The Platelet Fibrinogen Receptor: An Immunogold-Surface Replica Study of Agonist-induced Ligand Binding and Receptor Clustering

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Abstract. Platelet aggregation requires the binding of fibrinogen to its receptor, a heterodimer consisting of the plasma-membrane glycoproteins (GP) IIb and IIIa. Although the GPIIb-IIIa complex is present on the surface of unstimulated platelets, it binds fibrinogen only after platelet activation. We have used an immunogold-surface replica technique to study the distribution of GPIIb-IIIa and bound fibrinogen over broad areas of surface membranes in unstimulated, as well as thrombin-activated and ADP-activated human platelets. We found that the immunogold-labeled GPIIb-IIIa was monodispersed over the surface of unstimulated platelets, although the cell surface lacked immunoreactive fibrinogen. On thrombin-stimulated platelets, ∿65% of the GPIIb-IIIa molecules were in clusters within the plane of the membrane. Fibrinogen, which had been released from the α-granules of these cells, bound to GPIIb-IIIa on the cell surface and was similarly clustered. To determine whether the receptors clustered before ligand binding, or as a consequence thereof, we studied the surface distribution of GPIIb-IIIa after stimulation with ADP, which causes activation of the fibrinogen receptor function of GPIIb-IIIa without inducing the release of fibrinogen. In the absence of added fibrinogen, the unoccupied, yet binding-competent receptors on ADP-stimulated platelets were monodispersed. The addition of fibrinogen caused the GPIIb-IIIa molecules to cluster on the cell surface. Clustering was also induced by the addition of the GPIIb-IIIa–binding domains of fibrinogen, namely the tetrapeptide Arg-Gly-Asp-Ser on the α-chain or the γ-chain decapeptide γ402–411. These results show that receptor occupancy causes clustering of GPIIb-IIIa in activated platelets.

P LATELET aggregation requires the binding of fibrinogen to its membrane receptor, glycoproteins (GP)[1] IIb and IIIa (reviewed in reference 16). Fibronectin, von Willebrand factor, and vitronectin also bind to GPIIb-IIIa (12, 39, 45). The interaction of these ligands with the surface of platelets may potentiate aggregation and possibly the adherence of platelets to exposed vascular subendothelial surfaces (20, 55). GPIIb-IIIa is a complex consisting of two large, nonidentical glycoproteins, GPIIb and GPIIIa, which interact by a noncovalent, Ca^{2+}-dependent mechanism (23). Although the intact heterodimer is present in the plasma membrane of unstimulated platelets (11, 29, 38), the receptor does not bind fibrinogen or other ligands unless the cells have been activated by an agonist such as ADP or thrombin (3, 12, 39). This suggests that cell activation induces a con-

formational change in GPIIb-IIIa or "microenvironmental" changes in the plasma membrane that allow freer access of ligands to the receptor (9, 36, 51).

Previous investigators, using immunocytochemical and electron-microscopic techniques, have found that GPIIb-IIIa and fibrinogen cluster in the membrane of activated platelets. Polley et al. (43) reported co-clustering of GPIIb and GPIIIa in the membranes isolated from thrombin-stimulated platelets. Observations of thin sections of platelets, which permit visualization of only small amounts of membrane, have also revealed clustering of GPIIb-IIIa or fibrinogen (1, 21). These studies, however, are difficult to relate to the mechanism of receptor expression during platelet activation, because it was not determined whether clustering was an effect of platelet activation per se, the binding of fibrinogen to GPIIb-IIIa, or, in the study on isolated membranes (43), the membrane isolation procedure.

In the present study, we used a combined postfixation immunogold-surface replica technique to examine the distribution of the fibrinogen receptor and its bound ligand on the surface of unstimulated platelets and platelets activated with thrombin or ADP. This procedure allows the visualization of

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1. *Abbreviations used in this paper:* GP, glycoprotein; RGDS, Arg-Gly-Asp-Ser; RGES, Arg-Gly-Glu-Ser; γ402–411, Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val.

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macromolecules over large areas of membrane in intact cells. We found that clustering of GPIIb-IIIa in the membrane of activated platelets occurred only after fibrinogen or one of its GPIIb-IIIa binding-specific peptides had occupied the receptor.

Materials and Methods

Collection of Specimens

Platelets were isolated from the blood of aspirin-free human volunteers by the techniques described by Stenberg et al. (52), with one modification. Because GPIIb-IIIa is a calcium-dependent complex (11, 23), EDTA was omitted from all buffers. For thrombin-stimulation studies, blood was collected into acid-citrate-dextrose anticoagulant (8.5 × 10^{-2} M trisodium citrate, 6.5 × 10^{-2} M citric acid, 1.11 × 10^{-1} M dextrose, pH 4.5) in the ratio of one part of anticoagulant to six parts of blood. For ADP-stimulation studies, 1.05 × 10^{-1} M buffered citrate solution was used as the anticoagulant in a ratio of one part of citrate to nine parts of blood. Briefly, the platelets were isolated by a two-step differential centrifugation procedure. Prostaglandin E_1 (Sigma Chemical Co., St. Louis, MO) was included in the anticoagulant and wash buffers, at a concentration of 5 µg/ml, to inhibit platelet activation. The cells were washed twice in buffer containing 1.4 × 10^{-1} M NaCl, 2.0 × 10^{-2} M Hepes, 6.0 × 10^{-3} M glucose, pH 6.6; resuspended in a modified Tyrode's buffer containing 0.35% bovine serum albumin, without added Ca^{2+}; and allowed to equilibrate for 1 h at 37°C.

Thrombin Stimulation

After adjusting the calcium concentration to 0.001 M, we stimulated 2 × 10^9 platelets with 2 U of purified human α-thrombin (3,150 U/mg) (gift of Dr. John Fenton II, New York State Department of Health, Albany, NY). After 5 s, 30 s, 1 min, or 5 min of thrombin stimulation, 20 vol of fixative (either 2% paraformaldehyde + 0.05% glutaraldehyde, pH 7.2, or 4% paraformaldehyde-lysine-periodate [30]) were added to the platelets. Cells were fixed for 1 h at 37°C in the first fixative or for 4 h at 4°C in the second fixative. They were then washed in 0.1 M PBS with 0.05 M NH_4Cl, pH 7.2, to quench excess aldehyde groups. Fixed cells were next settled onto coverslips coated with poly-L-lysine (1 mg/ml distilled H_2O; Miles Scientific, Naperville, IL). For control studies, a sample of unstimulated cells was similarly processed in the absence of thrombin. Other samples of unstimulated and thrombin-activated platelets were reserved for use in immunochemical studies in suspension.

ADP Stimulation

Platelets isolated as described above were washed, resuspended in Tyrode's buffer containing 2.0% bovine serum albumin, allowed to equilibrate for 1 h at 22°C, and processed in one of the following ways, after adjustment of the final Ca^{2+} concentration to 0.001 M: (a) ligand-binding–competent cells, receptors unoccupied; no exogenous fibrinogen was added; (b) ligand-binding–competent cells, receptors occupied; 0.25 mg/ml fibrinogen (Kabi Diagnostica, Stockholm, Sweden) was added, and the cells were not stirred after ADP was added; (c) aggregating cells; 0.25 mg/ml fibrinogen was added, and the cells were stirred continuously after ADP was added. Before ADP stimulation, platelets in all three groups were diluted in Tyrode's buffer to a concentration of 250,000 cells/mm³ and incubated at 37°C for 5 min. Each sample was then stimulated with 20 µM ADP (Sigma Chemical Co.) diluted in Owren's veronal buffer (2.84 × 10^{-2} M sodium barbital in 1.25 × 10^{-1} M sodium chloride; American Dade, Aguado, Puerto Rico) for 5 min at 37°C. The platelets were then fixed as described above and processed in the same way as thrombin-stimulated platelets.

