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Role of non-kinase activity of myosin light-chain kinase in regulating smooth muscle contraction, a review dedicated to Dr. Setsuro Ebashi

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Abstract

Myosin light-chain kinase (MLCK) of smooth muscle consists of an actin-binding domain at the N-terminal, the catalytic domain in the central portion, and the myosin-binding domain at the C-terminal. The kinase activity is mediated by the catalytic domain that phosphorylates the myosin light-chain of 20 kDa (MLC20), activating smooth muscle myosin to interact with actin. Although the regulatory role of the kinase activity is well established, the role of non-kinase activity derived from actin-binding and myosin-binding domains remains unknown. This review is dedicated to Dr. Setsuro Ebashi, who devoted himself to elucidating the non-kinase activity of MLCK after establishing calcium regulation through troponin in skeletal and cardiac muscles. He proposed that the actin–myosin interaction of smooth muscle could be activated by the non-kinase activity of MLCK, a mechanism that is quite independent of MLC20 phosphorylation. The authors will extend his proposal for the role of non-kinase activity. In this review, we express MLCK and its fragments as recombinant proteins to examine their effects on the actin–myosin interaction in vitro. We also down-regulate MLCK in the cultured smooth muscle cells, and propose that MLC20 phosphorylation is not obligatory for the smooth muscle to contract.

Keywords: Myosin light-chain kinase; Domain structure of myosin light-chain kinase; Actin-binding activity; Myosin-binding activity; Kinase activity; Non-kinase activity; Calmodulin; Calcium regulation; Actin–myosin interaction; Contraction of smooth muscle

Research on smooth muscle regulation of actin–myosin interaction dates back to a 1966 report by Dr. Setsuro Ebashi and his colleagues [1]. They prepared crude actomyosin from chicken gizzard smooth muscle and monitored an ATP-dependent interaction between actin and myosin by inducing superprecipitation of the actomyosin. It superprecipitated faster in Ca^{2+} than in EGTA. They recognized that such sensitivity to Ca^{2+} is similar to that of the crude actomyosins from skeletal and cardiac muscles. However, once the smooth muscle actomyosin was desensitized to Ca^{2+}, it failed to superprecipitate, regardless of whether Ca^{2+} was present or not. In contrast, desensitized actomyosins from skeletal and cardiac muscles were able to superprecipitate to the same extent, irrespective of Ca^{2+} contractions.

Ten years later, Dr. Ebashi published an excellent method for the purification of actin and myosin from smooth muscle by using the aid of SDS–PAGE [2]. He con-

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firmed the failure of pure myosin to interact with pure actin, and proposed that the interaction must be activated by regulatory protein(s). To date, myosin light-chain kinase (MLCK) of smooth muscle is established as an enzyme to activate myosin by phosphorylating its 20-kDa light-chain of smooth muscle myosin (MLC20). Stimulation by agonists elevates the intracellular concentration of Ca^{2+} of smooth muscle, causing Ca^{2+} to bind to calmodulin (CaM). CaM in conjunction with Ca^{2+} (Ca/CaM) activates MLCK. Myosin, thus phosphorylated at MLC20 by MLCK, is in an active form and interacts with actin to induce contraction. The biochemical backgrounds and physiological implications are well documented in Ref. [3].

Dr. Ebashi made remarkable progress in his studies on troponin, a regulatory protein of skeletal and cardiac muscles; however, his other efforts in the search for regulatory protein(s) of smooth muscle remain unknown. He strongly believed that smooth muscle has activator(s) for actin–myosin interaction other than MLCK. After moving to the National Institute for Physiological Sciences at Okazaki, he devoted himself to the purification of such a protein. Conducting experiments by his own hand with great confidence, he finally arrived at the 155 kDa regulatory protein of the bovine stomach smooth muscle [4]. However, contrary to his belief, he detected the activity to phosphorylate MLC20 in the regulatory protein [4]. Its cDNA cloning revealed that its structure was common to the other MLCK molecules [5]. Fig. 1 depicts the domain structure of 155 kDa MLCK incorporating our data as reviewed in Refs. [6,7]. In short, MLCK is a fusion protein of the N-terminal actin-binding domain, the central catalytic domain, and the C-terminal myosin-binding domain. Dr. Ebashi took a renewed interest into how MLCK exerts a regulatory role in the actin–myosin interaction through its non-kinase activity derived from the actin-binding and myosin-binding domains [8].

The authors recently expressed the full-length MLCK from the above cDNA in *Escherichia coli* with the use of a cold-shock promoter. This wild-type (WT) MLCK was furnished with both kinase and non-kinase activities (Table 1) [9]. We also engineered ΔATP/MLCK that was devoid of kinase activity by replacing Gly residues with Ala in the ATP-binding motif [10] of X-Gly-X-Gly-X-X-Gly-X. We review here how MLCK activates the actin–myosin interaction through its non-kinase activity, including the provisional data of ΔATP/MLCK.

