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IMPORTANCE OF THE C-TERMINAL PART OF ACTIN IN INTERACTIONS WITH CALPONIN

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Summary: Native, modified or trypsin-truncated actin was used to study the impact of modifying the C-terminal part of actin (the last three amino acids) on interactions with calponin. We used three different techniques to show that these amino acids are essential for the actin-calponin interface. The locations of actin-calponin interaction sites on the actin crystal are discussed in terms of previously reported data.

Calponin (CaP) is a smooth muscle and nonmuscle specific protein which is present in different isoforms (1;2). It is preferentially localized in thin filaments and dense bodies with β-actin (3;4). These different localizations are probably due to different CaP isoforms (2). CaP is an actin, tropomyosin, myosin and calmodulin binding protein (5;6) which regulates actomyosin ATPase (7). This inhibition was demonstrated to be modulated by calcium and calmodulin. However, since the affinity of CaP for calmodulin does not seem to be physiologic, two other calcium binding proteins were recently found to modulate CaP-actin interactions with lower affinity than calmodulin S100 and caltropin (8;9). Binding of CaP to actin has been studied by EDC crosslinking and actin was found to bind CaP by its N-terminal region of residues 145-182 (10), and more recent studies using synthetized peptides revealed that this site is restricted to amino acids 145-163 (11). Two EDC crosslinking analysis studies of interaction sites on the actin molecule showed two different kinds of sites. In the first paper, Winder et al. used Cleveland mapping with V8 protease of the actin-CaP complex, and demonstrated involvement of the N-terminal part of actin between residues 1 to 226 (12). Secondly, Mezgueldi et al. showed that interaction with CaP occurs on the C-terminal part of actin at residues 326-355. We show here the importance of the last three residues of actin in the actin-CaP interaction.

MATERIAL AND METHODS

Protein preparations: Proteins were purified by previously described methods: skeletal myosin (13), chicken gizzard calponin (7), rabbit skeletal actin (14) with slight modifications (15), 1,5

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Abbreviations : ATPase, Adenosin-5'-triphosphatase; CaD, Caldesmon; CaP, Calponin; DTT, Dithiothreitol; EDC, 1 ethyl-3-[3-(dimethylamino)propyl] carbodiimide; ELISA, Enzyme linked immunosorbent assay; 1,5-IAEDANS, N-(iodoacetyl)-N'-(5-sulfo-1-naphthyl)-ethylenediamine; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; STI, Soybean trypsin inhibitor; V8 protease, *Staphylococcus aureus* V8 protease.

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IAEDANS-labelled actin (16) and trypsin-split actin (17). STI and trypsin treated TPCK were purchased from Sigma. All chemical reagents were of the highest analytical grade.

Cosedimentation assays: Cosedimentation assays were performed in 20 mM Tris-HCl, pH 7.5;2 mM $MgCl_2$; 1 mM DTT; 1 mM $CaCl_2$ and 1 mM ATP. Native actin, actin-IAEDANS or trypsin-truncated actin (20 µM) and CaP (4 µM) were mixed and incubated for 5 min. After centrifugation at 30 PSI in a Beckman Airfuge, the supernatant and pellet were analysed by SDS-PAGE.

Immunological techniques: ELISA was carried out according to the method of Engvall (18). F-actin was coated at 200 µg/mL on microtitre plate wells (Nunc), CaP was incubated for 1 h in decreasing concentrations. The first antibody used was a rabbit polyclonal antibody raised against CaP. The second antibody was an alkaline phosphatase conjugated anti-rabbit antibody (Jackson Immunoresearch).

Other methods: Protein concentrations were determined by spectrophotometric measurements in a Kontron Uvikon 930 using the following extinction coefficients: actin $\varepsilon^{1\%}_{280nm}$ = 11 (19); Myosin $\varepsilon^{1\%}_{280nm}$ = 5.5 (20); CaP $\varepsilon^{1\%}_{280nm}$ = 1,132 (7). ATPase activity was measured as described in (21).

