

Expression of Human Plasma Gelsolin in *Escherichia coli* and Dissection of Actin Binding Sites by Segmental Deletion Mutagenesis

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Abstract. Human plasma gelsolin has been expressed in high yield and soluble form in *Escherichia coli*. The protein has nucleating and severing activities identical to those of plasma gelsolin and is fully calcium sensitive in its interactions with monomeric actin. A number of deletion mutants have been expressed to explore the function of the three actin binding sites. Their design is based on the sixfold segmental repeat in the protein sequence. (These sites are located in segment 1, segments 2–3, and segments 4–6.) Two mutants, S1–3 and S4–6, are equivalent to the NH₂- and COOH-terminal halves of the molecule obtained by limited proteolysis. S1–3 binds two actin monomers in the presence or absence of calcium, it severs and caps filaments but does not nucleate polymerization. S4–6 binds a single actin monomer but only in calcium. These observations confirm and ex-

tend current knowledge on the properties of the two halves of gelsolin. Two novel constructs have also been studied that provide a different pairwise juxtaposition of the three sites. S2–6, which lacks the high affinity site of segment 1 (equivalent to the 14,000-*M_r* proteolytic fragment) and S1,4–6, which lacks segments 2–3 (the actin filament binding domain previously identified using the 28,000-*M_r* proteolytic fragment). S2–6 binds two actin monomers in calcium and nucleates polymerization; it associates laterally with filaments in the presence or absence of calcium and has a weak calcium-dependent fragmenting activity. S1,4–6 also binds two actin monomers in calcium and one in EGTA, has weak severing activity but does not nucleate polymerization. A model is presented for the involvement of the three binding sites in the various activities of gelsolin.

GELSOLIN is an 82,000-*M_r* calcium-dependent actin severing and capping protein found universally in vertebrate tissues (Yin et al., 1981; Kwiatkowski et al., 1988). A larger secreted form is readily isolated from blood plasma (Harris and Gooch, 1981). In addition to its severing activity, it accelerates actin polymerization by forming a stable nucleating complex with two actin monomers (Bryan and Kurth, 1984; Doi and Frieden, 1984; Weeds et al., 1986b).

Actin binding domains have been identified using limited proteolysis (Kwiatkowski et al., 1985; Bryan and Hwo, 1986). The protein is readily cleaved into two halves by a variety of proteases. The 45,000-*M_r* NH₂-terminal domain (CT45N using the nomenclature of Yin et al., 1988) severs actin filaments as effectively as gelsolin, is calcium insensitive, and has only weak nucleating activity (Bryan and Hwo, 1986; Chaponnier et al., 1986). The COOH-terminal domain of 38,000-*M_r* (CT38C) binds actin-Sepharose only in the presence of calcium, but neither severs nor nucleates (Kwiatkowski et al., 1985). Bryan (1988) has shown that this fragment binds a single actin monomer and inhibits actin polymerization at high molar ratios to actin, probably by sequestering monomers.

CT45N is further degraded to yield two smaller fragments, CT15N and CT28N, the former being NH₂-terminal (Chaponnier et al., 1986; Yin et al., 1988; Bryan, 1988). CT15N binds actin-Sepharose in a calcium-independent manner (Kwiatkowski et al., 1985) and forms a 1:1 high affinity complex with G-actin (Bryan, 1988). CT28N binds F-actin at a 1:1 stoichiometry with actin subunits (Yin et al., 1988; Bryan, 1988). Neither CT28N nor CT15N has severing activity.

These studies show that gelsolin is composed of stable structural domains connected by proteolytically sensitive regions. The cDNA derived amino acid sequence shows a strong tandem repeat, suggesting evolution by gene duplication (Kwiatkowski et al., 1986). Internal repeats have been reported for related actin severing proteins, fragmin (Ampe and Vanderkerckhove, 1987), severin (André et al., 1988), and villin (Bazari et al., 1988; Arpin et al., 1988). Our own analysis of all these proteins has revealed six large segmental repeats of ~15,000 *M_r* in gelsolin and villin and three similar repeats in severin and fragmin. The evidence suggests that this family of proteins has evolved from a precursor of ~130–150 amino acids (Way and Weeds, 1988). The boundaries of the six segments in gelsolin are consistent with the

fragmentation patterns reported earlier, but facile proteolytic cleavage appears to be restricted to sites between segments 1 and 2 and segments 3 and 4. The three actin binding domains are located in proteolytic fragments corresponding to segment 1, segments 2–3, and segments 4–6.

To understand the role of individual segments in the various actin binding activities of gelsolin, we have constructed and expressed in *Escherichia coli* a number of deletion mutants as well as whole gelsolin (Fig. 1). In the absence of an x-ray crystal structure for gelsolin or more importantly gelsolin-actin complexes, the mutants have been designed based on the chymotryptic digestion pattern of human plasma gelsolin (Chaponnier et al., 1986) and the sixfold segmental repeat in gelsolin (Way and Weeds, 1988). Our nomenclature for the mutants is based on their segmental content. Thus mutants S1–3 and S4–6, corresponding to CT45N and CT38C, have been made essentially as controls. Two mutants are described that cannot be made by proteolysis: S2–6 in which segment 1 (CT15N) is deleted and S1,4–6 which lacks segments 2 and 3 (CT28N). Both S2–6 and S1,4–6 have two actin binding sites but they behave very differently in severing and nucleation assays. Their properties provide new information about the roles of individual actin binding sites.

Materials and Methods

Construction of the Gelsolin Expression System

T4 DNA ligase, polynucleotide kinase, all restriction enzymes, and the non-phosphorylated Hind III linker (CAAGCTTG) were obtained from New England Biolabs (Beverly, MA), Klenow DNA polymerase from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). Methods for preparation and analysis of recombinant DNA and culturing of bacteria were those described by Maniatis et al. (1982). All DNA sequences were determined by a series of oligonucleotides evenly spaced along the sequence using the dideoxy chain termination method (Sanger et al., 1977).

Two overlapping but incomplete cDNA clones, MID and pU43a, which

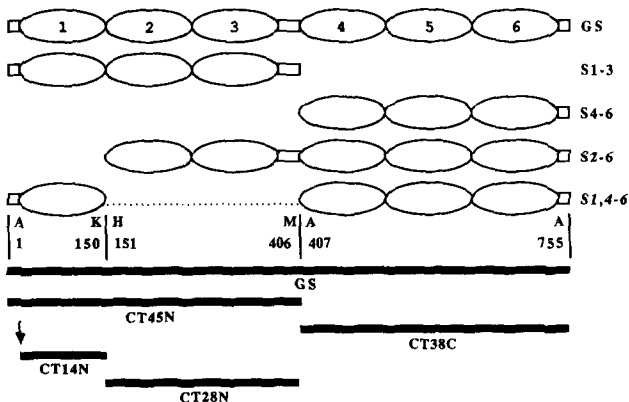


Figure 1. Schematic representation of the sixfold repeating sequence of gelsolin and the design of the deletion mutants. The NH₂- and COOH-terminal residues are indicated below in single letter code together with their positions in the amino acid sequence of human plasma gelsolin (Kwiatkowski et al., 1986). At the bottom of the figure the positions and notation of proteolytic fragments have been given for comparison. The vertical arrow indicates the position of Ser 24, the NH₂-terminal residue of CT14N (Yin et al., 1988).

together cover the entire coding region of human plasma gelsolin, were a kind gift from Drs. Kwiatkowski and Yin (Massachusetts General Hospital, Boston, MA; Kwiatkowski et al., 1986).

Nonphosphorylated Hind III linker (CAAGCTTG) was kinased and ligated into a unique Stu I site 64 bases 3' to the termination triplet of pU43a. Digestion with Bam HI and Hind III yielded a fragment containing the entire coding region of this clone. This fragment was ligated into the Bam HI and Hind III sites of the expression vector pLcII (Nagai and Thøgersen, 1984) and transformed into JM101. One clone pLcII/pU43a was picked ready to accept the required coding region from MID.

A 907-bp Eco RI-Bal I fragment from the 5' end of MID, containing the coding region absent in pU43a as well as a 613-bp overlap, was isolated and cloned into Eco RI-Hinc II-cleaved M13mp19. Several clones containing the insert were selected to produce single stranded DNA according to Carter et al. (1985). An oligonucleotide BamFXMID. (TCGCTGCCCGTCCGCGCGGGATCCATCGAGGGTAGGGCCACTGCGGTGCGGGGGGCGT) coding for Bam HI and Factor Xa recognition sites (underlined) was used to juxtapose the last codon of the Factor Xa sequence (Arg) and the first codon (Ala) of the mature human plasma gelsolin sequence. BamFXMID was kinased, annealed to the single stranded template, extended with Klenow DNA polymerase, and ligated according to Carter et al. (1985). The ligation mixture was transfected into calcium competent BMH 71-18 mutL cells and positive clones identified by hybridization with the mutagenic oligonucleotide. These were plaque purified and sequenced along their entire length. Using the engineered Bam HI site and a unique Not I site at position 394 in the 907 Eco RI-Bal I insert, a 294-bp fragment was isolated and ligated into pLcII/pU43a. The ligation mixture was transfected into QY13 and clones selected on ampicillin plates before restriction mapping and induction of the expression construct pLcIIFXGS (Fig. 2).

Construction of Mutants

A Bam HI and Hind III digest of pLcIIFXGS was used to obtain the entire coding sequence of human plasma gelsolin. This fragment was subcloned into M13mp19 and used to provide single stranded DNA for all subsequent mutagenesis. Mutagenesis was performed as described in the engineering of the Bam HI-Factor Xa recognition site in the 907-bp fragment from MID. A schematic representation of each gelsolin mutant along with positions changed is shown in Fig. 1. All mutant constructs were sequenced throughout their length before being excised from M13mp19 and engineered back into pLcII. Mutants with the correct construct were selected by restriction mapping before expression in QY13.

