Are the Conserved Sequences in Segment 1 of Gelsolin Important for Binding Actin?

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Abstract. The minimal region required for actin binding in the smallest of the three domains of gelsolin (termed Segment 1 or S1) was previously defined by deletion mutagenesis as residues 37–126. Further analysis of NH_2-terminal deletions here redefines the minimal functional core as residues 41–126. Amino acid substitutions within this core further elucidate the nature of the interaction of segment 1 with actin. Of 26 point mutants analyzed, 14 reduced the affinity for actin. The charged residues His 119, Arg 120, Glu 121, and Gln 123 appear to be involved in direct interaction with actin. Substitutions of Leu 108, Leu 112, and Val 117 by polar groups all affect the structural stability of segment 1 and thereby reduce binding affinity. In addition replacement of Glu 126 by aspartic acid modifies the physical properties of segment 1 and weakens binding. We have further shown that changing charged residues within the highly conserved pentapeptide sequence LDDYL (residues 108–112) has no effect on actin binding. This sequence, found in a number of different actin binding proteins, does not therefore constitute part of the interaction site. Similarly, substitution of the two acidic residues by basic ones within the DESG motif of segment 1 (residues 96–99, but also found near the COOH terminus of actin) does not impair binding. These results show the dangers of predicting functional sites on the basis of conserved sequences.

GELSOLIN is a calcium activated actin severing and capping protein universally distributed in vertebrate tissues (Kwiatkowski et al., 1988). It plays an important role in the rearrangements of the actin cytoskeleton that occur during division, differentiation, and locomotion (reviewed by Way and Weeds, 1990). Evidence for this comes from correlation of gelsolin expression with the behavior of cells in differentiation and locomotion (Kwiatkowski, 1988; Dieffenbach et al., 1989) and in transformation (Vandekerckhove et al., 1990; Chaponnier and Gabbiani, 1989). Furthermore, overexpression of gelsolin in fibroblasts enhances cell motility in proportion to the amount of total gelsolin in the cell (Cunningham et al., 1991).

The three different actin binding domains in gelsolin (Yin et al., 1988; Bryan, 1988) are distributed unevenly within the six repeating segments (S1–6) in the amino acid sequence (Way and Weeds, 1988). Monomer binding sites are present in segment 1 (S1)[1] and S4–6, while S2–3 contains the F-actin binding site. The roles of these three sites in the severing and nucleating activities of gelsolin have been studied by deletion mutagenesis (Kwiatkowski et al., 1989; Way et al., 1989). Severing requires only S1–3, but without S4–6 it is not calcium regulated. By contrast, S2–6 has the same nucleating capacity as gelsolin. Thus S1 is essential for severing but not for nucleation. The relationships of the three domains to binding sites on actin have been investigated by affinity chromatography (Pope et al., 1991).

S1, 150 residues in length in plasma and 125 in cytoplasmic gelsolin, contains a high affinity calcium-independent G-actin binding site (Yin et al., 1988; Bryan, 1988). We have localized this site to a minimal 89 amino acids (residues 37–126) and identified a region (residues 121–126) critical for binding (Fig. 1) (Way et al., 1990). We also demonstrated the presence of a calcium binding site in the complex that was not detectable in either protein on its own. This site was predicted because the affinity of S1 for actin is ~1,000-fold higher when calcium is present (Bryan, 1988), but it is not related to the calcium sites required for gelsolin activation. It probably accounts for the trapped calcium found in the EGTA stable binary complex of actin and gelsolin (Bryan and Kurth, 1984).

Residues 121–126 are COOH-terminally adjacent to a sequence of ~20 amino acids that is conserved in other actin monomer binding proteins (Fig. 2) (Vandekerckhove, 1989). The spatial proximity by this conserved region to actin has been demonstrated in the case of *Acanthamoeba* profilin by chemical cross-linking (Vandekerckhove et al., 1989): this sequence may play an important role in actin binding (Vandekerckhove, 1989; Vandekerckhove et al., 1989; Tellam et al., 1989; Haarer and Brown, 1990).

We have produced a series of 32 mutants in S1 in an attempt to identify residues involved in the interaction with actin. Our choice of residues was directed by four considera-

1. *Abbreviation used in this paper*: S1, Segment 1.

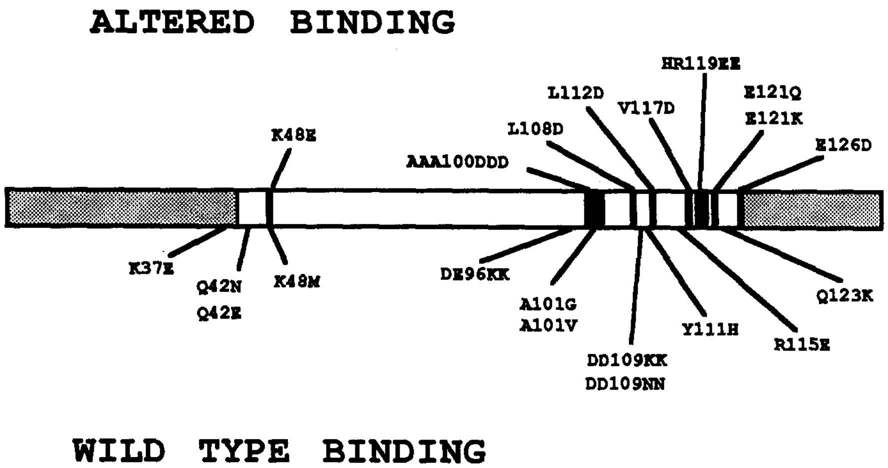

Figure 1. Schematic representation of S1 showing positions of substituted residues. Those above the box show altered actin affinity and those below are unchanged. The unshaded portion shows the minimal functional core based on NH_2- and COOH-terminal deletions.

