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35kDa FRAGMENT OF *h*-CALDESMON CONSERVES TWO CONSENSUS SEQUENCES OF THE TROPOMYOSIN-BINDING DOMAIN IN TROPONIN T

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SUMMARY: Using a tropomyosin-coupled affinity column, we have demonstrated a direct association between the chymotryptic 35kDa fragment of *h*-caldesmon, which is located at the C-terminal of the parent molecule, and gizzard tropomyosin. We have subsequently determined the nucleotide sequence of cDNA clones encoding the 35kDa fragment from the cDNA library prepared from chick embryo gizzards, and have deduced the amino acid sequence. Calculating from the predicted sequence, the 35kDa fragment is composed of 306 amino acid residues. In agreement with the tropomyosin-binding ability, the 35kDa fragment conserves two consensus sequences of the tropomyosin-binding domain in troponin T. These results suggest that the 35kDa fragment of *h*-caldesmon, at least in part, has a common property to the striated muscle troponin T.

Caldesmon was originally purified from chicken gizzard smooth muscles as a major calmodulin-binding protein which also interacts with actin filaments (1). Recently, two Mr forms of caldesmon (Mr's in the range of 120-150kDa and 70-80kDa) have been shown in a variety of tissues and cells (2-5). The 120-150kDa caldesmon (*h*-caldesmon) is predominantly present in the smooth muscles, and the 70-80kDa caldesmon (ℓ-caldesmon) in the nonmuscle tissues and cells (5). Accumulating evidences have led us to the conclusion that caldesmon is on important component in the smooth and nonmuscle actin-myosin interaction (see for a review, 6). There is, however, a dispute regarding the caldesmon-involved regulation in the actomyosin system (7-9). Despite these conflicting observations, we

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verified the regulatory mode of *h*-caldesmon, in which *h*-caldesmon regulates the actin-myosin interaction mediated through controlling the function of tropomyosin (10,11). This finding also suggested the direct or indirect association of *h*-caldesmon with tropomyosin. Actually, the possible interaction between *h*-caldesmon and tropomyosin has been reported from some laboratories (12-14). Therefore, one of the important subjects seems to be the regulatory mechanism of *h*-caldesmon in the tropomyosin-dependent actin-myosin interaction.

The chymotryptic 35kDa fragment of *h*-caldesmon, which is located at the C-terminal, binds to calmodulin and F-actin (15-17). It also retains an inhibitory effect on the tropomyosin-enhanced actomyosin ATPase (18). Here, we have demonstrated the interaction of the 35kDa fragment with tropomyosin. We have further determined the nucleotide sequence of the 35kDa fragment from its cDNA, and have deduced the amino acid sequence. From the present sequencing data, it is apparent that the 35kDa fragment retains two consensus sequences corresponding to the tropomyosin-binding domain in the troponin T molecule.

MATERIALS AND METHODS

Materials. Restriction enzymes (DNA- and RNA-modifying enzymes) and linker- and primer-DNAs were obtained from Takara Shuzo, Japan. Alkaline phosphatase-conjugated goat anti-rabbit IgG and [α-^{32}P]dCTP(400Ci/mM) were purchased from Cooper Biochemical and Amersham, respectively. *h*-Caldesmon was purified from chicken gizzard by the aid of heat treatment (19). The details of the purification procedure will be described in elsewhere (in preparation). Tropomyosin was prepared by the method of Ebashi *et al* (20). Production of *h*-caldesmon antiserum against New Zealand rabbits and purification of *h*-caldesmon-specific antibodies from antiserum using a *h*-caldesmon-coupled column were described in elsewhere (2). The 35kDa fragment of *h*-caldesmon was purified from the chymotryptic digests of *h*-caldesmon as described previously (16).

Purification of the 35kDa fragment-specific antibody. The 35kDa fragment was coupled to cyanogen bromide-activated Sepharose 4B, and the gels thus obtained were used for an affinity column. The *h*-caldesmon antibodies were applied to an affinity column and then washed with phosphate buffered saline (PBS), pH7.4. The bound antibody was eluted with 0.1M glycine•HCl buffer, pH2.3, followed by dialysis against PBS. Immunoblotting results revealed that the eluted antibody specifically cross-reacted with the 35kDa fragment, the 100kDa fragment containing the 35kDa fragment and the proteolytic small fragments derived from the 35kDa fragment (not shown).

