

Activation of Myosin Phosphatase Targeting Subunit by Mitosis-specific Phosphorylation

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Abstract. It has been demonstrated previously 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., S. Yamashiro, and F. Matsumura. 1994. *J. Cell Biol.* 124:129–137; Satterwhite, L.L., M.J. Lohka, K.L. Wilson, T.Y. Scherson, L.J. Cisek, J.L. Corden, and T.D. Pollard. 1992. *J. Cell Biol.* 118:595–605), 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 (two- to threefold) 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.

Key words: myosin • phosphorylation • phosphatase • mitosis • myosin binding

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 postmitotic 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 nonmuscle cells (see for review Kamm 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; Goeckeler and Wysolmerski, 1995), stress fiber assembly upon

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1. *Abbreviations used in this paper:* 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.

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 postmitotic 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; Murata-Hori 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 RMLC 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 RMLC 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 ~130 kD, a catalytic subunit of 38 kD, and a small subunit of 20 kD (Alessi et al., 1992; Shimizu et al., 1994; Shirazi et al., 1994). The catalytic subunit is the δ isoform of type 1 protein phosphatase (PP1c δ). 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 to 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 ATP γ S 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 ki-

nase 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 COOH-terminal region and this phosphorylation inhibited phosphatase activity (Kimura et al., 1996). It is known that Rho A is involved in the Ca²⁺-sensitization process in smooth muscle (see for review Somlyo 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 COOH-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, 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 RMLC 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 DME containing 10% newborn calf serum in an atmosphere of 5% CO₂ and 95% air at 37°C. CHO cells were maintained in F12 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 μ g/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% newborn 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 ³²P-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 (pAb; rabbit) raised against the NH₂-terminal 38 residues of chicken gizzard MYPT, termed Ab₁₋₃₈ (Berkeley Antibody Co.; Muranyi et al., 1998); pAb (rabbit) raised against residues 1–296 of gizzard MYPT, affinity purified using the MYPT fragment 1–296, termed Ab₁₋₂₉₆; and mAb (IgG1) raised against the gizzard phosphatase holoenzyme (Berkeley Antibody Co.; Trinkle-Mulcahy et al., 1995). Other antibodies were mAb against the type 1 phosphatase catalytic subunit, PP1c (Transduction Labs.), and mAb against the polyhistidine tag (Sigma Chemical Co.).

Protein Preparations

Trimeric myosin phosphatase was purified from chicken gizzard according to the method of 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 myosin light chain kinase (MLCK) and 5 µg/ml calmodulin in 30 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.1 mM ATP (with or without 0.1 mCi/ml [γ -³²P]ATP). Phosphorylated myosin was dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 1 M KCl, 1 mM MgCl₂, and 0.1 mM DTT. Recombinant protein phosphatase type 1 catalytic subunit, α isoform (PP1 α), was purchased from Calbiochem-Novabiochem.

Immunoprecipitation and Immunoblotting

Immunoprecipitation of MYPT was performed using two different buffers (buffer I and II). Buffer I contains 30 mM 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, 1 mM sodium vanadate, 1 mM DTT, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM 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 reduction 25 mM sodium pyrophosphate and 20 mM 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 16,000 g for 15 min. Cell lysates were stored at –80°C. After thawing quickly, the lysates were again centrifuged at 16,000 g for 15 min. Ab₁₋₂₉₆ or Ab₁₋₃₈ was added to the supernatants and incubated for 2 h at 4°C. The immunocomplex was precipitated with protein A-Sepharose (Pharmacia Biotech, Inc.) during a 1-h incubation. The immunocomplex was washed three 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% nonfat dried milk in PBS, and then incubated with the primary antibody (1:1,000 dilution) containing 0.3% BSA in PBS. Immunoreactive bands were detected with peroxidase-conjugated secondary antibody (1:1,000 dilution) using a chemiluminescence method (New England Nuclear).

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 (PP1 α from rabbit skeletal muscle; Calbiochem-Novabiochem) in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MnCl₂, 2 mM MgCl₂, 1 mM 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 MgCl₂, 0.1 mM CaCl₂, 5 mM EGTA, and 0.1 mg/ml cytochalasin D as described (Murray, 1991). Interphase ex-

tracts were prepared from mitotic extracts by the addition of 0.5 mM CaCl₂ followed by incubation at 20°C for 30 min to inactivate MPF.

