The Major Myosin-binding Site of Caldesmon Resides Near Its N-terminal Extreme

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The primary myosin-binding site of caldesmon was thought to be in the N-terminal region of the molecule, but the exact nature of the caldesmon-myosin interaction has not been well characterized. A caldesmon fragment that encompasses residues 1–240 (N240) was found to bind full-length smooth muscle myosin on the basis of co-sedimentation experiments. The interaction between myosin and N240 was not affected by phosphorylation of myosin, but it was weakened by the presence of Ca2+/calmodulin. To locate the myosin-binding site, we have designed several synthetic peptides based on the N-terminal caldesmon sequence. We found that a peptide stretch corresponding to the first 27 residues (Met-1 to Tyr-27), but not that of the first 22 residues (Met-1 to Ala-22), exhibited a moderate affinity toward myosin. We also found that a peptide containing the segment from Ile/Leu-25 to Lys-53 bound both myosin and heavy meromyosin more strongly and was capable of displacing caldesmon from myosin. Our results demonstrate that the sequence near the N-terminal extreme of caldesmon harbors a major myosin-binding site of caldesmon, in which both the nonpolar residues and clusters of positively and negatively charged residues confer the specificity and affinity of the caldesmon-myosin interaction.

Smooth muscle caldesmon (CaD)1 is an elongated, 89-kDa actin-associated protein (1). Its ability to reversibly inhibit the actomyosin ATPase activity in vitro has made CaD a good candidate to modulate smooth muscle contraction via a thin filament-based mechanism. North et al. (2) have previously reported that CaD is present in the “contractile zone” where both actin and myosin exist. In our recent immunogold electron microscopy studies (3), we further found that the majority of CaD is around myosin filaments in partially shortened gizzard muscles that exhibit banding patterns made up of alternating myosin-rich and myosin-deficient zones, known as “contraction bands” (4). This observation suggested that when myosin filaments aggregate, the CaD-bearing actin filaments, or portions thereof, maintain some kind of contact with the thick filaments. One possibility to explain this phenomenon is that CaD may weakly bind myosin.

Indeed, CaD has been known to interact with myosin (5, 6), but the functional significance of such interactions remains unclear. Since CaD also binds actin, and binding of actin does not prevent CaD from interacting with myosin, it has been suggested that CaD bridges actin and myosin and forms a ternary complex (7). In this ternary complex, CaD may act as a force-maintaining tether between the thick and thin filaments, which could explain the “latch” phenomenon found in many tonic smooth muscle tissues (8). There is no indication, however, what role the CaD-myosin interaction might play in phasic smooth muscles, such as gizzard, where the latch-type contractions are not observed under normal physiological conditions.

The precise location of the myosin-binding site(s) in CaD has not been determined. There seems to be a general consensus that the N-terminal portion of the CaD molecule contains a myosin-binding domain (7, 9–12), although a CaD fragment containing the C-terminal 288 residues was also reported to be able to bind myosin (13). The finding that phosphorylation of CaD in the N-terminal region by Ca2+/calmodulin-dependent protein kinase II abolishes its interaction with myosin (12, 14) is consistent with an N-terminal myosin-binding site. More recently, a deletion mutant of CaD lacking a 30-amino acid residue stretch (from 24 to 53) was shown to lose the capacity to co-sediment with myosin (11), further supporting the idea that the major myosin-binding site of CaD is in its N-terminal region.

Compared with the C-terminal part of CaD, relatively little is known about the functional domain of the N-terminal region. While C-terminal CaD fragments can be prepared by either proteolysis or by bacterial expression, the N-terminal fragments are more difficult to obtain. For unknown reasons, bacterial cells do not express well the N-terminal peptides. Here we report the preparation of a recombinant N-terminal CaD fragment that encompasses residues 1–240 (N240) by overexpression in insect cells. Using this recombinant fragment and synthetic peptides corresponding to segments in this region, we found that the myosin-binding site is localized within a relatively short stretch near the N terminus of CaD. Some of these peptides are capable of displacing full-length CaD from myosin and may therefore be useful in probing the functional significance of the myosin-binding property of smooth muscle CaD.

