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Cytoskeletal Domains in the Activated Platelet

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Abstract

Platelets circulate in the blood as discoid cells which, when activated, change shape by polymerizing actin into various structures, such as filopodia and stress fibers. In order to understand this process, it is necessary to determine how many other proteins are involved. As a first step in defining the full complement of actin-binding proteins in platelets, filamentous (F)-actin affinity chromatography was used. This approach identified >30 different proteins from ADP-activated human blood platelets which represented 4% of soluble protein. Although a number of these proteins are previously identified platelet actin-binding proteins, many others appeared to be novel. Fourteen different polyclonal antibodies were raised against these apparently novel proteins and used to sort them into nine categories based on their molecular weights and on their location in the sarcomere of striated muscle, in fibroblasts and in spreading platelets. Ninety-three percent of these proteins (13 of 14 proteins tested) were found to be associated with actin-rich structures in vivo.

Four distinct actin filament structures were found to form during the initial 15 min of activation on glass: filopodia, lamellipodia, a contractile ring encircling degranulating granules, and thick bundles of filaments resembling stress fibers. Actin-binding proteins not localized in the discoid cell became highly concentrated in one or another of these actin-based structures during spreading, such that each structure contains a different complement of proteins. These results present crucial information about the complexity of the platelet cytoskeleton, demonstrating that four different actin-based structures form during the first 15 min of surface activation, and that there remain many as yet uncharacterized proteins awaiting further investigation that are differentially involved in this process.

Keywords

actin-binding proteins; platelet activation; F-actin affinity chromatography

INTRODUCTION

Reorganization of the actin cytoskeleton in platelets during activation is a necessary event in blood coagulation [1]. Platelets are extremely rich in actin (estimated to be 20% of the soluble protein) and contain a wide range of actin-binding proteins [2]. During activation, these proteins interact with actin and each other to reorganize the actin filament structures into functional units that then mediate crucial events, such as: adherence to extracellular matrix and to other cells [3–5]; reorganization of the membrane skeleton [6]; extension of filopodia and lamellipodia to bring the activated platelet in contact with more distant cells [7]; contraction of the inner central cytoplasm facilitating the expulsion of granule contents such as growth factors and vasoactive substances [8]; and ultimately contractions of the

whole cell which constrict the clot into a tightly adherent complex structure called a “scab” in lay terms. Each of these activities must involve the participation of different structures whose common element is filamentous actin. However, the precise interactions between these proteins and actin, their spatial distribution before and after spreading, and how the actin filaments themselves form and become organized into these structures is not known.

Although a large number of actin-binding proteins have been found, there remain many, particularly those of low abundance, that remain undiscovered [1, 9–14]. Furthermore, it is still not understood how those proteins that have been identified interact to produce even one of the many aspects of platelet behavior. For example, while gelsolin has been extensively studied in vitro as a filament severing and barbed-end binding protein [14–16], how its activity is coordinated in vivo is not understood. Even the proposal that profilin and gelsolin together initiate the formation of filaments [16] does not address the question of how these filaments form at a particular place in the activated platelet, nor how these filaments become organized into a functional unit. It is only after a comprehensive overview of the many different interacting proteins has been obtained that the individual contributions of each of its components to cell function can be understood. Approaches that focus on the in vitro activity of a single protein, while extremely useful, fail to provide this overview.

The application of filamentous (F)-actin affinity chromatography to the study of the platelet cytoskeleton should provide such a comprehensive view of the numbers of actin-binding proteins present in the activated platelet. Those actin-binding proteins that have already been characterized can be eliminated from study either by their molecular weight or by Western blotting with antibodies to them, thus uncovering any novel, less abundant proteins likely to be isolated by this affinity approach. This technique has successfully identified large numbers of actin-binding proteins in other systems [11–14]. In yeast and *Drosophila*, where only a few actin-binding proteins were previously identified, some of these proteins were subsequently found to be homologues of known actin-binding proteins, while many others appear to be novel [17–19]. Immunofluorescence permitted preliminary assignment of each of these proteins to particular subcellular structures, which aids in focusing subsequent studies on their functional role [11–13]. A platelet protein identified by F-actin affinity columns, the Mab 2E4 antigen, colocalizes with actin in the lamellipodium of migrating cells and nerve growth cones, and binds the ends of actin filaments in vitro [20, 21], but is not significantly homologous to any other actin-binding protein (Bearer, manuscript in preparation). The Mab 2E4 monoclonal antibody was derived from the mouse producing antiserum 32 described in this report.

Within a single cell, actin filaments can form several distinctly different structures, such as microvilli and cell-cell adhesions [22–24]. This also appears to occur in platelets during activation [5, 25]. These different actin structures are known to be enriched in different actin-binding proteins. For example, adhesion plaques and stress fibers contain alpha-actinin [24], while microvilli contain villin and fimbrin [22, 23]. In the sarcomere of striated muscle, tropomyosin is distributed along the thin filaments, while gelsolin and CapZ are located at the Z-band, and myosin II forms the thick filaments that overlap but are not superimposable upon the actin [26].

Previous reports have used immunofluorescence, electron microscopy and video microscopy as a means to observe platelet shape change during activation [5–8, 25, 27–30]. These studies have individually revealed stress fibers, filopodial extensions and a contractile ring composed of microfilaments. None of these studies have looked comprehensively at the coordinated formation of these structures, nor have the actin filaments thought to be present in the lamellipodium that surround the platelet been visualized. Changes in distribution of individual proteins such as talin [4] and vinculin [5] have been elegantly described, but for

many actin-binding proteins identified biochemically in platelets, distributions remain unknown. As a by-product of this investigation it was found that during the first 15 min of activation on glass, platelets elaborate four different actin-based structures: filopodia, a contractile ring, lamellipodia, filopodia, and stress fibers. By staining with phalloidin during fixation, these structures could be more clearly delineated than has been possible in the past [5, 25]. Each of these four structures is directly related to a physiologic event in the homeostatic role of the platelet: shape change (filopodia, lamellipodia), adhesion (lamellipodia, adhesion plaques), degranulation (contractile ring) and contraction (stress-like fibers, filopodia).

The distinct visualization of these four structures provides a basis upon which to sort the actin-binding proteins isolated by F-actin affinity chromatography by categorizing them according to their localization to one or another of these structures as detected by immunofluorescence. A similar rationale has been used to categorize the actin-binding proteins identified by actin columns in *Drosophila* embryos [12]. The enrichment of a particular protein in one or another of these platelet actin structures would suggest a role for that protein in either the formation of that structure or its localized function during one of the particular events of platelet activation.

The two central aims of this report are: (1) to gain an insight into the full complement of actin-binding proteins present in the platelet, and (2) to evaluate the rapid and dramatic reorganization of actin and these proteins into distinct functional units during activation as a means to understand this cellular process, and as a tool to categorize the proteins involved in the formation and dynamics of each actin-rich structure.

