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α-Adducin dissociates from F-actin and spectrin during platelet activation

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A spectrin-based skeleton uniformly underlies and supports the plasma membrane of the resting platelet, but remodels and centralizes in the activated platelet. α-Adducin, a phosphoprotein that forms a ternary complex with F-actin and spectrin, is dephosphorylated and mostly bound to spectrin in the membrane skeleton of the resting platelet at sites where actin filaments attach to the ends of spectrin molecules. Platelets activated through protease-activated receptor 1, FcγRIIA, or by treatment with PMA phosphorylate adducin at Ser726. Phosphoadducin releases from the membrane skeleton concomitant with its dissociation from spectrin and actin. Inhibition of PKC blunts adducin phosphorylation and release from spectrin and actin, preventing the centralization of spectrin that normally follows cell activation. We conclude that adducin targets actin filament ends to spectrin to complete the assembly of the resting membrane skeleton. Dissociation of phosphoadducin releases spectrin from actin, facilitating centralization of spectrin, and leads to the exposure of barbed actin filament ends that may then participate in converting the resting platelet's disc shape into its active form.

Introduction

Platelets circulate in blood as discs where they must withstand high shear stresses. To tolerate such perturbations, the platelet has developed a highly organized cytoskeleton, which includes a peripheral microtubule coil and cross-linked actin filaments that fill the cytoplasmic space and connect to a membrane skeleton composed of spectrin and its associated proteins. In RBCs, the spectrin lattice forms a planar structure that laminates and supports the cytoplasmic face of the plasma membrane, providing an elastic matrix that returns shear-deformed red cells to a biconcave shape. The membrane skeleton of the platelet contributes to discoid shape (Fox et al., 1988; Hartwig and DeSisto, 1991). However, the platelet responds to vasculature damage by rapidly changing shape, first becoming round, and then extending filopodia and lamellae. These morphological changes require alterations in the membrane skeleton as well as actin remodeling.

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The membrane skeleton of the platelet is a planar network assembled primarily from spectrin molecules. In the erythrocyte, spectrin is joined into a network through attachments to short actin filaments. These 14-mers of actin are stabilized at their rapidly exchanging (barbed) ends by adducin (Kuhlman et al., 1996; Li et al., 1998), at their slow-exchanging (pointed) ends by tropomodulin (Ursitti and Fowler, 1994; Weber et al., 1994), and along their lengths by tropomyosin (Ursitti and Fowler, 1994). One significant difference between the platelet and erythrocyte is that the platelet contains considerably more actin, of which ~40% is assembled into long filaments measuring ~1 μm in length on average, providing platelets with 2,000–10,000 actin filaments (Hartwig, 1992). Whether or not platelets contain a specialized pool of short, membrane-associated actin filaments that cross-link spectrin molecules has been debated (Fox, 1993; Fox et al., 1988). If the ends of long actin filaments join the spectrin network, how these ends target the membrane skeleton requires an explanation.

Adducins comprise a protein family encoded by three genes (α, β, and γ). The α and γ isoforms (M_r of 103,000 and 84–86,000, respectively) are ubiquitously expressed in human cells; the β isoform (M_r of 97,000) is abundant in

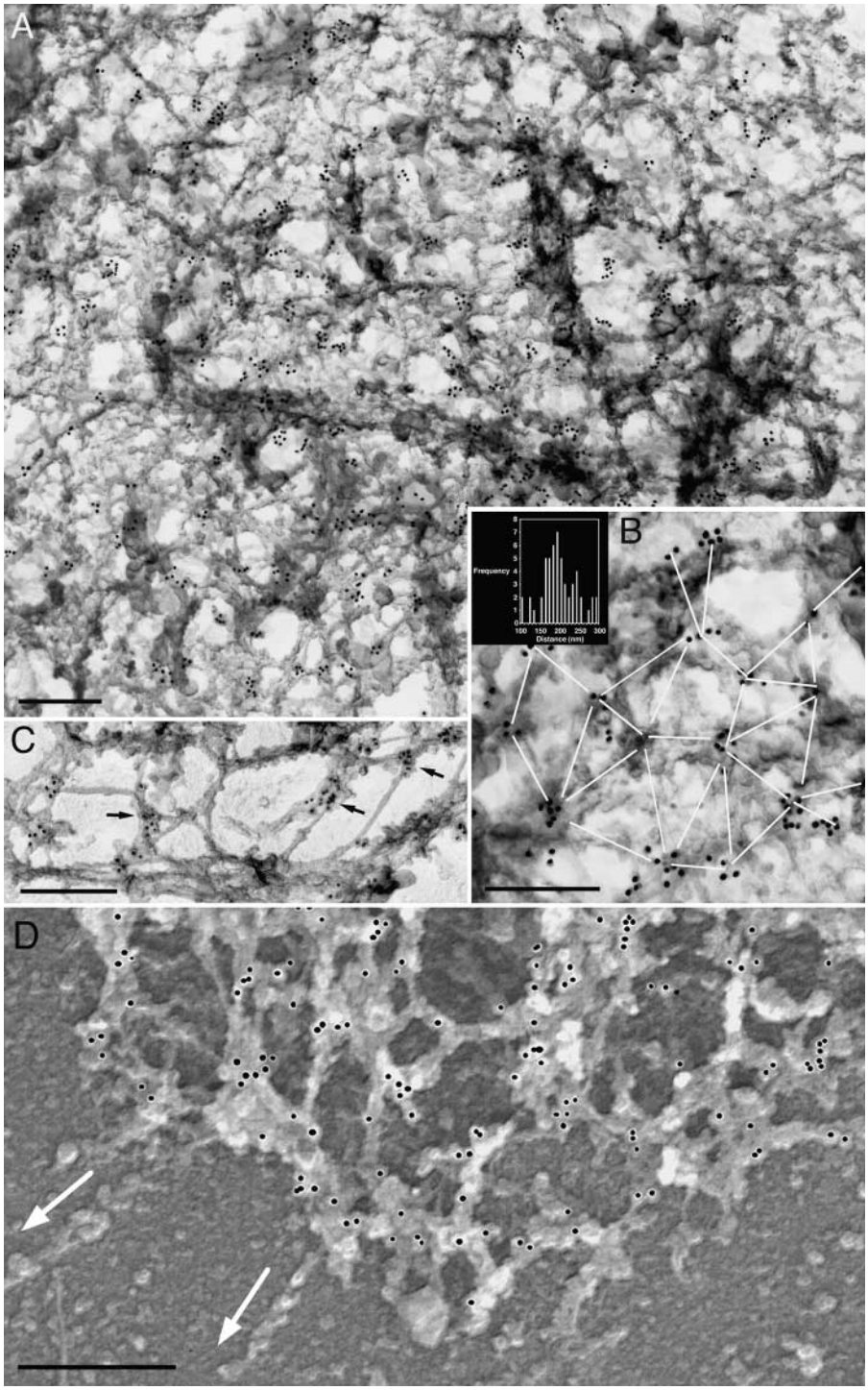

Figure 1. **α-Adducin localizes to periodic sites that are likely to be the ends of spectrin tetramers.** (A) Resting platelet cytoskeletons were labeled with 10 nm gold coated with goat anti–rabbit IgG. Gold is found in small clusters separated by ~200 nm at the ends of triangular pores (B) that are evenly distributed across the membrane skeleton. The distance between adjacent clusters is 201 ± 46 nm (n = 55). The inset shows the frequency length distribution of the intercluster distance. (C) A more open area of the membrane skeleton showing that anti-adducin labeling is found at the intersection of fibers. (D) Resting platelet cytoskeletons were sedimented onto glass coverslips without fixation to fracture the cytoskeleton and thus separate the spectrin-rich membrane skeleton from the core of F-actin. This image shows the membrane skeleton consisting primarily of spectrin filaments. Spectrin filaments are distinguished from actin, which has a rope-like appearance here, by virtue of labeling with myosin S1. Adducin is found almost exclusively in the spectrin-rich region. Arrows indicate the polarity of F-actin by pointing in the direction of the slow-growing or "pointed" end. Bars, 200 nm.

erythrocytes and the brain. Subunits assemble into heterodimers, or larger oligomers, through interactions in the globular amino-terminal head domains. In addition, assembly probably occurs through interactions near or within a myristoylated alanine-rich C-kinase substrate (MARCKS)* domain by the carboxyl-terminus (Hughes and Bennett, 1995), which contains PKC phosphorylation sites (Bennett et al., 1988) as well as binding sites for calcium/calmodulin (Gardner and Bennett, 1986) and possibly phospholipids. Adducin caps the barbed ends of actin filaments (Kuhlman et al., 1996; Li et al., 1998) and promotes their association with spectrin (Gardner and Bennett, 1987). However, phosphorylation of purified adducin by PKC diminishes its interaction with actin and spectrin (Matsuoka et al., 1998), whereas addition of exogenous calcium/calmodulin releases adducin from actin (Kuhlman et al., 1996). Together, these findings indicate that adducin may be involved in exposing

*Abbreviations used in this paper: Arp, actin-related protein; GF, GF 109203X; MARCKS, myristoylated alanine-rich C-kinase substrate; OG, *n*-octyl β-D-glucopyranoside; PAR, protease-activated receptor; TRAP, thrombin receptor–activating peptide.

actin filament barbed ends in motile cells after activation of certain signal transduction cascades. Consequently, adducin may be implicated in the development of actin assembly sites (Falet et al., 2002; Ichetovkin et al., 2002). Furthermore, signaling cascades that elevate intracellular calcium or activate PKC would destabilize the triadic interaction of adducin, actin filament barbed end, and spectrin, thereby potentially triggering perturbations in the spectrin network. Recent evidence suggests that adducin may also play a role in the dynamics of the platelet membrane skeleton; adducin is expressed in platelets (Gilligan et al., 1999) and becomes phosphorylated and proteolyzed after platelet activation (Gilligan et al., 2002). The works presented here link adducin's interactions with spectrin and actin filaments to modulation of the membrane skeleton organization in the resting and active platelet.

