Ca^{2+}-dependent Binding of Severin to Actin: A One-to-One Complex Is Formed

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ABSTRACT Severin is a protein from *Dictyostelium* that severs actin filaments in a Ca^{2+}-dependent manner and remains bound to the filament fragments (Brown, S. S., K. Yamamoto, and J. A. Spudich, 1982, *J. Cell Biol.*, 93:205–210; Yamamoto, K., J. D. Pardee, J. Reidler, L. Stryer, and J. A. Spudich, 1982, *J. Cell Biol.* 95:711–719). Further characterization of the interaction of severin with actin suggests that it remains bound to the preferred assembly end of the fragmented actin filaments. Addition of severin in molar excess to actin causes total disassembly of the filaments and the formation of a high-affinity complex containing one severin and one actin. This severin–actin complex does not sever actin filaments. The binding of severin to actin, measured directly by fluorescence energy transfer, requires micromolar Ca^{2+}, as does the severing and depolymerizing activity reported previously. Once bound to actin in the presence of >1 μM Ca^{2+}, severin is not released from the actin when the Ca^{2+} is lowered to <0.1 μM by addition of EGTA. Tropomyosin, DNase I, phalloidin, and cytochalasin B have no effect on the ability of severin to bind to or sever actin filaments. Subfragment 1 of myosin, however, significantly inhibits severin activity. Severin binds not only to actin filaments, but also directly to G-actin, as well as to other conformational species of actin.

Many important cellular functions, such as cytokinesis, amoeboid movement, phagocytosis, and substrate adhesion, require changes in the state of actin assembly and possibly in the rate of exchange between polymerized and nonpolymerized forms of actin in the cell. The molecular basis for this control is an area of great interest. Many investigators are studying proteins from nonmuscle cells that interact with actin and modify its polymerization properties (for reviews see references 6, 22, 35, and 45). In particular, several of these proteins can be thought of as constituting a group whose main property is an ability to fragment F-actin in a Ca^{2+}-sensitive manner.

Severin, a 40,000-dalton protein isolated from *Dictyostelium discoideum* (5, 48) is one of these proteins. Severin most closely resembles fragmin from *Physarum* (16–18, 40), but is also similar in many respects to gelsolin (49, 50), villin (3, 4, 7, 12, 27), and a 90,000-dalton protein from platelets (43) and plasma (15). Properties in common include a rapid effect on F-actin, Ca^{2+} dependence for activity, and under certain conditions the ability to increase the initial rate of actin polymerization. In addition, severin increases both the amount of unpolymerized actin in equilibrium with filaments and the extent of exchange between subunits in the unpolymerized pool and in filaments (48).

Previous studies (5, 48) suggested that Ca^{2+} is required for binding as well as for severing of filaments. After severing an actin filament in the presence of Ca^{2+}, severin appears to remain bound to the filament as judged by nanosecond fluorescence anisotropy measurements (48). To examine this Ca^{2+} requirement further, we used a direct binding assay measuring the extent of fluorescence energy transfer between labeled severin and labeled actin. Fluorescence energy transfer (39) has been applied to the study of actin–actin interactions and the exchange of actin monomers with actin in filaments because of its unique ability to provide information about neighboring monomers in a filament (30, 41, 44, 48).

Other aspects of the severin interaction with actin were also important to examine. Several of the other fragmentation proteins, including fragmin, gelsolin, villin, and the 90,000-dalton platelet protein, block polymerization from the barbed or preferred assembly end of actin filaments. It was of interest to ask if severin can also bind to a filament end and, if so, which end. In addition, because severin can interact with

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actin over a wide range of molar ratios, we wanted to know what the smallest complex formed would be if severin and F-actin were mixed in such a way that severin was present in molar excess. These and additional studies on the severin–actin interaction are presented in this report.

MATERIALS AND METHODS

Materials

Fluorescent probes, fluorescein-5-maleimide (FM),[1] fluorescein-5-isothiocyanate isomer I (FITC), and 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid (IAENS) were all purchased from Molecular Probes, Junction City, OR. Aprotinin was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Acrylamide, *bis*-acrylamide, and hydroxylapatite were from Bio-Rad Laboratories, Richmond, CA. DEAE cellulose (DE 52) was from Whatman Inc., Clifton, NJ. SDS was purchased from BDH Chemicals Ltd., Poole, England. ACS scintillation fluid was obtained from Amersham Corp., Arlington Heights, IL. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Cell-Culture: *Dictyostelium discoideum* (strain Ax3) was grown in suspension culture as described (37) and harvested at late log phase for the isolation of severin and mid-log phase for the isolation of actin.

Purification of Proteins: Rabbit skeletal muscle actin was prepared from acetone powder as described by Spudich and Watt (38) and further purified by the recycling procedure described by Pardee and Spudich (31). *Dictyostelium* actin was purified as described by Uyemura et al. (42), and [^{35}S]actin from *Dictyostelium* grown in [^{35}S]methionine was prepared as described by Simpson and Spudich (36). Subfragment 1 of myosin (S-1) was prepared from rabbit skeletal muscle myosin (21) according to the protocol of Weeds and Taylor (46) and purified by ammonium sulfate precipitation (40–55% saturation) and gel filtration on Bio-Gel A-1.5m in 40 mM KCl, 10 mM sodium phosphate at pH 7.0. Severin was isolated from *Dictyostelium* amoebae by the method of Brown et al. (5) as modified by Yamamoto et al. (48), except that EGTA was added to the lysis buffer to 3 mM final concentration. Severin was sometimes frozen in liquid N_2 and stored at −70°C after the DEAE step of the purification for 1–2 wk before finishing the purification. This storage did not result in loss of activity or change in SDS-acrylamide gel pattern. Rabbit muscle tropomyosin was made by the procedure of Spudich and Watt (38) with the additional hydroxylapatite chromatography described by Eisenberg and Kielley (9).

Fluorescent Labeling of Proteins: Rabbit skeletal muscle actin was labeled with FM or FITC as described by Pardee et al. (30) with the following variations for FITC labeling. Sometimes actin was labeled at 4 mg/ml instead of 2 mg/ml, and sometimes the labeling buffer used for dialysis before labeling was at pH 8.0, but labeling was always performed at pH 9.3.

