TYROSINE PHOSPHORYLATION AND ACTIVATION OF PYK2 IN PLATELETS: INVOLVEMENT IN PI 3-KINASE ACTIVATION

By

MOHAMED RABIE SAYED

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Department of Medicine

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Platelets play a central role in the hemostatic response. They also contribute significantly to the initiation of responses by other cells which occur concomitantly with, or as a necessary sequel to, the initial events that prevent loss of blood, and are directly involved in wide range of diseases. Platelets activate various intracellular signaling pathways in thrombus formation, upon blood vessel injury. These intracellular signaling events involve activation of protein-tyrosine kinases which play key regulatory roles in controlling platelet aggregation and in regulating the activity of p85/p110 form of PI 3-kinase. The exact mechanism leading to the activation of p85/PI 3-kinase through the G-protein-coupled receptor is not understood. We show, for the first time, a novel association of PYK2 with p85/PI 3-kinase. The activation of PYK2 and its association with PI 3-kinase were shown to be increased in a time dependent manner and uninhibited by RGDS peptide which blocks the interaction of ligands such as fibrin/fibrinogen with the platelet integrin GPIIb/IIIa. This was concomitant with increased PI 3-kinase activity and association in PYK2 immunoprecipitates. We also have shown that the PI 3-kinase inhibitor LY-294002 had no effect on the activity and association of PYK2 with PI 3-kinase. We suggest that a physical or functional association of PYK2 with PI 3-kinase may occur *in vivo* that is responsible for the initial activation of PI 3-kinase in thrombin-stimulated human platelets independently of integrin ligation. Furthermore, we observed major tyrosine phosphorylated proteins p115 and p102 were inducibly associated with p85/PI 3-kinase in PAF-stimulated rabbit and thrombin-stimulated human platelets respectively. These proteins were not immunoreactive with several antibodies in this molecular weight range known to be tyrosine phosphorylated and associate with PI 3-kinase on cell activation. We attempted to purify p115 which was shown to be composed of more than one polypeptide or different phosphorylation states of the same polypeptide. p102 was shown to be inducibly associated with PI 3-kinase, but was dependent upon

fibrinogen binding to the integrin. While there is some evidence of aggregation-dependent degradation of proteins in platelets involved a protein at 123 kD that immunoreacted with PYK2 antibody and could be a splice variant of PYK2, the exact mechanisms have not been clearly demonstrated. These events may influence the regulation of PYK2 and PI 3-kinase.

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
EDTA	ethylene diaminetetra-acetate
FPLC	fast protein liquid chromatography
G-protein	Guanine nucleotide-binding protein
GAP	GTPase-activating protein
GPIIb/IIIa	integrin α_{IIb}/β_3 = platelet fibrinogen receptor
IC	inhibitory concentration
IP3	inositol (1,4,5) trisphosphate
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
PAF	platelet-activating factor
PAGE	polyacrylamide gel-electrophoresis
PI	Phosphatidylinositol
PI 3-kinase	phosphoinositide 3-kinase
PI-(3,4,5)P3	phosphatidylinositol (3,4,5) triphosphate
PI-(3,4)P2	phosphatidylinositol (3,4) bisphosphate
PI-3-P	phosphatidylinositol (3) monophosphate
PLA2	phospholipase A2
PYK2	protein tyrosine kinase-2
p85	85 kD regulatory subunit of PI 3-kinase
p110	110 kD catalytic subunit of PI 3-kinase
p115/102	115/102 kD tyrosine-phosphorylated PI 3-kinase associating protein
3-PPIs	3-phosphoinositides
RGDS peptide	arginine-glycine-aspartic-serine
SH2/3	*src*-homology domain 2/3

SDS	sodium-dodecyl sulfate
TLC	thin-layer chromatography
TXA2	thromboxane A2

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INTRODUCTION

A. Platelets

A.1 Platelet physiology

A.1.1 Platelet ultrastructure and organization

Prior to stimulation, the resting circulating platelet formed by cytoplasmic fragmentation from megakaryocytes in bone marrow or lung [Herd and Page, 1994; Martin and Levine, 1991], is discoid in shape with an equatorial diameter of 3-4 μm and a thickness of approximately 1μm [White, 1987a]. A normal platelet count in human blood is from 1.3-4.0 x 10^8 per ml. The life time of platelets is about 10 days. Electron microscopy of this non-nucleated cell reveals extensive invagination of the surface membrane comprising an open canalicular system continuous with the plasma membrane surface, which forms an interconnecting network of canaliculi distributed throughout the cell. Unique to the platelet amongst circulating blood cells, and distinct from the intracellular dense tubular system, the canaliculi provide ready access to the interior of the platelet for plasma proteins and other substances, whilst also facilitating secretion from the amine and protein-storage granules during the release reaction [White, 1974]. Among the platelet-specific granules, the dense granules, are rich in pro-aggregatory substances including ATP, ADP, Ca^{2+}, pyrophosphate and serotonin. The more numerous α-granules contain vasoactive substances. Platelets contain numerous adhesive proteins and tissue repair factors; these include fibrinogen, fibronectin, thrombospondin, albumin, PDGF, plasminogen activator inhibitor I, von Willebrand factor (vWf), clotting factor VII, TGF-β, clotting factor V and the platelet specific proteins β-thromboglobulin and platelet factor IV. Organelles such as mitochondria, glycogen particles, lysosomes, and peroxisomes are also found in platelets [Rendu et al., 1987].

The human platelet surface membrane is a typical bilayer membrane composed of protein, lipid and carbohydrate. The membrane lipids are predominantly phospholipids (approximately 75%) and neutral lipid (20%) with a small amount of glycolipid also present [Shattil and Cooper, 1978; Tuffin, 1991] . At the surface membrane, the platelet possesses a substantial exterior coat or glycocalyx. This dense layer (20 nm thick) is rich in glycoprotein of at least 10 different types. Of these, glycoprotein Ib (GPIb), which exists as a complex of the disulfide-linked GPIbα and GPIbβ subunits with GPIX [Fox, 1988], whilst the GPIIb-IIIa complex is the most abundant that accounts for approximately 3% of the total platelet protein (i.e., 17% of the platelet membrane protein mass) [Calvete, 1995; Hack and Crawford, 1984]. It has become clear that the platelet surface glycoproteins play a primary role in the adhesion of platelets to exposed subendothelial matrix proteins, interaction with ligands such as collagen, thrombin, or PAF, and exposure of fibrinogen receptors to facilitate aggregation. Indeed, two inherited platelet functional disorders, Bernard-Soulier syndrome and Glanzmann's thrombasthenia, are caused by congenital deficiency in the surface membrane glycoproteins Ib and IIb-IIIa complex respectively [Nurden and Caen, 1978; Clemetson et al., 1982; Tuffin, 1991].

The cytoskeleton of the circulating platelet, responsible for both maintenance of normal morphometry (i.e. the discoid shape) in the unstimulated cell, and the alterations in cell shape induced by activation, is comprised predominantly of a circumferential band of microtubles [White and Sauk, 1984] and abundant cytosolic actin microfilaments [Boyles et al., 1985]. Tubulin, a heterodimer of two α and β-subunit polypeptides, each with molecular weight of approximately 55, 000, is the major microtubular protein. The tubulin subunits polymerize to form bundles which, in turn, aggregate to make up the characteristic tube-like tertiary structure of the protein. These are associated with a number of high molecular weight (approximately 300, 000) microtubule associated proteins (MAPs) related, but not identical, to those described in other tissues. Although the process is as yet poorly understood, cytosolic calcium concentration and calmodulin appear important in the

control of tubulin polymerization, with MAPs probably influencing the interaction of microtubules with other cytoskeletal proteins. Actin, in both its filamentous forms, has been estimated to constitute 20-30% of total platelet protein [Rosenberg et al., 1981; Fox et al., 1984]. It is present in quiescent platelets in both the polymeric F form and the soluble subunit G form, as is true of skeletal muscle actin; in contrast, the molar ratio of actin : myosin is much higher than in smooth or skeletal muscle tissue. However, a considerable proportion of the monomeric actin is associated in a stoichometeric 1:1 complex with the low molecular weight protein profilin. Termed profilactin, this complex effectively acts as a storage pool of actin monomer, dissociating rapidly when platelets are stimulated [Markey et al., 1981]. A considerable number of other structural proteins have been identified in the human platelet cytoskeleton, many of which are able to bind to actin filaments, including ABP (actin-binding protein), 235 k protein and α-actinin. It has been indicated that the organization (assembly) and function of actin microfilaments is largely dictated by the association of actin with the above proteins [Fox, 1987].

The stimulation of platelets by exposure to foreign surfaces, or following addition of excitatory agonists (e.g. thrombin) *in vitro*, causes a remarkable reorganization of both the surface and internal cell cytoskeleton. Shape change and aggregation can be studied with a platelet aggregometer, which records transmission of light through a stirred platelet suspension. The platelets rapidly lose their discoid shape, becoming more spherical in appearance, and extend short and long pseudopodia (i.e. filopodia) [White, 1987a; White, 1987b]. This transformation is largely driven by altered organization of the cytoskeletal actin filamentous network. The degree of actin polymerization increases, and in the presence of ABP (actin binding protein) and α-actinin, the actin molecules assemble into filaments. In turn, the filaments become crosslinked, aligned into bundles and associated with newly phosphorylated myosin [Zucker and Nachmias, 1985; Tuffin, 1991].

A.1.2 Platelets in Hemostasis

Platelets play an essential role in the first phases of the hemostatic process (Packham, 1994). They are involved in initiating the hemostatic plug (thrombus) formation at a site of vascular injury by promoting coagulation and subsequent wound healing [Gordon and Milner, 1976]. Activation of platelets at such sites results from their interaction with coagulation factors (e.g. thrombin), autocoids (e.g. platelet activating factor (PAF), adenosine diphosphate (ADP), thromboxane A_2), adhesion to non-platelet surfaces (e.g. collagen), or with other stimuli that arise under pathologic conditions. Among the latter group are antigen-antibody complexes or aggregated gamma globulin. A number of responses to stimulation are required if the platelet is to fulfill its hemostatic role. The nature of these responses, and the order in which they occur, is dependent on the nature of the stimulus.

A.1.2.1 Adhesion

Adhesion to newly exposed collagen fibrils in a damaged area of a blood vessel causes spreading of the platelet on this surface followed by thromboxane A_2 synthesis, secretion and phosphatidylserine exposure. In quiescent platelets, phosphatidylserine is almost exclusively located in the inner leaflet of the plasma membrane. Aggregation of other platelets on the adherent monolayer occurs as a consequence of their stimulation by the released autocoids. The increase in phophatidylserine content of the outer leaflet of the plasma membrane enhances the activation of Factor X and the conversion of prothrombin to thrombin and thus promotes fibrin deposition in the damaged area [Beavers et al., 1993; Cohen et al., 1982; Zucker and Nachmias, 1985]. The adhesion and aggregation of circulating blood platelets at sites of exposed vascular injury, is the first and critical event in the process of hemostasis, thrombus formation (initial plug), and ultimately, vessel repair.

The platelet response is dependent on a complex array of multiple-linked interactions which occur either directly between platelet surface membrane glycoproteins and vessel wall constituents, or indirectly via the vWf glycoprotein. When the endothelial lining of a blood vessel is damaged, platelets adhere and spread upon the exposed subendothelial matrix. The mechanism of adhesion is non-thrombogenic, dependent on the shear force (blood flow) at the site of injury and does not require active metabolism (ATP). At low shear forces in large vessels, the interaction of platelets with the vessel wall is predominantly vWf-independent and supported by primary platelet binding sites for collagen fibrils, fibronectin and laminin. The adhesion receptors involved at the platelet surface membrane are believed to be GPIa/IIa, GPIc/IIa ($\alpha_5\beta$) and GPIc/IIa ($\alpha_6\beta$) respectively. However, under conditions of high shear accompanied by turbulent flow at arterial bifurcations, damage sites, or in small vessels and capillaries, a secondary or reinforcing vWf-dependent mechanism assumes importance [Turitto et al, 1985; Tuffin, 1991]. Human vWf is a high molecular weight oligomeric plasma protein ranging in size from 500 kDa to 20,000 kDa, synthesized, stored and secreted by vascular endothelial cells, which can act as a bridge between the platelet membrane and subendothelial collagen. The platelet receptor GPIb/IX complex is considered to be the major binding site for vWf, and is primarily responsible for mediating adhesion under these conditions. However, in Bernard-Soulier syndrome platelets, which lack the entire GPIb/IX complex, vWf will still bind to the platelets suggesting a second interactive site unrelated to the GPIb/IX complex may present [Ruggeri et al., 1983].

A.1.2.2 Aggregation

Platelet shape changes and adhesion upon stimulation by a soluble agonist such as thrombin are generally followed by aggregation which, in contrast to adhesion, requires active metabolism. The aggregation response can often be resolved into two phases,

primary and secondary aggregation. The primary response involves the formation of a relatively unstable aggregate which results from agonist interaction with specific receptors on the platelet membrane, leading to the generation of second messengers by effector transducers such as phospholipase C. These signals transmit the initial stimulus back to the platelet surface, resulting in activation of the fibrinogen receptor (GPIIb/GPIIIa) then binding and cross-linking of adjacent platelets since fibrinogen as well as Ca^{2+}, is necessary to obtain this platelet-platelet interaction. Signal transduction pathways activated by platelet agonists as well as the functional responses they impinge upon are discussed in a later section. Without further stimulation platelets will dissociate from this loose aggregate thus producing a reversible response. Secondary, or irreversible, aggregation occurs as a consequence of further stimulation, or exposure to a more potent initial stimulus, and involves consolidation of the aggregate associated with secretion. The contents of the amine and protein storage granules contribute to aggregate consolidation by providing, respectively, autocoids (ADP, 5-hydroxytryptamine 5HT), which recruit further platelets, and proteins (fibrinogen) which enhance aggregate stability [Nachman et al., 1987]. Stimulation of phospholipase A_2 by agonists such as thrombin or PAF results in the generation and the secretion of thromboxane A_2 (TXA_2) which potentiates the aggregate stability by binding to specific platelet receptors. Other proteins released as a consequence of protein storage granule secretion promote wound-healing (platelet-derived growth factor) and leukocyte chemotaxis. Under some conditions lysosomal secretion can also occur but the physiological role of lysosomal enzyme release in hemostasis is unclear. For some agonists (ADP, adrenaline) at all concentrations and at suboptimal concentrations of others (collagen, PAF), secretion and secondary aggregation are dependent on arachidonate mobilization from membrane phospholipids and conversion of the fatty acid to TXA_2 via the cyclooxygenase pathway.

A.1.3 The platelet integrin (fibrinogen) receptor: GPIIb/IIIa

Fibrinogen has long been recognized as an essential cofactor for platelet aggregatory responses induced by agonists such as ADP. Patients suffering from congenital afibrinogenemia possess extended bleeding times and platelet aggregation is severely compromised. Similarly, platelets prepared as washed cell suspensions, or maintained in plasma depleted of fibrinogen, fail to respond normally in the presence of agonists, whilst the addition of fibrinogen restores sensitivity. Fibrinogen secreted from the platelet-storage granules in the presence of a powerful agonist such as thrombin, is also able to support a response even in washed suspensions. It has been shown that fibrinogen associates with the platelet surface on activation, and early electron microscopy studies established that fibrinogen acts as a 'bridge' between platelets during the aggregatory process. However, fibrinogen interaction with platelets appears to be reversible if the release reaction has not occurred (e.g. disaggregation of ADP-stimulated platelet by prostacyclin PGI_2 (a potent anti-aggregatory agonist) and drugs such as dipyridamole prevent fibrinogen binding despite the presence of shape change [Harfenist et al., 1981]. On quiescent discoid platelets, the majority of fibrinogen binding sites are unavailable. However, when platelets are stimulated by agonists such as thrombin, collagen, ADP or PAF, a specific high affinity fibrinogen binding site is rapidly exposed due to a conformational change in the fibrinogen receptor [Sims et al., 1991]. The initial evidence to indicate a role for the surface membrane GPIIb and GPIIIa molecules in fibrinogen binding, arose from the study of platelets from patients with Glanzmann's thrombasthenia, which revealed a dramatic decrease in the amount of these two glycoproteins [Nurden and Caen, 1978]. Platelets from such patients fail to bind fibrinogen and respond abnormally to a spectrum of agonists including ADP and thrombin. Although GPIIb and GPIIIa migrate as separate proteins on SDS-PAGE and are products of separate genes, they associate 1:1 stoichiometrically on the surface membrane as a non-covalently linked, calcium-dependent

heterodimeric complex [Jennings and Phillips, 1982]. Indeed, GPIIb and GPIIIa isolated from platelet membranes in Triton X-100 lysates dissociate on addition of EDTA, re-associate on addition of calcium, and bind fibrinogen in the presence of calcium only when complex formation has occurred [Fujimura and Phillipis, 1983].

In addition to fibrinogen, the platelet GPIIb/IIIa complex is able to bind fibronectin and vWf [Plow et al., 1985], such that thrombasthenic platelets also bind significantly reduced levels of these polypeptides. Despite the fact that fibrinogen, fibronectin and vWf are large multimeric proteins, it has become evident that short, discrete peptide recognition sequences are responsible for the interaction of these proteins with GPIIb/IIIa. Sequence data has revealed that fibrinogen, fibronectin and vWf each posses Arg-Gly-Asp (RGD) containing peptide domains. The RGD peptides bind directly to platelets and can be chemically crosslinked to both GPIIb and GPIIIa subunits in the complex. Such RGD-containing tetra or penta peptides inhibit fibrinogen binding to thrombin-stimulated platelets and appear to behave as competitive antagonists of adhesive protein binding, since their activity can be reversed by addition of fibrinogen [Gartner and Bennett, 1985; Plow et al., 1985; Shattil et al., 1998].

A.2 Signal transduction pathways and their interactions in platelets

In platelets, as in other cells, responses to changes in the environment are obtained by sensing these changes using plasma membrane receptors. The occupancy of such receptors by suitable agonists is transduced into appropriate responses via elaborate and interacting intracellular signaling, or signal transduction, mechanisms. Both the nature of the mechanisms present in a given cell, and the way in which they interact, differ widely. In platelets, it is accepted that most, if not all conventional excitatory agonists cause cellular activation but not with equal effect. Platelet agonists are commonly classified as strong and weak, but the distinctions between these are often blurred. By one set of definitions, strong

agonists are those which can trigger granule secretion even when aggregation is prevented by measures such as removing the extracellular Ca^{2+} needed for fibrinogen binding. Thrombin and collagen are examples of strong agonists. In contrast, weak agonists, such as ADP and epinephrine, require aggregation for secretion to occur. Presumably, the stronger platelet agonists play the primary role in platelet activation *in vivo*, while the weaker agonists play a supportive role, particularly ADP, which is released from platelet dense granules during the early phases of platelet aggregate formation. Another way to classify agonists is to consider the sets of intracellular effectors that are coupled to their receptors. By this classification, strong agonists are those which stimulate phosphoinositide hydrolysis and eicosanoid formation, raise the cytosolic free Ca^{2+} concentration and cause aggregation and secretion that are relatively unimpaired by inhibitors of TXA_2 formation. This suggests that their receptors are coupled to phospholipase C as well as phospholipase A_2. It also suggests that the phosphoinositide hydrolysis pathway is the dominant mediator of platelet responses to strong agonists. Weak agonists, on the other hand, have little or no ability to cause phosphoinositide hydrolysis and are more dependent on TXA_2 formation for their effects. This suggests that their receptors are linked to phospholipase A_2, but not to phospholipase C. The activation by excitatory agonists also stimulate protein-serine/threonine kinases (predominantly PKC) and protein- tyrosine kinases, which are important for the formation of platelet aggregates. Such activation is opposed by increases in intracellular cAMP. The latter response results from occupancy of plasma membrane receptors for inhibitory agonists which are linked to adenylyl cyclase.

In the following sections, I will summarize the physiological nature of the platelet agonists such as thrombin and PAF as well as their receptors, and the predominant signaling pathways involved that are thought to mediate platelet responses. Then I will consider in more detail phospholipid and protein-tyrosine kinase signaling pathways.

A.2.1 Agonist Receptors

Until seven years ago, far more was known about platelet responses to agonists than about the structure of the agonist receptors. The successful cloning of the receptors for TXA_2 [Hirata et al., 1991], platelet activating factor [Honda et al., 1991], and thrombin [Vu et al., 1991] has shown that each of these receptors resembles other G protein-coupled receptors, being comprised of a single polypeptide with 7 transmembrane domains. In the case of thrombin, receptor activation is thought to involve a unique mechanism in which thrombin cleaves its receptor, creating a new N-terminus that can serve as a tethered ligand. Peptides corresponding to the tethered ligand can mimic the effects of thrombin, while antibodies to the same domain inhibit platelet activation. Hung et al.(1992) were the first to demonstrate that the cloned thrombin receptor is coupled to phosphoinositide turnover. They showed that the activation of the thrombin receptor by thrombin or the tethered ligand peptide (T-14) leads to phosphoinositide hydrolysis and adenylate cyclase inhibition. Phosphoinositide turnover was pertusis-toxin insensitive but inhibition of adenylate cyclase was completely blocked by pertusis-toxin. The different responses may be modulated by two different proteins: Gq-like for phosphoinositide hydrolysis and G_i-like for adenylate cyclase inhibition [Hung et al., 1992]. The ability of thrombin to mediate platelet activation so efficiently may be due to both these effects which are known to promote platelet activation [Kroll and Schafer, 1989]. The main events in platelet activation are caused by the activation of numerous cellular signal transduction pathways including phospholipases A_2 and C, PI 3-kinase, protein kinase C, MAPK, protein-tyrosine kinases and Ca^{2+} channels leading to an increase in calcium influx as well as release from stores. G-proteins are involved in upstream regulation of many of these enzymes [Huang et al., 1991a; Pumiglia and Feinstein, 1993].

A.2.2 Thrombin

a. Physiology and Metabolism

Thrombin is a serine protease with multiple physiological effects [Stubbs and Bode, 1993]. It is generated from its zymogen, prothrombin, through the concerted action of several factors in the blood coagulation pathways. Unlike most coagulation zymogens (factors), prothrombin circulates in plasma at relatively high concentrations. Prothrombin has two homologous "kringle structures" indicative of ancestral gene duplication in the zymogen [Mann et al., 1981]. These structures are then followed by a connecting peptide segment which forms the thrombin A chain and joins to the much larger B chain which constitutes approximately half of the C-terminal polypeptide of the zymogen and is homologous with other serine proteases. The consequences of this assemblage is that the two halves of prothrombin are functionally unrelated. Whereas the function of the N-terminal moiety is the regulation of thrombin generation (e.g. zymogen biosynthesis, circulation, and activation), that of the C-terminal moiety is to carry out the regulatory functions of the activated zymogen (e.g., cleavage of fibrinogen and various other activities) [Fenton, 1988; Fenton and Bring, (1986)]. Prothrombin associates with negatively charged phospholipid surfaces in the presence of calcium. On the membrane, prothrombin is proteolytically cleaved in the presence of factor Xa by the prothrombinase complex which consists of proaccelerin (Va), Ca^{2+}, and membrane phospholipid, liberating the enzymatically active α-thrombin [Mann et al., 1990]. 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 is far more selective; it cleaves only particular Arg-Gly bonds on certain proteins [Stubbs and Bode, 1993].

Once thrombin binds to the thrombin receptor, it cleaves the N-terminus of the receptor, resulting in the release of a 41-amino acid peptide. The N-terminus created by this proteolytic event, the neo N-terminus, was shown to be the functional ligand. The fate and the function, if any, of the released peptide are unknown. A peptide as small as six amino acids representing the first six amino acids of the neo N-terminus is sufficient to activate the thrombin receptor in a cleavage-independent manner [Peterson and Lapentina, 1994].

b. The Biological effect of Thrombin and Signal Transduction

Thrombin is considered to be the most potent stimulator of platelet aggregation known, causing platelet adhesion, shape change, thromboxane production and secretion. In hemostasis, it mediates the conversion of fibrinogen to fibrin in plasma and the activation of clotting factors V, VIII, XIII and protein C. Activation of platelets by α-thrombin is marked by several biochemical changes. One of the earliest events is the activation of phospholipases C and A_2. Other well-characterized events include protein kinase C activation, phosphatidylinositol 3-kinase activation, actin polymerization, myosin light chain phosphorylation (a calcium-dependent event), activation of tyrosine kinases, activation of the Na^+/H^+ antiporter, and binding of fibrinogen to glycoprotein IIb-IIIa.

