Actin-Binding Peptide from Smooth Muscle Myosin Light Chain Kinase†

Satoshi Kanoh,‡ Masaaki Ito,§ Eiji Niwa,‡ Yasushi Kawano,‡ and David J. Hartshorne*,‖

Faculty of Bioresources and First Department of Internal Medicine, Mie University, Tsu 514, Japan, and Muscle Biology Group, Department of Biochemistry and Animal Sciences, University of Arizona, Tucson, Arizona 85721

Received April 1, 1993; Revised Manuscript Received June 7, 1993

ABSTRACT: The objective of this study was to localize the actin-binding site in the smooth muscle myosin light chain kinase. Limited proteolysis by thermolysin indicated that hydrolysis of the kinase at the N-terminal end of the molecule resulted in loss of actin-binding ability. Various methods of cleavage were investigated for the generation of a discrete actin-binding peptide. The method chosen was cleavage at the cysteine residues by the 5,5′-dithiobis(2-nitrobenzoic acid)–cyanide complex. This procedure yielded an actin-binding peptide of approximate M_r 17 000. The peptide was purified and shown to possess the actin-binding properties of the native myosin light chain kinase. The binding constant of the isolated peptide and parent enzyme to actin was estimated as 7.5×10^4 M^{-1}. From the amino acid composition of the peptide and comparison with the sequence of gizzard myosin light chain kinase, it was suggested that the actin-binding site is located within the N-terminal sequence 1–114. Comparison with other actin-binding proteins shows some similarities to gizzard α-actinin and caldesmon.

Phosphorylation of myosin is an important regulatory mechanism in smooth muscle and in nonmuscle cells (Hartshorne, 1987). The kinase responsible for phosphorylation of the two 20 000-dalton light chains of myosin is myosin light chain kinase (MLCK).[1] Contraction in smooth muscle is initiated by phosphorylation, and this event is thought to reflect an increased level of actin-activated ATPase activity for the phosphorylated myosin. The coupling of phosphorylation to intracellular Ca^{2+} transients occurs via the formation of the Ca^{2+}–CaM complex and its subsequent activation of MLCK.

Several studies have addressed the structure–function relationships of MLCK. Focusing on the smooth muscle and nonmuscle enzymes, these led to a general plan of the kinase in which the active site is central and is flanked on its C-terminal side by the CaM-binding site. Full-length cDNAs have provided derived sequences for the gizzard (Olson et al., 1990), rabbit uterine (Gallagher et al., 1991), bovine stomach (Kobayashi et al., 1992), and nonmuscle enzymes (Shoemaker et al., 1990). For the gizzard MLCK, the enzyme is composed of 972 residues (molecular mass 108 kDa), and the active site extends from Gly526 to Arg762. Between the active site and the CaM-binding site (Ala796 to Ser875; Olson et al., 1990) is a regulatory domain, and one hypothesis is that this contains a pseudosubstrate region (Pearson et al., 1988). An unusual feature of the smooth muscle and nonmuscle forms is the presence of motifs, designated I and II, that appear as repeats in twitchin, an unc-22 gene product (Benian et al., 1989). Similar motifs also are found in C-protein (Einheber & Fischman, 1990) and titin (Labiet et al., 1990). Gizzard MLCK contains three of the type II motifs and one type I (Olson et al., 1990). Motif II-3 is located at the C-terminal end of MLCK, and this region is also expressed as an independent protein, called telokin (Ito et al., 1989). Recently the crystal structure of telokin has been determined and shown to be a β-barrel structure very similar to the CH_2 domain of the immunoglobulin fragment Fc, termed the IgG fold (Holden et al., 1992). It was predicted by Takagi and Cox (1990) that MLCK would contain three IgG folds, the other two corresponding to II-1 (residues 147–240) and II-2 (residues 288–391). The function of these motifs in MLCK, or in other proteins, is not established. One possibility is that they are involved in the interaction of MLCK with the contractile apparatus, either myosin or actin. It was suggested for titin (Labiet et al., 1990) and C-protein (Einheber & Fischman, 1990) that these domains may be involved in interactions with myosin.

Some of the properties of MLCK are associated with defined sequences, for example, the active site and the CaM-binding site. However, one feature has not been assigned to a given structure, and this is the actin-binding site. By immunofluorescence, it was demonstrated that MLCK binds to stress fibers in nonmuscle cells (deLanerolle et al., 1981; Guerriero et al., 1981) and in addition staining of the nucleolus was observed (Guerriero et al., 1981). In skeletal and cardiac muscle, the antibody to MLCK reacted with a component(s) in the I band (Guerriero et al., 1981). These results suggested that MLCK binds to actin and this was later confirmed using isolated proteins (Dabrowska et al., 1982; Sellers & Pato, 1984). The purpose of this study is to identify the actin-binding region and to localize it in the MLCK molecule.

