Direct Observation of Actin Filament Severing by Gelsolin and Binding by gCap39 and CapZ

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Abstract. Dynamic behavior of actin filaments in cells is the basis of many different cellular activities. Remodeling of the actin filament network involves polymerization and depolymerization of the filaments. Proteins that regulate these behaviors include proteins that sever and/or cap actin filaments. This report presents direct observation of severing of fluorescently-labeled actin filaments. Coverslips coated with gelsolin, a multi-domain, calcium-dependent capping and severing protein, bound rhodamine-phalloidin–saturated filaments along their length in the presence of EGTA. Upon addition of calcium, attached filaments bent as they broke. Actophorin, a low molecular weight, monomer sequestering, calcium-independent severing protein did not sever phalloidin-saturated filaments. Both gCap 39, a gelsolin-like, calcium-dependent capping protein that does not sever filaments, and CapZ, a heterodimeric, non-calcium-dependent capping protein, bound the filaments by one end to the coverslip. Visualization of individual filaments also revealed severing activity present in mixtures of actin-binding proteins isolated by filamentous actin affinity chromatography from early *Drosophila* embryos. This activity was different from either gelsolin or actophorin because it was not inhibited by phalloidin, but was calcium independent. The results of these studies provide new information about the molecular mechanisms of severing and capping by well-characterized proteins as well as definition of a novel type of severing activity.

An intact and dynamic actin-based cytoskeleton is necessary for a wide range of cellular functions. However, although a number of proteins that sever actin filaments have been isolated and their in vitro activity described, the precise molecular mechanisms of how these proteins depolymerize actin filaments is as yet unknown. The traditional methods by which the severing of actin filaments have been studied rely upon indirect means, such as measuring the viscosity of a solution containing actin filaments (Yin and Stossel, 1979), or measuring the rate of decrease in fluorescence of pyrene-actin diluted below the critical concentration (Walsh et al., 1984). These methods do not permit direct observation of the effect of any protein upon an individual actin filament. In addition, in these assays the activity of any one protein could be obscured by the activity of some other protein with an opposite effect on the filaments when a mixture of proteins is used. To overcome this obstacle, I decided to develop an in vitro optical assay in which I could directly observe the effects of purified proteins and mixtures of proteins upon individual actin filaments.

Actin filaments are too small to be visualized directly by light microscopy. However, direct observations of their dynamic behavior in the presence of various binding proteins have recently become possible through the use of fluorescent labeling (Yanagida, et al., 1984; Kron and Spudich, 1986). Actin filaments saturated with rhodamine-labeled phalloidin have been used to observe the interactions between myosin and actin by fluorescence microscopy (Kron and Spudich, 1986), and this has provided detailed information about how the myosins move filaments about (some of these include Collins et al., 1990; Toyoshima et al., 1989). Direct labeling of the actin monomers with rhodamine also permits observation of the filaments by fluorescence (Honda et al., 1986). A fluorescence-microscope assay has been used to identify a microtubule severing activity present in *Xenopus* embryos (Vale et al., 1990), although the factor responsible for this activity has not yet been characterized.

In this report I show that severing and end-binding can also be easily monitored using labeled filaments. Intact gelsolin binds actin monomer, decreases the length of actin filaments in a calcium-dependent manner, and caps the barbed ends of filaments (Yin and Stossel, 1979; Yin et al., 1988; Kwiatkowski et al., 1989; Bryan, 1988; Way et al., 1989). In contrast, the low molecular weight actin-depolymerizing factors, which include actophorin (Cooper et al., 1986; Maciver et al., 1991), depactin (Mabuchi, 1983), cofilin (Yonezawa et al., 1988), and brain depolymerizing factor (Bamburg et al, 1980; Bamberg and Bray, 1988), are different from gelsolin: they do not require calcium, and they bind to filaments with low affinity. They decrease viscosity, and sedimentation of actin filaments too greatly to be explained on the basis of sequestration of monomeric actin (Cooper et al., 1986; Maciver, et al., 1991). This family of proteins is not known to cap the barbed ends of filaments. However,

proteins other than gelsolin do cap the barbed end of filaments restricting elongation without severing filaments. These include macrophage capping protein (gCap 39) which requires calcium (Southwick and Dinubile, 1986; Yu et al., 1990; Johnston et al., 1990; Young et al., 1990); and Cap Z, a heterodimeric protein that is calcium independent (Casella et al., 1986, 1987; Caldwell et al., 1989).

Binding to the barbed end of an existing filament is different from remaining associated with the barbed end of a severed fragment. Indeed, neither gelsolin nor severin have been shown to bind uniquely to the barbed end of an existing filament, but they have been shown to remain attached to the barbed end of an existing filament after they sever the filament (Giffard et al., 1984). After severing filaments, biotinylated severin will attach the fragments by their barbed ends to avidin-coated electron microscope grids. The free length of the filaments can then be oriented by the direction of flow of buffer. Thus, the direction of myosin-coated beads with respect to the polarity of the filaments could be determined (Spudich et al., 1985). Since the filaments in this study were not fluorescently labeled, they were not observed by light microscopy.

Each of these four severing-capping proteins has a different effect on phalloidin-stabilized filaments. It thus seemed likely that yet other types of severing behavior existed that could be identified by direct observation. To test this, I used mixtures of putative actin-binding proteins isolated by filamentous actin affinity chromatography of proteins extracted from early *Drosophila* embryos (Miller et al., 1989). This approach has been shown to identify a large number of proteins, many of which colocalize with actin in the embryo by immunofluorescence. Indeed, individual phalloidin-labeled filaments were observed to sever when incubated with actin column eluates. Severing occurred under different conditions than those required for either gelsolin or actophorin.

Materials and Methods

Materials

Rhodamine phalloidin (3.3 μM) was purchased from Molecular Probes, Inc. (Eugene, OR). mAb 2C4 which recognizes the carboxy terminus of gelsolin was from Sigma Chemical Co. (St. Louis, MO). gCap39 was a gift from Helen Yin (Southwestern Medical Center, Dallas, TX); actophorin was from Sutherland Maciver (Dept. Cell Biology, Johns Hopkins Medical School, Baltimore, MD); and CapZ was from John Cooper (Dept. Physiology and Cell Biology, Washington University School of Medicine, St. Louis, MO).

