Inhibitory Phosphorylation Site for Rho-associated Kinase on Smooth Muscle Myosin Phosphatase*  

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It is clear from several studies that myosin phosphatase (MP) can be inhibited via a pathway that involves RhoA. However, the mechanism of inhibition is not established. These studies were carried out to test the hypothesis that Rho-kinase (Rho-associated kinase) via phosphorylation of the myosin phosphatase target subunit 1 (MYPT1) inhibited MP activity and to identify relevant sites of phosphorylation. Phosphorylation by Rho-kinase inhibited MP activity and this reflected a decrease in $V_{\text{max}}$. Activity of MP with different substrates also was inhibited by phosphorylation. Two major sites of phosphorylation on MYPT1 were Thr$^{855}$ and Thr$^{856}$. Various point mutations were designed for these phosphorylation sites. Following thiophosphorylation by Rho-kinase and assays of phosphatase activity it was determined that Thr$^{856}$ was responsible for inhibition. A site- and phosphorylation-specific antibody was developed for the sequence flanking Thr$^{856}$ and this recognized only phosphorylated Thr$^{856}$ in both native and recombinant MYPT1. Using this antibody it was shown that stimulation of serum-starved Swiss 3T3 cells by lysophosphatidic acid, thought to activate RhoA pathways, induced an increase in Thr$^{856}$ phosphorylation on MYPT1 and this effect was blocked by a Rho-kinase inhibitor, Y-27632. In summary, these results offer strong support for a physiological role of Rho-kinase in regulation of MP activity.

It is accepted that phosphorylation of the 20-kDa myosin light chain (MLC20)$^1$ is an essential mechanism in regulating contractile activity in smooth muscle and non-muscle cells (1–3). Following stimulation by several agonists an increased MLC20 phosphorylation and resultant contraction frequently occurs. Since this is effective at submaximal Ca$^{2+}$ levels it is referred to as increased Ca$^{2+}$ sensitivity (3). The extent of MLC20 phosphorylation depends on the relative activities of myosin light chain kinase (4) and myosin phosphatase (MP) (5). The mechanism for Ca$^{2+}$ sensitization is thought to occur via inhibition of MP via a G-protein-linked pathway (3, 6, 7).

There is strong evidence that an additional component of the Ca$^{2+}$ sensitization pathway is RhoA. GTP$\gamma$S induces Ca$^{2+}$ sensitization in permeabilized smooth muscle fibers and this effect was blocked by ADP-ribosylation of Rho using an exoenzyme from Clostridium botulinum, C3, or Staphylococcus aureus, named epidermal differentiation inhibitor (8, 9). Addition of the GTP$\gamma$S-bound active form of RhoA (8) or a constitutively active mutant of RhoA (9) induced a Ca$^{2+}$ sensitization in various permeabilized smooth muscle preparations. A target for RhoA is Rho-kinase (10–12) and introduction of a constitutively active recombinant fragment of Rho-kinase into Triton X-100-permeabilized smooth muscle caused a Ca$^{2+}$-independent contraction via phosphorylation of MLC20 (13). A specific inhibitor of Rho-kinase, Y-27632, also was reported to inhibit the agonist-induced Ca$^{2+}$ sensitization of smooth muscle contraction (14). In non-muscle cells activation of RhoA promotes the formation of stress fibers and focal adhesion complexes (15). C3 blocked the formation of both structures in Swiss 3T3 cells treated with the Rho-activating stimulus, lysophosphatidic acid (LPA) (16). In addition, the expression of a constitutively active form of Rho-kinase induced stress fiber and focal adhesion formation in fibroblasts and an increase in the level of MLC20 phosphorylation (17). These results in smooth muscle and in non-muscle cells are attributed to an increase in MLC20 phosphorylation and this is thought to reflect the inhibition of MP.

MP is composed of three subunits: a catalytic subunit of type 1 phosphatase δ isoform, PP1cδ, and two non-catalytic subunits, 110 and 20 kDa. The 110-kDa subunit is a targeting molecule and thus has been termed myosin phosphatase target subunit 1 (MYPT1). MYPT1 is the key molecule involved in regulation of MP (for reviews, see Ref. 5).