Severin at 2×10^{-5} to 2×10^{-6} M was labeled with IAENS in 20 mM triethanolamine, 50 mM KCl, pH 7.4, 0.005% NaN_3 at a 10:1 dye:protein ratio for 6–22 h, in the dark on ice. The reaction mixture was then extensively dialyzed against the same buffer containing 0.5 mM 2-mercaptoethanol. Under these conditions 0.8–1.4 mol of IAENS was attached per mol of severin, as determined spectrophotometrically. The following extinction coefficients were used: ϵ^{M}_{490} FITC = 64,000 cm^{-1}; ϵ^{M}_{340} IAENS = 5,100 cm^{-1}; ϵ^{M}_{490} FM = 60,000 cm^{-1} (Pardee et al. [30]); $\epsilon^{mg/ml}_{290}$ actin = 0.62 (Houk and Ue [19]). Severin concentration was determined by the method of Schacterle and Pollack (34) using bovine serum albumin as a standard.

Direct Assay for Binding of Severin to Actin: Fluorescence energy transfer (FET) from IAENS-severin to FITC-actin or FM-actin was measured by monitoring IAENS fluorescence at 470 nm while exciting at 340 nm (Fig. 1). All measurements were made on a Spex Fluorolog spectrofluorometer (Spex Industries, Inc., Metuchen, NJ). In all cases the initial fluorescence of IAENS-severin was measured before the addition of 1/200 vol of a Ca^{2+} or EGTA stock solution. Fluorescence of the sample was checked again before addition of unlabeled or labeled actin. Fluorescence was then monitored continuously once all additions were made. Quench is defined as 100× (fluorescence of IAENS-severin − fluorescence of IAENS-severin with FITC-actin)/fluorescence of IAENS-severin. Maximum quench (maximum FET) was taken to represent 100% binding of severin to actin.

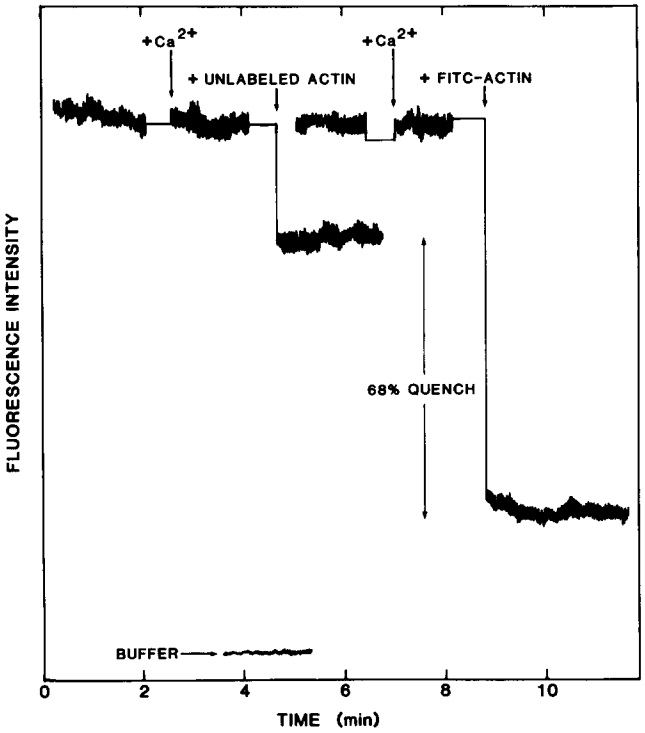

FIGURE 1 Fluorescence at 470 nm is monitored with time. The sample was excited with light at 340 nm. The left-hand trace shows the fluorescence of IAENS-severin alone, after the addition of 1/200 vol Ca^{2+} stock solution, and then after the addition of unlabeled actin. The right-hand trace shows the same sequence of additions except that the actin added at the last step is covalently labeled with FITC. In both cases the volume in which the actin is added is 0.25 of the final volume. In both cases actin was added at 8.4 mol/mol of severin. The quench in the right-hand trace is 68%.

IAENS-labeled severin can transfer as much as 70% of its fluorescence to FITC-labeled actin in the presence of 0.1 mM Ca^{2+} (Fig. 1). The addition of Ca^{2+} (0.1 mM) or EGTA (0.5 mM) to IAENS-severin does not change its fluorescence at 470 nm, nor does the mixing technique employed change the fluorescence. Furthermore, the drop in fluorescence of IAENS-severin upon the addition of unlabeled F-actin (Fig. 1, left-hand trace) is the amount expected by dilution of labeled severin. This indicates that there is no significant change in the intensity of IAENS-severin fluorescence at 470 nm upon binding to F-actin. The full extent of quench is often seen as quickly as the samples are mixed and returned to the fluorometer (e.g., Fig. 1). In some instances, perhaps indicating incomplete mixing, the fluorescence level decreases over the first few minutes to a plateau. In all cases this plateau level is taken to calculate quench.

To examine the behavior of this system, we measured FET as a function of the mole fraction of acceptor (FITC) at constant protein concentration of both actin and severin (Fig. 2). This was achieved by mixing unlabeled actin with FITC-actin to vary the dye:protein ratio. As expected, the amount of quench observed varies directly with the mole fraction of acceptor fluorophore. Qualitatively, the same results were obtained using FM-labeled F-actin, but since the maximum quench seen with that acceptor-labeled actin was only ~25%, FITC-actin labeled to the extent of 0.8–1.0 mol of FITC/mol of actin was used for all the experiments reported here.

Severin Activity Assay: Severin activity was measured as a decrease of viscosity of F-actin. A rolling ball viscometer (24) was used. The viscosity of muscle actin or *Dictyostelium* actin was measured at a final concentration of 0.2 mg/ml in F-buffer consisting of 20 mM triethanolamine, pH 7.4, 0.5 mM ATP, 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 0.005% NaN_3 unless otherwise noted below. Severin or buffer and Ca^{2+} or EGTA were added to the F-actin and incubated as described for each experiment. Samples were then loaded into 100-μl capillary tubes (Micropet, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ), a single microball (0.64 mm diameter; The New England Miniature Ball Co., Norfolk, CT) was added to each tube and the tubes sealed with plastacene. If the incubation was on ice the sample was

[1] *Abbreviations used in this paper*: DTT, dithiothreitol; FET, fluorescence energy transfer; FITC, fluorescein-5-isothiocyanate isomer I; FM, fluorescein-5-maleimide; HSS, high-speed supernatant; IAENS, 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid; S-1, subfragment 1 (myosin).

