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Identification of a region in segment 1 of gelsolin critical for actin binding

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The actin severing and capping protein gelsolin contains three distinct actin binding sites. The smallest actin binding domain of ~15 000 M_r was originally obtained by limited proteolysis and it corresponds to the first of six repeating segments contained in the gelsolin sequence. We have expressed this domain (here termed segment 1 or N150 to define its amino acid length) in *Escherichia coli*, together with a series of smaller mutants truncated at either N- or C-terminal ends, in an attempt to localize residues critical of actin binding. Limited truncation of segment 1 by 11 residues at its N-terminal end has no observable effect on actin binding, but on removal of a further eight residues, actin binding is totally eliminated. Although this loss of actin binding may reflect ablation of critical residues, we cannot rule out the possibility that removal of these residues adversely affects the folding of the polypeptide chain during renaturation. Truncation at the C-terminus of segment 1 has a progressive effect on actin binding. Unlike intact segment 1, which shows no calcium sensitivity of actin binding within the resolution of our assays, a mutant with 19 residues deleted from its C-terminus shows unchanged affinity for actin in the presence of calcium, but ~100-fold weaker binding in its absence. Removal of an additional five residues from the C-terminus produces a mutant that binds actin only in calcium. Further limited truncation results in progressively weaker calcium dependent binding and all binding is eliminated when a total of 29 residues has been removed. Although none of the expressed proteins on their own binds calcium, ^{45}Ca is trapped in the complexes, including the complex between actin and segment 1 itself. These results highlight a region close to the C-terminus of segment 1 that is essential for actin binding and demonstrate that calcium plays an important role in the high affinity actin binding by this domain of gelsolin.

Key words: actin binding/calcium/gelsolin

Introduction

Gelsolin is a calcium dependent actin severing and capping protein found universally in vertebrate tissues (Yin *et al.*, 1981; Kwiatkowski *et al.*, 1988). It also interacts with two actin monomers and nucleates the formation of capped filaments (Bryan and Kurth, 1984; Weeds *et al.*, 1986). Although the cellular function of gelsolin is unclear, a role in cell motility is suggested from the observation that levels of both gelsolin mRNA and protein increase drastically coincident with the cytoskeletal rearrangements that occur upon differentiation and locomotion (for review see Way and Weeds, 1990).

Amino acid sequence homologies between gelsolin, villin, fragmin and severin suggest that these proteins are members of a superfamily that have apparently evolved from a smaller 15 000 M_r repeating unit or segment (Kwiatkowski *et al.*, 1986; Ampe and Vandekerckhove, 1987; André *et al.*, 1988; Bazari *et al.*, 1988; Way and Weeds, 1988). The six segments (S1-6) in gelsolin (Way and Weeds, 1988) are distributed unevenly within the three actin binding domains identified by limited proteolysis (Bryan, 1988; Yin *et al.*, 1988). Segment 1 binds one actin monomer with high affinity; S2-3 bind stoichiometrically to F-actin subunits: both these activities are calcium independent. By contrast, S4-6 bind a single actin monomer but only in the presence of calcium. Studies with proteolytically derived fragments have shown that the severing activity of gelsolin requires both the G- and F-actin binding domains in the N-terminal half of the molecule (S1-3) (Bryan, 1988; Yin *et al.*, 1988). Further characterization of the three binding sites and their roles in the activities of gelsolin has been achieved using segmentally deleted mutants expressed in *Escherichia coli* (Way *et al.*, 1989) and C-terminal truncations expressed in Cos cells (Kwiatkowski *et al.*, 1989) respectively. These studies have provided much more information about the roles of the individual actin binding domains, but the exact locations of the actin binding sites have yet to be defined.

Segment 1 is the smallest domain of gelsolin that interacts with actin. Compared with other segments, segment 1 shows the highest level of homology within the family of actin severing and capping proteins (Way and Weeds, 1988). Furthermore, it contains a region of amino acid sequence homology with other actin monomer binding proteins: profilin, cofilin, destrin and actin depolymerization factor (Adams *et al.*, 1990; Matsuzaki *et al.*, 1988; Moriyama *et al.*, 1990; Vandekerckhove, 1989). This region of ~30 residues, at the C-terminus in *Acanthamoeba* profilin, is located close to actin in the complex, based on the observation that it can be cross-linked to the C-terminus of actin with a zero-length cross-linker (Vandekerckhove *et al.*, 1989). Vandekerckhove (1989) has suggested that this region of profilin may be an important conserved determinant in the actin binding properties of all these proteins.

Here we have begun to define the minimal region of segment 1 required for actin binding by examining the properties of a range of deletion mutants (Figure 1).

Results

Expression and purification of mutants

Pre-induction samples of total cell protein of N150 and all mutant expression constructs showed no obvious extra bands,