tions: (*a*) to test the hypothesis that conserved sequences are essential for function; (*b*) to investigate the role of charged residues close to the conserved COOH-terminal region; (*c*) to dissect the NH_2 terminus more extensively; (*d*) to examine the importance of lysine 48, based on the observation that it can be chemically cross-linked to acidic residues at the NH_2 terminus of actin. Results revealed a number of substitutions between residues 96 and 126 that weakened binding, either because the residues concerned are located at the contact site or because they are essential for structural stabil-

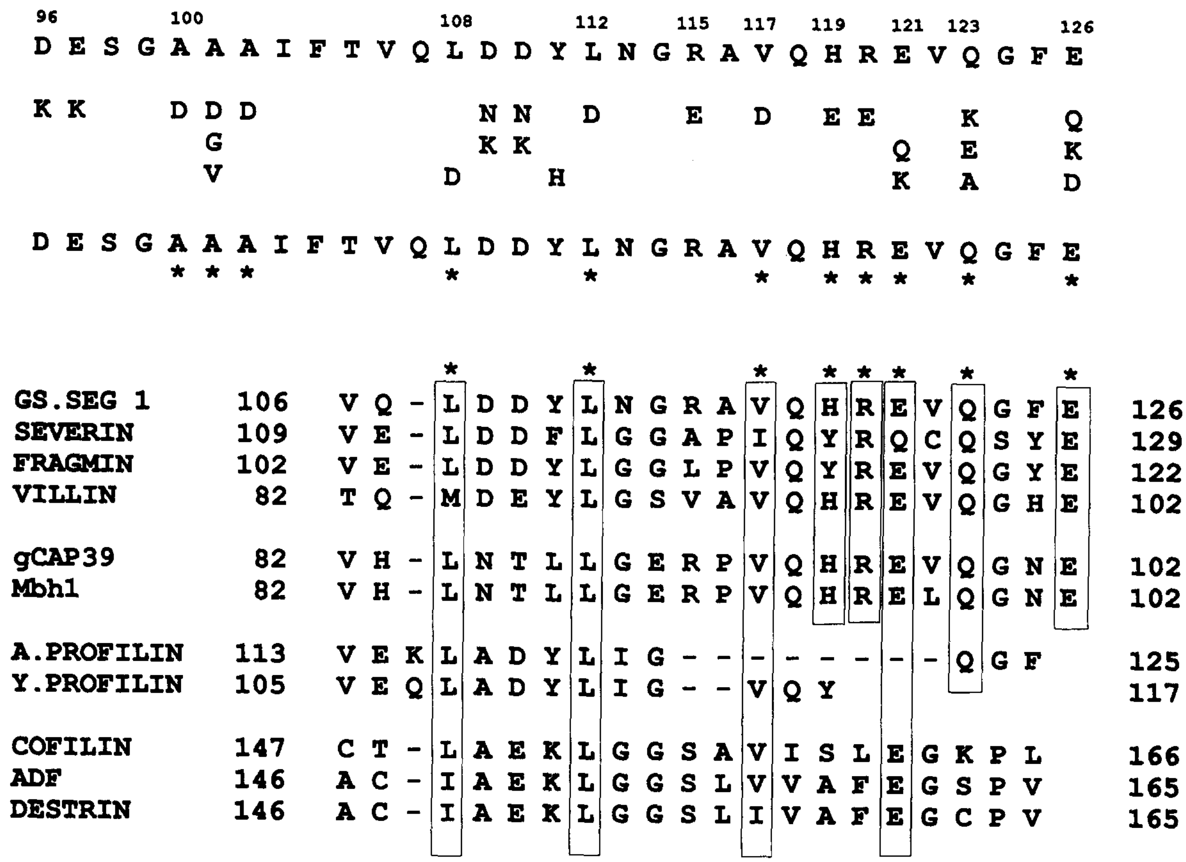

Figure 2. Upper part shows sequence of COOH-terminal part of S1 with positions of substitutions and their replacements indicated. * Indicates position where substitutions affected actin binding. Lower part shows sequence homologies in this region with other severing and capping proteins and with various monomer binding proteins. Sources of sequence data: gelsolin (Kwiatkowski et al., 1986); severin (André et al., 1988); fragmin (Ampe and Vandekerckhove, 1987); villin (Arpin et al., 1988; Bazari et al., 1988); gCAP 39 (a gelsolin-like capping protein of 351 amino acids [Yu et al., 1990]); Mbh 1 (myc basic motif homolog-1 [Prendergast and Ziff, 1991]); *Acanthamoeba* profilin (Ampe et al., 1985); yeast profilin (Oechsner et al., 1987); cofilin (Matsuzaki et al., 1988); ADF (actin depolymerizing factor (Adams et al., 1990)); and destrin (Moriyama et al., 1990).