DNA ligation. Ligation by T_4DNA ligase for processing of cDNA cloning using λ phage vector was done in the presence of 10% polyethylene glycol (PEG) 6000 and 0.75mM spermidine. The construction of plasmids for DNA sequencing was carried out in the presence of 5% PEG 6000 (21).

cDNA library construction and screening. Total cellular RNAs were prepared from chick embryo gizzards by the guanidine/cessium chloride methods (22). The poly(A)$^+$RNAs were purified by an oligo(dT)-cellulose column chromatography. cDNA synthesis was done by the method of Gubler and Hoffman (23), and a cDNA library was constructed in the expression vector λgt11 (24). Replica filters of this library were screened using the 35kDa fragment-specific antibody visualized with alkaline phosphatase-conjugated second IgG. The length of inserted cDNA into gt11 DNA was identified by the method of polymerase chain reaction (25) using λgt11 primers and DNA thermal cycler (Perkin Elmer Cetus).
DNA sequencing. The cDNA fragments released from the vector DNA were cloned into M13mp18RF-DNA and deletioned mutants were prepared as described in refs. 26 and 27. Single-stranded DNAs from recombinant plaques were sequenced by dideoxy chain termination method using [α-^{32}P]dCTP (28).
Others. The tropomyosin-coupled affinity chromatographies using the chymotryptic digests of *h*-caldesmon and the purified 35kDa fragment were carried out as described in the legend to Figure 1. N-Terminal amino-acid analysis of the 35kDa fragment was directly performed on an Applied Biosystems gas phase sequencer. The 35kDa fragment was further digested by α-chymotrypsin, and the 22, 18 and 14kDa fragments separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted onto polyvinylidine difluoride (PVDF) membrane. After electroblotting, PVDF membrane corresponding to the respective fragments were cut out and the sample pieces were sequenced with the aid of the above sequencer (29). Homology search was done by the computer-aided analysis system (DNASIS, Hitachi SK). SDS-PAGE was carried out by the method of Laemmli (30).

RESULTS

We examined the interaction of the chymotryptic fragments of *h*-caldesmon with tropomyosin using a tropomyosin-coupled affinity column. At low ionic strength, *h*-caldesmon, and the 100, 35 and 22kDa fragments in the chymotryptic digests bound to an affinity column, and were eluted by a high ionic strength buffer (Fig. 1A). The purified 35kDa fragment also bound to a column in a similar manner using the chymotryptic digests (Fig. 1B). These results indicate that like the parent molecule, the 35kDa fragment retains the tropomyosin-binding ability in addition to the calmodulin- and actin-binding activities.

We then determined the primary structure of the 35kDa fragment from its cDNA sequencing data as follows. Four colonies were selected which gave strongly immunoreactive positive signals with the 35kDa fragment-specific antibody. cDNAs from four colonies were cloned into M13 vector, and the cloned cDNAs were sequenced by the dideoxy method. Figure 2 shows the nucleotide sequence of the 35kDa fragment and its predicted amino acid sequence. The primary structure corresponding to the 35kDa fragment

