# The Inhibitory Complex of Smooth Muscle Caldesmon with Actin and Tropomyosin Involves Three Interacting Segments of the C-Terminal Domain 4[†]

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ABSTRACT: We have designed a series of recombinant peptides derived from the C-terminus of human caldesmon (amino acids 663−793, domain 4) to determine the structural basis of the multiple-sited caldesmon−actin−tropomyosin interaction. All the recombinant peptides are able to bind to actin and inhibit actin-activated myosin ATPase activity; 1 mol of peptide is bound per actin for >90% inhibition. However, equivalent inhibition of actin−tropomyosin activation of myosin ATPase requires less than one peptide per seven actin to be bound. We have found two sequences, H2 (amino acids 683−767) and H2+12 (amino acids 683−779), from the center of domain 4 which **potentiate** actin−tropomyosin filament activity; i.e., their effect is opposite to caldesmon. Maximum potentiation correlates with one H2 or H2+12 bound per four actin. This effect is completely dependent upon the presence of tropomyosin on the actin filament. H2 and H2+12 also increase actin−tropomyosin filament velocity in the *in vitro* motility assay. If the H2 sequence is extended by 20 amino acids at the N-terminal end to the N-terminus of domain 4, the peptide becomes an inhibitor. If H2 is extended by 19 amino acids at its C terminus, it becomes a tropomyosin-dependent inhibitor, and with a further extension of 7 amino acids to reach the C-terminus of human caldesmon (H2+26), inhibition is more potent. We conclude that three regions in domain 4 of caldesmon contribute to tropomyosin-dependent inhibition of actomyosin ATPase: a central segment [747−767 (690−710 in the chicken sequence)], which is essential but not sufficient for tropomyosin-dependent inhibition of actomyosin ATPase; and two actin binding segments N-terminal and C-terminal to this segment, 663−682 (606−625) and 770−793 (713−737). If only the central segment is present (H2, H2+12), the actin−tropomyosin−caldesmon peptide complex is not inhibitory, and its properties resemble actin−tropomyosin−caldesmon−$Ca^{2+}$·calmodulin.

Smooth muscle actomyosin is controlled by $Ca^{2+}$ acting upon both the thick filaments via myosin light chain phosphorylation and the thin filaments. Although myosin regulation plays a prime role in initiating smooth muscle contraction, there is evidence that the parallel thin filament-based regulation may be involved in relaxation (Barany & Barany, 1993; Gerthoffer, 1987; Khalil et al., 1995; Pfitzer et al., 1993). Studies have shown caldesmon to be the main regulatory protein of the smooth muscle thin filament, based on its ability to inhibit the actin−tropomyosin-activated myosin ATPase and filament motility, and the reversal of these effects by $Ca^{2+}$−calmodulin (Smith et al., 1987; Dabrowska et al., 1985; Sobue et al., 1982; Ngai & Walsh, 1984; Fraser & Marston, 1995b).

We have shown that the mechanism of caldesmon regulation of the thin filament is very similar to that of the troponin complex in striated muscle. Both proteins appear to control the strong binding interaction between actin and myosin by switching the thin filament between "ON" and "OFF" states through a cooperative mechanism that is dependent on and propagated by tropomyosin (Marston et al., 1994; Marston, 1995; Lehrer, 1994). This hypothesis is supported by the observation that the regulatory properties of troponin-I and caldesmon are indistinguishable. They both require actin to be associated with tropomyosin for effective inhibition, both inhibit the binding of strong myosin complexes such as S-1·ADP and S-1·AMP·PNP (Marston et al., 1994a), and both have no effect upon the weak myosin complex S-1·ADP·$P_i$ (Marston & Redwood, 1993). In the *in vitro* motility assay, troponin I and caldesmon "switch off" filament movement without affecting velocity (Fraser & Marston, 1995a,b).

The segments of the caldesmon amino acid sequence that are responsible for inhibition of actin−tropomyosin activation of myosin MgATPase have been investigated in detail using chemical methods of cleavage and expression of truncated mutants. It was established that the whole of the inhibitory property was contained within the C-terminal approximately 150 amino acids (out of the total 793 in human; see Figure 1), known as domain 4 (Szpacenko & Dabrowska, 1986; Hayashi et al., 1991; Wang et al., 1991; Redwood & Marston, 1993). Further studies with chicken gizzard caldesmon showed that full regulatory function was observed in a peptide containing the C-terminal 99 amino acids (Bartegi et al., 1990; Redwood & Marston, 1993), while with human caldesmon, the C-terminal 68 amino acids (expressed sequence H9) contain all the necessary sites for tropomyosin-dependent, $Ca^{2+}$−calmodulin-regulated inhibition (Marston

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et al., 1994a). This led to the division of domain 4 into domains 4a and 4b and the hypothesis that the C-terminal domain 4b alone is responsible for the inhibitory function of caldesmon (Bartegi et al., 1990; Redwood & Marston, 1993; Marston & Redwood, 1991).

Recently C-terminal deletions of the whole caldesmon molecule have been used in an attempt to define this regulatory sequence still closer. These experiments suggest that up to 20 amino acids may be removed from the C-terminus of the human caldesmon sequence (39 from the chicken sequence) before tropomyosin-dependent inhibition is lost (Wang et al., 1996), and a "strong actin binding site" associated with inhibition was proposed for the chicken sequence $^{718}$KRNLWE$^{723}$ (site CMB' in Figure 1, 775−780 in human) (Wang & Chacko, 1996). However, the concept of a single discrete site near the C-terminus is contradicted by studies indicating that there is a second inhibitory site, located in the N-terminal region of domain 4. Peptides such as the 7.3 kDa chymotryptic peptide described by Chalovich et al. (1992) (equivalent to 653−722 in the human sequence), and the human recombinant peptide H7 (622−767 human, 566−710 in chicken sequence) (Fraser, 1995; Marston et al., 1994a,b), lack the C-terminus of caldesmon and yet they are inhibitors. Furthermore, a number of studies have predicted both actin and tropomyosin binding sites in the N-terminal region of caldesmon domain 4 (Hayashi et al., 1991; Wang et al., 1991; Huber et al., 1995; Redwood & Marston, 1993).

In order to elucidate the number and location of actin binding site sequences involved in inhibition, it is necessary to study deletions of domain 4 from both the N- and the C-terminal end. We have previously demonstrated some properties of one such sequence, H2. This is an 85 amino acid peptide comprising the central two-thirds of human caldesmon domain 4 and lacking 20 N-terminal and 26 C-terminal amino acids (amino acids 683−767 in the human sequence, equivalent to 626−710 in the chicken sequence). H2 can bind to actin, and it can inhibit actin-activated myosin ATPase yet it potentiates the ATPase when tropomyosin is present (Marston et al., 1994a,b).

These results suggest a complex structure and function relationship in domain 4 of caldesmon that cannot be explained by a simple linear map of multiple independent functional sites. There is evidence from nuclear magnetic resonance studies that domain 4 of caldesmon, in contrast to the rest of the molecule, is highly folded with regions which are distant in the primary sequence being brought close together (Mornet et al., 1995).

