Casein Kinase II Phosphorylation of Caldesmon Downregulates Myosin−Caldesmon Interactions†

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ABSTRACT: It is well-known that caldesmon (CaD) is a substrate for casein kinase II (CKII), and the phosphorylation of CaD by CKII regulates the interaction of CaD with myosin. However, the functionally relevant CKII phosphorylation site(s) on CaD and the precise role of CaD phosphorylation by CKII in mediating CaD's function have remained elusive. In this study, we demonstrate that Ser-26 is the major CKII phosphorylation site on CaD, while Ser-73 is of relatively minor importance. Moreover, the phosphorylation of Ser-26 and Ser-73 reduced CaD's ability to bind myosin by 45% and 27%, respectively, suggesting that the interaction of CaD with myosin is downregulated, at least in part, by the phosphorylation of these serine residues by CKII. Our results also demonstrate that there are at least four myosin-binding motifs within the amino-terminal region of CaD, located between residues 1−23, 34−43, 44−53, and 86−115, respectively. The myosin-binding motif between residues 44−53 contributes to strong myosin binding, while the three other myosin-binding motifs are responsible for weak myosin binding. The sequences between residues 24−33 and 54−85 on CaD are not required for the binding of CaD to myosin; thus, both Ser-26 and Ser-73 are located outside of the myosin-binding motifs. It is therefore likely that the downregulation of myosin-CaD interactions by CKII phosphorylation is due to phosphorylation-induced conformational changes in the adjacent myosin-binding motifs on CaD, rather than by the direct modification of these myosin-binding motifs by CKII.

Caldesmon (CaD),[11] an actin-binding protein, has been thought to act as a regulator of actin−myosin interaction in a variety of cells (*1*−*3*). In smooth muscle, as one of the major components in thin filaments, CaD is implicated in the thin-filament-mediated regulation of smooth muscle contraction. Supportive evidence includes the inhibition by CaD of smooth muscle actomyosin ATPase (*1*−*9*), downregulation of the movement of actin filaments over myosin in an in vitro motility assay (*10*−*13*), and diminution of force generation in several skinned smooth-muscle fiber systems (*14*−*17*). The regulation of actomyosin ATPase by CaD is Ca^{2+}-dependent, since the CaD-induced inhibition of actomyosin ATPase can be released by the binding of calmodulin to CaD in the presence of Ca^{2+} (*1*−*3*, *7*, *18*). The presence of tropomyosin enhances this inhibition by CaD (*7*, *19*, *20*).

CaD is a substrate for several serine/threonine protein kinases (*21*−*26*), including Ca^{2+}-calmodulin-dependent protein kinase II (CDPKII) (*22*), casein kinase II (CKII) (*23*), protein kinase C (PKC) (*24*), mitogen-activated protein kinase (MAPK) (*21*), and p34cdc2 kinase (*25*, *26*). Phosphorylation of CaD by CKII has been reported to play a role in mediating the interaction between CaD and myosin (*23*, *27*). For example, Bogatcheva et al. reported that phosphorylation of CaD by CKII caused a 50% decrease in the binding of CaD to myosin (*27*), and Sutherland et al. (*23*) found that phosphorylation by CKII completely abolished the binding of CaD to immobilized myosin. Although it has been shown that the main CKII phosphorylation sites are in the amino-terminal region of CaD (*23*, *27*), it is still unclear which CKII phosphorylation site(s) is the functionally relevant site responsible for CKII-mediated downregulation of myosin binding by CaD.

CaD promotes the formation and stability of myosin filaments through direct binding to myosin, and it increases the myosin−actin interaction by tethering myosin to actin. It accomplishes this by binding myosin with its amino-terminal region and actin with its carboxyl-terminal region (*1*−*3*, *13*, *28*−*31*). CaD's actin-binding activity has been thought to play an important role in the regulation of cell motility and contraction for both smooth muscle and nonmuscle cells and in the regulation of force development and maintenance in smooth muscle cells (*1*−*3*). In addition to binding to actin and myosin (*28*, *29*, *32*), CaD interacts with a number of other proteins, including tropomyosin (*19*, *32*, *33*), calmodulin (*1*−*3*, *34*, *35*), tubulin (*36*), and caltropin (*37*).

Early attempts to localize CaD's myosin-binding sites involved the analysis of a series of CaD fragments obtained from limited proteolysis or chemical cleavage, which demonstrated that the myosin-binding site was located in the amino-terminal domain (*28*, *29*, *38*). Our recent studies using

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[1] Abbreviations: CaD, caldesmon; CKII, casein kinase II; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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several CaD internal deletion mutants refined this information, localizing the major myosin-binding site to the region between residues 24−53 on CaD (*13*). Consistent in part with our report is the study of Vorotnikov et al. (*39*) showing that the regions between residues 1−28 and 29−128 on CaD contribute to myosin binding. However, a study from Zhuang and Wang (*40*) indicated that the myosin-binding site on CaD lies in the sequence between residues 1−25, and not in the rest of CaD, because a CaD amino-terminal fragment lacking the region between residues 1−25 did not bind to myosin, a result that was inconsistent with our previous findings (*13*). Therefore it has become necessary to determine the locations of the myosin binding site(s) in the amino-terminal region of CaD more precisely.

In this study, we focus on the following questions: (1) What is the major CKII phosphorylation site on CaD? (2) Does the phosphorylation of CaD by CKII regulate the interaction of CaD with myosin? (3) How many myosin-binding motifs comprise the myosin-binding site in the amino-terminal region of CaD? (4) What is the structural relationship between the CKII phosphorylation site(s) and the myosin-binding site on CaD?