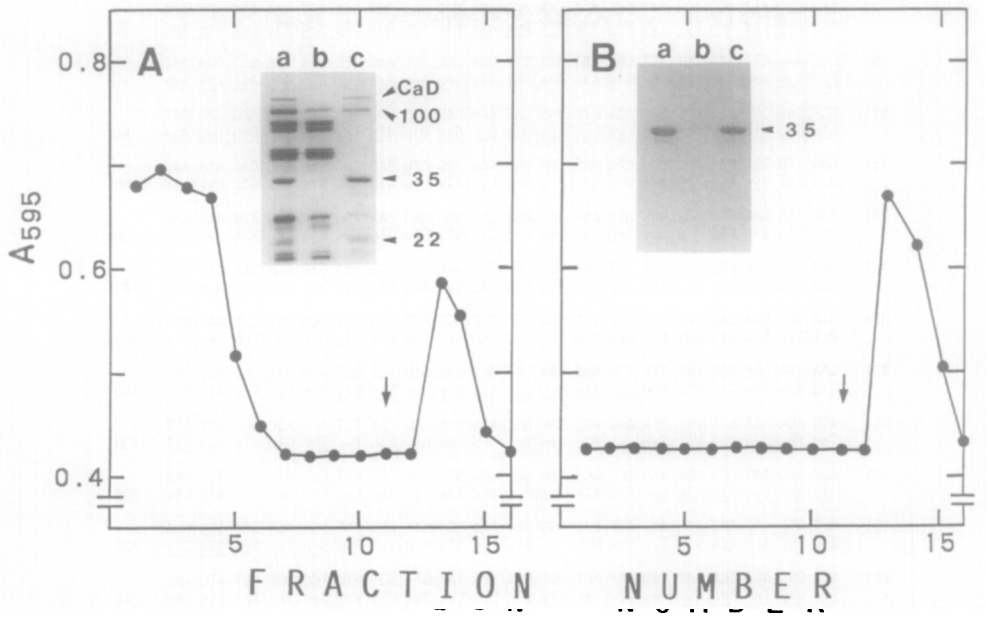

Fig. 1. Association between the chymotryptic digests of *h*-caldesmon or its 35kDa fragment and tropomyosin. The 0.9 x 2cm tropomyosin-coupled column was equilibrated with buffer A (20mM imidazole·HCl, pH7.0, 2mM $MgCl_2$ and 0.5mM dithiothreitol) and 3.5mg of the chymotryptic digests of *h*-caldesmon (A) or 1.0mg of the purified 35kDa fragment (B) in buffer A were applied to a column, respectively. After extensive washing, the proteins bound to a column were eluted with 200mM KCl in buffer A. Fractions were collected (0.8ml each) and their protein concentrations were determined by the method of Bradford (40). Insets show SDS polyacrylamide gel electrophoregrams. Each lanes indicate as follows: lane a, applied sample; lane b, flow through fraction; lane c, 200mM KCl eluate. CaD, the parent *h*-caldesmon; 100, 100kDa fragment; 35, 35kDa fragment; 22, 22kDa fragment.

predicts a protein of 305 amino acids with Mr of 34,000. The Mr of this fragment is in good agreement with that estimated by SDS-PAGE (35kDa). The amino acid composition of this fragment predicted from the nucleotide sequence agrees well with the direct amino acid composition data (Table I). To confirm the authenticity of the amino acid sequence, we compared the predicted sequence with the N-terminal sequences of the 35, 22, 18 and 14 kDa fragments obtained in this experiment and of the 15kDa fragment reported by Mornet *et al.* (31). The partial amino acid sequences are in excellent agreement with five regions of the predicted sequence.

In consideration of the regulatory mode of *h*-caldesmon on the actomyosin system, it is easy to speculate on the functional homology between caldesmon and troponins (11). Surprisingly, computer-assisted comparisons of the predicted amino acid sequence of the 35kDa fragment with

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1    GAT CGT AAA AGG GGA GTT CCA GAA CAA AAG GCA CAG AAT GGA GAA CGT GAA CTC ACT ACC
     Asp Arg Lys Arg Gly Val Pro Glu Gln Lys Ala Gln Asn Gly Glu Arg Glu Leu Thr Thr   20

61   CCC AAA CTT AAA TCT ACT GAA AAT GCT TTT GGA CGC TCC AAC TTG AAA GGA GCT GCA AAT
     Pro Lys Leu Lys Ser Thr Glu Asn Ala Phe Gly Arg Ser Asn Leu Lys Gly Ala Ala Asn   40

121  GCT GAG GCT GGC TCC GAG AAG CTG AAG GAG AAA CAG CAG GAG GCA GCT GTG GAG CTG GAT
     Ala Glu Ala Gly Ser Glu Lys Leu Lys Glu Lys Gln Gln Glu Ala Ala Val Glu Leu Asp   60

181  GAG CTG AAG AAA AGG CGG GAG GAG CGC CGG AAA ATC CTG GAG GAA GAG GAG CAG AAG AAG
     Glu Leu Lys Lys Arg Arg Glu Glu Arg Arg Lys Ile Leu Glu Glu Glu Glu Gln Lys Lys   80

241  AAA CAG GAG GAG GCT GAG AGA AAA ATC AGA GAG GAG GAG GAA AAG AAG AGG ATG AAG GAA
     Lys Gln Glu Glu Ala Glu Arg Lys Ile Arg Glu Glu Glu Glu Lys Lys Arg Met Lys Glu  100

301  GAA ATT GAA AGG AGA AGA GCT GAA GCT GCT GAG AAA CGT CAG AAA GTG CCA GAA GAT GGC
     Glu Ile Glu Arg Arg Arg Ala Glu Ala Ala Glu Lys Arg Gln Lys Val Pro Glu Asp Gly  120