The biological effects of thrombin, however, are not limited to hemostasis; the protease has also been implicated in inflammation and nerve cell function. Thrombin is chemotactic for monocytes and lymphocytes and mitogenic for lymphocytes and mesenchymal cells including vascular smooth muscle cells [Bizios et al., 1985; Mc Namara et al., 1993], fibroblasts and epithelial cells. Activation of T-lymphocytes by thrombin induces proliferation and interleukin-6 production [Naldini et al., 1993]. In endothelial cells, thrombin stimulates production of prostacyclin, platelet activating factor, plasminogen activator inhibitor I and platelet-derived growth factor. It also causes release of von Willebrand Factor and endothelial cell retraction leading to a potential selective

permeability of the vessel wall as well as inducing proliferation of endothelial cells [Dennington and Berndt, 1994]. Thrombin acts as a chemotactic stimulus for neutrophils and induces neutrophil adherence to the vessel wall by an endothelial cell-dependent mechanism [Lorant et al., 1991]. Finally, thrombin may have a neurotransmitter or neuromodulatory function and may be involved in neural development and plasticity as it is known to cause neurite outgrowth in primary neuronal cells [Dihanich et al., 1991].

A.2.3 Platelet-activating Factor (PAF)

Platelet activating factor (PAF, PAF-acether, AGEPC, 1-*O*--acetyl-2 -sn-glycerol-3-phosphocholine) is phospholipid with a broad range of biological activities in addition to platelet activation [Chao and Olson, 1993; Venable et al., 1993; Shukla, 1992; Nojima, 1991; Prescott et al., 1990]. PAF was originally described by Benveniste and his colleagues who showed that it was derived from antigen-stimulated, IgE-sensitized, rabbit basophils [Benveniste et al., 1972]. In addition, PAF can be formed by peritoneal macrophages [Henson, 1970], human and rabbit polymorphonuclear leukocytes, rabbit platelets, rabbit, rat and human alveolar macrophages, and human eosinophils [Kinlough-Rathbone and Mustard, 1987]. PAF is made available when cells are appropriately challenged, but does not exist as such in unstimulated cells. Thrombin-stimulated rabbit platelets form appreciable quantities of PAF but human platelets do not appear to produce significant quantities. [Demopoulos et al., 1979]. Responsiveness of platelets to PAF has been well described but the rate and extent of platelet aggregation in response to this mediator depend upon the species tested; rabbit platelets and guinea-pig are the most sensitive to PAF. They aggregate and release their granule contents in response to sub-nanomolar concentrations, whereas human platelets require much higher concentrations of this material and, in some cases, human platelets do not appear to respond significantly to

PAF [Benveniste et al., 1981]. The responsiveness of platelets to PAF is independent of released ADP or the formation of thromboxane A2 [Pinckard, 1983].

PAF is synthesized in cells by two routes, the remodeling and the *de novo* pathways. The remodeling pathway involves PLA_2 degradation of a 1-*O*-alkyl-2-acyl-sn-glycerol-3-phosphocholine (with liberation of arachidonic acid) followed by the action of an acetyltransferase to yield PAF. Stimulation of PAF production therefore is coupled to eicosanoid production with two pathways acting synergistically. PAF is degraded by pathways involving acetylhydrolase and mono-oxygenase metabolism [Snyder, 1995a; Snyder, 1995b].

PAF mediates many physiological responses in addition to platelet activation, including stimulation of glycogenolysis in liver, increased vascular permeability, hypotension, decreased cardiac output, smooth muscle contraction and activation of neutrophils, eosinophils and macrophages [Venable et al., 1993]. The most significant effect of PAF on platelets is thought to come about as a result of its formation in immunological reactions, such as allergic disorders, acute inflammation, asthma, convulsions, and endotoxin-induced and anaphylactic shock [O'Flaherty and Wykle, 1983].

PAF stimulation of platelets leads to phophoinositide turnover and activation of numerous signal transduction pathways including phospholipases A_2 and C and PI 3-kinase, protein kinase C, and calcium influx. PAF induces tyrosine phosphorylation of many proteins. It has been shown that pre-incubation of platelets with the tyrosine-kinase inhibitor erbstatin blocks numerous PAF-induced platelet responses [Salari et al., 1990]. The PAF receptor contains several tyrosine residues in its intracellular loops and tail suggesting perhaps a functional link between homologous down-regulation of the receptor and protein-tyrosine kinases. Experiments using vanadate suggest that protein-tyrosine phosphorylation may play a role in the regulation of the PAF receptor surface expression [Chao and Olson, 1993].

A.2.4 Phospholipase C

Physiological platelet stimuli, such as thrombin, collagen, ADP, and platelet-activating factor, induce rapid changes in platelet membrane phospholipids through the activation of specific phospholipases. Degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C appears to be the primary event in platelet activation [Heemskerk et al., 1993]. This leads to formation of the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These products remain inside the cell and mediate platelet activation since DAG and IP_3 can activate protein kinase C and cause elevation of cytosolic Ca^{2+} respectively [Lapetina and Siess, 1983]. Numerous studies have linked protein kinase C and Ca^{2+} to many platelet responses, especially aggregation and secretion. Agonist-induced activation of PLC is postulated to involve coupling to the receptor via a putative guanine nucleotide binding (G)-protein (Gq) [Berridge, 1987]. The most abundant isoforms of PLC detected in platelets are PLC $\beta 2$, PLC $\beta 3a$, and PLC $\gamma 2$. Whereas other isoforms, PLC $\beta 1$, PLC $\gamma 1$ and PLC $\beta 3b$ are present in smaller quantities [Banno et al., 1996; Okano et al., 1990]. Mechanisms for activation of these isoforms in platelets are similar to those described for other cell types [Okano et al., 1990].

A.2.5 Inositol 1,4,5-triphosphate and Calcium influx mechanisms

As in many cell types, the link between receptor occupation and the release of Ca^{2+} from intracellular stores in platelets seem to be the formation of the diffusible messenger, inositol 1,4,5-triphosphate. IP_3 is generated in response to most agonists and this messenger releases Ca^{2+} from the dense-tubular system [Rink, 1990; Berridge, 1989]. The IP_3 signal is rapidly terminated by either dephosphorylation to an inactive compound or phosphorylation to Ins (1,3,4,5)P4. IP4 is then rapidly de-phosphorylated to Ins (1,3, 4)P3

(a weak Ca^{2+} mobilizer) which comprises 90% of the IP_3 in thrombin-stimulated platelets [Daniel et al., 1987]. IP4 may also contribute to the transient nature of the increase in cytosolic Ca^{2+} by stimulating Ca^{2+} influx through the plasma membrane and/or resequestration to the dense-tubules [Ashby et al., 1990]. The increase in the cytosolic Ca^{2+} concentration occurs within seconds of agonist stimulation which is elevated from about 100 nM to low micromolar levels depending upon the agonist [Rink and Sage, 1990]. Ca^{2+} plays a key role in triggering many platelet functions and is perhaps the most important single intracellular mediator of cell function. The release of Ca^{2+} from the internal stores is closely associated with platelet shape changes, aggregation and secretion. The shape change caused by increased cytosolic Ca^{2+} results from the phosphorylation of one of the light chains of myosin by calcium-calmodulin-dependent kinase which enhances ATP-dependent interaction of actin and myosin and the generation of contractile forces [Daniel et al., 1984]. More extensive myosin phosphorylation has been related to granule centralization, a process required for secretion. Ca^{2+} also allows the association of the platelet integrin components, GPIIb and GPIIIa on the surface membrane that results in binding to fibrinogen [Fujimura and Phillipis, 1983]. Other targets for Ca^{2+} in activated platelets include a group of Ca^{2+}-dependent proteases called calpain which may be involved in cytoskeletal reorganization/aggregation [Yoshida et al., 1983]. In addition to protein kinase C, the intracellular Ca^{2+} has been involved in regulation of both phospholipase A_2 and PI-PLC [Kramer et al., 1986]. It has also been suggested that Ca^{2+} activates tyrosine kinase activity and that this is independent of the function of endoperoxides, ADP release or phosphoinositide hydrolysis [Takayama et al., 1991]. The regulation of protein tyrosine kinases in platelets will be discussed in a later section.

A.2.6 Diacylglycerol / Protein kinase C

Platelets exposed to certain stimulatory agonists generate 1,2-diacylglycerol, predominantly 1-stearoyl-2-arachidonyl-*sn*-–3-glycerol, as a consequence of phosphoinositide hydrolysis [Mauco et al., 1984]. The extent of DAG formation by human platelets exposed to thrombin was estimated at approximately 600 pmol/10^6 cells [Rittenhouse-Simmons, 1980]. DAG undergoes two routes of metabolism in platelets. It is converted to phosphatidic acid by the action of diacylglycerol kinase (DGK) or it is metabolized by diacylglycerol lipase to monoacylglycerol and stearic acid. At least three forms of DGK (DGK I, II, III) have been identified in platelets [Yada et al., 1990].

The concept that *sn* 1,2-diacylglycerols could function as intracellular bioregulators emerged from the elegant studies of Nishizuka and colleagues [Nishizuka, 1983]. With the knowledge that DAG was one of the immediate products of phospholipase C-catalyzed phosphoinositide hydrolysis, the role of DAG as the endogenous activator of protein kinase C (PKC) challenged the dogma of the period, which was that the signaling function of receptor-mediated phosphoinositide hydrolysis was a Ca^{2+} gating mechanism [Michell, 1975]. This bifurcation in the signaling functions of the products of phosphoinositide hydrolysis was accentuated by the demonstration that phorbol esters or DAG acted synergistically with elevated cytosolic Ca^{2+} to mediate platelet responsiveness [Yamanishi et al., 1983]. The essential foundation for this hypothesis was the observation that sub-maximal concentrations of a phorbol ester e.g. phorbol dibutyrate, and a Ca^{2+} ionophore, e.g. A23187, interact synergistically to stimulate secretion and aggregation.

Participation of the DAG/Ca^{2+}-activated PKC in intracellular signaling processes has been demonstrated in many cell types, including those of hematopoietic origin. PKC-mediated phosphorylation of numerous protein substrates is associated with a wide range of biological effects, including stimulus-secretion coupling, induction of cellular proliferation and differentiation, activation of nuclear transcription factors and cell surface

receptors and tumor promotion [Weinstein et al., 1997; Quest et al., 1997; Jarvis-David et al., 1994; Nishizuka et al., 1988]. PKC is expressed in mammalian systems as a family of diverse serine-threonine kinases, consisting of at least nine isoforms differing in both substrate specificity and dependence upon Ca^{2+} availability. Differential activation of PKC isoforms has been postulated to account for the divergent action of different enzyme activators [Hug and Sarre, 1993]. In platelets, DAG/Ca^{2+}- activated PKC has been associated with aggregation (without shape change), secretion and arachidonic acid metabolism. It has been proposed that PKC provides the 'inside-out' signal required for activation of the platelet integrin GPIIb/IIIa, allowing aggregation [Shattil and Cooper, 1987; Shattil et al., 1998]. The major substrate for PKC in platelets is pleckstrin (also designated p40 or p47) [Tyers et al., 1988]. Phosphorylation of pleckstrin is one of the earliest events observed to follow platelet (and PKC) activation in response to variety of agonists. It has been observed that brief treatment of platelets with β-PMA (β-phorbol myristate acetate) before exposure to the thrombin receptor agonist SFLIRN selectively inhibits Phophoinositide 3-kinase γ (PI3-Kγ) activity in intact platelets [Zhang et al., 1996]. This appears to constitute a form of feedback inhibition, because 3-phosphorylated phosphoinositides, similar to PLC-generated diacylglycerol, can promote pleckstrin phosphorylation by stimulating PKC [Zhang et al., 1995a].

A.2.7 Phospholipase A2

Phospholipase A_2 (PLA_2) is the second phospholipid-hydrolyzing enzyme in platelets whose activity may be regulated by G protein(s). There are two potential mechanisms by which this could be accomplished. One is the indirect activation of PLA_2 via the increase in cytosolic Ca^{2+} caused by Gp- or Gq-dependent phosphoinositide hydrolysis. A second mechanism is the direct activation of PLA_2 by a G protein. Current evidence suggests that PLA_2 is primarily located in the platelet cytosol and that arachidonate

release and metabolism occur in the dense tubular system [Laposata et al., 1987]. PLA_2 cleaves the sn-2 acyl bond of membrane phospholipids, yielding arachidonic acid (AA) and the corresponding lyso-phospholipid. AA is subsequently metabolized by cyclo-oxygenase and thromboxane synthetase to endoperoxides (EP) and thromboxane A_2 (TXA_2), which can act inside platelets or are released to the outside to activate other platelets [Lapetina and Siess, 1983]. The liberation and metabolism of AA has, however, an important function in initiating later phases of platelet activation, such as aggregation and the release reaction. EP and TXA2 could act as feedback promoters and induce further stimulation of PLC, leading to acceleration and amplification of platelet activation. PKC may indirectly potentiate PLA_2 activation by phosphorylating (and inhibiting) lipocortin, the endogenous PLA2 inhibitor [Kramer et al., 1986; Kramer et al., 1993].

A.2.7 Platelet Inhibition

The prostaglandins E_1 (PGE_1), D_2 (PGD_2) and I_2 (prostacyclin; PGI_2) and adenosine each demonstrate potent anti-aggregatory activity on human platelets. They are known to act through a specific receptor that is coupled to the heterotrimeric G protein, G_S, which in turn stimulates adenylate cyclase [Mills, 1982]. Adenylate cyclase in platelets is inhibited by excitatory agonists such as thrombin, ADP and adrenaline, whose receptors are coupled to the enzyme through G_i. However, these agonists do not stimulate platelet aggregation and secretion through reduction in cAMP since other adenyl cyclase inhibitors do not cause platelet activation [Haslam et al., 1987]. There is abundant evidence to support the postulate that an increase in intracellular cAMP depresses platelet aggregation and secretion [Crawford and Scrutton, 1987]. Agents that increase cAMP concentrations very effectively inhibit agonist-evoked rises in intracellular Ca^{2+} [Feinstein et al., 1983]. This inhibition is seen both in the absence and presence of external Ca^{2+}, suggesting that cAMP affects both internal discharge of Ca^{2+} and Ca^{2+} influx.

The mechanisms by which an increase in cAMP, acting presumably via protein kinase A, depresses platelet responsiveness are less well defined. Some possibilities have been described: (a) PKA phosphorylates a Ca^{2+} uptake system, enhancing Ca^{2+} removal from, or it shutting the input into, the cytosol [Feinstein et al., 1983]; (b) PKA mediates phosphorylation of myosin light chain kinase (MLCK), decreasing its affinity to Ca^{2+}-calmodulin and thereby decreasing MLCK activity resulting in abrogation of the shape change response [Conti and Adelstein, 1981]; (c) the activity of both PLC and PLA_2 are greatly reduced by an increase in cAMP, suggesting that other control mechanisms may exist to down-regulate PLC activity via cAMP-dependent PKA [Peterson and Lapentina, 1994]. Direct phosphorylation of PLC by cAMP-dependent PKA mechanisms has not been demonstrated in platelets. In short, almost all of the steps involved in the platelet-activation pathway are affected by increases in cAMP levels. Most of these can be directly explained by the inhibition of PLC-derived production of second messengers. However, the molecular mechanism(s) by which cAMP and PLC activity are related, is not understood.

B. Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI 3-kinase) plays an important role in transducing the mitogenic signals of various growth factors receptors and oncogene products. A diverse group of kinases, including protein kinase C (PKC) isoforms, p70 S6 kinase, and PKB/Akt have been shown to be regulated downstream of PI 3-kinase [Cantley et al., 1991; Chen and Guan, 1994b; Duronio et al., 1997]. More recently, PI 3-kinase has been implicated in a variety of other cellular responses, including membrane trafficking, actin reorganization, receptor internalization, glucose transport and inhibition of apoptosis [Fry, 1994 ; Scheid et al., 1995].

While the exact role of PI 3-kinase in platelets is not clear, it has been shown that PI 3-kinase activity promotes platelet aggregation and may play a role in cytoskeletal

reorganization. Studies with PI 3-kinase inhibitors wortmannin and LY294002 have indicated that PI 3-kinase is necessary for the initial agonist-induced activation of the fibrinogen receptor. Wortmannin was found to inhibit platelet aggregation induced by lyso-phosphatidic acid [Zhang and Rittenhouse, 1995b] and activation of the platelet integrin receptor GPIIb/IIIa [Gao and Shattil, 1995]. Our laboratory supports the idea that PI 3-kinase appears to contribute to the inside-out signal transduction that leads to the activation of the fibrinogen receptor [unpublished results]. Although, PI 3-kinase activation does not appear to be necessary for platelet secretion and the massive actin assembly that lead to platelet shape change [Kovacsovics et al., 1995], recent studies have shown that the products of PI 3-kinase, the 3-phosphorylated phosphoinositides, initiate actin assembly only for filopodia growth by generating protein C activation and stabilization of fibrinogen-binding to GPIIb/IIIa signaling [Hartwig et al., 1996; Shattil et al., 1998].

PI 3-kinase catalyzes the stereoselective transfer of a phosphate group from ATP to the 3'-position of the inositol ring of phosphatidylinositol [PI], phosphatidylinositol 4-phosphate [PI-4-P] and phosphatidylinositol 4,5-biphosphate [PI-(4,5)P_2], forming the putative second messengers phosphatidylinositol 3-phosphate [PI-3-P], phosphatidylinositol 3,4-biphosphate [PI-(3,4)P_2] and phosphatidylinositol 3,4,5-triphosphate [PI-(3,4,5)P_3] [Nakanishi et al., 1995]. These 3-phosphorylated phosphoinositides (3-PPI's) constitute a branch of phosphoinositide metabolism that is separately regulated and functionally distinct from the conventional polyphosphoinositide.

B1.1 Conventional polyphosphoinositide metabolism

The phosphoinositides, like other phospholipids in platelets, are components of membranes. Whereas PI-4-P and PI-(4,5)P_2 have been found to be exclusively located in the plasma membrane of platelets, PI appears to reside in both the plasma membrane and endoplasmic reticulum [Mauco et al., 1987]. Separate pools or compartmentalization of PI,

PI-4-P and PI-(4,5)P_2 have been proposed based on studies with rabbit platelets [Vickers and Mustard, 1986a; Vickers et al., 1986b].

The mass of PI in resting human platelets has been shown to range between 17-19 nmol/10^9 platelets. PI-4-P and PI-(4,5)P_2 are present at much lower amounts 3 and 1 nmol/10^9 platelets, respectively, such that they represent approximately 15% (for PI-4-P) and 5% (for PI-(4,5)P_2) of the total phosphoinositides present in human platelets [Verhoeven et al., 1987].

The formation of the conventional polyphosphoinositides PI-4-P and PI-(4,5)P_2 from the substrate PI are catalyzed by the enzymes PI 4-kinase and PI-4-P 5-kinase, respectively. On cell stimulation, the generation of DAG and IP3 by PLC involves PI-(4,5)P_2 exclusively, while generation of PI-(3,4,5)P3 result from phosphorylation of PI-(4,5)P2 by PI 3-kinase. The physiological role for the conventional polyphosphoinositides appears to ensure a sufficient supply of substrate for PLC and PI 3-kinase during cell stimulation [Carpenter and Cantley, 1990]. In addition, there is evidence to suggest, however, that these lipids play a role in cytoskeleton regulation [Rittenhouse, 1996; Fry, 1994; Adams and Pollard, 1989].

B.1.2 3-OH-Phosphorylated Phosphoinositides (3-PPIs) and their routes of synthesis

The discovery of PI 3-kinase activity in other cell types such as neutrophils led several groups to investigate the potential presence in platelets of phosphoinositides phosphorylated at the D-3 position on the inositol ring. Platelets indeed, form PI-(3,4)P_2 in response to thrombin [Nolan and Lapetina, 1990; Kucera and Rittenhouse, 1990]. Platelets also accumulate PI-(3,4,5)P_3; further more, both PI-(3,4)P_2 and PI-(3,4,5)P_3 increase in response to the thromboxane receptor agonist U46619 or to GTPγS, in permeabilized platelets [Kucera and Rittenhouse, 1990]. The use of an increase in ^{32}P-and ^{3}H-inositol-labeled PI-(3,4)P_2 as an indication of a change in mass has been validated for U46619-

stimulated platelets by mass measurements, and PI-(3,4)P_2 has been found to have the same distinctive fatty acid profile as that of the other phosphoinositides [Kucera and Rittenhouse, 1990]. The mass of PI-(3,4,5)P_3 that accumulates in platelets exposed to thrombin has been quantified directly, but based on the amount formed in equilibrium labeling experiments with ^{32}P, it has been estimated to be about 1% of the resting mass of PI-(4,5)P_2 or 8 to 10 pmol/10^9 platelets. It has been observed that the elevation in PI-(3,4,5)P3 precedes that in PI-(3,4)P2. No significant changes in PI-3-P are observed in intact platelets incubated with platelet agonists, and permeabilized platelets show only small increases in PI-3-P, which are minor in comparison with the increases seen in the more highly phosphorylated 3-PPIs [Kucera and Rittenhouse, 1990; Carter et al., 1994]. Stimulation of 3-PPIs accumulation can also be achieved with the thrombin receptor tethered ligand peptides, SFLLRNPNDKYEPF [Huang et al., 1991a] or SFLLRN [Zhang et al., 1995a], but it is not a consequence of platelet-derived growth factor (PDGF) secretion, because no form of PDGF is able to elicit this effect or to potentiate the effect of thrombin [King et al., 1991]. Other agonists tested thus far that increase 3-PPIs accumulation in platelets are lysophosphatidic acid (LPA) [Zhang et al., 1995b] and ADP [Zhang et al, 1996] and PAF [Lauener, 1997]. Each is effective, even when added to aspirin-treated platelets, although they are much weaker agonists than is thrombin.

While the pathway for formation and metabolism of these novel phospholipids remains controversial, recent evidence suggests that the formation of PI-(3,4,5)P_3 represents the primary output signal of activated PI 3-kinase. The observations of Stephens et al. [1991], and Hawkins et al. [1992] that the increases in labeled PI-(3,4,5)P_3 precede those in labeled PI-(3,4)P_2 and that labeled PI-3-P does not increase significantly in the period preceding (or during) that in which PI-(3,4,5)P_3 and PI-(3,4)P_2 levels increase, would both tend to favor the route of a stimulated PI 3-kinase acting on PI-(4,5)P_2, and activation of PI 3-kinase showing substrate preference for PI-(4,5)P_2 has been observed in several stimulated cell types [Cunningham and Majerus, 1990].

While the precise metabolism of PI-(3,4,5)P_3 has not been firmly established, it is clear that PI-(3,4,5)P_3 is not a substrate for phospholipase C and may perform a second messenger function *per se* [Cantley et al., 1991]. Growing evidence suggested that the potential signaling function of PI-(3,4,5)P_3 may be terminated by a PI-(3,4,5)P_3 5-phosphatase, generating PI-(3,4)P_2 [Stephens et al., 1991; Hawkins et al., 1992]. This concept has been confirmed in platelets by the identification of two PI-(3,4,5)P_3-hydrolyzing 5-phosphatases [Jackson et al., 1995] and the SH2 domain-containing inositol 5-phosphatase, SHIP [Giuriato et al., 1997]. In addition, two forms of inositol polyphosphate 3-phosphatase has been shown to be present in platelets [Jackson et al., 1995]. These two 3-phosphatases have been described in brain extract to have unusual property of hydrolyzing both inositol (1,3)P_2 and PI-3-P [Caldwell et al., 1991].