MATERIALS AND METHODS

Platelet Preparation

For biochemical experiments, platelets were isolated by centrifugation from freshly outdated platelet rich plasma (PRP) obtained from the Irwin Memorial Blood Bank in San Francisco or from the Providence Blood Bank in Rhode Island [14]. After the final centrifugation, the platelets were resuspended in Tyrode's solution (138 mM NaCl, 2.9 mM KCl, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM CaCl_2 and 0.4 mM MgCl_2 , pH 7.4) at a concentration of 10 ml per unit of PRP. All of the above centrifugations were at room temperature, and all solutions maintained at 37°C. The resultant washed platelets were incubated at 37°C in Tyrode's solution for 1 h. For activation, platelets were stimulated with 20 μM ADP for 5 min at the end of incubation. To prevent activation, platelets were treated with 20 μM prostaglandin E1 (Sigma, St. Louis, MO) during incubation in Tyrode's.

Aliquots of ADP-activated cells were either boiled directly in gel sample buffer (homogenates) or dripped before and after stimulation into 4% paraformaldehyde (EM Sciences, Fort Washington, PA) in 0.1 M Na cacodylate (Fischer Scientific, Springfield, NJ) buffer at pH 7.4, stained with 1 $\mu\text{g/ml}$ FITC-phalloidin (Sigma), in phosphate-buffered saline, and the presence or absence of filopodia observed by fluorescence microscopy.

Preparation of Platelet Extracts

Platelet extracts were prepared under conditions that maximized the depolymerization of endogenous actin: sonication and maintenance in a low salt buffer that favors G-actin and does not promote polymerization, freezing and thawing once, which partially denatures actin such that it is less ready to form filaments, and dilution to 1 mg/ml total protein concentration. The filamentous actin on the column was stabilized in low salt by saturation with phalloidin [11].

Platelets were disrupted by sonication (Branson Sonifier cell disrupter 350, with microtip output set at 4, 60% duty cycle, and pulsed bursts for 1 min) after addition of equal volume of 2× extract buffer (10 mM Tris-HCl (pH 7.5), 1 mM Na₃ EDTA, 1 mM Na₃ EGTA, 2% Nikkol (a monomolecular detergent similar to Triton X-100 but composed of a single molecular species, Nikkol Inc, Tokyo, Japan), 2:100 dilution of a protease inhibitor cocktail (0.1 M phenylmethyl sulphonyl fluoride, 1 mM benzamidine HCl, 1 mg/ml each of leupeptin, pepstatin A, phenanthroline and aprotinin in ethanol). After sonication, the sonicate was placed on ice and then centrifuged at 10,000 × g for 20 min at 4°C. The resulting supernatant was brought to 50 mM Tris-HCl pH 7.5, 5 mM DTT and 2 mM sodium pyrophosphate and then clarified by centrifugation at 100,000 × g for 1 h. By Coomassie staining of SDS polyacrylamide gels of each supernatant and pellet, about 80% of the actin was present in the high-speed supernatant, and all the major bands detected in the original homogenate were represented in the final high-speed supernatant. This highspeed supernatant was frozen in liquid nitrogen and then thawed, respun at 100,000 × g for 1 h, and the supernatant diluted to 1 mg/ml total protein concentration. Identical aliquots of the resulting extract were loaded onto parallel F-actin and bovine serum albumin (BSA, control) columns. One unit of platelet-rich plasma commonly resulted in 35 mg of solubilized protein.

Preparation and Use of F-Actin Columns

F-actin columns were constructed as described previously with minor modifications [11–13]. Rabbit skeletal muscle actin was purified and demonstrated to be >99% homogeneous on Coomassie blue-stained SDS polyacrylamide gels. Either 3 ml or 15 ml columns containing actin filaments or 1 mg/ml BSA were constructed according to published protocols [11]. For filamentous actin columns, the actin was stabilized by addition of phalloidin (10 µg/ml of column bed, i.e., molar ratio of phalloidin to actin of 10:1). Monomeric (G) actin columns were prepared as described [11]. G-actin columns were used once and prepared on that day.

Columns were pre-equilibrated with column buffer (50 mM Hepes (pH 7.5), 0.02% Nonidet-P40, 0.5 mM Na₃ EDTA, 0.5 mM Na₃ EGTA, 5 mM DTT, 1:1,000 protease inhibitor cocktail, and 10% glycerol). Platelet extracts diluted to 1.0 mg protein/ml with column buffer were brought to 10% glycerol, and then loaded onto the columns at a rate of one-third column volume per h.

After loading, the columns were washed with column buffer for at least 4–5 column volumes at a rate of not more than one column volume/h. No elution was begun until the wash fractions had dropped to less than 10 µg/ml of protein by Bradford protein assay (Bio-Rad, Hercules, CA). Typically, the actin columns took much longer to wash than the control columns, and had a higher background level of protein continuously leaking off the column. Once the protein concentration in the wash fractions had dropped to less than 10 µg/ml, the columns were eluted with column buffer containing 0.5 mM ATP and 3 mM MgCl₂, followed by 1.0 M KCl. Peak protein fractions were determined by the Bradford method and fractions pooled for gel electrophoresis. Usually the peak extended over a total fraction volume equal to that of the column bed.

To analyze eluted proteins, pooled fractions were acidified with 10% trichloroacetic acid (TCA) on ice for 15 min and sedimented in a microfuge. The pellet was resuspended in Laemmli gel sample buffer and loaded onto either an 8.5% or a 6–12% gradient SDS-polyacryl-amide gel [31].

Preparation of Antisera

Immunization of mice against gel-purified protein bands was performed by a slight modification of a published protocol [32]. Pooled elution peaks containing about 0.850 mg of protein from both ATP and salt eluates from F-actin columns loaded with activated cell extracts were precipitated in 10% TCA resuspended in sample buffer and electrophoresed in SDS through a 2×12 cm 6–12% polyacrylamide gradient gel. Protein bands were visualized by Coomassie blue staining and the gel slab photographed. After cross-linking with 2% aqueous glutaraldehyde, the gel was fixed in 7% acetic acid overnight, re-equilibrated in water, and the gel bands excised. Each slice was homogenized in 0.5 ml phosphate-buffered saline (PBS: 0.13 M NaCl, 2 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.2) in a loose-fitting teflon dounce homogenizer driven by a Waring blender and then frozen in liquid nitrogen and lyophilized. For immunizing mice, aliquots of this homogenate resuspended in phosphate-buffered saline were mixed with Freund's complete adjuvant (first injection), or with incomplete adjuvant (all subsequent injections). One mouse died. All but one of the surviving mice (13 of 14) produced antisera that recognized platelet protein by Western blot or by immunofluorescence.

