

Domain Structure in Actin-binding Proteins: Expression and Functional Characterization of Truncated Severin

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Abstract. Severin from *Dictyostelium discoideum* is a Ca^{2+} -activated actin-binding protein that severs actin filaments, nucleates actin assembly, and caps the fast growing ends of actin filaments. Sequence comparison with functionally related proteins, such as gelsolin, villin, or fragmin revealed highly conserved domains which are thought to be of functional significance. To attribute the different activities of the severin molecule to defined regions, progressively truncated severin polypeptides were constructed. The complete cDNA coding for 362 (DS362) amino acids and five 3' deletions coding for 277 (DS277), 177 (DS177), 151 (DS151), 117 (DS117), or 111 (DS111) amino acids were expressed in *Escherichia coli*. The proteins were purified to homogeneity and then characterized with respect to their effects on the polymerization or depolymerization kinetics of G- or F-actin solutions and their binding to G-actin. Furthermore, the Ca^{2+} binding of these proteins was investigated with a ^{45}Ca -overlay assay and by monitoring Ca^{2+} -dependent changes in tryptophan fluorescence. Bacterially expressed DS362 showed the same Ca^{2+} -dependent activities as native severin. DS277, missing the 85 COOH-terminal amino acids of severin, had lost its strict Ca^{2+} regulation and displayed a Ca^{2+} -independent capping activity, but was still Ca^{2+} dependent in its

severing and nucleating activities. DS151 which corresponded to the first domain of gelsolin or villin had completely lost severing and nucleating properties. However, a residual severing activity of $\sim 2\%$ was detectable if 26 amino acids more were present at the COOH-terminal end (DS177). This locates similar to gelsolin the second actin-binding site to the border region between the first and second domain. Measuring the fluorescence enhancement of pyrene-labeled G-actin in the presence of DS111 showed that the first actin-binding site was present in the NH_2 -terminal 111 amino acids. Extension by six or more amino acids stabilized this actin-binding site in such a way that DS117 and even more pronounced DS151 became Ca^{2+} -independent capping proteins. In comparison to many reports on gelsolin we draw the following conclusions. Among the three active actin-binding sites in gelsolin the closely neighbored sites one and two share the F-actin fragmenting function, whereas the actin-binding sites two and three, which are located in far distant domains, collaborate for nucleation. In contrast, severin contains two active actin-binding sites which are next to each other and are responsible for the severing as well as the nucleating function. The single actin-binding site near the NH_2 -terminus is sufficient for capping of actin filaments.

POLYMERIZATION of G-actin monomers into filaments, steady-state equilibrium between G- and F-actin as well as the three-dimensional organization of the filamentous meshwork are influenced by a variety of actin-binding proteins. These proteins are divided into different groups according to their mode of interaction with G- or F-actin (for review see Vandekerckhove, 1989).

Severin from *Dictyostelium discoideum* (Brown et al., 1982) belongs to the group of actin filament fragmenting proteins found in lower and higher eukaryotes. Other members of this group are the vertebrate proteins gelsolin (Yin and Stossel, 1979) and villin (Bretscher and Weber, 1979), or fragmin from *Physarum polycephalum* (Hasegawa et al., 1980). In the presence of micromolar Ca^{2+} these proteins interact with actin in at least three ways. First, they promote

actin assembly by generating nuclei. Second, they sever actin filaments by breaking the noncovalent bonds between actin subunits and third, once the filament is severed they remain bound to the barbed ends of the shortened filaments (Matsudaira and Janmey, 1988). The actin filament fragmenting proteins are remarkably similar with respect to their functional properties and primary structures. Sequence analysis revealed six large segmental repeats of ~ 15 kD in gelsolin and villin and three similar repeats in severin and fragmin (Kwiatkowski et al., 1986; Ampe and Vandekerckhove, 1987; André et al., 1988; Arpin et al., 1988). Sequence alignments suggest that the genes of vertebrate gelsolin and villin are derived from a duplication of an ancestor severin-like gene (Janmey and Matsudaira, 1988; Matsudaira and Janmey, 1988; Schleicher et al., 1988). Villin contains in ad-

dition to the six repeats found in gelsolin, the so-called "headpiece" domain (8.5 kD) at the COOH-terminus, which is unique and responsible for the bundling activity of villin (Glenney and Weber, 1981). The functions of complete and truncated gelsolin have been intensively studied with respect to their regulation, their capability to bind to actin, and their impact on the polymerization or depolymerization kinetics of G- or F-actin solutions (Bryan, 1988; Yin et al., 1988; Kwiatkowski et al., 1989; Way et al., 1989).

Here we describe functional assays with COOH-terminally truncated severin molecules. We have constructed five deletion mutants and expressed the mutant proteins and as a control, complete severin in *E. coli*. The properties of five COOH-terminal deletion mutants spanning amino acids 1-111 (DS111), 1-117 (DS117), 1-151 (DS151), 1-177 (DS177), or 1-277 (DS277) of severin (362 amino acids), have been studied in comparison to native severin with respect to their nucleation, capping, severing or G-actin-binding activity, and their ability to bind Ca^{2+} . The data suggest that the domain structure of severin is organized in a more simple fashion as compared to that of gelsolin. The two actin-binding sites of severin share the severing and nucleating functions, whereas in gelsolin these activities are split among three actin-binding sites. This might be the reason that gelsolin is a more efficient nucleator of actin polymerization than severin (Yin et al., 1990).

Materials and Methods

Construction of Truncated Severin cDNA

cDS4, a cDNA containing the complete coding sequence of *D. discoideum* severin, was cloned into the Eco RI site of pMS1 (Simon et al., 1988; André et al., 1989), an ATG-expression vector. The vector was linearized downstream of the cDS4 insert using Pst I and Sal I and a series of deletions was created by exonuclease III digest using the Erase-a-Base™ System (see Promega-Biotec [Madison, WI] 1987 Technical Manual). The digest was stopped at fixed time points and *E. coli* JM83 cells were transformed with religated plasmids. Clones expressing immunoreactive material were identified as described with a mixture of mAbs directed against severin (André et al., 1989). The 3' ends of three deletions were determined by DNA sequence analysis using an oligonucleotide (18-mer) specific for a vector sequence downstream of the deleted cDS4 as a primer for the dideoxy chain termination method (Sanger et al., 1977). In this paper we describe six constructs that contain either the complete coding region of 1,086 bp (cDS4) or truncated coding regions of 831 bp (cDS892), 531 bp (cDS592), 453 bp (cDS514), 351 bp (cDS412), and 333 bp (cDS394). The resulting proteins are designated according to the number of amino acids as DS362, DS277, DS177, DS151, DS117, and DS111, respectively. Six different primers, one specific for the 5'-end of cDS4 (37-mer) and the other five specific for the coding region upstream of the last severin-specific base of cDS892 (39-mer), cDS592 (37-mer), cDS514 (36-mer), cDS412 (36-mer), or cDS394 (38-mer), respectively, were synthesized and used in five polymerase chain reactions (PCRs).¹ All six primers contained 5' "add on" sequences (Scharf et al., 1986), comprising an Eco RI restriction site and for the 3' specific primers, in addition, a TAA stop codon immediately after the last severin-specific base. The PCR was essentially performed according to the manufacturer's instruction (Perkin Elmer Cetus, Norwalk, CT). Briefly, in each reaction ~0.05 ng of the template (cDS4 cloned into pMS1), 20 nmol of each dNTP, 100 pmol of the 5'-specific primer and 100 pmol of the corresponding 3'-specific primer and 4 U of Taq-polymerase (Amersham International, Amersham, UK) were used. 30 cycles, with 1 min denaturation at 92°C, 1 min annealing at 50°C, and 2 min extension at 72°C were performed. The denaturation step in the first cycle was extended to 2 min and the elongation step in the last cycle to 5 min. All cloning procedures were carried out according to Maniatis et al. (1982). DS277,

DS177, and DS111 were constructed by 3' deletion and by PCR, the characterization of both products gave the same results. This showed that the PCR did not lead to errors in the resulting polypeptides.

Purification of Severin and Truncated Severin Molecules

For purification of severin, *D. discoideum* cells were grown, harvested, opened, and the soluble extract was subjected to anion exchange chromatography essentially as described by Scheel et al. (1989). Solid ammonium sulfate was added to the flowthrough fraction and proteins precipitating between 60–80% of saturation were loaded after dialysis against MEDABP buffer (10 mM 2 [N-morpholino] ethane sulfonic acid, pH 6.5, 1 mM EGTA, 0.1 mM DTT, 0.02% $\text{Na}_2\text{S}_2\text{O}_8$, 1 mM benzamide, 0.5 mM PMSF) onto a phosphocellulose column (2.5 × 8 cm, equilibrated in MEDABP buffer). Severin was eluted with a linear salt gradient (2 × 150 ml, 0–250 mM NaCl in column buffer) at a conductivity between 4–6 mS. For final purification severin-containing fractions were dialyzed versus KEDABP buffer (10 mM potassium phosphate, pH 6.7, 1 mM EGTA, 0.2 mM DTT, 0.02% $\text{Na}_2\text{S}_2\text{O}_8$, 1 mM benzamide, 0.5 mM PMSF) and chromatographed on a hydroxylapatite column (2.5 × 5 cm, equilibrated in KEDABP buffer) using a linear KCl gradient (2 × 120 ml, 0–400 mM KCl). Pure severin, as judged by SDS-PAGE, was eluted between 19–24 mS.

