Phosphorylation of Myosin-binding Subunit (MBS) of Myosin Phosphatase by Rho-Kinase In Vivo

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Abstract. Rho-associated kinase (Rho-kinase), which is activated by the small GTPase Rho, phosphorylates myosin-binding subunit (MBS) of myosin phosphatase and thereby inactivates the phosphatase activity in vitro. Rho-kinase is thought to regulate the phosphorylation state of the substrates including myosin light chain (MLC), ERM (ezrin/radixin/moesin) family proteins and adducin by direct phosphorylation and by the inactivation of myosin phosphatase. Here we identified the sites of phosphorylation of MBS by Rho-kinase as Thr-697, Ser-854 and several residues, and prepared antibody that specifically recognized MBS phosphorylated at Ser-854. We found by use of this antibody that the stimulation of MDCK epithelial cells with tetradecanoylphorbol-13-acetate (TPA) or hepatocyte growth factor (HGF) induced the phosphorylation of MBS at Ser-854 under the conditions in which membrane ruffling and cell migration were induced. Pre-treatment of the cells with Botulinum C3 ADP-ribosyltransferase (C3), which is thought to interfere with Rho functions, or Rho-kinase inhibitors inhibited the TPA- or HGF-induced MBS phosphorylation. The TPA stimulation enhanced the immunoreactivity of phosphorylated MBS in the cytoplasm and membrane ruffling area of MDCK cells. In migrating MDCK cells, phosphorylated MBS as well as phosphorylated MLC at Ser-19 were localized in the leading edge and posterior region. Phosphorylated MBS was localized on actin stress fibers in REF52 fibroblasts. The microinjection of C3 or dominant negative Rho-kinase disrupted stress fibers and weakened the accumulation of phosphorylated MBS in REF52 cells. During cytokinesis, phosphorylated MBS, MLC and ERM family proteins accumulated at the cleavage furrow, and the phosphorylation level of MBS at Ser-854 was increased. Taken together, these results indicate that MBS is phosphorylated by Rho-kinase downstream of Rho in vivo, and suggest that myosin phosphatase and Rho-kinase spatiotemporally regulate the phosphorylation state of Rho-kinase substrates including MLC and ERM family proteins in vivo in a cooperative manner.

Key words: myosin-binding subunit of myosin phosphatase • Rho-kinase • Rho-kinase phosphorylation • cytokinesis

Evidence is accumulating that the small GTPase Rho plays crucial roles in the regulation of various cellular functions including stress fiber and focal adhesion formation (Ridley and Hall, 1992, 1994), smooth muscle contraction (Hirata et al., 1992; Gong et al., 1996), microvilli formation (Shaw et al., 1998), cytokinesis (Kishi et al., 1993; Mabuchi et al., 1993), cell migration (Takaishi et al., 1993, 1994), and gene expression (Hill et al., 1995). Rho cycles between GDP-bound inactive and GTP-bound active forms, and the GTP-bound form binds to specific targets and then exerts its biological functions (van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Kaibuchi et al., 1999). Numerous putative Rho targets have been identified, including protein kinase N (Amano et al., 1996b; Watanabe et al., 1996), Rho-kinase/ROKα/ROCK II (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996), myo-
sin-binding subunit (MBS)\(^1\) of myosin phosphatase (Kirmura et al., 1996), p140Dm (Watanabe et al., 1997), citron (Madaule et al., 1995), citron kinase (Madaule et al., 1998), rhophilin (Watanabe et al., 1996), rhoetokin (Reid et al., 1996), Kv1.2 (Cacherio et al., 1998), and phospholipase D (Singer et al., 1997). Rho-kinase (ROCK I) is an isomerase of Rho-kinase (Ishizaki et al., 1996; Leung et al., 1996).