In this study, we have investigated H2 and a series of recombinants in which either the N- or the C-terminus has been extended toward the ends of domain 4. Our results show that all the human caldesmon recombinant peptides are able to bind to actin and inhibit the actin-activated ATPase in the absence of tropomyosin, when one caldesmon peptide is bound per actin. However, in the presence of tropomyosin, inhibition requires less than one caldesmon peptide per seven actin to be bound, and the properties of the peptides are dependent on their position within domain 4. A sequence between 747 and 767 (690−710 in the chicken sequence) in the middle of domain 4 appears to be necessary, but not sufficient, for tropomyosin-dependent inhibition. Addition of sequence either C-terminal or N-terminal to this region confers inhibitory function. We propose that in the folded C-terminus of caldesmon, three regions contribute to regulation of the thin filament, and of these, two are required for effective inhibition.

## MATERIALS AND METHODS

*Construction and Expression of Recombinant Caldesmon Peptides.* Peptides representing sequence 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 previously described by Huber et al. (1993). Amplified DNA was ligated into the pMW172 plasmid (Way et al., 1989) and transfected into *Escherichia coli* BL21(DE3) cells. Caldesmon protein was expressed and purified as detailed by Redwood and Marston (1993) and Huber et al. (1993). The yields of purified expressed protein peptides are shown in Table 1. Amino acid numbering is according to the human caldesmon sequence of Humphrey et al. (1992). For comparison with other published work, the equivalent chicken numbering of Bryan et al. (1989) is also shown in Table 1 and in parentheses throughout the text.

*Purification of Proteins from Tissue.* Rabbit skeletal muscle actin and heavy meromyosin (HMM)[1] were prepared by standard procedures (Margossian & Lowey, 1982). HMM preparation for the motility assay was as described by Kron et al. (1991). Smooth muscle caldesmon and tropomyosin were prepared from sheep aorta by previously reported methods (Smith et al., 1987; Marston & Huber, 1995) while calmodulin was prepared from bovine brain acetone powder (Sigma) according to the procedure of Gopalakrishna and Anderson (1982). Protein concentrations were determined by the method of Lowry.

*Binding Assays.* The binding of caldesmon peptides to actin and actin−tropomyosin was determined by cosedimentation. Actin, or actin premixed with smooth muscle tropomyosin to a ratio of 1:0.4 (w/w), was mixed with caldesmon peptides at 25 °C in 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 1−2.5 mM $MgCl_2$, and 1 mM DTT in a total volume of 200 $\mu$L. An aliquot of 100 $\mu$L was taken for ATPase assay; then 25 $\mu$L of the mixture was removed, and the remaining 75 $\mu$L was centrifuged at 40000*g* for 60 min at 25 °C. A further 25 $\mu$L was removed, and the samples before and after sedimentation were separated by gel electrophoresis. The caldesmon bands were estimated by quantitative densitometry using a "Scanmaster 3" flat-bed scanner with "Quantity One" software (pdi Inc.). The band densities at increasing caldesmon concentrations before sedimentation produced a linear relationship, the equation of which was used to determine the free caldesmon concentration after sedimentation.

*Determination of ATPase Activity.* The effect of native caldesmon and recombinant caldesmon peptides on actin and actin−tropomyosin activation of the heavy meromyosin ATPase was determined at 25 °C in 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 1−2.5 mM $MgCl_2$, and 1 mM DTT with 1 $\mu$M rabbit skeletal HMM. Protein mixtures were prepared in a final volume of 100 $\mu$L, and the reactions were started by addition of MgATP to 5 mM. After termination of the reactions with 0.5 mL of 10% trichloroacetic acid, the $P_i$

[1] Abbreviations: $\phi$, rhodamine phalloidin; HMM, heavy meromyosin.

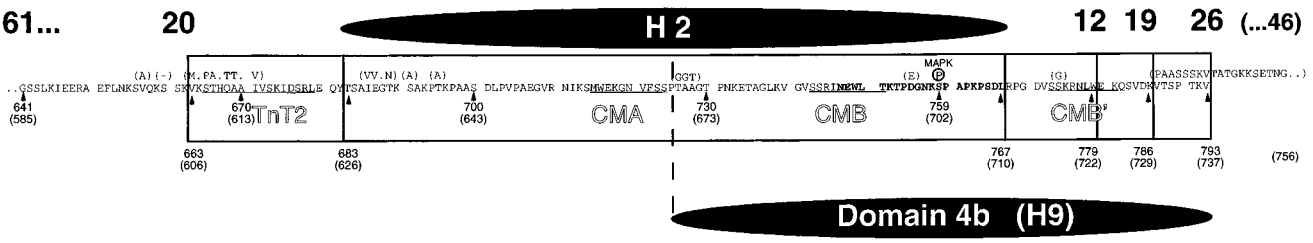


FIGURE 1: Amino acid sequence of caldesmon domain 4 and the position of the recombinant peptides. The amino acid sequence of the C-terminus of human caldesmon is shown. Residues which are different in the chicken sequence are shown above the human sequence in parentheses. Domain 4 is indicated by the box around the sequence. Divisions within the box indicate the termini of the recombinant peptides. The H2 sequence is indicated above, and the location of the N- and C-termini of the other recombinant peptides is indicated at either side of the bar. The minimum sequence of domain 4b, as defined by the H9 peptide, is shown below. The sequence in domain 4b proposed as an "essential" determinant for tropomyosin-dependent inhibition is shown in boldface type The calmodulin binding sites CMA, CMB, and CMB' (Huber et al., 1996; Marston et al., 1994) and the putative tropomyosin binding site with troponin T2 homology (TnT2) (Huber et al., 1995; Hayashi et al., 1991) are underlined. The MAP kinase phosphorylation site is indicated (Redwood et al., 1993).

Table 1: Number of Amino Acids, Sequences, Expression Yield, and Actin Binding Affinities of Recombinant Human Caldesmon Peptides[a]

| recombinant fragment | symbol on graph | total amino acids | sequence (human) | sequence (chicken) | yield (mg/L of culture) | actin binding affinity ($M^{-1} \times 10^6$) |
|---|---|---|---|---|---|---|
| caldesmon *h* | ⊞ | 793 | | | | 0.3 |
| H2 | ▲ | 85 | 683−767 | 626−710 | 7.3 | 0.039 |
| 61+H2 (H7) | □ | 146 | 622−767 | 566−710 | 1.1 | 0.196 |
| 20+H2 | ■ | 105 | 663−767 | 606−710 | 9.0 | 0.263 |
| H2+12 | △ | 97 | 683−779 | 626−722 | 19.0 | 0.109 |
| H2+19 | ◆ | 104 | 683−786 | 626−729 | 25.8 | 0.122 |
| H2+26 | ◇ | 111 | 683−793 | 626−737 | 8.0 | 0.144 |
| H9 | ● | 68 | 726−793 | 663−737 | 6.8 | 0.051 |

[a] The table also gives a key to the symbols used in the figures.

released was measured by the method of Taussky and Schorr (1953).

*In Vitro Motility Assay.* Rabbit skeletal muscle actin was labeled with rhodamine phalloidin ($\phi$) as described by Kron et al. (1991). Actin·$\phi$-tropomyosin and actin·$\phi$-tropomyosin−caldesmon peptide complexes were prepared at 10× assay concentrations and diluted immediately before use. The assay procedure and recording of filament movement were carried out according to our reported methods (Marston & Fraser, 1995b). Filament movement was analyzed using an automatic tracking program recently described by Marston et al. (1996).

