Mechanism of Interaction of *Dictyostelium* Severin with Actin Filaments

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ABSTRACT Severin, a 40,000-dalton protein from *Dictyostelium* that disassembles actin filaments in a Ca^{2+}-dependent manner, was purified 500-fold to >99% homogeneity by modifications of the procedure reported by Brown, Yamamoto, and Spudich (1982. *J. Cell Biol.* 93:205-210). Severin has a Stokes radius of 29 Å and consists of a single polypeptide chain. It contains a single methionyl and five cysteinyl residues. We studied the action of severin on actin filaments by electron microscopy, viscometry, sedimentation, nanosecond emission anisotropy, and fluorescence energy transfer spectroscopy. Nanosecond emission anisotropy of fluorescence-labeled severin shows that this protein changes its conformation on binding Ca^{2+}. Actin filaments are rapidly fragmented on addition of severin and Ca^{2+}, but severin does not interact with actin filaments in the absence of Ca^{2+}. Fluorescence energy transfer measurements indicate that fragmentation of actin filaments by severin leads to a partial depolymerization ($t_{1/2} \cong 30$ s). Depolymerization is followed by exchange of a limited number of subunits in the filament fragments wth the disassembled actin pool ($t_{1/2} \cong 5$ min). Disassembly and exchange are probably restricted to the ends of the filament fragments since only a few subunits in each fragment participate in the disassembly or exchange process. Steady state hydrolysis of ATP by actin in the presence of Ca^{2+}-severin is maximal at an actin:severin molar ratio of approximately 10:1, which further supports the inference that subunit exchange is limited to the ends of actin filaments. The observation of sequential depolymerization and subunit exchange following the fragmentation of actin by severin suggests that severin may regulate site-specific disassembly and turnover of actin filament arrays in vivo.

One of the central problems in cell motility is to understand the molecular basis for the transient nature of the nonmuscle contractile system. For instance, it is clear that cytokinesis, pseudopod formation, phagocytosis, and substrate adherence are dynamic events which demand both a spatial and temporal control of actin assembly and disassembly. Consequently, a pressing question has been to determine to what extent dynamic control over actin disassembly and subunit exchange is an inherent property of the filaments themselves. Extensive subunit exchange has been observed with purified actin filaments under some conditions (51, 52). However, recent experiments (39) indicate this may not occur under physiologically related ionic conditions. Under such conditions Pardee et al. (39) have shown by fluorescence energy transfer, ^{35}S-actin monomer exchange, and steady state nucleotide incorporation into filaments that highly purified actin filaments from both muscle and *Dictyostelium* amoebae undergo only limited subunit exchange. This observation poses a further question. Are there accessory proteins which can function to cause filament disassembly and enhance filament subunit exchange in response to metabolic signals?

Recent studies indicate that a number of proteins modulate actin filament behavior (for reviews see references 25, 42). Several cytosolic proteins cause fragmentation or depolymerization of actin filaments. Gelsolin, a 91,000-dalton protein, binds and shortens F-actin in the presence of Ca^{2+} (57, 58). Villin, a 95,000-dalton protein from microvilli of intestinal epithelia, bundles filaments in the absence of Ca^{2+} but fragments F-actin in the presence of Ca^{2+} (6, 7, 11). A 90,000-dalton human platelet protein with similar properties has also

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been isolated (50). Two different actin depolymerizing proteins that form complexes with actin filament subunits in a 1:1 molar ratio have been isolated from chick brain (2) and porcine plasma (17). In *Physarum polycephalum*, actin filament length may be controlled by a 42,000-dalton protein called fragmin (18) or actin-modulating protein (19, 20). In the presence of Ca^{2+}, fragmin binds both G-actin and F-actin, nucleates assembly, and fragments filaments into a population of oligomers of homogeneous length (18, 20). Final filament length is determined by the ratio of fragmin to actin. Most of these filament modifying proteins require Ca^{2+} for their effects, suggesting that they regulate actin-containing contractile complexes in vivo. However, the role of such proteins in governing cell motility is not understood.

Brown et al. (8) recently identified a 40,000-dalton protein from *Dictyostelium discoideum* amoebae which severs actin filaments in the presence of Ca^{2+}. This protein also accelerates actin assembly and causes partial depolymerization of filaments (8). Because these properties are Ca^{2+}-dependent and therefore potentially important for controlling the morphology of intracellular actin arrays, we studied the interaction of highly purified 40,000-dalton protein on purified actin filaments. Furthermore, because actin filaments appear to exchange filament subunits to only a limited extent in physiological ionic conditions (39), it was of interest to examine the effect of the 40,000-dalton protein on the mechanism and extent of filament subunit exchange. Considering the mechanism of action of this protein as elucidated here, we have named it severin.

In addition to electron microscopy, viscometry, and sedimentation, two fluorescence approaches were used to determine the interaction of severin with actin filaments. The first is nanosecond emission anisotropy, which provides information about the rotational motion of fluorescent chromophores (30, 34, 49, 54). The emission anisotropy kinetics depend on the size and shape of the labeled macromolecule (34) and also on its modes of flexibility (55). This technique can detect conformational changes within a protein as well as binding of a protein to filaments. The second experimental approach is fluorescence energy transfer, which monitors the proximity of donor-labeled and acceptor-labeled actin subunits (45, 46). Therefore, fluorescence energy transfer can be used to examine the effect of severin on filament assembly or disassembly and on filament subunit exchange. Filament assembly leads to energy transfer and a consequent decrease in the fluorescence intensity of the donor fluorochrome. Conversely, disassembly relieves this quenching of donor fluorescence.

Exchange of subunits between preassembled donor-labeled filaments and preassembled acceptor-labeled filaments is sensitively quantified by the decrease in donor fluorescence. An attractive feature of this latter energy transfer experiment is its sensitivity to subunit exchange but not to filament assembly or disassembly.

These fluorescence experiments provide a clear demonstration of the sequence of events which occur when severin is added to actin filaments in the presence of Ca^{2+}. On the basis of these results, we propose a model describing actin fragmentation, disassembly, subunit exchange, and enhanced steady state ATP hydrolysis by the action of *Dictyostelium* severin.

MATERIALS AND METHODS

Actin and Myosin Purification

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (44) and further purified with methods described by Pardee and Spudich (37). *Dictyostelium* actin was prepared as described by Uyemura et al. (48) and ^{35}S-methionine-labeled *Dictyostelium* actin as described by Simpson and Spudich (43). G-buffer in these experiments was 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.2 at 25°C, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 50 μM $CaCl_2$, and 0.005% NaN_3. F-buffer contained 50 mM KCl and 2 mM $MgCl_2$ in G-buffer. Myosin was prepared from rabbit skeletal muscle according to Kielley and Bradley (23).

