Location of smooth-muscle myosin and tropomyosin binding sites in the C-terminal 288 residues of human caldesmon

Pia A. J. HUBER,* Iain D. C. FRASER and Steven B. MARSTON

Department of Cardiac Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K.

We have produced nine recombinant fragments, H1 to H9, from a human cDNA that codes for the C-terminal 288 residues of caldesmon. The fragment H1, encompassing the 288 residues, is equivalent to domains 3 and 4 of caldesmon (amino acids 506–793 in human, 476–737 in the chicken gizzard sequence). It has been shown [Huber, Redwood, Avent, Tanner and Marston (1993) *J. Muscle Res. Cell Motil.* **14**, 385–391] to bind to actin, Ca^{2+} -calmodulin, tropomyosin and myosin. The fragments, H2 to H9, differ in length between 60 and 176 residues and cover the whole of domains 3 and 4 with many of the fragments overlapping. We have characterized the myosin and tropomyosin binding of these fragments. The binding of both tropomyosin and myosin is highly dependent on salt concentration, indicating the ionic nature of these interactions. The location of the myosin binding is an extended region encompassing the junction of

domains 3/4 and domain 4a (residues 622–714, human; 566–657, chicken gizzard). Tropomyosin binds in a smaller region within domain 4a of caldesmon (residues 663–714, human; 606–657 chicken gizzard). We confirmed predictions based on sequence similarities of a tropomyosin binding site in domain 3 of caldesmon; however, this site bound to skeletal-muscle tropomyosin and had little affinity for the smooth-muscle tropomyosin isoform. None of the protein fragments H2–H9 retained the affinity of the parent fragment H1 for either myosin or tropomyosin. This indicates the need for several interaction sites scattered over an extended region to attain higher affinity. The regions interacting with caldesmon in both tropomyosin and myosin are coiled-coil structures. This is probably the reason for their shared interaction sites on caldesmon and their similar natures of binding.

INTRODUCTION

Caldesmon is a component of the thin myofilaments of smooth-muscle and non-muscle cells. The smooth-muscle isoform of human caldesmon (Cal_h), has 793 residues and is an extremely elongated molecule (length *in vitro* 73 nm) that binds to actin, tropomyosin, myosin and calcium-binding proteins (see reviews in [1–4]). *In vivo* the thin filaments of smooth muscle are made up of actin, tropomyosin, caldesmon and a Ca^{2+} -binding protein in molar ratios of 14:2:1:1. When the four purified proteins are assembled *in vitro* they form Ca^{2+} -regulated thin filaments [5–7]. In native smooth-muscle thin filaments, caldesmon molecules are located in an extended conformation along the long pitch of the actin double helix, apparently in close side-by-side association with the tropomyosin molecules [8,9].

Cal_h has been divided into four structural domains (see Figure 1 [2,10]); actin and calmodulin binding are confined to the C-terminal 150 residues (domain 4) [10–13]. Tropomyosin binding sites have been identified in domains 1, 2 and 4 [10,14–17] and a binding site in domain 3 has been predicted on the basis of sequence similarity with troponin T [18–22]. Myosin binding has been demonstrated in domain 1 [17,23–25] and recently also in the C-terminus within domains 3 and 4 [26,27].

It has been suggested that the location of caldesmon in the thin filament is largely defined by caldesmon–tropomyosin interactions at specific multiple points along the molecules [10,28], and recent studies on caldesmon/tropomyosin co-crystals seem to support this view [29]. In this case, sites in domains 3 and 4, along with those in domains 1 and 2, would play a significant stabilizing role. It has also been shown that caldesmon, together with tropomyosin, stabilizes the actin filaments and prevents their depolymerization in non-muscle cells [30–32].

The dual binding interaction of caldesmon with both actin and

myosin can cross-link thick and thin filaments. This ‘tethering’ can promote thick–thin filament interaction, can impede filament movement and may contribute to the ‘latch’ state in smooth muscle [33–36]. In addition, the myosin–caldesmon interaction is believed to play a significant role in promoting the assembly of myosin into filaments and actomyosin lattices [37,38]. At present we do not know whether N-terminal (domain 1) and C-terminal (domain 3–4) myosin-binding sites in caldesmon are both involved in these processes or whether the two sites have different properties.

The location of the tropomyosin- and myosin-binding sites in domains 3 and 4 is currently unknown and their significance in comparison with the better characterized sites at the N-terminus has not been assessed. Tropomyosin and myosin may bind close to the known actin- and calmodulin-binding sites in domain 4. Current evidence indicates that tropomyosin-dependent inhibition of the activation of myosin MgATPase by actin does not require caldesmon–tropomyosin binding [39] and is not influenced by myosin binding to caldesmon [40]. However, there is some evidence that the binding of Ca^{2+} -calmodulin to caldesmon may affect the interaction of caldesmon with myosin (and tropomyosin) [41,42].