Purified Rho-kinase thiophosphorylated MYPT1 and induced an inhibition of MP activity (18, 19). In permeabilized smooth muscle, thiophosphorylation of MYPT1 was associated with a decreased activity of myosin phosphatase (20). Thus, a potential mechanism for inhibition of MP is via the phosphorylation of MYPT1 by Rho-kinase. Phosphorylation of MYPT1 and inhibition of MP was first observed with a gizzard isoform (M133) in which the major phosphorylation site for an endogenous kinase was Thr$^{856}$ (21). Using purified Rho-kinase the inhibitory site(s) on MYPT1 was found to be in the C-terminal part of MYPT1, i.e. Ser$^{897}$-Ile$^{1004}$ (19). Also, it is was suggested that the phosphorylation site(s) for Rho-kinase was in the sequence 753 to 1004 of gizzard MYPT1 (18).
In order to understand the regulation of MP it is essential to identify various functional regions of MYPT1. These include binding sites for PP1c and for the substrate, phosphorylated myosin, and also the inhibitory phosphorylation site(s). In the present study, we examined the effect of thiophosphorylation of MYPT1 on the enzymatic properties of MP and identified two sites, Thr695 and Thr850, on chicken MYPT1 as the major phosphorylation sites for Rho-kinase using point mutational analysis. Thr695, but not Thr850, was identified as the inhibitory phosphorylation site. Using a site- and phosphorylation state-specific antibody we found that Thr695 of MYPT1 was phosphorylated in Swiss 3T3 cells stimulated by LPA, which is known to activate Rho/Rho-kinase pathways (16).

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—Chemicals and vendors were as follows: [γ-32P]ATP (NEN Life Science Products Inc.); ATP, phorbol 12-myristate 13-acetate, i-α-phosphatidyl-s-serine, and i-α-lysophosphatic acid (1-oleoyl) (Sigma); ATP·S (Roche Molecular Biochemicals), nitrocellulose membrane (Advantec Toso Roshi, Tokyo, Japan); microcystin-LR (Wako Pure Chemical, Osaka, Japan); Dulbecco’s modified Eagle’s medium (Difco); (R)-(+)-trans-4-(4-pyridyl)-4-[(1-aminoethyl)cyclohexanecarboxamide dihydrochloride (Y-27632) was given by the Bristol-Myers Squibb Company; N-(3-aminopropyl)trimethoxysilane (Wako Pure Chemical, Osaka, Japan). All other chemicals were of the highest grade commercially available.

**Preparation of Proteins**—Rho-kinase and kinase-free myosin phosphatase (19) and PP1c (22) were prepared from chicken gizzard. Other preparations were as follows: MLC20 (23), myosin light chain kinase (24), and calponin (25) from chicken gizzard; the catalytic subunit of PKA from bovine heart (27); protein kinase C from human platelets (28); GST-RhoA (19). 32P-Labeled substrates were as follows: MLC20 (29), 32P-poly (30), and 32P-glycogen phosphorylase (31). The peptide corresponding to Lys689 to Thr689 of MYPT1 was synthesized and coupled with keyhole limpet hemocyanin by Sawady Co. (Tokyo, Japan). The rabbit polyclonal anti-phosphopeptide antibody was prepared and affinity-purified as described (33). This antibody is referred to as pM137T

**Production of Site- and Phosphorylation State-specific Antibody**—The cell lysates containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 and 95% humidified air at 37°C. Subconfluent cells were incubated in serum-free medium for 24 h, then 1 μl LPA or vehicle (phosphate-buffered saline) was added to the medium. In some experiments, serum-starved cells were pretreated for 30 min with 10 μM Y-27632 before stimulation with LPA. At each time indicated, reactions were terminated by addition of ice-cold trichloroacetic acid to a final 6% (v/v). Trichloroacetic acid was removed by centrifugation at 15,000 × g for 10 min and the precipitate was washed 3 times with cold acetone and dried. The dried cell powder was lysed in SDS sample buffer and analyzed by Western blotting using either pM137T or anti-MYPT1 antibody. The blots were exposed to films (Fuji RX) for various times (from 5 to 60 s) to obtain a linear response by the enhanced chemiluminescence method (Amersham Pharmacia Biotech). For pM137T the scans of Western blots were linear in the range of 10 to 500 ng of phosphorylated rG-MYPT1-11104. The amount of MYPT1 in the pertinent immunoreactive bands was estimated by densitometry (Densitograph, Atto, Japan).