Protein Preparation

Actin was purified from rabbit skeletal muscle according to Pardee and Spudich (1982). Gelsolin was purified from human blood platelets according to Yin (1986).

Platelet extracts were prepared from 5-d-old platelets purchased from Irwin Memorial Blood Bank (San Francisco, CA). The residual red blood cells were first removed by centrifugation at 150 *g* for 15 min, and then the platelets washed several times by pelleting at 270 *g* for 20 min, and resuspending in acid-citrate-dextrose (120 mM NaCl, 13 mM trisodium citrate, 30 mM dextrose, pH 7.0). Platelets were then incubated in Tyrode's solution (10 ml/U of platelets) (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 $MgCl_2$) at 37°C for 1 h, stimulated for 5 min with 20 μM ADP, and lysed by sonication at 4°C after addition to the cell suspension of an equal volume of a low ionic strength lysis buffer containing detergent (lysis buffer: 20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1% Nikkol, 2/100 PMSF, and 2/100 protease inhibitors. PMSF stock was 0.1 M phenylmethyl sulphonyl fluoride; protease inhibitors [Sigma Chemical Co.] were: 1 mM benzamidine, 1 mg/ml each of leupeptin, pepstatin A, phenanthroline, and aprotinin in ethanol). The sonicate was clarified by a low speed centrifugation (10,000 *g* for 15 min), the supernatant was brought to 50 mM Tris-HCl, pH 7.5, 5 mM DTT, and 2 mM sodium pyrophosphate and centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant was snap frozen in 0.5-ml aliquots for storage, and rapidly thawed before use. One unit typically yielded 35 mg of soluble protein.

Drosophila actin-binding proteins were isolated by filamentous actin affinity columns (15 ml–1 mg/ml) stabilized with phalloidin according to Miller et al. (1989), except that instead of eluting the column first with ATP, the column was initially eluted with 0.5 M KCl in column buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.02% NP-40, 5 mM DTT, 10% glycerol and 1:1,000 dilution of protease inhibitors), followed by elution with 0.5 mM ATP-3 mM $MgCl_2$. Peak protein fractions, usually totaling ~12 ml of 30–60 μg/ml protein were determined by Bradford protein assay (Bio-Rad Laboratories, Cambridge, MA), pooled, and used in the optical assay. 1 ml was precipitated with 10% TCA for protein gel analysis and resuspended in 0.1 ml gel sample buffer. To precipitate the myosin II, one half the eluate (5 ml) was dialyzed against 1 L of 10 mM Imidazole, pH 7.2, 5 mM $MgCl_2$, 1 mM DTT overnight at 4°C. The dialysate was centrifuged at 25,000 *g* for 30 min, and the pellet resuspended in 0.1 ml gel sample buffer. 1 ml of the 5 ml dialysate supernatant was TCA precipitated and resuspended in 0.1 ml gel sample buffer for protein gel analysis. An equal proportion of the total volume of eluate, supernatant and pellet were loaded onto the gel in parallel lanes. The supernatant was used for the optical assay.

Protein Gels and Western Blots

Platelet extract was mixed 1:1 with a 2× stock of Laemmli sample buffer (Laemmli, 1970), and 10 μl were electrophoresed through a 10% polyacrylamide SDS minigel gel and stained with Coomassie brilliant blue. Parallel gels loaded with the same extract were mounted in a Hoeffer Western blot apparatus and electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Blots were blocked in 5% BSA. Antigelsolin antibody was used at a 1:1,000 dilution, washed, and stained with affinity-purified antimouse alkaline phosphatase (Boeringer-Mannheim Biochemicals, Indianapolis, IN). TCA-precipitated column fractions were resuspended in gel sample buffer (Laemmli, 1970) and electrophoresed through a 10% polyacrylamide SDS gel. Protein bands were visualized with either Coomassie brilliant blue or silver staining (Accurate Chemical and Scientific Corp., Westbury, NY).

Optical Assay

This technique was developed as a variation of the motility assay (Kron and Spudich, 1986; Kron et al., 1991). Coverslips (No. 1, 18 mm square) were coated with 10 μl of 0.1% nitrocellulose in amylacetate (Fulam, Latham, NY) and allowed to dry in a fume hood. They were then mounted upside down over two slivers of No. 1 coverslip sealed onto a glass microscope slide with Apiezon vacuum grease (EM Sciences, Fort Washington, PA). The resultant perfusion chamber held a volume of ~50 μl. Purified proteins were perfused into the chamber directly. Gelsolin (2.0 μM) was suspended in assay buffer (25 mM Imidazole pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, and 5 mM DTT); platelet extract was diluted 1:5 with assay buffer; actophorin was used at a concentration of 2.6 μM; gCap 39 was diluted in assay buffer at a concentration of 2.0 μM; and CapZ was used at a concentration of 2 μM.

The effect of gelsolin on individual actin filaments was observed either using purified gelsolin or by using an antibody sandwich technique. Coverslips coated with nitrocellulose and mounted in a perfusion chamber were initially incubated either with purified gelsolin (2.0 μM) or first with mAb 2C4 (1:200 in assay buffer), which recognizes an epitope carried on the carboxy terminus of gelsolin (Yin et al., 1988). The antibody was applied twice in 50-μl aliquots, allowed to adhere for 5 min, and then the chamber washed with two changes of assay buffer, followed by two 50-μl aliquots of diluted platelet extract. After 5 min the chamber was again washed with assay buffer containing 0.5 mg/ml BSA, and actin filaments (1:50 dilution of the stock filaments into assay buffer-BSA with calcium or EGTA) were then perfused into the chamber. That the antibody recognized a single prominent band in the platelet extract was confirmed by Coomassie-stained gel and Western blotting (Fig. 1, *A* and *B*).

The antibody sandwich technique produced more consistent results than

the purified protein, with respect to the severing activity occurring across the coverslip and along any individual filament. This may be because the antibody presents the gelsolin in the right orientation to bind to filaments while gelsolin attached directly to the nitrocellulose is oriented randomly. It could also be because the platelet extract used with the antibody technique contained fresher protein, having taken only 1–2 h to prepare, while purification took several days. Antibodies have also proven useful for the attachment of myosin fragments to beads in other variations of the motility assay (Hynes et al., 1987).

