

Identification of a Membrane Skeleton in Platelets

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Abstract. Platelets have previously been shown to contain actin filaments that are linked, through actin-binding protein, to the glycoprotein (GP) Ib-IX complex, GP Ia, GP IIa, and an unidentified GP of M_r 250,000 on the plasma membrane. The objective of the present study was to use a morphological approach to examine the distribution of these membrane-bound filaments within platelets. Preliminary experiments showed that the Triton X-100 lysis buffers used previously to solubilize platelets completely disrupt the three-dimensional organization of the cytoskeletons. Conditions were established that minimized these post-lysis changes. The cytoskeletons remained as platelet-shaped structures. These structures consisted of a network of long actin filaments and a more amorphous layer that outlined the periphery. When Ca^{2+} was present, the long actin filaments were lost but the amorphous layer at the periphery remained; conditions

were established in which this amorphous layer retained the outline of the platelet from which it originated. Immunocytochemical experiments showed that the GP Ib-IX complex and actin-binding protein were associated with the amorphous layer. Analysis of the amorphous material on SDS-polyacrylamide gels showed that it contained actin, actin-binding protein, and all actin-bound GP Ib-IX. Although actin filaments could not be visualized in thin section, the actin presumably was in a filamentous form because it was solubilized by DNase I and bound phalloidin. These studies show that platelets contain a membrane skeleton and suggest that it is distinct from the network of cytoplasmic actin filaments. This membrane skeleton exists as a submembranous lining that, by analogy to the erythrocyte membrane skeleton, may stabilize the plasma membrane and contribute to determining its shape.

PLATELETS are small anucleate cells that have a characteristic discoid shape as they circulate in the blood. When they come into contact with damaged subendothelium at a site of injury, they change shape, extend filopodia, secrete their granular contents, express receptors for fibrinogen and coagulation factors, aggregate, and contract, thus retracting clots of externally bound fibrin. Many of these responses of stimulated platelets are thought to involve the contractile activity of actin filaments (18, 19). Several lines of evidence support this idea: bundles of actin filaments have been visualized in filopodia and surrounding the granules of activated cells; myosin associates with the actin filaments in stimulated platelets; clot retraction is dependent on a functional cytoskeleton; and cytochalasins inhibit several of the platelet's responses to activation.

The function of actin filaments in unstimulated, discoid platelets is less clear. Biochemical assays have shown that 40–50% of the total actin in discoid platelets is in a filamentous form (10, 12, 22). During the early stages of platelet activation, many of these filaments become cross-linked into structures that sediment from Triton X-100 lysates at low g forces (15,600 g for 4 min) (22). These filaments are not

linked to any detectable membrane glycoprotein (GP)¹ and are depolymerized when Ca^{2+} is present (10, 17, 36), perhaps as a result of the Ca^{2+} -dependent severing of filaments by gelsolin (50). Since these filaments undergo increased actin polymerization and associate with myosin during platelet activation, they may represent the contractile component of the cytoskeleton (17). We have also suggested recently that platelets contain a second pool of actin filaments (17). These filaments differ from the contractile filaments in that they require high g forces (typically 100,000 g for 3 h) to be sedimented, even from Triton X-100 lysates of activated platelets. They are selectively associated with membrane GPs and are resistant to Ca^{2+} -induced depolymerization (17). These results suggest that platelets contain membrane-associated actin filaments that are distinct from the contractile filaments of the cytoskeleton (17). We provided evidence that these membrane-associated filaments are associated with actin-binding protein (16, 17) and spectrin (25) and are linked through actin-binding protein to GP Ib-IX, GP Ia, GP IIa, and a GP of M_r 250,000 on the plasma membrane (16, 17).

1. Abbreviation used in this paper: GP, glycoprotein.

The objective of the present study was to use a morphological approach to characterize the subcellular distribution of the membrane-bound actin within platelets. In the past, the morphology of actin filaments in unstimulated platelets was difficult to visualize because of osmium-induced damage associated with the fixation techniques. However, several recent modifications of conventional techniques have demonstrated that actin filaments in unstimulated platelets exist both throughout the cytoplasm and at the periphery of the cell, where they parallel the plasma membrane and are found between the microtubule coil and the membrane (9, 15, 22, 33, 37). In the present study, we show that these actin filaments represent those that we have previously described as the contractile filaments. The membrane-associated actin, however, is a component of an additional structure. This structure has an amorphous appearance and lines the plasma membrane. Under conditions in which the cytoplasmic actin filaments are largely depolymerized, the membrane-associated filaments remain in a structure that retains the outline of the cell from which it originated. Thus, the platelet plasma membrane has a lining of membrane-bound actin that may function as a membrane skeleton, stabilizing the shape of the cell.

Materials and Methods

Isolation and Surface Labeling of Platelet Suspensions

Venous blood was drawn from healthy adult donors, and platelets were isolated from it by centrifugation as described (17). Platelets from patients with Bernard-Soulier syndrome were obtained as described elsewhere (24). To obtain platelets in which the surface GPs were ^3H -labeled, washed platelets were labeled by the sodium metaperiodate/sodium [^3H]borohydride method, as described previously (17). All platelets were resuspended at a final concentration of 1×10^9 platelets/ml at 37°C in a Tyrode's buffer containing 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM calcium chloride, and 0.4 mM magnesium chloride, pH 7.4. Platelets were incubated in this buffer for at least 2 h before use to allow them to recover from centrifugation-induced activation and to regain the discoid shape of unactivated platelets (22). Platelets that had been ^3H -labeled did not regain their discoid shape even 3 to 4 h after isolation and were therefore used as a source of non-discoid platelets.

Isolation and Analysis of Triton X-100-insoluble Residues

Platelets were lysed by addition of an equal volume of buffer containing 2% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4. Where indicated, 2 mg of leupeptin/ml (Vega Biotechnologies, Tucson, AZ), 100 mM benzamidine (Sigma Chemical Co.), and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) were included in the lysis buffer to inhibit proteolytic activity. This buffer is referred to as isotonic buffer. In some experiments, where indicated, EGTA was omitted from the lysis buffer to allow Ca^{2+} -dependent depolymerization of actin filaments (10, 17, 36). In some experiments, 0.1% alcian blue (Polysciences Inc., Warrington, PA), 100 μM phalloidin (Sigma Chemical Co.), or 0.66 mg of ristocetin/ml (Sigma Chemical Co.) were included to maintain the organization of the membrane-associated filaments. In other experiments, the membrane-associated filaments were stabilized by lysing the platelets with nine volumes of a buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 58 mM sodium borate, pH 8.0, or with nine volumes of a buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 5 mM sodium phosphate, pH 8.0. The latter two buffers are referred to as low-ionic-strength buffers. The concentration of protease inhibitors that they contained was such that the final concentration of the inhibitors in the Triton X-100 lysates was 1 mg

of leupeptin/ml, 50 mM benzamidine, and 1 mM PMSF. Triton X-100-insoluble and -soluble fractions were separated by centrifugation at 4°C , either for 4 min at 15,600 g or for 3 h at 100,000 g. Fractions were solubilized and analyzed on one-dimensional SDS-polyacrylamide gels by the method of Laemmli (34) or on two-dimensional gels as described by O'Farrell and modified by Ames and Nikaido (1). Polypeptides were detected by staining the gels with Coomassie Brilliant Blue. The relative amounts of actin in the fractions were determined by densitometric scans of stained, wet, one-dimensional gels, using a Hoefer scanning densitometer linked to an Apple IIe computer through which areas under peaks were integrated. ^3H -labeled GPs were detected by fluorography of gels treated with EN 3 HANCE (New England Nuclear, Boston, MA). The amounts of radioactivity in ^3H -labeled GPs were quantitated as described previously (17). Western blotting was performed by the method of Towbin et al. (48). Anti-tubulin antibodies were obtained from Miles Scientific (Naperville, IL), and anti-P235 was raised and characterized as described (23).

Preparation of Samples for Electron Microscopy

Suspensions of intact platelets or platelet lysates were fixed and prepared for electron microscopy as described previously (9). Briefly, samples were fixed by addition of a 10-fold volume of a fixative containing 0.2 M glutaraldehyde (10% glass-distilled solution, Electron Microscopy Sciences, Fort Washington, PA) and 40 mM lysine (Sigma Chemical Co.), in 60 mM sodium cacodylate buffer, pH 7.4. Samples were incubated at ambient temperatures for ~ 60 min, and the fixed material was then isolated by centrifugation for 15 min at 600 g. The material was washed twice in barbital buffer, pH 7.6, exposed for 15 min at 4°C to 0.05 M osmium tetroxide in the same buffer, rinsed three times in ice-cold distilled water, and stained overnight at 4°C in 0.05 M uranyl acetate in water. Fixed material was dehydrated in acetone and embedded in Epox 812 (Ernest F. Fullam, Inc., Schenectady, NY). For lysing and fixing platelets simultaneously, 1% Triton X-100 and 5 mM EGTA were added to the initial fixative. Sections were cut to a silver-grey thickness and stained with Reynold's lead citrate and uranyl acetate or were cut to 0.2 μm and left unstained. Samples were photographed at 80 kV in a JEOL 100 CX II microscope (JEOL, Peabody, MA).

Immunocytochemical Localization of Glycoprotein Ib and Actin-binding Protein

Antibodies against actin-binding protein (23) and the α -subunit of GP Ib (7) were raised, affinity-purified, and characterized as described previously. Affinity-purified antibodies against GP IIB were a generous gift of Dr. Laurence Fitzgerald of the Gladstone Foundation Laboratories (San Francisco, CA). Control rabbit IgG was obtained from Sigma Chemical Co.