To study these binding sites we prepared a set of recombinant fragments derived from the human caldesmon domain 3/4 fragment, H1 (residues 506–793 [22], equivalent to 476–737 in the chicken gizzard sequence [20]), described by Huber et al. [27]. The fragments cover the whole of domains 3 and 4 with many of the fragments overlapping (Figure 1). We have characterized myosin and tropomyosin binding by these fragments to locate the binding sites and assess their function. We have found that both tropomyosin and myosin bind in the same region, or at least in overlapping regions, of the C-terminus of caldesmon. Myosin

Abbreviations used: Cal_h , smooth-muscle caldesmon isoform (high molecular mass); Cal_l , non-muscle caldesmon isoform (low molecular mass); DTT, 1,4-dithio-DL-threitol; *f*, fluorescence emission.

* To whom correspondence should be addressed.

Expressed protein	Sequence (human numbers)	Sequence (gizzard numbers)	Calculated molecular mass (kDa)	Apparent molecular mass (kDa) (SDS/PAGE)	Domain structure	Actin binding	Calmodulin binding	Tropomyosin binding	Myosin binding	Myosin-rod binding	Reversal of myosin binding by Ca-CM	Reversal of H1 binding to Myosin*
Caldesmon ^h	1-793	1-756	93/89	146		1) 107-10 ⁸ M ⁻¹	1) 3x10 ⁶ M ⁻¹	1) 10 ⁶ M ⁻¹	1) ≥ 10 ⁶ M ⁻¹	+++	yes	n.d.
H1	506-793	476-737	32.5	38.4		2) +++ (5x10 ⁷ M ⁻¹)	3) +++ (4x10 ⁶ M ⁻¹)	+++ (3x10 ⁶ M ⁻¹)	+++ (3x10 ⁶ M ⁻¹)	++	yes	-
H2	683-767	626-710	9.0	8.0		++	3) +++	++	++	n.d.	n.d.	15
H3	506-566	476-510	7.0	7.2		0	nd	+	0	n.d.	n.d.	n.d.
H4	622-681	566-624	6.7	7.2		0	3) 0	++	++	n.d.	n.d.	10
H5	506-681	476-624	20.7	22.3		+	0	++	+	0	n.d.	20
H6	506-625	476-569	14.7	15.7		0	0	+	0	n.d.	n.d.	n.d.
H7	622-767	566-710	15.6	16.6		++	3) +++	++	++	+	n.d.	10
H8 (658C)	715-793	658-737 (658-756)	8.5 (10.6)	9.5		++	3) +++	+	0	n.d.	n.d.	n.d.
H9	726-793	669-737	7.3	6.4		++	3) +++	0	0	n.d.	n.d.	n.d.
N128	1-128	1-128	15.6	18.2		0	1) 0	+	++	+++	no	5
606C (see Ref. 10)	663-793...	606-756	16.0	18.0		+++	1) +++	++	+(+)	+	n.d.	n.d.

Figure 1 Domain structure, sequences and properties of recombinant human caldesmon fragments

Domains are indicated by 1, 2, 3 and 4. The N-terminal caldesmon fragment N128 was derived from a chicken gizzard caldesmon clone [10]. The C-terminal caldesmon fragments H1–H9 are derived from a human caldesmon clone. Sequence numbers refer to the human caldesmon sequence given by Humphrey et al. [22] or the chicken gizzard sequence of Bryan et al. [20]. The apparent molecular masses are derived from SDS/PAGE, see Figure 2. *, Molar excess of caldesmon fragment needed for 50% displacement of H1 bound to myosin. 70 ± 5% of H1 was bound to myosin in the absence of competing fragments. Binding affinities: + + +, strong binding (affinity comparable to Cal_h); + +, moderate binding (one-third to one-tenth of affinity of Cal_h); +, weak (one-twentieth to one-hundredth of affinity of Cal_h); 0, very weak or no binding (less than one-hundredth of affinity of Cal_h). References: ¹, Redwood and Marston [10]; ², Huber et al. [27]; ³, Marston et al. [13].

appears to bind to an extended region encompassing the domain 3/4 junction and domain 4a (residues 637–714 human, or 581–657 in chicken gizzard), whereas tropomyosin binds to a smaller region within domain 4a (residues 663–714 human, or 606–657 in chicken gizzard [20]).