Although the physiological target of the 3-PPIs is not well defined, their relevance to signaling events was implied by early findings that their synthesis [more specifically, that of PI-(3,4,5)P_3 and PI-(3,4)P_2] appears to be required in cells other than platelets for cell transformation or growth stimulation in response to mitogenic polyoma virus middle T antigen (mT) or PDGF [Carpenter and Cantley, 1990]. In these cases, the responsible kinase, PI 3-kinase, is found complexed in a phosphotyrosine-dependent manner via the phosphotyrosine-binding src-homology 2 (SH2) domains of the p85 subunit of one form of PI 3-kinase to mT/pp60 $^{c\text{-}src}$, or to the receptor tyrosine kinase, PDGF-R, respectively. Of interest as well is the observation that mT complex is associated with cytoskeletal focal contacts (the anchor points for actin stress fibers) and can be immunoprecipitated with anti-vinculin serum. Because cytoskeleton reorganization is one of the earliest changes observed in polyoma-infected cells or in neutrophils which have been shown to produce PI-(3,4,5)P_3 and PI-(3,4)P_2 upon stimulation by the chemotactic peptide f-Met-Leu-Phe (fMLP) [Stephens et al., 1991], a possible signaling target for the 3-PPIs has been thought to be the cytoskeleton. The platelet, of course, is another example of a cell that undergoes rapid reorganization of the cytoskeletal apparatus upon stimulation by physiologic agonists

such as thrombin, even if a proliferative response is not possible. However, Downes and Carter [1991] have proposed that 3-PPIs would be more effective as a modulator of the activity of an enzyme such as a kinase, that can amplify its signal, rather than acting stoichiometrically with cytoskeletal proteins. Studies with PKCs make this proposal increasingly plausible. Several members of the PKC family have been reported recently to be stimulated by PI-(3,4,5)P_3, including a mixture of rat brain PKC isozymes, PKCζ, and (by either PI-(3,4,5)P_3 or PI-(3,4)P_2) PKCε, δ, and η [Toker et al., 1994]. Studies in our laboratory is focused now on the role of PKCδ which was found to be phosphorylated and associated with PI 3-kinase in a human hematopoietic cell line, as well as in platelets upon cytokine and thrombin stimulation, respectively. [Ettinger et al., 1996].

B.2 Structural and functional characterization of phosphoinositide 3-kinases in platelets

The best known PI 3-kinase in platelets designated p85/PI 3-kinase, has been found to be a heterodimer consisting of 85-kD and 110-kD subunits [Carpenter and Cantley, 1990; Downes and Carter, 1991]. The p85 regulatory subunit (724 amino acids) contains a number of important motifs including an amino-terminal SH3 domain and two SH2 domains. The function of these domains will be discussed later in this section.

p85 does not have any kinase activity, and, is known to consist of two highly homologous proteins, p85α and p85β, and a partial protein which may exist, termed p85γ. The latter has been isolated from bovine brain which covers the C-terminal two-thirds of p85 protein sequence and is again highly homologous [Fry, 1994]. Western blots with antibodies to the p85 α subunit and the p85 β subunit have shown that the p85 α subunit is the more abundant isoform detectable in human platelet preparations [Zhang et al., 1996]. There is no known difference in the function of p85 α and p85 β.

Between the SH3 and the amino-terminal SH2 domain is a sequence homologous to the C-terminal part of the Break-cluster region (Bcr) gene product. This region of the Bcr

protein has been shown to exhibit homology with RhoGAP and it might be able to mediate a positive or negative regulatory interaction with a small G-protein [Fry, 1994]. Flanking the Bcr region in p85β, but not p85α, are two proline rich sequences which could mediate protein-protein interactions through binding of SH3 domains independent of tyrosine phosphorylation [Fry, 1994; Kepeller et al., 1994].

The 110-kD subunit contains the PI 3-kinase catalytic activity. There are at least three distinct but related 110 kDa catalytic subunits, p110 α , β were isolated from rat liver [Carpenter and Cantley, 1990], while p110 δ is in cells of hematopoietic lineage [Vanhaesebroeck et al., 1997], which associate with p85 to form heterodimeric PI 3-kinase. The p110 catalytic subunit also possesses intrinsic serine/threonine protein kinase activity which was originally thought to be a separate protein [Carpenter, 1993b]. However, all the attempts to separate this protein kinase activity from PI 3-kinase failed, and it was shown that in fact the serine/threonine protein kinase activity is intrinsic to the p110 subunit and the enzyme possesses a dual kinase specificity [Dhand et al., 1994]. This serine-directed protein kinase activity can phosphorylate the p85 subunit, resulting in inhibition of p85/PI 3-kinase activity. It has been suggested that this intrinsic negative regulation by the p110 subunit may serves to minimize generation of the 3-phosphorylated phosphoinositides in quiescent cells [Dhand et al., 1994].

Recently, a new form of PI 3-kinase has been identified in platelets after cloning and expression studies [Stoyanov et al., 1995; Zhang et al., 1996]. The molecular weight of this catalytic species (p110γ) is also 110 kD, but lacks a binding site for p85 subunits and, instead, contains an N-terminal pleckstrin homology (PH) domain. PI 3-Kγ is activated by βγ subunits of heterotrimeric G-proteins and can be inhibited by binding βγ with the PH domain-containing C-terminal peptide of a β-adrenergic receptor kinase (βARK-PH) lacking protein kinase activity. In mammalian and insect cells, it has been recently shown that PI 3-Kγ activation by G-protein βγ is mediated by a tightly bound adapter protein, p101 [Stephens et al., 1997]. Like p85/PI 3-kinase, PI 3-Kγ uses all three

phosphoinositide substrates [PI, PI-4-P and PI-(4,5)P_2] but displays a preference for PI-(4,5)P_2 [Abrams et al., 1996]. It has been shown recently that the platelet PI 3Kγ is much less sensitive than p85 / PI-3-kinase to the PI 3-kinase inhibitor wortmannin. Furthermore, the p85/PI 3-kinase, in preference to PI- 3Kγ, was found to contribute to the activation of GPIIb/IIIa when the thrombin receptor or PKC is stimulated [Zhang et al., 1996].

B.2.1 The SH2 domains

The SH2 domain was first identified as a discrete conserved protein module, approximately 100 amino acids in length, residing in the non-catalytic regions of cytosolic protein-tyrosine kinases [Fry, 1994]. Proteins possessing this domain have been divided into two sub-families; those which have an intrinsic enzymatic activity, e.g., PLCγ or GAP, and those which are composed of essential regulatory domains and presumably act as adapter or linker molecules, e.g., Sem-5/Grb-2, Nck, or Crk. The p85 proteins appears to fall into this latter group.

The role of the SH2 domain has now been precisely defined. The SH2 domains of p85 enable recruitment of the p110 catalytic subunit by binding phosphotyrosine in receptors, protein-tyrosine kinases or their substrates. The major high affinity binding site consensus for both the N- and C-terminal SH2 domains of p85α is Y-X-X-M motifs [Cantley and Songyang, 1994]. It has been suggested that the phosphotyrosine-p85 interaction acts as a coupling mechanism for membrane localization of p110 and that it may function to increase the specific activity of the enzyme through conformational changes transduced to the p110 subunit [Carpenter et al., 1993a].

A stretch of approximately 200 amino acids lie between the two SH2 domains of p85α and p85β that has been suggested to play an important function for this portion of the molecule. This domain exhibit a preference for binding phospholipids in the order PI-(4,5)P_2 > PI-4-P > PI [End and Gout, 1993]. The function of this putative lipid binding

domain is currently uncertain, but it has been suggested that it may play a role in forming a specific substrate binding pocket, thus increasing the affinity for PI-(4,5)P_2 [Fry, 1994].

B.2.2 The SH3 domain

A single SH3 domain is located very close to the amino terminus of both p85α and p85β isoforms. This domain forms a small protein module, 50-60 amino acids in length, that binds to protein sequences rich in proline residues. SH3 domains clearly function in controlling protein-protein interactions. It has been suggested that the SH3 domain of p85 may localize the PI 3-kinase to components of the cytoskeleton [Gout et al., 1993]. Studies with GST-SH3 domain fusion protein containing the SH3 domains of p85α has shown that PLCγ and Grb-2 bind with high-affinity [Gout et al., 1993; Fry, 1994]. It has also been shown that p85α is able to bind to the SH3 domains of non-receptor tyrosine kinases, Abl, Fyn and to the SH3 domain of p85α in vitro. Additionally, the v-Src and p85α SH3 domains have been shown to bind to a recombinant amino terminal fragment of p85α containing these proline-rich sequences [Kepeller et al., 1994; Fry, 1994]. The proline-rich sequences in p85 proteins may therefore have a role in both 'self' association and in association with other proteins, possibly in a regulatory capacity. There is also already evidence that these proline-rich binding site play a role in the interactions of PI 3-kinase with members of the Src-family of protein -tyrosine kinases [Pleiman, 1994]. Interestingly, in platelets, it has been reported that thrombin induces an association of p85 with $p60^{src}$ and $p59^{fyn}$ [Gutkind et al., 1990]. Furthermore, it has been shown that PI 3-kinase is significantly activated by the $p125^{FAK}$ proline-rich sequence binding to the SH3 domain of p85α in thrombin-stimulated human platelets [Gutkind et al., 1990]. The precise mechanism of PI 3-kinase regulation will be discussed below in greater detail.

C. Regulation of p85/PI 3-kinase in platelets.

All possible 3-phosphoinositide species have been identified in platelets. PI-3-P is found at low, but detectable levels in resting platelets, while PI-(3,4)P2 and PI-(3,4,5)P3 are produced only in response to agonist stimulation. There is some controversy as to which phosphoinositide, PI-3-P, PI-(3,4)P2 or PI-(3,4,5)P3 is synthesized initially following thrombin-stimulation. A clear route for the activation of p85/PI 3-kinase by the G-protein-coupled receptor or integrin clustering has not been yet defined, but it may involve protein kinase C and small GTP-binding proteins and involves tyrosine phosphorylation that is independent of integrin occupancy. Here, I will attempt to discuss the involvement of these proteins in the activation of p85/PI 3-kinase with an emphasis on the tyrosine phosphorylation which plays key regulatory role not only in regulating the activity of PI 3-kinase, but also in controlling platelet aggregation.

C.1 Role of PKC

The increase in 3-PPI levels that follows the activation of the thrombin receptor does not seem to be simply a consequence of PKC activation and/or Ca^{2+} mobilization, i.e., the activation of PLC is not sufficient for a full response. It has been shown that β-phorbol dibutyrate or β-phorbol myristate acetate (β-PMA), alone or together with Ca^{2+} ionophore, at concentrations that stimulate PKC activity and $[Ca^{2+}]$ increases equivalent to those activated by thrombin, does not produce a similar elevation of 3-PPI levels [Zhang et al., 1996]. Nevertheless, PKC stimulation does play a role in the process. Permeabilized platelets exposed to the pseudosubstrate peptide inhibitor of PKC, RFARKGALRQKNV, show inhibited accumulation of 3-PPIs in response either to thrombin or GTPγS, although this inhibitor does not affect the activity of purified PI 3-kinase [King et al., 1991]. Furthermore, only the PKC-activating β-isoform of PMA stimulates 3-PPI accumulation,

and activation of platelets with β-PMA causes a shift of α- and β-p85/ PI 3-kinase to the cytoskeleton [Zhang et al., 1996] . In contrast, PI 3-Kγ activity does not increase in the cytoskeleton of β-PMA-activated platelets. Thus, a portion (that which is due to p85/PI 3-kinase) of the 3-PPI response to agonists such as thrombin seems likely to be dependent on PKC activity. Of potential relevance here, β-PMA also rapidly stimulates tyrosine phosphorylation in human platelets, although the identities of the tyrosine kinases activated are not clear [Golden and Brugge, 1989].

Phosphorylation of the major PKC substrate in platelets, the p47 phosphoprotein, pleckstrin, has been shown to be partially dependent on PI 3-kinase as it was inhibited by wortmannin [Toker et al., 1995; Zhang et al., 1995a]. It has been shown that synthetic PI-(3,4)P_2 and PI-(3,4,5)P_3 when added to permeabilized platelets caused wortmannin insensitive phosphorylation of pleckstrin. These lipids were also able to overcome inhibition of pleckstrin phosphorylation by wortmannin in stimulated platelets. However, wortmannin was unable to inhibit phorbol ester mediated pleckstrin phosphorylation [Rittenhouse, 1996]. The conclusions reached suggest that pleckstrin phosphorylation in activated platelets is mediated by both PLC/DAG mediated activation of PKC and PI 3-kinase mediated activation of Calcium-independent PKC isoforms.

Although, the role of pleckstrin in platelet function is not well understood, recent data suggests that this protein may function to inhibit agonist-induced phosphoinositide hydrolysis mediated by various isoforms of phospholipase C [Abrams et al., 1995]. It has been shown that pleckstrin phosphorylation inhibited platelet PI 3-Kγ but not p85/PI 3-kinase [Abrams et al., 1996]. Furthermore, treatment of platelets with β-PMA before exposure to the thrombin receptor agonist SFLLRN, has been shown to selectively inhibit PI 3-Kγ activity in intact platelets [Zhang et al., 1996]. These evidences suggested that pleckstrin phosphorylation may constitute a form of a feedback inhibition of PI 3-kinase.

C.2 Role of GPIIb/IIIa

There is also a role for the platelet integrin GPIIb/IIIa in modulating the accumulation of 3-PPI in response to thrombin [Sorisky et al., 1992; Sultan et al., 1991]. Inhibition (90%) of fibrinogen binding to platelet integrin by RGDS inhibits by 40% to 50% the late (5 minutes) thrombin-induced increases in PI-(3,4)P_2 (this is the species that appears later than PI-(3,4,5)P_3), and a similar effect is achieved by omitting Ca^{2+} from the medium or when platelets from thrombasthenic (GPIIb/IIIa-deficient) patients are incubated with thrombin. However, RGDS does not inhibit 3-PPI accumulation in platelets exposed to β-PMA, [Zhang et al., 1996] implying that the activation of PI 3-kinase by PKC is not secondary to fibrinogen binding. Normal platelets and thrombasthenic platelets, when permeabilized in the presence of 0.1 mmol/L Ca^{2+} and exposed to thrombin or GTPγS form similar amounts of PI-(3,4)P_2 and PI(3,4,5)P_3, as do intact platelets exposed to thrombin in the absence of added Ca^{2+} [Rittenhouse, 1995]. These are conditions in which GPIIb/IIIa is not activated. Furthermore, the accumulation of PI-(3,4,5)P_3 and PI-(3,4)P_2 induced by exposure of platelets to LPA for 60 seconds is not affected by the presence of the disintegrin eristostatin, which very effectively blocks platelet aggregation [Zhang et al., 1995b]. It has been suggested that platelet integrin may modulate the activity of a PI-(3,4)P_2 phosphatase, PI-(3)P 4-kinase, and/or a PI 3-kinase [Sorisky et al., 1992], but, indeed the platelet integrin is not necessary for the initial activation of PI 3-kinase. Our results will show that the activation of PI 3-kinase is also independent of integrin activation, i.e., is not secondary to fibrinogen binding in thrombin-stimulated human platelets.

C.3 Role of GTP-binding Proteins

Activators of classical G-proteins, such as GTPγS, have been shown to activate PI 3-kinase in a dose-dependent manner in permeabilized platelets, thus implicating G-proteins in regulation of PI 3-kinase [Kucera and Rittenhouse, 1990]. Precisely which G-proteins are involved and whether this coupling is a direct or an indirect effect, remains to be determined. However, if there is one major G-protein responsible for the changes seen, it is apparently not of the G_i subclass that regulates adenylyl cyclase, because it has been shown that inactivation of G_i by pertussis toxin treatment causes only a minor inhibition of 3-PPI accumulation [King et al., 1991]. Similarly, epinephrine, although able to activate G_i via the α_2 adrenergic receptor, is neither able to stimulate significant increases in 3-PPIs or potentiate increases in 3-PPIs in conjunction with phorbol diester and Ca^{2+} ionophore [King et al., 1991]. One is still left with a wide variety of potential G-protein candidates for this regulatory function, including Gs, Gz, and Gq in the heterotrimeric class, as well as a full palette of small G proteins. Recently, it has been shown that the small Ras-related GTP-protein, RhoA, was able to activate PI 3-kinase in soluble preparations of platelets and the activation of PI 3-kinase by GTPγs can be blocked by C3 transferase which specifically ADP-ribosylates Rho. This inhibition can be overcome by the addition of exogenous Rho protein, but not the addition of the closely related G-protein, Rac, supporting a specific role for Rho in the activation process [Morii et al., 1992; Zhang et al., 1993]. In support of these data, a similar involvement of Rho in the activation of PI 3-kinase in cultured fibroblasts has been reported [Kumagai et al., 1993]. It has been suggested that activation of the platelet PI 3-kinase by RhoA occurred directly or indirectly [Zhang et al., 1993]. One argument in support of the direct route has been that PI 3-kinase possesses on its p85 subunit the Rho/GAP-like domain that has been necessary for the GTPase-activating function of Rho/GAP. However, studies designed to evaluate the direct

interaction between Rho and PI 3-kinase have indicated no direct binding of Rho to recombinant p85/PI 3-kinase [Zhang et al., 1992; Rittenhouse, 1996].

C.4 Role of Tyrosine phosphorylation

PI 3-kinase activity has been found in association with protein-tyrosine kinases after thrombin stimulation of platelets. The use of specific tyrosine-kinase inhibitors suggests a correlation between tyrosine phosphorylation and the synthesis of 3-PPIs in stimulated platelets. The tyrosine kinase inhibitor, tyrophostin AG-213 was shown to potently inhibit thrombin-stimulated generation of PI-(3,4)P_2. This inhibition was correlated with inhibition of platelet aggregation and secretion and the amount of p85 subunit detected in anti-phosphotyrosine immunoprecipitates [Guinebault et al., 1993; Yatomi et al., 1994]. Tyrosine kinase inhibitors also potently inhibit cytoskeletal association of PI 3-kinase; when platelets are activated with thrombin the membrane cytoskeleton (Triton X-100 insoluble fraction) becomes enriched in a number of signaling protein and lipid kinases including diacylglycerol kinase, PI 4-kinase, PI 4-P 5-kinase, phospholipase C, PKC, FAK, Src and PI 3-kinase [Zhang et al., 1992; Grodin et al., 1991; Guinebault et al., 1995]. It has been reported that up to 30% of p85/PI 3-kinase (immune-quantifiable enzyme) is translocated to the cytoskeleton representing about 70% of total platelet PI 3-kinase activity [Grodin et al., 1991; Zhang et al., 1996].

Although PI 3-kinase activity and p85 protein can be recovered in anti-phosphotyrosine immunoprecipitates from thrombin-stimulated platelets, there is no compelling evidence for direct tyrosine phosphorylation of any of the components of the PI 3-kinase in platelets [Guinebault et al., 1993; Mitchell et al., 1990; Zhang et al., 1992]. The most likely suggestion would therefore be that a fraction of the PI 3-kinase is probably binding directly to either a protein-tyrosine kinase, or to a tyrosine phosphorylated substrate molecule. This would be mediated via either SH2 or SH3 domains, as described

for the association of PI 3-kinase with Src-family kinases in other cell types [Carpenter and Cantely, 1990; Pleiman et al., 1994].

Ferrell and Martin [1988] demonstrated that in response to thrombin, sets of proteins are phosphorylated at tyrosine residues in human platelets which can be divided into three groups based on the kinetics of their phosphorylation. The first wave included four protein bands of a molecular weight mass of 70, 68, 34 and 27 kD within 5-20 s of thrombin treatment. The second wave included bands of 130, 115 and 105 kD and the putative $pp60^{c\text{-}src}$ band within 1-3 min. The third and the last wave of phosphorylation peaked within 3-5 min. after thrombin treatment and included 126, 100, 108 and 85 kD protein species. It has been suggested that the first peak is specific to thrombin treatment, the second peak is due to the activation and shape change, and the last one appears to correlate with aggregation [Feinstein et al., 1993; Dhar and Shukla, 1993]. Indeed, the platelet PI 3-kinase activity has been found to be associated with non-receptor tyrosine kinases including members of the Src family, $p60^{c\text{-}src}$ and $p59^{c\text{-}fyn}$ [Gutkind et al., 1990], and with the focal adhesion kinase, $p125^{FAK}$ [Guinebault et al., 1995]. The association of these protein tyrosine kinases (PTKs) with the activity of PI 3-kinase appears to be regulated by the events that control their stimulation. These events are distinguished by the temporal order of their activation and by their dependence on integrin binding to fibrinogen or on subsequent platelet aggregation. They are described in the following sections.

Early activation events are defined as those that are detectable within seconds after treatment of platelets with strong agonists, and by their insensitivity to treatments that prevent ligand binding to GPIIb/IIIa (for example, by incubation with RGDS, EDTA or function-blocking antibodies to GPIIb/IIIa) [Clark et al., 1994b]. Three PTKs are activated under these conditions: Syk, Src and the novel member of FAK gene family, PYK2.

$p72^{syk}$ is activated and tyrosine phosphorylated in platelets that are exposed to thrombin, collagen, TXA_2, or the low-affinity Fcγ receptor (FcγRIIA) [Fujii et al., 1994;

Chacko et al., 1994]. A portion of $p72^{syk}$ activation is insensitive to blockage of integrin function, but much of $p72^{syk}$ is phosphorylated/activated soon after agonist-induced or anti-LIBS (ligand induced binding site) Fab-induced binding of fibrinogen to GPIIb/IIIa [Clark et al., 1994a]. Syk is translocated to the cytoskeleton after thrombin-induced platelet activation [Law et al., 1994; Rittenhouse, 1996] and could thus participate in postintegrin as well as preintegrin signaling. Although there is no report of a direct association between PI 3-kinase and Syk, it has been shown that this tyrosine kinase contains three potential phosphorylation sites in the YXXM motifs known to be consensus binding sites for SH2 domains of p85/PI 3-kinase [Law et al., 1994] and p85/PI 3-kinase has been found in the immunoprecipitated activated FcγRIIA receptor containing $p72^{syk}$ [Chacko et al., 1996].

Src is the prototype for a family of kinases that is present in extremely high level in platelets, representing approximately 0.2-0.4% of total platelet protein [Golden and Brugge, 1989]. Following treatment with thrombin or other agonists there is a transient activation of Src in the detergent-soluble platelet fraction followed by a redistribution to the detergent-insoluble (cytoskeleton) fraction [Zhang et al., 1992; Clark et al., 1994b]. The activation of Src is independent of fibrinogen binding to GPIIb/IIIa and platelet aggregation. However, the localization of activated Src is dependent on GPIIb/IIIa, since it does not redistribute to the detergent-insoluble fraction in thrombin-treated Glanzmann platelets. It has been suggested that the GPIIb/IIIa-cytoskeleton complexes serve as the docking site for activated Src [Clark et al., 1994b]. There are four other members of the Src kinase family present in platelets, Fyn, Yes, Lyn and Hck, but at significantly lower levels than Src [Huang et al., 1991b].

Gutkind et al.[1990] reported that thrombin induced the association of PI 3-kinase with $p60^{src}$ and $p59^{fyn}$ in human platelets. There has no change in activities of Src and Fyn been detected in the PI 3-kinase immunoprecipitates upon thrombin stimulation and the mechanism of their association is not understood. It has been suggested that the translocation of Src and other proteins to the membrane and the possible cluster formation

or interactions of the SH2 domains of Src, PLC, ras-GAP and PI 3-kinase with membrane components (receptors) may play an important role in receptor signaling mechanisms in platelets [Dhar and Shukla, 1993].

Protein Tyrosine Kinase 2 (*PYK2*) [Lev et al., 1995] also known as RAFTK (Related Adhesion Focal Tyrosine Kinase) [Avraham et al., 1995] and the rodent cell adhesion kinase β (CAK-β) [Sasaki et al., 1995] were reported to have the closest homology (48% identity, 65% similarity) to FAK. Analysis of its deduced amino acid sequence also indicated that PYK2, like FAK, lacked a transmembrane region, myristylation sites, and SH2 and SH3 domains. In addition, like FAK, the PYK2 C-terminal domain contains a predicted proline-rich stretch of residues.

PYK2 has been involved in various signal transduction pathways, including those controlling cell growth, differentiation and activation. PYK2 is highly expressed in the central nervous system. It has been shown that PYK2 is involved in modulating calcium influx [Lev et al., 1995] and the activation of MAP kinase signaling pathway by acting with Src to link G_i-and Gq-coupled receptors with Grb2 and Sos and thereby MAP kinase in PC12 cells [Dikic et al., 1996]. In addition, activation of PYK2 was shown to couple with the c-jun N-terminal kinase signaling pathway due to stimulation by the inflammatory cytokine tumor necrosis factor and by stress signals such as ultraviolet light and osmotic shock [Tokiwa et al., 1996]. Therefore, PYK2 is a cell type specific, stress-sensitive mediator of the JNK signaling pathway [Tokiwa et al., 1996]. PYK2 is also expressed in various hematopoietic cells such as human megakaryocytes and platelets [Avraham et al., 1995; Li et al., 1996]. In megakaryocytes, a specific association of phosphorylated PYK2 with Src-family kinases (Src and Fyn) and the Grb2 adapter was demonstrated in an SH2-dependent manner after cellular integrin activation [Li et al., 1996]. PYK2 has been implicated in signaling events which link signals from cell surface to the cytoskeleton. It has been demonstrated that cytochalasin D, which disrupts the cytoskeleton, abolished the phosphorylation of PYK2 upon PMA and stem cell factor (SCF) stimulation [which causes

differentiation of megakaryocytic cell lines and potentiates the growth of megakaryocytes and their progenitors], indicating that PYK2 association with the actin cytoskeleton appears to be critical for its phosphorylation in CMK human megakaryocytic cells [Hiregowdara et al., 1996]. Furthermore, it has been suggested that PYK2 might be important in phosphotyrosine signaling events within focal adhesions. The colocalization of the PYK2 with vinculin, a focal adhesion protein, was observed upon fibronectin activation [Li et al., 1996]. In addition, an association of PYK2 with paxillin was also observed in CMK cells [Hiregowdara et al., 1996]. It has been shown that overexpression of a dominant-negative mutant of PYK2 significantly inhibited the tyrosine phosphorylation of paxillin upon PMA stimulation.

