

Identification of Membrane Proteins Mediating the Interaction of Human Platelets

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ABSTRACT Membrane glycoproteins that mediate platelet-platelet interaction were investigated by identifying those associated with the cytoskeletal structures from aggregated platelets. The cytoskeletal structures from washed platelets, thrombin-activated platelets (platelets incubated with thrombin in the presence of mM EDTA to prevent aggregation) and thrombin-aggregated platelets (platelets activated in the presence of mM Ca^{++}) were prepared by first treating platelet suspensions with 1% Triton X-100 and 5 mM EGTA and then isolating the insoluble residue by centrifugation. The readily identifiable structures in electron micrographs of the residue from washed platelets had the shape and dimensions of actin filaments. Analysis of this residue by SDS gel electrophoresis showed that it consisted primarily of three proteins: actin (mol wt = 43,000), myosin (mol wt = 200,000) and a high molecular weight polypeptide (mol wt = 255,000) which had properties identical to actin-binding protein (filamin). When platelets are activated with thrombin in the presence of EDTA to prevent aggregation, there was a marked increase in the amount of insoluble precipitate in the subsequent Triton extraction. Transmission electron microscopy showed that this residue not only contained the random array of actin filaments as seen above, but also organized structures from individual platelets which appeared as balls of electron-dense filamentous material $\sim 1 \mu\text{m}$ in diameter. SDS polyacrylamide gel analysis of the Triton residue of activated platelets showed that this preparation contained more actin, myosin and actin-binding protein than that from washed platelets plus polypeptides with mol wt of 56,000 and 90,000 and other minor polypeptides. Thus, thrombin activation appeared to increase polymerization of actin in association with other cytoskeletal proteins into structures that are observable after Triton extraction. The cytoskeletal structures from thrombin-aggregated platelets were similar to those from thrombin-activated platelets, except that the structural elements from individual platelets remained aggregated rather than randomly dispersed in the actin filaments. This suggested that the membrane components that mediate the direct interaction of platelets were in Triton residue from aggregated platelets. Only a small percentage of the membrane surface proteins and glycoproteins were found in the cytoskeletal structures from either washed platelets or thrombin-activated platelets. In contrast, the aggregated cytoskeletal structures from thrombin-aggregated platelets contained membrane glycoproteins IIb (26% of the total in pre-extracted platelets) and III (14%), suggesting that one or both of these glycoproteins participate in the direct interaction of platelets during aggregation.

In 1886, Eberth and Schimmelbusch (12) showed that blood platelets had the ability to "sort out" or aggregate to the exclusion of other blood cells. This aggregation reaction can occur *in vivo* at a site of vascular injury (which occurs during hemostasis) or in the absence of external trauma (which may result in a thrombotic lesion). Platelet aggregation *in vitro* offers a process especially suited to examination of cell-cell

interaction mechanisms, in that aggregation is tissue-specific, can be controlled by alteration of the medium, and involves cells that are isolated without the physical or enzymatic treatments usually required to dissociate a tissue.

For aggregation to occur, platelets must interact with a stimulus that activates them. Although several stimuli may be involved, e.g., adenosine diphosphate, collagen, or serotonin,

perhaps the most important biological stimulus is thrombin, a proteolytic enzyme (31). This enzyme binds to the plasma membrane of the platelet at both high and low affinity sites and is not internalized during stimulation (51). Although the receptor for thrombin on the membrane is not known, two membrane glycoproteins, glyocalycin and glycoprotein V, have been shown to interact with thrombin. Glyocalycin is an inhibitor of the proteolytic activity of thrombin (35), while glycoprotein V is a thrombin substrate (42). It is not known whether either of these interactions is required for thrombin activation of the platelet.

After interaction with thrombin, platelets characteristically lose their disk shape, produce many filopodia, and secrete the contents of their storage organelles. If Ca^{++} is present and the solution is agitated so that platelet collisions occur, activated platelets will spontaneously aggregate. It has been argued that platelet aggregation proceeds because activation modifies the plasma membrane, either by adding components to the membrane surface or by modifying preexisting components (15). Several studies have shown that the membrane surface of activated platelets differs from that of control platelets: (a) thrombin-stimulated platelets had more receptor sites for the lectin concanavalin A (38) and lentil-phytohemagglutinin (27) than did unstimulated platelets, (b) thrombin treatment of platelets also increased the number of factor X_a binding sites on the membrane surface, an event responsible for the procoagulant activity of platelets (30), (c) lactoperoxidase-catalyzed iodination disclosed that thrombin induced a change in the molecular organization of the platelet plasma membrane proteins because different proteins were labeled after thrombin stimulation (38), and (d) thrombin-activated platelets were found to express a hemagglutinin that was not active on unstimulated platelets (15). This last observation may be the most relevant to aggregation, as it demonstrated that the membrane surface of activated platelets was capable of binding specific receptors on other cells.

Although several postulates have been made to define the molecular mechanisms of platelet interaction (31), the molecules involved have not been identified. Our approach to identifying proteins that mediate platelet-platelet interaction was based on several observations which indicated that binding of macromolecules to their membrane receptors may render the receptor resistant to Triton solubilization if the receptor has interacted with cytoskeletal structures in the cell. For example, Triton extraction of cultured cells solubilizes most cellular proteins except for actin and other proteins in the cytoskeletal structure (6, 36, 52). Because the cytoskeletal structures of adhering cells remain attached to the substratum, it appeared that the membrane proteins mediating cell attachment were resistant to Triton solubilization because they were sandwiched between those actin cables directed to attachment points and the substratum. When these adhering structures were examined by SDS gel electrophoresis, they were found to contain fibronectin. This provided direct support for the postulate that cell-associated fibronectin mediates cell-substratum interaction (6). In another example, Flanagan and Koch (14) found that anti-Ig antibodies bound to their receptors on lymphocytes, induced an association between surface Ig and cellular actin, and rendered the Ig receptors resistant to Triton solubilization. Likewise, addition of concanavalin A to *Dictyostelium discoideum* amoebae caused the receptors for this lectin to remain in a structured organization in the presence of Triton (9).

We have now adapted this Triton extraction methodology to

platelets in an attempt to identify aggregation receptors. We reasoned that if the cytoskeletal structures of aggregated platelets remained associated after Triton extraction, then membrane proteins mediating this association could be identified. In this report, procedures are described for the preparation of cytoskeletal structures from platelets in solution. Further, we found that the cytoskeletal structures from aggregated platelets remained aggregated and that two plasma membrane glycoproteins, IIB and III, were associated with these complexes.

