A ROLE FOR PHOSPHATIDYLINOSITOL 3-KINASE IN PLATELET AGGREGATION

by

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We accept this thesis as conforming
to the required standard

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ABSTRACT

The aggregation of human platelets is an important physiological hemostatic event contingent upon receptor-dependent activation of the surface integrin GPIIb-IIIa and subsequent binding of fibrinogen. Previous studies have demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) is activated both prior to, and following, activation of GPIIb-IIIa in human platelets, but the role of PI 3-kinase in platelets has not been elucidated. The objective of the work contained in this thesis was to better understand the function of PI 3-kinase in platelets. I showed that the p85 subunit of PI 3-kinase associates with a tyrosine-phosphorylated 105 kDa protein. This association was dependent on GPIIb-IIIa activation and was not inhibited by PI 3-kinase inhibitors. Furthermore, the protein was not immunoreactive with several antibodies to known proteins in this weight range. PI 3-kinase inhibitors were also used to probe the dependence of thrombin-induced aggregation on PI 3-kinase. It was found that when platelets were stimulated with high concentrations of thrombin in the presence of LY294002, a competitive inhibitor of PI 3-kinase, the inhibitors had no affect on aggregation nor GPIIb-IIIa activation. However, with lower concentrations of thrombin, aggregation and GPIIb-IIIa activation were almost completely inhibited by LY294002. These results indicate that aggregation and GPIIb-IIIa activation is less dependent on PI 3-kinase activity when high concentrations of agonist are used, indicating that there are PI 3-kinase-independent pathways that can function under these conditions. Finally, the role of the SH2 domain containing inositol 5-phosphatase (SHIP) in regulating the products of PI 3-kinase in platelets was investigated using mice with a targeted gene disruption. It was found that the level of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃] was increased in SHIP⁻/⁻ platelets in response to Collagen Related Peptide (CRP) whereas formation of phosphatidylinositol 3,4-bisphosphate [PI(3,4)P₂] was reduced demonstrating a role for the 5-phosphatase in the metabolism of PI(3,4,5)P₃. I also demonstrated that the elevation of PI(3,4,5)P₃ was insufficient to
activate phospholipase C but it enhanced the elevation of the protein tyrosine kinase, Btk, and calcium influx.
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<table>
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<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CRP</td>
<td>collagen-related peptide</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>GP</td>
<td>glycoprotein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>protease activated receptor</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
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<tr>
<td>PGE₁</td>
<td>prostaglandin E₁</td>
</tr>
<tr>
<td>PGI₂</td>
<td>prostaglandin I₂</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PKG</td>
<td>protein kinase G</td>
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<tr>
<td>PMA</td>
<td>phorbol myristic acid</td>
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<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholipase A</td>
</tr>
<tr>
<td>RGDS</td>
<td>Arginine-Glycine-Aspartic Acid-Serine</td>
</tr>
<tr>
<td>RGES</td>
<td>Arginine-Glycine-Glutamic Acid-Serine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>src-homology-2</td>
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<tr>
<td>SHIP</td>
<td>SH2-domain containing 5'-inositol phosphatase</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>U</td>
<td>unit (National Institutes of Health unit definition)</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WM</td>
<td>wortmannin</td>
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Béatrice, tu es la lune et les étoiles dans mes nuits. Tu es le soleil dans mes journées.
To Nana
CHAPTER 1: INTRODUCTION

1.1. Overview

Our understanding of platelets has progressed considerably since they were first described in the middle of the 19th century as unknown dust particles on a blood smear. Despite this relatively late recognition, the critical role of platelets in hemostasis (the arrest of hemorrhage at the site of blood vessel injury), and in the pathogenesis of thrombosis and atherosclerosis has been irrefutably established. However, platelets are still better understood in macroscopic terms than in molecular terms, although this is changing rapidly.

Since platelets are relatively simple cell fragments, terminally differentiated and non-proliferative, they have become a useful system to study signal transduction. Over the past ten years research on platelet activation has demonstrated that, in response to a variety of agonists, platelets exhibit an exquisite coordination of several transduction pathways that regulate their activation. These pathways culminate in intricate physiological, morphological, and biochemical rearrangements to the platelet, that allow them to participate in hemostasis, along with endothelium, matrix proteins, and plasma proteins of the coagulation system.

Platelet signalling events have to be viewed as integrated networks. With this in mind, signal transduction research in platelets has endeavoured to delineate the components of these pathways in order to map out a more simplified scheme of how they work. In this vein, the work contained in this thesis has focused on understanding the role of phosphatidylinositol 3-kinase (PI 3-kinase) before and after the initiation of platelet aggregation. At the time these studies were undertaken, it was known that phosphatidylinositol 3-kinase played a role in platelet activation and aggregation, however the mechanism of its action was unknown.
1.2. Platelet Physiology

The uncomplicated morphological appearance of platelets belies the rather complex functional entities that they are [Marcus and Safier, 1994]. Biochemically they are highly active cells that possess mechanisms for the recognition of sites of injury for subsequent adhesion, spreading, activation, and aggregation [Ruggeri, 1997]. Platelets normally circulate in an unstimulated “resting” state as discrete smooth discs that do not adhere to normal blood vessels nor to other platelets. However, when platelets encounter a damaged blood vessel and become activated, they undergo a drastic change from the smooth disc shape to a spiny sphere, resulting in more efficient platelet-platelet contact and adhesion of platelets to the damaged vessel surface; thus initiating the gradual occlusion of the vessel by a platelet plug, or thrombus.

A number of physiological agonists interact with specific receptors on the platelet surface to initiate the production or release of several intracellular messenger molecules, including Ca\(^{2+}\) ions, products of phospholipase C-mediated phosphoinositide hydrolysis [inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG)], thromboxane A\(_2\) (TxA\(_2\)), and cyclic nucleotides (cAMP) [Majerus, 1992; Berridge, 1993; Lapetina, 1990]. These modulate the various discernible platelet responses of Ca\(^{2+}\) mobilization, protein phosphorylation, shape change, the extrusion of the contents of secretory organelles, aggregation through the formation of fibrinogen bridges between glycoprotein (GP) IIb-IIIa (see sec. 1.2.4. below) integrin molecules of adjacent platelets, and TxA\(_2\) synthesis.

Among the mediators to which platelets respond are ADP, TxA\(_2\), thrombin, platelet activating factor (PAF), and collagen, which vary in their relative abilities to induce various responses. Of the platelet agonists, thrombin is the most potent and arguably the most physiologically important [Davey, 1967]. Moreover, thrombin is an essential component in the hemostatic, proliferative, and inflammatory responses to injury [Coughlin, 1992].
1.2.1. Ultrastructure

Resting platelets circulate in blood as discoid anuclear cells, with a mean diameter of 3.6 μm, and a mean thickness of 0.9 μm; hence they are the smallest blood cells. Although classified as cells, platelets are truly cytoplasmic fragments derived from megakaryocytes, which are found primarily in the bone marrow, but also to a lesser degree in the heart, kidney, lung and spleen [Herd and Page, 1994]. Platelets are then formed via extension of cytoplasmic processes (proplatelets) from the megakaryocyte released through sinus endothelial cells in the marrow [Gewirtz and Poncz, 1991]. A normal platelet count ranges from 1.5 - 4.4 x 10^8/ml, and the lifetime of platelets in circulation is about 10 days. The precise mechanisms regulating platelet production, release and aging are still poorly understood.

The platelet is enveloped in a typical bilamellar plasma membrane that is composed predominantly of phospholipids [Tuffin, 1991]. It is extensively invaginated throughout the platelet, forming an interconnecting network of channels called the open surface-connected canalicular system (OSCCS). The OSCCS serves to tremendously increase the surface area of the plasma membrane [White, 1974]. Another distinguishing feature of platelets is the dense tubular system (DTS), which lies in close contact with the OSCCS. It appears to support the discoid shape of the platelet [White, 1987], and is the site where calcium is sequestered and where enzymes involved in prostaglandin synthesis are localized. Glycoproteins extrude through the phospholipid bilayer forming a dense matrix referred to as the glycocalyx. Glycoproteins, of which there are at least ten different types in platelets, play a primary role in the adhesion of platelets to exposed subendothelial matrix proteins, interaction with ligands, and exposure of fibrinogen receptors to facilitate aggregation. Their intracytoplasmic tails are in close contact with elements of the intraplatelet contractile system.
Upon activation, platelets lose their discoid shape, become relatively spherical in form and develop filopodial projections (slender pseudopodia), as well as bulky projections, that compose the spiny portion of the sphere [Nachmias, 1983]. This dramatic change in morphology is regulated by the actin cytoskeleton. Actin constitutes about 20% of total platelet protein [Fox et al., 1984], and upon activation this protein is rapidly polymerized to form filaments. The filopodia that extend from the surface of activated platelets contain polymerized actin. The organization of actin filaments is regulated by their association with proteins such as tropomyosin, actin-binding proteins, and α-actinin. In addition to mediating filopodia extension, the filaments control contractile events such as granule centralization, and clot retraction [Fox and Phillips, 1982]. Platelets also contain a membrane skeleton composed of short actin filaments crosslinked by actin-binding proteins, and connected to the plasma membrane via the association with cytoplasmic domains of certain membrane glycoproteins such as integrins. This membrane skeleton stabilizes the plasma membrane, and maintains platelet shape and plasticity. In addition to actin and actin-binding proteins, the unstimulated platelet contains a circumferential band of microtubules that lies just beneath the surface of the plasma membrane, and transverses the periphery several times [Boyles, 1985]. This microtubular coil is composed of tubulin, and contributes along with the actin membrane skeleton to maintaining the discoid shape of the unstimulated platelet [White, 1971].

Among the organelles found in platelets are the unique alpha (α) and dense (δ) granules, which are platelet storage granules [Rendu et al., 1987], and those in common with nucleated cells: glycogen particles, lysosomes (λ granules), peroxisomes, and a few mitochondria. α-granules serve as major storage modalities for proteins and peptides, which play a role as growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), and thrombospondin, [Zucker and Katz,
adhesive proteins such as fibrinogen, von Willebrand factor (vWF), fibronectin, and vitronectin; and coagulation factors such as factor V, factor XI, protein S, plasminogen activator inhibitor-1 (PAI-1), and high-molecular weight kininogen (HMWK). In addition, IgG, albumin, and a 140-kDa glycoprotein referred to as P-selectin (CD62) are also present. δ-granules are rich in pro-aggregatory substances like ADP, ATP, GDP, GTP, Ca$^{2+}$, Mg$^{2+}$, and serotonin (5-HT) [Rao, 1990].

When platelets are activated, the contents of the secretory granules are then extruded by fusion of granule membranes with those of the OS CCS. P-selectin is translocated to the platelet surface and diffuses into the plasma membrane, where it mediates adhesion of platelets to neutrophils, monocytes, and subsets of lymphocytes [Lorant et al., 1993]. Whereas dense granules are easily secreted, α-granule secretion requires higher agonist concentrations, while lysosomal or λ-granule translocation only occurs with potent activating agents such as thrombin or high doses of collagen. In addition to the contents of the three types of granules, strong agonists also induce platelets to produce and secrete TxA$_2$ and PAF during their activation and aggregation, establishing a positive feedback mechanism.

1.2.2. The Role of Platelets in Hemostasis

The hemostatic apparatus consists of blood vessels, platelets, and plasma coagulation and fibrinolytic factors and their inhibitors. This apparatus is usually quiescent but is set in motion within seconds after blood vessel injury. Three mechanisms essential for normal hemostasis act in concert at the site of injury to curtail bleeding: (1) vessel wall contraction, (2) platelet hemostatic plug formation, and (3) formation and maintenance of fibrin (blood coagulation). The initial response to the interruption of continuity of a blood vessel is defined as primary hemostasis. This near-instantaneous plugging of a hole in the
vessel wall, is achieved by a combination of vasoconstriction, and platelet adhesion and aggregation, but does not yet involve the proteins of the coagulation system [Ruggeri and Ware, 1993].

1.2.2.1. Adhesion

At a site of arterial injury, the endothelial barrier separating the subendothelial matrix (which consists of a variety of proteins such as collagens, fibronectin, laminin, and microfibrils) and the blood, is broken. Platelets instantly adhere to these subendothelial components, especially collagen. Adhesion and platelet aggregation as it occurs in vivo is shear stress-dependent. Under conditions of slow blood flow (low shear rates) adhesion is mediated primarily by platelets binding directly to collagen, fibronectin, surface-bound fibrinogen, and laminin, via their respective integrin receptors [Plow and Ginsberg, 1991]. Most of these adhesive proteins contain the RGD sequence as their cell recognition site [Ruoslahti and Pierschbacher, 1987]. Under conditions of high shear stress that occur in small vessels or in larger ones that are partially occluded, these interactions are insufficient to support adhesion and the presence of vWF is mandatory. vWF in the subendothelial matrix and vWF from the plasma are rapidly adsorbed onto the site of damage, where they mediate further platelet adhesion by interacting with the GP Ib-IX-V receptor complex [Ruggeri, 1993].

1.2.2.2. Aggregation

Aggregation, in contrast to adhesion, requires active metabolism and the activation of signalling pathways downstream of agonist receptors. It is commonly resolved into two phases, reversible and irreversible aggregation. Weak aggregating agents or low concentrations of stronger ones cause reversible aggregation, whereas stronger stimuli cause irreversible aggregation which is associated with TxA₂ synthesis and the release reaction. Reversible aggregation is initiated with the generation of an undefined signal by
an agonist that results in the binding of soluble fibrinogen to GPIIb-IIIa. In the resting state, GPIIb-IIIa can bind surface-bound fibrinogen, but cannot bind soluble fibrinogen. As a consequence of platelet activation, GPIIb-IIIa converts to an active conformation (via a process called inside-out signalling), leading to the bridging of proximal platelets via dimeric fibrinogen. On activation, GPIIb-IIIa also acquires the capacity to bind other adhesive proteins, including soluble vWF, and the surface-bound proteins fibronectin and vitronectin. The result is a relatively unstable local accumulation or aggregate of activated platelets, which is not dependent on granule secretion. Without further stimulation platelets will dissociate from this loose aggregate leading to the dissolution of the developing thrombus.

Irreversible aggregation is associated with (or dependent upon) secretion, and is thought to be due to stabilization of the fibrinogen bridges by thrombospondin released from α-granules [Leung and Nachman, 1986]. The activated platelet releasate is also responsible for the “recruitment phase” of thrombus formation which results in further platelet activation, enhancement of aggregation, and augmentation of the hemostatic plug as an occlusive entity for control of blood loss [Nachman et al., 1987]. The most important platelet agonists in the releasate are ADP and TxA$_2$ [Marcus and Safier, 1993]. ADP released from dense granules and TxA$_2$ produced via the eicosanoid pathway, serve as autocrine activators as well as activating and recruiting new platelets to the growing thrombus. In addition, metabolically intact erythrocytes arriving at the site of injury respond to the platelet releasate by producing pro-aggregatory substances themselves [Valles et al., 1993].

The dramatic shape change that platelets undergo after activation contributes in several ways to hemostasis. Pseudopodal extensions increase the effective collision diameter and the surface to volume ratio, increasing the probability of contact between platelets. Shape change also promotes rearrangement of platelet membrane
phospholipoprotein components which provides a catalytic surface for the assembly and activation of enzyme complexes of the coagulation cascade. These interactions culminate in the formation of thrombin, which amplifies the initial activating effect of collagen. Platelet activation and recruitment are maximally reinforced, resulting in further release, eicosanoid production, and the rapid generation of an insoluble meshwork of fibrin which is necessary to consolidate and confer permanence to the primary hemostatic plug.

1.2.3. Glycoprotein IIb-IIIa

The platelet fibrinogen receptor, glycoprotein IIb-IIIa or $\alpha_{\text{IIb}}\beta_{3}$, is a member of the integrin family of heterodimeric receptors that mediate cell-cell and cell-extracellular matrix interactions. A normal human platelet contains about 100,000 GPIIb-IIIa complexes, which represents approximately 1% to 3% of total platelet protein [Isenberg et al., 1987]. Of these complexes about 80% are randomly distributed on the external plasma membrane [Calvette et al., 1986]. The other GPIIb-IIIa pool is cryptic and located in the OS CCS and the inner membrane of the $\alpha$-granules, but becomes surface available upon platelet activation. It is interesting that, although a wide variety of receptors and ligands participate in platelet adhesion to the damaged vessel wall, GPIIb-IIIa is the only receptor for aggregation. The importance of GPIIb-IIIa is illustrated when GPIIb and GPIIIa are absent or their content greatly diminished in platelets. These patients suffer from Glanzmann’s thrombasthenia, a rare autosomal recessive congenital bleeding disorder characterized by the absence of macroscopic platelet aggregation in response to all physiologic stimuli.

1.2.3.1. Structure

The $\alpha_{\text{IIb}}$ (GPIIb) and $\beta_{3}$ (GPIIIa) subunits are type I transmembrane proteins, each with a relatively large extracellular domain, a single transmembrane domain, and a short
cytoplasmic tail. GPIIb consists of a heavy chain (125 kDa) that is disulfide-linked to a light chain (22 kDa). The heavy chain is externally oriented and contains four repeating segments that bind Ca\textsuperscript{2+}. The predicted transmembrane domain and 26 residue cytoplasmic domain are located within the light chain [Phillips et al., 1988]. GPIIIa is a single-chain protein of 95 kDa, with a large extracellular domain containing 56 disulfide-linked cysteine residues and a large disulfide-linked loop [Shattil, 1993]. Regions of the extracellular domains critical for subunit assembly and membrane expression are being identified. Mutations in the GPIIa heavy chain, including deletion of each Ca\textsuperscript{2+}-binding domain, allows heterodimer formation but not surface expression [Vinciguerra et al., 1996; Kahn et al., 1996; Basani et al., 1996]. These results demonstrate that heterodimer formation per se is not sufficient for surface expression, and that the Ca\textsuperscript{2+}-binding domains are not necessary for heterodimer formation but are required for surface expression. However, Ca\textsuperscript{2+} removal by chelation induces dissociation of GPIIb-IIIa and loss of function. Other elements in the N-terminal domain may also be important to stabilize the heterodimer [Lam, 1992].