361  GTA TCT GAA GAA AAG AAG CCA TTT AAA TGT TTC AGT CCT AAA GGT TCA TCT CTC AAG ATA
     Val Ser Glu Glu Lys Lys Pro Phe Lys Cys Phe Ser Pro Lys Gly Ser Ser Leu Lys Ile  140

421  GAA GAA CGA GCA GAA TTT TTG AAC AAA TCC GCT CAG AAG AGT GGT ATG AAA CCT GCC CAC
     Glu Glu Arg Ala Glu Phe Leu Asn Lys Ser Ala Gln Lys Ser Gly Met Lys Pro Ala His  160

481  ACG ACA GCA GTT GTC TCA AAG ATT GAC AGT AGA CTT GAG CAA TAT ACT AGC GCA GTT GTG
     Thr Thr Ala Val Val Ser Lys Ile Asp Ser Arg Leu Glu Gln Tyr Thr Ser Ala Val Val  180

541  GGC AAC AAG GCT GCA AAA CCC GCC AAA CCA GCA GCC TCT GAC CTT CCT GTT CCA GCT GAG
     Gly Asn Lys Ala Ala Lys Pro Ala Lys Pro Ala Ala Ser Asp Leu Pro Val Pro Ala Glu  200

601  GGT GTC CGT AAT ATC AAG AGC ATG TGG GAG AAA GGG AAT GTT TTT TCA TCA CCT GGG GGA
     Gly Val Arg Asn Ile Lys Ser Met Trp Glu Lys Gly Asn Val Phe Ser Ser Pro Gly Gly  220

661  ACA GGA ACA CCA AAT AAG GAA ACT GCT GGA CTG AAA GTT GGT GTC TCC AGT CGT ATC AAC
     Thr Gly Thr Pro Asn Lys Glu Thr Ala Gly Leu Lys Val Gly Val Ser Ser Arg Ile Asn  240

721  GAA TGG CTA ACC AAG ACC CCA GAG GGT AAC AAA TCG CCT GCT CCA AAA CCT TCT GAT TTA
     Glu Trp Leu Thr Lys Thr Pro Glu Gly Asn Lys Ser Pro Ala Pro Lys Pro Ser Asp Leu  260

781  AGA CCA GGA GAT GTA TCC GGC AAA CGT AAT CTC TGG GAG AAG CAG TCA GTT GAA AAG CCA
     Arg Pro Gly Asp Val Ser Gly Lys Arg Asn Leu Trp Glu Lys Gln Ser Val Glu Lys Pro  280

841  GCT GCT TCT TCT TCA AAG GTA ACA GCT ACG GGG AAA AAA TCA GAG ACT AAT GGT TTG AGA
     Ala Ala Ser Ser Ser Lys Val Thr Ala Thr Gly Lys Lys Ser Glu Thr Asn Gly Leu Arg  300

901  CAA TTT GAG AAA GAA CCG TAG AAG GCT ACT AAA GAC GCT GGA CCA ATC AGT TGG GGA AAA
     Gln Phe Glu Lys Glu Pro ***                                                     320

961  AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
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Fig. 2. The nucleotide sequence of the 35kDa fragment. The coding region of the cDNA has been translated into amino acids. Nucleotide sequence numbering appears on the left, amino acid sequence numbering on the right. The asterisks indicate the stop codon. Underlining of the predicted amino acid sequence indicates N-terminal amino acid sequences of five fragments determined by amino acid sequencer.

N-Terminal amino acid sequences of five fragments are as follows;

35kDa fragment·····Asp·Arg·Lys·Arg·Gly·Val·Pro·Glu·Gln·Lys·Ala·
Gln·Asn·Gly·Glu·Arg·Glu·Leu·Thr·Thr·Pro·Lys
22kDa fragment·····Leu·Asn·Lys·Ser·Ala·Gln·Lys·Ser·Gly·Met
18kDa fragment·····Thr·Ser·Ala·Val·Val·Gly·Asn·Lys·Ala·Ala
14kDa fragment·····Ser·Ser·Pro·Gly·Gly·Thr·Gly·Thr·Pro·Asn
15kDa fragment (Mornet *et al.* in ref.31)·····Ser·Asn·Leu·Lys·Gly·Ala·Ala·
Asn·Ala·Glu·Ala·Gly·Ser·Glu·Lys·Leu·Lys·Glu·
Lys·Gln·Gln·Glu·Ala·Ala·Val·Glu·Leu·Asp

that of the skeletal muscle troponin T reveal two homologous regions in both proteins (Fig. 3). One homologous region is found in residues 58-118 of the 35kDa fragment and residues 89-146 or 81-113 of rabbit fast muscle troponin T (32,33). It is noteworthy that residues 58-118 of the 35kDa fragment give a highly conserved consensus sequence of the tropomyosin-binding domain in a wide variety of the striated muscle troponin Ts.

