Characterization of Actin Filament Severing by Actophorin from *Acanthamoeba castellanii*

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Abstract. Actophorin is an abundant 15-kD actin-binding protein from *Acanthamoeba* that is thought to form a nonpolymerizable complex with actin monomers and also to reduce the viscosity of polymerized actin by severing filaments (Cooper et al., 1986. *J. Biol. Chem.* 261:477–485). Homologous proteins have been identified in sea urchin, chicken, and mammalian tissues. Chemical crosslinking produces a 1:1 covalent complex of actin and actophorin. Actophorin and profilin compete for crosslinking to actin monomers. The influence of actophorin on the steady-state actin polymer concentration gave a K_d of 0.2 μM for the complex of actophorin with actin monomers. Several new lines of evidence, including assays for actin filament ends by elongation rate and depolymerization rate, show that actophorin severs actin filaments both at steady state and during spontaneous polymerization. This is confirmed by direct observation in the light microscope and by showing that the effects of actophorin on the low shear viscosity of polymerized actin cannot be explained by monomer sequestration. The severing activity of actophorin is strongly inhibited by stoichiometric concentrations of phalloidin or millimolar concentrations of inorganic phosphate.

To understand the mechanisms that regulate the assembly and dynamics of the actin cytoskeleton, a catalog of more than 30 types of actin-binding proteins has been compiled in the hope that understanding the parts will give insight into the whole (Stossel et al., 1985; Pollard and Cooper, 1986). The redundancy of the system is striking at the biochemical level where a variety of proteins can have similar activities. In a given cell type more than one protein can sequester actin monomers or nucleate actin polymerization or cap actin filaments or crosslink actin filaments. Furthermore, an individual protein can have two or more of these activities. The actin filament severing proteins illustrate this redundancy.

The best characterized severing proteins are the ~90-kD gelsolin group (Yin and Stossel, 1979) and the ~42-kD fragmin/severin group (Hasegawa et al., 1980; Brown et al., 1982). Gelsolin requires Ca^{++} to sever actin filaments and is inhibited by phosphoinositides (Janmey and Stossel, 1987). It also caps the barbed end of actin filaments and forms nuclei for elongation by binding two actin monomers. These proteins consist of multiple functionally specialized domains sharing a common sequence motif. It is generally believed that both groups arose from a precursor of ~125 amino acids by a series of gene duplications resulting in fragmin/severin with three of these domains (Ampe and Vandekerckhove, 1987; Andre et al., 1988) and gelsolin/villin with six domains (Kwaitkowski et al., 1986; Way and Weeds, 1988; Bazari et al., 1988). The current models for severing by both groups of proteins involve the interaction of multiple domains with actin filaments.

The third class of severing proteins is distinct from the gelsolin and fragmin groups in most ways. This group includes actin depolymerizing factor (ADF) (Bamburg et al., 1980) and destrin (Nishida et al., 1985) from vertebrates, depactin from echinoderms (Mabuchi, 1983), and actophorin from *Acanthamoeba* (Cooper et al., 1986). They are 15,000–20,000 molecular weight, and have related sequences (Takaqi et al., 1988; Abe et al., 1990; Moiyama et al., 1990; Maciver, S. K., unpublished observations). They each form a tight complex with actin monomers that does not polymerize itself or nucleate actin polymerization. By EM and viscometry these proteins appear to sever actin filaments but do not require Ca^{++}. Since they are not known to have internal sequence repeats or multiple functional domains, they must have a different severing mechanism than the gelsolin group. Some even question whether they sever actin filaments (Stossel, 1989). No gene disruptions or other genetic manipulations have been made to test their role in live cells. Nevertheless, since actophorin is the second most abundant actin binding protein in the amoeba and since its relatives are present in high concentrations in a number of vertebrate organs (Bamburg and Bray, 1987), these proteins are likely to be important members of the actin system. *Dictyostelium* mutants deficient in severin are essentially normal (Andre et al., 1989), and since some cell types have been shown to contain

1. *Abbreviations used in this paper*: ADF, actin depolymerizing factor; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NHS, *N*-hydroxylsulfosuccinimide.

both types of severing proteins (Koffer et al., 1983, 1988) it is possible that this function is duplicated.

In this study we show that actophorin actually severs actin filaments and that severing is controlled by inorganic phosphate. This firmly places actophorin in the larger group of severing proteins that are thought collectively to contribute to the dynamics of actin filaments in cells. We also establish the stoichiometry and affinity of the complex of actophorin with actin monomers. These biochemical properties will provide the foundation for interpreting the mechanism of action when the three-dimensional structure of actophorin becomes available (Magnus et al., 1988). In the accompanying paper (Maciver et al., 1991), we show that the severing activity of actophorin can promote the formation of large, stiff bundles of actin filaments in the presence of alpha-actinin.

Materials and Methods

Protein Purification

Actin from rabbit skeletal muscle was prepared as described by MacLean-Fletcher and Pollard (1980), using gel filtration on Sephacryl S-300 in buffer-G (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 3 mM NaN_3). Actin was labeled with *N*-(1-pyrenyl) iodoacetamide (Molecular Probes Inc., Eugene, OR) by the method of Pollard (1984). For the steady-state critical concentration experiments, pyrenyl actin was further purified by dialysis against buffer-G overnight followed by gel filtration (S-100). The resulting actin did not give the apparently artifactual increase in fluorescence observed in mixtures of actophorin with pyrenyl actin monomers that was observed by Cooper et al. (1986). Actophorin was purified from *Acanthamoeba castellanii* exactly as described by Cooper et al. (1986). *Acanthamoeba* profilins I and II were a gift from Donald Kaiser (Johns Hopkins Medical School, Baltimore, MD). Phalloidin was purchased from Boehringer Mannheim Biochemical (Indianapolis, IN).

Video-microscopic Visualization of Microfilament Severing by Actophorin

The severing of actin filaments by actophorin was observed microscopically in a flow cell (Kron et al., 1991). One face of the cell was coated with nitrocellulose, which served as a substrate for the binding of *Acanthamoeba* myosin-II. Myosin-II was bound passively at a concentration of 0.012 mg/ml in Buffer-F (50 mM KCl, 12 mM Tris-HCl, pH 8.0, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 3 mM NaN_3). The remaining protein binding sites were blocked with a solution containing 0.5 mg/ml BSA (Sigma Chemical Co.) in buffer-F (Buffer-F/BSA). Rhodamine phalloidin-labeled actin (Rh-Ph-actin) was prepared by incubating 0.8 μM F-actin with 0.5 μM rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR). The flow cell was filled with 0.11 μM Rh-Ph-actin in buffer-F/BSA and the filaments were allowed to bind to the immobilized myosin-II. The fluorescence of the actin solution was protected from photobleaching by replacing the flow cell medium with antifade buffer (buffer-F/BSA containing 3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 50 mM DTT). Severing was initiated by replacement of the flow cell solution with 8.2 μM actophorin in antifade buffer.

