Multiple functional domains responsible for calmodulin (CaM) binding and actin-binding/actomyosin ATPase inhibition are present in the region between residues 598–756 of the chicken gizzard smooth muscle caldesmon (CaD) molecule. To precisely localize these functional domains and to further elucidate the structural basis of these domains, we analyzed a series of purified mutants of chicken gizzard smooth muscle CaD generated by internal deletions of amino acid sequences and expression in a baculovirus expression system. Our results demonstrate that, in addition to a strong actin-binding site sequence between residues 718–723 (Wang, Z., and Chacko, S. (1996) J. Biol. Chem. 271, 25707–25714), two weak actin-binding motifs are present in the regions between residues 690–699 and 650–666. These weak actin-binding regions function independently and are associated with weak actomyosin inhibitory activity. Analysis of the CaM-binding sites A (residues 658–666) and B (residues 690–695), the major CaM-binding sites in the C-terminal region of CaD, provided direct evidence for the involvement of both CaM-binding sites in the CaM-mediated reversal of the inhibition of actomyosin ATPase activity by CaD and for the functional independence of the two CaM-binding sites. Furthermore, the sequences between residues 598–649, upstream of CaM-binding site A, and 700–717, downstream of CaM-binding site B, appear to have no effect on either actin-binding or CaM-binding. The data also suggest that both CaM-binding sites A and B structurally overlap or lie in close proximity to the adjacent weak actin-binding sites and weak actomyosin ATPase inhibitory determinants.

Regulation of smooth muscle contraction is thought to occur primarily through myosin phosphorylation-dephosphorylation mechanisms (for review, see Refs. 1–3) complemented by a thin filament-mediated regulation that requires the actin/caldesmon-binding proteins, such as smooth muscle caldesmon (CaD)1 (4–9) and calponin (10, 11). It has been established that CaD inhibits the actin-activated ATPase activity of phosphorylated smooth muscle myosin in vitro motility assays (14–16), and down-regulates force generation in a chemically skinned fiber system (17). This CaD-induced inhibition of actomyosin ATPase activity is completely released by Ca\textsuperscript{2+}-binding protein calmodulin (CaM) or caltropin (18) in the presence of Ca\textsuperscript{2+}. Smooth muscle troponymosin enhances the inhibition (5–7, and for review, see Refs. 12 and 13). It has also been suggested that the inhibitory function of CaD is modulated by phosphorylation of the CaD C-terminal region by mitogen-activated protein kinase or cdc2 kinase, leading to partial reversal of the CaD-induced inhibition of actomyosin ATPase activity (19, 20). The exact inhibitory mechanism by which CaD inhibits actomyosin ATPase activity is unclear, but proposed models include competition of CaD with myosin for the same binding site in the actin molecule (21) and CaD-mediated effect on the catalytic step in the ATPase cycle (22, 23).

Functional analysis of CaD fragments produced from limited proteolysis, chemical cleavage, and bacterial expression systems has led to the localization of various functional domains in the CaD molecule. The major myosin-binding site was mapped to the NH\textsubscript{2}-terminal region of CaD (21, 24, 25), whereas the ATPase inhibitory determinants and the crucial actin-/calmodulin-binding sites were first mapped to a 38-kDa chymotryptic fragment in the C terminus (22, 26–28) and recently restricted to a smaller region between residues 658–756 (29, 30). More recently, our analysis of C-terminal deletion mutants of chicken gizzard CaD derived in our laboratory indicated the presence of multiple actin-binding sites and ATPase inhibitory determinants in the region between residues 658–756 (31). Further analysis of internal deletion mutants of chicken gizzard CaD identified a strong actin-binding motif of six amino acid residues from Lys\textsuperscript{716} to Glu\textsuperscript{723}, which also forms the core sequence for CaD-induced inhibition of actomyosin ATPase activity (32). Our findings also suggested that the region between residues 690 and 717 is associated with the weak inhibition of actomyosin ATPase activity. This weak inhibitory determinant between residues 690–717 and the strong inhibitory determinant between residues 718–756 function independently (32).