For immunocytochemical localization of platelet proteins in the Triton X-100-insoluble residues, 100- μl portions of platelets were lysed by addition of an equal volume of buffer containing 2% Triton X-100, 10 mM EGTA, 100 mM Tris-HCl, 4 mg of leupeptin/ml, 200 mM benzamidine, and 4 mM PMSF, pH 7.4. Antibodies (20 μl) were added to give a final concentration of 11.5 μg of anti-actin-binding protein/ml, 13 μg of anti-GP Ib/ml, 12.5 μg of anti-GP IIB/ml, or 10 μg of normal rabbit IgG/ml (Sigma Chemical Co.). Protein A coupled to 15-nm-diameter gold beads (200 μl) (Janssen Life Sciences Products, Beerse, Belgium) was added. In double-labeling experiments, antibodies were directly coupled to gold beads by conventional techniques (46). Samples were incubated at 37°C for 60 min, at which time they were fixed by addition of an equal volume of fixative and processed as described above.

Fluorescence Microscopy of Platelet Cytoskeletons

Platelets (100 μl) were lysed in 900 μl of a buffer containing 1% Triton X-100, 1 mM calcium chloride, 1 mg of leupeptin/ml, 50 mM benzamidine, 1 mM PMSF, and 5 mM sodium phosphate, pH 8. After the platelet lysate was incubated for 10 min at 4°C , 100 μl of the lysate was placed on poly-L-lysine-coated glass slides. The slides were incubated at ambient temperature for 40 min. Excess lysate was then removed with filter paper, and 100 μl of phosphate buffer containing 3.7% formaldehyde and 0.33 μM rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR) added. The slides were incubated at 4°C in the dark 30 min, rinsed three times with PBS, then examined and photographed with a Zeiss Universal microscope that was equipped with an epifluorescence rhodamine filter pack.

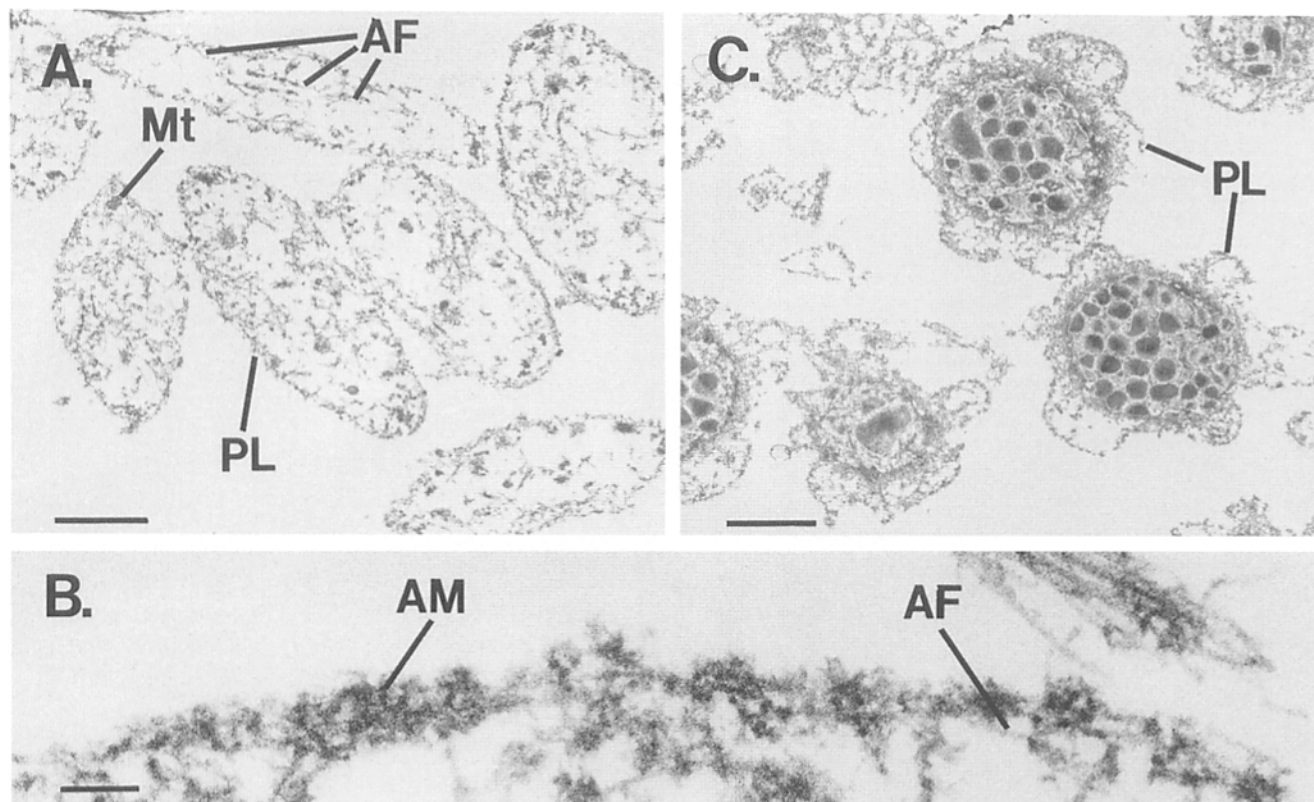


Figure 1. Thin sections of Triton X-100-insoluble residues from unstimulated, discoid platelets (*A* and *B*) or from platelets that had been activated with 0.1 NIH unit of thrombin/ml for 30 s (*C*). The platelets were lysed and fixed simultaneously. Thus, these images show the organization that the structural elements had in the intact cells. (*AF*) actin filaments; (*Mt*) microtubule coil; (*PL*) peripheral layer; (*AM*) amorphous material. Bars, (*A* and *C*) 1 μm ; (*B*) 0.1 μm .

Results

Isolation of Actin Filaments That Retain the Three-dimensional Organization That They Had in Intact Platelets

The major goal of this study was to determine the relationship between the membrane-bound actin filaments and the rest of the platelet cytoskeleton. Our strategy was to examine the organization of the cytoskeleton in intact platelets, then to identify the component that represented the membrane-bound actin filaments on the basis of its association with GP Ib and its resistance to Ca^{2+} -induced depolymerization in Triton X-100 lysates. The organization of the cytoskeleton in intact platelets can be visualized in cells that are treated with a fixative that includes Triton X-100 (9, 15). The cytoskeleton is composed primarily of filaments that have been well characterized as being actin (9, 15, 22, 33, 37). It has been reported that the actin filaments exist throughout the cytoplasm of unstimulated, discoid platelets and are concentrated in a continuous layer of Triton X-100-insoluble material at the periphery of the cell (*PL* in Fig. 1 *A*), between the microtubule coil and the solubilized plasma membrane (9, 15, 22, 33, 37). High-power images of the peripheral layer (Fig. 1 *B*) showed that while this layer is composed, at least in part, of structures that have the dimensions of actin filaments ($\sim 60\text{-\AA}$ wide), an additional, more amorphous mate-

rial (*AM* in Fig. 1 *B*) made up a considerable portion of the layer and in large part obscured the actin filaments.

When the platelets were activated, many of the actin filaments centralized around the granules (which were thus rendered more resistant to solubilization by the Triton X-100 present in the fixative (Fig. 1 *C*). However, as reported previously by others (15), a layer of material remained at the periphery of the cell (*PL* in Fig. 1 *C*). High-power images of this layer showed that it strongly resembled the peripheral layer from discoid cells (data not shown). While the peripheral layer from discoid cells followed the discoid outline of the intact platelet (Fig. 1 *A*), that from activated cells followed the irregular outline of these cells (Fig. 1 *C*). This suggested that the peripheral layer observed in the extracted cell followed the contours of the plasma membrane in the intact platelet.

To determine which component of the cytoskeletons contained the membrane-associated actin filaments, it was necessary to establish lysis conditions under which the cytoskeletons retained the organization that they had in the intact cells (i.e., that shown in Fig. 1 *A*). Platelets were therefore lysed in a Triton X-100-containing lysis buffer commonly used for the determination of actin filament content and cytoskeletal composition (4, 10–12, 31, 42). This buffer contains EGTA to chelate Ca^{2+} and thus prevents depolymerization of actin filaments (10, 17, 36). The resulting lysates

