The Structure and Function of the Actin-binding Domain of Myosin Light Chain Kinase of Smooth Muscle*

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Li-Hong Ye‡, Kohichi Hayakawa‡, Hiroko Kishi‡, Michihiro Imamura‡, Akio Nakamura‡, Tsuyoshi Okagaki‡, Takashi Takagi‡, Akiko Iwata‡, Takeshi Tanaka‡, and Kazuhiro Kohama‡

From the ‡Department of Pharmacology, Gunma University School of Medicine, Maebashi, Gunma 371 Japan, the §Biological Institute, Faculty of Science, Tohoku University, Sendai, Miyagi 980, Japan, and the ¶Research Division, Saitama Red Cross Blood Center, Yonan, Saitama 338, Japan

In addition to its kinase activity, the myosin light chain kinase (MLCK) of smooth muscle has an actin-binding activity through which it can regulate the actin-myosin interaction of smooth muscle (Kohama, K., Okagaki, T., Hayakawa, K., Lin, Y., Ishikawa, R., Shimmey, T., and Inoue, A. (1992) Biochem. Biophys. Res. Commun. 184, 1204–1211). In this study, we have analyzed the actin binding activity of MLCK and related it to its amino acid sequence by producing native and recombinant fragments of MLCK. Parent MLCK exhibited both calcium ion (Ca$^{2+}$) and calmodulin (Ca$^{2+}$/CaM)-sensitive and Ca$^{2+}$/CaM-insensitive binding to actin filaments. The native fragment, which consists of the Met$^1$–Lys$^{14}$ sequence (Kanoh, S., Ito, M., Niwa, E., Kawano, Y., and Hartshorne, D. J. (1993) Biochemistry 32, 8892–8897), and the recombinant NN fragment, which contains this 1–14 sequence, showed only Ca$^{2+}$/CaM-sensitive binding. An inhibitory effect of the NN fragment on the actin-myosin interaction was observed by assaying in vitro motility and by measuring the actin-activated ATPase activity of myosin. The recombinant NN/41 fragment, which is constructed without the Met$^1$–Pro$^{41}$ sequence of the NN fragment, lost both the actin binding activity and the inhibitory effect. We confirmed the importance of the 1–41 sequence by using a few synthetic peptides to compete against the NN fragment in binding to actin filaments. The experiments using recombinant fragments and synthetic peptides also revealed that the site for CaM-binding is the Pro$^{26}$–Pro$^{41}$ sequence. The site for the Ca$^{2+}$/CaM-insensitive binding, which is shown to be localized between the Ca$^{2+}$/CaM-sensitive site and the central kinase domain of MLCK, exerted no regulatory effects on the actin-myosin interaction.

Myosin light chain kinase (MLCK)$^1$ has an important regulatory role in smooth muscle contraction (see Ref. 1 for a review). MLCK phosphorylates the 20-kDa light chain of smooth muscle myosin together with calmodulin in the presence of Ca$^{2+}$ ions (Ca$^{2+}$/CaM), thereby activating the myosin, which can then interact with actin filaments to induce contraction.

In addition to this kinase activity, MLCK can act as an actin-binding protein; MLCK is present in association with the sarcomeric I-band (2), and it binds to actin filaments with a high affinity (3–5). We have shown that MLCK can inhibit the ATP-dependent interaction between actin and myosin by binding to actin filaments. This inhibition can be relieved by Ca$^{2+}$/CaM, as was demonstrated by avoiding the complication derived from its kinase activity (6–8), although the demonstration was limited to in vitro only.

Such an inhibition, however, is not peculiar to MLCK. Caldesmon (see Ref. 9 for a review) and calponin (10) are known to inhibit the actin-myosin interaction by binding to actin, an inhibition that is relieved by Ca$^{2+}$/CaM. Twenty years ago, Hartshorne et al. (11) obtained an inhibitory fraction from chicken gizzard in a preliminary form. The activity of the 130-kDa component of their fraction relates to the actin-binding property of MLCK. By measuring the content of MLCK, caldesmon, and calponin in the myofibril of smooth muscle (12), we have shown previously how the actin-binding property of MLCK participates in regulating smooth muscle contraction.

The relationship between the structure of MLCK and the function of the kinase activity in phosphorylating the myosin light chain and how Ca$^{2+}$/CaM modulates the activity has been well established (see Ref. 13 for a review). However, there has been little study of the structure-function relationship of the actin binding activity except for the purification of an actin-binding fragment from MLCK (14).

In this study, we have investigated which sequence of MLCK is responsible for the actin binding activity, which sequence inhibits the actin-myosin interaction, and which sequence binds CaM to relieve the inhibition. Our approach has been to cleave MLCK to prepare native fragments containing the actin binding activity (14), to design MLCK cDNA to express the recombinant fragments of actin binding activity in Escherichia coli, and then to analyze them biochemically. A few peptides have been synthesized to confirm these analyses. We have shown that: (i) MLCK has two actin-binding sites on the N-terminal side away from the central kinase domain, (ii) the binding site responsible for the inhibitory effect is at the Met$^1$–Pro$^{41}$ sequence, and (iii) CaM interacts with MLCK at the Pro$^{26}$–Pro$^{41}$ sequence.