MATERIALS AND METHODS

Preparation of Platelet Samples

Venous blood was obtained from normal healthy adult donors who had not taken any medication for the previous 2 wk. The platelets from freshly drawn blood were collected and washed as previously described (40). Platelet washing and subsequent experiments were performed at ambient temperatures ($22^\circ \pm 2^\circ\text{C}$) unless otherwise indicated. Platelet counts were determined by an Electrozone/Celloscope Counter (Particle Data, Inc., Elmhurst, Ill.). The washed platelets were suspended in either Tyrode's solution, ETS buffer, or calcium-, magnesium-free Tyrode's containing 0.001 M EDTA. Tyrode's solution contained 0.138 M sodium chloride, 0.0027 M potassium chloride, 0.012 M sodium bicarbonate, 0.00036 M sodium phosphate, 0.0018 M calcium chloride, 0.00049 M magnesium chloride, and 0.0055 M glucose, pH 7.4, and was used to prepare thrombin-activated platelets. ETS buffer contained 0.154 M sodium chloride, 0.001 M EDTA, and 0.01 M Tris, pH 7.6 (Tris [Trizma], Sigma Chemical Co., St. Louis, Mo.). ETS buffer and Ca^{++} , Mg^{++} -free Tyrode's with mM EDTA were selected to prepare thrombin-activated platelets because they allowed platelet activation and secretion by thrombin, but prevented aggregation. Both EDTA-containing buffers gave similar results; only data obtained using ETS buffer are presented in this manuscript. For the thrombin-treated preparations, 10^9 platelets were suspended in 1 ml of buffer and treated for 30 min with 1 NIH unit human thrombin (a generous gift of Dr. John Fenton II, Albany, N. Y.). During these incubations, the thrombin-activated platelets remained in suspension while the thrombin-activated platelets settled to the bottom of the tube.

Preparation of Cytoskeletal Structures

Washed platelets, suspended in either ETS buffer or Tyrode's solution, and the thrombin-treated preparations were treated for 5 min with an equal volume of the Triton extraction buffer (2% Triton X-100, Sigma Chemical Co., 0.01 M EGTA, and 0.1 M Tris, pH 7.4) and 2 NIH units/ml of hirudin (Sigma Chemical Co.). Separate measurements of the hydrolysis of the synthetic substrate S-2160 (Ortho Diagnostics, Inc., Raritan, N. H.) showed that this concentration of hirudin inhibited more than 98% of the thrombin activity. We found it necessary to include 5 mM EGTA in the Triton extracting solution for two reasons; (a) to make the divalent cation concentrations equivalent in all solutions, and (b) to prevent hydrolysis of the 255,000 protein by the calcium-dependent protease in platelets (43). The insoluble material (residue) was then collected by centrifugation of the solution for 4 min at 8,730 g in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting pellet was rinsed, without resuspension, with 1 ml of a solution that contained one part of the Triton extraction buffer to one part of the platelet suspending buffer. This solution was then recentrifuged for 4 min; in some cases, the supernate from the first 4-min centrifugation was further centrifuged at 100,000 g for 1 h.

Electron Microscopy

THIN SECTIONS: The samples were fixed in suspension by adding an equal volume of 4% glutaraldehyde in the sample buffer. The sample was then removed from solution by centrifugation in a Beckman microfuge for 4 min and the supernate replaced with 2% glutaraldehyde in 0.05 M sodium phosphate, pH 7.4. After a 5-min fixation, the solution was replaced by one containing 2% glutaraldehyde, 0.4% tannic acid, and 0.05 M sodium phosphate, pH 7.4. After 45 min, the sample was postfixated for 30 min in 0.1% OsO_4 , 0.05 M sodium phosphate, pH 6.0. The tannic acid treatment (3) and the postfixation technique (29) were used to preserve actin filaments. The sample was dehydrated in a graded series of ethanol solutions followed by propylene oxide and then embedded in Epon 812.

NEGATIVE STAINING: For electron micrographs of actin filaments, a drop of Triton-extracted platelets was placed on carbon-coated grids and stained with 2% phosphotungstic acid buffered to pH 7.2.

Electron micrographs of activated platelets were made on polylysine-coated grids. These were prepared by placing a drop of 5 mM polylysine (mol wt =

3,000, Sigma Chemical Co.) on a carbon-coated grid which had been glow-discharged. After 30 s, the grid was rinsed with distilled water. Each grid was then covered with a drop of washed platelets in Tyrode's solution and after adhesion, the platelets were extracted with a drop of the Triton extraction buffer for 1 min. The wet grid was then stained with 1% uranyl formate.

Light Microscopy

Acid-washed microscopy slides were coated according to the method of Fisher (13) in 5 mM poly-L-lysine (mol wt = 3,000), then rinsed in distilled water, and dried with nitrogen. The slide was then covered with several drops of washed platelets in Tyrode's solution, rinsed in Tyrode's solution for 30 s to remove nonadhering platelets, covered with a coverslip, and photographed with a Zeiss microscope with a $\times 100$ phase objective. After the coordinates of the field were recorded, the sample was removed, extracted with the Triton extraction buffer for 3 min, and the same field rephotographed.

Gel Electrophoresis

Washed platelets, thrombin-treated platelets, and the Triton residues were prepared for electrophoresis by solubilization in 2% SDS containing 2% vol/vol 2-mercaptoethanol. All samples were incubated at 100°C for 10 min to effect complete solubilization. For one-dimensional analysis, 10–50 μ g protein was electrophoresed through slab gels according to the method of Laemmli (23) using a resolving gel containing either 5% acrylamide or a 5–20% exponential gradient of acrylamide. All gels had 3% acrylamide in the stacking gel. The area allotted to each sample in the gel was 0.4 cm wide, 9 cm high, and 0.15 cm thick. Two-dimensional electrophoresis was performed by use of the method of O'Farrell (34). For the isoelectric focusing in the first dimension, the sample was prepared as reported by the method of Ames and Nikaido (1) and contained 1% vol/vol ampholines, 0.2% with a pH range of 3.5–10, and 0.8% with a pH range of 5–7 (LKB Produkter AB, Bromma, Sweden). The second dimension utilized a 5–10% exponential gradient acrylamide gel, prepared according to the procedure of Laemmli (23). The protein in all gels was stained with Coomassie brilliant blue. The relative amount of protein in individual bands of any sample was determined from scans of the stained, wet gel using an Ortec densitometer (Ortec Inc., Oak Ridge, Tenn.). Three copies of each scan were made and individual peaks were cut out and weighed. The value for each peak was an average of the three weights, which varied by <3%. The values reported here are the means (\pm SD) of the values from three different donors.

Platelets were radiolabeled with 125 I by lactoperoxidase-catalyzed iodination (the lactoperoxidase was a generous gift of Dr. Martin Morrison, Memphis, Tenn.) by the procedure previously reported (37, 41). For determination of the distribution of radioactivity, the stained gels of radiolabeled samples were dried under vacuum and stored next to RP Trimax X-ray film (3M Co., St. Paul, Minn.). After 1- to 3-d exposure, the film was processed as suggested by the manufacturer. The amount of radioactivity in individual bands was determined by cutting the bands from the dried gel and counting the gamma emissions in each band. The values reported are the means \pm SD of the values from six different donors.

