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# A Novel Regulatory Mechanism of Myosin Light Chain Phosphorylation via Binding of 14-3-3 to Myosin Phosphatase

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**Myosin II phosphorylation-dependent cell motile events are regulated by myosin light-chain (MLC) kinase and MLC phosphatase (MLCP). Recent studies have revealed myosin phosphatase targeting subunit (MYPT1), a myosin-binding subunit of MLCP, plays a critical role in MLCP regulation. Here we report the new regulatory mechanism of MLCP via the interaction between 14-3-3 and MYPT1. The binding of 14-3-3 $\beta$  to MYPT1 diminished the direct binding between MYPT1 and myosin II, and 14-3-3 $\beta$  overexpression abolished MYPT1 localization at stress fiber. Furthermore, 14-3-3 $\beta$  inhibited MLCP holoenzyme activity via the interaction with MYPT1. Consistently, 14-3-3 $\beta$  overexpression increased myosin II phosphorylation in cells. We found that MYPT1 phosphorylation at Ser472 was critical for the binding to 14-3-3. Epidermal growth factor (EGF) stimulation increased both Ser472 phosphorylation and the binding of MYPT1-14-3-3. Rho-kinase inhibitor inhibited the EGF-induced Ser472 phosphorylation and the binding of MYPT1-14-3-3. Rho-kinase specific siRNA also decreased EGF-induced Ser472 phosphorylation correlated with the decrease in MLC phosphorylation. The present study revealed a new RhoA/Rho-kinase-dependent regulatory mechanism of myosin II phosphorylation by 14-3-3 that dissociates MLCP from myosin II and attenuates MLCP activity.**

## INTRODUCTION

Myosin II is an actin-based motor protein that plays a critical role in diverse cell motile events such as muscle contraction, cell locomotion, cell division, and the maintenance of cell morphology. In vertebrate nonmuscle and smooth muscle cells, myosin II motor function is regulated by phosphorylation of the regulatory light chain (MLC; Kamm and Stull, 1989; Sellers, 1991; Tan *et al.*, 1992). Several protein kinases can phosphorylate myosin II in vitro but physiologically important kinases are thought to be different among the cells types. In smooth muscle, Ca<sup>2+</sup>/calmodulin-dependent MLC kinase (MLCK) is primarily responsible for myosin II phosphorylation. In nonmuscle cells, multiple kinases may phosphorylate myosin II upon various types of stimulation. On the other hand, recent studies have revealed that myosin II phosphorylation is also controlled by regulating the activity of MLC phosphatase (MLCP).

MLCP holoenzyme consists of three subunits, a myosin-binding large subunit (MYPT1), a 20-kDa small subunit, and a catalytic subunit of the type 1 protein serine/threonine phosphatase family, PP1 $\delta$  (Alessi *et al.*, 1992; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994). It has been reported that holoen-

zyme has an affinity for myosin (Shimizu *et al.*, 1994) and shows higher phosphatase activity than the isolated catalytic subunit (Alessi *et al.*, 1992; Shirazi *et al.*, 1994), suggesting that the binding of the regulatory subunits increases enzyme activity presumably related to the myosin-binding activity of MYPT1. It has been shown that MYPT1 can be phosphorylated by Rho-kinase, resulting in a decrease in MLCP activity in vitro (Kimura *et al.*, 1996). Rho-kinase phosphorylates MYPT1 at two sites in vitro, i.e., Thr641 and Thr799 (in rat MYPT1 sequence, corresponds to the Thr696 and Thr853 of human MYPT1, respectively), of which Thr641 is responsible for the inhibition of MLCP activity (Feng *et al.*, 1999). This raises the hypothesis that an activation of the Rho signaling pathway could phosphorylate MYPT1 by Rho-kinase, thus down-regulating MLCP.

The structure-function relationship of MYPT1 has been studied. Ichikawa *et al.* (1996) showed that the recombinant N-terminal two thirds of the large subunit contains a myosin-binding site. On the other hand, it has been reported that the C-terminal 291 residues of the large subunit, not the N-terminal fragment, bind to myosin. MYPT1 is critical to hold the three subunits together. The C-terminal 72 residues reside at the 21/20 kDa subunit binding site (Johnson *et al.*, 1997). The catalytic subunit binds to the large subunit at two sites, a relatively strong site in the N-terminal 38 residues and a weak site in the ankyrin repeat (residues 39–295; Hirano *et al.*, 1997). A binding site for phosphorylated MLC is also assigned to the ankyrin repeat.

Although isolated MLCP/MYPT1 binds strongly to myosin, intracellular localization of MYPT1 is not entirely colocalized with myosin filaments, and a significant fraction of MYPT1 is present throughout cytosol (Murata *et al.*, 1997). This raises the hypothesis that the association of MYPT1/MLCP to myosin in vivo is regulated.

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Abbreviations used: CBB, Coomassie blue staining; mcLR, microcystine LR; MLC, myosin light chain; MLCP, myosin light-chain phosphatase; MYPT1, myosin phosphatase targeting subunit 1; PP1 $\delta$ , type 1  $\delta$  protein phosphatase; siRNA, small interfering RNA.

In the present study, we found that 14-3-3 interacts with MYPT1, using yeast two-hybrid system. 14-3-3 is an acidic small-molecular-mass protein that is widely expressed in a variety of organisms, and it is thought that 14-3-3 play a role in various cellular processes such as signal transduction, cell cycle regulation, apoptosis, and cytoskeletal reorganization (Fu *et al.*, 2000; van Hemert *et al.*, 2001; Tzivion and Avruch, 2002; Aitken, 2006), although the function of 14-3-3 is still not completely understood. Here we report that 14-3-3 binds to MYPT1 and the binding attenuates MLCP phosphatase activity and induces dissociation of MYPT1 from myosin. The overexpression of 14-3-3 in COS7 cells abolished the localization of MYPT1 at the stress fiber, indicating that 14-3-3 controls the interaction between MLCP and myosin II, thus regulating myosin II phosphorylation in cells. Furthermore, we found that the phosphorylation of MYPT1 at Ser472 was critical for the binding to 14-3-3 and Rho-kinase-activated Ser472 phosphorylation in the epidermal growth factor (EGF) signaling pathway. The present study uncovers a new regulatory mechanism of MLCP function and thus the regulation of myosin II phosphorylation.

## MATERIALS AND METHODS

### Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed as described (Koga and Ikebe, 2005). Briefly, yeast AH109 strains were sequentially transformed with pGBKT7 coding for full-length rat MYPT1 and then with a human aorta cDNA library constructed in pACT2. Initial transformants were selected for being positive on synthetic complete plates lacking tryptophan-leucine-histidine-adenine and containing X- $\alpha$ -gal on the surface of the plates. Of 3.5 million initial transformants, two clones, termed clones TM27 and TM41, interacting with MYPT1 were identical.

### Vector and cDNA Constructs

The mutants were made by the site-directed mutagenesis strategy (Yano *et al.*, 1993). To delete the C-terminal residues of MYPT1, a stop codon was created at codons 39, 172, 297, and 517 for F38, K171, E296 and N516, respectively. pGBKT7/MYPT1 was digested by NcoI to excise a cDNA fragment encoding amino acids 1–376 of rat MYPT1. The digested MYPT1 was self-ligated and termed  $\Delta$ N. 14-3-3 $\beta$  cDNA in TM27 was subcloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and then subcloned into pGBKT7, pFBHTa, pGEX4T1, pDsRed2, pEGFP, and pGADT7. The entire coding region of 14-3-3 $\alpha$  and  $\zeta$  were amplified from human aorta cDNA library by PCR and then subcloned into pEGFP.

### Antibodies

Rabbit anti-green fluorescent protein (GFP) polyclonal antibody, mouse anti-14-3-3 $\beta$  mAb, anti-DsRed antibodies, and mouse anti-MLC mAb were purchased from MBL International (Woburn, MA), Calbiochem (La Jolla, CA), BD Biosciences (San Jose, CA), and Sigma (St. Louis, MO), respectively. Mouse anti-smooth muscle myosin mAb was produced as described (Hashihara *et al.*, 1989). The mouse mAb, which specifically recognizes phospho-Serine-19 of MLC, was prepared as described (Komatsu *et al.*, 2000). Rabbit anti-MYPT1 polyclonal antibody against the C-terminal peptide (TEDGSSKRDTQTD; amino acids 819–832 of Rat MYPT1) and rabbit anti-pSer472 polyclonal antibody (VIRSAphosphoSSPRLS; amino acids 467–477 of Rat MYPT1) were prepared by Genemed Synthesis (South San Francisco, CA) and purified by affinity chromatography as described (Niuro *et al.*, 2003). Alexa Flour 488 phalloidin and Texas-red phalloidin were purchased from Molecular Probes (Eugene, OR). Rabbit anti-PP1 $\delta$  and anti-Myosin IIB polyclonal antibodies were kindly supplied by Dr. Villa Moruzzi (University of Pisa, Italy) and by Dr. R. S. Adelstein (National Institutes of Health, Bethesda, MD), respectively.

