American Journal of Pathology, Vol. 141, No. 3, September 1992

Platelet Morphologic Changes and Fibrinogen Receptor Localization

Initial Responses in ADP-activated Human Platelets

Mary E. Hensler,* Mony Frojmovic,† Richard G. Taylor,* Roy R. Hantgan,* and Jon C. Lewis*

From the Departments of Pathology and Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina; and the Department of Physiology,† McGill University, Montreal, Quebec, Canada*

Platelet exposure to agonists results in rapid morphologic changes paralleled by fibrinogen binding and platelet aggregation. The current study used standardized stereology in conjunction with immunogold electron microscopy to correlate the initial morphologic changes with fibrinogen receptor localization on the surfaces of ADP-activated human platelets. A 45% increase in platelet circumference was observed after 3 seconds of activation (P = 0.001). Virtually all of this increase was due to a 13-fold increase in projection membrane, and the projections observed by stereo microscopy at this time were mostly blunt. Both blunt and long projections also accounted for the increase in platelet–platelet contacts at 10 seconds of activation. Immunogold electron microscopy using the monoclonal antibodies P2 and AP-2 against the fibrinogen receptor, glycoprotein IIb/IIIa (GP IIb/IIIa), showed relatively equivalent immunogold densities on projections compared with cell body during 30 seconds of activation. The activation-dependent anti-GP IIb/IIIa monoclonal antibody, 7E3, showed an immunogold density 37% greater on projections compared with cell body (P = 0.0001). Colocalization studies using 7E3 with a polyclonal antifibrinogen antibody showed bound fibrinogen in close proximity to the GP IIb/IIIa localized by 7E3 on projections. These studies support an important role for platelet projections during the earliest stages of fibrinogen binding and ADP-induced aggregation. (Am J Pathol 1992, 141:707–719)

The morphologic changes accompanying platelet exposure to an adhesive substratum or to a soluble aggregating agent such as adenosine diphosphate (ADP) have been studied extensively.[1–4] An event common to both adhesion and aggregation is the transition of platelets from smooth discocytes to discoechinocytes by the extension of pseudopods.[5] Platelets activated in plasma have been observed to extend elongated pseudopodia and become more spherical within seconds of exposure to ADP,[6,7] although it has been suggested that an intermediate form of discoechinocytes with blunt pseudopodia exists.[8] It is at this stage of early pseudopod formation that platelets begin to interact[9] in a fashion that subsequently leads to formation of complex aggregates.[10] Although it has been suggested that the extension of pseudopods during platelet shape change is important for the subsequent establishment of platelet–platelet contacts,[4,11,12] the actual contribution of pseudopods to aggregation remains unknown. Most importantly, the relationship between pseudopod extension and fibrinogen binding, which is central to platelet cohesion, remains poorly understood.[12,13]

Playing a key role in platelet–platelet interactions leading to aggregation is glycoprotein IIb/IIIa (GP IIb/IIIa), the platelet surface receptor for fibrinogen.[14–17] The importance of GP IIb/IIIa is documented in thrombasthenic patients, who have hemorrhagic diathesis with varying severity, which is related to deficiencies in fibrinogen binding due to decreased amounts or abnormalities in GPIIb/IIIa.[18,19] Glycoprotein IIb/IIIa is normally present as a calcium-dependent heterodimer[20] at very high density on the platelet surface, with approximately 40,000 to 50,000 copies per cell.[14,15] Recent data also suggest the existence of an internal pool of GP IIb/IIIa, possibly located in the open-channel system.[21] The fibrinogen

Supported by NIH grants RR 02722 for intermediate voltage electron microscopy, HL 14164 (Specialized Center of Research in Arteriosclerosis), HL 32349, CA 12197, and the Medical Research Council of Canada [M. Frojmovic]. M. E. Hensler was supported by NIH grant HL 07115, Institutional National Research Award.

Accepted for publication March 5, 1992.

Address reprint requests to Dr. Jon C. Lewis, Department of Pathology, Bowman Gray School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157-1092.

binding activity of GP IIb/IIIa is tightly regulated in that the receptor is capable of binding fibrinogen only after platelet activation.[22] The mechanism by which fibrinogen binding sites are exposed on the platelet surface is unclear. A number of studies, however, suggest that platelet activation is accompanied by conformational or microenvironmental changes involving existing GP IIb/IIIa such that fibrinogen binding sites may be exposed.[23–27]

Paralleling these activation-associated conformational changes appear to be changes in topographical distribution of GP IIb/IIIa on the cell surface.[28–33] Pseudopods may play an important role in such a relocation in that GP IIb/IIIa has been shown to cluster on pseudopods of suspended platelets after long-term thrombin activation[34] and on platelets adherent to artificial surfaces.[35] Although these observations are suggestive of a selective localization of the GP IIb/IIIa receptor complex, the association between pseudopod formation and the location of GP IIb/IIIa in its complexed form has not been studied at early activation times when cell–cell interactions are most critical. The goal of the current study was to correlate the earliest ADP-mediated membrane changes, manifest by the expression of pseudopods, with the topographical distribution of the GP IIb/IIIa receptor complex and fibrinogen. Our results document an overall diffuse localization of GP IIb/IIIa during platelet pseudopod formation; however, a subpopulation of "activated" GP IIb/IIIa appears to co-localize on pseudopods with bound fibrinogen. The results suggest that pseudopodial GP IIb/IIIa plays an important role in early platelet–platelet interactions.

Experimental Procedures

Materials

HEPES buffer, aprotinin, bovine serum albumin (fraction V), and ADP (grade IV) were purchased from Sigma Chemical Co. (St. Louis, MO). Human fibrinogen was obtained from IMCO (Stockholm, Sweden). A mouse monoclonal antibody specific for human GP IIb/IIIa in complex, P2,[36] was obtained from AMAC, Inc. (Westbrook, ME), and the monoclonal antibody against GP IIb/IIIa, AP-2,[37] was a gift of Dr. Thomas Kunicki (Milwaukee, WI). The activation-dependent GP IIb/IIIa specific mouse monoclonal antibody, 7E3,[24,25] was provided by Dr. Barry Coller (Stony Brook, NY). Rabbit polyclonal antibodies against human fibrinogen and human lactoferrin were both obtained from Organon-Teknika Corporation (West Chester, PA). Mouse monoclonal antibody against monocytes, MO_2, was obtained from Coulter Immunology (Hialeah, FL). Immunogold probes (Auroprobes goat anti-mouse and goat anti-rabbit, 5 and 15 nm) were purchased from Janssen Life Sciences (Olen, Belgium). All other chemicals were reagent grade.

Platelet Isolation and Activation

Some studies using platelets in plasma were done in the laboratory of Dr. Mony Frojmovic (Montreal) to compare early morphologic changes of gel-filtered platelets (GFP) with those of platelets in the more physiologic plasma environment. Whole blood was collected into 3.8% sodium citrate (9 volumes blood: 1 volume citrate). Platelet-rich plasma (PRP) was obtained by spinning at 150*g* for 15 minutes. Platelet-rich plasma was drawn off and stored at 37°C for 30 minutes in an atmosphere of 5% CO_2/95% air. The platelets in plasma were activated by addition of 10 μmol/l (micromolar) ADP to a 100 μl aliquot of PRP in a glass cuvette in an aggregometer (Payton Scientific Co.) with a one-half second stir at 1000 rpm. This was done to rapidly mix the activator without inducing significant aggregation.[12] At various times up to 60 seconds, 0.8% glutaraldehyde was added to stop the reaction. Platelet samples were transferred to 40 volumes glutaraldehyde (0.8%) and allowed to fix for 30 minutes. The platelets were rinsed free of fixative with Walsh-albumin buffer (0.137 mol/L [molar] NaCl, 0.003 mol/L KCl, 0.012 mol/L $NaHCO_3$, 0.0004 mol/L NaH_2PO_4, 0.002 mol/L $MgCl_2$, 0.0006 mol/L dextrose, 3.5% albumin, pH 7.4) and stored in this buffer for subsequent immunocytochemistry. To control for spontaneous activation of platelets, samples paralleling the ADP activation times were collected either from PRP that was maintained under static conditions or PRP that was stirred without the addition of ADP.

