Reversible Binding of Actin to Gelsolin and Profilin in Human Platelet Extracts

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Abstract. This paper documents the reversible appearance of high-affinity complexes of profilin and gelsolin with actin in extracts of platelets undergoing activation and actin assembly. Sepharose beads coupled to either monoclonal anti-gelsolin antibodies or to polyproline were used to extract gelsolin and profilin, respectively, from EGTA-containing platelet extracts and determine the proportion of these molecules bound to actin with sufficient affinity to withstand dilution (high-affinity complexes). Resting platelets (incubated for 30 min at 37°C after gel filtration) contained nearly no high-affinity actin/gelsolin or actin/profilin complexes. Thrombin, within seconds, caused quantitative conversion of platelet profilin and gelsolin to high-affinity complexes with actin, but these complexes were not present 5 min after stimulation. The calcium-dependent actin filament-severing activity of platelet extracts, a function of free gelsolin, fell in concert with the formation of EGTA-stable actin/gelsolin complexes, and rose when the adsorption experiments indicated that free gelsolin was restored. The dissociation of high-affinity complexes was temporally correlated with the accumulation of actin in the Triton-insoluble cytoskeleton.

AGENTS such as collagen, thrombin, or ADP activate blood platelets, altering both their metabolism and structure. The cells change in shape from a smooth disk to a sphere covered with spines. These spines contain actin bundles, which are not found in the unactivated cell (Gonnella and Nachmias, 1981). This change is accompanied by the polymerization of actin. While a major portion of the resting platelet's actin is unpolymerized, only ~20% of the actin of thrombin-stimulated cells is unpolymerized (Fox and Phillips, 1983). The molecular events leading to the initiation and promotion of actin polymerization are therefore of considerable interest.

Since the estimated actin concentration of platelets is 0.25–1.0 mM (Fox, 1986), which is at least 1,000–2,000 times above the critical concentration at which actin monomers spontaneously polymerize in vitro (Pollard and Cooper, 1986), control mechanisms are presumed to operate within the cell to keep the majority of the actin of the resting platelet in the unpolymerized state. One candidate for this regulatory mechanism is profilin, a protein that forms binary complexes with actin monomers (Carlsson et al., 1976). Another is gelsolin, which has several functions with respect to actin and is present in human platelets (Lind et al., 1982; Markey et al., 1982*b*; Kurth et al., 1983). It can sever actin filaments, thereby shortening them, and remains bound to the fast-growing ends of actin filaments. By so doing, gelsolin prevents monomer addition, but also creates new filament ends. In addition to its end-blocking properties, gelsolin, under certain conditions, can nucleate actin filament assembly (reviewed in Stossel et al., 1985 and Pollard and Cooper, 1986). Gelsolin therefore has the potential to affect the formation and length of actin filaments.

The interactions of these regulatory proteins with actin are complex. Lindberg and co-workers, who first discovered profilin, have documented the existence of profilin/actin complexes in extracts of cells of sufficiently high affinity that they resisted dissociation during gel filtration (Markey et al., 1982*a*). On the other hand, they showed that profilin was no longer associated with actin in extracts of thrombin-activated platelets, at a time when the actin had polymerized into filaments (Markey et al., 1981). This finding suggested that the profilin/actin interaction was somehow regulated in vivo. It has recently been reported that phosphatidylinositol 4,5-biphosphate (PIP_2)[1], which turns over rapidly during platelet activation, can promote dissociation of profilin/actin complexes in vitro (Lassing and Lindberg, 1985).

Others however, have consistently purified profilin from other cells in an uncomplexed form and have shown it to have relatively low affinity for monomeric actin (K_d = 1–11 μM). Such profilins have been relatively ineffective in sequestering actin monomers under polymerizing conditions in which actin filaments were present, a result predictable from the measured association constant of actin for profilin and for the

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1. *Abbreviations used in this paper:* ABP, actin-binding protein; PIP_2, phosphatidylinositol 4,5-biphosphate.

ends of actin filaments (Tseng and Pollard, 1982; Tobacman et al., 1983; Dinubile and Southwick, 1985). These findings have been interpreted to indicate that a simple mass action effect describes profilin's interaction with actin, and that active regulation of actin polymerization might be limited to factors (such as gelsolin) that influence the addition of monomers to the fast-growing ends of actin filaments.

Evidence that gelsolin is under active regulation is somewhat firmer, although the situation is also complicated. Calcium in micromolar concentrations is required for gelsolin to sever actin filaments, and to enhance both its ability to nucleate filament assembly or to bind to filament ends. The knowledge that cytosolic calcium rises during thrombin-induced platelet activation (Rink et al., 1982) suggests that gelsolin might be linked to actin assembly in thrombin-treated platelets. Bryan and co-workers have suggested, however, that gelsolin/actin complexes formed in the presence of calcium are not completely reversible, either in vitro or in extracts of platelets treated with a calcium ionophore (Kurth et al., 1983; Kurth and Bryan, 1984; Bryan and Kurth, 1984). Recent evidence indicates however, that the apparent irreversibility of the actin/gelsolin interaction seen in certain experimental situations may not apply to living cells (Chaponnier et al., 1987) and that PIP_2 dissociates actin/gelsolin complexes in vitro (Janmey and Stossel, 1987).

This paper describes the quantitative adsorption of profilin and gelsolin from platelet extracts prepared under different states of activation in an effort to document the existence of high-affinity complexes of these regulatory proteins with actin and to correlate their formation and dissociation with platelet actin assembly. The findings are consistent with active regulation of both profilin/actin and gelsolin/actin interactions.

Materials and Methods

Materials and Protein Preparation

Cyanogen bromide-activated Sepharose beads were obtained from Pharmacia, Inc., Piscataway, NJ. Polyproline, human thrombin, ADP, aprotinin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), leupeptin, DNase I, anti-fibrinogen antiserum, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO. Polyproline (average M_r 31,000) was coupled to Sepharose beads following the method of Tanaka and Shibata (1985) at a concentration of 5 mg/ml beads. DNase I was coupled to Sepharose at a concentration of 2 mg/ml. Monoclonal anti-gelsolin antibodies were prepared and coupled to Sepharose beads as previously described (Chaponnier et al., 1986). Sepharose beads conjugated to mouse monoclonal antibodies prepared to rabbit macrophage actin-binding protein (ABP) were the generous gift of Drs. Robert Ezzell, Sheila Egan, and John Hartwig (from our institution). Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (1971). Labeling of muscle actin with *N*-(1-pyrenyl)-iodoacetamide was carried out as described previously (Janmey et al., 1985).

