

## Dedicated Myosin Light Chain Kinases with Diverse Cellular Functions\*

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Cell signaling events that lead to increased  $[Ca^{2+}]_i$  in smooth and skeletal muscles activate  $Ca^{2+}$ /calmodulin-dependent MLCK. The kinase phosphorylates a specific site on the N terminus of the RLC of the molecular motor myosin II (1–3). RLC phosphorylation is sufficient to initiate contraction in smooth muscle, but in striated muscles, RLC phosphorylation potentiates the force and speed of contractions that are dependent on  $Ca^{2+}$  binding to troponin on actin-containing thin filaments. The only known physiological substrate for MLCK is myosin RLC; thus, it is a dedicated protein kinase.

Interest in RLC phosphorylation has increased substantially with recent reports implicating phosphorylation-dependent myosin II activity in many functions of nonmuscle cells. These include cell spreading and migration, neurite growth cone advancement, cytokinesis, cytoskeletal clustering of integrins at focal adhesions, stress fiber formation, platelet shape changes, secretion, transepithelial permeability, and cytoskeletal arrangements that affect ion currents or exchange at the plasma membrane (4–8). The potential importance of RLC phosphorylation in pathophysiological processes involving cell migration is apparent, but less obvious involvements may include cerebral vasospasm (9), increased endothelial permeability during inflammation (10), and asthma (11).

### Myosin Light Chain Kinase Family

In vertebrates there are two genes for MLCK (3). The skeletal muscle MLCK gene encodes a kinase catalytic core and regulatory segment containing autoinhibitory and  $Ca^{2+}$ /calmodulin-binding sequences (Fig. 1).

The smooth muscle MLCK gene expresses three transcripts in a cell-specific manner due to alternate promoters (12–14). Smooth muscle tissues normally have a short form of the kinase containing a catalytic core and regulatory segment that differ in sequence and catalytic specificity from skeletal muscle MLCK (Fig. 1). This kinase also contains three Ig modules, one Fn module as well as a PEVK repeat-rich region and an actin-binding sequence at the N terminus (Fig. 1). The Ig and Fn modules have  $\beta$  sandwich structures whereas the PEVK module is responsible for the titin-dependent elastic properties of striated muscle sarcomeres (15). The functions of these sequences in MLCK are not clear. However, the actin-binding sequence is both necessary and sufficient for high affinity binding *in vitro* and *in vivo* (16, 17). Although it was proposed that the actin-binding sequence resides in amino acids

1–41 of the short isoform (17), more recent evidence demonstrates the importance of three repeat motifs (DFRXXL) in residues 2–63, each of which may bind a single actin monomer in an actin filament (18, 19). Thus, the N terminus of MLCK may be anchored to actin thin filaments with extension of the catalytic core to myosin thick filaments for RLC phosphorylation. Smooth muscle MLCK is ubiquitous in all adult tissues with the highest amounts in smooth muscle tissues whereas the skeletal muscle kinase is tissue-specific (20).

Another transcript of the smooth muscle MLCK gene results in a longer form of the kinase. It contains all of the shorter MLCK in addition to an N-terminal extension with six Ig modules and two additional actin-binding motifs in tandem (Fig. 1). The N-terminal extension may be responsible for an increased affinity for actin-containing filaments (21). This kinase is not normally expressed in adult smooth muscle tissues but is found in smooth muscle cells in culture, in embryonic smooth muscle tissue, and in nonmuscle cells (12, 22, 23). The longer smooth muscle MLCK has been referred to as embryonic, nonmuscle, endothelial cell or the 210-kDa MLCK. However, the short form is also expressed in smooth muscle during embryogenesis and in some nonmuscle cells; moreover, the long form is not restricted to endothelial cells and is variable in size (20 kDa) in different animal species because of the number of PEVK repeat sequences (3, 12, 22–24). We thus propose a simplified terminology of long and short smooth muscle MLCK, which could also accommodate recently reported alternatively spliced transcripts (80).

