

REVIEW

Cell-cycle-dependent Regulation of Myosin Light Chain Kinase

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INTRODUCTION

In cell division of animal cells, the microfilament cytoskeleton undergoes dynamic reorganization [38, 41]. Microfilament bundles or stress fibers of cultured cells are disassembled during prophase concomitant with cell rounding. After chromosome segregation, the contractile ring is transiently formed perpendicular to the mitotic apparatus and is activated to separate the cytoplasm into two daughter cells. Then, as cells start to spread, microfilaments are reconstructed as stress fibers. However, the molecular mechanisms underlying these changes in microfilament organization are still unknown [for review see references 20, 28, 36, 37]. In particular, what controls the formation and activation of the contractile rings has been a primary objective for investigation.

Myosin light chain kinase (MLCK) has long been suspected to be a signal for the induction of cytokinesis. Although there is a lack of direct evidence, three factors argue for their important roles in activating the contraction of cleavage furrows. First, the regulatory light chain of cytoplasmic myosin II is the same as that of smooth muscle myosin II, which is substrate of MLCK. Second, in smooth muscle, strong physiological evidence has established that phosphorylation of Thr 18 and Ser 19 of the regulatory light chain is linked with the initiation of its actomyosin contraction [42]. Third, the MLCK which catalyzes this reaction is activated by calcium-calmodulin. Because there is growing evidence that Ca^{2+} acts as a second messenger in cytokinesis [14], it would provide a biochemical signal to activate MLCK.

In this short review, I will discuss roles of MLCK to understand mechanisms regulating cytokinesis.

A. FUNCTION OF MLCK

MLCK is activated in the presence of Ca^{2+} -calmodulin and phosphorylates the regulatory light chain of myosin II. MLCK is widely present not only in various skeletal, cardiac and smooth muscle cells [1, 5, 9, 23, 35, 48-50, 52] but also in non-muscle cells such as those in the spleen [4], brain [10, 18], Myxomycetes [45], aorta [47], platelets [11, 17], and sea

urchin eggs [7].

MLCK shows phosphorylation activity after binding to calmodulin at a ratio of 1:1 in the presence of Ca^{2+} ($K_d=0.3$ nM). However, MLCK detected in Myxomycetes lacks the calmodulin-binding site and shows calmodulin-independent phosphorylation activity [45]. The molecular weight of MLCK purified from smooth muscle, brain, and aorta is generally between 100 kD and 150 kD, but that of MLCK from Myxomycetes is about 35 kD due to the above reason.

MLCK is readily purified, and thus, there are many studies on the enzymatic properties of MLCK purified from the rabbit skeletal muscle or the smooth muscle of the chicken gizzard. The substrate specificity of MLCK is very high. No substrates other than the regulatory light chain (20 kD) of myosin II in muscle and non-muscle cells have been reported.

Previous studies using antibodies raised against smooth muscle MLCK found that MLCK was localized in the stress fibers of non-muscle cultured cells [6, 12], in the mitotic apparatus and midbody of mitotic cells and in the nucleolus of interphase cells [16]. It was also shown that MLCK was localized in the I bands of skeletal and cardiac muscle [6]. Several *in vitro* studies have shown binding of MLCK to actin filaments or myosin [6, 16]. The I-band location for MLCK, however, raises the possibility that MLCK is associated with actin filaments rather than its substrate, myosin, *in vivo*. Interestingly, there is no report showing MLCK localization in the contractile ring in dividing cells. The MLCK localization in this phase remains to be clarified.

MLCK is phosphorylated *in vitro* by several kinases such as cAMP-dependent protein kinase (A kinase) [8], cGMP-dependent protein kinase (G kinase) [31], Ca^{2+} -calmodulin-dependent protein kinase II (CaM kinase II) [22], and phospholipid-dependent protein kinase (C kinase) [23, 33]. Although the *in vitro* phosphorylation of MLCK by these kinases reduces the affinity for calmodulin, the possible role of the phosphorylation of MLCK *in situ* requires further study.