1.2.3.2. Ligand binding

GPIIb-IIIa recognizes adhesive proteins usually containing the Arg-Gly-Asp (RGD) motif, including fibrinogen, vWF, fibronectin, and vitronectin [Ruoslahti and Pierschbacher, 1986]. Since fibrinogen is present in high concentrations in plasma, it is the most important ligand for GPIIb-IIIa. It is a dimeric ligand containing three pairs of polypeptide chains (\(\alpha\), \(\beta\), and \(\gamma\)), and has four RGD sites, one at the N-terminal and one at the C-terminal end of each \(\alpha\) chain. GPIIb-IIIa also recognizes the H12 sequence, unique to fibrinogen, at the carboxy terminal of the fibrinogen \(\gamma\)-chain [Kloczczwiak et al., 1984]. The roles of the RGD sequences and the relationship between RGD and the H12 binding site on GPIIb-IIIa remain obscure. While RGD and H12 are essential for receptor-
specific binding, it is likely that other, yet unidentified, regions may also play roles in ligand/receptor interaction. Therapeutic strategies to inhibit platelet function have employed small peptides containing the RGD sequence, resulting in an effective inhibition of fibrinogen binding to GPIIb-IIIa and thus an effective inhibition of platelet aggregation [Plow et al., 1985]. Various snake venoms, most with a RGD/KGD motif, have proved to be a rich source of a wide variety of GPIIb-IIIa blocking peptides, the so-called disintegrins [Lefkovits et al., 1995].

Little or no fibrinogen is bound to resting platelets, but in the presence of agonists, at least 40 000 fibrinogen molecules bind to the platelet surface. The binding of soluble ligands can be detected within seconds of platelet activation and it reaches a steady-state within a few minutes. The initial phase of fibrinogen binding is reversible, but becomes progressively irreversible over 15-45 minutes [Fox, 1996]. Available evidence indicates that the initial, reversible phase of ligand binding to GPIIb-IIIa is due to affinity modulation of GPIIb-IIIa. Affinity modulation may result from some post-translational modification of a hypothetical regulatory protein that binds to one of the integrin cytoplasmic tails. This would lead to some change in the physical relationship between GPIIb and IIIa. Propagation of this change across the membrane would reorient the extracellular domains so as to increase access of fibrinogen to the receptor [Abrams et al., 1994]. The irreversible phase of ligand binding may be avidity modulation of GPIIb-IIIa, which implies a change in the functional affinity of the interaction between fibrinogen and GPIIb-IIIa due to rebinding or chelate effects. Avidity can be enhanced by ligand-induced changes intrinsic to the receptor [Peerschke, 1994], and receptor clustering [Fox, 1996] and internalization [Wencel-Drake et al., 1996]. Certain agents, exemplified by prostacyclin and nitric oxide, can inhibit and even reverse ligand binding. Although ligand-binding to surface-expressed GPIIb-IIIa is essential for initial, reversible platelet aggregation, the internal pools of GPIIb-IIIa can become exposed after cell activation and participate in the irreversible phase in which larger platelet aggregates form. In fact, the α-granule membrane pool of GPIIb-
IIIa may already be complexed with fibrinogen stored within these granules [Nurden, 1996]. If the surface pool of receptors on resting platelets becomes unable to bind ligand the α-granule pool may be able to support aggregation [Woods et al., 1986].

1.2.3.3. Regulation of Function

Since platelet aggregation is essential for primary hemostasis and the formation of occlusive platelet-rich thrombi, it is not surprising that the ligand binding function of GPIIb-IIIa is tightly regulated. This form of regulation, often referred to as “inside-out” signalling, involves a complex signalling network downstream of agonist receptors that focuses its output on the interconversion of GPIIb-IIIa from a receptor having low affinity/avidity for fibrinogen to one with high affinity/avidity. The tight regulation of the function of GPIIb-IIIa diminishes the likelihood of undesired platelet adhesion and aggregation in normal circumstances. While GPIIb-IIIa functions as a responsive adhesion receptor, under certain circumstances, it can also function as an initiating adhesion receptor. It mediates platelet adhesion to immobilized fibrinogen and fibrin without prior platelet activation [Savage et al., 1992]. Direct activation of GPIIb-IIIa by ligand recognition sequences is a potential mechanism for this activation-independent binding [Du et al., 1991]. However, GPIIb-IIIa does much more that just engage ligand. It also transduces signals in coordination with signals emanating from other plasma membrane receptors into the platelet resulting in post-ligand binding events, a process referred to as “outside-in” signalling [Juliano, 1996]. Outside-in signalling is triggered by ligand-induced oligomerization of GPIIb-IIIa because only multivalent ligands, such as fibrinogen, are capable of inducing the signal [Abrams et al., 1994]. This leads to secondary platelet responses such as the secretion of granule contents, the second wave of platelet aggregation, and cytoskeleton rearrangement [Peerschke, 1995].
1.3. Platelet Agonists and Receptors

In platelets, it is accepted that most, if not all conventional excitatory agonists cause platelet activation but not with equal effect. Platelet agonists are commonly referred to as either strong or weak, although the distinction between them is arbitrary. Generally, collagen, the particulate natural agonist, and thrombin, the soluble natural agonist, are considered to be the strongest direct platelet stimuli [Kroll, 1994]. Examples of agonists that can be classified as being weak, are ADP, epinephrine, and serotonin, as well as IgG. They, for the most part, play roles in the secondary aggregation induced by other aggregating agents, namely thrombin and collagen. Platelets can be rendered unresponsive to agonists by the action of inhibitory substances such as adenosine, PGI₂/PGE₁, and nitric oxide (NO).

Platelets can be acted upon by a number of physiological and pharmacological agents. Most of these agents are believed to exert their effects through the interaction with specific receptors on the platelet plasma membrane. All of the agonist receptors which interact with G proteins that have been identified to date consist of a single polypeptide with an extracellular N-terminal domain, seven hydrophobic transmembrane domains, and an intracellular C-terminal domain. Multiple sites in the cytoplasmic domains of these receptors contribute directly or indirectly to G protein coupling [Brass et al., 1997].

GPIIb-IIIa can also be activated by receptors that are not coupled to G proteins such as the Fc Receptors, and GPVI. In addition to specific interactions involving their Fab domains, IgG (secreted by α-granules) can interact with various membranes by their Fc region. Platelets possess a single class of Fcγ receptors, FcγRIIa. Clustering by this receptor induces shape change, secretion and aggregation, all typical platelet responses [Huang et al., 1992]. GPVI, which exists in a complex with FcRγ, can bind to collagen or
suitable triple-helical collagen-like peptides such as collagen-related peptide (CRP) activating platelets through a tyrosine kinase-dependent pathway [Moroi and Jung, 1997].

1.3.1. Thrombin

Thrombin, the principle and most potent soluble platelet agonist, is a multifunctional trypsin-like serine protease that exhibits both enzymatic and hormone-like properties. Several of thrombin’s functions involve cleavage of circulating substrates, such as the conversion of fibrinogen to fibrin monomers in the final stage of the coagulation cascade. [Davie et al., 1991]. In addition to its role as a potent activator of platelets [Davey and Luscher, 1967], thrombin can markedly influence the behaviour of a wide range of cell types. It causes endothelial cells to deliver P-selectin (the leukocyte adhesion molecule) to their surfaces [Hattori et al., 1989], to secrete vWF [Daniel et al., 1986], and to elaborate growth factors and cytokines [Colotta et al., 1994]. It is also a mitogen for fibroblasts and vascular smooth muscle cells [McNamara et al., 1992]. It is remarkable that one molecule can perform so many disparate roles considering the high degree of specificity necessary for the fine control of hemostasis.

1.3.1.1. Thrombin physiology and metabolism

Thrombin is synthesized in the liver and secreted into the blood as the zymogen prothrombin. Active thrombin is generated in the context of vascular injury when activation of the coagulation cascade triggers the proteolytic cleavage of prothrombin to an active protease. The resulting thrombin molecule is 39 kDa, about half the size of prothrombin, and consists of two disulfide-linked polypeptide chains; a 36 residue A (light) chain, and a 259 residue B (catalytic) chain. The B chain is homologous to trypsin but it is far more selective in that it cleaves only particular Arg-Gly bonds on certain proteins [Stubbs and Bode, 1993]. The boomerang-shaped A chain lies in a groove of the B-chain opposite to...
the active site. A noteworthy structural feature of thrombin is the anion-binding exosite patch. The patch is a series of amino acid residues remote from thrombin’s active site, which creates a positive field extending into the extramolecular space. It is able to preorient the approaching substrate by virtue of an ionic interaction with a negatively charged, acidic patch on fibrinogen, fibrin and thrombin receptors. Binding of these two areas leads to the rapid formation of a productive complex [Vu et al., 1991a].

1.3.1.2. Thrombin receptors

The preponderance of evidence supports the view that two different types of thrombin receptor are involved in thrombin-induced functions, certainly in the case of platelets. The two receptor types are now recognized to comprise of: (1) moderate affinity receptors that are well conserved and widely expressed G protein coupled, seven transmembrane domain receptors termed the protease-activated receptors (PAR); and (2) a selected population of GPIb-IX-V macrocomplexes which function as the high affinity thrombin receptor.

Several PAR receptors exist, but it seems that PAR-1 [Vu et al., 1991b] is the most important for activation of human platelets by thrombin. Two other PARs, PAR-3 and PAR-4 have been identified in mouse platelets, as well as human platelets [Ishihara et al., 1997; Xu et al., 1998]. Thrombin responses in PAR-3 deficient mice were markedly delayed and diminished but not absent, and PAR-4 activating peptides caused secretion and aggregation. Thus PAR-3 is necessary for normal thrombin responses in mouse platelets but a second PAR-4 mediated mechanism for thrombin signalling exists. Studies with PAR-activating peptides in human platelets indicate that PAR-4 also functions in human platelets, and that an analogous dual PAR receptor system consisting of PAR-1 and PAR-4 may operate in humans [Kahn et al., 1998]. A role for PAR-3 in humans has not been demonstrated.
In addition to the evidence that indicates that several PAR receptors participate in thrombin-mediated signalling, the GPIb-IX-V complex has also been implicated. Recent data show that this receptor is coupled to a phospholipase A\textsubscript{2} \( z \) isoform (a 14-3-3 protein) and signal transduction initiated by this receptor differs from that of the PAR receptors [Greco and Jamieson, 1991]. However, the role of this receptor and indeed, the identity of the high affinity receptor itself, is still a matter of debate in the literature. Recently it has been proposed that the high affinity receptor acts to regulate the local thrombin concentration at the platelet surface thus modulating platelet activation [Hayes and Tracy, 1999].

Of all the PAR receptors, PAR-1 is the most widely studied, but evidence indicates that all PAR receptors are activated in a similar way. The extracellular N-terminus of PAR-1 contains a site for cleavage by thrombin that is located between residues Arg\textsuperscript{41} and Ser\textsuperscript{42}. The region immediately C-terminal to the cleavage site forms a tethered ligand capable of activating the receptor [Vu \textit{et al.}, 1991b], apparently interacting with sites in the second extracellular loop and in the N-terminus near the first transmembrane domain [Gerszten \textit{et al.}, 1994]. The irreversibility of this proteolytic activation mechanism stands in contrast to the reversible agonist binding that activates prototypical G protein-coupled receptors. The 41 a.a. cleaved peptide that is released by this reaction has been shown to be a strong platelet agonist in its own right, however its mechanism of action is unknown [Furman \textit{et al.}, 1998]. Numerous studies with synthetic peptides (called Thrombin Receptor Activating Peptides or TRAPs) with the first 5 residues of the tethered ligand domain that bind and activate PAR-1, can mimic many of the effects of thrombin, but not all, indicating that coordinated effort of several thrombin receptors is required for a complete response to thrombin [Lau \textit{et al.}, 1994]. Activation of the thrombin receptor leads to the transduction of the signal via coupling to G proteins, and the stimulation a number of phospholipid-directed enzymes, generating both lipid-soluble and water-soluble second messengers. The structural determinants of the thrombin receptor's G protein specificity are unknown.
However, it appears that the second cytoplasmic loop confers coupling to $G_q$-like $G$ proteins [Verrall et al., 1997]. The details of these signal transduction pathways will be discussed in greater depth below with a prejudice for the effects mediated by thrombin.

1.4. Signal Transduction Pathways in Platelets

In platelets signalling occurs primarily through members of the seven transmembrane heterotrimeric ($\alpha\beta\gamma$) G protein-coupled family of receptors and through adhesion receptors (GPIIb-IIIa) that can cooperate or synergize functionally with the G protein-coupled receptors. Activation of platelets through these receptors lead to the various responses discussed above, namely cytoskeletal rearrangements, granule secretion, GPIIb-IIIa activation, and aggregation. Our knowledge of these signalling pathways is still far from complete, and although a full consideration of what is known about them is clearly beyond the scope of this review, those signalling pathways pertinent to GPIIb-IIIa activation and platelet aggregation, namely the inside-out and outside-in pathways, will be considered. As mentioned above a didactic distinction is often made between inside-out signalling, which denotes those reactions initiated by the binding of one or more agonists to their plasma membrane receptors leading to the activation of GPIIb-IIIa, and outside-in signalling which denotes reactions initiated by GPIIb-IIIa oligomerization. While it is difficult to determine the relative importance of inside-out and outside-in signalling in vivo, the former is clearly important in hemostasis, while the latter may play a role in both hemostasis and thrombosis by setting the ultimate size of the platelet thrombus.

1.4.1. Inside-out Signalling

Much remains to be elucidated about inside-out signalling and the details are likely to vary with the agonist. Since there is a discernible lag, albeit brief, between the addition
of an excitatory agonist and a change in the ligand-binding function of GPIIb-IIIa, inside-out signalling can be divided into two phases: 1) reactions that initiate and propagate the flow of information from agonists to integrin proximal effectors, which is presumably responsible for the lag; and 2) an integrin activation phase, which is presumably rapid, and mediated by integrin proximal proteins that are in direct contact with the integrin.

1.4.1.1. G proteins

The receptors for platelet activators couple to several G proteins, including the pertussis toxin-sensitive G, family, and the pertussis toxin insensitive families, G_q, G_{12} and G_{13}, which regulate different effectors [Neer, 1995]. Once activated, both G\alpha as well as G\beta\gamma become available. The main events in platelet activation are believed to be caused by activation of \beta isoforms of phospholipase C (PLC) resulting in the hydrolysis of phosphatidylinositol 4,5 bisphosphate [PI(4,5)P_2] to give inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The predominant PLC\beta isoforms in platelets are PLC\beta2 and PLC\beta3. Although both G_q-mediated release of \beta\gamma subunits [Banno et al., 1998] and the activation of G\alpha_q [Offermanns et al., 1997] can activate these PLC\beta isoforms in platelets, it would appear that G\alpha_q-mediated stimulation is the central pathway through which various platelet activators induce GPIIb-IIIa activation. Mouse platelets rendered null for G\alpha_q undergo shape change but fail to aggregate in response to thrombin, ADP, or TxA_2 receptor agonists, and the mice exhibit prolonged tail bleeding times [Offermanns et al., 1997].

Platelets contain both G proteins that are known to regulate adenylyl cyclase and therefore cAMP formation and cAMP-dependent protein kinase (PKA) activation: G_s and G_i. Traditionally, agents that increase cAMP levels in platelets, such as PGI_2, the potent
platelet activation and aggregation inhibitor, are described as having receptors that are coupled to Gs. Increased cAMP levels lead to a reduced Ca\(^{2+}\) mobilization in the DTS as well as decreased PI(4,5)P\(_2\) hydrolysis, and hence to the inhibition or reversal of platelet aggregation. Platelet aggregation is also inhibited by NO, which activates soluble guanylyl cyclase which leads to activation of cGMP-dependent protein kinase (PKG) [Freedman et al., 1997]. A common substrate for PKA and PKG is VASP, a 50-kDa protein that localizes to focal adhesion sites, binds F-actin and profilin, and regulates actin dynamics [Haffner et al., 1995]. While VASP phosphorylation correlates with the inhibition of aggregation [Eigenthaler and Walter, 1994], it is most likely that PKA and PKG exert their inhibitory effects on GPIIb-IIIa at multiple levels of signal transduction, indicating that there are several effectors of these protein-serine/threonine kinases in platelets [Shattil et al., 1998].

1.4.1.2. The role of tyrosine phosphorylation

Upon G protein activation three successive waves of protein tyrosine phosphorylation are generated. A role for tyrosine phosphorylation and tyrosine dephosphorylation in GPIIb-IIIa activation is indicated by observations that protein-tyrosine kinase inhibitors partially block fibrinogen binding [Jackson et al., 1996] and platelet aggregation, whereas inhibitors of protein-tyrosine phosphatases trigger platelet activation [Fox et al., 1996]. It has been noticed that complete inhibition of tyrosine phosphorylation was never obtained with protein-tyrosine kinase inhibitors such as tyrphostin or genistein but protein-serine/threonine phosphatase inhibitors [Fox et al., 1996]. Therefore these observations indicate that serine/threonine dephosphorylation is also necessary for platelets to be activated and would be involved in tyrosine phosphorylation [Levy-Toledano et al., 1997]. Activation of G protein-coupled receptors and an increase in tyrosine phosphorylation is associated with the temporal activation and subcellular relocation of a number of non-receptor tyrosine kinases (PTKs), including Src family
kinases (c-Src, c-Yes, Fyn, Lyn, Hck), Syk, and the FAK-related kinase, Pyk2 [Clark et al., 1994; Jackson et al., 1996; Raja et al., 1997]. Once phosphorylated many of these PTKs can then serve as scaffold proteins that bind to multiple cytoskeletal and signalling proteins [Aplin et al., 1998]. The mechanism by which G proteins couple to protein-tyrosine kinase cascades has not been determined, but the net result of the formation of these complexes is the tyrosine phosphorylation of a number of proteins including Vav (a guanine-nuclear exchange factor for the Rac GTPase) [Cichowski et al., 1996], cortactin (a cortical actin-binding protein) [Rosa et al., 1997], and PLCγ2 [Tate and Rittenhouse, 1993], which then mediate their own downstream cascades.