Mysin phosphatase is composed of MBS, a 37-kD type 1 catalytic subunit and a 20-kD regulatory subunit (Alessi et al., 1992; Hartsorne et al., 1998). Mysin phosphatase binds to phosphorylated myosin light chain (MLC) via MBS and dephosphorylates MLC. Rho-kinase phosphatase MBS, which leads to the inactivation of myosin phosphatase in vitro (Kirmura et al., 1996). Rho-kinase by itself phosphorylates MLC at the same site that is phosphorylated by MLC-kinase, and activates myosin ATPase (Alessi et al., 1992; Hartshorne et al., 1998). MLC phosphatase catalytic subunit and a 20-kD regulatory subunit (Alessi et al., 1992; Hartshorne et al., 1998). Myosin phosphatase interacts with both ERM family proteins and adducin through MBS, and dephosphorylates the phosphorylated ERM family proteins and adducin (Fukata et al., 1998; Kirmura et al., 1998). Mysin phosphatase activity toward ERM family proteins and adducin is inhibited when MBS is phosphorylated by Rho-kinase as described for MLC (Fukata et al., 1998). Moreover, we have found that the phosphorylation of moesin and adducin by Rho-kinase plays critical roles in the formation of microvilli-like structures in fibroblasts (Oshiro et al., 1998) and in membrane ruffling in MDCK cells (Fukata et al., 1998). However, it remains to be clarified how MBS phosphorylation is regulated in vivo. We found by use of this antibody that MBS was phosphorylated by Rho-kinase downstream of Rho in vivo, and that phosphorylated MBS was localized in the nucleus, cytoplasm and membrane ruffling area in TPA-stimulated MDCK cells, on stress fibers in interphase REF52 cells, and at the cleavage furrow in mitotic MDCK cells.

**Materials and Methods**

**Materials and Chemicals**

The expression plasmid of Botulinum C3 A DP-ribozym transferase (pGEX-C3) was kindly provided by Dr. A. H. al (University College London, London, UK). The MDCK cells and the cDNA-encoding mouse moesin (1–577 amino acids [aa]) were gifted from Dr. S. Tsukita (Kyoto University, Kyoto, Japan). M mononuclear anti-MBS A b (anti-MBS A b; antigen: 371–511 aa of MBS) was kindly provided by Dr. C. Hartshorne (University of Arizona, Tuscon, Arizona; Trinkle-Mulchan and Madaule, 1995). In addition, we have found that the phosphorylation of moesin and adducin was generated as follows. Glutathione-S-transferase-mouse moesin (GST-mouse moesin; 357–577 aa) was produced and purified from Escherichia coli as an antigen. The obtained antisera was then affinity-purified against mouse moesin (357–577 aa). Anti-ERM Ab specifically recognized ERM family proteins (data not shown). Protein kinase C (PKC) was prepared from rat brain as described (Kita and go, 1986). Phosphatidylinositol serine, bisbenzimide Hoechst, anti-MLC Ab, nocodazole, and N, N'-O dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cAMP) were purchased from Sigma Chemical Co. Anti-RhoA (1–20 GBq/mmol) was synthesized as described (Uehata et al., 1997). Human recombinant hepatocyte growth factor (HGF) was expressed in Spodoptera frugiperda cells in a baculovirus system and purified as described (Matsura et al., 1998a; Amano et al., 1997). Y-27832 was synthesized as described (Uehata et al., 1997). TPA and calyculin A were purchased from Wako Pure Chemical Industries, Ltd. All materials used in the nucleic acid study were purchased from Takara Shuzo Co. Other materials and chemicals were obtained from commercial sources.

**Preparation of Recombinant Proteins**

GST-catalytic domain of Rho-kinase (GST-CAT; 6–553 aa) and full-length RhoA (MBS) (1–1032 aa; Johnson et al., 1997) were produced in Spodoptera frugiperda cells in a baculovirus system and purified as described (Kirmura et al., 1997; Amano et al., 1996a). Myosin phosphatase interactions with both ERM family proteins and adducin through MBS, and dephosphorylates the phosphorylated ERM family proteins and adducin (Fukata et al., 1998; Kirmura et al., 1998). The phosphatase activity toward ERM family proteins and adducin is inhibited when MBS is phosphorylated by Rho-kinase as described for MLC (Fukata et al., 1998). Moreover, we have found that the phosphorylation of moesin and adducin by Rho-kinase plays critical roles in the formation of microvilli-like structures in fibroblasts (Oshiro et al., 1998) and in membrane ruffling in MDCK cells (Fukata et al., 1999), respectively. Based on these observations, we have proposed the dual regulation model in which myosin phosphatase and Rho-kinase cooperatively regulate the phosphorylation state of the substrates including MLC, adducin and moesin downstream of Rho. However, it remains to be clarified how MBS phosphorylation is regulated downstream of Rho in intact cells.