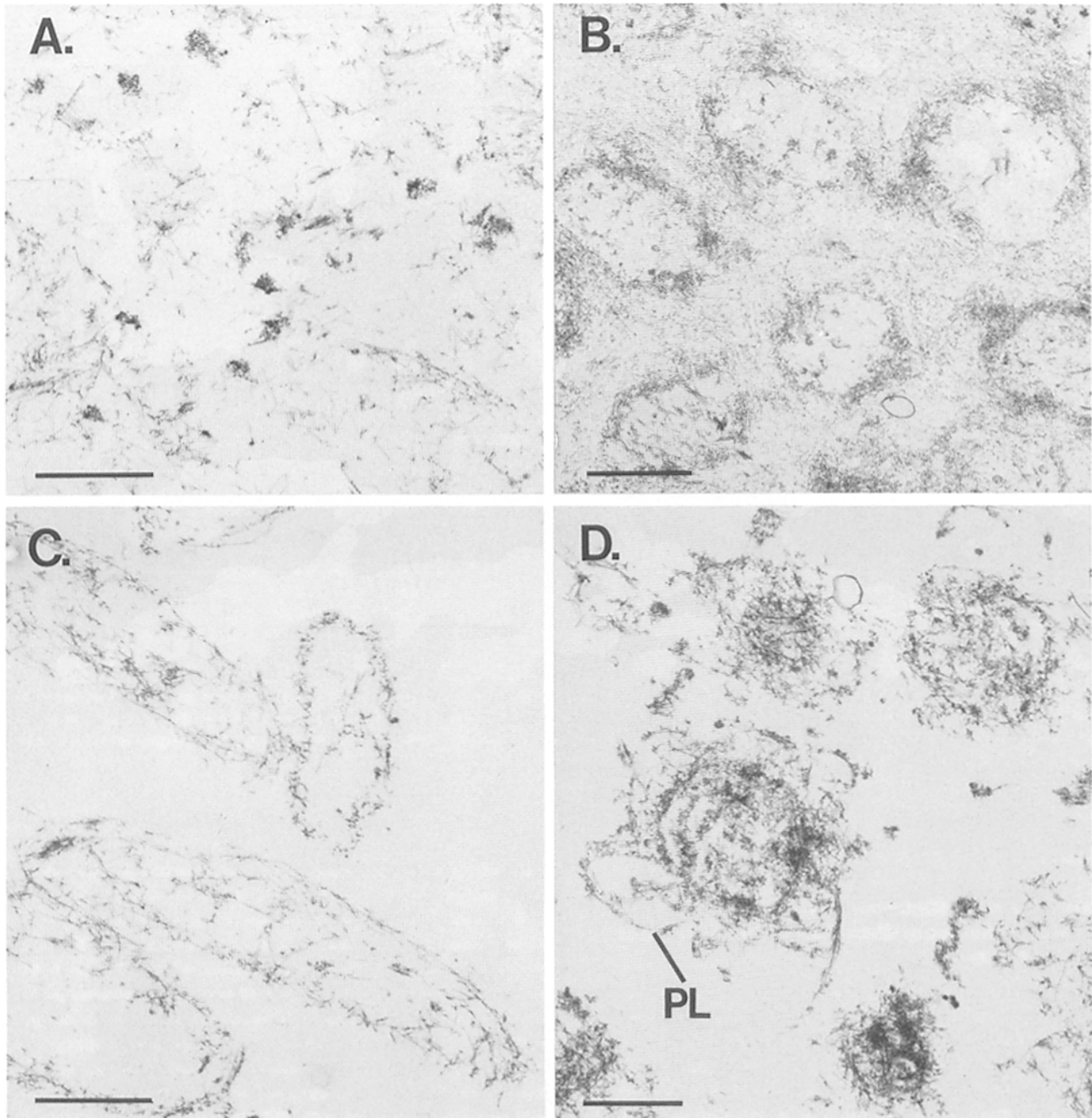


Figure 2. Thin sections of Triton X-100-insoluble residues that were fixed 4 min after solubilization of platelets with detergent. Unstimulated, discoid platelets (*A* and *C*) or platelets that had been activated with 0.1 units of thrombin/ml for 30 s (*B* and *D*) were solubilized by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, either in the absence (*A* and *B*) or presence (*C* and *D*) of protease inhibitors. In the absence of protease inhibitors, the organization of both microtubules and microfilaments was disrupted in the lysis buffer. In the presence of protease inhibitors, microtubules were still depolymerized but the actin filaments appeared to retain much of the organization that they had in the intact cell. (*PL*) peripheral layer. Bars, 1 μm .

were incubated at 4°C for 4 min and fixed, and the Triton X-100-insoluble residues were isolated by centrifugation. Fig. 2 *A* shows that the microtubule coil was solubilized in the lysis buffer. Analysis of the material that resisted solubilization on SDS-polyacrylamide gels showed that it consisted almost entirely of actin filaments (22, 31). However, these filaments did not retain the organization that they had had in the intact platelets. Filaments from discoid cells were recovered in a random array (Fig. 2 *A*). Filaments from activated

platelets were more resistant to disruption but still lost a considerable amount of the organization that they had had in the intact cell (Fig. 2 *B*). While the central filaments were often recovered as structures identifiable as such, the peripheral layer was no longer apparent (cf. Fig. 2 *B* with 1 *C*).

In an attempt to find lysis conditions that favored maintenance of the cytoskeletons in the organization that they had in the intact cell, protease inhibitors were included in the Triton X-100 lysis buffer. Fig. 2, *C* and *D*, shows that in the pres-

ence of PMSF, leupeptin, and benzamidine, the microtubule coil was still solubilized but the rest of the cytoskeleton retained much of its original organization (cf. Fig. 2 C with 1 A and Fig. 2 D with 1 C). The filamentous residues prepared in the presence of protease inhibitors were stable for at least 60 min at 37°C and for at least 16 h at 4°C. They were self-supporting (that is, they retained the outlines of the cells from which they originated) even in the absence of the microtubule coil, which was solubilized in the Triton X-100 lysis buffer (cf. Fig. 2 C with 1 A and Fig. 2 D with 1 C).

The platelet cytoskeleton is often defined as the material that sediments from Triton X-100 lysates at low g forces. We have pointed out, however, that while many of the actin filaments from activated platelets can be sedimented at low g forces, little of the total cytoskeletal material within *unstimulated* platelets sediments from Triton X-100 lysates at these g forces (22). Images such as those shown in Fig. 2 A suggest that this is because the networks of actin filaments that exist in the intact platelet are disrupted in the lysis buffer. Since the images presented in Fig. 2 show that inclusion of protease inhibitors in the lysis buffer decreases post-lysis changes in the organization of the cytoskeleton, we determined the effect of the modified lysis buffer on the sedimentation properties of actin filaments from platelet lysates. As reported previ-

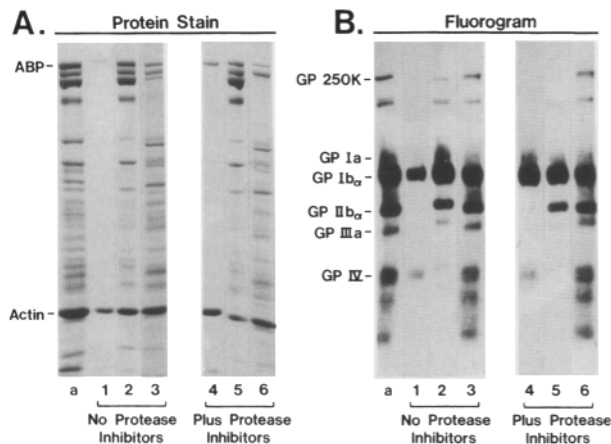


Figure 3. Sodium dodecyl sulfate SDS-polyacrylamide gels showing that the presence of protease inhibitors in the Triton X-100 lysis buffer maintains the platelet cytoskeletons in a form that can be sedimented from Triton lysates at low g forces. Unstimulated, discoid platelets (A) or those that had been partially activated as a result of radiolabeling by the sodium periodate/sodium [³H]borohydride method (B) were solubilized directly into an SDS-containing buffer (lanes a) or were solubilized by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, either in the absence (lanes 1–3) or presence (lanes 4–6) of protease inhibitors. Lysates were centrifuged at 15,600 g for 4 min and the pellets solubilized (lanes 1 and 4). The remaining low-speed supernatants were centrifuged at 100,000 g for 2.5 h and the pellets (lanes 2 and 5) and supernatants (lanes 3 and 6) solubilized. Samples were solubilized into an SDS-containing buffer in the presence of reducing agent and electrophoresed on SDS-polyacrylamide gels containing either 7.5% (A) or 5% (B) polyacrylamide. Proteins were detected by Coomassie Brilliant Blue (A), while ³H-labeled GPs were detected by fluorography (B). (ABP) actin-binding protein; (GP 250K) an unidentified plasma membrane GP of $M_r = 250,000$.

Table I. Effect of Protease Inhibitors on the Sedimentation of Actin-bound Glycoprotein Ib at Low and High g Forces

Inclusion in lysis buffer	GP Ib (% of total in platelets)*	
	Low speed pellet	High speed pellet
	15,600 g, 4 min	100,000 g, 2.5 h
None	8.5 ± 2.4	61.4 ± 8.4
Protease inhibitors	24.9 ± 4.2	41.1 ± 4.2

Platelets that had been partially activated as a result of radiolabeling by the sodium periodate/sodium [³H]borohydride method were solubilized by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, either in the absence or presence of protease inhibitors. Lysates were centrifuged at 15,600 g for 4 min, and the remaining low-speed supernatants were centrifuged for 2.5 h at 100,000 g.

* Values given are mean ± SE from three donors.

ously and shown in Fig. 3 A (lane 1), in the absence of protease inhibitors, few of the actin filaments from unstimulated discoid platelets could be sedimented from Triton X-100 lysates at low g forces. Most of the filaments (>70% of the total) required high g forces to be sedimented (Fig. 3 A, lane 2 and reference 22). In contrast, when protease inhibitors were included in the lysis buffer, 90.0 ± 2.3% (mean ± SE from three donors) of the filaments could be sedimented at low units of gravity (Fig. 3 A, lane 4); only 10.0 ± 2.3% of the filaments required high g forces to be sedimented (Fig. 3 A, lane 5).

We have reported (17) that the membrane-associated actin filaments have different sedimentation properties from the rest of the actin filaments in Triton X-100 lysates; in the absence of protease inhibitors, little of the actin-associated GP Ib was sedimented from Triton X-100 lysates at low g forces even from activated platelets. Again, it appeared possible that this may have resulted from disruption of the cytoskeletons in the lysis buffer. To determine whether the inclusion of protease inhibitors in the lysis buffer maintained the association between membrane-associated actin and other components of the cytoskeleton, we examined the effect of protease inhibitors on the sedimentation properties of GP Ib (the major membrane GP shown previously to be linked to the specific pool of membrane-associated actin [16, 17]). Fig. 3 B and Table I show that in the absence of protease inhibitors, only ~12% of the actin-bound GP Ib could be sedimented at low g forces, but that in the presence of protease inhibitors ~40% of the filament-associated GP Ib was maintained in a form that sedimented at low g forces.

