Mutagenesis Analysis of Functionally Important Domains within the C-terminal End of Smooth Muscle Caldesmon*

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The ability of chicken gizzard smooth muscle caldesmon (CaD) to inhibit actomyosin ATPase activity is due mainly to an inhibitory domain that resides within the C-terminal 67 amino acid residues of the CaD molecule. In the present study, a series of C-terminal truncation and internal deletion mutants of chicken gizzard smooth muscle CaD were systematically designed using a site-directed mutagenesis approach, and these mutant proteins were overexpressed in a baculovirus expression system. Analysis of actin binding and inhibition of actomyosin ATPase activity using these mutants identified a strong actin-binding motif of 6 amino acid residues (from Lys718 to Glu723), which also form the core sequence for CaD-induced inhibition of actomyosin ATPase. However, maximal inhibition by CaD requires the presence of residues 728–731, which are not associated with actin binding. Our data provide direct evidence for the requirement of actin binding to a specific region in CaD for CaD-induced inhibition of actin activation of smooth muscle myosin ATPase. Furthermore, our findings also show that the region between residues 690 and 717 is responsible for the weak inhibition of actomyosin ATPase and reveal that the inhibitory determinants located in the regions between residues 690 and 717 and residues 718 and 756 can function independently.

Smooth muscle caldesmon (CaD) is a multifunctional elongated molecule that is thought to play a role in modulating the interaction between myosin and actin (1–5; for review, see Refs. 6 and 7). Amino acid sequence and functional analyses have shown that the structure of smooth muscle CaD consists of an N-terminal domain, a helical middle region, and an C-terminal domain (6–8). The N-terminal domain contains the major myosin-binding core sequence that interacts with the S2 region of myosin (9–11), whereas the C-terminal domain is responsible for interaction with several proteins including actin, tropomyosin (9–11), whereas the C-terminal domain is responsible for interaction with several proteins including actin, tropomyosin, tubulin, calmodulin, and several other Ca²⁺-binding proteins (3, 6, 12–15). The middle region has been considered as a spacer, serving only a structural role (8).

The key functions of smooth muscle CaD in cell motility and contraction are its ability to down-regulate actomyosin ATPase activity, presumably by competing with myosin for actin binding (16, 17) and, as determined in an in vitro motility assay, to inhibit the movement of actin filaments over myosin heads, probably through a tethering pattern produced by the binding of the CaD N-terminus to the S2 region of myosin and the C-terminal region to actin (17, 18). The mechanism by which CaD inhibits actomyosin ATPase activity is not well understood, and it is rather complex, involving an enhancement of the inhibition by tropomyosin and reversal by Ca²⁺-calmodulin and/or phosphorylation of mitogen-activated protein kinase (1–5, 17, 18–22).

Domain mapping studies using CaD fragments obtained from limited proteolysis and chemical cleavage or using recombinant CaD fragments produced in bacterial expression systems have localized both the inhibitory domains and major actin/calmodulin binding sites to the C-terminal fragments with molecular masses ranging from ~20 to 40 kDa (8, 12, 19, 23–25). These functional domains have been further narrowed to a smaller peptide consisting of the 98 C-terminal amino acids from Trp659 to Pro756 (26, 27). Using a series of C-terminal truncated proteins of chicken gizzard smooth muscle CaD expressed in a baculovirus system, we showed that both the actin binding site(s) and the inhibitory domain(s) of CaD are composed of continuous multiple determinants located in the last 99 amino acids, whereas the main inhibitory determinants lie in the region between residues 690 and 756 (28). However, the precise localization of these functional determinants in the C-terminal end of CaD has remained unknown.

In this study, we generated a series of CaD C-terminal truncation mutants and internal deletion mutants using site-directed mutagenesis and polymerase chain reaction-based cloning strategies. Utilizing these mutants, we addressed the following questions. 1) Do the inhibitory or actin-binding motifs located within the last 67 amino acids of CaD function independently? 2) Do the actin binding and actomyosin ATPase inhibitory determinants overlap or do they juxtapose closely with each other? 3) What is the minimal sequence within the last 39 amino acids of the C terminus of CaD that is responsible for actin binding and inhibition of actomyosin ATPase?

