Location and functional characterization of myosin contact sites in smooth-muscle caldesmon

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Caldesmon interaction with smooth muscle myosin and its ability to cross-link actin filaments to myosin were investigated by the use of several bacterially expressed myosin-binding fragments of caldesmon. We have confirmed the presence of two functionally different myosin-binding sites located in domains 1 and 3/4a of caldesmon. The binding of the C-terminal site is highly sensitive to ionic strength and hardly participates in acto-myosin cross-linking, while the N-terminal binding site is relatively independent of ionic strength and apparently contains two separate myosin contact regions within residues 1–28 and 29–128 of chicken gizzard caldesmon. Both these N-terminal sub-sites are involved in the interaction with myosin and are predominantly responsible for the caldesmon-mediated high-affinity cross-linking of actin and myosin filaments, without affecting the affinity of direct acto-myosin interaction. Binding of caldesmon and its fragments to myosin or rod filaments revealed affinity in the micromolar

range. We determined various stoichiometries at maximal binding, which depended on the ionic strength and the concentration of Mg^{2+} ions. At 30 mM NaCl and 1 mM Mg^{2+} the maximum stoichiometry was 4 moles of caldesmon (or caldesmon fragment) per mole of myosin. At 130 mM NaCl/1 mM Mg^{2+} , or at 30 mM NaCl/5 mM Mg^{2+} it decreased to about two caldesmon molecules bound per myosin, while remaining 4:1 for individual caldesmon fragments, suggesting that all binding sequences on myosin were still fully capable of interaction. A further increase in the Mg^{2+} concentration led to a substantial decrease in both the affinity and maximum stoichiometry of caldesmon and the fragments binding to myosin. We suggest that caldesmon-myosin interaction varies according to the conformation of caldesmon in solution, that caldesmon-binding sites on myosin are not well defined and that their accessibility is determined by spatial organization and is blocked by divalent cations like Mg^{2+} .

INTRODUCTION

Smooth muscle caldesmon is associated with the thin filaments within the ‘contractile domain’ of smooth muscle cells [1,2]. It is a potent inhibitor of myosin ATPase activation by actin-tropomyosin [3–5]. This inhibition is regulated by Ca^{2+} -calmodulin, or another Ca^{2+} -binding protein, which renders smooth muscle thin filaments sensitive to Ca^{2+} [6–8].

A four-domain model, based on primary structure and functional analysis, has been proposed for caldesmon [9]. The association of caldesmon with the thin filaments is mainly due to strong interaction of the C-terminal domain 4 of caldesmon with actin. Domains 1–3 do not interact, or interact very weakly, with actin [10–12]. The extended thin-filament association of caldesmon may therefore be due to the interaction of caldesmon with tropomyosin, since binding sites for smooth muscle tropomyosin have been found in domains 1, 2 and 4 [13,14].

Caldesmon interacts with smooth muscle myosin [15,16] and two binding sites have been identified, one in either terminus of caldesmon [17–20]. Interaction of the C-terminal site is greatly diminished at high ionic strength [19] and its affinity for myosin may be decreased by phosphorylation [20]. The other N-terminal interaction site in caldesmon domain 1 is relatively insensitive to ionic strength and does not depend on the state of myosin light-chain phosphorylation [21–24]. Its appreciable affinity for myosin at physiological ionic strength and its low affinity for tropomyosin suggest a high probability that the N-terminus of caldesmon may project from thin filaments and bind myosin *in vivo*. Direct

electron microscopy of isolated native thin filaments showed caldesmon in an aligned position along the filament shaft [11,25]. Studies *in vitro*, however, failed to demonstrate association of the N-terminus of caldesmon with the actin filaments [26–28].