A stock solution of labeled actin filaments was prepared in the following manner: 94 μl of phalloidin (3.3 μM) was dried in a Savant speed vac, resuspended in 2 μl of ethanol, and then in 290 μl of assay buffer. To this was added 10 μl of 0.25 mg/ml actin in assay buffer. The labeling was allowed to proceed overnight. The filaments were usable for at least one week.

Actin filaments were used at a 1:50 dilution of the stock into assay buffer containing 0.5% BSA. After allowing filaments to adhere to the protein already bound to the nitrocellulose for 5 min, the chamber was again washed by two changes of assay buffer-BSA–containing and oxygen radical scavenging system: 0.018 mg/ml catalase, 0.1 mg/ml glucose oxidase, and 30 mg/ml glucose. DTT was added up to 50 mM to inhibit quenching due to oxidation.

To observe the effect of calcium on gelsolin-actin interactions, 50 μl of assay buffer-BSA containing from 0.05 to 4 mM $CaCl_2$ was perfused into the chamber while the filaments were under observation. For binding to gCap 39, 4 μl of filaments in 200 μl of assay buffer without EGTA were brought to 0.050 mM $CaCl_2$ before perfusion into the chamber. To release filaments from gCap 39, 2 mM EGTA in assay buffer-BSA was perfused into the chamber while the filaments were under observation. The same conditions were used for CapZ but had no effect on the number or orientation of the attached filaments.

Drosophila proteins eluting from F-actin columns were either used to coat the coverslip directly, or were first incubated for 1–2 min with actin filaments; 50 μl eluate or dialysate, and 4 μl of stock actin. Then 50 μl of assay buffer-BSA with oxygen radical scavengers was added, and the preparation directly applied to a glass coverslip. Actophorin (2 μM) was also mixed directly with actin filaments in the same way and the mixture mounted on coverslips.

Filaments did not adhere to nitrocellulose in the absence of binding proteins under these conditions. To adhere filaments to nitrocellulose directly, albumin was omitted from the perfusate. This approach was used successfully to observe severing of filaments that were mixed with a mixture of actin-binding proteins, but was unsuccessful because of the phalloidin saturation of the filaments for the detection of severing by actophorin.

Bound filaments were observed in either a Nikkon Microphot FX equipped with a 100 W mercury lamp, or in a Zeiss Axiophot with 100 W lamp. Micrographs were taken on TMax ASA 3200 with 2–12 sec exposures. Video microscopy was performed in the Zeiss facility at the Marine Biology Laboratory, Woods Hole, MA, using a Zeiss Axiophot with 100 Watt lamp, a Hamamatsu SIT camera, an Argus image processing unit, and a home VCR recorder. Photographs were taken from the screen after freezing images on Plus X film.

Results

Gelsolin Binds Actin Filaments along Their Length in the Absence of Calcium, and Severs Them Rapidly upon Addition of Calcium

Filaments adhered all along their lengths in EGTA to either gelsolin or antibody-gelsolin–coated glass surface (Fig. 2 *A*). When 4 mM $CaCl_2$ in the same buffer is injected into the chamber, intact filaments are rare, and instead the coverslip appears to be coated by linear arrays of dots that appear to be the severed fragments of filaments that still adhere to the gelsolin on the coverslip (Fig. 2, *B* and *C*, same preparation as *A* but different fields). This demonstrates that the lateral attachment of the filaments in EGTA was specifically mediated by gelsolin. Fragmentation of the filaments occurred in a wave across the coverslip from the point of calcium injection, and was complete within 20–30 s with 1 mM calcium. Rare fragmentation also appeared to occur before calcium perfusion.

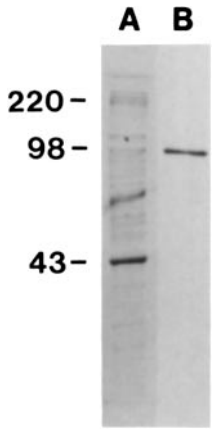

Figure 1. Coomassie-stained 10% polyacrylamide protein gel of platelet extract used for the antibody-sandwich technique, and (*B*) a corresponding Western blot of the same extract stained with mAb 2C4 against the carboxy terminus of gelsolin. Note there is only one band detected by the antibody. Molecular weight standards are indicated to the left.

Some filaments crinkled into zig-zags just before breaking as the wave of fragmentation passed by. Such bending frequently took place too rapidly to photograph, but could be recorded by video, and micrographs then taken from the monitor during play back. Filaments captured in the act of crinkling could be seen among other filaments that had already been severed (Fig. 2, *B* and *C*). Different filaments in the same field could have zig-zags in opposite directions, while the bending of any individual filament was often in the same direction along its length. The direction of bending was not related to the direction of flow of the buffer (Fig. 2, *B* and *C*, *large arrow* indicates buffer flow). Only very rarely were filaments seen to bend but not break.

When the angle of the zig-zag reached 45° normal to the long axis of the filament, a separation could be discerned between the part of the filament that was bent and the next point on the long axis. In this technique, what is visualized is not the actin filament itself but the light emitted by the rhodamine. Thus, separations cannot be detected until the two ends move far enough apart to be out of each others' light emission.

By video recording it was possible to photograph the fragmentation of individual filaments as the calcium effect took place (Fig. 2, *D* and *E*, same field photographed 20 s apart after calcium injection at time 0). After separation, one end of each fragment remained as a bright dot stuck to the coverslip, while the other end grew faint, as if it fell away from the focal plane or was depolymerized (Fig. 2, *B–E*). Thus, the fragments appear separated from each other. Often the remaining dots appeared to be spaced an equal distance apart (Fig. 2, *B* and *C*). Once filaments were severed into fragments and appeared as dots in the micrograph, further fragmentation was not observed. The site of breakage on the filament was just to one side of the bright dot, and was consistently seen on the same side of the dot along the length of the filament.

