BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 61–65 (1997)
ARTICLE NO. RC976402

Localization of a Calcium Sensitive Binding Site for Gelsolin on Actin Subdomain I : Implication for Severing Process

Jeanne Feinberg, Marie-christine Lebart, Yves Benyamin, and Claude Roustan[1]
Centre de Recherches de Biochimie Macromoléculaire (CNRS), Laboratoire de Recherche sur la Motilité Cellulaire (Ecole Pratique des Hautes Etudes), Université de Montpellier 1, B.P. 5051, 34033 Montpellier cedex, France

Received February 19, 1997

The binding of the N-terminal domain (S1) of gelsolin to monomeric actin has been extensively documented. In contrast, the location of the C-terminal calcium dependent domains (S4-6) interacting with the actin filament during the severing process remains uncertain. In this study, we have identified a new interface that supports calcium dependent gelsolin binding to actin. This site is located in a critical position towards actin-actin contact in the filament and in the vicinity of the phalloidin site. Using specific antibody and synthetic peptides derived from actin sequence within 105-132 residues, this interface was finally ascribed to the segment 112-120 on the actin subdomain-1.

The motility of eukaryotic and some prokaryotic cells (1) is mainly dependent on the dynamic properties of actin microfilaments. The association of actin molecules into filaments is regulated by numerous actin binding proteins (ABPs) and is accompanied by changes in the conformation of each monomer (2). The ABPs can be specific towards either G-actin (such as vitamin D binding protein, thymosin β4 or profilin) or F-actin (such as capZ or cross-linking protein family). In contrast, gelsolin as well as other proteins of the family of capping-severing proteins are able to bind both monomeric- and polymeric-actins (3). The *in vitro* basic activity of gelsolin includes binding to the barbed end of actin filament, its severing and the formation of actin nuclei which promote the growth of filaments (3). Although numerous studies have been made on the structural properties of gelsolin, little is currently known about how this molecule binds to actin filaments during the severing process.

Gelsolin activities are supported by at least three of the six domains (named S1-6) of the molecule (5,6). While the N-terminal half of gelsolin is enough for effective capping and severing activities (6,7), domain S4-6 is involved in calcium regulation (6) and its binding to actin is mainly supported by domain 4 (8).

Structural information on atomic resolution is currently limited to gelsolin domain 1 (S1) interacting with monomeric actin (9). This domain binds to the barbed end of actin, inhibiting actin polymerization. Domain 2 (S2) is more specific to F-actin, its interface being more probably located at the side of the filament (10,11) although interaction across the filament has been postulated (9). As X-ray crystallography can not be used for a direct visualization of severing complexes, a model has been proposed from electron microscopy (12). This model suggests a possible action of S4-6 during severing in the presence of calcium. S4 binds to the opposite side of S2 interface on F-actin, inducing a bent of the filament. However, interface between S4 and actin and the relation between S4, S5 and S6 are poorly documented. A more accurate knowledge of the S4-6 interface would help to better characterize the effect of this domain on the stability of the microfilament and the severing mechanism.

To further characterize the Ca^{2+} dependant gelsolin interaction sites, we have here investigated the interaction of gelsolin with various fragments or synthetic peptides derived from actin sequence. We have taken advantage of the calcium sensitivity of the studied interfaces to identify actin peptides which are in interaction with the S4-6 domains obtained after selective proteolysis. The data indicate that a sequence encompassing residues 112-120 of actin interacts with the C-terminal half of gelsolin (S4-6). The location of this sequence at the interface between actin monomers

[1] Correspondence should be addressed to C. Roustan, Centre de recherches de Biochimie Macromoléculaire (CNRS), B.P. 5051,34033 Montpellier cedex, France.Fax : 04 67 52 15 59.

Abbreviations: F-actin, filamentous actin; ELISA, enzyme-linked immunosorbent assay; IgG, Immunoglobulin G; 1,5-I-AEDANS, *N*-iodoacetyl-*N*-(5-sulfo-naphthyl) ethylenediamine.

0006-291X/97 $25.00

makes it critical for the stability of the filament. Binding of gelsolin to this site would contribute to the bending of the filament described by McGouch and Way (12) and therefore to the severing process.