MATERIALS AND METHODS

Construction of Recombinant Baculovirus Transfer Vectors—DNA oligonucleotide primers were synthesized using an Applied Biosystems DNA/RNA synthesizer. The C-terminal truncation mutants of chicken gizzard smooth muscle CaD cDNA were constructed by a polymerase chain reaction cloning approach as described (29) using the same up-stream oligonucleotide primer, 5’ACTGGGATCCCTATAAATATGGAT-GACTTTGAACGC-3’. The sequences of the downstream oligonucleotide primers used to generate these truncation mutants were as follows: CaD1–740, 5’GTCAGGATCCCTACGTAGCTGTTACCTT-3’; CaD1–717, 5’GTCAGGATCCCTATGAAGAAGAAGCAGC-3’; CaD1–735, 5’GTCAGGATCCCTATGAAGAAGAAGCAGC-3’; and CaD1–723, 5’GTCAGGATCCCTATGAAGAAGAAGCAGC-3’. The underlined sequences in each primer represent the complementary portion, and all the primers contain a BamHI restriction site. Polymerase chain reaction amplifica-
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**Fig. 1. The CaD-(Δ690–717) internal deletion mutation.** A, the open boxes indicate the central repetitive region consisting of a 13-amino acid sequence repeated eight times (8). The bent line represents the region deleted in the CaD mutant. The numbers indicate the amino acid residue numbers. The hatched areas indicate the sequence homology between CaD and tropinin T characterized previously (8). The two cysteine residues in the CaD molecule are presented as SH. The amino acid sequence of full-length CaD that is absent in the CaD mutant is shown by an interruption in the schematic structure. B, full-length CaD and CaD-(Δ690–717) were purified from the heat-stable fraction of the cell extract by one-step ion-exchange chromatography (DEAE-Sephasel column), and the purified full-length CaD and CaD-(Δ690–717) were analyzed by 4–12% gradient SDS-PAGE followed by Coomassie Blue staining. Lane 1, molecular mass standards (myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa); lane 2, CaD1–728; lane 3, CaD-(Δ690–717). The CaD mutants produced in some baculovirus expression systems show slight degradation (ranging from 3 to 7%), depending on the time between transfection and cell harvesting. We reported that the function (actin binding) of the baculovirus-generated full-length CaD, having slight degradation (5%), is indistinguishable from that of native CaD, suggesting that the presence of degradation in each of the purified baculovirus-generated recombinant CaD or CaD mutants has no significant effect on the binding data. It is possible that the cleaved site in the recombinant CaD is in its N terminus.

**Fig. 2. Binding of CaD-(Δ690–717) mutant to actin or tropomyosin-actin.** Binding of full-length CaD (○, ●) and CaD-(Δ690–717) (△, ▲) to smooth muscle actin (open symbols) or tropomyosin-actin (closed symbols) was measured as described (28, 29). Actin concentration remained constant at 25 μM, and tropomyosin was mixed with actin at a 1:4 molar ratio.

**Fig. 3. Inhibition of actin-activated myosin ATPase by CaD-(Δ690–717).** Actin or tropomyosin-actin-activated myosin ATPase activity was measured as described (38). Smooth muscle myosin and actin were kept constant at 1 and 25 μM, respectively, and tropomyosin was mixed with actin at a 1:4 molar ratio. Effect of CaD1–728 (○, ●) and CaD-(Δ690–717) (△, ▲) on actin (open symbols) or tropomyosin-actin (closed symbols)-activated myosin ATPase activity was determined.
incubation of agarose (without coupled calmodulin) with increasing concentrations of 14C-labeled full-length CaD or CaD mutants, and these values were subtracted from each point on the binding curve. The binding of full-length CaD and CaD mutants to actin was determined in the presence or absence of tropomyosin using 14C-labeled and unlabeled proteins as described (5, 28). All determinations of the binding assays were done in triplicate. The apparent dissociation constants for calmodulin, actin, and actin-tropomyosin bindings were determined by the method of Scatchard (36) and by weighted nonlinear least-squares curve fitting as described by Munson and Rodbard (37).

ATPase Assays—ATP assays were carried out at 25°C as described (38). Specific assay conditions are described in the figure legends.