RESULTS

We used skeletal native, IAEDANS-modified and trypsin-truncated actin to determine the importance of the C-terminal part of actin in interactions with CaP. At first, we cosedimented *different kinds of actins with CaP as described in the Material and Methods (Fig. 1). Quantitative* data were obtained by densitometric scanning of the gel shown in Fig.1. Only CaP remained in the supernatant (Lane 1), and with native actin all of the CaP completely cosedimented with actin in a 1/5 ratio (Lane 3). This ratio is an average of the maximum binding stochiometry determined between CaP and actin *in vitro* (1/3) versus that estimated in the tissue (1/7). The affinity of CaP for actin was identical to that shown previously (22). With modified actin, about 50% of CaP remained in the supernatant, indicating that IAEDANS prevented CaP-actin interactions (Lane 5). With truncated actin, all of the native CaP was found in the supernatant (Lane 7), showing that the last three amino acids of the molecule, removed by trypsin, are essential for actin-CaP

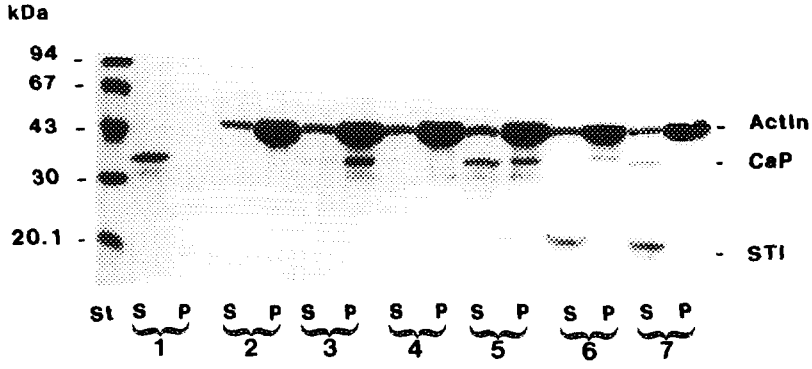

Figure 1. Binding of actin, actin IAEDANS and trypsin-truncated actin to calponin. Different kinds of actin were cosedimented with CaP at 200000 g for 30 min in a Beckman airfuge. Supernatant (S) and pellets (P) were analysed by SDS-PAGE (12.5%). St : Standards; Lane 1 : CaP alone; Lanes 2-3 : Native actin without or with CaP; Lanes 4-5 : Actin-IAEDANS without or with CaP; Lanes 6-7 : Truncated actin without or with CaP.

interactions. The quantity of CaP used was the same under all conditions. However, the presence of traces of trypsin in the actin preparation, even when washed several times and after the addition of a high quantity of soybean trypsin inhibitor (STI) as clearly evident on the gel, led to some CaP degradation. In our conditions, we clearly demonstrated that with native actin, all CaP is cosedimented, and with truncated actin, all of the remaining native CaP can be found in the supernatant.

ELISA was used to overcome this trypsin contamination, since this technique includes many additional washing steps. We coated different kinds of actin in the wells and incubated CaP at concentrations ranging from 1 µM/mL to 32 µM/mL, and CaP was detected with an anti-CaP antibody (Fig. 2). The results clearly showed that binding of CaP to IAEDANS or trypsin-truncated actin was less effective than with native actin. The signal was about 25% lower for IAEDANS actin and 40% lower for trypsin-truncated actin. Therefore, 60% of CaP binding to trypsin-truncated actin was observed whereas no binding was detected in cosedimentation experiments. This could be explained by the washing procedures used in the ELISA technique and the fact that actin coated the wells. Therefore no trypsin could remain in the wells during the procedure, indicating that both kinds of modified actin bind CaP less strongly than the native one.

