Platelet Membrane Topography: Colocalization of Thrombospondin and Fibrinogen With the Glycoprotein IIb–IIIa Complex

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PLATELET aggregation is a crucial step in normal hemostasis. Endothelial denudation leads rapidly to platelet adhesion, degranulation, and recruitment of additional platelets eventually resulting in the formation of a hemostatic plug and thrombus. Recent evidence suggests that the sequence of events culminating in platelet aggregation includes stimulation by an agonist followed by shape change, alpha granule secretion, and the membrane localization of several alpha granule proteins. Upon platelet stimulation, the platelet membrane glycoprotein complex IIb–IIIa (GPIIb–IIla) binds fibrinogen. This specific fibrinogen binding correlates with platelet aggregation. Thrombospondin (TSP), a major alpha granule protein with lectin-like activity, is secreted upon platelet stimulation and binds to the platelet membrane. TSP also binds to fibrinogen, and the formation of a macromolecular complex of TSP, fibrinogen, and GPIIb–IIla has been proposed as a major step required for platelet aggregation. Recent data reveal that the rate of fibrinogen dissociation from the activated platelet surface is enhanced in the presence of anti-TSP antibodies, supporting the premise that TSP serves to stabilize the binding of fibrinogen to the GPIIb–IIla complex. TSP appears to be essential for the secondary wave of platelet aggregation, making platelet aggregation irreversible. This model of the molecular mechanism of aggregation predicts a close topographic association between these proteins on the membrane of activated platelets.

Immunoelectron microscopy on platelet membranes isolated from thrombin-stimulated platelets has demonstrated macromolecular clustering of GPIIb and GPIIa. The possible role of thrombin-induced fibrin deposition as a factor initiating these clusters has not been fully clarified. In addition, bivalent antibody probes used to define molecular relationships in unfixed membrane preparations may themselves lead to focal clustering of antigens. Frozen thin-section immunoelectron microscopy allows an identification and localization of the critical proteins involved in platelet aggregation. An important advantage of this technique is that by labeling lightly fixed sectioned material, the distribution of labels observed is not influenced by the bivalency of the antibody probes. Recent studies using this technique have localized fibrinogen to the alpha granules in resting platelets. Following thrombin stimulation, membrane-associated fibrinogen has been visualized.

Using frozen thin-section immunoelectron microscopy, we present evidence of secretion of alpha granule proteins, and the macromolecular assembly of TSP and fibrinogen with the GPIIb–IIla complex present on the activated platelet surface. The complex localized to pseudopodia and interplatelet regions following platelet activation.

MATERIALS AND METHODS

Platelet Preparation

Venous blood was drawn from normal volunteers into plastic syringes containing one-sixth volume acid–citrate–dextrose with 100 mm of prostaglandin E1 (PGE1) and 100 μm of isobutylmethylxanthine. Following an initial centrifugation for 15 minutes at 90 g, the supernatant platelet-rich plasma was gel-filtered as previously described. The gel-filtered platelets were adjusted to pH 7.2 with a final Ca^{2+} concentration of 2 mmol/L and incubated for 15 minutes at 37°C prior to use. For thrombin stimulation, platelet suspensions were incubated with 1 U/mL final concentration for 90 seconds at 37°C without stirring. d-Phenylalanyl-prolyl-arginyl chloromethyl ketone (Calbiochem, La Jolla, Calif), 10^{-5} mol/L final concentration, was then added to stop further release. In other experiments, calcium ionophore, A23187 (Calbiochem), was added to a final concentration of 1 μmol/L for two minutes. Control platelet samples were not exposed to thrombin or ionophore. Following incubation, platelets were fixed at 22°C for two hours in 0.1 mol/L of phosphate buffer, pH 7.4, containing 4% paraformaldehyde and 0.05% to 0.5% glutaraldehyde. Fixed platelets were centrifuged for 15 minutes at 1,200 g and resuspended in Tyrode’s-Hepes buffer. Aliquots were centrifuged in a desktop Brinkman microfuge for five minutes into a cushion of 2 mol/L of sucrose, 5% gelatin, and quick-frozen in liquid nitrogen for frozen thin sectioning.