Western Blotting

Protein was transferred from SDS-PAGE to nitrocellulose [33]. Blots were blocked in 5% BSA in Tris-buffered saline (TBS). Primary antibodies were diluted at 1/200 in TBT (5% BSA, TBS and 0.2% Tween 20 (Bio-Rad)). After washing, the blot was incubated in affinity-purified goat anti-mouse IgG and IgM specific secondary antibody coupled to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and developed in nitro blue tetrazolium (Sigma) [33].

Antibodies to several well-characterized cytoskeletal proteins from platelets were obtained from the following sources: caldesmon and tropomyosin (Elizabeth der Terossian, Pasteur Institute, Paris, France), alpha-actinin and gelsolin (Sigma), macrophage actin-binding protein (John Hartwig, Harvard University Medical School, Boston, MA), glycoprotein IB (Joan Fox, Gladstone Institute, San Francisco, CA), spectrin and "Band 4.1" (Steve Shohet, University of California, San Francisco). All of these proteins were enriched in column eluates.

Immunofluorescence of Striated Muscle

Human skeletal muscle from surgical specimens received by the Pathology Department were frozen in O.C.T. Compound (Miles, Elkhart, IN), sectioned at -20°C and mounted on glass microscope slides. Slides were either allowed to air dry or were fixed for 90 s in 90% methanol, 10% acetone before drying. Slides were blocked in phosphate-buffered saline containing 1% BSA and 0.1% Triton X-100 (PBT) for 20 min, and then stained with different antisera diluted in PBT. After three washes, coverslips were counterstained with fluorescein-conjugated, affinity-purified goat anti-mouse IgG and IgM in PBT (Boehringer-Mannheim). When immunofluorescent banding patterns were difficult to align with striations as observed by phase, sections were double stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) at a concentration of $0.3 \mu\text{M}$ simultaneously with secondary antibody. Coverslips were then washed in PBS and mounted in 1 mg/ml 4-Diazabicyclo[2.2.2]-octane in glycerol (Aldrich Chem. Co. Milwaukee, WI) and viewed in a Nikon SA with a 100 Watt mercury bulb.

Immunofluorescence of Mouse Fibroblasts

NIH-3T3 cells were obtained from the Cell Culture Facility at UCSF and grown on sterilized coverslips in Dulbecco's Modified Medium supplemented with 10% calf serum.

After passage using trypsinization, cells were allowed to adhere to the coverslips for 36 hours. Sub-confluent cultures were selected and the growth media changed two hours prior to fixation. Petri dishes were fixed in warm Small's buffer [21] plus 0.25% Triton X-100 for 15 min and brought to room temperature. Petri dishes containing fixed coverslips were stored in Small's buffer at 4°C for not longer than 2 days before staining. Staining was performed as described for muscle sections above.

Immunofluorescence of Spreading Platelets

For immunofluorescence, fresh platelets were obtained from healthy volunteers [6, 14], and used on the day they were drawn. Washed platelets were incubated in Tyrode's solution for 1 h and then allowed to spread on clean #1 glass coverslips for 15 min at RT. Spread cells were fixed in 4% paraformaldehyde in Small's buffer [16] plus 0.25% Triton X-100 and 0.3 μ M rhodamine phalloidin (Molecular Probes, Eugene, OR). No structural changes were observed during this fixation as determined by high magnification video DIC of spread cells during fixation. The presence of rhodamine-phalloidin in the fixative greatly enhanced the intensity of fluorescence especially at the periphery of the lamellipodia without causing a detectable change in filamentous actin distribution. FITC-phalloidin used to assess shape change at the beginning of the preparation of the extract was not bright enough to image the lamellipodia but readily permitted evaluation of the presence of filopodia.

Coverslips were stained with antisera as described above for muscle. Platelets were photographed using a 60X objective with a 1.4 NA, a 1.25X optivar, and a 5X camera lens for a final magnification on the film of approximately 375X. Micrographs were printed at a 5- to 10-fold additional magnification.

RESULTS

Isolation of Proteins by F-Actin Affinity Chromatography

To understand how the platelet cytoskeleton is reorganized during activation, it is necessary first to identify as many actin-binding proteins as possible from platelets and then to determine to which actin-based structure they belong. As a first step, F-actin affinity chromatography was used because it identifies in a single step large numbers of actin-binding proteins solely by their ability to bind to filamentous actin [11, 12]. Application of this technique to human blood platelets revealed >30 different proteins comprising 2–4% of the total soluble pool (Figs. 1A–C, 2).

When extracts of ADP-activated human blood platelets were loaded onto F-actin and albumin (BSA, control) columns in parallel, approximately 2–4% of the total solubilized protein was retained by the F-actin column as compared to 0.2% retained by the albumin control column (Fig. 1A). Protein was first eluted with 0.5 mM Mg-ATP to release the myosins and any other ATP-sensitive actin-binding protein, and subsequently with 1 M KCl, which is expected to strip all other proteins from the column. The yield from a 12 ml (1 mg/ml of F-actin) column loaded with 18 mg of soluble protein (approximately one-half of a unit of platelet-rich plasma) was typically $\approx$ 1 mg of protein, while $\approx$ 200 μ g was obtained from smaller 3 ml column. Frequently, more protein eluted with ATP than with salt. When columns were run at slow speeds (<1 ml/hr) only small amounts of actin bled off the column.

One-third less protein eluted from F-actin columns loaded with extracts of prostaglandin E-1 (PGE-1-inhibited discoid cells) than from F-actin columns loaded in parallel with extracts from ADP-activated cells (Fig. 1B). PGE-1-inhibited cells eluted equally from the affinity column with salt or with ATP. More protein (2 to 3-fold) was retained and eluted from filamentous actin columns than from parallel columns made with globular (G) actin

monomers (Fig. 1C). Similar low amounts of proteins eluted from G-actin columns loaded with either activated or resting cell extracts.

SDS-PAGE analysis of the protein that eluted with ATP or salt from the actin column revealed multiple protein bands of molecular weights ranging from 17 kD to 260 kD (Fig. 2). Most of the bands present in the elutions were consistently observed from one preparation to the next: four independent extracts and their elutions from four different columns are shown in Figure 2. The major bands were significantly enriched in the column elution lanes as compared to their appearance in the extracts. That activation had occurred is demonstrated by a decrease in amount of protein in bands migrating at 235 kD (talin) and 260 kD (filamin). Proteolysis was consistent, producing the same effect each time. Since extracts were prepared in the presence of high levels of six different proteases and EDTA, this proteolysis is likely to occur during activation, as has been described [34–36]. Since this is an intracellular proteolysis that occurs during shape change, it is unlikely to cause the loss of a significant actin binding protein involved in that shape change.