MATERIALS AND METHODS

Proteins were isolated from the indicated sources and procedures: myosin light chain kinase from fresh chicken gizzards (Ikebe et al., 1987); actin from chicken skeletal muscle (Pardee & Spudich, 1982); calmodulin from frozen goat testes (Walsh et al., 1983); myosin from frozen turkey gizzards (Ikebe & Hartshorne, 1985); and the 20-kDa light chain from gizzard myosin (Hathaway & Haeberle, 1983).

The MLCK was assayed using light chains as substrate by the procedure of Ikebe et al. (1987).

Cleavage of MLCK. The DTNB–CN cleavage at cysteine residues was carried out essentially as described by Tobacman

† This work was supported by Grants HL 23615 and HL 20984 from the National Institutes of Health (to D.J.H.) and by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (to S.K.).

* Address correspondence to this author.
‡ Faculty of Bioresources, Mie University.
§ First Department of Internal Medicine, Mie University.
‖ University of Arizona.

[1] Abbreviations: MLCK, myosin light chain kinase; CaM, calmodulin; kDa, kilodalton(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/93/0432-8902$04.00/0

(1988) with minor modifications. MLCK (1–2 mg/mL) was dialyzed against 50 mM KCl, 25 mM Tris-HCl (pH 7.6). Solid guanidine hydrochloride was added to 4.7 M, and the sample was incubated at 37 °C for 30 min. DTNB was added to a 6-fold excess over total SH groups. After 20 min at 37 °C, the pH was increased by addition of Tris-HCl (pH 8.6) to 125 mM, and approximately a 600-fold excess (over DTNB) of KCN was added. After approximately 24 h at 4 °C, the mixture was dialyzed against 30 mM sodium acetate-HCl (pH 3.1) prior to SP-Toyopearl chromatography, or against 50 mM KCl, 25 mM Tris-HCl (pH 7.6) for actin-binding assays.

Actin-Binding Assays. An equal volume of F-actin (6–12 mg/mL) in 50 mM KCl, 25 mM Tris-HCl (pH 7.6) was added to the peptide or protein to be tested (in the same solvent) and left for 40 min at 4 °C. In some instances, bovine serum albumin was added at final concentrations from 2.5 to 8.4 mg/mL. The F-actin was sedimented by centrifugation for 15 min with a Beckman airfuge (30 psi) or a Hitachi microultracentrifuge (Model CS100) at 160000*g*. The pellets and supernatants were analyzed by SDS–PAGE.

Proteolysis of MLCK. Various proteases were monitored for their ability to release an actin-binding peptide from MLCK. Proteases were obtained from Sigma. Conditions of proteolysis were chosen to produce discrete fragments of MLCK, and these were established by SDS–PAGE. Proteases used were as follows: trypsin and α-chymotrypsin (Ikebe et al., 1987); *Staphylococcus aureus* protease [1:24 w/w ratio of protease to MLCK in 60 mM KCl, 25 mM Tris-HCl (pH 7.6)]; thermolysin (see figure legends); and subtilisin (1:24 w/w ratio of protease to MLCK).

Other Procedures. SDS–PAGE was carried out on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% SDS with the discontinuous buffer system of Laemmli (1970). Protein concentrations were estimated by the bicinchoninic acid protein assay reagent (Pierce Chemical Co.). Antibodies were raised in rabbits against intact gizzard MLCK and gizzard telokin (Ito et al., 1989). Conditions for Western blots are described previously (Ito et al., 1989). Sequence comparisons were carried out using either the Genetics Computer Group software package, version 7.2 (Wisconsin, 1992), or DNA-SIS-Mac.

RESULTS

Proteolysis. In an attempt to localize the actin-binding site on the MLCK molecule, the effect of limited proteolysis by thermolysin was investigated. At different times (15 s, 2 min, and 35 min), proteolysis was stopped (see Materials and Methods) and the digest mixed with F-actin. The samples were centrifuged to sediment F-actin, and SDS–PAGE was carried out as shown in Figure 1. For the nondigested sample, some of the MLCK bound to actin and was detected in the F-actin pellet (lane 5, Figure 1). After 15 s of proteolysis, several fragments of MLCK were visible. In general, these fragments did not bind to F-actin and were found predominantly in the supernatant fraction (lane 7, Figure 1). On further digestion, smaller fragments of MLCK were produced that predictably did not bind to F-actin. The limit peptide of thermolysin proteolysis of MLCK is the inactive fragment of 58 kDa (lanes 12 and 13, Figure 1). The N- and C-terminal amino acids of the 58-kDa fragment are V275 and A806, respectively (Pearson et al., 1991). Thus, since this fragment does not bind to F-actin, the actin-binding site on MLCK must be located in either the N- or the C-terminal flanking regions (residues 1–274 or 807–972, respectively).