Assay for Purification of Severin

^{35}S-labeled *Dictyostelium* actin was mixed with skeletal muscle actin. Fractions to be assayed were added to 0.4 mg/ml of F-actin in actin F-buffer containing either 0.2 mM $CaCl_2$ or 0.5 mM EGTA. The mixture, 100 μl, was incubated for 10 min at 25°C and centrifuged in a Beckman airfuge (Beckman Instruments, Menlo Park, CA) at 30 psi for 10 min. 50 μl of the supernatant was counted in a Beckman LS 7500 scintillation counter. To measure only the Ca^{2+}-sensitive activity, control assays with EGTA present were subtracted from those with Ca^{2+} present. One unit of activity is defined as the amount of protein which makes 50% of the F-actin nonsedimentable.

Severin Purification

Severin was purified at 4°C as described previously (8), with the following modifications. *D. discoideum* strain Ax-3 was grown in HL-5 medium (29) and was harvested in late log phase by centrifugation at 500 *g* for 7 min. Cells were washed in 5 vol (wt/vol) of 10 mM triethanolamine buffer, pH 7.5, 0.5 mM DTT, and collected by centrifugation at 500 *g* for 11 min. Washed cells (typically 200 *g*) were suspended in 2 vol (wt/vol) of lysis buffer which contained 10 mM triethanolamine, pH 7.5, 60 mM sodium pyrophosphate, 30% (wt/vol) sucrose, 0.01 mg/ml of Trasylol (Boehringer Mannheim, Indianapolis, IN) and 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). The cell suspension was sonicated on ice by five 30-s bursts at 60-s intervals with a Heat Systems W-220F sonicator (Ultrasonics, Inc., Plainview, NY) at position 7.

The lysate was centrifuged at 25,000 *g* for 15 min at 4°C; the resulting supernatant was further centrifuged at 100,000 *g* for 90 min. For each 100 ml of this high speed supernatant, 39 g of solid ammonium sulfate (60% saturation) were added slowly. The precipitate was removed by centrifugation at 25,000 *g* for 20 min. Then 14.3 g of solid ammonium sulfate (80% saturation) was added to each 100 ml of the resulting supernatant, and the precipitate was collected by centrifugation at 25,000 *g* for 30 min. The pellet was dissolved in 2 mM triethanolamine buffer, pH 7.5, 0.2 mM DTT (1/5 in vol to the starting number of grams of cells). The solution was dialyzed for 15 h against 2l of the same buffer with one buffer change. The dialysate was then mixed in a beaker with settled DEAE cellulose (DE52, Whatman Biochemicals, Ltd., Maidstone, England; 1/5 in volume to the starting number of grams of cells) that was equilibrated with 2 mM triethanolamine, pH 7.5, 0.2 mM DTT; the suspension was stirred every 15 min for 2 h. The protein that did not adhere to DEAE cellulose was separated from the resin by gravity filtration through Whatman 541 filter paper. The filtrate was applied to a hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) column (1.5 × 35 cm) equilibrated with 10 mM potassium phosphate, pH 6.7, and 0.2 mM DTT. Proteins which bound to the column were eluted in 2.6 ml fractions by a convex KCl gradient (0–0.3 M) in 10 mM potassium phosphate, pH 6.7, and 0.2 mM DTT. The convex gradient was constructed using a series of three buffer wells. Buffer flowed from a 0.3 M KCl well (150 ml) to a 0.15 M KCl well (150 ml) to a 0 M KCl well (60 ml) to the column. Activity eluted as a single peak at a KCl concentration of 0.17 M. This fraction was concentrated to 0.6 ml by vacuum dialysis in a 10,000-dalton cut-off collodion bag (Schleicher & Schuell, Keene, NH). Concentrated activity was applied to a Sephacryl S-200 column (1.5 × 73 cm) equilibrated with 20 mM triethanolamine, pH 7.5, 0.2 mM DTT, and 50 mM KCl. Activity was eluted in a 2.9 ml fraction as a single symmetrical peak with an effluent volume slightly larger than that of egg albumin.

Fluorescence Labeling of Proteins

IAENS-labeled F-actin was prepared by a modification of the procedure of Frieden et al. (14). FITC-labeled F-actin was prepared according to Pardee et al. (39). Fluorescein-5-isothiocyanate (FITC) and 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid (IAENS) were purchased from Molecular Probes, Inc. (Plano, TX). *Dictyostelium* severin at 10^{-5} M was reacted with 0.2 mM IAENS in 20 mM triethanolamine, pH 7.5 at 0°C, 50 mM KCl, and 1 mM $MgCl_2$ for 15 h in the dark and dialyzed against the same solution containing 0.2 mM DTT. Under these conditions, one to two IAENS molecules were attached to the protein without affecting its activity.

Fluorescence Energy Transfer

Fluorescence energy transfer between IAENS-labeled actin and FITC-labeled

actin was measured (39) with a Spex Fluorolog spectrofluorometer (Spex Industries, Inc., Metuchen, NJ). The extent of energy transfer from IAENS-labeled actin (fluorescent donor) to FITC-labeled actin (fluorescent acceptor) within filaments was determined by the quenching of IAENS fluorescence at 470 nm (λ_{ex} = 350 nm). Two types of transfer experiments were carried out. In the first, depolymerization of filaments after fragmentation by severin was measured. A 9:1 ratio of FITC-labeled actin monomers to IAENS-labeled actin monomers in G-buffer were co-assembled by the addition of 50 mM KCl and 2 mM $MgCl_2$. Assembly was followed by the increase in fluorescence quench at 470 nm. After full assembly was reached, unlabeled *Dictyostelium* severin in F-buffer was added to obtain final molar ratios of actin:severin of 20:1, 50:1, or 100:1. Net disassembly of filaments was monitored by the decrease in fluorescence quench at 470 nm. Control experiments in which 0.2 mM $CaCl_2$ was replaced by 0.5 mM EGTA were also performed.

The second energy transfer experiment measured filament subunit exchange rather than filament disassembly. To determine exchange, 10 parts of preassembled acceptor-labeled (FITC) filaments at 1.0 mg/ml were mixed with one part of donor-labeled (IAENS) filaments at 1.0 mg/ml in F-buffer, and subunit exchange between filaments in the absence of severin was quantified by the amount of IAENS-actin fluorescence quench observed (39). Unlabeled severin in F-buffer was then added to molar ratios of actin:severin of 20:1, 50:1, or 100:1, and the amount of subunit exchange induced by filament fragmentation was quantified by the increase in fluorescence quench at 470 nm (39). In both types of energy transfer experiment, the final actin concentration after addition of severin was between 0.7 and 1.1 mg/ml.