Platelets for gel filtration (GFP) were prepared from blood that had been drawn into acid-citrate-dextrose (9 volumes blood: 1 volume anticoagulant; acid-citrate-dextrose = sodium citrate 0.085 mol/l + dextrose 0.011 mol/l + citric acid 0.078 mol/l). All samples were from informed healthy human donors who had taken no aspirin for 2 weeks. The procedures used were examined and approved by the Clinical Research Practices Committee of the Bowman Gray School of Medicine, J. Kiffin Penry, M.D., Chairman. Platelet-rich plasma was obtained by spinning the blood at 400*g* for 15 minutes. To prevent spontaneous activation of the platelets, 150 μl acid-citrate-dextrose at 22°C was added to 1.5 ml PRP, and this was incubated at 37°C for 10 minutes just before gel filtration. The remaining PRP was stored at 22°C in an atmosphere of 5% CO_2/95% air for no more than 2 hours. Gel filtration of the cells was accomplished as previously described.[8] Briefly, 1 ml of the warmed PRP was layered over a 1.5 cm × 7 cm PD-10 column (Pharmacia, Piscataway, NJ) which had been packed with acetone-washed Sepharose 2B and equilibrated with a HEPES-

Tyrodes-albumin (HAT) buffer (0.13 mol/l NaCl, 0.0026 mol/l KCl, 0.01 mol/l HEPES, 0.0042 mol/l NaH_2PO_4, 0.1% aprotinin, 0.0055 mol/l dextrose, 3 mg/ml BSA, pH 7.2). Gel-filtered platelet counts were obtained using a Coulter ZM (Hialeah, FL), and the samples were diluted with HEPES-Tyrodes-albumin buffer to 80,000 to 120,000 cells/μl for use. The GFP were routinely stabilized for 15 to 30 minutes at 37°C before use, and all experiments were completed within 30 minutes of gel filtration.

Activation of GFP was done by allowing a 500-μl aliquot to stir at 37°C in the aggregometer (Chronolog Corporation, Havertown, PA) for 30 seconds; calcium (2 mmol/l [millimolar]) and in some cases fibrinogen (120 μg/mL) were added to the GFP suspensions. The suspensions of GFP were then stirred at 1100 rpm for at least 30 seconds to screen for spontaneous aggregation. Samples that aggregated under these conditions were not used for further experimentation. Gel-filtered platelets then were activated under stirring conditions by addition of 10 μmol/l ADP. Subsequent to activation, the samples were fixed at various times by addition of 500 μl 0.1 mol/l cacodylate-buffered 1% glutaraldehyde (pH 7.3). This preliminary fixation mixture was quickly transferred to 9 volumes fixative for an additional 10 to 20 minutes at 22°C. The samples were washed in cacodylate buffer and stored at 4°C. As was the case with PRP, control platelets included cells maintained under static conditions or cells that were stirred in the absence of ADP.

Immunocytochemistry

Immunolocalization of platelet surface antigens was done using the double-labeling technique previously applied in this laboratory.[35,38] Cells fixed with cacodylate-buffered (pH 7.3) glutaraldehyde (1.0%) were rinsed once at 22°C in Gey's balanced salts (pH 7.35), and this was followed by three washes in Gey's containing 0.1% glycine to quench free aldehyde groups and thereby minimize nonspecific binding of antibody. A final rinse in Gey's buffer alone was followed by incubation with the primary antibodies. The antibodies P2, AP-2, 7E3, and the negative control antibody, MO_2, were diluted to an IgG concentration of 10 μg/mL in Gey's salts for use. The polyclonal anti-fibrinogen and its negative control, anti-lactoferrin, were used at a protein concentration of 100 μg/mL. The primary antibodies were added to the fixed platelets, and the combination was incubated with slight agitation at 22°C for 45 minutes. After washing the platelets three times in Gey's salts to remove unbound antibody, the second-stage immunogold probes were added. Immunolocalization of GP IIb/IIIa was done using goat anti-mouse 15 nm immunogold, which was mixed with the nonspecific goat anti-rabbit 5 nm immunogold (1:1), and this solution was subdiluted 1:2 with Gey's salts. For the immunolocalization of fibrinogen or the co-localization experiments, goat anti-rabbit 15 nm immunogold was combined with 5 nm goat-anti-mouse immunogold as above. For each experiment, 100 μL of the mixture of immunogold probes was incubated with the platelets for 1 hour at 22°C with agitation. The platelets were subsequently rinsed three times in Gey's salts and then stored at 4°C.

Electron Microscopy and Stereology

Samples for transmission electron microscopy were postfixed with 1% OsO_4, stained *en bloc* with 2% uranyl acetate in 25% ethanol, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Thin sections of 60 to 80 nm were examined at 80 keV on a Philips TEM 400. Morphometric analyses of platelet surface changes and immunogold distribution was done on micrographs of 20 randomly selected platelet cross sections (on platelets 1.0 μ in diameter or larger). Platelet plasma membrane length (excluding open-channel system) was obtained on these micrographs by digitizing with a standard map wheel and calculating surface length. The average length of surface membrane per platelet is expressed in Results as mean circumference. Platelet surface length was divided into four categories: projection contact, projection noncontact, cell body contact, and cell body noncontact. Platelet surface was defined as a projection if the ratio of the length of projection surface membrane to the length of the projection base was 1.5 or greater; all other membrane was considered cell body. If opposing membranes were 20 nm or closer together, that portion of platelet surface accounted for by the closely positioned membranes was considered contact membrane[39]; all other membrane was noncontact. Immunogold density, expressed as amount of gold per micron membrane length, was calculated for projection and cell body categories (contact and noncontact). Gold was counted if it was 30 nm or closer to the plasma membrane on the cell exterior. This positioning was interpreted as representing specific localization of surface-associated glycoproteins. This distance was based on unpublished light scattering observations, which showed the total diameter of an immunoglobulin-coated gold probe having a 15-mm gold core to be in the range of 26 to 30 nm (R. Hantgan, March 1989). Binding of control nonspecific secondary immunogold probes in all experiments was less than one gold particle per platelet section. Statistical analyses of the membrane changes at the various times of activation were based on analysis of variance of the different

groups using the BMDP Statistical Software package (Berkeley, CA). Comparisons of projection and cell body immunogold labels were done using paired *t*-tests. All differences were considered significant at $P \leq 0.05$.

Samples for thick-section stereo electron microscopy were cut at a thickness of 1 to 2 μ and viewed at 300 kV using a Philips CM-30 intermediate-voltage electron microscope. Stereo pair micrographs were obtained at varying angles of specimen tilt for each sample. These stereo observations were highly instructive, for they provided an overview for assessing the distribution of projections and immunogold label. The thick-section observations were most important because the entire surfaces of intact platelets could be studied at electron microscope resolution.

Samples for scanning electron microscopy (SEM) were processed immediately after the primary fixation in glutaraldehyde. To accomplish this, the platelet and fixative suspension was drawn into a syringe and then collected on a 0.4-μ pore filter (Nuclepore, West Chester, PA), which then was transferred to a plastic holder designed for critical point drying. The samples were dehydrated through a graded series of ethanol and dried from CO_2 by the critical point method. The dried filters were placed on stubs and sputter-coated with gold-palladium (Denton Vacuum) for viewing on a Philips 501 SEM.