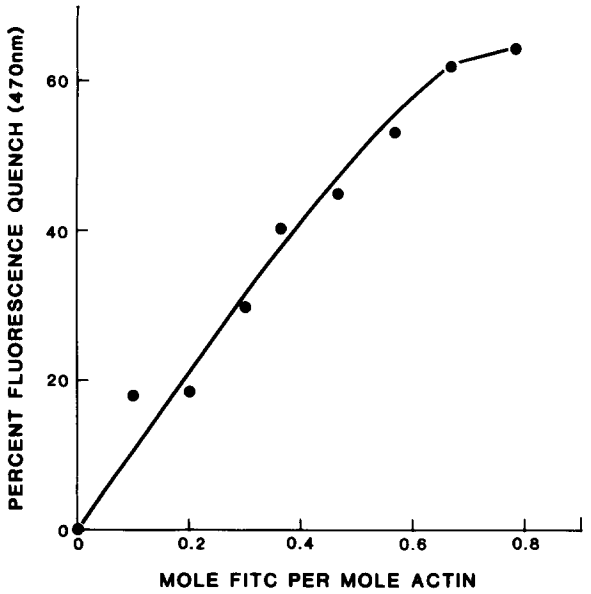

FIGURE 2 The efficiency of FET was measured as a function of mole fraction acceptor. The dye to protein ratio was varied by mixing unlabeled actin with actin labeled to 0.8 mol of FITC per mol of actin. Quench was then determined after mixing severin with actin at a molar ratio of 1.0:8.8.

allowed to come to 22°C. After several passes through the solution, the time for the ball to travel 6 cm through the solution with the tube at a 20° angle from horizontal was measured.

Effect of Severin on the Reannealing of F-Actin: The viscosity of either actin alone, or actin with severin present at a 7:1 ratio was measured in F-buffer containing either added 0.5 mM EGTA or added 0.1 mM Ca^{2+} (final concentrations) at 22°C using a rolling ball viscometer. One set of samples was used to measure initial viscosity. The second set of samples was then sonicated for 30 s at setting 5.5 (Kontes microultrasonic cell disrupter, Kontes Co., Vineland, NJ) to generate many filament ends, put into capillary tubes and the viscosity was followed with time.

Effect of Lowering the Ca^{2+} Concentration on Severin Bound to Actin: Severin was incubated with either muscle actin or *Dictyostelium* actin with either 0.4 mM Ca^{2+} or 0.4 mM EGTA. A sample of each was put into capillary tubes to measure the viscosity. To the remainder of each sample 0.66 mM EGTA was added if Ca^{2+} was present initially or 0.66 mM Ca^{2+} was added if EGTA was already present. The second set of samples was then put in capillary tubes and the viscosity of all the samples was followed with time.

Effect of Severin–Actin Complex on F-Actin: Either severin (at 1/100 the actin concentration) or buffer was added to F-actin (0.2 mg/ml final concentration) in 20 mM triethanolamine, pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 0.005% NaN_3, 0.2 mM ATP, 0.2 mM Ca^{2+}. Alternatively, the same amount of severin was mixed with a threefold excess of actin (3% of the total actin), preincubated for 10 min at 22°C and then added to the remaining actin. After loading into capillary tubes the viscosity of each of these three samples was followed with time using a rolling ball viscometer. This experiment was performed at 22°C.

Preparation of F-Actin "Seeds" Coated with S-1: F-actin from *Dictyostelium* (0.3 ml at 7.5 mg/ml) was pelleted in an airfuge at 100,000 *g* for 20 min. The actin was sonicated in a buffer containing 3 mM imidazole HCl, pH 7.5, 0.1 mM ATP, and 0.2 mM dithiothreitol (DTT) and left for 2 h to depolymerize. After further sonication, residual F-actin was removed by sedimentation at 100,000 *g* for 20 min, giving G-actin at 3.7 mg/ml. The G-actin was polymerized at 0.2 mg/ml in 10 mM imidazole–HCl, pH 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$, and 0.2 mM DTT (thereby reducing the ATP concentration to ~5 μM). After 30 min at 22°C, S-1 was added to the F-actin at a 1:1 molar ratio (final concentration 4.7 μM). Chemical cross-linking with glutaraldehyde was carried out to stabilize the complexes as described by Wang and Bryan (43). After dilution of the complex by an equal volume of the polymerization buffer, ice-cold glutaraldehyde was added to 0.5% (vol/vol) (~50 mM), and sodium borohydride was added 1 min later to 0.2 M. The cross-linked S-1-decorated actin was dialyzed overnight against 5 mM sodium phosphate pH 7.0, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT. Addition of sodium borohydride caused some foaming with the result that some of the protein was denatured in the froth. This was largely removed before dialysis, but after dialysis the protein was clarified in a microfuge. The concentration of actin complexed with S-1 in the supernatant was estimated by absorbance at 280 nm using an extinction coefficient for the complex $\epsilon_{280}^{1\%} = 8.44$ (based on the published extinction coefficients for actin and S-1 and assuming a 1:1 molar complex). Based on the dissociation constant for the complex of between 0.2 μM (26) and 0.1 μM (25), the S-1 bound under the cross-linking conditions should be between 80 and 90%.

Seeds were prepared from the decorated actin by sonication in a Kontes microultrasonic cell disrupter at power setting 0.5 for 2 × 6 s (watt density per square inch = 610 at 25 kHz). Samples were examined by electron microscopy using a Philips 200 microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) after staining with 1% uranyl acetate; these showed the expected arrowhead pattern. When the grids were washed with a solution containing ATP before staining there was no loss of S-1 showing that covalent linkage by the glutaraldehyde was complete. However, if the cross-linking was carried out at a 10-fold lower concentration of glutaraldehyde, there was substantial loss of S-1 decoration under these conditions. Although the lengths of the seeds were somewhat variable, an estimate could be made of their concentration based on the assumption that the average length was six actin periods (~0.2 μm), giving a concentration of ~0.01 μM.

