Localization of the Calcium-Sensitive Actin Monomer Binding Site in Gelsolin to Segment 4 and Identification of Calcium Binding Sites

Brian Pope, Sutherland Maciver, and Alan Weeds*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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ABSTRACT: Gelsolin is composed of six repeating segments of sequence (G1−6) and contains three distinct actin binding sites, two that bind to G-actin and one that binds to filaments. The calcium-dependent actin monomer binding site present in the carboxyl-terminal half of the protein (G4−6) plays a critical role both in the cooperative binding of actin by gelsolin and in its nucleating activity. Here we have localized this actin binding site to segment 4 (G4) by expressing the segments G4, G4−5, G5, and G5−6 in *Escherichia coli* and analyzing their actin binding properties. In addition we have measured their calcium binding. G4−5 and G5−6 each bind a single calcium ion, but there is no binding by G4 or G5. The affinity of binding by G5−6 is 10 times higher than that of G4−5, and calcium binding by G4−6 shows two sites of different affinity. Thus each actin binding site of gelsolin is restricted to a single segment (G1, G2, and G4), but the nonbinding segments G5 and G6 play an important role in the calcium regulation of actin binding and other activities of gelsolin.

Gelsolin is a calcium-activated actin filament severing and capping protein that is widely distributed in tissues of vertebrates and present also in blood plasma [reviewed Yin (1988) and Weeds and Maciver (1993)]. In addition to its severing activity, gelsolin binds to two actin monomers to produce a ternary complex that is a potent nucleator of polymerization (Doi & Frieden 1984; Janmey *et al.,* 1985; Weeds *et al.,* 1986b). Sequence analysis showed that gelsolin is composed of six repeating segments (G1−6),[1] on the basis of the pattern of its conserved residues (Way & Weeds, 1988). Although intact gelsolin binds two actins, three distinct actin-binding sites were identified in fragments obtained by chymotryptic digestion. These fragments correspond to G1 (containing a high-affinity monomer binding site), G2−3 (containing a filament binding site), and G4−6 (containing a calcium-dependent monomer binding site) (Chaponnier *et al.,* 1986; Bryan, 1988).

Analysis of the structural fold of G1 by X-ray crystallography showed that the pattern of conserved residues reflects the underlying apolar core of this domain (McLaughlin *et al.,* 1993) as originally predicted by Matsudaira and Janmey (Matsudaira & Janmey, 1988). It is therefore anticipated that the other five segments share a similar fold and that this fold may also occur in the segments of related proteins, including villin, severin from *Dictyostelium discoideum,* and fragmin from *Physarum polycephalum.* This prediction has recently been confirmed, using NMR techniques, in the case of segment 2 of severin by Schnuchel et al. (1995) as well as for villin segment 1 (Markus *et al.,* 1994).

On the basis of the fact that the high-affinity monomer binding site is contained within a single segment, we have explored the possibility that this is true for the other two binding sites. The F-actin binding site was recently located in G2, and the minimal severing unit was shown to be G1−2 (Way *et al.,* 1992b). Here we have expressed the individual segments G4 and G5 and pairs of segments G4−5 and G5−6 in *Escherichia coli* to localize the third actin binding site. Only constructs containing G4 were found to bind actin. Thus our results indicate that each of the three actin binding sites in gelsolin is restricted to a single segment.

The roles of the other segments of gelsolin have not been fully established. Some may act purely as spacers to bring the actin binding sites into the correct position and orientation for severing or nucleation to occur. Others are involved in calcium regulation. Here we show that G4−6 binds two calcium ions, like gelsolin (Yin & Stossel 1980; Bryan & Kurth 1984; Weeds *et al.,* 1986a), but unlike gelsolin, the affinities at the two sites are different. We further show that G4−5 and G5−6 each bind a single calcium ion, the latter with an order of magnitude higher affinity than the former. The possible role of these calcium sites is discussed.