Frozen Thin-Section Procedures

Thin sections (approximately 100 nm) of platelet pellets embedded in 2 mol/L of sucrose, 5% gelatin, were cut on a Sorvall (Newtown, Conn) MT-2 ultramicrotome with a frozen thin-section LTD-2 cryokit attachment as described. In some instances, slightly thicker sections provided better visualization of surface membrane events and are therefore included.
Immunocytochemical Procedures

Monospecific polyclonal rabbit antisera to human TSP was prepared according to previously published procedures. Monospecific polyclonal anti-human GPIIIa, and anti-human fibrinogen were raised in rabbits and F(ab')2 prepared as previously described. Goat antibody to mouse IgG Fc fragment, goat antibody to rabbit IgG, and goat antibody to human fibrinogen were obtained from Cappel Laboratories (Malvern, Pa). Mouse monoclonal antibody to human GPIb (AP-1) was a gift from Dr Thomas Kunicki (Milwaukee Blood Center). Mouse monoclonal antibody (SSA6) to GPIIIa was a gift from Dr Joel Bennett (University of Pennsylvania, Philadelphia). SSA6 was chosen because it does not inhibit fibrinogen binding to the GPIb-IIIa complex and thus would not be inhibited from binding by membrane-bound fibrinogen.

Colloidal gold particles were prepared according to methods previously described as 10- or 30-nm particles and conjugated to the antibody probes. Incubation, washing, and staining of frozen thin sections were carried out at room temperature similar to the methods described by Tokuyasu and Singer. Single-label studies involved incubation with a primary antibody for two hours followed by washing and then incubation with the appropriate secondary antibody linked to colloidal gold. Following the second incubation, washing and staining with 1% uranyl acetate was performed. In double-label studies, primary antibodies from different species were used (mouse monoclonals and rabbit polyclonals) followed by the appropriate second antibodies of goat origin linked to colloidal gold. In the double-label study of fibrinogen and TSP, TSP antisera derived from rabbit was followed by goat anti-rabbit antibody conjugated to 30 nm of colloidal gold and by goat anti-human fibrinogen conjugated to 10 nm of colloidal gold. The following protocol illustrates the major sequential steps in each of the separate experiments performed.

Single-label studies. (1) Rabbit anti-fibrinogen F(ab')2—goat anti-rabbit IgG—10 nm gold; (2) rabbit anti-TSP serum—goat anti-rabbit IgG—10 nm gold; (3) rabbit anti-GPIIIa serum—goat anti-rabbit IgG—10 nm gold; (4) rabbit anti-human albumin serum—goat anti-rabbit IgG—10 nm gold; (5) nonimmune rabbit serum—goat anti-rabbit IgG—10 nm gold; (6) mouse monoclonal anti-GPIb—goat anti-mouse IgG—10 nm gold.

Double-label studies. (7) Rabbit anti-TSP serum—goat anti-rabbit 30 nm gold plus goat anti-human fibrinogen—10 nm gold; (8) mouse monoclonal anti-GPIIIa plus rabbit anti-TSP serum—goat anti-mouse IgG—10 nm gold plus goat anti-rabbit IgG—30 nm gold; (9) mouse monoclonal anti-GPIb plus rabbit polyclonal anti-GPIIIa—goat anti-mouse IgG—10 nm gold plus goat anti-rabbit IgG—30 nm gold.

