This study investigates the involvement of actin microfilaments in fibrinogen receptor redistribution and cytoskeletal reorganization that takes place in fully spread, surface-activated platelets. Colloidal gold-labeled fibrinogen (Fgn-Au label) in conjunction with video-enhanced differential interference contrast light microscopy (VDIC) was used to identify fibrinogen binding sites, glycoprotein IIb/IIIa (GPIIb/IIIa), on fully spread platelets. Platelets were treated with cytochalasin D and E (5 x 10^-5 mol/L to 5 x 10^-4 mol/L) for 10 minutes, before or after incubation with Fgn-Au label. Results observed with VDIC were subsequently confirmed by high-voltage transmission and low voltage-high resolution scanning electron microscopic examination of the specimens. Preincubation of activated platelets with cytochalasin B caused decentralization of previously redistributed receptor-ligand complexes. Incubation of platelets with cytochalasin E 5 x 10^-4 mol/L prevented platelet activation and spreading. Thus, actin filaments appear necessary for platelet spreading from the discoid to the fully spread stage. The ligand-triggered, cytoskeletally directed movement of fibrinogen receptors in fully spread platelets appears to be dependent on the presence of intact, polymerized actin. This movement is distinct from the cytochalasin-insensitive accumulation of GPIIb/IIIa-ligand in the channels of the open canalicular system.

The role of cytoplasmic actin filaments in the movement of GPIIb/IIIa-ligand complexes on platelet surfaces is not entirely clear. Linkage of some platelet membrane receptors directly or indirectly to the cytoskeleton has been strongly suggested by several previous reports.13,16 Other recent reports suggest that movement of receptor ligand complexes into channels of the OCS is cytochalasin-sensitive and may be related to the membrane cytoskeleton rather than the cytoplasmic microfilament cytoskeleton.17,18 White and Escolar19 report that cytochalasin B does not block the centripetal movement of fibrinogen-receptor complexes on activated platelets. However, they also found that cytochalasin B caused decentralization of previously centralized receptor-ligand complexes accompanied by the formation of patches of labeled receptors.19 These patches were reportedly associated in some instances with remnants of the OCS. The centripetal movement of the ligand-bound GPIIb/IIIa in the fully spread platelet,20,21 in contrast to movement of ligand-bound receptors into the OCS on platelets at earlier stages of activation, may indicate an increase in the number of cytoskeletally linked GPIIb/IIIa complexes at this later stage of activation. In support of this view, Painter et al20 have reported linkage of actin to only 15% of GPIIb/IIIa in unactivated human platelets. Activation of platelets has been shown to result in increased actin polymerization21,22 and thrombin-induced platelet aggregation results in increased GPIIb/IIIa associated with actin filaments.16

The polymerization of globular actin to form actin
filaments is critical for many nonmuscle cell functions. Various studies suggest that the cytochalasins inhibit polymerization by binding to the growing ends of actin filaments and blocking the further addition of actin monomers.

Cytochalasin D has been shown to inhibit actin polymerization and induce depolymerization of actin filaments formed during thrombin-induced platelet shape change. It has been shown that cytochalasin D and E have a higher affinity than cytochalasin B for actin, and, unlike cytochalasin B, do not inhibit glucose transport into cells. Studies suggest that the cytochalasins inhibit poly-
gen. Aliquots were mixed into the boiling solution and the mixture refluxed for 30 minutes. During heating, the solution changed from a blue to an orange-red color indicating the formation of the monodispersed colloid. Inadequate stabilization of the colloid resulted in aggregation of the gold granules, as indicated by a color change from red to blue. The minimum amount of fibrinogen necessary to stabilize the gold colloid was 8 μg/mL of gold solution.

Ten milliliters of gold solution was added to a 10% excess of dialyzed fibrinogen (0.005 mol/L NaCl) with gentle stirring. After 5 minutes at room temperature, 0.5 mL of freshly prepared and prefilttered (Millipore 0.45 μm; Millipore Inc, Bedford, MA) 1% polyethylene glycol (molecular weight [MW] 20,000) was added to prevent aggregation. The gold-labeled fibrinogen was centrifuged in polycarbonate tubes at 11,000g for 30 minutes. The supernate was discarded and the concentrated red pool was resuspended to 1 mL with sterile-filtered (Millipore Millex-GS 0.2 micron filter unit) protein free Tyrode’s buffer supplemented with 1 mmol/L calcium. Tissue. Blood samples (10 mL) were collected in polypropylene tubes containing 10 EGTA (ethylene glycol-bis-[ amino ethyl ether]-N,N'-tetraacetic acid) and mixed by gentle inversion. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 180g for 15 minutes at room temperature (24°C ± 1°C) immediately after collection. PRP was removed with a polyethylene pipette, and platelets were separated from plasma proteins by passage through a Sepharose CL-2B (Sigma) column having a 40 mL bed volume. The column was equilibrated at room temperature with a calcium-free Tyrode’s buffer, pH 7.3. The buffer contained: 136 mmol/L NaCl, 2.7 mmol/L KC1, 0.42 mmol/L NaH₂PO₄, 0.12 mmol/L NaHCO₃, 2 mmol/L MgCl₂, 6H₂O, 1 g/L D-glucose, and 2 g/L bovine serum albumin. Platelets were collected in plastic tubes and kept at 37°C until used.

