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The Role of Thiols in the Activation of the Platelet Integrin $\alpha_{IIb}\beta_3$.

Sarah O'Neill BSc.

Thesis submitted to the University of Dublin for the degree of Doctor of Philosophy.

Research was carried out in the Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, under the supervision of Dr. Niamh Moran and the co-operation of Professor Luke O'Neill, Department of Biochemistry, Trinity College Dublin.

Department of Biochemistry

University of Dublin

Trinity College.

February 2001

DECLARATION

I hereby certify that this thesis, submitted to the University of Dublin for examination for the degree of Doctor of Philosophy, has not been submitted as an exercise for a degree at this or any other University. I also certify that this thesis is entirely my own work.

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Sarah O'Neill.

Sarah O'Neill.

February 2001.

"The horizon leans forward,
Offering you space
To place new steps of change.
Here, on the pulse of this fine day,
You may have the courage
To look up and out and upon me,
The Rock, the River, the Tree, your country.
No less to Midas than the mendicant.
No less to you now than the mastodon then."

by Maya Angelou

From "On the Pulse of Morning" from "The Complete Collected Poems (1995)"

For Paul and Conor

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Go raibh mile maith agaibh.

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SUMMARY

Platelets play a vital role in maintaining normal haemostasis. They circulate passively in the blood in an non-adhesive state as discrete smooth discs. When platelets encounter a vessel wall injury or are exposed to foreign surfaces they undergo a series of processes resulting in platelet activation. This leads to the formation of a localised haemostatic plug, which is stabilised by fibrin and ultimately prevents further blood loss from the vessel. These events are mediated through the interaction of adhesive macromolecules, such as plasma fibrinogen, with specific receptors present on the platelet membrane, most notably the platelet integrin $\alpha_{IIb}\beta_3$, in response to extracellular signals. This integrin receptor is the most abundant protein on the platelet surface. It is a cysteine-rich heterodimeric adhesion molecule that alters conformation in response to platelet activation and ligand binding. The mechanisms involved in these conformational changes are not fully elucidated. It is the purpose of this study to identify a physiological mechanism that permits $\alpha_{IIb}\beta_3$ to undergo conformational changes to make it competent to bind ligand upon platelet activation.

As already mentioned, this receptor is cysteine rich and the role of disulphide bonds in conformational changes inferred from studies using the reducing agent dithiothreitol (DTT). These studies were expanded here by examining the effects of DTT on platelet activation events such as platelet aggregation, fibrinogen binding, expression of ligand induced binding sites (LIBS) in the receptor, integrin secretory events and platelet signalling. It was established that disulphide bonds do indeed play a role in conformational changes of $\alpha_{IIb}\beta_3$ that lead to activation of this receptor. I hypothesised therefore, that the physiological

mediator of such changes may be the thiol/disulphide modifying enzyme protein disulphide isomerase (PDI; EC 5.3.4.1). It was established that PDI is present in the platelet by immunoblot analysis, flow cytometry and PCR. Consistent with this hypothesis, bacitracin, an inhibitor of thiol isomerase activity, inhibited many aspects of platelet integrin activation, including platelet aggregation and fibrinogen binding. However, in confocal microscopy co-localisation studies, PDI failed to co-localise with $\alpha_{IIb}\beta_3$. I therefore proposed an alternative hypothesis: that an endogenous thiol isomerase activity, associated with the Cys-Gly-X-Cys repeats in the integrin β-subunits, may be the mediator of integrin conformational changes (O'Neill et al. 2000). I established that the platelet integrin has thiol isomerase activity equivalent to PDI. A complete characterisation of this activity was performed. Thiol-isomerase activity in purified preparations of $\alpha_{IIb}\beta_3$ is time-dependent and saturable. It has a K_{cat} value of $2.17X10^{-4}$ mol $min^{-1}/\mu M$ enzyme compared to $2.45X10^{-4}$mol $min^{-1}/\mu M$ enzyme for PDI. The thiol isomerase activity of $\alpha_{IIb}\beta_3$ is maximal in the presence of EDTA and is inhibited by Ca^{2+} or Mg^{2+} divalent cations. In contrast, the activity is stimulated by Mn^{2+} at concentrations that induce integrin activation. The isomerase activity of PDI is unaffected by the presence or absence of divalent cations. RGD mimetics had no impact on this activity in $\alpha_{IIb}\beta_3$. Furthermore, isomerase activity was shown to be present in the integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$.

This thesis has provided the first demonstration of an enzymatic activity in integrins and offers a global mechanism for the activation of integrins. It suggests that disulphide bonds within the integrin or indeed its ligands may be locally modified during activation.

PUBLICATIONS.

The following publications are submitted in support of this thesis:

Sarah O'Neill, Aisling Robinson, Adele Deering, Michelle Ryan, Desmond, J. Fitzgerald and Niamh Moran. The Platelet Integrin $\alpha_{IIb}\beta_3$ Has an Endogenous Thiol Isomerase Activity. *Journal of Biological Chemistry*. 2000, (**275**): 36984-36990.

ABSTRACTS.

Sarah O'Neill, Aisling Robinson, Adele Deering, Desmond, J. Fitzgerald and Niamh Moran. Endogenous Thiol Isomerase Activity in the Platelet β_3 Integrin $\alpha_{IIb}\beta_3$ subunit. *Blood* 2000 (96):620a, Abstract #2666

O'Neill S., Fitzgerald D., Moran N.

The effects of an inhibitor of protein disulphide isomerase on platelet function assays. *Thrombosis and Haemostasis Supplement,* August 1999, Abstract #1651.

Fullard J., O'Neill S., Moran N., Murphy R., Fitzgerald D.

A compound heterozygote for the glycoprotein (GP) IIIa gene associated with Glanzmann Thrombasthenia. *Thrombosis and Haemostasis Supplement*, August 1999, Abstract #2352.

O'Neill S., Fitzgerald D., Moran N.

A possible role for a protein disulphide isomerase in conformational changes of GPIIb/IIIa. *Irish Journal of Medical Science*, July 1998, (**167**) No. 3.

O'Neill S., Stephens G., Fitzgerald D., Moran N:

Modulation of platelet GPIIb/IIIa affinity state: possible role for a protein disulphide isomerase. *Thrombosis and Haemostasis Supplement*, June 1997, Poster Session #2746.

Moran N., O'Neill S., Murphy N., Smith R., Stephens G., Fitzgerald D.

Role of disulphide bonds in recognition and signal transduction by platelet GPIIb/IIIa. *Tyrosine Phosphorylation and Cell Signalling*, August 1996, The Salk Institute.

PUBLICATIONS SUBMITTED AND IN PREPARATION:

John Fullard, Ronan Murphy, Sarah O'Neill, Niamh Moran, Brian Ottridge, Desmond J. Fitzgerald. A Val193Met Mutation in GPIIIa Results in a GPIIb/IIIa Receptor with a Constitutively High Affinity for a Small Ligand. *British Journal of Haematology*, 2001 (Submitted).

Sarah O'Neill, Desmond J. Fitzgerald and Niamh Moran. Regulation of the thiol isomerase activity in the platelet integrin $\alpha_{IIb}\beta_3$ (in preparation).

Sarah O'Neill, Desmond J. Fitzgerald and Niamh Moran. Bacitracin, an inhibitor of thiol isomerase activity, modulates the function of the platelet integrin $\alpha_{IIb}\beta_3$ (in preparation).

ABBREVIATIONS

abs	Absorbance
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
CHO	Chinese hamster ovary
cpm	Counts per minute
Cys	Cysteine
DAG	Diacylglycerol
DTS	Dense tubular system
dH_2O	Deionised, distilled water.
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate.
dpm	Disintegrations per minute
DTNB	Ellman's reagent, 5,5'-Dithio-bis(2-Nitrobenzoic acid)
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid

FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
Hel	Human erythroleukemic
HRP	Horse radish peroxidase
hrs	Hours
IP_3	Inositol 1,4,5-triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilodalton
LIBS	Ligand induced biding site
mAb	Monoclonal antibody
MeOH	Methanol
Mg^{2+}	Magnesium
MIDAS	Metal ion-dependent adhesion site
Mn^{2+}	Manganese
mRNA	Message ribonucleic acid
MTS	Microtubular system
NS	Not significant
OCS	Open canalicular system
OgFg	Oregon green labelled fibrinogen

PBS	Phosphate balanced salt solution
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase.
PGE_1	Prostaglandin E_1
PIP_2	Phosphatidylinositol triphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPP	Platelet Poor plasma
PRP	Platelet rich plasma
PVDF	Polyvinyl difluoride
RGD	Arg-Gly-Asp
rpm	Revolutions per minute
RT-PCR	Reverse transcribed polymerase chain reaction.
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS	Tris balanced salt solution
TRAP	Thrombin receptor activating peptide
TxA_2	Thromboxane A_2
vWf	von Willebrand factor

CHAPTER 1

Introduction

1.1. PLATELETS

1.1.1. Origin and production.

Platelets play a vital role in maintaining normal haemostasis. They originate as fragments of mature megakaryocytes in the bone marrow where they are liberated from the cytoplasmic protrusions of such a cell. Each megakaryocyte can produce approximately 4,000 platelets (Italiano et al. 1999). Platelets are non-nucleated and circulate passively in the blood in a non-adhesive state as discrete smooth discs. They are present at a concentration of between $1.5 - 4.0 \times 10^8$/ml of blood and are approximately 2-4 microns in diameter.

1.1.2. Structure.

Structurally, platelets are complex and their anatomy has been divided into four regions and the main structures are described in figure 1.1 (White 1994).

The **peripheral zone** consists of the platelet surface and includes the channels of the surface-connected open canalicular system (OCS), which are invaginations of the wall that burrow deeply into the cytoplasm. Such channels greatly increases the surface area of the platelet exposed to circulating plasma and provides a path for substances to be released by and reach far into the platelet. The exterior coat is extremely rich in glycoproteins that act as receptors involved in activation of the platelet and its interaction with other cells. Many of these receptors belong to the integrin super family with members of the β_1, β_2 and β_3 families present, all of which play a role in adhesion and aggregation. The middle layer of this zone is dense in phospholipids which provides

a surface necessary for the interaction with coagulant proteins. Lying just inside this is the submembrane region that consists of short-actin filaments associated with the cytoskeleton. This region is involved in the translation of surface received signals into chemical messages and physical alterations required for platelet activation.

The **sol-gel zone** is the cytoskeleton of the platelet containing detergent-resistant elements. Its fibre systems are responsible for the discoid shape of quiescent platelets and provides the contractile machinery involved in platelet shape change, contraction, secretion and pseudopodia protrusion.

The **organelle zone** contains mitochondria, electron-dense bodies, granules (α- and dense), peroxisomes and lysosomes. These organelles are involved in metabolic processes, storage of enzymes and agents such as non-metabolic adenine nucleotides, serotonin and calcium, which are secreted when the granules merge with the platelet membrane upon activation.

The **membrane systems** are the surface connected open canalicular system (OCS) described above and the dense tubular system (DTS). The DTS originates from pieces of the endoplasmic reticulum of the parent megakaryocyte. Both of these systems have been described as being equivalent to the sarcoplasmic reticulum of muscle cells (White 1994). The DTS acts as a store for calcium and has been shown to have a calcium pump activity involved in contractile events of the platelet. It is also the site of platelet prostaglandin synthesis (Gerrard et al. 1976).

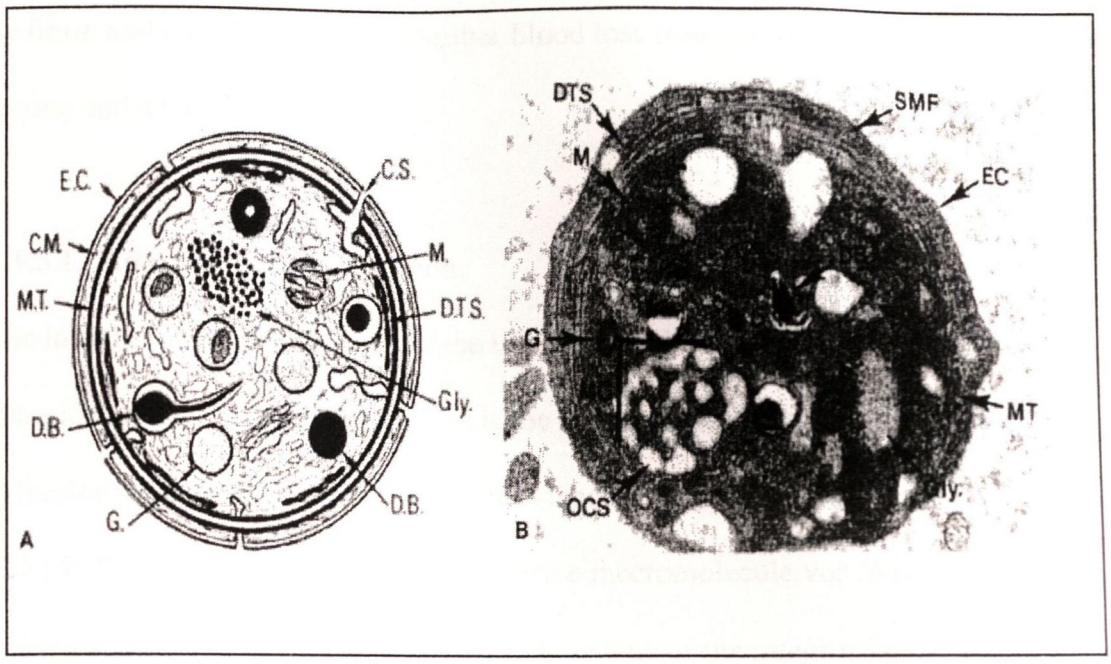


Figure 1.1. Schematic representation and electron micrograph of a platelet.

A. The diagram summarises the main structures observed in the platelet. The following components are identified: the exterior coat (EC), trilaminar unit membrane (CM), microtubules (MT), dense bodies (DB), granules (G), glycogen (Gly), dense tubular system (DTS), mitochondria (M), surface-connected canalicular system (CS).

B. An electron micrograph of such a platelet at a magnification of ×28,000.

Images were taken from Hemostasis and Thrombosis, Basic Principles and Clinical Practice, 3rd Edition, James G. White 1994.

1.1.3. Activation.

Activation of platelets occurs when they encounter a vessel wall injury or are exposed to thrombogenic surfaces or locally generated chemical agonists. They undergo a series of processes that include shape change, secretion, shedding of microvesicles, adhesion and

aggregation leading to the formation of a localised haemostatic plug, which is stabilised by fibrin and ultimately prevents further blood loss from the vessel. Figure 1.2 shows a resting and an activated platelet

1.1.3.1. Adhesion and aggregation.

The initial contact of platelets with the injured vessel wall is referred to as platelet adhesion and is a complex process. It is the first process in thrombus formation. Adhesion of platelets at the site of injury is mediated by the platelet surface glycoprotein (GP) Ib-IX-V complex that binds the adhesive macromolecule von Willebrand factor (vWF), specifically at the GPIbα subunit. Members of the integrin family are also involved, such as $\alpha_2\beta_1$, which binds collagen and has been shown to be involved in the early phase of adhesion under static conditions (Moroi et al. 1992). This binding of platelets to collagen induces the characteristic shape change of the activated platelet. Other integrins involved in platelet function are $\alpha_5\beta_1$, which binds fibronectin, and $\alpha_{IIb}\beta_3$ which binds fibrinogen, vWF and fibronectin. This tethering process of platelets via the vWF-GPIbα interaction occurs at both arterial and venous shear rates (Kulkarni et al. 2000). These adherent platelets become the reactive surface for continuing deposition of platelets. This interaction of platelets with each other is the second process in thrombus formation and is termed platelet aggregation.

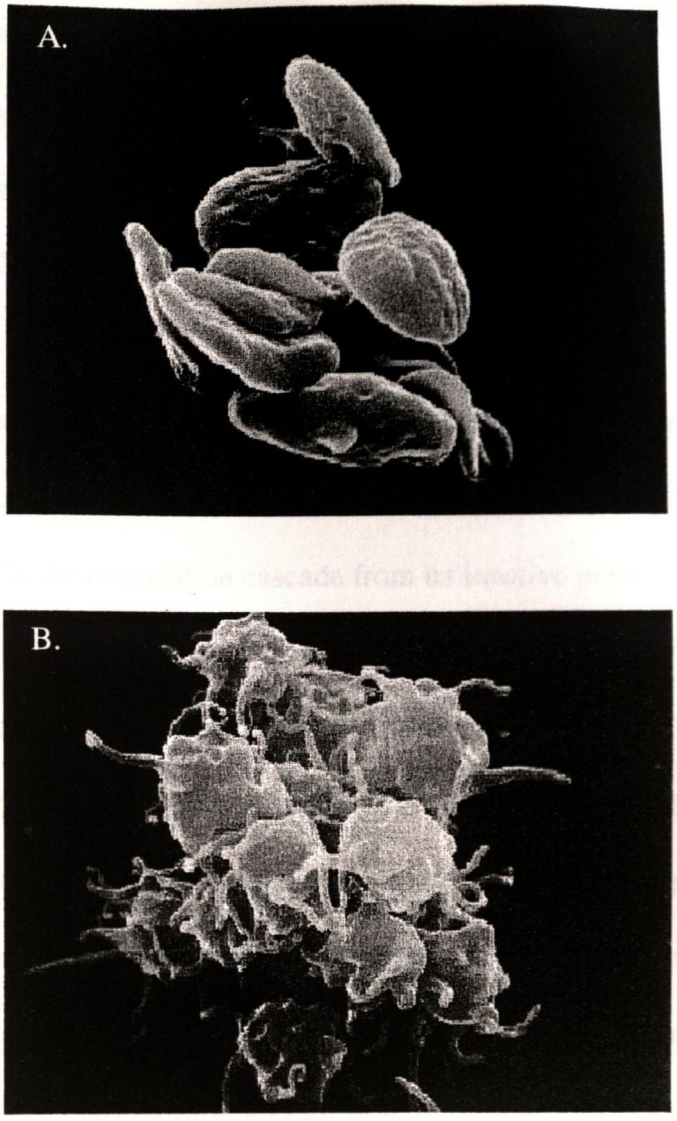


Figure 1.2. Scanning electron micrograph of resting and activated platelets.

A. Resting, unactivated platelets

B. Activated platelets with extended pseudopodia, adhering to each other.

Modified from URL: www.akh-wien.ac.at/biomed-research/htx/anatomy.html

Aggregation is dependent only on the GP-Ib-IX-V complex and $\alpha_{IIb}\beta_3$ (Ruggeri 2000). Fibrinogen plays an essential role in platelet aggregation and binds to $\alpha_{IIb}\beta_3$ bridging one platelet with another in the platelet aggregate (Peerschke 1985).

1.1.3.2. Agonists.

Thrombin, collagen and thromboxane A_2 (TxA_2) are considered strong agonists and cause platelet aggregation independent of granule secretion. Thrombin is a serine protease generated in the coagulation cascade from its inactive precursor prothrombin. It interacts with at least two types of receptors on the platelet surface. The first is a protease activated receptor (PAR), three have been identified: PAR-1, PAR-2, PAR-3 and PAR-4, with PAR-1 and PAR-4 present on the human platelet (Brass 1999) (Kahn et al. 1999). The second target for thrombin is a high affinity site on the GPIb subunit of the GPIb-IX-V complex (Jamieson 1997). The PARs belong to the family of proteins with seven transmembrane domains (7TM). Thrombin binds to and cleaves the extracellular tail of the PAR receptor and the newly exposed N-terminus acts as ligand for the receptor, activating the platelet . The liberated peptide may also have signalling functions (Furman et al. 1998).

Collagen is an extracellular matrix protein that is exposed at sites of endothelial damage and binds to platelets via $\alpha_2\beta_1$ and GPVI (Ichinohe et al. 1997) (Moroi and Jung 1997). TxA_2 is produced in platelets from arachidonic acid released from membrane phospholipids by the action of phospholipase A_2. Arachidonic acid is metabolised to the intermediate product prostaglandin $(PG)H_2$ by cyclooxygenase. PGH_2 is then metabolised to TxA_2 by thromboxane synthase and binds its platelet receptor of the 7TM

as seven transmembrane domain receptors (7TM) and are coupled to guanine nucleotide binding proteins or G-proteins. G-proteins are a family of heterotrimeric proteins consisting of distinct α, β and γ subunits in a non-covalent complex (Hawiger 1994). These proteins mediate the interactions between the 7TM receptor and intracellular effector systems such as adenyl cyclase, phospholipase C and phospholipase A_2. In the basal state the G protein trimer is associated with the intracellular domain of the receptor and GDP is tightly bound to the α subunit. Receptor occupation by an agonist releases the GDP molecule from the α subunit and is replaced with GTP to produce G_α-GTP. This results in a reduction of ligand affinity and the dissociation of G_α from $G_{\beta\gamma}$ G_α-GTP interacts with an effector molecule such as phospholipase C or adenyl cyclase. This process is terminated by the intrinsic GTP-ase activity of the α subunit which hydrolyses GTP to GDP and the α subunit then reassociates with the $\beta\gamma$ subunit. The subsequent signals are mediated through the specific effector systems. In the case of thrombin interaction with the platelet, for example, receptor activation results in the activation of phospholipase C_β and the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the secondary messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of calcium from intraplatelet stores and DAG activates protein kinase C (PKC). The increase in cytosolic calcium concentrations is believed to result in the activation of phospholipase A_2 (PLA_2) and activation of myosin light chain kinase resulting in the phosphorylation of myosin. PLA_2 releases arachidonic acid from membrane phospholipids. Arachidonic acid is metabolised to the intermediate product prostaglandin $(PG)H_2$ by cyclooxygenase. PGH_2 is then metabolised to TxA_2 by thromboxane synthase (Reilly and Fitzgerald 1993) (FitzGerald 1991). TxA_2 is a potent

platelet activator and its synthesis in response to agonist stimulation represents a major pathway of platelet positive feedback. Activation of PKC leads to phosphorylation of pleckstrin and other proteins. Occupancy of many G-protein coupled receptors also leads to the rapid activation of non-receptor protein tyrosine kinases such as members of the Src family, Syk and FAK (Golden et al. 1986) (Takeuchi et al. 1993) (Parsons et al. 1994). These kinases in turn phosphorylate substrates involved in recruiting complexes of signalling molecules (Fox 1996). Phosphorylation of these proteins occurs in waves and can be aggregation dependent or independent. Many of these tyrosine phosphorylated proteins associate with integrin-cytoskeletal complexes at sites of ligand bound receptors and are proposed to be the initiator of integrin-induced or outside-in signalling (Huang et al. 1993).

The precise molecular mechanisms involved in the activation of $\alpha_{IIb}\beta_3$ are not understood. In order to investigate these, a complete understanding of integrin structure in general and the structure of $\alpha_{IIb}\beta_3$ in particular is required. The final common pathway of these signalling events ultimately leads to activation of integrins on the platelet surface. Most noteworthy amongst these is the receptor for fibrinogen $\alpha_{IIb}\beta_3$.

1.2. INTEGRINS.

The integrins are a family of cell-surface heterodimeric receptors by which cells attach to extracellular matrices and other cell surface receptors, such as members of the immunoglobulin superfamily (Hynes 1992). All integrins are transmembrane glycoproteins composed of α and β subunits combined in a non-covalent manner. To date 16 α subunits and 8 β subunits have been identified which combine into more than

20 dimers and are summarised in figure 1.3 (Plow et al. 2000). The α subunits vary in size between 120 and 180 kDa and the β subunits between 90 and 110 kDa. Each dimer has a unique function and a limited number of ligands.

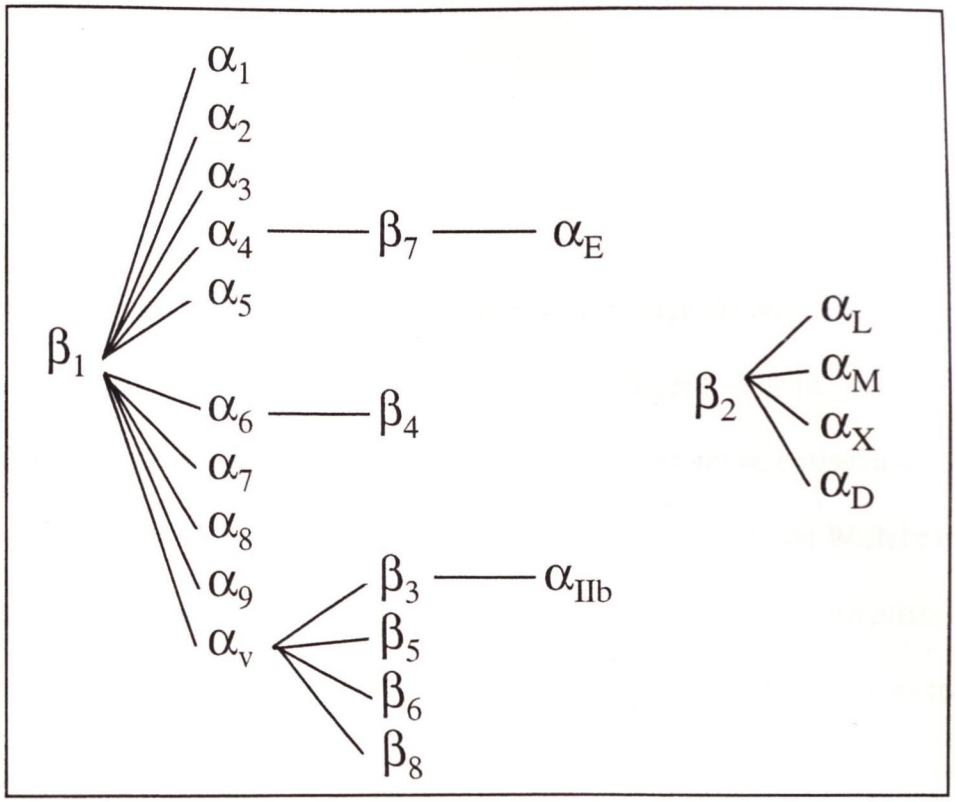


Figure 1.3. The integrin family

Shown are the known subunits and their heterodimeric complexes.

The diversity of the integrin family is further enhanced by alternative splicing. Several subunits have alternatively spliced cytoplasmic domains such as β_1 (Altruda et al. 1990), β_3 (van Kuppevelt et al. 1989), β_4 (Tamura 1990), α_3 (Tamura et al. 1991) and α_6 (Hogervorst et al. 1991). α_{IIb} can be alternatively spliced in the extracellular domain near to the membrane (Bray et al. 1990). The β_1 subfamily previously known as the

VLA (very late antigens) comprises of at least ten complexes. Those found on platelets include $\alpha_2\beta_1$, the collagen receptor, $\alpha_5\beta_1$, the fibronectin receptor and $\alpha_6\beta_1$, the laminin receptor (Humphries 2000). The β_2 and β_7 subfamilies are solely expressed on lymphocytes and include $\alpha_1\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_4\beta_7$ and $\alpha_E\beta_7$. The β_3 subfamily consists of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$. Little is known about the distributions and functions of the β_4, β_5, and β_6 families.

1.2.1. Ligands.

Individual integrins have the ability to recognise multiple ligands and furthermore individual ligands can be recognised by more than one integrin. The majority of ligands are extracellular matrix proteins and includes bone matrix proteins, collagens, fibronectin, fibrinogen, laminins, thrombospondin, vitronectin and von Willebrand factor. The interactions of these ligands with their respective integrins are primarily involved in cell adhesion. Integrins also mediate cell-cell interactions, such as the interaction of fibrinogen with the platelet receptor $\alpha_{IIb}\beta_3$ and by recognition of integral membrane proteins of the immunoglobulin family such as ICAM-1, ICAM-2 and VCAM-1. Which ligand binds its integrin is determined by relative affinity, availability within a specific microenvironment and the conformational state of the integrin.

A number of discrete recognition sites have been identified in integrin ligands. The first binding site to be described was the Arg-Gly-Asp (RGD) tripeptide sequence present in fibrinogen, fibronectin, vitronectin, collagen, thrombospondin and von Willebrand factor. This motif is recognised by the integrins $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_3\beta_1$ $\alpha_8\beta_1$, $\alpha_V\beta_1$ and

$\alpha_v\beta_6$. $\alpha_{IIb}\beta_3$ also recognises the motif His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (HHLGGAKQAGDV) in the γ-chain of fibrinogen. The sequence Asp-Gly-Glu-Ala (DGEA) in type I collagen is recognised by $\alpha_2\beta_1$, and $\alpha_4\beta_1$ binds Glu-Ile-Leu-Asp-Val (EILDV) in fibronectin. High resolution structures for several of these short recognition motifs indicate that they are presented on extended flexible loops between β strands (Main et al. 1992) (Jones et al. 1995).

1.2.2. Structure.

The subunits of integrins are glycoproteins, each with a single hydrophobic transmembrane segment. Electron microscopy studies show that the extracellular portions (>75 kDa for β subunits and >100 kDa for α subunits) form a globular head domain which is connected to the cell membrane by two stalks or cytoplasmic domains (Hantgan et al. 1999). Most integrin cytoplasmic domains are short consisting of approximately 50 amino acids or less. The exception is the tail of the β_4 integrin which is over 1,000 amino acids in length.

1.2.2.1. The α-subunit.

All integrin α subunits have seven homologous 60 amino acid repeats at the N-terminus. They are predicted to fold into a β-propeller structure (Springer 1997) with the blades oriented radially and pseudo-symmetrically around a central axis and parallel to the plasma membrane. Ligand contact points are located on the upper surface of this propeller. The lower face is thought to contain the calcium binding "EF-hand" motif. Several α subunits incorporate an additional folding domain of approximately 200

amino acids, termed the I (inserted) domain and is inserted between repeats 2 and 3. This domain is predicted to sit on "top" of the β-propellor and to be tethered by a hinge. The crystal structure of the I domain of an Mg^{2+}-occupied form of α_M has been determined (Lee 1995). The unique divalent cation co-ordination sphere located in this domain has been designated the metal ion-dependent adhesion site (MIDAS). It is the binding site for either Mg^{2+} or Mn^{2+} (Lee et al. 1995). The metal co-ordination site in the MIDAS is provided by a highly conserved motif of Asp-X-Ser-X-Ser (DXSXS) as well as a Thr and Asp residue from a non-contiguous region. The final co-ordination position is a glutamate side chain from another I domain molecule adjacent in the crystal lattice. It was proposed that this structure was similar to a ligand-receptor complex and that natural ligands would bind at this cation binding face. This led to the popular concept that integrins bind their ligand by "grabbing an Asp" (Bergelson and Hemler 1995). The importance of the involvement of this motif in ligand binding is demonstrated in mutation studies where mutation of any of the metal co-ordinating side chains of the I domain abolishes binding (Michishita et al. 1993).

There are currently no tertiary structure predictions for the C terminus portion of the extracellular domain of the α subunit. The cytoplasmic domains of the α subunits are highly conserved among different species. They play diverse roles in regulating integrin function and differ significantly in their ability to induce post-ligand events. The different α subunit cytoplasmic domains however show little homology to each other suggesting each has a unique role. For example, the cytoplasmic domains of the α subunits of $\alpha_5\beta_1$ and $\alpha_L\beta_2$ are not required for adhesion mediated by these integrins but

are required for adhesion mediated by the integrins $\alpha_2\beta_1$ and $\alpha_4\beta_1$ (Sastry 1993). Nevertheless conserved sequences are observed and the most striking of these is Gly-Phe-Phe-Lys-Arg (GFFKR). This motif is adjacent to the transmembrane domain and is conserved among all α subunits across species. The strict conservation of this sequence, its charge and location near the membrane suggest that it plays an important role in α subunit function. Furthermore deletion of mutation of this sequence leads to constitutive activation of some integrins suggesting an important functional role for this sequence. The sequence Thr-Ser-Asp-Ala (TSDA) at the carboxy terminus is conserved among the α_5, α_6, α_V and α_8 subunits but its relevance is not yet clear.

1.2.2.2. The β subunit.

All β subunits have a highly conserved region of approximately 250 residues near the amino terminus. The presence of the DXSXS sequence from the MIDAS co-ordination site of the α I domain in all β subunits suggest that these regions may be structurally and functionally related (Bajt and Loftus 1994). Mutations within this region in the β_3 subunit abolish ligand binding suggesting that this domain is important in ligand binding to the integrin (Loftus 1994). Furthermore, antibodies that inhibit ligand binding often map to this region. Mutations in the corresponding regions of other β integrins also inhibit ligand binding. Function blocking and activating monoclonal antibodies for β_1 map to a region within this domain (Takada and Puzon 1993). Mutational analysis of the β_1 (Puzon-McLaughlin et al. 1996) and β_2 (Goodman and Bajt 1996) subunits supports the notion that this region within the β subunit folds in an I domain like structure and may engage ligands via a MIDAS like motif. The N-terminal region

contains a 90 amino-acid repeat named a PSI (plexins, semsphorins and integrins) domain and is predicted to possess an α-helical character (Bork et al. 1999). The remainder of the extracellular portion of the β subunit comprises four cysteine rich repeats which share a significant degree of homology with the EGF-like modules in laminin (Yuan et al. 1990). The main difference is the presence of eight (seven in the first) rather than six cysteine residues.

The cytoplasmic domains of the β subunits are relatively short sequences of 40-60 amino acids in length. The exception is the β_4 subunit cytoplasmic domain that is approximately 1,000 amino acids in length (Tamura et al. 1990). The primary sequences of the cytoplasmic domains are highly conserved through evolution. The β_1 domains show 100% conservation across species. Conserved amino acid clusters are observed in the β_1, β_2, β_3, β_5, β_6, and β_7 domains. One of these clusters, for example, is a unique stretch of 11 amino acids His-Asp-Arg-Arg-Glu-Phe-Ala-Lys-Phe-Glu-Lys (HDRREFAKFEK). It is adjacent to the transmembrane domain and consists mainly of charged residues. In studies where a β_3 domain truncated below the Leu-Leu-Ile-Thr-Ile-His-Asp-Arg (LLITIHDR) is co-transfected with constitutively active α-subunit chimeras, the high affinity ligand binding of the integrin is abolished (Loh et al. 1996). This suggests a role for the β cytoplasmic domain in integrin affinity modulation. A second cluster consists of the sequence Asn-Pro-Ile-Tyr (NPIY), a conserved NPXY motif, recognised as a tyrosine phosphorylation motif. A role for this motif has shown to be involved in the internalisation of the low density lipoprotein (LDL) receptor (Chen et al. 1990). This motif has also been shown to be tyrosine phosphorylated in a

fibronectin receptor complex (Hirst et al. 1986). The third cluster contains an NPXY motif distal to the NPIY motif. In the β_1 and β_2 tails the sequence is NPXY/F while the other subunits contain a NXXY/F motif. Mutations in this motif in the β_1 and β_3 cytoplasmic domains abolished inside out signalling preventing the integrin supporting ligand binding (O'Toole et al. 1995). Expression of a truncated β_2 cytoplasmic domain lacking the two NPXY motifs resulted in a loss of internalisation (Rabb et al. 1993). In contrast the NPXY motifs of the β_3 were found not to be involved in the internalisation of $\alpha_{IIb}\beta_3$ (Chen et al. 1992). Mutation of the two tyrosine residues to Phe in the cytoplasmic tail of β_3, in a knockout mouse model, resulted in the formation of loose unstable aggregates, upon platelet activation with low dose thrombin. These aggregates eventually disaggregated into single platelets, implying that tyrosine phosphorylation of the β cytoplasmic tail is important for optimal and stable aggregation. Furthermore, the knockout mice demonstrated an in vivo bleeding defect and clot retraction was inhibited (Law et al. 1999).

The β_4 cytoplasmic domain is unique among β-subunits. It is very large containing approximately 1,000 amino acids and shares no homology with the shorter β cytoplasmic domains except for the presence of an NPXY motif (Suzuki and Naitoh 1990). It displays a cell type dependent proteolytic processing (Giancotti et al. 1992) and is associated with intermediate filaments (Stepp et al. 1990). A schematic representation of the domain structures of the integrin subunits is shown in figure 1.4.

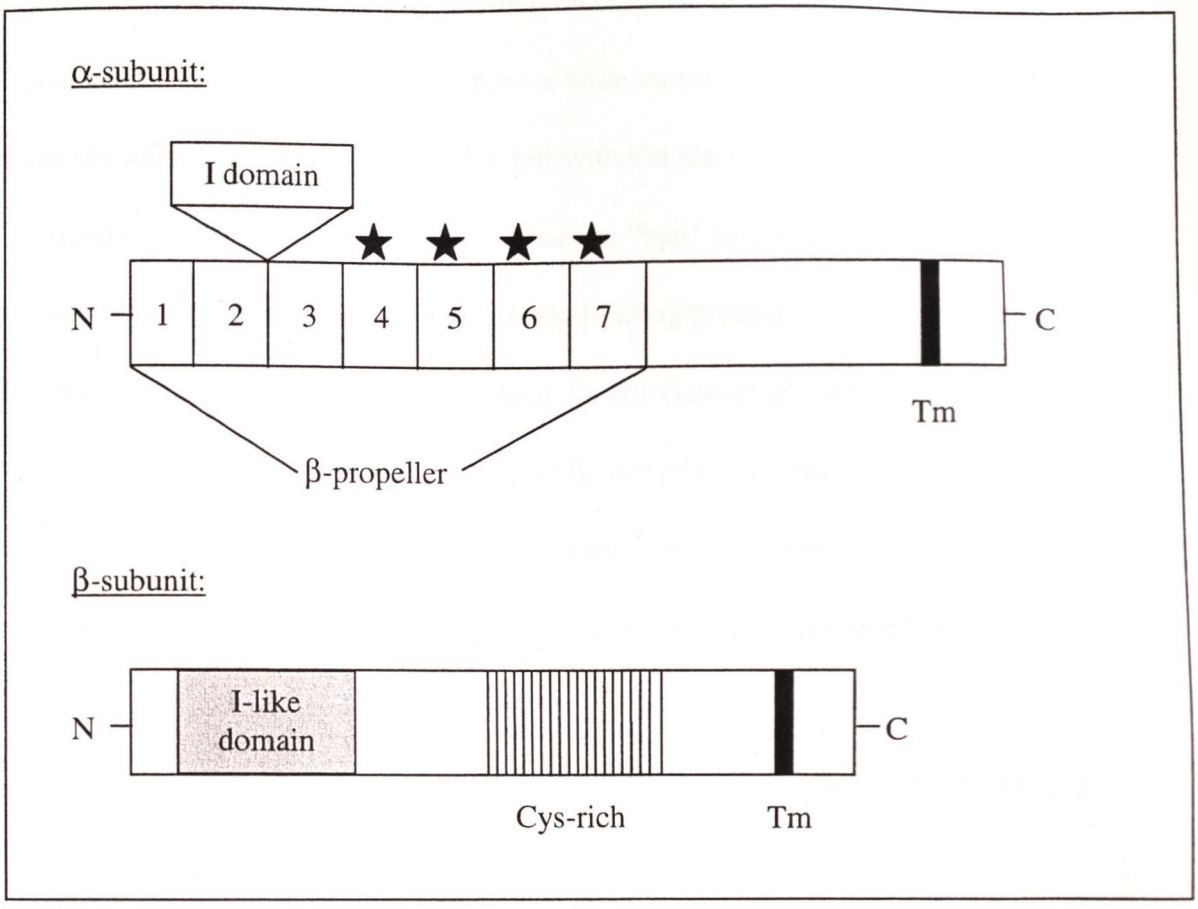


Figure 1.4. Schematic representation of the α and β subunits of integrins.

The seven homologous repeats of the α subunit are designated by the numbers 1-7. In those integrins that contain an I domain, this is inserted between repeats 2 and 3 of the α subunit. Stars denote putative Ca^{2+} binding sites. Hatched lines represents the cysteine rich region of β subunits. Tm represents the transmembrane domain. Modified from Leitinger B. et al. 2000.

1.2.3. Proteins interacting with the cytoplasmic domains of integrins.

Integrin cytoplasmic domains are the connection between engagement of the integrin by ligand and the cell's signalling machinery. Many proteins have been identified that interact with the cytoplasmic domains and are therefore proposed to be involved in the

regulation of integrin function. Some of these proteins can bind to α-tails and others to β-tails while some can bind to more than one type of α- or β- tails. Interestingly, the binding proteins identified thus far have a wide variation in their function, suggesting that the interaction of the cytoplasmic tail with the intracellular milieu is extremely complex. Using the β_1 cytoplasmic domain as "bait" in a yeast-two hybrid screen, filamin was identified as a specific integrin binding protein. It is a cytoskeletal protein which co-precipitates with β_1 in activated T-cells (Loo et al. 1998). It also co-precipitates with β_7 (Pfaff et al. 1998) and β_2 integrins (Sharma et al. 1995). The terminal end of this protein links with actin and thus is involved in the formation of actin networks. Its interaction with the β_2-tail suggests it may be involved in cell spreading.

Talin, another cytoskeletal protein, interacts with the tails of the β_1 and β_3 integrin subunits (Knezevic et al. 1996). The N terminus of this protein interacts with phospholipids and membrane components and its C terminus interacts the β-tails. It has been reported to also bind actin, vinculin and F-actin. α-actinin, an actin binding protein, has been shown to interact with the cytoplasmic domain of the β_1 integrin (Otey et al. 1993).

Proteins containing kinase activity bind to the tails of integrin subunits. One such protein is focal adhesion kinase (FAK). FAK is a 125kDa protein tyrosine kinase which is localised to focal adhesions (Schaller 1992). FAK can associate with the β_1, β_2 and β_3-tails and this was shown in studies using peptides derived from the sequences of these tails (Guan 1997). Another kinase shown to interact with the β-subunit cytoplasmic

domain is integrin-linked kinase (ILK). ILK is a 59kDa protein that contains a serine threonine kinase domain and was shown to phosphorylate the β_1 subunit tail. This phosphorylation event is thought to localise the β_1-tail to focal adhesion sites. An interaction between ILK and the β_2 and β_3 tails has also been demonstrated (Hannigan et al. 1996) (Huang and Wu 1999).

Paxillin is a multi-domain adapter protein that interacts with several structural and signalling proteins. Peptides corresponding to the β_1 cytoplasmic tail bound paxillin (Schaller et al. 1995). Recent studies have shown that paxillin associates directly with the α_4 subunit and not with any other α subunits (Liu et al. 1999). The adapter protein Shc has been shown to be involved in the activation of MAP kinase pathways by β_1 integrins (Wary et al. 1996). Calreticulin is a chaperone and calcium binding protein with multiple functions and diverse cellular locations. It can bind to the membrane-proximal region of α cytoplasmic tails (Coppolino et al. 1995). Members of the class of transmembrane proteins known as the tetraspanins have been shown to associate with integrins in immunoprecipitation experiments. The tetraspanin CD63 interacted with the VLA-3 and VLA-6 integrins (Berditchevski et al. 1995) and the tetraspanin CD9 and CD32 (F_c-γRII) were shown to interact with $\alpha_{IIb}\beta_3$ (Slupsky et al. 1997). A physical association between phosphatidylinositol 4-kinase, integrins and tetraspanins has been shown suggesting that a connection between integrins and phosphatidylinositide metabolism may exist (Berditchevski et al. 1997).