Statistical Methods

In single-label studies, analysis of the distribution of label in resting and stimulated platelets was performed using the Kolmogorov-Smirnov two-sample test. This method was chosen because it allows a comparison of two distributions without necessitating any assumptions about the nature of these distributions (ie, skewness). Our analysis was performed for each of the immunolabeled proteins in both the single-label and the double-label experiments. We have included statistical analysis only on the data we have presented (all micrographs shown). The distance between all membrane-associated labels was measured along the membrane surface to assess the distribution. This produced a large array of distances for each of the proteins. Clustered labels result in an array skewed toward smaller interlabel distances, while unclustered labels result in an array with a more random interlabel distance. Significant frequency differences...
were calculated according to the formula:

\[ D = \sqrt{(1/2)} - \frac{n1(\alpha/2)}{\sqrt{n1 + n2}} \cdot \sqrt{n1n2} \]

where \( D \) is the calculated significant unsigned difference at any \( P \) value (\( \alpha \)), and \( n1 \) and \( n2 \) are the number of observations in each sample distribution.

In double-label experiments, Kendall's nonparametric rank coefficient\(^{25} \) was used as a measure of correlation between dissimilar labels. As with the previous statistical method, no assumptions concerning the distributions are required. All of the surface membrane in the micrographs shown were scored according to the presence of each of the two labels in continuous 5-mm segments. The coefficient of correlation was calculated from the equation:

\[ T = \frac{S}{\sqrt{\frac{n(n-1)}{2}} - Tx} \cdot \frac{\sqrt{\frac{n(n-1)}{2}} - Ty}{\sqrt{\frac{n(n-1)}{2}}} \]

where \( S \) is an expression of the number of inversions, \( Tx \) and \( Ty \) are corrections for tied ranks and \( T \) is Kendall's nonparametric rank coefficient. The latter was compared with critical values for confidence.\(^{28} \)

**RESULTS**

**Topographical Distribution of Fibrinogen in Resting and Stimulated Platelets**

In unstimulated platelet sections, an intracellular distribution of the fibrinogen probe was found. In many sections, clusters of label were clearly seen over alpha granules. At concentrations of glutaraldehyde <0.05% and fixation times shorter than two hours, higher density of labeling was noted, but at the expense of morphological definition. Fibrinogen probes were seen infrequently on the platelet membrane of unstimulated platelets. Even in platelets minimally activated, as evidenced by some dilatation of the surface-connected canalicular system, very little label is associated with the platelet surface (Fig 1A). These findings are consistent with the findings of Sander et al.\(^{16} \) and Stenberg et al.\(^{17} \)

In thrombin-stimulated platelets, numerous pseudopodia were covered with fibrinogen probes (Fig 1B). The probes were also localized in interplatelet spaces and in the surface-connected canalicular system, consistent with secretion and membrane binding of endogenous platelet fibrinogen. In some thicker sections, the platelet body was seen in section while the pseudopodia were seen virtually whole with some intact membrane. This pattern of fibrinogen binding following thrombin stimulation is also in agreement with the results obtained by Stenberg et al.\(^{17} \)

In both resting and stimulated platelets, a very low level of nonspecific labeling was detected using pre-immune rabbit serum followed by goat anti-rabbit IgG-gold (Fig 2A). In resting platelets, albumin was detected over alpha granules. In thrombin-stimulated platelets, residual alpha granule albumin was detected, but there was no surface-associated albumin (Fig 2B), consistent with the findings of Sixma et al.\(^{29} \)

![Fig 2. Nonimmune and albumin controls (original magnification x63,000; current magnification x40,950).](image-url)
Topographic Distribution of TSP in Resting and Stimulated Platelets

In unstimulated platelets, TSP probes were localized over the alpha granules and very little was noted on the platelet surface (Fig 3A). This is consistent with previous biochemical data. Following thrombin stimulation, secretion of TSP into the surface-connected canalicular system and clustering of TSP on the platelet surface was evidenced by the distribution of the gold probes. In Fig 3B, several clusters of TSP are associated with the membrane of a large dilated portion of the surface-connected canalicular system. Clusters are also present on the external membrane of the platelets as well. In Fig 3C, TSP is noted between two adherent platelets. A section through two closely adherent platelets (Fig 3D) reveals TSP clustered on opposing platelet membranes.

