The Binding of Distinct Segments of Actin to Multiple Sites in the C-Terminus of Caldesmon: Comparative Aspects of Actin Interaction with Troponin-I and Caldesmon†

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ABSTRACT: Thin-filament-based regulation of the contractile response is considered to involve the interaction of actin with troponin-I in striated muscle and the interaction of actin with caldesmon in smooth muscle. The nature of the interaction with actin of these inhibitory proteins has been studied by proton magnetic resonance spectroscopy using segments of caldesmon and troponin-I which mimic their functional properties. Caldesmon is shown to interact with two distinct sites on the N-terminal residues 1−44 of actin subdomain 1 with corresponding contacts on caldesmon domain 3 and domain 4 at its C-terminus. We demonstrate that, whereas inhibition by the troponin-I fragment (residues 96−117) is effected by its interaction with the N-terminal region of actin, the separate inhibitory ability of different regions of the C-terminus of caldesmon (domains 4a and 4b) is mediated by interaction with noncontiguous segments on subdomain 1 of actin. Our studies of the spatial relationship of these actin contacts on caldesmon further suggest that one molecule of caldesmon may associate with two actin monomers. The demonstrated interactive nature of these caldesmon attachments to distinct regions of actin is relevant to the mechanism of calcium modulation of inhibition of actomyosin ATPase by caldesmon.

The demonstration in vitro of calcium-regulated inhibition of actomyosin ATPase by caldesmon has led to suggestions that this protein has a role in the thin-filament regulation of contractile activity in smooth muscle [see reviews by Chalovich (1988), Marston and Redwood (1991), and Bryan and Wang (1993)]. Numerous in vitro studies have shown caldesmon to be capable of interacting with all the other components of the contractile protein assembly of smooth muscle (Szpacenko & Dabrowska, 1986; Marston & Redwood, 1991; Huber et al., 1994). The multiplicity of these docking interactions potentially enables caldesmon to cross-link the actin and myosin filaments. These caldesmon−protein associations have been correlated with specific regions of the primary structure of caldesmon through the use of defined proteolytic and recombinant fragments. Accordingly, a domain model has been used in describing structure−function relationships for the caldesmon molecule (Marston & Redwood, 1991; Redwood & Marston, 1993).

The actin and Ca^{2+}-calmodulin binding sites that appear to be involved in regulating the actomyosin ATPase in vitro are found in the C-terminal 35 kDa fragment of caldesmon, comprising domains 3 and 4 (Mornet et al., 1988a,b; Wang et al., 1991; Chalovich et al., 1992). Domain 4b alone, comprising the extreme C-terminal fragment of about 100 residues, also shows these functional properties (Bartegi et al., 1990; Redwood & Marston, 1993). In this respect, the C-terminal domain 4b of caldesmon possesses similar properties to a much shorter peptide segment of the skeletal muscle troponin-I (TnI), the TnI inhibitory peptide, which also has been shown to be capable of tropomyosin-potentiated inhibition of actomyosin ATPase activity (Syska et al., 1976). In both muscle types, interaction with actin underlies the mechanism for effecting inhibition. The interaction site on actin for the TnI inhibitory fragment, residues 96−117 of troponin-I, has been shown by NMR experiments to involve two specific contacts that encompass residues 1−7 and 24−25 on subdomain 1 of the actin monomer (Levine et al., 1988). Similarly, the corresponding surface of actin interacting with the 35 kDa caldesmon fragment (i.e., domains 3 and 4) has been shown by NMR methods to involve interaction with actin residues 1−44 (Levine et al, 1990). These observations suggest that the N-terminal region of actin is a common target for the two inhibitory proteins. Delineation of the nature and topography of the inhibitory protein−actin contacts should enable comparison of the structural mechanisms which operate in the two muscle systems to control the interaction of actin and myosin leading to high MgATPase activity.

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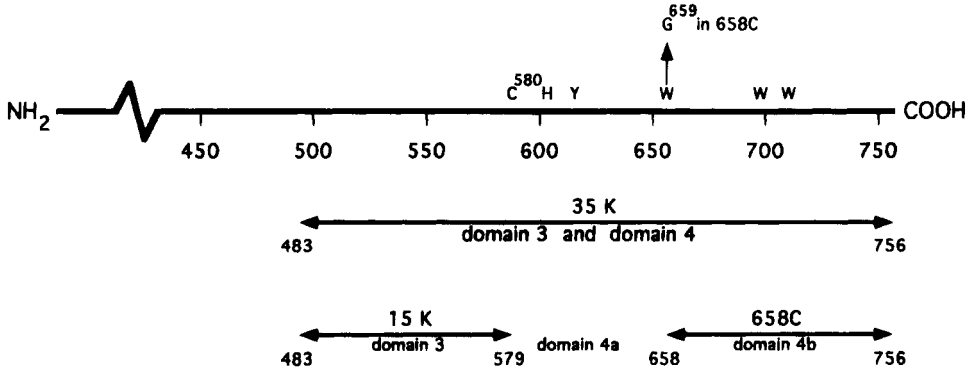

Thr	none	3	8
Trp	none	none	2 in 658C, 3 in native
Phe	1	2	1
Met	2	1	1
Tyr	none	1	none
His	none	1	none
Glu, Gln	31	7	11

FIGURE 1: Representation of the primary sequence of caldesmon showing the relationships of the C-terminal 35 kDa fragment and subfragments according to a proposed domain nomenclature (Redwood & Marston, 1993), with indications of the distribution of selected amino acids. These particular residue types are useful markers for identifying the locations of binding regions from NMR data.

Extending our earlier work (Levine et al., 1990), we have now gone on to define the second site of the two-pronged association between actin residues 1−44 and caldesmon. We report here on NMR spectral studies of the binding of a variety of caldesmon fragments to actin in the absence of tropomyosin (as the first step in the elucidation of the means by which tropomyosin brings about enhanced inhibition). These studies have enabled us to investigate the nature of the actin binding properties of inhibitory domain 4b (the 10 kDa C-terminal extremity of caldesmon) and the spatial relationship of these actin contacts to the sites on caldesmon involved in binding Ca^{2+}−calmodulin (CaM) with consequent release of inhibition. The data suggest that the actin interactions which underlie caldesmon's inhibitory activity in smooth muscle may entail bridging across two actin monomers. The data also enable us to compare the structural mechanism for the calcium-mediated relief of inhibition to that of the inhibitory segment of skeletal muscle troponin-I.

