Thrombin-Induced GPIb-IX Centralization on the Platelet Surface Requires Actin Assembly and Myosin II Activation

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In resting platelets, the GPIb-IX complex, the receptor for the von Willebrand factor (vWF), is linked to underlying actin filaments by actin-binding protein (ABP-280). Thrombin stimulation of human platelets leads to a decrease in the surface expression of the GPIb-IX complex, which is redistributed from the platelet surface into the open canalicular system (OCS). Because the centralization of GPIb-IX is inhibited by cytochalasin, it is believed to be linked to actin cytoskeletal rearrangements that take place during platelet activation. We have further characterized the mechanism of GPIb-IX centralization in platelets in suspension. Following thrombin stimulation, GPIb-IX shifts from the membrane skeleton of the resting cell to the cytoskeleton of the activated cell in a reaction sensitive to cytochalasin B. The cytoskeletal association of GPIb-IX involves ABP-280, as it correlates with the incorporation of ABP-280 into the activated cytoskeleton and because no dissociation of the ABP-280/GPIb-IX complexes is detected after thrombin activation. However, the incorporation of GPIb-IX into the cytoskeleton is complete within 1 minute, whereas GPIb-IX centralization requires 5 to 10 minutes for completion. The movement of GPIb-IX to the cytoskeleton of activated platelets is therefore necessary, but not sufficient for GPIb-IX centralization.

Once GPIb-IX has been aggregated into the center of the cell, it goes into the OCS and becomes inaccessible to surface labeling with antibodies. GPIb-IX centralization and removal from the cell surface is also induced by plasmin, adenosine diphosphate (ADP), or collagen in whole blood, and by phorbol esters. In contrast to rapid platelet responses, such as actin polymerization or secretion, which are maximally achieved within 1 minute of cell activation, the kinetics of GPIb-IX centralization are distinctly slow, starting 1 minute after activation and becoming maximal after 5 to 10 minutes. The molecular mechanism of this redistribution has not been established, but rearrangements of the actin cytoskeleton dependent on actin assembly following thrombin activation are necessary. The physiological relevance of the changes in GPIb-IX surface expression has not been established.

In the resting platelet, GPIb-IX complexes are linked to actin filaments by actin-binding protein (ABP-280) in an interaction between the cytoplasmic tail of GPIbα and the carboxyl terminus of ABP-280. ABP-280 is a component of both the membrane skeleton, a submembranous structure that coats the cytoplasmic surface of platelets, and the underlying actin cytoskeleton. The membrane skeleton is a planar lattice of spectrin, actin, and integral membrane proteins, such as GPIb-IX, which is thought to help maintain the resting platelet in a discoid shape.

The role of the actin cytoskeleton in mediating platelet shape changes has been well documented (for a review, see ). Platelet activation in response to agonists is accompanied morphologically by the rapid deployment of lamellipods and filopods filled with actin filaments. About 40% of the total actin in resting platelets is filamentous and activation by agonists leads, within 30 to 60 seconds, to a doubling of the platelet content of F-actin. Within 1 minute, the contractile protein myosin II is phosphorylated by myosin light chain kinases (MLCK) and inhibited by wortmannin. Wortmannin prevents myosin II association with the activated cytoskeleton and inhibits GPIb-IX centralization by 50%, without affecting actin assembly or the association of GPIb-IX to the cytoskeleton. Only micromolar concentrations of wortmannin, high enough to inhibit MLCK, prevent GPIb-IX centralization. These results indicate that thrombin-induced GPIb-IX centralization requires a minimum of two steps, one associating GPIb-IX to the activated cytoskeleton and the second requiring myosin II activation. The involvement of myosin II implies that GPIb-IX/ABP-280 complexes, linked to actin filaments, are pulled into the center, and that platelets may exert contractile tension on vWF bound to its receptor.

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kinase (MLCK) in a calcium-calmodulin–dependent pathway, associates to F-actin, and is capable of generating force. 26 Myosin activity is required for granule centralization and for platelet retraction. 27,28 Some of the molecular mechanisms leading to actin assembly in platelets and their relation to platelet shape changes have recently been characterized. 29,30 The nucleation sites on which actin assembly takes place are filament ends generated by two mechanisms: (1) a calcium-independent uncapping of preexisting filament ends, which results in the formation of filopods, and (2) a calcium-dependent fragmentation of long actin filaments into short ones, which occur under uncapping results in the protrusion of lamellipods.

We have now analyzed the mechanism of thrombin-induced GPIb-IX centralization and its relationship to the actin cytoskeleton. We report that GPIb-IX centralization involves two steps, (1) the association of the GPIb-IX/ABP-280 complexes to the activated cytoskeleton, and (2) the activation of myosin II and its incorporation into the activated cytoskeleton.

