Alignment of caldesmon on the actin–tropomyosin filaments

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We have reported previously that each smooth-muscle caldesmon binds predominantly to a region within residues 142–227 of tropomyosin, but a weaker binding site also exists at the N-terminal region of tropomyosin [Watson, Kuhn, Novy, Lin and Mak (1990) *J. Biol. Chem.* **265**, 18860–18866]. In view of recent evidence for the presence of tropomyosin-binding sites at both the N- and C-terminal domains of caldesmon, we have studied the binding of the N- and C-terminal fragments of human fibroblast caldesmon expressed in *Escherichia coli* to tropomyosin and its CNBr fragments. The N-terminal fragment, CaD40 (residues 1–152), binds tropomyosin, but the interaction is mostly abolished in the presence of actin. CaD40 binds strongly to

Cn1B(142–281) of tropomyosin, but weakly to Cn1A(11–127). The C-terminal fragment, CaD39, which corresponds to residues 443–736 of gizzard caldesmon, binds tropomyosin, and the interaction is enhanced by actin. CaD39 binds to both Cn1A(11–127) and Cn1B(142–281) of tropomyosin. Our results suggest that the N-terminal domain of caldesmon interacts with the C-terminal half of one tropomyosin molecule, whereas the C-terminal domain binds to both N- and C-terminal regions of the adjacent tropomyosin molecule along the actin filament. In addition, the binding of the N-terminal domain of caldesmon to the actin–tropomyosin filament is weak, which may allow this domain to project off the thin filament to interact with myosin.

INTRODUCTION

Caldesmon is present in both smooth-muscle and non-muscle cells, but absent from cardiac and skeletal muscle (for reviews see [1–3]). The smooth-muscle caldesmon (h-caldesmon) has a M_r of 87000 whereas the M_r of the lighter non-muscle isoform (l-caldesmon) is 57000. The h-caldesmon consists of three domains: an N-terminal domain (residues 1–230) containing the binding sites for myosin [4] and calmodulin [5], a helical middle domain (residues 231–400) which is absent from l-caldesmon [6], and a C-terminal domain (residues 401–765) housing the binding sites for actin [7–9], calmodulin [10,11] and tropomyosin [12,13]. Marston and Redwood [3] further subdivide the C-terminal domain into subdomain 3 (residues 509–565), which is similar to troponin-T, and subdomain 4 (residues 590–756), containing the actin- and calmodulin-binding sites.

Biochemical data have provided a general map of the functional domains of the caldesmon molecule; however, it is not clear how the caldesmon molecules are aligned along the thin filament. Exploiting the possibility that the tropomyosin polymers may act as a 'template' for the linear assembly of caldesmon on the thin filament, we have demonstrated that caldesmon binds to an extended region covering residues 11–227 of tropomyosin [14]. The major site of interaction, however, lies between residues 142–227 of tropomyosin, and a weaker caldesmon-binding site exists in the N-terminal half of tropomyosin. Knowing the stoichiometry of binding and the overall dimensions of caldesmon and tropomyosin [15], and incorporating electron-microscopy studies [16], we suggested that each caldesmon molecule spans two tropomyosin and 14 actin monomers such that the C-terminal region of caldesmon interacts with residues 142–227 of tropomyosin, and predicted that the N-terminal domain of caldesmon may interact with the adjacent molecule of tropomyosin [14].

Using a mixture of chymotryptic fragments of caldesmon, it has been shown recently that tropomyosin indeed binds to the N-terminal fragment of caldesmon [12,13,18], and this interaction is

inhibited by phosphorylation of the caldesmon fragment by casein kinase II [18]. Recently, Redwood and Marston [8] have demonstrated that tropomyosin binds to all three domains of smooth-muscle caldesmon, with different affinities. Since each h-caldesmon molecule can bind and span two tropomyosin molecules, it is possible that the N- and C-terminal domains of each h-caldesmon would interact with two adjacent tropomyosin molecules, involving residues 142–227 in each tropomyosin, or alternatively, involving different regions of the two tropomyosin molecules.

The objectives of this study are (1) to demonstrate definitively the presence of a second tropomyosin-binding site on the N-terminal domain of caldesmon, (2) to delineate the binding sites of caldesmon on tropomyosin, taking into account the presence of at least two tropomyosin-binding sites on caldesmon, and (3) to evaluate the strength of interaction between tropomyosin and the caldesmon fragments in the presence and absence of actin.

MATERIALS AND METHODS

Plasmid DNA constructs

cDNAs encoding the N-terminal (residues 1–243) and C-terminal (residues 244–538) fragments of human fibroblast caldesmon were obtained from plasmids pGEMCAD40 and pGEMCAD39 respectively [19]. These fragments were subcloned into pET expression vectors to yield pETCaD40 and pETCaD39, encoding the N- and C-terminal fragments, CaD40 and CaD39, respectively [20]. The subclones were verified by restriction-enzyme site analyses, insert size and nucleotide sequencing of the inserts.