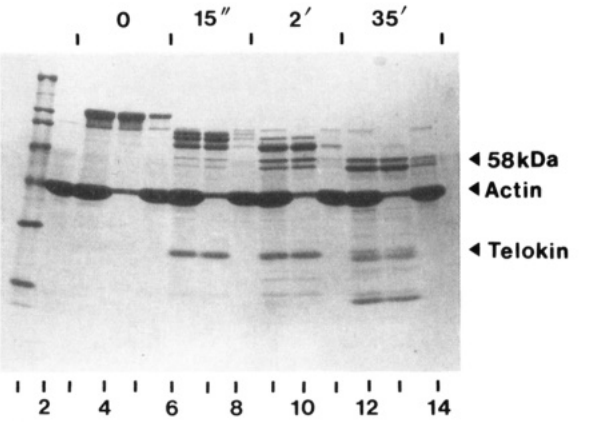

FIGURE 1: Actin binding of MLCK following proteolysis by thermolysin. MLCK (0.5 mg/mL) hydrolyzed at 25 °C with thermolysin (1:30, w/w) in 30 mM KCl, 1 mM NaCl, 0.3 mM $CaCl_2$, and 30 mM Tris-HCl (pH 7.5). Reaction was stopped by addition of EDTA to 5 mM at 15 s, 2 min, and 35 min. Samples were assayed for actin binding (at approximately 3.5 mg/mL F-actin), and SDS–PAGE was carried out on the initial mixture and the pellet and supernatant following centrifugation. Lane 1, molecular mass markers of 205, 116, 97.4, 66, 45, 29, and 14 kDa. Lane 2, actin. Lanes 3–5, 6–8, 9–11, and 12–14 show mixture, supernatant, and pellet, respectively, for 0-, 15-s, 2-min, and 35-min time points, respectively.

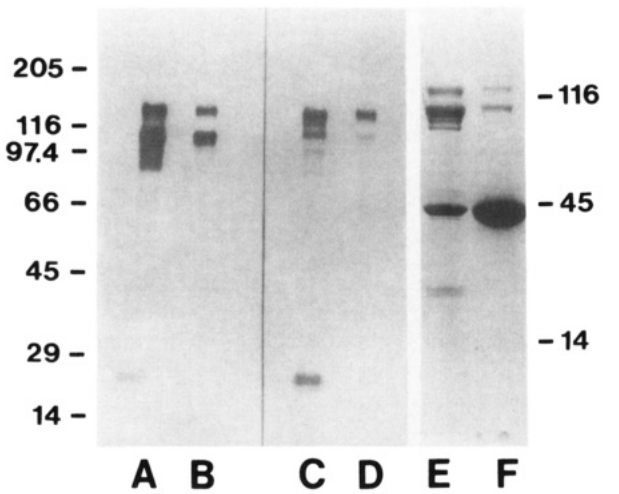

FIGURE 2: Effects of limited proteolysis of MLCK by thermolysin on actin binding. MLCK (0.7 mg/mL) hydrolyzed at 10 °C with thermolysin (1:120, w/w) in 50 mM KCl, 3 mM NaCl, 1.1 mM $CaCl_2$, and 30 mM Tris-HCl (pH 7.5). Reaction was stopped with EDTA at 20 s. Samples were assayed for actin binding. Lanes A and B, Western blots of supernatant and pellet, respectively, using antibody to native MLCK. Lanes C and D, Western blots of supernatant and pellet, respectively, using telokin antibody. Lanes E and F, SDS–PAGE of supernatant and pellet, respectively. Position of molecular mass markers (kDa) as indicated.

To examine which of the two regions might be involved in actin binding, MLCK was again subjected to mild proteolysis by thermolysin and assayed for actin binding (Figure 2). Western blots were carried out on the supernatants and pellets using polyclonal antibodies to MLCK and telokin [telokin is the C-terminal region of MLCK (Ito et al., 1989) corresponding to the sequence M816–E972 (Yoshikai & Ikebe, 1992)]. Using the antibody to MLCK, it was shown that only two components bound to actin. These were the native MLCK and a fragment of approximately 108 kDa (lane B, Figure 2). Cross-reaction with the antibody to telokin indicated that of these two components only the native enzyme contained the sequence corresponding to telokin (lane D, Figure 2). These results suggest, therefore, that the actin-binding site is located

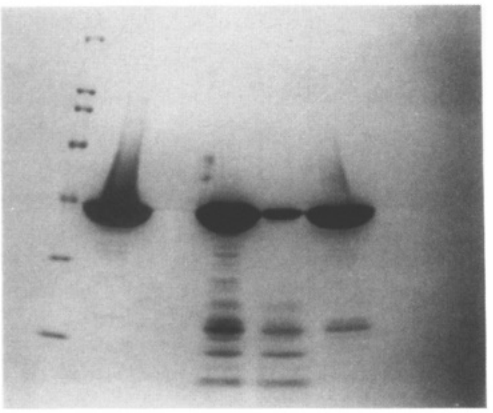

FIGURE 3: Binding to F-actin of a peptide released from MLCK by cleavage with DTNB–CN. MLCK was cleaved by DTNB–CN as outlined under Materials and Methods and assayed for actin binding. Final concentration of F-actin, 6 mg/mL. Samples were analyzed by SDS–PAGE. Lane A, F-actin. Lane B, actin plus MLCK sample before centrifugation. Lane C, supernatant from actin-binding assay. Lane D, pellet from actin-binding assay. On the left of the gel is shown the molecular mass markers (kDa) as in Figure 1.

at the N-terminal end of the MLCK molecule and should be contained within the sequence of residues 1–274.

