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Functional Role of the C-Terminal Domain of Smooth Muscle Myosin Light Chain Kinase on the Phosphorylation of Smooth Muscle Myosin

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Smooth muscle myosin light chain kinase (MLCK) is known to bind to thin filaments and myosin filaments. Telokin, an independently expressed protein with an identical amino acid sequence to that of the C-terminal domain of MLCK, has been shown to bind to unphosphorylated smooth muscle myosin. Thus, the functional significance of the C-terminal domain and the molecular morphology of MLCK were examined in detail. The C-terminal domain was removed from MLCK by α-chymotryptic digestion, and the activity of the digested MLCK was measured using myosin or the isolated 20-kDa light chain (LC20) as a substrate. The results showed that the digestion increased K_m for myosin 3-fold whereas it did not change the value for LC20. In addition, telokin inhibited the phosphorylation of myosin by MLCK by increasing K_m but only slightly increased K_m for LC20. Electron microscopy indicated that MLCK was an elongated molecule but was flexible so as to form folded conformations. MLCK was crosslinked to unphosphorylated heavy meromyosin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the absence of Ca^{2+}/calmodulin (CaM), and electron microscopic observation of the products revealed that the MLCK molecule bound to the head–tail junction of heavy meromyosin. These results suggest that MLCK binds to the head–tail junction of unphosphorylated myosin through its C-terminal domain, where LC20 can be promptly phosphorylated through its catalytic domain following the Ca^{2+}/CaM-dependent activation.

Key words: chicken gizzard, smooth muscle, smooth muscle myosin, smooth muscle myosin light chain kinase, telokin.

Smooth muscle myosin light chain kinase (MLCK) is a Ca^{2+}/calmodulin (CaM)-dependent protein kinase that phosphorylates the 20-kDa regulatory light chain (LC20) of smooth muscle myosin in response to an increase in the intracellular Ca^{2+} concentration. This phosphorylation initiates the contraction of smooth muscle through a marked increase in the actin-activated ATPase activity of myosin, thus activating its actin-translocating activity, while dephosphorylation induces relaxation of the muscle through the reverse of these activities to the basal levels (*1*). The phosphorylation-dependent regulation mechanism is also involved in the non-muscle actomyosin system (*2*). MLCK is thus one of the most important proteins to be characterized in detail as to structure and function in the smooth and non-muscle actomyosin system.

The domain organization of smooth muscle MLCK has been characterized based on the primary structure deduced from cDNA clones (*3*). Smooth muscle MLCK (the following residue numbers are for chicken gizzard MLCK) is composed of (i) an actin/thin filament-binding domain (residues 1–114), (ii) two immunoglobulin C-2–related domains (residues 147–240 and 288–381), and a fibronectin type III–related domain (residues 382–481), (iii) a catalytic domain (residues 526–762), (iv) a regulatory domain containing autoinhibitory and CaM-binding regions (residues 787–813), and (v) a C-terminal (telokin) domain (residues 816–972) containing a third immunoglobulin C-2–related domain (residues 857–951) (*4*). Immunofluorescence studies have shown that MLCK is co-localized with actin filaments and myosin-containing structures (*5*, *6*). Sellers and Pato (*7*) demonstrated that MLCK binds to actin filaments and unphosphorylated myosin filaments *in vitro*. MLCK was then shown to bind to actin filaments with a site located within its N-terminal 114 residues (*8*). The actin-binding site is further suggested to be restricted to within the N-terminal 41 residues (*9*, *10*), and the binding of actin with its site is reported to be inhibited by Ca^{2+}/CaM (*9*). The binding affinity of MLCK for actin, however, is low relative to the apparent high-affinity binding of MLCK to smooth muscle myofilaments and thin filaments (*11–13*). Recently, an N-terminal 75-residue structural motif, which comprises three tandem repeats of 25 residues containing a consensus sequence of DFRXXL, has been found to be responsible for the high-affinity binding of MLCK to myofilaments (*14*).

Although great progress has thus been made in characterizing the actin/thin filament-binding properties of MLCK, little is known about the binding to myosin. The C-terminal domain of MLCK is expressed as an independent protein, telokin (also called kinase-related protein or KRP), in smooth muscle tissues through the activity of a smooth muscle cell–specific second promoter located in an intron of

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Abbreviations: CaM, calmodulin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HMM, heavy meromyosin; LC20, 20-kDa regulatory light chain; MLCK, myosin light chain kinase.

the MLCK gene (*15–19*). Telokin has been shown to bind to the head–tail junction of unphosphorylated myosin and to stabilize myosin filaments under physiological conditions (*20, 21*). Telokin also inhibits the phosphorylation of heavy meromyosin (HMM) by MLCK (*20, 22, 23*). It has also been observed that a recombinant MLCK lacking the C-terminal domain (telokin domain) does not bind unphosphorylated myosin (*20*).

In this study, we examined the effects of proteolytic removal of the C-terminal domain of MLCK on the phosphorylation of myosin and the isolated LC20 in detail. We also examined the molecular morphology of MLCK and MLCK-myosin complex by means of electron microscopy. Based on the results, we proposed a structure of MLCK tethering unphosphorylated myosin filaments to the thin filaments in the resting state of smooth muscle, and discussed the possible regulatory role of telokin in smooth muscle contraction.

MATERIALS AND METHODS

Proteins—MLCK and CaM were prepared from chicken gizzard as described (*24–26*). Myosin was prepared from chicken gizzard as described by Yoshida and Yagi (*27*). The myosin preparation was almost completely unphosphorylated, as judged on urea-gel electrophoresis. HMM was prepared by digestion of chicken gizzard myosin with *Staphylococcus aureus* V8 protease according to the method of Ikebe and Hartshorne (*28*), and purified by gel filtration HPLC. Unphosphorylated LC20 was prepared from gizzard myosin (*27*) and purified by DE32 (Whatman) chromatography in the presence of 8 M urea (*29–31*). Telokin was prepared from chicken gizzard by the method of Ito *et al.* (*15*). Rabbit anti-telokin IgG was prepared and affinity-purified as described previously (*21*).

