

THROMBIN-INDUCED PLATELET MEMBRANE GLYCOPROTEIN IIb AND IIIa COMPLEX FORMATION

An Electron Microscope Study*

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Glycoprotein IIb (GPIIb)¹ and glycoprotein IIIa (GPIIIa) are major constituents of the platelet membrane (1). GPIIb and GPIIIa are markedly diminished or absent in platelets of patients with Glanzmann's thrombasthenia, a hereditary bleeding disorder characterized by an absence of fibrinogen binding and defective platelet aggregation (1-3). An IgG alloantibody obtained from a transfused patient with thrombasthenia interacts with GPIIb and GPIIIa and induces a thrombasthenia-like state in normal platelets (4, 5). Physiologic stimulants such as ADP, epinephrine, and thrombin induce fibrinogen binding to normal platelets, which correlates with platelet aggregability (2, 6, 7). The evidence thus suggests that these two glycoproteins are important in mediating platelet aggregation, probably by induction in the stimulated membrane of a fibrinogen receptor. We have recently reported the isolation of purified GPIIb and GPIIIa and the generation of monospecific antisera to these membrane proteins (8). GPIIb and GPIIIa are antigenically different and structurally distinct glycoproteins. In this study, we present direct morphologic evidence demonstrating macromolecular complex formation of GPIIb and GPIIIa in the platelet membrane after thrombin stimulation.

Materials and Methods

Preparation of Washed Platelets. A suspension of human platelets was obtained using the Ardlie buffer system modified as previously described (9).

Preparation of Antisera to Platelet Membrane Glycoprotein Ib (GPIb), GPIIb, and GPIIIa. Antisera to GPIb (10), GPIIb, and GPIIIa (8) were prepared in rabbits as described previously. The antisera to GPIIb and GPIIIa were absorbed with washed platelets from a patient with classical Glanzmann's thrombasthenia (8).

Preparation of F(ab')₂ Fragments of Antisera. The γ -globulin fraction was prepared from each monospecific antiserum by ammonium sulfate precipitation followed by triethylaminoethyl-cellulose column chromatography with phosphate buffer, pH 8.0, 0.0175 M. The γ -globulin fraction of goat anti-rabbit γ -globulin was obtained commercially (Miles Laboratories, Inc., Elkhart, Ind.). This fraction was further purified by triethylaminoethyl-cellulose chromatography using the same conditions as those used for the anti-glycoprotein antibodies. The purified γ -globulin fraction of each antibody was digested with pepsin by the method of Nisonoff et al.

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¹ Abbreviations used in this paper: GPIb, glycoprotein Ib; GPIIb, glycoprotein IIb; GPIIIa, glycoprotein IIIa; KLH, keyhole limpet hemocyanin.

(11). The $F(ab')_2$ fraction of each antibody was isolated by molecular sieve chromatography on Sephadex G-150 using normal saline buffered to pH 7.3.

Ferritin Conjugation of Goat Anti-Rabbit γ -Globulin. The $F(ab')_2$ fragment of the goat anti-rabbit γ -globulin was conjugated with ferritin by the method of Tawde and Ram (12). Free ferritin and unconjugated γ -globulin were separated from conjugated γ -globulin by Pevikon (Mercer Consolidated Corp., Yonkers, N. Y.) block electrophoresis (13).

Conjugation of Anti-GPIIb with Keyhole Limpet Hemocyanin (KLH). The $F(ab')_2$ fraction of anti-GPIIb was conjugated with KLH by the method of Karnovsky et al. (14).

Incubation of Platelets with Thrombin. Washed platelets were suspended at 200,000/ μ l in Ardlie buffer II in which bicarbonate was replaced with Tris (9). To 50 ml of the platelet suspension was added 12.5 U purified thrombin kindly supplied by Dr. J. Fenton II, New York State Department of Health, Albany, N. Y., and the mixture was incubated for 5 min at 37°C with constant shaking. A second aliquot of 50 ml of the washed platelet suspension was not incubated with thrombin. Both aliquots of platelets were then washed twice with the modified Ardlie II buffer, and each was then resuspended in the same buffer to 9 ml.

Treatment of Platelets with Specific Antisera (Table I)

EXPERIMENT 1. The thrombin-treated and untreated control platelets were divided into three samples (A, B, and C) of 3 ml each. Anti-GPIIIa [0.3 ml $F(ab')_2$ 20 μ g/ml] was added to two samples (A and C). KLH-conjugated anti-GPIIb [0.3 ml $F(ab')_2$ 14 μ g/ml] was added to the third sample (B). After incubation for 30 min at 37°C, all platelet samples were washed twice with the modified Ardlie II buffer. To each of the samples that had been incubated with anti-GPIIIa (A and C) was added 1.5 ml of the ferritin-conjugated $F(ab')_2$ fraction of the goat anti-rabbit γ -globulin (14.5 μ g/ml). The mixtures were incubated for 30 min at 37°C and the platelets were washed twice with the Ardlie buffer. One sample each (C) of thrombin-treated and control platelets that had been incubated with the ferritin antibody was then further incubated for 30 min at 37°C with the KLH-conjugated anti-GPIIb (14 μ g/ml) and washed twice with Ardlie buffer.