RESULTS

Two or More Functionally Independent Actomyosin ATPase Inhibitory Motifs Reside in the Last 67 Amino Acid Residues of CaD—We recently localized the major actin binding determinant(s) and inhibitory motifs for actomyosin ATPase in the chicken gizzard smooth muscle CaD molecule to its C-terminal 67 amino acid residues, presumably involving the regions between residues 690–717 and 718–756 (28). However, it is not known whether these two regions contain a functionally independent inhibitory motif(s) and where the functional domains in the C-terminal end of CaD precisely localize. Internal deletions of selected regions in the CaD molecule were made to investigate the dependence of the ATPase inhibitory determinants identified in the last 67 amino acids of the C terminus of CaD. Fig. 1A shows the schematic structure of the internal deletion mutant (CaD(-Δ690–717)) of chicken gizzard smooth muscle CaD lacking residues Asn690 to Gly717. This CaD mutant was overexpressed in a baculovirus expression system. The purity of CaD(-Δ690–717) used for our experiments was assessed by SDS-PAGE (Fig. 1B). As shown in Fig. 2, the binding of CaD(-Δ690–717) to actin began to level off at a stoichiometry of 0.08 mol of CaD per mol of actin, and the binding was saturated at a CaD concentration of 5 μm (molar ratio of CaD to actin, 1:4). CaD(-Δ690–717) binding to actin was higher in the presence of tropomyosin than in its absence (mol of CaD/mol of actin, 0.095 versus 0.08). However, the binding of CaD(-Δ690–717) to both actin and tropomyosin-actin was lower than that for the full-length CaD (CaD1–756). The apparent $K_d$ values for the actin-binding and tropomyosin-actin binding of CaD(-Δ690–717) obtained from Scatchard analysis (36, 37) were 0.63 ± 0.03 × 10^{-6} M and 0.34 ± 0.02 × 10^{-6} M, respectively, slightly higher than those for full-length CaD (0.52 ± 0.03 × 10^{-6} M for actin-binding; 0.28 ± 0.015 × 10^{-6} M for tropomyosin-actin binding).

Analysis of the inhibitory effect of CaD(-Δ690–717) on actomyosin ATPase activity revealed strong inhibition of actin or tropomyosin-actin-activated myosin ATPase (Fig. 3), although the inhibition was slightly lower than that produced by CaD1–756. These results, together with our previous data (28), suggest that the sequence from Asn690 to Gly717 contains a weak inhibitory motif and the stretch from Lys-718 to Pro-756 embraces a strong inhibitory motif, respectively. The two inhibitory motifs are functionally independent.

Presence of a Core Inhibitory Sequence in the Region between Residues 718 and 735—To delineate more precisely the inhibitory sequence in the region between residues 718 and 735.
CaD truncation mutants that have identical N-terminal ends but differ in their C-terminals due to the deletion of different portions from the C-terminus were constructed (Fig. 4A). All C-terminal truncation mutants were purified to >95% homogeneity (Fig. 4B). Actin binding of CaD1–740, CaD1–735, and CaD1–727, i.e. the CaD truncation mutants that progressively removed 16, 21, and 29 amino acids from the C-terminal end of CaD, was virtually indistinguishable from that of full-length CaD (Fig. 5A). In the presence of tropomyosin, the binding of CaD1–740, CaD1–735, and CaD1–727 were identical to those of full-length CaD as described above. Fig. 6 illustrates the ability of these truncation mutants to inhibit actomyosin ATPase activity. The inhibition of actin- or tropomyosin-actin-activated ATPase activity was unchanged with deletion of up to 21 amino acid residues (CaD1–740 and CaD1–735); however, further deletion of 8 (CaD1–727) amino acid residues resulted in an 8 and 10% reduction in the inhibition of the actin-activated myosin ATPase in the absence and the presence of tropomyosin, respectively, as compared with full-length CaD. The apparent Kd values of both actin binding and tropomyosin-actin binding for CaD1–740, CaD1–735, and CaD1–727 were identical to those of full-length CaD as described above.

The presence of tropomyosin increased the binding of CaD and the internal deletion mutants to actin (Fig. 8B). In the presence of tropomyosin, the apparent Kd values of actin binding for CaD-(Δ721–735) and CaD-(Δ718–735) were 0.72 ± 0.038 and 1.03 ± 0.047 × 10⁻⁶ M, respectively. The binding affinity of CaD-(Δ732–735), CaD-(Δ728–735), and CaD-(Δ724–735) for actin was very similar to that of full-length CaD.