Studies *in vitro* and *in situ* showed the ability of caldesmon to interact simultaneously with actin and myosin. Initially, caldesmon was suggested to directly affect the affinity of smooth muscle heavy meromyosin (HMM), complexed with nucleotide, for actin, because it enhanced the binding of this myosin fragment to actin [15]. Cross-linking of HMM to native thin filaments [29] and of the myosin rod to reconstituted actin filaments [23] was later observed, and caldesmon was determined to be the protein responsible. Motility data *in vitro* have indirectly supported a role of caldesmon in ‘tethering’ actin filaments to myosin [30–32]. Additionally, electron microscopy images of myosin rod filaments decorated with caldesmon showed their ‘frayed’ filament structure, and the presence of projecting ‘whiskers’ was attributed to caldesmon molecules [23]. Caldesmon was also reported to enhance myosin binding to actin in the cytoskeleton during platelet activation, and an N-terminal caldesmon fragment dissociated this complex [33]. The consequence of the simultaneous interaction of the C-terminus of caldesmon with actin and the N-terminus of caldesmon with myosin would be ‘tethering’ of actin filaments to myosin. If ‘tethering’ does occur *in vivo*, it might result either in enhanced force production when caldesmon inhibition of actomyosin ATPase is released by a Ca^{2+} -binding protein at increased intracellular Ca^{2+} , or passive force maintenance at low Ca^{2+} levels provided that caldesmon-

Abbreviations used: HMM, heavy meromyosin; SP.HMM, heavy meromyosin, thiophosphorylated by myosin light-chain kinase.

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mediated cross-bridges are load-bearing. The tethering by caldesmon may also play a role in the organization of actin and myosin filaments in smooth muscle cells.

The objectives of this study were: (1) to define the myosin-binding sites on caldesmon; (2) to characterize caldesmon interaction with smooth muscle myosin; and (3) to assess the role of individual myosin-binding sequences of caldesmon in its ability to cross-link actin filaments to myosin. We have produced mutant myosin-binding fragments of caldesmon that compete with caldesmon for interaction with myosin, and have obtained evidence that caldesmon directly mediates actin–myosin cross-linking. We show that the N-terminal myosin-binding site of caldesmon consists of at least two myosin contact regions which are solely responsible for the myosin binding in the actin–myosin cross-linking. A model of caldesmon interaction with smooth muscle myosin is suggested and discussed.

METHODS

Construction and expression of caldesmon mutants

DNA fragments encoding both N-terminal and C-terminal caldesmon mutants were obtained by PCR amplification using previously described human or chicken caldesmon constructs [13,19] as templates. Appropriate primer pairs were used to introduce start and stop codons and restriction enzyme sites. PCR products were then digested to create cohesive ends and ligated into the expression vector pMW172. The fragments, subcloned using the *NcoI* restriction site at the 5'-end (N128, N152 and N198), have the additional N-terminal tripeptide Met–Gly–Ser, where the methionine may be post-transcriptionally cleaved off. All the constructs were verified by double-stranded sequencing. The constructs were used to transform Ca^{2+} -competent BL21 (DE3) cells and the overexpressed caldesmon fragments were purified as described previously [13,14].

Protein preparations

Caldesmon was purified from chicken gizzards by the heat-treatment procedure of Bretscher [34]. Crude chicken gizzard myosin was isolated as described by Sellers et al. [35] and purified on a Sepharose CL-4B gel-filtration column (90 cm $\times$ 5 cm). For some binding experiments the crude myosin was used. Smooth muscle HMM was obtained by chymotryptic digestion of crude myosin, which was pre-phosphorylated with 2 $\mu\text{g}/\text{ml}$ of purified smooth muscle myosin light-chain kinase (a gift from Dr. J. R. Sellers, NHLBI, National Institutes of Health, Bethesda, MD, U.S.A.) in the presence of Ca^{2+} /calmodulin and adenosine 5'-[γ -thio]triphosphate to produce thiophosphorylated HMM [35]. Final purification of HMM from the kinase and associated phosphatase was achieved by gel-filtration on a Sephacryl S-300 column (90 cm $\times$ 5 cm) [36]. Smooth muscle rod was obtained by papain digestion of myosin [23]. Skeletal muscle actin was prepared according to the method of Straub [37] and skeletal muscle HMM as reported by Weeds and Taylor [38]. Protein concentrations were determined using the following molar absorption coefficients and molecular masses respectively: caldesmon, $A^{0.1\%}_{280} = 0.303$ [22], 89 kDa; G-actin, $A^{0.1\%}_{290} = 0.63$, 42 kDa; smooth muscle myosin, $A^{0.1\%}_{280} = 0.56$, 540 kDa [35]; HMM, $A^{0.1\%}_{280} = 0.65$ [36], 330 kDa. The concentration of actin and caldesmon fragments was determined by the method of Lowry et al. [39] using actin, determined by the Kjeldahl method, and chicken gizzard caldesmon respectively as standards.