MATERIALS AND METHODS

Construction and expression of caldesmon mutants

The cDNA of the C-terminal 33 kDa human caldesmon fragment H1 was derived from a human foetal liver library. It was subcloned into the pMW172 vector and expressed in *Escherichia coli*, as described by Huber et al. [27]. The plasmid construct pMW172/H1 was used as a template to generate eight mutant caldesmon DNA sequences by PCR amplification. Primer pairs were synthesized that allowed amplification of the appropriate cDNA sequence and introduced start and stop codons and restriction enzyme sites. This provided suitable termini for ligating the PCR products into the expression vector, as described by Redwood and Marston [10]. All the sequences were verified by double-stranded sequencing. The eight caldesmon fragments

(H2–H9) were expressed after transformation of the mutant DNA plasmid constructs into the *E. coli* strain BL21(DE3) [27]. The cells were extracted and the caldesmon peptides were purified by heat treatment as described by Redwood and Marston [10]. Final purification was achieved by ion-exchange chromatography on Q or S Sepharose Fast Flow (Pharmacia), depending on their calculated isoelectric point. The yield of purified fragment was 1–5 mg per litre of bacterial culture. Figure 1 shows the sequences of the caldesmon fragments H1–H9 and their locations in the domain model of caldesmon. The N-terminal fragment of caldesmon, N128, (Figure 1) was expressed and purified by N. Gusev [43] from the plasmid construct of C. S. Redwood [10].

Protein preparations

Caldesmon and smooth-muscle myosin were extracted from sheep aorta or chicken gizzard by procedures described by Taggart and Marston [44] and Sellers et al. [45] respectively. Myosin rod was produced by papain digestion as described by Marston et al. [46]. The rods were purified from the digest by the ethanol precipitation method of Margossian and Lowey [47]. Myosin and myosin rod were both stored in 50% (v/v) glycerol

at -20°C when not used immediately. Tropomyosin was prepared from sheep aorta or rabbit skeletal ether/ethanol powder made according to Bailey's protocol [48] and purified on a hydroxyapatite column (HA Ultrogel, BioSeptra, France) after Eisenberg and Kielley [49]. Calmodulin was prepared from bovine brain acetone powder (Sigma) as described by Gopalakrishna and Anderson [50]. Protein concentrations were determined by the method of Lowry [50a].

Binding assays

The binding of caldesmon fragments to filaments of smooth-muscle myosin or myosin rod was measured in sedimentation assays. Myosin and myosin rod were dialysed into 5 mM Pipes, adjusted to pH 7.2 with KOH solution at 22°C , 2.5 mM MgCl_2 , 1 mM dithiothreitol (DTT) and 5 mM KCl (buffer A) or 90 mM KCl (buffer B) for 20 h to form filaments. After dialysis the myosin-rod filaments were centrifuged at 40000 g for 20 min, the supernatant was removed and the pellets resuspended in the assay buffer. For the binding assays, fixed concentrations, usually $2\text{ }\mu\text{M}$, of caldesmon fragment were mixed with $0\text{--}8\text{ }\mu\text{M}$ myosin or myosin-rod filaments in buffer A or B to a total volume of $40\text{ }\mu\text{l}$. The mixtures were incubated at 22°C for 30 min and then centrifuged at 40000 g for 20 min. Aliquots ($20\text{ }\mu\text{l}$) of the supernatants were separated by gel electrophoresis on 8–18% (w/v) ExcelGels (Pharmacia), run for 80 min at 10°C , and stained with Page Blue 83. The bands were quantified by densitometry [Scanmaster (Howtek Inc.), 'pdi Quantity one' software].

Tropomyosin binding was measured with smooth-muscle or skeletal-muscle tropomyosin, conjugated under non-denaturing conditions with *N*-(1-pyrenyl)-maleimide at their cysteine residues as described by Lehrer et al. [51]. An aliquot ($0.25\text{ }\mu\text{M}$) of labelled tropomyosin was incubated with $0\text{--}4\text{ }\mu\text{M}$ caldesmon fragment in 5 mM Pipes, adjusted to pH 7.2 with KOH solution at 22°C , 2.5 mM MgCl_2 , 1 mM DTT and 30 mM KCl (total volume $150\text{ }\mu\text{l}$) at 22°C for 30 min and the fluorescence emission at 377 nm was measured with excitation at 339 nm (Perkin Elmer luminescence spectrometer LS-5) [52,53]. The change in the fluorescence intensity due to complexing of caldesmon with

tropomyosin is given as the percentage change relative to the fluorescence (f) of tropomyosin alone:

$$\Delta f = 100 \times (f_{\text{complex}} - f_{\text{tropomyosin}}) / f_{\text{tropomyosin}}$$

RESULTS

Expression of C-terminal caldesmon fragments

An SDS/PAGE separation of caldesmon and the nine recombinant fragments derived from a partial human cDNA sequence