Results

Adducin is a component of the resting platelet membrane skeleton where it links actin filament barbed ends to spectrin

Fig. 1 shows that immunogold particles recognizing anti-α–adducin IgG associate with the lattice-like membrane skeleton of the discoid platelet. It has been previously shown that spectrin is the main component of this fibrous lattice (Boyles et al., 1985; Hartwig and DeSisto, 1991). Detailed examination of anti-adducin immunogold-labeled specimens reveals that small clusters of 3–5 gold particles decorate the membrane skeleton uniformly at intersections composed of two or more strands: the thin fibers are likely to be spectrin, and the thicker (10 nm) filaments are actin. The spacing of individual gold clusters in Fig. 1 is consistent with α-adducin's association within the membrane skeleton near the ends of spectrin molecules at the points where actin filament ends insert. If platelet spectrin organizes as tetramers, as in the RBC membrane skeleton (Shotton et al., 1979; Liu et al., 1984), then the predicted distance between adducin molecules (gold clusters) bound to the β-spectrin amino-termini would be ~200 nm. As shown in Fig. 1 B, gold particle clusters are separated from neighboring clusters by an average distance of 201 ± 46 nm (mean ± SD, $n = 55$). Filament intersections and localization of adducin at these intersections are readily visible in Fig. 1 C (arrows), which depicts a distended region of the membrane skeleton.

We also localized α-adducin after labeling the membrane skeleton with myosin S1. After treatment with myosin S1, anti-α–adducin immunogold localizes near the end of undecorated strands (Fig. 1 D), but not to S1-decorated actin filaments. Actin filament barbed ends can be seen to associate primarily with the membrane skeleton (Fig. 1 D: arrows define polarity of filaments, with the direction of the arrows pointing toward the minus or pointed end).

Spectrin is centralized while adducin is released in active platelet cytoskeletons

In metal replicas, the center of the spread platelet cytoskeleton is composed of a dense region of compact fibrous material (Hartwig, 1992). This fibrous material is densely labeled with 10 nm anti-spectrin immunogold (Fig. 2 A), whereas only sparse gold labeling is found in the rest of the cytoskeleton (Fig. 2 B). This suggests that the fibrous material is the remnant of the membrane skeleton aggregating in the center during platelet spreading. Furthermore, although spectrin is redistributed into the center of active platelet cytoskeletons, immunoelectron microscopy analysis shows that little α-adducin remains associated with the active cytoskeleton (Fig. 2, C and D). Anti-adducin immunogold labeling is sparse in both the core of the cytoskeleton (Fig. 2 C) and in the cortex, where actin filaments are dense (Fig. 2 D). Quantitation of anti-adducin immunogold particles in electron micrographs of cytoskeletons revealed that there was 72% less gold label in the active versus resting cytoskeleton (11.4 ± 6.2 and 39.8 ± 17 gold particles/μm^2, respectively).

A comparison of immunofluorescent staining of fixed platelets and detergent-extracted cytoskeletons of platelets activated when sedimented onto glass further confirms that little α-adducin remains in detergent-permeabilized platelets (Fig. 3; cytoskeleton denotes platelets permeabilized with detergent before fixation). F-actin staining with Alexa® 488–phalloidin (green) delineates the cell boundaries in both intact cells (fixed before detergent) and cytoskeletons. These images also reveal the distribution of total adducin (mostly phosphoadducin in the active platelet). As detailed in the section entitled Adducin is phosphorylated by PKC in active platelets, the bulk of adducin becomes phosphorylated and soluble after platelet activation. Therefore, adducin remaining in the cytoskeleton is dephosphorylated, and some residual adducin staining in the dense center of the cytoskeleton occurs. In intact platelets, adducin staining (Texas red) is robust and found uniformly throughout the spread cell, as well as in the dense central area. To further understand the adducin–spectrin interaction, we compared the distribution of spectrin and adducin to F-actin in spread platelets (Fig. 3). In similar fashion to adducin, spectrin centralizes after platelet activation and spreading, although spectrin is concentrated in the center of the cells relative to adducin, which appears in both the cell center and the spread lamellae. After permeabilization to remove soluble phosphoadducin, both spectrin and adducin stain only the cytoskeletal centers of platelets. The centralization of spectrin and adducin requires actin assembly because centralization is greatly diminished in platelets treated with 2 μM cytochalasin B. This effect is most apparent in the centralization of spectrin (Fig. 3). Examination of adducin and spectrin localization during platelet spreading further delineates the membrane skeletal remodeling process (Fig. 4). Both adducin and spectrin localize throughout the resting platelet. By 1.5 min after the onset of surface activation, the earliest time we could reproducibly obtain adherent and active platelets, adducin and spectrin remain centrally located in an area corresponding to the previous boundaries of the resting platelet that is separate from the zone of new actin polymerization at the edge of the cell. As platelets continue to spread with longer times (5 min) on glass, F-actin staining in the center of the platelet becomes reduced as spectrin condenses into the cell center. A significant portion of the adducin remains centrally located at 5 min after activation, although some staining does extend into lamellae, which we believe represents soluble phosphoadducin.

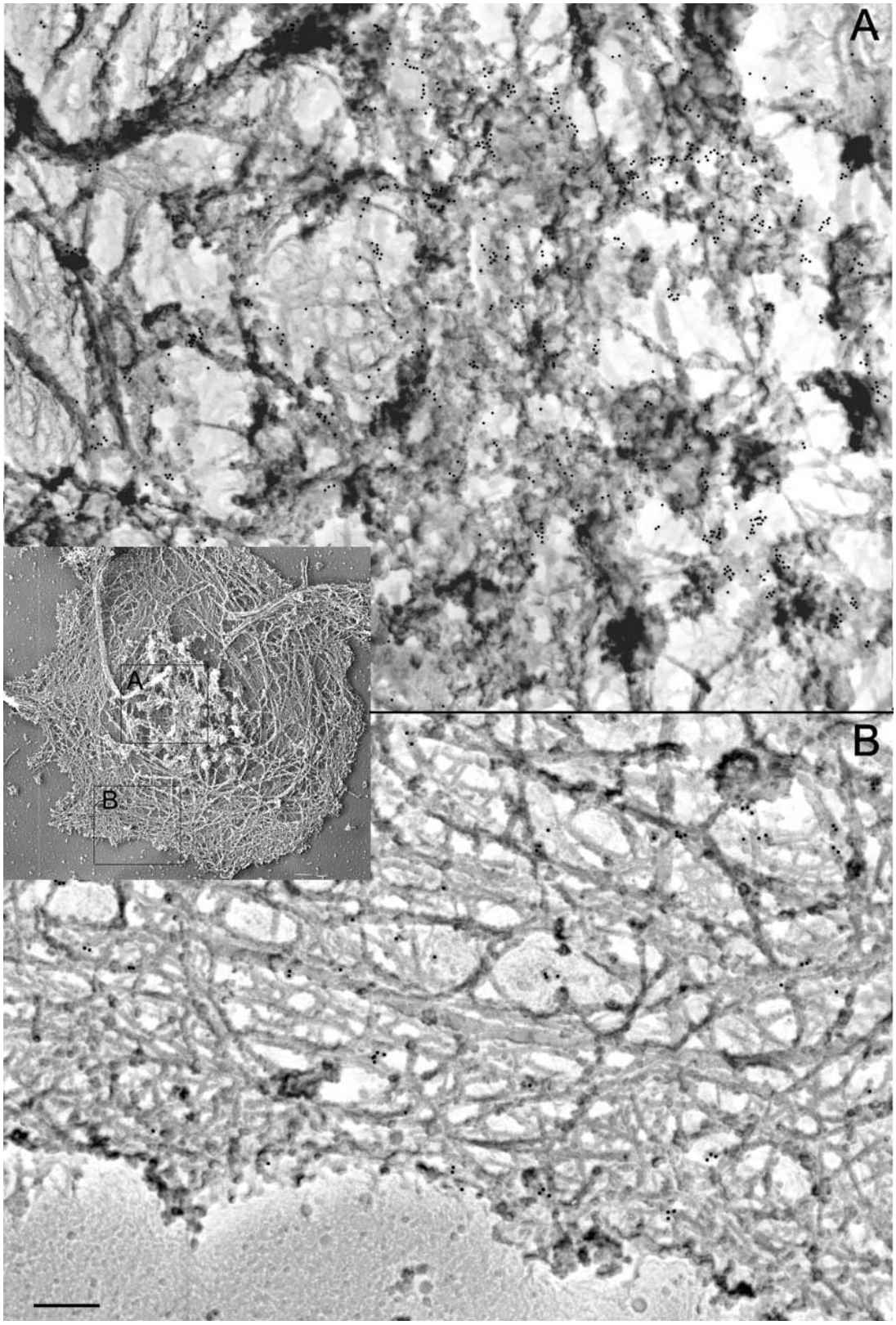

Figure 2. **Localization of spectrin in the cytoskeleton of a spread platelet by immunoelectron microscopy.** (A and B) high magnification images of those indicated in the inset. Dense binding of 10 nm immunogold anti-spectrin is found in the center of the cytoskeleton (A), whereas only sparse gold labeling is found in the rest of the cytoskeleton (B). (C and D) Immunogold labeling of platelet cytoskeletons shows little adducin remaining in the center (C) or in the cortex (D) after activation. Bars, 200 nm.