MLCK Cleavage. The next objective was to determine which method of cleavage would yield a discrete actin-binding peptide. Following cleavage under different conditions, the presence of an actin-binding peptide was screened, using the sedimentation procedure (see Materials and Methods). In general, proteolysis (by trypsin, α-chymotrypsin, thermolysin, subtilisin, and *S. aureus* protease) did not generate a suitable peptide in a reasonable yield. The method that was chosen was chemical cleavage at the cysteine residues using the DTNB–CN complex. The utility of this method is illustrated in Figure 3, and it is shown that one of the many peptides generated is sedimented in the F-actin pellet. The actin-binding peptide has a molecular mass of approximately 17 kDa.

Isolation of the Actin-Binding Peptide. Initially, purification of the actin-binding peptide was attempted by its cosedimentation with F-actin. However, the yield and contamination by actin were unacceptable, and the following isolation procedure was designed. MLCK was subjected to cleavage by DTNB–CN, and the resulting peptides were applied to chromatography on SP-Toyopearl. The elution profile is shown in Figure 4A. Fractions were monitored by their ability to bind F-actin in the sedimentation assay and by SDS–PAGE. Gel patterns for various fractions are shown in the inset of Figure 4A. The fractions that contained an actin-binding peptide (indicated by the hatched area of Figure 4A) were combined and applied to a TSK DEAE-5 PW column (Figure 4B). After removal of the nonbound protein, a linear NaCl gradient from 0 to 0.2 M was applied. Fractions were monitored as before, and the actin-binding peptide was detected as indicated in Figure 4B. The final step of purification was gel filtration. Fractions from anion-exchange chromatography containing the actin-binding peptide were combined, concentrated, and applied to a TSK G-3000 SW column. The actin-binding peptide was eluted as indicated in Figure 4C. This peptide appeared homogeneous by SDS–PAGE (inset, Figure 4C) and corresponded to a molecular mass of 17 kDa. The yield of the purified peptide was approximately 8 μg/mg of MLCK.

