Actin Filament Content and Organization in Unstimulated Platelets

JOAN E. B. FOX, JANET K. BOYLES, CLIFFORD C. REYNOLDS, and DAVID R. PHILLIPS

The Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute and Department of Pathology, University of California, San Francisco, San Francisco, California 94140

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ABSTRACT The extent of actin polymerization in unstimulated, discoid platelets was measured by DNase I inhibition assay in Triton X-100 lysates of platelets washed at 37°C by gel filtration, or in Triton X-100 lysates of platelets washed at ambient temperatures by centrifugation in the presence of prostacyclin. About 40% of the actin in the discoid platelets obtained by either method existed as filaments. These filaments could be visualized by electron microscopy of thin sections. Similar results were obtained when the actin filament content of discoid platelets was measured by sedimentation of filaments from Triton X-100 lysates at high *g* forces (145,000 *g* for 45 min). However, few of these filaments sedimented at the lower *g* forces often used to isolate networks of actin filaments from cell extracts. These results indicate that actin filaments in discoid cells are not highly crosslinked. Platelets isolated by centrifugation in the absence of prostacyclin were not discoid, but were instead irregular with one or more pseudopodia. These platelets also contained ~40–50% of their actin in a filamentous form; many of these filaments sedimented at low *g* forces, however, indicating that they were organized into networks. The discoid shape of these centrifuged platelets could be restored by incubating them for 1–3 h at 37°C, which resulted in the reversal of filament organization. High *g* forces were then required for the sedimentation of the actin. Approximately 80–90% of the actin in platelets washed at 4°C was filamentous; this high actin filament content could be attributed to actin polymerization during the preparation of the platelets at low temperatures. These studies show that platelet activation involves mechanisms for the structural reorganization of existing filaments, in addition to those previously described for mediating actin polymerization.

Actin is a major protein in most nonmuscle cells and is thought to be required for such diverse functions as cell division, motility, secretion, and phagocytosis (1). Actin within cells exists in two main forms, a monomeric, globular form (G-actin), which is often associated with profilin (2), and a filamentous form known as F-actin (1, 3). In that the functional form of actin within cells appears to be F-actin, one question that arises is: Are new actin filaments formed by the polymerization of G-actin at sites where filaments are required, or are preexisting filaments shuttled to these sites from other locations?

The observation of Bray and Thomas (3), that considerable actin is not sedimented from nonmuscle cells lysed with nonionic detergent whereas filamentous actin is sedimented, has led to the development of methods to measure the filament content of cells (4–6). In many cells, however, it is difficult to detect changes in filament content with these methods, possibly because changes in the filament content within localized regions of cells have little effect on total filament content. One exception, however, is in platelets. Platelets contain a large amount of actin (~15–20% of the total platelet protein is actin) and respond rapidly to stimulation by changing shape, secreting their granule contents, and retracting clots, processes that may require F-actin-associated contractile activity (7). Because these responses can be induced rapidly in purified cell preparations, platelets have provided an ideal system in which to investigate whether new actin filaments are formed during stimulation-induced responses.

The actin filament content of activated platelets has been studied in several laboratories, all of which have found that 70–80% of the total actin within activated platelets is filamen-

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tous (5, 6, 8, 9). Furthermore, it has been demonstrated that these filaments are associated with other proteins, including myosin and actin-binding protein, and thay they exist in structures that maintain their shape after platelet lysis (5). The filament content of unstimulated platelets, however, remains controversial. Rosenberg et al. (10) reported that almost all of the actin in unstimulated platelets is filamentous. Nachmias (11), on the other hand, reported that no filaments could be observed morphologically in individual unstimulated platelets, although a few short filaments were isolated from populations of platelets that were gel filtered in the presence of tetracaine (12). Others have found intermediate values (4–6, 8, 9, 13), and have shown that the filaments in unstimulated platelets are less structured than those in activated platelets (5).

Knowledge of the changes in actin filament content during activation is essential to understanding how the filamentous cytoskeleton of platelets forms during activation. This knowledge could also assist in the understanding of stimulus-induced, cytoskeletal changes within other cells. If actin in unstimulated platelets exists primarily in filaments, then activation need only involve a rearrangement of preexisting filaments to form the cytoskeletal structure of the activated platelet. If less F-actin is present in unstimulated platelets than in activated ones, activation must involve mechanisms to polymerize actin in addition to those required for its organization into a cytoskeletal structure. To address this question, we reevaluated the filament content of unstimulated, discoid platelets to provide the necessary reference point for mechanistic considerations. In addition, we investigated the reasons for the discrepancies in the reported filament content of unstimulated platelets.