MATERIALS AND METHODS

Preparation of Proteins—All procedures were carried out at 0–4 °C. The purity of proteins was routinely monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below) so...
that they were >95% pure. The purified proteins, except actin and myosin (see below), were divided into aliquots and stored at -80 °C until they were used. All columns (see below) were incorporated into a high performance liquid chromatography system (model L-6200; Hitachi, Tokyo, Japan). MLCK was prepared by the method of Adelstein and Klee (15), and was subjected to ammonium sulfate fractionation. After fractionation, MLCK was purified using column chromatography in DEAE-Toyopearl 650M columns and subsequently in SP-Toyopearl 650M (Tosoh, Tokyo, Japan); this MLCK was used in all experiments unless otherwise specified. We also purified smooth muscle myosin by SDS-PAGE followed by densitometry as described (23), respectively. The amino acid sequences of these fragments were determined using an amino acid sequencer according to the manufacturer’s instructions for the NN-, NC-, and NN/41 fragments. How-

**Expression and Purification of Recombinant MLCK Fragments**—For the expression of recombinant MLCK fragments, the pET system (Novagen, Madison, WI) was used according to the manufacturer’s instructions. Series of cDNA fragments (see Fig. 6 for their topology) encoding various domains of bovine stomach MLCK (23) were amplified by the polymerase chain reaction. The amplified fragments were subcloned into a pET21 vector and verified by DNA sequencing. The recombinant MLCK fragment was overproduced in *E. coli* BL21(DE3). The cells were grown to an absorbance at 600 nm of 0.5-0.6 in 2 liters of Luria broth medium containing ampicillin (50 μg/ml) and then were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. All of the fragments were soluble in low salt. Therefore, cells expressing the fragments were collected by centrifugation, and then were suspended in 40 ml of a solution of 14 mM 2-mercaptoethanol and 20 mM Tris-HCl (pH 7.5). The resulting supernatant was fractionated with ammonium sulfate. The fragments were purified from the supernatant by using a combination of DEAE-Toyopearl 650M and CM-Toyopearl 650M columns (Tosoh, Tokyo, Japan). When any minor contaminants were found in SDS-PAGE, they were removed by gel filtration with Superose 12HR (Pharmacia Biotech Inc.). The MLCK fragments were: NN-, NC-, and NN/41 fragments, 4.8 kDa; 42–80 peptide, 4.0 kDa. The molecular masses used for calculating concentrations of MLCK and its fragments under the conditions of 2 μM actin filaments, 0.5 mM ATP, 60 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA, and 20 mM Tris-HCl (pH 7.5) unless otherwise specified. The liberated phosphate was quantified in duplicate or triplicate by the malachite green method of Kodama et al. (26). We ensured that the rate of ATP hydrolysis was constant and that the specific activity of the ATPase did not differ from our previously reported values. Therefore, we expressed the activity as a normalized ATPase activity (27).

**Other Methods**—SDS-PAGE was carried out using the method of Laemmli (18) for a gel consisting of 12% acrylamide, 1.5% agarose, and 2.4% 10 wt% acrylamide and 0.8 wt% agarose, the purity (>95%) of MLCK and its fragments. Protein concentrations were determined using the methods of Bradford (29) and Lowry et al. (30) with bovine serum albumin as the standard.

The molecular masses used for calculating concentrations (M) of proteins and peptides were obtained from their amino acid sequences as follows: myosin, 440 kDa; actin, 41.8 kDa; CaM, 16.7 kDa; MLCK, 107.5 kDa; bovine stomach MLCK, 128.8 kDa; NTCP fragment, 12.4 kDa; NN fragment, 35.3 kDa; NC fragment, 44.4 kDa; NN/41 fragment, 30.7 kDa; NN/25 fragment, 33.3 kDa; 787–815 peptide, 3.4 kDa; 796–834 peptide, 4.1 kDa; 1–25 peptide, 3.0 kDa; 26–41 peptide, 1.7 kDa; 1–41 peptide, 4.8 kDa; 42–80 peptide, 4.0 kDa.

Homology of the specified amino acid sequences of MLCK and the fragments of these proteins was examined using MacDNASIS Pro 2000–90 (Hitachi Software Engineering, San Bruno, CA) to search the GenBank/NBRF-PIR/SWISS-PROT data bases. Isoelectric points of the synthetic peptides were calculated according to Skoog and Wichman (31).

**RESULTS**

**Actin-binding Properties of Recombinant Fragments of MLCK**—We designed a fragment so that it consisted of Met¹–Lys¹¹ sequence of bovine stomach MLCK (see Fig. 6 for its topology in MLCK), and subjected this NN fragment to an actin-binding assay. As shown in Fig. 1a by open circles, the amount of the NN fragment bound to actin filaments increased with increasing concentration of the NN fragment. The *Kₘ* of the binding of the NN fragment to actin filaments was 3.72 ± 0.20 × 10⁻¹ m⁻¹ (mean ± S.E., n = 3). The binding was abolished totally by CaM (Fig. 1b, open circles). Thus, we deduced that the 1–337 sequence contains a site for Ca²⁺/CaM-sensitive actin binding.