Platelet Preparation

Nine parts of whole blood were drawn into one part of a solution containing 86 mM trisodium citrate, 53 mM citric acid, and 111 mM glucose, pH 4.6, from healthy subjects. Blood was drawn by venipuncture using a silicone-coated butterfly needle and experiments were performed within 2–3 h of blood drawing. Platelet-rich plasma was prepared immediately by centrifuging the blood at 160 *g* for 10 min either at room temperature or 4°C. Platelet-rich plasma was passed through a 50-ml column of Sepharose 2B at room temperature that had been equilibrated with a buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.5 mM Na_2HPO_4, and 10 mM glucose, pH 7.4. The gel-filtered platelets were divided into 0.5-ml portions in plastic tubes and incubated at 37°C for 30 min. The preparation procedures facilitated analysis of small numbers of cells within hours of collection from single donors and did not require the addition of prostaglandin E_1, prostacyclin, aspirin, or local anesthetics to prevent platelet activation.

Experimental Design

Agonists were added in 50-μl volumes to 0.5-ml samples of the platelet suspensions, and the tubes were gently agitated by hand, but without mechanical stirring, for 5 s, and then placed in a 37°C heating block. At appropriate times the platelets were lysed by the addition of an equal volume of a lysis solution containing 120 mM Pipes, 50 mM Hepes, 20 mM EGTA, 4 mM $MgCl_2$, 10 mM glucose, 20 μg/ml leupeptin, 156 μg/ml benzamidine, 80 μg/ml aprotinin, 1 mM PSMF, 1.5% Triton X-100, pH 7.2 (2X-PHEM-Tx) (Schliwa and van Blerkom, 1981). After 2 min at room temperature, the extracts were centrifuged at 12,000 *g* at room temperature in an Eppendorf centrifuge (Brinkmann Instruments, Inc., Westbury, NY) for 10 min to sediment what is operationally designated the Triton-insoluble cytoskeleton. 500 μl of the resulting supernatant fluid was then added to 30 μl of a 50% (vol/vol) suspension of Sepharose beads conjugated either to anti-gelsolin antibodies ("anti-gelsolin beads") or to polyproline ("polyproline beads"). The mixtures were rotated end-over-end for 2 h at 4°C and then centrifuged at 12,000 *g* for 2 min. The beads were washed once with 1X-PHEM-Tx with 5 mM ATP, and incubated with a solution containing 0.3 M $MgCl_2$, 1 mM EGTA, 50 mM Tris, and 100 mM NaCl, pH 7.4, for 15 min at room temperature. After centrifugation, the beads were washed twice with a solution containing 50 mM Tris, 100 mM NaCl, 1 mM EGTA, pH 7.4. The incubation with 0.3 M $MgCl_2$ was included to depolymerize any actin filaments associated with the beads (Chaponnier et al., 1987). The beads were boiled in the gel sample buffer of Laemmli (Laemmli, 1970) and loaded onto 5–15% polyacrylamide minigels. After staining with Coomassie Blue, the gels were scanned with a Zeineh laser densitometer (Biomed Instruments, Inc., Fullerton, CA) and the relative peak areas quantified by a computer graphics program developed by Mr. Marc Kozam in our laboratory. The molar ratios of actin to gelsolin, or actin to profilin were thereby computed. Previous work has shown that under conditions where 1:1 actin/gelsolin complexes are formed, as assessed by equilibrium sedimentation (Coue and Korn, 1985), the Coomassie Blue-binding properties of gelsolin and actin immunoprecipitated by anti-gelsolin Sepharose beads are equal (Chaponnier et al., 1987). The Coomassie Blue dye-binding properties of profilin and actin were compared. Actin and profilin solutions were prepared in the same low-ionic strength buffer (2 mM Tris, 0.2 mM ATP, 2 mM $CaCl_2$, pH 7.8, buffer A) and the protein concentration of the solutions determined by the method of Lowry (Lowry et al., 1951). Equal amounts of the proteins (2–5 μg) were mixed before electrophoresis on a 5–15% SDS-polyacrylamide gel. Profilin bound Coomassie Blue 82% as well as actin on a weight basis, and actin/profilin ratios were calculated accordingly.

Determination of the Actin Filament-severing Activity of the Platelet Extracts

Since free gelsolin can sever actin filaments in a calcium-dependent manner, while actin/gelsolin complexes cannot (Janmey et al., 1985), the filament-severing capacity of platelet extracts was examined so that correlations could be made between this functional assay and the immunologic determinations of the state of the actin/gelsolin interaction made with the anti-gelsolin beads. As demonstrated previously (Walsh et al., 1984; Bryan and Coluccio, 1985; Coue and Korn, 1985; Janmey and Stossel, 1986; Northrup et al., 1986), pyrene-labeled actin filaments that are added to gelsolin, related actin-severing proteins, or gelsolin/actin complexes, before dilution to an actin concentration that is below the critical concentration of the slow-exchanging end of the filaments (but above that of the fast-exchanging end), depolymerize at a rate proportional to the number of these ends because the fast-exchanging (barbed) end of the filament is capped by gelsolin. The depolymerization rate therefore reflects the number of cuts made in the pyrene-labeled filaments by the gelsolin molecules originally present. Since gelsolin/actin complexes bind to, but do not sever actin filaments, this depolymerization assay measures only the concentration of free gelsolin and is insensitive to added gelsolin/actin complexes.

In these measurements, 30 μl of the Triton-soluble platelet extract supernatants (total protein concentration 0.4–0.8 mg/ml) were incubated with 5 μl of 12 μM pyrene-labeled F-actin for 10 s. To this mixture was then added 370 μl of buffer BC (20 mM Tris, 0.2 mM ATP, 1 mM $CaCl_2$, 0.2 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 150 mM KCl, pH 7.8) to reduce the pyrene/actin concentration to 150 nM. The initial rate of depolymerization was