Binding assays

The binding of caldesmon or its fragments to filamentous smooth muscle myosin, and of HMM to actin filaments was measured in sedimentation assays as previously described [40]. Proteins were dialysed into 20 mM Mops, pH 7.0, 1 mM MgCl_2 , 1 mM EGTA, 1 mM dithiothreitol and 30 or 50 mM NaCl. Caldesmon, its fragments and HMM were pre-spun separately immediately before the assays. Samples (50 μl) usually contained 1 μM HMM and 3–4 mM Mg-ATP, or 1–2 μM myosin without Mg-ATP, and various concentrations of other proteins as described in the Figure legends. Assays for actin–myosin cross-linking were started by the addition of Mg-ATP and conducted at room temperature. Samples were centrifuged for 20 min at 120 000 g and care was taken to ensure that not more than 30–40 % of total ATP was consumed. HMM binding was measured as previously described [29,41] as a ratio of the ammonium-EDTA ATPase activity of HMM not sedimented in the presence of actin to that in the absence of actin. To confirm the ATPase data, the composition of pellets was independently assessed by SDS/PAGE. To determine myosin binding, equal aliquots of the supernatants and pellets, after 15 min sedimentation at 100 000 g , were subjected to SDS/PAGE and the amount of bound caldesmon or caldesmon fragments was quantified by densitometry.

SDS/PAGE analysis [42] was performed using either 8–18 % ExcelGels (Pharmacia) or gels containing a gradient of 9–15 % acrylamide, methylenebisacrylamide (0.09–0.4 %) and urea (4.25–7.5 M) to improve separation of myosin (HMM) heavy chains from caldesmon. The protein bands were quantified by densitometry (Scanmaster; Howtek Inc., using 'pdi Quantity one' software, or a HP Scan III scanner, using 'NIH Image' software).

Determination of ATPase activity

Actin-activated SP.HMM (SP.HMM is HMM, thiophosphorylated by myosin light-chain kinase) ATPase was determined at 30 °C in 60 μl aliquots containing 1 μM SP.HMM and 5 μM actin in the buffer used for binding experiments containing 30 mM NaCl. Reactions were started by addition of up to 5 mM Mg-ATP and stopped with 0.5 ml of 10 % trichloroacetic acid. To measure ammonium-EDTA ATPase activity, aliquots of supernatants (25 μl) after HMM sedimentation were transferred to 0.5 ml of 0.3 mg/ml BSA/0.4 M NH_4Cl /20 mM EDTA/25 mM Tris/HCl/5 mM ATP/0.5 mM dithiothreitol, pH 8.0, and incubated for 15 min at 25 °C. After the reactions were terminated as above, the P_i released was measured by the method of Taussky and Schorr [43].

RESULTS

Site-directed mutagenesis has been used to produce three new N-terminal fragments (N152, N198, 29N152) and one C-terminal fragment (H12) of caldesmon (Figure 1). The fragments N128 and H1, described previously [13,19] were used in this study for comparison.

Characterization of myosin-binding fragments of caldesmon

Filamentous smooth muscle myosin was titrated with caldesmon and the three N-terminal fragments both at low (30 mM NaCl) and high (130 mM NaCl) ionic strength. The two sets of binding

Figure 1 Position of myosin-binding fragments in caldesmon structure

The top structure shows the four-domain model of caldesmon as proposed by Marston and Redwood [9]. Below are the fragments derived either from a chicken gizzard caldesmon clone (N-terminal, [44]) or from a human liver caldesmon clone (C-terminal [19]). The termini of the fragments are numbered according to the gizzard caldesmon sequence [44]. The approximate locations of myosin-, actin- and calmodulin-binding sites are indicated by arrows.

curves thus obtained (Figures 2A and 2B) show that all the fragments contain a myosin-binding sequence and have affinities for myosin in the range 10^5 – 10^6 M $^{-1}$ (see also Table 1). Their interaction with myosin was affected by ionic strength. The maximum binding of the fragments and intact caldesmon was determined to be 4 mol/mol of myosin at low ionic strength, which agrees with our previous measurements for intact caldesmon [23].