Previously, we investigated the importance of the sequence between Lys⁴² and Ala⁶⁰ to the inhibitory effect of MLCK by searching for a part of MLCK that is homologous to the actin-binding domain of caldesmon (6). We synthesized a peptide consisting of Lys⁴²–Ala⁶⁰ of MLCK and used it to compete against the actin binding activity of the NN fragment. How-
ever, as shown in Fig. 1c by the open circles, the binding was not at all affected.

We obtained another peptide, this time of the Met<sup>1</sup>–Pro<sup>41</sup> sequence, and found its inhibitory effect on the actin binding of the NN fragment as shown by the filled circles in Fig. 1c. Thus, the sequence responsible for the binding of the NN fragment to actin filaments, i.e. the Ca<sup>2+</sup>/CaM-sensitive site of MLCK, must be located at the N terminus of the MLCK molecule. We divided peptide Met<sup>1</sup>–Pro<sup>41</sup> into peptide Met<sup>1</sup>–Gly<sup>25</sup> and peptide Phe<sup>26</sup>–Pro<sup>41</sup>. Both of them failed to inhibit the actin binding (open and filled triangles in Fig. 1c), suggesting that there is a critical region for actin binding within the 1–41 residues.

To confirm this idea by another method, we produced the NN/41 fragment, which is devoid of the Met<sup>1</sup>–Pro<sup>41</sup> sequence of MLCK, and the NN/25 fragment which is devoid of the Met<sup>1</sup>–Gly<sup>25</sup> sequence of MLCK (see Fig. 6 for their topology in MLCK), and then assayed for their actin-binding activity. As shown by filled triangles in Fig. 1a, the binding of the NN/41 fragment to actin filaments remained at a basal level. Although the NN/25 fragment definitely bound to actin filament with $K_a = 1.01 \pm 0.53 \times 10^5$ M<sup>-1</sup> (mean ± S.E., $n = 3$), the affinity was much lower than that of the NN fragment. The length of the Pro<sup>26</sup>–Pro<sup>41</sup> sequence when added to the NN/41 fragment (i.e. the NN/25 fragment) is probably too short to endow it with an actin-binding ability as high as that of the NN fragment, confirming the importance of the 1–41 sequence as an actin-binding site.

We also produced the NC fragment, which comprises the Pro<sup>319</sup>–Val<sup>721</sup> sequence of bovine stomach MLCK (see Fig. 6 for its topology in MLCK). We assayed the NC fragment for its actin-binding ability. As shown in Fig. 1a by filled circles, it shows a definite actin binding activity with a $K_a = 1.83 \pm 0.47 \times 10^5$ M<sup>-1</sup> (mean ± S.E., $n = 3$). We used CaM in the presence of Ca<sup>2+</sup> to compete against the actin binding of the NC fragment, but failed to observe any effect of Ca<sup>2+</sup>/CaM (see Fig. 1b, filled circles). Considering the Ca<sup>2+</sup>/CaM-sensitive, actin-binding activity of the NN fragment, parent MLCK would be expected to bind to actin filaments in both Ca<sup>2+</sup>/CaM-sensitive and insensitive ways, which will be shown later and discussed (see Fig. 8a).

**Direct Detection of Interaction of Recombinant Proteins with CaM**—We immobilized CaM on an IAsys cubette surface, and allowed MLCK to interact with the surface. In the presence of Ca<sup>2+</sup>, the surface plasmon resonance of IAsys increased with the increase in the concentration of MLCK. However, in the presence of EGTA, the increase is only slight (data not shown). Such a difference indicates that the IAsys system could be reliably used to detect binding activity of MLCK and its fragments to CaM.

As shown in Fig. 2a by open (MLCK) and filled (bovine stomach MLCK) circles, the difference in the resonance between Ca<sup>2+</sup> and EGTA at the specified concentrations was readily increased at low concentrations of both MLCKs and approached saturation at their higher concentrations. This Ca<sup>2+</sup>-dependent change in the resonance of the NN fragment shown in Fig. 2a by filled squares is quite different from that of parent MLCK; it increased gradually. However, it was never saturated, indicating the low affinity of the fragments for CaM.

We assayed the NN/41 fragment with IAsys using the same surface of the cubette. As shown in Fig. 2a, the Ca<sup>2+</sup>-dependent changes in the resonance were not detected with this fragment (filled triangles), indicating that the CaM-binding site is not located in the C-terminal portion away from Lys<sup>42</sup>–Ala<sup>80</sup> (open circles). Ordinate, the bound amounts (%) of the NN fragment. Abscissa, the concentrations of the synthetic peptides (mM).
the actin-binding assay (see Fig. 1). Accordingly, the NC fragment did not cause any Ca$^{2+}$-dependent change in the resonance (Fig. 2a, open circles).

To identify the position of the CaM-binding site in the 1–41 sequence, we synthesized peptides of Met1–Gly25 and Pro26–Pro41. Because of their low molecular mass, their interaction with CaM could not be detected directly by resonance (data not shown). Therefore, their interaction with CaM was detected by observing the interaction of the NN fragment with the CaM-coated surface of an IAsys cubette in the presence of various concentrations of the synthetic peptide of Met1–Gly25 (open circles), Pro26–Pro41 (filled circles), or Met1–Pro41 (triangles). The responses (ordinate) relative to those in the absence of the peptides were plotted against the peptide concentrations.