## RESULTS

*Design of Recombinant Sequences from Caldesmon Domain 4.* Recombinant sequences were derived from a human caldesmon cDNA (Huber et al., 1993). Domain 4 is defined as the sequence from amino acid 663 (606 in the chicken sequence) to the C-terminus, since in previous work this sequence has been found to bind actin and tropomyosin and to inhibit actin and actin−tropomyosin activation of myosin ATPase in a manner indistinguishable from the intact caldesmon (Redwood & Marston, 1993). Recombinant sequences were based on the H2 sequence (see Figure 1 and Table 1). H2 was extended to the N-terminus of domain 4 in peptide 20+H2. The previously studied peptide H7 (61+H2 in this notation) was also investigated (Marston et al., 1994a). H2 was also extended toward the C-terminus by 12 amino acids (H2+12) to the C-terminus of the inhibitory peptide LW30 (Mezgueldi et al., 1994), by 19 amino acids (H2+19) to include the postulated "strong regulatory site" (Wang et al., 1996), and by 26 amino acids to the C-terminus (H2+26). For comparison, we also studied whole caldesmon, the whole of domain 4 as represented by the chicken recombinant peptide 606C (Redwood & Marston, 1993) (20+H2+46 in this notation) and domain 4b as represented by the peptide H9 (Marston et al., 1994b).

*Binding of Recombinant Caldesmon Peptides to Actin and Their Effect on the Actin-Activated HMM ATPase.* The actin binding properties of the caldesmon peptides were determined by cosedimentation. All the peptides were able to bind to actin with affinities in the range $(0.4-3) \times 10^5\ M^{-1}$ (Table 1). In general, the longer the sequence the higher the affinity; however, there were two notable exceptions: H2, representing the central two-thirds of domain 4, bound actin less well than other peptides of comparable size; and 20+H2 bound to actin with an affinity higher than the trend.

The effects of the peptides on the actin-activated myosin ATPase are shown in Figure 2A,B. All the peptides inhibited the ATPase, and the potency of inhibition paralleled the affinity for actin (compare with Table 1). Figure 3 shows the inhibition of actin activation related to the amount of caldesmon bound to actin for each peptide. Significantly, the inhibitory activity of all the peptides is the same and comparable to native caldesmon, with full inhibition being observed at approximately one caldesmon peptide bound per actin monomer. Thus, all the peptides, although containing different regions of domain 4, behave the same when bound to actin.

*Binding of Recombinant Caldesmon Peptides to Actin−Tropomyosin and Their Effect on the Actin−Tropomyosin Activated HMM ATPase.* All the peptides were found to bind actin−tropomyosin with a higher affinity than that determined with actin alone when the data were fitted to a simple binding curve. However, it has been demonstrated that the binding of caldesmon and caldesmon domain 4 fragments to actin−tropomyosin can be better accounted for on the basis of two independent binding sites on actin, one

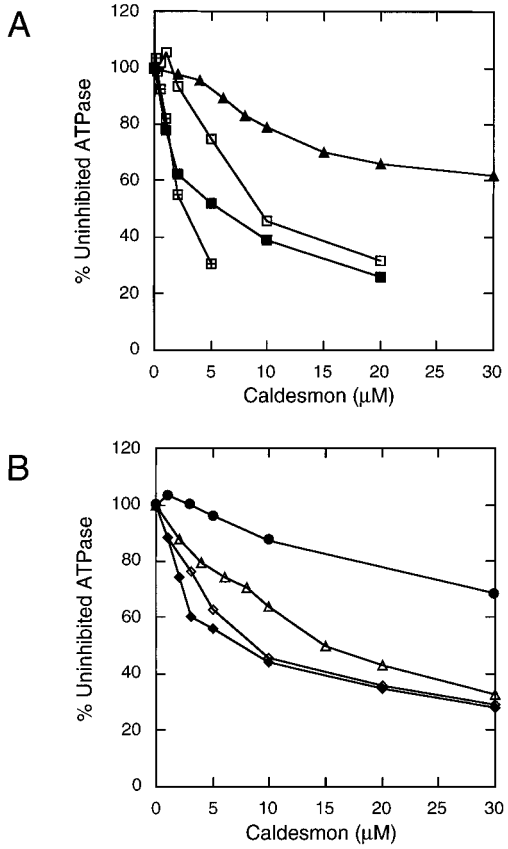


FIGURE 2: Effect of the recombinant peptides of human caldesmon on the actin-activated HMM Mg ATPase. (A) H2 (▲), 61+H2 (□), 20+H2 (■), and caldesmon *h* (open box with plus sign). (B) H9 (●), H2+12 (△), H2+19 (◆), and H2+26 (◇). Conditions: 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT, 25 °C, 1 $\mu$M skeletal muscle HMM, 12 $\mu$M actin, and 0−30 $\mu$M caldesmon peptide. Uninhibited ATPase rate was 2.0 $s^{-1}$.

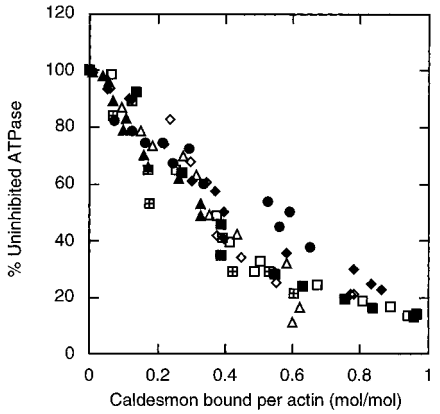


FIGURE 3: Relationship between inhibition of actin activation of HMM MgATPase by the recombinant caldesmon peptides and the amount of peptide bound to actin. Conditions: 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT, 25 °C, 1 $\mu$M skeletal muscle HMM, 12 $\mu$M actin, and 0−30 $\mu$M caldesmon peptide. In parallel with the measurement of ATPase activity, the amount of peptide bound to actin was determined by cosedimentation as described under Materials and Methods. Symbols are H2 (▲), 61+H2 (□), 20+H2 (■) , H2+12 (△), H2+19 (◆), H2+26 (◇), caldesmon *h* (open box with plus sign), and H9 (●).

with a stoichiometry of 1 per actin and an affinity similar to binding to actin in the absence of tropomyosin plus an additional low stoichiometry, high-affinity tropomyosin-dependent binding component (Smith et al., 1987; Yamakita et al., 1992; Redwood & Marston, 1993). Inspection of our binding curves showed that the points did not fit well to a single binding equation in the presence of tropomyosin; in Figure 4A, it is apparent that points tend to be to the left of the fitted line at low concentrations and to the right at higher concentrations as would be expected if the binding was to two independent classes of sites. Resolution of the two binding sites requires measurements to be made over a very large concentration range including concentrations down to 0.1 $\mu$M, which is only possible using radioactively labeled caldesmon. However, since previous experimentation has shown that the low stoichiometry tropomyosin-dependent binding is additional to and independent of binding to actin alone, it is legitimate to subtract the low-affinity actin binding from the binding to actin−tropomyosin in order to calculate the additional binding that occurs in the presence of tropomyosin. Figure 4A shows this calculation for H9 which, because of its low actin binding affinity, permits us to make measurements at a low level of saturation using the gel scanning assay. We found that an additional small component (0.15−0.2 caldesmon bound per actin) of high-affinity ($K_d$ < 1 $\mu$M) extra binding was present with all the recombinant peptides that were inhibitory (Figure 4B) with the exception of H2+19 which exhibited a larger component at lower apparent affinity (data not shown).