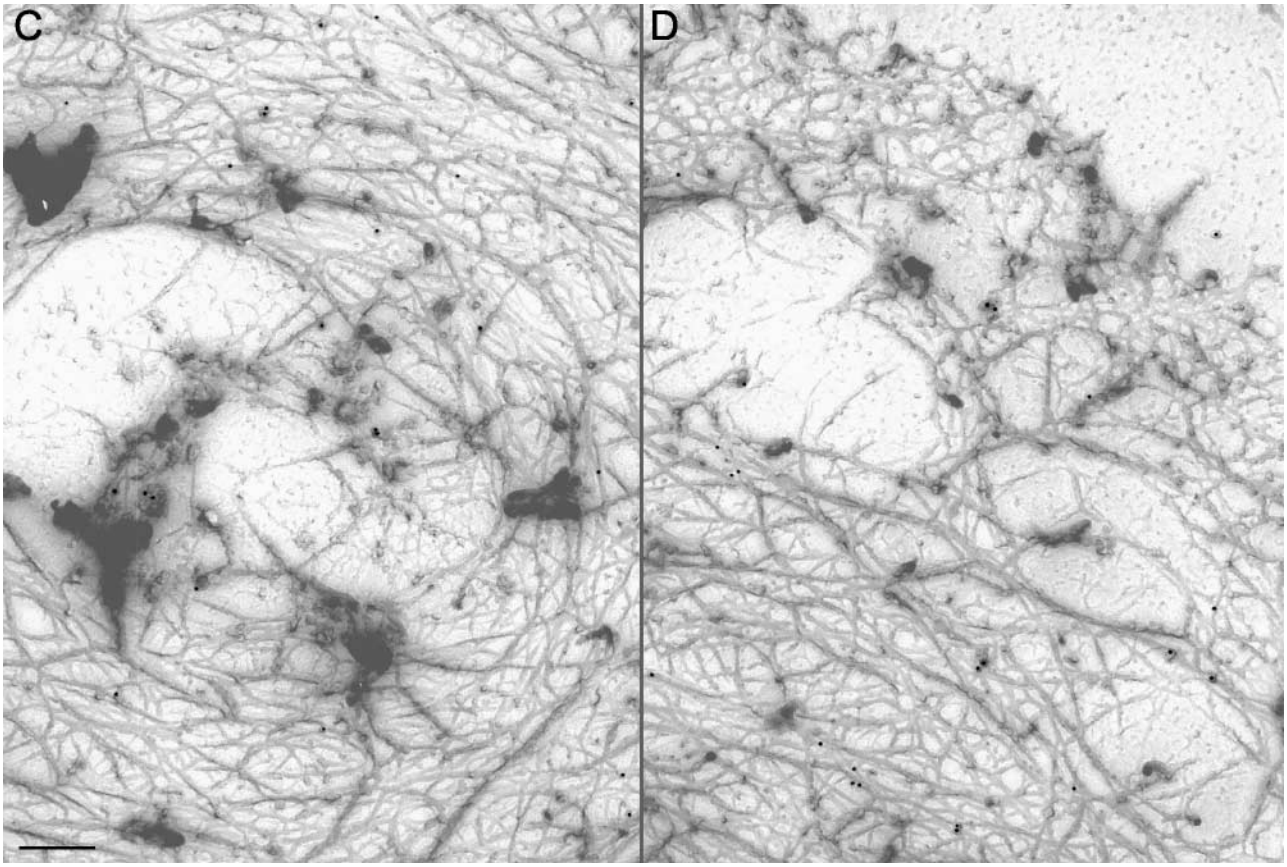

Figure 2 continued.

Centralization and dissociation of adducin and spectrin are inhibited by GF

To determine if spectrin centralization during platelet spreading involves PKC-mediated dissociation of adducin from spectrin, platelets were allowed to spread in the presence of 5 μM GF 109203X (GF), a selective inhibitor of PKC (Toullec et al., 1991). GF prevents the bulk release of adducin into the detergent-soluble fraction after stimulation with agonists (see section entitled Adducin is phosphorylated by PKC in active platelets). As shown in Fig. 3, inhibition of PKC slows the accumulation of adducin, spectrin, and F-actin in the platelet center; instead, actin and spectrin coalesce into a broad pericentral zone when platelets spread in the presence of GF. Adducin centralization is also affected, but to a lesser extent. GF does not affect the initial stages of adducin and spectrin remodeling as platelets spread on glass (1.5 min), as both proteins remain condensed in the cell center (Fig. 4). However, after longer times of spreading (5 min), adducin does not redistribute to the cell cortex (Fig. 4, compare untreated with +GF), and spectrin concentrates in a new ring structure along with F-actin in the middle of platelets treated with GF. 5 min after activation, PKC activity normally dissociates actin from spectrin. Hence, GF blocks the bulk export of spectrin from the platelet center. In an attempt to quantify these processes, we compared the platelet area cooccupied by actin and adducin or spectrin. Quantitative analysis of the fluorescent staining of platelets demonstrates that spectrin (Fig. 4, top graph) and adducin (Fig. 4, bottom graph) couple tightly to F-actin in platelets treated with GF, whereas the area of overlap declines over time in untreated platelets. However, these measurements do not take staining intensities into account, and therefore do not provide spatial protein concentrations.

Biochemical evidence indicates that the majority of adducin is associated with the cytoskeleton in resting platelets

The concentration of α-adducin in platelets was determined by quantitative immunoblotting. A pAb against α-adducin detected 2.7 ± 0.3 ng of adducin per μg of total platelet protein, equivalent to a platelet concentration of ~3 μM (unpublished data). When resting platelets permeabilized with Triton X-100 are centrifuged at high *g* forces (>100,000 *g*), proteins associated with F-actin pellet. As shown in Fig. 5 (D–F; time = 0), ~70–75% (2.15 ± 0.1 μM, $n = 6$) of α-adducin pellets in detergent-lysed resting platelets.

Adducin is phosphorylated by PKC in active platelets

To investigate adducin's PKC-dependent phosphorylation state in resting and activated platelets, we used mAb that recognizes the PKC phosphorylation site in α-adducin (p-Ser726) and detects phosphoadducin only in activated platelets. Fig. 5 shows the kinetics of α-adducin phosphorylation ($n = 3$) in platelets stimulated through protease-activated receptor 1 (PAR-1; Fig. 5 A), FcγRIIA (Fig. 5 B), and PMA

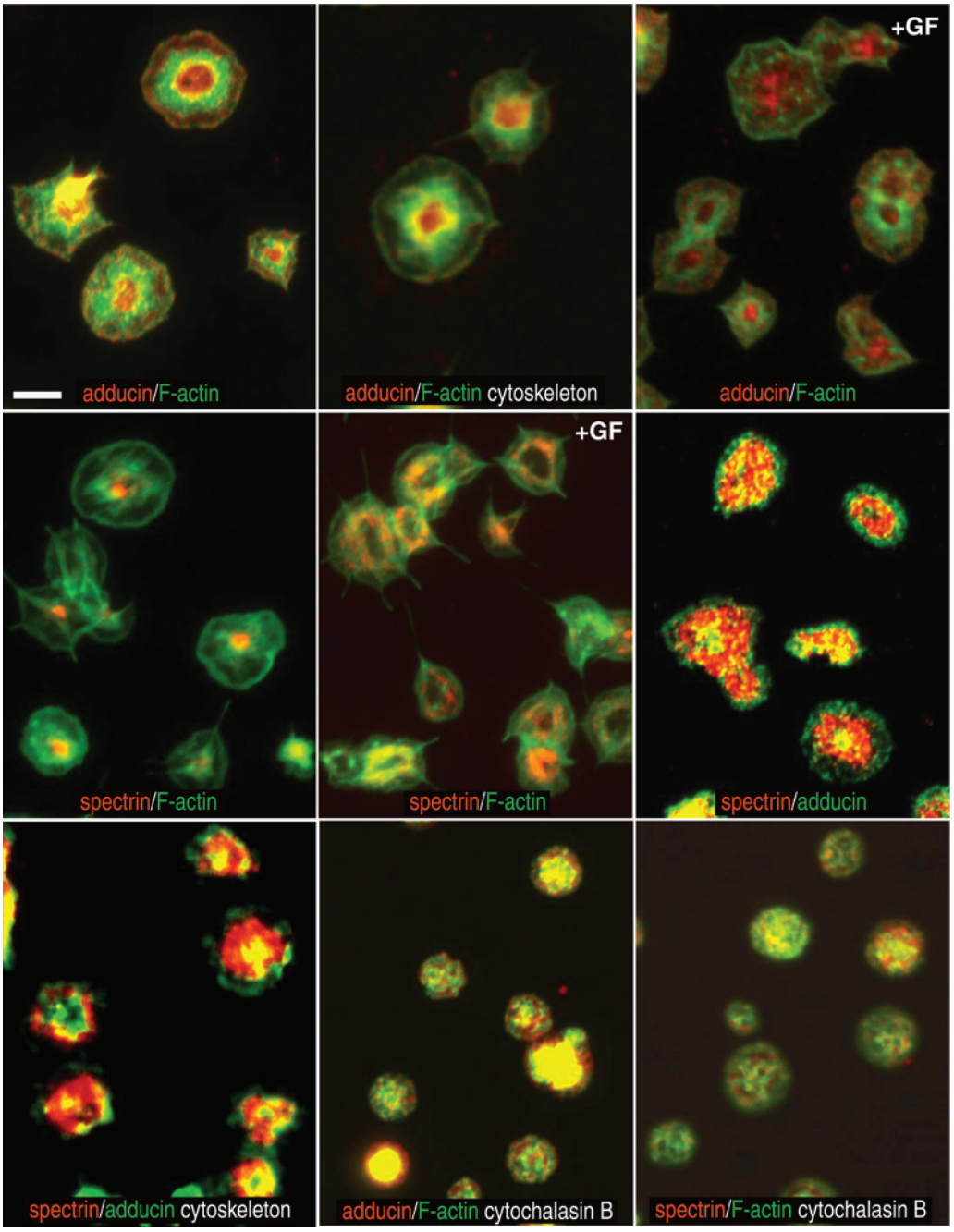

Figure 3. **Immunofluorescent localization of adducin, spectrin, and F-actin in platelets spread on glass.** The protein stained and fluorophore of the secondary antibody are indicated in each panel. Samples pretreated with 5 μM GF or 2 μM cytochalasin B are indicated. "Cytoskeleton" denotes platelets first extracted with Triton X-100 before fixation. All other panels show intact and fixed platelets. Bar, 5 μm.