For expression of complete (DS362) and COOH-terminally truncated severin molecules (DS277, DS177, DS151, DS117, and DS111), recombinant *E. coli* JM83 cells were grown at 37°C to an OD₅₈₀ of 0.5. The expression of *tac* promoter controlled products was induced with 1 mM IPTG and the cultures were further incubated for 2 h at 37°C. The cells were harvested by low speed centrifugation (4,000 g, 10 min), washed once with 1 mM Tris/HCl, pH 8.0, 1 mM EDTA, resuspended in a small volume of the same buffer, frozen in liquid nitrogen, and lyophilized. DS151- and DS117-expressing JM83 cells were resuspended in TEDABP buffer (10 mM Tris/HCl, pH 8.0, 1 mM EGTA, 1 mM DTT, 0.02% $\text{Na}_2\text{S}_2\text{O}_8$, 1 mM benzamide, 0.5 mM PMSF) and opened by ultrasonication. Insoluble material was pelleted (20 min, 30,000 g) and proteins were extracted from the pellet as described for the purification of DS362, DS277, DS177, and DS111. The lyophilized bacteria were extracted with acetone to remove membrane lipids and the remaining acetone-insoluble material was air-dried for 1–2 h. Proteins were extracted in a Dounce homogenizer with TEDABP buffer containing 30% sucrose and insoluble material was pelleted at 30,000 g for 20 min. This procedure was repeated with the same buffer, then once with TEDABP and 2 M urea, twice with TEDABP containing 6 M urea, and the final pellet was resuspended in 1% SDS, 1 mM β-mercaptoethanol and incubated for 3 min in a boiling waterbath. SDS-PAGE of aliquots of the supernatants showed that most of DS362, DS277, DS177, DS151, DS117, and DS111 was extracted with TEDABP containing 6 M urea. A minor amount of all four proteins was extracted with 2 M urea and with 1% SDS and a small amount of DS362 also with 30% sucrose.

DS362 and DS277 were further purified essentially as described for *D. discoideum* severin, with the exception that the buffers for the DEAE-, phosphocellulose-, and hydroxylapatite-column contained, in addition, 5 M urea and that the proteins were not precipitated with ammonium sulfate. From one liter of induced *E. coli* cells ~1.5 mg purified DS362 and 3.5 mg DS277 were obtained. DS177, DS151, DS117, or DS111 containing extracts were treated with ammonium sulfate (80% saturation), centrifuged (20,000 g, 20 min), the pellets dissolved in IEDABUP buffer (10 mM imidazole, pH 7.6, 1 mM EGTA, 1 mM DTT, 0.02% $\text{Na}_2\text{S}_2\text{O}_8$, 1 mM benzamide, 6 M urea, 0.5 mM PMSF), and then gel filtered on Sephacryl S200 (2.5 × 70 cm) using the same buffer.

To remove contaminating protein during the preparation of DS177, the pooled fractions from the gel filtration column were dialyzed versus TEDABP buffer containing 5 M urea and subjected to anion-exchange chromatography (DE52; 2.5 × 6 cm, equilibrated in the same buffer). Under the conditions used, DS177 did not bind to the resin. The flow-through was dialyzed against KEDABUP buffer (KEDABP buffer plus 5 M urea) and loaded onto a hydroxylapatite column (2.5 × 6 cm). The protein was step-eluted with 100 mM potassium phosphate in column buffer and ~2.5 mg pure DS177, as judged by SDS-PAGE, were obtained per liter of induced *E. coli* cells.

The DS151-containing fractions of the gel filtration column were dialyzed versus KEDABUP buffer and chromatographed on a hydroxylapatite column (2.5 × 5 cm, equilibrated in the same buffer) using a linear KCl gradient (2 × 140 ml, 0–200 mM KCl). Pure DS151, as judged by SDS-PAGE; was eluted between 4.0 and 6.0 mS and ~4 mg of DS151 were obtained per liter of induced *E. coli* cells.

1. Abbreviation used in this paper: PCR, polymerase chain reaction.

The DSI17-containing fractions of the gelfiltration column were dialyzed versus TEDABP buffer plus 5 M urea and chromatographed on a DEAE column (2.5 × 6 cm, equilibrated in the same buffer) using a linear NaCl gradient (2 × 140 ml, 0–300 mM NaCl). Pure DSI17, as judged by SDS-PAGE, was eluted between 2.0 and 4.0 mS. The final yield was ~2 mg DSI17 per liter of induced *E. coli* cells.

DSI11 was dialyzed against KEDABUP buffer and loaded onto a hydroxylapatite column (2.5 × 6 cm). The flow-through was collected, dialyzed against MEDABP buffer plus 5 M urea, loaded onto a phosphocellulose column (P11; 2.5 × 6 cm), and step-eluted with 400 mM NaCl in column buffer. About 1.5 mg pure DSI11 were obtained per liter of induced *E. coli* cells.

All six purified proteins were dialyzed in several steps against buffer A (2 mM Tris/HCl, pH 7.6, 0.2 mM Na₂ATP, 0.2 mM CaCl₂, 0.5 mM DTT, 0.01% NaN₃), containing decreasing molarities of urea and then stored at -70°C until they were used for the different assays. Under these conditions the proteins contained their activity at least up to 6 mo.

Low Shear Viscometry

Low shear viscometry was carried out after 20 min of incubation at 25°C in a falling ball viscometer (MacLean-Fletcher and Pollard, 1980). The reaction mixture (160 μl) contained usually 0.5 mg/ml rabbit skeletal muscle actin, and polymerization was started by addition of G-actin to buffered MgCl₂ (final concentration: 2 mM MgCl₂, 10 mM imidazole, pH 7.2, 1 mM ATP, 0.2 mM CaCl₂ or 1 mM EGTA). The data shown are the mean values of three experiments.

Fluorescence Spectroscopy

Actin was labeled with *N*-(1-pyrenyl)iodoacetamide (pyrene) (Kouyama and Mihashi, 1981) according to the modification of Cooper et al. (1983). Aliquots of pyrene-labeled actin were frozen in liquid nitrogen and stored at -70°C. Before use, the pyrene-labeled G-actin was thawed quickly and then centrifuged (10 min, 10,000 *g*) to remove larger precipitates. All fluorescence measurements were performed in a spectrofluorometer (model SFM 25; Kontron Instruments, Eching, FRG) in the ratio mode. For kinetic measurements the excitation wavelength was 365 nm and the emission wavelength was 386 nm. The temperature was 25°C, the sample volume was 800 μl. The data are plotted as arbitrary relative fluorescence values. Usually from three experiments the mean values were calculated either at fixed time points or from the slope of the plot of fluorescence versus time in the linear range of polymerization or depolymerization.

Nucleation of Actin Assembly. A mixture of pyrene-labeled G-actin and unlabeled G-actin in a molar ratio of 1:10 was polymerized at a final concentration of 4 μM in buffer B (buffer A containing 2 mM MgCl₂ and 150 mM KCl) with or without additional proteins. In the experiments without free Ca²⁺ the solution contained 2 mM EGTA. Fluorescence measurements were started 15 s after mixing and the slope of the plot of fluorescence versus time was calculated in the linear range of fluorescence increase.

Dilution Induced Depolymerization of F-actin. 8 μM pyrene-labeled G-actin was polymerized for 14 h at room temperature in buffer B in the presence of 40 nM DSI151, resulting in filaments precapped with DSI151. The precapped F-actin solution was diluted 20-fold into buffer B with or without 2 mM EGTA and with or without severin or truncated severin molecules. The solution was not vortexed but carefully mixed by shaking to minimize shearing and the measurement was started 15 s after diluting the filamentous actin to 0.4 μM, which is below the critical concentration of the "pointed" end (Wegner and Isenberg, 1983). The slope of the plot of fluorescence versus time was calculated in the linear range of fluorescence decrease. The decrease in fluorescence emission was shown to be proportional to the number of "pointed" filament ends (Bryan and Coluccio, 1985), therefore, this assay can be used for measuring the filament-severing activity of actin-binding proteins.

