Integrin-dependent Translocation of Phosphoinositide 3-Kinase to the Cytoskeleton of Thrombin-Activated Platelets Involves Specific Interactions of p85α with Actin Filaments and Focal Adhesion Kinase

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Abstract. Thrombin-induced accumulation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) but not of PtdIns(3,4,5,)P_3 is strongly correlated with the relocation to the cytoskeleton of 29% of the p85α regulatory subunit of phosphoinositide 3-kinase (PtdIns 3-kinase) and is accompanied by a significant increase in PtdIns 3-kinase activity in this subcellular fraction. Actually, PtdIns(3,4)P_2 accumulation and PtdIns 3-kinase, pp60$^{c\text{-}src}$, and p125^{FAK} translocations as well as aggregation were concomitant events occurring with a distinct lag after actin polymerization. The accumulation of PtdIns(3,4)P_2 and the relocalization of PtdIns 3-kinase to the cytoskeleton were both dependent on tyrosine phosphorylation, integrin signaling, and aggregation. Furthermore, although p85α was detected in anti-phosphotyrosine immunoprecipitates obtained from the cytoskeleton of thrombin-activated platelets, we failed to demonstrate tyrosine phosphorylation of cytoskeletal p85α. Tyrphostin treatment clearly reduced its presence in this subcellular fraction, suggesting a physical interaction of p85α with a phosphotyrosyl protein. These data led us to investigate the proteins that are able to interact with PtdIns 3-kinase in the cytoskeleton. We found an association of this enzyme with actin filaments: this interaction was spontaneously restored after one cycle of actin depolymerization–repolymerization in vitro. This association with F-actin appeared to be at least partly indirect, since we demonstrated a thrombin-dependent interaction of p85α with a proline-rich sequence of the tyrosine-phosphorylated cytoskeletal focal adhesion kinase, p125^{FAK}. In addition, we show that PtdIns 3-kinase is significantly activated by the p125^{FAK} proline-rich sequence binding to the src homology 3 domain of p85α subunit. This interaction may represent a new mechanism for PtdIns 3-kinase activation at very specific areas of the cell and indicates that the focal contact–like areas linked to the actin filaments play a critical role in signaling events that occur upon ligand engagement of α_{IIb}/β_3 integrin and platelet aggregation evoked by thrombin.

THE accumulation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2)[1] and PtdIns(3,4,5)P_3 in thrombin-stimulated human platelets is now well established (16, 24, 38, 41) and seems to be regulated differently (38). However, the exact mechanism of activation of phosphoinositide 3-kinase (PtdIns 3-kinase) in these cells is still obscure. The small G-protein rho (51), G-protein βγ subunit (44) protein kinase C (PKC) (26), Ca^{2+} (38), as well as tyrosine phosphorylation (19) may contribute to its activation. Possible ways of stimulation in other models also include physical association with specific proteins via the src homology 2 (SH2) domains or the proline-rich regions of the p85 subunit. This results in a conformational change of p85 that appears to be transmitted to the catalytic subunit (p110) as an activation signal (34, 37). Since PtdIns 3-kinase has been found to be associated with activated PDGF receptor (10) or pp60$^{v\text{-}src}$ (14), with a potentially very important role in mitogenesis (46) or oncogenic transformation (43), respectively, great interest has been elicited by its products (i.e., D3-phosphorylated phosphoinositides). Although their precise function is still unknown, these phospholipids have been suggested to play a role as second messengers activating downstream signaling enzymes like PKCζ isoform

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1. *Abbreviations used in this paper*: PtdIns, phosphatidylinositol; PKC, protein kinase C; SH3, src homology 3.

(29). Moreover, based on the homology of p110 with *Saccharomyces cerevisiae* protein Vps34p, PtdIns 3-kinase and/or its products have been recently suggested to play a role in intracellular sorting and down-regulating processes involving vesicle formation and targeting to the endosomal compartment (31). Another hypothesis is a possible role of PtdIns 3-kinase in regulating the polymerization and the organization of actin, as suggested by the simultaneous appearance of PtdIns(3,4,5)P_3 and actin polymerization of *N*-formyl peptide–stimulated human neutrophils (9).

In human platelets stimulated by thrombin, PtdIns(3,4,5)P_3 production occurs very rapidly (38), whereas the accumulation of PtdIns(3,4)P_2 is a late event dependent upon fibrinogen binding to the integrin receptor α_{IIb}/β_3 (38, 41, 42) and partly requires tyrosine phosphorylations (19, 48). Its production may be due to PtdIns(3,4,5)P_3 degradation by a 5-phosphatase (40), to the effect of a PtdIns(3)P 4-kinase (47), or to the direct action of PtdIns 3-kinase on PtdIns(4)P. Since accumulations of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 display different sensitivities to Ca^{2+}, integrin mobilization (38), and tyrosine phosphorylation (48), it is more likely that PtdIns(3,4)P_2 synthesis in platelet is due to the action of a 4-kinase on PtdIns(3)P or to the action of PtdIns 3-kinase on PtdIns(4)P, both of which would involve PtdIns 3-kinase. However, we and others have previously observed a translocation of PtdIns 3-kinase to the cytoskeleton of thrombin-activated platelets (18, 50). It is noteworthy that an association of PtdIns 3-kinase with the cytoskeleton has also been reported in other cells (32). In human platelets, thrombin stimulation leads to a dramatic reorganization of the cytoskeleton (15), which is possibly triggered in part by interaction of intetrin receptors with their ligands (21). Interestingly, several proteins involved in signal transduction are found relocated to the cytoskeleton of thrombin-activated platelets; among these are various enzymes of phosphoinositide metabolism (18) and $pp60^{c\text{-}src}$ (7, 18). This cellular protooncogene tyrosine kinase has been described to interact with PtdIns 3-kinase in different cell types (34). $pp60^{c\text{-}src}$ is constitutively present in large amounts in platelets and may be responsible for the phosphorylation and the regulation of different enzymes (8). A potential role of this tyrosine kinase in the integrin-dependent part of PtdIns 3-kinase activation in platelets is thus conceivable. In this model, thrombine induces the activation of $pp60^{c\text{-}src}$, which is then translocated to the cytoskeleton after α_{IIb}/β_3 integrin receptor mobilization and aggregation (7). However, other non–receptor tyrosine kinases whose activation is related to integrin signaling and cytoskeleton reorganization, like focal adhesion kinase ($p125^{FAK}$), may also play crucial roles in platelet activation (27).

Taken together, these observations point to a potentially important and dynamic function of the cytoskeleton in various signal transduction pathways. Therefore, to determine its possible functional relationships, we decided to study in detail the translocation of PtdIns 3-kinase to the platelet cytoskeleton during thrombin activation. Interestingly, kinetic studies revealed a strong correlation between the relocation of PtdIns 3-kinase and the accumulation of PtdIns(3,4)P_2. Since these events were dependent on both aggregation and α_{IIb}/β_3 integrin receptor engagement, our approach was to ask whether PtdIns 3-kinase activation and translocation were related to tyrosine phosphorylation promoted by integrin signaling. The data obtained indicated an important role for the integrin-dependent tyrosine kinase activation; however, cytoskeletal p85α was not tyrosine phosphorylated. This observation led us to investigate the proteins that may interact with PtdIns 3-kinase in the cytoskeleton of activated platelets. We found that thrombin activation of platelets leads to the formation of multienzymatic complexes, including PtdIns 3-kinase and tyrosine-phosphorylated $p125^{FAK}$ in areas tightly associated with the actin filament system corresponding to focal contact–like domains. Their formation appears to be dependent upon aggregation and is related to integrin-dependent tyrosine kinase activation. In investigating these associations further, we found that a proline-rich sequence of human $p125^{FAK}$ (residues 706–711) directly bound to the SH3 domain of p85α. Interestingly, micromolar amounts of a peptide corresponding to the proline-rich region of $p125^{FAK}$ (706–711) increased the specific activity of PtdIns-3 kinase. We therefore propose that this interaction may represent a new mechanism for PtdIns 3-kinase activation at very specific areas (i.e., focal contact points). The strong correlation observed between the formation of these focal adhesion–like domains and the accumulation of PtdIns(3,4)P_2 in this model may help to elucidate its functional relationships and emphasize the dynamic role of the cytoskeleton in signal transduction.

Materials and Methods

Antibodies and Fusion Proteins

The mouse anti-actin antibody was obtained from Amersham International (Buckinghamshire, UK). Mouse anti-$p125^{FAK}$, mouse anti-phosphotyrosine 4G10, and rat anti-p85α antibodies came from Upstate Biotechnology Inc. (New York). A highly specific antibody against the p85α subunit was kindly provided by Dr. Waterfield (Ludwig Institute for Cancer Research, London). Sheep polyclonal antiserum against $pp60^{c\text{-}src}$ was from Cambridge Research Biochemicals Inc. (Cambridge, UK). The mouse anti-phosphotyrosine 4G10 coupled to agarose, the GST- p85α–SH2 (NH_2) immobilized or not on agarose, and the anti-GST antibody were from TEBU (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). GST-p85α–SH3 was from PharMingen (San Diego, CA).