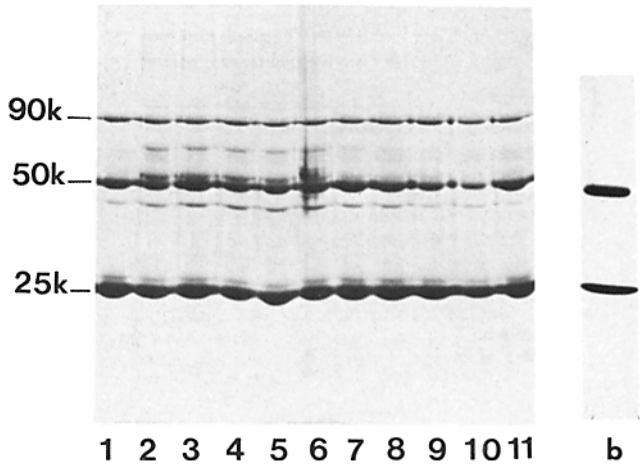

Figure 1. Time course of EGTA-stable gelsolin/actin complex formation in human platelet extracts after thrombin addition (1 U/ml). After removing the Triton-insoluble cytoskeleton, platelet extracts were added to anti-gelsolin beads. The beads were washed and the adhering proteins eluted and subjected to electrophoresis on 5–15% minigels which were stained with Coomassie Blue. Lane *1*, no thrombin added. Lanes *2–10*, postthrombin: Lane *2*, 5 s; lane *3*, 10 s; lane *4*, 15 s; lane *5*, 20 s; lane *6*, 30 s; lane *7*, 60 s; lane *8*, 90 s; lane *9*, 5 min; lane *10*, 20 min. Lane *11*, control platelets 20 min after the start of the experiment, no thrombin added. Lane *b*, anti-gelsolin Sepharose beads that were not incubated with a cell extract.

measured from the initial rate of fluorescence decrease, as previously described (Janmey and Stossel, 1986). Extracts were tested before and after incubation with the anti-gelsolin Sepharose beads.

Determination of Actin Incorporation into the Triton-insoluble Cytoskeleton

The actin content of the Triton-insoluble cytoskeleton was determined by removing the supernatant fluid from the pellet and then adding 20 μl of gel sample buffer. After boiling, the entire cytoskeleton was subjected to electrophoresis on 5–15% polyacrylamide gels in the presence of SDS. The gels were scanned and the relative amount of actin in each lane was determined.

Determination of the Actin, Profilin, and Gelsolin Content of Platelet Extracts

750 μl of gel-filtered platelets that had been incubated at 37°C for 30 min were added to an equal volume of 2X-PHEM-Tx. The mixture was centrifuged at 12,000 *g* for 10 min at room temperature. The Triton-insoluble pellet was taken up in gel sample buffer and subjected to electrophoresis on 5–15% SDS-polyacrylamide gels. 500 μl of the supernatant fluid was added to 60 μl of anti-gelsolin beads or 60 μl of polyproline beads. 500 μl of a 1:20 dilution of the supernatant (diluted in 1×-PHEM-Tx) was added to 60 μl of DNase-Sepharose beads. The mixtures were rotated at 4°C for 2 h, and then centrifuged at 12,000 *g* for 10 min. The supernatant fluids were added to a second set of beads (anti-gelsolin, polyproline, and DNase, respectively) and incubated with tumbling for 2 h at 4°C. Both sets of beads were then washed as described above, and subjected to electrophoresis on 5–15% polyacrylamide gels. The gels were scanned and the ratios of the proteins calculated. The amount of actin in the cytoskeleton was also determined and used to calculate the ratio of actin to profilin or gelsolin in the whole cell. Less than 5% of the amount of gelsolin, profilin, or actin bound to the first set of beads was bound to the second, indicating virtually complete adsorption.

Isolation and Characterization of the 14-kD Polyproline-binding Protein from Platelets

1 liter of outdated human platelets was obtained from the blood bank and the concentrates were centrifuged twice at 160 *g* for 20 min to remove red cells, white cells, and cellular debris. The platelets were then collected by centrifugation at 3,000 *g* for 20 min and suspended at a 30% concentration in a solution containing 50 mM Tris, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 50 μM beta-mercaptoethanol, 1 mM PMSF, 50 mM benzamidine, and 1 mg/ml aprotinin, pH 7.5. The cells were disrupted by sonication while in an ice bath with five 10-s bursts of sonication at maximum power in an Ultrasonics W 185 F apparatus (Heat Systems-Ultrasonics, Inc., Plainview, NY). After centrifugation at 65,000 *g* for 90 min, 20 ml of the resulting supernatant fraction was passed over a 10-ml column of polyproline-Sepharose. The column was washed with a solution containing 20 mM Tris, 150 mM NaCl, 0.5 mM ATP, pH 7.6 (TBS-ATP) until no eluting protein could be detected by UV absorbtion. The column was then washed with TBS-ATP containing 2 M urea, which eluted a protein that migrated as a single band of 43 kD on SDS-PAGE, and was presumed to be actin, in accordance with the observations of Tanaka and Shibata (1985). A subsequent wash with TBS-ATP containing 6 M urea eluted a protein that migrated with a molecular mass of 14 kD on 5–15% SDS-polyacrylamide gels. Fractions containing this protein were dialyzed for 3 d in 2 liters of buffer A, with four changes of buffer.

The amino acid composition of the 14-kD polypeptide was determined on an amino acid analyzer (model 6300; Beckman Instruments Inc., Palo Alto, CA) after hydrolysis with 6N HCl for 24 h at 110°C.

Isoelectric focussing of the isolated 14-kD polypeptide was carried out by the method of O'Farrell et al. (1977).

The functional effect of the 14-kD protein on actin assembly was determined by adding pyrene-labeled G-actin to different concentrations of the 14-kD protein, in buffer A. $MgCl_2$ and KCl were then added to final concentrations of 2 and 150 mM, respectively, to initiate actin polymerization. After a 2.5-min incubation to test for nucleating activity, platelet gelsolin was added to accelerate polymerization from the low-affinity filament end. The binding affinity of the 14-kD protein for actin was calculated from the steady state F-actin concentration determined from the final fluorescence levels (Janmey et al., 1985) using the expression $K_d = C_o [P]/[PA]$, where $[PA]$ is the concentration of the actin–14-kD protein complex (equal to the decrease in F-actin concentration brought about by the addition of the 14-kD protein), $[P]$ is the free 14-kD protein concentration (equal to the difference between the total 14-kD protein concentration and $[PA]$), and C_o is the critical actin concentration in the presence of gelsolin, taken to be 0.7 μM (Janmey and Stossel, 1986).

The 14-kD polyproline-binding protein was tested for actin filament-severing activity by adding various amounts of it to pyrene-labeled F-actin, diluted to 260 nM in buffer B. The initial rate of actin depolymerization under these conditions is proportional to the number of filament ends and is a sensitive measure of filament severing (Walsh et al., 1984; Bryan and Coluccio, 1985).