Figure 3 shows the influence of salt on myosin binding. An increase in ionic strength (NaCl) slightly changed the amount of the N-terminal fragments bound to myosin at fixed protein ratios and significantly decreased the binding of intact caldesmon. The stoichiometry at maximal binding of the fragments was unchanged by ionic strength, whereas the decrease in the affinity of caldesmon was associated with a decrease in its binding stoichiometry (compare Figure 3A with Figure 2 and Table 1). In contrast, we observed that the decrease in binding of caldesmon, as well as of all the caldesmon fragments, at increasing Mg $^{2+}$ concentration was associated with lowering of binding stoichiometry rather than with lowering of binding affinity (Figure 3B). Thus in the presence of 5 mM MgCl $_2$ and 30 mM NaCl only 2 moles of caldesmon or caldesmon fragment were maximally bound per mole of myosin (Figure 3B). We determined the dissociation constant of caldesmon from myosin under these conditions to be 3.9 μ M, which is thus unchanged from that at lower Mg $^{2+}$ concentrations. The affinity of the myosin–caldesmon or myosin–caldesmon fragment complex is however affected (in addition to the stoichiometry) when MgCl $_2$ is further increased to > 8 mM.

Table 1 Dissociation constants and maximum binding stoichiometry of caldesmon and its N-terminal fragments to smooth muscle myosin and the myosin rod

The values are calculated as means $\pm$ S.D. from the results of two experiments represented in Figure 2(A). Assays for evaluation of binding to rod filaments (1 μ M) were performed and quantified essentially as those for evaluation of binding to myosin. ND, not determined.

	Binding to myosin filaments		Binding to rod filaments	
	$B_{\max}$ (mol/mol)	K_d (μ M)	$B_{\max}$ (mol/mol)	K_d (μ M)
	30 mM NaCl		30 mM NaCl	
Caldesmon	4.00 ± 0.12	3.83 ± 0.33	ND	ND
29N152	4.08 ± 0.08	5.95 ± 0.41	4.0 ± 0.6	35.9 ± 9.6
N152	4.01 ± 0.05	8.46 ± 0.32	ND	ND
N198	3.49 ± 0.20	11.35 ± 1.51	ND	ND
	130 mM NaCl		100 mM NaCl	
Caldesmon	2.42 ± 0.67	14.65 ± 4.30	1.8 ± 0.2	9.4 ± 1.7
29N152	3.88 ± 0.18	13.87 ± 1.69	ND	ND
N152	4.28 ± 0.43	22.12 ± 4.50	3.9 ± 0.3	20.2 ± 2.8
N198	5.54 ± 0.51	48.66 ± 6.39	ND	ND

The caldesmon-binding site(s) have been located in the subfragment-2 portion of smooth muscle myosin [16,23]. This sequence is contained in the rod fragment of myosin, which we

Figure 2 Interaction of caldesmon and its N-terminal fragments with smooth muscle myosin

Caldesmon (■), N152 (●), 29N152 (△), and N198 (◇) were assayed for the binding with 1 μ M myosin in 10 mM Mops, pH 7.0, 1 mM MgCl $_2$, 1 mM EGTA, 1 mM dithiothreitol, and either 30 mM NaCl (A) or 130 mM NaCl (B). Representative curves are shown. The lines are fits of the data points to a simple binding equation. Arrows indicate the caldesmon (or caldesmon fragment) concentrations chosen for experiments described in Figure 3.

Figure 3 Effect of ionic strength (A) and Mg^{2+} (B) on the interaction of caldesmon and its N-terminal fragments with smooth muscle myosin

The molar ratios of caldesmon (or caldesmon fragment) to myosin ($1 \mu\text{M}$) in the assays were: 30 for 29N152 (Δ), 25 for N152 ($\circ$), 16 for N198 ($\diamond$), and 12 for caldesmon ($\blacksquare$). Concentrations of NaCl and Mg^{2+} were increased separately in each assay after mixing the proteins at 30 mM NaCl and 1 mM Mg^{2+} .

