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広島大学学位請求論文

The Regulatory Mechanism of Myosin Phosphorylation during Mitosis: The Cell Cycle-Dependent Control of Myosin Light Chain Kinase and Myosin Phosphatase.

（細胞分裂期におけるミオシンリン酸化の制御機構：細胞周期依存的なミオシン軽鎖キナーゼおよびミオシンフォスファターゼの活性調節機構）

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目次

1. 主論文

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The Regulatory Mechanism of Myosin Phosphorylation during Mitosis: The Cell Cycle-Dependent Control of Myosin Light Chain Kinase and Myosin Phosphatase.

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Table of Contents

1. General Abstract

Phosphorylation of regulatory light chain of myosin II (MRLC) is thought to regulate the contractility of actomyosin in smooth muscle and non-muscle cells, and may play important roles in controlling the reorganization of the actomyosin cytoskeleton in many physiological cell events. It has been previously demonstrated that during mitosis the sites of myosin phosphorylation are switched between the inhibitory sites, Ser-1/2, and the activation sites, Ser-19/Thr-18 (Yamakita, Y., Yamashiro, S., Matsumura, F. J. Cell Biol. 124: 129-137. 1994; Satterwhite, L.L., Lohka, M.J., Wilson, K.L., Scherson, T.Y., Cisek, L.J., Corden, J.L., Pollard, T.D. J. Cell Biol. 118: 595-605. 1992), suggesting a regulatory role of myosin phosphorylation in cell division. The phosphorylation of MRLC is controlled by the balance of two enzymatic activities, i.e. myosin light chain kinase and myosin phosphatase. Thus it is important to examine spontaneously both enzymatic activities. To explore the functions of myosin phosphorylation in cell division, I have examined the activities of both myosin light chain kinase and phosphatase in cell division.

First, I have examined the kinase activities capable of phosphorylating MRLC in unfertilized and fertilized sea urchin egg extracts (see chapter 1). It was found that total kinase activity phosphorylating MRLC *in vitro* did not fluctuate throughout the first cell cycle. It was shown that MRLC was phosphorylated at two different sites, myosin light chain kinase (MLCK) and protein kinase C (PKC) phosphorylation sites, respectively. While MRLC phosphorylation at MLCK sites (Ser-19/Thr-18) showed no significant changes during the first cell cycle, the activity of the kinase(s) responsible for phosphorylation at PKC sites (Ser-1/2 and Thr-9) showed a significant increase at metaphase. Butyrolactone I, a selective inhibitor of $p34^{cdc2}$ kinase, specifically inhibited the activity of the kinase(s) for PKC sites at metaphase, indicating that the increase in MRLC phosphorylation at PKC sites may be induced by $p34^{cdc2}$ kinase. Thus, it is suggested that $p34^{cdc2}$ kinase may be involved in the regulation of MRLC phosphorylation during cell division.

Second, I have investigated that the possibility that myosin phosphatase activity may be altered during cell division (see chapter 2). It was found that the myosin phosphatase targeting

subunit (MYPT) undergoes mitosis-specific phosphorylation *in vivo* in cultured cells and is reversed during cytokinesis. MYPT phosphorylated either *in vivo* or *in vitro* in the mitosis-specific way showed higher binding activity to myosin II (2 to 3 fold) compared to MYPT from cells in interphase. One of the mitosis-specific phosphorylation sites of MYPT was determined at Ser-430 of chicken MYPT which corresponds to Ser-435 of rat MYPT. Furthermore, the activity of phosphorylated myosin phosphatase was increased more than twice and it is suggested this reflected the increased affinity of myosin binding. These results indicate the presence of a unique positive regulatory mechanism for myosin phosphatase in cell division.

The activation of myosin phosphatase during mitosis would enhance dephosphorylation of the MRLC at MLCK sites, thereby leading to the disassembly of stress fibers during prophase. Together, MRLC phosphorylation at PKC sites would support the disassembly of stress fibers. The mitosis-specific effect of phosphorylation of MYPT is lost on exit from mitosis and the resultant increase in myosin phosphorylation at MLCK sites may act as a signal to activate cytokinesis.

2. General Introduction

At mitosis, normal animal cultured cells show profound reorganization of the microfilament cytoskeleton. During prophase, both stress fibers and focal adhesions are disassembled, resulting in cell rounding. At cytokinesis, microfilaments form a contractile ring that contracts to generate daughter cells. During post-mitotic cell spreading, microfilaments reassemble into stress fibers and focal adhesions are formed. The molecular mechanisms underlying these drastic alterations in the microfilament organization during cell division are largely unknown.

Phosphorylation of the regulatory light chain of myosin II (MRLC) is thought to regulate actomyosin contractility in smooth muscle and in many non-muscle cells (see for reviewKamm and Stull, 1985; Moussavi, et al., 1993; Somlyo and Somlyo, 1994). MRLC is phosphorylated by myosin light chain kinase at two sites, a primary site, Ser-19, and a secondary site, Thr-18. It can also be phosphorylated (for example by protein kinase C) at Ser- 1 or Ser-2 and Thr-9. *In vitro*, the phosphorylation at Ser-19 activates actin-activated ATPase activity of myosin II (Sellers, 1991; Trybus, 1991) whereas phosphorylation at Ser-1/2 and Thr-9 can inhibit ATPase activity of myosin phosphorylated at Ser-19 (Bengur, et al., 1987; Ikebe and Reardon, 1990). *In vivo*, phosphorylation of Ser-19 is correlated with a variety of contractile processes including smooth muscle contraction (Sellers, 1991; Trybus, 1991), contraction of cultured cells upon serum stimulation or treatment with certain drugs (Giuliano, et al., 1992; Kolodney and Elson, 1993; Goeckeleer and Wysolmerski, 1995), stress-fiber assembly upon serum stimulation of starved 3T3 cells (Chrzanowska-Wodnicka and Burridge, 1996), and cytokinesis of higher eukaryotic cells (Yamakita, et al., 1994; Post, et al., 1995; DeBiasio, et al., 1996; Jordan and Karess, 1997; Matsumura, et al., 1998).

Previously, it was demonstrated that during cell division the sites of phosphorylation on MRLC changed (Satterwhite, et al., 1992; Yamakita, et al., 1994). The major phosphorylation site of interphase cells is Ser-19. When cells enter mitosis, Ser-19 is no longer phosphorylated, but Ser-1/2 become the major phosphorylation sites. During cytokinesis, Ser-1/2 phosphorylation is switched back to Ser-19, and Ser-19 phosphorylation persists during post-mitotic cell spreading, suggesting that Ser-19 phosphorylation may activate contractile rings and is required for stress

fiber reassembly. These biochemical studies are consistent with the observations that myosin II phosphorylated on Ser-19 localized in cleavage furrows (Post, et al., 1995; DeBiasio, et al., 1996; Matsumura, et al., 1998). Further, mutational analysis of a Drosophila *spaghetti squash* gene encoding MRLC revealed that phosphorylation of Drosophila MRLC on Ser-21 (which corresponds to Ser-19 of vertebrate MRLC) is essential for cell division (Jordan and Karess, 1997). A notable exception is myosin II of *Dictyostelium discoideum*, where heavy chain phosphorylation, but not light chain phosphorylation, is critical for the regulation of its cell motility and cytokinesis (Uyeda and Spudich, 1993; Hammer, 1994; Ostrow, et al., 1994).

Phosphorylation of MRLC at Ser-19 is controlled by the balance of two enzymatic activities, i.e. myosin light chain kinase(s) and myosin phosphatase. While recent efforts have focused on the functions of myosin phosphatase in the regulation of smooth muscle contraction, the functions of myosin phosphatase in nonmuscle cell motility are unclear. A trimeric myosin phosphatase is accepted as the major protein phosphatase that is responsible for dephosphorylation of MRLC in smooth muscle and perhaps in nonmuscle cells (see for review Hartshorne, et al., 1998). The holoenzyme consists of three subunits: a large subunit of about 130 kDa, a catalytic subunit of 38 kDa and a small subunit of 20 kDa (Alessi, et al., 1992; Shimizu, et al., 1994; Shirazi, et al., 1994). The catalytic subunit is the δ isoform of type 1 protein phosphatase (PP1cδ). The small subunit may be a regulatory subunit, but its function is unclear. The large subunit is known as the myosin phosphatase targeting subunit (MYPT) also referred as M130/133 (Shimizu, et al., 1994), M110 (Chen, et al., 1994) or myosin binding subunit (Okubo, et al., 1994; Kimura, et al., 1996a). MYPT can bind to both the catalytic subunit and myosin, and thus, will target the substrate, myosin, with the phosphatase. Without MYPT, PP1c showed low phosphatase activity toward myosin, indicating a critical role of MYPT in myosin dephosphorylation (Alessi, et al., 1992; Hirano, et al., 1997; Johnson, et al., 1997).

Recently there have been several reports that phosphorylation of MYPT may modulate phosphatase activity. It was shown that MYPT was phosphorylated by an unknown kinase copurified in the phosphatase holoenzyme preparations from smooth muscle and that this phosphorylation inhibited phosphatase activity (Ichikawa, et al., 1996). Subsequently, Rho-

kinase was shown to phosphorylate MYPT in the C-terminal region and this phosphorylation inhibited phosphatase activity (Kimura, et al., 1996a). It is known that Rho A is involved in the Ca^{2+}-sensitization process in smooth muscle (see for reviewSomlyo and Somlyo, 1994; Hartshorne, et al., 1998) and the possibility that this is due to inhibition of myosin phosphatase via phosphorylation of MYPT by Rho-kinase is attractive (Uehata, et al., 1997). It has also been suggested that a similar mechanism of phosphatase inhibition could initiate activation of contractile rings during cytokinesis (Amano, et al., 1996). Finally, the *in vitro* phosphorylation of MYPT in its C-terminal region by protein kinase A resulted in a decreased binding to acidic phospholipids (Ito, et al., 1997). This observation led to the hypothesis that within the cell the binding of MYPT to the cell membrane may be regulated by cAMP.

The changes in the phosphorylation states of MRLC during cell division suggest the presence of regulatory mechanisms of MRLC phosphorylation that involve kinases and/or phosphatases. In this work, I demonstrate the regulation of both kinase and phosphatase activities that are involved in MRLC phosphorylation during cell division. First, the kinase activity which phosphorylates MRLC at Ser-1/2 and Thr-9 is increased significantly at metaphase. This mitosis-specific phosphorylation of MRLC may be induced by $p34^{cdc2}$ kinase. Second, MYPT of myosin phosphatase is phosphorylated in a mitosis-specific way, resulting in the activation of phosphatase. Unlike phosphorylation by other kinases reported so far, the mitosis-specific phosphorylation provides a positive regulatory effect on phosphatase activity. The activation of myosin phosphatase, together with MRLC phosphorylation at Ser-1/2 and Thr-9, would promote the disassembly of stress fibers during prophase. The reduced level of phosphatase activity in cytokinesis may then lead to increased myosin phosphorylation at Ser-19 and activation of contractile rings.

3. Chapter 1

Mitosis-specific Phosphorylation of Smooth Muscle Regulatory Light Chain of Myosin II at Ser-1 and/or -2 and Thr-9 in Sea Urchin Egg Extract

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Footnotes

Abbreviations: MRLC, regulatory light chain of myosin II; MLCK, myosin light chain kinase; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DAPI, 4, 6-diamidino-2-phenyl indole; DW, distilled water.

ABSTRACT. We analyzed the kinase activities capable of phosphorylating the regulatory light chain of myosin-II (MRLC) from chicken gizzard in unfertilized and fertilized sea urchin egg extracts. Total kinase activity phosphorylating MRLC *in vitro* did not fluctuate throughout the first cell cycle. Phosphopeptide mapping analysis showed that MRLC was phosphorylated at two different sites corresponding to myosin light chain kinase purified from chicken gizzard (MLCK) and protein kinase C (PKC) phosphorylation sites, namely MLCK and PKC sites, respectively. The activity of the kinase(s) responsible for phosphorylation of MRLC at PKC sites showed a significant increase at metaphase. Phosphoamino acid analysis revealed that this increase in MRLC phosphorylation was due to phosphorylation at serine residue (Ser-1 and/or Ser-2) and at threonine residue (Thr-9). This increase in phosphorylation at PKC sites is occurred concomitantly with an increase in histone H1 kinase activity. In contrast, MRLC phosphorylation at MLCK sites showed no significant changes during the first cell cycle. Butyrolactone I, a selective inhibitor of $p34^{cdc2}$ kinase, inhibited the activity of the kinase(s) responsible for phosphorylation of MRLC at PKC sites at metaphase. These results suggest that the increase in MRLC phosphorylation at PKC sites (Ser-1 and/or -2, and Thr-9) at metaphase may be induced by $p34^{cdc2}$ kinase. Thus, $p34^{cdc2}$ kinase may be involved in the regulation of MRLC phosphorylation during cell division.

INTRODUCTION

In eukaryotic cells, marked changes in cell shape and structure occur during mitosis and cytokinesis (26, 29). In cultured cells, microfilaments bundle or stress fibers disassemble during prophase with cell rounding. Subsequently the microfilaments are transiently reorganized to form the contractile ring around the mitotic apparatus during anaphase. In telophase, constriction of the cleavage furrow occurs and finally divides the cell into two daughter cells. While the morphological aspects of these changes are well documented, the molecular mechanisms that control microfilament reorganization during cytokinesis are largely unknown (20, 24, 28). Myosin-II and actin accumulate in the contractile ring (7, 25), and myosin-II is thought to function as a motor for cytokinesis (6, 16, 19). In smooth muscle, phosphorylation of the regulatory light chain of myosin-II (MRLC) by myosin light chain kinase (MLCK) is thought to be a trigger for initiation of the constriction (30). Phosphorylation of MRLC by MLCK results in activation of actin-activated myosin-II Mg^{2+}-ATPase and an increase in the stability of myosin-II filaments. Therefore, phosphorylation of MRLC has been suspected to be a signal for the induction of cytokinesis (9).

Satterwhite *et al.* compared the phosphorylation state of MRLC *in vitro* in Xenopus oocyte extracts at interphase with those at metaphase (27). The sites of phosphorylation in interphase corresponded to those phosphorylated by MLCK. In metaphase, MRLC was phosphorylated at the sites where protein kinase C (PKC) phosphorylates. Further, $p34^{cdc2}$ kinase phosphorylated MRLC at PKC sites which are known to inhibit its actin-activated ATPase activity and to reduce the stability of myosin-II filaments (2, 13, 21). Thus, they proposed that MRLC phosphorylation at PKC sites by $p34^{cdc2}$ kinase inhibits myosin-II activity and delays cytokinesis until the onset of anaphase. Yamakita *et al.* (32) investigated the phosphorylation states of MRLC *in vivo* in cultured cells. In metaphase, MRLC was phosphorylated mainly at PKC sites. After the release of mitotic arrest, the phosphorylation level of MLCK sites gradually increased as with the progression of cell division. This suggests that MLCK is transiently inactive at metaphase. Thus, the changes in phosphorylation state of MRLC may play an important role in signaling cytokinesis (8, 27, 32).

We analyzed MRLC kinase activities in unfertilized and fertilized sea urchin egg extracts. Our results suggest that at metaphase $p34^{cdc2}$ kinase may be involved in the increase of MRLC phosphorylation at PKC sites (Ser-1 and/or -2, and Thr-9). Thus, $p34^{cdc2}$ kinase may play important roles in the regulation of MRLC phosphorylation during cell division.

RESULTS

MRLC kinase activities in sea urchin eggs. We first examined the histone H1 kinase activities in unfertilized and fertilized of sea urchin eggs. After insemination, the eggs were cultured and egg extracts were prepared at the indicated times. As shown in Fig. 1A, histone H1 kinase activity was increased at metaphase (70-90 min after fertilization). Then we examined the MRLC kinase activities. The total MRLC kinase activity was higher in the presence of Ca^{2+}/calmodulin than in their absence (Fig. 1B). There was no significant change in the extent of MRLC phosphorylation during the first cell cycle either in the presence or absence of Ca^{2+}/calmodulin.

