Redistribution of the Fibrinogen Receptor of Human Platelets after Surface Activation

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ABSTRACT   We investigated the whole cell distribution of the platelet membrane receptor for fibrinogen in surface-activated human platelets. Fibrinogen-labeled colloidal gold was used in conjunction with platelet whole mount preparations to visualize directly the fibrinogen receptor. Unstimulated platelets fail to bind fibrinogen, and binding was minimal in the stages of activation immediately following adhesion. The amount of fibrinogen bound per platelet increased rapidly during the shape changes associated with surface activation until 7,600 ± 500 labels were present at saturation. Maximal binding of fibrinogen was followed by receptor redistribution. During the early stages of spreading, fibrinogen labels were uniformly distributed over the entire platelet surface, including pseudopodia, but the labels become progressively centralized as the spreading process continued. In well spread platelets, labels were found over the central regions, whereas peripheral areas were cleared of receptors. Receptor redistribution during spreading was accompanied by cytoskeletal reorganization such that a direct correlation was seen between the development of specific ultrastructural zones and the distribution of surface receptor sites suggesting a link between the surface receptors and the cytoskeleton. The association of fibrinogen receptors with contractile elements of the cytoskeleton, which permits coordinated receptor centralization, is important to the understanding of the role of fibrinogen in normal platelet aggregation and clot retraction.

Platelet aggregation is essential for normal hemostasis. Recent investigations have indicated there is a strong correlation between fibrinogen binding to activated platelets and aggregation (9, 30, 37, 45, 47). Fibrinogen is a required co-factor in platelet aggregation induced by ADP, since suspensions of gel-filtered or washed human platelets fail to aggregate in response to ADP in the absence of fibrinogen (9, 21, 29, 33). Further evidence for a role of fibrinogen in aggregation is illustrated by the observation that platelets in citrated plasma from afibrinogenemic patients aggregate poorly in response to ADP and epinephrine (19, 48, 49). The mechanism by which fibrinogen mediates platelet aggregation is unknown, but it is most likely a function of the association of fibrinogen with the platelet surface, since aggregation involves the surface membranes of adjacent platelets (30, 49). The direct association of fibrinogen with specific membrane glycoproteins is supported by studies with platelets from Glanzmann's thrombasthenia. Platelet membrane glycoproteins IIb (GPIIb) and IIIa (GPIIIa) are markedly diminished in this inherited bleeding disorder (39), and platelets from these patients do not bind fibrinogen and do not aggregate (3, 8, 22, 31). Recent

studies by Marguerie and co-workers (24, 25), Bennet and Vilaire (3), and Plow and Marguerie (41) have confirmed that fibrinogen binds to specific saturable receptor sites on the platelet membrane. In these studies, using radiolabeled fibrinogen, binding is not detectable on unstimulated platelets, but fibrinogen binding is induced by activation with ADP and epinephrine. Peerschke et al. (37) showed binding of radiolabeled fibrinogen to activated platelets correlates well with the time course of ADP-induced aggregation. The interaction of fibrinogen with its membrane receptor has been characterized with regard to the effects of temperature, pH, and the requirement for divalent cations (26). Association constants have been estimated by Scatchard plot analysis of saturable binding curves (3, 24, 26, 37, 41).

In this study, we report the use of fibrinogen-labeled colloidal gold (FGN-Au)[1] to visualize directly the human platelet membrane receptor for fibrinogen by scanning and high-

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[1] *Abbreviations used in this paper:* FGN-Au, fibrinogen-labeled colloidal gold; HVEM, high-voltage electron microscopy; PHEM, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$.

voltage electron microscopy (HVEM). The temporal changes in the whole cell distribution of the fibrinogen receptor correlate with the cytoskeletal reorganization that accompanies the shape change or spreading process that platelets exhibit in response to a variety of stimuli including an artificial surface. The results support an association between the fibrinogen receptor and the cytoskeleton and provide additional insight into the possible mechanisms of action of fibrinogen in platelet aggregation and clot retraction.

MATERIALS AND METHODS

Preparation of Colloidal Gold: A stock solution of colloidal gold granules having an average diameter of 18 nm were prepared by reducing HAuCL$_4$ with trisodium citrate (12–14, 17). A 4% solution of HAuCL$_4$ (0.5 ml) was added to 200 ml of deionized distilled water and brought to a boil. A freshly prepared solution of 1% trisodium citrate (5 ml) was rapidly added to the boiling solution and the mixture refluxed for 30 min. The formation of the monodisperse colloidal particles was indicated by a dark blue to red color change. The colloidal solution was cooled, filtered through a microporous filter (Millipore Millex-GS 0.22-μm filter unit), and stored at 4°C under sterile conditions.