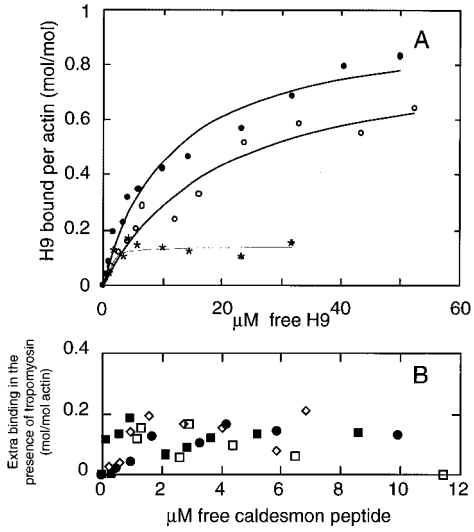


FIGURE 4: Binding of the H9 fragment to actin and actin−tropomyosin and the tropomyosin-dependent high-affinity binding. Conditions were as described for Figure 3 except smooth muscle tropomyosin was incorporated at a 0.4:1 (w/w) ratio with actin. (A) H9 binding was determined by cosedimentation in the absence (○) and presence (●) of tropomyosin. The curved lines are the best fits of the data to the simple binding equation $B = B_{max}$[caldesmon]/($K_d$ + [caldesmon]). For actin binding, $B_{max} = 0.86 \pm 0.06$ mol of H9/mol of actin and $K_d = 19.7 \pm 4.9$ $\mu$M. The data for binding in the presence of tropomyosin deviate from the fitted curve, indicating two classes of binding sites. The difference data (*) were calculated as the difference between the amount of H9 bound in the presence of tropomyosin and the curve fitted to the data for H9 binding to actin. A simple binding equation fit (fine line) yields $B_{max} = 0.14 \pm 0.01$ per actin and $K_d = 0.93 \pm 0.58$ $\mu$M. (B) The high-affinity tropomyosin-dependent binding component for 61+H2 (□), 20+H2 (■), H2+26 (◇), and H9 (●) was calculated as described for panel A.

The caldesmon peptides had different effects on the actin−tropomyosin-activated HMM ATPase activity. The H2 peptide had an unexpected effect in that it increased the ATPase in the presence of smooth muscle tropomyosin

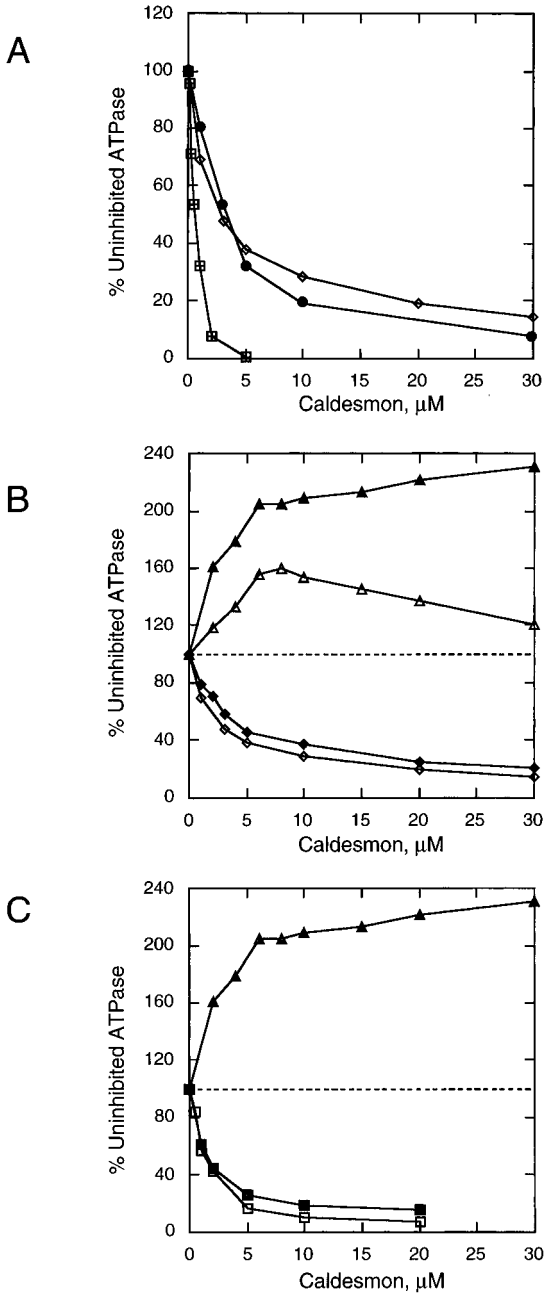


FIGURE 5: Effect of the recombinant peptides of human caldesmon on the actin−tropomyosin activated ATPase. (A) Peptides with intact C-terminus, caldesmon *h* (open box with plus sign), H9 (domain 4b) (●), and 606C (domain 4: 20+H2+46) (◇). (B) H2 (▲) and C-terminal extensions H2+12 (△), H2+19 (◆), and H2+26 (◇). (C) H2 (▲) and N-terminal extensions 20+H2 (■) and 61+H2 (□). Conditions: 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT, 25 °C, 1 $\mu$M skeletal muscle HMM, 12 $\mu$M actin−tropomyosin (w/w ratio 1:0.4), and 0−30 $\mu$M caldesmon peptide. Uninhibited ATPase rate was 3.5 $s^{-1}$.

(Figure 5B). Maximal ATPase activity was observed with 30 $\mu$M added H2 where the rate was increased 2.3-fold. Another peptide, H2+12, was also able to increase ATPase activity, but maximal increase in activation was less at 1.6-fold and ATPase activity began to decrease at concentrations above 8 $\mu$M added.

The inhibitory property of caldesmon was restored in peptide H2+19 (Figure 5B). This peptide inhibited the actin−tropomyosin-activated ATPase almost as well as H2+26 which contains the native C-terminus of human caldesmon. Figure 5A shows inhibition by caldesmon, H9

