Characterization of the functional properties of smooth muscle caldesmon domain 4a: evidence for an independent inhibitory actin–tropomyosin binding domain

Mohammed EL-MEZGUELDI¹, O'Neal COPELAND, Iain D. C. FRASER, Steven B. MARSTON and Pia A. J. HUBER

Cardiac Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, U.K.

Recent analysis has shown the presence of three sequences in the C-terminal 170 amino acids of human caldesmon (domain 4) which are involved in actin binding and tropomyosin-dependent inhibition of actomyosin ATPase. Two are in domain 4b (amino acids 715–793) and one is in domain 4a (amino acids 636–714). In the present work we have compared recombinant peptides containing either domain 4a or domain 4b to address the question as to whether domain 4a alone has any inhibitory activity. We have produced three new recombinant fragments containing domain 4a: H10 [622–708], H12 [506–708] and H13 [622–726] and we have characterized their functional properties. All three fragments bound to actin and tropomyosin. Caldesmon, but not domain 4b, was able to displace the fragments H10, H12 and

H13 from actin. Thus the isolated caldesmon domain 4a peptides bind to the same region on actin as in the whole molecule while domains 4a and 4b occupy different sites on the actin molecule. Unlike domain 4b, none of the domain 4a fragments inhibited the actomyosin ATPase in the absence of tropomyosin. However both domain 4a and 4b fragments displayed an inhibitory activity in the presence of tropomyosin. H13 and H12 were more potent inhibitors than H10. Ca²⁺-calmodulin bound to H13 and reversed the inhibitory activity of this fragment but did not bind to H10 and H12. We conclude that domain 4a can act as an independent inhibitory actin–tropomyosin binding domain, but its properties are very different from the extreme C-terminal domain 4b.

INTRODUCTION

Caldesmon is a thin filament-associated protein first isolated from smooth muscle and later shown to be a component of a wide range of non-muscle cells [1]. It binds to many contractile proteins including actin, tropomyosin and myosin [2–4]. Caldesmon also binds to Ca²⁺-binding proteins such as calmodulin [2,5]. The binding of caldesmon to actin leads to the inhibition of the actin activation of myosin ATPase. This inhibition is greatly amplified by tropomyosin [6,7] and reversed by Ca²⁺-binding proteins at high Ca²⁺ concentrations [8–10]. These properties have led to the proposal that caldesmon may regulate the thin filament interaction with myosin in smooth muscle and non-muscle cells [1,11].

An important feature in caldesmon regulatory function is that it is mediated by tropomyosin [12,13]. Caldesmon, at *in vivo* ratios to actin–tropomyosin, switches the thin filament between an 'on' and an 'off' state through a co-operative mechanism that is dependent on and propagated by tropomyosin [14]. Therefore definition of sites involved in actin binding and in the tropomyosin-dependent inhibition is essential for understanding caldesmon function.

Caldesmon is a long molecule able to span 7 to 14 actin monomers when incorporated into the thin filament. In 1991, Marston and Redwood proposed a four-domain model for the caldesmon molecule [15]. There is general agreement that the inhibitory function of caldesmon is located near its C-terminus. All the actin-binding sequences and the entire tropomyosin-dependent inhibitory property are fully contained within the C-terminal 288 amino acids, approximately corresponding to domain 3 and 4 (reviewed in [16]). Many studies have shown that

there is a minimal tropomyosin-dependent, Ca²⁺-calmodulin regulated actin inhibitory domain at the extreme C-terminus of caldesmon [17,18]; the smallest C-terminal peptide reported to retain this function is 68 amino acids [19]. Within this short sequence, named domain 4b [18], two inhibitory actin binding sequences which overlap Ca²⁺-calmodulin binding sites have been identified [20,21].

Although isolated domain 4b contains a functional inhibitory property which resembles whole caldesmon it binds to actin relatively weakly and does not bind to tropomyosin at all [14,17–20,22]. In contrast, the binding of domain 4 as a whole quite closely resembles the intact caldesmon molecule [18,20], thus indicating that the N-terminal part of domain 4 (domain 4a) and perhaps parts of domain 3, contribute to caldesmon binding to actin and tropomyosin and to the consequent inhibition of the actomyosin ATPase.