Nanosecond Emission Anisotropy Measurements

Nanosecond emission anisotropy was measured using the apparatus described by Reidler et al. (41). Samples containing 0.01 to 0.1 mg/ml IAENS-labeled severin in the presence or absence of F-actin were excited at 325 nm with the frequency-doubled output of a mode-locked cavity-dumped argon-ion/rhodamine 6G dye laser to obtain rotational correlation times. The decay of IAENS fluorescence anisotropy was measured from 5 to 100 ns. The anisotropy emission kinetics were fit to a sum of two exponential decays, corresponding to rapid (ϕ_1) and slow (ϕ_2) rotational motions.

ATPase Activity

The ATPase activity of F-actin in the presence and absence of severin was measured using [γ-^{32}P] ATP according to the method of Clarke and Spudich (9).

DNase I Activity Determinations

DNase I activity was measured according to Harris et al. (16). DNA (200 μg/ml) in 0.125 M triethanolamine, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 mM NaN_3 was maintained at 20°C. 10 μl of F-actin (0.4 mg/ml), with or without severin, were added to 90 μl of DNase I (20 μg/ml in 15% glycerol, 30 mM NaCl, 20 mM triethanolamine, pH 7.5) in a cuvette at 20°C. 900 μl of DNA solution were added and mixed quickly with a plastic bar. OD_{285} was recorded at a chart speed of 10 in/min for 1 min. DNase I activity was determined from the slope of the linear part of the recorded curve. G-actin concentration was calculated assuming that 4.2 μg of G-actin inactivates 3.1 μg of DNase I.

Viscosity Measurement

The viscosity of F-actin was measured either by an Ostwald type viscometer (Cannon Instrument Co., State College, PA) or by a rolling ball viscometer (40). The flow rate of water in the Ostwald type viscometer was 112 s at 25°C. For rolling ball viscometry, the time required for a small stainless steel ball (Microball Co., Peterborough, NH) to roll 6 cm in a capillary (100 μl micropipette, Becton, Dickinson, & Co., Parsippany, NJ) at a declination of 30°C was measured. The time was converted to viscosity using a glycerol-water solution as a standard.

Amino Acid Analysis

Protein was hydrolyzed in 6N HCl at 110°C for 24 h and analyzed in a Hitachi 835 amino acid analyzer. Sulfhydryl content of the protein was determined separately using 5,5'-dithiobis-(2-nitrobenzoic acid) (12). Protein was denatured at 25°C in 0.35 M Tris-HCl, pH 8.0, 1% SDS, and 10 mM EDTA to expose sulfhydryl groups to the reagent. A 200-fold molar excess of 5,5'-dithiobis-(2-nitrobenzoic acid) was added and color development at 412 nm was measured. The extinction coefficient of thionitrobenzoate anion at 412 nm was taken as 1.36×10^4 M^{-1} cm^{-1}.

Stokes Radius Determination

The effluent volume of severin on a Sephacryl S-200 column (1.5 × 73 cm) was compared with those of proteins whose Stokes radii are known. 0.3 ml of protein was loaded onto the column and 1 ml fractions were collected. The protein standards used were bovine serum albumin (BSA) (35.5 Å), ovalbumin (30.5 Å), chymotrypsinogen (20.9 Å), and ribonuclease A (16.4 Å). Stokes radius was determined by interpolation of the linear plot of $(-\log k_{av})^{1/2}$ vs. Stokes radius (26).

Molecular Weight Determination by SDS Gel Electrophoresis

SDS PAGE was carried out according to Laemmli and Favre (27) using a 1.5 mm thick slab gel. Protein standards used to determine the molecular weight of severin were phosphorylase b (94,000), BSA (67,000), glutamic dehydrogenase (53,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

Other Methods

Protein concentration was measured by the Bradford method (4), using BSA as a standard. Free Ca^{2+} concentration was calculated assuming a K_d for Ca^{2+} and EGTA of 2×10^{-7} M (47). For electron microscopy, samples were negatively stained with 1% (wt/vol) uranyl acetate and observed with a Philips 201 electron microscope at 80 kV.

RESULTS

Purification of Severin

Table I summarizes a typical purification of severin. Loss of activity at the Sephacryl S-200 column step is not due to the column itself but to the adsorption of protein to the collodion membrane during concentration. The final recovery of activity was 15% of that in the high speed supernatant and was enriched about 500-fold. Severin therefore, represents ~0.2% of the total protein in the high speed supernatant. This value is reasonable since we can hardly observe the band corresponding to severin on SDS polyacrylamide gels loaded with 35 μg of high speed supernatant protein (Fig. 1). An average yield of severin is 2.5 mg from 200 g of wet cells.

Fig. 1 shows SDS PAGE band patterns at each step of the procedure. Because the limit of detection for Coomassie-Blue-stained protein bands on a 1.5 mm thick slab gel is ~0.05 μg/band (37), the absence of a visible band other than severin with a load of 8 μg protein suggests that the final product is >99% pure.

Structural Properties of Severin

The subunit molecular weight of severin determined by SDS PAGE is 40,000 and its elution position on Sephacryl S-200

TABLE I

Purification of Severin

	Volume	Protein*	Activity‖	Specific activity	Yield
	ml	*mg*	*U*	*U/mg*	*%*
HSS§	430	8,600	8,500	1.0	100
AS	51	300	4,400	15	52
DEAE	61	35	3,300	94	39
HAP	53	4.7	2,200	470	26
S-200	26	2.5	1,300	520	15

* Starting material was 200 g of wet *D. discoideum*.

‖ The assay and details of the purification procedure are described in Materials and Methods.

§ Abbreviations used are the same as in Fig. 1.

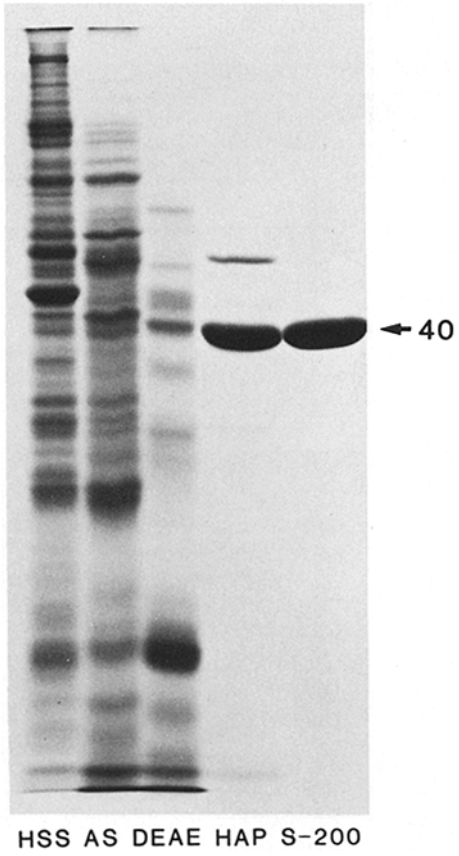

FIGURE 1 SDS PAGE of severin purification fractions. High speed supernatant (HSS), 35 μg. 60–80% ammonium sulfate pellet (AS), 25 μg. That fraction which does not adhere to DEAE cellulose (DEAE), 17 μg. Pooled fractions from hydroxyapatite column (HAP), 8 μg. Pooled fractions from Sephacryl S-200 column (S-200), 8 μg. Molecular weight, × 10^{-3}.