Table 1
Bacterial expression of recombinant full-length MLCK (WT MLCK) and its kinase-dead mutant (ΔATP MLCK) [9]

	Non-kinase activity		Kinase activity
	Actin-binding	Myosin-binding	
WT MLCK	+	+	+
ΔATP MLCK	+	+	–

Recombinant full-length MLCKs and the kinase-dead mutant were expressed in a bacterial expression system using a cold-shock promoter (*Csp A promoter*). To create wild-type (WT) MLCK, the open reading frame of bovine stomach MLCK cDNA [5] was subcloned into the NdeI/EcoRI site of the cold-shock vector pCold I. This WT MLCK exhibited both kinase activity (+) and non-kinase activities derived from actin-binding (+) and myosin-binding (+). ΔATP MLCK was created by substitution to Ala from three Gly residues of the X-Gly-X-Gly-X-X-Gly-X motif [10] of the ATP-binding site of bovine MLCK. [N.B. The ΔATP MLCK has non-kinase activity derived from actin-binding (+) and myosin-binding (+); however, it does not have kinase activity (−).]

Examples of the dissociation of MLC20 phosphorylation from contractile/migratory activities of smooth muscle cells

Some pharmacologists were interested in smooth muscle contraction under the extreme conditions reviewed in Ref. [11]. Uchida and his colleagues induced the contraction of uterine smooth muscle in Ca^{2+}-free medium by using oxytocin as an agonist, and observed a contraction that was not associated with MLC20 phosphorylation [12]. Karaki and his colleagues reported that phorbol ester was similarly able to contract a few arterial smooth muscles in Ca^{2+}-free medium without an increase in the MLC20 phosphorylation [13].

Platelet-derived growth factor (PDGF) induces chemotaxis of smooth muscle cells and myofibroblasts. Because the collagen matrix populating them indicated contractile activity in the presence of agonists [14], it was assumed that a similar activating mechanism for the actin–myosin inter-

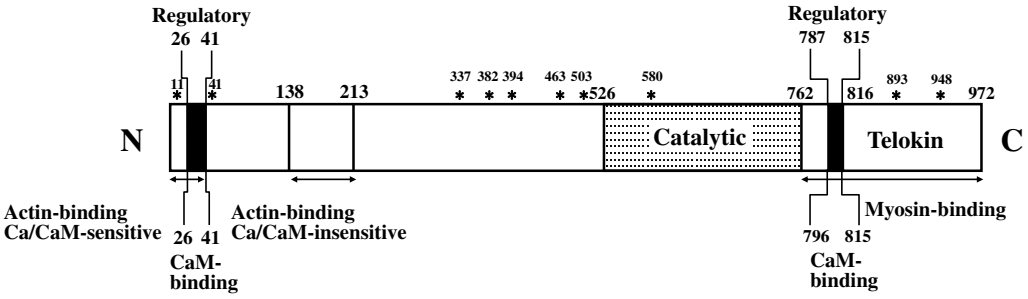

Fig. 1. Domain structure of MLCK. The numbers refer to the amino acid sequence deduced from cDNA coding for MLCK from chicken gizzard smooth muscle [30]. The assignment of the structure was carried out with MLCKs of both chicken gizzard and bovine stomach smooth muscles. The latter MLCK contains 12 residue repeats of unknown function in addition to gizzard MLCK [5]. For the convenience of readers, the repeats were deleted from the scheme. The single nucleotide polymorphisms that may cause missense mutation of MLCK are marked by asterisks (see Perspectives for details). They were detected in human MLCK, but the numbers were modified for gizzard MLCK.

action was involved. We [15,16] and others [17] observed migration to PDGF without alteration in MLC20 phosphorylation. The role of the actin-binding proteins through Rho and the Rho kinase system is briefly reviewed in Ref. [18].

MLCK as an actin-binding protein

MLCK has been known for many years to be present in association with thin filaments of sarcomeres in skeletal and cardiac muscles [19]. This actin-binding property was then analyzed biochemically [20–22] as follows. MLCK purified from chicken gizzard smooth muscle was mixed with actin filaments; the mixture was centrifuged, and the precipitate was subjected to SDS–PAGE. The amount of MLCK precipitated with actin filaments was calculated and then plotted against the concentration of actin. When a similar experiment was carried out in the presence of Ca/CaM, the binding of MLCK to actin was antagonized [22]. We confirmed the antagonism, but it was partial, suggesting that MLCK contains two classes of actin-binding sites (i.e., Ca/CaM-sensitive and Ca/CaM-insensitive sites) [23].