Rat MYPT was prepared from interphase REF-2A cells by immunoprecipitation with Ab₁₋₂₉₆-conjugated Sepharose beads (cross-linked with dimethylpiperimidate; Pierce Chemical Co.). Buffer I and 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 1 mM ATP (with or without 1 mCi/ml [γ -³²P]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 421–442 of rat MYPT (which corresponds to Val 416–Lys 437 of chicken MYPT) was commercially synthesized (Bio-Synthesis Inc.). The peptide (20 µg) was phosphorylated with *Xenopus* mitotic extracts in the presence of 1 mCi/ml [γ -³²P]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 MYPT304–511 was subcloned into a pQE32 vector (QIAGEN, Inc.) with a hexahistidine tag at the NH₂ terminus as described (Hirano et al., 1997). NH₂- and COOH-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 *Escherichia coli* and purified by a metal affinity column (Sigma Chemical Co.) as described (Hirano et al., 1997).

Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene). For the mutation of Ser 427 to Asp, a forward primer, 5'-GAAGAGAGGAAAGATGAAGATCCTGCTTCGTGGAGGTTAG-3', and a reverse primer, 5'-CTAACCTCCACGAAGCAGGATCTTCATCTTCTCTCTTC-3', were used. For the mutation of Ser 430 to Glu, a forward primer, 5'-GAAAGATGAATCTCCTGCTGAGTGGAGGTTAGGTTCTTCG-3', and a reverse primer, 5'-CGAAGACCTAACCTCCACTCCAGCAGGAGATTCATCTTTC-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 Ab₁₋₂₉₆ with 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 MgCl₂, 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 16,000 g 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 Ab₁₋₃₈ with buffer II and phosphorylated (without radioactive ATP) in vitro us-

ing *Xenopus* mitotic or interphase extracts as described above. After extensive washing, immobilized myosin phosphatase was incubated at 30°C with ³²P-labeled myosin (0.5 μM) in 30 mM Tris-HCl (pH 7.5), 0.1 M KCl, 2 mM MgCl₂, 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 radioactivities of the supernatants were determined by Cerenkov counting. The reaction time was adjusted so that ~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 in Boyle et al. (1991). Phosphopeptides were detected by autoradiography. SDS-PAGE was performed as described by 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 (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 mAb specific for MYPT. As Fig. 1 a 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 pAb, Ab₁₋₃₈, 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 PP1c. The phosphatase treatment completely restored the reactivity of mitotic MYPT with the mAb (Fig. 1 b; 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. 1 b; 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 cells, 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

