Adhesion Receptor Activation of Phosphatidylinositol 3-Kinase

VON WILLEBRAND FACTOR STIMULATES THE CYTOSKELETAL ASSOCIATION AND ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE AND pp60<sup>src</sup> IN HUMAN PLATELETS

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The cytoskeleton participates in the coordinated regulation of intracellular signaling molecules, following agonist stimulation of cells. We have demonstrated that von Willebrand factor (vWF) induced the cytoskeletal association and activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) in human platelets. The activation of PtdIns 3-kinase coincided with the tyrosine phosphorylation of multiple platelet proteins, as assessed by anti-phosphotyrosine immunoblotting. One of these tyrosine-phosphorylated proteins, pp60<sup>src</sup>, became specifically enriched in the cytoskeletal fraction of vWF-stimulated platelets. The vWF-stimulated cytoskeletal association of PtdIns 3-kinase and pp60<sup>src</sup> required platelet stirring and aggregation, was specifically blocked by an anti-GPIb monoclonal antibody, and was not observed in platelets lacking the glycoprotein Ib/IX complex (Bernard-Soulier syndrome). Pretreatment of normal platelets with 5 mm EDTA (37°C for 90 min) or RGDS (2 mma), which disrupts the binding of various adhesive proteins to platelet integrins and inhibits fibrinogen-mediated platelet aggregation, did not alter the vWF-stimulated activation and cytoskeletal association of PtdIns 3-kinase and pp60<sup>src</sup>. Pretreatment of platelets with acetylsalicylic acid (1 mm) completely abolished vWF-stimulated production of thromboxane A<sub>2</sub>, dense granule release, and the activation of protein kinase C, without altering the activation and cytoskeletal translocation of PtdIns 3-kinase and pp60<sup>src</sup>. Our results suggest that vWF binding to the platelet adhesion receptor glycoprotein Ib/IX can mediate activation and translocation of both tyrosine and lipid kinase(s) independent of other agonists.

Cellular adhesion is essential for inflammation, immune recognition, and hemostasis. The cell surface receptors responsible for cell-cell and cell-matrix adhesive interactions belong to distinct receptor classes, including members of the integrin, immunoglobulin, leucine-rich glycoprotein, cadherin, and selectin gene families (for review, see Hynes, 1992; Springer, 1990; Takeichi, 1991). Recent evidence suggests that a number of these cellular receptors not only participate in cellular adhesion, but transduce signals from the extracellular environment to specific cytoplasmic signaling enzymes (Hynes, 1992).

Phosphatidylinositol 3-kinase (PtdIns 3-kinase) is a recently described signaling enzyme involved in the metabolism of inositol phospholipids (Whitman et al., 1988). The binding of specific hormones and growth factors to tyrosine kinase or G-protein-linked receptors is associated with the activation of PtdIns 3-kinase (Auger et al., 1989; Trayan-Moray-Kaplan et al., 1988). This lipid kinase phosphorylates inositol phospholipids at the D-3 position of the inositol ring, forming phosphatidylinositol 3-phosphate (PtdIns(3)P<sub>2</sub>), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) (for review, see Cantley et al., 1991; Majerus et al., 1990). The association of PtdIns 3-kinase with several growth factor receptors and oncogene products is essential for their mitogenic potential (Carpenter and Cantley, 1990). However, a role for PtdIns 3-kinase in cellular processes distinct from mitogenesis is suggested by the observation that terminally differentiated cells such as platelets rapidly produce PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> following thrombin stimulation (Kucera and Rittenhouse, 1990). Recent studies have demonstrated a thrombin-dependent interaction of PtdIns 3-kinase with pp60<sup>src</sup> in human platelets (Gutkind et al., 1990) raising the possibility that non-receptor tyrosine kinases are involved in the activation of PtdIns 3-kinase.

Thrombin-stimulated platelet aggregation is associated with the activation and cytoskeletal translacation of both PtdIns 3-kinase and pp60<sup>src</sup> (Grondin et al., 1991; Zhang et al., 1992). Various other signaling enzymes, including phospholipase C, protein kinase C, diacylglycerol kinase, and phosphatidylinositol 4-kinase have also been identified within the cytoskeletal matrix of stimulated platelets (Grondin et al., 1991; Zhang et al., 1992). Collectively, these observations suggest a role for the cytoskeleton in the assembly of platelet signaling complexes.