Preparation and Activation of Platelets

Platelet concentrates provided by the local blood bank (Centre Régional de Transfusion Sanguine de Toulouse) were used to prepare platelets as previously described (19) according to Ardlie et al. (2). For inositol lipid analysis, platelets were labeled with 0.3 mCi/ml ^{32}Pi (Amersham International) during 60 min in Ca^{2+}-free Tyrode's buffer (pH 6.5, 0.2 mM EGTA) at 37°C. After a washing step in the same buffer minus EGTA, platelets were resuspended in Tyrode's buffer containing 2.5 mM $CaCl_2$ (3×10^9 or 5×10^8 cells per ml when the tetrapeptide RGDS was used) (42). In some experiments, before the stimulation by human thrombin (Sigma Chemical Co., St. Louis, MO), platelets were preincubated for 5 min in the presence of 100 μM tyrphostin AG-213 (supplied by Dr. A. Levitzki, Hebrew University, Jerusalem, Israel) or for 15 s with 500 μM RGDS (Sigma Chemical Co.). Activation by thrombin was performed with shaking. Aggregation was followed in parallel by turbidimetry using an aggregometer (Chronolog, Havertown, PA) (3×10^9 or 5×10^8 cells per ml as indicated, 600 rpm.).

Lipid Extraction and Analysis

Reactions were stopped by addition of chloroform/methanol (vol/vol), and the lipids were extracted following a modified procedure of Bligh and Dyer (3). Lipids were immediately deacylated and analyzed using an HPLC technique on a Partisphere SAX column (Whatman International Ltd., UK) as previously described (41).

Cytoskeleton Extraction

Reactions were stopped, and cytoskeleton was immediately isolated by adding 1 vol of ice-cold CSK buffer containing 100 mM Tris-HCl, pH 7.4, 20 mM EGTA, 2 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF (Sigma Chemical Co.), and 2% (vol/vol) Triton X-100 to control or activated platelet suspensions (33). After a 10-min incubation at 4°C, the cytoskeletal and Triton X-100–soluble fractions were separated by centrifugation (12,000 *g*, 10 min, 4°C). For further use, cytoskeletons were washed as described by Grondin et al. (18) and subsequently resuspended in suitable buffers by sonication (20 kHz for 2 × 10 s) using an ultrasonic cell disruptor.

Isolation of Polymerized Actin and the Actin-Binding Protein–Rich Fraction

Actin filaments were isolated as previously described by Payrastre et al. (32). Briefly, cytoskeletons from resting or activated platelets were solubilized in 10 ml of buffer A containing 0.6 M KI, 100 mM Pipes, pH 6.5, 100 mM KCl, 10 μg/ml leupeptin, 1 mM PMSF, and 100 μM Na_3VO_4 for 20 min at 4°C with gentle shaking and centrifuged at 40,000 *g* for 20 min at 4°C. The supernatant, containing actin, was dialyzed at 4°C for 3 h against a buffer containing 10 mM Pipes, pH 6.8, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 2 μg/ml aprotinin, and 100 μM Na_3VO_4. Actin was then repolymerized, and this suspension was centrifuged at 12,000 *g* for 10 min at 4°C. The pellet, corresponding to actin filaments and the actin-binding protein–rich fraction, was resuspended in 50 mM Tris-HCl (pH 7.3) and immediately used for the PtdIns 3-kinase assay or Western blotting.

PtdIns 3-Kinase Assay

PtdIns 3-kinase activity was measured in a final volume of 100 μl containing 50 mM Tris-HCl, pH 7.4, 1.5 mM DTT, 100 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 5 μM ATP, exogenous lipid vesicles (100 μM phosphatidylinositol plus 200 μM phosphatidylserine, prepared by sonication in 50 mM Tris-HCl, pH 7.4), and proteins from the different fractions. Reactions were started by adding 10 μCi of [γ-^{32}P]ATP (Amersham International) and were performed at 37°C with shaking for 10 min. Reactions were stopped by adding a mixture of chloroform/methanol (vol/vol), and lipids were extracted and analyzed as previously described (41).

Immunoprecipitation and Immunopurification

Cytoskeletons were resuspended in 1 ml of a lysis buffer containing 40 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 mM NaF, 10 mM EDTA, 40 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 0.2% SDS, and 1% (vol/vol) Triton X-100. After sonication (20 kHz for 2 × 10 s), shaking for 20 min, and centrifugation (12,000 *g* for 10 min at 4°C), the soluble fraction was collected and subsequently precleared for 30 min at 4°C with protein A–Sepharose CL-4B or protein G–Sepharose 4B fast flow, depending on the subclasses and the origin of the antibodies used (Sigma Immuno Chemicals). Precleared suspensions were then incubated for 120 min at 4°C with the different antibodies: anti-phosphotyrosine antibody 4G10 coupled to agarose, anti-p125^{FAK} antibody (dilution 1:200), or anti-p85α antibody (dilution 1:200). After the first 60 min, 50 μl of 10% (wt/vol) protein A– or protein G–Sepharose resuspended in lysis buffer was added. The immunoprecipitates were then washed three times as described (19). With anti-phosphotyrosine immunoprecipitates, the phosphotyrosyl proteins were eluted as previously described (19).

Gel Electrophoresis and Western Blotting

Proteins were solubilized in electrophoresis sample buffer (100 mM Tris-HCl, pH 6.8, 15% (vol/vol) glycerol, 25 mM DTT, 3% SDS), boiled for 5 min and separated on 7.5% SDS-PAGE. Proteins were then blotted onto nitrocellulose (Bio Rad Laboratories, Hercules, CA) as described previously (19). The nitrocellulose was blocked for 60 min at room temperature in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% (vol/vol) Tween 20 (TBST) containing 5% milk powder or 2% BSA (Sigma Chemical Co.) when phosphotyrosyl proteins were analyzed. Immunodetection was performed with different antibodies as indicated in the figures. Antibody reaction was visualized using alkaline phosphatase– or peroxidase-conjugated secondary antibodies. Nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate were used as reagents (Sigma Chemical Co.) for alkaline phosphatase; the ECL chemiluminescence system (Amersham International) was used for peroxidase. Quantification of the different bands was performed using a densitometric analyzer (CRIS, Ramonville, France), which determines the pixel volume in each area.

Overlay Assay

Total cytoskeletons isolated from thrombin-activated platelets (0.7 IU/ml for 5 min) or immunopurified p125^{FAK} were subjected to 5–15% gradient SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose was incubated with 1% BSA, 1% milk powder in TBST (0.1% Tween 20) for 5 h and subsequently incubated overnight at 4°C in 50 mM Tris-HCl (pH 7.5) containing 12 mM 2-mercaptoethanol, 0.2 M NaCl, and 0.1% milk powder in the presence of GST-p85α–SH3, GST-p85α–SH2 (NH_2), or GST (at 15 μg/ml). Nitrocellulose was then washed in TBST (0.1% Tween 20) containing 1% milk powder for 1 h to remove unbound material and then probed with anti-GST mAb as previously described.

Peptide Synthesis

The peptides KPPRPG, KVVRVG, and KPPRPG were prepared by solid-phase synthesis on an automated peptide synthesizer (model 430A; Applied Biosystem Inc., Foster City, CA) according to *N-tert*butyloxycarbonyl (t-Boc)-amino acids and symmetric anhydride or hydroxybenzotriazole ester as activation chemistry. The products were removed from the resin and simultaneously deprotected by reaction with liquid anhydrous hydrofluoric acid in the presence of anisol and m-cresol as a cation scavenger at −5°C. The purity of the final products (97%) was assessed by analytical reverse-phase liquid chromatography (column Aquapore RP 300, L8, 220 mm, 4.6 mm) and fast atom bombardment mass spectrometry (MH+, 651.2) on a ZAP-HS double focusing spectrometer (VG Analytical, UK).

Affinity Column

The proline-rich peptide from p125^{FAK} was coupled to CNBr-activated Sepharose 4B beads at a ratio of 4 mg of peptide per ml of activated beads according to the instructions provided by the manufacturer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Cytoskeletons were resuspended in 1 ml of lysis buffer as described for the immunoprecipitation method, precleared with Sepharose 4B, and incubated or not in the presence of a mixture of two different mAbs to the SH3 domain of p85α (obtained from Dr. Waterfield's Laboratory and Dr. Matsuda). 50 μl of 10% (wt/vol) beads coupled to the peptide were added during 120 min at 4°C. The beads were then washed three times as described for the immunoprecipitation method, resuspended in sample buffer, and separated by SDS-PAGE (7.5%). Proteins were transferred to nitrocellulose and probed with antibodies to p85α.