Capping of Actin Filaments. Two approaches were used, to show capping of actin filaments. (1) A mixture of labeled and unlabeled G-actin (see nucleation of actin assembly) was polymerized for 14 h at room temperature in buffer B in the presence or absence of Ca²⁺ and with or without additional protein, the fluorescence emission of the polymerized solution was determined and the amount of F-actin was calculated as described (Janmey and Stosel, 1986). (2) Preformed unlabeled actin filaments were used as nuclei (800 nM final concentration) for the polymerization of actin monomers (3 μM final concentration, 20% pyrene labeled). Because the fragmenting activity of severin creates additional "pointed" ends for polymerization, only truncated severin molecules lacking or retaining only min-

imal severing activity, were used in this assay. The assay was performed essentially as described (Pollard, 1983), using buffer A containing 50 mM KCl with or without 1 mM EGTA.

Interaction with Pyrene-labeled G-actin. The fluorescence enhancement assay described by Lee et al. (1988), was used to monitor binding of DSI17 and DSI11 (2.4 μM each) to pyrene-labeled G-actin (160 nM) under nonpolymerizing conditions. The emission scan was performed at an excitation wavelength of 342 nm and the excitation scan by measuring the emission at 386 nm. The excitation and emission spectra were recorded in buffer A containing 1 mM CaCl₂. The quantitative changes in fluorescence shown were corrected for buffer and peptide values.

Binding of Ca²⁺. Changes in tryptophan fluorescence were exploited for monitoring binding of Ca²⁺ to severin or severin derivatives. The emission scan was performed at an excitation wavelength of 290 nm and the excitation scan by measuring emission at 350 nm. The spectra were recorded in buffer A plus 1 mM EGTA ("Ca²⁺-free" buffer) or buffer A plus 1 mM EGTA and 2 mM CaCl₂ with 2 μM protein and corrected for buffer fluorescence. The free Ca²⁺-concentration, which was 1 nM in the case of the Ca²⁺-free buffer and 1 mM in the case of the Ca²⁺-containing buffer, was calculated using the apparent dissociation constant of Ca-EGTA, determined by Harafuji et al. (1980).

⁴⁵Ca Overlay

Purified proteins were bound onto a nitrocellulose membrane via a slot blot apparatus. The nitrocellulose was washed twice for 15 min with overlay buffer (10 mM imidazole, pH 6.8, 40 mM NaCl, 5 mM MgCl₂), containing 1 mM EGTA to remove Ca²⁺ bound to the proteins. Then the filter was washed three more times for 15 min with overlay buffer to remove the EGTA. The ⁴⁵Ca-overlay was carried out essentially as described (Maruyama et al., 1984).

Binding to G-actin Beads

For the preparation of actin from *D. discoideum*, the soluble extract was chromatographed on a DE52 column (5 × 30 cm) using a linear salt gradient of 0–350 mM NaCl (2 × 750 ml) in TEDABP buffer, pH 8.0, containing 0.1 mM ATP. Under the conditions used, actin was eluted at a conductivity between 10–14 mS, the appropriate fractions were pooled and subjected to ammonium sulfate precipitation. The protein fraction which was precipitated by 0–37% of saturation was dialyzed extensively against buffer A, cleared by centrifugation (1 h, 100,000 *g*), and brought to 2 mM MgCl₂, 50 mM KCl, and 1 mM ATP. After 4 h at 4°C, the polymerized actin was collected by ultracentrifugation (3 h, 150,000 *g*), depolymerized by extensive dialysis versus buffer A, cleared by ultracentrifugation (3 h, 150,000 *g*), and chromatographed on a Sephacryl S300 column (2.5 × 60 cm) equilibrated in buffer A. Coupling of G-actin to Affi-Gel 10 (Bio-Rad Laboratories, Cambridge, MA) was done essentially as described by Kwiatkowski et al. (1985). Briefly, 3 vol of G-actin (2 mg/ml) in buffer P (1 mM NaHCO₃, pH 7.6, 0.1 mM CaCl₂, 0.2 mM ATP) were added to 1 vol of Affi-Gel 10 beads in the same buffer and rotated overnight at 4°C. Then, 0.1 M ethanolamine, pH 8.0, was added to saturate free binding sites and the beads were rotated for another 2 h at 4°C. The beads were washed four times with buffer A to remove unbound G-actin and stored in the same buffer at 4°C. Usually the beads prepared in this manner were used the same or the following day. Severin and truncated severin molecules (2.5 μM) were incubated for 15 min at 20°C with a twofold excess of BSA to minimize nonspecific binding and either with or without free G-actin (5 μM) as specific competitor. Then the beads (50 μl) were added and the suspensions (total volume of 200 μl) were shaken vigorously for 30 min at 20°C. The beads were pelleted in an Eppendorf centrifuge and supernatants and pellets were analyzed by SDS-PAGE.

Miscellaneous

Actin was prepared from rabbit skeletal muscle (Spudich and Watt, 1971) and gel filtered on Sephacryl S300. The concentration of actin and pyrene-labeled actin was determined as described (Cooper et al., 1983). All other protein concentrations were determined by the method of Bradford (1976) using BSA as a standard. SDS-PAGE was performed on minislab gels (110 × 83 × 0.5 mm) using the buffer system of Laemmli (1970).

Reagents

DEAE-cellulose (DE52) and phosphocellulose (P11) were purchased from

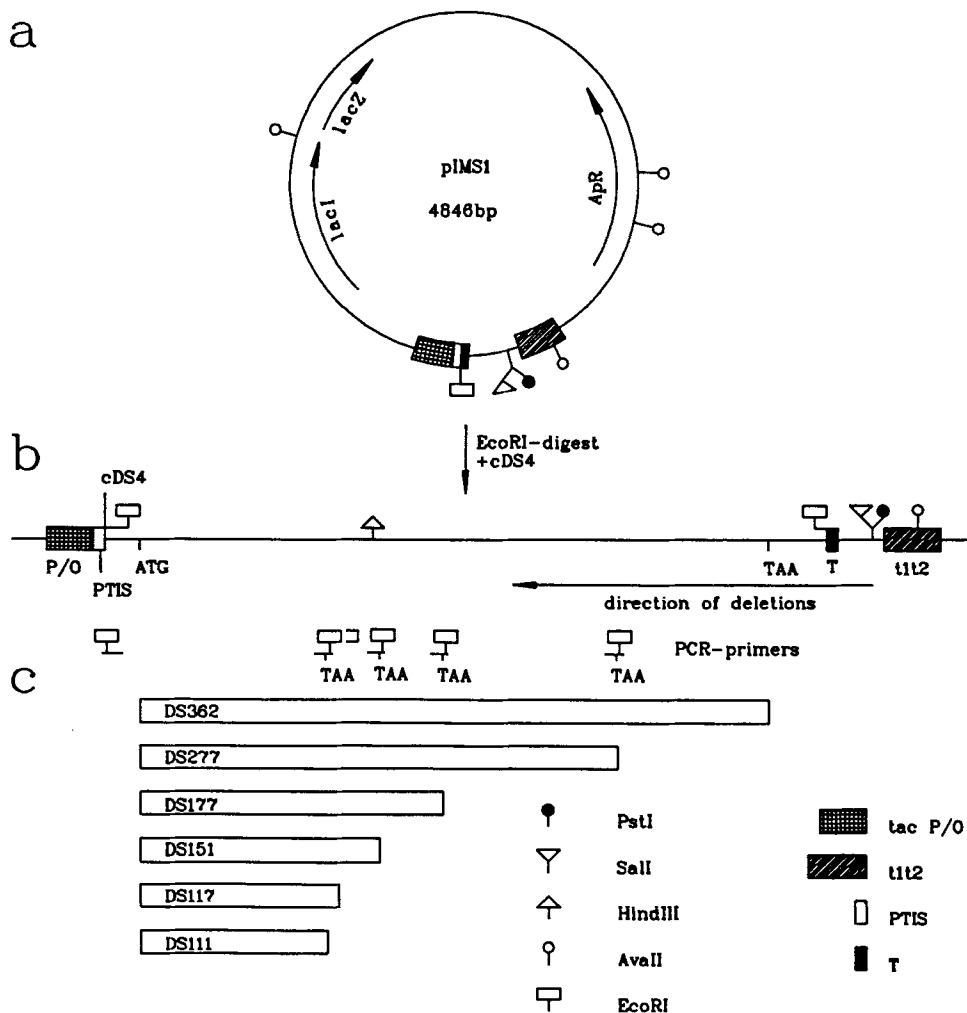


Figure 1. Topography of the expression vector, the cDS4 coding sequence, and the truncated severin molecules. (a) Presentation of the pIMS1 expression vector. Important restriction endonuclease recognition sites, regulatory elements for the expression of proteins (*tac P/O*, *tac* promoter and operator; *PTIS*, portable translation initiation site; *T*, synthetic translational terminator; *t1,t2*, transcriptional terminators), and the ampicilline resistance gene (*Ap^R*) for the selection of transformed bacteria are shown. The vector was cleaved with *EcoRI* and *cDS4*; coding for complete severin was cloned into this site. (b) The unique *PstI* and *SalI* restriction endonuclease recognition sites were used to create directed deletions in 5' direction. Start and stop codon of *cDS4*, the internal *HindIII* site of *cDS4*, the direction of the deletions, and the regulatory elements for the expression of proteins are indicated. (c) PCR primers were used to design truncated *cDS4* constructs coding for peptides containing only severin-specific amino acids. The relative sizes of complete severin and the five constructs DS277, DS177, DS151, DS117 and DS111 are schematically presented.