The platelet adhesion receptor, GPIb/IX, is a member of the leucine-rich glycoprotein gene family which binds the subendothelial ligand, von Willebrand factor (vWF), thereby anchoring platelets to sites of vascular damage (Ruggeri and Zimmerman, 1987). In this report, we demonstrate that the binding of vWF to platelet GPIb/IX stimulates the activation and cytoskeletal localization of PtdIns 3-kinase and pp60<sup>src</sup>.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P<sub>2</sub>), phosphatidylinositol, phenylmethylsulfonyl fluoride, Triton X-100, sodium vanadate, ADP, and arginine-glycine-aspartic acid-serine (RGDS) were from Sigma. Bovine thrombin and adrenaline was from Parke Davis. Ristocetin was supplied by Paudsett and Levee Inc. (Germany). Protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology. Enhanced chemiluminescence Western blotting detection kit was obtained from Amersham (United Kingdom). All radiochemicals were from NEN Dupont. Transfer polyvinylidene difluoride membranes were

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<sup>1</sup>The abbreviations used are: PtdIns 3-kinase, phosphatidylinositol 3-kinase; vWF, von Willebrand factor; mAb, monoclonal antibody.
obtained from Immobilon, Millipore. Sodium dodecyl sulfate was from Bio-Rad. Collagen was obtained from Chrono-Log Corp. All blood products were donated by Red Cross Blood Bank (Melbourne, Australia).

Antibodies—Anti-phosphotyrosine mAb 4G10 and anti-p85 (p85 subunit of PtdIns 3-kinase) polyclonal antibodies were from Upstate Biotechnology Inc. mAb 327 to pp60c-src was a kind donation from Dr. Joan Brugge (University of Pennsylvania). Anti-phosphotyrosine mAb PY20 were from DAKO (Denmark), and both anti-mouse and anti-rabbit peroxidase-conjugated IgG were from Silenus (Australia). mAb AK2 to GPIb/IX was from DAKO and kindly donated by Dr. Michael Berndt (Baker Medical Research Institute, Melbourne).

Preparation of Washed Platelets—Platelets were obtained from healthy volunteers who had not taken anti-platelet medication in the preceding 2 weeks and washed using a modified method of Baenzerger and Majerus (1974). Whole blood was anticoagulated, 6 volumes of blood to 1 volume of acid-citrate-dextrose (90 mM sodium citrate, 7 mM citric acid, pH 4.6, 140 mM dextrose, supplemented with 70 mM theophylline). Platelet-rich plasma was obtained by centrifugation of whole blood to settle for 3 h at room temperature, inclined at 45°. These platelets were sedimented during washing at 500 x g for 10 min, whereas normal platelets were pelleted by centrifugation at 2000 x g for 10 min. The platelet pellets were resuspended in buffer, 4.3 mM NaH₂PO₄, 24.3 mM Na₂HPO₄, 4.3 mM K₂HPO₄, pH 6.5, 113 mM NaCl, 5.5 mM glucose, 0.5% bovine serum albumin, and 10 mM theophylline, washed twice, then finally resuspended in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM glucose. In some experiments, platelets were resuspended in buffer containing 5 mM EDTA, 2 mM RGDS, or 5 μg/ml anti-GPIb/IX mAb, AK2. In other experiments, platelets were incubated for 90 min at 37°C with either EDTA (5 mM) or acetylsalicylic acid (ASA) (10 μM) prior to final resuspenion of the platelets in buffer. ATP (5 μM) was added to each reaction mixture, and the enzyme assay incubated for 20 min at room temperature. Reactions were stopped with 100 μl of 1.0 M HCl and phospholipids extracted with 200 μl of chloroform/methanol (1:1) and 500 μl of 2.0 M KCl. PtdIns 3-kinase assays were performed in the linear range with respect to protein concentration and time. 32P-Labeled inositol phospholipids were resolved by thin layer chromatography with a 2.0 M acetic acid/n-propanol (35:65 v/v) solvent system and detected by autoradiography, as described (Carpenter et al., 1990). Individual phospholipids were scraped from the thin layer chromatography plate and quantitated by liquid scintillation counting. The authenticity of each phospholipid was confirmed by HPLC analysis of the deglycerolipid product as described previously (Auger et al., 1989; Divecha et al., 1991).