**RESULTS**

**Effects of Thiophosphorylation of Myosin Phosphatase on Enzymatic Properties**—To confirm and extend the earlier observation that phosphorylation of MYPT1 by Rho-kinase inhibits MP activity (18, 19), the influence of phosphorylation on kinetic parameters and substrate specificity were examined. ATP·S rather than ATP was used because of the resistance of the thiophosphate group to phosphatase (there is some phosphatase activity toward phosphorylated MYPT1 in the holoenzyme (21)). The effects of thiophosphorylation of MYPT1 by Rho-kinase are shown in Table I. The dominant effect is on

**Table I**

| Phosphorylation of MYPT1 | MP activity | Vmax |
|--------------------------|-------------|------|
| Nonphosphorylated         | 0.83 ± 0.07 | 544 ± 32 |
| Thio-MYPT1                | 1.24 ± 1.23 | 162 ± 16 |

1 μM EGTA, 100 μg/ml phosphatidyl-s-serine, 100 ng/ml phorbol 12-myristate 13-acetate, 1 μM microcystin-LR, 250 μM (γ-32P)ATP, and 30 μg/ml rG-MYPT1-11104. Phosphorylation of MP by endogenous kinase(s) was carried out at 30°C for 60 min in 20 μM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM dithiothreitol, 1 mM MgCl2, 1 μM microcystin-LR, 250 μM (γ-32P)ATP, and 30 μM MP holoenzyme containing the endogenous kinase (21).
Vmax which is decreased by about 70% following thiophosphorylation. There is a slight, but probably insignificant, effect on Km.

The effects of thiophosphorylation also was investigated using various substrates. Two additional substrates for PP1 were chosen and compared with MLC20, namely glycogen phosphorylase (31) and calponin (30). Thiophosphorylation of MP by Rho-kinase resulted in inhibition of phosphatase activity for each substrate tested (Table II). The extents of inhibition were approximately the same (about 75%).

**Determination of Phosphorylation Sites on MYPT1 for Rho-kinase**—From previous studies (19) it was established that the phosphorylation sites for Rho-kinase were in the C-terminal part (Ser667 to Ile1004) of MYPT1. To identify the phosphorylated residues, the MYPT1 in the kinase-free MP holoenzyme was phosphorylated to about 2 mol of P/mol of MYPT1 by Rho-kinase, as described previously (19). Phosphoamino acid analysis of the phosphorylated MYPT1 indicated that Thr was the major site of phosphorylation (Fig. 1A). Phosphoserine was detected as a minor component. Two recombinant proteins, full-length MYPT1 (rG-MYPT11–1004), and a point mutant (rG-MYPT1 T850A) decreased phosphorylation levels similar to wild-type MYPT1. These results support the contention that the inhibition of phosphatase activity is due primarily to the phosphorylation of Thr695 by Rho-kinase.

**TABLE II**

| Substrate                  | Phosphatase activity | 
|---------------------------|----------------------|
|                           | Nonphosphorylated MP | Thiophosphorylated MP |
|                           | nmol/min/mg          |                       |
| 32P-MLC20 (3.4 μM)        | 41.6 ± 15.4          | 108.1 ± 6.2           |
| 32P-Calponin (3.5 μM)     | 361.3 ± 13.4         | 95.1 ± 3.7            |
| 32P-Glycogen phosphorlase (5.5 μM) | 54.3 ± 4.7 | 12.4 ± 0.8 |

**Effect of thiophosphorylation on MP activity toward various substrates**

Values given are mean ± S.E. (n = 4).

**Fig. 1. Determination of phosphorylation sites on MYPT1 by Rho-kinase.** A, phosphoamino acid analysis of MYPT1. MP was phosphorylated by Rho-kinase to 1.7 mol of P/mol of MYPT1. 32P-MYPT1 was eluted from excised gel slices and subjected to two-dimensional phosphoamino acid analysis. The position of free phosphate (P), phosphoserine (P-Ser), phosphothreonine (P-Thr), and partially hydrolyzed residues (arrowhead) are indicated on the right. B, stoichiometry of phosphorylation of MYPT1 mutants by Rho-kinase. rG-MYPT11–1004 (●), rG-MYPT1T695A (○), rG-MYPT1T850A (▲), rG-MYPT1T695A/T850A (●), and rG-MYPT1T695A/T850A (□) were phosphorylated by Rho-kinase and 32P incorporation was determined as described under “Experimental Procedures.” Error bars indicate mean ± S.E. (n = 3).
Phosphorylation of Myosin Phosphatase by Rho-kinase