A number of well-characterized proteins were present in the eluates as detected by immunoblots. As expected, myosin II (200 kD) eluted with ATP, recognized as a prominent band migrating at 200 kD, while filamin (260 kD), alpha-actinin (105 kD), gelsolin (95 kD), caldesmon, (80 kD), and tropomyosin (34 kD) were enriched in salt eluates as detected in Western blots (Fig. 3). Glycoprotein IB (150 kD) which binds to actin via filamin [34, 38] is detected in blots (Fig. 3) but is not readily visualized by Coomassie staining of protein gels and thus appears to be retained at low levels. However, the presence of small amounts of this protein in eluates suggests that either tertiary interactions occurred in the column bed or that complexes were not completely disassembled during extraction procedures. This phenomena has been shown to occur using microtubule affinity chromatography [39]. This does not mean that the column is nonspecific, but rather that complexes can also potentially be isolated in which only one component binds to actin. However, vinculin (135 kD) was not consistently enriched in column eluates, possibly also because it does not bind actin directly in the native state [40, 41] and talin (235 kD) was never detected (data not shown). Other proteins known or suspected to be present in platelets but which were not investigated include CapZ, scinderin, and low molecular weight tropomyosin. The molecular weights of these proteins are known; thus, antibodies were not raised against bands predicted to be composed of these or other known actin-binding proteins.

As a control for nonspecific retention by the F-actin column, extracts and eluates were probed in Western blots with anti-tubulin antibodies. Although extracts contained abundant tubulin, none was detected in eluates. Furthermore, as demonstrated (Fig. 1), control columns with either G-actin, albumin, or insulin (not shown) did not retain appreciable amounts of protein.

In addition to these proteins, many other proteins were present migrating at molecular weights ranging from 30 kD to 150 kD which do not correspond to the molecular weights of previously described actin-binding proteins found in platelets. Prominent bands appear at approximately 110, 95, 70, 45, and 43 kD in ATP eluates, and 105, 80, 55, 45, and 34 kD in salt eluates.

The molecular weights of actin-binding proteins from resting cells were compared to those from activated cells by SDS-PAGE. While there was a significant difference in the quantity of protein there was no consistent difference in the molecular weights of proteins in silver-stained gels that were loaded with identical amounts of total protein, although there were differences in the relative amounts of protein in several bands as detected by Coomassie staining. Platelets are synthetically silent—they do not synthesize protein to any significant

degree. Thus, there is unlikely to be any new protein synthesized during ADP activation, although changes in affinity of proteins for actin caused by post-translational modifications, and calcium-dependent proteolysis of actin-binding proteins are known to occur during aggregation [35, 36]. The major differences in column eluates from activated cells as compared with resting cells was an increase in the relative amount of a 200 kD band (myosin II), and a moderate decrease in the 260 kD band (filamin) as well as the presence of breakdown products of filamin as detected by Western blot. Because proteins were analyzed in relatively high percentage gels, whether or not the low molecular weight monomer binding proteins such as profilin and thymosin B4 were present in eluates from G-actin columns was not studied.

Approaches for Categorizing These Proteins Into “Complementation” Groups

Sorting on the basis of molecular weight—The large number of apparently unknown proteins that eluted from the actin affinity column posed a challenge: what do all these different proteins do, and how is their activity coordinated during activation? To address this, antibodies were raised to 14 of these proteins and used to sort them into groups according to the following criteria: (1) their molecular weight in Western blots, and (2) their immunolocalization in the sarcomere of striated muscle, (3) in cultured fibroblasts, and (4) in spreading blood platelets. Fourteen different polyclonal antibodies were raised to gel-purified protein bands from column eluates. These bands and their respective antisera are numbered in order of decreasing molecular weight of the band used as immunogen. These 14 bands were deliberately chosen from among those proteins likely to be novel, either because their molecular weight was not that of a previously characterized actin-binding protein, or because they failed to be detected in Western blots probed with antibodies to characterized actin-binding proteins.

It was deliberately chosen to make antibodies to these proteins as a next step in their characterization rather than microsequencing them, because sequence only provides in-depth information if the protein turns out to be one that has already been sequenced. The belief was that these proteins were novel. Since antibodies are readily available to virtually all the actin-binding proteins previously identified in platelets and could be used to identify them in column eluates, it was not necessary to obtain microsequences for this purpose. While amino acid sequence will provide tools for cloning each of these proteins in the future, with >30 proteins to choose from, the project was not yet at a stage in which cloning was an appropriate next step. In contrast to short amino acid sequences, antibodies provide tools to characterize the molecular weight and the cellular distribution of novel proteins, which have proven successful in characterizing these proteins. Furthermore, antibodies will provide probes for a purification strategy. Clearly, full length purified protein is ultimately more useful than a short peptide sequence.

Antisera were tested for their ability to recognize proteins in platelet homogenates, in extracts, and in F-actin column eluates by Western blot. Examples of 8 of these Western blots are shown in Figure 4. Results for all 14 antisera are compared in Table I. Antisera 7, 14, 16, 21, 26, and 28 recognized proteins as single bands that were abundant enough to be detected in homogenates of whole cells (Fig. 4A, Table I). In contrast, antisera 6, 18, 19, 30, 32, and 33 only detected protein in the enriched F-actin column eluate (Fig. 4B, Table I), demonstrating that these proteins were of relatively low abundance in the platelet, but were enriched in the affinity column eluate. Since five-fold less total protein was loaded in each lane for the F-actin column eluate compared to the homogenate, these results also demonstrate that these antigens are greatly enriched in the column eluate.

Seven of the 13 antisera reacted with a single band of the same molecular weight as the immunogen, while 5 reacted with single bands that were of higher molecular weight (see

Table I), suggesting that the original immunogen was a breakdown product. Six different column eluates were pooled to acquire enough protein for immunization. Therefore, minor breakdown products that could have been used for immunization would not appear in blots. In other blots (not shown) both bands were visible. Conversely, antiserum 6, raised against a 100 kD band, recognizes no bands in homogenates and a lower, 65 kD band in eluates, suggesting that the original 100 kD immunogen was the intact protein which is more often proteolyzed to 65 kD in column eluates. Antiserum 21 did not recognize a single band but rather a smear of proteins. In four instances, two different antisera reacted with bands of similar molecular weight. Numbers 14 and 16 appear similar, as do 26 and 28, 6 and 18, and 19 and 20. Thus, the 13 different antisera recognize at least eight unique proteins. Since the antisera for these proteins was raised against gel-purified bands that migrate close together on 6–12% gradient gels, it is possible that the antibodies that blot to bands with similar apparent molecular weights actually recognize different proteins that are not well-resolved in the Western blot. This was further supported by the immunofluorescence staining pattern of the different antisera detailed below.

