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Location of the calmodulin- and actin-binding domains at the C-terminus of caldesmon

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Digestion of caldesmon with carboxypeptidase Y is accompanied by loss of its ability to inhibit actomyosin ATPase activity and to bind actin and calmodulin. Similarly, carboxypeptidase Y digestion of a terminal 40 kDa chymotryptic fragment of caldesmon abolishes its inhibition of the actomyosin ATPase and binding to actin and calmodulin. This represents the first direct demonstration that these functional domains of caldesmon are located close to the carboxy-terminus of the molecule.

Actin; Calmodulin; Actin-binding protein; (Smooth muscle)

1. INTRODUCTION

Caldesmon is an actin- and calmodulin-binding protein present in smooth muscle and in a number of non-muscle cells [1,2]. Its binding to actin results in inhibition of the actin-activated ATPase activity of myosin; this inhibition can be blocked, at least partially, by calmodulin in the presence of Ca^{2+} [3-5] or by Ca^{2+}/calmodulin-dependent phosphorylation of caldesmon [6,7]. These properties of caldesmon suggest that it may be involved in regulating actin-myosin interaction in a variety of contractile systems.

Electron micrographs of rotary-shadowed caldesmon reveal a flexible, extended morphology [8,9]. Sedimentation velocity measurements indicate a length of 740 Å [10]. To localize the actin- and calmodulin-binding domains on the caldesmon molecule, limited proteolysis with chymotrypsin combined with calmodulin affinity chromatography and sedimentation experiments have been exploited [11,12]. All these biochemical activities of caldesmon, i.e. its ability to bind actin and calmodulin and to inhibit actomyosin ATPase activity, are retained in a small fragment of 18 kDa [11]. This fragment is derived from a larger one of 40 kDa which appears early during proteolysis [11-14]. The 40 kDa fragment of caldesmon has been tentatively placed at the C-terminus [12,15].

Here, we present direct evidence that the F-actin- and calmodulin-binding domains of caldesmon are located near the C-terminus of the caldesmon molecule.

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2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Caldesmon was prepared from fresh chicken gizzards according to Bretscher [16]. Chymotryptic fragmentation and isolation of the 40 kDa fragment were performed as described [11]. The 18 kDa chymotryptic fragment of caldesmon was prepared as in [11] and further purified by extraction of the peptide from polyacrylamide gels after electrophoresis according to Laemmli [17] or by calmodulin-Sepharose affinity chromatography and CM-Sephadex cation-exchange chromatography. Removal of SDS from the gel-purified peptide was performed as described in [18]. Rabbit skeletal muscle myosin was prepared according to Perry [19] and further purified following the procedure of Kielley and Bradley [20]. Rabbit skeletal muscle actin and chicken gizzard tropomyosin

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were prepared according to [21]. The purity of all these proteins was checked by SDS-polyacrylamide gel electrophoresis.

2.2. *Protein cleavage*

Digestion of caldesmon or its 40 kDa fragment with carboxypeptidase Y was performed at a protein concentration of approx. 1 mg/ml, at a 1:10 (w/w) ratio of enzyme to substrate in 75 mM KCl, 20 mM imidazole-HCl (pH 7.0), 1 mM 2-mercaptoethanol, 10 mM NaN_3 at 30°C. The reaction was terminated by boiling of the withdrawn aliquots. Digestion of caldesmon with α-chymotrypsin to produce 40 and 18 kDa fragments was performed at 30°C at an enzyme/substrate (w/w) ratio of 1:1000 for the time giving the maximal yield of required fragment (usually 2 min for the 40 kDa peptide and 10 min for that of 18 kDa). For analytical purposes, a lower concentration of enzyme was also used as indicated in the figure legends. The reaction, performed in the same buffer as used for carboxypeptidase Y cleavage, was stopped by addition of phenylmethanesulfonyl fluoride (10× the weight of chymotrypsin).

2.3. *ATPase assay*

ATPase activity of rabbit skeletal muscle actomyosin (reconstituted from 30 μg/ml F-actin and 120 μg/ml myosin) was assayed in the absence or presence of chicken gizzard tropomyosin (at a 1:7 molar ratio to actin monomer) and intact or digested caldesmon or 40 kDa fragment in 50 mM KCl, 10 mM imidazole-HCl (pH 7.0), 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM EGTA at 30°C. The amount of P_i liberated was determined as in [22].

2.4. *Amino acid analysis*

Triplicate samples of the 18 kDa chymotryptic fragment of caldesmon, purified by the two methods indicated in section 2.1, were dialyzed overnight against 2 × 10 l of 10 mM NH_4HCO_3 and hydrolyzed in vacuo at 110°C in 6 N HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for 24, 48 and 72 h prior to composition analysis by ninhydrin detection using a Beckman model 6300 amino acid analyzer equipped with a Spectra-Physics SP4270 2-channel integrator.