Results

Human platelets while in circulation are primarily discoid in shape and have relatively smooth surfaces; however, it is known that cells containing surface projections exist under conditions of nonstimulation.[5] In the current study, phase-contrast and SEM were used to assess the relative proportions of discocytes and discoechinocytes either in plasma or after gel filtration. Approximately 63% of platelets in plasma and 59% of GFP were discocytes before ADP stimulation, whereas the remaining were generally spheroid in configuration and were characterized by the presence of a few, short, blunt microspikes that projected from otherwise smooth plasma membranes (Figure 1). This shape profile was altered only slightly when control cells were maintained under stirring conditions; on stirring for as long as 1 minute, the percentage of platelets having short projections was increased by less than 10%. Although short projections were observed by both phase contrast and SEM on the stirred, control platelets, the cells clearly were not stimulated to interact, for more than 95% of unstimulated control platelets existed as single cells when observed by Nomarski differential interference contrast microscopy. The remaining 5% were primarily doublets in which the paired platelets had limited contact.

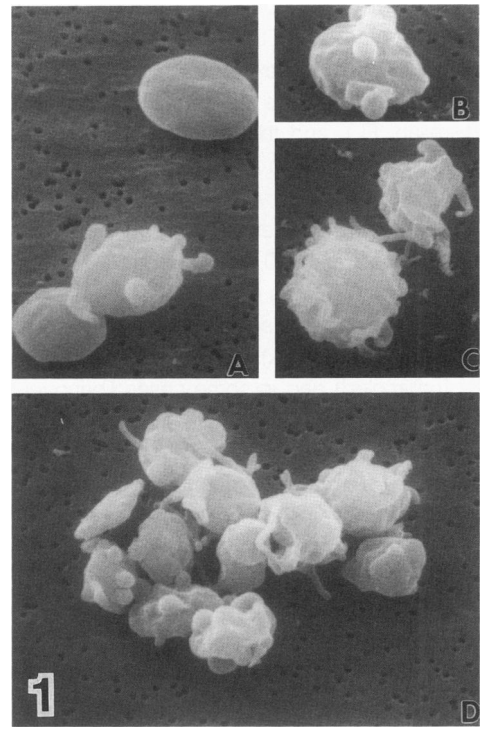


Figure 1. *Scanning electron micrographs demonstrating morphology of gel-filtered platelets at different stages of short-term activation with 10 μmol/l ADP.* **A:** *GFP before ADP activation. The discoid shape and smooth plasma membrane characteristic of unstimulated cells is illustrated (×5,000).* **B:** *Platelets exposed to ADP for 3 seconds. Illustrated are the expression of blunt projections that elaborate from an irregular plasma membrane (×5,000).* **C:** *GFP, activated for 5 seconds. Shown are the abundant projections, many of which contribute to initial contacts between the cells (×5,000).* **D:** *Platelets exposed to ADP for 10 seconds. Note the early aggregate containing platelets of varying morphologies, most of which are characterized by multiple projections (×5,000).*

The morphology of platelets was significantly altered on stimulation with ADP. First, there was a marked transition from discocyte to discoechinocyte. In marked contrast to resting cells having few projections, this was characterized by the extension of numerous microspikes projecting from irregular cell surfaces (Figure 1B, C). The early projections induced by ADP treatment varied in shape from the short, blunt balloonlike membrane protrusions found with resting cells to elongate filopodia. This transition from platelets having predominantly smooth to highly irregular surfaces was rapid, observed after only 1 second of activation. After 3 to 5 seconds of ADP stimulation, approximately 80% of the cells had various-sized surface projections. This represented a reduction by 65% in the number of discoid, smooth-surfaced cells. The initial activation of platelets was essentially complete between 3 and 10 seconds; projections were observed on more than 85% of the cells at the 10-second time. The

second major change on ADP stimulation was cellular interaction resulting in a shift from predominantly single cells to a population composed of platelet microaggregates (Figure 1C). This platelet–platelet association after short-term activation was more pronounced for platelets in whole plasma than for platelets after gel filtration, yet the general pattern was consistently observed. When the cells were stimulated in plasma for 3 seconds, fixed, and then observed by Nomarski optics, approximately 25% of observed particles were small aggregates composed of two and three platelets. Interestingly, even at this early time, approximately 4% of the particles were microaggregates containing between four and 10 cells. The formation of microaggregates in plasma continued over the 30-second experimental time; by 10 seconds, more than 10% of the platelet clusters contained at least three cells, and at 30 seconds this had increased to approximately 20%. Essentially, the pattern of progressive cell association was observed for both GFP and those activated in plasma.

The rapid extension of surface projections that preceded microaggregate formation raised questions concerning the amount of membrane on the platelet surface before and during the transformation. Therefore, thin-section transmission electron microscopy stereology was used to quantitatively address this issue. Based on previous data,[4,40] it was hypothesized that the elaboration of projections over the 30-second experimental time should be accompanied by a concomitant increase in surface membrane. When micrographs of ADP-activated GFP were digitized, it was found that the circumference of the cells increased from a mean of 6.7 ± 0.12 μ to 9.8 ± 0.28 μ ($P = 0.001$) during the initial 3 seconds. This 45% net increase in surface was comparable to a 42% increase quantitated for platelets stimulated in plasma (data not shown). Surprisingly, the surface increase was mostly complete by 3 seconds, and large changes in total surface membrane after this initial period were not observed.

Although total surface was maximal after the 3-second activation, it was found during the microaggregation stage (3 to 30 seconds) that the distribution of membrane on the platelet surface did not remain static, but rather the distribution underwent continuous alterations. To better define these changes in surface membrane distribution, platelet membrane before digitization was categorized as either surface projection or cell body, and then the amount of plasma membrane for each category on each platelet was determined (Table 1). The initial and rapid change in the amount of surface membrane during the first 3 seconds was accounted for by a 13-fold increase in the amount of projection membrane ($P = 0.001$), and by 10 seconds the quantity of projection membrane had increased 20-fold (Table 1). Although

Table 1. *Length of Plasma Membrane (μm)*

Activation time (sec)	Projection	Cell body
0	0.31 ± 0.08	6.42 ± 0.11
3	4.00 ± 0.25*	5.78 ± 0.21
10	6.12 ± 0.36	4.02 ± 0.19†
30	5.06 ± 0.26	4.38 ± 0.14

* Indicates greater than projection membrane at 0 seconds and less than at 10 seconds ($P = 0.001$).
† Indicates less than cell body membrane at 3 seconds ($P = 0.001$).
Numbers represent mean ± standard error (n = 60 at each time).

marginal during the immediate stages of activation, a reduction in cell body membrane was pronounced between 3 and 10 seconds, when a 30.5% reduction was found ($P = 0.001$). Cell body membrane remained at this level over the ensuing 20 seconds of activation.

The data above document an initial increase in plasma membrane, and this increase in surface membrane appears to be specific to the development of projections, which was accompanied by microaggregation. To determine whether the formation of projections and increase in surface at different times after ADP stimulation correlated with platelet-to-platelet cohesion (formation of adhesive contacts), platelet surface membrane was digitized and then categorized as contact if the apposing membranes were 20 nm or closer[39] (Figure 2). Using this criterion, approximately 6% of the platelet surface in preparations of resting cells was characterized as contact surface. Although by definition the resting cells were in limited contact, the association probably resulted from centrifugation and packing of the cells during preparation for electron microscopy. If this is the situation, the 6% contact observed with the resting cells represents experimental background (Table 1). Notably, the amount of cell contact was unchanged with 3 seconds of activation; but at this early time there occurred a shift in the location of contact areas, with the newly formed projections accounting for 40% of the contact sites. An approximate twofold increase in adhesive contact-associated platelet membrane was observed at 10 seconds, and as summarized in Table 2, a significant proportion of this increase was directly related to the formation of projections. One and one-half times as much projection membrane (0.701 ± 0.08 μ) as compared with cell body (0.43 ± 0.05 μ) membrane was involved in these early ADP-induced platelet–platelet interactions. This general trend of increased adhesive contact continued, and by 30 seconds 19% of the platelet circumference was involved. As opposed to the earlier times, contact membrane at 30