Sorting on the basis of location in the sarcomere of human striated muscle—

Antisera raised against platelet actin-binding proteins were screened for reactivity and location in frozen sections of human striated muscle. The rationale was that since the sarcomere of striated muscle is a highly organized, almost crystalline structure, it could be exploited to determine whether a given protein localizes with the barbed ends of actin filaments (Z-band), along the length of filaments (I-band or actin-like), or with myosin II (A-band). Many isoforms of other actin-binding proteins are present in muscle, including gelsolin and CapZ in the Z band, and tropomyosin along the length of the thin filaments. For an antibody to recognize the muscle isoform, it must react with conserved epitopes, and therefore, not all antibodies cross-react with all tissue-specific isoforms. Hence, this is a good test if it works, but negative results do not necessarily mean that a muscle isoform does not exist.

Seven of the antisera reacted with frozen sections of human striated muscle. Five of these gave unique staining patterns by immunofluorescence, and two stained muscle diffusely (Fig. 5 and Table I). The spatial relationship between the antibody staining and the sarcomeric striations was determined by using phase contrast microscopy of the same area of the specimen as shown, or by double labeling with fluorescent phalloidin (not shown). Figure 5 demonstrates examples of three of these staining patterns: Z-band (antiserum 7), myosin-like (A-band, antiserum 32), and both Z and A band staining (antiserum 16). Overlap of stain with the striations visualized by phase is readily done at the microscope by flipping back and forth between images, and is not as easy to see in micrographs. The patterns obtained for the other 4 antisera which were equally sharp are listed in Table I for comparison.

Sorting on the basis of location in subconfluent cultures of mouse fibroblasts

—Antisera were further characterized by staining patterns in fibroblasts, since these cells have actin-rich structures not found in striated muscle. Nine of the fourteen antibodies stained mouse fibroblasts. Representative examples of the six different staining patterns observed for these antibodies is shown in Figure 6. Two (7 and 32) were enriched in the lamellipodium, an area of the cell where actin filaments are rapidly turning over. One of these stained the lamellipodium in a brightly diffuse pattern (7), while the other stained the outer third of the actin fringe (32). The staining pattern of Mab 2E4, a monoclonal antibody derived from the same mouse whose sera is labeled 32 in this paper, also stains the outer third of the lamellipodium in motile fibroblasts [21]. Two others (6 and 18) were concentrated around the nucleus. One of these (18) also appears to stain the outermost ends of stress fibers. Two (14 and 16) stained stress fibers only. Three gave a nonspecific diffuse

pattern (18, 21, and 33). Several of those that stained human muscle failed to stain mouse fibroblasts (Table I).

Sorting of proteins on the basis of the staining pattern in human platelets—

It was not originally expected that subcellular localization by immunofluorescence would be possible in platelets, since these cells are very small. However, a high energy light source coupled with the very bright staining afforded by including rhodamine phalloidin in the fixative made it possible to distinguish the separate actin structures in platelets by fluorescence microscopy without resorting to electron microscopy. Using this method, it was discovered that when platelets were allowed to spread on glass for 15 min and then fixed, four distinct actin structures in each individual platelet could usually be identified: filopodia, lamellipodia, contractile ring, and stress-like fibers.

Two examples of phalloidin-stained platelets and a schematic representation of these four actin structures in an idealized platelet is shown in Figure 7. The contractile ring is not visible in Figure 7A because it is out of the plane of focus. Figure 7B shows a different platelet with the focal plane slightly above the surface of the coverslip where the ring is more easily observed. Filopodial extensions are not visible in either of these platelets because the filopodia have been completely submerged by the advancing lamellipodium which surrounds each of these cells. A bundle of actin apparently remaining from a former filopodium is indicated by an arrow in Figure 7A. Filopodial extensions are shown in several of the platelets in Figure 8.

Comparisons of staining patterns of the different antibodies were made between platelets that had reached the typical morphology of the fully spread stage, after the formation of stress-like fibers and before the dissolution of the contractile ring. The majority of platelets on the coverslip at 15 min have this morphology, although platelets at other stages of activation are also present.

Five patterns of staining by these antibodies were observed in discoid and in spread platelets double-labeled for actin filaments. Shown in Figure 8 are representative images of each of the five different staining patterns: (1) peripheral staining in the discoid, resting cell, and, after activation on glass, a punctate staining along the outer edge of the lamellipodium with no cytoplasmic staining (antiserum 19); (2) diffuse staining in the discoid cell and thick filamentous staining of the contractile ring after activation (antiserum 16). The ring is in a slightly higher focal plane than the lamellipodium, and the intensity of the actin staining in the central region obscures the ring itself in the micrograph of phalloidin staining. This ring has been shown to form in spreading platelets [7–8]. If the staining were due to the greater thickness of the cell at the center, it should not have the central unstained region. Hence, this staining pattern is most likely due to association of these proteins with the contractile ring; (3) granule-like staining in the discoid cell, and staining throughout the full thickness of the lamellipodium as well as some diffuse central staining after spreading (antiserum 32). This antibody stained only the outer portion of the lamellipodium in fibroblasts. This difference could result from a difference in the organization of the actin in the lamellipodium in these two different cell types, or since the platelet lamellipodium is more shallow, a sublamellipodial difference may not be appreciable; (4) peripheral staining of the discoid cell and staining of fine filopodia and weak granular staining of the cytoplasm after activation (antiserum 21). These filopodia are so fine that phalloidin staining of them cannot be imaged relative to the brightly fluorescent cell center; and (5) peripheral staining of the discoid cell, and staining of lamellipodia and cytoplasm but no staining of even the most prominent filopodia after activation (antiserum 7). Since the staining patterns of the other antisera were similar to these five basic patterns, they are listed in Table I and not shown.

DISCUSSION

Actin filament reorganization is crucial for each step in the biologic function of the platelet in blood clotting—shape change, aggregation and adhesion, degranulation and retraction [1, 35, 37]. The four distinct actin structures described here are likely to make individual functional contributions to each of these behaviors: filopodia and lamellipodia for shape change and aggregation, stress fibers for adhesion, and the contractile ring and stress fibers in degranulation and retraction. The actin filaments in each of these four structures behave differently. For example, filopodia extend from the cell outwards and contain bundles of actin filaments oriented with uniform polarity and possibly tethered together by bundling proteins [30]. In contrast, stress fibers are bundles of filaments with both polarities that attach to adhesion plaques and exert tension inwards [10, 35]. The contractile ring is a more loosely aligned array of actin filaments that apparently contracts as a circlet in the center of the cell [8]. Finally, unlike these more stable actin filaments, actin in the lamellipodium is rapidly polymerizing [7]. Thus, it is not unexpected that each structure should be composed of different actin-binding proteins.

Platelets have been used as a source for the biochemical purification of many different actin binding proteins [2]. All of these proteins have been found because they were highly enriched in the anucleate platelet, which is not surprising since 20% of the soluble protein in platelets is estimated to be actin [2]. In contrast to traditional methods which rely on relative abundance, affinity chromatography enriches for low abundant proteins and thus provides a relatively comprehensive view of the full complement of actin-binding proteins within the cell [11–13]. In this paper, F-actin affinity columns permitted the identification of >30 proteins, many of which were not abundant. The restricted location of these less abundant proteins (6, 18, 19, 20, 30, 32, and 33) to specific actin structures suggests that they are functionally significant even though they are not abundant. Furthermore, the presence of cross-reactive antigens in skeletal muscle and in fibroblasts suggests that some of these proteins are likely to be involved in basic functions of actin that transcend type-specific cell behavior.