MATERIALS AND METHODS

Construction and Expression of Human Gelsolin Segments. Constructs were prepared by further engineering of the cDNA clone for the C-terminal residues 407−755 of human plasma gelsolin (Way *et al.,* 1989). Segments G4 (defined as amino acid residues Ala^{407} − Gly^{528}), G5 (Gly^{529} − Lys^{634}), and G6 (Met^{635} − Ala^{755}) plus the double segments G4−5 (Ala^{407} − Lys^{634}) and G5−6 (Gly^{529} − Ala^{755}) were prepared by PCR, with insertion of a *Bam*H1 site before the first codon and a TAG stop and a *Hin*dIII site after the last. All constructs were then *Bam*H1−*Hin*dIII digested and ligated into the pMW172 expression vector (Way *et al.,* 1990). These manipulations yielded constructs with a Met-Gly-Ser leader sequence, where the Met may or may not have been processed, and a flush C-terminal.

E. coli strain BL21(DE3) was transformed with these constructs, and the pLysS and pLysE variants were similarly

* Address correspondence to this author at MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K. Telephone: 011 44 223 402401. Fax: 011 44 223 213556.

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[1] Abbreviations: G1-6, the six repeating segments of sequence in gelsolin; IPTG, isopropyl β-D-thiogalactoside.

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transformed with G4 and G6. A fourth *E. coli* strain, HMS 174, was also transformed with G6. G4 and G5−6 required an initial growth of a 10-mL seed culture to mid-log phase before centrifugation and reseeding of 50−100-mL cultures, which were again grown to mid-log phase. Following centrifugation, fresh ampicillin medium including IPTG was added for immediate induction ("full seeding protocol"). G4−5 was prepared by the same method but without the final centrifugation and replenishment of the medium before IPTG induction. G4−6 was expressed and purified as described previously (Pope *et al.*, 1991).

Cell pellets were frozen, and the soluble fraction or the inclusion bodies were prepared (Way *et al.*, 1992a). G4 was isolated from inclusion bodies and purified by chromatography on DEAE-cellulose (Whatman DE52) in 10 mM Tris-HCl, pH 8.0, 0.2 mM EGTA, and 1 mM sodium azide (buffer A) with a gradient to 0.2 M NaCl. G5 and G4−5 were isolated from the soluble fraction and dialyzed against 5 mM sodium phosphate, pH 6.0, 0.2 mM EGTA, and 1 mM sodium azide before being loaded onto a column of Whatman CM52 resin. Elution for each was with gradients to 0.2 M phosphate. G5−6 was present both in the soluble fraction and in inclusion bodies. Preparation from inclusion bodies followed the method used for G4, while G5−6 in the soluble phase was fractionated first on Whatman DE-52 in 0.2 mM EGTA (eluting at 85 mM NaCl) and then pooled, dialyzed against buffer A in which 1 mM $CaCl_2$ replaced the EGTA, and further chromatographed on DE-52 in buffer containing calcium [purification based on Bryan (1988)]. The G5−6 eluted at 50 mM NaCl under these conditions.

Proteins were concentrated on Amicon Centricon units and filtered through a 0.45-μm membrane (Millipore). Protein concentrations were calculated from absorbance measurements at 280 nm, based on the tyrosine and tryptophan content of each construct (Gill & von Hippel, 1989). Values for A_{280} = 1.0 cm^{-1} were G4 = 49.2 μM, G5 = 71.7 μM, G4−5 = 29.2 μM, G5−6 = 22.2 μM, and G4−6 = 15.5 μM.