Figure 3 The binding of caldesmon and H1 to smooth-muscle myosin at low and high ionic strength

Caldesmon or H1 ($2\text{ }\mu\text{M}$) was mixed with $0\text{--}8\text{ }\mu\text{M}$ smooth-muscle myosin in 5 mM Pipes, pH 7.2, containing 2.5 mM MgCl_2 , 1 mM DTT at 25°C and 5 mM KCl (buffer A) (filled symbols) or 90 mM KCl (buffer B) (open symbols). Binding was determined by co-sedimentation with myosin. Data are fitted to the equation: fraction bound = $[\text{CD}]/(K + [\text{CD}])$, where CD = caldesmon or H1. $\bullet$, $\circ$, H1; $\blacksquare$, $\square$, caldesmon. Solid lines represent best fit curves. For caldesmon in 5 mM KCl, $K = (1.2 \pm 0.17) \times 10^{-6}\text{ M}$; for caldesmon in 90 mM KCl, $K = (1.1 \pm 0.28) \times 10^{-5}\text{ M}$; for H1 in 5 mM KCl, $K = (1.1 \pm 0.14) \times 10^{-6}\text{ M}$; for H1 in 90 mM KCl, $K = (1.5 \pm 0.25) \times 10^{-5}\text{ M}$.

Figure 2 SDS/PAGE of caldesmon and recombinant fragments H1–H9

Purified fragments ($0.5\text{--}1.0\text{ }\mu\text{g}$) were run on an 8–18% ExcelGel (Pharmacia) at 10°C for 80 min and stained with Page Blue 83. Standards of high molecular masses (M_h) and low molecular masses (M_l) are in the first and last lane, respectively.

Figure 4 Binding of H1-H9 to smooth-muscle myosin

The binding was measured in buffer A (5 mM KCl) as described in Figure 3. The best fit curve of the H1-myosin interaction is displayed for comparison. Data points represent means $\pm$ S.E.M. from 3-7 experiments.

Figure 5 Binding of caldesmon (CDh), H1, H5, H7 and N128 to smooth-muscle myosin-rod filaments

Conditions and analysis were as described in Figure 4.

Figure 6 Effect of Ca^{2+} calmodulin on caldesmon (CDh), H1 and N128 binding to smooth-muscle myosin filaments

Caldesmon, H1 or N128 ($2 \mu\text{M}$) was incubated in buffer A (see Figure 3), with $4 \mu\text{M}$ myosin and 0-16 μM calmodulin in the presence of Ca^{2+} (0.1 mM). The highest concentrations of calmodulin were also assayed in EGTA (0.1 mM) (open symbols).

[27] is shown in Figure 2. The location and characteristics of these fragments are shown in Figure 1. The fragments H1, H2, H7, H8 and H9 (containing or representing part of domain 4) have been shown to bind to actin and Ca^{2+} -calmodulin and H1,

H7, H8 and H9 are tropomyosin-dependent inhibitors of actin-activated myosin MgATPase [13,54]. As the parent sequence, H1, also binds to tropomyosin and smooth-muscle myosin [26,27], we constructed a further series of recombinants to

Figure 7 Fluorimetric titration of *N*-(1-pyrenyl)maleimide-labelled tropomyosin with caldesmon fragments H1-H9

Labelled tropomyosin ($0.25 \mu\text{M}$) was incubated with $0\text{--}4 \mu\text{M}$ caldesmon fragments in 5 mM Pipes, $\text{pH } 7.2$, containing 2.5 mM MgCl_2 , 1 mM DTT and 30 mM KCl at 25°C . The fluorescence emission at 377 nm was measured with excitation at 339 nm . Data are expressed as percentage increase over pyrene-labelled tropomyosin alone. The fluorescence of pyrene-labelled tropomyosin was enhanced by fragments H7, H5, H4 and H2. K_d and maximum fluorescence increase were determined for H1 by a non-linear fit to the equation $\Delta f = \Delta f_{\text{max}}[H1]/(K_d + [H1])$: $\Delta f_{\text{max}} = +29.8 \pm 2.7\%$; $K_d = 0.4 \pm 0.13 \mu\text{M}$. Data points represent means $\pm$ S.E.M. from 3 or 4 experiments, where different preparations of tropomyosin and caldesmon fragments were used.

investigate these binding sites. These fragments, H3, H4, H5 and H6, contain sequences from domain 3 and the beginning (N-terminus) of domain 4. The expressed and purified caldesmon peptides were identified by their size on SDS/PAGE (see Figure 2) and by Western blots with a polyclonal antibody raised against sheep aorta caldesmon (results not shown). Whereas the fragment H5 showed a weak interaction with actin, none of the other fragments, H3, H4 or H6 bound to actin at all (Figure 1 and [55]).