### Purification of Proteins

*Escherichia coli* expressing glutathione S-transferase (GST)-14-3-3 $\beta$  were lysed in phosphate-buffered saline (PBS) with 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10  $\mu$ g/ml leupeptin. The homogenate was centrifuged at 10,000  $\times$  g for 20 min at 4°C, and the supernatant was subjected to reduced glutathione (GSH)-Sepharose 4B chromatography. After extensive wash, the GST-fusion proteins were eluted by 10 mM glutathione, 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM PMSF, and 10  $\mu$ g/ml leupeptin. Smooth muscle myosin and MLCK were prepared as described (Ikebe and Hartshorne, 1985; Ikebe *et al.*, 1987). *Xenopus* oocyte calmodulin was purified as described (Ikebe *et al.*, 1994b). Recombinant MLCP holoenzyme was purified from coinfecting

Sf9 cells with rat His MYPT1, rat PP1 $\delta$ , and rat M21 expressing viruses as described (Takizawa *et al.*, 2002). Recombinant MYPT1 was also purified according to the same procedure. Flag MYPT1 was purified using anti-Flag affinity chromatography and eluted with Flag peptide containing 30 mM Tris-HCl, pH 7.5, 150 mM NaCl. His 14-3-3 $\beta$  was expressed in Sf9 cells and purified by Ni-NTA chromatography according to the manufacturer's protocol (Qiagen, Chatsworth, CA).

### In Vitro Binding Assay

His MYPT1 (75  $\mu$ g/ml) was mixed with GST-14-3-3 $\beta$  (60  $\mu$ g/ml) or GST alone (60  $\mu$ g/ml) in the buffer A containing 30 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.5% NP-40 for 1 h at 4°C. GSH-Sepharose was added and the solution was further incubated for 0.5 h. Similarly, GST 14-3-3 $\beta$  (75  $\mu$ g/ml) with or without Flag MYPT1 (60  $\mu$ g/ml) was incubated in the buffer A for 1 h at 4°C, and then the mixture was further incubated with Flag agarose for 0.5 h. The GSH-Sepharose or the Flag agarose was washed three times with buffer A. The bound proteins were eluted and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue (CBB). One micromolar myosin, 2 nM MYPT1, and 6  $\mu$ M 14-3-3 $\beta$  were mixed in 30 mM Tris-Cl, pH 7.5, 0.3 M KCl for 3 h at 4°C, and mouse anti-myosin antibody was added and incubated for 3 h at 4°C. The mixture was further incubated for 1 h at 4°C with protein A-Sepharose. Protein A-Sepharose was washed three times with 30 mM Tris-Cl, pH 7.5, 0.3 M KCl, and 1% NP40 and subjected to SDS-PAGE, followed by Western blotting. Flag MYPT1 was purified in the presence of 1  $\mu$ M mCLR, microcystine LR (mCLR) and used for the binding assay of MYPT1 and PP1 $\delta$  in the presence or absence of 14-3-3 $\beta$ . Flag-MYPT1 (50  $\mu$ g/ml), PP1 $\delta$  (60  $\mu$ g/ml), and GST or GST 14-3-3 $\beta$  (300  $\mu$ g/ml) were incubated with Flag agarose in buffer A containing 100 nM mCLR, for 1 h at 4°C. Flag agarose was then washed with buffer A containing 100 nM mCLR, and the bound fraction was eluted as described above.

### Western Blotting

The samples were subjected to 7.5–20% gradient SDS-PAGE, and then the proteins were transferred to nitrocellulose membrane as described (Komatsu *et al.*, 2000).

### Immunoprecipitation

COS7 or NIH3T3 cells were washed with cold PBS three times and then scraped in lysis buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.1% Triton-X, 1 mM PMSF, 50 mM NaF, 10  $\mu$ g/ml leupeptin, and 1  $\mu$ M mCLR). The cells were lysed through a 26-gauge needle and centrifuged at 14,000  $\times$  g for 5 min at 4°C. Supernatants were incubated with protein A-Sepharose to absorb nonspecific binding proteins for 1 h at 4°C, and the supernatants were incubated with control IgG or specific antibody for 3 h at 4°C and then further incubated with protein A-Sepharose for 1 h at 4°C. Immunoprecipitates were washed with lysis buffer containing 100 mM NaCl three times and subjected for SDS-PAGE, followed by Western blotting.

### Phosphatase Assay

The phosphatase assay was carried out using the phosphorylated myosin as a substrate as described (Koga and Ikebe, 2005).

### Kinase Assay and Autoradiography

Phosphorylation of smooth muscle myosin was carried out at 25°C for 60 min with 1 mg/ml myosin in 30 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mM CaCl<sub>2</sub>, and 0.2 mM ATP in the presence or absence of 100 nM mCLR. The kinase reaction was started by the addition of cell lysate prepared as described above. The phosphorylation level of MLC was detected by Western blotting followed by densitometry analysis.

### Cell Culture and Transfection

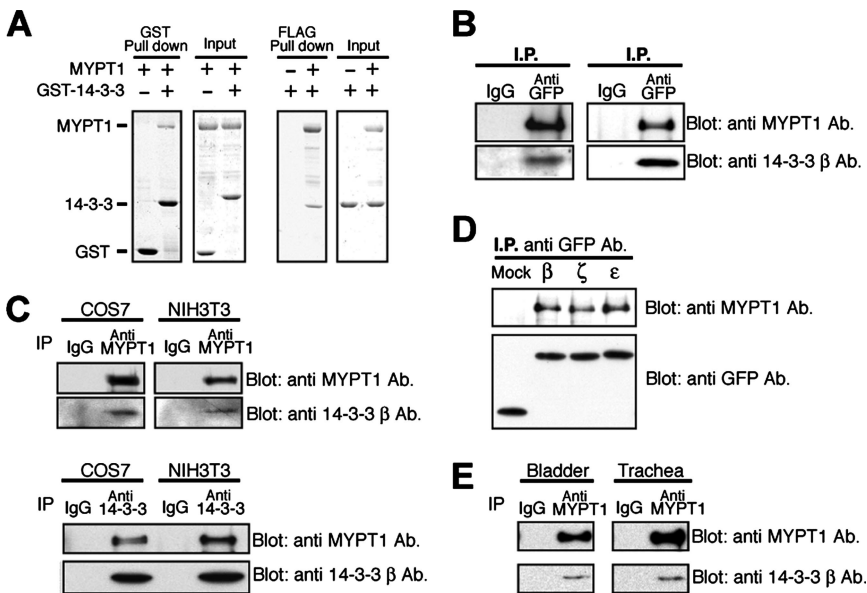
COS7 and NIH3T3 cells were cultured with DMEM, containing 10% fetal bovine serum. Cells were transfected using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's protocol.

### Small Interfering RNA Transfections

Control small interfering RNA (siRNA) was purchased from Dharmacon (Boulder, CO). siRNA sequences against Rock-I and Rock-II were also from Dharmacon (siGenome reagents D-003536 (GCCAATGACTTACTAGGA) and D-004610 (GCAAATCTGTAACTACTCG), respectively). HeLa cells were transfected with 50 nM siRNA using X-tremeGene siRNA reagent (Roche). After 72-h transfection, cells were starved for 24 h and stimulated with 25 ng/ml EGF.

### Immunofluorescence staining and image processing

Immunocytochemistry was performed as described (Komatsu *et al.*, 2000, 2002).



**Figure 1.** Interaction of 14-3-3 proteins with MYPT1 in vitro (A, B, and D) and in vivo (C and E). (A) Binding of the purified 14-3-3β with MYPT1. Left, His MYPT1 (75 μg/ml) and GST-14-3-3β 60 μg/ml or GST (60 μg/ml) were mixed and subjected to binding assay. Right, GST 14-3-3β (75 μg/ml) with or without Flag MYPT1 (60 μg/ml) were incubated with Flag agarose. The bound proteins were eluted and subjected to SDS-PAGE. Gels were stained with CBB. (B) Coimmunoprecipitation of expressed MYPT1 and 14-3-3β. The expressed GFP MYPT1 or GFP 14-3-3β in COS7 cells was immunoprecipitated with control IgG or anti-GFP antibody. Left, coimmunoprecipitation of 14-3-3β with GFP-MYPT1; right, coimmunoprecipitation of MYPT1 with GFP 14-3-3β. (C) Coimmunoprecipitation of endogenous MYPT1 and 14-3-3β. Untransfected COS7 or NIH3T3 cell lysates were used for immunoprecipitation with control IgG, anti-MYPT1 antibody or anti-14-3-3β antibody. (D) Coimmunoprecipitation of expressed 14-3-3β, ε, and ζ and MYPT1. The expressed GFP 14-3-3β, ε, and ζ in COS7 cells was immunoprecipitated with anti-GFP antibody. The immunoprecipitates were subjected to Western blotting.