Preparation of Other Proteins and Binding Assays. Actin and pyrene-labeled actin were prepared from rabbit skeletal muscle (Weeds *et al.*, 1986b; Pope *et al.*, 1994). Inhibition of polymerization was carried out using 4 μM actin (containing 10% pyrene−actin) in the presence of 40 nM gelsolin (to accelerate polymerization) essentially as described previously (Way *et al.*, 1990). G-actin was mixed with various concentrations of the segments in 10 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol, and 1 mM NaN_3 for >1 h before 3 mM $MgCl_2$ and 100 mM NaCl were added to polymerize the actin. Fluorescence measurements at excitation and emission wavelengths of 366 and 384 nm, respectively, were made after >3 h of polymerization. The critical concentration of the actin is measured separately, and the concentration of complex, [AG], is calculated from $[A]_{total} - [A]_0 -$ [F-actin], where $[A]_0$ is the critical concentration. K_d values are estimated from plots of [complex] against [gelsolin segment] by nonlinear least squares fitting using Enzfitter (Biosoft, Cambridge, U.K.) on an IBM PC or Kaleidagraph (Synergy Software, Reading, PA) on an Apple Macintosh.

Actin sedimentation assays were carried out as follows: Segments were clarified for 30 min at 100 000 rev/min (av 390000*g*) in a Beckman TL100 centrifuge before incubation with F-actin in F-buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 100 mM NaCl, 0.1 mM ATP, 0.2 mM dithiothreitol, 3 mM NaN_3, and either 0.1 mM $CaCl_2$ or 0.2 mM EGTA). Actin (5−20 μM) was mixed with F-buffer (100 μL final volume) before the binding protein was added. The mixture was left at 4 °C overnight and then centrifuged for 20 min at 75 000 rev/min (av 220000*g*). Supernatants were carefully removed, and 20 μL of SDS gel buffer was added to both supernatant and pellet. In addition, 97 μL of F-buffer was added to the pellets to equate volumes of supernatants and pellets for SDS−PAGE analysis.

Densitometry of gels was performed on a Molecular Dynamics gel densitometer and the relative distribution of actin between pellet and supernatant was determined. The gelsolin segments were all found in the supernatant. The concentrations of complex were calculated from the actin released into the supernatant; hence the concentrations of bound and free segment were calculated assuming that only 1:1 complexes with actin can be formed. From these values the dissociation constant (K_d) is estimated. However, at low segment:actin ratios, errors can be large. The method proved unsuitable to quantitate the tight binding of G4−6, as measurements were complicated by the increased monomeric actin in the supernatant due to barbed-end capping (Weber *et al.*, 1991).

An alternative method to determine K_d was reported by Tabacman and Korn (Tabacman & Korn, 1982). The F-actin concentration $[A]_F$ in the presence of monomer binding protein [G] is given by [F-actin] = $[A]_{total} - [A]_0 -$ [AG] (where $[A]_0$ = actin critical concentration). Since the dissociation constant for the complex in the presence of F-actin is $K_d = [A]_0[G]/[AG]$, therefore

$$[A]_F = [A]_{total} - [A]_0 - [G]_{total}\{[A]_0/([A]_0 + K_d)\} \quad (1)$$

where $[G]_{total}$ is the total concentration of the segment. Plotting $[A]_F$ against $[G]_{total}$ gives the slope $[A]_0/([A]_0 + Kd)$, from which Kd and $[A]_0$ can be estimated by least squares analysis. Thus values for both Kd and the critical concentration in the presence of the segments are obtained.

Calcium Binding Studies. Proteins (1 mL at 20 μM) were dialyzed against 20 mM imidazole−HCl, pH 7.0, 0.05 M NaCl, 1 mM $MgCl_2$, and 0.02 mM EGTA for 48 h to equilibrate the EGTA to this low concentration. Dialysis was then carried out against 50 mL of the same buffer containing radiolabeled $CaCl_2$ (5−70 μM). After 36 h, the samples were changed to a second dialysis vessel containing the same solutions and dialyzed a further 48 h. Protein concentrations were remeasured, and concentrations of calcium in the samples and dialysates were determined from the radioactivity. Using the apparent binding constant of EGTA and calcium (Harafugi & Ogawa, 1980), the free and bound calcium concentrations were calculated. From these values, dissociation constants and numbers of binding sites were estimated by nonlinear least squares analysis as above.