Other Methods

Actin in the Triton residues was adsorbed to deoxyribonucleate 5'-digonucleotidohydrolase (EC 3.1.4.5) (DNase-I) affinity columns as described by Lazarides and Lindberg (24) after the actin filaments were first solubilized with 0.5 M KI (52). Protein concentrations were estimated by the method of Lowry *et al.* (25) using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. With samples containing Triton, the modifications reported by Dulley and Grieve (11) were employed to prevent interference by this detergent in the protein assay.

RESULTS

Our strategy for identifying membrane proteins mediating platelet aggregation was to first determine whether cytoskeletal structures from aggregated platelets remained aggregated and then to identify membrane glycoproteins associated with those aggregates. Achievement of this objective relied on our ability to prepare stable cytoskeletal structures when suspended platelets were extracted with Triton. Although reports indicated that the organized cytoskeleton of individual platelets was a stable structure after Triton extraction of platelets that were adhering to a solid substratum (32, 33), our initial efforts showed that

such structures were not present after extraction of platelets in suspension. Accordingly, we first had to determine the conditions required for the preparation of stable cytoskeletal structures.

Triton Solubilization of Washed and Thrombin-Activated Platelets

When washed, unactivated platelets were treated with 1% Triton X-100; they immediately dissolved but within minutes a flocculent precipitate formed which remained suspended in solution but was barely visible (Fig. 1a). Thrombin-activated platelets were similarly extracted (Fig. 1b). Thrombin-activated platelets were prepared by adding thrombin to washed platelets in the presence of EDTA. Such platelets were activated (i.e., they changed shape, produced filopodia, and secreted the contents of the storage organelles), but did not aggregate because aggregation requires the presence of Ca^{++} (58). The thrombin-activated platelets always yielded a larger amount of precipitate after Triton solubilization than did washed platelets (Table I).

Fig. 2 shows electron micrographs of these Triton precipitates (or "residues"). The residue from washed platelets (Fig.

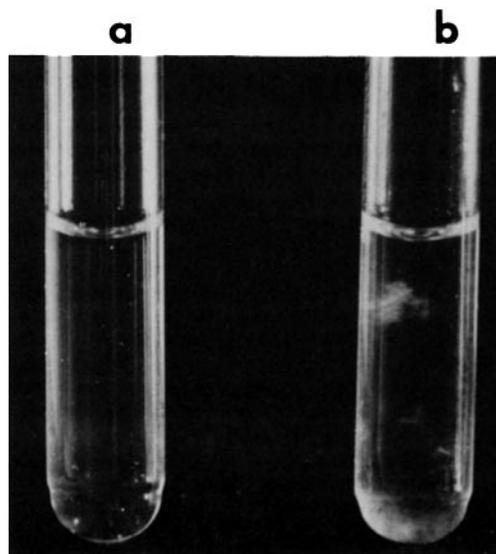


FIGURE 1 Triton extraction of washed and thrombin-activated platelets. Washed platelets (a) and thrombin-activated platelets (b) in ETS buffer were treated for 5 min with 1% Triton X-100. Note the presence of the flocculent precipitate which formed in the thrombin-activated sample.

TABLE I

Amount of Protein in Triton Residues from Washed Platelets and Thrombin-Activated Platelets

Platelets	Before extraction	Triton residue
Washed	2.09 \pm 0.07*	0.41 \pm 0.02* = 19.6 \pm 1.6%‡
Thrombin activated	2.05 \pm 0.08	0.73 \pm 0.04 = 35.6 \pm 3.3%

Washed platelets, suspended in ETS buffer, and thrombin-activated platelets were treated with Triton extraction buffer and the insoluble residue (see Fig. 1) was isolated by centrifugation. The Triton residue was then solubilized in SDS and diluted to the original volume of platelets. The values are an average of determinations for triplicate samples.

* mg Protein per ml (\bar{x} \pm SD).

‡ Percent of total platelet protein (\bar{x} \pm SD).