Aggregometry

Routine aggregometry was performed on washed platelets from each of the three ADP-stimulation groups at a cell concentration of 250,000 platelets/mm³ and a final ADP concentration of 20 µM. Aggregation was monitored in a Bio/Data Platelet Aggregation Profiler, model PAP-2A (Bio/Data Corp., Willow Grove, PA).

Synthetic Peptides

Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES) were purchased from Peninsula Laboratories, Belmont, CA. The decapeptide γ402–411 was synthesized by the Biomolecular Resource Center at the University of California, San Francisco. The compounds were tested for cell lysis using a lactate dehydrogenase assay (Sigma Chemical Co.) and found to be nonlytic at concentrations of 500 µM for the tetrapeptides and 1 mM for the decapeptide. The peptides were used at concentrations that inhibited fibrinogen-mediated aggregation, as assessed by aggregometry. These concentrations were 200 µM for RGDS and RGES and 835 µM for the decapeptide, although RGDS and the decapeptide could effectively inhibit aggregation at concentrations of 50 and 200 µM, respectively, if the bovine serum albumin percentage was decreased from 2.0% to 0.35%. However, for comparison with the previous ADP-stimulation studies, the albumin concentration was kept at 2.0%. The peptides were supplied to platelets in place of fibrinogen before ADP stimulation, as described above.

Immunochemical Procedures

Cells in Suspension. Fixed platelets were immunolabeled according to the procedures of Stenberg et al. (52), with rabbit anti–GPIIIa (1:100 dilution) (6) as the primary antibody and protein A-gold (5 nm diam) (Janssen Pharmaceuticals, Beerse, Belguim) as the electron-dense marker. The platelets were then postfixed in glutaraldehyde and osmium tetroxide and processed for thin-sectioning electron microscopy as described previously (52).

Platelets Settled on Coverslips. Fixed unstimulated, thrombin-stimulated, or ADP-stimulated platelets that had attached to the poly-L-lysine-coated coverslips were rinsed with PBS containing 0.2% gelatin. The coverslips were then incubated overnight at 4°C with one of four primary antibodies at the dilutions or protein concentrations given: polyclonal anti–GPIIIa (1:100) (6), monoclonal Tab (0.039 mg/ml) (28), monoclonal T10 (0.02 mg/ml) (29), polyclonal antifibrinogen (1:5,000) (Cappel Laboratories, West Chester, PA). Preimmune rabbit serum was used as a control. The coverslips were then rinsed in PBS + gelatin, and incubated for 1 h at 22°C with protein A-gold, 10 nm diam, at a dilution of 1:50 (Janssen Pharmaceuticals). The cells were fixed for 15 min at 25°C in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), rinsed in the same buffer, postfixed for 15 min at 4°C in veronal acetate-buffered 1% osmium tetroxide, and dehydrated through graded ethanols at 4°C.

Preparation of Surface Replicas

Our modifications of a previously described procedure (47) for preparing surface replicas are given in detail elsewhere (22). Briefly, dehydrated poly-L-lysine-coated coverslips with adherent, immunolabeled platelets were placed in a critical-point-drying apparatus (Polysciences, Inc., Warrington, PA) and processed using bone-dry carbon dioxide as a transition fluid. After critical-point drying, the coverslips were mounted on the stage of a Balzers 360M freeze-fracture apparatus (Balzers Union, Hudson, NH) and the bell jar was evacuated. Rotary-shadowed surface replicas were made using platinum-carbon (<40°), followed by carbon (<90°), electrodes. The replicas were floated onto 5.25% NaClO (Purex Corp., Lakewood, CA) for a brief digestion period. They were then washed in distilled water and picked up on uncoated grids. Specimens were examined and photographed with a JEOL 100CX electron microscope at an accelerating voltage of 80 kV.

Quantitation of Receptors

Gold particles, existing as either singletons or members of a cluster of two or more particles, were counted over a measured area of the plasma membranes of platelets from activated and unstimulated samples. The number of receptors per platelet was determined by multiplying the particle count by a factor derived from the work of Mosher et al. (32), who have calculated the surface areas of unstimulated and stimulated platelets to be 22 × 10^{-12} m² and >32 × 10^{-12} m², respectively.

Results

To assess the surface distribution of the platelet fibrinogen receptor and its ligand on intact platelets, we initially examined thin sections of immunocytochemically labeled unstimulated and thrombin-activated cells. In unstimulated platelets,

the label for GPIIb-IIIa was distributed along the plasma membrane, in no apparent pattern, at a density of 6.4 ± 3.8 gold particles/μm of membrane (Fig. 1). Thrombin stimulation resulted in prominent change in shape and aggregation of the platelets; the density of gold particles increased to 9.0 ± 1.0/μm of plasma membrane (Fig. 2), but the clustering observed by other investigators was not seen. This discrepancy was probably due to our use of protein A-gold as the immunoprobe; previous investigators used IgG-gold, a reagent that is often inherently clumped (i.e., more than one gold particle per IgG molecule). To determine whether platelet activation might cause changes in the surface distribution of GPIIb-IIIa not appreciable in conventional thin sections,