Protein purification

Tropomyosin was prepared from chicken breast as described in [21], with further purification on a DEAE-cellulose column to remove nucleic acids. CNBr digestion and purification of the fragments, Cn1A(11–127) and Cn1B(142–281), were performed as described previously [14]. Actin was prepared from acetone-

Abbreviations used: l-caldesmon, low-molecular-mass form of caldesmon; h-caldesmon, high-molecular-mass form of caldesmon; DTT, dithiothreitol; HMM, heavy meromyosin; ANM, *N*-(4-anilino-1-naphthyl)maleimide.

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dried powder prepared from rabbit skeletal muscle as described in [22]. Smooth-muscle caldesmon and tropomyosin were prepared from chicken gizzards as described in [23]. Rabbit skeletal-muscle heavy meromyosin (HMM) was prepared as described in [24]. Chicken gizzard myosin was prepared as in [25]. Recombinant N- and C-terminal fragments of human fibroblast caldesmon were expressed in *Escherichia coli* as described in [14,20]. The *E. coli*-expressed caldesmon fragments were purified as described in [14], except that a 0–55% satd.-(NH₄)₂SO₄ precipitation was performed for the C-terminal fragment. The N-terminal fragment was applied to an FPLC Mono Q column and eluted with a 10–300 mM NaCl gradient. Protein concentrations were determined by the method of Lowry et al. [26], with BSA as standard.

Fluorescence studies

Chicken breast tropomyosin was reduced with 10 mM dithiothreitol (DTT), and the excess DTT was removed by gel filtration on a Sephadex G-10 column. The reduced tropomyosin was labelled with *N*-(1-pyrenyl)maleimide (Polyscience) as described previously [27,28], except that excess maleimide was removed by gel filtration on a Sephadex G-10 column equilibrated with buffer (10 mM KCl, 10 mM Mops, pH 7.2). The stoichiometry of labelling was determined by using the absorption coefficient of $2.3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 343 nm for pyrene [28]. Fluorescence measurements were done as described in [27]. Known samples of caldesmon fragments or intact caldesmon were added to a solution of pyrene-labelled tropomyosin (0.2 μM) in 10 mM Mops, pH 7.2, 1.0 mM EGTA, 0.01% NaN₃ and 2 mM DTT.

Tropomyosin labelling with *N*-(4-anilino-1-naphthyl)-maleimide (ANM) was performed as described previously [14,27].

Actin sedimentation with intact caldesmon, and the N- and C-terminal domains of caldesmon with or without tropomyosin

Sedimentation experiments were done as described in [29]. Briefly, actin (3 μM) in the presence or absence of tropomyosin (0.86 μM) was incubated with different amounts of CaD40 and CaD39 at 25 °C for 1 h in 20 mM Tris/HCl (pH 7.5)/100 mM KCl/2 mM MgCl₂/1 mM ATP/1 mM DTT. Samples (0.1 ml) were then centrifuged at 109000 *g* for 1 h at 2 °C. The amounts of actin, tropomyosin and caldesmon fragments in the pellets and supernatants were analysed by SDS/PAGE and densitometry. The amounts of actin and caldesmon used for the gel assay were within the range of linear relationship between dye-staining and protein quantity.

To measure the binding of intact caldesmon, CaD40 and CaD39 to tropomyosin in the presence of actin, caldesmon or its fragments were mixed with tropomyosin (1:1 molar ratio) under conditions such that they would interact (50 mM NaCl, 20 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 mM ATP, 1 mM DTT), and actin (3–7 μM) was added to the reaction mixtures. The amounts of tropomyosin, actin and caldesmon fragments sedimented in the pellets were analysed by SDS/PAGE and densitometry.

Actin-activated HMM ATPase activity

Effects of caldesmon fragments CaD40 and CaD39 on actin-activated rabbit skeletal HMM ATPase activity were assayed by the method outlined in [30]. Assay conditions were: 1.2 μM skeletal-muscle HMM, 3.3 μM skeletal-muscle actin, 0.6 μM chicken gizzard tropomyosin, in 10 mM Mops/5 mM MgCl₂/5 mM KCl/1 mM DTT, pH 7.0 at 37 °C. The concentration of the caldesmon fragments was varied from 0 to 3.3 μM . To study the release of myosin-ATPase inhibition by calmodulin, the same

assay conditions were used, except that the concentration of CaD39 was 0.34 μM and the calmodulin concentration was varied from 0 to 30 μM .