It should be noted that the immunoprecipitation experiments were carried out in the presence of mCLR. (E) Binding of MYPT1-14-3-3 in the smooth muscle tissues. Rabbit bladder or porcine trachea lysates were used for immunoprecipitation with control IgG or anti-MYPT1 antibody.

## RESULTS

### Yeast Two-Hybrid Screening for Binding Partners of MYPT1

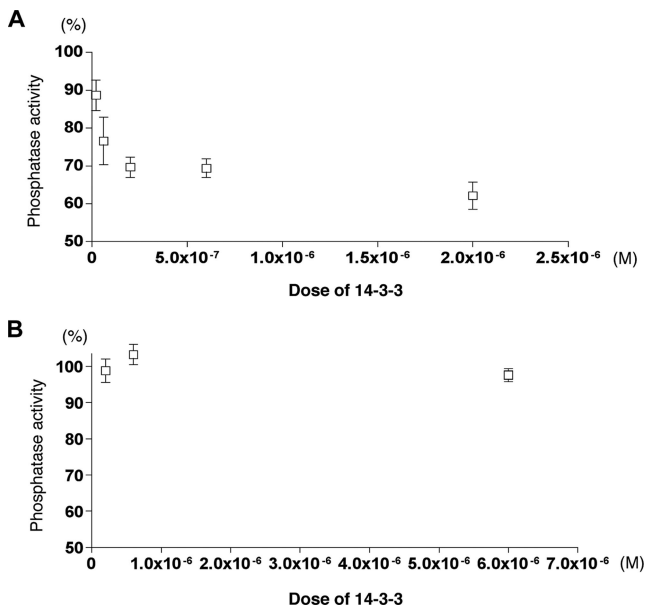
To identify the novel binding partners of MYPT1, a yeast two-hybrid screening was performed using full-length rat MYPT1 as bait. p116<sup>Rip</sup> (Koga and Ikebe, 2005) and 14-3-3β were identified as the binding proteins of MYPT1. As shown in Figure 1A, His MYPT1 was coprecipitated with GST-14-3-3β but not GST alone (left panel). On the other hand, GST 14-3-3β was also coprecipitated with Flag MYPT1 using Flag agarose (right panel). The results indicate that 14-3-3β directly binds to MYPT1. Furthermore, endogenous 14-3-3β was coimmunoprecipitated with GFP-MYPT1 (Figure 1B, left panel) and endogenous MYPT1 was coimmunoprecipitated with GFP-14-3-3β (Figure 1B, right panel). The binding between endogenous MYPT1 and 14-3-3β in untransfected NIH3T3 and COS7 cells was also demonstrated (Figure 1C). Furthermore, the interaction between MYPT1 and 14-3-3β was found in smooth muscle tissues such as rabbit bladder and porcine trachea (Figure 1E). It has been known that the targeting proteins of 14-3-3 may interact with multiple 14-3-3 isoforms (Freed *et al.*, 1994; Mills *et al.*, 2000). We examined if MYPT1 binds to other 14-3-3 isoforms. We examined the isoforms, 14-3-3ζ and 14-3-3ε, which have the highest (87%) and the lowest (65%) homology with 14-3-3β sequences, respectively. As shown in Figure 1D, GFP 14-3-3ζ or ε bound to MYPT1 as well as GFP 14-3-3β in COS7 cells transfected with 14-3-3β, ζ, or ε. However, the binding of MYPT1-14-3-3ε or ζ was not detected in untransfected COS7 cells (not shown) unlike 14-3-3β. Therefore we concentrated our efforts on 14-3-3β in the following experiments. It should be noted that the binding activity was detected in the presence of a potent protein phosphatase inhibitor, mCLR, in the lysis buffer for the immunoprecipitation experiments. The result suggests that phosphorylation is involved in the binding between MYPT1 and 14-3-3β.

### Effect of 14-3-3β on MLCP Activity

As the association of MYPT1 to the catalytic subunit enhances the myosin phosphatase activity (Alessi *et al.*, 1992; Shirazi *et al.*, 1994), we examined whether the binding of 14-3-3β to MYPT1 influences the phosphatase activity of MLCP. The MLCP activity of the holoenzyme was measured by using phosphorylated smooth muscle myosin by MLCK as a substrate. As shown in Figure 2A, the phosphatase activity of MLCP was inhibited by 14-3-3β in a dose-dependent manner. Similar inhibition of the phosphatase activity was also observed with isolated MLC as a substrate (not shown). In contrast, 14-3-3β did not inhibit the phosphatase activity of PP1δ, the catalytic subunit of MLCP (Figure 2B). The result indicates that the inhibition of the phosphatase activity by 14-3-3β is not due to the direct inhibition of the catalytic subunit, but is mediated by the binding of 14-3-3β to MYPT1. Because the dissociation of MYPT1 from the catalytic subunit is anticipated to result in the decrease in the phosphatase activity, we examined whether MYPT1 dissociates from the catalytic subunit in the presence of 14-3-3β. 14-3-3β was incubated with Flag-MYPT1 and His PP1δ, and then the proteins bound to Flag-affinity resin were analyzed (Figure 3A). As expected, 14-3-3β was coeluted with Flag-MYPT1. On the other hand, the amount of PP1δ coeluted with MYPT1 was not affected by the binding of MYPT1-14-3-3β. Interestingly, the interaction between MYPT1/PP1δ and 14-3-3β in the absence of microcystin LR (mCLR), a potent Ser/Thr phosphatase inhibitor, was not detected by Coomassie blue staining (CBB; Figure 3B), suggesting that phosphorylation is involved in the interaction.

We also examined the effect of 14-3-3β overexpression on the association between MYPT1 and PP1δ. The cell lysates expressing 14-3-3β were subjected to immunoprecipitation using anti-MYPT1 antibody, followed by Western blotting. The amount of PP1δ in MLCP coimmunoprecipitated with MYPT1 was not affected by the 14-3-3β overexpression (not shown). These results suggest that the binding of 14-3-3β to MYPT1 does not hamper the binding of MYPT1 to the





**Figure 2.** The effect of 14-3-3 $\beta$  on MLCP (A) or PP1 $\delta$  (B) activity. Various concentrations of His 14-3-3 $\beta$  and 2.5 nM MLCP (A) or 30 nM PP1 $\delta$  (B) were mixed, and then the dephosphorylation reaction was started by adding 0.75  $\mu$ M <sup>32</sup>P-labeled myosin. The prepared myosin was free of contaminated phosphatase activity. It should be noted that MLCP used in this experiment was not phosphorylated. Phosphatase assay was performed as described in *Material and Methods*. Values are mean  $\pm$  SEM of three independent experiments and expressed as 100% of the phosphatase activity in the absence of 14-3-3 $\beta$ .

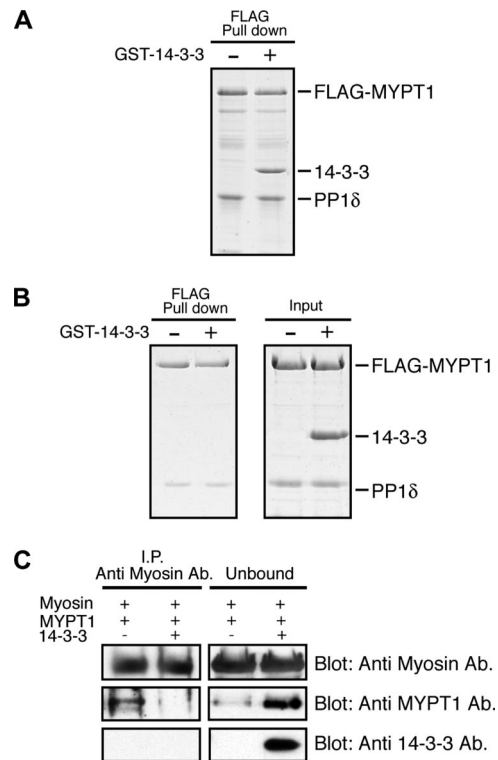
catalytic subunit. In other word, 14-3-3 $\beta$  induced the inhibition of MLCP activity is not due to the dissociation of MYPT1 from the holoenzyme.