It has been assumed that Ca\textsuperscript{2+}-CaM is involved in the regulation of CaD function because Ca\textsuperscript{2+}-CaM completely reverses the inhibition of actomyosin ATPase activity by CaD and, to some extent, displaces CaD bound to actin (4–9, and for review, see Refs. 12 and 13). Comparison of data from biochemical studies using synthetic and chymotryptic peptides of CaD has defined two major CaM-binding sites in the C terminus of CaD, which have been precisely localized to a sequence between residues 658–666 (CaM-binding site A) (33) and a sequence between residues 687–695 (CaM-binding site B) (34). Analysis of C-terminal truncated proteins of CaD further localized CaM-binding site B to a six-residue stretch from Asn\textsuperscript{690} to
Lys<sup>695</sup> (31). However, the functional role of the two calmodulin-binding sites in the interaction with Ca<sup>2+</sup>-CaM remains controversial.

To better understand the weak actin-binding and actomyosin ATPase inhibitory domains and the relationship between the CaM-binding sites and actin-binding/actomyosin ATPase inhibitory domains in the CaD molecule, we generated a series of internal deletions in the regions that encompass the weak actin-binding sites, and analyzed these mutants for their actin-binding, inhibition of actomyosin ATPase, and CaM binding. Our data clearly demonstrate that 1) two weak actin-binding/actomyosin ATPase inhibitory motifs are located in the regions between residues 650–666 and 690–699, respectively; that 2) both CaM-binding sites A and B are functionally involved in CaM-induced reversal of CaD-mediated inhibition of actomyosin ATPase activity; and that 3) both CaM-binding sites A and B overlap or are in close proximity with the adjacent weak actin-binding/ATPase inhibitory determinants.

MATERIALS AND METHODS

Construction of Recombinant Baculovirus Transfer—Experiments were designed to produce CaD mutants that lack specific targeted amino acid sequences while preserving the rest of the CaD sequence. The full-length chicken gizzard smooth muscle CaD cDNA (a generous gift from Dr. Joseph Bryan) (35) was subcloned into the pBluescript SK<sup>−</sup> plasmid, and sense single-stranded pBluescript CaD cDNA was made as a template for a site-directed mutagenesis reaction as described (32). The following antisense mutagenesis oligonucleotides were synthesized using an Applied Biosystems DNA/RNA synthesizer:

**-CCAGAGATTACGTTTGATACGACTGGAGAC-3**<sup>9</sup>

**-GTCAGAGGCTGCTGGTGCACTAGTATATTG-3**

**-CTTGTTGCCCAACAGGCCAGGTTCTTACACC-3**

**-TGTTCCCCCAGGTGATGCACTAGTATATTG-3**

**-TTTACGGAGCATATGG-3**

**-CCAGAGATTACGTTTACCCTGGGGTCTT-3**

**-TGTTCCCCCAGGTGATGCACTAGTATATTG-3**

**-TTTACGGAGCATATGG-3**

**-CTTGTTGCCCAACAGGCCAGGTTCTTACACC-3**

Internal deletion mutants of CaD were generated as described (32) and confirmed by sequencing the mutagenized regions. After subcloning into the PvuI941 vector for expression in Sf9 cells, each CaD mutant was checked for correct orientation, and sequences were reconfirmed by DNA sequencing (U. S. Biochemical Corp.).

Transfection and Isolation of Recombinant Baculovirus—Production of baculovirus recombinants by co-transfecting Sf9 cells with a mixture of recombinant baculovirus vector and wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) DNA and isolation of the recombinants were carried out as described (31, 32, 36, 37).

Protein Purification—Recombinant full-length CaD and CaD mutants were prepared as described (36). Smooth muscle actin, CaD, myosin, and tropomyosin were purified from chicken gizzard (38–40). Calmodulin was isolated from bovine brain acetone powder (41). The concentrations of full-length CaD and CaD mutants were determined by the method of Lowry (42), and the concentrations of other proteins were measured spectrophotometrically using the following extinction coefficients: actin, ε<sub>280</sub> = 6.3; calmodulin, ε<sub>287</sub> = 1.9; tropomyosin, ε<sub>280</sub> = 1.9; and myosin, ε<sub>280</sub> = 0.647.

Binding Assays for Calmodulin and Smooth Muscle Actin—CaM-coated agarose (Sigma) was used to determine the binding of full-length CaD or CaD mutants to CaM according to our published method (31). Non specific binding was estimated by 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.