Preparation of Fibrinogen, Fibrinogen-Gold, and Antibody-Gold Complexes: Fibrinogen was a gift of Dr. Deane Mosher (University of Wisconsin). It was purified from fresh human citrated plasma by precipitation with 25% saturated ammonium sulfate followed by DEAE-cellulose chromatography (28). The final product was dialyzed against 0.01 M Tris and 0.14 M NaCl, pH 7.4 Aliquots of 10–15 mg/ml were stored at −70°C.

Absorption isotherms (13, 16) were performed to determine the minimum amount of fibrinogen necessary to stabilize the colloidal gold. Previous investigations have indicated that optimum absorption occurs at or slightly basic to the pI of the protein. The gold solution was adjusted to pH 6.5 with 0.2 N K$_2$CO$_3$ as measured by gel-filled combination electrode (No. 9115, Orion Research, Inc., Cambridge, MA). A series of fibrinogen solutions of increasing concentration were made up to 1 ml and added to 5 ml of colloidal gold. After 1 min at room temperature, 1 ml of 10% NaCl solution was added to the fibrinogen-gold complex. Inadequate stabilization of the colloid results in flocculation of the gold granules with flocculation indicated by a color change from red to blue, which can be judged visually or spectrophotometrically. The minimum amount of fibrinogen necessary to prevent flocculation was 8 μg/mg of gold solution.

10 ml of gold solution (pH 6.5) was added to a 10% excess of dialyzed fibrinogen (0.005 M NaCl) and mixed by gentle inversion. After 5 min at room temperature, 0.5 ml of freshly prepared and prefiltered (Millipore 0.45 μm) polyethylene glycol 20,000 mol wt was added to prevent aggregation. The FGN-Au was concentrated and excess fibrinogen removed by centrifugation in polycarbonate tubes at 10,000 rpm for 30 min. The supernate was discarded, and the concentrated red pool was resuspended to 1 ml with sterile filtered (Millipore 0.2 μm) protein-free Tyrodes buffer supplemented with 1 mM Ca^{++}.

The monoclonal antibody 10E5, which is directed against the GPIIb-IIIa receptor complex, was a gift from Dr. Barry Coller (State University of New York at Stonybrook Health Sciences Center). The specificity of 10E5 has been fully characterized in a previous report (7). The minimum amount of antibody necessary to stabilize the gold particles was determined by absorption isotherms as described above. After conjugation and centrifugation, the antibody-gold complex (10E5-Au) was resuspended to 1 ml with sterile filtered (Millipore 0.2 μm) 0.1 M phosphate buffer; pH 7.4.

Platelet Preparation and Gold Labeling: Platelets were obtained from normal healthy adult volunteers by venous puncture. Blood samples (10 ml) were collected in polypropylene tubes containing 10 mM EGTA and mixed by gentle inversion. Platelet-rich plasma was prepared by centrifugation of whole blood at 180 g for 10 min at room temperature. Platelets were separated from plasma proteins by passage through a Sepharose C1-2B column (46) having a 40-ml bed volume. The volume was equilibrated at room temperature with a calcium-free Tyrode's buffer, pH 7.3 (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH$_2$PO$_4$, 12 mM NaHCO$_3$, 2 mM MgCl$_2$, 1 g/liter dextrose and 2 g/liter albumin). Platelets were collected in the void volume and deposited on Formvar filmed Ni maxtiform grids and allowed to settle and adhere at 37°C in a moist chamber. The extent of spreading was monitored by phase-contrast microscopy, and at various stages of spreading, grids containing adherent platelets were removed and washed with protein-free buffer. The time frame for platlet spreading after surface activation has been well documented (2). From the time of contact with the Formvar film, the entire spreading process requires ~12–

15 min. Maximum incubation time was 20 min.

Platelet cytoskeletons were prepared by previously described extraction procedures (43). The extraction buffer, PHEM, consistedof 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl$_2$. Well spread platelets were washed with PHEM buffer then lysed with 0.15% Triton X-100 in PHEM buffer for 1 min. Grids were thoroughly washed in buffer and prepared for electron microscopy as described below.

Platelets were labeled by incubating individual grids in 20 μl of the FGN-Au or 10E5-Au suspension for 5 min at room temperature. In control experiments, platelets were incubated with albumin-labeled colloidal gold, colloidal gold conjugated to the IgG fraction of normal serum, or with a large excess of soluble fibrinogen (2 mg/ml) before incubation in the FGN-Au suspension. Grids were thoroughly washed in buffer to remove unbound label and processed for electron microscopy.