In platelets, PYK2 was shown to be phosphorylated as early as 10 seconds after stimulation by thrombin and several agonists such as collagen, ADP, epinephrine and calcium ionophore A23187 [Raja et al., 1996]. Unlike FAK, PYK2 was found to be tyrosine phosphorylated during early phase of platelet activation by integrin independent mechanism and is not dependent on platelet aggregation. It has been shown that treatment of platelets with thrombin in the absence of stirring, or pretreatment of platelets with RGDS peptide, did not prevent PYK2 phosphorylation. Furthermore, phosphorylation of PYK2 did not require integrin engagement since platelets treated with the 7E3 inhibitory antibodies that block fibrinogen binding to GPIIb/IIIa did not inhibit PYK2 phosphorylation. Similarly, platelets treated with LIBS6 antibodies, which specifically activate GPIIb/IIIa, did not induce PYK2 phosphorylation [Raja et al., 1996]. While the role of PYK2 in platelets has yet to be elucidated, it has been shown that RAFTK is associated with the actin filaments of the cytoskeleton, which is suggested to be critical for its phosphorylation [Raja et al., 1996].

Events dependent on fibrinogen binding to GPIIb/IIIa. The tyrosine phosphorylation events that are dependent on GPIIb/IIIa can be divided into two distinct phases: first, those that take place in the absence of platelet aggregation and are dependent

on cross-linking and oligomerization of GPIIb/IIIa; and second, those that require fibrinogen-dependent platelet aggregation. The only PTK that has been shown to be activated upon GPIIb/IIIa crosslinking is Syk, which is also phosphorylated on tyrosine under these conditions [Clark et al., 1994a].

Events dependent on platelet aggregation. These events are defined not only by their absence in platelets treated with agonist under conditions that block fibrinogen binding to GPIIb/IIIa, but also by their dependence on aggregation in thrombin-treated platelets. Several PTKs may be involved in these events: (1) FAK (focal adhesion kinase) is implicated by its activation following aggregation, and (2) Src family kinases and Syk are implicated by their redistribution to integrin-dependent cytoskeletal complexes [Clark, 1994a; Clark, 1994b; Fry, 1994; Shattil et al., 1998].

FAK is a tyrosine kinase that localizes with integrins at the cytoskeleton via a C-proximal focal adhesion targeting (FAT) sequence and an N-proximal sequence that binds to the cytoplasmic domains of β-integrins [Clark et al., 1995]. In platelets, activation (and tyrosine-phosphorylation) of FAK is stimulated by integrin cross-linking in a manner dependent on PKC activation [Shattil et al., 1994]. The coordination of signaling in platelets triggers changes in the cytoskeleton which appears necessary for the tyrosine phosphorylation of FAK [Shattil et al., 1994].

It has been shown that thrombin activation of platelets leads to formation of a multienzymatic complex of PI 3-kinase and tyrosine-phosphorylated $p125^{FAK}$ in an area tightly associated with the actin filament system corresponding to the focal contact-like domains [Guinebault et al., 1995]. Their formation appears to be dependent upon aggregation and is related to integrin-dependent tyrosine kinase activation. In investigating these associations, it has been found that a proline-rich sequence of the human FAK (residues 706-711) directly bound to the SH3 domain of p85α subunit. Increased PI 3-kinase activity was observed due to that reaction. It has been suggested that FAK may be responsible for the increased PI 3-kinase activity in the cytoskeleton and the accumulation

of PI-(3,4)P_2 in the late phase of platelet activation that is dependent on both aggregation and GPIIb/IIIa integrin receptor engagement [Guinebault et al., 1995].

D. Current problems and objectives

As outlined in the above discussion, the exact mechanism leading to the activation of p85/PI 3-kinase by agonist-occupied G-protein-coupled receptors is poorly understood, but it is believed that protein-tyrosine kinases play a key modulatory role in regulating the activity of p85/PI 3-kinase.

The goals of this project were:

1. To characterize unidentified proteins that associate with, and may regulate, p85/PI 3-kinase in thrombin and/or PAF-stimulated platelets.
2. To identify the mechanism in which p85/PI 3-kinase is initially activated in thrombin-stimulated platelets.

METHODS

A. Platelet Isolation

A.1 Human Platelets

Human blood was withdrawn from healthy, drug-free volunteers. Approximately 100 ml of the freshly drawn blood was mixed directly with the anticoagulant ACD solution (25 g trisodium citrate dihydrate, 14 g citric acid and 20 g glucose per liter) at a ratio of 9:1. Platelets were immediately isolated according to a previously described method [Kinlough-Rathbone et al., 1970] with some modifications. Briefly, platelet-rich plasma was first separated from the blood by centrifugation at 190 x g for 15 min. and the platelet pellet was obtained by centrifugation of platelet-rich plasma at 2500 x g for 15 minutes. The platelet pellet was gently resuspended in 10 ml of Tyrode's buffer 1 (136.9 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.35 g albumin and 0.1 g dextrose in 100 ml volume, pH 7.35) supplemented with apyrase (2 units/ml) and heparin (50 units/ml). After incubating at 37 ^oC for 15 minutes, the solution was centrifuged at 1200 x g for 10 minutes. The supernatant was decanted and the platelets resuspended gently in 10 ml of the Tyrode's buffer 2 (the same as Tyrode's 1 without heparin) and incubated at 37 ^oC for 10 minutes. Platelets were then pelleted at 1200 g for 10 minutes and resuspended at room temperature in Tyrode's buffer 3 (the same as Tyrode's 2 except with only 0.2 unit/ml apyrase). The platelet suspension was counted in an automated Coulter counter. Erythrocyte or Leukocyte contamination was never greater than 0.1%. For experiments, platelets were suspended at a concentration of 4-5 X 10^8/ml.

A.2 Rabbit Platelets

Platelets were isolated as previously described by Pinckard et al.[Pinckard et al., 1979]. Whole blood from New Zealand white rabbits was anticoagulated with the ACD anticoagulant at a ratio of 9:1 and used immediately. Anticoagulated blood (30 ml) was diluted with 10 ml Tyrode's buffer pH 6.50 containing 0.1 mM EGTA and centrifuged for 12 minutes at 190 x g. The platelet-rich plasma (8 ml) was withdrawn and underlayered with 2 ml Histopaque solution (57 mg/ml polysucrose, 90 mg/ml sodium diatrizoate, density 1.077 g/ml, Sigma Chemical Co.) then spun at 600 x g for 12 minutes. Platelets, which formed a band at the Histopaque/plasma interface were removed, diluted with Tyrode's buffer + EGTA and the Histopaque separation repeated once more. The platelet suspension was diluted again in Tyrode's + EGTA. pelleted at 600 x g then washed three times in Tyrode's buffer + EGTA. For experiments, platelets were gently resuspended in Tyrode's buffer pH 7.2 containing 1 mM Ca^{2+} at a concentration of 2 X 10^9/ml. All subsequent manipulations were at room temperature.

B. Platelet Activation

Prior to the addition of agonists, aliquots of washed platelets (2 X 10^9/ml) were preincubated in a Lumiaggregometer (Biodata Corp) at 37 ^oC for 2 minutes without stirring. Platelets were stirred at 37 ^oC for 2 minutes in the absence or presence of the following agonists: platelet-activating factor (PAF) (10 nM; Sigma) in case of rabbit platelets, thrombin (1 unit/ml; Sigma) in the case of human platelets. At the end of the activation period, platelets were lysed in an equal volume of ice-cold TX-100 solubilization buffer (2% Triton X-100, 20 mM Tris-HCl pH 8, 137 mM NaCl, 20% glycerol, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM Na_3MoO_4, 2 mM phenylmethylsulfonyl fluride, 20 µg/ml leupeptin, 2 µg/ml aprotinin, 20 mM NaF, 2 µM pepstatin, 20 µg/ml soybean trypsin

inhibitor). Platelet lysates were left on ice for 30 minutes and then sonicated, using 3 X 2 seconds pulses with a probe sonicator. Detergent-insoluble fractions were removed by centrifugation for 15 min. at 15,000 rpm in the cold (4 °C). Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce).

In some experiments, platelets were pretreated with one of the following prior to stimulation with thrombin: RGDS (0.5-1 mM; Sigma), EDTA for 2 min. at 37 °C (4 mM), the PKC inhibitor, bisindolylmaleimide for 1 hour at room temperature (12 μM; Calbiochem) and the PI 3-kinase inhibitor; LY-294002 for 10 min. at 37 °C (25 μM; Sigma). The stock solution of LY-294002 was prepared in DMSO at a concentration of 50 mM and stored at -70 °C. Immediately before use, 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.

C. Isolation of the Platelet Plasma Membrane

Protease and tyrosine phosphatase inhibitors (2 mM EDTA, 1 μM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, 1 μM pepstatin, 10 μg soybean trypsin inhibitor, 1 mM Na orthovanadate and 1 mM molybdate) were added to the rabbit platelet suspension (2 X 10^9/ml) in Tyrode's buffer pH 7.2 containing 1 mM Ca^{2+} . Platelets were either left unstimulated or stimulated with PAF (10 nM; 2 min.). The reaction was stopped by gentle sonication on ice, 5 X 3 seconds pulses with probe sonicator. The unbroken cells were centrifuged at low speed (2000 rpm in SS34 rotor) for 10 min. at 4 °C. The supernatant was recovered and spun at 34,000 x g (15,000 rpm in SS34) for 15 min. The pellet (crude membranes) was isolated, washed twice with cold PBS, pH 7.4, resuspended in 10 mM Hepes, pH 7.4, with protease and tyrosine phosphatase inhibitors (as indicated above) and then lysed with TX-100 solubilization buffer. The Triton-insoluble

membrane fractions were sonicated 3 X 3 seconds pulses for complete solubilization. The supernatant (cytosol) and the membrane suspensions were either immunoprecipitated with anti-p85 polyclonal antibody or with an equal volume of 2 X SDS-PAGE sample buffer containing 1% β-mercaptoethanol (2-Me) and boiled for 3 min.

D. Immunoprecipitation of platelet lysates

Platelet lysates (50-300 μg) were immunoprecipitated with a rabbit antisera to the p85 sununit of PI 3-kinase (p85β and p85α (polyclonal), a mouse monoclonal anti-p85α, a rabbit polyclonal anti-PYK2 (Upstate Biotechnology Inc. (UBI), a mouse monoclonal anti-phosphotyrosine antibody (4G10; UBI) or with a goat anti-PYK2 raised against either the N terminus or C terminus of PYK2 (Santa Cruz) for 2 hr or overnight at 4 °C. The immunocomplexes were immobilized on 25 μl packed beads of protein A-Sepharose (in the case of immunoprecipitation with anti-p85 antibody (polyclonal) or protein G-Sepharose for additional 1 hr at 4 °C. In some experiments, platelet lysates were incubated with protein G- or protein A-sepharose in the absence of antibodies to deterimne any non-specific associations. The immunoprecipitates were washed 4 X with ice-cold 1 X solubilization buffer containing protease and tyrosine phosphatase inhibitors (mentioned above) and resuspended in 30-35 μl of 2 X SDS sample buffer containing 1% 2 Me.

E. GST-SH2 affinity-binding

200-1000 μl of the fusion protein, glutathione S-transferase (GST) containing the N- or C-terminus of the SH2 domain of p85/PI 3-kinase (bacterial expression plasmids kindly provided by Melanie Welham; University of Bath, Bath, U.K.) was immobilized on Glutathione-Sepharose beads (20-100 μl of packed beads; Pharmacia) and incubated for 1 h at 4 °C. The GST-beads complex was washed twice with GST buffer (150 mM NaCl, 25

mM Tris; pH 7.5, and 10 mM β mercaptoethanol) + 0.5% Tween-20 and then incubated with rabbit platelet lysates (50-1000 μg) from unstimulated or PAF- stimulated cells for 2 h at 4 °C. The precipitates were washed 4 X with ice-cold 1 X solubilization buffer containing protease and tyrosine phosphatase inhibitors (described above) and resuspended in 20-50 μl of 2 X SDS sample buffer + 1% 2 Me. Proteins were eluted from the beads and subjected to electrophoresis on 7.5 % SDS-polyacrylamide gel, 2-D electrophoresis, or column chromatography for protein purification.

F. Protein Purification of an unidentified protein (p115 kDa)

F.1 Two-Dimension Electrophoresis

The platelet proteins (50 μg) eluted from the glutathione beads or protein A-Sepharose (in the case of p85 immunoprecipitation) were diluted 1:1 with the first dimension sample buffer (9.5 M urea, 20% TX-100, 5% 2-Me, 1.6% Bio-Lyte 5/7 ampholyte, and 0.4% Bio-Lyte 3/10 ampholyte) and separated according to their IEF (first dimension) by the Mini-Protean II 2-D tube-cell (Bio Rad) exactly as described by the Bio Rad instruction manual for Mini-Protean II (catalog numbers 165-2960, 165-2961 and 165-2965) and then layered onto SDS-PAGE (7.5%) for the second dimension separation. The gel was transferred to nitrocellulose and immunoblotted by anti-phosphotyrosine (4G10) antibody.

F.2 Separation by Column Chromatography on FPLC system

F.2.1 Mono Q anion exchange Column

The column (Pharmacia) was equilibrated with the Mono Q buffer; solution A (50 mM Tris; pH 7.4, 1 mM NaF, 1 mM EDTA + the protease and tyrosine phosphatase inhibitors). The platelet proteins eluted from the glutathione beads were diluted 1:1 with the Mono Q buffer and loaded onto the column. The flow rate was set at 1 ml/min. and a linear gradient of 0-1.0 M NaCl (solution B, contained 1 M NaCl in buffer A) over 12 ml was programmed. The protein fractions were collected, subjected to SDS-PAGE (7.5%) and transferred to be immunoblotted using anti-phosphotyrosine antibody (4G10).

F.2.2 Superose-12 Gel Filtration Column

The Superose-12 column (Pharmacia) was first equilibrated in column buffer (0.15 M NaCl and 0.05 M phosphate, pH 7.2). Cytochrome C (2 mg/ml) was used to determine the void volume and washing was continued overnight with the Superose buffer . The platelet proteins eluted from the glutathione beads (300-1000 µg) were diluted 1:1 with 100 mM Tris, pH 7.4 and 0.1% Triton X-100 + the protease and tyrosine phosphatase inhibitors. The lysates (100 µl) were then loaded onto the column and eluted at a flow rate of 0.4 ml/min. The protein fractions were collected, aliquots were mixed with 5 X SDS sample buffer containing 2 Me, and separated by SDS-PAGE (7.5%) to be immunoblotted against anti-phosphotyrosine antibody. Alternatively, precipitation with 10% trichloroacetic acid (TCA) in the presence of ovalbumin was used to concentrate the proteins. The void volume was 7 ml.

F.2.3 Protein Precipitation and Silver Staining

Samples collected from the gel filtration (Superose-12 column) were incubated with 10% TCA in the presence of ovalbumin (10 µg/ml) and left on ice for 2 h. The precipitates were recovered after centrifugation at 15,000 rpm for 10 min. at 4 ^{0}C, and washed 1 X

with ethanol (95%). The samples were spun again and the supernatant was carefully aspirated. The protein pellet was air dried for few seconds and resuspended in 50 mM Tris, pH 7.4 + 0.1% Triton X-100. The protein suspension was mixed with 5 X SDS sample buffer containing 2 Me, boiled and separated by SDS-PAGE (7.5%). The gel was silver stained according to the protocol of Morrisey [1981].

G. Protein Gel Electrophoresis and Immunoblotting

Platelet proteins were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were blocked for 1 h. or overnight with 10 mM Tris; pH 8, 150 mM NaCl (TBS) containing either 5% milk powder or bovine serum albumin and 0.05% azide. The primary antibody was diluted in TBS containing 1% bovine serum albumin and 0.02% azide and incubated with the membrane overnight or for 2 hr at room temperature. After extensive washing with TBS containing 0.05% Tween 20 (TBST), the membranes were incubated for 1 h. at room temperature with horseradish peroxidase-coupled secondary antibody in TBST. Bound antibodies were detected using enhanced chemiluminescence (Amersham Corp.). In some instances, blots were re-probed with other antibodies after stripping with 62.5 mM Tris; pH 6.8, 2% SDS, 100 mM 2-Me at 50 ^{o}C for 30 min.

H. PI 3-kinase Assay

Platelet lysates (300 μg) from unstimulated or thrombin stimulated (1 U/ml; 2 min.) aliquots were immunoprecipitated with a monoclonal anti-p85α, anti-PYK2 (UBI and Santa Cruz), or a monoclonal anti-phosphotyrosine (4G10, UBI) antibodies for 3 hr or overnight at 4 ^{o}C and the immune complexes collected on protein G-Sepharose (25 μl packed beads). The precipitates were washed twice with 1 X solubilization buffer and three

times with buffer consisting of 10 mM Tris, pH 7.4. Ten µg sonicated PI (Avanti Polar Lipids, Alabaster, AL) was mixed with the beads and kept for 10 min. on ice. The kinase reaction was initiated by adding 40 µl of kinase buffer (50 µM ATP, 30 mM Hepes; pH 7.4, 30 mM $MgCl_2$, 200 µM adenosine) containing 10 µCi of [γ-^{32}P] ATP. After 15 min. at room temperature, 0.1 ml of 1 N HCl and 0.2 ml of chloroform:methanol (1:1 v:v) were added to stop the reaction. The samples were vortexed then the lower organic layer (70 µl) transferred to new tubes and kept at -20 °C until analysis.

^{32}P-labeled PI(3)P was separated from residual [γ-^{32}P] ATP (retained at the origin) by chromatography on oxalate-treated TLC plates using a solvent system of chloroform:methanol:water:28% ammonium hydroxide (90:70:15:5, v:v:v:v). TLC plates were exposed to X-ray film at -80 °C then radioactivity incorporated into PI(3)P (PI 3-kinase activity) was either measured by excising the spot from the plate followed by liquid scintillation counting or by phospho-imaging, using the molecular imager (Bio Rad).

I. PYK2 Kinase Assay

The immunoprecipitated complexes obtained by immunoprecipitating platelet lysates with anti-PYK2 antiserum (UBI) were washed 3 X with lysis buffer (50 mM Tris; pH 7.4, 10% glycerol, 1% NP-40, 100 mM NaCl, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml microcystin-LR, 20 µg/ml leupeptin, 2 µM pepstatin, 1 mM Na_3VO_4 and 1mM Na_3MoO_4) and twice in kinase buffer (20 mM Hepes; pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_3VO_4 and 20 µM ATP). The kinase assay was initiated by incubating the immune complex in the kinase buffer containing 25 µg of poly (Glu / Tyr, 4:1; 20-50 kD; Sigma Chemical Co.) and 5 µCi [^{32}P] ATP at room temperature for 30 min. Reaction were terminated by spotting onto p81 paper, followed by extensive washing in 1.0% phosphoric acid. PYK2 kinase activity was measured by liquid scintillation counting.

RESULTS

A. Characterization of Tyrosine-Phosphorylated Proteins associated with PI 3-Kinase

Since association of PI 3-kinase (via the p85 subunit) with tyrosine-phosphorylated proteins is a major mechanism for activation of this enzyme [Carpenter et al., 1993a], we set out to look for tyrosine-phosphorylated proteins that associate with PI 3-kinase in agonists (such as PAF or thrombin)-stimulated platelets.

Rabbit or human platelet lysates from unstimulated or stimulated aliquots were immunoprecipitated with anti-p85/PI 3-kinase antibody. Proteins were eluted by boiling beads in SDS-sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with anti-phosphotyrosine (4G10) antibody to detect the tyrosine-phosphorylated proteins associated with PI 3-kinase. Heavily tyrosine phosphorylated proteins of apparent molecular weight 115 kD (p115) and 102 kD (p102) were shown to be associated with PI 3-kinase in PAF-stimulated rabbit and thrombin-stimulated human platelets respectively (Figure 1). p115 was also shown in thrombin-stimulated rabbit platelets (results not shown). p115/p102 were shown to be the major substrates that were consistently observed and which were phosphorylated almost exclusively in response to agonists. Attempts to identify these proteins by myself and my colleague Ron Lauener, used antibodies against a number of potential tyrosine phosphorylated proteins having a similar molecular weight and which are associated with PI 3-kinase in platelets or in other cell systems. These experiments ruled out Gab-1, the 110 kD catalytic subunit of PI 3-kinase, p120 ras-GAP, JAK-2, cbl, FAK or integrin subunit β1 as possible candidates. Because of the easier availability of rabbit blood, we first attempted to characterize p115 in PAF-stimulated rabbit platelets.

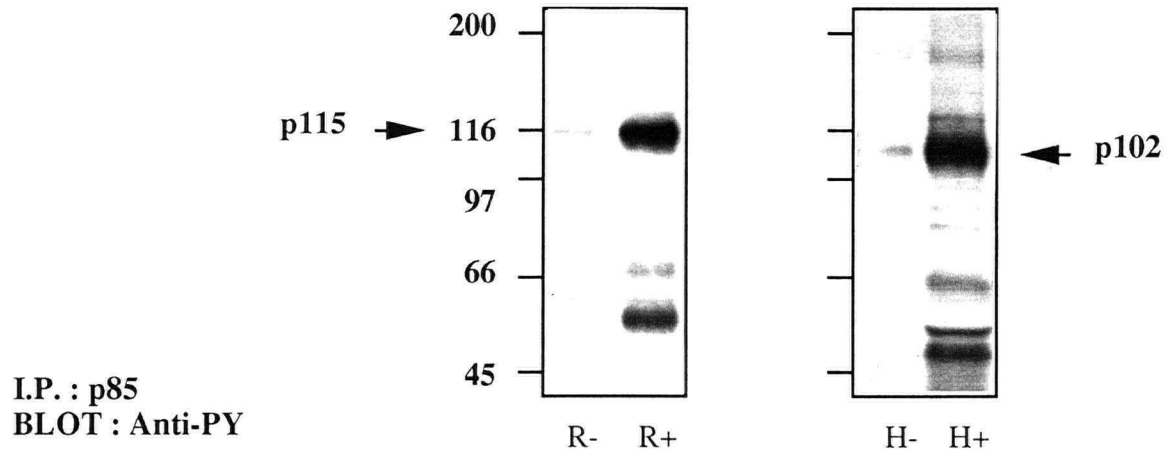


Figure 1

A major tyrosine-phosphorylated protein is shown to be associated with p85/PI 3-kinase in PAF-stimulated rabbit (p115) and thrombin-stimulated human (p102) platelets. Rabbit (R) and human (H) platelets were either stimulated with 10 nM PAF (R+) and 1 U/ml thrombin (H+) for 2 min., or left unstimulated, (R-) and (H-) respectively. The reaction was terminated by adding 1 volume of 2 X TX-100 solubilization buffer. After sonication and centrifugation, the supernatants from detergent lysed platelets were immunoprecipitated (I.P.) with polyclonal antisera to the p85/PI 3-kinase and captured on protein A-Sepharose. Eluted proteins were separated by SDS/PAGE, transferred to nitrocellulose then blotted with monoclonal antibody (4G10) to phosphotyrosine (PY). Positions of molecular weight standards are shown on the left and the arrows indicate the positions of p115 and p102 proteins.