MATERIALS AND METHODS

Rabbit skeletal muscle actin was prepared as described in (13). Actin was selectively cleaved by *S. aureus* V8 protease and the obtained fragments isolated as previously described (14). Bovine plasma gelsolin was purified as described by Soua et al. (15). Gelsolin domains were obtained by chymotryptic cleavage (16). Synthetic peptides were obtained by solid phase synthesis with a Milligen Pepsynthetizer TM 9050 (Milligen Division, Wadford hertz, UK) as previously described (17). Peptide 112-125 was labeled with 1,5-I-AEDANS (16). Synthetic peptides 105-120 and 119-132 were coupled to Sepharose-4B by CNBr procedure as previously reported (17). Antisera to gelsolin and to 105-120 actin sequence were elicited in rabbits (15,17). The antibodies directed against 105-120 actin sequence were purified by affinity chromatography (18). Their specificity have been previously established (18). Anti-IgG antibodies labeled with alkaline phosphatase were purchased from Biosys (Compiègne, France).

Fluorescence experiments were carried out using a Perkin Elmer Luminescence Spectrometer LS 50. Spectra for dansylated peptide were performed in 10 mM Tris buffer, pH 7.5 with the exitation wavelength set at 340 nm. Fluorescence changes were deduced from the area of emission spectra.

ELISA was previously reported in details (19). Microtiter-plates were coated with actin (5 μg/ml), actin fragment (5 μg/ml) or synthetic peptide (5 μg/ml), then saturated with gelatin (0.5%) and gelatin hydrolysate (3%). Binding was followed at 405 nm using alkaline-phosphatase-labeled anti-IgG. Nonspecific absorption was determined for each sample using uncoated wells. Each assay was performed in triplicate.

SDS PAGE were carried out as described in (20). Protein concentrations were determined by UV absorbance (21).

RESULTS

Involvement of Actin Subdomain 1 in the Interaction of the Ca^{2+}-Dependent Domains of Gelsolin

Binding experiments (direct ELISA) in the presence of Ca^{2+} show (Fig. 1) that gelsolin binds to 1-226 actin fragment. This interaction does not occur when gelsolin is incubated in the presence of excess of EGTA (10 mM), added to the incubation mixture before interaction. This result clearly indicates that ELISA is suitable to monitor the Ca^{2+} dependent binding of gelsolin. In addition, if EGTA is added after the formation of gelsolin-actin fragment complexes, the extent of the interaction is greatly decreased. As shown in Figure 1, the binding capacity is two fold lower when EGTA is present, while the apparent Kd is similar. Thus, two gelsolin interfaces, characterized by a different Ca^{2+} sensitivity are located in the 1-226 actin sequence. A calcium independent site, implicated in the binding of gelsolin domain 2 (S2) to the side of the microfilament, was previously evidenced in the N-terminal region of actin (1-28 sequence) (11). Further experiments were then performed to identify the second and new calcium-dependant interface. Since 96-132 sequence in actin subdomain-1 is important for the binding of numerous associated proteins such as cross-linking proteins, myosin head as well as for actin-actin contacts in the filament (2), we have tested the possible competition between a specific antibody directed against this region (i.e. 105-120 sequence) and gelsolin molecule for their interaction with actin. Figure 2 shows that gelsolin, (which presents a higher affinity for actin than the antibody), induces the release of the 105-120 antibody (18) from coated actin. A new gelsolin interface being thus roughly delineated, a more accurate location was attempted using affinity chromatography.