Filaments are flexible and will often assume various sinuous outlines on the coverslip. The correlation between the curvature of the filament and the probability of severing was quantitated in two ways. First, I measured the radius of curvature at 1.0-μm intervals along 102 μm of total filament length before calcium infusion in micrographs from three separate experiments. Within 1 min of calcium perfusion, 76 breaks occurred, all at places where the filament had a radius of curvature of more than 2 μm. Filaments were severed at 1–3-μm intervals apparently without relationship to the pre-

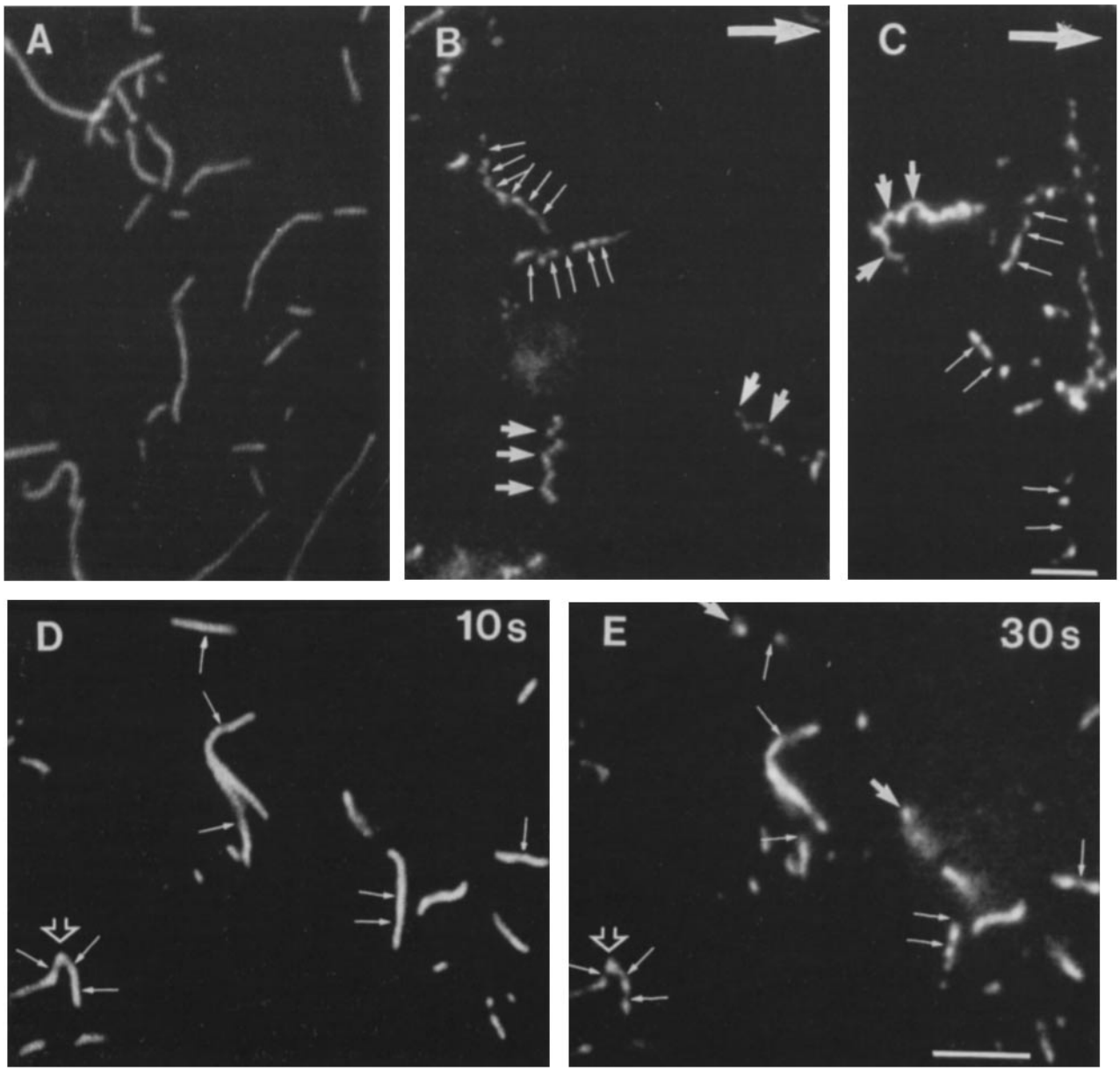

Figure 2. Gelsolin severing of rhodamine-phalloidin actin filaments. (*A*) Filaments adhere to gelsolin-coated coverslip in 1 mM EGTA. (*B* and *C*) Different fields on the same coverslip as A after calcium. 30 s after infusion of 4 mM $CaCl_2$ in assay buffer containing 1 mM EGTA, filaments are rare and instead the coverslip displays rows of fluorescent dots (*small arrows*) apparently spaced an equal distance apart. Some filaments are apparently captured in the act of breaking and appear as zig-zags (*medium arrows*). The direction of the zig-zag is not related to the direction of buffer flow during infusion (*large arrow* is direction of buffer flow). (*D* and *E*) Same field photographed at a 20-s interval during calcium perfusion: 10-s after calcium, some filaments have already begun to break (*D*). Small arrows identify sites of breakage in the same filaments present in both micrographs. A large clear arrow identifies a highly curved section of a filament that is not severed at the apex of the curve. Medium-sized arrows in *E* identify the ends of filaments that remain visible as bright dots on the coverslip after the breakage has occurred. Micrographs taken from the video monitor during live recording. Bars: (*A–C*) 10 μm; (*E*) 10 μm.

existent curvature of the filament except at highly curved segments, which were not severed. However, only five highly curved segments with radii of curvature <2 μm were present within the 102 μm. Thus, it appeared that severing was independent of curvature except for highly curved regions of which there were too few for statistical analysis. Therefore, I measured the tangential angle of the filaments at 34 sites where severing occurred and compared this angle with angles measured at 3-μm intervals on intact actin filaments selected at random. The distribution of angles along unsevered filaments was the same as that of the angles at sites where severing occurred (mean = 10.3 and 9.7, respectively). The null hypothesis (that severing was random) returned a high probability ($P = 0.8$) in a *t* test. Thus, except for rare places where filaments are highly curved, severing occurs independently of the preexistent curvature of the filament.