Video microscopy. Live platelets were observed with a video-enhanced differential-interference-contrast (VDIC) light microscope. Formvar-coated grids were placed on no. 1.1 coverslips (typically 24 × 40 mm) and affixed with thin strips of double-sided tape overlying the edges of the grid. Layers of glass strips cut from coverslips were placed on two sides of the grids as shims. A single coverslip (18 × 18 mm, no. 1) was placed on top of the shims and sealed in place with melted VALAP, a 1:1 mixture of vaseline, lanolin, and paraffin. The resultant chamber was open at two ends to enable introduction and exchange of media by capillary filling.

Column-purified platelets were allowed to surface activate for 20 minutes on formvar-coated grids at 24°C ± 1°C. They were treated with concentrations of cytochalasin D or E between 5 × 10⁻⁴ mol/L and 5 × 10⁻⁶ mol/L, for 5 minutes or 10 minutes before, or after incubation with colloidal gold-labeled fibrinogen for 10 minutes (final concentration of DMSO in contact with the platelets was 1%, 0.1%, 0.01%, and 0.001% respectively, in Tyrode’s buffer). The effects of the cytochalasins on fibrinogen receptor redistribution were monitored by VDIC throughout the experiments. For control experiments, surface-activated platelets were treated with 1%, 0.1%, 0.01%, or 0.001% DMSO in protein-free Tyrode’s buffer for 10 minutes before or after incubation of the platelets with Fgn-Au labels.

The effect of cytochalasin E on platelet activation and shape change was also investigated. Column-purified platelets were treated with 5 × 10⁻⁴ mol/L cytochalasin E, and then allowed to spread on formvar-coated grids for 10 minutes. The grids were rinsed with 0.1% DMSO in Tyrode’s buffer and specimens were observed on the microscope for another 5 minutes before fixation with glutaraldehyde. In another set of experiments, platelets were allowed to activate on formvar-coated grids for 10 minutes, then treated with cytochalasin E, 5 × 10⁻⁴ mol/L, for another 10 minutes. Specimens were subsequently prepared for electron microscopy.

Electron microscopy. All specimens were labeled while living, subsequent fixation was in 1% glutaraldehyde (Tousimis Research Corp, Rockville, MD) buffered with 0.1 mol/L HEPES containing 0.2% tannic acid and 0.05% saponin (pH 7.3) for 30 minutes at 24°C ± 1°C. The specimens were postfixed in 0.1 mol/L HEPES-buffered 0.05% osmium for 15 minutes at room temperature and stained with aqueous 1% uranyl magnesium acetate for 15 minutes. Samples were dehydrated through a graded series of ethyl alcohol (15%, 20%, 30%, 50%, 70%, 80%, 85%, 90%, 95%, for 3 minutes each) to absolute ethanol, itself dried by storage over molecular sieve. The specimens were dried in a critical point dryer equipped with an in-line molecular sieve filter (no. 8782; Tousimis Research Corp) and a hydrophobic, water-excluding filter to remove particulates, oils, and traces of water from the liquid CO₂, then stored over molecular sieve until examined with the AEI EM7 1 MeV electron microscope of the University of Wisconsin-Madison, IMR facility. Stere pair micrographs were taken at tilt angles appropriate for specimen thickness and magnification. The speci-
mens were later examined with a modified Hitachi S900 lowvoltage high-resolution scanning electron microscope (LV-HR-SEM; Hitachi Ltd., Tokyo, Japan).