Induction, Expression, and Purification of Mutants

Cultures of QY13 containing the vector pLcIIFXGS or mutated constructs were grown and induced as described in Nagai and Thøgersen (1987). Post-heat shock flasks were grown at 37°C for 3 h before centrifugation of cells at 4°C (accelerating up to 10,000 g and immediately turning off the centrifuge). Pellets were frozen at -20°C before resuspension at room temperature in 50 mM Tris HCl pH 8.0, 25% sucrose, and 1 mM EDTA (20 ml/liter original culture). 5 mg lysozyme (10 mg/ml in distilled water) was added to 20 ml suspension to lyse the cells and digestion proceeded for 30 min at room temperature. The suspension was clarified by centrifugation at 150,000 g for 20 min and the supernatant dialyzed overnight at 4°C against 10 mM Tris HCl pH 8.0, 100 mM NaCl, 0.1 mM CaCl₂, and 3 mM NaN₃. fxgelsolin was purified by actin-Sepharose affinity chromatography (Weeds et al., 1986a). The fusion protein was subsequently digested with Factor Xa (kindly provided by Dr. K. Nagai [Medical Research Council Laboratory of Molecular Biology]) in 25 mM Tris HCl pH 8.0, 100 mM NaCl, 0.1 mM CaCl₂, and 1 mM NaN₃ at a substrate to enzyme ratio of 50:1 (wt/wt) for 5 h at room temperature or 100:1 (wt/wt) overnight at 4°C to yield native protein.

Unlike fxgelsolin, not all mutants were soluble but were found in the inclusion body fraction. Inclusion body preparations were purified as described in Nagai and Thøgersen (1987) and solubilized in 8 M urea. The urea was removed by dialysis against 10 mM Tris HCl pH 8.0, 100 mM NaCl, 0.1 mM CaCl₂, and 3 mM NaN₃. The dialysate was clarified by further centrifugation at 2,500 g for 10 min followed by filtration through a 0.2 μm-filter Millipore Continental Water Systems (Bedford, MA). Further purification of mutants was performed by actin-Sepharose chromatography (Weeds et al., 1986a). Calcium dependence was examined using EGTA-containing buffers. Mutant concentrations were calculated from the tyrosine and tryptophan content (Bryan, 1988) or using the Folin reaction as described previously (Weeds et al., 1986a).

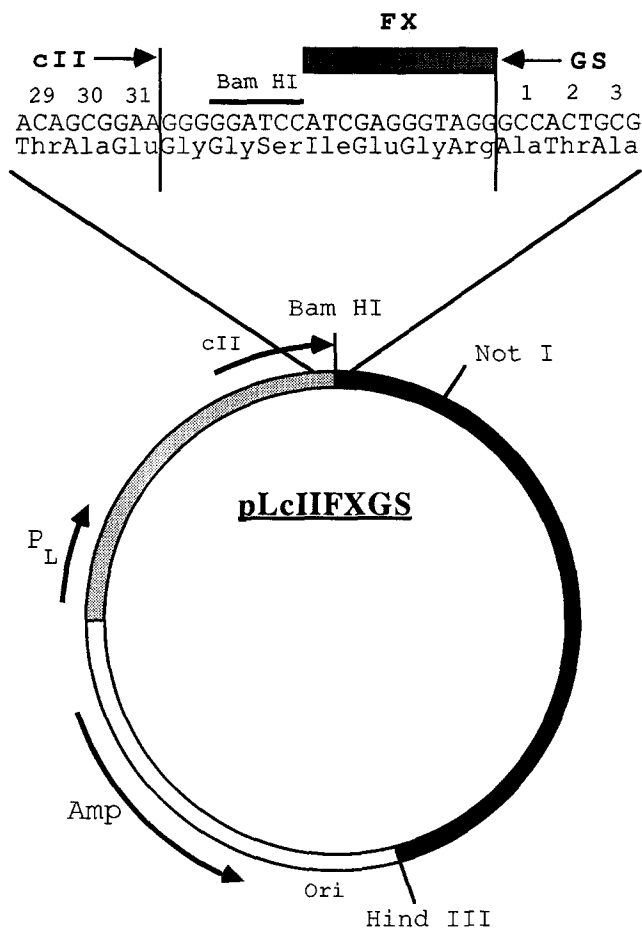


Figure 2. Organization of the cleavable fusion protein expression vector, pLcIIFXGS. The plasmid directs synthesis of a hybrid protein consisting of the first 31 amino-terminal residues of lambda cII protein, the Factor Xa recognition sequence (FX), and human plasma gelsolin. PL, Lambda promoter; Amp, ampicillin-resistance gene; and Ori, the origin of replication of the vector.

Assay Methods

All assays have been performed on mutant proteins purified on actin-Sepharose with the cII leader peptide present. fxgelsolin is as active as pig plasma gelsolin both with and without the cII leader peptide. There is no reason to believe that the fusion peptide is detrimental to the activity of gelsolin.

The DNase I inhibition assay (Harris et al., 1982) was used to compare fxgelsolin with pig plasma gelsolin and to analyze the mutants.

Preparation of Fluorescently Labeled Actin and Monomer Binding Assays

Titration with NBD-Actin and PI-Actin. Preparation of PI-actin¹ reacted on Cys 374 with *N*-(1-pyrenyl)iodoacetamide and NB-actin reacted with *N*-ethylmaleimide on Cys 374 then on Lys 373 with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole were as described (Weeds et al., 1986b). Titrations with NBD-actin were carried out using a constant total protein concentration (200 or 400 nM) with continuous variation of both gelsolin and NBD-actin in G' buffer (10 mM Tris HCl pH 8.0, 0.2 mM ATP and dithiothreitol, 0.1

1. **Abbreviations used in this paper:** NBD-actin, actin reacted with *N*-ethylmaleimide on Cys 374 then on Lys 373 with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PI-actin, actin reacted on Cys 374 with *N*-(1-pyrenyl)iodoacetamide.

mM CaCl₂, and 1 mM NaN₃) as described previously (Weeds et al., 1986b). BSA (0.1 mg/ml) was added to all samples to minimize losses due to adsorption at surfaces. Samples were incubated at 4°C overnight in the dark before measuring the fluorescence at 21°C. Similar experiments were also carried out under polymerizing conditions (G' buffer + 2 mM MgCl₂ and 0.1 M KCl, with either 0.1 mM CaCl₂ or 1 mM EGTA). For experiments in the absence of calcium, both G-actin and mutant were preincubated at 2 μM in 0.25 mM MgCl₂, 1 mM EGTA before mixing.

Titration were also carried out with 1 μM PI-actin and various concentrations of SI-3.

Nucleation Assay. 4 μM PI G-actin in G' buffer (containing varying concentrations of fxgelsolin in the range 10–120 nM) was polymerized by addition of 1:20 vol of 40 mM MgCl₂ and 2 M KCl. The fluorescence increased exponentially with time: a semi-Ln plot of the approach to equilibrium gives a straight line of slope $k^+ [N]$ where k^+ is the association rate constant at the pointed end of filaments and $[N]$ the concentration of gelsolin. (Under the ionic conditions used, $[nuclei] = [gelsolin]$ [Janmey and Stossel, 1986; Selve and Wegner, 1986].) The nucleating efficiency of mutants is calculated relative to that of gelsolin.

Inhibition of Polymerization. 2.8 μM PI-actin was polymerized in the presence of 30 nM gelsolin to give nucleated filament assembly. The concentration of polymerized actin was determined from the final fluorescence and measurement of the critical concentration (method in Harris and Weeds, 1983). The effects of increasing mutant concentration on the maximum fluorescence enhancement were monitored and the concentration of actin bound to mutant was determined from the percent inhibition of fluorescence enhancement compared with controls.

Airfuge Assay. Assays carried out as described previously, using 18 μM actin and different concentrations of mutants (Pope and Weeds, 1986). Samples of supernatant and pellets resuspended to the original volume were analyzed by SDS-PAGE and densitometry was performed using a densitometer (Comag Electrophoresis Scanner; Cambridge Instruments Ltd., Cambridge, UK).

Viscometry. A capillary flow viscometer was used at 22°C with 14.9 μM F-actin (Pope and Weeds, 1986). Flow times were 91.5 ± 0.5 s for buffer and 138 ± 1 s for 14.9 μM F-actin. Measurements were made initially in 0.2 mM EGTA after addition of fxgelsolin or mutant. The same samples were then assayed in 1.6 mM excess calcium. Results are expressed as specific viscosity. Additional assays were performed for S2-6 and SI,4-6 using "capped" filaments with an average length of ~400 monomers prepared by polymerizing 54 μM G-actin in the presence of 13.6 nM fxgelsolin.

Severing Assay. The severing assay was adapted from that originally described for villin by Walsh et al. (1984) and used for gelsolin by Bryan and Coluccio (1985). Precapped filaments were used throughout to avoid problems due to variation in the extent of capping and eliminate monomer dissociation from free barbed ends.

25 μM G-actin in G' buffer (containing 4.2 μM PI-actin) was polymerized in the presence of 0.125 or 0.25 nM gelsolin by addition of 5 mM MgCl₂ and 100 mM NaCl to produce precapped filaments with an average length of ~200 or 100 monomers, respectively. (The length distribution is initially Poisson [Oosawa and Asakura, 1975].) Polymerization is complete in <2 min. The filaments were diluted to 200 nM in 10 mM Tris HCl pH 8.0, 50 mM NaCl, 3 mM MgCl₂, 0.2 mM ATP, 0.2 mM dithiothreitol, and 0.1 mM CaCl₂ (conditions used by Bryan and Coluccio, 1985) or G' buffer, using a cut Gilson tip to prevent shearing, and mixed by inverting the cuvette three times. Under these conditions, below the critical concentration, capped filaments depolymerize at a constant rate from their "pointed" ends, giving a linear fluorescence change over at least 10 min of the assay (the maximum time used, to minimize photobleaching). The rate of actin monomer dissociation is obtained by dividing this initial rate by the total fluorescence change for depolymerization and the off-rate (k^-) estimated on the assumption that the filament concentration equals the gelsolin concentration (Janmey and Stossel, 1986; Selve and Wegner, 1986).

