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LOCALIZATION OF A NEW α-ACTININ BINDING SITE IN THE COOH - TERMINAL PART OF ACTIN SEQUENCE

M-C Lebart, C. Méjean, M. Boyer, C. Roustan and Y. Benyamin *

LP 8402, Centre de Recherches de Biochimie Macromoléculaire (CNRS), U. 249 (INSERM), Laboratoire de Biochimie et Ecologie des Invertébrés Marins (EPHE), Université de Montpellier I, B.P. 5051, F-34033 Montpellier Cédex, France

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Summary : The interaction of filamentous actin with α–actinin, an actin cross-linking protein,is well established. On the other hand, monomeric actin - α–actinin interaction has been a subject of controversy. In this report, we have characterized the interaction of monomeric actin, coated on plastic plates under conditions of non - polymerization, with α–actinin in presence of magnesium. Using specific polyclonal anti - actin antibodies, with the whole molecule or purified peptides, we have localized two sites of interaction on actin molecule : one near Thr-103 and a new one in the twenty last amino acids.

α–Actinin belongs to the actin-binding super family of proteins. It is a homodimer (MW = 200 kD) with subunits forming an antiparallel side-by-side association in agreement with the rod-shaped and dumbbell-like structure visualized by electron microscopy. α-Actinin has a wide spread distribution : it was found in both vertebrate and invertebrate, muscle and non muscle cells (for review, see 1). Its capacity to cross-link actin filaments was pointed out, inducing network or bundles of filamentous (F)-actin depending on the α-actinin / actin ratio (2) as well as a limited activation of the actomyosin ATPase (3, 4). α-actinin was thus proposed to have a fundamental role in muscle contraction in anchoring actin filaments in Z disks of sarcomeres and in non muscle cell motility processes in the organization of the sub-membrane cytoskeleton (1).

The comparison of the α–actinin sequence with other proteins revealed a striking homology in the NH_2 segment, which has been experimentally shown to contain the actin-binding domain (5, 6), with the product of dystrophin gene responsible for a muscular disease (7). Likewise, it should be mentioned that this conservative actin-binding domain has recently been found in different proteins including α-actinin, filamin, 120 kD gelation factor, spectrin and dystrophin speculating that they could belong to the same family (8).

It has been shown that α-actinin, which competes with tropomyosin (9, 10), may interact only with F-actin ; studies on the interaction between α–actinin and monomeric or

* To whom correspondence should be addressed : CRBM (CNRS) Route de Mende B. P. 5051 F-34033 Montpellier Cédex, France.

Abbreviations : 1-7 antibodies, anti-actin-(1-7)-sequence antibodies ; E.L.I.S.A., enzyme-linked-immunosorbent assay ; 1, 5- IAEDANS : N- iodoacetyl-N-(5-sulfo-1-naphtyl) ethylenediamine ; kD, kiloDalton ; $K_{d\ app}$, apparent dissociation constant ; DNase I, deoxyribonuclease I.

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globular (G)-actin have provided controversial results (9, 11-13). However, the presence of Mg^{2+} ions was shown to be important for the α-actinin - actin interaction (12, 14). This can be interpreted in terms of the necessity for actin monomers to transconform in the F-conformation which is afford in the actin filament. Moreover, it has previously been shown that α-actinin which frequently interacts with F-actin in a 1 : 14 actin monomers ratio (2) could also interact in a 1: 2-3 ratio with a high excess of α-actinin (15).

In the present study, we have further investigated the interaction of α-actinin with plastic coated monomeric actin in the presence of Mg^{2+} ions. Using E.L.I.S.A. technique with anti - actin polyclonal antibodies of various specificities, we have precisely located a part of the α-actinin - actin interface in the actin structure.

MATERIALS AND METHODS

Smooth muscle α-actinin was obtained from chicken gizzard as described (16). Rabbit skeletal muscle actin was isolated from acetonic powder (17), and further purified by gel filtration using an S300 column (2,5 x 90 cm) equilibrated with 50 mM Tris, 0.5 mM $CaCl_2$, 0.2 mM ATP, 5 mM β mercaptoethanol, 5 mM $Na_2S_2O_3$ and 600 mM KI buffer, pH = 7.5. G-actin was obtained from KI purified actin after exhaustive dialysis against G-buffer (2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP, pH = 7.8-8) and ultracentrifugation (3 hours at 150 000 g). F-actin was obtained by polymerization of G-actin in 100 mM KCl, 2 mM $MgCl_2$ at 20°C during 3 hours and ultracentrifugation (2 hours at 150 000 g). Actin was labelled at Cys-374 by 1,5-IAEDANS (18).The 10 kD fragment (285-375 sequence) was obtained and purified as previously described (19). The cyanogen bromide peptide CB9 (355-375 sequence) was isolated from dansylated actin using a High-Performance-Liquid-Chromatography Deltapak C4 column running with acetonitrile gradient (0 to 60 %) (20). The selected pic (49 % acetonitrile) was monitored at 232 and 340 nm (data not shown) and the purified peptide CB9 identified by its amino acid composition which totally agreed with the theoretical composition of 355-375 actin sequence.