MATERIALS AND METHODS

Preparation of Discoid Platelet Suspensions: Venous blood from healthy adult donors was collected into acid citrate/dextrose, and the erythrocytes were removed by centrifugation as previously described (14). Platelets were isolated from platelet-rich plasma by two different methods. In the first method, previously described by Nachmias (11), platelet-rich plasma was filtered at 37°C through Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ; 3 ml through a column of 1.6 × 30 cm), which was preequilibrated with 136 mM sodium chloride, 2 mM potassium chloride, 10 mM sodium carbonate, 0.5 mM sodium phosphate, and 4 mM magnesium chloride, pH 7.4. Fractions (1.5 ml) were collected at a flow rate of 12 ml/h. Peak fractions ($2–4 \times 10^8$ platelets/ml) were pooled and incubated with added glucose (4.4 mM) at 37°C for at least 30 min before the actin filament content was measured. In many experiments, as indicated in the text, prostacyclin (PGI_2),[1] supplied by Dr. J. Pike of the Upjohn Co. (Kalamazoo, MI), was included in the anticoagulant (50 ng/ml) and in the column buffer (10 ng/ml).

In the second method, platelets were obtained from blood that had been drawn into anticoagulant that contained 50 ng/ml of PGI_2. They were separated from plasma by centrifugation at 730 *g* for 20 min and washed by resuspension in the original plasma volume of buffers that were preequilibrated to 37°C and contained 10 ng/ml of PGI_2. The first two washes included 120 mM sodium chloride, 13 mM trisodium citrate, and 30 mM dextrose, pH 7.0. The third wash was with 154 mM sodium chloride, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4. After each wash, platelets were isolated by centrifugation for 10 min at ambient temperatures at 2,000 *g*. The washed platelets were resuspended in a buffer containing 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, and 1 mM EDTA, pH 7.4, at a concentration of $1–3 \times 10^8$ platelets/ml, and were incubated for at least 30 min before the actin filament content was measured.

Other Methods of Preparing Washed Platelet Suspensions: Platelet suspensions were also prepared by two other methods that have been described previously by others. In one method, described by Phillips and Agin (14), platelets were isolated from plasma by centrifugation at 730 *g* for 20 min, washed two times by resuspension and centrifugation in 120 mM sodium chloride, 13 mM trisodium citrate, and 30 mM dextrose, pH 7.0, and once by resuspension and centrifugation in 154 mM sodium chloride, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4. Platelets were resuspended in 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, and 1 mM EDTA, pH 7.4, at a concentration of $1–3 \times 10^8$ platelets/ml. All buffers and centrifugation steps were at ambient temperatures. In some preparations, cytochalasin D (Sigma Chemical Co., St. Louis, MO) was added to the platelet-rich plasma and to the washing buffers (final concentration 2.5×10^{-5} M cytochalasin, 0.5% ethanol).

In other experiments, platelets were isolated as described by Rosenberg et al. (10). This method also involved centrifugation, but all steps were performed at 4°C. Platelets were isolated from platelet-rich plasma by centrifugation at 1,000 *g* for 15 min at 4°C. These platelets were washed two times in ~40 times the pellet volume of a buffer containing 126 mM sodium chloride, 5 mM potassium chloride, 0.3 mM EDTA, and 10 mM sodium phosphate, pH 7.4, and finally resuspended in about two times the pellet volume of the same buffer. All platelet counts were determined using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

Determination of Actin Filament Content: Platelets in suspension were lysed by the addition of an equal volume of buffer containing 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, at 22 ± 2°C (5). The actin filament content was determined either by DNase I inhibition assay as described previously (6), or by sedimentation of Triton X-100-insoluble actin filaments at 145,000 *g* for 45 min at 4°C in a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). Sedimented material was washed once in a 1:1 mixture of platelet suspension buffer and lysis buffer and then solubilized in SDS (5). Solubilized material was electrophoresed through slab gels according to the method of Laemmli (15) using a 5–20% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel (5). Protein was stained with Coomassie Brilliant Blue, and the actin content was determined by densitometry of the M_r 43,000 polypeptide (which we have previously shown to be actin [16]), using a Beckman DU8 densitometer (Beckman Instruments, Inc.). The amount of sedimented actin was expressed as a percentage of that actin in total platelet extracts, which were solubilized in SDS, electrophoresed, and quantitated in the same way as the sedimented material.

Analysis of Triton X-100-Insoluble Filamentous Material: Platelets in suspension were lysed by the addition of an equal volume of buffer containing 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, at 22 ± 2°C (5). Triton X-100-insoluble material was isolated immediately by sedimentation at 15,600 *g* for 4 min in an Eppendorf microfuge (Brinkmann Instruments Inc., Westbury, NY). The sedimented material was washed once in a 1:1 mixture of platelet suspension buffer and lysis buffer. In some experiments, the platelet suspension was lysed by the addition of 10 vol of an ice-cold buffer containing 1% Triton X-100, 40 mM potassium chloride, 10 mM imidazole-chloride, 10 mM EGTA, and 2 mM sodium azide, pH 7.0 (10). These lysates were left at 4°C for 12 min, and the material was then isolated by centrifugation at 15,600 *g* for 2 min at 4°C. Triton X-100-insoluble material was solubilized in SDS, and the polypeptide composition was determined by SDS PAGE.