Affinity chromatography

Chicken breast tropomyosin, caldesmon or its fragments was coupled to CNBr-activated Sepharose 4B (Pharmacia) by the manufacturer's recommended procedure. The column (~1 ml) was equilibrated with 10 mM imidazole (pH 7.0)/1 mM EGTA/10 mM NaCl/0.01% NaN₃/1 mM DTT. Proteins were eluted with a linear 10–400 mM NaCl gradient, in 20 ml, at a flow rate of 0.6 ml/min and collected in 1 ml fractions.

Binding of caldesmon and its fragments to a calmodulin-Sepharose 4B affinity column was performed essentially as described previously [31]. Intact caldesmon and the N- and C-terminal fragments were applied to a calmodulin-Sepharose column in the presence of 1 mM CaCl₂ and 10 mM NaCl and washed with the same buffer. The bound protein was eluted with a 10–400 mM NaCl gradient with 1 mM EGTA instead of Ca²⁺.

Interaction between myosin and the caldesmon fragments

Sedimentation experiments were done as outlined in [32]. Depolymerized chicken gizzard myosin (2 μM) was incubated with 10 μM CaD40 or CaD39 and dialysed overnight against the polymerizing buffer (1 mM ATP, 10 mM imidazole, pH 7.0, 30 mM NaCl, 5 mM MgCl₂ and 1 mM DTT at 4 °C). Samples (0.2 ml) were centrifuged for 5 min in a bench-top centrifuge. Supernatants and pellets were analysed by SDS/PAGE and densitometry.

RESULTS

Assignment of the N- and C-terminal fragments of I-caldesmon expressed in *E. coli*

The purity of the N-terminal (CaD40) and C-terminal (CaD39) fragments of human fibroblast caldesmon was evaluated by SDS/PAGE as shown in Figure 1. A yield of about 2 mg and

Figure 1 SDS/PAGE of CaD39 and CaD40

Lane 1, low-*M_r* (LMW) standards: hen egg-white ovalbumin (45000); bovine carbonic anhydrase (31000); soybean trypsin inhibitor (21500). Lane 2, *E. coli*-expressed CaD39. Lane 3, *E. coli*-expressed CaD40.

Table 1 Amino acid composition of caldesmon fragments CaD40 and CaD39

ND, not determined.

Amino acid	Composition (mol/mol)			
	CaD40		CaD39	
	Calculated*	Observed	Calculated†	Observed
Asx	20	19	19	20
Thr	12	10	17	17
Ser	5	5	26	24
Glx	42	40	54	53
Pro	3	4	17	18
Gly	2	4	16	18
Ala	14	14	22	23
Cys	0	ND	1	ND
Val	7	8	14	15
Met	3	3	4	3
Ile	2	3	7	8
Leu	10	11	19	19
Tyr	1	1	1	1
Phe	3	3	6	6
Lys	10	9	40	40
His	0	0	3	3
Trp	0	0‡	3	3‡
Arg	27	25	26	25

*Values are based on the amino acid sequence for residues 1–152 of human fibroblast caldesmon [19] plus the 9 residues of mis-sense mutation at the C-terminal end.

†Values correspond to residues 244–538 of human fibroblast caldesmon [19].

‡Trp was determined after hydrolyses of the peptides at 160 °C for 1 h with 4 M methanesulphonic acid and confirmed spectrophotometrically as described by Spande and Witkop [41].

1 mg per litre of *E. coli* culture was routinely obtained for the N- and C-terminal fragments respectively.

The cDNA insert in pETCaD40 corresponds to residues 1–243 of human fibroblast caldesmon. The M_r of this fragment as predicted by the amino acid sequence [19] is 29 500. Since the ratio of apparent M_r :actual M_r for a caldesmon fragment, N128 (residues 1–128), from the N-terminal domain has been estimated to be about 1.5 [8], CaD40 is expected to migrate as a band of M_r 44 000 on SDS/PAGE. Instead, its apparent M_r was only 29 000, as shown in Figure 1, suggesting that the *E. coli*-expressed CaD40 fragment is smaller than expected. It was confirmed by mass spectroscopy that CaD40 has a mass of 19.2 kDa. To ascertain if the CaD40 insert in the pETCaD40 plasmid was intact, the plasmid was purified from an *E. coli* colony and the complete cDNA insert was sequenced. A single mutation was detected at residue 78, where a Glu in the wild type was mutated to a Val. A single deletion of adenosine at nucleotide 568 (corresponding to amino acid residue 153 [19]) caused a frame shift resulting in the introduction of a stop codon TAG at nucleotides 596–598 and a mis-sense sequence of 9 residues, Val-Lys-Val-Ala-Lys-Lys-Asp-Thr-Arg, at the C-terminal end. This assignment of CaD40 was confirmed by N-terminal sequencing and amino acid composition analyses. N-terminal sequence analysis of the purified CaD40 fragment showed that the N-terminal sequence was Met-Asp-Asp-Phe-Glu, as reported [19]. Amino acid composition analysis of this fragment is shown in Table 1. Taking all the evidence together, the CaD40 fragment corresponds to residues 1–152 of human fibroblast caldesmon, with an additional 9 mis-sense residues at the C-terminal end to give a molecule of 161 residues.