The severing capacity of fxgelsolin or mutant was measured from the fluorescence kinetics after addition of mutant (made ~30 s after dilution of the capped filaments to provide a control of the initial fluorescence of each diluted actin sample). Since filaments are cut at random, the resulting length distribution is exponential. Thus depolymerization follows exponential kinetics according to the equation: $A(t) = A^0 e^{-k_{obs} t}$, where $k_{obs} = G/A^0 k^-$, $A(t)$ is the concentration of F-actin at time t , $A^0 = 0.2 \mu M$ F-actin, G the gelsolin concentration, and k^- the off-rate for monomers from the pointed end of filaments. A semi-Ln plot of $[F(t) - F(\infty)]$ gives a straight line with slope = k_{obs} , where $F(\infty)$ = fluorescence at the end of the reaction and $F(t)$ = that at time t . On the assumption that every gelsolin mole-

cule severs and caps an actin filament, a plot of k_{obs} as a function of gelsolin concentration gives a slope = k_-/A^2 . The value of k_- should equal that obtained in the control depolymerization assays using capped filaments. A lower value indicates less efficient severing. If the extent of severing was small, the severing activity was determined by comparing the initial slope of the fluorescence decrease in the presence of mutant with controls.

Electron Microscopy. F-actin (2.4 μ M) was incubated for various times with mutants at concentrations up to 2.4 μ M in 2 mM TrisHCl pH 7.5, 0.5 mM MgCl₂, 0.1 M KCl, 0.5 mM ATP, and either 0.2 mM CaCl₂ or 1 mM EGTA. Samples were prepared for microscopy as described by Valentine et al. (1968).

Calcium Binding. Experiments were done using a rapid calcium binding assay developed by Koch et al., 1986. Three 9-cm filter circles (Nos. 54 or 541; Whatman Inc., Clifton, NJ) were washed in a Buchner funnel with 10 mM imidazole pH 7.0, 0.1 M NaCl, and 1.0 mM MgCl₂ (washing buffer). All buffers were made up in BDH Analar water (<1.25 μ M Ca²⁺) and stored in plastic to avoid contaminating calcium from glassware. A grid-*ded* nitrocellulose filter (BA 85/20 Schleicher & Schull, Inc., Keene, NH) was placed on top of the filter circles and rinsed with washing buffer. The presence of the three filter circles ensures that the vacuum is sufficient but not excessive. 50–200 pmol of protein in washing buffer were spotted on to the nitrocellulose with the system under vacuum. After applying protein, the nitrocellulose filter was overlaid in a rocking petri dish for 15 min with 5–10 ml of washing buffer containing 1–2 mCi/ml ⁴⁵Ca²⁺ and 0–40 μ M carrier ⁴⁰Ca²⁺. Surplus calcium buffer was removed from the filter by vacuum. The nitrocellulose was washed in 50 ml washing buffer for 20 s in a petri dish on a rotary shaker and dried again. Although bound calcium can exchange during the rinsing stage, this procedure gave reproducible background counts (~7,000 counts/min) without apparent loss of protein-bound calcium. After drying, the filter was cut into squares and radioactivity measured in a scintillation counter. Negative controls (cytochrome c) and buffer blanks were included on each filter (these gave similar counts). Assays were carried out at different protein concentrations and free calcium concentrations between 0.1 and 40 μ M, but mostly at 10 or 40 μ M. The free calcium concentration was based on Harafuji and Ogawa (1980).

Calcium binding was also measured by equilibrium dialysis as described previously (Weeds et al., 1986a), using 0.3-ml samples containing ~10 μ M protein and dialyzing against 50 ml calcium buffer containing 5–40 μ M total calcium (0.131–20.3 μ M free calcium). Protein concentrations were measured after dialysis by absorbance at 280 nm (and calibrated using Bradford and Folin assays). Binding data were fitted using a nonlinear least squares fit (Press et al., 1988) to the equation $B = n[Ca]/(K_d + [Ca])$, where B is the bound calcium per mol mutant, $[Ca]$ is the free calcium concentration, n the maximum number of binding sites, and K_d the dissociation constant.

Results

Expression and Purification of Gelsolin and Mutants

There were no obvious bands corresponding to gelsolin (or mutants) before induction (Fig. 3), but samples of postinduction cell protein revealed a band that comigrated with plasma gelsolin. Western blots showed that only this band cross-reacted with an affinity-purified polyclonal antibody against plasma gelsolin (data not shown). f_xgelsolin, synthesized at 10–15% of total cell protein, was found in the soluble fraction after cell lysis. It was purified on actin-Sepharose (Fig. 3) (Weeds et al., 1986a). NH₂-terminal sequence analysis (by the method of Matsudaira [1987]) of f_xgelsolin digested with factor Xa, confirmed that the first 12 amino acids were identical to those of human plasma gelsolin (Kwiatkowski et al., 1986).

Expression levels of mutants varied both between mutants and with different preparations (Fig. 4). However, the overall levels of expression were generally as good as that observed for f_xgelsolin (compare Fig. 3 and Fig. 4). Unlike f_xgelsolin, the mutants were found mainly in inclusion bodies, although S4–6 and S1,4–6 were also present in significant concentrations in the soluble fraction. These two mutants have been purified from both soluble and insoluble fractions and shown

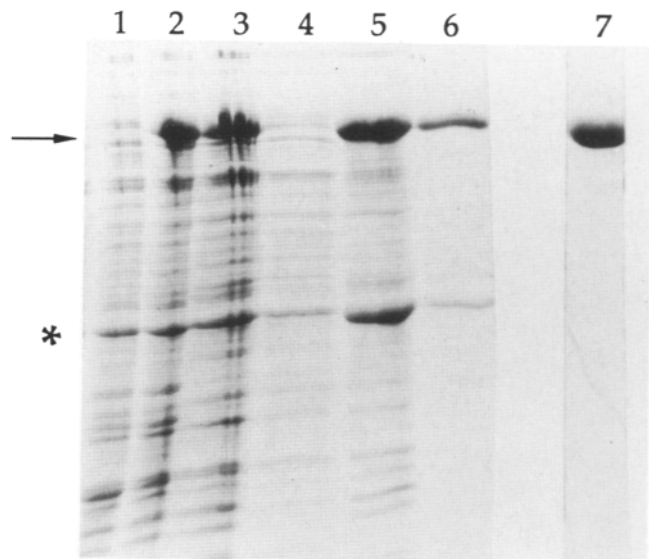


Figure 3. Expression and purification of f_xgelsolin. Lanes 1–7, SDS-PAGE. Lane 1, preinduction total cell protein; 2, 3-h postinduction total cell protein; 3, soluble fraction; 4, protein not bound to actin-Sepharose; 5, actin-Sepharose-bound protein; 6, actin-Sepharose bound after washing with 1 M MgCl₂; 7, purified f_xgelsolin eluted with 4 M MgCl₂. The arrow indicates the position of f_xgelsolin and the asterisk indicates actin (also the position of a protein in *E. coli* that comigrates with actin).

to behave identically. S2–6 was also present in very small amounts in the soluble fraction (Fig. 4). Inclusion bodies were readily solubilized in 8 M urea. Removal of urea by dialysis precipitated much of the contaminating protein, leaving relatively pure mutant in the supernatant (Fig. 4).

Activities of f_xgelsolin and Mutants

Actin-Sepharose Binding. f_xgelsolin and all deletion mutants bound to actin-Sepharose in 20 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 0.2 mM ATP, 2 mM NaN₃, and 0.1 mM CaCl₂. S2–6 and S4–6 were eluted in the same buffer containing 0.5 M NaCl or in 1 M MgCl₂ (a step included in the purification of plasma gelsolin to remove immunoglobulins), but f_xgelsolin, S1–3, and S1,4–6 required 4 M MgCl₂ for elution.

The calcium dependence of binding to actin-Sepharose was also analyzed by loading columns in 0.1 mM CaCl₂ and washing with the same buffer plus 1 mM EGTA. S2–6 was completely eluted while S1,4–6 remained fully bound (Fig. 5). S4–6 behaved like S2–6 while S1–3 and f_xgelsolin were like S1,4–6 (data not shown). When the mutants were applied to actin-Sepharose in the presence of 1 mM EGTA, S1,4–6 bound but S2–6 did not (Fig. 5).

Binding to Monomeric Actin

NBD-Actin Titrations. The continuous variation method is the best means of determining the stoichiometry of complexes in NBD-actin titrations, provided the affinity is high and there is significant fluorescence enhancement on complex formation (Weeds et al., 1986b). Experiments using f_xgelsolin showed maximum fluorescence enhancement in calcium at two actin monomers per gelsolin (Fig. 6A). Simi-