Electron Microscopy: All samples were fixed in 0.1 M HEPES buffered 1% glutaraldehyde, 0.2% tannic acid, 0.05% Saponin, pH 7.2, for 30 min at room temperature (27). Specimens were postfixed in buffered 0.05% OsO$_4$ for 20 min and stained in 1% UMgAc (aq.) for 10 min. Specimens were dehydrated through a graded series of alcohol to absolute alcohol, itself dried by storage over molecular sieve. Samples were dried by the critical point procedure in a critical point dryer equipped with an in-line molecular sieve filter and a hydrophobic water-excluding filter. Samples were evaporatively coated with a thin layer of carbon and stored over molecular sieve until examined with the AEI EM 7 1 MeV electron microscope of the Madison HVEM facility. Stereo pair micrographs were taken at tilt angles appropriate for specimen thickness and magnification (18). Some samples were evaporatively or sputter coated with ~10 nm of gold or gold palladium and examined on a JEOL JSM 35C scanning electron microscope at 10–20 kV accelerating voltage. A tilt angle of 7° was used for SEM stereo pairs.

The number of FGN-Au labels bound per platelet at saturation was determined by direct counting of labels on individual platelets. Micrographs of randomly selected well spread platelets from three separate experiments were enlarged to a final print magnification of 100,000. Individual labels were counted and marked on a transparent overlay.

RESULTS

Fibrinogen-labeled colloidal gold is a suitable particulate marker for the detection and localization of the platelet membrane receptor for fibrinogen by both scanning and high voltage electron microscopy. The individual FGN-Au labels are visible as discrete markers, relatively homogeneous in size and shape (Fig. 1). Control preparations, those in which platelets were incubated with albumin-labeled colloidal gold, colloidal gold conjugated to the IgG fraction of normal serum, or with an excess of soluble fibrinogen before incubation with FGN-Au, lacked significant labeling.
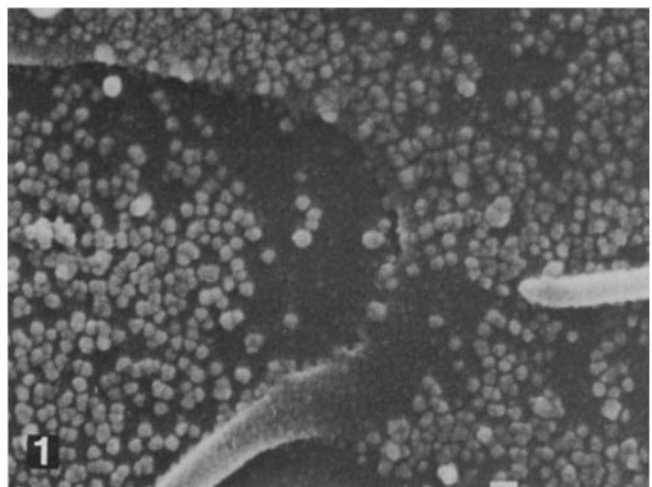

FIGURE 1 Individual FGN-Au labels on the surface of a human platelet as seen by scanning electron microscopy. Bar, 0.1 μm. × 35,500.

In the initial stages of activation immediately after adhesion, minimal spreading of the hyalomere produces characteristically dendritic shaped platelets possessing several long thin pseudopodia. During this stage of spreading, FGN-Au labels are present in limited numbers (Fig. 2). There is a rapid, marked increase in the amount of FGN-Au bound per platelet as spreading proceeds as illustrated in Fig. 3. FGN-Au labels are distributed uniformly over the entire surface of platelets including the pseudopodia. The binding of fibrinogen continues to increase during the intermediate stages of spreading (Fig. 4). Further extension of the cytoplasm produces larger, more oval platelets that possess numerous short pseudopodia. During this stage, FGN-Au labels remain evenly distributed; however, the labels are generally absent from the majority of the pseudopodia. Platelets in the end stages of spreading possess a flattened, predominately round to oval shape with a raised granulomere area (Figs. 5 and 6). No further increase in the amount of fibrinogen bound per platelet occurs; however, a centralization of the FGN-Au levels is seen. In this well spread form, which represents saturation of the fibrinogen

receptor, there are 7,600 ± 500 labels per platelet. These labels are found primarily overlying the central regions of well spread platelets while the peripheral areas are relatively free of labels (Fig. 5). In very late forms of spread platelets, this centralization is pronounced with labels tightly clustered over the central areas (Fig. 6).

Whole mount preparations of unlabeled platelets in the well spread form show the presence of four distinct ultrastructural zones (23). The cytoskeleton underlying each of these zones is exposed following Triton X-100 detergent extraction (Fig. 7). HVEM examination of labeled, well spread platelets reveals the same binding pattern seen by scanning electron microscopy on platelets during the same stage. FGN-Au labels are found clustered over the inner filamentous zone and granulomere while absent from the peripheral web and outer filamentous zone (Fig. 8). The sharp delineation of the fibrinogen labels corresponds to the transition from the loosely woven array of filaments in the outer filamentous zone to the more homogeneous "trabecular-like" inner filamentous zone. Well spread platelets labeled with the monoclonal antibody-gold conjugate directed against the IIb-IIIa glycoprotein complex (fibrinogen-receptor) demonstrated the same receptor distribution (Fig. 9).