MATERIALS AND METHODS

Protein Preparations. Actin, fragments of actin (residues 1−18 and 16−41), the sequence-randomized homologue of actin residues 1−18, and the TnI inhibitory peptide (residues 96−115) were prepared and purified by the methods previously described (Levine et al., 1988). The proteolytically-derived 35 and 15 kDa C-terminal fragments of caldesmon were prepared and purified as previously described (Levine et al., 1990). Fragment 658C, comprising 99 residues (658−756) at the C-terminus of caldesmon, was produced by mutagenesis and bacterial expression techniques and purified as previously described (Redwood & Marston, 1993). Fragment 658C is functionally equivalent to a 10 kDa cyanogen bromide cleavage product from native caldesmon studied by Bartegi et al. (1990) even though Gly was substituted for Trp at position 659 to simplify NMR spectra and permit closer study of the involvement of Trp-692 and Trp-722 in protein−protein interactions. Residues are numbered according to the sequence of chicken gizzard caldesmon (Bryan et al., 1989).

Wheat germ calmodulin was prepared, purified, and spin-labeled at Cys-27 as previously described (Gao et al., 1992). The 35 kDa caldesmon fragment was spin-labeled at Cys-580 by the method used to spin-label calmodulin (Gao et al., 1992).

Nuclear Magnetic Resonance Experiments. One- and two-dimensional proton NMR spectra were obtained at 500 MHz on a Bruker AMX spectrometer using quadrature detection and at a sample temperature of 25 °C in ^2H_2O solution unless otherwise stated. The pH values quoted are direct meter readings using an Ingold Model 6030 electrode. Binding titrations were carried out by addition of small aliquots of a concentrated stock solution in order to keep dilution effects below 5%. Protein−protein interaction was generally detected by difference spectroscopy in order to highlight line broadening effects. In some titrations, two-pulse (180°−t−90°−t) spin-echo methods (t = 60 ms) were also used to enhance the ability to distinguish and classify by the coupling pattern closely spaced resonances in a one-dimensional spectrum.

RESULTS

Underlying much current work on caldesmon is a "mimetic" approach aimed at the definition of the minimal protein "domain" required to effect functional properties. The caldesmon fragments used in our present study, depicted in Figure 1, are a 35 kDa proteolytic fragment (domains 3 and 4), a 15 kDa proteolytic fragment (domain 3), and a recombinant fragment, 658C (domain 4b). While all these caldesmon analogues have been shown to interact with actin (Redwood & Marston, 1993), only the 35 kDa and 658C fragments are able to mimic the parent molecule's ability to inhibit actomyosin ATPase activity. In order to understand the mechanism of the actin−caldesmon system, it is necessary to define with precision the sites on actin and the regions of these different caldesmon fragments involved in the interaction between the two proteins.

Sites on the N-Terminal Region of Actin That Interact with Caldesmon. In our previous study (Levine et al., 1990), we found that the actin peptide spanning residues 1−44 interacts

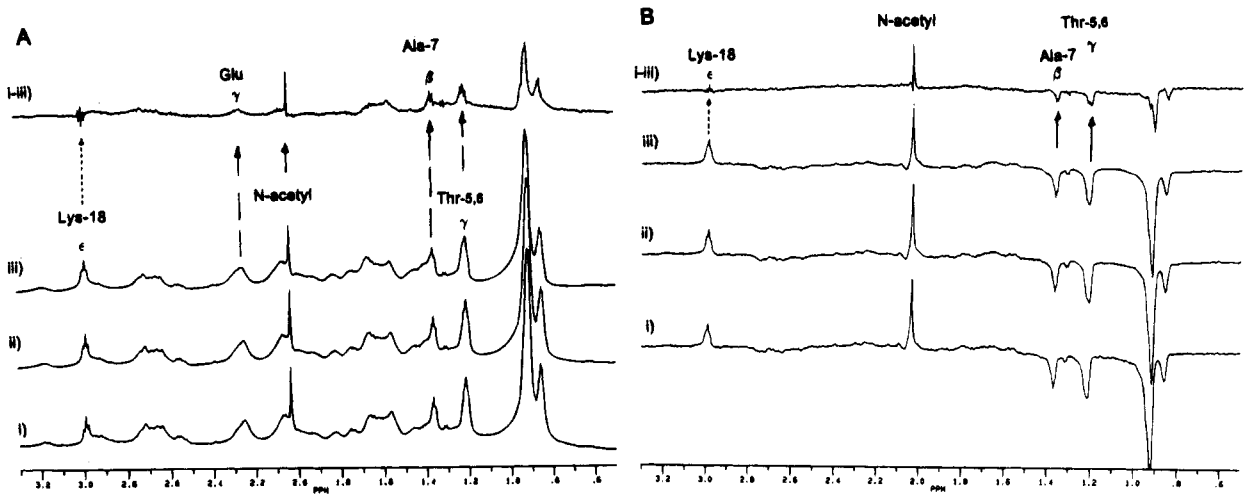

FIGURE 2: Binding of the C-terminal 35 kDa fragment of caldesmon to actin residues 1−18 involves contacts with the N-terminal extremity of the actin peptide. (A) Bottom trace (i): proton NMR spectrum of the peptide corresponding to residues 1−18 of actin (400 μM, pH 7.2). Middle traces (ii, iii): spectra after addition of substoichiometric amounts of the 35 kDa fragment of caldesmon (30 and 55 μM total concentration, respectively). Top trace: difference spectrum (i − iii) showing resonances from the actin peptide perturbed by the interaction with the caldesmon fragment, which correlate with specific N-terminal residues (e.g., the *N*-acetyl moiety, Ala-7, and Thr-5 and/or -6). (B) Parallel set of two-pulse spin-echo spectra, which discriminate in favor of signals deriving from molecular features having the greater mobility. Inverted signals in the bottom and middle traces are doublets, while upright signals are singlets or triplets.

with caldesmon, and that NMR signals from amino acid residues located at both the N- and the C-terminal regions of the peptide are perturbed. These results suggested a two-site attachment between actin residues 1−44 and caldesmon. One of these contacts was localized to domain 3 of caldesmon with the corresponding site on actin identified by the study of smaller actin peptides (residues 1−18, 16−41, and 28−56) to be in the region of residues 20−41. The location on the C-terminus of caldesmon of the interaction site for the N-terminus of actin residues 1−44 was not precisely defined in that previous study.

Titration of actin residues 1−18 with the 35 kDa fragment comprising caldesmon domains 3 and 4 results in the perturbation of a limited number of actin signals (Figure 2) which derive from the N-terminal region of the peptide sequence (Ac-DEDETTALV-). These observations confirm previous results obtained using actin residues 1−44 and are indicative of specific contacts with the 35kDa caldesmon fragment involving the N-terminal residues of this actin peptide. The residues making these contacts, including Ala-7 and Thr-5 and/or -6, are readily distinguished since they possess relatively high mobility in the free actin peptide (Figure 2B) and are observed to incur some loss of mobility as a result of the interaction. In order to validate the specificity of this peptide−protein interaction indicated by the differential relaxation effects that reflect the binding of the N-terminal region of the actin peptide to the 35 kDa caldesmon fragment, we have made use of a control peptide having the same amino acid composition as actin residues 1−18 but with its sequence randomized. No spectral perturbation was detectable upon titration of this control peptide, lending credance to the conclusion that the differential effects observed using actin residues 1−18 result from its specific interaction with the 35kDa caldesmon fragment. These data are in keeping with our previous observations on the two-site binding of actin residues 1−44 to caldesmon. We therefore conclude that caldesmon domains 3 and 4 which retain inhibitory function interact with separate sites at the N-terminal of actin.