Assay of PtdIns 3-Kinase in the Presence of the Proline-rich Peptide from p125^{FAK}

PtdIns 3-kinase was immunopurified from the Triton X-100–soluble fraction of resting platelets and was assayed essentially as previously described except that the final concentration of ATP was 50 μM and the exogenous lipid vesicles used were 150 μM phosphatidylinositol 4-monophosphate/300 μM phosphatidylserine or 150 μM phosphatidylinositol/300 μM phosphatidylserine. After addition of the peptide (solubilized in 50 mM Tris-HCl, pH 7.4), samples were preincubated for 10 min at 4°C. The reaction was then started by adding 10 μCi of [γ-^{32}P]ATP and was performed at 37°C with shaking for 5 min. The lipids were immediately extracted, analyzed, and quantified by HPLC (41). Under these assay conditions, PtdIns(3)P or PtdIns(3,4)P_2 formation was linear with time for 10 min.

Results

PtdIns(3,4)P_2 but Not PtdIns(3,4,5)P_3 Synthesis Parallels Translocation of Activated PtdIns 3-Kinase to the Cytoskeleton

We and others have previously shown that upon thrombin stimulation, both p85α and PtdIns 3-kinase activity significantly relocate to the cytoskeleton of human platelets (18, 50). Quantitative immunoblotting (Fig. 1) showed that under resting conditions, p85α was hardly detectable in the

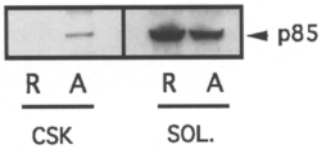

Figure 1. Quantitative immunodetection of p85α in the cytoskeleton. Cytoskeletons were extracted as indicated in Materials and Methods from resting (*R*) or thrombin-stimulated (0.7 IU/ml for 5 min) (*A*) platelets. Proteins from the cytoskeleton (*CSK*) and the corresponding Triton X-100–soluble (*SOL.*) fractions obtained from 1.6×10^8 platelets were separated by SDS-PAGE (7.5%) blotted onto nitrocellulose, and probed with the anti-p85α antibody.

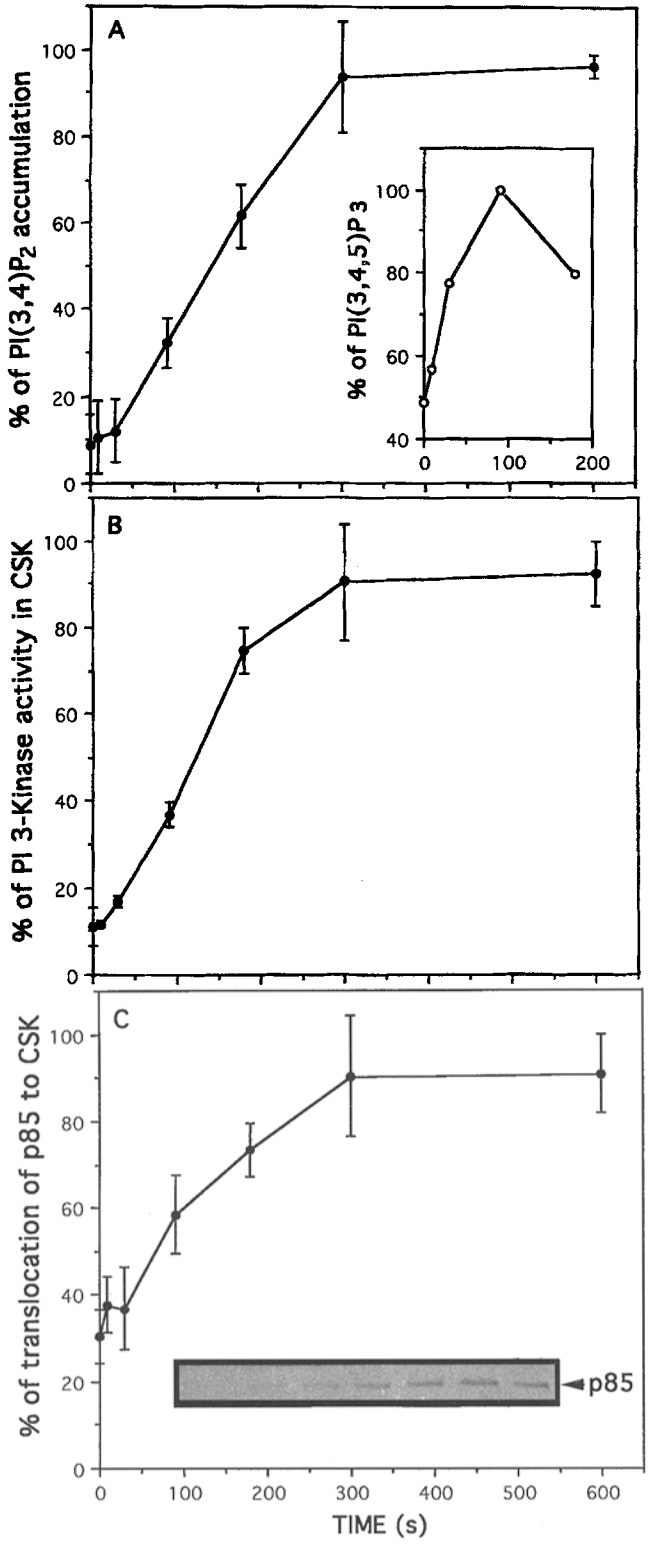

Figure 2. Comparison of the time courses of PtdIns 3-kinase translocation and 3D-phosphorylated inositol lipids accumulation in whole cells upon thrombin stimulation. ^{32}P-labeled platelets (3×10^9 cells/ml) were activated with 0.7 IU/ml thrombin for increasing periods of time. Reactions were stopped by addition of chloroform/methanol (vol/vol). Lipids were immediately extracted and deacylated. [^{32}P]PI(3,4)P_2 (●) and [^{32}P]PI(3,4,5)P_3 (○) were subsequently separated and quantified using HPLC (*A*) as described in Materials and Methods. In parallel, platelets from the same preparation were incubated under similar conditions (1.6×10^8 cells per assay) and lysed by addition of CSK buffer. Cytoskeletons were immediately extracted and assayed for PtdIns 3-kinase activity (*B*) or analyzed for the presence of p85α by Western blotting (*C*). Results are expressed as percent control and are means ± SD of three to five independent experiments.

cytoskeleton, whereas up to 29 ± 9% was translocated to this compartment after 5 min of activation using 0.7 IU/ml thrombin. A parallel decrease in the Triton X-100–soluble fraction was observed. The use of a highly specific antibody from Dr. Waterfield's laboratory unambiguously identified platelet p85 as the α isoform (data not shown).

In agreement with Zhang et al. (50), when PtdIns 3-kinase was assayed in the homogenate and in the cytoskeleton obtained from thrombin-stimulated cells, its specific activity (picomoles per minute per p85α) was increased by eightfold in the cytoskeleton.

To investigate further a possible functional role for the relocation of PtdIns 3-kinase, we checked whether the kinetics of the production of D3-phosphorylated phosphoinositides were correlated with the redistribution of the enzyme. Interestingly, Fig. 2 shows a striking correlation between PtdIns-(3,4)P_2 accumulation in whole platelets, increasing PtdIns 3-kinase activity in the cytoskeleton, and translocation of p85α to the cytoskeleton (Fig. 2, *A*, *B*, and *C*, respectively). In contrast, the PtdIns(3,4,5)P_3 signal (Fig. 2 *A*, *inset*) appeared to be more rapid than PtdIns(3,4)P_2 synthesis, as previously reported (24, 38). Thus, the accumulation of PtdIns(3,4)P_2 but not of PtdIns(3,4,5)P_3 temporally correlated with the translocation of PtdIns 3-kinase to the cytoskeleton.

Tyrosine Phosphorylation, Integrin Engagement, and Aggregation Are Required for Association of PtdIns 3-Kinase with the Cytoskeleton and for PtdIns(3,4)P_2 Accumulation

Thrombin-induced platelet aggregation is accompanied by a number of biochemical events, among them pp60$^{c\text{-}src}$ translocation to the cytoskeleton, tyrosine phosphorylation of several cytoskeletal proteins, and actin polymerization. Fig. 3, *A* and *B* show that translocation of p85α, pp60$^{c\text{-}src}$, and p125^{FAK} correlated well and may be considered late events, since they were significantly detectable only after 30 s of stimulation. In addition, Fig. 3 *B* indicates that translocation of p85α, p125^{FAK}, and pp60$^{c\text{-}src}$ to the cytoskeleton was not due to an artifactual trapping of proteins during actin polymerization, which was very rapidly measurable. Indeed, the F-actin content increased about two times within 10 s of stimulation. Moreover, it is noteworthy that p85α, p125^{FAK}, and pp60$^{c\text{-}src}$ relocalization was significantly measurable only when 25% of aggregation (ΔT max%) was reached.