Results

Identification of High-Affinity Actin/Gelsolin or Actin/Profilin Complexes in Platelet Extracts

SDS-PAGE analysis of anti-gelsolin IgG-conjugated beads revealed polypeptides with mobilities corresponding to M_r 25,000 and 50,000, which were the light and heavy chains of the antibody, respectively, as shown in Fig. 1. No Coomassie Blue–stainable bands were seen on SDS-PAGE of beads conjugated to polyproline. As shown in Fig. 1, anti-gelsolin beads precipitated distinct polypeptides from platelet extracts. The major additional bands in resting platelet extracts incubated with anti-gelsolin beads had mobilities identical to the proteins actin and gelsolin. When polyproline beads were used, the major bands were noted with mobilities of 14 and 42 kD (Fig. 2). With certain types of activation, additional bands, believed to be fibrin derived, were also associated with the beads (see below).

The 14-kD polyproline–binding polypeptide was identified as profilin because: (*a*) it has an affinity for polyproline-Sepharose beads and could be eluted from the beads with a polyproline solution (average M_r 6,000, 10 mg/ml), as described for chick embryo profilin (Tanaka and Shibata, 1985); (*b*) it was present when platelet extracts were added to DNase

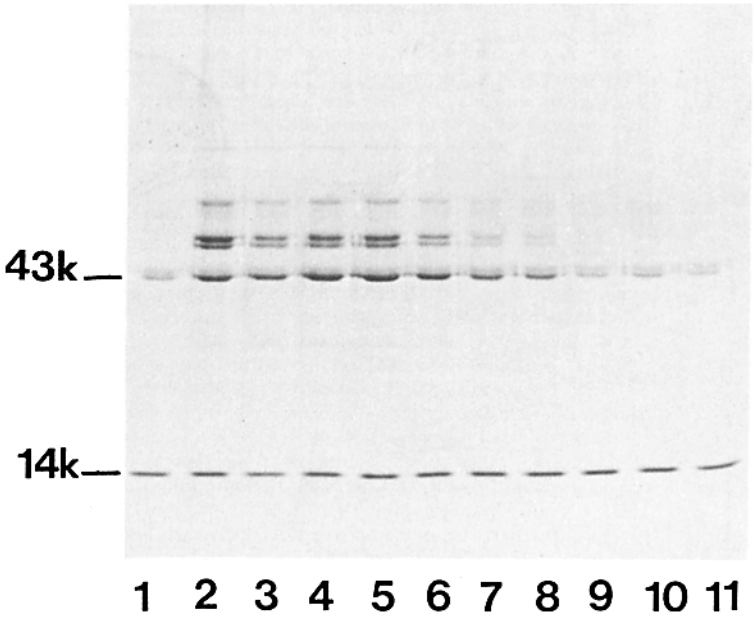

Figure 2. Formation of actin/profilin complexes in human platelet extracts after the addition of thrombin (1 U/ml). After removing the Triton-insoluble cytoskeleton, platelet extracts were added to polyproline-Sepharose beads. The beads were washed and the adhering proteins eluted and subjected to electrophoresis on 5–15% minigels that were stained with Coomassie Blue. Lane *1*, no thrombin added. Lanes *2–10*, post-thrombin: lane *2*, 5 s; lane *3*, 10 s; lane *4*, 15 s; lane *5*, 20 s; lane *6*, 30 s; lane *7*, 60 s; lane *8*, 90 s; lane *9*, 5 min; lane *10*, 20 min. Lane *11*, control platelets 20 min after the start of the experiment, no thrombin added.

beads 10 s after thrombin addition; (*c*) it had an amino acid composition similar to that of conventionally purified platelet profilin (Table I); (*d*) it had a pI of 7.1; (*e*) it neither accelerated actin filament assembly nor severed actin filaments (Fig. 3); (*f*) its addition to actin monomers before the addition of salts inhibited actin polymerization in a manner consistent with other profilins, with a K_d for actin of 3.7 and 4.5 μM for the two experiments shown in Fig. 3. The 14-kD polypeptide is thus distinguished from other low molecular mass actin-binding proteins such as depactin (Mabuchi, 1983) or actophorin (Cooper et al., 1986), which either accelerate filament assembly or sever filaments.

The 42-kD polypeptide was identified as actin by its electrophoretic mobility and by the finding that if platelet extracts were mixed with Sepharose beads conjugated to DNase I and the fluid phase subsequently added to the polyproline beads, only the 14-kD polypeptide was associated with the beads. This finding indicates that the 42-kD peptide has the property of binding DNase I, as does actin.

No gelsolin bound to the polyproline beads after incubation with cell extracts, nor was the 14-kD polypeptide associated with anti-gelsolin beads after extract addition, indicating that a ternary gelsolin/actin/profilin complex was not formed. If calcium (final free concentrations, 2 mM) was added to the lysis and washing solutions, the molar ratio of actin to gelsolin associated with the anti-gelsolin beads was 2:1. Since gelsolin binds two actin molecules in the presence of micromolar Ca^{2+} in vitro, this is an expected result and indicates that the washing procedures removed any actin oligomers that might have been present and that the proteins bind Coomassie Blue dye equivalently.

The quantitative removal of gelsolin or profilin from the depleted extracts was assessed by adding the extracts to a second set of beads after their initial incubation with beads. No gelsolin or profilin bound to the second set of beads (data not shown). Although these extracts still contained large amounts of actin, no actin bound to the second set of beads, indicating the adequacy of the washing procedures and the validity of the process used to assess the interactions of actin with gelsolin and profilin.

Further confirmation that actin in the extracts was not binding to the beads directly was obtained by adding the extracts to Sepharose beads conjugated to mouse monoclonal antibodies with specificity towards rabbit macrophage ABP. As shown in Fig. 4, the beads immunoprecipitated ABP, but, after the washing procedures little actin was seen, despite the fact that ABP was tightly bound to the beads. This result does not imply that ABP is not bound to actin in the cell or in the extract, but rather, that protein/protein complexes of moderate affinity (in the micromolar range; Hartwig and Stossel, 1981) may dissociate during this assay.

Table I. Amino Acid Composition of Profilins and Human Platelet 14-kD Polyproline-binding Protein

Amino acid	Calf spleen profilin	Human platelet profilin	Human platelet 14-kD polyproline-binding peptide
Asx	10.56	10.6	10.42
Thr	8.45	8.2	7.72
Ser	6.34	8.2	7.42
Glx	6.34	6.9	4.63
Pro	2.82	3.4	4.38
Gly	11.97	12.0	11.45
Ala	7.75	7.6	7.49
Cys	2.11	ND	ND
Val	7.75	8.2	9.68
Met	4.23	3.1	2.99
Ile	4.93	4.1	3.78
Leu	7.75	10.7	9.73
Tyr	2.82	2.7	3.89
Phe	3.52	2.4	3.18
Lys	6.34	6.9	8.44
His	1.41	1.4	0.57
Arg	3.52	3.4	4.23
Trp	1.41	ND	ND

Data for calf spleen profilin is from Nystrom et al. (1979). Data for human platelet profilin is calculated from data of Markey et al. (1978). Data for human platelet 14-kD polyproline-binding polypeptide is from this report.