Use of Seeds to Nucleate Actin Assembly: Various concentrations of seeds, G-actin, and severin were tested to obtain optimal filament growth. The requirements were: (*a*) spontaneous nucleation should be minimal; (*b*) polymerization carried out in the absence of severin should show filament growth at both ends of the seeds; and (*c*) in the presence of severin (which was expected to inhibit growth at one end of the filament) growth at the unblocked filament end should be readily detected. It was also important that the relative concentration of severin to seeds should be sufficiently high to ensure that most of the seeds had bound severin. The conditions described gave minimal F-actin unattached to S-1 decorated seeds, and controls showed that >90% of these seeds showed growth at their barbed ends (Table I). Growth from the pointed end was ~30% in these controls, but the extent of growth at this end of the filament would be expected to be small because of the lower association constant at the pointed end of the filament (33).

Conditions for growing actin filaments from the ends of the seeds were as follows: 25 μl of seeds were mixed with 5 μl of 0.30 μM severin and buffer (20 mM NaCl, 1 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0) and G-actin (added at a concentration of 1.1 or 2.2 μM). Polymerization was initiated by adding salt solution to a final concentration of 0.1 M KCl, 1 mM $MgCl_2$, with either 0.2 mM Ca^{2+} or 0.5 mM EGTA. The total volume was 50 μl. Control polymerizations were carried out in the absence of severin. In other experiments severin was added after polymerization to ensure that it was actively breaking filaments. Polymerization times were usually 2 min but times up to 10 min were also tested. The polymerization was terminated by applying 5-μl samples to carbon-coated grids and washing with 20 mM NaCl, 1 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0 (8) and then with 1% uranyl acetate.

TABLE I

Severin Blocks Polymerization from the Barbed End of Actin Filaments

Components (in order of addition)*	Percentage of filaments counted‡ with growth at: Barbed end only	Pointed end only	Both ends	Total number of filaments counted
	%	%	%	
F-actin, EGTA, G-actin	67	7	26	57
F-actin, Ca^{2+}, G-actin	70	4	26	46
F-actin, severin, EGTA, G-actin	55	10	35	62
F-actin, severin, Ca^{2+}, G-actin	25	64	11	138
F-actin, Ca^{2+}, G-actin, severin	62	10	28	102

* Conditions were as described in Materials and Methods. For the experiment shown in the last line, filament elongation was allowed to occur for 10 min before severin was added.

‡ Those filaments with no growth at either end were not counted because many of these may represent decorated fragments inactivated by the glutaraldehyde treatment.

Ca^{2+}/EGTA Buffers: To analyze the Ca^{2+} requirement for the severin interaction with actin, Ca^{2+}/EGTA buffers were used. Ca^{2+} and EGTA were mixed to produce concentrated stock solutions with Ca^{2+}/EGTA ratios of 2.0, 1.5, 1.2, 1.1, 1.0, 0.85, 0.35, and 0.2. The Ca^{2+}/EGTA buffers were then added to the protein samples so the final EGTA concentration was 0.5 mM. The free Ca^{2+} concentration was calculated using the apparent affinity constant determined by Harafuji and Ogawa (14).

S200 Gel Filtration: A 1-cm × 50-cm S200 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) eluted at 4°C was used to analyze the product of a 4:1 molar mixture of severin and actin in the elution buffer. The mixture was incubated 10 min at 22°C before loading onto the column. The elution buffer was 20 mM triethanolamine, pH 7.5, 0.2 mM DTT, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM Ca^{2+}, 0.5 mM ATP, 0.01% NaN_3. Aldolase, transferrin, bovine serum albumin, and egg albumin were each run separately as molecular weight markers. K_{av} is (elution volume − void volume)/(total volume − void volume). The column eluate was analyzed by SDS PAGE on 12% gels according to Laemmli (23) as modified by Ames (1). Severin and actin were quantitated by scanning the gel after Coomassie Brilliant Blue staining with a Transidyne RFT scanning densitometer (Transidyne General Corp., Ann Arbor, MI). The peak weights of scanned column fraction bands were compared to standards of the same proteins run on the same gel to convert to weight of protein.

Interaction of Severin with Different Conformational Species of Actin: Actin was recycled by pelleting stored F-actin, resuspending the actin pellet in G-buffer (2 mM triethanolamine, pH 7.4, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.005% NaN_3) and dialyzing in a collodion bag (Sartorius GmbH, Göttingen, Federal Republic of Germany) against the same buffer for 2 h at 4°C. The depolymerized actin was clarified by high-speed centrifugation for either 60 min at 150,000 *g* or 20 min at 30 psi (130,000 *g*) in an airfuge. This clarified actin was used directly for assays requiring G-actin. For F-actin and the HSS-actin (i.e., the nonfilamentous actin remaining in the high-speed supernatant [HSS] after centrifugation), salt was added and the actin allowed to polymerize at 22°C for 2 h. One of three buffers was used: 0.1 M KCl alone or 0.1 M KCl and 1 mM Mg^{2+} or 0.1 M KCl and 1 mM Ca^{2+} each in G-buffer. The polymerized actin was used directly for assays requiring F-actin. Part of each sample was centrifuged again for 20 min at 30 psi to pellet the filaments and the supernatant was used in experiments requiring HSS-actin. The protein concentration of these supernatants was determined (2) using actin (the concentration of which was determined spectrophotometrically) as a standard. F-actin and G-actin were at 0.25 mg/ml final concentration at a 13:1 molar excess over severin. For the HSS-actin experiments, however, because it was not possible to concentrate the actin, adding the maximum volume resulted in the following molar ratios of actin:severin—For the KCl only buffer 2.4:1, for KCl + Mg^{2+} 2.1:1, and for KCl + Ca^{2+} 3.3:1.