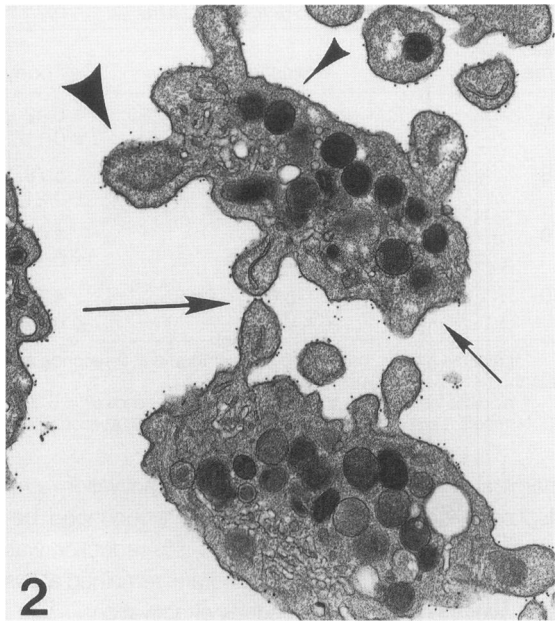


Figure 2. *Thin-section transmission electron micrograph illustrating the parameters used to categorize platelet plasma membrane. These platelets were from a sample that had been immunogold-stained for GP11b/111a after ADP activation. Surface membrane was categorized as: projection (large arrowhead) or cell body (small arrowhead). Platelet plasma membrane that was positioned 20 nm or closer in apposition to other platelet membrane was further categorized as contact (large arrow); all remaining membrane was considered noncontact (small arrow) (×22,800).*

seconds was equally divided between projection and cell body. When GFP were activated in medium to which fibrinogen was not added, a decrease in the amount of contact membrane was observed. This reduction of cohesion in the absence of fibrinogen was most pronounced for projections, and the phenomenon is consistent with the role of fibrinogen as a molecular bridge between platelets. Interestingly, although fibrinogen was omitted, selected contacts did form. These contacts most likely were stabilized by the small amount (less than 10 μg/ml) of fibrinogen remaining in the medium after gel filtration.

Given the dramatic and immediate increase in projection membrane and cohesive contacts that is found with ADP stimulation, it was of interest to study relationships between the distribution of fibrinogen and its receptor, GP IIb/IIIa, on the platelet surface during projection formation and microaggregation. Platelets for the receptor localization studies were fixed in suspension and probed with either P2 or AP-2, mouse monoclonal antibodies

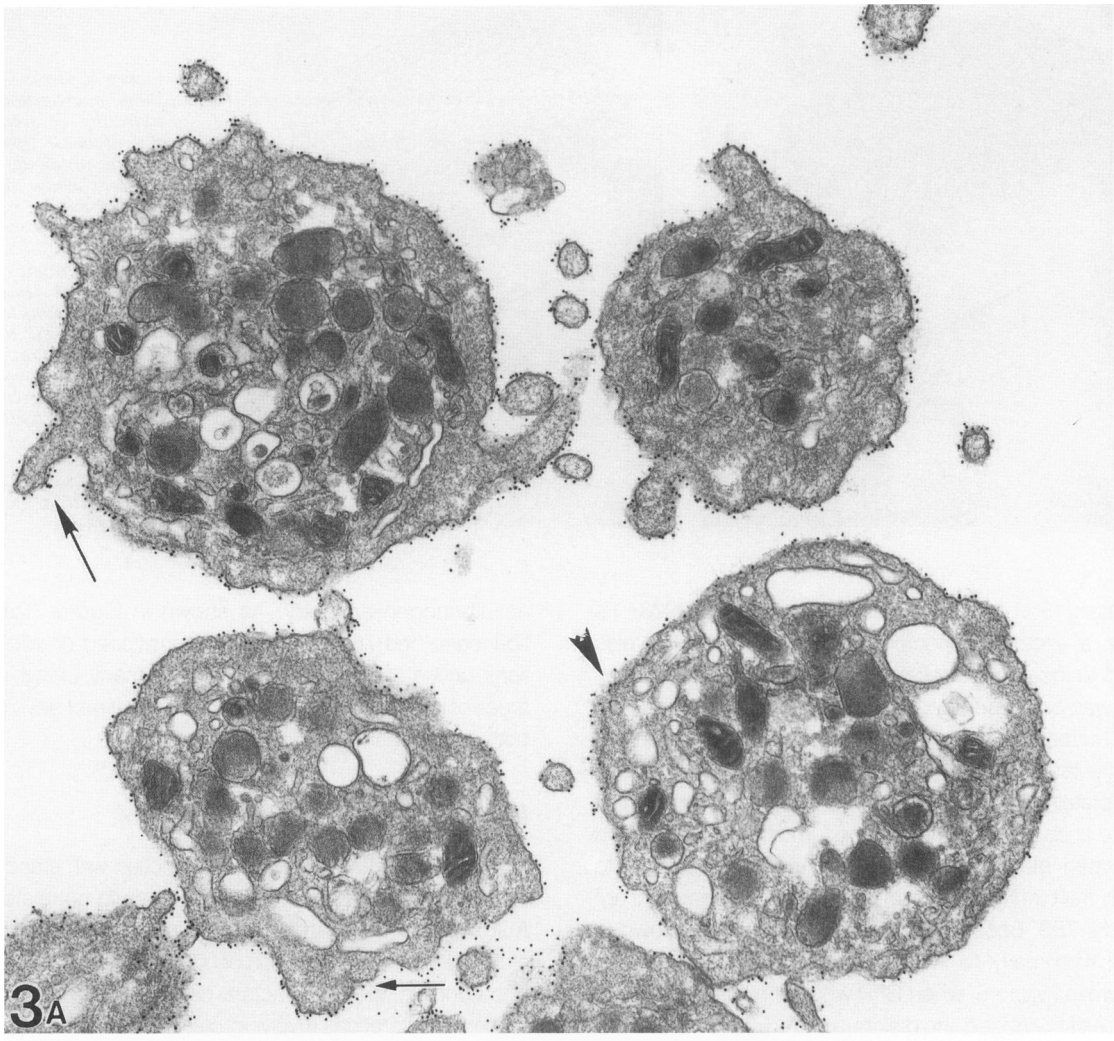
Table 2. *Percentage of Platelet Membrane Involved in Contacts*

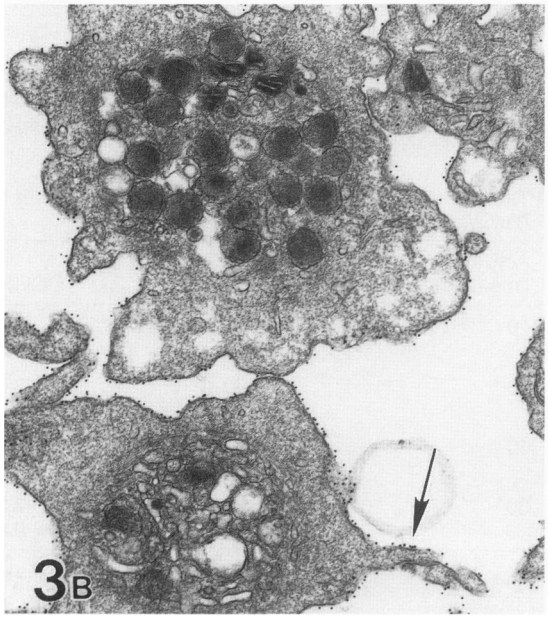
Activation time (sec.)	Membrane Location	
	Projection	Cell body
0	0.15%	6.38%
3	2.55	3.35
10	6.75	4.39
30	9.19	10.1
30(-fibrinogen)	5.14	8.52