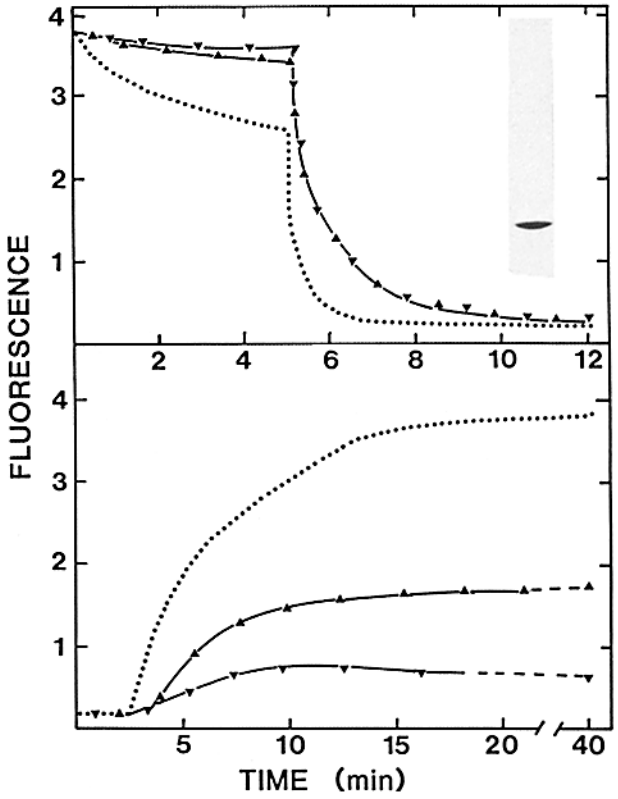

Figure 3. Effect of 14-kD polyproline-binding (platelet profilin) on actin assembly and disassembly. (*Top*) Pyrene-labeled F-actin was diluted to 260 nM in solutions containing buffer B alone (*dotted line*) or with 15 (▲) or 30 μM (▼) platelet profilin. 5 min after dilution, 160 μM gelsolin was added to sever actin filaments and accelerate their depolymerization. A Coomassie Blue–stained SDS-PAGE gel of the 14-kD protein preparation used is shown in the inset. (*Bottom*) Pyrene-labeled G-actin (3.5 μM) was added to buffer B in the absence (*dotted line*) or presence of 10 (▲) or 20 μM (▼) platelet profilin. After 2.5 min of incubation in salt-containing solutions, 0.02 μM gelsolin was added to accelerate polymerization. Fluorescence is plotted on an arbitrary scale with a baseline signal from samples containing no pyrene/actin subtracted for each concentration of profilin.

Effects of Various Procedures on the Detection of Gelsolin/Actin Complexes in Platelet Extracts

Platelet Isolation Procedure. Anti–gelsolin beads incubated with platelet extracts removed both free gelsolin and EGTA-stable actin/gelsolin complexes. Extracts prepared immediately after gel filtration of platelets contained a variable proportion of the platelet gelsolin bound to actin in an EGTA-stable complex. However, further incubation of the gel-filtered platelets for 30 min at 37°C resulted in a change in the mean actin/gelsolin ratio of the extracts from 0.66 ± 0.28 to 0.32 ± 0.26 (mean and standard deviation of five experiments). This finding is likely due to the fact that platelet isolation procedures cause some degree of activation of the cells, but not in an all-or-none fashion, since expected cellular responses to agonists can be obtained after such manipulations if the appropriate conditions are used and the cells allowed to reequilibrate (Fox et al., 1984). The decrease in the actin/gelsolin ratio after gel filtration suggests that the EGTA-stable gelsolin/actin complex can be dissociated

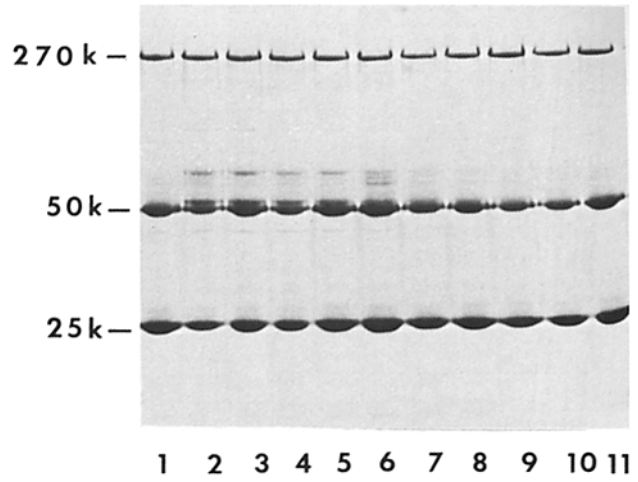

Figure 4. Association of actin with anti-ABP-Sepharose beads incubated with platelet extracts. The samples were processed as described for Figs. 1 and 2 and subjected to electrophoresis on 5–15% minigels. Lane *1*, no thrombin added. Lanes *2–10*, postthrombin: lane *2*, 5 s; lane *3*, 10 s; lane *4*, 15 s; lane *5*, 20 s; lane *6*, 30 s; lane *7*, 60 s; lane *8*, 90 s; lane *9*, 5 min; lane *10*, 20 min. Lane *11*, control platelets 20 min after the start of the experiment, no thrombin added.

and free gelsolin generated after mild platelet stimulation, though the standard deviations of the measurements are large.

Effect of Thrombin. Within 5 s after the addition of thrombin (1 U/ml) to unstirred platelet suspensions, EGTA-stable complexes were noted (Figs. 1 and 5). The proportion of gelsolin present in these complexes peaked within 15–20 s thereafter and then declined to near baseline levels by 5 min. Platelets extracted several minutes after thrombin stimulation contained no more EGTA-stable complexes than did ei-

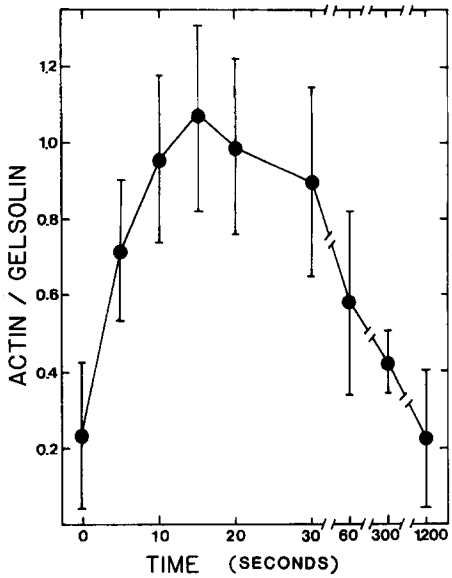

Figure 5. Time course of formation of EGTA-stable actin/gelsolin complexes after thrombin addition. The ratio of actin/gelsolin in each sample was calculated after scanning gels such as that shown in Fig. 1. Mean values of 11 experiments are shown. Error bars indicate the standard deviation of identical time points from different experiments.