TABLE II

Amino Acid Composition of Severin

Asp	32.0
Thr	19.0
Ser	29.1
Glu	32.1
Pro	16.8
Gly	25.5
Ala	24.2
Val	17.0
Cys*	4.9
Met	1.0
Ile	17.2
Leu	27.7
Tyr	11.0
Phe	16.7
Lys	29.8
His	6.2
Arg	9.0

* Sulfhydryl content was determined separately by using 5,5'-dithiobis-(2-nitrobenzoic acid) as described in Materials and Methods.

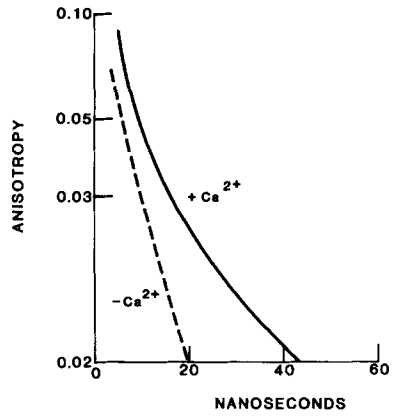

FIGURE 2 Ca^{2+} alters the nanosecond emission anisotropy kinetics of IAENS-labeled severin. In F-buffer, which contains Ca^{2+} (——), the rotational correlation times are 6 ns (ϕ_1) and 31 ns (ϕ_2) for IAENS-labeled severin at 0.1 mg/ml. When 0.2 mM $CaCl_2$ was replaced by 0.5 mM EGTA (- - -), the mobility of the probe increased: ϕ_1 = 2 ns and ϕ_2 = 8 ns.

coincides with this molecular weight, suggesting that its native molecular weight is also 40,000. Severin is globular since its Stokes radius (29 Å; see Materials and Methods) is comparable to the value of 25 Å calculated for a rigid hydrated spherical protein with a molecular weight of 40,000 assuming a partial specific volume of 0.75 cm^3/g and a degree of hydration of 0.2 cm^3/g (31).

The amino acid composition of severin is shown in Table II. It contains five cysteinyl residues and one methionine residue. Since it does not adhere to DEAE cellulose at pH 7.5 and low ionic strength, and is eluted from hydroxyapatite by a KCl gradient alone, severin behaves like a basic protein. This is not clear from the amino acid composition presented in Table II, presumably because glutamine and asparagine have been hydrolyzed to glutamic acid and aspartic acid, respectively, during the determination.

Ca^{2+} Induces a Conformational Change in Severin

Ca^{2+} is required for all known effects of severin on F-actin (8; also see below). We therefore examined the effect of Ca^{2+} on the conformation of severin. As shown in Fig. 2, the nanosecond emission anisotropy kinetics of IAENS-labeled severin are different in the presence and absence of Ca^{2+}. The rotational correlation time (ϕ_2) increases from 8 to 31 ns in the presence of Ca^{2+}. A fluorochrome rigidly attached to a spherical 40,000-dalton protein is expected to have a ϕ_2 of ~25 ns (34). Thus, the relaxation time of 8 ns indicates that the fluorescent probe covalently attached to severin shows a high degree of rotational mobility in the absence of Ca^{2+}. Severin becomes more rigid (or the probe has less rotational freedom) on binding Ca^{2+}.

Severin Binds to F-Actin in Ca^{2+}

In the presence of EGTA, the emission anisotropy decay curve for IAENS-labeled severin added to muscle F-actin was nearly the same as that of the severin alone (ϕ_2, 8 ns) (Fig. 3, dashed line). When severin was added to F-actin in the presence of Ca^{2+} (Fig. 3, unbroken line), a very long rotational correlation time of >400 ns was observed. Since this slow rotational motion is characteristic of actin filaments (38), the result indicates that severin binds to F-actin in the presence of Ca^{2+}. Severin appears to be bound to the "barbed" end of actin filaments since addition of G-actin to the "barbed" end, but not to the "pointed" end, is inhibited by severin (A. G. Weeds

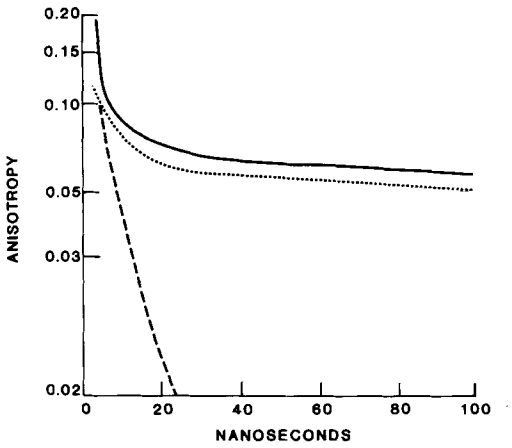

FIGURE 3 Effect of Ca^{2+} on the nanosecond emission anisotropy of IAENS-labeled severin in the presence of F-actin. 0.01 mg/ml severin plus 1 mg/ml F-actin in 0.2 mM $CaCl_2$ (——). Severin plus F-actin in $<10^{-8}$ M Ca^{2+} (+0.5 mM EGTA) (- - -). EGTA added to a final concentration of 4 mM to the severin-F-actin complex formed in the presence of 0.2 mM $CaCl_2$ (·····). Samples were in F-buffer.

and J. A. Spudich, unpublished observations). Once bound, severin does not readily dissociate upon addition of EGTA. The emission anisotropy decay curve remains the same even when a 20-fold molar excess of EGTA over Ca^{2+} is added to the F-actin solution containing bound severin (Fig. 3, dotted line).