A.1 p115 was found to be associated with p85/PI 3-kinase in the cytosol

While the mode of the translocation of PI 3-kinase to membranes following its activation is unclear, it has been suggested that translocation is probably brought about by a direct interaction with the membrane-spanning or membrane-associated protein tyrosine kinase involving the SH2 or SH3 domains of the p85 subunit, or via associating to adapter proteins that are predominantly cytoplasmic [Fry, 1994; Holgadomadruga et al., 1996; Weinder et al., 1996]. To investigate the localization of the associated p115, the plasma membranes from the sonicated aliquots of unstimulated and PAF-stimulated rabbit platelets (10 nM; 2 min.) were prepared by ultracentrifugation. The isolated membrane and cytosolic fractions were immunoprecipitated with polyclonal anti-p85 antiserum and immobilized on protein A- Sepharose. The proteins eluted from the beads were resolved by SDS-PAGE and blotted by anti-phosphotyrosine (4G10) antibody. p115 was shown to be predominantly cytosolic in both control and stimulated platelets with no detectable localization in the membrane (Figure 2).

A.2 p115 was associated with the SH2 domain of p85 in vitro

To test whether p115 may bind to the SH2 domain of p85, we prepared GST fusion proteins having either the C-terminal or N-terminal-SH2 of p85 to perform in vitro binding experiments. Sonicated platelet lysates from unstimulated and PAF-stimulated (10 nM; 2 min.) samples were incubated with the GST fusion proteins captured on Glutathione-Sepharose beads. After washing, the bound proteins were eluted, separated on SDS-PAGE and detected by western blotting with 4G10 antibody (Figure 3). p115 was shown to be associated to both the N- or C-terminal of the GST-SH2. However, p115 binding to GST-C-SH2 was slightly stronger than that to GST-N-SH2. Compared to the

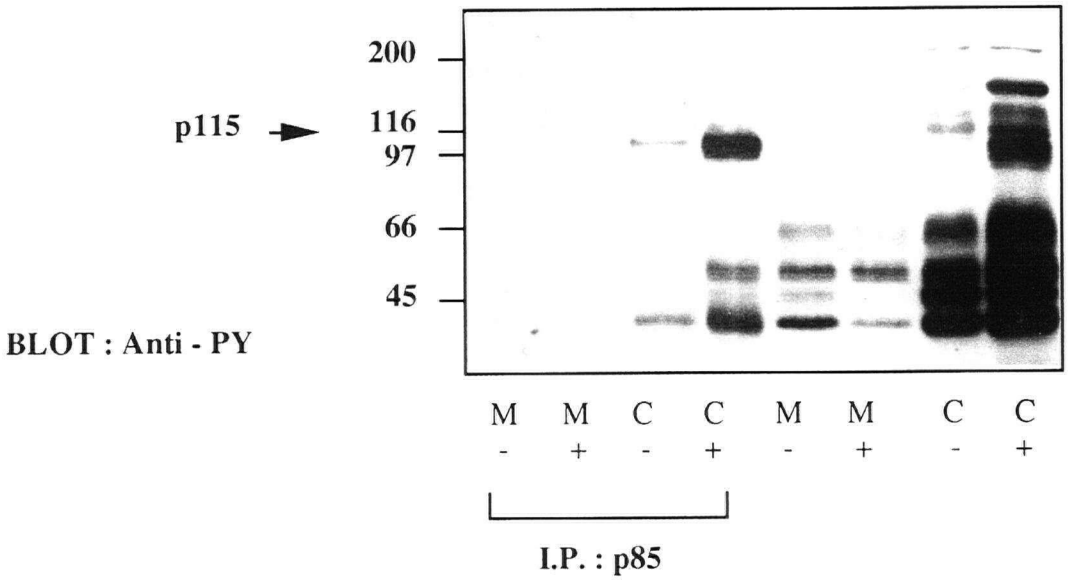


Figure 2

p115 is associated with p85/PI 3-kinase in the cytosol. Isolated rabbit platelets were either left unstimulated (-) or stimulated with 10 nM PAF (+). The reactions terminated after 2 min. by sonication in an ice bath. Cytosol (C) and membranes (M) were isolated by ultracentrifugation and immunoprecipitated with polyclonal antisera to p85/PI 3-kinase. Some aliquots from the isolated cytosol and membranes prior to immunoprecipitation, were mixed with 1 volume of 2 X SDS-sample buffer containing 2 Me. Eluted proteins were separated by SDS/PAGE, transferred and blotted by anti-PY antibody. The arrow indicates the position of p115 protein.

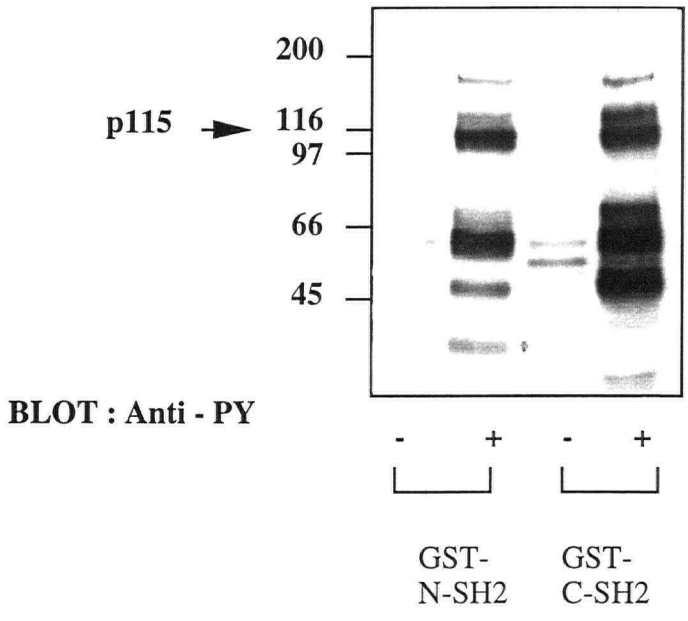


Figure 3

p115 is interacting with the SH2 domains of p85/PI 3-kinase *in vitro*. Glutathione S-transferase (GST) proteins coupled to N-SH2 and C-SH2 domains of p85 subunit were immobilized on Glutathione-Sepharose beads and incubated with platelet lysates from PAF-stimulated (10 nM; 2 min.(+) and unstimulated (-) samples. Proteins eluted from the beads, separated by SDS/PAGE and blotted with anti-PY antibody.

anti-p85 co-immunoprecipitation, the SH2 fusion proteins bound several additional tyrosine phosphorylated proteins.

B. Characterization and purification of p115 from PAF-stimulated rabbit platelets

B.1 Two-dimensional gel electrophoresis

The first method we used to separate p115 from other tyrosine phosphorylated proteins associated with p85 was two-dimensional gel electrophoresis because of its high resolution. The denatured proteins eluted from immunoprecipitated samples with anti-p85 antibody or from affinity precipitation with GST-C-SH2 protein were subjected to 2-D electrophoresis. Two spots were detected at approximately 115 kD in p85 immunoprecipitates, which indicate that p115 may consist of more than a single protein (Figure 4). However, these could also represent different phosphorylation states of the same protein. At present these results are not conclusive.

B.2 Column purification

We attempted to use column chromatography in order to separate p115 by size or charge. The protein could then be subjected to further purification by using two-dimensional gel electrophoresis.

Platelet lysates (800-1000 μg) from PAF-stimulated samples were captured on glutathione beads that contained GST-C-SH2 fusion proteins. The denatured proteins eluted from the beads by boiling in SDS sample buffer, were loaded onto a Mono Q column, and a wide range of fractions were separated on SDS-PAGE (7.5%) and immunoblotted using 4G10 antibody. p115 was shown to elute over a broad range of salt concentrations around 0.5 M of the NaCl gradient (results not shown). This technique was

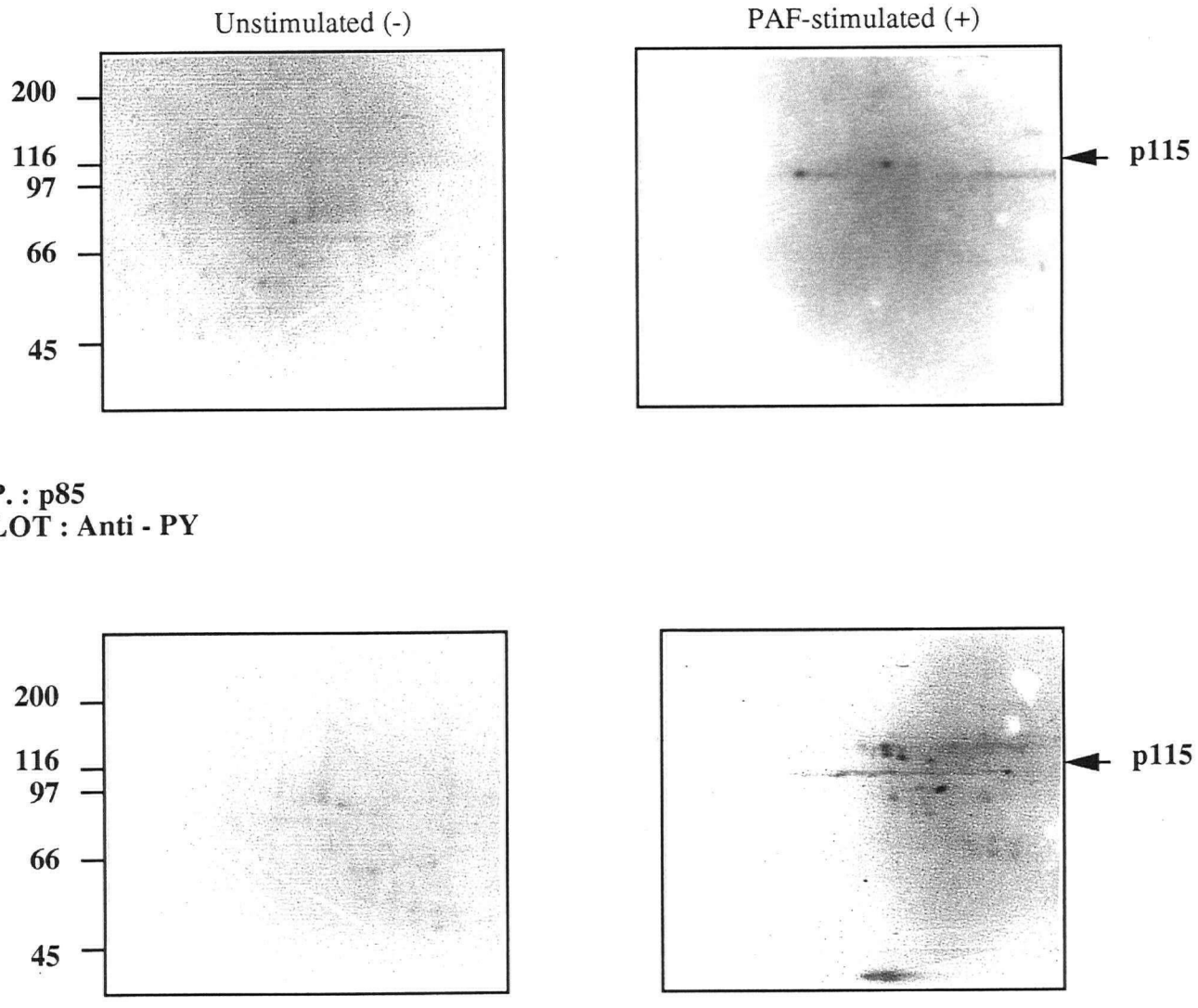
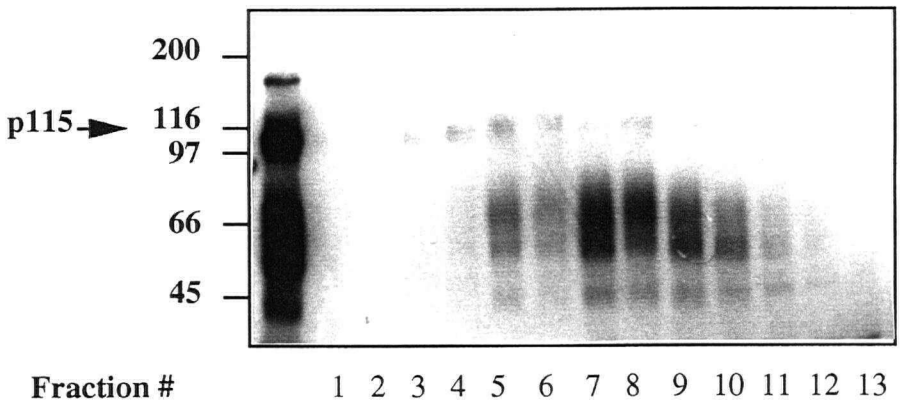


Figure 4

p115 may consist of more than one protein. Platelet lysates (50 μg) from PAF-stimulated (10 nM; 2 min. (+) and unstimulated (-) samples were either immunoprecipitated with polyclonal anti-p85 antibody or affinity precipitated (A.P.) with GST-C-SH2 fusion proteins. Eluted proteins were mixed 1:1 with the first dimension sample buffer, separated by the Mini-Protean II 2-D gel and then casted onto SDS/PAGE for second dimension separation. The gel was transferred and blotted with anti-PY antibody. Arrows indicate the position of p115 protein or proteins.

not successful in separating p115 from the other tyrosine phosphorylated proteins. Therefore, we decided to dialyze the denatured proteins in a buffer containing 0.1% TX-100 and 25 mM Tris-HCl; pH 7.4, and loaded it instead on a gel filtration column.

Superose-12 gel filtration was partially successful, although p115 migrated over a broad range. At fractions # 2,3 and 4 following 7 ml flow through, the p115 band was separated from most of the other tyrosine-phosphorylated proteins (Figure 5). The fractions # 2 to 4, which were most enriched with p115, were collected and precipitated by TCA. 5% and 15% of the concentrated proteins, were eluted in SDS-sample buffer, separated by SDS-PAGE (7.5%) and were either blotted by anti 4G10 antibody or silver stained (Figure 6). These results demonstrate that p115 could be separated from the other proteins. However, whether p115 was composed of one protein or more, remained to be determined. Furthermore, it has not yet been clearly established whether the band detected with anti-phosphotyrosine immunoblotting corresponds precisely to the band detected by silver staining.



A.P. : C-SH2-(p85)
BLOT : Anti-PY

Figure 5

Separation of p115 from the other tyrosine phosphorylated proteins. Supernatant (1 mg) from detergent-lysed and sonicated PAF-stimulated platelets (10 nM; 2 min.) were affinity precipitated with GST-C-SH2 fusion proteins and the eluted proteins were fractionated by superose-12 column on FPLC. After 7 ml flow through, the fractions were collected, separated by SDS/PAGE and blotted with anti-PY antibody. The first lane is an aliquot of the proteins bound to the GST fusion protein and subsequent lanes represent aliquots of sequential fractions. The arrow indicates the position of the p115 protein.

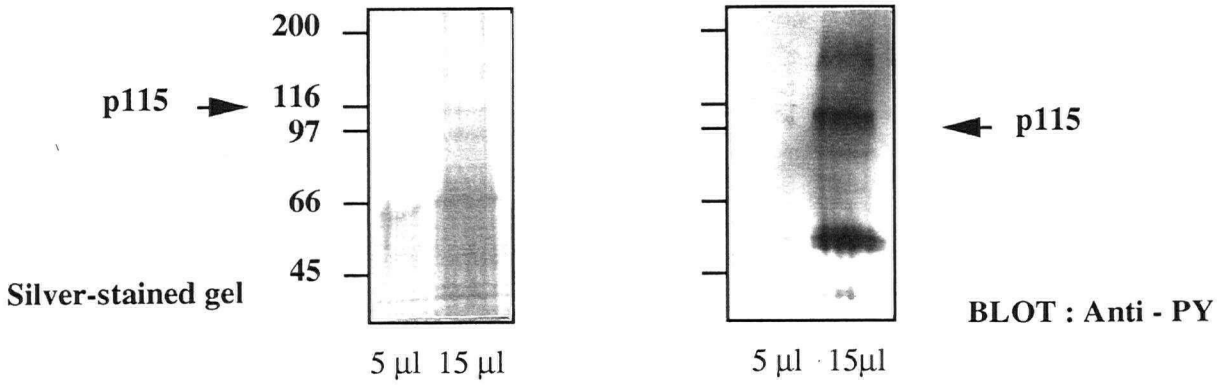


Figure 6

The fractions # 2 to 4 collected from the gel filtration column were concentrated by TCA, separated by SDS/PAGE and either silver stained (left panel) or immunoblotted with anti-PY antibody (right panel). In each case, the first lane are molecular weight standards and the next two samples are 5 µl and 15 µl of the concentrated fractions. Arrows indicate the position of p115 in the immunoblot and the position of a band that may represent p115 in the silver-stained gel.

C. PYK2 Involved in PI 3- kinase regulation in Thrombin-Stimulated human Platelets

FAK has previously been shown to associate with PI 3-kinase activity which was dependent on both platelet aggregation and GPIIb/IIIa integrin engagement [Chen and Guan, 1994a; Guinebault et al., 1995]. A new member of the FAK gene family, PYK2, was another candidate tyrosine kinase activated in human platelets that we next investigated to determine if it may correspond to p102. An antibody to PYK2 became commercially available recently which facilitated this work.

C.1 The activity of PI 3-kinase was not secondary to fibrinogen binding to platelet integrin and involved association with PYK2.

We first examined the activity of PI 3-kinase upon thrombin (THR) stimulation of human platelets in the absence and presence of the aggregation inhibitor, RGDS peptide (0.5 mM), for 2 min. (Figure 7). As shown previously [Gutkind et al., 1990] the concentration of thrombin used (1 U / ml), was shown to cause maximal platelet shape change and aggregation. A monoclonal antiserum (mAb) against the p85 subunit of PI 3-kinase was used for immunoprecipitation of platelet lysates. Compared with lysates prepared from unstimulated (UN) platelets, the activity of PI 3-kinase was increased by 3.8 ± 0.35 (mean ± S.E.) fold (Figure 7). A similar increase in the activity, 3.85 ± 0.29 fold (Figure 7), was observed in the lysates from platelet pretreated with RGDS peptide which inhibits platelet aggregation (90%) by competing off fibrinogen binding to the platelet integrin.

Immunoprecipitation with the monoclonal anti-p85/PI 3-kinase followed by blotting with anti-PYK2 serum showed that both platelet lysates from unstimulated (UN) or pretreated with RGDS contained PYK2 protein, whereas, lysates from platelets stimulated with thrombin (THR) alone or in the presence of RGDS peptide, showed increased association of PYK2 protein (Figure 8 A). Figure (8 B) demonstrates that nearly equal

amounts of p85 was immunoprecipitated in each case. These results demonstrated that PI 3-kinase was associated with PYK2, the association was found to be increased with increased PI 3-kinase activity, and both the association of PYK2 and the activity of PI 3-kinase was independent of fibrinogen binding to the integrin GPIIb/IIIa. We were unable to detect any presence of PYK2 in PAF- or thrombin-stimulated rabbit platelets which might be due to the lack of cross reactivity with the antibodies purchased from UBI and Santa Cruz.

C.2 PI 3-kinase activity was associated with PYK2 and was independent of fibrinogen-binding.

We further investigated the association of PYK2 with PI 3-kinase activity by performing time-course experiments (Figure 9 and 10) under the reciprocal conditions in which PI 3-kinase co-immunoprecipitated with anti-PYK2 antiserum was tested. Lysates from unstimulated (UN) and stimulated platelets (1U / ml) at various times (0.5', 1', and 2'), were immunoprecipitated by a polyclonal antiserum against PYK2 and in parallel, aliquots of the lysates were incubated with the protein G-Sepharose in the absence of the antibody to determine the background CPM. The activity of PI 3-kinase associated was then quantified (Figure 9). Compared with lysates from unstimulated platelets, *in vitro* kinase activity assays using phosphatidylinositol as substrate, showed 1.5 ± 0.28, 3.5 ± 0.09, and 3.9 ± 0.1 fold increase in PI 3-kinase associated with PYK2 at each time point indicated above respectively. The amount of PI 3-kinase activity associated with PYK2 in stimulated platelets (THR; 2 min.) pretreated with RGDS peptide (0.5 mM; 2 min.) was increased by 4.0 ± 0.08 fold compared with unstimulated platelets (UN) in the presence of RGDS (Figure 10 A and B). These results demonstrated that PI 3-kinase activity in association with PYK2 was increased in a time-dependent mechanism, reaching maximum activity at 2 min. Also, treatment with RGDS peptide, which inhibits fibrinogen-binding to the integrin (GPIIb/IIIa), had no effect on the activity of PI 3-kinase associated with PYK2. Furthermore, the amount of PI 3-kinase activity recovered in association with PYK2 was

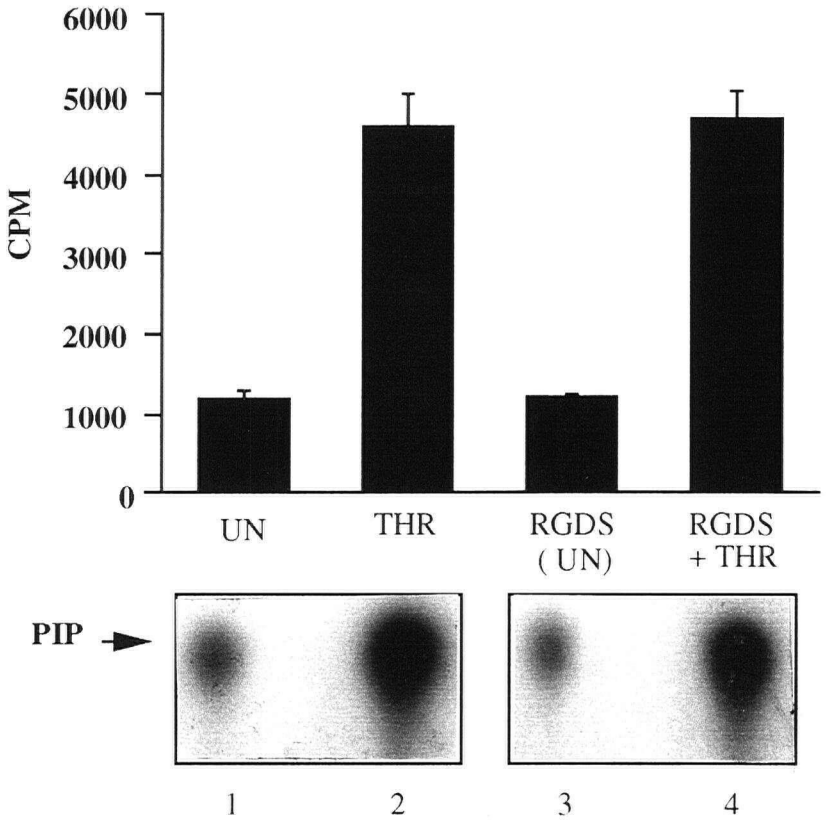


Figure 7

Activation of PI 3-kinase occurs independently of fibrinogen-binding. Human platelets were either unstimulated (UN) or stirred in the presence of thrombin (THR; 1 unit / ml) alone or with the tetra peptide RGDS (0.5 mM) for 2 min. and then lysed in TX-100 solubilization buffer. Equal amount of lysates were immunoprecipitated with anti-p85 (mAb). The immunoprecipitated proteins were immobilized on protein G-agarose and subjected to *in vitro* PI 3-kinase assay in which phosphatidylinositol was used as a substrate. The activity of PI 3-kinase was resolved and analysed on thin layer chromatography (TLC). The product of PI 3-kinase, phophatidylinositol 3-phosphate (PIP), was autoradiographed as indicated above (lanes 1- 4 show representative samples from an autoradiogram) and measured by scintillation counting following scraping of spots from the TLC plates (top graph). Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments.

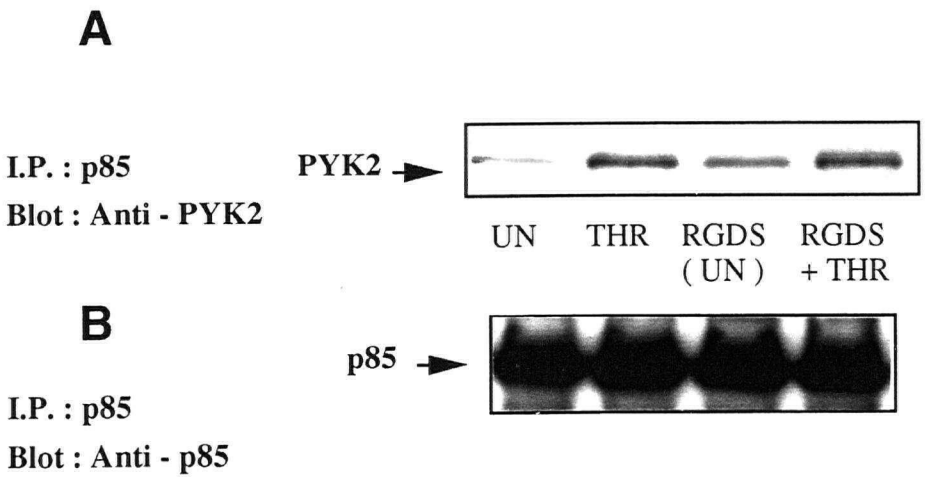


Figure 8

(A) aliquots of the anti-p85 immunoprecipitates were resolved by SDS/PAGE (7.5%), and the separated proteins were transferred to nitrocellulose membrane and probed with anti-PYK2 or (B) with anti-p85 antibodies.