Cleaving MLCK with cyanogen bromide (CNBr) at Met residues revealed that the actin-binding core of MLCK lies in the sequence Asp^{2}-Met^{213} (see Fig. 2 for its localization in the MLCK molecule). Like parent MLCK, the CNBr fragment was composed of Ca/CaM-sensitive and Ca/CaM-insensitive sites for actin binding [24,25]. However, the fragment obtained by cleavage with 2-nitro-5-thiocyanatobenzonic acid (NTCB) at Cys residues, which were composed of the Met^{1}-Lys^{114} sequence (Fig. 2), bound to actin only in a Ca/CaM-dependent manner [24,25].

To confirm the presence of two classes of actin-binding sites in MLCK, we expressed various fragments of bovine stomach MLCK cDNA [5] in *E. coli* (see Fig. 2 for the constructs) and examined their actin-binding activity. The N-fragment of MLCK bound actin filaments by both Ca/CaM-sensitive and Ca/CaM-insensitive mechanisms. Ca/CaM-sensitive binding was detected in the NN-fragment located in the N-terminal half of the N-fragment, and Ca/CaM-insensitive binding was observed in the NC-fragment located in the C-terminal half of the N-fragment.

To narrow the Ca/CaM-sensitive region for actin-binding in the NN-fragment, we deleted the Met^{1}-Pro^{41}

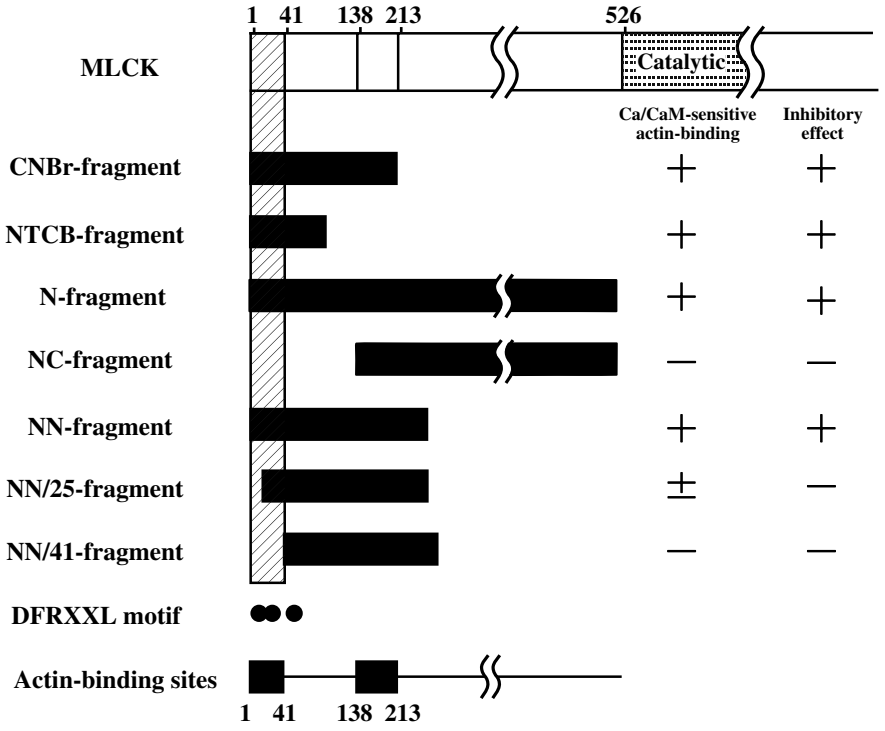

Fig. 2. Topology of localization and N-terminal regions of MLCK. NTCB fragments were obtained by cleavage of gizzard MLCK with NTCB, and CNBr fragments were obtained by cleavage of gizzard MLCK with CNBr [25]. The other N-terminals of MLCK were expressed in *E. coli* at various lengths by the use of cDNA coding bovine stomach MLCK, which are denoted by the bars [23]. The resulting recombinant fragments were purified, and their inhibitory effect (right column) was examined by measuring the actin-activated ATPase activity of smooth muscle myosin and the movement of actin filaments on a glass surface coated with myosin. In this assay, myosin was used after its MLC20 was fully phosphorylated. In the left column, + denotes the Ca/CaM-sensitive actin-binding activity of the fragments, and − denotes the absence of fragment activity. The right column depicts the inhibitory effects of the fragments: + indicates a fragment exhibiting an inhibitory effect; − indicates a fragment exhibiting no effect. At the bottom, Ca/CaM-sensitive (1–41) and Ca/CaM-insensitive (138–213) actin-binding sites are expressed by the boxes. The solid circles (●) denote the DFRXXL motif for the proposed actin-binding sequence [27]. (N.B. The numbers were modified for gizzard MLCK. Our analysis indicated that the actin–myosin interaction was inhibited through the Ca/CaM-sensitive site of 1–41).

sequence from this fragment [25]. The resulting NN/41 fragment failed to bind actin filaments. The actin-binding activity of the NN/25-fragment, devoid of the Met^{1}-Gly^{25} sequence, was very much reduced (Fig. 2). We concluded that the 1–41 sequence was responsible for the Ca/CaM-sensitive actin binding [25].

