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

By Tibor J. Kovacsics and John H. Hartwig

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. Blockage of cytosolic calcium increases induced by thrombin by loading with the cell permeant calcium chelator Quin-2 AM inhibited GPIb-IX centralization by 70%, but did not prevent its association with the activated cytoskeleton. Quin-2 loading did, however, decrease the incorporation of myosin II into the activated cytoskeleton. The role of myosin II was further probed using the myosin light chain kinase (MLCK) inhibitor wortmannin. Wortmannin prevents myosin II association to 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 cell 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. Myosin activity is required for granule centralization and for platelet retraction. Some of the molecular mechanisms leading to actin assembly in platelets and their relation to platelet shape changes have recently been characterized. 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, after 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 complex to the activated cytoskeleton, and (2) the activation of myosin II and its incorporation into the activated cytoskeleton.

MATERIALS AND METHODS

Reagents. P-orthophosphoric acid, Renaissance chemiluminescence reagents and autoradiography film were purchased from New England Nuclear Corp, Boston, MA; DNase I from Boehringer Mannheim Corp, Indianapolis, IN; leupeptin from VEGA Biotechnological Inc, Tucson, AR; benzamidine, aprotinin, phenylmethylsulfonyl fluoride (PMSF), human thrombin, protastaglandin E1 (PGE1), 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 glycoprotein IIb (GPIIb), from Amgen Inc, Westbrook, ME; fluoroescin isothiocyanate (FITC)-labeled Fab¿ fragments of goat antimag IGF from Tago Immunologicals, BioSource International, Camarillo, CA; horseradish peroxidase-labeled goat antimag I antibodies from Kirkegaard and Perry, Gaithersburg, MD; Protein A Sepharose CL-4B, CNBr-activated Sepharose 4B and Sepharose 2B from Pharmacia-LKB, Piscitaway, NJ. A rabbit antiserum to glycocalicin (GPIIb) was kindly provided by Dr Joan 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.

Isolation and activation of platelets. Human platelets were purified as described previously, 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 200 g for 15 minutes. A total of 1 ¡mol/L PGE1 was added to platelet rich plasma and the plasma was gel-filtered over a Sepharose 2B column equilibrated with platelet buffer. The Triton-insoluble and -soluble fractions from an equivalent volume of 4% paraformaldehyde in platelet buffer were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. The solubilized samples were fractionated on 10% SDS-PAGE, stained with Coomassie Blue on SDS-PAGE gel. The resultant high speed centrifugation (platelet supernatant) was boiled in SDS-PAGE buffer. The soluble phase of the high speed supernatant was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. The solubilized proteins were transferred to Immobilon P membranes (Millipore Corp, Bedford, MA) according to the method of Towbin et al. Because GPIb was detected by immunoblotting using S22, a mouse antimag I antibody, which recognizes the GPIb chain, at a 1:2000 dilution. GPIb was visualized using MoAb1 antibody. Primary antibody binding was visualized with a horseradish peroxidase-labeled goat antimag I 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 and digitizing the gel using a flat-bed scanner and analyzing the images within the linear range using the NIH Image program.

Immunoprecipitation of GPIb. 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, 1% Triton X-100, 1% sodium deoxycholate, and 2% NP-40, 0.1% SDS, and 0.1% sodium doxycholate. The lysates were centrifuged at 10,000 g for 1 hour, and the supernatant was collected for immunoprecipitation. 

Flow cytometry studies. Platelets (2 × 10^7/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 Hepes 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 antimag I antibody, at a 1:2000 dilution, and their fluorescence was read on 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).
body and 20 μL protein A Sepharose. Controls included Sepharose CL4B beads coupled to mouse IgG. The beads were washed three times with TBS for 1 minute in 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 x 10^6/mL and incubated for 1 hour at 37°C with 1 mg/mL of 1P 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% glutaraldehyde 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 5 x 10^6 cells/mL, placing 250 μL of the cell suspension in wells of a 96-well microtiter plate, each containing a polylysine-coated coverslip, and centrifuging the plate at 1,460g for 5 minutes at 20°C. 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 in 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 a solution composed of 1 mg/mL of sodium borohydride in 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 anti-glycoprotein IIb-IIIa monoclonal antibody to IgG for 90 minutes. Unbound antirabbit IgG for 90 minutes. Unbound gold was removed by a 1-minute wash in PBS/BSA, and then incubated with 1.8 nm of platinum and 10 nm of 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. Because GPIb-IX is linked to ABP-280 in resting platelets, 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. 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 GPIb-IX. In agreement with previous findings, the majority of platelet ABP-280, α-actinin, and GPIb-IX 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 GPIb-IX from the membrane skeleton to the activated cytoskeleton. Densitometric analysis of the SDS-PAGE and immunoblots showed that ~40% of this 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 GPIb-IX 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 platelets was 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. Figure 2 shows that comparable amounts of ABP-280 coprecipitated with GPIb-IX in both resting and activated lysates. This was confirmed by densitometry analysis of the immunoblot, which showed the ratio of GPIb-IX 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-IX (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-IX into the activated cytoskeletal fraction (Fig 3). Treatment of platelets with cytochalasins inhibits assembly of new actin filaments, incorporation of actin with ABP-280 into the activated cytoskeleton, and the surface redistribution of GPIb-IX. 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. Cytochalasin B treatment also decreased the incorporation of GPIb-IX 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, at which time, GPIb-IX centralization is beginning.
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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 15,600 g and at 100,000 g 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-GPIbα IgG (B). P designates total platelet lysate. This experiment is representative of four experiments.

shown on Fig 4A. We compared the time-courses of GPIb-IX centralization to its association with the activated cytoskeletons. As shown in Fig 4B, the incorporation of ABP-280, myosin I1, and GPIbα into the activated cytoskeletons of suspended platelets was complete 1 minute after treatment with thrombin. Although the binding of myosin II decreased at later time points, some myosin II remained associated to the cytoskeleton, as previously shown.25 The discrepancy between the kinetics of GPIb-IX centralization and its association to the activated cytoskeleton indicates that although actin assembly initiates GPIb-IX centralization, additional processes are required to complete it.

