Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho-kinase)*

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Mutsuki Amano, Masaaki Itot, Kazushi Kimura, Yuko Fukata, Kazuyasu Chihara, Takeshi Nakamoto, Yoshiharu Matsuura, and Kozo Kaibuchi

From the Division of Signal Transduction, Nara Institute of Science and Technology, Takayama, Nara 630-01, the 2nd Department of Internal Medicine, Mie University School of Medicine, Tsu 514, the 3rd Department of Internal Medicine, Kyoto University Faculty of Medicine, Kyoto 606, and the 1st Department of Virology II, National Institute of Health, Tokyo 162, Japan

The small GTPase Rho is implicated in physiological functions associated with actin-myosin filaments such as cytokinesis, cell motility, and smooth muscle contraction. We have recently identified and molecularly cloned Rho-associated serine/threonine kinase (Rho-kinase), which is activated by GTP-Rho (Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) EMBO J. 15, 2208–2216). Here we found that Rho-kinase stoichiometrically phosphorylated myosin light chain (MLC). Peptide mapping and phosphoamino acid analyses revealed that the primary phosphorylation site of MLC by Rho-kinase was Ser-19, which is the site phosphorylated by MLC kinase. Rho-kinase phosphorylated recombinant MLC, whereas it failed to phosphorylate recombinant MLC, which contained Ala substituted for Thr-18 and Ser-19. We also found that the phosphorylation of MLC by Rho-kinase resulted in the facilitation of the actin activation of myosin ATPase. Thus, it is likely that once Rho is activated, then it can interact with Rho-kinase and activate it. The activated Rho-kinase subsequently phosphorylates MLC. This may partly account for the mechanism by which Rho regulates cytokinesis, cell motility, or smooth muscle contraction.

Rho is a small GTPase, which exhibits both GDP/GTP binding and GTPase activities (for reviews, see Refs. 1 and 2). Rho has GDP-bound inactive and GTP-bound active forms, which are interconvertible by GDP/GTP exchange and GTPase reactions (1, 2). Rho is implicated in the cytoskeletal responses to extracellular signals including lysophosphatidic acid and certain growth factors, which form stress fibers and cause focal adhesion (3, 4). Rho is also implicated in other physiological functions associated with cytoskeletal rearrangements such as cell morphology (5), cell aggregation (6), cell motility (7), and cytokinesis (8, 9). Recent studies indicate that Rho is also involved in the regulation of phosphatidylinositol 3-kinase (10–12), phosphatidylinositol 4-phosphate 5-kinase (13), and c-fos expression (14). Upon stimulation with certain extracellular signals, GDP-Rho may be converted to GTP-Rho, and then it can bind to specific targets and cause its effects. We have recently purified three putative targets for Rho (p128, p138, and p164) from the bovine brain (15, 16). p128 was identified as serine/threonine kinase, protein kinase N (15). p138 was identified as the MBS1 of myosin phosphatase (16). p164 was identified as a novel serine/threonine kinase, named Rho-kinase (17), which is also known as ROK (18).

MLC phosphorylation plays pivotal roles in smooth muscle contraction (for reviews, see Refs. 19–21) and in the actin-myosin interaction for stress fiber and contractile ring formation in non-muscle cells (for a review, see Ref. 22). This also has an effect on cytokinesis and cell motility (22). MLC kinase primarily phosphorylates MLC at Ser-19 (19–21, 23). Any protein kinases so far obtained, besides specific kinases such as MLC kinase, do not phosphorylate this site (24). When smooth muscles are stimulated by agonists such as vasoconstrictors, Ca2+ is mobilized into the cytoplasm. Ca2+ activates the calmodulin-dependent MLC kinase. The MLC phosphorylation induces myosin-actin interaction and thereby activates myosin ATPase (19–21), which then induces smooth muscle contraction (19–21). However, the cytosolic Ca2+ level is not always proportional to the contraction level, and an additional mechanism that can regulate the Ca2+ sensitivity of the smooth muscle contraction has been proposed (25). Since GTP-γS, a non-hydrolyzable GTP analog, lowers the Ca2+ concentrations necessary for the contraction of permeabilized smooth muscles, a GTP-binding protein was presumed to regulate the Ca2+ sensitivity (26, 27). Rho has been shown to be involved in the GTP-enhanced Ca2+ sensitivity of the smooth muscle contraction (28). Recent evidence suggests that GTP-γS increases MLC phosphorylation at submaximal Ca2+ concentrations presumably by inhibiting myosin phosphatase through Rho (29). We have recently shown that GTP-Rho activates Rho-kinase, and then Rho-kinase phosphorylates MBS and thereby inactivates myosin phosphatase (16). This may increase MLC phosphorylation and induce the consequent contraction of the smooth muscles.