The tetrapeptide RGDS and tyrphostin AG-213 are commonly used to inhibit the binding of fibrinogen to integrin α_{IIb}/β_3 receptor and to inhibit tyrosine phosphorylation, respectively. Both compounds have been previously shown

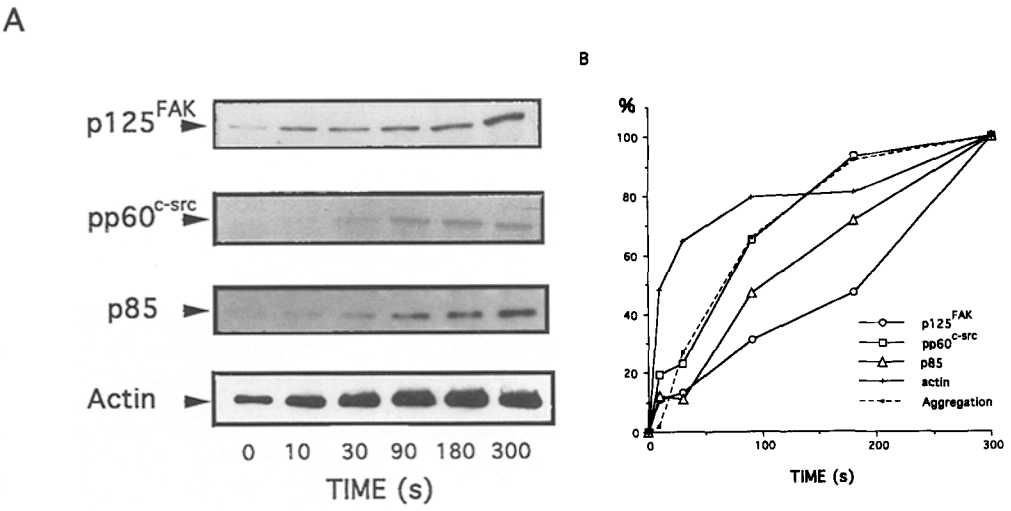

Figure 3. Analysis and comparison of different events occurring in cytoskeletons of thrombin-stimulated platelets. Platelets were stimulated with 0.7 IU/ml thrombin for increasing periods of time as indicated in the figure. The reactions were stopped by addition of CSK buffer, and cytoskeletons were immediately extracted. Cytoskeletal proteins (corresponding to 1.6×10^8 platelets) were separated by SDS-PAGE (7.5%), blotted onto nitrocellulose, and probed with different antibodies as indicated in the figure. Alkaline phosphatase detection was used for pp60$^{c\text{-}src}$ analysis, and the ECL system was used for p85α, p125^{FAK}, and actin detection (*A*). (*B*) Time course of quantitated Western blot by densitometric analysis; data are expressed as a percentage of maximal translocation for each protein and are representative of three different experiments.

to reduce significantly aggregation as well as PtdIns(3,4)P_2 accumulation induced by thrombin (19, 42). Here, we show that tyrphostin (Fig. 4 *A*) and RGDS (Fig. 4 *B*) were able to inhibit both the translocation of p85α to the cytoskeleton and PtdIns(3,4)P_2 synthesis (Table I), with RGDS being somewhat more potent. The translocation of pp60$^{c\text{-}src}$ has already been shown to depend on α_{IIb}/β_3 mobilization (22, 30); therefore, we used it as an internal control. Its relocalization was also clearly inhibited by RGDS (Fig. 4 *B*), whereas tyrphostin had a clear but weaker inhibitory effect. Table I indicates that the percentage of inhibition of aggregation (ΔT max %) induced by tyrphostin or RGDS varied in the same way as the percentage of inhibition of p85α translocation to the cytoskeleton, as well as the percentage of inhibition of PtdIns(3,4)P_2 accumulation. However, RGDS again had a more pronounced effect on aggregation. Moreover, EGTA (5 mM) was also able to inhibit thrombin-induced aggregation by 80 $\pm$ 3%, and interestingly, p85α relocalization and PtdIns(3,4)P_2 synthesis were both inhibited in the same range (85 $\pm$ 5%). Finally, the absence of shaking during thrombin stimulation abolished these events by about the same percentage as the inhibition observed with EGTA, clearly indicating that they are aggregation dependent. Therefore, integrin engagement, aggregation, and tyrosine phosphorylation are crucial for PtdIns(3,4)P_2 accumulation as well as p85α translocation to the cytoskeleton. To investigate these relationships further, we have immunopurified the phosphotyrosyl proteins from the cytoskeleton and probed them with anti-p85α and anti-pp60$^{c\text{-}src}$ antibodies. Interestingly, both proteins were absent in the pool of phosphotyrosyl proteins obtained from the cytoskeleton of resting cells, whereas they were clearly detected in the immunoprecipitates obtained from the cytoskeleton of thrombin-activated platelets (Fig. 5). Again, this was inhibited in the presence of tyrphostin. An interesting question then concerned the tyrosine phosphorylation state of cytoskeletal p85α.

Cytoskeletal Activated PtdIns 3-Kinase Is Not Tyrosine Phosphorylated on p85α

Anti-p85α immunoprecipitates were prepared from the cytoskeleton of resting or thrombin-activated platelets. The presence of p85α in such immunoprecipitates was analyzed by PtdIns 3-kinase assay and Western blotting (Fig. 6 *A*). Both PtdIns 3-kinase activity and p85α were clearly detected in the anti-p85 immunoprecipitate obtained from the cytoskeleton of activated platelets (Fig. 6 *A*), indicating the

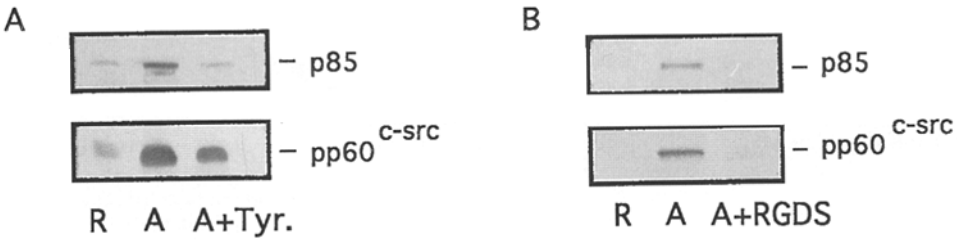

Figure 4. Effect of tyrphostin AG-213 and RGDS on p85α and pp60$^{c\text{-}src}$ relocalization. Cytoskeletons were prepared from resting (*R*) or thrombin-activated (0.7 IU/ml, 5 min) (*A*) platelets preincubated or not with tyrphostin (100 μM for 5 min) (panel *A*) or with RGDS (500 μM for 15 s) (panel *B*). Cytoskeletal proteins (corresponding to 1.6×10^8 platelets) were separated by SDS-PAGE (7.5%), blotted onto nitrocellulose, and probed with anti-p85α or anti-pp60$^{c\text{-}src}$ antibodies as indicated in the figure.

Table I. Effect of Tyrphostin and RGDS Treatment on Several Platelet Responses

Platelet responses	Percent of inhibition by tyrphostin (100 μM)	Percent of inhibition by RGDS (500 μM)
PtdIns(3,4)P_2 synthesis	53.0 ± 5.6	60.3 ± 6.9
p85 translocation to CSK	40.4 ± 12.0	54.2 ± 6.4
Aggregation ΔT max %	39.2 ± 10.5	81.5 ± 0.8

Platelets were preincubated or not with tyrphostin (100 μM) or RGDS (500 μM) and activated for 5 min by thrombin as indicated in Materials and Methods. PtdIns(3,4)P_2 synthesis was quantified using an h.p.l.c. technique. The translocation of p85α to the cytoskeleton (CSK) was detected by Western blotting experiments using anti-p85α antibody as a probe. Quantification was performed by densitometric analysis. Platelet aggregation was measured by turbidimetry using a Chronolog aggregometer (600 rev/min). Results are representative of three to five experiments, $P < 0.001$, unpaired t test.