MATERIALS AND METHODS
Construction of Recombinant Baculovirus

Cloning the CaD-N240 cDNA into pBlueBacIII Transfer Vector—CaD-N240 (chicken gizzard residues 1–240) cDNA fragment was obtained by using the PCR techniques with the full-length CaD cDNA as a template. Primers of the following sequences were used: 5′-CGG GGA TCC ATG GAT GAC TTT GAA CGC CGT AGA-3′ and 5′-CCC AAG CTT TCA ATC AGT TAT ACT CTG CTC CCC TTC TGC-3′, where the upstream
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primer contains the CaD translation start codon ATG and the BamHI restriction site, and the downstream primer contains the translation stop site and HindIII restriction site. The PCR product was inserted into pBlueBacIII (Invitrogen) between the BamHI and HindIII sites. The sequence of the resulting vector, pBlueBacIII/CaDN240, was confirmed by double-stranded DNA sequencing over the region of insertion.

Transfection and Recombinant Virus Purification—3 μg of pBlueBacIII/CaDN240 DNA was cotransfected into SF9 cells with 1 μg of linear AcMNPV DNA (Invitrogen) by the technique of cationic liposome-mediated transfection according to the protocol provided by Invitrogen. To amplify the recombinant pBlueBacIII/CaDN240 baculoviruses, SF9 cells in a 100-mm dish (1.5 × 10⁶ cells) were infected with 10-fold dilutions of virus inoculum (from cotransfections) in the range of expected titer and overlaid with 5 ml of 0.7% agarose containing 150 μg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside). The recombinants were clearly distinguishable within 5–6 days postinfection as blue plaques, which were picked with a Pasteur pipette. Several rounds of plaque assay enabled us to obtain the pure recombinant baculovirus, and the plaque stock was used to infect SF9 cells with a multiplicity of infection (MOI) of 0.01.

Expression of N240 was confirmed by immunonephelometry reaction and partial amino acid sequencing as described in the Invitrogen instruction manual. Expression of N240 and the time course for production of recombinant protein were also carried out as described in the Invitrogen instruction manual. Expression of N240 was confirmed by immunonephelometry reaction and partial amino acid sequencing.

Expression and Purification of Recombinant N240

Expression of recombinant N240 in large scale was performed in High-Five cells as previously described (15). 48 h after infection, the maximally expressing cells were pelleted by centrifugation, resuspended with 50 ml of buffer A (50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.5 mM EDTA, 0.25% phenylmethylsulfonyl fluoride, 5 μM leupeptin), frozen and thawed twice, and centrifuged at 42,000 rpm for 40 min. The supernatant was collected and loaded onto a DE-52 column. After washing the column with buffer A, the bound proteins were eluted with a 50–500 mM NaCl gradient in buffer A; fractions containing N240 were identified by SDS-polyacrylamide gel electrophoresis. The overall yield was ~80 mg/liter culture.

Preparation of Other Proteins

Smooth muscle myosin was prepared from chicken gizzard according to published procedures (16). Phosphorylation of myosin was carried out by using purified gizzard myosin light chain kinase (a kind gift from Dr. Phil Graceffa) in the presence of Ca²⁺/CaM and 2 mM ATP (17). HMM and S-1 were prepared by cleavages of chicken gizzard myosin with chymotrypsin and papain, respectively (18). Actin was prepared from rabbit skeletal muscles (19). Recombinant chicken gizzard CaD was purified from baculovirus-transfected insect cells without heat or detergent treatments (data not shown).