In this study, we determined the sites of phosphorylation of MBS by Rho-kinase, and prepared antibody that specifically recognized MBS phosphorylated at Ser-854, which was the specific site for Rho-kinase, to understand how MBS phosphorylation is regulated in vivo. We found by use of this antibody that MBS was phosphorylated by Rho-kinase downstream of Rho in vivo, and that phosphorylated MBS was localized in the nucleus, cytoplasm and membrane ruffling area in TPA-stimulated MDCK cells, on stress fibers in interphase REF52 cells, and at the cleavage furrow in mitotic MDCK cells.
and 1 mM DTT) containing 100 μM γ-<sup>32</sup>P]ATP (1-20 GBq/mmol), 5 pmol of PK C, and 20 pmol of full-length MBS. After an incubation for 15 min at 37°C, the reaction products were boiled in SDS sample buffer and aliquots of the reaction products were subjected to SDS-PAGE. The radiolabeled bands were visualized and estimated by an image analyzer (BA S-2000; Fuji).

To determine the sites of phosphorylation of MBS, the recombinant full-length MBS (1 nmol of protein) was phosphorylated with GST-CAT (200 pmol of mRNA in 1 ml of kinase buffer A containing 100 μM γ-<sup>32</sup>P]ATP for 1 h at 30°C, and the reaction product was digested with Achromobacter protease I at 37°C for 20 h. The obtained peptides were applied onto a C18 reverse phase column (SG120; 4.6 × 250 mm; Shimadzu) and eluted with a linear gradient of 0-48% acetonitrile for 100 min at a flow rate of 1.0 ml/min by high-performance liquid chromatography (System Gold; Beckman). The radioactive peptides were separated and phosphoamino acid sequencing was carried out with a peptide sequencer (PPSQ-10; Shimazu). The fractions obtained from each E domain degradation cycle were degraded for 3P in a Beckman liquid scintillation counter.

### Production of Site- and Phosphorylation State-specific Antibody for MBS

A rabbit polyclonal antibody against MBS phosphorylated at Ser-854 (anti-pS854 A b) was prepared as described (Nigakage et al., 1997). The phosphopeptide Cys-Arg<sup>460</sup> Glu-Lys-A-Arg-Ag-phosphoSer<sup>854</sup>-Thr-Gly<br>Val-Ser-Phe<sup>859</sup> was chemically synthesized as an antigen and bound to the carrier protein, keyhole limpet hemocyanin at the NH<sub>2</sub>-terminal cysteine residue, by Peptide Institute Inc. The obtained antiserum was then affinity-purified against the phosphopeptide.

### Cell Culture

MDCK cells were grown in DME containing 10% fetal bovine serum, 5% CO<sub>2</sub>, 50 μM DTT, 50 mM Na<sub>2</sub>EDTA, 50 mM KCl, and 1 mM MgCl<sub>2</sub>, 10 μM Ca<sub>2+</sub>, and 20 μg/ml of leupeptin, 50 μg/ml PM SF, 1 mM DTT, and 0.1 μM calyculin A with divnuced homogenizer. The homogenates were loaded onto 1 M sucrose in buffer H, and centrifuged at 1,600<br>g for 10 min. The resulting supernatants were used as cytosolic fraction. The resulting precipitates were subjected to immunoblotting analysis.

### Detection of Phosphorylated MBS in MDCK Cells by Immunoblot Analysis

We employed the conditions for C3 treatment as described (Nishiki et al., 1999). MDCK cells were seeded at a density of 3 × 10<sup>5</sup> cells/cm<sup>2</sup> in 10-cm dishes.

### Subcellular Fractionation

MDCK cells were seeded at a density of 1.2 × 10<sup>5</sup> cells in 10-cm dishes. Non- and TPA-stimulated MDCK (5 × 10<sup>5</sup>) cells were fractionated as described (Chen et al., 1992) with slight modifications. In brief, cells were washed and homogenized in buffer H (20 mM Hepes at pH 7.4, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 50 mM NaF, 30 mM sodium pyrophosphate, 20 μg/ml leupeptin, 50 μg/ml PM SF, 1 mM DTT, and 0.1 μM calyculin A) with a Dounce homogenizer. The homogenates were loaded onto 1 M sucrose in buffer H, and centrifuged at 1,600 g for 10 min. The resulting supernatants were used as cytosolic fraction. The resulting precipitates were subjected to immunoblotting analysis.