RESULTS

Preparation and Purification of Segments. Expression levels of G5 were at high levels similar to those reported for the other gelsolin segments, G1 and G2 (Way *et al.*, 1992a). Recoveries of 90 mg/L were achieved by placing a streak of cells into 1 L of 2XTY and leaving the culture to grow overnight at 37 °C before harvesting the cells without induction (not shown). G4 and G6 did not express when

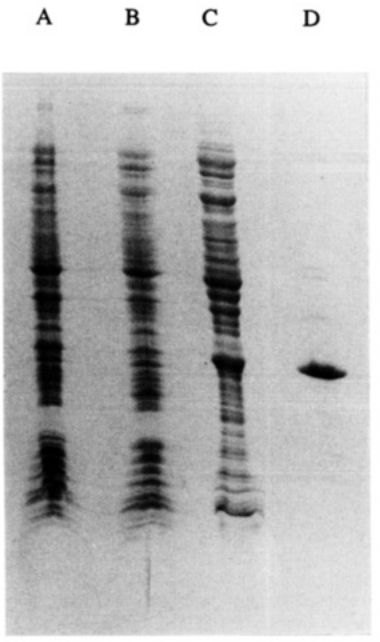

FIGURE 1: Purification of G5−6 analyzed by SDS−PAGE. (A) Complete cell homogenate after expression in *E. coli*; (B) soluble extract from (A); (C) G5−6 pool from gradient on DEAE column chromatographed in EGTA; (D) pool from second DEAE column in calcium.

this method was used. However, when the full seeding and induction procedure was used as described in Materials and Methods, low levels of G4 (~1.5 mg/L) were obtained. Attempts to express G6 in all three BL21(DE3) strains were unsuccessful, as was that using transcription by the T7 RNA polymerase produced during infection of HMS174 with CE6λ phage. However, DNA sequencing confirmed the inserts to be correct. Also the requisite lysogen- and G6-containing plasmids were shown to be present in plasmid DNA prepared from pLysS and pLysE strains of BL21(DE3) containing G6 in the pMW172 vector, which had failed to show expression on induction with IPTG. Further plating experiments confirmed that the cells had lost the ability to express the target protein even though they retained the G6 plasmid.

As G5 was readily expressed at high levels, we combined this segment with either G4 or G6 to facilitate expression of these segments. Using the full seeding protocol for G5−6 and the normal induction method for G4−5, it was possible to purify 3−4 mg of each from 1 L of culture. G5−6 expression was best performed in 50−100-mL volumes and was rarely detected in 1-L cultures. Bacterial growth virtually ceased following induction, suggesting that the expressed protein is toxic to the cells. The expression and purification of G5−6 are shown in Figure 1. Taken together, these results demonstrate the toxic effects of both G4 and G6 on the expression system.

Polymerization Inhibition Assays. Figure 2 shows complex formation (based on reduction of pyrenyl F-actin fluorescence) when G4−6 was mixed with G-actin prior to polymerization. Complex formation is tight, giving a $K_d \sim$ 23 nM, consistent with published values (Bryan, 1988; Way *et al.*, 1989). Similar experiments with G4−5 showed very little inhibition of F-actin polymerization under these conditions, and there were no inhibitory effects by G4, G5, or G5−6 (not shown).

Earlier work has shown that the affinity of G1 for actin is much greater in the presence of calcium than in its absence