EXPERIMENT 2. GPIb, another major platelet membrane glycoprotein not involved in platelet aggregation, was used as a control (1). The same procedure was used as in experiment 1. Instead of using anti-GPIIIa antibody, 0.3 ml of the $F(ab')_2$ fraction of anti-GPIb was used (46.5 μ g/ml). The same KLH-conjugated anti-GPIIb was used at the same concentration as in experiment 1. All platelet samples that had been incubated with anti-GPIb were further incubated with the ferritin-conjugated anti-rabbit γ -globulin. For the platelets that were to be double-labeled, the sequence of addition was anti-GPIb, ferritin-conjugated anti-rabbit γ -

TABLE I
Sequence of Addition of Antisera for the Morphologic Demonstration of Distribution of GPIb, GPIIb, and GPIIIa in the Unstimulated and Thrombin-stimulated Platelets

Platelets	Sample	Incubation step		
		1	2	3
Experiment 1 Thrombin-treated and untreated control	A	Anti-GPIIIa*	Anti-rabbit γ -globulin (Fe)‡	—
	B	Anti-GPIIb (KLH)§	—	—
	C	Anti-GPIIIa	Anti-rabbit γ -globulin (Fe)	Anti-GPIIb (KLH)
Experiment 2 Thrombin-treated and untreated control	A	Anti-GPIb	Anti-rabbit γ -globulin (Fe)	—
	B	Anti-GPIIb (KLH)	—	—
	C	Anti-GPIb	Anti-rabbit γ -globulin (Fe)	Anti-GPIIb (KLH)

* Anti-GPIIIa: $F(ab')_2$ fraction of rabbit antibody to platelet membrane GPIIIa.

‡ Anti-rabbit γ -globulin (Fe): ferritin-conjugated $F(ab')_2$ fraction of goat antibody to rabbit γ -globulin.

§ Anti-GPIIb (KLH): KLH-conjugated $F(ab')_2$ fraction of rabbit antibody to platelet membrane GPIIb.

|| Anti-GPIb: $F(ab')_2$ fraction of rabbit antibody to platelet membrane GPIb. For methodology, see text.

globulin, and KLH-labeled anti-GPIIb (Table I). All incubation times and wash steps were the same as for experiment 1.

Preparation of Membranes for Electron Microscopy. A sample of platelets from each aliquot was removed and was frozen and thawed three times. The resultant platelet membranes were washed three times in a 1:10 dilution of normal saline. Samples to which the ferritin-conjugated antibody had been added were applied to a copper grid coated with collodion and carbon, and stained with bismuth subnitrate (15); those that had been incubated with the KLH-conjugated antibody were stained with 2% uranyl acetate and applied to the grids. Samples that contained both markers were first stained with uranyl acetate, and after application to the grid were further stained with bismuth subnitrate. The grids were viewed under a Philips 301 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.) at 80 kV.

Results

Morphologic Demonstration of GPIIIa Distribution in the Unstimulated and Thrombin-stimulated Platelet Membranes. Fig. 1A demonstrates the distribution of GPIIIa in the unstimulated platelet membrane as evidenced by the indirect ferritin-labeling procedure. The ferritin molecules were scattered randomly over the platelet surface. There was a very small amount of clustering, as indicated by occasional clumps of three to five ferritin molecules. The distribution of GPIIIa in the platelet membrane after stimulation of the platelet with thrombin is shown in Fig. 1B. Large clumps of the ferritin label (indicated by white arrows) were visualized, indicating that membrane perturbation had resulted in the clustering of the GPIIIa molecules.

Morphologic Demonstration of GPIIb Distribution in the Unstimulated and Thrombin-stimulated Platelet Membrane. Fig. 2A demonstrates the distribution of KLH-conjugated anti-GPIIb in the unstimulated platelet membrane. Most of the KLH molecules were randomly scattered over the surface of the membrane, but in addition there was a small amount of clumping. Fig. 2B shows the distribution of the same label when the platelets were stimulated with thrombin. Very large clumps of KLH molecules can be seen (indicated by white arrows), indicating that as was the case with GPIIIa, membrane perturbation resulted in clustering of the GPIIb molecules.