Figure 4 Dissociation of the caldesmon–myosin complex by myosin-binding caldesmon fragments

The binding of $3 \mu\text{M}$ caldesmon to $1.3 \mu\text{M}$ smooth muscle myosin in the presence of different concentrations of N152 ($\circ$, $\bullet$), 29N152 (Δ), and H12 ($+$, $\boxplus$) was quantified as described in the Materials and methods section. The assays were carried out either at 50 mM NaCl ($\bullet$, Δ , $\boxplus$), or at 130 mM NaCl ($\circ$ and $+$). For the N-terminal fragments mean values $\pm$ S.D. ($n = 3$) are shown.

additionally used in binding experiments. As demonstrated in Table 1, the affinities of caldesmon and its fragments for binding to the myosin rod were close to those of their interaction with myosin at high ionic strength. The affinity of 29N152 for the myosin rod was 7-fold lower than that for myosin at low ionic strength.

We attempted to characterize further the location of the C-terminal myosin-binding site of caldesmon using H12, a fragment of human caldesmon, corresponding to residues 476–663 (complete domains 3/4a) of chicken gizzard caldesmon [44]. H12 bound to myosin in a very similar manner to H1, a previously described C-terminal caldesmon fragment (amino acids 476–737) [14,19], indicating that residues 664–737 (domain 4b) are not involved in binding to myosin. This interaction, although difficult to quantify precisely in our assay because of the tendency of H12 to aggregate and sediment by itself, was highly salt-dependent. We estimated that up to 3–4 moles of H12 could be bound to

Figure 5 Effect of caldesmon on the binding of smooth muscle (A) and skeletal muscle (B) HMM to actin

Actin filaments (open symbols) or actin filaments reconstituted with caldesmon (closed symbols) were assayed for the binding with $1 \mu\text{M}$ of smooth muscle SP.HMM (circles) and $1 \mu\text{M}$ unphosphorylated HMM (squares) in the presence of 3.3 mM Mg-ATP. The caldesmon/actin molar ratio was 1:8. Representative curves are shown.

1 mole of myosin at low ionic strength (10–30 mM NaCl; results not shown).

Dissociation of the caldesmon–myosin complex by myosin-binding fragments of caldesmon

Competition assays were performed in order to investigate the mode of caldesmon–myosin interaction. The different sensitivity of the C- and N-terminal myosin-binding sites of caldesmon to ionic strength was used to discriminate their contribution to the interaction. Figure 4 shows that the two fragments containing the N- or C-terminal myosin-binding sites, N152 and H12 respectively, were able to dissociate caldesmon from its complex with myosin at low ionic strength. At 130 mM NaCl, only N152 was an effective competitor, while H12 did not dissociate caldesmon from myosin. These results indicate that at low ionic strength both sites of caldesmon can be involved in binding to

Figure 6 The tight binding of smooth muscle HMM to actin is mediated by caldesmon

SP.HMM ($1 \mu\text{M}$; ●) or HMM ($1 \mu\text{M}$; ○) was titrated with caldesmon, and $1 \mu\text{M}$ SP.HMM was titrated with the C-terminal caldesmon fragment H1 (▲) in the presence of 3.3 mM Mg-ATP. The binding of the complexes formed was compared with the inhibition of acto-SP.HMM Mg-ATPase inhibition by caldesmon (◇). Conditions are the same as in Figure 2, except for the HMM binding curve which was obtained at 50 mM NaCl. The uninhibited ATPase rate was 0.66 s^{-1} .

myosin and engage the same sites on myosin, while under physiological ionic conditions only the N-terminal binding site of caldesmon is likely to mediate interaction with myosin.

The extreme N-terminal sequence of caldesmon seems to play a significant role in interaction with myosin. 29N152, lacking the first 28 residues, was much less potent in competing with caldesmon for myosin binding when compared with N152 (Figure 4). The potent displacement of caldesmon from myosin by N152 was additionally accompanied by a parallel increase in bound fraction of the fragment. No binding of 29N152 to the caldesmon–myosin complex was detected in this competition assay (results not shown). This indicates that although these individual fragments had similar affinities for myosin (Table 1), the first 28 residues of caldesmon contribute to the interaction.