Anti - α-actinin sera were induced in rabbits using 100 μg of protein per injection (21). Anti - α-actinin antibodies were purified on sepharose 4B - α-actinin immunoadsorbant column (22). Purified anti - actin antibodies directed against epitopes located in 1-7 (23), 40-113 (24), 195-226 (24) and 285-375 (25) sequences were previously described. Sheep anti - dansyl antibodies were obtained as in (19).

E.L.I.S.A. technique (26) was used to monitor the interaction between α-actinin and actin or derivates. Each well was coated with 50 ng of G-actin, 50 μg of F-actin, 3 μg of 10 kD (285-375 sequence) or 150 ng of CB9 (355-375 sequence) peptides under the conditions previously described (27). The assays were carried out using 10 mM Tris, 10 mM KCl, 2 mM $MgCl_2$ and 0.05 % Tween 20, pH = 7.4. The incubations were all made at 20°C. In the direct method, anti - α-actinin antibodies (7 μg/ml) were added after α-actinin incubation. In competition method, anti - actin antibodies were incubated in presence of α-actinin. Anti - sheep and anti - rabbit immunoglogulins (1 : 3000) were obtained from Biosys (Compiègne, France). Aditional details are given in the figure legends.

RESULTS

The interaction of α-actinin with coated actin was investigated using direct E.L.I.S.A. technique with anti - α-actinin antibodies. The results (Fig. 1) clearly show that α-actinin can bind to coated monomeric and filamentous actins with a similar affinity (K_d app = 2. 10^{-6} M) in the presence of Mg^{2+} and K^+.This similar apparent affinity between G- and F- actin can be explained by the presence of 2 mM $MgCl_2$ (12, 14) which modifies the conformation of G-actin

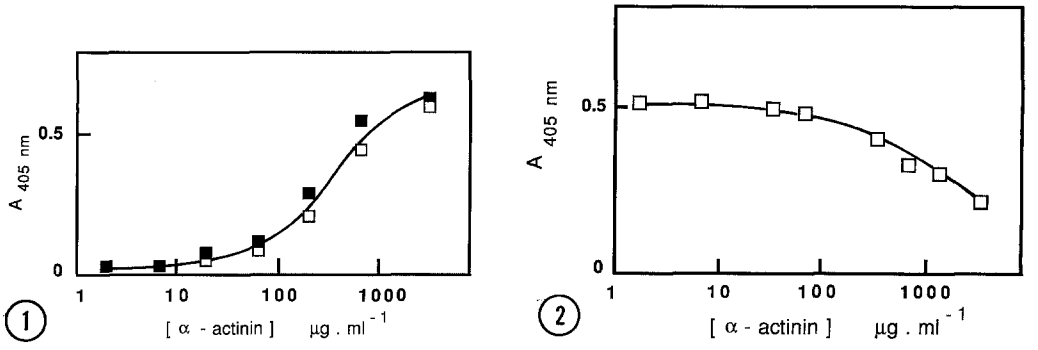

Fig. 1 . **α–actinin binding on actin conformers.** Direct E.L.I.S.A was carried out as described in the Materials and Methods section. G- (□) or F- (■) actin was coated on plastic plates, α–actinin was incubated at various concentrations (0 to 3.3 mg.ml^{-1}). Anti - α–actinin (7 µg.ml^{-1}) antibodies were used to follow the reaction.

Fig. 2 . **Effect of α–actinin on 40-113 antibody - G-actin interaction.** Increasing amounts of α–actinin (0 to 3.3 mg.ml^{-1}) were incubated in presence of constant concentration (1 µg.ml^{-1}) of 40-113 antibodies, the binding of which was monitored at 405 nm.

(coated at 330 ng/ml in absence of Mg^{2+}, below the critical concentration), while its polymerization is impeded due to its immobilization on plastic plates (24). Thus, it appears that in the presence of 2 mM $MgCl_2$, coated monomeric actin also binds α–actinin.