#### The Effect of 14-3-3 $\beta$ on the Binding of MYPT1 to Myosin

One of the important functions of MYPT1 is its binding ability to myosin (Sellers and Pato, 1984), and it has been thought that this property bears the specificity of MLCP holoenzyme. Therefore, we examined the effect of 14-3-3 $\beta$  on the binding activity of MYPT1 to myosin. Smooth-muscle myosin, MYPT1, and the 14-3-3 $\beta$  mixture was subjected to immunoprecipitation using anti-myosin antibody. MYPT1 was coimmunoprecipitated with myosin (Figure 3C) consistent with the previous reports (Ichikawa *et al.*, 1996; Hirano *et al.*, 1997; Johnson *et al.*, 1997). Quite interestingly, 14-3-3 $\beta$  markedly inhibited the binding of MYPT1 to myosin (Figure 3C). 14-3-3 $\beta$  was not coimmunoprecipitated with myosin, therefore, the inhibition of coimmunoprecipitation of MYPT1 with myosin by 14-3-3 $\beta$  is not due to the competition of the MYPT1 and 14-3-3 $\beta$  at the binding site on the myosin molecule.

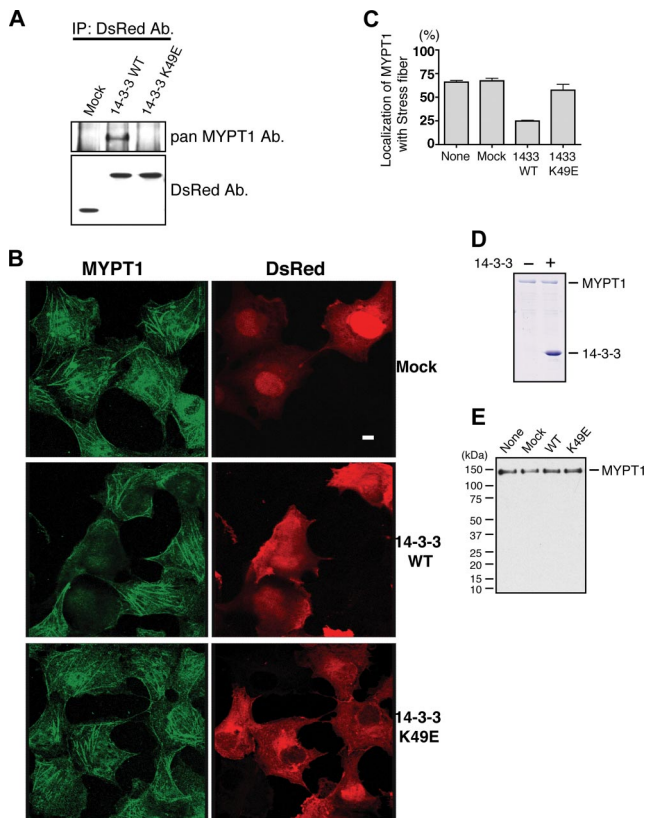
#### 14-3-3 $\beta$ Affects the Localization of MYPT1 on Stress Fiber

Because the binding of 14-3-3 $\beta$  to MYPT1 dissociates MYPT1 from myosin *in vitro*, we examined whether 14-3-3 $\beta$  affects MYPT1 localization in cells. To address this question, we examined the effect of the DsRed 14-3-3 $\beta$  overexpression on the MYPT1 localization in the cells. We used the 14-3-3 $\beta$  K49E mutant as a negative control because it has been reported that lysine 49 of 14-3-3 $\zeta$  is essential for the interaction with targeting proteins (Zhang *et al.*, 1997). To confirm that 14-3-3 $\beta$  K49E does not bind to the targeting protein, we performed immunoprecipitation using DsRed 14-3-3 $\beta$  wild-



**Figure 3.** The effect of 14-3-3 $\beta$  on the binding of MYPT1 to PP1 $\delta$  or Myosin. (A) 14-3-3 $\beta$  does not change the binding between MYPT1 and PP1 $\delta$ . Flag-MYPT1 (50  $\mu$ g/ml), PP1 $\delta$  (60  $\mu$ g/ml), and GST or GST 14-3-3 $\beta$  (300  $\mu$ g/ml) were incubated with Flag agarose in the buffer A containing 100 nM mCLR and incubated for 1 h at 4°C. Flag agarose was washed and the bound fraction was eluted and then subjected to SDS-PAGE. Gels were stained with CBB. (B) 14-3-3 $\beta$  does not interact with MYPT1/PP1 $\delta$  in the absence of the phosphatase inhibitor. FLAG-MYPT1 and PP1 $\delta$  were incubated with or without GST 14-3-3 $\beta$  and without mCLR and then subjected to pull-down assay using FLAG agarose, followed by CBB staining. (C) 14-3-3 $\beta$  dissociates MYPT1 from myosin. The mixture of Myosin, MYPT1 with or without 14-3-3 $\beta$  were subjected to immunoprecipitation using anti-myosin antibody. The immunoprecipitates were subjected to Western blotting. Left and right, the bound and the unbound fractions to anti-myosin antibody, respectively.

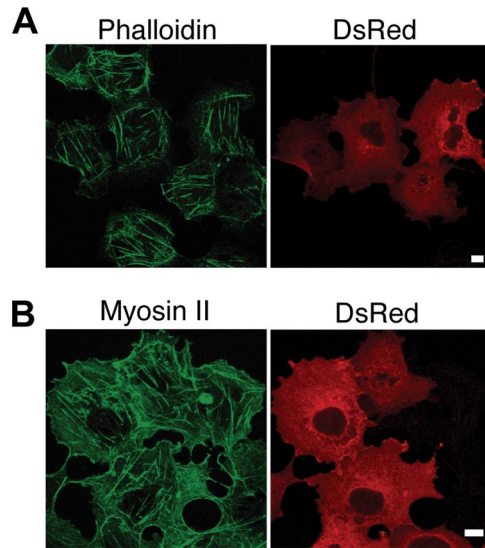
type (WT) or K49E-transfected COS7 cells. As shown in Figure 4A, MYPT1 was coimmunoprecipitated with 14-3-3 $\beta$  WT but not with the K49E mutant, suggesting that K49E mutation abolished the binding of 14-3-3 $\beta$  to MYPT1. It was shown that MYPT1 localizes at the stress fiber in COS7 cells (Koga and Ikebe, 2005). Although the overexpression of 14-3-3 K49E did not diminish the stress fiber localization of MYPT1, the expression of DsRed 14-3-3 WT dramatically attenuated the stress fiber localization of MYPT1 (Figure 4B). Approximately 65% of the tested cells showed the stress fiber localization of MYPT1 in untransfected cells as well as mock- or 14-3-3 K49E-transfected cells, whereas the number of cells showing the stress fiber localization of MYPT1 was significantly decreased in 14-3-3 WT transfected COS 7 cells (Figure 4C). It should be noted that purified 14-3-3 did not facilitate the degradation of MYPT1 (Figure 4D). 14-3-3 overexpression also did not have an effect of MYPT1 degradation in COS7 cells (Figure 4E). Figure 5B shows the localization of myosin II in COS7 cells. The cells were transfected with DsRed14-3-3 $\beta$  WT and immunostained with anti-nonmuscle myosin IIb antibody. The overexpression of 14-3-3 $\beta$  showed no effect



**Figure 4.** The effect of 14-3-3 $\beta$  overexpression on MYPT1 localization at the stress fiber. (A) The binding between DsRed 14-3-3 WT or K49E mutant and endogenous MYPT1. The cell lysates of COS7 cells transfected with DsRed 14-3-3 WT or K49E or of mock-transfected cells were used for immunoprecipitation using anti-DsRed antibody. The immunoprecipitates were subjected to Western blotting. (B) Representative images of the localization of MYPT1 in the 14-3-3 $\beta$ -transfected cells. COS7 cells were transfected with 14-3-3 $\beta$  WT or K49E or were mock transfected. MYPT1 in COS7 cells were immunostained with anti-MYPT1 antibody and visualized by FITC-conjugated anti-rabbit IgG (green). DsRed-conjugated 14-3-3 $\beta$  (red) were visualized with DsRed fluorescence signal. The cells expressing DsRed-14-3-3 WT do not show the stress fiber localization of MYPT1. Bar, 10  $\mu$ m. (C) Fraction of the cells showing the stress fiber localization of MYPT1. More than 600 individual cells were counted from three independent experiments. Results are shown as means  $\pm$  SEM of triplicate experiments. (D) Effect of 14-3-3 $\beta$  on the MYPT1 degradation. Purified MYPT1 and 14-3-3 $\beta$  were incubated at 25°C for 3 hrs, then the mixture was subjected to SDS-PAGE, followed by C.B.B. staining. (E) Effect of 14-3-3 $\beta$  overexpression on the MYPT1 degradation in cells. COS7 cell lysates transfected with mock 14-3-3 WT or 14-3-3 K49E were analyzed by Western blotting using anti-MYPT1 antibodies as probes. Molecular masses were indicated on the left.

on the stress fiber localization of myosin IIb. The result is consistent with the *in vitro* finding that 14-3-3 $\beta$  has no binding activity to myosin II.