Effect of Other Actin Binding Proteins: To look for possible effects of proteins or compounds that bind to actin on the ability of severin to interact with actin, the protein or compound to be tested was preincubated with actin. Muscle myosin S-1, cytochalasin B, DNase 1 (Worthington Biochemical Corp., Freehold, NJ) or phalloidin (Boehringer Mannheim Biochemicals) was mixed with F-actin at a 1.5:1 molar ratio. Muscle tropomyosin was tested at a 0.3 molar ratio to actin. F-buffer was used. ATP was omitted from the buffer in the case of S-1. A 10 mM cytochalasin B stock solution in DMSO and a 1 mM phalloidin stock solution in 0.14 M KCl, 2% DMSO were used.

RESULTS

Severin Binds to the Preferred Assembly End of Actin Filaments

To answer the question of where severin binds to actin filaments, we used S-1-decorated actin filaments as seeds for assembly of G-actin in the presence or absence of severin. Only seeds with growth at one or both ends were scored in that no growth at either end could result from denaturation of the seeds by the glutaraldehyde treatment. In the absence of severin there is considerably more growth from the barbed end (~90%) than from the pointed end (~30%; Table I). In the presence of severin and Ca^{2+}, however, only 36% of the seeds have barbed end growth compared with 75% with pointed end growth. With severin in the absence of Ca^{2+}, ~90% of the filaments still show growth from the barbed end. The results show that severin does not inhibit the association of G-actin monomers to the pointed end of actin filaments either in the presence or absence of 0.2 mM Ca^{2+}, but assembly at the barbed end is markedly inhibited provided Ca^{2+} is present. When severin was added to filaments preassembled on seeds, preferential filament growth (90% of filaments showed growth at the barbed end) was the same as the control but the filaments were shorter, due to severing activity. This result shows that severin does not sever filaments preferentially at the S-1-free/S-1-decorated junction; rather, severin must inhibit G-actin addition to the barbed end. These results suggest that severin binds to the barbed or preferred assembly end of actin filaments.

Severin Does Not Affect Reannealing of F-Actin after Sonication in EGTA

The following viscosity experiment was done to determine if any effect of severin could be detected at low ($<10^{-8}$ M) Ca^{2+} concentrations when many filament ends were present. As can be seen in Fig. 3, the recovery of viscosity after sonication is the same in rate and extent for actin + severin in the presence of EGTA as for actin alone. In contrast, the viscosity of the sample of actin + severin in Ca^{2+} has the viscosity of buffer before and after sonication. Thus severin in EGTA does not interfere with reassembly or reannealing of actin after sonication, in marked contrast to the effect of severin on actin in 0.1 mM Ca^{2+}.

Effect of Severin on Actin in Ca^{2+} Is Not Reversed by Addition of EGTA

The viscosity decrease of F-actin caused by severin in Ca^{2+} is not reversed within 22 h by the addition of EGTA (Fig. 4). *Dictyostelium* actin gave the same result as muscle actin. F-actin without added severin was also tested in these Ca^{2+} and EGTA conditions and had the viscosity of filaments in all cases (data not shown).

Preformed Severin–Actin Complex Does Not Sever F-Actin

The viscosity of F-actin alone, actin plus severin, or actin plus preformed actin–severin complex was followed with time (Fig. 5). The sample of F-actin to which complex was added is indistinguishable from actin alone. This is in contrast to the F-actin to which severin alone was added, which shows a low viscosity as quickly as measured and is constant over 2 h.

Severin Forms a 1:1 Complex with Actin When Severin is in Excess

We used fluorescence energy transfer to measure directly the severin interaction with actin. The effect of varying the actin to severin ratio on the extent of energy transfer is shown in Fig. 6. At molar ratios of 2 actin: 1 severin and higher, maximum quench was observed. Below 2:1 the amount of quench decreased. At 1:1 ~69% of maximum quench was seen. This is compatible with the formation of one to one complexes (demonstrated by gel filtration chromatography in Fig. 7). Less than 100% quench at a 1:1 ratio is best explained in one of two ways. Either the second actin molecule changes the conformation of the 1:1 actin:severin complex so that the two probes are brought closer together in the 2:1 complex, or the presence of a second actin molecule labeled with an

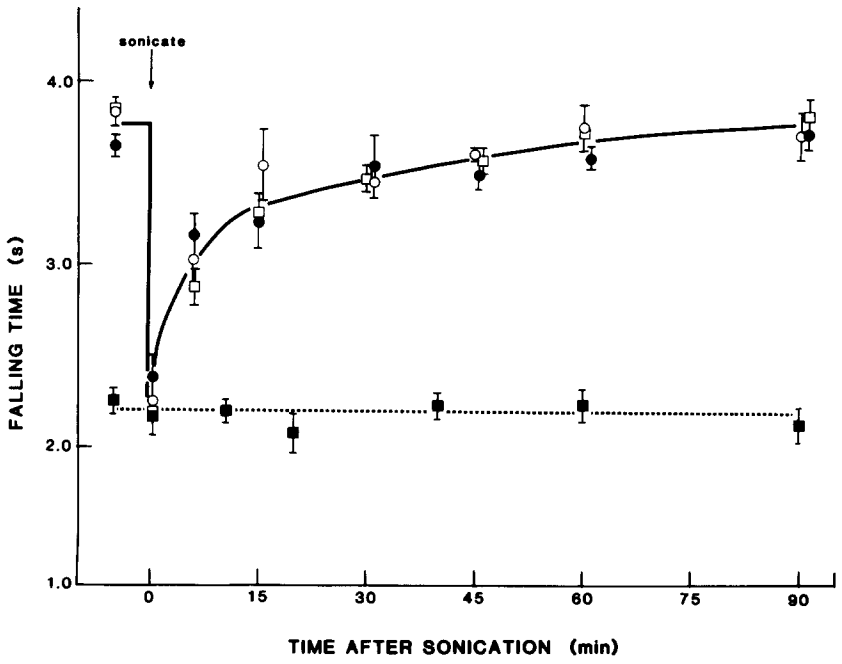

FIGURE 3 The viscosity of F-actin in the presence of severin before and after sonication at time = 0, in 0.2 mM Ca^{2+} or 1.0 mM EGTA, was measured as a function of time. The molar ratio of actin:severin was 7:1. (■) Actin with severin in Ca^{2+}. (□) Actin with severin in EGTA. (●) Actin alone in Ca^{2+}. (○) Actin alone in EGTA.

