Antigenic probes locate a serum-gelsolin-interaction site on the C-terminal part of actin

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The implication of part of the C-terminal of actin (within the 285–375 sequence) in the interaction of serum gelsolin was investigated by the use of specific antibodies. These antibodies were directed against two or three discrete epitopes, one of which was specific for skeletal-muscle actin. Some of these epitopes were found to be near the serum gelsolin–actin interface. Thus it can be assumed that part of the C-terminal of actin is exposed at the barbed end of the actin filament. The interaction between tropomyosin and actin was also studied.

INTRODUCTION

Actin is one of the most abundant proteins in muscle and non-muscle cells. It may act as a contractile or structural protein, and is characterized by its ability to interact with a large number of associated proteins (ABPs) and by its very conserved sequence. Specifically, six isoactins have been described in higher vertebrates, and only 6% mutation occurs between actins in an invertebrate (scallop) and in rabbit skeletal muscles (Vandekerckove & Weber, 1984).

These mutations are located in precise regions of the sequence. The N-terminal part (1–7 sequence) appears to be the most variable region and is characteristic of each isoform. In G- and F-actins, this region is highly exposed (Méjean *et al.*, 1987b), chemically reactive (Sutoh, 1982) and strongly antigenic (Benyamin *et al.*, 1986). It is characterized by a cluster of negative charges that allow chemical cross-linking between actin and different ABPs such as myosin head (Sutoh, 1982), depactin (Sutoh & Mabuchi, 1984) and fragmin (Sutoh & Hatano, 1986), which raises the question of the specificity of these ABPs for this variable sequence.

In previous papers (Boyer *et al.*, 1985; Méjean *et al.*, 1987a) we reported a different approach using anti-actin antibody populations directed against a limited part of the actin sequence as potential competitors with a given ABP. In this way, the constant 18–28 sequence (and not the 1–7 sequence) was identified as being part of the actin–myosin head rigor complex interface (Méjean *et al.*, 1987a).

The C-terminal part (260–375 sequence) of the actin molecule is also subject to several discrete mutations and has also been implicated in the binding of various ABPs. Myosin light chain and depactin can be cross-linked to the 359–375 sequence (Sutoh, 1982; Sutoh & Mabuchi, 1984); the chemical reactivity of some lysine residues (Lys-326, -328 and -336) appears to decrease after tropomyosin binding (Lu & Szilagyi, 1981). Finally, removal of the C-terminal residue (Phe-375) appears to

abolish profilin binding (Malm *et al.*, 1983), which suggests that there may be a monomer–monomer interaction site on the C-terminal part of actin. The topology of actin interactions can be investigated by the use of polyclonal antibodies to the C-terminal 285–375 sequence (Boyer *et al.*, 1985) to locate the corresponding antigenic epitopes and to test their reactivity in the actin–ABP complex.

In the present paper we report the presence of two or three epitopes in the C-terminal region, one of which is specific for skeletal-muscle actin. Their antigenic reactivity appears to be unaffected in the F-actin–tropomyosin complex, but is strongly decreased in the G-actin–gelsolin complex. Moreover, the corresponding antibodies were found to abolish the gelsolin–actin interaction.

MATERIALS AND METHODS

Actins, ABP and derivatives

Rabbit skeletal-muscle and scallop muscle actions were obtained from acetone-dried powders (Spudich & Watt, 1971). Bovine aortic actin (Cavadore *et al.*, 1985a) was a gift from Dr. J. C. Cavadore. Unfolded skeletal-muscle actin was proteolysed with trypsin; 1.4 mg of S-carboxymethylated actin (Benyamin *et al.*, 1979) in 1 ml of 0.1 M-sodium bicarbonate buffer, pH 8.8, was incubated with trypsin (proteinase/actin ratio 1:20, w/w) for 2 h at 20 °C. The absence of residual large fragments was verified by SDS/polyacrylamide-slab-gel electrophoresis. CNBr cleavage of skeletal-muscle actin was performed as described elsewhere (Elzinga *et al.*, 1973). Lysine residues in actin were modified by reaction with citraconic anhydride (Atassi & Habeeb, 1972); 1.4 mg of S-carboxymethylated actin in 1 ml of 0.1 M-sodium borate buffer, pH 9, was treated with a 30-fold excess of reagent per lysine residue. Tyrosine residues were nitrated with tetranitromethane (Sokolovsky *et al.*, 1966); all tyrosine residues were modified, as determined spectrophotometrically. Tropomyosin from rabbit skeletal muscle was