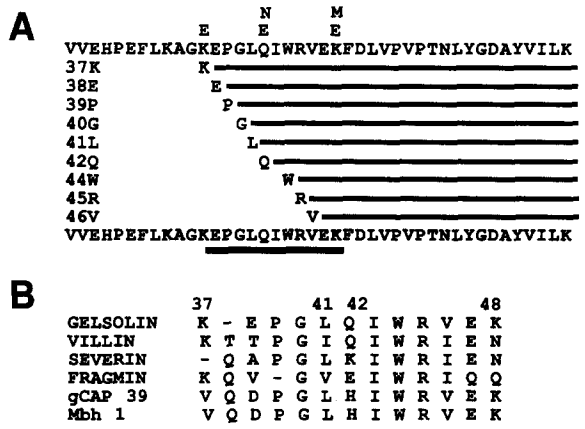

Figure 3. (*A*) Sequence of NH_2-terminal half of S1 (cytoplasmic form) with point substitutions and NH_2-terminal deletion mutants, numbered from the start of the plasma form, indicated. Underlined sequence refers to that shown in *B*, in which the homologies with other members of the severing and capping class of proteins are shown. Sources of sequences as in Fig. 2.

ity. In addition, loss of actin binding occurs when Leu 41 or residues beyond it are deleted from the NH_2 terminus (Fig. 3).

Materials and Methods

Nomenclature, Construction, and Purification of the Mutants

NH_2 terminal deletion mutants are named from the first position in the plasma gelsolin sequence. Point mutants are named according to the original residue, its position, and replacement. For example K48E is a substitution of lysine 48 by glutamic acid. Where more than one residue in a contiguous series has been changed, the number of only the first residue is given. Other mutants have been named individually, particularly those containing substitutions that are not adjacent.

All S1 mutants were engineered, sequenced, and subcloned into pMW-172 as described by Way et al. (1990). Most were expressed as previously, but more recently a streak of BL21(DE3) containing the expression construct is inoculated directly into 1 liter of medium and grown at 34°C. After overnight growth, cell densities are >2 A_{600} cm^{-1} and SDS-PAGE has always showed high levels of expression in the absence of induction by isopropyl β-D-thiogalactoside. Thus cells can be harvested immediately.

Mutants were purified from inclusion bodies, except where specified in Table I. Frozen cell pellets were resuspended in 25 ml 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and 25% sucrose to which was added 240 μl 1 M $MgCl_2$, 24 μl 1 M $MnCl_2$, and 10 μl 10 mg/ml Sigma D 4527 deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO). After incubation for 20–30 min at 22°C, the suspension was centrifuged for 10 min at 12,000 *g* and the supernatant retained for isolation of mutants present in the soluble fraction.

In previous preparations (Way et al., 1989, 1990), the pellets from this centrifugation were washed extensively with buffers containing 1% Triton X-100 and 1% sodium deoxycholate to remove cell debris from inclusion bodies (Nagai and Thøgersen, 1987). Triton X-100 contaminates any proteins not retained on DEAE-cellulose and was difficult to remove by dialysis. However, it precipitates at <25% ammonium sulphate saturation while proteins were collected in the 25–60% saturation range. Because some mutants bound Triton X-100 very tightly, cell pellets were resuspended without Triton in 50 ml of 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 1% sodium deoxycholate, and 2 mM EGTA for 20–30 min at 22°C. Deoxycholate binds to DEAE-cellulose and any residual detergent is removed by dialysis, since it has both a much higher critical micelle concentration and lower micelle molecular weight than Triton X-100 (Furth et al., 1984).

Inclusion bodies were pelleted at 12,000 *g* for 10 min and washed up to three times with 50 ml 10 mM Tris-HCl, pH 8.0, 1 mM EGTA until the supernatant was clear. They were solubilized in 10 mM Tris-HCl, pH 8.0, 0.2 mM EGTA, and 1 mM NaN_3 (buffer A) plus 8 M urea. After dilution of the urea to 6 M and centrifugation at 12,000 *g* for 20 min, supernatant fractions were applied to DEAE-cellulose equilibrated in buffer A. Mutants that bound to the column were eluted with a salt gradient. This procedure was used for all the mutants here, except those shown in Table I, for which 6 M urea was present throughout the fractionation to prevent aggregation.

The majority of the purified mutants showed no turbidity at 320 nm when concentrated to A280 values > 1.0 on Centricon membranes (Amicon Corporation, Danvers, MA). All mutants purified on DEAE-cellulose in urea were subsequently gel filtered on Sephacryl S200 in buffer A in the absence of urea and only monomeric fractions analyzed. Sephacryl fractionation, used on a random basis to examine mutants not purified in urea, showed that these were completely monomeric. The presence of self-association was monitored either by turbidity at 320 nm or by light scattering measurements in the fluorimeter (the most sensitive assay), or, when very extensive, was evident by precipitation. Self-association was frequently observed for mutants requiring urea during DEAE-cellulose fractionation, but was not detected for other mutants except where stated.

S1 is very stable in the presence of trypsin, chymotrypsin, or V8 protease. A simple test for native conformation was to assess protease sensitivity by SDS-PAGE after incubation in the presence of 1:100 wt/wt ratios with chymotrypsin, trypsin or 1:20 wt/wt ratio of V8 protease for up to 10 h.