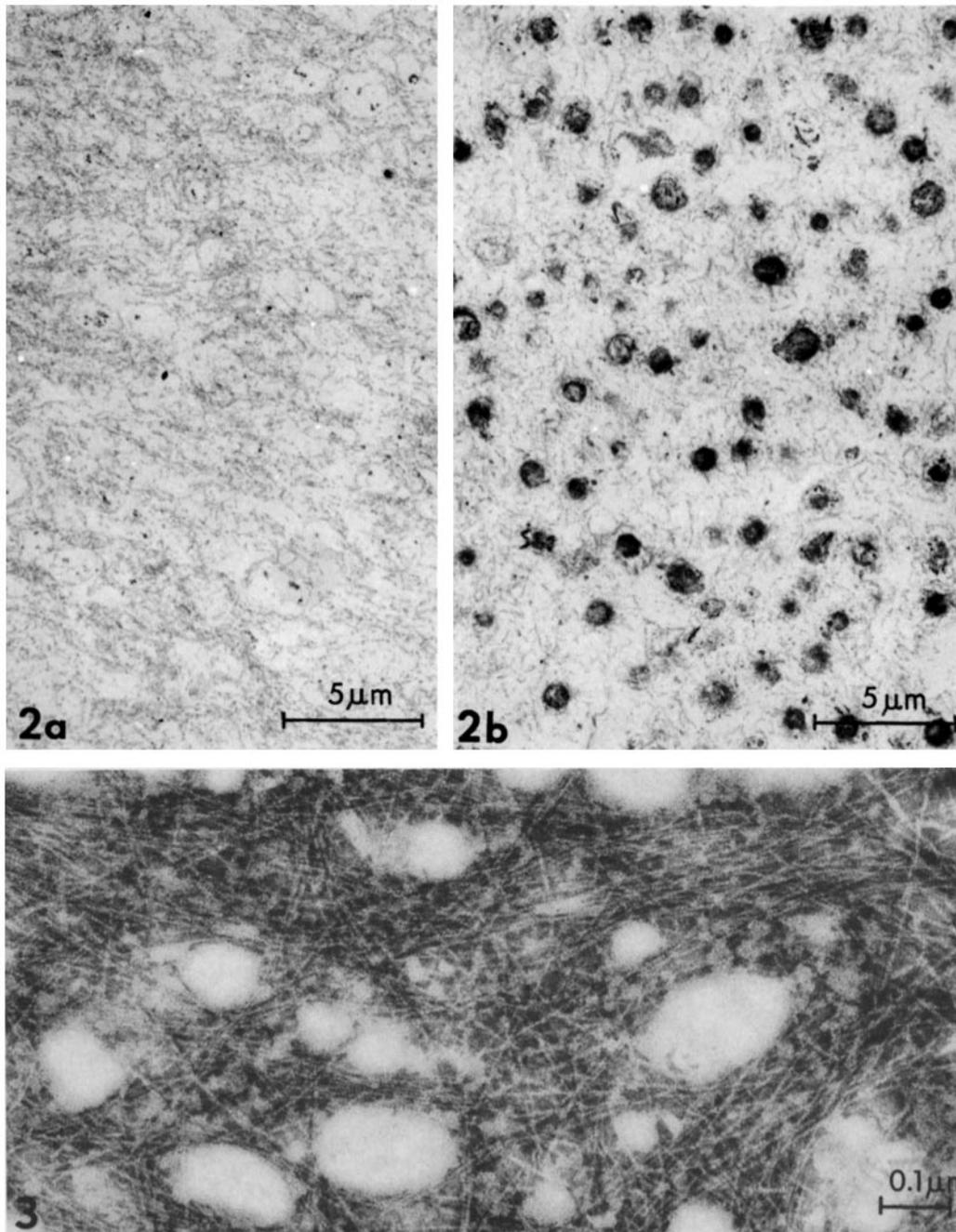


FIGURE 2 Thin sections of Triton residues from washed platelets. $\times 4,000$. The Triton-insoluble material from washed platelets (a) contained only filaments, whereas the residue from thrombin-activated platelets (b) contained not only filaments but dense balls of filamentous material which appeared to be derived from individual platelets.

FIGURE 3 Negative staining of the filaments in the Triton residue from washed platelets. $\times 101,000$.

2a) was composed entirely of filamentous material without structures that resembled the cytoskeletons of individual platelets. In contrast, the residue from stimulated platelets (Fig. 2b) had filamentous material and numerous electron-dense balls $\sim 1 \mu\text{m}$ in diameter that were randomly distributed throughout the field. A previous study showed that microtubules are solubilized by 1% Triton (52) and, indeed, structures resembling microtubules were not present after Triton was added to washed platelets. It appeared that the initial centrifugation removed all filamentous material because recentrifugation of the initial supernate at 100,000 g for 1 h did not remove any additional filaments.

Identification of Actin in the Triton Residues

On the basis of physical dimensions of the filaments in the Triton residue and a previous report that actin filaments are resistant to Triton solubilization (5), we thought that the filaments in the Triton residue of washed platelets were made of actin. This was confirmed by several observations: (a) examination of the Triton residue from washed platelets by negative staining (Fig. 3) showed that the filaments were 60 Å in diameter, which is similar to the previously reported diameter of actin filaments (21), (b) two-dimensional electrophoresis of this residue (Fig. 4) employing isoelectric focusing in the first

dimension and SDS electrophoresis in the second dimension as reported by O'Farrell (34) showed that the major protein present had an identical apparent molecular weight (43,000) and isoelectric point (5.75) as previous reports for platelet actin (8, 19), and (c) when the filaments were solubilized in 0.5 M KI, the 43,000 mol wt protein was adsorbed onto a DNase-I column (data not shown), a specific adsorbant of actin (24).

Polypeptide Composition of the Triton Residue from Thrombin-Activated Platelets

Fig. 5 shows a comparison of the polypeptides in the Triton residues from washed and thrombin-activated platelets. Because these samples represent the Triton residue from an equal number of platelets, these gels reflect the quantitative protein

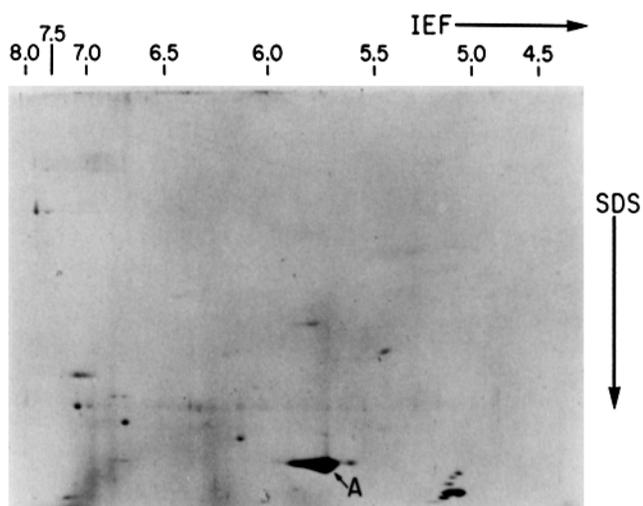


FIGURE 4 Two-dimensional electrophoresis of the Triton residue from washed platelets. The actin filaments from a preparation similar to that in Fig. 1 *b* were collected by centrifugation, solubilized in SDS, and analyzed by two-dimensional electrophoresis as described by O'Farrell (34). The pH gradient of the first-dimension isoelectric focusing (IEF) is indicated at the top of the figure. The primary spot in this figure is actin (A) which had an isoelectric point of 5.75.

TABLE II
Percent of Actin, Myosin, and Actin-binding Protein in the Triton Residues from Washed Platelets and Thrombin-Activated Platelets

Platelets	Actin	200,000 protein (myosin)	255,000 protein (actin-binding protein)
(a) Platelets in ETS buffer			
Washed	40.9 ± 5.2*	17.4 ± 1.2*	23.1 ± 3.1*
Thrombin activated	64.2 ± 8.7	63.9 ± 9.3	34.5 ± 5.7
(b) Platelets in Tyrode's solution			
Washed	43.6 ± 3.7	< 0.01	29.6 ± 11.2
Thrombin aggregated	63.9 ± 9.1	33.0 ± 11.2	55.2 ± 11.8

Washed platelets and the Triton residues from the preparations indicated were solubilized in SDS and electrophoresed on one-dimensional gels as described by Laemmli (23). The Coomassie Blue-stained gels were scanned with a densitometer and the areas under the actin, myosin, and actin-binding protein peaks determined. The values are the average of determinations for three separate platelet preparations.

* Percent of each protein in the Triton Residue ($\bar{X} \pm SD$).