In order to locate the α–actinin binding site on the actin sequence, we used purified polyclonal antibodies of various specificities towards known actin sequences in competition with α-actinin. We observed that the α–actinin - G-actin interaction did not modify the 1-7, 195-226 and 285-355 antibody activities. In contrast, the reactivity of 40-113 antibodies, specific to the 95-113 sequence (28), near the Thr-103 residue (24), was decreased in the presence of α-actinin (Fig. 2). This result locates the α–actinin - actin interface near the segment 95-113 of actin. Finally, using dansyl labeled G-actin, we observed that anti - dansyl antibodies and α-actinin competed with each other for G-actin (Fig. 3A and 3B). This result points out a proximity between residue Cys-374 and the α-actinin interface.

The eventual participation of the C-terminal part of actin in α-actinin interface was further explored using the 10 kD peptide (285-375 sequence) in direct E.L.I.S.A. experiments. As shown in Fig. 4A, α-actinin interacts with the coated peptide with a significant affinity ($K_{d\ app}$ = 3. 10^{-6} M). This interaction was corroborated with the coated cyanogen bromide peptide CB9 (355-375 sequence) (Fig. 4B). Thus, it appears that the binding properties of the C-terminal part of actin towards α–actinin lies in the twenty last residues of actin sequence. This conclusion was strengthened by the negative result obtained with 285-375 antibodies (Fig. 2) which interact near Met-299, Met-325, and Met-355 and therefore not within the CB9 peptide (25). Moreover, the reciprocal effect between anti - dansyl antibodies and α-actinin towards dansylated Cys-374 of actin (Fig. 3) was not observed when the dansylated CB9 peptide was coated in place of dansylated monomeric actin (data not shown). This may implicate a spatial proximity between Cys-374 and α-actinin binding site since CB9 peptide can be only partially refolded.

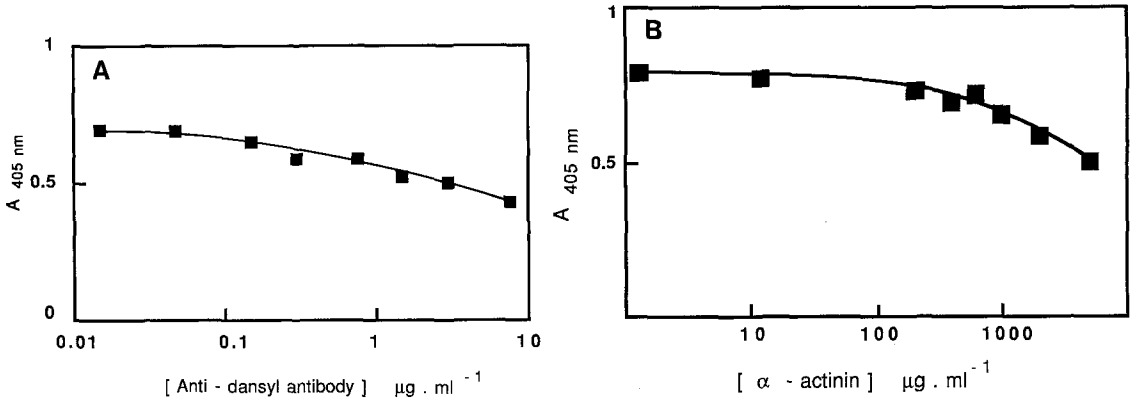

Fig. 3 . **Implication of the COOH - terminal part of actin in α–actinin binding.** A : Effect of anti - dansyl antibodies on α–actinin - G-actin interaction. Increasing amounts of sheep anti - dansyl antibodies (0 to 7.5 $\mu g.ml^{-1}$) were incubated in presence of constant concentration of α–actinin (670 $\mu g.ml^{-1}$). α–actinin interaction was followed using rabbit anti - α–actinin antibodies. B : Effect of α–actinin on anti - dansyl antibodies - labelled G-actin interaction. Increasing amounts of α–actinin (0 to 5 $mg.ml^{-1}$) were incubated in presence of constant concentration (20 $\mu g.ml^{-1}$) of anti - dansyl antibodies, the binding of which was monitored at 405 nm.

DISCUSSION

We have studied the interaction between α-actinin and monomeric actin using E.L.I.S.A. technique. We have previously shown that coated G-actin is able to interact with gelsolin (25), myosin head (29) and DNase I (28) but, in contrast to coated F-actin (27), is unable to bind tropomyosin (data not shown).We found that α-actinin can bind not only F- but also G-actin (coated on plastic plates under conditions of non polymerization) with a $K_{d\ app} = 2.\ 10^{-6}$ M in presence of $MgCl_2$. Other authors (9, 13) have reported controversial results concerning such an