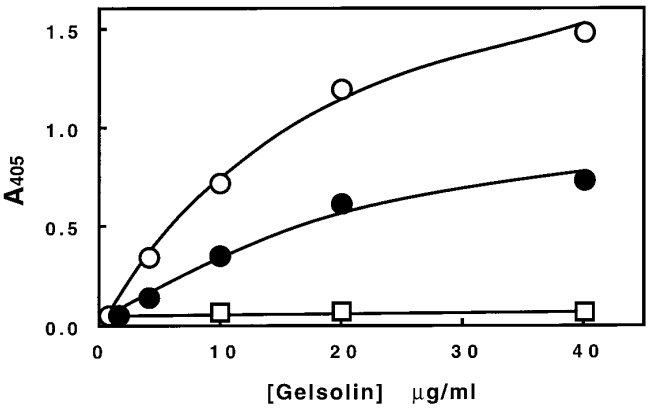

FIG. 1. Interaction of gelsolin with the N-terminal fragment (amino acids 1-226) of actin monitored by ELISA. Increasing amount of gelsolin was incubated with coated actin fragment (5 μg/ml) in 50 mM Tris buffer pH 7.8 containing either 4 mM $CaCl_2$ (○) or 10 mM EGTA (□). In another experiment (●), gelsolin was incubated with coated actin fragment in the presence of $CaCl_2$, followed by another incubation with 10 mM EGTA. Binding was detected by using anti-gelsolin antibodies and monitored at 405 nm.

Identification of Actin Sequence Implicated in the Interaction

Calcium dependent gelsolin-actin interface was delineated using affinity chromatography. Actin peptides 105-120 and 119-132 coupled to Sepharose were tested for their ability to bind gelsolin. Gelsolin was loaded onto the column in the presence of Ca^{2+} and the bound material was desorbed by EGTA. A typical affinity chromatography profile obtained with the 105-120 column is shown in Figure 3. In the same conditions, an unrelated protein such IgG and an actin binding protein such DNAse-1 did not bind to the peptide (19). Elution and electrophoretic patterns show that only peptide 105-120 and not 119-132 is able to interact with gelsolin. Therefore, the new characterized interface is located within the 105-120 sequence. The role of calcium and the selectivity of the two peptide resins together with controls obtained with DNAse-1 and IgG, demonstrate the specificity of the method.

The location was further restricted by performing

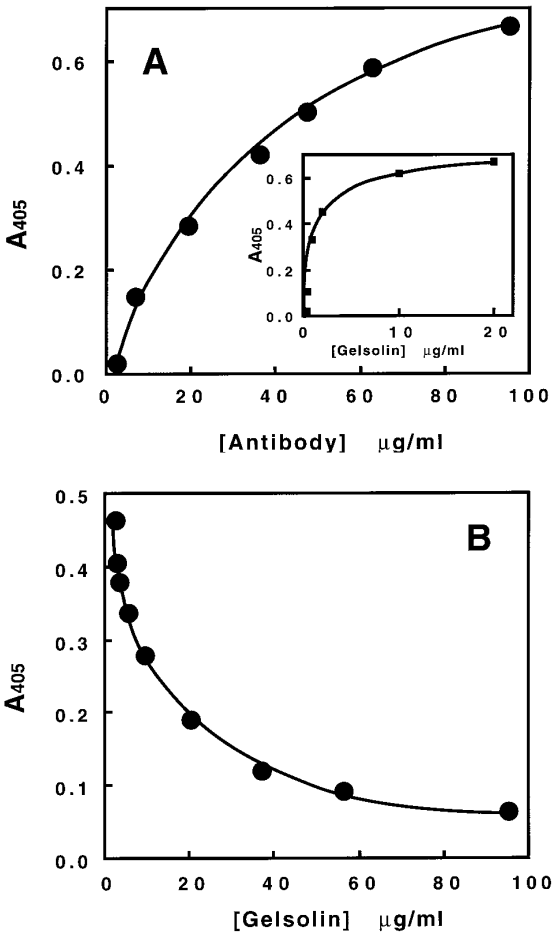

FIG. 2. Interaction of purified antibodies directed against 105-120 sequence with actin. (A) The antibody (0-100 μg/ml) binding to coated actin was carried out by direct ELISA. Inset: binding of gelsolin (0-20 μg/ml) with coated actin. This result was reported to compare the respective activities of gelsolin and antibodies towards actin. (B) Binding of antibodies at 30 μg/ml to coated actin in the presence of increasing concentrations of gelsolin (0-100 μg/ml). Binding was monitored at 405 nm.

