The Platelet Cytoskeleton Stabilizes the Interaction between α_{IIb}β_{3} and Its Ligand and Induces Selective Movements of Ligand-occupied Integrin

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Joan E. Fox§, Sanford J. Shattil, Raelene L. Kinlough-Rathbun*, Mary Richardson‡, Marian A. Packham§§, and David A. Sanan¶¶

From the ‡Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, §Children’s Hospital, Oakland Research Institute, Oakland, California 94609, ¶¶University of Pennsylvania, Philadelphia, Pennsylvania 19104, *McMaster University, Hamilton, Ontario, ¶¶Queens University, Kingston, Ontario, §§Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, and ¶¶Gladstone Institutes, San Francisco, California 94141

Previously, we showed that a subpopulation of the major platelet integrin, α_{IIb}β_{3}, co-sediments from detergent lysates with talin and other membrane skeleton proteins. Once α_{IIb}β_{3} has bound adhesive ligand in a platelet aggregate, the detergent-insoluble α_{IIb}β_{3} redistributes (along with the detergent-insoluble membrane skeleton proteins and a variety of signaling molecules) to a fraction that contains cytoplasmic actin filaments. Concomitantly, certain signaling molecules are activated. The present study shows that, in intact platelets, α_{IIb}β_{3} forms clusters when occupied by ligand and is selectively moved into the open canalicular system; α_{IIb}β_{3} that has not bound ligand remains diffusely distributed at the periphery of the cell. When cytoplasmic actin filaments are depolymerized by cytochalasins, the ability of α_{IIb}β_{3} to bind ligand is decreased, and the movement of ligand-occupied α_{IIb}β_{3} is prevented. Together with the previous findings, these results suggest that (i) membrane skeleton-associated α_{IIb}β_{3} is selectively induced to bind ligand in activated platelets, (ii) ligand-induced transmembrane signaling causes an altered association of membrane skeleton-associated α_{IIb}β_{3} with the cytoplasmic component of the cytoskeleton, (iii) ligand-induced cytoskeletal reorganizations stabilize the interaction between ligand and integrin, and (iv) ligand-occupancy triggers cytoskeletal reorganizations that result in selective movements of occupied ligand.

Integrins are a family of transmembrane glycoproteins that bind adhesive molecules and play important roles in mediating cell-cell and cell-matrix interactions (1). Several integrins are unable to bind their ligand unless the cells are activated (2–6). One such integrin is the glycoprotein IIb-IIIa complex (α_{IIb}β_{3}) on platelets (7). When platelets are activated, unidentified intracellular events act on α_{IIb}β_{3} to induce binding of fibrinogen to the extracellular domain of the receptor; by cross-linking α_{IIb}β_{3} molecules on adjacent platelets, fibrinogen is thought to mediate the formation of platelet aggregates. Although binding of the adhesive ligand is initially reversible, it becomes irreversible after several minutes (8, 9). The binding of adhesive ligand to α_{IIb}β_{3} in a platelet aggregate induces a number of intracellular events, including phosphorylation of specific proteins on tyrosine residues (10–12), activation of calpain (13), the calpain-induced hydrolysis of cytoskeletal proteins (13–16), and activation of Na+/K+ exchange (17). How α_{IIb}β_{3} is induced to bind adhesive ligand, how binding is rendered irreversible, or how binding of adhesive ligand to the extracellular domain of α_{IIb}β_{3} activates intracellular signaling molecules are all unanswered questions.

