules and actomyosin systems (Even-Ram *et al.*, 2007). All three isoforms of class II myosins are present in platelets –myosin IIA (P35579- 96,900 copies per platelet), myosin IIB (P35580 – 13,900 copies per platelet) and myosin IIC (Q7Z406 – 11,400 copies per platelet) (Burkhart *et al.*, 2012). Non muscle myosin isoforms studied in 3T3 fibroblast cells suggest that all isoforms have distinct subcellular localisation – myosin IIA was found enriched in the Golgi apparatus whereas myosin IIB was more peripherally located (Togo and Steinhardt, 2003), evidence for which is not available in platelets.

Myosin IIA is 230 kDa protein and the holoenzyme consists of two identical heavy chains and two light chains, which regulate the activity and stability of the protein. In platelets, myosin IIA has been shown to generate the force required for clot retraction. After stimulation of platelets, integrins get tethered to the underlying actin filaments by binding several adhesion site proteins, like talin, tensin, filamin, zyxin, paxillin, vinculin, α-actinin and moesin. Once tethered to actin, myosin is the motor that applies the contractile forces on actin filaments to cause clot retraction (Michelson, 2012).

Myosin IIA self-aggregates into smaller bipolar filaments, each containing 28 molecules. Activity of myosin IIA is regulated by 20 kDa light chain which promotes conformational changes in the protein that favours filament assembly. Phosphorylation of myosin light chain kinases by calcium ions (Ca^{2+}) generates the contractile forces applied on actin molecules (Michelson, 2012). Myosin IIA contains two different light chains (called essential and regulatory light chains); both are Ca^{2+}-binding proteins but differ from calmodulin, a Ca^{2+}-binding regulatory subunit found in many enzymes, in their Ca^{2+}-binding properties (Lodish 2000).

Magnesium ion (Mg^{2+}) driven concerted ATP hydrolysis and movement of the myosin heads along adjacent actin filaments generates a sliding motion that results in contraction of the interlinked actin filaments (Betapudi, 2014).

Myosin IIA was predominantly found to be present in the cytosol of resting platelets and in spread platelets some myosin IIA appears to be present peripherally (Figure 5.11 A). Myosin IIA association with the membrane fraction was not affected by depolymerisation of F-actin, which suggests that the membrane association of myosin IIA is actin independent (Figure 5.11 B). Antibody was previously characterised and used in our laboratory (Grimm-Gunter *et al.*, 2009).

When its association with detergent insoluble fraction of resting platelets was analysed (Figure 5.11 C) it was interesting to observe that myosin IIA was found only in the high speed isolation and under conditions of F-actin depolymerisation

was completely absent from the pellet. This meant that the minimal association of myosin IIA to the detergent insoluble high speed pellet was entirely dependent on its association with F-actin.

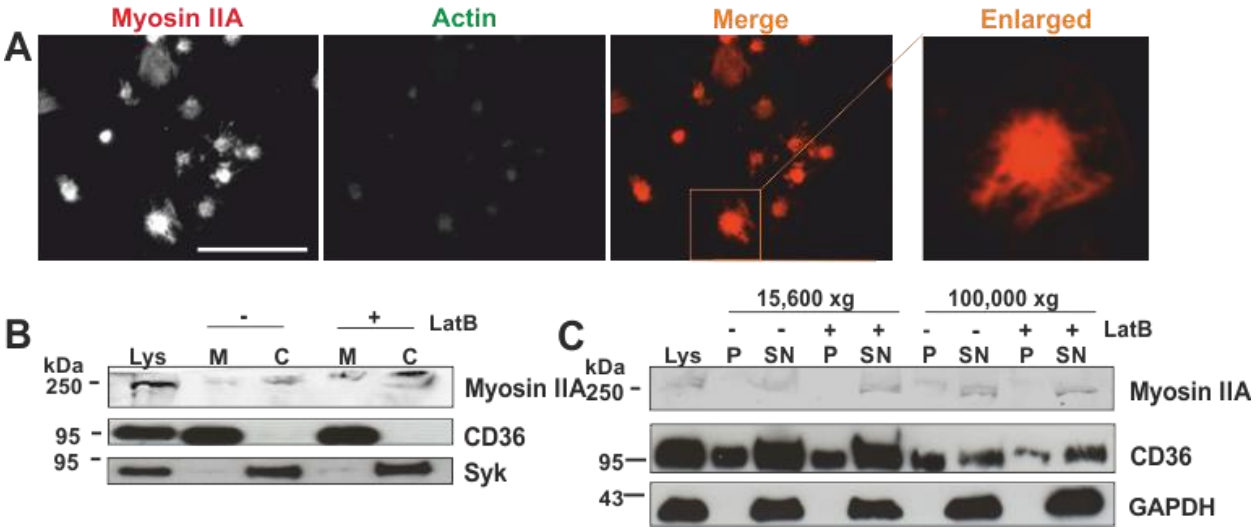

Figure 5.11 Subcellular localisation and distribution of myosin IIA. (A) Subcellular visualisation of mysosin IIA: Human blood platelets (2 x 10^7 platelets) were allowed to spread on fibrinogen coated coverslips. Platelets were fixed, immunostained for myosin IIa (Alexa 568, secondary antibody, red) and counterstained for actin filaments (FITC-phalloidin, green) for visualisation using a fluorescent microscope. Scale bar = 10µm. **(B) Subcellular distribution of myosin IIA:** Human blood platelets (8 x 10^8/mL platelets) were lysed by freeze-thaw in liquid nitrogen. Samples were then spun at 100,000xg for 1 hour to separate membrane and cytosolic fractions. The cytosolic (C) and membrane (M) fractions were normalised by volume for Western blotting. CD36 was used as a membrane marker, whereas Syk was used as a cytosolic marker for resting platelets. **(C) Association of myosin IIA to actin in detergent insoluble pellet.** Human blood platelets (8 x 10^8/mL platelets) were lysed in the presence

of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. (A) The supernatant (SN) and pellet (P) were normalised by volume for Western blotting. CD36 was used as marker for the pellet fraction, whereas GAPDH was used as a marker for the supernatant fraction.

All samples from (B and C) were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-myosin IIA. Latrunculin B (LatB; 20µM, 20 min) was used to depolymerise F-actin prior to lysis.

5.2.8 Association of tropomyosin to the detergent insoluble pellet in resting platelets

Tropomyosins make up 1.6% of total platelet proteins and are composed of two similar, non-identical dimeric subunits – αα and ββ. Non-muscle tropomyosin found in platelets lacks 37 amino acids in the amino terminal region compared to their muscle protein counterparts and the carboxyl-terminal is markedly different (Gerhards, DiGirolamo and Hitchcock-DeGregorij, 1985). Although sequence similarities exist in the other amino residues, the terminal-end differences lead to different properties and consequently differences in function of the tropomyosins. While muscle tropomyosin polymerizes head-to-tail to form long filaments that lie along actin grooves, platelet tropomyosin polymerises poorly owing to the difference in the carboxyl-terminal (Michelson, 2013). Additionally, platelet tropomyosin binds weakly to actin filaments and shows low affinity for skeletal muscle troponin (troponin is absent in platelets) (Gerhards, DiGirolamo and Hitchcock-DeGregorij, 1985; Crabos *et al.*, 1991). Taken together, this leads to the conclusion that it is unlikely for platelet tropomyosin to regulate the actin-myosin interactions and probably have a novel role in platelets (Michelson, 2013).

Tropomyosin in fibroblasts is only associated to actin filaments that are organised into stable bundles and absent from the filaments that in a dynamic state of flux and is thought to confer rigidity and stability to actin filaments in non-muscle cells (Bryce *et al.*, 2003; Khaitlina and Hinssen, 2014). A similar function in platelets can be hypothesised owing to the lack of troponin.

A preliminary assessment of tropomyosin association with detergent insoluble fraction of resting platelets provides information that tropomyosin is present in soluble and insoluble fractions at both speeds of isolation (Figure 5.12). A number of bands appear on the blot when probed with the anti-tropomyosin antibody. The bands represent multiple tropomyosin isoforms, as the antibody from Chemicon, identifies tropomyosin isforms 1, 2, 3, 5a, 5b, and 6 and it was previously characterised and used in our lab (Grimm-Gunter *et al.*, 2009).

An interesting observation was that in the low speed isolation, tropomyosins appeared predominantly in the soluble fraction in control samples, but remarkably absent in the detergent insoluble fraction when F-actin is depolymerised. Furthermore, tropomyosins appear predominantly in the high speed detergent pellet fraction, whereas the amounts in the pellet reduce when F-actin is depolymerised using Lat B. Observations from detergent insoluble pellets from both isolations suggest a strong interaction between tropomyosins and detergent insoluble pellet in resting platelets, although the interaction is not entirely dependent on actin binding. We performed immunostaining using anti-tropomyosin as we did for other cytoskeletal proteins, but due to time constraints, it was not possible to optimise the antibody for use in visualising in platelets and reproduce them successfully.