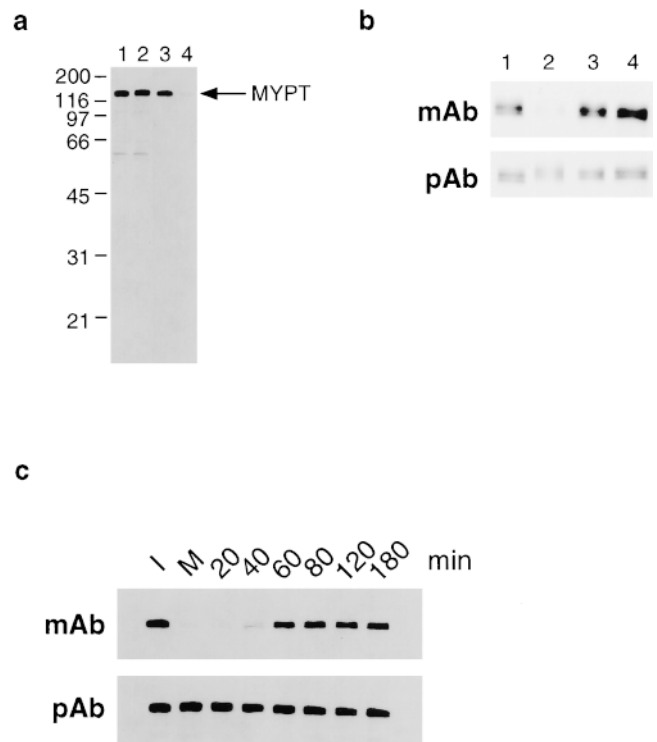


Figure 1. Mitosis-specific phosphorylation of MYPT. (a) Mitotic MYPT loses a reactivity to the mAb specific to MYPT. Total cell lysates of interphase (lanes 1 and 3) or mitotic cells (lanes 2 and 4) were immunoblotted with rabbit pAb (lanes 1 and 2) or mouse mAb (lanes 3 and 4). The arrow indicates the position of MYPT (130 kD). Molecular mass markers are indicated in kD 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) or mitotic cells (lanes 2 and 4). Half of the immunoprecipitate was treated with PP1c (lanes 3 and 4). Both treated or untreated samples were immunoblotted with the mAb or pAb. (c) Time course of mitosis-specific modification of MYPT during cell division. Total cell lysates were prepared from interphase cells (lane I), mitotic cells (lane M), and cells at different stages of cell division (time in minutes after the release of mitotic arrest), and blotted with either the mAb or the pAb. Cytokinesis occurred 40–60 min after release of mitotic arrest.

on the cell cycle stage. As Fig. 1 c (upper panel) 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 postmitotic 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. 1 c, 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 40 min and then was reversed at 60 min. These results again indicate that MYPT was dephosphorylated during cytoki-