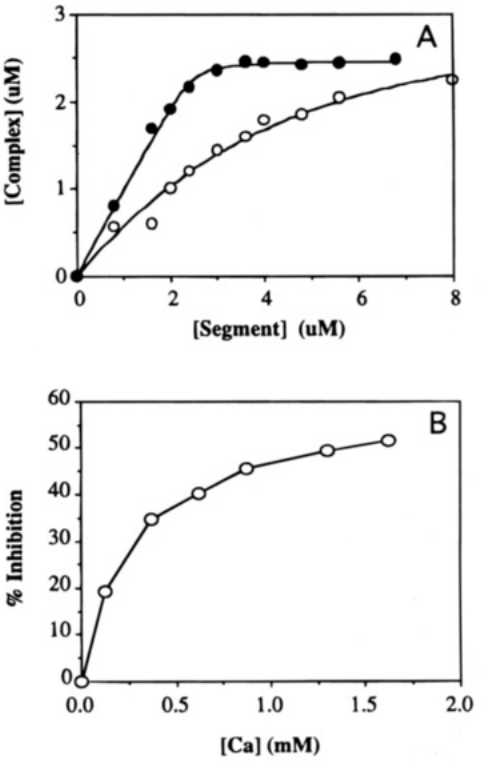

FIGURE 2: (A) Binding of G4−6 and G4−5 to 4 μM G-actin. Complex formation was measured by inhibition of fluorescence of PI-actin by G4−6 (●, in 0.2 mM $CaCl_2$) and G4−5 (○, in 1.2 mM $CaCl_2$). K_d values from nonlinear least squares fitting are 23 nM (G4−6) and 1.6 μM (G4−5). (B) Inhibition of polymerization of 4 μM actin containing 10% PI-actin by 8 μM G4−5 at various concentrations of free calcium ions.

(Bryan, 1988), and following deletion of 19 residues from the C-terminus of G1, binding became completely dependent on the presence of calcium (Way *et al.*, 1990). We therefore tested the effects of increased calcium concentration on the binding of G4−5 and G5−6. The inhibitory activity of G4−5 increased with increasing calcium concentration and appeared to saturate at concentrations in excess of 1 mM (Figure 2B). There was no effect of added calcium on G5−6 binding (not shown). Least squares analysis of complex formation for two preparations of G4−5 in the presence of 1.2 mM $CaCl_2$ gave a mean stoichiometry of 1.1:1 (G4−5:actin) and a K_d of 1.6 μM (Figure 2A). Thus G4−5 binds to G-actin about 50 times more weakly than does G4−6 and only when the calcium concentration is increased. Because of the limited availability of G4, we were unable to quantitate its binding using fluorescence methods.

Sedimentation Assays. When F-actin was mixed with G4−6 and pelleted immediately, >95% of the G4−6 was found in the supernatant and >95% of the actin was in the pellet. However, when centrifugation was delayed after the proteins were mixed, increased amounts of the actin were found in the supernatant, sequestered by the G4−6. Steady state was reached after about 4 h at room temperature or 8 h at 4 °C. The standard procedure was to mix F-actin with the expressed proteins for 16 h at 4 °C in the presence of either 0.2 mM EGTA or 1 mM calcium before centrifugation (Figure 3).

Figure 4A shows the decreased F-actin concentration in the presence of G4−5. Plotting the F-actin concentration against the total concentration of segment according to eq 1 gave a K_d for G4−5 of 1.8 μM. When the data for G4−5

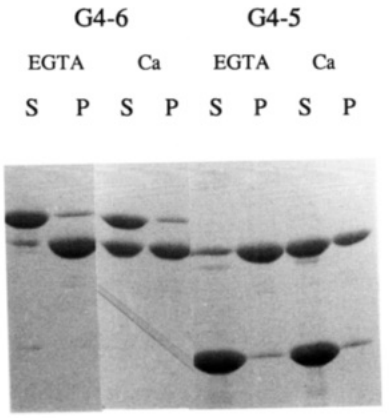

FIGURE 3: Sedimentation of overnight incubations of G4−6 and G4−5 with F-actin assayed by SDS−PAGE. 4.8 μM G4−6 or 12 μM G4−5 was mixed with 6 μM F-actin in the presence of 0.2 mM EGTA or 1 mM $CaCl_2$. The results demonstrate calcium-dependent binding of 0.5:1 and 0.7:1 for G4−6 and G4−5, respectively.