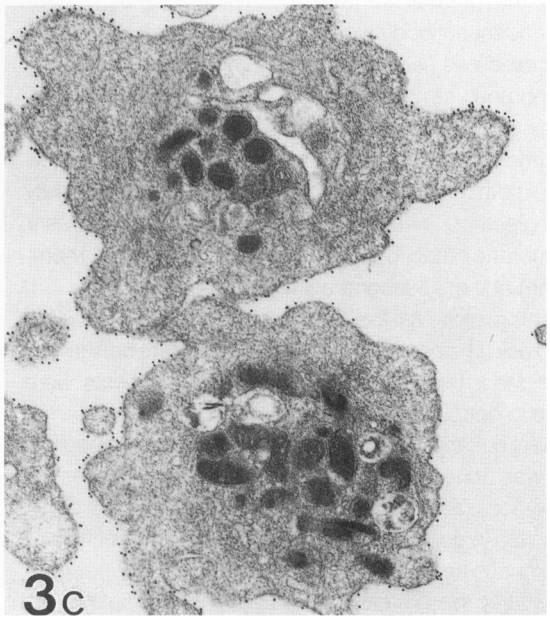
specific for GP IIb/IIIa in complex.[36,37] Glycoprotein IIb/IIIa, localized by P2, appeared to be diffusely distributed on the irregular cell body membrane as well as on short projections at 3 seconds of ADP activation (Figures 3 and 4). This generalized pattern of immunogold distribution was most evident in three-dimensional micrographs of thick sections where the entire cell surface could be observed (Figure 4). When the P2 antibody immunogold density was quantitated from thin sections at this short time of stimulation, the distribution was about equal for projections and cell body (Table 3). To address whether GP IIb/IIIa might undergo a redistribution with more prolonged activation, immunogold labeling for the receptor was also quantified on platelets activated for 10 and 30 seconds. As summarized in Table 3, GP IIb/IIIa at 30 seconds was localized by P2 at a slightly greater density (19%) on projection compared with cell body ($P = 0.0001$); although slight, this shift was statistically significant. In contrast to an average immunogold density for P2 of approximately eight gold particles per micron of membrane, nonspecific labeling with the monoclonal antibody MO_2, which is specific for monocytes, was found to be less than 1 gold particle per 10 microns of membrane.

To address whether competition from bound fibrinogen or the tightness of platelet contacts might be blocking receptor sites for the P2 antibody, immunolocalization was done on platelets activated for 30 seconds in the absence of exogenous fibrinogen. This, as already noted, limited the amount of tight contacts among platelets. Under these activation conditions, the overall immunogold density was increased 36% compared with platelets activated for 30 seconds in the presence of fibrinogen. This increase in immunogold density in the absence of the ligand was evident on both projections and cell body ($P \leq 0.0002$); however, under these conditions, the immunogold density on projections was still only slightly

Figure 3. *Immunogold localization of glycoproteins IIb and IIIa on ADP-stimulated gel-filtered platelets.* **A**: *Following 3 seconds of platelet stimulation, the monoclonal antibody, P2, was uniformly distributed to the cell body (arrowhead), blunt projections (small arrow), and pseudopods (large arrow), X = 25,000.* **B**: *Cells stimulated with ADP for 10 seconds followed by P2 localization. Immunogold density was slightly increased on the pseudopods (arrow) when compared to cell body; however, the shift was subtle, X = 17,250.* **C**: *Cell stimulated with ADP for 30 seconds prior to immunochemistry with AP-2. Although some areas of the cell body have less label than pseudopods and projections, a diffuse pattern of labeling was found, X = 17,250.*


3A


3B


3C

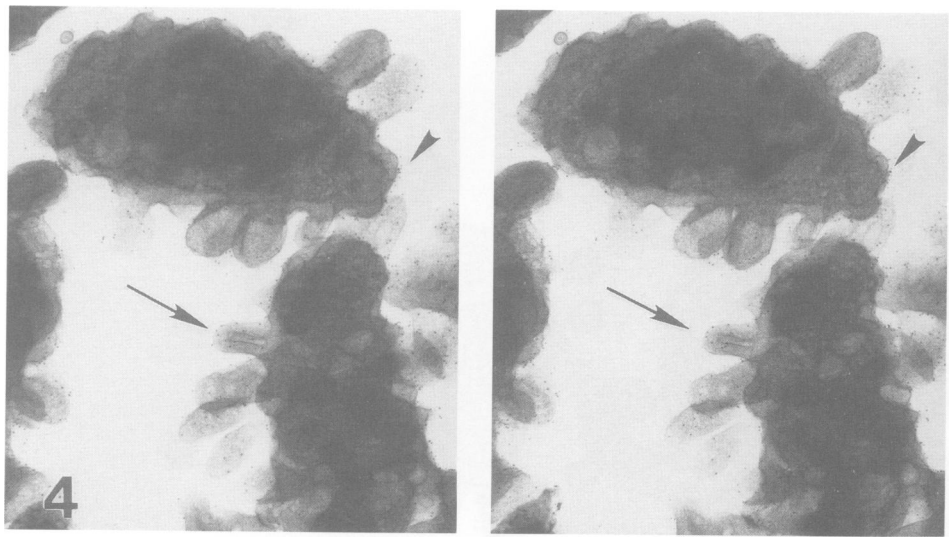


Figure 4. *Stereo (3-D) IVEM micrograph illustrating the distribution of the GP IIb/IIIa-specific antibody, P2. The cells shown in this micrograph are representative of platelets activated for 3 seconds in plasma with 10 μmol/l ADP. Immunogold (15 nm) label, indicating P2 antibody localization, was distributed on blunt membrane projections (arrow) as well as along the shelf of cell body membrane (arrowhead) from which the projections extend (×17,000).*

greater (18%) than that on cell body ($P = 0.0008$). Finally, a second monoclonal antibody, AP-2, was also used to localize the receptor complex, and the distribution was comparable to that found with P2 (Figure 3).

The results obtained with the P2 and AP-2 antibodies suggested an overall diffuse distribution of GP IIb/IIIa on the platelet surface during microaggregation. Both of these antibodies, however, bind to both unactivated and activated platelets.[36,37] It was of interest, therefore, to study the binding pattern of the activation-dependent antibody, 7E3, because this antibody has been shown to bind maximally to ADP-stimulated platelets.[24,25] As shown in Figure 5, when GFP were stimulated in the absence of fibrinogen, the distribution of 7E3 was not diffuse over the platelet surface, but rather the activation-dependent antibody was preferentially located on platelet projections, with many areas of the main cell body membranes remaining devoid of gold. When the distribution of 7E3 was quantitated, as had been done for P2, the immunogold density was increased approximately threefold after ADP stimulation. Furthermore, the density was greater ($P = 0.0001$) on projections (2.2 particles/μ membrane) than on cell body (1.4 particles/μ membrane) at the 30-second activation time.

This preferential localization of 7E3 on projections led us to ask whether the ligand fibrinogen might be similarly positioned; therefore, co-localization experiments were done to correlate receptor and ligand distribution on the platelet surface. Figure 6 illustrates that when colocalization was done using the antibody P2, GP IIb/IIIa was diffusely localized on both projections and the cell body. This was in contrast to the more focal location of fibrinogen at contact points and projections. To more specifically verify this preferential localization of the ligand, co-localization experiments were done using 7E3 and the anti-fibrinogen antibody. As shown in Figure 7, anti-fibrinogen and 7E3 were similarly positioned on projections and at contact areas, whereas many uninvolved areas of the platelet cell body surface remained devoid of both antibodies.

Discussion

Immunogold cytochemistry in conjunction with standard stereology was used in the current study to correlate on ADP-stimulated platelets the earliest morphologic changes with the distribution of fibrinogen receptors. It is apparent from the ultrastructural data reported herein that numerous alterations involving platelet membrane occur before the establishment of platelet–platelet interactions leading to the formation of aggregates. One alteration was a 45% increase in the amount of external surface membrane after 3 seconds of ADP stimulation. This finding that platelets initially undergo ADP-mediated shape

Table 3. *Density of Immunogold Label for P2 (Gold Particles/μm of Cell Surface)*

Activation time (sec)	Projection	Cell body
0	—	8.09 ± 0.37
3	9.30 ± 0.47	8.69 ± 0.42
10	8.54 ± 0.45*	7.67 ± 0.48
30	8.92 ± 0.47†	7.23 ± 0.43

* Indicates greater than cell body ($P = 0.03$).
† Indicates greater than cell body ($P = 0.0001$).
Numbers represent mean ± standard error (n = 60 at each time).