(Fig. 5 C). Resting platelets (time = 0) have little phosphoadducin in either their cytoskeletons (Fig. 5, A–C, p) or soluble protein fractions (Fig. 5, A–C, s). Phosphoadducin is detectable by 15 s and maximal 30–60 s after treating platelets with 25 μM thrombin receptor–activating peptide (TRAP) to ligate PAR-1 (Fig. 5 A). Platelets activated through FcγRIIA have maximal levels of phosphoadducin 2–3 min after activation (Fig. 5 B). When platelets are activated with 100 nM PMA, phosphoadducin is evident at 1–5 min, but the bulk of PKC-dependent phosphorylation requires 10–15 min of exposure to PMA (Fig. 5 C). Phosphorylation of α-adducin induced by agonists is inhibited by 5 μM GF (Fig. 5, A and C).

Dissociation of α-adducin from the activated platelet cytoskeleton

Once phosphorylated at Ser726, adducin no longer sediments with the F-actin cytoskeleton. Furthermore, after platelet activation through PAR-1 (Fig. 5 E), FcγRIIA (Fig. 5 E), or by PMA (Fig. 5 F), phosphoadducin is recovered only in the soluble fraction after centrifugation at 100,000 *g*. However, the kinetics of α-adducin release are different for the three ago-

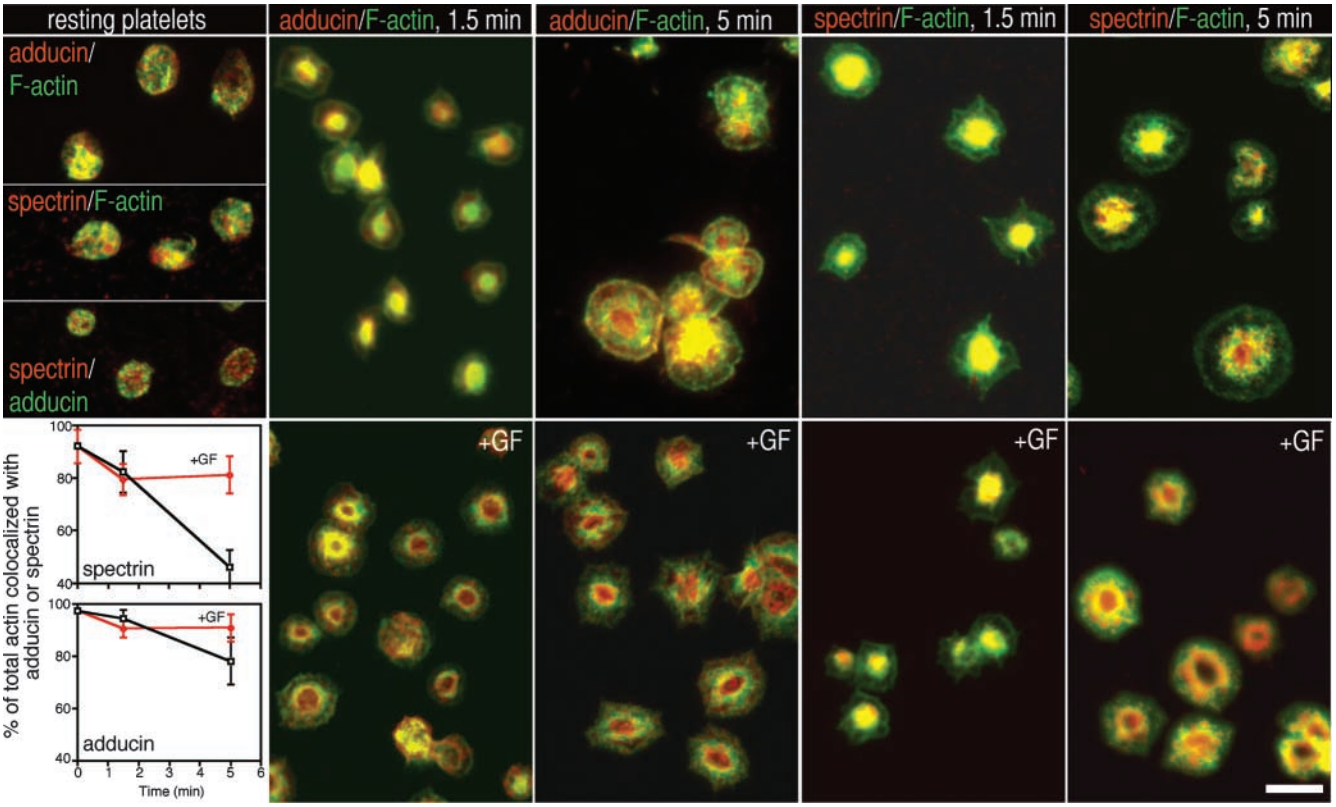

Figure 4. **Immunofluorescent localization of adducin, spectrin, and F-actin as platelets spread for 1.5 or 5 min.** The protein stained and fluorophore of the secondary antibody is indicated in each panel. Samples treated with GF are indicated. Panels showing resting platelets are located in the top left. Bar, 5 μm. GF treatment reduces the separation of actin from spectrin and adducin. The two graphs in the bottom left quantify the amount of colocalization of F-actin staining with spectrin (top graph) or adducin (bottom graph) staining during normal platelet spreading (squares with black lines) or spreading in the presence of 5 μM GF109203X (diamonds with red lines) with respect to the incubation time (mean ± SD, $n = 10$).

nists. Stimulation through PAR-1 leads to the most rapid release of adducin, which begins within seconds and is maximal by 2 min after activation. In contrast, treatment with PMA induces the slowest response, requiring 15 min for the maximal release of adducin. α-Adducin release in FcγRIIA-ligated platelets is intermediate, with maximal release requiring 3–4 min. In all cases, 10–25% of the total α-adducin remains unphosphorylated and associated with the detergent-insoluble F-actin cytoskeleton of the activated platelet.

Adducin releases from spectrin during platelet activation

Biochemical experiments further support the finding that adducin physically associates with spectrin in the membrane skeleton. To specifically examine the effects of phosphorylation on the interaction of adducin with spectrin, total adducin was immunoprecipitated from lysates of resting and PAR-1–ligated platelets and immunoblotted with anti-spectrin antibodies. Quantitative immunoblots revealed that 75 ± 12% ($n = 4$) of the total spectrin in platelets associates with adducin in the resting cell (Fig. 6 A, squares, time = 0). After ligation of PAR-1, less spectrin was found in association with adducin in immunoprecipitates prepared from platelet lysates (Fig. 6 A, squares). Spectrin dissociation from adducin was rapid and reached a maximal value by 1 min, at which point 65–70% of the spectrin had dissociated. We also examined the effect of GF on the spectrin–adducin interaction and found that GF prevents the release of spectrin from adducin (Fig. 6 A, diamonds). The membrane skeleton and actin filament core of the platelet can also be separated into distinct biochemical populations after detergent permeabilization using differential centrifugation (Fox et al., 1988). The core cytoskeleton, composed of long cross-linked actin filaments, sediments at 15,000 *g*. In contrast, the membrane skeleton containing the spectrin lattice and its associated proteins, including some actin filaments, sediments only at higher forces of ≥100,000 *g*. Adducin's sedimentation profile follows that of the membrane skeleton; ~75% of adducin resides in the pellet sedimented at ≥100,000 *g* (Fig. 6 B, hp) and only a small amount of adducin is associated with the low speed pellet (Fig. 6 B, lp). We used differential centrifugation to determine if adducin releases from the platelet membrane skeleton after addition of PMA. As shown in Fig. 6 B, there was a bulk transition of α-adducin out of the high speed pellet, which contains spectrin and the membrane skeleton, and into the high speed supernatant. The small amount of adducin in the 15,000 *g* pellet, which contains the core cytoskeleton, did not change with activation.

What signaling components participate in adducin release from the cytoskeleton?