A number of authors have proposed actin binding sites within domain 4a and several peptides which contain domain 4a, but do not contain the extreme C-terminus have been shown to be tropomyosin dependent, Ca²⁺-calmodulin regulated inhibitors of actomyosin [20,23–25]. Moreover, Chalovich and co-workers [26] showed that a 7.3 kDa chymotryptic peptide corresponding to the human sequence 653–722 is able to inhibit actomyosin ATPase. However, large quantities of this peptide were needed to obtain maximum inhibition. In their recent paper, Fraser et al. [20] proposed that the actin binding site in domain 4a was one of three interacting segments needed for tropomyosin-dependent inhibition, but left open the question as to whether the segment in domain 4a had any autonomous inhibitory activity, as suggested by Chalovich et al. [26], or is only functional in concert with the segments in domain 4b.

Abbreviations used: S-1, chymotryptic myosin subfragment; DTT, dithiothreitol.

¹ To whom correspondence and reprint requests should be addressed (e-mail: m.elmezgueli@ic.ac.uk).

In order to determine the function of domain 4a, we have designed a series of recombinant caldesmon peptides with different lengths and sequences covering domains 3 and 4a (see Figure 1). These have been studied in comparison with caldesmon and our previously investigated peptides covering domains 3, 4a and 4b. Collectively, our results show unambiguously that the polypeptide stretch 622–708 (chicken sequence 566–651), corresponding to domain 4a, represents a functionally critical part of the caldesmon molecule. Domain 4a binds to actin and tropomyosin and is an autonomous tropomyosin-dependent inhibitor of actomyosin ATPase. Moreover, it exhibits certain unique features not observed in other regions of the caldesmon molecule, which may be crucial components of the caldesmon regulatory activity.

MATERIALS AND METHODS

Construction and expression of recombinant caldesmon peptides

Peptides representing sequences from the C-terminal region of caldesmon were designed and generated using a PCR-based cloning strategy. Oligonucleotide primers were designed to introduce start and stop codons and restriction enzyme sites at the desired location in the sequence of the M13mp18/human caldesmon construct of Huber et al. [27] and were used to amplify the cDNA, which was then ligated into the pMW172 plasmid [28]. The final constructs were verified by double stranded sequencing. Caldesmon peptides were expressed in the *Escherichia coli* strain BL21(DE3) and purified as detailed by Redwood and Marston [18] and Huber et al. [22]. Amino acid numbering is according to the human caldesmon sequence of Humphrey et al. [29]. To allow comparison with other published work the equivalent numbering of Bryan et al. [30] for chicken caldesmon is shown also, in parentheses, throughout the text.

Purification of proteins from tissue

Rabbit skeletal muscle F-actin and chymotryptic myosin subfragment 1 (S-1) were prepared by standard methods [31,32]. Smooth muscle caldesmon and tropomyosin were prepared from sheep aorta as reported previously [20,22]. Calmodulin was prepared from bovine brain acetone-dried powder (Sigma) according to the procedure of Thomas et al. [33]. Protein concentrations were determined by the Lowry method.

Binding assays

The binding of caldesmon peptides to actin and actin-tropomyosin was determined by co-sedimentation. Actin or actin pre-mixed with smooth muscle tropomyosin 1:0.4 (w/w), was mixed with caldesmon peptides at 25 °C in 5 mM Pipes pH 7.0/10 mM KCl/2.5 mM MgCl₂/1 mM dithiothreitol (DTT) in a final volume of 100 µl. After centrifugation (60 min at 50000 g and 25 °C, Sorvall SM24 rotor) the supernatants were carefully removed and the pellets were dissolved in electrophoresis sample buffer [4 % (w/v) SDS/10 % (v/v) 2-mercaptoethanol/200 mM Tris/HCl (pH 8.9)/20 % (v/v) glycerol]. Samples from the supernatant and the pellet were separated by SDS/PAGE (8–18 % acrylamide gradient) (Excel Gel, Pharmacia Biotech, Inc.). The amount of bound protein was determined by scanning densitometry of the gels (Howtek Scanmaster 3 and PDI system I, version 2.2 software). A calibration curve was generated by scanning known quantities of the recombinant peptides loaded on to the same gel. All points were within the linear range. The quantity of protein bound in the actin pellet was normalized by the equation: area of the recombinant fragment/area of actin.

The binding of the recombinant peptides to calmodulin in the presence and absence of Ca²⁺ was determined by co-sedimentation with calmodulin coupled to CNBr-activated Sepharose as described previously [7].

Caldesmon binding to *N*-pyrenylmaleimide-labelled tropomyosin was measured fluorometrically [3,22].

Competition experiments

Mixtures of actin (12 µM)–tropomyosin (1:0.4, w/w), caldesmon domain 4a fragments (H10, H12 and H13) (12 µM) and various concentrations of caldesmon or H9 were incubated for 20 min at 25 °C in 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM MgCl₂/1 mM DTT in a final volume of 100 µl. After centrifugation (50000 g for 60 min at 25 °C; Sorvall SM24 rotor) the supernatants were carefully removed and the pellets were dissolved in electrophoresis sample buffer and analysed as above.