Furthermore, these results suggest that the change in the MYPT1 localization at stress fiber by 14-3-3 $\beta$  in cells is neither due to the change in stress fiber structure (Figure 5A) nor the change in myosin II localization at the stress fiber (Figure 5B), indicating that the function of 14-3-3 is specific to the binding of MYPT1 to myosin, but not the disruption of the cytoskeletal structure. These results are consistent with the results of *in vitro* binding experiments and support the idea that 14-3-3 $\beta$  interferes with the binding between MYPT1 and myosin.



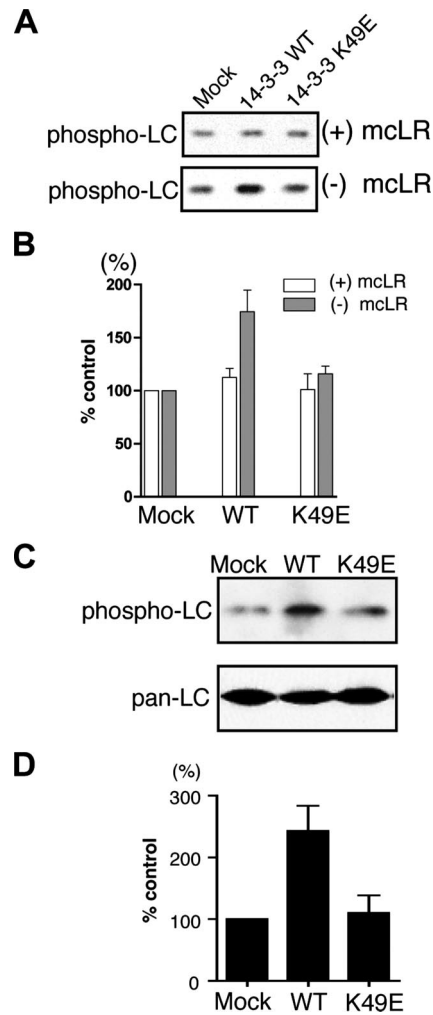
**Figure 5.** The effect of 14-3-3 $\beta$  overexpression on stress fiber (A) or myosin II localization at the stress fiber (B). 14-3-3 $\beta$  was visualized with DsRed fluorescence signal. (A) Stress fiber was identified with Alexa-conjugated phalloidin (green). (B) Myosin was immunostained with anti-Myosin IIb antibody and visualized by FITC-conjugated anti-rabbit IgG (green). Bar, 10  $\mu$ m.

#### The Effect of 14-3-3 $\beta$ on Myosin II Phosphorylation in Cells

As described above, 14-3-3 $\beta$  attenuated MLCP activity *in vitro*. To see whether 14-3-3 $\beta$  affects myosin II phosphorylation *in vivo*, we determined the phosphorylation level of myosin II in cells. The cells were transfected with DsRed 14-3-3 WT or K49E or were mock transfected. The cells were harvested and subjected to Western blot using anti-phospho-Ser19 specific MLC antibody. The phosphorylation level of myosin II in the 14-3-3 WT-transfected cells was  $\sim$ 2.4 times higher than that of mock- or 14-3-3 K49E-transfected cells (Figure 6, C and D). To examine whether the increase in MLC phosphorylation is due to the increase in MLCK activity or the decrease in MLCP activity, we measured the effect of 14-3-3 $\beta$  overexpression on MLC phosphorylation in the presence and absence of the MLCP inhibitor, mCLR, using phosphorylation site-specific antibodies against Ser19 of MLC as a probe (Komatsu *et al.*, 2000). The isolated myosin II was incubated with the total cell lysates in the presence or absence of mCLR, and MLC phosphorylation was monitored by Western blotting and quantified by densitometry. If MLCK activity is affected by 14-3-3 $\beta$ , MLC phosphorylation should be altered by 14-3-3 $\beta$  in the presence of mCLR. As shown in Figure 6, A and B, MLC phosphorylation was increased by 14-3-3 $\beta$  WT overexpression in the absence of mCLR, whereas the phosphorylation was unaffected by 14-3-3 $\beta$  WT overexpression in the presence of mCLR. The results suggest that 14-3-3 increased myosin II phosphorylation by affecting the phosphatase activity but not the kinase activity.

#### Determination of the Element of MYPT1 Responsible for the Binding to 14-3-3

To determine the 14-3-3 binding element of MYPT1, yeast strain AH109 was sequentially transformed with the pG-BKT7 vector containing various fragments of MYPT1 and then with the pGADT7 vector containing the 14-3-3 $\beta$ . The transformants were subjected to nutrient selection (Figure



**Figure 6.** The effect of 14-3-3 $\beta$  overexpression on myosin phosphorylation. (A) Effect of 14-3-3 $\beta$  WT overexpression on the MLCK and MLCP activities. MLC phosphorylation activity in the cell lysates was determined in the presence or absence of mCLR, a potent MLCP inhibitor. DsRed 14-3-3 WT-, K49E-, or mock-transfected COS7 cell lysates were incubated with myosin II for 60 min as described in *Materials and Methods*. Phosphorylated myosin II was subjected to SDS-PAGE, followed by Western blotting using phospho-Ser19-MLC antibodies. (B) Quantitative analysis of the increase in myosin phosphorylation by 14-3-3 $\beta$  WT. The relative intensities of MLC phosphorylation were shown. The phosphorylation level by mock-transfected cell lysates was taken as 100%. Values are mean  $\pm$  SEM of three independent experiments. (C) The effects of 14-3-3 $\beta$  overexpression on the MLC phosphorylation level. The phosphorylation level was determined by Western blotting using anti-phospho-specific MLC antibody. NIH3T3 cells transfected with DsRed 14-3-3 WT or K49E or mock-transfected were collected in 10% TCA. After sedimentation at  $5000 \times g$  for 5 min, the pellet was washed with H<sub>2</sub>O three times and then subjected to SDS-PAGE followed by Western blotting. (D) Histogram showing the increase in MLC phosphorylation by 14-3-3 $\beta$ . Bands of Western blotting were subjected to densitometry. Values are mean  $\pm$  SEM of three independent experiments. The phosphorylation levels (signal of anti-phospho-MLC/signal of anti-pan-MLC) in mock-transfected cells were taken as 100%.

7A). The N-terminal 516, but not 296 fragment, showed a positive signal, suggesting that the region Lys297-Asn516 contains the 14-3-3 binding site. Furthermore, the vector containing the C-terminal fragment Met377-Lys976 was

found to be positive. These results defined Met377-Asn516 to be the 14-3-3 binding site. It has been known that the binding of 14-3-3 required phosphorylation of target protein (Furukawa *et al.*, 1993; Michaud *et al.*, 1995; Muslin *et al.*, 1996). We examined whether MYPT1 phosphorylation is required for the binding to 14-3-3 $\beta$ . The cell lysate was incubated with PP1 $\delta$  or mCLR and then subjected to immunoprecipitation followed by Western blotting. Endogenous MYPT1 was coimmunoprecipitated with endogenous 14-3-3 $\beta$  when the sample was treated with mCLR but not with PP1 $\delta$  (Figure 7B). The result strongly suggests that MYPT1 phosphorylation is required for the binding to 14-3-3 $\beta$ .

