Localization of Protein Regions Involved in the Interaction between Calponin and Myosin*

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Calponin is a 33-kDa smooth muscle-specific protein that has been suggested to play a role in muscle contractility. It has previously been shown to interact with actin, tropomyosin, and calmodulin. More recently we showed that calponin also interacts with myosin (Szymanski, P. T., and Tao, T. (1993) FEBS Lett. 331, 256–259). In the present study we used a combination of co-sedimentation and fluorescence assays to localize the regions in myosin and calponin that are involved in the interaction between these two proteins. We found that recombinant chicken gizzard α-calponin co-sediments with myosin rod and, to a lesser extent, with light meromyosin. Fluorescently labeled recombinant calponin shows interaction with heavy meromyosin and myosin subfragment 2 but not subfragment 1. A fragment comprising residues 7–182 and a synthetic peptide spanning residues 146–176 of calponin co-sediments with myosin, but fragments comprising residues 7–144 and 183–292 do not. Our results indicate that there are calponin binding sites in the subfragment 2 and light meromyosin regions of myosin, and that the region comprising residues 145–182 of calponin mediates its interaction with myosin.

Calponin (CaP) is a 33-kDa smooth muscle-specific, thin filament-associated protein that has been suggested to play a role in muscle contractility (2, 3). It is capable of binding to actin (2, 4–6), tropomyosin (7–10), and calmodulin (4, 6, 11). The binding sites for these proteins are all located in the NH2-terminal portion of CaP (residues 7–182) (12).

CaP inhibits the actin-activated myosin ATPase in solution (3, 13), unloaded shortening velocity, and, to a lesser extent, isometric force in permeabilized smooth muscle fibers (14–17). It is commonly assumed that these effects are exerted via CaP interactions with both actin and myosin. Previous studies showed that CaP indeed binds to isolated smooth muscle myosin (1, 20). This interaction was found to be reversible upon the addition of Ca2+-calmodulin (1) and partially abolished upon phosphorylation of CaP by protein kinase C.2

In this study we further investigated the interaction between CaP and myosin. Using a combination of co-sedimentation and fluorescence assays, we localized the regions in these two proteins that are involved in their interaction. Fragments of chicken gizzard myosin and recombinant chicken gizzard α-CaP (RoCaP) were generated by proteolytic digestion. This allowed us to isolate the major functional portions of these two proteins, viz. S1, S2, rod, HMM, and LMM of myosin and the NH2-terminal fragment (residues 7–182), the NH2-terminal fragment without the central portion (residues 7–144), and the COOH-terminal fragment (residues 183–292) of CaP.

Our data show that there are CaP binding sites in the S2 and LMM regions of myosin and that the region in CaP that contains a so-called actin-binding domain (residues 145–176) (4) is primarily involved in the interaction of CaP with myosin.

EXPERIMENTAL PROCEDURES

Materials—α-Chymotrypsin, papain, and other commonly used proteolytic agents were from Sigma. Precast polyacrylamide gradient (4–20%) Tris-glycine gels were from Novex. The rapid-Ag-stain kit was from ICN, and other materials for gel electrophoresis were from Bio-Rad.

Proteins—Chicken gizzard myosin was prepared according to Ikebe et al. (21). Fragmentation of myosin into rod and S1 by digestion with papain and into LMM and HMM by digestion with α-chymotrypsin was performed as described (22). S2 was generated from HMM by α-chymotrypsin cleavage (23).

Expression and purification of RoCaP was as described previously (24). Digestion of RoCaP with α-chymotrypsin using a protease to substrate weight ratios of 1:1000 and 1:100 were performed according to Mezgueldi et al. (4). Labeling of RoCaP with N-iodoacetyl-N′-(5-sulfophenyl)ethylene~amine (Aldrich) was carried out by incubating RoCaP (40 μM) with a 3-fold molar excess of N-iodoacetyl-N′-(5-sulfophenyl)ethylene~amine in 20 mM Heps, 0.1 M NaCl, pH 7.5, for 5 h at room temperature. Dithiothreitol (1 mM) was added to quench the reaction, followed by dialysis to remove excess reagents.

The polypeptide EKQQRQRQPEKLReGRNIIQLQGTNKFAC corresponding to residues 146–176 of CaP and a COOH-terminal Cys was synthesized on an ABI protein synthesizer and purified by high pressure liquid chromatography using conventional methods.

Protein concentrations were determined spectrophotometrically at 280 nm, using A1%1 cm values of 7.4 for RoCaP, 4.5 for smooth muscle myosin, 2.2 for rod, 3.0 for LMM, 6.5 for HMM, 7.0 for S2, and 7.7 for S1; an extinction coefficient of 5600 M–1 cm–1 was used for the synthetic polypeptide.

Binding Assays—For sedimentation binding assays RoCaP or its fragments were incubated with myosin or its insoluble fragments (rod or LMM) in 20 mM Heps, 50 mM NaCl, 2 mM NaN3, 1 mM dithiothreitol, pH 7.5, for 20 min at 4 °C and then centrifuged at 80,000 rpm for 20 min.