Effect of Recombinant Proteins on the Actin-Myosin Interaction—As shown in Fig. 3a by filled circles, the motility of actin filaments on a myosin-coated glass surface was inhibited with the increase in the concentration of the NN fragment. Half-maximal inhibition was observed in the presence of 41.1 ± 5.5 nM (mean ± S.E., n = 3) of the NN fragment. The inhibition was relieved by Ca$^{2+}$/CaM (Fig. 3a, open circles). Actin-activated ATPase activities of myosin (Fig. 3b, filled circles) were reduced with the increase in the concentration of the NN fragment. Half-maximal inhibition was observed in the presence of 0.276 ± 0.028 μM (mean ± S.E., n = 3). Ca$^{2+}$/CaM effectively relieved the inhibition; the inhibition caused by 1 μM NN fragment was abolished by an 8–10-fold molar excess of CaM over the NN fragment (Fig. 3c, filled circles).

It must be noted that the concentration required for inhibiting the activity half-maximally was about 6.7-fold higher than the concentration required for inhibiting the motility. Such a discrepancy has been noted by Sato et al. (8) by using aortic MLCK. The difference in the concentrations of actin filaments used for the motility and ATPase assays may be one possible explanation.

The regulatory activity of the NN/41 fragment was also tested both with motility and ATPase assays. As expected from the absence of actin binding activity (Fig. 1a), we failed to find any effect, irrespective of the presence or absence of Ca$^{2+}$/CaM (Fig. 3, a–c, squares). Taking the absence of the actin binding activity of the NN/41 fragment (Fig. 1a) into consideration, the
absence of any effect indicates that Met¹–Pro⁴¹ is the sequence of MLCK that is responsible for the inhibition of myosin through actin binding activity. The NN/25 fragment, which showed a weak actin binding activity (Fig. 1a), failed to inhibit the effect in the motility and ATPase assays (Fig. 3, a and b, asterisks), suggesting that the residues on both N- and C-terminal sides of Gly²⁵ are required for inhibiting the actin-myosin interaction.

The NC fragment, on the other hand, bound to actin filaments in a Ca²⁺/CaM-insensitive manner (Fig. 1b). As shown in Fig. 3 (a and b) by triangles, the NC fragment did not exert any regulatory activity as monitored both by the motility and ATPase assays. The absence of activity was confirmed in the presence of various concentrations of Ca²⁺/CaM (Fig. 3c, triangles). Thus, we conclude that the Ca²⁺/CaM-insensitive binding site of MLCK has no regulatory activity.

**Confirmation with Parent MLCK and Its Native Fragment**—We allowed parent MLCK to bind to actin filaments in a similar way to its recombinant fragments. In the presence of 1 mM EGTA, MLCK bound to actin filaments at its high affinity site at 0.15 ± 0.02 mol/mol actin (mean ± S.E., n = 3) with an affinity constant \(K_a = 2.16 ± 0.65 \times 10^6 \text{ M}^{-1}\) (mean ± S.E., n = 3). At its low affinity site, MLCK bound to actin filaments with a \(K_a = 4.67 ± 0.17 \times 10^5 \text{ M}^{-1}\) (mean ± S.E., n = 3). In the presence of 1 mM Ca²⁺ and CaM at an 8-fold molar excess over MLCK, binding of MLCK to the actin filaments had only a single \(K_a\) of 3.1 ± 10⁵ M⁻¹. Because this value is closer to the \(K_a\) of the low affinity site, we conclude that the actin binding at the high affinity site is Ca²⁺/CaM-sensitive.

As we describe below (see Fig. 5a), the Ca²⁺/CaM-insensitive binding is confirmed by the persistence of actin binding activity in MLCK when the concentration of CaM was increased. Sellers and Pato (4) showed Ca²⁺/CaM-sensitive binding of MLCK to actin filaments. However, we conclude that parent MLCK binds to actin filaments in both Ca²⁺/CaM-sensitive and -insensitive ways, confirming the Ca²⁺/CaM-sensitive and -insensitive binding of the NN- and NC fragments, respectively.

We produced the NTCB fragment from MLCK by chemical cleavage. In the presence of 1 mM EGTA, the NTCB fragment showed actin binding activity, confirming the results of Kanoh et al. (14). Scatchard plots of the data showed that the NTCB fragment bound to actin filaments maximally at 0.2 mol/mol actin with a single \(K_a\) of 4.8 ± 10⁵ M⁻¹. In the presence of 1 mM Ca²⁺ and CaM at a 9-fold molar excess over MLCK, we detected no binding activity of the NTCB fragment to actin filaments. We consider that the actin binding of the fragment is very similar to that of the NN fragment, i.e. Ca²⁺/CaM-sensitive, although we are aware that the \(K_a\) of the NTCB fragment is lower than that of the Ca²⁺/CaM-sensitive site of parent MLCK (\(K_a = 2.16 ± 10^6 \text{ M}^{-1}\)). The difference is discussed in more detail under "Discussion."

**DISCUSSION**

This report analyzes two classes of actin-binding site of MLCK, i.e. Ca²⁺/CaM-sensitive and Ca²⁺/CaM-insensitive sites as shown in Table I. The former is involved in the inhibitory effect of MLCK on the actin-myosin interaction. The latter site has no regulatory activity.