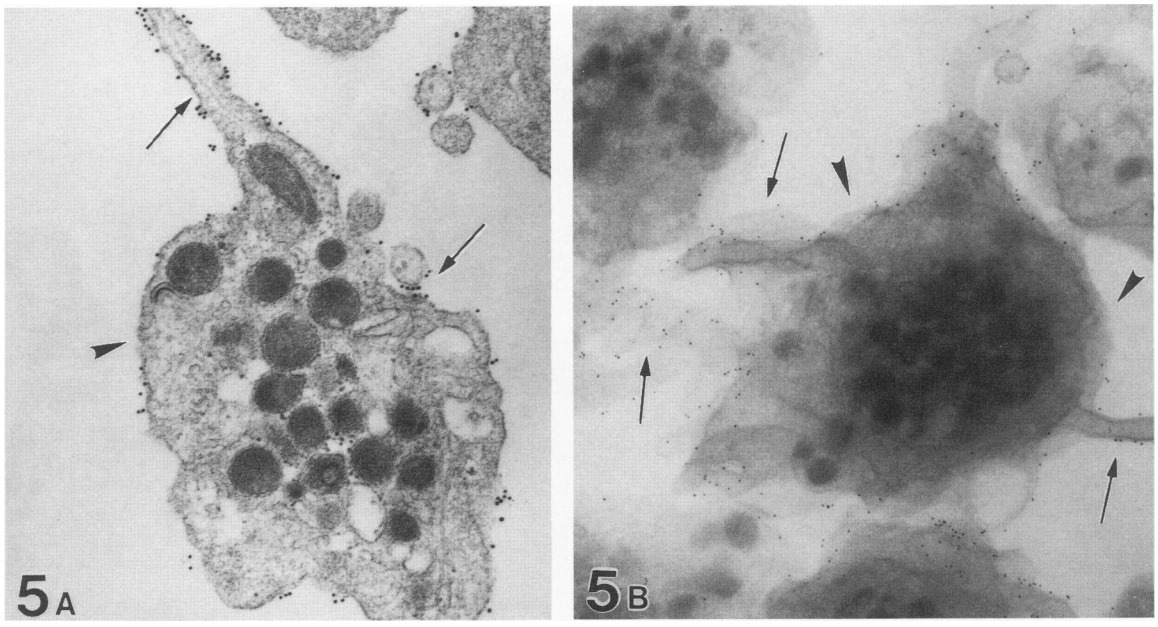


Figure 5. *Electron micrographs illustrating the distribution of 7E3 immunogold on GFP that had been activated for 30 seconds in the absence of fibrinogen. Immunogold label for the activation-dependent antibody was commonly found on membrane projections (arrows) with a relative lack of gold on cell body membrane (arrowheads) (×25,500).* **A:** *Conventional thin-section TEM micrograph (×26,300).* **B:** *Thick-section IVEM micrograph (×25,500).*

change by increasing surface membrane is in agreement with photomicroscopy results reported earlier by Frojmovic and associates.[4,41] It is known that platelets contain various internal sources of membrane, including cisternae of the open canalicular system (OCS), alpha granules, and elements of the dense tubular system. There is not evidence in the literature suggesting incorporation of dense tubular system membrane into the plasma membrane, and alpha-granule release is characteristically minimal after ADP stimulation. Although the specific data have not been included in this report, radioimmunoassay for β-thromboglobulin was used in our studies to monitor the platelet release reaction, and under the conditions reported in this study, alpha granule re-

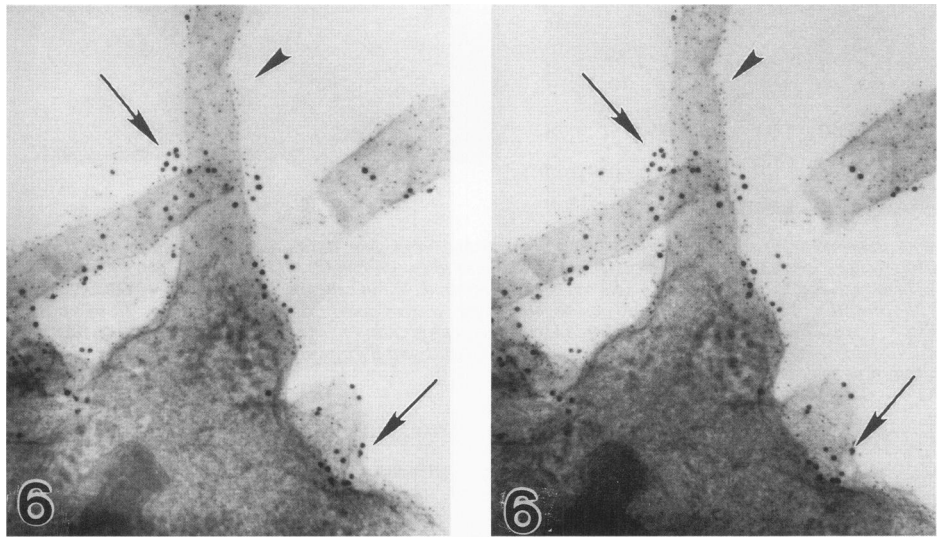


Figure 6. *Three-dimensional stereo pair illustrating simultaneous localization of GP IIb/IIIa (by the mouse monoclonal antibody, P2) and fibrinogen (by the rabbit polyclonal anti-fibrinogen antibody) on GFP which had been activated for 30 seconds in the presence of fibrinogen. The diffuse location of the smaller gold label (5 nm), indicative of P2 (GP IIb/IIIa) distribution (arrowhead), is contrasted by the more focal localization of fibrinogen (larger, 15 nm gold) on projections and at points of contact (arrows) (×58,800).*