we studied surface replicas of immunolabeled unstimulated and thrombin-activated platelets.
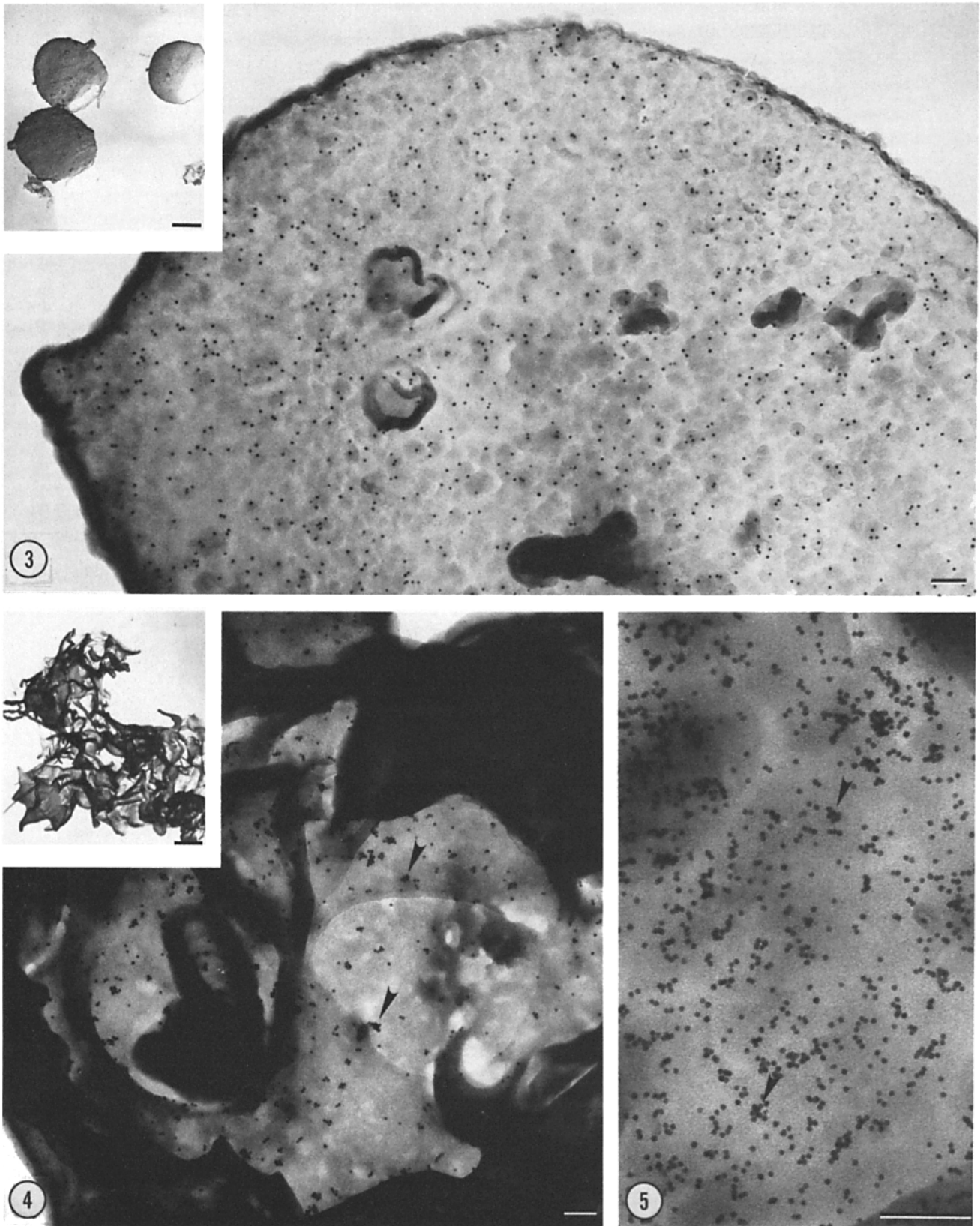
Surface Replicas

Fibrinogen Receptor Glycoproteins. On the surfaces of unstimulated platelets incubated with anti–GPIIIa, gold particles were uniformly distributed in an unclustered pattern (Fig. 3). 95% of the gold particles were monodispersed, and the remainder were either doublets (4%) or triplets (1%).

Thrombin stimulation caused several significant changes in the platelets. Prominent filopodial projections appeared and the platelets aggregated (Fig. 4, *inset*). Although the amount of immunolabeling for GPIIIa on the surfaces of
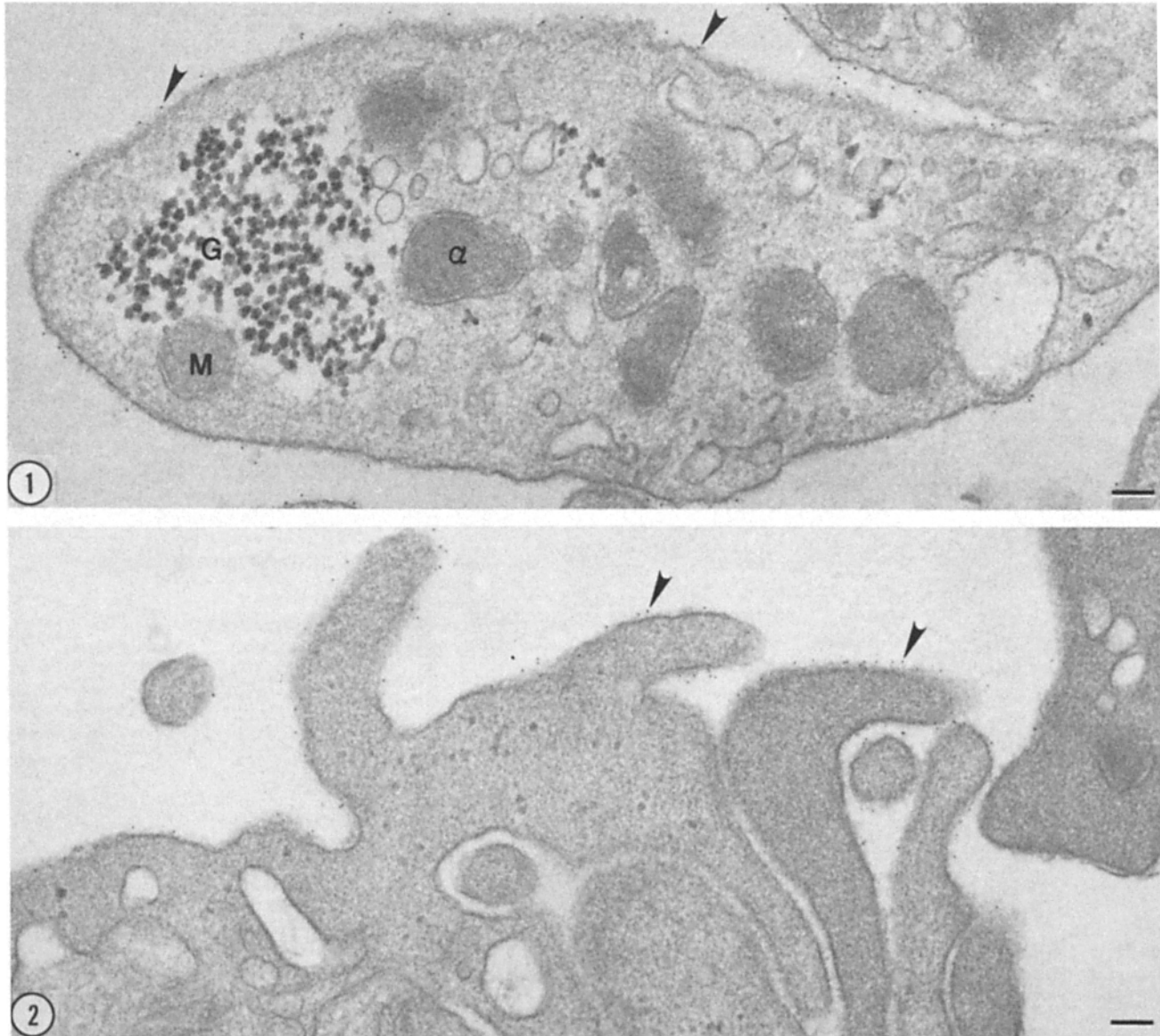
Figures 1 and 2. (Fig. 1) Thin section of a fixed, unstimulated platelet incubated with anti–GPIIIa antiserum. The immunogold label (*arrowheads*) is distributed over the entire plasma membrane of the cell. However, as has been previously shown (56), fixation renders the GPIIb-IIIa located on surface-connected canalicular membranes inaccessible to the immunoprobes. (α) α-Granule; (*M*) mitochondrion; (*G*) glycogen. Bar, 0.1 μm. (Fig. 2) Thin section of aggregated platelets activated with thrombin for 1 min and immunolabeled for GPIIIa as in Fig. 1. The gold probe (*arrowheads*) is distributed as in unstimulated platelets, with no apparent clustering. Because of the close apposition of neighboring platelets, the immunoprobes could not reach membranes within the aggregates. Bar, 0.1 μm.