DISCUSSION

In this study, we used FGN-Au to illustrate the temporal changes in the amount of fibrinogen bound per platelet and the redistribution of the fibrinogen receptor during the activation process of human platelets. The binding of FGN-Au to the fibrinogen receptor is specific, saturable, and time-dependent. FGN-Au binding is minimal during the initial stages of adhesion and activation that is consistent with results from radiolabeling studies reported by others that show that while non-stimulated platelets in suspension fail to bind fibrinogen there is a rapid increase in the amount of fibrinogen bound per platelet following stimulation by ADP or epinephrine (3, 24, 26, 41). After surface activation, the normally discoid platelets undergo a well documented sequence of morphological changes including formation of pseudopodia, spreading of the hyalomere, and granule release (2, 50, 51). The binding of FGN-Au to surface-activated platelets in-
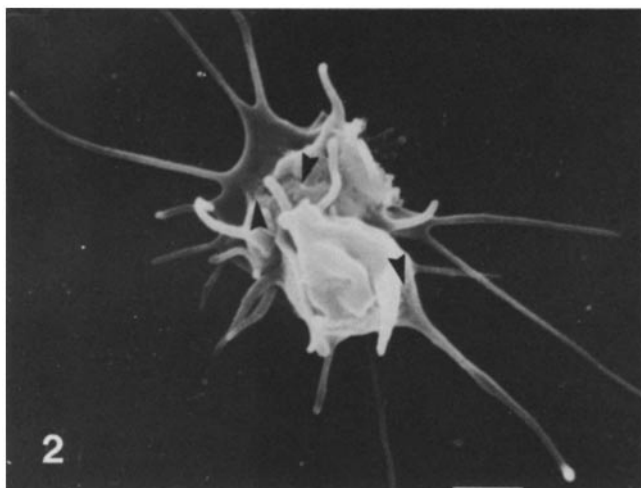

FIGURE 2 Platelets in the initial stages of the spreading process following adhesion. Platelets possess several long pseudopodia. Few FGN-Au labels (arrows) are present. Bar, 1 μm. × 9,100.
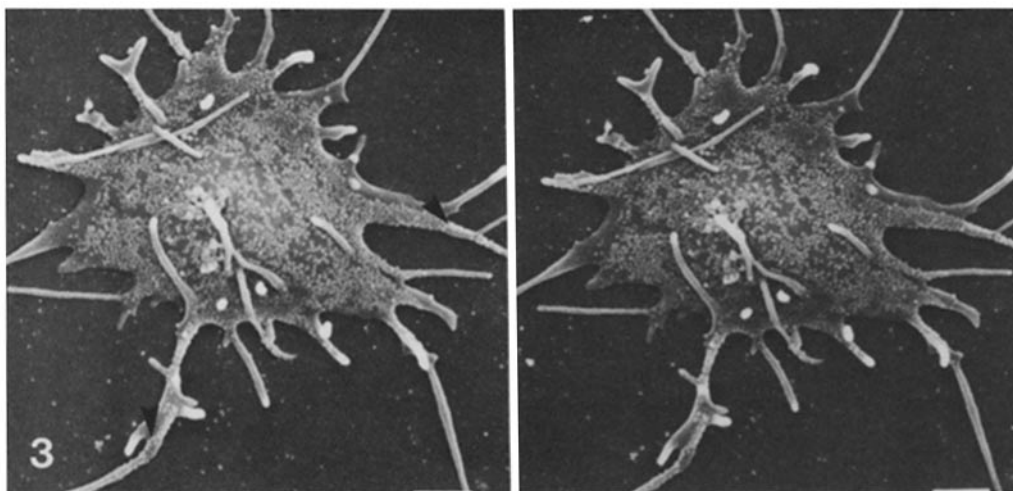