Table I. Summary of Properties and Purification of Point Mutants

Altered actin binding		Wild-type actin binding	
Bound to DEAE	Not bound	Bound to DEAE	Not bound
AAA100DDD	E121Q	K37E	Q42N
HR119EE	E121K	Q42E	DE96KK
L108D*	E126Q*	K48M	A101G
L112D‡	E126K*	R115E	A101V
V117D‡	E126D		DD109NN‡
Triple§			DE109KK‡
K48E			Y111H
Q123K (N126)			Q123K
Q123E (N126)			
Q123A (N126)			

Mutants have been classified into two groups based on their actin binding properties. In each case those that bind to DEAE-cellulose are distinguished from those that do not. The point mutants were isolated from inclusion bodies (with the exception of those marked ‡, which were obtained from *E. coli* soluble fraction). * denotes proteins purified in urea on DEAE-cellulose. NH_2-terminal deletion mutants (not shown in the table) were isolated exclusively from inclusion bodies; they all bound to DEAE-cellulose and were fractionated in urea. They were subsequently purified on S-200 Sephacryl in the absence of urea. § The triple mutant contains all three substitutions L108D, L112D, and V117D.

Assays for Interaction with Actin

Preparation of PI-actin (actin reacted on Cys 374 with *N*-[1-pyrenyl]iodoacetamide) and NBD-actin (actin reacted with *N*-ethyl maleimide on Cys 374 then on Lys 373 with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole) have been described earlier (Weeds et al., 1986). Fluorescence measurements were made using a spectrofluorimeter (Model LS50; Perkin Elmer Corp., Norwalk, CT) with excitation and emission wavelengths for PI-actin of 366 and 384 nm and for NBD-actin of 468 and 520 nm, respectively. Light-scattering measurements were carried out in the fluorimeter using excitation and emission wavelengths of identical value, equal to the emission wavelengths above. Data were analyzed using nonlinear least squares analysis using the algorithm of Marquart (Press et al., 1988) with Enzfitter (distributed by Biosoft, Cambridge, UK).

Monomer binding was assessed using three methods: (*a*) fluorescence enhancement of NBD-actin when mutants were mixed with 100 nM NBD-actin; (*b*) inhibition of actin polymerization using ~4 μM actin (containing 15% PI-actin); (*c*) binding to actin-Sepharose. All assays were carried out in either 0.2 mM or <0.01 μM $CaCl_2$ as detailed in Way et al. (1990). Each

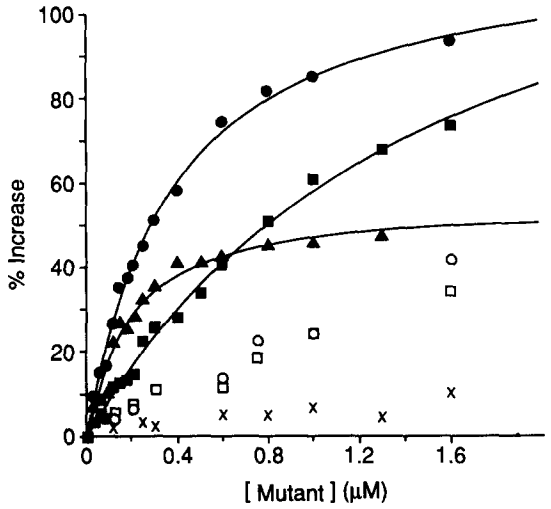

Figure 4. Fluorescence enhancement of 100 nM NBD-actin with increasing concentrations of L108D (▲), L112D (●), V117D (■), and the triple mutant containing all three substitutions (×). Lines show fitted curves corresponding to K_d values of 0.13 μM (L108D), 0.3 μM (L112D), and 1.5 μM (V117D). Closed symbols in calcium and open in EGTA. The maximum fluorescence enhancement by L108D in EGTA was <10%, similar to that of the triple mutant shown in calcium.

assay has advantages and disadvantages. (*a*) Enhancement of fluorescent NBD-actin provides the greatest sensitivity for determining low K_d values (between 0.01 and 1.0 μM). Although not all mutants enhance the fluorescence of NBD-actin and the extent of enhancement varies between mutants (Way et al., 1990; Fig. 4), we regard NBD-actin titrations as providing the most reliable K_d values, except where the extent of fluorescence enhancement < 25% or K_d > 1 μM. (*b*) The inhibitory effect of mutants on the extent of actin polymerization is particularly useful for estimating K_d values in the range 0.1–10 μM. It does not accurately assess K_d values much lower than 0.1 μM, because it is not possible to work at very low actin concentrations—adding gelsolin as a nucleator increases the rate of polymerization but also the critical concentration, thereby limiting the minimum concentration of actin that can be used. The presence of gelsolin also eliminates detection of capping filament ends by the mutants themselves. (*c*) Affinity chromatography provides a quick and effective means of assessing binding. However, mutants with a high K_d may be dissociated from the column during washing stages and those showing self-association on the column will give misleadingly high levels of binding, as reported by Pope et al. (1991).

Results

Purification of Mutants

Expression levels of point mutants were similar to our previous results (Way et al., 1990), but modification to overnight growth and expression gave reproducibly higher yields without the need for induction by isopropyl β-D-thiogalactoside. Table I summarizes the protein isolation procedure both from inclusion bodies and soluble fractions. The purity of the mutants was >95% based on SDS-PAGE.