1.2.4. The effects of cations on ligand binding.

The binding of ligands to integrins is without exception bivalent-cation dependent. For many integrins ligand binding is stimulated by Mn^{2+}, as was first shown for $\alpha_5\beta_1$ (Gailit and Ruoslahti 1988). An increase in the affinity of the integrin $\alpha_2\beta_1$ for ligand occurred in the presence of Mg^{2+} when Ca^{2+} was chelated with EGTA (Staatz et al. 1989) (Hu et al. 1995). The platelet integrin $\alpha_{IIb}\beta_3$ was shown to have a single high-affinity binding site for Ca^{2+} with a K_d of 80nM and 3-4 medium-affinity Ca^{2+}-binding sites with a K_d of 40μM (Rivas and Gonzalez-Rodriguez 1991). Five binding sites of relatively high affinity for cations were detected in $\alpha_{IIb}\beta_3$ using terbium luminescence spectroscopy, four on the α-subunit and one on the β-subunit (Cierniewski et al. 1994). Binding of fibrinogen to $\alpha_{IIb}\beta_3$ is supported by millimolar concentrations of either Ca^{2+} or Mg^{2+} (Phillips and Baughan 1983). Fibrinogen binding was optimal in a mixture of 1mM Ca^{2+} and 1μM Mg^{2+} suggesting the presence of two different cation binding sites, one specific for Ca^{2+} and the other for Mg^{2+}. Cations also have specific effects on the binding capacity of fibronectin to $\alpha_5\beta_1$ (Mould et al. 1995). Mn^{2+} promoted high levels of ligand binding, Mg^{2+} promoted low levels of binding and Ca^{2+} failed to support binding. Ca^{2+} acted as a non-competitive inhibitor of Mn^{2+}-supported binding suggesting that each cation recognised different sites in the integrin. In contrast, Ca^{2+} acted as a competitive inhibitor of Mg^{2+}-supported ligand binding, implying that Ca^{2+} can displace Mg^{2+} from the integrin.

1.2.5. Integrins as signalling molecules.

Integrins are necessary for vascular and haematopoietic cell development, angiogenesis, cell migration in response to injury and extracellular matrix assembly (Shattil and Ginsberg 1997). Specific integrins such as platelet $\alpha_{IIb}\beta_3$ are crucial in the maintenance of normal haemostasis. Previously, integrins were thought of as just adhesion molecules simply bridging cells to each other or cells with a matrix but it is now accepted they have a greater role. There is much evidence supporting the hypothesis that these molecules act as signalling molecules, once occupied by their ligands. This role of the receptor involves transmitting information in both directions across the plasma membrane. Upon binding of the integrin by ligand and the concomitant clustering of the integrins at focal adhesion sites, this outside-in signal results in changes of cell shape, reorganisation of the cytoskeleton and generation of a variety of biochemical signals inwards into the cell. These signals include activation of protein tyrosine kinases such as src, FAK and Syk and the activation of phosphatidylinositol 3-kinase and MAP kinases. Furthermore, ligand binding to integrins can change the affinity and avidity of the receptor. In platelet aggregation, affinity modulation seems to be responsible for the initial binding of soluble fibrinogen or vWF to $\alpha_{IIb}\beta_3$ (Shattil et al. 1997). A modification of true affinity implies a structural change that is characteristic of integrins resulting in a greater strength of ligand binding. Different affinity states have been described in the β_1, β_2, β_3 and β_7 subunits reflecting conformational changes within and between the subunits (Faull et al. 1993), (Lollo et al. 1993), (Berlin et al. 1993). These conformational changes affect the shape and the accessibility of the integrin surface to the ligand. Avidity modulation implies a change in the functional affinity of the receptor.

Rebinding of ligand or chelation of cations may affect the interaction of ligand with the integrin. The altered avidity of integrins is likely to be as a result of their clustering or multimerisation in the plane of the plasma membrane. Clusters of ligand occupied integrins have been observed in platelets, endothelial cells and vascular smooth muscle cells. These clusters can be involved in small focal complexes which form in the assembly of filopodial and lamellipodial extensions (Nobes 1995). The formation of these clusters and extensions are regulated by members of the Rho family of GTPases implying a relationship between Rho signalling and integrin function (Tapon and Hall 1997).

1.3. THE PLATELET INTEGRIN $\alpha_{IIb}\beta_3$.

The platelet glycoprotein integrin $\alpha_{IIb}\beta_3$ (in integrin nomenclature), also termed GPIIb/IIIa in platelet nomenclature or CD41/CD61 in immunology nomenclature, has been identified as the key mediator of platelet aggregation. It was originally suggested to be the receptor for fibrinogen because patients with the autosomal recessive disorder Glanzmann's thrombasthenia, whose platelets failed to aggregate to any agonists were found to lack glycoproteins IIb and IIIa (Mustard and Packham 1970), (Hagen et al. 1980), (Nurden et al. 1979). It was not until 1978 that a specific association between stimulated platelets and fibrinogen was observed (Mustard et al. 1978). $\alpha_{IIb}\beta_3$ forms a non-covalent, calcium dependent heterodimer on the platelet surface as was determined by crossed immunoelectrophoresis (Kunicki et al. 1981). It is unique in that its expression is limited to blood platelets and megakaryocyte lineage. It is a member of the integrin family and the most abundant receptor on the platelet membrane with estimates of between 40,000 and 80,000 copies per platelet (Faraday et al. 1994) (Xia 1996)

(Wagner et al. 1996). In addition, an intracellular pool of $\alpha_{IIb}\beta_3$ within the platelet α-granules can be become expressed on the surface upon activation, increasing the amount of functional receptors by 30-50% (Cramer et al. 1990). On resting unstimulated platelets, $\alpha_{IIb}\beta_3$ is randomly dispersed on the surface and is only capable of binding immobilised fibrinogen. In response to platelet activation, however, $\alpha_{IIb}\beta_3$ becomes a receptor for several adhesive proteins, the binding of which is mediated in part by an Arg-Gly-Asp (RGD) recognition motif commonly found on integrin ligands. Binding of two of these proteins, fibrinogen and von Willebrand factor, which are prominent ligands for $\alpha_{IIb}\beta_3$, cause platelets to aggregate (Phillips et al. 1991). Upon engagement of ligand $\alpha_{IIb}\beta_3$ transmits information across the platelet membrane to instigate post-ligand binding events such as tyrosine phosphorylation of specific proteins and reorganisation of cytoskeletal proteins in the process known as "outside-in" signalling.

1.3.1. Structure.

The α_{IIb} and β_3 subunits each have a large extracellular domain, a transmembrane domain and a short cytoplasmic domain. The α_{IIb} subunit in its mature form consists of a heavy (alpha) chain that is disulphide bonded to a light (beta) chain and the β subunit is a single polypeptide. α_{IIb} has an apparent molecular weight of 136kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with the heavy chain having a weight of 125kDa and the light chain 23kDa. The apparent molecular weight of the β_3 subunit is 95kDa on a non-reduced gel and upon reduction the apparent molecular weight increase to 114kDa (Jennings and Phillips 1982) (Phillips and Agin 1977).

1.3.2. Primary Structure.

The genes for α_{IIb} and β_3 are physically linked on the q21-22 region of the long arm of human chromosome 17 (Bray et al. 1987). The DNA sequence for the human platelet α_{IIb} subunit was obtained by initial screening of a λgt11 HEL expression library with a polyclonal anti-human platelet GPIIb IgG and subsequent screening with a 401-bp fragment of the 5' end of the 1.9kb cDNA used in the study (Poncz et al. 1987). A total of 3.3 kilobases of cDNA was sequenced and revealed a continuous open reading frame. The cDNA encoded a total of 1039 amino acids of which 871 made up the larger subunit, 137 the smaller subunit and 30 amino acids constituted an amino terminal signal peptide of the α_{IIb} protein. The smaller subunit contained a 26-residue hydrophobic sequence that potentially represented a transmembrane domain. The larger subunit contained four stretches of 12 amino acids that are similar to the calcium binding sites of calmodulin and troponin. The amino acid sequence obtained showed 74% and 63% homology with the vitronectin and fibronectin α-subunits respectively. The subunit is translated as a single polypeptide and then cleaved into two disulphide linked subunits (Bray et al. 1986). The subunits are connected to each other by a single disulphide bond between α_{IIbH} Cys^{9} and α_{IIbL} Cys^{826} (Calvete and Gonzalez-Rodriguez 1986). The amino acid sequence for α_{IIb} is shown in figure 1.5.

```
        10         20         30         40         50         60
        |          |          |          |          |          |
MARALCPLQA LWLLEWVLLL LGPCAAPPAW ALNLDPVQLT FYAGPNGSQF GFSLDFHKDS
        70         80         90        100        110        120
        |          |          |          |          |          |
HGRVAIVVGA PRTLGPSQEE TGGVFLCPWR AEGGQCPSLL FDLRDETRNV GSQTLQTFKA
       130        140        150        160        170        180
        |          |          |          |          |          |
RQGLGASVVS WSDVIVACAP WQHWNVLEKT EEAEKTPVGS CFLAQPESGR RAEYSPCRGN

       190        200        210        220        230        240
        |          |          |          |          |          |
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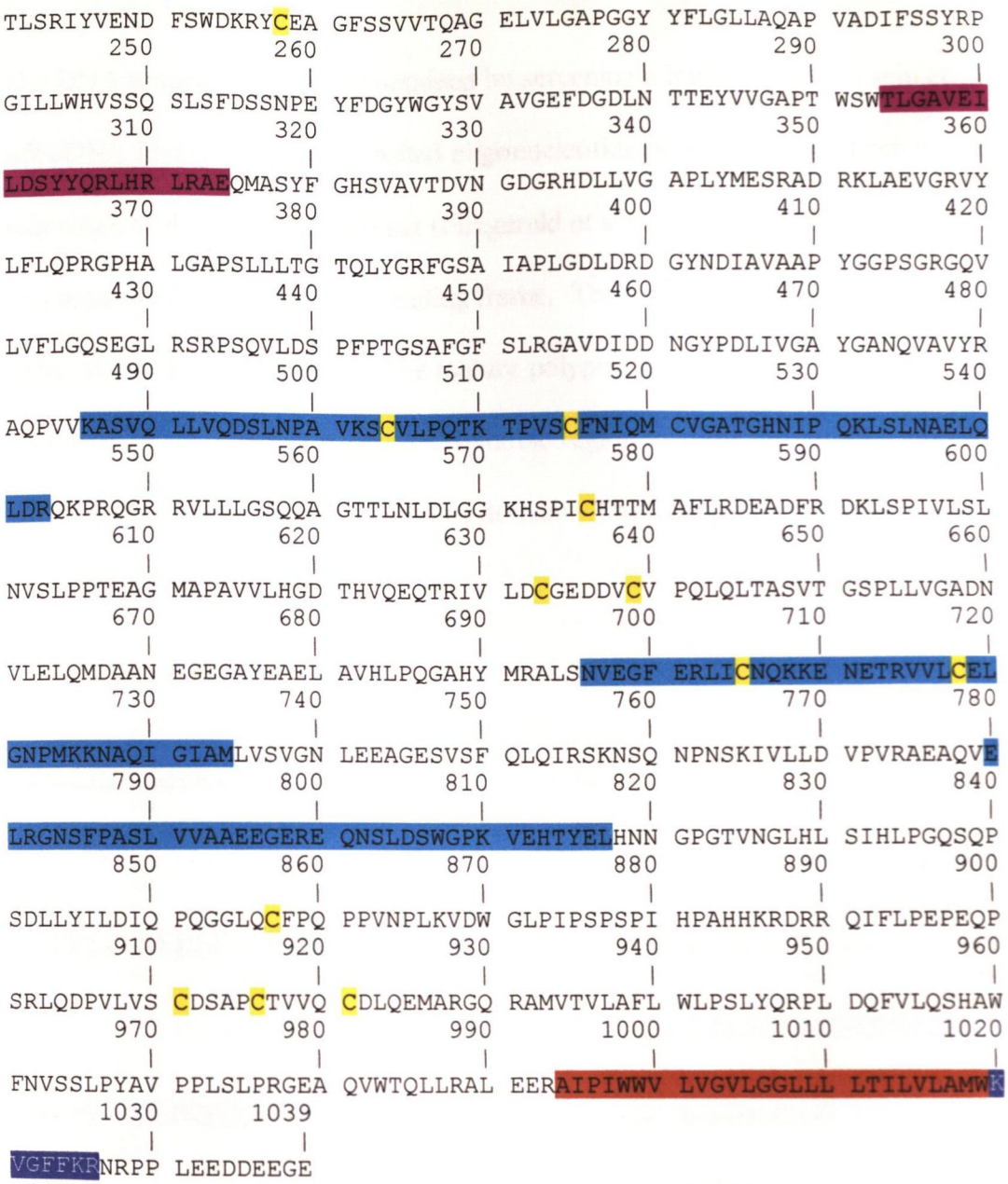


Figure 1.5. The amino acid sequence of the α_{IIb} subunit.

Residues 1-31, in green correspond to the signal sequence peptide. Residues 32-993 correspond to the extracellular domain. Residues 994-1019, in red, correspond to the transmembrane domain. Residues 1020-1039 correspond to the cytoplasmic domain. The cysteine residues are highlighted in yellow. The residues involved in the interface with the β_3 subunit are in turquoise. A probable fibrinogen binding site is shown in pink. The conserved KVGFFKR sequence in the cytoplasmic domain is highlighted in blue. This sequence was obtained from the National Centre for Biotechnology Information (NCBI) Entrez site, with the accession code P08514.

The DNA sequence for β_3 was obtained by screening a human umbilical vein endothelial cell cDNA library using radiolabelled oligonucleotide probes designed from peptide sequences of the platelet β_3 subunit (Fitzgerald et al. 1987). A total of 3.17 kilobases was sequenced in a single open reading frame. The first 26 amino acids represents an amino terminus signal peptide. The mature polypeptide consists of 762 amino acids. A stretch of 29 residues in a strong hydrophobic region towards the carboxy terminus probably represents the transmembrane domain. The amino acid sequence for the β_3 subunit is shown in figure 1.6

```
        10         20         30         40         50         60
        |          |          |          |          |          |
MRARPRPRPL WVTVLALGAL AGVGVGGPNI CTTRGVSSCQ QCLAVSPMCA WCSDEALPLG
        70         80         90        100        110        120
        |          |          |          |          |          |
SPRCDLKENL LKDNCAPESI EFPVSEARVL EDRPLSDKGS GDSSQVTQVS PQRIALRLRP
       130        140        150        160        170        180
       |          |          |          |          |          |
DDSKNFSIQV RQVEDYPVDI YYLMDLSYSM KDDLWSIQNL GTKLATQMRK LTSNLRIGFG
       190        200        210        220        230        240
       |          |          |          |          |          |
AFVDKPVSPY MYISPPEALE NPCYDMKTTC LPMFGYKHVL TLTDQVTRFN EEVKKQSVSR
       250        260        270        280        290        300
       |          |          |          |          |          |
NRDAPEGGFD AIMQATVCDE KIGWRNDASH LLVFTTDAKT HIALDGRLAG IVQPNDGQCH
       310        320        330        340        350        360
       |          |          |          |          |          |
VGSDNHYSAS TTMDYPSLGL MTEKLSQKNI NLIFAVTENV VNLYQNYSEL IPGTTVGVLS
       370        380        390        400        410        420
       |          |          |          |          |          |
MDSSNVLQLI VDAYGKIRSK VELEVRDLPE ELSLSFNATC LNNEVIPGLK SCMGLKIGDT
       430        440        450        460        470        480
       |          |          |          |          |          |
VSFSIEAKVR GCPQEKEKSF TIKPVGFKDS LIVQVTFDCD CACQAQAEPN SHRCNNGNGT
       490        500        510        520        530        540
       |          |          |          |          |          |
FECGVCRCGP GWLGSQCECS EEDYRPSQQD ECSPREGQPV CSQRGECLCG QCVCHSSDFG
       550        560        570        580        590        600
       |          |          |          |          |          |
KITGKYCECD DFSCVRYKGE MCSGHGQCSC GDCLCDSDWT GYYCNCTTRT DTCMSSNGLL
       610        620        630        640        650        660
       |          |          |          |          |          |
CSGRGKCECG SCVCIQPGSY GDTCEKCPTC PDACTFKKEC VECKKFDREP YMTENTCNRY
       670        680        690        700        710        720
       |          |          |          |          |          |
CRDEIESVKE LKDTGKDAVN CTYKNEDDCV VRFQYYEDSS GKSILYVVEE PECPKGPDIL
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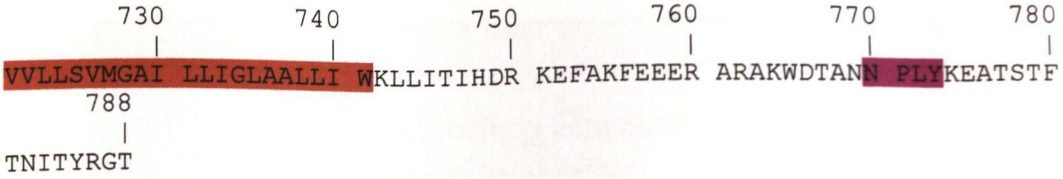


Figure 1.6. The amino acid sequence of β_3.

Residues 1-26, in green, correspond to the signal sequence peptide. Residues 27-718 correspond to the extracellular domain. Residues 719-741 correspond to the transmembrane domain and residues 742-788 correspond to the cytoplasmic domain. The cysteines are highlighted in yellow. The residues involved in the interface with the α_{IIb} subunit are in turquoise. The residues involved in the MIDAS like domain of the β subunit are bolded in red (residues 145-149) and include the highly conserved motif DXSXS. The fibrinogen binding site is highlighted in bright green. The highly conserved NPXY tyrosine phosphorylation motif is highlighted in pink. This sequence was obtained from the National Centre for Biotechnology Information (NCBI) Entrez site, with the accession code P05106.

A schematic representation of each subunit is shown in figures 1.7 and 1.8.

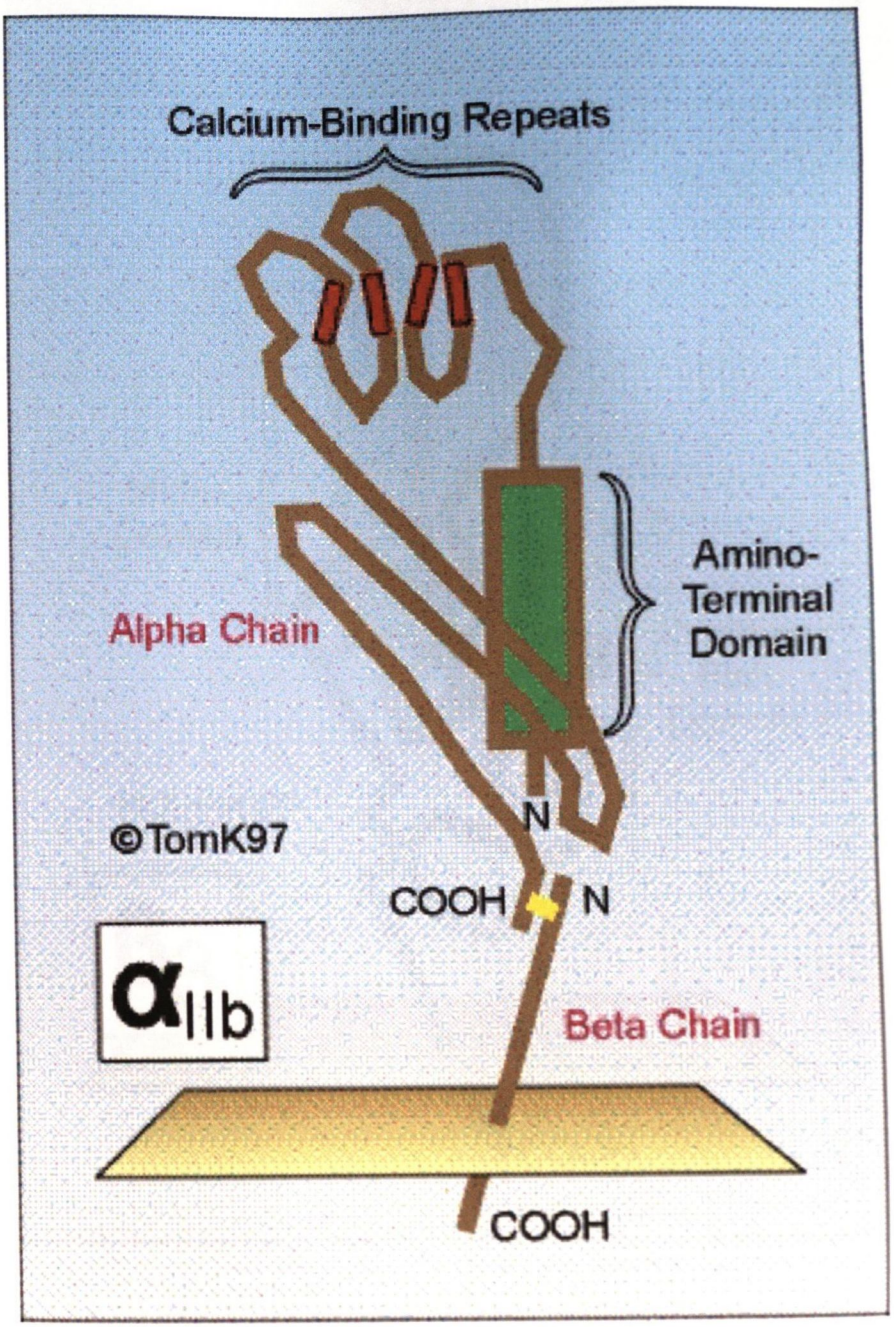


Figure 1.7. A schematic representation of the structure of the α_{IIb} subunit (Tom Kunicki, http://www.scripps.edu/mem/eht/kunicki/a2bb3.html).

The subunit is composed of a heavy (alpha) and a light (beta) chain that are linked by a single disulphide bond, shown in yellow. The calcium binding motifs are highlighted in red.

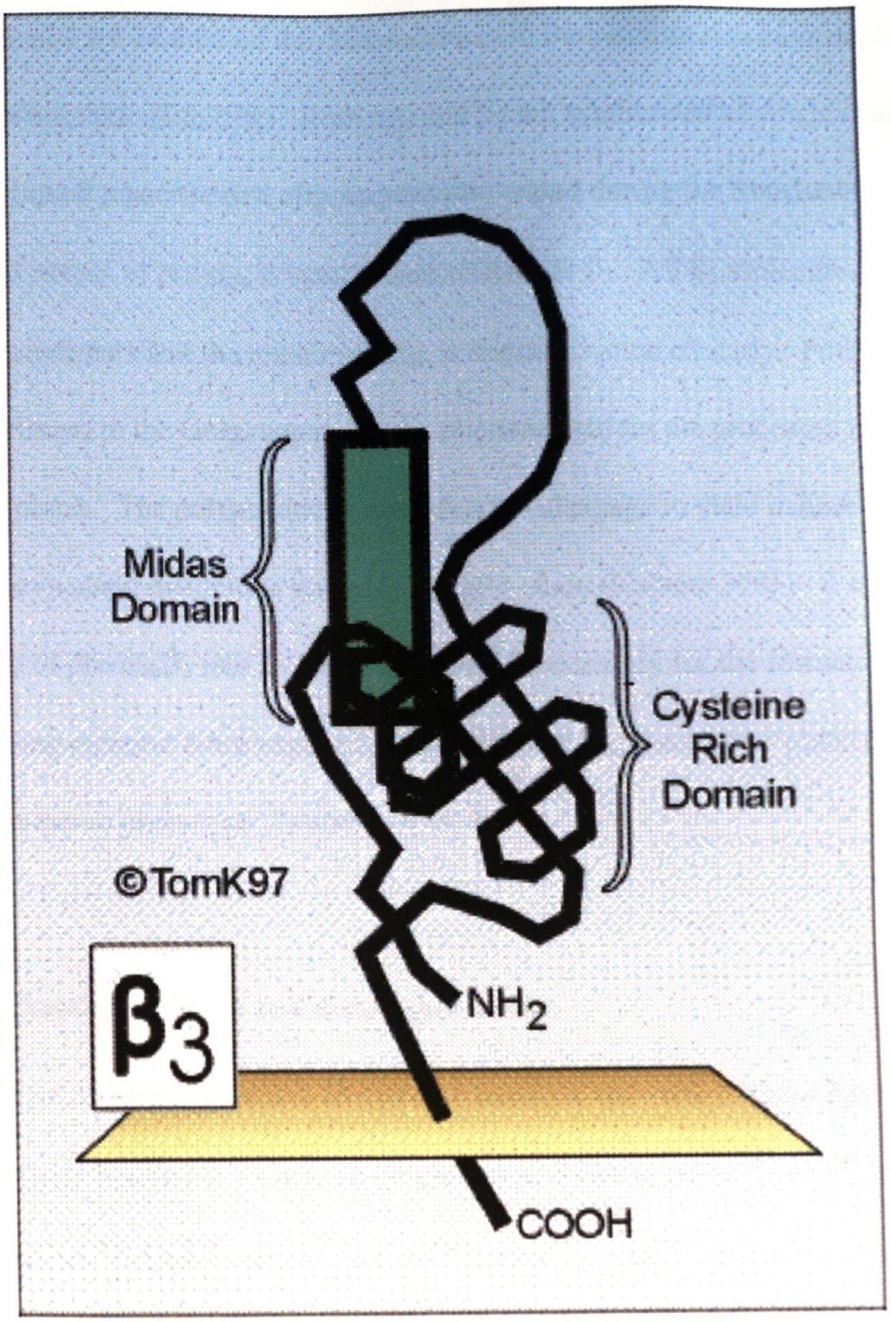


Figure 1.8. A schematic representation of the structure of the β_3 subunit.

(Tom Kunicki http://www.scripps.edu/mem/eht/kunicki/a2bb3.html).

The β_3 subunit has a large extracellular region that contains a cysteine rich domain and the cation binding site similar to a MIDAS domain.

The subunits are assembled into heterodimers in the calcium rich environment of the endoplasmic reticulum (ER). Both α_{IIb} and β_3 are synthesised as single chain precursors with N-linked mannose rich oligosaccharides added during the translation process. A five fold excess of pre-α_{IIb} is synthesised relative to β_3. All β_3 molecules are assembled into heterodimers and the remaining α_{IIb} is degraded intracellularly. Following transportation to the Golgi apparatus the oligosaccharides are processed into complex carbohydrates. The polypeptide chains undergo cleavage to yield mature α_{IIb} composed of its heavy chain disulphide linked to its light chain (Calvete 1995). It appears that cleavage of pre-$\alpha_{IIb}\beta_3$ into a mature molecule is necessary for the formation of a functioning receptor since expression of uncleaved α_{IIb} results in a platelet Glanzmann thrombasthenic phenotype (Iwamoto et al. 1994).

1.3.3. Cysteine residues and disulphide bonds.

The mature α subunit contains 18 cysteine residues and each of these has been shown to be disulphide bonded to its nearest neighbour as follows: αCys^{56}– Cys^{65}; αCys^{107}– Cys^{130}; αCys^{146}– Cys^{167}; αCys^{473}– Cys^{484}; αCys^{490}– Cys^{545}; αCys^{602}– Cys^{608}; αCys^{674}– Cys^{687}; $\alpha_H Cys^{826}$– $\alpha_L Cys^{9}$ and $\alpha_L Cys^{14}$– Cys^{19} (Calvete et al. 1989). This nearest-neighbour cysteine residue pairing results in relatively short disulphide bonds which may function mainly to stabilise the short range folding of the molecule.

The β subunit of $\alpha_{IIb}\beta_3$ contains 56 cysteines and the subunit has been divided into four main structural domains: the N-terminal cysteine rich domain; the fibrinogen binding domain; the cysteine-rich proteinase-resistant core and the C-terminal domain

comprising the transmembrane and cytoplasmic domains (Calvete et al. 1991). The N-terminal cysteine-rich domain comprises the first 100 amino acids and contains 7 cysteine residues (Calvete et al. 1988). In this domain it was found that Cys^{5} disulphide bonds with Cys^{435} in the N-terminal side of the proteinase resistant core. Other disulphide bond pairings in this domain were found to be Cys^{13}– Cys^{23}, Cys^{16}– Cys^{49} and Cys^{26}– Cys^{38}. Attempts to cleave both the fibrinogen binding and proteinase-resistant domains proved difficult. Therefore, assumptions were made about the cysteine pairings in these domains. For example, it is suggested that Cys^{521} is disulphide bonded to any one of the cysteine residues within the 501-508 stretch of Cys-Leu-Cys-Gly-Gln-Cys-Val-Cys (CLCGQCVC). The cysteine residues in the four cysteine rich region (CRR) tandem repeat units are highly conserved and appear in all other β subunits of integrins. Fitzgerald's analysis states that the first cysteine repeat is incomplete in that it only contains seven of eight cysteines and the next three repeats each have a consensus sequence of Cys-X-Cys-X-X-Cys-X-Cys (Fitzgerald et al. 1987). This first cysteine in the first repeat is replaced by either an Phe, Met, Leu or Tyr. Since these motifs are common to all the β integrin subunits it is hypothesised that these repeats represent an important additional functional domain of the subunit.

1.3.4. Ligand binding.

$\alpha_{IIb}\beta_3$ is a multi-functional receptor and binds at least four adhesive proteins on activated platelets: fibrinogen, fibronectin, vitronectin and von Willebrand factor (vWf). The binding of soluble ligands to $\alpha_{IIb}\beta_3$ can be detected within seconds of platelet activation and it reaches a steady state within minutes (Bennett and Vilaire 1979). The binding of

fibrinogen to $\alpha_{IIb}\beta_3$ is required for aggregation and although the major function of $\alpha_{IIb}\beta_3$ is in platelet aggregation, it also plays a role in platelet adhesion. Under conditions of high shear, platelet adhesion and aggregation may be mediated by the binding of vWf to $\alpha_{IIb}\beta_3$ (Ikeda et al. 1991). Fibrinogen binds to the receptor via the Arg-Gly-Asp (RGD) amino acid recognition sequence. This sequence is present on all four adhesive proteins, being originally identified in fibronectin (Pierschbacher and Ruoslahti 1984). Fibrinogen contains two RGD sequences, both located in the Aα-chain, one near the N-terminus and a second near the C-terminus (Doolittle et al. 1979). A second sequence has been identified on the fibrinogen molecule which interacts with $\alpha_{IIb}\beta_3$ and is located at the carboxyl end of the γ chain (Kloczewiak et al. 1989). It is a 12-amino-acid dodecapeptide sequence (HHLGGAKQAGDV) and is unique to fibrinogen. Upon platelet activation, $\alpha_{IIb}\beta_3$ undergoes conformational changes and now becomes a competent, high affinity receptor for plasma fibrinogen. Soluble fibrinogen does not bind to resting $\alpha_{IIb}\beta_3$ and the mechanism which causes these conformational changes to occur within the receptor upon activation are not fully elucidated. These conformational changes can be monitored using specific monoclonal antibodies (Kouns et al. 1990). The enhanced rate of binding of one of these monoclonal antibodies, 7E3, to $\alpha_{IIb}\beta_3$ following platelet activation suggests that a change in the conformation of the $\alpha_{IIb}\beta_3$ complexes occurs, allowing $\alpha_{IIb}\beta_3$ to become competent to bind fibrinogen (Coller 1985). It has also been found that when some RGD-peptides bind to purified $\alpha_{IIb}\beta_3$ similar conformational changes are induced in the receptor, equivalent to those observed upon ligand binding and platelet activation (Parise et al. 1987).

1.3.5. Conformational Changes.

The activation-induced conformational change in $\alpha_{IIb}\beta_3$ was confirmed by the production of a monoclonal antibody that specifically recognised the activated integrin (Shattil et al. 1985). The antibody is designated PAC-1 and it binds specifically to $\alpha_{IIb}\beta_3$ on western blots and also inhibits platelet aggregation. In a separate study, changes in fluorescence resonance energy transfer that occurred between differentially labelled α_{IIb} and β_3 with antibodies after platelet stimulation indicated that a conformational change had occurred in the receptor (Sims et al. 1991). These conformational changes are thought to result in the exposure of the fibrinogen-binding site, which is cryptic in the inactive receptor. Furthermore, $\alpha_{IIb}\beta_3$ on unstimulated platelets can bind immobilised fibrinogen. Like the binding of activated platelets to soluble fibrinogen, this event requires divalent cations and can be inhibited by RGD containing peptides or monoclonal antibodies against $\alpha_{IIb}\beta_3$ (Savage et al. 1992). Why unactivated $\alpha_{IIb}\beta_3$ can interact with immobilised but not soluble fibrinogen, and activated $\alpha_{IIb}\beta_3$ can interact with both is not clear cut. Perhaps the immobilised fibrinogen adopts a conformation recognisable by the unactivated receptor allowing it to bind.

The control of $\alpha_{IIb}\beta_3$ affinity for ligand is apparently controlled by signals transmitted through the cytoplasmic domains of each subunit. The amino acid sequences of the α_{IIb} and β_3 cytoplasmic domains are shown in figure 1.9

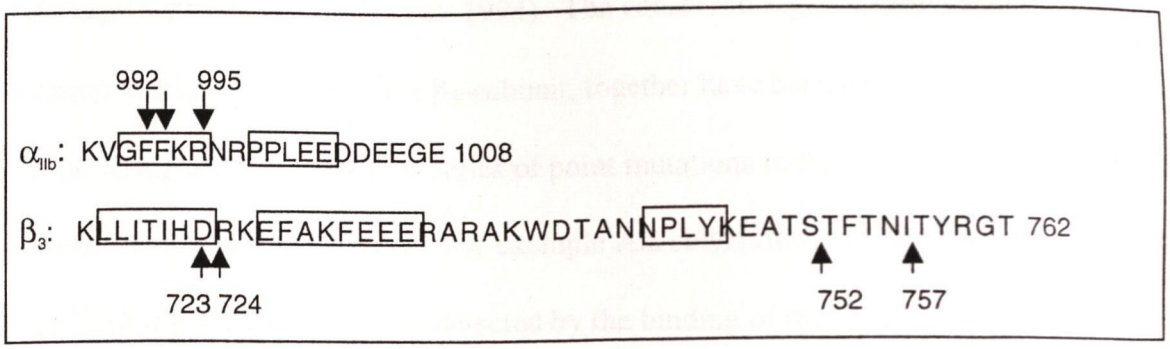


Figure 1.9. The amino acid sequences of the cytoplasmic tails of α_{IIb} and β_3. Sequences conserved among α and β subuints of integrins are boxed. The arrows and numbers indicate the position of mutations made in each subunit and their impact on $\alpha_{IIb}\beta_3$ function is described below.

Evidence for the role of the cytoplasmic tails in integrin activation comes from the analysis of naturally-occurring or engineered mutations in these regions. A mutation generated in the β_3 tail resulted in a Glanzmann's thrombasthenia phenotype that abrogated $\alpha_{IIb}\beta_3$ function, indicating the relevance of this β-cytoplasmic domain in activation of the receptor. The mutation introduced a stop codon at position Arg^{724}. This shortening of the tail suggests that the sites important for integrin activation are located in the deleted region (Wang et al. 1997). It is noteworthy that both consensus tyrosine phosphorylation motifs (NXXY) are in this region. In a separate study the insertion of a stop codon at Ile^{757} in the cytoplasmic tail of β_3 prevented the cells from mediating cell spreading, following transient co-expression with wild-type α_{IIb} in CHO cells. Again this suggests an important regulatory role for this region, and for the second NXXY motif, in particular (Ylanne et al. 1995). In contrast, truncation of the α_{IIb} tail at Gly^{991} proximal to the conserved GFFKR motif resulted in the formation of a

constitutively active integrin, highlighting the importance of this region in the regulation of integrin affinity (O'Toole et al. 1994). The conserved regions, GFFKR in the α_{IIb}-subunit and LLITIHDR in the β_3-subunit, together have been described as the "integrin hinge" (Hughes et al. 1996). A series of point mutations in this "hinge" results in the formation of an active receptor. For example Ala substitutions of Phe^{992}, Phe^{993} and Arg^{995} of α_{IIb} activated $\alpha_{IIb}\beta_3$ as detected by the binding of the monoclonal antibody PAC-1. Moreover, the observation was made that the relative positioning of α_{IIb} Arg^{995} and β_3 Asp^{723} may allow the cytoplasmic domains to interact via a salt bridge. Charge reversal mutants were constructed and each mutation resulted in the formation of a receptor with a high affinity state as determined by the binding of PAC-1. It was therefore concluded that an interaction between the conserved motifs of the two subunits regulates the affinity of the integrin for ligand and constrains the receptor in a low affinity state. Furthermore, we have shown that short peptide sequences of the KVGFFKR region of α_{IIb} can induce receptor activation, TxA_2 formation and platelet aggregation emphasising the importance of this region in activation of the receptor (Stephens et al 1998). However, the ligand binding capacity of the GFFK-mutated integrin can be reversed when the β_3 tail is truncated at position 724 or when Ser^{752} is replaced by a Pro residue suggesting an additional regulatory role for the C-terminal region of the β_3 tail (O'Toole et al. 1994).

Recently, the NMR structure of a lipid modified peptide corresponding to the α_{IIb} cytoplasmic tail as well as that of a peptide with the Phe^{992} and Phe^{993} residues substituted with Ala has been described (Vinogradova et al. 2000). Both the wild type

and mutant peptides have α-helical features in their N-terminal part. The wild type peptide adopted a closed conformation while that of the mutant adopted an open confirmation. A proposed mechanism for the regulation of ligand binding was put forward by these authors: In resting platelets $\alpha_{IIb}\beta_3$ is maintained in an inactive state through interaction of the two cytoplasmic tails with each other and/or the interaction of a cellular protein. The cytoplasmic tail adopts a closed conformation with its N-terminus interacting with its acidic C-terminal loop. This loop acts as a switch to open or close the conformation of the α_{IIb} cytoplasmic tail thereby activating the receptor. Opening of the switch may occur by a protein-protein interaction as a result of a conformational change that exposes the N-terminal helix of the α_{IIb} tail and activates the receptor. However, even a complete understanding of the regions and cytoplasmic proteins involved in the regulation of the integrin response to cellular activation, fails to inform us of precisely how the extracellular regions of the integrin undergo conformational changes such that they alter their affinity for their ligands. In order to understand this mechanism a knowledge of the ligand binding regions of integrins in general, and $\alpha_{IIb}\beta_3$ in particular, is necessary.

1.3.6. Ligand binding surfaces.

The fine structure of $\alpha_{IIb}\beta_3$ has not been established as crystallising such a heavily glycosylated molecule is difficult in the extreme. Nonetheless, a model has emerged in which the ligand binding pocket of $\alpha_{IIb}\beta_3$ is discontinuous and is formed by structures from both the α and β subunits (Plow et al. 1992) (Calvete et al. 1992). These structures remain cryptic in the resting, unactivated receptor. It is thought that a major relative

reorientation along the subunit interface occurs during the conformational transition of the integrin (O'Toole 1991). In this study a comparison of the proteolytic degradation patterns of non-RGD binding (inactive) and RGD-binding (active) conformers of purified $\alpha_{IIb}\beta_3$ was made. The results revealed that in the activated state, the N-terminal half of α_{IIb} protected itself and residues 100-348 in β_3 from proteolysis, while the C-terminal portion of α_{IIb} was more susceptible to proteolysis. So with platelet activation the binding pocket of the receptor opens up. The subunit interface is thought to be composed of residues 217-298 and 324-366 of β_3 and residues 486-553, 696-734 and 780-817 of α_{IIb} and the membrane proximal region of the α subunit (Calvete 1995; 1994).

1.3.6.1. Ligand binding sites on β_3 subunit of the platelet integrin.

RGD containing peptides bind to residues Asp^{109}-Glu^{171} in β_3 implicating this stretch of residues in ligand binding (D'Souza et al. 1988). The receptor sites recognised by these peptides have been hypothesised to be separate yet overlapping (Santoro and Lawing 1987). Monoclonal antibodies directed against this region inhibited ligand binding and platelet aggregation (Calvete et al. 1991) (Andrieux et al. 1991). This sequence of β_3 from residues 109-171 is highly conserved in all β subunits of integrins. In an analysis of β_3 from a thrombasthenic patient it was found that substitution of Asp 119 $\rightarrow$ Tyr resulted in complete loss of ligand binding and failure of platelets to aggregate thus indicating the importance of this residue in ligand binding (Loftus et al. 1990). In a separate study it was shown that the stretch of sequence between residues 118 and 131 on β_3 possess both ligand and cation binding properties (D'Souza et al. 1994).

Furthermore, it was shown that ligand displaced cation from this region of intact $\alpha_{IIb}\beta_3$ and altered the affinity of integrin for cation. Consequently, the binding of ligands and cations to $\alpha_{IIb}\beta_3$ are closely linked. Critical functional roles were assigned to Ser^{121} and Ser^{123} in ligand binding function (Bajt and Loftus 1994). Together Asp^{119}, Ser^{121} and Ser^{123} compose a DXSXS motif that is absolutely conserved in all β integrins. High resolution crystal studies established that this motif constitutes a portion of the metal co-ordination site known as the MIDAS domain (Lee 1995). This motif in β_3 has been shown to function as a MIDAS-like domain (Tozer et al. 1996) and that the residues Asp^{217} and Glu^{220} play a role in ligand binding function via a likely contribution to a metal co-ordination sphere. Thus, it would appear that the cation binding MIDAS domain and the ligand binding region are the same or, at least, overlapping. Another binding site for Arg-Gly-Asp (RGD) peptides lies within this region (D'Souza et al. 1988). Peptides corresponding to the β_3 residues Ser^{211}-Gly^{222} also bound fibrinogen and blocked its binding to $\alpha_{IIb}\beta_3$ implicating this region in ligand binding (Charo et al. 1991). Notably, this region overlaps with the MIDAS co-ordinating residues at Asp^{217} and Glu^{220} (Tozer et al. 1996). Mutation of Arg^{214} into a Trp residue prevents fibrinogen binding to $\alpha_{IIb}\beta_3$ in response to platelet activation agonists but interestingly does not affect the interaction of an RGD-peptide with the receptor in solution (Lanza et al. 1992) (Djaffar and Rosa 1993). A spontaneous mutation of Arg^{214} to Gln resulted in the disassembly of the two subunits even in the presence of Ca^{2+} and an inability to bind ligand (Bajt et al. 1992). These substitutions of Arg^{214} therefore appear to maintain the integrin in an inactive conformation that prevents activation to allow ligand binding. This implies that this residue is critical for maintaining the correct tertiary structure of

the ligand binding site on β_3. It is also of interest to note that I have been involved with a study of an Irish Glanzmann's Thrombasthenia patient in whom a mutation was identified at Val^{193}. This patient shows a similar phenotype to the Arg^{214} mutation, in that the resulting integrin fails to bind fibrinogen but is capable of binding a small RGD-mimetic (Fullard 2001). Thus, it would appear that the N-terminal portion of the β_3 subunit may be divided into two domains: the N-terminal half appears to be critical for ligand binding and the C-terminal half plays a role in both subunit association and regulation of the expression of the active site (Calvete 1995).

1.3.6.2. Ligand binding sites on α_{IIb} subunit of the platelet integrin.

Peptides derived from the carboxy terminus of the γ-chain of fibrinogen cross-linked to a region of α_{IIb} defined by residues Ala^{294}- Met^{314} identifying this region as a ligand binding domain (D'Souza et al. 1990). In addition, the recombinant fragment α_{IIb}171-464 binds to immobilised fibrinogen in a ratio of 2.2:1 and this binding was optimal in the presence of 0.5mM Ca^{2+}, when all four Ca^{2+} binding sites on the fragment were occupied (Gulino et al. 1992). Domain swapping experiments indicate that the amino terminal one third (residues 1-334) of α_{IIb} defines the ligand recognition specificity of the integrin $\alpha_{IIb}\beta_3$ (Loftus et al. 1996). The over-lap of these regions clearly identifies residues 171-334 as important in ligand binding. Consistent with this, mutation of α_{IIb} residues Gly^{184}, Tyr^{189}, Tyr^{190}, Phe^{191} and Gly^{193} to alanines significantly blocked binding of $\alpha_{IIb}\beta_3$ to soluble fibrinogen (Kamata et al. 1996). The loop of α_{IIb} (residues 147-166) defined by the disulphide bond between Cys^{146}- Cys^{167} has been implicated in

the ligand binding function of $\alpha_{IIb}\beta_3$. Substitution of Asp^{163} by Ala abolished the ligand binding function of the integrin (Honda et al. 1998).