Abbreviations used: ABP, actin-binding protein; anti-(10 kDa fragment) antibodies, anti-[actin fragment (sequence 285–375)] antibodies; e.l.i.s.a., enzyme-linked immunosorbent assay.

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Fig. 1. Antigenic reactivity of electrophoretic blotting from various actins or actin derivatives revealed with anti-(10 kDa fragment) antibodies

(a) Electrophoretic blotting from rabbit skeletal-muscle actin (lane 1), *S. aureus*-V8-proteinase digest (lane 2) and purified 285-375 fragment (lane 3). (i) Coomassie Blue-stained polyacrylamide gel after electrophoresis; (ii) electrophoretic replicates revealed with purified anti-(10 kDa fragment) antibodies (20 µg/ml). N and C indicate the N-terminal (1-225 sequence) and C-terminal (226-375 sequence) peptides of actin. (b) Electrophoretic blotting from scallop muscle actin (lane 1), bovine aortic actin (lane 2) and rabbit skeletal-muscle actin (lane 3). Electrophoretic replicates were revealed with purified anti-(10 kDa fragment) antibodies (20 µg/ml).

purified by the procedure of Cavadore *et al.* (1985b). Serum gelsolin was purified as described by Soua *et al.* (1985). Actin was digested with *Staphylococcus aureus* V8 proteinase as previously described (Roustan *et al.*, 1985).

Antibodies

Antibodies to serum gelsolin were obtained in rabbits as previously described (Soua *et al.*, 1985). Anti-IgG antibodies labelled with alkaline phosphatase were from Biosys (Compiègne, France). Anti-[actin fragment (sequence 285-375)] [anti-(10 kDa fragment) antibodies were obtained in sheep as previously reported (Boyer *et al.*, 1985). The antigen used was the 285-375 fragment from skeletal-muscle actin coupled to haemocyanin. Its specificity was checked by immunoblotting of the purified 285-375 fragment and *S. aureus*-V8 proteinase hydrolysate (Fig. 1). The results obtained agree with the fact that only the purified fragment was injected into the sheep. Consequently, the antibodies reacted with the 10 kDa (sequence 285-375) and 16 kDa (sequence 227-375) fragments, but not with the N-terminal 27 kDa (sequence 1-226) fragment (Fig. 1). It was also checked that these antibodies do not react with tropomyosin or gelsolin. Moreover, pre-immune serum did not react at all (results not shown).

Antibody populations were purified by affinity chromatography (Andersson *et al.*, 1978).

Analytical methods

Protein concentrations were determined by absorbance as described previously (Woods, 1967; Méjean *et al.*, 1986; Porte & Harricane, 1986). SDS/polyacrylamide-slab-gel electrophoreses were run according to the procedure of Laemmli (1970). Immunoblotting was performed as previously described (Roustan *et al.*, 1982). The peroxidase reaction was detected on nitrocellulose sheets by using 4-chloro-1-naphthol as the chromophoric substrate. E.l.i.s.a. (Engvall, 1980) was used to demonstrate the interaction between actin, ABP and antibody populations. Details of the direct and competitive methods have been reported in previous papers (Méjean *et al.*, 1987a,b). Only the specific conditions are given in the Figure legends.