B. MECHANISM OF EXPRESSION OF MLCK ACTIVITY

Recently, the entire amino acid sequence of smooth

muscle MLCK has been clarified, and the relationship between the structure and function of this enzyme has been studied in detail [34]. Comparison with kinases other than MLCK and ATP-binding proteins has suggested that the glycine-rich region in about middle of the MLCK molecule functions as the catalytic site of this enzyme. In addition, the peptides containing the site phosphorylated by A kinase has been shown to strongly bind to calmodulin. This suggests that the calmodulin binding site is present near the site phosphorylated by A kinase. This area corresponds to Ala 796-Leu 813 near the C terminal in MLCK (Fig. 1). Bagchi *et al.* [2] produced various point mutants using the *E. coli* expression system and suggested the importance of Gly811 and Arg812 at the calmodulin binding site for binding to calmodulin.

On the other hand, Kemp *et al.* [25] reported that a sequence resembling the sequence at the site of myosin 20 kD light chain phosphorylation by MLCK is present near the N terminal of the calmodulin binding site. The synthetic peptide containing the sequence of this region (Ala783-Gly804)

inhibited MLCK activity and was termed a pseudo-substrate inhibitor since its structure resembles that of the 20 kD light chain. Ikebe *et al.* [24] partially degraded MLCK with trypsin and evaluated in detail the relationship between the amino acid sequence of each fragment and activity. They confirmed the presence of a site inhibiting calmodulin-dependent kinase activity of MLCK near the N terminal of the calmodulin binding site and showed the primary importance of Arg797-Lys799 at this site for inhibiting activity.

These findings suggest the following mechanism of the expression of MLCK activity. When MLCK is not bound with calmodulin, the pseudo-substrate inhibitor site is bound with or close to the catalytic site of MLCK. Therefore, the catalytic site cannot come into contact with the myosin light chain, and MLCK cannot phosphorylates the myosin light chain. On the other hand, when MLCK is bound with calmodulin, the pseudo-substrate inhibitor site dissociates from the catalytic site, and MLCK phosphorylates the myosin light chain.