The third transcript of the smooth muscle MLCK gene is the C-terminal Ig module, which results in the expression of the telokin protein in phasic smooth muscle tissues (14). Telokin may contribute to  $Ca^{2+}$  desensitization of smooth muscle force by cyclic nucleotides (25).

Other kinases are related to the MLCK family (15). Titin, a molecular template for sarcomere assembly and passive elasticity in vertebrate striated muscles, contains a single kinase domain (26). The 3.0-MDa titin polypeptide contains 132 Fn and 166 Ig modules (Fig. 1). The titin kinase has a two-step activation mechanism involving tyrosine phosphorylation in the active site followed by  $Ca^{2+}$ /calmodulin binding (26), leading to phosphorylation of telethonin, a Z-disc protein required for sarcomere formation.

Kinases related to vertebrate skeletal and smooth muscle MLCKs are also found in invertebrates. A single *Drosophila* gene produces from multiple internal promoters a number of transcripts with overlapping ends (15). Small transcripts (3.2–5.2 kb) encode kinase proteins similar in size to the vertebrate MLCKs whereas larger transcripts (13–25 kb) encode giant proteins similar to mammalian titins. The largest 25-kb transcript encodes a 926-kDa stretchin MLCK that has multiple structural modules (Fig. 1). *Caenorhabditis elegans* and *Aplysia* express the related kinase, twitchin. The crystal structure of the catalytic region of twitchin kinase shows an autoinhibitory segment binding to the catalytic core. It does not contain a classical calmodulin-binding sequence, but it is activated by the  $Ca^{2+}$ -binding protein, S100A1<sub>2</sub> (27). The *Dictyostelium* MLCK is structurally the simplest related kinase containing a catalytic core and a regulatory segment that must be phosphorylated for activation (Fig. 1) (28).

### Activation by $Ca^{2+}$ /Calmodulin

Upon complex formation with a calmodulin-binding peptide derived from MLCK,  $Ca^{2+}$ /calmodulin undergoes a conformational collapse with its two domains wrapping around the peptide through the bending of a flexible central helix (29–31). The calmodulin-binding sequences of both smooth and skeletal muscle MLCKs then form an amphiphilic  $\alpha$ -helix.

Kinetic and equilibrium studies of the binding interactions among  $Ca^{2+}$ , calmodulin, and MLCK, as well as small-angle x-ray and neutron scattering results are consistent with an ordered se-

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The abbreviations used are:  $[Ca^{2+}]_i$ , intracellular calcium concentration; MLCK, myosin light chain kinase; RLC, regulatory light chain of myosin II; Fn, fibronectin; PEVK, repeat sequences rich in Phe, Glu, Val, and Lys residues; kb, kilobase(s);  $K_{CaM}$ , concentration of  $Ca^{2+}$ /calmodulin required for half-maximal activation; CaMK II,  $Ca^{2+}$ /calmodulin-dependent protein kinase II; PAK, p21-activated kinase; MAPK, mitogen-activated protein kinase; GTP $\gamma$ S, guanosine 5'-3-O-(thio)triphosphate; MEK, MAPK/extracellular signal-regulated kinase kinase; uPA, urokinase-type plasminogen activator.

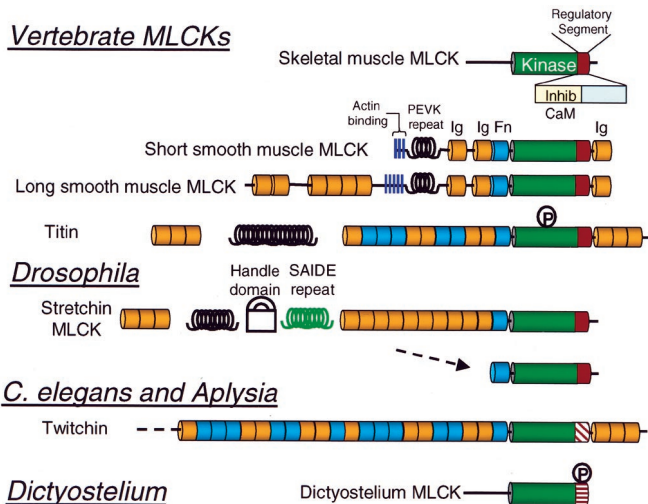
Vertebrate MLCKsDrosophilaC. elegans and AplysiaDictyostelium