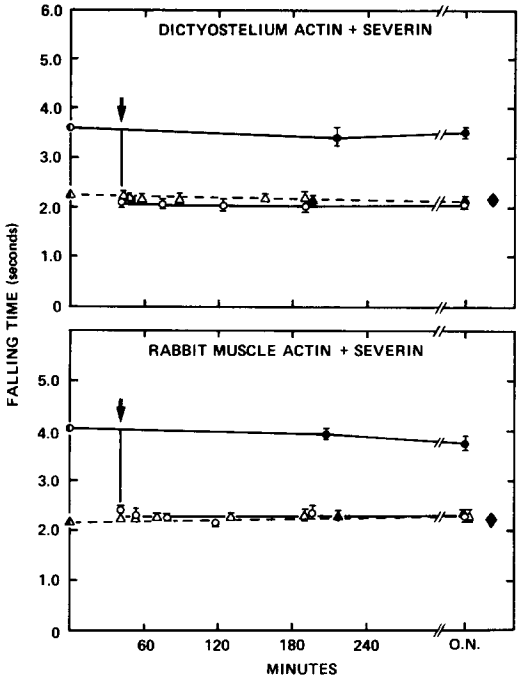

FIGURE 4 The effect of changing the Ca^{2+} concentration on the viscosity of either *Dictyostelium* actin or muscle actin in the presence of severin was measured. To half of each sample of actin with severin in 0.4 mM EGTA Ca^{2+} was added to a final concentration of 0.66 mM at the arrow (○), and viscosity was followed with time. The viscosity of the remaining half of each sample (with only EGTA) was also monitored with time (●). A second set of samples was a mixture of actin and severin in 0.4 mM Ca^{2+} (▲). At the arrow EGTA was added to half of each of these two samples (△), to a final concentration of 0.66 mM, and the viscosity of all four samples was followed with time. The proteins were present at a 7:1 molar ratio of actin to severin. The viscosity of buffer (◆) is also shown. *O.N.*, overnight.

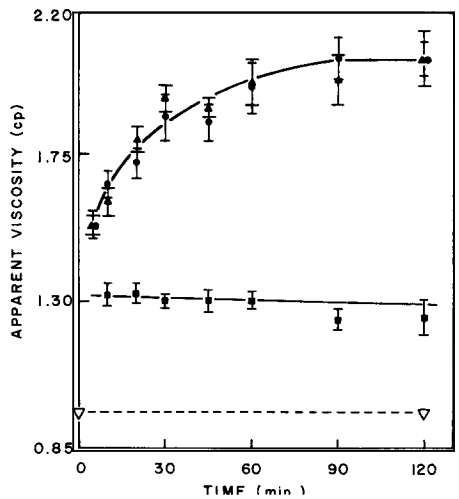

FIGURE 5 The effect of added severin or severin-actin complex on the low-shear viscosity of F-actin was followed with time. Actin was at 0.2 mg/ml final concentration. Severin was added at a 1:100 ratio with actin. To generate the severin-actin complex, we preincubated severin with a threefold molar excess of actin (3% of the total actin) for 10 min at 22°C. The complex was then added to the remaining 97% of the actin. (△) Buffer; (■) severin added to F-actin; (▲) severin-actin complex added to F-actin; (●) F-actin alone. Viscosity measured as falling time in the rolling ball viscometer was converted to centipoise (*cp*) by calibrating the viscometer using standard glycerol and water mixtures (24). Each curve represents sequential readings made on a single sample which remained in the capillary tube for the duration of the experiment.

acceptor probe contributes additional quench directly. Still larger complexes do not show a further increase in the amount of quench. This is reasonable since energy transfer can only occur efficiently over distances of 40–60 Å or less (39). A 2 actin:1 severin limit complex is unlikely because that would predict ≤50% quench at a 1:1 ratio, which is not observed.

Gel filtration chromatography was used to analyze directly the size of the limit complex formed when a molar excess of severin is added to actin. Severin and actin were mixed in a 4:1 molar ratio in the presence of Ca^{2+} and chromatographed on an S200 gel filtration column. The elution fraction (K_{av}) of the peak of complex was compared with the elution fraction of molecular weight standards (*inset* of Fig. 7). The complex elutes at a position corresponding to a molecular weight of

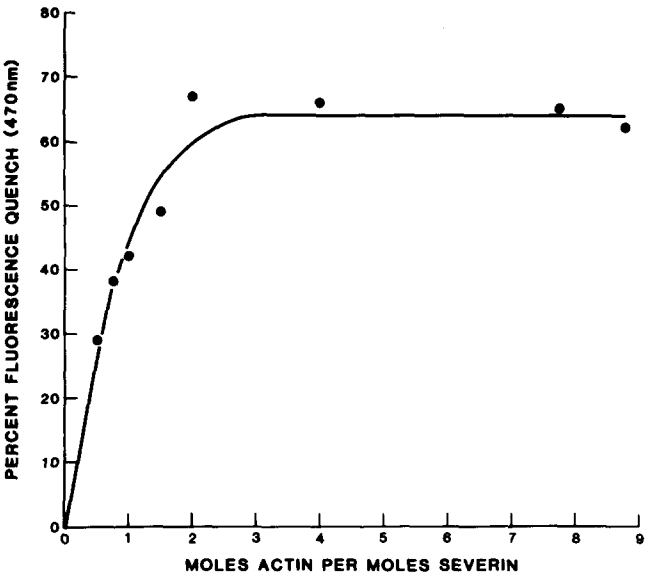

FIGURE 6 Percent quench was measured as a function of the molar ratio of actin to severin. For all points the final actin concentration was 0.4 mg/ml, and the concentration of severin varied to give the indicated molar ratios. The actin was fully polymerized before being added to severin for fluorescence measurements. The buffer contained 20 mM triethanolamine, pH 7.4, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.005% NaN_3, 120 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$.