Myosin binding

We have identified a binding site for smooth-muscle myosin in the C-terminus of caldesmon, within fragment H1 [27]. Myosin binding was previously thought to be confined to the N-terminus of caldesmon [23]. The affinity of H1 for smooth-muscle myosin and for myosin-rod filaments was very similar to that for whole caldesmon in a paired experiment at low ionic strength. Figure 3 shows the binding of caldesmon and H1 to smooth-muscle myosin at salt concentrations of 5 and 90 mM KCl. Both H1 and caldesmon showed a decrease in affinity with increasing salt concentration, indicating the involvement of ionic bonds in this interaction. The apparent affinity of caldesmon and H1 for myosin in 5 mM KCl was $(0.8\text{--}0.9) \times 10^6/\text{M}$, similar to previous determinations [27,46].

To locate the myosin binding sequence in the C-terminus of caldesmon we tested our set of truncated recombinants in the

myosin-binding assay at low ionic strength (Figure 4). None of the fragments bound as well as H1 (represented in each plot by the best-fitting curve taken from Figure 3). The central fragments H2, H7 and H4 bound to myosin but with one-third to one-tenth of the affinity of H1 or caldesmon. The C-terminal fragments of H1, i.e. H8 and H9, and the N-terminal fragment H6 showed very low affinity ($K_{\text{app}} < 10^4/\text{M}$).

We also tested the relative affinities of our fragments for myosin by competition assays. We incubated myosin and H1 at fixed concentrations with increasing amounts of fragments H2, H4, H5, H7 and N128. N128 is an N-terminal chicken gizzard caldesmon fragment previously shown to bind myosin [10] (see Figure 1). The ability of these fragments to compete with H1 for myosin decreased in the order $\text{N128} > \text{H7} \geq \text{H4} > \text{H2} > \text{H5}$ (Figure 1). In a different experiment, H1 and N128 were only able to partially displace whole caldesmon from myosin, even at 48 times molar excess (results not shown).

Caldesmon binds to the myosin molecule within the S2 region [23,25,41]. We have used myosin-rod filaments containing the S2 part of myosin in our binding assays (Figure 5). N128 bound to myosin rod as well as full-length caldesmon, whereas binding of H1 was slightly lower. H7 showed an even weaker affinity for myosin rod, whereas H5 showed virtually no binding. The affinities of H7 and H5 for myosin rod, relative to H1, were comparable with those observed with whole myosin.

Binding of caldesmon and H1 to myosin at low ionic strength was abolished by Ca^{2+} -calmodulin (Figure 6). In contrast, the

Figure 8 The binding of caldesmon domain 3 (H6) to smooth-muscle (a) and skeletal-muscle (b) tropomyosin

Skeletal- and smooth-muscle tropomyosin were labelled with *N*-(1-pyrenyl)maleimide as described in Materials and methods. The fluorescence was measured as described in Figure 7. Derived parameters for binding to skeletal tropomyosin are: H1, $\Delta f_{\max} = +81.1 \pm 6.4\%$, $K_d = 2.4 \pm 0.47 \mu\text{M}$; H6, $\Delta f_{\max} = +61.2 \pm 4.4\%$, $K_d = 2.3 \pm 0.4 \mu\text{M}$.

binding of the N-terminal fragment, N128, to myosin was only marginally reduced by Ca^{2+} -calmodulin.

Tropomyosin binding

The binding of the C-terminus of caldesmon to tropomyosin was measured by using tropomyosin labelled at its cysteine residues with *N*-(1-pyrenyl)maleimide [51]. A change in the fluorescence intensity of pyrene-labelled tropomyosin has been observed on titration with caldesmon [52]. Similarly, H1 induced a fluorescence enhancement of up to 30% with an apparent affinity of $2.5 \times 10^6/\text{M}$. Figure 7 shows the observed fluorescence change of the pyrene-labelled tropomyosin in titrations with the fragments H1 to H9. The plots show the means with standard errors from three or four separate experiments with different tropomyosin preparations. In most cases the data were not consistent enough to justify any curve-fitting. The fragments H8 and H9, from the C-terminal domain 4b, induced only minor fluorescence changes in pyrene-labelled tropomyosin, which agrees with the findings reported for the equivalent chicken gizzard sequence, 658C [10]. The fragment H7, representing the N-terminus and middle of domain 4, induced a fluorescence enhancement of up to 15%,

whereas a minor fluorescence change of up to 10% was observed with fragments H2, H4 and H5.