The cytoplasmic face of the plasma membrane of platelets is coated by a membrane skeleton that associates with the cytoplasmic domains of transmembrane glycoproteins and with underlying cytoplasmic actin filaments (18–20). When platelets are lysed with Triton X-100, the cytoplasmic actin filaments can be sedimented by low speed centrifugation. The membrane skeleton separates from the cytoplasmic filaments; the fragments of membrane skeleton and associated membrane glycoproteins require higher forces to be sedimented (19). Recently, we showed that some α_{IIb}β_{3} sedimented with fragments of the membrane skeleton from lysates of unstimulated platelets (21). Tyrosine kinases (pp60^{src} and pp62^{yes}) were also recovered in this detergent-insoluble fraction (21). Once α_{IIb}β_{3} had bound to fibrinogen in a platelet aggregate, α_{IIb}β_{3} along with membrane skeleton proteins (21), tyrosine kinases (21), tyrosine-phosphorylated proteins (21), and a variety of additional signaling molecules (e.g., phosphoinositide 3-kinase and protein kinase C) (22) were recovered in the detergent-insoluble fraction that contained the cytoplasmic component of the cytoskeleton. We suggested that the membrane skeleton may play a role in regulating the activation of α_{IIb}β_{3} that binds to ligand in the membrane skeleton-associated α_{IIb}β_{3} causes the integrin-skeleton complexes to undergo an altered association with cytoplasmic actin forming “focal contact-like” structures, and that these focal contact-like structures may in turn play an important role in positioning signaling molecules and in regulating the ability of such molecules to mediate integrin-induced signaling events (21, 23).

Our previous study (21) was based on the co-sedimentation of the integrin with detergent-insoluble fractions. In the present...
study, we provide evidence that membrane skeleton-associated α11β3 binds ligand and is incorporated into complexes with cytoplasmic actin filaments in intact cells, that the formation of these integrin-cytoskeletal complexes results in stabilization of integrin-ligand interactions and a selective movement of ligand-occupied integrin into the surface-connected open canalicular system.

MATERIALS AND METHODS

Antibody Production and Characterization—PAC-1 and ASG8 are both monoclonal antibodies of the immunoglobulin (Ig) M class that recognize epitopes on α11β3. PAC-1 is similar to fibrinogen in that it binds only to activated platelets (4). ASG8 binds to both unstimulated and activated platelets and does not cross-react with fibrinogen binding antibodies. Monoclonal and monoclonal antibodies against α11β3 were provided by Dr. David Phillips of COR Therapeutics (San Francisco, CA), polyclonal antibodies against fibrinogen were from Calbiochem (San Diego, CA) and those against glycoprotein Ib were raised as described previously (18).

Isolation and Analysis of Platelet Suspensions—The venous blood of healthy adult donors was collected into acid citrate dextrose solution, and platelet-rich plasma was prepared (19). Platelets were isolated by gel filtration (24) or centrifugation (19) and resuspended at a concentration of 1 × 10^9 platelets/ml in Tyrode’s buffer (19). Platelets were activated with thrombin (a gift of Dr. J. ohn W. Fenton II of the New York Department of Health, Albany, NY) or ADP (Sigma). Unless otherwise stated, activation was induced without stirring. In some experiments, platelets were preincubated with cytochalasins (Sigma), which were added in a final concentration of 0.1% dimethyl sulfoxide (Me2SO). Control incubations also contained Me2SO. Platelet aggregation and secretion of ATP were assessed in a lumigraphometer (Chrono-Log Corporation, Havertown, PA) (25). Western blotting was performed by the method of Towbin et al. (26). Antigen-antibody complexes were detected with 125I-labeled anti-IgG (DuPont NEN).

Binding of PAC-1 to Platelets—Monoclonal antibody PAC-1 was labeled with fluorescein isothiocyanate (FITC) (24) and incubated with platelets in the presence of an agonist. At intervals, platelets were diluted into suspension buffer, and the amount of bound PAC-1 was determined by flow cytometry (24). Becton Dickinson FACSFACS 440 cytometer, San Jose, CA). For analysis of the reversible and irreversible components of PAC-1 binding, 45-μl aliquots were added to 50 μl of buffer or to the same buffer containing 100 μM EDTA; after 10 min, samples were diluted 3-fold, and the amount of bound PAC-1 was determined.