### Preparation of Interphase, Early Mitotic, and Later Stages of Cell Division Cells

MDCK cells in interphase, early mitotic (metaphase), and later stages of cell division were prepared as described (Bauer et al., 1998; Goto et al., 1998) with slight modifications. MDCK cells were seeded at a density of 1.2 × 10<sup>5</sup> cells in a 10-cm dish, and cultured for 24 h. Nocodazole was added directly to the medium at 0.33 μM, and then cells were cultured for additional 12 h. Mitotic cells were collected with PBS containing 4 mM EGTA and 0.33 μM nocodazole by mechanical shake off, and adherent cells were used as interphase cells. Mitotic cells were rinsed twice and suspended with nocodazole free medium, and plated onto the dishes. Cells at 0, 30, 60, or 90 min after removal of nocodazole were used as early mitotic and later stages of cell division cells. These cells were treated with 10% (wt/vol) trichloroacetic acid as described above.

### Immunofluorescence Analysis

MDCK cells were fixed with 3.0% formaldehyde in PBS for 20 min and treated with PBS containing 0.2% Triton X-100 for 10 min on ice or acetone for 10 min at −20°C (for anti-pS65 A b). R E F 5 2 cells were fixed with 3.0% formaldehyde in PBS for 10 min and treated with PBS containing 0.2% Triton X-100 for 10 min at room temperature. A titer being washed with PBS three times, the cells were incubated with anti-pS854 A b, anti-pMBS A b, anti-pTR558 A b, anti-ER-M A b, anti-pP2B A b, or anti-M-LC A b at 4°C overnight, TM71 for 2 h at room temperature, or anti-MBS A b for 1 h at room temperature. The cells were washed with PBS three times, then incubated with fluorescent isothiocyanate (FITC)-conjugated anti-rabbit or -mouse Ig G A b, or -rat Ig G A b for 1 h at room temperature or tetramethylrhodamine B isothiocyanate (TRITC)-phalloidin for 30 min at room temperature. DNA's were stained with 1 μg/ml of bisbenzimide Hoechst 33342 for 3 min at room temperature. Fluorescently labeled cells were examined with a Zeiss LSM 510 (Carl Zeiss). In some experiments, fluorescent images (Figs. 4 and 5) were taken with PXL cooled CCD camera (Photometrics) with Delta Vision processing software (Aplied Precision Inc.). Exposure time was adjusted to obtain FITC and Texas red or rhodamine images with roughly equal intensities in nonstimulated MDCK cells (see Fig. 4 A and B, A and B, and B, respectively; checked by histogram analysis). Under the same condition, the images of TPA- or HGF-stimulated MDCK cells were taken. The images of Figs. 4 A and 5 A, or Figs. 4 B and 5 B were treated with same establishment of contrast and brightness, respectively. Grayscale FITC and Texas red images were converted into green and red images, respectively, and then merged to synthesize RGB color images. A ratio image was created using an Image-Pro image processing system (Media Cybernetics). A grayscale image of anti-pS854 A b was divided by a corresponding grayscale image of anti-MBS A b, and the resultant image was multiplied by 50.

### Microinjection

R E F 5 2 cells were seeded at a density of 3 × 10<sup>5</sup> cells per 13-mm cover glass in 6-cm dishes and incubated for 48 h. M B P (5 mg/ml), C 3 (0.1 mg/ml), C 3 (0.1 mg/ml) plus G TP-γ S (0.4 mg/ml), or M B P R • B PH (TT) (5 mg/ml) was microinjected along with a marker protein (rabbit or mouse Ig G at 1 mg/ml) into the cytoplasm of cells. A titer injection, the cells were incubated at 37°C for 30 min, and fixed as described above.