Severin Fragments F-Actin in a Stoichiometric Manner

Electron microscopy showed that severin converts long actin filaments to short fragments in the presence but not the absence of Ca^{2+} (Fig. 4*A* and *B*). As indicated earlier (8) the effect of severin on actin filaments is stoichiometric rather than enzymatic. The fragmentation of actin filaments occurs within a few seconds of addition of severin, and can be measured by a viscosity decrease (Fig. 4*C*). At a ratio of severin to actin of 1:20, the reduced viscosity was decreased by 10 times to <1 g/dl.

Ca^{2+} Is Required in the 1–10 μM Range for Severin Action

As previously reported (8), the activity of severin requires Ca^{2+}. In Fig. 5, the effect of severin on F-actin viscosity, F-actin sedimentability, and on the contractility of reconstituted actomyosin were measured as a function of free Ca^{2+} concentration. By all of these assays severin is active in the presence of 1–10 μM Ca^{2+}. By the criterion of viscosity, filament severing is maximal at 10^{-6}–10^{-5} M Ca^{2+}. The sedimentation assay shows that at maximum fragmentation of filaments by severin, a significant proportion of the actin has depolymerized to oligomers of less than about 10 subunits[1] and perhaps even to monomers. Severin also inhibits the ATP-mediated contraction of actomyosin. At low free Ca^{2+} concentrations, contraction of actomyosin occurs normally as evidenced by the contraction of an actomyosin gel into a tight clot. However, at free Ca^{2+} concentrations of from 1–10 μM, clot formation is severely inhibited when severin is present in a 1:100 ratio to actin (Fig. 5).

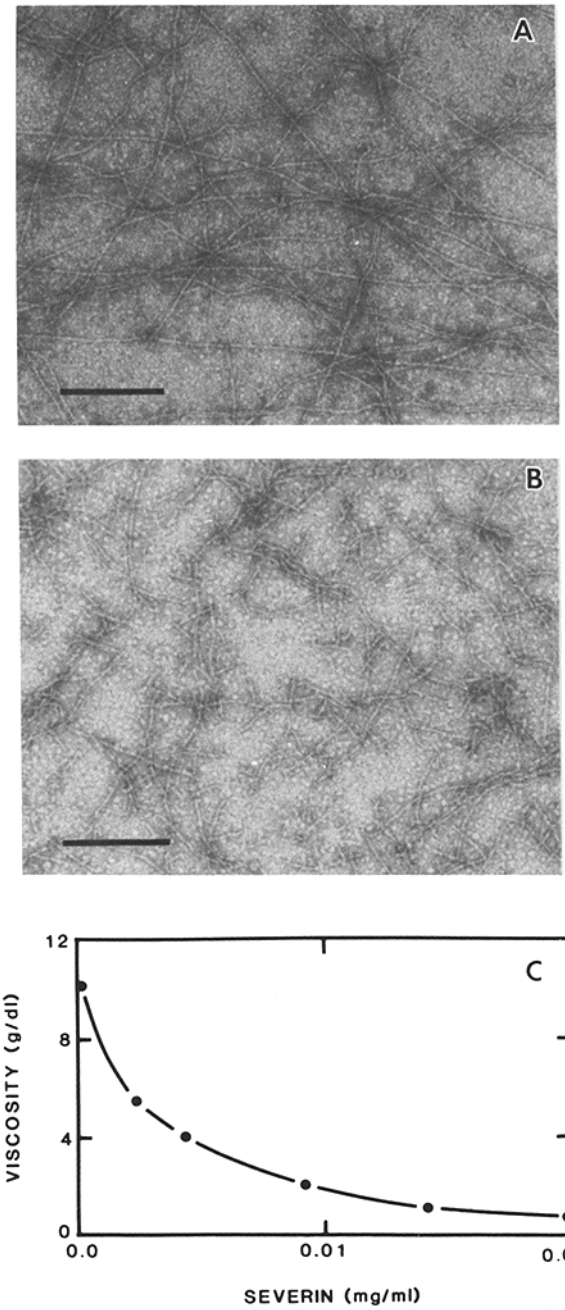

FIGURE 4 Fragmentation of actin filaments by severin. Electron micrographs of muscle F-actin (*A*) and muscle F-actin in the presence of severin at 1:20 (wt/wt) severin:actin (*B*). Bar, 0.2 μm. × 70,000. (*C*) Effect of severin on F-actin viscosity. F-actin (0.4 mg/ml) in F-buffer was incubated with various concentrations of severin and the resulting final viscosity determined with an Ostwald type viscometer at 25°C.

Severin Interaction with F-Actin Leads to Partial Disassembly of the Actin Filaments

The viscometry, sedimentation, and electron microscopy studies shown above indicate that actin filaments are reduced in length by severin in the presence of Ca^{2+}. By applying the technique of fluorescence energy transfer, it has been possible

[1] Sedimentation assays in a Beckman airfuge operating at 30 psi for 5–30 min using 100-μl mixtures of 0.5 mg/ml cross-linked actin oligomers (24) indicated that oligomers containing <10 subunits did not sediment under the assay conditions used.

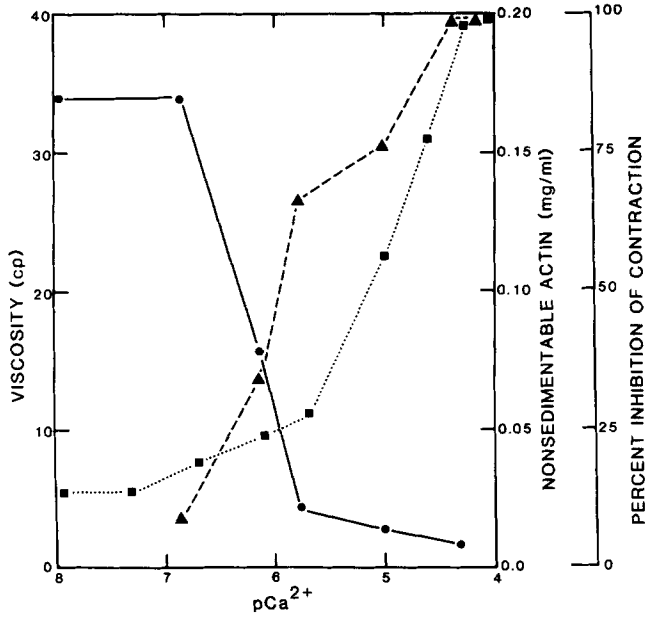

FIGURE 5 Ca^{2+} concentration dependence of severin activity on F-actin. Filament severing activity was assayed by the viscosity change of F-actin (●). F-actin (400 μg/ml) was mixed with 12 μg/ml severin in actin F-buffer containing various free Ca^{2+} concentrations. Viscosity was measured by a rolling ball viscometer. Filament depolymerizing activity (■) was measured by the sedimentability of F-actin in a Beckman airfuge at 130,000 g_{ave} for 30 min. Buffer and protein concentrations were identical to the viscometry assays. Effect of severin on the extent of contraction of an actomyosin gel (▲). Actin (1.2 mg/ml), myosin (0.15 mg/ml), and severin (0.012 mg/ml) were mixed in 50 mM KCl, 20 mM triethanolamine, pH 7.5, 0.04 mM $MgCl_2$ at various free Ca^{2+} concentrations. Contraction at 22°C was initiated by adding ATP to give a final concentration of 3 mM. In the absence of Ca^{2+}, the gel contracted to ~8% of its initial volume. Percent inhibition was calculated by comparing the volumes of contracted gels.