NH_2-Terminal Deletion and Point Mutations

The advantages and disadvantages of the various assays has been outlined in Materials and Methods. As will be seen, there is reasonable agreement between the quantitative results for the two types of fluorescence assay (see Table III). We have taken K_d values <0.01 μM as indistinguishable from wild type, values between 0.05 and 0.2 μM as not necessarily different from each other, but different from wild type, because the true wild-type value is much lower than 0.01 μM. K_d values much greater than 0.2 μM show greatly reduced binding affinity.

Fig. 3 *A* shows a series of sequential NH_2-terminal deletions from Lys 37 to Val 46. S1 remains active with deletion to Leu 41, but further truncation causes inactivation (Table II). Gel filtration of these mutants showed an increasing shift into the void volume with a correspondingly smaller monomer peak. The proportion of monomer was 100% (37K and 38E), 80% (39P), 60% (40G and 41L), 20% (42Q), and 0% (45R).

No point mutations in the NH_2-terminal half of the molecule affected actin binding, with the single exception of K48E, which showed weaker binding in EGTA but not in calcium (Fig. 3 *A* and Table III).

COOH Terminal Point Mutants

COOH-terminal point mutants can be classified as (*a*) indistinguishable from segment 1 (Table II); (*b*) binding with altered affinity (Table III); and (*c*) inactive (Table II).

Three of the most strongly conserved residues in the sequence common to severing and monomer binding proteins are Leu 108, Leu 112, and Val 117 (Fig. 2). Changing each of these to aspartic acid greatly reduced binding affinity (Fig. 4 and Table III). Differences observed in the extent of fluorescence enhancement suggest variation in the quantum yield for the individual complexes in calcium and EGTA (Fig. 4).

Mutation of buried hydrophobic residues to polar ones can adversely affect protein conformation giving rise to altered structure and physical properties. L108D, L112D, and V117D were all 100% monomeric on gel filtration. L112D and V117D showed resistance to V8 protease similar to S1 (L108D was not tested). L112D, but not V117D, showed limited susceptibility to chymotrypsin, as evidenced by the gradual disappearance of the 17,500-mol weight band and the concomitant appearance of three weak bands of molecular weight 14,000–16,500.

When all three mutations were combined in the same construct, the fluorescence assays showed virtually no binding (Table III and Fig. 4). The apparently strong binding to

Table II. Mutants with Actin Binding Properties Identical to S1 and Mutants that Show No Binding

Type	Bind actin		No binding
NH_2-terminal deletions	38E	39P	42Q
	40G	41L	44W
Single point mutants	K37E		E126Q*
	Q42E	Q42N	E126K*
	K48M		
	A101G	A101V	
	Y111H	R115E	
	Q123K*		
Multiple point mutants	DE96KK		AAA100DDD
	DD109KK	DD109NN	

* These mutants precipitated on purification.

Table III. Mutants with Altered Affinity for Actin

Mutant	PI-actin K_d		NBD-actin K_d		Actin-Sepharose binding	
	Ca	EGTA	Ca	EGTA	Ca	EGTA
	(μM)		(μM)			
S1	0.01	0.01	0.01	0.05	+++	+++
E121Q	0.01	ND	0.01	0.08	+++	++
E121K	0.01	~4	0.01	>2	+++	+
E126D	0.08	0.7	0.02	0.5	+++	+++
HR119EE	0.06	0.5	0.02	–	+++	–
L108D	0.1	~4	0.1	–	+++	+
L112D	0.4	0.9	0.3	>1.4	++	++
V117D	1.0	2.6	1.5	>1.5	++	+
Triple*	~4	ND	–	–	ppt*	ppt*
K48E	0.01	ND	0.01	0.27	+++	++
Q123E‡	0.1	–	0.15	–	++	–
Q123A‡	2	–	–	–	+	–
Q123K‡	~5	–	–	–	+	–

Strength of binding to actin-Sepharose shown semi-quantitatively compared to S1: +++ (>95%); ++ (65–90%); + (30–45%); – (<10%).
* The triple mutant contains all three substitutions L108D, L112D, and V117D. It showed much greater binding to actin-Sepharose than S1, indicating precipitation on the column.
‡ Substitutions made in deletion mutant N126, containing the first 126 residues of segment 1.

actin-Sepharose implies self-association, which was evident also on Sephacryl S200 (>50% of the protein eluting in the void volume). The purified monomer peak precipitated on storage at 4°C and it bound strongly to Sepharose coupled to S2–3, (which does not bind S1 [Pope et al., 1991]), confirming extensive self association.

Substituting the two basic residues 119 and 120 with glutamic acid eliminated binding in EGTA, as measured by affinity chromatography. There was no enhancement of NBD-actin fluorescence by this mutant in EGTA (maximum enhancement at a 10-fold excess of mutant was <10%) (Fig. 5). The K_d calculated from polymerization inhibition in EGTA also indicated greatly weakened affinity and there was reduction in affinity also in calcium (Table III, Fig. 6).