2.5. *Polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out in 7–20% gradient slab gels according to Laemmli [17]. For calculation of molecular mass, the following marker proteins were used: rabbit skeletal muscle myosin heavy chain (205 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (66 kDa), actin (42 kDa), troponin I (21 kDa) and calmodulin (16.7 kDa).

2.6. *Protein determination*

Protein concentration was determined by measuring UV light absorbance using the following values of extinction coefficients and molecular mass: caldesmon, $E_{276} = 0.30$, 140 kDa [16]; G-actin, $E_{290} = 0.63$, 42 kDa [23]; myosin, $E_{279} = 0.54$, 470 kDa [24]; chicken gizzard tropomyosin, $E_{278} = 0.22$, 72 kDa [25]; or by the method of Lowry et al. [26].

3. RESULTS AND DISCUSSION

As shown in fig.1c, during chymotryptic treatment of caldesmon under very mild conditions (enzyme/substrate: 1:20000, w/w), the 40 kDa fragment appears after 1 min digestion, in parallel with high molecular mass fragments of 110 and 80 kDa. Only the 40 kDa fragment retains the ability of native caldesmon to bind actin and calmodulin and inhibit actomyosin ATPase activity [11]. This suggests that the 40 kDa fragment originates from a terminal part of the caldesmon molecule. On the other hand, this fragment is not formed when caldesmon is first treated with carboxypeptidase Y and then with chymotrypsin (fig.1,d), suggesting that it is the C-terminal peptide.

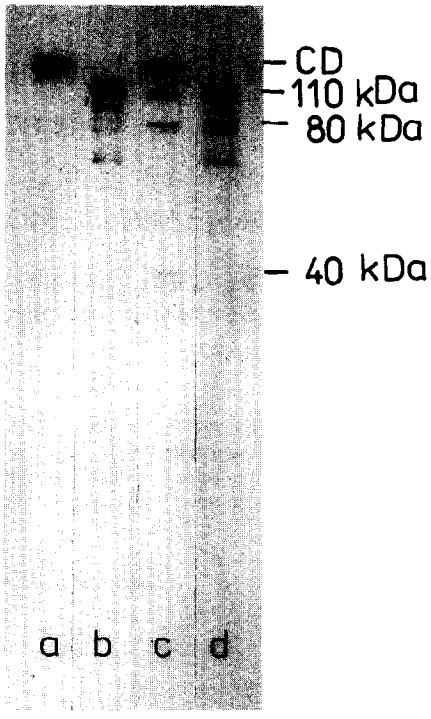

Fig.1. SDS-polyacrylamide gel electrophoresis of intact caldesmon (a), caldesmon digested with carboxypeptidase Y (b), caldesmon digested with α-chymotrypsin (c), and carboxypeptidase Y-treated caldesmon digested with α-chymotrypsin (d). Caldesmon (1 mg/ml) was digested with carboxypeptidase Y at an enzyme/substrate ratio of 1:10 (w/w) for 180 min at 30°C. The digestion with α-chymotrypsin was performed for 1 min under the same conditions but at a 1:1000 (w/w) ratio of enzyme to substrate.

Studying the effect of carboxypeptidase Y on caldesmon, we have found that degradation of this protein to 115 kDa and lower molecular mass fragments is accompanied by a gradual decrease of