FIG. 1. A schematic of structural and functional elements in myosin light chain kinase and related protein kinases.

quence of events culminating in MLCK activation (3, 24, 32–37). The autoinhibitory sequence folds back on the surface of the large lobe of the MLCK catalytic core and prevents RLC but not ATP binding in the catalytic cleft. Results from protein fragmentation complementation analyses indicate that the principal autoinhibitory motif is contained within the sequence between the catalytic core and the calmodulin-binding sequence (36) consistent with previous results obtained with truncation mutants (38). Residues C-terminal to the calmodulin-binding sequence (including the Ig module) are not functional components of the regulatory segment (36).

In the presence of  $\text{Ca}^{2+}$ , the C-terminal domain of calmodulin binds to the N terminus of the calmodulin-binding sequence in MLCK with the subsequent binding of the N-terminal domain to the C terminus of the calmodulin-binding sequence (Fig. 2). Ensuing calmodulin interactions with the catalytic core *per se* appear to be necessary for activation (3, 33, 39, 40). Although calmodulin binding and activation processes are basically similar for smooth and skeletal muscle MLCKs, mutated calmodulins with abilities to activate one or the other kinase indicated distinct differences in target-specific interactions (33, 41). The regulatory segment is subsequently displaced from the catalytic site with calmodulin collapsed at a position near the end but adjacent to the catalytic core (Fig. 2). The exposed catalytic site of the kinase allows the N terminus of RLC to bind with closure of the cleft and transfer of phosphate from ATP to RLC resulting in the reorientation of calmodulin.

**Phosphorylation of Smooth Muscle MLCK *In Vitro***

The activity of smooth muscle MLCK can be modulated by phosphorylation at specific sites that lead to increased  $K_{\text{CaM}}$  or  $V_{\text{max}}$  values (Fig. 3); however, both nonphosphorylated and phosphorylated MLCKs are tightly regulated by  $\text{Ca}^{2+}$ /calmodulin binding, and no evidence exists for physiologically relevant  $\text{Ca}^{2+}$ /calmodulin-independent activity. The most well documented effect of MLCK phosphorylation is a 10-fold increase in  $K_{\text{CaM}}$  that occurs upon phosphorylation of one of two serine residues in the C terminus of the calmodulin-binding sequence. Several protein kinases phosphorylate this site *in vitro*, including protein kinase A (42), protein kinase C (43), CaMK II (44), and PAK (45). MLCK contains several phosphorylation consensus sites for proline-directed protein kinases, and phosphorylation of either of two sites outside of the catalytic core and regulatory segment by members of the MAPK family increases  $V_{\text{max}}$  with no change in  $K_{\text{CaM}}$  (46, 47).

**Some Biological Functions of Smooth Muscle MLCKs**

Because of its dedicated nature, MLCK figures prominently in efforts to define the regulation of actomyosin-dependent functions in both smooth and nonmuscle cells where all isoforms of myosin II are activated by RLC phosphorylation (2, 5). The extent of RLC phosphorylation represents a balance between the relative activi-

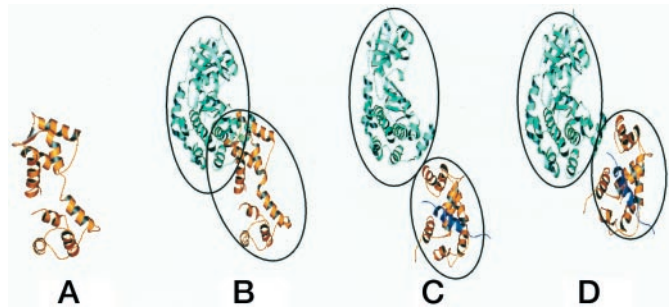


FIG. 2. Model for calmodulin activation of MLCK. Calmodulin (A) is an elongated molecule that initially binds with its C domain to the calmodulin-binding sequence of MLCK (B). Calmodulin then collapses and translocates the regulatory segment exposing the catalytic cleft (C). Binding of substrates results in closure of the cleft and reorientation of calmodulin. Calmodulin structures are shown in gold, modeled structures of the kinase core are green, and the MLCK calmodulin-binding peptide is blue. The ellipsoids represent the structures of MLCK and calmodulin obtained from x-ray and neutron scattering experiments (32, 35).