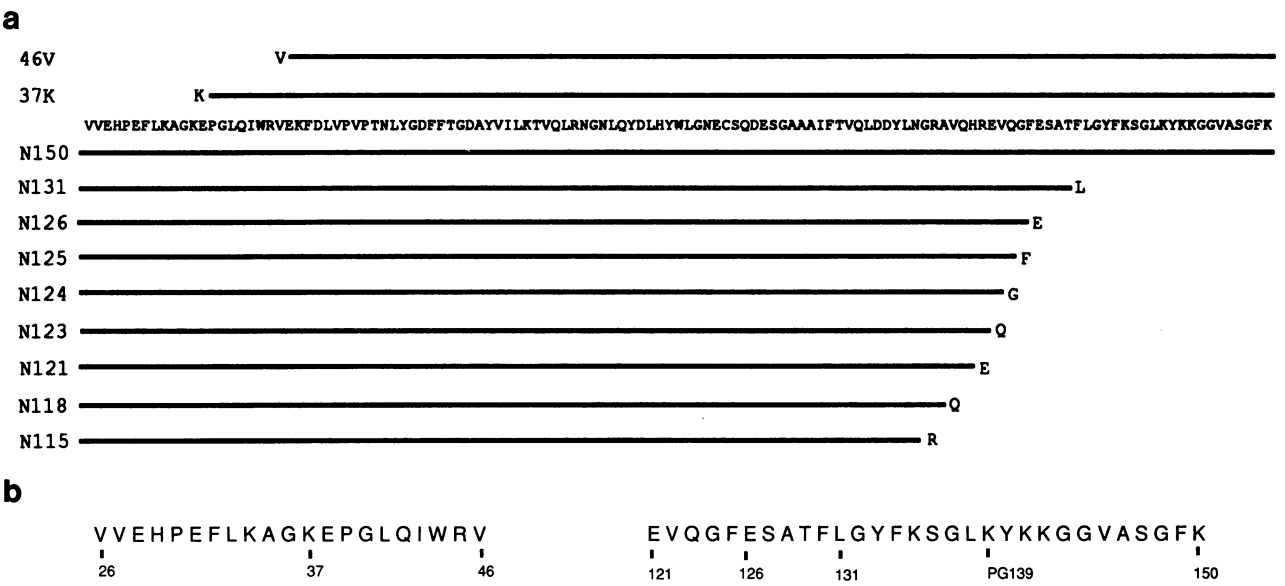

Fig. 1. Indicates the complete amino acid sequence of segment 1 from the beginning of the cytoplasmic form of gelsolin, i.e. the 25 residue plasma extension sequence has been omitted. All deletion mutants are shown schematically in relation to this sequence along with their terminal amino acid residue and name. The N- and C-terminal sequences are shown enlarged below.

but post-induction samples showed strong bands corresponding to the presence of proteins of the expected size. Figure 2 shows the purification of N150. Expression levels and purification of all mutants were similar to those of N150, although smaller C-terminal deletion mutants tended to give slightly lower yields (standardly in excess of 100 mg pure protein per litre culture).

Actin–Sepharose binding

N150 bound strongly in the presence or absence of calcium as did 37K (Figure 3 and Table I). N131 gave similar binding to N150 in calcium but showed weaker binding in EGTA. Further truncations from the C-terminus down to N121 resulted in a gradual decrease in binding in the presence of calcium and virtually complete loss of binding in EGTA. There was insignificant binding of N118 and N115 even in calcium.

Inhibition of polymerization

Increasing concentrations of N150 reduced the amount of polymerized actin (Figure 4A) but did not affect the rate of gelsolin-nucleated assembly (data not shown). Total inhibition of polymerization occurred at a mutant/actin ratio of 1:1 both in the presence and absence of calcium. This is consistent with the high affinity of binding observed earlier with segment 1 prepared by proteolysis (Bryan, 1988). Identical results were obtained with 37K. N131 also showed equally strong binding in calcium, but higher concentrations of N131 were needed to inhibit polymerization in the absence of calcium, suggesting much weaker binding affinity (Figure 4A).

N126 showed complete calcium dependence in the inhibition of polymerization assay (Figure 4B) and mutants smaller than this were similarly inactive in the absence of calcium (data not shown). Although N124–N126 showed similar effects on actin polymerization, N123 appeared to be much less active and N121 had no inhibitory effect on actin polymerization, indicating negligible interaction under these conditions. N118 and N115 were also negative in this assay (results not shown).

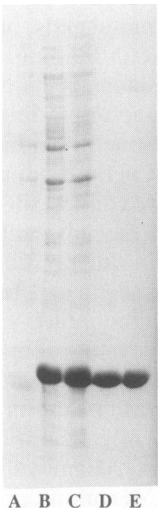

Fig. 2. Expression and purification of N150 on SDS–PAGE. **A**, pre-induction total cell protein; **B**, post-induction total cell protein; **C**, inclusion body preparation; **D**, protein not retained on DE-52; **E**, purified protein from CM-52.

Similar conclusions were obtained from measurements of the critical concentration of actin in the presence of the mutants (Figure 5). The increase in critical concentration was identical using N124–N126 (1.2 mol actin/mol mutant), whereas N123 gave an increase of 0.88 mol actin/mol, consistent with a lower binding affinity. N121 had no effect on the critical concentration. Fluorescence measurements of N126 in EGTA were also coincident with control values (not shown).

NBD–actin titrations

Titration with NBD–actin gives a more sensitive assay of binding affinity, provided that interaction affects the quantum

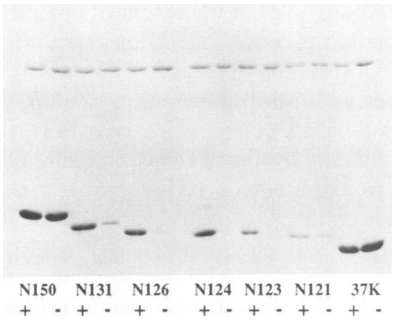

Fig. 3. Binding of mutants to actin–Sepharose ± calcium. Resin samples analysed by SDS–PAGE. The upper band (actin) shows that loadings were equivalent for all samples. Thus relative intensities reflect extent of binding of the different mutants. + with calcium; −, without calcium.