RESULTS

Colloidal gold-labeled fibrinogen can be seen by VDIC to bind near the periphery of spread platelets and move across the platelet surface in the plane of the membrane, forming a band of label surrounding the granulomere.\textsuperscript{2,6,9,36} Individual 18-nm gold labels, although they are too small to be actually resolved by the light microscope, can be detected, and their movement followed, by their inflated diffraction image.\textsuperscript{9,36} Concentrations of gold on the platelet surface or in the OCS appear as black areas in the VDIC (Fig 1, a through d). Subsequent observation by low-voltage high-resolution scanning electron (LV-HR-SEM) and high-voltage electron (HVEM) of the same platelets as were observed by VDIC during labeling allows confirmation of the identity and final locations of the gold labels and correlation of the positions of the labels with platelet ultrastructure (Fig 1e). Treatment of platelets with 1.0% or 0.1% DMSO (the cytochalasin vehicle) in Tyrode’s buffer for 10 minutes, either before or after incubation with colloidal gold-labeled fibrinogen, did not inhibit or reverse movement of the ligand-receptor complexes to the IFZ (Fig 1, a through e). The velocity of the ligand-receptor complexes is 20 nm/s over a 2.0 to 2.5 μm distance, depending on how near the platelet margin initial attachment occurs.\textsuperscript{9,40}

Both cytochalasin D and E inhibited the centripetal movement of Fgn-Au across the surface of fully spread platelets, incubated with colloidal gold-labeled fibrinogen, in a concentration-dependent manner.\textsuperscript{41} Treatment of platelets with (5 × 10^{-5} mol/L and 5 × 10^{-6} mol/L) cytochalasin D or E before incubation with Fgn-Au did not prevent binding of the ligand to its receptor; however, receptor redistribution was inhibited in 97% to 100% of the platelets (Table 1). In 100 randomly counted, fully spread, surface-activated platelets, pretreatment with 5 × 10^{-7} mol/L cytochalasin D resulted in only a slight inhibition

![Fig 1. VDIC-LM micrographs of spread platelets (a) and (b) before addition of Fgn-Au label, (a) before incubation with DMSO, (b) after 7 minutes of incubation with DMSO, (c) and (d) at 4% and 9 minutes, respectively, after labeling. Specimen was treated with 0.1% DMSO for 10 minutes before incubation with colloidal gold-labeled fibrinogen. As ligand-receptor complexes move centripetally in the plane of the membrane (c and d), gold particle density increases. (e) HVEM stereo pair whole mount of the spread platelet in (a through d). Zonal organization of the cytoskeleton is apparent: proceeding inward from the cell margin the zones are: (PW) a non-substrate-attached peripheral web of fine filaments; (OFZ) an outer filamentous zone consisting of an open meshwork of microfilaments; and (IFZ) the inner filamentous zone: a densely packed, overlapping network of actin filaments which encircles the granulomere region (G) consisting of granules suspended within a microfilament meshwork. Ligand-receptor complexes (small dark spheres) are localized on the membrane overlying the IFZ. Bar = 1 μm.](image-url)
(2.7% ± 0.88%). Greater inhibition of receptor movement (13.7% ± 3.5%) was seen with cytochalasin E, 5 × 10⁻⁸ mol/L (Table 1). Pretreatment with 5 × 10⁻⁸ mol/L cytochalasin D or E for 10 minutes, followed by incubation with colloidal gold-labeled fibrinogen, did not inhibit redistribution of ligand-receptor complexes on fully spread platelets.

After treatment of platelets with cytochalasin D or E (5 × 10⁻⁵ mol/L or 5 × 10⁻⁸ mol/L) the distinct ultrastructural zones characteristic of the cytoskeleton of fully spread platelets could not be clearly distinguished. The IFZ, a region of dense highly anastomotic actin filaments, was disrupted (Fig 2a). Treatment of spread platelets with cytochalasin E is not inhibited by cytochalasin B. Hence, movement of gliand-receptor complexes was determined. The effects of cytochalasins D and E were determined. These agents that have been reported to inhibit actin polymerization and are capable of inducing depoly-
CYTOCHALASIN EFFECTS ON PLATELET RECEPTORS

Fig 2. (a) HVEM (1 MeV accelerating voltage) stereo pair whole mount of a fully spread platelet treated for 10 minutes with cytochalasin D, $5 \times 10^{-6}$ mol/L, before incubation of the specimen with colloidal gold-labeled fibrinogen. The distinct ultrastructural zones characteristic of fully spread platelets as seen in Fig 1e cannot be clearly defined following treatment with this agent. The label (small dark spheres) is seen individually and in clusters over the entire platelet membrane. Bar = 1 pm. (b) 20 keV accelerating voltage LV-HR-SEM of the same platelet as in (a). Note the uniform distribution of label, small white spheres, and the scattered granules (arrows) over platelet. Bar = 1.15 pm.