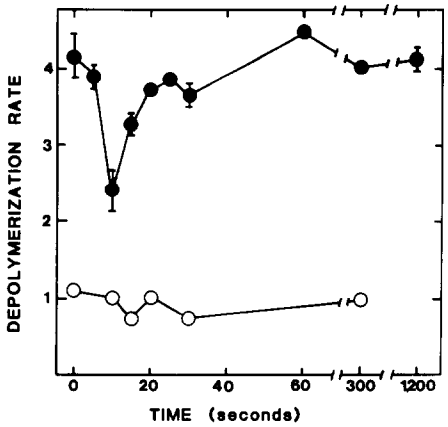

Figure 6. Change in actin filament-severing activity of platelet extracts after thrombin stimulation. 30 μl of platelet extract was added to pyrene-labeled actin filaments, and the mixture diluted 10 s later to a final pyrene/actin concentration of 150 nM. The fluorescence of the diluted mixture was recorded as a function of time. The initial rate of fluorescence decrease is proportional to the rate of actin filament depolymerization, and to the number of filament ends. The greater the filament-severing capacity of the extract, the larger the number of filament ends, and hence the greater the depolymerization rate. The rates of depolymerization are shown for platelet extracts prepared at different time points after the addition of thrombin either before (*solid circles*) or after (*open circles*) depletion of gelsolin by incubating the extracts with anti-gelsolin beads.

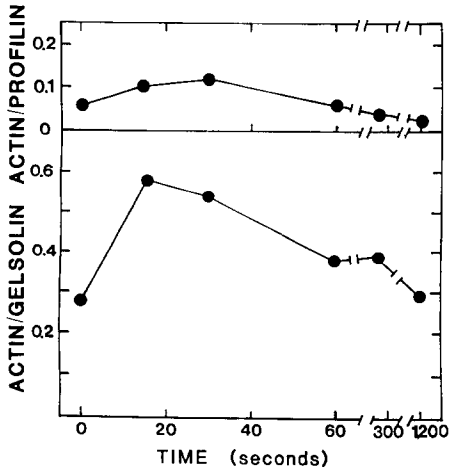

Figure 7. Formation of actin/profilin and EGTA-stable actin/gelsolin complexes after stimulation of platelets with ADP. A representative experiment is shown in which ADP (5 μM) was added to gel-filtered platelets for the indicated times before lysis with a Triton-containing buffer. EGTA-stable actin/gelsolin complexes form transiently but to a lesser extent than when thrombin is used to stimulate the platelets. Little change in the proportion of profilin bound to actin is noted.

ther unstimulated platelets incubated for the same period of time, or platelets reequilibrated after gel filtration. Anti-gelsolin beads recovered from extracts of thrombin-stimulated platelets contained several additional polypeptides in the range of 50–70 kD. These polypeptides, believed to result from the action of thrombin on fibrinogen, were not present if ADP was used as the stimulus. Immunoblotting (Towbin et al., 1979) indicated that the peptides were cross-reactive with an anti-fibrinogen antibody (data not shown). These polypeptides were also present on Sepharose anti-ABP beads (Fig. 4) incubated with the same extract used in the experiments shown in Figs. 1 and 2, though no actin was associated with the beads. These findings suggest that the actin associated with the anti-gelsolin beads was not bound to fibrin. Further support for this conclusion is found in the fact that the actin/gelsolin ratio was observed to change in response to stimuli at times when these polypeptides were not found adsorbed to the beads (i.e., immediately after gel filtration and after ADP stimulation). The fibrin is likely derived from fibrinogen contained within the platelets' alpha granules and/or plasma fibrinogen trapped within the invaginations of the plasma membrane that form the open canicular system of the platelet.

As shown by the solid circles in Fig. 6, platelet extracts prepared before thrombin stimulation contained actin filament-severing activity (reflected by a high rate of depolymerization of exogenous pyrene-labeled actin filaments). This activity fell after thrombin stimulation, reaching a nadir 15 s after thrombin addition, coincident with the peak of EGTA-stable complexes as determined by the immunologic assay. This was followed by a return to the previous level. This depolymerizing activity was not seen if EGTA was added to the reaction cuvette, a finding consistent with the known calcium requirement of gelsolin. Further evidence that the loss of filament severing capacity was related to changes in gelsolin was found by examining the platelet supernatants after incubation with the anti-gelsolin beads. As shown by the open symbols in Fig. 6, the majority of the filament-severing capacity of the extracts was removed by the immunoprecipitation with anti-gelsolin beads. Of note is the presence of residual filament-severing activity in the extracts after gelsolin depletion. Its source is not currently known.

Effects of ADP. The platelet agonist ADP (5 μM) also induced EGTA-stable complex formation in platelets, although the proportion of gelsolin present in EGTA-stable complexes was never as great as with thrombin (Fig. 7). If ADP was again added to the platelets 20 min after the first challenge, a second cycle of EGTA-stable complex formation was noted, although the magnitude of the increase was lower than with the initial stimulation. Addition of a second dose of thrombin, however, did not induce a second cycle of complex formation (data not shown).

Effects of Various Treatments on the Detection of High-Affinity Profilin/Actin Complexes in Platelet Extracts

Platelet Preparation. Platelets extracted immediately after gel filtration contained more profilactin complexes than platelets incubated for an additional 30 min at 37°C, suggesting that the affinity of profilin for actin changed during this interval, possibly because gel filtration caused some degree of cell activation and shape change. (The actin/profilin ratio fell from 0.25 ± 0.23 to 0.14 ± 0.12 in four experiments.) On two occasions the lysis buffer was added directly to platelet-rich plasma without subjecting the platelets to gel filtration. The actin/profilin ratio of these minimally perturbed cells was 0.10 and 0.03, suggesting that the gel filtration step was not responsible for the low actin/profilin ratio found in resting gel-filtered cells.