Quantitation of PtdIns 3-Kinase Activity—PtdIns 3-kinase activity was quantitated on platelet extracts diluted 1/1000-fold by measuring the enzymatic incorporation of 32P into PtdIns4,5P₂, forming PtdIns[3-32P,4,5P₂], as described previously (Susu et al., 1992). The specific activity of PtdIns 3-kinase in the Triton X-100-soluble and cytoskeletal fraction of platelets was determined by comparing total enzyme activity with the total amount of p85 in each fraction. Quantitation of p85 was determined by performing densitometry on p85 immunoblots.

p85 (p85 Subunit of PtdIns 3-Kinase) and p60c-src Immunoblots—Platelet cytoskeletons were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with specific antibodies to p85 and p60c-src.
Adhesion Receptor Activation of PtdIns 3-Kinase

pp60<sup>src</sup> → pp60<sup>src</sup>

**Fig. 2.** Ristocetin-induced binding of vWF to platelets stimulates the cytoskeletal association of pp60<sup>src</sup>. a, washed platelets were exposed to control buffer (lane 1); vWF (10 μg/ml) and ristocetin (1 mg/ml) without stirring (lane 2); thrombin (1 unit/ml) (lane 3); vWF and ristocetin (lane 4); vWF and ristocetin, in the presence of 2 mM RGDS, (lane 5) or b) control buffer (lane 1); ristocetin alone (lane 2); vWF and ristocetin (lane 3); or bovine plasma (diluted 1:4) (lane 4). Each of the reaction mixtures were stirred for 5 min (except where otherwise indicated) at room temperature. The Triton X-100-insoluble cytoskeletal fraction was prepared as described under “Experimental Procedures,” electrophoresed by 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes prior to immunoblotting with a monoclonal antibody to pp60<sup>src</sup> (mAb 327).

**Fig. 3.** Phosphatidylinositol 3-kinase translocates to the cytoskeletal fraction of vWF-stimulated platelets. a-d, washed platelets were stirred for 5 min at room temperature in the presence of control buffer (CONT), thrombin (1 unit/ml) (THR), or vWF (10 μg/ml) and ristocetin (1 mg/ml) (vWF). Platelet fractionation was performed as described under “Experimental Procedures.” a and b represent the Triton X-100-soluble fraction of platelets, and c and d refer to the cytoskeletal fraction of platelets. PtdIns 3-kinase assays were performed on a 1/200 (A) or 1/1000 (B) dilution of each platelet fraction. Platelet extracts were incubated with PtdIns(4,5)P<sub>2</sub> (0.2 mg/ml) and phosphatidylserine (0.2 mg/ml), for 20 min at room temperature, in the presence of [γ-<sup>32</sup>P]ATP. After lipid extraction, [γ-<sup>32</sup>P]-labeled phospholipids were resolved by thin layer chromatography and detected by autoradiography. PtdIns 3-kinase activity was quantitated by measuring the formation of PtdIns(3,4,5)P<sub>3</sub> as described under “Experimental Procedures.” In b and d: lane 1, resting platelets; lane 2, thrombin; lane 3, vWF and ristocetin.