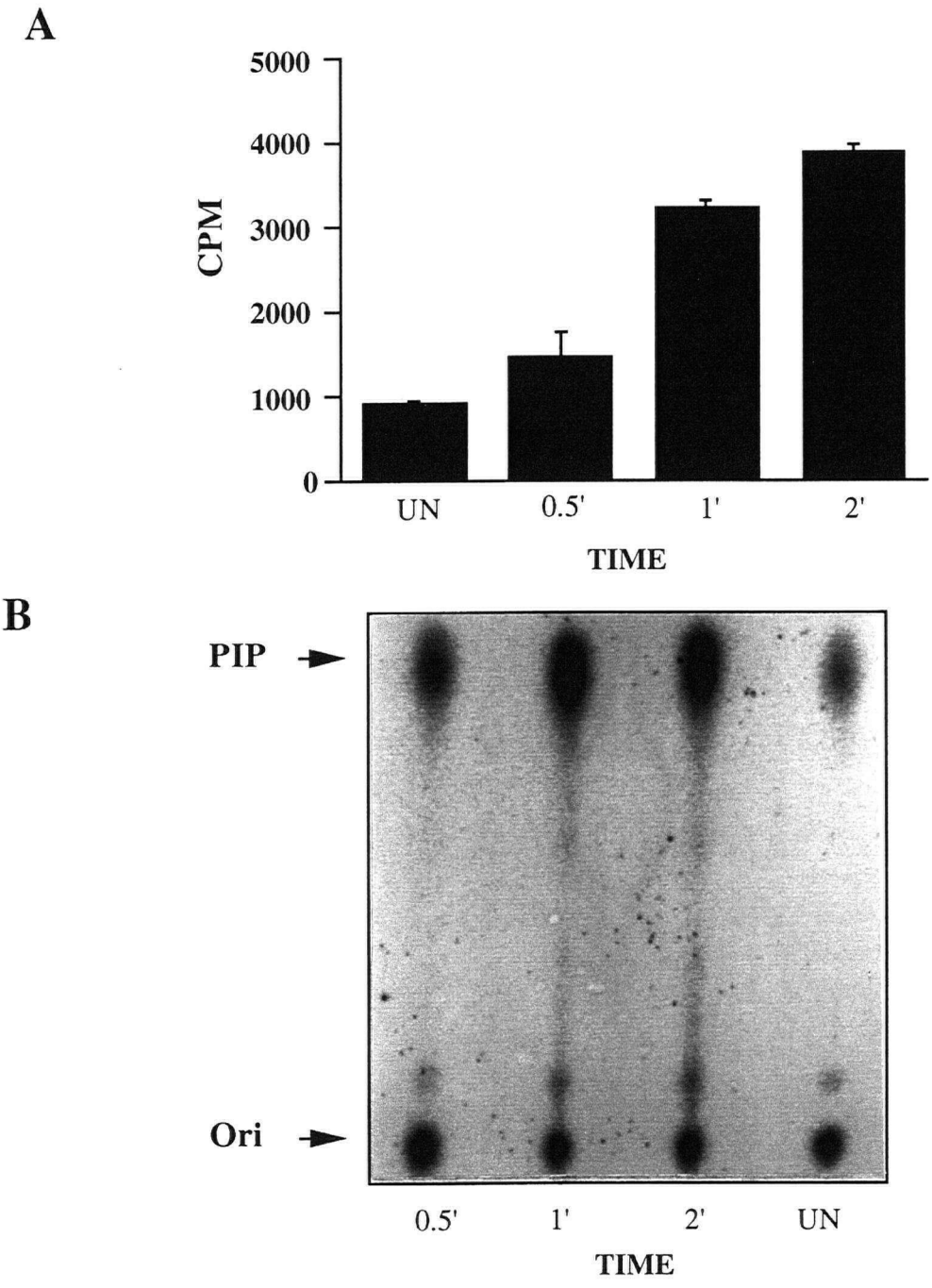


Figure 9

Association of PI 3-kinase activity with PYK2 in a time-dependent manner. Platelets were either unstimulated (UN) or stirred in the presence of thrombin (1 U/ml) for the indicated time shown above. Equal amounts of lysates were immunoprecipitated with anti-PYK2 antibodies and assayed for PI 3-kinase activity. The location of the origin (Ori) and phosphatidylinositol 3-phosphate(PIP) are indicated at the lower (B). The CPM values were measured following scraping of the spots from the TLC plates and plotted as indicated in the graphe (A). Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments.

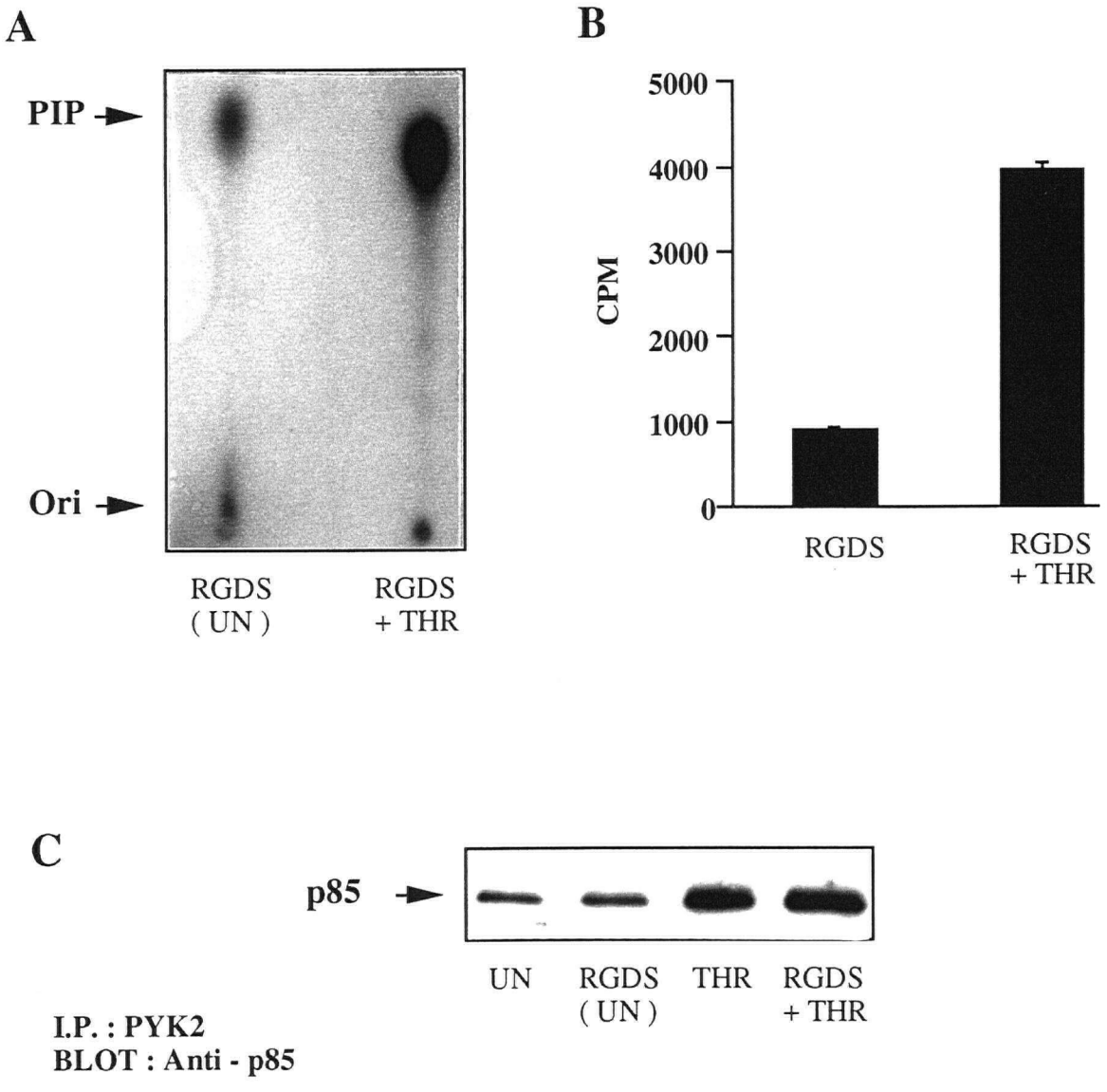


Figure 10

Association of PI 3-kinase activity with PYK2 is independent of fibrinogen-binding. Platelets were either unstimulated (UN) or pretreated with RGDS (0.5 mM; 2 min.) and stirred in the presence of thrombin (THR;1 U/ml) for 2 minutes. Equal amounts of lysates were immunoprecipitated with anti-PYK2 antibodies and assayed for PI 3-kinase activity. The location of the origin (Ori) and phosphatidylinositol 3-phosphate (PIP) are indicated at the representative autoradiogram shown in panel(A). The CPM values were measured following scraping of the spots from the TLC plates and plotted as indicated in (B). Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments. Aliquots of anti-PYK2 immunocomplex from samples treated as indicated (C) were immunoblotted using anti-p85 antibody.

significantly increased in thrombin-stimulated platelets. PI 3-kinase activity in PYK2 immunoprecipitation was about 70-75% of the total activity recovered using immunoprecipitation with monoclonal anti-PI 3-kinase antibody (shown above, Figure 7). The amount of p85 associated with PYK2 was shown to be increased in the lysates immunoprecipitated with anti-PYK2 antibody and blotted with anti-p85/PI 3-kinase serum from platelets stimulated with thrombin alone (THR; 2 min.) or in the presence of RGDS (0.5 mM; 2 min.) (Figure 10 C). The western blot also confirmed that the increased association of p85/PI 3-kinase with PYK2 was independent of fibrinogen-binding.

C.3 Increase in PYK2 activity and tyrosine phosphorylation correlate with increase PI 3-kinase association.

It is plausible that the activity of PYK2 may play a role in PI 3-kinase association. To test this possibility, a polyclonal antiserum against PYK2 was used for immunoprecipitation of platelet lysates prepared from unstimulated (UN) and thrombin-stimulated platelets (1U / ml) at various times (0.5', 1', and 2'). *In vitro* kinase assays were performed using poly (Glu:Tyr, 4:1) as substrate (Figure 11 A). The increase in the activity of PYK2 was found to be, 3.1± 0.7 , 3.4 ± 0.65, and 3.8 ± 0.8 - fold at the times indicated above, respectively. Western blotting with anti-PY also showed corresponding increases in tyrosine phosphorylation of PYK2 at the above mentioned times (Figure 12 A). The maximum activity and phosphorylation of PYK2 was reached at 1- 2 min. The maximum phosphorylation of PYK2 was maintained until 5 minutes (results not shown). To investigate the association of PI 3-kinase with PYK2, we stripped and reprobed the anti-PY blot with anti-p85 serum. Increase in p85 association was concomitant with increased PYK2 activity and phosphorylation, reaching maximal association at 2 min. (Figure 12 B). The same blot was reprobed with anti-PYK2 serum to show equal amounts of PYK2 were immunoprecipitated in each case (Figure 12 C). We also investigated the activity of PYK2 in

the presence of the aggregation inhibitor RGDS. Lysates from unstimulated (UN) platelets or pretreated with RGDS (0.5 mM; 2 min.) followed by addition of thrombin (1U / ml ; 2 min.) were used. *In vitro* kinase assay of the PYK2 immunoprecipitates showed similar increases in the activity of PYK2 in the presence of RGDS (Figure 11 B). These results demonstrated the close correlation between PI 3-kinase increased association with PYK2 and increases in the activity or/and the tyrosine phosphorylation of PYK2 which was shown also to be independent of platelet aggregation, or more accurately, independent of fibrinogen binding to the platelet integrin GPIIb/IIIa.

C.4 PI 3-kinase inhibitor (LY) had no effect on the activity and association of PYK2 with PI 3-kinase .

Based on the pervious observation by Rankin et al. [1996], who suggested that PI 3-kinase activity was required for PDGF-stimulated phosphorylation of FAK, we investigated the possibility of whether PI 3-kinase or its products might be regulating the activity of PYK2. Platelets were incubated with LY-294002 (25 μM) for 10 min. prior to stimulation by thrombin (1U / ml; 2 min.). This concentration of LY-294002 used was found to potently inhibit PI 3-kinase activation by us and others, (unpublished results), [Kovacsovics et al., 1995]. *In vitro* tyrosine kinase assay was performed on lysates immunoprecipitated by anti-PYK2 antiserum, from platelets incubated with LY-294002, without stimulation (UN) and in the presence of thrombin (THR) (Figure 13 A). LY-294002 had no effect on thrombin-stimulated activation of PYK2 tyrosine kinase. Aliquots of immunoprecipitates using anti-PYK2 antiserum were immunoblotted by anti-PY (Figure 13 B) and reprobed by anti-p85 serum (Figure 13 C). Results showed that the PI 3-kinase inhibitor also had no effect on the increased phosphorylation of PYK2 and its association with PI 3-kinase upon thrombin stimulation. Clearly, PYK2 is not downstream of PI 3-kinase activity and the association of PI 3-kinase with PYK2 is a consequence of PYK2 activation or tyrosine phosphorylation which allows the association, presumably via the p85 subunit.

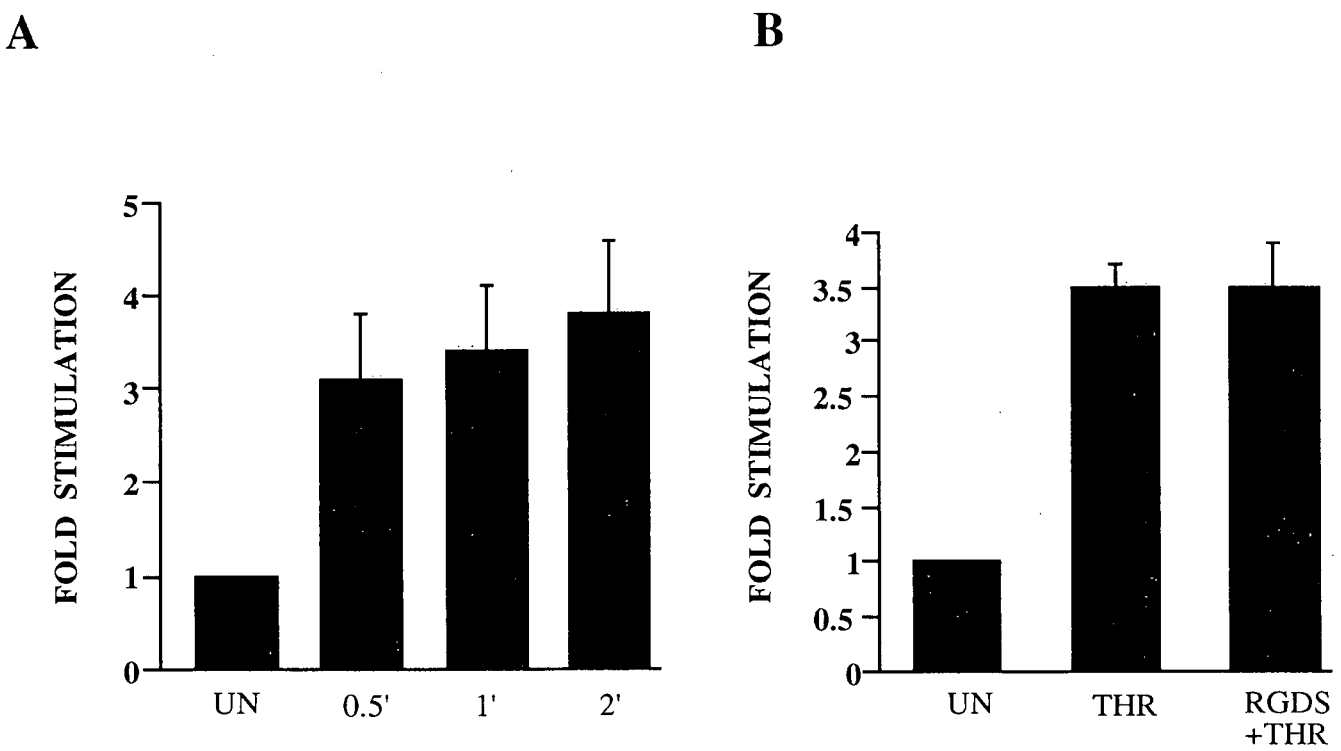


Figure 11

Effect of tyrosine phosphorylation and activation of PYK2 on the association of PI 3-kinase. (A) Platelets were left unstimulated (UN) or stirred in the presence of thrombin (THR; 1 U/ml) for the indicated times. PYK2 was immunoprecipitated from detergent-solubilized lysates and subjected to an *in vitro* tyrosine kinase assay using poly (Glu:Tyr, 4:1) as a substrate. (B) The activity of PYK2 was also determined following treatment with thrombin in the presence or absence of RGDS peptide (0.5 mM; 2 min.) 2 min. Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments.

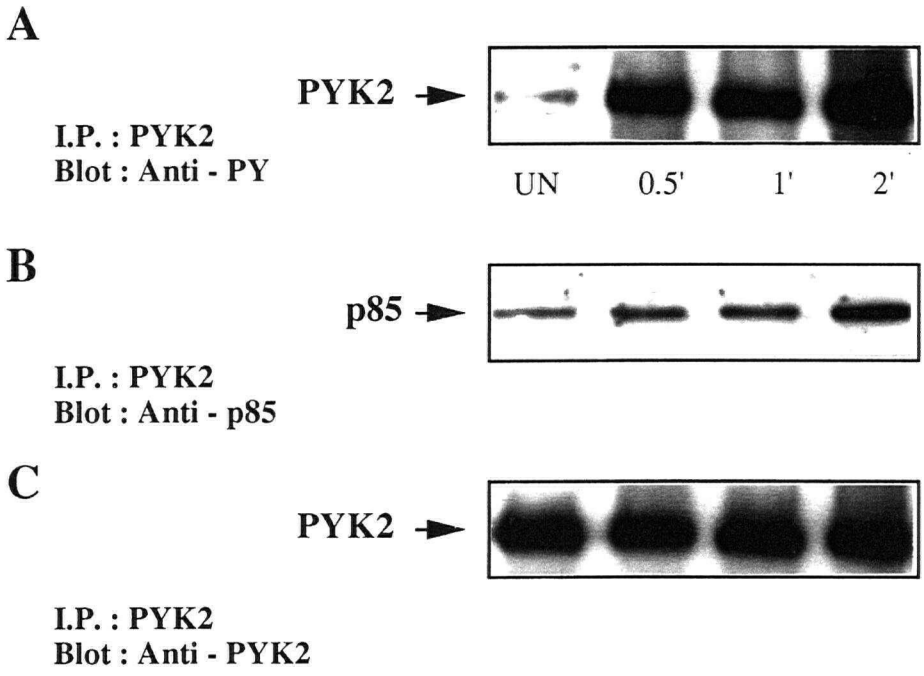


Figure 12

Aliquots from PYK2 immunoprecipitates (Figure 11 A) were immunoblotted with anti-PY antibody to determine the level of the tyrosine phosphorylation of PYK2 (A). Blot was stripped and probed with anti-p85 antibody (B), and (C) with anti-PYK2 to show equal amounts immunoprecipitated.

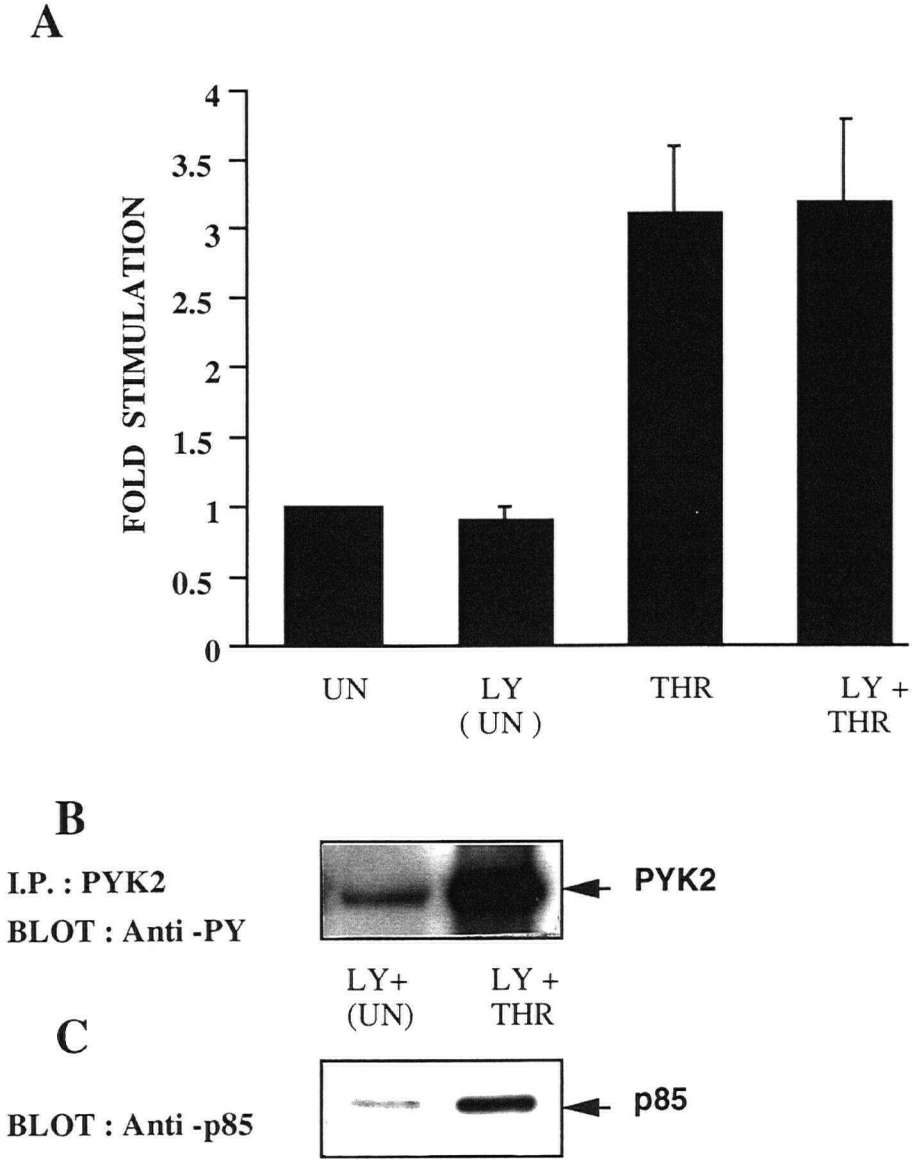


Figure 13

Effect of the PI 3-kinase inhibitor (LY) on the activity and the associaton of PYK2. (A) platelets were incubated with LY-294002 (25 μM; 10 min.) and stirred with thrombin (1 U/ml; 2 min.) or left unstimulated. The tyrosine kinase activity of PYK2 was determined as in Fig. 11. (B) Aliquots of PYK2 immunocomplex were immunoblotted with anti-PY and (C) anti-p85 antibodies as in Fig. 12. Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments.

C.5 Tyrosine phosphorylation and mobility shift of PYK2 are observed in p85/PI 3-kinase immunoprecipitates.