In another series of experiments, a site-directed mutagenesis approach was used to generate internal deletion mutants of chicken gizzard smooth muscle CaD, in which 4–18 amino acid residues in the region from Lys718 to Ser735 were progressively nest-deleted (Fig. 7A). These mutants were used to identify the core sequence necessary for inhibition of actomyosin ATPase activity. All CaD internal deletion mutants were overexpressed in a baculovirus expression system and purified to >95% homogeneity (Fig. 7B), although their yields varied slightly. As expected, CaDΔ732–735 and CaDΔ728–735, lacking 4 and 8 amino acid residues, respectively, bound to actin or tropomyosin-actin as efficiently as full-length CaD (Fig. 8A and 8B). The absence of residues 724–727 also did not impair the binding of CaD to actin (Fig. 8A). However, further deletion of 6 amino acid residues between Lys718 and Glu723 caused a considerable decrease in the binding of CaD to actin (Fig. 8A). Apparently, the 6-amino acid stretch, KRNLWE, contributes to the strong actin-binding motif located within the last 39 amino acid residues of CaD. The apparent dissociation constants of CaD-(Δ721–735) and CaD-(Δ718–735) were 0.72 ± 0.038 and 1.03 ± 0.047 × 10⁻⁶ M, respectively. The binding affinity of CaD-(Δ732–735), CaD-(Δ728–735), and CaD-(Δ724–735) for actin was very similar to that of full-length CaD.

![Fig. 6. Inhibition of actin-activated myosin ATPase by the CaD C-terminal truncation mutants.](image)
respectively, suggesting that deletion of residues 732–735 and 724–727 has no effect on CaD-induced inhibition of actin-activated myosin ATPase. These results demonstrate that there are two inhibitory determinants between residues 718–723 and 728–731 that are separated by 4 amino acid residues from Lys724 to Val727. These two determinants directly participated in producing the strong inhibitory activity by smooth muscle CaD because deletion of residues 721–735 resulted in a reduction by 24% in the inhibition of actomyosin ATPase as compared with the full-length CaD, and further removal of three residues from Lys718 to Asn720 caused a dramatic decrease by 61% (Fig. 9A). Apparently, the core sequence is composed of the 6 amino acid residues between Lys718 and Glu723. Our data also suggest that residues 728–731 that are irrelevant to the binding of CaD to actin (as shown in Fig. 8) are associated with a weak inhibition of actomyosin ATPase.

In the presence of tropomyosin, the inhibition by both full-length CaD and these internal deletion mutants was enhanced (Fig. 9B).

Reversal of the Inhibition of Actomyosin ATPase by CaD Truncated and Internal Deletion Mutants in the Presence of Ca\(^{2+}\)-Caldesmon.—We previously obtained some evidence to suggest that the region between residues 690 and 717 is responsible for the high affinity binding to calmodulin (28). To obtain more accurate information about the binding affinity of CaD to calmodulin, we examined the binding of CaD-(Δ690–717) to calmodulin using calmodulin-coupled agarose and \(^{14}C\)iodoacetamide-labeled or unlabeled full-length CaD and CaD-(Δ690–717). Troponyosin, which does not bind to calmodulin, was used as a negative control. Nonspecific binding was considered as background. As shown in Fig. 10, A and B, the absence of CaD residues 690–717 caused a 41% reduction in the binding to calmodulin, but under the same assay conditions, tropomyosin did not bind to calmodulin-coupled agarose. The binding of both CaD\(^{1–756}\) and CaD-(Δ690–717) increased upon raising their molar concentrations (Fig. 10C). Interestingly, the calmodulin binding for both CaD\(^{1–756}\) and CaD-(Δ690–717) leveled off at a stoichiometry of 1 mol of CaD/mol of calmodulin, although the maximal calmodulin-binding of CaD-(Δ690–717) was significantly lower than that of CaD\(^{1–756}\). The apparent dissociation constant of CaD-(Δ690–717) was 1.96 ± 0.058 \(\times\) 10\(^{-6}\) M, much higher than that of full-length CaD (0.98 ± 0.061 \(\times\) 10\(^{-6}\) M). Our results provide unequivocal evidence that a second strong calmodulin-binding motif is located in the residues 690–717, presumably involving residues Asn690 to Lys695.