RESULTS

Stimulation of Platelet Aggregation by vWF and Ristocetin in the Presence of EDTA or Anti-GPIb mAb AK2—The attachment and immobilization of vWF to the subendothelial matrix is essential for its interaction with the platelet adhesion receptor GPIb/IX (Roth, 1991). In contrast the binding of soluble vWF to GPIb/IX under conditions of low shear stress requires the presence of cationic molecules such as the macrolide antibiotic ristocetin or the snake venom protein, botrocetin (Ruggeri and Zimmerman, 1987). Fig. 1a demonstrates vWF (10 μg/ml) induced washed platelet aggregation in the presence of ristocetin (1 mg/ml). Aggregation required platelet stirring and was specifically blocked by the anti-GPIb/IX mAb, AK2 (Fig. 1a). Soluble vWF also promotes platelet aggregate formation by binding to GPIIb/IIIa on the surface of activated platelets (Savage et al., 1992). To examine the role of GPIIb/IIIa in vWF-induced platelet aggregation, we abolished the ligand binding...
compétency of GPIb/IIIa by pretreating platelets with 5 mM EDTA. This pretreatment of platelets completely inhibited ADP (10 μM), thrombin (1 unit/ml), and collagen (5 μg/ml) induced platelet aggregation (data not shown) but had minimal effect on aggregation stimulated by vWF (Fig. 1a). These experiments indicate that under the assay conditions employed the binding of vWF to GPIb/IX is sufficient for platelet aggregation.

vWF-induced Platelet Aggregation Stimulates Tyrosine Phosphorylation of Platelet Proteins—In order to examine the relationship between vWF-induced platelet aggregation and protein tyrosine phosphorylation, we performed anti-phosphotyrosine immunoblots on vWF-stimulated platelets. As described previously in unstimulated platelets, a major 60-kDa phosphotyrosine-containing protein was observed (Fig. 1b, lane 1) (Ferrell and Martin, 1988). This protein was recognized by antibodies against pp60^{c-src} (data not shown). In the presence of ristocetin (1 mg/ml) and vWF (10 μg/ml), tyrosine phosphorylation of platelet proteins corresponding to molecular masses of 118, 75, 67, 65, 46, 41, 35, and 29 kDa was observed (Fig. 1b, lane 3). A similar set of proteins is also tyrosine phosphorylated following thrombin stimulation of platelets (Ferrell and Martin, 1988), although the degree of phosphorylation of individual proteins differs between the two agonists (Fig. 1b, lane 2).

Cytoskeletal Localization of pp60^{c-src} in vWF-stimulated Platelets—Previous studies have implicated the src family of tyrosine kinases in the agonist-stimulated phosphorylation of platelet proteins (Ferrell and Martin, 1988; Golden and Brugge, 1989). Approximately 40% of total platelet pp60^{c-src} redistributes to the 15,000 x g cytoskeletal fraction of thrombin-stimulated platelets (Hovarth et al., 1992). This cytoskeletal localization is associated with a transient increase in the enzyme activity of pp60^{c-src} (Clark and Brugge, 1993). We investigated whether vWF-induced platelet aggregation is associated with the cytoskeletal translocation of pp60^{c-src}. pp60^{c-src} was not detectable in the resting platelet cytoskeleton by immunoblot analysis (Fig. 2a, lane 1); however, the cytoskeletal fraction of both vWF and thrombin-stimulated platelets contained comparable quantities of pp60^{c-src} (Fig. 2a, lanes 3 and 4). The vWF-stimulated cytoskeletal association of pp60^{c-src} required platelet stirring and aggregation (Fig. 2a, lane 2) but was not dependent on ligand binding to GPIb/IIIa, as 2 mM RGDG had no inhibitory effect on pp60^{c-src} translocation (Fig. 2a, lane 5). In control experiments, ristocetin alone (1 mg/ml) did not stimulate the cytoskeletal association of pp60^{c-src} (Fig. 2b, lane 2). Furthermore, the exposure of platelets to bovine plasma (which induces platelet aggregation in the absence of ristocetin) resulted in a similar increase in the cytoskeletal content of pp60^{c-src} (Fig. 2b, lane 4), as observed with purified vWF and ristocetin (Fig. 2b, lane 3).

In further studies, we examined the enzyme activity of cytoskeletal-associated pp60^{c-src} by performing in vitro kinase assays on pp60^{c-src} immunoprecipitates. No detectable autophosphorylated pp60^{c-src} was observed in the cytoskeleton of resting platelets; however, cytoskeletal extracts from vWF- or thrombin-stimulated platelets contained comparable quantities of autophosphorylated pp60^{c-src} (data not shown). These studies indicate that vWF-induced platelet aggregation is associated with the cytoskeletal localization of enzymatically active pp60^{c-src}.