MATERIALS AND METHODS

Construction of Recombinant Baculovirus Transfer Vectors. Chicken gizzard smooth muscle cDNA was provided by Dr. Joseph Bryan (Baylor College of Medicine). A series of CaD internal deletion and point mutants were generated using site-directed mutagenesis as described (*41*, *42*). Oligonucleotide primers used to create the structures of the CaD mutants were as follows: CaDΔ24−33, 5′-CGCCTTGAAGCAGAGGAGGAAGAAGCTGCC-3′; CaDΔ24−43, 5′-CGCCTTGAAGCAGAGCGAGCTCGACAGGAA-3′; CaDΔ24−53, 5′-GATACATCTCCTTCTTCCTCTGCTTCAAGGCGC-3′; CaDΔ54−85, 5′-CCAACAATGCAGCTTCCTTTTGCCGCAGCCTTTCC-3′; CaDΔ86−115, 5′-ACGTAGTACAGATGATCCAACGATCACAGAT-3′; CaDΔ116−159, 5′-TCAAAAGGAATTTGACACAGTTACCAAATCGT-3′; CaDΔ160−200, 5′-ATTGAGGAAACAGAAAAAGACAACAAGGAT-3′; CaDS26A, 5′-AGCAGAGAGACTGGCCTACCAGAGAAATG-3′; CaDS73A, 5′-AATGCCCAGAACGCTGTGGCAGAAGAA-3′; and CaDT83A, 5′-GAAACGAAACGTAGTGCAGATGATGAAGCTGCA-3′. The underlined sequences indicate the mutated codons. Point mutations in CaD were generated by replacing threonine or serine residue with alanine. Each mutant cDNA was then subcloned into the baculovirus expression vector pVL1392 for expression in Sf9 or High-Five insect cells. The correct orientation and construction of each CaD mutant were confirmed by both restriction mapping and DNA sequencing.

Transfection and Isolation of Recombinant Baculovirus. *Spodoptera frugiperda* (Sf9) insect cells were cotransfected with a mixture of wild-type AcNPV DNA and recombinant baculovirus pVL1392 vector containing either wild-type CaD or one of the CaD mutants, using the $CaPO_4$ transfection procedure as described previously (*41*, *42*). Recombinant baculoviruses were isolated according to our published method (*41*, *42*). Expression of recombinant CaD and the CaD mutants in Sf9 cells was verified by immunoblotting with a polyclonal antibody against chicken gizzard smooth muscle CaD (Sigma, MO).

Protein Purification. Recombinant CaD and CaD mutants were isolated using a modified protein purification strategy (*43*). Smooth muscle myosin was purified from chicken gizzard smooth muscle tissue as described (*13*, *44*). Phosphorylated smooth muscle myosin was prepared using smooth muscle myosin light chain kinase purified from chicken gizzard. The concentrations of full-length CaD and the CaD mutants were determined by the method of Lowry (*45*), and an extinction coefficient of $E(1\%)_{277} = 0.647$ was used to determine the concentration of smooth muscle myosin.

Binding Assays for Smooth Muscle Myosin. Full-length CaD and the CaD mutants were labeled with [^{14}C]iodoacetamide (*46*). The binding of CaD or its mutants to smooth muscle myosin was measured using sedimentation assays, as described previously (*13*, *46*). The amounts of bound and unbound CaD were determined by measuring the radioactive counts present in the pellets and supernatants, respectively (*46*). Each experiment was performed in triplicate. The apparent dissociation constants for myosin binding were determined by Scatchard analysis (*47*).

Caldesmon Phosphorylation. Purified CaD and CaD mutants (0.6 μM) were phosphorylated by CKII (0.2 μg) in 50 μL of 10 mM 3-[*N*-morpholino]propanesulfolic acid (MOPS; pH 7.2), 5 mM $MgCl_2$, 30 mM NaCl, 2 mM EGTA, 0.4 μM protein kinase A inhibitor, 10 mM β-glycerol phosphate, 0.4 mM sodium orthovanadate, 0.5 mM dithiothreitol, 100 μM ATP, and 100 μCi [γ-^{32}P]ATP (∼3000 Ci/mmol). After incubation for 5−120 min at 30 °C, 20-μL aliquots of each sample were spotted onto numbered P81 phosphocellulose filters (Upstate Biotechnology, NY) and allowed to bind for 30 s. The reactions were stopped by adding 20 μL of 40% TCA to each sample. The filters were then washed and processed as described (*48*). In other experiments, equimolar amounts of CaD (0.6 μM) or each CaD mutant were phosphorylated as described above. The reactions were stopped by adding SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 20% SDS, 1 mM EDTA, 10% glycerol, 5 mM DTT, and 0.45 mM bromophenol blue) and subjected to SDS−PAGE and autoradiography. The protein bands were excised and counted in a liquid scintillation counter (Beckman Instruments).

Phosphoamino Acid Analysis. The CKII phosphorylation of CaD and CaD mutants was performed as described above. After incubation with CKII, CaD or its mutants were isolated using heat treatment and SDS−PAGE followed by Coomassie Blue staining. The CaD bands were cut out and subjected to phosphoamino acid analysis. Phosphoamino acid analysis was carried out according to our published procedure (*49*).

RESULTS

To identify the functionally relevant CKII phosphorylation site(s) and to determine the structural relationship between the CKII phosphorylation site(s) and the myosin-binding site on CaD, we generated a series of CaD internal deletion mutants in which only the targeted regions were deleted (Figure 1). All the mutant proteins were purified according to our published method (*43*). The yield for each mutant varied from 50 to 100 μg/10^6 cells (data not shown).