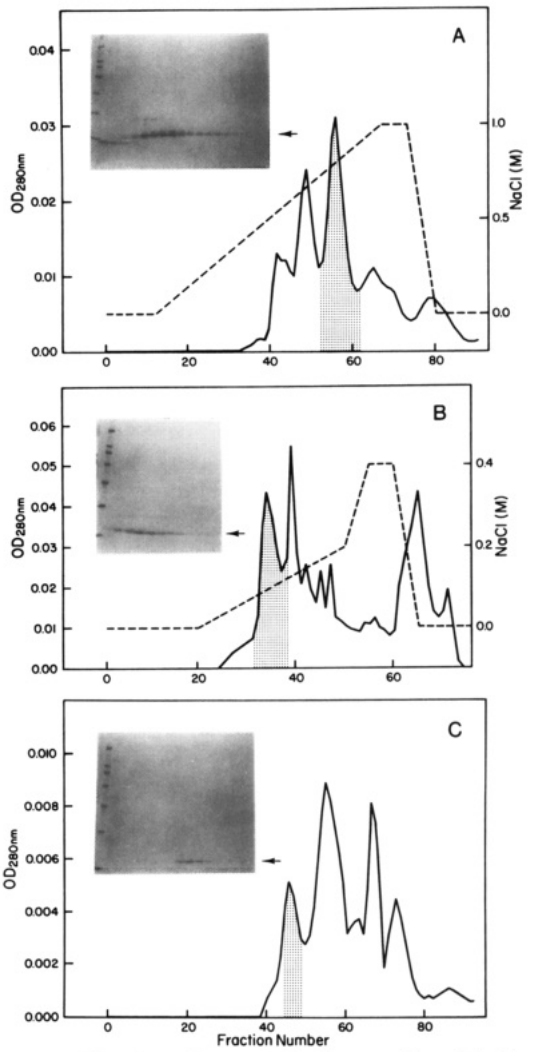

FIGURE 4: Purification of the actin-binding peptide. (A) Chromatography on SP-Toyopearl. The cleavage products of MLCK (approximately 8 mg) were applied to a 1.6 × 12.5 cm SP-Toyopearl column attached to a Pharmacia FPLC system and washed for 30 min with 30 mM sodium acetate-HCl (pH 3.1), and a linear NaCl gradient, 0–1 M, was applied, as indicated by the dashed line. Protein eluted during the washing stage not shown. Flow rate, 2 mL/min. Fractions were monitored by SDS–PAGE (lanes 2–20 of inset show fractions 49–67) and assayed for actin binding. Actin-binding peptide (as indicated by arrow on inset) was contained in fractions 53–62 (indicated by hatched area of chromatography profile). Lane 1 of inset shows molecular mass markers (kDa) as in Figure 1. (B) Chromatography on DEAE-5PW. The combined fractions from the SP-Toyopearl column were dialyzed against 30 mM Tris-HCl (pH 8.9) and applied to a TSK DEAE-5PW column, 0.75 × 7.5 cm, attached either to the FPLC system or to a Japan Spectroscopic 880-PU HPLC system. The column was washed with 30 mM Tris-HCl (pH 8.9) for 20 min and a linear NaCl gradient, 0–0.2 M, applied. A second gradient to 0.4 M NaCl then was applied. Gradients indicated by the dashed line. Protein eluted during the washing stage not shown. Flow rate, 1 mL/min. Fractions were monitored as in (A). The actin-binding peptide (indicated by arrow in inset) was found in fractions 33–39 (indicated by hatched area). (C) Chromatography on G-3000. The combined fractions from the DEAE column were concentrated to approximately 1 mL (with Centricon 10 microconcentrators, Amicon Co.) and applied to a TSK G-3000 SW column, 0.75 × 60 cm, equilibrated with 50 mM KCl, 0.3 M NaCl, and 25 mM Tris-HCl (pH 7.6). The column was eluted at a flow rate of 0.5 mL/min, and fractions were monitored as in (A). The actin-binding peptide (indicated by arrow in inset) was found in fractions 46–49 (indicated by hatched area). These fractions were combined and concentrated with Centricon 10 filters.

Characterization of the Actin-Binding Peptide. Binding of the isolated peptide to F-actin is illustrated in Figure 5. Using the sedimentation assay, it is clear that the peptide and

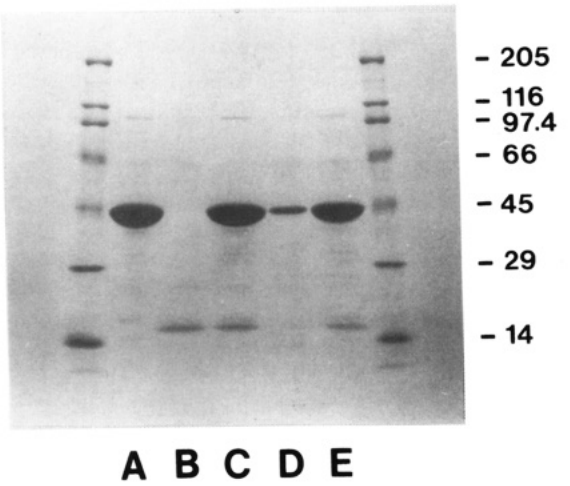

FIGURE 5: Binding of the purified actin-binding peptide to F-actin. The peptide (33 μg; 5 μM) was added to F-actin (0.9 mg; 58 μM) in a final volume of 0.37 mL and after 40 min at 4 °C was assayed for actin binding. Solvent: 50 mM KCl, 25 mM Tris-HCl (pH 7.6). Samples were analyzed by SDS–PAGE. (A) F-Actin; B, actin-binding peptide; C, mixture of actin and peptide; D and E, supernatant and pellet, respectively, from actin-binding assay. At both sides of the gel are shown the molecular mass markers (in kDa) as in Figure 1.

F-actin are cosedimented. In the presence of bovine serum albumin (up to 8.4 mg/mL), binding of the peptide to F-actin still occurred, thus reducing the possibility of nonspecific interaction. In addition, it was shown earlier (Figure 3) that many of the peptides generated by DTNB–CN cleavage of MLCK did not bind to F-actin, and this also argues against nonspecific binding.

An approximate binding constant (K_B) was estimated from the data shown in Figure 6. Binding of the purified peptide and parent MLCK was estimated from the sedimentation assay at varying concentrations of F-actin. The binding curves are shown in Figure 6A and for the isolated peptide and the native enzyme are similar. At greater than a 20–40-fold molar excess of actin, saturation binding occurred. The data in Figure 6A are shown as a double-reciprocal plot in Figure 6B. From this plot, a K_B of approximately 7.5×10^4 M^{-1} was estimated for both the peptide and native MLCK. This value is similar to that determined earlier by Sellers and Pato (1984) for MLCK, namely, 2.5×10^5 M^{-1}. These experiments indicate that the actin-binding properties of the parent molecule are retained in the isolated peptide.

The amino acid composition of the isolated peptide is given in Table I. This is compared to the theoretical amino compositions of the four large peptides predicted from cleavage at cysteine residues for the gizzard MLCK. [The amino acid composition of the predicted peptides is based on the sequence derived from cDNA (Olson et al., 1990).] The isolated peptide is similar in composition to the peptide corresponding to the sequence 1–114. Since cleavage by DTNB–CN modifies the cysteine [i.e., the generated N-terminal residue (Jacobson et al., 1973; Degani & Patchornik, 1974)], the presence or absence of free α-NH_2 groups cannot be used diagnostically. It is concluded, therefore, on the basis of amino acid composition and evidence presented above from thermolysin proteolysis, that the actin-binding peptide of MLCK is contained within the N-terminal sequence 1 through 114.