The changes in the phosphorylation sites of MRLC during cell cycle of sea urchin eggs. For comparison, MRLC was phosphorylated by MLCK, PKC, and the egg extract. The phosphorylated MRLC bands were processed for one-dimensional phosphopeptide mapping, and then the phosphorylation sites were analyzed (Fig. 2A). The phosphopeptide pattern of MRLC phosphorylated by MLCK showed three major spots (lane 1, M). In contrast, that of MRLC phosphorylated by PKC showed two higher mobility spots (lane 2, C1 and C2). Lane 3 shows the phosphopeptide map of MRLC phosphorylated by the egg extract prepared at metaphase. The phosphopeptide pattern demonstrated that MRLC was phosphorylated at both MLCK sites and PKC sites by the egg extract. The same results were obtained when each peptide was processed for two-dimensional phosphopeptide mapping analysis (data not shown).

We analyzed the changes in the levels of phosphorylation at each site (C1, C2, and M, respectively) during the cell cycle. MRLC was phosphorylated by each egg extract as shown in Fig. 1B, and then processed for phosphopeptide mapping analysis. Fig. 2B and C showed that at metaphase (70-90 min after fertilization) the levels of MRLC phosphorylation at PKC sites (C1 and C2) were increased either in the presence or absence of Ca^{2+}/calmodulin. This increase in phosphorylation at PKC sites occurred concomitantly with an increase in histone H1 kinase activity (Fig. 1A). Densitometric analysis of the autoradiograms showed that the extent of phosphorylation at PKC sites by the egg extract prepared at 80 min after fertilization was about three times than that by unfertilized egg extract (data not shown). In contrast, the changes in phosphorylation level at

MLCK sites (M) did not show a significant change in the presence of Ca^{2+}/calmodulin during the cell cycle (Fig. 2B). However, in the absence of Ca^{2+}/calmodulin there was a slight increase in phosphorylation at MLCK sites accompanied by the onset of mitosis, the level of phosphorylation being kept almost constant thereafter (Fig. 2C). The extent of phosphorylation at MLCK sites was much greater in the presence than in the absence of Ca^{2+}/calmodulin.

Phosphoamino acid analysis of the PKC site phosphorylation at metaphase. We investigated the phosphoamino acid residues in the C1 and C2 peptides. As shown in Fig. 3, phosphoamino acid analysis revealed that C1 peptide was phosphorylated at threonine residues but not at serine residues (lanes 1 and 3). In contrast, C2 peptide was phosphorylated at serine residues but not at threonine residue (lanes 2 and 4). It was reported that phosphorylation of MRLC by PKC occurred at Ser-1, Ser-2, and Thr-9 (2). Thus, C1 and C2 peptides may be phosphorylated at Thr-9 and at Ser-1 and/or Ser-2, respectively. These results suggest that the increase of MRLC phosphorylation at metaphase was due to phosphorylation at Ser-1 and/or -2, and Thr-9.

Inhibitory effect of Butyrolactone I against the kinase activity that phosphorylates MRLC at PKC sites. We analyzed the kinase activity responsible for phosphorylation of MRLC at PKC sites at metaphase. It has been shown that PKC sites are phosphorylated by PKC or $p34^{cdc2}$ kinase (27, 32). As described above, the increase of MRLC phosphorylation at PKC sites occurred simultaneously with the increase in histone H1 kinase activity. To investigate whether the phosphorylation at PKC sites was mediated by $p34^{cdc2}$ kinase, the inhibitory effect of butyrolactone I, a selective inhibitor of $p34^{cdc2}$ kinase, was examined. As shown in Fig. 4, butyrolactone I inhibited histone H1 kinase activity in the egg extract prepared at 80 min after fertilization (IC_{50}= 5 μM), with a sensitivity similar to that reported against purified $p34^{cdc2}$ kinase (15). Fig. 5A shows the phosphopeptide map of MRLC phosphorylated in the presence of butyrolactone I. Butyrolactone I also inhibited MRLC phosphorylation at PKC sites in the egg extract prepared at 80 min after fertilization. The inhibitory effect showed higher sensitivity against phosphorylation at PKC than at MLCK sites. The relative ratios of phosphorylation of each peptide (C1, C2, and M, respectively) were quantified and are shown in Fig. 5B. The IC_{50} value of

butyrolactone I against the phosphorylation at PKC sites was about 3 μM. This inhibitory effect of butyrolactone I against the kinase responsible for phosphorylation at PKC sites shows a similar sensitivity to that against histone H1 kinase activity (compare Figs. 4 and 5). In contrast, the inhibitory effect of butyrolactone I against phosphorylation at PKC sites was about 100-fold higher than that against MLCK sites (Fig. 5B). These results suggest that the increase of phosphorylation at PKC sites at metaphase may be mediated by $p34^{cdc2}$ kinase.

DISCUSSION

Our results demonstrated that the states of MRLC phosphorylation at PKC sites show a significant change in a cell cycle-dependent manner in sea urchin eggs. MRLC phosphorylation at PKC sites is increased at metaphase in the first cell cycle (Fig. 2). Then, the phosphorylation at PKC sites decreases at anaphase. Furthermore, we showed that MRLC is phosphorylated not only at PKC sites but also at MLCK sites at metaphase. MRLC phosphorylation by PKC is known to inhibit actin-activated Mg^{2+}-ATPase activity and to reduce the stability of myosin-II filaments (2, 13). It has also been reported that additional MRLC phosphorylation by PKC after initial phosphorylation by MLCK results in a decrease of actin-activated Mg^{2+}-ATPase activity of myosin II (22). These results suggest that myosin II Mg^{2+}-ATPase activity is relatively low at metaphase as compared with that at other mitotic stages.

Satterwhite *et al.* (27) reported that in metaphase MRLC was phosphorylated at PKC sites by $p34^{cdc2}$ kinase in *Xenopus* egg lysates, but not in interphase, in which phosphorylation was detected only at MLCK sites. Thus, they proposed that MRLC phosphorylation at PKC sites by $p34^{cdc2}$ kinase inhibits myosin-II activity during pro/metaphase and delays cytokinesis until the onset of anaphase. Yamakita *et al.* (32) reported phosphorylation states of MRLC *in vivo* in cultured cells. At metaphase, MRLC was phosphorylated mainly at PKC sites. After the release of mitotic arrest, the phosphorylation level of MLCK sites gradually increased as cells underwent cell division. They confirmed the results by Satterwhite *et al.* and suggested that the changes in phosphorylation of MRLC from PKC sites to MLCK sites may be a signal for induction of cytokinesis. These results are consistent with those obtained here from sea urchin eggs with regard to the phosphorylation level of PKC sites.

In this study, we investigated MRLC phosphorylation in detail during the cell cycle. The increase in MRLC phosphorylation at PKC sites in the presence of Ca^{2+}/calmodulin occurred in parallel with the activation of histone H1 kinase activity (Figs. 1 and 2). This phosphorylation at PKC sites took place even in the absence of Ca^{2+}/calmodulin. These results suggest that the phosphorylation

at PKC sites is not caused by PKC. Thus, to investigate whether the phosphorylation at PKC sites is caused by $p34^{cdc2}$ kinase, the effect of butyrolactone I, a selective inhibitor of $p34^{cdc2}$ kinase, were examined. Butyrolactone I inhibited the phosphorylation at PKC sites as well as histone H1 kinase activity in the egg extract (Figs. 4 and 5), with as IC_{50} value was of about 2-5 μM. In contrast, its IC_{50} value against the phosphorylation at MLCK sites was about 100-fold higher than that against phosphorylation at PKC sites. The IC_{50} values of butyrolactone I against $p34^{cdc2}$ kinase and PKC were reported to be 0.68 and 160 μM, respectively (15). Therefore, it is reasonable to assume that phosphorylation at PKC sites at metaphase is induced by $p34^{cdc2}$ kinase. It was also reported that butyrolactone I inhibits cdk 2 kinase, a cdc2 family kinase (15). Thus, it is also possible that MRLC phosphorylation at PKC sites is induced by other enzymes involved in cyclin-dependent kinase family.

Recently, Mishima and Mabuchi reported that MRLC phosphorylation is increased at mitotic phase in sea urchin egg (21). They demonstrated that $p34^{cdc2}$ kinase is not responsible for MRLC phosphorylation at metaphase because of the small effect of butyrolactone I against the total MRLC kinase activity. However, in their experiments, the inhibitory effects of butyrolactone I against phosphorylation at each site (MLCK or PKC sites) are unclear. In this study, we analyzed the inhibitory effects of butyrolactone I in detail (Fig. 5). The results obtained here indicate that $p34^{cdc2}$ kinase may be responsible for the phosphorylation at PKC sites but not at MLCK sites. However, further investigations are required to determine whether MRLC phosphorylation at PKC sites at metaphase is induced by $p34^{cdc2}$ kinase.

Interestingly, the phosphorylation at MLCK sites occurred even in the absence of Ca^{2+}/calmodulin (Fig. 2). It has been reported that *Dictyostelium* MLCK is Ca^{2+}/calmodulin independent (31). Recently, we reported that mitogen-activated protein kinase activated protein kinase 2 (MAPKAP kinase 2) phosphorylates MRLC at MLCK sites in the absence of Ca^{2+}/calmodulin (17). However, it has not been determined whether the Ca^{2+}/calmodulin-independent MLCK is MAPKAP kinase 2. Further investigations are necessary to identify the

kinase(s) responsible for phosphorylation of MRLC at MLCK sites in the absence of Ca^{2+}/calmodulin in sea urchin eggs.

Although the phosphorylation at MLCK sites is elevated in the presence of Ca^{2+}/calmodulin, this activation was not so marked (about 2-3 fold) as compared with the case of smooth muscle MLCK (1). Sea urchin MLCK has not been identified, but it has been reported that a multifunctional Ca^{2+}/calmodulin-dependent kinase has MRLC phosphorylating activity in sea urchin eggs (5). It is possible that sea urchin MLCK is different from conventional Ca^{2+}/calmodulin-dependent MLCKs (e.g. smooth muscle or non-muscle MLCK). Thus, purification of MLCK from sea urchin eggs remains to resolve these problems. Our preliminary results in identifying sea urchin MLCK showed that the enzyme in sea urchin eggs has a smaller molecular mass than conventional MLCKs.

As mentioned above, the regulation of MRLC phosphorylation in sea urchin eggs seems to be quite complex. In this study, the MRLC phosphorylation at MLCK sites showed no significant change during the first cell cycle in the presence of Ca^{2+}/calmodulin (Fig. 2). However, the localization of the phosphorylated MRLC during the cell cycle is largely unclear. It is important to investigate the spatial and temporal localization of MLCK site- and PKC site-phosphorylated MRLC. For this purpose, phosphorylation site-specific antibodies are useful tools to analyze the regulation of MRLC phosphorylation. Indeed, we developed a specific antibody that recognizes only MLCK site-phosphorylated MRLC, and the antibody showed a restricted staining pattern in dividing HeLa cells (our unpublished results). Thus, it is likely that the regulation of phosphorylation state of MRLC plays important roles in cytokinesis.

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LEGENDS FOR FIGURES

Fig. 1. Histone H1 and MRLC kinase activity during the first cell cycle. Histone H1 and MRLC were phosphorylated with egg extracts that prepared at the indicated times after fertilization. (A) Developmental stages of fertilized eggs were determined by observation of eggs staining with DAPI. Histone H1 kinase activity was assayed using histone H1 as a substrate. (B) MRLC kinase activity was assayed using MRLC as a substrate. Assays were carried out in the presence (open circles) or absence (closed circles) of Ca^{2+}/calmodulin.

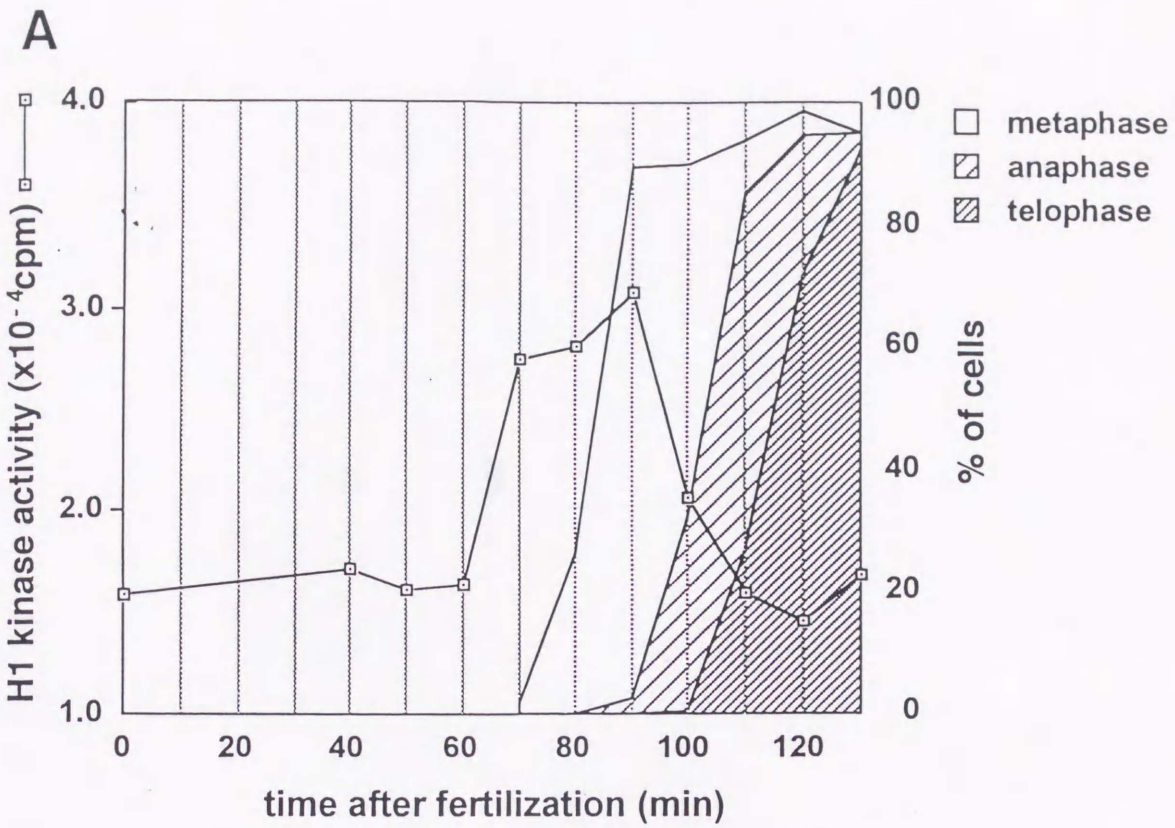
Fig. 2. Autoradiogram of one-dimensional phosphopeptide map of MRLC. Tryptic phosphopeptides of MRLC were prepared as described under MATERIALS AND METHODS. Samples were processed for electrophoresis (vertical dimension bottom to top). The origin is indicated as ori. (A) MRLC was phosphorylated by MLCK (lane 1), PKC (lane 2), or the egg extract (lane 3). The phosphopeptides that were phosphorylated at MLCK sites are indicated as M. C1 and C2 indicate the peptides phosphorylated at PKC sites. (B and C) MRLC was phosphorylated by egg extracts prepared at the indicated time after fertilization as shown in Fig. 1B. MRLC was phosphorylated in the presence (B) or absence (C) of Ca^{2+}/calmodulin.