Determination of ATPase activity

The effect of native caldesmon and recombinant caldesmon peptides on actin and actin–tropomyosin activation of myosin ATPase was determined in 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM MgCl₂/1 mM DTT at 25 °C. Protein mixtures (12 µM actin, 3 µM tropomyosin and 1 µM S-1) were prepared in a final volume of 100 µl and the reactions were started by the addition of MgATP to a final concentration of 5 mM. The reactions were terminated with 0.5 ml of 10 % (w/v) trichloroacetic acid and the P_i released was measured by the method of Taussky and Schorr [34].

RESULTS

Design, expression and purification of the recombinant fragments

Three new recombinant peptides covering domains 3 and 4 of caldesmon have been expressed in *E. coli*. Figure 1 shows the location of these peptides and previously studied peptides in the caldesmon sequence. The purity of the peptides is illustrated in Figure 2 (first lane on the left for each peptide) and their sequences and properties are listed in Table 1. H5 and H12 contained breakdown products which did not bind to actin. The peptide H12 [human 506–708 (chicken 476–651)] contains the whole of domain 3 and domain 4a but does not include the Ca²⁺-calmodulin and weak actin binding sequence 708–726 (chicken sequence 651–665) described by Zhan et al. [35] (site A on Figure 1). The peptides H10 and H13 both start at amino acid 622, which is also the starting point of the peptide H7, which we have studied previously. H7 is known to bind strongly to actin and tropomyosin and to be inhibitory [14,20,22]. These peptides contain domain 4a and the linking regions between domain 3 and 4, since domain 4 is considered to start at 636 (597 in the chicken sequence). H10 terminates at 708, like H12, and H13 terminates at 726 and thus includes Ca²⁺-calmodulin binding site A; H7 terminates at 767 (chicken 710) and includes one of the actin binding segments of domain 4b [20]. H10 and H13 were designed to contain more N-terminal sequence than the chymotryptic 7.3 kDa fragment of Chalovich et al. [26], since very large quantities of this peptide were needed to get maximum inhibition. In order to investigate the contribution of the C-terminus of domain 4a on the properties of H12 and H13, we studied the previously characterized tropomyosin binding fragment H5 [22], which corresponds to domain 3 plus the N-terminal part of domain 4a [human 506–681 (chicken 476–624)]. For comparison we used the whole of domain 3 and 4, H1 [human 506–793

Figure 1 Schematic representation of the various caldesmon recombinant peptides used in this work

H10, H12 and H13 are the newly produced peptides. H5 was characterized previously as a tropomyosin binding peptide [22]. H1, H7 and H9 are actin binding and inhibitory peptides [19,20,27]. The ovals C, B and B' correspond to the three actin binding sequences identified by Fraser et al. [20]. CMA, CMB and CMB' are the calmodulin binding sites identified previously [19,35,47,48]. TnT2 represents a putative tropomyosin binding sequence with homology with the troponin T2 fragment [22,37]. In domain 3, the shaded area represents the weak actin binding sequence identified by Morinet et al. [48]. Oval A is the weak actin binding sequence proposed by Wang et al. [25] and Zhan et al. [35]. Also shown is the 7.3 kDa chymotryptic peptide characterized by Chalovich et al. [26]. Amino acid numbering is for human caldesmon [29] with the numbering for chicken caldesmon proposed by Bryan et al. [30] given in parentheses.

Figure 2 Interaction between F-actin-tropomyosin and the recombinant peptides

The binding of the recombinant peptides H5, H9, H10, H12 and H13 to F-actin (Actin) in the presence of smooth muscle tropomyosin (Tm) was determined by co-sedimentation followed by PAGE of the supernatant (S) and the pellet (P) for each peptide. The two left lanes for each peptide are the peptide alone and the two lanes to the right are the peptide with actin-tropomyosin. M, molecular mass marker. The concentrations used were: 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM $MgCl_2$ /0.1 mM DTT containing 12 μ M actin, 3 μ M tropomyosin and 6 μ M of each recombinant peptide.

(chicken 476–737)] and the domain 4b recombinant peptide H9 [human 726–793 (chicken 669–737)] previously characterized [20,27].

Binding of the recombinant fragments to actin

The binding of the recombinant fragments to skeletal muscle F-actin was analysed by co-sedimentation and competition experiments. Figure 2 shows the co-sedimentation with actin-tropomyosin of the peptides H5, H10, H12 and H13 from domains 3 and 4a compared with H9, which corresponds to

domain 4b. In the presence of actin-tropomyosin all of the fragments sedimented with the actin-tropomyosin complex. Binding to actin also took place in the absence of tropomyosin (see Table 1).