Changing either of the acidic residues Glu 121 or Glu 126 had very different effects on the properties of S1. Replacement of Glu 121 (which is highly conserved [Fig. 2]) by lysine substantially weakened binding in EGTA (Table III), but substitution by glutamine had little effect. Changing Glu 126 to either glutamine or lysine greatly reduced the solubility of the protein. Both mutants showed extensive precipitation on dialysis after chromatography on DEAE-cellulose in urea and there was further precipitation subsequently on

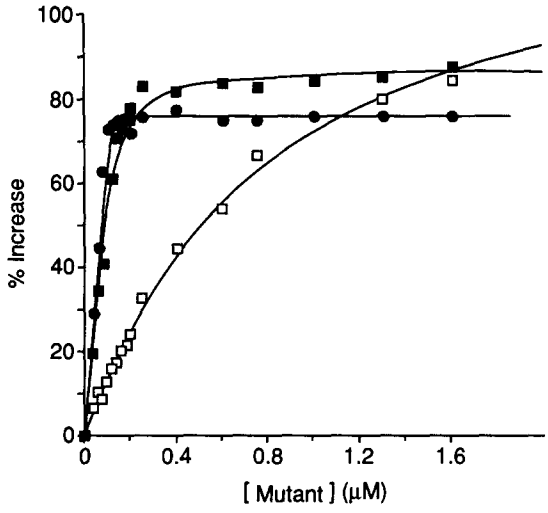

Figure 5. Fluorescence enhancement of 100 nM NBD-actin with increasing concentrations of HR119EE (●) and E126D (■). Lines show fitted curves corresponding to K_d values of 0.02 μM (closed symbol, E126D in calcium) and 0.5 μM (open symbol, E126D in EGTA). Straight lines have been drawn through the data for HR119EE in calcium to show maximum enhancement at a 1:1 molar ratio.

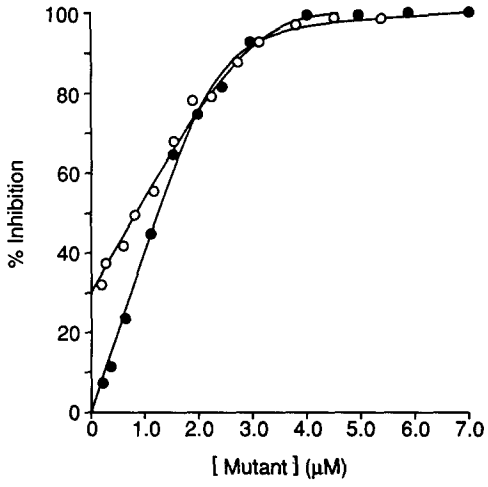

Figure 6. Percent inhibition of actin polymerization using 3.4 μM actin with HR119EE in calcium (●) and EGTA (○). Fitted curves are for K_d = 0.06 μM (calcium) and 0.5 μM (EGTA). Note the apparent finite inhibition of polymerization in EGTA in the absence of gelsolin.

Sephacryl S200. Fluorescence assays on the pure protein (<10% of the expressed material) showed very high levels of light scattering. Even substitution by aspartic acid at this position reduced solubility: this mutant could not be concentrated above 10 μM because of aggregation on Centricon membranes. However, it showed no increase in light scattering in fluorescence assays: its binding to actin, although similar to S1 in calcium, was much weaker in EGTA (Fig. 5). The unaltered binding to actin-Sepharose in EGTA probably reflects self-association on the column (Table III).

Analysis of COOH-terminal deletion mutants suggested that Gln 123 might be important for actin interaction (Way et al., 1990). Changing this residue to lysine did not weaken binding (Fig. 7 and Table II). However, we further examined substitutions of Gln 123 in N126, a truncated mutant containing only the first 126 residues of S1 that binds actin much more weakly than S1 in calcium and not at all in EGTA (Way et al., 1990). The most conservative change tested (Q123E) did not affect actin affinity, but substitution by either alanine or lysine gave appreciably weaker binding (Fig. 7).

Finally, although substitution of Ala 101 by valine or glycine had no effect, when the three adjacent alanine residues were all converted to aspartic acid, the protein was completely inactive. Nevertheless it was fully soluble and stable to proteolysis by chymotrypsin and trypsin.

