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Lys-373 of actin is involved in binding to caldesmon

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Limited proteolysis of actin with trypsin removes its two or three C-terminal amino acid residues [Proc. Natl. Acad. Sci. USA 81 (1984) 3680–3684]. Carboxypeptidase B-treatment of G- and F-actin previously digested with trypsin revealed that in the first case preferential release of three and in the second two C-terminal amino acid residues takes place. Tryptic removal of three but not two C-terminal amino acid residues of actin causes weakening of its interaction with caldesmon and lowering of the caldesmon-induced inhibitory effect on actomyosin ATPase activity. Therefore, it is concluded that the third amino acid residue from the C terminus of actin, Lys-373, is important for the interaction with caldesmon.

Caldesmon; C terminus of actin; Binding; Actomyosin ATPase

1. INTRODUCTION

Three recent publications [1–3] from different laboratories pointed out the importance of C-terminal amino acids, in addition to N-terminal region [4–6] of actin, for the interaction of this protein with caldesmon. However, studies on the binding of caldesmon to actin in which C-terminal amino acid residues were removed by trypsin treatment has led to contradictory results. Whereas in our experiments the interaction of caldesmon with truncated actin was weaker than that with intact one [2], Crosbie et al. [1] found only different binding of caldesmon to intact and 1,5-IAEDANS-labeled (at Cys-374), but not to truncated actin. Since in our investigations G-actin was proteolysed with trypsin whilst Crosbie et al. [1] proteolysed F-actin it was important to identify C-terminal amino acid residues released under both conditions and to correlate this release with the binding of actin to caldesmon.

2. MATERIALS AND METHODS

Caldesmon was isolated from chicken gizzards according to the method of Bretscher [7]. Rabbit skeletal muscle myosin was prepared according to Perry [8] and further purified following the procedure of Kielley and Bradley [9]. Rabbit skeletal muscle actin and chicken gizzard tropomyosin were prepared according to the procedures described elsewhere [10].

Digestion of Mg-G-actin with trypsin was carried out in buffer containing 0.1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM EGTA, 1 mM DTT, 0.02% NaN_3 and 2 mM HEPES, pH 7.6 at 1:50 (w/w) enzyme to substrate ratio for 60 min at 25°C, as described in detail in [2]. Digestion of Ca-F-actin was performed similarly as in [1] in a medium composed of 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM DTT, 100 mM KCl, 0.02% NaN_3 and 2 mM HEPES, pH 7.6, at 1:5 (w/w) trypsin to actin ratio for 20 min at 25°C. In both cases reaction was terminated by adding a 5-fold (w/w) excess of soybean trypsin inhibitor over trypsin.

Both preparations of actin were then purified from trypsin, trypsin inhibitor and released actin peptides, by two cycles of polymerization with 100 mM KCl, ultracentrifugation and depolymerization by homogenization and dialysis against G-buffer (0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM DTT, 0.02% NaN_3 and 2 mM HEPES, pH 7.6).

Carboxypeptidase B treatment of purified actin truncated with trypsin in either Ca-F-form or Mg-G-form was carried out, after their transformation to Ca-G-form, in G-buffer at 1:10 molar ratio of enzyme to substrate for 20 h at 4°C. Digested preparations were purified again by two polymerization–depolymerization cycles. Trichloroacetic acid (final concentration 10%) was added to the supernatants obtained after first ultracentrifugation and the precipitated protein was removed by centrifugation. The concentrated supernatants were analysed for the presence of amino acids released by carboxypeptidase B treatment on a Beckman 119 CL analyser.

The purity of all proteins was checked by SDS-PAGE on 7.5–20% gradient mini-slab gels according to Laemmli [11].

Protein concentration was determined by measuring UV light absorbance with following values of extinction coefficients and molecular masses: G-actin, $E^{1\%}_{290\ nm} = 6.3$, 42 kDa [12]; myosin, $E^{1\%}_{279\ nm} = 5.4$, 470 kDa [13]; chicken gizzard tropomyosin, $E^{1\%}_{277\ nm} = 1.9$, 68 kDa [14]; caldesmon, $E^{1\%}_{280\ nm} = 3.8$, 87 kDa [15].

Sedimentation experiments were performed at room temperature in buffer comprising 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 2 mM β-mercaptoethanol and 20 mM imidazole-HCl, pH 7.0. Samples prepared by mixing of caldesmon with F-actin (in proportions indicated in the figure legends), were incubated for 30 min under very mild shaking in water bath. Following ultracentrifugation for 30 min in a Beckman Airfuge, the resuspended pellets were subjected to SDS-PAGE. Gels were stained with Coomassie brillant blue G-250 and the amount of caldesmon bound to actin in the pellets was quantified by laser scanning densitometry.