Table I. Binding of mutants to actin–Sepharose ± calcium and calcium binding to mutants and their complexes with actin

Mutant	% Binding to actin–Sepharose		% Ca sensitivity	^{45}Ca binding	
	Ca	EGTA		Mutant	Complex
N150	100	100	4	0.13	0.63
N131	76	28	63	<0.1	0.55
N126	68	17	76	<0.1	0.45
N125	69	21	70	<0.1	<0.1
N124	53	13	77	<0.1	<0.1
N123	51	13	75	<0.1	<0.1
N121	28	15	46	n.d.	n.d.
N118	12	7	42	n.d.	n.d.
N115	18	14	25	n.d.	n.d.
37K	100	81	17	<0.1	0.54
46V	39	41	0	n.d.	n.d.

n.d. = not determined.
At least four separate calcium determinations were made for complexes with N150, N131, N126, N125 and 37K.

yield of the fluorophore. Both N150 and N131 showed a sharp transition at a stoichiometry of 1:1 in their fluorescence enhancement measured as a function of mutant concentration in the presence of calcium, consistent with a K_d value in the nM range (Figure 6A). The extent of fluorescence enhancement with N150 in EGTA was similar to that in calcium, though the binding affinity appeared to be marginally weaker, based on the initial slope of the plot. By contrast, N131 gave no fluorescence enhancement of NBD–actin in EGTA. Although the affinity of this mutant for actin is weaker than that of N150 in EGTA (Figure 4A), this total absence of fluorescence enhancement was unexpected. It is noteworthy that 37K, which showed similar binding properties to N150 in the polymerization assay, also failed to increase the fluorescence of NBD–actin, both using the standard titration assay of Figure 5 and when the two proteins were mixed at 10 μM concentration of each.

N123–N126 enhanced the fluorescence of NBD–actin in calcium, but the marked curvature of the plots indicates much weaker binding affinity (Figure 6B). The fitted curves in Figure 6B assume 1:1 complex formation and suggest K_d values for N124–N126 of 0.25–0.48 μM, with maximum fluorescence enhancement of 84% (similar to that in

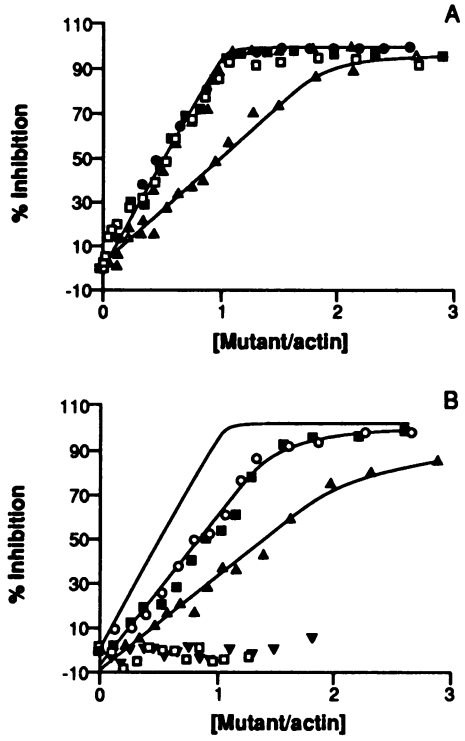

Fig. 4. Percent inhibition of actin polymerization related to the ratio of the concentration of mutant to polymerizable actin. **A**, N150 (●); N131 (▲); 37K (■); solid symbols in calcium and open symbols in EGTA. **B**, N126 (■); N124 (○); N123 (▲); N121 (▼) (all in calcium); N126 in EGTA (□). The solid line in B is taken from N150 values in A. The lines through the data points in A correspond to K_d values of 10 nM and 750 nM: those in B to 600 nM (N124, N126) and 1.3 μM (N123), with a line corresponding to 10 nM also shown.

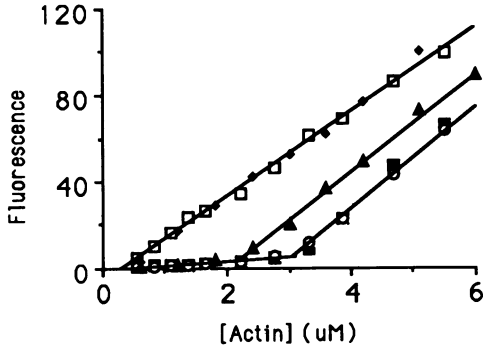

Fig. 5. Effects of mutants on the steady state concentration of polymerized actin measured by fluorescence of pyrene actin. Control actin nucleated in calcium with gelsolin (□); nucleated polymerization in the presence of 2.25 μM N126 (■); N124 (○); N123 (▲); N121 (◆).

Figure 6A). The extent of fluorescence enhancement with N123 is much less and the data are consistent with a maximum enhancement of 46% and K_d = 0.73 μM. No fluorescence enhancement was expected in EGTA, since these mutants do not bind actin in the absence of calcium. Nor was there any fluorescence enhancement in calcium by the smaller mutants N121, N118 and N115 which showed no binding in other assays.

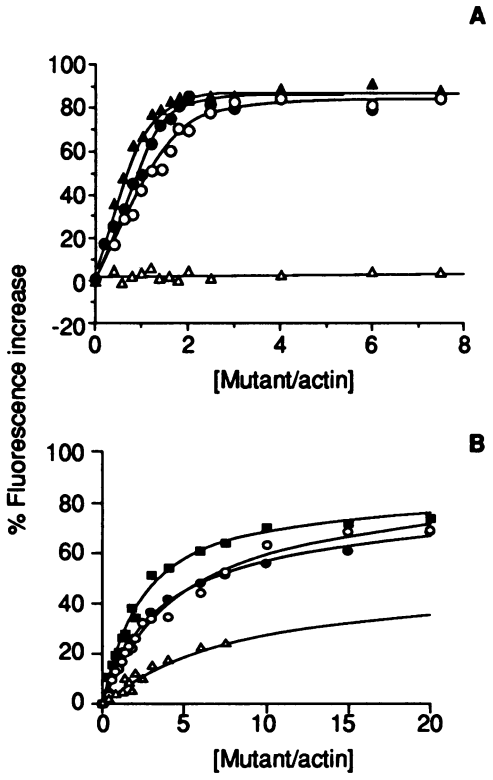

Fig. 6. Fluorescence enhancement at increasing mutant concentrations using 100 nM NBD−actin. **A.** N150 in calcium (●); N150 in EGTA (○); N131 in calcium (▲); N131 in EGTA (△). **B** N126 (■); N125 (●); N124 (○); N123 (△) all in calcium. The lines through the data points in A correspond to K_d values of 5 nM, 10 nM and 40 nM respectively; those in B to 250 nM (N126), 340 nM (N125), 480 nM (N124) and 730 nM (N123).