CaD1−550, which lacks most of the carboxyl terminal region of CaD, was used as a control (data not shown). The

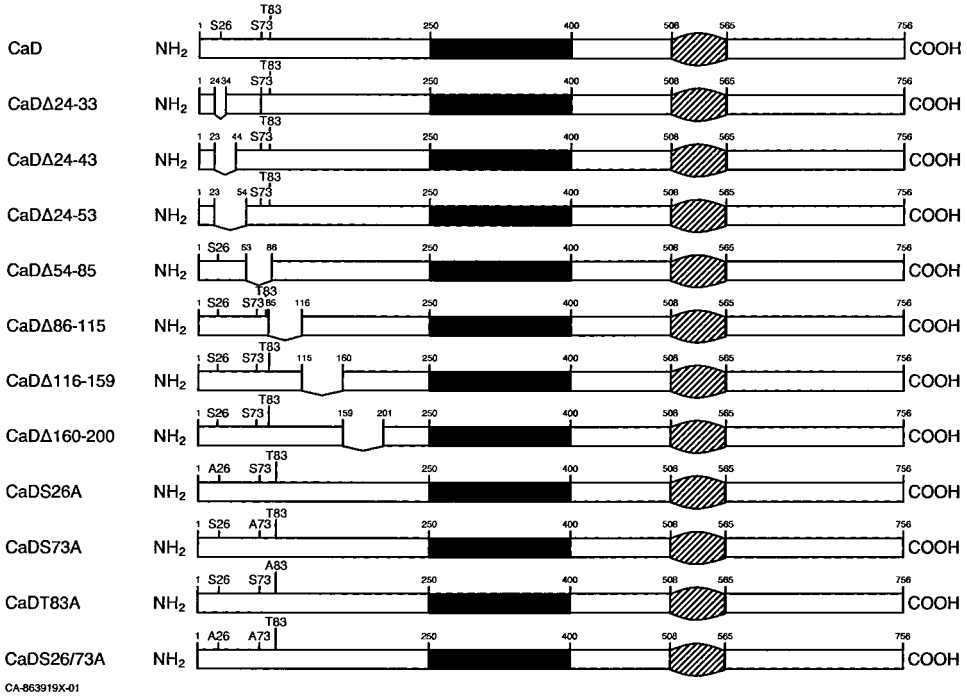

FIGURE 1: Schematic representation of CaD internal deletion and point mutants. Black boxes represent the central repetitive region consisting of a 13-amino acid sequence repeated eight times (*54*). Hatched areas indicate regions of sequence homology between CaD and troponin T, as determined by amino acid sequence alignment (*54*). V-Shaped areas indicate the regions deleted in the CaD mutants. Numbers represent the amino acid positions. S and A indicate serine and alanine, respectively.

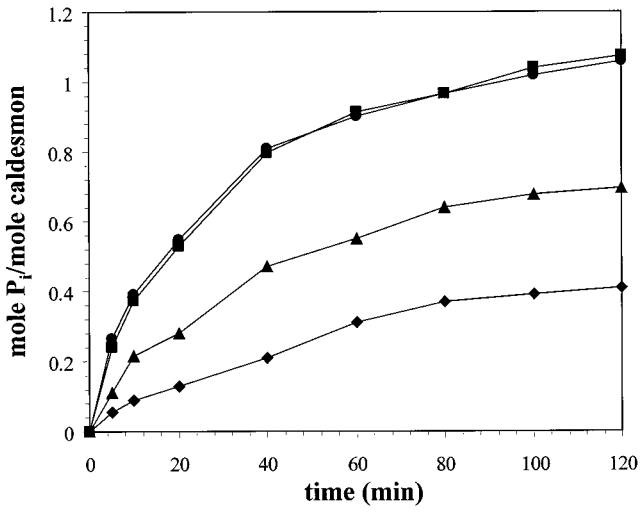

FIGURE 2: Phosphorylation of CaDΔ24−53, CaDΔ54−85, and CaDΔ86−115 by CKII. CKII phosphorylation of CaD and each CaD mutant was performed. The assay conditions are described in Materials and Methods. The number of moles of CaD bound per mole of Pi is plotted as a function of incubation time. The symbols ●, ■, ▲, and ♦ represent full-length CaD, CaDΔ86−115, CaDΔ54−85, and CaDΔ24−53, respectively.

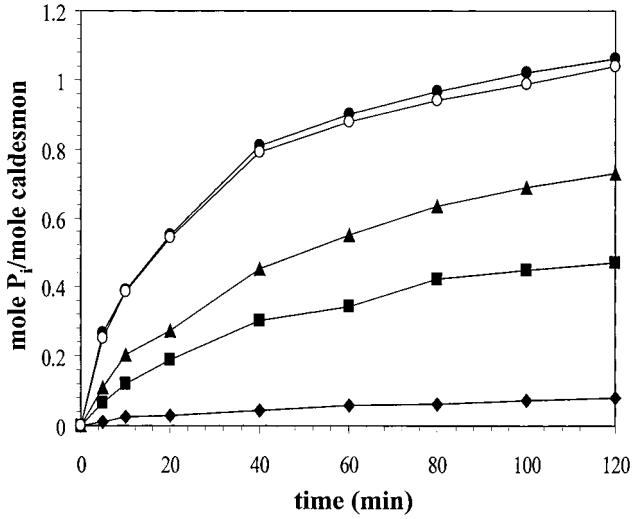

FIGURE 3: Phosphorylation of CaDS26A, CaDS73A, CaDT83A, and CaDS26/73A by CKII. Phosphorylation of CaD or each mutant by CKII was performed. The assay conditions were the same as stated in Figure 2. The symbols ●, ■, ▲, ♦, and ○ represent full-length CaD, CaDS26A, CaDS73A, CaDS26/73A, and CaDT83A, respectively.

phosphorylation level of CaD1−550 by CKII was similar to that of full-length CaD (data not shown), suggesting that the sequence between residues 551−756 does not contain phosphorylation sites for CKII. By contrast, CaDΔ24−53 was only weakly phosphorylated by CKII (Figure 2). Deletion of the region between residues 54−85 only contributed to a slight decrease in the phosphorylation by CKII (Figure 2), and deletion of the sequences between residues 86−115, 116−156, and 160−200 did not alter the degree of CKII phosphorylation (data not shown). These results indicated that Ser-26 and Ser-73 are the CKII phosphorylation sites; however, Ser-26 appears to be the major site.