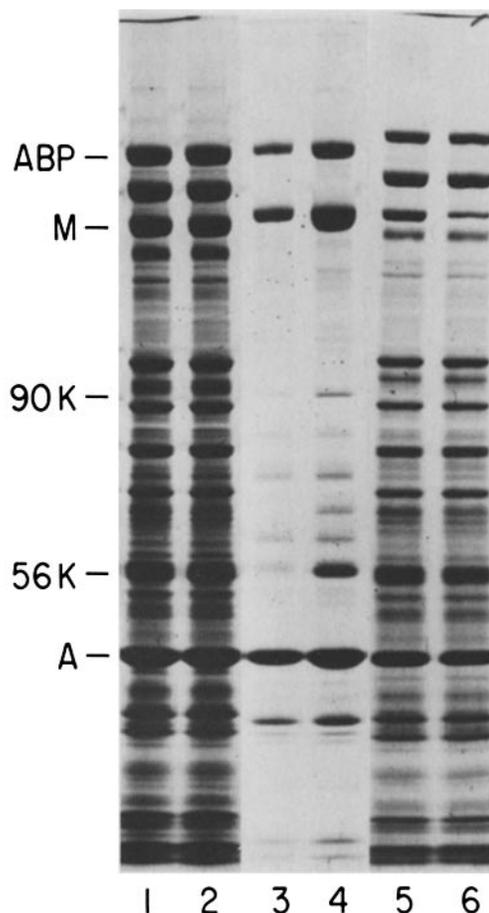


FIGURE 5 SDS polyacrylamide gel electrophoresis of the whole platelets, Triton residues, and Triton supernates from washed platelets and thrombin-activated platelets. Washed platelets and thrombin-activated platelets, suspended in ETS buffer, were treated with 1% Triton X-100 for 5 min and the Triton-insoluble material (Triton residues, which contained actin filaments) separated from solution by centrifugation. The proteins were solubilized in SDS, separated on a 5-20% exponential gel, and stained by Coomassie Blue. The samples are: whole platelet samples from washed platelets, gel 1, and thrombin-activated platelets, gel 2; Triton residue from washed platelets, gel 3, and thrombin-activated platelets, gel 4; Triton supernates from washed platelets, gel 5, and thrombin-activated platelets, gel 6. The presence of actin-binding protein (ABP), myosin (M), and actin (A) are visible in all samples. The 56,000 and 90,000 mol wt proteins are also indicated.

changes seen in Table I. The three most prevalent polypeptides present in the Triton residues of washed platelets (lane 3) had apparent mol wt of 43,000 (actin), 200,000, and 255,000. Incubation of this residue with a crude preparation of platelet calcium-dependent protease (43) resulted in the selective hydrolysis of the 255,000 protein (data not shown). The residue from activated platelets (lane 4) had ~50% more actin and 255,000 protein as did that from washed platelets. In addition, the amount of 200,000 protein increased about fourfold (Table II *a*). Polypeptides with mol wt of 56,000 and 90,000 were also present in increased concentration in the residue from thrombin-activated platelets as were other minor polypeptides. These polypeptides were not characterized further.

Triton Extraction of Adhering Platelets

The electron-dense balls in the Triton residue from activated platelets were readily observed by phase-contrast light micros-

copy. None were observed when Triton was added to washed platelets but, after thrombin-activated platelets were Triton extracted, they appeared as numerous as the platelets. To determine whether each ball represented a cytoskeleton of an individual platelet, we allowed platelets to adhere to polylysine-coated glass coverslips before extraction with Triton X-100. We reasoned that because platelets will adhere tightly to glass (16) and become activated because of interaction with glass and the polylysine (28), we should be able to detect the cytoskeletons of individual platelets after Triton extraction. As shown by phase-contrast light microscopy (Fig. 6*a*), platelets

adhere to and spread out on the glass surface coated with polylysine. When the same field is examined after extraction of adhering platelets with Triton (Fig. 6*b*), each platelet produced material that remained associated with the polylysine-coated surface.

Fig. 7 shows the structure of the adhering (activated), extracted platelet after negative staining. This preparation was made by extracting platelets that adhered to polylysine-coated carbon film. The structure of the cytoskeletal filaments is readily observed by this procedure. In many cases, bundles of filaments could be observed extending out from the central

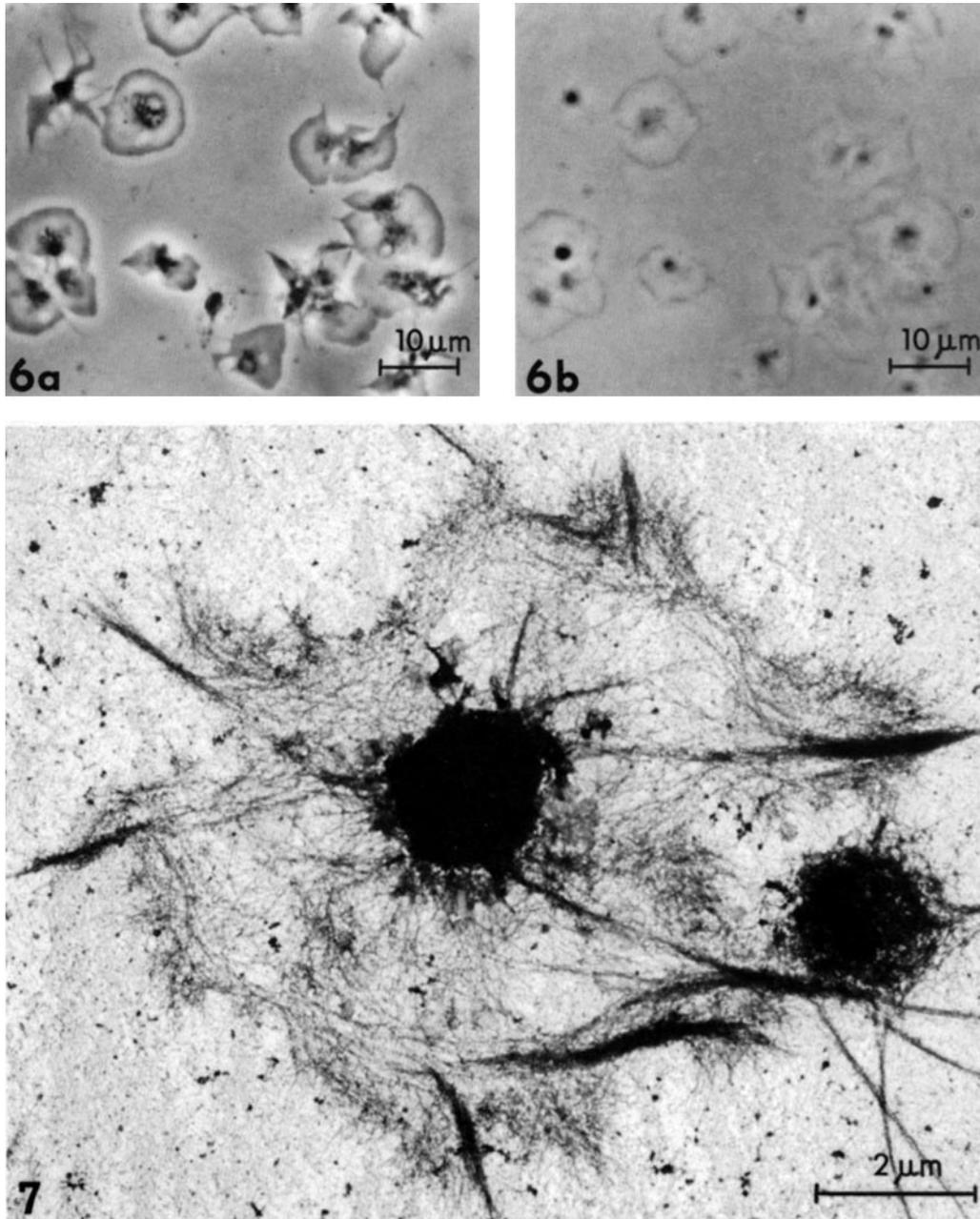


FIGURE 6 Phase-contrast microscopy of platelets adhering to a polylysine-coated glass slide before (*a*) and after (*b*) Triton extraction. The one-to-one correspondence between pre-extracted platelets and the resulting "balls" with attached cytoskeletons is evident as the two micrographs have the identical field of view. $\times 1,090$.