Whatman (Maidstone, Kent, England), hydroxylapatite and Affi-Gel 10 from Bio-Rad Laboratories (Richmond, California), Sephacryl S200 and S300 from Pharmacia (Uppsala, Sweden), and N-(1-pyrenyl)iodoacetamide from Molecular Probes Inc. (Junction City, OR). All chemicals were of analytical grade.

Results

Purification of Complete and Truncated Severin Molecules from *E. coli*

Five COOH-terminally truncated severin molecules with apparent molecular masses of 32 kD (DS277), 22 kD (DS177), 18 kD (DS151), 15 kD (DS117), and 14 kD (DS111) were purified and characterized in addition to complete severin (Fig. 1). Exonuclease III digests starting from the *SalI*-site in 5' direction also deleted the translational terminator. Therefore, translation stopped only at randomly occurring stop codons in the vector sequence, and the truncated severin molecules contained, depending on the reading frame, vector-specific amino acids at the COOH-terminus. Sequencing of the 3' ends of the truncated cDNAs of DS277, DS177, and DS111 revealed that 14 or 17 vector-specific amino acids were added at the COOH-terminus of these pro-

teins. To exclude any interference of these additional amino acids, we engineered the 3' ends in such a way that translation stopped with the last severin-specific amino acid. This was achieved using primers for the PCR reaction, which contained a *TAA* stop codon and an *EcoRI* restriction site as 5' "add on" sequence (Scharf et al., 1986). Both types of proteins behaved identically in the functional assays. All results presented in this paper were obtained with PCR-derived constructs. Fig. 2 shows total protein of *E. coli* transformants and the corresponding purified severin derivatives after SDS-PAGE.

Loss of Strict Ca^{2+} Regulation after Removal of 85 COOH-terminal Amino Acids from Severin

The effects of complete and truncated severin on the viscosity of an F-actin solution were assayed by low shear viscometry. Severin and DS362 lowered the viscosity dramatically in the presence of Ca^{2+} . In the absence of Ca^{2+} even high concentrations of both proteins decreased the viscosity only weakly (Fig. 3, A and B). In contrast, DS277, DS177, and DS151 were active in the presence and in the absence of Ca^{2+} (Fig. 3, C-E). While DS117 could decrease the vis-

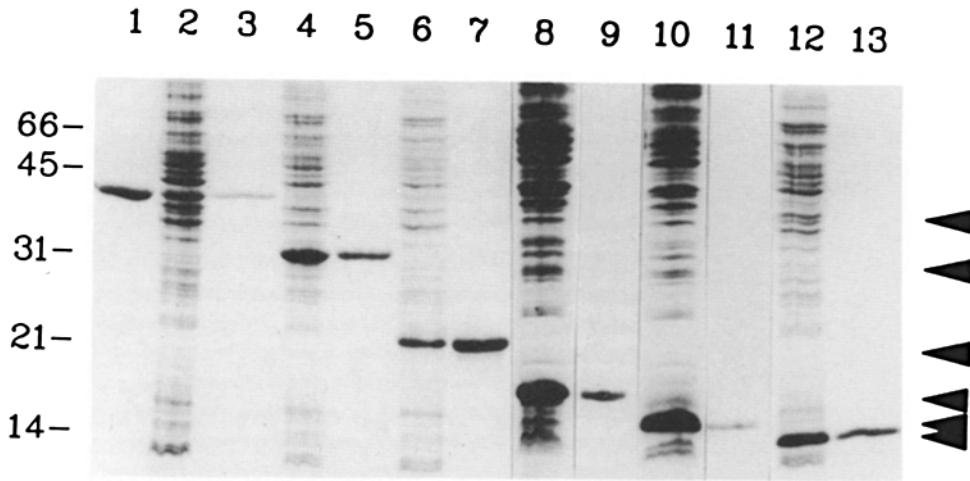


Figure 2. Gel electrophoresis of total cell homogenates and purified severin molecules. Transformed *E. coli* cells were induced with IPTG, aliquots of total cell homogenates (lanes 2, 4, 6, 8, 10, and 12) or purified proteins (lanes 1, 3, 5, 7, 9, 11, and 13) were subjected to SDS-PAGE (15% acrylamide) and stained with Coomassie blue (lanes 2/3, DS362 = complete severin; lanes 4/5, DS277; lanes 6/7, DS177; lanes 8/9, DS151; lanes 10/11, DS117; lanes 12/13, DS111). Purified severin from *D. discoideum* is shown in lane 1. The positions of complete severin and of truncated severin molecules from bacteria are indicated with arrowheads. The sizes of the molecular mass markers in kD are shown on the left.

cosity substantially (Fig. 3 F), even high concentrations of DS111 did not affect the viscosity of the F-actin solution (Fig. 3 G). In the low shear viscometry assays we found always a more pronounced viscosity-decreasing activity in the pres-

ence of Ca^{2+} . Considering similar observations with the Ca^{2+} -independent *D. discoideum* capping protein Cap 32/34 (Schleicher et al., 1984) and the gelsolin-actin complex from human platelets (Kurth et al., 1983), the reason for this

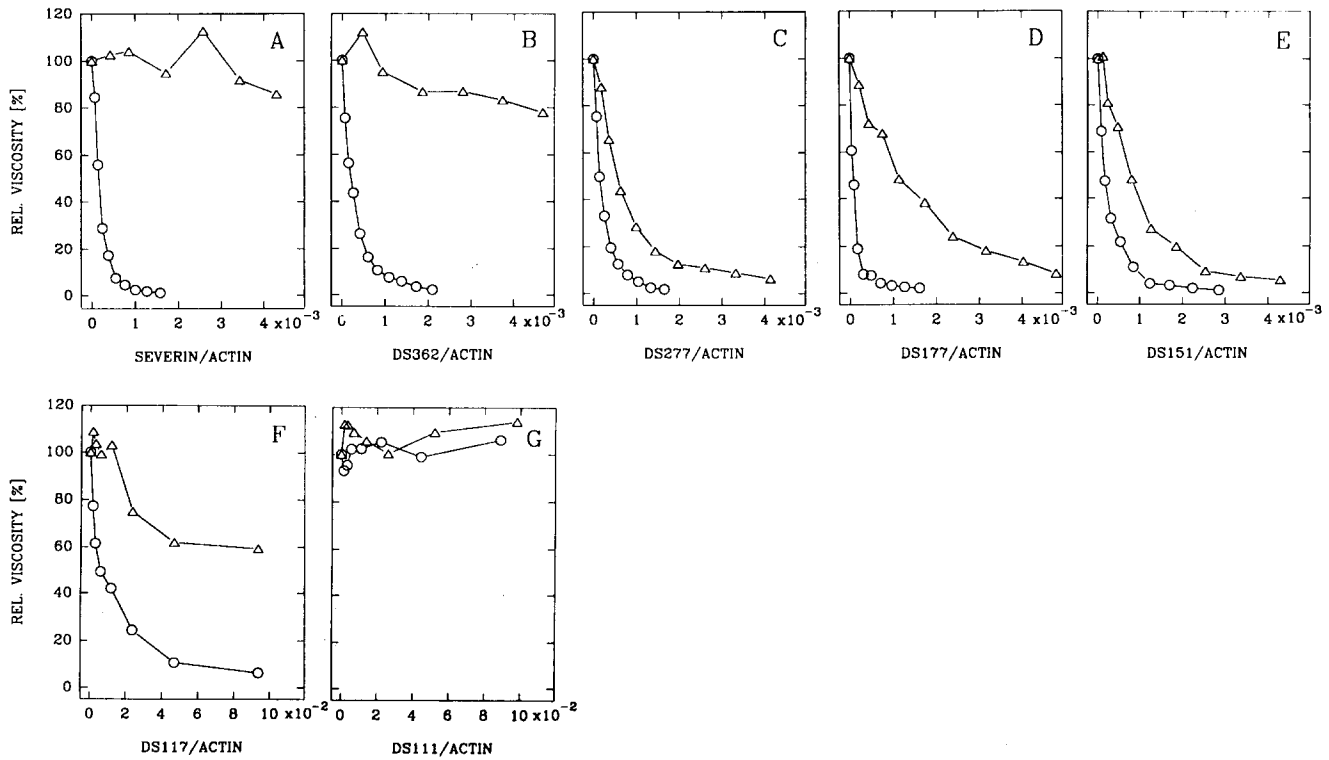


Figure 3. Low shear viscometry in the presence (O) or absence (Δ) of Ca^{2+} . $12 \mu M$ G-actin was polymerized for 20 min at $25^\circ C$ with increasing concentrations of severin or truncated severin molecules. The ratios of actin-binding protein to actin (mol/mol) and the relative viscosities were calculated. The panels show the properties of severin from *D. discoideum* (A), of bacterially expressed complete severin (DS362; B), and of the truncated severin molecules DS277 (C), DS177 (D), DS151 (E), DS177 (F) and DS111 (G). Please note the different scale of the X-axis in F and G.