experiments using solid phase (ELISA) and solution assays (fluorescence) on a synthetic peptide derived from actin sequence 112-125 which overlaps the sequences 105-120 and 119-132. The experiments carried out by fluorometry (Fig. 4) provide a saturation curve for increasing concentrations of gelsolin. We have observed indeed a calcium dependant fluorescence enhancement (about 15%) when gelsolin was added to the dansylated peptide. The interaction only occurs in the presence of Ca^{2+}. The addition of EGTA in the reaction mixture before adding gelsolin prevents the interaction. When EGTA was added after gelsolin-peptide reaction, we have observed gelsolin dissociation which substantiates that domains S4-6 are implicated in this association. The effect of EGTA on the fluorescence of the dansylated peptide in absence of gelsolin was tested as a negative control (not shown). The binding of gelsolin to 112-125 sequence was also confirmed by ELISA (Fig. 4, inset).

Determination of the Gelsolin Domains Involved in the Interface

Bovine plasma gelsolin as well as human gelsolin can be cleaved by chymotrypsin into three major fragments (16), an N-terminal one of about 15 kDa (S1), a middle one of 30 kDa (S2-3) and a large C-terminal segment of 47 kDa (S4-6) (21). In order to determine the domain which is implicated in the interface, a chymotryptic digest of gelsolin was passed through two affinity columns, each prepared with a peptide resin of sequence 105-120 and 119-132. The retained fragments of gelsolin, were eluted by EGTA in the same conditions described above for the whole molecule. The eluted material was analyzed by SDS PAGE. Using the column

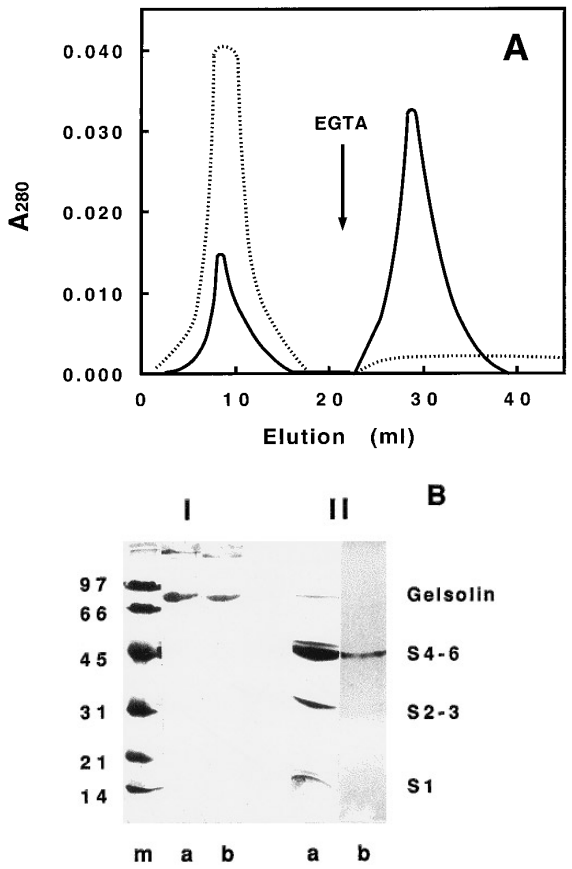

FIG. 3. Binding of gelsolin or gelsolin hydrolysate to actin peptides 105-120 and 119-132 coupled to Sepharose-4B . (A) Gelsolin (250 μg/ml) was passed through columns (1.4 × 5 cm) of Sepharose-4B-linked peptides 105-120 (—) or 119-132 (· · · · ·) (1 mg peptide/1 ml resin). The bound material was eluted by 50 mM Tris buffer pH 7.5 supplemented by 10 mM EGTA. (B) SDS/PAGE analysis of retention of gelsolin (I) or gelsolin chymotryptic digest (II) by insolubilized peptide 105-120. Lane m, molecular weight markers (kDa), lanes a, samples load onto the column and lanes b, fractions eluted by EGTA. Gels were stained by Coomassie blue.