As the peptides bound to actin we explored whether they bound to the same region on actin as did the parent caldesmon. Figure 3A shows that caldesmon displaced H10, H12 and H13 from actin. Around 50% of the peptide bound to actin was displaced by 5 μ M of caldesmon. In contrast, high concentrations (up to 60 μ M) of the domain 4b peptide, H9, did not displace the domain 4a peptides H10, H12 and H13 from actin (Figure 3B).

Table 1 Properties of recombinant caldesmon peptides

Sequences (human according to [29], chicken according to [30]), length of peptides, affinity for actin, inhibitory properties and the calmodulin- and tropomyosin-binding properties of the peptides (+, fragment displays the property described; 0, fragment does not possess the property; –, a small amount of H12 sedimented with calmodulin both in the presence and absence of Ca²⁺). *, affinities for actin taken from [20,27,38a,50]. n.d., not done.

Caldesmon peptide	Symbol on graphs	Human sequence	Chicken sequence	Length of peptide (human)	Affinity for actin (M ⁻¹ × 10 ⁶)	Binding to Ca ²⁺ -calmodulin	Binding to tropomyosin	Inhibition of actin–myosin ATPase	Inhibition of actin–tropomyosin–myosin ATPase
Caldesmon		1–793	1–756	793	3*	+	+	+	+
H1	×	506–793	476–737	288	1*	+	+	+	+
H9	◆	726–793	669–737	68	0.051*	+	0	+	+
H5		506–681	476–624	176	n.d.	0	+	0	0
H10	○	622–708	566–651	83	0.1	0	+	0	+
H12	□	506–708	476–651	203	2	–	+	0	+
H13	△	622–726	566–669	105	0.34	+	+	0	+

Figure 3 Displacement of H10, H12 and H13 from actin by caldesmon or H9

Co-sedimentation assays were performed as described in the Materials and methods section using 12 μM actin, 12 μM H10 (○), H12 (□) or H13 (△) and increasing concentrations of caldesmon (A) or H9 (B) as shown on the abscissa. After centrifugation, the pellets were separated by PAGE, and the amount of bound recombinant peptides was measured by scanning densitometry. In the absence of caldesmon or H9, 4.8 μM of H10, 6.6 μM of H12 and 7.2 μM of H13 bound to actin. Those values were considered as 100%. The conditions used were: 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM MgCl₂/0.1 mM DTT.

Inhibition of the actin-activated myosin ATPase

In the absence of tropomyosin none of the peptides had any effect on the actin activation of S-1 ATPase (Figure 4) In the presence of tropomyosin increasing concentrations of H12 and H13 inhibited ATPase activity. At a 1:1 molar ratio of peptide/actin (12 μM each) around 90 % inhibition was obtained for both H12 (Figure 4B) and H13 (Figure 4C), consistent with their

Figure 4 Effect of the recombinant peptides on the acto-S-1 ATPase activity

ATPase assays were carried out as described in the Materials and methods section using increasing concentrations of H10 (A), H12 (B) and H13 (C) in the absence (closed symbols) or presence (open symbols) of smooth muscle tropomyosin. The concentrations used were: 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM MgCl₂/0.1 mM DTT containing 12 μM actin, 1 μM S-1 and 3 μM smooth muscle tropomyosin, when present. Uninhibited ATPase activity was 6.0 s⁻¹ and 1.4 s⁻¹ in the presence and absence of tropomyosin respectively.

Figure 5 Relationship between the inhibition of acto-S-1 ATPase activity by the recombinant peptide and the quantity of the peptide bound to actin

In parallel with the measurement of ATPase activity, the quantity of H12 (A) or H13 (B) bound to actin was determined by co-sedimentation as described in the Materials and methods section. Closed symbols, actin; open symbols, actin-tropomyosin.

high binding affinities. H10 also inhibited actin-tropomyosin activation of S-1 ATPase (Figure 4A) but the inhibition was significantly less than with H12 and H13. Even at the highest concentration of H10 used (24 μM) only 50% inhibition occurred.