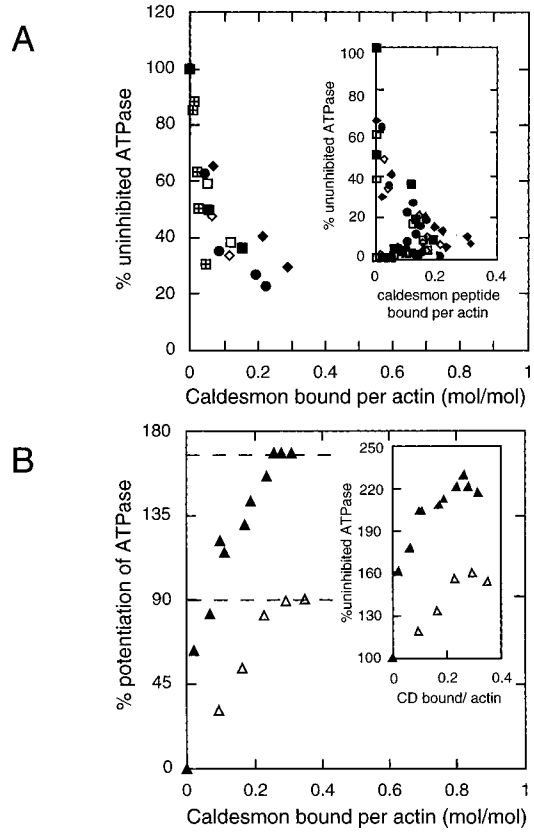


FIGURE 6: Relationship between inhibition (A) and potentiation (B) of actin−tropomyosin activation by the caldesmon peptides and the amount of peptide bound to actin−tropomyosin. In parallel with the measurement of ATPase activity, the amount of peptide bound to actin−tropomyosin was determined by cosedimentation as described under Materials and Methods. For key to symbols, see Table 1. (A) Inhibition vs binding for caldesmon *h* (open box with plus sign), H9 (●), 61+H2 (□), 20+H2 (■) , H2+19 (◆), and H2+26 (◇). The main figure shows inhibition plotted against the total caldesmon bound per actin. The inset shows inhibition plotted against the tropomyosin-dependent binding, calculated as in Figure 4. (B) Potentiation vs binding for H2 (▲) and H2+12 (△). The "% potentiation" values for H2 and H2+12 were derived by subtraction of the actin-activated ATPase rate at each peptide concentration from the actin−tropomyosin-activated ATPase rate. Dotted lines show the plateau level of activation. Conditions: 10 mM KCl, 5 mM $K_2$PIPES, pH 7.0, 1−2.5 mM $MgCl_2$, 1 mM DTT, 25 °C, 1 $\mu$M skeletal muscle HMM, 12 $\mu$M actin−tropomyosin (w/w ratio 1:0.4), and 0−30 $\mu$M caldesmon peptide. Inset: Actin−tropomyosin-activated ATPase activity plotted against binding of H2 and H2+12.

(domain 4b), and 606C (20+H2+46) in comparison. Inhibitory function was also restored by addition of sequence to the N-terminus of H2. 20+H2 and 61+H2 were both found to be potent inhibitors of the actin−tropomyosin-activated ATPase (Figure 5C).

Caldesmon has been shown to be capable of inhibiting actin−tropomyosin activation at a very low stoichiometry of 1 caldesmon bound for every 10−14 actin monomers over a wide range of conditions (Marston & Redwood, 1993). Figure 6A shows the relationship of inhibition to binding for the recombinant caldesmon peptides that retain inhibitory function. Inhibition does not correlate with such a low caldesmon peptide:actin ratio as with native caldesmon, but it is significantly lower than in the absence of tropomyosin. 61+H2, 20+H2, H2+26, and domain 4b (H9) inhibit >70% in the range of one caldesmon peptide for every seven actin monomers, whereas for H2+19 the stoichiometry is ap-

proximately 2-fold higher. The data shown in Figure 6A plot the total caldesmon peptide bound which is made up of actin binding plus the high-affinity tropomyosin-dependent binding. The decreasing slope of the trend on the points at high percentage inhibition is likely to be due to a large component of caldesmon peptide−actin binding since with most of these peptides the difference in affinities of the two classes of binding sites are less than with intact caldesmon. When inhibition data were plotted against the tropomyosin-dependent binding alone, the data were shifted such that 80−90% inhibition correlated with a lower stoichiometry more comparable with that shown for whole caldesmon (see inset to Figure 6A).

*Characterization of the Potentiating Property of H2 and H2+12. (i) Stoichiometry of Potentiation.* The tropomyosin-dependent activation caused by H2 and H2+12 appears to be maximal at concentrations less than the actin concentration (Figure 5B). At higher caldesmon peptide concentrations, the ATPase activity decreased in parallel with the inhibition observed without tropomyosin (compare Figure 2A,B with Figure 5B). Thus, in the presence of tropomyosin, the ATPase activity appears to be the net result of actin-dependent inhibition and actin−tropomyosin-dependent potentiation. Additional evidence for the independence of activating and inhibiting effects is given by our previous observation that when actin−tropomyosin activation of ATPase is fully activated (potentiated) by the addition of NEM·S-1 H2 cannot activate but only inhibits in a manner exactly parallel to the effect of H2 on actin alone (Marston et al., 1994a). When we subtracted the actin-activated ATPase rate from the actin−tropomyosin-activated rate at each peptide concentration, the difference, which we have termed potentiation, increased to a plateau at about 30 $\mu$M H2 and 15 $\mu$M H2+15 and was maintained at higher concentrations. Figure 6B shows this net tropomyosin-dependent activation related to the amount of H2 or H2+12 bound to actin−tropomyosin. In both cases, the ATPase reaches the plateau of activation at the relatively low stoichiometry of approximately one caldesmon bound per four actin monomers. It should be noted that if the total ATPase activity is plotted in this way, the peak activation is still reached at a low ratio of caldesmon peptide bound to actin−tropomyosin (inset to Figure 6B).

*(ii) Functional Competition between Activating and Inhibiting Peptides.* It has been demonstrated previously that the mechanism by which H2 activates actin−tropomyosin activation is similar to that mediated by NEM−S-1. H2 switches the actin−tropomyosin filament to the "on" state, while the inhibitory action of caldesmon is a directly opposing switch of the thin filament to the "off" state (Marston et al., 1994a). The results shown in Figure 7A suggest that activating peptides like H2 may interact with the same sites on actin−tropomyosin that are involved in caldesmon inhibition. In the presence of tropomyosin, H2 is shown to be capable of reversing the inhibition of ATPase activity by native caldesmon, 61+H2, and H9. In the absence of tropomyosin, however, H2 did not reverse caldesmon or 61+H2 inhibition (Figure 7B), and, in agreement with H2 being an inhibitor of actin activation in its own right (Figure 2A), it reduced the ATPase further from its partly inhibited level.

*(iii) Effect of Activating Peptides on in Vitro Motility.* Smooth muscle tropomyosin has been shown to activate actin