Preparation of Samples for Electron Microscopy: For examination of platelet morphology, suspensions were fixed for 30–90 min at ambient temperatures in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), 50 mM lysine (Sigma Chemical Co.), 5 mM EGTA, and 50 mM sodium cacodylate, pH 7.4. Cells were sedimented in an Eppendorf microfuge, and the fixation was continued for 30 min to 3 h in a solution of 2% glutaraldehyde and 50 mM sodium cacodylate, pH 7.4. The cells were washed once, exposed to cold 1% osmium tetroxide in uranyl acetate buffer, pH 7.6, for 15 min, rinsed in cold, distilled water, and stained overnight in 2% uranyl acetate in water. Fixed material was dehydrated in acetone and embedded in Epox 812 (Ernest F. Fullam, Inc., Schenectady, NY). Sections were cut and stained with Reynold's lead citrate and uranyl acetate and photographed in a JEOL 100 CX II microscope (JEOL USA, Peabody, MA). For examination of actin filaments in lysed preparations, suspensions were lysed and fixed simultaneously at ambient temperatures with a solution containing 1% Triton X-100, 2% glutaraldehyde, 5 mM EGTA, 50 mM lysine, and 50 mM sodium cacodylate, pH 7.4 (17). Filaments were sedimented in an Eppendorf microfuge, and the fixation was continued as described above.

RESULTS

Actin Filament Content of Unstimulated, Discoid Platelets

The actin filament content of platelets can be measured by DNase I inhibition assay (6) or by high-speed centrifugation

[1] *Abbreviations used in this paper:* PGI_2, prostacyclin.

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(5) after lysis of the platelets with Triton X-100. Since plasma contains a factor that can rapidly depolymerize actin filaments in Triton X-100 lysates (8), these measurements must be made on platelets that have been washed free of plasma proteins. Although platelets can be washed by several methods, the procedures used can activate the platelets to varying degrees.

In this study, two methods were used to isolate unstimulated platelets to free them of contaminating plasma protein. In the first method, platelet-rich plasma was passed through a column of Sepharose 2B at 37°C (11). In the second method, platelets were centrifuged from plasma at ambient temperatures and resuspended in a series of washing buffers (14), which were preequilibrated to 37°C and contained PGI_2 (10 ng/ml) to prevent the activation of the platelets during centrifugation.

Electron microscopy was used to confirm that the platelets were not activated. Unstimulated platelets are discoid and are clearly identified as such in electron microscope images. Activated platelets lose this shape and produce numerous blebs and filopodia (18). Most of the platelets isolated by both of these procedures were discoid shaped; we found, as did Nachmias (11), that the nondiscoid platelets present in either preparation could be converted to discoid platelets by incubating the suspensions at 37°C with glucose. Gel-filtered platelets were normally restored to the discoid shape within 30 min, while centrifuged platelets required ~90 min of incubation. Fig. 1 shows typical preparations of discoid platelets isolated either by gel filtration (Fig. 1 *a*) or by centrifugation (Fig. 1 *b*). We found that the inclusion of PGI_2 was absolutely essential for the recovery of significant numbers of discoid cells by the centrifugation method. Although discoid cells could usually be prepared by gel filtration in the absence of PGI_2, we routinely included PGI_2 in the preparation when the actin filament content was to be measured.

The actin filament content of discoid platelets obtained by the two methods was measured in Triton X-100 lysates. Table I shows that platelets prepared by either method contained ~40% of their actin in a filamentous form, as measured either by the DNase I inhibition assay (6) or by sedimentation of filaments at high *g* forces (5). These filaments did not result from the extension of the few filopodia present in these platelet preparations (Fig. 1); a similar filament content existed in platelet preparations that contained many more filopodia as a result of their isolation in the absence of PGI_2 (see below). When the filament content of discoid platelets was measured by sedimentation at the lower *g* forces used previously in many laboratories (~12,000 *g*) (2, 5, 10, 12, 19), much lower values were obtained (Table I), indicating that few of the filaments present in lysates of discoid cells sedimented at these *g* forces.

To visualize the actin filaments in the discoid platelets, we lysed cells with Triton X-100 and simultaneously fixed in the presence of lysine, which prevents damage of actin filaments by osmium tetroxide (17). This method allowed for the removal of detergent-soluble cellular components. The remaining material consisted of scattered remnants of granules in an extensive network of long actin filaments (50–70Å) and microtubules (200–230Å) (Fig. 2). This Triton X-100 insoluble material maintained the discoid shape of the unextracted cells (Fig. 2); this observation, together with the finding that every Triton-insoluble structure examined contained similar networks of filaments, provided further evidence for the conclusion that the actin filaments measured biochemically in the platelet suspensions were distributed throughout the platelet population and were not present only in the few platelets that had extended filopodia.