To verify these results and to see if Thr-83 on CaD is also phosphorylated by CKII, we generated several CaD point mutants using site-directed mutagenesis, in which the threonine or serine residue was replaced by alanine (Figure 1). Analysis of these mutants showed that replacement of Ser-73 resulted in a 32% decrease in phosphorylation by CKII (Figure 3). As expected, the mutation of Ser-26 caused a greater reduction, 52%, in CKII phosphorylation (Figure 3). The phosphorylation level of the dual CaD point mutant (CaDS26/73A) was only about 8% of that of full-length CaD

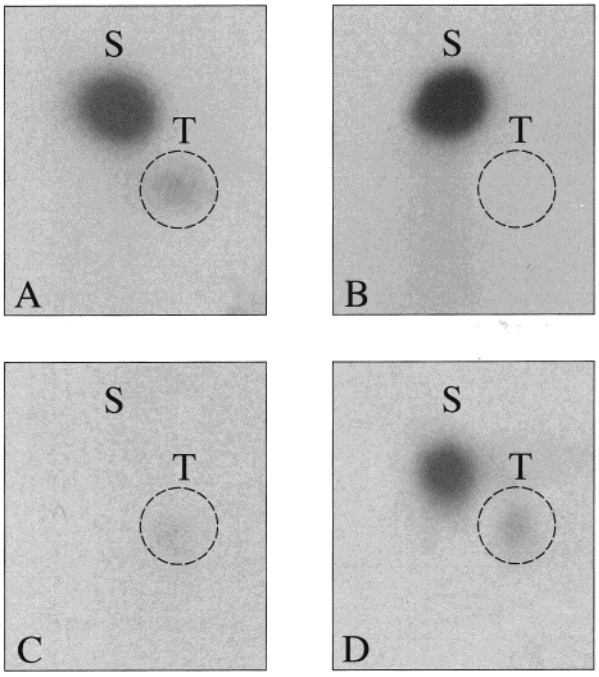

FIGURE 4: Phosphoamino acid analysis of CaD, CaD1−550, CaDS26/73A, and CaDT83A. S and T represent phosphoserine and phosphothreonine, respectively. The thin-layer cellulose plates were exposed to X-ray film for 5−10 days: (A) full-length CaD, (B) CaD1−550, (C) CaDS26/73A, and (D) CaDT83A.

(Figure 3). The phosphorylation level of CaDΔ24−33 was similar to that of CaDΔ24−43 but much lower than that of full-length CaD (data not shown). Interestingly, the mutation of Thr-83 had no effect on the level of CKII phosphorylation when compared with that of full-length CaD (Figure 3). Figure 4 shows the phosphoamino acid analysis of full-length CaD, CaD1−550, CaDS26/73A, and CaDT83A. Phosphorylation of full-length CaD by CKII resulted in a strong ^{32}P-labeled spot and a weak ^{32}P-labeled spot comigrating with the phosphoserine and phosphothreonine standards, respectively (Figure 4A). The weak ^{32}P-labeled spot was not affected by mutation of Thr-83 (Figure 4D), and the deletion of the sequence between residues 551−765 abolished the weak threonine phosphorylation by CKII (Figure 4B), suggesting that the C-terminal region of CaD contains a threonine which can be weakly phosphorylated by CKII. As expected, the CaDS26/73A mutant was not serine-phosphorylated (Figure 4C). These results not only confirm our results described above but also indicate that Thr-83 is not phosphorylated by CKII in vitro.

As shown in Figures 2 and 3, incorporation of approximately 1 mol of phosphate/mol of CaD resulted from the incubation of full-length CaD or CaDT83A with CKII for 2 h. In contrast, 0.695 mol of P_i/mol of CaDΔ54−85 and 0.41 mol of P_i/mol of CaDΔ24−53 were incorporated under the same conditions (Figure 2). As we expected, the P_i incorporation level of CaDΔ86−115 was similar to that of full-length CaD (Figure 2). The incorporation of P_i into CaDS26A, CaDS73A, and CaDS26/73A was 0.47 mol of P_i/mol of CaDS26A, 0.73 mol of P_i/mol of CaDS73A, and 0.08 mol of P_i/mol of CaDS26/73A, respectively (Figure 3). These data clearly demonstrate that the level of S26 phosphorylation is greater than that of S-73 phosphorylation.

Phosphorylation of CaD by CKII reduces its ability to bind to myosin (*23*, *27*). To determine which CKII phosphorylation site on CaD is functionally relevant, we tested the effect of CKII phosphorylation on the binding of full-length CaD and CaD mutants to smooth muscle myosin. In these experiments, the unphosphorylated CaD and CaD mutants were used as controls. The effect of phosphorylation by CKII on the myosin binding of CaD is shown in Figure 5. Phosphorylation by CKII resulted in a significant decrease (67%) in the binding of CaD to myosin (Figure 5), consistent with the previous results (*23*, *27*). By contrast, phosphorylation by CKII caused a smaller effect on the ability of either CaDS26A or CaDS73A to bind myosin (Figure 5), and no effect was seen on the binding ability of CaDS26/73A (Figure 5). Scatchard analysis showed that the apparent K_d values of myosin binding for CKII-phosphorylated CaDS26A and CaDS73A were $0.94 \pm 0.067 \times 10^{-6}$ and $1.21 \pm 0.089 \times 10^{-6}$ M, respectively. The apparent K_d value of CKII-phosphorylated CaDS26/73A was identical to that of unphosphorylated CaD. The low-level binding of CKII-phosphorylated CaD to myosin precluded a reliable determination of the apparent dissociation constant in this case.