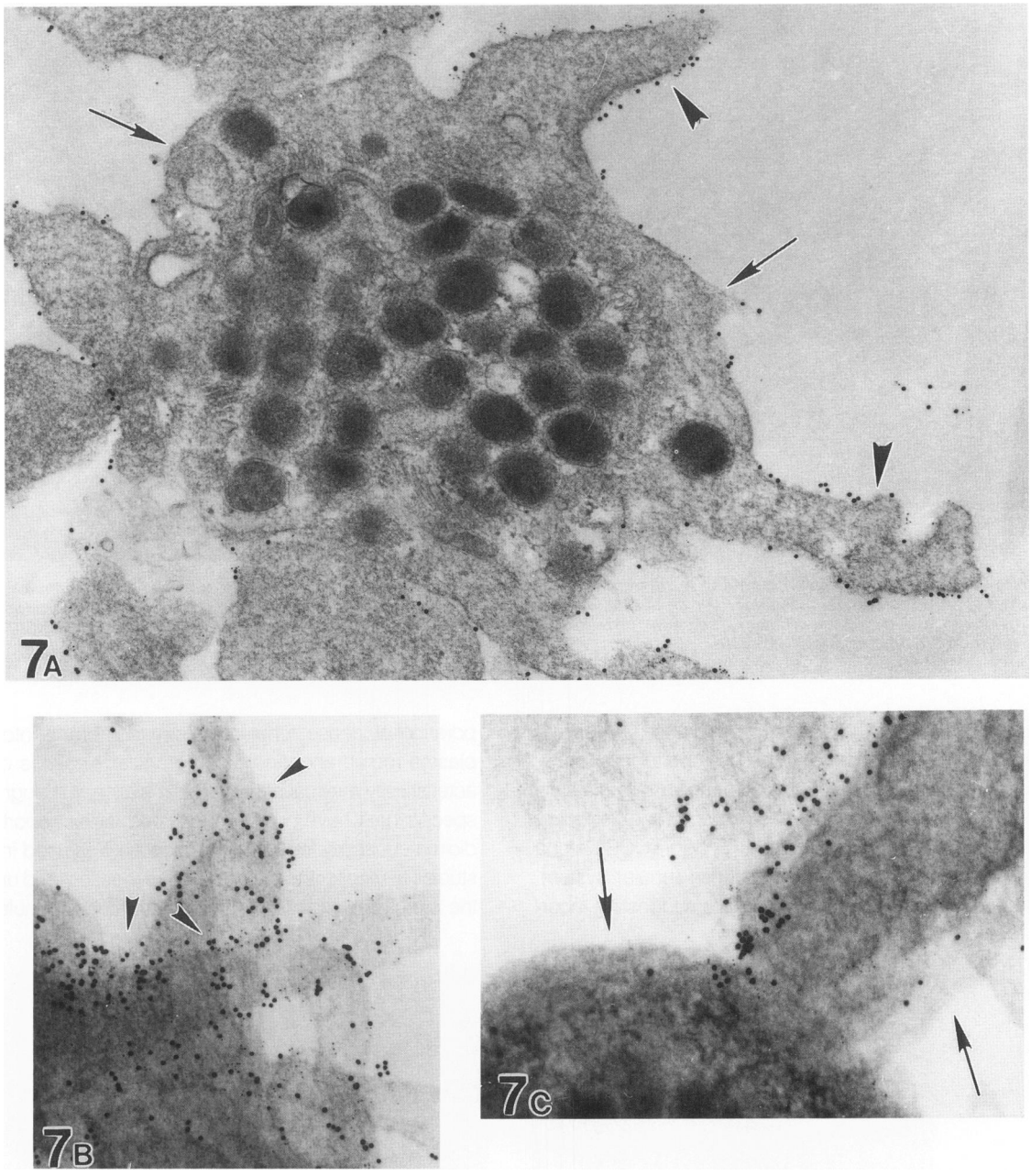


Figure 7. *Platelets that had been stimulated for 30 seconds with ADP and then simultaneously immunostained using 7E3 and anti-fibrinogen.* **A:** *Thin-section overview of a platelet on which both 7E3 (smaller, 5 nm gold) and anti-fibrinogen (larger, 15 nm gold) are more concentrated on pseudopods and blunt projections (arrowheads) than on the main cell body (arrows) X = 39,000.* **B:** *Stereo pair illustrating distribution of 7E3 (smaller, 5 nm gold) and anti-fibrinogen (larger, 15 nm gold) on GFP activated for 30 seconds in the presence of fibrinogen. Both the 7E3 and anti-fibrinogen antibodies co-localized on projections, many of which were in contact (arrowheads) (×46,200).* **C:** *Thick-section 2-D micrograph illustrating relative lack of both 7E3 and anti-fibrinogen immunogold labels on cell body membrane that is not participating in platelet contacts (arrows) (×55,100).*

lease was negligible. In view of these observations, the most likely source for the rapidly externalized membrane is the canalicular system,[41] which has long been known to be in continuity with the plasma membrane.[42] Evagination of OCS membrane has clearly been related to the morphologic changes that occur during platelet adhesion and spreading on various substrata,[43–45] and the absence of a well-developed channel system has been related to the limited spreading of bovine platelets to artificial surfaces.[43]

The presence of GP IIb/IIIa within a variety of membrane compartments has been documented by several investigators,[21,46,47] and fibrinogen receptor complexes have been identified in membranes of both the OCS and

alpha granules.[47] An important finding from the current studies was that the overall density of GP IIb/IIIa, as identified by immunogold electron microscopy, did not change after the ADP-stimulated increase in platelet surface membrane. It is conceivable that this lack of change in receptor density may represent the addition of new GP IIb/IIIa complexes to the platelet surface by OCS evagination after ADP stimulation. The additional fibrinogen-binding sites made available during activation could play an important role in mediating platelet aggregation.

Interestingly the distribution of immunogold label for GP IIb/IIIa in complex remained random on the platelet surface during short-term activation. This diffuse distribution was characterized also by a lack of localized clustering of the receptor in the membrane. Studies by Isenberg et al,[32] however, have documented clustering of GP IIb/IIIa on suspension-activated platelets. These earlier studies were based on observations of platelets that had been activated for 5 minutes, a time sufficiently long to form tight aggregates and to initiate platelet participation in clot retraction. Redistribution of GP IIb/IIIa and the formation of receptor–ligand clusters may be necessary at such later times to stabilize the aggregates, to juxtapose fibrinogen monomers in a fashion that facilitates fibrin polymerization, and ultimately to provide focal supports for clot retraction.[48,49]

Although the overall distribution of GP IIb/IIIa complexes remained random during the early times of platelet activation, the interaction of fibrinogen with its receptor was more focal in nature and was found on pseudopods and at regions of cell–cell interaction. The localization of fibrinogen on pseudopods and at contact points is consistent with the SEM data of Oliver and Albrecht,[13] who showed fibrinogen bound to the pseudopods of ADP-activated platelets. In the current study, the localization of the ligand on pseudopods was interesting, for the amount of membrane present in both pseudopods and in cell–cell contacts increased with time; this was consistent with functional fibrinogen receptors on these membranes. Initially, the projections were blunt and were extended from an otherwise discoid cell body. When GFP were activated for longer times, such as 30 seconds, the blunt projections were no longer observed, and at these later times elongate filopodia were most common. Technically, it was not possible for us to show progressive generation of the elongate filopodia from the early blunt projections, but it is possible that one serves as the precursor for the other. Predictably, the rapid generation of pseudopods in the turbulent environment of circulation or in an aggregometer cuvette would increase the probability of platelet–platelet collisions, leading to membrane interactions.[12,50] This possibility was indeed substantiated by our data, which document a progressive increase in the participation of projections in platelet contacts. In fact, virtually all of the newly formed contacts involved projection membrane during the first 10 seconds of activation. These observations are important, for they give credence to the possible role for filopodial-bound fibrinogen in subsequent platelet aggregation.[11–13]

The present data also suggest that pseudopods may have a membrane system that is specialized for the purpose of facilitating platelet interaction. In support of this suggestion is the observation that the density of the activation-dependent, anti-GP IIb/IIIa antibody, 7E3, was significantly greater on projections. This preferential localization of "activated" GP IIb/IIIa to projections occurred in spite of the fact that the general distribution of GP IIb/IIIa, as recognized by P2, remained uniformly distributed after activation. Conceivably, the somewhat selective identification of projection-related GP IIb/IIIa complexes by 7E3 and of the ligand, fibrinogen, may indicate localization of a GP IIb/IIIa subpopulation that participates in platelet–platelet interaction. Topographical differences in the distribution of receptor subpopulations previously have not been demonstrated for platelets activated in suspension, but such specialization is consistent with the heterogeneity in GP IIb/IIIa structure[51] and expression[52] that have recently been reported.

Acknowledgments

The authors thank the EM staff at MICROMED, NIH Resource for IVEM, Bowman Gray School of Medicine for excellent technical work, and Mrs. Barbara Lindsay for manuscript preparation.

References

1. Allen RD, Zacharski LR, Widirstky ST, Rosenstein R, Zaitlin LM, Burgess DR: Transformation and motility of human platelets. J Cell Biol 1979, 83:126–142
2. Lewis JC, White MS, Prater T, Taylor RG, Davis KS: Ultrastructural analysis of platelets in non-human primates: III. Stereo microscopy of microtubules during platelet adhesion and the release reaction. Exp Mol Pathol 1982, 37:370–381
3. White JG: Fine structural alterations induced in platelets by adenosine diphosphate. Blood 1968; 31:604–622
4. Frojmovic MM, Milton JG: Physical, chemical, and functional changes following platelet activation in normal and "giant" platelets. Blood Cells 1983; 9:359–382
5. Barnhart MI, Walsh RT, Robinson JA: A three-dimensional view of platelet responses to chemical stimuli. Ann NY Acad Sci 1972, 201:360–390
6. Milton JG, Frojmovic MM: Turbidometric evaluations of platelet activation: Relative contributions of measured shape change, volume, and early aggregation. J Pharmacol Methods 1983, 9:101–115
7. Gear ARL: Rapid platelet morphological changes visualized