DISCUSSION

An actin-binding peptide has been isolated from gizzard MLCK and found to correspond to the N-terminal part of the

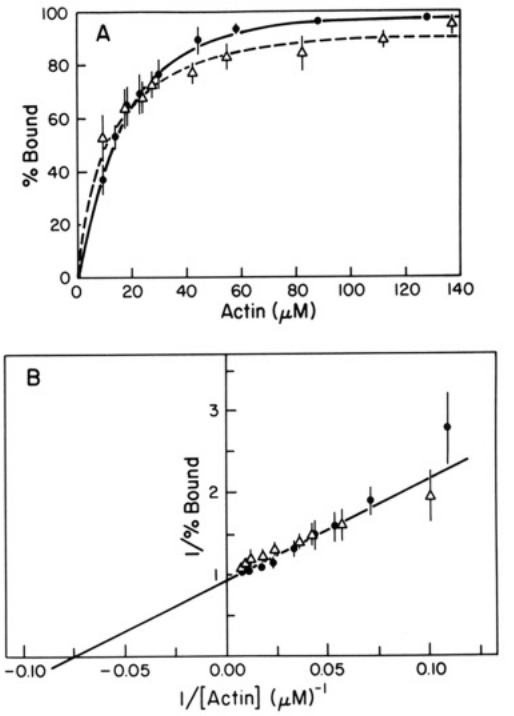

FIGURE 6: Binding curves for the purified peptide and F-actin. Varying amounts of F-actin (0–138 μM) were added to the purified peptide (4.6 μM) or native MLCK (1.1 μM, assuming a molecular mass of 108 kDa) in 50 mM KCl/25 mM Tris-HCl (pH 7.6). After 40 min at 4 °C, the samples were assayed for actin binding and the supernatants analyzed by SDS–PAGE. The amount of nonbound peptides was calculated from scans of the gels, using a Shimadzu dual-wavelength chromatoscanner, CS-910. (A) Binding curves for the peptide (●) and native MLCK (△). (B) Data from (A) expressed as a double-reciprocal plot. Each point represents the mean ± SD (n = 5). Data were fit using the Lotus Freelance R3J program.

Table I: Amino Acid Compositions (Residues/100) of the Actin-Binding Peptide Isolated from MLCK and the Four Large Peptides Predicted from the Sequence of MLCK

	peptide[a]	theor 1–114	theor 236–321	theor 438–569	theor 769–880
D[b]	8.8 ± 0.56	8.8	2.3	9.9	8.2
E[b]	13.1 ± 0.24	12.3	10.5	22.1	10.9
S	8.0 ± 0.34	6.1	7.0	7.6	9.1
G	8.4 ± 1.84	3.5	2.3	6.1	5.5
H	0.9 ± 0.25	0.9	0	0	2.7
R	5.7 ± 0.45	6.1	2.3	5.3	6.4
T	5.5 ± 0.20	5.3	14.0	4.6	4.5
A	11.7 ± 0.29	12.3	11.6	5.3	10.0
P	12.7 ± 0.60	14.9	14.0	0.8	4.5
Y	0.3 ± 0.14	0	0	3.8	1.8
V	6.5 ± 0.21	7.9	9.3	9.9	5.5
M	0.6 ± 0.09	0.9	0	0.8	6.4
I	0.7 ± 0.21	0	2.3	4.6	3.6
L	4.9 ± 0.32	4.4	4.7	3.8	5.5
F	2.4 ± 0.13	2.6	2.3	4.6	2.7
K	10.3 ± 1.55	14.0	17.4	10.7	12.7

[a] Data are means ± SD (n = 6). [b] D and E represent Asp + Asn and Glu + Gln, respectively.

molecule. The actin-binding properties of the parent molecule are retained in this peptide. Peptides from MLCK were generated by cleavage at cysteine residues, and the N-terminal peptide resulted from cleavage at C115 (Olson et al., 1990). It is likely that the actin-binding site(s) is (are) represented by a sequence within this 114-residue peptide, but such has not been identified. This N-terminal sequence does not contain a repetitive unc motif, and the unc II-1 region begins at G147 (Olson et al., 1990). It is unlikely, therefore, that the IgG fold is functional as an actin-binding domain. It is interesting

that the N-terminal 75 residues of the 126-kDa rabbit uterine (Gallagher et al., 1991) and the 155-kDa bovine stomach (Kobayashi et al., 1992) MLCKs are similar to the corresponding gizzard MLCK sequence (9 and 7 of the 75 residues being different, respectively). Assuming that the actin-binding property in mammalian MLCK is preserved, this may indicate that the actin-binding site is contained within the first 75 residues. In the 140-kDa MLCK from nonmuscle cells, chick embryo fibroblasts, the N-terminal sequence is distinct, and the exact sequence corresponding to gizzard residues 1–114 is found in the fibroblast MLCK at residues 286–399 (Shoemaker et al., 1990). The MLCKs from rabbit (Takio et al., 1986) and rat (Roush et al., 1988) skeletal muscle do not show marked similarities in sequence to the N-terminal region of gizzard MLCK.