**PtdIns 3-Kinase Translocates to the Cytoskeletal Fraction of vWF-activated Platelets—**PtdIns 3-kinase has previously been shown to associate with non-receptor tyrosine kinases and to translocate from the cytosol to the membrane cytoskeleton, following thrombin stimulation of platelets (Gutkind et al., 1990; Zhang et al., 1992). To investigate whether vWF can also stimulate the cytoskeletal association of PtdIns 3-kinase, we measured PtdIns 3-kinase activity in diluted Triton X-100-soluble and -insoluble (cytoskeletal) extracts by measuring the phosphorylation of PtdIns(4,5)P2 as described under "Experimental Procedures." We demonstrated the formation of PtdIns(3,4,5)P3 using 1/200 or 1/1000 dilutions of Triton X-100-soluble extract (Fig. 3a). The authenticity of the 32P-labeled phospholipids was established by HPLC analysis of the deacylated and deglycosylated products (data not shown). At a 1/200 dilution of Triton X-100 extract, greater than 50% of the generated PtdIns(3,4,5)P3 was subsequently dephosphorylated to a less polar lipid. This phospholipid comigrated with a commercial PtdIns(3,4,5)P3 standard on thin layer chromatography and was identified as PtdIns(3,4,5)P3 by HPLC analysis (data not shown). The generation of PtdIns(3,4,5)P3 suggests the presence of a PtdIns(3,4,5)P3 5-phosphomonoesterase in the Triton X-100-soluble extract, as described previously (Stephens et al., 1991; Susa et al., 1992). The quantitation of PtdIns 3-kinase activity was therefore determined on platelet extracts diluted 1000-fold. Under these conditions significantly less breakdown of PtdIns(3,4,5)P3 was observed (Fig. 3a). Both thrombin and vWF stimulation of platelets resulted in a 40–45% decrease in PtdIns 3-kinase enzyme activity in the Triton X-100-soluble fraction (Fig. 3, a and b) (14.9 ± 2.6 [32P]PtdIns(3,4,5)P3 cpm x 10^4 (n = 4) in resting platelets versus 8.5 ± 1.3 and 8.4 ± 1.2 [32P]PtdIns(3,4,5)P3 cpm x 10^4 (n = 4) in thrombin and vWF-stimulated platelets, respectively). This decrease in Triton X-100-soluble activity was associated with a 4.5–5-fold increase in cytoskeletal enzyme activity (Fig. 3, c and d) (1.5 ± 0.3 [32P]PtdIns(3,4,5)P3 cpm x 10^4 (n = 3) in resting platelets versus 7.4 ± 1.2 and 7.9 ± 0.9 [32P]PtdIns(3,4,5)P3 cpm x 10^4 (n = 3) in thrombin and vWF-stimulated platelets, respectively).

**Detection of the pp85 Subunit of PtdIns 3-Kinase in the Cytoskeletal Fraction of vWF-stimulated Platelets—**Immunodetection of the pp85 subunit of PtdIns 3-kinase has previously been demonstrated to correlate closely with thrombin and vWF-stimulated platelets using an anti-p85 polyclonal antibody confirmed the translocation of PtdIns 3-kinase (Fig. 4a, lanes 3 and 4). 