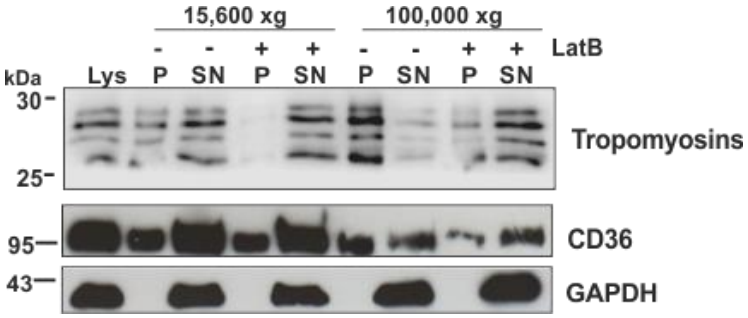

Figure 5.12 Association of tropomyosins to actin in detergent insoluble pellet. Human blood platelets (8 x 10^8/mL platelets) were lysed in the presence of 1% TX-100 and lysates spun at low speed (15,600xg) for 20 min and high speed (100,000xg) for 1 hour. (A) The supernatant (SN) and pellet (P) were normalised by volume for Western blotting. CD36 was used as a marker for the pellet fraction, whereas GAPDH was used as a marker for the cytosolic fraction. All samples were resolved on 12% SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-tropomyosin. Latrunculin B (LatB; 20µM, 20 min) was used to depolymerise F-actin prior to lysis.

5.3 Discussion

5.3.1 Proteomics of the platelet cytoskeleton: Limitations

Our proteomics meta-analysis revealed that actin binding proteins within the cytoskeleton form the biggest proportion of total cytoskeleton proteins and it has previously been shown that cytoskeleton-related proteins are the most abundant proteins in platelets (Burkhart *et al.*, 2014). We investigated phosphorylation of platelet cytoskeletal proteins upon thrombin stimulation in a meta-analysis which covered research articles that used various methodologies and platelet proteome enrichment protocols like use of phospho-protein enrichment beads (IMAC beads) (Zahedi *et al.*, 2008; Qureshi *et al.*, 2009), stable isotope labelling (SIL) and specific ion-exchange columns in the LC/MS (Marcus, Moebius and Meyer, 2003; Qureshi *et al.*, 2009). The methods used in the papers we assessed, included assessment of dot intensities of proteins on 2D-PAGE that are prone to errors – especially since complex spots can contain more than one protein and subject to false-positive protein identifications (Senis and García, 2012; Burkhart *et al.*, 2014). Moreover, the use of LC/MS techniques has overcome some of these limitations and increased the speed of analysis. It is possible to identify and quantify thousands of proteins within hours to a pre-set false discovery rate, where the unwanted identifications of random proteins are controlled. The main drawback of this approach though, is that quantitation of individual protein isoforms, which is done at the peptide level, is skewed and may not be a true representation of the protein abundance. A key example of this limitation was that CAP1 was identified in platelet proteomics studies but

CAP2 was not, although investigations from our lab and our collaborator, Dr Peche, suggest that CAP2 is present in platelets (unpublished). We identified a similar trend in our meta-analysis where, L-plastin isoform, which is the most abundant isoform of plastin in haematopoietic cells, is not represented in the copy numbers obtained from the proteomics studies, where T-plastin is the most abundant (Grimm-Gunter *et al.*, 2009; Burkhart *et al.*, 2012). Since, the three isoforms of plastins are similar, differing only in their N-terminus (Shinomiya, 2012), it may be presumed that software randomly recognises one isoform over another as a hit when assessing the MS data. Thus, it might misrepresent the true abundance of a protein isoform during protein identification.

In addition to these limitations, a key to understanding platelet proteome would lie in standardisation of platelet preparation, activation and subsequent protein purification protocols when studying platelets to give an accurate and consistent platelet proteomic profile (Harrison *et al.*, 2011) which would further enable true quantitation of the platelet proteome. Although, recent evidence from Burkhart *et al*. (2014) suggests that platelet proteome is stable across healthy donors and reproducible over multiple blood donations using LC/MS, an observation which reinforces the observations by *Winkler et al*. (2008), where they assessed 500 protein dots using 2D-PAGE.

An understanding of the phospho-proteome and the quantitative changes occurring therein would provide important clues in evaluating platelet signalling pathways and in identification of novel drug targets based on the patient's platelet proteome, a step towards the personalising of treatments in platelet

disorders or disease pathogenesis (Burkhart *et al.*, 2014; Vélez and García, 2015).

5.3.2 Actin-binding protein in platelets

Proteins that interact with actin to regulate the polymerization or depolymerization of individual filaments, or the organisation of groups of filaments into higher order structures may play several roles during different stages of platelet shape change. Although most proteins have only one specific type of interaction with actin, the state of the actin-molecule (whether globular, or filamentous and whether bound to ADP or ATP) and the presence of other associated proteins can influence how this specific activity affects the cytoskeleton of the cell as a whole. Thus a single activity can produce many different outcomes when viewed from the perspective of the whole platelet during its many morphological changes (Hartwig, Steffen and Cell, 1992; Bearer, Prakash and Li, 2002). An overview of localisation of a few actin binding proteins in resting platelets is presented in this chapter.

5.3.2.1 Arp2/3 and CAP1 have distinct distribution patterns

Arp2/3 complex in platelets has been characterised to be present in the platelet cortical regions in spread, activated platelets (Calaminus *et al.*, 2008). Arp2/3 in resting platelets is thought to be dormant and its activation is considered as the promoting step in platelet shape change upon stimulation (Li, Kim and Bearer,

2002). The observations in resting platelets indicated that most Arp2/3 is cytosolic and the association to the membrane is partially dependent on its association to actin. Further, Arp2/3 association to the detergent insoluble pellet was entirely dependent on its association to actin, confirming the evidence shown by (Fox *et al.*, 1988).

CAP1 and Arp2/3 are postulated to play a role in plant development either in a common regulatory pathway or in co-ordination as a complex regulating actin dynamics (Deeks *et al.*, 2007). Further, owing to commonality that CAP1 and Arp2/3 have a role in co-ordinating actin dynamics and have a common interacting partner in profilin (Bearer, Prakash and Li, 2002; Balcer *et al.*, 2003), we investigated if there was any underlying interaction between the two proteins. It was observed in the preliminary assessment in spread platelets that although both proteins localise cortically, there was no evidence of the two proteins interacting, or co-localising in the platelet cortex when spread on both, collagen and fibrinogen matrices. This evidence ruled out obvious possibilities that Arp2/3 and CAP1 potentially interact, but a lack co-immunoprecipitation of the two proteins would have provided more conviction to the conclusion.

5.3.2.2 Complex interactions and associations exist between actin binding proteins

Arp2/3 and coronin 1a have been established to have influence on actin nucleation. Phosphorylation of coronin 1a mediated by PI3K in macrophages is

known to affect its Arp2/3 binding (Gandhi and Goode, 2013). Coronin 1a was found to have membrane associations independent of its association to actin. Research in coronin 1a deficient mice has shown that coronin 1a influences cAMP and PKA regulation via $G\alpha_s$ in neuronal cells (Jayachandran *et al.*, 2014), which might explain its tethering to the G-protein coupled receptors at the platelet membrane under resting or activation inhibitory conditions. Research in our lab using PGI_2 -related PKA activation to understand the role of coronin 1a in platelet function is currently underway.

Presence of short actin filaments are known to activate Arp2/3-related nucleation along the sides of actin filaments. Tropomyosins inhibit this nucleation by Arp2/3 (Blanchoin, Pollard and Hitchcock-DeGregori, 2001). The lack of Arp2/3 in the high speed detergent insoluble pellet upon actin depolymerisation and a corresponding increase in tropomyosins may be indicative of this inverse relation between the two proteins. Since a precise mechanism of action of tropomyosins in platelets is to yet be defined, owing to the lack of troponins in platelets (Michelson, 2012), speculative investigations may reveal a unique function for tropomyosins in regulation of Arp2/3 activity and a role in a broader regulation of actin turnover.

Tropomyosins inhibit association of F-actin to its binding protein, villin, a protein commonly known to have a critical role in the microvilli of epithelial cells (Khurana and George, 2008). The presence of villin in platelets is unexpected as it is normally only found in epithelial cells but its presence may provide additional reinforcement to the cytoskeletal machinery that requires rapid

transformation upon activation due to the multiple actin dynamic functions that are attributed to the protein (in epithelial cells).