To determine whether the strong calmodulin-binding site in the region between residues 658 and 689 (26), which is further
narrowed down to residues 658–666 (8), is functionally involved in down-regulating CaD-induced inhibition of actin-activated myosin ATPase, we analyzed the calmodulin-induced reversal of the inhibition by either full-length CaD or CaD-(Δ690–717). Reversal of the inhibition of actomyosin ATPase activity by CaD-(Δ690–717), shown in Fig. 3, by calmodulin is depicted in Fig. 11. The inhibition caused by both CaD1–756 and CaD-(Δ690–717) decreased upon increasing the concentration of calmodulin, and the percentage of reversal by calmodulin for the inhibition caused by CaD-(Δ690–717) and full-length CaD was similar. This result indicates that the CaM-binding site between 658 and 689 is important for calmodulin to reverse the inhibition of actomyosin ATPase activity by CaD. Furthermore, tropomyosin had no effect on the calmodulin-induced reversal of inhibition by either full-length CaD or CaD-(Δ690–717) (Fig. 11). At a stoichiometry of 0.075 mol of CaD/mol of calmodulin, the inhibitions caused by both CaD-(Δ690–717) and CaD1–756 were completely reversed.

Reversal of the inhibition of actomyosin ATPase by CaD truncated and internal deletion mutants (shown in Figs. 4 and 7) in the presence of Ca\(^{2+}\)-calmodulin was also examined. The inhibition by these truncation mutants was reversed by calmodulin in the presence of Ca\(^{2+}\) in a pattern similar to that seen with full-length CaD (data not shown). A stoichiometry of 0.075 mol of CaD/mol of calmodulin was required for the complete reversal of the inhibition by either full-length CaD or these truncation mutants (data not shown). Additionally, the inhibition by these mutants described in Fig. 9 was completely reversed at about 13:1 molar ratio of calmodulin to CaD (data not shown).

**DISCUSSION**

Our previous study indicated that the region necessary for inhibition of actomyosin ATPase was localized mainly in the C-terminal 67 amino acid residues (28). The 67-amino acid stretch also contains two or more functional motifs for actin binding and one high affinity calmodulin binding site (28). However, the precise localizations of these functional domains and the exact relationship between them remain unclear. In the present study, we generated a series of C-terminal truncation mutants and internal deletion mutants that, unlike bacterially expressed CaD peptides, have intact C-terminal domains and central helical regions but different C-terminal ends. Thus, the major regions of the CaD molecule and the structural relationships during protein-protein interaction remain intact, a feature that is impossible to obtain with CaD fragments derived from limited proteolysis or bacterial expression systems (8, 12, 19, 23–27). Using the CaD truncation mutants and internal deletion mutants expressed in the baculovirus expression system, we extend our previous study and have precisely determined the major ATPase inhibitory domain and one strong actin-binding motif located in the last 39 amino acid residues of chicken gizzard smooth muscle CaD.

We show here that the region between residues 690 and 717 is responsible for weak inhibitory activity and weak actin binding, whereas the region between residues 718 and 756 contributes to strong inhibitory activity and strong actin binding. Evidently, the regions between residues 690–717 and 718–756 each contain at least one inhibitory determinant for the CaD-induced inhibition of actomyosin ATPase, and these inhibitory determinants can function independently. However, the deletion of one binding site is likely to affect not only the affinity but also the stoichiometry as shown in Figs. 2, 5, and 8. There are two possible explanations for the decrease in the stoichiometry of binding. 1) The conformation of CaD is changed by the deletion of either one of the actin-binding sites. 2) The conformation of the actin upon binding of CaD may vary depending on the presence or absence of either one of the actin-binding sites on the CaD. Hence, the difference in the stoichiometry of binding observed upon deletion of one binding site without affecting the other may be attributed to these confor-
mational changes, and it is possible that the same phenomenon may apply for the binding of CaM mutants deficient in CaM-binding site to CaM, as shown below.