MATERIALS AND METHODS

Reagents. 32P-orthophosphoric acid, Renaissance chemiluminescence reagents and autoradiography film were purchased from New England Nuclear Corp, Boston, MA; DNase I from Boehringer Mammheim Corp, Indianapolis, IN; leupeptin from Vela Biotechnological Inc, Tucson, AR; benzamidin, aprotinin, phenylmethylsulfoxyl fluoride (PMSF), human thrombin, prostaglandin El (PGE,), Quin-2 AM, cytochalasin B, wortmannin, phallacidin, EGTA, Triton X-100, bovine serum albumin (BSA), and miscellaneous chemical reagents from Sigma Chemical Corp, St Louis, MO; S22, a mouse monoclonal antibody to glycoplasticin (GPIba), from Amac Inc, Westbrook, ME; fluorescein isothiocyanate (FITC)-labeled F(ab'), fragments of goat antimouse IgG from Tago Immunologicals, BioSource International, Camarillo, CA; horseradish peroxidase-labeled goat antimouse antibody from Kierkegaard and Perry, Gaithersburg, MD; Protein A Sepharose CL-4B, CNBr-activated Sepharose 4B and Sepharose 2B from Pharmacia-LKB, Piscitaway, NJ; A rabbit antibody to glycoplasticin (GPIsbs) was kindly provided by Dr. E.B. Fox, Children’s Hospital Oakland Research Institute, Oakland, CA; Monoclonal antibody (MoAb5) and MoAb1, mouse monoclonal antibodies against human, ABP-280, have been previously characterized. 30,31

Isolation and activation of platelets. Human platelets were purified as described previously, 21 with a few modifications. Briefly, human blood was drawn from volunteers, who had not ingested aspirin for at least 10 days, into 0.1% volume of Aster-Jandl anticoagulant and centrifuged at 200g for 15 minutes. A total of 1 ml of platelet PGE, was added to platelet rich plasma and the plasma was gel-filtered over a Sepharose 2B column equilibrated with platelet buffer (10 mmol/L Hepes, 145 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L MgCl2, 0.5 mmol/L NaH2PO4, 10 mmol/L glucose, 0.3% BSA, pH 7.4) at 37°C. Gel-filurated platelets were allowed to rest for 60 minutes at 37°C before use. The platelet concentration, determined on a Coulter counter (Coulter Corp, Dade County, FL), was typically 2.5 to 3.0 x 1010/ml. Immediately before testing, EGTA was added to a final concentration of 2.5 mmol/L. Platelets were activated by the addition of 1 unit of thrombin/mL. In experiments using metabolic inhibitors, gel purified platelets were preincubated at 37°C with 40 mmol/L Quin-2 AM for 25 minutes, with 10 mmol/L wortmannin for 15 minutes, or with 10 mmol/L cytochalasin B for 2 minutes. These inhibitors were added to platelets from stock solutions in dimethylsulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1% in all experiments. Controls including DMSO carrier alone were included.

Flow cytometry studies. Platelets (2 x 109/mL) were fixed by the addition of 1 volume of 4% paraformaldehyde in platelet buffer for 30 minutes at 37°C and washed twice with Heps buffer (5 mmol/L Hepes, 140 mmol/L NaCl, pH 7.4) for 5 minutes. They were incubated for 1 hour at room temperature with S22, an antigglobulin antibody, at saturating conditions in Heps buffer containing 0.3% BSA. Platelets were washed twice, incubated for 45 minutes at room temperature with FITC-labeled F(ab'),2 fragments of goat antimouse IgG at the dilutions recommended by the manufacturer, washed twice, and their fluorescence read in a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The samples were gated for platelets, and aggregates were excluded based on forward- and side-scatter profiles. A total of 10,000 events were recorded and the results were analyzed using Lysis II software (Becton Dickinson).

Isolation and analysis of platelet cytoskeletons. Low speed (15,600g) and high speed (100,000g) pellets of detergent-lysed platelets, corresponding to the cytoplasmic cytoskeleton and to the membrane skeleton respectively, were obtained according to published methods, 22 with a few modifications. Platelets were lysed by adding an equal volume of lysis buffer containing 2% Triton X-100, 100 mmol/L Tris HCl, 20 mmol/L EGTA, 5 mmol/L phallacin, pH 7.4, supplemented with 2 mmol/L PMSF and 100 μg/mL each of leupeptin, aprotinin, and benzamidin. All subsequent steps were performed at 4°C. After 5 minutes, the lysates were spun at 15,600g for 5 minutes at 4°C in an Eppendorf Microfuge. The resulting low speed pellets were washed once in 100 mmol/L Tris, 20 mmol/L EGTA, 1% Triton X-100, 5 mmol/L phallacin, 1 mmol/L PMSF, pH 7.4, and twice in Tris-buffered saline (TBS; 10 mmol/L Tris HCl, 140 mmol/L NaCl, pH 7.4), solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer in the presence of β-mercaptoethanol, and boiled for 5 minutes. The supernatant from the first 15,600g centrifugation was spun at 100,000g for 2.5 hours at 4°C in a Beckman ultracentrifuge. The resulting high speed pellets were washed in similar fashion to the slow speed pellets and solubilized in SDS-PAGE buffer. The soluble phase of the 100,000g centrifugation (platelet supernatant) was boiled in SDS-PAGE buffer. The Triton-insoluble and -soluble fractions from an equivalent number of platelets were analyzed by SDS-PAGE on 5% to 15% polyacrylamide gradient gels. For immunoblot analysis, proteins were transferred to Immobilon P membranes (Millipore Corp, Bedford, MA) according to the method of Towbin et al. 32 Because GPIbα stains poorly with Coomasie Blue on SDS-PAGE, 32 it was detected by immunoblotting using S22, a mouse antigglobulin MoAb, which recognizes the GPIbα chain, at a 1:2000 dilution. ABP-280 was visualized using MoAb1 antibody. 33 Primary antibody binding was visualized with a horseradish peroxidase-labeled goat antimouse antibody at a 1:4000 dilution. The immunoblots were developed according to the enhanced chemiluminescence method. The intensities of the bands of the SDS-PAGE and the Western blots were analyzed by densitometry after digitizing the gel using a flat-bed scanner and analyzing the images within the linear range using the NIH Image program.