Further evidence for the tyrosine phosphorylation-integrin activation correlation comes from the study of FcγRIIa and GPVI. Both FcγRIIa and the FcRγ subunit associated with GPVI possess an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail [Reth, 1989]. ITAM sequences contain two variably spaced tyrosine residues. Clustering of membrane receptors promotes the phosphorylation, presumably by Src kinases, of the two tyrosine residues of the ITAM. This allows specific anchoring of the tandem SH2 domains of Syk. Syk then induces a complex set of signalling events leading to the eventual tyrosine phosphorylation and activation of PLCγ2 [Gratacap et al., 1998], and the activation of the MAP kinases via an upstream cascade involving Grb2-SOS and the Ras family of GTPases [Zhang et al., 1998b]. A number of proteins play a critical role in the activation of PLCγ2 downstream of Syk although the precise sequence of events is not established. For example, tyrosine phosphorylation and activation of PLCγ2 is inhibited completely in mice deficient in the adaptor molecule LAT (an adaptor protein critical for T-cell signalling) [Sarkar, 1998]. Furthermore, PLCγ2 activation is also reduced in platelets deficient in Btk (a Tec family protein-tyrosine kinase), a phenomenon that is regulated by PI 3-kinase [Quek and Watson, 1998].
1.4.1.3. Consequences of phospholipase activation

Most platelet agonists that activate GPIIb-IIIa will induce the hydrolysis of PI(4,5)P$_2$, forming IP$_3$ and DAG, either through Go$_q$ and PLC$_{\beta}$, or through tyrosine kinases and PLC$\gamma$ [Brass et al., 1997]. IP$_3$ functions to increase the cytoplasmic free Ca$^{2+}$ by binding to a specific receptor on the dense tubular system and initiating the release of Ca$^{2+}$ [Rink and Sage, 1990]. However this alone is not sufficient to activate GPIIb-IIIa, and it has been suggested that a Na$^+/Ca^{2+}$ exchanger may play a role in changing the sensitivity of GPIIb-IIIa to agonist [Shiraga et al., 1996]. Ca$^{2+}$ has several other targets in platelets, including calmodulin and thus myosin light chain kinase (MLCK) [Yoshida et al., 1983], calpains, and phospholipase A$_2$ (PLA$_2$) [Kramer et al., 1986].

The initial wave of DAG formation is a result of PLC activation; however, there is a second wave of DAG formation that is attributable to the hydrolysis of phosphatidylcholine (PC) by phospholipase D. The role of PLD activation in platelet activation is uncertain, but it is proposed that it has a role in the regulation of PLA$_2$ activity [Exton, 1997]. DAG, in association with Ca$^{2+}$, activates conventional PKC isoforms ($\alpha$, $\beta$, $\gamma$), leading to the GPIIb-IIIa activation, a response blocked by PKC inhibitors. Activation of PKC and phosphorylation of its intracellular substrates precede the activation of GPIIb-IIIa. Moreover, the cytoplasmic domain of GPIIIa can be phosphorylated by PKC in activated platelets in vitro. However, there is no good correlation that exists between the phosphorylated state and the activation of GPIIb-IIIa [van Willigen et al., 1996]. The major substrate for PKC in platelets is pleckstrin [Tyers et al., 1988]. Phosphorylation of pleckstrin is one of the earliest events observed to follow platelet activation in response to a variety of agonists. Phosphorylation of pleckstrin by PKC prevents the self-association of pleckstrin dimers and trimers. This in turn allows the interaction of pleckstrin with other proteins and lipids. Pleckstrin possesses regions, now referred to as pleckstrin homology domains (PH) [Haslam et al., 1993], that are present in more than 100 other proteins.
These form a major structural motif for protein:protein or protein:lipid interactions, with potential targets that include $G_{\beta\gamma}$ and $PI(4,5)P_2$ [Abrams et al., 1996; Harlan et al., 1994]. Furthermore the N-terminal PH domain of pleckstrin has been shown to inhibit $PLC_{\beta}$ and $PLC_{\gamma}$-mediated phosphoinositide hydrolysis [Toker et al., 1995]. A recent report correlates impaired inside-out signalling to GPIIb-IIIa in a patient who had platelets with defective pleckstrin phosphorylation [Gabbeta et al., 1996]. So, while no functional link between pleckstrin and GPIIb-IIIa has been established, pleckstrin phosphorylation appears to be an important event in GPIIb-IIIa activation.

1.4.1.4. The Ras and Rho family pathways

The Ras and Rho families are small GTP-binding proteins that appear to control the interplay between the actin cytoskeleton and other platelet functions [Wolthuis et al., 1998]. Platelets appear to contain most of the Ras-related proteins and proteins downstream of Ras that have been detected in other cells, including Raf-1, mitogen-activated protein kinases (MAPKs) (also known as Extracellular signal Regulated Kinases or ERKs), and PI 3-kinase. Circumstantial evidence has implicated the Rho family of GTPases, among them Cdc42, Rac, and Rho in particular, in inside-out signalling. Rac, for example, regulates thrombin-induced actin polymerization in platelets [Hartwig et al., 1995]. It had been reported by Morii et al. [1992] that incubation of platelets with C3 exoenzyme, an inhibitor of RhoA, inhibited thrombin-induced platelet aggregation. However, subsequent studies have shown that RhoA inactivation had no effect on either agonist-induced affinity modulation of GPIIb-IIIa or primary platelet aggregation. It did however, decrease the adhesion of agonist-stimulated platelets to fibrinogen, and the formation of focal adhesions during platelet spreading [Leng et al., 1998]. Given its demonstrated effects on the cytoskeleton, it is conceivable that RhoA may help to regulate the formation of large,
irreversible platelet aggregates through modulating integrin clustering [Machesky and Hall, 1996].

1.4.1.5. The cytoplasmic tails of GPIIb-IIIa

So far it has been difficult to determine how each of the molecular events discussed above switch GPIIb-IIIa into a high affinity state. While the identity of the proximate intracellular regulator of GPIIb-IIIa function has not been identified, it is known that the cytoplasmic domains of GPIIb-IIIa are critical for the activation of the integrin via intracellular signalling pathways. Structural correlations have identified potential cytoplasmic motifs in GPIIIa that regulate its activation. A single point mutation at the GPIIIa cytoplasmic domain can result in abrogation of GPIIb-IIIa activation: serine to proline inhibits GPIIb-IIIa signalling [Chen et al., 1994]. Second, site-directed mutagenesis of an aspartic acid in GPIIIa or deletions of membrane-proximal residues, disrupt potential salt bridges between GPIIb and GPIIIa, resulting in a constitutively active GPIIb-IIIa receptor [Wang et al., 1997]. Thus, it appears that changes in charge in the cytoplasmic portion of GPIIb-IIIa may have important consequences with respect to the structure and function of GPIIb-IIIa. Phosphorylation of GPIIIa on a threonine residue, which would change the charge in GPIIIa has been implicated in exposing binding sites on GPIIb-IIIa, although the role that this phosphorylation plays in GPIIb-IIIa function is unknown [Hillery et al., 1991; Lerea et al., 1999]. These results indicate a model in which the membrane-proximal portions of GPIIb and IIIa normally interact, possibly through a salt bridge, to form a hinge through which signals impacting on membrane distal tail residues are propagated across the membrane to modulate receptor affinity. Certain mutations or deletions break this hinge, leaving the receptor in a permanent high-affinity state [Hughes et al., 1996]. In unstimulated platelets the GPIIb might bind a negative regulator or interact with GPIIIa in such a way as to prevent the action of a positive
regulator. In stimulated platelets, a change in these relationships would either relieve the negative constraint or trigger the function of the positive regulator.

Several subcellular proteins have been suggested to interact with the cytoplasmic domain of GPIIb-IIIa, and a number have actually been shown to bind directly to integrin cytoplasmic tails, at least in vitro: Calreticulin [Rojiani et al., 1991] and CIB, a calcium-binding protein [Naik et al., 1997], both bind to GPIIb; β2-agonist binds to the GPIIIa cytoplasmic tail and, when overexpressed, causes fibrinogen-dependent cell aggregation [Kashiwagi et al., 1997]. GPIIb-IIIa co-immunoprecipitates with a membrane protein, integrin-associated protein (CD47) [Lindberg et al., 1994]. CD47 may be involved in GPIIb-IIIa-mediated Ca\(^{2+}\) influx [Schwartz et al., 1993]. CD9, a member of the tetraspan family of transmembrane proteins colocalizes with GPIIb-IIIa in platelet α-granule membranes and filopodia. Antibodies to CD9 can stimulate aggregation in an Fc receptor-independent manner [Slupsky et al., 1989]. Whether all these proteins are involved in the regulation of GPIIb-IIIa remains to be elucidated and will hopefully be resolved in future investigations.

1.4.2. Outside-In Signalling

A number of platelet responses are dependent on the initial binding of an adhesive ligand to GPIIb-IIIa. These include actin rearrangements to form filopodia and focal adhesions [Hartwig et al., 1996], conversion of small platelet aggregates into larger ones, spreading on a vascular adhesive matrix [Weiss et al., 1989], clot retraction [Schoenwaelder et al., 1997], and with some agonists, exocytotic secretion and vesiculation [Gemmell et al., 1993]. Even less is known about outside-in signalling than inside-out signalling, but it is clear that some of the same molecules or pathways involved in inside-out signalling are involved in outside-in signalling. Once GPIIb-IIIa becomes
engaged with fibrinogen, it can act as a nidus for the assembly of signalling complexes, that collaborate with agonist-induced pathways to effect the final platelet responses.

1.4.2.1. The role of protein tyrosine phosphorylation

The initial contact of resting platelets with soluble or immobilized fibrinogen or vWF leads to a detectable activation of Syk, Vav, and tyrosine phosphorylation of several uncharacterized proteins coincident with filopodia extension [Hartwig et al., 1996; Miranti et al., 1998]. Tyrosine phosphorylation of Syk can be induced by the simple dimerization of GPIIb-IIIa and precedes the platelet aggregation process [Clark et al., 1994]. These responses are triggered by fibrinogen binding and GPIIb-IIIa oligomerization because inhibitors of fibrinogen binding, such as RGDS or disintegrins, abolish these responses [Abrams et al., 1994]. After filopodia extension, the platelet begins to spread, flatten out, and form microaggregates, coincident with the activation of the pp60^src protein-tyrosine kinase. At this intermediate stage, punctate clusters of GPIIb-IIIa are discernable by immunofluorescence microscopy on the basal surfaces of adherent cells [Shattil et al., 1997].

Full platelet spreading or aggregation is characterized by the formation of focal adhesions, a complex protein/lipid structure that connects the extracellular matrix with the intracellular actin network via GPIIb-IIIa. Focal adhesion proteins talin [Knezevic et al., 1996] and α-actinin [Otey et al., 1990] have been shown to directly interact with the cytoplasmic domains of GPIIb-IIIa. Full spreading or aggregation is associated with the activation of a protein-tyrosine kinase called pp125^FAK (Focal adhesion kinase), as well as the tyrosine phosphorylation of several additional substrates including LAT [Sarkar, 1998], Tec (A PH containing tyrosine kinase) [Laffargue et al., 1997], and SHIP (an SH2 containing inositol 5-phosphatase) [Giuriato et al., 1997]. At this stage, one target of tyrosine phosphorylation might be the GPIIIb tail itself [Law et al., 1996]. Tyrosine
phosphorylation of FAK and the other proteins is dependent on platelet aggregation [Shattil et al., 1994], and can be inhibited by cytochalasin D, an inhibitor of RhoA-mediated actin polymerization, indicating that the activation of these proteins is dependent on an intact cytoskeleton [Lipfert et al., 1992]. Unlike integrin mediated activation of FAK and its downstream effectors, GPIIb-IIIa induced Syk and Vav tyrosine phosphorylation were found to be insensitive to inhibition of actin polymerization. Together Syk and Vav trigger lamellipodia formation in fibrinogen-adherent cells expressing GPIIb-IIIa and both Syk and Vav colocalized with GPIIb-IIIa in lamellipodia but not in focal adhesions. As multiple proteins are activated downstream of Syk and Vav, these proteins may regulate a unique integrin signalling pathway that differs from the FAK pathway in its proximity to GPIIb-IIIa. It is possible that a function of this pathway may be to promote actin polymerization, and cytoskeletal rearrangements by acting on downstream effectors like Rac and PI 3-kinase. The multiple outputs from this pathway would be expected to collaborate with other signalling pathways to effect the full range of anchorage-dependent responses of platelets.

Eventually there is a decrease in the tyrosine phosphorylation of many substrates, due to cytoskeletal recruitment and activation of protein-tyrosine phosphatases [Jackson et al., 1996], as well as calpain, and other Ca^{2+}-dependent cytoplasmic cysteine proteases that are translocated to the cytoskeleton and activated upon platelet stimulation. Platelet protein-tyrosine phosphatases (PTP) include PTP 1B, PTP H1 or MEG, SHP-1, and SHP-2 [Frangione et al., 1993; Jackson et al., 1996]. Studies have shown that pervanadate (a potent PTP inhibitor) induces platelet aggregation [Levy-Toledano et al., 1997]. The platelet stimulation is accompanied by a huge increase in tyrosine phosphorylated substrates, indicating that tyrosine phosphatase activity is essential for either maintaining platelets in an inactive state or to downregulate the activated state. Although phosphatases are implicated in negative regulation, a positive role of SHP-1 during platelet stimulation has been suggested. This might involve the activation of Src upon dephosphorylation, and indeed SHP-1 was recently found to associate with Src in thrombin-stimulated platelets.
[Falet et al., 1996]. These events are not necessarily strictly outside-in signalling events, as the involvement of phosphatases in inside-out signalling has been reported.

Although tyrosine phosphorylation has been emphasized in the above section, in reality a growing list of protein-serine/threonine protein kinases and phosphatases, adaptor molecules and other proteins have been identified in focal adhesions or have been shown to translocate to the detergent-insoluble or core cytoskeleton of aggregated platelets. For example, Rac and RhoA, members of the Rho family of small GTPases involved in actin assembly, partition to the cytoskeleton [Hartwig et al., 1995]. It can be speculated that one function of these small GTPases in outside-in signalling may be to promote an appropriate actin-based microenvironment for FAK activation [Leng et al., 1998], and certain other biochemical events such as PI 3-kinase activation [Zhang et al., 1995a] (PI 3-kinase is discussed in greater detail below.)

1.4.2.2. Focal adhesion kinase

After platelet activation FAK translocates to the cytoskeleton at focal adhesions where it serves as a docking site for signalling proteins. FAK requires both GPIIb-IIIa ligation and co-stimulatory signals from Ca\textsuperscript{2+} and PKC downstream of agonist-receptor engagement for its activation [Shattil et al., 1994]. The subcellular localization of FAK is dictated by a focal adhesion targeting region (FAT) and a possible binding site for GPIIIa [Clark and Brugge, 1995]. The protein contains six phosphorylation sites. Autophosphorylation of Tyr\textsuperscript{398} which can serve as high affinity binding sites for the SH2 domain of Src family kinases. The binding of Src also leads to the phosphorylation of Tyr\textsuperscript{925}, thereby creating a docking site for the adaptor protein Grb2 that is known to mediate Ras activation by binding of the GDP/GTP exchange factor Sos, linking FAK to the Ras/MAP kinase pathway. PI 3-kinase is also able to bind to FAK via its SH2 domain, as well as to proline-rich sequences in FAK via its SH3 domain [Guinebault et al., 1995; Kovacsovics et al., 1995b]. FAK also complexes through its proline-rich motifs with the
SH3 domains of the adaptor proteins p130cas and paxillin, as well as a Rho-GTPase activating protein GRAF [Shattil et al., 1998]. Both paxillin and p130cas are phosphorylated as a result of this interaction. Hence, in platelets FAK may play a central role in signal transduction after GPIIb-IIIa ligation or in platelet adhesion, thereby strengthening ligand-receptor interaction and coordinating further signalling.

1.4.3. Phosphatidylinositol 3-kinase and Platelets

One of the most important research fronts today for work on signal transduction in platelets concerns the factors governing the metabolism of the products of phosphatidylinositol (phosphoinositide) 3-kinases (PI 3-kinase). The PI 3-kinases comprise a family of lipid kinases that catalyzes the stereoselective transfer of a phosphate group from ATP to the D-3 position of the inositol ring in inositol phospholipids and were first identified through their association with viral oncoproteins and activated protein-tyrosine kinases [Whitman et al., 1988]. Several forms of PI 3-kinase have been identified in platelets: 1) p85/p110 [Rittenhouse, 1996], 2) p110γ [Tang and Downes, 1997] and 3) HsC2-PI 3-kinase [Zhang et al., 1998a]. Activation of PI 3-kinases is an important signalling event that has been linked causally to a variety of platelet functions, particularly aggregation. The second messengers involved in these events are thought to be PI(3,4)P₂ and PI(3,4,5)P₃, which are capable of stimulating the activity of protein kinases [Shattil et al., 1998], some PKC isoforms [Toker et al., 1994], and may act in additional ways.

1.4.3.1. Accumulation and synthesis of 3-phosphorylated phosphoinositides

The discovery of PI 3-kinase in other cell types led several groups to investigate the presence of D-3 phosphoinositides in platelets. Thrombin activation of platelets triggers synthesis of PI(3,4)P₂ and PI(3,4,5)P₃ [Kucera and Rittenhouse, 1990]. There does not
seem to be a significant change in the levels of PI(3)P in intact platelets when stimulated with thrombin. PI(3,4,5)P₃ is produced very rapidly, peaking within 20 sec of stimulation in a Ca²⁺ and GPIIb-IIIa independent manner [Sorisky et al., 1992]. The most abundant product of PI 3-kinase is PI(3,4)P₂ which is only detectable 30 sec after thrombin addition, with a rapid phase before 90 sec that is independent of GPIIb-IIIa activation, followed by a late phase (peaks 3-4 min) that is associated with GPIIb-IIIa engagement [Sultan et al., 1991]. The evidence above indicates that the levels of PI(3,4,5)P₃ and PI(3,4)P₂ are regulated differently [Guinebault et al., 1995]. Thus an important question to ask is, what proportion of D-3 phosphoinositides is due to initial agonist interaction with its platelet receptor, or GPIIb-IIIa activation and aggregation?

Although there is no consensus on the route of D-3 phosphoinositide synthesis downstream of agonist receptor activation, the weight of the literature indicates that PI(3,4,5)P₃ is generated by PI 3-kinase acting on PI(4,5)P₂ [Stephens et al., 1991; Hawkins et al., 1992; Carter et al., 1994]. The delayed generation of PI(3,4)P₂ is attributed either to a 5-phosphatase-mediated hydrolysis of PI(3,4,5)P₃ or to direct action of PI 3-kinase on PI(4)P. Activation of a PI 3-kinase with a substrate specificity for PI(3,4)P₂ has been demonstrated in several cell types, including platelets, supporting this conclusion. These studies are contrasted by a series of older studies that reported the route of synthesis to be PI(3)P to PI(3,4)P₂ to PI(3,4,5)P₃ [Cunningham et al., 1990; Cunningham and Majerus, 1990]. While a PI(3)P 4-kinase has been described in platelets [Yamamoto et al., 1990], so far no 5-kinase activity that uses PI(3,4)P₂ as a substrate has been described. It has been argued that the methodologies used by this group resulted in a misinterpretation of their results, and these studies have been ascribed less importance in recent years.

