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Two of the three actin-binding domains of gelsolin bind to the same subdomain of actin

Implications for capping and severing mechanisms

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Gelsolin binds two monomers in the nucleating complex with G-actin in calcium and caps actin filaments. However, 3 actin-binding domains have been identified within its 6 repeating sequence segments corresponding to S1, S2–3 and S4–6. S1 and S4–6 bind only G-actin whereas S2–3 binds specifically to F-actin. Two of the three domains (S2–3 and S4–6) are required for nucleation and a different pair (S1 and S2–3) for severing. Here we show for the first time that the domains unique to nucleation (S4–6) or severing (S1) compete for the same region on subdomain 1 of G-actin. We further show that S2–3 binds actin monomers weakly in G-buffer conditions and that this interaction persists when S1 or S4–6 are also bound. Thus gelsolin associates with two distinct regions on actin. Since S2–3 does not bind monomeric actin in F-buffer, we suggest that its high affinity 1:1 stoichiometry for filament subunits reflects interaction with two adjacent subunits.

Gelsolin; Actin binding site

1. INTRODUCTION

Gelsolin is a calcium-dependent modulator of actin filament structure [1] containing six repeating segments (S1–6) in its amino acid sequence [2]. It is cleaved by chymotrypsin into 3 stable domains: a 14 000 M_r fragment (corresponding to S1), a central portion of 26 000 M_r (S2–3) and a C-terminal domain of 38 000 M_r (S4–6), each of which binds actin [1,3].

A model for the regulation of gelsolin activity [4] postulated that association with actin is prevented by internal interaction between S1 and S4–6 in the absence of calcium. We proposed that these two domains 'open up' in calcium and that severing and capping occur through the F-actin affinity of S2–3 combined with the disruptive power of S1. We further suggested that S1 and S4–6 might bind to the same actin subunit in the complex between gelsolin and 2 actin subunits so that all 3 binding sites would be occupied.

Here we have investigated the binding properties S1, S2–3, S4–6 and combinations of these domains (prepared following expression in *Escherichia coli* [4,5]) using Sepharose resins coupled to actin, or to each of the domains, or to DNase I. Based on these experiments it is clear that (i) S1 and S4–6 cannot bind to the same monomer or filament subunit; (ii) there is no direct interaction between these two domains in the absence of calcium (unless their individual properties are modified by constraints imposed in intact gelsolin); (iii) S2–3 binds monomeric actin in G-buffer, but not in F-buffer, and this binding occurs simultaneously with that of either S1 or S4–6; and (iv) DNase I does not interfere with the binding of any of the gelsolin domains. A revised model is proposed for the interactions of gelsolin with actin.

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2. MATERIALS AND METHODS

S1 (residues 1–150) of human plasma gelsolin was expressed in and purified from *E. coli* [5] as also were the segmental deletion mutants, S2–6 and S1,4–6 (gelsolin lacking S1 and S2–3, respectively) [4]. S4–6, (residues 407–755) was purified from *E. coli* on Whatman DE52 resin in 10 mM Tris-HCl, pH 8.0, 0.2 mM EGTA, 1 mM NaN_3 (buffer A), with a linear gradient to 0.4 M NaCl [4]. S2–3 (residues 151–406) was engineered into pMW172 [5] using restriction fragments that incorporated the N-terminal start of S2–6 and C-terminal stop of S1–3. S2–3, solubilised from inclusion bodies, was not retained on Whatman DE52 in Buffer A and final purification was achieved on Whatman CM52 in 10 mM sodium succinate, pH 6.0, 0.2 mM EGTA, 1 mM NaN_3, eluting with a gradient to 0.2 M NaCl. All gelsolin domains were dialysed into Buffer A and stored at −20°C. Protein concentrations were determined as described previously [5].

Actin was coupled to Sepharose at approximately 1 mg/ml final bed resin [6]. 1 ml columns containing 100–500 μl samples of resin were equilibrated with 10 volumes of the standard buffer: 'G-buffer-Ca' (2 mM Tris-HCl, pH 8.0, 0.1 mM ATP, 3 mM NaN_3 and 0.1 mM $CaCl_2$). The same buffer was used containing 75 μM $MgCl_2$ + 0.2 mM EGTA in place of 0.1 mM $CaCl_2$ to test binding in the absence of calcium. F-Buffers contained 10 mM Tris-HCl, pH 8.0, 0.1 mM ATP, 3 mM NaN_3, 0.1 M NaCl, 1 mM $MgCl_2$ and either 1 mM $CaCl_2$ or 0.2 mM EGTA. Proteins were diluted to 1.5 ml in the appropriate

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buffer, filtered through 0.2 μm Acrodiscs (Gelman Sciences, Ann Arbor, MI) and loaded to a 5-fold molar excess over the coupled actin. (Concentrations of coupled actin were estimated from the concentration of protein taken for coupling less that remaining in the resin washes and divided by the final bed volume.) Columns were then washed with 5 column-volumes of the relevant buffer, before removing 75 μl samples for analysis on SDS-PAGE.