Morphologic Demonstration of GPIIb-IIIa Complex Formation in the Thrombin-stimulated Platelet Membrane. Fig. 3A demonstrates the distribution of GPIIb and GPIIIa in the unstimulated platelet as indicated by an indirect ferritin marker assay for anti-GPIIIa and KLH conjugated to anti-GPIIb. Both labels were randomly scattered over the membrane surface. However, after stimulation of the platelet with thrombin, large mixed clumps of ferritin and KLH were seen (KLH is indicated by the white arrows and ferritin is indicated by black/white arrows in Fig. 3B), which indicate macromolecular complex formation (co-clustering) between GPIIb and GPIIIa.

Morphologic Demonstration of GPIb Distribution in the Unstimulated and Thrombin-stimulated Platelet Membrane. Fig. 4A demonstrates the distribution of GPIb in the membrane of the unstimulated platelet as evidenced by the indirect ferritin assay. Most of the ferritin marker is seen in clusters of 10–20 molecules, indicating that unlike GPIIb and GPIIIa, which appear to be mainly randomly distributed in the platelet membrane, the distribution of GPIb is primarily focal. After platelet stimulation with thrombin, very large clumps of ferritin (indicated by white arrows) were visualized, which indicated marked clustering of the GPIb molecules in the membrane (Fig. 4B).

Lack of Demonstrable Complex Formation between GPIb and GPIIb after Platelet Stimulation with Thrombin. Fig. 5A demonstrates the distribution of GPIb and GPIIb in the unstimulated platelet membrane, as evidenced by an indirect ferritin assay for anti-