**TABLE I
Specific activity of PtdIns 3-kinase in platelet subcellular fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity (cpm x 10^4/pp85 unit)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 soluble</td>
<td>24.4 ± 3.5 (n = 4)</td>
<td>122.5 ± 8.2 (n = 4)</td>
</tr>
<tr>
<td>15,000 x g cytoskeleton</td>
<td>26.9 ± 3.8 (n = 3)</td>
<td>149.1 ± 9.4 (n = 3)</td>
</tr>
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![Fig. 4. Translocation of the pp85 subunit of PtdIns 3-kinase to the cytoskeletal fraction of vWF-stimulated platelets. a, washed platelets were exposed to control buffer (lane 1); vWF (10 μg/ml) and ristocetin (1 mg/ml) without stirring (lane 2); thrombin (1 unit/ml) (lane 3); vWF and ristocetin (lane 4); vWF and ristocetin in the presence of RGDS (2 mM) (lane 5); or b, control buffer (lane 1); ristocetin alone (1 mg/ml) (lane 2); vWF and ristocetin (lane 3) or bovine plasma (diluted 1:4) (lane 4). Each reaction mixture was stirred for 5 min (except where otherwise indicated) at room temperature. Platelet cytoskeletons were extracted and Western blots were performed using anti-p85 polyclonal antibodies as described under "Experimental Procedures."](image-url)
Adhesion Receptor Activation of PtdIns 3-Kinase

The Effect of Acetylsalicylic Acid on vWF-stimulated Cytoskeletal Translocation and Activation of PtdIns 3-Kinase and pp60^c-scr—Acetylsalicylic acid (ASA) is known to inhibit platelet aggregation. However, its effect on the cytoskeletal localization of PtdIns 3-kinase and pp60^c-scr has not been thoroughly investigated.

**Figure 5.** Cytoskeletal association of PtdIns 3-kinase and pp60^c-scr in platelets deficient in GPIb/IX or platelets pretreated with acetylsalicylic acid, EDTA, or anti-GPIb mAb. Normal washed platelets were incubated with control buffer, acetylsalicylic acid (1 mM), EDTA (5 mM), or anti-GPIb mAb AK2 (5 ng/ml) prior to stimulation with vWF (10 μg/ml) and ristocetin (1 mg/ml). Platelets were lysed and separated into Triton X-100-soluble and -insoluble (cytoskeletal) fractions. Immunoblots were performed on cytoskeletal extracts using antibodies against PtdIns 3-kinase and pp60^c-scr. The figure shows the relative band intensities of PtdIns 3-kinase and pp60^c-scr in various conditions.

**Legend:**
- Lane 1: Unstimulated platelets
- Lane 2: vWF and ristocetin stimulation of platelets
- Lane 3: vWF and ristocetin stimulation of platelets in the presence of anti-GPIb mAb AK2

**Results:**
- In control conditions, both PtdIns 3-kinase and pp60^c-scr are present in the cytoskeletal fraction of vWF-activated platelets.
- Pretreatment with acetylsalicylic acid or EDTA reduces the cytoskeletal association of PtdIns 3-kinase and pp60^c-scr.
- Anti-GPIb mAb AK2 blocks the cytoskeletal association of PtdIns 3-kinase and pp60^c-scr.

**Conclusion:**
Acetylsalicylic acid and EDTA decrease the cytoskeletal localization of PtdIns 3-kinase and pp60^c-scr, which suggests that these agents may interfere with the cytoskeletal signaling pathways activated by vWF.

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*Note: The diagram and table are not included in the text.*
in agonist-stimulated activation of pp60^c-src and PtdIns 3-kinase (Sultan et al., 1991; Horvath et al., 1991; Oda et al., 1992). While a subset of integrins bind adhesive proteins via specific RGDs consensus sequences, other integrins, including GP Ia/IIa, bind collagen in an RGDs-independent manner (Hyemen, 1992). We have shown that RGDs does not have a inhibitory effect on vWF-induced cytoskeletal association of PtdIns 3-kinase and pp60^c-src. To exclude the possibility that other integrin adhesive receptors may be required for vWF-stimulated translocation of PtdIns 3-kinase and pp60^c-src, we incubated platelets with 5 mM EDTA for 90 min, at 37 °C, during the platelet washing process, and performed all platelet activation studies in the presence of 5 mM EDTA, as described under "Experimental Procedures." These conditions disrupt the ligand binding capacity of all integrins but do not affect the ability of vWF to bind GPIb/IX and stimulate platelet aggregation (Smyth et al., 1993). vWF stimulation of EDTA-treated platelets resulted in the cytoskeletal association of both pp60^c-src (Fig. 5a, lane 4) and PtdIns 3-kinase (Fig. 5b, lane 4). Furthermore, activation of cytoskeletal PtdIns 3-kinase was unaffected by EDTA pretreatment of platelets (specific activity of 138 ± 15.7 [32P]PtdIns(3,4,5)P3 cpm x 10^6/p85 unit (n = 2) versus 149.1 ± 9.4 [32P]PtdIns(3,4,5)P3 cpm x 10^6/p85 unit (n = 3) for untreated platelets). However, pretreatment of platelets with the anti-GPIb/IX mAb AK2 (Fig. 5a, lane 5) or stimulation of platelets deficient in the GPIb/IX complex (Bernard-Soulier syndrome) (Fig. 5d, lane 3) by vWF, markedly reduced the cytoskeletal localization of pp60^c-src. Similar results were also obtained with PtdIns 3-kinase (see Fig. 5b, lane 5 and Fig. 5c, lane 5). These results indicate that vWF binding to GPIb/IX is necessary and sufficient for the cytoskeletal translocation of activated PtdIns 3-kinase and pp60^c-src.