FIGURE 3 Platelet in pseudopodial stage of spreading. The number of FGN-Au labels bound has increased significantly over the number present in Fig. 2. Labels are uniformly distributed over the membrane including the pseudopodia (arrows). Bar, 1 μm. × 7,900.
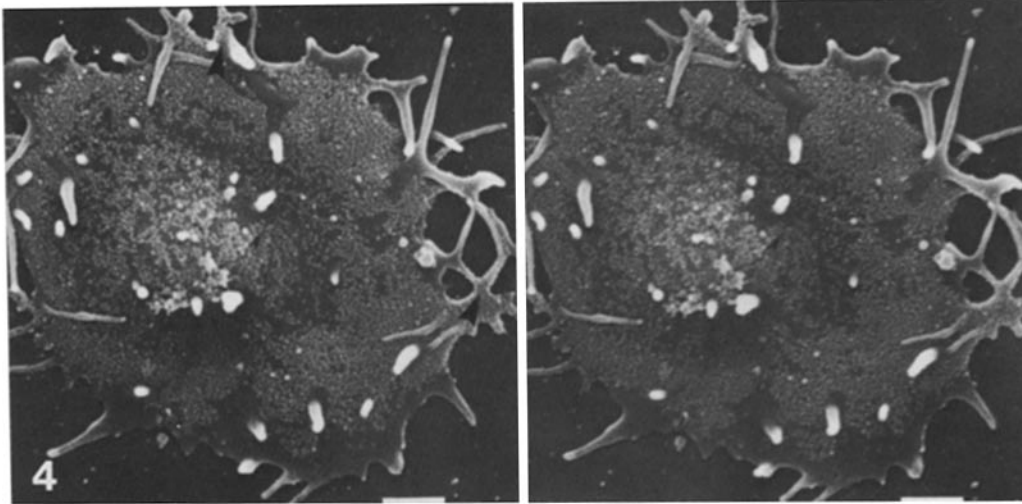
FIGURE 4 Platelet in an intermediate stage of spreading possesses many short pseudopodia. FGN-Au labels are still present on some pseudopodia (arrows); however, the majority of pseudopodia lack labels. Bar, 1 μm. × 8,200.
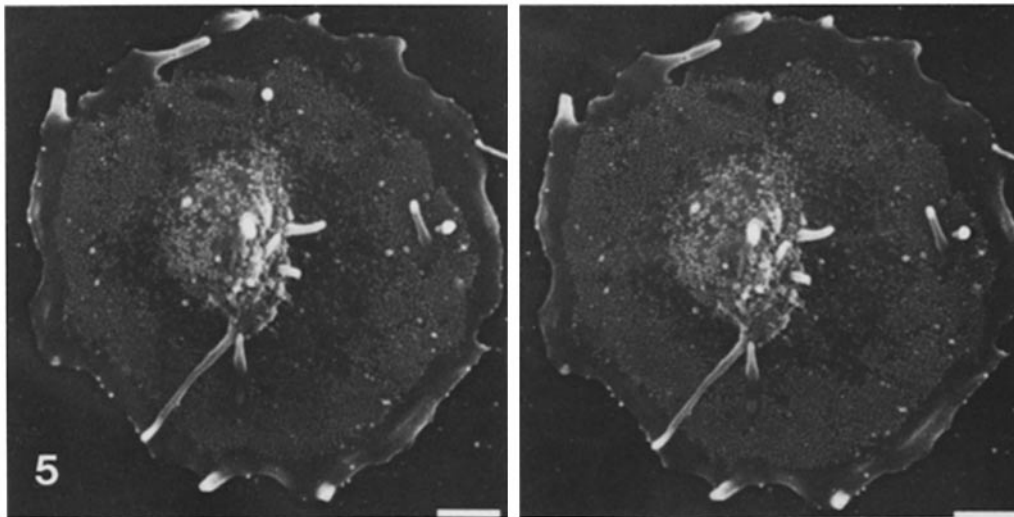

FIGURE 5 Platelet in the late stage of spreading has a more rounded shape with few pseudopodia. FGN-Au labels are found predominately over the central areas while the peripheral areas possess few labels. Bar, 1 μm. × 8,200.
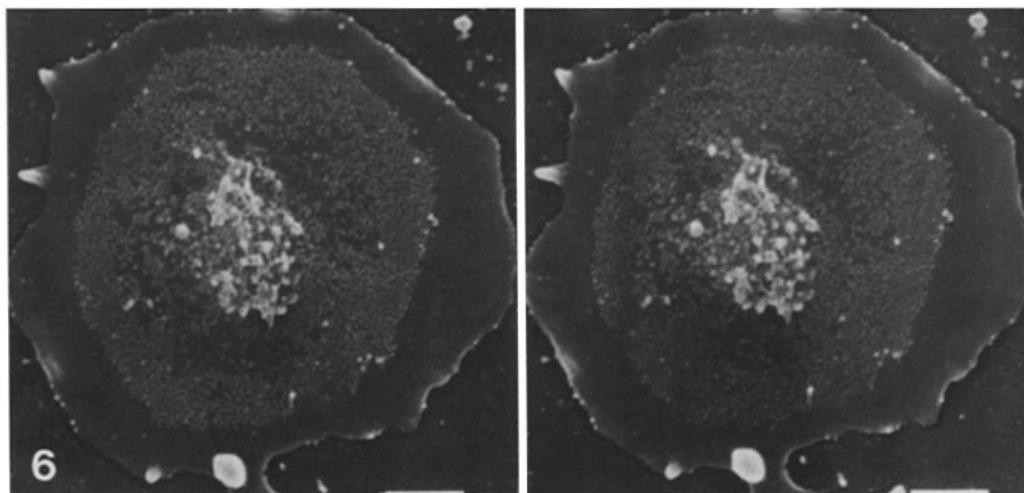