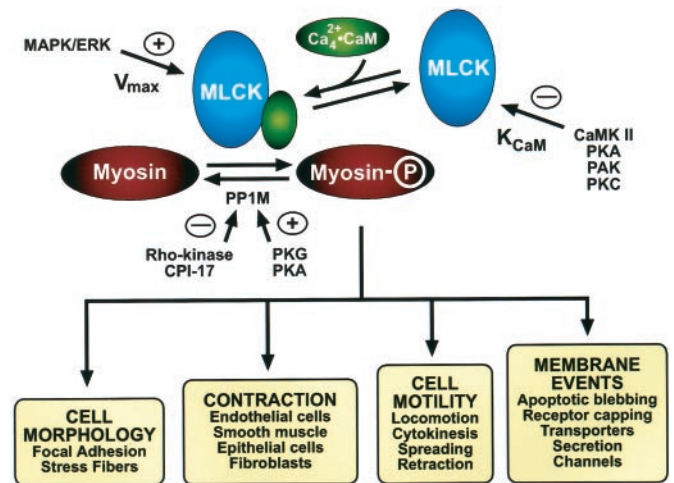


FIG. 3. Schematic for regulation and modulation of myosin II phosphorylation. Both  $\text{Ca}^{2+}$ /calmodulin-dependent MLCK and myosin phosphatase activities are modified by phosphorylations resulting from a network of cell signaling pathways. Myosin II phosphorylation mediates a variety of cell responses. ERK, extracellular signal-regulated kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, cGMP-dependent protein kinase.

ties of MLCK and myosin phosphatase, both of which are subject to extensive regulation (Fig. 3).

**Smooth Muscle**—In both intact and permeable smooth muscle fibers, increases in  $[\text{Ca}^{2+}]_i$  result in increased RLC phosphorylation and force (1, 2). The sufficiency of RLC phosphorylation for contraction is shown by treatment of both intact cells and skinned fibers with proteolytically activated MLCK in the absence of elevated  $[\text{Ca}^{2+}]_i$  (2). The dependence of force on steady state RLC phosphorylation is described by a unique relation for most contractile and dilatory agents, with a few exceptions that may point to thin filament-linked or other collateral types of regulation (48). The dependence of RLC phosphorylation on  $[\text{Ca}^{2+}]_i$  in differentiated smooth muscle arises from the  $\text{Ca}^{2+}$ /calmodulin-dependent activity of short MLCK bound to myofilaments (49).

The sensitivity of RLC phosphorylation to  $[\text{Ca}^{2+}]_i$  can be modulated by the action of different signaling pathways that modify MLCK or myosin phosphatase activities (Fig. 3).  $\text{Ca}^{2+}$  sensitization of RLC phosphorylation is observed where  $[\text{Ca}^{2+}]_i$  remains constant and RLC phosphorylation increases in response to application of agonists that lead to GTP-dependent inhibition of myosin phosphatase (50). Smooth muscle myosin phosphatase is a type 1 serine/threonine phosphatase consisting of a 110–130-kDa myosin-binding subunit, a 37-kDa catalytic subunit (PP1c), and a 20-kDa subunit with unknown function. Inhibition is effected by changes in subunit interactions occurring in response to phosphorylation of the myosin-binding subunit by Rho kinase (51) or other unidentified kinases (52). Inhibition can also be brought about by the action of CPI-17 when phosphorylated by protein kinase C (53).