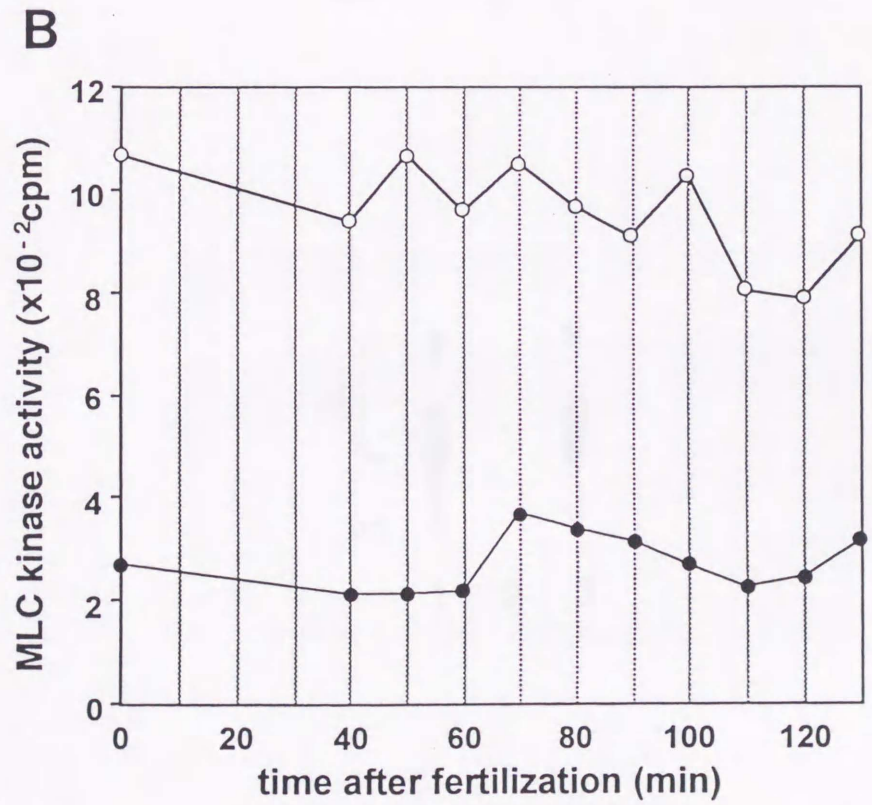
Fig. 3. Phosphoamino acid analysis of C1 and C2 peptides. MRLC was phosphorylated by the egg extract prepared at 80 min after fertilization, then C1 and C2 peptides were prepared as described in the legend to Fig. 2. Phosphoamino acid analysis was performed as described under MATERIALS AND METHODS. Lanes 1 and 2, marker phosphoamino acids detected by ninhydrin. Lanes 3 and 4, corresponding autoradiography. Lanes 1 and 3, C1 peptide. Lanes 2 and 4, C2 peptide.

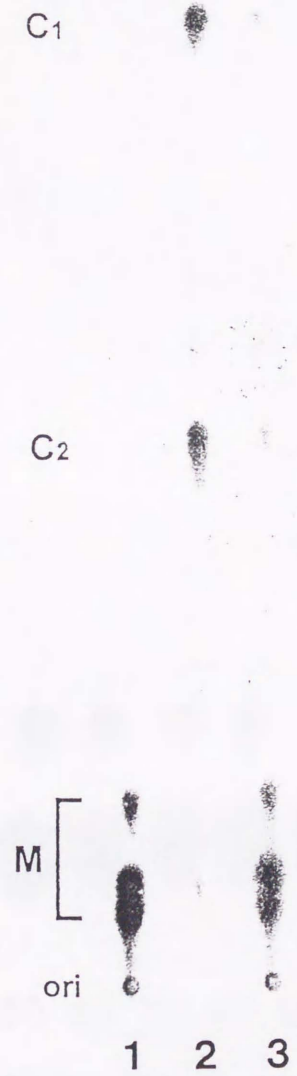
Fig. 4. Inhibitory effect of butyrolactone I against histone H1 kinase activity. Histone H1 was phosphorylated by egg extract prepared at 80 min after fertilization with various concentrations of butyrolactone I. Phosphorylation of histone H1 was quantified by densitometric analysis of autoradiograms.

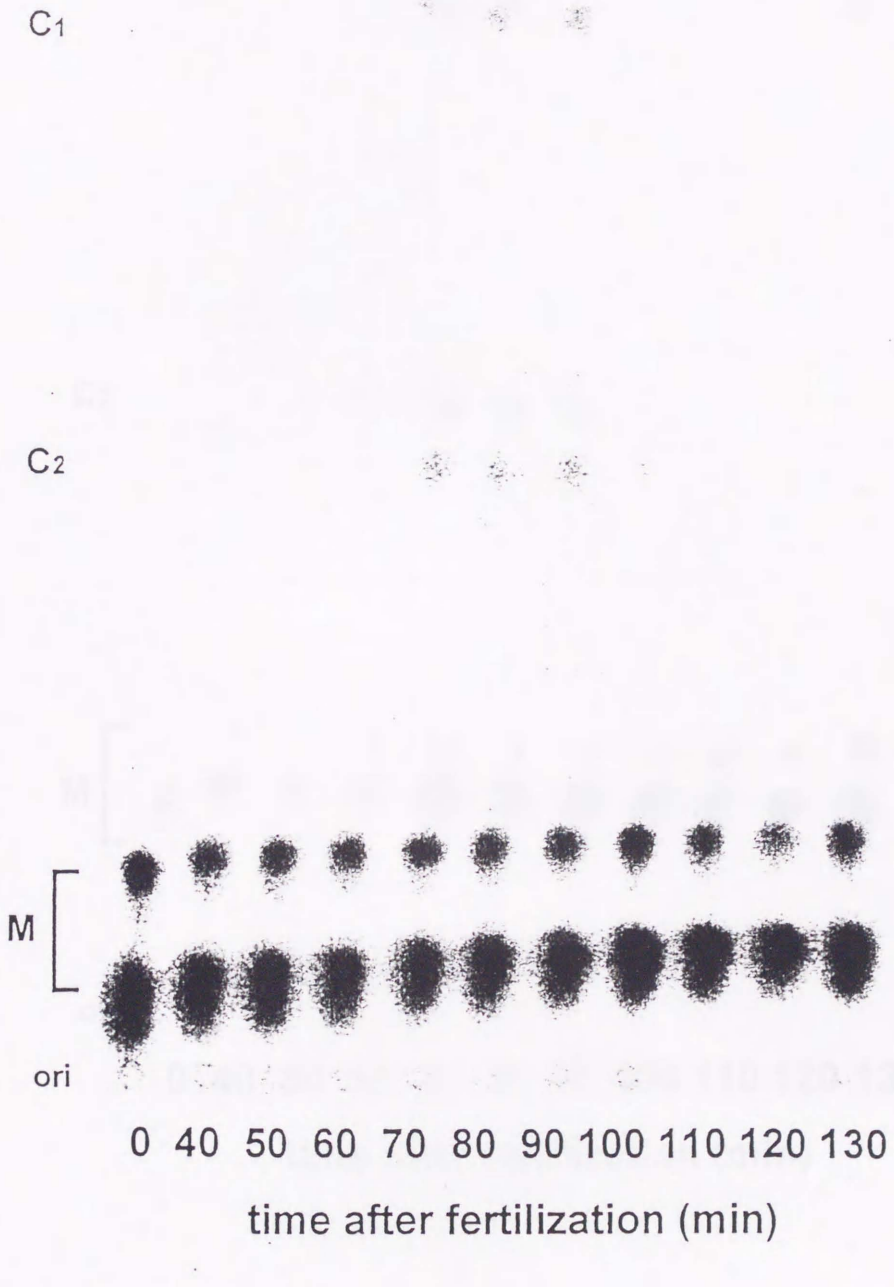
Fig. 5. Inhibitory effect of butyrolactone I against MRLC phosphorylation. MRLC was phosphorylated by egg extract prepared at 80 min after fertilization with various concentrations of butyrolactone I. Phosphorylated MRLC was subjected to one-dimensional phosphopeptide

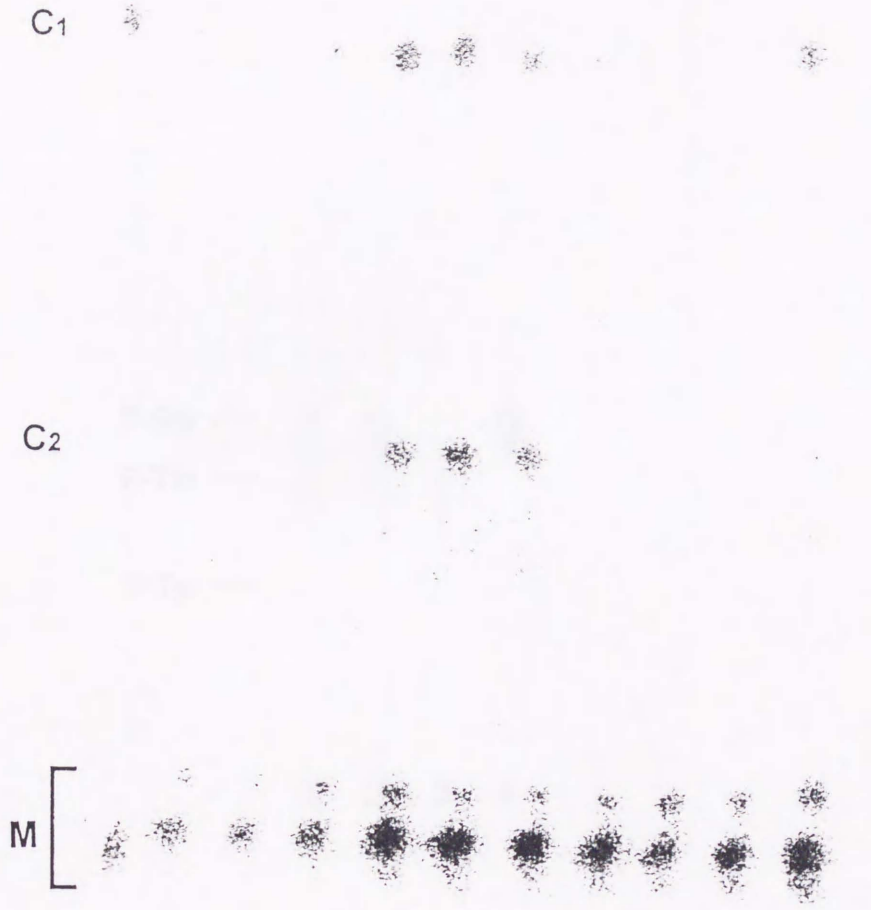
mapping. (A) Autoradiogram of phosphopeptide map of MRLC phosphorylated in the presence of butyrolactone I. (B) Quantitative analysis of phosphorylation at MLCK and PKC sites. Each phosphopeptide shown in A was quantified by densitometric analysis. Closed circles indicate the extents of phosphorylation at MLCK sites (M), and open circles and boxes indicate phosphorylation at PKC sites, C1 and C2 peptides, respectively.

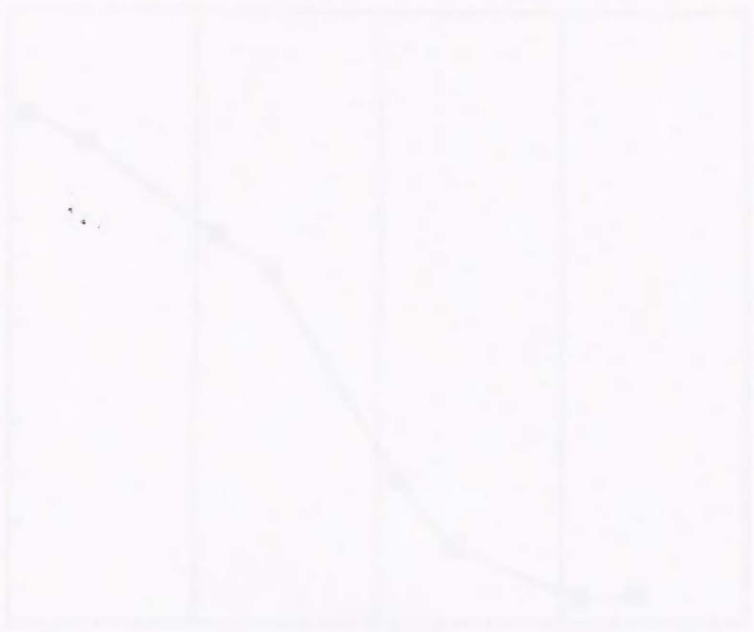

A
H1 kinase activity (x10⁻⁴cpm)
4.0
3.0
2.0
1.0
100
80
60
40
20
0
% of cells
metaphase
anaphase
telophase
0
20
40
60
80
100
120
time after fertilization (min)


B
MLC kinase activity (x10⁻²cpm)
12
10
8
6
4
2
0
0
20
40
60
80
100
120
time after fertilization (min)


C1
C2
M
ori
1
2
3


C1
C2
M
ori
0 40 50 60 70 80 90 100 110 120 130
time after fertilization (min)


C1
C2
M
ori
0 40 50 60 70 80 90 100 110 120 130
time after fertilization (min)



P-Ser —

P-Thr —

P-Tyr —

1 2 3 4

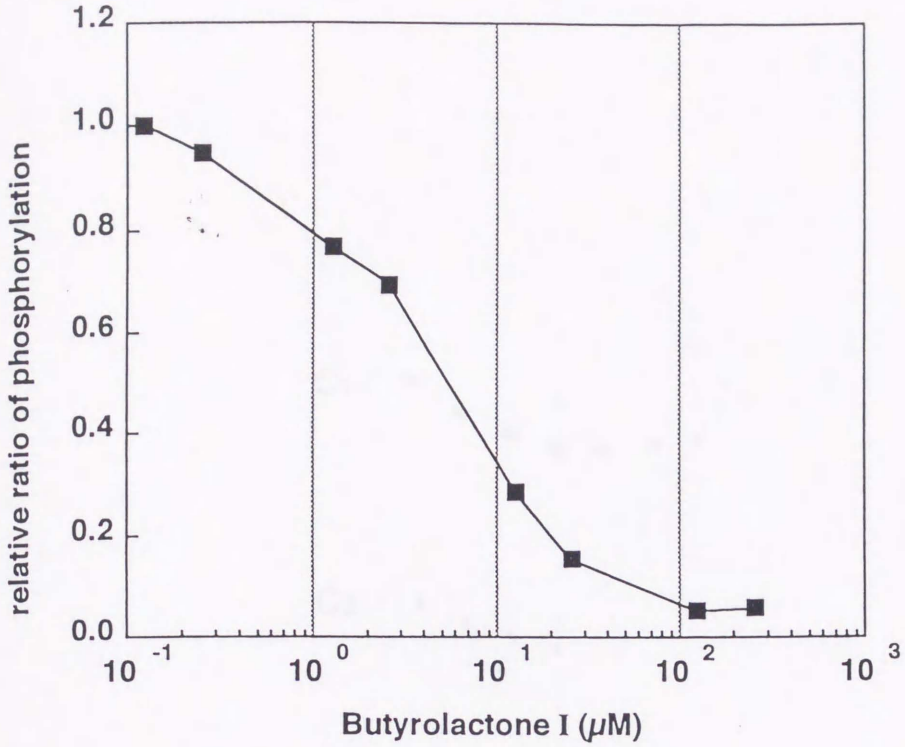
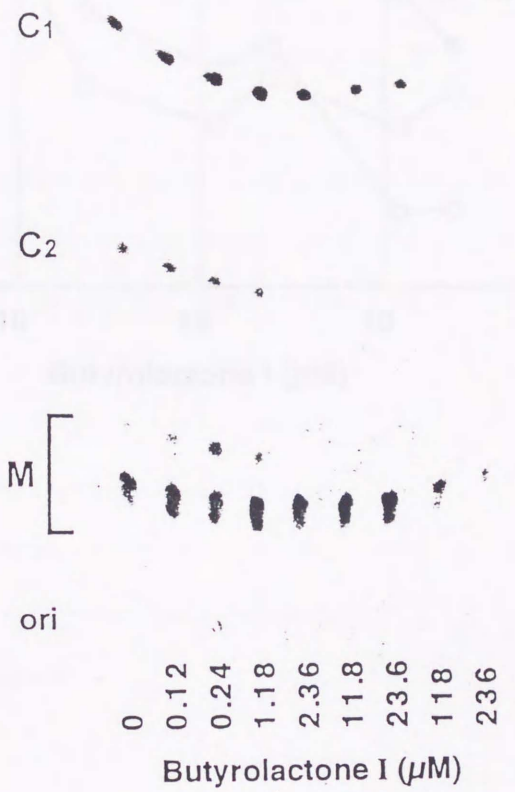
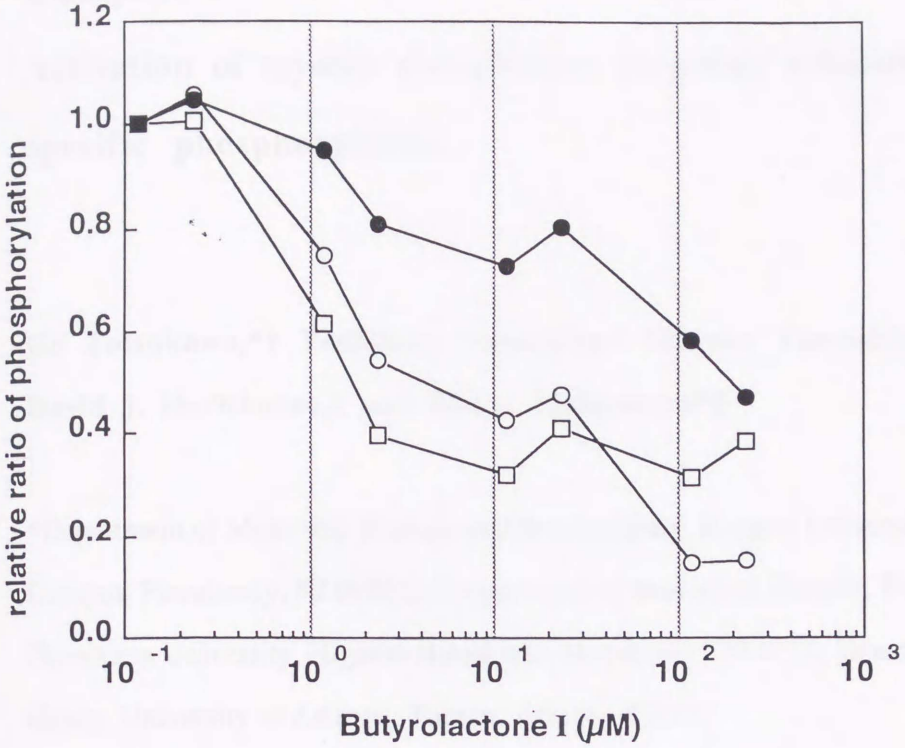