Immunoprecipitation of GPIbα. Platelets were lysed by adding an equal volume of lysis buffer, containing 2% Triton X-100, 100 mmol/L Tris HCl, 20 mmol/L EGTA, 5 mmol/L phallacin, pH 7.4, supplemented with 2 mmol/L PMSF and 100 μg/mL each of leupeptin, aprotinin, and benzamidin and 2 mg/mL DNase I, to depolymerize cytoskeletal actin filaments, as described by Fox. 34 After incubation for 30 minutes at 4°C, the lysate was centrifuged for 15 minutes at 100,000g. Clarified lysates were incubated for 2 hours at 4°C with Sepharose CL4B beads coupled to MoAb5 antibody, a mouse anti–ABP-280 MoAb, 35 or with rabbit antigglobulin anti-
body and 20 μL protein A Sepharose. Controls included Sepharose CL-4B beads coupled to mouse IgG. The beads were washed three times with TBS for 1 minute at an 15,600g at 4°C in an Eppendorf Microfuge and resuspended in SDS-PAGE buffer. The samples were analyzed by SDS-PAGE and immunoblotting, as described.

Platelet phosphorylation. Platelets isolated by two cycles of centrifugation at 800xg in the presence of 1 μmol/L PGE, were resuspended in platelet buffer at a concentration of 5 × 10^6/mL and incubated for 1 hour at 37°C with 1 μCi/mL of 32P orthophosphoric acid. Unbound 32P was separated from platelets by gel filtration in the presence of 1 μmol/L PGE, over a Sepharose 2B column. The platelets were allowed to rest for 1 hour at 37°C before use. The platelets were solubilized in SDS-PAGE buffer and analyzed by SDS-PAGE on a 13% polyacrylamide gel. After drying, the gels were analyzed by autoradiography on a Renaissance film.

Immunoelectron microscopy studies. GPIb-IX on the surface of resting and activated platelets (thrombin, 1 U/mL) was labeled. Suspended cells were fixed with 1% gluteraldehyde in platelet buffer lacking added albumin for 10 minutes at 37°C. Fixed cells were attached to glass coverslips by diluting them to a concentration of 1.8 nm colloidal gold particles coated with affinity purified antibody. After attachment of the cells, unreacted aldehydes were blocked with a 1-minute wash of a solution composed of 1 mg/mL of sodium borohydride and platelet buffer. Blocking solution was removed and the cells washed into phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA). The PBS/BSA wash solution was replaced with 25 μL of 10 μg/mL of affinity purified rabbit antitrypsin-nectin in PBS/BSA for 60 minutes. This solution was removed, the coverslips washed three times with PBS/BSA, and then incubated with 10 nm colloidal gold particles coated with affinity purified goat antirabbit IgG for 90 minutes. Unbound gold was removed by washing in PBS after which the IgG-gold complexes were fixed to the cell surface using 1% glutaraldehyde in PBS. The coverslips were washed in distilled water, rapidly frozen, freeze-dried, and rotary coated with 1.8 nm of platinum and 2.5 nm of carbon without rotation in a Cressington CFE-50 apparatus (Cressington, Watford, UK).

RESULTS

GPIb-IX/ABP-280 complexes bound to actin move from the membrane skeleton into the cytoskeleton after thrombin stimulation. In response to agonist-stimulation, platelets form an activated cytoskeleton, which results from the assembly of soluble actin monomers into new filaments, and incorporation of the actin cross-linking proteins ABP-280, α-actinin, and the contractile protein myosin II.4,5.6.7 The formation of this actin cytoskeleton has been extensively studied by fractionating cells in the presence of the detergent Triton X-100 into three components: a 15,000g pellet (thereafter called the cytoskeleton); a 100,000g pellet (the membrane skeleton); and a 100,000g supernatant (soluble protein fraction).7 Because GPIb-IX is linked to ABP-280 in resting platelets,4,5 we determined whether GPIb-IX remains with ABP after activation and associates with the activated cytoskeleton. Platelets were lysed with Triton X-100 in the presence of 5 μmol/L phallacidin to stabilize existing actin filaments.4 As shown in Fig 1, the cytoskeleton of resting platelets was composed predominately of actin filaments and of small amounts of ABP-280, α-actinin, and GPIbα. In agreement with previous findings,4.5 the majority of platelet

ABP-280, α-actinin, and GPIbα were bound to actin filaments present in the membrane skeletal fraction. Myosin II was also found predominately in the membrane skeletal fraction. Thrombin stimulation resulted in a marked increase in the amount of F-actin in the cytoskeletal fraction and in a shift of ABP-280, α-actinin, and GPIbα from the membrane skeleton to the activated cytoskeleton. Densitometric analysis of the SDS-PAGE and immunoblots showed that >40% of these latter proteins became associated to the activated cytoskeleton as a result of thrombin activation. A large percentage of GPIb-IX remained associated with the membrane skeleton in activated platelets. Very little GPIbα was soluble in either resting or activated cells, indicating it is in constant association with actin filaments.