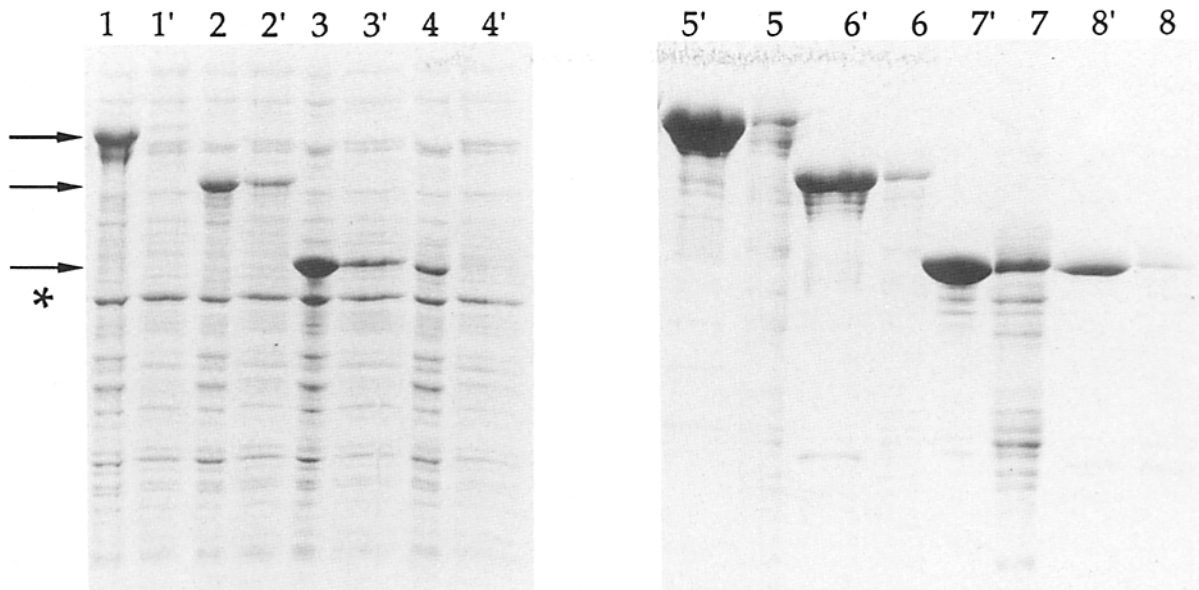


Figure 4. Expression and distribution of gelsolin mutants 3-h postinduction in *E. coli*. Lanes 1, 1', 5', and 5, S2-6 (I, total cell protein; I' soluble fraction, 5', solubilized after removal of 8 M urea; 5, insoluble after removal of urea). Lanes 2, 2', 6', and 6, S1,4-6 (lanes as described for S2-6 above). Lanes 3, 3', 7', and 7, S4-6 (lanes as above). Lanes 4, 4', 8', and 8, S1-3 (lanes as above). The arrows mark the positions of S2-6, S1,4-6, and S4-6 or S1-3, while the asterisk shows the *E. coli* protein that comigrates with actin (the intensity of this band shows that gel loadings are similar for all mutants).

lar experiments in the absence of calcium showed no fluorescence enhancement. Comparable experiments with the mutants are also shown in Fig. 6. The inflexion point in Fig. 6 B indicated a binding stoichiometry of 1.8 actin per S1-3 both in calcium and EGTA, but curvature of the graph makes the accuracy less precise. S4-6 gave no fluorescence enhancement in calcium or EGTA (Fig. 6 D). S2-6 gave a profile similar to that of fxgelsolin, with maximum enhancement on binding two actin monomers in calcium (Fig. 6 D) and no enhancement in EGTA (data not shown). S1,4-6 gave a similar fluorescence enhancement plus or minus calcium and a stoichiometry close to 1:1 may be extrapolated from Fig. 6 C. The maximum enhancement with this mutant in calcium was much less than that for all other mutants. A lower quantum yield associated with segment 1 was also inferred from

titrations of S1,4-6 with NBD-actin using either fixed actin concentrations and variable S1,4-6 or vice versa (the mean value for maximum enhancement in eight different experiments under the three assay conditions was $51 \pm 4.5\%$).

Experiments carried out with S1-3, S4-6, and S1,4-6 under polymerizing conditions but below the critical concentration showed no differences in either the extent of fluorescence enhancement or inflexion points of the titrations (data not shown).

The binding stoichiometry of S1-3 was further assessed from the fluorescence increase on mixing this mutant with $1 \mu\text{M}$ PI-actin in G'-buffer (an effect not observed with S4-6 or S1,4-6). Maximum enhancement occurred at ~ 1.8 actin/S1-3. The binding studies are summarized in Table I.

Inhibition of Polymerization

Monomer binding by mutants was investigated by inhibition of actin polymerization as shown in Fig. 7. Increasing concentrations of S4-6, S1-3, or S1,4-6 reduced the maximum fluorescence enhancement, but did not affect the rate constant for gelsolin-nucleated polymerization. By contrast, S2-6 increased the rate of polymerization (see below). The concentration of mutant-bound actin was calculated from the percent inhibition assuming a maximum concentration of monomeric actin available for polymerization equals $1.9 \mu\text{M}$ (based on a critical concentration of $0.9 \mu\text{M}$). Binding is very tight (Fig. 7). The binding stoichiometry was determined from the linear slope at low mutant concentrations, giving 1.0 for S4-6, 2.12 for S1-3, and 1.81 for S1,4-6. A Scatchard plot of the results shown in Fig. 7 for S4-6 suggests a dissociation constant of ~ 25 nM.

Nucleation

When $4 \mu\text{M}$ actin was polymerized in the presence of fxgel-

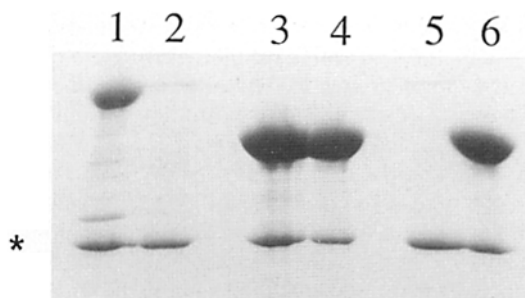


Figure 5. Binding to actin-Sepharose of S2-6 (lanes 1, 2, and 5) and S1,4-6 (lanes 3, 4, and 6). Lanes 1 and 3 show bound samples after loading in calcium; lanes 2 and 4 show material remaining bound after elution with 10 column volumes of buffer containing 1 mM EGTA; lanes 5 and 6 show bound mutant after loading column in EGTA. Asterisk indicates the position of actin.

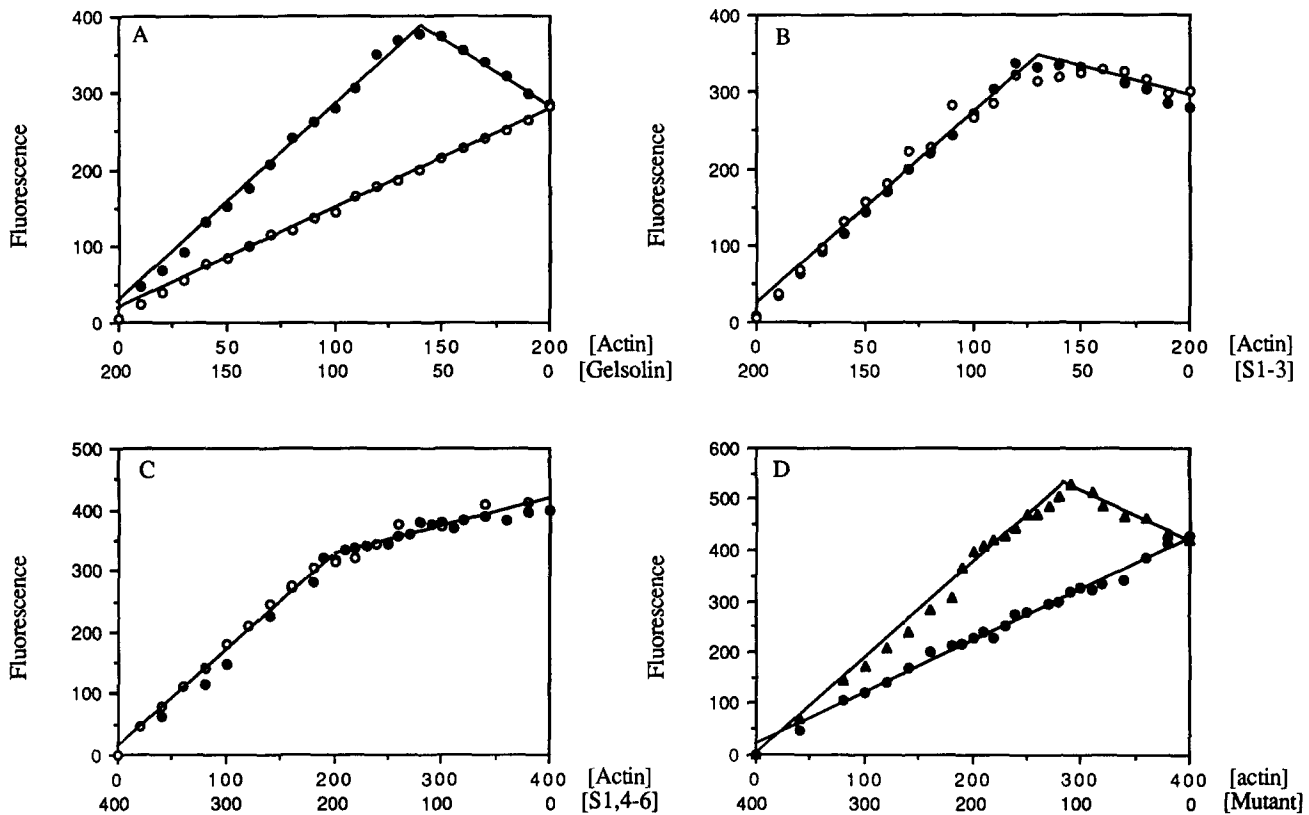


Figure 6. Fluorescence titrations at constant total protein concentration (in nanomolar) with continuous variation of both NBD-actin and gelsolin (or mutant). In all cases, solid symbols represent analyses in the presence of 0.1 mM calcium, while open circles show experiments in the absence of calcium. (A) fxgelsolin; (B) S1-3; (C) S1,4-6; (D) triangles represent S2-6 and circles S4-6. Data for S2-6 and S4-6 in the absence of calcium are not shown: they were identical to those of fxgelsolin in EGTA or S4-6 in calcium.

solin, the fluorescence intensity increased exponentially with time after a lag of 20 s or less and reached a maximum within 3–10 min depending on gelsolin concentration. In the absence of gelsolin the fluorescence rise was <2% of the total change on polymerization in the first 2 min and <15% in the first 10 min, showing that spontaneous nucleation was very slow. The rate constant obtained from semi-Ln plots increased linearly with gelsolin concentration (10–100 nM), giving a value of k^+ for monomer addition at the pointed ends of filaments of $0.179/\mu\text{M}/\text{s}$ (Fig. 8). S2-6 gave a similar value for $k^+ = 0.165/\mu\text{M}/\text{s}$. By contrast, S4-6, S1-3, and S1,4-6 at concentrations up to 120 nM did not increase the rate of fluorescence rise above controls.