Localization of Proteins by Immunofluorescence—Platelets were activated in the presence of monoclonal antibody PAC-1 or ASG8. Incubations were terminated by addition of 9 volumes of a solution containing 4% paraformaldehyde in 150 mM sodium chloride, 10 mM Tris-HCl, pH 7.4, and platelets were allowed to settle onto poly-L-lysine-coated glass slides (19). Fixed platelets were permeabilized by addition of a detergent-containing buffer, and the amount of bound PAC-1 was determined, discoid platelets (Fig. 1A) eventhough

RESULTS

Ligand-induced Association of α11β3 With Cytoplasmic Actin in the Intact Platelet—Based on studies in detergent-lysed platelets, we have previously suggested that α11β3 can associate with a submembranous component of the cytoskeleton and that binding of adhesive ligand to α11β3 leads to altered association of the integrin and membrane skeleton proteins with cytoplasmic actin filaments. If this model is correct, then α11β3 that had bound its adhesive ligand would be expected to show a different distribution within intact platelets from that of α11β3 that had not bound ligand. Further, any ligand-induced change in distribution that occurred as a consequence of association of the integrin with cytoplasmic actin would be prevented by disrupting the network of cytoplasmic actin filaments.

To determine whether ligand-occupied α11β3 had a different distribution from unoccupied integrin, platelets were incubated with the fibrinogen-mimetic monoclonal antibody, PAC-1. At intervals following thrombin addition, platelets were fixed, permeabilized with Triton X-100, and incubated with a polyclonal α11β3 antibody that bound to both ligand-occupied and unoccupied α11β3. The distribution of PAC-1-occupied and total α11β3 was visualized by dual-label confocal microscopy. As reported previously (4), virtually no PAC-1 bound to unstimulated, discoid platelets (Fig. 1A) even though α11β3 was over all of the surface of the platelets (Fig. 1B). As the platelets were activated with thrombin, PAC-1 binding occurred. At early times after platelet activation (e.g. 60 s), PAC-1-occupied α11β3 was clustered in a few discrete areas (Fig. 1C). However, α11β3 antibodies revealed that the rest of the α11β3 was still present over all of the surface of the activated platelet (Fig. 1D). At later times after platelet activation, clusters of PAC-1-occupied α11β3 became concentrated toward the center of the platelet (Fig. 1E). Polyclonal antibodies revealed that α11β3 that had not bound PAC-1 was still present in a relatively uniform distribution at the periphery of these platelets (Fig. 1F). The diameters of two representative platelet profiles (1 and 2) in Fig. 1, E and F, have been marked by pairs of arrows. When comparing, profiles 1 + 2 (PAC-1 distribution) in Fig. 1E with profiles 1 + 2 (α11β3 distribution) in Fig. 1F, it is obvious that the PAC-1 labeling pattern has a much smaller diameter than the α11β3 labeling pattern in each case, small enough, in fact, to fit within the peripheral α11β3 labeling pattern. The platelets shown in Fig. 1 had been activated with thrombin at a platelet concentration of 1 × 10^9 platelets/ml. A similar distribution of PAC-1-occupied α11β3 was detected on platelets that were activated at a concentration of 1 × 10^8 platelets/ml (data not shown). Another monoclonal antibody of the IgM class, ASG8, binds to a site on α11β3 other than that to which

1 The abbreviations used are: Ig, immunoglobulin; FITC, fluorescein isothiocyanate.
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adhesive ligand binds; it recognized $\alpha_{IIb}\beta_3$ on both unstimulated and activated platelets and showed a distribution similar to that detected with the polyclonal $\alpha_{IIb}\beta_3$ antibody (data not shown). Thus, the clustering and centralization of PAC-1-occupied $\alpha_{IIb}\beta_3$ presumably results from occupancy of the ligand binding site rather than from the binding of an IgM. Further evidence for this came from experiments in which platelets were incubated with $40 \mu$g/ml PAC-1 for 2 min and then with 1.0 unit/ml thrombin for the indicated times. Incubations were terminated by the addition of paraformaldehyde. Platelets were permeabilized and incubated with polyclonal anti-$\alpha_{IIb}\beta_3$ antibodies. The distribution of PAC-1 and polyclonal antibodies were detected by confocal microscopy as described under "Materials and Methods." The images in the right-hand panels show the same platelets as are shown in the corresponding left-hand panels. In Panel F the outline of the platelets is revealed by labeling with $\alpha_{IIb}\beta_3$ antibodies; in Panel E, the location of PAC-1 in the same platelets is shown; comparison of the platelets indicated with arrows in the two panels shows that PAC-1 is concentrated toward the center of the platelets.