Table I. Comparison of the amino acid composition of the 35kDa fragment determined by the direct measurement with that the deduced sequence

	24h hydrolysate[a]		sequencing data	
	Count	Mol %	Count	Mol %
Gly	22.0	(7.0)	21	(6.86)
Ala	30.2	(9.6)	28	(9.15)
Val	15.8	(5.0)	16	(5.23)
Leu	16.8	(5.3)	16	(5.23)
Ile	7.3	(2.3)	7	(2.29)
Ser	23.1	(7.3)	26	(8.50)
Thr	13.6	(4.3)	14	(4.58)
Cys	0.6	(0.2)	1	(0.33)
Met	2.8	(0.9)	3	(0.98)
Asp	21.0	(6.6)	7	(2.29)
Asn			13	(4.25)
Glu	61.4	(19.4)	45	(14.71)
Gln			11	(3.59)
Arg	23.5	(7.4)	22	(7.19)
Lys	47.7	(15.1)	46	(15.03)
His	1.0	(0.3)	1	(0.33)
Phe	6.2	(2.0)	6	(1.96)
Tyr	1.1	(0.4)	1	(0.33)
Trp	2.3	(0.7)	3	(0.98)
Pro	19.5	(6.2)	19	(6.21)
Total	315.9	(100)	306	(100)
Mr	35000		34000	

[a]Results described in (16).

Residues 156-171 of the 35kDa fragment are also the homologous region of the C-terminal domain in troponin T (residues 243-259), that is the second binding domain for tropomyosin (34).

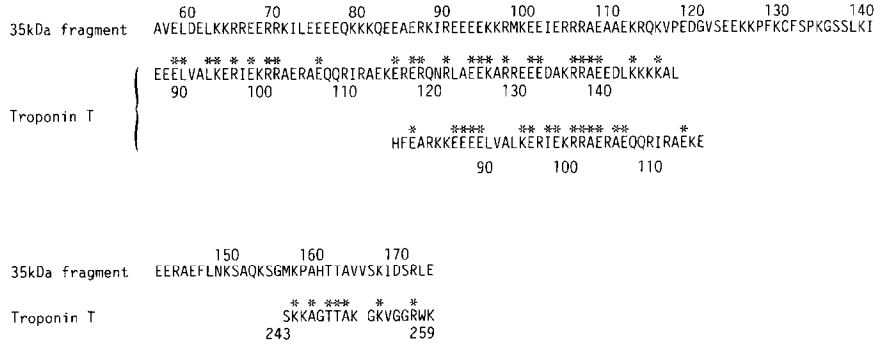

Fig. 3. Comparison of highly homologous regions of the 35kDa fragment in *h*-caldesmon with those in troponin T. Numbers indicate the amino acid residues counting from each N-terminal end. The asterisks show the identical amino acid residues in two proteins.

DISCUSSION

Most recently, Fujii *et al.* have demonstrated the association of the partial purified 35kDa fragment with tropomyosin (17). In this paper, we have shown direct evidence for the purified 35kDa fragment binding to tropomyosin and have deduced the amino acid sequence of the 35kDa fragment containing two consensus sequences of the tropomyosin-binding domain in troponin T. As mentioned previously, the calmodulin- and actin-binding domain in *h*-caldesmon is also restricted within this fragment (15-17). In the case of troponin I, the actin-binding domain is located in the C-terminal portion of CN4 fragment (residues 103-115) containing the arginine- and lysine-rich sequence (35,36). Residues 63-97 and 104-158 of the 35kDa fragment contain a similar sequence to residues 103-115 of troponin I. Recent studies make clear the primary structure of some calmodulin-dependent enzymes (37-39). Although there is no consensus sequence of the calmodulin-binding domain in these calmodulin-dependent enzymes, the calmodulin-binding regions commonly form clusters composed of basic amino acid residues. From the present predicted sequence, residues 1-60 and 221-300 of the 35kDa fragment are prime candidates for such calmodulin-binding regions. We are now intending to determine the precise binding regions for tropomyosin, calmodulin and actin in the 35kDa fragment.

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