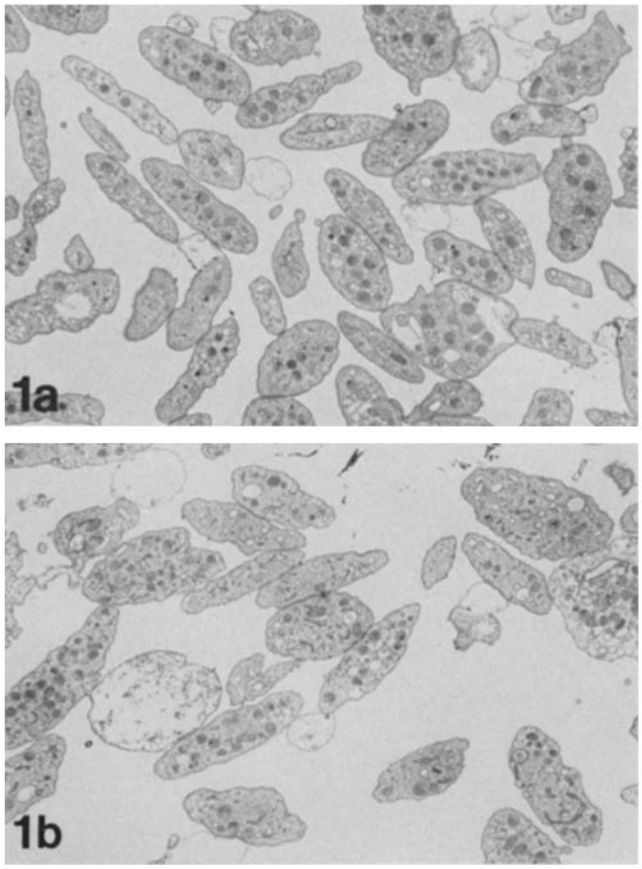

FIGURE 1 Electron micrographs of washed platelet suspensions. Platelets were washed (*a*) by gel filtration at 37°C or (*b*) by centrifugation at ambient temperatures in the presence of PGI_2 (final concentration of 50 ng/ml in the anticoagulant and 10 ng/ml in all the buffers). Gel-filtered platelets were incubated at 37°C with added glucose for 30 min, while centrifuged platelets were incubated at 37°C for 90 min before preparation for electron microscopy. × 5,000.

TABLE I

Actin Filament Content of Discoid Platelets

Method of platelet isolation	Actin filament content		
	Measured by DNase I inhibition	Measured by high-speed centrifugation*	Measured by low-speed centrifugation‡
		% *of total*	
Gel filtration	37.9 ± 1.5§	41.9 ± 4.4§	6.8 ± 2.5§
Centrifugation	44.0 ± 3.3	42.1 ± 3.9	17.7 ± 2.6

Discoid platelets were isolated in the presence of PGI_2, either by gel filtration or by centrifugation as described in the text.
* 145,000 *g* for 45 min.
‡ 15,600 *g* for 4 min.
§ Mean ± SE from three donors.

Filament Content of Platelets Isolated by Other Methods

To investigate the reason for the discrepancies in the previously reported values for the actin filament content of