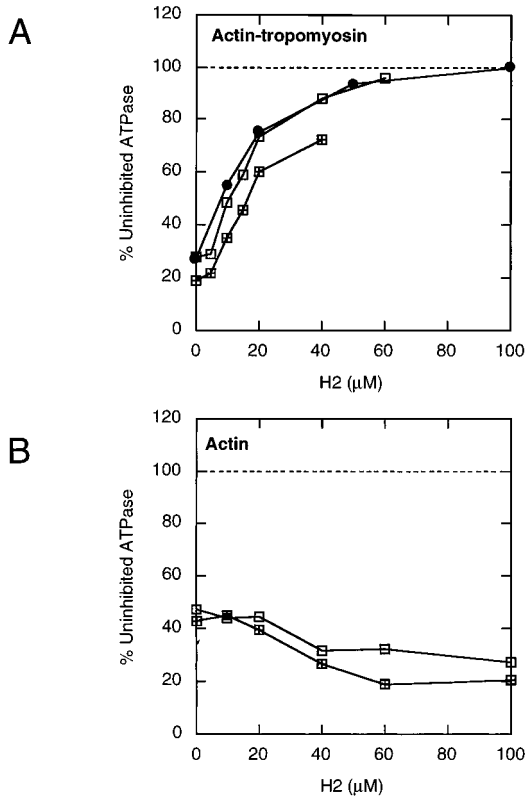


FIGURE 7: Effect of H2 on inhibition of ATPase activity by caldesmon, 61+H2, and H9. Conditions: 10 mM KCl, 5 mM $K_2$-PIPES, pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT, 25 °C, 1 $\mu$M skeletal muscle HMM, 12 $\mu$M actin or actin−tropomyosin (w/w ratio 1:0.4). (A) In the presence of tropomyosin. The uninhibited ATPase rate was 3.0 $s^{-1}$. 1 $\mu$M native caldesmon, 5 $\mu$M 61+H2 (□), and 16 $\mu$M H9 (●) inhibited the ATPase rate by 70−80%. Addition of H2 reversed inhibition. (B) In the absence of tropomyosin. The uninhibited ATPase rate was 2.9 $s^{-1}$. 2 $\mu$M native caldesmon (open box with plus sign), and 10 $\mu$M 61+H2 (□) inhibited the ATPase rate by 66 and 52%, respectively. Addition of H2 caused further slight inhibition parallel to the effect of H2 alone (see Figure 2).

activation of the myosin ATPase under most conditions *in vitro*, and our results demonstrate that both H2 and H2+12 can increase this activation further (Figure 5B). In the *in vitro* motility assay, the activating property of smooth muscle tropomyosin is manifested as an increase in velocity of actin−tropomyosin filaments relative to actin alone and that velocity can be increased still further by adding NEM·S-1. In contrast caldesmon and its inhibitory peptides decrease the fraction of actin−tropomyosin filaments motile with a slight reduction of velocity (Fraser & Marston, 1995b).

We compared the effect of H2, H2+12, and H2+26 on actin−tropomyosin filament motility using the same procedure as in our previous work. Figure 8 shows that H2+12 was able to increase actin−tropomyosin filament velocity by 44% from 3.5to 5.0 $\mu$m/s at 200 nM, while H2 increased the velocity to 4.3 $\mu$m/s at 600 nM, reflecting its weaker binding. A decrease in velocity was observed at higher H2+12 concentrations, which mirrored the effect of H2+12 on the actin−tropomyosin-activated ATPase shown in Figure 5B. H2 and H2+12 had little effect on either the proportion of filaments motile or the density of filaments attached to the immobilized myosin. In contrast, H2+26 behaved exactly like whole caldesmon: it decreased the fraction of filaments motile at a constant or slightly reduced velocity.

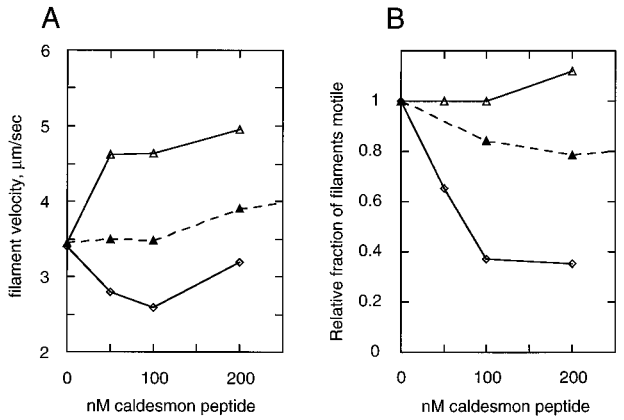


FIGURE 8: Effect of H2, H2+12, and H2+26 on actin−tropomyosin filament motility parameters in the *in vitro* motility assay. Actin·$\phi$-tropomyosin−H2 (▲), −H2+12 (△), and −H2+26 (◇) complexes were formed at a range of caldesmon peptide concentrations and infused into a motility cell containing immobilized skeletal muscle HMM. MgATP was added, and the movement of filaments was recorded. Filament motility was analyzed as described by Marston et al. (1996). Approximately 500 filament vectors were measured. The filament velocities had a Gaussian distribution with a standard deviation approximately one-third of the average velocity. Average filament velocity, nm/s (A), and the change in fraction of filaments motile expressed relative to the value for actin−tropomyosin (B) are plotted in this figure. Actin·$\phi$-tropomyosin filament velocity was increased significantly by incorporation of H2+12 up to 200 nM and by higher concentrations of H2 with little change in the fraction of filaments motile. H2+26 reduced the fraction of filaments motile but did not enhance velocity in the same way as intact caldesmon.

## DISCUSSION

The C-terminal 150 amino acids of caldesmon (domain 4) form an inhibitory complex with actin and tropomyosin, which is regulated by $Ca^{2+}$ and calmodulin (Sobue & Sellers, 1991; Marston & Huber, 1996). We have used bacterially expressed recombinant peptides of human caldesmon to provide insight into the structural organization of this regulatory domain. Previous work has defined a single site near the C-terminus of caldesmon which is essential for actin binding and inhibition (Wang et al., 1996; Wang & Chacko, 1996), but there is evidence for additional essential sites within caldesmon domain 4 (Hayashi et al., 1991; Wang et al., 1991; Marston et al., 1994; Chalovich et al., 1992) which the technique of successive deletions from the C-terminus cannot define. Furthermore, structural evidence indicates that domain 4 is likely to be a highly folded and flexible structure with several separate segments contributing to the actin binding site (Mornet et al., 1995; Czurylo et al., 1993; Huber et al., 1995). This complex relationship between the sequence represented by a peptide and its function suggests the involvement of up to three separate and interdependent regions of domain 4. In this case, making internal deletions, as in Wang and Chacko's experiments (1996), would be inappropriate since they could disrupt structure remote from the site of deletion. Therefore, in order to locate the regulatory segments and to understand how they interact, we have studied a set of long peptides derived from caldesmon domain 4 with deletions from both the N-terminus and the C-terminus, and we have related the functional effects of these peptides to their binding to actin and actin−tropomyosin. The starting point for this study is H2, an 85 amino acid actin binding segment from the center of domain 4 (see Table 1, Figure 1), since it is known to have lost its ability to inhibit actin−tropomyosin activation of myosin ATPase (Marston et al., 1994a,b).

*Actin−Caldesmon Interaction.* It is generally agreed that all the actin binding sites of caldesmon in the $10^5-10^6$ $M^{-1}$ affinity range are located within domain 4 [reviewed in Marston and Huber (1995)]. Most of caldesmon's contacts with actin have been mapped to actin subdomain 1 (Mornet et al., 1995; Crosbie et al., 1994; Levine et al., 1990; Hodgkinson et al., 1997). This binding is proposed to block the attachment of myosin·ADP·$P_i$ and thereby inhibit the actomyosin ATPase cycle, and it is necessary to bind caldesmon to every actin to completely inhibit ATPase activation in this way (Marston et al., 1994a; Horiuchi et al., 1991; Marston & Redwood, 1993; Chalovich et al., 1987; Velaz et al., 1989). Our results indicate that this is also true for all the recombinant peptides of domain 4 that we tested; thus, they all occupy the same site on actin.