All villin that was found to associate with the detergent insoluble pellet of resting platelets was dependent on its association with actin. This emphasises a potential role for villin in platelet actin cytoskeleton. A role for villin in platelets can be further investigated by using Ca^{2+} and its chelators, as villin exists in an autoinhibited conformation and its association to calcium releases it, like a hinge mechanism, which makes it available for interaction with F-actin (Hesterberg and Weber, 1986). Actin capping function of villin increases in presence of Ca^{2+} and decreases with PIP_2 (Khurana and George, 2008). Additional regulation of villin activity occurs via tyrosine phosphorylation (Tomar, 2004) and preliminary investigations in platelets can be carried out in signalling pathways initiated by Src-family kinases to identify if villin truly has a role to play in actin dynamics in platelets.

Association of actin binding proteins to PIP_2 is an important regulator of actin dynamics and selectively promotes or inhibits these proteins. An important actin binding protein, profilin, when associated to PIP_2 cannot bind actin monomers, whereas hydrolysis of PIP_2 allows profilin to bind actin monomers (Lambrechts *et al.*, 1997). CAP1 associates with this free profilin in the cytoplasm and co-ordinates assembly of actin filament at the barbed end. Additionally, Arp2/3 associates with profilin as it nucleates filaments at 70˚ angle to the existing, growing actin filament generating branched, three dimensional actin networks. As seen, in Figure 4.4 all profilin was found to be present in the detergent

soluble fraction of resting platelets, a testament to its specificity for binding actin monomers.

L-plastin is thought to be sufficient to promote changes in integrin-mediated adhesion in lymphocytes. L-plastin depleted cells showed reduction in talin recruitment and actin polymerisation (Morley, 2012). Talin is an important component of focal adhesions and has been shown to localise at the actin nodules, which are podosome-like structures mediating platelet-substrate and platelet-platelet interactions (Calaminus *et al.*, 2008; Poulter *et al.*, 2015). Another important feature of talin is its ability to activate integrin-mediated inside-out signalling. Additionally, L-plastin, when phosphorylated localises to focal adhesion sites and increases attachment to F-actin, but it is still unclear if L-plastin performs this function as a part of a complex or as an adapter protein linking integrins to the actin cytoskeleton (Morley, 2012).Together with observations that talin interacts with CAP1 in HeLa cells (Haitao Zhang *et al.*, 2013), and with L-plastin in lymphocytes (Morley, 2012), it can be postulated that L-plastin, talin and CAP1 are most likely responsible for influencing the adhesive capacity of a cell and would be an interesting observation to make in platelets. Experimental set-up that investigated co-immunoprecipitation in platelet lysates and co-immunostaining in spread platelets displaying actin nodules and probed for proteins including talin, CAP1 and L-plastin would reveal interesting preliminary insights into the feasibility of the adhesion hypothesis.

The key to critically investigating any protein in platelets is by use of deficient or knockout mouse models, some of which are already available and described in

literature – of the proteins investigated in the current chapter, mouse models are available for coronin1a (Föger *et al.*, 2006), IQGAP2 (Chiariello *et al.*, 2012), CAP2 (Peche *et al.*, 2007), villin (Pinson *et al.*, 1998), and L-plastin (Grimm-Gunter *et al.*, 2009) deficiency, but there are some others that cannot be generated owing to their critical role in cell regulation and KO strategies are lethal in cofilin1 (Bamburg and Bernstein, 2010), profilin 1 (Witke *et al.*, 2001), ARPC (Suraneni *et al.*, 2012) and myosin IIa deficient mice (Even-Ram *et al.*, 2007) and yet others like CAP1 and tropomyosins have not yet been created.

Based on the preliminary observations made in the current chapter, we hypothesise that there are several actin associated protein candidates that can be investigated further and their interactions can reveal how the platelet cytoskeleton undergoes dynamic changes in an efficient manner.

5.3.3 IQGAP1 does not appear to interact with CAP1

An interaction between CAP1 and IQGAP1 was considered probable owing to presence of suitable known interacting domains on the two proteins – interaction between proline-rich domains in CAP1 with WW domain of IQGAP proteins. This probability was validated when some CAP1 was co-immunoprecipitated with IQGAP1 WW domain, in transfected cells, which was hypothesised to interact with proline-rich region in CAP1, but the IQGAP protein itself did not show any interaction.

Since there was no solid evidence of direct interaction between the likely candidates for domain interaction, a lack of overall interaction between the two proteins was assumed. Two conditions arise from this assumption – there is no interaction between the proteins or that the method has limitations which fail to recognise the interactions as the conditions under which this is studied may present ionic strengths that predispose proteins to different protein folding patterns and therefore failing to interact.

The proline-rich region of CAP1 is conserved across evolution and is thought to be vital for the role of CAP1 in its intracellular cytoskeletal remodelling. IQGAPs, have been characterised as scaffolding proteins that affect cytoskeletal reorganisation in a Rac1 and Cdc42-dependent manner via secondary signals like Ca^{2+}/calmodulin release (Smith, Hedman and Sacks, 2015). Since there was a lack of change in CAP1 and IQGAP1 interaction in presence or absence of Ca^{2+}, it was clear that any potential interaction did not occur in the calmodulin-dependent signalling pathways.

Phosphorylation of CAP1 has been shown to be an important event in regulating the function of CAP1 (Zhou *et al.*, 2014), and a speculation that conformational changes in the proline-rich region of CAP1 ensuing from phosphorylation may promote interaction between IQGAP1 and CAP1. As seen in figure 5.13, there are several putative sites of phosphorylation present within the proline-rich domain of CAP1 protein, of which Y164, T307, S308 and S310, have been confirmed in site-specific mutational analysis (http://www.phosphosite.org/). Phosphorylation of CAP1 at any of the sites within the proline-rich domains

could lead to conformational change in the protein structure, affecting the activation status of the protein and its interaction with presumed binding partners such as IQGAP1.

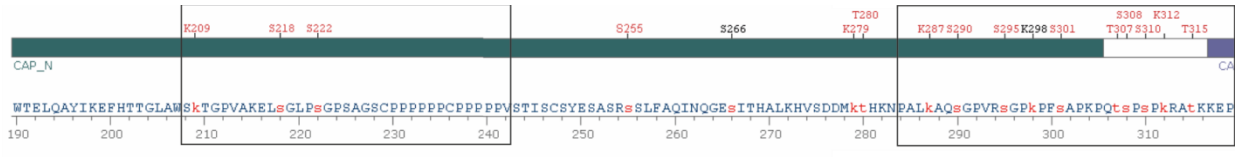

Figure 5.13 Phosphorylation sites on the human CAP1. Sites identified as phosphorylatable, based on having site-specific methods for identification include Y164, T307, S308 and S310, and most other sites identified lie within the proline-rich central region of the protein, as indicated by the outlines. (Source: http://www.phosphosite.org/)

Calcium chelating agents that bind intracellular calcium like BAPTA/AM (1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester) can provide interesting insights into the mechanism of IQGAP1 and CAP1 interaction under stimulatory conditions (where CAP1 phosphorylation state is altered) to completely rule out any potential for interaction between the domains of the two proteins.

An independent study with site-specific mutations at the phosphorylation sites on CAP1, co-transfected in cell lines as in the present investigation, could

present a profile of interactions between the two proteins, thus providing more insights into any underlying mechanisms of action. Additionally, producing recombinant proteins and testing them using surface plasmon resonance (SPR) strategies would enable assessing any possible direct interaction between the two proteins. If a confirmation of interaction is observed, proximity ligation assays could help visual assessment of the interaction within platelets.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE OUTLOOK

The research surrounding MetS and its associated co-morbidities like CVD have highlighted the role of adipokines in the disease progression. Novel observations are reported in this thesis regarding the potential role of resistin in platelet activation, thus contributing towards the building of a preliminary disease model *in vitro*. This study enhances the knowledge in the field by showing that resistin blunts thrombin mediated platelet aggregation and dense granule secretion, and that it affects the cytoskeleton in addition to activating downstream effectors of PI3K and MAPK pathways. Interestingly, these investigations into the proposed resistin receptor, CAP1, have brought to the forefront the phenomenon of CAP1 translocation upon agonist stimulation. Explorations in the platelet model have revealed that CAP1 translocates to the cytosol when stimulated with thrombin, while it translocates towards the membrane when inhibited with PGI_2. Additionally, this study also found that resistin caused a translocation of CAP1 away from the membrane, albeit this observation was only preliminary. Furthermore, the subcellular localisation of several actin cytoskeletal proteins was assessed, where the limitations of working with platelets became more apparent in elucidating the precise role of cytoskeletal proteins.