The incorporation of GPIb-IX into the activated cytoskeleton correlates with incorporation of ABP-280, suggesting that GPIb-IX and ABP-280 remain linked after thrombin activation. To confirm the stability of this association, we determined if the interaction between GPIb-IX and ABP-280 in resting platelets13,14,20,32 is altered by cell activation. GPIb-IX was immunoprecipitated from lysates of resting or activated platelets and the amount of coprecipitated ABP-280 was determined. All lysates were treated with DNAse I to depolymerize actin filaments and release the GPIb-IX/ABP-280 complex.32 Figure 2 shows that comparable amounts of ABP-280 coprecipitated with GPIbα in both resting and activated lysates. This was confirmed by densitometry analysis of the immunoblot, which showed the ratio of GPIbα to ABP-280 to be 2 to 1 in resting and activated cells. Similar results were obtained by immunoprecipitating ABP-280 and analyzing the amount of coprecipitated GPIb (data not shown). These results indicate no detectable dissociation of the GPIb-IX/ABP-280 complex during thrombin stimulation.

Actin assembly is necessary, but not sufficient, for GPIb-IX centralization. We also investigated whether the known inhibitory effect of cytochalasin B on the centralization of GPIb-IX in activated platelets correlates with the incorporation of GPIb into the activated cytoskeletal fraction (Fig 3). Treatment of platelets with cytochalasin inhibits assembly of new actin filaments, incorporation of actin with ABP-280 into the activated cytoskeleton,37 and the surface redistribution of GPIb-IX.4,6,9,10 Figure 3 shows that 10 μmol/L cytochalasin B, which blocked GPIb-IX centralization as determined by flow cytometry (data not shown), prevented the incorporation of actin, ABP-280 and α-actinin into the cytoskeletal fraction. It did not, however, alter the movement of myosin II into the activated cytoskeleton, as previously shown by Carroll et al.37 Cytochalasin B treatment also decreased the incorporation of GPIb into the cytoskeletal fraction, indicating that thrombin-induced GPIb-IX centralization requires the addition of new actin filaments to the activated cytoskeleton and the association of GPIb-IX to the cytoskeleton.

Although actin assembly is required for GPIb-IX centralization in activated cells, the kinetics for actin assembly and GPIb-IX centralization differ. Thrombin-induced actin assembly and cell shape change are complete in 1 minute,38 at which time, GPIb-IX centralization is beginning.4.6 as
Thrombin stimulation results in the association of GPIb-IX to the activated cytoskeleton. Resting platelets or platelets activated for 10 minutes with 1 U/mL thrombin were lysed in 100 mmol/L Tris-HCl, 20 mmol/L EGTA, 1% Triton X-100, and 10 μmol/L phallacidin. The lysates were sequentially centrifuged at 15,600g and at 100,000g to yield the low-speed pellet (C), the high-speed pellet (membrane skeleton, M) and the resulting supernatant containing soluble proteins (S), which were analyzed by SDS-PAGE (A) and by immunoblotting using anti-GPIIb IgG (B). P designates total platelet lysate. This experiment is representative of four experiments.

Fig 1. Thrombin stimulation results in the association of GPIb-IX to the activated cytoskeleton. Resting platelets or platelets activated for 10 minutes with 1 U/mL thrombin were lysed in 100 mmol/L Tris-HCl, 20 mmol/L EGTA, 1% Triton X-100, and 10 μmol/L phallacidin. The lysates were sequentially centrifuged at 15,600g and at 100,000g to yield the low-speed pellet (C), the high-speed pellet (membrane skeleton, M) and the resulting supernatant containing soluble proteins (S), which were analyzed by SDS-PAGE (A) and by immunoblotting using anti-GPIIb IgG (B). P designates total platelet lysate. This experiment is representative of four experiments.

Fig 2. GPIb-IX and ABP-280 are not dissociated in thrombin activated cells. Resting platelets or platelets activated with 1 U/mL thrombin for 1 or 10 minutes were lysed in 100 mmol/L Tris-HCl, 20 mmol/L EGTA, 1% Triton X-100 containing 1 mg/mL DNAse I. GPIbα was immunoprecipitated using rabbit antihydrocalcin IgG and protein A-Sepharose bead conjugates. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted for GPIbα and ABP-280. The ratio of ABP-280 to GPIbα, as determined by densitometry, is shown above each lane. This immunoblot is representative of four experiments.