The relationship between the inhibitory activity of the caldesmon peptides and the amount of the peptide bound to actin was then investigated (Figure 5). Up to 0.8 molecule of H12 (Figure 5A) and H13 (Figure 5B) bound to actin with no change in the actin-activated ATPase in contrast to peptide H9, which approached 100% inhibition at 1 molecule of peptide bound/molecule of actin [20]. In the presence of tropomyosin, 90% inhibition was obtained with about 0.4 molecule of H12 or H13 bound/molecule of actin (Figure 5), whereas, in previous experiments, 90% inhibition required only 0.1 molecule of caldesmon, domain 4 or domain 4b peptide bound/molecule of actin-tropomyosin [13,18,20]. H10 did not bind strongly enough to actin-tropomyosin for a satisfactory measurement to be made.

Interaction of the peptides with Ca^{2+} -calmodulin:

Figure 6(A) shows the binding of the caldesmon peptides H9, H10 and H13 to Ca^{2+} -calmodulin assessed by co-sedimentation with calmodulin-Sepharose. H9 and H13 bound to Ca^{2+} -calmodulin but H10 did not bind within the range tested. This result is as expected, since H10 does not incorporate any of the previously identified Ca^{2+} -calmodulin binding sequences (see Figure 1).

We then assessed the effect of Ca^{2+} -calmodulin on the inhibitory activity of the domain 3 and 4a peptides, H12 and H13, which strongly inhibited the actin-tropomyosin activation of S-1 ATPase. As shown in Figure 6(B), Ca^{2+} -calmodulin reversed the inhibition obtained by H13 and the domain 4b fragment, H9,

Figure 6 Binding of recombinant peptides to Ca^{2+} -calmodulin and the effect of Ca^{2+} -calmodulin on peptide inhibition of acto-S-1 ATPase activity

(A) Binding of the recombinant peptides to Ca^{2+} -calmodulin. Calmodulin-Sepharose (Pharmacia-LKB Biotechnology Inc.) was mixed with 0–14 μM of H13 (Δ) or H10 ($\circ$) in a total volume of 100 μl and the Sepharose was pelleted. The amount of each fragment bound to calmodulin was calculated as the difference between the amount in the supernatant before (total peptide) and after (free peptide) centrifugation and was measured by SDS/PAGE and scanning densitometry. H9 ($\blacklozenge$) was used as a control. The buffer used was: 5 mM Pipes (pH 7.0)/10 mM KCl/2.5 mM MgCl_2 /0.1 mM DTT/0.5 mM CaCl_2 . (B) Effect of Ca^{2+} -calmodulin on inhibition by H9, H12 and H13. ATPase assays were carried out as described in the Materials and methods section. The ATPase activities were inhibited by 8 μM H13 (Δ , 47% inhibition) or H12 ($\square$, 64% inhibition) and increasing amounts of calmodulin were added. H9 8 μM ($\blacklozenge$, 64% inhibition) was used as a control. The conditions used were: 5 mM Pipes (pH 7.0)/30 mM KCl/2.5 mM MgCl_2 /0.1 mM DTT/0.5 mM CaCl_2 containing 12 μM actin, 1 μM S-1 and 3 μM tropomyosin. Uninhibited ATPase activity was 2.0 s^{-1} .

which was used as a control. In the absence of calcium, calmodulin had no effect on H9 and H13 inhibition (results not shown). However, calmodulin had a very small effect on the inhibition by H12, both in the presence and absence of calcium.

Binding to tropomyosin

The binding of the caldesmon domain 4a peptides to tropomyosin was studied by measuring the change in fluorescence of pyrene attached to Cys-190 of smooth muscle tropomyosin. Figure 7 shows the effect of H10, H12 and H13 on the fluorescence of pyrene-labelled smooth muscle tropomyosin. The largest peptide H12, corresponding to domain 3 and domain 4a, gave around 35% enhancement of the fluorescence of pyrene-labelled tropomyosin and was indistinguishable from H1, which corresponds to the whole of domains 3 and 4. The shorter peptides H10 and H13, which lack domain 3, induced an increase in the fluorescence of pyrene-labelled tropomyosin, which was saturating, but the increase in pyrene fluorescence was only 20%. Fitting the data to a simple binding equation indicated that all the peptides had an affinity in the range $(0.2\text{--}0.5) \times 10^6 \text{ M}^{-1}$.

Figure 7 Effect of the recombinant peptides on the fluorescence of pyrene-labelled tropomyosin

Caldesmon peptide H1 (x), H9 (◆), H10 (○), H12 (□) or H13 (△) was added to 1 μ M *N*-pyrenyl-maleimide-labelled smooth muscle tropomyosin and pyrene fluorescence (f) was measured as described by Huber et al. [22]. The results are expressed as the change in f/f_0 due to the peptide, where f_0 is the initial fluorescence of pyrene-labelled tropomyosin.