To extend these observations, we have examined if Rho-kinase phosphorylates MLC. We found that Rho-kinase stoichiometrically phosphorylated MLC at the site that is phosphorylated by MLC kinase, which causes the activation of myosin ATPase.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—MLC (30), Myosin and MLC kinase (23) were purified from the frozen chicken gizzard. F-actin was purified from the rabbit skeletal muscle (31). Rho-kinase was purified from the bovine brain (17). [γ-32P]ATP was purchased from Amersham Corp. All mate-

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† To whom correspondence should be addressed. Tel.: 81-7437-2-5440; Fax: 81-7437-2-5449; E-mail: kaibuchi@bs.aist-nara.ac.jp.
The Small GTPase Rho and Myosin Light Chain

The DNA encoding the catalytic fragment of Rho-kinase (6–553 amino acids) was inserted into the BamHI site of pAcY1M1-GST to produce GST-Rho-kinase. GST-Rho-kinase was purified from Sf9 cells by use of a baculovirus system (32) by means of a glutathione-Sepharose column (17). GST-Rho-kinase was constitutively active. The DNA fragment encoding MLC was amplified by polymerase chain reaction from rat brain Quick clone cDNA (Clontech) with the primers 5’-ATTAGGATCCTGAT- CATCTTTTGCTTGCTGTC-3’ and 5’-ATAAGGATCTCGATGATCCTACCGC-3’. The apparent affinity of isolated MLC for Rho-kinase was estimated by measuring the phosphorylation of various concentrations of MLC (Fig. 1B). The apparent $K_m$ values for MLC in the presence and absence of GTP-$\gamma$S-GST-RhoA were 2.6 ± 0.4 and 12.6 ± 1.6 μM, and the molecular activities were 0.26 ± 0.03 and 0.15 ± 0.02 s⁻¹, respectively. Thus, it is likely that GTP-$\gamma$S-GST-RhoA increases the affinity of Rho-kinase for MLC and produces the maximum velocity of the phosphorylation reaction. The apparent $K_m$ value and molecular activity of GST-Rho-kinase were 0.91 ± 0.07 μM and 0.67 ± 0.09 s⁻¹, respectively. The apparent $K_m$ value and molecular activity of MLC kinase for MLC were 52.1 ± 7.1 μM and 2.0 ± 0.36 s⁻¹, respectively, under the conditions. The $K_m$ values for MLC kinase was lower than that of Rho-kinase, indicating that Rho-kinase phosphorylates myosin at lower concentrations, whereas the molecular activity of Rho-kinase was lower than that of MLC kinase. The lower molecular activity of Rho-kinase than that of GST-Rho-kinase may be explained by the fact that Rho-kinase lost its activity to some extent during the purification.

MLC is phosphorylated primarily at Ser-19 and secondarily at Thr-18 by MLC kinase (23), and the phosphorylation of Ser-19 is essential to facilitate actin activation of myosin ATPase (39, 40). MLC is phosphorylated at Ser-1, Ser-2, and Thr-9 by protein kinase C, and this phosphorylation by protein kinase C inhibits the actin activation of myosin ATPase (41–43). To determine the primary phosphorylation site of MLC by Rho-kinase, we performed peptide mapping of the phosphorylated MLC by either Rho-kinase, MLC kinase, or protein kinase C in vitro. The pattern of two-dimensional peptide mapping of MLC phosphorylated by Rho-kinase was identical to that produced by MLC kinase and different from that produced by

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2 A detailed analysis concerning recombinant Rho-kinase will be described elsewhere.
protein kinase C (Fig. 2A). A phospoamino acid analysis revealed that phosphorylation occurred mainly on the serine residue and partially on the threonine residue of the MLC that was phosphorylated by Rho-kinase, and that the phosphorylation occurred only on the serine residue of the MLC that was phosphorylated by the MLC kinase. It may be noted that the MLC kinase preferentially phosphorylates MLC at Ser-19 in these conditions. Essentially identical results were obtained when GST-Rho-kinase was used instead of Rho-kinase.

We fused GST proteins with wild type MLC and with MLC containing a substitution for the alanine residues for Thr-18 and Ser-19, and then we examined if Rho-kinase and MLC kinase could phosphorylate these recombinant proteins. Rho-kinase, GST-Rho-kinase, and MLC kinase phosphorylated GST-MLC but did not phosphorylate GST or GST-MLCA18A19 (Fig. 2B). Protein kinase C phosphorylated both GST-MLC and GST-MLCA18A19 (data not shown). These results indicate that Rho-kinase phosphorylates MLC mainly at Ser-19, which is the same site phosphorylated by MLC kinase. To examine whether Rho-kinase functions equivalently to MLC kinase in a cell-free system, we performed the actin-activated MgATPase assay. Purified intact myosin was phosphorylated to 1 mol/mol by GST-Rho-kinase, and then the actin-activated MgATPase activity was measured. The MgATPase activity of the phosphorylated myosin increased in a F-actin-dependent manner to the extent similar to that increased by MLC kinase (Fig. 3). The apparent $K_m$ values for actin and the molecular activity of the phosphorylated myosin were $0.56 \pm 0.05 \mu M$ and $0.18 \pm 0.02 s^{-1}$, respectively. These values were roughly the same as those for the myosin phosphorylated by MLC kinase. We used GST-Rho-kinase instead of native Rho-kinase in this experiment because high concentrations of myosin were necessary to detect the myosin ATPase activity and the stoichiometrical phosphorylation of myosin by native Rho-kinase was difficult under the conditions.