1.2
1.0
0.8
0.6
0.4
0.2
0.0
relative ratio of phosphorylation
10 -1
10 0
10 1
10 2
10 3
Butyrolactone I (μM)

Fig 5A




relative ratio of phosphorylation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10 -1
10 0
10 1
10 2
10 3
Butyrolactone I (μM)

4. Chapter 2

Activation of myosin phosphatase targeting subunit by mitosis-specific phosphorylation

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Running Title: Mitosis-specific phosphorylation of myosin phosphatase
Key words: myosin, phosphorylation, phosphatase, mitosis, myosin binding

1. Abbreviations used in this paper: mAb, monoclonal antibody; MLCK, myosin light chain kinase; MYPT, myosin phosphatase targeting subunit; pAb, polyclonal antibody; PP1c, catalytic subunit of type 1 protein phosphatase; RMLC, regulatory myosin light chain.

Abstract

It has been previously demonstrated that during mitosis the sites of myosin phosphorylation are switched between the inhibitory sites, Ser 1/2, and the activation sites, Ser 19/Thr18 (Yamakita, Y., Yamashiro, S., Matsumura, F. J. Cell Biol. 124: 129-137. 1994; Satterwhite, L.L., Lohka, M.J., Wilson, K.L., Scherson, T.Y., Cisek, L.J., Corden, J.L., Pollard, T.D. J. Cell Biol. 118: 595-605. 1992), suggesting a regulatory role of myosin phosphorylation in cell division. To explore the function of myosin phosphatase in cell division, the possibility that myosin phosphatase activity may be altered during cell division was examined. We have found that the myosin phosphatase targeting subunit (MYPT) undergoes mitosis-specific phosphorylation and that the phosphorylation is reversed during cytokinesis. MYPT phosphorylated either *in vivo* or *in vitro* in the mitosis-specific way showed higher binding to myosin II (2 to 3 fold) compared to MYPT from cells in interphase. Furthermore, the activity of myosin phosphatase was increased more than twice and it is suggested this reflected the increased affinity of myosin binding. These results indicate the presence of a unique positive regulatory mechanism for myosin phosphatase in cell division. The activation of myosin phosphatase during mitosis would enhance dephosphorylation of the myosin regulatory light chain, thereby leading to the disassembly of stress fibers during prophase. The mitosis-specific effect of phosphorylation is lost on exit from mitosis and the resultant increase in myosin phosphorylation may act as a signal to activate cytokinesis.

At mitosis, normal animal cultured cells show profound reorganization of the microfilament cytoskeleton. During prophase, both stress fibers and focal adhesions are disassembled, resulting in cell rounding. At cytokinesis, microfilaments form a contractile ring that contracts to generate daughter cells. During post-mitotic cell spreading, microfilaments reassemble into stress fibers and focal adhesions are formed. The molecular mechanisms underlying these drastic alterations in the microfilament organization during cell division are largely unknown.

Phosphorylation of the regulatory light chain of myosin II (RMLC)[1] is thought to regulate actomyosin contractility in smooth muscle and in many non-muscle cells (see for reviewKamm and Stull, 1985; Moussavi, et al., 1993; Somlyo and Somlyo, 1994). RMLC is phosphorylated by myosin light chain kinase at two sites, a primary site, Ser 19, and a secondary site, Thr 18. It can also be phosphorylated (for example by protein kinase C) at Ser 1 or Ser 2 and Thr 9. *In vitro*, the phosphorylation at Ser 19 activates actin-activated ATPase activity of myosin II (Sellers, 1991; Trybus, 1991) whereas phosphorylation at Ser 1/2 and Thr 9 can inhibit ATPase activity of myosin phosphorylated at Ser 19 (Bengur, et al., 1987; Ikebe and Reardon, 1990). *In vivo*, phosphorylation of Ser 19 is correlated with a variety of contractile processes including smooth muscle contraction (Sellers, 1991; Trybus, 1991), contraction of cultured cells upon serum stimulation or treatment with certain drugs (Giuliano, et al., 1992; Kolodney and Elson, 1993; Goeckeleer and Wysolmerski, 1995), stress-fiber assembly upon serum stimulation of starved 3T3 cells (Chrzanowska-Wodnicka and Burridge, 1996), and cytokinesis of higher eukaryotic cells (Yamakita, et al., 1994; Post, et al., 1995; DeBiasio, et al., 1996; Jordan and Karess, 1997; Matsumura, et al., 1998).

Previously, it was demonstrated that during cell division the sites of phosphorylation on RMLC changed (Satterwhite, et al., 1992; Yamakita, et al., 1994; Totsukawa, et al., 1996). The major phosphorylation site of interphase cells is Ser 19. When cells enter mitosis, Ser 19 is no longer phosphorylated, but Ser 1/2 become the major phosphorylation sites. During cytokinesis, Ser 1/2 phosphorylation is switched back to Ser 19, and Ser 19 phosphorylation persists during post-mitotic cell spreading, suggesting that Ser 19 phosphorylation may activate contractile rings and is required for stress fiber reassembly. These biochemical studies are consistent with the

observations that myosin II phosphorylated on Ser 19 localized in cleavage furrows (Post, et al., 1995; DeBiasio, et al., 1996; Matsumura, et al., 1998). Further, mutational analysis of a Drosophila *spaghetti squash* gene encoding RMLC revealed that phosphorylation of Drosophila RMLC on Ser 21 (which corresponds to Ser 19 of vertebrate RMLC) is essential for cell division (Jordan and Karess, 1997). A notable exception is myosin II of *Dictyostelium discoideum*, where heavy chain phosphorylation, but not light chain phosphorylation, is critical for the regulation of its cell motility and cytokinesis (Uyeda and Spudich, 1993; Hammer, 1994; Ostrow, et al., 1994). Phosphorylation of MRLC at Ser 19 is controlled by the balance of two enzymatic activities, i.e. myosin light chain kinase(s) and myosin phosphatase. While recent efforts have focused on the functions of myosin phosphatase in the regulation of smooth muscle contraction, the functions of myosin phosphatase in nonmuscle cell motility are unclear. A trimeric myosin phosphatase is accepted as the major protein phosphatase that is responsible for dephosphorylation of MRLC in smooth muscle and perhaps in nonmuscle cells (see for reviewHartshorne, et al., 1998). The holoenzyme consists of three subunits: a large subunit of about 130 kDa, a catalytic subunit of 38kDa and a small subunit of 20kDa (Alessi, et al., 1992; Shimizu, et al., 1994; Shirazi, et al., 1994). The catalytic subunit is the δ isoform of type 1 protein phosphatase (PP1cδ). The small subunit may be a regulatory subunit, but its function is unclear. The large subunit is known as the myosin phosphatase targeting subunit (MYPT) also referred as M130/133 (Shimizu, et al., 1994), M110 (Chen, et al., 1994) or myosin binding subunit (Okubo, et al., 1994; Kimura, et al., 1996). MYPT can bind to both the catalytic subunit and myosin, and thus, will target the substrate, myosin, with the phosphatase. Without MYPT, PP1c showed low phosphatase activity toward myosin, indicating a critical role of MYPT in myosin dephosphorylation (Alessi, et al., 1992; Hirano, et al., 1997; Johnson, et al., 1997).

Recently there have been several reports that phosphorylation of MYPT may modulate phosphatase activity. Initially, it was found that incubation of α-toxin permeabilized rabbit portal vein with ATPγS caused inhibition of phosphatase activity and concomitant thiophosphorylation of MYPT (Trinkle-Mulcahy, et al., 1995). Next it was shown that MYPT was phosphorylated by an

unknown kinase copurified in the phosphatase holoenzyme preparations from smooth muscle and that this phosphorylation inhibited phosphatase activity (Ichikawa, et al., 1996). The major site of phosphorylation of MYPT was T654 or T695 for the M130 and M133 MYPT isoforms, respectively. Subsequently, Rho-kinase was shown to phosphorylate MYPT in the C-terminal region and again this phosphorylation inhibited phosphatase activity (Kimura, et al., 1996). It is known that Rho A is involved in the Ca^{2+}-sensitization process in smooth muscle (see for reviewSomlyo and Somlyo, 1994; Hartshorne, et al., 1998) and the possibility that this is due to inhibition of myosin phosphatase via phosphorylation of MYPT by Rho-kinase is attractive (Uehata, et al., 1997). It has also been suggested that a similar mechanism of phosphatase inhibition could initiate activation of contractile rings during cytokinesis (Amano, et al., 1996). Finally, the *in vitro* phosphorylation of MYPT in its C-terminal region by protein kinase A resulted in a decreased binding to acidic phospholipids (Ito, et al., 1997). This observation led to the hypothesis that within the cell the binding of MYPT to the cell membrane may be regulated by cAMP.

The changes in the phosphorylation states of RMLC during cell division (Satterwhite, et al., 1992; Yamakita, et al., 1994; Totsukawa, et al., 1996; Matsumura, et al., 1998) suggest the presence of regulatory mechanisms of MRLC phosphorylation that involve kinases and/or phosphatases. Based on the above discussion, it was thought that one reasonable possibility was the phosphorylation of MYPT and modification of myosin phosphatase activity. In this paper, we demonstrate that MYPT is phosphorylated in a mitosis-specific way. Unlike phosphorylation by other kinases reported so far, the mitosis-specific phosphorylation provides a positive regulatory effect on phosphatase activity. Phosphorylated MYPT shows an increased myosin binding ability, resulting in the activation of phosphatase during mitosis. Such activation would increase the dephosphorylation of RMLC and thus promote the disassembly of stress fibers during prophase. The mitosis-specific modifications, i.e. phosphorylation of MYPT and activation of phosphatase activity, are lost as the cell enters cytokinesis. The reduced level of phosphatase activity may then lead to increased myosin phosphorylation and activation of contractile rings.

Materials and methods

Cell culture

SV-40 transformed rat embryo cells (REF-2A) were maintained in Dulbecco's modified Eagle's medium (DME) containing 10 % newborn calf serum in an atmosphere of 5 % CO_2 and 95 % air at 37 °C. Chinese hamster ovary (CHO) cells were maintained in F 12 medium containing 10 % fetal bovine serum. REF-2A cells at mitotic and later stages of cell division were prepared as described previously (Yamashiro, et al., 1990; Hosoya, et al., 1993; Yamakita, et al., 1994). Briefly, cells were first treated for 3 h with 0.25 μg/ml nocodazole, and mitotic cells (prometaphase) were collected. After washing with ice-cold DME to remove nocodazole, cells were plated in fresh culture dishes and incubated at 37 °C in DME containing 10 % new born calf serum to allow cell cycle progression. Mitotic cells recovered spindles at 10-20 min after release of nocodazole arrest, and underwent cytokinesis at 40-60 min. In some experiments, REF-2A cells at each mitotic stage were labeled with ^{32}P-orthophosphoric acid as described previously (Yamashiro, et al., 1990; Hosoya, et al., 1993; Yamakita, et al., 1994).

Antibodies

The following antibodies against MYPT were used: a polyclonal antibody (rabbit) raised against the N-terminal 38 residues of chicken gizzard MYPT, termed Ab_{1-38} (BAbCo., Richmond, CA) (Muranyi, et al., 1998); a polyclonal antibody (rabbit) raised against residues 1-296 of gizzard MYPT, affinity purified using the MYPT fragment 1-296, termed Ab_{1-296}; a monoclonal antibody (IgG1) raised against the gizzard phosphatase holoenzyme (BAbCo.) (Trinkle-Mulcahy, et al., 1995). Other antibodies were: a monoclonal antibody against the type 1 phosphatase catalytic subunit, PP1c, (Transduction Labs., Lexington, KY); and a monoclonal antibody against the polyhistidine tag (Sigma, St Louis, MO).

Protein preparations

Trimeric myosin phosphatase was purified from chicken gizzard according to the method of Alessi et al. (Alessi, et al., 1992). Nonmuscle myosin II was purified from bovine lung as described (Sellers, 1991). For the preparation of Ser 19-phosphorylated myosin, purified myosin was incubated for 30 min at 25 °C with 10 μg/ml MLCK and 5 μg/ml calmodulin in 30 mM Tris-HCl

(pH 7.5), 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM ATP (with or without 0.1 mCi/ml [γ-^{32}P] ATP). Phosphorylated myosin was dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 1 M KCl, 1 mM $MgCl_2$ and 0.1 mM DTT. Recombinant protein phosphatase type1 catalytic subunit, α isoform (PP1cα) was purchased from Calbiochem (La Jolla, CA).

Immunoprecipitation and Immunoblotting

Immunoprecipitation of MYPT was performed using two different buffers (buffer I and II): Buffer I contains Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5 % NP-40, 25 mM NaF, 100 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 1mM sodium vanadate, 1 mM DTT, 1 mM benzamidine, 10μg/ml leupeptin, 10 μg/ml aprotinin and 1mM PMSF. Due to the high salt concentration of buffer I, MYPT was immunoprecipitated without the catalytic subunit. Immunoprecipitation of MYPT with buffer I was used for the myosin binding experiments. Buffer II was a lower ionic strength and was the same as buffer I except for omission of NaF and sodium vanadate and 25mM sodium pyrophosphate and 20mM β-glycerophosphate. Using buffer II MYPT was immunoprecipitated in the myosin phosphatase complex and was used to determine myosin phosphatase activity.