Actin filaments in conjunction with myosin and actin-associated proteins were isolated from smooth muscle as myofibrils, although myofibrils do not possess a sarcomeric structure. The binding activity of MLCK to myofibrils was much higher than to actin filaments [26]. However, the mutant MLCK, of which residues 1–141 were deleted from the N-terminal, failed to bind myofibrils and actin filaments [26]. To identify the actin-binding motif in MLCK, various amino acid residues of the actin-binding region of MLCK were replaced by Ala, and the mutants were allowed to bind to myofibrils. This alanine-scanning mutagenesis revealed that the Asp-Phe-Arg-X-X-Leu motif plays a major role in the binding of MLCK to myofibrils ([27], see also Ref. [28] for review).

Table 2
Two distinct sites for CaM-binding in parent MLCK [23]

	Regulation of actin-binding[a]	Regulation of kinase activity[b]
Pro^{26}-Pro^{41} peptide	+	−
Ser^{787}-Ser^{815} peptide	−	+

[a] Actin-binding of the NN-fragment was monitored in the presence of Ca/CaM and various concentrations of the 26–41 peptide and 787–815 peptide of gizzard MLCK. Only the former was effective to prevent actin-binding.

[b] Smooth muscle myosin was phosphorylated by MLCK in the presence of Ca/CaM. This reaction was carried out in the presence of various concentrations of the 26–41 peptide or 787–815 peptide. The latter inhibited the reaction.

Two distinct sites for CaM-binding in parent MLCK

We split the 1–41 sequence mentioned above into Met^{1}-Gly^{25} and Pro^{26}-Pro^{41} peptides [23]. The 26–41 peptide interfered with the binding of Ca/CaM to the NN-fragment, where the IQ motif, a consensus sequence of CaM-binding protein [29], could be assigned. We identified the 26–41 sequence of MLCK as a CaM-binding region that regulates the binding of MLCK to actin [23]. Ca/CaM is also known to bind at the Ala^{796}-Ser^{815} sequence to regulate the kinase activity MLCK (see Ref. [30] for review). We synthesized Ser^{787}-Ser^{815} peptide, and examined it in the presence of Ca/CaM to determine if it interferes with MLC20 phosphorylation by MLCK and the actin-binding of the NN-fragment (Table 2). The 787–815 peptide affected MLC20 phosphorylation but not actin binding. The opposite results were obtained for the 26–41 peptide (Table 2). Thus, the site in MLCK that regulates its kinase activity must be totally different from the one that regulates its actin-binding activity (see also Fig. 8 in Ref. [23]).

Regulation of actin–myosin interaction by MLCK through its non-kinase activity

Fig. 3A depicts the detection of actin–myosin interaction with an in vitro motility assay [31]. We allowed the actin filaments to move in an ATP-dependent manner on a glass surface coated with myosin, the MLC20 of which was fully phosphorylated, and observed the velocity of the movement in the presence of various concentrations of MLCK. As indicated in Fig. 3B, the mean velocity of the movement in the absence of MLCK was 0.58 μm/s. The velocity decreased as the MLCK concentration increased (open circles), and 16 nM MLCK eliminated the movement. The effect of Ca/CaM was to retard this inhibition (solid circles). The myosin used for the assay was free from phosphatase contamination, and the MLC20 phosphorylation of the myosin was carried out