Synthesis of Myosin-binding Peptides

Peptides were synthesized on an automated peptide synthesizer (ABI-431A) as described previously (20). For peptides containing N terminus residues (i.e. MY27C and MA22C), acetylated Met was used. The synthesized peptides were purified by reversed phase HPLC using a C8 column (Si-O-Si-(CH₂)₇-C₂H₄ from Phenomenex, Torrence, CA). The purity of the peptides was ascertained by amino acid composition and sequence analyses, as well as mass spectrometry (PerSeptive Biosystems, Voyager Elite). For fluorescence titration studies, purified peptides were labeled at the C-terminal Cys with a DE-52 column. After washing the column with buffer A, the bound proteins were eluted with a 50–500 mM NaCl gradient in buffer A; fractions containing N240 were identified by SDS-polyacrylamide gel electrophoresis. The overall yield was ~80 mg/liter culture.

Electron Microscopic Studies

Rotary shadowing electron microscopy was carried out as described previously (22). Antibody labeling was necessary for the identification of N240 when bound to HMM. To do this, N240 and monoclonal antibody, C18, of which the epitope locates in the N-terminal CaD (23), were incubated at concentrations of 5 μM each overnight in 50 mM ammonium acetate and 30% glycerol, pH 7, at room temperature. The mixture was further incubated with gizzard HMM at 0.5 μM each. CaD (0.2 μM) or N240 (0.5 μM), gizzard skeletal HMM (0.5 μM), and F-actin (10 μM) were mixed in 2 mM MgATP, 0.5 mM dithiothreitol, 30% glycerol, and 0.1 mM ammonium acetate, pH 7. All of these mixtures were then processed for visualization. Since it was expected that binding of N240 to HMM or that of HMM to F-actin in the presence of ATP was rather weak, we adopted a recently modified shadowing method (3) that allowed us to use HMM and actin concentrations at near micromolar ranges, as compared with ~10 μM in the conventional methods. In order to minimize the bundling of F-actin induced by CaD, proteins were adsorbed on mica within 10 s after the addition of CaD. N240 alone did not cause bundling of F-actin.

RESULTS AND DISCUSSION

Binding of N240 to Myosin—The recombinant N-terminal CaD fragment N240 showed binding to unphosphorylated smooth muscle myosin, as evidenced by co-sedimentation experiments (Fig. 1A). To estimate the binding affinity of N240 for myosin, we have carried out a series of centrifugations by mixing a fixed amount of myosin and increasing amounts of N240. The Coomassie-stained N240 bands in the pellet were then digitized, normalized against the myosin light chain band, and fitted to a binding equation (see “Materials and Methods”). The fitting yielded a binding constant of 1.2 × 10⁶ M⁻¹. Phosphorylation of the myosin light chain had no effects on the apparent binding (data not shown).

Inclusion of Ca²⁺/CaM in the mixture slightly decreased, but did not eliminate, the amount of N240 co-precipitated with myosin, and this effect was reversible by the addition of EDTA (Fig. 1B). CaM appeared to modulate the binding between the N-terminal region of CaD and myosin in a Ca²⁺-dependent manner; this is in agreement with an earlier report that showed that myosin was retained to CaD-Sepharose to a much lesser extent in the presence of Ca²⁺/CaM than in its absence (5). The N-terminal region of CaD does not have a well-characterized CaM-binding site as does in the C-terminal region of CaD; however, it contains a weak CaM-binding site, as shown previously by photocross-linking and affinity chromatography (24, 25). It is possible that this secondary site around Cys-153 is responsible for the observed CaM-dependent interaction with myosin. The physiological role of such a modulatory effect awaits further study.

Electron Microscopic Visualization of CaD-Myosin Interaction—Binding of N240 to myosin was further studied by electron microscopy. At concentrations of 0.5 μM each, we observed only occasional complex formation between N240 and HMM (Fig. 2). The site of binding, based on the positions of the monoclonal antibody molecules (asterisks), was predominantly...
at the C-terminal end of the S2 region with a few exceptions, in which case N240 appeared to bind at the myosin heads (see inset). A rough statistical tally revealed that 22 out of ~350 HMM molecules were bound at the S2 region, while five bound at the head. Although the S2-binding site agrees well with the biochemical data (5), the significance of N240 binding at the myosin head is not clear, considering the relatively low affinity between the two proteins. However, regardless of the specificity of such binding, the presence of multiple binding sites could explain the ability of CaD to promote myosin filaments formation (26) or aggregation (see below). The 22 complexed HMM out of ~350 molecules could be translated to an affinity constant of ~1.5 × 10^5 M^-1; thus, the frequency of bound molecules observed by EM is in good agreement with the results of the co-sedimentation experiment (see above).