Thirteen of 14 antigens to which polyclonal sera were raised colocalize with specific actin structures in the spread platelet. Colocalization of these proteins with actin filaments in cells demonstrates that these proteins associate with actin *in vivo*, and thus confirms that their isolation by F-actin affinity chromatography is due to a real affinity for actin filaments and not to nonspecific binding induced by *in vitro* conditions. Confirmation of their ultrastructural interactions with actin will require subsequent studies of each antigen at the electron microscope level. Since apparently all of these proteins bind to actin filaments to some extent even when isolated from resting cells, it is possible either that the extraction conditions activates actin binding, or that they are always active but have only a limited amount of actin to interact with in resting cells.

Since these antigens can be viewed as a random sample of the 30 proteins retained and eluted from filamentous actin columns, it seems likely that the majority of the proteins eluting from the columns are actin-associated in the cell or are breakdown products of actin-binding proteins. Furthermore, it remains possible that even this approach has not identified all of the platelet actin-binding proteins. Clearly, as judged by the results of the F-actin chromatography, there remains a wealth of actin-associated proteins yet to be characterized, despite the fact that many have already been found in platelets.

The proteins described in this paper are likely to be novel, based on their molecular weight and their localization in the platelet. For example, although 7 has a molecular weight similar to alpha-actinin, it cannot be this protein since it elutes with ATP from actin columns and is

not found in stress fibers or adhesion plaques in fibroblasts, as is alpha actinin. Similarly, 19/20 cannot be the fibronectin receptor GPIIbIIIa, since that receptor is diffusely localized over the spread platelet [28] and not concentrated at the outer edge, as is 19/20. Conversely, CAP (Asp 56) could be 28, since the behavior of that protein in platelets has not been studied in-depth enough for a comparison to be made and the molecular weights are suggestive.

By analyzing the distribution of these 13 antigens in three cell types, it was possible to group them into nine categories. These categories are shown in Table II. From these categories two equally interesting functional roles can be envisioned: (1) these proteins could be involved in the formation of specific actin structures; or, conversely, (2) the association of these proteins with particular actin-rich structures, such as the contractile ring or the filopodia, could mediate the subsequent function of that structure, for example, the contraction of the contractile ring, or the tethering of membrane or cytosolic components to actin filaments, thereby creating domains of differing molecular composition within the contiguous cytoplasm.

One potential drawback to using affinity chromatography as an approach is that it identifies proteins merely on their binding to actin filaments and reveals nothing about whether that binding affects the behavior of actin. Thus, the data presented here cannot differentiate between the two possibilities listed above. Indeed, binding to actin could be a means to stabilize proteins in the cytoplasm and not have any relevance to actin dynamics. In fact, F-actin affinity chromatography was originally developed to find proteins that tethered developmental cues to actin in the cytoplasm of the *Drosophila* embryo and not for the discovery of proteins involved in actin dynamics. It is noteworthy that to date no such tethering proteins have been found, and instead the affinity approach has isolated proteins with profound effects on actin dynamics [16, 19]. Hence, it is to be expected that the proteins reported here are more likely to be involved in actin dynamics than in tethering cytoplasmic components to actin. The location of these proteins in structures whose behavior is known provides clues as to what type of dynamic interaction they might each have with actin filaments.

The reorganization of the actin and these actin-associated proteins into four distinct structures during the initial 15 min of activation raises a number of interesting questions: for example, how are the structures constructed? Do the actin binding proteins unique to each contribute to their formation, to their subsequent function or to both? Because the platelet reorganizes its cytoskeleton rapidly (within 15 min) under experimental control, it provides an ideal model system in which these questions can be studied. Because of its analogy to other cells, investigation of the reorganization of the actin cytoskeleton of the platelet into these four distinct structural, functional, and molecular domains addresses biological questions that transcend the role of cytoskeleton in platelet physiology. Which functional role each protein plays can only be definitively proven after rigorous biochemical and genetic studies of each protein in vitro and in vivo.

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References

1. Fox JEB, Philips DR. Polymerization and organization of actin filaments within platelets. *Sem Hematol.* 1983; 20:243–260.
2. Fox JE. Regulation of platelet function by the cytoskeleton. *Adv Exp Med Biol.* 1993; 344:175–185. [PubMed: 8209786]
3. O'Halloran T, Beckerle MC, Burridge K. Identification of talin as a major cytoplasmic protein implicated in platelet activation. *Nature (Lond).* 1985; 317:449–451. [PubMed: 2995830]
4. Beckerle MC, Miller DE, Bertagnoli ME, Sarah Locke. Activation redistribution of the adhesion plaque protein, talin, in intact human platelets. *J Cell Biol.* 1989; 109:3333–3346. [PubMed: 2513330]
5. Nachmias VT, Golla R. Vinculin in relation to stress fibers in spread platelets. *Cell Motil Cytoskel.* 1991; 20:190–202.
6. Bearer EL. Platelet membrane skeleton revealed by quick freeze deep etch. *Anat Rec.* 1990; 227:1–11. [PubMed: 2368921]
7. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol.* 1992; 118:1421–1442. [PubMed: 1325975]
8. Stark F, Golla R, Nachmias VT. Formation and contraction of a microfilamentous shell in saponin-permeabilized platelets. *J Cell Biol.* 1991; 112:903–913. [PubMed: 1900299]
9. Bearer EL. Role of actin polymerization in cell locomotion: Molecules and models. *Am J Resp Cell Molec Biol.* 1993; 8:582–591.
10. Luna EJ. Molecular links between the cytoskeleton and membranes. *Curr Opin Cell Bio.* 1991; 3:120–126. [PubMed: 1854476]
11. Miller KG, Alberts BM. F-actin affinity chromatography: Technique for isolating previously unidentified actin-binding proteins. *Proc Nat Acad Sci USA.* 1989; 86:4808–4812. [PubMed: 2740328]
12. Miller KG, Field CM, Alberts BM. Actin binding proteins from *Drosophila* embryos: A complex network of interacting proteins detected by F-actin affinity chromatography. *J Cell Biol.* 1989; 109:2963–2975. [PubMed: 2512303]
13. Drubin DG, Miller KG, Botstein D. Yeast actin binding proteins identified by filamentous actin affinity chromatography. *J Cell Biol.* 1988; 107:2551–2561. [PubMed: 3060468]
14. Bearer EL. Direct observation of actin filament severing by gelsolin and binding by gCap 39 and CapZ. *J Cell Biol.* 1991; 115:1629–1638. [PubMed: 1661732]
15. Yin HL. Gelsolin: Calcium- and polyphosphoinositide-regulated actin-modulating protein. *Bio Essays.* 1987; 7:176–179.
16. Stossel TP. The machinery of blood cell movements. *Blood.* 1994; 84:367–379. [PubMed: 8025265]
17. Adams AE, Botstein D, Drubin DG. Requirement of yeast fimbrin for actin organization and morphogenesis *in vivo*. *Nature.* 1991; 354:404–408. [PubMed: 1956405]
18. Drubin DG, Mulholland J, Zhu Z, Botstein D. Homology of a yeast actin-binding protein to signal transduction proteins and myosin-1. *Nature.* 1990; 343:288–291. [PubMed: 2405279]
19. Kellerman KA, Miller KG. An unconventional myosin heavy chain gene from *Drosophila melanogaster*. *J Cell Biol.* 1992; 119:823–834. [PubMed: 1429838]
20. Bearer, EL. Actin and actin-associated proteins in *Xenopus* eggs and early embryos. In: Pederson, R., editor. *Cytoskeleton in Development*. Vol. 26. New York: Academic Press; 1992a. p. 34-52.
21. Bearer EL. An actin-associated protein present in the microtubule organizing center and the growth cones of PC-12 cells. *J Neurosci.* 1992b; 12:750–761. [PubMed: 1372044]
22. Mooseker MS. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu Rev Cell Biol.* 1985; 1:209–241. [PubMed: 3916317]
23. Bretscher A. Microfilament structure and function in the cortical cytoskeleton. *Annu Rev Cell Bio.* 1991; 7:337–374. [PubMed: 1839710]
24. Bertagnoli ME, Beckerle MC. Regulated membrane-cytoskeleton linkages in platelets. *Ann NY Acad.* 1994; 714:88–100.