Topographic Distribution of GPIIIa in Resting and Stimulated Platelets

GPIIIa appeared diffusely distributed over the platelet surface and was also seen over the platelet body in a pattern consistent with canalicular system location. Coincident alpha granule location could not be entirely excluded (Fig 4A). Upon thrombin stimulation, GPIIIa probes clustered in patches on the platelet surface especially on pseudopodia (Fig 4B and C). The distances between all of the membrane-associated label in the micrographs shown were measured and nonparametric analysis was performed. The Kolmogorov-Smirnov method was used to calculate unsigned differences between the two distributions (resting vs stimulated). The analysis confirmed that they differed significantly (P < .05).

Topographic Distribution of GPIb in Resting and Stimulated Platelets

In both resting and thrombin-stimulated platelets, GPIb was distributed diffusely over the platelet surface. No clusters of probes were seen (data not shown).

Topographic Distribution of TSP and Fibrinogen in Double-Label Studies in Resting and Stimulated Platelets

As in single-label experiments, TSP and fibrinogen were distributed intracellularly within alpha granules in resting platelets and appeared on the platelet membrane in platelets stimulated with thrombin or the calcium ionophore A23187. With both agonists, there was colocalization of the labels in patches found on the platelet surface, on pseudopodia, and between adherent platelets. In Fig 5A and B, adherent thrombin-stimulated platelets are bridged by fibrinogen/fig 3. Topographic distribution of thrombospondin (original magnification × 83,000; current magnification × 41,580). (A) Unstimulated platelet section incubated with rabbit anti-TSP serum followed by goat anti-rabbit–colloidal gold. Label is seen over the platelet body clustered over alpha granules. Very little label is present on the platelet surface. (B) Thrombin-stimulated platelet section incubated with rabbit anti-TSP serum followed by goat anti-rabbit–colloidal gold. Clusters of label are seen on the platelet surface as well as within the surface-connected canalicular system. (C) Thrombin-stimulated platelet section incubated with rabbit anti-TSP serum followed by goat anti-rabbit–colloidal gold. TSP is noted between two adherent platelets. (D) Thrombin-stimulated platelet section incubated with rabbit anti-TSP serum followed by goat anti-rabbit–colloidal gold. Two closely adherent platelets with TSP clustered on opposing platelet membranes.
Both thrombin- and A23187-stimulated platelets exhibited coclustering of fibrinogen and TSP. A23187 causes platelet aggregation and release without fibrinogen-to-fibrin conversion. The ratio of fibrinogen to TSP was greater in the thrombin-stimulated platelets (Fig 5A and B) than in the A23187-stimulated platelets (Fig 5C and D). Thus, we cannot exclude the possibility that a small amount of the label in the thrombin-stimulated platelets was in fact secondary to fibrin deposition. Nevertheless, the ratio of fibrinogen to TSP in the A23187-stimulated platelets was approximately 1:1, making fibrin deposition an unlikely explanation for our findings.

Topographic Distribution of GPIIIa and TSP in Double-Label Studies in Resting and Stimulated Platelets

Distribution of labels in resting platelets was similar to that observed in single-label experiments. Upon thrombin stimulation, coclustering of GPIIIa and TSP probes was seen on the platelet surface, especially on pseudopodia and between adherent platelets. In Fig 6A, all of the membrane-associated label is clustered at one pole of the platelet. In Fig 6B, a pseudopod is covered with clustered GPIIIa and TSP. Figure 6C depicts two adherent platelets with GPIIIa and TSP in the interplatelet space. By statistical analysis, the two labels belonged to the same distributions (P < .05).

Topographic Distribution of GPIIb and GPIIIa in Resting and Stimulated Platelets

In resting platelets, both labels were distributed diffusely over the platelet surface, consistent with our findings in single-label experiments. Intracellular GPIIIa was also detected in a distribution similar to that obtained with polyclonal antibody in the single-label experiments. In Fig 7A, clusters of GPIIIa are seen only over the body of the platelet. No membrane-associated label is clustered. Intracellular GPIb was detected at a lower density than GPIIIa. Upon thrombin stimulation, clustering of GPIIIa was detected over the platelet membrane, but GPIb remained diffusely distributed (Fig 7B and C). In comparison with the unstimulated platelet, less GPIIIa was seen within the platelet body, suggesting that some of the internal pool of GPIIIa was externalized upon platelet stimulation. By nonparametric analysis, the two labels did not colocalize.