~90,000 for a spherical protein. The relative amounts of actin and severin were analyzed by quantitative densitometry of Coomassie Blue stained gels of the column fractions. As shown in Fig. 7, the peak fractions of the complex contain approximately a 1:1 molar ratio of severin:actin.

Severin Binding to Actin Requires Micromolar Ca^{2+}

Severin activity has been shown to be sensitive to Ca^{2+} concentration (5, 48). The Ca^{2+} concentration dependence for severin binding to actin was measured using the FET assay (Fig. 8). The binding of severin to actin was found to require 1–5 μM Ca^{2+}. This is a Ca^{2+} concentration similar to that needed for severin activity as found previously (5, 48), and as observed again in this study in a parallel experiment to that shown in Fig. 8 using [^{35}S]actin to measure activity (11) instead of FET (data not shown).

Estimation of the Affinity of Severin for Actin

Maximum energy transfer is seen with severin at 5×10^{-7} M (20 μg/ml) and actin at 1×10^{-6} M (40 μg/ml) suggesting a lower limit of 4×10^{7} M^{-1} for the association constant if 95% of the severin is bound under these conditions. Another calculation can be made which gives an even higher estimate for the association constant. In a different experiment IAENS-severin was reacted with FITC-actin in Ca^{2+} and maximum FET was seen. Then a small volume of concentrated unlabeled actin was added so that unlabeled actin was present in fivefold molar excess over labeled actin. Over the course of 1 h no relief of quench was seen. This slow rate of dissociation would be consistent with an association constant of $>10^{10}$ M^{-1} assuming a diffusion limited on rate of 10^{7}–10^{9} s^{-1} M^{-1} (10).

Severin Binds to a Variety of Forms of Actin

Actin is known to exist in several forms (28, 29, 32). At steady state the nonfilamentous actin remaining in the high speed supernatant after centrifugation (HSS-actin) appears to be different species depending on the salts used to induce polymerization (29). We used the FET assay to test whether severin can bind to these different conformational species (Table II). For all the various species of actin examined, energy transfer was observed in Ca^{2+} and the extent of quench was ≥80% of that observed with F-actin. For the three different HSS-actin species, 79–83% of the quench of F-actin was measured, whereas G-actin in low salt buffer showed 91% of the quench seen with F-actin. In all cases maximum quench could be obtained in these samples by the subsequent addition of excess FITC–F-actin. These results suggest that severin can interact with all of these different forms of actin with high affinity.

Many Actin-binding Factors Do Not Affect Severin Binding, but S-1 Stabilizes the Actin Filaments

To assess the effects of other known actin-binding proteins and compounds on the interaction of severin with actin, FITC-actin was incubated with a 1.5 molar excess of muscle S-1, DNase 1, cytochalasin B, or phalloidin, or a 0.3:1 molar ratio of muscle tropomyosin to actin. The ability of these samples to quench IAENS-severin fluorescence was then tested. The only sample which was significantly different from actin alone was the actin that was preincubated with S-1 (Table III). With S-1, quench was reduced to ~60% of that seen without S-1, indicating an inhibition of binding of severin

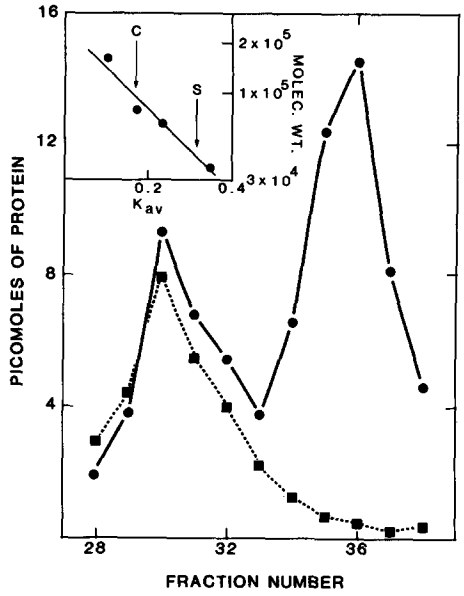

FIGURE 7 Severin and actin at a molar ratio of four severin per actin were chromatographed on a Sephacryl 200 column in the presence of Ca^{2+}. Each fraction from the column was analyzed by SDS PAGE to quantitate the amounts of severin (●) and actin (■) in each fraction. The amounts of protein determined by densitometer scans of the gel are plotted for each fraction. The elution fractions (K_{av}) of (from large to small) aldolase, transferrin, bovine serum albumin and egg albumin are indicated in the inset. The elution fractions of the complex (c) and severin alone (s) are indicated by arrows.

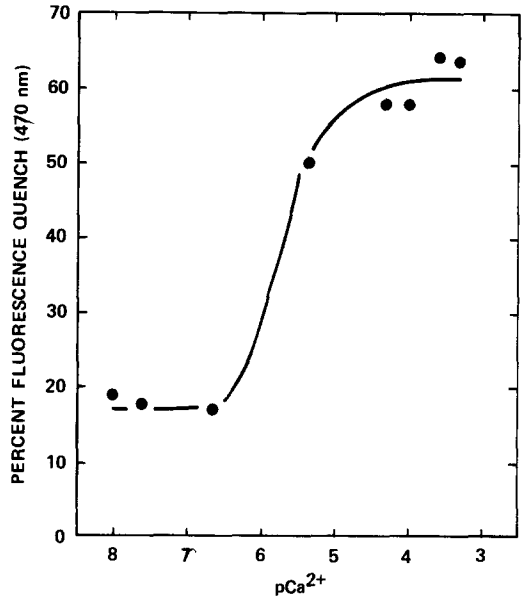

FIGURE 8 Severin binding to actin, measured as quench in the FET assay, is plotted as a function of the free Ca^{2+} concentration. A small volume of 1 mg/ml actin was added to the severin in Ca/EGTA containing buffer (final concentrations were severin 7×10^{-7} M; actin 5.8×10^{-6} M) and the fluorescence at 10 min was used to calculate percent quench. A transition was observed in the range of 5×10^{-7} to 5×10^{-6} M free Ca^{2+}, using the apparent K_d of EGTA at pH 7.5 for Ca^{2+} of 3.98×10^{-8} M established by Harafuji and Ogawa (14).

to actin. This 60% level of quench was reached within 5 min and remained constant over the next 20 min. The ability of these proteins and compounds to bind to actin was checked before use in the fluorescence experiment. The absence of ATP in the buffer used for the S-1-decorated actin sample did not affect the binding of severin to F-actin in control samples (data not shown).