Morphological Demonstration That Platelets Contain a Distinct Pool of Membrane-bound Actin

We conclude that structures such as those shown in Fig. 2, C and D, contain the cytoplasmic actin filaments and at least some of the filaments that we have characterized previously (17) as being a distinct pool of membrane-bound actin. To determine the relationship between these two pools of actin, the Triton X-100-insoluble structures were incubated with affinity-purified antibodies against GP Ib and gold-labeled Protein A. The antibodies labeled the structures, confirming that GP Ib-IX was a component of the cytoskeleton (Fig. 4 A). In general, the antibodies showed little association with

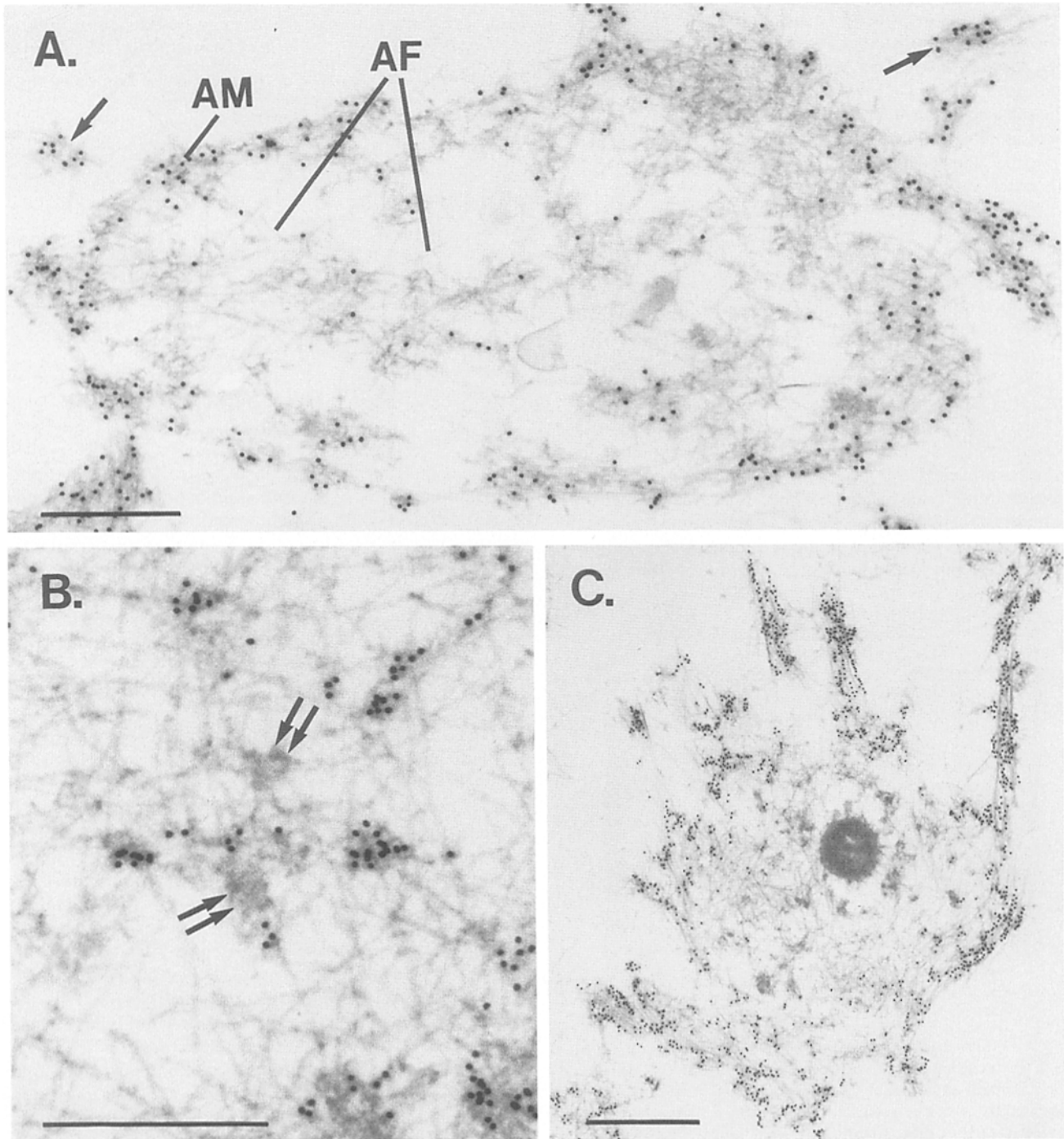
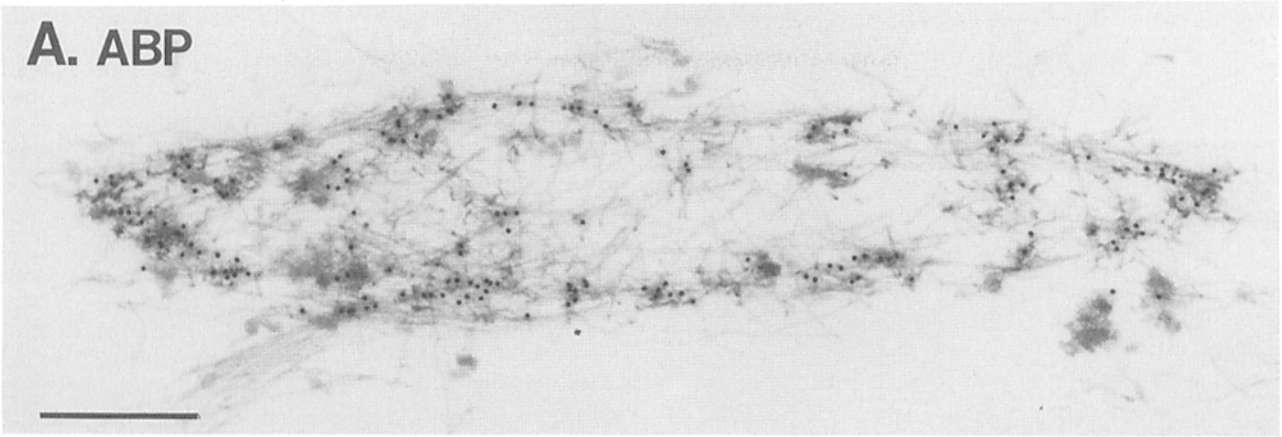


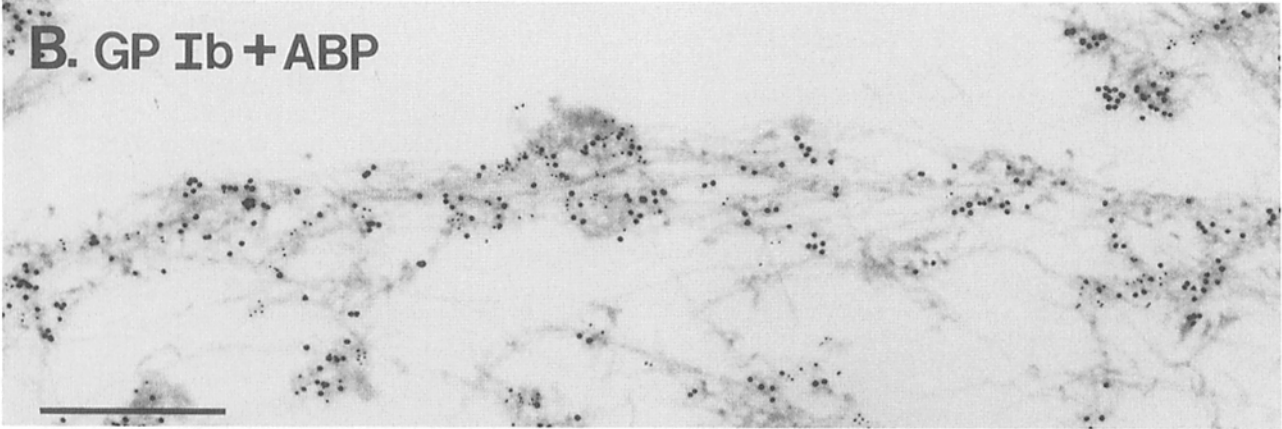
Figure 4. Electron micrographs of the Triton X-100-insoluble residues from platelets showing the presence of amorphous material that contains GP Ib. Unstimulated platelets (*A* and *B*) or platelets that had been stimulated with 0.1 NIH unit of thrombin/ml for 15 s (*C*) were lysed with Triton X-100 in the presence of EGTA and protease inhibitors. Lysates were incubated at 37°C for 1 h with anti-GP Ib and gold-labeled Protein A. Samples were then fixed and prepared for electron microscopy. The distribution of gold particles shows that little GP Ib was associated with visible actin filaments (*AF*). Glycoprotein Ib was associated almost entirely with the dense amorphous material (*AM*) that tended to be located at the periphery of the Triton X-100-insoluble residues. In many images, amorphous material such as that indicated by arrows in *A* appeared to have "peeled off" from the cytoskeletons. The double arrows in *B* indicate amorphous material that had not become labeled with antibodies. Bars, (*A* and *B*) 0.5 μm ; (*C*) 1 μm .