To determine whether the clustering and centralization of $\alpha_{IIb}\beta_3$ was induced by cytoplasmic actin filaments, platelets were preincubated with cytochalasins. Cytochalasins inhibit the burst of actin polymerization that occurs when platelets are activated (28, 29). Thin section electron microscopy (Fig. 3) revealed that concentrations of cytochalasin higher than those needed to inhibit actin polymerization also disrupted the pre-existing network of cytoplasmic actin filaments. Fig. 3A shows an untreated platelet with intact cytoplasmic actin filaments, whereas Fig. 3B shows that the membrane skeleton remained intact after cytochalasin treatment but that the cytoplasmic actin network was disrupted. In addition, biochemical experiments revealed that, while the amount of actin sedimenting from detergent lysates at low g forces (i.e. networks of cytoplasmic actin) was markedly reduced in cytochalasin-treated cells, the amount of membrane skeleton proteins and $\alpha_{IIb}\beta_3$ that sedi-

Fig. 1. Immunofluorescence confocal images showing the distribution of ligand-occupied $\alpha_{IIb}\beta_3$ (left column) and total $\alpha_{IIb}\beta_3$ (right column) in thrombin-stimulated platelets. Platelet suspensions were incubated with 40 $\mu$g/ml PAC-1 for 2 min and then with 1.0 unit/ml thrombin for the indicated times. Incubations were terminated by the addition of paraformaldehyde. Platelets were permeabilized and incubated with polyclonal anti-$\alpha_{IIb}\beta_3$ antibodies. The distribution of PAC-1 and polyclonal antibodies were detected by confocal microscopy as described under "Materials and Methods." The images in the left-hand panels show the same platelets as are shown in the corresponding right-hand panels. In Panel F the outline of the platelets is revealed by labeling with $\alpha_{IIb}\beta_3$ antibodies; in Panel E, the location of PAC-1 in the same platelets is shown; comparison of the platelets indicated with arrows in the two panels shows that PAC-1 is concentrated toward the center of the platelets.

Fig. 2. Immunofluorescence images showing the distribution of fibrinogen in activated platelets. A platelet suspension was agitated gently with 1.0 unit/ml thrombin and 200 $\mu$g/ml fibrinogen for 5 min. Platelets were then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in the presence of detergent. Fibrinogen was detected with polyclonal antibodies (A) and $\alpha_{IIb}\beta_3$ with a monoclonal antibody that binds to a site other than that occupied by fibrinogen (B). Panel A shows immunolabeled fibrinogen clustered in each individual platelet. Panel B shows the same field of platelets immunolabeled for $\alpha_{IIb}\beta_3$ which covers the entire surface of the cells. Compare the groups of platelets marked with arrows in the two panels. Clearly the fibrinogen (A) is more central in each platelet while the $\alpha_{IIb}\beta_3$ (B) extends right to the peripheries of the platelets.