To directly determine if PKC-mediated phosphorylation could release adducin from platelet actin, the purified cata-

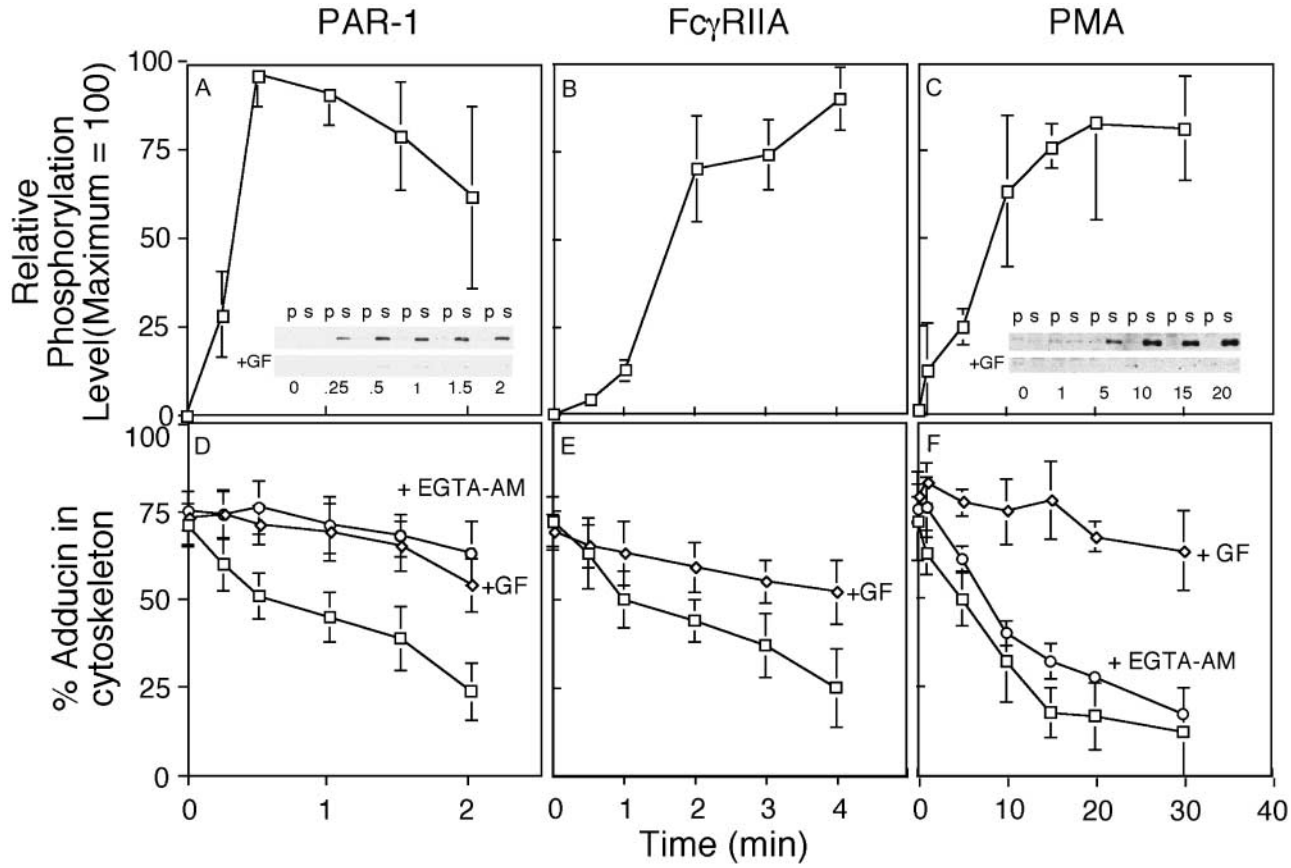

Figure 5. **α-Adducin is bound to the actin cytoskeletons of resting platelets and is phosphorylated and released from the cytoskeletons of active platelets.** (A–C) Phosphorylation of adducin at Ser726 follows platelet activation by PAR-1 (A), FcγRIIA (B), and PMA (C). The kinetics of adducin phosphorylation are plotted (mean ± SD, $n = 3$). Insets (A and C) show representative Ser726 phosphoimmunoblots ± GF for PAR-1 and PMA activation. (D–F) The amount of adducin associated with F-actin in 215,000 *g* pellets of permeabilized platelets (squares) activated by PAR-1 (D), FcγRIIA (E), or PMA (F) is compared with platelets treated before permeabilization with either GF (diamonds, D–F) or EGTA-AM (circles, D and F).

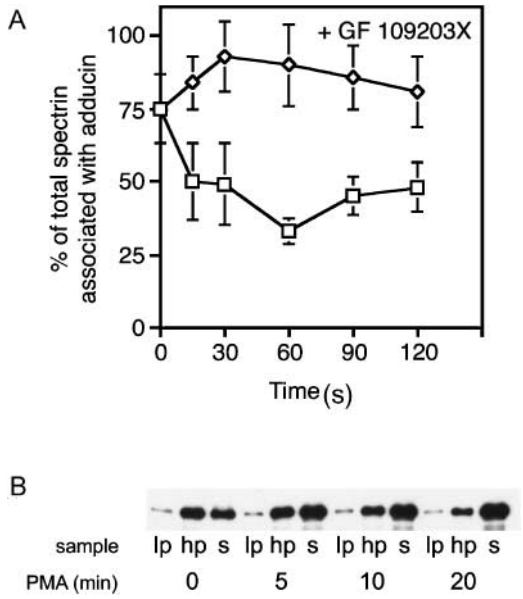

Figure 6. **α-Adducin associates with spectrin in resting platelets, but dissociates during platelet activation.** (A) Percentage of total spectrin that immunoprecipitates with adducin in resting platelets or platelets activated with 25 μM TRAP (squares). Less spectrin associates with α-adducin after platelet activation. Dissociation of spectrin from adducin is prevented when platelets are incubated with the PKC inhibitor GF 109203X (diamonds). (B) Distribution of adducin in the cytoskeleton (lp, 15,000 *g* low speed pellet), membrane skeleton (hp, 215,000 *g* high speed pellet), and soluble (s, high speed supernatant) phase of PMA-stimulated platelets permeabilized with Triton X-100 and detected by immunoblot.

lytic subunit of PKC was added to detergent-permeabilized, washed platelets. As shown in Fig. 7 A, treatment with purified PKC releases phosphoadducin from the membrane skeleton into a 215,000 *g* soluble fraction. Because many actin filament barbed-end capping proteins are released from actin by polyphosphoinositides and because the MARCKS domain of other proteins also interacts with phospholipids, we examined whether α-adducin could also be released by polyphosphoinositide micelles in *n*-octyl β-D-glucopyranoside (OG)-permeabilized and washed platelets. We found that 75 μM $PI_{4,5}P_2$ or $PI_{3,4}P_2$ (or $PI_{3,4,5}P_3$, unpublished data) releases α-adducin from these permeabilized platelets, but the same concentration of phosphatidylserine has no effect on adducin release (Fig. 7 B).

Because the MARCKS domain of adducin has calcium/calmodulin binding sites, we additionally sought to determine whether cytosolic calcium transients play a role in ad-

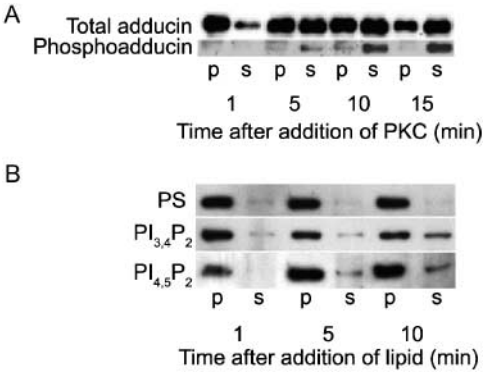

Figure 7. **Treatment of permeabilized, washed platelets with the catalytic subunit of PKC or $PI_{3,4}P_2$ micelles releases α-adducin from the cytoskeleton.** (A) Platelets were extracted with 0.4% OG and washed twice in PHEM without OG. They were treated with the catalytic subunit of PKC (Sigma-Aldrich) at the indicated times, and then centrifuged to generate pellet (p) and supernatant (s) fractions. Immunoblots of total cytoskeletal α-adducin (top immunoblot) or Ser726 phospho–α-adducin (bottom immunoblot) are shown. (B) Immunoblots of total α-adducin in cytoskeletal pellets (p) and soluble fractions (s) of permeabilized platelets treated with phosphatidylserine (PS), $PI_{3,4}P_2$, or $PI_{4,5}P_2$.

ducin release. Accordingly, platelets were treated with 40 μM EGTA-AM to chelate calcium. Chelation inhibits the release of adducin from the platelet membrane skeleton after PAR-1 stimulation (Fig. 5 D, circles). However, in PMA-treated platelets, which do not normally undergo intracellular calcium transients (Falet and Rendu, 1994), EGTA-AM has little effect on the release of adducin from actin (Fig. 5 F, circles).

A recent report indicated that adducin is cleaved by calpain, a calcium-dependent cytoplasmic protease, in platelets activated by thrombin (Gilligan et al., 2002). However, we find that treatment of platelets with calpeptin, an inhibitor of calpain's proteolytic activity, does not alter the kinetics of adducin release (unpublished data). Our experiments do not indicate that α-adducin is cleaved by calpain in platelets activated by a PAR-1–specific activating peptide, cross-linking of FcγRIIA, or by phorbol esters.

Furthermore, because it has been reported that adducin is phosphorylated by rho kinase, an event that affects adducin–actin interactions (Kimura et al., 1998; Fukata et al., 1999), we examined the effect of the rho kinase inhibitor, Y-27632, on the association of adducin with actin. Adducin release was unaffected in platelets that were pretreated with 5 μM Y-27632 and activated by PAR-1, FcγRIIA, or PMA (unpublished data). Nonetheless, we confirmed that 5 μM Y-27632 blocked LPA-induced stress fiber formation in serum-starved fibroblasts (unpublished data), a process mediated by rho kinase (Amano et al., 1997).