Table 1. Binding of Monomeric Actin to fxgelsolin and Mutants

Activity	fxgelsolin	S1-3	S4-6	S2-6	S1,4-6
Actin-Sepharose calcium	+	+	+	+	+
Actin-Sepharose EGTA	–	+	–	–	+
NBD-actin calcium	2	2	0	2	1
NBD-actin EGTA	0	2	0	0	1
Inhibition of polymerization	ND	2	1	ND	2
Nucleation	+	–	–	+	–
Total sites calcium	2	2	1*	2	2*
Total sites EGTA	0	2	0	0	1

Fluorescently "silent" sites.

Sedimentation Assay

The effects of mutants on F- and G-actin were studied using cosedimentation assays at various mutant:actin ratios (e.g., 1:2 in Fig. 9). fxgelsolin, S1,4-6, S1-3, and S4-6 were all found predominantly in the supernatant both in calcium and EGTA. By contrast, over 80% of S2-6 was in the pellet when sedimentation was carried out in calcium within a few minutes of mixing, but after longer incubation (30 min), increasing amounts of this mutant were also found in the supernatant. In EGTA S2-6 was always predominantly in the pellet.

The actin distribution between supernatant and pellet was measured by gel densitometry. Actin increased in the supernatant with all mutants except S2-6 (Fig. 9). Incubation of S4-6 with F-actin for <30 min in calcium gave a ratio of actin/S4-6 = 0.4 this increased to >0.7 after 4-h incubation, showing the effects of monomer sequestration on filament depolymerization. In EGTA there was no increase in supernatant actin concentration. Incubations of S1,4-6 with F-actin for 30 min or 4 h gave a ratio of actin/S1,4-6 = 1.8 in calcium and ~0.7 in EGTA, consistent with the binding stoichiometries obtained by other methods. Experiments with S2-6 showed an increasing proportion of the actin in the supernatant in calcium for incubation times >30 min; no such increase occurred in EGTA.

Similar sedimentation experiments using G-actin under polymerizing conditions showed: (a) the ratio in the supernatant using S4-6 was 1.1 in calcium, but in EGTA all the actin polymerized; (b) with S1,4-6, the actin/mutant ratio in

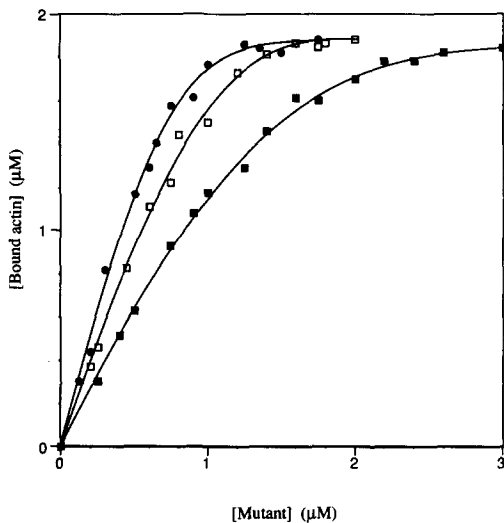


Figure 7. Bound actin, calculated from inhibition of polymerization of 2.8 μM PI-actin (containing 1.9 μM polymerizable actin) by S1,4-6 (open squares), S1-3 (solid circles), and S4-6 (solid squares). Values for stoichiometry obtained from linear portion of slopes (with concentration range of mutant used in parenthesis): S4-6 = 1.00 (0-1.4 μM); S1-3 = 2.12 (0-0.75 μM); S1,4-6 = 1.81 (0-0.8 μM).

the supernatant was 1.8 in calcium and 1.1 in EGTA; (c) when S2-6 was mixed with G-actin at low concentrations under polymerizing conditions, most of the actin and mutant were in the pellet.

DNase Inhibition Assay

The DNase inhibition assay showed that fxgelsolin is active in the cell supernatant. After purification on actin-Sepharose fxgelsolin has an activity of 27-29 U/nmol similar to that of plasma gelsolin (Weeds et al., 1986b). This activity is unchanged after digestion of the fusion protein with Factor Xa. S1-3 gave a similar activity, S1,4-6 an intermediate value of

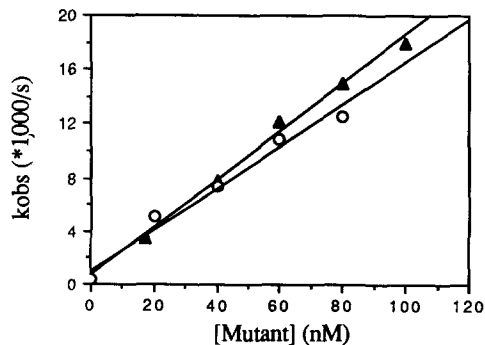


Figure 8. Effect of mutant concentration on rate constant for polymerization of 4 μM PI-actin in the presence of fxgelsolin (solid triangles) and S2-6 (open circles). Slope for fxgelsolin = 0.179/ $\mu\text{M}/\text{s}$ and S2-6 = 0.165/ $\mu\text{M}/\text{s}$.

14-18 U/nmol, while S4-6 and S2-6 showed no activity in this assay.

Viscometry

The effects of fxgelsolin on the specific viscosity of F-actin in calcium and EGTA are shown in Fig. 10 A with comparative results for plasma gelsolin. The viscosity decreases steeply in calcium and reaches a minimum of fxgelsolin/actin ratios of $\sim 1:30$. Assays using several different preparations, with or without the fusion peptide, gave similar profiles to Fig. 10 A. fxgelsolin has no significant effect on the viscosity of actin filaments in EGTA.

Fig. 10, B-D shows the effects of the mutants on actin viscosity. S4-6 had no effect on specific viscosity. S1-3 reduced the viscosity both in the presence and absence of calcium, but the decrease was not as marked as that observed with gelsolin (Fig. 10 B). The effect of S2-6 was quantitatively similar to gelsolin, but the final viscosity significantly higher (Fig. 10 C). The behavior of S1,4-6 was very different from the other mutants. The viscosity did not drop sharply at low concentrations of S1,4-6, but decreased approximately linearly

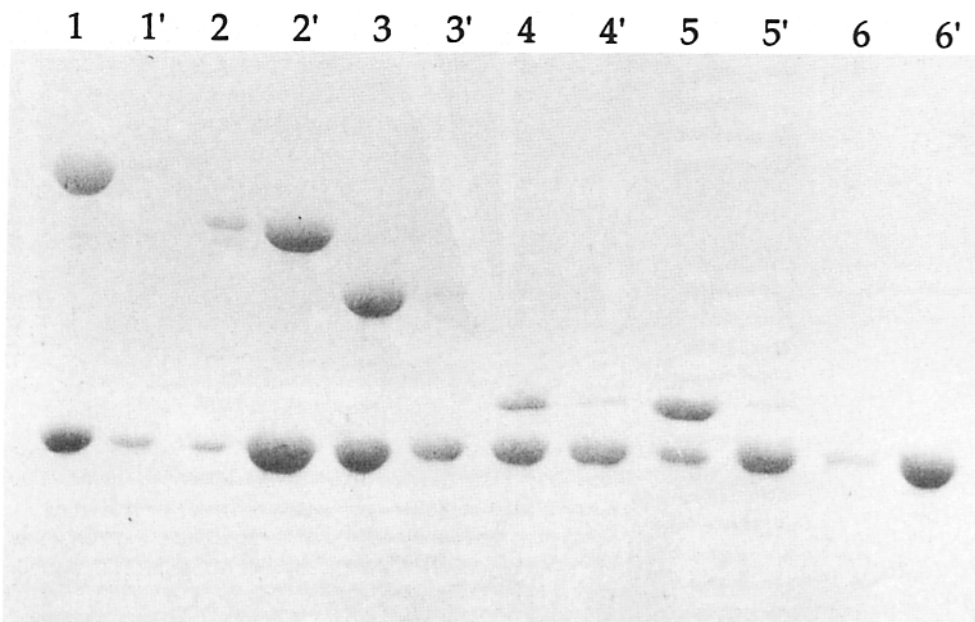


Figure 9. Cosedimentation of mutants with F-actin, mixed at 1:2 molar ratio. Lanes 1-6, supernatant fractions; lanes 1'-6', pellets resuspended to same volume as supernatants. Lanes 1 and 1', fxgelsolin; lanes 2 and 2', S2-6; lanes 3 and 3', S1,4-6; lanes 4 and 4', S1-3; lanes 5 and 5', S4-6; lanes 6 and 6', control.