Table 2 Binding of caldesmon and caldesmon fragments to immobilized tropomyosin (TM) and calmodulin (CaM)

	[NaCl] at which the protein peak is eluted		
	TM column	CaM column	
		in 1 mM Ca^{2+}	in 1 mM EGTA
Intact caldesmon	140–250 mM	100% Bound	100 mM
CaD40	200 mM	100% Bound	20–40 mM
CaD39	125 mM	100% Bound	100 mM

Based on the cDNA sequence, the C-terminal fragment CaD39 corresponds to residues 244–538 of human fibroblast caldesmon [19] or residues 443–736 of chicken gizzard β -caldesmon [33]. It has a calculated M_r of 33 200, which agrees with a mass of 33.25 kDa determined by mass spectroscopy. The apparent M_r of CaD39 on SDS/PAGE (Figure 1) is 40 000, giving a ratio of apparent M_r :actual M_r of 1.2. The amino acid composition of CaD39 (Table 1) also confirms that it corresponds to residues 244–538 of human fibroblast caldesmon, as reported previously [19,20].

Functional characterization of the CaD40 and CaD39 fragments

Firstly, we determined that the *E. coli*-expressed CaD40 and CaD39 fragments of l-caldesmon were fully functional before using them for subsequent binding studies.

The N-terminal fragment, CaD40, retains its ability to bind gizzard myosin with a stoichiometry of about 1 mol of CaD40/mol of myosin (results not shown). The C-terminal fragment, CaD39, did not bind myosin under the same conditions, although interaction between similar C-terminal fragments of caldesmon and myosin has been reported by some [35,36], but not by others [4,34]. CaD40 does not interact with actin (Figure 4a), as reported by others [3]. The CaD40 fragment was retained by a calmodulin-Sepharose column in the presence of 1 mM Ca^{2+} and was eluted between 20 and 40 mM NaCl when Ca^{2+} was removed from the column, as shown in Table 2. These results suggest that there may be a weak binding site for calmodulin in the N-terminal domain of caldesmon, as previously reported [5].

The C-terminal fragment, CaD39, binds actin (Figure 4a) and calmodulin as strongly as intact gizzard caldesmon (Table 2). In addition, CaD39 inhibits the actin-activated HMM ATPase activity by about 70% at a molar ratio of CaD39:actin of 0.5:1.0, and the inhibition was reversed by calmodulin in the presence of Ca^{2+} . Under the same conditions, CaD40 has no effect on the acto-HMM ATPase activity.

Interaction between the CaD40 and CaD39 fragments and intact tropomyosin

We chose to use pyrene-labelled chicken breast α -tropomyosin in the present studies, because each α -tropomyosin chain contains a single Cys at residue 190 which is in close proximity to the other in the non-staggered and parallel coiled coil [28]. We have shown previously that chicken gizzard and chicken breast tropomyosin have identical affinity for caldesmon [27], indicating that the caldesmon-binding sites are conserved in both tropomyosin isoforms, and therefore it is justifiable to use chicken breast α -tropomyosin instead of smooth-muscle tropomyosin in this study. An incorporation of > 0.9 mol of pyrene per mol of chain

Figure 2 Binding of pyrene-labelled tropomyosin to intact caldesmon, CaD40 and CaD39

(a) Fluorescence emission spectra of pyrene-tropomyosin in the presence and absence of intact caldesmon, CaD39 or CaD40. Pyrene-labelled tropomyosin ($0.2 \mu\text{M}$) in 10 mM Mops, $\text{pH } 7.2$, 1.0 mM EGTA and 0.01% NaN_3 (—). The molar ratio of pyrene-tropomyosin/caldesmon was $4:1$ for intact caldesmon (.....), $6:1$ for CaD40 (---), and $3:1$ for CaD39 (----). The excitation wavelength was at 340 nm . Monomer peaks are at 385 nm and 405 nm and the excimer peak is at 490 nm . (b) Binding curves for pyrene-labelled tropomyosin and intact caldesmon, CaD39 and CaD40. Buffer conditions were the same as in (a). ΔF = the change in excimer/monomer tropomyosin fluorescence intensity at each addition of intact caldesmon (∇), CaD39 ($\square$) or CaD40 ($\circ$); F_0 = initial fluorescence of tropomyosin.

(M_r 33000) of α -tropomyosin was obtained. As shown in Figure 2(a), the two emission maxima at 385 and 405 nm corresponded to monomeric fluorescence, and a strong excimer fluorescence peak at 490 nm was also observed, due to the close proximity of the pyrene rings attached to Cys-190 [28]. Binding of intact gizzard h-caldesmon, CaD40 and CaD39 to pyrene-labelled tropomyosin increases the monomer emission slightly, but decreases the excimer fluorescence by more than 50% at saturation, indicating a localized unwinding of the tropomyosin coiled coil at the pyrene probes.