ATPase activity of rabbit skeletal muscle actomyosin (reconstituted from 80 μg/ml F-actin and 200 μg/ml myosin) in the presence of 22 μg/ml chicken gizzard tropomyosin, was assayed at 30°C in a medium containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA and 10 mM imidazole-HCl, pH 7.0. The concentration of caldesmon is given in the figure legends. The amount of P_i liberated was measured by the method of Fiske and SubbaRow [16].

3. RESULTS

Actins treated with trypsin both in Mg-G- and Ca-F-forms were further digested with carboxypeptidase B.

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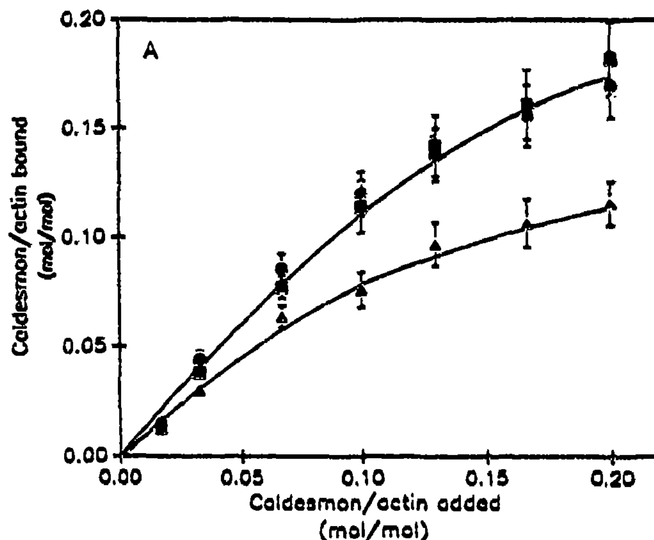
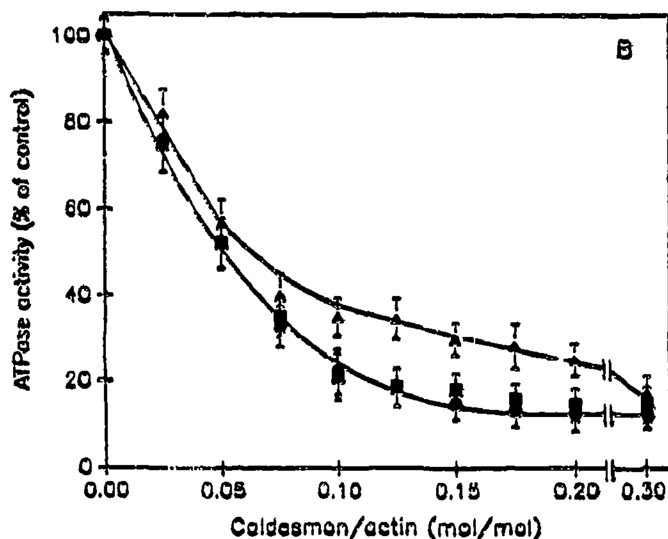
This digestion resulted in release of the two amino-acid residues: arginine (0.95 mol/actin monomer) and lysine (0.85 mol/actin monomer) in the former case, and arginine (0.93 mol/actin monomer) and only traces (less than 0.1 mol/actin monomer) of lysine in the latter case. Since in the actin sequence arginine and lysine are 372 and 373 residues, respectively, the results show that limited trypsin digestion of actin in F-form [1] leads to preferential removal of 374–375 dipeptide, whereas digestion of actin in G-form removes 373–375 tripeptide.

Binding of caldesmon to tryptically cleaved actins consisting of amino-acid residues 1–373 and 1–372 as well as to the intact one, was assessed by a high-speed co-sedimentation. As shown in Fig. 1A actin devoid of two C-terminal amino acid residues exhibited essentially the same degree of caldesmon binding when compared with the intact one, while binding of caldesmon to actin devoid of three C-terminal amino acid residues was weaker. This indicates that the presence of Lys-373 in actin molecule is important to the interaction of caldesmon with C-terminal part of actin.

Fig. 1B shows the effect of caldesmon on ATPase activity of actomyosin reconstituted from skeletal muscle myosin and actin, either intact or devoid of two or three amino-acid residues. All measurements were performed in the presence of smooth muscle tropomyosin. The profiles of the titration with caldesmon of the ATPase activity of actomyosin containing intact and devoid of two amino-acid residues actin revealed practically no difference. In the case of actin devoid of three C-terminal amino-acid residues, the inhibition of actomyosin ATPase activity by caldesmon was about 20% lower than that observed with intact actin at saturating level of caldesmon. Since increase of the concentration of caldesmon results in diminishing this difference one can suppose that removal of three C-terminal amino acids of actin decreases its binding affinity to caldesmon. Fig. 1C showing the relationship of caldesmon binding to activation of myosin Mg^{2+} ATPase by actin truncated in the G-form and the intact one (in the presence of smooth muscle tropomyosin) supports this view. The weakening of the inhibitory effect of caldesmon on the ATPase activity is well correlated with caldesmon-actin binding and seems not to depend on the changes in actin–myosin interaction reported earlier [2].