Some clarity has been garnered from results generated by looking at PI(3,4)P₂ synthesis downstream of GPIIb-IIIa activation. The synthesis of an antibody, anti-ligand-induced binding site antibody 6 (LIBS), that can directly activate GPIIb-IIIa without the involvement of inside-out signalling has enabled researchers to better understand the
synthesis of the D-3 phosphoinositides [Frelinger et al., 1991]. LIBS activated platelets demonstrate a time dependent, transient accumulation of PI(3)P, preceding an accumulation of PI(3,4)P₂. There is no increase in PI(3,4,5)P₃. Moreover, there is an increase in PI(3)P 4-kinase, PI 3-kinase and PI(4)P 3-kinase activities. However, there is no increase in PI(4,5)P₂ 3-kinase or PI 4-kinase activities. Thus it appears, most of PI(3,4)P₂ synthesis in response to aggregation by LIBS is generated via PI to PI(3)P to PI(3,4)P₂ [Banfic et al., 1998]. A model then emerges in which early generation of PI(3,4)P₂ (before GPIIb-IIIa activation) caused by thrombin receptor stimulation proceeds primarily via PI(4,5)P₂ to PI(3,4,5)P₃ to PI(3,4)P₂ and/or PI(4)P to PI(3,4)P₂. When GPIIb-IIIa is activated and engages fibrinogen, a novel pathway is activated, and PI(3,4)P₂ is generated via PI to PI(3)P to PI(3,4)P₂.

1.4.3.2. Structure and functional characterization of PI 3-kinases

The first form of PI 3-kinase to be identified in platelets was a heterodimer composed of a 110 kDa catalytic subunit with a tightly bound regulatory subunit of 85 kDa [Otsu et al., 1991], that was activated in a Rho-dependent manner. The p85 subunit possesses a number of regions with homology to recognized signalling proteins. These include a Bcr homology domain, a Src homology (SH) 3 domain, and two SH2 domains and two proline-rich regions. SH2 domains function to couple PI 3-kinase to tyrosine phosphorylated proteins containing the specific consensus sequences YMXM or YXXM [Songyang et al., 1993]. The activity of p110 appears to be regulated by its localization to the plasma membrane where its substrate is located [Kelly et al., 1993], and by allosteric modifications by the p85 subunit once p85 is bound to tyrosine phosphorylated proteins [Giorgetti et al., 1993].

Heterotrimeric G-protein regulated forms of PI 3-kinase have also been identified in platelets following observations that activation of G protein-coupled receptors in platelets
caused a rapid accumulation of PI(3,4,5)P$_3$. A Gβγ-responsive PI 3-kinase has been cloned and characterized in platelets, and is a p110γ-related PI 3-kinase (PI 3-Kγ) [Tang and Downes, 1997]. This enzyme is immunologically and biochemically distinct from the p85/PI 3-kinase, and appears to be less sensitive to the PI 3-kinase inhibitor wortmannin than p85/PI 3-kinase [Zhang et al., 1996]. It has been shown that in mammalian and insect cells the p110γ subunit exists in a heterodimer with a 101 kDa protein [Stephens et al., 1997]. There is strong evidence that the p101 "adaptor" allows liberated Gβγ subunits to activate the lipid kinase in the p110γ subunit. The PI 3-Kγ lacks the binding site for p85 and instead, contains an N-terminal PH domain. PI 3-Kγ phosphorylates all three potential phosphoinositide substrates but with a marked preference for PI(4,5)P$_2$ [Abrams et al., 1996]. Approximately 60% of thrombin-receptor-activated D-3 phosphoinositide accumulation can be attributed to PI 3-Kγ [Zhang et al., 1996].

As mentioned above, it has recently been observed that aggregation of platelets caused by the activation of GPIIb-IIIa and its consequent binding of fibrinogen, stimulates a novel pathway for synthesis of PI(3,4)P$_2$ [Zhang et al., 1998a]. Such synthesis depends upon both the generation of PI(3)P, which is sensitive to wortmannin and calpain inhibitors, and the phosphorylation of PI(3)P by a PI(3)P 4-kinase. The authors surmise from these data that a recently characterized PI 3-kinase of the Type II subclass, a C2-domain containing PI 3-kinase isoform (HsC2-PI 3-K) is present in platelets and is likely to be responsible for the stimulated synthesis of PI(3)P. Therefore, although little is known about the role of this PI 3-kinase in platelets, it appears that HsC2-PI 3-K may be an important effector for integrin-dependent signalling.
1.4.3.3. Regulation of PI 3-kinase in platelets

Several different regulatory mechanisms can act simultaneously on PI 3-kinases to control their activity in response to extracellular stimuli. The complexity of upstream regulatory (activating and inhibitory) pathways impinging on PI-3 kinase has increased greatly in the last several years. This has been compounded by the discovery of several additional isoforms of PI-3 kinase in platelets which are differentially regulated, and the fact that the regulatory mechanisms for PI 3-kinase observed in other cell systems may not readily apply to platelets. In addition, as different platelet agonists elicit different responses from platelets, it is reasonable to assume PI 3-kinase regulation may vary among agonists.

1.4.3.3.1. The role of tyrosine phosphorylation and PI 3-kinase localization

The best characterized mechanism of regulation of PI 3-kinases is the interaction of the p85 subunit bound to p110 with tyrosine phosphorylated sequences in other proteins. The use of protein-tyrosine kinase inhibitors in platelets indicates an intimate relationship between tyrosine phosphorylation and the synthesis of D-3 phosphoinositides in stimulated platelets. These inhibitors can potently inhibit the generation of PI(3,4)P\(_2\), but not PI(3,4,5)P\(_3\), as well as the cytoskeleton (more specifically, the membrane skeleton that underlies the plasma membrane) association of PI 3-kinase. This inhibition correlates with inhibition of GPIIb-IIIa activation, platelet aggregation, as well as a dramatic decrease in the amount of the p85 subunit of p85/PI 3-kinase detected in anti-phosphotyrosine immunoprecipitates. Likewise, inhibition of tyrosine phosphatases in platelets promotes PI(3,4)P\(_2\) production. Therefore tyrosine phosphorylation is necessary for PI 3-kinase to be active, and to translocate to the membrane skeleton where it has access to its membrane embedded substrates [Zhang et al., 1995a]. In the activated platelet, it is estimated that 30% of p85/PI 3-kinase is translocated to the membrane skeleton, representing about 70% of total PI 3-kinase activity [Grodin et al., 1991]. Furthermore, this activity is not dependent on newly polymerized actin because it is not blocked by cytochalasin D.
Although PI 3-kinase activity and p85 protein can be recovered from anti-phosphotyrosine immunoprecipitates from thrombin-stimulated platelets, there is no evidence that p85/PI 3-kinase is tyrosine phosphorylated itself [Guinebault et al., 1993]. The most compelling explanation of these results is that PI 3-kinase, by virtue of the SH2 domains of the p85 subunit, is being regulated by proteins that become tyrosine phosphorylated in response to agonists. Consistent with this idea, PI 3-kinase can be coprecipitated with Src [Gutkind et al., 1990], Syk [Yanagi et al., 1994], and Cbl [Saci et al., 1999] from lysates of activated platelets. The interaction of p85/PI 3-kinase with tyrosine phosphoproteins may therefore regulate PI 3-kinase activity by translocating the enzyme to the membrane skeleton where it is proximal to its substrate lipid, and also by allosteric regulation of the kinase activity, possibly by alteration of the relative orientation of the two SH2 domains.

The role of the SH3, proline-rich, and Bcr homology domains in p85 have also been implicated in regulating p85/PI 3-kinase. p85/PI 3-kinase can be activated directly by interactions between the SH3 domain and proline-rich domain of p85, and a proline-rich and SH3 domain of FAK. A GST fusion protein containing the p85 SH3 domain bound with high affinity to the microtubule-binding protein, dynamin, as well as PLCγ and Grb2 [Gout et al., 1993; Fry, 1994]. The proline-rich sequences of p85 may play a role in the interactions of PI 3-kinase with members of the Src-family of protein-tyrosine kinases [Gutkind et al., 1990]. The presence of both the SH3 domain and the proline-rich sequences in p85 subunit indicates that self-association may occur in vivo. This could represent another level of regulation. Indeed, it has been shown in vitro that the SH3 domain associates with full-length recombinant p85 and that binding of an SH3 domain to p85 promotes activation of the lipid kinase activity [Pleiman et al., 1994]. No function has yet to be attributed to the p85 Bcr homology domain. This sequence is related to those found in rhoGAP and n-chimerin, which have been shown to stimulate the hydrolysis of
GTP on Rho, Rac, and/or Cdc42 [Otsu et al., 1991]. It is tempting to speculate that the N-terminal third of p85 encompassing the SH3 domain and the Bcr regions may be involved in processing signals to or from members of the family of small G proteins.

1.4.3.3.2. The role of GTP-binding proteins

Activators of GTP-binding proteins, such as GTPγS, a non-hydrolyzable GTP analogue, have been shown to activate the accumulation of D-3 phosphoinositides in saponin-permeabilized platelets incubated with [γ-32P]-ATP [Kucera and Rittenhouse, 1990]. Subsequent work demonstrated that a small GTP-binding protein, Rho, is primarily responsible for the GTP-dependent activation of PI 3-kinase in platelets [Rittenhouse, 1995]. Rho is a GTPase that has been found to regulate certain actin rearrangements in platelets but not those required for protrusive and force-generating events [Leng et al., 1998]. These events are blocked if Rho is ADP-ribosylated by the exoenzyme C3 transferase, and overcome by the injection of exogenous Rho but not by the related small G-protein Rac [Morii et al., 1992]. Rho is immunoprecipitated from platelet cytosol by p85/PI 3-kinase-directed antibodies, but it has been shown that there is no direct binding of Rho to p85/PI 3-kinase. Therefore it is most likely that some sort of complex is present involving molecules in addition to Rho and PI 3-kinase [Fry, 1992]. Identification of specific roles for Rho is complicated because it is unclear whether Rho mediates its effects upstream or downstream of GPIIb-IIIa activation, and agonist and integrin pathways may converge at several levels. In fibroblasts it has been shown that there is a causative link between Rho and tyrosine phosphorylation of focal adhesion proteins, such as FAK. As mentioned before, in platelets FAK activation requires integrin ligation and actin polymerization and occurs concomitantly with full aggregation and spreading, conditions in which actin rearrangement by Rho has already occurred. Therefore, one can speculate that
Rho might promote an appropriate actin-based microenvironment or complex for FAK activation and therefore the activation of p85/PI 3-kinase [Gachet et al., 1997].

1.4.3.3.3. The role of PKC and pleckstrin

PKC appears to be required for the normal accumulation of the D-3 phosphoinositides in platelets [King et al., 1991]. The addition of a specific pseudosubstrate peptide, which prevents PKC activation, partially inhibits the appearance of D-3 phosphoinositides in response to either thrombin or GTPγS. This pseudosubstrate had no effect on purified PI 3-kinase. However, a partial inhibition of PI 3-kinase activity with this inhibitor indicates that the elevation of [Ca^{2+}] and the activation of PKC subsequent to receptor-mediated PLC activation are not sufficient to fully activate PI 3-kinase [King et al., 1991]. Furthermore, β-phorbol myristate acetate (βPMA), a powerful PKC activator, stimulates D-3 phosphoinositide accumulation and the shift of p85/PI 3-kinase to the membrane skeleton. In contrast PI 3-Kγ activity does not increase in the membrane skeleton in response to βPMA [Zhang et al., 1996]. Thus the activation of p85/PI 3-kinase in response to agonists such as thrombin, seems to be dependent on PKC.

βPMA treatment of platelets also promotes the rapid tyrosine phosphorylation of a number of proteins [Golden and Brugge, 1989]. Although the identities of the protein-tyrosine kinases activated in this manner are not known, it is conceivable that PKC is exerting its effects on D-3 phosphoinositide accumulation via a protein-tyrosine kinase, because tyrosine phosphorylation is an important modulator of PI 3-kinase activity [Carpenter and Cantley, 1990]. As mentioned above, in addition to tyrosine phosphorylation, one of the earliest events observed to follow platelet and PKC activation is the phosphorylation of pleckstrin. When pleckstrin is phosphorylated it greatly inhibits PI 3-Kγ activity, but not p85/PI 3-kinase activity. Pleckstrin-mediated inhibition of PI 3-Kγ
is overcome by excess Gβγ and is restricted to PI(4,5)P₂ as substrate, i.e. pleckstrin does not inhibit phosphorylation of PI(4)P or PI [Abrams et al., 1996]. Consistent with this, activation of PKC by βPMA prior to platelet lysis causes inhibition of Gβγ-stimulatable PI 3-kinase activity only with respect to PI(4,5)P₂ substrate. Thus phosphorylation of pleckstrin may constitute an important inhibitory mechanism for PI 3-Kγ mediated signalling.

1.4.3.3.4. The role of GPIIb-IIIa

GPIIb-IIIa seems to have an integral role in modulating the accumulation of PI(3,4)P₂ in response to thrombin [Sultan et al., 1991]. It was noted above that inhibition of fibrinogen binding to GPIIb-IIIa with RGD containing peptides or omission of Ca²⁺ from the medium inhibits the late thrombin-induced increases in PI(3,4)P₂ by approximately 50%. However, this effect is not seen when platelets are stimulated with βPMA implying that the activation of PI 3-kinase by PKC is a GPIIb-IIIa-independent event, or at least not secondary to fibrinogen binding. GPIIb-IIIa may thus modulate the activity of PI 3-kinase, but is not necessary for the initial activation of PI 3-kinase.

Several proteins with potential roles in the regulation of the D-3 phosphoinositides are activated in a GPIIb-IIIa-dependent fashion, including SHIP and calpains. Upon thrombin stimulation SHIP is tyrosine-phosphorylated and relocated to the membrane skeleton in an aggregation and GPIIb-IIIa-mediated mechanism [Giuriato et al., 1997]. There is an observed temporal correlation between the mobilization of SHIP in the pool of tyrosine-phosphorylated proteins, its tyrosine phosphorylation, its relocation to the membrane skeleton, and the production of PI(3,4)P₂ occurring upon thrombin stimulation. This indicates that SHIP is a potential candidate for the hydrolysis of PI(3,4,5)P₃, producing a significant amount of the PI(3,4)P₂ that accumulates. In light of the recent
observations that an HsC2 PI 3-K is active in platelets, and generates PI(3,4)P₂ from PI, the relative impact of SHIP on the accumulation of PI(3,4)P₂ is unknown. Besides SHIP, two other PI(3,4,5)P₃ 5-phosphatases have been described in platelets, which may also participate in the regulation of the level of D-3 phosphoinositides [Jackson et al., 1996]. Recent observations also implicate SHIP in mediating PI(3,4,5)P₃ regulated receptor-dependent Ca²⁺ signals. PI(3,4,5)P₃ acts as an upstream activation signal for Tec kinases, such as Btk, resulting in Tec kinase-dependent PLCγ activation and IP₃ production. This pathway is blocked when SHIP is engaged [Scharenberg et al., 1998; Pasquet et al., unpublished results].

In thrombin-stimulated platelets GPIIb-IIIa engagement triggers calpain translocation to the membrane skeleton and activation. The activation of calpains results in the selective hydrolysis of several platelet proteins including cytoskeletal proteins and signalling enzymes, and have been implicated in cytoskeleton reorganization. Activation but not redistribution of calpains requires GPIIb-IIIa engagement and platelet aggregation. Inhibition of calpain, using calpeptin, a cell permeant calpain inhibitor, in conditions where GPIIb-IIIa engagement was preserved, results in a dose dependent inhibition of PI(3,4)P₂ synthesis. In these conditions the maximal calpeptin inhibition value of the level of PI(3,4)P₂ (about 60%) was close to that obtained with RGDS alone [Montsarrat et al., 1997]. This evidence indicates that calpains are an early step in GPIIb-IIIa-mediated signalling and are involved in the regulation of GPIIb-IIIa-dependent PI(3,4)P₂ accumulation. Moreover, since calpeptin inhibits the activation of Hsc2-PI 3-K [Zhang et al., 1998a], it is possible that Hsc2-PI 3-K is a substrate for calpain. Hsc2-PI 3-K is strongly implicated in a novel PI(3,4)P₂ synthesis pathway, in conjunction with a PI 3-P 4-kinase. Calpains may directly hydrolyze and activate Hsc2-PI 3-K, therefore stimulating the synthesis of PI(3,4)P₂. However, other mechanisms involving calpain targets are equally likely to contribute to the stimulation.
1.4.3.4. Function of PI 3-kinase in platelets

Relatively little is understood about the function(s) of the PI 3-kinases in platelets. It is probable that PI 3-kinase may serve to augment or contribute to a function(s) controlled by one of the many other pathways activated by agonists in platelets. A number of lines of evidence bespeak a function for the D-3 phosphoinositides as second messengers. Central questions then are what is the relative role of PI 3-kinase in platelet function compared to the other more established activation pathways, and is activation of PI 3-kinase indispensable to any function or can its absence be compensated for by another pathway?