Sometimes, sites of fragmentation occurred just next to the point of maximum curvature, and the piece of filament at the apex of such a curved section remained intact. This

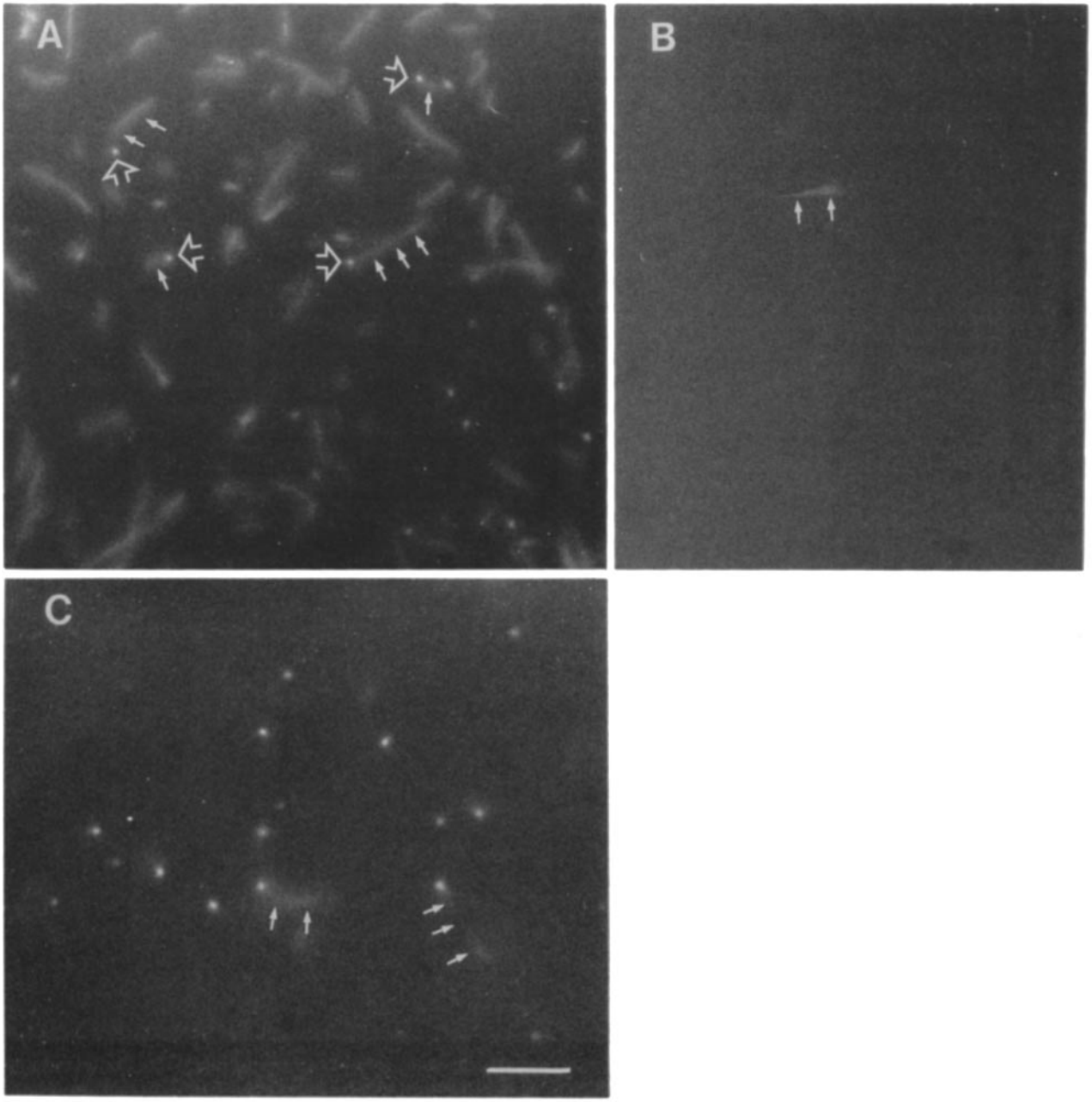

Figure 3. Macrophage capping protein (gCap 39) and CapZ adhere filaments by one end to the coverslip. (*A*) Actin filaments appear like bright dots (*open arrows*) with out-of-focus tails (*arrows*) when attached in the presence of calcium (1 mM) to coverslips coated with purified gCap 39. (*B*) Same field one minute after infusion of 1 mM EGTA. An out-of-focus filament is drifting away (*arrow*). (*C*) Actin filaments attached to coverslips coated with purified CapZ. Unattached, out-of-focus tails (*arrows*) are moving too fast to expose slow micrograph film. Micrographs taken on the microscope without video processing. Bar, 10 μm.

is graphically demonstrated by a filament shown in Fig. 2, *D* and *E* (*open arrow*).

Fragmentation was not a result of photodamage, since filaments could be photographed multiple times without any fragmentation in the absence of calcium. Photon damage only causes the filaments to fragment after significant quenching has occurred such that the filaments are too dim to photograph.

Direct Observation of Association of Proteins to the Ends of Actin Filaments

Macrophage capping protein, or gCap 39, a gelsolin-like capping protein (Southwick and DiNubile, 1986; Yu, 1990) adhered intact filaments by one end to the glass coverslip. Indeed, instead of seeing noodles lying lengthwise parallel to the coverslip, bright dots were observed on coverslips coated with purified gCap 39, or on coverslips coated with anti-gCap 39 antibody-antigen complex perfused with actin filaments in a buffer containing 4 mM calcium/1 mM EGTA (Fig. 3 *A*). The unattached end of the filament could be seen dangling below the focal plane (Fig. 3 *A*, *small arrows*). Often the rapid movements of the flexible unattached portion of the filament prevented it from forming an image on the slow micrograph film. Slowly, after several minutes, the fila-

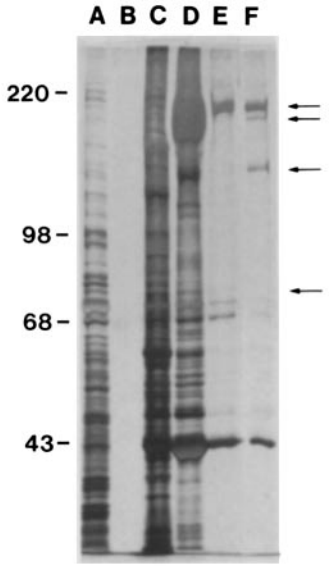

Figure 4. Silver-stained 10% polyacrylamide protein gel of proteins present in F-actin column eluates loaded with extracts from *Drosophila* embryos. (Lane *A*) extract; (lane *B*) last wash before elution; (lane *C*) TCA precipitate of 0.5 M KCl eluate; (lane *D*) TCA precipitate of ATP eluate; (lane *E*) low ionic strength dialysis supernatant; (lane *F*) low salt dialysis pellet. Arrows identify bands of interest: the 200-kD band distributes equally between the supernatant and pellet; the 190- and 150-kD protein removed from the low salt supernatant and found only in the low salt pellet, and the largest of the three remaining visible bands at 80, and 68/70 kD remain in supernatant. The 43-kD band is presumably composed primarily of actin. Molecular mass standards are to the left.