Platelets were pretreated with RGDS (0.5 mM; 2 min.), left unstimulated (UN), or stimulated with thrombin (THR; 1U / ml; 2 min.). The lysates were immunoprecipitated by anti-p85 serum and western blotted by anti-PY. A tyrosine phosphorylated protein was shown to coincide exactly at the same molecular weight (114 kD) as PYK2 in the lysates from platelets treated with RGDS and stimulated with thrombin. However, in the lysates of platelets stimulated with thrombin in the absence of the aggregation inhibitor (RGDS), we detected the appearance of a major phosphorylated protein at mol. wt. between 114 and 100 kD at approximately 102 kD (Figure 14 A). We believe that this tyrosine-phosphorylated protein which associates with p85/PI 3-kinase during platelet aggregation is either a tyrosine kinase or a tyrosine phosphorylated substrate associate with PI 3-kinase in an aggregation dependent manner because it appears only in the presence of RGDS, or it might be a proteolytic degraded product due to platelet aggregation as suggested from results discussed below. When we reprobed the anti-PY blot with anti-PYK2 serum, we confirmed that a tyrosine phosphorylated protein which was observed at mol. wt. 114 kD, was PYK2. In this example, we observed a decrease in mobility of PYK2 on the SDS polyacrylamide gel in the lysates from platelets stimulated with thrombin, which is consistent with modification of the enzyme by phosphorylation (Figure 14 B). In some cases, a more subtle mobility shift was observed (e.g. see Figure 8 A). These results also indicate that the association of p102 with p85/PI 3-kinase was dependent upon aggregation, unlike the association of PYK2. Furthermore, the results also showed an increase in the amount of PYK2 co-immunoprecipitated with PI 3-kinase in the presence and absence of RGDS upon thrombin stimulation as shown above.

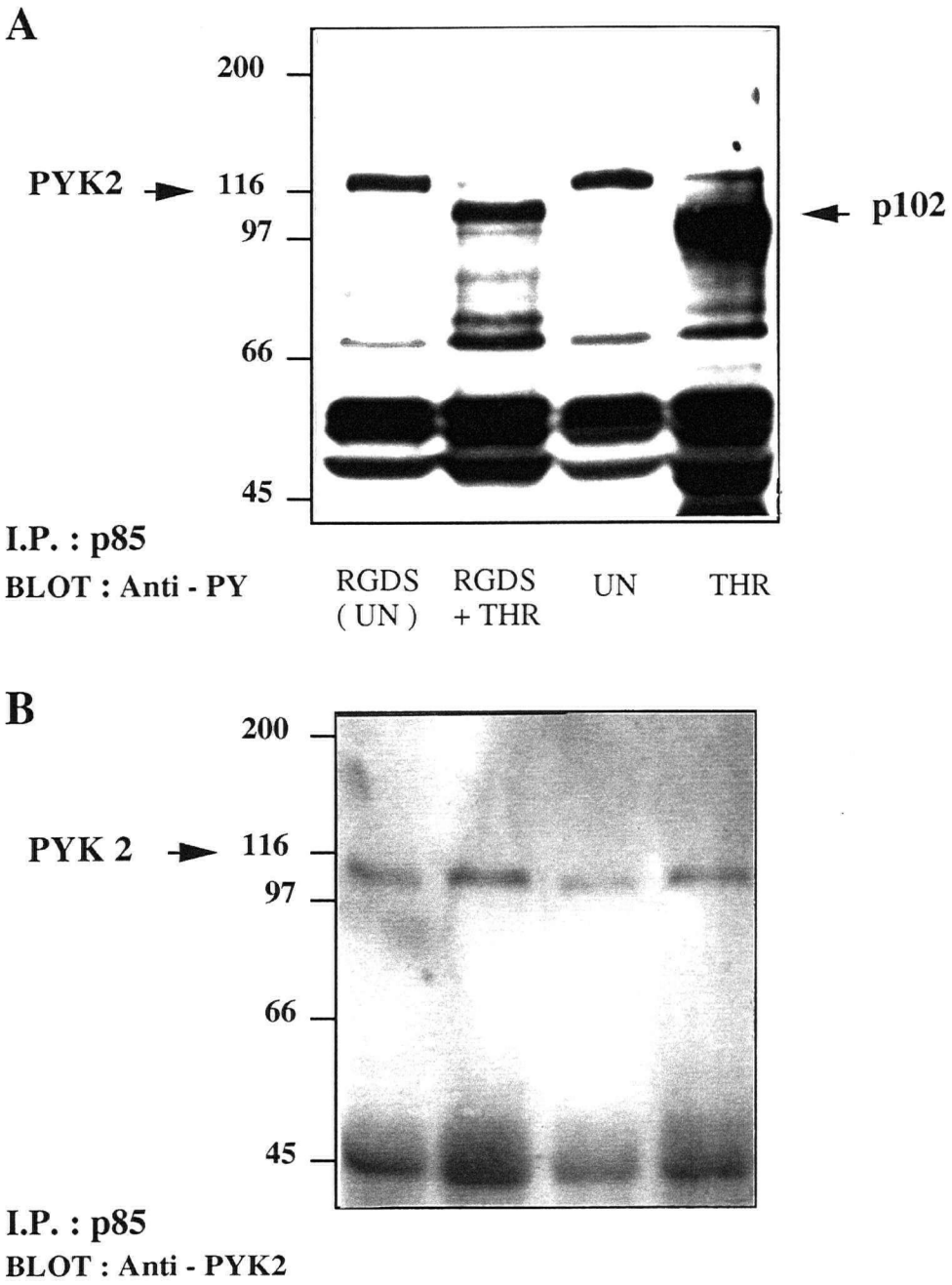


Figure 14

Detection of tyrosine phosphorylation of PYK2 in p85 immunoprecepitates. (A) Isolated platelets were treated with thrombin (THR; 1 U/ml) or/and RGDS (0.5 mM) for 2 min., or left unstimulated (UN). The p85 subunit of PI 3-kinase was immunoprecipitated with anti-p85 antibody from detergent-solubilized lysates and captured by protein A-sepharose. The tyrosine phosphorylated proteins associated with p85 were determined by immunoblotting the immunoprecipitates with anti-PY antibody. (B) Blot was reprobed with anti-PYK2 antibody. Arrows indicate the positions of PYK2 and p102 protein.

C.6 Possible proteolytic degradation of a tyrosine phosphorylated protein at molecular weight of 123 kD during platelet aggregation.

Total cell lysates from platelets were unstimulated (UN), stimulated by thrombin (1U / ml; 2 min.), or pretreated with RGDS (0.5 mM; 2 min.) without or with thrombin (THR). The reaction was stopped by solubilization buffer in samples treated with RGDS, unstimulated or thrombin treated (lanes 1, 2, 3 and 4). The last two, unstimulated and thrombin-stimulated samples (lanes 5 and 6) were mixed directly with hot SDS-sample buffer containing 2-mercaptoethanol (Figure 15 A). The total proteins were resolved on SDS/PAGE and immunoblotted using 4G10 anti-phosphotyrosine antibody. We observed the appearance of several tyrosine phosphorylated proteins between the 116 and 97 kD standards upon thrombin stimulation from samples lysed using solubilization buffer, and these were reduced when the stimulated platelets were stopped directly in hot SDS-sample buffer. The presence of proteins in this region was inhibited when platelets were pretreated with the aggregation inhibitor (RGDS) and was concomitant with increases in a tyrosine-phosphorylated protein at 123 kD (lane 2; Figure 15 A).

The anti-PY blot was reprobed with anti-PYK2 serum. PYK2 protein was seen to migrate in duplicates on the SDS/PAGE which was minimally reduced (or no decrease) due to aggregation and solubilization (Figure 15 B). Results also showed a 123 kD protein was weakly detected by anti-PYK2 antibody, and this was absent in the case of stimulated platelets lysed with solubilization buffer (lane 4; Figure 15 B). This may correspond to a higher molecular weight of form of PYK2 described by others [Raja et al., 1996] These results demonstrate a possible proteolytic degradation of a phosphoprotein at approximately 123 kD that immunoreacted with anti-PYK2 antibody, which was due to platelet aggregation and at least partially due to solubilization conditions. At present, it is not clear whether the presence of the 123 kD band detected in anti-PYK2 blots is related to the changes in tyrosine phosphorylated proteins observed at the same molecular weight.

C.7 Regulation of PI 3-kinase activity by PYK2 and other associated tyrosine-phosphorylated proteins may be mediated by calcium and PKC.

The possible involvement of calcium and PKC in regulating the tyrosine phosphorylated proteins associated with PI 3-kinase activity was investigated. Lysates from unstimulated platelets (UN), stimulated by thrombin (THR; 1U / ml) and pretreated with the agonists, PKC inhibitor bisindolylmaleimide (Bis; 12 μM), or EDTA (4 mM) in the presence and absence of thrombin response were immunoprecipitated by a monoclonal phosphotyrosine antiserum (4G10 antibody). The immunoprecipitates were subjected to *in vitro* PI 3-kinase assay and the associated activity was quantified. The activity of PI 3-kinase increased 4.9 ± 0.3 fold in response to thrombin, while in the presence of PKC inhibitor (Bis) or EDTA, the activity was considerably reduced to, 2.3 ± 0.5 and 1.5 ± 0.1-fold, respectively (Figure 16 A). Aliquots of the 4G10 immunoprecipitates were resolved on SDS/PAGE and immunoblotted by either anti-p85/PI 3-kinase or by anti-PYK2 serum. The amount of p85 and PYK2 were shown to be reduced in the presence of PKC inhibitor (Bis) and reached a minimum in the presence of EDTA (Figure 16 B). These results suggest that PKC and calcium-dependent pathways may mediate the activity of PI 3-kinase by regulating PYK2 and other tyrosine-phosphorylated proteins associated with p85.

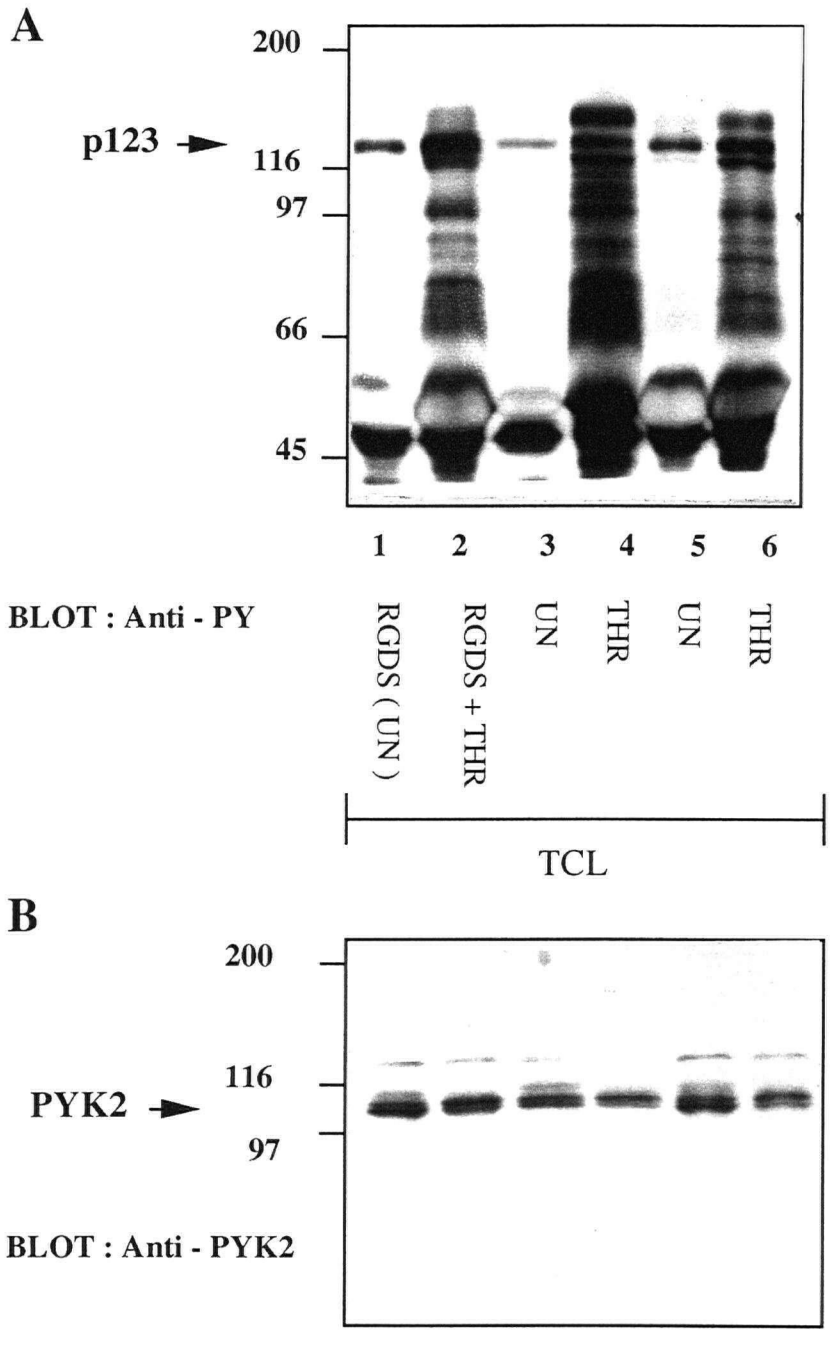


Figure 15

Potential proteolytic degredation of the 123 kD phosphoprotein during platelet aggregation. (A) Platelets unstimulated (UN) or stimulated in the presence of thrombin (THR; 1 U/ml), or / and RGDS (0.5 mM) for 2 min. The reaction was stopped by either 1 volume of 2 X solubilization buffer (1, 2, 3 and 4) or with hot 1 volume of 2 X sample buffer (2 % SDS) (lanes 5 and 6). The total cell lysates (TCL) were immunoblotted and probed with anti-PY antibody. (B) Blot was reprobed with anti-PYK2 antibody.

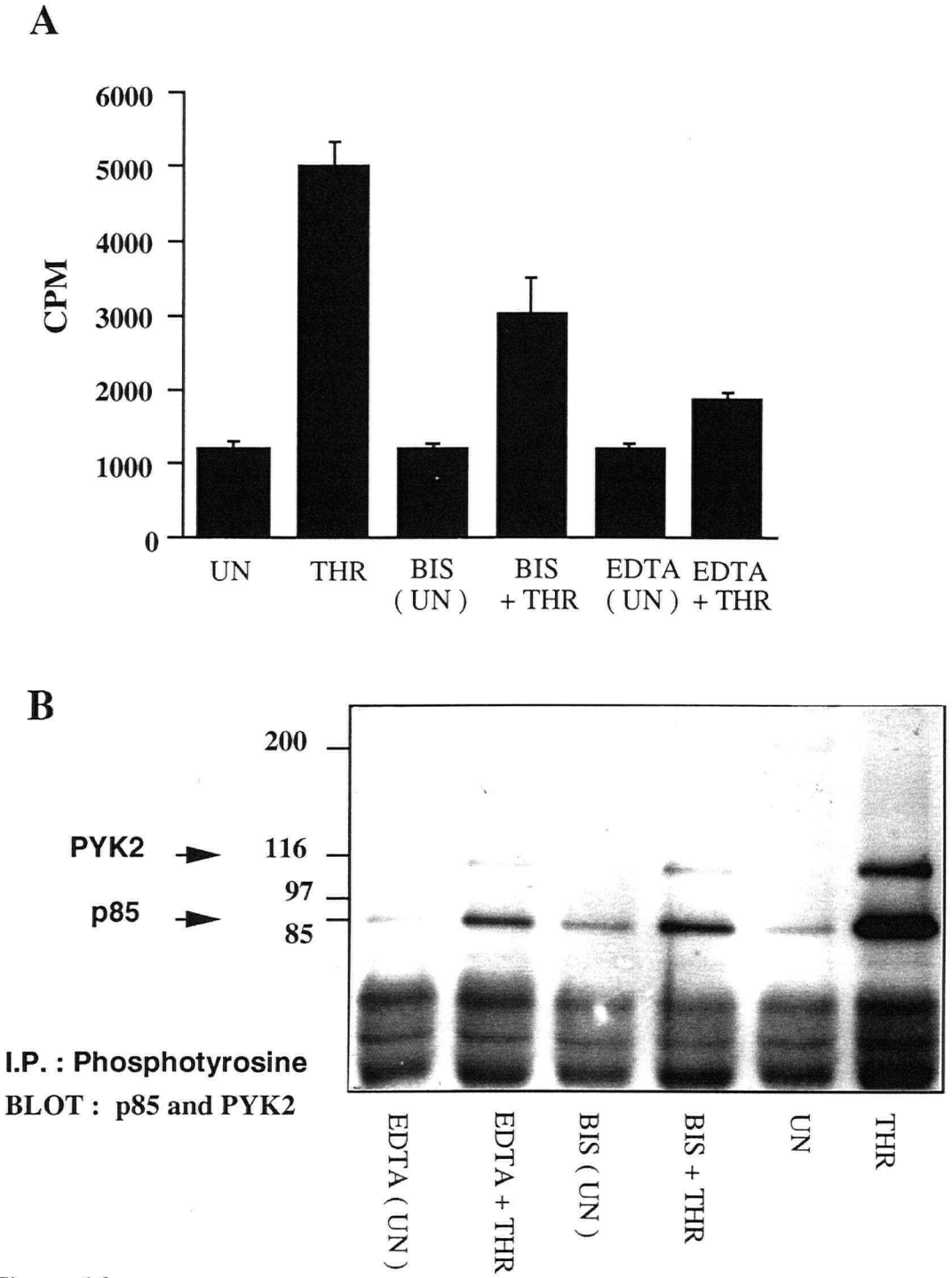


Figure 16

The effect of PKC inhibitor and Calcium chelator on the tyrosine phosphorylated proteins associated with PI 3-kinase. (A) Platelets were unstimulated (UN) or stimulated with thrombin (THR; 1 U/ml) for 2 min., or pretreated with bisindolylmaleimide (BIS; 12 μM) for 1 h, or with EDTA (4 mM) for 2 min., before stimulation with thrombin. Equal amount of lysates were immunoprecipitated. with anti-PY antibody and subjected to PI 3-kinase assay. Results in the graph are expressed as average of three sets of duplicates ± S.E. assayed in three independent experiments. (B) Aliquots of 4G10 immunoprecipitates were western blotted and probed with either anti-p85 and anti-PYK2 antibodies.

DISCUSSION

A. Characterization of tyrosine-phosphorylated proteins associated with PI 3-kinase

PI 3-kinase activity has been shown to interact with a large number of both receptor and non-receptor protein tyrosine kinases [Cantley et al., 1991]. In platelets, PI 3-kinase activity has been shown to be associated with the non-receptor protein tyrosine kinase FAK [Guinebault et al., 1995]. Increases in PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates have been observed in both thrombin-stimulated human platelets and in PAF-stimulated rabbit platelets. It has been shown that in platelets stimulated with agonists, such as thrombin and PAF, PI 3-kinase inducibly associated with tyrosine-phosphorylated proteins which may serve as a major mechanism for PI 3-kinase activation in platelets [Lauener, 1997; Torti et al., 1995; Yatomi et al., 1994; Guinebault et al., 1995].

The major tyrosine-phosphorylated substrate increased in anti-p85/PI 3-kinase immunoprecipitates from PAF-stimulated rabbit and thrombin-stimulated human platelets has an apparent molecular weight of 115 and 102 kD respectively (Figure 1). These two proteins have been found to be inducibly associated with PI 3-kinase with the same kinetics as the increase in PI 3-kinase protein or PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates [Lauener, 1997]. Therefore it is plausible that inducible association of p115 and p102 with PI 3-kinase is a mode for activation of PI 3-kinase in PAF and thrombin-stimulated platelets.

We attempted to identify these substrates using antibodies against a number of potential tyrosine-phosphorylated proteins having a similar molecular weight and that have been shown to be associated with PI 3-kinase in other systems or in platelets. Tyrosine-phosphorylated ras-GAP has been found to co-immunoprecipitate with PI 3-kinase in IGF-1 stimulated rat HTC cells and PDGF stimulated CHO cells [Sanchez-Margalet et al., 1995; Kaplan et al., 1990]. JAK-2 forms a complex with PI 3-kinase and other signaling

proteins in IL-11 stimulated hematopoietic cells [Fuhrer and Yang, 1996]. FAK has been found to associate with PI 3-kinase not only in thrombin-stimulated human platelets but also in polyoma middle-T transformed fibroblasts and PDGF stimulated fibroblasts [Bachelot et al., 1996; Chen and Guan, 1994b]. In activated lymphoid cells (A20 B-cells or jurkat T-cells) the most prominent tyrosine-phosphorylated substrate associated with PI 3-kinase has been identified as the product of the c-cbl oncogene, $p120^{c\text{-}cbl}$ [Hartley et al., 1995; Meisner et al., 1995]. Immunoblotting experiments with these anti-sera in either thrombin- or PAF-stimulated platelets ruled out the identity of p115 and p111 as the 110 kD subunit of PI 3-kinase, p120 ras-GAP, JAK-2, cbl, FAK or integrin β.

Another candidate for this protein was the recently identified Gab 1 (Grb2-associated binder-1) protein which is tyrosine-phosphorylated and associates with the PI 3-kinase p85 subunit in EGF-stimulated A431 cells [Holgadomadruga et al., 1996; Weinder et al., 1996]. This protein bears significant sequence and domain homology to IRS-1 and may serve as an adapter between certain receptor tyrosine-kinases such as the EGF or the c-Met receptor and downstream pathways. From attempts to immunoprecipitate p85/PI 3-kinase by the anti-serum against Gab 1 or immunoblotting p85 immunoprecipitates with anti-Gab 1, it was concluded that no association of Gab 1 with PI 3-kinase occurs in platelets. Therefore we ruled out Gab 1 as a potential candidate for p115 or p102.

A.1 p115 was found to be associated with p85 in the soluble fraction of PAF-stimulated rabbit platelets.

In resting cells PI 3-kinase activity is found mainly in a soluble cytosolic fraction. Its substrates, however, reside in cell membranes and so some form of translocation must be required upon stimulation to bring the two into contact. The importance of the subcellular localization of PI 3-kinase for its correct functioning has been clearly demonstrated by non-transforming mutants of both the polyoma-virus middle T antigen and

the v-Abl oncogene [Fry, 1994]. While the mechanism of recruitment of PI 3-kinase to membranes is not completely clear, translocation of PI 3-kinase has been recorded following its activation by G-protein linked receptors [Fry, 1994]. In the platelet system both p85 protein and PI 3-kinase activity were observed to be translocated from a soluble fraction into a Triton-insoluble membrane skeleton (cytoskeleton) fraction following exposure to thrombin [Zhang et al., 1992]. It has been suggested that PI 3-kinase recruitment by the membrane receptor tyrosine kinases such as the EGF and the c-Met receptor occurs via binding to the tyrosine-phosphorylated cytoplasmic protein Gab 1, in EGF-stimulated A431 cells [Holgadomadruga et al., 1996; Weinder et al., 1996]. Taking all this evidence together, we investigated the localization of p115 which might be a PI 3-kinase adapter, mediating its activation or translocation to the plasma membrane. Our results showed that p115 was cytosolic and was the most prominent protein associated with the p85 subunit in PI 3-kinase immunoprecipitates (Figure 2). Tyrosine phosphorylation of p115 was remarkably increased in the cytosolic fraction of PAF-stimulated samples. No traces of p115 was detected in the membrane fractions. These results may indicate that p115 serves as a substrate or a docking protein for PI 3-kinase in the cytosol, mediating its activation or/and transient translocation to the platelet membrane. However, we can not rule out the possibility that p115 may be loosely associated with the cytoskeleton or membranes which may released into the cytosolic fraction during the isolation of the platelet membranes following sonication and centrifugations. The localization of p102 protein in the human platelets has not been investigated.

A.2 p115 was associated with the SH2 domain of p85/PI 3-kinase

A major mechanism for cellular activation of PI 3-kinase occurs by ligation of the SH2 domains of the p85 regulatory subunit with specific sequences in proteins containing phosphotyrosine residues [Carpenter et al., 1993a]. Thus it was plausible that the tyrosine

phosphorylated 115 kD might be interacting with the SH2 domain of p85. Indeed, *in vitro* affinity binding assay with GST fusion proteins demonstrated that p115 associates with p85/PI 3-kinase via the SH2 domains (Figure 3). p115 binding to GST proteins fused with the C-terminal of the SH2 domain of p85 was shown to be slightly stronger than that to the N-terminal. The association of p115 with the SH2 domain of p85/PI 3-kinase may be the means by which PI 3-kinase is activated upon PAF stimulation. While the mechanism of PI 3-kinase translocation to the platelet cytoskeleton is unknown, it is believed to involve either SH2 or SH3 domain interaction with a membrane associated Src-family kinase or a membrane-bound substrate of a protein tyrosine kinase [Fry, 1994]. Therefore, we suggest that p115 may be involved in the translocation of PI 3-kinase to the cytoskeleton by serving as a docking protein or a linker to a membrane-bound tyrosine kinase or a membrane bound substrate, allowing PI 3-kinase the physical interaction of PI 3-kinase with the membrane.