Domain 3 of caldesmon contains a sequence similar to troponin T (T1 fragment) at residues 564–621 [22] (508–565 in the chicken gizzard sequence [20]), which was predicted to bind tropomyosin [18]. H6 contains this sequence but induced only a minor fluorescence change in pyrene-labelled smooth-muscle tropomyosin (Figure 8a). In contrast, when we tested the binding of H1 and H6 to pyrene-labelled skeletal-muscle tropomyosin, both fragments enhanced pyrene fluorescence by up to 75% and with similar apparent affinities (Figure 8b).

DISCUSSION

Caldesmon binds to tropomyosin and myosin. Our recent work indicates that both proteins bind to multiple sites in the extended caldesmon molecule. Tropomyosin has been found to bind in domains 1, 2 and 4 of caldesmon, whereas myosin-binding sites are located in domains 1 and 3/4 [10,27]. In this report we have studied the sites for myosin and for tropomyosin binding within the C-terminus of smooth-muscle caldesmon by using a series of recombinant fragments. These were derived from the human caldesmon domain 3/4 fragment, H1 (residues 506–793 [22], equivalent to 476–737 in the chicken gizzard sequence [20]), described by Huber et al. [27]. The fragments cover the whole of domains 3 and 4 with many of the fragments overlapping (Figure 1). Caldesmon fragments produced by bacterial expression have a high probability of taking up the natural folding and characteristics of the equivalent sequence in the full-length protein. This has been shown in functional and binding studies in our and other laboratories [7,10,19,26,27,53].

Myosin binding

The caldesmon fragment H1, representing domains 3 and 4, bound to myosin and myosin-rod filaments, confirming previous measurements [26,27]. We were able to locate the main interaction site in the domain 3/4 junction and in domain 4a (residues 622–714 human [22], 566–657 chicken gizzard [20]) on the basis of significant binding of H7 and 606C, and the lack of binding by fragments H8, H9 and 658C (see Figure 4 and Redwood and Marston [10]). All these fragments seem to be structurally equivalent to the respective sequences in the full-length caldesmon molecule, on the basis of their ability to bind actin and calmodulin and to show tropomyosin-dependent inhibition of the myosin ATPase (see Figure 1) [13,54]. The interaction of myosin with H1 subfragments is, however, weaker than with H1 and thus seems to depend not only on the location but also on the extension of the represented sequence. This indicates that a full myosin interaction in the C-terminus of caldesmon requires a long, uninterrupted sequence. This is further confirmed by our observation that minor modification within H1 can affect myosin binding without having an effect on actin and calmodulin interactions. Labelling of cysteine 636 (580 in the chicken gizzard sequence [20]) with iodoacetamide or *N*-hydroxysuccinimide abolishes the H1–myosin interaction (results not shown). Similarly, Yamakita et al. [26] reported that a 38 kDa C-terminal recombinant fragment lost its affinity for myosin on mitosis-specific phosphorylation by cdc2 kinase, and Katayama [56] reported that proteolysis of caldesmon by α -chymotrypsin impairs or abolishes myosin binding. Because the N-terminal myosin-binding site of caldesmon is also believed to involve an extended stretch of the sequence, as reported by Velaz et al. [23], we conclude that the interaction of caldesmon and myosin requires a number of contacts distributed over an extended region of both molecules.

Figure 9 Location of caldesmon–tropomyosin and caldesmon–myosin binding sites

The α -helices that make up domains 1, 2 and 3 of caldesmon are represented by bars, and the C-terminal domain 4 is outlined as a folded loop structure (see [4,12] for justification). Tropomyosin is shown bound antiparallel to caldesmon; the numbers denote residues in the sequence. The location and length of the sequences coded for by exon 2 (residues 39–80) and exon 6 (residues 189–213) are indicated [68]. One caldesmon extends over parts of three tropomyosin molecules, similar to those formed by Ca_h and Ca_l (the non-muscle caldesmon isoform, of low molecular mass) [46]. H1 is, however, unable to cross-link actin and myosin filaments as Ca_h and Ca_l do, suggesting that the sites for actin and myosin in H1 cannot be occupied simultaneously.

Caldesmon is able to cross-link actin and myosin filaments. This is probably due to the C-terminal actin and the N-terminal myosin-binding sites in caldesmon [46,57]. We have observed by electron microscopy that H1 forms whiskers when bound to smooth-muscle myosin-rod filaments, similar to those formed by Ca_h and Ca_l (the non-muscle caldesmon isoform, of low molecular mass) [46]. H1 is, however, unable to cross-link actin and myosin filaments as Ca_h and Ca_l do, suggesting that the sites for actin and myosin in H1 cannot be occupied simultaneously.