Individual actin filaments were imaged with an Olympus inverted microscope (IMT-2) in the fluorescence mode. The fluorescence images in a field were enhanced using analogue signal processing and recorded onto 1/2 inch VHS tape at 30 full frames per second. 10 frames of a recorded field were averaged and photographed (Polaroid Freeze-frame). The field width was calibrated with a stage micrometer subsequent to the data collection.

Myosin-II was prepared from *Acanthamoeba* (Sinard and Pollard, 1989); this procedure was shown previously to yield myosin-II with a slow turnover of ATP due to endogenous phosphorylation (Collins and Korn, 1981). We confirmed that the myosin-II used in these experiments bound actin filaments but did not move or sever the filaments in the presence of ATP, presumably because the myosin was phosphorylated endogenously.

Low Shear Viscometry

Apparent viscosity of actin filament solutions at low shear was measured with a falling ball in a 100 μl capillary tube (Corning Glass, New York, NY) (Maclean-Fletcher and Pollard, 1980). Actin was polymerized in the capillary by adding 20 μl of 10× KME buffer (500 mM KCl, 10 mM $MgSO_4$, 10 mM EGTA, 100 mM Tris-HCl, pH 8.0) to 180-μl samples immediately before loading the sample. Samples were incubated in a temperature regulated water bath at 25°C. Unless otherwise stated the final actin concentration was 10 μM, and the incubation time was greater than two hours.

Kinetic Studies

Polymerization of actin was measured by changes in fluorescence of *N*-(1-pyrenyl) iodoacetamide covalently attached to cysteine 374 of actin or by 90° light scattering of unlabeled actin at 400 nm using a fluorescence spectrophotometer (model 650-10S; Perkin-Elmer Corp., Pomona, CA).

The number concentration of filaments was determined by measuring the rate of polymer elongation or shortening. The rate of actin filament elongation is directly proportional to the number concentration of actin filaments and can be either positive or negative depending on the concentration of actin monomers:

$$\text{Rate} = k_+[A_1][\text{ends}] - k_-[\text{ends}]$$

where k_+ is the association rate constant, k_- is the dissociation rate constant, A_1 is the actin monomer concentration and "ends" is the number concentration of filaments. These rate constants have been measured (Pollard, 1983; Pollard and Cooper, 1986). When the actin concentration makes the value of the term $k_+(A_1) > k_-$ the filaments grow longer; otherwise the filaments shorten. Either way, knowledge of the actin monomer concentration and the rate of change of the polymer concentration provide a direct measure of the concentration of ends. The depolymerization variant of this assay was first used by Walsh (1984) and by Bryan and Coluccio (1985) to document severing of actin filaments by villin and gelsolin. The actin monomer sequestering activity of actophorin or other actin binding proteins must, of course, be taken into account when making these calculations. Since actophorin has equal effects on elongation at both ends of filaments (Cooper et al., 1986), we used the sum of the rate constants in our calculations of the polymer number concentration. Depolymerization experiments were performed semi-automatically using a stopped flow rapid mixing device (SFA-II Rapid Kinetics Accessory; Hi-Tech Scientific, England).

Chemical Cross-linking

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxylsulfosuccinimide (NHS) were obtained from Pierce Chemical Co. (Rockford, IL) and were dissolved in 2 mM potassium phosphate buffer pH 7.5 immediately before use (NHS was brought to pH 7.5 with 1 M NaOH). Chemical cross-linking was performed according to Vandekerkhove et al. (1989). Briefly, EDC and NHC were added to samples of rabbit muscle actin, profilin and actophorin all at 10 μM, in 2 mM potassium phosphate to give final concentrations of 1 mM each. After 10 min, more EDC and NHS were added to give a new concentration of 2 mM. After a further 10 min the reaction was stopped with the addition of 200 mM glycine stock (pH 7.5) to give a final concentration of 9 mM. Products were analyzed by SDS-PAGE. After separating proteins by SDS-PAGE, proteins were electrophoretically transferred onto nitrocellulose, incubated with a mAb raised to actophorin (Kaiser and Maciver, unpublished), and detected with peroxidase-labeled goat anti-mouse Ig (Tago, Burlingame, CA).

Results

Stoichiometry of the Complex of Actin with Actophorin

Chemical crosslinking of mixtures of actin monomers and actophorin with the water soluble carbodiimide, EDC, produced a new species of ~60 kD (Fig. 1). A 1:1 complex would have a molecular mass of 58 kD. Both proteins had to be present with the crosslinker to produce the 60-kD species. Antibodies to actophorin reacted with the 60-kD band on immunoblots (Fig. 1 *B*). As in the case of profilin crosslinking to actin (Vandekerckhove et al., 1989), the yield of the actin-actophorin complex was much higher in the presence of NHS, a reagent that stabilizes reactive intermediates