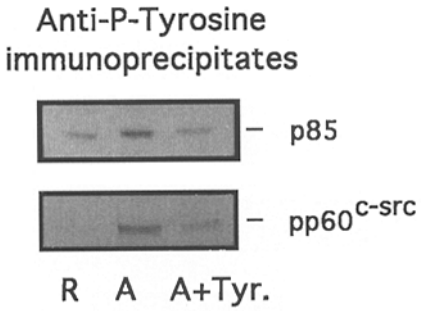

Figure 5. Detection of p85α and pp60$^{c\text{-}src}$ in anti-phosphotyrosine immunoprecipitates obtained from cytoskeleton of thrombin-stimulated platelets. Cytoskeletal proteins obtained from 1.5 × 10^9 resting (*R*) or thrombin-activated (0.7 IU/ml for 5 min) platelets, preincubated (*A+Tyr.*) or not (*A*) with 100 μM tyrphostin, were immunopurified using the agarose-coupled anti-phosphotyrosine antibody 4G10 and immunoblotted as indicated in each panel.

validity of the procedure. When these immunoprecipitated proteins were then probed with an anti-phosphotyrosine mAb (Fig. 6 *A*, *right panel*), we found that p85α present in the cytoskeleton of activated platelets was itself virtually not tyrosine phosphorylated. However, possibly only a small population of p85α is phosphorylated. Obviously, the best candidate would be p85α recovered in the anti-phosphotyrosine immunoprecipitate obtained from the cytoskeleton of thrombin-stimulated platelets. To check this possibility, we first estimated, using nonlimiting amounts of antibody, the p85α content recovered in the anti-phosphotyrosine immunoprecipitate from the cytoskeleton of activated platelets (Fig. 6 *B*, lane *2*) compared with the content of p85α recovered in the anti-p85α immunoprecipitate from a similar cytoskeleton (Fig. 6 *B*, lane *1*). The data indicate that only a subpopulation of p85α was recovered in the anti-phosphotyrosine immunoprecipitate. Finally, when the immunopurified phosphotyrosyl proteins were then probed with an anti-phosphotyrosine antibody (Fig. 6 *B*, lane *3*), no 85-kD band could be detected, indicating that, after a 5-min stimulation, the pool of p85α recovered in the anti-phosphotyrosine immunoprecipitate was virtually not tyrosine phosphorylated. These data raised the question of the identification of the proteins interacting with PtdIns 3-kinase in the cytoskeleton.

Translocation of PtdIns 3-Kinase Is Directed to the Actin Filament System

As a first attempt to localize PtdIns 3-kinase in the cytoskeleton, we performed a selective extraction procedure. By solubilizing cytoskeleton in potassium iodine followed by in vitro polymerization of actin as previously described (32), we selectively obtained the actin filament system consisting of actin and actin-binding proteins. As indicated in Fig. 7, actin was indeed present in these fractions, with a significant increase in F-actin content upon thrombin stimulation. As shown in Fig. 7, p85α was hardly detectable in the F-actin–rich fraction obtained from resting platelets, whereas in thrombin-activated platelets, p85α was clearly present in the F-actin fraction. Interestingly, p125^{FAK} and pp60$^{c\text{-}src}$ were also strongly present in this fraction (Fig. 7). Finally, a significant PtdIns 3-kinase activity was measured in the isolated actin filamental system of stimulated cells (Fig. 7), indicating that both p85α and the catalytic subunit p110 were associated with actin or with an actin-binding protein in stimu-

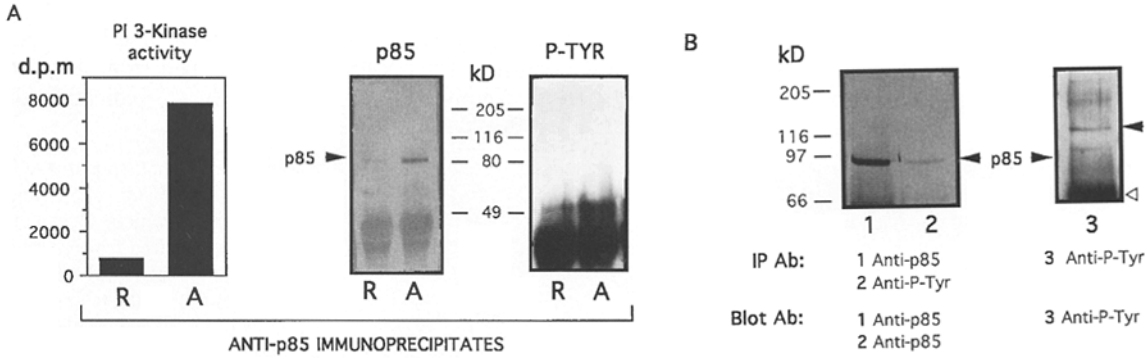

Figure 6. Analysis of the state of tyrosine phosphorylation of cytoskeletal p85α. (*A*) Cytoskeletal proteins obtained from 1.5 × 10^9 resting (*R*) or thrombin-activated (0.7 IU/ml for 5 min) (*A*) platelets were immunoprecipitated using the anti-p85α antibody. The immunoprecipitates were checked for the presence of PtdIns 3-kinase by measuring its lipid kinase activity and by Western blotting using anti-p85α antibody or the anti-phosphotyrosine antibody 4G10 as probes as indicated in the figure. (*B*) Cytoskeletons obtained from 1.5 × 10^9 thrombin-activated (0.7 IU/ml for 5 min) platelets were used for immunoprecipitations using the anti-p85α antibody (lane *1*) or the agarose-coupled anti-phosphotyrosine antibody 4G10 (lanes *2* and *3*). Immunoprecipitated proteins were then separated by SDS-PAGE (7.5%), blotted onto nitrocellulose, and probed with anti-p85α antibody (lanes *1* and *2*) or the anti-phosphotyrosine antibody 4G10 (lane *3*). The closed arrow on the upper right corresponds to 125 kD. The open arrowhead below shows the reactivity of immunoglobulin heavy chain.

lated platelets. Moreover, this result shows that PtdIns 3-kinase was still able to bind to the actin filament system after one cycle of depolymerization–repolymerization of actin in vitro. These data do not prove a direct association of PtdIns 3-kinase with actin, since several proteins may be candidates to link this enzyme to the actin filament system. Our previous data suggested an association of p85α with a cytoskeletal tyrosine-phosphorylated protein; pp60$^{c\text{-}src}$ is a possible candidate (20). A 125-kD protein is also strongly recognized by anti-phosphotyrosine antibodies in the cytoskeleton of activated platelets (Fig. 6 *B*, lane *3*). Since platelet aggregation induces focal contact-like areas, p125^{FAK} may thus be a good candidate as well (36). Therefore, we investigated the possible interactions between PtdIns 3-kinase and these two non-receptor tyrosine kinases.

Cytoskeletal PtdIns 3-Kinase Is Associated with Tyrosine-Phosphorylated p125^{FAK}

Despite the use of various antibodies and methodologies, we were unable to demonstrate the presence of pp60$^{c\text{-}src}$ in the anti-p85α immunoprecipitate (or the other way around) obtained from cytoskeleton (data not shown). Thus, we have no evidence for a direct interaction between PtdIns 3-kinase and pp60$^{c\text{-}src}$ in the cytoskeleton of activated platelets.

We then prepared anti-p125^{FAK} immunoprecipitates from the cytoskeleton of resting and thrombin-stimulated platelets. They were probed with anti-phosphotyrosine and anti-p85α antibodies. Fig. 8 shows that p125^{FAK} present in the cytoskeleton of resting platelets was weakly phosphorylated, whereas significant tyrosine phosphorylation was observed upon thrombin stimulation. It is noteworthy that a protein of ~190 kD was also recognized and may be a putative p125^{FAK}-binding phosphotyrosyl protein or a member of the focal adhesion complex. When anti-p85α antibody was used as a probe, no signal appeared in the anti-p125^{FAK} immunoprecipitate obtained from the cytoskeleton of resting platelets, whereas a clear and single 85-kD protein was detected upon thrombin stimulation. These data indicate a thrombin-dependent physical interaction between the regulatory subunit (p85α) of PtdIns 3-kinase and the tyrosine-phosphorylated p125^{FAK} in the cytoskeleton of activated platelets. This interaction appears specific, since under similar conditions, p85α was not recovered when nonimmune IgG1 from mouse was used instead of the anti-p125^{FAK} antibody (Fig. 8, lane *C*). Moreover, in agreement with our previous data (Fig. 6), the cytoskeletal p125^{FAK}-associated p85α was virtually not tyrosine phosphorylated, since no 85-kD protein was revealed in the anti-p125^{FAK} immunoprecipitate with the anti-phosphotyrosine antibody (Fig. 8, *left panel*).