By measuring the change in excimer/monomer fluorescence ratios, $F(490 \text{ nm})/F(405 \text{ nm})$, with respect to added caldesmon or its fragments, binding curves were constructed, as shown in Figure 2(b). The affinity of intact caldesmon, CaD40 and CaD39

for pyrene-labelled tropomyosin is similar, with K_d values of $1.1 \times 10^{-7} \text{ M}$, $1.4 \times 10^{-7} \text{ M}$ and $9.2 \times 10^{-8} \text{ M}$ respectively. One mol of caldesmon or its fragments binds 0.5 – 1.5 mol of tropomyosin.

The interactions between tropomyosin and the CaD40 and CaD39 fragments were confirmed by using tropomyosin immobilized on Sepharose resins, as summarized in Table 2. The N- and C-terminal caldesmon fragments were retained by the tropomyosin-Sepharose column at 10 mM NaCl and were eluted at 200 and 125 mM NaCl respectively. Intact caldesmon was eluted between 140 and 250 mM NaCl . BSA did not bind to the same tropomyosin-Sepharose column.

Interaction of CaD39 and CaD40 fragments with tropomyosin in the presence of actin

Although both CaD40 and CaD39 interact with tropomyosin (Figure 2), it is important to determine whether the caldesmon fragments can interact with actin-bound tropomyosin. As shown in Figure 3(a), we measured the effect of intact caldesmon, CaD40 or CaD39 ($0.9 \mu\text{M}$ each) on the fluorescence intensity of ANM-labelled tropomyosin which was bound to actin. Both intact caldesmon and CaD39 increased the ANM fluorescence of tropomyosin by about 35% and 25% , respectively, at the emission maximum, suggesting that both proteins interacted with actin-bound tropomyosin. On the other hand, CaD40 had an insignificant effect on the ANM-tropomyosin fluorescence, suggesting that CaD40 interacts very weakly, if at all, with actin-bound tropomyosin. This observation is confirmed by titration of the ANM-labelled tropomyosin-actin complex with increasing amounts of caldesmon, CaD39 or CaD40, as shown in Figure 3(b). It is noteworthy that, if DTT is not included in the reaction buffer, caldesmon causes the aggregation of the tropomyosin-actin filaments, presumably by bundling, resulting in cloudiness.

Another possible interpretation of the results in Figure 3 is that CaD40 interacts with the actin-bound tropomyosin, but the binding does not affect the ANM-labelled Cys in tropomyosin. To test this hypothesis, we have measured direct binding, using the actin-tropomyosin sedimentation method. As shown in Figure 4(a), in the absence of tropomyosin a negligible amount of the CaD40 fragment was sedimented with the actin pellets, confirming that the N-terminal domain of caldesmon does not bind actin [8]. In the presence of tropomyosin, CaD40 still failed to co-sediment with the actin-tropomyosin complex, indicating that the CaD40-binding site on tropomyosin may be blocked by actin. On the contrary, CaD39 binds actin and tropomyosin-actin, but, more importantly, its affinity for the tropomyosin-actin complex is higher than that for actin alone.

It has been previously reported [17] that tropomyosin enhances the binding of intact caldesmon to actin; thus our results indicate that this enhanced interaction of caldesmon with actin-tropomyosin is contributed by the C-terminal domain of caldesmon. We have used another approach to test this hypothesis. Intact caldesmon, CaD40 or CaD39 was mixed with tropomyosin in a $1:1$ molar ratio under conditions where they interacted. Actin (3 – $7 \mu\text{M}$) was added to the reaction mixtures, and the amount of caldesmon or its fragments sedimented with the tropomyosin-actin pellets was measured. In a separate control experiment, tropomyosin was absent from the reaction mixture. As shown in Figure 4(b), free or tropomyosin-bound caldesmon was sedimented in the actin pellets, and the amount of caldesmon bound to the actin-tropomyosin filaments was higher than that to actin alone. Similarly, both free and tropomyosin-bound CaD39 interacted with actin, and its affinity for actin was higher when tropomyosin was present. As expected,

Figure 3 Effects of caldesmon, CaD39 and CaD40 on the fluorescence of ANM-labelled tropomyosin bound to actin