Sites on Caldesmon Fragments with Inhibitory Activity That Interact with the N-Terminal Region of Actin. Titration of the caldesmon 35 kDa fragment with F-actin confirms that the actin contact sites involve distinct sequence locations on caldesmon domains 3 and 4 because signals from only certain residue types in caldesmon are perturbed by the binding of actin (Figure 3). Note, for example, the absence of effects on signals from His, Phe, or Tyr residues which occur in a variety of positions in the primary structure of domain 4. The interaction must involve domain 4, however, because of the contribution of signals from Trp and Thr, two residue types present only in this domain. In addition, domain 3, previously shown by us to interact with actin, is here confirmed to be involved because of the contribution of signals from three Met residues, only two of which occur in domain 4 (see Figure 1). Also implicated in this interaction with actin are other caldesmon residue types whose spectral signals are perturbed (e.g., Ala, Arg, Glu/Gln, Lys), which may derive from either N- or C-terminal regions of the 35 kDa fragment. In this context, it is interesting to note that Glu/Gln residues are much more abundant in domain 3 (33%) than in domain 4 (10%). The NMR assays of the binding of F-actin and actin peptides to the 35 kDa fragment therefore indicate that interaction of the C-terminus of caldesmon with F-actin entails specific contacts for actin's N-terminus on domains 3 and 4 of caldesmon.

From the fact that actin residues 20−41 interact specifically with caldesmon domain 3, while residues 1−18 fail to bind (Levine et al., 1990) but are here shown to interact with the 35 kDa fragment, we conclude that it is caldesmon domain 4 which provides the site of interaction for the N-terminal extremity of actin. In the structure of actin (Kabsch et al., 1990), residues 20−41 are depicted as receding from residues 1−7. The residue groupings identified as participating in actin's association with domains 3 and 4 of caldesmon thus derive from topographically distant zones on actin. Given the structural organization of domains 3 and 4 inferred from NMR studies, these data suggest that the 35 kDa fragment adopts a well-defined conformation that enables it to achieve such spatially distinct contacts on actin.

In order to shed light on the topography of the 35 kDa fragment that facilitates not only actin binding by caldesmon

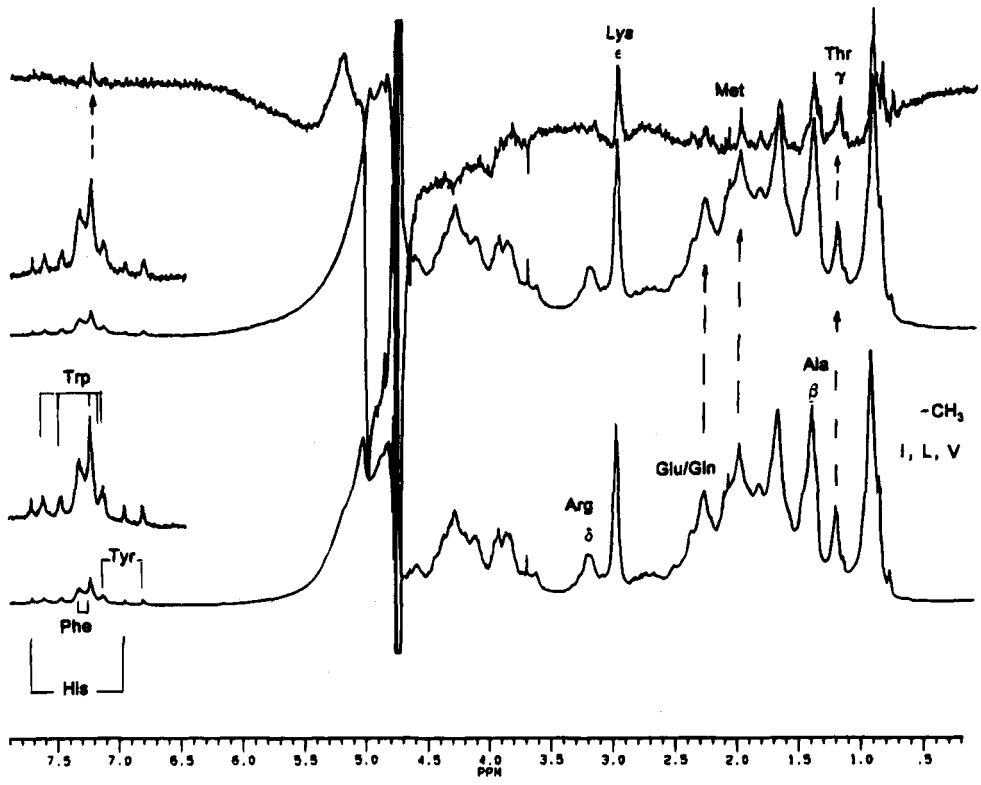

FIGURE 3: Binding of F-actin to the C-terminal 35 kDa fragment of caldesmon involves specific residue types in the latter. Bottom trace: proton NMR spectrum of the 35 kDa fragment of caldesmon (200 μM, pH 7.2). Middle trace: spectrum after addition of a substoichiometric amount of F-actin. Top trace: difference spectrum (bottom minus middle) showing resonances of the caldesmon fragment perturbed by the interaction with actin, which derive from a limited range of residue types.