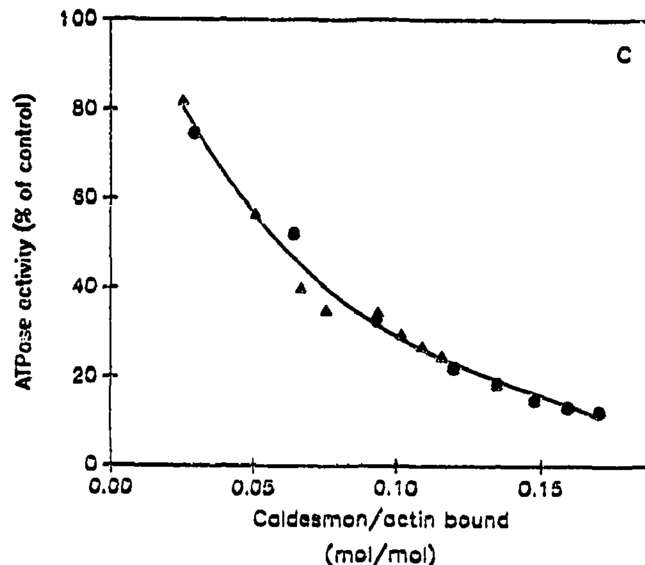


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Fig. 1. Characteristics of the interaction of caldesmon with intact actin (●) and 1–372 (▲) and 1–373 (■) polypeptide chains of actin. A. inding of caldesmon to intact and truncated actins. Binding was determined by co-sedimentation of caldesmon with respective actin followed by analysis of the pellets by PAGE. B. The effect of caldesmon on ATPase activity of actomyosin containing intact and truncated actins. ATPase activity was determined as described in section 2. C. The relationship between binding of caldesmon to intact actin and actin devoid of three C-terminal amino-acid residues and inhibition of activity of actomyosin ATPase containing intact and truncated actins.

4. DISCUSSION

The location of caldesmon-binding site(s) in the N-terminal region of actin is well established [4–6]. Recently, several lines of experimental evidence implicated also C-terminal part of primary sequence of actin as providing site of contact with caldesmon. It was shown that: (1) 5,5'-dithiobis(2-nitrobenzoic acid) is able to form a disulfide cross-link between Cys-374 of actin and Cys- 580 of caldesmon [3], the residue which is located in proximity to actin-binding sites of caldesmon; (2) actin devoid of three C-terminal amino acid residues shows weaker interaction with caldesmon than intact one and, in consequence, ATPase activity of actomyosin reconstituted from myosin and truncated actin is less inhibited by caldesmon [2]; (3) acting with fluorescein-, pyrene- or 1,5-IAEDANS-modified Cys-374 has lower affinity for caldesmon [1]. Crosbie et al. [1] reported, however, that removal of three C-terminal amino acids by trypsin completely restored the ability of actin of caldesmon binding.

In the present paper we provide evidence that the discrepancy between our earlier results [2] and those of Crosbie et al. [1] are caused by different conditions of actin digestion with trypsin and, in consequence, differently truncated actins used in experiments. The C-terminal sequence of actin (-Arg^{372}-Lys-Cys-Phe-COOH) contains two sites potentially recognized by trypsin: one between Arg-372 and Lys-373 and the other between Lys-373 and Cys-374 [17,18]. Our present results, in agreement with those obtained earlier by Suck et al. [19] and very recently by O'Donoghue et al. [20] and Mossakowska et al. [21], indicate that limited proteolysis of F-actin with trypsin preferentially releases two (but not three as suggested in [1]) C-terminal amino acids (Cys-374 and Phe-375), whereas during digestion of G-actin Lys-373 is also released. It suggests that the peptide bond between Arg-372 and Lys-373 is better protected from cleavage by intermonomer interactions in the actin filament than bond between Lys-373 and Cys-374. We conclude therefore, that inconsistency between our results [2] and those of Crosbie et al. [1] is due to the presence of substantial amounts of Lys-373 in their trypsin-treated actin which is not present in our preparation of truncated actin. There is a possibility that this negatively charged residue creates part of the N-terminal caldesmon-binding site(s) of actin, since both N and C termini are located in close proximity in the tertiary structure of actin [22], however, it cannot be excluded that it forms additional independent electrostatic bonds with caldesmon.

Subsequent to the completion of the present work Crosbie et al. [31] reported that removal of more than three C-terminal amino acids weakens actin–caldesmon interaction. In our opinion, the cluster of basic amino-acid residues including Lys-373 is involved in the interaction of the C terminus of actin with caldesmon.

Caldesmon binding to actin was reported to interfere with the binding of several other actin-binding proteins like filamin [23], calponin [24], profilin [25], and myosin [26,27]. At least the two latter of these proteins are also able to bind to C-terminal region of actin [28–30]. Therefore, the C-terminal caldesmon-binding site of actin may be in part responsible for this interference that seems to have functional significance.

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