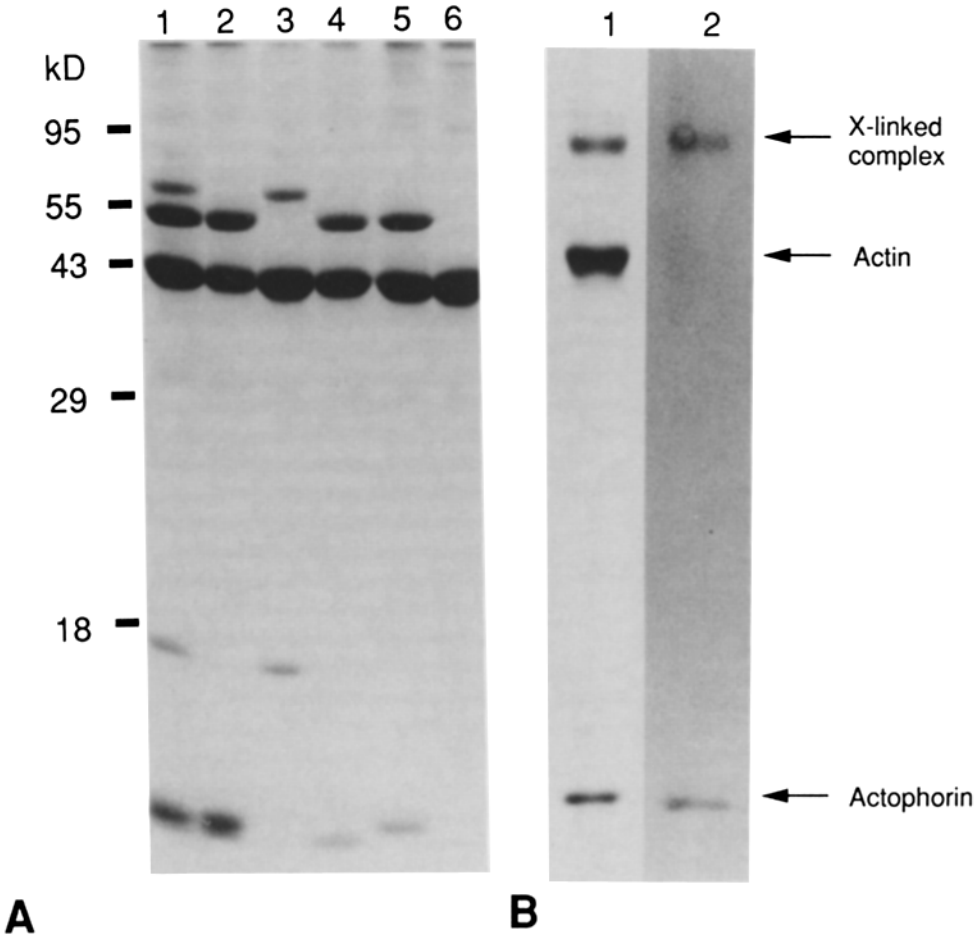

Figure 1. SDS-PAGE of the products of chemical crosslinking of actin to actophorin and profilins-I and -II. The proteins (each at 10 μM) were reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in the presence of *N*-hydroxylsulfosuccinimide (NHS) for 30 min at room temperature. Conditions; 2 mM potassium phosphate pH 7.0. (*A*) Lane 1, actin, *Acanthamoeba* profilins I and II and actophorin. Lane 2, actin and profilins I and II. Lane 3, actin and actophorin. Lane 4, actin and profilin I. Lane 5, actin and profilin-II. Lane 6, actin alone. (*B*) Lane 1, actin and actophorin. Lane 2, immunoblot of Lane 1 probed with an antibody to actophorin.

formed by reaction of carboxyls with the carbodiimide. These reactions yield a zero length crosslink between amine and carboxyl groups of the two proteins.

In mixtures of actin, actophorin, and an *Acanthamoeba* profilin, a single actin monomer can be crosslinked to either one of the profilins or to an actophorin molecule, but not to both types of actin binding protein simultaneously (Fig. 1). The simplest interpretation is that the binding sites for actophorin and profilin on actin overlap, precluding the binding of both molecules simultaneously.

Affinity of Actophorin for Actin Monomers

Using light scattering and pyrenyl-actin fluorescence to measure the steady-state concentration of polymerized actin over a range of actin concentrations, we obtained an estimate of the dissociation constant for the complex of actophorin with actin monomers. Actophorin reduces the polymer signal by the same amount at every actin concentration above the critical concentration (Fig. 2). In interpreting these data we assumed (Tobacman and Korn, 1982; Tseng and Pollard, 1982) that once steady state is reached, the critical concentration of actin (obtained from the break in the control plot) is in equilibrium with complexes and that all other actin molecules are polymerized. The differences in the critical concentrations in the presence of various concentrations of actophorin provide a direct measure of the concentration of complex and gives a K_d of between 0.1 and 0.2 μM.

Experimental Evidence That Actophorin Severs Actin Filaments

Five different experimental strategies, three qualitative and two quantitative, have provided consistent evidence that actophorin severs actin filaments. Most convincingly we directly visualized the severing of rhodamine-phalloidin–labeled actin filaments by actophorin in the light microscope (Fig. 3). This confirmed observations by EM (Cooper et al., 1986 and additional data not illustrated) that the filaments were shorter in the presence of actophorin. Two quantitative assays, an actin filament elongation assay (Figs. 4 and 5) and an actin filament depolymerization assay (Fig. 6), showed that the number concentration of polymer ends is a function of the concentration of actophorin. Finally, actophorin reduces the viscosity of polymerized actin far out of proportion to its effect on polymer concentration (Fig. 7).

The video microscopy assay provides direct evidence for severing as well as evidence for a possible mechanism. Severing began 10–20 s after introduction of 8 μM actophorin into the flow cell (Fig. 3 *B*) and continued for 60–120 s until depolymerization and severing reduced the lengths of some of the filaments below the resolution of the microscope (Fig. 3 *C*). Severing frequently, but not exclusively, occurred at bends. We analyzed the relationship between filament curvature and frequency of severing in two ways. First, we measured the radius of curvature at 0.5-μm intervals along 28 filaments from two experiments having a total length of 119

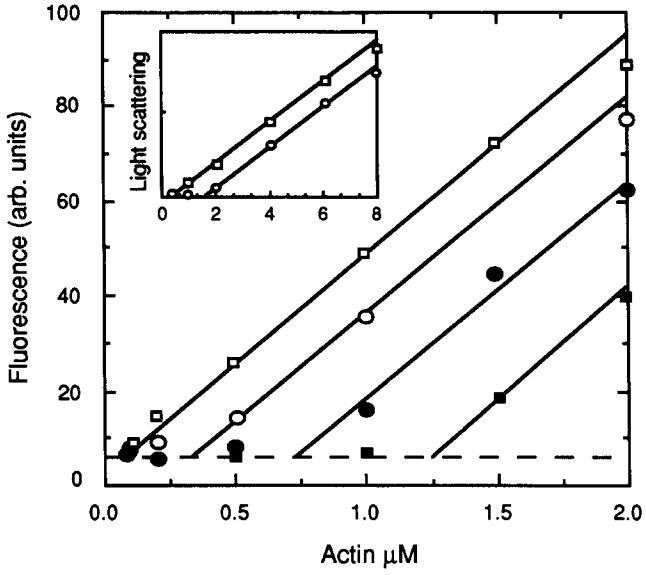

Figure 2. The effect of actophorin on the critical concentration for the polymerization of rabbit muscle actin. The steady-state concentration of polymerized actin was assayed by pyrenyl-actin fluorescence or 90° light scattering (*inset*). Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at room temperature. Actin was 10% pyrene labeled. Actophorin concentrations: 0.0 (□); 1.0 μM (○); 2.0 μM (●; 4.0 μM (■). The intersection on the baseline (*dashed line*) gives a K_d of 0.1–0.2 μM assuming a 1:1 complex of actin with actophorin is formed. We calculated the following K_ds: 0.15 μM for 1 μM actophorin, 0.12 μM for 2 and 0.14 μM for 4. A K_d of 0.1 μM was calculated from the light scattering data (inset).

μm. During the first minute with 8 μM actophorin, highly curved segments with a radius of curvature <1.5 μm had a higher probability of severing ($P = 0.28$) than segments with lesser curvature ($P = 0.16$). Second, we measured angles tangential to the sites of 21 severing events and compared these angles to similarly measured angles at 21 other sites on the same filament population chosen in an unbiased manner. The null hypothesis (severing is random) returned a low probability ($P = 0.0005$) in a t test. Therefore, although severing can occur at any site along a filament, it occurs more frequently at bends as illustrated in Fig. 3.