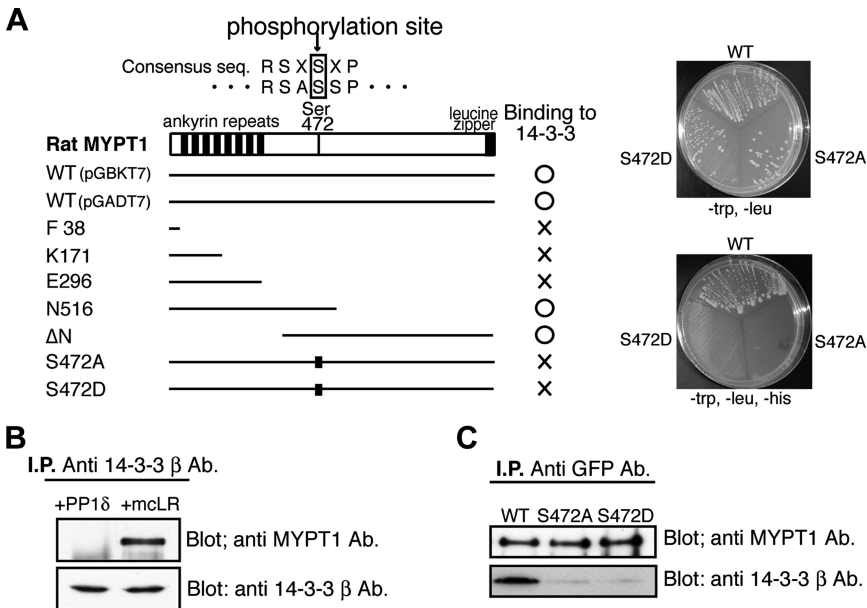
Using synthetic phosphopeptides, it was shown previously that a motif, RSXphosphoSXP (where X represent any amino acid), is critical for the binding of target proteins to 14-3-3 (Muslin *et al.*, 1996). We searched this motif in MYPT1 sequence and found the RSASSP sequence at Arg469-Pro474, which lies within the 14-3-3 binding region (Figure 7A). To examine whether the Ser472 MYPT1 phosphorylation is critical for the binding to 14-3-3 $\beta$ , we produced mutant MYPT1 in which Ser472 is replaced by Ala or Asp, respectively, and the mutants were subjected to yeast two-hybrid assays. As shown in Figure 7A, both mutants failed to grow in the nutrient selection plate, suggesting that Ser472 is essential for the binding of MYPT1-14-3-3 $\beta$ . To further ensure this issue, GFP MYPT1 mutants were expressed in COS7 cells and the binding of the mutants to 14-3-3 $\beta$ , as well as the binding of WT MYPT1-14-3-3 $\beta$  was examined by coimmunoprecipitation assay (Figure 7C). The amount of coimmunoprecipitated 14-3-3 $\beta$  was markedly diminished by the mutation of Ser472 to Ala or Asp, respectively, indicating that Ser472 is critical for the binding of MYPT1-14-3-3 $\beta$ . As shown in Figure 4, 14-3-3 $\beta$  overexpression diminished the stress fiber localization of MYPT1. We transfected S472A MYPT1 into COS7 cells to see whether the mutation of MYPT1 at S472 affects the stress fiber localization of MYPT1 in 14-3-3 $\beta$ -overexpressing cells. As shown in Figure 8, 14-3-3 $\beta$  failed to diminish the stress fiber localization of S472A MYPT1 unlike WT MYPT1. The result is consistent with the notion that S472 phosphorylation is critical for the binding of 14-3-3 $\beta$  to MYPT1.

#### *The Phosphorylation of MYPT1 at Ser472 and Its Effect on the Interaction between MYPT1 and 14-3-3 $\beta$*

Because the isolated MYPT1 binds to 14-3-3 $\beta$ , the question is if the Ser472 of the isolated MYPT1 is phosphorylated by endogenous kinase in Sf9 cells. To answer this question, we produced the antibody specifically recognizing the phosphorylated-Ser472 of MYPT1, termed pS472 antibody. The isolated MYPT1 was preincubated with PP1 $\delta$  as a negative control because PP1 $\delta$  treatment diminished the binding of MYPT1-14-3-3 $\beta$  (Figure 7B). The pS472 antibody recognized the untreated MYPT1 but not the dephosphorylated MYPT1, suggesting that the isolated MYPT1 is partially phosphorylated at Ser472 (Figure 9A). The result shows that the binding of 14-3-3 to isolated-MYPT1 is because of the presence of phosphorylated MYPT1 at Ser472. The Ser472 phosphorylation level of MYPT1 was significantly increased by Rho-kinase. This is consistent with previous report that Ser472 is one of the Rho-kinase induced phosphorylation sites in vitro (Kawano *et al.*, 1999). It should be noted that pS472 antibody did not recognize Ser472A MYPT1 phosphorylated by Rho-kinase. These results demonstrate that the pS472 antibody is specific to the phosphorylated Ser472 of MYPT1.

To see the effect of MYPT1 phosphorylation at Ser472 on the binding to 14-3-3 $\beta$ , MYPT1 was phosphorylated by Rho-kinase and then subjected to GST pulldown assay using GST





**Figure 7.** The determination of the element of MYPT1 responsible for the binding to 14-3-3β. (A) Schematic diagram of rat MYPT1 including 14-3-3 protein binding motifs and, its deletion constructs used in this study. AH109 Yeast strain was simultaneously transformed with a pGBKT7 fused to MYPT1 cDNA and a pGADT7 fused to 14-3-3β cDNA. The strain was also cotransformed with a pGADT7 fused to MYPT1 cDNA and a pGBKT7 fused to 14-3-3β cDNA. Transformants were streaked on the plate lacking leucine, tryptophan, and then the produced colonies were restreaked on the plate lacking leucine, tryptophan and histidine containing X-α-Gal on the surface of the plate. (B) Effect of the phosphorylation of MYPT1 on the binding to 14-3-3β. The COS7 cell lysates were treated with 75 nM PP1δ or 100 nM mCLR for 30 min at 25°C. The lysates were used for immunoprecipitation with anti-14-3-3β antibody and then subjected to Western blotting. (C) Effect of the mutation of S472 MYPT1 on the binding to 14-3-3β. COS7 cells were cotransfected with GFP MYPT1 (WT), (S472A) or (S472D), and 14-3-3, respectively. The lysates were immunoprecipitated with anti-GFP antibody in the presence of 100 nM mCLR and the precipitates were subjected to Western blotting.

14-3-3β. The binding of MYPT1-14-3-3β was increased by MYPT1 phosphorylation (Figure 9B). Because it is known that Rho-kinase phosphorylates T641 and T799 of MYPT1, we examined whether MYPT1 phosphorylation at these sites affects the binding of 14-3-3β to MYPT1. The mutation of MYPT1 at T641 and T799 (T641A/T799A MYPT1) did not significantly influence the phosphorylation-dependent binding of MYPT1-14-3-3β, while the additional mutation at Ser472 to Ala diminished the binding of MYPT1-14-3-3β. These results indicate that Thr641 and Thr799 are not responsible for the binding of MYPT1-14-3-3β.

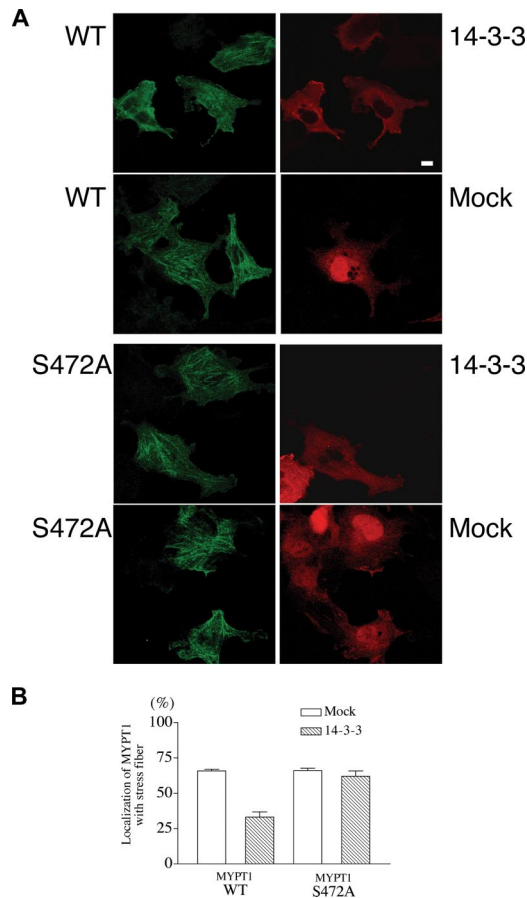
An important issue is whether or not Ser472 MYPT1 phosphorylation is regulated by agonist stimulation in cells, unlike Thr641 MYPT1 phosphorylation (Niuro *et al.*, 2003). We examined a change of Ser472 phosphorylation after EGF stimulation, which activates the RhoA-signaling pathway (Koga and Ikebe, 2005). Figure 9C shows that EGF stimulation significantly increased Ser472 phosphorylation in cells, unlike the Thr641 site. EGF-induced Ser472 phosphorylation was abolished by Y-27632, a Rho-kinase specific inhibitor (Figure 9D). Consistently, immunoprecipitation assay using anti-14-3-3β antibody revealed that EGF stimulation up-regulated the binding of 14-3-3 to MYPT1 in cells correlated with the increase in Ser472 phosphorylation, and this binding was dramatically inhibited by Y-27632 (Figure 9E). Furthermore, the elimination of Rho-kinase I and II in cells by siRNA treatment significantly decreased the phosphorylation level of Ser472 MYPT1 and MLC (Figure 9F). These results suggest that Rho-kinase is involved in the phosphorylation of MYPT1 at Ser472. It should be noted that the EGF-induced increase in the Thr799 MYPT1 phosphorylation was not notably changed by the elimination of Rho-kinase (Figure 9F). Next we performed an in vitro phosphatase assay using WT MLCP and T641A/T799A MLCP to see if the effect of 14-3-3β on the MLCP activity is influenced by the possible phosphorylation of these sites (Figure 9G). Both WT MLCP and T641A/T799A MLCP activities were similarly inhibited by 14-3-3β. Taking these results together, we concluded that Rho-kinase is involved in Ser472 MYPT1 phosphorylation and regulates the binding of MLCP to 14-3-3 and MLC phosphorylation in cells.