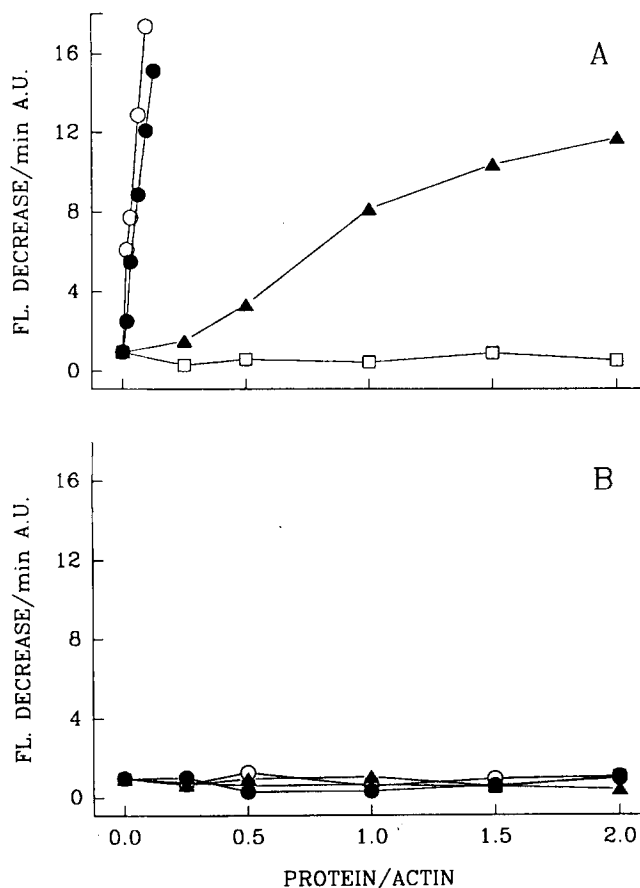


Figure 4. Severing activity in dilution induced depolymerization assays with (A) or without Ca^{2+} (B). $8 \mu\text{M}$ pyrene-labeled F-actin was diluted 20-fold into buffer B with or without severin or truncated severin molecules and the slopes of the plot of fluorescence versus time in the linear range of fluorescence decrease (A.U., arbitrary units) were determined. The influence of increasing ratios of actin-binding protein to actin (mol/mol) on the depolymerization rate of 400 nM pyrene-labeled F-actin was investigated in comparison to the depolymerization rate without additional protein. (○) Severin; (●) DS277; (▲) DS177; (□) DS151.

result is rather based on the generation of more but shorter actin filaments in the presence of EGTA. Therefore, slight differences in this assay might reflect the calculation to different control values ($+/-\text{Ca}^{2+}$) and not the actual Ca^{2+} dependency of the actin-binding protein.

No Severing Activity after Removal of Domains II and III

A truncated severin molecule might decrease the viscosity of an actin solution due to its F-actin fragmenting or capping activity. Using low shear viscometry it is not possible to decide which one of these two activities would be responsible for the decrease in viscosity. Dilution of the actin concentration below the critical concentration of the "pointed" end results in dissociation of actin monomers from this end until a new equilibrium is reached. Fragmentation of F-actin creates additional pointed ends and as the depolymerization rate is proportional to the number of pointed ends (Bryan and Coluccio, 1985), it is possible to demonstrate fragmenting activity of actin-binding proteins. In the presence of Ca^{2+} ,

severin and DS277 showed comparable F-actin fragmenting activity, while DS177 retained only a weak severing activity ($\sim 2\%$ of severin). DS151, missing another 26 amino acids, had completely lost the severing activity and no influence on the depolymerization rate of F-actin was seen (Fig. 4 A). This result shows that similar to gelsolin (Kwiatkowski et al., 1989), important amino acids for the severing activity are located at the border region between the first and second domain of severin. DS362 displayed an activity similar to DS277 (data not shown). In the absence of Ca^{2+} , neither severin nor the truncated severin molecules could fragment F-actin (Fig. 4 B).

DS277, DS177, and DS151 Capped Actin Filaments Independently of Ca^{2+}

G-actin was polymerized either with or without severin and severin derivatives, the fluorescence emission at steady state was determined, and the fraction of F-actin was calculated according to Janmey and Stossel (1986). Capping of the "barbed" end of the actin filament would increase the G-actin concentration at steady state due to the lower affinity of G-actin monomers for the pointed end of the actin filament. In the presence of Ca^{2+} all proteins except DS117 and DS111 decreased the fraction of F-actin at steady state, while in the absence of Ca^{2+} a decrease in the fraction of F-actin was only observed with DS277, DS177, and DS151 (Table I).

In a second approach, the elongation of unlabeled preformed nuclei with pyrene-labeled G-actin monomers was investigated. If the barbed end is capped, only the pointed end is available for elongation and in this case the elongation rate is drastically reduced. As severin and DS362 fragment F-actin filaments in the presence of Ca^{2+} , thereby raising the number of filaments and creating additional nuclei, they were not investigated in this capping assay. The influence of DS277 on the elongation rate of preformed actin nuclei was only tested in the absence of Ca^{2+} , because DS277 like severin and DS362 contains a Ca^{2+} -dependent F-actin

Table I. Capping of Actin Filaments

Protein	[F-actin]*		% [F-actin]†	
	Ca^{2+}	EGTA	Ca^{2+}	EGTA
Severin	3.2	4.1	80	102.5
DS362	3.4	3.9	85	97.5
DS277	3.3	3.3	82.5	82.5
DS177	3.5	3.2	87.5	80
DS151	3.4	3.3	84	82
DS117‡	3.9	3.9	97.5	97.5
DS111	4.0	3.9	100	97.5

The solutions containing $4 \mu\text{M}$ G-actin (20% pyrene-labeled) and 25 nM (Ca^{2+}) or 50 nM (EGTA) severin or severin derivatives, were polymerized in buffer B for 14 h and the fluorescence emission was determined. The final F-actin content was calculated relative to the control.

* The concentration of polymeric actin [F-actin] was calculated from the fluorescence using the equation: $[\text{F-actin}] = [\text{actin}]_{\text{tot}} \times (f - G)/(F - G)$, where $[\text{actin}]_{\text{tot}}$ is the total molar concentration of actin subunits, F and G are the fluorescence intensities expected if all of the actin was in the polymeric or monomeric state, respectively, and f is the sample fluorescence (Janmey and Stossel, 1986).

† The percentage of polymeric actin, % [F-actin], was calculated as $([\text{F-actin}]_{\text{with additional protein}}/[\text{F-actin}]_{\text{without additional protein}}) \times 100$.

‡ In deference to DS111, DS117 showed at high concentrations ($1 \mu\text{M}$) weak capping activity ($\sim 90\%$ F-actin).

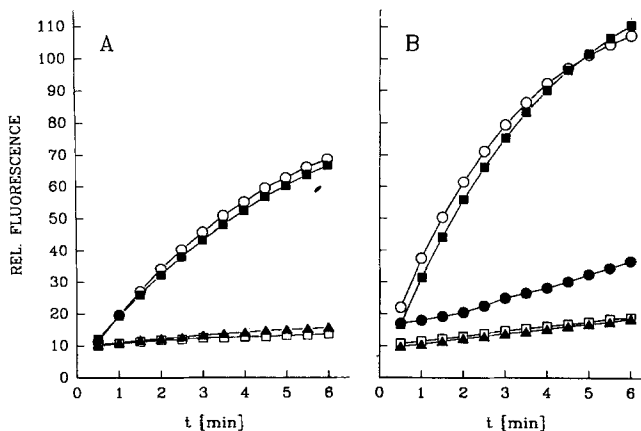


Figure 5. Elongation of F-actin nuclei in the presence of truncated severin molecules. The polymerization of 3 μM G-actin (20% pyrene-labeled) in buffer A with or without severin derivatives was started by addition of 800 nM F-actin as nuclei for polymerization. The increase in fluorescence emission was monitored and plotted versus time. Elongation of unlabeled F-actin nuclei with labeled G-actin monomers was assayed in the presence of 0.2 mM Ca^{2+} (A) or in the presence of 0.2 mM Ca^{2+} plus 1 mM EGTA (B) without severin derivatives (\circ , control), with DS277 (\bullet , only in B), with DS177 (\blacktriangle), with DS151 (\square) or with DS111 (\blacksquare). The final concentrations of the severin derivatives were 25 nM in A and 50 nM in B.

severing activity. In the presence of Ca^{2+} , DS177 and DS151 reduced the elongation rate dramatically in comparison to the elongation rate of nuclei in the control; DS111 did not reduce the elongation rate (Fig. 5 A). This suggests that DS177 and DS151 are capping the barbed ends of the filaments. A slight reduction of the elongation rate was observed with high concentrations of DS117, but not with DS111 (1.5 μM each), suggesting that DS117 retained a weak capping activity (data not shown). In the absence of Ca^{2+} , DS277, DS177, and DS151, but not DS111, reduced the elongation rate in comparison to the elongation rate of nuclei in the control sample (Fig. 5 B). Similar data were obtained with Cap32/34, a protein from *D. discoideum* that is known to cap but not to sever actin filaments (Schleicher et al., 1984).