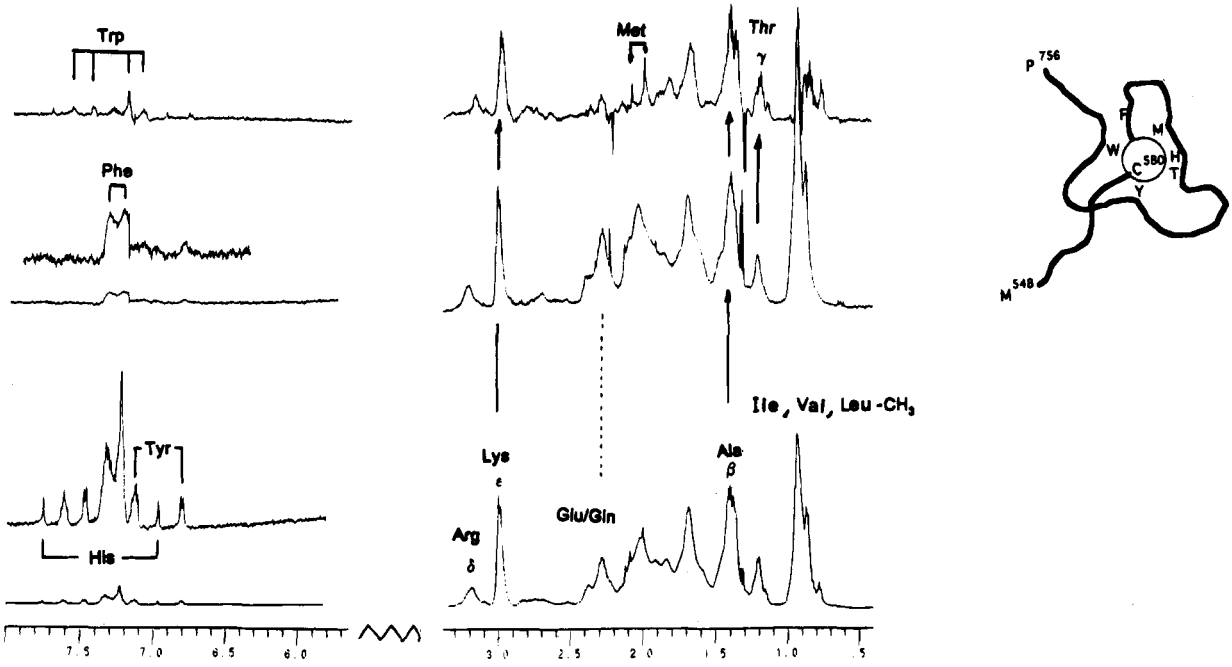

FIGURE 4: Spin-labeling of the 35 kDa fragment of caldesmon shows that sequentially remote residues lie relatively close to Cys-580. Bottom trace: proton NMR spectrum of the unlabeled 35 kDa fragment of caldesmon. Middle trace: proton NMR spectrum of the 35 kDa fragment of caldesmon spin-labeled at Cys-580. Top trace: difference spectrum (bottom minus middle) showing resonances from groups closest in three-dimensional space from the spin-label; these include signals from His-610, Tyr-625, at least one Trp residue, at least two Met residues, and multiple Thr residues, all of which appear to be perturbed more than those from Phe residues. Inset: schematic diagram of a folded structure for domain 4 of caldesmon, summarizing proximities of nonsequential locations inferred from these data.

but also its inhibitory activity, we have made use of a paramagnetic spin-label introduced at the sole cysteine residue, Cys-580. Comparison of the spectra of this fragment with and without the covalently attached probe (Figure 4) directly reveals the residues in the locale of the probe since the induced signal relaxation is proportional to r^{-6} from the spin-label. The resonances thus perturbed permit mapping about the probe as origin. The spectral readout shows that sequentially remote residues (e.g., His-610, Tyr-625, and at least one of Trp-659,692,722) are disposed within 1.0−1.5 nm from the probe, indicative of a well-defined polypeptide configuration for the C-terminal region of the molecule.

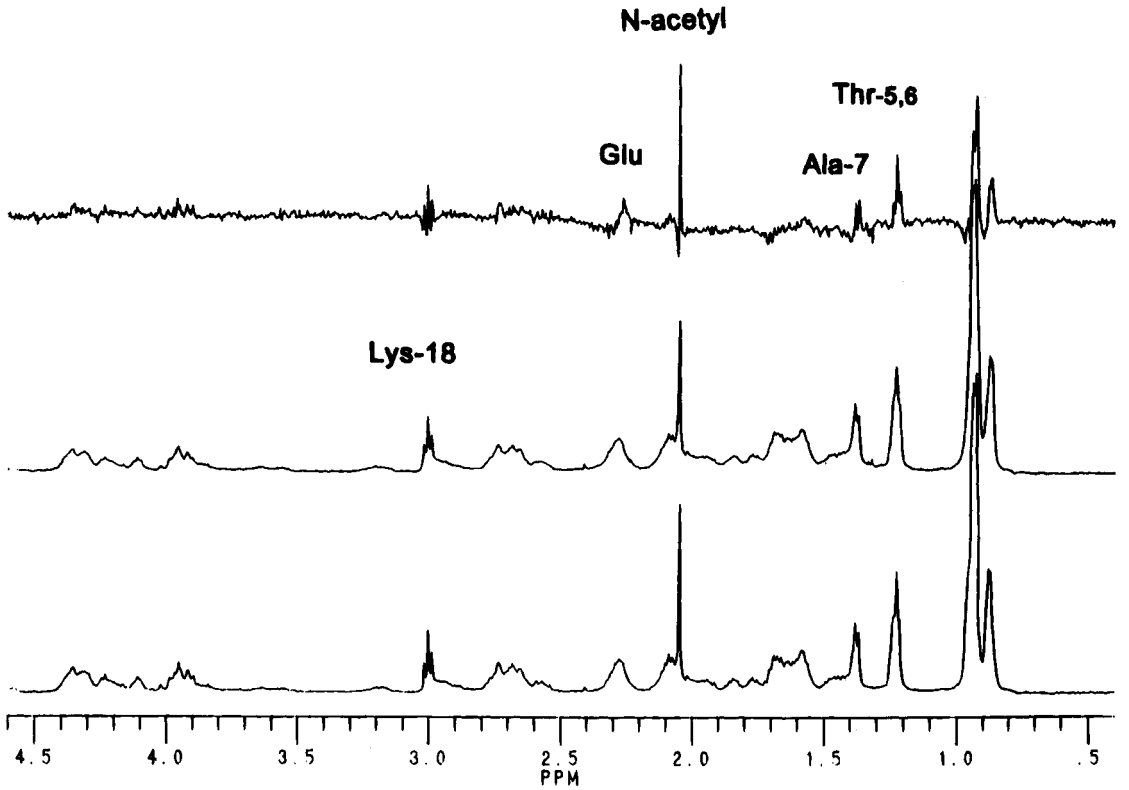

FIGURE 5: Binding of actin residues 1−18 to the C-terminal 35 kDa fragment of caldesmon spin-labeled at Cys-580 shows that signals from certain groups (e.g., the *N*-acetyl moiety, Ala-7, and Thr-5 and/or -6) are specifically perturbed by the spin-label. Bottom trace: proton NMR spectrum of actin residues 1−18 (400 μM, pH 7.2). Middle trace: spectrum after addition of the spin-labeled 35 kDa fragment of caldesmon to a total concentration of 8 μM. Top trace: difference spectrum (bottom minus middle) showing resonances from groups in the bound actin peptide closest in three-dimensional space from the spin-label.