ANM-labelled tropomyosin (1 μ M) was incubated with actin (4 μ M) in 20 mM Tris/HCl (pH 7.5)/50 mM KCl/5 mM MgCl₂/1 mM ATP/1 mM DTT at 25 °C for 30 min and sedimented for 30 min at 100 000 *g*. The actin-tropomyosin pellet was redissolved in the same buffer to give a final concentration of 2 μ M actin and 0.3 μ M tropomyosin. ANM fluorescence-emission spectra were obtained after each addition of known portions of caldesmon or its fragments. Excitation wavelength was at 356 nm. (a) Fluorescence emission spectra of ANM-labelled tropomyosin + actin (—), ANM-tropomyosin + actin + intact gizzard caldesmon (---), ANM-tropomyosin + actin + CaD39 (· · · · ·) and ANM-tropomyosin + actin + CaD40 (— · — · —). (b) Binding of gizzard caldesmon, CaD39 or CaD40 to actin-bound tropomyosin. $\Delta F = F - F_0$ = difference in fluorescence intensity at 456 nm of ANM-tropomyosin-actin in the presence (*F*) and absence (*F*₀) of caldesmon or its fragments. Intact caldesmon (○); CaD40 (□); CaD39 (▽).

CaD40 did not bind to actin alone. Furthermore, CaD40 originally bound to tropomyosin did not co-sediment with the tropomyosin-actin filament, confirming that the CaD40-binding site on tropomyosin is blocked by actin.

In one study, Bogatcheva et al. [18] observed some binding of an N-terminal fragment to tropomyosin-actin when a mixture of N- and C-terminal chymotryptic fragments of caldesmon was used for sedimentation studies. To investigate whether the C-terminal fragment may enhance the interaction between the N-terminal fragment of caldesmon and actin-tropomyosin, the binding of a mixture of CaD40 and CaD39 fragments to actin-tropomyosin was determined. In the presence or absence of CaD39, a negligible amount of CaD40 bound to the actin-tropomyosin pellet (< 5%).

Figure 4 Direct measurement of intact gizzard caldesmon, CaD39 and CaD40 bound to actin in the presence or absence of tropomyosin by sedimentation analyses

(a) Actin (3 μ M) with or without tropomyosin (0.86 μ M) was incubated with different amounts of caldesmon fragments (0–10 μ M) at 25 °C for 1 h in 20 mM Tris/HCl (pH 7.5)/100 mM KCl/2 mM MgCl₂/1 mM ATP/1 mM DTT. CaD39 (● and ○); CaD40 (■ and □). White and black symbols represent binding curves in the presence and absence of tropomyosin respectively. (b) Intact caldesmon, CaD40 or CaD39 was mixed with tropomyosin in a 1:1 molar ratio under conditions such that they would interact (as in a, except that 50 mM NaCl was used) and actin (3–7 μ M) was added to the reaction mixtures. The amounts of actin, tropomyosin and caldesmon in the pellets were analysed by SDS/PAGE and densitometry. Gizzard caldesmon (△, ▲), CaD39 (○, ●), CaD40 (□, ■). White and black symbols represent the presence and absence of tropomyosin respectively.

Table 3 Binding of tropomyosin (TM) and tropomyosin fragments to immobilized caldesmon fragments

	[NaCl] at which the protein peak is eluted	
	CaD40 column	CaD39 column
Intact TM	110 mM	150 mM
Cn1A	40 mM	150 mM
Cn1B	110 mM	130 mM
BSA	10 mM	10 mM

Interaction of the N- and C-terminal domains of caldesmon with fragments of tropomyosin

In order to locate the binding site(s) on tropomyosin for the N- and C-terminal fragments independently, we have studied the binding of tropomyosin and its N- and C-terminal CNBr fragments, Cn1A(11–127) and Cn1B(142–281) respectively, to immobilized CaD40 and CaD39. As summarized in Table 3,

intact tropomyosin and Cn1B(142–281) bind to the N-terminal fragment of caldesmon, whereas Cn1A(11–127) binds weakly to the same CaD40–Sepharose column. The immobilized C-terminal fragment of caldesmon, on the other hand, interacts strongly with intact tropomyosin as well as with Cn1A(11–127) and Cn1B(142–281). These results indicate that the binding site for the N-terminal fragment of caldesmon resides within the C-terminal half of tropomyosin whereas the C-terminal caldesmon fragment binds to both N- and C-terminal regions in tropomyosin.

DISCUSSION

Both N- and C-terminal fragments of human fibroblast caldesmon expressed in *E. coli* retain their known functions, and therefore it is justifiable to use these fragments in the present studies. The N-terminal fragment, CaD40, binds myosin, but not actin, as previously reported by others [3] using smooth-muscle h-caldesmon fragments. We have also shown that CaD40 interacts weakly with calmodulin in the presence of Ca^{2+} , as reported previously by Wang et al. [5]. The significance *in vivo* of this weak calmodulin-binding site is not clear at present. The C-terminal fragment, CaD39, binds actin, tropomyosin and Ca^{2+} /calmodulin, and inhibits actin-activated HMM ATPase activity.