Caldesmon-mediated cross-linking of smooth muscle HMM to actin

Actin filaments or actin filaments reconstituted with caldesmon were mixed with the soluble myosin fragment, HMM, in the presence of an excess of ATP, and the fraction of unbound HMM was quantified by measuring ammonium-EDTA ATPase activity in the supernatant after rapid sedimentation of actin. The amount of direct binding of HMM–ADP– P_i to actin under these conditions depends on whether the light chains of HMM are phosphorylated [41]. Usually 5–10% of unphosphorylated HMM and 25–40% of SP.HMM were sedimented with pure actin in the presence of ATP (see Figure 5). However, when caldesmon was present on actin filaments, a much higher fraction of the myosin fragment was pelleted with actin (Figure 5). This caldesmon-mediated tight-binding of HMM–ADP– P_i to actin filaments was independent of the state of HMM phosphorylation (Figure 5; [23]) and ionic strength (results not shown), but was strictly specific for the type of myosin. When skeletal muscle HMM–ADP– P_i was used instead of smooth muscle HMM, no enhancement of its binding to actin was detected in the presence of caldesmon (results not shown).

To quantify this ‘tethering’ we have directly measured the interaction of $1 \mu\text{M}$ HMM with $10 \mu\text{M}$ actin in the presence of ATP as a function of increasing caldesmon concentration. Two representative curves, shown in Figure 6, demonstrate that caldesmon enhances the amount of both unphosphorylated and phosphorylated HMM bound to actin in a dose-dependent manner. Similar results were obtained under various ionic conditions, including near physiological values of 130 mM NaCl (results not shown). In the absence of tropomyosin, caldesmon was shown to sterically inhibit the activation of HMM ATPase by actin, thereby providing a linear relation between the extent of inhibition and the amount of caldesmon bound to actin [4,45]. Therefore we assessed the fraction of actin-bound caldesmon by measuring the inhibitory activity of caldesmon upon acto-HMM ATPase (Figure 6). The fraction of actin-associated HMM–ADP– P_i was simultaneously determined in the same assay (Figure 6). Maximum binding of caldesmon to actin (1 molecule per 2–3 actins) and full inhibition of ATPase were associated with the maximum amount of cross-linked HMM. Since the apparent affinity of the tight binding seemed to be

Figure 7 Reversal of caldesmon-mediated acto-SP.HMM (A) and acto-HMM (B) cross-linking by myosin-binding fragments of caldesmon

(A) SP.HMM ($1 \mu\text{M}$) or (B) HMM ($1 \mu\text{M}$) was assayed for cross-linking with $10 \mu\text{M}$ actin in buffer, as in Figure 3, in the presence of 3.3 mM Mg-ATP and either 30 mM NaCl and $2 \mu\text{M}$ caldesmon (A), or 50 mM NaCl and $4 \mu\text{M}$ caldesmon (B). The values of the weak binding (in the absence of caldesmon and caldesmon fragments) are indicated by horizontal arrows. Only N-terminal fragments 29N152 (▲), N152 (●), N198 (◇), and N128 (◻) were used. In (A) representative curves are shown, in (B) the data are the means $\pm$ S.D. of three separate experiments.

extremely high, it allowed reasonable fitting of this data to a simple binding equation. The calculated value of the (actin–caldesmon)–HMM dissociation constant was about $1\ \mu\text{M}$, and the net value of HMM that was maximally cross-linked was about 80 %, independently of its state of phosphorylation.

In order to test whether the C-terminal myosin-binding site of caldesmon was contributing to this high affinity we titrated the actin–HMM–ADP– P_i mixture with H1, the C-terminal caldesmon fragment containing all actin-binding sites but only the C-terminal myosin-binding site of the parent protein. Although the assay was carried out at 10 mM NaCl, the ionic strength most favourable for H1–myosin binding [14], we have detected no H1-induced actin–SP.HMM cross-linking, although we did observe the displacement of SP.HMM–ADP– P_i weakly bound to actin (Figure 6). The dissociation of this myosin fragment from actin by H1 is likely to be due to the steric blocking of myosin-binding sites on actin by saturating amounts of bound H1. These results and the similar tight binding obtained at low and high ionic strength clearly demonstrate that only the N-terminal myosin-binding site of caldesmon is likely to be involved in HMM binding and ‘tethering’ to actin.

Reversal of caldesmon-induced actin–HMM cross-linking

To obtain evidence for caldesmon being a physiological actin–myosin cross-linker, the full set of caldesmon N-terminal fragments (see Figure 1) was used to dissociate the ternary actin–caldesmon–SP.HMM–ADP– P_i (Figure 7A) and actin–caldesmon–HMM–ADP– P_i (Figure 7B) complexes. In both cases the fragments competed with caldesmon for HMM binding similarly to the competition for myosin binding (Figure 4). Although the fraction of dissociated SP.HMM was quite low and comparable with the value of weak actin–SP.HMM–ADP– P_i interaction, the tightly bound unphosphorylated HMM was almost totally displaced by N152 and N198. The truncated fragment 29N152 had little reversal effect, which is in good agreement with the myosin-binding results (Figure 4).