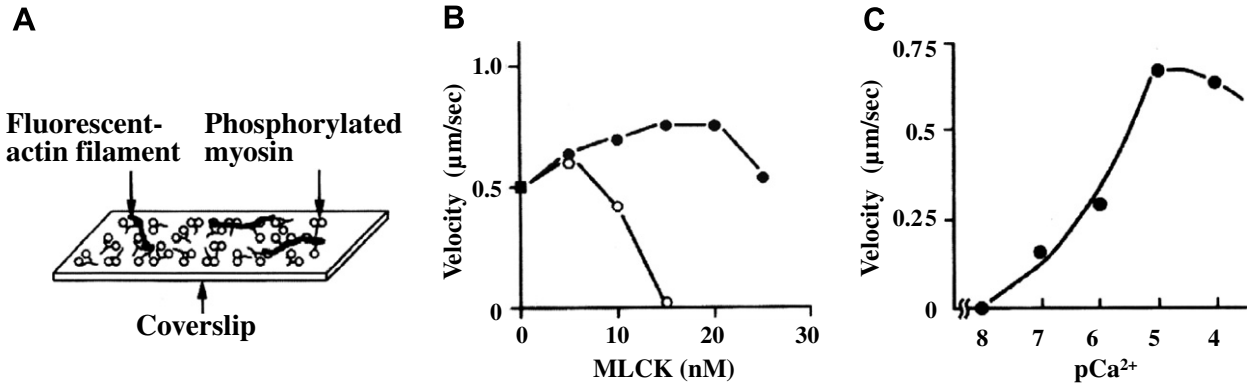

Fig. 3. Inhibitory effect of MLCK in an in vitro motility assay on a myosin-coated glass surface coated with phosphorylated myosin [31]. (A) Fluorescent actin filaments (∼, thick lines) in MgATP were placed on a coverslip coated with myosin (♀, double-headed symbols) and allowed to move. (B) The movement of actin filaments was observed under a fluorescence microscope in the presence of CaM, MLCK, and Ca^{2+} (●, solid circles) or EGTA (○, open circles). The velocity of movement was plotted against MLCK concentrations. (C) A similar assay was carried out in the presence of MLCK, CaM, and various concentrations of Ca^{2+}.

to saturation. Therefore, we concluded that a mechanism other than kinase activity regulated the actin–myosin interaction; we referred to this novel mechanism as the non-kinase activity of MLCK.

The effect of Ca^{2+} concentration on the movement is depicted in Fig. 3C. We allowed actin filaments to move on a myosin-coated surface in the presence of MLCK, CaM, and various concentrations of Ca^{2+} [31]. The Ca^{2+} concentration that enabled half-maximal velocity of the movement was in the micro molar range, supporting the physiological relevance of non-kinase activity of MLCK in terms of Ca^{2+} concentrations. The inhibitory effect of MLCK through non-kinase activity was confirmed by measuring the actin-activated ATPase activity of phosphorylated myosin [23].

Next, we sought to determine the effect of non-kinase activity on unphosphorylated myosin. Because in vitro motility assay is difficult using a glass surface coated with unphosphorylated myosin, we tested using only actin-activated myosin ATPase activity [32]. The effect was striking: the ATPase activity was increased threefold with K_m of 1–2 μM. The effect could not be attributed to MLC20 phosphorylation by the kinase activity of MLCK because neither Ca^{2+} nor CaM was included in the assay.

The activating effect of non-kinase activity was confirmed by expressing MLCK from cDNA coding bovine stomach MLCK with the use of a cold-shock expression vector (Table 1). The wild-type (WT) MLCK exhibited actin-binding and myosin-binding activities. Here, V_{max} of kinase activity of WT MLCK was comparable to V_{max} of purified MLCK, although K_m of WT MLCK was higher than K_m of purified MLCK [9]. By mutating Gly residues to Ala in the ATP-binding site of X-Gly-X-Gly-X-X-Gly-X [10], we engineered ΔATP MLCK that was devoid of kinase activity [9]. We confirmed the stimulatory effect through the non-kinase activity of MLCK by the mutant ΔATP MLCK: the mutant was able to stimulate actin-activated ATPase activity threefold with K_m of 1–2 μM (Fig. 4D).

In summary, MLCK exerts an inhibitory effect on the actin–myosin interaction when myosin is phosphorylated but exerts an activating effect when myosin remains