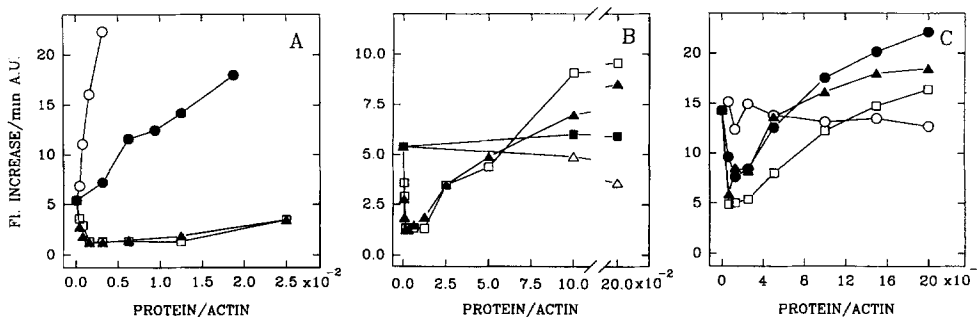


Figure 6. Comparison of the nucleation activity of severin, DS277, DS177, DS151, DS117, and DS111. The purified proteins were added to buffer B and the polymerization was started with 4 μM G-actin (10% pyrene-labeled). The assay mixture contained 0.2 mM CaCl_2 (A and B), or in addition 2 mM EGTA (C). The fluorescence increase versus time (A.U., arbitrary units)

was monitored and the slope per minute in the linear range of fluorescence increase was calculated for different ratios of actin-binding protein to actin (mol/mol). The influence of increasing concentrations of severin (\circ , only in A and C), DS277 (\bullet , only in A and C), DS177 (\blacktriangle), DS151 (\square), DS117 (\triangle , only in B), or DS111 (\blacksquare , only in B) on the polymerization rate is shown. Please note the different scale of the Y-axis in B. The X-axes in B and C show up to ~ 10 -fold higher concentrations of selected severin derivatives, demonstrating the pseudonucleation of DS277 (\bullet), DS177 (\blacktriangle), and DS151 (\square).

Nucleation of Actin Assembly

In the presence of micromolar Ca^{2+} , low concentrations of severin (molar ratios between 1:2,560 and 1:320) and DS277 (molar ratios between 1:320, and 1:53) nucleated, while DS177 and DS151 (molar ratios between 1:2,560 and 1:40) retarded the assembly of G-actin in comparison to the control. Maximal retardation of polymerization was observed at molar ratios of 1 molecule DS177 or DS151 to 640 molecules actin (Fig. 6 A). However, we observed an increase in the rate of assembly with increasing concentrations of DS177 or DS151. At molar ratios of 1:10 or 1:5 the rate of assembly in the presence of DS177 or DS151 was faster than the rate of assembly in control samples (Fig. 6 B). The same was true for DS177, DS151, and in addition for DS277 (molar ratios between 1:160 and 1:5) in the absence of Ca^{2+} , while identical concentrations of severin had no significant influence on the rate of actin assembly under these conditions (Fig. 6 C). Similar observations were made with the villin 44T fragment in the presence of Ca^{2+} (Janmey and Matsudaira, 1988), with the gelsolin-actin complex from human platelets (Kurth et al., 1983) and with Cap100 from *D. discoideum* (M. Schleicher, unpublished observations). High concentrations of DS111 had no influence on the rate of actin assembly, but high concentrations of DS117 (molar ratios of 1:10 and 1:5) slightly retarded the rate of assembly. This again argues for a weak capping activity which is still present in DS117 but not in DS111 (Fig. 6 B, see also Fig. 3, F and G).

Binding of G-actin

To identify G-actin-binding domains, the proteins were incubated with *D. discoideum* G-actin coupled to Affi-Gel 10 beads either in the presence or in the absence of free G-actin. After 30 min of incubation, the beads were pelleted and supernatants and pellets analyzed on SDS-PAGE as shown in Fig. 7 A. With severin, DS277, DS177, and DS151 nearly complete binding to the beads was found and binding was strongly reduced when a twofold molar excess of free G-actin was present as a specific competitor. In this type of assay the binding properties of DS117 and DS111 were ambiguous, probably due to a lower affinity for G-actin (data not shown). Therefore, fluorescence enhancement of pyrene-

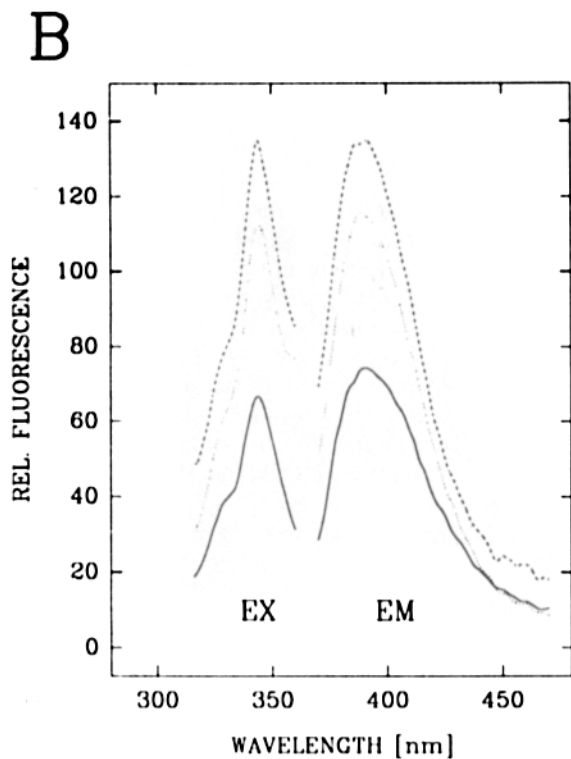
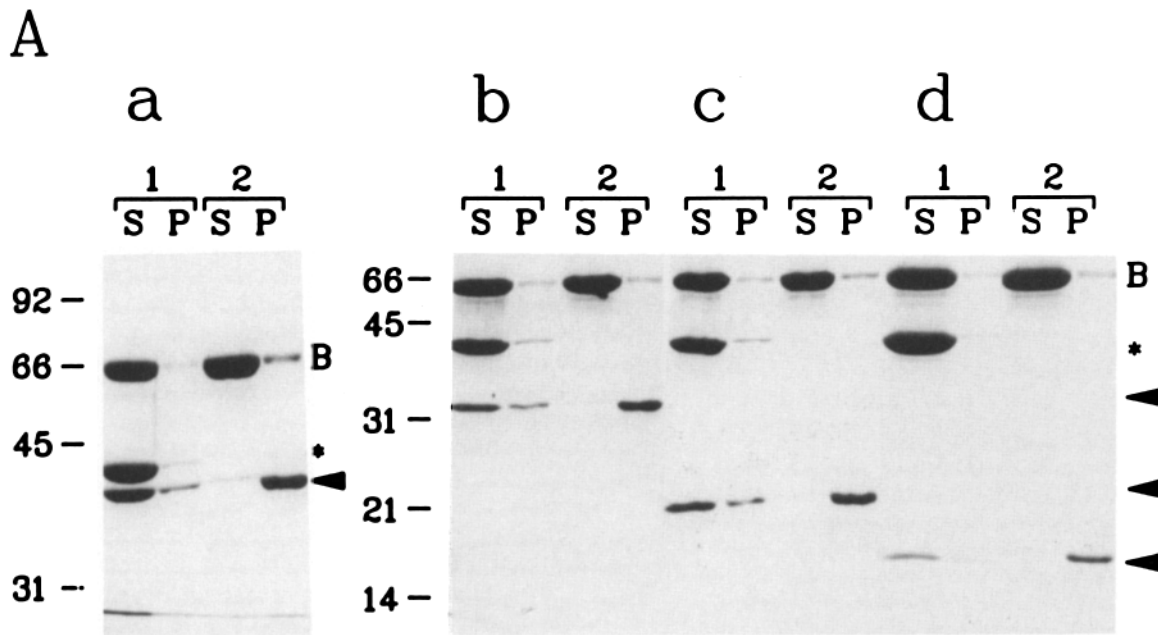