FIGURE 7 Negative staining of two Triton-extracted platelets adhering to a carbon-coated and polylysine-coated grid. The cytoskeleton of the extracted platelet resembles the shape of the activated platelets of Fig. 6. The bundles of fibers appear to represent the individual filopodia. $\times 11,000$.

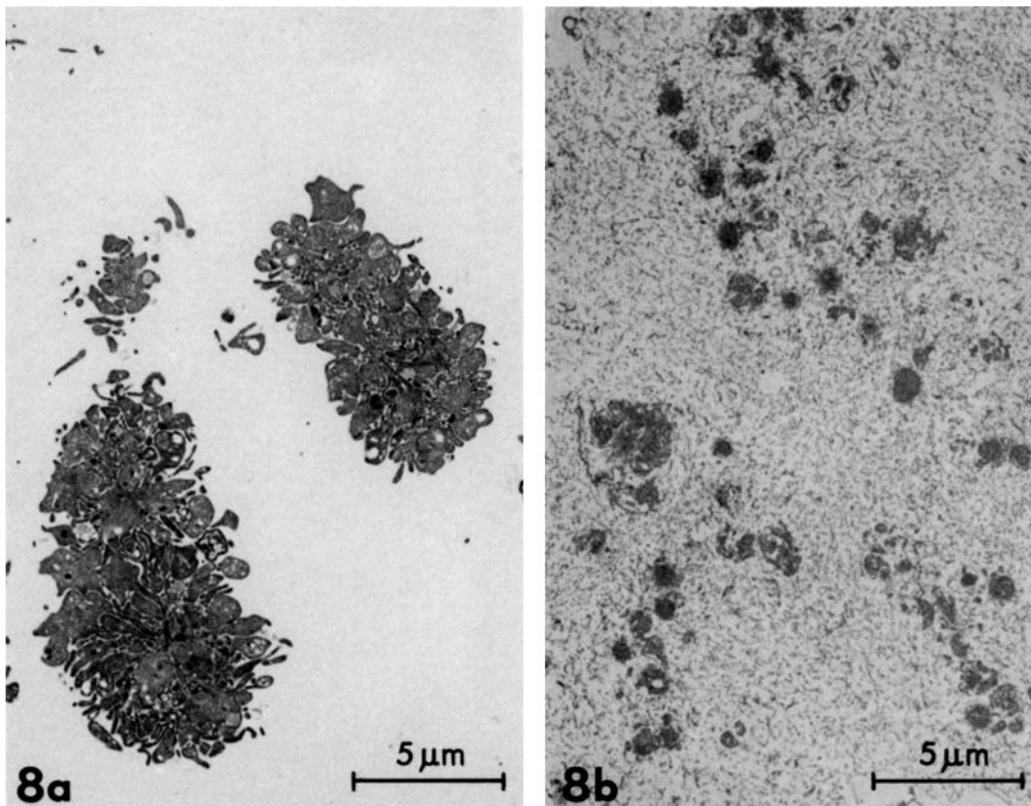


FIGURE 8 Thin sections of aggregated platelets (a) and Triton-extracted aggregated platelets (b). It may be noted that unlike the extraction of thrombin-activated platelets (Fig. 2 b), where the cytoskeletal structures of individual platelets were dispersed, those of thrombin-aggregated platelets remained aggregated. $\times 4,000$.

body of the platelet in what appeared to be remnants of filopodia. The data show that cytoskeletal structures of individual platelets were identifiable after extraction of adhering platelets with Triton and that their shape resembled that of the unextracted platelets.

Triton Extraction of Aggregated Platelets

Washed platelets will aggregate after treatment with thrombin in a Ca^{++} -containing buffer. Fig. 8a shows an electron micrograph of thrombin-aggregated platelets. These platelets have an irregular shape (unstimulated platelets are disk shaped) and have few storage organelles (31).

When aggregated platelets were treated with 1% Triton, a flocculent precipitate was obtained which had a macroscopic appearance similar to that of the precipitate shown in Fig. 1 b. The Triton residues of thrombin-aggregated platelets contained $39.9 \pm 6.2\%$ ($n = 3$) of the total platelet protein, while the residues of washed platelets in Tyrode's solution contained $20.7 \pm 2.7\%$ ($n = 3$). Examination of this precipitate by electron microscopy (Fig. 8 b) showed that the precipitate from aggregated platelets contained cytoskeletal structures similar to those present after the extraction of thrombin-activated platelets. However, unlike the activated platelet preparation, where the cytoskeletons were dispersed in the actin filaments, the cytoskeletons from aggregated platelets remained associated.

Because of this difference, we examined the two preparations for the presence of membrane proteins to see if the filaments in the aggregated preparation were interacting directly or if another component(s) was mediating their interaction. Because platelet aggregation results from the interaction of membrane

surfaces, we thought it most likely that the aggregated structures were interacting through one or more of the membrane surface components. To detect membrane surface proteins and glycoproteins in this preparation, we radiolabeled intact platelets with ^{125}I by lactoperoxidase-catalyzed iodination and analyzed the membrane components in the Triton residues after SDS gel electrophoresis. Preliminary studies (data not shown) showed that iodinated platelets aggregated like control platelets.

Fig. 9a shows the polypeptide composition of the Triton residues of washed platelets (lane 3) and thrombin-aggregated platelets (lane 4). Also included in this figure for comparison are the gels of residues from washed platelets in ETS buffer (lane 1) and thrombin-activated platelets (lane 2). Quantitation of individual bands in these samples, Table II b, showed that there was $\sim 50\%$ more actin and 255,000 protein in the residue of thrombin-aggregated platelets than in washed platelets in Tyrode's solution. Although these amounts were similar to those present in platelets in ETS buffer (compare to Table II a), the amounts of 200,000 protein were markedly lower. No explanation is apparent for the different effects of the two buffers on 200,000 protein distribution because all preparations were subsequently solubilized into a buffer containing 5 mM EGTA.