The concentrations of myosin, LC20, CaM, and telokin were determined from the absorbance at 280 nm using absorption coefficients of 0.48, 0.20, 0.21, and 0.78 $(mg/ml)^{-1}$ cm^{-1}, respectively. The concentrations of MLCK and HMM were determined by the biuret method using bovine serum albumin as a standard (*32*). The molecular weights used for MLCK, CaM, myosin, HMM, LC20, and telokin were 110,000, 17,000, 500,000, 340,000, 20,000, and 17,000, respectively.

Western Blot Analysis—Proteins separated on an SDS–polyacrylamide gel were transblotted onto a nitrocellulose membrane in a solution containing 10% methanol and 20 mM Tris-glycine (pH 8.3). The membrane was blocked with 3% gelatin, and reacted with rabbit anti-telokin IgG and then goat anti–rabbit IgG antibodies conjugated with horseradish peroxidase. Proteins bound with the anti-telokin IgG were detected by means of the peroxidase reaction using 4-chloro-1-naphthol as a color substrate.

Chymotryptic Digestion of MLCK—The digestion of MLCK was carried out using α-chymotrypsin (1:1,000, w/w) for 5–30 min at 25°C in 0.1 M NaCl, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). The digestion was initiated by the addition of α-chymotrypsin to MLCK (0.2 mg/ml) preincubated for 5 min at 25°C, and terminated with 1 mM diisopropyl fluorophosphate.

Amino Acid Sequence Analysis—Protein bands separated on an SDS–polyacrylamide gel were transblotted onto a polyvinylidene difluoride membrane. The protein bands transferred to the membrane were stained with Coomassie Brilliant Blue G-250 and excised, followed by application to an Applied Biosystems 492 protein sequencer at the Center for Instrumental Analysis of Hokkaido University.

Measurement of MLCK Activity—The phosphorylation reaction of MLCK was carried out using 1 nM MLCK and 60 nM CaM in a reaction mixture containing 4–20 μM myosin or 5–50 μM LC20 as a substrate, 0–120 μM telokin, 0.1 M NaCl, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 1 mM DTT, and 20 mM imidazole (pH 7.0). The MLCK activity was measured according to the method of Corbin and Reimann (*33*). If necessary, MLCK and CaM were diluted with a solution containing 2 mg/ml BSA, 1 mM DTT, and 20 mM imidazole (pH 7.0).

Cross-Linking of MLCK to HMM—MLCK (3.8 μM) and HMM (1.0 μM) were incubated for 15 min at 25°C with 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 15 mM imidazole (pH 7.0). The cross-linking reaction was terminated by the addition of 10 mM DTT. The reaction mixture was subjected to gel filtration HPLC to remove uncross-linked MLCK.

Gel Electrophoresis—SDS-PAGE was carried out on a slab gel composed of a 4% stacking gel and a 12.5% separating gel, according to the method of Laemmli (*34*). The gel was stained with Coomassie Brilliant Blue G-250.

Gel Filtration HPLC—Gel filtration HPLC was performed at room temperature on a TSKgel G5000PW_{XL} column (7.8 × 300 mm) with a TSK guard column PW_{XL} (6.0 × 40 mm) attached to a JASCO Gulliver series HPLC system. Elution of proteins was performed at 0.5 ml/min with 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM imidazole (pH 7.0), and monitored as to the absorbance at 280 nm.

Electron Microscopy—Protein samples were diluted to ~5 μg/ml with 70% glycerol and 0.4 M ammonium acetate, sprayed onto freshly cleaved mica, rotary-shadowed with platinum, and then observed under a Hitachi H-800 electron microscope operated at 75 kV (*35*).

RESULTS

Digestion of MLCK by α-Chymotrypsin—In order to examine the functional role of the C-terminal domain of smooth muscle MLCK, MLCK was digested with α-chymotrypsin to remove its C-terminal domain (Fig. 1). The digestion of MLCK produced polypeptides of apparent molecular masses of 95 and 24 kDa, as reported previously (*36*). The 24-kDa peptide exhibited similar mobility to telokin and was identified as the C-terminal domain of MLCK by immunoblotting with anti-telokin antibodies (data not shown). The activity of MLCK digested for 30 min was still completely Ca^{2+}/CaM dependent, like that of the native enzyme, indicating that the 95-kDa fragment contained intact autoinhibitory and calmodulin-binding domains (*37–40*). In the early stage of the digestion, bands with mobilities lower than that of the 95-kDa band were observed (Fig. 1), suggesting proteolysis at the N-terminal region of MLCK. Then, the 95-kDa material was electroblotted onto a polyvinylidene difluoride membrane and sequenced. The sequence of the N-terminal 20 residues was GAKKKP-PAENGSASTPAPNA, which is identical to that of residues 64–83 of chicken gizzard MLCK. These results indicated

that the 95-kDa fragment retained neither the N-terminal 63 residues involved in an actin/thin filament-binding domain (*8–10*, *13*, *14*) nor the C-terminal domain corresponding to the telokin sequence of MLCK.