Figure 5. Electron micrographs showing the presence of actin-binding protein (*ABP*) in the peripheral layer of the Triton X-100-insoluble residues from platelets. Unstimulated platelets were lysed with Triton X-100 and incubated at 37°C for 1 h with anti-actin-binding protein (*A*), normal rabbit IgG (*C*), or antibodies against the platelet plasma membrane glycoprotein GP IIb (*D*). The distribution of these antibodies was detected with gold-labeled Protein A, as described in the legend to Fig. 4. The lysate shown in *B* was incubated with both anti-actin-binding protein and anti-GP Ib. Anti-actin-binding protein was directly coupled to gold particles of ~ 15 -nm diameter. Anti-GP Ib was coupled to gold particles of ~ 6 -nm diameter. The distribution of gold particles shows that actin-binding protein was present almost entirely at the periphery of the cell, where it was associated with the same dense amorphous material as GP Ib. Normal rabbit IgG and antibodies against GP IIb had little affinity for any component of the Triton X-100-insoluble structures. Bars, 0.5 μm .

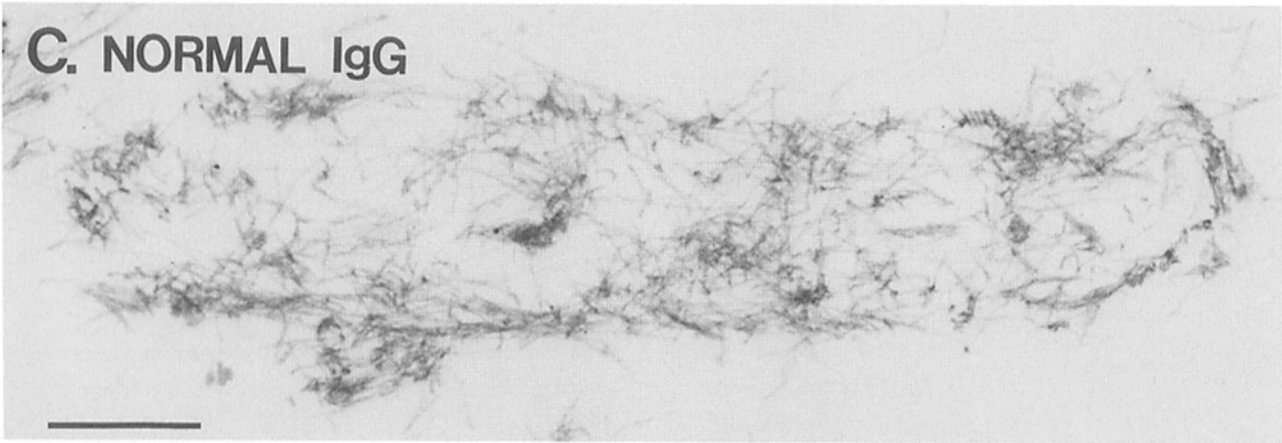
A. ABP



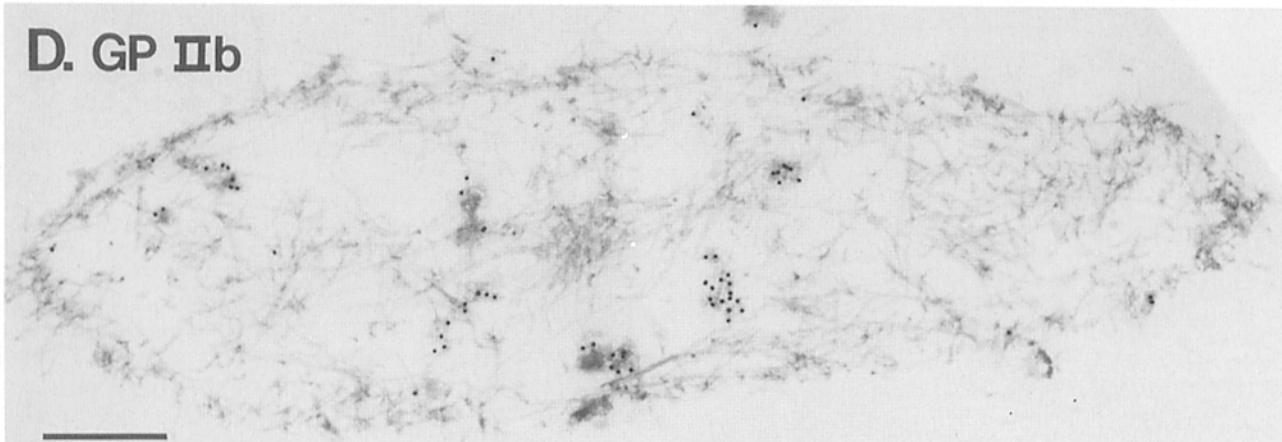
B. GP Ib + ABP



C. NORMAL IgG



D. GP IIb



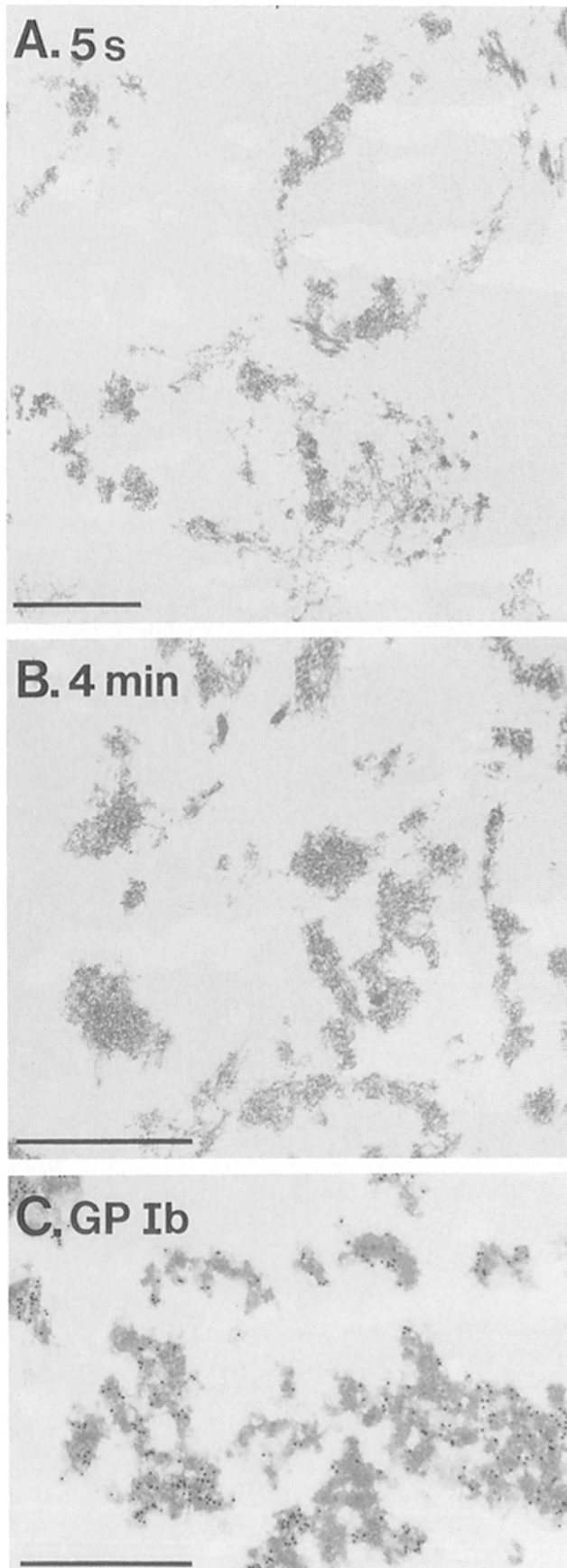


Figure 6. Electron micrographs of the Triton X-100-insoluble material remaining when Ca^{2+} was present in the platelet lysates. Un-

visible actin filaments, but appeared to label dense amorphous material that was concentrated toward the periphery of the cell (Fig. 4, *A* and *C*). This is seen more clearly in high power images such as Fig. 4 *B*. The amorphous material did not result from the presence of antibodies since amorphous material that had not become labeled with antibodies was often observed (e.g., indicated by double arrows in Fig. 4 *B*). Whereas the amorphous layer was relatively continuous in platelets lysed and fixed simultaneously (Fig. 1 *B*), it appeared to be present in patches on the structures that were fixed 1 h after lysis (Fig. 4, *A* and *B*). This suggests that considerable reorganization of the amorphous material still occurred in the lysis buffer even in the presence of protease inhibitors. In support of this idea, amorphous material was often visualized “peeling off” of the cytoskeleton or adjacent to it. Examples of this are indicated by arrows in Fig. 4 *A*. The reorganization of the GP Ib-bound actin is consistent with the observation that while protease inhibitors increased the amount of GP Ib that sedimented with the bulk of the actin filaments at low gravitational forces (Fig. 3 *B* and Table I), considerable amounts of actin-bound GP Ib still remained in the supernatant obtained by centrifugation at low speed.

Antibodies against actin-binding protein, the linkage protein between GP Ib-IX and the membrane-associated actin filaments (16), also labeled the amorphous material; little labeling of visible actin filaments was observed (Fig. 5 *A*). Dual labeling—using GP Ib antibodies that were coupled to gold particles of ~ 6 -nm diameter and actin-binding protein antibodies that were coupled to gold particles of 15-nm diameter—showed that GP Ib-IX and actin-binding protein were components of the same amorphous material (Fig. 5 *B*). Control experiments showed that normal rabbit IgG did not label any component of the Triton X-100-insoluble residues (Fig. 5 *C*). Similarly, antibodies against the plasma membrane glycoprotein GP IIb showed little labeling of the structures (Fig. 5 *D*). The small amount of labeling observed with the latter antibodies is consistent with reports that while GP IIb-IIIa becomes associated with the cytoskeleton in aggregating platelets, only small amounts are recovered in association with the Triton X-100-insoluble residues isolated from suspensions of unstimulated platelets (17, 40, 41).