Fig. 9b shows the individual ^{125}I -labeled membrane components in these preparations. Lane 5 is for references and is of iodinated, unextracted platelets. The major labeled components, which are glycoproteins, are identified by the nomenclature previously used (41). The other lanes in this figure show the iodinated membrane glycoproteins in the Triton residue of washed platelets in ETS buffer (lane 6), thrombin-activated

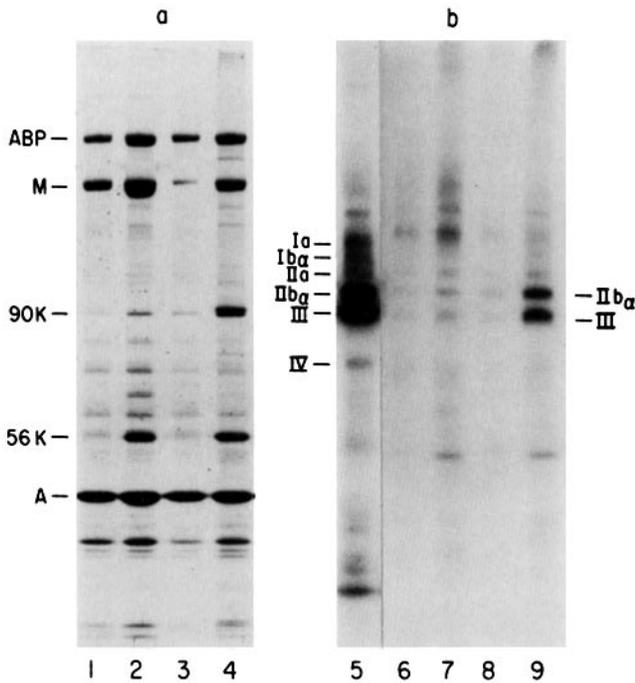


FIGURE 9 Distribution of ^{125}I -labeled membrane glycoproteins in the Triton-extracted samples. Washed platelets were radiolabeled using lactoperoxidase-catalyzed iodination and suspended in ETS buffer or Tyrode's solution. After thrombin treatment, we added an equal volume of Triton extraction buffer, isolated the insoluble residues by centrifugation, solubilized it in SDS, and electrophoresed it on SDS gels. The gels in *a* are protein-stained gels; those in *b* are autoradiograms of dried gels and show the ^{125}I distribution in the gel. The samples are: washed platelets in ETS buffer, lanes 1 and 6; thrombin-activated platelets, lanes 2 and 7; washed platelets in Tyrode's solution, lanes 3 and 8; thrombin-aggregated platelets, lanes 4 and 9; unextracted platelets, lane 5. The protein identifications in *a* were described in the legend to Fig. 5. The glycoproteins identified in *b* use the nomenclature previously described (40). Glycoproteins IIb and III appeared to be selectively associated with the Triton residue from aggregated platelets.

platelets (lane 7), washed platelets in Tyrode's solution (lane 8), and thrombin-aggregated platelets (lane 9). We routinely observed that of the major glycoproteins, the amounts of glycoproteins IIb and III appeared to be selectively increased in the Triton residues from thrombin-aggregated platelets. Table III gives the percentages of each of these glycoproteins in the four platelet preparations. About 26% of glycoprotein IIb and about 14% of glycoprotein III were in the residue from thrombin-activated platelets, while the amounts of these two glycoproteins in the other three preparations were barely above background levels. Although the glycoprotein III in the residue from aggregated platelets had a similar electrophoretic mobility to the 90,000 protein in the 5–20% acrylamide gels (Fig. 9), close examination of these gels and of others stained for carbohydrate, suggested that glycoprotein III and the 90,000 protein could be separated by suitable electrophoretic procedures. To increase separation of this molecular weight region, lower percentage acrylamide gels were used. With 5% acrylamide gels, one band was identified as glycoprotein III because it co-electrophoresed with glycoprotein III in a purified preparation containing glycoproteins IIb and III on both reduced and nonreduced gels (41), was labeled on intact cells by lactoperoxidase, stained for carbohydrate by carbohydrate-staining

procedures, and was identical in electrophoretic mobility to a band missing in thrombasthenic platelets (40). The reduced, 5% gel in Fig. 10 shows the Triton residue of aggregated platelets (lane 2) and a mixture of glycoproteins IIb and III

TABLE III
Percent of Glycoproteins IIb and III in the Triton Residues from Washed Platelets, Thrombin-Activated Platelets, and Thrombin-aggregated Platelets

Platelets	IIb	III
Washed in ETS buffer	$5.4 \pm 2.1^*$	$2.4 \pm 0.9^*$
Thrombin activated	7.2 ± 4.0	3.4 ± 1.3
Washed in Tyrode's solution	3.7 ± 2.1	1.9 ± 0.5
Thrombin aggregated	26.1 ± 7.2	14.3 ± 5.4

Washed platelets were radiolabeled by lactoperoxidase-catalyzed iodination and suspended in either ETS buffer or Tyrode's solution. After thrombin treatment, we added an equal volume of Triton-solubilizing buffer and isolated the insoluble residues by centrifugation. The Triton residues and the unextracted platelets were solubilized in SDS, electrophoresed on one-dimensional gels, and the amounts of radioactivity associated with glycoproteins IIb and III were determined. The values are the average of determinations for six separate platelet preparations.

* Percent of each glycoprotein in the Triton residue ($\bar{x} \pm \text{SD}$).

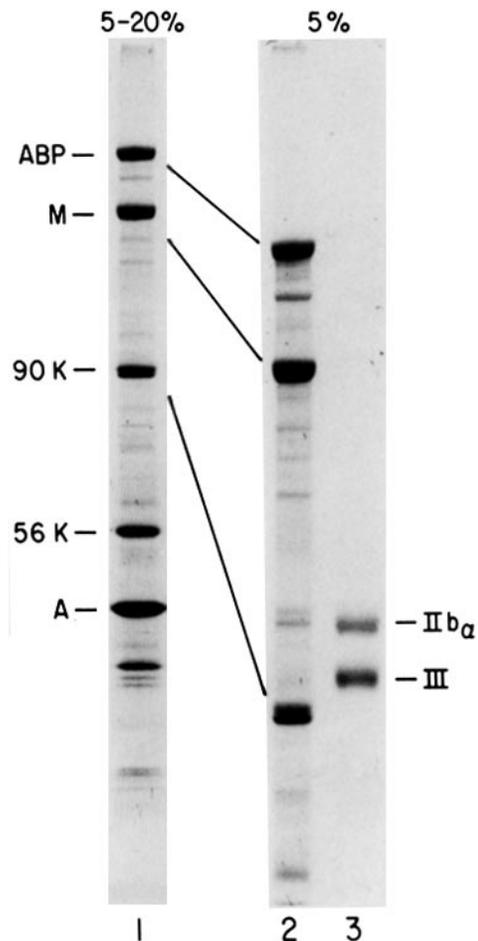


FIGURE 10 Comparison of the Triton-insoluble material from aggregated platelets to purified glycoproteins IIb $_{\alpha}$ and III. The Triton-insoluble residue from aggregated platelets was solubilized in SDS and electrophoresed on either a 5–20% exponential gradient of acrylamide (lane 1) or a 5% gel (lane 2). Also shown on the 5% gel is a sample containing 2.3 μg protein of a mixture of glycoprotein IIb $_{\alpha}$ and III (lane 3), purified by a procedure to be reported (L. K. Jennings and D. R. Phillips). The 5% gel allows for separation of glycoprotein III from the protein-staining material in the 90,000 region of the gel.

(lane 3) which demonstrates that glycoprotein III is distinct from the protein-staining band at 90,000, a band clearly comprised of two proteins as visualized on the 5% gel, but not on the 5–20% gel (lane 1).