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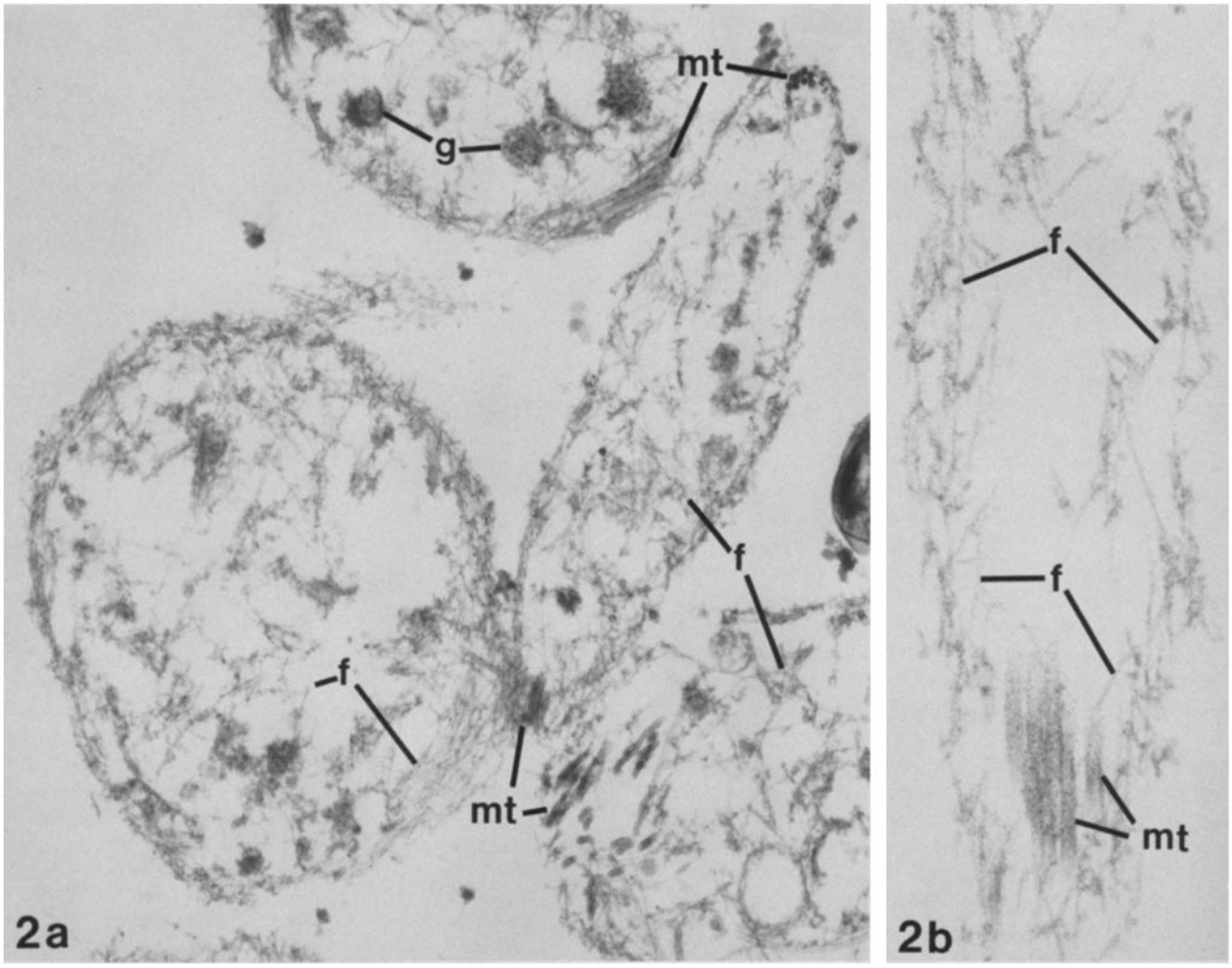

FIGURE 2 Electron micrograph of actin filaments in Triton X-100-solubilized, discoid platelets. Discoid platelets were obtained by gel filtration at 37°C as described by Nachmias (11). Platelets were incubated with added glucose for 30 min, then fixed simultaneously with lysis by the method of Boyles (17). *g*, granules; *f*, actin filaments; *mt*, microtubules. × 31,000. *Inset* shows detailed structure of filaments. × 108,000.

platelets, we isolated platelet suspensions by two other methods described in the literature. In the first method, platelets were isolated by centrifugation at ambient temperatures as described by Jennings et al. (5). It has been suggested that the platelet actin filament content of 40–50% observed using this method is a result of centrifugation-induced activation (11). Fig. 3*a* shows an electron micrograph of platelets isolated in this way. The platelets lost their discoid shape and were spherical, often with one or more pseudopodia. Table II shows that 48.6 ± 1.6% (mean ± SE; $n = 7$) of the actin in these platelets existed in filaments, as measured by the DNase I inhibition assay. As reported previously (5), similar values were obtained when the filament content was measured by high-speed centrifugation. In a comparison of the filament content of these platelets with that of discoid platelets, it was apparent that there had been only a minor increase in actin filament content (48% vs. 40%), even though the platelets had changed shape and extended many filopodia as a result of centrifugation. In contrast to the actin filaments in discoid platelets, however, ~60–70% of the filaments present in the centrifuged platelets sedimented at the lower *g* forces, indicating that they were organized in networks. The discoid shape of the centrifuged platelets could be restored by incubating them for 1–3 h at 37°C (data not shown). During this incubation, the F-actin content was unaltered, but the percentage of filaments that could be sedimented at low *g* forces decreased, showing that the networks dissociated (data not shown).

To confirm that there had been little polymerization of actin as a result of the centrifugation of platelets, we included 2.5×10^{-5} M cytochalasin D in the platelet-rich plasma and all the washing buffers. This drug inhibits stimulus-induced actin polymerization without affecting the preexisting filament content of cells (9, 20). Table II shows that the filament content of platelets isolated in the presence of cytochalasin D was only slightly lower than that of platelets isolated without the drug. The filaments present in centrifuged platelets isolated either in the presence or absence of cytochalasin could be visualized by electron microscopy of lysed, fixed platelets (data not shown). We have therefore concluded that most filaments present in centrifuged platelets already exist and do not arise from centrifugation-induced activation.

An exception to this conclusion was found in platelets isolated by centrifugation as described by Rosenberg et al. (10). These researchers observed that platelets contain 80–90% of their actin in a filamentous form prior to stimulation.