Calcium dependence of actin binding

Based on its effects on actin polymerization and the extent of binding to actin−Sepharose (Figures 3 and 4), N123−N126 showed negligible interaction with actin in the absence of calcium. The concentration of calcium required for interaction was determined from measurements of the extent of inhibition of actin polymerization for N126 as a function of free calcium concentration. 50% binding activity occurred at ~0.6 μM free calcium.

Calcium binding to mutants and their complexes with actin

Although negligible ^{45}Ca binding was detected using the rapid calcium binding assay for N150 or any of the mutants, complexes with actin showed significant binding, particularly N150, N131, N126 and 37K (Table I). The amount of calcium bound varied with the extent of washing of the filters. Values for complexes with N150 approaching 1 were obtained with mimimal washing, but the background counts were very much higher, (>70% of the counts for N150 complexes compared with <20% for samples with washing).

Calcium binding to complexes with N150, N131 and N126 was also measured by equilibrium dialysis, using G-actin containing 15% NBD−actin to assess complex formation. In two experiments with N150, the bound calcium was 1.11 and 0.98 mol/mol complex, compared with a value of 0.26 mol/mol for actin alone and 0.0 for N150 (a net binding of ~0.8 mol/mol complex). Similar experiments for complexes with N131 and N126 gave net binding of 0.54 and 0.18 mol/mol respectively, but the extent of complex formation measured by fluorescence enhancement was 75% for N131 and ~25% for N126.

Complexes with N150 prepared in calcium and dialysed into the same buffer containing excess EGTA showed no reversal of binding by fluorescence measurements, but the net bound calcium was reduced somewhat, to 0.54 mol/mol complex. By contrast, complexes with N131 treated similarly showed almost complete reversal of the fluorescence enhancement, consistent with dissociation of the complex and the bound calcium was also totally removed.

Discussion

Our results show that segment 1, the smallest actin binding domain of gelsolin, can be truncated to a limited extent at either end without loss of actin binding. Removal of 11 residues of the N-terminal cytoplasmic sequence in 37K does not affect monomer binding as measured by inhibition of polymerization or affinity chromatography. The very sharp transition seen in Figure 4A for 37K is indistinguishable from that of N150 and suggests high affinity binding. However, there was no fluorescence enhancement of NBD−actin by 37K. This may be explained either because NBD−actin differs from unlabelled actin in not binding 37K, or because the quantum yield of the NBD−actin is unchanged in the complex. Although there is precedent for chemical modification at the C-terminus of actin having an adverse effect on the binding of profilin (Malm, 1984), we can see no reason why 37K should not bind NBD−actin, when it clearly binds PI−actin. Thus it is likely that loss of fluorescence enhancement is caused by a difference in the local environment of the NBD reporter group due to deletion of these residues.

Deletion of an additional nine residues in 46V abolished actin binding altogether. The sequence deleted between 37K and 46V includes a tryptophan residue, conserved in segments 1 and 4 of gelsolin and present also in villin, severin and fragmin. Bazari *et al.* (1988) suggested that this residue might be of structural or functional significance. Furthermore, lysine 48, also in this region of segment 1 has been chemically coupled to the N-terminus of actin with a zero-length cross-linker suggesting close proximity in the complex (Vancompernolle *et al.*, 1990). Although loss of actin binding may indicate removal of essential residues in this region, it may equally reflect incorrect folding of the truncated protein caused by the absence of important secondary structural features. Tsou (1988) has recently suggested that if folding of nascent proteins is co-translational, removal of N-terminal sequences may severely disrupt the refolding of denatured proteins. At present it is not possible to distinguish between these two possibilities without further experiments to study the conformation of the proteins.

A more extensive series of deletion mutants at the C-terminal end has highlighted a region of segment 1 crucial for actin interaction. N131 showed binding properties in calcium virtually indistinguishable from N150 under all conditions tested. The abrupt transition in the NBD−actin titration for N131 and N150 is consistent with high affinity binding: assuming a 1:1 complex, the data can be fitted to a K_d of 5−10 nM. However, N131 differs from N150 in showing much lower affinity for actin in EGTA, as seen

both from inhibition of polymerization and affinity chromatography. Using the simple model described by Tabacman *et al.* (1983) for profilin binding, the data in Figure 4A can be fitted to a K_d value of 750 nM. (A similar K_d value was obtained using a more complex analysis taking into account also the co-operativity of actin polymerization as outlined by Oosawa and Asakura, 1975.) Thus removal of the C-terminus of segment 1 has reduced the affinity of N131 for actin in EGTA by a factor of 100 compared with its binding in calcium.

N131 shows no fluorescence enhancement when mixed with NBD−actin in EGTA (Figure 6A). Assuming a K_d of 0.75 μM, the maximum enhancement expected at a 10-fold excess of mutant is ~47%, which is ten times greater than that observed. Thus the absence of any fluorescence increase is not due to the higher K_d value, but may reflect changes in the local environment of the NBD reporter group, as also appears to be the case for 37K. It is surprising that deletions from both N- and C-termini of segment 1 affect the environment of the NBD residue of Lys374 of actin, since it suggests that the two ends of segment 1 are close together. However, this is also the case for actin itself (Kabsch *et al.*, 1990). It is also surprising that N131 should give no fluorescence increase in EGTA, when it enhances the fluorescence of NBD−actin in calcium as also do mutants containing more extensive truncations. However, quantum yields can be greatly affected by local conformational changes, solvent cage relaxation and any processes coupled to translational or rotational motion that affect the lifetime of the singlet state. Such changes can be relatively small and would not necessarily affect the affinity of interaction, particularly if the surface interface between the two proteins is extensive.