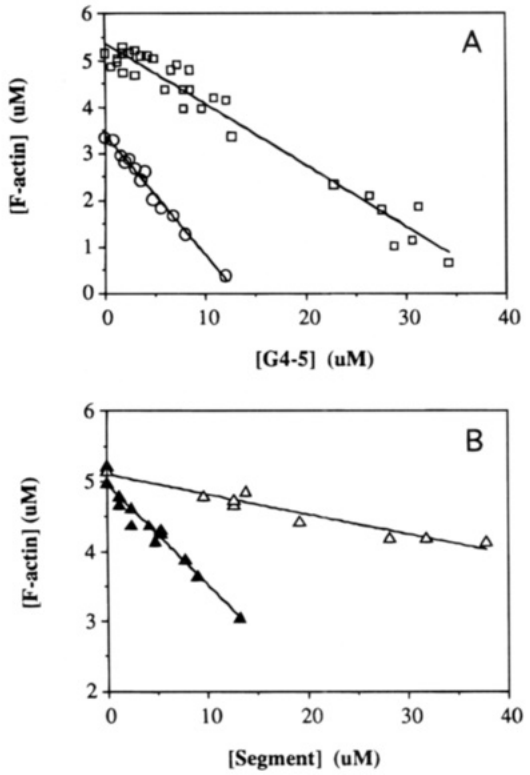

FIGURE 4: (A) Sedimentation of 6 μM F-actin after mixing with G4−5 analyzed according to eq 1 (□). The open circles (○) show the data for G4−5 using 4 μM actin, taken from Figure 2A and replotted according to eq 1. K_d values were 1.8 and 0.9 μM, respectively. (B) Sedimentation analysis for G4 (▲, K_d = 1.8 μM) and G5−6 (△).

from Figure 2A were plotted in the same manner, a K_d of 0.9 μM was obtained (Figure 4A). There was no displacement of the lines in Figure 4A extrapolated to zero G4−5 concentration from the measured F-actin concentration in the absence of G4−5. Similar experiments with G4−6 showed a displacement corresponding to 0.9 μM actin (data not shown). This displacement corresponds to the increase in critical concentration of the actin due to capping by G4−6 (Weber *et al.*, 1991). Thus it appears that G4−5 does not cap the barbed ends of filaments.

Similar analyses of experiments with G4 and G5−6 are shown in Figure 4B. Even with a 6-fold excess of G5−6,

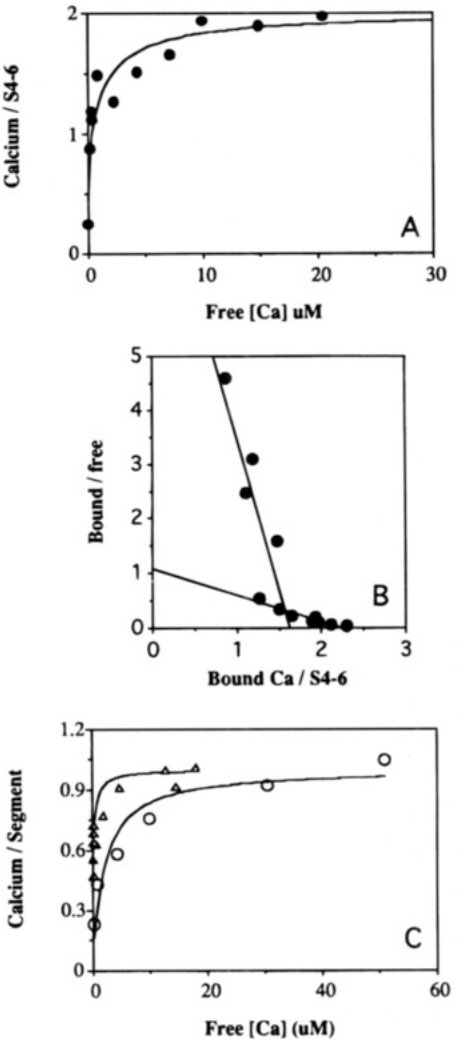

FIGURE 5: (A) Calcium binding by G4−6 analyzed by nonlinear least squares fitting for two independent binding sites ($K_d \sim 0.1$ and 2 μM). (B) Scatchard plot of the same data, which gave K_d values of 0.18 and 2.1 μM. (C) Calcium binding by G4−5 (○) and G5−6 (△) giving K_d values of 2.0 and 0.19 μM, respectively.

the decrease in F-actin concentration was less than 1 μM, suggesting that there is negligible monomer binding by G5−6. A K_d value in excess of 30 μM was estimated from the data in Figure 4B. The K_d value for G4 was 1.8 μM, similar to that for G4−5. As in the case of G4−5, there was no evidence for significant capping activity.