1.3.6.3. Cation regulation of ligand binding on $\alpha_{IIb}\beta_3$.

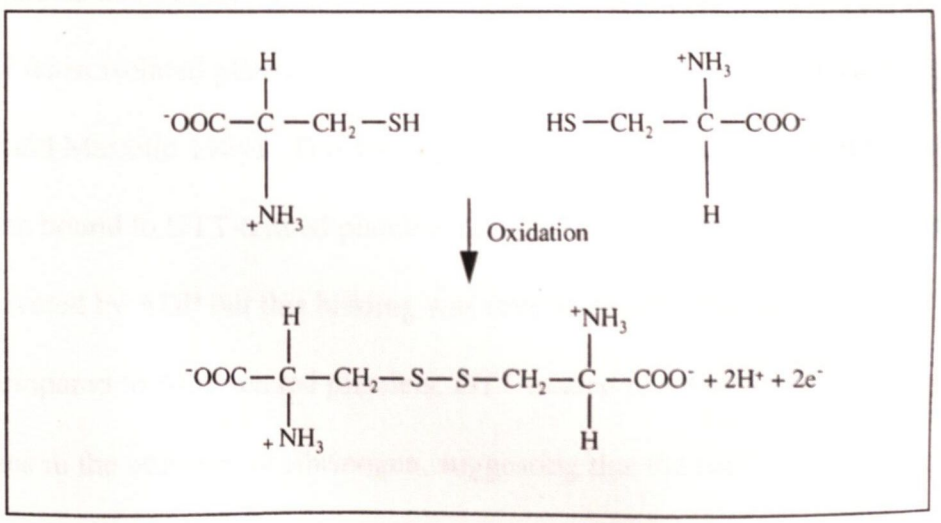
Divalent cations can bind to up to five sites on integrins and can promote (Smith et al. 1994) or suppress (Hu et al. 1995) ligand binding. Two classes of cation binding sites in the β_3 subunit have been identified (Hu et al. 1996). The first class of sites promote ligand binding to the integrin when occupied by divalent cations. A recombinant fragment of β_3 (residues 95-373) bound fibrinogen in the presence of Mn^{2+} and low concentrations of Ca^{2+} whereas high concentrations of Ca^{2+} prevented this interaction. This finding suggests that a high affinity Ca^{2+} site is present within this region. This site is proposed to be functionally identical to a ligand-competent site (Mould et al. 1995) which can bind Ca^{2+}, Mg^{2+} or Mn^{2+} and must be occupied for ligand to bind integrin. The second class of sites are allosteric to the ligand binding site, are highly specific for Ca^{2+} and when occupied increase the dissociation rate between RGD and integrin (Hu et al. 1996). Four EF-hand Ca^{2+} binding domains are present in the α_{IIb} subunit (Cierniewski et al. 1994). Mutations in these calcium binding domains of α_{IIb} in a Glanzmann's thrombasthenic phenotype demonstrated the importance of these domains in the intracellular transport of the heterodimer. This indicates that these domains play a structural rather than a ligand binding role (Basani et al. 1996) (Poncz et al. 1994).

1.3.7. Ligand induced binding sites (LIBS).

The binding of fibrinogen and other ligands to activated $\alpha_{IIb}\beta_3$ causes additional conformational changes on the integrin. In addition, peptides corresponding to the dodecapeptide and the RGD sites of the γ- and α- chains of fibrinogen, respectively, change the conformation of $\alpha_{IIb}\beta_3$ (Parise et al. 1987). Conformational changes in $\alpha_{IIb}\beta_3$ result in the expression of neo-epitopes referred to as ligand induced binding sites (LIBS) and are detectable by monoclonal antibodies. D3GP3 is such a LIBS antibody (Kouns et al. 1990). It was generated by using β_3 as an immunogen and found to bind specifically to the β subunit of $\alpha_{IIb}\beta_3$. It did not bind to reduced β_3 suggesting that the epitope for the antibody is non-linear and dependent on the disulphide bonding of the subunit. Furthermore platelet binding of D3GP3 could be greatly enhanced by occupancy of the receptor by RGDS peptides or by treatment with EDTA. Analysis of the binding of this antibody to $\alpha_{IIb}\beta_3$ concluded that this receptor exists in multiple states of conformation. In one conformation, the epitope for D3GP3 is not exposed. In a second conformation, the receptor is unoccupied and the epitope is exposed and in the third conformation the receptor is occupied and again the epitope is exposed. The epitope for D3GP3 has been assigned to region of β_3 contained within the residues 422 to 692 which contains the tandem cysteine rich repeats (Kouns et al. 1991). Treatment of purified $\alpha_{IIb}\beta_3$ with the reducing agent dithiothreitol (DTT) activates the receptor and exposes ligand induced binding sites independent of ligand binding (Kouns 1993). Chelation of the cations using EDTA appears to have a similar effect (Kouns et al. 1990). This is a complex phenomenon in that it requires conditions such as time (> 15minutes) and temperature (37°C) that suggest that simple chelation of calcium, which

could occur within seconds of EDTA addition, is only part of the explanation. However, this observation clearly highlights the issue that divalent cations play a critical role in the conformational changes that occur in integrin activation.

Treatment of purified $\alpha_{IIb}\beta_3$ with the reducing agent dithiothreitol (DTT) activates the receptor and exposes ligand induced binding sites independent of ligand binding (Kouns 1993). Indeed, numerous studies have shown that cysteine/thiol modifying compounds activate platelets. The amino acid cysteine is a sulphur containing protein. The sulphur atom of the sulphidryl group (-SH) can be polarised making this group chemically reactive. This occurs as a result of the electrons in the outer orbitals being easily distorted by electric fields of other polar molecules. The sulphidryl groups of two cysteine molecules can be oxidised to produce a disulphide called cystine. The reaction proceeds as follows:



Platelet membranes have free sulphidryl and disulphide groups. The integrity of the platelet and certain of its biological functions are dependent on these thiol groups (Aledort et al. 1968) (Ando et al. 1974).

The reducing agent dithiothreitol (DTT) can cause platelet aggregation in preparations of PRP when used in concentrations greater than 3mM while lower concentrations inhibited platelet aggregation, suggesting the existence of two populations of disulphide bonds on the platelet surface (MacIntyre 1974). Platelet aggregation by DTT did not occur in a washed platelet preparation unless exogenous fibrinogen (0.1mg/ml) was added. Pre-incubation of fibrinogen with DTT did not alter the profile of the aggregation implying DTT was acting directly on the platelets. Furthermore, DTT induced platelet aggregation was not inhibited by PGE_1 (1µg/ml) (MacIntyre 1974). DTT causes platelets to bind fibrinogen in a specific and saturable manner but not to the same extent as that caused by ADP. A fluctuation in the number of thiol groups was observed when isolated platelet membranes were exposed to adenosine diphosphate (Zucker and Masiello 1984). This finding was supported by Peerschke who found that fibrinogen bound to DTT-treated platelets in a manner similar to that when platelets were activated by ADP but this binding was reversible after the addition of EDTA. When compared to ADP treated platelets, DTT treated platelets formed only small aggregates in the presence of fibrinogen, suggesting that the conformation of $\alpha_{IIb}\beta_3$ in DTT treated platelets did not support fibrinogen binding to the same extent as agonist activated platelets (Peerschke 1995).

The sulphidryl inhibitor thimerosal also induced platelet aggregation in PRP over a wide range of concentrations (Leone et al. 1976). Addition of L-cysteine to PRP inhibited platelet aggregation induced by thimerosal suggesting that this compound was binding directly to sulphidryl groups and that the binding of SH groups was an important step in platelet aggregation. In a separate study addition of thimerosal to FURA-2 loaded platelets caused a sharp increase in the intraplatelet level of calcium. This mobilisation of calcium may result from the oxidation of SH groups of an internal calcium transport system which is not necessarily a Ca^{2+}-pump or IP_3-channel (Hecker et al. 1989). Alternatively, it might be explained by a direct effect on the platelet integrin.

Four major classes of sulphidryl groups have been identified on the platelet surface. A rapid burst of reduction occurred when platelets were spin-labelled with ADP and was inhibited by adenosine and sulphidryl blocking compounds (Robey et al. 1979). More evidence for an important role for thiol bonds in platelet activation comes from studies with the bimane compound monobromobimane, which is membrane permeable and forms a fluorescent adduct with thiol groups. This compound inhibited ADP, epinephrine and collagen induced platelet aggregation and had no effect on shape change in platelets in PRP. The number of fibrinogen sites on ADP activated platelets was decreased but the affinity for ligand remained unchanged (Zucker and Mauss 1986). Platelet aggregation immobilises clustered fibrinogen receptors so that they associate with the cytoskeletal core. Monobromobimane reversed this association by changing the conformation of $\alpha_{IIb}\beta_3$ necessary for its binding of fibrinogen and so reversed clustering suggesting a specific effect on the platelet integrin (Puszkin et al. 1989).

The importance of the cysteines and disulphide bonds in the β subunit in the activation of $\alpha_{IIb}\beta_3$ is further emphasised by the mapping of epitope sites of the LIBS antibodies. Many LIBS antibodies map to regions that are clustered at the carboxy terminus of the β subunit (Frelinger et al. 1990) (Du et al. 1993) (O'Toole et al. 1990). Exceptions are D3GP3, AP5 and AP6. The epitope for AP5 lies at the amino terminus of the β subunit within residues 1-6 and that of D3GP3 within residues 422-490. The existence of LIBS at the opposite extremes of the molecule substantiates the postulation that activation-related events induce global conformational changes within this subunit of $\alpha_{IIb}\beta_3$. These changes may be assisted by long range disulphide bonds and the bond between Cys^5 and Cys^{435} (Calvete et al. 1991) which permit physical proximity of the terminus of the subunit with distant regions such as the CRRs (Honda et al. 1995). In fact, disruption of the Cys^5- Cys^{435} bond in β_3 resulted in an increase in affinity of $\alpha_{IIb}\beta_3$ for ligand (Liu 1997). Another LIBS antibody, AP6, binds to residues 211-221 in the β_3 subunit (Nurden et al. 1996). The epitope for this monoclonal antibody appears only when $\alpha_{IIb}\beta_3$ is occupied by intact fibrinogen and cannot be induced by either RGD peptides or the dodecapeptide of the fibrinogen γ-chain. This suggests that the receptor undergoes different conformational changes that allow whole molecule fibrinogen to bind to the subunit. The extent of these conformational changes may vary during platelet activation depending on the agonist used. Additional studies with D3GP3 revealed that the interaction of different ligands with $\alpha_{IIb}\beta_3$ results in different conformational changes of the receptor. Binding of vitronectin to $\alpha_{IIb}\beta_3$ did not induce expression of the epitope for this antibody suggesting the expression of LIBS sites is ligand dependent (Mondoro et al. 1996). Mutation of the residue Tyr^{562}, which lies within the cysteine rich region of

the β_3 subunit, to an Asn resulted in enhanced ligand binding of $\alpha_{IIb}\beta_3$. In addition to the antibodies described above other LIBS antibodies bind to the cysteine rich region of β_3 and include LIBS2, LIBS3 and LIBS6 (Frelinger et al. 1991) (Du et al. 1993). These antibodies alter receptor affinity, specificity and function. These findings suggest that the cysteine rich repeats have a very important regulatory role in integrin β_3 functions. This is supported by the finding that a select group of cysteines located in the cysteine rich domain of β_3 remain unpaired and exhibit the properties of a redox site (Yan and Smith 2000). The redox status of these free cysteines can directly influence the activation state of the integrin. In this study, purified activated $\alpha_{IIb}\beta_3$, which was found to have 4-5 free cysteines, could be converted to a resting conformer, shown to have 2-3 free cysteines, in the presence of nitric oxide and reduced gluthathione as a result of mild reduction. This suggests that the regulation of $\alpha_{IIb}\beta_3$ can be controlled by events occurring extracellularly bypassing those events that occur at the cytoplasmic tails post ligand binding known as outside in signalling.

1.3.8. Signalling inwards across the platelet membrane.

"Outside-in" signalling is signalling through $\alpha_{IIb}\beta_3$ as a result of ligand binding to the receptor. This event instigates a myriad of intracellular signals that lead to cytoskeletal rearrangement, pseudopodia formation, spreading on solid phase fibrinogen, increased Ca^{2+} mobilisation, enhanced TxA_2 synthesis, activation of kinases including FAK and increased cytoskeletal reorganisation accompanying changes in platelet shape (Phillips et al. 1980) (Yamaguchi et al. 1990). This signalling is instigated by interactions of the cytoplasmic tails of the subunits, signalling molecules, such as kinases and phosphatases

and structural cytoskeletal proteins including talin, α-actinin and vinculin (Shattil et al. 1998).

Experiments have shown a regulatory role for the α_{IIb} cytoplasmic domain in ligand binding while the cytoplasmic domain of β_3 appears to be involved in both ligand binding and its signalling response (Hughes et al. 1995) (Ylanne et al. 1995) (Ylanne et al. 1993). Ligand binding to the extracellular portion of $\alpha_{IIb}\beta_3$ results in the exposure of LIBS on the α_{IIb} cytoplasmic tail between residues Lys^{994} and Asp^{1003} (Leisner et al. 1999). This ligand induced conformational change in the integrin cytoplasmic domain may play a part in the intracellular assembly of cytoskeletal proteins found at focal adhesions. Furthermore, site directed mutagenesis of this cytoplasmic region, Pro^{998} for Ala and Pro^{999} for Ala, resulted in a constitutively activated integrin that expressed an increased affinity. This study demonstrated that the extracellular ligand binding sites and this cytoplasmic LIBS epitope on α_{IIb} are functionally coupled and suggests bi-directional modulations of $\alpha_{IIb}\beta_3$ conformations across the membrane of the platelet.

Within seconds of binding ligands such as fibrinogen and vWF, platelets extend filopodia in conjunction with activation of Syk and phosphorylation of proteins including β_3 itself (Hartwig et al. 1996) (Law et al. 1996). These responses are triggered by fibrinogen binding since these phosphorylation events are not observed in the presence of inhibitors or the binding of monomeric ligands (Abrams et al. 1994). The β_3 cytoplasmic domain contains two tyrosine residues at positions 747 and 759 and are part of a motif common to all β integrins known as the integrin cytoplasmic tyrosine (ICY)

motif (van der Geer 1995). Phosphorylation of these two residues occurs as a general consequence of platelet aggregation and allows the β tail to interact with cytoskeletal components (Jenkins et al. 1998). For example, phosphorylated β_3 associates with myosin and the signalling proteins Shc and Grb2 (Law et al. 1996). Mutation of these two tyrosine residues to Phe in a knockout mouse model resulted in the formation of loose unstable aggregates upon platelet activation with low dose thrombin. These aggregates eventually disaggregated into single platelets implying that tyrosine phosphorylation of the ICY motif is important for optimal and stable aggregation (Law et al. 1999). Furthermore, the knockout mice demonstrated an in vivo bleeding defect and clot retraction was inhibited. At the next stage of activation, platelets spread out if adhering to an immobilised matrix or form small aggregates. Again, phosphorylation of a number of platelet proteins such as Src and FAK occurs, and clusters of $\alpha_{IIb}\beta_3$ form on the basal side of the platelet (Shattil et al. 1998). The proteins Src, Yes and p21rasGTPase-activating protein have been shown to interact with $\alpha_{IIb}\beta_3$ (Fox et al. 1993). The clustered integrin can also activate protein kinase C and induce FAK phosphorylation (Haimovich et al. 1996). The molecular adapter SLP-76 also plays a role in communicating cytoskeletal rearrangements in platelets. SLP-76 in association with Syk, associates with and activates the adapter Nck and the Rac exchange factor Vav1 (Obergfell et al. 2000). Due to cytoskeletal recruitment and activation of phosphatases there is also a decrease in tyrosine phosphorylation of many other substrates. However, the precise molecular pathways in the over all scheme of outside-in signalling remains unclear.

1.4. $\alpha_{IIb}\beta_3$ ANTAGONISTS.

Since platelet aggregation is irrevocably dependent on the engagement of ligands by $\alpha_{IIb}\beta_3$, inhibition of this binding was recognised as having the potential to provide an effective therapy of arterial thrombosis. Antagonists developed so far include monoclonal antibodies to the receptor, peptides containing the RGD motif called disintegrins from venomous snakes, synthetic peptides and non peptides that mimic the fibrinogen binding site.

1.4.1. Disintegrins.

Disintegrins are a family of cysteine-rich, low molecular weight proteins found in the venom of various snakes. They selectively promote or inhibit platelet aggregation by targeting GPIb-IX-V, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, GPVI or their respective ligands and bind with a high affinity. They belong to either the C-type lectin family or the metalloproteinase-disintegrins (Andrews and Berndt 2000). The inhibitory activity of disintegrins is largely determined by RGD/KGD sequences within the disulphide bond-dependent peptide conformation of the molecule (Huang et al. 1987) (Scarborough et al. 1991) (Calvete et al. 1991). The structure of disintegrins consists of overlapping loops and loops within loops (Jaseja et al. 1993). The RGD sequence is located at the most exposed end of a mobile loop protruding 14-17 Å from the protein core (Brockel et al. 1992). Four disintegrins, albolabrin, bitistatin, echistatin and eristostatin bind to the integrin β_3 subunit between residues 217-302 in a RGD dependent manner (Calvete et al. 1994). Regions within this sequence form part of the subunit interface (Plow et al. 1992) and an activation dependent epitope (Piotrowicz 1990). Disintegrins selectively

interact with integrins and this interaction is dependent on the amino acids adjacent to RGD, the pattern of intramolecular disulphide bonds and regions of the disintegrin molecule beyond the RGD loop (Marcinkiewicz et al. 1997). For example, eristostatin and echistatin bind with different affinities to receptors on the platelet surface. Eristostatin is a more potent inhibitor of platelet aggregation, binding with high affinity to both resting and activated platelets. In contrast, the binding of echistatin to resting platelets is significantly lower than its binding to ADP activated platelets (McLane et al. 1994). The interaction of these two disintegrins with platelets results in the expression of LIBS on $\alpha_{IIb}\beta_3$ and extensive conformational changes of the receptor (Du et al. 1993). Again, the effect of eristostatin was stronger than that of echistatin (Marcinkiewicz et al. 1996).

1.4.2. Clinically-approved $\alpha_{IIb}\beta_3$ antagonists.

The first $\alpha_{IIb}\beta_3$ antagonist to be approved for use in clinical trials was the monoclonal antibody abciximab or 7E3 (Coller 1986). Abciximab is in fact, a Fab fragment of the monoclonal antibody and a potent inhibitor of platelet aggregation (Mascelli et al. 1998). Its effect in the prevention of complications at the time of coronary angioplasty has been proven in many clinical trials (Lefkovits and Topol 1995) (Investigators 1997) (Kong et al. 1998). It binds to platelets with a high affinity and has a slow dissociation rate (Tcheng et al. 1994). This antibody also binds to the $\alpha_V\beta_3$ integrin on endothelial cells, smooth muscle cells and activated $\alpha_M\beta_2$ on leukocytes (Coller 1999).

Eptifibatide or integrelin is a cyclic heptapeptide based on the Lys-Gly-Asp (KGD) amino acid sequence (Scarborough et al. 1993). Tirofiban or aggrastat is a tyrosine-derivative non-peptide mimetic and the compound lamifiban is also a non-peptide mimetic. These compounds are competitive inhibitors of $\alpha_{IIb}\beta_3$ with low affinities and high dissociation rates (Harrington et al. 1995) (Kereiakes et al. 1996) (Alig et al. 1992). They are potent inhibitors of platelet aggregation in vitro. However, in general, the intravenous forms of the peptide and non-peptide mimetics have not been as effective clinically as abciximab (Theroux et al. 1996) (Investigators. 1997) (Kong et al. 1998). Thrombocytopenia is an infrequent (<1% of cases) but well documented complication of the administration of $\alpha_{IIb}\beta_3$ antagonists (Lincoff et al. 2000). The mechanism is unknown but may be as a result of expression of LIBS on the receptor (Peterson et al. 1998). The effectiveness of these fibrinogen antagonists may be limited as they only block fibrinogen binding to its receptor whereas the inhibition of platelet secretion is agonist dependent (Nobiletti 1997). Moreover, some of these antagonistic compounds have been shown to act as partial agonists inducing platelet aggregation at low doses (Peter et al. 1998; Cox et al. 2000). In our laboratory, Cox et al have shown that Orbofiban has a distinct partial agonist activity at low doses of drug and that this is associated with increased mortality in the OPUS TIMI-16 trial (Cox et al. 2000). In another study, continued TxA_2 formation occurred despite the administration of an $\alpha_{IIb}\beta_3$ antagonist to patients undergoing coronary angioplasty, suggestive of partial agonist activity (Byrne et al. 1997). TxA_2 synthesis in platelets is activated during outside-in signalling. The fact that TxA_2 synthesis can continue in the presence of an $\alpha_{IIb}\beta_3$ antagonist suggests that the activated integrin is still capable of promoting platelet activation events even though platelet aggregation has been inhibited. Alternatively, it

suggests that the RGD-mimetics are acting as partial agonists. In either case, an agent that could block integrin activation as opposed to integrin occupancy might address some of these issues and offer a new therapeutic concept to supersede existing strategies of competitive antagonism of ligand binding through RGD-mimetic drugs.

1.5. AIMS.

It is established that $\alpha_{IIb}\beta_3$ undergoes conformational changes as a result of both inside-out signalling in the platelet and subsequent ligand binding to the receptor. The mechanisms by which these conformations occur have not been fully elucidated. Following cellular activation, they undergo conformational changes to become high affinity receptors for their specific ligand(s). There is overwhelming evidence to suggest that the cysteine residues and disulphide bond arrangements within the receptor are somehow involved. Treatment of platelets with the reducing agent, dithiothreitol (DTT) can induce an activation-like state in many integrins suggesting that disulphide bonds are important for the receptor's tertiary structure and may be involved in activation-induced conformational changes. The 'switch' mechanism whereby integrins are converted from their resting conformation is critically important to their cellular function. The precise molecular mechanisms involved in this 'switch' mechanism constitutes the main aim of this thesis.

To establish that a role for changes in the disulphide bond arrangement is involved in the activation of $\alpha_{IIb}\beta_3$, the effects of DTT on various platelet functions were examined. These studies compliment and verify similar studies completed thus far, and verify that DTT treatment of the platelet has a specific effect on the activation state of $\alpha_{IIb}\beta_3$.

Having established that disulphide bond rearrangement results in activation of the receptor I investigated the potential role(s) the thiol isomerase enzyme, protein disulphide isomerase (PDI) may have in conformational changes of $\alpha_{IIb}\beta_3$. A variety of techniques were employed, including confocal microscopy to examine co-localisation of $\alpha_{IIb}\beta_3$ and PDI in the platelet. The effects of a thiol isomerase inhibitor on platelet functions were examined to investigate a role for PDI in the conformational changes of the receptor. Alternate sources of thiol isomerase activity were thoroughly investigated in an attempt to establish the existence of a platelet specific isomerase.

CHAPTER 2

Materials and Methods

2.1. MATERIALS

2.1.1. General reagents were obtained from the following suppliers:

Abbott Laboratories, Sligo, Ireland.

Butterfly® 19-gauge needle.

Amersham Pharmacia Biotech UK Ltd., Buckinghamshire England.

Enhanced-chemiluminescence (ECL) kit, protein G beads.

Bio-Rad Laboratories Ltd., Hertfordshire, England.

Bradford assay, Bradford DC assay.

Calbiochem-Novabiochem, (U.K.) Ltd., Nottingham, England.

Ribonuclease A (RNase), protein disulfide isomerase (PDI), fibrinogen, protease inhibitor cocktail.

Chemicon International, Ltd., Harrow, England.

Purified $\alpha_V\beta_3$ and $\alpha_5\beta_1$

Dako, Carpinteria, CA, U.S.A.

Fluorescent mounting medium.

Molecular Probes Europe BV, Leiden, Netherlands.

Oregon green labelled fibrinogen.

Peninsula laboratories, Merseyside, England.

Thrombin receptor activating peptide (TRAP).

Schleicher and Schuell, Keane, New Hampshire, U.S.A.

Centrex filters 10kD MWCO, 100mm polystyrene plates.

Sigma Aldrich Irl., Tallaght, Ireland.

3-(n-morpholino) propanesulfonic acid (MOPS), acrylamide, adenosine diphosphate (ADP), bacitracin, citric acid anhydrous, crystallised BSA, cytidine 2',3'cyclic monophosphate (cCMP), dextrose, dithiothreitol (DTT), ethylenediaminetetra acetic acid (EDTA), GFB filters, glass slides pre-coated with poly-L-lysine, guanidine hydrochloride, Kodak (Biomax ML) film, PBS, polyvinyldifluoride (PVDF), problot, Prostaglandin E_1, protein A peroxidase, Sephadex G25 column, sepharose 2B-300, sodium chloride, sodium citrate, sodium dodecyl sulphate (SDS), sodium fluoride, sodium pyrophosphate, sodium orthovanadate, thrombin, Triton X-100 and trizma base.

2.1.2. Antibodies were obtained from the following:

Dr. Eric Quéméneur, Département d'Ingéniérie et d'Etudes des Protéines, Centre d'Études Saclay, Gif-sur-Yvette, France.

A kind gift of polyclonal rabbit anti-human PDI.

Dr. Lisa Jennings, University of Tennessee, Tennessee, U.S.A.

A kind gift of monoclonal anti-LIBS D3GP3.

Becton Dickinson, Oxford, England.

Polyclonal anti-mouse IgG-FITC.

Immunotech, Marseilles, France.

CD41a, CD62P.

Molecular Probes, Leiden, Netherlands.

Goat anti-mouse Alexa-488 conjugated IgG, goat anti-rabbit Alexa 546 conjugated IgG.

Stressgen Biotechnology Corporation, CA, U.S.A.

Polyclonal rabbit anti-PDI.

2.1.3. Molecular biology reagents were obtained from the following:

Promega, Southampton U.K.

1X reverse transcription buffer, DNA polymerase, Moloney murine leukaemia virus, reverse transcriptase dNTPs random hexamers, Taq, Total RNA Isolation System, Wizard DNA isolation kit, Wizard DNA mini prep kit.

Invitrogen BV, Leek, Netherlands.

The Original Cloning ® Kit.

ABI Prism, Perkin Elmer, Foster City, CA, U.S.A.

Dye Terminator Cycle Sequencing Ready Reaction kit

2.1.4. Radioligand was obtained from Searle, Skokie, IL, U.S.A.

[^{3}H]-SC52012.

2.1.5. Reagents for 2D Electrophoresis were obtained from the following:

BDH AnalaR (B&H, Northern Ireland)

Urea, thiourea.

Boehringer Mannheim (East Sussex, UK).

Acrylamide.

Pharmacia Biotech (Upsala, Sweden).

IPG buffer pH3-10, agarose, Immobiline Drystrip gels pH1-10 (18cm), IPG cover fluid

2.1.6. Water used in experiments was of the highest purity grade.

2.2. METHODS

2.2.1. Platelet functional assays.

2.2.1.1. Isolation of platelets from whole blood.

Venous whole blood was drawn from normal healthy volunteers, free from aspirin and other drugs that may affect platelet function for 7-10 days. Blood was drawn from the antecubital vein using a Butterfly® 19-gauge needle and anti-coagulated with 0.1 volume of 3.8% sodium citrate. The blood was aliquoted into 5ml aliquots in plastic tubes. Platelet rich plasma (PRP) was obtained by centrifugation at 170 x g for 12 minutes at room temperature. The blood separates into two fractions, the top being PRP. The top two thirds of this fraction was gently removed, using a plastic transfer pipette, into fresh plastic tubes for experiments. Platelet poor plasma (PPP) was obtained by centrifuging the remaining blood at 1,000 x g for 10 minutes at room temperature and removing the top fraction to a fresh plastic tube.

2.2.1.2. Preparation of Gel Filtered Platelets.

Venous whole blood was drawn from normal healthy volunteers, free from aspirin and other drugs that may affect platelet function for 7-10 days. Blood was drawn from the antecubital vein using a Butterfly® 19 gauge needle and anticoagulated with 0.15 volume of acid:citrate:dextrose (ACD: 38mM citric acid anhydrous, 75mM sodium citrate, 124mM dextrose) (Mendelsohn 1991). The blood was centrifuged at 170 x g for 12 minutes at room temperature. The PRP was removed to a 50ml plastic tube, acidified to pH6.5 with ACD and prostaglandin E_1 (PGE_1) (1μM) was added. The platelets were

pelleted through the plasma by centrifugation at 1,000 x g for 12 minutes at room temperature. The supernatant was removed and the platelets were resuspended in buffer A (130mM NaCl, 10mM trisodium citrate, 9mM $NaHCO_3$, 6mM dextrose, 0.9mM $MgCl_2$, 0.81mM KH_2PO_4, 10mM Tris pH 7.35). The platelet suspension was applied to a packed Sepharose 2B-300 column (5-6ml of packed sepharose per ml of applied platelets in a column 1.5 cm in diameter) equilibrated with buffer A and filtered. 0.5ml fractions were collected and the platelet count in each fraction was assessed using a Sysmex K-1000 counter (Toa Medical Electronics Company Ltd., Japan). The fractions with the highest platelet count were pooled and calcium chloride ($CaCl_2$) at a final concentration of 1.8mM was added. Platelet aliquots were then used for various experimental conditions.

2.2.1.3. Platelet Aggregation.

Platelet aggregations can be examined by measuring light transmission through stirred platelet suspensions. Stirring is required for the platelet-platelet contact that initiates the formation of aggregates. As these are formed, light scattering increases and therefore light transmission increases. Platelet aggregations were monitored using a BioData PAP-4 aggregometer (Horsham, PA, USA). PRP or gel filtered platelets were aliquoted into siliconised glass tubes in a final volume of 500µl, incubated at 37°C and stirred at 1,000 rpm. PPP, which is devoid of platelets, was used to set a 100% baseline for PRP aggregations and buffer A (section 2.2.1.2) was used to set the baseline for gel filtered platelet aggregations. Platelets were allowed to stir for 20 seconds to check for spontaneous aggregation before the addition of agonists. The resulting aggregations were followed for various time intervals.

2.2.1.4. Platelet adhesion assay.

Gel filtered platelets were prepared as outlined in section 2.2.1.2. Platelet adhesion to fibrinogen and fibrin were studied in 100mm polystyrene plates. Plates were coated with either 3mls of 0.5mg/ml fibrinogen or 5mg/ml crystallised BSA in sodium bicarbonate buffer pH 9.6, at room temperature overnight with very gentle agitation. Fibrin plates were obtained by incubating a fibrinogen coated plate with 3mls of 1U/ml thrombin in phosphate balanced salt (PBS) buffer pH 7.4 for 1 hour at 37°C. All plates were washed 3 times with 5ml aliquots of PBS. These were blocked with 3mls of 5mg/ml crystallised BSA in sodium bicarbonate buffer pH 9.6 for 2 hours at room temperature. Plates were washed with buffer A (section 2.2.1.2) for 5 minutes at room temperature. The platelet count was adjusted to 2-3 x 10^8 platelets/ml. 1ml aliquots of either untreated or treated platelets were added to either a BSA, fibrinogen or fibrin coated plate and incubated at room temperature for 30 minutes. Non-adhered platelets were removed and transferred to 1.5ml eppendorfs, centrifuged in a microcentrifuge at maximal speed for 20 seconds to pellet the platelets. The supernatant was removed using a transfer pipette and the platelet pellet was solubilised in 100µl of radio-immunoprecipitation (RIPA) buffer (1% Triton X-100, 50mM NaCl, 1mM Na_3VO_4, 12mM Na pyrophosphate, 50mM NaF, 0.2% SDS, 20mM Tris pH7.4, 5mM EDTA, protease inhibitor cocktail). Adhered platelets were scraped into 100µl aliquots of warm RIPA buffer and transferred to 1.5ml eppendorfs. The protein concentration of both the adhered and non-adhered fractions was determined using the Biorad Bradford assay. Briefly, 20µl of platelet lysate was incubated with 200µl 1:4 dilution of Bradford solution in a 96-well plate, incubated at room temperature for 15 minutes and the

absorbance read at 630nm. A standard curve was generated using bovine serum albumin (BSA) ranging from 0-15μg. In general, 1×10^9 platelets contain 2mg of protein.

2.2.2. Flow Cytometry Analysis.

Flow cytometry allows the analysis of surface expressed molecules on the surface of cells and platelets. Cell or platelet suspensions are taken up in a stream of buffer and drops containing single cells or platelets pass one by one through the path of a laser beam. The effects of this passage are monitored. Cell size is based on the scattering effects of the beam and the intensity of fluorescently labelled tags determines the number of labelled cells in the population being monitored. All experiments were carried out at room temperature on a Becton-Dickinson FACScan (Flow Activated Cell Sorter) flow cytometer at 488/510 nm. Data acquisition and analysis was performed with the Lysis II programme. Platelet populations were gated and mean fluorescent histograms were generated for each sample. Statistical analysis was performed on the geometric scale. Results are expressed as mean fluorescence.

2.2.2.1. Fibrinogen Binding Assay.

Fibrinogen labelled with the fluorescent tag, oregon green (OgFg), was used in all assays. A dose dependent fibrinogen binding assay was first carried out, to establish that OgFg (0.125mg/ml - 3.0mg/ml) bound to ADP activated platelets in a specific and saturable manner. This assay is a modification of the fibrinogen binding assay of Xia *et al* (Xia 1996). Binding of OgFg to resting platelets was carried out in the presence of the RGD mimetic Integrilin (2μg/ml). In a typical binding assay, PRP was diluted 10

fold in buffer A (section 2.2.1.2) supplemented with 1.8mM $CaCl_2$. Platelets were activated with 10μM adenosine diphosphate (ADP). The binding assay was performed at room temperature for 20 minutes in subdued lighting. The reaction was stopped by the addition of 2mls of buffer A. Based on the results of the binding curve (Figure 2.1), one point assays were subsequently done using OgFg at a final concentration of 1mg/ml. The diluted PRP was incubated with various agents for 5 minutes at room temperature before the addition of OgFg. Samples were read immediately on the FACS.

2.2.2.2. Ligand Induced Binding Site (LIBS) Assay.

Citrated PRP was prepared as outlined in section 2.2.1.1. 100μl aliquots of PRP were incubated with one of the following: PGE_1 (56nM), Integrilin, an RGD mimetic (2μg/ml), EDTA (5mM), ADP (20μM), TRAP (20μM), respectively, for 30 minutes at 37°C. Each reaction was stopped by the addition of 0.5mls PBS. 50μl aliquots of each of these treatments were incubated with the anti-LIBS antibody, D3GP3 (20μg/ml), for 10 minutes (a gift of Dr. Lisa Jennings, University of Tennessee). The secondary antibody, polyclonal anti-mouse IgG-FITC (Fluorescein isothiocyanate) (20μg/ml) was added to each tube for 10 minutes. The platelets were fixed with 1% paraformaldehyde for 10 minutes. 1ml of PBS was added to each tube and samples were analysed on the FACS. Control samples were prepared in which the primary antibody was omitted to assess the autofluorescence of the assay. All experiments were performed at room temperature.

2.2.2.3. P-selectin Assay.

Citrated PRP was prepared as outlined in section 2.2.1.1. 100µl aliquots of PRP were incubated with and without ADP (20µM) for 3 minutes at 37°C. 500µl PBS was added to each aliquot to stop the reaction. 50µl aliquots of each of these treatments were incubated with anti-p-selectin (CD62P) antibody (20µg/ml) for 10 minutes. The secondary antibody, polyclonal anti-mouse IgG-FITC (20µg/ml) was added to each tube for 10 minutes. The platelets were fixed with 1% paraformaldehyde in PBS for 10 minutes. 1ml of PBS was added to each tube and samples were analysed on the FACS. Control samples were prepared in which the primary antibody was omitted to assess the autofluorescence of the assay. All experiments were performed at room temperature.

2.2.2.4. Glycoprotein $\alpha_{IIb}\beta_3$ Assay.

Citrated PRP was prepared as outlined in section 2.2.1.1. 100µl aliquots of PRP were incubated with and without ADP (20µM) for 3 minutes at 37°C. 500µl PBS was added to each aliquot to stop the reaction. 50µl aliquots of each of these treatments were then incubated with anti-$\alpha_{IIb}\beta_3$ antibody (CD41a) (20µg/ml) for 10 minutes. The secondary antibody, polyclonal anti-mouse IgG-FITC (20µg/ml) was added to each tube for 10 minutes. The platelets were then fixed with 1% paraformaldehyde in PBS for 10 minutes. 1ml of PBS was added to each tube and samples were analysed on the FACS. Control samples were prepared in which the primary antibody was omitted to assess the autofluorescence of the assay. All experiments were performed at room temperature.

2.2.2.5. Detection of Protein Disulfide Isomerase.

A 100µl aliquot of citrated PRP was centrifuged at 20,000 x g for 10 seconds. The supernatant was removed and the platelet pellet was resuspended in either 100µl of PBS pH 7.4 or PBS containing 0.1% Triton X-100. Samples sat on ice for 20 minutes. 1ml of PBS was added and sample was centrifuged at 20,000 x g for 10 seconds. The supernatant was removed and the platelets resuspended in 50µl 1:500 dilution of rabbit anti-human PDI (a gift from Dr. Eric Quéméneur, Département d'Ingéniérie et d'Etudes des Protéines, Centre d'Études Saclay, Gif-sur-Yvette, France) for 30 minutes at room temperature. 1ml of PBS was added and sample was centrifuged at 10,000 x g for 3 minutes. The supernatant was removed and the platelets resuspended in 50µl of 1:1,000 dilution of polyclonal anti-rabbit IgG-FITC for 30 minutes at room temperature. 1ml of PBS was added and sample was centrifuged at 10,000 x g for 3 minutes. The supernatant was removed and the platelets resuspended in 1ml of 1% paraformaldehyde in PBS. A control sample was prepared in which the primary antibody was omitted to assess the autofluorescence of the assay. Samples were analysed on the FACS.

2.2.3. Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting.

SDS-PAGE is primarily used for the determination of the molecular weight of polypeptides (Pitt-Rivers and Impiombato 1968). Under appropriate conditions all reduced polypeptides bind the same amount of sodium dodecyl sulphate (SDS) on a weight basis (1.4g SDS/g polypeptide) and so separate out in a gel matrix based on the molecular weight of the polypeptide.

2.2.3.1. Preparation of gels.

One-dimensional SDS-PAGE was performed by the method of Laemmli using a slab gel in a discontinuous buffer system (Laemmli 1970). The following buffers were used:

30% acrylamide: 29.2g acrylamide and 0.8g bisacrylamide in 100ml of deionised water (dH_2O). This is referred to as a 30%T, 2.6% C stock where T = (g acrylamide + g bisacrylamide)/100ml and C is g bisacrylamide/100g total monomers.

Stacking buffer (4X): 0.4% SDS in 0.5M Tris pH 6.8.

Separating buffer (4X): 0.4% SDS in 1.5M Tris pH 8.8.

Stacking gel: 4% acrylamide, 1X stacking buffer.

Separating gel: 7%, 10%, 12.5% or 15% acrylamide, 1X separating buffer.

Both the stacking and separating gel mixtures were heated at 37°C for a couple of minutes and degassed under vacuum, to remove oxygen, an inhibitor of polymerisation. To polymerise the gels, temed (0.5-1µl/ml) and ammonium persulfate (0.05%) were added to the gel mixtures. The separating gel was poured first and allowed to polymerise before the stacking gel was poured on top of it. Generally, a 10 lane stacking gel was poured and all gels were 1mm in thickness.

2.2.3.2. Sample Preparation and Separation of Proteins.

The protein concentration of lysates was determined using the Bradford method as described in section 2.2.1.4. If detergents present in the lysate interfered with this assay, the Bradford DC assay was used to determine the protein concentration. This assay is a microtiter plate assay and was carried out as follows: 20µl of solution S was added to 1ml of solution A to make solution A′. 5µl of either sample or standard was added to

wells of a 96 well plate in triplicate. 25µl of solution A′ and 200µL of solution B were added to each well. The plate was allowed to stand at room temperature for 15 minutes and then read on a plate reader at 630nm (Wallac Victor^2 1420 Multilabel Counter, EG&G Wallac, Wallac, Oy, Turku, Finland.). A standard curve was generated using BSA ranging in concentration from 0-15µg. Once the concentration of proteins in the lysate was determined, an appropriate volume of protein lysate was mixed with an equal volume of 2X sample buffer (125mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue). The samples were heated at 100°C for 5 minutes and loaded onto the gel at the required concentration. This generally ranged from 5-30µg. It was always ensured the same concentration was present in each lane, to allow direct comparison from lane to lane. The proteins were separated through the gel by applying a constant current of 25mA for a mini gel. A lane containing molecular weight standards was always included in the gel. The gel was allowed to run until the gel front was approximately 0.2cm from the bottom of the gel.

2.2.3.3. Blotting.

Blotting is the transfer of protein molecules from the gel matrix in which they have been separated onto the surface of an immobilising membrane such as nitrocellulose, polyvinyldifluoride (PVDF) or problot. A wet system was used in which the gel and membrane were clamped into a cassette between filter paper and sponges and immersed in buffer. The buffer used was a modified Towbin buffer (25mM Tris, 192mM glycine, 20% (v/v) MeOH, 0.2% SDS). The proteins were blotted onto the membrane overnight at a constant voltage of 30V at 4°C or 100V for 1 hour in the presence of an ice pack. This ice pack prevented overheating of the system derived from Joule heating during the

transfer. Upon completion of the transfer the membranes were removed from the cassettes and stained with the reversible protein stain Ponceau-S (0.1% Ponceau-S in 1% acetic acid) to check for the efficiency of transfer and mark the molecular weight standards. The membranes were destained with dH_2O in preparation for probing with antibodies. If membranes were not probed immediately they were allowed to air dry and stored in a plastic folder until required.

2.2.3.4. Probing with antibodies.

Membranes were blocked in a blocking buffer of 5% carnation milk in PBS containing 0.05%-1% tween-20 or for anti-phosphotyrosine immunoblots 5% bovine serum albumin (BSA) in 170mM NaCl, 0.2% non-idet P40 (v/v), 50mM Tris pH 7.4 for 1 hour at room temperature with gentle agitation. The membrane was washed with buffer for 5 minutes four times. After washing the membrane was incubated with the primary antibody for 2 hours at room temperature with gentle rocking. Again, the membrane was washed with buffer for 5 minutes four times. Incubation with the appropriate secondary antibody was for 1 hour at room temperature with gentle rocking. Membranes were washed with buffer for 5 minutes four times and for 10 minutes once. Development of membrane was performed using an enhanced-chemiluminescence kit (ECL) and Kodak (Biomax ML) film.