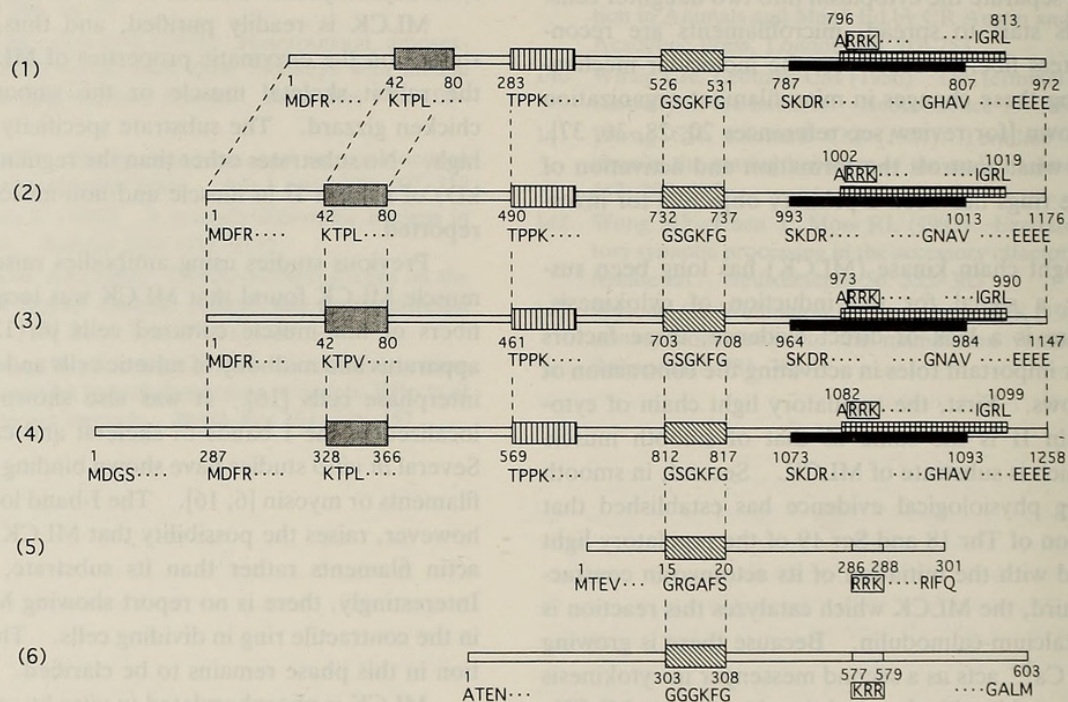


FIG. 1. Schematic representation of regulatory and putative structural domains of MLCKs. (1), chicken gizzard smooth muscle MLCK [34]; (2), bovine stomach smooth muscle MLCK [26]; (3), rabbit uterine smooth muscle MLCK [15]; (4), chick embryo fibroblast MLCK [43]; (5), *Dictyostelium* MLCK [46]; (6), rabbit skeletal muscle MLCK [44]. The amino-terminal sequence of about 80 residues is a highly conserved region among smooth muscle MLCKs. A significant similarity between a sequence from Lys42 to Ala80 (fine lattice) in amino-terminal portion of these four smooth muscle MLCKs and that from Lys695 to Ala731 in the carboxyl-terminal portion of chicken gizzard caldesmon was found [27]. The corresponding sequence in caldesmon is characterized as the actin- and CaM-binding domain [19]. This similarity confirms the assumption that the amino-terminal sequence in the smooth muscle MLCKs plays an important role in the regulation of actin-myosin interaction. Comparison with the ATP binding site in ATP binding proteins suggests that the catalytic site of the chicken gizzard MLCK is present in the glycine-rich region starting Gly526 (diagonal stripes). The sequence of the region from Ser787 to Val807 is called substrate inhibitory domain (black box). The pseudosubstrate hypothesis for the regulation of the smooth muscle MLCK was based on the observation that this region bore a remarkable similarity to the phosphorylation site sequence in the regulatory light chain of myosin II [25]. This region is considered to be bound with the catalytic site when not bound with calmodulin. The calmodulin binding site is present on its right side (C terminal side) (cross stripes). Ikebe *et al.* [24] showed the primary importance of three basic residues (Arg797 to Lys799; clear box) at this site for inhibiting MLCK activity. When this site is bound with calmodulin, the pseudosubstrate inhibitor site on the N terminal is impaired, making binding to the catalytic site impossible. This results in expression of MLCK activity. Vertical stripes represent the putative phosphorylation site by cdc2 kinase (Hosoya, unpublished data).

C. CELL CYCLE AND MLCK

As described above, the function and structure of MLCK in the smooth muscle have been extensively studied. In addition, analysis of amino acid sequences has shown that the structure of MLCK in non-muscle cells is similar to that in smooth muscle (Fig. 1). This section outlines the role of MLCK in non-muscle cells in each phase of the cell cycle based on results of previous studies.

Shoemaker *et al.* [43] introduced antisense DNA of MLCK into cultured chick embryo fibroblasts or 3T3 cells and observed a marked morphological changes in the cells. Both cells exhibited a more rounded morphology, reminiscent of the morphological changes seen in V-src-transformed cells. In addition to the effect on morphology, there appears to be an effect of antisense DNA on cell proliferation. These findings indicate that MLCK is indispensable to the progression of the cell cycle (cell division). The marked morphological changes also suggest an important role of MLCK in the maintenance and control of the cytoskeleton such as stress fibers.

Fishkind *et al.* [13] degraded MLCK by trypsin and obtained a fragment without calmodulin sensitivity. They microinjected this fragment into cultured cells during mitosis under a microscope and evaluated its effects on cell division. If MLCK regulates contraction of cleavage furrows, microinjection of this fragment would affect the kinetics of cytokinesis and the formation of cleavage furrows. However, the microinjection neither changed the rate of contraction of cleavage furrows nor affected the formation of cleavage furrows. After microinjection of this fragment, many projections formed near the cell surface, and they repeated protrusion and retraction during and after metaphase. Interestingly, there was a significant delay in the transit time from nuclear envelope breakdown to anaphase onset. These results suggest the involvement of MLCK in the function of the mitotic apparatus but not in the contraction of cleavage furrows.

Pharmacological approaches using MLCK inhibitor have shown interesting results. Mabuchi and Takano-Ohmuro [29] evaluated the action of ML-7 and ML-9 known as MLCK inhibitors on sea urchin eggs and observed inhibition of cytokinesis. We analyzed in detail the time point of action of MLCK using Wortmannin (WM), a MLCK inhibitor recently reported [30]. Cytokinesis was inhibited by addition of 2 to 5 μ M of WM at any point of time before mitotic apparatus formation, but not after the mitotic apparatus formation (Hosoya, unpublished data). In the presence of WM below 1 μ M, no effect was observed. These results suggest that the site of action of MLCK is present at the time of mitotic apparatus formation but not during contraction of the cleavage furrows (Fig. 2). In other words, MLCK may be involved in the mitotic apparatus formation itself.

Then, how does MLCK activity change during each process of the cell cycle?