Our finding that both N- and C-terminal fragments of caldesmon bind to tropomyosin is in agreement with other reports [8,12,13,18]. Redwood and Marston [8] suggested that there are three tropomyosin-binding sites on caldesmon. One of their fragments, N128 (residues 1–128), binds tropomyosin weakly, whereas our study shows that CaD40 (residues 1–152) binds tropomyosin as strongly as the intact h-caldesmon. Although these results suggest that the major tropomyosin-binding site on the N-terminal domain of caldesmon may reside in a region within residues 129–152 of caldesmon, further experiments are required before a definitive conclusion can be made. Redwood and Marston [8] have shown that the tropomyosin-binding site on the C-terminal domain of caldesmon lies at a region between residues 606 and 658. This is consistent with our finding that CaD39, which corresponds to residues 443–736 of chicken gizzard caldesmon, binds strongly to tropomyosin.

We have previously demonstrated that the major caldesmon-binding site on tropomyosin is located in the region spanning residues 142–227 [14]. A weaker caldesmon-binding site was also detected at the N-terminal half of tropomyosin. In this study, we have shown that the N-terminal domain of caldesmon represented by CaD40 interacts with the C-terminal fragment of tropomyosin, Cn1B(142–281), but this interaction is suppressed by the presence of actin. The C-terminal fragment of caldesmon, CaD39, binds to both N- and C-terminal fragments of tropomyosin, Cn1A(11–127) and Cn1B(142–281) respectively. Furthermore, the CaD39–tropomyosin interaction is enhanced by actin. Based on these new findings, and our previous data indicating that the region spanning from residue 227 to the C-terminal end of tropomyosin does not appear to be involved in binding to caldesmon [14], a diagram depicting the possible arrangement of caldesmon on the thin filament is shown in Figure 5. The C-terminal domain of caldesmon is in contact with an extended region of tropomyosin within residues 11–227. The major binding site, however, is located within residues 142–227, which has also been identified as the major Ca^{2+} -sensitive troponin-T-binding site on tropomyosin [42]. Although the N-terminal domain of caldesmon can interact strongly with tropomyosin within residues 142–227 of an adjacent tropomyosin molecule, this interaction is largely inhibited while tropomyosin is bound

Figure 5 Schematic representation of the possible alignment of caldesmon on the tropomyosin–actin filament

Each caldesmon molecule spans two tropomyosin and 14 actin monomers. The C-terminal domain of caldesmon interacts with an extended region of one tropomyosin molecule covering residues 11–227. A strong caldesmon-binding site (shown by hatching) occurs at residues 142–227 of tropomyosin, and a weaker site (shown by stippling) lies at the N-terminal half of tropomyosin covering residues 11–142. A weak interaction occurs between the N-terminal domain of caldesmon and the C-terminal region of an adjacent tropomyosin molecule within residues 142–227.

to actin. Such an arrangement may allow the N-terminal domain of caldesmon to interact with myosin at the S2 region.

At present it is not clear what role the caldesmon–tropomyosin interaction plays in the thin-filament structure. One possibility is that the tropomyosin polymer may act as a template for the alignment of caldesmon along the thin filaments. Several studies *in vitro* have suggested that tropomyosin may also play a significant role in the regulation of smooth-muscle contraction. Horiuchi et al. [37] showed that caldesmon decreased the k_{cat} of acto-myosin ATPase in the presence of tropomyosin, and Hemric et al. [38] found that tropomyosin enhanced the effectiveness of caldesmon inhibition of actomyosin ATPase activities by affecting the K_{ATPase} rather than the k_{cat} . Marston and Redwood [39] have demonstrated that caldesmon inhibits a rate-limiting step in the actin activation of S1 MgATPase activity in the presence of tropomyosin, and the inhibition is co-operative. Shirinsky et al. [40] have shown that tropomyosin enhances caldesmon inhibition of movements of actin filaments on immobilized myosin [40].