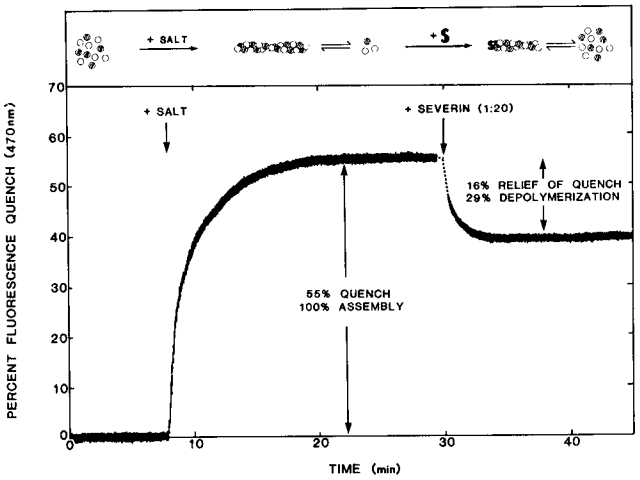

FIGURE 6 Actin filament fragmentation by severin induces partial filament disassembly. A 10:1 ratio of rabbit skeletal muscle FITC-G-actin to IAENS-G-actin in G-buffer containing 20 mM triethanolamine, pH 7.4, was coassembled by addition of KCl to 50 mM and $MgCl_2$ to 2 mM. Total actin concentration was 0.75 mg/ml. $CaCl_2$ was added to 0.2 mM, followed by one part of severin added to 20 parts of assembled actin. Relief of fluorescence quench was monitored at 470 nm.

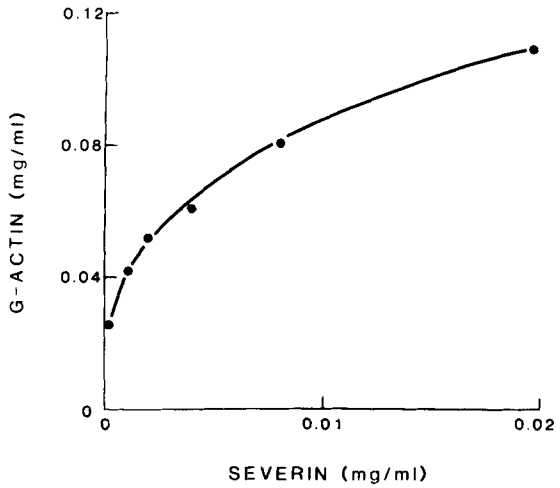

FIGURE 7 Depolymerization of F-actin by severin. The final G-actin concentration in F-actin solutions treated with various concentrations of severin was determined by DNase I inhibition assays. The initial muscle F-actin concentration was 0.4 mg/ml in F-buffer for each assay.

to both kinetically follow the sequence of events which occur subsequent to filament fragmentation, and to quantify the amounts of filament disassembly and filament subunit exchange. As shown in Fig. 6, coassembly of donor-actin and acceptor-actin can be followed by quench of the donor-actin fluorescence. Complete assembly (critical actin concentration ≤20 μg/ml) is equivalent to 55% quench of donor fluorescence. After filaments were completely assembled, addition of severin in 0.2 mM Ca^{2+} caused rapid depolymerization of filaments to a new steady state level (Fig. 6). In this case, at an actin:severin ratio of 20:1, 29% of the filament subunits disassembled. Depolymerization occurred with a half-time ($t_{1/2}$) of ~30 s and no net reassembly was observed. Addition of severin in $<10^{-8}$ M Ca^{2+} (+ 0.5 mM EGTA) caused no fluorescence changes (data not shown). Since filament fragmentation takes place within a few seconds after severin addition, the demonstration of a filament depolymerization halftime of ~30 s indicates that depolymerization occurs either simultaneously with or immediately following filament fragmentation.

In a separate experiment, the extent of depolymerization of actin filaments in the presence of severin (+Ca^{2+}) was assayed by the DNase I assay described by Blikstad et al. (3). This assay is believed to measure G-actin specifically (3). For F-actin at 0.4 mg/ml, addition of severin to 0.02 mg/ml (severin:actin of 1:20) resulted in net depolymerization of 28% of the F-actin (Fig. 7). This value, which was obtained by using unlabeled actin, is in excellent agreement with that obtained by the fluorescence energy transfer assay (Fig. 6).

Severin Interaction with F-actin Leads to an Increase in Filament Subunit Exchange

To determine if filament fragmentation by severin had any effect on filament subunit exchange, a fluorescence energy transfer exchange assay using preassembled donor-labeled filaments (IAENS-F-actin) and acceptor-labeled filaments (FITC-F-actin) was performed (39). As shown in Fig. 8, when an excess of acceptor-labeled actin filaments was added to donor filaments in F-buffer containing 0.2 mM $CaCl_2$ but no severin, virtually no subunit exchange was detected. This agrees with our earlier observation of severely limited actin filament subunit exchange in physiologically related salt conditions (39).

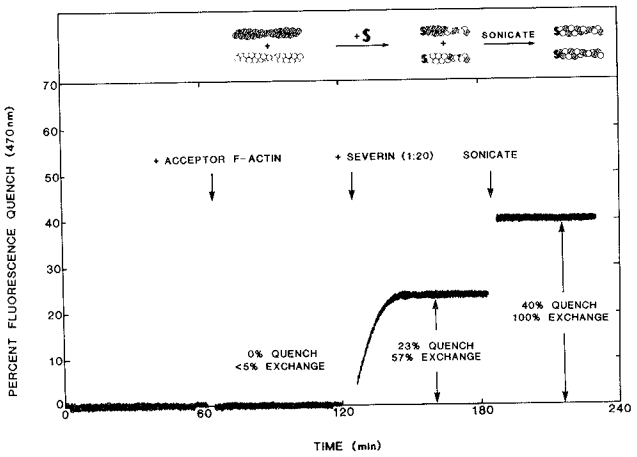

FIGURE 8 Severin enhances actin subunit exchange between filaments. Separately assembled donor- and acceptor-labeled muscle F-actins were mixed to final concentrations of 0.1 mg/ml donor and 0.9 mg/ml acceptor in F-buffer containing 20 mM triethanolamine, pH 7.4, and 0.2 mM $CaCl_2$. Fluorescence quench was monitored at 470 nm for 1 h at 25°C. One part of severin was added to 20 parts of assembled actin and the increase in quench monitored. After the plateau exchange value was reached, the sample was sonicated to completely randomize the subunits between the donor and acceptor filaments (100% exchange).