Biochemical experiments performed by several groups have established that most of the actin filaments present in platelet lysates are depolymerized when Ca^{2+} is present (10, 17, 36). Depolymerization may result, at least in part, from the action of gelsolin, a Ca^{2+} -sensitive protein that severs filaments and thereby induces their depolymerization (50). We have reported previously that the membrane-associated actin filaments differ from the majority of the actin filaments

stimulated platelets were lysed with Triton X-100 in the presence of protease inhibitors but in the absence of a Ca^{2+} chelator. Platelets were fixed 5 s (*A*) or 4 min after lysis (*B*). The Triton X-100-insoluble material in *C* was incubated with anti-GP Ib and gold-labeled with Protein A for 1 h before fixation. Most of the actin filaments in platelets were instantly depolymerized when a Ca^{2+} chelator was absent. The Ca^{2+} -insensitive residues that remained appeared to originate from the periphery of the cell (*A*) but rapidly collapsed in the lysis buffer (*B*). The residues had an amorphous appearance and contained GP Ib (*C*). Bars, 1 μm .

in platelets in that they are resistant to Ca^{2+} -induced depolymerization (17). Therefore, as a further test of the idea that the peripheral layer of amorphous material contains the membrane-associated actin filaments, we compared the effect of Ca^{2+} on visible filaments of the Triton X-100-insoluble structures with its effect on the amorphous material. Fig. 6 *A* shows that essentially all of the actin filaments long enough to be visualized by thin-section electron microscopy were lost when Ca^{2+} was present (cf. Fig. 1 *C* with Fig. 6 *A*). Presumably these filaments represent those that are severed and depolymerized in the presence of Ca^{2+} . Loss of these filaments occurred extremely rapidly, so that within 5 s of lysis the peripheral amorphous material was the primary component of the cytoskeleton that remained (Fig. 6 *A*). While the amorphous material continued to retain the outline of the platelet at 5 s after lysis (Fig. 6 *A*), by 4 min (Fig. 6 *B*) it had lost this organization and appeared to collect into aggregates. This result is consistent with the presence of the amorphous material in a continuous layer in the Triton X-100-insoluble structures from platelets lysed and fixed simultaneously (Fig. 1 *B*), and with its disruption and aggregation into patches in the structures from platelets that were fixed several minutes after lysis (Fig. 4). Biochemical analysis of the amorphous material isolated on SDS-polyacrylamide gels revealed that actin and actin-binding protein were major components and that all of the GP Ib that was bound to actin filaments was present in this amorphous material (17). Immunolabeling confirmed that the amorphous material contained GP Ib (Fig. 6 *C*) and actin-binding protein (data not shown).

Aggregation of the amorphous material was prevented if the highly cationic agent, alcian blue, was included in the Triton X-100 lysis buffer (Fig. 7). By including this agent, we were able to demonstrate that the amorphous layer of material that remained in the presence of Ca^{2+} was a continuous layer at the periphery of the cytoskeleton. Alcian blue stabilized the peripheral layer of amorphous material without affecting the solubilization of phospholipids or other membrane GPs (data not shown). The peripheral layer was also stabilized by the inclusion of phalloidin (Fig. 7 *C*) or ristoctin (Fig. 8 *A*) (an agent thought to bind to negatively charged molecules on the platelet surface [13]) in the lysis buffer. Platelets from patients with Bernard-Soulier syndrome lack GP Ib (one of the major components of the peripheral layer), but contain actin, actin-binding protein, GP Ia, GP IIa, and the *M*, 250,000 GP (26). The peripheral layer remaining when platelets from these patients were lysed with Triton X-100 in the presence of Ca^{2+} showed little tendency to col-

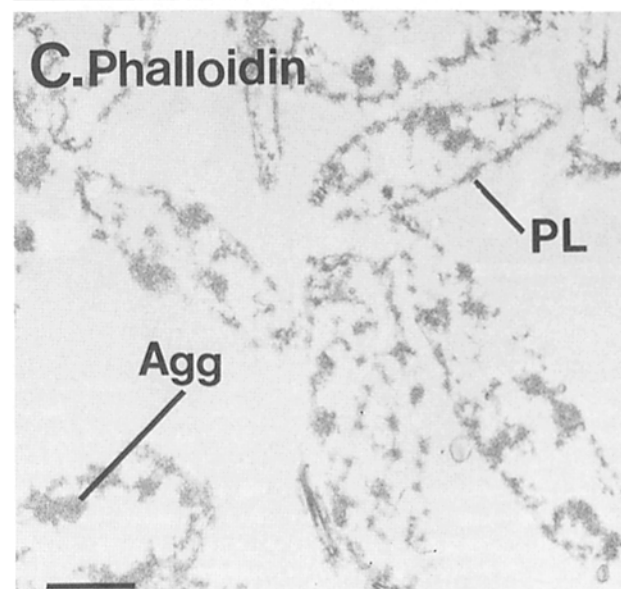
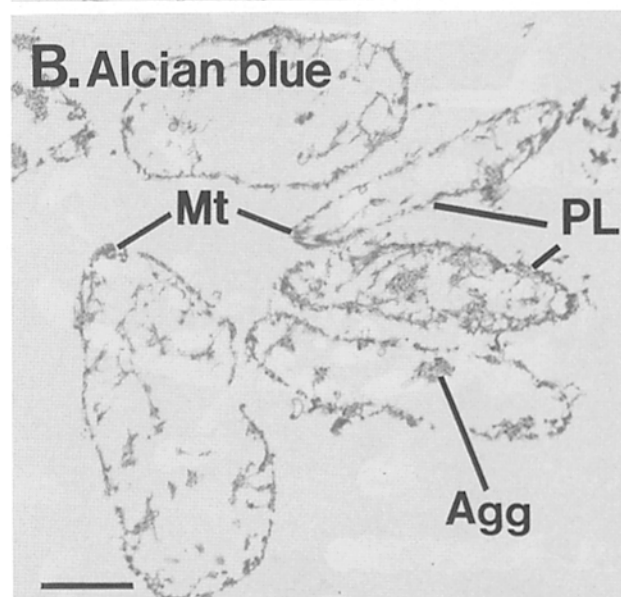
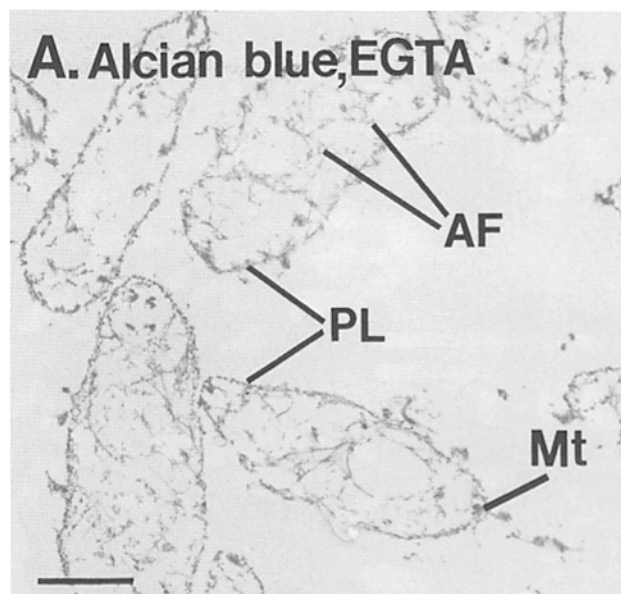


Figure 7. Electron micrographs showing stabilization of the peripheral layer of Ca^{2+} -insensitive material by alcian blue or phalloidin. Platelets were lysed with Triton X-100 in the presence of protease inhibitors and 0.05% alcian blue (*A* and *B*) or 50 μM phalloidin (*C*). When Ca^{2+} was chelated (*A*), cytoplasmic actin filaments (*AF*), the peripheral layer of Triton X-100-insoluble material (*PL*), and the microtubule coil (*Mt*) were retained with the organization that they had in the intact cell. When Ca^{2+} was not chelated (*B* and *C*), most of the visible actin filaments were depolymerized. The peripheral layer, however, still retained the continuous discoid shape of the cell from which it originated. (*Agg*) Insoluble aggregates of undefined proteins. Bars, 1 μm .