Fig. 3. Electron micrographs showing the selective disruption of the cytoplasmic actin filaments by cytochalasin B. Platelets were preincubated alone (A) or in the presence of 2.5 $\times$ 10^{-4} M cytochalasin B (B) prior to lysis and preparation of the detergent-insoluble cytoskeletons for electron microscopy. In Panel A, the membrane skeleton is shown as a cortex enclosing cytoplasmic actin cables. This image is representative of about 20 different experiments. Panel B shows that cytochalasin B disrupted the cytoplasmic component of the cytoskeleton, but left the membrane skeleton intact.
m ented at high g forces (i.e. in the membrane skeleton fraction) was not detectably altered (data not shown). To determine the effect of disruption of the network of cytoplasmic actin filaments on the ligand-induced redistribution of \( \alpha_{IIb}\beta_3 \), cytochalasin-treated platelets were incubated with thrombin in the presence of PAC-1, and the distribution of PAC-1 was determined. Ligand-occupied \( \alpha_{IIb}\beta_3 \) still appeared to be present in clusters in the cytochalasin-treated cells (Fig. 4A). However, in contrast to the non-cytochalasin-treated cells (as seen in Figs. 1 and 2), the clusters of ligand-occupied integrin did not move inward but remained at the periphery of the cell (Fig. 4A). Fig. 4B shows the same field as seen in Fig. 4A, but now the platelet periphery is delineated by immunolabeled total \( \alpha_{IIb}\beta_3 \). These results suggest that in non-cytochalasin-treated platelets, ligand-occupied \( \alpha_{IIb}\beta_3 \) associates with cytoplasmic actin and that this association leads to the inwards movement of the occupied integrin.

**Localization of Ligand-occupied \( \alpha_{IIb}\beta_3 \)**—The images shown in Figs. 1 and 2 were obtained when platelets were permeabilized with Triton X-100 prior to the addition of the secondary antibodies. Although the intensity of the fluorescence was often weaker, the same clustering and centralization of ligand-occupied \( \alpha_{IIb}\beta_3 \) was detected when the permeabilization step was omitted (Fig. 5) (control experiments using antibodies to actin-binding protein, a protein known to be present only on the cytoplasmic side of the membrane, confirmed that the platelets were not permeabilized inadvertently in these experiments) (data not shown). These results suggested that the membranes of the surface-connected open canalicular system were the most likely location of the centralized receptor since the immunolabeling antibody could have gained access via the open channels. To determine if this was the case, thin-section electron microscopy of platelets that had been activated in the presence of PAC-1 and subsequently fixed, sectioned, and incubated with anti-mouse antibodies coupled to 5 nm of colloidal gold was performed. Because the PAC-1 was added prior to sectioning the amount of label in any one section was small. However, some PAC-1 was detected on the surface of the cell (where it would initially bind); any nonperipheral PAC-1 was present in the open canalicular system (Fig. 6, Panels A and B, arrows). When 125 gold particles were counted (representing staining of 127 platelets), 21 were present on the surface of the platelets, 93 in the open canalicular system, and 11 in intracellular areas. In platelets that had not been activated, a total of 13 gold particles were counted in 130 platelets; 3 particles on the surface, 3 in the open canalicular system, and 7 in the cytoplasm (data not shown). These data suggest that the cytoplasmic labeling represents background levels. The finding that ligand-occupied \( \alpha_{IIb}\beta_3 \) is present primarily in the open canalicular system, is consistent with the observation by others that fibrinogen is localized in the open canalicular system of activated platelets (30).

**Regulation of Fibrinogen Binding by the Cytoskeleton**—To determine whether the cytoplasmic actin filaments play a role...
in regulating the binding of ligand to \(\alpha_{\mathrm{IIb}}\beta_3\), platelets were incubated with cytochalasin under conditions in which the network of cytoplasmic actin filaments was disrupted and the subsequent ADP-induced binding of PAC-1 to platelets visualized by immunofluorescence (Fig. 7). The images in Panels A, B, and C were generated using similar exposures so that the relative amounts of PAC-1 binding to the platelets in cytochalasin-treated (Panel C) and -untreated cells (Panel B) could be visualized. Comparison of these images revealed that cytochalasin decreased the binding of PAC-1 to ADP-activated platelets. Similar results were obtained when platelets were activated with thrombin (data not shown).