Interaction of a Proline-rich Sequence of p125^{FAK} (706–711) with the SH3 Domain of p85α as a New Mechanism of PtdIns 3-Kinase Activation

To determine the domain of interaction between p125^{FAK} and p85α, we first investigated the potential binding of the SH2 domain of p85α to the tyrosine-phosphorylated region of p125^{FAK}. Using the immobilized p85α–SH2 (NH_2) domain, we were unable to precipitate p125^{FAK} significantly. More-

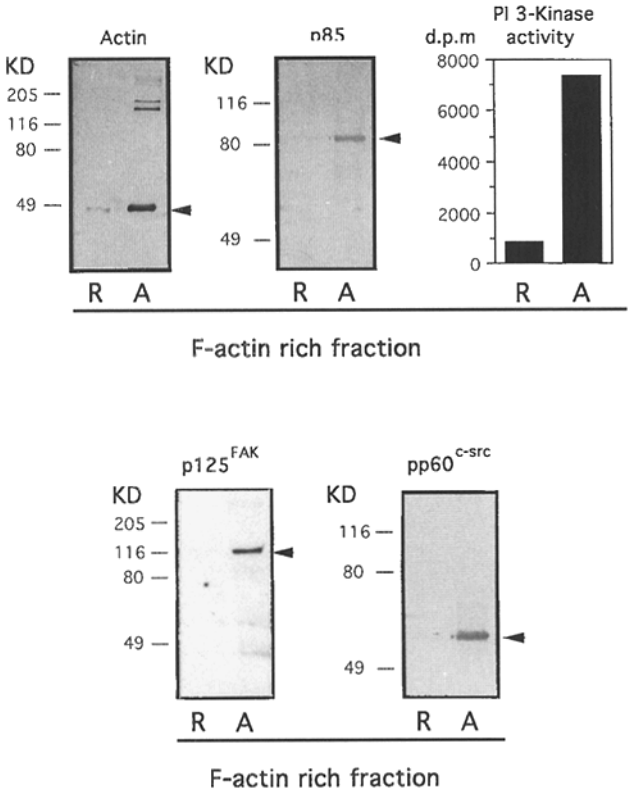

Figure 7. Association of p85α with the actin filament system. Proteins of the F-actin–rich fraction corresponding to 8 × 10^8 resting or activated-platelets were separated by SDS-PAGE (7.5%), and analyzed by Western blotting. The presence of actin, p85α, p125^{FAK}, and pp60$^{c\text{-}src}$ was checked using specific antibodies as indicated above each panel.

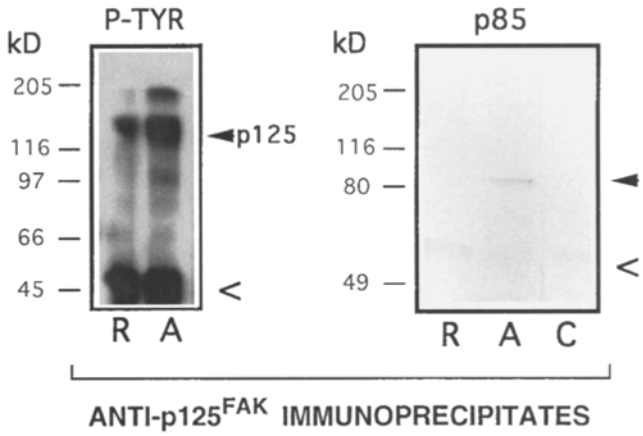

Figure 8. Presence of p85α in anti-p125^{FAK} immunoprecipitates from cytoskeletons of thrombin-stimulated platelets. Anti-p125^{FAK} immunoprecipitates were prepared from cytoskeletons of 1.5 × 10^9 resting (*R*) or thrombin-stimulated (0.7 IU/ml for 5 min) (A) platelets. After SDS-PAGE (7.5%), the proteins were analyzed by Western blotting with the anti-phosphotyrosine antibody 4G10 or the anti-p85α antibody as indicated above each panel. To evaluate the specificity of the association, a control in which nonimmune IgG1 from mouse was used instead of anti-p125^{FAK} antibodies is included (lane *C*). The open arrowhead shows the position of immunoglobulin heavy chain.

over, overlay technique did not indicate binding of GST-p85α-SH2 (NH_2) to the immunopurified tyrosine-phosphorylated p125^{FAK} on nitrocellulose (data not shown). These negative data are in agreement with the fact that p125^{FAK} does not possess the typical consensus p85-SH2 binding domain in the potential tyrosine phosphate-containing sequence found near its catalytic domain.

Another possibility was the binding of a proline-rich domain of p125^{FAK} to the SH3 domain of p85α. We therefore synthesized a peptide, KPPRPG, corresponding to a proline-rich sequence of human p125^{FAK} (residues 706–711). Once immobilized on Sepharose beads, this peptide could significantly bind cytoskeletal p85α (Fig. 9). This direct interaction seems to be specific, since PLCγ1, another SH3-containing enzyme of the phosphoinositide metabolism present in platelets, was not found to bind to this peptide (data not shown). In addition, preincubation of resuspended cytoskeleton, obtained from activated platelets, with specific antibodies directed to the SH3 domain of p85α significantly prevented this association (Fig. 9, *A* and *B*, lane *3*), strongly suggesting that the proline-rich sequence of p125^{FAK} (701–711) interacts with the SH3 domains of p85α. As a control, unrelated antibodies (nonimmune IgG1 from mouse or mAbs directed against the EGF receptor, which is not present in platelets) used instead of anti-p85α-SH3 antibodies were not able to reduce the association significantly (data not shown). Moreover, Fig. 9 *C* shows that the p85α-SH3 domain used as GST-p85α-SH3 was also able to bind directly to the peptide KPPRPG in vitro NH_2. Fig. 10 *A* indicates that p85α-SH3 but not p85α-SH2 (NH_2) was able to bind in vitro to the immunopurified p125^{FAK} obtained from cytoskeleton of activated platelets. Since weak interactions may be favored in these experiments, we have done overlay assay in which blots of immunopurified p125^{FAK} (Fig. 10 *B*, lane *1*) or total cytoskeletal proteins obtained from activated platelets (lanes *2* and *3*) were probed with GST-p85α-SH3 (lanes *1* and *3*) or with GST alone as a control (lane *2*) and then detected with an anti-GST antibody. Under these conditions, p85α-SH3 was able to bind to immunopurified p125^{FAK} (Fig. 10 *B*, lane *1*) and to a 125-kD protein of the cytoskeleton of activated platelets that matches p125^{FAK} (Fig. 10 *B*, lane *3*). In addition, p85α-SH3 was found to bind more weakly to three other proteins (Fig. 10 *B*, lane *3*) of ~93, 64, and 68 kD, which have yet to be identified.

Finally, we assessed the effect of the binding to p85α of the proline-rich sequence of p125^{FAK} on PtdIns 3-kinase activity. As shown in Fig. 11, the peptide KPPRPG significantly increased the PtdIns 3-kinase activity of immunopurified enzyme by 2.5-fold. Half-maximal activation was obtained in the presence of 10 μM peptide. Preincubation of the enzyme with antibodies directed to the SH3 domain of p85α before addition of the peptide reduced this activation by ~40% (data not shown). Finally, a control peptide, KVVRVG (Fig. 11) or KPPRVG (data not shown), was unable to activate PtdIns 3-kinase significantly under similar conditions.

To prove definitively that the direct binding of the peptide is responsible for the activation of PtdIns 3-kinase, we preincubated the immunopurified enzyme with or without 50 μM peptide for 120 min in lysis buffer lacking SDS, washed the immunocomplex twice to remove the unbound peptide, and finally assayed for PtdIns 3-kinase. Again, a twofold increase in activity was observed, indicating that the direct binding of the peptide is responsible for the activation of PtdIns 3-kinase.

Discussion

In agreement with Zhang et al. (50), we have demonstrated that ~30% of p85α is translocated to the cytoskeleton of thrombin-stimulated platelets. The translocation of p85α is