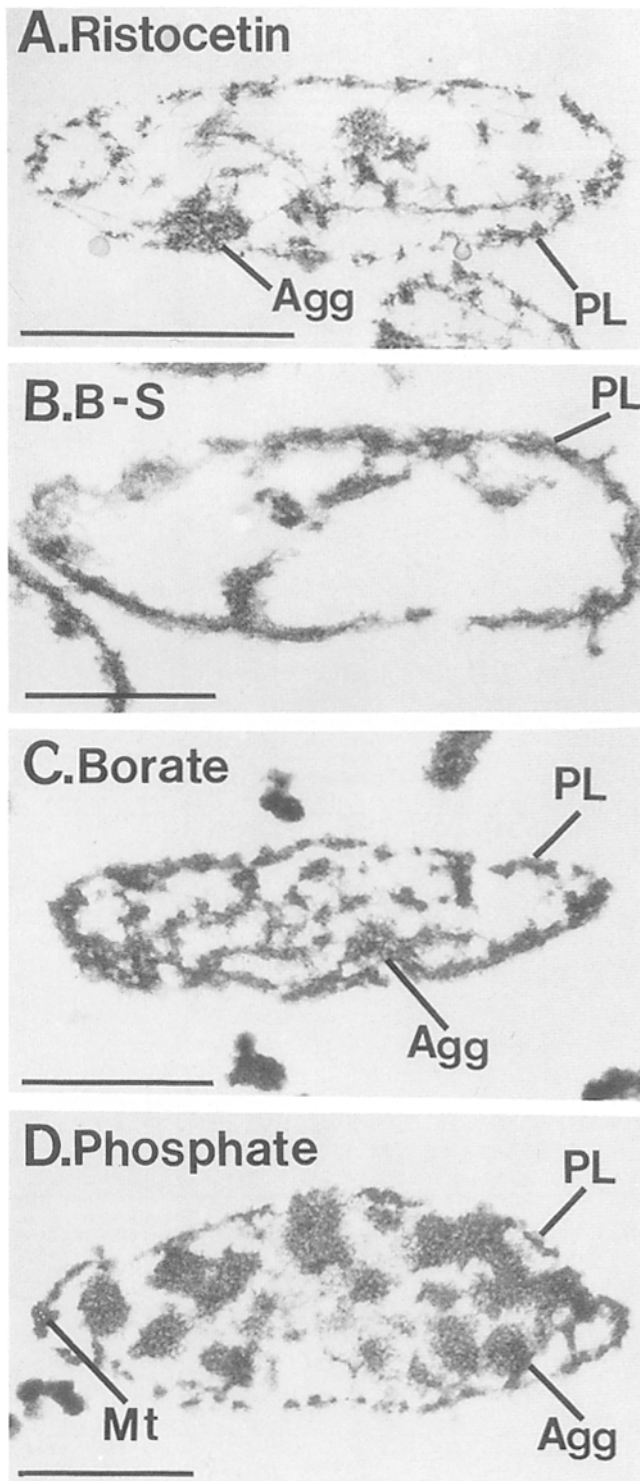


Figure 8. Electron micrographs of the peripheral layer of Ca^{2+} -insensitive material obtained from platelets incubated in a variety of lysis buffers for 4 min before fixation. Each lysis buffer induced depolymerization of most of the cytoplasmic actin filaments yet retained the continuous peripheral layer of actin. In each case, the peripheral layer appeared to maintain the discoid shape of the cell from which it originated. (A) Suspensions of platelets were lysed with an equal volume of 2% Triton X-100, protease inhibitors, 100 mM Tris-HCl, and ristocetin, pH 7.4. (B) Suspensions of Bernard-Soulier (B-S) platelets were lysed with an equal volume of buffer containing 2% Triton X-100, protease inhibitors, and 100 mM Tris-HCl, pH 7.4. (C) Suspensions of platelets were lysed with 9 vol-

umes of buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 58 mM sodium borate, pH 8.0. (D) Suspensions of platelets were lysed with nine volumes of buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 5 mM sodium phosphate, pH 8.0. (PL) peripheral layer; (Agg) insoluble aggregates of undefined proteins; (Mt) microtubule coil. Bars, 1 μm .

lapse in the lysis buffer (Fig. 8 B). Finally, when platelets were lysed under low-ionic-strength conditions, in buffers commonly employed for isolation of the erythrocyte membrane skeleton (35, 51), the visible cytoplasmic actin filaments were largely depolymerized but the amorphous material remained and retained the shape of the unextracted cell (Fig. 8, C and D). Some of the lysis buffers used in these experiments retained aggregates of insoluble material (Agg in Figs. 7 and 8) as well as the peripheral layer of Ca^{2+} -resistant actin. In addition, the microtubule coil was retained in some of the buffers. The microtubule coil was most effectively stabilized in the buffers containing alcian blue (Fig. 7 B) and ristocetin (Fig. 8 A). In the low-ionic-strength borate (Fig. 8 C) and phosphate (Fig. 8 D) buffers, the microtubule coil was retained only in some platelets. The microtubule coil was solubilized when Bernard-Soulier platelets were extracted with the isotonic lysis buffer. Even when the microtubule coil was solubilized, the peripheral layer of material retained the smooth outline of the unstimulated platelet from which it originated (e.g., Fig. 8, B and C). Moreover, the irregular shape characteristic of activated platelets was retained by the peripheral layer of amorphous material when activated platelets were solubilized (data not shown). Thus, the membrane-bound actin is a component of a continuous layer of amorphous material at the periphery of the platelet. This layer follows the contours of the plasma membrane and is self-supporting even in the absence of the microtubule coil and the majority of the cytoplasmic actin filaments.

The GP Ib-IX-bound actin has previously been characterized as being filamentous (on the basis of its sedimentation at high g forces and depolymerization by DNase I [17]). However, the present study shows that GP Ib-IX is bound to material that has an amorphous appearance (i.e., the material shown in Fig. 6 C and at the periphery of the structures shown in Figs. 7 and 8). Few filaments could be visualized in this material. As a test of the idea that the peripheral layer contained actin filaments that were simply too short to visualize by the techniques used in this study, the structures shown in Fig. 8 D were incubated with rhodamine-labeled phalloidin. This molecule, which only binds to actin that is in a filamentous form, bound to the peripheral layer of the Triton X-100-insoluble structures (Fig. 9), supporting the idea that filamentous actin is present.

Previously, we have shown that the Triton X-100-insoluble residues remaining when platelets are lysed in the presence of Ca^{2+} (i.e., lysed as in Fig. 6) contain actin filaments, actin-binding protein, and all of the actin-bound GP Ib-IX complex (17). In an attempt to identify additional, potential components of the peripheral layer of membrane-bound actin, the Triton X-100-insoluble residues remaining under conditions in which the membrane-bound actin was preserved as a continuous layer (i.e., lysed as in Figs. 7 and 8)

umes of buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 58 mM sodium borate, pH 8.0. (D) Suspensions of platelets were lysed with nine volumes of buffer containing 1% Triton X-100, protease inhibitors, 1 mM calcium chloride, and 5 mM sodium phosphate, pH 8.0. (PL) peripheral layer; (Agg) insoluble aggregates of undefined proteins; (Mt) microtubule coil. Bars, 1 μm .

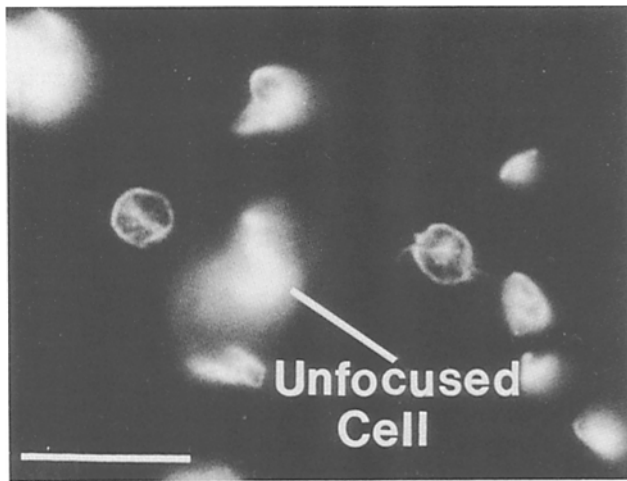


Figure 9. Immunofluorescence image of the Triton X-100-insoluble residues obtained when platelets lysed with low-ionic-strength phosphate buffer that contained Ca^{2+} and protease inhibitors were incubated with rhodamine-labeled phalloidin. Although the peripheral layer of Triton X-100-insoluble material that outlined the shape of the platelet had an amorphous appearance when viewed by thin-section electron microscopy (Figs. 5, 6, and 7), it bound phalloidin, indicating the presence of actin filaments. Bar, 100 μm .

were isolated by centrifugation and analyzed on SDS-polyacrylamide gels (Fig. 10). Some of the proteins that sedimented were identified as tropomyosin, gelsolin, and α -actinin (on the basis of their isoelectric points and molecular weights), fibrinogen and thrombospondin (on the basis of their molecular weights), and P235 (on the basis of its reactivity with anti-P235 on Western blots). Tubulin (identified on Western blots) was present under those conditions in which intact microtubules were observed morphologically.

Discussion

We have shown previously that in Triton X-100 lysates some of the actin filaments in platelets are associated with plasma membrane GPs (primarily the GP Ib-IX complex, GP Ia, GP IIa, and an unidentified GP of M_r 250,000) (16, 17). These membrane-associated filaments have sedimentation properties different from those of the rest of the actin filaments in Triton X-100 lysates. Further, these filaments differ from the rest of the actin filaments in that they are resistant to the depolymerization that is induced when Ca^{2+} is present. The present report shows that this membrane-associated actin is a component of a submembranous lining that is linked to membrane GPs and is distinct from the cytoplasmic actin filaments. In addition, our study suggests that the submembranous structure may function as a skeleton, determining the contours of the plasma membrane.