Comparison of pp60^c-src Cytoskeletal Translocation in Platelets Activated by vWF, Thrombin, Collagen, or ADP/Adrenaline—In addition to thrombin and vWF, we observed that collagen and ADP/adrenaline stimulation of platelets results in the cytoskeletal translocation of pp60^c-src (Fig. 6a). These results raise the possibility that pp60^c-src translocation represents a common intracellular signaling event associated with platelet aggregation. Consistent with this hypothesis, we found that EDTA treatment of platelets completely abolished platelet aggregation and the cytoskeletal translocation of pp60^c-src by thrombin, collagen, and ADP/adrenaline (Fig. 6b, lanes 2, 4, and 5). Collectively, these results suggest that platelet aggregation and the cytoskeletal translocation of pp60^c-src and PtdIns 3-kinase are closely linked phenomena. Furthermore, our studies with vWF indicate that both pp60^c-src and PtdIns 3-kinase are able to associate with the cytoskeleton under conditions in which ligand binding to platelet integrins is abolished.

**DISCUSSION**

There are five major receptor families which mediate cell adhesion: (i) cadherins, (ii) members of the immunoglobulin superfamily, (iii) selectins, (iv) integrins, and (v) the leucine-rich glycopolypeptide family of which the receptor for vWF, GPIb/IX, is a member. There is increasing evidence that the transfer of information by adhesion receptors involves the activation of tyrosine kinases (Juliano and Haskill, 1993). These observations have been well demonstrated with the platelet integrin GPIb/IIa, which regulates the tyrosine phosphorylation of platelet proteins following platelet activation (Shattil and Brugge, 1991). Studies reported in this paper demonstrate that a member of the leucine-rich glycopolypeptide family can also mediate the cytoskeletal association of pp60^c-src and stimulate tyrosine phosphorylation of platelet proteins. In addition, this adhesion receptor can induce the activation and cytoskeletal translocation of PtdIns 3-kinase. These signaling events mediated by vWF have the following characteristics: (i) they are directly induced by vWF binding to GPIb/IX and do not require the presence of other platelet agonists such as thrombin; (ii) they occur independent of ligand (i.e. fibrinogen) binding to GPIb/IIa; (iii) they are not affected by conditions which abrogate other known platelet activating pathways, including phosphatidylinositol turnover and prostaglandin metabolism; (iv) they require platelet stirring and aggregation and are therefore likely to represent a post-aggregation event; (v) they do not require secretion of other potential activators of PtdIns 3-kinase, such as platelet-derived growth factor.

We have shown that vWF and thrombin induce the cytoskeletal translocation of pp60^c-src and PtdIns 3-kinase to a similar extent. Furthermore, both agonists stimulate a comparable increase in the specific activity of cytoskeletal PtdIns 3-kinase compared with the cytosolic form, suggesting that the enzyme is not only recruited to the cytoskeleton but is also activated. These studies are surprising in that other platelet responses including dense granule release, thromboxane A_2 production, activation of protein kinase C, and the mobilization of intracellular calcium are stimulated to a much greater extent by thrombin than vWF (Kroll et al., 1991). These observations are consistent with the hypothesis that vWF-mediated activation and translocation of PtdIns 3-kinase and pp60^c-src are dependent on the cytoskeletal signaling pathways.