We were unsuccessful in initial attempts to observe severing by actophorin using coverslips coated with myosin II at 0.2 mg/ml and actin filaments treated with equimolar rhodamine-phalloidin. However, by reducing the rhodamine-phalloidin/actin ratio to 1:2 and the myosin II concentration to 0.012 mg/ml severing of actin filaments was observed (Fig. 3). This severing required actophorin. No actin filaments were severed by anti-fade buffer alone. Thus filaments were not spontaneously broken, for example by the excitation light, as observed for microtubules (Vigers et al., 1988).

The elongation assay establishes that actophorin increases the number concentration of actin filaments at all stages of spontaneous polymerization (Fig. 4) and at steady state (Fig. 5). After a lag, at the outset of spontaneous polymerization during which the first long polymers formed, samples with actophorin had a higher concentration of filaments than the control (Fig. 4). After the steady-state polymer concentration is reached, the polymer number concentration is constant over 12 h (2.6 nM after 24 min (Fig. 4 *B*) and 2.5 nM after 12 h (Fig. 5 *A*)). At steady state (12 h after polymerization was initiated) the number concentration of filaments was directly proportional to the actophorin concentration (Fig. 5 *A*). Consequently, the log mean length of the filaments was an inverse function of the actophorin concentration (Fig. 5 *B*). Compared with control filaments that average 22 μm in length, the filaments are <1.0 μm long in 5 μm actophorin. If these short filaments are constantly annealing (Murphy et al., 1988), there must also be continued severing.

Actophorin increases the rate of depolymerization of filaments diluted well below the critical concentration (Fig. 6); this provides another line of evidence that, compared to controls, the number concentration of filaments is higher, the av-

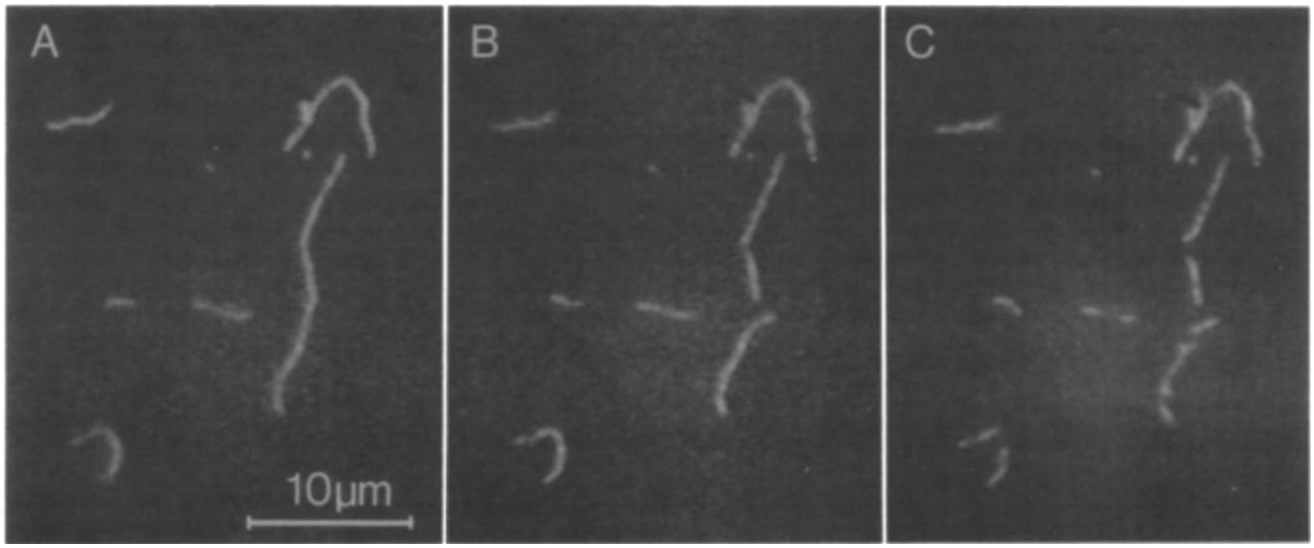

Figure 3. Videomicrograph of actin filaments being severed by actophorin. (*A*) Field of filaments before the introduction of 8.2 μM actophorin. (*B*) Same field 15 s after the end of the flow. (*C*) Field after 40 s of actophorin.

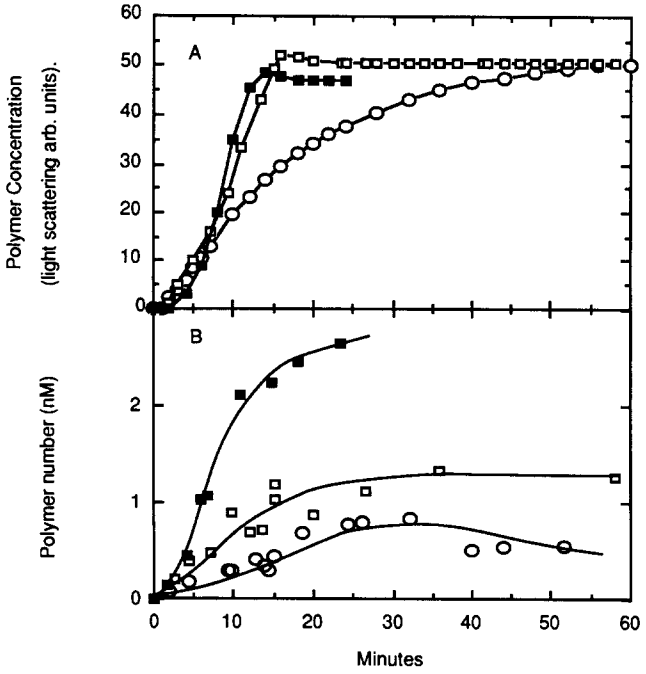

Figure 4. Actophorin accelerates the spontaneous polymerization by increasing the number concentration of filaments. The polymer concentration was measured by light scattering. Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MGSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C. (*A*) Time course of spontaneous polymerization of 5 μM actin alone (○), or with 1 μM actophorin (□) or 2 μM actophorin (■). (*B*) At various times during spontaneous polymerization, actin monomers were added to increase the total actin concentration to μM and the new rate of change of light scattering was recorded. The concentration of filaments was calculated from the increment in the rate of polymerization immediately after the addition of fresh monomer, taking into account the free actin monomer concentration. Symbols are the same as in *A*.