1.4.3.4.1. Protein recruitment

PI(3,4)P$_2$ and PI(3,4,5)P$_3$ are both localized and embedded in the plasma membrane and transduce signals, at least in part, by binding proteins via their specific PH, and SH2 domains, and recruiting them to the membrane skeleton. Several proteins are recruited in this way, such as the PH domain-containing Akt/PKB [Burgering et al., 1997], a protein-serine/threonine kinase, Grp1 (General receptor of phosphoinositides), a cytohesin-like protein that has guanine nucleotide exchange activity against the ARF-GTPase family members and is known to control the avidity of cell surface integrins [Klarulund et al., 1997], and Btk [Vármai et al., 1999]; or SH2 containing proteins Src, PLCγ1 and PLCγ2 [Gratacap et al., 1998], or p85 itself [Toker and Cantley, 1997]. A number of these proteins, such as Btk and PLCγ2, or Btk and Src have been shown to interact with one another and regulate each other's activity. Therefore D-3 phosphoinositides may modulate the assembly of signalling complexes, bringing kinases into contact with their substrates, and stimulating the activation of a number of signalling pathways.
1.4.3.4.2. GPIIb-IIIa activation

Some data on the possible functions of PI 3-kinase in platelet activation have emerged recently with the availability of the PI 3-kinase inhibitors, wortmannin and LY294002. Platelets extend filopods from their surface when acted upon by various agonists. This process is initiated by the removal of capping proteins from the ends of actin filaments (referred to as uncapping). Filopodia extension is associated with GPIIb-IIIa mediated platelet aggregation. It has been reported that outside-in signalling to actin by GPIIb-IIIa requires the activity of PI 3-kinases and that D-3 phosphoinositide production is critical for actin uncapping [Hartwig et al., 1996]. Another function of the D-3 phosphoinositides may be to sustain GPIIb-IIIa in an active form after the burst of DAG formed by the PLC pathways fades [Zhang et al., 1996]. The inhibition of PI 3-kinase activity with wortmannin fails to alter the ability of GPIIb-IIIa to bind fibrinogen after platelet activation but diminishes by about 60% the ability of PAC-1, an IgM antibody that recognizes only the active form of GPIIb-IIIa, to bind the receptor [Rittenhouse, 1996]. This finding indicates that D-3 phosphoinositides sustain GPIIb-IIIa activation by participating in receptor clustering, which promotes outside-in signalling. Whereas inhibitors of PI 3-kinase partially block agonist-induced activation of GPIIb-IIIa and platelet aggregation, alluding to a role for PI 3-kinase in inside-out signalling and GPIIb-IIIa activation [Zhang et al., 1996], it has been suggested that D-3 phosphoinositides function more to stabilize fibrinogen binding rather than activate it, therefore modulating irreversible aggregation [Kovascovics et al., 1995a]. Furthermore, accumulation of PI(3,4)P₂ is dependent on fibrinogen binding to GPIIb-IIIa, more consistent with a role for this particular lipid in outside-in signalling. The use of PI 3-kinase inhibitors has highlighted a number of platelet responses, including platelet aggregation, that appear to be controlled, at least in part, by PI 3-kinases. However, there is yet to be agreement on the underlying mechanism that is employed.
1.4.3.4.3. Effect of PI 3-kinase on PKC activation and pleckstrin phosphorylation

The D-3 phosphoinositides are also involved in modulating PKC activation and a late phase of pleckstrin phosphorylation in activated platelets by stimulating protein kinase activity [Toker et al., 1995]. When synthetic PI(3,4)P$_2$ and PI(3,4,5)P$_3$ are added to permeabilized platelets they cause wortmannin insensitive phosphorylation of pleckstrin. These lipids are also able to overcome inhibition of pleckstrin phosphorylation by wortmannin in stimulated platelets [Zhang et al., 1995b]. The pattern of pleckstrin phosphorylation activated by PI(3,4,5)P$_3$ is not distinguishable from that of pleckstrin phosphorylation in intact platelets exposed to βPMA. These findings implicate a D-3 phosphoinositide-activated protein kinase in pleckstrin phosphorylation. As mentioned earlier phosphopleckstrin may well have inhibitory consequences for Gβγ-activated PI(4,5)P$_2$-requiring signals [Abrams et al., 1996]. Thus phosphopleckstrin may cause a feedback inhibition of PI 3-Kγ-mediated production of PI(3,4,5)P$_3$.

Several studies have shown that PI(3,4)P$_2$ and PI(3,4,5)P$_3$ can activate a number of PKC isoforms, particularly the novel and atypical PKCs [Toker et al., 1994; Nakanishi et al., 1993], as well as the PKC-related kinase PRK1 [Palmer et al., 1995]. The activation of PKC in this manner may provide an alternate route to the classic pathway of PKC activation via PLC, and DAG formation. This may serve a redundant function or it may result in a sustained PKC response after DAG has been metabolized. However, the physiological relevance of activating PKC downstream of PI 3-kinase is yet to be determined.
1.5. Current Problem

As outlined above, platelets are terminally differentiated cells that play an integral role in hemostasis and wound repair activated by numerous agonists, including thrombin. The functional responses to agonist-induced platelet activation are well characterized, but the signal transduction pathways regulating these responses are not. It is being increasingly appreciated that the activation of the novel lipid kinase, the phosphatidylinositol 3-kinase, is involved in regulating an important platelet function i.e. aggregation, and is also, in turn, affected by this function. Currently little is known, however, about the mechanism of PI 3-kinase action and its relative importance in platelet aggregation. The analysis of the molecular mechanisms of PI 3-kinase activation during thrombin stimulation and the identification of targets of PI(3,4)P₂ and PI(3,4,5)P₃ in platelets will provide valuable information on the mechanism of GPIIb-IIIa regulation and platelet activation and may have implications for the development of novel intracellular strategies aimed at controlling platelet activation in vivo.

1.6. Objectives

The goals of this project were:

1. To investigate the mechanism of phosphatidylinositol 3-kinase effects on platelet aggregation.
2. To correlate inhibition of PI 3-kinase in thrombin-stimulated platelets with inhibition of aggregation to ascertain its PI 3-kinase dependency.
3. To characterize proteins that associate with, and may regulate, p85/PI 3-kinase during platelet activation.
CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and their sources

Acetic Acid
Adenosine 5’-triphosphate salt
Ammonium bicarbonate
Ammonium hydroxide
Ammonium persulphate
Ammonium phosphate
Bromophenolblue
Bovine Serum Albumin
n-butyl alcohol
Calcium chloride
CHAPS (3-[cholamidopropyl]dimethylammonio]-1-propane sulfonate
Chloroform
Coomassie Brilliant Blue R-250
Dimethylpimelimidate
Dimethyl sulfoxide
Dithiothreitol
Ethanolamine
Ethylene diamine tetraacetic disodium salt (EDTA)
Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)
Ethyl formate
Formaldehyde-solution (37%)
Glutathione-Sepharose
Glycerol
Glycine
HEPES
Hydrochloric Acid
Iodoacetamide
IPG buffer
Isopropyl-1-thio-β-D-galactopyranoside (IPTG)
LY294002
Magnesium chloride
2-Mercaptoethanol
Methanol
Methylamine (25%)
Nitric Acid (65%)
Nonidet P-40 (10% solution)
Orthophosphate, H$_3$[32P]O$_4$
Petroleum ether
Ponceau S concentrate
Potassium chloride
Potassium oxalate
Silver nitrate
Sodium azide
Sodium bicarbonate
Sodium carbonate

Fisher Scientific
Sigma
Fisher Scientific
Fisher Scientific
Fisher Scientific
Fisher Scientific
Roche-Boehringer
Fisher Scientific
Fisher Scientific
Fisher Scientific
Fisher Scientific
BioRad
Sigma
Fisher Scientific
Roche-Boehringer
Fisher Scientific
Sigma
Fisher Scientific
Sigma
Fisher Scientific
Sigma
Fisher Scientific
Pharmacia-Amersham
Roche-Boehringer
ICN
Sigma
Sigma
Fisher Scientific
Fisher Scientific
Fisher Scientific
Calbiochem
NEN radiochemicals
Fisher Scientific
Sigma
Sigma
Fisher Scientific
Fisher Scientific
Fisher Scientific
Sigma
Fisher Scientific
Fisher Scientific
Fisher Scientific
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<td>Tris(hydroxymethyl)aminomethane base</td>
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2.1.2. Platelet-specific reagents

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2.1.3. Consumables

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<tr>
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2.1.4. Protease Inhibitors

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<td>Leupeptin - Serine protease inhibitor</td>
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<tr>
<td>Pepstatin A</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phenylmethylsulphonylfluoride (PMSF) - serine protease inhibitor</td>
<td>Sigma</td>
</tr>
<tr>
<td>Soybean trypsin factor - trypsin and factor Xa</td>
<td>Sigma</td>
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2.1.5. Antibodies

4G10
CD42a
anti-p85
PAC-1

Pharmingen
Becton-Dickinson
Upstate Biotech. Inc.
Becton-Dickinson

2.1.6 Gifts

7E3 monoclonal anti-GPIIb-IIIa antibody

Collagen Related Peptide (CRP)

([GCP*{GPP*}]_{10}GCP*G_\text{N}; P* = hydroxyproline)

pGEX plasmids with GST-p85/PI 3-kinase SH2 domains

Dr. B. Coller, Mt. Sinai Medical Centre, NY
Drs. M. Barnes and G. Knight, Oxford University, UK
Dr. Melanie Welham, University of Bath, UK
2.2. Methods

2.2.1. Platelet Preparation

2.2.1.1. Human platelet preparation

Freshly drawn venous blood from healthy volunteers (with informed consent), who claimed not to have taken any medication in the previous 10 days, was collected into 0.1 vol. of anticoagulant ACD solution (25 g trisodium citrate dihydrate, 14 g citric acid and 20 g glucose per liter). The first 5 ml of drawn blood was discarded. Platelets were immediately isolated as described previously [Mustard et al., 1989]. Briefly, platelet-rich plasma was separated from citrated blood by centrifugation at 190 g for 15 min at room temperature. Prostaglandin E\textsubscript{1} was added to the platelet-rich plasma to a final concentration of 10 nM, and the platelet-rich plasma was centrifuged at 1000 g for 10 min at room temperature. The platelet-poor plasma was discarded, and the sedimented platelets were gently resuspended in a small volume (2 ml) of Tyrode's Buffer (136.9 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO\textsubscript{3}, 0.42 mM NaH\textsubscript{2}PO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5.5 mM dextrose, and 0.35% albumin) acidified to pH 6.5 with ACD, and supplemented with apyrase (3 U/ml), heparin (50 U/ml), and indomethacin (10 μM). An aliquot of the platelet suspension was taken to determine the platelet density using an automated Coulter Counter. Erythrocyte or leukocyte contamination was never greater than 0.1%. The platelet suspension was centrifuged at 1000 g for 10 min at room temperature and resuspended at a concentration of 1 x 10\textsuperscript{9} cells/ml in Tyrode's buffer (pH 7.35) containing 10 μM indomethacin. Platelets were incubated at 37°C for 30 min without stirring prior to experimentation.
2.2.1.2. Mouse platelet preparation

Murine blood was taken by cardiac puncture following carbon dioxide asphyxiation. Blood was collected into 0.1 vol. of anticoagulant ACD solution. Platelet-rich plasma was separated from citrated blood by centrifugation at 190 g for 15 min at room temperature. Platelets were isolated from platelet rich plasma by centrifugation at 200 g for 10 min in the presence of PGE₅ (10 μM) and resuspended in a modified Tyrodes’s-HEPES buffer (134 mM NaCl, 0.34 mM NaH₂PO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 1 mM MgCl₂, 5 mM glucose) acidified to pH 6.5 with ACD, and supplemented with PGE₅ (10 μM). Platelets were centrifuged at 1000 g for 10 min and resuspended at a concentration of 5 x 10⁸ cells/ml in Tyrode’s-HEPES buffer (pH 7.3) containing EGTA (1 mM) and indomethacin (10 μM). Platelets were incubated at 37°C for 30 min without stirring prior to experimentation.

2.2.2. Platelet Activation

Aggregation was determined by the turbidimetric method in a Lumiaggregometer (BioData Corp., Haverstown, PA). Platelet suspensions (0.5 ml) were transferred to a siliconized glass cuvette containing a small magnetic stir bar. After two minutes preincubation at 37°C the cuvette was placed in a measurement chamber at a stirring speed of 1100 rpm. The following agonists were added in a small volume (5 μl) and light transmission at 609 nm was recorded continuously with a potentiometric pen recorder: thrombin (final concentration 1 U/ml or 0.2 U/ml), or Collagen Related Peptide (CRP) (final concentration 1 μg/ml).

In some experiments, platelets were pretreated with one or more of the following prior to stimulation with thrombin or CRP: RGDS (0.5 mM) for 2 min at 37°C, RGES (0.5
mM) for 2 min at 37°C, 7E3 (50 μg/ml) for 30 min at 37°C, LY294002 (25 or 50 μM) for 10 min at 37°C. The stock solution of LY294002 was prepared in DMSO at a concentration of 50 mM and stored at -70 °C. Immediately before use, the stock solution was diluted in ice-cold water to provide the desired 50X working solution. The concentration of DMSO vehicle in platelet experiments never exceeded 0.1%, which by itself had no effect on the parameter being determined.

2.2.3. Expression and purification of p85/PI 3-kinase SH2 domains

The pGEX plasmid containing the N-SH2 or C-SH2 domains of p85/PI 3-kinase fused with Glutathione S-transferase (GST) were transformed into Escherichia coli DH5α. Cells were grown to an OD$_{600}$ of 0.6-0.7, and incubated with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 26°C. After pelleting, cells were lysed with 10 mg/ml lysozyme and successive freeze/thaw cycles between liquid nitrogen and a 37 °C water bath, in resuspension buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol, and 80 μg/ml phenylmethylsulfonyl fluoride and 20 μg/ml soybean trypsin inhibitor). When the solution became viscous, 1 M MgCl$_2$ and 10 mg/ml DNAase I were added for 15 min at room temperature. NP-40 was added to 1% in the lysate and the lysate was rotated for 30 min at 4 °C. Particulates were removed by centrifugation at 15 000 rpm for 30 min. The clarified lysate was incubated with glutathione-Sepharose beads for 2 h at 4 °C, washed three times with ice-cold phosphate-buffered saline (PBS) containing 0.5% NP-40, 100 mM NaCl and 5 mM 2-mercaptoethanol. The GST-fusion proteins were analyzed by resolving them on 10% SDS-PAGE gels, which were then stained with Commassie Blue. The GST-fusion proteins were stored at 4°C in PBS with 0.05% NaN$_3$. 

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2.2.4. General Protein Biochemistry Techniques

2.2.4.1. Protein quantitation

A series of protein standards was prepared by adding 0 to 2 mg/ml of BSA to wells containing 200 μl of a 50:1 mixture of solutions A and B from the bicinchoninic acid protein assay kit. Concurrently, 10 μl of each sample to be quantitated was added to wells containing 200 μl of a 50:1 mixture of solutions A and B. After incubating the wells at 37 °C for 30 min, the optical density was measured at 450 nm and the readings were analyzed by linear regression.

2.2.4.2. Immunoprecipitation

At the end of the activation period, platelets were lysed in an equal volume of ice-cold NP-40 solubilization buffer (2% [v/v] NP-40, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 20% glycerol, 4 mM EDTA, 20 mM NaF, 0.4 mM Na₃VO₄, 2 mM Na₃MoO₄, 80 μg/ml phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μM pepstatin, 20 μg/ml soybean trypsin inhibitor). Platelet lysates were rotated at 4 °C for 30 min and then sonicated, using 3 x 2 second pulses with a probe sonicator. Detergent-insoluble fractions were removed by centrifugation for 15 min at 15,000 rpm in the cold (4 °C).

Platelet lysates (500 μg) were precleared by mixing with a protein A-Sepharose, protein G-sepharose mixture for 1 h at 4 °C. Following rotation for 1 h, the beads were pelleted and the platelet lysates transferred to new microfuge tubes. Platelet lysates were immunoprecipitated with a rabbit polyclonal anti-p85α overnight at 4°C. The immunocomplexes were immobilized on 30 μl of packed protein G-Sepharose beads for an
additional hour at 4 °C. (For two-dimension electrophoresis the polyclonal anti-p85α was covalently cross-linked to protein G-sepharose using dimethylpimelimidate, and ethanolamine.) The immunoprecipitates were washed 3X with ice-cold 1X solubilization buffer containing the aforementioned protease and tyrosine phosphatase inhibitors, resuspended in 40 μl of 2X Laemelli’s Buffer containing 1% 2-mercaptoethanol, and then boiled for 5 min.

2.2.4.3. GST-p85/PI 3-kinase SH2 affinity-binding

The GST-p85 N-SH2 or C-SH2 fusion proteins were washed twice with GST buffer (150 mM NaCl, 25 mM Tris pH 7.4, and 10 mM 2-mercaptoethanol) and 0.5 % Tween-20, and then incubated with lysates from resting or thrombin-stimulated (1 U/ml) human platelets for 2 h at 4 °C. The precipitates were washed 3X with ice-cold solubilization buffer with the aforementioned protease and phosphatase inhibitors, and resuspended in 40 μl of 2X Laemelli’s Buffer containing 1% 2-mercaptoethanol, and then boiled for 5 min.

2.2.4.4. Immunoblotting

Proteins were resolved by electrophoresis on 7.5% SDS-polyacrylamide gels, and silver stained according to Morrissey [1981], or transferred to nitrocellulose membranes. The nitrocellulose membranes were then blocked with TBS (20 mM Tris-Cl; pH 7.4, 150 mM NaCl), containing 3% Bovine Serum Albumin (BSA) and 0.05% NaN₃ overnight. The primary antibody, a mouse monoclonal anti-phosphotyrosine antibody (4G10) or polyclonal anti-p85 were diluted in TBS containing 1% BSA, and 0.05% NaN₃, and incubated with the membrane for 3 h at room temperature. After extensive washing with TBS and TBS-T (0.05% Tween 20), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-coupled goat anti-mouse or goat anti-rabbit
secondary antibody diluted 1:10 000 in TBS-T. Bound antibody was detected using an enhanced chemiluminescence (ECL) detection system.

2.2.4.5. Autoradiography

The membrane to be autoradiographed was wrapped in cellophane to protect the film from moisture and contamination. The wrapped membrane was placed into a cassette with an intensifying screen. The X-ray film was exposed from 10 s to several minutes depending on the intensity of the bands and the amount of background.

2.2.4.6. Re-probing Western blots

For ECL blots, the previously developed, antibody coated membranes were stripped of bound antibody using 62.5 mM Tris-Cl; pH 6.8, 2% [w/v] SDS, 100 mM 2-mercaptoethanol, at 50°C for 30 min with intermittent agitation. The membranes were then washed with TBS-T several times and reblocked overnight with 3% BSA and 0.05% NaN₃ in TBS. The number of times the proteins on a nitrocellulose membrane were stripped did not exceed two.

2.2.5. Flow Cytometry

In the standard assay, unstirred samples (10 μl) of washed platelets (1 x 10⁸ platelets/ml) in Tyrode’s buffer supplemented with PGE₁ (10 μM) were incubated with 1 μl of a saturating concentration of either FITC conjugated PAC-1 antibody (25 μg/ml), which recognizes the activated form of GPIIb-IIIa or PerCP conjugated CD42a antibody (25 μg/ml), which recognizes both the inactive and active form. Some samples were treated with agonist (0.5 μl), in the presence of 1 mM RGDS or 1 mM RGES. The samples were
incubated for 5 min in the dark at room temperature without stirring. Samples were then
diluted twenty-fold in Tyrode’s buffer. No washing steps were required. Platelets were
analyzed using a Coulter Epics XL-MCL Cell Analysis System (Beckman-Coulter).