To test calcium sensitivity, columns were either loaded in calcium and washed in EGTA, or 2 separate columns were used, one developed in a calcium-containing buffer and the other in EGTA. Competition experiments were carried out by loading one protein then a second and sometimes a third, with the relevant washing procedure and removal of samples for gel-analysis at the intermediate stages. Additional experiments were carried out with gelsolin domains coupled directly to Sepharose using the same procedures as for actin.

The terminology used to distinguish non-covalently bound protein from that covalently linked involes a "-" for covalent coupling and ":" for non-covalent complex, e.g. actin:DNase I-Sepharose or S1:actin:DNase I-Sepharose indicate that the actin is noncovalently bound to DNase I-Sepharose in the former case and that S1 is in turn retained by actin:DNase I-Sepharose in the latter. Thus the first protein named in the complex is the last that was applied to the affinity column.

Binding was quantitated by densitometry using a Camag flat bed electrophoresis scanner (Cambridge Instruments Ltd, Cambridge, UK) by reference to the trace levels of Sepharose-coupled proteins released on treatment with SDS. More accurate quantitation of both actin and the relevant gelsolin domain was obtained using Sepharose coupled to DNase I. Non-specific binding in G-buffers was tested by including 0.1% gelatin in the buffers [6]. This procedure was not used routinely because it gave rise to background staining that interfered with gel-densitometry and because no differences were observed in the levels of bound protein.

Sedimentation assays were performed in the airfuge as described previously [6]. Gelsolin domains were mixed at a molar ratio of 0.8:1 with 20 μM actin in F-buffers. Pelleted samples of actin saturated with S2-3 were prepared for electron microscopy as described previously [5] and examined in a Philips CM12 electron microscope.

3. RESULTS

3.1. *S1 and S4-6 compete for the same site on actin*

Both S1 and S4-6 bind actin-Sepharose in calcium, but only S1 binds in EGTA. When S1 was loaded after S4-6, there was virtually complete displacement of S4-6 (to <5% of S1 level; compare Fig. 1,lanes G and I). (Although these gels also show the effects of S2-3, similar results were obtained in the absence of S2-3.) When S1 was loaded before S4-6, <5% S4-6 was bound by the actin (compare Fig. 1, lanes A and D). Table I shows quantitation of these results. Experiments using DNase I-Sepharose showed that S4-6 binds to the actin:DNase I-Sepharose more weakly than S1 (0.8-0.9 moles per mole of actin compared to 1.2 for S1), consistent with their different binding affinities [3]. Addition of S1 to S4-6:actin:DNase I-Sepharose displaced the S4-6 (to <10% of the level of S1) and, as expected, S4-6 did not bind to S1:actin:DNase I-Sepharose (Table I). When the individual gelsolin domains were chemically linked to Sepharose, similar results were obtained: S1-Sepharose bound actin well, but this complex did not bind S4-6 (Fig. 2, lanes B and D). As expected, S1 displaced actin from actin:S4-6-Sepharose (Table I).

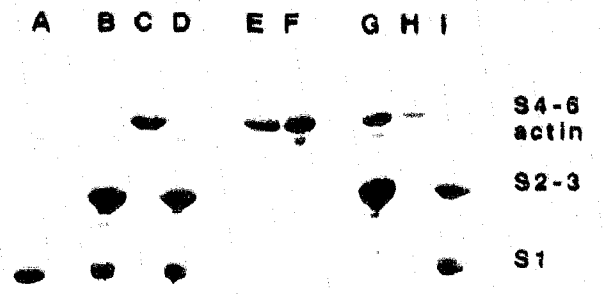

Fig. 1. SDS-PAGE of two actin-Sepharose affinity columns equilibrated in G-buffer-Ca. *Column 1*: A-D, (A), (B) and (D) resin samples after sequential loading of S1, S2-3 and S4-6 respectively; (C) flowthrough after loading S4-6. *Column 2*: E-I, (F), (G) and (I) resin samples after sequential loading of S4-6, S2-3 and S1. (E) and (H) flowthrough samples after loading S4-6 and S1.