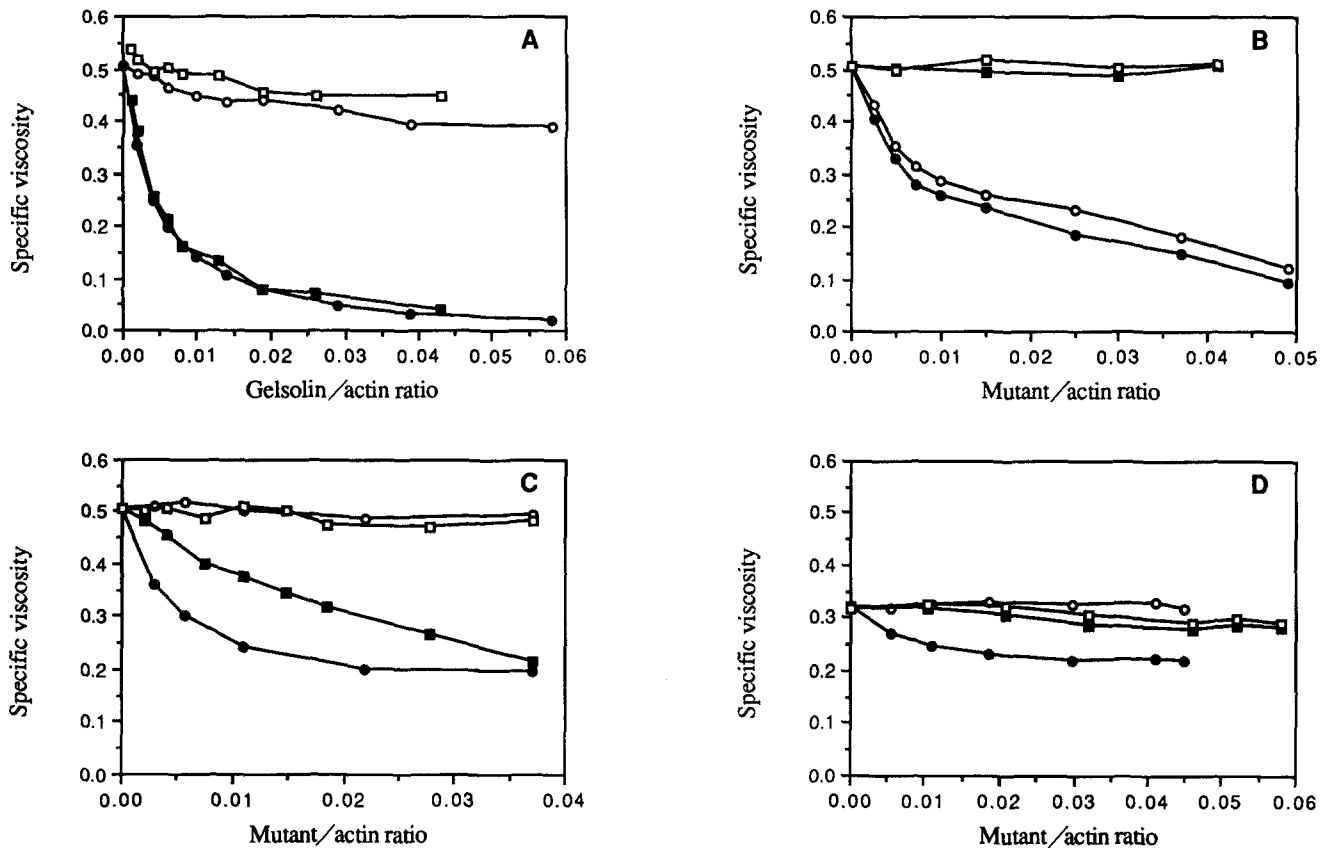


Figure 10. Effects of gelsolin and mutants on the viscosity of 15 μ M F-actin. Measurements were made within 3 min of mixing in either 0.1 mM calcium (*solid symbols*) or 0.2 mM EGTA (*open symbols*). (A) Pig plasma gelsolin (*circles*) and fxgelsolin (*squares*); (B) S1-3 (*circles*) and S4-6 (*squares*); (C) S2-6 (*circles*) and S1,4-6 (*squares*); (D) S2-6 (*circles*) and S1,4-6 (*squares*) but with capped filaments prepared by nucleating actin in the presence of 0.25% gelsolin (on a molar basis).

with increasing concentration of mutant in a calcium-dependent manner (Fig. 10 C). This effect was reproducible over several different preparations.

The viscosity of F-actin may be reduced either by internal severing or by monomer dissociation from filament ends. To test the possible involvement of the "barbed" end of actin filaments on the effects observed for S1,4-6, viscometric assays were performed using capped filaments. These assays are less sensitive because the starting viscosity is much lower. However, it is clear from Fig. 10 D that S2-6 reduced the specific viscosity of capped filaments in a calcium-dependent manner, but S1,4-6 has little effect.

Severing Assay

The fluorescence decrease in the severing assay was exponential with time giving a rate constant, k_{obs} , which increased linearly with gelsolin concentration. A value of $k_{obs} = 0.068/s$ was obtained from the plot in Fig. 11. These values compare with a mean value of $0.058 \pm 0.014/s$ from the initial depolymerization rate of capped filaments obtained from >20 control experiments using at least four different preparations of PI-actin and seven of gelsolin.

S4-6 had no effect on the depolymerization rate even at a 1:2 molar ratio to actin. Assays using the other mutants are shown in Fig. 11. S1-3 had a severing activity similar to that of gelsolin based on the apparent k_{obs} value, while the other two mutants had activities 10–20% of this value. (Assays car-

ried out on three different preparations of each mutant confirmed these results.)

Electron Microscopy

The effects of all the mutants on F-actin were examined in

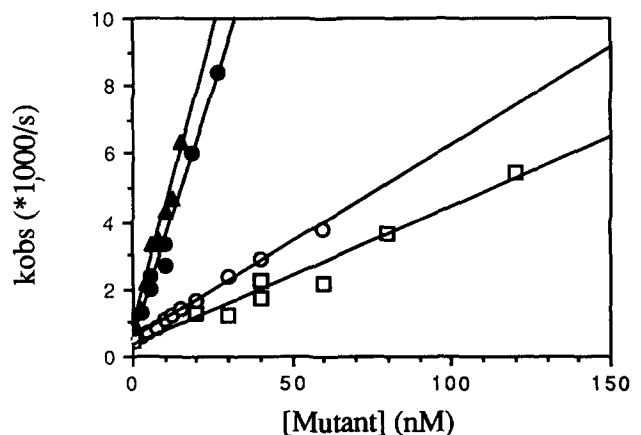


Figure 11. Effect of mutants on the rate constant for F-actin depolymerization in the severing assay, using fxgelsolin (*solid triangles*), S1-3 (*solid circles*), S2-6 (*open circles*), and S1,4-6 (*open squares*). Values of k_{obs} calculated from the slopes are: fxgelsolin = $0.068 s^{-1}$; S1-3 = $0.059 s^{-1}$; S2-6 = $0.0115 s^{-1}$ (17% of gelsolin value); S1,4-6 = $0.0082 s^{-1}$ (12% of gelsolin value).

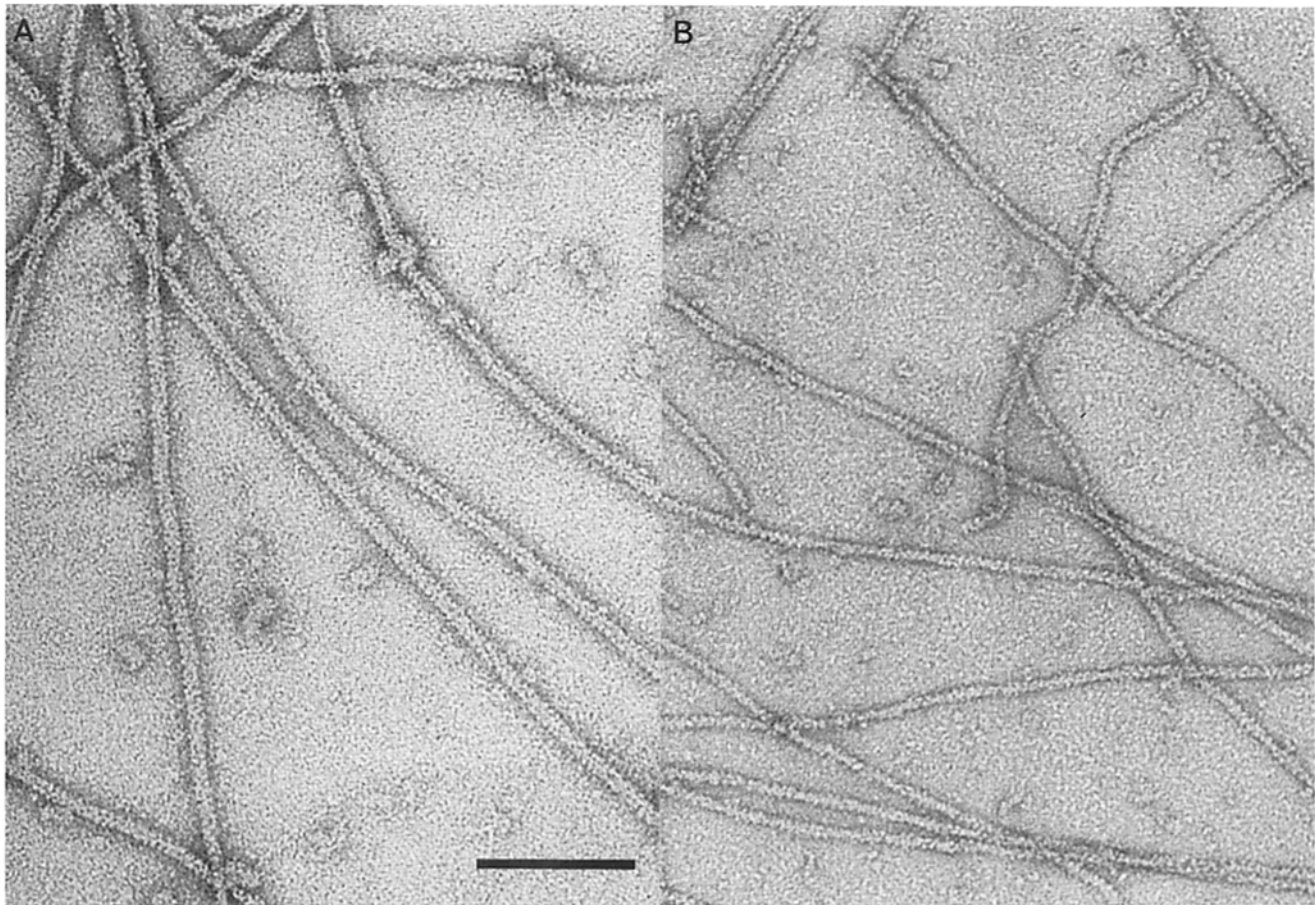


Figure 12. Electron micrographs of (A) F-actin incubated with S2-6 at 1:1 molar ratio and sampled within 2 min and (B) actin control. Bar, 100 nm.

the electron microscope. fxgelsolin and S1-3 rapidly severed filaments. S4-6 had no effect on filaments even at 1:1 molar ratios. Long filaments were clearly visible when samples of S2-6 and F-actin were mixed at 1:1 molar ratios in calcium, but within 5 min, there were only short filaments. The mean length was ~ 53 nm ($n = 330$, $SD = 28$ nm). These filaments were stable up to 3 h and did not reanneal. The most striking observation was that filaments had a diameter about twice that of controls and the helical pitch was less marked (Fig. 12). In EGTA filaments appeared broadened but no shortening was observed.