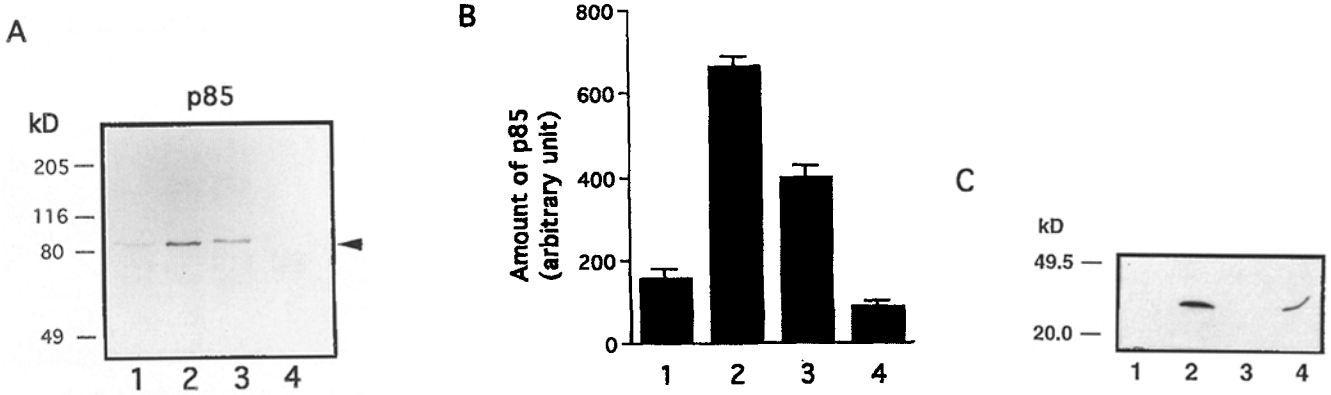

Figure 9. Association of the immobilized proline-rich sequence of p125^{FAK} (706–711) with the SH3 domain of p85α. Proteins from cytoskeleton of resting (lane *1*) or thrombin-activated (0.7 IU/ml for 5 min) (lane *2*) platelets were adsorbed onto Sepharose beads coupled to the p125^{FAK} proline-rich region (706–711) as described in Materials and Methods. Alternatively, proteins from cytoskeleton of activated platelets were preincubated for 30 min with antibodies directed to the SH3 domain of p85α and then adsorbed onto Sepharose beads coupled to the p125^{FAK} proline-rich region (706–711) (lane *3*). Finally, proteins from cytoskeleton of activated platelets were adsorbed to Sepharose beads alone as a control (lane *4*). After SDS-PAGE (7.5%), the proteins adsorbed were analyzed by Western blotting with the anti-p85α antibody (*A*). (*B*) Quantification by densitometric analysis of the Western blot. Data are expressed in arbitrary units (pixel points) and are means ± SEM of three different experiments. Finally, the direct binding of p85α-SH3 to the proline-rich region of p125^{FAK} (706–711) was analyzed in *C*. Purified GST-p85α-SH3 was diluted in lysis buffer to 5 μg/ml and incubated for 1.5 h with Sepharose beads alone (lane *1*) or with Sepharose beads coupled to the peptide (lane *2*). As a control, GST alone was incubated with the Sepharose beads coupled to the peptide (lane *3*). After extensive washing, the adsorbed proteins were analyzed by Western blotting with the anti-GST antibody. Lane *4* shows the position of GST-p85α-SH3 (37 kD) on the blot.

likely accompanied by a functional p110 catalytic subunit, since PtdIns 3-kinase activity was measured in the cytoskeleton of activated platelets and exhibited a higher specific activity than in the homogenate. Interestingly, PtdIns(3,4)P_2 but not PtdIns(3,4,5)P_3 synthesis paralleled the translocation of both p85α and PtdIns 3-kinase activity to the cytoskeleton, strongly suggesting a relationship between these two events. In thrombin-stimulated platelets, PtdIns(3,4,5)P_3 is produced very rapidly, whereas PtdIns(3,4)P_2 accumulates at a later stage (24, 38). Although PtdIns(3,4)P_2 has been shown to be a degradation product of PtdIns(3,4,5)P_3 in neutrophils, Sorisky et al. (38) have demonstrated that the production of these two phospholipids is regulated differently in thrombin-stimulated platelets. In this model, in contrast to the PtdIns(3,4)P_2 response, the accumulation of PtdIns(3,4,5)P_3 is unaffected by Ca^{2+} or the tetrapeptide RGDS (38, 42). Our results suggest that the redistribution of PtdIns 3-kinase to the cytoskeleton may be of importance for the accumulation of PtdIns(3,4)P_2. Detailed time course studies of PtdIns(3,4)P_2 accumulation and PtdIns 3-kinase, p125^{FAK}, and pp60$^{c\text{-}src}$ relocalization revealed that these events are in fact concomitant and significantly detectable only when 25–30% of aggregation (ΔT max %) is reached. Similar data have been obtained by Torti et al. (45) for the low molecular weight GTP-binding protein Rap2B. In contrast, actin polymerization is very rapidly detected, since double the amount of F-actin is observed within 10 s of stimulation. One may note that this time course of polymerization fits rather well with the PtdIns(3,4,5)P_3 signal as previously shown in fMLP-stimulated neutrophils (9). Moreover, it is noteworthy that the state of actin polymerization may in turn influence the phosphorylation state of several cytoskeletal proteins, including the focal adhesion kinase p125^{FAK} (23).

On the other hand, we have previously shown that PtdIns(3,4)P_2 accumulation is inhibited by RGDS (42). Here we demonstrate that the redistribution of p85α to the actin cytoskeleton and aggregation (ΔT max %) are inhibited in a similar manner by RGDS. In addition, EGTA (5 mM)

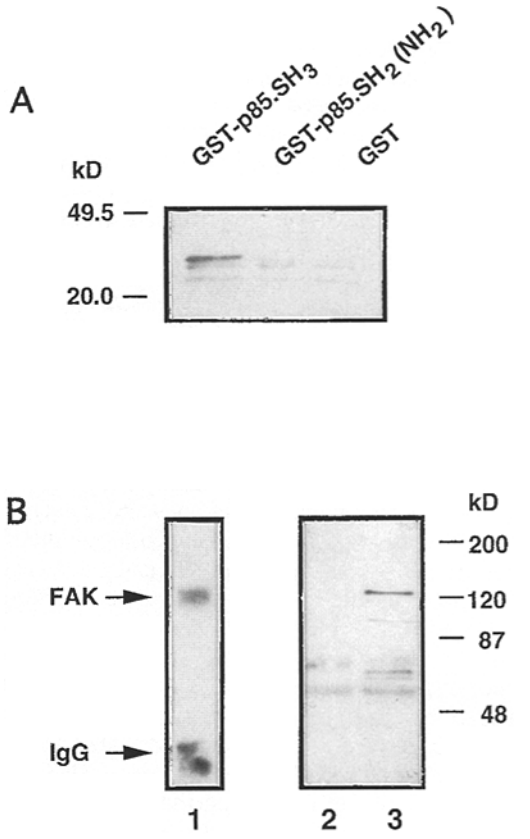

Figure 10. Direct binding of p85α–SH3 to cytoskeletal p125^{FAK}. p125^{FAK} was immunopurified from cytoskeleton of thrombin-activated platelets (0.7 IU/ml for 5 min) as described in Materials and Methods. The immunocomplex was then incubated in lysis buffer at 4°C for 1.5 h in the presence of 15 μg/ml GST-p85α–SH3, GST-p85α–SH2 (NH2), or GST alone, as indicated in the figure. After two washes in lysis buffer and three washes in 50 mM Tris-HCl, pH 7.35, 150 mM NaCl, the adsorbed proteins were analyzed by Western blotting using the anti-GST antibody as a probe (*A*). (*B*) Immunopurified p125^{FAK} (lane *1*) or total cytoskeletal proteins prepared from activated platelets (lanes *2* and *3*) were resolved by 7.5% or 5–15% SDS-PAGE, respectively, and blotted onto nitrocellulose for overlay assay as indicated in Materials and Methods. Proteins on nitrocellulose were then probed with GST-p85α–SH3 (lanes *1* and *3*) or with GST alone (lane *2*) and subsequently detected with anti-GST antibody using the ECL system. The nitrocellulose of lane *3* has then been stripped according to the procedure provided in the ECL kit and reprobed with the anti-p125^{FAK} antibody. The position of p125^{FAK} is indicated by the arrowhead.

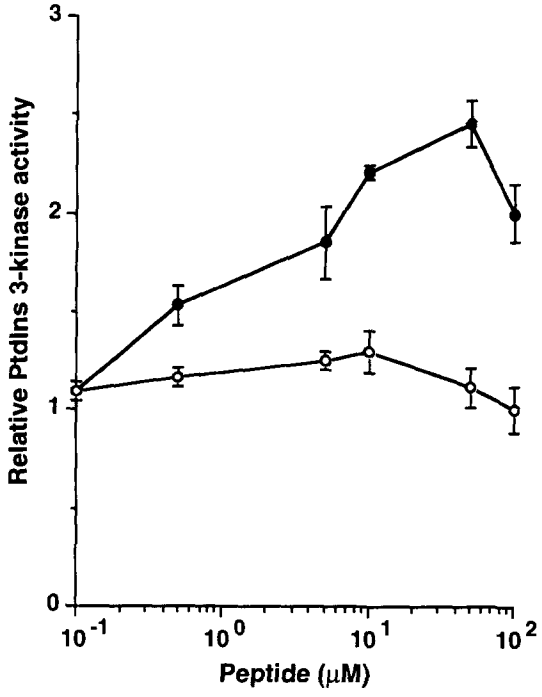

Figure 11. Activation of PtdIns 3-kinase by the peptide containing the proline-rich sequence of p125^{FAK} (706–711). Immunopurified PtdIns 3-kinase was assayed in the presence of increasing concentrations of the peptide corresponding to the proline-rich sequence of p125^{FAK} (KPPRPG) (●) or a control peptide with the same number of charges (KVVRVG) (○). Products of PtdIns 3-kinase were quantitated by HPLC as described in Materials and Methods. Results are expressed as PtdIns 3-kinase activity relative to that measured in the absence of peptide, which was taken as 1. The results shown are mean ± SD of three to five different experiments.