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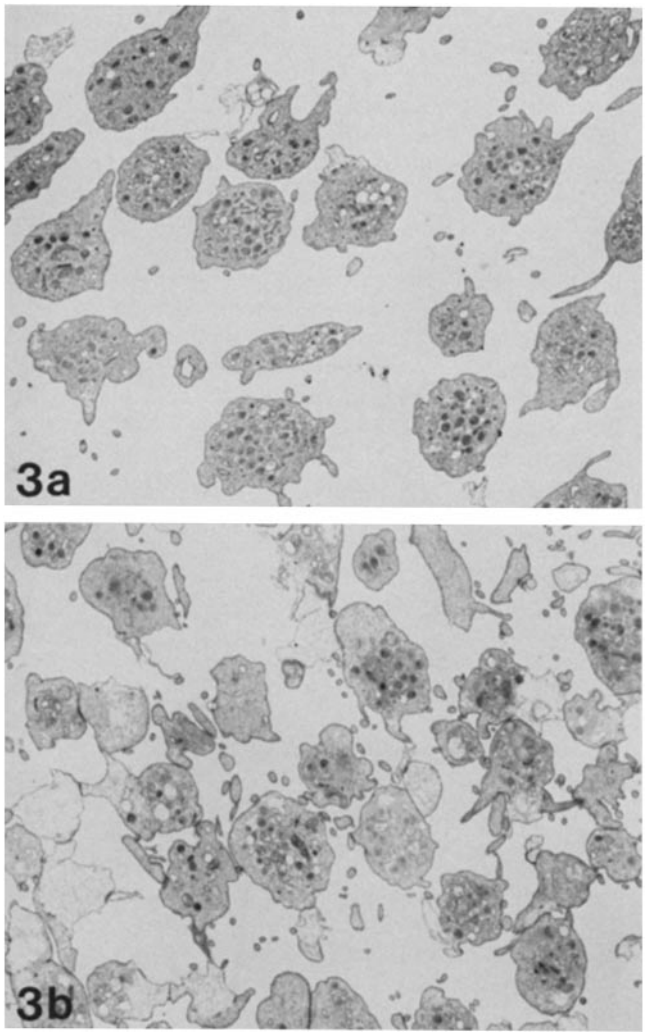

FIGURE 3 Electron micrographs of washed platelet suspensions. Platelets were washed (*a*) by centrifugation at ambient temperature (5) or (*b*) by centrifugation at 4°C (10). Suspensions were prepared for electron microscopy as described in the text. × 5,000.

TABLE II

Effect of Cytochalasin D in the Washing Buffers on the Actin Filament Content of Centrifuged Platelets

Inclusion in washing buffers	Actin filament content*
	% *of total*
None	48.6 ± 1.6‡
Cytochalasin D	43.5 ± 3.0§

Platelets were isolated by centrifugation (5) in the presence or absence of cytochalasin D (2.5×10^{-5} M).
* Determined by DNase I inhibition assay.
‡ Mean ± SE from seven donors.
§ Filament content was significantly lower than that of platelets prepared without cytochalasin D; $P < 0.05$ by two-sided, paired *t*-test.

They also found that more actin-binding protein, myosin, and α-actinin are associated with actin filaments than has been described by others (5). This is illustrated in Fig. 4, which compares the protein composition of Triton X-100-insoluble filaments isolated as described by Rosenberg et al. (10) with those isolated as described above (5). As summarized in Table III, filamentous material sedimenting at low *g* forces from Triton lysates of platelets isolated as described above contained mainly actin, as well as small amounts of actin-binding protein and myosin. Filamentous material sedimenting from

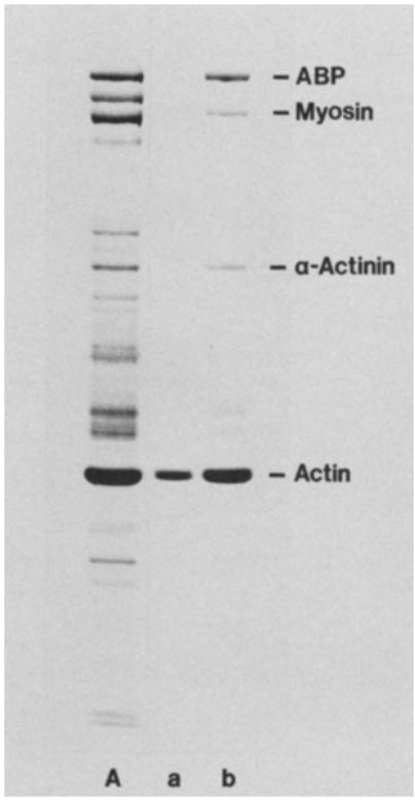

FIGURE 4 Protein composition of Triton X-100-insoluble, filamentous material from platelets washed by two centrifugation methods. Triton X-100–insoluble material was prepared from (lane *a*) platelets (2.6×10^8 platelets/ml) washed by centrifugation at ambient temperatures (5) and lysed as described by Jennings et al. (5), or from (lane *b*) platelets (8.7×10^9 platelets/ml) washed by centrifugation at 4°C (10) and lysed as described by Rosenberg et al. (10). Each track contains the amount of Triton X-100–insoluble material obtained from 7.8×10^6 platelets. Lane *A* contains 7.8×10^6 platelets prepared by centrifugation at ambient temperatures (5) and solubilized directly into SDS. There were no visible differences between the protein composition of platelets isolated by the two methods: *ABP*, actin-binding protein.