Effects of Thrombin. After the addition of thrombin the

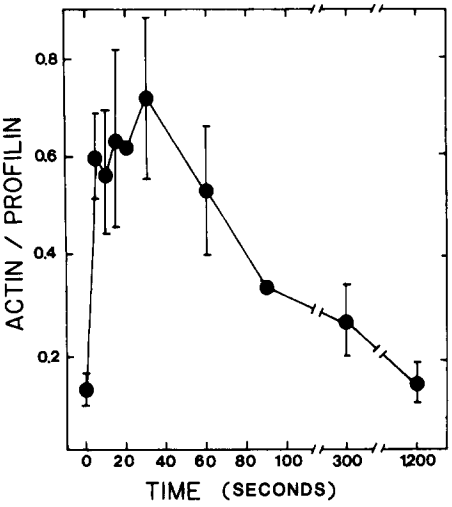

Figure 8. Time course of formation of actin/profilin complexes after stimulation of human platelets with thrombin (1 U/ml). The ratio of actin/profilin in each sample was calculated after scanning gels such as that shown in Fig. 2. The mean and standard deviations of five experiments are shown.

actin/profilin ratio increased within 10 s, and peaked at ~30 s. This ratio declined to near-baseline values by 5 min after thrombin addition, as shown in Figs. 2 and 8. Restimulation with thrombin did not lead to a second cycle of complex formation.

Effects of ADP. Only a small change in the actin/profilin ratio of extracts was noted after the addition of ADP (5 μM) (Fig. 7).

Cytoskeletal Actin Content of Stimulated Platelets

As shown in Fig. 9, the amount of actin present in Triton-insoluble cytoskeletons of thrombin-activated platelets increased after thrombin stimulation with the majority of the material appearing in the Triton-insoluble cytoskeleton during the time period (15–30 s post-thrombin) where changes

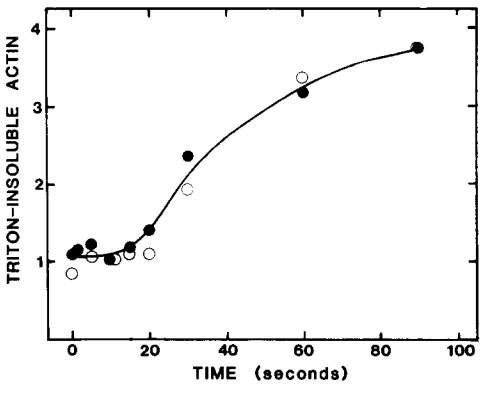

Figure 9. Incorporation of actin into the Triton-insoluble cytoskeleton of human platelets after the addition of thrombin (1 U/ml). The Triton-insoluble cytoskeleton of platelets was subjected to electrophoresis on 5–15% SDS-polyacrylamide gels which were stained with Coomassie Blue. The relative amount of actin present in each cytoskeleton was determined by gel densitometry. The different symbols indicate values from two separate experiments.

in the actin-binding properties of gelsolin and profilin were noted. The rate of actin accumulation in the cytoskeletons, a consistent finding during the course of these experiments, is somewhat slower than that reported by others (Jennings et al., 1981), who have described almost complete skeleton formation within 15 s.

Determination of the Actin/Profilin and Actin/Gelsolin Ratio of Platelets

Platelet extracts were added to an excess of anti-gelsolin, polyproline, and DNase beads. PAGE and gel densitometry were used to determine the relative amounts of actin, profilin, and gelsolin adsorbed to the beads and the amount of actin present in the Triton-insoluble cytoskeleton of resting cells.

In two experiments the actin/profilin ratio of the Triton-soluble supernatant was 4.3:1 and 4.1:1, while the actin/profilin ratio of the whole cell (taking the amount of actin in the pellet into account) was 6.5:1 and 5.4:1 (mean, 6:1). The actin/gelsolin ratio of the Triton-soluble supernatant was 24.3:1 and 26:1, and the ratio of the whole cell was 37.3:1 and 34.7:1 (mean, 36:1). 25 and 35% of the cell's total actin was present in the Triton-insoluble pellet.

The protein concentration of the extracts was found to range between 0.43 and 0.71 mg/ml. If actin accounts for 20% of the platelet's protein content, the actin concentration in such extracts is 2–3.3 μM. The calculated profilin and gelsolin concentrations in the uncentrifuged extracts are in the range of 333–550 and 55–92 nM, respectively. Since the profilin present in high-affinity profilin/actin complexes (calf spleen profilactin) has a K_d with respect to actin of $\sim 10^{-8}$ M (Lassing and Lindberg, 1985), and the K_d of gelsolin for actin in Ca^{2+} is 10 nM or less (Coue and Korn, 1985), neither gelsolin/actin nor (high-affinity) profilin/actin complexes present in the extracts initially would be expected to dissociate under the conditions of these experiments.

Discussion

One strategy used in studying the regulation of actin assembly is to determine if regulatory proteins bind to actin at times of cell activity. The existence of relatively high-affinity interactions between actin and some of these proteins facilitates this approach. This study examines the interactions of gelsolin and profilin with actin in the period immediately after platelet stimulation and describes the actin-binding properties of platelet gelsolin and profilin in extracts of detergent lysed cells.

High- and Low-Affinity Binding of Profilin to Actin

Profilin was initially isolated from calf spleen (Carlsson et al., 1976), and subsequently from human platelets (Harris and Weeds, 1978; Markey et al., 1978). The results reported here indicate that profilin can exist in at least two states in a single cell type, with regard to its affinity for actin. The existence of the high-affinity state, analogous to the profilin/actin complexes described by Carlsson and his associates (1976), is clear from the analysis of extracts prepared in the first 30 s after the addition of thrombin. These complexes persist despite the dilution necessarily imposed by the lysis and washing buffers. While some have suggested, based on

the analysis of kinetic data, that profilin can bind to actin filament ends (Pollard and Cooper, 1984), we believe that we have excluded this possible explanation for our findings by choosing conditions that cause actin filaments to depolymerize, though our kinetic data (Fig. 3) are consistent with this hypothesis.

The failure to recover high-affinity profilin/actin complexes in extracts prepared from resting platelets or recovered platelets does not necessarily indicate that profilin is not complexed to actin in the cells themselves. A relatively low-affinity interaction between actin and profilin would not be expected to result in detectable complexes in this assay, given the amount of dilution that results from cell lysis and washing of the polyproline-Sepharose beads. Thus, the experimental procedures used cannot distinguish free profilin from profilin bound to actin with low affinity, and cannot be used to test whether low-affinity profilin has mass action–related regulatory effects on actin assembly in this cell type. They do suggest however that the actin-binding properties of profilin can change rapidly, in a manner consistent with a dependence upon intracellular metabolic changes, such as those suggested by Lassing and Lindberg (1985). Further, they suggest that there is not enough profilin (assuming that all profilin molecules bind to polyproline) to complex with all the unpolymerized actin in platelets, and that other factors may well be important in regulating actin assembly.