Fig 3. Cytochalasin B inhibits thrombin-induced GPIbα association to the activated cytoskeleton. The Triton-insoluble activated cytoskeleton (C) and membrane skeleton (M) fractions of platelets activated with 1 U/mL of thrombin in the presence or absence of 10 μmol/L cytochalasin B were analyzed by SDS-PAGE (A) and by immunoblot for GPIbα (B), as described in Materials and Methods. The results are representative of four experiments.
cells exposed to thrombin for 1 minute, at which time shape changes have taken place. When GPIb-IX receptors were labeled with anti-GPIb-IX immunogold at 1 minute, anti-GPIb-IX gold bound avidly to the cell center, but failed to label an ~0.5 μm zone at the cell margin (Fig 6A). Filopods and other surface protrusions also labeled poorly with anti-GPIb gold (Fig 6B). During subsequent minutes, GPIb-IX molecules underwent a progressive aggregation to the cell center. Figure 5B shows the altered surface of a representative cell activated in suspension, 10 minutes after treatment with thrombin. The lack of anti-GPIb immunogold labeling of the cell surface demonstrates that most of the GPIb-IX complex was removed from the surface, although a small number of gold particles remain in the cell body.

**GPIb-IX centralization is calcium-dependent.** To characterize further the mechanisms involved in GPIb-IX centralization, we investigated the role of intracellular calcium. A rapid rise in cytoplasmic calcium concentrations is one of the first responses induced by thrombin39-41 and remodels the resting cytoskeleton by activating actin filament severing proteins. Calcium transients also lead to the activation of myosin II, which is regulated by phosphorylation. Calcium/calmodulin complexes bind to and activate MLCK, which phosphorylates the myosin 20 kD light chain.23,25,26 To maintain resting intracellular calcium levels at nmol/L levels during exposure of cells to thrombin, platelets were incubated with 40 μmol/L Quin-2 AM for 30 minutes before exposure to agonist. This concentration and time of Quin-2 AM loading results in a final concentration of Quin 2 in platelets that is millimolar.13 As shown in Fig 7A, loading of platelets with Quin-2 AM diminished by 70% ± 20%, the ability of cells to centralize GPIb-IX after thrombin stimulation. Analysis of the activated cytoskeletons showed only a minor reduction in the incorporation of actin, ABP-280, and GPIbα, which therefore did not account for the reduction in GPIb-IX centralization, but showed a marked decrease of the movement of myosin II into the activated cytoskeletal fraction (Fig 7B).

Intracellular calcium chelation alters the morphology of activated cells, which fail to develop lamellipodial extensions and present short, abnormal filopods.21 Quin-2 AM loading not only prevented GPIb-IX centralization, but also changed the distribution of GPIb-IX on the platelet surface. Figure 5C shows the distribution of anti-GPIb immunogold on the surface of a representative Quin-2 AM loaded platelet, 10 minutes after exposure to agonist. GPIb-IX remained dispersed across the surface of the cell including the surface of the abnormal filopodia.

**GPIb-IX centralization requires myosin II association to the cytoskeleton.** To investigate further myosin II involvement of GPIb-IX centralization, we employed wortmannin, a fungal metabolite that irreversibly inhibits myosin light chain.42,43 By flow cytometry, treatment of platelets with 10 μmol/L wortmannin inhibited thrombin-induced GPIb-IX centralization by 52% ± 20% (Fig 7A). The effect of wortmannin treatment on the composition of the activated cytoskeletons was also determined (Fig 7B). Compared with platelets activated in the absence of inhibitor, wortmannin did not affect actin assembly or the association of actin, α-actinin, ABP-280, or GPIbα to the activated cytoskeletons. In contrast, wortmannin decreased by 90% the incorporation of myosin II into the activated cytoskeletons, suggesting that myosin II activation and its interaction with the cytoskeleton are required for GPIb-IX centralization. The inhibitory effect of wortmannin on GPIb-IX centralization was confirmed by immunoelectron microscopy. Figure 5D shows that 10 μmol/L wortmannin did not prevent the shape changes induced by thrombin; this result was expected, as wortmannin does not
Fig 5. Location of GPIb-IX on the surface of resting cells and cells activated with 1 U/mL of thrombin for 10 minutes. The location of GPIb-IX complexes on the surface of fixed cells was determined using affinity purified rabbit anti-GPIbα and 10 nm gold particles coated with goat antirabbit IgG. (a) Distribution of anti-GPIbα immunogold particles on the surface of a representative resting platelet. There are many examples of linear aggregates of gold on the surface of this cell (arrowheads). (b) Localization of GPIb-IX on the surface of cell exposed to thrombin for 10 minutes. Gold labels the surface weakly, although some gold is present in the central portion of the cell. No gold label is associated with the surface of filopods. (c) Effect of Quin-2 loading on the distribution of the GPIb-IX receptors on the surface of a cell exposed to thrombin for 10 minutes after loading 40 μmol/L Quin-2 AM for 25 minutes before the addition of agonist. Gold label is abundant and covers all regions of the cell including the abnormal filopodia formed by these cells. (d) Effect of 10 μmol/L wortmannin on cell activation and GPIb-IX distribution. Cells were incubated with wortmannin for 15 minutes before thrombin treatment. Cells were fixed after exposure to thrombin for 10 minutes. Cells treated with wortmannin are able to extend normal filopods in suspension, but do not efficiently clear GPIb-IX from their surfaces. Immunogold is restricted to the cell body and is not found on the surfaces of filopods. The bars are 0.5 μm.
Fig 6. Distribution of GPIb-IX on the surface of cells fixed after 1-minute exposure to thrombin. Platelets were stimulated with 1 U/mL thrombin for 1 minute before fixation with 1% glutaraldehyde. The location of GPIb-IX complexes on the surface of fixed cells was determined using affinity-purified rabbit anti-GPIbα and 10 nm gold particles coated with goat antirabbit IgG. (A) Region of a cell that has extended a broad lamellipodia. Gold particles visualizing GPIb-IX are restricted from the outermost 0.5 μm band of the cell surface. Note that the gold particles have been aggregated into linear clusters (arrowheads) in the center of the cell. (B) Region of a cell that has extended a single filopod. GPIb-IX labeling is restricted to the surface of the filopod and its basal region. The bars are 0.1 μm.