In the case of preincubation of actin with S-1, the filaments were examined in the electron microscope before and after the addition of severin. Long decorated filaments were seen before the addition of severin, and short decorated filaments ~0.1 μm long were seen after. Because severin was added at a 1:1 ratio to actin, no filaments would have been seen in the absence of S-1. Thus, we suspect that there were regions of the F-actin that were not saturated by S-1 and were therefore vulnerable to disassembly by severin, whereas other segments were saturated with S-1 and were therefore resistant to severin action.

DISCUSSION

The rapid effect of severin on F-actin is probably due to a direct action of the protein on filaments; i.e., severin may bind along the length of the filament and induce fragmentation. A second mechanism, which suggests that severin nucleates new filament formation and thereby generates many new ends for assembly, appears less likely. This mechanism proposes that actin monomers from the long actin filaments would shuttle to the severin nuclei by exchange, resulting in predominantly new short actin filament fragments. This possibility is ruled out by two types of experiment. Yamamoto et al. (48) observed only limited exchange of monomers after fragmentation with severin and not the nearly 100% exchange predicted by this model. Furthermore, this model would predict that the severin–actin complex would be as effective as severin alone in its effect on filaments, and this is not observed (see Fig. 5). Since no effect was detected over 2 h after addition of severin–actin complex to filaments, the rate of redistribution of actin subunits must be a fairly slow process, unable to account for the rapid change in average length of the actin filament population. This result is also in agreement with the results of Pardee et al. (30), who found that the extent of exchange of purified actin under similar salt conditions is extremely low.

Regarding the site of binding of severin to F-actin, we were able to localize the binding to the barbed or preferred assembly end of the filament. The technique we used, described by Woodrum et al. (47), was first used to characterize an actin-binding protein by Isenberg et al. (20) studying *Acanthamoeba* capping protein. Similar results have been obtained for the platelet 90,000-dalton protein (43), for fragmin (40), and for gelsolin from macrophage (49). Glenney et al. (13) concluded that villin binds at the barbed end from an experiment in which actin was grown from beads coated with villin and the F-actin polarity was determined by S-1 decoration. Thus far all of the Ca^{2+}-sensitive fragmentation factors that have been studied in this way bind the barbed ends of filaments. Capping protein from *Acanthamoeba* (20) also blocks growth of filaments at the barbed end, but this protein is not Ca^{2+} sensitive and has not been reported to fragment filaments.

The Ca^{2+} requirement for binding as measured by the FET assay is the same as for activity, in the micromolar range. This is similar to the Ca^{2+} requirement of gelsolin (50), villin (12), and fragmin (18).

TABLE II

*Severin Interacts with Different Forms of Actin**

	Quench			
	G-buffer	KCl buffer	KCl, Mg^{2+} buffer	KCl, Ca^{2+} buffer
	%	%	%	%
G-actin‡	68	—	—	—
F-actin‡	—	73	74	73 (75)‖
HSS-actin§	—	61 (71)‖	59 (75)‖	62 (67)‖

* See details in Materials and Methods.
‡ F-actin or G-actin was added at 13 mol/mol of severin.
§ HSS-actin was 2–4 mol/mol of severin; see Materials and Methods.
‖ Numbers in parentheses are the values of quench obtained when FITC-F-actin was added to 3.8 mol/mol of severin in the reaction mixture.

TABLE III

Effect of Other Actin-binding Proteins and Compounds on the Binding of IAENS Severin to FITC actin

Components added to IAENS-severin	Quench*	Final molar ratio (added agent: actin:severin)
	%	
FITC-actin	100	0:1:1
FITC-actin + S-1	59	1.5:1:1
FITC-actin + TM	103	0.3:1:1
FITC-actin + DNase 1	105	1.5:1:1
FITC-actin + CB	93	1.5:1:1
FITC-actin + phalloidin	106	1.5:1:1

*Normalized to 100% for IAENS-severin + Ca^{2+} + FITC-actin.

Although the complex formed by severin and actin is not covalent, we have not found conditions under which severin bound to actin in the presence of Ca^{2+} is released by the addition of excess EGTA. Thus, we do not see relief of quench if EGTA is added to the severin–actin complex formed in the presence of Ca^{2+}, even after 22 h. This result extends earlier observations where restoration of monomeric anisotropy values (monitoring IAENS-severin) was not seen after the addition of EGTA to severin bound to F-actin in Ca^{2+} (48). Similarly the activity of *Physarum* fragmin is not readily reversible by the addition of excess EGTA (18).

As observed for gelsolin and villin (12, 13, 50), complexes can be formed by severin with G-actin. Furthermore, all the conformational species tested—G-actin in low-salt buffer, the nonsedimentable actin (HSS-actin) present in fully polymerized actin solutions, and F-actin—are able to bind severin in the FET assay. Whether severin binds these different conformational species of actin with different affinities is a question raised by the less than maximal quench seen, but the actual affinities remain to be determined. Another possible explanation for the lower quench seen with HSS-actin is that, in this different conformation, the probes may be further apart when severin is bound, lowering the maximum quench possible. However, the relative contributions of these two effects remains to be determined.

The role of severin in the cell is not clear. One can suggest that a change in Ca^{2+} concentration may be used by the cell to trigger severin activity, but it is unlikely to be an on/off switch because severin is not rapidly released when the Ca^{2+} concentration drops. Furthermore, the ability of severin to interact with actin is likely to be modulated by the presence of other actin-binding proteins in the cell, because, for example, myosin S-1 can interfere with severin binding. The evidence so far is consistent with severin being a component of the cytosol (11) with a major effect of fragmenting actin filaments, but understanding severin's role in the complex scheme that allows the cell exquisite control of its motility must await more experiments.

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