S1,4-6 eliminated all filaments at high molar ratios, but at lower ratios (1:20 or 1:50) filaments appear to be shorter. Capped filaments (1:400 gelsolin/actin) treated with S1,4-6 are more stable, but at 1:1 molar ratios, they disappear within a few minutes.

Calcium Binding

Calcium binding values in the presence of 50–200 pmol of mutants were increased to between 10,000 and 35,000 counts/min compared with controls of 7,000. Table II shows the calcium-binding stoichiometry for fxgelsolin and the four mutants. fxgelsolin and S2-6 binds 2 mol of calcium each and S1-3 and S4-6 1 mol. Because S1,4-6 gave an intermediate value using the rapid binding assay, equilibrium dialysis was carried out to assess the binding affinity (Fig. 13).

The binding stoichiometries of the three mutants tested (together with K_d values in parenthesis from nonlinear least squares fit) were as follows: S4-6 = 1.24 (0.8 μ M); S2-6 = 2.3 (0.55 μ M); S1,4-6 = 1.67 (0.8 μ M).

Discussion

Properties of Purified fxgelsolin

fxgelsolin is active in the cell supernatant as judged by the DNase I inhibition assay. It behaves like human platelet gelsolin (Bryan and Kurth, 1984; Kurth and Bryan, 1984), bovine plasma gelsolin (Coué and Korn, 1985), and pig stomach gelsolin (Hinssen, H., A. G. Weeds, unpublished data)

Table II. Calcium Binding to fxgelsolin and Deletion Mutants

Protein	Different preparations <i>n</i>	Total samples <i>n</i>	SD	Mean value (mol/mol protein)
Fxgelsolin	7	36	0.36	1.96
S2-6	6	30	0.30	1.91
S4-6	9	42	0.19	0.99
S1-3	5	20	0.05	0.86
S1,4-6	8	36	0.16	1.61

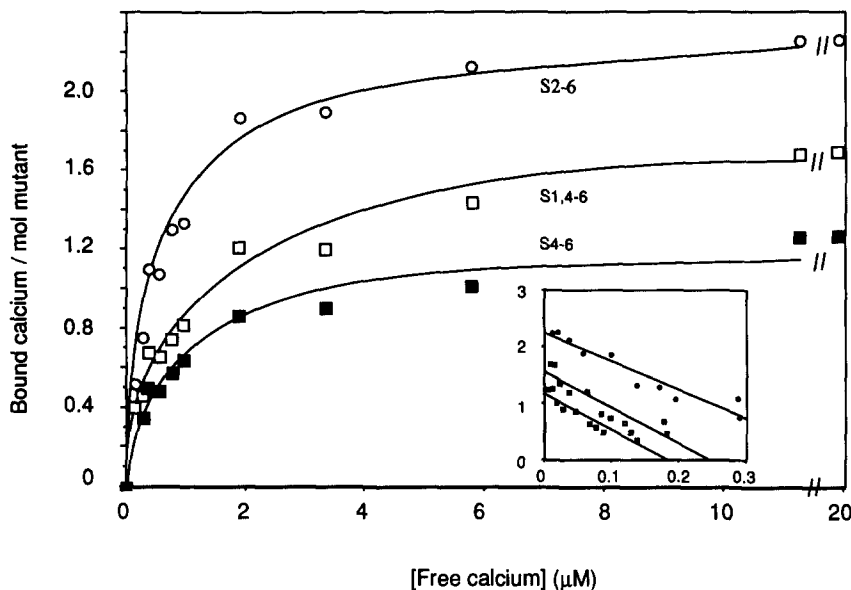


Figure 13. Calcium binding to S4-6 (solid squares), S2-6 (open circles), and S1,4-6 (open squares) measured by equilibrium dialysis. The binding stoichiometries (together with K_d values in parenthesis) were as follows: S4-6 = 1.24 ± 0.05 ($0.8 \pm 0.1 \mu\text{M}$); S2-6 = 2.3 ± 0.06 ($0.55 \pm 0.05 \mu\text{M}$); S1,4-6 = 1.67 ± 0.07 ($0.08 \pm 0.1 \mu\text{M}$). (Inset) Scatchard plot with bound calcium per mole mutant on the ordinate. K_d values were $\sim 0.6 \mu\text{M}$ in every case.

by showing no interaction with G-actin in the absence of calcium. fxgelsolin binds 2 mol calcium ions per mole and reduces the specific viscosity of F-actin in a calcium-dependent manner as seen with other gelsolins (Yin et al., 1980; Hinssen, 1987).

The elongation rate constant in nucleation assays, measured in 2 mM MgCl_2 , 100 mM KCl , was $0.18/\mu\text{M/s}$ (Table I). A similar experiment using 5 mM MgCl_2 gave a value of $0.22/\mu\text{M/s}$. These small differences and those reported by others (Weeds et al., 1985; Janmey and Stossel, 1986; Carlier and Pantaloni, 1988) are not unexpected, because this rate constant varies with ionic conditions (Doi and Frieden, 1984). The rate constant for monomer dissociation calculated from the severing assay is identical to that measured for the depolymerization of gelsolin-nucleated filaments. Similar values have been obtained using plasma gelsolin (Weeds, A. G., and J. Gooch, unpublished data) and from measurements of off-rates reported by Carlier and Pantaloni (1988). Differences with other published values (Janmey and Stossel, 1986) are consistent with the stabilization of filaments by salt (Bryan and Coluccio, 1985).

Properties of Gelsolin Mutants

Interactions with Monomeric Actin. All mutants were found to bind to actin-Sephadex in calcium. Both S1-3 and S4-6 show actin binding properties identical to the corresponding proteolytic fragments. S2-6 binds only in calcium, comparable to S4-6, while S1,4-6 shows calcium-independent binding similar to S1-3 and fxgelsolin. The conversion from calcium-independent to calcium-dependent binding therefore mirrors the loss of segment 1, which contains the high affinity actin binding site (Kwiatkowski et al., 1985; Yin et al., 1988, Bryan, 1988).

The results here provide clear evidence for actin binding without fluorescence enhancement (i.e., "silent sites"). S4-6 has a single calcium-dependent binding site, based on its association properties with actin-Sephadex, its effects on F-actin and G-actin in sedimentation assays, and its inhibition

of actin polymerization, but it does not enhance the fluorescence of NBD-actin or PI-actin (Fig. 6; Table I). This is consistent with earlier observations using the COOH-terminal half of plasma gelsolin (Way et al., 1988).

S1-3 forms a 1:2 complex with actin based on monomer sequestration during actin polymerization and fluorescence enhancement experiments with both PI-actin and NBD-actin (Table I). These findings are fully consistent with those of Bryan and Hwo (1986) who suggest a 1:2 complex on the basis of gel filtration and fluorescence measurements, although the curvature in their fluorescence experiments indicated much weaker binding.

S2-6 gives identical results to fxgelsolin (Table I). The calcium-independent actin binding site in segments 2-3 (Bryan, 1988; Yin, 1988) becomes calcium sensitive in this mutant, showing that monomer binding is modulated by segments 4-6.

S1,4-6 shows two binding sites in calcium by the inhibition assay and from sedimentation experiments, but only one in EGTA. However, NBD-actin titrations suggest one site in calcium or EGTA (Table I). Thus this mutant differs from both S2-6 and gelsolin in showing only a single binding site in calcium by fluorescence enhancement.

Since S1-3, S2-6, and S1,4-6 each bind two actin monomers in calcium, the question is raised as to whether all three sites in gelsolin might be occupied simultaneously or if not, which two sites are occupied in the ternary complex? The observation that S2-6 (but not S1-3 or S1,4-6) nucleates actin polymerization as effectively as gelsolin indicates that segment 1 is not required to create a fully competent nucleus. However, segment 1 appears to be occupied in the ternary complex based on (a) the formation of a high affinity binary complex in EGTA from the ternary complex (Bryan and Kurth, 1984; Coué and Korn, 1985; Weeds et al., 1986b; Selve and Wegner, 1987) and (b) inhibition of nucleotide exchange (Harris, 1985; Tellam, 1986; Bryan, 1988; Harris, 1988). Our evidence supports this since only mutants containing segment 1 bind actin to EGTA. These results can

most easily be reconciled if the actin monomer bound to segments 4–6 is also associated with segment 1.

The stoichiometry of actin binding by S2–6 in calcium suggests that the fluorescently silent site in S4–6 is occupied first, i.e., binding is cooperative (see Weeds et al., 1986b, for details of the arguments). By contrast the two binding sites in S1,4–6 are not coupled, since NBD-actin fluorescence shows only a single site, corresponding to segment 1. Cooperative binding by gelsolin has been reported elsewhere (Janmey et al., 1986; Selve and Wegner, 1987). Thus it appears that in the absence of calcium the high affinity sites are inaccessible to G-actin: calcium facilitates monomer binding initially on segments 4–6 and subsequently at the other sites.

Interactions with F-Actin

The methods used here differ in ionic conditions and the actin concentrations. Differences in results may therefore reflect the actin affinities of the mutants and or differences in stability of the F-actin. Before considering the results, it is important to appreciate the differences between the methods.

Viscometry provides a very sensitive measure of severing because it is most strongly influenced by the longest filaments and these have the highest probability of being severed.