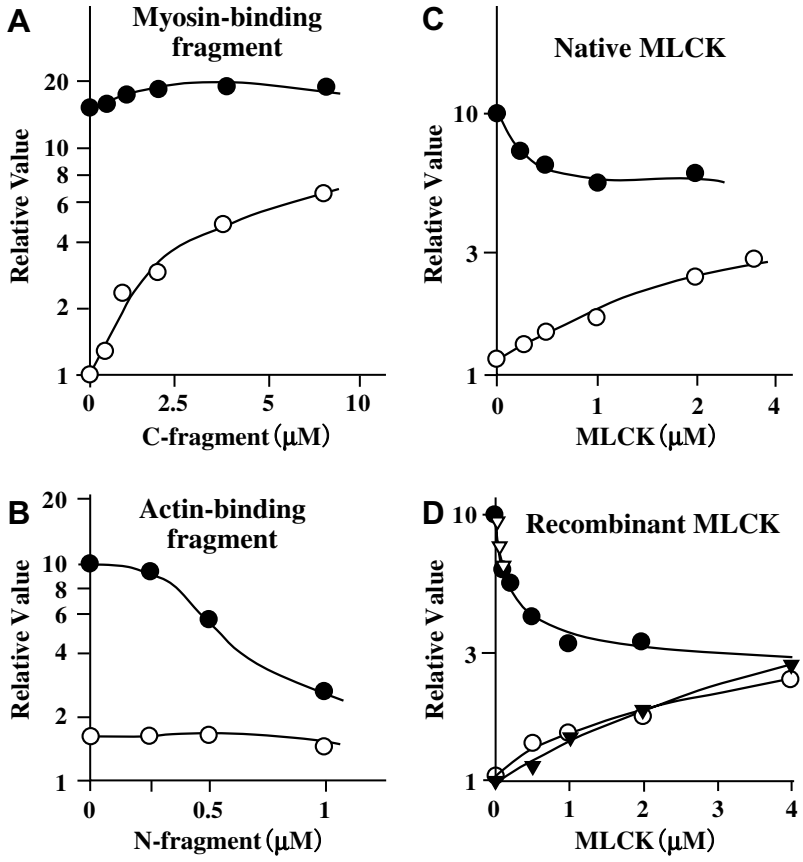

Fig. 4. Distinct effects of MLCK and its fragments according to the state of MLC20 phosphorylation [7]. The actin-activated ATPase activities of myosin in both phosphorylated (solid symbols) and unphosphorylated (open symbols) forms were measured in the presence of various concentrations of C-terminal 777–972 fragment (A), N-terminal 1–515 fragment (B), purified native MLCK of chicken gizzard (C), and the recombinant full-length MLCK (D). As indicated in Table 1, the recombinant full-length MLCK was expressed from cDNA of bovine stomach MLCK, where circles denote the WT MLCK without any mutations, and triangles, the ΔATP MLCK that was mutated to eliminate kinase activity. The relative values of the activity (ordinates) were plotted against the concentrations of the fragments and MLCK (abscissas). For the constructs of the C-fragment and N-fragment, see Figs. 2 and 5. (N.B. The numbers were modified for gizzard MLCK. When myosin is phosphorylated beforehand, the non-kinase activity of MLCK exerts inhibition through actin binding. The non-kinase activity activates the unphosphorylated myosin through myosin binding.)

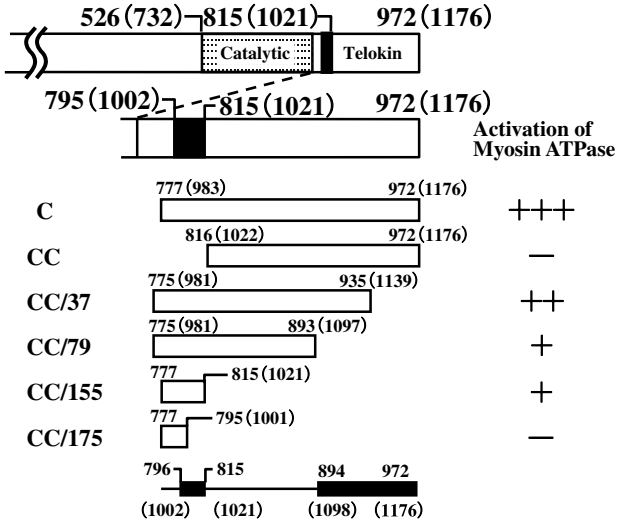

Fig. 5. Localization and effect of various C-terminal fragments of MLCK. C, CC, C/37, and C/79 were recombinant fragments. C/155 and C/175 were synthetic peptides. Although the design is based upon the amino acid sequence of bovine stomach MLCK [5], we modified the sequence numbers for chicken gizzard MLCK [30] for the convenience of readers. The numbers of bovine MLCK [5] are denoted in parentheses. The column indicates the activating effect of the fragments on the actin-activated ATPase activity of unphosphorylated myosin: + indicates fragments exhibiting activating effect; – indicates fragments exhibiting no such effect. The boxes at the bottom indicate the sequences (i.e., 796–815, 894–972) that are responsible for the activation.

unphosphorylated. As explained below, the inhibition is imposed by the N-terminal of MLCK (Figs. 2 and 4), and activation is exerted by the C-terminal of MLCK (Fig. 5).