Platelets are known to be inherently difficult cells to manipulate as they are not amenable to microinjection due to their small size. Also, their lack of a nucleus precludes genetic manipulation, and slight changes in their environment can result in activation (Patel-Hett *et al.*, 2008). These limitations have prevented direct visualization of cellular dynamics in resting and activated platelets. Insights into cytoskeletal structures have come primarily from microscopic analyses and assessments of the detergent insoluble fractions of resting and activated platelets (Hartwig *et al.*, 1999; Fox, 2001; Patel-Hett *et al.*, 2008; Senis and García, 2012). The solution to overcoming these limitations comes with proteomic analyses and protein assessments in gene knockdown models to gain further insight into the regulation of the platelet cytoskeleton (Patel-Hett *et al.*, 2008; Senis and García, 2012).

The results achieved here have presented with possibilities of further discussion where CAP1 and resistin might have relative roles that are in tandem rather than ones previously suggested in a ligand-receptor relation. It has therefore opened up avenues for additional exploration in the field, which follows in the sections ahead.

6.1 Does resistin affect platelet receptors?

G-protein coupled receptors are regulated by two main families of protein kinases – GRK (G-protein coupled receptor kinases), which phosphorylate agonist-occupied GPCRs to mediate homologous receptor desensitisation (Figure 6.1 A) and secondary messenger (Ca^{2+}) activated kinases, such as PKC, which phosphorylate ligand-bound and inactive GPCRs in a heterologous manner (Figure 6.1 B) (Hardy *et al.*, 2005). PAR1 signalling was desensitised using its receptor agonist peptide at subthreshold levels, where PAR1 was not internalised but the signalling cascade including Ca^{2+} mobilisation, PKC signalling and α and dense-granule secretion were downregulated. This desensitisation of the signalling pathway was resensitised by subthreshold level doses of PAR4 peptide (Ubl, Sergeeva and Reiser, 2000; Fälker *et al.*, 2011). PKC-mediated desensitisation of purinergic GPCRs like $P2Y_1$ and $P2Y_{12}$ on platelets, when exposed to ADP in a concentration and time dependent manner is previously reported by (Hardy *et al.*, 2005).

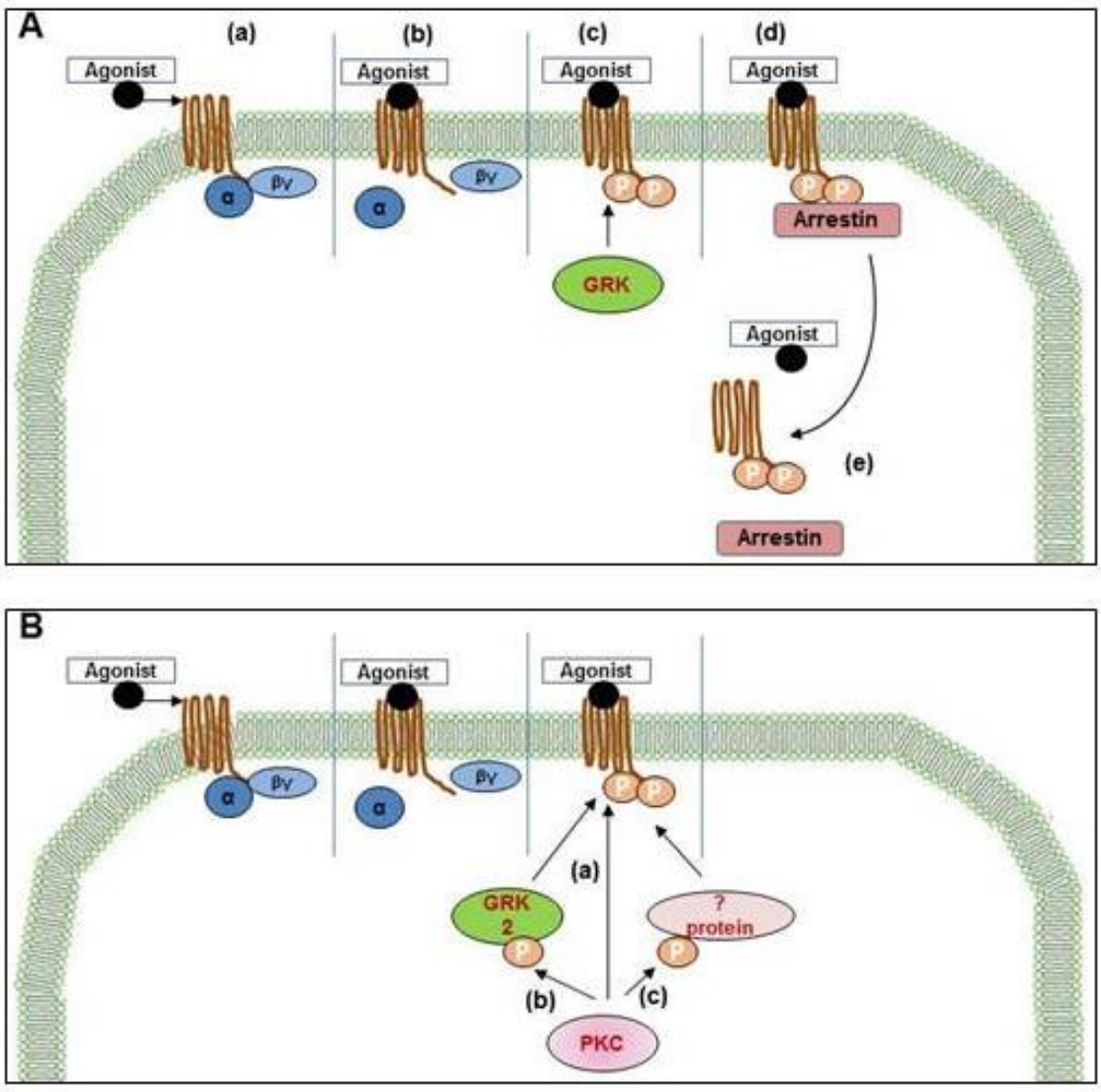

Figure 6.1 Model mechanisms of G-protein coupled receptor desensitisationin platelets. (A) Classical model. Upon stimulation of platelets by an agonist (a) The GPCR is activated by agonist leading to (b) G protein coupling and effector modulation, where the α and βγ subunits dissociate, allowing phosphorylation by GRK. (c) The agonist-occupied GPCR is subsequently phosphorylated by GRK, and (d) arrestin binds to the phosphorylated GPCR, leading to receptor desensitisation, thus preventing additional GPCR mediated signalling and (e) is followed by internalisation of the receptor. **(B) Secondary messenger dependent-kinase model.** The first events in classical model after agonist stimulation are similar and involve GPCR

coupling and subsequent dissociation of the α and βγ subunits. The difference is that arrestins do not play a role in GPCR desensitisation, instead alternative events may follow which are dependent on secondary messenger proteins that are also activated during agonist stimulation: (a) Activated protein kinase C (PKC) can directly phosphorylate and desensitise the GPCR, or (b) PKC can phosphorylate and activate GRK2, which consequently has an enhanced ability to phosphorylate the GPCR, or (c) PKC can phosphorylate other, as yet unidentified, regulatory proteins, which then effect GPCR desensitisation. Desensitisation of GPCR render them unavailable for triggering their respective downstream signalling pathways (Adapted from (Kelly, Bailey and Henderson, 2008)

The hypothesis that is presented here is one of resistin-mediated pre-activation of platelets. A variation on the hypothesis that has not been explored as a part of this study is receptor desensitisation. The identity of the precise thrombin receptor which is activated in response to resistin treatment is still unknown, an assessment of effect of resistin on platelet aggregation responses upon stimulation with receptor agonist peptides would provide preliminary clues about the receptor involved. PAR1 and PAR4 receptor agonist peptides (SFLLRN and AYPGKF, respectively) can be used to determine if there is any dose dependent desensitisation of receptors (Kim *et al.*, 2002). Additionally, flow cytometric experimental evidence to determine the change in surface expression of PAR1 or PAR4 can be assessed to rule out the possibility of receptor internalisation owing to exposure of platelets to resistin. Since PKC activation occurs upon activation of PARs, a fura-2 fluorescence-based assay using pan-PKC inhibitors [Ro31-8425 and Ro31-8220 (Fälker *et al.*, 2011)] as controls when characterising intracellular Ca^{2+} mobilisation (Kong and Lee, 1995) would help outline if signalling downstream of PAR was affected by resistin. Together these experiments would clarify which PAR is activated in response to resistin and if the blunting of platelet aggregation that was noted was a result of receptor internalisation or receptor desensitisation. Above experimental set-up with additional control by using PPARγ-inhibitors, which are described as resistin inhibitors (Steppan and Lazar, 2004), can help account for resistin-mediated activity of receptors and reveal the existence of additional mechanisms of resistin action on platelet function.