Sutoh and Yin (1989) reported that segment 1 could be cross-linked to a region containing the first 18 residues of actin. Although the residues concerned in both actin and segment 1 were not identified, they suggested that the acidic residues at the N-terminus of actin, which are highly exposed at the surface (Kabsch *et al.*, 1990), were prime candidates for this chemical attachment. This high negative charge density in actin might interact with a similar high density of basic residues in segment 1. The 19 residues deleted in N131 form the major part of a sequence of 24 residues containing no acidic groups and is unique in N150 in having four closely spaced basic residues in the short sequence KSGLKYKK (Figure 1). Clearly this region of high basic charge is not essential for actin interaction. In a recent report, Vancompernolle *et al.* (1990) have demonstrated cross-linking between Lys48 and one of the three acidic residues at the N-terminus of actin. An alternative possible role for this region of highly concentrated basic charge might be in binding the hypothetical actin-like pseudo substrate DDD (residues 737−739 of gelsolin), the presence of which is thought to stop all actin interactions in the absence of calcium (Kwiatkowski *et al.*, 1989).

Deletion beyond N131 produced dramatic changes in actin binding. N126, N125 and N124 were totally calcium dependent in their actin binding and showed significantly lower affinities than N131. K_d values of 250−500 nM were obtained for all three mutants from the NBD−actin titrations, and values around 600 nM from polymerization inhibition measurements. N123 has an even weaker affinity for actin: a K_d ~700 nM was calculated from the fluorescence data in Figure 6B (with a corresponding maximum enhancement of 46%). However, if the true maximum is 80−90%, as observed with N124−N126, N131 and N150, then the dissociation constant is much higher. This conclusion is supported by the polymerization inhibition measurements which can be fitted to K_d of 1.3 μM (Figure 4B). Further truncation to N121 results in total loss of actin interaction when measured by fluorescence assays. Residual apparent weak binding on actin−Sepharose is close to non-specific levels.

These results highlight the importance of residues between Val122 and Leu131 for calcium dependent actin binding and show that truncation of the C-terminus of segment 1 converts it from an apparently calcium independent form to a calcium dependent one. The concentration of calcium required for half-maximal binding for N126 was ~0.6 μM, similar to that required to activate gelsolin nucleating and severing activities. This is also the value of the dissociation constant of calcium from gelsolin (Way *et al.*, 1989), but it differs considerably from the calcium binding affinity of actin alone (Carlier *et al.*, 1986; Zimmerle *et al.*, 1987). However, no calcium binding was detected with N150, N131 or N126 using equilibrium dialysis against 23 μM $^{45}CaCl_2$. This result is not unexpected based on earlier measurements of calcium binding to gelsolin, S1−3, S4−6 and S2−6 (Way *et al.*, 1989).

A study of ^{45}Ca binding to complexes using a rapid filter assay showed that the complex between N131 and actin retained ~0.55 mol bound calcium/mol. A similar value was obtained in a less extensive study using equilibrium dialysis, but complex formation, assessed by fluorescence enhancement of NBD−actin, was only ~75%, suggesting that the calcium bound in the complex was >0.7 mol/mol. Thus the low values obtained for a number of the mutants in Table I reflect incomplete complex formation as well as loss of bound calcium during washing procedures. Although intact segment 1 shows little difference in affinity for actin in our assays [the K_d for the NBD−actin titration of N150 (Figure 6A) is ~10 nM in calcium and ~40 nM in EGTA], we found a net ~0.6 mol/mol bound ^{45}Ca using the rapid filter assay and a value >0.8 by equilibrium dialysis. This provides strong evidence that calcium plays a part in complex formation by segment 1 itself. Complexes formed by N150 in calcium were not dissociated in EGTA (<10 nM free Ca^{2+}), as evidenced by fluorescence enhancement of NBD−actin, and although there was a reduction in bound calcium to ~0.54 mol/mol, much of the calcium remained trapped.

These results suggest that interaction between segment 1 and actin creates an additional calcium binding site, distinct from the calcium sites in gelsolin. This was confirmed in control experiments on ^{45}Ca binding to ternary complexes of gelsolin with two actin monomers which gave 2.8 mol calcium bound, compared with 2.0 for gelsolin alone.

Previous studies have demonstrated calcium 'trapping' in the EGTA stable gelsolin−actin binary complex (Bryan and Kurth, 1984). It is now clear that the actin in this complex binds uniquely to segment 1 (Bryan, 1988; Way *et al.*, 1989). The question arises whether segment 1 shows calcium dependent actin binding, that could not be detected within the resolution of these assays. Bryan (1988) reported a lower apparent affinity of NT14 for actin in EGTA, based on a more rapid off-rate for etheno-ATP dissociation from the

complex in the absence of calcium, but suggested that the calcium might stabilize nucleotide binding rather than complex formation itself. Nevertheless, his experiments suggested a K_d for actin ~1 nM in EGTA compared with a value of ~5 pM in calcium. Recent measurements of association and dissociation rate constants for complex formation have suggested a K_d for NT14 of 10 mM in EGTA and ~1000-fold lower in calcium (Weber *et al.*, 1990). Thus the calcium sensitivity 'recovered' in N131 is not a new activity: truncation of segment 1 has simply reduced the actin affinity to a range where the calcium dependence can readily be measured.