Platelets were distinguished from any contaminating erythrocytes and WBCs on the
basis of their forward- and side-light scatter profile. Debris or “machine noise”,
demonstrating a scatter profile distinctly smaller than that of platelets, was excluded from
the analysis by setting the appropriate forward-scatter threshold. A gate was set around the
platelets and 10 000 cells were analyzed for FITC fluorescence (fluorescence-1 [FL1]
channel) to quantitative the amount of platelet-bound PAC-1 or for PerCP fluorescence
(fluorescence-3 [FL3] channel) to quantitate the amount of platelet-bound CD42a antibody.
PAC-1 binding was expressed as the percentage of platelets positive for PAC-1. PAC-1-
positive cells were defined as those platelets with a fluorescence intensity >99.0% to
99.5% of unstimulated platelets. CD42a staining was expressed in arbitrary fluorescence
units as the mean fluorescence intensity per platelet. The light scatter and the fluorescence
signals were set in logarithmic gain.

2.2.6. Detection and Analysis of Intracellular Phosphoinositides

2.2.6.1. Labelling of platelets with $^{32}$P-orthophosphate

Platelets, isolated as above, were resuspended at 5 x 10$^9$ platelets/ml in phosphate
and calcium free Tyrode’s-HEPES buffer, pH 7.0 and were incubated with 1 mCi/ml of
H$_3$[$^{32}$P]O$_4$ for 1 h at 37 °C with occasional mixing. Excess label was removed by
centrifugation at 1 000 g, and the platelets washed twice at room temperature with fresh
medium. The platelets were then resuspended at 1 x 10$^9$ platelets/ml in phosphate
containing Tyrode’s-HEPES buffer + 1 mM Ca$^{2+}$ + 10 μM indomethacin, and allowed to
“rest” for 15 min at 37°C. Platelet suspensions were stimulated with agonist at 37 °C, and
reactions were terminated by adding 50 μl of 1 N HCl followed by chilling in an ice-bath for 15 min.

2.2.6.2. Extraction of total cellular lipids

Total platelet phospholipids were extracted by a modification of a method previously described by Gold et al.[1994]. Briefly, chilled platelet suspensions were transferred to 15 ml polypropylene tubes then vortexed for 1 min with 1.88 ml 2:1 MeOH:CHCl₃ (v:v) and allowed to stand at room temperature for 20 min. Phases were separated by vortexing the mixture with 0.63 ml H₂O, then 0.63 ml CHCl₃, followed by centrifugation. The lower phase was recovered and the residual upper phase re-extracted with 0.5 ml 2:1 CHCl₃:MeOH (v:v) and the organic layers combined. Two washes of the organic layer with 0.5 ml 1:0.9 MeOH:0.1 M EDTA (v:v) to remove divalent cations were performed. The extract was transferred to glass tubes and dried under nitrogen.

2.2.6.3. De-acylation and high performance liquid chromatography (HPLC) analysis of glycerophosphoinositides

To measure phosphoinositides, the lipid film was dissolved in 1.8 ml of methylamine reagent (MeOH:25% methylamine:n-butyl alcohol; 45.7:42.8:11.4 v:v:v) and incubated for 50 min at 53 °C. The methylamine reagent deacylates the lipids leaving the polar glycerophosphoinositide head group. The mixture was dried in vacuo, resuspended in water, and dried a second time. The dried samples were re-dissolved in 2 ml of water, extracted three times with 2 ml of n-butyl alcohol:light petroleum ether:ethyl formate (20:4:1; v:v:v) retaining the aqueous layer each time, dried again, then resuspended in 160 μL of water and stored at -80°C until HPLC analysis. Ammonium phosphate, pH 3.8 was added to each sample to a final concentration of 10 mM. The deacylated samples were then separated on a Partisil 10 SAX ion exchange column. Following injection, the column was
washed with water for 15 min, then eluted with a 60 min linear gradient of 0-0.25 M ammonium phosphate pH 3.8 followed by a 50 min linear gradient of 0.25 -1.0 M ammonium phosphate pH 3.8 at a flow rate of 1 ml/min. $^{32}$P radioactivity was quantitated by scintillation counting of 1.0 ml column fractions using scintillation fluid. The identity of $^{32}$P-glycerophosphoinositide (3,4) bisphosphate and $^{32}$P-glycerophosphoinositide (3,4,5) trisphosphate were confirmed by their elution time (72 min and 99 min, respectively) relative to those for ADP and ATP (internal standards).

2.2.6.4. Thin-layer chromatography (TLC) analysis of phosphatidic acid

For separation of phosphatidic acid (PA), the lipid film was dissolved in 100 µl 95:5 CHCl$_3$: MeOH and applied to the origin of a 20 cm x 20 cm oxalate-treated silica-gel TLC plate. After drying briefly, the plate was developed to the top in the following solvent system; CHCl$_3$: MeOH:10N HCl (174:26:1) (v:v). This solvent system only allows the PA to migrate. The plates were dried thoroughly in a flow hood, then exposed to autoradiography film typically for 2-4 h at -80 °C. The PA spots were excised from the TLC plate and quantified by scintillation counting. Values were routinely normalized to total $^{32}$P dpm in the extract.

2.2.7. Pleckstrin Phosphorylation

Platelets, isolated as above, were resuspended at 5 x 10$^9$ platelets/ml in phosphate and calcium free Tyrode’s-HEPES buffer, pH 7.0 and were incubated with 1 mCi/ml of H$_3$[32P]O$_4$ for 1 h at 37 °C with occasional mixing. Excess label was removed by centrifugation at 1 000g, and the platelets washed twice at room temperature with fresh medium. The platelets were then resuspended at 1 x 10$^9$ platelets/ml in phosphate containing Tyrode’s-HEPES buffer + 1 mM Ca$^{2+}$ + 10 µM indomethacin, and allowed to
"rest" for 15 min at 37°C. Platelet suspensions were stimulated with agonist at 37°C, and reactions were terminated by adding an equal volume of ice-cold NP-40 solubilization buffer (2% [v/v] NP-40, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 20% glycerol, 4 mM EDTA, 20 mM NaF, 0.4 mM Na₃VO₄, 2mM Na₃MoO₄, 80 µg/mL phenylmethysulphonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µM pepstatin, 20 µg/ml soybean trypsin inhibitor). Detergent-insoluble fractions were removed by centrifugation for 15 min at 15,000 rpm in the cold (4°C). A 40 µl aliquot of the lysate was resuspended in 40 µl of 2X Laemelli’s Buffer containing 1% 2-mercaptoethanol, and then boiled for 5 min. Proteins were resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. The gel was dried down, and pleckstrin phosphorylation analyzed on a BioRad Molecular Imager FX with BioRad Multiphor software.

2.2.8. Two Dimensional Electrophoresis

The platelet proteins eluted from protein G-Sepharose after α-p85 immunoprecipitation were diluted 1:1 with first dimension sample buffer (9.5 M urea, 2% [w/v] CHAPS, 1% [w/v] dithiothreitol, and bromophenol blue). The sample was mixed with and a rehydration solution (8 M urea, 2% [w/v] CHAPS, 0.5% [v/v] IPG buffer, 2% [w/v] DTT, and bromophenol blue), and allowed to absorb into an Immobiline ® DryStrip precast immobilized pH gradient (IPG) gel. The platelet proteins were then fractionated in the IPG strip according to their isoelectric point (pI) using a Multiphor II Electrophoresis System by Pharmacia-Amersham (first dimension). After IEF, the IPG strip was saturated with a reducing equilibration buffer (50 mM Tris-Cl, pH 6.8, 6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 2% [w/v] DTT, bromophenol blue), and then washed in an alkylating equilibration buffer (50 mM Tris-Cl, pH 6.8, 6 M urea, 30% [v/v] glycerol, 2%
[w/v] SDS, 2.5% [w/v] iodoacetamide, bromophenol blue). The IPG strip was then layered onto SDS-PAGE (10%) to separate proteins according to their molecular mass (second dimension). The gel was then either transferred to nitrocellulose and immunoblotted by anti-phosphotyrosine (4G10) antibody, or silver stained according to Morrissey [1981].
CHAPTER 3: ROLE OF PI 3-KINASE IN PLATELET AGGREGATION

3.1. Rationale and Hypothesis

PI 3-kinase has been shown to be activated in platelets in response to thrombin [Huang et al., 1991]. While the exact role of PI 3-kinase in platelets is not known, a recent study has suggested that the activation of PI 3-kinase in platelets stimulated via the thrombin receptor agonist peptide (TRAP) is important for irreversible aggregation [Kovacsovics et al., 1995b]. The activation of the thrombin receptor can be mimicked by TRAPs, but they do not evoke all of the responses seen with thrombin. Thrombin induced PI 3-kinase activation and its effect on aggregation might therefore present some differences from that of TRAP.

Many of the proteins that are tyrosine phosphorylated upon thrombin stimulation of platelets remain to be identified. Since association of PI 3-kinase with tyrosine-phosphorylated proteins is a major mechanism for activation of this enzyme, characterizing proteins that may associate with PI 3-kinase might lead to a greater understanding of its role and mechanism of action in platelets.

3.2. Results

3.2.1. Functional studies

Effect of PI 3-kinase inhibitors on human platelet aggregation - LY294002 is a potent and specific PI 3-kinase inhibitor. It has an IC<sub>50</sub> of 1.4 μM, and concentrations of 50 μM and above are known to completely inhibit PI 3-kinase activity [Vlahos et al., 1994]. We first determined the dose response, for the inhibitory effect of LY294002 on
thrombin-induced aggregation. The platelet aggregation procedure was performed on a turbidometric aggregometer. In this instrument, changes in aggregation are recorded as platelet-rich plasma and aggregating reagents are stirred together in a cuvette. The aggregometer serves as a standardized spectrophotometer. As aggregation proceeds, more light passes through the sample. Stirred platelets, pre-incubated with increasing doses of LY294002 for 15 min, were stimulated in an aggregometer with increasing concentrations of thrombin. An effect of LY294002 on aggregation was found to be very dependent upon the concentration of thrombin used (Figure 3.1). When platelets were stimulated with high concentrations of thrombin (i.e. greater than 1 U/ml) they aggregated in the presence of all concentrations of LY294002 used. This platelet aggregation was irreversible. For example, when 1 U/ml thrombin was used, no inhibition of platelet aggregation was detected at concentrations of LY294002 of up to 100 μM. However, at low concentrations of thrombin (0.25 U/ml and lower) increasing the dose of LY294002 led to a progressive and eventual abrogation of platelet aggregation. For example when platelets were stimulated with 0.25 U/ml thrombin in the presence of 50 μM LY294002, the percent light transmission (percent aggregation) was less than 10%. It appeared that in the presence of concentrations of LY294002 that are known to completely inhibit PI 3-kinase activity, low doses of thrombin (less than 0.25 U/ml) were not sufficient to induce platelet aggregation, as compared to high doses (greater than 0.5 U/ml).

Effect of LY294002 on PI 3-kinase activity - LY294002 was tested on platelets for its ability to block the thrombin-induced increase in PI(3,4)P₂ and PI(3,4,5)P₃. Platelets were labelled for 60 min with ³²P-orthophosphate in phosphate free Tyrode’s buffer, and preincubated with 50 μM LY294002 for 15 min. Platelets were activated by 0.2 U/ml thrombin or 1.0 U/ml thrombin for 2 min under aggregating conditions, and the optical density of the platelet suspension recorded (Figure 3.2). Consistent with the dose response
Figure 3.1. Effect of PI 3-kinase inhibition on thrombin-induced human platelet aggregation. Washed platelets were preincubated with varying doses of LY294002 for 15 min. Platelets were activated with the indicated concentrations of thrombin under stirring conditions. The curves were generated from aggregometer tracings and are representative of five experiments. Results are shown as a mean ± standard deviation.
LY294002 LIM
Thrombin Doses:

- □ 2 U/ml
- ▲ 1 U/ml
- ○ 0.5 U/ml
- ▼ 0.25 U/ml
- ▣ 0.125 U/ml
- ◇ 0.0625 U/ml
Figure 3.2. Inhibition of aggregation by LY294002 depends on the dose of thrombin. Washed human platelets were preincubated with 50 μM LY294002 for 15 min. Aggregation was started by addition of either 1 U/ml thrombin or 0.2 U/ml thrombin under stirring conditions. A representative set of tracings is shown, but similar tracings have been obtained in at least 10 independent experiments. In some cases, the extent of aggregation using 0.2 U/ml thrombin did not reach the same maximal level, depending on the individual donor, but the effect of LY294002 was similar. Abbreviations are as follows: thrombin (Thr); LY294002 (LY).
data, the aggregometer tracings showed that at 1 U/ml thrombin, there was no effect of PI 3-kinase inhibitors, and the aggregation was irreversible. When 0.2 U/ml thrombin was used, aggregation was almost to the same extent, and irreversible aggregation was achieved. However, preincubation with 50 μM LY294002 could almost completely inhibit aggregation induced by 0.2 U/ml thrombin.

Stimulations were conducted for 2 min before the reaction was stopped and the lipids extracted, to allow production of PI(3,4)P$_2$, the major lipid product of PI 3-kinase activation in response to thrombin (Kucera and Rittenhouse, 1990). Following solvent extraction and deacylation, the water soluble glycerophosphoinositides were separated by HPLC as described in the Materials and Methods. Figures 3.3 and 3.4 represent the typical elution profiles for PI(3,4)P$_2$ and PI(3,4,5)P$_3$ generated in response to the experimental conditions. Preincubation of platelets with LY294002 led to the abrogation of thrombin-stimulated PI(3,4)P$_2$ and PI(3,4,5)P$_3$, irrespective of the concentration of thrombin used to stimulate the platelets (Figure 3.5). These results indicate that micromolar LY294002 totally inhibits detectable PI 3-kinase activation in response to both high and low doses of thrombin in human platelets.

Role of PI 3-kinase in the activation of GPIIb-IIIa - The sequence of events leading to platelet aggregation includes first an agonist-induced change in the extracellular domain of GPIIb-IIIa, enabling fibrinogen binding, which is followed by platelet aggregation, and outside-in signalling. To determine if the alteration of aggregation results from an inhibition of GPIIb-IIIa, we tested whether LY294002 inhibits the conformational change of GPIIb-IIIa in flow cytometry studies using the PAC-1 monoclonal antibody [Shattil et al., 1987]. This method detects changes in the final activation of GPIIb-IIIa, but not its kinetics. Human platelets were preincubated with 50 μM LY294002, and then stimulated with either...
Figure 3.3. LY294002 inhibits PI(3,4)P₂ production in human platelets. Partisil 10 SAX HPLC elution profile of deacylated PI(3,4)P₂. The asterisk indicates the elution position of PI(3,4)P₂.
Figure 3.4. LY294002 inhibits PI(3,4,5)P₃ production in human platelets. Partisil 10 SAX HPLC elution profile of deacylated PI(3,4,5)P₃. The asterisk indicates the elution position of PI(3,4,5)P₃.
Figure 3.5. LY294002 inhibits PI 3-kinase activation in thrombin-stimulated human platelets. $^{32}$P-labelled washed platelets were preincubated with 50 μM LY294002 for 15 min. Platelets were activated with the indicated concentration of thrombin under stirring conditions. The reaction was stopped after 2 min by addition of HCl. Lipids were extracted and analyzed as described under "Material and Methods." The results are expressed as a mean ± standard deviation from three experiments. Abbreviations are as follows: untreated (Un); thrombin (Thr); LY294002 (LY).
1 U/ml thrombin or 0.2 U/ml thrombin. When platelets were stimulated with 1 U/ml thrombin, LY294002 had no effect on GPIIb-IIIa activation (Figure 3.6A); it was only when a lower concentration of thrombin (0.2 U/ml) was used that blocking PI 3-kinase completely inhibited activation of GPIIb-IIIa. This indicates that PI 3-kinase has an important role in the inside-out signaling events leading to GPIIb-IIIa activation, but it is only essential at low doses of thrombin. The tetrapeptide RGDS or the monoclonal antibody 7E3, which competitively inhibit PAC-1 and fibrinogen binding, inhibited GPIIb-IIIa activation as expected. RGES, a tetrapeptide which does not prevent PAC-1 nor fibrinogen binding, did not inhibit GPIIb-IIIa activation. Total GPIIb-IIIa was measured using CD42a, a monoclonal antibody that detects both the inactive and active form of GPIIb-IIIa (Figure 3.6B). Although LY294002 inhibited GPIIb-IIIa activation by 0.2 U/ml thrombin, it did not affect up-regulation of GPIIb-IIIa exposure, indicating that the differences observed between 1 U/ml thrombin and 0.2 U/ml thrombin were not due to total surface expression of GPIIb-IIIa.

3.2.2. p105

Characterization of tyrosine-phosphorylated proteins associated with PI 3-kinase in human platelets - Platelet stimulation is accompanied by a large increase in tyrosine phosphorylated substrates as compared to resting platelets. Many of these tyrosine phosphorylated proteins remain to be identified, and it is possible that some may associate with p85/PI 3-kinase. To identify those tyrosine phosphorylated proteins that associate with p85/PI 3-kinase in thrombin stimulated platelets, lysates from human platelets stimulated with 1 U/ml thrombin or from resting platelets were immunoprecipitated with anti-p85 antibody. Western blotting with anti-phosphotyrosine (4G10) antibody showed
Figure 3.6. LY294002 inhibits the activation of GPIIb-IIIa by low doses of thrombin but not the upregulation of GPIIb-IIIa. Washed human platelets were preincubated with the indicated concentrations of LY294002 for 15 min at 37°C. Activated GPIIb-IIIa was detected by the binding of FITC-PAC-1 (A). Total GPIIb-IIIa was analyzed using CD42a (B). Staining was assessed by flow cytometry, as described under "Material and Methods." Results are expressed as the percentage of platelets positive for PAC-1 or they are expressed in arbitrary fluorescence units as the mean fluorescence intensity per platelet. The data for (A) is represented as the mean ± standard deviation from three experiments. Abbreviations are as follows: untreated (Un); LY294002 (LY).
that several proteins associated with p85/PI 3-kinase in thrombin stimulated human platelets (Figure 3.7). One of the most prominent tyrosine phosphorylated bands had an apparent molecular mass of 105 kDa. Attempts to identify this protein using antibodies against a number of potential tyrosine phosphorylated proteins having a similar molecular weight and which are known to be associated with p85/PI 3-kinase in platelets and other cell systems, have been unsuccessful. These earlier experiments ruled out Gab-1, the 110 kDa catalytic subunit of PI 3-kinase, p120 Ras-GAP, JAK-2, cbl, FAK, or integrin subunit β1 as possible candidates. Further characterization of p105 has been attempted by 2D-gel electrophoresis. 2D-gel electrophoresis separates proteins both by mass and charge, and can result in a high degree of resolution of proteins. However, technical problems with 2D-gel electrophoresis have spurred us to pursue characterizing and identifying p105 using mass spectrometry instead. p105 will be resolved by SDS-PAGE, silver stained, excised from the gel, and then trypsinized. The fragments generated by trypsinization can then be subjected to mass spectrometry to generate a mass "footprint" that is unique to each protein as well as an amino acid sequence. The mass footprint and amino acid sequence will then be cross-checked with available databases to determine whether p105 is known or has homology to known proteins.