Non-kinase, inhibitory activity of N-terminal, actin-binding domain of MLCK

We sought to determine if inhibition of the movement (Fig. 3) of actin filaments on a surface coated with phosphorylated myosin is attributable to the actin-binding sites of MLCK. If so, we also wanted to investigate whether relief of the inhibition by Ca/CaM (Fig. 3) is brought about by the Ca/CaM-sensitive actin-binding site of MLCK. To address these issues, we examined the effect of N-terminal actin-binding fragments of MLCK containing only the Ca/CaM-sensitive site (i.e., native NTCB-fragment and recombinant NN-fragment) [23]. Both fragments inhibited the movement, and the inhibition was relieved by Ca/CaM. However, the NC-fragment, which contains the Ca/CaM-insensitive site, failed to modify movement (Fig. 2). The truncated forms of the NN-fragment (i.e., the NN/41-fragment), which is devoid of the 1–41 sequence of the NN-fragment, and the NN/25-fragment, which is devoid of the 1–25 sequence of the NN-fragment, also failed to modify the movement (Fig. 2) [23]. Therefore, we concluded that the inhibitory effect of MLCK is exerted through the Ca/CaM-sensitive 1–41 sequence for actin binding, which includes the Asp-Phe-Arg-X-X-Leu motif proposed for actin binding [27]. This conclusion was confirmed by measuring the actin-activated ATPase activity of smooth muscle myosin in the phosphorylated form; the NN-fragment inhibited the activity, but the NN/41- and NN/25-fragment did not. The NC-fragment, which is composed of the Ca/CaM insensitive actin-binding site, failed to exert a regulatory role. Taken together, our data stress the active role of the 1–41 sequence of MLCK in its actin-linked inhibition (Fig. 2).

The inhibitory effect of unphosphorylated myosin was not obvious. The fragment that modifies the actin-activated ATPase activity is a C-terminal myosin-binding fragment (open circles in Fig. 4A and B). This modification is not attributable to the kinase activity of MLCK, because the myosin-binding fragment does not contain the kinase domain of MLCK (see below).

Non-kinase, activating effect of C-terminal, myosin-binding domain MLCK

MLCK is composed of an N-terminal actin-binding domain, a central kinase domain, and a C-terminal telokin domain (Fig. 1). The telokin domain is responsible for causing the MLCK to bind to myosin [22]. The region between the kinase and telokin domains has been characterized as the regulatory domain for kinase activity (Table 2). To investigate these C-terminal regions of MLCK, we expressed its 983–1176 fragment in *E. coli* as a recombinant protein using cDNA coding bovine stomach MLCK (Fig. 5). When the fragment is numbered for gizzard MLCK, it corresponds to the 777–972 fragment [30].

Because in vitro motility assay does not work with unphosphorylated myosin, we adopted the conventional assay of the actin-activated ATPase activity of myosin. The C-fragment, a construct of the telokin domain extending to the regulatory domain for the kinase activity of MLCK, exerted an activating effect on the ATPase activity (Fig. 4A). Although the effect was slight when the myosin was phosphorylated (Fig. 4A), the activation for unphosphorylated myosin was significant (i.e., $V_m = 7.36$-fold with $K_m = 1.06$ μm) [32].

We expressed a CC-fragment that was identical to the telokin domain by deleting the N-terminal portion extending to the regulatory domain from the C-fragment (Fig. 5). The CC-fragment failed to exert the activity. We also deleted 37 amino acids from the C-fragment by 37 amino acids to express C/37, and by 79 amino acids to express C/79 fragment (Fig. 5). Both fragments stimulated the activity. Using a peptide synthesizer, we additionally produced C/155, which was composed of Asp^{777}-Ser^{815} of gizzard MLCK, and C/175 fragments, which were composed of Asp^{777}-Met^{795} of gizzard MLCK. We observed that C/175 effectively stimulated the activity, but C/155 failed to do so. We concluded that both 796–815 and 893–972 sequences of gizzard MLCK (1002–1021 and 1097–1176

sequences of bovine stomach MLCK) are required for the activation.

Search for the physiological relevance of the non-kinase activity of MLCK

The concentration of MLCK required for exerting the regulatory role (i.e., K_m for an activating effect through myosin-binding, and IC_{50} for an inhibitory effect through actin-binding) is not low enough to explain a physiological role, if we adopt the cellular concentrations of MLCK, actin and myosin [20]. Therefore, it is still too early to derive the physiological significance from our in vitro data.

Several kinases have been reported to phosphorylate MLCK [33–39]. For example, protein kinase A (PKA) phosphorylates MLCK in its regulatory domain at Ser^{815} and Ser^{828} [33,40] (Ser^{1021} and Ser^{1034} if we convert to bovine stomach MLCK). The kinase activity of MLCK phosphorylated at Ser^{815} is reduced, whereas that of MLCK phosphorylated at Ser^{828} is not reduced. We found that MLCK phosphorylated at Ser^{828} inhibited the actin-activated ATPase activity of phosphorylated myosin more strongly than did the control MLCK [40]. Our preliminary data further demonstrated that MLCK phosphorylated at Ser^{828} stimulated the ATPase activity of unphosphorylated myosin more effectively than the control MLCK (i.e., with $V_{max} = 20$-fold and $K_m = 0.1$ μM) [41]. These changes in inhibiting and activating effects must be established for determining the physiological relevance of the non-kinase activity of MLCK.