Binding of [14C]-labeled CaD mutants to actin and tropomyosin-actin was determined by co-sedimentation using an Airfuge (Beckman Instruments) as described (31). The amounts of bound and unbound CaD mutants were estimated using a liquid scintillation counter (Beckman Instruments). The [14C]-labeled full-length CaD and CaD mutants were also used to determine actin binding in the presence or absence of tropomyosin, as described (31). All the binding assays were done in triplicate. The apparent dissociation constants for calmodulin, actin, and actin-tropomyosin bindings were determined by Scatchard analysis (43) and by weighted nonlinear least-squares curve fitting as described by Munson and Rodbard (44).

ATPase Assay—ATPase assays were carried out at 25 °C as described (45). Specific assay conditions are described in the figure legends. ATPase assays for each experimental parameter were repeated four times using four different protein preparations. Data from a representative experiment are shown in each figure.

RESULTS

Localization of the Weak Actin-binding Motif and Weak ATPase Inhibitory Determinant—Our previous studies suggested that the region between residues 690–717 in the CaD molecule is associated with weak actin binding, weak inhibition of actomyosin ATPase activity, and strong CaM binding (31, 32). However, the exact location of the functional motifs in the sequence between residues 690–717 and its structural relationship with CaM-binding site B are unknown (36). To further define the functional motifs, we constructed several internal deletion mutants of chicken gizzard smooth muscle CaD, which progressively lacked the targeted sequences within residues 690–717, and overexpressed them in a baculovirus system (36). Fig. 1 illustrates the schematic structures of these internal mutants. All the mutant proteins were purified to near homogeneity (>95% purity) using our published method (36).

Binding of [14C]-labeled CaD mutants to smooth muscle actin was determined by a co-sedimentation assay using full-length CaD as a control. The actin-binding of both CaD<sub>700–717</sub> and CaD<sub>710–717</sub>, which lack 18 and 8 amino acids, respectively (see in Fig. 1), was similar to that of full-length CaD (Fig. 2A). By contrast, binding of CaD<sub>690–717</sub> and CaD<sub>690–699</sub> to actin was slightly less (13% and 9%, respectively) than that of full-length CaD (Fig. 2A). The actin-binding of both full-length CaD and CaD mutants was saturated at a CaD concentration of 5 μM (molar ratio of CaD to actin, 1:4). As expected, the presence of smooth muscle tropomyosin increased the binding of both full-length CaD and the internal deletion mutants to actin or tropomyosin-actin (43, 44) showed that CaD<sub>690–717</sub> and CaD<sub>690–699</sub> bound to actin with lower affinity (0.65 ± 0.033 × 10<sup>−6</sup> m and 0.64 ± 0.037 × 10<sup>−6</sup> m, respectively) than did full-length CaD (0.5 ± 0.03 × 10<sup>−6</sup> m), whereas CaD<sub>710–717</sub> and CaD<sub>700–717</sub> bound to actin with an affinity (0.51 ± 0.03 × 10<sup>−6</sup> m and 0.51 ± 0.035 × 10<sup>−6</sup> m, respectively) similar to that of intact CaD. In the presence of smooth muscle tropomyosin, the apparent K<sub>d</sub> values of actin-binding for CaD<sub>1–756</sub>, CaD<sub>710–717</sub>, CaD<sub>700–717</sub>, and CaD<sub>690–699</sub> were 0.27 ± 0.01, 0.27 ± 0.013, 0.28 ± 0.011, 0.36 ± 0.02, and 0.35 ± 0.025 × 10<sup>−6</sup> m, respectively.

Effects of full-length CaD and the internal deletion mutants on the inhibition of the activation of smooth muscle myosin ATPase activity by smooth muscle actin or tropomyosin-actin were measured (in Fig. 3). CaD<sub>710–717</sub>, which lacks residues Leu<sup>710</sup> to Gly<sup>717</sup> inhibited the actin or tropomyosin-actin activated ATPase hydrolysis of myosin as efficiently as did full-length CaD, whereas CaD<sub>700–717</sub> and CaD<sub>690–699</sub> were 0.27 ± 0.01, 0.27 ± 0.013, 0.28 ± 0.011, 0.36 ± 0.02, and 0.35 ± 0.025 × 10<sup>−6</sup> m, respectively.