by scanning-electron microscopy: Kinetics derived from a quenched-flow approach. Br J Haematol 1984, 56:387–398
8. Hantgan RR: A study of the kinetics of ADP-triggered platelet shape change. Blood 1984, 64:896–906
9. Gear ARL: Rapid reactions of platelets studied by a quenched-flow approach: Aggregation kinetics. J Lab Clin Med 1982, 100:866–886
10. Frojmovic MM, Milton JG, Duchastel A: Microscopic measurements of platelet aggregation reveal a low ADP-dependent process distinct from turbidometrically-measured aggregation. J Lab Clin Med 1983, 101:964–976
11. Milton JG, Frojmovic MM: Adrenaline and adenosine diphosphate-induced platelet aggregation require shape change. J Lab Clin Med 1984, 104:805–815
12. Frojmovic MM, Longmire K, van de Ven TG: Long-range interactions in mammalian platelet aggregation: II. The role of platelet pseudopod number and length. Biophys J 1990, 58:309–318
13. Oliver JA, Albrecht RM: Colloidal gold labelling of fibrinogen receptors in epinephrine- and ADP-activated platelet suspensions. Scanning Microsc 1987, 1:745–756
14. Bennett JS, Vilaire G: Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 1979, 64:1393–1401
15. Plow EF, Marguerie GA: Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP. J Biol Chem 1979, 255:10971–10977
16. Marguerie GA, Plow EF, Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 1979, 254:5357–5363
17. Parise LV, Phillips DR: Reconstitution of the purified platelet fibrinogen receptor. J Biol Chem 1985, 260:10698–10707
18. George JN, Nurden AT, Phillips DR: Molecular defects in interactions of platelets with the vessel wall. N Engl J Med 1984, 311:1084–1098
19. Nurden AT: Abnormalities of platelet glycoproteins in inherited disorders of platelet function, Platelets in Biology and Pathology. Vol III. Edited by D MacIntyre, J Gordon. New York, Plenum Press, 1988, pp 37–94
20. Kunicki TJ, Pidard D, Rosa J-P, Nurden AT: The formation of Ca^{++}-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. Blood 1981, 58:268–278
21. Woods VL, Wolff LE, Keller DM: Resting platelets contain a substantial centrally located pool of glycoprotein IIb/IIIa complex which may be accessible to some but not other extracellular proteins. J Biol Chem 1986, 261:15242–15251
22. Peerschke EIB, Zucker MB, Grant RA, Egan JJ, Johnson MM: Correlation between fibrinogen binding to human platelets and platelet aggregability. Blood 1980, 55:841–847
23. Shattil SJ, Hoxie J, Cunningham M, Brass LF: Changes in the platelet membrane glycoprotein IIb/IIIa complex during platelet activation. J Biol Chem 1985, 260:11107–11114
24. Coller BS: 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 1985, 76:101–108
25. Coller BS: Activation affects access to the platelet receptor for adhesive glycoproteins. J Cell Biol 103:451, 1986.
26. Shattil SJ, Motulsky HJ, Insel PA, Flaherty L, Brass LF: Expression of fibrinogen receptors during activation and subsequent desensitization of human platelets by epinephrine. Blood 1986, 68:1224–1231
27. Niiya K, Hodson E, Bader R: Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation: Relationship to the binding of fibrinogen and platelet aggregability. Blood 1987, 70:475–483
28. Loftus JC, Albrecht RM: Redistribution of the fibrinogen receptor of human platelets after surface activation. J Cell Biol 1984, 99:822–829
29. Peerschke EIB, Zucker MB: Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. Blood 1981, 57:663–670
30. Polley MJ, Leung L, Clark FY, Nachman RL: Thrombin-induced platelet membrane glycoprotein IIb and IIIa complex formation: An electron microscopic study. J Exp Med 1981, 154:1058–1068
31. Santoso S, Zimmermann U, Neppert J, Mueller-Eckhardt C: Receptor patching and capping of platelet membranes induced by monoclonal antibodies. Blood 1986, 67:343–349
32. Isenberg WM, McEver RP, Phillips DR, Shuman MA, Bainton DF: An immunogold-surface replica study of agonist-induced ligand binding and receptor clustering. J Cell Biol 1987, 104:1655–1663
33. Shiba E, Fossel E, Lindon J, McEver RP, Salzman EW: Glycoprotein IIb/IIIa clustering in activated platelets demonstrated by nuclear magnetic resonance. Circulation 1988, 78:II-309
34. Asch AS, Leung LL, Polley MJ, Nachman RL: Platelet membrane topography: Colocalization of thrombospondin and fibrinogen with the glycoprotein IIb/IIIa complex. Blood 1985, 66:926–934
35. Lewis JC, Hantgan RR, Stevenson SC, Thornburg T, Kieffer N, Guichard J, Breton-Gorius J: Fibrinogen and glycoprotein IIb/IIIa localization during platelet adhesion: Localization to the granulomere and at sites of platelet interaction. Am J Pathol 1990, 136:239–252
36. McGregor JL, McGregor L, Bauer A-S, Catimel B, Brochier J, Dechavanne M, Clemetson KJ: Identification of two distinct regions within the binding sites for fibrinogen and fibronectin on the IIb/IIIa human platelet membrane glycoprotein complex by monoclonal antibodies P2 and P4. Eur J Biochem 1986, 159:443–449
37. Pidard D, Montgomery RR, Bennett JS, Kunicki TJ: Interaction of AP-2, a monoclonal antibody specific for the human platelet glycoprotein IIb/IIIa complex, with intact platelets. J Biol Chem 1983, 258:12582–12586
38. Breton-Gorius J, Lewis JC, Guichard J, Kieffer N, Vainchenker W: Monoclonal antibodies specific for human platelet membrane glycoproteins bind to monocytes by focal absorption of platelet membrane fragments. Leukemia 1987, 1:131–141
39. French JE, Poole JCF: Electron microscopy of the platelets in artificial thrombi. Proc R Soc Lond [Biol] 1962, 157:170–176

40. Frojmovic MM, Milton JG: Human platelet size, shape, and related functions in health and disease. Physiol Rev 1982, 62:185–261
41. Frojmovic MM, Wong T, Wylie J, White JG: Platelet external surface membrane is osmotically doubled irrespective of size or species (human bovine): Dynamics and membrane sources. Thromb Haemost 1987, 58:302
42. White JG, Krumwiede M: Further studies of the secretory pathway in thrombin-stimulated human platelets. Blood 1987, 69:1196–1203
43. White JG, Krumwiede M: Platelet open canalicular system (OCS): A two-way street. Circulation 1988, 78:II-546
44. Escolar G, Leistikow E, White JG: The fate of the open canalicular system in surface and suspension-activated platelets. Blood 1989, 74:1983–1988
45. Grouse LH, Rao GHR, Weiss DJ, Perman V, White JG: Surface-activated bovine platelets do not spread, they unfold. Am J Pathol 1990, 136:399–408
46. Hourdille P, Benabdallah S, Belloc F, Nurden AT: Distribution of glycoprotein IIb/IIIa complexes in the surface membranes of human platelets and megakaryocytes. Br J Haematol 1985, 59:171–182
47. Wencel-Drake JD, Plow EF, Kunicki TJ, Woods VL, Keller DM, Ginsberg MH: Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. Am J Pathol 1986, 124:324–334
48. Lewis JC, Johnson C, Ramsamooj P, Hantgan RR: Orientation and specificity of fibrin protofibril binding to ADP-stimulated platelets. Blood 1988, 72:1992–2000
49. Cohen I, Gerrard JM, White JG: Ultrastructure of clots during isometric contraction. J Cell Biol 1988, 93:775–787
50. Marsh S, Jones G: Microspike function in cell aggregation. Eur J Cell Biol 1982, 28:278
51. Bray PF, Leung CS, Shuman MA: Human platelets and megakaryocytes contain alternately spliced GPIIb mRNAs. J Biol Chem 1990, 265:9587–9590
52. Jackson CW, Jennings LK: Heterogeneity of fibrinogen receptor expression on platelets activated in normal plasma with ADP: Analysis by flow cytometry. Br J Haematol 1989, 72:407–414