Mitotic or interphase REF-2A cells were lysed in either immunoprecipitation buffer I or II. Cells were homogenized with a Dounce homogenizer and clarified by centrifugation at 16,000g for 15 min. Cell lysates were stored at –80 °C. After thawing quickly, the lysates were again centrifuged at 16,000g for 15 min. $Ab_{1\text{-}296}$ or $Ab_{1\text{-}38}$ was added to the supernatants and incubated for 2 hrs at 4 °C. The immunocomplex was precipitated with Protein A-Sepharose (Pharmacia, Piscataway, NJ) during a 1 hr incubation. The immunocomplex was washed 3 times with each buffer, once with PBS, and analyzed by SDS-PAGE followed by Western blotting. The immunoprecipitated MYPT also was used for myosin binding or phosphatase assays.

Immunoblotting was performed as follows: Polyvinylidene difluoride (PVDF) membranes were blocked with 5 % non-fat dried milk in PBS, and then incubated with the primary antibody (1:1000 dilution) containing 0.3 % BSA in PBS. Immunoreactive bands were detected with peroxidase-

conjugated secondary antibody (1:1000 dilution) using a chemiluminescence method (NEN, Boston, MA).

Protein phosphatase treatment of MYPT

Rat MYPT was prepared by immunoprecipitation using buffer I as described above. Half of the immunoprecipitate was treated for 30 min at 30 °C with one unit of recombinant serine/threonine phosphatase (PP1cα from rabbit skeletal muscle, Calbiochem) in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MnCl_2$, 2 mM $MgCl_2$, 1mM DTT, 0.1 mg/ml BSA and 1 mM PMSF. Both treated and untreated samples were analyzed by SDS-PAGE followed by Western blotting.

Phosphorylation of MYPT with *Xenopus* egg extracts

Mitotic or interphase extracts of *Xenopus* eggs were used to reconstitute cell cycle-dependent phosphorylation of MYPT. Mitotic extracts were prepared from *Xenopus* unfertilized eggs in an XB buffer containing 20 mM Hepes (pH 7.7), 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA and 0.1 mg/ml cytochalasin D as described (Murray, 1991). Interphase extracts were prepared from mitotic extracts by the addition of 0.5 mM $CaCl_2$ followed by incubation at 20°C for 30 min to inactivate MPF.

Rat MYPT was prepared from interphase REF-2A cells by immunoprecipitation with $Ab_{1\text{-}296}$-conjugated Sepharose beads (crosslinked with dimethylpimelimidate; Pierce, Rockford, IL). Buffer I and buffer II, were used for myosin binding studies and myosin phosphatase assays, respectively. Purified chick gizzard myosin phosphatase was also bound to the same antibody-conjugated beads. MYPT-bound beads were washed once with XB buffer (without cytochalasin), mixed with an equal volume of mitotic or interphase extracts and incubated at 25 °C for 30 min in the presence of 1mM ATP (with or without 1 mCi/ml [γ-^{32}P] ATP). The beads were washed extensively with buffer I or II, and then subjected to SDS-PAGE, immunoblotting, two-dimensional tryptic phosphopeptide mapping, myosin binding or phosphatase assays.

A peptide (NH2-ISPKEEERKDESPASWRLGLRKC-COOH) corresponding to residues 421 to 442 of rat MYPT(which corresponds to Val 416-lys 437 of chicken MYPT) was commercially synthesized (Bio-Synthesis inc., Lewisville, TX). The peptide (20 μg) was phosphorylated with

Xenopus mitotic extracts in the presence of 1 mCi/ml [γ-^{32}P] ATP as described above.

Trichloroacetic acid was added to 10% to precipitate proteins and the phosphorylated peptide was recovered by centrifugation in the supernatant. The peptide was then separated by Tricine-SDS PAGE (Schagger and von Jagow, 1987). The phosphorylated peptide was detected by autoradiography, excised from Tricine-SDS gels and digested with TPCK-treated trypsin followed by two-dimensional phosphopeptide mapping.

Construction of mutants of MYPT

cDNA encoding chicken MYPT 304-511 was subcloned into a pQE32 vector (Quiagen, Santa Clarita, CA) with a hexahistidine tag at the N-terminus as described (Hirano, et al., 1997). N- and C-terminal truncations were made by PCR amplification with pQE32-MYPT304-511 as a template. The sense and antisense primers were designed to contain BamHI and SalI sites at 5' and 3' ends, respectively, to ligate the PCR products unidirectionally into the pQE32 vector. After digestion of the PCR products with BamHI and SalI, they were inserted into the BamHI- and SalI-digested pQE32 vector. The truncation mutants obtained were MYPT304-410, MYPT304-444, MYPT421-511, and MYPT432-511. These proteins were expressed in E. coli and purified by a metal affinity column (Sigma) as described (Hirano, et al., 1997).

Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the mutation of Ser 427 to Asp, a forward primer, 5'-GAAGAGAGGAAAGATGAAGATCCTGCTTCGTGGAGGTTAG-3', and a reverse primer, 5'-CTAACCTCCACGAAGCAGGATCTTCATCTTTCCTCTCTTC-3', were used. For the mutation of Ser 430 to Glu, a forward primer, 5'-GAAAGATGAATCTCCTGCTGAGTGGAGGTTAGGTCTTCG-3', and a reverse primer, 5'-CGAAGACCTAACCTCCACTCAGCAGGAGATTCATCTTTC-3', were used. PCR was performed with pQE32-MYPT304-511 as a template with Pfu polymerase, according to the manufacturer's instructions (Stratagene).

Myosin binding assay

This assay was performed as follows. Rat MYPT was immunoprecipitated from mitotic or interphase cells using buffer I, and eluted from the Sepharose beads by incubating for 2 min with

0.1 M glycine, pH 2.3, containing 0.1 mg/ml BSA, followed by immediate neutralization with 2 M Tris base. The eluted MYPT was mixed with phosphorylated myosin (0.1 μM) in 30 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA and 0.5 mM ATP. Samples were incubated at 4 °C for 10 min, and myosin was precipitated by centrifugation at 16,000g for 10 min. Both pellet and supernatant were added to an equivalent volume of SDS sample buffer and subjected to SDS-PAGE followed by immunoblotting analysis. The amount of MYPT was estimated densitometrically by scanning immunoreactive bands using purified chicken MYPT as a standard.

Myosin binding was also examined with *in vitro* phosphorylated MYPT. Rat MYPT was immunoprecipitated from interphase cells using buffer I and phosphorylated *in vitro* with *Xenopus* mitotic or interphase extracts as described above. Phosphorylated MYPT was eluted from the immunocomplex and used for myosin binding, as described above.

Myosin phosphatase Assay

Rat MYPT was immunoprecipitated from interphase cells using buffer II and phosphorylated (without radioactive ATP) *in vitro* using *Xenopus* mitotic or interphase extracts as described above. After extensive washing, immobilized myosin phosphatase was incubated at 30 °C with ^{32}P-labeled myosin (0.5 μM) in 30 mM Tris-HCl (pH 7.5), 0.1 M KCl, 2 mM $MgCl_2$, and 0.1 mg/ml BSA. The reaction was terminated by the addition of trichloroacetic acid and BSA to final concentrations of 10 % and 3 mg/ml, respectively. After centrifugation, the radioactivity of the supernatants were determined by Cerenkov counting. The reaction time was adjusted so that about 10-20 % of the substrate was dephosphorylated. In some experiments, the gizzard myosin phosphatase was used to examine effects of phosphorylation on myosin phosphatase activity.

Other procedures

Two-dimensional tryptic phosphopeptide mapping was performed using cellulose thin layer plates as described (Boyle, et al., 1991). Phosphopeptides were detected by autoradiography. SDS-PAGE was performed as described by Blatter et al. (Blatter, et al., 1972) using 12.5 % polyacrylamide gel and the Laemmli buffer system (Laemmli, 1970). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using BSA as standard.

Results

Mitosis-specific phosphorylation of MYPT

Initially a mitosis-specific modification of MYPT was observed by immunoblotting the mitotic cell lysates with the monoclonal antibody (mAb) specific for MYPT. As Fig. 1a shows, the mAb did not detect a band in the total cell lysates of mitotic cells (lane 4) but the same mAb reacted strongly with MYPT from interphase cells (lane 3). The lack of reactivity in mitotic cell lysates was not due to degradation of MYPT during mitosis because the polyclonal antibody, $Ab_{1\text{-}38}$, (pAb) reacted equally well with MYPT from either interphase (lane 1) or mitotic (lane 2) cells. In addition, in cells rounded by trypsin treatment the reactivity of MYPT with the mAb was retained (data not shown). Thus it is suggested that the loss of recognition of the mAb for MYPT was due to a modification of MYPT incurred during mitosis.

One possibility was that the lack of reactivity to the mAb was due to mitosis-specific phosphorylation. To test this idea, mitotic and interphase MYPT were immunoprecipitated, and divided into two aliquots. One aliquot was used as a control and the other was incubated with PP1cα. The phosphatase treatment completely restored the reactivity of mitotic MYPT with the mAb (Fig. 1b; compare lanes 2 and 4 of the upper panel), indicating that the lack of reactivity to the mAb is due to mitosis-specific phosphorylation. It should also be noted that MYPT from mitotic cells showed a subtle but significant upward shift of mobility on SDS-PAGE (compare lanes 1 and 2 of the lower panel), and that the same phosphatase treatment eliminated this shift (see lanes 2 and 4 of the lower panel). In contrast, incubation with phosphatase of MYPT from interphase cells did not affect its reactivity with the mAb (Fig. 1b; compare lanes 1 and 3). These results indicate that serine/threonine phosphorylation is responsible for the mitosis-specific modification of MYPT. Next a time course of the mitosis-specific phosphorylation during cell division was examined. Total cell lysates were prepared from interphase, mitotic cells and cells at different stages of cell division (see Materials and Methods). MYPT was analyzed in each preparation by immunoblotting using the same mAb and pAb. Immunoblots with the mAb clearly demonstrated that a mitosis-specific phosphorylation of MYPT occurred and was dependent on the cell-cycle stage. As Fig. 1c

(upper panel, labeled "mAb") shows, mitotic MYPT (lane M) exhibited complete loss of reactivity against the mAb whereas interphase MYPT (lane I) showed a strong reaction to the same antibody. The lack of reactivity continued until 40 min after release of mitotic arrest and then was recovered at 60 min, at which point cytokinesis occurred. At later phases when the cell was involved in post-mitotic spreading (80-180 min), the reactivity of MYPT to the mAb was similar to that of interphase cells.

The lack of reactivity to the mAb parallels the mobility shift observed with the pAb. An immunoblot with the pAb (Fig. 1c, lower panel) reveals that MYPT showed a slight upward shift in mobility during mitosis (compare lanes I and M). The upward shift was apparent until 40min and then was reversed at 60 min. These results again indicate that MYPT was dephosphorylated during cytokinesis. We have also observed similar modifications of MYPT during mitosis of other cells including Chinese hamster ovary (CHO) cells (data not shown).

The sites of mitosis-specific phosphorylation differ from those observed during interphase

To examine whether net phosphate incorporation into MYPT was increased during mitosis, MYPT was immunoprecipitated from interphase and mitotic cells after *in vivo* labeling with ^{32}P-orthophosphate. As Fig. 2a shows, the level of phosphorylation of mitotic MYPT (lane 2) is similar to that of interphase MYPT (lane 1). One explanation for the lack of reactivity to the mAb could be that different sites were phosphorylated in the two stages, rather than a net increase in phosphorylation during mitosis.

To examine this possibility, two-dimensional phosphopeptide mapping was performed. MYPT was again immunoprecipitated from mitotic and interphase cells labeled *in vivo* with ^{32}P-orthophosphate, digested with trypsin, and analyzed by peptide mapping. Fig. 2b shows the phosphopeptide maps generated with interphase (panel I) and mitotic (panel M) MYPT. To identify which spots are mitosis-specific, a mixture of mitotic and interphase MYPT samples was subjected to peptide mapping (panel Mix). The map of mitotic MYPT (panel M) revealed four mitosis-specific phosphopeptide spots (indicated by M1-4). In addition, one spot (M5) showed a considerably higher intensity in mitotic MYPT. On the other hand, the interphase map (panel I)

gives two interphase-specific spots (indicated by I). There are four spots (indicated by C) which were observed commonly in both mitotic and interphase MYPT maps. These results demonstrate that the sites of phosphorylation were different between mitotic and interphase MYPT. We also examined the phosphopeptide pattern of MYPT prepared from cells at 120 min after the release of mitotic arrest. This pattern was identical to that shown with interphase cells (data not shown). This is consistent with the result that the reactivity of MYPT to the mAb at 120 min was similar to that of MYPT from interphase cells.

In vitro reconstitution of mitosis-specific phosphorylation MYPT

To further characterize mitosis-specific phosphorylation, an attempt was made to reconstitute the mitosis-specific phosphorylation *in vitro*. Mitotic *Xenopus* egg extracts were used as a kinase fraction. MYPT was immunoprecipitated from interphase cultured rat cells using buffer I, and aliquoted into two. One-half was incubated with mitotic *Xenopus* extracts in the presence of Mg-ATP. As a control, the other half was incubated with interphase *Xenopus* extracts that had been prepared from mitotic extracts following the addition of Ca^{2+} (which converted mitotic extracts into interphase extracts; see (Murray, 1991) and "Materials and Methods"). The incubation with the mitotic extracts eliminated the reactivity of MYPT against the mAb (Fig. 2c; upper panel: lane 2). At the same time, the mobility of MYPT showed an upward shift (Fig. 2c; lower panel: lane 2) when compared with the mobility of untreated MYPT (lane 1). In contrast, incubation with the interphase extracts did not alter the reactivity to the mAb nor did it induce the mobility shift (lane 3). These results suggest that *Xenopus* mitotic extracts are able to reconstitute the mitosis-specific phosphorylation.

To confirm the reconstitution of MYPT, the phosphorylation sites of MYPT were analyzed by two-dimensional phosphopeptide mapping. As indicated in Fig. 2d, the phosphopeptide map of MYPT phosphorylated *in vitro* by mitotic *Xenopus* extracts (panel X) was similar to that phosphorylated *in vivo* in mitotic cells (compare panel M, Fig. 2b). For comparison, a mixture of *in vivo* and *in vitro* phosphorylated MYPT was subjected to phosphopeptide mapping (panel Mix, Fig. 2d). The map of MYPT phosphorylated by *Xenopus* mitotic extracts showed four (M1-3, M5) out of the five mitotic-specific spots, although two interphase specific spots (I) appeared

simultaneously. The spot M4 could be seen only after prolong exposure of the autoradiograph (data not shown). On the other hand, MYPT phosphorylated by interphase extracts yielded a map similar to that of interphase MYPT (data not shown).

Identification of a mitosis-specific phosphorylation site

The loss of reactivity to the mAb indicated that the epitope of the mAb may contain a site of mitosis-specific phosphorylation. It was known that the epitope to the mAb was between residues 371 and 511 (Hartshorne, unpublished results) and to define more precisely the epitope a series of truncation mutants were analyzed. In Fig. 3a it is shown that fragment 421-511 has a positive reaction with the mAb while 432-511 was negative, indicating that the epitope is localized between residues, 421 and 431. There are two serines (no threonine) in this sequence, Ser 427 and Ser 430 (Fig. 3b), and these two residues were mutated to Asp and Glu, respectively. The resultant point mutants were expressed in bacteria, and the reactivities of these two mutants to the mAb were examined by immunoblotting. It was found that mutation of Ser 430 to Glu (S430E) resulted in complete loss of reactivity (lane 3 of Fig. 3c). In contrast, the mutant replacing Ser 427 with Asp (S427D) still showed a strong reactivity to the mAb, though the reactivity was weaker than the control (lane 2 of Fig. 3c). A reasonable conclusion from these results is that Ser 430 of MYPT is a site phosphorylated during mitosis although it is possible that simultaneous phosphorylation at Ser 427 may also occur.