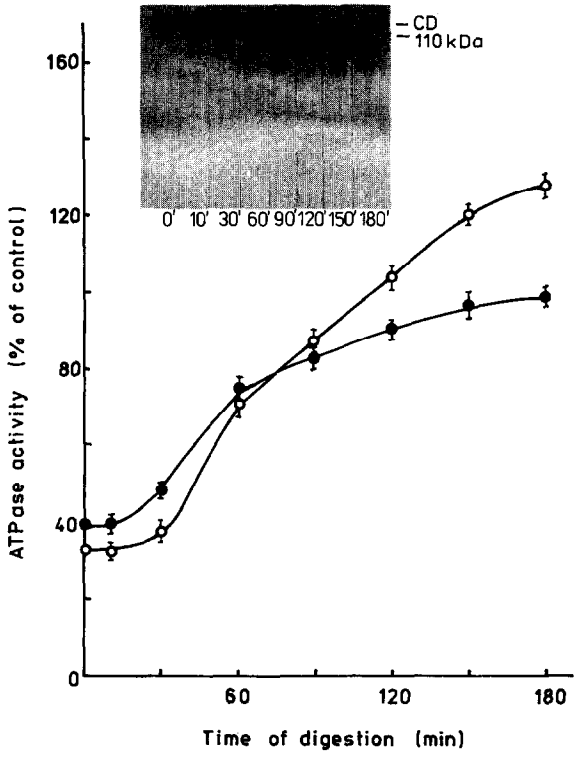

Fig.2. Effect of carboxypeptidase Y-treated caldesmon on the ATPase activity of rabbit skeletal muscle actomyosin in the absence (●) and presence (○) of chicken gizzard tropomyosin. Molar ratio of caldesmon to actin monomer was 1:10. Control ATPase activity of actomyosin in the absence of caldesmon was 0.38 μmol $P_i \cdot$ mg myosin$^{-1} \cdot$ min^{-1} in the absence of tropomyosin and 0.61 μmol $P_i \cdot$ mg myosin$^{-1} \cdot$ min^{-1} in the presence of tropomyosin. SDS-polyacrylamide gel electrophoresis shows the time course of digestion of caldesmon with carboxypeptidase Y at a 1:10 (w/w) enzyme/substrate ratio.

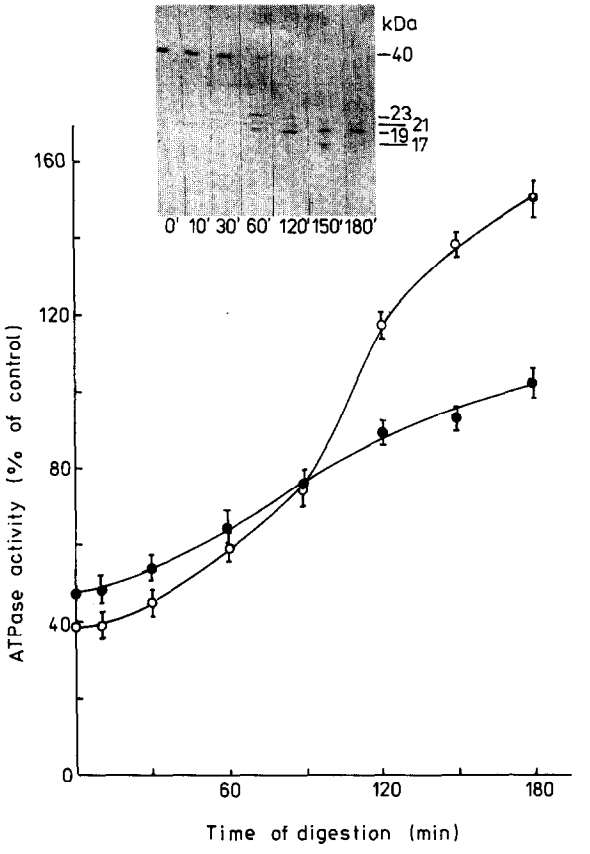

Fig.3. The effect of carboxypeptidase Y-treated 40 kDa chymotryptic fragment of caldesmon on the ATPase activity of rabbit skeletal muscle actomyosin in the absence (●) and presence (○) of chicken gizzard tropomyosin. Molar ratio of 40 kDa fragment of caldesmon to actin monomer was 1:10. Control ATPase activities of actomyosin in the absence and presence of tropomyosin are given in the legend to fig.2. SDS-polyacrylamide gel electrophoresis shows the time course of digestion of the 40 kDa fragment of caldesmon with carboxypeptidase Y at a 1:10 (w/w) enzyme/substrate ratio.

its ability to inhibit actomyosin ATPase. The inhibition is completely abolished in both the presence and absence of tropomyosin after disappearance of the parent caldesmon molecule (fig.2). Following digestion in the presence of tropomyosin, actomyosin ATPase activity is actually significantly (approx. 20%) above the control value. Degradation products bind neither to F-actin in sedimentation experiments nor to an affinity column of calmodulin-Sepharose 4B (not shown).

Moreover, carboxypeptidase Y treatment of the 40 kDa fragment of caldesmon caused its degradation to a 20 kDa polypeptide which again was inactive with respect to the inhibition of actomyosin ATPase (fig.3), as well as to the binding to actin and calmodulin (not shown). Since caldesmon devoid of the 20 kDa C-terminal region loses actin- and calmodulin-binding properties and actomyosin ATPase inhibition, it can be concluded that these functional domains are located in this fragment.

Prolonged digestion of caldesmon with α-chymotrypsin results in degradation of the 40 kDa peptide to an 18 kDa fragment [11–13]. The amino acid composition of this peptide, purified by two different methods, is listed in table 1. It is a basic peptide at pH 7.5. Elucidation of the complete

Table 1

Amino acid composition of the 18 kDa caldesmon fragment

Amino acid	Residues/mol
Asp + Asn	13.3
Thr	9.2
Ser	16.6
Glu + Gln	23.8
Por	7.0
Gly	17.1
Ala	15.5
Val	9.0
Met	1.3
Ile	4.0
Leu	9.1
Tyr	0.8
Phe	3.2
His	0.8
Lys	8.0
Arg	4.9

amino acid sequence of caldesmon will allow more precise definition of this peptide.

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