FIG. 2. Effects of phosphorylation of MYPT1 mutants on phospha-
tase activity. Effects of phosphorylation of rG-MYPT1–1004 (A), rG-MYPT1T695A (B), and rG-MYPT1T850A (C) on the ability to activ-
ate PPIc. Each MYPT1 mutant was incubated with Rho-kinase in the presence (●) or absence (○) of ATP·S for 60 min without microcys-
tin-LR under conditions similar to those in Fig. 1B. Various amounts of each mutant were then incubated with PPIc at 30 °C for 5 min and the reconstituted phosphatase activity assayed as described under “Experimental Procedures.” The activity of PPIc alone was expressed as 1. D, effects of Rho-kinase on reconstituted MP. rG-MYPT1–1004 (●), rG-
MYPT1T695A (○), and rG-MYPT1T850A (△) were incubated with PPIc8 (molar ratio 3:1:mutant:PPIc6) at 30 °C for 5 min. Then the reconsti-
tuted phosphatases were phosphorylated by Rho-kinase for the indicated time and phosphatase activity measured as described under “Experimental Procedures.” Error bars indicate mean ± S.E. (n = 3).

Production and Characterization of a Site- and Phosphoryl-
aton-dependent Antibody for Thr695—The potential impor-
tance of Thr695 phosphorylation has been documented above. If phosphorylation of this residue could be monitored (for exam-
ple, in cell extracts) it would provide a valuable tool for studying
Rho-kinase activity relative to MYPT1. To this end a rabbit
polyclonal antibody was prepared that recognized phosphoryl-
ation-dependent Antibody for Thr 695—The antibody was evalu-
ated by Western blots. As shown in Fig. 3, rG-MYPT1–1004 (lane 2) and rG-
MYPT1T695A (lane 4) both phosphorylated by Rho-kinase were
recognized. Nonphosphorylated rG-MYPT1–1004 (lane 1) and rG-
MYPT1T695A phosphorylated by Rho-kinase (lane 3) were not
detected. rG-MYPT1–1004 was phosphorylated by protein kinase C, but this was not detected (lane 6). A weak reaction was detected for rG-MYPT1–1004 phosphorylated by protein kinase A (PKA; lane 5) but rG-MYPT1T695A phosphorylated by PKA was not recognized (lane 7). Also, pM133T695 did not detect MYPT1 following phosphorylation of the native holoen-
zyme by PKA (lane 10, see figure legend). The holoenzyme was
phosphorylated by Rho-kinase (lane 9) and the endogenous kinase (lane 12), and in both cases the MYPT1 was recognized by pM133T695. The nonphosphorylated forms were not detected (lanes 8 and 11). These results suggest that pM133T695 recog-
nizes phosphorylation at Thr695 of MYPT1. The kinases responsi-
sible for this phosphorylation are Rho-kinase and the endoge-
nous kinase in the MP holoenzyme.

LPA-Induced In Vivo Phosphorylation of MYPT1 at Thr695—To determine if the antibody could be used to detect
MYPT1 phosphorylation under in vivo conditions the effect of
LPA on serum-starved Swiss 3T3 cells was monitored. It was
reported that in this system LPA stimulates stress fiber and focal
adhesion formation and involves activation of RhoA (16).

The serum-starved cells were incubated with 1 μM LPA for
different times, as indicated, and cell lysates were analyzed by
Western blots using pM133T695 (see “Experimental Proce-
dures”). Even in the absence of LPA some phosphorylation of
Thr695 was detected (Fig. 4A). Following LPA stimulation, the
to the catalytic subunit of PKA (2.9 mol of P/mol of MYPT1; lane 11,
MYPT1 in MP holoenzyme containing the endogenous kinase; lane 12,
MYPT1 in MP holoenzyme phosphorylated by the endogenous kinase
(1.5 mol of P/mol of MYPT1). Samples (150 ng in each lane) were
subjected to SDS-PAGE followed by Western blotting analyses with
pM133T695 (upper panels). The blots were stained with Coomassie Blue as shown in the lower panels and the MYPT1 band is shown.

DISCUSSION

There is considerable evidence that Ca2+ sensitization in
smooth muscle reflects inhibition of myosin phosphatase (for
reviews, see Refs. 3 and 5). This occurs at low, but finite, levels of Ca2+ where the activity of myosin light chain kinase does not
overide the phosphatase activity. However, the mechanism of
MP inhibition is still not established. Initially, it was thought
that the Ca2+–sensitizing effect of arachidonic acid was due to
dissociation of the MP complex (37) or, as subsequently sug-
gested, the activation of an atypical protein kinase C isoform or
an unknown kinase (38). Other theories include: phosphoryla-
tion of MYPT1 by an endogenous kinase (21) or, by Rho-kinase
(18, 19); and inhibition by a phosphorylation-dependent pro-
tein, CPI17 (39, 40). However, there is compelling evidence
that RhoA is involved in Ca2+ sensitization (8, 9) and any proposed
mechanism should include consideration of this point. A logical
link to RhoA is Rho-kinase (10–12) and thus an attractive
hypothesis is that phosphorylation of Rho-kinase is an impor-
tant component of the Ca2+–sensitization mechanism (18). This
does not exclude other kinases/phosphatases but suggests that
such are involved they may be linked to the RhoA cascade.