The affinities of the different peptides are roughly related to their size (Table 1) except for an indication that the center of domain 4 (the H2 peptide) binds actin relatively poorly while regions at the C-terminus and more particularly at the N-terminus (the extra 20 amino acids, 663−682, present in 20+H2) show stronger binding. This is in accord with earlier reports of two separate actin binding regions at either end of domain 4 (Hayashi et al., 1991; Wang et al., 1991; Chalovich, 1992).

*Inhibition of Actin−Tropomyosin Activation of Myosin ATPase.* The inhibitory interaction of caldesmon with actin−tropomyosin filaments is fundamentally different from inhibition of actin filaments. The role of caldesmon is to switch the actin−tropomyosin filament between an active, ON conformation, and an inhibited OFF conformation and biochemically this mechanism is indistinguishable from that of troponin. Moreover, the peptides 606C, 61+H2, and H9 have been shown to inhibit the strong myosin binding site and regulate filament motility like whole caldesmon (Marston et al., 1994a; Marston & Huber, 1995; Fraser & Marston, 1995b). All the inhibitory peptides from domain 4 exert their maximum inhibition at low stoichiometry like whole caldesmon and whole domain 4 (606C: 20+H2+46) (Figure 6A), and they all show a small component (0.1−0.2 per actin) of high-affinity, tropomyosin-dependent binding (Figure 4B). Clearly the peptides are binding to the complex $(actin_7 \cdot Tm)_n$ with the stoichiometry being defined by a specific site on tropomyosin (Phillips et al., 1986; Marston & Redwood, 1993; Hnath et al., 1996). Thus, when actin−tropomyosin is inhibited by caldesmon peptide bound at 1 per 7−10 actins, most actin monomers are not occupied by caldesmon peptide and can still bind further caldesmon peptide with an affinity the same as to pure actin, although this does not necessarily lead to further inhibition.

Although all the peptides examined showed tropomyosin-enhanced regulation of actin activation of ATPase activity, there is no correlation with their binding to tropomyosin. Thus, H2 (activator) and 61+H2 (inhibitor) have a high affinity for tropomyosin while H9 (inhibitor) has no detectable binding (Huber et al., 1995). If caldesmon can bind to actin and influence the state of the tropomyosin strand without direct association, it must induce some type of conformational change in the actin (Wu et al., 1997). A mechanism of this type could also account for the unusual properties of peptides H2 and H2+12.

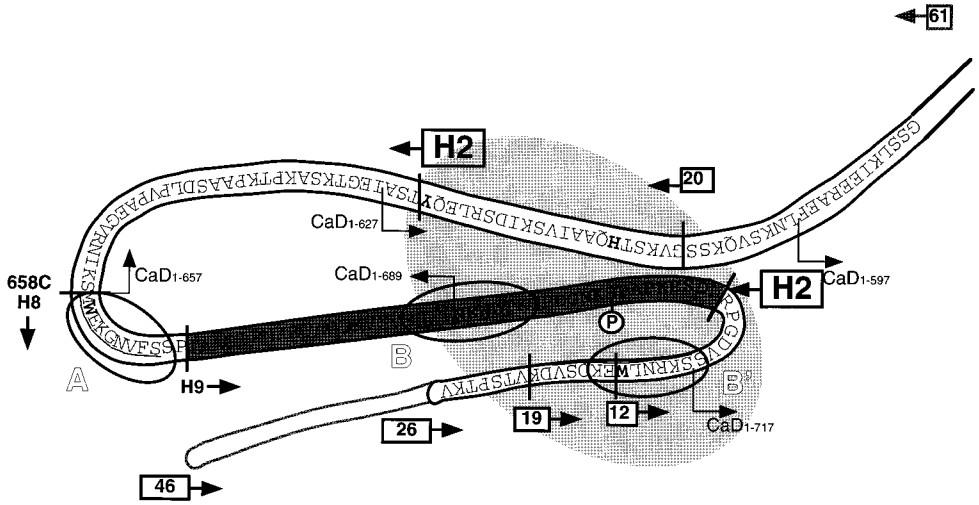


FIGURE 9: Model for the structure of the inhibitory conformation of domain 4 of caldesmon. The termini of the peptides are indicated by lines on the sequence and identified in the boxes. The locations of Wang et al.'s C-terminal deletions in this region [$CaD_{1-657}$ etc. (Wang et al., 1996)] are also indicated. The shaded region of the sequence indicates the region common to all the recombinant peptides, and the boldface sequence within it corresponds to the "essential" central sequence described under Discussion. Calmodulin binding sites A, B, and B' are circled. The gray shaded circle indicates the suggested boundary of the actin binding site. The site of MAP kinase phosphorylation is indicated, and the C-terminal extension present in chicken caldesmon is outlined in gray. Individual boldface amino acids refer to those residues found to be close to Cys 636 (580) (Mornet et al., 1995).

*Potentiation of Actin−Tropomyosin by the Central Segment of Domain 4.* The effect of H2 and H2+12 on actin-activated myosin ATPase activity is just like whole caldesmon and all the other peptides from domain 4 which bind and inhibit fully at a stoichiometry of around 1:1 (Figures 2 and 3). When added to actin+caldesmon mixtures which are partially inhibited, H2 and H2+12 increase the inhibition (Figure 7B). This suggests that, like whole caldesmon, they block the weak myosin binding site (Marston & Redwood, 1993).

The unusual behavior of H2 and H2+12 is manifested only when tropomyosin is present on the actin filament. They are not simply unable to inhibit; rather they potentiate actin−tropomyosin activation of myosin ATPase and increase the velocity of filaments in the motility assay (Figures 5B and 8). Since the activation is absolutely dependent upon tropomyosin and saturates at a low level of occupancy like tropomyosin-dependent inhibition (one H2 or H2+12 per four actins, Figure 6B), the most probable mechanism for activation is likely to be mediated by control of the same actin−tropomyosin ON−OFF equilibrium (Marston et al., 1994a; Fraser, 1995). We suggest that H2 and H2+12 increase the probability that actin−tropomyosin will be in the ON state, whereas the inhibitory peptides increase the probability that actin−tropomyosin will be in the OFF state. This idea is supported by several lines of evidence.

The competition experiments showed that adding H2 to actin−tropomyosin inhibited by caldesmon, H9, or 61+H2 reverses the inhibition (Figure 7A); thus, H2 appears to compete for a site on actin which is essential for tropomyosin-dependent inhibition. In addition, both the H2 activation effect and the tropomyosin-dependent inhibition by domain 4 fragments are strongly dependent upon salt concentration, whereas inhibition of actin is not (Fraser, 1995; Fraser & Marston, 1996a).

Direct evidence is provided by comparison with the effects of *N*-ethylmaleimide-modified subfragment-1 (NEM·S-1). Both H2 and NEM·S-1 potentiate actin−tropomyosin activation at substoichiometric concentrations but are slightly inhibitory upon actin activation. We propose that H2 and H2+12 act in the same way as NEM·S-1 since if actin−tropomyosin activation is first potentiated by substoichiometric NEM·S-1 and then H2 is added activation is absent and H2 inhibits in parallel with its inhibition of pure actin (Marston et al., 1994a). It is well established that NEM·S-1 acts as a rigor crossbridge in the presence of ATP and thereby switches actin−tropomyosin toward the ON (or potentiated) state (Nagashima & Asakura, 1982).