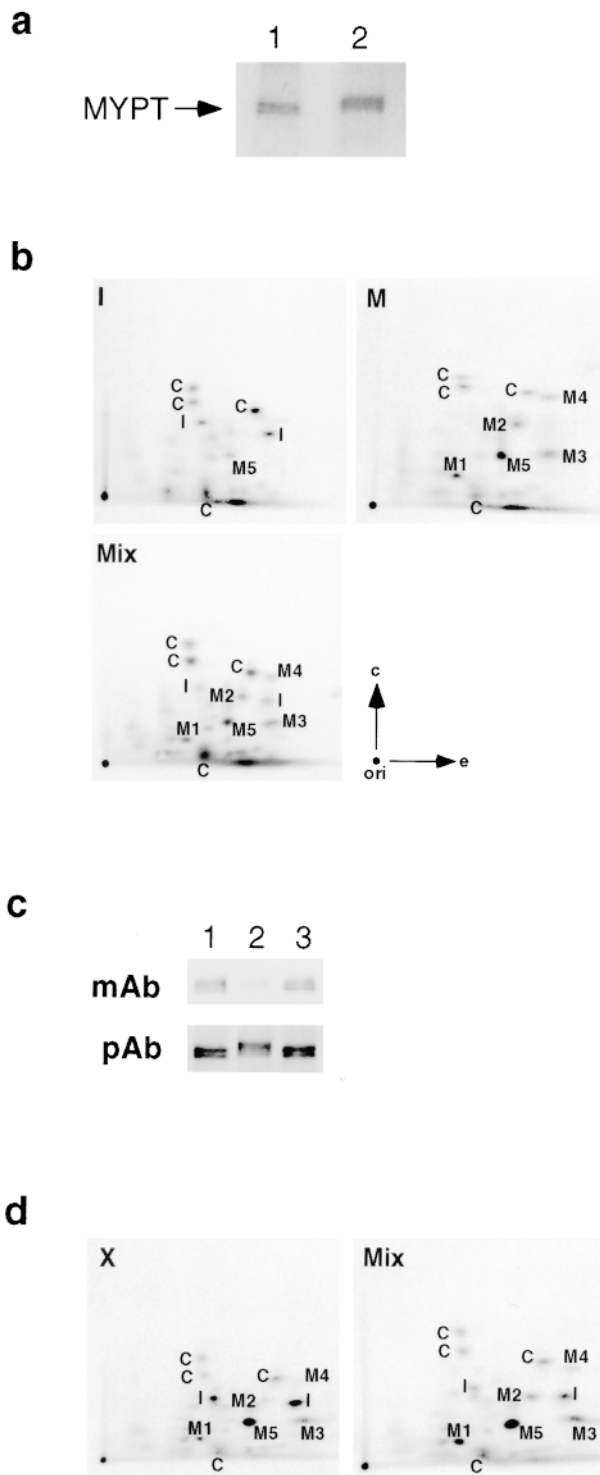


Figure 2. Phosphopeptide analysis of mitotic and interphase MYPT and in vitro reconstitution of mitosis-specific phosphorylation of MYPT. (a) In vivo phosphorylation of MYPT from interphase or mitotic cells. MYPT was isolated by immunoprecipitation from interphase (lane 1) or mitotic cells (lane 2) that had been labeled with ^{32}P -orthophosphate. ^{32}P -labeled MYPT was separated by SDS-PAGE followed by autoradiography. (b) Two-dimensional tryptic phosphopeptide mapping analysis of in vivo phosphorylated MYPT. I, MYPT isolated from interphase; M, MYPT from mitotic cells; 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

nesis. We have also observed similar modifications of MYPT during mitosis of other cells including 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. 2 a 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. 2 b shows the phosphopeptide maps generated with interphase (I) and mitotic (M) MYPT. To identify which spots are mitosis-specific, a mixture of mitotic and interphase MYPT samples was subjected to peptide mapping (Mix). The map of mitotic MYPT (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 (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,

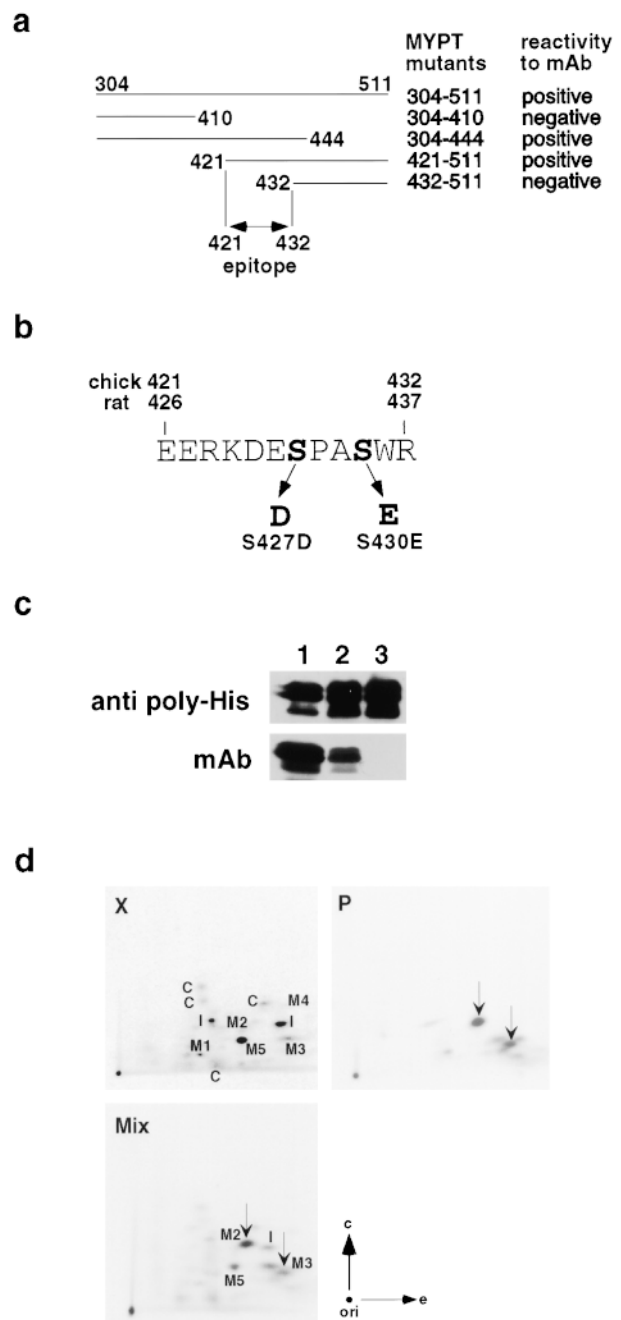
map. Phosphopeptide spots specifically observed in interphase map and spots commonly observed in both interphase and mitotic MYPT are labeled with I and C, respectively. Arrow with e, electrophoretic dimension; arrow with c, chromatographic dimension; ori, the origin. (c) In vitro reconstitution of mitotic 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 or pAb. 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 (X). For comparison, a mixture of in vivo and in vitro phosphorylated MYPT (Mix) is shown. The mitosis-specific spots are indicated by M.