Calcium Binding Assays. Calcium binding to G4−6 indicated a maximum stoichiometry of two calcium ions per mole of protein. The results in Figure 5A were best fitted using two different K_d values (1.9 and 0.09 μM). The presence of two different binding sites can be more readily appreciated from the Scatchard plot (Figure 5B), where the two slopes suggest K_d values of 2.1 and 0.18 μM, respectively. Similar equilibrium dialysis experiments were carried out with the other expressed segments. Both G4−5 and G5−6 bound one calcium ion, and nonlinear least squares fitting suggested K_d values of 2.0 and 0.19 μM, respectively (Figure 5C). No calcium binding was detected by either G4 or G5 on their own.

DISCUSSION

The results here demonstrate that the third actin binding site of gelsolin is located in segment 4. The dissociation

Table 1: Binding Sites for Actin and Calcium on Gelsolin Segments

protein	sites for actin	K_d (μM)	sites for calcium	K_d (μM)
G1–6	2	cooperative[a]	2[a]	1[a]
G1	1	5×10^{-6} [a]	0	
G4–6	1	0.03	2	0.2 and 2
G4–5	1	1.6	1	2
G5–6	0	>30	1	0.2
G4	1	1.8	0	
G5	0		0	

[a] Binding sites for G1–6 and G1 are taken from papers cited in the text. Cooperative binding of actin by gelsolin was reported earlier (Janmey *et al.*, 1986; Weeds *et al.*, 1986b).

constants for G4 and G4–5 are in the low micromolar range, about 60 times weaker than that of G4–6 (K_d = 25 nM) (Figure 4; Table 1). Furthermore, following removal of G6, actin binding by G4–5 requires a higher calcium concentration than the 0.2 mM routinely used in these experiments (Figure 2B). Saturation of binding using fluorescence methods occurred at ~1 mM calcium concentration. These results suggest that, although actin binding occurs only with segment 4, the presence of G6 appears to be necessary for high-affinity interaction. This is analogous to the effects of G3, which although not needed for severing activity (Way *et al.*, 1992b), is nevertheless required for strong capping by G1–3 (Sun *et al.*, 1994).

The requirement for calcium at millimolar concentration cannot be explained from the calcium binding by G4–5 itself, which is in the micromolar range (Table 1). Whether this calcium requirement reflects trapping of additional calcium in the complex with actin as has been demonstrated for G1 remains to be tested (Way *et al.*, 1990). It is clear from the structure of the G1 complex that a calcium ion is involved directly in the binding between G1 and actin and enhances the affinity of interaction (McLaughlin *et al.*, 1993).

Calcium binding measurements showed that G4–6, like gelsolin, binds 2 mol of calcium per mole of protein. However, whereas the Scatchard plots for gelsolin appeared linear, suggesting identical K_d values (Yin *et al.*, 1980; Bryan & Kurth, 1984; Weeds *et al.*, 1986a), the two binding sites in G4–6 have different affinities (Figure 5B; Table 1). The two values were identical to those calculated for G4–5 and G5–6 as independent units; the latter bound calcium an order of magnitude more tightly than the former. The fact that neither G4 nor G5 alone binds calcium suggests that interaction between segments is required for calcium binding. Taken together, our actin and calcium binding measurements suggest that the high-affinity calcium binding site in G5–6 modulates both the affinity of actin binding and the calcium sensitivity of G4 or G4–5.