This data suggests that dense granule release is inhibited by resistin treatment of platelets, whereas an evaluation of α- granules is pending. The report by Qiu *et al*. (2014) suggesting an increase in P-selectin expression upon resistin treatment lacks key controls that enable assessment of α- granule release and a repetition including the controls will clarify if there is any differential regulation of granule release upon exposure of platelets to resistin.

6.2 CAP1 in actin nodule-mediated platelet adhesion

Actin nodules are podosome-like structures that develop in spreading platelets and form a network of actin structures that are thought to play a key role in platelet-substrate and platelet-platelet interactions (Calaminus *et al.*, 2008; Schachtner *et al.*, 2013; Poulter *et al.*, 2015). It has been reported that actin nodules are enriched in cytoskeletal proteins like talin, cortactin, Arp2/3 and vinculin (Calaminus *et al.*, 2008), whereas the centre of the actin nodules appears devoid of integrin β3, only appearing as clusters on the periphery of the nodules (Poulter *et al.*, 2015). Interestingly, CAP1 localising at the actin nodules in spread platelets was observed. In its role in cell motility and cell adhesion, CAP1 was shown to co-IP with FAK and talin in HeLa cells (Haitao Zhang *et al.*, 2013). Talin, which is a key component of focal adhesions, regulates integrin-mediated inside-out signalling (Nieswandt, Varga-Szabo and Elvers, 2009; Ciobanasu, Faivre and Le Clainche, 2013) along with several other cytoskeletal proteins like zyxin, vinculin, VASP, α-actinin, skelemin, L-plastin, and others (Calderwood, Shattil and Ginsberg, 2000). CAP1 association

with talin and the observations that CAP1 localises at actin nodules, together is suggestive of the role CAP1 might play in signal transduction from integrins, during activation, to the actin cytoskeleton, or from the cytoskeleton enabling integrin activation, a role similar to that of talin and L-plastin (Morley, 2012; Ciobanasu, Faivre and Le Clainche, 2013; Haitao Zhang *et al.*, 2013).

In order to assess if CAP1 played a role in adhesion in platelets, a preliminary step would be performing co-immunoprecipitation in platelet lysates and assessing association of CAP1 with proteins like integrinβ3, talin, and FAK. Simultaneously, observations in spread platelets can be sought by co-immunostaining for CAP1 along with integrinβ3, talin, and FAK to replicate and identify any co-localisation of CAP1 with talin and other indicated proteins. CAP1-talin association would suggest that CAP1 was involved in regulating integrin inside-out signalling, and CAP1-integrinβ3 association would further help corroborate the possibility that CAP1 plays a role in platelet adhesion by means of actin nodules.

Actin nodules are also sites of phosphorylation of tyrosine residues (Poulter *et al.*, 2015). Phosphorylation of Tyr397 on FAK is critical for FAK activity and promotes its association to Src (Schlaepfer, Mitra and Ilic, 2004). It would be interesting to find if FAK phosphorylation affected CAP1 localisation to actin nodules, but unfortunately it would be difficult to perform experiments in platelets to elucidate it, owing to the lack of phospho-CAP1 antibodies, lack of CAP1 KO mice and the impracticality of silencing protein expression in platelets.

6.3 CAP1 and resistin in apoptosis

Elevated levels of CAP1 and resistin have both been correlated with multiple cancer pathologies as described in sections 1.3.3 and 1.6.2. Additionally, matrix-metalloproteinase-9 (MMP-9) elevation in response to resistin-mediated PKCε activation (Ding *et al.*, 2011) and MMP-9 specific degradation of CAP1 as a substrate (Cauwe *et al.*, 2008), juxtaposes MMP-9 as a factor that might potentially regulate the interaction of the two proteins *in vivo*.

6.3.1 Novel role for resistin in clearing extracellular CAP1

The main criticism of Lee *et al*. (2014), where they first presented evidence of CAP1 and resistin interaction is the demonstration of protein-protein interaction using recombinant proteins, without sufficient evidence *in vivo*. The critique also includes the lack of extracellular CAP1 domain required for binding extracellular resistin, in addition to the absence of resistin internalisation. Interestingly, CAP1 was found in the urine samples of patients with systemic lupus erythromatosus and associated as an endothelial cell related autoantigen, suggestive of the importance of clearing all extracellular CAP1 (Frampton *et al.*, 2000; Cauwe *et al.*, 2008). Extracellular CAP1 may be required to prevent actin polymerisation or block free ADP sites on F-actin. One study notes that mice that were given intravenous G-actin showed pulmonary venous obstruction by actin filaments in addition to pulmonary microthrombi. ADP bound to F-actin (in 1:1 ratio) acts as a more potent platelet agonist compared to free ADP alone owing to its multivalency and can lead to wanton thrombus formation when F-actin is found

in circulation (Scarborough, Bradford and Ganguly, 1981; Lee and Galbraith, 1992) thus highlighting the need to block ADP sites or be degraded effectively in circulation.

The fact that CAP1 is present in the extracellular milieu owing to cytolysis (Cauwe *et al.*, 2008) presents an alternative, where CAP1 and resistin might potentially interact with each other in the extracellular domain without the need for resistin internalisation. The circulating levels of CAP1 and resistin might determine the efficiency of the interaction, if any. Since resistin causes an elevation in MMP-9 expression in vascular smooth muscle cells (Ding *et al.*, 2011), it might suggest a novel role for resistin in healthy subjects, in clearing extracellular CAP1, removing its actin regulatory effects, thus preventing actin toxicity resulting from cytolysis and protecting against systemic autoimmune diseases (Lee and Galbraith, 1992; Cauwe *et al.*, 2008).

6.3.2 A pro-inflammatory complex of CAP1-resistin in promoting apoptosis

Another avenue of investigation could be along the lines of CAP1-mediated apoptosis in platelets (Leytin *et al.*, 2006; Gyulkhandanyan *et al.*, 2012, 2013; Leytin, 2012; Posch *et al.*, 2013; Zharikov and Shiva, 2013). CAP1 has been established as a pro-apoptotic protein that shuttles actin to mitochondria in response to endogenous apoptosis inducers in mammalian cells. CAP1 provides a direct link from the actin cytoskeleton to the mitochondria (site of apoptosis) by functioning as an actin shuttle in response to signals that cause

apoptosis (Wang *et al.*, 2008). Resistin on the other hand, has been demonstrated to be a survival factor in mammalian cells, responding to ER stress (Gao *et al.*, 2009; Suragani *et al.*, 2013; Rak *et al.*, 2015). Contrasting to this, elevated levels of resistin are also confirmed to increase the production of MMP-9. MMP-9 when overexpressed, is pro-apoptotic (Bergers *et al.*, 2000; Mannello *et al.*, 2005; Ding *et al.*, 2011). MMP-9 also helps decrease CAP1 levels *in vivo* (Xie *et al.*, 2014) which is anti-apoptotic. Owing to such contrasting observations in relation to these three proteins *in vitro* and *in vivo*, a hypothesis emerges where these proteins have distinct roles under varying physiological conditions. Extrapolating the evidence, it can be speculated that intracellular CAP1 is pro-apoptotic, but when released extracellularly after cytolysis, it forms a complex with resistin forming a pro-inflammatory complex that promotes cell-death or carcinogenesis. Further adding to the speculations, MMP-9-mediated degradation of CAP1 may be prevented if it forms a complex with resistin and can help explain the distinct observations in relation to resistin and MMP-9, with respect to CAP1, where free resistin is anti-apoptotic, and supposedly, CAP1-resistin complex increases MMP-9 expression leading to tumourigenesis.