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at 4 °C in a Beckman Instruments TL100 ultracentrifuge using the TL100.2 rotor. Reaction mixtures before centrifugation and pellets solubilized with Laemmli buffer (25) were subjected to gradient (4–20%) SDS-PAGE. The amounts of materials were quantified by densitometry of Coomassie Blue- or silver-stained gels using a laboratory-built image-analysis system as described earlier (1). Standard curve for RoCaP was constructed to establish the linear concentration range.

For binding assays in solution, DAN-RoCaP (1 μM) was incubated with increasing concentrations (0–5 μM) of the soluble myosin fragments (HMM, S1, and S2) in 20 mM Hapes, 50 mM NaCl, 2 mM NaOAc, 1 mM dithiothreitol, pH 7.5, for 20 min at 4 °C. Fluorescence-intensity measurements were carried out on an I.S.S. K2 fluorometer (Champaign, IL) using wavelengths of 377 and 480 nm for excitation and emission, respectively.

Statistical Analysis—Student’s t test was used for statistical analysis, and a confidence level of p < 0.05 was chosen as an indication of a statistically significant difference.

RESULTS AND DISCUSSION
Fluorescence Titration—Addition of increasing concentrations of HMM to a solution containing DAN-RoCaP produced a concentration-dependent and saturable increase of the label’s fluorescence intensity (Fig. 1). In contrast, S1 did not cause any change in the fluorescence of DAN-RoCaP. These data indicate that CaP interacts with HMM but not with S1 and indirectly implicate the S2 region of myosin as a CaP binding site. The fluorescence titration curve of S2 is very similar to that of HMM (Fig. 1). When the data were fitted by a nonlinear regression method (26), we obtained apparent binding constants of 4.0 ± 0.3 × 10^6 M⁻¹ (n = 3) and 4.7 ± 0.4 × 10^6 M⁻¹ (n = 3) (all uncertainty values are S.E.; n refers to the number of determinations) for HMM and S2, respectively. Thus, isolated S2 binds DAN-RoCaP with virtually the same affinity as HMM, providing more direct evidence that the S2 region of myosin contains a CaP binding site.

Since it is possible for S1 to bind DAN-RoCaP without affecting the label’s fluorescence, steady-state polarization and anisotropy decay measurements were also carried out; neither showed any changes with S1 (data not shown). Also, the binding of DAN-RoCaP to myosin was found to be the same as that of unlabeled RoCaP using the co-sedimentation assay (see below; data not shown), indicating that the label does not affect the capacity of RoCaP to interact with myosin.

Co-sedimentation—Addition of increasing concentrations of myosin, myosin rod, and LMM to a solution of RoCaP followed by high speed centrifugation produced a concentration-dependent increase in sedimentation of RoCaP that approaches saturation at high concentrations of added proteins (Fig. 2). As was found previously for myosin (1), some amounts of RoCaP remain in the supernatant even at the highest concentrations of the proteins used, and about 10–20% of RoCaP sedimented in the absence of added proteins. Nonlinear regression analysis of the data in Fig. 2 yielded apparent binding constants of 2.6 ± 0.3 × 10^6 M⁻¹ (n = 6), 1.0 ± 0.3 × 10^6 M⁻¹ (n = 7), and 1.5 ± 0.5 × 10^6 M⁻¹ (n = 5) for LMM, intact myosin, and rod, respectively. These binding constants are not statistically different (p < 0.05) from each other. We noted, however, that the fraction of RoCaP bound at high concentrations of added proteins is significantly lower for LMM compared with intact myosin and rod. The analysis procedure yielded maximal fraction bound values of 0.3, 0.7, and 0.5 for LMM, myosin, and rod, respectively. We stress that our experimental conditions were such that the binding curves have not reached saturation, so that the fraction of RoCaP bound at saturation could only be determined approximately.

The co-sedimentation results described above show that the CaP binding function of myosin resides entirely in the rod segment, a conclusion that is consistent with our finding that S1 does not bind CaP. One of the CaP binding sites in the rod must be in the S2 region, as we concluded from the fluorescence titration results. Furthermore, we found that LMM, which lacks the S2 segment, also binds CaP. Thus, there must be another CaP binding site in the LMM portion of the myosin rod.

Our conclusion that there are two CaP binding sites in myosin or myosin rod and only one in LMM is consistent with our finding that the maximal amount of bound RoCaP for LMM is roughly half the amount for myosin or rod.

Binding of RoCaP Fragments to Myosin—As reported previously for gizzard CaP (4), limited digestion of RoCaP with α-chymotrypsin at a low protease to substrate weight ratio of 1:1000 for 17 min at 25 °C produced two major fragments (Fig. 3A, lane 4). Their molecular masses are 215 and 13.7 kDa based on electrophoretic mobilities. These two fragments remain stable up to about 20 min of digestion, and then further cleavage takes place (Fig. 3A, lane 5). The 21.5-kDa fragment represents the NH₂-terminal portion of RoCaP spanning the region between Asn 7 and Tyr 182, and the 13.7-kDa fragment spans the region between Gly 183 and the COOH terminus of intact RoCaP (4).