DISCUSSION

Location of myosin-binding sites in caldesmon

Using both proteolysis and bacterial expression techniques two myosin-binding regions in domains 1 and 3/4a of caldesmon have previously been determined [14,16,19–22,24,27]. In order to gain detailed information on the size and location of these myosin-binding sites we constructed three N-terminal and one C-terminal caldesmon fragment (Figure 1).

All the fragments with an intact N-terminus were very similar in their binding to myosin (Figure 2) and in competition with caldesmon for interaction with myosin or HMM–ADP– P_i (Figures 4 and 7). These and previous studies [13,14,22,27] suggest that the C-terminal boundary of this myosin-binding site is located before Cys-153, probably around residue 128 of caldesmon.

In contrast, the fragment lacking the N-terminal 28 residues showed weaker interaction with myosin fragments than with whole myosin, according to our rod binding and competition experiments (Table 1, Figure 7). This suggests that the N-terminal 28 residues are important for tight interaction with myosin and may even form a separate second binding site in domain 1. We have previously suggested that two stretches in the N-terminus of caldesmon may form the α -helices of several heptades, with a periodic distribution of charged residues. These were found within residues 2–39 and 76–113 of caldesmon respectively, and have been suggested to form a triple-stranded

coiled-coil with target protein sequences [21]. This, and our present experiments, indicating two N-terminal myosin contact sites, are further supported by our earlier finding where a 27 kDa chymotryptic fragment of duck gizzard caldesmon (residues 1–175) was found to interact more strongly with myosin than with its further-degraded fragment of 25 kDa [21,46]. It is likely that the 25 kDa and the 27 kDa chymotryptic fragments differ in their N-terminus, but not in their C-terminus [21,22,24].

The lack of ability of the fragment 29N152 to compete with intact caldesmon in binding and cross-linking experiments (Figures 4 and 7) is unlikely to be due to improper conformation of this fragment in solution, since it binds myosin filaments similarly to other fragments and to intact caldesmon (Figure 1). In addition, we have found that the time-course of its phosphorylation by casein kinase II *in vitro* is the same as that of N152, and the phosphorylation similarly affects the myosin-binding properties of these fragments, indicating proper folding (A. V. Vorotnikov, S. B. Marston and P. A. J. Huber, unpublished work). We thus conclude that the inability of 29N152 to compete with intact caldesmon for myosin binding is due to the reduction of contact sites rather than misfolding of the fragment.

The C-terminal myosin-binding site of caldesmon seems to cover an even longer stretch, encompassing sequences in domains 3 and 4a of caldesmon. H12 (203 amino acids) binds myosin with an apparently equal affinity to that of its parent fragment, H1, whereas smaller fragments from domains 3 and 4a (60–176 amino acids) had a substantial loss in binding affinity [14]. Thus H12 was useful to assess the mode of caldesmon–myosin interaction and the contribution of two myosin-binding regions to the ability of caldesmon to cross-link actin filaments to myosin.

Mode of caldesmon interaction with myosin

All N-terminal caldesmon fragments bound to myosin with a stoichiometry of 4 to 1 (probably 2 per myosin heavy chain), and this interaction did not depend to any significant extent on ionic strength (Figures 2 and 3A). The C-terminal fragments, H1 and H12, also seemed to have the same maximum stoichiometry, but their interaction strongly depended on salt concentration ([14] and this work). Nevertheless, C-terminal and N-terminal fragments must engage the same binding sequences on myosin, because when used separately they were able to efficiently displace caldesmon from the complex with myosin (or HMM) at low ionic strength (see Figures 4 and 7). This indicates that at low ionic strength and 1 mM Mg^{2+} , i.e. under conditions favourable for these two sites of caldesmon to interact, four caldesmon molecules could be bound to one myosin, presumably each through any one of the myosin-binding sites. Mg^{2+} ions strongly inhibit interaction of both caldesmon and its fragments with myosin, primarily affecting the binding stoichiometry rather than the affinity (perhaps by blocking the binding interface). In this respect there is good agreement of our data with those of Chalovich and co-workers [24,47] who determined a stoichiometry of two caldesmon molecules bound per myosin at 5 mM Mg^{2+} and low (30 mM NaCl) ionic strength. The same stoichiometry of the interaction was also obtained under similar conditions by Mani and Kay [48]. It seems possible that even higher stoichiometries than 4:1 for the caldesmon–myosin interaction in the absence of Mg^{2+} could be achieved. It would be reasonable to speculate that there are no well-defined caldesmon-binding sites in the myosin subfragment-2 structure and a maximum stoichiometry of the protein interaction would depend on the spatial availability of the particular myosin region for caldesmon.

