Role of Myosin Light Chain Phosphorylation in the Regulation of Cytokinesis

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ABSTRACT. Phosphorylation of regulatory light chain (RMLC) of myosin II at Ser19/Thr18 is likely to play important roles in controlling the morphological changes seen during cell division of cultured mammalian cells. Phosphorylation of RMLC regulates the activity of myosin II, an essential motor for cytokinesis, and phosphorylation of RMLC shows dramatic changes during mitosis. Two enzymes, myosin phosphatase and kinase, control phosphorylation of RMLC. Myosin phosphatase is activated during mitosis, apparently as a result of mitosis-specific phosphorylation of the myosin phosphatase targeting subunit (MYPT). This activation of myosin phosphatase is likely to result in RMLC dephosphorylation, causing the disassembly of stress fibers and focal adhesions during prophase. The phosphorylation of MYPT is lost in cytokinesis, which would decrease myosin phosphatase activity. At the same time, ROCK (Rho-kinase) probably phosphorylates MYPT at its inhibitory sites, further decreasing the activity of myosin phosphatase. These changes in MYPT phosphorylation would raise RMLC phosphorylation, leading to the activation of myosin II for cytokinesis. RMLC phosphorylation is also regulated by several RMLC kinases including ROCK (Rho-kinase), MLCK and citron kinase, all of which are localized at cleavage furrows. Future studies should examine whether these multiple kinases are redundant or whether they control distinct aspects of cell division.

Key words: Rho/ROCK (Rho-kinase)/citron kinase/myosin phosphatase/myosin light chain phosphorylation

At mitosis in vertebrate cultured cells, profound changes occur in microfilament structure and function. When normal adherent cells such as fibroblasts enter into prophase, cells become rounded and microfilament bundles (stress fibers) are disassembled. Focal adhesions, specialized structures for cell adhesions, to which microfilament bundles anchor, are also disrupted, causing reduced adhesion to extracellular matrix (ECM). Cell migration is also inhibited. After cytokinesis, daughter cells reverse these morphological alterations, re-assembling stress fibers and focal adhesions, and re-attaching to and spreading on the ECM.

Phosphorylation of regulatory myosin light chain (RMLC) appears to play important roles in the regulation of these morphological changes. First, myosin II is an essential motor for cytokinesis (DeLozanne and Spudich, 1987; Mabuchi and Okuno, 1977) and cell spreading (Cramer and Mitchison, 1995). Second, the activity of myosin is known to be regulated by phosphorylation of myosin light chain (RMLC) (Kamm and Stull, 1985; Moussavi et al., 1993; Sellers, 1991; Somlyo and Somlyo, 1994; Trybus, 1991). In vivo phosphorylation of Ser19 is correlated with a variety of contractile processes in smooth muscle and nonmuscle cells (Chrzanowska-Wodnicka and Burridge, 1996; DeBiasio et al., 1996; Giuliano et al., 1992; Jordan and Karess, 1997; Matsumura et al., 1998; Murata-Hori et al., 1998; Post et al., 1995; Schaller et al., 1995; Sellers, 1991; Trybus, 1991; Yamakita et al., 1994). Third, the formation of focal adhesions and stress fibers depends on phosphorylation of RMLC (Chrzanowska-Wodnicka and Burridge, 1996; Totsukawa et al., 2000). Thus the disassembly of stress fibers and focal adhesions during mitosis would involve RMLC dephosphorylation. Finally, mutational analysis of the Drosophila spaghetti squash gene, which encodes RMLC, revealed that phosphorylation of Drosophila RMLC on Ser 21 (which corresponds to Ser19 of vertebrate RMLC) is essential for cell division (Jordan and Karess, 1997).

The importance of RMLC phosphorylation in cell division is reinforced by the findings by Kaibuchi and
coworkers (Amano et al., 1996; Kimura et al., 1996). They demonstrated that Rho-kinase or ROCK (one of the Rho effector molecules) phosphorylated MYPT, resulting in the inhibition of myosin phosphatase, and that ROCK (Rho-kinase) directly phosphorylates RMLC, both of which activities lead to an increase in RMLC phosphorylation. This finding is significant because Rho has been shown to be essential for cytokinesis. In this mini-review, we focus on the roles of RMLC phosphorylation in cytokinesis of mammalian cultured cells.