Association of the Region between Residues 650 and 666 with Weak Actin-binding and Weak Inhibition of Actomyosin ATPase Activity—Previous studies using a 7.3-kDa CaD C-terminal peptide produced from limited proteolysis indicated that the region between residues 597–665 in the chicken gizzard smooth muscle CaD molecule is critical for both actin-
binding and inhibition of actomyosin ATPase activity (46). However, studies using truncated CaD mutants suggested that the region between residues 658–689 is only associated with weak actin-binding and weak inhibition of actomyosin ATPase activity and that the region between residues 598–657 has no detectable activity for inhibition of actomyosin ATPase (31). To provide more precise analysis of the weak actin-binding and inhibitory domains, we generated various CaD internal deletion mutants (Fig. 1), focusing on the region between residues 609–666.

Deletion of the region between residues 609–628 did not alter the binding of CaD to actin, nor did removal of residues 629–639 (CaDΔ629–639) or residues 629–649 (CaDΔ629–649) (Fig. 4uA). However, further deletion of a segment between residues 650–666 resulted in a 16% decrease in the binding to actin compared with the actin-binding produced by full-length CaD (Fig. 4A). Moreover, the binding of CaDΔ650–666 to actin was similar to that of CaDΔ629–666, and dual deletions of residues 650–666 and 690–699 (CaDΔ650–666\690–699) resulted in an additive effect on the decreased level of binding to actin (Fig. 4A). Apparently, the region between residues 650–666 contains a functional motif responsible for weak actin-binding. The apparent dissociation constants of CaDΔ609–628, CaDΔ629–639, and CaDΔ629–649 were identical to that of full-length CaD, whereas the apparent $K_d$ value for CaDΔ629–666 was $0.68 \pm 0.037 \times 10^{-6}$ M, indicating a 36% increase over that of full-length CaD. The apparent $K_d$ value of the actin-binding of CaDΔ650–666\690–699 was $1.07 \pm 0.07 \times 10^{-6}$ M. The presence of tropomyosin increased the binding of full-length CaD and the CaD mutants to actin (Fig. 4B). The apparent $K_d$ values of CaDΔ629–666 and CaDΔ650–666\690–699 for tropomyosin-actin binding were $0.4 \pm 0.027$ and $0.61 \pm 0.053 \times 10^{-6}$ M, respectively. The apparent $K_d$ values of CaDΔ650–666 for both actin binding and tropomyosin-actin binding were indistinguishable from those of CaDΔ629–666.

Analysis of the ability of the internal deletion mutants to inhibit actomyosin ATPase activity revealed a similar degree of inhibition for both actin and tropomyosin-actin activated myosin ATPase activities with CaDΔ1–756, CaDΔ629–639, and CaDΔ629–649, whereas deletion of residues 629–666 caused a 37 and 19% decrease in the inhibition of actin-activated and tropomyosin-actin-activated myosin ATPase activity, respectively, as compared with that of full-length CaD (Fig. 5, A and B). In the presence of CaDΔ650–666, the inhibition of actin-activated and tropomyosin-actin-acti-
vated myosin ATPase activities was similar to that of CaDΔ629–666 (Fig. 5, A and B). The CaD dual internal deletion mutant, CaDΔ650–666/690–699, caused a less inhibitory effect on both actin-activated and tropomyosin-actin-activated myosin ATPase activities compared with those generated by either CaDΔ629–666 or CaDΔ690–717. A CaD internal deletion mutant lacking residues 597–608 was also generated using the baculovirus expression system; this mutant showed the same actin binding and inhibitory function as full-length CaD (data not shown). Thus, the weak inhibitory determinant is present in the amino acid stretch from Glu650 to Ser666.

Functional Involvement of Both CaM-binding Sites A and B in CaM-induced Reversal of Inhibition of CaD-induced Actomyosin ATPase Activity—As shown in Fig. 6, A and B, deletion of the regions between residues 690–699 and between residues 650–666 led to a 40 and 24% decrease, respectively, in binding to CaM as compared with that of full-length CaD. CaDΔ650–666/690–699 lacking residues 650–666 and 690–699 was unable to bind to CaM (Fig. 6B). The CaM-binding capacity of CaDΔ650–666 was similar to that of CaDΔ629–666 (data not shown). The sequences between residues 700–717 and residues 609–649 were not responsible for the binding of CaD to CaM because CaDΔ609–628, CaDΔ629–639, CaDΔ629–649, CaDΔ710–717, and CaDΔ700–717 bound to CaM as tightly as full-length CaD. Moreover, deletion of the sequence between residues 680–689 did not affect the binding of CaD to CaM.
Scatchard analysis showed that the CaD subdomains CaD700–717, CaD629–639, CaD629–649, CaD629–666, and CaD650–666 bound to CaM with the same affinity as full-length CaD (0.98 ± 0.061 × 10⁻⁶ M). In contrast, the affinity of both CaD650–666 and CaD690–717 to CaM (2.01 ± 0.063 and 1.96 ± 0.058 × 10⁻⁶ M, respectively) was reduced ~2-fold compared with that generated by full-length CaD. As expected, CaD629–666 displayed a markedly reduced affinity for CaM (1.45 ± 0.048 × 10⁻⁶ M). The apparent Kᵣ value of the CaM-binding of CaD650–666 was indistinguishable from that of CaD629–666.