The effect of H2 and H2+12 upon *in vitro* movement of actin−tropomyosin filaments is to increase filament velocity while not affecting the fraction of filaments that are motile or displacing filaments from the myosin surface. Again the effect is absolutely dependent upon tropomyosin. We have observed that the only way in which actin−tropomyosin filament velocity can be increased in these conditions is by agents which switch filaments toward the ON state, i.e., troponin at pCa5 and NEM·S-1 (Fraser & Marston, 1995a; Marston & Fraser, 1996b).

The activating property of the H2 and H2+12 peptides is unique among the peptides of caldesmon, while tropomyosin-dependent inhibition is observed with peptides having additional C- or N-terminal sequence. This indicates that three interacting actin binding segments of domain 4 are involved in inhibition.

*The Central Actin Binding Segment.* An actin binding segment essential for inhibition is located in H2, and the domain 4b peptide H9 is inhibitory and so must also contain this sequence. The sequence common to H2 and H9 is 726−767 (663−710), which corresponds to the sequence coded by exon 11 in the genomic sequence (Hayashi et al., 1992) and is located at the C-terminal end of the H2 peptide (shaded area in Figure 9).

It is possible to identify a still shorter "essential" sequence, amino acids 747−767 (690−710), within this segment since Wang et al. (1996) found that on shortening the sequence from 1−774 (mutant $CaD_{1-717}$) to 1−746 (mutant $CaD_{1-689}$) tropomyosin-dependent inhibition was abolished. In addition, the inhibitory peptide LW30, described by Mezgueldi

et al. (1994), contains much of this sequence. The sequence is shown in boldface in Figures 1 and 9. In support of the functional importance of this segment, it should be noted that the "essential" sequence includes $Ser^{759(702)}$ that is the principal site for phosphorylation by MAP kinase. Phosphorylation of this serine, or mutation to aspartic acid, can affect inhibition of actin−tropomyosin activation (Redwood et al., 1993; Adam, 1996). The "essential" sequence also corresponds to the region 752−771 (695−714), which is similar to the actin binding sequence of myosin light chain kinase (Kobayashi et al., 1992), and it is particularly proline-rich, a motif identified as common to many protein−protein binding sites (Wiliamson, 1994).

*The C-Terminal Actin Binding Segment*. Recently Wang et al. identified a "strong regulatory site" spanning residues $^{718}$KRNLWE$^{723}$ of chicken caldesmon (775−780 in human) near the C-terminus of domain 4 (Wang & Chacko, 1996). This corresponds to the site shown as "CMB′" in Figures 1 and 9. Our results support this proposal since H2+12 does not contain this complete sequence and is not an inhibitor while H2+19, which does include the sequence, is an inhibitor. H2+26 is a more potent inhibitor than H2+19, and it needs less peptide bound per actin−tropomyosin for equivalent inhibition (Figure 6A). The seven amino acid extension contains the sequence $^{785}$DKVT$^{788}$ equivalent to chicken residues $^{728}$EKVT$^{731}$ which were proposed as necessary for maximal inhibition (Wang & Chacko, 1996). In chicken, there are a further 19 C-terminal amino acids not present in human caldesmon that appear to have no regulatory function. Thus, the C-terminal segment involved in actin binding and tropomyosin-dependent inhibition is in the region 770−793 (713−737) and includes the "strong regulatory site" [775−780 (718-723)].

*The N-Terminal Actin Binding Segment.* We have identified a further inhibitory actin binding sequence, 663−682 (606−625), at the N-terminus of domain 4 since addition of 20 amino acids N-terminal to H2 produces a peptide, 20+H2, which is inhibitory and extension by a further 41 amino acids (61+H2) does not increase the inhibitory potency or actin binding affinity (Table 1, Figure 5C). We have already noted that there is likely to be a strong actin binding site in this segment; furthermore, this segment has a region of homology with the T2 peptide of skeletal muscle troponin T (see Figure 1) and has been shown to include the only tropomyosin binding region in domain 4 (Huber et al., 1995; Hayashi et al., 1991).

This assignment is compatible with other laboratories. Wang et al. (1996) found a decrease in actin and actin−tropomyosin binding when the mutant 1−653(597) ($CaD_{1-597}$) was compared with the mutant 1−684(627) ($CaD_{1-627}$) (the locations of Wang et al.'s mutants are shown in Figure 9). Similarly, domain 4 fragments (606C, 20K) have a higher affinity for actin−tropomyosin than the shorter domain 4b fragments (658C, H9, 10K) (Redwood & Marston, 1993). The 7.3 kDa fragment of Chalovich et al. (1992), which can inhibit ATPase activity at high concentrations, also contains this sequence.

*A Model for the Regulatory Interaction of Three Segments of Domain 4 with Actin, Tropomyosin, and $Ca^{2+}$·Calmodulin.* Domain 4 of caldesmon is a compact but flexible structure (Czurylo et al., 1993; Levine et al., 1990). Although there is little evidence for extensive secondary structure, it is known that the peptide chain is folded since nuclear magnetic resonance measurements have shown that amino acids from all three putative inhibitory segments are within 1.5 nm of cysteine 636(580) that is at the junction between domains 3 and 4 (Mornet et al., 1995). Presumably caldesmon binding to actin−tropomyosin produces a structure in which caldesmon is stabilized and the conformation of actin is altered so that the tropomyosin is fixed in the OFF state.

Figure 9 shows a model of how the peptide chain of domain 4 might be folded when it is bound to actin−tropomyosin. We propose the three regions which contribute to inhibition are positioned to form a single actin−tropomyosin binding zone. The "essential" sequence between $Asn^{747(690)}$ and $Leu^{767(710)}$ is positioned in the center, and we suggest that the presence of either the N-terminal or the C-terminal actin binding segment in this zone is sufficient to maintain the inhibitory conformation. In the absence of N- and C-terminal segments, the H2−actin−caldesmon conformation is not constrained and is clearly functionally different in its interaction with tropomyosin. H2 may bind in a completely different and unphysiological manner from caldesmon. Alternatively, its bound structure may resemble the actin−tropomyosin−caldesmon structure when inhibition is reversed due to the $Ca^{2+}$ binding protein of the thin filaments (Pritchard & Marston, 1993) since the functional properties are very similar.

The reversal of caldesmon inhibition by $Ca^{2+}$·calmodulin is readily explained in this model since the important $Ca^{2+}$·-calmodulin binding sequence known as site B is located in the central "essential" sequence and calmodulin binding site B′ corresponds to the C-terminal "strong regulatory" sequence (Marston et al., 1994; Huber et al., 1996) (see Figures 1 and 9). $Ca^{2+}$·calmodulin binding, therefore, will profoundly affect the inhibitory actin binding conformation of domain 4. In support of this model, we have recently tested a mutant of caldesmon domain 4b in which the sequence of site B (part of the "essential" sequence), $^{748}$EWLTKT$^{753}$, is replaced by a nonsense sequence, PGHYNN. Binding to actin, actin−tropomyosin, and $Ca^{2+}$·calmodulin is little changed, but the coupling of binding to actin−tropomyosin inhibition is partially lost and the coupling of $Ca^{2+}$−calmodulin binding to reversal of inhibition is completely lost (Huber et al., 1997). Thus, sites that can be occupied either by actin or by $Ca^{2+}$·calmodulin could provide the regulatory switch of caldesmon.

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