ments appeared to zipper onto the coated glass surface beginning at the attached end, finally coming to lie parallel to it. When EGTA was perfused into the chamber, within 1 min the filaments completely detached (Fig. 3 *B*, same field as *A*, 1 min after EGTA). An out of focus filament moving across the field provides assurance that the correct focal plane has been maintained. Identical results were obtained using the antibody sandwich technique.

Purified Cap Z also adhered filaments by one end to the coverslip (Fig. 3 *C*). Addition of calcium made no statistical difference to the number of attached filaments or to their orientation of binding (16 filaments/field in EGTA, $n = 30$ fields; versus 15.5 filaments/field in 1 mM $CaCl_2$, $n = 30$).

Severing Activity Is Present in ATP-Mg Eluates from F-Actin Columns Loaded with Extracts from Drosophila Embryos

A large number of proteins are specifically retained and eluted from F-actin columns loaded with extracts of early (0–5 h) *Drosophila* embryos as previously described (Miller et al., 1989). In this experiment, the extract (Fig. 4, silver-stained protein gel, lane *A*) was loaded onto the column, the column washed extensively (lane *B*), and then eluted first with 0.5 M KCl in extract buffer (lane *C*), followed by 2 mM ATP-4 mM $MgCl_2$ in extract buffer (lane *D*). The ATP eluate (lane *D*) contains a large amount of protein migrating at ~200 kD that is presumably myosin II, as well as prominent bands at 150, 80, 68/70, 43, and 34/32 kD approximate molecular masses. After low salt dialysis, approximately half of the 200-kD band (*arrows* at high molecular mass) and all of the 150-kD species (*arrow*) are sedimented by centrifugation (Fig. 4, lane *E*, supernatant, and lane *F*, pellet after low salt dialysis). In the supernatant, there are two major bands of 200- and the 43-kD species. By Western blot, these are at least partially composed of actin and myosin (data not shown). It is possible that this 200-kD band contains additional proteins that could be responsible for the severing activity. In addition to these two bands, three other bands are visible by silver staining in a 10% polyacrylamide SDS gel: a faint band at 80 kD (*arrow*), and the 70/68-kD "doublet".

No movement of filaments could be induced by the infusion of ATP into chambers coated with the ATP-eluting proteins. I attributed this to the presence of nonmotor proteins that attach the filaments by their sides to the glass and prevent the myosins from moving them. Filaments did writhe in the presence of ATP, and would come on and off the glass at different points along their length. No other activity was detected.

Severing was readily apparent when filaments were first mixed with the ATP column eluate and then perfused into the chamber. The attached filaments were observed to break in place, without being pulled apart as can occur with myosin motors. Thus, the severing was unlikely to be due to a myosin. The broken lengths of the filaments remained visible and did not fall away from the glass or depolymerize further. The severing activity was independent of calcium since it occurred in buffers containing an 80-fold excess of EGTA (1 mM) over the calcium (12 μM) that was left over from the final polymerization of the actin filaments during purification. It was also insensitive to phalloidin, since the labeled filaments were made in a 10-fold molar excess of phalloidin. Furthermore, it was not a result of photon damage, since these filaments could be observed and repeatedly photographed in the absence of column eluate without breaking.

This activity was further purified by dialysis of the ATP eluate in a low ionic strength buffer followed by centrifugation to pellet the myosins. The supernatant (Fig. 4, lane *E*), which contains five different protein species by silver staining, had a twofold higher incidence of severing events per total filament length than did the eluate: 52 events/64 μm for ATP eluate (0.9 severing events per micron); and 142 events/81 μm for the low salt dialysate supernatant (1.8 severing events per micron) (Fig. 5 *B*). This suggests that there is an inhibitory factor that has been lost. Some filaments are severed at many points along their length and others are not severed at all. This severing occurred in the absence of ATP.

In contrast, the same amount of total eluate from F-actin columns with 0.5 M salt which preceded the ATP elution had no detectable severing activity under these conditions. This eluate had a 1.5-fold higher total protein concentration (150 μg/ml) than the ATP eluate (100 μg/ml).

Finally, to see whether the *Drosophila* severing factor was different than the low molecular weight calcium independent severing proteins, I examined the effect of actophorin on rhodamine-phalloidin–saturated filaments. No severing activity was detected, although filaments made from rhodamine-labeled actin depolymerized rapidly under the same conditions. These results further support the hypothesis that the *Drosophila* severing factor was also different from the low molecular weight actin severing proteins. Furthermore, they confirmed that severing by gelsolin was less sensitive to phalloidin than severing by actophorin.

Discussion

By direct observation of rhodamine-phalloidin–saturated actin filaments, I have been able to visualize the events of severing of actin filaments by gelsolin, and the association with one end of preexisting actin filaments by its relative, gCap