To further test whether Ser 430 is one of the mitosis-specific phosphorylation sites, a peptide containing Ser 430 (from residues 421 to 442 of rat MYPT) was synthesized and phosphorylated *in vitro* using *Xenopus* mitotic extracts. The peptide was digested with trypsin and subjected to two-dimensional phosphopeptide mapping. A map from the phosphorylated synthetic peptide (panel P of Fig. 3d) yielded several spots, apparently due to incomplete trypsin digestion (the peptide has multiple lysine and arginine residues). To examine whether any of these spots matched the mitotic spots, the map generated from the phosphorylated synthetic peptide was compared with a map from MYPT phosphorylated with *Xenopus* extracts (panel X), and with a map of a mixture from the peptide and MYPT samples (panel Mix). It was found that two major spots (indicated by arrow) of the map generated from the synthetic peptide correspond to two

mitotic spots, M2 and M3. These results, together with the mutational analyses, indicate that the spots, M2 and M3, are derived from mitosis-specific phosphorylation at Ser 430. Again, there is the possibility that Ser 427 may also be phosphorylated. It should be noted that there are other mitosis-specific phosphorylation sites corresponding to the mitotic spots of M1, M4 and M5.

Increased myosin binding ability of mitotic MYPT and higher phosphatase activity shown by mitotic myosin phosphatase

In order to explore the functional significance of mitosis-specific phosphorylation of MYPT, the myosin binding activities of MYPT from mitotic or interphase cells was compared. Mitotic and interphase MYPT were immuno-affinity purified, and their myosin binding abilities were examined using phosphorylated myosin in the presence of Mg-ATP, as described in "Materials and Methods". As Fig. 4a shows, the amount of mitotic MYPT bound to phosphorylated myosin was about 3 fold higher than MYPT from interphase cells.

Because the quantity of MYPT isolated from mitotic cells was limited, the *in vitro* reconstitution system was used to prepare mitotic and interphase MYPT thus allowing a more extensive evaluation of the myosin-binding properties of MYPT, i.e. to use a wider range of MYPT concentrations. Rat MYPT immunoprecipitates (using buffer I) were phosphorylated by either mitotic or interphase extracts, as described above, and then MYPT was eluted from the immunocomplexes. Varying concentrations of eluted MYPT (2-25nM) were mixed with phosphorylated myosin in the presence of Mg-ATP and their binding was examined. As Fig. 4b shows, the amount of MYPT bound to phosphorylated myosin was 2-3 times higher using MYPT phosphorylated with mitotic extracts compared to the MYPT phosphorylated by interphase extracts. At 25nM MYPT, it was found that about 68 % of MYPT phosphorylated by mitotic extracts bound to myosin while only 31 % of MYPT phosphorylated by interphase extracts bound to myosin. These results support the data obtained for *in vivo* phosphorylation of MYPT (compare with Fig. 4a).

The enhanced myosin binding activity of mitotic MYPT suggested that myosin phosphatase activity may be increased during mitosis. To test this possibility, MYPT was immunoprecipitated using buffer II (to retain the catalytic subunit in complex with MYPT), phosphorylated with either

Xenopus mitotic or interphase extracts and used to assay phosphatase activities. Again, the MYPT phosphorylated with *Xenopus* mitotic extracts showed loss of reactivity to the mAb (lane 2 of the upper panel of Fig. 5a), indicating that mitosis-specific phosphorylation of MYPT occurred. It was also confirmed, by immunoblotting with the pAb against MYPT and with the mAb to PP1c (middle and lower panel of Fig. 5a), that essentially identical amounts of MYPT and the catalytic subunit were present in the immunoprecipitates treated with mitotic or interphase *Xenopus* extracts. As Fig. 5b shows, the myosin phosphatase treated with mitotic extracts had approximately twice the activity than that treated with interphase extracts. Similar results were obtained with chick gizzard myosin phosphatase (data not shown). These results indicate that the enhanced myosin binding ability was accompanied by a higher phosphatase activity.

Discussion

In this paper we have demonstrated that MYPT is phosphorylated in a mitosis-specific way and that phosphorylation increases its myosin binding ability, as well as myosin phosphatase activity. The activation of myosin phosphatase by mitosis-specific phosphorylation is unique as it provides a positive regulatory mechanism for myosin phosphatase distinct from previous reports on phosphorylation of MYPT. For example, phosphorylation by Rho-kinase (Kimura, et al., 1996) or by endogenous kinase (Ichikawa, et al., 1996) was reported to inhibit the activity of myosin phosphatase. Phosphorylation by protein kinase A was found to decrease the association of MYPT with lipids (Ito, et al., 1997), which was suggested as a mechanism to regulate association of MYPT with membranes. Activation of myosin phosphatase via the cGMP-dependent protein kinase has been proposed (Wu, et al., 1996; Lee, et al., 1997) but the mechanism of activation has not been established.

Mechanism of activation of myosin phosphatase by mitosis-specific phosphorylation

Ser 430 (which corresponds to Ser 435 in rat MYPT) was identified as one of the mitosis-specific phosphorylation sites. It should be noted that the sequence surrounding Ser 430 is well conserved among different species including human, rat and chicken although Ser 430 is changed to Thr 435 in human (see for reviewHartshorne, et al., 1998). In fact, chicken and rat MYPT have an identical sequence of 42 amino acids around this phosphorylation site. There are two genes that express MYPT, denoted MYPT1 and MYPT2 (Takahashi, et al., 1997; Fujioka, et al., 1998). The isoform examined in this article is MYPT1 and MYPT2 is found in heart and brain. The putative mitosis-specific phosphorylation site also is found in MYPT2 at Ser 437. The sequence around this site, 428 to 447 of MYPT2 is 90% identical to 421 to 440 of MYPT1. Thus, it is possible that phosphorylation of this site on MYPT is a general mechanism for activation of myosin phosphatase activity. It is interesting that Ser 430 does not have a consensus sequence for cdc2 kinase. Although cdc2 kinase can phosphorylate MYPT *in vitro*, a phosphopeptide pattern generated by cdc2 is different from the peptide map of MYPT phosphorylated *in vivo* during mitosis (data not shown). Likewise, NIM A kinase (Pu, et al., 1995) does not seem to be responsible because a

phosphopeptide map generated by NIM A kinase is quite different from the mitotic pattern (data not shown). Clearly, identification of the kinase responsible for the mitosis-dependent phosphorylation is a priority for future studies.

It is not clear at present how mitotic phosphorylation influences the binding of MYPT to myosin. One of the mitosis-specific phosphorylation sites, Ser 430 (Ser 427 may be phosphorylated simultaneously) is located toward the middle of the MYPT molecule. There is controversy regarding the location of the myosin-binding sites on MYPT. It has been reported that the ankyrin repeats at the N-terminal portion of MYPT are involved (Hirano, et al., 1997) and also that a C-terminal sequence is implicated (Johnson, et al., 1997). Neither part of the molecule is close (in terms of linear sequence) to Ser 430. A direct influence of Ser 430 on either of the putative myosin-binding sequences therefore is not possible unless the MYPT molecule bends, or folds, to accommodate such an interaction. It is possible that other mitosis-specific phosphorylation sites on MYPT may be close to one of the myosin binding sites and contribute to the myosin-binding effect. Alternatively, phosphorylation of Ser 430 may induce a longer-range conformational change at the myosin-binding site. Clearly, the solution to this problem requires the identification of all of the mitosis-specific phosphorylation sites as well as the region(s) of MYPT involved in interaction with myosin.

It has been reported that cdc2 kinase phosphorylates PP1c, and that phosphorylation inhibits its activity toward phosphorylase *a* (Dohadwala, et al., 1994). This seems to be contradictory to the results presented here. However, PP1c is involved in the dephosphorylation of several proteins, and the activity toward each protein is dependent on various targeting molecules. For example, the activity of PP1c toward phosphorylase *a* is considerably decreased in the presence of MYPT (Alessi, et al., 1992; Johnson, et al., 1996; Hirano, et al., 1997). The presence of target molecules may also affect the accessibility of PP1c for phosphorylation by cdc2 kinase. It is thus possible that phosphorylation of PP1c by cdc2 kinase may not inhibit myosin phosphatase activity during mitosis. This is based on the following two reasons: First, an activation of myosin phosphatase, rather than inhibition, was found following phosphorylation with the *Xenopus* mitotic extracts (Fig. 5b). If phosphorylation of PP1c by cdc2 kinase and resultant inhibition was

a dominant mechanism it should have been detected in these experiments. Second, the phosphorylation of PP1c of chick myosin phosphatase by *Xenopus* mitotic extracts was not observed (data not shown).

Physiological significance

Mitosis-specific phosphorylation of MYPT may play a significant role in the regulation of microfilament reorganization during cell division of cultured cells. The enhanced myosin phosphatase activity would increase the probability for dephosphorylation of RMLC during prophase, leading to the disassembly of stress fibers and cell rounding. This notion is consistent with our previous results showing that Ser 19 phosphorylation is decreased when cells enter mitosis (Yamakita, et al., 1994). The increased activity of myosin phosphatase and resultant disassembly of stress fibers also are compatible with the localization of MYPT during mitosis. MYPT was reported to show a diffuse localization during mitosis (Ito, et al., 1997) while it is associated with microfilament structures such as stress fibers and adhesion belts during interphase (Inagaki, et al., 1997; Murata, et al., 1997).

In addition, the reversal of mitosis-specific phosphorylation, i.e. dephosphorylation, during cytokinesis negates the activation of myosin phosphatase and thus would favor a higher level of Ser 19 phosphorylation for the activation of contractile rings. This idea again is consistent with previous results in that the phosphorylation sites on RMLC change from S1/2 to Ser 19 during cytokinesis (Satterwhite, et al., 1992; Yamakita, et al., 1994). This is supported by our recent immunolocalization data using a Ser 19 phosphorylation specific antibody, in which prometaphase cells showed a lower level of Ser 19 phosphorylation than cells at telophase (Matsumura, et al., 1998). These changes in phosphatase activity would explain a previous observation by (Fishkind, et al., 1991) that microinjection of a catalytic fragment of MLCK (constitutively active MLCK) delayed the onset of anaphase but did not alter the rate of progression of cytokinesis. Perhaps, the increased activity of myosin phosphatase during prometaphase could counteract phosphorylation of Ser 19 by the catalytic fragment of MLCK before cytokinesis. The decrease in myosin phosphatase activity during cytokinesis would then be able to regulate cleavage furrow contraction.

If these speculations are correct then the mitosis-specific phosphorylation of MYPT may play a pivotal role in the control of cell division.

The positive regulatory mechanism of myosin phosphatase described above contrasts to the negative regulation by phosphorylation of MYPT with Rho-kinase. It is possible that both mechanisms collaborate to regulate the massive re-organization of microfilaments during cell division. Our current model for regulation of myosin phosphatase during cell division is shown in Fig. 6. This incorporates two phosphorylation steps: an activation via the mitosis-specific kinase(s) and an inhibition via Rho-kinase (Amano, et al., 1996; Kimura, et al., 1996). It is therefore reasonable to suggest that there are at least two functional phosphorylation sites to reflect the positive and negative regulatory effects. When cells enter prophase it is proposed that the mitosis-specific phosphorylation occurs (via an unknown kinase(s)) and this causes activation of myosin phosphatase and a decrease in the level of myosin phosphorylation (at Ser 19). The result is disassembly of stress fibers and cell rounding. On exit from mitosis and prior to, or during cytokinesis, the activating site(s) on MYPT are dephosphorylated. At the same phase of the cell cycle it is suggested that inhibition of myosin phosphatase occurs via phosphorylation of MYPT by Rho-kinase. Here the next result would be an increase in the level of myosin phosphorylation (again at Ser 19) and activation of myosin for cell division. There are important components of this scheme that must be identified before a plausible mechanism can be established and these include the kinase(s) and phosphatase(s) involved at the mitosis-specific stage.

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Figure Legends

Figure 1. Mitosis-specific phosphorylation of MYPT. (a) Mitotic MYPT loses a reactivity to the mAb specific to MYPT. Total cell lysates of interphase (lanes 1 and 3) or mitotic cells (lanes 2 and 4) were immunoblotted with rabbit pAb (lanes 1 and 2) or mouse mAb (lanes 3 and 4) antibodies. The arrow indicates the position of MYPT (130 kDa) Molecular weight markers are indicated in kDa at the left of the figure. (b) Treatment with PP1c restores the reactivity of mitotic MYPT to the mAb. MYPT was immunoprecipitated from interphase (lanes 1 and 3, Fig. 1b) or mitotic cells (lanes 2 and 4). Half of the immunoprecipitates was treated with PP1c (lanes 3 and 4, Fig. 1b). Both treated or untreated samples were immunoblotted with the mAb (upper panel, labeled with "mAb") or pAb (lower panel, labeled with "pAb"). (c) Time course of mitosis-specific modification of MYPT during cell division. Total cell lysates were prepared from interphase (lane I), mitotic cells (lane M), and cells at different stages of cell division (time in min. after the release of mitotic arrest is shown on the top of each lane), and blotted with either the mAb (upper panel, labeled with "mAb") or the pAb (lower panel, labeled with "pAb"). Cytokinesis occurred 40-60 min after release of mitotic arrest.

Figure 2. Phosphopeptide analysis of mitotic and interphase MYPT and *In vitro* reconstitution of mitosis-specific phosphorylation of MYPT. (a) *In vivo* phosphorylation of MYPT from interphase or mitotic cells. MYPT was isolated by immunoprecipitation from interphase (lane 1) or mitotic cells (lane 2) that had been labeled with ^{32}P-orthophosphate. ^{32}P-labelled MYPT was separated by SDS-PAGE followed by autoradiography. (b) Two dimensional tryptic phosphopeptide mapping analysis of *in vivo* phosphorylated MYPT. Panel I, MYPT isolated from interphase; panel M, MYPT from mitotic cells; panel Mix, a mixture of mitotic and interphase MYPT. M1-4, phosphopeptide spots specifically observed in mitotic map; M5, a spot whose intensity is increased in mitotic map. Phosphopeptide spots specifically observed in interphase map and spots commonly observed in both interphase and mitotic MYPT are labeled with "I" and "C", respectively. Arrow with e, electrophoretic dimension; arrow with c, chromatographic dimension; ori, the origin. (c) *In vitro*

reconstitution of mitotic phosphorylation. Interphase MYPT was prepared by immunoprecipitation and phosphorylated with *Xenopus* mitotic or interphase extracts. Both samples were separated by SDS-PAGE followed by immunoblotting using the mAb (upper panel) or pAb (lower panel). Lane 1, a control without addition of *Xenopus* extracts; lane 2, MYPT treated with mitotic extracts; lane 3, MYPT treated with interphase extracts. (d) Phosphopeptide mapping analysis of *in vitro* phosphorylated MYPT. MYPT was phosphorylated *in vitro* using *Xenopus* mitotic extracts and analyzed by phosphopeptide mapping (panel X). For comparison, a mixture of *in vivo* and *in vitro* phosphorylated MYPT (panel "Mix") are shown. The mitosis-specific spots are indicated by M.