2.2.3.5. Immunoprecipitation of specific proteins from lysates.

Platelet lysates were prepared as outlined in section 2.2.1.4. The lysates were precleared with a 1:50 dilution of protein G beads in precipitating buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton-X 100) for 1 hour at 4°C. The lysate was centrifuged in a

microcentrifuge at maximal speed for 30 seconds to pellet the beads. The supernatant was transferred to a fresh eppendorf and the precipitating antibody (2µg/ml) was added and incubated overnight at 4°C with end over end agitation. The precipitated proteins were harvested with protein G beads for 1 hour at 4°C, with end over end agitation. The preparation was centrifuged in a microcentrifuge at maximal speed for 30 seconds to pellet the beads. The supernatant was removed and the beads were washed in precipitating buffer four times. After the final wash the beads were resuspended in 50µl of 1X sample buffer (section 2.2.4.2.) and heated at 100°C for 5 minutes. Samples were separated on the appropriate % gel and transferred to PVDF. Membranes were probed with the required primary antibodies. The secondary antibody used in these assays was a 1:1500 dilution of protein A peroxidase. Membranes were developed with an ECL kit.

2.2.4. Radiolabelled Binding Assay.

This assay was used to examine the interactions of a radiolabelled RGD mimetic with its receptor, $\alpha_{IIb}\beta_3$, on the surface of resting and activated platelets. The radiolabelled ligand used in these assays was the tritiated compound [^{3}H]-SC52012, with a specific activity of 1.0 Ci/mmol (Zablocki et al. 1993). The binding assay was performed in triplicate at room temperature for 30 minutes. 50µl aliquots of gel filtered platelets with a count of 0.25-0.5X10^8 platelets/ml in buffer A (section 2.2.1.2.) were incubated with concentrations of [^{3}H]-SC52012 ranging from 1-500nM. The platelet agonist ADP (20µM) and various agents were introduced into a final volume of 100µl. Unlabeled SC52012 (50µM) was added to one of the triplicates. After the 30 minute incubation, free ligand was separated from bound ligand by rapid filtration onto GFB filters in a

Brandel M-24RP cell harvester (Gaithersburg, MD, USA). Filters were rapidly washed with 10mls ice cold 10mM Tris pH7.4 over 10 seconds. Filters were dropped into 5ml of ecoscint and counted in a LKB Wallac 1214 rackbeta scintillation counter. Specific binding was determined by subtracting counts present in excess unlabeled ligand from total binding and analysed using Ligand version 3.0 (McPherson et al. 1983) or Delta graph 4.0.

2.2.5. Molecular Biology Techniques.

Extraction and isolation of RNA from cells has to be performed rapidly because of the labile nature of RNA and the presence of active ribonucleases in cells. A lysing environment is employed where the chemical nature of the isolation procedure results in the denaturation of these ribonucleases.

2.2.5.1. Preparation of RNA from platelets.

Citrated PRP was prepared as outlined in section 2.2.1.1. The platelets were centrifuged through the plasma at 2,000 x g for 7 minutes at room temperature to form a pellet. Total RNA was isolated using RNAgents® Total RNA Isolation System. The plasma supernatant was removed and the platelet pellet was resuspended with 0.5ml of chilled denaturing buffer. Platelets were lysed by repeat pippeting. 50µl of sodium acetate (2M) pH 4.0 was added to the lysed platelet sample and mixed thoroughly by inverting the tube 4-5 times. 500µl of phenol:chloroform:isoamyl alcohol (125:24:1) pH 4.7 was added to the sample that was mixed by inversion 3-5 times, shaken vigorously for 10 seconds and chilled on ice for 15 minutes. Sample was centrifuged at 10,000 x g for 20

minutes at 4°C. The top two thirds of the aqueous phase, which contains the RNA, was transferred to a fresh tube. An equal volume of isopropanol was added to the aqueous phase and the sample was incubated at -20°C for 40 minutes. The sample was centrifuged at 10,000 x g for 10 minutes at 4°C to pellet the RNA. The pellet was washed with 1ml of ice-cold 75% ethanol and centrifuged at 10,000 x g for 10 minutes at 4°C. The ethanol was carefully removed using a sterile RNase-free pipet tip and the pellet was air dried in an RNase-free environment. The pellet was resuspended in 20µl of nuclease-free water and stored at -80°C. Long term storage of RNA in solution is not recommended as it is likely to be broken down by ribonucleases introduced during the isolation procedure.

2.2.5.2. Polymerase Chain Reaction (PCR).

The polymerase chain reaction is an *in vitro* method for the amplification of specific DNA sequences by the extension of complementary strands of DNA (cDNA) by primers. cDNA is obtained by a reverse transcriptase PCR of template RNA isolated from cells or tissue.

2.2.5.2.1. Reverse transcription of RNA.

Platelet RNA was denatured at 65°C for 10 minutes and incubated at 37°C overnight in the presence of 1X reverse transcription buffer, 400 units of Moloney murine leukaemia virus reverse transcriptase, 25µM deoxynucleoside triphosphates (dNTPs) and 100ng of random hexamers in a final volume of 20µl. The reaction was stopped by heating at

95°C for 5 minutes and the newly generated complementary DNA (cDNA) was used as template in the polymerase chain reaction (PCR).

2.2.5.2.2. Primer Design.

Primer sequence design was based on the known nucleotide sequence of the protein of interest. Primers were designed either by hand or using a primer design programme in Pedro's Biomolecular Research Tools (www.ucl.ac.uk/lmcb/research_tools.html). It was ensured that the GC content of each primer was similar and the sequence of each was not complementary to the other. The sense and anti-sense primers were either 24 or 21mers and were synthesised by Genosys Biotechnologies, Cambridgeshire, England.

2.2.5.2.3. The Polymerase Chain Reaction.

The PCRs were performed in a final volume of 50µl with 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.25µg of each sense and antisense primer, 2 units of Taq DNA polymerase and 5µl of cDNA (as prepared in section 2.2.5.2.1.). The reaction cycles were as follows: denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 68°C for 1.5 minutes for 40 cycles. The PCR products were separated on a 1.2% agarose gel containing 0.5µg/ml of ethidium bromide and visualised on a ultraviolet transilluminator. Bands were carefully cut out from the gel with clean scalpel blades and the PCR products were then prepared for sequencing.

2.2.5.3. Sequencing of PCR Products.

Sequencing of PCR products was performed in a Perkin Elmer Applied Biosystems automated fluorescent sequencer. This sequencer detects fluorescence from four different dyes that are used to identify the nucleotides A, C, G and T. An argon ion laser excites each dye at a different wavelength and each base can be distinguished in a single reaction.

2.2.5.3.1. Extraction of DNA from agarose.

Extraction of the PCR product from the agarose slices was performed using a Wizard DNA isolation kit. The agarose slice was mixed with 1ml of resin and heated at 65°C for 5 minutes or until the agarose was melted. The resin /DNA mix was transferred to the barrel of a 2ml syringe attached to a mini-column and pushed onto the column. It was very important to work quickly at this stage to maintain a high yield. The mini column was detached from the barrel and the plunger was removed. The column was reattached and 2mls of 80% isopropanol was pushed onto the column. The column was detached and transferred to a 1.5ml eppendorf and centrifuged at 10,000 x g for 2 minutes to dry out the resin. The column was transferred to a fresh 1.5ml eppendorf and 50µl of nuclease free water was added to the column for 1-5 minutes at room temperature and centrifuged at 10,000 x g for 20 seconds. The eluted DNA was now prepared for ligation.

2.2.5.3.2. Ligation into pCR™II vector.

Ligation of the eluted DNA into the cloning vector pCR™II was performed using the Original Cloning ® Kit. The PCR products were ligated into the vector in the following

reaction: 6µl fresh PCR product, 1 µl 10X ligation buffer, 2 µl pCR™II vector (25ng/ml), 1µl T4 DNA ligase (4.0 Weiss units) and 1 µl sterile water. The ligation reaction was incubated at 14°C overnight.

2.2.5.3.3. Transformation of Ligation into Competent Cells.

The vial of 0.5M β-mercaptoethanol and one 50µl vial of competent cells per reaction was removed from the kit and thawed on ice. 2µl of β-mercaptoethanol was pipetted into the competent cells and it was extremely important not to mix by pipetting up and down. 2µl of each ligation reaction was added and vials were then incubated on ice for 30 minutes. Each vial was heat shocked at 42°C for exactly 30 seconds and placed on ice for 2 minutes. 450µl SOC medium (2% bacto tryptone, 0.5% bacto yeast extract, 0.05% NaCl, 0.25mM KCl, 10mM $MgCl_2$, 20mM glucose) was added to each vial and shaken horizontally at 37°C for 2 hours at 225 rpm in a rotary shaking incubator. 200µl of each transformation was spread onto an LB agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 50µg/ml ampicillin. (Plates were made ahead of time and 40µl of 40mg/ml X-Gal was spread onto them). Plates were inverted and placed in a 37°C incubator overnight.

2.2.5.3.4. Restriction Analysis of Transformations.

2-3 white colonies were picked from each plate and grown up in 4mls of LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50mg/ml ampicillin overnight at 37°C at 225 rpm in a rotary shaking incubator. The plasmids are isolated using a Wizard DNA mini prep kit. 2mls of cells are centrifuged at 10,000 x g for 2 minutes at room

temperature. Supernatant was removed and the cell pellet was resuspended in 200µl resuspension buffer, to which 200µl of lysis buffer was added. Samples were mixed by inverting tubes 4-5 times. 200µl of neutralising solution was added and mixed by inverting 4-5 times. Lysates were centrifuged at 10,000 x g for 5 minutes at room temperature. A mini column was then attached to a 2ml syringe, from which the barrel was removed. 1ml of resin was pipetted into the barrel to which the cell lysate was added. Plunger was inserted and mix was pushed onto the column. Column was removed from the syringe and then the plunger was removed. The column was reattached and 2mls of wash buffer was added which was then pushed onto the column with the plunger. The column was removed from the syringe and placed in a fresh 1.5ml eppendorf and centrifuged at 10,000 x g for 2 minutes to dry the resin. Column was then transferred to a second fresh eppendorf and 50µl of nuclease free water was added for 1 minute. The column was centrifuged for 20 seconds at 10,000 x g to elute the DNA. An EcoRI digest reaction was set up as follows: 16µl DNA, 2µl 10X buffer H and 2µl enzyme. The reaction tubes were incubated at 37°C for 1 hour. The resulting digests were separated on a 1.2% agarose gel to determine the correct insert size.

2.2.5.3.5. Sequencing Analysis.

Samples, with the correct insert size, were prepared for sequencing by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit as follows: 8µl terminator ready reaction mix, 0.4µg double stranded DNA, 3.2 pmoles primer (Universal M13 reverse or forward primers) in 20µl using PCR grade water. Samples were placed in the thermal sequencer and the following programme was used: rapid thermal ramp to 96°C;

96°C for 10 seconds; rapid thermal ramp to 50°C; 50°C for 5 seconds; rapid thermal ramp to 60°C, 60°C for 4 minutes for 25 cycles. To remove any excess dyes, samples were ethanol precipitated. Each reaction was added to 2µl 3M sodium acetate, pH4.6 and 50µl 95% ethanol, vortexed and allowed to sit on ice for 10 minutes. Samples were centrifuged at maximum speed for 30 minutes at 4°C. The supernatant was carefully removed and the pellet washed with 250µl 70% ethanol. The pellet was dried in a vacuum centrifuge and resuspended in 25 µl of template suppression reagent. Samples were vortexed, centrifuged and heated at 90°C for 2 minutes. Samples were then loaded on to the sequencer.

2.2.6. Purification of $\alpha_{IIb}\beta_3$ from platelets.

2.2.6.1. Platelet Preparation.

The Blood Transfusion Service Board, Pelican House, Dublin, Ireland generously donated outdated platelet concentrates. Approximately 100 units of platelet concentrates were pooled into 0.5 litre bottles and centrifuged at 300 x g for 5 minutes at 4^0C to clear contaminating red blood cells. The supernatant was removed and platelets were pelleted through the plasma by centrifugation at 1800 x g for 15 minutes at 4^0C. The supernatant was removed and platelets were washed three times in a buffer containing 150mM NaCl, 1mM EDTA, 20mM Tris pH 7.4. The final platelet pellet was resuspended in lysis buffer (1% (w/v) Triton X-100, 150mM NaCl, 1mM $CaCl_2$, 10µM leupeptin, 10mM Tris pH 7.2) and platelets were lysed on ice for 30 minutes and stored at -80^0C.

2.2.6.2. Glycoprotein Purification.

The platelet lysates were removed from storage and thawed rapidly by immersion in a water bath at 37^0C. Lysates were pooled and centrifuged at 30,000 x g for 15 minutes at 4^0C to remove insoluble material. The supernatant was removed and applied to a 1.5 X 10 cm column of concavalin A equilibrated with buffer B (0.1% (w/v) Triton X-100, 150mM NaCl, 1mM $CaCl_2$, 0.05% (w/v) NaN_3, 10mM Tris pH 7.4) at a flow rate of 1-2 ml/min at room temperature. The retained glycoproteins were washed with buffer B and eluted with buffer B containing 100mM α-methyl-D-mannoside at a flow rate of 0.5ml/min into 2-2.5 ml fractions. Detection of $\alpha_{IIb}\beta_3$ in the collected fractions was carried out by dot blotting aliquots onto PVDF membrane and probing with anti-$\alpha_{IIb}\beta_3$ antibody (CD41a, Immunotech, Marseilles, France). The $\alpha_{IIb}\beta_3$ containing fractions were pooled and applied to a 1.5 X 10 cm column of heparin sepharose equilibrated with buffer B at a flow rate of 1-2 minutes at room temperature into 2-2.5 ml fractions. The column retains thrombospondin and the flow through fractions contains α_{IIb} and β_3. The $\alpha_{IIb}\beta_3$ containing fractions were detected by dot blot. These fractions were pooled and concentrated by ultrafiltration using PM30 filters (Amicon Corp. Danvers, MA USA) to a volume of 8-10 mls. This was applied to a 2.5 X 110 cm column of sephacryl S-300 equilibrated with buffer B at a flow rate of 50-80 ml/hour at room temperature into 5-5.5 ml fractions. Again detection of $\alpha_{IIb}\beta_3$ containing fractions was carried by dot blot analysis. These fractions were pooled and concentrated to a volume of 8-10 mls and applied a second time to the sephacryl S-300 column. The $\alpha_{IIb}\beta_3$ containing fractions were aliquoted and rapidly frozen by immersion in a dry ice methanol bath and stored at

-80^0C. Aliquots were thawed rapidly in a 37^0C water bath before use in experiments. The concentration of the purified protein was determined using the Bradford DC assay.

2.2.7. Isomerase assay.

The basis of this assay was to test the ability of an isomerase to catalyse the refolding of a disulphide rich containing protein from its unfolded reduced form. The protein used in these studies was ribonuclease (RNase) which contains eight cysteines and four disulphide bonds. The substrate for RNase was cyclic cytidine 2',3'-phosphate and RNase hydrolyses the phosphate at the 2' position. This hydrolysis was monitored at 284nm.

2.2.7.1. Preparation of reduced and denatured RNase (rdRNase).

30mg of purified RNase was reduced and denatured in the presence of 0.15M dithiothreitol and 6M guanidine HCl in a final volume of 1.5 ml in 0.1M Tris, pH 8.6 at room temperature for 18 hours. The preparation was desalted on a sephadex G25 column equilibrated with 0.01N HCl and 0.5ml fractions were collected. The concentration of rdRNase in each fraction was determined from Beer's law, which states that the absorbance of a solution A is directly proportional to its concentration c and the path length l through the spectrophotometer. This law is expressed as $A=c\varepsilon l$ where ε is a proportionality constant called the molar extinction coefficient ($M^{-1}cm^{-1}$). The extinction coefficient of rdRNase is $9200M^{-1}\ cm^{-1}$ at 275nm. Briefly, a 1:10 dilution was made from each fraction and its absorbance was read at 275nm when blanked

against 0.01N HCl. Fractions with the highest concentrations were aliquoted and stored under argon at -80°C before use in experiments.

2.2.7.2. Reactivation of reduced and denatured RNase.

The reduced and denatured enzyme was diluted into 0.1M Tris-HCl pH 7.4 with 1mM EDTA at a final concentration of 30μM containing the isomerase of interest. The extent of reactivation of rdRNase was monitored by removing aliquots at various time points and measuring recovered RNase activity by examining changes in absorbance at 284nm in a Unicam split beam spectrometer when the enzyme was added to its substrate (0.44mM cytidine 2',3' cyclic monophosphate (cCMP) in 0.1M 2-(N-morphlino) propanesulfonic acid (MOPS) pH 7.0). A typical assay consisted of 1.4μM RNase acting on 0.44mM cCMP. In a parallel assay the activity of native RNase acting on cCMP was monitored at each time point. The recovered activity of rdRNase was then expressed as a percentage of this native RNase control.

2.2.8. Confocal Microscopy.

Analysis of immunofluorescently labelled cells using confocal microscopy allows the determination of co-localisation of molecules. In these studies this method was employed to examine the site of the integrin $\alpha_{IIb}\beta_3$ and protein disulfide isomerase on the surface of resting and activated platelets. Glass slides pre-coated with poly-L-lysine or slides coated with fibrinogen (20μg/ml) for 2 hours were blocked with 1% BSA in tris buffered saline (TBS, pH 7.4) for 1.5 hours in a humidified staining tray. Gel filtered platelets (GFP), prepared as described in section 2.2.1.2 were diluted to 2.0×10^5 /ml

and allowed to adhere to the poly-L-lysine coated slides for 15 minutes and to the fibrinogen-coated slides for 30 minutes. Slides were washed with TBS and fixed in ice-cold methanol for 7 minutes. Slides were blocked with 1% BSA for 20 minutes. Primary antibodies (1:200 dilution for monoclonal anti-$\alpha_{IIb}\beta_3$ (CD41a) complex specific and 1:50 for polyclonal anti-protein disulfide isomerase) were incubated for 45 minutes in humidified staining trays. Slides were washed and incubated with secondary antibodies as follows: a 1:400 dilution of goat anti-mouse Alexa-488 conjugated IgG and goat anti-rabbit Alexa 546 conjugated IgG in TBS were incubated for 10 minutes. Slides were washed and mounted in fluorescent mounting medium. Images were analysed on a Zeiss Axioplan 2 confocal microscope. All experiments were carried out at room temperature.

2.2.9. 2D Electrophoresis.

2.2.9.1. First Dimension.

Gel filtered platelets were prepared as outlined in section 2.2.1.2, to a concentration of $2x10^9$ and were lysed in lysis buffer (9.5M urea, 4% CHAPS, 40mM Tris base, 0.08% IPG buffer, 1% DTT, 1mM PMSF, 0.1% RNase) in the presence of 1mM EDTA. 80µg of lysed protein was added to the gel rehydration solution (7M urea, 2M thiourea, 2% CHAPS, 0.01% bromophenol blue, 0.5% IPG buffer, 0.05% DTT). Samples were loaded into ceramic holders, the IPG (Immobilised pH Gradient) strips™ inserted and overlaid with IPG cover fluid. Holders were placed into the IPGphor Isoelectric Focusing Unit™ (Pharmacia Biotech, Upsala, Sweden) and rehydrated overnight for 12hrs. Gel strips were focused for 60,000Vh.

2.2.9.2. Second Dimension

IPG strips™ were equilibrated for 15 minutes in a DTT containing solution (50mM Tris-HCL pH8.8, 6M urea, 30% v/v glycerol, 2% w/v SDS, 1%w/v DTT) and for a further 15 minutes in an iodoacetamide containing solution (50mM Tris-HCL pH8.8, 6M urea, 30% v/v glycerol, 2% w/v SDS, 4.8% w/v iodoacetamide). IPG strips™ were then sealed on top of a 10% polyacrylamide gel using agarose sealing solution (25mM Tris, 192mM glycine, 0.1%w/v SDS, 0.5% w/v agarose, bromophenol blue) and the gels run at 20mA in a Hoefer DALT ™(Pharmacia Biotech, Upsala, Sweden) 10 gel system at 10°C until the dye front had just run off.

2.2.9.3. 2D Protein Transfer.

Separated proteins were transferred to PVDF membrane in transfer buffer (25mM Tris, 192mM glycine, 20%v/v methanol, 1% w/v DTT). Transfer was carried out in a DALT tank for 20 hours at 4°C. Transfer efficiency was determined using the reversible stain Ponceau-S and destained using dH_2O.

Further methodological procedures and statistical analysis are described in subsequent chapters, as appropriate to the experiments being carried out.

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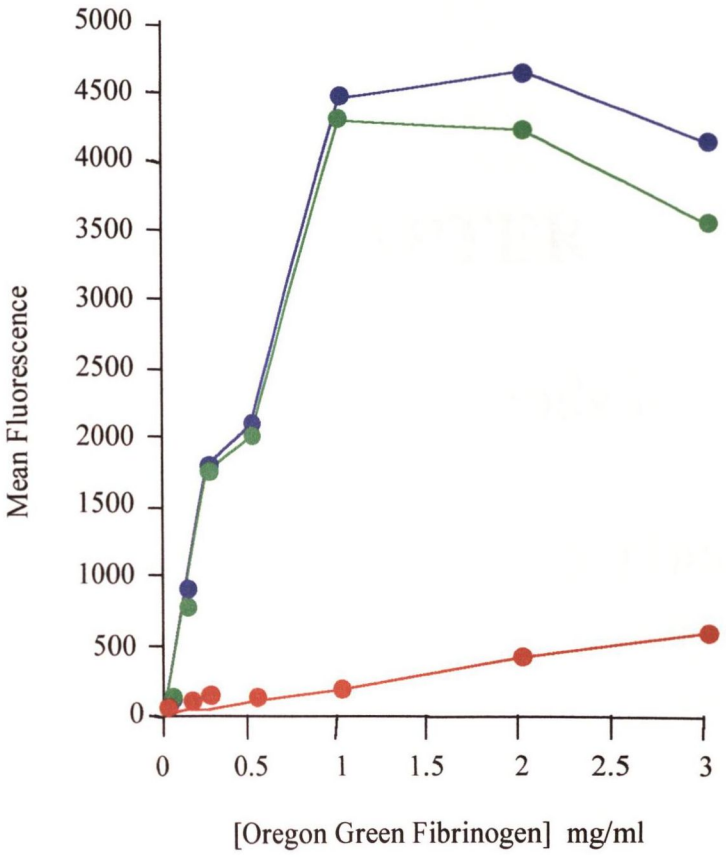


Figure 2.1. Oregon green labelled fibrinogen binds to platelets in a specific manner.

Oregon green labelled fibrinogen binds to ADP activated platelets in a specific manner (●). Specific binding was determined by the subtraction of binding in resting platelets (●) from total binding to activated platelets (●). Data shown are the mean of n = 2 experiments.

CHAPTER 3

A role for disulphide bonds in recognition and signal transduction by platelet $\alpha_{IIb}\beta_3$

3.1. INTRODUCTION.

Upon platelet activation, $\alpha_{IIb}\beta_3$ undergoes conformational changes in which the receptor goes from a resting to an activated state that is competent to bind soluble fibrinogen. The mechanisms by which platelet activation causes these conformational changes within the receptor have not been elucidated. $\alpha_{IIb}\beta_3$ is the most abundant platelet protein accounting for approximately 20% of the platelet surface area. Furthermore, it is a cysteine rich protein with a total of 18 cysteines in the alpha sub-unit and 56 cysteines in the extracellular domain of the beta subunit (Calvete 1995). These cysteine residues are highly conserved in all the beta subunits of the human integrin family and indeed across species. The cysteine content of the beta subunit is unusually high for a protein and the resulting disulphide bonds are important for the structural integrity of the receptor. The presence of so many cysteines in such a concentrated manner in receptors on the platelet surface suggests a functional role for these residues. This chapter focuses on the evidence in support of such a role for cysteines of $\alpha_{IIb}\beta_3$ during platelet activation.

The reducing agent, dithiothreitol (DTT) can cause platelet aggregation in preparations of PRP when used in concentrations greater than 3mM, while lower concentrations inhibit platelet aggregation (MacIntyre 1974). This effect of DTT is mediated, at least in part, via a direct effect on $\alpha_{IIb}\beta_3$ as it induces binding of fibrinogen in a specific and saturable manner (Zucker and Masiello 1984). Also, treatment of purified $\alpha_{IIb}\beta_3$ with DTT activates the receptor and exposes ligand induced binding sites independent of ligand binding, suggesting that the effect of DTT is to mimic a functional activation of the platelet integrin (Kouns 1993). DTT treatment of platelets can therefore mimic

various aspects of platelet activation, which include aggregation, fibrinogen binding and the exposure of ligand induced binding sites in purified $\alpha_{IIb}\beta_3$.

The purpose of the following studies was to confirm and expand the findings described above and to verify that the effects of DTT on platelets are mediated through $\alpha_{IIb}\beta_3$. To investigate if modification of the receptor disulphide bond pattern by DTT leads to any novel signalling events in the platelet that may reflect conformational changes in the platelet integrin.

3.2. METHODS.

3.2.1. DTT induced platelet aggregations.

Aggregation studies were performed using the reducing agent dithiothreitol (DTT) to confirm the work of MacIntyre et al. Gel filtered platelets were prepared as outlined in section 2.2.1.2. DTT at a final concentration of either 0.5mM or 20mM was added to platelets stirring at 1,000 rpm at 37°C in a final volume of 500µl in a BioData aggregometer. The resulting aggregation was monitored for 4-6 minutes. The DTT aggregations were also performed in the presence of exogenous human fibrinogen (1mg/ml) and PGE_1 (2µM).

3.2.2. Binding of fibrinogen to platelets.

This assay was carried out as described in section 2.2.2.1. Increasing concentrations of oregon green labelled fibrinogen (OgFg, 0.125mg/ml-3.0mg/ml) was added to ADP (10µM) activated platelets in PRP diluted 1:10 with buffer A. Non-specific binding was determined in the presence of the $\alpha_{IIb}\beta_3$ antagonist Integrilin (2µg/ml). Based on these results, subsequent assays were carried out using labelled fibrinogen at a single concentration of 1mg/ml. Diluted PRP was incubated with DTT at a final concentration of either 0.5mM or 20mM for 5 minutes before the addition of OgFg. The binding reaction was for 20 minutes at room temperature and was stopped by the addition of 2mls of PBS. Samples were read immediately on the FACS. ADP (10µM) activated platelets were used as a positive control for this assay. Data were expressed as mean fluorescence.

3.2.3. Determination of ligand induced binding sites (LIBS) on $\alpha_{IIb}\beta_3$.

Gel filtered platelets were prepared as outlined in section 2.2.1.2. Platelets were treated in the absence or presence of various concentrations of DTT (0.5mM-50mM) for 5 minutes at room temperature. Aliquots of each treatment were either fixed immediately in 1% formaldehyde or incubated for a further 30 minutes at 37°C in the presence of either PBS, 5mM EDTA or 20μM TRAP. At 30 minutes, platelets were washed with PBS containing 1% BSA and then fixed with 1% formaldehyde. Samples were washed again and 50μl aliquots were incubated with 0.4μg/ml of anti D3GP3, (gift of Dr. L. Jennings, University of Tennessee), for 30 minutes at room temperature. The samples were washed and resuspended in 100μl of PBS/1% BSA containing 40μl of goat anti mouse antibody conjugated to a FITC molecule. After incubation for a further 30minutes, samples were washed and resuspended in 1% formaldehyde. Control assays were prepared omitting the anti-D3GP3 antibody to assess the amount of auto fluorescence in samples. Samples were analysed for fluorescence on a Becton Dickinson FACS-Scan at 488/510nm. Treatment of platelets with 5mM EDTA exposes maximal D3GP3 epitopes and was used as a positive control in all assays. Data were expressed as mean fluorescence.

The platelets in these experiments were gel-filtered to remove exogenous fibrinogen. To confirm that the expression of the ligand induced binding sites obtained were not as a result of the binding of residual fibrinogen in the preparation, the experiments were performed in the presence of the $\alpha_{IIb}\beta_3$ antagonist RO44-9883 (3μM, n=3), which itself does not induce LIBS.

3.2.4. Binding of the RGD mimetic ^{3}H-SC52012 to normal and Glanzmann's Thrombasthenic platelets.

Binding of ^{3}H-SC52012 was performed as described in section 2.2.5. Gel filtered platelets (0.25×10^{8}/ml), both normal and those obtained from a Glanzmann's Thrombasthenic patient (n = 1) were incubated with 20mM DTT for 5 minutes at room temperature. Activation of platelets with and without 20μM ADP were used as controls for the assay.

3.2.5. Tyrosine phosphorylation events in DTT induced platelet aggregation.

Gel filtered platelets were prepared as outlined in section 2.2.1.2. Platelet aggregations were performed as described in section 2.2.1.3. Briefly, platelets were allowed to aggregate to thrombin at a final concentration of 1U/ml for 30 seconds, 3 minutes and 6 minutes or to DTT at a final concentration of either 0.5mM or 20mM for 6 minutes at 37°C at 1100 rpm. To evaluate the effects of RGD mimetics on phosphorylation events in thrombin and DTT aggregations the following study was carried out: gel filtered platelets were aggregated in the presence of either buffer (control), 2μg/ml Integrilin or 1μM RO44-9883, for 3 minutes at 37°C at 1100 rpm. Spinning platelets in a microcentrifuge, at maximum speed for 10 seconds stopped the aggregation. The supernatant was removed and the platelet pellet was resuspended in 50μl of 1X RIPA buffer (section 2.2.1.4.). The protein concentration of lysates was determined as described in section 2.2.1.4 and proteins were separated on SDS-PAGE gels and transferred to PVDF as described in section 2.2.4. An anti-phosphotyrosine western was performed on the membrane as described in section 2.2.4.4. Two primary antibodies

were used, a combination of anti-4G10 and anti-PY20 at a final concentration of 1μg/ml each. The secondary antibody was a 1:1500 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP). The blots were developed with ECL.

3.2.6. The effects of DTT on tyrosine phosphorylation events in platelet adhesion.

Platelet adhesion was performed as described in section 2.2.1.4. Gel filtered platelets were incubated with either buffer or 20mM DTT for 5 minutes at room temperature before being placed on either BSA, fibrinogen or fibrin coated plates. The concentration of lysates was determined as described in section 2.2.1.4 and proteins were separated on SDS-PAGE gels and probed for phosphotyrosine as described above.

3.3. RESULTS.

3.3.1. Platelet aggregation induced by DTT.

Addition of 20mM DTT to platelets in a stirred system caused them to aggregate to approximately 12% over four minutes. The aggregation started after a lag period of 1-2 minutes (Figure 3.1a). Upon the addition of exogenous human fibrinogen (1mg/ml) DTT induced platelet aggregation occurred immediately without a lag period. The extent of aggregation was enhanced with platelets aggregating to approximately 25% (Figure 3.1b). Platelet aggregation to DTT was unaffected by PGE_1 (2μM) (Figure 3.1c). DTT (0.5mM) had no effect on platelet aggregation.

3.3.2. Fibrinogen binding to platelets activated with DTT.

Treatment of platelets with 20mM DTT, induced fibrinogen binding as measured by the binding of OgFg in a FACS assay (Figure 3.2). This binding was less than that obtained when platelets were activated with the agonist ADP (10μM, n=9). No OgFg binding was observed following treatment with 0.5mM DTT. This data indicates that DTT has a specific effect on the platelet $\alpha_{IIb}\beta_3$ to enhance fibrinogen binding.

3.3.3. The effect of DTT on the expression of LIBS in $\alpha_{IIb}\beta_3$.

Platelets treated with DTT (0.5mM and 50mM for 5 minutes) showed an increase in the expression of LIBS epitopes (Figure 3.3a) as indicated by the increased binding of the LIBS antibody D3GP3. The response to DTT was dose dependent. It was found that maximal binding of the antibody occurred when platelets were treated with 20mM DTT

for 5 minutes. However, when the DTT-incubation time was extended by 30 minutes, the LIBS binding sites were lost implying that high doses of DTT first expose LIBS sites and then destroys them (Figure 3.3b). In contrast, coincubation of platelets with DTT and 5mM EDTA resulted in a uniform increase in LIBS expression when compared to treatment of platelets with DTT alone (Figure 3.3c). This suggests that this concurrent treatment protects the LIBS sites and in some manner exposes possibly more cryptic sites on the receptor. Interestingly, when platelets were coincubated with DTT and the agonist TRAP no such protection occurred, the level of LIBS expression observed was similar to that obtained with DTT treatment alone (Figure 3.3d).

The platelets in these experiments were gel-filtered to remove exogenous fibrinogen. To confirm that the expression of LIBS observed were solely attributable to DTT treatment and not as a result of the binding of residual fibrinogen in the preparation, the experiments were repeated in the presence of the $\alpha_{IIb}\beta_3$ antagonist RO44-9883 (3μM), an RGD mimetic. Interaction of this compound with the receptor does not result in the expression of LIBS. In the presence of this antagonist, expression of LIBS epitopes was obtained when platelets were treated with DTT (Figure 3.4).

3.3.4. The effect of DTT on the binding of [^{3}H]-SC52012 to normal and Glanzmann's Thrombasthenic platelets.

SC52012 is a non-peptide antagonist of $\alpha_{IIb}\beta_3$. It binds to the RGD binding site on the integrin and competes with fibrinogen for binding. It binds to resting and ADP activated platelets in a saturable and time dependent manner (Figure 3.5). The total number of

binding sites (B_{max}) was similar for both resting and activated platelets with estimated values of 38,860±5,037 (n=3) per resting platelet and 35,072±4,327 (n=3) per activated platelet. The affinity (K_d) for ligand in the resting platelet was 108.2±15.5nM. This increased dramatically upon platelet activation with ADP to 31.3±10.4nM. When platelets were treated with DTT (20mM) both the B_{max} and K_d appeared to increase to 59,235±9,081 and 11.5±2.0nM respectively (n=3). Statistical analysis (Instat Version 4.0) revealed that the DTT B_{max} value was not significantly different from that of activated and resting platelets whereas the K_d value was significantly different.

The affinity for this ligand in resting platelets obtained from a Glanzmann's Thrombasthenic patient was found to have a value of 23nM and a B_{max} of 29,387 per platelet (Figure 3.6). Treatment of these platelets with 20μM ADP and 20mM DTT resulted in K_d values of 10.3nM and 16nM respectively. Activation of the Glanzmann's Thrombasthenic platelets with ADP did not appear to change the B_{max}, which had a value of 29,242 but in contrast treatment with DTT decreased this value to 21,438.

The data so far suggests that DTT is having a specific activating effect on the platelet integrin $\alpha_{IIb}\beta_3$, causing binding of fibrinogen, expression of LIBS, and enhanced affinity for small ligands. PGE_1, a physiological antagonist of platelet activation, had no effect on DTT-induced platelet aggregation, suggesting the effects of DTT are extracellular. However, since DTT treatment seemed to be inducing an active conformation in $\alpha_{IIb}\beta_3$, it seemed appropriate to investigate whether DTT was inducing platelet activation. I therefore examined the effects of DTT treatment on tyrosine phosphorylation events in platelet aggregation and adhesion.

3.3.5. The effect of DTT on tyrosine phosphorylation events in platelet aggregation.

Platelet activation by agonists results in an increase in the phosphorylation of tyrosine residues of particular proteins in the platelet. As can be seen in figure 3.7 this increase in phosphorylation was observed in certain proteins (marked with red arrows in lanes 3 and 4) when platelets were activated with 1U/ml thrombin. The molecular weight of these proteins are 130, 125, 101/105, 77-80, 64, 60, 52 and 48 kDa and are typical of such phosphorylation events in the platelet. It is noted that maximal phosphorylation occurred 3 minutes after activation with thrombin.

Activation of platelets with 0.5mM and 20mM DTT (lanes 5 and 6) resulted in a level of phosphorylation that was similar to that obtained in resting platelets (lane 1) or that obtained with 30 seconds of thrombin activation (lane 2). Activation of platelets with DTT in the presence of 2mg/ml fibrinogen (lanes 7 and 8) showed an increase in the phosphorylation of p130 and p77-80 similar to that obtained in lanes 3 and 4. Unfortunately, the fibrinogen bands at 45,55 and 65 kDa masked any events that may have occurred in proteins with molecular mass less than 66 kDa in these lanes.

In an attempt to rectify this, the aggregations were performed in the presence of the RGD mimetics Integrilin and R044-9883 (Figure3.8). Looking at proteins in the molecular mass range 40- 60 kDa from platelets activated with thrombin resulted in the phosphorylation of two proteins with estimated masses of 52 and 48 kDa respectively (lane 4). In the presence of Integrilin (lane 5) and RO44-9883 (lane 6) both proteins were dephosphorylated. Activation of platelets by thrombin in the presence of DTT

(lane7) saw a reduction in the level of phosphorylation of these two proteins. Addition of the RGD mimetics resulted in complete dephosphorylation of these proteins (lanes 8 and 9).

3.3.6. The effect of DTT on tyrosine phosphorylation events in platelet adhesion.

Resting unactivated platelets adhere to and spread upon a fibrinogen or fibrin matrix. Such adhesion results in the induction of many tyrosine phosphorylation events (Figure 3.9) when compared to platelets that adhere to BSA. Most notable among these events was the phosphorylation of proteins 125, 105/101, 85, 72, 58, 50, 42, 38 kDa (indicated by red arrows). The phosphorylation events that occurred as a result of this adhesion were markedly reduced in platelets treated with DTT. It was also noted that there was no phosphorylation in non-adhered platelets.

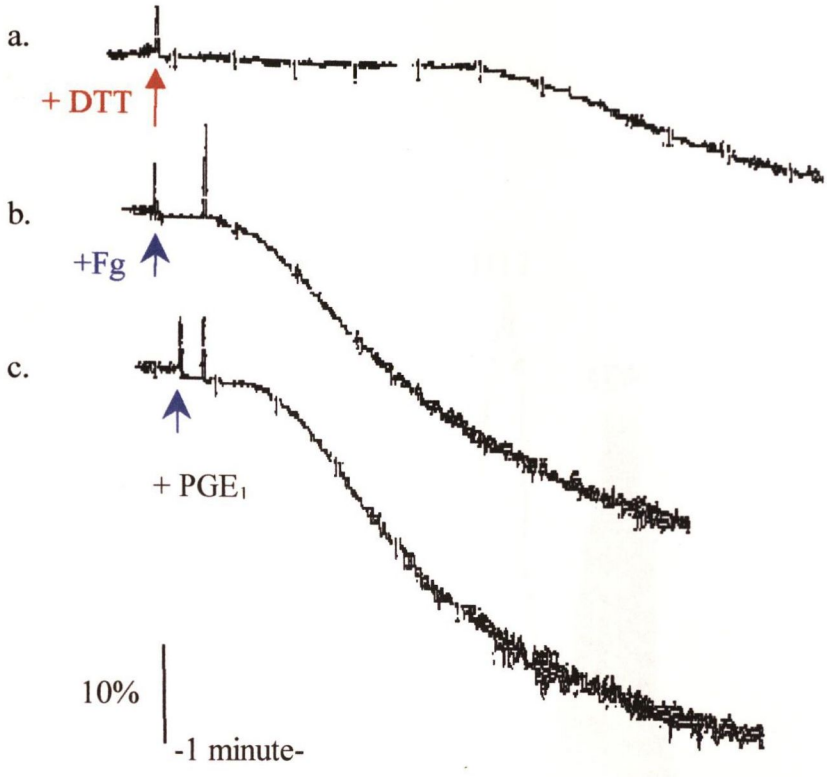


Figure 3.1. Platelet aggregation induced by DTT.

a)DTT at a final concentration of 20mM was added to gel-filtered platelets in a BioData aggregometer. The reaction was monitored for four minutes at which time the platelets had aggregated to an extent of approximately 12%, after an initial lag period of approximately two minutes. b)This aggregation was enhanced upon the addition of exogenous fibrinogen (1mg/ml) and occurred immediately. c)When platelets were preincubated with PGE_1 (2μM), they still supported aggregation induced by DTT in the presence of exogenous fibrinogen. These data represents one of n = 5 experiments.

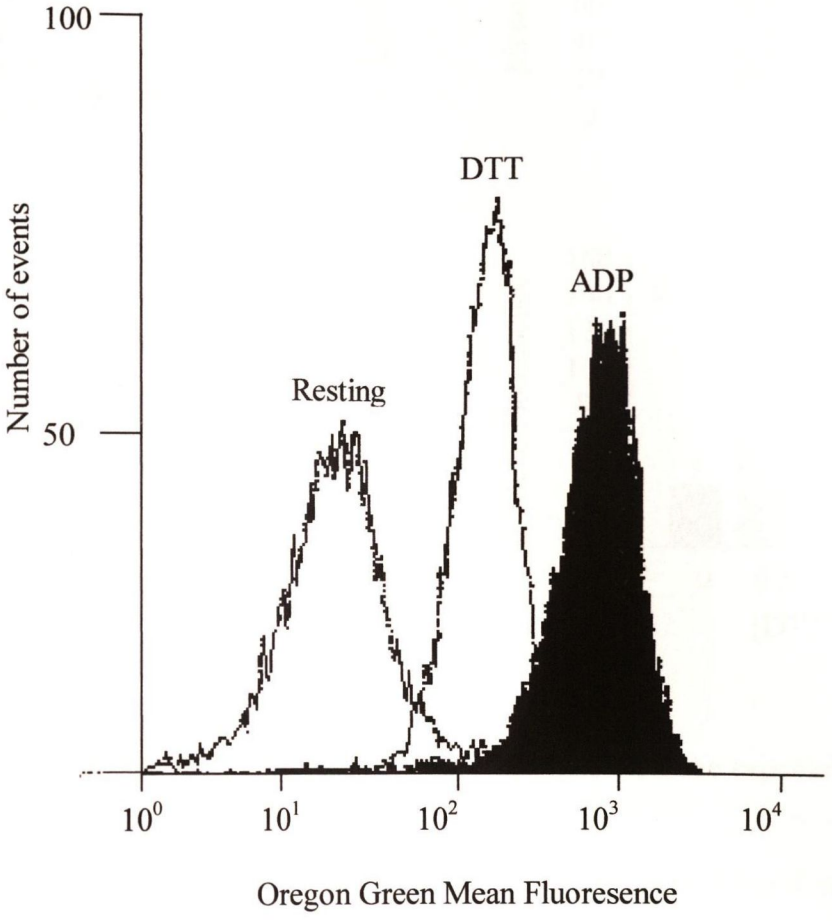


Figure 3.2. DTT induces fibrinogen binding to platelets.

In resting unactivated platelets, a low basal background binding of OgFg is indicated by the low level of fluorescence. When platelets are activated with ADP there is a dramatic increase in mean fluorescence indicating the binding of fibrinogen. When platelets were treated with 0.5mM DTT it was found that the level of binding of fibrinogen was equivalent to background binding (data not shown). Treatment of platelets with 20mM DTT induced significant binding of fibrinogen to the platelets. This binding was less than that obtained when platelets were activated with 10μM ADP. These data represents one of n = 4 experiments.