Adducin phosphorylation by PKC and its release from the cytoskeleton coincide with actin assembly and barbed-end exposure

Regardless of how platelets are activated, phosphorylation of α-adducin at Ser726 coincides with actin filament assembly and actin filament barbed-end exposure. Release of adducin from the platelet membrane skeleton might contribute to barbed-end exposure and the onset of actin assembly in active platelets. Accordingly, we examined the role of PKC-mediated adducin phosphorylation in the actin assembly reaction of platelets stimulated either through PAR-1, FcγRIIA, or by the addition of PMA. Platelets activated through PAR-1 show a small but reproducible reduction in actin assembly and barbed-end exposure when treated with GF, as compared with untreated controls (Fig. 8, A and B). This small effect on barbed ends and actin assembly is consistent with the GF-insensitive, barbed-end nucleation activity of gelsolin and actin-related protein 2/3 (Arp2/3; see section entitled Adducin releases from spectrin during platelet activation). Barbed-end exposure and actin assembly in platelets activated through the FcγRIIA receptor are more sensitive to PKC inhibition. In FcγRIIA-activated platelets, GF inhibits actin assembly by ~70% (Fig. 8 C) and suppresses barbed-end exposure by ~40–80% at early time points (Fig. 8 D). As expected, inhibition of PKC in platelets exposed to PMA blocks >95% of both actin assembly (Fig. 8 E) and barbed-end exposure (Fig. 8 F).

Because other proteins can regulate actin filament barbed-end exposure, we investigated the effect of GF on the translocation of gelsolin and capZ, two actin filament barbed-end capping proteins, as well as on the actin-nucleating Arp2/3 complex. As shown in Fig. 9, GF affects neither the reversible entry of gelsolin nor the incorporation of the Arp2/3 complex and capZ into the cytoskeletons of platelets activated through PAR-1. In addition, the association of the Arp2/3 complex and gelsolin with actin does not change in PMA-treated platelets. Nonetheless, capZ does associate with the actin cytoskeleton of PMA-treated platelets, as expected from the increase in the number of barbed ends. The association of capZ with actin is blocked when platelets are treated with GF (Fig. 9).

Discussion

Here, we confirm that platelets express α-adducin (Gilligan et al., 1999) and show it is a structural protein of the resting cytoskeleton, bound to both spectrin and F-actin. After platelet activation, phosphorylation of adducin at Ser726 releases it from spectrin and F-actin. Release of adducin from spectrin facilitates the centralization of the membrane skeleton during platelet spreading. Release of adducin from F-actin exposes actin barbed ends that contribute to the actin assembly reactions in lamellae and filopodia.

Adducin is believed to self-associate into heterodimers and heterotetramers (Hughes and Bennett, 1995; Li et al., 1998) composed of α, β, or γ isoforms. Because most mammalian cells express little of the erythroid β isoform, adducin molecules in platelets are α and γ assemblies (based on an α-adducin concentration of 3 μM and a vol of 7 fl, each platelet expresses ~12,600 α-adducin subunits). Depending on whether adducin is a heterodimer (1α:1γ) or heterotetramer, each platelet would have either 12,600 or ≤6,300 adducin molecules, respectively. The theoretical maximal number of adducin binding sites at spectrin ends is 4,000 because each platelet has ~2,000 spectrin tetramers (Hartwig and DeSisto, 1991). As ~70%

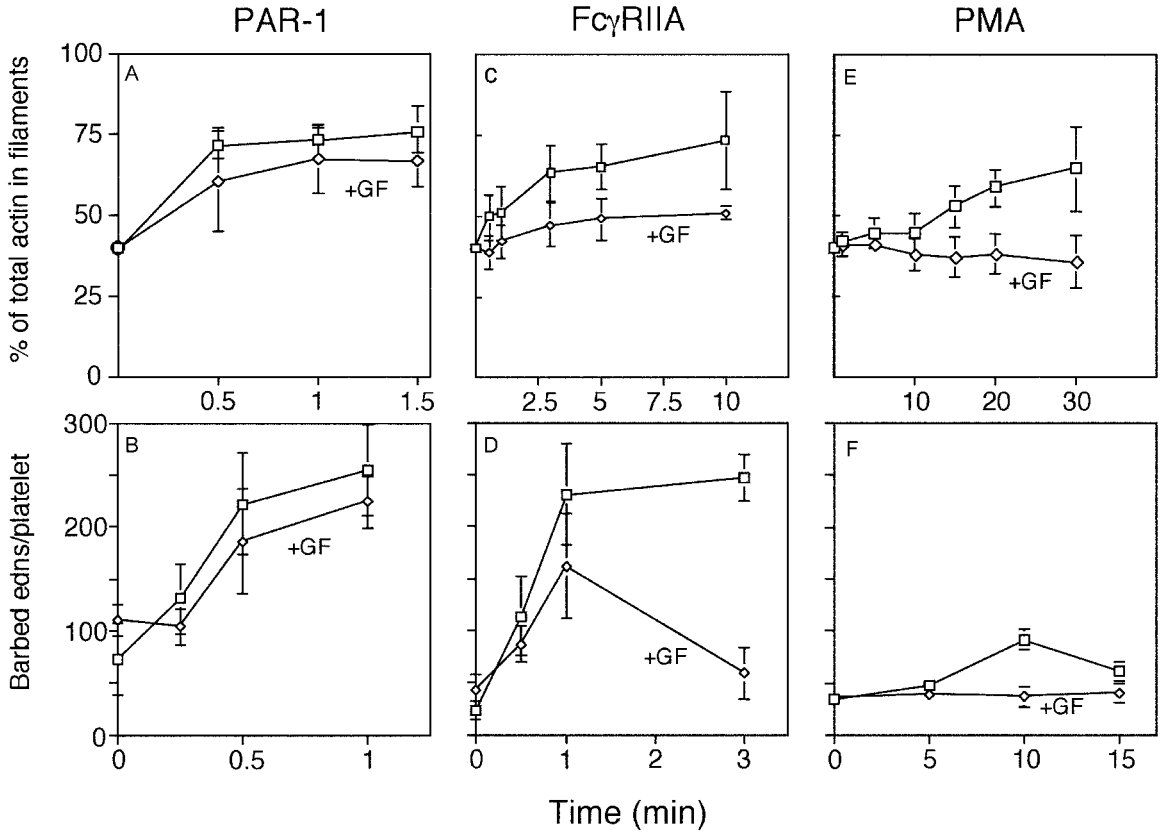

Figure 8. **Actin assembly and barbed-end exposure are diminished by inhibiting PKC.** (A and B) Cytoskeletal changes are temporally linked to the phosphorylation and release of adducin from the cytoskeleton. (A) F-actin content and (B) barbed-end exposure in resting platelets and platelets stimulated through PAR-1 in the absence (squares) or the presence (diamonds) of GF. (C and D) Comparison of F-actin content (C) and free barbed ends (D) in platelets activated through FcγRIIA in the absence (squares) or presence (diamonds) of GF. Both are reduced by 50–70% in platelets stimulated through FcγRIIA when PKC is inhibited. (E and F) Platelets stimulated by 100 nM PMA exhibit marked inhibition of F-actin assembly (E) and barbed-end exposure (F) when pretreated with GF (diamonds), compared with controls (squares).

of adducin is bound to spectrin in the resting platelet, the number of bound adducin complexes is ~4,400, which is close to this theoretical maximum. On the other hand, the number of actin filaments in a resting platelet has been estimated at 2,000–10,000. Therefore, each resting platelet has a sufficient amount of adducin to cap many of these actin filaments and to target them to spectrin in the membrane skeleton. In addition, resting platelets have a significant portion of their actin filament barbed ends capped by capZ. We have previously reported that ~7,000 capZ molecules associate with the resting cytoskeleton (Barkalow et al., 1996). If the higher estimate for actin filaments is accurate, then the adducin and capZ bound to actin filament ends would account for the >98% capping of the barbed ends (Hartwig, 1992). However, if the lower estimate for actin filaments is correct, a significant portion of the adducin and capZ would be associated with other Triton X-100–insoluble elements, such as lipid rafts that are rich in polyphosphoinositides.

The association of adducin with both actin filament barbed ends and spectrin is regulated by PKC-dependent phosphorylation of adducin (Matsuoka et al., 1998). Adducin associates with the platelet membrane skeleton, but dissociates when phosphorylated by PKC at Ser726, consistent with the finding that phosphorylation by PKC within adducin's MARCKS domain separates it from both actin filaments and spectrin. In addition, Gilligan et al. (2002) reported that adducin releases from detergent-insoluble cytoskeletons of thrombin-activated platelets, and that its release coincides with phosphorylation by PKC. Adducin also has a calcium/calmodulin binding site (Gardner and Bennett, 1986), and treatment with exogenous calcium/calmodulin releases adducin from RBC ghosts (Kuhlman et al., 1996). For most agonists, maximal actin filament barbed-end exposure or production after platelet activation requires a transient increase in intracellular calcium. Thus, it seems likely that calcium/calmodulin binding may help release adducin from the barbed ends of actin filaments in platelets. Platelets treated with PMA do not undergo fluctuations in intracellular calcium (Falet and Rendu, 1994), yet do manifest a slow but significant dissociation of adducin from actin that is unaffected by treatment with EGTA-AM. Furthermore, actin nucleation is independent of gelsolin (Fig. 9) because gelsolin does not become transiently activated to move into the PMA-activated cytoskeleton. Together, these findings suggest that release of adducin from the membrane skeleton does not require binding of calcium/calmodulin.