FIGURE 6 Platelet in a very late stage of spreading illustrating the marked redistribution of the fibrinogen receptors to the central region of the platelet. A wide peripheral band is free of labels. Bar, 1 μm. × 10,300.

creases markedly during the early stages of this shape change and rapidly reaches saturation. The number of labels present on the platelets after saturation is less than the mean number of receptors as calculated by Scatchard analysis for platelets in suspension (3, 25, 41) for several reasons. The calcium-dependent GPIIa-IIIb complex has an apparent Stokes radius of 7.1 nm as calculated by Jennings and Phillips (20). With a mean size of 30 nm (gold granule + fibrinogen exclusive of
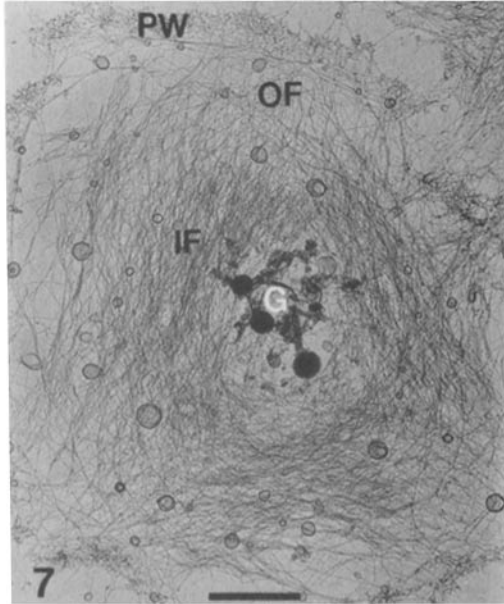

FIGURE 7 HVEM whole mount. Triton-insoluble cytoskeleton of a well spread platelet. The peripheral web (PW) consists of a dense meshwork of filaments. Filaments in the outer filamentous zone (OF) are present within a loosely woven array while those within the inner filamentous (IF) zone are very densely packed and surround the remains of the granulomere. (G). Bar 1 μm. × 12,155.

conductive metal coating), a single FGN-Au label can cover several individual receptor sites depending on the spatial orientation of the receptor complexes within the membrane following induction. Steric hinderance might also inhibit binding of the label to discrete receptor sites. The adhesive face of the platelet must also be considered. In certain cultured cell systems, cells are able to modulate their receptors away from the face in contact with the substrate towards the exposed upper surface (52). It is unknown whether adherent platelets are capable of this activity; if not, it is possible that a number of receptors remain on the adhesive face and unavailable for binding.

Nonactivated platelets and platelets in the initial stages of adhesion lack fibrinogen labels. Small numbers of labels first appear during the early pseudopodial stage. The maximal binding of fibrinogen occurs during the late pseudopodial stage when the platelet cytoskeleton consists of a homogeneous network of microfilaments throughout the cytoplasm and microfilament bundles that extend into the pseudopodia. After maximal binding of fibrinogen, the receptors become centrally located. The receptors are cleared first from the pseudopodia followed by peripheral regions in a progressive process. Centralization of the fibrinogen receptor is coincident with the development of the four distinct ultrastructural zones present only in well spread platelets (1, 23). Platelets in the pseudopodial stages, which possess uniform labeling, lack these distinct structural zones, which suggests that reorganization of contractile cytoskeleton elements may play a major role in receptor movement. Pollard et al. (42), Debus et al. (10), and Painter and Ginsberg (35) have shown, through immunofluorescent techniques, there is a redistribution of myosin during platelet spreading. Myosin is initially uniformly distributed throughout the cytoplasm but becomes more centralized in stimulated platelets. The same authors also show a high concentration of actin in platelet pseudopods, while in later stages the actin distribution appears more uni-
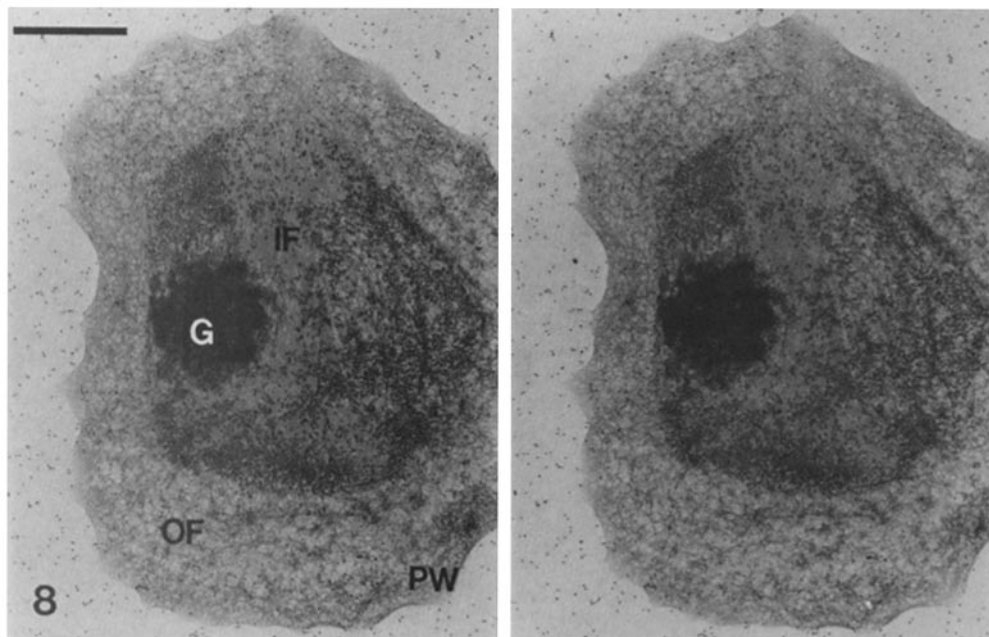