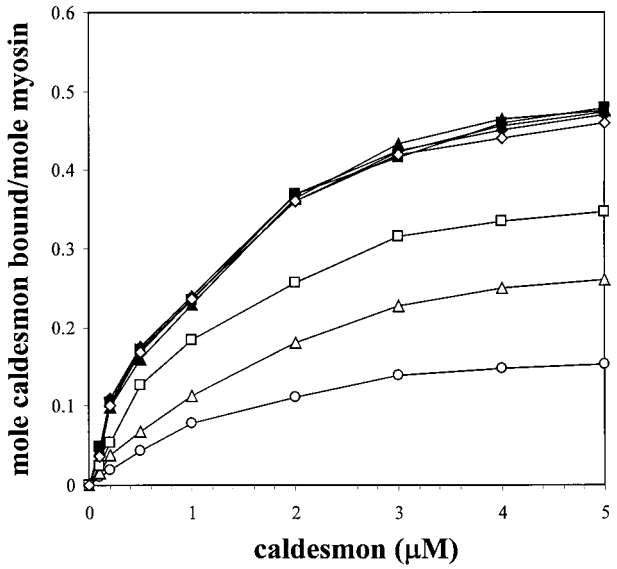

FIGURE 5: Effect of CKII phosphorylation on the binding of CaD or CaD mutants to smooth muscle myosin. CaD and each CaD mutant were incubated for 2 h at 30 °C under the conditions for the in vitro phosphorylation assay as described in Materials and Methods in either the absence or presence of CKII. The binding of the phosphorylated (open symbols) or unphosphorylated (closed symbols) CaD or CaD mutants to smooth muscle myosin was performed according to our published method (*13*, *46*). The ○,●, □,■, △,▲, and ◇,◆ represent full-length CaD, CaDS26A, CaDS73A, and CaDS26/73A, respectively.

The deletion of the sequence between residues 24−53 strikingly weakened the binding of CaD to myosin, consistent with our previous report (*13*) that the major myosin-binding motif on CaD is located between residues 24−53 (Figure 6A). In addition, CaDΔ54−85, CaDΔ116−119, and CaDΔ160−200 bound to myosin as strongly as did full-length CaD (Figure 6A), suggesting that Ser-73 does not directly contribute to the binding of CaD to myosin. On the other hand, the myosin binding of CaDΔ86−115 was 85% of that of full-length CaD. These data demonstrate that the sequence between residues 86−115 is involved in weak binding of CaD to myosin.

To further map the major myosin-binding site in the region between residues 24−53, we tested the binding capacity of

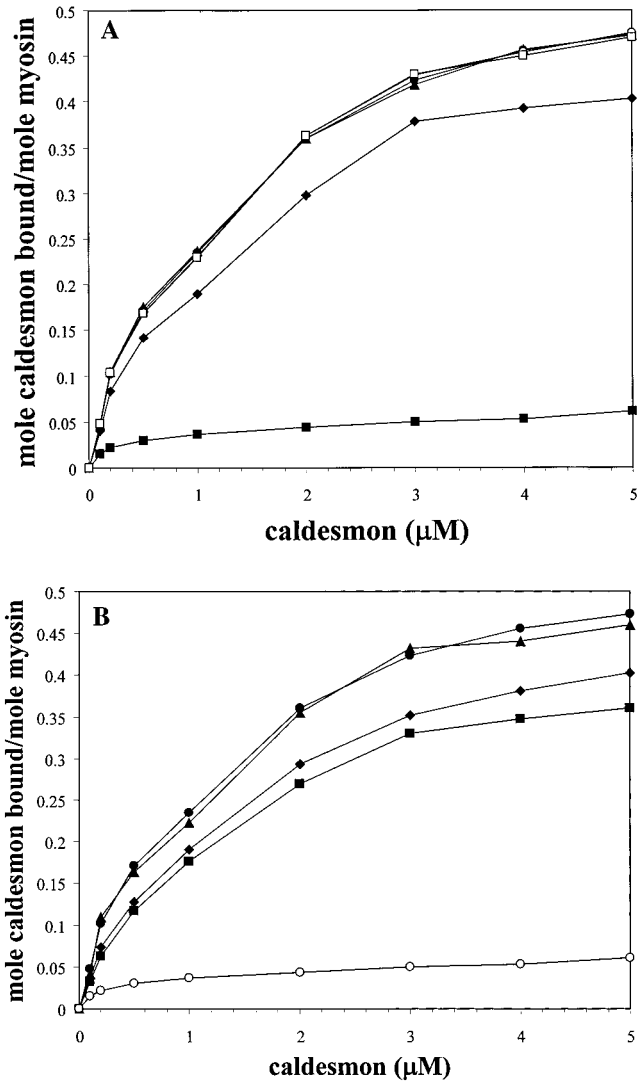

FIGURE 6: Binding of CaD internal deletion mutants to smooth muscle myosin. The assay conditions were the same as stated in Figure 5: (A) binding of full-length CaD (●), CaDΔ24−53 (■), CaDΔ54−85 (▲), CaDΔ86−115 (♦), CaDΔ116−159 (○), and CaDΔ160−200 (□) to smooth muscle myosin; (B) binding of full-length CaD (●), CaDΔ1−23 (■), CaDΔ24−33 (▲), CaDΔ24−43 (♦), and CaDΔ24−53 (○) to smooth muscle myosin.

CaDΔ24−33, CaDΔ24−43, and CaDΔ24−53 to smooth muscle myosin. As shown in Figure 6B, the deletion of residues 24−33 did not affect the binding of CaD to myosin. Clearly, Ser-26 is located outside of the myosin-binding site on CaD. On the other hand, deletion of residues 24−43 reduced CaD's binding to myosin up to 15%, compared with full-length CaD. Importantly, the deletion of an additional 10-amino acid sequence (residues 24−53) resulted in a more than 80% decrease in myosin binding. Furthermore, the amino-terminal deletion of the first 23 residues also caused a 23% decrease in myosin binding (Figure 6B). These data demonstrate that the major myosin-binding site lies in a 10-amino acid sequence between residues 44−53, and the sequences between residues 1−23 and 24−43 are involved in weak myosin binding. The apparent K_d values of myosin binding for full-length CaD, CaDΔ24−43, CaDΔ86−115, and CaDΔ1−23 were $0.7 \pm 0.04 \times 10^{-6}$, $0.86 \pm 0.048 \times 10^{-6}$, $0.84 \pm 0.054 \times 10^{-6}$, and $0.92 \pm 0.062 \times 10^{-6}$ M, respectively. The apparent dissociation constants of CaDΔ54−85, CaDΔ116−159, CaDΔ160−200, and CaDΔ24−33 were identical to that of full-length CaD. We failed to make any reliable determination of the apparent K_d value for CaDΔ24−53, since the amount of CaDΔ24−53 that bound to myosin was very low.