Additional support for the physiological relevance was obtained by down-regulating MLCK. To block MLCK expression, GbaSM-4, a cell line from the basilar artery of guinea pigs, was infected by an adenovirus vector carrying MLCK cDNA in an anti-sense orientation. When we measured the contraction of the collagen fiber [14] after allowing it to populate the infected cells, we found that its contraction was very much reduced. However, the MLC20 phosphorylation after agonist stimulation proceeded to the same extent as the control GbaSM-4 cells, which were transfected by the vector carrying a stuffer (Fig. 4 in Ref. [42]). We suggested that kinase(s) other than MLCK phosphorylated MLC20 [42]. The replacement of the activity of MLC20 phosphorylation was also recognized in mice with MLCK knocked out [43].

Our study of the down-regulation of the 210 kDa long isoform of MLCK, however, was incomplete [42]. Down-regulation of both long and short isoforms of MLCK was carried out with GbaSM4 by another strategy: we used RNAi constructed based upon the kinase domain sequence that is shared by both MLCKs. We observed that the migratory activity toward chemoattractants was impaired, but that the MLC20 phosphorylation remained unaffected (unpublished observation). To establish the effect of down-regulation, it is necessary to conduct an experiment to express the N-terminal fragment and/or C-terminal fragment in the MLCK-down-regulated GbaSM4 cells.

Perspectives

Dr. Ebashi's last paper, published in 1998 [8], proposed that "the 155 kDa protein may have a dual function" by comparing the extent of MLC20 phosphorylation with the rate of superprecipitation, an indication that MLCK may have both kinase and non-kinase activities. Dr. Ebashi also treated the 155 kDa protein in various ways to find the discrepancy between the two activities. Our data indicates that phosphorylation of MLCK at Ser^{815} and Ser^{828} by PKA modified kinase activity and non-kinase activity, respectively [41]. These results confirm Dr. Ebashi's idea of dual function.

Based on our works [6,7,11,44,45], we have reviewed multiple ways in which MLCK exerts its regulatory role in the actin–myosin interaction (Fig. 4). When myosin is unphosphorylated, MLCK phosphorylates MLC20 in association with Ca/CaM to activate myosin ATPase activity [3]. In addition to this well-established mode of action, MLCK binds to myosin through the 796–815 sequence together with the 892–972 sequence as shown by the boxes in Fig. 5, and then activates the myosin ATPase activity. When myosin is phosphorylated beforehand, MLCK exerts an inhibitory role through the 1–41 sequence of the actin-binding domain (Figs. 3 and 4).

It must be noted that non-kinase activity is detectable not only for the actin-binding and myosin-binding domains, but also for the catalytic domain as follows [46,47]. We produced a few fragments of the catalytic domain by proteolysis of gizzard MLCK. Regardless of whether they were constitutively active or active only in Ca/CaM [48], all fragments exerted an inhibitory activity without requiring Ca/CaM to the movement of actin filaments on a glass surface coated by the phosphorylated myosin. We confirmed that the myosin could not undergo further phosphorylation. Therefore, we interpreted that the catalytic domain is able to exert the non-kinase activity to the phosphorylated myosin and proposed that the binding of a part of the catalytic domain to the head of myosin may inhibit the movement of actin filament [46,47].

Both WT MLCK and ΔATP MLCK were expressed as recombinant proteins (Table 1) and are expected to be free from post-transcriptional modifications such as phosphorylation. Therefore, testing the changes in kinase and non-kinase activities after phosphorylating MLCK by various kinases is quite intriguing. With the advances in mass spectrometry, we can determine which residues of MLCK are phosphorylated after allowing an agonist to elicit contraction of smooth muscle. Relating the site(s) phosphorylated in vitro with the site(s) phosphorylated in vivo will reveal the role of kinase and non-kinase activities in the actual contraction. For example, tonic smooth muscles develop tension as long as an agonist is present. Whereas MLC20 phosphorylation is detectable just after stimulation by the agonist, myosin in the remaining period of contraction is mostly in the unphosphorylated form. Such a contraction, which has been called a latched contraction [49],

might be induced by non-kinase activity, especially by myosin-binding activity localized in the boxes shown in Fig. 5.

The other modulation in vivo will be single nucleotide polymorphisms (SNPs) of MLCK. Our search revealed 23 SNPs [50,51]. Among them, 10 missense mutations are listed in Fig. 1. After mutating WT MLCK at the SNPs site, followed by the expression in *E. coli*, we are able to detect changes in the kinase and non-kinase activities of MLCK and relate them with symptoms of diseases. This approach may open the door to the involvement of MLCK in hereditary vascular diseases.

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