The conclusion reached here, that a high percentage of platelet profilin is in a low-affinity state with respect to actin, differs from that reached by Markey and colleagues (1981) who found more free profilin in platelet extracts after thrombin stimulation than before. The use by these workers of a washing technique to prepare platelets may have caused them to examine cells that had a higher baseline level of high-affinity complexes (because of platelet activation during centrifugation) than cells prepared by gel filtration. The re-equilibration step used in the studies reported here (incubating cells for 30 min at 37°C after gel filtration) may have further aided the detection of low-affinity complexes in resting cells.

The finding that resting cells (which contain a relatively small amount of polymerized actin) contain the low-affinity form of profilin suggests a number of possibilities: (*a*) low-affinity profilin may be adequate to maintain actin in a monomeric state; (*b*) other as yet undefined regulators are involved in preventing actin polymerization; (*c*) there are few free fast-growing (barbed) ends; and/or (*d*) the ends that do exist are not accessible to monomers, either due to capping by filament end–blocking proteins, or because of other constraints, such as blocking of the filament ends by the plasma membrane or associated proteins. A low affinity interaction between actin and profilin would not be expected to prevent filament formation in the presence of free fast-growing filament ends, since such ends have a higher affinity for actin monomers (K_d = 0.1 μM) than does low-affinity profilin (K_d = 5 μM), as noted by others. It seems likely therefore, that the fast-growing ends of the filaments are not free to bind monomers, and may be capped by end-blocking proteins, perhaps gelsolin. If this is the case, filament formation requires either that the fast-growing ends of the filaments become accessible to monomers and/or that filament formation occurs on newly formed nuclei.

It is not clear what purpose is served by the apparent change in profilin's affinity for actin, for such a change would work to prevent filament polymerization at a time when filaments are known to form. This apparent paradox may indicate that the formation of the high-affinity complex is not directly related to the control of actin polymerization per se, but is a marker of other intracellular events, such as PIP_2 hydrolysis. Alternatively, actin molecules may not be equally capable of binding to profilin. If profilin binds preferentially to a particular (as yet undefined) subset of actin monomers, the finding of high-affinity complexes may signal an increase in the amount of this (hypothetical) actin species present in the cell. Alternatively, high-affinity complexes may form because binding to profilin confers some special property on an actin monomer. This possibility is not obviated by the net polymerization of actin that occurs with stimulation for there is a large excess of actin relative to profilin present in the cells, as assessed by these experiments, and all the profilin could be bound to actin without depleting the cell of monomers.

Reversible Formation of Actin/Gelsolin Complexes

These experiments indicate that factors other than calcium are involved in the regulation of the gelsolin/actin interaction in platelet extracts, and demonstrate that actin/gelsolin complexes can be dissociated by cell extracts. Recent work showing that PIP_2 can regulate the binding of actin to gelsolin (Janmey and Stossel, 1987) suggests that PIP_2 can reverse actin/gelsolin complexes formed in the presence of Ca^{2+}. If metabolic intermediates play a role in regulating actin assembly, it seems likely that the particular experimental conditions used to isolate and study actin and its associated regulatory proteins will have a significant effect upon the apparent interactions of these proteins with actin. Thus, the inability of Kurth and Bryan (1984) to demonstrate free gelsolin in platelet extracts after ionophore stimulation might be related to the length of time (1 h at room temperature) that passed between stimulation of the cells and centrifugation of the cell extracts. The experiments reported here, conducted by lysing the cells rapidly and incubating the extracts with Sepharose beads at 4°C, may have lessened the consumption of a regulatory metabolic intermediate.

Unlike the results obtained when pure solutions of gelsolin and actin are examined in vitro (where chelation of calcium does not result in the formation of free gelsolin molecules), platelets can apparently regenerate free gelsolin after actin/gelsolin complexes have formed, as can rabbit macrophages (Chaponnier et al., 1987). This is noteworthy because it is now possible to consider that gelsolin may play a role in actin filament assembly, a conjecture difficult to sustain in the absence of evidence that free gelsolin could be formed after the formation of actin/gelsolin complexes.

While free gelsolin might be thought to play a role in filament growth (by forming a nuclei and/or by severing filaments, thereby creating new ends to which monomers can bind), such actions would result in filaments whose fast-growing (barbed) ends are blocked by gelsolin and whose growth is conducted at the pointed end of the filament. Similarly, while EGTA-stable actin/gelsolin complexes can also nucleate filament assembly, they remain bound to the barbed ends of filaments regardless of the Ca^{2+} concentration (Janmey et al., 1985) and would promote filament assembly via

pointed-end monomer addition. Experiments using cytochalasins (Casella et al., 1981; Fox et al., 1981) suggest however, that most of the actin filament assembly in platelets occurs via barbed-end growth. Without a mechanism to generate free gelsolin from gelsolin-capped filaments, thereby allowing filament elongation to proceed from the barbed end, the action of gelsolin cannot reasonably be invoked in explaining filament formation.

The experiments reported here obviate this objection to the possible role of gelsolin in promoting the growth of actin filaments by showing that gelsolin can indeed be dissociated from actin. It is therefore possible to suggest that gelsolin may promote filament assembly. Free gelsolin, or gelsolin/actin complexes, could bind to actin monomers as a consequence of a rise in intracellular Ca^{2+}, forming nuclei and oligomers, and then dissociate from the newly formed filaments' barbed ends, thereby allowing rapid filament assembly to proceed. Alternatively, free gelsolin might sever filaments already present in the cytoplasm and then dissociate from the filament end, creating a new barbed and a new pointed end. Gelsolin's binding to, and subsequent dissociation from, actin may follow as a result of the hydrolysis and subsequent synthesis of PIP_2 after platelet stimulation (Kito et al., 1986).

The changes in the actin-binding properties of gelsolin and profilin reported here may reflect diverse changes in the cell that are important for understanding actin assembly. Multiple factors, such as the number of free filament ends, the concentration of nucleotides (reviewed by Pollard and Cooper, 1986), and the binding of lipids to proteins (Burn et al., 1985; Lassing and Lindberg, 1985; Janmey and Stossel, 1987) are likely to play important roles in determining the net effects of agonists on actin filament assembly in platelets and other cells. Transient changes in the binding of key regulatory proteins, such as profilin and gelsolin, to actin may be important in explaining the dynamic nature of actin filament assembly and structure in nonmuscle cells.

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