Addition of severin triggered immediate subunit exchange. An initial period of exchange exhibiting a half-time of ~5 min was followed by leveling off to a constant limited exchange value. Addition of a 1:20 ratio of severin:actin caused 57% exchange of filament subunits contained within the filament fragments. Sonication of the sample, which is known to cause complete randomization of subunits under these conditions (39, 46), resulted in complete (100%) exchange of filament subunits. In this case 40%, rather than 55%, quench is equivalent to complete subunit exchange because the actin fragments had partially depolymerized before exchange occurred (Fig. 6).

It was possible to obtain an estimate of the number of subunits that disassemble and exchange per filament fragment by quantifying disassembly and exchange by fluorescence energy transfer in the presence of various ratios of severin:actin (Table III). As the proportion of severin:actin was increased, corresponding increases in both limited disassembly and limited subunit exchange were observed (Table III). However, the total number of actin subunits that disassembled or exchanged per actin fragment remained constant. In each case, the value was of the order of 10 actin subunits per filament fragment (see Table III).[2] The total amount of subunit disassembly or exchange therefore appears to be directly proportional to the number of filament ends.

TABLE III

Effect of Increasing Severin on Actin Depolymerization and Exchange

Actin: severin*	Filament disassembly	Subunits disassembled per fragment‖	Filament subunit exchange	Subunits exchanged per fragment§
mol/mol	%	*Average*	%	*Average*
100:1	6	6	7	7
50:1	11	6	17	8
20:1	29	6	57	8

* Severin added to 0.75 mg/ml F-actin in F-buffer.

‖ Calculated assuming random fragmentation by 100% of the added severin and that one severin molecule produces one filament break.[2]

§ Exchange calculated after correcting fragment size for subunits disassembled after fragmentation (e.g., 20 subunits/fragment minus 6 subunits disassembled equals 14 subunits/fragment remaining; 14 subunits/fragment times 57% exchange equals 8 subunits exchanged per filament fragment).

Filament Fragmentation Increases the Steady State ATPase Activity of F-actin

Another way to measure filament subunit exchange is by the hydrolysis of ATP that occurs for every subunit exchange event (32, 36, 39). In the scheme shown in Fig. 9, most of the filament subunits are not available for exchange (unshaded actin subunits) and the rate of ATP hydrolysis is limited by the number of filament ends. As the number of filament ends increases due to fragmentation by severin, the rate of ATP hydrolysis should increase. The rate should be maximal at an actin:severin ratio where the number of subunits not available for exchange approaches zero. For example, the rate might be maximal at an actin:severin ratio of 15:1 where the number of subunits available for exchange is 8 (15 subunits per fragment before disassembly minus 6 subunits that disassemble minus 1 subunit in a nonexchangeable tight complex with severin; see Table III).[2] At lower actin:severin ratios the total amount of exchangeable actin decreases due to an increasing proportion of actin subunits bound in tight nonexchangeable complexes with severin. Thus as the actin:severin ratio decreases below 15:1, the rate of ATP hydrolysis would be expected to eventually diminish to zero as the actin:severin ratio approaches 1. The experimental results are consistent with this interpretation (Fig. 9).

DISCUSSION

In this study, we provide evidence that severin, a 40,000-dalton protein from *Dictyostelium*, changes conformation on binding Ca^{2+}, and only the Ca^{2+}-bound form interacts with F-actin. Severin apparently does not dissociate readily from F-actin even at very low free Ca^{2+} concentrations ($<10^{-8}$M) once it is bound to F-actin in the presence of 0.2 mM $CaCl_2$.

The experiments reported here allow an interpretation of the sequence of events that follow Ca^{2+}-mediated interaction of severin with actin filaments (Fig. 10). In the presence of Ca^{2+}, severin binds intact actin filaments. Within 30 s, filaments are completely fragmented. Filament fragments then partially depolymerize with a half-time of ~30 s, leading to an increase in the size of the subunit pool (critical actin concentration, C_A).

[2] This value indicates that the number of subunits involved in exchange per filament is indeed small. The actual values shown in Table III must be adjusted depending on the validity of the following assumptions used in their calculation: (*a*) 100% of the severin molecules are active; (*b*) one severin molecule produces one filament break; (*c*) the affinity of severin for actin filaments is high so that all of the severin added is bound in the experiments described; and (*d*) only one end of the filament is involved in exchange in these experiments since the other end is blocked by the presence of bound severin (Weeds and Spudich, unpublished observations). At present the important conclusions are that the number of subunits involved in exchange is directly proportional to the number of filament ends and that the number is small. The value is also in agreement with subunit exchange observed with filaments in the absence of severin (39).

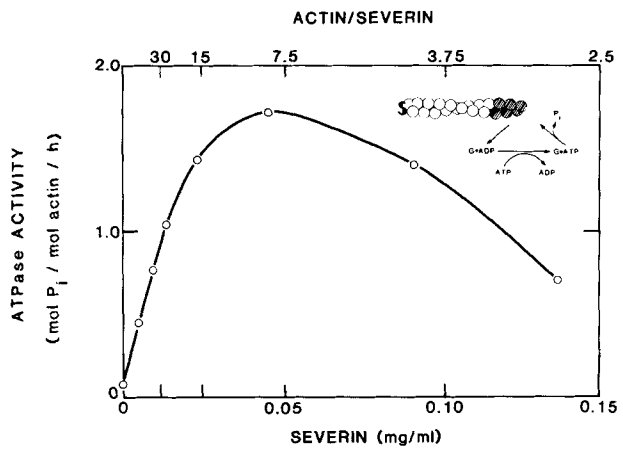

FIGURE 9 Effect of severin-induced fragmentation on the ATPase activity of F-actin. The steady state ATPase activity of muscle F-actin at 0.4 mg/ml was measured in F-buffer containing 20 mM triethanolamine, pH 7.4, at 25°C, and 0.2 mM $CaCl_2$ in the presence of increasing amounts of severin. Severin alone demonstrated no ATPase activity under these conditions. (*Inset*) Model of steady state ATP hydrolysis restricted to actin filament ends.