DISCUSSION

CaD can be phosphorylated by several serine/threonine kinases, including CDPKII (*22*), CKII (*23*), PKC (*24*), MAPK (*21*), and $p34^{cdc2}$ kinase (*25*, *26*). MAPK and $p34^{cdc2}$ kinase preferably phosphorylate serine/threonine residues located in the carboxyl-terminal region of CaD (*1*−*3*), while the preferred phosphorylation sites by CKII and CDPKII on CaD are in the amino-terminal region (*23*, *27*). Although CaD is phosphorylated by many protein kinases, CKII is the major endogenous CaD kinase in smooth muscle (*50*). Wawrzynow et al. (*51*) suggest that the predominant phosphorylation site by CKII on CaD is Ser-73. On the other hand, analysis of several CaD fragments obtained from chemical cleavage by Sutherland et al. (*23*) showed that both Ser-73 and Thr-83 on CaD can be preferentially phosphorylated by CKII. Interestingly, a synthetic CaD peptide containing both Ser-73 and Thr-83 can be phosphorylated by CKII only at Thr-83 (23). Apparently, the main CKII phosphorylation site(s) on CaD remained to be determined. Here, we tested the ability of CKII to phosphorylate several CaD internal deletion mutants (Figure 1). On the basis of the studies described above, we assumed that the main CKII phosphorylation site would be Ser-73 and/or Thr-83. Surprisingly, the deletion of residues 24−53 on CaD resulted in a striking decrease in the level of CKII phosphorylation (Figure 2), whereas deletion of residues 54−85 caused only a minimal decrease in phosphorylation (Figure 2). These observations strongly suggest that Ser-26 is the main CKII phosphorylation site and that Ser-73 or Thr-83 is a minor CKII phosphorylation site. To rule out the possibility that the decreased CKII phosphorylation of either CaDΔ24−53 or CaDΔ54−85 resulted from deletion-induced conformational changes, we generated several CaD single-point mutations in which Ser-26, Ser-73, or Thr-83 was replaced with alanine and one dual-point mutant in which both Ser-26 and Ser-73 were replaced with alanine. The results obtained using these constructs further confirm that Ser-26 is the preferred site of CKII phosphorylation (Figure 3). In addition, we observed that the dual-point mutant (CaDS26/73A) was not significantly phosphorylated by CKII (Figure 3), even after an extended incubation time (data not shown) and that the rate of CKII phosphorylation for CaDT83A was similar to that for full-length CaD (Figure 3). Using phosphoamino acid analysis, we further confirmed that Thr-83 is not phosphorylated by CKII (Figure 4). The discrepancy between our observations and those of others (*23*, *51*) is most likely due to differences in the assay strategies, experimental conditions, and sources of CaD (*23*). It should be noted that the studies from both Sutherland et al. (*23*) and Wawrzynow et al. (*51*) used isolated CaD peptides obtained from the chemical cleavage or proteolysis of CKII-phosphorylated CaD. Since these CaD peptides lack a major portion of the CaD molecule, they may not have the three-dimensional structure that is necessary for interacting with CKII. On the other hand, in this study, we used both purified CaD internal deletion mutants and CaD point mutants to identify the CKII

phosphorylation sites on CaD. Although we cannot rule out the possibility that the internal deletion of CaD induced a conformational change, it is less likely that the replacement of Ser-26, Ser-73, or Thr-83 with alanine caused a conformational change that had an effect on the interaction between CaD and CKII or that altered the substrate specificity for CKII. Alanine was selected as the replacement residue because a variety of studies have shown that it is compatible with all types of secondary structures and that it does not impose new structural effects associated with hydrogen bonding, unusual hydrophobicity, or steric bulk (*52*),

Myosin−CaD interactions can be downregulated by the phosphorylation of CaD by CKII. However, it has been unclear which CKII phosphorylation site(s) on CaD is responsible for this downregulation. In this report, analysis of several CaD point mutants (Figure 5) allowed us to determine the involvement of the CKII phosphorylation sites Ser-26 and Ser-73 in the CKII-mediated regulation of CaD myosin binding. We observed that phosphorylation of CaD by CKII resulted in a 67% decrease in CaD binding to myosin, consistent with previous reports (*23*, *27*). Although phosphorylation of both Ser-26 and Ser-73 by CKII was required for the downregulation of myosin binding, Ser-26 was the major functionally relevant phosphorylation site because the reduction in the myosin binding of CaD by the CKII phosphorylation of Ser-73 is only about one-half of that seen for Ser-26 (Figure 5).

Because of technical difficulties (i.e., CaD can be phosphorylated by many serine/threonine kinases in smooth muscle tissue, and it is hard to maintain a differentiated phenotype in smooth muscle cells in culture), it is unknown whether CaD can be phosphorylated by CKII in vivo or whether the binding of CaD to myosin can be downregulated by CKII phosphorylation. However, our present study, together with others (*23*, *50*, *51*), provides evidence that, in vitro, CaD can be phosphorylated by CKII and the binding of CaD to myosin can be downregulated by CKII phosphorylation. Interestingly, the major CKII phosphorylation site on CaD is Ser-26, which is adjacent to the myosin-binding motifs (residues 1−23 and 34−53) on CaD. These data support the possibility that the binding of CaD to myosin could be mediated by CKII phosphorylation in vivo. As CaD can modulate muscle contraction through binding to myosin (*1*−*3*), it is possible that CKII can mediate smooth muscle contraction through the phosphorylation of CaD. In the future, we will examine this issue further using skinned smooth muscle fibers and CKII-phosphorylated CaD.