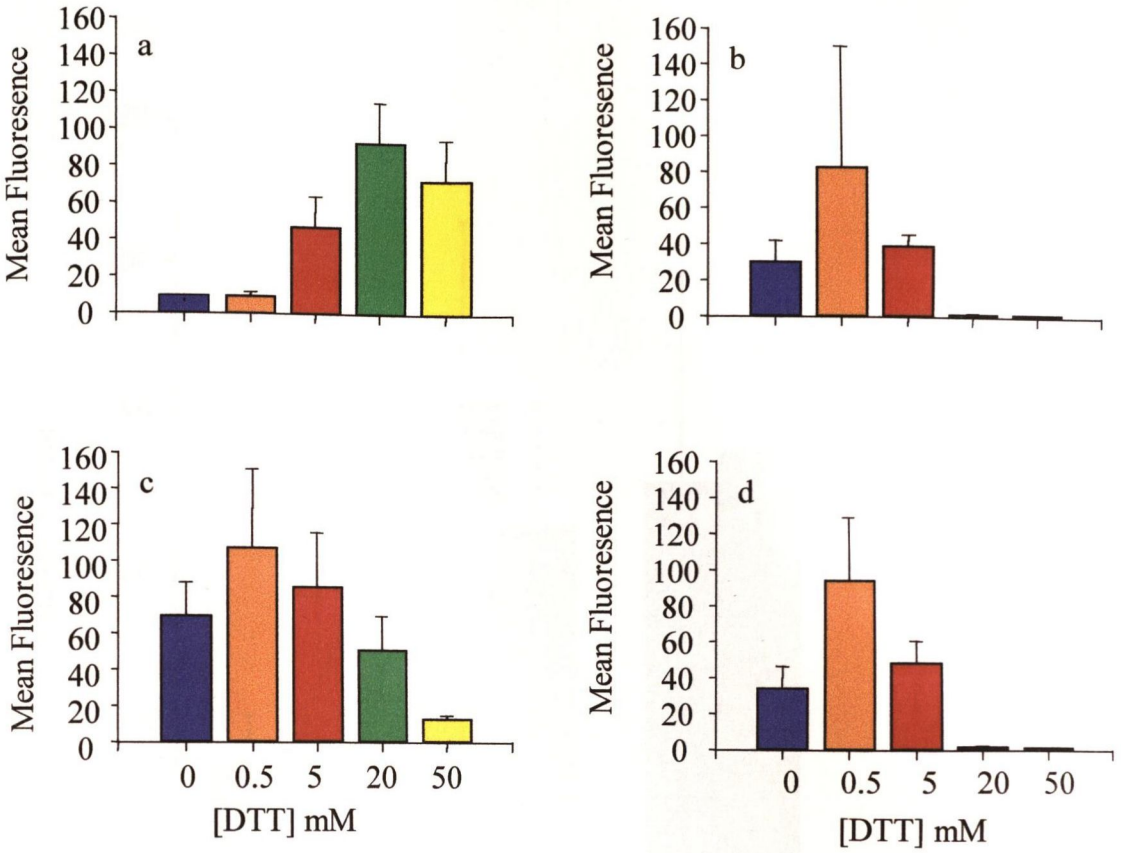


Figure 3.3. The effect of DTT treatment of platelets on the expression of LIBS in $\alpha_{IIb}\beta_3$.

a)Treatment of platelets with DTT at concentrations between 0.5mM and 50mM for 5 minutes shows a dose dependent increase in the expression of ligand induced binding sites in $\alpha_{IIb}\beta_3$ as detected by the antibody anti-D3GP3. b)When platelets were treated with the same range of concentrations for a further 30 minutes, a transient expression of LIBS was observed. Maximal expression occurred at 0.5mM DTT and rapidly disappeared thereafter. c)The level of expression of LIBS observed with 30 minutes of DTT is enhanced when the platelets are coincubated with 5mM EDTA. d)Coincubation of DTT treated platelets with TRAP (20μM) resulted in the same level of LIBS expression as that obtained with DTT treatment alone, figure 3b. Data shown are the mean fluorescence and expressed as the mean ± SD of n = 4 experiments.

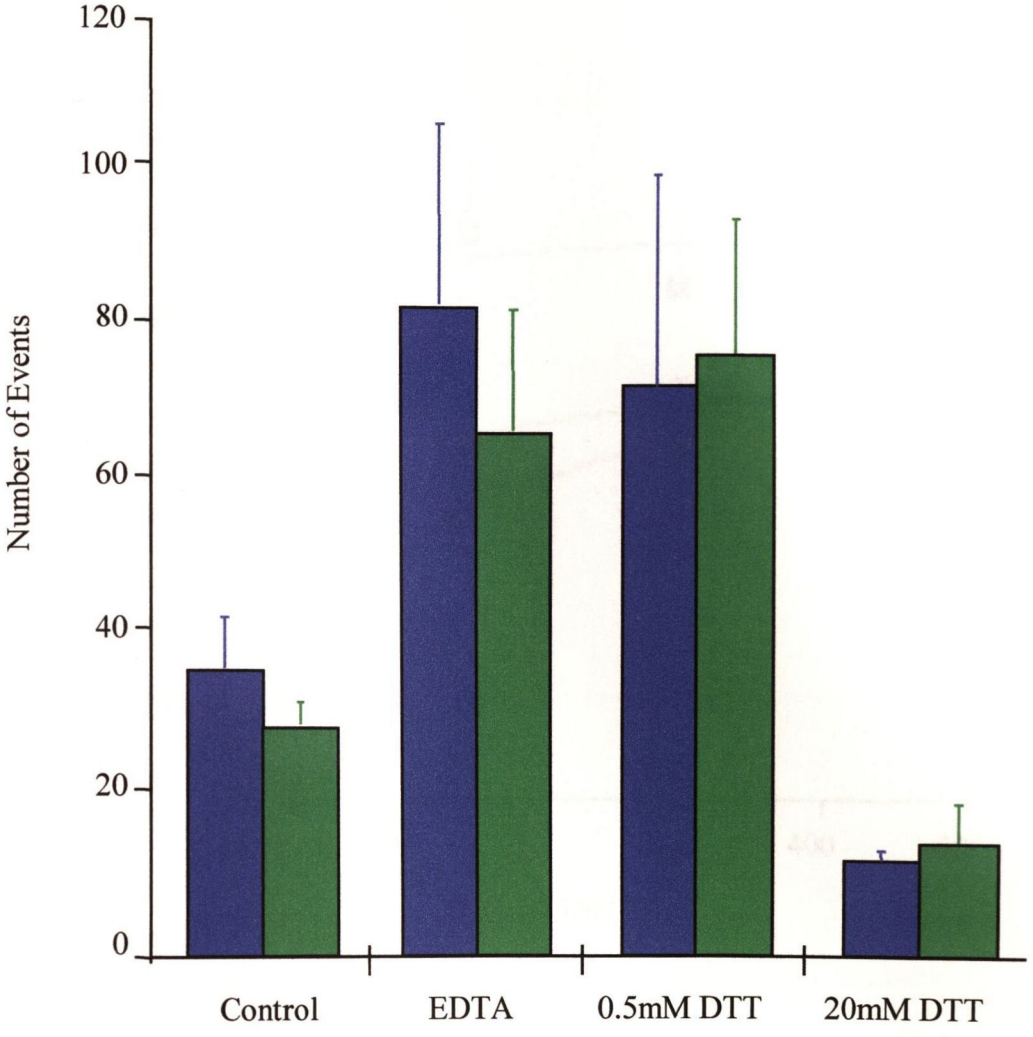


Figure 3.4. DTT induced LIBS in $\alpha_{IIb}\beta_3$ in the presence of the RGD mimetic RO44-9883.

Platelets were untreated (control) or treated with EDTA (5mM), 0.5mM DTT and 20mM DTT for 30 minutes at 37°C in the absence (■) or presence (■) of RO44-9883 (3μM). LIBS expression was comparable in the presence or absence of the mimetic regardless of platelet treatment. Data shown are the mean ± SD of n =3 experiments.

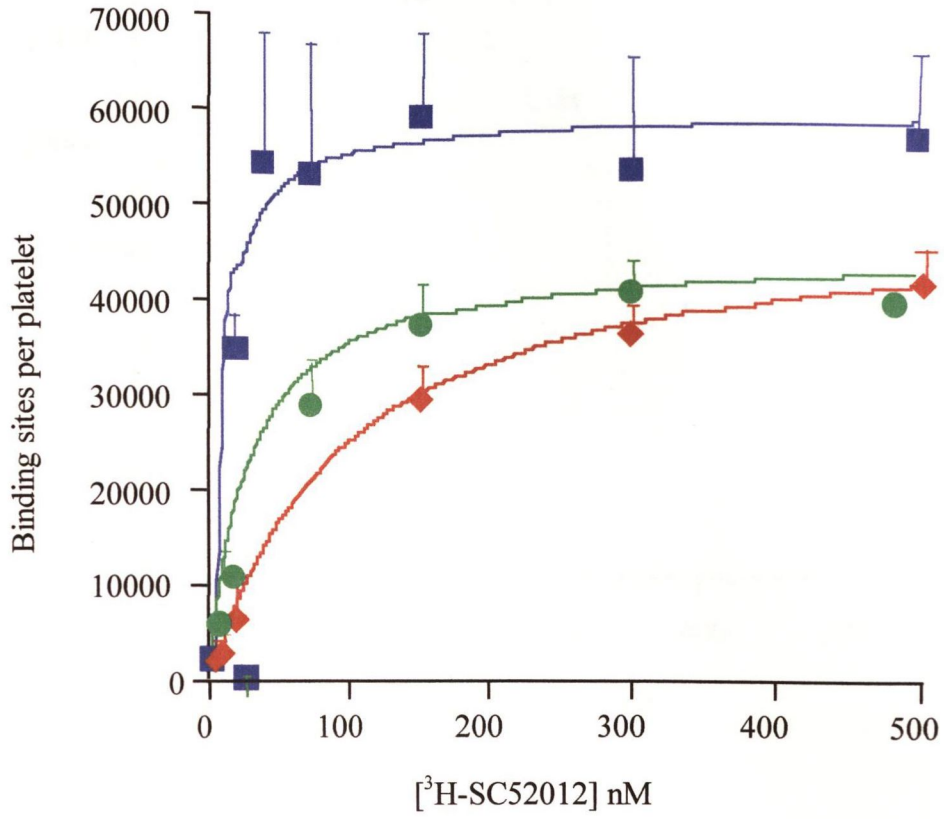


Figure 3.5. The effect of DTT on the binding of [^{3}H]-SC52012 to platelets.

[^{3}H]-SC52012 binds to resting unactivated platelets in a saturable manner (◆) with a K_d of 108.2±15.5nM and a B_{max} of 38,860±5,037. Upon platelet activation with ADP (20μM) (●) the K_d increases to 31.3±10.4nM and the B_{max} is 35,072±4,327. This compound binds to DTT (20mM) (■) treated platelets in a saturable manner. The K_d increased dramatically to 11.5±2.0nM. The apparent B_{max} of the DTT treated platelets had a value of 59,235±9,081. Data were analysed with Deltagraph 4.0 and are expressed as the mean ± S.D of n =3 experiments.

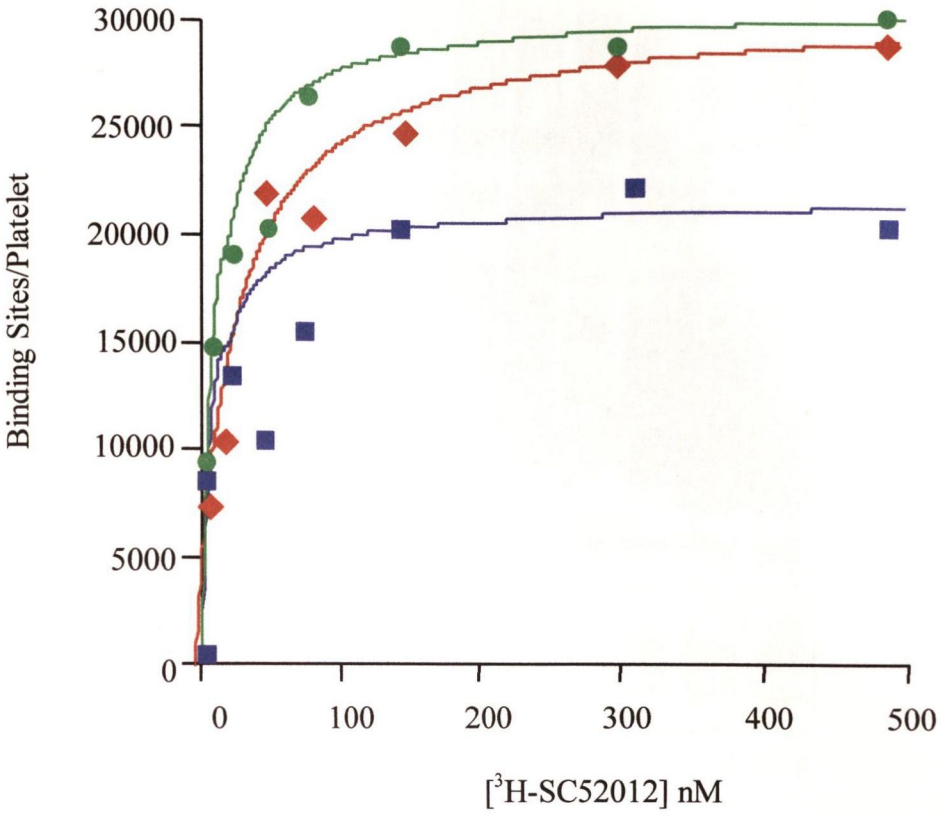


Figure 3.6. Binding of [^{3}H]-SC52012 to platelets from a Glanzmann's Thrombasthenic patient.

[^{3}H]-SC52012 bound to these platelets in a saturable manner. The K_d for this ligand in resting platelets was found to have a value of 23nM and a B_{max} of 29,387 sites per platelet (◆). Treatment of platelets with 20μM ADP (●) resulted in a K_d value of 10.3nM and a B_{max} of 29,242 sites per platelet. 20mM DTT (■) resulted in a K_d value of 16nM and a B_{max} of 21,438 sites per platelet. Data were analysed with Deltagraph 4.0 and shows n =1 experiment.

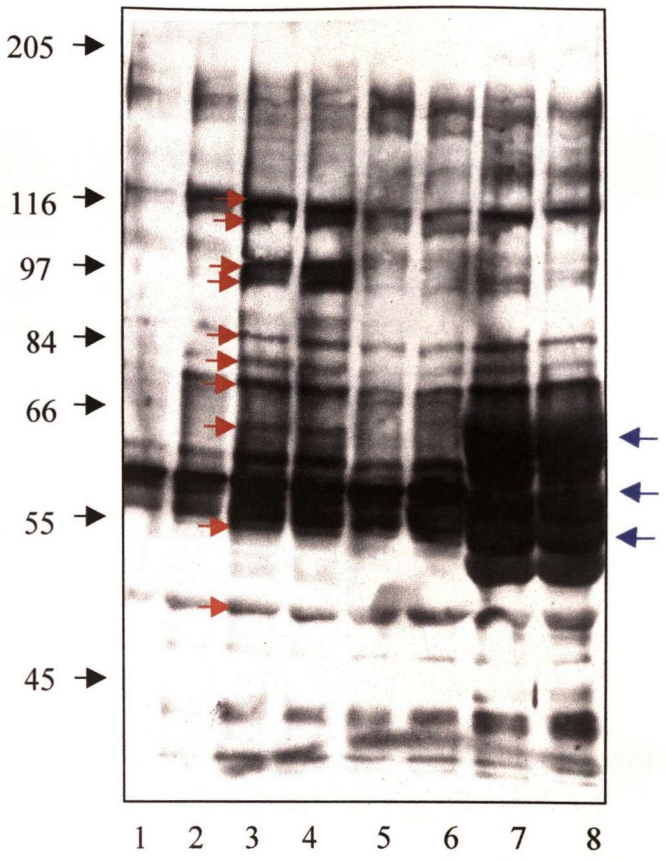


Figure 3.7. The effects of DTT on tyrosine phosphorylation events in platelet aggregation.

Gel filtered platelets were aggregated with buffer (lane 1), 0.1U/ml thrombin for 30 seconds (lane2), 3 minutes (lane 3) and 6 minutes (lane 4). Platelets were treated with 0.5mM DTT (lane 5), 20mM DTT (lane 6), 0.5mM DTT + 2mg/ml Fg (lane7) and 20mM DTT + 2mg/ml Fg (lane8). Platelet lysates were separated on a 7.5% SDS-PAGE gel and tyrosine phosphorylation events were detected by immunoblot analysis. Molecular weight markers are indicated in kDa on the left of the figure. Red arrows indicate phosphorylation events that occurred during activation. Blue arrows indicate the presence of fibrinogen bands at 50, 55 and 65kDa. These data represents one of 3 independent experiments.

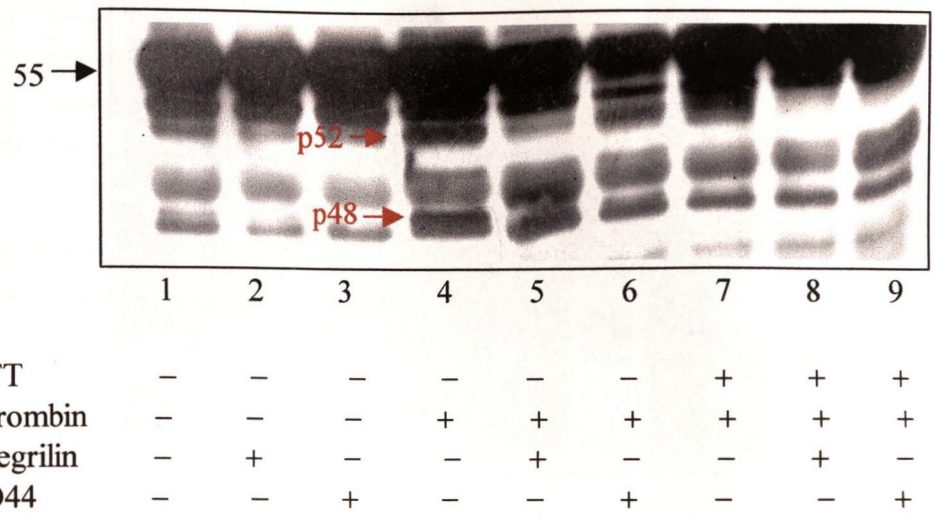


Figure 3.8. The effects of RGD mimetics and DTT on thrombin induced phosphorylation events in platelet aggregation.

Gel filtered platelets were aggregated for 3 minutes in the presence of buffer (lane 1); 2μg/ml Integrilin (lane 2); 1μM RO44-9883 (lane3); 1u/ml thrombin (lane 4); 1u/ml thrombin + Integrilin (lane 5); 1u/ml thrombin + RO44-9883 (lane 6); 1u/ml thrombin + 20mM DTT (lane 7); 1u/ml thrombin + 20mM DTT + 2μg/ml Integrilin (lane 8); 1u/ml thrombin + 20mM DTT + 1μM RO44-9883 (lane9). Platelet lysates were separated on a 7.5% SDS-PAGE gel and tyrosine phosphorylation events were detected by immunoblot analysis. Molecular weight markers are indicated in kDa on the left of the figure. Red arrows indicate proteins phosphorylated during activation. These data represents one of 3 independent experiments.

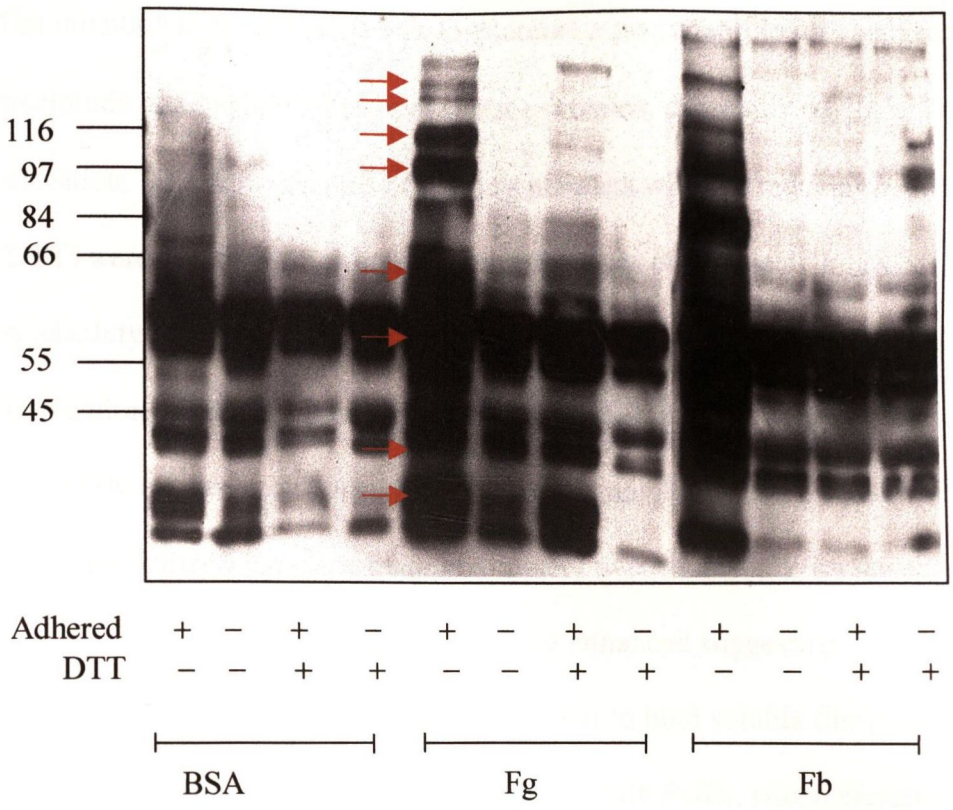


Figure 3.9. The effect of DTT treatment on platelet phosphorylation in adhesion. Gel filtered platelets were treated with either buffer (–) or 20mM DTT (+) for 5 minutes before adhesion to plates coated with BSA, fibrinogen (Fg) or fibrin (Fb). Platelet lysates were prepared from both adherent (+) and non-adhereent (–) platelets and separated on a 7.5% SDS-PAGE gel. Tyrosine phosphorylation was detected by immunoblot analysis. Molecular mass markers are indicated in kDa on the left of the figure. Red arrows indicate proteins phosphorylated during activation. These data represents one of 3 independent experiments.

3.4. DISCUSSION.

The intention of these studies was to examine a potential role for changes in the disulphide bond pattern of platelet surface proteins, especially $\alpha_{IIb}\beta_3$, on platelet activation. The consequences of treating platelets with the reducing agent dithiothreitol (DTT) were examined in various functional assays of platelets. It has been established by MacIntyre *et al.* that low concentrations of DTT (< 3mM) inhibited platelet aggregation, while higher concentrations caused platelet aggregation after a lag phase. This study has been confirmed here and shows that 20mM DTT causes platelet aggregation after a lag phase of approximately two minutes. With the addition of exogenous fibrinogen these aggregations are enhanced suggesting that the receptor has undergone conformational changes that allow it to bind soluble fibrinogen. It was also confirmed that when platelets were pre-treated with PGE_1, which elevates intraplatelet levels of cAMP resulting in the formation of a quiescent platelet, they could still support aggregation. These data suggests, therefore, that DTT acts directly on $\alpha_{IIb}\beta_3$ and that the resulting disulphide bond reduction caused a change in conformation of the receptor, making it competent to bind fibrinogen.

The ability of the reduced receptor to bind fibrinogen was confirmed in the fibrinogen binding assay. The results of these experiments showed that soluble fibrinogen do indeed bind to platelets treated with DTT. This binding was less than that observed in platelets activated with ADP and correlated with the relative extent of platelet aggregation.

DTT also caused an increase in expression of LIBS epitopes on $\alpha_{IIb}\beta_3$ in intact platelets. This confirms the work of Kouns et al, who established that DTT induces LIBS in purified preparations of $\alpha_{IIb}\beta_3$. It is noted that the LIBS antibody, anti-D3GP3, used in these studies binds to the cysteine rich core of the β_3 subunit that is remote from the RGD binding pocket (Kouns et al. 1990). Exposure of platelets to high concentrations of DTT ($\geq$ 5mM) for a short period of time (5 minutes) induces LIBS expression, but after a prolonged period of time (30 minutes) the LIBS epitopes are lost. This may indicate that the region of the β_3 subunit that is recognised by the D3GP3 antibody has been disrupted as a result of excessive disulphide bond reduction. Treatment of platelets with a lower concentration of DTT (0.5mM) for 30 minutes, however, exposes LIBS maximally. This suggests therefore, the existence of different sets of disulphide bonds that are reduced at different rates, which in turn determines the resulting conformational change(s) and the level to which the receptor is activated. Again this data supports the hypothesis that more than one population of disulphides/thiols are present on the platelet surface. Chelation of calcium by EDTA possibly results in the dissociation of the subunits of $\alpha_{IIb}\beta_3$ and allows maximal detection of LIBS by antibodies within the receptor. When platelets are treated with DTT and EDTA simultaneously, the level of expression of LIBS observed was increased over and above that observed with DTT alone. This implies that a removal of calcium from binding sites and a change in disulphide bond patterns within the cysteine rich core exposes cryptic ligand sites within the receptor. Interestingly, coactivation of platelets with DTT and the potent platelet agonist TRAP had no significant effect on LIBS expression when compared to DTT treatment alone. Since these experiments were carried out on gel filtered platelets no

extracellular fibrinogen was available to bind the receptor and so express LIBS as a result of out-side in signalling.

When the RGD binding pocket of $\alpha_{IIb}\beta_3$ is blocked by the fibrinogen receptor antagonist RO44-983, which does not itself induce LIBS, DTT treatment of these platelets nevertheless resulted in the expression of LIBS in the receptor. This implies that DTT induced LIBS was not secondary to DTT induced fibrinogen binding but was as a direct effect of thiol reduction and the rearrangement of disulphide bonds within the receptor.

Agonist activation of platelets results in an increased affinity of $\alpha_{IIb}\beta_3$ for fibrinogen. This can be quantified by measuring the binding capacity of platelet receptors for a small molecule RGD mimetic, SC52012. DTT treatment of platelets increases this affinity of $\alpha_{IIb}\beta_3$ for this molecule over and above that observed with agonist activation of the platelet. A possible explanation for this is that DTT forces a conformation on the receptor such that it binds ligand more tightly as a result of disulphide bond rearrangement in and around the RGD pocket. Again this study is identifying a difference between conformational changes as a result of disulphide bond pattern changes and those caused by activation of the platelet by an agonist. The mutation identified in the Glanzmann's Thrombasthenic patient has been identified as a substitution of a Met residue for a Val at position 193 (Fullard 2001). The affinity for ^{3}H-SC52012 of this patient's platelets had a value of 26nM, which is similar to the value obtained when normal platelets were activated with ADP (31.3nM). Thus, the affinity of the Glanzmann's Thrombasthenic receptor at rest is equivalent to that of a normal activated receptor. Treatment of the Glanzmann's Thrombasthenic platelets with both

DTT and ADP increased the affinity of the receptor further to 10.3nM and 16nM respectively. This result suggests that the receptor is in an altered affinity state that is equivalent to an activated state. However, both DTT and ADP can affect this conformation to further alter the affinity of the receptor for ligand. This suggests that $\alpha_{IIb}\beta_3$ can go through a series of conformational changes, and not just a single one that ultimately determines the affinity of the receptor for ligand.

Agonist induced platelet activation is associated with the phosphorylation of numerous platelet proteins on tyrosine residues, including β_3 itself. The level of tyrosine phosphorylation observed in DTT treated platelets were similar to that obtained in resting platelets. This may be attributed to the fact that DTT treated platelets only aggregate to 12% compared to approximately 60% with thrombin. It has been established that platelet aggregates obtained in the presence of DTT are looser and less compact than those obtained when platelets are activated with an agonist (Peerschke 1995). It would be expected, therefore, that the level of phosphorylation in these platelets would be different when compared to that in agonist treated platelets. Activation of platelets by thrombin in the presence of DTT resulted in a phosphorylation profile that was similar to that obtained when platelets were activated by thrombin in the presence of an RGD mimetic. This suggests that the conformational change induced by DTT is similar to a conformation that may occur as a result of having the RGD pocket occupied.

Interestingly, although DTT treated platelets are capable of binding immobilised fibrinogen, such platelets appear not to signal inwards. This implies that the

conformational changes necessary to permit fibrinogen binding are not the same as those required for signal transduction.

In conclusion, the data presented here suggests that disulphide bond rearrangement is a physiological mechanism by which $\alpha_{IIb}\beta_3$ undergoes conformational changes. Such conformational changes result in the formation of an active conformation of the receptor that can support platelet aggregation, fibrinogen binding and expression of ligand induced binding sites. However, the DTT-induced conformation of $\alpha_{IIb}\beta_3$ is not equivalent to an activated receptor, as it is incapable of transmitting a signal inward into the platelet. It is possible however, that the DTT-induced conformation of $\alpha_{IIb}\beta_3$ represents an intermediate conformation of the integrin formed during the activation process. This intermediate conformation has an increased affinity for ligands but cannot transmit a signal inward into the platelet. A further conformational change in the integrin (such as the formation of novel disulphide bonds) might be necessary to complete the activation cycle.

Work from the laboratory of Calvete initially indicated that each cysteine in $\alpha_{IIb}\beta_3$ was involved in a disulphide bond and hence no free thiols were present (Calvete et al. 1989) (Calvete et al. 1991). Recently, it has been shown that free thiols do exist in resting and activated conformations of the integrin (Yan et al. 2000). Moreover, there are two additional free thiols in the activated conformation than in the resting state. The cysteines yielding these free thiols were not identified but the data suggested that the long range disulphide bond (Cys^5-Cys^{435}) in the β_3 subunit be altered during activation. From a physiological point of view, a possible mediator of such events may be the

enzyme protein disulfide isomerase (PDI). PDI is a ubiquitous thiol-modifying enzyme present in all cells. It is probable therefore, that if platelet PDI is involved in disulphide bond rearrangement within the platelet integrin $\alpha_{IIb}\beta_3$, that an inhibitor of this enzyme would also inhibit receptor mediated events. One such inhibitor is bacitracin and its effects on various platelet functions are examined in the next chapter.

CHAPTER 4

A potential role for platelet PDI in the modulation of the function of $\alpha_{IIb}\beta_3$

4.1. INTRODUCTION.

It is established that treatment of platelets with the reducing agent DTT results in the activation of the $\alpha_{IIb}\beta_3$ integrin. This suggests that a corresponding physiological rearrangement of disulphide bonds within the receptor allow it to convert from a resting, inactive conformation to an active conformation that is competent to bind soluble fibrinogen and other ligands. From a physiological viewpoint, a mediator of such events may be a thiol isomerase. It has been reported that enzymes that catalyse disulphide exchanges, such as protein disulfide isomerase (PDI) are present on the cell surface of various tissues and involved in various aspects of cell surface biology. PDI associates with human corneal stroma cells (Ahmad and Church 1991), is involved in multimerisation of proteins at the surface of the thyroid (Berndorfer et al. 1996), (Couet et al. 1996) and catalyses disulphide reductions at the plasma membrane of Chinese hamster ovary cells (Mandel et al. 1993). I therefore explored the hypothesis that PDI could modify integrin function on the surface of platelets.

PDI has a primary role in the correct formation of disulphide bonds in nascent proteins in the endoplasmic reticulum. It is one of the most abundant residents of this organelle and is so named because it catalyses thiol:disulphide interchange reactions which can lead to the "shuffling" or isomerisation of intramolecular disulphide bonds. It is believed to accelerate the folding of proteins by catalysing this interchange reaction, a rate-limiting step during protein folding (Freedman 1989). It exhibits a broad specificity and has been used to catalyse the *in vitro* folding of a variety of proteins. Its primary structure is highly conserved between species and is approximately 0.4% of total cellular protein (Hillson et al. 1984).

PDI is very widely distributed and has been detected in many tissues. It was first isolated and identified as a microsomal enzyme from rat liver homogenate, capable of accelerating the conversion of reduced bovine pancreatic RNase to its enzymatically active form (Goldberger 1964). The sequence of cloned rat liver PDI cDNA predicted a protein with two distinct regions homologous with *Escherichia coli* thioredoxin, a known co-factor in oxidation-reduction reactions (Edman et al. 1985). Molecular cloning of the beta subunit of human prolyl-4-hydroxylase, from a hepatoma λgt11 cDNA expression library suggested that this subunit and PDI were products of the same gene. A comparison of the cDNA sequence obtained with that of PDI from rat liver revealed an 84% homology at the nucleotide level and a 94% homology at amino acid level (Pihlajaniemi et al. 1987). PDI was also identified as a human cellular thyroid hormone binding protein, present in the endoplasmic reticulum. A 98% sequence homology to the beta subunit of prolyl-4-hydroxylase and 85% sequence homology to rat PDI were found (Cheng et al. 1987). The microsomal triglyceride-transfer protein, which catalyses the transport of triglyceride and cholesteryl ester between membranes, is a complex composed of two proteins having apparent molecular weights of 58,000 and 88,000 Da. The 58,000 molecular mass component of this protein has been identified as protein disulfide isomerase. A 1:1 ratio of PDI to the 88,000 molecular component was found forming a stable tightly associated protein complex (Wetterau et al. 1991). Human liver endoplasmic reticulum p58 was characterised as a PDI isoform (Bourdi et al. 1995). In human placental tissue the protein previously identified as PLCα was shown to be an isoform of PDI (Charnock-Jones et al. 1996). A 57kDa protein from human spleen was identified as a substrate for the tyrosine kinase Lyn and as a protein related to PDI (Donella-Deana et al. 1996). It can be concluded that PDI has a wide

distribution and has many functions in addition to its recognised function in the folding of nascent proteins in the endoplasmic reticulum. In addition it often forms functional complexes with other proteins.

The major features of PDI are conserved across eukaryotes. PDI is composed of approximately 500 amino acid residues. It contains a number of regions of sequence showing internal repetition or homology to other known proteins suggesting that PDI is a modular protein. Native mammalian PDI is a homodimer (Freedman et al. 1994). The protein is composed of domains a, e, b, b' a' and c as shown in figure 4.0

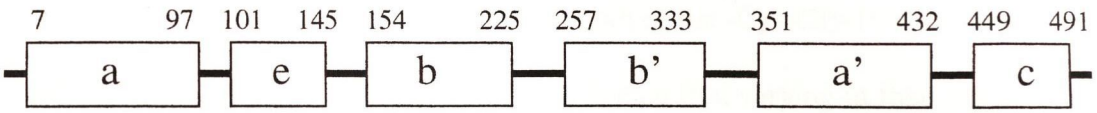


Figure 4.0. The inferred domain structure of human protein disulfide isomerase. The a- and a'- domains are thioredoxin like domains, e is a region with homology to oestrogen receptors, encoded by a single exon, b and b' represent an internal duplication, and c is a highly acidic putative Ca^{2+}-binding region. Open boxes represent inferred domains, the residue numbers indicating the minimum domain boundaries. Linking bars indicate ambiguous or interdomain regions. Modified from Freedman R.B. (Freedman et al. 1994).

Comparison of regions a and a' with *E. coli* thioredoxin revealed a considerable sequence homology. There is a 37% identity between the first 1-100 residues of PDI and residues 330-440 of thioredoxin. Residues 150-245 show 55% identity with thioredoxin's residues 250-345. Each of these regions contains the presumed active site sequence: a vicinal dithiol/disulphide couple formed by two cysteine residues in the

sequence Trp-Cys-Gly-His-Cys-Lys. Sequencing analysis of PDI cDNAs have shown that the active sites are highly conserved through evolution (Edman et al. 1985).

Model-building studies show that the thioredoxin like domains of PDI can fold in a similar manner to that of thioredoxin. These domains can function as an independent oxidoreductase with an interconvertible dithiol/disulfide group. Since PDI contains two such domains and is a homodimer in solution, this implies there are four active groups per PDI molecule (Freedman et al. 1988).

Both catalytic sites have been shown to act independently of one another. Site-directed mutagenesis was used to determine if one or both of the -Cys-Gly-His-Cys- sites acted as catalytic sites. Three expression plasmids of human PDI varying in their signal sequences were constructed and expressed in *E. Coli.* In plasmid number one the first Cys of the N-terminal site was mutated to a serine, in plasmid number two the first Cys of the C-terminal was mutated to a serine and in the third plasmid the first Cys of both sites were mutated to a serine. The PDI activity of plasmids one and two was found to be 50% of wild type PDI while that of plasmid three had no activity. This implies that the two -Cys-Gly-His-Cys- sites of PDI represent the catalytic sites for isomerase activity and that each acts independently of one another (Vuori et al. 1992).

Different isoforms of PDI are associated with different tissues. A platelet PDI has been identified and a PDI activity was released from platelets activated with a calcium ionophore. It was localised to the sub membranous canalicular channels of platelets. Further characterisation of this protein in platelets revealed that it has a similar apparent

mass, pI and N-terminal sequence to other human PDIs (Chen et al. 1995). There is evidence that thrombospondin I, a large multifunctional protein released from activated platelets and which functions as an extracellular matrix protein, is a substrate for platelet PDI. It has been suggested that there is a role for intrareceptor disulphide bond formation in the collagen receptor $\alpha_2\beta_1$ and changes the affinity of the receptor for its ligand (Gofer-Dadosh et al. 1997). It is possible, therefore, that a thiol isomerase may be involved in conformational changes of integrin receptors allowing them to bind their respective ligands. The evidence suggests, therefore, that a PDI exists in platelets and that various platelet proteins may be modified by a thiol isomerase-like activity, such as thrombospondin, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$.

If a thiol isomerase is involved in disulphide bond rearrangement within integrin receptors, an inhibitor of this enzyme would be expected to inhibit integrin mediated events. A known inhibitor of PDI is the antibiotic bacitracin. Bacitracin polypeptides were first identified as a strain from *Bacillus licheniformis* (Johnson 1945). The commercial form of bacitracin consists of a mixture of more than 22 different polypeptides (Tsuji and Robertson 1975). The isoforms bacitracin A and B account for most of the antibiotic activity and amount to about 60% of the mixture by weight (Medina 1993). Bacitracin partially blocked the arrest in proliferation of HT1080 fibrosarcoma cells associated with contact inhibition suggesting that PDI have a role to play in the events mediating contact inhibition (Clive and Greene 1994). The inhibitor was used in other studies to imply a role for PDI in the shedding of human thyrotropin receptor ectodomain (Couet et al. 1996). Bacitracin and antibodies against PDI inhibited a reductive function of the plasma membrane (Mandel et al. 1993). It also selectively

inhibited β_1 and β_7 integrin mediated lymphocyte adhesion (Mou et al. 1998). Thus, there is strong evidence to support bacitracin as an inhibitor of PDI. To examine the potential role PDI may have in the conformational changes of $\alpha_{IIb}\beta_3$ the effects of bacitracin on various platelet functions were examined in experiments reported in this chapter. It is reasonable to suggest that if an inhibitor of PDI inhibits integrin function, then PDI must play a direct role in integrin activation.

4.2. METHODS

4.2.1. Detection of PDI in platelets by immunoblot analysis.

Platelet lysates were prepared as outlined in section 2.2.1.4. Purified PDI (2µg) was resuspended in 1X sample buffer (section 2.2.3.2). Samples were prepared for electrophoresis and run out on a 7.5% SDS-PAGE gel. The separated proteins were transferred to PVDF membrane as described in section 2.2.3. Immuno detection was carried out as outlined in section 2.2.3.4. The primary antibody used was a 1:1,000 rabbit polyclonal anti-PDI antibody (Stressgen Corporation) and the secondary was a 1:5,000 dilution of donkey anti-rabbit antibody conjugated to horseradish peroxidase (HRP). Membranes were developed using an ECL kit.

4.2.2. Detection of PDI on the surface of platelets using flow cytometry.

A 100µl aliquot of citrated PRP was centrifuged at 20,000 × g for 10 seconds. The supernatant was removed and the platelet pellet was resuspended in 100µl of PBS or PBS containing 0.1% Triton X-100 and allowed to sit on ice for 20 minutes. 1ml of PBS was added and sample was centrifuged at 20,000 × g for 10 seconds. The supernatant was removed and the platelets were resuspended in 50µl 1:500 dilution of a rabbit anti-human PDI antibody, (a gift from Dr. Eric Quéméneur, Département d'Ingéniérie et d'Etudes des Protéines, Centre d'Études Saclay, Gif-sur-Yvette, France) for 30 minutes at room temperature. 1ml of PBS was added and samples were centrifuged at 10,000 × g for 3 minutes. The supernatant was removed and the platelets were resuspended in 50µl of 1:1,000 dilution of polyclonal anti-rabbit IgG-FITC for 30

minutes at room temperature. 1ml of PBS was added and sample was centrifuged at 10,000 × g for 3 minutes. The supernatant was removed and the platelets were resuspended in 1ml of 1% formaldehyde in PBS. A control sample was prepared in which the primary antibody was omitted to assess the autofluorescence of the assay. Samples were analysed on the FACScan flow cytometer.

4.2.3. RT-PCR of platelet cDNA using PDI primers.

The first 33 amino-acid residues of protein disulphide isomerase isolated from human platelets are similar to the sequence for the β-subunit of prolyl-4-hydroxylase, also known as PDI (Chen et al. 1995). Primer sequence design was based on the sequence obtained for PDI from a λgt blood library (Tasanen 1988). Primers were designed so that they spanned the total length of the protein, especially in highly conserved regions, most notably the regions coding for the two catalytic sites of the enzyme (underlined below). At an amino acid level the primers designed are in Italics. The sequence was obtained from National Center of Biotechnolgy Infomation Entrez site: http://www.ncbi.nlm.nih.gov using accession code P07237.

N2→

MLRRALLCLA VAALVRADAP EEEDHV***LVLR*** ***KSNF***AEALAA HKYLLVEFYA PWCGHCKALA

PEYAKAAGKL KAEGSEIRLA KVDATEESDL AQQYGVRGYP TIKFFRNGDT ASPKEYTAGR

EADDIVNWLK KRTGPAATTL RDGAAAESLV ESSEVAVIGF FKDVESDSAK QFLQAAEAID

DIPFGITSNS DVFSKYQLDK DGVVLFKKFD EGRNNFEGEV TKENLLDFIK HNQLPLVIEF

TEQTAPKIFG GEIKTHILLF LPKSVSDYDG KLSNFKTAAE SFKGKILFIF IDSDHTDNQR

F2→

ILEFFGLKKE ECPAVRLITL EEEM***TKYKPE*** ***S***EELTAERIT EFCHRFLEGK IKPHLMSQER

PC2←

R11←

AGDWDKQP***VK*** ***VPVGKNF***EDV AFDEKKNVFV EFYAPW**CGHC** KQLAPIWDKL GE***TYKDHENI***

VIAKMDSTAN EVEAVKVHSF PTLKFFASAD RTVIDYNGER TLDGFKKFLE SGGQGAGDDD

DLEDLEEAEE PDMEEDDDQK AVKDEL

At a nucleotide level the primer sequences were as follows:

Primer	Sequence
PDI N2 (sense)	5'-GTCCTGGTGCTGCGGAAAAGCAAC-3'
PDI F2 (sense)	5'-ACCAAGTACAAGCCCGAATCG-3'
PDI pC2 (antisense)	5'-GTTCTTCCCAACAAGCACCTTGACA-3'
PDI R11 (antisense)	5'-GATGTTCTCATGGTCCTTGTACGT-3'

PCRs were performed in a final volume of 50µl with 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.25ng of each sense and antisense primers, 2 unit Taq DNA polymerase (Promega) and a 5µl volume of the reverse transcription reaction as outlined in section 2.2.6.2.

Primers were paired as follows:

Primer Pair	Expected size (bp)
N1, pC2	1127
F2, R11	291

The reaction cycles were as follows: denaturation at 94°C for 1 minute, annealing at 50° C for 1 minute and extension at 68 °C for 1.5 minutes for 40 cycles. Products were separated on a 1.2% agarose gel and visualised on an ultraviolet transilluminator. Bands were carefully cut out from the gel with clean scalpel blades and the PCR products were then prepared for sequencing.

4.2.4. Sequencing of PDI PCR Products.

The PCR products were prepared for sequencing as described in section 2.2.5.3.

4.2.5. Detection of isoforms of PDI in platelets by 2D Electrophoresis.

2D electrophoresis and transfer was performed as described in section 2.2.9. The PDI immunoblot was carried out as described in section 4.2.1.