Many of the actin filaments in unstimulated platelets are concentrated close to the periphery of the cell, where they can be visualized as filaments up to 1- μm long (9). The present report shows that most of these filaments were rapidly depolymerized when Ca^{2+} was present in the lysates; they therefore represent the filaments that we characterized as the cytoplasmic actin filaments involved in the contractile responses of the platelet (17). This interpretation is supported

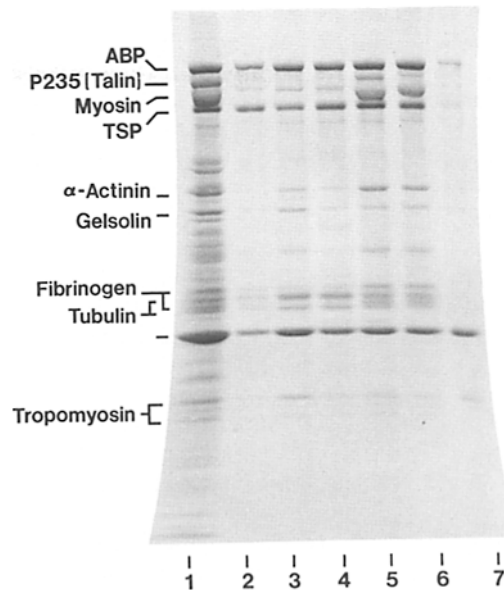


Figure 10. SDS-polyacrylamide gels of whole platelets and the Triton X-100-insoluble material isolated from platelets lysed in various lysis buffers. Unstimulated platelets were solubilized directly into an SDS-containing buffer (lane 1) or were lysed in a Triton X-100-containing buffer. Lysates were incubated at 4°C for 4 min, and the Triton X-100-insoluble structures were isolated by centrifugation at 15,600 g for 4 min. All samples were solubilized in an SDS-containing buffer in the presence of reducing agent and electrophoresed on SDS-polyacrylamide gels containing a 5–20% gradient of polyacrylamide. (Lane 2) Platelets were lysed in the isotonic buffer containing 2% Triton X-100, protease inhibitors, and 100 mM Tris-HCl, pH 7.4 (i.e., as in Fig. 6 B). (Lane 3) Platelets were lysed in the isotonic buffer, but in the presence of alcian blue (i.e., as in Fig. 7 B). (Lane 4) Platelets were lysed in the isotonic buffer, but in the presence of ristocetin (i.e., as in Fig. 8 A). (Lane 5) Platelets lysed in a low ionic strength phosphate buffer (i.e., as in Fig. 8 D). (Lane 6) Platelets lysed in a low ionic strength borate buffer (i.e., as in Fig. 8 C). (Lane 7) Bernard-Soulier platelets lysed in the buffer containing 2% Triton X-100, protease inhibitors, and 100 mM Tris-HCl, pH 7.4 (i.e., as in Fig. 8 B). (ABP) actin-binding protein; (TSP) thrombospondin; (P235) platelet polypeptide of M_r = 235,000 that appears to be similar to talin (39).

by the tendency of the filaments to centralize around the granules during platelet activation.

An additional layer of a more amorphous material was observed at the periphery of the cytoskeletons. Other researchers have reported that the peripheral layer of platelet cytoskeletons is composed, in part, of an amorphous material (15, 33) that is closely adjacent to the lipid bilayer (15), but this material has not been described in detail. In the present study, the amorphous material was visualized as an apparently continuous layer in platelets that were simultaneously lysed with Triton X-100 and fixed. The material was distinct from the visible actin filaments of the cytoskeleton, and several lines of evidence suggest that it contains the pool of actin filaments that we previously identified in Triton X-100 lysates as being membrane-associated. First, the amorphous material became labeled with antibodies against GP Ib and actin-binding protein, whereas the visible actin filaments showed little labeling. Second, like the membrane-associated actin filaments described previously (17), the amorphous material

at the periphery remained insoluble in Triton X-100 under conditions in which most of the actin filaments were depolymerized. Finally, analysis of the amorphous material on SDS-polyacrylamide gels showed that it was composed primarily of actin and actin-binding protein and contained 100% of the actin-bound GP Ib-IX complex. Actin, actin-binding protein, and the GP Ib-IX complex are the proteins that we identified previously as the major components of the actin-membrane linkage mechanism, based on their co-sedimentation at high g forces, their co-isolation on sucrose gradients of platelet lysates, and their status as the major components of the Triton X-100-insoluble residues obtained by extraction of isolated platelet membrane vesicles (17).

The amorphous layer of Triton X-100-insoluble material clearly is a continuous layer that follows the contours of the platelet from which it originates. Thus, it appears to line the plasma membrane. Since this layer is attached to the plasma membrane, it seems likely that it would stabilize the membrane, preventing the membrane from fragmenting. Because the amorphous layer retained the original contours of the platelet under a variety of lysis conditions in which the lipid bilayer, the microtubule coil, and the majority of the cytoplasmic actin were solubilized, this layer is probably a self-supporting structure. We suggest, therefore, that the amorphous layer of Triton X-100-insoluble material at the periphery of the platelet cytoskeleton represents a membrane skeleton. Like the membrane skeleton of the RBC (5), the platelet membrane skeleton may provide a skeletal framework that contributes to the determination of the shape of the platelet. This is not to argue that the microtubules or the cytoplasmic actin filaments do not also participate in this process.

The platelet membrane skeleton shows several other similarities to the erythrocyte membrane skeleton. Both skeletons are unstable in isotonic buffer but remain intact in low-ionic-strength buffers (45). Both skeletons contain actin that is presumed to be in a filamentous form because it is insoluble in Triton X-100, depolymerized by DNase I, and binds phalloidin. However, in both cases, few actin filaments are visualized by thin-section electron microscopy. Recently, the RBC membrane skeleton has been studied by negative-stain techniques (45). The resolution achieved with this method has allowed the visualization of actin filaments that are only ~33-nm long. Thus, the actin filaments in the RBC membrane skeleton are much shorter than an individual spectrin molecule (~200 nm [5]) or an actin-binding protein molecule (~150 nm [23, 29]), and their smaller size would preclude their visualization by the techniques used in the present study. It is likely that the platelet membrane skeleton comprises short actin filaments comparable in length to those of the erythrocyte membrane skeleton. In the RBC, the short actin filaments are cross-linked by spectrin, which also serves to link the skeleton to plasma membrane GPs (5). A major difference between the platelet and the RBC membrane skeletons is that, in platelets, spectrin is a very minor component of the skeleton (25) and actin-binding protein is a major component. Actin-binding protein has a morphology similar to that of spectrin and, like spectrin, exists as a head-to-head dimer with free tails that bind to actin filaments (29). Furthermore, like spectrin, actin-binding protein associates with specific plasma membrane GPs (16). Thus, it appears

likely that actin-binding protein may function in platelets much like spectrin in erythrocytes.

One concern about biochemical experiments that detect associations by their existence in lysed cells is that association may have occurred in the lysis buffer. The immunocytochemical experiments in this study show that GP Ib and actin-binding protein are associated primarily with the amorphous material that exists at the periphery of the cytoskeletons of both unstimulated and thrombin-activated platelets, providing strong evidence that the interactions among GP Ib, actin-binding protein, and actin exist in intact cells. However, the morphological images show that insoluble aggregates of material, apparently unrelated to the cytoskeletons, are retained to varying degrees in different lysis buffers. These images emphasize, therefore, that insolubility of proteins in Triton X-100 does not necessarily bear any relationship to cytoskeleton association. Clearly, a combined morphological and biochemical approach must be used before a protein can be conclusively shown to be a component of the cytoskeleton. Because noncytoskeletal proteins were present in the Triton X-100-insoluble residues, we were not able to identify additional components of the platelet membrane skeleton unequivocally. Although potential components were identified (e.g., tropomyosin, gelsolin, α -actinin), immunocytochemical studies will be necessary to determine whether any of these proteins are selectively associated with the membrane skeleton.

The images presented in this study emphasize that associations can also be disrupted in cell lysates. The platelet cytoskeleton was shown to be disrupted in the lysis buffers most commonly used for its isolation. Thus, reports that proteins such as P235 (14), vinculin (43), caldesmon (42), or α -actinin (42) are not associated with the cytoskeleton of unstimulated platelets may be based on experiments in which there is lysis-induced dissociation of these proteins from actin filaments. Further, the networks of actin filaments in unstimulated platelets are disrupted more readily than are those from activated platelets. It appears likely, therefore, that activation-induced changes in components of the cytoskeletons that have been measured as an increased sedimentation of proteins at low g forces (4, 11, 31, 42) might be a direct result of the increased resistance of cytoskeletons from activated platelets to post-lysis disruption rather than a result of a true change in protein associations in the intact cell.

The present study suggests that platelets contain both cytoplasmic actin filaments and a membrane skeleton. There have been reports of membrane skeletons in other cell types. For example, submembranous structures that show morphological similarities to the membrane skeleton of the RBC have been visualized as a lining to the lipid bilayer in several cell types (30). In addition, Triton X-100-insoluble residues that contain plasma membrane proteins and retain the shape of the unextracted cells have been isolated (2, 3, 6, 32). Although the protein composition of such structures has not been described in detail, the observation that actin-binding protein is present, often in a submembranous location, in many other cell types (8, 47, 49) suggests that other cells may contain a membrane skeleton similar to that in the platelet.

The studies described here suggest that the platelet membrane skeleton stabilizes the plasma membrane and plays a

role in determining its contours. It is conceivable that, as in the RBC, such a skeleton could also regulate the properties of the membrane GPs to which it is attached. Preliminary evidence suggests that the platelet membrane skeleton may regulate the lateral distribution of GP Ib (20). Further, there is evidence that linkage of the GP Ib-IX complex to the membrane skeleton regulates the ability of GP Ib to bind von Willebrand factor (21). Finally, it appears possible that the membrane skeleton is involved in transmembrane signaling. In support of this idea, receptors for the agonists thrombin (GP Ib [28]) and collagen (thought to be GP Ia [38, 44]) are both transmembrane proteins that are associated with the membrane skeleton (16, 17). It is of interest in this regard that several enzymatic activities have been shown to be associated with the components of the erythrocyte membrane skeleton (27). The availability of methods for isolating and visualizing the intact platelet membrane skeleton should permit a detailed study of the potential role of the skeleton in these functions.

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