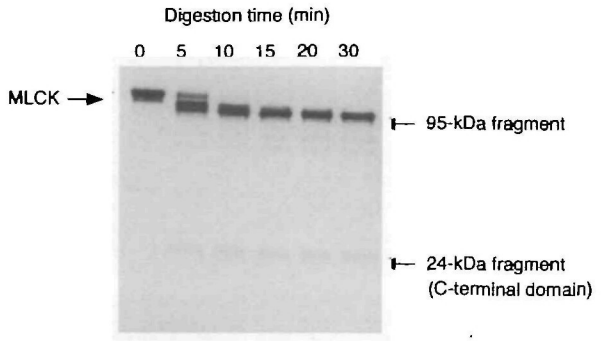

Fig. 1. **Chymotryptic digestion of MLCK.** MLCK (0.2 mg/ml) was digested at 25°C for 0–30 min with α-chymotrypsin (1:1,000, w/w) in 0.1 M NaCl, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). Aliquots were taken at the indicated times and subjected to SDS-PAGE, and then the proteins were stained with Coomassie Brilliant Blue G-250. A 24-kDa fragment generated on the digestion was identified as the C-terminal domain of MLCK by Western blotting with anti-telokin antibodies.

Effect of Digestion of MLCK on Its Kinase Activity—The effect of proteolysis of MLCK on the phosphorylation of the isolated LC20 or myosin was examined. The rate of phosphorylation of LC20 decreased with an increase in the digestion time and reached ~60% of the original level in 30 min (Fig. 2A). The phosphorylation rates were measured with various concentrations of LC20, and the results proved that the decrease in the phosphorylation rate was mainly due to a decrease in V_m (Fig. 2B). The K_m for LC20 was essentially unaffected by the digestion. On the other hand, the rate of phosphorylation of myosin decreased to less than 40% of the original level with an increase in the digestion time (Fig. 3A). The phosphorylation rates were measured with various myosin concentrations, and the results showed that the decrease was due to a marked increase in K_m and a small decrease in V_m (Fig. 3B). The increase in K_m may be due to the loss of the C-terminal domain since telokin, whose sequence is identical to that of the C-terminal domain of MLCK (*15–17*), binds to unphosphorylated myosin (*20*, *21*).

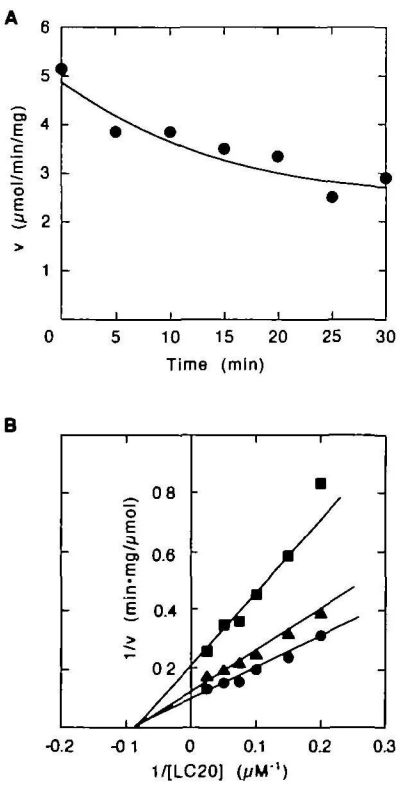

Fig. 2. **Effect of digestion of MLCK on the phosphorylation of the isolated LC20.** Digestion of MLCK was carried out as described in the legend to Fig. 1 and was terminated with 1 mM diisopropyl fluorophosphate. The rate of phosphorylation of LC20 by MLCK was measured at 25°C with 1.0 nM digested MLCK, 60 nM calmodulin, and 8 μM LC20 (A) or 5–40 μM LC20 (B) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 1 mM DTT, and 20 mM imidazole (pH 7.0). A: The rate of phosphorylation by MLCK was plotted against the digestion time. B: The rates of phosphorylation by MLCK digested for 0 (●), 5 (▲), and 30 min (■) were plotted against the LC20 concentration. The apparent V_m and K_m values obtained for these digested MLCKs are 10 μmol/min/mg and 12 μM, 8 μmol/min/mg and 12 μM, 5 μmol/min/mg and 12 μM, respectively.

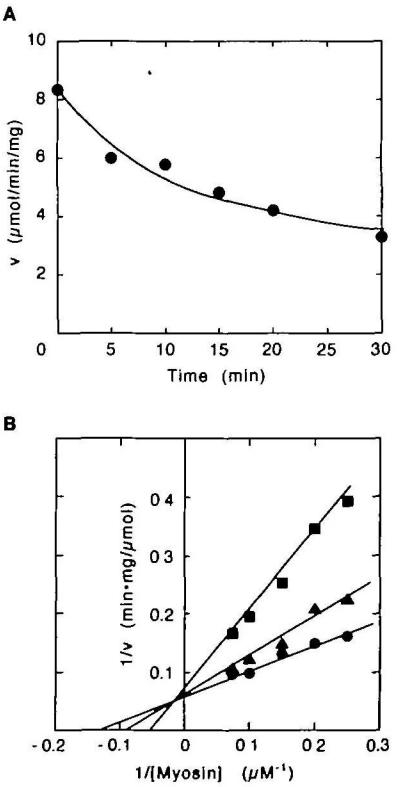

Fig. 3. **Effect of digestion of MLCK on the phosphorylation of myosin.** Digestion of MLCK was carried out as described in the legend to Fig. 1 and was terminated with 1 mM diisopropyl fluorophosphate. The rate of phosphorylation of myosin by MLCK was measured at 25°C with 1.0 nM digested MLCK, 60 nM calmodulin, and 4 μM myosin (A) or 4–14 μM myosin (B) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 1 mM DTT, and 20 mM imidazole (pH 7.0). A: The rate of phosphorylation by MLCK was plotted against the digestion time. B: The rates of phosphorylation by MLCK digested for 0 (●), 5 (▲), and 30 min (■) were plotted against the myosin concentration. The apparent V_m and K_m values obtained for these digested MLCKs are 19 μmol/min/mg and 7 μM, 16 μmol/min/mg and 11 μM, 15 μmol/min/mg and 22 μM, respectively.