4.2.6. Platelet aggregations in the presence of bacitracin.

PRP was prepared as outlined in section 2.2.1.1. Platelets were preincubated with bacitracin at a final concentration of 1.75mM, 3.5mM or 7mM for 5 minutes at 37°C. The platelet agonists thrombin (1U/ml), ADP (20μM) or U46619, a thromboxane mimetic (1μM) were added to the bacitracin treated platelets in a final volume of 500μl. Aggregations were monitored with stirring at a rate of 1,000 rpm for 4 minutes at 37°C in a BioData aggregometer as described in section 2.2.1.3.

4.2.7. The effects of bacitracin on fibrinogen binding to ADP activated platelets.

The binding assay was performed as described in section 2.2.2.1. The diluted PRP was incubated with bacitracin at a final concentration of 1.75mM, 3.5mM, or 7mM for 5 minutes before the addition of oregon green labelled fibrinogen and ADP (10μM). Binding reaction was for 20 minutes at room temperature and was stopped by the addition of 2mls buffer A (section 2.2.1.4.). Samples were read immediately on the FACScan flow cytometer.

4.2.8. The effects of bacitracin on the expression of ligand induced binding sites (LIBS) in $\alpha_{IIb}\beta_3$.

PRP was prepared as outlined in section 2.2.1.1. 1ml aliquots of a 1:1 dilution of PRP in PBS were incubated with and without 7mM bacitracin for 5 minutes at 37°C. 100µl aliquots of each of these treatments were then incubated with one of the following: PGE_1 (56nM), Integrilin (2µg/ml), EDTA (5mM) or ADP (20µM), respectively, for 30 minutes at 37°C. The assay was completed as described in section 2.2.2.2.

4.2.9. The effects of bacitracin on surface expression of platelet $\alpha_{IIb}\beta_3$ in resting and activated platelets.

PRP was prepared as outlined in section 2.2.1.1. 1ml aliquots of a 1:1 dilution of PRP in PBS were incubated with and without bacitracin at a final concentration of either 7mM or 14mM for 5 minutes at 37°C. 100µl aliquots of each of these treatments were incubated with and without ADP (20µM) for 3 minutes at 37°C. The assay was completed as described in section 2.2.2.4.

4.2.10. The effects of bacitracin on the binding of the RGD mimetic, ^{3}H- SC52012 to resting and activated platelets.

This assay was performed as described in section 2.2.5. Platelets were incubated with 7mM bacitracin for 5 minutes at 37°C before the addition of ADP and the tritiated compound.

4.2.11. The effects of bacitracin on the expression of P-selectin (CD62P) on the surface of activated platelets.

PRP was prepared as outlined in section 2.2.1.1. 1ml aliquots of a 1:1 dilution of PRP in PBS were incubated with and without bacitracin at a final concentration of either 7mM or 14mM for 5 minutes at 37°C. 100µl aliquots of each of these treatments were incubated with and without ADP (20µM) for 3 minutes at 37°C. The assay was completed as described in section 2.2.2.3.

4.2.12. The effects of bacitracin on tyrosine phosphorylation events in resting and activated platelets.

Gel filtered platelets were prepared as outlined in section 2.2.1.2. Platelets were incubated with 7mM and 14mM bacitracin for 5 minutes at 37° before activation with 1u/ml thrombin for 3 minutes in an aggregometer. Reactions were stopped by the addition of RIPA buffer (section 2.2.1.4.). Samples were prepared for SDS-PAGE and transfer to PVDF membrane as described in section 2.2.4. The membranes were detected with anti-phosphotyrosine antibodies as outlined in section 3.2.5.

4.2.13. Analysis of the surface expression of PDI and $\alpha_{IIb}\beta_3$ on resting and adhered platelets by confocal microscopy.

Gel filtered platelets were prepared as outlined in section 2.2.1.2. and layered onto slides coated with poly-L- lysine and fibrinogen. These were then stained for both $\alpha_{IIb}\beta_3$ and

PDI as described in section 2.2.8. The stained slides were examined for evidence of co-localisation of these two proteins.

4.3. RESULTS.

4.3.1. PDI is present in platelets as detected by immunoblot analysis

Immunoblot analysis of total platelet lysate with antibodies against PDI revealed the presence of a single band with an estimated size of 57kDa (Figure 4.1). This band coincided with purified PDI and the estimated size of the immuno-reactive bands corresponds to the predicted molecular mass of protein disulfide isomerase.

4.3.2. Detection of PDI in platelets using flow cytometry.

PDI was not detected on the surface of intact platelets as the level of fluorescence obtained was similar to that in platelets labelled with secondary antibody alone (Figure 4.2). When platelets were permeabilised with 0.1% Triton X-100, an increase in mean fluorescence indicated the presence of PDI in the platelet, which was not accessible on the platelet surface.

4.3.3. RT-PCR Analysis of Platelet PDI.

PCR products of the expected size were amplified from platelet cDNA using low stringency conditions (Figure 4.3). The PCR reactions were repeated a number of times using cDNA isolated from different donors. These products were excised from the agarose gels and sequenced.

4.3.4. Sequence analysis of platelet PDI-PCR products and the detection of platelet isoforms of PDI.

The sequence data obtained confirm that PDI in platelets contains the two highly conserved catalytic sites of other PDIs. Figure 4.4a shows the sequence data of a region spanning the second catalytic site, using primers F2 and R11. The alignment programme BLAST revealed 95-100% identity with the sequence of human prolyl-4-hydroxylase. The translation programme GRAIL showed that there was approximately 92% identity at an amino acid level when compared to the sequence for human proyly-4-hydroxylase. This suggests that perhaps an additional PDI sequence is present in the platelet in addition to the one identified by Chen *et al.* In an analysis of a 2D immunoblot of platelet lysate, using a polyclonal antibody against PDI two proteins were identified (Figure 4.4b).

4.3.5. Bacitracin inhibits platelet aggregation.

It was found that bacitracin inhibited platelet aggregation caused by the potent platelet agonist thrombin (0.1U/ml) in a dose dependent manner (Figure 4.5). Bacitracin at a final concentration of 7mM completely inhibited thrombin induced platelet aggregation while concentrations of 3mM and 1.75mM inhibited this aggregation by approximately 60% and 50% respectively. Bacitracin also completely inhibited U46619 (2μM) induced aggregation but at a lower concentration (1.75mM) (Figure 4.6). In separate studies in my laboratory, bacitracin also inhibited aggregation to collagen, ADP and epinephrine (data not shown).

4.3.6. Bacitracin inhibits fibrinogen binding to activated platelets.

Bacitracin dose dependently inhibited the binding of OgFg to ADP (10μM) activated platelets (Figure 4.7). In the presence of 7mM bacitracin, the level of fibrinogen binding obtained was similar to that in resting unactivated platelets. Bacitracin at concentrations of 3.5mM and 1.75mM inhibited fibrinogen binding by approximately 85% and 60% respectively. There was no difference in the binding of labelled fibrinogen to resting unactivated platelets in the presence or absence of bacitracin.

4.3.7. The effect of bacitracin on the expression of LIBS in platelet $\alpha_{IIb}\beta_3$.

Maximal expression of D3GP3 LIBS epitopes is obtained following a 30 minute incubation of platelets at 37°C in the presence of 5mM EDTA. D3GP3 sites can also be induced in platelets by incubation with RGD mimetics or by platelet agonists such as ADP. In resting, unactivated platelets treated with PGE_1 (0.56nM) a very low expression of LIBS was detected by the monoclonal antibody D3GP3 (Figure 4.8). Bacitracin had no effect on this level of expression. Ligand induced binding sites, exposed upon receptor engagement with Integrilin, were also unaffected by bacitracin. In contrast, bacitracin significantly reduced LIBS exposure in response to EDTA (5mM) expression to a level equivalent to that obtained when platelets were treated with Integrilin. Bacitracin also caused a reduction in the level of LIBS expression in response to ADP (20μM) and although this failed to achieve significance, the trend towards a reduction in expression is implied.

4.3.8. Bacitracin affects the level of activation induced level of $\alpha_{IIb}\beta_3$ complex on the surface of resting and activated platelets.

Activation of intact platelets with ADP (20μM) or TRAP (20μM) resulted in an increased number of $\alpha_{IIb}\beta_3$ complexes on the platelet surface as indicated by the increase in reactivity to the complex specific antibody used in this assay (Figure 4.9). The antibody used was CD41a, which recognises the α_{IIb} subunit only when it is complexed to the β_3 subunit. The source of this increase in $\alpha_{IIb}\beta_3$ is presumed to be from the platelet α-granules and the stimulus for its movement to the platelet surface is probably via integrin mediated outside-in signalling. When platelets were treated with either 7mM or 14mM bacitracin, this activation induced increase in CD41a reactivity was not observed and remained at the same level observed in unactivated platelets. Bacitracin had no effect on basal levels of $\alpha_{IIb}\beta_3$ complex on the surface of resting platelets.

4.3.9. The effect of bacitracin on the binding of ^{3}H[SC52012] to resting and activated platelets.

In a series of binding assays the effect of bacitracin was examined on the binding of ^{3}H-[SC52012] to resting and ADP activated platelets (Figure 4.10). The binding parameters are summarised in Table 4.1. There was no significant difference between the number of binding sites (B_{max}) per platelet for resting and ADP treated platelets, with values of 43,472±11,021 and 34,632±7,433 respectively. With bacitracin treatment, however, the number of sites apparently increased to values of 71,077±14,438 per resting platelet and 58,221±6,986 per ADP activated platelet, implying that bacitracin facilitated the binding of the mimetic by exposing new binding sites on the surface of $\alpha_{IIb}\beta_3$. Statistical

analysis revealed that the difference between untreated and bacitracin treated control platelets was not quite significant, $p = 0.053$. In contrast, the effect of bacitracin on B_{max} in ADP treated platelets was significant $p < 0.005$. The affinity (K_d) of the ligand in the resting platelet was found to be 67.1±13.4 nM and in the activated platelet this increased to 23.9±8.7 nM. Bacitracin had no significant effect on the affinity of the receptor for the ligand, resulting in K_d values of 78.1±28 nM for resting platelet and 42.6±7.0 nM for ADP activated platelets.

4.3.10. Bacitracin has no effect on platelet α-granule secretion.

When platelets are activated the contents of two types of granules are secreted and redistributed from the interior to the exterior of the platelet. P-selectin, also known as GMP-140, is found within the alpha granules of resting, unactivated platelets. Upon platelet activation, the granules fuse with the platelet membrane and P-selectin is then expressed on the platelet surface. Analysis by flow cytometry revealed that P-selectin is expressed on the platelet surface when platelets are activated with ADP (20μM) and TRAP (20μM). Bacitracin had no effect on P-selectin expression on the platelet surface in either resting or activated platelets (Figure 4.11). Thus, the effects of bacitracin appear to be confined to conformational effects on the platelet integrin $\alpha_{IIb}\beta_3$ as it does not have any effect on the activation of platelet secretory events as measured by P-selectin surface expression.

4.3.11. The effects of bacitracin on tyrosine phosphorylation events in the platelet.

Upon platelet activation a considerable number of proteins become phosphorylated on tyrosine residues as indicated in lane 4 when compared to resting, unactivated, platelets in lane 1. It would appear that incubation of both resting (lanes 2 and 3) and activated platelets (lanes 5 and 6) with bacitracin inhibited all of these events (Figure 4.12).

4.3.12. PDI does not co-localise with $\alpha_{IIb}\beta_3$ in platelets.

In indirect immnuofluorescence studies using confocal microscopy it was found that, regardless of the activation state of the platelet, PDI did not co-localise with $\alpha_{IIb}\beta_3$ (Figure 4.13). Activated platelets, which spread on a fibrinogen-coated surface, or resting platelets, immobilised on poly-L-lysine, stain for both $\alpha_{IIb}\beta_3$ and for PDI. However, these proteins do not co-localise at any stage as shown in the overlay images (Figure 3D, H) where colocalisation would be detected by the appearance of yellow pixels.

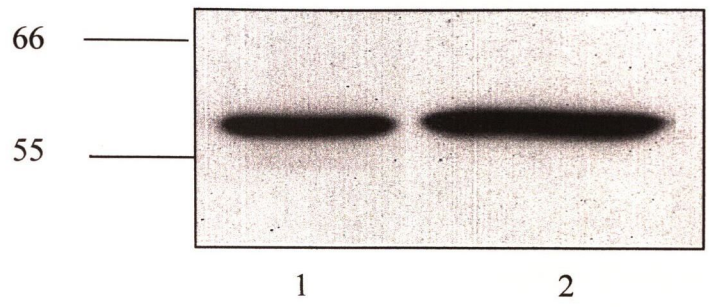


Figure 4.1. Immunoblot analysis of platelet PDI.

2μg of purified bovine PDI (lane 1) and 20μg of total platelet lysate (lane 2) were separated on a 7.5% SDS gel and proteins were transferred to a PVDF membrane. Membrane was probed with a polyclonal anti-PDI antibody (Stressgen Biotech. Corp.). A single band at approximately 57kDa was present in both lanes, the estimated molecular mass for PDI.

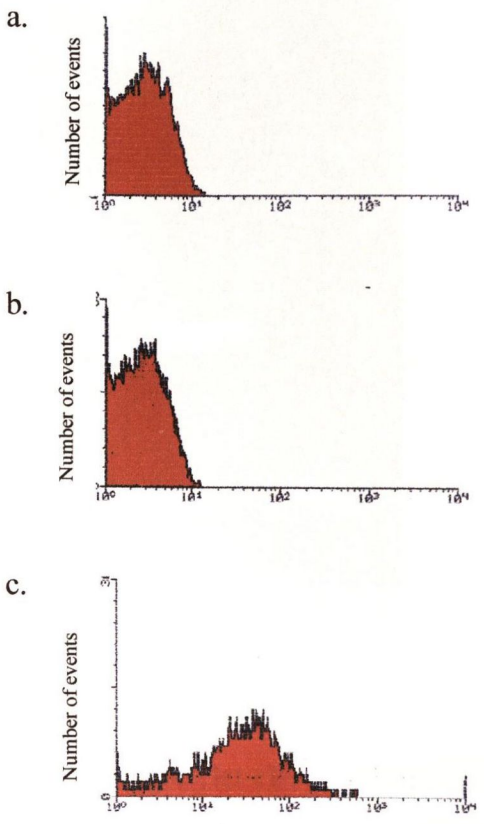


Figure 4.2. Analysis of platelet PDI by flow cytometry analysis.
Shown here are flow cytometric histograms with mean fluorescence on the x-axis and number of events of the y-axis. (a) Binding of the secondary antibody (goat anti-mouse Ig FITC) to platelets, indicating the level of autofluorescence of the samples. (b) Intact platelets were incubated with a rabbit anti-human PDI antibody. PDI was not detectable on the platelet surface, as the mean fluorescence was similar to that obtained in the autofluorscent sample in (a). (c) Permeabilised platelets were incubated with the same antibody as in (b). An increase in mean fluorescence shows the presence of PDI in the platelet. These data represent one of n = 3 independent experiments.

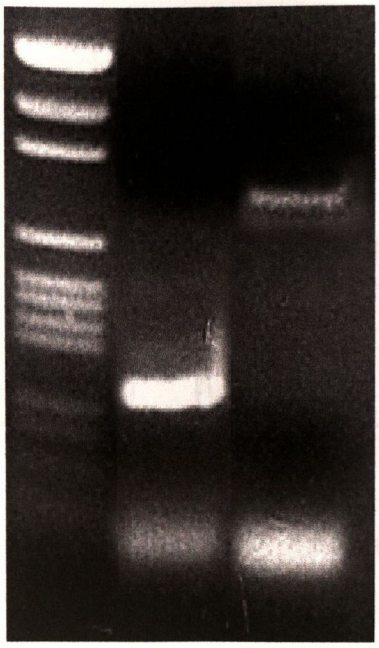


Figure 4.3. PCR products of platelet cDNA using PDI primers.

Shown are the PCR products obtained from platelet cDNA using primers based on PDI sequence. Lane 1 shows pGEM markers with values of 2645, 1605, 1198, 676, 517, 460 and 396 bp. Lane 2 shows the PCR product obtained using the primer pair F2, R11 with an estimated size of 291bp. Lane 3 shows the PCR product obtained using the primer pair N2, pC2 with an estimated size of 1052bp. The lower band in lanes 2 and 3 is the primer dimer of each sample. These data represent one of n = 7 independent experiments.

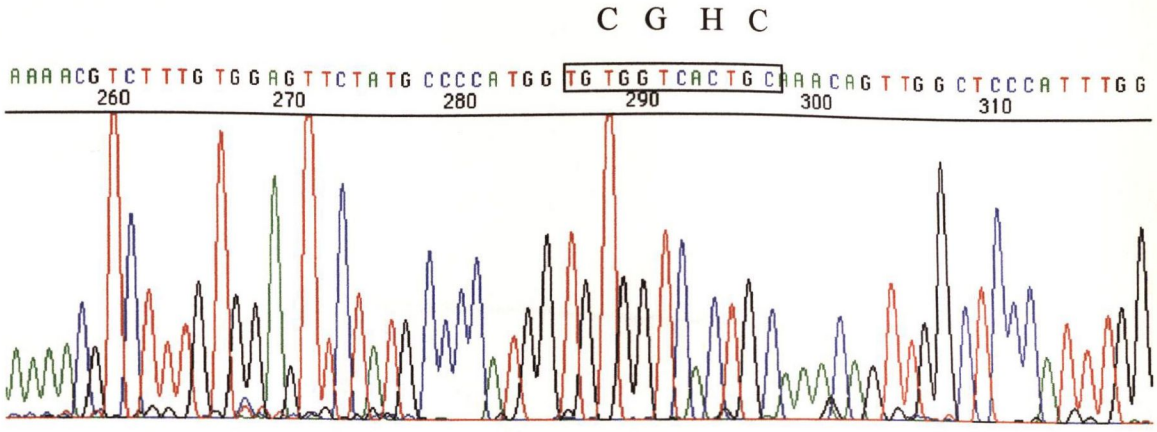


Figure 4.4a. Sequence data of PDI PCR product.

Shown is a section of the sequence histogram obtained from the PDI PCR product using primers F2 and R11. The sequence coding for the second catalytic site is boxed with the corresponding single code amino acids above.

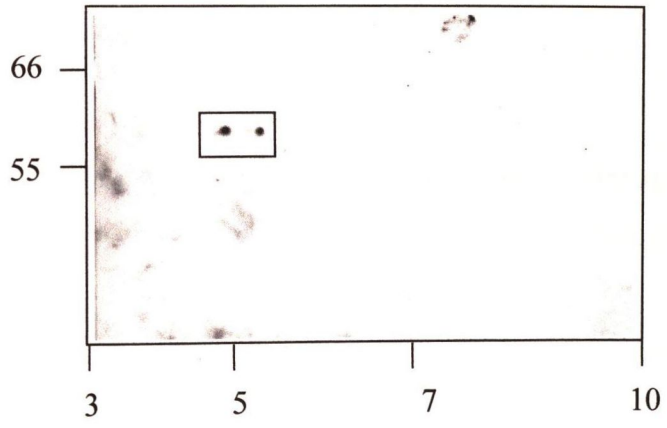


Figure 4.4b. Identification of isoforms of PDI in platelets.

80μg of total platelet lysate was loaded onto an IPG strips (non-linear pH range 3-10, shown on the bottom of the gel) in the first dimension. For the second dimension a vertical 7.5% SDS-PAGE gel was used. Separated proteins were transferred to a PVDF membrane. Membrane was probed with a polyclonal anti-PDI antibody (Stressgen Biotech. Corp.). Two spots (boxed) at approximately 57kDa were visualised, at a molecular mass estimated for PDI.

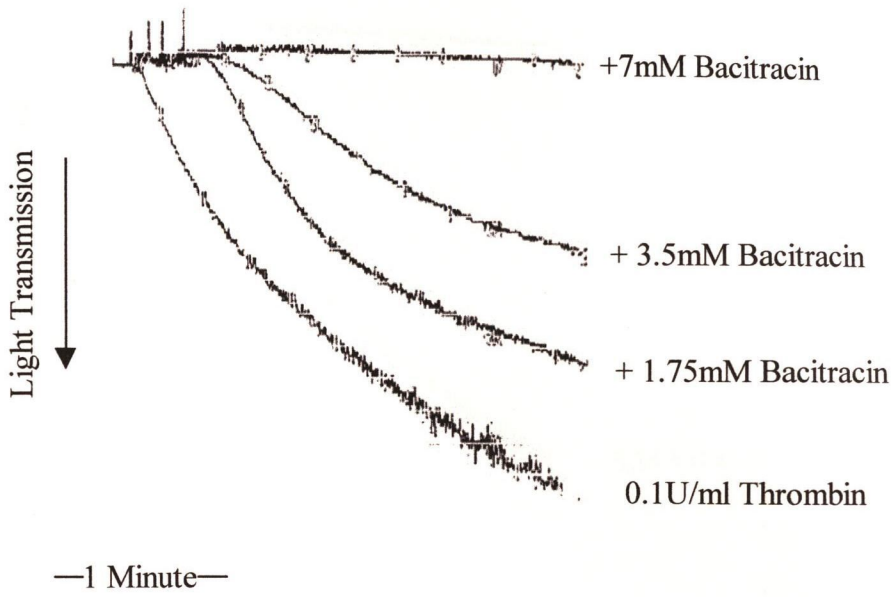


Figure 4.5. Bacitracin inhibits thrombin induced platelet aggregation.
Platelets activated by the potent agonist thrombin (0.1U/ml) aggregate to an extent of approximately 80% as indicated by the increase in light transmission. Preincubation of platelets with bacitracin, at the concentrations indicated, for 5 minutes at 37°C results in inhibition of aggregation in a dose dependent manner. Maximal inhibition occurs with 7mM bacitracin. These data represent one of n = 5 experiments.

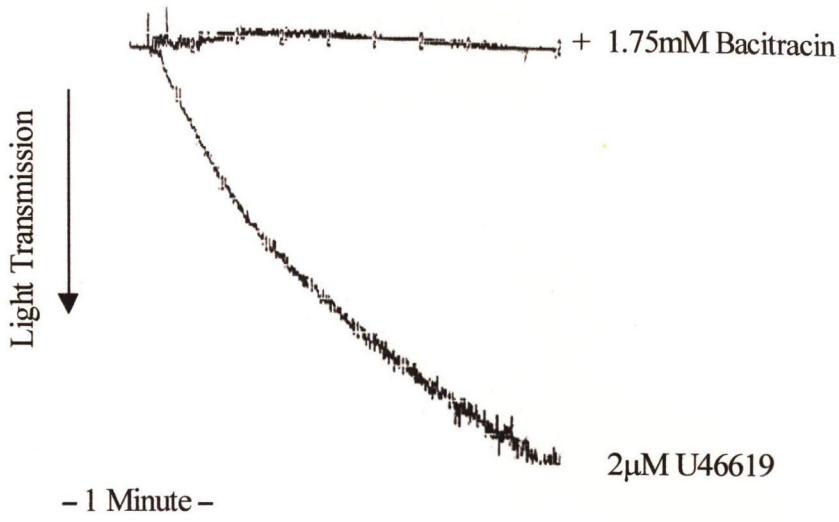


Figure 4.6. Bacitracin inhibits U46619 induced platelet aggregation.

Platelets activated by the thromboxane mimetic U46619 (2μM) aggregated to an extent of approximately 70% as indicated by an increase in light transmission. Preincubation of platelets with 1.75mM bacitracin for 5 minutes at 37°C completely inhibited this U46619 induced aggregation. These data represent one of n = 3 independent experiments.

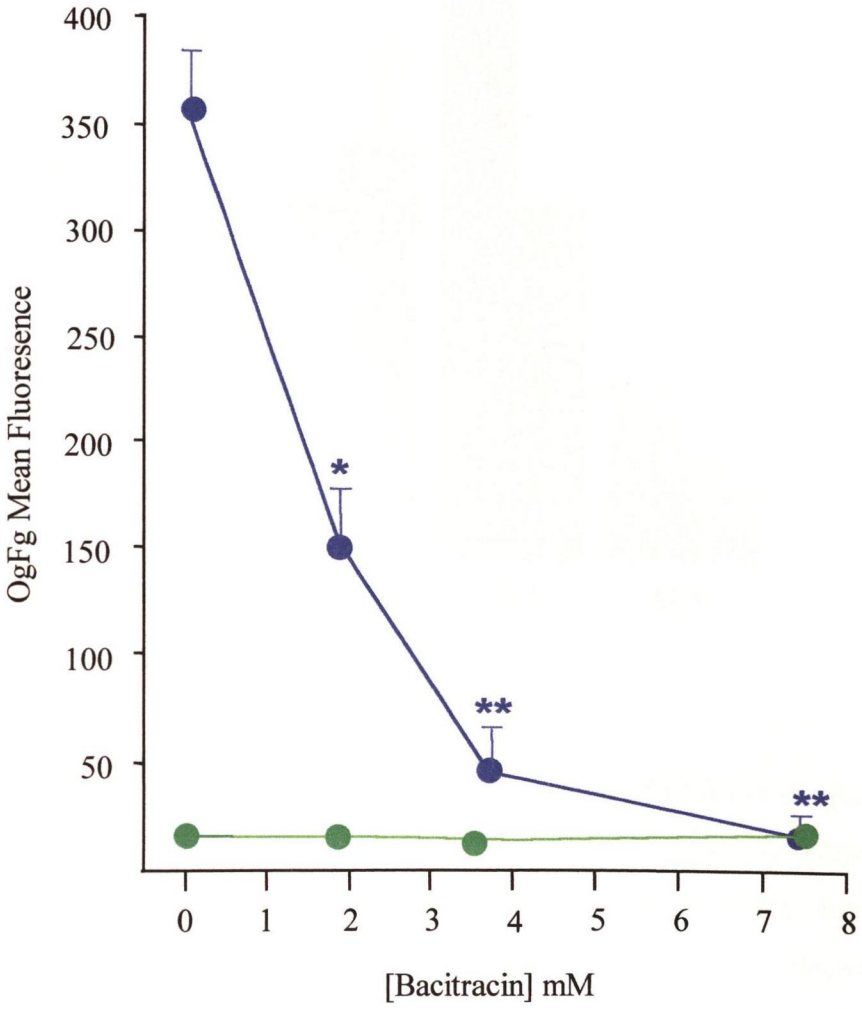


Figure 4.7. Bacitracin dose dependently inhibits fibrinogen binding.

Bacitracin at concentrations of 1.75mM, 3.5mM and 7mM, dose dependently inhibited oregon green labelled fibrinogen (OgFg) binding to ADP (10μM, ●) activated platelets. Bacitracin had no effect on the binding of OgFg to resting unactivated platelets (●). Data shown are the mean ± S.D. of n = 4 experiments.

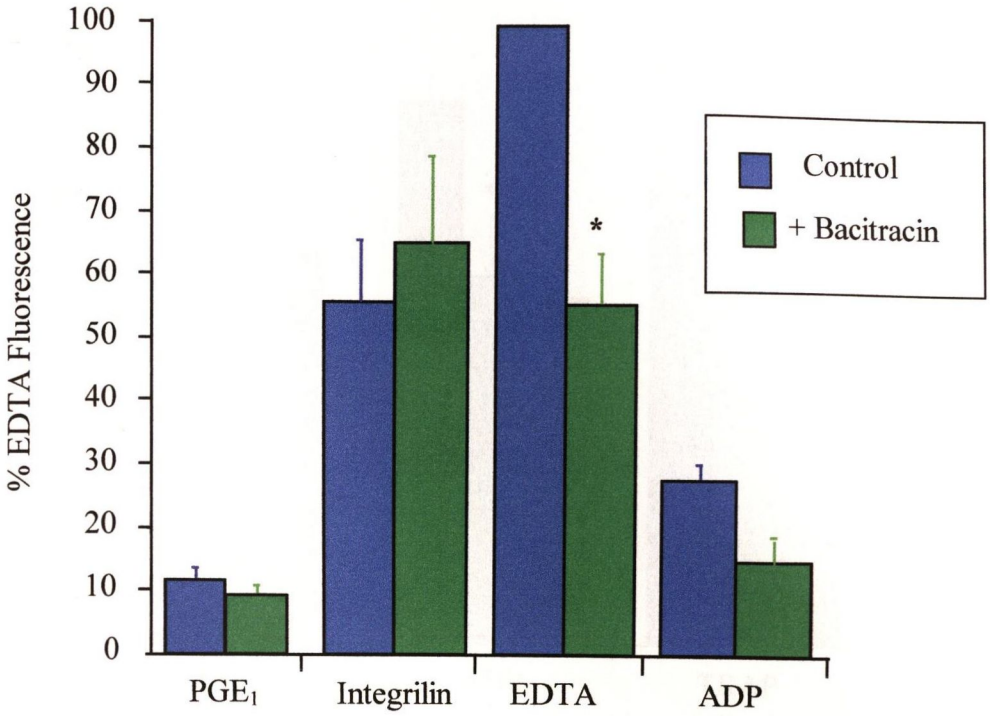


Figure 4.8. The effects of bacitracin on the expression of LIBS in $\alpha_{IIb}\beta_3$.
Treatment of platelets with PGE_1 results in the formation of a quiescent platelet in which expression of LIBS was suppressed and bacitracin (7mM) had no effect. Engagement of the receptor by Integrilin (2μg/ml) resulted in the expression of LIBS. Bacitracin had no significant effect on this expression. In the presence of 5mM EDTA, maximal expression of LIBS was obtained. Here bacitracin significantly reduced this level of expression of LIBS and was comparable to that obtained in the presence of Integrilin. ADP (20μM) activation of platelets resulted in LIBS expression but not to the same extent as that obtained with either Integrilin or EDTA. Bacitracin had no effect on this ADP induced expression of LIBS. The data shown here is presented as a % of maximal expression of LIBS obtained with 5mM EDTA and are the mean ± S.D. of n = 5 experiments.

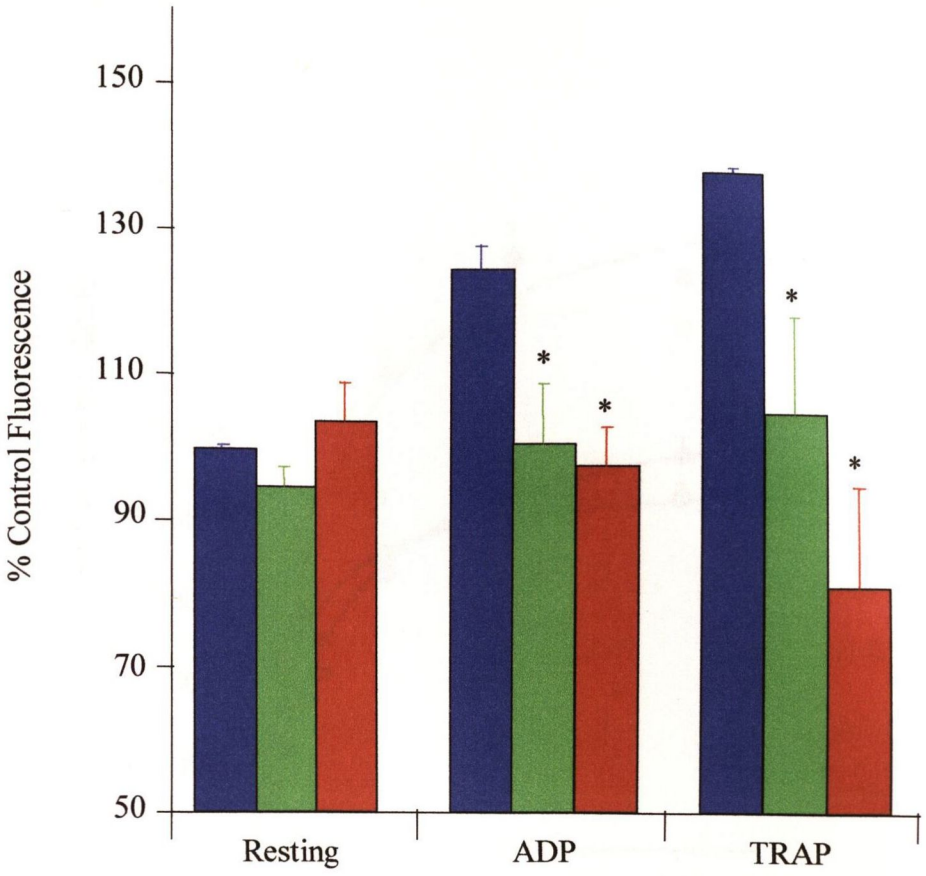


Figure 4.9. The effects of bacitracin on the expression of $\alpha_{IIb}\beta_3$ on the surface of resting and activated platelets.

Levels of $\alpha_{IIb}\beta_3$ on the surface of resting and activated platelets are detected by the binding of the antibody anti-CD41a to platelets by flow cytometry analysis. In resting platelets $\alpha_{IIb}\beta_3$ is detected on the platelet surface (■) and this level of expression increases upon activation with the agonists ADP (20μM) and TRAP (20μM). Incubation of platelets with bacitracin at concentrations of 7mM (■) and 14mM (■) had no effect on levels of $\alpha_{IIb}\beta_3$ in the resting platelet. However, bacitracin prevented this agonist-induced elevation of receptor expression on the surface of the activated platelet. The data shown here are the mean ± SD of n = 3 experiments.

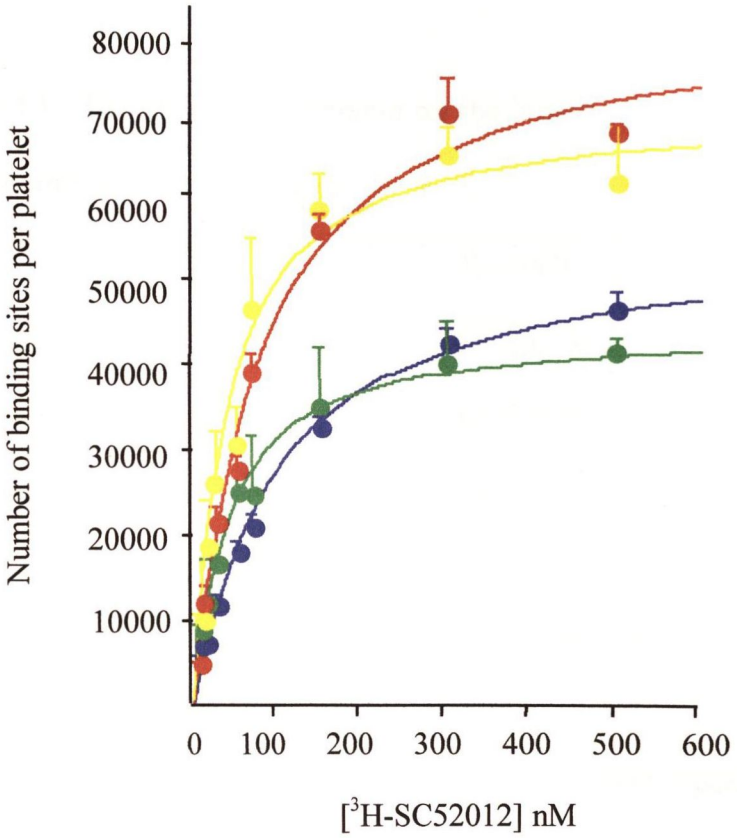


Figure 4.10. The effects of bacitracin on the binding of the RGD mimetic SC52012 to resting and activated platelets.

^{3}H-SC52012 bound to resting (●) and activated platelets (●) in a saturable manner. In the presence of 7mM bacitracin binding of this tritiated RGD mimetic to resting (●) and (●) activated platelets also occurred in a saturable manner. The data shown here are the mean ± S.D. of n = 4 independent experiments.

Table 4.1. The effects of bacitracin on the binding parameters of ^{3}H-SC52012 to resting and activated platelets.

	K_d (nM)	B_{max}
Resting	67.1±13.4	43472±11021
20μM ADP	23.9±8.7	34632±7433
7mM Bacitracin	78.1±7.7	71077±14438
7mM Bacitracin + 20μM ADP	42.6±7.0	58221±6986

The data shown are the mean ± sem of n = 4 independent experiments. Statistical analysis was determined using a Student t test (two tail).

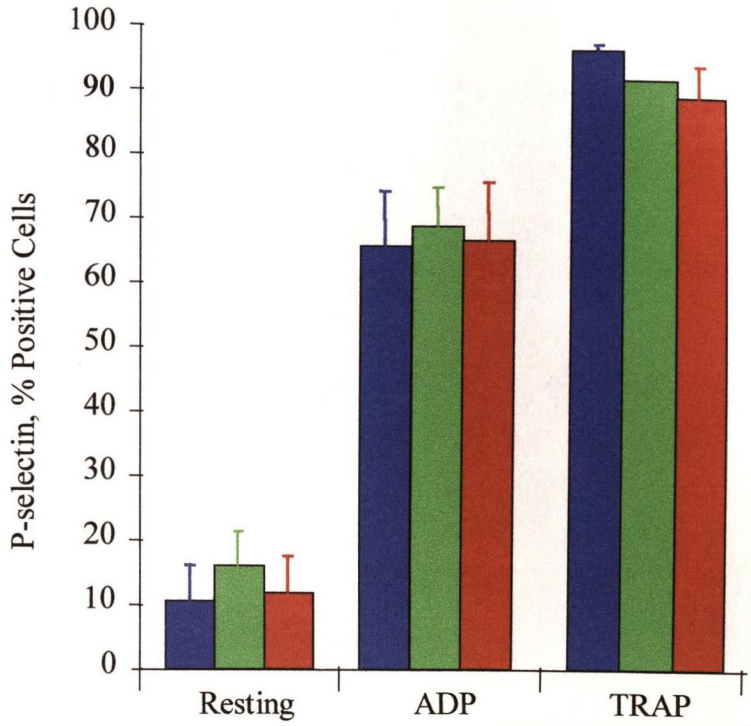


Figure 4.11. The effects of bacitracin on the expression of P-selectin on the surface of resting and activated platelets.

Levels of P-selectin on the surface of resting and activated platelets were detected by the binding of the antibody CD62P to platelets by flow cytometry analysis. In resting platelets p-selectin is detected on the platelet surface (●) and this level of expression increased dramatically upon activation with the agonists ADP and TRAP. Incubation of platelets with bacitracin at concentrations of 7mM (●) and 14mM (●) had no effect on these levels in either resting or activated platelets. The data shown here are the mean ± SD of n = 3 experiments.

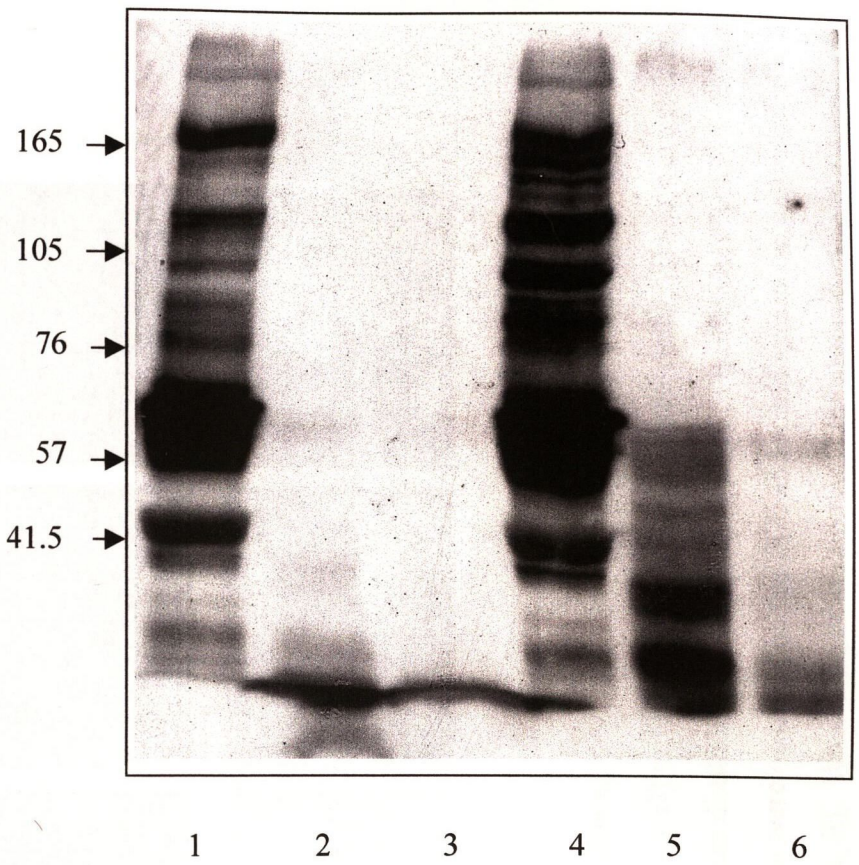


Figure 4.12. The effects of bacitracin on tyrosine phosphorylation events in platelet aggregation.

Gel filtered platelets were treated with buffer (lane 1), 7mM bacitracin (lane2), 14mM bacitracin (lane 3), 0.1U/ml thrombin (lane 4), 7mM bacitracin + 0.1U/ml thrombin (lane 5) and 14mM bacitracin + 0.1U/ml thrombin (lane 6). Platelet lysates were separated on a 7.5% SDS-PAGE gel and tyrosine phosphorylation events were detected by immunoblot analysis. Molecular mass markers are indicated in kDa on the left of the figure. These data represents one of 2 independent experiments.

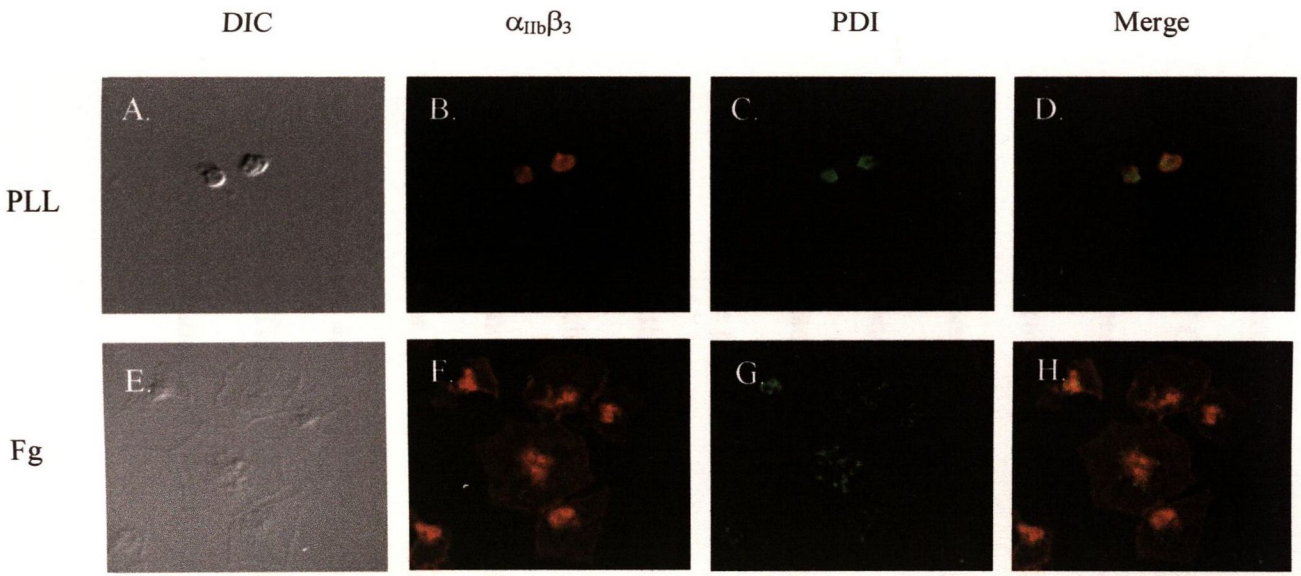


Figure 4.13. PDI does not co-localise with $a_{IIb}b_3$ in adhered platelets.