When the limited α-chymotryptic RoCaP digest was centrifuged together with an equimolar concentration of myosin,
76.6 ± 3.2% (n = 6) and 12.2 ± 1.0% (n = 6) of the 21.5- and 13.7-kDa fragments, respectively, were sedimented (Fig. 3B, lanes 10–12). When the same RαCaP digest was centrifuged without myosin, 17.2 ± 0.9% (n = 6) and 11.3 ± 1.8% (n = 6) of 21.5- and 13.7-kDa fragments, respectively, were found in the pellets (Fig. 3B, lanes 7–9). Thus, although a significant amount (55%) of the 21.5-kDa fragment sedimented via binding to myosin, virtually none of the 13.7-kDa fragment did. These data indicate that the myosin binding function of CaP resides within residues 7–182 and not residues 183–292. It is interesting to note that actin (4, 5), tropomyosin (4, 5, 8, 9), calmodulin (5, 6, 11), and caltropin (27) binding sites are located in the same NH2-terminal portion of CaP.

As for native CaP (4), digestion of RαCaP with α-chymotrypsin at a high protease to substrate weight ratio (1:100) produced further cleavage (Fig. 4A). After digestion for 30 min at 25 °C, the 13.7-kDa COOH-terminal fragment was completely digested. The 21.5-kDa NH2-terminal fragment (residues 7–182) was cleaved between Tyr144 and Ala145 into a 15.5-kDa fragment that spans residues 7–144 and smaller segments. When this extensive chymotryptic digest of RαCaP was centrifuged with an equimolar concentration of myosin, 15.4 ± 1.4% (n = 6) of the 15.5-kDa fragment was sedimented compared with the total unsedimented material (Fig. 4B, lanes 12–14). When the same digest was centrifuged without myosin, 13.5 ± 1.0% (n = 6) of the 15.5-kDa RαCaP fragment was sedimented (Fig. 4B, lanes 9–11). These data clearly indicate that the RαCaP fragment that spans residues 7–144 does not
bind myosin. Since the 21.5-kDa NH₂-terminal fragment (residues 7–182) does bind myosin, our data suggest that the myosin binding site in CaP is located within residues 145–182.

When a synthetic peptide that contains residues 146–176 of CaP was centrifuged with and without myosin, 51.0 ± 2.2% (n = 6) and 14.7 ± 0.9% (n = 5), respectively, of the peptide was sedimented (Fig. 5A). These data show more directly that the central region of CaP spanning residues 145–182 is responsible for binding to myosin.

To ascertain whether the binding of our synthetic CaP peptide to myosin is specific, we centrifuged the preformed complex between myosin and the peptide (each at 3 μM) in the presence of increasing concentrations of RoCaP. As shown in Fig. 5B (lanes 8–12), addition of RoCaP resulted in a concentra-
tration-dependent displacement of the synthetic peptide from myosin. Specifically, the amount of peptide that sedimented with myosin decreased from 48.3 ± 2.1% (n = 3) in the absence of RoCaP to 29.7 ± 2.0% (n = 3) and 21.8 ± 1.5% (n = 3) in the presence of 1.5 and 3.0 μM RoCaP, respectively. Our data show that the peptide competes with RoCaP for binding to myosin, indicating that the interaction between the peptide and myosin is specific.

CONCLUSIONS

Our results show that there are CaP binding regions in the S2 and LMM portions of myosin and that the CaP segment comprising residues 146–182 constitutes a myosin binding region. We noted that caldesmon (28–31) and telokin (32), two proteins that have been shown to regulate smooth muscle contractility (33) and myosin assembly (34), also bind to myosin in the S2 region. More interestingly, the same stretch of residues (145–182) in CaP has previously been shown to be a region of interaction with actin (4, 5). Thus, myosin and actin share (or partially share) a common binding region in CaP, so it is not likely that CaP functions as a linker between myosin and actin.

The physiological significance of the interaction between CaP and myosin is not clear at this point. It was recently reported that CaP is primarily localized in the cytoskeletal regions of chicken gizzard cells (35). However, it was recognized that some of the CaP are found near myosin filaments, suggesting that CaP may serve as a link between cytoskeletal extensions and contractile regions (35). We may speculate that a physiological function of CaP might be to link a component of the cytoskeleton (e.g., desmin intermediate filaments) with myosin in the contractile region. Under such a circumstance the local concentrations of both CaP and myosin are likely to be quite high, so that the interaction might be able to occur in situ even though it is relatively weak at physiological NaCl concentrations in vitro (1). Clearly, much additional work will be required before the true physiological role of CaP can be understood.

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