Effect of CaD on the Actomyosin Interaction, Electron Microscopic Views—Marston (27) previously reported that in the presence of ATP, CaD potentiated binding of gizzard HMM to actin filaments. He calculated that each CaD molecule caused the binding of ~20 HMM molecules, thus suggesting a mechanism other than direct cross-linking of HMM to actin by CaD. We have tried to reproduce his experimental conditions within the limit of electron microscopy and indeed observed very similar phenomena. Gizzard HMM did not show much binding to F-actin in the presence of ATP (Fig. 3A); however, the addition of 0.2 μM CaD enhanced the binding of HMM to F-actin (B). It appeared that aggregation of HMM and bundling of F-actin had occurred. When N240 was used in lieu of CaD, similar phenomena, albeit in much smaller scale, were observed (C). Bar, 0.2 μm.

in the presence of CaD and ATP could be an artifact and not take place in situ, since myosin molecules do not aggregate. The molecular mechanism of how CaD causes HMM to aggregate; nevertheless, is an intriguing question in view of the fact that CaD indeed facilitates myosin filament formation.

N240 did not induce as drastic an enhancement of HMM binding as in the case of full-length CaD. A lesser extent of HMM aggregation, albeit at a much lower frequency, and highly HMM-decorated F-actin was nevertheless observed (Fig. 3C). The difference in the degree of enhancement may be explained by the lack of the C-terminal CaD sequence in N240.
Fig. 4. A comparison of the N-terminal sequence among CaD isoforms. The sequences for chicken (ch), A33430; rabbit (rab), L37147; human (hum), M83216; and rat CaD (A55887) are from GenBank. The sequence for mouse (mus) CaD is from this laboratory (H. Guo and C.-L. A. Wang, unpublished results). Also shown are the sequences of the peptides used in this study. The consensus sequence corresponding to that of IK29C is underlined. Also shown are the sequences of the peptides used in this study.

Their similarity in causing HMM aggregation and enhancement of HMM binding to F-actin, on the other hand, could be attributed to their common myosin-binding properties. It remains uncertain whether or not the observed aggregation requires N240 to bind F-actin, but such ability should conceivably facilitate the binding of HMM to F-actin.

Identification of Myosin-binding Site—A comparison of the N-terminal amino acid sequence of several CaD isoforms (Fig. 4) revealed that the peptide stretch from residue Tyr-27 to Lys-53 (chicken gizzard numbering system) represents a segment of identical sequence for both mammal and avian CaD. Assuming that myosin binding is a common property of CaD, and that all CaD isoforms target at a conserved sequence in the myosin molecule, this homologous segment would be a good candidate for the myosin-binding site. Consistent with this hypothesis is the finding that a CaD mutant with this segment deleted indeed lost myosin binding capacity (11). One caveat for this negative results derived from the internal deletion mutation is that the observed lack of binding for the mutant may be due to an altered conformation resulting from the deletion rather than the loss of these residues per se. To show that the deleted segment in fact contains the myosin-binding site of CaD, we have prepared a synthetic peptide, IK29C, corresponding to this N-terminal common sequence.

IK29C (25IAYQRNDDEEAAAAERERRRARQERLRQK53-C) was found to co-sediment with gizzard myosin in a dose-dependent manner, whereas the scrambled peptide, IK29CX1 (REAERARQREIDLRAREKQRAERYQDQRND-C) only displayed very weak, basal binding (Fig. 5, A and B). To quantitate the binding, we have carried out fluorescence titration using NBD-labeled peptides and gizzard HMM. The binding constants were estimated to be 3.83 × 10^6 M^-1 and 1.14 × 10^6 M^-1 for IK29C and MY27C, respectively (Fig. 6).