affect the actin assembly reaction. Surface labeling with anti-GPIb IgG-gold complex showed gold dispersed evenly across the cell bodies of platelets. However, filopods on thrombin-stimulated cells in the presence of wortmannin did not stain for GPIb-IX.

Although micromolar wortmannin irreversibly inhibits MLCK, wortmannin inhibits phosphatidylinositol 3-kinase (PI 3-K) in the nanomolar range. To determine if the effect of wortmannin on GPIb-IX centralization results from an inhibition of PI 3-K and/or MLCK, we determined the effects of a wortmannin dose-response on thrombin-induced GPIb-IX centralization (Fig 8A) and the composition of activated cytoskeletons (Fig 8B). Wortmannin inhibited GPIb-IX centralization maximally at 10 μmol/L with a half-maximum of ~0.3 μmol/L, consistent with the IC₅₀ of 0.3 μmol/L for MLCK determined by Nakanashi et al. Analysis of activated cytoskeletons shows that myosin II incorporation was abrogated only at wortmannin doses >1 μmol/L. Taken together, these results suggest that the effect of wortmannin on GPIb-IX centralization is related to the inhibition of MLCK and not PI 3-K.

Wortmannin and Quin-2 AM inhibit myosin light chain phosphorylation. To further show that the inhibition of GPIb-IX centralization by Quin-2 AM or wortmannin is related to myosin II, we determined whether treatment with these agents prevented the phosphorylation of myosin light chains in response to thrombin (Fig 9). Platelets were analyzed 30 seconds following thrombin activation, corresponding to the maximal phosphorylation of pleckstrin and myosin light chains. Myosin light chain phosphorylation was totally blocked by wortmannin and mildly reduced by Quin-2 AM. The pattern of inhibition of myosin light chain phosphorylation in the presence of wortmannin and Quin-2 AM correlated with the levels of incorporation of myosin II into the cytoskeletal fractions (Fig 9). Pleckstrin phosphorylation was not affected by Quin-2 AM and was only slightly reduced by wortmannin when assayed 30 seconds after cell activation.

DISCUSSION

After stimulating platelets with thrombin, most groups have found a reduction in the surface expression of GPIb-IX receptors, present on the surface of the resting cells, which results from a redistribution to the OCS. There is, however, one published report to the contrary, and we do not yet understand the basis of the experimental differences that result in GPIb-IX remaining on the activated platelet surface in this study. In our work, GPIb-IX was removed from the cell surface as determined by flow cytometry and electron microscopy. We have now characterized some aspects of the mechanism of centralization of GPIb-IX, which requires not only actin assembly, but also myosin II activation and association with the cytoskeleton. This suggests that GPIb-IX redistribution is a contractile event and may be employed to exert contractile tension on the substratum during platelet retraction.

GPIb-IX is stably connected to actin filaments by ABP-280: Activation of cells shifts the GPIb-IX/ABP-280-actin complexes from the membrane skeleton to the newly formed activated cytoskeleton. Our results show first that the ma-
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Fig 7. GPIb-IX centralization is inhibited by Quin-2 AM and wortmannin. Platelets activated with thrombin after loading with 40 μmol/L Quin-2 AM or incubation with 10 μmol/L wortmannin were analyzed for GPIb-IX staining by flow cytometry (A) or by SDS-PAGE and immunoblotting for GPIbα of activated cytoskeletons (B and C). (A) Platelets were incubated with 40 μmol/L Quin-2 AM for 25 minutes or 10 μmol/L wortmannin for 15 minutes before exposure to thrombin. Platelets were then activated with 1 U/mL thrombin for 10 minutes, fixed with 2% paraformaldehyde, and surface content of GPIbα analyzed by flow cytometry. The data represent the mean ± SD of six experiments. (B and C) Low-speed pellets of resting platelets or activated cells preincubated with or without 40 μmol/L Quin-2 AM or 10 μmol/L wortmannin were isolated and analyzed by SDS-PAGE (B) and immunoblot for GPIbα (C). The results are representative of four experiments.