Figures 3–5. (Fig. 3) Surface replica of an unstimulated platelet immunolabeled with anti–GPIIIa. The antigen is uniformly distributed over the entire surface of the cell. Bar, 0.1 μm. (*Inset*) The discoid nature of unstimulated platelets. Bar, 1 μm. (Fig. 4) Surface replica of a thrombin-stimulated platelet immunolabeled with anti–GPIIIa antibody. At all periods of stimulation examined, the extent of labeling in individual cells varied. Most platelets showed approximately the same number of gold particles seen in unstimulated cells, in addition to clustering of the antigen (*arrowheads*). The blackened areas at the corners of the figure represent long filopodial projections that have fallen back onto the surface of the cell. Since such projections were often not extracted in the bleach digestion step, assessment of receptor
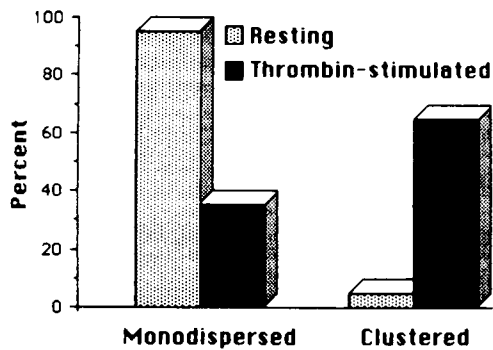
Figure 6. Patterns of gold particles on surface replicas of unstimulated and thrombin-activated platelets. Gold particles were counted on 60 platelets in each group.

these activated platelets varied greatly, most cells showed approximately the same number of gold particles per unit area as unstimulated cells, or about 10,000 gold particles per platelet (Fig. 4). However, on all cells ~65% of the gold particles were now clustered. Binding studies using radiolabeled antibodies to the receptor have shown that each platelet bears ~40,000–50,000 GPIIb-IIIa complexes (29, 38). The diminution in number of sites with the surface-replica procedure may be due to steric hindrance of the immunoprobes used, fixation sensitivity of the antigen, or other factors. Indeed, Griffiths and Hoppeler (18) have reported only a 14% label-

ing efficiency of plasma-membrane proteins for immuno-electron-microscopic methods in comparison to biochemical assays. The clustering of the gold particles was more clearly apparent at higher magnification (Fig. 5). The patterns of immunolabeling with two monoclonal antibodies, Tab, which recognizes GPIIb, and T10, which recognizes an epitope on the GPIIb-IIIa complex in the presence of Ca^{2+}, were indistinguishable from the pattern with anti-GPIIIa. No immunogold label was observed when preimmune rabbit serum was used in place of antibodies to the receptor glycoproteins. The differences in GPIIb-IIIa-labeling patterns on unstimulated and thrombin-activated platelets are summarized in Fig. 6.

The Physiological Ligand, Fibrinogen. The virtual absence of label on the surfaces of unstimulated platelets immunolabeled with antifibrinogen antiserum (Fig. 7) showed that there was no immunoreactive fibrinogen bound to these cells. Thrombin stimulation caused the secretion of α-granule contents and the subsequent binding of the endogenous fibrinogen to its cell-surface receptors (Fig. 8). At higher magnification, the immunoprobe for the ligand showed clustering similar to that seen with antibodies to the glycoproteins of the receptor (Fig. 5).
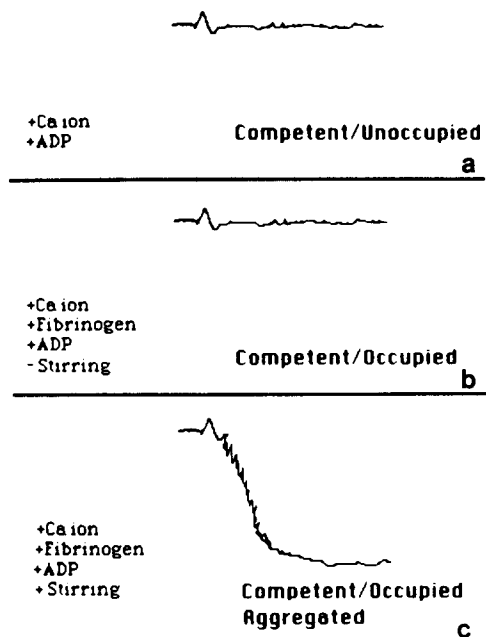
ADP Stimulation

From the experiments with thrombin, we concluded that both fibrinogen and its receptor clustered in the plane of the plasma membrane after physiologic activation of platelets.
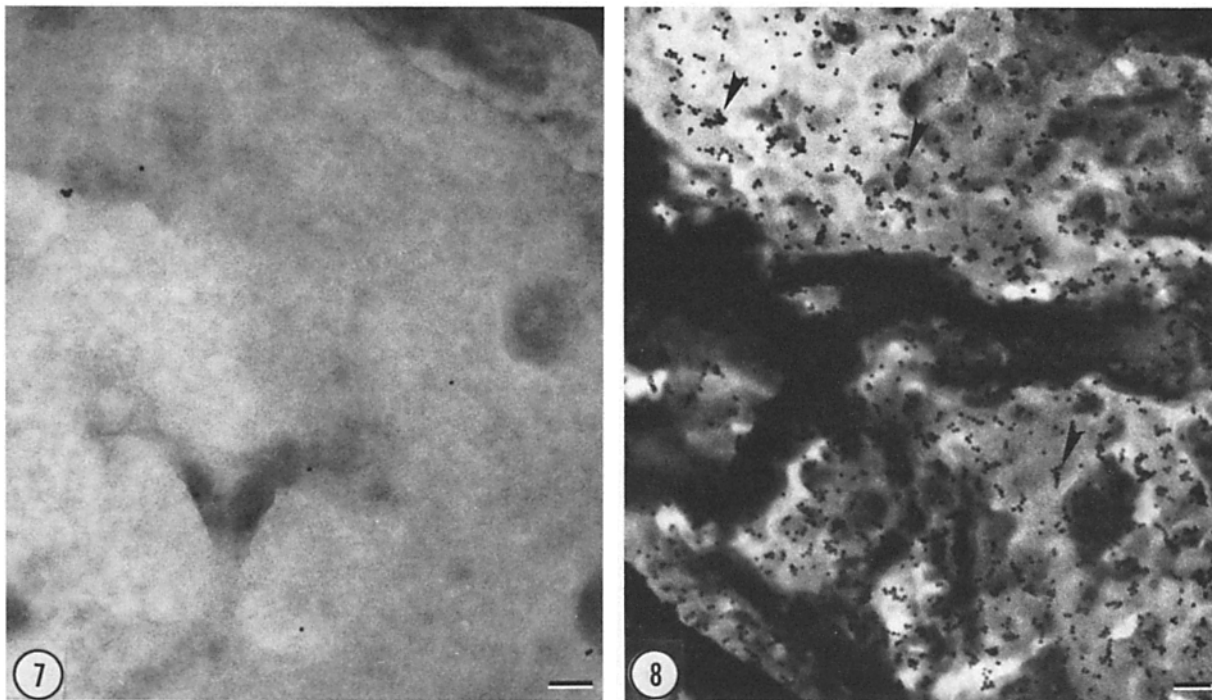
Figures 7 and 8. (Fig. 7) Surface replica of an unstimulated platelet immunolabeled with antifibrinogen antibody. There is virtually no immunoreactive ligand bound to the surface of the cell. Bar, 0.1 μm. (Fig. 8) Surface replica of a thrombin-activated platelet immunolabeled with antifibrinogen antibody. The gold probe is present in high concentrations and is clustered (*arrowheads*). Bar, 0.1 μm.

and ligand distribution at these sites is difficult. Bar, 0.1 μm. (*Inset*) Low-power view of a surface-replicated microaggregate of thrombin-activated platelets. The shape change seen on thin-section profiles in Fig. 2 is also apparent here. Bar, 1 μm. (Fig. 5) Magnified view of surface replica of a thrombin-activated platelet immunolabeled with anti-GPIIIa. Clustering of the immunoprobe is indicated by arrowheads. Bar, 0.1 μm.