Figure 7. (A) Binding of severin and truncated severin molecules to G-actin coupled to Affi-Gel 10 beads. 2.5 μM severin (a), DS277 (b), DS177 (c), or DS151 (d) were preincubated with a twofold excess of BSA either with (lanes 1) or without (lanes 2) free G-actin. After incubation with G-actin beads and centrifugation, the proteins from the supernatants (S) and pellets (P) were subjected to SDS-PAGE (a, 10% acrylamide; b-d; 15% acrylamide). The positions of actin (*) and BSA (B) and of severin, DS277, DS177, and DS151 (arrowheads) are indicated at the right and the positions of the molecular mass markers at the left. (B) Fluorescence enhancement of pyrene-labeled G-actin (solid line) upon binding of DS117 (dashed line) or DS111 (dotted line) in the presence of 1 mM Ca^{2+} . The excitation scans (EX) or emission scans (EM) of either pyrene-labeled G-actin alone (160 nM) or of pyrene-labeled G-actin (160 nM) together with DS117 (2.4 μM) or with DS111 (2.4 μM) were recorded and corrected for either the buffer fluorescence or for the fluorescence of the buffer plus the proteins, DS117 and DS111 (2.4 μM). The excitation spectra were recorded at the emission wavelength of 386 nm and the emission spectra with excitation at 342 nm.

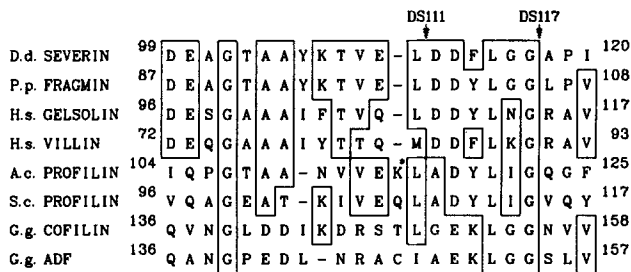


Figure 8. Sequence comparison of the putative actin-binding region of severin to different actin-binding proteins. The COOH-terminal ends of DS111 and DS117 are indicated. The asterisk in the sequence of *A. castellanii* profilin marks Lys¹¹⁵ which was chemically cross-linked to Glu³⁶⁴ of actin (Vandekerckhove et al., 1989). Four or more identical residues are boxed.

labeled G-actin upon binding of a ligand (Lee et al., 1988) was exploited to demonstrate binding of DS117 and DS111 to G-actin. Fig. 7 B shows that both truncated severin derivatives significantly increased the fluorescence of pyrene-labeled G-actin in excitation as well as emission scans.

Since DS117 displayed in addition to the G-actin-binding activity a weak capping activity (Fig. 3 F), we assumed that the region between amino acids 117 and 111 could be of some functional significance in the severin molecule. Sequence comparisons of this region with other actin-binding proteins revealed very high homology to fragmin from *Physarum polycephalum* (Ampe and Vandekerckhove, 1987), and to human gelsolin and villin (Kwiatkowski et al., 1986; Arpin et al., 1988). We also found substantial homology to profilin from *Acanthamoeba castellanii* and *Saccharomyces cerevisiae* (Ampe et al., 1985; Oechsner et al., 1987) and to a smaller degree to cofilin and actin depolymerizing factor from chicken (Abe et al., 1990) (Fig. 8). Interestingly Vandekerckhove et al. (1989) found in *A. castellanii* profilin that Lys¹¹⁵, which is located in this region, could be chemically crosslinked to Glu³⁶⁴ of actin.

Binding of Ca²⁺

Direct binding of Ca²⁺ to severin and truncated severin derivatives was assayed in ⁴⁵Ca²⁺ overlays and by fluorometry. ⁴⁵Ca²⁺-binding was most obvious in severin, DS362, and

DS277; almost no label was found with DS177 and DS151, while the label of DS117 and DS111 was clearly above background (data not shown). Taking into account that this type of assay might lead to erroneous results due to different affinities between proteins and nitrocellulose or disturbed conformations of the peptides, we chose also tryptophan fluorescence for testing direct Ca²⁺-binding in solution (Fig. 9). Tryptophan is highly fluorescent compared to the other aromatic amino acids and its fluorescence is very sensitive to a wide variety of environmental conditions (Schmid, 1989). Severin contains four, DS277 three, and DS177, DS151, DS117, and DS111 contain two tryptophan residues. In excitation and emission scans, a fluorescence enhancement of severin, DS277, DS117, and DS111 occurred in the presence of Ca²⁺ indicating binding of Ca²⁺ to these proteins. In DS177 and DS151 only very weak changes were observed. These data strongly indicate an NH₂-terminal Ca²⁺-binding site which is still present in DS111 and would correspond to the cryptic Ca²⁺-binding site found in gelsolin (Kwiatkowski et al., 1989). Considering the Ca²⁺-dependent activities and the changes in tryptophan fluorescence in longer severin derivatives beyond 151 amino acids, we cannot exclude a second or even a third Ca²⁺-binding site.

Discussion

In this paper we describe functional studies on bacterially expressed truncated severin polypeptides that have been purified to homogeneity. Table II summarizes the results of the functional assays. The activities of severin and of shortened polypeptides suggest a domain structure with distinct functional regions for nucleation, severing, capping of actin filaments, and for Ca²⁺ binding. Native severin from *D. discoideum* cells and DS362, the full-length polypeptide purified from *E. coli*, showed in all assays comparable activities, and their effects on polymerization or depolymerization kinetics of actin solutions were strictly Ca²⁺ dependent.

Actin-binding Regions in Severin

As severin is homologous to both halves of gelsolin (57% similarity to domains 1-3, 49% to domains 4-6), it is of interest to compare our results to data obtained with gelsolin derivatives. Gelsolin contains three actin-binding sites, two of which are located in the NH₂-terminal half, the third is

Table II. Summary of the Activities of Severin or Severin Derivatives

Protein	Severing		Capping		Nucleating		Monomer binding	Ca ²⁺ binding*	
	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	Trp	⁴⁵ Ca ²⁺
Severin	+	-	+	-	+	-	‡	++	++
DS362	+	-	+	-	+	-	ND	ND	+++
DS277	+	-	+	+	+	-	‡	++	++
DS177	(+)§	-	+	+	(-)	-	‡	+/-	+/-
DS151	-	-	+	+	-	-	‡	+/-	+/-
DS117	-	-	(+)§	(+)§	-	-	†	++	+
DS111	-	-	-	-	-	-	†	+++	++

* Ca²⁺ binding was investigated by monitoring tryptophan fluorescence (Trp) and with a ⁴⁵Ca²⁺-overlay assay (⁴⁵Ca²⁺).

‡ Binding to G-actin Sepharose.

§ Only at very high concentrations.

|| Residual nucleating activity might be obscured by strong capping activity.

† Fluorescence enhancement of pyrene-labeled G-actin. ND, not determined.

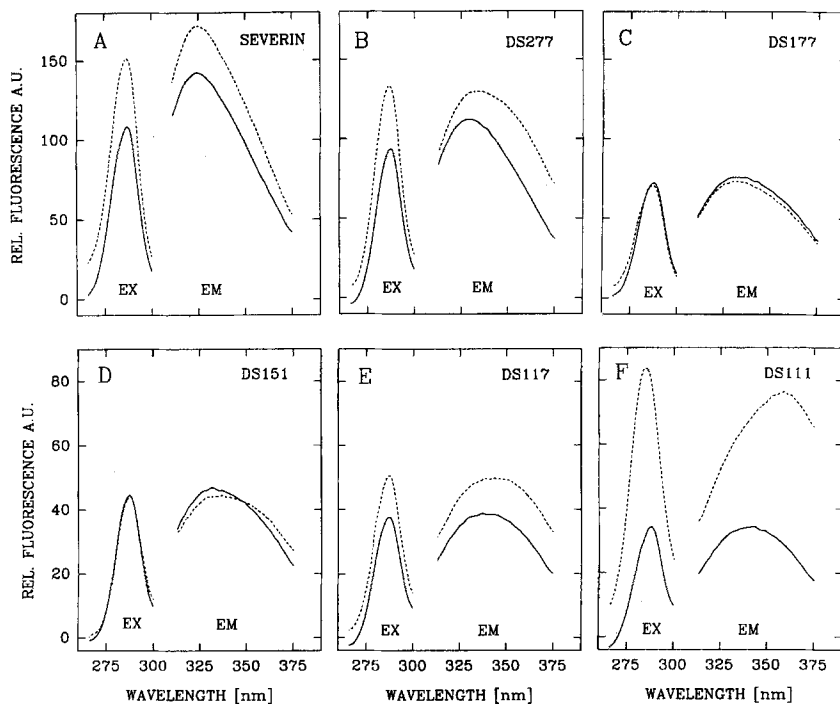


Figure 9. Tryptophan fluorescence of severin and truncated severin derivatives. Emission (EM) and excitation (EX) spectra of severin and severin derivatives ($2 \mu\text{M}$ each) were recorded in the absence (solid line) and the presence (1 mM) of free Ca^{2+} (dashed line) and corrected for buffer fluorescence. The excitation spectra were recorded at the emission wavelength of 350 nm and the emission spectra with excitation at 290 nm . Please note the different scales of the Y-axes in D–F. (A.U., arbitrary units).

located in the COOH-terminal half of the molecule (Bryan, 1988). Most likely the nucleation of actin assembly requires the cooperation of the second and third actin-binding sites, since the loss of one of these sites results in a drastically decreased nucleation activity. For the severing function of gelsolin the cooperation of the first and second actin-binding sites seems to be necessary (Way et al., 1989). In analogy to the results obtained with gelsolin, we argue that also in the severin molecule the severing as well as the nucleating activities require the cooperative interaction of two actin-binding domains.