DISCUSSION

Polley et al have previously studied the membrane topography of GPIIb and GPIIIa in isolated membranes of resting and thrombin-stimulated human platelets and demonstrated clustering of GPIIb and GPIIIa in thrombin-stimulated platelet membranes. However, fibrin as well as fibrinogen can bind to the platelet surface, thus we must address the possibility of thrombin-induced fibrin deposition resulting in the observed clustering of GPIIIa–IIIa. Also, in experiments in which the membrane is probed with bivalent antibodies prior to fixation, crosslink-induced clustering of bivalent probes may occur. The present study addresses these questions by using fixed frozen thin-sectioned material and subsequent immunoelectron microscopy. Frozen thin-sec-
Fig 5. Topographic distribution of TSP and fibrinogen: double-label experiment (A and B) Thrombin-stimulated platelet sections incubated with goat anti-fibrinogen-10 nm of gold and with rabbit anti-TSP serum followed by goat anti-rabbit-30 nm of gold. Following stimulation, coclusters of TSP and fibrinogen probes are found on the platelet surface. (A original magnification ×63,000; current magnification ×50,400. B, original magnification ×63,000; current magnification ×41,580). (C and D) A23187-stimulated platelet sections showing similar distribution of TSP and fibrinogen probes. In c, the thickness of the section shows a surface membrane cluster to advantage but does not show intracellular detail. (Original magnification ×63,000; current magnification ×40,950).

mentioned material is only lightly fixed, altering antigenicity minimally, and is embedded in a water-permeable medium such as gelatin, making the antigen more accessible to antibody probes than in plastic-imbedded specimens. Since the immunocytochemistry follows sectioning in these experiments, the bivalency of the probes will not affect the distribution of label. Morphological detail of thrombin- or ionophore-treated platelets was, in general, less optimal than for resting platelets. In some cases, though intracellular detail was less precise, sections thicker than 100 nm provided the best visualization of membrane events. Our study provides direct evidence for the presence of a macromolecular assembly of fibrinogen and TSP with the GPIIb-IIIa membrane complex following activation. In addition, there appears to be redistribution of the complex to pseudopodia and membrane areas between adjacent platelets.

We have observed secretion and membrane localization of fibrinogen following thrombin stimulation (Fig 1B). This is in agreement with previously published results with similar techniques showing fibrinogen localization within the alpha granule in resting platelets and on the platelet membrane following thrombin stimulation. Further, a similar distribution was seen following ionophore A23187 stimulation. A23187 results in platelet aggregation and release without fibrinogen-to-fibrin conversion. Thus, fibrin formation and deposition is not an unlikely explanation for these results. TSP, a major alpha granule protein, was similarly distributed in the resting platelet and colocalized with fibrinogen on the platelet membrane following stimulation with either thrombin or A23187. In contrast, albumin, another alpha granule constituent, did not appear to become membrane-bound following thrombin stimulation. These findings provide evidence for the close topographic association of TSP with platelet-bound fibrinogen in stimulated platelets. Previous studies of TSP-fibrinogen interactions are consistent with these findings.

Using both polyclonal and monoclonal antibodies to GPIIIa, we have shown a redistribution of surface GPIIb-IIIa from a diffuse pattern, to one of patches seen over pseudopodia and in interplatelet spaces. The clustering of GPIIb-IIIa in patches following platelet stimulation confirms previous studies showing clustering of GPIIb-IIIa.
following thrombin stimulation using unfixed whole-membrane mounts. Our findings with a monoclonal antibody to GPIb revealed that glycoprotein's distribution is unchanged by thrombin stimulation. In separate double-label studies, both fibrinogen and GPIb–IIIa colocalized with TSP in patches that were again seen on pseudopodia and between platelets, strongly suggesting the assembly of a macromolecular complex between these proteins. These observations provide additional support for the proposed role of TSP in stabilizing the fibrinogen–GPIIb-IIIa complex. The lateral mobility of the GPIIb–IIIa complex upon platelet stimulation may allow for the polarization of the platelet-adhesive surface and migration of the complex to areas that oppose similar patches on adjacent platelets.