Our study using Hela cells showed that MLCK is phos-

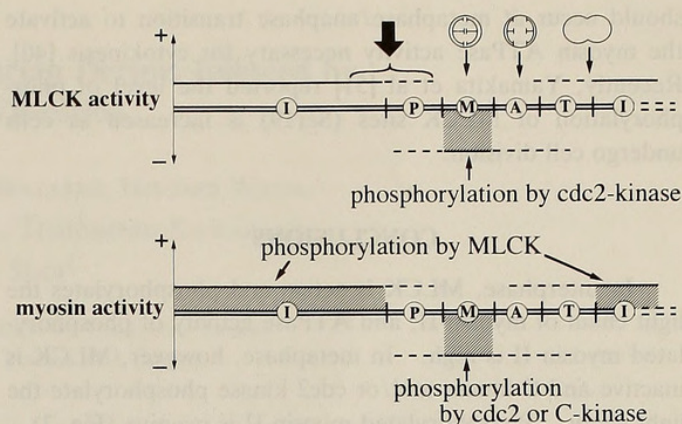


FIG. 2. MLCK activity and presence or absence of phosphorylation of myosin light chain as the substrate in each phase of the cell cycle. I, P, M, A, and T indicate the interphase, prophase, metaphase, anaphase, and telophase, respectively. In the upper figure, the areas above and below the two lines indicate high and low MLCK activity, respectively. In the lower figure, the areas above and below the two lines indicate that myosin is activated and not activated, respectively. In both figures, the shaded area represents that MLCK or myosin light chain is phosphorylated. In the upper figure, the thick arrow indicates the time at which first cell division is not inhibited by WM. At present, changes in phosphorylation and activity of MLCK and myosin in the borderline of the prophase, anaphase, and telophase are unclear, and therefore, the time of phosphorylation is indicated by dotted lines.

phorylated in mitotic cells (metaphase), and the activity of MLCK phosphorylation is high in metaphase cells but low in interphase cells [21]. After phosphorylation, the affinity of MLCK for calmodulin slightly decreases. As described above, MLCK is a substrate for many kinases (A kinase, G kinase, CaM kinase II and C kinase). However, studies have clarified that kinase that phosphorylates MLCK in metaphase is not these kinases but is cdc2 kinase (Hosoya, unpublished data). When MLCK is phosphorylated by cdc2 kinase in metaphase, MLCK activity may be decreased compared with that in interphase.

Satterwhite *et al.* [39] compared the phosphorylation state of myosin regulatory light chain in *Xenopus* oocyte extracts between metaphase and interphase. In both metaphase and interphase extracts, the light chain was phosphorylated. The sites of phosphorylation in interphase corresponded to those of phosphorylation by MLCK. In metaphase, the site of phosphorylation corresponded to a part of the phosphorylation site (Ser1 and/or Ser2 and Thr9) by C kinase or cdc2 kinase but not to that by MLCK. This result suggests that MLCK is transiently inactive in metaphase, because phosphorylation of these residues by C kinase inhibits the actin-activated ATPase of smooth muscle myosin previously phosphorylated on Ser19 [3, 32]. The inactivation of myosin in metaphase is consistent with our result.

Since phosphorylation of regulatory light chain at the sites (Ser1 and/or Ser2 and Thr9) in metaphase results in reduction of actin-activated myosin ATPase activity, they considered that dephosphorylation at the inhibition sites

should occur at metaphase/anaphase transition to activate the myosin ATPase activity necessary for cytokinesis [40]. Recently, Yamakita et al [51] reported the level of phosphorylation of MLCK sites (Ser19) is increased as cells undergo cell division.

CONCLUSIONS

In interphase, MLCK is active and phosphorylates the light chain of myosin II, and ATPase activity of phosphorylated myosin II is high. In metaphase, however, MLCK is inactive and C kinase and/or cdc2 kinase phosphorylate the light chain. Phosphorylated myosin II is inactive (Fig. 2).

The switching of sites of phosphorylation from Ser1 and/or Ser2 to Ser 19 is likely to be a signal to activate contractile rings during cell division. Which kinase is responsible for the phosphorylation of the MLCK sites at metaphase/anaphase transition? A natural candidate is MLCK. However, how is MLCK reactivated at metaphase/anaphase transition? How is MLCK involved in mitotic apparatus formation and expression of its function? Further studies are needed to elucidate these questions.

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