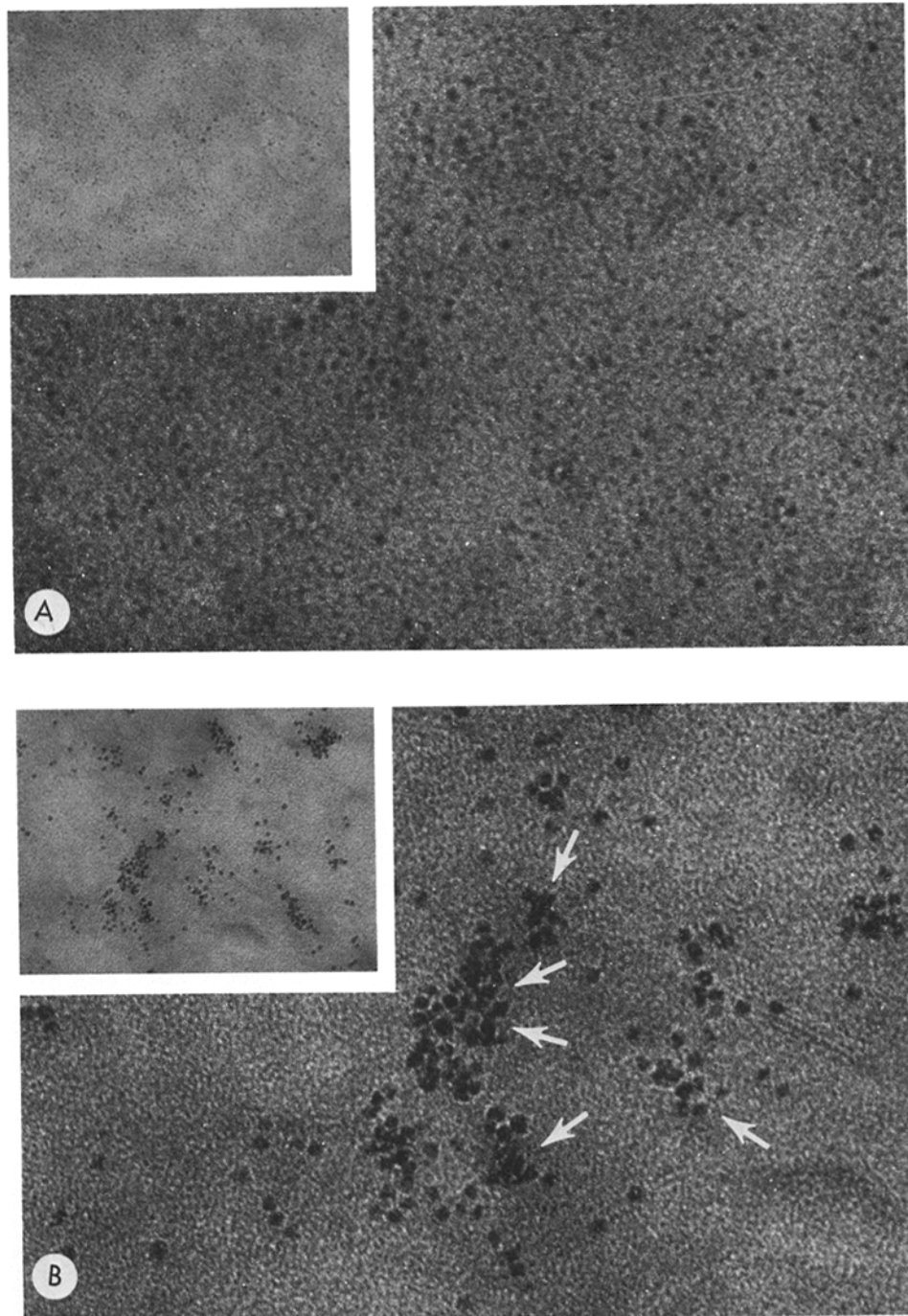


FIG. 1. Platelets interacted with rabbit antibody to platelet membrane GPIIIa followed by ferritin-conjugated goat anti-rabbit γ -globulin. (A), untreated platelets; (B), thrombin-treated platelets, stained with bismuth subnitrate. White arrows in (B) indicate clusters of ferritin molecules. $\times 278,400$. Inserts $\times 87,000$.

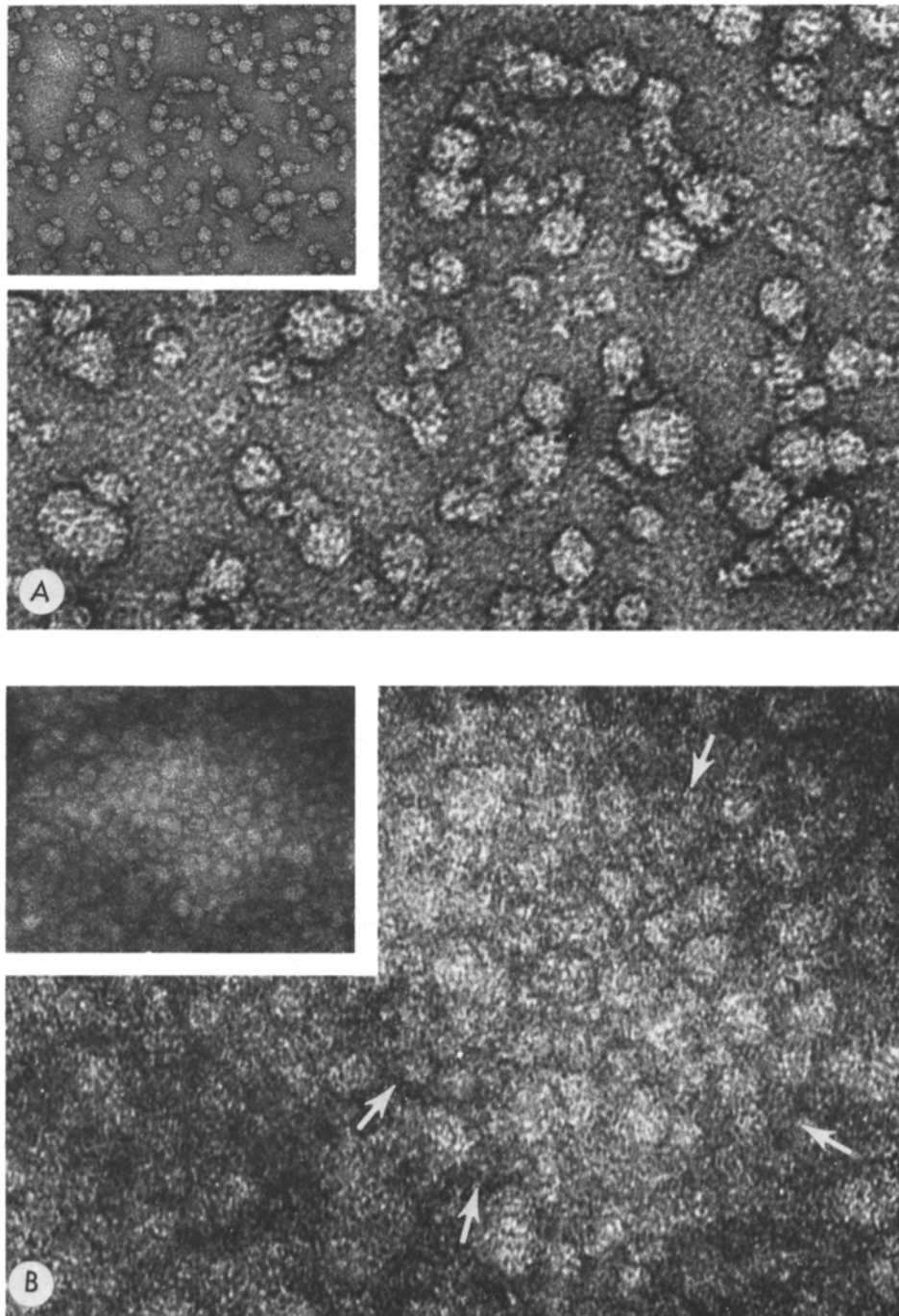


FIG. 2. Platelets interacted with KLH conjugated to anti-platelet GPIIb. (A), untreated platelets; (B), thrombin-treated platelets, stained with uranyl acetate. White arrows in (B) indicate a typical large cluster of KLH molecules. $\times 278,400$. Inserts $\times 87,000$.