The role of calcium in complex formation is seen best with the truncated mutants N123–N126 which show no actin binding in EGTA. These mutants have actin binding behaviour similar to that of the equivalent N-terminal fragment of villin, termed 14T by Janmey and Matsudaira (1988). This proteolytic fragment inhibits actin polymerization in calcium but not in its absence. Futhermore, its affinity for actin is markedly less than the N-terminal severing domain of villin from which it was obtained. It is noteworthy that the severing domain of villin retains calcium sensitivity unlike the equivalent domain of gelsolin. Since segment 1 is essential for the severing activity of gelsolin (Way *et al.*, 1989), the difference in calcium sensitivity between these two proteins probably arises because of the very large difference in binding affinities of their respective segment 1 components. This could be tested if a modified form of S1–3 of gelsolin containing a truncated segment 1 was shown to have calcium dependent severing activity.

The recovery of calcium sensitivity in segment 1 parallels a similar finding in C-terminal truncations of S1–3 (Kwiatkowski *et al.*, 1989). The calcium independent severing activity of S1–3 was retained in a truncated mutant, PG260, the C-terminus of which coincides with the end of segment 2. However, further truncation to PG237 gave a restoration of calcium dependence, which was retained down to the size of PG160. Interestingly PG237 in segment 2 has a C-terminal alignment corresponding exactly to the critical Gly residue 124 of segment 1 (Way and Weeds, 1988). Thus recovery of calcium dependence occurs with deletions at the same position of motif C in both segments 1 and 2 of gelsolin, suggesting that this motif is an important determinant for actin binding.

Although profilin does not contain a region corresponding to motif C, there are three residues of motif C, QGF, critical for actin binding in segment 1, which are found at the C-terminus of *Acanthamoeba* profilin (Vandekerckhove, 1989). It will be interesting to find out whether removal or alteration of these residues will affect actin binding to profilin. We are currently investigating the contribution of these and other individual residues in this critical region of segment 1.

Materials and methods

Nomenclature of the mutants

Mutants of segment 1 truncated at their C-terminal ends are defined on the basis of their terminal residue e.g. N150 (corresponding to intact segment 1) contains residues 1–150 of human plasma gelsolin, plus an additional N-terminal 7 residues (MGSIEGR) retained as a consequence of re-engineering from the earlier expression system (Way *et al.*, 1989). 37K and 46V are named from their first amino acid residue, but contain only MGS at their N-terminus.

Unless otherwise stated all DNA manipulations were performed according to standard procedures (Maniatis *et al.*, 1982). The expression vector pRK172 (McLeod *et al.*, 1987) based on the original pET vectors of Studier (Studier *et al.*, 1990) containing a modified polylinker (pKN172 Figure 7)

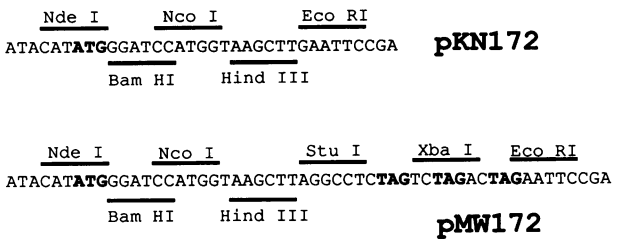

Fig. 7. Shows the polylinkers in the vectors pKN172 and pMW172. In both cases the initial Met codon is indicated in bold, as are the terminating codons in all three phases in pMW172.

was the kind gift of Kiyoshi Nagai. This vector was further modified by the insertion of a blunt restriction enzyme site (*StuI*) and stop codons in all three phases as follows: two complementary oligonucleotides MWA 5′-GATCCATGGTAAGCTTAGGCCTCTAGTCTAGACTAG-3′ and MWB 5′-AATTCTAGTCTAGACTAGAGGCCTAAGCTTACCATG-3′ were kinased, annealed to each other and ligated into pKN172 that had been previously digested with *Bam*HI and *Eco*RI. pMW172 (Figure 7) containing the modified polylinker was initially selected by restriction mapping prior to confirmation by sequencing.

Construction of N150 (segment 1) and N131

The oligonucleotide MWN150 5′-GCATCAGGATTCAAGTGAAAGCTT-CACGTGGTACCCAAC-3′ was used to insert stop-*Hin*dIII site (underlined) after amino acid residue 150 as previously described (Way *et al.*, 1989). N150 was sequenced throughout its entire length before being subcloned into *Bam*HI–*Hin*dIII cleaved pKN172 vector, transformed and expressed in the *E.coli* strain BL21(DE3) (Studier and Moffatt, 1986).

The expression construct N150 was uniquely cleaved by *Avr*II, treated with Mung Bean nuclease (Pharmacia) and re-ligated in the presence of kinased *Xba*I/stop linkers (CTAGTCTAGACTAG) (New England Biolabs). (This was performed before pMW172 became available.) N131 was selected by restriction mapping prior to sequencing throughout its entire length. Although the first stop codon is not in frame, the second TAG adds on an additional Leu and Ala at the C-terminus compared with Leu and Gly in the wild type; amino acid residue 131 being a Leu in the native sequence.

Construction of mutants

The entire coding region of N150 was subcloned into M13mp19 on a *Bam*HI and *Hin*dIII fragment and grown in CJ236 to produce single stranded *dut ung* DNA template (Kunkel *et al.*, 1987). Mutagenesis was performed as previously described (Way *et al.*, 1989) with the exception that the final ligated reaction was transformed into JM101 and positive clones identified by sequencing rather than by hybridization with the mutagenic oligonucleotide. All C-terminal deletion mutants produced by the insertion of a stop codon (TAG) and *Stu*I restriction site were subcloned into pMW172 as *Bam*HI–*Stu*I fragments. N-terminal deletion mutants were engineered by the insertion of a *Bam*HI restriction site and subcloned as *Bam*HI–*Hin*dIII fragments. All mutants (Figure 1) were sequenced throughout their entire length before expression in BL21(DE3).