We also did the cosedimentation experiments in the presence of CaD, under which condition IK29C showed weakened binding to myosin. Binding of the peptide was found to be accompanied by concomitant dissociation of CaD (Fig. 7), indicating that IK29C effectively competes with CaD for myosin binding, and thus that IK29C must contain the major myosin-binding sequence in CaD. As a control, the scrambled peptide, IK29CX1, did not co-sediment with myosin; nor was CaD displaced from the pellet.

It is noteworthy that the IK29C sequence contains mainly charged residues with a cluster of negative charges (DDDEEEE) followed by a cluster of positive charges (RRRRR). We have designed another “scrambled” peptide, IK29CX2 (EEEDID-EAAAAAIYNRERRRRRQKQQ-C), in which the two clusters of opposite charges were preserved and augmented. Curiously, IK29CX2 sedimented by itself, apparently resulting from self-aggregation because of the highly polarized charges and the hydrophobic core. In the presence of added bovine serum albumin, the aggregation was much alleviated, and the peptide no longer precipitated in significant amounts, either by itself or in the presence of myosin. Thus, the specific sequence and the separation of clustered charges, rather than the charges alone seem to be the determinant for myosin binding.
In addition to the charged residues, there are also three hydrophobic residues (Ile-Ala-Tyr) at the N terminus of IK29C. To test whether these nonpolar residues play any role in myosin-binding, we have synthesized two peptides, MY27C, which corresponds to the first 27 residues in the gizzard sequence (1MDDFERRRELRRQKREEMRLEAERLSY27-C), and MA22C, corresponding to the first 22 residues of the same sequence except that the C-terminal 5 residues, including the three nonpolar ones, are truncated (1MDDFERRRELRRQKREEMRLA22-C). Both peptides were prepared with acetylation at the N terminus and purified by HPLC.

We found that MY27C exhibited a weak affinity toward myosin; only at a relatively high concentration (≥20 μM) was there an appreciated amount of peptide detected in the pellet when co-sedimented with myosin. MA22C, on the other hand, did not show any significant binding to myosin (Fig. 5B). Thus, the 3 uncharged residues (Ile-Ala-Tyr in the mammalian sequence, or Leu-Ser-Tyr in the avian sequence) most likely contribute to the specific interaction between CaD and myosin, although the downstream charged residues are also needed for the observed affinity. This finding is in good agreement with a report by Vorotnikov et al. (28) who predicted that one of the myosin-binding sites is within the N-terminal 28-residue peptide stretch on the basis that a fragment lacking this stretch (29N152) was unable to displace intact CaD from myosin filaments. Interestingly, such a truncated fragment nevertheless exhibited nonspecific binding toward myosin with a nearly identical affinity to that of intact CaD, suggesting the specificity for the CaD-myosin interaction stems from residues other than the charged clusters.

While the major myosin-binding site is located at the N terminus, we cannot rule out the presence of other, weaker myosin-binding sites in the CaD molecule. Huber et al. (13) found that a 288-residue C-terminal CaD fragment (H1) co-sedimented with smooth muscle myosin and therefore proposed a myosin-binding site in the region between residues 506 and 793 of the human sequence. This binding site, however, may not be significant under physiological conditions, because the binding occurred only at low salt (5 mM KCl) and became negligible at higher ionic strength (90 mM; see Ref. 13).
Possible functional roles of CaD-myosin interactions postulated thus far include (i) to stabilize unphosphorylated myosin filaments (26); (ii) to maintain force at no cost of energy (latch) (8); (iii) to keep thick filaments in an organized form during contraction (3); and (iv) to enhance binding of myosin to actin filaments (29). In view of the in situ localization of CaD in smooth muscle cells, we further propose that (v) interactions between CaD and myosin may play a role in determining the positioning of CaD along the actin filament. It remains to be seen which if any of these possibilities are the true functions. If the CaD-myosin interaction indeed plays a physiological role, the question then is whether or not such an interaction is regulated and how. These issues are ultimately linked to the function of CaD. The myosin-binding peptides (IK29C and MY27C) should provide us with a useful tool for the design of experiments to find these answers.