The binding of PAC-1 was quantitated by flow cytometry. As shown in Fig. 8, cytochalasin inhibited the binding of PAC-1 to thrombin-activated platelets in a dose-dependent manner. Cytochalasin also inhibited binding of the natural ligand, fibrinogen, as shown by an inhibition of the thrombin-induced aggregation of a stirred platelet suspension (Fig. 9, top panel). This was a specific inhibitory effect, as shown by the lack of an inhibitory effect on the secretion of ATP (Fig. 9, bottom panel). The concentrations of cytochalasins required to inhibit aggregation (Fig. 9) were comparable to those required to inhibit PAC-1 binding (Fig. 8).

The binding of ligand to \(\alpha_{\mathrm{IIb}}\beta_3\) initially occurs in a reversible manner (it can be reversed by addition of EDTA), but with time it becomes irreversible (8, 9). To determine whether the cytoplasmic actin filaments are involved in regulating the reversible or irreversible component of binding, platelets were preincubated in the presence or absence of cytochalasin and activated with thrombin in the presence of PAC-1; after either 5 or 45 min, one aliquot was diluted into buffer while another was diluted into buffer containing EDTA. The irreversible component of binding was defined as the PAC-1 that remained bound to the platelets following dilution in EDTA, while the reversible binding was the difference between that which remained bound in EDTA and that which remained bound in buffer alone. As shown in Fig. 10, cytochalasin E inhibited the reversible binding of PAC-1 by approximately 50%. However, it almost completely inhibited the irreversible binding of PAC-1 to activated platelets.

**DISCUSSION**

The transmembrane signaling that occurs across integrins is of critical importance in a variety of events such as inflammation, embryonic development, arterial thrombosis, and hemo-

![Image](http://www.jbc.org/content/284/13/7008/F6.large.jpg)

**Fig. 6.** Electron micrographs showing the distribution of ligand-occupied \(\alpha_{\mathrm{IIb}}\beta_3\) in thrombin-stimulated platelets. Platelet suspensions were incubated with 40 \(\mu\)g/ml PAC-1 for 2 min and then incubated with 0.5 unit/ml thrombin for 15 min. Platelets were fixed with paraformaldehyde and embedded in LR White resin. Ultrathin sections were stained with immunogold to localize PAC-1. Panels A and B represent two different fields of the same sample. The arrows indicate the invaginations of the open canalicular system. Note the localization of PAC-1 to the open canalicular system.

![Image](http://www.jbc.org/content/284/13/7008/F7.large.jpg)

**Fig. 7.** Effect of cytochalasin on the PAC-1 binding to ADP-activated platelets. Suspensions of platelets were incubated for 20 min with 0.1% Me\(_2\)SO (Panels A and B) or with \(10^{-4}\) M cytochalasin E (CE) (Panel C). PAC-1 was then added to a concentration of 20 \(\mu\)g/ml. After 2 min, incubation with buffer (Panel A) or with 20 \(\mu\)g/ml ADP (Panels B and C) was initiated. Incubations were terminated after 3 min by the addition of paraformaldehyde. Platelets were lysed, and the distribution of PAC-1 detected with fluorescently labeled secondary antibodies as described under "Materials and Methods."
bilizing the integrin-ligand interaction. Taken together with our previous results in detergent lysates, these studies indicate that the cytoskeleton plays an important role in regulating the two-way signaling across aIIb3 in platelets.

Association of aIIb3 with the Cytoskeleton in Intact Cells—It has been known for many years that a subpopulation of aIIb3 cosediments with cytoplasmic actin filaments from detergent lysates of platelets that have aggregated (34, 35). It has been assumed that this sedimentation results from an association of aIIb3 with cytoplasmic actin filaments. The present study shows that this is the case. Thus, a subpopulation of aIIb3 clustered and moved into the depths of the open canalicular system in activated platelets; this movement was induced by the cytoplasmic actin filaments because if these filaments were disrupted with cytochalasins, the movement was prevented.