The data presented in this study are consistent with direct
phosphorylation of MYPT1 by Rho-kinase as an inhibitory
mechanism.

MYPT1 is known to interact with several components and
some of the regions of interaction have been identified. The
N-terminal one-third of MYPT1 can bind to both PP1c and phosphorylated myosin and activate phosphatase activity (22, 29, 41). The ankyrin repeats region (residues 39–295) is important in this regard, but also the N-terminal 38 residues are involved in the PP1c interaction. Residues 35 to 38, Lys-Val-Lys-Phe, and flanking charged residues form the PP1c consensus-binding motif and these are critical for the binding of PP1c (42, 43). In addition, residues 1 to 16 are thought to be involved in activation of PP1c (22). The C-terminal half of MYPT1 contains binding sites for M20 (44, 45), RhoA (18), acidic phosphoprotein (37), and phosphatase activity toward glycogen phosphorylase would increase. The two sites identified on MYPT1 for Rho-kinase are Thr695 and Thr580 and the sequences N-terminal to the phosphorylated Thr are identical, i.e. Arg-Arg-Ser-Thr. This sequence is a putative target for PKA, although Ser is preferred to Thr (54). This probably accounts for the low extent of phosphorylation by PKA of rG-MYPT11–1004 at Thr695, although the MP holoenzyme was not phosphorylated. In addition, it was shown previously that phosphorylation of MP by PKA did not affect the phosphatase activity (32). Thus, it is proposed that the phosphorylation of Thr695 in vivo is not achieved by PKA. Protein kinase C may also be eliminated. Based on the limited evidence provided here it is suggested that the consensus sequence for Rho-kinase, and the endogenous kinase, is similar to that for PKA and requires basic residues at the −3 and −2 positions.

Several earlier studies carried out under “physiological” conditions (α-toxin permeabilized smooth muscle (20), platelets (55, 56); and NIH 3T3 cells (18)) had suggested a correlation between phosphorylation of MYPT1 and inhibition of MP activity, but the site(s) of phosphorylation were not identified. The only study to identify the inhibitory phosphorylation site, i.e. Thr695, was done with isolated proteins (21). The objective of the LPA-Swiss 3T3 cell experiment was to determine if Thr695 was phosphorylated under in vivo conditions with a regime that activated RhoA. This was achieved. LPA induced about a 4-fold increase in Thr695 phosphorylation and this stimulation was blocked by a Rho-kinase inhibitor, Y-27632 (14). These results add confidence to accepting the idea that Rho-kinase under physiological conditions may modify phosphatase activity via phosphorylation of MYPT1 at Thr695. However, there are several aspects that remain to be resolved. For example, which kinase is responsible for phosphorylation of MYPT1 in Swiss 3T3 cells in the non-stimulated state (Fig. 4) and what is the role of this phosphorylation? In addition, it was proposed that phosphorylation of MYPT1 may induce activation as well as inhibition of phosphatase activity. It was shown that MYPT1 was phosphorylated by a mitosis-specific kinase and activated MP activity (57). Also the activation of MP in vascular smooth muscle by elevated cGMP levels has been suggested (58, 59). Thus, several features of MYPT1 and possibly MYPT2, must be considered to obtain a more complete picture of its regulation of PP1c activity.

In summary, the results presented above are supportive of a physiological role for Rho-kinase in the regulation of MP activ-

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2 A. Murányi, M. Ito, and D. J. Hartshorne, personal communication.
RhoA pathway is thought to be activated, Y-27632, blocked the LPA-induced phosphorylation of Thr695. Binding sites at the N-terminal end of MYPT1. Theoretically, at least in terms of the primary structure, from the PP1c-Km antibody was developed for phospho-Thr695 and its flanking sites are inhibited. Thus, it is likely that the inhibitory effect is due to direct phosphorylation of the MYPT1 subunit by Rho-kinase. The activity is indicated and the major site involved in inhibition of phosphorylation of Thr695 occurred under in vivo conditions where the RhoA pathway is thought to be activated, i.e., following LPA stimulation of Swiss 3T3 cells. The Rho-kinase inhibitor, Y-27632, blocked the LPA-induced phosphorylation of Thr695.

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