TABLE III

Protein Composition of Triton X-100-Insoluble, Filamentous Material from Platelets Isolated by Two Methods

Platelet isolation procedure	Amount of Triton X-100–insoluble polypeptide*		
	Actin	Myosin heavy chain	Actin-binding protein
		% *of total*	
a	28.5 ± 6.1‡	12.6 ± 2.6‡	7.0 ± 1.7‡
b	86.3 ± 2.3	27.0 ± 1.9	58.2 ± 3.0

Platelets were (*a*) washed by centrifugation at ambient temperatures and lysed as described by Jennings et al. (5) or (*b*) washed by centrifugation at 4°C and lysed at 4°C as described by Rosenberg et al. (10). Triton X-100–insoluble material was isolated (*a*) by sedimentation at 15,600 *g* for 4 min and (*b*) by sedimentation at 15,600 *g* for 2 min after 12 min on ice.
* Determined by SDE PAGE of material sedimented at 15,600 *g*.
‡ Mean ± SE from three different donors.

lysates of platelets isolated by the method of Rosenberg et al. (10) contained much more actin; it also contained more actin-binding protein and myosin than did the other preparation. In addition, it contained several other proteins (Fig. 4, lane *b*), the most prominent of which was one of an apparent molecular weight of 105,000, which has recently been shown to be an α-actinin-like protein (21).

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TABLE IV

Effect of Lysis Conditions on the Triton Insolubility of Platelet Polypeptides

Lysis buffer	Amount of Triton-insoluble polypeptide*		
	Actin	Myosin	Actin-binding protein
	% of total		
a	29.1 ± 2.4‡	16.3 ± 2.5‡	8.5 ± 2.1‡
b	25.3 ± 1.2‡	12.7 ± 0.9	12.0 ± 0.9

The platelet suspension (2.6×10^8 platelets/ml) was prepared by centrifugation at ambient temperature (5) and lysed with (*a*) an equal volume of 2% Triton X-100, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.4, or (*b*) 10 vol of ice-cold, 10 mM imidazole chloride, 40 mM potassium chloride, 10 mM EGTA, 1% Triton X-100, and 2 mM sodium azide, pH 7.0. Triton-insoluble material from (*a*) was isolated immediately by centrifugation at 15,600 *g* for 4 min, while that in (*b*) was isolated by centrifugation for 2 min after 12 min on ice.

* Determined by SDS PAGE of material sedimented at 15,600 *g*.

‡ Mean ± SE from three different donors.

To investigate the reasons for these discrepancies, we examined the ramifications of the two different lysis conditions and methods of platelet isolation. Table IV shows that the lysis conditions had no effect on the amount of actin that sedimented. Although platelets lysed with the buffers described by Rosenberg et al. (10) showed slightly higher amounts of actin-binding protein and myosin associated with their actin filaments, the differences were small (Table IV).

The main difference between the two methods of platelet isolation was that Rosenberg et al. centrifuged platelets at 4°C, while others have used ambient temperatures (2, 5). Since cold-induced activation of platelets has been described experimentally (22, 23), we examined the morphology of platelets isolated at the two temperatures. Electron microscopy revealed that platelets isolated at 4°C are highly irregular in shape, with numerous filopodia, and they often appear in microaggregates (Fig. 3*b*). In comparison, platelets isolated at ambient temperatures lose their discoid shape, but are less irregularly shaped than those isolated at 4°C and show no indication of aggregation (Fig. 3*a*).

DISCUSSION

It has been argued that the actin filaments detected in platelets washed by centrifugation may exist as a result of the activation of the platelets during their preparation (11). In the present study, various isolation procedures and inhibitors of platelet activation have been used to obtain unactivated, discoid platelets. Furthermore, a combined morphological and biochemical approach has been taken to examine the actin filaments within these cells. Platelets were obtained either by gel filtration or by centrifugation and were determined to be unstimulated on the basis of having the discoid form they are thought to display in circulating plasma. Activation of platelets during their isolation was prevented by including PGI_2 in all preparations; this agent has been shown to inhibit platelet activation, presumably by increasing intracellular cyclic AMP levels (24). Discoid platelets isolated by gel filtration or by centrifugation in the presence of PGI_2 contained ~40% of their actin in a filamentous form, as determined either by high-speed centrifugation of filaments from Triton X-100 lysates or by DNase I inhibition assay.

As an additional test of whether this 40% value was correct, platelets were isolated by centrifugation in the presence of cytochalasin D. This drug inhibits activation-induced polymerization of actin in platelets without affecting preexisting filament content (9, 20). The inclusion of cytochalasin D in all of the washing solutions had little effect on the filament content of platelets isolated by centrifugation. Since these platelets also contained ~40% of their actin in a filamentous form, it has been concluded that the filaments measured in isolated platelets already existed in unstimulated, circulating cells and did not result from the activation of these platelets during the isolation procedure.