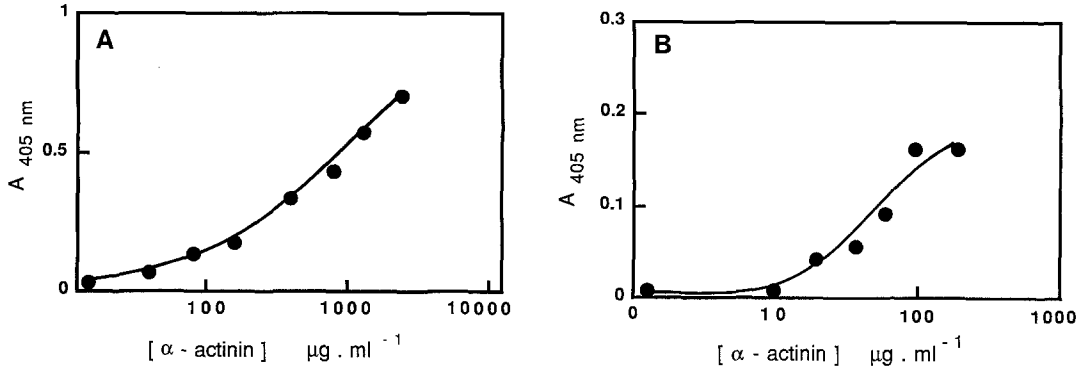

Fig. 4 . **Interaction of α–actinin with actin peptides.** Interaction of α–actinin (0 to 2 $mg.ml^{-1}$) with coated 10K peptide (285-375 sequence) (A) and coated CB9 peptide (355-375 sequence) (B) was followed at 405 nm with rabbit anti - α–actinin antibodies.

interaction, depending on the technique (analytical ultracentrifugation and gel filtration chromatography) and on the experimental conditions (presence or not of $MgCl_2$ in buffers) employed. One can speculate that the α-actinin - G-actin interaction was not detected due to the weakness of the interaction as well as to the necessity (data not shown) of $MgCl_2$ (12, 14) which likely induces conformational changes in actin monomers (24).

The location of α-actinin binding site(s) on G-actin sequence was investigated. We have found that α-actinin interacts in two segments of actin sequence : the first one located near the 103 residue and the second in the twenty last amino acids of actin sequence. These results partially agree with previous studies using 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide (E.D.C) catalyzed cross-linking experiments (5) which located two α-actinin-binding sites on actin sequence : site I (segment 1-12) and site II (segment 87-119 or 87-123). It would be noteworthy that the 1-12 sequence of actin has often been shown to cross-link to other actin-binding proteins due to its acidic amino acid composition. One can mention that proteins such as gelsolin, which has previously been cross-linked to the NH_2 segment of actin using the same technique (30), was not found to interact with this part of actin using immunological approach (31). In addition, the high variability of this sequence during evolution (32) would contrast with the presence of a conservative actin-binding domain on α-actinin (8).

The recent resolution of the 3-D structure of actin via actin-DNase I complex allows us to locate the interaction sites of α-actinin in the sub-domain 1 of actin.This domain has been reported to interact with several actin-binding proteins in part due to its accessibility in the filamentous structure of actin (33). The sequences 1-12 and 89-119 or 113, as defined by (5), appear to be located on opposite sides of actin molecule. This observation would implicate that α-actinin has two distinct actin-binding sites. New investigations concerning the interface on α-actinin molecule will probably elucidate this point.

Moreover, it should be noticed that myosin sub-fragment -1 interacts with residues 1-28 and around residue 89 of actin (29) which includes the interface on one side of sub-domain 1 of actin. α-Actinin, which has been shown to increase the actomyosin ATPase two to three fold, is unlikely to bind to the same side which would impede S-1 binding. Therefore, one can speculate that α-actinin binding sites are on the opposite side of actin.

It has been demonstrated that α-actinin and tropomyosin compete with each other for binding on F-actin (9, 10). In this context, previous studies have implicated Arg-95 (34) and the COOH extremity of actin sequence (35) in the tropomyosin - F-actin interface. So, the location of two binding sites for α-actinin on actin sequence, one site near 103 residue and the other in the twenty last amino acid residues, could easily explain these results.

Lastly, studies carried out with profilin have shown that α-actinin can promote polymerization of actin from profilactin complex (36). This can be explained by a direct competition between the two proteins. Moreover, profilin has been reported to interact near Glu-364 in actin sequence (37). These results emphasize the presence of an α-actinin binding site in the C-terminal part of actin.

In conclusion, we have found two sites of interaction for α-actinin on actin sequence : one near Thr-103 in agreement with previous studies (5) and a new one in the twenty last amino

acids. Whether these sites are structurally independant or correspond to two different sub-sites of a unique interface is still unknown. A more precise location of these two binding domains is now been investigated using synthetic peptides. The extension of this study to filamin and dystrophin, which belong to the same family as α-actinin (8) and may possess equivalent binding site(s) on actin molecule, is in progress.

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