FIGURE 8 HVEM whole mount. Platelet in a similar stage of spreading as that in Fig. 6. FGN-Au labels are found overlying the inner filamentous (IF) and granulomere (G) zones while labels are nearly absent from the outer filamentous (OF) zone and peripheral web (PW). Stereo imaging reveals that the background labels are on the backside of the Formvar film. Bar, 1 μm. × 15,000.
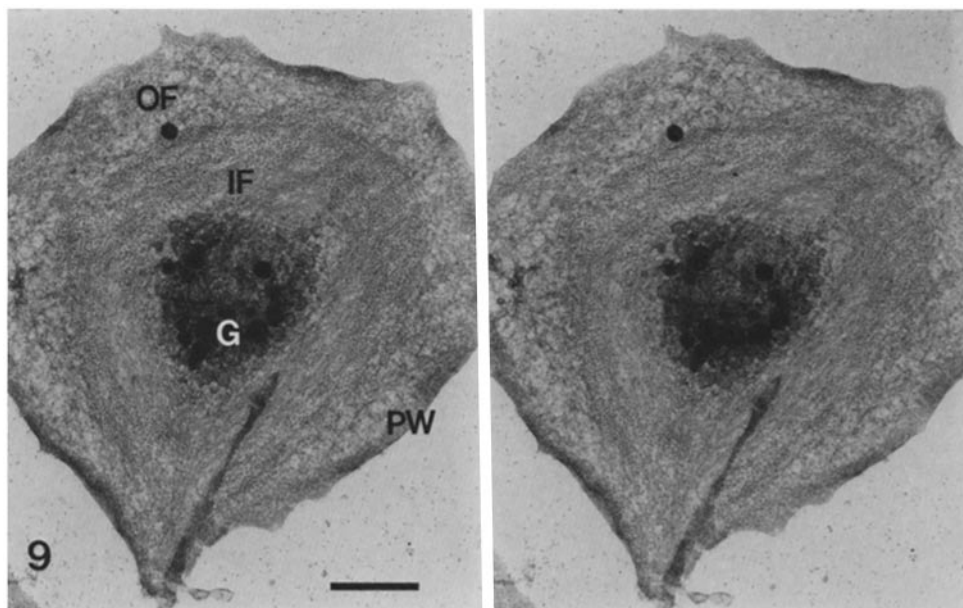
FIGURE 9   HVEM whole mount. Well spread platelet labeled with colloidal gold conjugated to a antibody directed against the GP IIb-IIIa receptor complex. Labels exhibit the same centralized distribution as the FGN-Au labels in Fig. 8. Bar, 1 μm. × 11,800.

form. This is consistent with our findings demonstrating actin bundles in pseudopods while in the later fully spread forms, actin filaments are seen both in the outer filamentous zone and in somewhat higher density in the peripheral web and in the inner filamentous zone that surrounds the granulomere (23). This segregation could provide the mechanism for force generation and directed movement inward of receptors. The sharp delineation between the outer and inner zones together with the nearly exclusive localization of FGN-Au labels over the inner filamentous zone suggests a mechanism in which the receptor glycoproteins are linked to the reorganizing cytoskeletal elements.