Fig 8. Concentration dependence of the inhibitory effect of wortmannin on thrombin-induced GPIb-IX centralization. Platelets preincubated with increasing doses of wortmannin and activated with 1 U/mL thrombin were analyzed for GPIb-IX staining by flow cytometry (A) or by SDS-PAGE of activated cytoskeletons (B). (A) Platelets were preincubated with increasing concentrations of wortmannin for 15 minutes before thrombin activation. Platelets were then activated with 1 U/mL thrombin for 10 minutes, fixed with 2% paraformaldehyde, and surface content of GPIbα analyzed by flow cytometry. The results are expressed as percent maximal inhibition of GPIb-IX redistribution and represent the mean ± SD of six experiments. (B) Low-speed pellets of resting platelets or activated cells under the same conditions were isolated and analyzed by SDS-PAGE (B). The results are representative of three experiments.

The majority of GPIb-IX is always connected to actin filaments. The platelet cytoskeleton has been further differentiated into two subfractions, a membrane skeleton and an actin-rich cytoskeleton, based on their differential sedimentation.19 In the resting platelet, GPIb-IX is found predominantly in the membrane skeleton. Following thrombin activation, a portion of the GPIb-IX/ABP-280 complex is shifted from the membrane skeleton to the activated cytoskeletons, in parallel with α-actinin and myosin II (Fig 1). In an initial report describing the association of GPIb-IX and ABP-280, Fox12 did not detect an increase in GPIbα in activated cytoskeletons.

However, in a subsequent report by Zhang et al.,20 GPIbβ, another component of the GPIb-IX complex, was incorporated into activated cytoskeletons in a cytochalasin B sensitive-fashion. The initial failure of Fox to detect GPIb-IX association to the activated cytoskeletons was probably due to the absence of phallacidin in the lysis buffer because phallacidin stabilizes actin filaments21 and prevents the disassembly of actin filaments in permeabilized platelets.18

Three lines of evidence demonstrate that the association between GPIb-IX and ABP-280 persists in activated cells and is necessary for GPIb-IX to interact with the cytoskeleton. First, the integrity of the GPIb-IX/ABP-280 complex persists after thrombin activation, as demonstrated by co-
precipitation experiments (Fig 3). Second, GPlb-IX association, or lack of association to activated cytoskeletons in cytochalasin B-treated cells, correlates with incorporation of ABP-280. Third, the absence of ABP-280 degradation products co-precipitating with GPIbα indicates that calpain cleavage of ABP-280 does not occur during GPlb-IX centralization. Calpain activation is unlikely to take place under the nonaggregating conditions used to follow GPlb-IX centralization, as it requires GPIIb-IIIa occupancy by fibrinogen.

The connection of GPlb-IX to actin is essential for its centralization. New actin filament assembly is also required, as cytochalasin B treatment prevents GPlb-IX centralization and the shift of GPIbα to the cytoskeletons. However, a second step in addition to actin assembly is necessary to centralize GPlb-IX on the platelet surface. While actin assembly and GPIbα association to the activated cytoskeletons was complete within a minute, GPlb-IX centralization reached a maximum only 5 to 10 minutes following activation, suggesting that additional processes are involved in the centralization. This second step is presumably also linked to cytoskeletal rearrangements because it only occurs after GPlb-IX becomes linked to the cytoskeleton.

Role of calcium metabolism in GPlb-IX centralization. The marked inhibition of GPlb-IX centralization by Quin 2 indicates that it is dependent on a transient increase in intracellular calcium. Processes mediated by calcium transient include myosin II activation and regulation of actin assembly, and our results suggest that both are required for optimal GPlb-IX centralization. The inhibition of myosin II activation in the presence of Quin-2 AM was demonstrated by a decrease in the extent of phosphorylation of myosin light chain and by a parallel reduction in the amount of myosin II incorporated into the activated cytoskeletal fraction.

Quin-2 AM treatment of platelets reduced not only the degree of GPlb-IX centralization, but also affected its surface distribution. GPlb-IX remained at the periphery of the Quin-2 loaded cells and was strikingly present on the membrane periphery and the surface of filopods, structures normally devoid of GPlb-IX staining after thrombin-stimulation. An increase in intracellular calcium results in actin filament severing, presumably by gelsolin, fragmenting long actin filaments into short ones. Because the interaction of GPlb-IX with actin filaments does not change during activation, GPlb-IX/ABP-280 complexes remain attached to the underlying actin filament fragments. It is these actin filament fragments which, when uncapped, become the templates onto which the soluble actin monomers assemble and generate membrane lamellipodia and filopodia. These filament fragments would remain at the base of the extension, because they are cross-linked to the cytoskeleton. Little GPlb-IX would be expected to be on the surface overlaying the new filament portions, as all the GPlb-IX is bound to the preexisting filament fragments and little is available to be bound along the sides of the new filament portions. This hypothesis is supported by the poor GPlb-IX staining on the newly formed extensions and suggests that actin assembly indirectly restricts GPlb-IX from the cell surface (Fig 10C). The altered pattern of GPlb-IX staining in the presence of Quin-2 AM shows that the calcium-dependent severing of submembranous actin filaments is necessary for the spatial restriction of GPlb-IX from the new areas of filament assembly during this first phase of GPlb-IX redistribution.