Binding studies in F-buffer also showed differences in binding affinity between S1 and S4-6. S1 bound equally strongly to actin-Sepharose in G or F-buffer, but S4-6 bound at about 70% of the level found in G-buffer and only in calcium. This difference in affinity was also seen when S4-6:actin-Sepharose loaded in G-buffer was washed extensively with F-buffer: most of the S4-6 was washed slowly from the column. Similar washing of S1:actin-Sepharose did not release the S1.

3.2. *S2-3 bind actin simultaneously with S1 or S4-6*

S2-3 bound to both actin-Sepharose and actin:DNase I-Sepharose in G-buffer ± calcium. When S2-3 was added alone or as the final component in a series of additions, the stoichiometry of S2-3:actin was slightly over 2 after the standard 5 column volume washing procedure, even in the presence of 0.1% gelatin to prevent non-specific binding. By contrast S2-3 showed no binding to actin-Sepharose in F-buffer (<0.1 mol/mol actin) and virtually all the S2-3 bound in G-buffer was displaced when the resin was washed in F-buffer.

Experiments to test the binding of S2-3 to S1:actin-Sepharose and to actin:S1-Sepharose showed that S2-3 and S1 bind simultaneously to the same actin (Fig. 1, lanes D and I; Fig. 2, lane E). Similarly S2-3 and S4-6 bind simultaneously to the same actin (Fig. 1, lane G). However, the level of S2-3 bound was reduced to 1:1 when resins containing S2-3 were subsequently treated with S1 or S4-6 (Fig. 1, lane D and I; Table I). The binding of S2-3 is still weak, since most of it was eluted when the columns were washed with F-buffer. The binding of the S2-3 domain to actin-Sepharose was explored further using larger segmental mutants [4]. S2-6 bound to actin:S1-Sepharose and to S1,4-6:actin-Sepharose in G-buffer conditions. Since S1 and S4-6 cannot bind together, association of S2-6 must occur through the S2-3 domain. Similarly S1-3 bound to

Table I

Summary of binding properties of gelsolin segments

Sepharose resin	Load 1	Load 2	Load 3	Ratio on resin 1	2	3
Actin	(S4-6)	S2-3	S1	0.05	1.0*	1.0
Actin	S1	S2-3	S4-6	1.0	1.0	0.05
Actin	S1,4-6	S1-3		1.2	1.0	
DNase I	A	S4-6		1.0	0.9	
DNase I	A	S1	S4-6	1.0	1.3	0.06
DNase I	A	S1,4-6	S2-6	1.0	1.1	1.0
S4-6	(A)	S1		0	0	

Proteins were loaded onto the relevant resins in G-buffer-Ca in the order given and the ratio of bound protein determined by densitometry of SDS-polyacrylamide gels. Domains in brackets are bound initially, but subsequently displaced by addition of S1. *S2-3 binds <0.1 in F-buffers.

S1,4-6:actin-Sepharose without displacing S1,4-6, again consistent with binding through the S2-3 domain. The stoichiometry of S1,4-6:S1-3 was 1.2:1 (Table I). When S1-3 was loaded before S1,4-6, only S1-3 bound, showing that the S1 site was fully saturated by the first mutant (data not shown). These experiments demonstrate that the S2-3 binding site on actin is accessible even when larger mutants are bound at other sites. It is noteworthy that the high stoichiometry observed with S2-3 alone was not found with S2-6 or S1-3, even though both bind through the S2-3 domain. This suggests that the high values seen with S2-3 are anomalous. Densitometry of the complex S2-6:S1,4-6:actin on DNase I-Sepharose was 1.0:1.1:1, indicating that all three proteins associate simultaneously with the same actin monomer (Table I).

When F-actin was centrifuged in the presence of each of the individual domains, only S2-3 co-sedimented. No differences were observed in the level of binding ± calcium. Electron microscopy of pelleted S2-3 saturated F-actin showed filaments that were essentially indistinguishable from native F-actin (not shown).