Validation of this speculative hypothesis of CAP1-resistin-MMP-9 complex formation would be extremely difficult using only platelets as the key cell model of investigation. As such, MMP-9 is known to have inhibitory role in platelets (Wrzyszcz and Wozniak, 2015). The use of endothelial cell-lines, megakaryocytic cell lines, since these closely affect platelet behaviour, would enable the convenience of various knockdown tools in resolving potential

protein-protein interactions. An ideal candidate for this investigation would be endothelial cells, owing to CAP1-related auto-immune pathology (Cauwe *et al.*, 2008) and resistin-mediated effects on endothelial cell signalling (Dandona *et al.*, 2006; Manduteanu *et al.*, 2009). Additional tools that could be used would include anti-CAP1 antibody to clear extracellular CAP1, kinase-inhibitors to prevent downstream effectors of apoptosis, TZDs to dampen resistin-related outcomes and Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) which prevents MMP-9 expression would be deemed as effective controls during *in vivo* investigations. In order to delineate the role of CAP1 in regulating the actin cytoskeleton (Hubberstey and Mottillo, 2002; Ono, 2013; Zhou, Zhang and Field, 2014) and its role in apoptosis, which is shown to be actin-independent (Wang *et al.*, 2008), the use of actin depolymerising agents or inhibitors of actin polymerisation like latrunculins and cytochalasins would aid in distinguishing the two roles and in firmly assessing how CAP1 interacts with other proteins, like resistin, independently. Positive tests of apoptosis would include mitochondrial toxins and potent agonists like thrombin, which are known to induce apoptosis in platelets (Lopez *et al.*, 2008; Gyulkhandanyan *et al.*, 2013), as well as endothelial cells (Bae *et al.*, 2009). These tests would reveal changes in CAP1 and MMP-9 expression, upon apoptosis induction in the presence or absence of resistin. Since the dose of thrombin (high/low) determines which of the distinct PAR1 and PAR4-mediated downstream activatory pathways are triggered (Kahn *et al.*, 1999; Holinstat, 2006; Duvernay *et al.*, 2013), it would be interesting to assess if there are concentration dependent effects on the

expression and interaction amongst the proteins based on varying degrees of thrombin-induced inflammation or apoptosis. In addition to using thrombin for promoting apoptosis, PAR1 and PAR4 peptides could be used to further resolve the activation pathway involved and further aid in elucidating the CAP1, MMP-9 and/or resistin-related paradigm.

This preliminary understanding of how CAP1, resistin and MMP-9 interact, would form the basis of a bigger investigation that would firmly establish the role of these proteins in disease states that they are correlated and enhance our understanding if they form inflammatory complexes which influence their behaviour *in vitro* and *in vivo*, thus improving the understanding of inflammation and disease progression.

6.4 The role of CAPs and resistin in cardiac myopathy

Resistin has been correlated withIR, obesity and T2DM and consequently, also with CVD (Jung, Park, *et al.*, 2006; Meshkani and Adeli, 2009; Jamaluddin *et al.*, 2012; Huang and Yang, 2015). It has been established that increased levels of resistin in diabetic patients lead to increase of sarcomere contraction in the cardiac tissue affecting its contractility in addition to promoting cardiac hypertrophy by stimulation of AMP-activated kinases in response to IRS-1/MAPK phosphorylation (See Figure 1.2) (Kim *et al.*, 2008; Kang *et al.*, 2011). Interestingly, CAP2 is known to localise near the M-band of the sarcomere and its deficiency also leads to a disarray of sarcomeric actin filaments initiating

cardiac hypertrophy (Peche *et al.*, 2013; Kosmas *et al.*, 2015). Also of interest is that cardiac myopathy related mortality in CAP2 KO mice, where female mice presented better outcomes compared to their male counterparts, highlighting the male versus female differences (Peche *et al.*, 2013) . When this is noted in the light of the observation that circulating levels of resistin have also been shown to differ between males and females (Steppan and Lazar, 2004; Chen *et al.*, 2009), taken together, this is suggestive of a link between CAP1 and resistin could have implications in cardiac myopathies too, but prior to that any interactions between CAP2 and resistin remains to be established.

6.5 Potential for a relation between IQGAP and CAP via resistin?

IQGAPs are multidomain cytoplasmic scaffolding proteins that juxtapose Rho GTPases Rac1 and Cdc42, Ca^{2+}/calmodulin signals and cytoskeletal reorganization events. In platelets, functionally distinct roles for IQGAP1 and IQGAP2 have been suggested. IQGAP1 was proposed to modulate the procoagulant function of platelets by regulating the secretory pathway of α-granule exocytosis (Schmidt, 2012b), whereas IQGAP2 has been shown to act as a scaffolding protein linking thrombin activation to platelet cytoskeleton (Schmidt *et al.*, 2003). Ablation of IQGAP2 (*Iqgap2 -/-*) in a mouse model has shown the mice to be hypoglycaemic and protected from hepatic steatosis andIR, supporting the notion that IQGAP2 may be involved in causing IRin liver cells (Chiariello *et al.*, 2012).

Although the investigations stated in (Chapter 5) show no interaction of CAP1 with IQGAP1 it may be interesting to study if IQGAP1 expression was altered when platelets were pre-treated with resistin, and extending the investigation to gauge if CAP1 and IQGAP1 interaction occurred in response to external stimuli such as treatment with resistin. The supporting precursors for the above study are available in literature which show that IQGAP2 deficiency leads to inhibition of GSK3β kinase activity (Schmidt, 2012a), which in turn has been shown to affect CAP1 phosphorylation (Zhou *et al.*, 2014) and resistin involvement in Akt upregulation, which is regulated by GSK3β (Cho *et al.*, 2001; Woulfe *et al.*, 2004b). An investigation similar to the one carried out in (chapter 3 and 4) and probing for IQGAP1 instead can reveal if resistin affects the localisation profile

of the protein, as observed in the case of CAP1. Additional investigations to include GSK3 inhibitors will shed more light on the involvement of the kinase in resistin-related changes in IQGAP1, if any. Since *Iqgap1 -/-* mice are available (Li *et al.*, 2000), they can be used as a model to understand IRowing to resistin and the role of IQGAP1 therein. Public availability of phospho-CAP1 (Zhou *et al.*, 2014) antibody would improve the study significantly in the data produced to assess mechanisms of IR.

6.6 Bioenergetics influencing platelet cytoskeletal reorganisation

Actin polymerisation and depolymerisation are governed strictly by nucleotide exchange that provide the energy required for cell movements (Bugyi and Carlier, 2010). The energy requirements are thought to be higher in platelets undergoing cytoskeletal reorganisation and granule secretion, evidence of which was first described in 1985 by Verhoeven *et al.* It was noted that metabolic stress regulates cytoskeletal reorganisation in cancer cells and it raises the probability that similarly β-oxidation also regulates actin dynamics in spreading platelets (Caino *et al.*, 2013). Mitochondrial function assessment as an indicator of energetics within platelets was carried out recently, providing an insight that cytoskeletal reorganisation upon activation may indeed be governed by the metabolic stress experienced by platelets (Chacko *et al.*, 2013; Kramer *et al.*, 2014). In MetS, blood cells experience metabolic stress owing to the presence of adipokines and impaired glucose metabolism and it may affect the platelet cytoskeleton, a trend we observed when platelets were exposed to elevated

levels of resistin. An investigation of the effect of resistin on metabolic behaviour of platelets using an extracellular flux analyser which measures oxygen consumption rate and extracellular acidification rate and records mitochondrial respiration and glycolysis as a function of metabolic vigour, will enable the understanding if resistin affects platelet metabolism. Additional controls using insulin and insulin sensitizers would clarify potential mechanisms underlying the effect of resistin on platelet function, if any. A correlation can then be sought between metabolism of resistin-treated and untreated platelets to assess how resistin pre-treatment affects shape change upon treatment with platelet agonists in a time course, as we have preliminary evidence from aggregation data to support the metabolic observations. This experiment would also require the incorporation of controls where resistin itself is inhibited (TZDs – insulin sensitizers), the platelet actin cytoskeleton is depolymerised (latrunculins, cytochalasins), thus inhibiting shape change, in addition to respiratory complex inhibitors (rotenone, antimycin, oligomycin, cyanides.), alongside simultaneous aggregation and spreading experiments to gain a clearer mechanistic overview of effect of resistin on platelet metabolism during cytoskeletal reorganisation.

6.7 Ethnic and racial variation in circulating resistin is yet to be established

Variation in the concentration of circulating resistin in male and female population and its correlation to risk of diabetes is well documented in clinical studies (Steppan and Lazar, 2004; Chen *et al.*, 2009). The key disease states that are predicted by elevated resistin are IR, atherosclerosis, and diabetes (Lehrke *et al.*, 2004; Reilly *et al.*, 2005). There is clear evidence of certain racial groups being predisposed to IR and diabetes established since 1990's (Suchindran *et al.*, 2009; Tuchman, 2011; Spanakis and Golden, 2013). Resistin, as predictor of atherosclerosis in males was established by Reilly *et al*. (2005). A recent clinical study examined if plasma resistin levels were an effective predictor of atherosclerosis and CVDs, and they reported that the association of resistin to CVD risk was greatest in the Hispanic ethnic group (Muse *et al.*, 2015). A study carried out in normal, pregnant females based on ethnic differences and distinct patterns were observed in the circulating levels of adipokines (Chen and Scholl, 2015). Clinical data is not available for plasma resistin concentrations within specific ethnic communities related to IR but it can be presumed that such variations potentially exist affecting their predisposition to IR and diabetes, as it did for CVD. A clinical study is required where multi-ethnic population which is age and gender matched, are measured for multiple indicators of IR like HOMA index, QUICKI, and McAuley index (to avoid bias towards IR measurement) can then be associated with plasma resistin levels to accurately identify any racial/ethnic correlations and associations between IR

and plasma resistin concentrations. Platelet response to agonists along with IR predictors will add to the observations of the clinical study and help reveal association of plasma resistin to thrombosis states.