Multiple Thr residues, which occur only in domain 4 (Figure 1), and at least two Met residues are also disposed close to the probe. Conversely, very few Glu/Gln residues are close to the probe, suggesting that most of domain 3 (where Glu/Gln residues are very abundant) is at least 1.5 nm distant from the spin-label at Cys-580. An outline fold generated from these data (inset, Figure 4) suggests that domain 4b, consistent with the predominantly nonpolar residue composition in this region of the molecule, forms a comparatively compact scaffold which is capped by domain 4a to enable the relative disposition of these different locations about the probe at Cys-580.

The spin-label probe has also provided the means by which to map the actin−caldesmon interfaces. Actin residues 1−7 are observed to dock in the vicinity of the spin-label as indicated by the specific pattern of line broadening induced upon titration of the actin peptide comprising residues 1−18 with the 35 kDa species labeled at Cys-580 (Figure 5; cf. Figure 1). The conclusion that actin residues 1−7 dock at the topographically compact core of the C-terminal 35 kDa fragment and that the N-terminal region of this caldesmon fragment (domain 3) interacts with actin residues 20−41 is supported by the interaction shown for the intact molecules, i.e., that actin binding results in the perturbation of signals corresponding to domain 4 as well as domain 3 of caldesmon (Figure 3). Thus the two N-terminal sites on actin bind to specific sites of the C-terminus of caldesmon.

The importance of both of these actin contacts to the inhibitory properties of the 35 kDa fragment is suggested by the observation of cooperative effects that underlie the double-sited attachment to actin. Such cooperativity is illustrated in Figure 6. This shows that the addition of the peptide comprising actin residues 1−18 at substoichiometric ratios to the 35 kDa caldesmon fragment affects the binding of this caldesmon fragment to actin residues 16−41. For example, signals from this latter actin peptide deriving from Val-30 and His-40 are progressively perturbed upon binding of the N-terminal actin peptide to the caldesmon fragment. The marked cooperativity of binding of these two actin peptides to the caldesmon fragment (domains 3 and 4) confirms the specificity of their individual interactions and further indicates that interaction of the N-terminal extremity of actin (residues 1−7) with domain 4 of caldesmon elicits a change of conformation in the latter that alters the nature of the interaction between actin and domain 3 of caldesmon. This conclusion, i.e., that the interfaces between the C-terminus of caldesmon and actin are interactive, is relevant to the means by which calmodulin antagonizes the binding of caldesmon to actin, since it suggests that release of the interaction between domain 4 of caldesmon and residues 1−7 of actin could directly modulate other caldesmon−actin contacts.

Caldesmon−Calmodulin Interactions. Actin binding to the C-terminal region of caldesmon is weakened by Ca^{2+}−calmodulin. The binding of calmodulin at this region of caldesmon appears to involve at least two contacts located at the C-terminal residues of the 35 kDa fragment (Marston et al., 1994; Mezgueldi et al., 1994). This conclusion is based on studies with mutant forms of the fragment as well as those with small caldesmon peptides comprising the sequences about Trp-659 and Trp-692. Calmodulin has also been observed to cross-link to Cys-580 of caldesmon (Wang, 1988), while the region encompassing Trp-659 of caldesmon has been suggested to comprise a calmodulin contact site (Wang et al., 1991). We therefore proceeded to probe the nature of the interaction between calmodulin and the 35 kDa

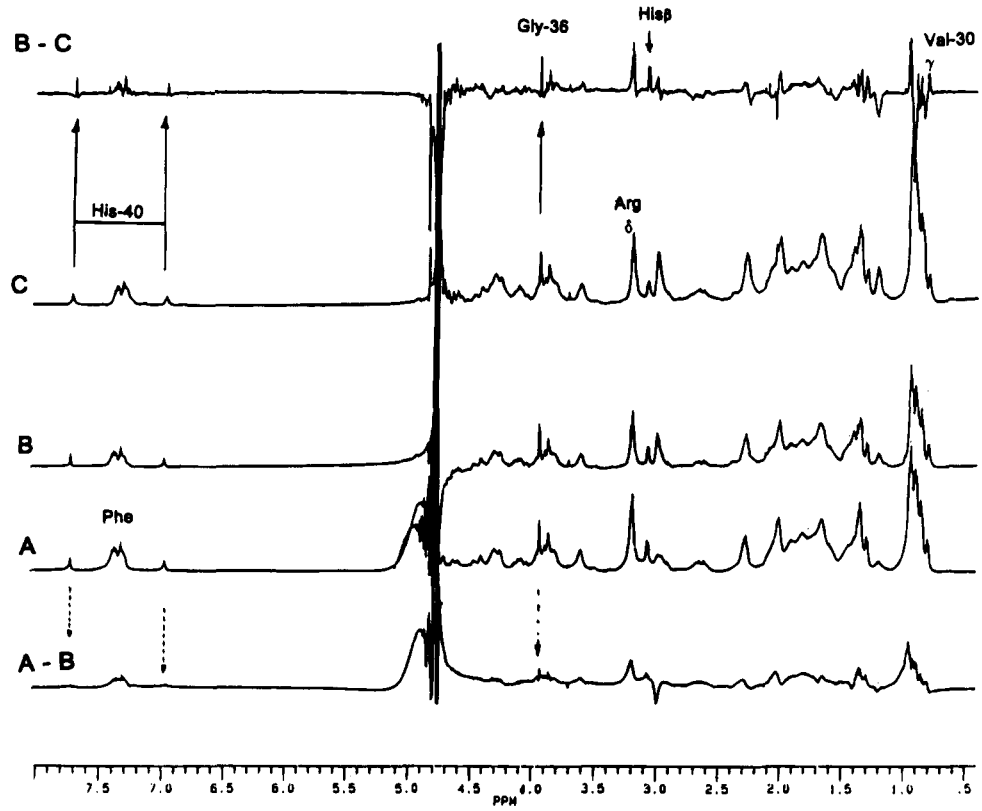

FIGURE 6: Binding of two N-terminal actin peptides to the 35 kDa fragment of caldesmon exhibits cooperative effects. (A) Proton NMR spectrum of peptide corresponding to residues 16−41 of actin (400 μM, pH 7.2). (B) Spectrum after addition of the 35 kDa fragment of caldesmon to a total concentration of 45 μM. (C) Spectrum after addition of the peptide corresponding to residues 1−18 of actin to a total concentration of 120 μM. Bottom trace: difference spectrum (A − B) showing resonances from actin peptide 16−41 perturbed by the interaction owing to a loss of mobility of specific residues. Top trace: difference spectrum (B − C) showing further spectral perturbations associated with actin residues 16−41 (e.g., His-40 and Val-30) that result from the formation of a ternary complex.

fragment of caldesmon by NMR using wheat germ calmodulin that contains a reactive Cys at position 27.