By analyzing several CaD internal deletion mutants generated using a baculovirus expression system, we recently demonstrated that the major myosin-binding site on CaD resides in a 30-amino acid stretch between residues 24−53 (13). A recent study from Vorotnikov et al. (*39*) using several CaD fragments containing the sequences between residues 1−128, 1−152, and 29−152 also suggested the possibility that the myosin-binding site in the amino-terminal region of CaD contains two separate myosin-binding motifs within residues 1−28 and 29−128. Subsequently, a report from Zhuang and Wang (*40*) showed that a synthetic peptide containing the sequence between residues 1−25 binds to a myosin-affinity column, indicating that the region between residues 1−25 contains a myosin-binding motif required for CaD to bind to myosin. More recently, it has been suggested that a synthetic peptide containing the sequence between residues 25−53 of CaD induces the in vivo contraction of single permeabilized smooth muscle cells from the ferret portal vein, probably by competing with the myosin-binding domain of endogenous CaD (*53*). Here, we tested a series of CaD internal deletion mutants for their ability to bind to smooth muscle myosin. These mutants, unlike CaD fragments or CaD peptides, retain the entire structure of CaD, except for the targeted sequences. We found that the myosin-binding site in CaD consists of at least four myosin-binding motifs located in the amino-terminal regions between residues 1−23, 34−43, 44−53, and 86−115. The core sequence responsible for the strong myosin binding lies between residues 44−53 on CaD, whereas the myosin-binding motifs in residues 1−23, 34−43, and 86−115 on CaD contribute to weak myosin binding (Figure 6A,B). Moreover, we have demonstrated that residues 24−33, 54−85, 116−159, and 160−200 on CaD are not required for myosin binding (Figure 6A).

Consistent with our previous study (*43*), the maximum myosin binding of CaD in this study was 0.5 mol of CaD/mol of myosin, suggesting that, under our assay conditions, one CaD molecule binds to two myosin molecules. It is, however, unclear how one CaD molecule binds to two myosin molecules. It is known that CaD binds to myosin by interacting with the S1/S2 junction of myosin (*28*, *30*, *33*, *54*). It has been reported that the stoichiometry of CaD binding to myosin ranges from 1 to 3 mol of CaD/mol of myosin (*1*). The differences in the reported stoichiometry of the myosin binding of CaD in different studies may arise from differences in the assay conditions.

Our present findings that the deletion of neither residues 24−33 nor residues 54−85 had any effect on the ability of CaD to bind to myosin indicate that both Ser-26 and Ser-73 are structurally located outside of the myosin-binding motifs on CaD. Because of this, the downregulation of myosin−CaD interaction by CKII phosphorylation cannot be caused by the direct modification of the myosin-binding motifs by CKII phosphorylation. However, both Ser-26 and Ser-73 are downstream or upstream of the core myosin-binding motifs (residues 1−23, 34−43, 44−53, and 86−115) on CaD. Therefore, it is likely that phosphorylation of either Ser-26 or Ser-73 induces conformational changes in the adjacent myosin-binding motifs, thus altering their ability to bind to myosin.

In summary, we have identified Ser-26 but not Ser-73 to be the major site of CKII phosphorylation on CaD and shown that phosphorylation of both Ser-26 and Ser-73 is involved in modulating the interaction between CaD and myosin. Analysis of a series of CaD internal deletion and point mutants suggests that Thr-83, which Sutherland et al. (*23*) proposed as a CKII phosphorylation site, is not phosphorylated by CKII. In addition, we further refined the region containing the major myosin-binding site of CaD from residues 24−53 (*13*) to be the sequence between residues 44−53. Our data also demonstrated that there are at least three functional motifs within the amino-terminal region of CaD that are responsible for weak myosin binding, located between residues 1−23, 34−43, and 86−115. The localization of the CKII phosphorylation sites (i.e., Ser-26 and Ser-73) and the myosin-binding motifs on CaD and our understanding of the structural relationships among these sites will

help us to understand how CKII phosphorylation mediates the downregulation of myosin binding by CaD.