The binding of H1 to myosin is readily disrupted by Ca^{2+} -calmodulin (see Figure 6 and [42]). Calmodulin is also an accepted Ca^{2+} -dependent competitor for caldesmon–actin interaction [1,4,6]. Several discrete Ca^{2+} -calmodulin binding sites, centred on the regions around tryptophans 716, 749 and 779 in domain 4 of caldesmon, have been described [13,58,59]. The tryptophans are not located in the proposed myosin-binding sequence, but it has been shown in cross-linking experiments by Wang [60] and with NMR by Mornet et al. [12] that Cys-636, which is involved in myosin binding, is physically close to these tryptophans. If caldesmon domain 4 is folded, as proposed by Mornet et al. [12] and outlined in Figure 9, the interference of Ca^{2+} -calmodulin and myosin in their interaction with caldesmon becomes obvious. In addition, Hemric et al. [42] reported that Ca^{2+} -calmodulin also interferes with the N-terminal binding site, but only in a complex where the Ca^{2+} -calmodulin binding sites are linked with the N-terminus of caldesmon, i.e. in unpurified digested caldesmon but not in the purified myosin-binding fragment. This result suggests some linkage of the Ca^{2+} -calmodulin-induced conformation of the C-terminus of caldesmon to the conformation of its N-terminus. This is possible in the structural model of the thin filament where N- and C-termini are close and may even overlap. Such an interaction has been suggested by Tsurada et al. [61].

The site on myosin for the binding of caldesmon has been ascribed to the S2 region [41], but it has not been possible to isolate an S2 fragment from smooth-muscle myosin that binds caldesmon. We have observed that chymotryptically produced HMM from chicken gizzard myosin binds to caldesmon, whereas chymotryptically produced HMM from sheep aorta myosin often does not. A difference in the sequence in the light-meromyosin/S2 junction of the two myosin types is probably responsible for their difference in the chymotryptic cleavage sites and may account for the difference in their caldesmon interaction [62,63]. This suggests that the light-meromyosin/S2 hinge region is an important site. H1 interacts more weakly with myosin rod

than does the full-length caldesmon molecule or the N128 fragment. The H1–myosin binding site might therefore be extended beyond the head/rod junction into S1, whereas the N-terminal site seems exclusively within S2 (see Figure 9). This would be compatible with reported weak binding of smooth S1 to whole caldesmon [64].

The binding of the C-terminus of caldesmon to myosin complements the N-terminal site and has been suggested in structural studies of the interaction of smooth-muscle thin filaments with myosin heavy meromyosin by Marston [40] and more recently by Katayama et al. [57]. Myosin binding gives caldesmon the ability to cross-link thick and thin filaments. This might be important in the regulation of myosin–actin interaction and the C-terminal site could render a Ca^{2+} dependency to this function.

Tropomyosin binding

In addition to sites in domains 1 and 2, tropomyosin binds to the C-terminus of caldesmon in domain 4a and in particular to the sequence of residues 663–714 (equivalent to 606–657 in chicken gizzard). This is based on the high affinity of 606C, the binding of H7 and the lack of binding of H8 and H9. It is interesting to note that all these fragments are potent and reversible inhibitors of the actin–tropomyosin-activated myosin ATPase [54]. The binding is contained in the H7 fragment and partly in H4 and H2 (Figures 1 and 7). The binding of tropomyosin to domain 4a has previously been suggested by Redwood and Marston [10] and by Hayashi et al. [15], where two recombinant fragments, 513–734 (483–677) and 692–793 (635–756) retained their binding to tropomyosin, implying the involvement of the overlapping sequence.

A comparison of the sequences of caldesmon with skeletal-muscle troponin T suggests a more discrete site, as pointed out by Hayashi et al. [18]. Domain 4a of caldesmon contains a sequence, residues 665–679 (608–622), with a high degree (50%) of identity to a region in troponin T, residues 244–257, contained in the cyanogen bromide fragment T2 (residues 159–259 of troponin T) [65]. The T2 fragment interacts with tropomyosin in the region of Cys-190 of tropomyosin, and this interaction is Ca^{2+} -sensitive [65–67]. The same region in tropomyosin has been proposed as a strong binding site for the C-terminus of caldesmon [28,61]. Exon 6 of the tropomyosin gene encodes the region around Cys-190 (residues 189–213). Skeletal and smooth muscles have an alternatively spliced exon 6 in both α and β tropomyosin

genes but retain an identity of 36–44% [68]. Colote et al. [69] analysed the nucleotide sequences of exons 6 of skeletal- and smooth-muscle tropomyosin and suggested that the unexpectedly small divergence of the sequences could indicate that interacting proteins in skeletal and smooth muscles have exerted selection pressure on these sequences. They consequently postulated the existence of a protein in smooth muscle, presumably caldesmon, that may be the counterpart of skeletal-muscle troponin. In support of this idea, Hnath and Phillips [29] have recently demonstrated a strong binding site between caldesmon and tropomyosin in tropomyosin–caldesmon co-crystals, which is at the same place on tropomyosin as the major troponin T binding site.