With respect to the centralized receptor redistribution in the fully spead platelets, it is possible the fibrinogen receptors are simply being restrained in the membrane at their site of formation while receptor free membrane continues to spread outward as the spreading process proceeds. However, a mechanism involving receptor redistribution based on movement within the plane of the membrane is perhaps more likely for the following reasons. First, if receptors are simply restrained in the membrane, pseudopodial labeling patterns should be retained in well spread platelets that are formed by outward membrane flow into the area between pseudopods. These patterns are never observed in well spread forms. Second, many labeled receptor sites are at a greater distance from the center of the platelet in early spreading forms than in well spread forms. This is particularly true of the early pseudopodial stage, in which labels are found far out on the pseudopodia. This distance is greater than the distance at which the farthest labels are found in the very late stage spread platelet in which all labels are tightly packed in the smaller area underlaid by the inner filamentous zone of the cytoskeleton. Third, in preliminary studies, in which well spread platelets are lightly fixed, then labeled with the monoclonal antibody directed against the glycoprotein IIb-IIIa receptor complex, receptors are not found tightly clustered over the central area as when living platelets are labeled, but rather are found predominantly over peripheral regions including the peripheral web. This data and its implications with respect to the

movement of occupied and unoccupied receptor complexes will be considered in more detail in a manuscript now in a preparation.

Further evidence that supports the interaction of the fibrinogen receptor with cytoskeletal elements comes from studies involving platelet-membrane glycoproteins that have identified GPIIb and GPIIIa as the specific components that complex with fibrinogen (32). Both glycoproteins are integral proteins of the platelet membrane and are solubilized only by detergent disruption of the membrane (4). Furthermore, both glycoproteins apparently span the membrane since neither one is affected by thrombin treatment of intact platelets, yet both are hydrolyzed by thrombin treatment of isolated platelet membranes (38). Biochemical studies have also demonstrated these glycoproteins to be directly associated with the cytoskeleton. Phillips and Agin (39) reported that the amounts of GPIIb and GPIIIa were selectively increased in the Triton X-100 residues from thrombin aggregated platelets, while the amounts in Triton X-100 residues from unstimulated platelets are not significantly above background levels. Painter and Ginsberg (36) reported that concanavalin A induces a linkage of GPIIa and GPIIIb to Triton X-100-insoluble structures, and under conditions that promote the depolymerization of actin, both glycoproteins were released from the Triton X-100 residue indicating a direct association with actin. Other mechanisms, some involving actin binding proteins as an intermediate between glycoproteins and actin, have also been proposed (11, 40). The signal for centralization of these receptor glycoproteins is generated following their interaction with the natural ligand or with antibody since the gold-labeled monoclonal antibodies directed against the nonligand filled GPIIb-IIIa receptor complex exhibited a similar receptor distribution pattern to FGN-Au on platelets in the end stages of activation.

The mechanism through which receptor-bound fibrinogen mediates aggregation is unknown. While a reduction of surface charge after fibrinogen binding (44) may facilitate the association of membrane components on adjacent platelets, it has also been proposed that the cross-linking of exposed

receptors on adjacent platelets by dimeric fibrinogen may play a more important role (15, 19, 34). Similarly, platelet-fibrinogen bridges are of probable importance in clot retraction. A model proposed by Cohen (5, 6) relies upon the extension and retraction of pseudopods bound to fibrin to gather fibrin strands into the developing platelet-fibrin clumps. This would be the case in the pseudopodial stages in which we have shown the fibrinogen receptors to be distributed over the entire membrane including the pseudopodia. The subsequent inward movement of receptors with attacked fibrinogen (fibrin) strands provides a mechanism whereby linked adjacent platelets would be drawn closer together and could overlap. In support of this mechanism, R. D. Allen and co-workers (personal communication) have observed strands of fibrin extending out radially from areas corresponding to the inner filamentous and granulomere zones platelets to similar areas on adjacent fully spread platelets. This mechanism is consistent with the abnormalities both in platelet aggregation and in clot retraction seen in thrombasthenic patients whose platelets lack the fibrinogen receptor.

A more precise localization of myosin with respect to actin and the exact nature of the receptor-cytoskeleton link remains to be determined as does the distribution, movement, and interaction with the other receptor glycoproteins important in platelet function. Considering the size of the receptor complexes and the number of various different receptors thus far described, the ratio of receptors/surface area in the platelet is extremely high when compared with most nucleated cell types. Thus, spatial factors may prove to be very important. The movement, concentration, and surface area occupied by the fibrinogen receptor, as demonstrated in the present study, has to be considered in the context of numerous other receptor molecules also located on the platelet surface and possibly associated with the platelet cytoskeleton. The availability of receptors may depend not only on the presence of an active receptor on the platelet surface but on whether or not steric blocking of this receptor by adjacent other types of receptors or receptor-ligand complexes occurs. In the latter situation, it will be of particular interest to determine if a "first come, first served" strategy is employed with respect to ligand, or if some method of ensuring a supply of unblocked receptors, for some or all ligand types, is at work, such as sequential presentation or surface compartmentalization.

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