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REFERENCES

1. Marston, S. B., and Redwood, C. S. (1991) *Biochem. J. 279*, 1−16.
2. Sobue, K., and Sellers, J. R. (1991) *J. Biol. Chem. 266*, 12115−12118.
3. Matsumura, F, and Yamashiro, S. (1993) *Curr. Opin. Cell Biol. 5*, 70−76.
4. Sobue, K., Morimoto, K., Inui, M., Kanda, K., and Kakiuchi, S. (1982) *Biomed. Res. 3*, 188−196.
5. Ngai, P. K., and Walsh, M. P. (1984) *J. Biol. Chem. 259*, 13656−13659.
6. Marston, S. B., and Smith, C. W. (1985) *J. Muscle Res. Cell Motil. 6*, 669−708.
7. Horiuchi, K. Y., Miyata, H., and Chacko, S. (1986) *Biochem. Biophys. Res. Commun. 136*, 962−968.
8. Lash, J. A., Sellers, J. R., and Hathaway, D. R. (1986) *J. Biol. Chem. 261*, 16155−16160.
9. Chalovich, J. M., Cornelius, P., and Benson, C. E. (1987) *J. Biol. Chem. 262*, 5711−5716.
10. Shirinsky, V. P., Biryukov, K. G., Hettasch, J. M., and Sellers, J. R. (1992) *J. Biol. Chem. 267*, 15886−15892.
11. Horiuchi, K., and Chacko, S. (1995) *J. Muscle Res. Cell Motil. 16*, 11−19.
12. Fraser, I. D. C., and Marston, S. B. (1995) *J. Biol. Chem. 270*, 19688−19693.
13. Wang, Z., Jiang, H., Yang, Z. Q., and Chacko, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11899−11904.
14. Wagner, A. S. J., Dabrowska, R., and Rüegg, J. C. (1985) *FEBS Lett. 192*, 9−12.
15. Pfitzer, G., Zeugner, C., Troschka, M., and Chalovich, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5904−5908.
16. Kraft, T., Chalovich, J. M., Yu, L. C., and Brenner, B. (1995) *Biophys. J. 68*, 2404−2418.
17. Malmqvist, U., Arner, A., Makuch, R., and Dabrowska, R. (1996) *Eur. J. Physiol. 432*, 241−247.
18. Smith, C. W. J., and Marston, S. B. (1985) *FEBS Lett. 184*, 115−119.
19. Dabrowska, R., Goch, A., Galazkiewicz, B., and Osinska, H. (1985) *Biochim. Biophys. Acta 842*, 70−75.
20. Sobue, K., Takahash, K., and Wakabayash, I. (1985) *Biochem. Biophys. Res. Commun. 132*, 645−651.
21. Childs, T. J., Watson, M. H., Sanghera, J. S., Campbell, D. L., Pelech, S. L., and Mak, A. S. (1992) *J. Biol. Chem. 267*, 22835−22859.
22. Ikebe, M., and Reardon, S. (1990) *J. Biol. Chem. 265*, 17607−17612.
23. Sutherland, C., Renaux, B. S., Mckay, D. J., and Walsh, M. P. (1994) *J. Muscle Res. Cell Motil. 15*, 440−456.
24. Vorotnikov, A. V., Gusev, N. B., Hua, S., Collins, J. H., Redwood, C. S., and Marston, S. B. (1994) *J. Muscle Res. Cell Motil. 15*, 37−48.
25. Yamashiro, S., Yamakita, Y., Ishikawa, R., and Matsumura, F. (1990) *Nature 344*, 675−678.
26. Yamashiro, S., Yamakita, Y., Hosoya, H., and Matsumura, F. (1990) *Nature 349*, 169−172.
27. Bogatcheva, N. V., Vorotnikov, A. V., Birukov, K. G., Shirinsky, V. P., and Gusev, N. B. (1993) *Biochem. J. 290*, 437−442.
28. Sutherland, C., and Walsh, M. P. (1989) *J. Biol. Chem. 264*, 578−583.
29. Hemric, M. E., and Chalovich, J. M. (1990) *J. Biol. Chem. 265*, 19672−19678.
30. Ikebe, M., and Reardon, S. (1988) *J. Biol. Chem. 263*, 3055−3058.
31. Haeberle, J. R., Trybus, K. M., Hemric, M. E., and Warshaw, D. M. (1992) *J. Biol. Chem. 267*, 23001−23006.
32. Katayama, E., Horiuchi, K. Y., and Chacko, S. (1989) *Biochem. Biophys. Res. Commun. 160*, 1316−1322.
33. Fujii, T., Imai, M., Rosenfeld, G. C., and Bryan, J. (1987) *J. Biol. Chem. 262*, 2757−2763.
34. Zhan, Q., Wong, S. S., and Wang, C. L. A. (1991) *J. Biol. Chem. 266*, 21810−21814.
35. Marston, S. B., Fraser, I. D. C., Huber, P. A. J., Pritchard, K., Gusev, N. B., and Torok, K. (1994) *J. Biol. Chem. 269*, 8134−8139.
36. Ishikawa, R., Kagami, O., Hayashi, C., and Kohama, K. (1992) *Cell Motil. Cytoskeleton 23*, 244−251.
37. Mani, R. S., McCubbin, W. D., and Kay CM. (1992) *Biochemistry 31*, 11896−11901.
38. Velaz, L., Ingraham, R. H., and Chalovich JM. (1990) *J. Biol. Chem. 265*, 2929−2934.
39. Vorotnikov, A. V., Marston, S. B., and Huber, P. A. (1997) *Biochem. J. 328*, 211−218.
40. Zhuang, S., and Wang, C.-L. A. (1997) *Mol. Biol. Cell 8*, 156a, #904.
41. Wang, Z., and Chacko, S. (1996) *J. Biol. Chem. 271*, 25706−25714.
42. Wang, Z., Yang, Z.-Q., and Chacko, S. (1997) *J. Biol. Chem. 272*, 16896−16903.
43. Wang, Z., Horiuchi, K. Y., Jacob, S. S., Gopalakurup, S., and Chacko, S.(1994) *J. Muscle Res. Cell Motil. 15*, 646−658.
44. Chacko, S. (1981) *Biochemistry 20*, 702−707.
45. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
46. Wang, Z., Horiuchi, K. Y., and Chacko, S. (1996) *J. Biol. Chem. 271*, 2234−2242.
47. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci. 51*, 660−672.
48. Vorotnikov, A. V., Shirinsky, V. P., and Gusev, N. B. (1988) *FEBS Lett. 236*, 321−324.
49. Wang, Z., Danielsen, A. J., Maihle, N. J., and McManus, M. J. (1999) *J. Biol. Chem. 274*, 33807−33813.
50. Orotnikov, A. V., Gusev, N. B., Hua, S., Collins, J. H., Redwood, C. S., and Marston, S. B. (1993) *FEBS Lett. 334*, 18−22.
51. Wawrzynow, A., Collins, J. H., Bogatcheva, N. V., Vorotnikov, A. V., and Gusev, N. B. (1991) *FEBS Lett. 289*, 213−216.
52. Wells, J. A.(1991) *Methods Enzymol. 202*, 390−411.
53. Lee, Y.-H., Gallant, C., Guo, H, Li, Y., Wang, C.-L. A., and Morgan, K. G. (2000) *J. Biol. Chem. 275*, 3213−3220.
54. Ball, E. H., and Kovala, T. (1988) *Biochemistry 27*, 6093−6098.
55. Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G., and Lin WG. (1989) *J. Biol. Chem. 264*, 13873−13879.

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