Purification and expression of mutants

One litre cultures of BL21(DE3) containing segment 1 constructs were grown at 37°C and induced by the addition of IPTG (Sigma) to a final concentration of 0.6 mM at a cell density of A_{600} = 1.0 ± 0.1. Cells were harvested 3 h post-induction and processed as described in Way *et al.* (1989) with the exception that lysozyme was omitted because it co-migrated with some of the mutants in the purification.

All mutants were purified from inclusion bodies except N150 which was purified from both soluble and inclusion body fragments. Inclusion body preparations were solubilized in 10 mM Tris–HCl pH 8.0, 0.2 mM EGTA, 1 mM NaN_3 (buffer A) containing 8 M urea and subsequently clarified at 30 000 *g* for 15 min. Following dilution of the urea to 6 M using buffer A, the mutants were loaded onto a column of Whatman DE52 resin equilibrated in buffer A. All mutants, with the exception of N150 bound to the resin and were eluted with a linear salt gradient to 0.3 M NaCl. N150 was collected in the flow through leaving impurities bound and subsequently fractionated on CM-52 at pH 6.0 with a salt gradient (Pope *et al.*, 1989). All purified mutants were dialysed into buffer A and stored either frozen or in 50% glycerol at −20°C.

Protein concentrations were based on A_{280} values calculated on the basis of their tyrosine and tryptophan content. These values were confirmed by Bradford or Lowry protein determinations. Final yields of purified protein varied between 100 and 250 mg/l of original cell culture. SDS–PAGE was carried out as described previously (Weeds *et al.*, 1986).

Assays for interaction with actin

(i) The effects of the mutants on the extent of actin polymerization were assayed essentially as described previously (Way *et al.*, 1989). When 4 μM G-actin containing 0.6 μM PI−actin in G′ buffer (10 mM Tris−HCl pH 8.0, 0.2 mM ATP, 0.2 mM dithiothreitol, 1 mM NaN_3 and 0.2 mM $CaCl_2$) was polymerized in the presence of 60 nM gelsolin by addition of 3 mM $MgCl_2$ and 0.1 M NaCl, reaction was complete within 10 min as assessed by fluorescence enhancement. The same reaction mixture incubated for 20 min in the presence of various concentrations of segment 1 mutants results in a reduction of the polymerizable actin, in proportion to the amount of monomer sequestered by the mutant. The concentration of polymerized actin was calculated from the fluorescence yield and percent inhibition of polymerization plotted against the ratio of the concentration of mutant to polymerized actin. The fluorescence increment per μM F-actin was determined from the slope of the critical concentration plot [see (iv)]. Similar experiments were carried out in the same buffer but with 0.3 mM $MgCl_2$ and 0.2 mM EGTA in place of calcium.

Calcium dependence of actin interaction was measured at pH 7.4 in buffers containing 0.5 mM EGTA and free calcium concentrations between 0.05 and 85 μM, based on the dissociation constants for Ca−EGTA of Harafuji and Ogawa (1980).

(ii) Titrations with 100 nM NBD−actin were carried out as described in Weeds *et al.* (1986) in G′ buffer, or in the same buffer containing 0.3 mM $MgCl_2$ and 0.2 mM EGTA in the place of calcium. Bovine serum albumin was present at 0.1 mg/ml in all samples to prevent losses due to adsorption to surfaces. K_d values were estimated from single site binding curves using the Marquardt non-linear fitting method (Press *et al.*, 1988).

(iii) Binding to actin−Sepharose was performed as follows: monomeric actin was coupled to Sepharose at 1 mg/ml bed volume (Pope and Weeds, 1986) and 200 μl poured into 1 ml disposable syringes plugged with glass wool. Columns were equilibrated with F-buffer (10 mM Tris−HCl pH 8.0, 0.1 M NaCl and 1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.2 mM $CaCl_2$ and 1 mM NaN_3) or the same buffer containing 1 mM EGTA. Mutant proteins were filtered through a 0.2 μM Millipore filter before use and loaded at 5-fold molar excess over coupled actin. Columns were washed with 10−30 volumes of the loading buffer and 100 μl samples of resin removed for analysis by polyacrylamide gel electrophoresis in SDS. Checks for non-specific binding were made either using Sepharose resin coupled to N150 or by inclusion of 0.1% gelatin in the buffers. Quantitation was by densitometry using a Camag flat bed electrophoresis scanner. Bound mutant is related to the actin concentration on the gel. Although all the actin should be covalently bound to the Sepharose, a small proportion is always seen on the gels and this provides a useful means of normalizing the extent of binding of the different mutants. Values are normalized to N150. Per cent calcium sensitivity is based on the extent of binding ± calcium for each mutant: {100 * (mutant bound in calcium−mutant bound in EGTA)/(mutant bound in calcium)}.

(iv) The critical concentration of actin polymerization was measured as the inflection point of fluorescence vs. total actin concentration as described by Cooper *et al.* (1984). Samples containing 0−7.0 μM actin (including 15% PI−actin) were polymerized overnight in the presence or absence of 2−3 μM mutant before the steady state fluorescence was measured.

Preparations of PI−actin (actin reacted on Cys374 with N-(1-pyrenyl) iodoacetamide) and NBD−actin (actin reacted with N-ethyl maleimide on Cys374 then on Lys373 with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole) and fluorescence measurements were made as described previously (Weeds *et al.*, 1986).

Calcium binding assays

Rapid calcium binding assays were carried out on nitrocellulose filters as described previously (Way *et al.*, 1989). Values for complexes with actin were corrected for calcium binding to actin controls on the same filters.