In our previous study, we found that aIIb3 only redistributed to the low speed detergent-insoluble fraction in platelets in which ligand had bound to the integrin; moreover, ligand binding alone was not sufficient and the platelets needed to aggregate, presumably because ligand-induced cross-linking of the integrin was needed (36). In the present study, we used the fibrinogen mimetic monoclonal antibody PAC-1 (which is a pentameric molecule and therefore induces signaling comparable to that induced by fibrinogen in a platelet aggregate rather than that induced by fibrinogen in a nonaggregating suspension) (37). Dual-labeled immunofluorescence allowed us to show that only aIIb3 that had bound ligand moved inward. The rest of the aIIb3 remained at the periphery of the cell. We conclude, therefore, that the association of an aIIb3 molecule with cytoplasmic actin in aggregating platelets is a direct consequence of ligand binding to that molecule of integrin. One can envisage a physiological mechanism in which the selective movement of ligand-occupied integrin inward into the open canalicular system allows the externally bound fibrin clot to be pulled inward.

Others have used fibrinogen-coated gold beads or soluble fibringen to study movements of aIIb3 on adherent platelets (38–49). The distribution of gold beads on platelets activated in suspension has also been studied (40, 41, 50–52). These studies have revealed that aIIb3 that had bound gold beads moves inward. Although the physiological relevance of the movement of gold beads on the platelet surface has been questioned (53) and the functional state of integrins or signaling molecules on surface-activated platelets as compared to platelets activated with a physiological agonist is completely unknown, it is of interest that in all cases only the occupied receptor moves inward.

Fig. 8. Effect of cytochalasin E (CE) on the PAC-1 binding to platelets. Suspensions of platelets (1 × 10⁸ platelets/ml) were incubated with 0.1% Me₂SO or with the indicated concentrations of cytochalasin E in the presence of 0.1% Me₂SO for 30 min. Platelets were subsequently incubated in the presence of 20 µg/ml FITC-labeled monoclonal antibody PAC-1, either with no further addition (Control) or with the addition of 0.1 unit/ml thrombin. Following an incubation of 60 s, samples were diluted 100-fold with a Tyrode’s solution, and the amount of FITC-labeled PAC-1 bound to the platelets was detected by flow cytometry. This figure is representative of the results of six different experiments.

Fig. 9. Dose-dependent inhibition of aggregation by cytochalasin E. Suspensions of platelets (3 × 10⁸ platelets/ml) were preincubated for 10 min in the presence or absence of cytochalasin E at the concentrations shown. Cytochalasin was added in a final volume of 0.1% Me₂SO, which was also present in the control incubation. Luciferin-luciferase reagent was then added and the platelet suspensions stirred with 1.0 NIH unit/ml thrombin in an aggregometer. The aggregation of platelets was detected as an increase in the transmittance of light through the suspension (top panel); secretion of ATP was detected as an increased luminescence (bottom panel).
Selective Association of Membrane Skeleton-associated α₁bβ₃ with Cytoplasmic Actin—In our previous biochemical experiments (21), we noticed that in unstimulated platelets a subpopulation of α₁bβ₃ co-sedimented with membrane skeleton proteins at high forces (about 30% of the total platelet α₁bβ₃). As platelets bound ligand, it was this population that initially redistributed to the low speed detergent-insoluble fraction. The present study supports the idea that only a subpopulation of α₁bβ₃ is induced to bind ligand and to associate with cytoplasmic actin filaments in the intact cell. Taken together with the previous biochemical findings, this suggests that the α₁bβ₃ that sediments with membrane skeleton proteins from unstimulated platelets represents a pool of integrin that can be selectively induced to bind ligand. The finding that the α₁bβ₃ that bound ligand became clustered even when the networks of cytoplasmic filaments were depolymerized with cytochalasins (which did not disrupt the membrane skeleton) is also consistent with the possibility that it is the α₁bβ₃ that is associated with membrane skeleton proteins that is selectively induced to bind ligand. Because the membrane skeleton is in close contact with the plasma membrane and associates with both signaling molecules (21) and α₁bβ₃ (21), it appears possible that this structure serves to localize signaling molecules that are involved in the activation of the integrin. Similarly, the association of the integrin with this structure may be important in allowing the selective association of ligand-occupied integrin with underlying cytoplasmic actin filaments. Future work will be needed to find out how the integrin associates with submembranous skeletal proteins and to directly test the hypothesis that this association allows the activation and ligand-induced association of the integrin with cytoplasmic actin.