Analysis to determine whether both CaM-binding sites A and B were functionally involved in CaM-induced reversal of CaD-mediated inhibition of actomyosin ATPase activity showed that inhibition by both CaD629–666 and CaD690–717 was reduced with increasing concentrations of Ca²⁺-CaM (Fig. 7). At 1:1 molar ratios of CaM to actin or tropomyosin-actin, the percentage of reversal by CaM for the inhibition caused by CaD629–666 was slightly lower than that of both full-length CaD and CaD690–717. At a stoichiometry of 0.075 mol CaD/mol CaM, the inhibition induced by both CaD629–666 and CaD690–717 was completely reversed, similar to that for full-length CaD (Fig. 7). Ca²⁺-CaM also reversed the inhibition by CaD629–666 in a pattern similar to that of CaD690–717. Additionally, tropomyosin did not affect the CaM-induced reversal of the inhibition by either full-length CaD or the internal deletion mutants consistent with our recent findings (31, 32). Together, the results indicate that both CaM-binding sites A and B are functionally involved in the CaM-reversal for inhibition of actomyosin ATPase activity by CaD and both function independently.
Based on our previous studies (31, 32), we postulated the presence of a weak actin-binding motif in the region between residues 690–717. The present study supports this suggestion and further defines the weak actin-binding motif to a 10-residue sequence from Asn690 to Gly 699. The sequence between residues 700–717 does not contribute to actin binding since removal of this region had very little effect on the binding of CaD to actin (Fig. 2). We therefore conclude that the 67-amino acid region between residues 690–756 in the C terminus of CaD contains two discontinuous actin-binding motifs, i.e., the strong actin-binding motif consisting of six residues from Lys718 to Glu723 (32) and the weak actin-binding motif located between residues 690–699. The two functional motifs are separated by an 18-residue stretch from Asn700 to Gly717 (Fig. 2). The presence of tropomyosin did not modify these binding sites except for the increased amounts of CaD bound per actin (Fig. 2B). The 10-residue sequence from Asn690 to Gly699 was also associated with very weak inhibition of actomyosin ATPase activity (Fig. 3). Interestingly, the region between residues 700–709, which is not required for actin-binding, appears to be necessary for the weak inhibition of actomyosin ATPase activity because deletion of residues 710–717 (CaDΔ710–717) had no effect on the inhibition of actomyosin ATPase activity by CaD, and further deletion of the stretch from Asn700 to Asp709 (CaDΔ700–717) slightly lowered the inhibition of actomyosin ATPase activity by CaD (Fig. 3). Again, tropomyosin enhanced this effect (Fig. 3B). These results strongly suggest that the weak inhibitory determinant is located in the 20-residue stretch from Asn690 to Asp709.

Our previous analysis of CaD C-terminal truncated mutants (31) showed that the region between residues 598–657, responsible for weak actin-binding, has no detectable activity on inhibition of actomyosin ATPase, whereas residues 658–689 are needed for both weak actin-binding and weak inhibition of actomyosin ATPase activity (31). Experiments using CaD C-terminal internal deletion mutants in the region between residues 629–666 (Fig. 1) showed that deletion of the segment from Met658 to Ser 666. Moreover, the region between residues 629–649 (Figs. 4 and 5), upstream of the segment from Glu650 to Ser666,
is not involved in either actin-binding or inhibition of actomyosin ATPase activity since the actin-binding and inhibitory activities of CaD were unchanged with deletion up to 21 residues from Val<sup>620</sup> to Ala<sup>649</sup>.