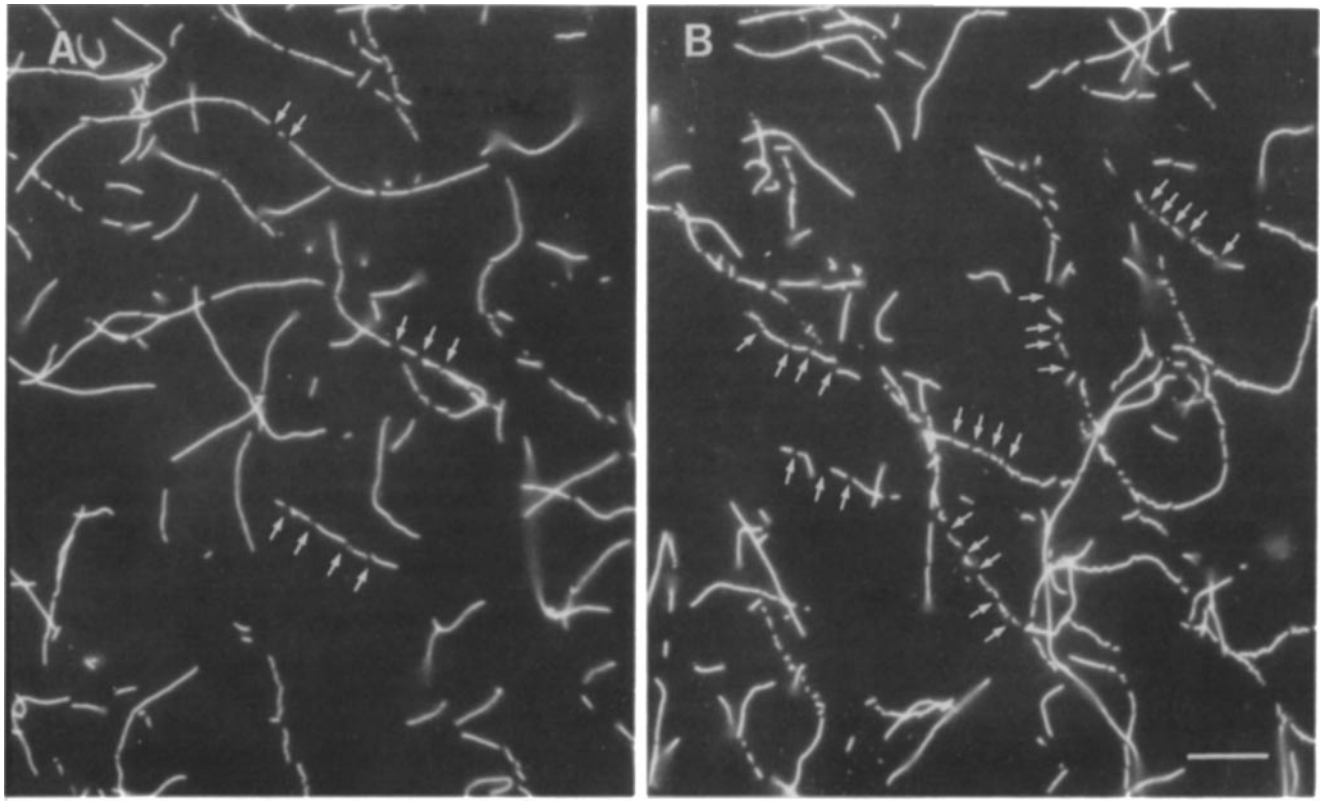

Figure 5. Severing activity is present in the ATP eluate and enhanced in the low salt dialysis supernatant. (*A*) Actin filaments were mixed with proteins eluting with ATP from F-actin columns after first eluting the column with 0.5 M KCl. Discontinuities in the filaments can readily be seen (*arrows*). While some filaments have multiple breaks, others are not severed at all. (*B*) After low ionic strength dialysis and centrifugation, severing activity is more pronounced (*arrows*), and all but a few filaments appear to be susceptible. Bar, 10 μm.

39. In addition, direct observation of labeled filaments revealed the presence of an unusual severing activity in mixtures of actin-binding proteins isolated by F-actin-affinity chromatography from early *Drosophila* embryos.

Labeled Filaments Permit Direct Observation of End-binding and Severing

This assay demonstrates definitively that gCap 39 physically associates with one end of an intact filament without either severing it or nucleating new filaments from it, at least during the initial phases of its association with actin filaments. Such an association has already been elegantly demonstrated for CapZ by immunoelectron-microscopy (Casella et al., 1987). The association with the sides of filaments by gelsolin observed by this method was therefore not due to an inability of this technique to detect end-binding. End-binding can only be detected in this assay when the protein associates uniquely with the ends of filaments. In mixtures of proteins that contain both side-binding and end-binding activities, or with a protein like gelsolin that binds both to the sides and the ends, end-binding is obscured.

In contrast to gCap 39, in EGTA gelsolin adhered filaments along their length to the coated coverslip, as has previously been described for myosin (Kron and Spudich, 1986). The filaments were flexible and assumed various configurations on the glass. Upon infusion of calcium, a wave of severing spread across the filaments. Often filaments crinkled during severing. Severing was apparently random with respect to the curvature of the filaments, but rare sharp bends were not severed. After severing, one end of the filament remained associated with the glass, appearing as a bright dot. Rows of such dots and fragments left evidence as to the original outline of the filament.

Mechanism of Gelsolin Severing

The antibody binds the carboxy terminus of gelsolin (Yin et al., 1988), thus orienting it appropriately on the coverslip. The antibody-stabilized gelsolin would then bind by its amino terminus to the sides of the actin filament. That it is the amino terminus, in particular domain 2, that binds to the side of the filament is based on the observation that proteolytic fragments containing this part of gelsolin bind actin filaments with a 1:1 stoichiometry, but do not sever filaments (Yin et al., 1988; Kwiatkowski, et al., 1989; Bryan, 1988; Way et al., 1989; Janmey and Matsudaira, 1988). This binding has not before been demonstrated for intact gelsolin, but it could not sever filaments if it did not bind to their sides. The homologous domain in villin does bind to the sides of filaments (Bretscher and Weber, 1980; Matsudaira, et al., 1985*a*,*b*; Janmey and Matsudaira, 1988; reviewed in Matsudaira and Janmey, 1988; André et al. 1988; Bazari et al., 1988). It has been proposed that intact gelsolin fails to bind to filaments in sedimentation assays in EGTA because the side-binding site is masked in a calcium-dependent manner (Way et al., 1989). It is possible that the gelsolin used in this assay was already partially activated by calcium present in the Tyrode's solution or by the small amount of chelated calcium present in the actin preparation. It is also possible that

this assay is more sensitive to low affinity binding than solution chemistry, as will be further discussed below.