Immunofluorescence experiments have revealed that in cultured cells, binding of an integrin to its ligand in the extracellular matrix causes it to cluster and become incorporated into complexes of cytoskeletal proteins and signaling molecules known as focal contacts (54). The selective clustering of ligand-occupied α₁bβ₃ and its association with cytoplasmic actin filaments in platelets is reminiscent of the formation of focal contacts. Several of the proteins that co-sediment with α₁bβ₃ from detergent lysates of unstimulated platelets and redistribute with α₁bβ₃ into the low-speed detergent-insoluble fraction from activated platelets are proteins that have been found to co-localize with integrins in focal contacts (e.g. vinculin, talin, and pp60c-src) (54). Additional components of focal contacts (e.g. protein kinase C) incorporate into the low-speed pellet in aggregating platelets (22). Thus, the cytoskeletal reorganizations that are induced as a consequence of ligand binding to α₁bβ₃ in platelets may be similar to those that form as a consequence of integrin-ligand interactions in cultured cells, and similar signaling mechanisms may be involved.

Regulation of Transmembrane Signaling—α₁bβ₃ can exist in a number of different affinity states, and several steps may be involved in the activation of the integrin and subsequent stabilization of ligand binding (55–57). As discussed above, the present immunofluorescence findings combined with the previous findings in detergent lysates (21) indicate that the membrane-skeleton associated α₁bβ₃ is selectively induced to bind ligand. However, the finding that concentrations of cytochalasins that disrupted the cytoplasmic actin filaments inhibited ligand binding suggests that the cytoplasmic actin filaments also play an important role in regulating ligand binding to α₁bβ₃. Previously, variable effects of cytochalasins on fibrinogen binding (58, 59), aggregation (58), and fibrinogen-gold distribution (40, 41, 44) have been reported. These variations may have resulted from the use of cytochalasins at concentrations that had variable effects on the cytoplasmic actin filaments. In the present study, we determined concentrations of cytochalasins that were needed not only to inhibit the increased polymerization of actin that occurs when platelets were activated (28, 29) but also to disrupt the preexisting networks of cytoplasmic actin (60). These concentrations of cytochalasins almost totally prevented the conversion of the binding of fibrinogen from reversible to irreversible. In addition, they partially inhibited the reversible component of the binding. In cultured cells, the formation of focal contacts plays a role in stabilizing integrin-ligand interactions (54). Thus, in platelets, the formation of the “focal contact-like” integrin-cytoskeletal complexes may be important in rendering the binding of ligand irreversible. Associations between the cytoplasmic actin filaments and components of the membrane skeleton probably exist even in the unstimulated platelets (19, 20). Thus, one possible mechanism by which the cytoplasmic actin filaments could exert their regulatory influence on the earlier, reversible stages of ligand binding might be their association with membrane skeleton...
functions which in turn might associate with α1β2 and signaling molecules.

Summary—Taken together with the results of our previous biochemical studies, the present studies indicate that the subpopulation of α1β2 that is associated with the membrane skeleton is preferentially activated, that it subsequently becomes incorporated into complexes with cytoplasmic actin filaments, and that the formation of these integrin-rich cytoskeletal complexes plays a role in stabilizing the ligand-integrin interactions, inducing a selective redistribution of occupied integrin, and inducing activation of signaling molecules. Future studies will be needed to elucidate the molecular nature of the interactions between α1β2 and the membrane skeleton and to identify the mechanisms by which integrin-cytoskeletal complexes regulate the post-occupancy events in activated platelets.

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