The Effect of Telokin on MLCK Activity—The effect of telokin on the phosphorylation of myosin by MLCK was then examined to clarify whether or not the C-terminal domain was responsible for the binding of MLCK to myosin. The rate of phosphorylation of native MLCK decreased with an increase in the concentration of telokin and reached ~30% of the original level with 120 μM telokin (Fig. 4A). In contrast, the rate of phosphorylation of the digested MLCK was originally as low as that of the native enzyme with 120 μM telokin, and decreased only slightly on the addition of telokin. The phosphorylation rates with various concentrations of myosin in the presence and absence of telokin showed that telokin decreased the apparent affinity of MLCK for myosin (Fig. 4B). The inhibition by telokin was not observed for the phosphorylation of isolated LC20 (data not shown). These results suggest that MLCK binds to myosin through its C-terminal domain and phosphorylates LC20, but not to the isolated LC20.

Morphology of MLCK Molecules Revealed by Electron Microscopy—The molecular morphology of MLCK under high salt conditions was examined by electron microscopy

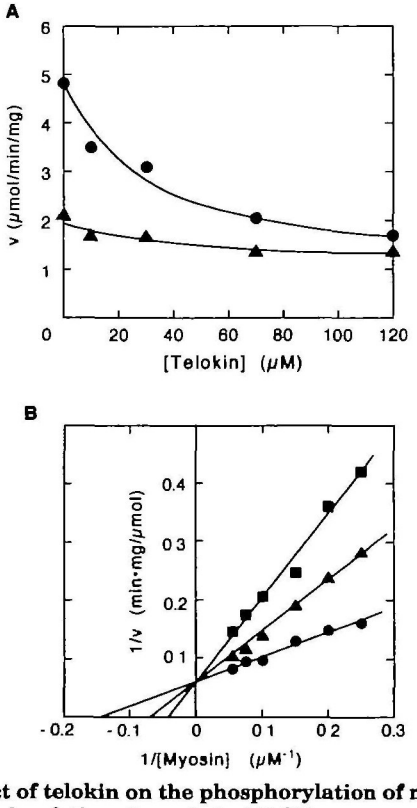

Fig. 4. **Effect of telokin on the phosphorylation of myosin.** The rate of phosphorylation of myosin by MLCK was measured at 25°C with 1.0 nM MLCK, 60 nM calmodulin, and 4 μM myosin and 0–120 μM telokin (A) or 4–18 μM myosin, and 0, 20, or 50 μM telokin (B) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 1 mM DTT, and 20 mM imidazole (pH 7.0). A: The rate of phosphorylation by MLCK digested for 0 (●) and 30 min (▲) was plotted against the concentration of telokin. B: The rates of phosphorylation by MLCK in the presence of 0 (●), 20 (▲), and 50 μM (■) telokin were plotted against the myosin concentration. The apparent V_m and K_m values obtained for MLCK with these concentrations of telokin are 16 μmol/min/mg and 7 μM, 16 μmol/min/mg and 14 μM, and 16 μmol/min/mg and 24 μM, respectively, and the apparent inhibition constant (K_i) of telokin obtained from the K_m values is 23 μM.

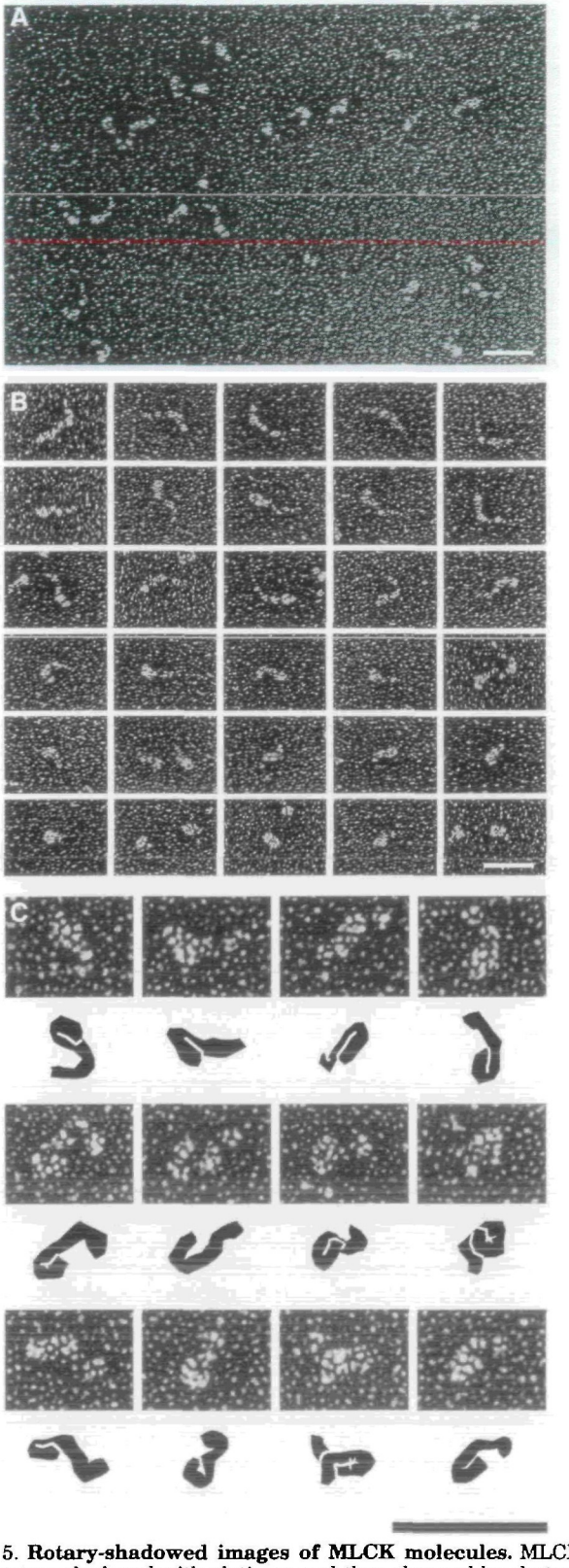

Fig. 5. **Rotary-shadowed images of MLCK molecules.** MLCK was rotary-shadowed with platinum and then observed by electron microscopy. A: Field of MLCK molecules. B: Selected images of MLCK molecules in various conformations. Molecules in the extended to folded conformations are shown from top to bottom, respectively. C: Selected images, at a higher magnification, of MLCK molecules in the various folded conformations. A structure judged as most probable by eye is drawn under each electron micrograph. The scale bars represent 50 nm.