The role of the cytoskeleton in this redistribution process remains an important unanswered question. Platelet stimulation leads to extensive cytoskeletal reorganization coincident with platelet shape change and pseudopodia formation. Recent data suggest that, upon thrombin stimulation,
GP Ib–IIa becomes bound to the cytoskeleton while GP Ib may be cleaved from cytoskeletal attachments by calcium-activated protease. Pseudopodia contain tightly packed bundles of microfilaments whose attachment to the complex at the ends of pseudopodia, as we have observed. It is possible that the cytoskeleton may play a role in orienting more adhesive platelet surfaces to one another or to the substrate by regulating the redistribution of the GP Ib–IIa–fibrinogen–TSP complex. In addition, the striking concentration of the complex on pseudopodia may reflect GP Ib–IIa that becomes newly exposed as a result of shape change or alpha granule secretion. Newly expressed GP Ib–IIa may result from the fusion of granule membranes with canicular membranes during secretion or by the externalization of membrane of the surface-connected canalicular system. The random distribution of GP Ib—a platelet membrane glycoprotein that appears to function primarily in platelet adhesion rather than aggregation—even after thrombin stimulation, is consistent with diffusion of an unanchored membrane protein. When platelets adhere to a substratum of injured vessel wall or a platelet plug, GP Ib may exhibit a different membrane distribution than we have observed in this system.

In these experiments, monoclonal anti-GP Ib antibodies as well as monoclonal anti-GP IIa were used to follow the distribution of the GP Ib–IIa complex. The relationship of GP Ib to GP IIa in the membrane of resting platelets is uncertain, but recent data suggest that the two exist as a heterodimer which, upon stimulation, acquires the capability of binding fibrinogen. The use of anti-GP IIa antibodies for these experiments was based on the fact that SSA6 does not inhibit fibrinogen binding and therefore detection of the GPII b–IIa complex would not be obscured by bound fibrinogen. Of some interest is the intracellular distribution of GP II a. This is consistent with recent evidence supporting the presence of a pool of intraplatelet GPIIb–IIa that becomes exposed following thrombin stimulation. Our own data do not allow us to answer unequivocally the subcellular location of intraplatelet GPIIb–IIa; the intracellular distribution we have observed is consistent with surface canalicular GPIIb–IIa but we cannot exclude the presence of some intragranule GPIIb–IIa as well. The presence of GPIIb–IIa in alpha granules has been reported by Gogstad et al and Wencel-Drake et al. Our findings are further morphological evidence for the presence of intracellular GPIIb–IIa. The function of this intracellular pool remains to be determined.

GP Ib was also distributed intracellularly (Fig 7). There was less intense labeling than with the GPIIIa probe. In both instances, label was seen over areas of the platelet body that were not clearly alpha granule related, suggesting that the glycoproteins labeled may be associated with membrane of the surface-connected canalicular system. The functional significance of this localization is unclear.

We have demonstrated the colocalization of platelet-derived fibrinogen and TSP with the GPIIb–IIa complex on the surface of thrombin- and A23187-stimulated human platelets, lending support to the premise that a macromolecular assembly of these proteins forms during platelet aggregation. It should be emphasized that these experiments were performed under unstimred conditions, in which any polarity of the membrane induced by platelet–platelet or platelet–substrate contact would not be observed and thus the findings represent intrinsic properties of these proteins and the platelet membrane. Changes that are likely to occur as a result of direct platelet–platelet contact are the subject of further investigations. The membrane distribution of other alpha granule constituents—such as von Willebrand factor and fibronectin—in platelet aggregation also remains to be studied.

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