The inhibitory effect of MLCK on the actin-myosin interaction is brought about by its actin binding activity. This actin-linked nature was first demonstrated with a Nitella-based motility assay (see Ref. 25 for the method), where we allowed MLCK to bind to the actin cables that run on the inner surface of the plasma membrane of Nitella internodal cells (6). With the myosin-coated motility assay used in the present study, Sato et al. (8) suggested the actin-linked nature of the effect after observing that the inhibitory effect was obscured with the increase in the concentrations of actin filaments. The present study (Table I) demonstrates the actin-linked nature of the effect by using actin-binding fragments of MLCK which were produced (i) by purifying the NTCB fragment with conventional protein chemistry and (ii) by producing recombinant fragments with the pET expression system.

**Fig. 4. Effect of synthetic peptides on the actin binding activity of the NTCB fragment.** Competitive binding of the NTCB fragment (3 μM) to actin filaments was examined in the presence of various concentrations of peptide Met¹–Gly²⁵ (open triangles), peptide Pro²⁶–Pro⁴¹ (filled triangles), peptide Met¹–Pro⁴¹ (filled circles), or peptide Lys²³–Ala⁴⁰ (open circles). The bound amounts relative to those in the absence of the peptides were plotted against the concentrations of the peptides (μM).

The inhibitory effects of MLCK and the NTCB fragment on the actin-myosin interaction are shown in Fig. 5. MLCK effectively inhibited the motility of actin filaments in the presence of EGTA. However, there was no inhibition in the presence of Ca²⁺/CaM. Similar inhibition and its relief by Ca²⁺/CaM were observed for the NTCB fragment, confirming the results obtained with the NN fragment (Fig. 3). The higher concentration of the NTCB fragment compared with that of parent MLCK (Fig. 5, compare a and b) is attributable to their different affinities to actin filaments.

Taken together with the CaM binding activity of the NTCB fragment (see Fig. 3, asterisks), the above data for the NTCB fragment confirm those for the NN fragment. We consider that the NN fragment is a recombinant form of the NTCB fragment and that it contains the whole sequence (Met¹–Lys¹¹⁴) of the NTCB fragment (see Fig. 6).
MLCK, with respect to its actin-linked regulatory role as follows. The Ca\(^{2+}\)/CaM-sensitive, actin-binding site is localized at the extreme N terminus of MLCK, consisting of Met\(^1\)–Pro\(^{41}\) (Fig. 6, a and b). The localization was determined (i) by comparing actin binding activity of the recombinant NN fragment containing only the Ca\(^{2+}\)/CaM-sensitive site with that of the NN/41 fragment, which is devoid of the 1–41 sequence of the NN fragment (Fig. 1c); and (ii) by using a synthetic peptide of Met\(^1\)–Pro\(^{41}\) to compete against the actin binding of the native NTCB fragment (Fig. 4) and its recombinant form, the NN fragment (Fig. 1c).

Calponin (10) and caldesmon (9) are actin-binding proteins in smooth muscle that inhibit the actin-myosin interaction in a similar way to MLCK. Recent biochemical studies have narrowed the length of amino acid sequence responsible for actin binding activity down to 37 amino acids for calponin (33), and 32 and 46 amino acids for caldesmon (34). In agreement with these values, a length of 41 amino acids was shown to be the Ca\(^{2+}\)/CaM-sensitive, actin-binding site. This was demonstrated as follows. (i) When the peptide of Met\(^1\)–Pro\(^{41}\) was split into Met\(^1\)–Gly\(^{25}\) and Pro\(^{26}\)–Pro\(^{41}\), both peptides lost the antagonism for the actin binding of the NTCB (Fig. 4) and NN fragments (Fig. 1c). (ii) The extension of fragment length from the NN/41 fragment to the NN/25 fragment failed to restore full actin binding activity (Fig. 1a). This weak actin-binding ability of the NN/25 fragment suggests the possibility that some residues within the 1–41 sequence may not be involved in the actin binding.

It must be noted that the calculated pI of the sequence of Met\(^1\)–Pro\(^{41}\) is 10.67. Because actin is a highly acidic protein, we wonder whether the alkaline property of the sequence could cause MLCK to bind to actin nonspecifically. As shown in Fig.

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**FIG. 5.** Regulatory role of MLCK and its NTCB fragment as examined by *in vitro* motility assay. An *in vitro* motility assay was conducted in the presence of various concentrations of MLCK or NTCB fragments as described in the legend to Fig. 3. The velocities of the movements (\(\mu\)m s\(^{-1}\)) were plotted against their concentrations (nM). Panel a, effect of MLCK was examined in the presence (open circles) and absence (filled circles) of Ca\(^{2+}\) and 20 nM CaM. Panel b, effect of the NTCB fragment was examined in the presence (open circles) and absence (filled circles) of Ca\(^{2+}\) and 150 nM CaM.