CaP is known to inhibit actomyosin ATPase (Fig. 3). Skeletal muscle acto-myosin was used to investigate the ability of CaP to inhibit actomyosin ATPase activity since this system is active without phosphorylation of the myosin head, contrary to the smooth actomyosin system. CaP inhibited actomyosin activity by 80% at 1 µM, while actin-IAEDANS inhibited it by 15% at 1 µM CaP. Trypsin-truncated actin did not inhibit this activity at all.

DISCUSSION

In this study, we demonstrated the importance of the last three amino acids of actin in CaP-actin interactions. This point was investigated since EDC crosslinking of CaP with actin-

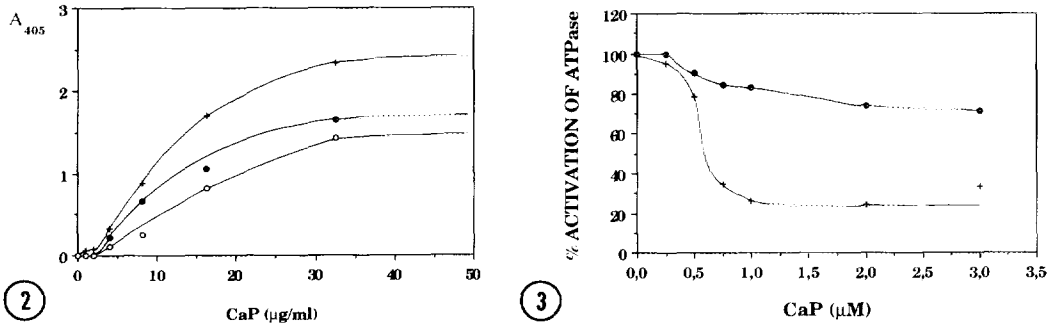

Figure 2. Interaction of actins with calponin. In direct ELISA experiments, interaction of decreased calponin concentrations (1 to 32 µM/mL) with different kinds of actins was analysed at 405 nm with an anti-CaP polyclonal antibody. Native actin (+), Actin-IAEDANS (●), Trypsin-truncated actin (○).

Figure 3. CaP inhibition of skeletal actin-activated myosin magnesium ATPase. Actomyosin ATPases activities were measured as previously described (21)with 0.2 mM myosin and a molar ratio of actin/myosin = 50. Native actin (+), Actin-IAEDANS (●).

IAEDANS gave a very poor product yield (data not shown). We thus used other techniques to determine whether there was actually a decrease in actin affinity for calponin due to the presence of IAEDANS. Cosedimentation, ELISA and ATPase were used and demonstrated a decrease in affinity. We wanted to determine whether this decrease was due to sterical hindrance of cysteine 374, or whether these last three residues were directly implicated in the interaction. F-actin was thus submitted to limited trypsin proteolysis and it cleaved after residue 373. This actin is known to be stable because it was the one used to obtain the first actin crystal (23). This technique was previously used by Kolakowski et al. (24) to study involvement of the C-terminal part of actin in binding with caldesmon (CaD). They demonstrated that lysine 373 was essential for this interface.

Under two different experimental conditions, the same chemical crosslinker (EDC) revealed two CaP interaction sites on actin. The first one is located in the N-terminal part of actin, between residues 1-226, excluding residues 1 to 12 and lysine 61 (12;25), and the second zone is in the C-terminal part between residues 326-355 (10). In the first case, Cleveland mapping with V8 protease revealed that the 30 kDa N-terminal part of actin binds to CaP (split V8 protease on actin at residue 226)(17). This fragment is very large and mainly corresponds to subdomain 1 and subdomain 2 of actin (Fig.4). Therefore the site cannot be located more