25. Debus E, Weber K, Osborn M. The cytoskeleton of blood platelets viewed by immunofluorescence microscopy. *Eur J Cell Biol.* 1981; 24:45–52. [PubMed: 7016544]
26. Lu MH, DiLullo C, Schultheiss T, Holtzer S, Murray JM, Choi J, Fischman DA, Holtzer H. The vinculin/sarcomeric- α -actinin/ α -actinin nexus in cultured cardiac myocytes. *J Cell Biol.* 1992; 117:1007–1022. [PubMed: 1577864]
27. Allen RD, Zacharski LR, Widirstky ST, Rosenstein R, Zaitlin LM, Burgess DR. Transformation and motility of human platelets. *J Cell Biol.* 1979; 83:126–142. [PubMed: 511936]
28. Loftus JC, Albrecht RM. Redistribution of the fibrinogen receptor of human platelets after surface activation. *J Cell Biol.* 1984; 99:822–829. [PubMed: 6088559]
29. Albrecht RM, Goodman SL, Simmons SR. Distribution and movement of membrane-associated platelet glycoproteins: Use of colloidal gold with correlative video-enhanced light microscopy, low voltage high-resolution scanning microscopy, and high-voltage transmission electron microscopy. *Am J Anat.* 1989; 185:149–164. [PubMed: 2773810]
30. Nachmias VT. Cytoskeletons of human platelets at rest and after spreading. *J Cell Biol.* 1980; 86:795–802. [PubMed: 6157694]
31. Laemmli UK. Cleavage of the structural proteins of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
32. James TC, Elgin S. Identification of a non-histone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. *Molec Cell Biol.* 1986; 6:3862–3872. [PubMed: 3099166]
33. Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor; 1988.
34. Rosenberg S, Stracher A, Lucas RC. Isolation and characterization of actin and actin-binding protein from human platelets. *J Cell Biol.* 1981; 91:201–211. [PubMed: 7197680]
35. Beckerle MC, O'Halloran T, Burridge K. Demonstration of a relationship between talin and p235, a major substrate of calcium-dependent protease in platelets. *J Cell Biochem.* 1986; 30:259–270. [PubMed: 3009504]
36. Fox JEB, Reynolds CC, Phillips DR. Calcium-dependent proteolysis occurs during platelet aggregation. *J Biol Chem.* 1983; 258:9973–9981. [PubMed: 6309792]
37. Fox JEB, Phillips DR. Inhibition of actin polymerization in blood platelets by cytochalasins. *Nature.* 1981; 292:650–652. [PubMed: 6894786]
38. Fox JE. Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. *J Clin Invest.* 1985; 76:1673–1683. [PubMed: 2932470]
39. Kellogg DR, Field CM, Alberts BM. Identification of microtubule-associated proteins in the centrosome, spindle and kinetochore of the early *Drosophila* embryo. *J Cell Biol.* 1989; 109:2977–2991. [PubMed: 2574177]
40. Johnson RP, Craig SW. An intramolecular association between the head and tail domains of vinculin modulates talin binding. *J Bio Chem.* 1994; 269:12611–12619. [PubMed: 8175670]
41. Wood CK, Turner CE, Jackson P, Critchley DR. Characterization of the paxillin-binding site and the C-terminal focal adhesion targeting sequence in vinculin. *J Cell Sci.* 1994; 107:709–717. [PubMed: 8207093]

Fig. 1. Elution profiles of F-actin, G-actin, and control (albumin) columns loaded with extracts from activated or resting platelets. In each case, the first arrow indicates the 0.5 mM Mg-ATP elution, and the second arrow indicates the subsequent 1.0 M KCl elution. **A:** Comparison of F-actin and control (BSA) columns loaded with extracts from ADP-activated platelets. The protein content of each fraction of 1.5 ml is plotted against the fraction number in each case. In this experiment, 30 ml of extract (1.2 mg/ml) was loaded onto two parallel columns, one a 3 ml F-actin (1 mg/ml actin) and the other a 3 ml control (albumin, 3 mg/ml) column. **B:** Comparison of resting versus activated platelet extracts chromatographed on F-actin columns. The profile for the ADP-extract eluting from F-actin column shown in A is compared to a profile from a parallel F-actin column loaded with

extract from PGE-1 inhibited cells. The amount of protein in the extract from the PGE-1-inhibited cells was adjusted so that both columns were loaded with same amount of protein.

C: Comparison of F-actin versus G-actin columns loaded with ADP-activated cell extracts. Twenty ml of soluble platelet protein at a concentration of 1 mg/ml from three units of platelet-rich plasma was loaded in parallel onto two columns: one, a 3 ml column of F-actin (2.5 mg/ml actin) and the other, a 3 ml column of G-actin (3.3 mg/ml actin). The protein concentration in each 1.5 ml fraction is plotted against the fraction number. Similar low levels of binding to the G-actin column were obtained for columns loaded with resting cell extracts.

Fig. 2.