Addition of calmodulin spin-labeled at position 27 to the 35 kDa caldesmon fragment led to the enhanced broadening of specific caldesmon resonances as a result of the relaxation induced by the paramagnetic probe upon calmodulin−caldesmon interaction. The nature of the signals perturbed by the spin-label (Figure 7) shows that complex formation indeed involves the proximity of Trp-659, -692, and -722 in caldesmon to the N-terminal domain of calmodulin on which the probe is attached. The lack of involvement of the N-terminal region of the 35 kDa caldesmon fragment in calmodulin binding is demonstrated by the observation that the resonances primarily perturbed by the spin-label probe show marked homology to those deriving from the C-terminal domain 4b. The nature of the signals of groups in the vicinity of the bound probe in the complex is highlighted by difference spectroscopy (Figure 7) and can also be seen to correlate with the perturbations induced in the spectrum of the isolated 35 kDa fragment when labeled at Cys-580 (cf. Figure 4). These observations are in agreement with the finding that Cys-580 in caldesmon can be cross-linked to calmodulin. It thus appears that the topography of the 35 kDa fragment (inset, Figure 4) is such as to act as a scaffold which presents separate contact regions for calmodulin arrayed in the vicinity of the actin binding site(s). This structural organization and its conformational flexibility allow the cooperativity of the binding effects to actin and may be due in part to the proline-rich segments that occur in the C-terminal end of the molecule, acting as spacers/hinges.

The N-Terminus of Actin Is the Common Target for Caldesmon and Troponin-I Inhibitory Function. The extreme N-terminal residues of actin contribute to the actin contacts made by the calcium-mediated inhibitory-competent segment of caldesmon, the C-terminal 35 kDa fragment comprising domains 3 and 4. Residues 1−7 of actin are also known to be involved in the docking of the inhibitory peptide of troponin-I, which is much shorter than the caldesmon fragments studied here. In this regard, it is of interest that two separate regions derived from the C-terminus of caldesmon (domain 4), encompassing residues 597−629 (Wang et al., 1991) and residues 693−722 (Mezgueldi et al., 1994), have been shown to possess tropomyosin-potentiated inhibition. Only the latter of these short residue segments occurs in the recombinant caldesmon fragment 658C (comprising residues 658−756), which has been shown to possess to a good degree the inhibitory capacity of the intact molecule (Redwood & Marston, 1993). We therefore undertook the comparative study of the binding to actin of caldesmon fragment 658C and the TnI inhibitory peptide. Here it was helpful that there are NMR signals from only two tryptophan residues in the C-terminal end of the caldesmon fragment (Trp-692 and -722), since Trp-659 had been mutated to Gly in 658C, a change which does not influence the binding/inhibitory properties of this caldesmon analogue. Addition of F-actin to the fragment led to the progressive relaxation of a variety of signals (Figure 8), most notably those deriving from the spectral reporter groups for the N- and C-terminal ends of the 658C moiety, Met-658 and Trp-692 and -722, respectively. It has recently been demonstrated that only a relatively short segment of the 658C

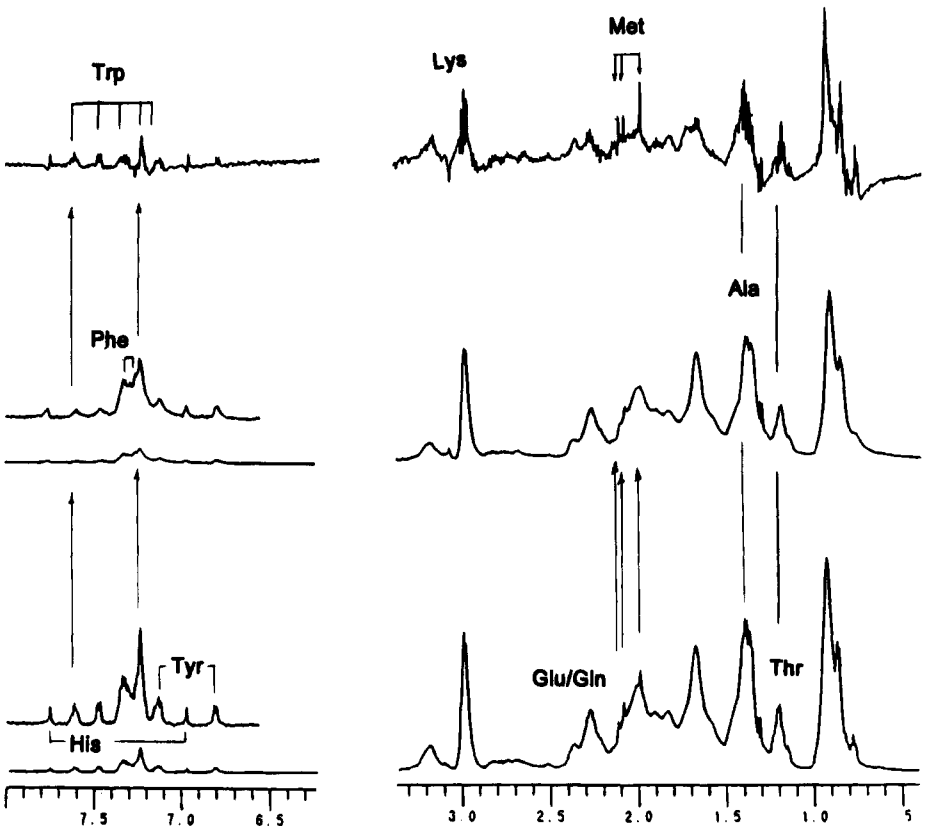

FIGURE 7: Addition of calmodulin spin-labeled at Cys-27 to C-terminal fragments of caldesmon shows domain 4 site(s) to be involved in the interaction. Bottom trace: proton NMR spectrum of 35 kDa fragment of caldesmon (200 μM, pH 7.2). Middle trace: spectrum after addition of 40 μM spin-labeled CaM. Top trace: difference spectrum (bottom minus middle) showing resonances from caldesmon fragment that are perturbed, including those from Trp (cf. little effect on Phe), Thr (solely in domain 4), and Met.