Gel filtered platelets were allowed to adhere to poly-L-lysine (PLL, A-D) or fibrinogen (Fg, E-H) coated slides. These were stained for $\alpha_{IIb}\beta_3$, using a monoclonal complex specific antibody (CD41a; B, F), and for protein disulfide isomerase (anti-PDI; C, G) using a polyclonal antibody with the appropriately labelled Alexa secondary antibodies. Resting platelets were observed on the PLL slides and spread or 'activated' platelets on the Fg coated slides using differential interference contrast (DIC) mode (A, E). When images were merged the absence of yellow pixels indicates that $\alpha_{IIb}\beta_3$ did not co-localise with protein disulfide isomerase (D, H). Images were made on a Zeiss Axioplan 2 confocal microscope with a 40X oil-immersion lens (1.4n/a). The data shown represents one of n=3 independent experiments.

4.4. DISCUSSION.

PDI is present in platelets as confirmed by immunoblot analysis using a polyclonal antibody. This result is substantiated by the sequencing data obtained from PCR products of platelet cDNA using primers specifically designed against PDI. Interestingly, the results obtained from the flow cytometry experiments do not support the observation of Chen *et al* that platelet PDI is on the surface of the platelet, as it was not possible to detect PDI on intact platelets. In these experiments, PDI appeared only upon gentle permeabilisation of the platelet by Triton X-100. In the studies of Essex *et al*, showing PDI present on the external surface of platelets, fluorescent microscopy at a magnification of only ×660 was employed. The conclusion that PDI is surface bound was due to the presence of a halo of fluorescence at the perimeter of the platelet (Essex et al. 1995). Since platelets are only in the order of 2-4 microns in diameter and because of the intricacy of the platelet surface, it is difficult to be certain of the exact position of a protein under such experimental conditions. The data obtained in my study suggest that PDI is in fact not on the surface. It may be postulated therefore that PDI resides in the open cannicular system just below the membrane of the platelet. It is released upon platelet activation, as already described (Chen et al. 1995) and only then can PDI act on target substrates. Therefore, it is expected that bacitracin will inhibit events associated with platelet activation and the results obtained from these inhibitory studies are outlined below.

Bacitracin is an antibiotic that also inhibits PDI. It has been used in many studies to implicate thiol isomerase activity (Clive and Greene 1994) (Couet et al. 1996) (Mandel

et al. 1993). In chapter 3, I showed that thiol groups are important for the activation of the platelet integrin and hypothesised that PDI may be the physiological mediator. To support this it is necessary to show that an inhibitor of PDI can inhibit platelet function in general and $\alpha_{IIb}\beta_3$ activation in particular. I demonstrated that bacitracin, at concentrations that are known to inhibit PDI activity, inhibited platelet aggregation to a variety of agonists in a dose dependent manner. This suggests the receptor was unable to undergo conformational changes as a result of platelet activation. It could, therefore, not bind the fibrinogen necessary to support platelet aggregation. This result was confirmed when bacitracin also dose dependently inhibited binding of soluble fibrinogen to activated platelets.

Interestingly, bacitracin, at a concentration of 7mM had no significant effect on ligand, in this instance Integrilin, induced expression of LIBS in $\alpha_{IIb}\beta_3$ as detected by the binding of the antibody D3GP3. It did, however, reduce significantly the level of EDTA induced LIBS expression, which corresponds to a maximal expression of LIBS. $\alpha_{IIb}\beta_3$ is a calcium dependent heterodimer and upon ligand binding to the receptor calcium is hypothesised to be released from its binding sites. Chelation of this cation by EDTA, therefore, mimics ligand binding and results in the exposure of LIBS and the concomitant conformational changes of the receptor. It is noted however, that the observed EDTA-induced conformational changes in $\alpha_{IIb}\beta_3$ requires a 30 minute incubation at 37°C. This implies that simple chelation of calcium or other divalent ions do not cause such conformational changes, as these effects would be observed immediately. The implications are that a secondary process is occurring, although I have no theories yet as to what this might be. An interesting parallel exists with another

platelet glycoprotein thrombospondin. This is a major glycoprotein of platelet α-granules which is released upon platelet activation and undergoes similar conformational changes in the presence of EDTA (Sun et al. 1992). It forms covalent complexes with itself and with other proteins in reactions that require isomerisation of disulphide bonds in the absence of calcium. It appears that the EDTA stabilised conformation of thrombospondin seems to be identical to its active conformation (Huang et al. 1997). In parallel, the EDTA stabilised conformation of $\alpha_{IIb}\beta_3$ is equivalent to its active conformation. The data presented here therefore suggests that an isomerisation of labile disulphide bonds occurs within $\alpha_{IIb}\beta_3$ when calcium is removed leading to a conformational change and a dramatic increase in LIBS expression over and above that of ligand induced LIBS. It is these events that the bacitracin is inhibiting thereby preventing the receptor adopting an EDTA stabilised conformation.

Upon platelet activation, the number of $\alpha_{IIb}\beta_3$ complexes on the surface increases. Bacitracin inhibits this increase without effecting the number of complexes in resting, unactivated platelets. The antibody used in these studies recognises the α_{IIb} subunit only when it is complexed to the β_3 subunit. Upon platelet activation, internal stores of $\alpha_{IIb}\beta_3$ are mobilised to the platelet surface thereby increasing the observed numbers of the receptor. Activation induced conformational changes in the receptor may act as the signal leading to this secretory phase of platelet activation. Again, bacitracin would appear to be inhibiting these changes that lead to this activation increase in complex $\alpha_{IIb}\beta_3$ on the platelet surface. Thus, by inhibiting the activation of the platelet integrin, bacitracin is also inhibiting integrin mediated outside-in signals.

Bacitracin had no effect on the expression of P-selectin in agonist activated platelets. This would suggest that integrin conformational changes are not involved in this particular marker of platelet activation. In a Glanzmann's Thrombasthenic patient with defective $\alpha_{IIb}\beta_3$ function, P-selectin expression occurs in agonist stimulated platelets (Fullard 2001). P-selectin expression and by implication, α- and dense granule secretion, is therefore independent of integrin function. The fact that bacitracin has no effect on this marker of platelet activation emphasises that bacitracin is not a non-specific inhibitor of platelet function, but in fact a specific inhibitor of $\alpha_{IIb}\beta_3$ mediated events.

A somewhat contrasting result was obtained from the experiments looking at the effects of bacitracin on the binding of the RGD mimetic SC52012 to control and activated platelets. Statistical analysis of the number of binding sites B_{max} revealed a not quite significant difference between untreated and bacitracin treated control platelets. A significant increase in the number of binding sites occurred in ADP activated platelets in the presence of bacitracin when compared to ADP treated platelets alone ($p<0.05$). There was a slight indication of the existence of more than one binding site per receptor upon scrutiny of the ligand analysis, which may explain this apparent increase in B_{max}. Bacitracin had no effect on the affinity of the mimetic for the receptor in either resting or activated platelets. This data suggests that a series of conformational changes occurs within the receptor to uncover only subpopulations of binding sites for this RGD mimetic upon platelet activation. By preventing some of these conformational changes at a particular stage in platelet activation, the action of bacitracin on the receptor may in

fact uncover more binding sites for the mimetic. Alternatively, this may be an artefact of the experimental conditions and will be discussed below.

Platelet activation with the platelet agonist thrombin results in the phosphorylation of tyrosine residues on many proteins. Treatment with bacitracin saw a peculiar loss of these events in both resting and activated platelets. Since completion of these experiments it has been reported that a protease contaminant is present in all commercial preparations of bacitracin (Rogelj et al. 2000). The results obtained here, therefore, may be as a result of this protease activity. In this study, it was established that in experiments performed in plasma, as is the case for the majority of experiments here, the inherent protease inhibitory effects of the plasma would abolish protease activity of bacitracin. This was confirmed by experiments done in my own laboratory. In all the experiments detailing aggregation, OgFg binding, LIBS expression, CD41a binding and P-selectin expression, there was no evidence for protease activity as detected by immunoblot analysis of $\alpha_{IIb}\beta_3$. I am therefore confident that the inhibitory effects observed in these *in vitro* experiments using plasma are due to the isomerase inhibiting properties of bacitracin. With regards to the effects of bacitracin on the binding of SC52012, the protease activity may cleave parts of the receptor and thereby expose more sites that bind the mimetic. The lack of tyrosine phosphorylation events in the presence of bacitracin may be because bacitracin proteolyses the platelet integrin directly or because it has proteolytic activity on the phosphotyrosine containing proteins.

All in all, the data presented here suggests strongly a role for disulphide bond isomerisation that results in conformational changes leading to activation of the receptor.

The evidence is that the mediator of such events may be a protein disulphide isomerase because bacitracin inhibited many aspects of both platelet and receptor activation. Indeed, it has been recently shown that PDI mediates platelet aggregation and secretion in the later stages of platelet activation (Essex and Li 1999). Nonetheless, the results of the confocal microscopy studies are provocative in that it is clearly evident that PDI does not co-localise with $\alpha_{IIb}\beta_3$ in either the resting or activated platelet. Furthermore, the identification of more than one isoform of PDI in the platelet by the same antibody used in the confocal studies lends support to the premise that PDI and $\alpha_{IIb}\beta_3$ do not co-localise on the platelet surface. Mou *et al* also identified an inhibitory role for bacitracin on integrin-mediated events but concluded that these events were independent of PDI (Mou et al. 1998). The implications are that bacitracin is acting on a separate target, perhaps in tandem with PDI itself, in inhibiting activation of $\alpha_{IIb}\beta_3$. The possibility that a separate thiol isomerase activity exists in platelets will be explored in the next chapter.

CHAPTER 5

Identification and characterisation of the endogenous thiol isomerase activity in the platelet integrin $\alpha_{IIb}\beta_3$.

5.1. INTRODUCTION.

The conformational changes in $\alpha_{IIb}\beta_3$ are the composite result of at least two processes. Firstly, intracellular signals converge on the cytoplasmic tails of the integrin that result in activation of the receptor permitting it to bind ligand. Secondly, the extracellular domains respond with an increased affinity for ligand and an altered display of neo-epitopes suggestive of altered protein folding.

The subunits of integrins are rich in cysteine residues. The pairing of these residues in disulphide bonds, particularly in the platelet specific integrin $\alpha_{IIb}\beta_3$, have been shown to be important in the structural integrity of the receptor (Calvete et al. 1991) (Calvete 1999). However, the observation has been made that no free cysteines exist in either the α_{IIb} or β_3 subunits (Calvete 1995). This conclusion has been used to dismiss any potential role for disulphide bonds in integrin activation although there is ample evidence that reduction of bonds by the reducing agent DTT induces an active conformation in $\alpha_{IIb}\beta_3$ and other integrins (Zucker and Masiello 1984) (Peerschke 1995) (Gofer-Dadosh et al. 1997) (Edwards et al. 1995). Moreover, selective mutation of critical cysteines in the platelet integrin $\alpha_{IIb}\beta_3$ results in the induction of a constitutively active form of the receptor lending further support to the proposal that altered dithiol bonding is implicated in integrin activation (Liu 1998) (Edwards et al. 1995) (Davis and Camarillo 1993).

In chapters 3 and 4 of this thesis, it has been demonstrated that a role exists for thiols in the activation of the platelet integrin but that a functional role for platelet PDI in this event does not exist. Alternatively, the receptor itself may have intrinsic isomerase

activity. The catalytic sites of PDI contain the highly conserved motif Cys-Gly-His-Cys. Other proteins that contain a similar tetrapeptide motif include the chaperone molecules Erp^{61} and Erp^{72} (Lundstrom-Ljung et al. 1995), the gonadotropins, lutropin and follitropin (Boniface and Reichert 1990) (Chew et al. 1995), insulin-like growth factor binding protein 3 (IGFBP-3) (Koedam 1994) and fibronectin (Langenbach 1999). All these proteins have demonstrable endogenous isomerase activity.

Analysis of the integrin cysteine patterns reveals that there are nine tetrapeptide motif (Cys-X-X-Cys) repeats in each β-integrin subunit. The specific Cys-Gly-X-Cys sequence is repeated four times, once in each of the regions identified as the cysteine-rich repeat regions (CRRs) (Fitzgerald et al. 1987). This strongly suggested that $\alpha_{IIb}\beta_3$ may also have its own intrinsic isomerase activity. Therefore, I examined purified integrin $\alpha_{IIb}\beta_3$ for the presence of endogenous thiol isomerase activity. Furthermore, a biochemical and pharmacological profile of this activity in the integrin was performed.

5.2. METHODS.

5.2.1. Comparison of the amino acid sequences of the beta subunits of the integrin family.

The multiple sequence alignment programme CLUSTALW at EMBL Outstation, European Bioinformation Institute (www.ebi.ac.uk/clustalw/) was used to compare the amino acid sequences of the beta subunits across species.

5.2.2. Purification of $\alpha_{IIb}\beta_3$.

The platelet integrin $\alpha_{IIb}\beta_3$ was purified from outdated units of human platelets as described in section 2.2.6. The purified protein was stored at –80°C in a buffer containing 20mM Tris pH 7.4, 1mM $CaCl_2$ and 0.1% Triton-X-100 and was thawed rapidly at 37°C before use in experiments. The protein was concentrated to approximately 4mg/ml by filtration through centrex filters (10,000 Da molecular weight cut off filters) in a microcentrifuge at 4°C at 14,000 x g for 30 minutes.

5.2.3. Immunoprecipitation of PDI from purified $\alpha_{IIb}\beta_3$.

2µg/ml of either purified $\alpha_{IIb}\beta_3$ or protein disulfide isomerase in 50mM Tris pH 7.4 with 150mM NaCl and 1% Triton-X 100 was precipitated with a rabbit polyclonal anti human PDI antibody (a kind gift of Dr. Eric Quemeneur, Centre d'Etudes Saclay, Gif-sur-Yvette, France). Precipitates were harvested with protein G beads. Samples were separated on 12.5% SDS-PAGE and transferred to PVDF as described in section 2.2.3.

Membrane was probed with 1:1000 dilution of anti-PDI antibody (rabbit anti-protein disulfide isomerase polyclonal antibody from Stressgen Biotech Corporation) followed by 1:1500 dilution of Protein A peroxidase. Peroxidase activity on the membrane was developed with ECL.

5.2.4. Preparation of reduced and denatured RNase (rdRNase).

This was prepared as outlined in section 2.2.7.1.

5.2.5. Reactivation of reduced and denatured RNase: time and dose dependent studies.

These experiments were carried out as outlined in section 2.2.7.2. Reduced denatured enzyme at a final concentration of 30μM was diluted into 0.1M Tris-HCl pH 7.4 with 1mM EDTA containing either 5μM bovine PDI, purified $\alpha_{IIb}\beta_3$ at concentrations of either 0.5μM, 5μM or 15μM, 5μM ovalbumin or no isomerase. The extent of reactivation of rdRNase was monitored by removing aliquots at various time points over a 65 hour period and measuring recovered RNase activity. This was measured by monitoring the changes in absorbance at 284nm in a Unicam split beam spectrophotometer when enzyme or 'recovering' enzyme (RNase) was added to substrate (0.44mM cytidine 2',3' cyclic monophosphate (cCMP)). A typical assay consisted of 1.4μM RNase and 0.44mM cCMP in 0.1M 2-(N-morphlino) propanesulfonic acid (MOPS) pH 7.0. Activity was expressed as a percentage of a native RNase control.

5.2.6. The effects of pH on the isomerase activity of $\alpha_{IIb}\beta_3$.

The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$ and PDI at a final concentration of 1.5μM. The following buffers were used 0.1M Tris pH 6.8/1mM EDTA, 0.1M Tris pH 7.4/1mM EDTA and 0.1M Tris pH 8.0/1mM EDTA. Recovered RNase activity was monitored at 24 hours.

5.2.7. The effects of temperature on the isomerase activity of $\alpha_{IIb}\beta_3$.

The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$ and PDI at a final concentration of 1.5μM in a buffer containing 0.1M Tris pH 7.4/1mM EDTA. At T = 0 hours, samples were placed at 4°C, room temperature or 37°C. Recovered RNase activity was monitored at 24 hours.

5.2.8. Determination of the enzymatic parameters of $\alpha_{IIb}\beta_3$ and PDI.

To determine these parameters, the RNase refolding assay described in section 2.2.7.2 was employed. Concentrations of reduced denatured RNase ranging from 7.5 to 75 μM was used in (i) the absence of isomerase, (ii) the presence of $\alpha_{IIb}\beta_3$ (1.5μM) or (iii) PDI (1.5μM). The recovered activity of rdRNase was determined by measuring the initial velocity over 1 minute at 284nm after a 48 hour incubation. This velocity or rate was automatically calculated by a kinetic's programme in the spectrophotometer. Plots of substrate concentration versus initial velocity were fit to the Michaelis-Menten equation using Deltagraph. A direct comparison of the abilities of $\alpha_{IIb}\beta_3$ and PDI to catalyse the reactivation of rdRNase was made by determining values for the K_m, which is equivalent

to the substrate concentration that yields half maximal velocity of the enzyme; the k_{cat}, a measure of the number of molecules of substrate converted to product per unit time when the enzyme is working at maximal efficiency and the ratio of k_{cat}/K_m, a measure of catalytic efficiency and enyzme specificity. The initial rates of the uncatalaysed reaction, which were determined in the absence of a source of isomerase activity, were subtracted from the catalysed rates determined in the presence of PDI or integrin. This enabled a truer value for these kinetics to be determined. A standard curve was generated from the initial rates of various concentrations (0-4μM) of native RNase. A value for μM of RNase regenerated per minute for V_{max} was then determined from the slope of this curve.

5.2.9. The effects of bacitracin on the isomerase activity of $\alpha_{IIb}\beta_3$ and PDI.

The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$ and PDI at a final concentration of 1.5μM. At time (T) = 0 hours bacitracin at concentration of either 0 μM, 0.1 μM, 1 μM, 3.3 μM, 10 μM, 33 μM, 100μM was added. RNase activity was monitored at 24 hours and the results were expressed as % of maximal recovery of rdRNase in the absence of bacitracin.

5.2.10. The effects of cations on the isomerase activity of $\alpha_{IIb}\beta_3$.

The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$ at final concentrations of 1.0μM or 1.5μM. At time (T) = 0 hours, either 1mM $MgCl_2$ in 0.1M Tris pH 7.4, 1mM $MnCl_2$ in 0.1M Tris pH 7.4, EDTA free buffer, or buffer containing

1mM EDTA were added to the samples. It is noted that the first three samples all have a final concentration of 100μM $CaCl_2$, as a result of the dilution of purified $\alpha_{IIb}\beta_3$ into assay, which is in a buffer containing 1mM $CaCl_2$. Recovered RNase activity was monitored at 48 hours.

5.2.11. The effects of RGD mimetics on the isomerase activity of $\alpha_{IIb}\beta_3$.

The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$ and PDI at a final concentration of 1.5μM. The following mimetics were added at T = 0 hours: RGDS (0.5mM), Integrilin (2μg/ml), SC52012 (1μM) and ReoPro (10μg/ml). Recovered RNase activity was monitored at 48 hours.

5.2.12. The isomerase activity of other integrins.

Purified $\alpha_V\beta_3$ and $\alpha_5\beta_1$ was obtained from Chemicon Corporation. The isomerase assay was set up as described in section 5.2.5 using $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$ and PDI at a final concentration of 0.5μM in 0.1M Tris pH7.4/1mM EDTA. Recovered RNase activity was monitored at 48 hours.

5.3. RESULTS.

5.3.1. Analysis of the integrin cysteine patterns.

Analysis of the integrin cysteine patterns reveals that there are four highly conserved cysteine rich repeats (CRRs) in each β-integrin subunit. In figure 5.1 the alignment of the second cysteine-rich-repeat from human β_3 is compared with the same region from other β-integrins ranging from sponge to mammalian sequences. There is 44-63% sequence identity within this region increasing to 100% for the β_3 subunits. The cross-species homology index is further increased when conservative amino-acid substitutions are accounted for, giving a value of 56-83% homology. The cysteine residues are 100% conserved. Similar homology scores are obtained when the first, third or fourth CRRs are aligned. The four CRRs of β_3 subunits from human, mouse and dog are 90-94% identical. The alignments also reveal the presence of two motifs in each repeat: motif I has the sequence 'GXCXCXXCXC' and is separated by 5-12 amino acids from motif II which has the sequence 'GXXCXC' (Figure 5.2). In all integrins, the first CRR is imperfect with the first cysteine of Motif I replaced by a Phe, Met, Leu or Tyr. Similarly, the fourth CRR is incomplete in all integrins, lacking the second motif but having a similar GXXCXXCXXC in β_1, β_3, and $\beta_{5\text{-}8}$ integrins. β_8 integrins are an exception to the integrin family and have very corrupted CRRs, the first CRR containing a CXXC, but not the flanking residues of motif I. It lacks motif II. The second CRR also lacks motif II. The third CRR lacks Cys^2 in motif I but has a normal motif II. The fourth CRR, like all other integrins, lacks a true motif II.

Motif I contains the active site sequence Cys-X-X-Cys (CXXC) of the ubiquitous thiol modifying enzymes, PDI and thioredoxin. Each β integrin subunit contains nine repeats of this CXXC tetrapeptide sequence; two in the amino terminal region, one in each of the four CRRs, and two in the modified motif II of the fourth CRR. The final CXXC is immediately C-terminal to the fourth CRR. The four CXXC repeats found in the four CRRs of the β_3-integrins have a Gly in their most N-terminal 'X' position in common with PDI and many of the other proteins known to have endogenous thiol isomerase activity. A comparison of the CXXC motifs from various proteins is shown in figure 5.3.

5.3.2. Purified $\alpha_{IIb}\beta_3$.

Platelet $\alpha_{IIb}\beta_3$ was purified as described in section 2.2.6. Coomassie blue staining of an SDS-PAGE gel shows the presence of $\alpha_{IIb}\beta_3$ run under reducing conditions, with two bands corresponding to the beta subunit β_3 and the heavy chain of the alpha subunit α_{IIbH} at 95kDa and 115kDa, respectively. The light chain of the alpha subunit is observed at 25kDa. There is no evidence for any additional co-purifying or contaminating proteins in this preparation (Figure 5.4).

5.3.3. Immunoprecipitation of PDI from the $\alpha_{IIb}\beta_3$ purification preparation.

To ascertain that platelet PDI had not copurified with $\alpha_{IIb}\beta_3$, the purified integrin preparation was checked for the presence of PDI immune-reactive proteins by immunoprecipitation as outlined in section 5.2.3. PDI, with a molecular mass of 57kDa on SDS-PAGE, is not apparent in coomassie blue stained gels or in western blots of

purified $\alpha_{IIb}\beta_3$ (Figure 5.4), nor was it precipitated from the purified $\alpha_{IIb}\beta_3$ preparation (Figure 5.5). It is therefore concluded that the integrin preparation is devoid of contaminating platelet PDI.

5.3.4. Isomerase activity in $\alpha_{IIb}\beta_3$.

Purified $\alpha_{IIb}\beta_3$ (15μM) was shown to have equivalent thiol isomerase capacity to PDI (5μM). In contrast, ovalbumin, a cysteine-rich protein that lacks a Cys-X-X-Cys motif, had no isomerase activity above baseline levels when compared to an uncatalysed reaction (Figure 5.6). This activity in $\alpha_{IIb}\beta_3$ was shown to be saturable in both a time and concentration dependent manner. (Figure 5.7).

5.3.5. Increasing pH and isomerase activity in $\alpha_{IIb}\beta_3$ and PDI.

Increasing the pH of the assay buffer form pH6.8 to pH 8.0 increased the isomerase activity of both $\alpha_{IIb}\beta_3$ and PDI (Figure 5.8). Increasing the pH of a solution increases the number of hydroxyl ions (OH^-). These ions target thiol groups (SH) resulting in their oxidation and the formation of disulphide bonds. Thiol disulphide exchange reactions are maximal when the pKa of the thiol equals the pH of the solution. A typical cysteine has a side chain pKa of 8.7. Therefore a buffer at pH 8.0 would support maximal disulphide bond formation and this is reflected in the results obtained here. Subsequent assays were therefore performed at pH 7.4.

5.3.6. Increasing temperature and its effect on isomerase activity in $\alpha_{IIb}\beta_3$ and PDI.

Increasing the temperature of the assay from room temperature to 37°C had no effect on the isomerase activity of PDI. Curiously, this increase in temperature inhibited the isomerase activity of $\alpha_{IIb}\beta_3$ (Figure 5.9). Subsequent assays were therefore performed at room temperature. This was not explored further and all assays were performed at room temperature. This is the standard procedure in all RNase refolding assays.

5.3.7. A comparison of the kinetics of $\alpha_{IIb}\beta_3$ and PDI.

A direct comparison of the abilities of $\alpha_{IIb}\beta_3$ and PDI to catalyse the reactivation of rdRNase was made by determining values for the k_{cat} and K_m of each protein from Michaelis-Menten plots of kinetic data (Figure 5.10 and Table 5.1). Both proteins showed typical equilibrium kinetics. The affinity (K_m) of the integrin for its substrate (RNase) is less for $\alpha_{IIb}\beta_3$ than for PDI although the catalytic capacity of the enzymes (k_{cat}) is equivalent. Thus, the intrinsic thiol isomerase activity within $\alpha_{IIb}\beta_3$ is potent and has high capacity, equal to that observed in PDI.

5.3.8. Bacitracin inhibits the isomerase activity of purified $\alpha_{IIb}\beta_3$ and PDI.

To examine the pharmacological profile of this activity, we looked at the effects of the PDI inhibitor bacitracin. Bacitracin dose dependently inhibited the isomerase activity of both PDI and $\alpha_{IIb}\beta_3$ (Figure 5.11). The IC_{50} value determined for $\alpha_{IIb}\beta_3$ is 11μM and 4μM for PDI. Maximal inhibition of $\alpha_{IIb}\beta_3$ activity is observed at 100μM bacitracin.

5.3.9. Cations affect the isomerase activity of $\alpha_{IIb}\beta_3$.

The observed isomerase activity of $\alpha_{IIb}\beta_3$ occurs in the presence of the calcium chelator EDTA (1mM). When the assay is performed in the presence of 100μM $CaCl_2$, $\alpha_{IIb}\beta_3$ lost the ability to reactivate rdRNase while the enzymatic activity of PDI was unaffected (Figure 5.12). The combination of magnesium and calcium had an inhibitory effect on the isomerase activity of $\alpha_{IIb}\beta_3$. Intriguingly, the combination of calcium and manganese increased the isomerase activity close to that obtained in the presence of EDTA (Figure 5.13). The concentration of $MnCl_2$ used in this assay is the same as that used to induce integrin activation in many studies (Yan et al. 2000).

5.3.10. The effects of RGD mimetics on the isomerase activity of $\alpha_{IIb}\beta_3$.

Incubation of $\alpha_{IIb}\beta_3$ with the RGD mimetics had no effect on the isomerase activity (Figure 5.14). It is not known whether mimetics actually bind to the purified protein in this assay.

5.3.11. Isomerase activity in other integrins.

Both $\alpha_V\beta_3$ and $\alpha_5\beta_1$ were found to have isomerase activity (Figure 5.15). The activity of $\alpha_V\beta_3$ is greater than that of $\alpha_{IIb}\beta_3$. This data suggests that the activity does indeed reside in the common β_3 subunit of these two integrins. $\alpha_5\beta_1$ also expresses isomerase activity but, under the conditions tested, its appears less potent than the β_3 integrins. Perhaps the conditions of time, temperature, pH and Ca^{2+} concentrations optimised for $\alpha_{IIb}\beta_3$ are not ideal for $\alpha_5\beta_1$.

Accession No.	Protein	Second cysteine rich repeat
P05106	human β_3	RGECLCGQCVCHSSDFG–KIT–GKYCECDDFSCVRYKGEMCSG
P11835	mouse β_3	RGECLCGQCVCHSSDFG–KIT–GKYCECDDFSCVRYKGEMCSG
g5733735	dog β_3	RGECLCGQCVCHSSDFG–KIT–GKYCECDDFSCVRYKGEMCSG
P18084	human β_5	RGDCSCNQCSCFESEFG–KIY–GPFCECDNFSCARNKGVLCSG
Q07441	baboon β_5	RGDCSCNQCSCFESEFG–KIY–GPFCECDNFSCARNKGVLCSG
P18564	human β_6	RGDCYCGQCICHLSPYG–NIY–GPYCQCDNFSCVRHKGLLCGG
P26010	human β_7	KGHCQCGRCSCSGQSS-------GHLCECDDASCERHEGILCGG
P26013	rabbit β_8	RGVCVCGKCLCHKMKLG–KVY–GKYCEKDDFSCPYHHGSLCAG
P26012	human β_8	RGVCVCGKCSCHKIKLG–KVY–GKYCEKDDFSCPYHHGNLCAG
P05556	human β_1	NGECVCGQCVCRKRDNTNE–IYSGKFCECDNFNCDRSNGLICGG
P05107	human β_2	LGDCVCGQCLCHTSDVPGKLIY–GQYCECDTINCERYNGQVCGG
P53714	pig β_2	LGDCICGQCVCHTSDVPNKKIY–GQFCECDNMNCERFDGQVCGG
P11584	drosoph β	RGHCVCGACECHKRPNP–IEIISGKHCECDNFSCERNRNQLCSG
AF059607	greenurch	RGDCVCGLCECDTRGDP–NRVITGEFCQCDNYLCPRSGGKVCGG
g2335165	spon-coral	RGECACGECVCRVEQGQ–RFY–GKLCECNDFSCPEYEGNLCGG
g3789800	mullosc β	RGSCVCGECLCNTRSSDSAQTFSGPYCECDDYSCNQFNEQICGG

Figure 5.1. A comparison of the amino acid sequence of the second cysteine rich repeats (CRR) of the human β_3 integrin subunit with the equivalent region from other beta integrin subunits.

The subunits are identified by their Swiss Prot or genbank accession numbers. Identical residues are highlighted in yellow. Similar residues are highlighted in grey. The Cys-X-X-Cys motif in each repeat is shown in red.

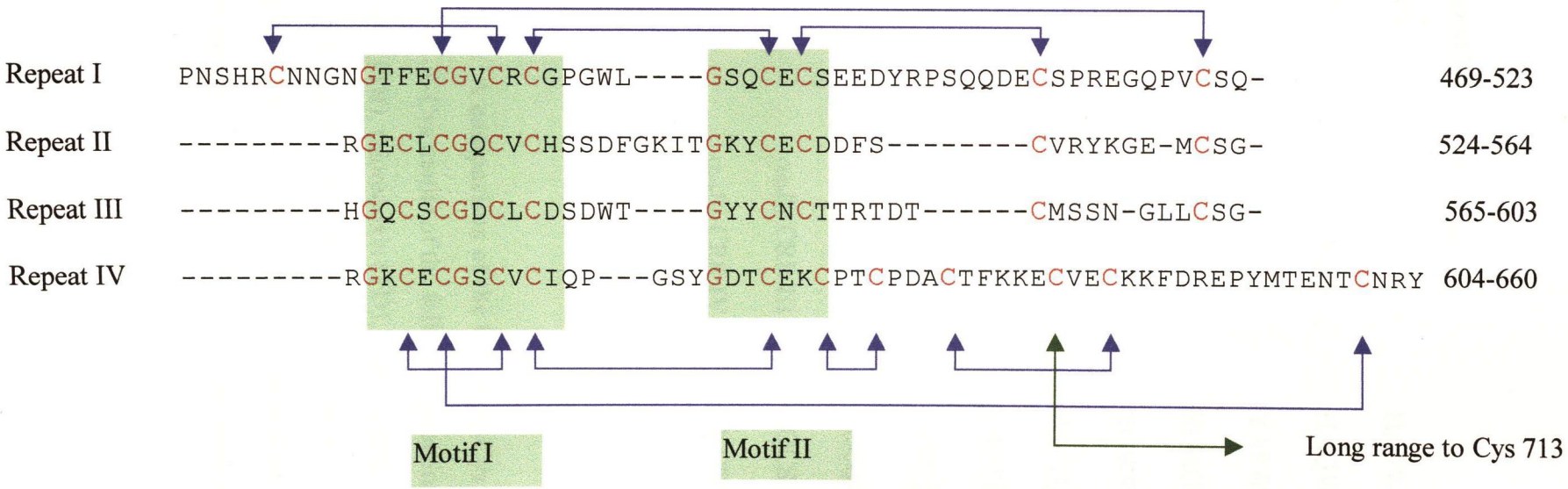


Figure 5.2. Alignment of the four cysteine rich repeats of human β_3 protein.

The assumed disulphide bonds are indicated by the arrows, taken from the study by Calvete (Calvete et al. 1991). Identical amino-acids are shown in red text. Motifs I and II are boxed in green. The sequence was obtained from the sequence for β_3 using the accession number Swiss Prot P05106.

Accession No.	Protein	
P07237	PDI	HAPW**CGHC**KALAP
Q99757	Thioredoxin	HAQW**CGPC**KILGP
P13667	ERP 72	YAPW**CGHC**KQLEP
P20959	IGFBP-3	RDAG**CGCC**LTCAL
P02751	Fibronectin	RGWR**CDNC**RRPGG
P01225	FSH	TQCH**CGKC**DSDST
P04275	vWF	KAPT**CGLC**EVARL
P05106	$\alpha_{IIb}\beta_3$ CRR I	GTFE**CGVC**RCGPG
	$\alpha_{IIb}\beta_3$ CRR II	GECL**CGQC**VCHSS
	$\alpha_{IIb}\beta_3$ CRR III	GQCS**CGDC**LCDSD
	$\alpha_{IIb}\beta_3$ CRR IV	GKCE**CGSC**VCIQP

Figure 5.3. A comparison of the Cys-X-X-Cys motifs of various proteins.
The Cys-X-X-Cys motifs of the following proteins are shown here: protein disulphide isomerase (PDI), thioredoxin (TD), endoplasmic-reticular protein 72 (ERP-72), insulin-like growth factor binding protein-1 (IGFBP-1), fibronectin (Fn), follotropin (FSH), von Willebrand Factor (vWF) and the cysteine rich repeats (CRRs) I-IV of human $\alpha_{IIb}\beta_3$. The motif is highlighted in yellow with the cysteine residues in red.

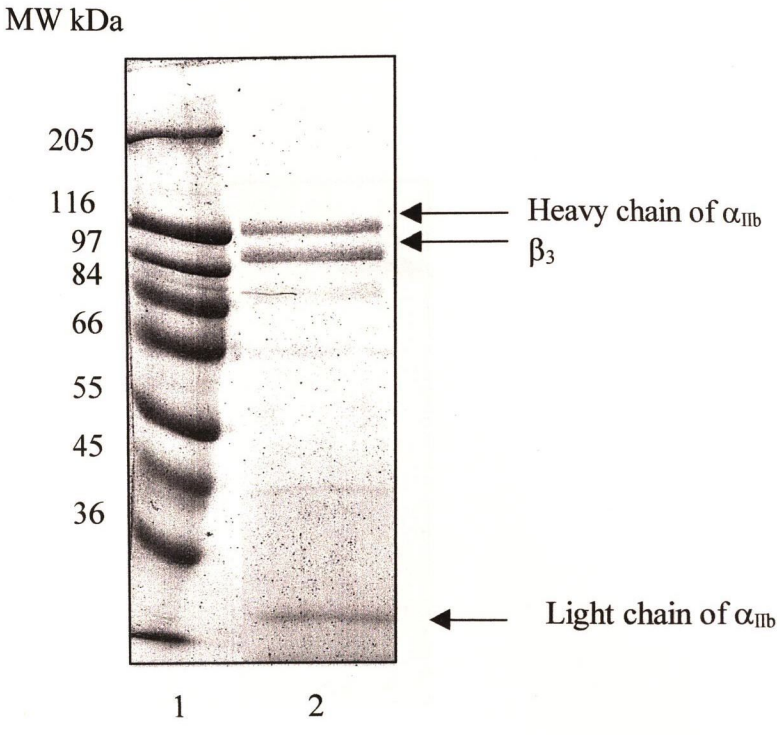


Figure 5.4. Coomassie blue stain of purified $\alpha_{IIb}\beta_3$.

The purified protein was separated on a 7.5 % SDS-PAGE gel. Lane 1 contains high molecular weight (MW) markers. Their sizes are indicated to the left in kilodaltons (kDa). Lane 2 shows purified platelet $\alpha_{IIb}\beta_3$.The heavy chain of the alpha subunit α_{IIb} is present at a molecular weight of 115 kDa and the beta subunit β_3 at 95 kDa. The light chain of the alpha subunit is observed at 25 kDa.

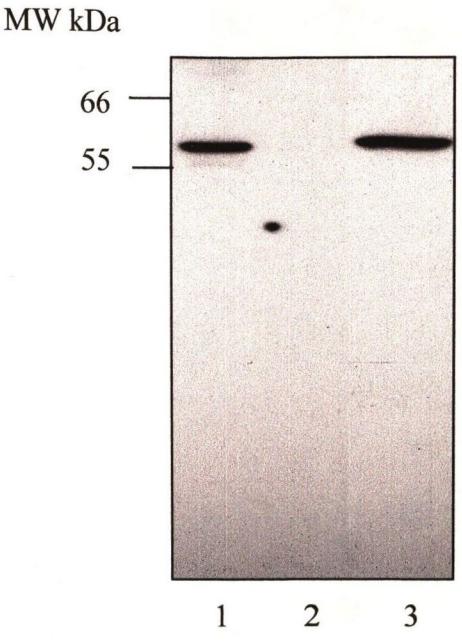


Figure 5.5. Immunoprecipitation of PDI from purified $\alpha_{IIb}\beta_3$.

To ascertain that there is no contaminating PDI in the purified preparation of $\alpha_{IIb}\beta_3$, purified PDI (2μg/ml, lane 1) and $\alpha_{IIb}\beta_3$ (2μg/ml, lane 2) were immunoprecipitated with a rabbit anti-human protein disulfide isomerase polyclonal antibody (a gift of Dr. E. Quemeneur), separated on 12.5% SDS-PAGE gel and immunoblotted with rabbit anti-protein disulfide isomerase polyclonal antibody from Stressgen. Lane 3 contains 10μg total platelet lysate, which serves as the positive control.

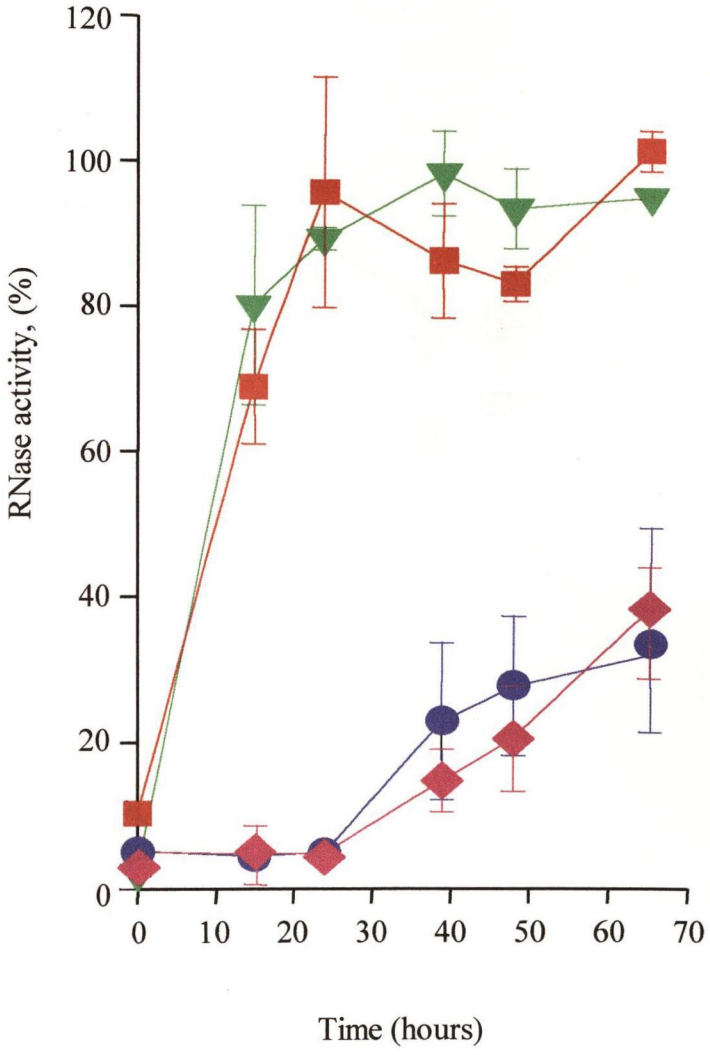


Figure 5.6. Endogenous isomerase activity in $\alpha_{IIb}\beta_3$.

Native RNase was reduced and denatured (rdRNase, 30μM) as described in section 2.2.8.1, and incubated with 5μM PDI (■), 15μM αIIbβ3 (▼), 5μM ovalbumin (†) or no enzyme (◆) in 0.1M Tris-HCl buffer, pH 7.4 containing 1mM EDTA. Recovery of RNase activity was monitored at the indicated time points. Results are expressed as a percentage of the activity observed in native RNase preparations. Data are the means ± S.D. of n = 3 experiments.

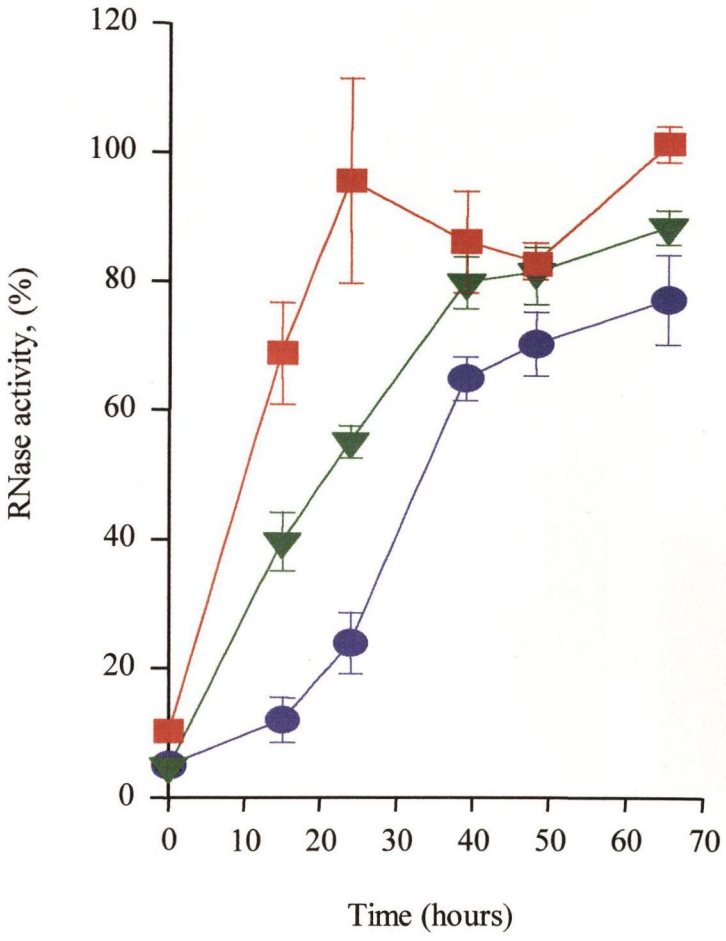


Figure 5.7 Isomerase activity in $\alpha_{IIb}\beta_3$ is both time and dose dependent.