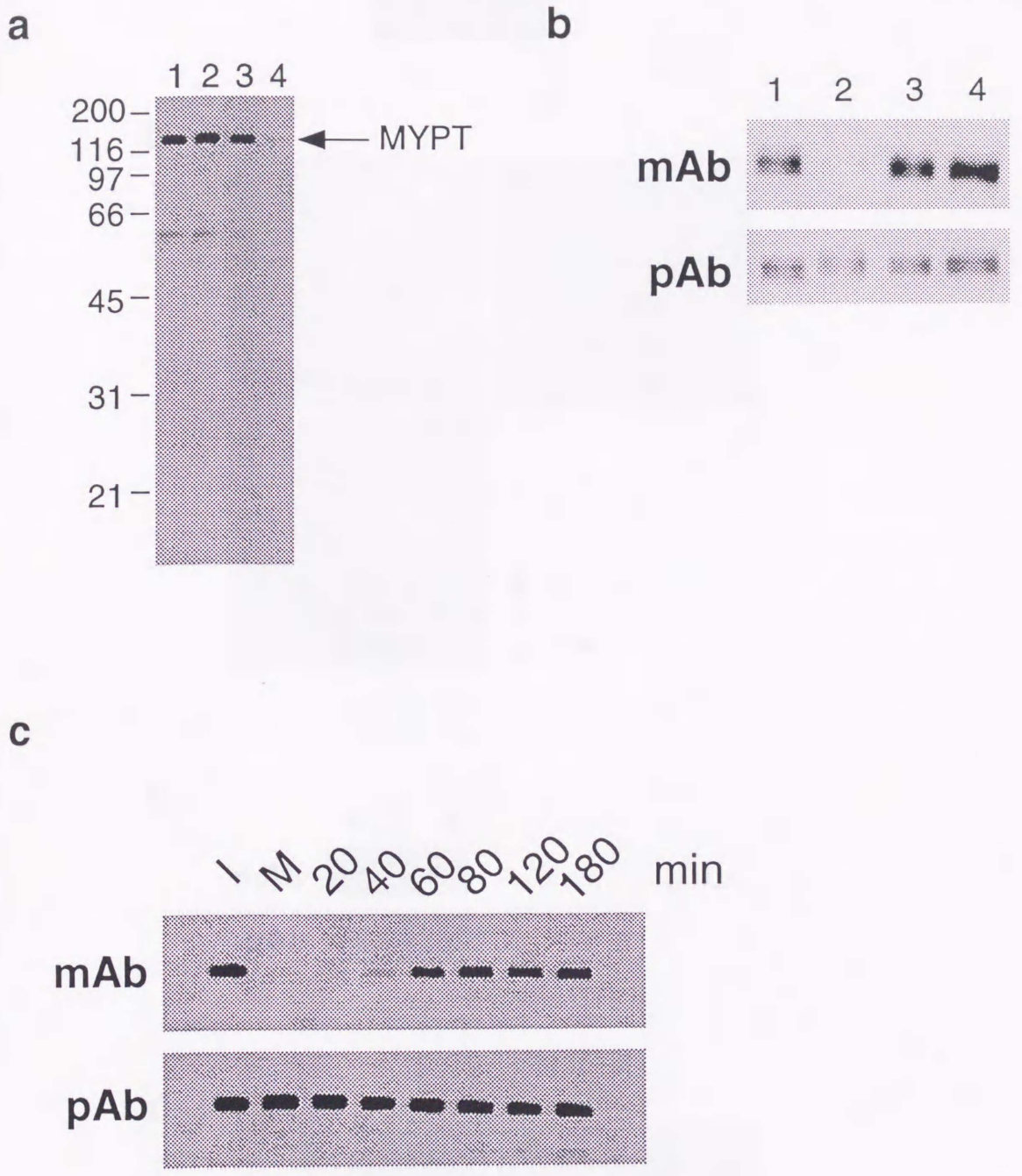
Figure 3. Identification of one of the mitosis-specific phosphorylation sites of MYPT. (a) Schematic presentation of the truncated mutants of chick MYPT. The reactivities to the mAb of truncated mutants are shown. MYPT truncated mutants were immunoblotted with the mAb against MYPT. The protein expression in bacteria was confirmed with an anti-polyhistidine antibody. The epitope of the mAb should be located between 421 and 432. (b) The epitope region of MYPT to the mAb. The sequence shown is Glu 421 to Arg 432 of chick MYPT (which corresponds to Glu 426 to Arg 432 of rat MYPT), and contains two serine residues of serine 427 and serine 430. To mimic phosphorylation, serine 427 and serine 430 were mutated to Asp (S427D) and Glu (S430E), respectively and the mutants were expressed in bacteria. (c) Immunoblot analysis of the phosphorylation-mimicking mutants of MYPT. The S427D and S430E mutants were separated by SDS-PAGE followed by immunoblotting analysis using the anti-polyhistidine antibody (upper panel) or the mAb against MYPT (lower panel). Lane 1, wild type MYPT304-511; lane 2, S427D mutant; lane 3, S430E mutant. (d) Phosphopeptide mapping analysis of a synthetic peptide (NH2-ISPKEEERKDESPASWRLGLRKC-COOH) containing Ser 430. Panel P, peptide map of the synthetic peptide phosphorylated by *Xenopus* mitotic extracts; Panel X, map of rat MYPT phosphorylated by *Xenopus* mitotic extracts; Mix, a map of a mixture of the synthetic peptide and MYPT. The two spots in panel P (indicated by arrow) match M2 and M3 of mitotic spots.

Figure 4. Increased myosin binding ability of mitotic MYPT. (a) Mitotic or interphase MYPT was prepared by immuno-affinity purification and myosin binding activity was determined by cosedimentation assay with phosphorylated myosin as described in "Materials and Methods". The figure shows myosin binding of MYPT prepared from interphase cells (I) or mitotic cells (M). The values shown are the means ± S. E. from three independent experiments. (b) Myosin binding activity of *in vitro* phosphorylated MYPT. MYPT was phosphorylated *in vitro* with *Xenopus* mitotic or interphase extracts and myosin binding ability was examined as described in "Materials and Methods". MYPT phosphorylated with mitotic extracts (■);MYPT phosphorylated with interphase extracts (○).

Figure 5. Myosin phosphatase activity of *in vitro* phosphorylated myosin phosphatase. (a) Immunoblotting analysis of *in vitro* phosphorylated myosin phosphatase. Interphase myosin phosphatase was prepared by immunoprecipitation and phosphorylated *in vitro* with *Xenopus* interphase (lane 1) or mitotic extracts (lane 2). After extensive washing, phosphorylated samples were analyzed by immunoblotting with the mAb (upper panel, labeled with "mAb") or pAb against MYPT (middle panel, labeled with "pAb"), or anti-PP1c antibody (lower panel). (b) Myosin phosphatase activity of the *in vitro* phosphorylated myosin phosphatase. Left column, myosin phosphatase phosphorylated with interphase extracts; right column, myosin phosphatase phosphorylated with mitotic extracts. The values shown are means ± S. E. from three independent experiments.

Figure 6. Model of the regulation of myosin phosphatase during cell division. MYPT has two functionally different phosphorylation sites: One is the mitosis-specific phosphorylation site(s) including Ser 430 that activate myosin phosphatase activity, and the other is Rho-kinase site(s) that inhibit myosin phosphatase activity. See the text for details.

Fig. 1


a
1 2 3 4
200
116
97
66
45
31
21
MYPT
b
1 2 3 4
mAb
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I M 20 40 60 80 120 180 min
mAb
pAb

Fig. 2

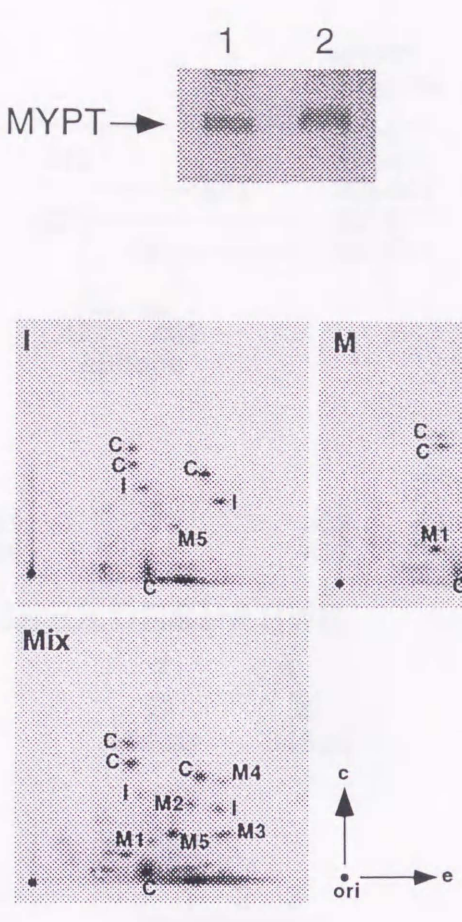
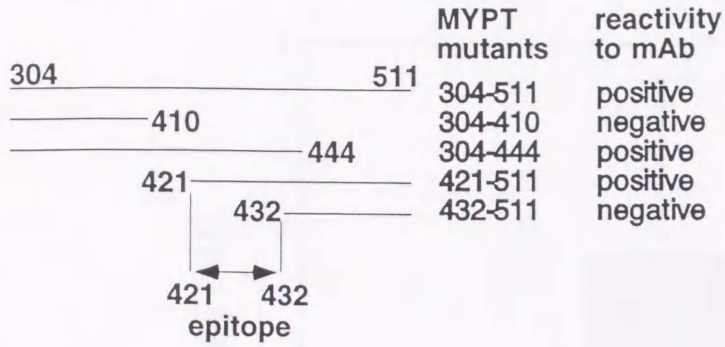


Fig. 3

a


MYPT mutants
reactivity to mAb
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511
304-511 positive
410
304-410 negative
444
304-444 positive
421
421-511 positive
432
432-511 negative
421 432
epitope

b


chick 421
rat 426
432
437
EERKDESPASWR
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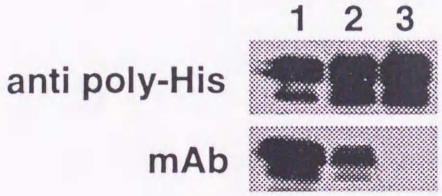
E
S427D
S430E

c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mAb

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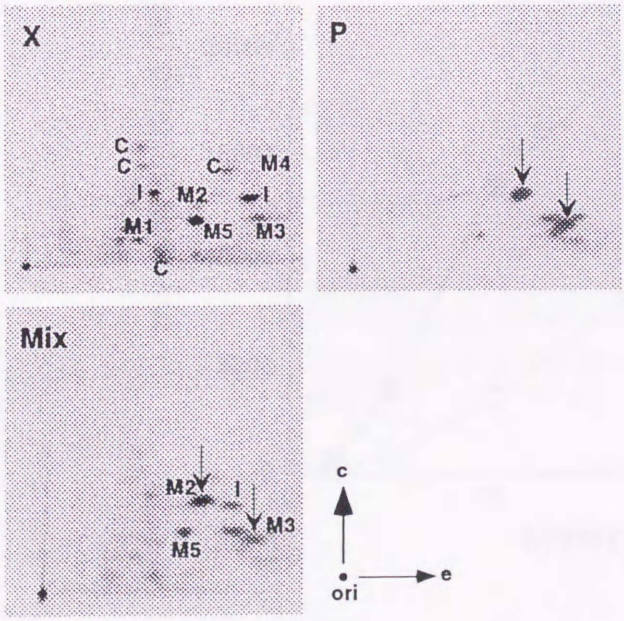
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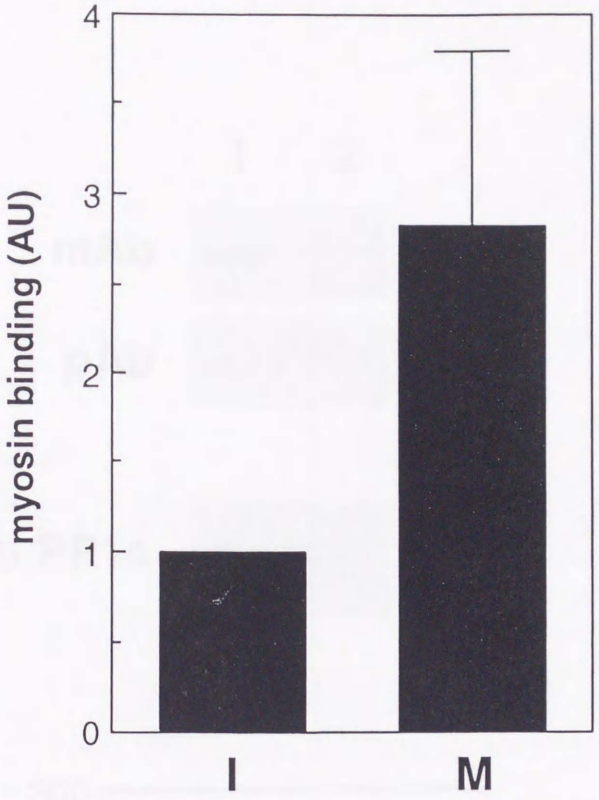
P
Mix
M1
M2
M3
M4
M5
c
e
ori

Fig. 4

a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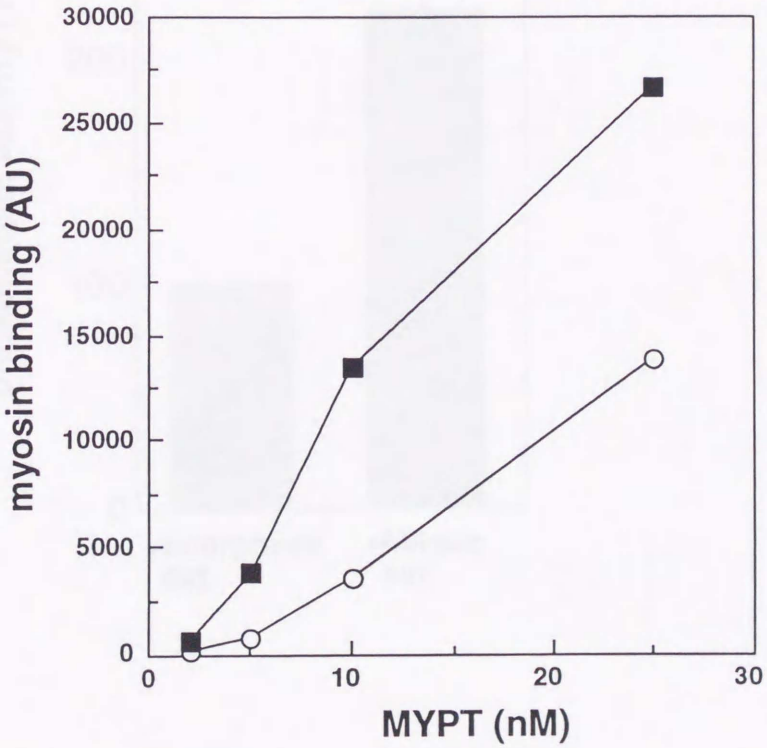
1
2
3
4
I
M

b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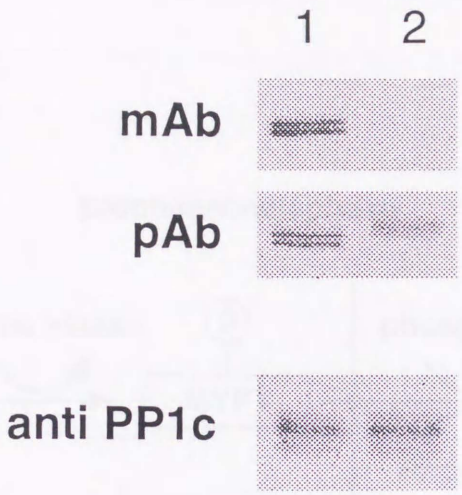
30000
0
10
20
30
MYPT (nM)