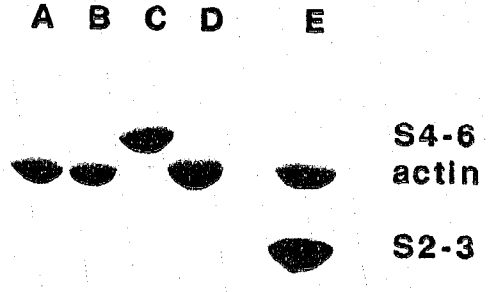

Fig. 2. SDS-PAGE of S1-Sepharose affinity chromatography in G-buffer-Ca. (A) and (C) flowthrough samples after loading actin and S4-6 sequentially. (B), (D) and (E) resin samples after loading actin, S4-6 and S2-3 sequentially.

S1-Sepharose, S4-6-Sepharose and S2-3-Sepharose were also used to test for direct interaction between the individual domains e.g. by loading S2-3 or S4-6 to S1-Sepharose etc., but we were unable to demonstrate any binding between the individual domains.

4. DISCUSSION

Based on displacement of S4-6 from actin-Sepharose and of S1:actin from S4-6-Sepharose, it is clear that S1 and S4-6 compete for the same region of monomeric actin and that this region is well removed from the DNase I binding site (Table I and Fig. 3). S1 is always expected to displace S4-6 because the K_d of S1:actin << S4-6:actin [3]. Larger mutants containing these two domains show similar displacement behaviour. These results are consistent with chemical cross-linking studies [7] showing S1 linked to residues 1–18 of actin and S4-6 to residues 356–375, since the 3D structure shows close spatial proximity between residues N- and C-termini of actin in subdomain 1 [8].

S2-3 showed significant binding to actin-Sepharose in G-buffer ± calcium, but negligible binding in F-buffer (Table I). These results are consistent with the negative findings of Yin et al. [9] in F-buffer and with gel-filtration experiments using mixtures of S2-3 and actin in G-buffer [3], which showed production complexes of very high M_r ($>2 \times 10^6$). Bryan's results suggested that S2-3 forms large aggregates with actin in G-buffer, even though it elutes at a position corresponding to the monomer in the absence of actin [3]. This property of S2-3 to aggregate actin, probably through self-association of the complexes, may explain the anomalously high stoichiometry of S2-3 bound to actin observed in our experiments. Interaction of S2-3 with actin always resulted in stoichiometries >2 per actin, suggesting either more than one binding site on monomeric actin or self-association of S2-3 in the complex. The latter interpretation is considered more likely because larger mutants containing S2-S3 (e.g. S1-3 and S2-6) gave 1:1 stoichiometries.

The stoichiometry of S2-3:actin was reduced to 1:1

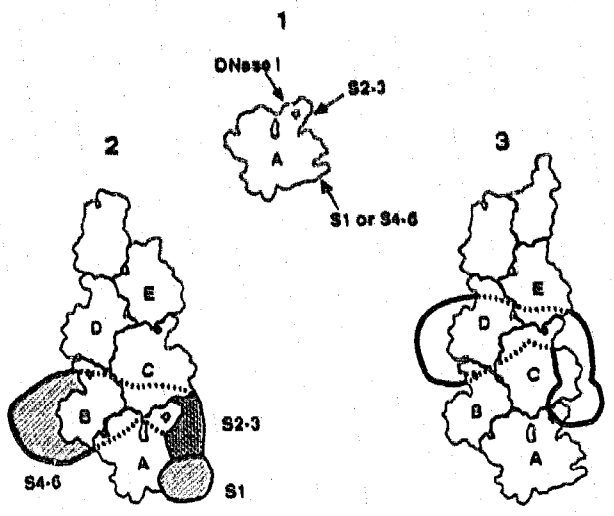

Fig. 3. Schematic model for the interaction of the three domains of gelsolin with actin. (1) Shows the binding site for DNase I based on [8], together with the sites for S1 or S4-6 on subdomain 1 and a possible binding location of S2-3 between these two sites. (2) Shows the position of the gelsolin cap at the barbed end of a filament (the structure of which is taken from [12]). The three domains are distinguished by the different shading, with S1 bound to subdomain 1 of subunit A, S4-6 bound to the equivalent domain of subunit B and S2-3 binding at the interface between subunits B and C. Subunits A and B are adjacent on the one-start helix and A and C on the two-start helix. The dotted lines indicate where gelsolin wraps round the back of the actin helix. (3) Shows gelsolin in the process of severing; S2-3 binds between subunits C and E thereby locating S1 to its position on subdomain 1 of C. Severing occurs releasing subunits A and B at the pointed-end of new filament and a capped filament with subunits C and D at the barbed-end equivalent to that shown in (2).

when other gelsolin domains (e.g. S1,S1-3 or S1,4-6) were added subsequently (Table I). In each case binding of the second component must have occurred through S1, showing that S1 and S2-3 bind to the same monomer simultaneously. It is noteworthy that simultaneous binding of S1 and S2-3 to actin has also been observed using electrophoresis under non-denaturing conditions [10]. Using DNase I-Sepharose we obtained a quaternary complex of S2-6:S1,4-6:actin:DNase I, confirming that the binding sites on actin for S1, S2-3 and DNase I are all distinct (Fig. 3,1).