F-actin columns consistently enrich for particular proteins. Silver-stained 10% polyacrylamide gel of extracts and eluates from four different ADP-activated platelet extracts (X, **lanes 1–4**) run on four different 3 ml F-actin columns eluted with 0.5 mM Mg-ATP in column buffer (ATP, **lanes 1–4**) followed by 1.0 M KCl in the same buffer (KCl, **lanes 1–4**). Protein peaks were determined by Biorad Bradford protein assay, the peak fractions pooled and precipitated in 10% TCA and the pellet solubilized in SDS-containing gel sample buffer. One-tenth of the total elution peak from a 3 ml F-actin column was loaded onto each lane. S, molecular weight standards.

Fig. 3. Elution of previously identified actin-binding proteins from F-actin columns. Western blots probed with (1) anti-macrophage actin-binding protein (filamin); (2) anti-glycoprotein IB; (3) anti-alpha-actinin; (4) anti-gelsolin; (5) anti-caldesmon; (6) anti-tropomyosin. Large arrows to the right indicate the migration of the antigens. Molecular weight standards indicated by small arrows on the left: myosin (200 kDa), phosphorylase a (98 kDa), albumin (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Fig. 4. Western blots showing antigens recognized by antisera raised against gel-purified bands from F-actin column eluates. Antisera number is indicated at the top of each lane; C indicates a control blot that was incubated in parallel but without primary antibody. Antisera in **A** (C, 7, 16, 28, and 30) are blotted against whole platelet homogenates at 40 μ g per lane, while antisera in **B** (6, 19, 32, and 33) are blotted against F-actin column eluates containing 8 μ g per lane. Molecular weight standards are indicated by arrows to the left: myosin (200 kDa), phosphorylase a (98 kDa), albumin (68 kDa), and ovalbumin (45 kDa).

Fig. 5. Platelet actin-binding proteins are found in the Z-band, and the A band in striated muscle. Frozen sections of human skeletal muscle stained with: (A) immunofluorescence of antiserum 7 and (B) same field, phase contrast; (C) antiserum 32; and (D) corresponding phase of the same field; (E) antiserum #16, and (F) corresponding phase image. Magnification: 2,400 X.

Fig. 6. Staining patterns of platelet antigens in cultured fibroblasts compared to filamentous actin distribution by double label immunofluorescence. Double images of individual cells double-labeled with one of the antisera (**left** panels) and rhodamine phalloidin (**right** panels). Antisera are as indicated at the top of each set. Bar = 10 μ m.

Fig. 7.

Four distinct actin domains in spread platelets. **A** and **B**: Two examples of platelets fixed and stained with rhodamine phalloidin 15 minutes after exposure to a glass coverslip. Arrow in **A** indicates a residual filopodial extension that has been engulfed by the advancing lamellipodium which surrounds the cell. In **A** the focal plane is on the glass surface; in **B** the focal plane is slightly above the glass revealing the contractile ring more clearly. **C**: An idealized schematic of the actin filament structures displayed in spread platelets: (1) leading edge; (2) filopodium; (3) lamellipodium; (4) contractile ring; (5) stress-like fibers. (In previous studies using electron microscopy, the cytoplasmic terminal of the filopodial actin bundles has been seen to connect with filaments that run the circumference of the cell [6, 21]. By immunofluorescence as described in this paper, that connection was not detected, and thus has been omitted from this diagram.)

Fig. 8. Distribution of actin-binding proteins in discoid platelets and after activation. Discoid platelets (**left** column) stained with five different antisera as indicated to the left. Spread platelets fixed 15 min after exposure to glass coverslip and double-labeled by immunofluorescence with different antisera (**middle** column, antisera indicated to the far left) and with phalloidin (**right** column, same cell as in middle column). Arrows indicate filopodia (antiserum 21). Bar = 5 μ m.

The Molecular Weights and Spatial Distribution of Platelet Actin-Associated Proteins as Determined by Western Blot and by Immunofluorescence of Spread Platelets, Skeletal Muscle, and Cultured Cells

TABLE I

Ab no. ^a	Size of immunogen	Size on blot			Skeletal msl	Fibro-blasts	Location in platelet				
		H ^b	E ^b				D	LE	F	L	CR
6 ^f	100	ND ^c	68		-	N					++ ^d
7 ^{e,f,g}	95	95	95		Z	LE/PN	+	+++			
14	70	70	70		D	ND				+	+++
16 ^{e,f,g}	68	68	68		Z/A	SF/LE					++++
18	65	ND	68/55		-	N/D			++	++	
19 ^e	63	ND	110		-			+++	+		
20 ^{e,f}	60	ND	110		-	F			++++		
21 ^f	58	many	many		-	L/R		+	++++		
26	57	75	75		ND		+	++		++	
28	55	75	57		Z	-		++		++	
30	35	ND	72		D	-	+		+++		
32 ^{e,f,g}	27	ND	43		A	L/PN	+			++++	
33	25	ND	130		I	D		+++			

^a Ab no: The number assigned to each antiserum based on molecular weight of the original antigen in column eluate.

^b H: Homogenate was used for Western blot; E: Pooled elutions from F-actin columns were used for Western blot.

^c Abbreviations listed in the order they appear: ND: not detected; D: diffuse; LE: leading edge (outermost border of lamellipodia); F: filopodia; L: lamellipodia; CR: contractile ring; N: nuclear; PN: perinuclear; SF: stress fibers; R: ruffles; Z: Z-band (barbed ends of actin filaments); A: A-band (myosin-like); I: I-band (actin-like).

^d The number of + indicates the relative intensity of staining.

^e See Figure 8 for example of staining pattern.

^f See Figure 6 for example of staining pattern.

^g See Figure 5 for example of staining pattern.

TABLE II

Categories of New Actin-Binding Proteins According to Their Molecular Weight and Staining Patterns

Category	Staining patterns	Ab no.	Mol. wt. (kDa)
1.	Contractile ring in platelets	6	65
	Perinuclear staining in fibroblasts	18	65
	Nonreactive to muscle		
2.	Leading edge in platelets	7	95
	Leading edge and perinuclear in fibroblasts		
	Z-band in muscle		
3.	Contractile ring in platelets	14	68
	Stress fibers in fibroblast	16	70
	Both Z and A bands in muscle		
4.	Leading edge and filopodia in platelets	19	110
	Filopodia in fibroblasts	20	110
	Nonreactive in muscle		
5.	Leading edge in platelets	21	many bands
	Diffuse in fibroblasts		
	Nonreactive in muscle		
6.	Lamellipodium/diffuse in platelets	26	75
	Nonreactive with fibroblasts	28	75
	Z-band/diffuse in muscle		
7.	Filopodia in platelets	30	72
	Nonreactive with fibroblasts		
	Diffuse in muscle		
8.	Lamellipodium in platelets	32	43
	Lamellipodium in fibroblasts		
	Myosin-like (A-band) in muscle		
9.	Leading edge in platelets	33	130
	Diffuse in fibroblasts		
	Actin-like (I-band) in muscle		