The endogenous thiol isomerase activity of $\alpha_{IIb}\beta_3$ is concentration and time dependent. This graph shows reactivation of rdRNase by $\alpha_{IIb}\beta_3$ at concentrations of 0.5μM(●), 5μM(▼) and 15μM(■). Results are the means ± S.D. of n = 3 experiments.

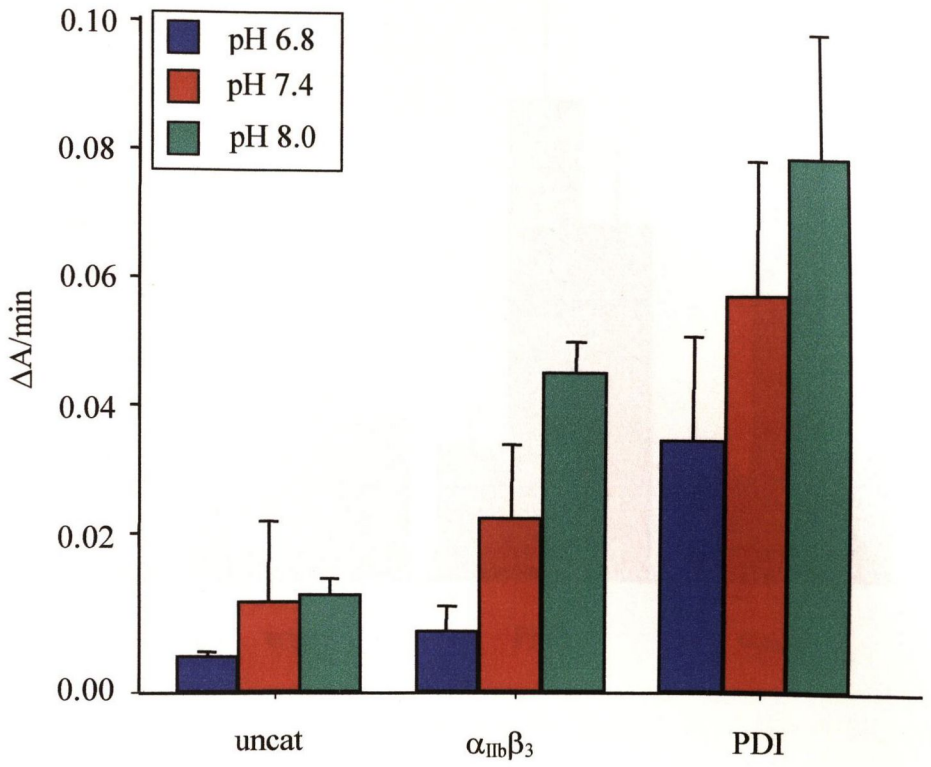


Figure 5.8. The effects of increasing pH on the isomerase activity in PDI and $\alpha_{IIb}\beta_3$.

rdRNase was incubated with no enzyme (uncat), PDI (1.5μM) and $\alpha_{IIb}\beta_3$ (1.5μM) in 0.1M Tris buffer containing 1mM EDTA. The pH of the buffer was altered in each assay as indicated: pH 6.8 (■), pH 7.4 (■) and pH 8.0 (■). Recovery of RNase activity was monitored at 24 hours. The results are expressed as the initial rate of absorbance and are the mean ± S.D. of n = 3 experiments in duplicate.

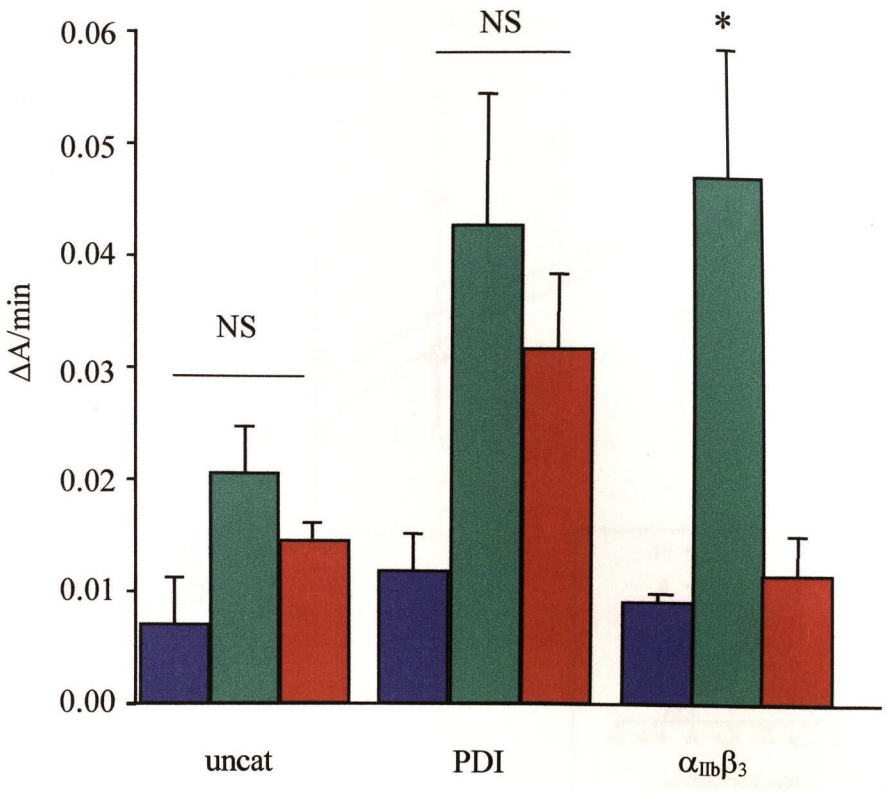


Figure 5.9. Increasing temperature and isomerase activity in PDI and $\alpha_{IIb}\beta_3$. rdRNase was incubated with no enzyme (uncat), PDI (1.5μM) and $\alpha_{IIb}\beta_3$ (1.5μM) in 0.1M Tris buffer containing 1mM EDTA. Samples were incubated for 24 hours at 4°C (■), 22°C (■) and 37°C (■). Recovery of RNase activity was then monitored. The results are expressed as the initial rate of absorbance and are the mean ± S.D. of n = 2/3 experiments in duplicate. Statistical significance was determined using Student t test. * $p < 0.05$ compared to 22°C, NS = non significance compared to 22°C.

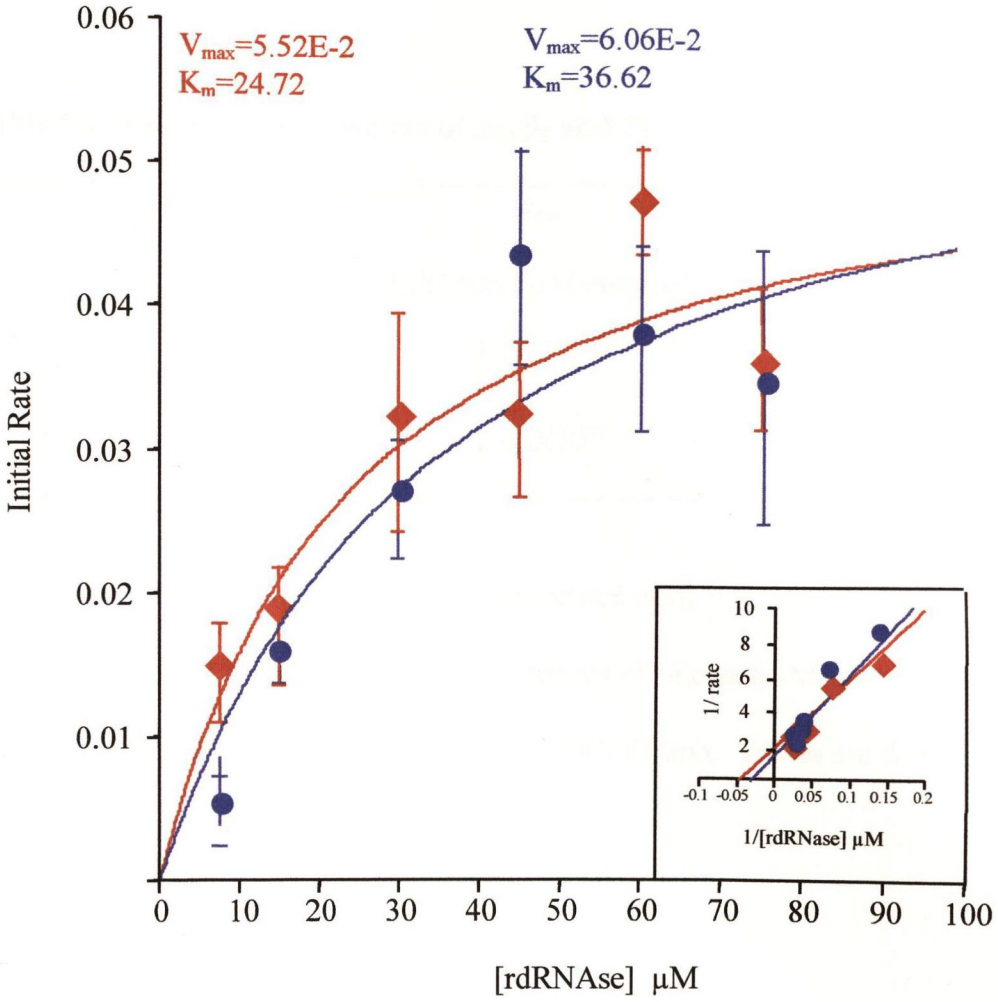


Figure 5.10. Kinetics of endogenous thiol isomerase activity in purified $\alpha_{IIb}\beta_3$. rdRNase was incubated with 1.5µM PDI (◆), or 1.5µM $\alpha_{IIb}\beta_3$ (●) in 0.1M Tris-HCl buffer, pH7.4, containing 1mM EDTA. Recovery of RNase activity was monitored at 48hrs. Plots of substrate concentration *versus* initial velocity were fit to Michaelis-Menten equation using Deltagraph 4.0 to determine V_{max} and K_m. Data are the means ± S.D. of n = 4 separate experiments. The Lineweaver-Burke transformation of this data is shown in the inset-figure.

Table 5.1. Enzymatic parameters of $\alpha_{IIb}\beta_3$ and PDI.

	K_m (μM)	k_{cat} ($\mu M\ min^{-1}/\mu M$ enzyme)	k_{cat}/K_m ($\mu M^{-1}\ min^{-1}$)
$\alpha_{IIb}\beta_3$	36.62	$2.17X10^{-4}$	$5.93X10^{-6}$
PDI	24.72	$2.45X10^{-4}$	$9.91X10^{-6}$

The standard RNase refolding assay was performed using 7.5-75μM rdRNase in the presence of 1.5μM PDI or 1.5μM $\alpha_{IIb}\beta_3$. Analysis of Michaelis-Menten data was performed using the curve-fitting programs of DeltaGraph. Values are derived from the mean $\pm$ S.D. of n = 4 separate experiments.

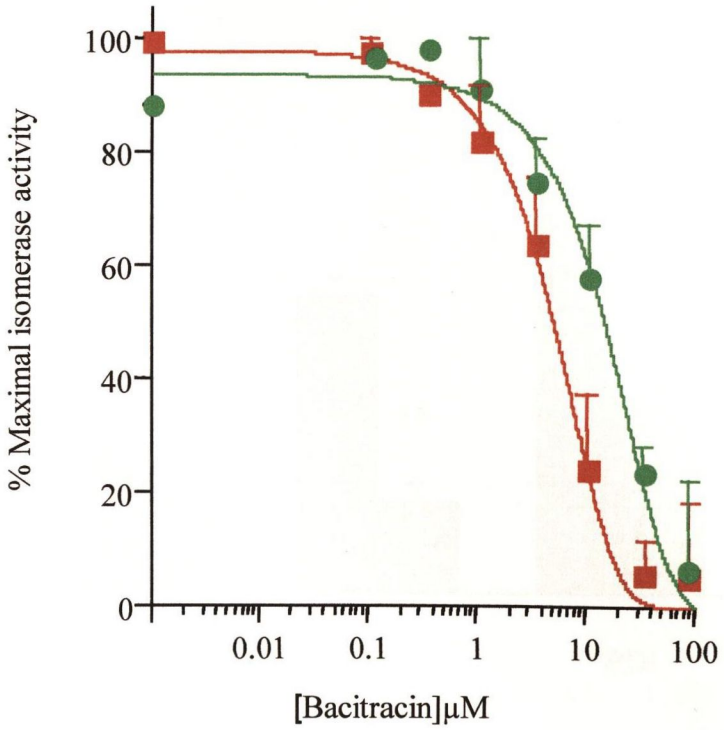


Figure 5.11. Bacitracin inhibits the endogenous thiol isomerase activity of $\alpha_{IIb}\beta_3$. 5μM each of PDI (■) and $\alpha_{IIb}\beta_3$ (●) were incubated with the PDI inhibitor, bacitracin, at the doses indicated for 24 hours. The results are expressed as % of maximal recovery of the refolding activity of rdRNase observed in the absence of bacitracin and are shown as mean ± S.D. of n = 3 experiments.

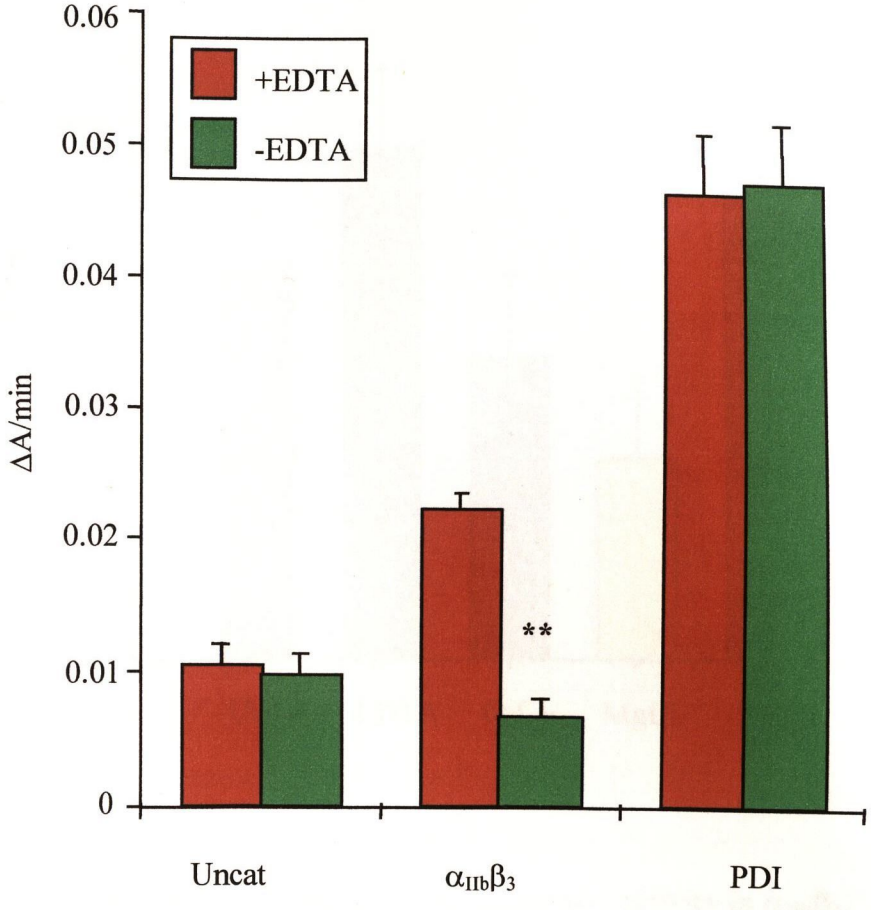


Figure 5.12. The effects of the calcium chelator EDTA on the isomerase activity of $\alpha_{IIb}\beta_3$ and PDI.

Refolding of rdRNase was monitored in the absence of a catalyst (uncat) or in the presence of PDI (1.5μM) or $\alpha_{IIb}\beta_3$ (1.5μM) for 24 hours. Assays were performed in the presence (■) or absence (■) of 1mM EDTA. The results are expressed as the change in absorbance of the initial rate of recovered RNase activity over 1 minute (ΔA/min). Data are shown as mean ± S.D. of n = 4 experiments. Statistical significance was determined using paired Student-t test. ** indicates a p value <0.05.

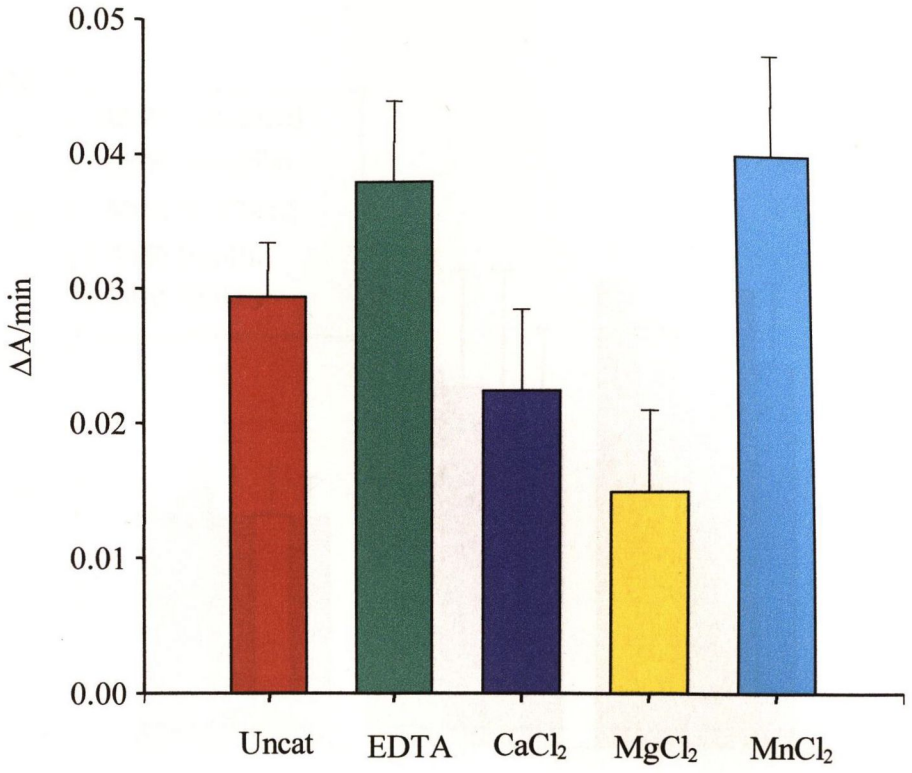


Figure 5.13. The effects of cations on the isomerase activity in $\alpha_{IIb}\beta_3$.
Refolding of rdRNase was monitored after 48 hours in the absence (uncat, ■) or presence of either 1mM EDTA (■), 1mM $CaCl_2$ (■), 1mM $MgCl_2$ (■) or 1mM $MnCl_2$ (■). As the purified integrin is in a buffer containing 1mM $CaCl_2$, assays performed in the absence of EDTA will have 100μM $CaCl_2$ present in addition to the indicated cation, present at 1mM. The results are expressed as the change in absorbance of the initial rate of recovered RNase activity over 1 minute (ΔA/min). Data are shown as mean ± S.D. of n = 3 experiments.

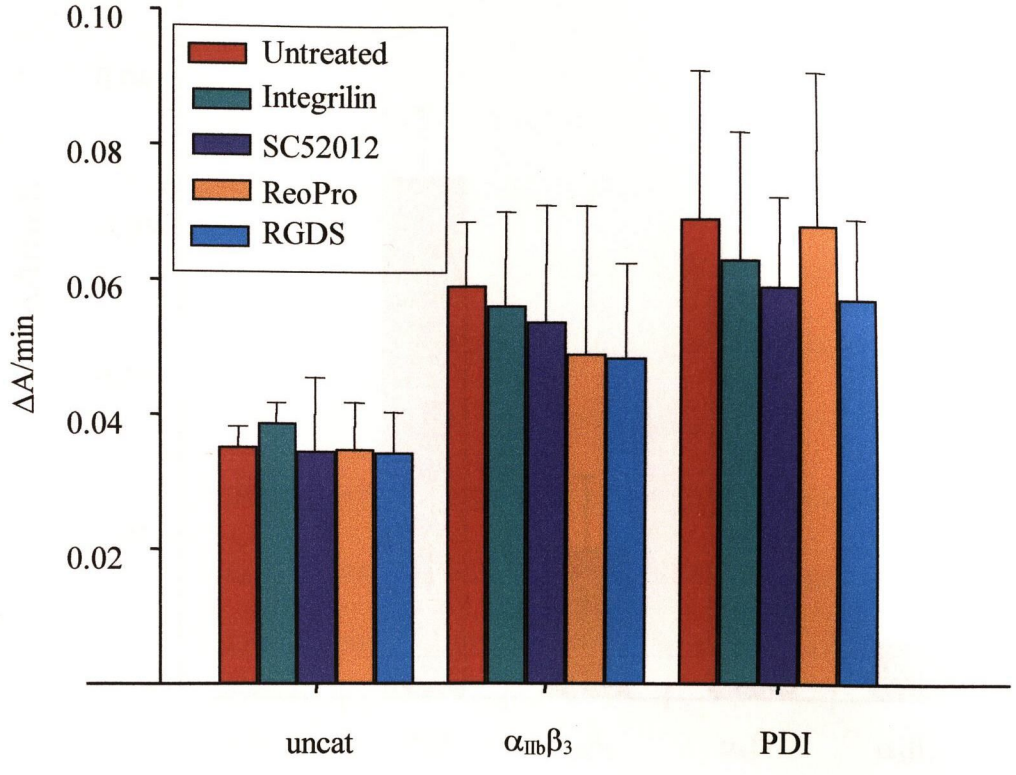


Figure 5.14. RGD mimetics have no effect on the isomerase activity of $\alpha_{IIb}\beta_3$. Incubation of $\alpha_{IIb}\beta_3$ and PDI with the RGD mimetics at the following concentrations Integrilin (2μg/ml), SC52012 (1μM), ReoPro (10μg/ml) and RGDS (0.5mM) had no effect on the isomerase activity. Data shown is the mean ± S.D. of n = 3 experiments. There is no significant difference between any of the treated samples compared to the untreated controls.

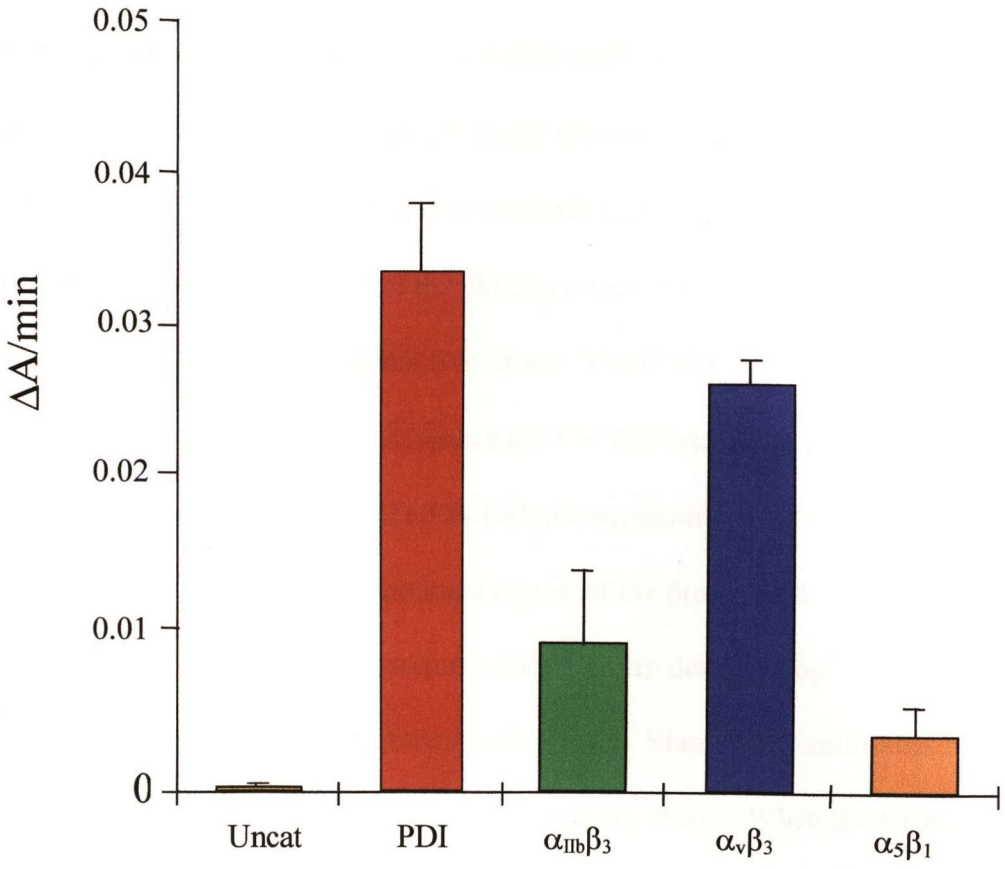


Figure 5.15. Thiol isomerase activity in other integrins.

Refolding of rdRNase was monitored at a 24 hours in the absence of a catalyst (uncat) or in the presence of PDI (0.5μM), $\alpha_{IIb}\beta_3$ (0.5μM), $\alpha_v\beta_3$ (0.5μM) or $\alpha_5\beta_1$ (0.5μM). The results are expressed as the change in the initial rate of the RNase reaction over 1 minute (ΔA/min) monitored at 24 hours. Data shown are the the mean ± S.D. of n = 3 experiments.

5.4. DISCUSSION.

It was proposed at the end of chapter 4 that $\alpha_{IIb}\beta_3$ could contain an endogenous thiol isomerase enzymatic activity and there are several reasons to suggest this. The active site sequence common to PDI and thioredoxin Cys-Gly-X-Cys (CGXC) is repeated four times within the beta subunit of the platelet integrin while the less specific Cys-X-X-Cys sequence is present an additional five times. The CGXC repeat forms part of the larger motif GXCXCGXCXC, present within each of the four CRRs in β_3. These repeats correspond to the CRRs identified by Calvete suggesting that this region arose by duplication of an important functional region of the protein. Moreover, this repeat motif is common to all integrin beta subunits identified to date lending support to the theory that it represents an important functional domain. Many LIBS antibodies bind to sites within the CRRs (Honda et al. 1995) (Kouns et al. 1991). When the CRRs are either deleted or mutated, such integrins cannot be regulated between high- and low-affinity states. Only one mutation to date has been characterised that directly involves a cysteine residue in the CRR of $\alpha_{IIb}\beta_3$ (Ruan et al. 1999). In this case, the surface expression of the integrin is affected suggesting a role for the CRRs in the assembly, stability or transport of the intact integrin to the platelet surface. In contrast, when the entire CRRs are deleted from $\alpha_{IIb}\beta_3$, normal levels of integrin expression in Sf9 insect cells are obtained implying that the CRRs are not involved in the assembly, stability or surface expression of the integrin complex (Wippler et al. 1994). In a recent study it was demonstrated that $\alpha_{IIb}\beta_3$ is activated by a series of specific conformational rearrangements in the ectodomain that increase the rate of ligand association. Such changes are focused at a disulphide-bonded knot of β_3 adjacent to the CRRs and are

independent of signal-induced modifications of the integrin such as phosphorylation of the cytoplasmic tail. A peptide extending from residues 393 to 423 appears in the active conformation of the purified $\alpha_{IIb}\beta_3$. This segment is directly adjacent to Cys^{435}, which forms a disulphide bond with Cys^5 and it encompasses Cys^{405}, which bonds to Cys^{655}. This suggests that changes in disulphide bond patterns within the receptor are involved in its conformational changes (Yan et al. 2000) (Yan and Smith 2000). These observations indicate that the CRRs, while not important for integrin assembly, expression or ligand recognition, are critical for the regulation of the integrin affinity state and its inducible capacity to bind ligand.

As shown in chapter 4, there is no evidence to support the co-localisation of PDI with the platelet integrin. Therefore, it is attractive to propose an endogenous thiol isomerase activity. It was also shown in chapter 4, the PDI inhibitor bacitracin, inhibited integrin-specific events such as aggregation and fibrinogen binding in *in-vitro* assays of platelet function. Similar studies show that bacitracin inhibits β_1 and β_7 integrin-mediated adhesive function in B-lymphocytes by a PDI-independent mechanism (Mou et al. 1998).

In this chapter, I unambiguously demonstrate the presence of thiol isomerase activity in the platelet integrin $\alpha_{IIb}\beta_3$. Its activity is time-dependent, dose-dependent and saturable. Like PDI, it is inhibited in a dose-dependent manner by bacitracin, with a similar IC_{50}. However, this particular result is noted with caution because of the probable protease contaminant in the bacitracin preparation as discussed in chapter 4 (Rogelj et al. 2000). Nonetheless, bacitracin exerted a functional response in platelets (in plasma), namely the

inhibition of fibrinogen binding and platelet aggregation. The inhibition of an endogenous thiol isomerase activity of $\alpha_{IIb}\beta_3$ by bacitracin therefore provides a biochemical mechanism to explain this antiplatelet effect.

A direct comparison of the abilities of $\alpha_{IIb}\beta_3$ and PDI to catalyse the reactivation of rdRNase was made by determining values for the k_{cat}, and K_m of each protein. Both proteins showed typical equilibrium kinetics. The catalytic capacity of each protein was the same implying that the isomerase activity within $\alpha_{IIb}\beta_3$ is as potent catalytically, as bovine PDI at renaturing the artificial substrate, rdRNase.

Unlike PDI, however, the catalytic activity of $\alpha_{IIb}\beta_3$ is Ca^{2+} sensitive and is maximal in the presence of the calcium chelator EDTA. Integrins are calcium-dependent heterodimers and are therefore dependent on the presence of Ca^{2+} to maintain their dimeric structure. In the presence of 1mM EDTA, the α_{IIb} and β_3 subunits can be expected to dissociate. In contrast, at 100μM Ca^{2+}, the heterodimer is intact. The lack of thiol isomerase activity in the presence of Ca^{2+} may be explained by an inhibitory effect exerted by the α_{IIb} chain on the enzymatic activity of the β_3 subunit. Such a hypothesis is attractive, as the integrin α-subunit has been shown to interact with cytoplasmic signalling proteins in an inducible manner during cellular activation (Stephens 1998) (Hannigan et al. 1996) (Naik et al. 1997). Alternately, local concentrations of Ca^{2+} may exert a direct inhibitory effect on the function of the integrin. Divalent cations can bind up to five sites on integrins and can promote (Smith et al. 1994) or suppress ligand binding (Hu et al. 1995). Binding sites with a high affinity for

Ca^{2+} are present on the β_3 integrin and when bound by cation increase the dissociation rate between RGD and integrin (Hu et al. 1996). As ligand binds to the receptor Ca^{2+} is displaced from these high affinity sites. This displacement of cation from these sites may be the trigger to initiate the isomerase activity of the receptor. The presence of Mn^{2+} in the isomerase assay recovered the activity of $\alpha_{IIb}\beta_3$ in the presence of Ca^{2+} to the levels observed in the presence of EDTA. This ties in very nicely with the observation that a recombinant fragment of β_3 (residues 95-373) bound fibrinogen in the presence of Mn^{2+} and low concentrations of Ca^{2+}, whereas high concentrations of Ca^{2+} prevented this interaction (Mould et al. 1995).

Binding of RGD mimetics and the Fab fragment of the antibody 7E3 (ReoPro) had no effect on the isomerase activity of either $\alpha_{IIb}\beta_3$ or PDI. Since the RGD binding pocket is suppose to be remote from the CRR region of the integrin, occupancy of this pocket would not necessarily inhibit the isomerase activity. The cysteines of the core are still available to interact in a catalytic reaction with a target substrate which in this instance is rdRNase. Also, as this assay is a cell free assay it is difficult to gauge if these compounds have actually interacted with the purified protein. However, since some of these compounds can induce an activated conformation in the purified integrin, it was of interest to investigate their effect on the isomerase activity of the integrin.

The observed increase in isomerase activity in increasing pH was expected as the redox enviroment of the assay was changed. Since the pKa (8.7) of the reactive side chain of cysteine is close to pH8 it was likely an increase in isomerase activity would be found in this buffer as the reaction would favour disulphide bond formation and the refolding of

rdRNase would be accomplished more rapidly. Curiously, increasing the ambient temperature from room temperature to 37°C had no effect on the activity of PDI but it was lost in $\alpha_{IIb}\beta_3$. This suggests that the optimal temperature for this assay to detect isomerase activity in $\alpha_{IIb}\beta_3$ is room temperature.

Other proteins, which do not belong to the thiol redox family, contain vicinyl cysteine residues. Such proteins include the gonadotrophins, lutropin and follitropin (Boniface and Reichert 1990) (Chew et al. 1995), retinal cognin (Pariser et al. 1998) fibronectin (Langenbach 1999), insulin-like growth factors (IGFs) and IGF binding protein-3 (Koedam et al 1994). All of these proteins have endogenous thiol isomerase activity that has been attributed to the presence of the CXXC motif. vWF also has vicinyl cysteines, but no attempts were made to measure thiol isomerase activity in this protein (Mayadas and Wagner 1992). The presence of vicinyl cysteines in $\alpha_{IIb}\beta_3$ and other integrins strongly suggested, therefore, the presence of an endogenous thiol isomerase activity in this family of adhesion molecules. Because of the highly conserved nature of the putative isomerase sites within all integrin β-subunits, other integrins may be predicted to have a similar activity. Purified vitronectin receptor ($\alpha_V\beta_3$), which shares a common β-subunit with $\alpha_{IIb}\beta_3$ also displays endogenous thiol isomerase activity suggesting that the β_3 chain contains the enzymatic activity.

The function of the endogenous integrin thiol isomerase activity remains unknown. However, it is attractive to suggest that it plays a physiological role in the activation-induced conformational changes of the integrins.

This is the first demonstration of an enzymatic activity within an integrin subunit. The potential for disruption of integrin function through specific inhibition of this endogenous isomerase activity offers a new therapeutic concept to supercede existing strategies of competitive antagonism of ligand binding through RGD-mimetic drugs.

CHAPTER 6

General Discussion

By virtue of the high concentrations of both fibrinogen and platelets in the plasma, the platelet integrin $\alpha_{IIb}\beta_3$ must be maintained in a resting or inactive state to ensure proper flow of blood through blood vessels. Therefore, an understanding of the activation mechanism of $\alpha_{IIb}\beta_3$ is of the utmost importance. The precise mechanisms by which integrins are activated have not been fully elucidated. Both subunits of $\alpha_{IIb}\beta_3$, but particularly β_3, are unusually cysteine rich in their residue content. The contribution of these cysteines in disulphide bonds to the integrity of integrin structure and function has been proposed by many groups (Humphries 2000), (Calvete 1999), (Kouns et al. 1994). It was therefore the basis of this thesis to explore a potential role for the involvement of the disulphide bonds of $\alpha_{IIb}\beta_3$ in activation mechanisms of this receptor. $\alpha_{IIb}\beta_3$ is an excellent model for exploring mechanisms involved in the activation and deactivation of the integrin family.

In platelet functional assays, it has been shown that treatment with the reducing agent DTT, causes platelet aggregation, fibrinogen binding and $\alpha_{IIb}\beta_3$ conformational changes as was confirmed in chapter 3. Platelet preparations readily aggregated in response to DTT after a lag phase of about two minutes. Platelets activated in this manner did not aggregate to the same extent as platelets activated with a physiological agonist such as ADP. Nonetheless, DTT induced aggregation was enhanced upon the addition of exogenous fibrinogen. This result suggests that $\alpha_{IIb}\beta_3$ had undergone the conformational changes required to allow soluble fibrinogen to bind to the receptor. This was confirmed from the results of the fibrinogen binding assay. Conformational changes were detected in the receptor, upon treatment with DTT, by the binding of the LIBS antibody D3GP3,

which only binds to the receptor once neo-epitopes are exposed as a result of conformational changes within the receptor. It was established that DTT was acting directly on $\alpha_{IIb}\beta_3$ as PGE_1 treated platelets supported aggregation induced by this reducing agent. The DTT induced conformation(s) of $\alpha_{IIb}\beta_3$ is not equivalent to that of an activated receptor, as it is incapable of transmitting a signal into the platelet. Phosphorylation events that occur as the result of ligand bound induced conformational changes, were not observed in either DTT induced platelet aggregation or adhesion. Interestingly, DTT treatment of platelets resulted in an increase in the affinity of $\alpha_{IIb}\beta_3$ for an RGD mimetic over and above that observed with agonist activation of the platelet. Furthermore, the phosphorylation profile obtained from platelets activated with thrombin in the presence of DTT was similar to that in platelets activated by thrombin in the presence of an RGD mimetic. The implications of these results are that the rearrangement of disulphide bonds caused by this chemical reduction imposes a conformation that mimics a receptor bound by ligand at the RGD site and that the affinity of this pocket is altered. All in all, the results obtained in chapter 3 strongly support the hypothesis that a rearrangement of disulphide bonds within $\alpha_{IIb}\beta_3$ are involved in conformational changes that yield a receptor competent to bind ligand upon platelet activation.

However, this scenario still begs the question how these changes occur within the receptor? An approach to this requires consideration of the endoplasmic reticulum. In the endoplasmic reticulum, the final conformation of a protein is determined not only by its amino acid sequence but also by the involvement of a cellular component of this organelle, the enzyme protein disulphide isomerase (PDI).

Protein disulphide isomerase (PDI) catalyses thiol/disulphide interchange reactions and, depending on the target polypeptide substrate and the imposed environmental redox conditions, promotes disulphide bond formation, isomerisation or reduction (Freedman 1989). PDI does not determine the polypeptide's folding pathway, but instead makes possible the formation of the correct set of disulphide bonds by promoting rapid reshuffling of incorrect disulphide pairings. PDI is a likely candidate enzyme for rearrangement of disulphide bonds which has the potential to be a mediator of conformational changes in $\alpha_{IIb}\beta_3$. Evidence in favour of its involvement is that PDI is actually present in platelets (Essex et al. 1995) and is released from the platelet in response to platelet activation (Chen et al. 1995). The presence of PDI in platelets was confirmed in chapter 4 using both immunochemistry and molecular biology techniques. It was important to establish that PDI had a role to play in events that resulted in platelet activation and the consequent activation of $\alpha_{IIb}\beta_3$. The approach taken was to use a pharmacological inhibitor of PDI and investigate its effects on platelet functional assays.

The PDI inhibitor used in these studies was bacitracin. It was expected that bacitracin would inhibit platelet aggregation and fibrinogen binding to activated platelets assuming PDI has a role to play in the activation of the platelet integrin. This indeed was the case, as shown in chapter 4, with bacitracin inhibiting both platelet aggregation and fibrinogen binding to platelets in a dose dependent manner. Thus, bacitracin was having an effect at the cellular level and it would therefore be anticipated to do so at the integrin level. This did not happen. Bacitracin had no effect on the expression of LIBS on $\alpha_{IIb}\beta_3$ that occur as a result of the binding of the antagonist Integrilin. Interestingly, bacitracin significantly reduced the level of expression of LIBS obtained when platelets were

treated with the chelator EDTA and tended to reduce agonist (ADP) induced LIBS. It is noted here that EDTA treatment of platelets results in maximum expression of LIBS in $\alpha_{IIb}\beta_3$. It is thought that integrin bound cations are required to present the ligand binding sites in a competent conformation. Following the initial capture of ligand by the receptor, the cation present at the ligand binding site is no longer required and may be displaced by the ligand (D'Souza et al. 1994). Chelation of cations by EDTA can therefore mimic ligand binding, resulting in the conformational changes that lead to a level of expression of LIBS on the receptor over and above that achieved by the binding of the mimetic. This is supported by studies that show ligand binding and cation binding sites are adjacent and overlapping, and that the binding of ligand and cation is mutually exclusive. It implies that cryptic sites are exposed as a result of substantial conformational changes upon the removal of all the calcium by EDTA and hints at a secondary process that bacitracin is actually inhibiting. These were the first clues suggesting that something else was happening at the receptor level. Unfortunately, the finding that a protease contaminant is present in commercial preparations of bacitracin, means that the results of the RGD mimetic binding studies have to be considered cautiously, since these experiments were carried out on gel filtered platelets, in the absence of plasma protease inhibitors. If the isomerase inhibiting capabilities of bacitracin are greater than that of the protease activity these results are intriguing. In the presence of bacitracin the RGD mimetic could still bind to the platelets and moreover had a similar affinity to that in untreated platelets. In the ADP treated platelets the presence of bacitracin increased the number of binding sites for ligand. This data suggests that the action of bacitracin on the receptor is uncovering additional binding sites. Again there is an inference that more than one process is happening here.

At this juncture I felt it necessary to establish that there was a physical interaction between PDI and $\alpha_{IIb}\beta_3$ at the platelet surface. The approach taken was to examine this interaction by confocal microscopy. In this study it was shown that PDI did not co-localise with $\alpha_{IIb}\beta_3$ in either resting or activated platelets. This result supported the notion that the action of bacitracin on platelets was indeed revealing secondary processes. Proteins having a tetrapeptide motif containing vicinyl cysteines similar to the catalytic active site of both PDI and thioredoxin, but not members of the thiol redox family, all had intrinsic isomerase activity. This led to a closer re-examination of the sequences of both the α_{IIb} and β_3 subunits which revealed that nine such motifs were actually present in the beta subunit. Furthermore, of these nine, four were found in the highly conserved cysteine rich repeats and four of these motifs had the sequence Cys-Gly-X-Cys, identical to that of the active sites of PDI and thioredoxin. Extending this analysis to the entire integrin family it showed that each beta subunit contained up to nine such motifs, all highly conserved. These motifs are peresent in all beta subunits across species. Is it possible that $\alpha_{IIb}\beta_3$, and indeed all integrins, have enzymatic activity in the form of an isomerase activity?

To answer this question, I purified $\alpha_{IIb}\beta_3$ from platelets. Subsequently, using an RNase refolding assay designed to demonstrate the thiol isomerase capability of a protein, I showed the presence of a thiol isomerase activity in the platelet integrin $\alpha_{IIb}\beta_3$, as outlined in chapter 5. This activity is dose dependent and saturable. The catalytic capacity of $\alpha_{IIb}\beta_3$ is equivalent to that of PDI. Unlike PDI however, the isomerase activity of $\alpha_{IIb}\beta_3$ is calcium sensitive and is maximal in the absence of this cation. An

intimate connection exists between ligand and cation binding for integrin function. Activation of integrins may favour a ligand-receptor complex rather than a cation-receptor complex. A calcium bound receptor has no isomerase activity and adopts a particular conformation that does not allow the binding of soluble ligand. Once calcium is not bound to its binding site the constraint on the receptor to maintain it in a default resting state is gone. The receptor can now undergo conformational changes by means of this isomerase activity and change the local physical environment of the ligand binding pockets so that they can now bind ligand.

Furthermore, this activity is also present in two other integrins, the vitronectin receptor $\alpha_V\beta_3$ and the fibronectin receptor $\alpha_5\beta_1$. Since $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ share a common beta subunit, the implication is that the isomerase activity is present in this subunit and consequently may be assumed to be present in all beta subunits.

This characterisation of an endogenous thiol isomerase activity in the platelet integrin $\alpha_{IIb}\beta_3$ is the first demonstration of an enzymatic activity in an integrin. It suggests that disulphide bonds can be altered locally during $\alpha_{IIb}\beta_3$ activation, resulting in a receptor that is competent to bind ligand. This isomerase activity can be now be a novel target for drug therapy. By inhibiting this activity, the potential to disrupt integrin function by preventing its activation may well take the place of the competitive antagonism of RGD-mimetic drugs in the treatment of thrombosis and other integrin mediated pathologies.

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