The DNase inhibition assay is carried out in 15% glycerol, conditions under which actin filaments do not depolymerize below the critical concentration (Harris et al., 1982). Were depolymerization to occur, the extent of DNase inhibition would be much greater than that observed: an inhibitory activity equivalent to two actin monomers is produced by gelsolin (Weeds et al., 1986b). The most plausible explanation of this stoichiometry is that DNase binds to the two actin subunits at the pointed ends of filaments and is thereby inhibited. In support of this Podolski and Steck (1988) have shown that DNase I binds to the pointed ends of protofilaments in red cell cytoskeletons.

The severing assay measures the number concentration of free pointed ends. Because gelsolin severs filaments very rapidly and caps their barbed with high affinity (Selve and Wegner, 1986), there is a 1:1 correlation between the gelsolin concentration and number of filaments. The agreement in values of k_{-} between severing assays and control depolymerization experiments with nucleated filaments supports this conclusion. A lower activity may reflect weaker binding affinity, a defective severing mechanism or reduced monomer dissociation rate constant (e.g., actin filaments are stabilized by tropomyosin [Bernstein and Bamberg, 1982]).

S1–3 and S4–6. All assays show that these mutants behave identically to the NH₂- and COOH-terminal proteolytic fragments of gelsolin (Kwiatkowski et al., 1985; Chaponnier et al., 1986; Bryan, 1988; Yin et al., 1988).

S2–6. Viscometric analysis suggests that S2–6 severs filaments in a calcium-dependent manner, but the magnitude of the effect is small (e.g., the fall in viscosity using at 1:200 mutant/actin ratio, where the assay is most sensitive, is only 36% the value obtained with gelsolin). The severing assay also suggests an activity <20% that of f_xgelsolin (Fig. 11). Sedimentation experiments in calcium also showed binding to filaments and a time-dependent appearance of both actin and S2–6 in the supernatant. Electron microscopy showed a calcium-sensitive time-dependent reduction in filament length and prolonged incubation gave short filaments of 19

± 10 monomers long at 1:1 molar ratios of S2–6/actin. The most striking effect of S2–6 was to increase the diameter of the filaments (Fig. 9), indicating lateral association along their entire length. The calcium dependence seen in the interaction of S2–6 with G-actin does not exist with F-actin, based on sedimentation and electron microscopy in EGTA. One possible explanation is that the affinity of the actin binding site in segments 2–3 for F-actin subunits is much higher than that for G-actin monomers (Bryan, 1988; Yin et al., 1988).

These results show that S2–6 has a high affinity for the sides of filaments and that severing occurs relatively slowly as compared to gelsolin. One property of this mutant that may be relevant in understanding its severing mechanism is that it caps filament ends. This is based on its nucleating activity, the absence of reannealing of shortened filaments, and an inhibitory activity of elongation of actin protofilaments in red cell ghosts 77% that of gelsolin (unpublished observations using the method of Pinder et al., [1986]). Association to the sides of filaments may affect their flexibility and facilitate the formation of a cap, i.e., fragmentation. Such “capping-induced severing” is very different from the activity of gelsolin, because it occurs much more slowly and not at every binding site.

S1,4–6. All the assays show that S1,4–6 lacks the potent severing activity of gelsolin. Viscometry and the severing assay suggest even lower activities than S2–6. One striking contrast between S2–6 and S1,4–6 is in the sedimentation assay, in which S2–6 appears with F-actin in the pellet while S1,4–6 is always in the supernatant (complexed with two actin monomers in calcium and one in EGTA). Thus S1,4–6 does not bind strongly to filaments. Nevertheless, electron microscopy showed clear evidence of shortened filaments at S1,4–6/actin ratios 1:50 or 1:20 and at higher ratios filaments disappeared almost instantaneously. However, when capped filaments were incubated with S1,4–6 they were more refractile. This was clearly evident in electron microscopy, but also apparent in both viscometric and DNase inhibition assays. We are unable at present to explain these observations but monomer sequestration alone cannot account for the 20% reduction in specific viscosity when this mutant is added to F-actin at 1:100 molar ratio. Nor can it explain the results of the severing assay (Fig. 11) which is carried out below the critical concentration.

Localization of Calcium Binding Sites

Gelsolin binds 2 mol calcium per mole protein. One binding site is fully contained within segments 4–6; the other in segments 1–3 (Table II). Binding studies with S2–6 suggest that the site in S1–3 is localized in segments 2–3. However, S1,4–6 gives an intermediate value of 1.6–1.7 both in the rapid assay and in equilibrium dialysis (Fig. 13). Binding affinities appear to be identical at all sites based on K_d values of 0.6–0.8 μ M from equilibrium dialysis (similar to those previously reported for gelsolin [Weeds et al., 1986a]). The apparent paradox in these observations may be resolved if the additional binding site in segment 1 of S1,4–6 corresponds to the cryptic binding site identified in a COOH-terminal truncated mutant containing the first 160 residues of gelsolin (Kwiatkowski et al., 1989).

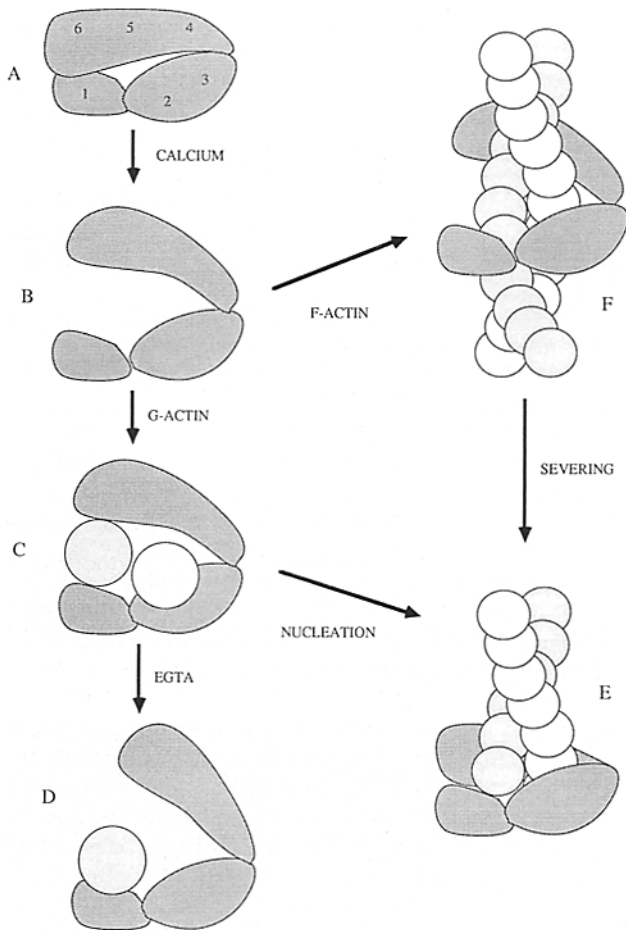


Figure 14. Schematic model to show the interaction of gelsolin with actin. In the absence of calcium, actin binding is prevented by the close association of segment 1 and segments 4–6. Addition of calcium facilitates binding of two G-actin monomers, one to segments 2–3 and the other initially to segments 4–6 but also to segment 1. (This ternary complex is the nucleating species under polymerizing conditions.) On removal of calcium, binding by segments 2–3 and 4–6 is lost, leaving a single monomer tightly associated with segment 1. In the presence of F-actin, gelsolin binds to the sides of filaments via segments 2–3 and both severing and capping occur by the interaction of segment 1 and probably segments 4–6 with an adjacent actin subunit.

Model and Summary

Our experiments have been designed to explore the role of the different actin binding sites in gelsolin. The results resolve some of the uncertainties from earlier work and provide new insights about the functions of individual actin binding domains. We propose a hypothesis to account for the properties of gelsolin shown schematically in Fig. 14.

In the absence of calcium, gelsolin is locked in a conformation that prevents all actin binding. This is achieved by an interaction between segments 1 and 4–6, based on the observations that fgelsolin binds neither F-actin nor G-actin in EGTA, but all mutants containing either segment 1 or segments 4–6 bind either monomeric or polymeric actin.

Addition of calcium opens the molecule to interact with actin. Segmental flexibility between the domains is evident from the proteolytic susceptibility of gelsolin in calcium, but

not in EGTA (Bryan and Hwo, 1986; Gooch, J., and A. G. Weeds, unpublished observations).

Binding to monomeric actin occurs initially in segments 4–6 and formation of the ternary complex appears to involve all three sites. Both gelsolin and S2–6 nucleate polymerization. However, the cap formed by S2–6, unlike that of gelsolin, is not stable in the absence of calcium. Recent experiments on the disassembly of actin filaments capped with S2–6 have shown a depolymerization rate in G' buffer about sevenfold higher in EGTA than in calcium, when compared with similar experiments with gelsolin-capped filaments (Weeds, A. G., unpublished work). This provides strong support for the hypothesis that segment 1 binding is essential for stable capping after removal of calcium.

Gelsolin interacts with the sides of actin filaments via segments 2–3 and severing occurs by the disruptive power of segment 1. The weak severing activity of S2–6 shows the importance of segment 1 in the severing process, but it also suggests an involvement of segments 4–6 in the fragmenting process, since CT28N does not sever on its own.

Further work is in progress to elucidate the details of the severing process and other mutants are under construction, but ultimately a full understanding of the interactions with actin will require the crystallographic structure of gelsolin and its complexes.

We would like to thank Mr. T. Smith for synthesizing oligonucleotides and Drs. D. Kwiatkowski and H. Yin for providing cDNA clones of human plasma gelsolin. We also thank Drs. Kwiatkowski, Janmey, and Yin for information about calcium binding studies on gelsolin mutants prior to publication. We are very grateful to Dr. A. Wegner for providing mathematical proof of length distributions when filaments are severed randomly; to Dr. W. Gratzer for helpful discussions; to Dr. Stuart Berger and Mr. N. Weeds for writing computer programs and Miss L. Richards for proofreading this manuscript.

We dedicate this paper to the memory of Dr. Sandy MacLeod, whose scientific excellence, commitment, and generosity we sadly miss.

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