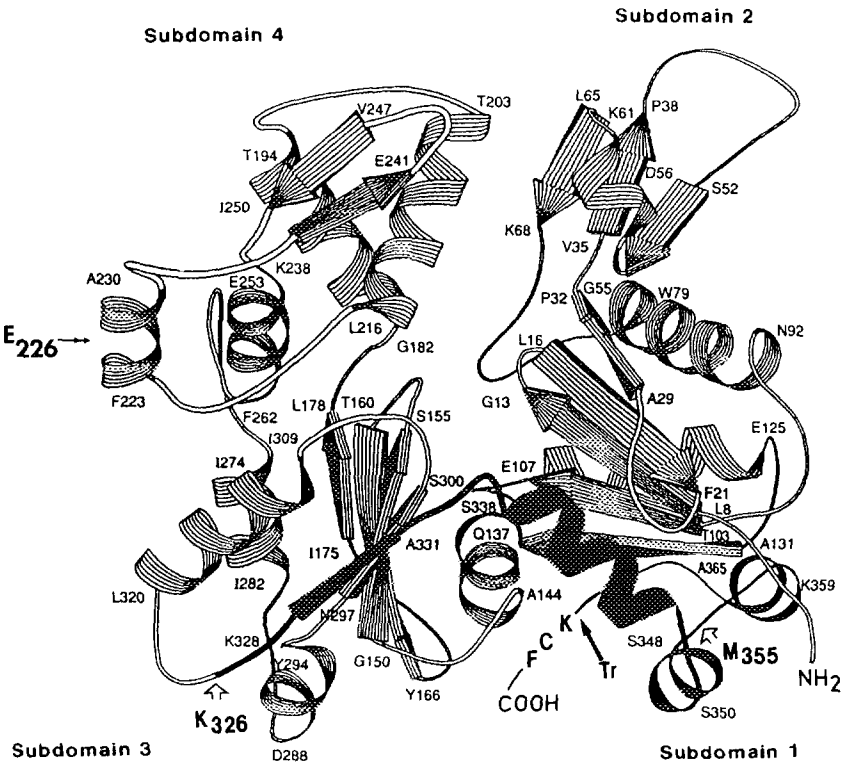

Figure 4. Secondary structure representation of the G-actin crystal structure (From 23). The fragment extending from 326 to 355 (as pointed out by white arrows) is noted in black. The trypsin cleavage site shown with a black arrow (Tr) and the three last residues (which are missing in the crystal), K-C-F, have been added. Position of residue 226 (the cleavage site of the V8 protease) is also shown with a double-headed arrow. Reprinted with permission from *Nature* (23). Copyright 1990 Macmillan Magazines Limited.

precisely. In the second case, the product of actin-calponin crosslinking by EDC was submitted to total hydrolysis with CNBr and the C-terminal segment of residues 326-355 was shown to link the N-terminal calponin region. Relative to the actin crystal (Fig. 4), this 326-355 region overlaps on two subdomains of actin, subdomain 3 and subdomain 1. The last three residues of actin reside in subdomain 1 and can integrate one of the two sites stated above, but will always be located in subdomain 1.

The involvement of these three residues in the CaP-actin interaction was also supported by two observations. First CaD, which bound actin by its C-terminal part and its N-terminal part (residues 1-12), was displaced by CaP, which did not bind actin by its twelve N-terminal residues, from its complex with F-actin (26-28). Secondly CaP decreased the fluorescence intensity of pyrene-labeled F-actin (labelled on its cystein 374) (29).

Regulatory proteins CaD and CaP both provide smooth muscle regulation. This study clearly revealed the importance of the last residues of actin in interactions with CaP. As compared to CaD, C-terminal integrity of actin is essential for CaD-actin interactions but is not affected by the 374 fluorescent labelling of actin (28;30) whereas covalent crosslinking between CaP and actin is significantly abolished when this actin labelling is used. This difference observed in chemical reactivity towards modified actin by these two regulatory proteins indicates a possible variability in the regulation pathway process during smooth muscle contraction. This would explain the need for two regulatory proteins in smooth muscles whose properties are very similar and their association in distinct thin filaments and their specific cell compartimentation (4;26).

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