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APPENDIX 1

Classification of cytoskeletal proteins: Classification and their abundance (copy numbers) in platelets identified from proteomics studies including the changes in phosphorylation of proteins upon thrombin stimulation (Average ratio of activated/resting phosphorylation of protein). Information charts from (Marcus, Moebius and Meyer, 2003; Zahedi *et al.*, 2008; Qureshi *et al.*, 2009; Burkhart *et al.*, 2012) is used to compile the table.

Actin and actin associated proteins				
Protein accession number	**Estimated copy number**	**Cytoskeletal protein**	**MW [kDa]**	**Average ratio (Activated/ Resting)**
P60709	795,000	ACTB_HUMAN Actin, cytoplasmic 1	41.7	1.06
P63261	791,000	ACTG_HUMAN Actin, cytoplasmic 2	41.8	1.63
P62736	600,000	ACTA_HUMAN Actin, aortic smooth muscle	42	1.03
P07737	503,000	PROF1_HUMAN Profilin	15	0.99
A8MW06	320,000	TMSL3_HUMAN Thymosin beta-4-like protein 3	5.1	0.15
P23528	244,000	COF1_HUMAN Cofilin	18.5	1
Q562R1	220,000	ACTBL_HUMAN Beta-actin-like protein 2	42	1.18
A9Z1Y9	159,000	TMSL6_HUMAN Putative thymosin beta-4-like protein 6	5.1	-
P37802	130,000	TAGL2_HUMAN Transgelin-2	22.4	0.99
Q9Y490	116,000	TLN1_HUMAN Talin	269.6	1.03
P67936	107,000	TPM4_HUMAN Tropomyosin alpha-4 chain	28.5	0.95
Q9Y281	93,200	COF2_HUMAN Cofilin-2	18.7	-
P12814	92,100	ACTN1_HUMAN Alpha-actinin	103	1.04
P21333	87,700	FLNA_HUMAN Filamin-A	280.6	1
Q01518	41,700	CAP1_HUMAN Adenylyl cyclase-associated protein 1	51.9	1.01
P61158	30,600	ARP3_HUMAN Actin-related	47.3	1.05

		protein 3		
P61160	30,300	ARP2_HUMAN Actin-related protein 2	47.3	
Q99439	29,500	CNN2_HUMAN Calponin-2	33.7	0.94
O15145	27,500	ARPC3_HUMAN Actin-related protein 2/3 complex subunit 3	20.5	1.13
P47756	26,400	CAPZB_HUMAN F-actin-capping protein subunit beta	31.3	1.02
P59998	26,000	ARPC4_HUMAN Actin-related protein 2/3 complex subunit 4	19.7	1.02
P09493	26,000	TPM1_HUMAN Tropomyosin alpha chain	32.7	0.93
P35609	25,800	ACTN2_HUMAN Alpha-actinin-2	103	-
P31146	23,400	COR1A_HUMAN CoroninA	51	0.98
Q9ULV4	23,300	COR1C_HUMAN CoroninC	53.2	1
P06753	23,100	TPM3_HUMAN Tropomyosin alpha-3 chain	32.8	0.98
O15511	22,900	ARPC5_HUMAN Actin-related protein 2/3 complex subunit 5	16.3	1.01
P52907	20,900	CAZA1_HUMAN F-actin-capping protein subunit alpha	32.9	0.98
O15143	19,100	ARC1B_HUMAN Actin-related protein 2/3 complex subunit 1B	40.9	1.03
O15144	17,400	ARPC2_HUMAN Actin-related protein 2/3 complex subunit 2	34.3	1.07
P47755	16,400	CAZA2_HUMAN F-actin-capping protein subunit alpha-2	32.9	0.98
P35241	15,500	RADI_HUMAN Radixin	68.5	0.9
Q14315	15,500	FLNC_HUMAN Filamin-C	290.8	0.26
Q08495	14,500	DEMA_HUMAN Dematin	45.5	0.98
P60981	14,400	DEST_HUMAN Destrin	18.5	1.04
Q9Y4G6	13,700	TLN2_HUMAN Talin-2	271.4	1.1
P15311	13,300	EZRI_HUMAN Ezrin	69.4	1.15
Q27J81	7,500	INF2_HUMAN Inverted formin-2	137.5	-
P63313	5,500	TYB10_HUMAN Thymosin beta0	50	-
Q01082	4,600	SPTB2_HUMAN Spectrin beta chain, brain 1	274.4	1
P13797	4,500	PLST_HUMAN Plastin-3	70.8	1.18
O94929	2,100	ABLM3_HUMAN Actin-binding LIM protein 3	77.8	1.05
P13796	2,000	PLSL_HUMAN Plastin-2	70.2	0.84
Q9NZ32	2,000	ARP10_HUMAN Actin-related	46.3	1.01

		protein 10		
Q9UHB6	1,900	LIMA1_HUMAN LIM domain and actin-binding protein 1	85.2	1.07
O00399	1,900	DCTN6_HUMAN Dynactin subunit 6	20.7	1.94
P09327	1,600	VILI_HUMAN Villin	92.6	0.98
P53814	1,600	SMTN_HUMAN Smoothelin	99.4	1.21
Q14651	1,600	PLSI_HUMAN Plastin	70.2	
Q8N556	1,400	AFAP1_HUMAN Actin filament-associated protein 1	110	-
O14639	1,100	ABLM1_HUMAN Actin-binding LIM protein 1	87.6	0.86
Q12792	1,000	TWF1_HUMAN Twinfilin	40.3	1.15
P46940	1,000	IQGA1_HUMAN Ras GTPase-activating-like protein IQGAP1	189.1	0.94
P57737	760	CORO7_HUMAN Coronin-7	100.5	0.95
P02549	650	SPTA1_HUMAN Spectrin alpha chain, erythrocyte	279.8	0.69
O95425	620	SVIL_HUMAN Supervillin	247.6	0.96
P35221	Low	CTNA1_HUMAN Catenin alpha-1	100	1.35
O75369	Low	FLNB_HUMAN Filamin-B	278	0.99
Q27J81	Low	INF2_HUMAN Inverted formin-2	135.5	0.97
P62328	Low	TYB4_HUMAN Thymosin beta-4	5.1	-
A4UGR9	Low	XIRP2_HUMAN Xin actin-binding repeat-containing protein 2	198.5	-
Tubulin and microtubule associated proteins				
Protein accession number	**Estimated copy number**	**Cytoskeletal protein**	**MW [kDa]**	**Average ratio (Activated/ Resting)**
P68366	185,000	TBA4A_HUMAN Tubulin alpha-4A chain	49.9	1.14
Q9BQE3	174,000	TBA1C_HUMAN Tubulin alphaC chain	49.9	1.09
Q9H4B7	144,000	TBB1_HUMAN Tubulin beta chain	50.3	1.06
Q9NY65	128,000	TBA8_HUMAN Tubulin alpha-8 chain	50.1	1.05
Q9H853	125,000	TBA4B_HUMAN Putative tubulin-like protein alpha-4B	50	-
P07437	115,000	TBB5_HUMAN Tubulin beta chain	49.6	0.98
Q13748	110,000	TBA3C_HUMAN Tubulin alpha-3C/D chain	49.9	0.85
P68371	106,000	TBB2C_HUMAN Tubulin beta-2C	49.8	1.04