The three domains (I–III) of severin roughly span the regions between amino acids 1–151, 152–262, and 263–362, respectively (Way and Weeds, 1988). Nearly complete removal of domain III does not change severing and nucleating activities, thus locating two actin-binding sites to domains I and II. After removing also domain II, the resulting protein (DS151) lost the ability to sever and to nucleate. This indicates the presence of an actin-binding site that most likely binds to the side of actin-filaments, in domain II. Because the extension of domain I by only 26 amino acids (DS177) restores residual severing activity, the presence of the second actin-binding site in the border region between domains I and II is highly suggestive. A more detailed location of the actin-binding site in domain I was possible by using sensitive fluorescence measurements on pyrene-labeled G-actin (Lee et al., 1988). The drastic increase of intrinsic fluorescence in excitation and emission spectra clearly showed that the shortest severin derivative (DS111) interacted with G-actin. Activity studies on DS111 were not as sensitive as fluorescence measurements. However, it required only six additional amino acids (DS117) for unequivocal detection of an F-actin capping activity. This region (amino acid 99 to 120 of severin) shows very high homology to different actin-binding proteins (Fig. 8).

Ca²⁺-binding Regions in Severin

For better insights into the location of potential Ca^{2+} -binding regions we addressed this question in three ways: functional assays, overlay experiments with $^{45}\text{Ca}^{2+}$, and changes of tryptophan fluorescence upon addition of Ca^{2+} . On the track of progressively truncated severins, the first change in Ca^{2+} regulation occurred after deletion of 85 COOH-terminal amino acids with the appearance of Ca^{2+} -independent capping activity. However, severing and nucleating activities always remained Ca^{2+} dependent. In $^{45}\text{Ca}^{2+}$ overlay experiments and in fluorescence measurements a biphasic pattern was observed (see also Table II). In DS151 and in DS177 the least Ca^{2+} -binding could be detected, whereas in the short peptides DS111 and DS117 as well as in the longer ones, DS277 and DS362 Ca^{2+} binding seemed evident. From these data we assume that (a) severin contains a Ca^{2+} -binding site in the NH_2 -terminal 111 amino acids; and (b) the appearance of a clearly Ca^{2+} -dependent severing activity in DS177 as compared to DS151 locates a putative second Ca^{2+} -binding site in or close to the actin-binding region of domain II. The observed Ca^{2+} -independent capping activity in the peptides DS277, DS177, DS151, and DS117 does not necessarily require a third Ca^{2+} -binding site and could be explained by the removal of 85 COOH-terminal amino acids as a regulatory domain (see also Fig. 10). To elucidate whether domains II or III also contain an active Ca^{2+} -binding site, work is in progress to express single domains separately and to test their Ca^{2+} -binding properties.

Nucleation vs. Pseudonucleation

Studies with genetically engineered actin-binding proteins offer the opportunity to gain a better knowledge about principal questions of actin polymerization. It is widely assumed that nucleation is favored if proteins like gelsolin or severin

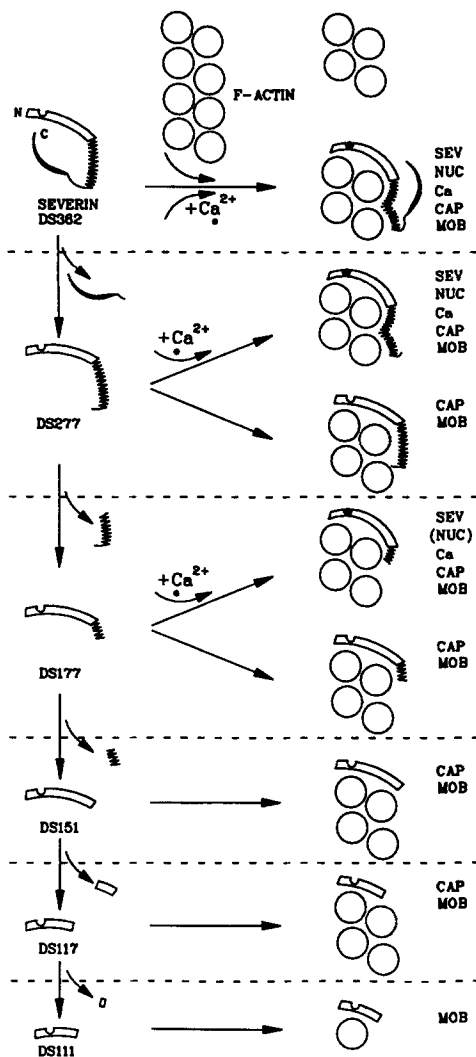


Figure 10. The functions of severin and truncated severin derivatives in a schematic overview. Five different functions have been studied: severing (SEV), nucleation (NUC), Ca^{2+} -binding (Ca), capping (CAP), and actin-monomer-binding (MOB). In the complete severin molecule (SEVERIN or DS362) all five activities are activated after binding of Ca^{2+} (first panel). The removal of 85 COOH-terminal amino acids led to a truncated molecule (DS277) that exposed independently of Ca^{2+} two actin-binding sites which were able to cap actin filaments. However, severing and nucleation could be achieved only after a putative conformational change upon binding of Ca^{2+} (second panel). Loss of another 100 amino acids (DS177) did not alter the Ca^{2+} -independent capping activity, but the Ca^{2+} -dependent severing activity was drastically reduced. Together with the residual severing activity, we postulate a residual nucleating activity that is masked by the strong capping activity and cannot be demonstrated in nucleation assays (third panel). DS151, comprising the first domain of native severin, has lost the residual severing activity of DS177. However, it was still able to cap actin filaments and bind actin monomers in the presence and absence of Ca^{2+} . This locates similar to gelsolin, the second actin-binding site to the border region between the first and second domains (fourth panel). Removal of additional 34 amino acids resulted in DS117 which displayed a weak capping and monomer-binding activity (fifth panel). DS111 missing six more amino acids still could bind G-actin and Ca^{2+} , which locates a G-actin and a Ca^{2+} -binding site to the first 111 amino acids of severin (sixth panel).

bind two actin monomers, generating a capped actin dimer that acts as a nucleus for elongation at the pointed end. DS151 turned out to be a Ca^{2+} -independent capping protein that contains only one actin-binding site but shows an unusual nucleation activity (see also Fig. 6 B). At low concentrations the onset of elongation is delayed as it is typical for capping proteins without nucleation activity (Ankenbauer et al., 1989). At higher concentrations an activity is observed that we would like to call pseudonucleation. We suggest that a capping protein with only one actin-binding site drastically increases at elevated concentrations the number of open pointed ends that are available for elongation. Viscosity in these samples is low because many and short filaments are generated. This pseudonucleation activity has also been observed in the *D. discoideum* capping proteins Cap32/34 and Cap100 (Schleicher, M., unpublished observations). It might be the basis of the unusual nucleation behavior of the macrophage capping protein (Young et al., 1990), the villin 44T-fragment (Janmey and Matsudaira, 1988), the *A. castellanii* capping protein (Cooper and Pollard, 1985), and the gelsolin-actin complex (Kurth et al., 1983).

Conclusions

On the basis of the domain model we draw the following conclusions: severin contains two actin-binding sites, one each in domains I and II, and at least one Ca^{2+} -binding site which is located in the 111 NH_2 -terminal amino acids. Compared to gelsolin, in severin only two actin-binding sites are responsible for capping, severing, and nucleation. A direct comparison of the nucleation activity of gelsolin and severin showed that gelsolin is a better nucleator than severin, but both proteins sever actin filaments equally well (Yin et al., 1990). Based on the sequences, the distances between the actin-binding sites which are responsible for nucleation are much larger in gelsolin than in severin. This suggests, (a) that in gelsolin as well as in severin the filament-fragmenting properties reside in two closely neighbored actin-binding sites; and (b) that the putative gene duplication during evolution led to an "improved" actin-binding protein whose enhanced nucleation activity might be caused by a more relaxed conformation during this reaction.

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