RESULTS

Antigenic properties of the 285-375 sequence (C-terminal part) of actin

Antibody-specificity for actin conformers. The reactivity of purified anti-(10 kDa fragment) antibodies was tested by direct e.l.i.s.a. As shown in Fig. 2, their reactivities with unfolded, monomeric or filamentous actin were almost identical. The apparent dissociation constants were about $1.5 \times 10^{-8} \pm 0.3 \times 10^{-8}$ M, as determined by analysis of the plot of absorbance versus antibody concentration. On the basis of this result, it can be assumed that antigenic epitopes are accessible on both native and unfolded actin conformations.

Antibody-specificity for isoactins. The specificities of the antibodies for the three muscle actin isoforms (skeletal muscle, aortic smooth muscle and scallop muscle) were tested by immunoblotting. As shown in Fig. 1, the

Fig. 2. Reactivity of anti-(10 kDa fragment) antibodies for unfolded skeletal-muscle G- and F-actins

Direct e.l.i.s.a. was carried out as described previously (Méjean *et al.*, 1987a,b). The binding of the antibodies was monitored at 405 nm by using anti-(sheep IgG) antibody labelled with alkaline phosphatase. Results are expressed as percentage binding. Specific antibodies were incubated with coated S-carboxymethylated actin (●), G-actin (□) or F-actin (△).

Fig. 3. Antigenic reactivity of different actins or actin derivatives by competition e.l.i.s.a.

S-Carboxymethylated skeletal-muscle actin (50 ng/well) was coated on micro-titre plates. Purified anti-(10 kDa fragment) antibodies (4 μg/ml) were incubated with the coated unfolded actin in the presence of increasing antigen concentrations up to 200 μg/ml. (a) Reactivity of various S-carboxymethylated actins: rabbit skeletal-muscle actin (●), scallop muscle actin (■) and bovine aortic actin (▲). (b) Reactivity of skeletal-muscle actin derivatives: actin (●), citraconylated actin (○), nitrated actin (■), tryptic digest (▲) and CNBr digest (□).

antibodies cross-reacted with scallop and smooth-muscle actins. However, the homologous antigen was more reactive on the basis of a comparison of band intensities.

The first conclusion was confirmed by competitive e.l.i.s.a. (see the Materials and methods section). In these experiments the wells of micro-titre plates were coated with S-carboxymethylated actin and the antibodies were incubated in the presence of various concentrations of the selected actin isoform, so that coated and free actin were in competition for the antibodies. The results show (Fig. 3a) that the scallop and aortic actins, in contrast with homologous actin, afforded only partial competition with coated actin. About half of the antibodies were released at high aortic or scallop actin concentrations (about 70 μg/ml). Thus it can be concluded that the unaffected antibody populations were directed against a specific skeletal-muscle sequence.

Antibody-specificity for modified or fragmented actins.

To obtain further information on the location of antigenic determinants in the 285–375 sequence, the interaction between antibodies and chemically modified

Fig. 4. Reactivity of anti-(10 kDa fragment) antibodies in the presence of serum gelsolin or tropomyosin, monitored by direct e.l.i.s.a.

Results are expressed as percentage effect. Anti-(10 kDa fragment) antibodies (8 μg/ml) were incubated with coated skeletal-muscle G-actin in the presence of increasing ABP concentrations up to 4.7 μM: ○, tropomyosin; ●, serum gelsolin.

or hydrolysed actin was monitored by competitive e.l.i.s.a., as described above. In the case of the tryptic digest (unfolded actin was digested as described in the Materials and methods section), all of the antibody populations were displaced by actin peptides (Fig. 3b). However, the apparent affinity of the antibodies was lower than for the whole actin molecule, probably because of the small size of the fragments. Thus lysine and arginine residues do not appear to be included in the antigenic epitopes. Furthermore, the exclusion of lysine residues was confirmed by the following result. As shown in Fig. 3(b), modification of actin lysine residues by citraconylation, which changes a side chain from positive to negative, did not alter antibody binding.