**DISCUSSION**

In the present study, we found that 14-3-3β directly binds to MYPT1. It has been shown that 14-3-3 can interact with various proteins and influence the localization of target molecules and/or regulate the enzymatic activity (Aitken, 1995; Fu *et al.*, 2000; Muslin and Xing, 2000; van Hemert *et al.*, 2001; Tzivion and Avruch, 2002; Aitken, 2006). We found that 14-3-3β attenuates the phosphatase activity of MLCP, but not the catalytic subunit. Because the binding of 14-3-3β to MYPT1 does not interfere with the intersubunit binding of the MLCP holoenzyme, the 14-3-3β-induced decrease in the phosphatase activity would be due to the change in the conformation at the binding sites between MYPT1 and the catalytic subunit. It is known that MYPT1 significantly increases the myosin phosphatase activity of PP1δ and that the Rho-kinase induced phosphorylation attenuates the elevated phosphatase activity without dissociation of MYPT1 from the holoenzyme (Feng *et al.*, 1999). These results suggest that the inhibition mechanism of MYPT1 by 14-3-3β is similar to the inhibition by MYPT1 phosphorylation in which the inhibition is achieved by attenuating the activator function of MYPT1. It is plausible that the binding of 14-3-3β to MYPT1 changes the binding interface between MYPT1 and PP1δ thus abolishing the critical interaction between MYPT1 and PP1δ that affects the catalytic activity.

Quite interestingly, we found that 14-3-3β binding to MYPT1 induces the dissociation of MYPT1 from myosin. Consistently, the overexpression of 14-3-3β in cells markedly diminished the localization of MYPT1 at the stress fiber. Because MYPT1 associates with myosin II in the stress fiber, the result can be explained by the finding that the binding of 14-3-3β to MYPT1 induces dissociation of MYPT1 from myosin II in stress fiber. Because 14-3-3β does not break the MLCP subunit structure, it is anticipated that 14-3-3β dissociates the MLCP holoenzyme from myosin by breaking the interaction between MYPT1 and myosin. The dissociation of MLCP from myosin is expected to result in decrease in the dephosphorylation rate of myosin, thus increasing the myosin phosphorylation level. It should be noted that 14-3-3 inhibited the phosphatase activity of MLCP even though the





**Figure 8.** The effect of 14-3-3 $\beta$  overexpression on the localization of MYPT1 (WT) and MYPT1 (S472A) at stress fiber. (A) Representative images of GFP MYPT1 (WT) and MYPT1 (S472A) in the cells transfected with 14-3-3 $\beta$ . Note that DsRed 14-3-3 $\beta$  diminished stress fiber localization of MYPT1 (WT) but not MYPT1 (S472A). The untransfected cell showed normal stress fiber localization of MYPT1 (WT; top panel). COS7 cells were cotransfected with GFP-MYPT1 (WT) or (S472A) and DsRed 14-3-3 $\beta$  or were mock transfected. Bar, 10  $\mu$ m. (B) Fraction of the cells showing stress fiber localization of MYPT1. The stress fiber localization of WT MYPT1 was significantly diminished by 14-3-3 $\beta$ , but that of S472A MYPT1 was not affected. More than 300 individual cells were observed from three independent experiments. Values are mean  $\pm$  SEM of three independent experiments.

isolated MLC was used as a substrate, suggesting that the inhibition of the phosphatase activity *in vitro* is not directly due to the dissociation of MLCP from myosin. Therefore, it is anticipated that 14-3-3 inhibits MLCP activity by dual mechanism in cells, i.e., the decrease in the enzymatic activity and the segregation of the substrate (myosin) from the enzyme (MLCP). Supporting this view, we actually found that the expression of 14-3-3 $\beta$  increased the MLC phosphorylation level in cells (Figure 6), and this is due to the down-regulation of myosin phosphatase, but not the activation of myosin kinase activity.

It has been puzzling that although isolated MLCP strongly binds to myosin, the majority of MLCP in cell is localized throughout cytosol, and only a part of the MLCP colocalized with the structure where myosin II is present, such as stress fiber (Murata *et al.*, 1997). The present results suggest that 14-3-3 $\beta$  is at least, in part, responsible for the cytosolic localization of MLCP. The question is how 14-3-3 $\beta$  controls

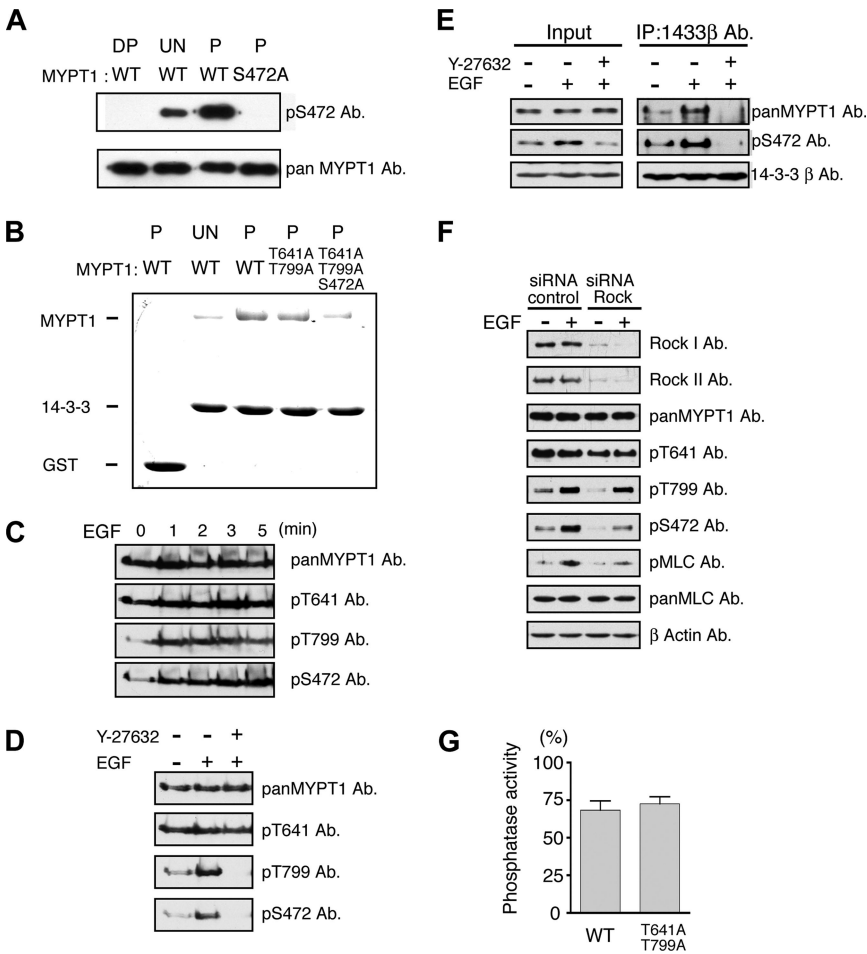
MLCP localization at the myosin II-containing structure *in vivo*. To address this question, we examined the effect of the protein phosphatase treatment of MYPT1 on the binding to 14-3-3 $\beta$ . The result suggested that the phosphorylation of MYPT1 is critical for the binding of MYPT1-14-3-3 $\beta$ .