DISCUSSION

Caldesmon is a component of Ca^{2+} -regulated thin filaments in smooth muscle and numerous studies have shown that it is involved in regulating the actin filament by Ca^{2+} -calmodulin regulated, tropomyosin-enhanced inhibition of the actin activation of myosin ATPase activity [11,15,16]. Inhibition of actin activation appears to be a consequence of caldesmon displacing myosin from weak binding sites on actin, whereas inhibition of actin-tropomyosin involves a co-operative process in which one caldesmon molecule can inhibit up to 14 actin molecules [7,13]. Previous work has shown that the inhibitory properties of caldesmon reside in the C-terminal 170 amino acids, domain 4 (residues 36–38). Within domain 4, three separate actin-binding inhibitory sequences have been identified (C, B and B' in Figure 1) and recent work suggests that the three segments work together in the intact molecule, since peptides containing two of the three sequences are inhibitors with properties comparable with caldesmon [20].

Two of the proposed actin binding sequences (B and B') are contained within the C-terminal 68 amino acids of human caldesmon (peptide H9) as well as in the 99 amino acid C-terminal segments of chicken caldesmon (10K in [17] or peptide 658C), and have been well characterized [17,18,19,20]. The contribution made to inhibition by the proposed actin binding segment in domain 4a is less clear. Caldesmon has been shown to inhibit the actin-tropomyosin activation of myosin ATPase at low and high ionic strengths. The affinity of caldesmon and caldesmon peptides to actin-tropomyosin is affected by ionic strength but the relationship between binding and inhibition is not (i.e. the amount of caldesmon bound to actin needed to get maximum inhibition is independent of ionic strength in the range of 0.017–0.15) [7,12]. Peptides which contain domain 4a bind strongly to actin and are less affected by salt concentration than domain 4b peptides, indicating at least some distinctive properties of domain 4a [20,38a]. Hitherto, the only peptide exclusively derived from domain 4a is the 7.3 kDa chymotryptic fragment described by Chalovich et al. [26].

We have investigated the properties of peptides containing domain 4a but not 4b. The data lead to the unambiguous finding that the isolated domain 4a binds to actin and tropomyosin. The peptide H13, which contains the known Ca^{2+} -calmodulin binding

sequence ($^{715}\text{MWEKGNVFSS}^{724}$, site A on Figure 1) [19,35,39], also binds Ca^{2+} -calmodulin. These findings are compatible with previous assignments of a strong actin binding segment, 663–682 (chicken sequence 606–625) [20], and an overlapping tropomyosin binding segment, 665–679 (chicken sequence 608–622) [22,23], within domain 4a. All of the peptides described in the present study bound more strongly than the 7.3 kDa peptide described by Chalovich et al. [26], indicating that the N-terminal sequence may be needed to stabilize the actin binding segment. It is particularly interesting to note that competition experiments show that domain 4a could bind to a site on actin which is displaced by the native caldesmon molecule, but that domains 4a and 4b do not compete and so bind at separate sites on actin. This is in accord with NMR measurements, which assigned separate contact regions on actin for domains 4a and 4b [40]. It has recently been suggested that domain 4b binds near the C-terminus of actin, while domain 4a may bind near the N-terminus [40,41]. In contrast, Wang et al. [21] have reported that domain 4a (chicken sequence 598–649) was considered not to be involved in actin binding. The substantially higher actin binding affinity of H13 compared with H10 suggests that there is also an actin binding determinant in the region 708–726 (chicken sequence 651–669), which corresponds to calmodulin binding site A, in agreement with previous studies [35,42].

Our direct experimentation indicates that isolated domain 4a not only binds to actin but is also capable of inhibiting actin-tropomyosin activation of myosin ATPase; however, the inhibitory properties differ radically from whole caldesmon or isolated domain 4b. There was no detectable inhibition of actin activation even when > 80 % of actin was occupied by domain 4a, thus domain 4a appears to bind to actin without interfering with weak myosin binding. This property has also been demonstrated for the binding of calponin to actin [43]. In contrast, whole caldesmon, domain 4b and the peptide H7, which contains domain 4a, and the first half of domain 4b, all block weak binding sites when each actin molecule is occupied [13,14]. This functional distinction between domains 4a and 4b supports the hypothesis that the domain 4a binding site on actin is separate from the domain 4b site.