The mechanism by which calcium binding to G4–6 regulates the severing activity located in G1–3 of gelsolin is not known. The most common hypothesis is that calcium opens the molecule, thereby making the actin binding sites accessible (Way *et al.*, 1989). The doubling of hydrodynamic volume when calcium binds gelsolin is consistent with this (Patkowski *et al.*, 1990). There is evidence for communication between the two halves of the molecule both from the cooperativity of complex formation (Janmey *et al.*, 1986; Weeds *et al.*, 1986b) and from studies of actin binding by G2–6 (Way *et al.*, 1989). Although the F-actin binding site of G2 shows no requirement for calcium in either G1–3 or G2–6, G2–6 binds cooperatively to two actin monomers only in the presence of calcium. Thus calcium regulation of gelsolin depends on communication between the actin binding sites in G4–6 and those in G1 or G2–3.

The importance of G6 for calcium regulation was evident from earlier experiments in which deletion of only 23 residues from the C-terminus of gelsolin resulted in loss of calcium requirement for severing activity (Kwiatkowski *et al.*, 1989). If we assume that G6 has a fold similar to G1, deletion of these residues would remove critical apolar amino acids that in G1 help stabilize the core structure and pin the C-terminal α-helix against the central β-sheet (McLaughlin *et al.*, 1993). More extensive truncation of G6, by 95 residues, resulted in almost complete loss of nucleating activity (Kwiatkowski *et al.*, 1989). This deletion is equivalent to removal of almost all of the central core of G1. On the basis of our identification of G4 as the third actin binding site and evidence that nucleation requires only the two sites in G2–6 (Way *et al.*, 1989), this result may seem paradoxical. However, the experiments reported here demonstrating that G6 enhances both calcium and actin binding show the importance of this segment for transmitting the calcium signal to all other segments.

Differences have been reported between the calcium requirement for the capping and cutting activities of villin (Northrop *et al.*, 1986) and for the capping and monomer binding activities of macrophage capping protein (Young *et al.*, 1994). The calcium requirement for gelsolin activation is controversial. Several independent measurements of calcium binding, using equilibrium dialysis, indicated a K_d ~ 1 μM (Yin & Stossel, 1980; Bryan & Kurth, 1984; Weeds *et al.*, 1986a). In earlier work we showed that 50% activation of nucleation occurred at ~1 μM calcium (Harris & Weeds, 1983), and Selve and Wegner reported that the rate of formation of the ternary complex was maximal at 1 μM calcium (Selve & Wegner, 1987). By contrast, a K_d of 20 μM calcium was determined from measurements of the polarization of tryptophan fluorescence (Kilhoffer & Gérard, 1985), and recent work has suggested that half-maximal activation of nucleation and severing occurs at 10 μM calcium at pH 7.4 (Lamb *et al.*, 1993). Unfortunately the calcium-induced conformational changes measured by dynamic light scattering for gelsolin (Patkowski *et al.*, 1990) and G4–6 (Hellweg *et al.*, 1993) have not been correlated with specific calcium concentrations. Further work will be needed to resolve these differences, but it is clear that the calcium requirement for severing is overridden at lower pH values (Lamb *et al.*, 1993).

In conclusion, the three actin binding sites of gelsolin are each contained within single segments: the two monomer binding sites are in G1 and G4 [the two segments showing closest sequence homology (Way & Weeds, 1988)], and the filament site is in G2. Thus, as in the case of severin, each actin binding site is restricted to a single segment (Eichinger & Schleicher, 1992). However, a major difference between gelsolin and severin is that severin has little nucleating activity (Yin *et al.*, 1990): the actin binding sites may be too close together or wrongly oriented to bring adjacent actins into the right proximity for nucleation. The secrets of severing in gelsolin may indeed reside in the first two segments (Way *et al.*, 1992b), but the non-actin binding segments enhance the binding activities of neighboring segments, facilitate the orientation of bound actins for

nucleation (Hesterkamp *et al.,* 1993), and bring about the communication between segments that is essential for calcium regulation.

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