Fig. 5

a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mAb
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anti PP1c

b

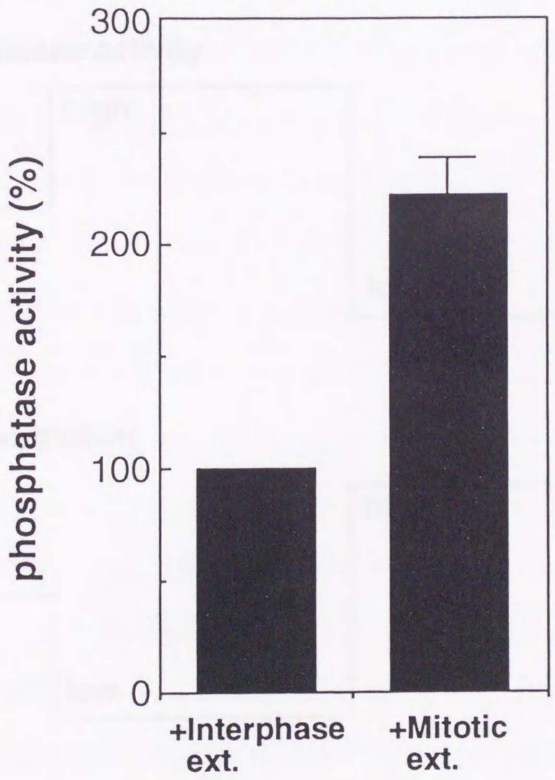
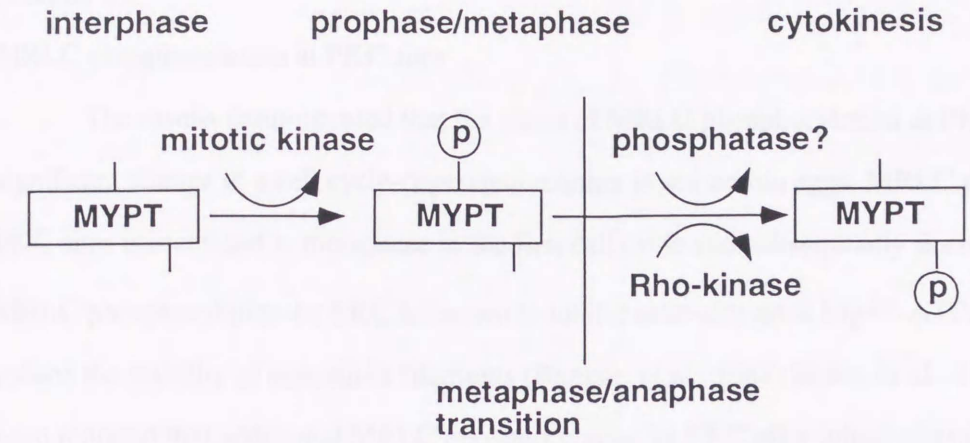
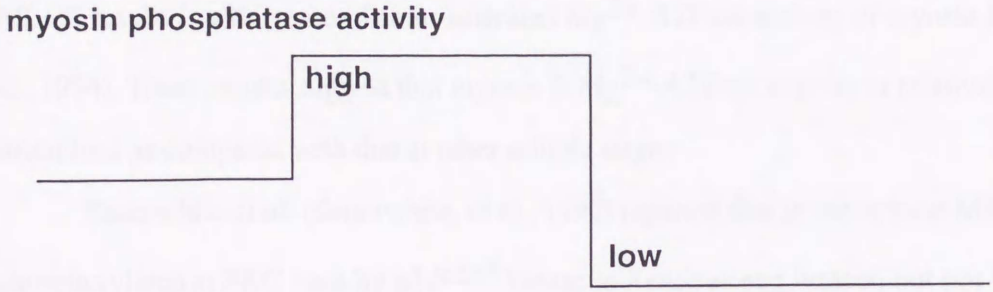
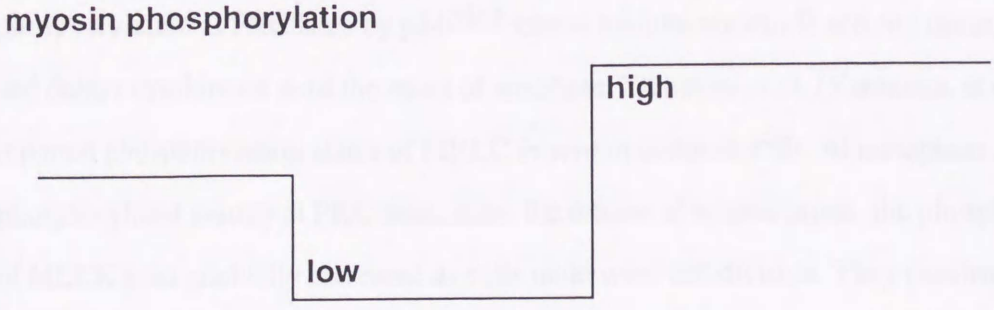

300
200
100
0
phosphatase activity (%)
+Interphase ext.
+Mitotic ext.

Fig. 6


interphase
prophase/metaphase
cytokinesis
mitotic kinase
P
phosphatase?
MYPT
MYPT
MYPT
Rho-kinase
P
metaphase/anaphase
transition


myosin phosphatase activity
high
low


myosin phosphorylation
high
low

5. General Discussion

In this work I have demonstrated that MRLC phosphorylation at PKC sites is significantly increased at metaphase of fertilized sea urchin eggs (chapter 1). Furthermore, I have shown that MYPT of myosin phosphatase is phosphorylated in a mitosis-specific way in cultured cells and that phosphorylation increases its myosin binding ability, as well as myosin phosphatase activity (chapter 2).

MRLC phosphorylation at PKC sites

The results demonstrated that the states of MRLC phosphorylation at PKC sites show a significant change in a cell cycle-dependent manner in sea urchin eggs. MRLC phosphorylation at PKC sites is increased at metaphase in the first cell cycle and subsequently decreased at anaphase. MRLC phosphorylation by PKC is known to inhibit actin-activated Mg^{2+}-ATPase activity and to reduce the stability of myosin-II filaments (Bengur, et al., 1987; Ikebe, et al., 1987). It has also been reported that additional MRLC phosphorylation by PKC after initial phosphorylation by MLCK results in a decrease of actin-activated Mg^{2+}-ATPase activity of myosin II (Nishikawa, et al., 1984). These results suggest that myosin II Mg^{2+}-ATPase activity is relatively low at metaphase as compared with that at other mitotic stages.

Satterwhite *et al.* (Satterwhite, et al., 1992) reported that in metaphase MRLC was phosphorylated at PKC sites by $p34^{cdc2}$ kinase in *Xenopus* egg lysates, but not in interphase, in which phosphorylation was detected only at MLCK sites. Thus, they proposed that MRLC phosphorylation at PKC sites by $p34^{cdc2}$ kinase inhibits myosin-II activity during pro/metaphase and delays cytokinesis until the onset of anaphase. Yamakita *et al.* (Yamakita, et el., 1994) reported phosphorylation states of MRLC *in vivo* in cultured cells. At metaphase, MRLC was phosphorylated mainly at PKC sites. After the release of mitotic arrest, the phosphorylation level of MLCK sites gradually increased as cells underwent cell division. They confirmed the results by Satterwhite *et al.* and suggested that the changes in phosphorylation of MRLC from PKC sites to MLCK sites may be a signal for induction of cytokinesis. These results are consistent with those obtained here from sea urchin eggs with regard to the phosphorylation level of PKC sites.

The increase in MRLC phosphorylation at PKC sites occurred in parallel with the activation of histone H1 kinase activity. This phosphorylation at PKC sites took place even in the absence of Ca^{2+}/calmodulin. These results suggest that the phosphorylation at PKC sites is not caused by PKC. Thus, to investigate whether the phosphorylation at PKC sites is caused by $p34^{cdc2}$ kinase, the effect of butyrolactone I, a selective inhibitor of $p34^{cdc2}$ kinase, were examined. Butyrolactone I inhibited the phosphorylation at PKC sites as well as histone H1 kinase activity in the egg extract, with as IC_{50} value was of about 2-5 μM. In contrast, its IC_{50} value against the phosphorylation at MLCK sites was about 100-fold higher than that against phosphorylation at PKC sites. The IC_{50} values of butyrolactone I against $p34^{cdc2}$ kinase and PKC were reported to be 0.68 and 160 μM, respectively (Kitagawa, et al., 1993). Therefore, it is reasonable to assume that phosphorylation at PKC sites at metaphase is induced by $p34^{cdc2}$ kinase. In fact, we have isolated $p34^{cdc2}$ kinase from sea urchin and shown that purified $p34^{cdc2}$ kinase can phosphorylate MRLC at PKC sites. It should be noted that butyrolactone I also inhibits cdk 2 kinase, a cdc2 family kinase (Kitagawa, et al., 1993). Thus, it is possible that MRLC phosphorylation at PKC sites is induced by other enzymes involved in cyclin-dependent kinase family. Further investigations are required to determine whether MRLC phosphorylation at PKC sites at metaphase is induced by $p34^{cdc2}$ kinase.

MLCK activities during cell division

In contrast to phosphorylation at PKC site, MLCK sites phosphorylation of MRLC showed no significant change during the first cell cycle in the presence of Ca^{2+}/calmodulin. Although the phosphorylation at MLCK sites is elevated in the presence of Ca^{2+}/calmodulin, this activation was not so marked (about 2-3 fold) as compared with the case of smooth muscle MLCK (Adelstein and Klee, 1981). Sea urchin MLCK has not been identified, but it has been reported that a multifunctional Ca^{2+}/calmodulin-dependent kinase has MRLC phosphorylating activity in sea urchin eggs (Chou and Rebhun, 1986). It is possible that sea urchin MLCK is different from

conventional Ca^{2+}/calmodulin-dependent MLCKs (i.e. smooth muscle or non-muscle MLCK). It should be necessary to purify MLCK from sea urchin eggs to resolve these problems.

Recently, it has been shown that Rho-kinase, which is one of the effector molecule of small G protein Rho, can phosphorylate MRLC at MLCK sites (Amano, et al., 1996). Rho has been shown as an key molecule participating in the reorganization of actin cytoskeleton (Hall, 1998; Narumiya, et al., 1998). It is also suggested that Rho is involved in cell division of fertilized oocytes from sea urchin (Mabuchi, et al., 1993) and *Xenopus* (Kishi, et al., 1993). It is very attractive that Rho regulates MRLC phosphorylation in cell division through Rho-kinase. On the other hand, we reported that mitogen-activated protein kinase activated protein kinase 2 (MAPKAP kinase 2) phosphorylates MRLC at MLCK sites (Komatsu and Hosoya, 1996) and, furthermore, isolated MAPKAP kinase 4 from sea urchin, which is capable of phosphorylating MRLC at MLCK sites (Komatsu, et al., 1997). It is also reported that MAP kinase can phosphorylate directly MLCK resulting an activation MLCK ( ). These results suggest that MAP kinase cascade may be involved in myosin phosphorylation. Further investigations are necessary to characterize the precise factors responsible for the temporal and spatial control of myosin phosphorylation.

Myosin phosphatase activity during cell division

The activation of myosin phosphatase by mitosis-specific phosphorylation is unique as it provides a positive regulatory mechanism for myosin phosphatase distinct from previous reports on phosphorylation of MYPT. For example, phosphorylation by Rho-kinase (Kimura, et al., 1996) or by endogenous kinase (Ichikawa, et al., 1996) was reported to inhibit the activity of myosin phosphatase. Phosphorylation by protein kinase A was found to decrease the association of MYPT with lipids (Ito, et al., 1997), which was suggested as a mechanism to regulate association of MYPT with membranes. Activation of myosin phosphatase via the cGMP-dependent protein kinase has been proposed (Wu, et al., 1996; Lee, et al., 1997) but the mechanism of activation has not been established.

Mechanism of activation of myosin phosphatase by mitosis-specific phosphorylation

Ser-430 (which corresponds to Ser-435 in rat MYPT) was identified as one of the mitosis-specific phosphorylation sites. It should be noted that the sequence surrounding Ser-430 is well

conserved among different species including human, rat and chicken although Ser-430 is changed to Thr-435 in human (see for reviewHartshorne, et al., 1998). In fact, chicken and rat MYPT have an identical sequence of 42 amino acids around this phosphorylation site. There are two genes that express MYPT, denoted MYPT1 and MYPT2 (Takahashi, et al., 1997; Fujioka, et al., 1998). The isoform examined in this article is MYPT1 and MYPT2 is found in heart and brain. The putative mitosis-specific phosphorylation site also is found in MYPT2 at Ser-437. The sequence around this site, 428 to 447 of MYPT2 is 90% identical to 421 to 440 of MYPT1. Thus, it is possible that phosphorylation of this site on MYPT is a general mechanism for activation of myosin phosphatase activity. Clearly, identification of the kinase responsible for the mitosis-dependent phosphorylation is a priority for future studies.

It is not clear at present how mitotic phosphorylation influences the binding of MYPT to myosin. One of the mitosis-specific phosphorylation sites, Ser-430 (Ser-427 may be phosphorylated simultaneously) is located toward the middle of the MYPT molecule. There is controversy regarding the location of the myosin-binding sites on MYPT. It has been reported that the ankyrin repeats at the N-terminal portion of MYPT are involved (Hirano, et al., 1997) and also that a C-terminal sequence is implicated (Johnson, et al., 1997). Neither part of the molecule is close (in terms of linear sequence) to Ser-430. A direct influence of Ser-430 on either of the putative myosin-binding sequences therefore is not possible unless the MYPT molecule bends, or folds, to accommodate such an interaction. It is possible that other mitosis-specific phosphorylation sites on MYPT may be close to one of the myosin binding sites and contribute to the myosin-binding effect. Alternatively, phosphorylation of Ser-430 may induce a longer-range conformational change at the myosin-binding site. Clearly, the solution to this problem requires the identification of all of the mitosis-specific phosphorylation sites as well as the region(s) of MYPT involved in interaction with myosin.

Physiological significance

Mitosis-specific phosphorylation of MYPT may play a significant role in the regulation of microfilament reorganization during cell division of cultured cells. The enhanced myosin phosphatase activity would increase the probability for dephosphorylation of MRLC during

prophase, leading to the disassembly of stress fibers and cell rounding. The increased activity of myosin phosphatase and resultant disassembly of stress fibers also are compatible with the localization of MYPT during mitosis. MYPT was reported to show a diffuse localization during mitosis (Ito, et al., 1997) while it is associated with microfilament structures such as stress fibers and adhesion belts during interphase (Inagaki, et al., 1997; Murata, et al., 1997).

In addition, the reversal of mitosis-specific phosphorylation, i.e. dephosphorylation, during cytokinesis negates the activation of myosin phosphatase and thus would favor a higher level of Ser-19 phosphorylation for the activation of contractile rings. This idea again is consistent with previous results in that the phosphorylation sites on MRLC change from Ser-1/2 to Ser-19 during cytokinesis (Satterwhite, et al., 1992; Yamakita, et al., 1994). This is supported by recent immunolocalization data using a Ser-19 phosphorylation specific antibody, in which prometaphase cells showed a lower level of Ser-19 phosphorylation than cells at telophase (Matsumura, et al., 1998; Murata-Hori, et. al.,1998).

It was reported that microinjection of a catalytic fragment of MLCK (constitutively active MLCK) significantly delayed the onset of anaphase but did not alter the rate of progression of cytokinesis (Fishkind, et al., 1991). This results suggest that the increased activity of myosin phosphatase is important for normal progression of cell cycle during prometa/metaphase since myosin phosphatase activity should be counteracted by phosphorylation of Ser-19 by the catalytic fragment of MLCK before cytokinesis. The decrease in myosin phosphatase activity during cytokinesis would then be able to regulate cleavage furrow contraction. If these speculations are correct then the mitosis-specific phosphorylation of MYPT may play a pivotal role in the control of cell division.

The positive regulatory mechanism of myosin phosphatase described above contrasts to the negative regulation by phosphorylation of MYPT with Rho-kinase. It is possible that both mechanisms collaborate to regulate the massive re-organization of microfilaments during cell division. This incorporates two phosphorylation steps: an activation via the mitosis-specific kinase(s) and an inhibition via Rho-kinase (Amano, et al., 1996; Kimura, et al., 1996). It is therefore reasonable to suggest that there are at least two functional phosphorylation sites to reflect

the positive and negative regulatory effects. When cells enter prophase it is proposed that the mitosis-specific phosphorylation occurs (via an unknown kinase(s)) and this causes activation of myosin phosphatase and a decrease in the level of myosin phosphorylation (at Ser-19). The result is disassembly of stress fibers and cell rounding. On exit from mitosis and prior to, or during cytokinesis, the activating site(s) on MYPT are dephosphorylated. At the same phase of the cell cycle it is suggested that inhibition of myosin phosphatase occurs via phosphorylation of MYPT by Rho-kinase. Here the next result would be an increase in the level of myosin phosphorylation (again at Ser-19) and activation of myosin for cell division. There are important components of this scheme that must be identified before a plausible mechanism can be established and these include the kinase(s) and phosphatase(s) involved at the mitosis-specific stage.

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2．公表論文

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（2）Activation of Myosin Phosphatase Targeting Subunit by Mitosis-specific Phosphorylation.

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