Role of myosin II in GPlb-IX centralization. An important role for myosin II activation in GPlb-IX centralization is suggested by inhibition experiments using Quin-2 AM and wortmannin. Both of these agents interfere with the activation of MLCK, which is required for myosin II stimulation. As mentioned previously, Quin 2 partially inhibited myosin light chain phosphorylation and myosin II’s association with activated cytoskeletons. Wortmannin, on the other hand, completely inhibited phosphorylation of the light chain of myosin II and incorporation of myosin II into the cytoskeletal fraction. Several lines of evidence suggest that the inhibitory effect of wortmannin on GPlb-IX centralization is related specifically to its inhibition of MLCK. First, although wortmannin is also a potent inhibitor of PI 3-K, participation of PI-3 K activity in GPlb-IX centralization was ruled out by the observation that concentrations of wortmannin that completely inhibit PI 3-K do not greatly affect GPlb-IX movements on the cell surface. An effect of wortmannin on protein kinase C, which is controversial, is also unlikely as pleckstrin phosphorylation was only slightly inhibited by wortmannin at these doses. Finally, wortmannin does not affect thrombin-induced actin assembly, which we
confirmed here by evaluating the composition of activated cytoskeletons and by its lack of effect on platelet shape change (Fig 5D).

On phosphorylation by MLCK, myosin II can bind actin filaments. It is likely that the shift of myosin II from the membrane skeleton to the activated cytoskeleton results from the binding of myosin II to actin filaments. This suggests that myosin II is linked to the membrane skeleton by an interaction with proteins other than actin filaments. Once activated, myosin II can exert contractile tension, which is thought to be required for platelet secretion and is necessary for clot retraction. Myosin II activation aggregates cytoplasmic granules preceding their fusion with the membranes of the OCS. Retraction of protrusions such as filopods occurs later in the activation process and requires actin assembly, myosin II activation, platelet-to-platelet contacts between fibrin polymers, and GPIb-IIIa receptors on the platelet surface. Myosin II filaments are believed to apply tension on actin bundles connected to GPIb-IIIa-fibrin complexes. GPIb-IX centralization also involves the association of GPIb-IX to actin filaments, requires myosin II activation, and has slow kinetics. The central aggregation of GPIb-IX, therefore, appears functionally similar to the platelet retraction mediated by GPIb-IIIa receptors.

Based on our morphological and functional data, we propose the following model for GPIb-IX centralization (Fig 10). In resting platelets, GPIb-IX is joined to underlying long actin filaments by ABP-280 (Fig 10A). Thrombin activation of cells leads initially to an increase in cytosolic calcium concentrations. Increased calcium activates gelsolin, which fragments the long cortical actin filaments (Fig 10B). The actin fragments subsequently become uncapped, allowing soluble actin monomers to assemble onto their barbed ends. This shifts soluble actin, stored in complexes with β4-thymosin, into the cytoskeleton and generates membrane expansions. The surfaces of these cellular extensions are depleted of GPIb-IX complexes because the GPIb-IX/ABP-280 complexes have remained attached to fragmented portions of the filaments that are restricted to the previous boundaries of the resting cell (Fig 10C). Once the activated cytoskeleton has been assembled, activated myosin II motors can contract actin filaments into the cell center. This process drags the actin filament-linked GPIb-IX/ABP-280 complexes into the cell center (Fig 10D).

Implications for the role of GPIb-IX centralization. The role of GPIb-IX centralization in platelet function has remained elusive. It was originally suggested that the decrease in surface expression of GPIb-IX following thrombin stimulation may transform platelets from a proadhesive to a proaggregatory state. However, our finding that GPIb-IX redistribution is a contractile process, analogous to that mediated by GPIb-IIIa, suggests that GPIb-IX could be also involved in linking the contractile tension generated by platelets to the substratum as they adhere and move on it. In support of this hypothesis, vWF linked to GPIb-IX is centralized along with GPIb-IX following thrombin-stimulation.
may cooperate with the GPIIb-IIIa receptor in this process. vWF is a polymeric protein that interacts with both GPIb-IX and activated GPIIb-IIIa receptors on the surface of human platelets and can, when bound to GPIb-IX, induce activation of GPIb-IIIa. Furthermore, vWF binds to fibrin, and, under high-shear stress conditions, contributes to the incorporation of platelets into clots (reviewed in ). Therefore, because vWF incorporated into clots binds potentially both GPIb-IX and GPIIb-IIIa, the generation of contractile tension between cells could involve both receptors.

ACKNOWLEDGMENT

We would like to thank Lance Taylor for help in preparing the platelets, Rebecca Winokur for help in with the electron micrograph pictures, Peter Lopez, and his collaborators at the Core Flow Cytometry Facility of the Dana Farber Cancer Institute (Boston, MA) for performing flow cytometry studies. We express our gratitude to Thomas P. Stossel for his continuous support, his stimulating ideas, and his critical reading of the manuscript.

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ACTOMYOSIN-MEDIATED GPII-IX MOVEMENT


Thrombin-induced GPIb-IX centralization on the platelet surface requires actin assembly and myosin II activation

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