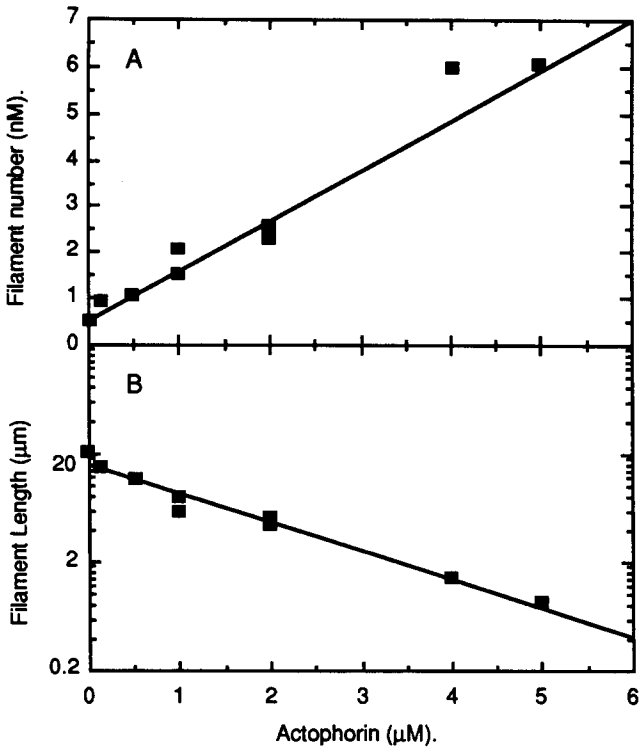

Figure 5. Actin filament elongation assay to evaluate the dependence of the steady-state actin filament number concentrations and length on the concentration of actophorin. Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C. (*A*) 5 μM actin was polymerized with actophorin for 12 h and fresh actin monomers were added to increase the final actin concentration to 10 μM. The filament number concentration was measured from the initial rate of polymerization, taking into account the effect of actophorin on the concentration of monomers. (*B*) The data shown in *A* were used to calculate the average length of the filaments taking into account the critical concentration and the actin bound to actophorin assuming a K_d of 0.1 μM.

erage filament is shorter and the ends are not blocked. The polymer number concentration was roughly proportional to the actophorin concentration (Fig. 6 *B*), and the number of filaments estimated by this assay was in good agreement with the elongation assay. The number of filaments calculated from the initial rate of depolymerization (Fig. 6) was 0.15 nM with 7.5 μM actin alone, 0.35 nM with 0.3 μM actophorin, and 1.2 nM with 0.6 μM actophorin. Given similar concentrations of reactants the polymerization assay produced 0.5 nM with 5 μM actin, 0.8 nM with 0.3 μM actophorin, and 1.1 nM with 0.6 μM actophorin.

The Reduction in Viscosity of Actin Filaments Is Not Attributable to Monomer Sequestration

Although actophorin reduces the concentration of polymerized actin (Fig. 2), this makes only a minor contribution to the dramatic effect of actophorin on the low shear viscosity of actin (Fig. 7; Cooper et al., 1986). This is further evidence that the filaments are shortened by actophorin. The data for Fig. 7 were gathered by varying the concentration of actin or actophorin, but are plotted as a function of the concentration of polymerized actin to emphasize that the apparent viscosity in the presence of actophorin can be lower by two orders of magnitude than expected from the polymer concentration.

Two Different Agents That Stabilize Actin Filaments Inhibit the Severing Action of Actophorin

We used three different assays to examine the effects of phalloidin and inorganic phosphate on the severing of actin filaments by actophorin. The acceleration of the late stage of spontaneous polymerization (Cooper et al., 1986) is a sensitive indicator of severing, low shear falling ball viscometry is a sensitive assay for polymer length and light microscopy images severing directly.

Phalloidin altered all of the effects of actophorin on spontaneous polymerization in the direction of control samples (Fig. 8). Alone, phalloidin increased the initial rate of polymerization and reduced the time taken to reach steady state. Phalloidin with actophorin reduced the late stage acceleration, reduced the maxima overshoot, and increased the steady-state polymer concentration, largely eliminating the effects seen with actophorin alone (Fig. 8). Phalloidin also inhibited the reduction in the low shear viscosity of polymerized actin caused by actophorin (Fig. 8, *inset*). Saturation of the actin filament with rhodamine phalloidin completely prevented severing visualized by fluorescence microscopy.

Inorganic phosphate strongly inhibited the ability of actophorin to reduce the viscosity of polymerized actin (Fig.

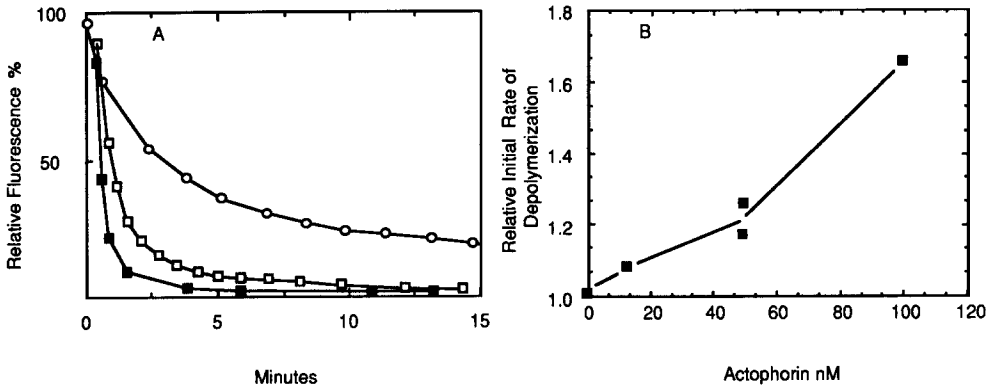

Figure 6. Actin depolymerization assay to evaluate the effect of actophorin on the relative number concentrations of filaments. (*A*) Depolymerization of actin filaments diluted to the critical concentration (0.1 μM) in the presence of various concentrations of actophorin. Dilution (at time 0) of F-actin solutions into buffers containing stated concentrations of actophorin was performed semi-automatically by a stopped flow device (details in Materials and Methods). Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$ 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C. (○) 0.1 μM (final concentration) actin alone. (□) 0.1 μM (final concentration) actin with 0.3 μM (final concentration) actophorin. (■) 0.1 μM actin with 0.6 μM actophorin. (*B*) Actophorin concentration dependence of the initial rate of depolymerization with respect to the rate of diluted control filaments in the absence of actophorin.

9, *A* and *B*) and the effect of actophorin on the kinetics of actin polymerization (Fig. 9, *C* and *D*). These effects required millimolar concentrations of phosphate with 50% protection at 5–10 mM phosphate (Fig. 9 *A*). In the presence of 25 mM phosphate, 2 μM actophorin had no effect on the critical concentration of actin (Fig. 9 *D*, *inset*), so actophorin may not bind actin under these conditions. Pyrophosphate was not as effective, and sulfate had no effect at a concentration of 25 mM (Fig. 9 *B*).