We showed here that Rho-kinase phosphorylated both iso-

![Fig. 2](image-url)

**Fig. 2.** Identification of the phosphorylation site of MLC by Rho-kinase. A, a phosphopeptide mapping analysis of MLC. MLC (0.5 μg of protein) was phosphorylated by Rho-kinase, MLC kinase, or protein kinase C. Phosphorylated MLC was digested with trypsin, and each sample was loaded onto a silica gel plate. Phosphopeptides were separated by electrophoresis (horizontal dimension) and chromatography (vertical dimension) and then were visualized by an image analyzer. Asterisks denote origins. B, phosphorylation of recombinant MLC. MLC, GST, GST-MLC, or GST-MLCA18A19 (2 μM each) was phosphorylated by Rho-kinase (20 ng of protein), GST-Rho-kinase (10 ng of protein), or MLC kinase (10 ng of protein) as indicated. Lanes 1–4, by Rho-kinase; lanes 5–8, by GST-Rho-kinase; lanes 9–12, by MLC kinase. Lanes 1, 5, and 9, MLC; lanes 2, 6, and 10, GST; lanes 3, 7, and 11, GST-MLC; lanes 4, 8, and 12, GST-MLCA18A19. The results are representative of three independent experiments.

![Fig. 3](image-url)

**Fig. 3.** Effect of phosphorylation of myosin by Rho-kinase on the MgATPase activity that was activated by actin. Myosin was incubated with GST-Rho-kinase (●), with MLC kinase (▲), or without kinase (▲). After incubation, the ATPase activity was measured at the various concentrations of F-actin. The values shown are means ± S.E. of triplicates.

![Fig. 4](image-url)

**Fig. 4.** Model for the regulation of MLC phosphorylation by Rho, Rho-kinase, and myosin phosphatase. Cat, catalytic subunit of myosin phosphatase.

lated MLC and MLC of intact myosin in a GTP-Rho-dependent manner. The primary phosphorylation site of MLC by Rho-kinase was at Ser-19, which is the same site phosphorylated by MLC kinase. The phosphorylation of MLC of intact myosin increased the MgATPase activity of myosin. These results indicate that Rho-kinase phosphorylates the MLC of intact myosin and activates its MgATPase activity in a GTP-Rho-dependent manner. The $K_m$ value of Rho-kinase for MLC was lower than that of MLC kinase, but the molecular activity of Rho-kinase was lower than that of MLC kinase. This indicates that Rho-kinase efficiently phosphorylates MLC at lower concentrations.

When smooth muscles are stimulated by an agonist such as vasoconstrictors, Ca$^{2+}$ is mobilized into the cytoplasm. Ca$^{2+}$ activates the calmodulin-dependent MLC kinase. Because
smooth muscles contain large amounts of myosin (about 10% of the total protein, about 50 μM) and MLC kinase (about 0.1% of the total protein) (19). MLC kinase is believed to phosphorylate MLC in smooth muscles. However, non-muscle tissue such as liver contains much smaller amounts of myosin and MLC kinase (data not shown). Rho-kinase is ubiquitously expressed in various tissues (17). We have speculated that Rho-kinase phosphorylates the MLC of intact myosin and activates MgATPase in a GTP-Rho-dependent manner when non-muscle cells are stimulated by an agonist such as lysophosphatidic acid and certain growth factors and that the phosphorylated myosin interacts with actin leading to stress fiber and contractile ring formation (Fig. 4). In fact, we have recently found that overexpression of dominant activated RhoA (RhoAV14) in NIH3T3 cells results in an increase in MLC phosphorylation as well as stress fiber formation (16). This may be partly explained by the fact that GTP-RhoA interacts with Rho-kinase and MBS of myosin phosphatase and activates Rho-kinase; the activated Rho-kinase subsequently phosphorylates the MBS, thereby inactivating myosin phosphatase (Fig. 4) (16). We have assumed that phosphorylation of MLC by Rho-kinase also contributes to the increased level of MLC phosphorylation in these cells. Further studies are necessary to understand the roles of Rho-kinase in controlling MLC phosphorylation.

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