Changes in RMLC phosphorylation during cell division

We, as well as others, have demonstrated that the sites of phosphorylation on RMLC change during cell division (Satterwhite et al., 1992; Yamakita et al., 1994). When cells enter mitosis, dephosphorylation at Ser19 occurs, and Ser1/2 becomes the major phosphorylation site. During cytokinesis, Ser1/2 phosphorylation is switched back to Ser19, and Ser19 phosphorylation persists during post-mitotic cell spreading. We, as well as others, have developed an antibody (Ab) specific to Ser19-phosphorylated RMLC, and demonstrated that Ser19-phosphorylated RMLC shows distinct localization within dividing and motile cells (Matsumura et al., 1998; Murata-Hori et al., 1998). These results suggest that RMLC phosphorylation is involved in the reorganization of microfilaments during mitosis.

It has been suggested that cdc2 kinase is responsible for the Ser1/2 phosphorylation (Komatsu et al., 1997; Mishima and Mabuchi, 1996; Satterwhite et al., 1992). Because Ser1/2 phosphorylation inhibits activity of myosin II, the switching from Ser 1/2 to Ser 19 has been proposed to activate myosin II for cytokinesis (Satterwhite et al., 1992). This is an attractive model because MPF activity would prevent myosin from premature activation before metaphase-anaphase transition, which would go toward explaining the regulation of timing of cytokinesis. However, as the activity of cdc2 to phosphorylate Ser 1/2 is rather weak in vitro (Yamakita et al., 1994), other kinases may be involved in the Ser1/2 phosphorylation. Furthermore, it has been reported that in sea urchin eggs, continued activation of MPF by nondegradable cyclin could not block cytokinesis if mitotic spindles were moved close to the cortex (Shuster and Burgess, 1999). Thus, it is still an open question whether Ser1/2 phosphorylation plays a functional role in regulating cytokinesis.

It is noteworthy that RMLC phosphorylation does not seem to play a major role in regulating cell division in some types of cells such as Dictyostelium. Data derived from mutational analyses of Dictyostelium myosin II suggest that neither phosphorylation of RMLC nor the binding of RMLC to myosin heavy chain is required for cell motility and cytokinesis in this organism (Ostrow et al., 1994; Uyeda and Spudich, 1993; Yumura and Uyeda, 1997). It is also worthy of note that Dictyostelium does not have Rho (Prokopenko et al., 2000), which is essential for the regulation of RMLC phosphorylation. Perhaps, Dictyostelium has developed a different system in regulating the activity of myosin. For example, it has been shown that heavy chain phosphorylation of Dictyostelium myosin II is more critical for the regulation of myosin II activity (Egelhoff et al., 1993; Hammer, 1994).

Roles of myosin phosphatase in cell division

RMLC phosphorylation (at Ser19) is controlled by the balance of two groups of enzymes, myosin light chain kinases and myosin phosphatases. A trimeric myosin phosphatase is the major phosphatase responsible for dephosphorylation of MRLC in both smooth muscle and nonmuscle cells (Hartshorne et al., 1998). It consists of three subunits: a large subunit of about 130 kDa (referred here as the myosin phosphatase targeting subunit, MYPT), a catalytic subunit of 38kDa (the δ isoform of type 1 protein phosphatase, PP1c) and a small subunit of 20kDa with unknown function (Alessi et al., 1992; Shimizu et al., 1994; Shirazi et al., 1994). MYPT (also referred as M130/133 (Shimizu et al., 1994), M110 (Chen et al., 1994) or myosin binding subunit (Kimura et al., 1996; Okubo et al., 1994) can bind both to the catalytic subunit of PP1c and to myosin, and thus can act to target the phosphatase to the substrate, myosin. Without MYPT, PP1c shows low phosphatase activity toward myosin, indicating a critical role for MYPT in myosin dephosphorylation (Alessi et al., 1992; Hirano et al., 1997; Johnson et al., 1997).