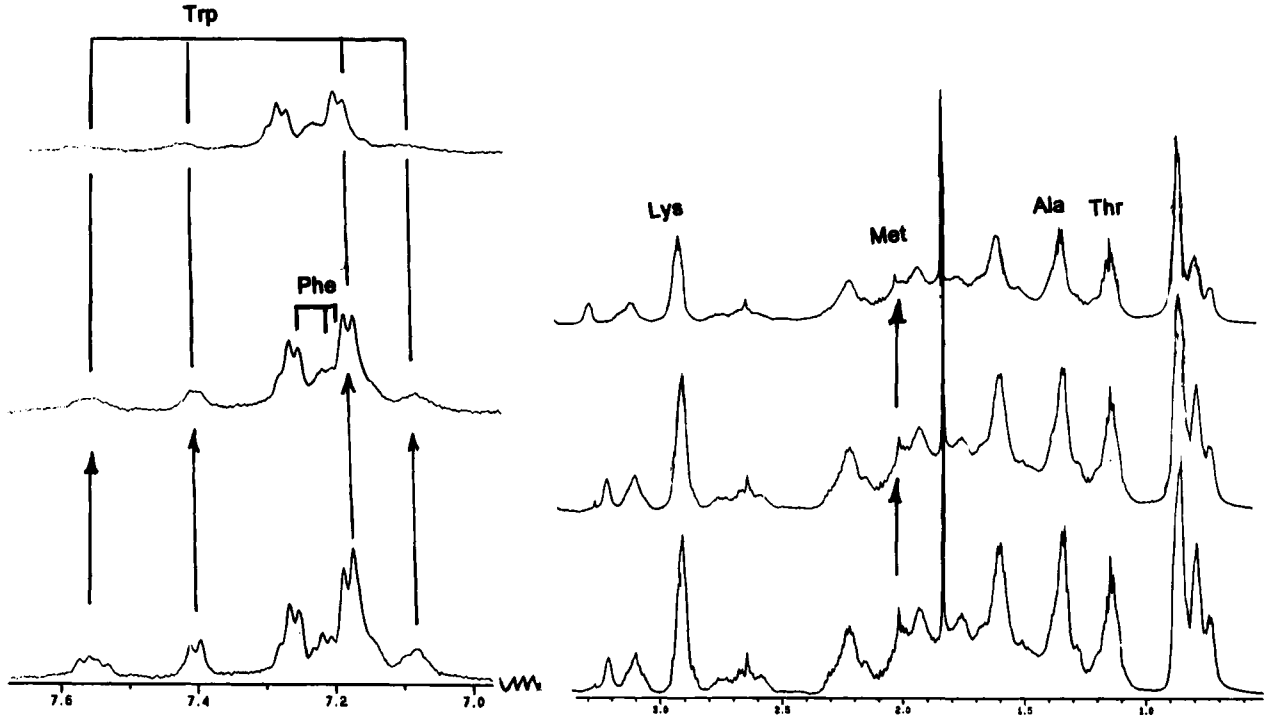

FIGURE 8: Binding of F-actin to the C-terminal caldesmon fragment 658C results in spectral changes for residues at both ends of the latter. Bottom trace: proton NMR spectrum of fragment 658C (400 μM, pH 7.2). Middle traces: spectra after addition of F-actin to total concentrations of 20 and 60 μM. Top trace: difference spectrum (bottom minus 60 μM) showing resonances of the caldesmon fragment perturbed by the interaction, including residue types Met, Thr, and Trp.

molecule, i.e., residues 693−722 (Mezgueldi et al., 1994), is directly involved in the binding of this C-terminal fragment of caldesmon to actin. The extensive nature of the spectral perturbations for 658C here observed is therefore likely to arise from the conformational changes upon complex formation with F-actin. This conclusion, rather than the possibility of an extensive surface of contact between 658C and actin, is supported by the observation that actin does not protect

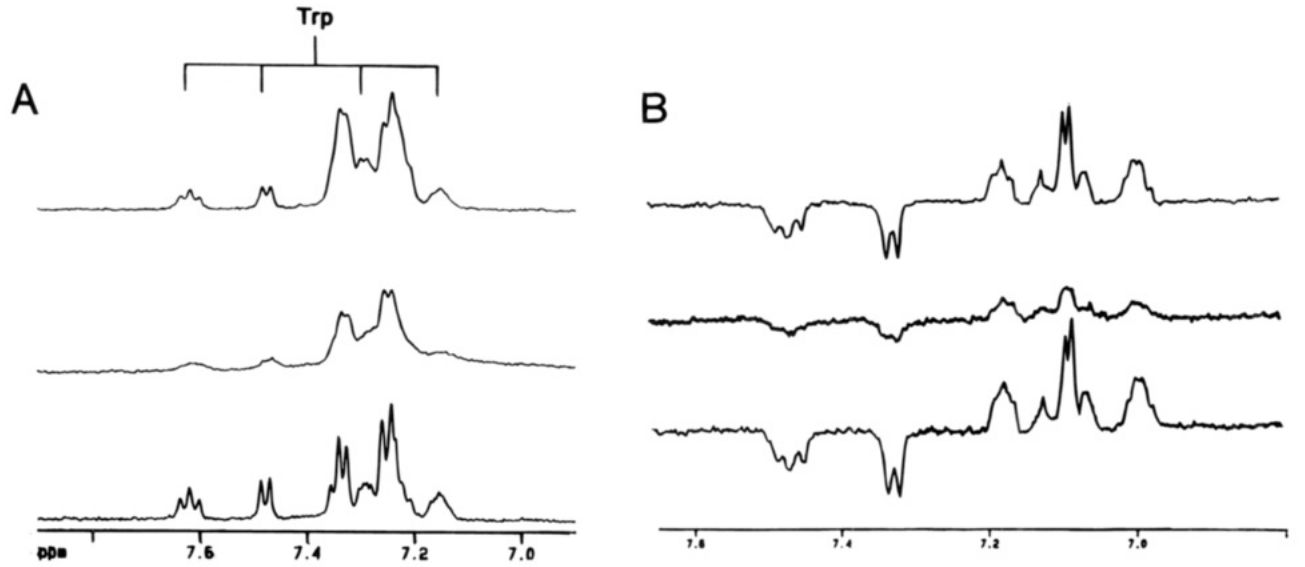

FIGURE 9: Inhibitory TnI peptide readily displaces caldesmon fragment 658C from F-actin. (A) Bottom trace: aromatic region of proton NMR spectrum of fragment 658C (400 μM, pH 7.2). Middle trace: spectrum after addition of F-actin (160 μM), where broadening of previously sharp signals indicates a marked loss of mobility for the tryptophan side chain groups from which they derive. Top trace: spectrum obtained during titration with TnI inhibitory peptide (150 μM vs 400 μM caldesmon fragment), showing restoration of intensity for all signals of the caldesmon fragment. (The TnI peptide is bound to F-actin rather than free in solution since no new sharp signals appear in the difference spectrum.) (B) Parallel set of two-pulse spin-echo spectra (cf. Figure 2B), which discriminate in favor of signals deriving from molecular features having the greater mobility. Inverted signals in the bottom and middle traces are doublets, while upright signals are singlets or triplets.

this region of the molecule against proteolytic digestion while the cleavage product is still able to interact with F-actin (Mezgueldi et al., 1994).