The bending of the filaments as they break is one of the more intriguing observations presented here. This must be interpreted with caution, however. In this fluorescent technique, the light emitted by the phalloidin label appears larger than it actually is. Thus, separations between two ends of filaments that are still within the same light halo cannot be resolved. Hence, it is not possible to tell whether the break in the filament occurs before, during, or after the bending. Therefore, the zig-zag appearance of some filaments during severing could either be caused by gelsolin bending the filament until it snaps, or it could be a consequence of gelsolin-induced breaks that permit the ends to move sideways in opposite directions. One possible scenario is that the gelsolin severs one of the protofilaments in the double-stranded actin helix, rendering the filament more flexible until the other side separates. While the bend-snap hypothesis is attractive, it must be more rigorously tested by more high resolution structural studies.

Phalloidin Does Not Prevent Gelsolin Severing

The filaments used in this assay were stabilized and labeled with a tenfold excess of rhodamine phalloidin. Phalloidin has been known to inhibit the depolymerization of filaments (Cooper, 1986). While phalloidin does inhibit the severing activity of cofilin (Yonezawa et al., 1984) and actophorin (Maciver et al., 1991), it does not inhibit either the rate or the degree of gelsolin-mediated decreases in viscosity (Verkhovsky et al., 1984), nor does it inhibit the severing activity of severin, a *Dictyostelium* gelsolin-like protein (Giffard et al., 1984). In the assay reported in this paper, phalloidin did not prevent gelsolin severing, but whether it blocks further depolymerization of the severed fragments could not be determined. Thus, phalloidin would not be expected to inhibit gelsolin activity in assays that measure the size of filaments, such as viscosity or sedimentation as has been reported (Verkhovsky et al., 1984). However, if further depolymerization is inhibited, this would be detected in assays that measure loss of total filamentous actin, like measurements of pyrene-actin fluorescence, which is directly proportional to the total number of actin molecules in filaments (Kouyama and Mihashi, 1981; Walsh et al., 1984). That further depolymerization is inhibited by phalloidin is suggested by the result that phalloidin partially decreases both the rate and extent of severing by fragmin, a gelsolin-like homologue (Hinssen, 1981).

That actophorin and cofilin are inhibited by phalloidin while gelsolin and severin are not further supports the contention that the mechanism of severing by these proteins is different. Thus, it is not surprising that while actophorin seems to have a predilection for places where the filament is already bent (Maciver et al., 1991), gelsolin does not.

It must be remembered that phalloidin may subtly alter the molecular mechanism of severing, and that this alteration cannot be evaluated from these studies. Detailed comparisons between the effect of gelsolin on rhodamine-labeled actin versus the effect on rhodamine-phalloidin–labeled filaments will be necessary to evaluate this possibility. Conjugation of fluorescent probes directly to actin may also effect the binding or the activity of these proteins.

Correlation with Traditional Assays Is Complicated

Direct observation provides new information about the molecular mechanisms of the interaction of severing and capping proteins with individual actin filaments. However, results gained this way cannot be expected to correlate exactly with information gained by solution chemistry. One reason for this is that the coverslip is two dimensional, while solution chemistry is three dimensional. Thus, on a glass surface, once an actin filament has bound, the concentration of filaments in the vicinity of the bound filament approaches infinity and is not proportional to the concentration of filaments in the buffer. In this way, low affinity binding not preserved in the test tube can be detected on the coverslip. Examples of this are the association of gelsolin to the sides of filaments. A drawback of the two-dimensional nature of the assay is that it complicates kinetic measurements and calculations of binding constants because the concentration of proteins will vary according to the microenvironment along the surface of the coverslip, the viscosity of the buffer, and the flexibility of the protein or filament in suspension.

Identification of Unusual Severing Activities in Mixtures of Proteins

Because direct observation provides new information about severing not gained from previous techniques, it was of some interest to see if it would identify novel severing activity in crude extracts. Indeed, an unusual severing activity was detected by this assay in mixtures of proteins extracted from *Drosophila* embryos. This activity was different from that of the two classes of severing proteins isolated from other systems: gelsolin and actophorin. The *Drosophila* factor is unique in several ways: it apparently associates with actin filaments in an ATP-sensitive manner, since it elutes from F-actin columns with ATP even when columns are preeluted with 0.5 M salt; it is not inhibited by phalloidin, which inhibits the low molecular weight severing proteins; and it does not require calcium, which is required by the gelsolin-villin family of capping-severing proteins whose severing activity is not prevented by phalloidin.

This factor was further purified from ATP eluates by low salt dialysis which removed many of the other protein species found in the eluate, including an apparent inhibitory factor and at least half of the 200-kD band which is at least in part composed of myosin II. This inhibitory factor could be myosin II, which is partially removed by low salt precipitation and is known to inhibit the severing of filaments by severin (Giffard et al., 1984). Whatever its nature, an inhibitory factor would have obscured detection of this severing factor in traditional assays. Furthermore, traditional assays use an F-actin buffer containing ATP, and do not use phalloidin-stabilized filaments. Thus, the activity of other severing proteins, such as actophorin, would overwhelm detection of this severing activity.

It is puzzling how a severing factor would be retained on an F-actin column rather than chopping up the filaments and washing out along with the filament fragments. One explanation for this might be that the factor, like gelsolin, remains associated with the filament after severing it. The severed filament fragment would not wash off the column if the filament were initially conjugated to the beads at many points along their lengths, or were stuck to each other by bundling

proteins. Subsequent elution of the severing factor with ATP could mean that the binding of the factor to actin filaments is ATP-sensitive, like the myosins. However, this cannot be confirmed until the purified protein is obtained and in vitro binding studies are performed.

No actin filament severing protein has yet been identified in *Drosophila* embryos. Although a large number of putative actin-binding proteins have been found (Miller et al., 1989), their effect on actin filaments has not yet been characterized. The early *Drosophila* embryo offers a genetic system in which to study the contribution of the actin cytoskeleton to both developmental and cellular events, such as the redistribution of positional cues known to accompany fertilization in other species (reviewed in Bearer, 1991), or the elaboration of the cleavage furrow. Therefore, the isolation and purification of such a severing factor as described in this report would potentially provide a useful tool for such future studies.

Conclusion

Direct observation of filaments can be expected to lead to the identification and purification of such novel severing factors as that reported here in *Drosophila* embryos or perhaps to other types of severing or depolymerizing proteins, as well as to the elucidation of the molecular mechanisms of severing employed by proteins that have already been extensively studied by other means.

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