Phosphorylation of MYPT has been reported to modulate phosphatase activity. For example, MYPT was phosphorylated by an unknown kinase copurified in phosphatase preparations from smooth muscle and this phosphorylation inhibited phosphatase activity (Ichikawa et al., 1996). The unknown kinase has been recently identified as ZIP-like kinase (MacDonald et al., 2001). ROCK (Rho-kinase) was also shown to phosphorylate MYPT (in the C-terminal region) and this phosphorylation also inhibited phosphatase activity (Kimura et al., 1996). Recent study has demonstrated that phosphorylation at Thr 695 is responsible for the inhibition of myosin phosphatase by Rho-kinase (ROCK) (Feng et al., 1999). Finally, the in vitro phosphorylation of MYPT by protein kinase A resulted in a decreased binding to acidic phospholipids (Ito et al., 1997), which would be expected to decrease the binding of MYPT to the cell membrane.

We have demonstrated that MYPT is phosphorylated in a mitosis-specific way (Totsukawa et al., 1999). Unlike phosphorylation by other kinases reported so far, this mitosis-specific phosphorylation provides a positive regulatory effect on phosphatase activity. Phosphorylated MYPT shows increased myosin binding, which appears to reflect the activation of this phosphatase during mitosis.
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activation should result in the dephosphorylation of RMLC (at Ser19) and disassembly of stress fibers during prophase. The mitosis-specific phosphorylation is lost upon exiting mitosis, and the resultant increase in RMLC phosphorylation (again at Ser19) may act as a signal activating cytokinesis.

We are currently investigating the role of myosin phosphatase in cell division by expressing constitutively active myosin phosphatase in tissue culture cells. We speculated that the NH2-terminus of MYPT spanning residues 1 to 296 (MYPT1-296) would behave as a “constitutively active” mutant for the following reasons. First, MYPT1-296 when combined with PP1cδ has increased myosin phosphatase activity compared to PP1c alone (Hirano et al., 1997). This reflects the binding of MYPT1-296 to both myosin and PP1c. Second, MYPT1-296 may be able to associate with PP1c because cells have a large pool of PP1c (Andreassen et al., 1998; Fernandez et al., 1992). Third and most importantly, MYPT1-296 does not contain the inhibitory phosphorylation sites by ROCK. Thus MYPT1-296 would not be subjected to regulation by ROCK. The following results have demonstrated that MYPT1-296 indeed acts as “constitutively active” myosin phosphatase mutant (Totsukawa et al., 1999). MYPT1-296 was able to bind PP1c in vivo and showed myosin phosphatase activity.

Microinjection of MYPT1-296 caused MLC dephosphorylation in a variety of cells including 3T3 fibroblasts, rat embryo fibroblasts, PtK2 cells, and normal rat kidney cells along with disruption of both actin stress fibers and focal adhesion. Furthermore, MYPT1-296 inhibits stress fibers and focal adhesion formation upon serum stimulation in serum-starved 3T3 cells, indicating that RMLC phosphorylation is necessary for the formation of both stress fibers and focal adhesions.

Our preliminary data have suggested that MYPT1-296 inhibited cell division. Microinjection of MYPT1-296 into rat kangaroo kidney (PtK2) cells at prometaphase resulted in a delay of cytokinesis longer than 2hrs in about 70% of injected cells. Of those 90% cells showed abnormal cytokinesis. Consistent with this observation, we were unable to isolate a stable cell line expressing MYPT1-296. These results suggest that regulation of myosin phosphatase is important for cell cycle progression.

What are the kinases that phosphorylate RMLC during cytokinesis?