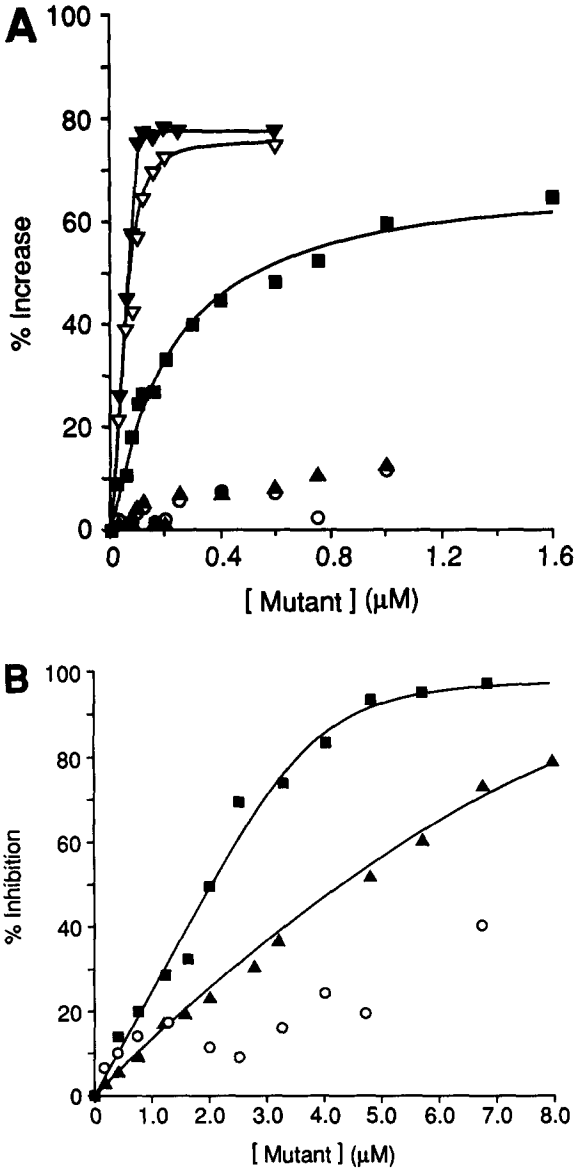

Figure 7. (*A*) Fluorescence enhancement of 100 nM NBD-actin with increasing concentrations of Q123K (▼) in S1. Solid symbols, calcium and open ones, EGTA. Curve in EGTA shows fit corresponding to K_d = 0.014 μM. These results are identical to control S1 (Way et al., 1990). Other results are for substitutions in deletion mutant N126 in calcium: Q123K (○), Q123A (▲), and Q123E (■), with curve fitted for K_d = 0.15 μM. (*B*) Percent inhibition of actin polymerization using 3.9 μM actin for substitutions in deletion mutant N126 in calcium: Q123K (○), Q123A (▲), and Q123E (■), with curves fitted for K_d = 2 μM and 0.1 μM for the latter two, respectively.

Discussion

We have previously shown that the K_d of S1 for actin is 5–10 nM, based on NBD-actin titrations and polymerization inhibition experiments. However this value greatly exceeds the value of ~5 pM, based on the association and dissociation rate constants of etheno-ATP exchange (Bryan, 1988), but which could not be measured by equilibrium methods. The dissociation rate constant for the complex was ~200-fold greater in EGTA than in calcium, indicating a K_d ~1 nM (Bryan, 1988). Our earlier experiments using COOH-terminal truncation of S1 confirmed this calcium dependence and identified a calcium binding site present only in the complex (Way et al., 1990). Using K_d values of 5 pM in calcium and 1 nM in EGTA and a limiting sensitivity in our assays of ~10 nM, it is clear that mutations resulting in a decrease of affinity <1,000-fold will be readily observed in EGTA, but not in calcium. K_d changes >1,000-fold will also be detected in calcium, but binding in EGTA will be virtually eliminated. (Interactive systems such as these have been analyzed in detail by Weber [1972], who showed that when ligands promote each other's binding, they necessarily stabilize the same conformation.) Hence by assaying binding in the presence and absence of calcium, it is possible to estimate changes in K_d over a total range >10^5. This corresponds to a standard free energy change of over 6 kcal/mol. Such a change exceeds that of hydrophobic interactions in the apolar core of proteins, where the loss of free energy of folding is 1.0–1.6 kcal/mol per methylene group removed (Kellis et al., 1989). It is also in excess of the free energy of a salt bridge in an apolar environment (3–5 kcal/mol) (Anderson et al., 1990). Hence, changing a single critical residue at the interaction site is sufficient to account for a change of K_d of 10^5.

NH_2 Terminal Mutants

Actin binding is lost when 45 residues are deleted from the NH_2-terminal end of S1, but not when 36 residues are removed (Way et al., 1990). Here we have shown that actin binding is retained until the Leu 41 is removed. Conservation of a hydrophobic residue at this position always holds true (Fig. 3). We cannot discriminate between loss of bind-

ing due to elimination of critical residues or incorrect folding of the polypeptide chain, but we favor the latter interpretation because the greatest decrease in the proportion of monomer (from 60 to 20%) occurred on removal of Leu 41. Of the point mutations tested in the NH_2-terminal region, only K48E showed reduced affinity for actin in EGTA. This suggests that Lys 48 may be involved in actin binding, which is consistent with the observation that in the complex this residue is cross-linked to one of the acidic residues at the NH_2 terminus of actin (K. Vancompernolle and J. Vandekerckhove, Laboratory for Genetics, State University Ghent, Belgium, personal communication).

COOH-Terminal Mutants

The amino acid sequence homologies between the COOH terminus of S1 and other actin severing or monomer binding proteins, especially the conservation of the sequence LDDYL (Fig. 2), have led to the suggestion that this region may be an important determinant in actin binding (Vandekerckhove, 1989; Vandekerckhove et al., 1989; Tellam et al., 1989). This conclusion is supported by COOH-terminal truncation of severin (Eichinger et al., 1991). Results based on elongation kinetics suggested that F-actin capping occurred when the related sequence LDDFL was present but not in its absence. However, this effect was observed only when high concentrations of mutant were used, showing that the capping affinity was very weak. This may explain our inability to demonstrate similar effects with COOH-terminally deleted mutants of gelsolin: there was no detectable actin binding when S1 had been truncated to Glu 121, which is nine residues COOH-terminal of the LDDYL sequence (Way et al., 1990). Different structural requirements for capping and monomer binding may also account for these observations. Nevertheless we have explored the importance of this and other sequences in the COOH-terminal region using point mutations, many of which had no detectable effect (Table II).

The LDDYL sequence of S1 might be functionally related to LLDYL in DNase I (Tellam et al., 1989). The atomic structure of actin-DNase I complex shows that the aspartic acid in this sequence forms a salt bridge with arginine 39 in an apolar pocket of actin (Kabsch et al., 1990). Since S1 and DNase I bind simultaneously to actin, their contact sites are different (Pope et al., 1991). Nevertheless, if the acidic residues in the LDDYL sequence of S1 were in contact with actin, substitution by basic ones would radically affect binding. No such effect was observed when the three central residues within the LDDYL sequence were changed. This is not unexpected based on the recent finding that gCAP39 (Yu et al., 1990) and Mbh1 (Prendergast and Ziff, 1991) contain no acidic residues in this region (Fig. 2).