**TABLE I**

Summary of binding to actin filaments of MLCK and its fragments and their inhibitory effects on the actin-myosin interaction

| MLCK and its fragments | Actin binding | Inhibitory effects |
|------------------------|--------------|--------------------|
| MLCK (full-length, native) | + | + |
| NTCB fragment (native) | + | + |
| NN fragment (recombinant) | + | + |
| NC fragment (recombinant) | - | - |
| NN/41 fragment (recombinant) | - | - |
| NN/25 fragment (recombinant) | - | - |

*Fig. 6.* Schematic diagram of constructs of native and recombinant fragments and domain structure of MLCK. Panel a, the proposed actin-binding and CaM-binding sequences responsible for the Ca\(^{2+}\)/CaM-sensitive actin binding of MLCK are shown together with other domains of bovine stomach MLCK (23). The asterisk (*) shows the Ca\(^{2+}\)/CaM-insensitive, actin-binding sequence, which exerts no regulatory role in the actin-myosin interaction. Panel b, the recombinant fragments, i.e. NN, NC, NN/25, and NN/41 fragments, were constructed using cDNA of bovine stomach MLCK as described under "Materials and Methods." The NTCB fragment was produced by the chemical cleavage of chicken gizzard MLCK by the method of Kanoh et al. (14). Peptides of Met\(^1\)–Pro\(^{41}\), Met\(^1\)–Gly\(^{25}\), Lys\(^{42}\)–Ala\(^{80}\), and Pro\(^{26}\)–Pro\(^{41}\) were synthesized with an automated synthesizer. These fragments and peptides are shown by black bars. Because chicken gizzard MLCK is devoid of 240 residues made up of 12-residue repeats (23), the NTCB fragment is interrupted by the dotted lines. The proposed Ca\(^{2+}\)/CaM-sensitive, actin-binding site, which is responsible for the actin-linked inhibition by MLCK of the actin-myosin interaction, is indicated by the shaded area.

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**FIG. 6.** Schematic diagram of constructs of native and recombinant fragments and domain structure of MLCK. Panel a, the proposed actin-binding and CaM-binding sequences responsible for the Ca\(^{2+}\)/CaM-sensitive actin binding of MLCK are shown together with other domains of bovine stomach MLCK (23). The asterisk (*) shows the Ca\(^{2+}\)/CaM-insensitive, actin-binding sequence, which exerts no regulatory role in the actin-myosin interaction. Panel b, the recombinant fragments, i.e. NN, NC, NN/25, and NN/41 fragments, were constructed using cDNA of bovine stomach MLCK as described under "Materials and Methods." The NTCB fragment was produced by the chemical cleavage of chicken gizzard MLCK by the method of Kanoh et al. (14). Peptides of Met\(^1\)–Pro\(^{41}\), Met\(^1\)–Gly\(^{25}\), Lys\(^{42}\)–Ala\(^{80}\), and Pro\(^{26}\)–Pro\(^{41}\) were synthesized with an automated synthesizer. These fragments and peptides are shown by black bars. Because chicken gizzard MLCK is devoid of 240 residues made up of 12-residue repeats (23), the NTCB fragment is interrupted by the dotted lines. The proposed Ca\(^{2+}\)/CaM-sensitive, actin-binding site, which is responsible for the actin-linked inhibition by MLCK of the actin-myosin interaction, is indicated by the shaded area.
4, the peptide of the Lys$^{42}$-Ala$^{60}$ sequence did not affect the ability of the NTCB fragment to bind to actin filaments, although its $pI$ was similarly alkaline ($pI = 10.51$). Therefore, the interaction between MLCK and actin filaments at the site of Met$^1$-Pro$^{41}$ is not attributable to the nonspecific binding between the acidic and alkaline sequences. We searched for homology of Met$^1$-Pro$^{41}$ with other actin-binding proteins and found sequences that show a 35% identity within the actinin sequence, a 32.3% identity within the dystrophin sequence, and a 28.1% identity within the villin sequence. The 30.0% identity within the villin sequence, and a 28.1% identity within the dystrophin sequence, a 32.3% identity within the dystrophin sequence, and a 28.1% identity within the coronin sequence.

Unlike the Ca$^{2+}$/CaM-sensitive, actin-binding site, the position of the Ca$^{2+}$/CaM-insensitive, actin-binding site was not determined precisely (asterisk in Fig. 6a). What we can conclude is that the Ca$^{2+}$/CaM-insensitive site is included in the NC fragment as shown in Fig. 6. The sequence of Pro$^{319}$-Lys$^{337}$ in the NC fragment overlapped with the NN fragment and is obviously devoid of the Ca$^{2+}$/CaM-insensitive site (compare the NC fragment with the NN fragment in Fig. 6b). Therefore, the Ca$^{2+}$/CaM-insensitive site must be present somewhere in the Gly$^{338}$-Val$^{721}$ sequence of bovine stomach MLCK.

Is there any other sequence in MLCK that binds to actin filaments in a Ca$^{2+}$/CaM-sensitive manner? The NN/41 and NC fragments cover the N-terminal portion of MLCK except for the 1–41 sequence. Neither the NN/41 fragment nor the NC fragment showed Ca$^{2+}$/CaM-sensitive binding to actin filaments (Table I, Fig. 1). We obtained the central kinase domain by proteolyzing MLCK$^2$ and the C-terminal myosin-binding domain by purifying telokin (32). Because these domains failed to show actin binding activity, the 1–41 sequence is the sole sequence responsible for the Ca$^{2+}$/CaM-sensitive binding of MLCK that allows it to exert its regulatory role in the actin-actinomyosin interaction.