A recent report (Pumiglia and Feinstein, 1993) which examined the ability of thrombin or ristocetin and cryoprecipitate to induce the cytoskeletal association of pp60^c-src was unable to demonstrate cytoskeletal enzyme translocation when cryoprecipitate and ristocetin were used as the platelet agonists. An explanation for this apparent discrepancy with our results is not immediately obvious. However, our studies were performed using fresh platelets in a purified component assay, whereas Pumiglia and Feinstein used platelets which were up to 24 h old and were activated by ristocetin in the presence of cryoprecipitate. It is not clear what concentration of vWF was used in this study and whether the aggregation process was GPIb/IX-dependent.

Fibrinogen binding to GPIb/IIa appears to play an important role in the ability of several platelet agonists to stimulate the cytoskeletal association of pp60^c-src and for the production of 3-phosphorylated phosphoinositides in thrombin-stimulated platelets (Oda et al., 1992; Horvath et al., 1992; Sultan et al., 1991). vWF-stimulated cytoskeletal translocation of both pp60^c-src and PtdIns 3-kinase appears to occur independently of fibrinogen binding to GPIb/IIa and is paradoxically increased by RGDS or EDTA pretreatment of platelets. An obvious explanation for this increase is not immediately apparent. The ability of RGDS and EDTA to block vWF binding to GPIb/IIa may indirectly enhance vWF binding to GPIb/IX and thereby stimu-

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late increased enzyme translocation. An alternative and more interesting possibility is that some form of signaling cross-talk may exist between integrins and GPIb/IX, such that ligand binding to integrins negatively regulates the cytoskeletal translocation of signaling enzymes in vWF-stimulated platelets.

The mechanism by which vWF activates PtdIns 3-kinase remains to be established. Various adhesion receptors, including members of the cadherin, integrin, and leucine-rich glycoprotein gene families, associate with cytoskeletal proteins (Hynes, 1992; Takeichi, 1991; Roth, 1991). The association of GPIb/IX with the cytoskeleton, through its attachment to actin-binding protein, may be essential for vWF-induced signaling. The cytoplasmic domain of GPIb/IX does not share any of the characteristics of other signal generating receptors, such as kinase or phosphatase domains, or sequences for interacting with G-proteins (Roth, 1991). However other members of the adhesion receptor family, as exemplified by the integrins, also lack such features and are capable of transducing similar signaling events (Hynes, 1992). We have not been able to demonstrate complex formation between immunoprecipitated GPIb/IX and PtdIns 3-kinase. It is possible that the activation and translocation of PtdIns 3-kinase is mediated indirectly by p60<sup>src</sup> or other non-receptor tyrosine kinases. According to this model, the cytoskeletal localization of p60<sup>src</sup> results in the phosphorylation of cytoskeletal-associated proteins (Fox et al., 1993) as a necessary step for PtdIns 3-kinase recruitment.

The formation of cytoskeletal signaling complexes may then serve to localize activated PtdIns 3-kinase in close proximity to its relevant lipid substrates (Zhang et al., 1992).

The binding of subendothelial vWF to GPIb/IX anchors platelets to sites of vessel wall damage. Once anchored, platelets undergo major cytoskeletal reorganization, leading to shape change, spreading, and adhesion with other activated platelets, to form a stable hemostatic plug (George and Shattil, 1991). Our results raise the possibility that the initial adhesion process results in the stimulation of both tyrosine and lipid kinase(s). This adhesion-induced activation of PtdIns 3-kinase may stimulate the production of 3-phosphorylated phosphoinositides as a necessary step for cytoskeletal reorganization (Kucera and Rittenhouse, 1990; Eberle et al., 1990; Wynn and Arcaro, 1994). While the functional significance of our findings remains to be established, they nevertheless indicate a role for adhesion receptors in the activation of PtdIns 3-kinase and extend the potential signaling role of this lipid kinase to include adhesion-related cellular events.

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