Our conclusion that S1 and S2-3 bind simultaneously to the same actin monomer does not mean that these domains interact with the same actin in intact gelsolin or in S1-3. On the contrary, we showed that S1-3 bound to 2 actin monomers with high affinity in both G-buffer and F-buffer [4]. Although our earlier experiments do not permit accurate evalution of dissociation constants, the data are consistent with K_d values for S2-3 ~20-50 nM, much lower than the values that can be estimated from affinity chromatography using isolated S2-3. Thus the affinity of S2-3 for actin is much greater when monomers are bound in close proximity in complexes with S1-3 or gelsolin. One possible explanation is that S2-3 binds at the interface between two adjacent monomers, corresponding to a site at low radius close to the centre of the filament. This is consistent with the appearance of S2-3 decorated F-actin since examination of a large number of filaments decorated with S2-3 showed no significant differences from F-actin controls, in contrast to a 50% increase in diameter of filaments decorated with S2-6 [4].

These results provide new information about how gelsolin severs and caps filaments and nucleates polymerization. It is clear that if S1 and S4-6 bind simultaneously to the nucleating complex, they must do so at similar positions on the two different monomers. Because of the rotational symmetry of subunits in the filament, we suggest that these domains contact two adjacent subunits in the one-start helix (A and B Fig. 3,2). This model is consistent with the uniform thickening seen when S2-6 decorates F-actin [4], showing that the C-terminal half of gelsolin wraps around the filament. It is noteworthy that nucleation occurs with gelsolin and S2-6, but not with S1-3, suggesting that S4-6 is essential for binding the second monomer in the correct orientation for nucleation.

The two monomers bound by S1-3 form a stable cap even though they do not nucleate polymerization. This would arise if S1-3 binds to 2 subunits along the axis of the filament (i.e. A and C on the two-start helix in Fig. 3,2). We therefore suggest that gelsolin contacts 3 actin subunits in the filament, even though only 2 actins remain bound under depolymerizing conditions. Gelsolin binding to three actin subunits has recently been reported elsewhere [11].

The structural model of F-actin suggests that strong hydrophobic interactions between neighbouring molecules along the two-start helix contribute most to the stability of the filament [12]. Severing activity by S1-3 or gelsolin must break these contacts (Fig. 3,3). We believe that binding S2-3 to the sides of filaments targets S1 to its site on subdomain 1, which weakens the contacts between neighbouring actin subunits and results in severing. A more detailed mechanism of the interactions of gelsolin domains with actin must await further structural information, from image processing of S2-6 decorated actin filaments (Way and Amos, unpublished work) and the crystal structure of gelsolin and its various complexes with actin.

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REFERENCES

[1] Yin, H.L. (1988) Bioessays 7, 176-179.
[2] Way, M. and Weeds, A.G. (1988) J. Mol. Biol. 203, 1127-1133.
[3] Bryan, J. (1988) J. Cell Biol. 106, 1553-1562.

[4] Way, M., Gooch, J., Pope, B. and Weeds, A.G. (1989) J. Cell Biol. 109, 593–605.
[5] Way, M., Pope, B., Gooch, J., Hawkins, M. and Weeds, A.G. (1990) EMBO J. 9, 4103–4109.
[6] Pope, B. and Weeds, A.G. (1986) Eur. J. Biochem. 161, 85–93.
[7] Sutoh, K. and Yin, H.L. (1989) Biochemistry 28, 5269–5275.
[8] Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature 347, 37–44.
[9] Yin, H.L., Iida, K. and Janmey, P.A. (1988) J. Cell Biol. 106, 805–812.
[10] Safer, D. (1989) Anal. Biochem. 178, 32–37.
[11] Edgar, A.J. (1990) J. Muscle Res. Cell Motility 11, 323–330.
[12] Holmes, K.C., Popp, D., Gebhard, W. and Kabsch, W. (1990) Nature 347, 44–49.