The maximum binding of caldesmon to myosin and myosin rods is reduced at high ionic strength. Assuming that myosin and the myosin rod are still fully capable of interaction with the N-terminus of caldesmon with maximum stoichiometry (Table 1), we suggest that changes in the conformation of caldesmon at high ionic strength may be responsible for the difference. It has been shown that caldesmon can form a folded 'hairpin' structure which provides communication between the N- and C-terminal domains of caldesmon [49–51]. An increase in ionic strength or binding of calmodulin or nucleotide were shown to cause caldesmon to unfold and to decrease the caldesmon–myosin binding stoichiometry ([14,47]; see also Table 1). It is reasonable to assume that an extended caldesmon molecule is able to cover more binding surface on myosin than a folded molecule. If caldesmon-binding sites on myosin are not well defined, as suggested by our results, the spatial availability of the binding sequence on myosin would depend on the conformation of bound caldesmon. This effect is restricted to stoichiometry and was not observed in terms of affinity [50]. The spatial restriction will be diminished in the case of relatively short myosin-binding fragments that are insensitive to conformational changes (Figure 3A).

Cross-linking of actin filament to myosin by caldesmon

Caldesmon enhances the binding of HMM to actin. The effect is strictly specific for the smooth muscle myosin fragment [18,29]), but is independent of the state of HMM phosphorylation (Figure 6; [15,24]). The irrelevance of the phosphorylation state confirms and agrees well with the location of the caldesmon-binding region in subfragment-2 of myosin [16,18]. Using N-terminal caldesmon fragments in competition experiments we obtained evidence that caldesmon physically cross-links HMM to actin rather than increasing the affinity of direct acto–myosin interaction [15].

The cross-linking is mediated exclusively by the N-terminal myosin-binding and the C-terminal actin-binding domains of caldesmon. This is strongly supported by the observation that: (1) the C-terminal binding site of caldesmon has very low affinity for myosin at high ionic strength, as shown by its inability to dissociate the caldesmon–myosin complex (Figure 4 and [14]); and (2) that H1, containing only the C-terminal myosin binding site, is not able to link HMM to actin even at low ionic strength (Figure 6). The latter is presumably caused by spatial proximity or even overlap of the myosin- and actin-binding sites in this region of the molecule. The N-terminal 28 amino acids of caldesmon are important for the tightening of the interaction with myosin, which is indicated by the inability of 29N152 to dissociate the link between actin and HMM formed by caldesmon (Figure 7). We also observed that tropomyosin slightly potentiated the cross-linking (results not shown), probably because it enhances caldesmon–actin interaction [13,52] and does not compete with myosin for the N-terminus of caldesmon [27].

A striking peculiarity in the caldesmon-mediated actin–myosin cross-linking effect is its extremely high affinity for myosin (Figure 5). It is unlikely to derive from the co-operativity between the two myosin-binding regions inside the N-terminal myosin-binding site of caldesmon, since the affinity of direct caldesmon–myosin interaction is of the same order of magnitude as the affinity of its individual fragments (Table 1, Figure 2). The ability of certain caldesmon fragments to dissociate the cross-linked complex almost completely (Figure 7B), although at high molar excess, argues against the suggestion that caldesmon binding to myosin may indirectly affect the affinity of myosin heads for

actin [15]. It does not, however, disagree with our earlier suggestion that actin-bound caldesmon may cooperatively interact with the thin filament and the myosin filament, while actin-free caldesmon does not bind cooperatively [29]. We hope that future work will illuminate the nature of the high affinity of acto–myosin cross-linking by caldesmon and that it will clarify whether cross-linking plays a role in tonic and latch contraction specific to smooth muscle.

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