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REFERENCES
1. Marston, S. B., and Huber, P. A. J. (1996) in Biochemistry of Smooth Muscle Contraction (Barany, M., ed) pp. 77–90, Academic Press, Inc., San Diego
2. North, A. J., Gimona, M., Cross, R. A., and Small, J. V. (1994) J. Cell Sci. 107, 437–444
3. Mabuchi, K., Li, Y., Tao, T., and Wang, C.-L. A. (1996) J. Muscle Res. Cell Motil. 17, 243–260
4. Draeger, A., Amos, W. B., Ikebe, M., and Small, J. V. (1990) J. Cell Biol. 11, 2463–2473
5. Ikebe, M., and Reardon, S. (1988) J. Biol. Chem. 263, 3055–3058
6. Hemric, M. E., and Chalovich, J. M. (1988) J. Biol. Chem. 263, 1878–1885
7. Velaz, L., Ingraham, B. H., and Chalovich, J. M. (1990) J. Biol. Chem. 265, 2929–2934
8. Marston, S. B. (1989) J. Muscle Res. Cell Motil. 10, 97–100
9. Katayama, E., Horieuchi, K. Y., and Chacko, S. (1989) Biochem. Biophys. Res. Commun. 160, 1316–1322
10. Wang, C.-L. A., Carlos, A., and Lu, R. C. (1990) Biophys. J. 57, 162 (abstr.)
11. Wang, Z., Jiang, H., Yang, Z. Q., and Chacko, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11899–11904
12. Sutherland, C., and Walsh, M. P. (1989) J. Biol. Chem. 264, 578–583
13. Huber, P. A. J., Fraser, I. D. C., and Marston, S. B. (1995) Biochem. J. 312, 617–625
14. Hemric, M. E., Lu, F. W. M., Shrager, R., Carey, J., and Chalovich, J. M. (1993) J. Biol. Chem. 268, 15305–15311
15. Zhuang, S., Mabuchi, K., and Wang, C.-L. A. (1996) J. Biol. Chem. 271, 30242–30248
16. Ikebe, M., Alba, T., Onishi, H., and Watanabe, S. (1978) J. Biochem. (Tokyo) 85, 1643–1655
17. Zhuang, S., Wang, E., and Wang, C.-L. A. (1995) J. Biol. Chem. 270, 19964–19968
18. Seidel, J. C. (1980) J. Biol. Chem. 255, 4355–4361
19. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
20. Wang, E., and Wang, C.-L. A. (1996) Arch. Biochem. Biophys. 339, 156–162
21. Morris, E. F., and Lehrer, S. S. (1984) Biochemistry 23, 2214–2220
22. Mabuchi, K. (1991) J. Struct. Biol. 107, 22–28
23. Lin, J. J.-C., Davis-Nanthakumar, E. J., Jin, J.-P., Lourim, D., Novy, R. E., and Lin, J. L.-C. (1991) Cell Motil. Cytoskeleton 20, 95–108
24. Wang, C.-L. A. (1988) Biochem. Biophys. Res. Commun. 156, 1033–1038
25. Wang, C.-L. A., Wang, L.-W. C., and Lu, R. C. (1989) Biochem. Biophys. Res. Commun. 162, 746–752
26. Katayama, E., Scott-Woo, G., and Ikebe, M. (1995) J. Biol. Chem. 270, 3919–3925
27. Marston, S. (1989) Biochem. J. 259, 303–306
28. Vorotnikov, A. V., Marston, S. B., and Huber, P. A. J. (1997) Biochem. J. 328, 211–218
29. Haeberle, J. R., Trybus, K. M., Hemric, M. E., and Warshaw, D. M. (1992) J. Biol. Chem. 267, 23901–23906
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