Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 239-245

INTERACTION OF CALDESMON AND MYOSIN SUBFRAGMENT 1 WITH THE C-TERMINUS OF ACTIN

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Received March 2, 1992

SUMMARY: The interactions of caldesmon and S1 with the C-terminus of actin were examined in co-sedimentation experiments using proteolytically trunctated actin. It is shown that removal of 6 residues from the C-terminus of actin reduces the binding of caldesmon by about 50% while improving the binding of S1 to actin. We also show that S1 protects actin's C-terminus from enzymatic cleavage. Both S1 and caldesmon binding to actin are decreased in the presence of an actin C-terminal peptide. These results emphasize the importance of the C-terminus of actin in binding to S1 and caldesmon.

Caldesmon is a flexible, elongated molecule that binds tightly and simultaneously to actin and smooth muscle myosin (reviewed in 1) and inhibits the actin activated ATPase of smooth and skeletal myosins and their subfragments (2-6). These characteristics of caldesmon make it a possible candidate for regulation of actomyosin interactions. Caldesmon also competes with myosin fragments for the binding to actin in rigor (5). The competition between caldesmon and myosin for the binding to actin suggests at least partial overlap of their actin binding sites and could be the basis of the regulatory function of caldesmon.

Recent studies have revealed that caldesmon (7-9) and myosin.ATP (10-12) interact directly with the N-terminus of actin (7-9). The C-terminus of actin, adjacent to its N-terminus, has also been implicated in the interaction with caldesmon (13,14) as well as the actomyosin ATPase activity (Dabrowska et al., personal communication). Binding and fluorescence experiments with Cys-374 labeled actin revealed a communication between the caldesmon-binding site on actin and actin's C-terminal region (13).

0006-291X/92 $1.50

Moreover, Graceffa and Janscó (14) demonstrated that a disulfide cross-link could be formed between actin's C-terminal Cys-374 and Cys-580 of caldesmon, suggesting a direct contact between these sites. In the present study, we further characterize the interaction between the C-terminus of actin and caldesmon as well as that between the C-terminus of actin and S1. The latter interaction has been indicated by cross-linking experiments (15), actin structure considerations (16), and spectroscopic studies (17, 18).

EXPERIMENTAL PROCEDURES

Preparation of Proteins and Antisera-Subfragment 1 (S1), rabbit skeletal muscle actin, and caldesmon were prepared as described previously (6,13). 1,5-IAEDANS modified actin at Cys-374 (13) contained between 0.6 and 1.0 dye/actin. The concentrations of modified actin and caldesmon were measured as before (13).

The antiserum against the N-terminus of S1 (acetyl-Ser-Ser-Asp-Ala-Asp-Met-Ala-Val-Lys) was a generous gift from Dr. T. Chen. The antiserum against caldesmon was a generous gift from Dr. C.-L. A. Wang. The synthetic actin C-terminal peptide (Lys-Gln-Glu-Tyr-Asp-Glu-Ala-Gly-Pro; MW= 1,179 Da) was purchased from the custom peptide synthesis facility at the University of California, San Diego.

Airfuge Binding Experiments-The co-sedimentation experiments were performed as described earlier (13). Unmodified and labeled G-actin (5.0μM) were polymerized with 40mM NaCl and 2mM $MgCl_2$ in G-buffer and subsequently incubated with either caldesmon (from 1μM to 3μM) or S-1 (from 1μM to 3μM). Samples for co-sedimentation were pelleted in a Beckman Airfuge, run on SDS gels, Coomassie stained, and analyzed by densitometry. Caldesmon and S1 binding to actin were expressed in terms of percentage of their binding to unmodified actin, which was normalized to 100%. The actual stoichiometries of either caldesmon or S1 binding to actin varied between 0.2 and 0.6, depending on protein concentrations.

Proteolytic Removal of Labeled Cys-374 from Actin-In order to remove the last 3 C-terminal residues of actin, F-actin modified at Cys-374 with 1,5-IAEDANS was cleaved by trypsin (5:1 molar ratio of actin:trypsin) at 25°C for approx. 20 mins. (19) and as described previously (13). Chymotryptic removal of the 6 C-terminal residues of actin was achieved in a similiar manner except that the chymotrypsin:actin molar ratio was 1:2.4 and the reaction was performed at 35-37°C for approx. 20 mins. The chymotryptic cleavage was stopped with PMSF dissolved in ethanol. The progress and the completion of tryptic and chymotryptic cleavages of the C-terminal residues on F-actin was monitored as before (13).