The results obtained in these experiments are consistent with a model of filamentous actin-dependent fibrinogen receptor movement in spread platelets. Incubation of spread platelets with cytochalasin D or E ($5 \times 10^{-6}$ mol/L and $5 \times 10^{-7}$ mol/L) caused inhibition of centripetal fibrinogen receptor movement and decentralization of previously redistributed fibrinogen-receptor complexes, in both cases with disruption of the platelet cytoskeletal reorganization. The effect was relatively rapid and could be observed as early as 2 minutes after exposure of the platelets to cytochalasin. Ligand-receptor complexes are observed by VDIC light microscopy to drift out of the IFZ individually or as small clusters or “islands” of receptor complexes. Correlative examination of the same platelets by LV-HR-SEM and HVEM showed these islands of receptors to sometimes be attached to remaining short strands of poorly organized microfilaments. These concentrations of cytochalasins D and E are similar to those that bind to actin filaments and cause actin depolymerization in platelets. A previous report showed that addition of cytochalasin D ($5 \times 10^{-6}$ mol/L) to platelets which had previously been stimulated with thrombin resulted in a return within 3 minutes to G-actin levels similar to those of unstimulated platelets and a decrease in the amount of actin incorporated into the triton-insoluble residue.

Thus, movement as well as maintenance of the final position of the fibrinogen-GPIIb/IIIa complex on the surfaces of fully spread platelets appears to require linkage of the fibrinogen-GPIIb/IIIa complex to the intact actin filament network of the cytoplasmic cytoskeleton. Disruption of the actin filament network by cytochalasin does not appear to prevent fibrinogen binding to GPIIb/IIIa but blocks subsequent ligand-triggered movement. In addition, when movement of receptor-ligand complex has occurred and final location has been reached, disruption of the actin network leads to the “drifting” of ligand-receptor complexes to apparently random positions throughout the membrane. Although the specimens were prepared for electron microscopy by methods that provided good preservation of both cytoskeletal elements and membranes, we were unable to confirm an earlier report of the associations...
between these clusters of receptors and remnants of OCS where these persisted. By using the VDIC light microscopy to track the movement of the Au-fgn-receptor in living platelets, we have observed that the OCS, if present, fills with Au-fgn at the same time as the centralization process. Subsequent addition of cytochalasin causes decentralization of receptor-ligand complexes over the platelet surface.

As previously noted, some receptor-ligand complexes move on the surface as “islands” associated with subsurface cytoplasmic remnants. No change in the amount of filled OCS or in the number of receptor-ligand complexes in the OCS after cytochalasin treatment is observed. That is, receptor-ligand complexes do not move out of the OCS. The VDIC allows tracking the dynamic nature of these...
Fig 4. (a) HVEM stereo pair permits viewing of label relative to underlying internal ultrastructural features. Specimen was incubated with Fgn-Au label for 10 minutes followed by treatment with cytochalasin E, $5 \times 10^{-8}$ mol/L. Previously redistributed Fgn-Au-labeled receptors have decentralized and are distributed over the entire platelet surface. Ligand-receptor complexes that entered the channels of the open canalicular system (arrows) appear insensitive to cytochalasin treatment and have not moved. Bar = 1 μm. (b) 20 keV LV-HR-SEM of same platelet as in (a). Atomic number contrast apparent at this accelerating voltage enables clear identification of the gold label on the surface and in the OCS. Bar = 1 μm. (c) 1.2 keV LV-HR-SEM of same platelet as in (a and b). Visualization of the surface localization of the label is facilitated at this lower accelerating voltage. Bar = 0.83 μm.
movements before electron microscopic observation while procedures using addition of Fgn-label and visualization of only the final result by electron microscopy alone, may be more difficult to interpret. Movement of ligand-receptor complexes into the OCS takes place over a relatively short range. The receptors are not attracted to openings into the OCS from the entire platelet surface, but rather are cleared from the membrane immediately surrounding the openings.

The decentralization of previously centralized receptor-ligand complexes in response to cytochalasin treatment of spread platelets appears to be due to the disruption of the cytoplasmic actin filament network to which the receptors are tethered at this stage of platelet activation. It is not clear whether surface receptors become totally “unlinked” from the cytoskeletal elements, or remain linked to disorganized cytoskeletal remnants which diffuse in the cytoplasm beneath the membrane, as reflected by ligand-receptor movement in the plane of the membrane. Both individual label and small clusters can be seen. In the case of clusters, they often appear over areas of subjacent microfilament remnants. Disruption of microfilament structure would also eliminate the possibility of movement of the receptor-ligand complex via an actin-myosin interaction. This may occur by actin filament breakdown and/or by inhibition of the actin-myosin interaction. Previous studies in our laboratory indicate that agents which prevent myosin phosphorylation also inhibit the ligand-receptor movement.