In contrast, after CNBr cleavage of actin at the methionine residues (Fig. 3b), the antibody populations did not react at all with the corresponding fragments. Thus methionine residues appear to be included in all the epitopes.

Finally, modification of all the tyrosines by nitration with tetranitromethane led to an intermediate result. About half of the antibodies (as in the case of the isoactin study) reacted near this residue.

Involvement of the 285–375 sequence in tropomyosin and serum gelsolin interactions

Purified anti-(10 kDa fragment) antibodies were used to study the interaction of tropomyosin and gelsolin by direct e.l.i.s.a. As these antibodies are equally reactive with G-actin and F-actin, it was possible to follow any displacement of the antibodies by tropomyosin or gelsolin respectively.

Tropomyosin-F-actin interface. In a previous paper (Méjean *et al.*, 1987a) we reported that tropomyosin is able to interact with F-actin adsorbed on the plastic of microtitre plates, and affects the binding of anti-actin antibodies specific for the F-actin conformation.

In the present work, the binding of anti-(10 kDa fragment) antibodies to coated F-actin was measured in the presence of various tropomyosin concentrations. As

Fig. 5. Interaction of serum gelsolin with G-actin monitored by direct e.l.i.s.a.

Gelsolin was incubated in 2 mM-Tris/HCl buffer, pH 7.4, containing 2 mM- CaCl_2 and 0.2 mM-ATP in the presence of coated G-actin. After incubation with purified anti-gelsolin antibodies (0.15 $\mu\text{g/ml}$), then with anti-(rabbit IgG) antibodies labelled with alkaline phosphatase, the gelsolin incorporation was monitored at 405 nm.

Fig. 6. Effect of anti-(10 kDa fragment) antibody on gelsolin binding to coated G-actin

Gelsolin (1.3 $\mu\text{g/ml}$) was incubated with coated G-actin in micro-titre plates in the presence of various anti-(10 kDa fragment) antibody concentrations (as indicated). The bound gelsolin was monitored at 405 nm with the use of anti-gelsolin antibodies (elicited in rabbits) and anti-(rabbit IgG) antibodies labelled with alkaline phosphatase.

shown in Fig. 4, the interaction with antibodies was not modified, whatever the tropomyosin concentration (up to 270 $\mu\text{g/ml}$). Thus it can be assumed that antigenic determinants in the 285–375 region are excluded from the tropomyosin–F-actin interface.

Serum gelsolin–G-actin interface. Interaction of gelsolin with coated G-actin was tested by the use of anti-gelsolin antibodies. Fig. 5 shows the saturation curve obtained. Its analysis gives the apparent K_D of gelsolin for coated G-actin ($K_D = 2.2 \times 10^{-9} \pm 0.5 \times 10^{-9}$ M). This constant is of the same order of magnitude as the K_D (10^{-9} M) reported for gelsolin interaction with actin in the presence of Ca^{2+} (Janmey *et al.*, 1986).

In a first experiment, the effect of gelsolin binding upon the anti-(10 kDa fragment) antibody interaction

was investigated. Antibody binding (used at a fixed concentration of 8 $\mu\text{g/ml}$) was monitored at 405 nm in the presence of various gelsolin concentrations (0 to 380 $\mu\text{g/ml}$). As shown in Fig. 4, more than half of the antibodies were released at saturating gelsolin concentrations. In a second experiment, we investigated the binding of gelsolin (at a fixed concentration of 1.3 $\mu\text{g/ml}$) to coated G-actin in the presence of increasing anti-(10 kDa fragment) antibody concentrations (Fig. 6). The gelsolin interaction was monitored by the use of anti-gelsolin antibodies. The binding of gelsolin to anti-G-actin complex was in this case almost completely abolished.