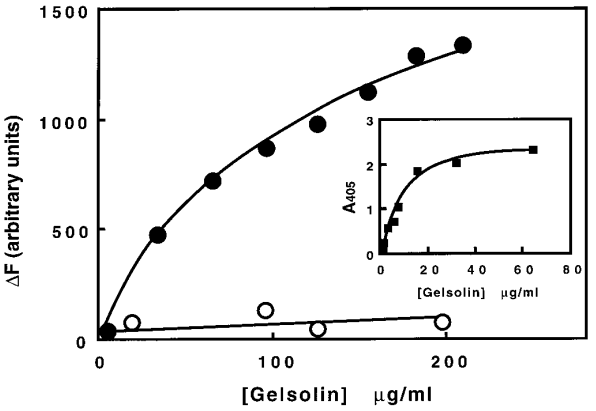

FIG. 4. Enhancement of dansylated peptide 112-125 fluorescence upon gelsolin binding. Peptide (2 μg/ml) was mixed with increasing amounts of gelsolin in the presence of $CaCl_2$ (4 mM) (●) or EGTA (10 mM) (○) and the area of the emission spectra were measured. Inset: Binding of gelsolin to coated 112-125 peptide evidenced by ELISA. Binding was monitored at 405 nm.

containing peptide 105-120, a major band corresponding to the 47 kDa fragment was visualized (Fig. 3B). In contrast, no material was eluted from the 119-132 column.

DISCUSSION

In recent studies describing the severing activity of gelsolin, the occurrence of important conformational changes in the actin molecule has been reported. In the last step of the severing process, gelsolin remains linked to the barbed end of the actin filament. The formation of this complex implicates a long range cooperative conformational change which affects the whole actin filament (23). It has also been observed from electronic microscopy that during the severing process, gelsolin or S2-6 fragment induces a bending of the actin filament in the presence of calcium (12). This structural rearrangement seems to destabilize F-actin. This can probably be ascribed to the activity of the S4-6 domains, since the S1-3 domain was not describe to produce such an effect (12). This rearrangement implicates the interaction of gelsolin with some critical residues involved in the actin-actin interface. Among these interfaces, some are located in actin subdomain 1(2,24) as sequence 110-112 which has been implicated in contacts along the left handed genetic helix (24).

We report for the first time evidence for the involvement of the 112-120 actin sequence in gelsolin binding. We have found that a large N-terminal fragment of actin (sequence 1-226) which includes the major part of actin subdomain 1 contains two binding sites for gelsolin. A calcium independent site located within 1-28 sequence of actin is specific for the binding of gelsolin S2-3 domains (11). In contrast, the second and new interacting site appears to be Ca^{2+} regulated, and therefore implicated in the specific binding of S4-6 domains, since the two other domains (S1 and S2-3) are known to be calcium independent (16,22). The binding of antibodies directed against 105-120 actin sequence is decreased by gelsolin interaction suggesting that the second binding site of the 1-226 fragment is located at or near this actin sequence. From the two synthetic peptides tested (sequences 105-120 and 119-132), only the peptide of sequence 105-120 displayed significant actin binding ability with calcium dependency, as judged by affinity chromatography. Furthermore, we have found as demonstrated by ELISA and fluorescence measurements that sequence 112-120 is an essential structure involved in the calcium dependent binding of gelsolin. The implication of the S4-6 domains in this new interface was then confirmed by affinity chromatography. Among the gelsolin domains obtained by chymotrypsic digestion, only the S4-6 one presented a calcium dependent interaction with the 105-120 sequence.

It is noteworthy that this interface is located near the sites characterized for crosslinking proteins such α-actinin and filamin which specifically bind to the side of actin filament. Filamin indeed interacts with the 105-113 segment (17,19). α-actinin, in contrast to filamin and gelsolin, does not bind to the same actin region (i.e., 105-120) (17). In fact, α-actinin displays a binding capacity towards the C-terminal part of the α-helix encompassing residues 112-125 (17). The difference in the location of these interfaces seem to correlate with the specificity of the activities of these proteins, lateral crosslinking for α-actinin and filamin, destabilization of adjacent actin monomers for S4-6 domains of gelsolin. Sequence 112-120 would support gelsolin binding in close proximity of actin-actin interaction, since residues 110-112 of one actin monomer has been shown to contact residues 195-197 of the adjacent monomer (2, 24). Furthermore, phalloidin, which is known to stabilize the actin filament not only binds in the vicinity of residues 117-119 as demonstrated by crosslinking experiments (2,25), but is also in competition with gelsolin for F-actin binding (26). All together, these results evidence the importance of the 112-120 sequence, in addition with the already identified sites (9), for the severing activity of gelsolin.