		chain		
P04350	96,000	TBB4_HUMAN Tubulin beta-4 chain	49.6	-
P68371	94,700	TBB2C_HUMAN Tubulin beta-2C chain	49.8	1.04
Q15555	7,600	MARE2_HUMAN Microtubule-associated protein RP/EB family member 2	37	0.96
P23258	2,300	TBG1_HUMAN Tubulin gamma chain	51.1	1.05
P23258	2,000	TBG1_HUMAN Tubulin gamma chain	51.1	1.05
Q86UP2	1,800	KTN1_HUMAN Kinectin	156.2	1.07
P27816	1,700	MAP4_HUMAN Microtubule-associated protein 4	120.9	0.54
Q96PK2	1,500	MACF4_HUMAN Microtubule-actin cross-linking factor 1, isoform 4	670.1	-
Q66K74	1,400	MAP1S_HUMAN Microtubule-associated protein 1S	112.1	0.95
Q9UPN3	1,300	MACF1_HUMAN Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	620	0.98
Q96PK2	1,100	MACF4_HUMAN Microtubule-actin cross-linking factor 1, isoform 4	670.1	-
Q9BSJ2	1,000	GCP2_HUMAN Gamma-tubulin complex component 2	102.5	1.01
Q96CW5	850	GCP3_HUMAN Gamma-tubulin complex component 3	103.5	0.97
P78559	750	MAP1A_HUMAN Microtubule-associated protein 1A	305.3	0.87
Q99867	<500	TBB4Q_HUMAN Putative tubulin beta-4q chain	51	-
Q9BVA1	<500	TBB2B_HUMAN Tubulin beta-2B chain	48.7	-
Q3ZCM7	<500	TBB8_HUMAN Tubulin beta-8 chain	49.9	-
Q71U36	<500	TBA1A_HUMAN Tubulin alphaA chain	49.9	
P46821	Low	MAP1B_HUMAN Microtubule-associated protein 1B	270.5	-
A6NHL2	Low	TBAL3_HUMAN Tubulin alpha chain-like 3	49.9	-

Q13885	Low	TBB2A_HUMAN Tubulin beta-2A chain	49.9	1.06
Intermediate filament proteins				
Protein accession number	**Estimated copy number**	**Cytoskeletal protein**	**MW [kDa]**	**Average ratio (Activated/ Resting)**
Q13884	5,700	SNTB1_HUMAN Beta-syntrophin	58	1.1
P13645	3,700	K1C10_HUMAN Keratin, type I cytoskeletal 10	66	-
P08670	2,400	VIME_HUMAN Vimentin	53.6	0.95
P35527	1,800	K1C9_HUMAN Keratin, type I cytoskeletal 9	66	-
P35908	1,600	K22E_HUMAN Keratin, type II cytoskeletal 2 epidermal	66	-
P02545	1,200	LMNA_HUMAN Prelamin-A/C	74.1	0.83
P02533	780	K1C14_HUMAN Keratin, type I cytoskeletal 14	66.5	-
P13647	Low	K2C5_HUMAN Keratin, type II cytoskeletal 5	47	-
P13646	Low	K1C13_HUMAN Keratin, type I cytoskeletal 13	66	-
P08779	Low	K1C16_HUMAN Keratin, type I cytoskeletal 16	66	-
Q86Y46	Low	K2C73_HUMAN Keratin, type II cytoskeletal 73	66	-
P78386	Low	KRT85_HUMAN Keratin, type II cuticular Hb5	66	-
Myosins				
Protein accession number	**Estimated copy number**	**Cytoskeletal protein**	**MW [kDa]**	**Average ratio (Activated/ Resting)**
P60660	229,000	MYL6_HUMAN Myosin light polypeptide 6	16.9	0.9
P35579	96,900	MYH9_HUMAN Myosin-9	226.4	0.97
P19105	88,100	ML12A_HUMAN Myosin regulatory light chain 12A	19.8	0.96
P24844	88,000	MYL9_HUMAN Myosin regulatory light polypeptide 9	19.8	0.95
O14950	86,900	ML12B_HUMAN Myosin regulatory light chain 12B	19.8	-
P14649	20,900	MYL6B_HUMAN Myosin light chain 6B	22.7	1
P35749	15,300	MYH11_HUMAN Myosin1	227.2	0.92

P35580	13,900	MYH10_HUMAN Myosin0	228.9	0.88
Q7Z406	11,400	MYH14_HUMAN Myosin4	200	-
P05976	10,900	MYL1_HUMAN Myosin light chain 1/3, skeletal muscle isoform	16.7	-
Q15746	4,200	MYLK_HUMAN Myosin light chain kinase, smooth muscle	210.6	1.01
O00159	2,700	MYO1C_HUMAN Myosin-Ic	121.6	0.97
Q86YV6	2,400	MYLK4_HUMAN Myosin light chain kinase family member 4	211	-
Q9Y4I1	1,800	MYO5A_HUMAN Myosin-Va	215.3	1
Q92614	1,700	MY18A_HUMAN Myosin-XVIIIa	233	0.93
Q13459	1,400	MYO9B_HUMAN Myosin-IXb	243.4	0.95
P12829	1,300	MYL4_HUMAN Myosin light chain 4	21.6	-
B0I1T2	1,000	MYO1G_HUMAN Myosin-Ig	116.4	1.23
Q9UM54	690	MYO6_HUMAN Myosin-VI	149.6	1.04
O00160	640	MYO1F_HUMAN Myosin-If	124.8	1.06
O00160	590	MYO1F_HUMAN Myosin-If	124.8	1.06
Q9Y4I1	<500	MYO5A_HUMAN Myosin-Va	215.3	1
Q9UKX3	<500	MYH13_HUMAN Myosin3	200	-
Q9ULV0	Low	MYO5B_HUMAN Unconventional myosin-Vb	210	-
P13533	Low	MYH6_HUMAN Myosin-6	150	-
Q13402	Low	MYO7A_HUMAN Myosin-VIIa	150	-
Other motor proteins				
Protein accession number	**Estimated copy number**	**Cytoskeletal protein**	**MW [kDa]**	**Average ratio (Activated/ Resting)**
Q14203	3,600	DCTN1_HUMAN Dynactin subunit 1	141.6	1
Q9UJW0	3,000	DCTN4_HUMAN Dynactin subunit 4	52,3	1.01
O75935	3,000	DCTN3_HUMAN Dynactin subunit 3	21.1	1.07
Q9NQT8	1,700	KI13B_HUMAN Kinesin-like protein KIF13B	202.5	1
Q9H1H9	1,000	KI13A_HUMAN Kinesin-like protein KIF13A	195.8	-
Q9BVG8	820	KIFC3_HUMAN Kinesin-like protein KIFC3	92.7	1.06
Q13409	640	DC1I2_HUMAN Cytoplasmic dynein 1 intermediate chain 2	71.4	1.09

O43896	550	KIF1C_HUMAN Kinesin-like protein KIF1C	195.8	-
Q9UFH2	<500	DYH17_HUMAN Dynein heavy chain 17, axonemal	515	-
Q9P225	Low	DYH2_HUMAN Dynein heavy chain 2, axonemal	507.4	0.85
Q8TE73	Low	DYH5_HUMAN Dynein heavy chain 5, axonemal	528.7	-
Q96JB1	Low	DYH8_HUMAN Dynein heavy chain 8, axonemal	514.3	-

APPENDIX 2

Complete details of antibodies including their usage.

Antibody	Supplier (Details)	Western blotting (Dilution in TBS-T)	Immunostaining (Dilution in PBS)
CAP1	Abcam (ab133655)	1:8000	1:300
β-actin	Abcam (ab20272)	1: 500	-
GAPDH	Calbiochem (6C5-CB1001)	1:6000	-
CD36	Santa Cruz (H-300 - SC-9154)	1:1000	-
Cofilin	Received as a gift from Cologne	1:500	-
Profilin	Received as a gift from Cologne	1:500	-
Coronin 1a	Abcam (ab72212)	1:1000	1:100
pAKT	Cell Signalling (9271)	1:1000	-
p38	Abcam (ab7952)	1:1000	-
pERK1/2	Cell Signalling (4370)	1:1000	-
pVASP Ser 157	Cell Signalling (3111)	1:1000	-
pVASP Ser 239	Cell Signalling (3114)	1:1000	-
Syk	Santa Cruz (4D10-SC1240)	1:1000	-
IQGAP1	Santa Cruz (H-109- SC-10792)	1:1000	-
Arp2/3 complex	Millipore (ARPC2/p34-Arc)	1:200	1:20
myc	Generated in lab previously (Grimm-Gunter, 2009)	Non-diluted	-
GFP	Generated in lab previously (Grimm-Gunter, 2009)	Non-diluted	-
p(Ser) PKC substrate	Cell Signalling (2261)	1:1000	-
Villin	Generated in lab previously (10_2C_3) (Grimm-Gunter, 2009)	1:1000	1:300
L-plastin	Generated in lab previously (Grimm-Gunter, 2009)	1:1000	1:300
Myosin IIa	Novus Biologicals (NBP1-31733)	1:1000	1:300
Tropo-myosin	Chemicon (AB5441 - 1,2,3,5a,5b,6)	1:1000	1:100