Discussion

The evidence reported here strengthens the conclusion that actophorin not only forms a tight 1:1 complex with actin monomers but also severs actin filaments. In the following paragraphs we summarize the strengths and weaknesses of possible mechanisms of action of actophorin and compare its properties with those of the other members of its family and those of the larger severing proteins. One of the main conclusions is that there must be at least two fundamentally different ways for proteins to sever actin filaments, one used by actophorin and another used by the larger severing proteins.

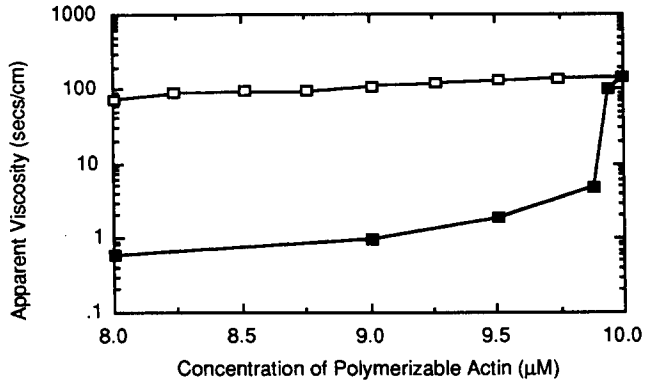

Figure 7. The reduction in the low shear viscosity of actin filaments by actophorin is not attributable to monomer sequestration. Actin concentrations between 8.0 and 10.0 μM polymerized alone (□). Actin at 10 μM with actophorin at 0, 0.05, 0.1, 0.5, 1.0, and 2.0 μM (■). The concentrations of polymer were calculated assuming a K_d of 0.1 μM for the actophorin/actin complex. Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C.

Actophorin Binds to Actin Monomers

Three independent lines of evidence show that actophorin forms a complex with actin monomers that alters the ability of the actin to polymerize. Actophorin can be chemically crosslinked to actin monomers (Fig. 1). The crosslinking experiments suggest that the stoichiometry is 1:1 but cannot rule out the binding of additional actophorin molecules. The experiments also show that profilin and actophorin compete in some way for binding to an actin molecule. Simple steric interference is the most likely explanation, but conformational changes in the actin cannot be ruled out. Steady-state polymerization experiments provide independent evidence for sequestration of actin monomers in a complex with actophorin and the only available information about the binding affinity. The dissociation constant calculated from the effect of actophorin on the polymer concentration is in the range of 0.1–0.2 μM, more than 20 times stronger than the affinity of *Acanthamoeba* profilin for actin (Lee et al., 1988).

All of the evidence suggests that complexes of actin monomers with actophorin do not participate in the assembly of actin filaments. In particular, these complexes do not appear to nucleate actin polymerization like gelsolin and other capping proteins.

Other members of the actophorin family also form a 1:1 complex with actin. ADF was crosslinked to cysteine 374 on actin through the 5 Å sulfhydryl reactive agent *N,N'-p*-phenylenedimaleimide forming a stoichiometric complex (Giuliano et al., 1988). The K_d of the actin-ADF complex is 0.05–0.1 μM. Sutoh and Mabuchi (1984, 1989) observed the stoichiometric interaction of depactin and actin by crosslinking with EDC and similar stoichiometric interactions have been reported for destrin (Nishida et al., 1985) and an 18 kD ADF-like protein from ascites hepatic cell line (Ohta et al., 1984).

Actophorin Binds Weakly to Filaments and Does Not Appear to Cap Them

Given the actin filament severing ability of actophorin dis-

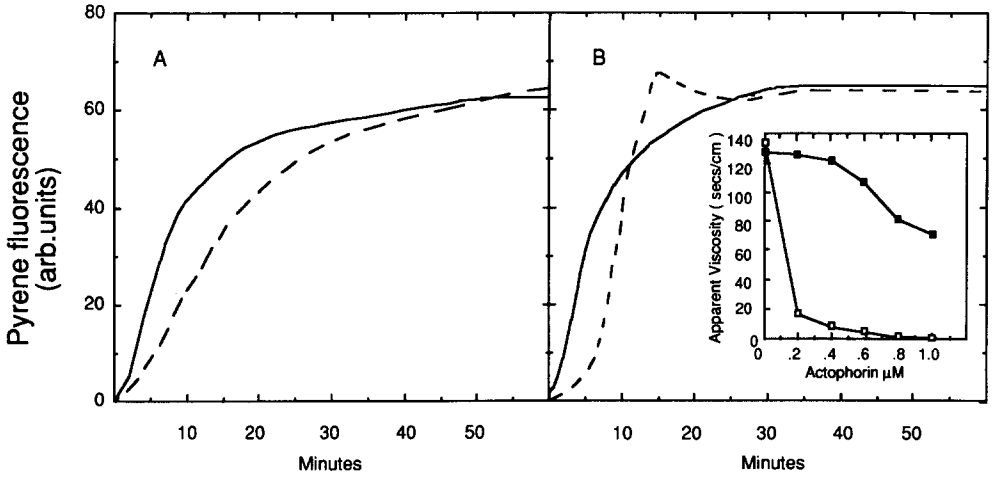

Figure 8. Phalloidin inhibits the effects of actophorin on the time course of the spontaneous polymerization of actin. The polymer concentrations were measured by the enhancement of fluorescence of pyrenyl-actin. Conditions: 4 μM pyrene actin (2.5% labeled), 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C. (*A*) Actin alone (- - -); actin with 2 μM phalloidin (—). (*B*) Actin with 2 μM actophorin (- - -); actin with 2 μM actophorin and 2 μM phalloidin (—). (*Inset*) Phalloidin inhibits the reduction of the low shear viscosity (measured by falling ball viscometry) of actin-filaments by actophorin. Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C; 10 μM actin alone (□); 10 μM actin with 10 μM phalloidin (■).

cussed below, actophorin must bind to the filaments in some way, but the reaction is poorly understood. No binding of actophorin to actin filaments was detected by pelleting (Cooper et al., 1986), but these experiments were complicated by the severing of the filaments by actophorin. At the high concentrations of actophorin that would be required to detect weak binding, the filaments are so short that they pellet poorly. The available data are consistent with either weak binding of actophorin to actin filaments, with a $K_d > 10^{-5}$ M, or with a very small number of sites being available on an actin fila-