*p105 association with p85/PI 3-kinase is increased upon thrombin stimulation* - To examine whether p105 association with p85/PI 3-kinase is constitutive or induced by thrombin stimulation of human platelets, p85 was immunoprecipitated from resting or thrombin-stimulated platelet lysates. The samples were resolved on an SDS-PAGE gel and the gel was then cut into two parts. The first half was immunoblotted with 4G10 (Figure 3.8A) and the other half was silver stained (Figure 3.8B). Silver staining showed that there is a large increase in the amount of p105 associated with p85/PI 3-kinase upon thrombin stimulation of platelets compared to that of resting platelets. There also appears to be a small amount of p105 associated with p85/PI 3-kinase in resting platelets. Furthermore the increase in association of p105 with p85/PI 3-kinase upon thrombin stimulation correlated.
Figure 3.7. p105 is a major p85/PI 3-kinase associated tyrosine-phosphorylated protein. Washed human platelets were either left untreated or treated with 1 U/ml thrombin for 2 min under constant stirring. At the end of the stimulation period, samples were solubilized, resolved on 7.5% SDS-PAGE gels, transferred to a nitrocellulose membrane, and blotted with 4G10 anti-phosphotyrosine antibody or a non immune antibody. Positions of molecular weight standards are shown on the left and the arrow indicates the position of p105. Results are representative of five experiments.
1U/ml Thrombin

116.3
97.4
66.2
45

kDa

p105

IP: αp85 control Ab

Blot: 4G10
Figure 3.8. p105 association with p85/PI 3-kinase increases upon thrombin stimulation. Washed human platelets were either left untreated or treated with 1 U/ml of thrombin for 2 min under constant stirring. At the end of stimulation period, samples were solubilized, immunoprecipitated with anti-p85 antibody, resolved on a 7.5% SDS-PAGE gel, and the gel cut in half. One half was transferred to a nitrocellulose membrane, and blotted with 4G10 anti-phosphotyrosine antibody, and the other half was silver stained. Positions of molecular weight standards are shown in the centre, and the arrows indicate the position of p105. Results are representative of three experiments.
with the observed increase in tyrosine phosphorylation of this protein with thrombin treatment.

*p105 is associated with the SH2 domain of p85 in vitro* - As p105 is heavily tyrosine phosphorylated upon thrombin treatment, the potential binding of p105 may bind to the SH2 domain of p85 was investigated. GST fusion proteins having either the C-terminal or N-terminal-SH2 domain of p85 were captured on glutathione-Sepharose beads and then incubated with lysates from human resting platelets or thrombin-stimulated platelets. After washing, the bound proteins were eluted, separated on SDS-PAGE and detected by immunoblotting with 4G10 antibody (Figure 3.9). p105 was shown to be associated with both the N-terminal and C-terminal SH2 domains of p85. GST alone did not bind any significant amount of protein non-specifically. p105 binding to the GST-C-SH2 was slightly stronger than that to GST-N-SH2. These results indicate that in vitro p105 associates with the SH2 domains of p85, although it is still possible that an intermediate protein tightly bound to p105 binds directly to the p85 SH2 domains.

*p105 tyrosine phosphorylation is coupled to GPIIb-IIIa engagement* - Addition of RGDS to thrombin-stimulated platelets abolishes aggregation by preventing fibrinogen binding to GPIIb-IIIa. This treatment inhibited the bulk of p105 phosphorylation occurring at 2 min of thrombin stimulation (Figure 3.10A). Moreover, when platelets were treated with RGES, which does not block fibrinogen binding to GPIIb-IIIa, the tyrosine phosphorylation of p105 was not inhibited. These data clearly indicate that p105 phosphorylation is dependent on platelet aggregation via GPIIb-IIIa engagement. The level of immunoprecipitated p85 was revealed by immunoblotting using anti-p85 antibody (Figure 3.10B).

*The tyrosine phosphorylation of p105 is upstream of PI 3-kinase* - The tyrosine phosphorylation of p105 promoted by thrombin was not inhibited when platelets were
Figure 3.9. Binding of p105 to GST fusion proteins of p85/PI 3-kinase. GST or GST fusion proteins containing p85 N-terminal SH2 domain (N-SH2) or p85 C-terminal SH2 domain (C-SH2) coupled to agarose beads were added to lysates of both resting and thrombin stimulated platelets (1 U/ml). The samples were resolved on SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibody. The arrow indicates the position of p105.
Figure 3.10. Tyrosine phosphorylation of p105 is dependent on GPIIb-IIIa activation. Washed human platelets were either untreated or treated with 1 U/ml thrombin under constant stirring conditions. In some cases, 0.5 mM RGDS or 0.5 mM RGES was also added to the platelets 2 min prior to stimulation with thrombin. Platelets were lysed with Nonidet P-40 buffer and immunoprecipitated with anti-p85 antibody. After resolution of the immunoprecipitated proteins using 7.5% SDS-PAGE, proteins were transferred to nitrocellulose and immunoblotted with either anti-phosphotyrosine antibody (A) or anti-p85 antibody (B). Results are representative of three experiments. Abbreviations are as follows: untreated (Un); thrombin (Thr).
A

IP: αp85
Blot: 4G10

B

IP: αp85
Blot: αp85

Un   Thr   Thr + RGDS   Thr + RGES

p105

p85
Figure 3.11. Tyrosine phosphorylation of p105 is not inhibited by LY294002. Washed human platelets were either preincubated with 50 μM LY294002 for 15 min at 37°C. Platelets were left untreated or treated with 1 U/ml thrombin for 2 min under constant stirring conditions. Platelets were lysed with Nonidet P-40 buffer and immunoprecipitated with anti-p85 antibody. After resolution of the immunoprecipitated proteins using 7.5% SDS-PAGE, proteins were transferred to nitrocellulose and immunoblotted with either anti-phosphotyrosine antibody (upper panel) or anti-p85 antibody (lower panel). Results are representative of three experiments.
IP: αp85
Blot: 4G10

IP: αp85
Blot: αp85

Thrombin       -  +  +  -
LY294002       -  -  +  +
preincubated with 50 μM LY294002 (Figure 3.11). At this concentration LY294002 is known to completely inhibit PI 3-kinase activity. These results indicate that PI 3-kinase products are not involved in the pathway that results in the tyrosine phosphorylation of p105.

3.3. Discussion

3.3.1. Functional Studies

Upon platelet activation the inside-out signalling pathways act on GPIIb-IIIa to induce binding of fibrinogen and the subsequent formation of platelet aggregates. The engagement of GPIIb-IIIa by fibrinogen leads to outside-in signalling through the integrin, which regulates a host of platelet functions. It has been demonstrated that PI 3-kinase is involved in both inside-out and outside-in signalling pathways in platelets. Several studies have indicated that blocking PI 3-kinase activation in response to agonists such as phorbol esters, lyso-phosphatidic acid, serotonin/epinephrine and TRAP can at least partially block aggregation [Kovacsiovics et al., 1995b; Yatomi et al., 1992; Zhang et al., 1995c; Shah et al., 1995]. However, there is a paucity of information about how PI 3-kinase is activated and what specific events or responses are controlled by this pathway. In these studies we have investigated the role of PI 3-kinase in human platelets aggregating in response to thrombin. Human platelets were examined for their response to thrombin by measuring aggregation, GPIIb-IIIa activation, and lipid production in terms of their sensitivity to the PI 3-kinase inhibitor, LY294002. We show that aggregation and GPIIb-IIIa activation in response to thrombin is sensitive to LY294002, but only at low or suboptimal concentrations of thrombin. We also show that when the proteins associated with PI 3-kinase in anti-p85 immunoprecipitates were analyzed by anti-phosphotyrosine
immunoblots, a tyrosine phosphorylated protein of approximately 105 kDa was present. The tyrosine phosphorylation of this protein was dependent on fibrinogen binding to GPIIb-IIIa, and on PI 3-kinase activation.

The role of PI 3-kinase activity in signal transduction can be studied by the use of selective inhibitors of this enzyme, LY294002, and wortmannin (WM). These two inhibitors are structurally and mechanistically distinct. WM is a covalent, irreversible inhibitor of PI 3-kinase [Nakanishi et al., 1992], whereas LY294002 is a competitive antagonist for the ATP-binding site of PI 3-kinase [Vlahos et al., 1994]. Interpretation of early studies using WM is complicated by the use of very high concentrations that are known to inhibit other enzymes such as myosin light chain kinase, PLD, or PI 4-kinase. Furthermore, conflicting reports have been published on the inhibition of wortmannin of pleckstrin phosphorylation in platelets, thus the issue of potential inhibition of PKC by wortmannin is a critical one [Yatomi et al., 1992; Hashimoto et al., 1992]. Although both studies failed to detect direct inhibition of PKC in vivo, Yatomi et al. reported a reduction in pleckstrin phosphorylation in response to concentrations of thrombin or PMA which failed to induce aggregation. WM was not used in the studies conducted in this thesis. Although we cannot rule out the possibility that LY294002 inhibits signalling molecules other than PI 3-kinase, several lines of evidence indicate that the functional data presented in this thesis result from a direct inhibition of PI 3-kinase. Although it is an antagonist for ATP-binding LY294002 has been shown in vivo not to inhibit other ATP-requiring enzymes, including PKC, PI 4-kinase and tyrosine kinases [Vlahos et al., 1994]. Furthermore, the fact that LY294002 and WM, two unrelated metabolic inhibitors, have been shown to have the same effects in platelets [Kovacsovics et al., 1995; Lauener et al., unpublished results], argues strongly for the selectivity of LY294002 when used at appropriate concentrations.
When the effect of LY294002 was tested on platelet aggregation, it was apparent that at 50 μM LY294002, a concentration that has been shown to completely block PI 3-kinase activity, there was little or no effect on aggregation induced by high or maximal doses of thrombin. When lower concentrations of thrombin were used, the inhibition of PI 3-kinase by LY294002 resulted in an inhibition of aggregation. Although measurement of platelet aggregation by a lumiaggregometer is an established technique, it is also an insensitive technique. Approximately 20-30% of the total platelets in suspension must form aggregates before there is a significant change in light transmission. A more sensitive technique is to measure aggregation indirectly by examining the activation state of GPIIb-IIIa. Therefore we tested whether the same parameters would have a similar effects on the conformational change in GPIIb-IIIa in flow cytometry studies using PAC-1 monoclonal antibody, which recognizes the conformational change induced in GPIIb-IIIa when platelets are activated. The binding of PAC-1 was abolished when a low dose of thrombin was used to stimulate platelets in the presence of 50 μM LY294002. There was no effect of LY294002 on GPIIb-IIIa activation induced by high doses of thrombin. The results from these two experiments indicate that PI 3-kinase plays a role in events leading to platelet aggregation, but it is a role that can be circumvented, presumably by parallel signalling pathways, when higher concentrations of thrombin are used.

The most extensive work to date using PI 3-kinase inhibitors to examine the role of PI 3-kinase in platelet aggregation has been conducted by Hartwig's group [Kovacsovics et al., 1995b]. In this work, stirred platelets, preincubated with increasing doses of WM and LY294002, were activated with 25 μM TRAP. They did not examine the effect of PI 3-kinase inhibitors on aggregation induced by a range of TRAP concentrations in this study. They observed that aggregation in the presence of WM and LY294002 was characterized by an initial aggregation response followed by a progressive disaggregation of platelets or reversible aggregation. Furthermore, they observe that there is reduced
binding of PAC-1 to GPIIb-IIIa of TRAP-stimulated platelets in the presence of WM. These authors concluded that PI 3-kinase does not seem to be required for the initiation of GPIIb-IIIa exposure, since aggregation started in the presence of WM, but rather it is required for the mechanism(s) of sustained GPIIb-IIIa activation, and thus is necessary for irreversible aggregation. Therefore our studies regarding the role of PI 3-kinase in thrombin-induced platelet aggregation appear to contrast with their findings. Parallel studies conducted in our lab have also tested the effects of PI 3-kinase inhibitors on the aggregation on human platelets in response to TRAP and PAF [Lauener et al., unpublished results]. These studies demonstrated that the effect of LY294002 on reversible aggregation observed by Kovacsovics et al. with 25 μM TRAP could be reproduced. However when low concentrations of TRAP were used, it was clear that LY294002 was able to completely block aggregation. The results with PAF are very similar to those with thrombin. Therefore we do not find that PI 3-kinase is required for irreversible aggregation, particularly in response to strong agonists such as thrombin. From our results, we conclude that PI 3-kinase activity plays a vital role in the inside-out signalling events leading to GPIIb-IIIa activation and aggregation in response to low concentrations of platelet agonists. The later events leading to irreversible aggregation may also be partially dependent on PI 3-kinase, as in the case of the TRAP studies, but again this can be bypassed by other signalling pathways.

There are several possible explanations for the differences we observe between thrombin and TRAP with regard to their dependence on PI 3-kinase in the signalling pathways leading to aggregation. It has been suggested that these differences are due to the fact that thrombin generates a fibrin net from fibrinogen released from platelets, whereas TRAP does not. It is essentially impossible to reverse aggregation when this fibrin net forms. While we cannot ignore this observation, the fact that PAF demonstrated similar effects to thrombin, and that PAF is incapable of generating fibrin from fibrinogen, it is unlikely that the differences between thrombin and TRAP are due to fibrin net formation.
Secondly one might speculate that the effects via the different thrombin receptors could provide a possible explanation. It has been demonstrated that TRAP, which only activates PAR-1, may not evoke all of the responses or even necessarily the same pattern of responses as those seen with thrombin, which activates multiple PAR receptors as well as a high affinity receptor that has not yet been identified [Brass et al., 1997]. Although much has to be learned about these differences, it must be stressed that we have demonstrated that the high/low agonist dose effects can also be observed with PAF, and the same arguments do not apply regarding the various thrombin receptors.

In short, the fundamental point of our work is that when PI 3-kinase activity and D-3 phosphoinositide production are blocked, the dependence of aggregation on these lipids can be bypassed or substituted for. However, when a less potent agonist, such as TRAP or low doses of thrombin are used, the activity of PI 3-kinase is indispensable for aggregation. Explanations for this phenomenon abound, but it is possible that the activity of a downstream enzyme regulated by PI 3-kinase is necessary for aggregation. A candidate for this enzyme might be one of the PKC family of kinases that are potentially regulated by phosphorylation pathways dependent upon PI 3-kinase, as was shown recently [Legood et al., 1998]. Additional evidence has shown that PI(3,4,5)P$_3$ is involved in the regulation of non-conventional PKC isoforms [Nakanishi et al., 1993], although this observation has not been fully substantiated [Palmer et al., 1995]. It is possible that the PKC isoform(s) may be regulated by multiple phosphorylation pathways, and thus at a certain threshold they can circumvent PI 3-kinase inhibition. An interesting concept at the receptor level was recently proposed as a possible explanation for the differences in platelet activation between high and low doses of thrombin [Hayes and Tracy, 1999]. These authors suggest that the platelet high affinity receptor binding site for thrombin allows for the regulation of local thrombin concentrations at the platelet surface thus modulating platelet activation. Thrombin sequestration at this site may provide an essential regulatory mechanism in vivo such that a threshold level of thrombin is required to initiate explosive
platelet activation responses. By sequestering low levels of thrombin at the platelet surface this site may prevent unwanted cleavage of the PAR receptors, preventing platelet activation and thrombus formation.

It should be mentioned that the role of other PI 3-kinase isoforms, notably the gamma isoform of PI 3-kinase (p110γ), were not investigated in this study. The potential contribution of this lipid kinase to the formation of the D-3 phosphoinositides cannot be overlooked. However, several studies have suggested that p85/PI 3-kinase, in preference to p110γ, contributes to activation of GPIIb-IIIa when the thrombin receptor is stimulated [Zhang et al., 1996]. It is also clear from the studies with LY294002 that the production of PI 3-kinase products was being inhibited, therefore if p110γ was playing an important role in platelet aggregation, it too was being inhibited by LY294002. Thus our arguments regarding the role of PI 3-kinase can encompass all of the isoforms.

3.3.2. p105

Activation of the thrombin receptor on human platelets leads to an extremely rapid series of events that includes the activation of tyrosine kinases. The net result is tyrosine phosphorylation of a myriad of proteins, a number of which may be expected to bind to p85/PI 3-kinase, by virtue of its SH2 domains. Indeed p85/PI 3-kinase has been shown to co-immunoprecipitate with several proteins including FAK, src, and syk [Gutkind et al., 1990; Yanagi et al., 1994; Guinebault et al., 1995].

When the proteins associated with PI3-kinase, in anti-p85 immunoprecipitates of thrombin-stimulated human platelet lysates, were analyzed by anti-phosphotyrosine immunoblots, a major tyrosine phosphorylated protein which migrated at an apparent weight of 105 kDa was identified. The increase in p105 in anti-p85 immunoprecipitates closely paralleled the elevation in anti-phosphotyrosine-associated PI 3-kinase activity.
[Lauener, unpublished results]. The tyrosine phosphorylation of this protein was dependent on GPIIb-IIIa activation and platelet aggregation, as blocking fibrinogen binding to GPIIb-IIIa with RGDS resulted in the inhibition of p105 tyrosine phosphorylation. Some tyrosine phosphorylation of this protein was seen in anti-p85 immunoprecipitates from resting platelet lysates. Despite the care taken during platelet preparation, one cannot fully exclude the occurrence of a minor non-specific activation that may explain the weak basal tyrosine phosphorylation level observed in resting conditions. This protein has not been characterized in the current literature, and is not immunoreactive with several antibodies to known proteins in this molecular weight range.