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+Ca ion
+ADP            Competent/Unoccupied
                                        a
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+Ca ion
+Fibrinogen
+ADP
-Stirring       Competent/Occupied
                                        b
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+Ca ion
+Fibrinogen
+ADP
+Stirring       Competent/Occupied
                Aggregated
                                        c
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Figure 9. Aggregometry tracings of the three groups of platelets in the ADP-stimulation studies. (*A*) Binding-competent, receptors unoccupied; (*B*) binding-competent, receptors occupied, nonaggregated; and (*C*) binding-competent, receptors occupied, aggregated. The conditions of preparing the platelets are indicated in the lower left corner of each panel.

However, because thrombin also causes the release of α-granule contents (including fibrinogen), we could not determine whether receptors that are competent for ligand binding cluster before or after they bind fibrinogen. We therefore performed experiments using ADP. Although at low doses this agonist does not induce α-granule secretion, it does cause platelets to change shape, bind exogenous fibrinogen, and, if stirred, aggregate (27) (Fig. 9). The binding-competent receptor, therefore, remains unoccupied unless fibrinogen is added. By comparing the topographic distribution of GPIIb-IIIa on ADP-activated platelets prepared with and without added fibrinogen, we could ascertain whether receptor clustering occurs prior to, or as a consequence of, ligand binding.

Binding-competent but Unoccupied by Ligand. Platelets that were stimulated with ADP in the absence of fibrinogen underwent shape change but did not aggregate as assessed by aggregometry (Fig. 9 *a*) and morphologic study. They showed monodisperse surface distribution of fibrinogen receptors (Fig. 10) and lacked surface immunoreactive fibrinogen (Fig. 11).

Binding-competent and Occupied by Ligand. When platelets were stimulated with ADP in the presence of added fibrinogen, but not stirred, they changed shape, but did not aggregate (Fig. 9 *b*). They showed clustering of fibrinogen receptors, with 55% of the gold particles per cell in clusters (not illustrated). Studies with antifibrinogen antibody showed that the ligand was also clustered in such cells, although there was less immunolabel for fibrinogen than in thrombin-stimulated cells.

When platelets were processed in the same way as the unstirred cells, but were stirred after the addition of ADP, they underwent aggregation as assessed by aggregometry (Fig. 9 *c*). They showed the same surface distribution of fibrinogen

receptors and ligand as the unstirred cells. Thus, ligand binding, but not aggregation, is required for receptor clustering.

Binding-competent and Occupied by GPIIb-IIIa-binding Domains of Fibrinogen. To determine whether GPIIb-IIIa clustering is due to the polyvalent nature of the fibrinogen molecule, or merely a function of receptor occupancy, we substituted RGES, RGDS, or γ402-411 for fibrinogen in ADP-stimulation studies. RGDS and γ402-411 have been shown to be the GPIIb-IIIa-binding domains on the α- and γ-chains, respectively, of the fibrinogen molecule (25, 35, 41, 45). Gartner et al. (14, 15) have demonstrated that RGES does not compete with fibrinogen binding to platelets as does RGDS, even though it differs chemically from RGDS by only one methyl group. Therefore, RGES was used as a control peptide. Incubation of ADP-stimulated platelets with RGDS (Fig. 12) or the decapeptide (not illustrated) also caused clustering of GPIIb-IIIa. The GPIIb-IIIa molecules remained monodispersed when RGES was substituted for fibrinogen in ADP-activated platelets (not illustrated).

Discussion

In this study, we have used surface replication of immunolabeled intact platelets to examine the topographic distribution of fibrinogen and its receptor on unstimulated, thrombin-activated, and ADP-activated cells. In unstimulated platelets, GPIIb-IIIa was uniformly monodispersed over the surface of the cell. GPIIb-IIIa does not bind fibrinogen on unstimulated platelets, and accordingly, there was no immunoreactive fibrinogen on the plasma membranes of these cells. After thrombin stimulation, ∼65% of the immunolabeled GPIIb-IIIa was clustered in the plane of the plasma membrane. Thrombin stimulation also causes platelet α-granules to secrete fibrinogen, which subsequently binds to GPIIb-IIIa on the cell surface. This bound fibrinogen was similarly clustered. To ascertain whether GPIIb-IIIa clustered before, or as a consequence of, ligand binding in activated platelets, we also analyzed ADP-activated platelets. ADP induces the expression of fibrinogen receptors on the platelet plasma membrane, but it does so without causing α-granule secretion (27). On the surfaces of ADP-stimulated cells whose receptors were ligand-binding-competent, but unoccupied, GPIIb-IIIa remained monodispersed. However, when fibrinogen was added, either in its native form or as the RGDS or γ402-411 active GPIIb-IIIa-binding peptides, the GPIIb-IIIa on ADP-stimulated cells was clustered.