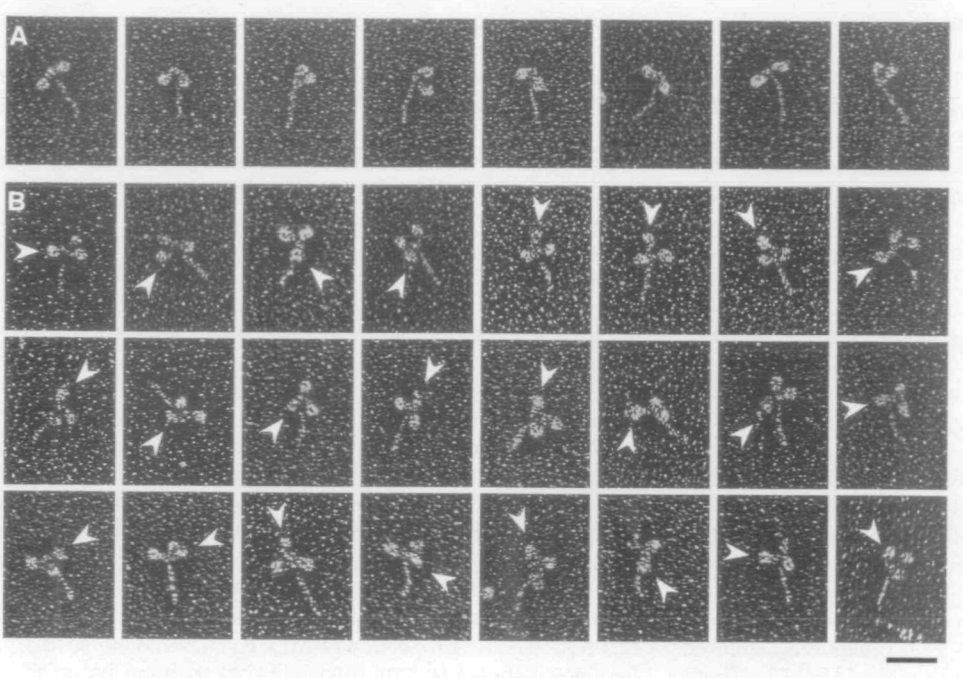

Fig. 6. **Visualization of MLCK binding to HMM.** Complexes of MLCK and unphosphorylated HMM were cross-linked with a zero-length cross-linker, EDC, rotary-shadowed with platinum, and then observed by electron microscopy. A: Uncross-linked unphosphorylated HMM. B: MLCK-unphosphorylated HMM complex. Arrowheads indicate structures presumed to be MLCK. The scale bar represents 50 nm.

(Fig. 5). Rotary-shadowed images of MLCK showed the divergent molecular morphology of this enzyme. Some were elongated rod-like images of ~40 nm long, some globular images of 10–20 nm in diameter, and others images composed of globular head-like and thin tail-like parts of intermediate sizes (Fig. 5, A and B). Examination of these molecules under higher magnification, however, suggested that the MLCK molecule had an elongated but flexible rod-like structure (Fig. 5C). Some molecules appeared to comprise several domain-like small globular parts, which might be connected by flexible joints. Thus the molecules bend or fold from either end to form various conformations, as described above. The unitary elongated structure of the MLCK molecules was determined to be 41 ± 4 nm long and 5 ± 1 nm wide (n = 101). Images of the chymotrypsin-digested MLCK appeared to be similar to those of the native enzyme except for the length of 36 ± 3 nm (n = 149).

Visualization of the Binding of MLCK to HMM—To determine if MLCK binds to the head–tail junction of myosin like telokin does (*21*), MLCK was cross-linked to unphosphorylated HMM with a zero-length cross-linker, EDC. MLCK was mixed with unphosphorylated HMM in 50 mM NaCl in the absence of Ca^{2+}/CaM and ATP to avoid the binding of MLCK to HMM through its active site, and then the mixture was treated with EDC. After uncross-linked MLCK was removed by gel filtration HPLC, the cross-linked sample was examined by electron microscopy (Fig. 6). An extra structure presumed to be MLCK was observed at the head–tail junction of HMM in the cross-linked sample (Fig. 6B). Such a structure was not observed when uncross-linked HMM was examined (Fig. 6A) or when the chymotrypsin-digested MLCK was used for cross-linking instead of the native MLCK (data not shown). These results suggest that MLCK binds to the head–tail junction of HMM through its C-terminal domain. Most MLCK bound to HMM had a globular appearance, which might be due to the folding of the MLCK molecule, as described above. When the fraction of uncross-linked MLCK separated by gel filtration HPLC was examined by electron microscopy, most of the molecules showed globular images similar to those cross-linked to HMM, indicative of intramolecular cross-linking of MLCK in the folded conformation (data not shown).