Although parent MLCK shares the 1–41 sequence with the native NTCB fragment and the recombinant NN fragment, there is a difference in $K_o$ values. When the Ca$^{2+}$/CaM-dependent site was separated from parent MLCK ($K_a = 2.16 	imes 10^6$ M$^{-1}$) as the NTCB fragment, its $K_o$ was reduced to 4.86 $10^5$ M$^{-1}$. The $K_o$ of the NN fragment, a recombinant form of the NTCB fragment, is also low, i.e., 3.72 $10^5$ M$^{-1}$. Such a reduction in the affinity upon cleavage from a parent molecule has also been noticed for the actin binding activity of the $\alpha$-actinin family (35). On the other hand, affinity to actin filaments of the Ca$^{2+}$/CaM-insensitive site is affected only slightly upon its separation from the parent molecule. The NC fragment, which carries only this site, binds to actin filaments with a $K_o = 1.83 	imes 10^5$ M$^{-1}$ (Fig. 1a), an affinity that is comparable with the $K_o$ values of the Ca$^{2+}$/CaM-insensitive sites of MLCK ($K_o = 4.67 	imes 10^5$ M$^{-1}$ as measured in the absence of the Ca$^{2+}$/CaM, and $K_o = 3.1 	imes 10^5$ M$^{-1}$ as measured in the presence of Ca$^{2+}$/CaM).

According to Dabrowska et al. (3), the concentrations of MLCK and actin filaments in chicken gizzard smooth muscle cells are estimated to be 3 $\mu$M and 830 $\mu$M, respectively, and MLCK in the cells is adequately absorbed by the actin filaments. Our estimation of affinity for actin of the Ca$^{2+}$/CaM-sensitive site of MLCK explains their data, indicating that 0.36% of actin filaments in the cells are in the MLCK-bound form. The concentration of MLCK that gives half-maximal inhibition of the motility of actin filaments is 2.1 nM (Fig. 5a). Using a 3 nM concentration of actin filaments in the motility assay, we calculate that 0.42% of actin filaments would be in the MLCK-bound form. A comparable figure has also been calculated from the concentration of MLCK that gives half-maximal inhibition of the actin-activated ATPase (see Fig. 2 in

![Fig. 7. Comparison of amino acid sequences of CaM-binding proteins.](image-url) Amino acid sequences conforming to the consensus IQ motif were aligned. The homologous residues are shown in bold letters.

![Fig. 8. Two distinct sites for CaM binding in parent MLCK.](image-url) Panel a, effect of CaM on Ca$^{2+}$/CaM-sensitive actin binding of MLCK as examined by the four experiments was examined in the absence (filled circles) and presence of peptide Pro$^{39}$-Pro$^{41}$ at 100 $\mu$M (open circles) or peptide Ser$^{97}$-Ser$^{115}$ at 100 $\mu$M (triangles), as described in the legend to Fig. 1. a and b. Panel b, as described under "Materials and Methods," smooth muscle myosin was phosphorylated by MLCK and Ca$^{2+}$/CaM in the presence of various concentrations of peptide Pro$^{39}$-Pro$^{41}$ (filled circles), peptide Ser$^{97}$-Ser$^{115}$ (open circles), or peptide Ala$^{96}$-Ser$^{104}$ (triangles).
Ref. 6). To establish the physiological role of the actin binding activity of MLCK (36), it remains to be demonstrated whether or not such a small percentage of actin filaments in smooth muscle cells could participate in regulating their contraction. Alternatively, the in vivo binding of MLCK to actin filaments may serve not to activate the actin-myosin interaction per se but simply to localize the kinase activity to the vicinity of its substrate.

Actin binding of calponin (10) and caldesmon (9) is regulated by Ca\(^{2+}\)/CaM in a similar way to MLCK (see Fig. 1). The sites that bind Calponin in calponin (10) and caldesmon (9) are located close to the sites for their actin binding. Therefore, the CaM-binding site that regulates the actin binding of MLCK would be expected to be close to the actin-binding site. Accordingly, we demonstrated that the CaM-binding site is included in the actin-binding site, i.e. Met\(^1\)–Pro\(^{41}\) sequence, by showing (i) that the NN/41 fragment that is devoid of the Met\(^1\)–Pro\(^{41}\) sequence fails to bind CaM (Fig. 2a) and (ii) that the Met\(^1\)–Pro\(^{41}\) peptide competes against the NN fragment for CaM binding (Fig. 2b). Further, when we divided the 1–41 peptide into two peptides of Met\(^1\)–Gly\(^{26}\) and Pro\(^{26}\)–Pro\(^{41}\), the sequence that competes for CaM binding is the latter (Fig. 2b). The calculated pI values of the former (pI = 10.25) and the latter (pI = 10.82) are similar. Therefore, the failure of the former peptide to bind to CaM suggests that the interaction between CaM and the latter peptide was not caused by the non-specific binding of an alkaline peptide to an acidic protein such as CaM. As shown in Fig. 7, the IQ motif, a consensus sequence in CaM-binding proteins, is located near the IQ motif (a consensus sequence in CaM-binding proteins) in its absence and presence of peptide Ser\(^{787}\)–Ser\(^{815}\) (Fig.8, filled triangles), exerted no effect. Thus, we concluded that parent MLCK has at least two classes of CaM-binding sites, which rules out the possibility that the CaM-binding activity of the 26–41 sequence was produced upon fragmentation of MLCK.