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. 2 c, upper panel, lane 2). At the same time, the mobility of MYPT showed an upward shift (Fig. 2 c, 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. 2 d, the phosphopeptide map of MYPT phosphorylated *in vitro* by mitotic *Xenopus* extracts (X) was similar to that phosphorylated *in vivo* in mitotic cells (compare M, Fig. 2 b). For comparison, a mixture of *in vivo* and *in vitro* phosphorylated MYPT was subjected to phosphopeptide mapping (Mix, Fig. 2 d). 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 prolonged 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, D.J., unpublished results) and to define more precisely the epitope a series of truncation mutants was analyzed. In Fig. 3 a 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 432. There are two serines (no threonine) in this sequence, Ser 427 and Ser 430 (Fig. 3 b), 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 immuno-



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 437 of rat MYPT), and contains two serine residues of Ser 427 and Ser 430. To mimic phosphorylation, Ser 427 and Ser 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 antipolyhistidine antibody or the mAb against MYPT. Lane 1, wild-type MYPT304-511; lane 2, S427D mutant; lane 3, S430E mutant. (d) Phosphopeptide mapping analysis of a synthetic peptide (NH₂-ISPKEERKDESPASWRLGLRKC-COOH) containing Ser 430. P, peptide map of the synthetic peptide phosphorylated by *Xenopus* mitotic extracts; 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 P (indicated by arrows) match M2 and M3 of mitotic spots.

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 antipolyhistidine antibody. The epitope of the mAb should be located between 421 and 432. (b)

blotting. It was found that mutation of Ser 430 to Glu (S430E) resulted in complete loss of reactivity (lane 3 of Fig. 3 c). 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. 3 c). 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 421–442 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 (P, Fig. 3 d) 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 (X), and with a map of a mixture from the peptide and MYPT samples (Mix). It was found that two major spots (indicated by arrows) 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

To explore the functional significance of mitosis-specific phosphorylation of MYPT, the myosin binding activities of MYPT from mitotic or interphase cells were 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. 4 a shows, the amount of mitotic MYPT bound to phosphorylated myosin was about threefold 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, 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 (2–25 nM) were mixed with phosphorylated myosin in the presence of Mg-ATP and their binding was examined. As Fig. 4 b shows, the amount of MYPT bound to phosphorylated myosin was two to three times higher using MYPT phosphorylated with mitotic extracts com-

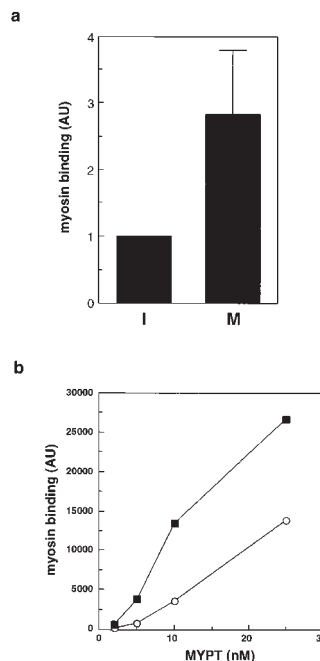


Figure 4. Increased myosin binding ability of mitotic MYPT. (a) Mitotic or interphase MYPT was prepared by immunoaffinity purification and myosin binding activity was determined by cosedimentation assay with phosphorylated myosin as described in Materials and Methods. The figure shows myosin binding of MYPT prepared from interphase cells (I) or mitotic cells (M). The values shown are the means \pm SEM 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 (○).

pared to the MYPT phosphorylated by interphase extracts. At 25 nM MYPT, it was found that ~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. 4 a).