Polyphosphoinositides may also influence the adducin–actin–spectrin interaction by interacting with adducin's

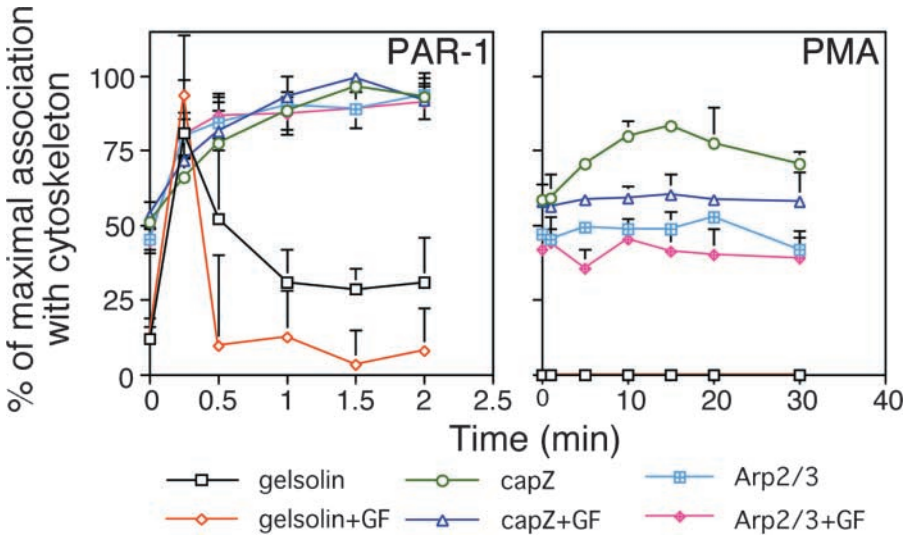

Figure 9. **Inhibition of PKC by GF does not alter the activation of gelsolin, capZ, or Arp2/3 in PAR-1–ligated platelets.** Results from platelets stimulated by PAR-1 ligation or addition of PMA are shown. PMA does not induce an association of gelsolin or Arp2/3 with the cytoskeleton, but capZ does associate with the cytoskeleton in a PKC-dependent fashion. All data are expressed as a percentage of maximal association with the cytoskeleton (mean ± SD, $n = 3$).

MARCKS domain. Polyphosphoinositides bind to a motif within the MARCKS protein (aa 151–175, referred to as the MARCKS domain in other proteins containing this motif) and are sequestered from PLC when bound (Glaser et al., 1996). Phosphorylation of MARCKS by PKC dissociates the lipid from MARCKS, releasing this constraint. Polyphosphoinositides also bind to actin filament barbed-end binding proteins such as gelsolin (Janmey and Stossel, 1987) and capZ (Schafer et al., 1996), and prevent/reverse their binding. In light of these findings, we tested the ability of polyphosphoinositides to modulate adducin binding in the platelet cytoskeleton, and found that $PI_{3,4}P_2$, $PI_{4,5}P_2$ (Fig. 7), and $PI_{3,4,5}P_3$ (unpublished data) each dissociated a portion of the adducin bound to the cytoskeleton.

Platelet activation, which can be triggered by various agonists, initiates platelet shape changes that are driven by forces generated from polymerization at the barbed ends of actin filaments. Different receptor-mediated signaling cascades may invoke actin regulation with variable efficiencies using a host of barbed-end capping proteins. In PAR-1–stimulated platelets, the majority of free actin filament barbed ends originate from gelsolin-driven severing and uncapping amplified by Arp2/3 (Witke et al., 1995; Falet et al., 2002); however, adducin does contribute some (~10%) of the total barbed ends generated. In platelets activated through FcγRIIA and PMA, adducin contributes 60–100% of the new actin filament barbed ends. This greater dependence on adducin reflects the inactivity of gelsolin and Arp2/3 under these signaling conditions. CapZ's association with the actin cytoskeleton in PMA-activated platelets is not surprising because its primary function in active platelets is to cap free barbed ends (Barkalow et al., 1996). Although adducin-mediated nucleation activity may contribute only a small portion of barbed ends, the removal of adducin from the membrane skeleton may be important for efficient lamellar extension. Membrane destabilization would occur as actin filament ends are released, thereby disrupting the integrity of the spectrin lattice as adducin strengthens the actin–spectrin interaction. Destabilizing the membrane skeleton would in turn facilitate membrane extension during platelet spreading. Consistent with this hypothesis is the observation that adducin releases from spectrin during platelet activation in a manner dependent on its phosphorylation at Ser726 by PKC. Furthermore, the localization of spectrin at the platelet center is severely hindered when PKC activity is blocked in active platelets. Therefore, we propose that the binding of α-adducin to spectrin in resting platelets is important in maintaining the membrane skeleton, and that remodeling of the membrane skeleton during activation is achieved in part by α-adducin release from spectrin.

Our structural and biochemical studies provide information to refine the model of the membrane skeleton in resting platelets and to begin to elucidate the dynamic changes after platelet activation. As illustrated in Fig. 10, the membrane skeleton of the resting platelet is a network of spectrin tetramers that interconnect at actin filament ends. Adducin molecules strengthen the spectrin–actin connections and target actin filament barbed ends to spectrin. Because these actin filaments are part of and are cross-linked to the actin cytoskeleton, adducin acts as a bridge between the membrane skeleton and the actin cytoskeleton. Platelet activation alters both the actin cytoskeleton and the membrane skeleton. Within seconds after platelet activation (Fig. 10, early event), Ca^{2+}-gelsolin severs preexisting actin filaments into small fragments. The subsequent release of gelsolin from the barbed ends of these filament fragments generates templates onto which the Arp2/3 complex binds, and new actin filaments are polymerized (Fig. 10, early events, new actin assembly zone; Falet et al., 2002). Actin filaments grow off of Arp2/3 as well as the free barbed ends and fill lamellae. In a slower process (30 s–1 min), adducin releases from actin and spectrin (Fig. 10, early events), freeing spectrin and allowing the membrane skeleton to become centralized during platelet spreading (Fig. 10, later events). However, GF-treated platelets do not release adducin from spectrin and actin. Platelet activation in the presence of GF leads to structural abnormalities; stabilization of the spectrin network prohibits its centralization, leading to accumulation of spectrin and actin filaments in a zone equivalent to the boundaries of the resting cell (Fig. 10, late events). Nonetheless, actin filaments remain susceptible to severing by Ca^{2+}-gelsolin.

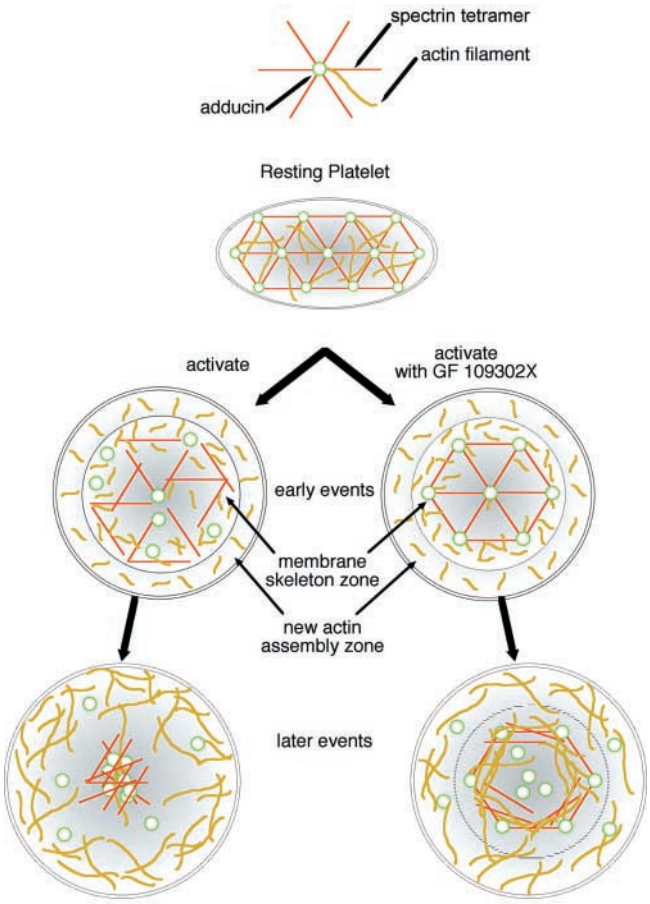

Figure 10. **Diagram summarizing the reorganization of F-actin, spectrin, and adducin during platelet spreading.** (Left) Normal spreading. The resting platelet has a spectrin lattice that laminates the cytoplasmic face of its plasma membrane. Adducin molecules sit at the intersections of spectrin tetramers and bind F-actin barbed ends. Changes in the adducin–actin–spectrin interaction occur as platelets become active. Platelet activation (early events) results in gelsolin-mediated F-actin severing and adducin release from spectrin–actin, which destabilizes the membrane skeleton. (Later events) Actin polymerization and centralization of spectrin–dephosphoadducin–F-actin follow the fragmentation phase of platelet activation. (Right) PKC inhibition slows spectrin centralization. F-actin is severed by gelsolin, but adducin is not phosphorylated and cannot release from spectrin–actin. Actin nucleation sites remain associated with the membrane skeleton because F-actin fragments remain bound to spectrin. This enhances actin assembly in the region of the membrane skeleton, which does not condense and remains dispersed in the center of the activated platelet. Spectrin, actin, and adducin are defined in the diagram.