DISCUSSION

The following conclusions can be derived from our data: (a) the cytoskeletal structures of washed platelets as revealed by Triton extraction consist only of a random array of actin filaments, while those from activated platelets consist of organized filamentous structures from individual platelets, (b) there is a 50% increase in the amount of actin in the cytoskeletal structures of thrombin activation of platelets, and (c) glycoproteins IIb and III are involved in the formation of platelet-platelet interactions.

Cytoskeletal Structures of Activated Platelets

Our findings disclosed that the cytoskeletal structures revealed by Triton extraction from thrombin-activated platelets were different from those of control platelets. The insoluble material from the extracted, unstimulated platelets contained only filaments with the physical dimensions and protein composition of actin filaments with no recognizable structures of individual platelets. By contrast, if platelets were activated by thrombin or by adhesion to the polylysine-coated surfaces, the cytoskeletal structures remaining after Triton extraction consisted of filaments which maintained the dimensions of the unextracted platelet. These observations seem to contradict a previous report by Nachmias *et al.* (32) who showed that extraction of platelets adhering to carbon-coated grids left cytoskeletal structures from individual platelets. However, platelets can be “activated” by interaction with almost any surface independent of its polylysine content (47). In view of the present findings, it seems likely that the cytoskeletal structures described by Nachmias’ study were those from platelets activated by adhesion.

Thrombin-induced Actin Polymerization

Bray and Thomas (5) found that the filamentous actin within nonmuscle cells is resistant to Triton solubilization while the unpolymerized actin is Triton soluble. This distinction has led investigators to conclude that filamentous actin represents about one-half of the total actin in fibroblasts (5, 6, 35, 50), and two-thirds and one-third of the actin in amoebae nuclei and cytoplasm, respectively (46). In the present study, ~40% of the platelet actin of unstimulated platelets is resistant to Triton solubilization. Since this residue exists in structures with the dimensions of actin filaments, it would appear that the amount of actin in this residue represents the filamentous actin in washed platelets. This value is in remarkable agreement with that reported by Blikstad *et al.* (4) who also concluded that ~40% of the total platelet actin was filamentous because 60% was in a form which was able to inhibit DNase I. We have now found that ~60% of the actin from thrombin-activated platelets is resistant to Triton solubilization, a 50% increase of that present from unstimulated platelets. Because filopodia produced by activated platelets contain actin filaments (56, 59) and because the Triton-insoluble actin from thrombin-activated platelets appears to represent filamentous actin, the increase in Triton-insoluble actin may indicate the amount of actin polymerized during platelet activation.

The other two polypeptides present in high concentrations

in the Triton residue of unstimulated platelets had apparent mol wt of 200,000 and 255,000. Circumstantial evidence permits tentative identification of these polypeptides. The 200,000 protein appeared to be platelet myosin because it had the same electrophoretic mobility as platelet myosin isolated by the procedure of Pollard *et al.* (44), and because it remained with the cytoskeletal structures. The 255,000 protein was judged to be actin-binding protein (20), also termed platelet filamin (54) by the following criteria: (a) its apparent molecular weight was similar to that of platelet actin-binding protein (26, 54), (b) it was selectively hydrolyzed by the calcium-dependent protease in platelets as is platelet actin-binding protein (10, 43), and (c) it isolated with the actin filaments, a property shared with actin-binding protein (22, 48, 50, 55). The increased Triton insolubility of myosin and actin-binding protein in thrombin-stimulated platelets may indicate an association between these proteins and the newly formed actin filaments.

Membrane Proteins Mediating Cell-Cell Interaction

Glycoproteins IIb and III are prominent glycoproteins in the platelet plasma membrane; together they account for ~25% of the material stained by Coomassie blue on SDS gels of platelet plasma membranes (37). Both glycoproteins contain sialic acid (2), appear to span the thickness of the membrane (39) and, like most plasma membrane glycoproteins, are oriented with their carbohydrate-containing regions exposed to the outside of the cell (37). Glycoprotein III has been shown to be antigenetically similar to porcine skeletal muscle α -actinin (17), suggesting that it could serve as an attachment site for actin on the platelet plasma membrane.

Although glycoproteins IIb and III are isolated with platelet plasma membranes (37), they are readily extracted when isolated membranes are treated with 1% Triton X-100 (H. R. Prasanna and D. R. Phillips, unpublished observations). We now have shown that this concentration of detergent also solubilizes these glycoproteins from either washed platelets or thrombin-activated platelets. However, a significant amount of these glycoproteins remained with the cytoskeletal structures when aggregated platelets were extracted.

An analogous difference in solubility occurs in other cells whose plasma membrane components are restricted by interactions on the membrane surface. For example, the large external transformation-sensitive (LETS) protein (fibronectin) is soluble in Triton when tissue culture cells are in suspension, but is resistant to solubilization after adhesion (18). This property of Triton insolubility led to the early suggestion that the LETS protein mediates the attachment of cells to substrata. In another example, Smith and Koch (49) showed that membrane-associated IgG was Triton soluble, but that the addition of anti-IgG to the cell rendered this receptor Triton insoluble, presumably because of a complex between the receptor, antibody, and actin cables. We found that when aggregated platelets are extracted with Triton, the resulting cytoskeletal structures remain aggregated. What is holding these structures together? Either the filamentous material in each structure is interacting directly with the filaments in an adjacent structure, or the interaction is mediated by a membrane component(s). Because the major proteins in isolated cytoskeletons are not labeled on intact cells by lactoperoxidase iodination, they may not be expressed on the membrane surface (38, 42). This finding is consistent with the observation of Pollard *et al.* (45) who also found that actin is not on the outer surface of the

platelet plasma membrane. It appears likely, therefore, that a membrane component is mediating the interactions of the aggregated cytoskeletal structures. Two membrane glycoproteins, IIb and III, were selectively retained with these aggregates. Because these glycoproteins are not retained with the cytoskeletons of activated platelets when prepared under similar conditions (i.e., extracted in the presence of 5 mM EGTA), we suggest that they are involved in the direct interaction of platelets during aggregation and that these glycoproteins become Triton insoluble because of macromolecular associations between the membrane surfaces and the actin cables.

In support of a role for glycoproteins IIb and III as aggregation sites on the platelet membrane, platelets from patients with Glanzmann's thrombasthenia, an inherited bleeding disorder, lack glycoproteins IIb and III, while other membrane components are present in apparently normal amounts (40). Functional analysis of these platelets has consistently shown that these platelets will not aggregate. Other functions are normal, e.g., adherence to exposed subendothelium (53), activation by ADP, thrombin, and collagen, and secretion by thrombin and collagen (7, 57). The abnormal clot retraction is no doubt linked to the failure to aggregate. The specific absence of glycoproteins IIb and III in platelets that have an inherited aggregation defect supports the suggestion that platelet-platelet interaction is mediated by one, or both, of these glycoproteins.

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