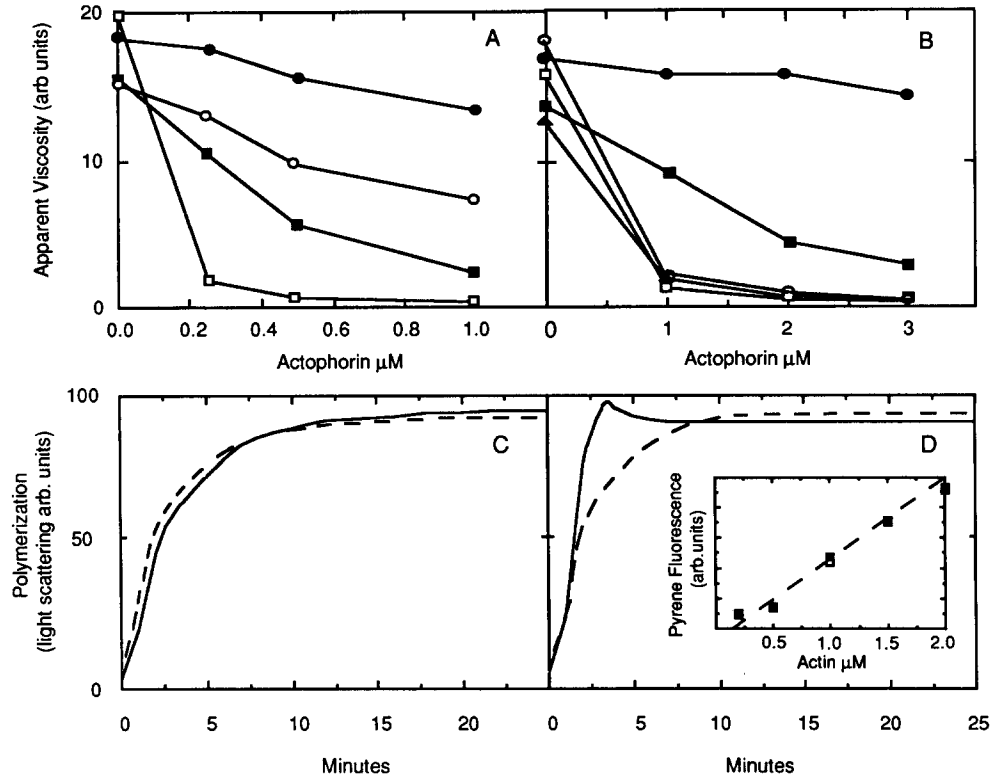

Figure 9. Phosphate inhibits the effects of actophorin on the low shear viscosity (*A* and *B*) and polymerization (*C* and *D*) of actin. Conditions: 12 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at 25°C. Stocks of potassium phosphate, sodium pyrophosphate, and potassium sulfate were adjusted to pH 8.0. 10 μM actin alone (□); 10 μM actin with 5 mM (■), 10 mM (○) and 25 mM phosphate (●). (*B*) 10 μM actin alone (□); 10 μM actin with 25 mM phosphate (●), 25 mM pyrophosphate (■), 25 mM sulfate (○) or 5 mM sulphate (▲). (*C* and *D*) The concentration of polymerized actin was assayed by 90° light scattering. (*C*) 7 μM actin alone (-). 7 μM actin with 10 mM potassium phosphate, pH 8.0 (- - -). (*D*) 7 μM actin, 1 μM actophorin, and 10 mM PO_4 (- - -). 7 μM actin and 1 μM actophorin (—). Inset is a critical concentration experiment in the presence of 25 mM phosphate, with and without 2 μM actophorin (same conditions as Fig. 2).

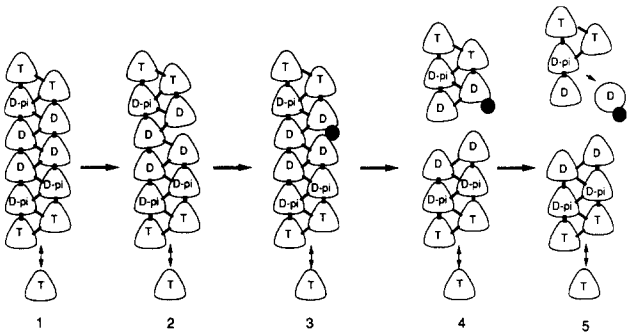

Figure 10. Model for severing by actophorin. To display the actin subunits and their postulated contacts we have adopted the approach introduced by Erickson (1989). The actin filaments are "unwound" and projected on a 2-D plane (*1*). Actin subunits (*triangular shapes*) with bound ATP, ADP with bound Pi and ADP are indicated by T, D-pi, and D, respectively. Subunits within the filament are held by four contacts, two longitudinal and two diagonal. According to this mechanism, thermal motion of the filament reveals an otherwise buried site close to a longitudinal contact (*2*), an actophorin molecule is then able to bind this site on an actin subunit with bound ADP (*3*). This destabilizes the longitudinal bond, leaving the filament held together by only one longitudinal, and one weak diagonal contact, thus, at this point the filament is more likely to break (*4*) than elsewhere.

ment for higher affinity binding of actophorin. Alternatively, the binding site may be fully exposed in filaments but the conformation of assembled actin subunits may reduce their affinity for actophorin. There is no way to distinguish these alternatives at the present time. Proteins related to actophorin either bind weakly (Koffer et al., 1988) or not at all (Nishida et al., 1984) to actin filaments.

There is no evidence that actophorin affects one end of actin filaments preferentially. Actophorin inhibits assembly at both ends (Cooper et al., 1986) and this can be explained by monomer sequestration alone. There could also be subtle effects on one end, but it is clear that there is not a strong bias in these effects as observed for both the gelsolin group and the fragmin/severin group of severing proteins which tightly cap the barbed ends of filaments. The fact that actophorin prolongs the lag phase of spontaneous polymerization rather than shortening it, supports the argument that actophorin does not nucleate actin filaments like capping protein (Cooper and Pollard, 1985). Also, the elongation assay shows no nucleating activity at early time points.

Actophorin Severs Actin Filaments

Five different approaches including direct observation by light microscopy have provided evidence that actophorin severs and thereby reduces the length of actin filaments. We have used the technique pioneered by Vale (1991) for microtubules, to directly visualize severing of actin microfilaments. Other attempts (Bearer, 1991) to demonstrate actophorin-dependent severing in the light microscope were not successful, most probably because stoichiometric labeling of the filaments with rhodamine phalloidin inhibited severing. Light microscopy is the most direct, but the elongation assay (Fig. 4) for measuring polymer number concentration provides the most conclusive quantitative data for severing. Actophorin increases the rate at which diluted pyrene-actin filaments depolymerize, again indicating severing action. Finally, the effects of actophorin on the electron microscopic appearance of filaments (Cooper et al., 1986) and on the low shear viscosity (Fig. 8) are also consistent with severing. Enhanced nucleation of filaments is an alternative mechanism that could give some of the same results, as observed for *Acanthamoeba* capping protein (Cooper and Pollard, 1985). This is highly unlikely in the case of actophorin, because none of the observations are consistent with nucleation of filaments by actophorin. Furthermore, such a mechanism cannot account for the rapid reduction in length observed when actophorin is mixed with preformed actin filaments.