We conclude that GPIIb-IIIa clustering occurs on activated platelets as a consequence of receptor occupancy. That not all of the receptors clustered after thrombin stimulation may be due to the experimental conditions. The thrombin studies were performed on washed platelets, so all of the fibrinogen available for binding had been secreted from α-granules. Plow et al. (40) have calculated that there is sufficient platelet-derived fibrinogen to occupy ∼30% of the GPIIb-IIIa molecules. Therefore, ligand concentration may determine the degree of receptor clustering. Similar ligand-induced receptor clustering has been described for the epidermal growth factor receptor on intact (19) and plasma membrane vesicles (58) of A431 cells. These findings suggest that receptor occupancy alone may be sufficient to promote homotypic receptor interactions in some cell types. However, there is no evidence that the GPIIb-IIIa/fibrinogen complex
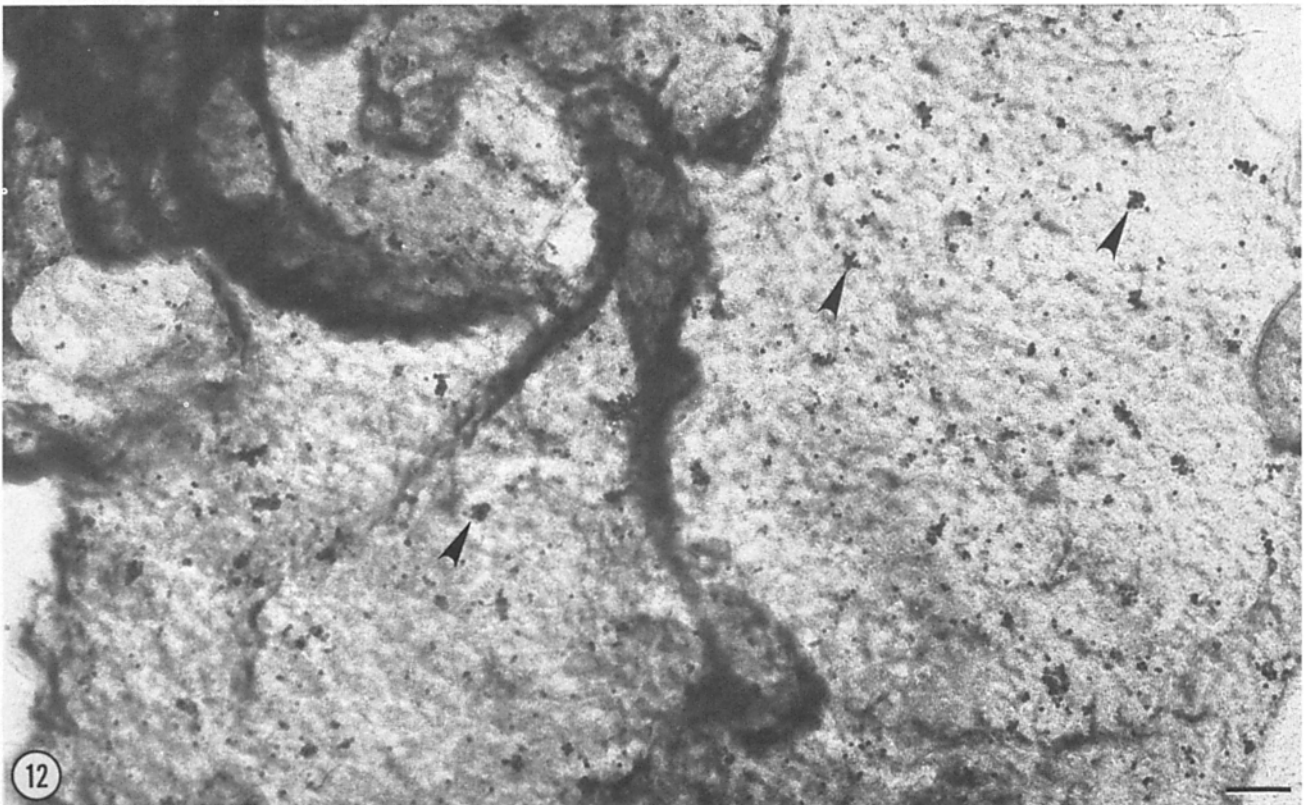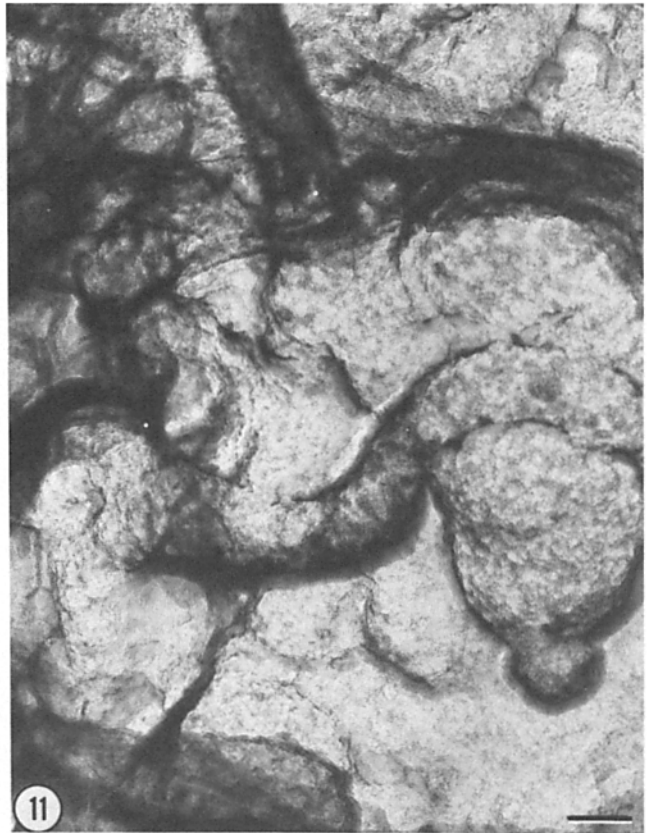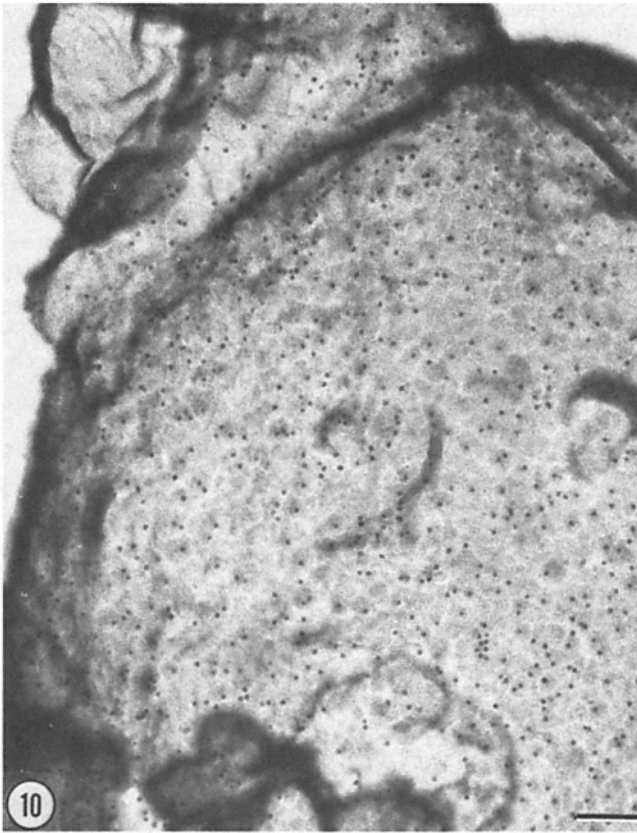
Figures 10–12. (Fig. 10) Surface replica of a washed platelet stimulated with ADP. Because fibrinogen was not added, the receptors were unoccupied, even though they were binding-competent. The cell was fixed, immunolabeled with the monoclonal antibody Tab, and surface-replicated. The gold probe, bound to GPIIb, is monodispersed over the platelet surface. Bar, 0.1 μm. (Fig. 11) Surface replica of a platelet stimulated with ADP as described in Fig. 10 and exposed to antifibrinogen. No immunoreactive fibrinogen is present. Bar, 0.1 μm. (Fig. 12) Replica of the immunolabeled surface of a platelet stimulated with ADP in the presence of RGDS, and probed with Tab, the monoclonal antibody to GPIIb. The gold probe is clustered at several sites (*arrowheads*). Bar, 0.1 μm.

is internalized for subsequent intracellular processing, as is the case with most other receptor–ligand complexes.

The significance of GPIIb-IIIa clustering in platelets is unknown; however, clustering is known to be an antecedent to biological responses for a variety of receptors, including those for epidermal growth factor (49, 50, 54), insulin (24), and IgE (31). In the study on IgE receptors, the investigators concluded that the association of the IgE receptor with Ca^{2+} channels, a second messenger system common to many cell types, including the platelet (4, 46), may depend upon clustering. It remains to be determined whether GPIIb-IIIa clustering after fibrinogen binding serves the purpose of (a) signal transduction, (b) ion channel modulation, as proposed by several investigators (2, 5, 44), or (c) establishing microdomains within the plasma membrane for cytoskeletal anchoring (34, 37). However, not all glycoproteins that are expressed on the surface of the activated platelet cluster. We have recently reported on the surface distribution of GMP-140, an α-granule integral membrane glycoprotein in the resting platelet that is expressed on the plasma membrane after physiologic (22, 53) or pathologic (17) activation. Even at the latest time point analyzed, 5 min after thrombin activation, ~90% of the immunogold probe for GMP-140 was monodispersed. This glycoprotein, then, serves as an important control, indicating that clustering of gold-labeled glycoproteins is not merely an epiphenomenon associated with physiologic changes in platelet shape and/or membrane fluidity.