B. Initial attempts to purify p115 from PAF-stimulated rabbit platelets

The fundamental question of how we can isolate p115 from the other tyrosine phosphorylated proteins that immunoprecipitated with p85 and whether p115 contained one or more polypeptide protein was addressed by using 2-D gel electrophoresis which can be a potential method to separate polypeptide proteins by their isoelectric point using IEF in the first dimension, followed by separation according to molecular weights in the second dimension SDS-gels. p115 that either immunoprecipitated with p85/PI 3-kinase antibody or affinity precipitated with GST-C-SH2 fusion proteins was shown to be separated by the 2-D gel into more than one polypeptide protein (Figure 4). There may be more than one protein co-migrating with similar or very close apparent molecular weight, or these spots may represent different charged species of the same polypeptide. However, while there was some promise in using 2-dimensional gels, we attempted to test whether p115 could be

isolated by using column chromatography and then if necessary, it could be subjected to two dimentional gel electrophoresis.

Mono Q column purification was not successful in separating p115 from the other tyrosine-phosphorylated proteins because the polypeptide was eluted together with the bulk of proteins. This lead us to attempt a strategy in which the SDS-denatured proteins were first dialyzed, then separated by gel filtration column and then if necessary, the concentrated proteins could be further purified by the Mono Q column.

p115 was shown to be separated from the low molecular weight proteins in fraction # 2, 3 and 4 after a flow through volume of 7 ml by using superose-12 column (Figure 5). Examination of the flow through fractions by using GST fusion proteins to affinity precipitate p115 showed no evidence of p115 protein. 5% and 15% of the concentrated fractions # 2 to 4 were silver stained and immunoblotted with anti-PY (Figure 6). p115 was shown to be separated at least partially from the other proteins and it would be expected that several sets of samples could be processed and either combined to obtain enough material for sequencing, or subjected for further purification by two-dimensional gel electrophoresis.

These attempts to purify p115 have set parameters in our lab which are currently being used to further characterize the p115 protein.

C. Involvement of PYK2 in PI 3-kinase regulation in thrombin-stimulated platelets

During our effort to characterize the tyrosine phosphorylated proteins that may regulate PI 3-kinase activity in platelets, we examined PYK2 as a potential mediator. PYK2 has been previously described to be phosphorylated on tyrosine residues in thrombin-stimulated platelets independently of the events induced by fibrinogen binding to GPIIb/IIIa [Raja et al., 1996]. Phosphorylation of PYK2 has been observed to be induced as early as 10 seconds, after addition of thrombin, reaching a maximum at 2 minutes and tapering off

by 10 minutes [Raja et al., 1996]. Interestingly, in parallel studies, PI 3-kinase has been shown to be activated during the same time course in thrombin and PAF-stimulated platelets [Sorisky et al., 1992; Lauener, 1997]. In this study, we examined the activity of PI 3-kinase in the presence of the platelet aggregation inhibitor peptide, RGDS, followed by 2 minutes stimulation with thrombin. Our results indicate that fibrinogen binding to the platelet integrin is not required for PI 3-kinase activation (Figure 7). In agreement with studies on platelets from thrombasthenic patients [Rittenhouse, 1995], and platelets treated with RGDS or disintegrin eristostatin which effectively blocks platelet aggregation [Zhang and Rittenhouse, 1995b; Zhang et al., 1996], these results suggest that PI 3-kinase activation occurs upstream to platelet aggregation and is independent of platelet integrin activation. The kinetics of PI 3-kinase activity was found to closely correlate with a novel association with PYK2 (Figure 8 A). The increases in PI 3-kinase activity and the increase in the level of PYK2 associated with PI 3-kinase were independent of fibrinogen-binding to the platelet integrin. These results suggest that PI 3-kinase may serve as a direct or indirect target for PYK2, which may contribute to its activation.

In examining the activity of PI 3-kinase in PYK2 immunoprecipitates, we observed a time-dependent increase in PI 3-kinase activity associated with PYK2 reaching its maximum by 2 min. (Figure 9). Increases in the activity of PI 3-kinase and the amount of p85 associated with PYK2 were shown to be independent on fibrinogen binding to the platelet integrin (Figures 10). The amount of PI 3-kinase activity recovered by 2 minutes thrombin stimulation in PYK2 immunoprecipitates was found to represent 75% of the maximum PI 3-kinase activity in p85/PI 3-kinase immunoprecipitates (Figures 7 and 9). Approximately a 4.2 fold increase in PI 3-kinase activity was observed with the monoclonal antibody to PI 3-kinase, compared with a 4 fold increase in PI 3-kinase activity in PYK2 immunoprecipitates following thrombin stimulation for 2 minutes. These results clearly indicate that a large proportion of the activity of PI 3-kinase in the immunoprecipitates using the α-p85 monoclonal antibody is associated with PYK2.

The cellular activity of PI 3-kinase occurs due to not only a physical association with tyrosine phosphorylated substrates, but also due to its association with a functionally activated tyrosine kinases which triggers signal transduction events [Carpenter et al., 1993a; Fry, 1994]. In mammalian cells, PI 3-kinase associates with activated growth factor receptors and oncogene-encoded tyrosine kinases upon an appropriate stimulus, which correlates with cell proliferation and transformation [Cantley et al., 1991]. In platelets and fibroblasts, PI 3-kinase has been shown to be associated with activated FAK in response to thrombin and cell adhesion respectively, in an integrin-dependent manner which perhaps leads to cell adhesion, proliferation or aggregation [Guinebault et al., 1995; Chen and Guan, 1994a].

To answer the question whether PYK2 is activated during a time course upon thrombin stimulation which may correlate with increases in PI 3-kinase activity recovered from PYK2 immunoprecipitates during such times, we performed *in vitro* tyrosine kinase assay on PYK2 immunoprecipitates in a time -dependent manner. Furthermore, using the aggregation inhibitor, RGDS peptide, which separates the early activation signals from those that are dependent upon integrin activation. Quantitative immunoprecipitation of detergent lysates from thrombin stimulated platelets with polyclonal antibody to PYK2, followed by an *in vitro* kinase assay showed that the increased activity and phosphorylation of PYK2 (Figure 11 and 12) was consistent with the increased level of PI 3-kinase association (Figure 9) in a time-dependent mechanism. The results show a striking correlation between PI 3-kinase activity and PYK2 activity which was independent of fibrinogen-binding upon thrombin stimulation. The time-courses show both PI 3-kinase and PYK2 activation follow approximately the same kinetics with a maximum of 3.8 fold increase by 2 minutes. These results strongly suggest that the PI 3-kinase activation in the early phase of platelet activation that occurs independently of fibrinogen binding, may be due to either physical or functional association with PYK2.

It has been previously shown that LY-294002, a potent inhibitor of PI 3-kinase, inhibited PDGF-stimulated FAK tyrosine phosphorylation [Rankin et al., 1996]. Therefor, the possibility of whether PYK2 may serve as a substrate for PI 3-kinase or its products was investigated by using LY-294002. LY-294002 (a derivative of the flavonoid, quercetin) is a competitive inhibitor of the kinase ATP-binding site. The IC_{50}'s we obtained for LY (25 μm) inhibition of thrombin-stimulated PI 3-kinase (results not shown) are in good agreement with data obtained using PAF, thrombin receptor directed agonist peptide (TRAP), or β-PMA [Lauener, 1997; Kovacsovics et al., 1995; Toker et al., 1995; Zhang et al., 1996]. Results indicate that the PI 3-kinase inhibitor has no effect on the activation of PYK2 as well as the association of the p85 subunit of PI 3-kinase (Figure 13) suggesting that PYK2 is not downstream of PI 3-kinase and the association of PI 3-kinase with PYK2 is likely to be a consequence of PYK2 activation and phosphorylation facilitating the association. These results, together with the above, suggest that PYK2 may mediate signal transduction by the G-protein-coupled receptor (thrombin) in a manner similar to that of receptor tyrosine kinases. In this model, thrombin receptor / ligand binding would activate the tyrosine kinase activity of PYK2, resulting in its autophosphorylation. This would then recruit PI 3-kinase and other signaling molecules to form a multimolecular complex.

We consistently observed a major phosphoprotein from human platelets which migrated as a broad band on SDS/PAGE gel with apparent molecular range between 114 and 100 kD, which we refer to as p102, in the p85/PI 3-kinase immunoprecipitates upon thrombin stimulation. This may be the human homolog of the p115 protein in rabbit platelets which was discussed above. The appearance of this phosphoprotein which we have been trying to characterize, was found to be greatly reduced due to treatment with the aggregation inhibitor RGDS (Figure 14 A). The appearance of only a single band of a deduced molecular weight of 114 kD in p85 immunoprecipitates treated with RGDS was shown to correspond to the PYK2 protein (Figure 14 B). These results suggest that the thrombin-induced p102 may be an aggregation dependent protein associated with p85/PI 3-

kinase that partially co-migrates with PYK2 on SDS/PAGE gel. Since results showed that PYK2 mediates PI 3-kinase activation that occurs independently of platelet aggregation or more accurately, independently of fibrinogen-binding to the platelet integrin, we believe that p102 is a tyrosine kinase or a tyrosine phosphorylated substrate that may serve as an adapter protein to mediate PI 3-kinase translocation to the cytoskeleton in thrombin-stimulated platelets. However, there is a possibility that p102 may be a degraded product of PYK2 that is undetectable by the commercially available PYK2 antibodies. Recently, a new PYK2 isoform with an apparent mol. wt. of 106 kD has been characterized in hematopoietic cells [Dikic et al., 1998]. These authors suggested that different PYK2 isoforms may be present in hematopoietic cells. It has also been shown that PYK2 and FAK undergo sequential proteolytic cleavage in thrombin, collagen, and calcium ionophore A23187-stimulated platelets [Raja et al., 1996; Cooray et al., 1996]. The possible proteolytic degradation of PYK2 and other tyrosine phosphorylated proteins due to solubilization conditions or/and platelet aggregation were observed (Figure 15) and is discussed below.

On the other hand, the level of the immunoreactive PYK2 protein associated with PI 3-kinase in thrombin induced platelets in the presence and absence of RGDS was observed to be increased concomitant with its slower mobility on SDS/PAGE which clearly indicates that the increased level of PYK2 associated with PI 3-kinase was due to its phosphorylation upon thrombin stimulation.

In this study, we observed an appearance of multiple tyrosine phosphorylated proteins between 116 and 97 kD upon thrombin stimulation in parallel with a decrease in the level of a tyrosine-phosphorylated protein at approximately 123 kD (Figure 15 A) which was shown to co-migrate with a polypeptide detected with PYK2 antibody (Figure 15 B). These protein modifications were completely blocked or reduced in thrombin-stimulated platelet samples pretreated with RGDS, or mixed directly with hot SDS-sample buffer, respectively. However, only a very minor decrease in the level of PYK2 (114 kD)

(or no decrease) was observed during platelet aggregation and solubilization. These results indicate that a possible protein degradation may be occurred due to platelet aggregation and partially due to the solubilization conditions. PYK2 has been reported to have an apparent molecular weight of 123 kD which undergoes a proteolytic degradation due to thrombin stimulation with an appearance of two molecular mass fragments of approximately 80 and 75 kD [Raja et al., 1996]. Although we observed a decrease in the level of 123 kD which was only detected slightly with anti-PYK2 antibodies, we have not observed the two fragments. We believe that this 123 kD protein could be a splice variant of PYK2. Dikic, et al., [1998] have described a new isoform of PYK2 (PYK2-H) generated by alternative RNA splicing. In agreement with Ganju et al., [1997], Astier et al., [1997] and Dikic et al., [1998], we observed PYK2 as a doublet at approximately 114 kD, suggesting that different PYK2 isoforms may be expressed [Dikic et al., 1998]. It is possible that the difference between our observation and the others [Raja et al., 1996] is due to the difference in platelet isolation and solubilization techniques which results in difference in protein modification resolved on SDS/PAGE. Results indicate that the protein fragmentations that were observed upon thrombin stimulation could be due to protein degradation [Kinlough-Rathbone and Mustard, 1987] by calpain and other proteases [Fox et al., 1990; Fox et al., 1991; Fox et al., 1993; Cooray et al., 1996; Fox et al., 1985] or due to redistribution to the actin-rich cytoskeletal complexes upon their phosphorylation [Clark et al., 1994b]. The issue of whether PYK2 undergoes degradation upon thrombin-stimulated aggregation, or during platelet solubilization has not been resolved. Evidence from the whole cell lysates suggests that aggregation and solubilization conditions can affect the appearance of tyrosine phosphorylated proteins. Part of this effect may be due to degradation of proteins, but this may also be an involvement of dephosphorylation by phosphatases.

Platelet activation occurs through a rise of cytoplasmic Ca^{2+} level, and this Ca^{2+} mobilization is accompanied by the activation of a number of kinases among which the serine/threonine kinase C (PKC) and tyrosine protein kinases. Tyrosine protein

phosphorylation is observed during platelet activation by thrombin [Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura, 1989], PAF [Salari et al., 1990; Clark et al., 1994b], ionophore A23187 and PKC activators [Golden and Brugge, 1989; Takayama et al., 1991]. These phosphorylations are dependent upon tyrosine protein kinases such as $pp60^{c\text{-}src}$ [Ferrell and Martin, 1988; Golden and Brugge, 1989; Rendu et al., 1989], pp125FAK [Lipfert et al., 1992], and $p72^{Syk}$ [Fujii et al., 1994]. It has been shown that the addition of EDTA reduced protein tyrosine phosphorylation induced by all three agonists, suggesting that platelet tyrosine kinase activity is controlled by intracellular Ca^{2+} [Falet and Rendu, 1994]. Although, direct activation of PKC has been demonstrated to induce protein tyrosine phosphorylation, it has been suggested that Ca^{2+} mobilization is essential for tyrosine protein kinase activation in human platelets and occurs downstream of PKC only when PKC is directly activated [Falet and Rendu, 1994].

Studies with PYK2 in PC-12 cells and platelets has demonstrated that PYK2 phosphorylation was regulated by the intracellular Ca^{2+} [Lev et al., 1995; Raja et al., 1996]. In thrombin-stimulated platelets, PYK2 tyrosine phosphorylation has been shown to be reduced by the PKC inhibitors calphostin C and bisindolymaleimide, suggesting that the activation of PYK2 in platelets is mediated by PKC [Raja et al., 1996]. On the other hand, the accumulation of the 3-PPI levels in platelets has been shown to be sensitive to Ca^{2+} mobilization and PKC activation [Sorisky et al., 1992; King et al., 1991]. In agreement with these studies, our results show that omission of Ca^{2+} from the medium or inhibition of PKC resulted in reduction in the level of PYK2 and PI 3-kinase immunoprecipitated with anti-phosphotyrosine antibody (Figure 16 B). The reduced amount of PYK2 and p85 levels showed more sensitivity to omission of Ca^{2+} than inhibition of PKC, which suggests, in agreement with Falet and Rendu, [1994], that protein tyrosine phosphorylation by calcium mobilization occurs down stream of PKC activation. These results were closely correlated with the activity of PI 3-kinase detected in the anti-phosphotyrosine immunoprecipitates (Figure 16 A) which indicate that PI 3-kinase

activity is mediated by tyrosine phosphorylated proteins that are directly regulated by the intracellular calcium and indirectly by PKC activity.

As mentioned above (Introduction) PI 3-kinase has been found to be associated with the protein tyrosine kinases, FAK, Syk and Src. It has been suggested that FAK might be responsible for the increased PI 3-kinase activity (30%) in the Triton-insoluble fraction (cytoskeleton) and the accumulation of PI-(3,4)P2 in the late phase of platelet activation that is dependent on both aggregation and fibrinogen binding to the GPIIb/IIIa integrin receptor [Guinebault et al., 1995]. Although there is no report of direct binding between Syk and PI 3-kinase, Syk activity has been found in PI 3-kinase immunoprecipitates, reaching maximum activity by 10 seconds in thrombin-stimulated porcine platelets and subsequent decrease to the basal level by 2 minutes [Yanagi et al., 1994]. While the mechanism of association of Src and Fyn with PI 3-kinase is not understood, it has been suggested that the activated PI 3-kinase might be a possible substrate for Src which may mediate its translocation to the membrane-linked receptors [Gutkind et al., 1990; Dhar and Shukla, 1993; Fry, 1994]. Based on these results, one is left with our novel finding of the association of PYK2 with PI 3-kinase activity that was shown in agreement with Zhang and Rittenhouse [1995b], Rittenhouse [1996] and Zhang et al. [1996] to be activated independently of fibrinogen binding to the platelet integrin. We suggest that PYK2 may be responsible for the initial activation of PI 3-kinase in the early phase of platelet activation upon thrombin stimulation.

The implication of the stimulatory effect of GTPγS is that 3-PPI accumulation is under the control GTP-binding protein(s) [Kucera and Rittenhouse, 1990]. Studies with PYK2 in PC-12 cells have suggested that the phosphorylation of PYK2 might be partly mediated by Src, thus generating docking sites for additional signaling molecules that are recruited by PYK2. It has also been proposed that PYK2 acts with Src to link Gi- and Gq-coupled receptors with MAP kinase signaling pathway [Tokiwa et al., 1996; Dikic et al., 1996]. Similarly , we propose that there might be a role for Src which has been suggested

to serve as a docking site for PI 3-kinase in platelets [Clark et al., 1994b] and PYK2 in linking the G-protein-coupled receptors (thrombin) with PI 3-kinase activation.

CONCLUSION

A clear route for PI 3-kinase activity through G-protein-coupled receptors is not understood, but it is believed to be regulated by a tyrosine kinase(s) that is independent of integrin occupancy [Rittenhouse, 1996; Fry, 1994]. Here for the first time we report that the activity of PI 3-kinase in human platelets may be regulated by the association with the novel member of the focal adhesion kinase gene family, PYK2. The association and the activity of PYK2 were shown to be increased in a time dependent manner upon thrombin stimulation and were uninhibited by RGDS peptide which blocks the interaction of ligands such as fibrin/fibrinogen with the platelet integrin GPIIb/IIIa. This study demonstrated that the increased activity and phosphorylation of PYK2 were strikingly correlated with the increased activity of PI 3-kinase in the early stages of platelet activation independently of integrin engagement. We therefore propose that a physical or a functional association of PYK2 with PI 3-kinase may occur *in vivo* that is responsible for the initial activation of PI 3-kinase in thrombin-stimulated human platelets and it may be regulated by calcium mobilization and indirectly by PKC activity. Based on results from Dikic, et al., [1996], suggesting that PYK2 with Src may link signal molecules to the plasma membrane G-protein-coupled receptors, we also suggest that PYK2 may link PI 3-kinase to the membrane receptors (see Figure 17 for complete illustration of this hypothesis).

Furthermore, we report the inducible association of a previously unreported 115 kD tyrosine-phosphorylated protein with PI 3-kinase in PAF-stimulated rabbit and p102 in thrombin-stimulated human platelets. It has been shown that p115 protein associates with PI 3-kinase in close correlation with the increase in PI 3-kinase activity / protein levels in anti-phosphotyrosine immunoprecipitates [Lauener, 1997]. We could not identify this

protein by immunoreactivity to antibodies directed against a number of tyrosine phosphorylated signaling proteins in this molecular weight range. Initial attempts at purification suggest that the p115 protein may consist of more than one polypeptide, or different phosphorylation states of the same polypeptide. In parallel, we investigated another protein that is inducible in thrombin-stimulated human platelets at 102 kD exhibiting the same characteristics as p115 in rabbit platelets. Studies with the platelet aggregation inhibitor RGDS, which completely blocked the tyrosine phosphorylation of p102 protein upon thrombin stimulation, suggest that this protein is may be an aggregation-dependent tyrosine kinase or a tyrosine phosphorylated substrate that associates with PI 3-kinase during platelet integrin engagement. We suggest that p102 may serve as an adapter or docking protein to enable recruitment of PI 3-kinase enzyme to the platelet cytoskeleton. However, we observed protein degradation dependent on platelet aggregation and solubilization conditions in which we showed a decrease in the level of a protein at 123 kD immunoreacted with anti-PYK2 antibody. This was concomitant with appearance of number of tyrosine phosphorylated proteins between 116 and 97 molecular range. Therefore, it is possible that p102 may be a degraded polypeptide, and there is a possibility that it is a product of PYK2 during platelet aggregation that is undetectable by the commercially available PYK2 antibodies. Investigation is underway in our lab to determine of whether p102 is a novel protein that associate with PI 3-kinase in an aggregation-dependent manner, or it is a degraded product of PYK2.

PI 3-kinase Signal Transduction Pathway
In Thrombin-Stimulated Platelets

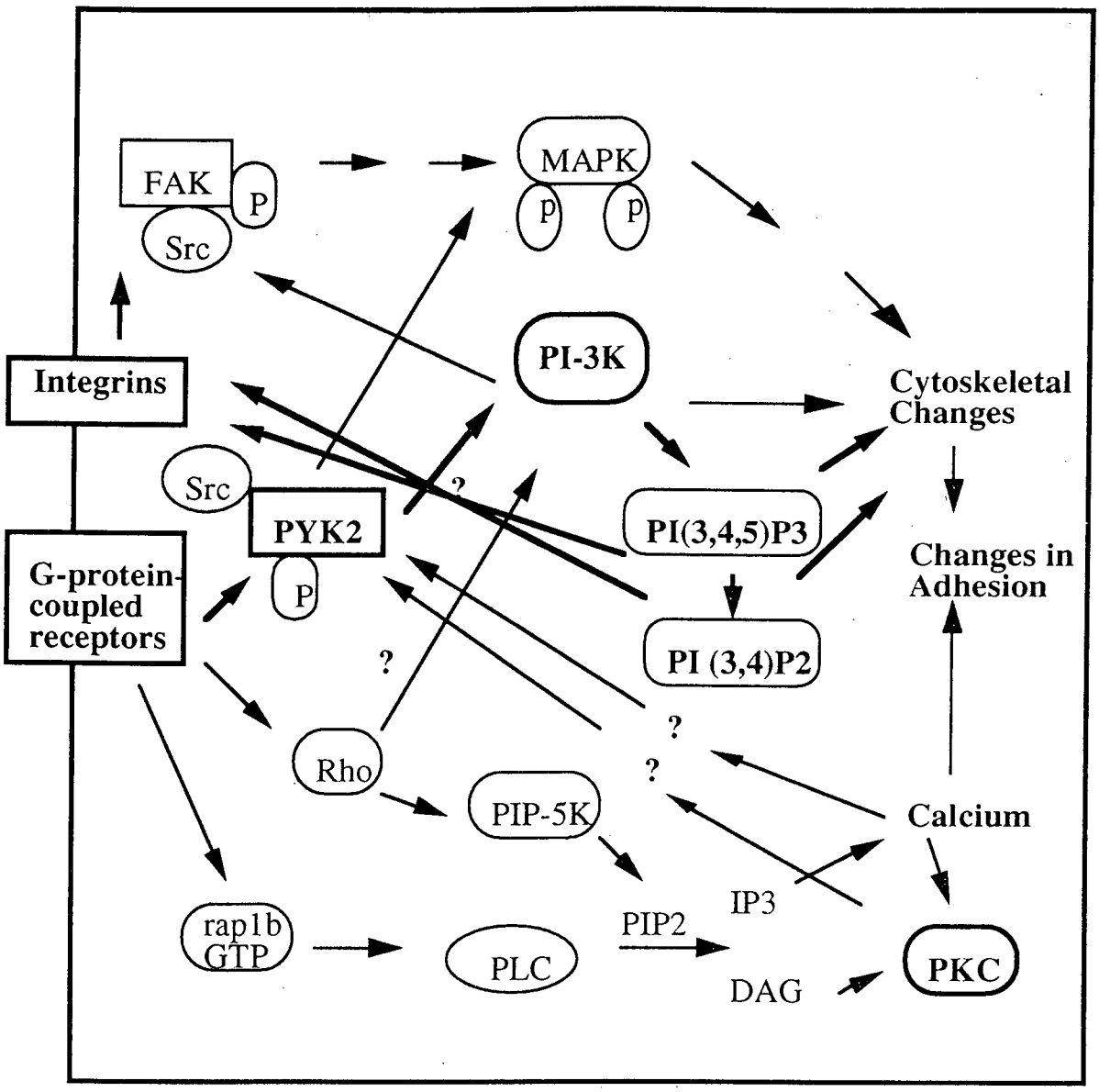


Figure 17

Potential PI 3-kinase regulation through G-protein-coupled receptors. The pathways outlined here are hypothetical, and are compiled from our results, studies by Shattil et al., [1998] and several studies discussed above.

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