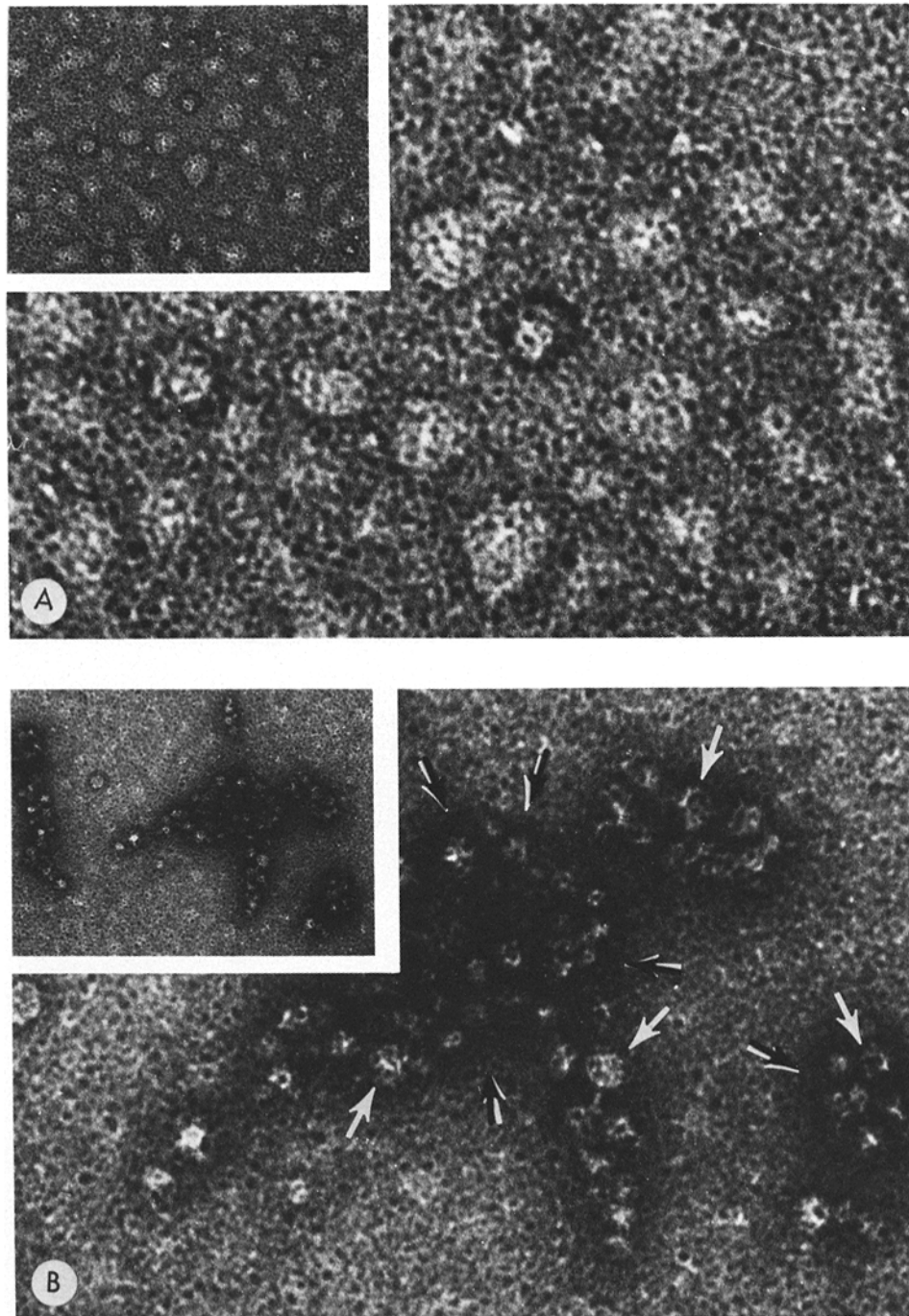


FIG. 3. Platelets interacted with anti-GPIIIa, ferritin conjugated goat anti-rabbit γ -globulin; KLH-conjugated anti-GP IIb. Stained with uranyl acetate and bismuth subnitrate. (A), untreated platelets; (B), thrombin-treated platelets. In (B) can be seen mixed macromolecular co-clusters of ferritin molecules (black/white arrows) and KLH molecules (white arrows). $\times 278,400$. Inserts $\times 87,000$.