or the absence of shaking during activation significantly inhibited the association of p85α with the cytoskeletons as well as the accumulation of PtdIns(3,4)P_2. Therefore, aggregation via the binding of adhesive proteins to their receptors, especially fibrinogen binding to α_{IIb}/β_3, is required for both the production of PtdIns(3,4)P_2 and the translocation of PtdIns 3-kinase and pp60$^{c\text{-}src}$ to the actin cytoskeleton. The similarity both in the time course and in the sensitivity of tyrphostin and RGDS of PtdIns(3,4)P_2 synthesis and PtdIns 3-kinase translocation to the cytoskeleton suggests that these events might be tightly linked. This is not the case for PtdIns(3,4,5)P_3 production, which is not regulated in the same way (38, 48). Such a difference would be explained by the presence in human platelets of a PtdIns 3-kinase activated by G-protein $\beta\gamma$ subunits (44), which was recently identified in other hematopoietic cells (39). Our present data and those previously obtained on Glanzmann's thrombastenic platelets (42) may suggest a role for the α_{IIb}/β_3-linked actin cytoskeleton in the compartmentalization of activated PtdIns 3-kinase. The inhibitory effect of tyrphostin that we observed is in agreement with the fact that when RGDS blocks the binding of fibrinogen and other ligands to α_{IIb}/β_3, thrombin-induced tyrosine phosphorylation of several substrates is also blocked (11, 17). The chronology of this cascade of events may also explain why RGDS has a more potent inhibitory effect than tyrphostin. Thus, both fibrinogen binding to α_{IIb}/β_3 and the subsequently induced tyrosine phosphorylations are crucial events for the translocation of PtdIns 3-kinase to the actin cytoskeleton and the production of PtdIns(3,4)P_2 in thrombin-stimulated platelets. The role of tyrosine kinases in this mechanism is confirmed by the presence of a fraction of p85α in anti-phosphotyrosine immunoprecipitates obtained from the cytoskeleton of thrombin-activated platelets. However, we have found that the pool of cytoskeletal p85α is virtually not tyrosine phosphorylated, even though only a subpopulation of p85α is recovered in the anti-phosphotyrosine immunoprecipitate. Furthermore, we were also unable to show the tyrosine phosphorylation of p85α in the Triton X-100–soluble fraction of activated platelets (data not shown). Therefore, its presence in the pool of phosphotyrosyl proteins is possible only if p85α is physically associated with another tyrosine-phosphorylated protein. This result stimulated us to investigate the proteins that may interact with PtdIns 3-kinase in the cytoskeleton of activated platelets. We found that the relocalization of PtdIns 3-kinase and pp60$^{c\text{-}src}$ is preferentially directed to the actin filament system consisting of F-actin and actin-binding proteins. Functional PtdIns 3-kinase is still able to bind to the actin filaments even after one cycle of depolymerization–repolymerization of actin in vitro. Our observations are consistent with the recent data obtained by Fox et al. (13). These authors have observed that the Triton X-100–insoluble membrane skeleton components sedimented at 100,000 *g*, such as spectrin, vinculin, or talin, as well as pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$, and p21^{ras}-GAP, are redistributed to the Triton X-100–insoluble low speed fraction (15,000 *g*) during thrombin-induced aggregation. In this respect, it is noteworthy that a number of proteins that have been identified in the platelet membrane skeleton are actin-binding proteins (12, 13). Our data do not prove a direct association with actin, since actin-binding proteins may link PtdIns 3-kinase to F-actin and since we found a population of cytoskeletal p85α associated with a tyrosine-phosphorylated protein. The kinase pp60$^{c\text{-}src}$ is found in the actin-rich fraction and has been described to interact weakly with PtdIns 3-kinase in the Triton X-100–soluble fraction of thrombin-activated human platelets (20). Therefore, we have investigated such a possibility in the cytoskeleton. However, using both Western blot and enzymatic assays, we have failed to find a clear interaction between PtdIns 3-kinase and pp60$^{c\text{-}src}$ under our conditions (data not shown). The association between p85α and src-like kinases has been described in other models and seems to be due to SH3-mediated interactions (35); this mechanism might be minor in platelets (20).

However, another interesting candidate is p125^{FAK}, a protein tyrosine kinase involved with integrin signaling that is phosphorylated and activated upon thrombin stimulation of platelets (27). Moreover, actin-dependent cytoskeletal interactions seem to be necessary for these events (27). Interestingly, p125^{FAK} is not activated in platelets from Glanzmann's thrombasthenia (27), which also fail to produce PtdIns(3,4)P_2 (42). Our results show both the presence of a tyrosine-phosphorylated form of p125^{FAK} in the cytoskeleton of thrombin-activated platelets and an interaction between tyrosine-phosphorylated p125^{FAK} and non–tyrosine-phosphorylated p85α in this cytoskeleton. Furthermore, the amount of p85α found associated with p125^{FAK} is comparable to the amount of p85α recovered in the anti-phosphotyrosine immunoprecipitate. This association may therefore explain the presence of non–tyrosine-phosphorylated p85α in such immunoprecipitates and the increase in its ability to be precipitated with the anti–phosphotyrosine antibody after thrombin stimulation. It is noteworthy that the potential tyrosine phosphate–containing domain found near the catalytic domain of p125^{FAK} (1) does not exhibit the consensus motif known to bind the SH2 domain of p85α, and using different approaches, we were unable to demonstrate an association by this way. However, two proline-rich domains (564–576 and 694–714) containing the PPXP motif (6, 49) are found in the sequence of p125^{FAK} (1). Here we show that a peptide corresponding to residues 706–711 of human p125^{FAK} binds to the SH3 domain of non–tyrosine-phosphorylated p85α. This binding seems to be specific, since the p85α SH3 domain was also able to bind to immunopurified p125^{FAK} in vitro or to a 125-kD cytoskeletal protein that matches p125^{FAK} on nitrocellulose.

Moreover, this binding led to a significant activation of PtdIns 3-kinase. This activation possibly results from the induction of a conformational change leading to the activation of the p110 catalytic subunit, which is comparable to the activation described for PtdIns 3-kinase by the tyrosine-phosphorylated PDGF receptor (10), IRS-1 (28) and the *src* family kinase SH3 (34). Our data strongly suggest a new mode of PtdIns 3-kinase regulation in which binding of proline-rich sequence of p125^{FAK} (706–711) to the SH3 domain of p85α increased enzymatic activity of this lipid kinase. This mechanism is operative during thrombin-induced aggregation and may be important for enzyme access to its substrate at very specific areas of the cell (i.e., focal contacts). The role of 3D-phosphorylated phospholipids at these particular points has now to be investigated in detail. However, with respect to the data published recently, it is possible

to suggest that these lipids may play a role in the organization of actin polymerization, leading to the formation of actin filament bundles linked to focal contact points.

We propose that p125^{FAK} is responsible, at least in part, for the indirect binding of PtdIns 3-kinase to actin microfilaments and plays a critical role in activating and directing this lipid kinase to an appropriate location. Since we demonstrate here a role of aggregation, integrin receptor engagement and subsequent tyrosine phosphorylation in PtdIns 3-kinase translocation, and concomitant PtdIns(3,4)P_2 accumulation, it is tempting to suggest that p125^{FAK} may be a key enzyme in coupling integrin receptor signaling with these events. During the reviewing process of this manuscript, Chen and Guan (4, 5) described an interaction of p125^{FAK} with PtdIns 3-kinase in fibroblasts. Interestingly, this association was induced by cell adhesion to fibronectin-coated culture dishes (5) or by PDGF treatment (4). Our results also suggest that PtdIns 3-kinase likely displays different modes of interaction with cytoskeletal elements since only ~25% of cytoskeletal p85α is found associated with p125^{FAK}. This physical interaction may be transient, and PtdIns 3-kinase may then be compartmentalized in other cytoskeletal areas possibly to be down-regulated. The presence of the SH2 and SH3 domains as well as SH3-binding motifs in the p85 subunit (25) may explain the different targeting of these associations. Further work is necessary to identify all of the different cytoskeletal proteins with which PtdIns 3-kinase may be associated and their relevant biochemical and functional relationships.

In conclusion, focal contact-like areas induced upon thrombin stimulation of platelets, involving α_{IIb}/β_3, p125^{FAK}, as well as other phosphotyrosyl proteins and actin microfilaments, are very important for PtdIns 3-kinase relocation and PtdIns(3,4)P_2 accumulation. Whether this inositol lipid may in turn play a role in stabilizing the clot remains to be established.

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