Cleavage Time Course of Modified Actin in the Presence and Absence of S-1-1,5-IAEDANS modified F-actin (24μM) was incubated with S1 (22μM) in 40mM NaCl and 10mM Imidazole solution, pH 7. Proteolytic removal of the C-terminus of actin by trypsin or chymotrypsin was performed as described above except that the reactions were stopped at 5, 15, and 25 minutes. The reaction products were run on a 10% polyacrylamide SDS gel, fixed and photographed under UV illumination. After photography, the gels were Coomassie stained. The densitometric traces of Coomassie-stained actin bands and the fluorescent actin bands were analyzed to determine the percentage of actin cleavage in the presence and absence of S1. The percentage cleavage at each time point was

determined from ratios of fluorescent intensity to Coomassie intensity relative to a similar ratio for uncleaved actin.

Immunochemical Assays-The ELISA assay was performed as described previously (20,21). Either 0.25 mg/ml of caldesmon or 0.25 mg/ml of S1 in PTB buffer were added to microtiter plates coated with F-actin. The actin C-terminal peptide was added in quadruplicate wells in concentrations from 0.16nM to 5mM. Antisera against caldesmon and the N-terminus of S1 were used to detect caldesmon and S1 binding to actin, respectively. The developed color (see 20 and 21 for ELISA assay conditions) was measured at 410nm in a Molecular Devices Vmax microplate reader. No interference of actin and caldesmon binding was detected for the caldesmon antiserum. The titer for both the anti-N-terminal S1 sera and the anti-caldesmon sera was 1:3000.

RESULTS AND DISCUSSION

Binding Studies- We have previously shown that tryptic cleavage of the 3 C-terminal residues on actin, including the removal of 1,5-IAEDANS probe on Cys-374 of actin reverses the modification-induced decrease in the binding of caldesmon to actin (13). This suggests that although Cys-374 on actin and Cys-580 can be linked via disulfide bond (14), the 3 C-terminal residues on actin do not contribute significantly to the binding between these proteins. In the present study, we examine the effect of chymotryptic removal of 3 additional residues from actin's C-terminus (19) on the binding of caldesmon and S1 to actin. Co-sedimentation experiments of caldesmon with chymotryptically cleaved actin revealed a substantial reduction of caldesmon binding to actin, by about 50% (Table 1). The large effect on caldesmon binding after the removal of 6 C-terminal residues on actin can be understood in terms of: 1) proteolysis indirectly affecting the main caldesmon binding site at the N-terminus of actin or 2)

TABLE 1
BINDING OF CALDESMON AND S1 TO ACTIN (%)

Actin	% BINDING	
	Caldesmon	S1
Unmodified actin	100[a]	100[a]
1,5-IAEDANS-actin	75±15(5)[b]	94±2(3)
Actin (1-372) tryptically cleaved	115±10(5)[b]	115±3(4)
Actin (1-369) chymotryptically cleaved	55±10(10)	117±5(3)

[a]A binding value of 100% was assigned to the binding of caldesmon and S1 to control, unmodified actin. The actual stoichiometries of caldesmon and S1 binding to actin varied between 0.2 and 0.6.
[b]Values taken from reference 14.
The number of experiments in each set is given in parentheses.

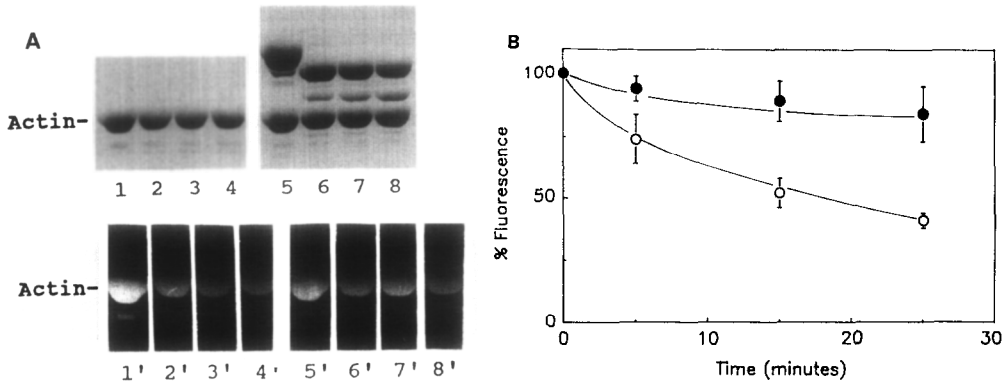

FIG. 1. Effects of S1 binding on the cleavage of C-terminus of actin. 1,5-IAEDANS modified actin (24.0 μM) in the presence (•) and absence (○) of S1 (22.0 μM) was cleavaged by trypsin as described under "Expermental Prodecures". A, lanes 1 to 4, Coomassie stained gels of modified actin cleavage by trypsin (for 0, 5, 15, and 25 mins., respectively) in the absence of S1. Lanes 5 to 8, actin cleavage in the presence of S1 (for 0, 5, 15, and 25 mins., respectively). Lanes 1'to 8', correspond to the same bands viewed under UV illumination. B, time course of tryptic cleavage of modified actin in the presence (•) and absence (○) of S1. The progress of digestion was monitored as described in "Experimental Procedures". Standard deviation (n=3) is indicated by error bars.

removal of a secondary caldesmon contact site at the C-terminus. The possibility of any major structural perturbations of actin by proteolysis can be discounted since the truncated protein polymerizes and binds S1 well.