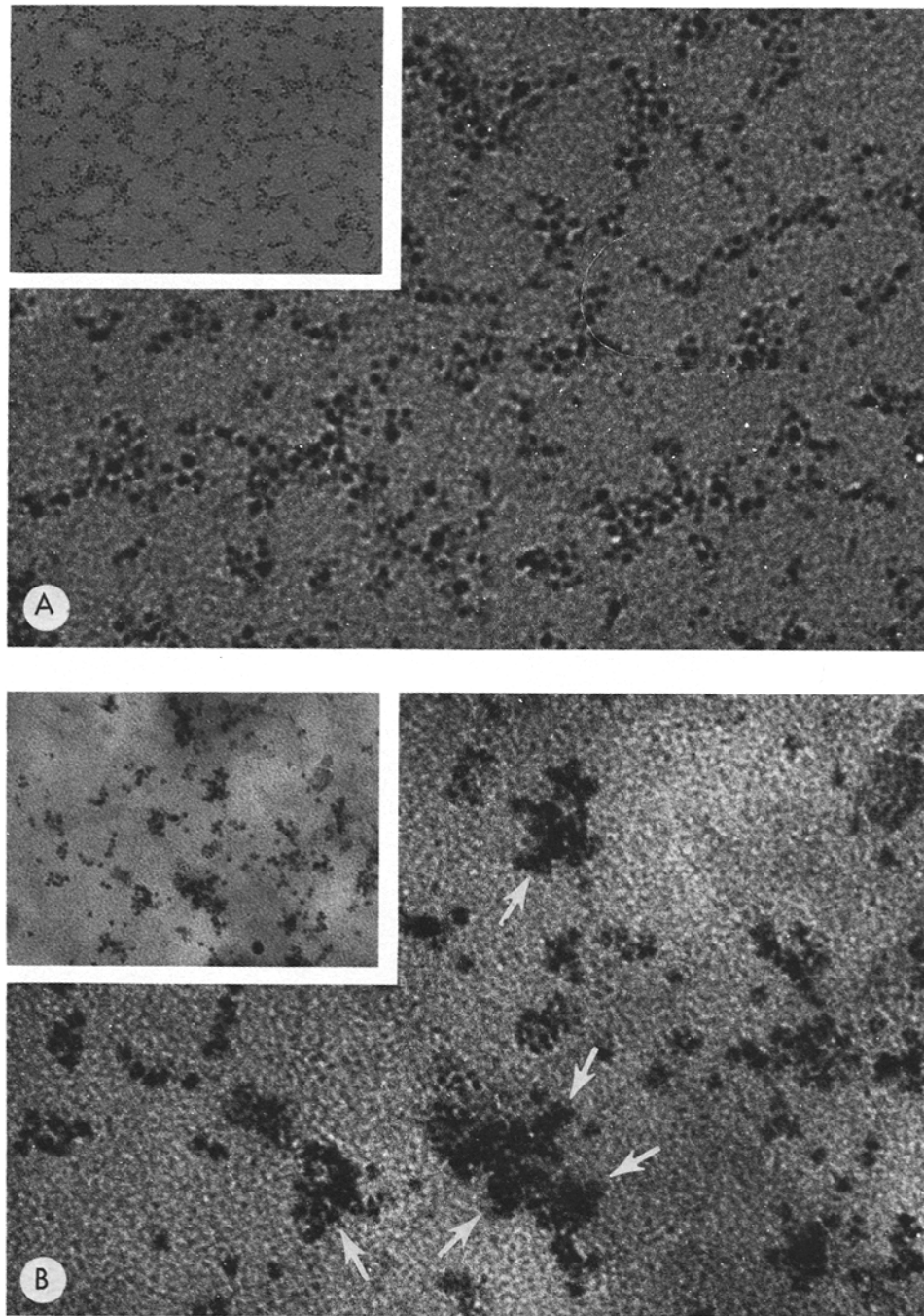


FIG. 4. Platelets interacted with rabbit antibody to platelet membrane GPIb (anti-GPIb), followed by ferritin-conjugated goat anti-rabbit γ -globulin. (A), untreated platelets; (B), thrombin-treated platelets. Stained with bismuth subnitrate. White arrows in (B) indicate typical clusters of ferritin molecules. $\times 278,400$. Inserts $\times 87,000$.