Calcium binding was also measured by equilibrium dialysis as described previously (Weeds *et al.*, 1986), using 1 ml samples containing ~10 μM actin, mutant or complexes of the two and dialysing against 100 ml of modified G′-buffer (containing 23 μM $^{45}CaCl_2$ ± 100 μM EGTA and 50 μM $MgCl_2$ (giving a free [Ca] in EGTA of <10 nM). At 50 μM magnesium concentration, actin does not polymerize, and although much of the magnesium is chelated by ATP, the free magnesium concentration is ~13 μM, sufficient to saturate the high affinity site on the actin (Gershman *et al.*, 1986).

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References

Adams,M.E., Minamide,L.S., Duester,G.L. and Bamburg,J.R. (1990) *Biochemistry*, **29**, 7414−7420.
Ampe,C. and Vandekerckhove,J. (1987) *EMBO J.*, **6**, 4149−4157.
André,E., Lottspeich,F., Schleicher,M. and Noegel,A. (1988) *J. Biol. Chem.*, **263**, 722−728.
Bazari,W.L., Matsudaira,P., Wallek,M., Smeal,T., Jakes,R. and Ahmed,Y. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4986−4990.
Bryan,J. (1988) *J. Cell Biol.*, **106**, 1553−1562.
Bryan,J. and Kurth,M.C. (1984) *J. Biol. Chem.*, **259**, 7480−7487.
Carlier,M.F., Pantaloni,D. and Korn,E.D. (1986) *J. Biol. Chem.*, **261**, 10778−10784.
Cooper,J.A., Blum,J.D. and Pollard,T.D. (1984) *J. Cell Biol.*, **99**, 217−225.
Gershman,L.C., Selden,L.A. and Estes,J.E. (1986) *Biochem. Biophys. Res. Commun.*, **135**, 607−614.
Harafuji,H. and Ogawa,Y. (1980) *J. Biochem.* (Tokyo), **87**, 1305−1312.
Janmey,P.A. and Matsudaira,P.T. (1988) *J. Biol. Chem.*, **263**, 16738−16743.
Kabsch,W., Mannherz,H.G., Suck,D., Pai,E.F. and Holmes (1990) *Nature*, **347**, 37−44.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367−382.
Kwiatkowski,D.J., Stossel,T.P., Orkin,S.H., Mole,J.E., Colten,H.R. and Yin,H.L. (1986) *Nature*, **323**, 455−458.
Kwiatkowski,D.J, Mehl,R., Izumo,S., Nadal-Ginard,B. and Yin,H.L. (1988) *J. Biol. Chem.*, **263**, 8239−8243.
Kwiatkowski,D.J., Janmey,P.A. and Yin,H.L. (1989) *J. Cell Biol.*, **108**, 1717−1726.
Malm,B. (1984) *FEBS Lett.*, **173**, 399−402.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McLeod,M., Stein,M. and Beach,D. (1987) *EMBO J.*, **6**, 729−736.
Matsuzaki,F., Matsumoto,S., Yahara,I., Yonezawa,N., Nishida,E. and Sakai,H. (1988) *J. Biol. Chem.*, **263**, 11564−11568.
Moriyama,K., Nishida,E., Yonezawa,N., Sakai,H., Matsumoro,S., Iida,K. and Yahara,I. (1990) *J. Biol. Chem.*, **265**, 5768−5773.
Oosawa,F. and Asakura,S. (1975) In *Thermodynamics of the Polymerization of Protein.* Academic Press Ltd, London, pp. 30−31.
Pope,B. and Weeds,A.G. (1986) *Eur. J. Biochem.*, **161**, 85−93.
Pope,B., Gooch,J., Hinssen,H. and Weeds,A.G. (1989) *FEBS Lett.*, **259**, 185−188.
Press,W.H., Flannery,B.P., Teukolsky,S.A. and Vetterling,W.T. (1988) *Numerical Recipes in C.* Cambridge University Press, Cambridge, UK.
Sutoh,K. and Yin,H.L. (1989) *Biochemistry*, **28**, 5269−5275.
Studier,F.W. and Moffatt,B.A. (1986) *J. Mol. Biol.*, **189**, 113−130.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60−89.
Tabacman,L.S., Brenner,S.L. and Korn,E.D. (1983) *J. Biol. Chem.*, **258**, 8806−8812.
Tsou,C.-L. (1988) *Biochemistry*, **27**, 1809−1812.
Vancompernolle,K., Van Damme,J., Puype,M., Korn,E. and Vandekerckhove,J. (1990) Abstracts of Sixth Meeting of the European Cytoskeletal Club, to be published in *J. Muscle Res. Cell Motil.*
Vandekerckhove,J. (1989) *Curr. Opinion Cell Biol.*, **1**, 15−22.
Vandekerckhove,J.S., Kaiser,D.A. and Pollard,T.D. (1989) *J. Cell Biol.*, **109**, 619−626.
Way,M. and Weeds,A.G. (1988) *J. Mol. Biol.*, **203**, 1127−1133.
Way,M. and Weeds,A.G. (1990) *Nature*, **344**, 292−294.
Way,M., Gooch,J., Pope,B. and Weeds,A.G. (1989) *J. Cell Biol.*, **109**, 593−605.
Weber,A., Pring,M., Lin,S.-L. and Bryan,J. (1990) *Biochemistry*, (submitted).
Weeds,A.G., Harris,H.E., Gratzer,W.B. and Gooch,J. (1986) *Eur. J. Biochem.*, **161**, 77−84.
Yin,H.L., Hartwig,J.H., Maruyama,K. and Stossel,T.P. (1981) *J. Biol. Chem.*, **256**, 9693−9697.
Yin,H.L., Iida,K. and Janmey,P.A. (1988) *J. Cell Biol.*, **106**, 805−812.
Zimmerle,C.T., Patane,K. and Frieden,C. (1987) *Biochemistry*, **26**, 6545−6552.

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