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REFERENCES

- 1 Sobue, K. and Sellers, J. R. (1991) *J. Biol. Chem.* **266**, 12115–12118
- 2 Matsumura, F. and Yamashiro, S. (1993) *Curr. Opin. Cell Biol.* **5**, 70–76
- 3 Marston, S. B. and Redwood, C. S. (1991) *Biochem. J.* **279**, 1–16
- 4 Velaz, L., Ingraham, R. H. and Chalovich, J. M. (1990) *J. Biol. Chem.* **265**, 2929–2934
- 5 Wang, C.-L. A., Wang, L. W. C. and Lu, R. C. (1989) *Biochem. Biophys. Res. Commun.* **162**, 746–752
- 6 Ball, E. H. and Kovala, T. (1988) *Biochemistry* **27**, 6093–6098
- 7 Bartegi, A., Fattoum, A., Derancourt, J. and Kassab, R. (1990) *J. Biol. Chem.* **265**, 15231–15238
- 8 Redwood, C. S. and Marston, S. B. (1993) *J. Biol. Chem.* **268**, 10969–10976
- 9 Mezgueldi, M., Derancourt, J., Calas, B., Kassab, R. and Fattoum, A. (1994) *J. Biol. Chem.* **269**, 12824–12832
- 10 Zhan, Q., Wong, S. S. and Wang, C.-L. A. (1991) *J. Biol. Chem.* **266**, 21810–21814
- 11 Marston, S. B., Fraser, I. D. C., Huber, P. A. J., Pritchard, K., Gusev, N. B. and Torok, K. (1994) *J. Biol. Chem.* **269**, 8134–8139
- 12 Hayashi, K., Fujio, Y., Kato, I. and Sobue, K. (1991) *J. Biol. Chem.* **266**, 355–361
- 13 Velaz, L., Chen, Y.-D. and Chalovich, J. M. (1993) *Biophys. J.* **65**, 892–898
- 14 Watson, M. H., Kuhn, A. E., Novy, R. E., Lin, J. J.-C. and Mak, A. S. (1990) *J. Biol. Chem.* **265**, 18860–18866
- 15 Graceffa, P., Wang, C.-L. A. and Stafford, W. F. (1988) *J. Biol. Chem.* **263**, 14196–14202
- 16 Moody, C., Lehman, W. and Craig, R. (1990) *J. Muscle Res. Cell Motil.* **11**, 176–185

- 17 Horiuchi, K. Y. and Chacko, S. (1988) *Biochemistry* **27**, 8388–8393
- 18 Bogatcheva, N. V., Vorotnikov, A. V., Birukov, K. G., Shirinsky, V. P. and Gusev, N. B. (1993) *Biochem. J.* **290**, 437–442
- 19 Novy, R. E., Lin, J. L.-C. and Lin, J. J.-C. (1991) *J. Biol. Chem.* **266**, 16917–16924
- 20 Novy, R. E., Sellers, J. R., Liu, L.-F. and Lin, J. J.-C. (1993) *Cell Motil. Cytoskeleton* **26**, 248–261
- 21 Sanders, C. and Smilie, L. B. (1984) *Can. J. Biochem. Cell Biol.* **62**, 443–448
- 22 Pardee, J. D. and Spudich, J. A. (1982) *Methods Enzymol.* **85**, 164–181
- 23 Bretscher, A. (1984) *J. Biol. Chem.* **259**, 12873–12880
- 24 Margossian, S. S. and Lowey, S. (1982) *Methods Enzymol.* **85B**, 55–71
- 25 Persechini, A. and Hartshorne, D. (1983) *Biochemistry* **22**, 470–476
- 26 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 27 Watson, M. H., Kuhn, A. E. and Mak, A. S. (1990) *Biochim. Biophys. Acta* **1054**, 103–113
- 28 Graceffa, P. and Lehrer, S. S. (1980) *J. Biol. Chem.* **255**, 11296–11300
- 29 Mak, A. S., Watson, M. H., Litwin, C. M. E. and Wang, J. H. (1991) *J. Biol. Chem.* **266**, 6678–6681
- 30 Pollard, T. D. (1982) *Methods Enzymol.* **85B**, 123–130
- 31 Childs, T. J., Watson, M. H., Sanghera, J. S., Campbell, D. L., Pelech, S. L. and Mak, A. S. (1992) *J. Biol. Chem.* **267**, 22853–22859
- 32 Hemric, M. E. and Chalovich, J. M. (1990) *J. Biol. Chem.* **265**, 19672–19678
- 33 Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G. and Lin, W. G. (1989) *J. Biol. Chem.* **264**, 13873–13879
- 34 Sutherland, C. and Walsh, M. P. (1989) *J. Biol. Chem.* **264**, 578–583
- 35 Yamakita, Y., Yamashiro, S. and Matsumura, F. (1992) *J. Biol. Chem.* **267**, 12022–12029
- 36 Huber, P. A. J., Redwood, C. S., Avent, N. D., Tanner, M. J. A. and Marston, S. B. (1993) *J. Muscle Res. Cell Motil.* **14**, 385–391
- 37 Horiuchi, K. Y., Samuel, M. and Chacko, S. (1991) *Biochemistry* **30**, 712–717
- 38 Hemric, M. E., Freedman, M. V. and Chalovich, J. M. (1993) *Arch. Biochem. Biophys.* **306**, 39–43
- 39 Marston, S. B. and Redwood, C. S. (1993) *J. Biol. Chem.* **268**, 12317–12320
- 40 Shirinsky, V. P., Biryukov, K. G., Hettasch, J. M. and Sellers, J. R. (1992) *J. Biol. Chem.* **267**, 15886–15892
- 41 Spande, T. F. and Witkop, B. (1967) *Methods Enzymol.* **11**, 498–506
- 42 Mak, A. S. and Smilie, L. B. (1981) *J. Mol. Biol.* **149**, 541–550