Further analysis revealed that the motif RSXSXP present in the MYPT1 sequence is responsible for the interaction between 14-3-3 $\beta$  and MLCP, because 1) the mutation of S472 of MYPT1 inhibited the growth of the 14-3-3 $\beta$ -transfected yeast on the nutrient selection plates in yeast two-hybrid experiments and 2) the S472A and S472D mutant of MYPT1 failed to coimmunoprecipitate with 14-3-3 $\beta$  in contrast to the WT MYPT1. This view is supported by the finding that the localization of the S472A mutant of MYPT1 at the stress fiber in COS7 cells was not influenced by 14-3-3 $\beta$  expression, whereas the stress fiber localization of WT MYPT1 was diminished. It has been reported that 14-3-3 protein bind to the phosphorylated proteins and the RSXSXP motif is critical for the binding to partner proteins such as Raf (Muslin *et al.*, 1996) and CDC25 (Zha *et al.*, 1996; Mils *et al.*, 2000). The present result agrees with the notion found in other 14-3-3 binding proteins.

Because the mutation of S472 interferes with the interaction between 14-3-3 $\beta$  to MYPT1, the phosphorylation site critical for the binding should be S472. To clarify this issue, we produced the antibody that specifically recognize the phosphorylated Ser472 of MYPT1. The antibody recognized MYPT1 phosphorylated by Rho-kinase, but not MYPT1 dephosphorylated by PP1 $\delta$ . Furthermore, the mutation of Ser472 completely abolished the interaction of these antibodies with MYPT1. These results indicate that the produced antibodies are specific to the phosphorylated Ser472 of MYPT1. Interestingly, the isolated MYPT1 was recognized with anti-phospho-Ser472 antibody, indicating that MYPT1 expressed in Sf9 cells is partially phosphorylated at Ser472. Therefore, it is thought that the binding of 14-3-3 to the expressed MYPT1 is because of the presence of phosphorylated MYPT1 at Ser472.

It is interesting that S472D MYPT1 also failed to interact with 14-3-3 $\beta$ , indicating that the introduction of the negative charge at the position of Ser472 does not mimic the phosphorylation effect. The introduction of the acidic residues has been used to determine the phosphorylation sites of proteins assuming that the negative charges of acidic amino acid resemble the phosphate moiety, thus mimicking the phosphorylation effect. However, the 3D position of the phosphate moiety is different from that of the side chains of the acidic residues, and it is anticipated that the acidic residues do not always mimic the phosphorylation effect. In fact, the replacement of Ser19 of MLC by Asp does not mimic the phosphorylation effect on myosin motor function, although it mimics the phosphorylation effect on filament formation of myosin (Ikebe *et al.*, 1994a; Kamisoyama *et al.*, 1994). It was also shown that mutation of S175 of calponin to Asp shows a dephosphorylated phenotype rather than the phosphorylated phenotype (Tang *et al.*, 1996). We think that the introduction of a phosphate moiety at a proper 3D configuration is essential to induce the binding of MYPT1-14-3-3 $\beta$ .

Using the phosphorylation site specific antibody against Ser472, we found that Rho-kinase phosphorylates Ser472 of MYPT1 in cells, and this is consistent with the previous report that Rho-kinase can phosphorylate this site *in vitro* (Kawano *et al.*, 1999). Furthermore, we found that Ser472 MYPT1 phosphorylation is increased by EGF stimulation, and the Ser472 phosphorylation is inhibited by the Rho-kinase inhibitor. Therefore, it is plausible that the activation

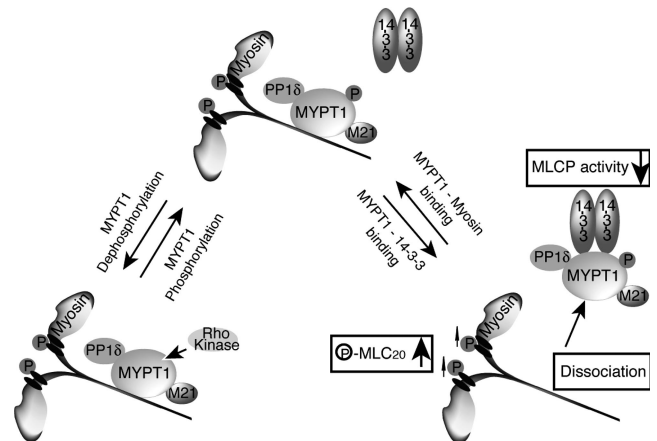


**Figure 9.** Agonist (EGF) induces the phosphorylation of MYPT1 at Ser472 by Rho-kinase in the cells, which enhances the binding of MYPT1-14-3-3β. (A) Specificity of the phosphorylation site-specific antibody against Ser472 of MYPT1. The purified MYPT1 (Un) was dephosphorylated by PP1δ (DP) or phosphorylated by Rho-kinase (P). Samples were subjected with SDS-PAGE, followed by Western blotting using pSer472 and pan MYPT1 antibodies, respectively. (B) The effect of MYPT1 phosphorylation by Rho-kinase on the binding to 14-3-3β. MYPT1 WT, T641A/T799A, or S472A/T641A/T799A mutant was phosphorylated by Rho-kinase, incubated with GST 14-3-3β, and then subjected to GST-pulldown assay. (C) EGF-induced phosphorylation of MYPT1 at Ser472 in cells. HeLa cells were stimulated with 25 ng/ml EGF after serum starvation for 24 h. MYPT1 phosphorylation levels were analyzed by Western blotting using the indicated antibodies. (D) Effect of Y-27632 on the EGF-induced MYPT1 phosphorylation. MYPT1 phosphorylation levels in HeLa cells was examined by Western blotting with or without pretreatment with 10 μM Y-27632 before the EGF stimulation. (E) Inhibitory effect of Y-27632 on the EGF-induced binding of MYPT1-14-3-3. HeLa cells treated as described in D were subjected to the immunoprecipitation assay in the presence of mCLR and Y-27632 with anti-14-3-3β antibody. The samples were then examined by Western blotting. (F) Silencing of Rho-kinase I and II inhibited Ser472 MYPT1 phosphorylation and MLC phosphorylation. HeLa cells were transfected with control or Rho-kinase I and II siRNA for 72 h and stimulated with 25 ng/ml EGF for 5 min after the serum starvation for 24 h. P, phosphorylated; Un, untreated; DP, dephosphorylated. (G) Effect of 14-3-3β on the WT

MLCP and T641A/T799A MLCP activities. A fixed amount of 14-3-3β (0.06 μM) was added to WT MLCP and T641A/T799A MLCP, respectively, in the phosphatase assay solution. A phosphatase assay was performed as described in *Materials and Methods*. Values are mean ± SEM of three independent experiments and expressed as 100% of the phosphatase activity in the absence of 14-3-3β.

of the RhoA pathway induces the phosphorylation of MYPT1 at Ser472, which enhances the interaction between 14-3-3β and MYPT1. Surprisingly, the elimination of endogenous Rho-kinase I and II by Rho kinase-specific siRNA did not affect EGF-induced Thr799 MYPT1 phosphorylation, whereas the siRNA-induced gene silencing diminished both the EGF-induced increase in the Ser472 MYPT1 phosphorylation and MLC phosphorylation. Consistently, 14-3-3β inhibited T641A/T799A MLCP activity to the same potency as the inhibition of WT MLCP activity. These results indicate that Ser472 but not Thr641/Thr799 MYPT1 phosphorylation is predominantly regulated by Rho-kinase in the EGF signaling, thus regulating MLCP activity and MLC phosphorylation. Further studies are required to clarify the pathways responsible for the regulation of MYPT1 phosphorylation at Thr641/Thr799 sites.

On the basis of the present study, we propose the following scenario for the regulatory function of 14-3-3/MYPT1. The agonist stimulates RhoA/Rho-kinase pathway, which phosphorylates Ser472 MYPT1. The phosphorylation enhances the interaction between 14-3-3β and MYPT1, which results in the decrease in the myosin dephosphorylation rate due to the inhibition of MLCP activity and the dissociation of MLCP from myosin, thus increasing in myosin phosphor-



**Figure 10.** A model for the regulation of MLCP by 14-3-3. MLCP is associated with myosin II via MYPT1/myosin II heavy-chain interaction. Once MYPT1 is phosphorylated at Ser472, 14-3-3β binds to MYPT1, dissociating MLCP from myosin II heavy chain and decreasing the myosin phosphatase activity.

ylation (Figure 10). It has been known that the activation of RhoA pathway increases myosin phosphorylation due to the inhibition of myosin dephosphorylation activity (Kimura *et al.*, 1996). The present study reveals the novel mechanism of RhoA-dependent MLCP regulatory mechanism and suggests that the binding of 14-3-3 to MYPT1 is, in part, responsible for the down-regulation of MLCP, thus enhancing myosin II-based motor activity in mammalian cells.

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