As a major mechanism for the activation of PI 3-kinase occurs through the association of p85 with tyrosine phosphorylated proteins through its SH2 domains, it was plausible that p105 might be interacting with the SH2 domain of p85. Indeed, in vitro affinity binding assays with GST fusion proteins demonstrated that p105 associates with p85/PI 3-kinase via both the N-terminal and C-terminal SH2 domains when platelets were stimulated with thrombin. p105 binding to the C-terminal SH2 domain of p85 was shown to be slightly stronger than that to the N-terminal SH2 domain. As the catalytic activity and subcellular localization of the p110 subunit of p85/PI 3-kinase are regulated through protein-protein interactions of p85, it is plausible that p105 modulates p85/PI 3-kinase in response to platelet agonists or in response to GPIIb-IIIa activation.

We could not exclude, however, that p105 is associated with p85/PI 3-kinase in resting platelets. p85/PI 3-kinase can associate with proteins by the SH3 or polyproline domains of p85. To determine whether p105 is associated with p85/PI 3-kinase prior to its tyrosine phosphorylation upon thrombin activation we analyzed anti-p85 immunoprecipitates by silver staining. Silver staining demonstrated that there is a slight association of a polypeptide co-migrating with p105 in anti-p85 immunoprecipitates in resting platelets. However upon thrombin stimulation of platelets there is a dramatic increase in this association. Furthermore, this association correlated with the tyrosine
phosphorylation of p105, supporting the notion that the interaction between p105 and p85/PI 3-kinase is mediated through the p85 SH2 domain after platelet activation and p105 tyrosine phosphorylation. As mentioned above, it is difficult to avoid some non-specific activation of platelets during their preparation, thus it is impossible to distinguish between what may be a basal association of p105 through the p85 SH3 or polyproline domains or a weak level of association mediated by SH2 domains. It remains to be elucidated whether there is some basal association between these two proteins.

To determine whether p105 tyrosine phosphorylation and association with PI 3-kinase occurred before PI 3-kinase activation, we used LY294002 to inhibit PI 3-kinase activity. We found that LY294002 had no effect on p105 tyrosine phosphorylation or on p85/PI 3-kinase association induced by thrombin, which indicates that the two events occurred upstream or independently of the lipid kinase. These data and the data mentioned above indicate that p105 may represent a novel protein, perhaps acting as an adaptor protein that may modulate PI 3-kinase activity, similar to the function of Gab-1, IRS-1 or IRS-2 in other cells. That p105 tyrosine phosphorylation was dependent on GPIIb-IIIa activation and platelet aggregation, attests to a role for this protein in GPIIb-IIIa mediated events such as full platelet spreading or aggregation of platelets. p105 may therefore be involved in the modulation of PI 3-kinase events that are not directly regulated by thrombin receptors.

A greater understanding of this protein will come from a more detailed characterization of the protein. The results presented here are still considered preliminary. Our results from attempts to purify this protein using 2D-gel electrophoresis are not definitive, and refinements are required before the results warrant further discussion. It has recently come to light that a rapid analysis of p105, excised from a single dimension gel, by mass spectrometry is now possible. We are currently exploring this avenue.
CHAPTER 4: SHIP IS A NEGATIVE REGULATOR OF PI (3,4,5)P₃ IN PLATELETS

4.1. Rationale and Hypothesis

In platelets, the production of D-3 phosphoinositides upon stimulation has been well documented. These D-3 phosphoinositides have been shown to mediate specific effects in human platelets, and therefore it is crucial to understand the mechanisms involved in their regulation. One obligatory step in their biosynthesis is the phosphorylation of the D-3 position of the inositol ring by PI 3-kinases. However, besides the PI 3-kinases, little is known about the enzymes that regulate the levels of D-3 phosphoinositides. SHIP is a 5’ phosphoinositide phosphatase which may have a direct role in PI lipid turnover. SHIP-mediated conversion of PI(3,4,5)P₃ to PI(3,4)P₂ may then have an overall positive or negative affect on platelet responses. (The following studies were the contribution of this author to a collaborative effort between our laboratory and Dr. Jean-Max Pasquet and Dr. Steve Watson at the University of Oxford, as well as the laboratory of Dr. Gerald Krystal at the University of British Columbia which provided the SHIP⁻¹ knockout mice. Only the results garnered from this author’s work will be presented, but work performed by Dr. Pasquet at Oxford will be referred to in the discussion to put the results into a contextual framework.)

4.2. Results

Phosphatidylinositol (3,4,5) trisphosphate formation is potentiated in SHIP⁻¹ platelets - To discern the importance of SHIP as a regulator of PI 3-kinase generated signals, platelets from SHIP⁻¹ knockout mice were used to measure PI(3,4)P₂ and PI (3,4,5)P₃ levels following CRP stimulation. The platelets were derived from knockout mice generated previously (Helgason et al., 1998) and have been found to have no
functional SHIP. However, the importance of other 5' phosphatases, such as SHIP2, in the hydrolysis of PI(3,4,5)P3 remains to be elucidated. To address the question of whether SHIP1 is an important component of PI(3,4,5)P3 turnover, 32P-orthophosphate labelling was performed to directly label the ATP pool used by PI 3-kinase to phosphorylate its substrates. Following solvent extraction and deacylation, the water soluble glycerophosphoinositides were separated by HPLC as described in the Materials and Methods. Figure 4.1 represents a typical elution profile of PI(3,4)P2 and PI(3,4,5)P3 generated in response to CRP treatment of SHIP+/− and SHIP+/− platelets. As can be seen CRP stimulated formation of PI(3,4,5)P3 in mouse platelets is consistent with observations in human platelets (Pasquet et al., unpublished results). The basal level of PI(3,4,5)P3 was increased by a factor of three in platelets, whereas the level of PI(3,4)P2 was slightly reduced (Figure 4.2). PI(3,4,5)P3 levels were significantly enhanced in response to CRP in platelets whereas PI(3,4)P2 were significantly reduced but not completely inhibited. The level of PI(4,5)P2 was similar in control and treated platelets. This demonstrates that PI(3,4)P2 is partly formed by metabolism of PI(3,4,5)P3 through the action of SHIP1. The differences in radioactivity most likely reflects increases in mass, as opposed to differences in specific activity of the labelled products between the SHIP+/− and SHIP+/− platelets.

PLC activity and pleckstrin phosphorylation in SHIP+/− platelets - The PI 3-kinase pathway has been shown to play an important role in the regulation of PLCγ2 activation. PI(3,4,5)P3 is thought to support recruitment or association of PLCγ2 with the plasma membrane through binding to the PH domain and/or the tandem SH2 domains of the phospholipase. To investigate if the increased level of PI(3,4,5)P3 influences PLC activity, we measured the level of the DAG metabolite, phosphatidic acid (PA), and the substrate for PKC, pleckstrin, in platelets prelabelled with 32P. 32P-PA formation (Figure 4.3) and 32P-pleckstrin phosphorylation (Figure 4.4A and B) are not significantly altered in SHIP+/− platelets under basal conditions or following stimulation with high and low concentrations
Figure 4.1. SHIP elevates PI(3,4)P\(_2\) and decreases PI(3,4,5)P\(_3\). Partisil 10 SAX HPLC elution profiles of deacylated PI(3,4)P\(_2\) (A) and PI(3,4,5)P\(_3\) (B) from SHIP\(^{-}\) and SHIP\(^{+/+}\) platelets following 2 min with ( ) and without ( ) 0.1 µg/ml CRP. The asterisk indicates the elution position of PI(3,4)P\(_2\). The profiles are representative of 2 separate experiments of duplicates.
A

SHIP -/-

SHIP +/-

Elution time (min)

CPM

Basal

CRP

B

SHIP -/-

SHIP +/+ -

Elution time (min)

CPM

Basal

CRP
Figure 4.2. PI(3,4)P$_2$ (A) and PI(3,4,5)P$_3$ (B) metabolism in SHIP$^{-}$ mouse platelets in response to CRP. $^{32}$P-labelled washed mouse platelets (4 x 10$^8$/ml) were stimulated at 37°C for 2 min with 0.1 μg/ml CRP. The reaction was stopped after 2 min by addition of HCl. Lipids were extracted and analyzed as described under "Material and Methods." The results are expressed as a mean ± range from 2 separate experiments of duplicates. Abbreviations are as follows: untreated (Un); collagen-related peptide (CRP).
Figure 4.3. Phosphatidic acid metabolism in SHIP⁺ mouse platelets in response to CRP and thrombin. ³²P-labelled washed mouse platelets (4 x 10⁸/ml) were stimulated at 37°C for 2 min with 0.1 and 10 μg/ml CRP and 0.001 and 0.1 U/ml thrombin. The reaction was stopped after 2 min by addition of HCl. PA formation was analyzed after phospholipid extraction by TLC and quantified by scintillation counting. The results are expressed as a mean ± range from 2 separate experiments of triplicates. Where error bars are absent indicates that they are too small to be represented on the graph. Abbreviations are as follows: untreated (Un); thrombin (Thr); collagen-related peptide (CRP).
Phosphatidic Acid Formation
Figure 4.4. Pleckstrin phosphorylation in SHIP−/− mouse platelets in response to thrombin. 32P-labelled washed mouse platelets (4 x 10^8/ml) were stimulated at 37°C for 2 min with 0.1 U/ml thrombin. Platelets were lysed with Nonidet P-40 buffer and resolved on 7.5% SDS-PAGE gels. The gels were dried down and exposed to X-ray film at -80°C and developed (A) or analyzed on a BioRad Molecular Imager FX using BioRad Multiphor software (B). The results are expressed as a mean ± range from 2 separate experiments of duplicates. Where error bars are absent indicates that they are too small to be represented on the graph. Abbreviations are as follows: untreated (Un); thrombin (Thr).
of CRP or thrombin demonstrating there was no major change in PLC activity.

4.3. Discussion

SHIP has recently been shown to be present in human platelets and may be involved in platelet activation evoked by thrombin, as well as a number of other agonists, including CRP [Giuriato et al., 1997; Pasquet et al., unpublished results]. The objectives of the study presented here were an effort to establish a role for SHIP_1 in PI(3,4,5)P_3 regulation in platelets, and whether this regulation influences downstream targets activated by PI 3-kinase.

To investigate the role of SHIP_1 in platelets, we have used a mouse model in which the SHIP_1 gene has been disrupted. The significance of SHIP_1 in mice development has been previously characterized [Helgason et al., 1998]. The mice are viable and fertile, but suffer from splenomegaly, myeloid infiltration of the lungs, wasting and shortened life span. We show that PI(3,4,5)P_3 is elevated slightly under basal conditions and markedly in response to CRP in SHIP_1^−/− platelets. Confirmation of the role of SHIP_1 in the metabolism of PI(3,4,5)P_3 is provided by the corresponding decrease in the level of PI(3,4)P_2 under resting and stimulated conditions in SHIP_1^−/− platelets. The residual formation of PI(3,4)P_2 could be derived by synthesis from PI(4)P by the recently described class II PI 3-kinases that show a strong preference for PI or PI(4)P [Zhang et al., 1998a].

This study was part of a larger, collaborative effort with Dr. Jean-Max Pasquet and Dr. Steve Watson at Oxford University. The focus of this larger study was to examine the role of SHIP_1 in platelet signalling by CRP. CRP binds to and activates platelets via GPVI, a collagen receptor on the surface of platelets. CRP binding triggers a series of reactions resulting in the activation of PLCγ2, and therefore the production of DAG and IP_3. The PI 3-kinase pathway is though to play an important role in the regulation of PLCγ2 as
PI(3,4,5)P3 is required for full activation of PLCγ2 by GPVI. Two mechanisms have been proposed to account for the role of PI 3-kinase pathway in the regulation of PLCγ2, namely via activation of the Tec family kinases or by direct interaction of PI(3,4,5)P3 with the phospholipase. We observe that PLC activity is not altered in SHIPr-deficient platelets under basal conditions or in response to CRP. This demonstrates that the elevation of PI(3,4,5)P3 alone is insufficient to activate PLC and that the level of the phospholipid is not rate limiting in the response to GPVI. It therefore seems that only a fraction of the generated PI(3,4,5)P3 is required for regulation of the PLC in response to CRP. This may explain why near-maximal concentrations of WM and LY294002 are required to inhibit PLC activation by CRP [Pasquet et al., unpublished results].

Parallel experiments conducted at Oxford demonstrated that tyrosine phosphorylation of Btk, a Tec family member, is dramatically increased under basal conditions and in response to CRP in SHIPr-deficient platelets. This confirms earlier reports that tyrosine phosphorylation of Btk is downstream of PI(3,4,5)P3 [Scharenberg et al., 1998]. Btk and the other Tec family kinases are implicated in the potentiation of Ca2+ influx into the platelet. This group of kinases contain a PH domain in their N-terminal regions which selectively binds to PI(3,4,5)P3 [Scharenberg et al., 1998]. This is consistent with a model in which elevation of PI(3,4,5)P3 is sufficient to induce Btk recruitment to, and activation at, the membrane skeleton, leading to increased Ca2+ influx. The data implicate SHIP as an important negative regulator of sustained calcium signalling, at least in part through its ability to lower PI(3,4,5)P3 levels, and the consequent deactivation of Tec kinases.
CHAPTER 5: GENERAL DISCUSSION

5.1. The role of PI 3-kinase in platelet aggregation

PI 3-kinase inhibitors were used to probe the dependence of thrombin-induced human platelet aggregation on PI 3-kinase. LY294002 inhibited aggregation at low concentrations of thrombin, but aggregation at high concentrations of thrombin was unaffected by LY294002. These studies show that PI 3-kinase plays an important role in the aggregation response of human platelets at lower activation states, or in response to low concentrations of agonist. PI 3-kinase signalling may be of less importance at higher activation states or higher agonist concentrations due to contributions from other signalling pathways that may impinge on a common downstream enzyme.

Future studies should have two principle focuses. One will be to examine the activity of kinases dependent on PI 3-kinase activity, and determine how they are regulated at various agonist concentrations, in the presence of absence of PI 3-kinase inhibitors. Particular attention will be given to the PKC, and its isoforms. Secondly, peptide agonists for the thrombin-activated PAR receptors expressed on human platelets have recently become available. These peptides can specifically activate a particular PAR receptor without significantly activating the other thrombin receptors. These peptides will enable us to explore the effect of PI 3-kinase inhibitors on GPIIb-IIIa activation and platelet aggregation downstream of particular PAR receptors.

We have also described and preliminarily characterized p105, a major tyrosine phosphorylated substrate associating with PI 3-kinase in thrombin-activated human platelets. p105 tyrosine phosphorylation is dependent on GPIIb-IIIa activation, and it lies upstream of PI 3-kinase activation. Further characterization could reveal a major new mechanism for PI 3-kinase in platelets. Therefore a key focus of future experiments is to purify p105 sufficiently such that mass spectrometry can be attempted to identify this
protein. If mass spectrometry reveals that p105 is a known protein, we may be able to use the tools currently available for study of the protein in other cells systems to explore the role of this protein in PI 3-kinase modulation. However, currently there are several avenues available to characterize this protein further. As many proteins in platelets are translocated to the membrane skeleton upon activation, including PI 3-kinase, it is logical to inquire where the location of p105 is in both resting and activated platelets i.e. is it translocated, as one would expect, to the membrane skeleton upon thrombin activation? Furthermore does the tyrosine phosphorylation of p105 correlate with translocation of this enzyme to the membrane skeleton, if indeed it does translocate? Secondly, since the tyrosine phosphorylation of p105 is dependent on GPIIb-IIIa activation and is therefore involved in outside-in signalling, it would be important to specifically investigate the function of this protein in outside-in signalling without the impact of inside-out signalling pathways. This can be achieved with LIBS antibody which activates GPIIb-IIIa independently of agonist receptors and inside-out signalling.

5.2. SHIP is a negative regulator of PI(3,4,5)P3 in platelets

The role of SHIP in the turnover of PI 3-kinase generated PI(3,4)P2 and PI(3,4,5)P3 was assessed using platelets derived from SHIP- deficient mice. The levels of PI(3,4,5)P3 in SHIP+ platelets were much greater following stimulation with CRP compared with wild type platelets. This contrasts with a decrease in the levels of PI(3,4)P2 in SHIP+ platelets, indicating that one of SHIP's functions is to generate this lipid. Furthermore, there was no change in the formation of phosphatidic acid or phosphorylation of the protein kinase C substrate pleckstrin in SHIP+ platelets, whereas there was a dramatic increase in tyrosine phosphorylation of Btk under basal conditions in SHIP+ platelets. These results demonstrate that the elevation of PI(3,4,5)P3 in SHIP- platelets is insufficient to activate PLC and that its level is not rate limiting in the response to CRP.
These results further support a physiological role for PI(3,4,5)P\(_3\) in the regulation of Ca\(^{2+}\) influx through Btk.

SHIP-deficient mice have tremendous promise to reveal much more about D-3 phospholipid metabolism in mice platelets. No functional studies have been done as of yet on SHIP-deficient platelets. It would be of great interest to examine the aggregation response of these platelets compared to control platelets when challenged with agonist. Moreover, as the route of PI(3,4)P\(_2\) formation in post-integrin signalling in platelets is still a matter of contention, performing time courses of D-3 phosphoinositide formation in SHIP-deficient platelets may provide some clarity to this debate. If for example, PI(3,4)P\(_2\) formation is unaffected when SHIP-deficient platelets are activated by agonist, this would indicate that PI(3,4)P\(_2\) is being formed during outside-in signalling from the action of PI 3-kinase on PI(4)P, and not the dephosphorylation of PI(3,4,5)P\(_3\). However, as there are other phosphatases that are activated during outside-in signalling, one would not be able to draw definitive conclusions from the time courses of D-3 phosphoinositide formation.

In platelets it has been shown that Btk is phosphorylated downstream of Ca\(^{2+}\) and downstream of PI(3,4,5)P\(_3\). It is therefore unclear which of these mechanisms underlies the increase in phosphorylation of Btk, as seen in the SHIP-deficient mice. Answers to this question may arise from the use of PI 3-kinase and Btk inhibitors, as well as microinjection studies of PI(3,4,5)P\(_3\). Do PI 3-kinase inhibitors block Ca\(^{2+}\) entry in response to thrombin in control mice and is this lost in SHIP-deficient mice? Do the recently described Btk inhibitors block Ca\(^{2+}\) entry and prevent the actions of PI 3-kinase inhibitors on Btk phosphorylation? Finally, does microinjection of PI(3,4,5)P\(_3\) potentiate Ca\(^{2+}\) flux in control megakaryocytes? These questions are currently being addressed.

In closing, the work presented here should help in our understanding of the role of PI 3-kinase and its products in platelet function. Eventually this may contribute to the development of treatments for conditions with which platelets are intimately linked such as thrombosis and atherosclerosis.
REFERENCES


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