The data from the present study also suggested that the actin-binding motifs present in residues 650–666 and 690–699 function independently since deletion of either residues 650–666 or 690–699 reduced the binding of CaD to actin. Furthermore, the CaD dual internal deletion mutant, CaD<sub>650–666\669–699</sub>, had a lower binding capacity to actin when compared with those generated by either CaD<sub>650–666</sub> or CaD<sub>669–699</sub> (Fig. 4).

While the major CaM-binding sites in CaD molecule are well established, there is controversy about the functional significance of CaM-binding sites A and B in CaM-induced reversal for inhibition of actomyosin ATPase activity by CaD (34, 47). The data from Marston et al. (34) favor CaM-binding site B, but not CaM-binding site A, as the function-associated CaM-binding site because a synthetic peptide corresponding to the sequence Ser<sup>657</sup>-Gly<sup>670</sup> did not release the CaM-induced reversal of the inhibition of actomyosin ATPase activity by CaD. By contrast, Zhuang et al. (47), using a similar research approach, concluded that CaM-binding site A, but not CaM-binding site B, is involved in the CaM-CaD interaction for reversal of the CaD-induced inhibition. One reason for this discrepancy may be the lack of amino acids adjacent to CaM binding sites A or B needed for the protein conformation required for the protein-protein interaction in these studies. To clarify this issue, we analyzed the CaD internal deletion mutants in which CaM-binding sites A and B were nest-deleted, respectively, for their functional significance of CaM-binding site A was first demonstrated by Wang and co-workers (33), and high-affinity CaM-binding site B was derived from our present and previous data (31) and from the data of Mezgueldi et al. (48). The low affinity CaM-binding site, the tropomyosin-binding site at the C-terminal end of CaD, and the actin-binding/inhibitory site 3 are suggested by our previous data (31). Actin-binding/inhibitory sites 1 and 2 are demonstrated in the present study.

The discrepancies between our observations and those of others most likely rests in the use of synthetic CaD peptides, including VG29C (47) and M73 (34) that lack the necessary conformation for functional and structural interaction with CaM. Our present data also indicate that CaM-binding sites A and B each structurally overlap or lie in close proximity to the adjacent weak actin-binding motifs and weak inhibitory domains. The structural features of both CaM-binding sites A and B support the assumption that CaM competes with actin for binding to the same common contact sites in the CaD molecule. Note that the NMR measurements of bacterially generated human CaD fragments by Marston et al. (34) suggested the presence of a CaM-binding motif in a region corresponding to the sequence between residues 717–725 in the chicken gizzard smooth muscle CaD molecule. However, our previous results show that the deletion of the sequence between residues 718–756 has no effect on the binding of CaD to CaM (31). Moreover, a CaD internal deletion mutant lacking the region between residues 717–725 bound to CaM with the same affinity as did full-length CaD (data not shown). These results directly rule out the possibility that a CaM-binding site is present in the region between residues 717–725. Thus, it is unlikely that CaM-induced reversal of inhibition of actomyosin ATPase activity by CaD is caused in part through interaction of residues 717–725 in the CaD molecule with CaM.

The presence of three functional domains for actin-binding/actomyosin ATPase inhibition in the regions between residues 718–731, 690–699, and 650–666 (Fig. 8) is consistent with the multiple-site model for actin-binding/actomyosin ATPase inhibitory domains proposed by us and others (28, 31, 32). Both the regions between residues 650–666 and 690–699 consist of 70% uncharged and 30% charged amino acid residues, suggesting that, along with charge-charge interaction and hydrogen bonding, hydrophobic interaction may be involved in the interaction of the CaD and actin that is essential for inhibition of actomyosin ATPase activity by CaD.

In summary, the last 99 amino acid residues at the C terminus of CaD contain three functionally independent motifs responsible for actin-binding/actomyosin ATPase inhibition. The three functional domains are structurally discontinuous and are located in the regions between residues 718–731, 690–699, and 650–666, in which the regions between residues 690–699 and 650–666 each also contain a strong CaM-binding site. This precise localization should help to better understand the regul-
latory mechanisms involved in the interaction of CaD with actin and the regulation of actin-mycosin interaction and of actomyosin ATPase.

Acknowledgments—We are grateful to R. Rasmus and the Tyson Co. for providing fresh chicken gizzards, Marina Hoffman for editorial assistance, and Dr. Joseph Bryan for the chicken smooth muscle caldesmon cDNA, which was used originally in constructing the recombinant baculovirus vector. Photographic work was done by Jamie Hayden (Bio-Graphics).

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