Trypsin and chymotrypsin cleaved actins were also employed to examine the importance of the C-terminus to S1 binding. Co-sedimentation experiments were performed with S1 and unmodified actin, 1,5-IAEDANS modified actin (at Cys-374), tryptically cleaved actin (1-372), and chymotryptically cleaved actin (1-369). The results (Table 1) show that the modification of the penultimate residue on actin causes an insignificant decrease in S1 binding (94% ± 2%). However, the binding of S1 to trypsin or chymotrypsin treated actin was improved by approximately 15% compared to intact actin.

Effect of S1 Binding on the Cleavage of the C-terminus of Actin- The effect of S1 on the C-terminus of actin was examined also by monitoring the C-terminal tryptic cleavage of 1,5-IAEDANS-modified actin (24μM) in the absence and presence of S1 (22μM). The cleaved samples were run on SDS gels and the UV-illuminated fluorescent actin bands and Coomassie-stained actin bands were quantified by densitometry (Fig. 1, A). The resulting ratios of fluorscent band intensities to those of Coomassie bands yield the information on the cleavage in the C-terminal region of actin.

Fig. 1 shows an inhibition in the rate of C-terminal actin cleavage by trypsin in the presence of S1. The fact that S1 was cleaved by trypsin to the 25, 70 (and to smaller extent also 50 and 20) fragments did not prevent the protection of actin from proteolysis. Sedimentation of the cleaved samples revealed the presence of tryptic S1 fragments in the pellet, together with actin. Most likely, S1 and tryptic S1 are able to protect C-terminus of actin from cleavage by either 1) steric blockage of the C-terminus or 2) communication with the C-terminus, rendering it less available for cleavage. Identical results were found for chymotryptic cleavage of actin in the presence of S1 (data not shown). Similar experiments with caldesmon were unsuccessful because of the rapidity of caldesmon digestion by trypsin and chymotrypsin.

Effect of Actin C-terminal Peptide on the Binding of Caldesmon or S1 to Actin- The present study has provided additional evidence for a communication between actin's C-terminus and caldesmon and S1. It appears that COOH region may be either a minor binding site or an area of indirect interaction with caldesmon and S1. A possible overlap between S1 and caldesmon binding residues on the C-terminus of actin may occur in the cluster of negative residues 358-366, which can be cross-linked to S1 (15). A synthetic peptide corresponding to this C-terminal site on actin was used in ELISA experiments in which actin was coated to microtiter wells and allowed to reach equilibrium with either 1) caldesmon and the peptide 2) S1 and the peptide 3) caldesmon alone 4) S1 alone. Caldesmon and S1 binding were detected with antisera to these proteins as described under "Experimental Procedures". The results were normalized to $O.D._{410}$ measured in the absence of the peptide. The peptide inhibited both S1 and caldesmon binding to actin by about 15% and 30%, respectively (Fig. 2). These results suggest that the extended C-terminal region of actin (358-374) plays a part in the contact site for both caldesmon and S1. Caldesmon is more sensitive than S1 with respect to both the actin peptide and to modification of Cys-374 as well as to chymotryptic cleavage of actin (Table 1). While the competition of caldesmon and S1 for actin binding may involve both the N- and C-terminal regions of actin, the latter region may have different contributions to caldesmon and S1 binding. It also appears that the primary binding sites for S1 and caldesmon are outside the C-terminal region since neither the peptide nor proteolytic removal of up to 6 C-terminal residues on actin abolished these interactions.

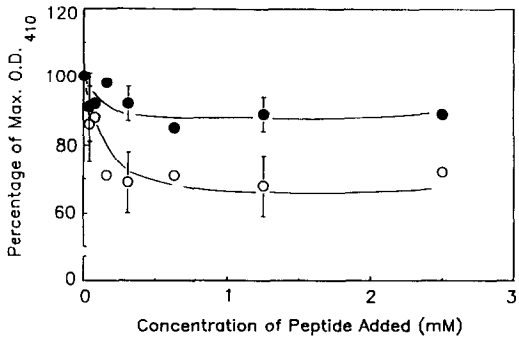

FIG. 2. Effect of actin C-terminal peptide on the binding of caldesmon and S1 to actin. Actin C-terminal peptide (0-5mM) was added together with caldesmon (0.25mg/ml) (o) or S1 (0.25mg/ml) (•) to triplicate wells of microtiter plates coated with actin. Binding of CDS and S1 was detected as described under "Experimental Procedures". Optical density readings at 410nm monitor the amounts of CDS or S1 bound to actin. The results are normalized to the binding in the absence of the peptide.

ACKNOWLEDGMENTS

This work was supported by UPSH grants AR22031 (to E.R.) and AR35216 and AR40540-01A1 (to J.M.C.).

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