A longer sequence similarity between caldesmon and troponin T in domain 3 of caldesmon shown by Hayashi et al. [18,19] and Bryan et al. [20] has provoked predictions for tropomyosin binding in this part of caldesmon. The caldesmon sequence, 564–621 (508–565 in chicken gizzard [20]) has 43% identical residues plus an even more marked conservation of the pattern of acidic and basic amino acids when compared with residues 89–146 of skeletal-muscle troponin T. This sequence is contained in a cyanogen bromide-cleaved fragment CB2 (residues 71–151 of troponin T), described by Pearlstone and Smillie [66], that binds to the end-to-end overlapping region of tropomyosin polymers in a Ca^{2+} -independent manner. Surprisingly, we found only a very weak interaction between smooth-muscle tropomyosin and caldesmon's domain 3 (e.g. H6) containing the sequence similar to CB2. This lack of interaction seems to be caused by isoform diversity in the tropomyosin. The region of skeletal tropomyosin involved in the interaction with the CB2 fragment of troponin T probably includes the sequence, residues 39–80, encoded by the exon 2 of the tropomyosin gene. This exon is alternatively spliced (2a or 2b) in α -tropomyosin such that there is only 33% identity retained between the sequences encoded in smooth muscle by exon 2a and in skeletal muscle by exon 2b [68]. When we examined the binding of H6 to skeletal muscle tropomyosin we found a high affinity (Figure 8b). This is consistent with the finding of Pearlstone and Smillie [65], who showed that the CB1 fragment, residues 1–151 of skeletal troponin T, thus containing the CB2 fragment, binds to skeletal-muscle but not to smooth-muscle tropomyosin.

The presence of a sequence with similarity to a skeletal-muscle tropomyosin-binding protein and affinity for skeletal-muscle tropomyosin in domain 3 of smooth-muscle caldesmon thus seems to be a curious coincidence. However, the domain 3 sequence is common to smooth-muscle and non-muscle caldesmon and it is noted that exon 2b of α -tropomyosin is expressed in skeletal and non-muscle tropomyosin molecules (see review in [68]). This suggests that the potential tropomyosin binding site in domain 3 could be important for the non-muscle isoform of caldesmon.

The tropomyosin-binding sites in the C-terminus of caldesmon are clearly discrete but extended sites, and the other sites in domain 1 and 2 could be similar. Corresponding discrete sites on tropomyosin have been identified by Watson et al. [61], and the two sets of sites may be incorporated into a self-consistent structural model, as outlined in Figure 9. This model is similar to models recently proposed by Watson and co-workers [61] and by Hnath et al. [29]. Chalovich and colleagues [70,71] have highlighted a potential 'parking problem' for caldesmon binding to the smooth-muscle thin filament. One caldesmon is bound at a regular spacing of 1 in every 14 actins in thin filaments. Yet every actin is seen as identical when caldesmon binds to actin. The presence of additional discrete tropomyosin-binding sites reduces the problem considerably because one caldesmon mol-

ecule tries to bind to every two tropomyosin molecules and this may explain why the fitted binding parameters derived by Chalovich et al. indicate that there is no parking problem in practice.

In conclusion, we have located the sites in the C-terminus of caldesmon that interact with smooth-muscle myosin and tropomyosin. Tropomyosin and myosin interact in the same region, or at least in overlapping regions, of caldesmon, and these interactions are highly dependent on salt concentration. We note that the regions on myosin and tropomyosin involved in caldesmon interaction both have coiled-coil structures that may be responsible for the similarity of the location and properties of the interaction with caldesmon. We have found that smooth-muscle tropomyosin is able to compete with smooth-muscle myosin for the binding to caldesmon at higher concentrations (results not shown), whereas Ca^{2+} -calmodulin weakens the caldesmon–myosin interaction (Figure 6 and [42]) and the caldesmon–tropomyosin interaction [72]. This interference is not due to an overlap of binding sites between Ca^{2+} -calmodulin and myosin or tropomyosin but may be explained by a folded structure of the C-terminus of caldesmon, thus reducing the distances of remote sites in the primary structure (see Figure 9). Consequently tropomyosin, myosin, actin and Ca^{2+} -calmodulin binding sites in domain 4 seem to be physically very close to each other. It will be the task of future studies to determine the involvement of these multiple interactions in the function of caldesmon within smooth-muscle cells.

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