Substitutions of either of the two leucines in the LDDYL motif and also of Val 117 by aspartic acid profoundly affected actin binding (Table III). Based on the resistance of V117D to chymotrypsin of V8 protease, this mutant is structurally similar to S1. L112D showed limited susceptibility to chymotrypsin, consistent with exposure of hydrophobic residues close to COOH-terminal end of the mutant. (We can rule out cleavage at the NH_2-terminal end because there are no residues labile to chymotrypsin that would give the observed pattern of products). When all three mutations were combined, actin binding was abolished. This triple mutant showed extensive self-association, indicating altered protein conformation. It seems likely that these apolar residues form part of the internal scaffold of S1: their substitution by charged residues prevents actin binding through disruption of protein structure rather than by direct involvement in actin binding.

S1 contains a tetrapeptide sequence DESG that is also found in β-actin, and related to DEAG in α-actin (Kwiatkowski et al., 1986). The glutamate in this sequence has been chemically cross-linked to Lys115 of *Acanthamoeba* profilin (Vandekerckhove et al., 1989). Tellam et al. (1989) suggested that DESG in S1 might be located at the actin binding site, based on the hypothesis that this sequence in F-actin was located at the subunit contact site and that gelsolin behaved as a pseudosubstrate. We found no diminution of actin binding when both Asp 96 and Glu 97 were changed simultaneously to lysine. Furthermore, the structural model of F-actin places the DESG sequence on the outside of the filament where it does not participate in subunit interactions (Holmes et al., 1990).

Charge substitutions at locations between residues 119 and 126 reduced actin binding. Glu 126 appears to be important for the stability and function of S1 (Table II and III). Even the most conservative substitution by aspartic acid reduced protein solubility and weakened actin binding. Earlier studies using COOH-terminal truncation showed no difference in actin affinity whether Glu 126 was present at the COOH terminus or removed (Way et al., 1990). Taken with our findings here, we suggest that Glu 126 is important to the stability of S1, but does not form a contact with actin. The highly conserved Glu 121 is more tolerant of change. Replacement by glutamine had little effect, but substitution by lysine caused dramatic loss of actin binding EGTA. Because charge substitutions at positions 119 and 120 also weakened actin binding, we suggest that residues 119–121 play a part in the contact site with actin.

The observation that COOH-terminal deletion of Gln 123 resulted in total loss of actin binding (Way et al., 1990), together with the similarities in this region with profilin (Vandekerckhove, 1989), suggests that Gln 123 might be involved at the actin contact. However, changing this residue in S1 to lysine did not weaken binding. Nevertheless, changes in binding affinity can occur that are undetected in our assays if the K_d remains below 0.01 μM. As an example of this, substitution of Gln 123 by alanine or lysine in N126 (a COOH-terminally truncated mutant showing weaker binding in calcium and none in EGTA) reduced actin affinity a further 20–50-fold, suggesting that this residue is involved in interaction.

Capping by S1

One striking difference observed in the inhibition of polymerization assays is that in calcium the curves extrapolate to zero in the absence of mutant, whereas in EGTA there is significant residual "inhibition" (Fig. 6). This suggests capping of the barbed ends of the filaments by S1 (this would not be detected in calcium because gelsolin is added as a nucleator). The mean displacement from zero in experi-

ments using S1 and 8 of the point mutants was 22 ± 5%, corresponding to an actin concentration of ~0.8 μM. This value compares with a difference in critical concentration from polymerizations measured in (*a*) calcium + gelsolin and (*b*) EGTA − gelsolin of 0.70 μM. This interpretation was supported by experiments in calcium but without gelsolin, which also showed finite inhibition of polymerization at zero mutant concentration and it was confirmed by critical concentration measurements ± calcium, with and without HR119EE. In the absence of mutant, the critical concentration was 0.1 μM ± calcium, but in the presence of 3 μM mutant, the values were 3.8 μM in calcium and 2.3 μM in EGTA. Assuming that capping by the mutant increases the critical concentration to 0.8 μM, the additional unpolymerized (complexed) actin (3.0 μM in calcium and 1.5 μM in EGTA) is consistent with K_d values for complex formation of <0.05 μM and 0.8 μM, respectively (values similar to those in Table III).

With the single exception of L108D, all mutants capped actin filaments in EGTA. Weber et al. (1991) have recently reported capping by S1 derived from gelsolin by proteolysis. Capping must occur by addition of complex, because complex formation takes place before polymerization is initiated and the concentration of free S1 is very low. Mixing 4 μM actin with 0.2 μM S1 gives <0.5% free S1 based on K_d = 0.01 μM. Capping by S1 actin complex is not unexpected, since Selve and Wegner (1986) have reported that binary complexes of gelsolin and actin bind the barbed ends of actin filaments in EGTA with a capping affinity of $>10^{10}$ M^{-1}.

In summary, over half the substitutions tested had no effect on actin binding. Most of these involved replacement of charged residues, probably located at the surface of S1. However, we have identified residues in the vicinity of His 119-Arg 120-Glu 121 as possibly being located at the interaction site with actin. Proof of our interpretation must await solution of the three-dimensional structure of the complex.

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