In addition to its ability to bind the RGDS domain on the α-chain of fibrinogen, the GPIIb-IIIa molecular complex has broader interest as a member of a family of Arg-Gly-Asp-specific cell adhesive protein receptors (45, 48). Fibrinogen, fibronectin, vitronectin, and von Willebrand factor all contain at least one RGDX sequence. Gardner and Hynes (13) have shown that the cell-attachment domain of fibronectin that binds to fibroblasts also binds to an inducible receptor on activated platelets, GPIIIa being the glycoprotein identified. With the finding of GPIIb-IIIa–related proteins on endothelial cells (10, 26), smooth muscle cells, fibroblasts (8, 42), and leukocytes (7, 42), a clearer understanding of cell-adhesive events at the molecular level in physiologic and pathologic thrombosis becomes a realistic goal.

We have determined that receptor occupancy alone is sufficient to induce clustering, and that the ligand need not be polyvalent to cross-link GPIIb-IIIa in the plane of the membrane. Other investigators (33, 57) have used concanavalin A, a tetravalent ligand that activates platelets and induces interactions between GPIIb-IIIa and the platelet cytoskeleton. Painter et al. (33) concluded that this association between the fibrinogen receptor glycoproteins and the actin cytoskeleton required clustering of GPIIb-IIIa molecules, and that the polyvalency of concanavalin A mediated such clustering. However, Phillips and co-workers (37) found substantial amounts of GPIIb-IIIa only in the Triton-insoluble cytoskeletons of "aggregated" thrombin-activated platelets, indicating that activation of the fibrinogen binding function of GPIIb-IIIa (with subsequent binding of the polyvalent fibrinogen molecule), is temporally distinct from cytoskeleton-mediated aggregation of platelets. Because our ADP-activated platelets showed clustering of receptors even in the absence of aggregation, we conclude that the cytoskeleton does not direct receptor clustering, but rather, that ligand-induced

GPIIb-IIIa clustering, mediated by receptor occupancy alone, promotes subsequent association with the actin cytoskeleton. Therefore, we propose the following model for ligand–receptor–cytoskeleton interaction at the platelet plasma membrane after physiologic activation: (a) ADP or thrombin induces a change in the GPIIb-IIIa complex that makes the receptor fibrinogen-binding-competent; (b) when available, as in thrombin-stimulated platelets, or when exogenously supplied to ADP-activated platelets, fibrinogen binds to GPIIb-IIIa; (c) the occupied receptors cluster and establish a microenvironment in the plasma membrane that facilitates interaction between the clustered glycoproteins and the platelet cytoskeleton.

References

1. Asch, A. S., L. L. K. Leung, M. J. Polley, and R. L. Nachman. 1985. Platelet membrane topography: colocalization of thrombospondin and fibrinogen with the glycoprotein IIbIIIa complex. Blood. 66:926-934.
2. Banga, H. S., E. R. Simons, L. F. Brass, and S. E. Rittenhouse. 1986. Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. Proc. Natl. Acad. Sci. USA. 83:9197-9201.
3. Bennett, J. S., and G. Vilaire. 1979. Exposure of platelet fibrinogen receptors by ADP and epinephrine. J. Clin. Invest. 64:1393-1401.
4. Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature (Lond.). 312: 315-321.
5. Brass, L. F. 1985. Ca^{2+} transport across the platelet plasma membrane. A role for membrane glycoproteins IIb and IIIa. J. Biol. Chem. 260:2231-2236.
6. Bray, P. F., J. P. Rosa, V. R. Lingappa, Y. W. Kan, R. P. McEver, and M. A. Shuman. 1986. Biogenesis of the platelet receptor for fibrinogen: evidence for separate precursors for glycoproteins IIb and IIIa. Proc. Natl. Acad. Sci. USA. 83:1480-1484.
7. Burns, G. F., L. Cosgrove, T. Triglia, J. A. Beall, A. F. Lopez, J. A. Werkmeister, C. G. Begley, A. P. Haddad, A. J. F. d'Apice, M. A. Vadas, and J. C. Cawley. 1986. The IIb-IIIa glycoprotein complex that mediates platelet aggregation is directly implicated in leukocyte adhesion. Cell. 45:269-280.
8. Charo, I. F., L. A. Fitzgerald, B. Steiner, S. C. Rall, L. S. Bekeart, and D. R. Phillips. 1986. Platelet glycoproteins IIb and IIIa: evidence for a family of immunologically and structurally related glycoproteins in mammalian cells. Proc. Natl. Acad. Sci. USA. 83:8351-8355.
9. Coller, B. S. 1985. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. J. Clin. Invest. 76: 101-108.
10. Fitzgerald, L. A., I. F. Charo, and D. R. Phillips. 1985. Human and bovine endothelial cells synthesize membrane proteins similar to human platelet glycoproteins IIb and IIIa. J. Biol. Chem. 260:10893-10896.
11. Fitzgerald, L. A., and D. R. Phillips. 1985. Calcium regulation of the platelet membrane glycoprotein IIbIIIa complex. J. Biol. Chem. 260:11366-11374.
12. Fujimoto, T., S. Ohara, and J. Hawiger. 1982. Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor VIII/von Willebrand factor on human platelets. J. Clin. Invest. 69:1212-1222.
13. Gardner, J. M., and R. O. Hynes. 1985. Interaction of fibronectin with its receptor on platelets. Cell. 42:439-448.
14. Gartner, T. K., and J. S. Bennett. 1985. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. J. Biol. Chem. 260:11891-11894.
15. Gartner, T. K., J. W. Power, E. H. Beachey, J. S. Bennett, and S. J. Shattil. 1985. The tetrapeptide analogue of the alpha chain and decapeptide analogue of the gamma chain of fibrinogen bind to different sites on the platelet fibrinogen receptor. Blood. 66:305a. (Abstr.)
16. George, J. N., A. T. Nurden, and D. R. Phillips. 1984. Molecular defects

in interactions of platelets with the vessel wall. *N. Engl. J. Med.* 311: 1084–1098.

17. George, J. N., E. B. Pickett, S. Saucerman, R. P. McEver, T. J. Kunicki, N. Kieffer, and P. J. Newman. 1986. Platelet surface glycoproteins: studies on resting and activated platelets and platelet membrane microparticles in normal subjects and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J. Clin. Invest.* 78:340–348.

18. Griffiths, G., and H. Hoppeler. 1986. Quantitation in immunocytochemistry: correlation of immunogold labeling to absolute number of membrane antigens. *J. Histochem. Cytochem.* 34:1389–1398.

19. Hopkins, C. R., K. Miller, and J. M. Beardmore. 1985. Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. *J. Cell Sci.* (Suppl.)3: 173–186.

20. Houdijk, W. P. M., K. S. Sakariassen, P. F. E. M. Nievelstein, and J. J. Sixma. 1985. Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. *J. Clin. Invest.* 75:531–540.

21. Hourdillé, P., M. Hasitz, F. Belloc, and A. T. Nurden. 1985. Immunocytochemical study of the binding of fibrinogen and thrombospondin to ADP and thrombin-stimulated human platelets. *Blood.* 65:912–920.