This study supports the conclusion that calcium dependent gelsolin interface included a critical region of the actin-actin interfaces located within sequence 112-120.

ACKNOWLEDGMENTS

This work was supported by grants from the *Association Française contre les Myopathies* and the *Institut Français de Recherches pour l'exploitation de la mer.*

REFERENCES

1. Labbé, J. P., Harricane, M. C., Boyer, M., Derancourt, J., Roustan, C., and Benyamin, Y. (1996) *Comp. Biochem. Physiol.* **114B,** 287–293.
2. Sheterline, P., Clayton, J., and Sparrow, J. C. (1995) *Protein Profile* **2,** 1–103.
3. Weeds, A. G., and Maciver, S. (1993) *Opin. Cell. Biol.* **5,** 63–69.
4. Yin, H. L., Zaner, K. S., and Stossel, T. P. (1980) *J. Biol. Chem.* **255,** 9494–9500.
5. Kwiatkowski, D. J., Stossel, T. P., Orkin, S. H., Mole, J. E., Colten, H. R., and Yin, H. L. (1986) *Nature* **323,** 455–458.
6. Way, M., Gooch, J., Pope, B., and Weeds, A. G. (1989) *J. Cell. Biol.* **109,** 593–605.
7. Way, M., Pope, B., and Weeds, A. G. (1992) *J. Cell. Biol.* **119,** 835–842.
8. Pope, B., Maciver, S., and Weeds, A. G. (1995) *Biochemistry* **34,** 1583–1588.
9. McLaughlin, P. J., Gooch, J., Mannhertz, H. G., and Weeds, A. G. (1993) *Nature* **364,** 685–692.
10. Way, M., and Matsudaira, P. (1993) *Current Biology* **3,** 887–890.
11. Feinberg, J., Benyamin, Y., and Roustan, C. (1995) *Biochem. Biophys. Res. Commun.* **209,** 426–432.
12. McGouch, A., and Way, M. (1995) *J. Struct. Biol.* **115,** 144–150.
13. Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* **246,** 4866–4871.
14. Roustan, C., Benyamin, Y., Boyer, M., Bertrand, R., Audemard, E., and Jauregui-Adell, J. (1985) *FEBS Lett.* **181,** 119–123.
15. Soua, Z., Porte, F., Harricane, M. C., Feinberg, J., and Capony, J. P. (1985) *Eur. J. Biochem.* **153,** 275–287.
16. Feinberg, J., Capony, J. P., Benyamin, Y., and Roustan, C. (1993) *Biochem. J.* **293,** 813–817.
17. Lebart, M. C., Méjean, C., Roustan, C., and Benyamin, Y. (1993) *J. Biol. Chem.* **268,** 5642–5648.
18. Labbé, J. P., Boyer, M., Mejean, C., Roustan, C., and Benyamin, Y. (1993) *Eur. J. Biochem.* **215,** 17–24.
19. Méjean, C., Lebart, M. C., Boyer, M., Roustan, C., and Benyamin, Y. (1992) *Eur. J. Biochem.* **209,** 555–562.
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
21. Labbe, J. P., Boyer, M., Roustan, C., and Benyamin, Y. (1994) *Biochem. J.* **284,** 75–79.
22. Bryan, J. (1988) *J. Cell. Biol.* **106,** 1553–1562.
23. Prochniewiez, E., Zhang, Q., Janmey, P. A., and Thomas, D. D. (1996) *J. Mol. Biol.* **260,** 756–766.
24. Holmes, K. C., Popp, D., Gebhard, W., and Kabsch, W. (1990) *Nature* **347,** 44–49.
25. Vandekerckhove, J., Deboben, A., Nassal, M., and Wieland, T. (1985) *EMBO J.* **4,** 2815–2818.
26. Allen, P. G., and Janmey, P. A. (1994) *J. Biol. Chem.* **269,** 32916–32923.