The tropomyosin-dependent inhibition shown by domain 4a peptides is also different from intact caldesmon and domain 4b, since full inhibition requires at least twice as much domain 4a bound to actin as caldesmon [13,20]. Thus although domain 4a is an autonomous inhibitor, its inhibitory properties are unlike caldesmon. Domain 4a peptides H10 and H12 contain no Ca^{2+} -calmodulin binding sites and so inhibition is not reversed by Ca^{2+} and calmodulin. However the addition of 18 amino acids, containing the Ca^{2+} -calmodulin binding site A, to domain 4a can confer Ca^{2+} -calmodulin binding and regulation on domain 4a peptides (compare H12 and H10 with H13).

Extensive data on the mapping of caldesmon actin binding and inhibitory sites are now available. The convergent idea is that the actin-caldesmon interaction involves multiple sites localized in the C-terminal domain 3, 4a and 4b [18,20,21,24,40,42,44]. The properties of these domains can be separated. Domain 3 binds weakly to actin but has no effect on ATP hydrolysis. Domain 4a binds actin and tropomyosin and exhibits an abnormal, completely tropomyosin-dependent inhibition. Domain 4b binds to actin more weakly than caldesmon, does not bind to tropomyosin and inhibits actomyosin ATPase both in the absence and presence of tropomyosin. Furthermore, the inhibitory activity of domain 4b is reversed by Ca^{2+} -calmodulin in a similar manner to whole caldesmon.

Fraser et al. [20] proposed that caldesmon inhibition involves three actin binding segments, one in domain 4a (site C in Figure

1) and two in domain 4b (sites B, B' in Figure 1). We have proposed also that a peptide must span at least two adjacent sites for normal inhibitory behaviour to occur. Recent NMR analysis of domain 4b has confirmed directly that two-site docking on actin, involving the tryptophan residues at sites B and B', is an essential feature of the caldesmon domain 4b-actin interaction [44,45]. Our study of domain 4a is compatible with this model; domain 4a contains one actin binding sequence and does not give normal inhibition, whereas the peptide H7 contains sites C and B and is an inhibitor [14,20]. Nevertheless, domain 4a does make an important contribution to caldesmon function, since it contains strong actin and tropomyosin binding sites. Fragments of caldesmon, which contain domains 4a and 4b, bind to actin at least 15 times more strongly than domain 4b alone and the difference becomes much greater as salt concentration is increased [18,20,46,38a]. It is probable that the three-point docking on actin provided by the binding sites in domains 4a and 4b is necessary for caldesmon to bind and inhibit at physiological salt concentrations.

This research was supported by a grant from the Wellcome Trust.