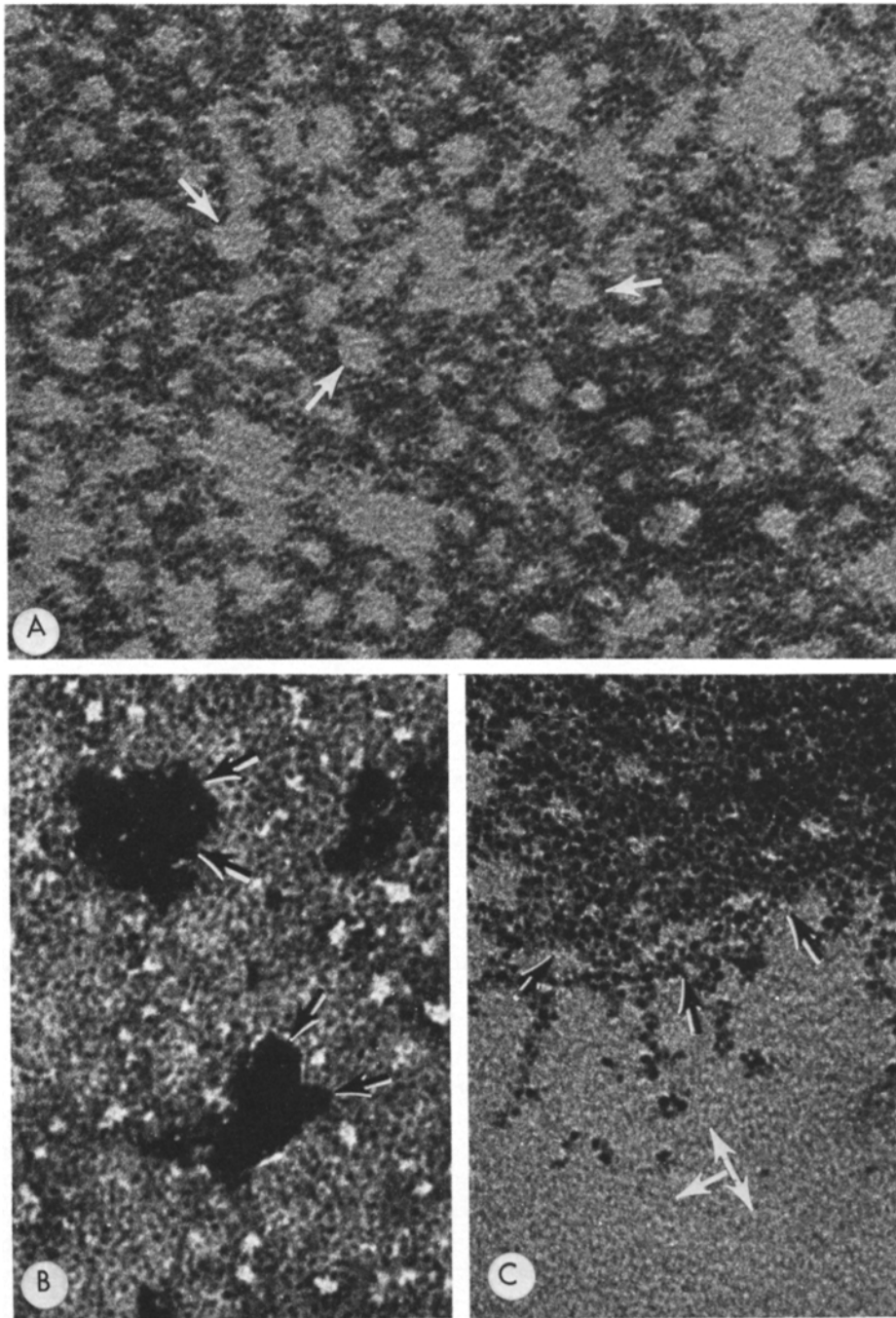


FIG. 5. Platelets interacted with anti-GPIIb; ferritin-conjugated goat anti-rabbit γ -globulin; KLH-conjugated anti-GPIIb. Stained with uranyl acetate and bismuth subnitrate. (A), untreated platelets; (B) and (C), thrombin-treated platelets. Typical KLH molecules in (A) are indicated by white arrows. In (B) can be seen clusters of ferritin molecules (black/white arrows) with some randomly distributed KLH molecules. In (C) another area of the same preparation is shown. In this area very large clusters of ferritin (black/white arrows) adjacent to very large clusters of KLH (white arrows) are shown. No mixed macromolecular complexes of ferritin and KLH were seen. $\times 278,400$.

GPIb and KLH conjugated to anti-GPIIb. Fig. 5 B and C demonstrates the distribution of the two markers in the platelet membrane after thrombin stimulation. In Fig. 5 B, discrete clumps of ferritin (indicated by black/white arrows) were seen with no associated KLH molecules. In the same figure, some randomly distributed KLH molecules are shown. In another sample of the same preparation, very large clusters of ferritin (black/white arrows) adjacent to very large clusters of KLH (white arrows) were seen (Fig. 5 C). No mixed clusters indicating complex formation between the two glycoproteins were seen in any of the areas of the preparation subjected to ultrastructural analysis.