Comparison of the gizzard sequence 1–114 to several other actin-binding proteins did not reveal any striking homology. Little similarity was detected between the MLCK peptide and gelsolin, actin-binding protein 1, actin-binding protein 280, Cap 42, hisactophilin, tropomyosin of smooth and skeletal muscle, and gizzard myosin heavy chains. For smooth muscle α-actinin (Baron et al., 1987), some similarity was found. With the N-terminal 134 residues of α-actinin, there is 33% similarity and 20% identity to the isolated MLCK peptide. Kuhlman et al. (1992) suggest that an actin-binding site in α-actinin is represented by residues 120–134. This region matches the MLCK sequence 52–63 with 50% similarity and 8% identity. Similarity between caldesmon and MLCK also was found. Recent studies on gizzard caldesmon have suggested the presence of three actin-binding sites, all within the C-terminal half of the molecule (Wang et al., 1991). At the N-terminal side of actin site II (Wang et al., 1991), there is a matching sequence. The pentapeptide PAPKP is identical for residues 703–707 and 99–103 of caldesmon and MLCK, respectively. Since the assignment of the boundaries of the actin-binding sites in caldesmon is tentative (Wang et al., 1991), the significance of this sequence may need to be reevaluated. Previously it was suggested that a short (tetrapeptide) sequence may be part of the actin-binding domains of gelsolin (Kwiatkowski et al., 1986) and Cap 42(a) (Ampe & Vandekerckhove, 1987). Similarity of the N-terminal part of gizzard MLCK and caldesmon also was noted by Kohama et al. (1992). They found that the sequence K42–A80 of gizzard MLCK was 38.5% homologous with the caldesmon sequence K695–A731, which represents much of the actin site II (Wang et al., 1991). The corresponding sequence of bovine stomach MLCK also shows a 38% identity with this C-terminal portion of caldesmon (Kobayashi et al., 1992).

It was estimated previously that in nonvascular smooth muscle the molar ratio of MLCK to actin is between 200 and 300 (Dabrowska et al., 1982). This would correspond to about two molecules of MLCK per 1-μm length of thin filament. In vascular muscle (femoral artery; Cong et al., 1992), the concentration of MLCK is lower, and thus the stoichiometry of MLCK to actin would be reduced. Even allowing for the possible preferential location of MLCK in contractile domains, as opposed to structural domains (Small et al., 1986), this still limits the localization of MLCK to a few molecules per thin filament. [Sellers and Pato (1984) showed that MLCK also bound to dephosphorylated myosin. However, if a direct competition is assumed for the two possible locations, then most of the MLCK would be associated with actin since its concentration is much higher than myosin.]

The following points are raised. Is there a preferred binding site on the thin filament for MLCK, or is the binding random? Intuitively, the latter does not seem acceptable, but on the other hand, the only distinct locations could be at either the pointed or barbed ends of the filament. There is no evidence for MLCK as a capping protein, and the limited data available on MLCK localization are not consistent with binding at the dense body or Z-line (deLanerolle et al., 1981; Guerriero et al., 1981; Cavadore et al., 1982). Obviously, if MLCK remains attached to the thin filament during contraction, this could limit access to the myosin cross bridges. Accepting that the binding site to actin is located in the N-terminal 114 residues of MLCK, does this leave enough distance for the active site to "reach" the 20-kDa light chains of myosin? The distance between the end of the N-terminal sequence and the ATP-binding site (G526) is 412 residues. If an α-helical extended conformation is assumed, this would correspond to approximately 64 nm. It is known that the sequence 115–527 contains the unc II-1, unc II-2, and unc I-1 domains. If each of these is considered to be similar in shape to unc II-3 [i.e., telokin, with a long dimension of 6.4 nm (Holden et al., 1992)], this would reduce the connecting distance to about 40 nm, still adequate to reach myosin. For example, in the electron micrographs of rabbit portal-anterior mesenteric vein (Somlyo, 1980), it is evident that (even allowing for shrinkage) several thin filaments are within 10–20 nm of the thick filament surfaces. It is also possible that MLCK attaches and detaches from the thin filament during different phases of the contractile cycle. If access to myosin is increased with the detached form of MLCK, the detachment should coincide with the increase of intracellular [Ca^{2+}] and subsequent binding of Ca^{2+}–calmodulin to MLCK. Indeed, Sellers and Pato (1984) reported about a 3-fold reduction in the binding constant for the binding of MLCK to actin in the presence of Ca^{2+} plus calmodulin. It is not known if this change in K_B is adequate to provide an on–off mechanism. Thus, several questions remain to be answered, and the initial emphasis is to establish whether MLCK remains bound to the thin filament during all phases of smooth muscle activity.

ACKNOWLEDGMENT

We are grateful to Dr. V. Guerriero, Jr., for many valuable discussions and for his help in the computer comparisons of protein sequences.

REFERENCES

Ampe, C., & Vandekerckhove, J. (1987) *EMBO J. 6*, 4149–4157.

Baron, M. D.; Davison, M. D., Jones, P., & Critchley, D. R. (1987) *J. Biol. Chem. 262*, 17623–17629.

Benian, M. G., Kiff, J. E., Neckelmann, N., Moerman, D. G., & Waterston, R. H. (1989) *Nature (London) 342*, 45–50.