REFERENCES

1. Kamm, K. E., and Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 595–620
2. Guerraiero, V., Jr., Bowley, D. R., and Mean, A. R. (1981) Cell 27, 449–458
3. Dobrowolska, R., Hinkins, S., Walsh, M. P., and Hartshorne, D. J. (1982) Biochem. Biophys. Res. Commun. 107, 1524–1531
4. Sellers, J. R., and Pate, M. D. (1984) J. Biol. Chem. 259, 7749–7766
5. Yamazaki, K., Ito, K., Sobe, K., Mori, T., and Shibata, N. (1987) J. Biochem. (Tokyo) 101, 1–9
6. Kohama, K., Okagaki, T., Hayakawa, K., Lin, Y., Ishikawa, K., Shimen, T., and Inaue, A. (1992) Biochem. Biophys. Res. Commun. 184, 1204–1211
7. Ye, L.-H., Hayakawa, K., Lin, Y., Okagaki, T., Fujita, K., and Kohama, K. (1994) J. Biochem. (Tokyo) 116, 1377–1382
8. Sato, M., Ye, L.-H., and Kohama, K. (1995) J. Biochem. (Tokyo) 118, 1–3
9. Sobe, K., and Sellers, J. R. (1991) J. Biol. Chem. 266, 12115–12118
10. Takekoshi, K., and Nadal-Ginard, B. (1991) J. Biol. Chem. 266, 13284–13288
11. Hartshorne, D. J., Abrams, L., Aksoy, M., Dobrowolska, R., Driska, S., and Sharkey, K. (1977) in The Biochemistry of Smooth Muscle (Stephens, N. L., ed.) pp. 513–532, University Press Park, Baltimore, MD
12. Allen, B. G., and Walsh, M. P. (1994) Trends Biochem. Sci. 19, 362–368
13. Kanoh, S., Ito, M., Niwa, E., Kawano, Y., and Hartshorne, D. J. (1993) Biochemistry 32, 8902–8907
14. Adelstein, R. S., and Klee, C. B. (1981) Annu. Rev. Pharmacol. Toxicol. 21, 227–257
15. Lin, Y., Ishikawa, R., Okagaki, T., and Kohama, K. (1994) in Regulation of the Contractile Cycle in Smooth Muscle (Nakano, T., and Hartshorne, D. J., eds) pp. 159–173, Springer-Verlag, Tokyo
16. Kowayama, H., Suzuki, M., Koga, R., and Ebashi, S. (1988) J. Biochem. (Tokyo) 104, 862–866
17. Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kempt, B. E., and Means, A. R. (1990) Proc. Natl Acad. Sci. U.S.A. 87, 2284–2288
18. Metsumura, F., and Lin, J. C.-C. (1982) J. Biol. Chem. 257, 163–171
19. Nakamura, S., and Nonomura, Y. (1984) J. Biochem. (Tokyo) 96, 575–578
20. Emash, S. (1976) J. Biochem. (Tokyo) 79, 229–231
21. Okagaki, T., Higashi-Fujime, S., Ishikawa, K., Takano-Ohmuro, H., and Kohama, K. (1991) J. Biochem. (Tokyo) 109, 858–866
22. Kobayashi, H., Inoue, A., Mikawa, T., Kawaiama, H., Hotta, Y., Masaki, T., and Ebashi, S. (1992) J. Biochem. (Tokyo) 112, 786–791
23. Ishikawa, R., Okagaki, T., Higashi-Fujime, S., and Kohama, K. (1991) J. Biol. Chem. 266, 21784–21790
24. Warrick, H. M., and Spudich, J. A. (1987) Annu. Rev. Cell Biol. 3, 379–421
25. Toda, M., Takeda, K., and Kometani, K. (1986) J. Biochem. (Tokyo) 99, 1465–1472
26. Lin, Y., Ishikawa, R., Okagaki, T., Ye, L.-H., and Kohama, K. (1994) Cell Motil. Cytoskel. 29, 250–258
27. Laemmli, U. K. (1970) Nature 227, 680–685
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
29. Skoog, B., and Wichman, A. (1986) Trends Anal. Chem. 5, 82–83
30. Shirinsky, V. P., Vrotsnikov, A. V., Brakov, K. G., Nanaev, V. K., Collinge, M., Lukas, T. J., Sellers, J. R., and Wattersen, D. M. (1993) J. Biol. Chem. 268, 16578–16583
31. Megguelid, M., Fattoum, A., Derancourt, J., and Kassab, R. (1992) J. Biol. Chem. 267, 15943–15953
32. Wang, C.-L. A., Wang, L.-W. C., Xu, X. A., Lu, R. C., Saavedra-Alanis, V., and Bryan, J. J. (1991) J. Biol. Chem. 266, 9166–9172
33. Inoue, N., and Asano, A. (1986) J. Biochem. 102, 1087–1092
34. Okagaki, T., and Kohama, K. (1995) in Smooth Muscle Contraction (Kohama, K., and Saida, K., eds) pp. 17–35, Sarger, Basel
35. Halsall, D. J., and Hammer, J. A. (1990) FEBS Lett. 267, 126–130