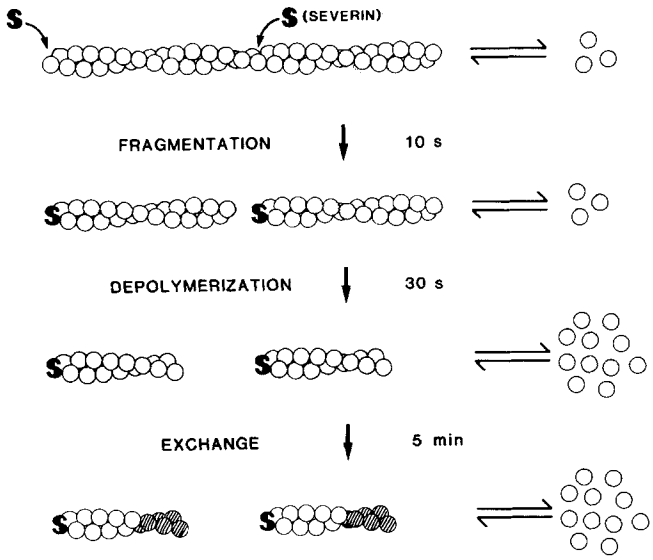

FIGURE 10 Proposed model for interaction of severin with actin filaments. Actin filaments are fragmented within 30 s of addition of severin in the presence of 0.2 mM Ca^{2+}. Severin remains bound to actin filaments in the presence of Ca^{2+}. A limited number of subunits then disassemble from the filament fragment end, with a $t_{1/2}$ = 30 s. Partial depolymerization is followed by exchange of a limited number of subunits at the fragment end with the C_A actin pool ($t_{1/2}$ = 5 min).

Each fragment is partially shortened in the process. A limited number of fragment subunits subsequently exchange with the C_A subunit pool with a half-time of ~5 min. Because exchange is limited, we infer that exchange is restricted to a specific domain of the filament fragment. A likely site for both limited exchange and depolymerization would be the filament end. By this end-only hypothesis, we suggest that the rise in critical concentration and filament subunit exchange is a direct result of the increased concentration of filament ends generated during fragmentation. Although it is not known if one or both fragment ends participate in the exchange process, the number of subunits that depolymerize or exchange from each fragment appears to be independent of fragment length (Table III). Under the assumption that severin reacts randomly along the filament length to produce an average length fragment population, the number of subunits which disassemble or exchange from each fragment remains constant at 6–8 subunits regardless of the filament fragment length. If only 50% of the severin is competent to bind F-actin, or if two severin molecules are required at each breakage site, then the number of exchanging or depolymerizing subunits at the fragment end would be 12–16.[2]

It is important to note that in these fluorescence energy transfer experiments, filament fragmentation per se has little effect on the amount of energy transfer. At an acceptor:donor ratio of 10:1, fluorescence donor actin subunits in the fragment interior are surrounded by the same array of acceptor subunits both before and after severing. Furthermore, energy transfer efficiency curves for various ratios of IAENS-actin donor and FITC-actin acceptor subunits within filaments indicate that donor subunits occurring at the ends of fragments would experience ~82% of maximum transfer to the penultimate subunit (39). Since the probability of a donor subunit appearing at the end of a 20 subunit oligomer is maximally 4 in 20, we expect the reduction in quench due to production of a 20 subunit oligomer to be ~1.9%. The 16% quench reduction observed for a 20:1 actin:severin ratio is therefore due almost entirely to disassembly. Moreover, DNase I assays confirm the value of 29% actin depolymerization obtained by fluorescence energy transfer.

Several different proteins that shorten the length of actin filaments have now been isolated from various kinds of nonmuscle cells (2, 6–8, 11, 16–20, 33, 35, 50, 56–58). These proteins also function to solate actin gels (21, 33, 56, 57), nucleate assembly of actin filaments (7, 8, 11, 15, 20, 21), and cause net depolymerization of actin filaments (2, 8, 11, 16–18, 21). An actin filament fragmentation protein similar to severin called fragmin (18) or actin-modulating protein (19) has previously been isolated from *Physarum*. Fragmin has an apparent molecular weight by SDS PAGE of 42,000, and in the presence of $>10^{-6}$M Ca^{2+} rapidly fragments filaments. Of the many actin-associating proteins now described, *Dictyostelium* severin is most similar to *Physarum* fragmin. Further studies will reveal the extent of the resemblance of these two proteins. Currently, two differences are apparent. Fragmin is indistinguishable from actin by SDS PAGE and contains no cysteine residues (18, 19). In contrast, severin migrates ahead of actin on SDS PAGE at a molecular weight of 40,000 and contains 5 mol cysteine per mole severin.

Unlike the action of gelsolin (56–58), fragment reassembly is not readily obtained by removing free Ca^{2+} after reaction with severin. This suggests that repolymerization of actin after fragmentation and disassembly by severin may require the action of yet another accessory protein to dissociate the actin-severin complex.

The biological function of an actin filament fragmentation factor within actively motile cells is not yet known, but there are several intriguing possibilities. In view of the observations that highly purified actin filaments are quite stable to filament disassembly and exhibit only limited subunit exchange under physiologically relevant salt concentrations (32, 39), factor-induced disassembly and oligomer formation may provide the biological pathway through which actin is transformed from filamentous to nonfilamentous form in vivo. Abramowitz et al. (1) and Bray and Thomas (5) first observed large stores of unpolymerized actin in nonmuscle cells, and it is now generally

agreed that in many nonmuscle cells between 50% and 70% of the total cellular actin remains unpolymerized or in short oligomer form (for review, see reference 28). Translocation of actin from site to site in response to metabolic demands for such events as cytokinesis, cell motility, and endocytosis is thought to occur by coordinating actin recruitment and assembly at the location of contraction with disassembly or dismantling of previously formed contractile arrays at a distant site (10). For instance, Wohlfarth-Bottermann and his colleagues (13, 15, 22, 53) have shown that actin in *Physarum* is quickly assembled and disassembled during contraction-relaxation cycles in this organism. With the knowledge that Ca^{2+}-dependent filament severing also initiates dynamic actin transformations such as disassembly and subunit exchange, the proposed requirement for a severin type protein to regulate actin functions in vivo takes on heightened interest.

This work was supported by research grants from the National Institute of General Medical Sciences (GM 25240 to J. A. Spudich and GM 24032 to L. Stryer), National Institutes of Health Postdoctoral Fellowship to J. D. Pardee, a Postdoctoral Fellowship to K. Yamamoto from the Muscular Dystrophy Association of America, and a Cystic Fibrosis Foundation Fellowship to J. Reidler.

Received for publication 1 March 1982, and in revised form 16 July 1982.

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