Together, these findings can be interpreted to suggest a nonmuscle actin-myosin force-generating system which causes the movement of receptors that are tethered to actin filaments. Another possible mechanism for this receptor-ligand movement is an actin treadmilling process. By decreasing the average filament length through a change in the steady state balance between the net polymerizing ends, actin monomers can flow through an actin filament treadmill and thereby provide a mechanism for movement of proteins that may be attached to the actin subunits. Margolis and Wilson have proposed a very similar mechanism for the polymerization of microtubules and for its inhibition by colchicine. Evidence has also been provided to show that cytochalasin D blocked the rearward movement of surface-bound particles that are linked to the cytoskeleton while internal organelle movements persisted, thus showing that the integrity of the actin filament is required for retrograde transport of surface-bound particles.
The short-range, cytochalasin-independent movement of labeled receptors into the OCS occurs in platelets activated in suspension that have not attached to a surface and in adherent platelets that have not reached the fully spread stage. In adherent, fully spread platelets, ligand-induced receptor movement is generally the longer range, surface redistribution, from the periphery to the center of the platelet, which is cytoplasmic actin-dependent. The OCS is often not present or present in reduced amount in the fully spread platelet. This suggests that the movement of gold-labeled receptors into the OCS differs from the centripetal surface movement seen on spread platelets both in mode of action and in physiological function. Clearing of fibrinogen-bound receptors into the OCS does not require the assembly of cytoplasmic actin and can therefore occur on platelets that are not fully activated. Round or dendritic platelets in suspension have considerable OCS, thus most areas of the platelet surface are not far from openings to the OCS. This form of movement is therefore particularly effective for the clearing of particulate material and the secretory function associated with this stage of platelet activation.

Once a platelet is adherent and becomes fully spread, the OCS is greatly diminished or absent. Most areas of the membrane are far from openings into the OCS. The long-range, cytoplasmic cytoskeletonally directed centripetal movement, facilitates platelet-platelet interactions, important at this stage of activation. The interaction of nonadherent, dendritic platelets in suspension, with fully spread adherent platelets appears to occur by a fibrinogen bridging mechanism. The triggering of directed, centripetal movement of the Fgn-receptor complexes on the adherent platelets, moves and facilitates clustering of dendritic platelets, providing a mechanism for the initiation of thrombus formation. This is particularly evident in thrombus forming on biomaterial surfaces.

It is also important to note that incubation of platelets with cytochalasin E (5 × 10^-6 mol/L) inhibited platelet activation and the progress of spreading. The results obtained in this study suggest that this effect of cytochalasin E is dependent on its ability to inhibit actin polymerization and hence significantly inhibit the initial platelet shape change from discoid to the spread dendritic stage. While 83% of the platelets remained discoid or spherical, only 17% progressed to the dendritic stage. Treating platelets...
with this agent after the initiation of spreading prevented most of the platelets from reaching the fully spread stage; 6% of treated platelets became fully spread as compared with 38% of control, DMSO-treated platelets. White et al.,\textsuperscript{17,18} using cytochalasin B, have previously reported findings similar to those we have obtained regarding the effect of cytochalasin E on platelet spreading.

Many laboratories have reported that 70% to 80% of actin in thrombin-activated platelets is filamentous\textsuperscript{19,20} while only 40% to 50% of the actin in unactivated platelets is in the filamentous form.\textsuperscript{21,22} Activation involves mechanisms that increase polymerization of monomeric actin to filaments. Results from the present study suggest that these mechanisms are inhibited by treatment of platelets with cytochalasin E. Progress of platelet shape change from the dendritic to the fully spread stage involves reorganization of actin filaments.\textsuperscript{23} Our results involving platelets treated with cytochalasin E after spreading has begun to indicate that this agent inhibits the progress of platelet spreading from the dendritic to the fully spread stage by preventing reorganization of actin filaments, or by causing depolymerization of existing filaments.

We feel the studies reported herein clearly demonstrate significance of polymerized cytoplasmic actin in the process of platelet spreading as well as the peripheral to central movement of receptor-ligand-Au seen in fully spread adherent platelets.

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