The precise localization of the strong actin binding motif between residues 718 and 756 has been determined in our present study. Deletion of 29 amino acid residues from the C-terminal end of CaD had no effect on the binding of CaD to actin, whereas further deletion of residues spanning from Lys$^{718}$ to Val$^{727}$ markedly reduced the ability of CaD to bind to actin, suggesting that this 10-amino acid stretch harbors a strong actin-binding motif. The data in Fig. 8 further define the actin-binding motif as a 6-amino acid sequence from Lys$^{718}$ to Glu$^{723}$. Thus, our findings rule out the possibility that the activity of the C-terminal actin-binding motif depends on a single amino acid residue. The actin-binding motif, KRNLWE, contains amino acids with positively and negatively charged side chains. An uncharged nonpolar amino acid, tryptophan, is also present in the composition of the actin-binding motif. In fact, tryptophan has been found to be the key residue associated with calmodulin binding in other proteins (39). Thus, it seems likely that tryptophan and the three charged amino acid residues act in concert to form the actin-binding pocket.

The last 21 amino acid residues from the C-terminal end of CaD do not participate in the CaD-induced inhibition of actomyosin ATPase. The inhibitory motif actually consists of two discontinuous epitopes represented by a 6-amino acid sequence from Lys$^{718}$ and Glu$^{723}$ and a 4-amino acid stretch from Lys$^{728}$ to Ala$^{731}$. The 6-amino acid sequence, KRNLWE, contains the main inhibitory activity since the deletion of this sequence caused a more significant decrease in the CaD-induced inhibitory activity. On the other hand, the 6-amino acid sequence also encompasses the overall sequence responsible for the strong actin binding. This finding suggests that the inhibitory domain largely overlaps with the actin-binding motif, consist-

FIG. 10. Cosedimentation of CaD-(Δ690–717) with calmodulin-coated agarose. Binding of CaD$^{1–756}$ and CaD-(Δ690–717) to calmod-

FIG. 11. Effect of calmodulin on the inhibition induced by CaD$^{1–756}$ and CaD-(Δ690–717). Conditions and symbols are the same as described in Fig. 3, except that CaD and actin concentrations were kept constant (0.15 mol of CaD per mol of actin).

ul was determined as described under “Materials and Methods.” A, lane 1, molecular mass standards (see Fig. 1B, lanes 2, 4, 6, CaD-(1–756), CaD-(Δ690–717), and tropomyosin from washing, respectively; lanes 3, 5, 7, CaD$^{1–756}$, CaD-(Δ690–717), and tropomyosin from elution, respectively. B, histogram of the relative levels of bound (hatched) and unbound (open) proteins from the binding studies determined by scanning densitometry. C, bindings of calmodulin to CaD$^{1–756}$ (●) and CaD-(Δ690–717) (○) were determined at varying concentrations of 14C-labeled proteins. Moles of CaD bound per mol of calmodulin are plotted as a function of CaD concentration.
ent with the requirement for binding of CaD to actin for CaD-induced inhibition of actomyosin ATPase. Note, however, that the maximal inhibition by CaD requires the presence of the sequence between residues 728 and 731 (Fig. 9), a region not associated with actin binding. Most likely, the sequence between residues 728 and 731 is required in maintaining the conformation of the inhibitory domain. Future studies with smooth muscle myosin subfragment-1 and these mutants or new CaD C-terminal point mutants will help us to define the precise nature of these interactions and shed light on the mechanism by which CaD down-regulates actin-activated myosin ATPase.

The C-terminal region of smooth muscle CaD contains at least two calmodulin-binding sites, as demonstrated by several investigators using synthetic peptides and bacterially expressed fragments (27, 28, 40, 41). One calmodulin-binding site, designated CaM-binding site A, has been localized in a 7-residue segment from Trp659 to Phe665 (40). The other site, CaM-binding site B, was mapped to the region between residues 675 and 695 (41) and has recently been narrowed to a 6-residue stretch from Asn690 to Lys695 (28). Using indirect peptide-competitive binding assays, Marston et al. (41) showed that site B (residues 675–695), but not site A (residues 658–666), is closely associated with the reversal of CaD-induced inhibition of actomyosin ATPase. By contrast, A. Wang and colleagues (42) recently reported that the functionally related CaM-binding motif in site B lies between residues 690–695 (28). Our present data indicating that calmodulin reverses the inhibition induced by CaD (Δ690–717), which lacks the site B sequence, are in accord with the report of A. Wang and colleagues (42). However, we find that the binding of this mutant to calmodulin occurs at a much lower level than that for full-length CaD. Thus, the importance of site B in reversing CaD-induced inhibition by calmodulin cannot be ruled out.

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