Blanchard, A., Ohanian, V., & Critchley, D. (1989) *J. Muscle Res. Cell Motil. 10*, 280–289.

Cavadore, J.-C., Molla, A., Harricane, M.-C., Gabrion, J., Benyamin, Y., & Demaille, J. G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3475–3479.

Cong, M. C., Cohen, P., Kitazawa, T., Ikebe, M., Masuo, M., Somlyo, A. P., & Somlyo, A. V. (1992) *J. Biol. Chem. 267*, 14662–14668.

Dabrowska, A., Hinkins, S., Walsh, M. P., & Hartshorne, D. J. (1982) *Biochem. Biophys. Res. Commun. 107*, 1524–1531.

Degani, Y., & Patchornik, A. (1974) *Biochemistry 13*, 1–11.

deLanerolle, P., Adelstein, R. S., Feramisco, J. R., & Burridge, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4738–4742.

Einheber, S., & Fischman, D. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2157–2161.
Gallagher, P. J., Herring, B. P., Griffin, S. A., & Stull, J. T. (1991) *J. Biol. Chem. 266*, 23936–23944.
Guerriero, V., Jr., Rowley, D. R., & Means, A. R. (1981) *Cell 27*, 449–458.
Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) 2nd ed., pp 423–482, Raven Press, New York.
Hathaway, D. R., & Haeberle, J. R. (1983) *Anal. Biochem. 135*, 37–43.
Holden, H. M., Ito, M., Hartshorne, D. J., & Rayment, I. (1992) *J. Mol. Biol. 227*, 840–851.
Ikebe, M., & Hartshorne, D. J. (1985) *J. Biol. Chem. 260*, 13146–13153.
Ikebe, M., Stepinska, M., Kemp, B. E., Means, A. R., & Hartshorne, D. J. (1987) *J. Biol. Chem. 260*, 13828–13834.
Ito, M., Dabrowska, R., Guerriero, V., Jr., & Hartshorne, D. J. (1989) *J. Biol. Chem. 264*, 13971–13974.
Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583–6591.
Kobayashi, H., Inoue, A., Mikawa, T., Kuwayama, H., Hotta, Y., Masaki, T., & Ebashi, S. (1992) *J. Biochem. (Tokyo) 112*, 786–791.
Kohama, K., Okagaki, T., Hayakawa, K., Lin, Y., Ishikawa, R., Shimmen, T., & Inoue A. (1992) *Biochem. Biophys. Res. Commun. 184*, 1204–1211.
Kuhlman, P. A., Hemmings, L., & Critchley, D. R. (1992) *FEBS Lett. 304*, 201–206.
Kwiatkowski, D. J., Stossel, T. P., Orkin, S. H., Mole, J. E., Colten, H. R., & Yin, H. L. (1986) *Nature 323*, 455–458.
Labiet, S., Barlow, D. P., Gautel, M., Gibson, T., Holt, J., Hsieh, C.-I., Francke, U., Lednard, K., Wardale, J., Whiting, A., & Trinick, J. (1990) *Nature (London) 345*, 273–276.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., & Means, A. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2284–2288.
Pardee, J. D., & Spudich, J. A. (1982) *Methods Enzymol. 85*, 164–181.
Pearson, R. B., Wettenhall, R. E. H., Means, A. R., Hartshorne, D. J., & Kemp, B. E. (1988) *Science 241*, 970–973.
Pearson, R. B., Ito, M., Morrice, N. A., Smith, A. J., Condron, R., Wettenhall, R. E. H., Kemp, B. E., & Hartshorne, D. J. (1991) *Eur. J. Biochem. 200*, 723–730.
Roush, C. L., Kennelly, P. J., Glaccum, M. B., Helfman, D. M., Scott, J. D., & Krebs, E. G. (1988) *J. Biol. Chem. 263*, 10510–10516.
Sellers, J. R., & Pato, M. D. (1984) *J. Biol. Chem. 259*, 7740–7746.
Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J., & Watterson, D. M. (1990) *J. Cell Biol. 111*, 1107–1125.
Small, J. V., Fürst, D. O., & Demey, J. (1986) *J. Cell Biol. 102*, 210–220.
Somlyo, A. V. (1980) in *Handbook of Physiology, Section 2* (Bohr, D. G., Somlyo, A. P., & Sparks, H. V., Eds.) Vol. II, pp 33–67, American Physiological Society, Bethesda, MD.
Takagi, T., & Cox, J. A. (1990) *J. Biol. Chem. 265*, 19721–19727.
Takio, K., Blumenthal, D. K., Walsh, K. A., Titani, K., & Krebs, E. G. (1986) *Biochemistry 25*, 8049–8057.
Tobacman, L. S. (1988) *J. Biol. Chem. 263*, 2668–2672.
Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.
Wang, C.-L. A., Wang, L.-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V., & Bryan, J. (1991) *J. Biol. Chem. 266*, 9166–9172.
Yoshikai, S.-I., & Ikebe, M. (1992) *Arch. Biochem. Biophys. 299*, 242–247.