Therefore, gelsolin-dependent actin nucleation as well as lamellar extension, which drive platelet spreading, persist in GF-treated platelets.

The binding of adducin to platelet spectrin and its localization in the platelet membrane skeleton places adducin at an important site to link actin filament barbed ends to spectrin and provides an explanation for how these actin filament ends find spectrin (Hartwig and DeSisto, 1991). Because adducin, spectrin, and actin filaments exist in all mammalian cells, our findings may be relevant for the membrane–cytoskeletal interfaces of other cell types as well. For example, in translocating cells, the formation of adducin, actin, and spectrin complexes may reversibly assemble at defined sites to facilitate directional motility.

Materials and methods

Antibodies

Anti–human α-adducin serum was obtained by immunizing New Zealand white rabbits with aa 347–737 of human α-adducin as a GST fusion protein. A phosphoserine 726–specific mAb was generated in mice by injecting the phosphopeptide phe-arg-thr-pro-ser (phospho)-phe-leu-lys-lys. The mice were processed for mAb production by isolating cells from the spleen, and fusing them with mouse myeloma cells (SB 2) to produce hybridoma cells. The cells were then screened for antibody production specific to the phosphopeptide.

Isolation of platelets

Human platelets were isolated from anti-coagulated blood. Platelet-rich plasma was obtained by centrifuging the blood at 200 *g* for 15 min. Platelets were separated from the plasma by gel filtration over a Sepharose-2B column preequilibrated with platelet buffer (145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.3% BSA, 0.5 mM NaH_2PO_4, and 5 mM KCl, pH 7.4). The platelets were allowed to stand at 37°C for 30 min before further treatment.

Platelet activation

Resting platelets were activated by (1) PAR-1 ligation with 25 μM TRAP; (2) 3 μg/ml anti-FcγRIIA, followed by 30 μg/ml goat anti–mouse $F(ab')_2$ at 37°C; or (3) 100 nM PMA (phorbol 12-myristate 13-acetate). In some experiments, platelets were pretreated with either 40 μM EGTA-AM for 30 min or with 5 μM GF 109203X, an inhibitor of PKC (Toullec et al., 1991), for 1 min. At the appropriate times, platelets were lysed with 1/10 vol of 10× lysis buffer (120 mM Pipes, 60 mM Hepes, 100 mM EGTA, 20 mM $MgCl_2$, 1% Triton X-100, 10 μM phallacidin, and 0.5 mg/ml each of leupeptin, aprotinin, and benzamidine, pH 6.9). Samples were centrifuged for 30 min at 215,000 *g* in a centrifuge (model TL-100; Beckman Coulter). Platelet samples treated with PKC or $PI_{3,4}P_2$ micelles were extracted in lysis buffer with 0.5% OG instead of Triton X-100. Supernatants and pellets were mixed in equal volumes of SDS-PAGE sample buffer. All samples were incubated at 37°C overnight.

Immunoprecipitation of adducin

Platelets at rest or activated with TRAP were extracted with lysis buffer. Anti-adducin polyclonal serum was added at a 1:200 dilution, along with 20 μl of packed GammaBind™ G Sepharose (Amersham Biosciences). Samples were mixed at 4°C overnight. The Sepharose was sedimented at 1,000 *g* for 5 min. The supernatants were recovered and saved for SDS-PAGE and immunoblotting. The Sepharose pellet was washed 5× with platelet buffer and prepared for SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting

SDS-PAGE was performed using a modified 8 or 15% acrylamide Laemmli slab gel (Laemmli, 1970). Proteins were transferred for 75–90 min to PVDF membranes (Millipore) using a Trans-Blot® cell (Bio-Rad Laboratories) at 1 A. PVDF membranes were blocked in PBS with 0.02% Tween 20 and 5% Carnation nonfat dry milk, pH 7.4. Blocked membranes were incubated with primary antibody for 2 h at RT, washed three times with PBS, and probed with a 1:4,000 dilution of goat anti–rabbit IgG HRP conjugate (Bio-Rad Laboratories) for 1 h. Washed membranes were again washed and developed using chemiluminescence (SuperSignal®; Pierce Chemical Co.).

Electron microscopy

Resting platelets were extracted with 0.75% Triton X-100, 0.1% glutaraldehyde, PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9), and 1 μM phallacidin (glutaraldehyde is omitted if fracturing of the cytoskeleton is desired as in Fig. 1 D), and attached by centrifugation to poly-L-lysine–coated coverslips (Hartwig and DeSisto, 1991). The adhered cytoskeletons were washed, and free aldehydes were blocked with 0.1% sodium borohydride in PHEM. For analysis of activated platelet cytoskeletons (e.g., Fig. 2), platelets were first centrifuged onto coverslips, then detergent permeabilized (Hartwig, 1992). Myosin S1 labeling of cytoskeletons was achieved by incubating samples with 2 mg/ml myosin S1 for 5 min. The cytoskeletons were washed in PBS/1% BSA to block nonspecific protein binding, incubated for 1.5 h at 22°C with anti-spectrin pAb di-

luted to ~10 μg/ml in PBS/BSA, washed six times with PBS/BSA, and incubated for 1.5 h with anti–rabbit IgG 10-nm colloidal gold conjugates. The labeled cytoskeletons were extensively washed, fixed with 1% glutaraldehyde in PBS for 10 min, washed into distilled water, rapidly frozen, freeze-dried, and rotary shadowed with 1.2 nm tungsten-tantalum at 45° followed by 3 nm of carbon at 90° without rotation. Replicas were photographed with an electron microscope (model 1200-EX; JEOL USA, Inc.) at a 100-kV accelerating voltage.

Immunofluorescence

Resting platelets were centrifuged at 200 *g* for 5 min onto poly-L-lysine–coated glass coverslips in platelet buffer with or without 5 μM GF 109203X or 2 μM cytochalasin B. Adherent platelets were fixed with 2% formaldehyde in platelet buffer for 10 min, followed by 0.5% Triton X-100 in platelet buffer for 10 min. The coverslips were blocked with 0.5% albumin and 0.05% NaN_3 in platelet buffer (blocking buffer) overnight, then incubated at RT for 2 h with mouse anti-spectrin (#S3396; Sigma-Aldrich) or rabbit anti-α–adducin, each at a dilution of 1:250. The coverslips were washed four times with blocking buffer, and incubated for 1 h at RT with a 1:200 dilution of Texas red–labeled anti–rabbit IgG for adducin combined with F-actin staining; Alexa® 488–labeled anti–rabbit IgG for adducin combined with spectrin staining; and Alexa® 568–labeled anti–mouse IgG for all spectrin staining (Molecular Probes, Inc.). The coverslips were exposed to 1 μM phalloidin-Alexa® 488 (Molecular Probes, Inc.) for F-actin staining, washed four times with blocking buffer, and mounted (Aqua Polymount; Polysciences) onto microscope slides. Images were recorded digitally with a cooled CCD camera (Orca; Hamamatsu Corporation) on a microscope (Eclipse PE2000; Nikon) using a 60× oil immersion objective and MetaMorph® software (Universal Imaging Corp.).

To monitor the effects of activation on the localization of spectrin, adducin, and F-actin, a time-course experiment was performed. Resting platelets were centrifuged at 200 *g* for 1 min onto poly-L-lysine–coated coverslips in platelet buffer with or without 5 μM GF109203X. At times of 1.5 min or 5 min after the onset of centrifugation, the samples were then fixed and extracted. Unstimulated platelets (time = 0) were generated by fixing resting platelets for 10 min, then centrifuging them onto poly-L-lysine–coated coverslips. To quantitate the extent of colocalization of spectrin and adducin with F-actin in platelets treated with or without GF 109203X, ratiometric imaging was performed using MetaMorph® analysis software (Universal Imaging Corp.). The images were digitally thresholded with a software function that subtracts background signals below a critical pixel density. Individual cells ($n = 10$) for each condition and time point were randomly selected, and the total area of fluorescence signal for actin, spectrin, and/or adducin was measured for each platelet. The extent of association between spectrin or adducin and F-actin is expressed as a percent ratio of the overlapped area to the total area of actin signal.

Platelet actin assembly assay and FACS® analysis

The F-actin content of platelets at various times after stimulation was quantitated by flow cytometry of FITC-phalloidin–labeled platelets in a FACScan™ (Beckton Dickinson). TRAP, IV.3 antibody, or PMA were used to activate the platelets, in the presence or absence of 5 μM GF 109203X. After activation, the platelets were fixed for 30 min at the desired time point by the addition of an equal vol of 6.8% formalin. A 1/40 vol of 4% Triton X-100 containing 40 μM FITC-phalloidin was used to extract platelets and label the F-actin. Forward and side scattering were used to identify platelets and measure the mean fluorescence of 10,000 cells.

Actin filament barbed-end exposure in platelets

The number of barbed ends per platelet was obtained in a nucleation assay using pyrene-labeled actin (Hartwig, 1992; Hartwig et al., 1995). 90 μl of platelets at a concentration of 2×10^8/ml were activated with TRAP, IV.3 antibody, or PMA. Triton X-100–extracted platelets were diluted with buffer B (0.1 M KCl, 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM ATP, 10 mM Tris, and 0.5 mM β-mercaptoethanol, pH 7.4) and 1 μM monomeric pyrene-labeled actin. The fluorescence data were gathered using a fluorimeter (model LS-50B; PerkinElmer) with an excitation and emission wavelength of 366 and 386 nm.

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