Discussion

Because methods are now available for the isolation and purification of the platelet membrane glycoproteins GPIIb and GPIIIa, and monospecific antisera have been produced to them (8), it was possible to use an immunologic probe to study the spatial organization of these two glycoproteins in the platelet membrane. For the ultrastructural studies, two immunologic markers were used: one was an indirect assay in which ferritin was conjugated to goat anti-rabbit γ -globulin and used to localize the rabbit antibody to GPIIIa. The second ultrastructural label was KLH conjugated directly to anti-GPIIb. When both markers were used separately, the distribution of GPIIb and GPIIIa in the membrane of the unstimulated platelet was shown to be random with occasional small foci. After stimulation of the platelet with thrombin, large clusters of either ferritin or KLH were visualized, indicating that membrane perturbation had resulted in the clustering of GPIIb and GPIIIa molecules.

In a double-label experiment, it was possible to determine the topographic relationship of the two separate glycoproteins to each other. In the unstimulated platelet membrane, random distribution of both labels was visualized. However, after stimulation of the platelet with thrombin, large mixed clusters of ferritin and KLH were seen. This observation indicated that thrombin treatment of the platelet had resulted in the formation of large clusters of GPIIb-GPIIIa complexes.

The spatial organization of GPIb in the unstimulated platelet was different from that seen for GPIIb and GPIIIa. The GPIb glycoprotein was organized in microclusters in the resting platelet membrane surface. Thrombin treatment of the platelet membrane resulted in the formation of large clusters of GPIb molecules. In a double-label experiment, ferritin was used as the probe with anti-GPIb and KLH was used with anti-GPIIb. Large separate clumps of ferritin molecules and of KLH molecules were visualized. However, no mixed clusters of ferritin and KLH were seen, indicating that even though membrane perturbation induced by thrombin stimulation had resulted in the formation of large clusters of GPIb molecules, no complex between GPIb and GPIIb was formed. The data suggest that the formation of the GPIIb-GPIIIa macromolecular complex is a specific phenomenon associated with membrane stimulation. Other studies have also suggested a close association of GPIIb and GPIIIa in the stimulated platelet membrane. A monoclonal antibody made to thrombin-stimulated platelets detected a complex of the two proteins in Triton X-100-solubilized platelet membranes (16). An identical result has been reported on crossed immunoelectrophoresis of solubilized platelet membrane proteins using a polyspecific rabbit antibody (5).

Recent studies (17) suggest that receptor complex formation and clustering are important for signal generation after membrane stimulation. In some circumstances, ligand-induced membrane receptor clustering forms part of a membrane recycling pathway (18). In view of the absence of fibrinogen binding by thrombasthenic platelets and the concomitant deficiency of GPIIb and GPIIIa in the disorder, it seems logical to assume that the thrombin-activated GPIIb-GPIIIa complex may function as the fibrinogen receptor in platelets. Preliminary studies in fact demonstrate complex formation of GPIIb-GPIIIa with purified human fibrinogen.²

Because GPIIb and GPIIIa are normally exposed on the unstimulated platelet surface (1), which does not bind fibrinogen (2, 3, 6), platelet membrane perturbation should initiate new spatial and topographic relationships between these two glycoproteins, which are probably necessary for the generation or induction of the fibrinogen receptors. Peerschke and Zucker (19) have shown that membrane fluidity and receptor clustering are necessary for fibrinogen binding and platelet aggregation.

We suggest that macromolecular complex formation of GPIIb and GPIIIa is necessary for the induction of the fibrinogen receptor in stimulated platelets. Further studies are currently under way to determine morphologically the spatial organization of fibrinogen to the GPIIb-GPIIIa complex in the activated platelet.

Summary

The topographic relationships of platelet membrane glycoprotein IIb and glycoprotein IIIa have been studied in stimulated and unstimulated human platelets using immunoelectron microscopy. An indirect approach with ferritin-conjugated goat anti-rabbit γ -globulin was used to localize the rabbit antibody to glycoprotein IIIa. The second ultrastructural label was keyhole limpet hemocyanin conjugated directly to antibody to glycoprotein IIb. Using the double labels, it was demonstrated that glycoprotein IIb and glycoprotein IIIa were distributed randomly in the unstimulated platelet membrane. After platelet stimulation with thrombin, large clusters of glycoprotein IIb-glycoprotein IIIa complexes were formed. No complex formation between glycoprotein Ib and glycoprotein IIb was observed in control experiments. These observations suggest that thrombin stimulation initiates the specific glycoprotein IIb-glycoprotein IIIa macromolecular complex formation on the platelet surface, which may act as the active fibrinogen-binding site required for normal platelet aggregation.

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