22. Isenberg, W. M., R. P. McEver, M. A. Shuman, and D. F. Bainton. 1986. Topographic distribution of a granule membrane protein (GMP-140) that is expressed on the platelet surface after activation: an immunogold-surface replica study. *Blood Cells (Berl.).* 12:191–204.

23. Jennings, L. K., and D. R. Phillips. 1982. Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIbIII complex. *J. Biol. Chem.* 257: 10458–10466.

24. Kahn, C. R., K. L. Baird, D. B. Jarrett, and J. S. Flier. 1978. Direct demonstration that receptor crosslinking or aggregation is important in insulin action. *Proc. Natl. Acad. Sci. USA.* 75:4209–4213.

25. Kloczewiak, M., S. Timmons, T. J. Lukas, and J. Hawiger. 1984. Platelet receptor recognition site on human fibrinogen: synthesis and structure relationship of peptides corresponding to the carboxy-terminal segment of the γ-chain. *Biochemistry.* 23:1767–1774.

26. Leeksma, O. C., J. Zandbergen-Spaargaren, J. C. Giltay, and J. A. van Mourik. 1986. Cultured human endothelial cells synthesize a plasma membrane protein complex immunologically related to the platelet glycoprotein IIb/IIIa complex. *Blood.* 67:1176–1180.

27. Marguerie, G. A., E. F. Plow, and T. S. Edgington. 1979. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 254:5357–5363.

28. McEver, R. P., N. L. Baenziger, and P. W. Majerus. 1980. Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. *J. Clin. Invest.* 66: 1311–1318.

29. McEver, R. P., E. M. Bennett, and M. N. Martin. 1983. Identification of two structurally and functionally distinct sites on human platelet membrane glycoprotein IIbIIIa using monoclonal antibodies. *J. Biol. Chem.* 258:5269–5275.

30. McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.

31. Menon, A. K., D. Holowka, W. W. Webb, and B. Baird. 1986. Crosslinking of receptor-bound IgE to aggregates larger than dimers leads to rapid immobilization. *J. Cell Biol.* 102:541–550.

32. Mosher, D. F., D. M. Pesciotta, J. D. Loftus, and R. M. Albrecht. 1986. Secreted alpha granule proteins. The race for receptors. *In* Platelet Membrane Glycoproteins. J. N. George, A. T. Nurden, and D. R. Phillips, editors. Plenum Publishing Corp., New York. 171–191.

33. Painter, R. G., M. Ginsberg, and Jaques, B. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:565–573.

34. Painter, R. G., K. N. Prodouz, and W. Gaarde. 1985. Isolation of a subpopulation of glycoprotein IIbIII from platelet membranes that is bound to membrane actin. *J. Cell Biol.* 100:652–657.

35. Parise, L. V., and D. R. Phillips. 1985. Reconstitution of the purified platelet fibrinogen receptor: fibrinogen binding properties of the glycoprotein IIb–IIIa complex. *J. Biol. Chem.* 260:10698–10707.

36. Peerschke, E. I., and M. B. Zucker. 1981. Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. *Blood.* 57:663–670.

37. Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* 86:77–86.

38. Pidard, D., R. R. Montgomery, J. S. Bennett, and T. J. Kunicki. 1983. Interaction of AP2, a monoclonal antibody specific for the human platelet glycoprotein IIbIIIa complex, with intact platelets. *J. Biol. Chem.* 258: 12582–12586.

39. Plow, E. F., and M. H. Ginsberg. 1981. Specific and saturable binding of plasma fibronectin to thrombin stimulated human platelets. *J. Biol. Chem.* 256:9477–9482.

40. Plow, E. F., M. H. Ginsberg, and G. A. Marguerie. 1986. Expression and function of adhesive proteins on the platelet surface. *In* Biochemistry of Platelets. D. R. Phillips and M. A. Shuman, editors. Academic Press, Inc., Orlando, FL. 225–256.

41. Plow, E. F., A. H. Srouji, D. Meyer, G. Marguerie, and M. H. Ginsberg. 1984. Evidence that three adhesive proteins interact with a common recognition site on activated platelets. *J. Biol. Chem.* 259:5388–5391.

42. Plow, E., J. Loftus, E. Levin, D. Fair, and M. Ginsberg. 1985. The cytoadhesins: a family of related cell surface molecules expressed by a variety of cells. *Blood.* 66:311a. (Abstr.)

43. Polley, M. J., L. L. K. Leung, F. Y. Clark, and R. L. Nachman. 1981. Thrombin-induced platelet membrane glycoprotein IIb and IIIa complex formation: an electron microscope study. *J. Exp. Med.* 154:1058–1068.

44. Powling, M. J., and R. M. Hardisty. 1985. Glycoprotein IIb–IIIa complex and Ca^{2+} influx into stimulated platelets. *Blood.* 66:731–734.

45. Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559–1562.

46. Rink, T. J., and T. J. Hallam. 1984. What turns platelets on? *Trends Biochem. Sci.* 9:215–219.

47. Robenek, H., G. Schmitz, and G. Assmann. 1984. Topography and dynamics of receptors for acetylated and malondialdehyde-modified low-density lipoprotein in the plasma membrane of mouse peritoneal macrophages as visualized by colloidal gold in conjunction with surface replicas. *J. Histochem. Cytochem.* 32:1017–1027.

48. Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell.* 44:517–518.

49. Schechter, Y., L. Hernaez, J. Schlessinger, and P. Cuatrecasas. 1979. Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. *Nature (Lond.).* 278:835–838.

50. Schreiber, A. B., T. A. Libermann, I. Lax, Y. Yarden, and J. Schlessinger. 1983. Biological role of epidermal growth factor–receptor clustering: investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.* 258:846–853.

51. Shattil, S. J., J. A. Hoxie, M. Cunningham, and L. F. Brass. 1985. Changes in the platelet membrane glycoprotein IIbIIIa complex during platelet activation. *J. Biol. Chem.* 260:11107–11114.

52. Stenberg, P. E., M. A. Shuman, S. P. Levine, and D. F. Bainton. 1984. Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. *J. Cell Biol.* 98:748–760.

53. Stenberg, P. E., R. P. McEver, M. A. Shuman, Y. V. Jacques, and D. F. Bainton. 1985. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J. Cell Biol.* 101: 880–886.

54. Wakshull, E. M., and W. Wharton. 1985. Stabilized complexes of epidermal growth factor and its receptor on the cell surface stimulate RNA synthesis but not mitogenesis. *Proc. Natl. Acad. Sci. USA.* 82:8513–8517.

55. Weiss, H. J., V. T. Turitto, and H. R. Baumgartner. 1986. Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIbIIIa, Ib, and storage granules. *Blood.* 67:322–330.

56. Wencel-Drake, J. D., E. F. Plow, T. J. Kunicki, V. L. Woods, D. M. Keller, and M. H. Ginsberg. 1986. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am. J. Pathol.* 124:324–334.

57. Wheeler, M. E., J. M. Gerrard, and R. C. Carroll. 1985. Reciprocal transmembranous receptor-cytoskeleton interactions in concanavalin A-activated platelets. *J. Cell Biol.* 101:993–1000.

58. Zidovetzki, R., Y. Yarden, J. Schlessinger, and T. M. Jovin. 1986. Microaggregation of hormone-occupied epidermal growth factor receptors on plasma membrane preparations. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:247–250.