Analysis of the distribution of GPIb-IX on the cell surface by immunoelectron microscopy during thrombin-stimulation (Fig 5 and 6) confirmed that GPIb-IX centralization is slow relative to actin assembly. Figure 5A shows the distribution of 10 nm colloidal anti-GPIb gold particles on the surface of a resting platelet. Gold particles are dispersed across its surface and, at many points form linear rows, as shown previously.18,38 Figure 6 shows representative portions of

Molecular ratio

<table>
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<tr>
<th>ABP-280</th>
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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 antiligycocalcin 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.
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 thrombin 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. 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. 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 filopodia. Quin-2 AM loading not only prevented GPIb-IX centralization, but also 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 with the cytoskeleton.** To investigate further myosin II involvement of GPIb-IX centralization, we employed wortmannin, a fungal metabolite that irreversibly inhibits myosin light chain. 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-GPIba and 10 nm gold particles coated with goat antirabbit IgG. (a) Distribution of anti-GPIba 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.
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.49 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 IC_{50} of 0.3 μmol/L for MLCK determined by Nakanashi et al.43 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.25 30 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,47 present on the surface of the resting cells, which results from a redistribution to the OCS.6 8 There is, however, one published report to the contrary:21 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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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. In the resting platelet, GPIb-IX is found predominantly in the membrane skeleton. Following thrombin activation, a portion of the GPIb-IX/ABP-280 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, Fox did not detect an increase in GPIbα in activated cytoskeletons.

However, in a subsequent report by Zhang et al., 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 filaments and prevents the disassembly of actin filaments in permeabilized platelets.

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-

![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.](image)

![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.](image)
then activated with 1 U/mL thrombin for 30 seconds and the reaction was stopped by addition of SDS-PAGE buffer. The samples were analyzed by SDS-PAGE on a 13% polyacrylamide gel and analyzed by autoradiography. The positions of pleckstrin and myosin light chain kinase are shown. The results are representative of two experiments.

Fig 9. Effect of Quin-2 AM and wortmannin on thrombin-induced myosin light chain kinase and pleckstrin phosphorylation. P-labeled platelets were preincubated with with 40 μmol/L Quin-2 AM for 25 minutes or 10 μmol/L wortmannin for 15 minutes. Platelets were then activated with 1 U/mL thrombin for 30 seconds and the reaction was stopped by addition of SDS-PAGE buffer. The samples were analyzed by SDS-PAGE on a 13% polyacrylamide gel and analyzed by autoradiography. The positions of pleckstrin and myosin light chain kinase are shown. The results are representative of two experiments.

Precipitation experiments (Fig 3). Second, GPIb-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 coprecipitating with GPIbα indicates that calpain cleavage of ABP-280 does not occur during GPIb-IX centralization. Calpain activation is unlikely to take place under the nonaggregating conditions used to follow GPIb-IX centralization, as it requires GPIb-IIIα occupancy by fibrinogen.

The connection of GPIb-IX to actin is essential for its centralization. New actin filament assembly is also required, as cytochalasin B treatment prevents GPIb-IX centralization and the shift of GPIbα to the cytoskeletons. However, a second step in addition to actin assembly is necessary to centralize GPIb-IX on the platelet surface. While actin assembly and GPIbα association to the activated cytoskeletons was complete within a minute, GPIb-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 GPIb-IX becomes linked to the cytoskeleton.

Role of calcium metabolism in GPIb-IX centralization. The marked inhibition of GPIb-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 GPIb-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 GPIb-IX centralization, but also affected its surface distribution. GPIb-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 GPIb-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 GPIb-IX with actin filaments does not change during activation, GPIb-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 GPIb-IX would be expected to be on the surface overlaying the new filament portions, as all the GPIb-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 GPIb-IX staining on the newly formed extensions and suggests that actin assembly indirectly restricts GPIb-IX from the cell periphery (Fig 10C). The altered pattern of GPIb-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 GPIb-IX from the new areas of filament assembly during this first phase of GPIb-IX redistribution.

Role of myosin II in GPIb-IX centralization. An important role for myosin II activation in GPIb-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 GPIb-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 GPIb-IX centralization was ruled out by the observation that concentrations of wortmannin that completely inhibit PI-3 K do not greatly affect GPIb-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, and actin-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 34-thromosin, 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 GPIIb-IX and activated GPIIb-IIIa receptors on the surface of human platelets\(^7\) and can, when bound to GPIb-IX, induce activation of GPIIb-IIIa.\(^8\) Furthermore, vWF binds to fibrin, and, under high-shear stress conditions, contributes to the incorporation of platelets into clots (reviewed in \(^9\)). 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.

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

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