DISCUSSION

In this study we examined the functional and structural significance of the C-terminal (telokin) domain of smooth muscle MLCK by means of proteolytic removal of this domain. The C-terminal domain was removed by digestion with α-chymotrypsin (Fig. 1), as previously reported (*36*). The generated MLCK fragment lacking the C-terminal domain (a 95-kDa peptide) was proved to have further lost the N-terminal 63 residues. On the phosphorylation of isolated LC20, the digestion induced a considerable decrease in V_m but did not affect the K_m value (Fig. 2). Similar results have been reported for truncated recombinant MLCKs using a peptide corresponding to residues 11–24 of LC20 or the isolated LC20 as a substrate (*11*, *39*). On the phosphorylation of myosin, the digestion induced a marked increase in K_m in addition to a small decrease in V_m (Fig. 3). The phosphorylation of myosin by intact MLCK was inhibited by telokin due to competitive binding of MLCK and telokin to a myosin site (apparent K_i = 23 μM) (Fig. 4). In contrast, the rate of phosphorylation by the digested MLCK was at the same level as that with the intact enzyme in the presence of 120 μM telokin, and showed no further decrease on the addition of telokin (Fig. 4A). These results indicate that MLCK binds to myosin through its C-terminal domain but does not bind to the isolated LC20. No inhibition by telokin was actually observed for the phosphorylation by intact MLCK of the isolated LC20 or myosin subfragment 1, a single head fragment of myosin (data not shown), in agreement with the previous results (*20–23*). On the other hand, the decrease in V_m induced by the digestion

suggests interaction of the catalytic domain with the N-terminal segment or the C-terminal domain in the MLCK molecule. The N-terminal 63-residue segment is involved in a region responsible for the binding to actin/thin filaments (*8–10*, *13*, *14*) and may be spatially apart from the catalytic domain, since two immunoglobulin-related domains and a fibronectin-related one are in between the N-terminal segment and the catalytic domain (*3*, *4*, *13*). In contrast, the C-terminal domain is in close proximity to the catalytic domain: only an autoinhibitory/CaM-binding sequence is between them. Thus the C-terminal domain is rather likely to be responsible for the interaction with the catalytic domain. These results thus suggest that MLCK binds to myosin through its C-terminal domain, which may also interact with the catalytic domain, and phosphorylates LC20 in the myosin without dissociation.

The MLCK molecules observed on electron microscopy appeared to be morphologically divergent (Fig. 5). However, the divergence may be due to the flexible nature of the unitary elongated structure (41 nm in length and 5 nm in width) of MLCK molecules. The elongated molecules of MLCK often appeared to be composed of 5–6 globular domains, some of which might be immunoglobulin-related domains and a fibronectin-related domain (*3*, *4*, *13*). These domain-like structures are possibly connected with flexible joints, so that the molecules would be flexible enough to fold into more globular conformations (Fig. 5C). Cross and Sobieszek (*41*) previously presented rotary-shadowed images of pig stomach MLCK, the images being only globular ones similar to those described above. This might be due to the low salt-conditioned sample they used for rotary shadowing. They also estimated the molecular weight of MLCK to be 160,000 by gel filtration using globular proteins for calibration, but this large value rather fits our elongated images of MLCK. Recently, the length for rabbit uterus smooth muscle MLCK was estimated to be 54 nm, based on sedimentation velocity data (*12*, *42*). Since a 66-residue segment comprising residues 76–141 in chicken gizzard MLCK is replaced by a 245-residue segment in rabbit uterus MLCK (*4*, *43*), the length may be compatible with our measured value for gizzard enzyme.

In MLCK molecules, each end of the rod-like structure often appeared to bend back toward the other end, having a globular head-like appearance. If one end of the molecule bends back and the other end is extended, the molecule appears to have the structure of a globular head with a tail. Such images were often observed actually, and thus one might think that the structure of smooth muscle MLCK is consistent with a suggested structure of skeletal muscle MLCK composed of a C-terminal head with a N-terminal tail (*44*, *45*). However, in smooth muscle MLCK, the tail-like part does not always seem to be N-terminal and the head-like part does not always contain the C-terminus, as mentioned above. When the N-terminus of the molecule was mapped with an anti-MLCK monoclonal antibody (Sigma, #M7905), which binds to a region within residues 64–129 of chicken gizzard MLCK, the end opposite to the mapped end did not always appear to be the head-like globular part (data not shown). In detail, the modeled structure of rabbit skeletal MLCK has a total length of ~20 nm, with a ~2-nm wide head and a ~1-nm wide tail. It may be difficult to distinguish such a difference in the widths of the tail and head domains in rotary-shadowed images. Rotary-shadowed images are generally 1–2 nm thicker than the original structure because of the shadowed metal layer. Thus the proposed structure for skeletal MLCK may be compatible with the structure revealed by electron microscopy (Fig. 5).

The binding of MLCK to the head–tail junction of myosin was demonstrated by electron microscopy of the cross-linked complex of MLCK with HMM (Fig. 6). The site of myosin is that reported for telokin-binding (*20*, *21*), in agreement with the kinetic results (Fig. 4). The MLCK bound to HMM had a rather globular appearance, probably because of intramolecular cross-linking(s), indicative of possible folding of MLCK molecules in the bound state.

MLCK has been shown to be localized in actomyosin-containing filaments in smooth muscle and non-muscle cells (*5*, *6*, *11*, *12*), and to bind tightly to thin filaments through its N-terminal domain (*13*, *14*). In smooth muscle, the surface-to-surface distance from thin to thick filaments has been estimated to be ~15 nm (*46*). We showed that chicken gizzard MLCK is a flexible rod-like molecule of ~40 nm long and binds to the head–tail junction of unphosphorylated myosin through its C-terminal domain. In light of these molecular properties of MLCK, the enzyme seems to be able to phosphorylate myosin LC20 without dissociation from thin and thick filaments in smooth muscle cells. Smooth muscle MLCK molecules, which are anchored on thin filaments through their N-terminal domains, may bind to unphosphorylated myosin and tether thick filaments near thin filaments. Thus the enzyme is ready for the phosphorylation of LC20 in myosin in response to the Ca^{2+}/CaM signal. It has actually been shown that MLCK can phosphorylate LC20 in myosin without dissociation from the stress fibers in smooth muscle cells (*12*). When myosin was phosphorylated, MLCK may be dissociated from thick filaments, leading to the direct interaction of thick filaments with thin filaments for initiation of the contraction of smooth muscle.