While the present study indicates that filaments present in washed platelets isolated by several techniques do not arise from the activation of cells during isolation, one other study using cells isolated by centrifugation (10) offered contradictory results. We confirmed the results of Rosenberg et al. (10), finding that most of the actin in Triton X-100 lysates of platelets isolated by centrifugation at 4°C is filamentous. However, low temperatures are known to activate platelets, as judged by changes in shape (22), exposure of fibrinogen receptors (23), increased phosphorylation of proteins within platelets (22), and increased polymerization of actin (25).

Several lines of evidence suggest that platelets isolated by centrifugation at 4°C become activated. First, the level of F-actin found in these platelets is comparable to that level existing after activation of platelets with thrombin (5, 6, 8, 9, 20, 25) or ionophore A23187 (20). Second, increased amounts of myosin were associated with actin filaments from platelets isolated at 4°C. Low temperatures have previously been found to induce the phosphorylation of the myosin light chain (22), an event that also occurs during activation of platelets with thrombin (26, 27), collagen (28), or ionophore A23187 (28); this activity increases the affinity of myosin for actin filaments (29). Third, as with thrombin-activated platelets (5, 30), increased amounts of actin-binding protein were associated with actin filaments from platelets isolated at 4°C. Finally, electron microscopy showed that platelets isolated at 4°C change shape, extend filopodia, and form aggregates. We have concluded, therefore, that the high content and organization of F-actin in platelets washed at 4°C as reported by Rosenberg et al. (10) does not represent that level found in unstimulated platelets, but resulted from the activation of platelets during centrifugation at 4°C.

In the past, actin filaments in unstimulated platelets have been difficult to visualize by electron microscopy because of problems in preserving the filaments during osmium tetroxide fixation. The fixation procedure used in the present study included lysine in the glutaraldehyde fixative to preserve actin filament morphology (17). The addition of this fixative to the discoid platelets, in conjunction with the simultaneous addition of detergent, resulted in the removal of detergent-soluble cellular components and the clear visualization of filaments, while at the same time preventing postlysis changes in filament organization. It was demonstrated that filaments observed in this way originated from discoid cells, as evidenced by the fact that they retained the discoid shape of unextracted platelets. The visualization of extensive arrays of filaments in unstimulated, discoid platelets is consistent with biochemical data indicating that ~40% of the actin in these platelets is filamentous. In contrast, Nachmias (11) was unable to visualize F-actin by negative staining of extracted, discoid platelets. Although the reason for this discrepancy is not entirely clear, it is possible that insufficient extraction prevented the exposure of these filaments in the latter study.

Understanding the functioning of actin filaments during

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platelet activation requires an understanding of how actin filament content and organization differs in unstimulated and thrombin-activated platelets. The concentration of actin within platelets is thought to be between 0.25 and 1.0 mM (4, 31). Because this is much higher than the critical concentration—~30 μM (7)—the finding in the present study that only 40% of the actin in unstimulated platelets is filamentous indicates that mechanisms exist for preventing the polymerization of actin. One such mechanism may be provided by profilin, which binds to actin monomers and acts as a buffer preventing their polymerization into filaments (2, 7, 32); an additional mechanism may be provided by proteins that bind to the ends of preexisting actin filaments, preventing further addition of monomers (for review, see reference 7). In that 70–80% of the actin in activated platelets is filamentous (5, 6, 8, 9, 20), mechanisms must also exist for inducing the polymerization of actin during platelet activation. This polymerization probably results from the formation of new actin nuclei to which actin monomers add on from the profilin-bound pool. These actin nuclei may be proteins such as actin-binding protein. Alternatively, polymerization may be induced by the release of barbed end-capping proteins from preexisting filaments (for review, see reference 7). The evidence also indicates that a reorganization of actin filaments occurs during platelet activation. In Triton X-100 lysates of discoid platelets, <20% of the actin filaments sedimented at low speeds, whereas in lysates of platelets that had changed shape, >60% of the filaments sedimented. Because purified F-actin does not sediment at low *g* forces, while networks of cross-linked actin filaments do (33, 34), the most likely explanation for these findings is that shape change is associated with the reorganization of F-actin, possibly into networks of filaments. Since there is little net polymerization of actin filaments during the shape change process, this reorganization presumably involves preexisting filaments. Further evidence for a reorganization of filaments during platelet activation comes from the morphological demonstration that filaments isolated from isolated Triton X-100 lysates of thrombin-activated platelets are organized into condensed balls, while those isolated from unstimulated platelets exist in random arrays (5). Because there are more filaments in activated platelets, and the filaments are more highly cross-linked than those of discoid platelets, we have concluded that platelet activation involves mechanisms for inducing actin polymerization and for causing the reorganization of existing filaments.

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