Since the number concentration of actin filaments is stable for hours in the presence of actophorin and since short actin filaments at these concentrations rapidly anneal (Murphy et al., 1988), the actophorin must either sever actin filaments at steady state or block annealing. Neither mechanism can be ruled out, but we favor continuous severing, since the proteins that block annealing are generally strong capping proteins and would be expected to cosediment with actin.

Mechanism of Actin Filament Severing

We hypothesize that the most likely severing mechanism is that actophorin destabilizes filaments by intercalating between subunits (Fig. 10). The mechanism is based on the large difference in the affinity and/or the number of actophorin binding sites of monomeric and polymeric actin. We suggest that the actophorin binding sites of subunits is partially buried but can be exposed rarely by thermal distortion or strain in the bonds between subunits in filaments. The higher frequency of severing at preexisting bends in actin (Fig. 3) supports this hypothesis. Actophorin binds to actin monomers with about the same affinity as the actin subunits have for the end of a filament, so with an intercalated actophorin (1989), the probability of polymer breakage would be much higher at the site of intercalation than elsewhere along the polymer.

The validity of the intercalation hypothesis should be tested rigorously by the structural studies of actophorin that are now in progress (Magnus et al., 1989). Depactin can be crosslinked to both NH_2- and COOH-terminal peptides of actin (Mabuchi and Sutoh, 1984, 1989) and ADF can be crosslinked to cys374 (Guiliano et al., 1988). This places part of the binding site for this class of proteins at the barbed end of the actin molecule (Kabsch et al., 1990) on the outer surface of the filament near the longitudinal bonds of the long-pitch helix (Holmes et al., 1990). The structure of actophorin and the identity of the actin and actophorin residues crosslinked by EDC should reveal whether part of the actophorin binding site is usually buried within the filament.

Gelsolin (and fragmin/severin) may use a different mechanism to sever actin filaments. Gelsolin is a more potent severing protein than actophorin, requires Ca^{2+}, caps the severed end, and nucleates actin polymerization. Although studied in less detail, fragmin and severin appear to have similar properties. The severing mechanism is not fully understood, but plausible models, based on studies of domains produced by proteolysis (Kwiatkowski et al., 1985; Bryan and Hwo, 1986) or by expression of recombinant cDNAs (Kwiatkowski et al., 1989; Way et al., 1989) propose that all six domains of the 82-kD protein collaborate in the severing process by encompassing the entire circumference of the fila-

ment. Weak severing ability remains in a small gelsolin fragment which may function in a manner similar to that of the actophorin group.

Stabilizing Agents Protect Actin Filaments from Severing by Actophorin

The proposed intercalation mechanism is consistent with protection of the filaments by phalloidin. Phalloidin stabilizes actin filaments against a variety of disruptive agents, as well as spontaneous fragmentation (Estes et al., 1981). By reducing the likelihood of intercalation sites, they could also protect filaments from actophorin. Phalloidin also inhibits severing by fragmin (Hinssen, 1981). Tropomyosins inhibit the severing of filaments by gelsolin, (Ishikawa et al., 1989), fragmin (Hinssen, 1981), villin (Burgess et al., 1987), and ADF (Bernstein and Bamburg, 1982).

Phosphate Is a Potential Physiological Regulator of Severing by Actophorin

Millimolar concentrations of phosphate inhibit the binding of actin to actophorin and severing of actin filaments by actophorin. This is the concentration range required for phosphate binding to a low affinity site on polymerized actin subunits with bound ADP (Carlier and Pantaloni, 1988). Since binding of phosphate to these sites causes the polymer to behave much like filaments with ATP bound to each subunit (Rickard and Sheterline, 1986), it is generally thought to represent the reformation of the actin-ADP-P_i intermediate that forms transiently during the assembly of filaments from actin-ATP monomers (Carlier and Pantaloni, 1988). Since subunits newly incorporated into actin filaments are largely the actin-ADP-P_i intermediate that forms transiently during the assembly of filaments from actin-ATP monomers (Carlier and Pantaloni, 1988), rapidly growing filaments should be much less susceptible to severing by actophorin than older filaments, unless the cytoplasmic concentration of phosphate is greater than ~5 mM, when all filaments should be resistant.

Interpretation of the Effects of Actophorin on Spontaneous Polymerization

It is remarkable that a protein, which sequesters actin monomers and which reduces the length of actin filaments 10-fold by severing, has only a subtle effect on the time course of spontaneous polymerization (Figs. 4, 8, and 9). In bulk samples there is little effect on the initial lag but the rate of polymerization increases late in the reaction. We believe that the sum of several antagonistic reactions can explain the observations. The reactions are the formation of the actin monomer/actophorin complex, the severing of actin filaments by actophorin, and the normal actin polymerization reactions including nucleation, elongation, ATP hydrolysis, and P_i dissociation.

Under the initial conditions used in our experiments (micromolar concentrations of actophorin with an excess of actin monomers), much of the actophorin and part of the actin are tied up in complexes. This reduces the concentration of free actophorin available for severing filaments and minimizes the apparent severing activity. Furthermore, the sequestration of actin in these complexes should retard polymerization, especially since nucleation is so dependent on the actin monomer concentration. Unexpectedly, actophorin has only a small effect on the initial lag. This result can only be explained by a higher concentration of polymer ends in the presence of actophorin; the increase in ends just offsets the inhibition of nucleation and elongation by sequestration of monomers! This interpretation is confirmed by the elongation assay (Fig. 4 *B*) showing that the polymer number concentration in the presence of 1 μM actophorin was higher than controls as early as 2.5 min after the initiation of polymerization when only 6% of the free actin had polymerized (Fig. 4 *A*). This difference in polymer number concentration can be explained by severing. It is calculated that at this early stage in the polymerization process the mean length of the filaments in the presence of 1 μM actophorin is 1.6 μm, however, because of the large amount of monomeric actin present, only 0.21 μM actophorin is expected to be uncomplexed and so presumably able to sever. Another possible explanation is that actophorin can efficiently sever filaments only above a certain length.

After the lag phase, the bulk polymerization rate with actophorin exceeds the control in spite of the monomer sequestration, due to the high concentration of filaments. We attribute this to severing. We cannot yet explain the "overshoot" in polymer concentration prior to attaining steady state, but note that others have observed similar overshoots in stirred or disrupted samples (Cooper and Pollard, 1982; Cerven, 1987). At steady state the polymer concentration is less than controls due to sequestration of monomers in a nonpolymerizable complex with actophorin.

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