$\text{Ca}^{2+}$  desensitization of RLC phosphorylation occurs upon phosphorylation of MLCK at the C terminus of the calmodulin-binding sequence and subsequent increase in  $K_{\text{CaM}}$ . Although the predominant response of smooth muscles to dilatory agents such as  $\beta$ -adrenergic agonists or nitric oxide is diminished  $[\text{Ca}^{2+}]_i$ , inhibition of RLC phosphorylation can be brought about without reductions in  $[\text{Ca}^{2+}]_i$  such as when cAMP is elevated in depolarized muscles (54). In this case, phosphorylation of MLCK is increased but not in the site that increases  $K_{\text{CaM}}$  (55, 56), suggesting that  $\text{Ca}^{2+}$  desensitization of RLC phosphorylation may result from the cAMP-dependent activation of myosin phosphatase (25, 54, 55). Surprisingly, MLCK is phosphorylated on the C terminus of the calmodulin-binding sequence in a  $\text{Ca}^{2+}$ -dependent manner during smooth muscle contractions by CaMK II, resulting in desensitization of RLC phosphorylation to  $[\text{Ca}^{2+}]_i$  (49, 55, 56). Although recent pharmacological studies with inhibitors of CaMK II proposed MLCK phosphorylation did not contribute to desensitization (57, 58), the observed decreases in force and RLC phosphorylation likely arose from inhibition of CaMK II activity on  $\text{Ca}^{2+}$  channels resulting in decreased  $[\text{Ca}^{2+}]_i$  (59, 60).  $[\text{Ca}^{2+}]_i$  thus acts in two ways to regulate  $\text{Ca}^{2+}$ -dependent RLC phosphorylation; it acts positively to increase RLC phosphorylation by activating MLCK, and at greater concentrations it acts negatively on RLC phosphorylation by stimulating CaMK II phosphorylation of MLCK. MLCK is likely to be dephosphorylated *in vivo* by myosin phosphatase as its phosphorylation is potentiated by agents known to inhibit myosin phosphatase such as agonists and GTP $\gamma$ S (61). This may represent feedback inhibition of the Rho-kinase-mediated  $\text{Ca}^{2+}$  sensitization pathway.

**Nonmuscle Cells**—Phosphorylated myosin II is an important effector of cytoskeletal activities in a number of cellular functions (Fig. 3). Many of these functions arise in response to extracellular signals that dictate temporally and spatially coordinate increases in  $[\text{Ca}^{2+}]_i$  and rearrangements of the actin cytoskeleton (62).  $\text{Ca}^{2+}$ -independent cell signaling pathways also regulate target effectors including a host of actin-binding proteins and the motor protein myosin II (4, 63). These pathways generally couple ligand-bound receptors to the cytoskeleton through activation of small GTP-binding proteins including Cdc42 and Rac with effector kinases, PAK and MEK kinase, and Rho with its effector kinase, Rho kinase (64). There is growing interest in the role of MLCK in regulating myosin II ATPase activity during various motile processes and modulation by these  $\text{Ca}^{2+}$ -independent signaling pathways. Some examples of these current topics follow.

Myosin II is regulated by the  $\text{Ca}^{2+}$ /calmodulin-dependent MLCK with the necessary kinetic properties that dictate the rate and direction of cell movement in response to temporally and spatially complex extracellular signals. Photolysis of caged inhibitory peptides directed to either calmodulin or MLCK results in blockade of cell locomotion, granule flow, and forward motion of the leading lamellipod within a few seconds in motile, polarized eosinophils (65). However, the formation of phase dark ruffles and lamellar extensions, processes believed to be dependent on actin polymerization, continues. In the smooth muscle cell line SM3, decrease of MLCK by antisense mRNA inhibited both motility and lamellipodia formation (66). Motility in eosinophils and fibroblasts is associated with elevated  $[\text{Ca}^{2+}]_i$ , and  $\text{Ca}^{2+}$ /calmodulin-dependent MLCK appears to be a major molecular control mechanism for cell locomotion (65). However, these conclusions cannot be generalized to all locomoting cells as polarization and chemotaxis occur in some cell types without changes in  $[\text{Ca}^{2+}]_i$  (62).