DISCUSSION

In recent studies describing the binding of associated proteins on the actin surface, some domains have been located (Hambly *et al.*, 1986). One of them is involved in the DNAase I interaction. X-ray-diffraction studies show that DNAase I binds near the cleft between the two lobes of the actin molecule (Suck *et al.*, 1981). This localization has been narrowed down to the 50–69 sequence by cross-linking experiments (Sutoh, 1984). Moreover, by using specific antibodies, we have shown the involvement of a region in the 168–225 sequence (Boyer *et al.*, 1985). Thus the DNAase I interface appears to include two distinct regions in separate actin sequences.

The C-terminal part of actin also constitutes a potential location for an ABP interface. Profilin (Malm *et al.*, 1983), depactin (Sutoh & Mabuchi, 1984) and Gc protein (Goldschmidt-Clermont *et al.*, 1986) appear to be good candidates for binding here. The cases of tropomyosin and gelsolin are less well documented, but gelsolin is competitive with Gc protein (Coue *et al.*, 1986) and is DNAase-I-independent (Janmey *et al.*, 1986). Consequently, it is reasonable to suppose that it interacts on the C-terminal part of actin. We have tested this hypothesis by using the immunological approach that successfully delimited the S-1 protein–actin interface (Méjean *et al.*, 1987a). This approach was useful not only in excluding certain regions but also in more precisely locating the ABP–actin interface.

The antibodies were elicited by using a protein fragment (285–375 sequences of skeletal-muscle actin) as the antigen. Their reactivity did not depend upon the actin conformation and they appeared to be essentially specific for continuous epitopes. The epitopes can be assumed to be at the surface of both G-actin and F-actin molecules, located in discrete sequences outside the trypsin-cleavage targets (arginine and lysine residues). Their number and location were determined by studying the antigenic reactivity of actin isoforms and skeletal-muscle actin derivatives. As shown in Fig. 3, antibody reactivity with both scallop and aortic actins was partially lost. Thus a portion of the antigenic determinants on the 285–375 fragment must be located on mutation points (Fig. 7) common to the two actins (Met/Leu-299, Thr/Ser-358) and the other portion on constant sequences. Furthermore, these antigenic sequences include methionine residues and at least one tyrosine residue, as shown by CNBr cleavage and chemical modification by tetranitromethane.

On the basis of these remarks (Fig. 7), and keeping in mind the size of an antigenic epitope (6–8 amino acid

Fig. 7. Location of the potential antigenic epitopes in the 285–375 fragment of actin

residues), it can be assumed that two or three epitopes are located around Met-299 and near Met-325 and/or Met-355, as these three positions comply with the above restrictions.

With regard to tropomyosin binding on F-actin, it appears (Fig. 4) that the antigenic regions are not situated near or at the binding interface. This may be because the interface is in a groove between actin monomers in unexposed regions, which is generally not the case of antigenic regions. Another explanation can be derived from results of ^1H -n.m.r. investigations, which show that the purified CNBr-cleavage peptide CB9 (355–375 sequence) interacts with tropomyosin (Moir & Levine, 1986). Thus the C-terminal sequence involved in the tropomyosin interaction would not affect the antibody interaction, which occurs elsewhere.

The actin binding interface for serum gelsolin appears to be on some part of the C-terminal sequence. Our results clearly show that at least half of the antibodies are released by gelsolin, and that they can completely displace gelsolin from actin. This indicates that gelsolin and a specific antibody population are in competition. One region of the 285–375 sequence appears to be implicated.

This conclusion is in agreement with a previous study showing that anti-(10 kDa fragment) antibodies are unable to compete with DNAase I for G-actin (Boyer *et al.*, 1985). Consequently, the corresponding antigenic epitopes are outside the DNAase I interface. The finding that the same antibody populations compete with serum gelsolin is a clear structural confirmation of the topological independence of the DNAase I and gelsolin interfaces and of the existence of the ternary DNAase I-actin-gelsolin complex (Thorstensson *et al.*, 1982). Finally, these results suggest that some of the epitopes in the 285–375 sequence are exposed at the barbed end of the microfilament, the extremity capped by gelsolin (Janmey & Stossel, 1986).

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