Two RMLC kinases have been suggested so far. One is

A. Effects of citron kinase activation

![Diagram of citron kinase activation]

B. Effects of ROCK (Rho-kinase) activation

![Diagram of ROCK activation]

**Fig. 1.** Schematic diagram of the regulation of RMLC phosphorylation by citron kinase and ROCK. Unlike ROCK, citron kinase does not block turnover of RMLC phosphorylation.
MLCK and the other is ROCK (Rho-kinase). In addition to conventional MLCK found in smooth muscle, nonmuscle cells have a higher Mr MLCK with an N-terminal extension (Garcia et al., 1997). It has been reported that the long isoform of MLCK is localized in cleavage furrows and its activity is regulated by mitosis-specific phosphorylation (Poperechnaya et al., 2000). ROCK, as well as Rho, is also localized in cleavage furrows. ROCK can directly phosphorylate RMLC in vitro (Amano et al., 1996) and in vivo (Totsukawa et al., 2000). At the same time, ROCK inhibits myosin phosphatase by phosphorylating the myosin target-subunit of myosin phosphatase. Both activities lead to an increase in RMLC phosphorylation. An attractive model has thus been proposed: RhoA activates ROCK (Rho-kinase), which increases RMLC phosphorylation, thereby activating contractile rings for cytokinesis (Amano et al., 1996).

This model has yet to be proven. Narumiya and coworkers suggested that citron kinase, another RhoA effector molecule, is a target of Rho in cytokinesis for the following three reasons: First, citron kinase is localized in cleavage furrows. Second, a specific inhibitor of ROCK, Y-27632, is not effective in blocking cytokinesis. Third, they found that the overexpression of mutants of citron kinase inhibited cytokinesis (Madaule et al., 1998). Additional evidence of the role of citron kinase in cytokinesis has been presented in a study with citron kinase knock-out mice. Citron kinase-deficient mice showed cytokinesis defects in certain specific neuronal precursors even though ROCK is abundantly present (Di Cunto et al., 2000). It is thus important to identify the substrates of citron kinase.

We have found that citron kinase phosphorylates RMLC at Ser19 and Thr 18 (unpublished results). Unlike ROCK, however, citron kinase does not phosphorylate MYPT. Expression of the delta3 mutant of citron kinase (containing the kinase domain alone) induces stress fibers and increases diphosphorylation of MLC in the presence of a ROCK inhibitor, Y-27632.

It should be worth pointing out the differences in the activities of these three kinases. First, citron kinase does not inhibit myosin phosphatase. This indicates that citron kinase, unlike ROCK, does not block the turnover of RMLC phosphorylation (Fig. 1A). The activation of ROCK, on the other hand, would convert most RMLC into phosphorylated forms of RMLC (Fig. 1B). Turnover of RMLC phosphorylation appears to be important for cell motility: RMLC phosphorylation can be increased by inhibiting myosin phosphatase via microinjection of a function-blocking anti-

Fig. 2. Immunofluorescent localization of mono- and diphosphorylated RMLC in dividing PTK2 cells. Merged image of monophosphorylated RMLC (green), diphosphorylated RMLC (red) and DNA (blue).
body or decreased by expressing constitutively active myosin phosphatase. In either case, we have observed the blockage of cell motility (unpublished results). Second, citron kinase generates diphosphorylated form of RMLC more efficiently than does MLCK. Because diphosphorylated myosin shows higher contractile activity than does monophosphorylated myosin (Ikebe et al., 1988), diphosphorylated myosin may play a distinct role in regulating cytokinesis. Indeed, immunofluorescence using specific antibodies against mono- or diphosphorylated RMLC (Sakuda et al., 1994; Sakurada et al., 1998) has revealed that diphosphorylated RMLC is localized in a more constricted region of cleavage furrows (Fig. 2). Hosoya and coworkers have also reported that ROCK (Rho-kinase) generates diphosphorylated RMLC (personal communication). Future studies should be directed at identification of the role of diphosphorylation in cell motility including cytokinesis.

It is likely that citron kinase, together with ROCK and MLCK, provide multiple systems to control MLC phosphorylation during cell division. While the presence of the multiple regulatory systems reinforces the importance of MLC phosphorylation in cell division control, future studies should examine whether these kinases are redundant or whether they control different aspects of cell division. If they do have distinct functions, it is important to determine how they are regulated. Such studies will shed light on why citron kinase deficient mice show cytokinesis defects in certain neuronal precursors.

References


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