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. 5 a), 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. 5 a), that essentially identical amounts of MYPT and the catalytic subunit were present in the immunoprecipitates treated with mitotic or interphase *Xenopus* extracts. As Fig. 5 b 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 phos-

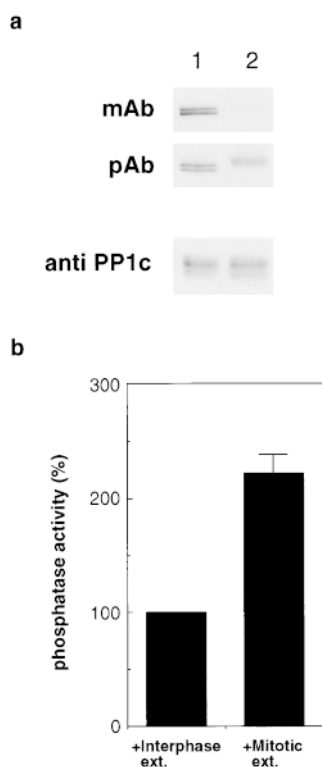


Figure 5. Myosin phosphatase activity of in vitro phosphorylated myosin phosphatase. (a) Immunoblotting analysis of in vitro phosphorylated myosin phosphatase. Interphase myosin phosphatase was prepared by immunoprecipitation and phosphorylated in vitro with *Xenopus* interphase (lane 1) or mitotic extracts (lane 2). After extensive washing, phosphorylated samples were analyzed by immunoblotting with the mAb, pAb against MYPT, or anti-PP1c antibody. (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 \pm SEM from three independent experiments.

phorylated 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 humans (see for review Hartshorne 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, 428–447 of MYPT2 is 90% identical to 421–440 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 NH₂-terminal portion of MYPT are involved (Hirano et al., 1997) and also that a COOH-terminal sequence is implicated (Johnson et al., 1997). Neither part of the molecule is close (in terms of linear sequence) to Ser 430. Therefore, a direct influence of Ser 430 on either of the putative myosin-binding sequences 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. 5 b). 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 phos-

phorylation 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 the negative regulation by phosphorylation of MYPT with Rho-kinase. It is possible that both mechanisms collaborate to regulate the massive reorganization 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 unknown kinase) 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 before, 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 (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, these include the kinase(s) and phosphatase(s) involved at the mitosis-specific stage.

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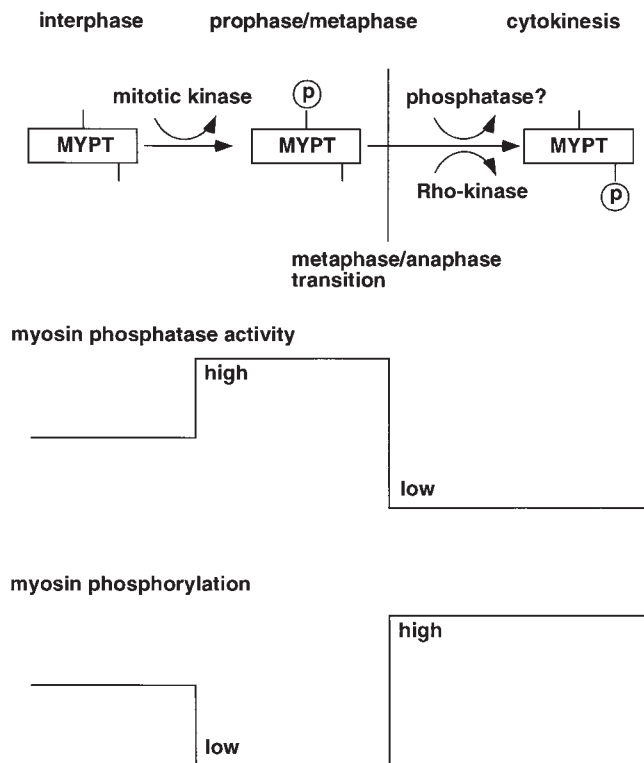


Figure 6. Model of the regulation of myosin phosphatase during cell division. MYPT has two functionally different phosphorylation sites. One is the mitosis-specific phosphorylation site(s) including Ser 430 that activate myosin phosphatase activity, and the other is Rho-kinase site(s) that inhibit myosin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

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