Short and long isoforms of smooth muscle MLCK may have distinct cellular functions. Fluorescence imaging of green fluorescent protein-tagged long MLCK showed this isoform to be localized to the cleavage furrow of dividing HeLa cells, whereas the green fluorescent protein-tagged short form was diffusely distributed in the cytoplasm (67). Localization to the cleavage furrow required the five actin-binding motifs and the N-terminal extension, whereas localization of long or short MLCK to actin filaments during interphase required only the actin-binding motifs.

MLCK activity may be modulated by signaling pathways known to regulate cytoskeletal morphology. Phosphorylation of MLCK with an associated decrease in RLC phosphorylation and disassembly of stress fibers was observed in fibroblasts injected with cAMP-dependent protein kinase (68). These results are consistent with

the well documented role of myosin II activity in stress fiber assembly (69) and also with cAMP-mediated desensitization of MLCK (Fig. 3). Nevertheless, some caution is warranted because of the findings that activation of the cAMP pathway in smooth muscle did not increase  $K_{\text{CaM}}$  (1, 3).

MLCK is also a target of the Rho family of GTPases in signaling to the cytoskeleton (45, 70–72). MLCK phosphorylation by PAK is associated with decreased MLCK activity, inhibition of RLC phosphorylation, and inhibition of cell spreading or contraction (45, 70). PAK is strongly implicated in cell locomotion, and other studies have shown that constitutively activated PAK can induce motility/contractility and RLC phosphorylation, potentially through its ability to phosphorylate myosin RLC directly (71–73). The RhoA pathway leads to stress fiber assembly and focal adhesion formation by inhibiting myosin phosphatase activity and thereby enhancing RLC phosphorylation (4, 63). Interestingly, Rho kinase may directly phosphorylate myosin RLC in stress fibers whereas MLCK phosphorylates RLC in cortical actin bundles in fibroblasts (74). It is clear from these and other studies that localization of MLCK will play an important role in its biological functions.

The  $\text{Ca}^{2+}$  and calmodulin-dependent MLCK signaling pathway may represent one of several parallel pathways for regulation of myosin II activity in response to growth factors and adhesion proteins. Treatment of MCF-7 breast cancer cells with uPA, an activator of the MAPK pathway, stimulated cell migration, phosphorylation of MLCK, and myosin RLC, all of which were inhibited by a MEK inhibitor (75). These results are consistent with MAPK-mediated stimulation of MLCK activity by phosphorylation and resultant increases in cell migration (46). Interestingly, the ability of uPA to stimulate MCF-7 cell migration above basal values depended upon the engagement of specific integrins; a second class of integrins stimulated migration that was refractory to both uPA and MLCK inhibitors, indicating an alternate signaling pathway (75).

Growth factor signaling may lead to tyrosine phosphorylation of MLCK (76). The N terminus of long MLCK contains an SH2-binding domain and a consensus tyrosine phosphorylation site for Src. Treatment of endothelial cells with diperoxovanadate decreased barrier function and was associated with Src binding to cortactin and tyrosine phosphorylation of MLCK (77, 78). Long MLCK containing phosphotyrosine was also isolated from fibroblasts transfected by constitutively activated epidermal growth factor receptor, v-Erb-B (79).

### Concluding Remarks

MLCK has served as a model for defining the enzyme activation mechanism by the ubiquitous  $\text{Ca}^{2+}$  receptor, calmodulin, and a wealth of biochemical, biophysical, and cellular information provides insights into involved molecular processes. Recently identified distinct isoforms of smooth muscle MLCK may regulate specific functions of motility depending upon their respective intracellular locations and  $\text{Ca}^{2+}$  sensitivities. The future holds exciting prospects for identifying how networks of signaling pathways that regulate the actin cytoskeleton control functionally important cellular properties of long and short MLCKs.

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**Note Added in Proof**—A recent report shows phosphorylation of specific tyrosine residues in the N-terminal extension of long MLCK by p60<sup>src</sup> increases kinase activity (81).

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