REFERENCES

- Sobue, K., Kanda, K., Tanaka, T. and Ueki, N. (1988) *J. Cell Biochem.* **37**, 317–325
- Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5652–5655
- Horiuchi, K. Y. and Chacko, S. (1988) *Biochemistry* **27**, 8388–8393
- Ikebe, M. and Reardon, S. (1988) *J. Biol. Chem.* **263**, 3055–3058
- Shirinsky, V. P., Bushueva, T. L. and Frolova, S. I. (1988) *Biochem. J.* **255**, 203–208
- Dabrowska, R., Goch, A., Galazkiewicz, B. and Osinska, H. (1985) *Biochim. Biophys. Acta* **842**, 70–75
- Smith, C. W., Pritchard, K. and Marston, S. B. (1987) *J. Biol. Chem.* **262**, 116–122
- Pritchard, K. and Marston, S. B. (1991) *Biochem. J.* **277**, 819–824
- Pritchard, K. P. and Marston, S. B. (1993) *Biochem. Biophys. Res. Commun.* **190**, 668–673
- Ngai, P. K. and Walsh, M. P. (1984) *J. Biol. Chem.* **259**, 13656–13659
- Sobue, K. and Sellers, J. R. (1991) *J. Biol. Chem.* **266**, 12115–12118
- Marston, S. B. and Redwood, C. S. (1992) *J. Biol. Chem.* **267**, 16796–16800
- Marston, S. B. and Redwood, C. S. (1993) *J. Biol. Chem.* **268**, 12317–12320
- Marston, S. B., Fraser, I. D. C. and Huber, P. A. J. (1994) *J. Biol. Chem.* **269**, 32104–32109
- Marston, S. B. and Redwood, C. S. (1991) *Biochem. J.* **279**, 1–16
- Marston, S. B. and Huber, P. A. J. (1996) in *Biochemistry of Smooth Muscle Contraction* (Barany, M. and Barany, K., eds.), pp. 77–90, Academic Press, San Diego
- Bartegi, A., Fattoum, A., Derancourt, J. and Kassab, R. (1990) *J. Biol. Chem.* **265**, 15231–15238
- Redwood, C. S. and Marston, S. B. (1993) *J. Biol. Chem.* **268**, 10969–10976
- 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
- Fraser, I. D. C., Copeland, O., Wu, B. and Marston, S. B. (1997) *Biochemistry* **36**, 5483–5492
- Wang, Z., Yang, Z.-Q. and Chacko, S. (1997) *J. Biol. Chem.* **272**, 16896–16903
- Huber, P. A. J., Fraser, I. D. C. and Marston, S. B. (1995) *Biochem. J.* **312**, 617–625
- Hayashi, K., Kanda, K., Kimizuka, F., Kato, I. and Sobue, K. (1989) *Biochem. Biophys. Res. Commun.* **164**, 503–511
- Wang, Z., Horiuchi, K. Y. and Chacko, S. (1996) *J. Biol. Chem.* **271**, 2234–2242
- Wang, C.-L. A., Wang, L.-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V. and Bryan, J. (1991) *J. Biol. Chem.* **266**, 9166–9172
- Chalovich, J. M., Bryan, J., Benson, C. E. and Velaz, L. (1992) *J. Biol. Chem.* **267**, 16644–16650
- Huber, P. A. J., Redwood, C. S., Avent, N. D., Tanner, M. J. A. and Marston, S. B. (1993) *J. Musc. Res. Cell Motil.* **14**, 385–391
- Way, M., Gooch, G., Pope, B. and Weeds, A. G. (1989) *J. Cell Biol.* **109**, 593–605
- Humphrey, M. B., Herrera-Sosa, H., Gonzalez, G., Lee, R. and Bryan, J. (1992) *Gene* **112**, 197–205
- Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G. and Lin, W. G. (1989) *J. Biol. Chem.* **264**, 13873–13879
- Margossian, S. S. and Lowey, S. (1982) *Methods Enzymol.* **85**, 55–71
- Straub, F. B. (1942) *Studies from the Institute of Medical Chemistry, University of Szeged* **2**, 3–16
- Thomas, T. P., Gopalakrishna, R. and Anderson, W. B. (1987) *Methods Enzymol.* **141**, 399–428
- Taussky, H. H. and Schorr, E. (1953) *J. Biol. Chem.* **202**, 675–685
- Zhan, Q., Wong, S. S. and Wang, C.-L. A. (1991) *J. Biol. Chem.* **266**, 21810–21814
- Szpacenko, A. and Dabrowska, R. (1986) *FEBS Lett.* **202**, 182–186
- Hayashi, K., Fujio, Y., Kato, I. and Sobue, K. (1991) *J. Biol. Chem.* **266**, 355–361
- Riseman, V. M., Lynch, W. P., Nefsky, B. and Bretscher, A. (1989) *J. Biol. Chem.* **264**, 2869–2875
- Fraser, I. D. C. (1996) PhD Thesis, London University
- EL-Mezgueldi, M., Derancourt, J., Calas, B., Kassab, R. and Fattoum, A. (1994) *J. Musc. Res. Cell Motil.* **15**, 220
- Mornet, D., Bonet-Kerrache, A., Strasburg, G. M., Patchell, V. B., Perry, S. V., Huber, P. A. J., Marston, S. B., Slatter, D. A., Evans, J. S. and Levine, B. A. (1995) *Biochemistry* **34**, 1893–1901
- Hodgkinson, J. L., Marston, S. B., Craig, R., Vibert, P. and Lehman, W. (1997) *Biophys. J.* **72**, 2398–2404
- Wang, Z. and Chacko, S. (1996) *J. Biol. Chem.* **271**, 25707–25714
- EL-Mezgueldi, M. and Marston, S. B. (1996) *J. Biol. Chem.* **271**, 28161–28167
- Huber, P. A. J., Levine, B. A., Fraser, I. D. C., Copeland, O., EL-Mezgueldi, M. and Marston, S. B. (1997) *Biochemistry* **37**, 2314–2325
- Gao, Y., Copeland, O., Huber, P. A. J., Marston, S. B. and Levine, B. A. (1997) *J. Musc. Res. Cell Motil.* **18**, 201
- EL-Mezgueldi, M., Derancourt, J., Callas, B., Kassab, R. and Fattoum, A. (1994) *J. Biol. Chem.* **269**, 12824–12832
- Huber, P. A. J., EL-Mezgueldi, M., Grabarek, Z., Slatter, D. A., Levine, B. A. and Marston, S. B. (1996) *Biochem. J.* **316**, 413–420
- Mornet, D., Audemard, E. and Derancourt, J. (1988) *Biochem. Biophys. Res. Commun.* **154**, 564–571
- Reference deleted
- Smith, C. W. J. and Marston, S. B. (1985) *FEBS Lett.* **184**, 115–119