When the intracellular concentration of Ca^{2+} returns to its resting level, the corresponding dephosphorylation of thick filaments leads to the re-binding of MLCK to the thick filaments. The slow relaxation of tonic muscles might be due to the re-binding of MLCK whose N-terminus has been anchored on thin filaments. In contrast, phasic muscles contain abundant telokin (*16*, *18*), which might inhibit the re-binding of MLCK, leading to the rapid relaxation of phasic muscles. Unlike the inhibitory action for MLCK-binding to myosin, telokin was recently suggested to enhance myosin light chain phosphatase activity (*47*). The enhanced phosphatase activity may also induce rapid relaxation. However, either effect of telokin must be canceled when muscles are activated for contraction, thus the potential functioning of telokin in the contraction-relaxation cycle needs further regulatory factors, which remains to be investigated.

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REFERENCES

1. Kamm, K.E. and Stull, J.T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* **25**, 593–620
2. Tan, J.L., Ravid, S., and Spudich, J.A. (1992) Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* **61**, 721–759
3. Gallagher, P.J., Herring, B.P., and Stull, J.T. (1997) Myosin light chain kinases. *J. Muscle Res. Cell Motil.* **18**, 1–16
4. Olson, N.J., Pearson, R.B., Needleman, D.S., Hurwitz, M.Y., Kemp, B.E., and Means, A.R. (1990) Regulatory and structural motifs of chicken gizzard myosin light chain kinase. *Proc. Natl. Acad. Sci. USA* **87**, 2284–2288
5. de Lanerolle, P., Adelstein, R.S., Feramisco, J.R., and Burridge, K. (1981) Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in nonmuscle cells. *Proc. Natl. Acad. Sci. USA* **78**, 4738–4742
6. Guerriero, V., Jr., Rowley, D.R., and Means, A.R. (1981) Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. *Cell* **27**, 449–458
7. Sellers, J.R. and Pato, M.D. (1984) The binding of smooth muscle myosin light chain kinase and phosphatases to actin and myosin. *J. Biol. Chem.* **259**, 7740–7746
8. Kanoh, S., Ito, M., Niwa, E., Kawano, Y., and Hartshorne, D.J. (1993) Actin-binding peptide from smooth muscle myosin light chain kinase. *Biochemistry* **32**, 8902–8907
9. Ye, L.-H., Hayakawa, K., Kishi, H., Imamura, M., Nakamura, A., Okagaki, T., Takagi, T., Iwata, A., Tanaka, T., and Kohama, K. (1997) The structure and function of the actin-binding domain of myosin light chain kinase of smooth muscle. *J. Biol. Chem.* **272**, 32182–32189
10. Gallagher, P.J. and Stull, J.T. (1997) Localization of an actin binding domain in smooth muscle myosin light chain kinase. *Mol. Cell. Biochem.* **173**, 51–57
11. Lin, P., Luby-Phelps, K., and Stull, J.T. (1997) Binding of myosin light chain kinase to cellular actin-myosin filaments. *J. Biol. Chem.* **272**, 7412–7420
12. Lin, P., Luby-Phelps, K., and Stull, J.T. (1999) Properties of filament-bound myosin light chain kinase. *J. Biol. Chem.* **274**, 5987–5994
13. Stull, J.T., Lin, P.J., Krueger, J.K., Trewhella, J., and Zhi, G. (1998) Myosin light chain kinase: functional domains and structural motifs. *Acta Physiol. Scand.* **164**, 471–482
14. Smith, L., Su, X., Lin, P., Zhi, G., and Stull, J.T. (1999) Identification of a novel actin binding motif in smooth muscle myosin light chain kinase. *J. Biol. Chem.* **274**, 29433–29438
15. Ito, M., Dabrowska, R., Guerriero, V., Jr., and Hartshorne, D.J. (1989) Identification in turkey gizzard of an acidic protein related to the C-terminal portion of smooth muscle myosin light chain kinase. *J. Biol. Chem.* **264**, 13971–13974
16. Gallagher, P.J. and Herring, B.P. (1991) The carboxyl terminus of the smooth muscle myosin light chain kinase is expressed as an independent protein, telokin. *J. Biol. Chem.* **266**, 23945–23952
17. Collinge, M., Matrisian, P.E., Zimmer, W.E., Shattuck, R.L., Lukas, T.J., Van Eldik, L.J., and Watterson, D.M. (1992) Structure and expression of a calcium-binding protein gene contained within a calmodulin-regulated protein kinase gene. *Mol. Cell. Biol.* **12**, 2359–2371
18. Herring, B.P. and Smith, A.F. (1996) Telokin expression is mediated by a smooth muscle cell-specific promoter. *Am. J. Physiol.* **270**, C1656–C1665
19. Birukov, K.G., Schavocky, J.P., Shirinsky, V.P., Chibalina, M.V., Van Eldik, L.J., and Watterson, D.M. (1998) Organization of the genetic locus for chicken myosin light chain kinase is complex: multiple proteins are encoded and exhibit differential expression and localization. *J. Cell. Biochem.* **70**, 402–413
20. Shirinsky, V.P., Vorotnikov, A.V., Birukov, K.G., Nanaev, A.K., Collinge, M., Lukas, T.J., Sellers, J.R., and Watterson, D.M. (1993) A kinase-related protein stabilizes unphosphorylated smooth muscle myosin minifilaments in the presence of ATP. *J. Biol. Chem.* **268**, 16578–16583
21. Masato, T., Numata, T., Katoh, T., Morita, F., and Yazawa, M. (1997) Crosslinking of telokin to chicken gizzard smooth muscle myosin. *J. Biochem.* **121**, 225–230
22. Silver, D.L., Vorotnikov, A.V., Watterson, D.M., Shirinsky, V.P., and Sellers, J.R. (1997) Sites of interaction between kinase-related protein and smooth muscle myosin. *J. Biol. Chem.* **272**, 25353–25359
23. Nieznanski, K. and Sobieszek, A. (1997) Telokin (kinase-related protein) modulates the oligomeric state of smooth muscle myosin light-chain kinase and its interaction with myosin filaments. *Biochem. J.* **322**, 65–71
24. Yazawa, M., Sakuma, M., and Yagi, K. (1980) Calmodulins from muscles of marine invertebrates, scallop and sea anemone. *J. Biochem.* **87**, 1313–1320
25. Yoshida, M., Minowa, O., and Yagi, K. (1983) Divalent cation binding to wheat germ calmodulin. *J. Biochem.* **94**, 1925–1933
26. Yoshida, M. and Yagi, K. (1986) Purification and characterization of a phosphoprotein phosphatase that dephosphorylates myosin and the isolated light chain from chicken gizzard smooth muscle. *J. Biochem.* **99**, 1027–1036
27. Yoshida, M. and Yagi, K. (1988) Two kinds of myosin phosphatases with different enzymatic properties from fresh chicken gizzard smooth muscle. Purification and characterization. *J. Biochem.* **103**, 380–385
28. Ikebe, M. and Hartshorne, D.J. (1985) Proteolysis of smooth muscle myosin by *Staphylococcus aureus* protease: Preparation of heavy meromyosin and subfragment 1 with intact 20000-dalton light chains. *Biochemistry* **24**, 2380–2387
29. Kondo, S. and Morita, F. (1981) Smooth muscle of scallop adductor contains at least two kinds of myosin. *J. Biochem.* **90**, 673–681
30. Minowa, O., Matsuda, S., and Yagi, K. (1983) Ca^{2+}-induced conformational changes of 20,000 dalton light chain of vertebrate striated muscle myosins. *J. Biochem.* **94**, 25–35
31. Hasegawa, Y., Ueno, H., Horie, K., and Morita, F. (1988) Two isoforms of 17-kDa essential light chain of aorta media smooth muscle myosin. *J. Biochem.* **103**, 15–18
32. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751–766
33. Corbin, J.D. and Reimann, E.M. (1974) Assay of cyclic AMP-dependent protein kinases. *Methods Enzymol.* **38**, 287–290
34. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
35. Katoh, T. and Morita, F. (1996) Roles of light chains in the activity and conformation of smooth muscle myosin. *J. Biol. Chem.* **271**, 9992–9996
36. Foyt, H.L., Guerriero, V., Jr., and Means, A.R. (1985) Functional domains of chicken gizzard myosin light chain kinase. *J. Biol. Chem.* **260**, 7765–7774
37. Pearson, R.B., Wettenhall, R.E.H., Means, A.R., Hartshorne, D.J., and Kemp, B.E. (1988) Autoregulation of enzymes by pseudosubstrate prototopes: myosin light chain kinase. *Science* **241**, 970–973
38. Ikebe, M., Stepinska, M., Kemp, B.E., Means, A.R., and Hartshorne, D.J. (1987) Proteolysis of smooth muscle myosin light chain kinase. Formation of inactive and calmodulin-independent fragments. *J. Biol. Chem.* **260**, 13828–13834
39. 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., and Watterson, D.M. (1990) Use of DNA sequence and mutant analyses and antisense oligodeoxynucleotides to examine the molecular basis of nonmuscle myosin light chain kinase autoinhibition, calmodulin recognition, and activity. *J. Cell Biol.* **111**, 1107–1125
40. Ito, M., Guerriero, V., Jr., Chen, X., and Hartshorne, D.J. (1991) Definition of inhibitory domain of smooth muscle myosin light chain kinase by site-directed mutagenesis. *Biochemistry* **30**,