The inhibitory competence of domain 4b of caldesmon is generally taken to occur by a mechanism similar to that intrinsic to the TnI inhibitory peptide. In order to probe the mechanistic similarity of the inhibitory properties displayed by the TnI peptide and by fragment 658C, we undertook actin binding competition experiments. Both domain 4b of caldesmon (Bartegi et al., 1990) and the TnI peptide (Levine et al., 1988) possess comparable affinity for actin (10^6 and 10^5, respectively). The ability of the inhibitory peptide of troponin-I to compete with caldesmon fragment 658C was studied by titration of this peptide (TnI residues 96−117) into a solution containing both F-actin and the caldesmon fragment.

Displacement of fragment 658C by the TnI peptide was directly monitored by the reduced perturbation of the tryptophan residues of 658C, indicating a decrease of its complex formation with actin (Figure 9). The titration data show significant competition by the TnI peptide; i.e., displacement of the caldesmon 658C fragment from F-actin is readily achieved by markedly substoichiometric concentrations of the TnI peptide. These observations on binding propeties to actin show that, since both TnI and caldesmon inhibitory fragments separately possess comparable overall affinities for actin, the nature of their respective interactions with actin must differ. The mechanism for inhibition operating in the case of caldesmon residues 693−722 in domain 4b must involve an indirect effect on the N-terminal region of actin, a contact site utilized by the entire caldesmon domain 4 as identified by the spin-label probe (cf. Figure 5).

DISCUSSION

We have demonstrated that two sites on the N-terminal region of actin (residues 1−44) are involved in the interaction

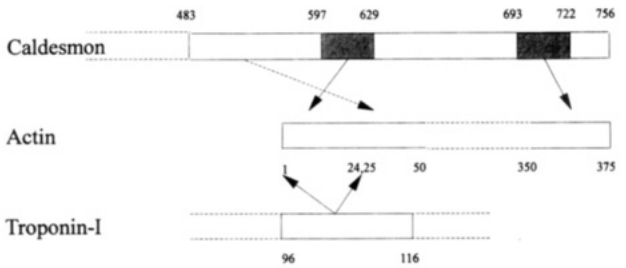

FIGURE 10: Schematic representation of complementary regions of interaction between actin and the inhibitory segments of the two regulatory proteins, caldesmon and troponin-I.

with caldesmon. The extreme N-terminal site on actin (residues 1−7) interacts with domain 4 of caldesmon, whereas the other N-terminal site (actin residues 20−41) interacts with domain 3 of caldesmon (Figure 10). Evidence from studies on defined fragments suggests that the inhibitory function of caldesmon is associated with residues 597−722, i.e., domain 4. Thus, it can be concluded that, as in the case of troponin-I, interaction with the N-terminus of actin at residues 1−7 is an important aspect of the inhibitory function of caldesmon on the actomyosin ATPase.

In view of the expectation that the action of caldesmon in regulating the actomyosin ATPase might resemble that of troponin-I, it is of interest to compare the interaction of actin with minimum inhibitory fragments of the two proteins. Clearly there is a difference. It has been shown elsewhere (Levine et al., 1988) that actin interacts with the inhibitory peptide of TnI (residues 96−117) with two regions of its N-terminus of actin, residues 1−7 and residues 24−25. In contrast, the site in the region encompassing actin residues 20−41 interacts with caldesmon domain 3 that has no inhibitory activity and presumably is not directly involved in inhibiting the actin−myosin interaction. The TnI peptide, however, proved to be a remarkably competent inhibitor of the binding of recombinant fragment 658C of caldesmon to F-actin. These results underline the functional capacity of the N-terminal residues of actin as target sites for inhibitory

interactions. The special significance of the N-terminus of actin in caldesmon inhibitory function has recently been demonstrated (Crosbie et al., 1994) since, though complex formation with caldesmon was not greatly affected, mutation of the extreme N-terminal residues of actin greatly impaired the ability of caldesmon to inhibit actomyosin ATPase.

Inspection of the subdomain structure of the G-actin monomer (Kabsch et al., 1990) indicates that the N-terminal regions of actin that interact with caldesmon are some 4−5 nm apart and therefore spatially distinct. The distance between the interacting sites on caldesmon must be similar as is indicated by our data which show that the regions of caldesmon here involved in actin binding can maximally span a distance of 4 nm (i.e., extremities from the probe). Such a distance raises the possibility that the complex with F-actin spans two actin monomers. The derived topography of one caldesmon C-terminal (domains 3 and 4) across two actin monomers is supported by previous cross-linking data which point to a 1:2 complex, while it has been shown that Cys-580 can be cross-linked to the C-terminal region (Cys-374) of actin (Graceffa et al., 1993). Thus, unlike the case with the inhibitory TnI peptide, it is possible that the binding of caldesmon's C-terminus involves attachment across two actin monomers in its structural mechanism for inhibiting the acto−S1 interaction.

The cooperativity of binding of caldesmon domains 3 and 4 to the two distinct segments on subdomain 1 of actin (residues 1−7 and 20−41) reflects the complexity of the mechanism for acto−S1 inhibition. The interactive nature of binding to different regions of subdomain 1 of actin has previously been described for myosin light chain 1 (LC1) (Grand et al., 1983). The light chain is readily displaced from its binding site on the C-terminal region of subdomain 1 of actin (residues 350−375) by the binding of the inhibitory segment of TnI to residues 1−28 of this actin subdomain. The similarly ready displacement of caldesmon domain 4b by the TnI peptide from F-actin indicates that these two inhibitory fragments do not bind to the same site. Inhibition by the minimal caldesmon inhibitory fragment (domain 4b) therefore occurs by a mechanism that does not directly involve interaction with the N-terminal regulatory switch region of actin utilized by troponin-I (residues 1−7). Rather, the NMR spectral data showing the binding of this region of actin close to the spin-label on caldesmon residue 580 suggest that it is the caldesmon actin binding region associated with domain 4a (caldesmon residues 597−629) which involves the interaction site on actin subdomain 1 utilized by the troponin-I inhibitory segment (Figure 10). It thus appears that multiple actin contacts underlie the mechanism by which caldesmon mediates actin−myosin interaction.

Relief of the inhibitory capability of the TnI peptide by calcium in skeletal muscle involves the switch of attachment of this short region from actin to troponin-C. In the case of caldesmon, our data show that relief by calmodulin binding to domain 4 of caldesmon not only will result directly in detachment of caldesmon from actin residues 1−7 but also will mediate the cross-monomer interaction of caldesmon with F-actin by antagonizing the binding of caldesmon to actin residues 20−41. Thus, the calcium-modulated inhibitory capacity of the caldesmon C-terminal differs from that of its skeletal TnI inhibitory peptide counterpart both in terms of the manner of association with F-actin and with regard to the way in which association with the calcium receptor alters attachment to actin.

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