3498–3503
41. Cross, R.A. and Sobieszek, A. (1985) Influence of smooth muscle myosin conformation on myosin light chain kinase binding and on phosphorylation. *FEBS Lett.* **188**, 367–374
42. Mabuchi, Y., Stafford, W.F., and Grabarek, Z. (1998) Calmodulin induces long range conformational transitions in smooth muscle myosin light chain kinase. *Biophys. J.* **74**, A38
43. Gallagher, P.J., Herring, B.P., Griffin, S.A., and Stull, J.T. (1991) Molecular characterization of a mammalian smooth muscle myosin light chain kinase. *J. Biol. Chem.* **266**, 23936–23944
44. Mayr, G.W. and Heilmeyer, L.M.G., Jr. (1983) Shape and substructure of skeletal muscle myosin light chain kinase. *Biochemistry* **22**, 4316–4326
45. Mayr, G.W. and Heilmeyer, L.M.G., Jr. (1983) Skeletal muscle myosin light chain kinase. A refined structural model. *FEBS Lett.* **157**, 225–231
46. Somlyo, A.P., Devine, C.E., and Somlyo, A.V. (1973) Filament organization in vertebrate smooth muscle. *Philos. Trans. R. Soc. Lond.* **265**, 223–229
47. Wu, X., Haystead, T.A.J., Nakamoto, R.K., Somlyo, A.V., and Somlyo, A.P. (1998) Acceleration of myosin light chain dephosphorylation and relaxation of smooth muscle by telokin. Synergism with cyclic nucleotide-activated kinase. *J. Biol. Chem.* **273**, 11362–11369