### Results

#### Determination of the Sites of Phosphorylation of MBS by Rho-Kinase

We first determined the sites of phosphorylation of MBS by Rho-kinase in vitro as follows. Full-length MBS was expressed in Spodoptera frugiperda cells in a baculovirus system and purified. Purified MBS was then incubated with the GST-catalytic domain of Rho-kinase (GST-CAT; 6–553 aa). CAT has been previously shown to be constitutively active in vitro and in vivo (A mano et al., 1996a, 1997). Phosphorylated MBS was digested with A chromobacter protease I and subjected to high-performance liquid chro-
Table I. Amino Acid Sequences of Phosphopeptides Derived from Rho-Kinase Phosphorylated Rat3 MBS

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Amino acid sequence*</th>
<th>Phosphorylated residues†</th>
<th>Relative amount of phosphate in peptide§ % total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>NASRIESLEQEK</td>
<td>Ser-332</td>
<td>3.4</td>
</tr>
<tr>
<td>(residue 330–341)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-2</td>
<td>DTAGVIRSAASSPRLLSDLN</td>
<td>Ser 472</td>
<td>8.5</td>
</tr>
<tr>
<td>(residue 463–483)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-3</td>
<td>ARSRQARQSRSTQGTVLTDLQEA</td>
<td>Ser-693, Ser-696, Thr-697</td>
<td>23.5</td>
</tr>
<tr>
<td>(residue 685–710)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-4</td>
<td>RRSTGVSFQTDSENQERQSDLGEDQSK</td>
<td>Ser-854, Thr-855</td>
<td>22.0</td>
</tr>
<tr>
<td>(residue 852–881)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-5</td>
<td>TGSSYGLAEITASKEAQK</td>
<td>Thr-443, Ser-445</td>
<td>6.0</td>
</tr>
<tr>
<td>(residue 443–460)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-6</td>
<td>RDIQTDVSRYDSSSTSSDRLGASYSYLEERK (residue 882–920)</td>
<td>Thr-884, Ser-894, Ser-905</td>
<td>11.5</td>
</tr>
<tr>
<td>AP-7</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-8</td>
<td>YSRTRYDEYARYGPSTSSSTTPSSSLSTLGSLYASSQNLRPNSLVGITSAYRGLTK (residue 759–818)</td>
<td>Thr-762, Ser-774</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*Residue numbers correspond to Rat3 MBS.
†The residues that showed the highest radioactivity in each peptide are underlined.
§Determined from radioactivity in the HPLC analysis as shown in Fig. 1. Total is not 100% because the amount of minor peaks is excluded.

Production and Characterization of the Site- and Phosphorylation State-specific Antibody for MBS

To investigate how the phosphorylation of MBS by Rho-kinase is regulated in vivo, we prepared the site- and phosphorylation state-specific antibody for MBS. As shown in Table I, Rho-kinase phosphorylated multiple sites of MBS in vitro. Thr-697 was one of the major sites of phosphorylation of MBS by Rho-kinase. However, we cannot distinguish the phosphorylation of Thr-697 by Rho-kinase and the endogenous kinase in vivo as described above. Ser-854, which was also one of the major sites of phosphorylation of MBS by Rho-kinase, is the phosphorylation site specific to Rho-kinase among known MBS kinases in vitro (We confirmed that PKC did not phosphorylate MBS at Ser-854: see below). The phosphorylation at Ser-854 can serve as a pertinent indicator to study MBS phosphorylation by Rho-kinase in vivo. Thus, we prepared the rabbit polyclonal antibody (anti-MBS-854) and tested its ability to recognize the phosphorylated form of MBS. The antibody was found to be highly specific for the phosphorylated form of MBS and did not cross-react with the non-phosphorylated form of MBS.

![Figure 1](https://via.placeholder.com/150)

Figure 1. Determination of the sites of phosphorylation of MBS by Rho-kinase. Purified full-length MBS was incubated with GST-CAT in kinase buffer A containing 100 μM γ-[32P]ATP for 1 h at 30°C, and the reaction product was digested with Achromobacter protease I. The obtained peptides were applied onto a C18 reverse phase column as described in Materials and Methods.
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Figure 2. Specificity of phosphorylation site-specific antibody (anti-pS854 Ab). (A) Recognition of MBS phosphorylated at Ser-854. A total of 200 fmol of GST-MBS-CT (CT: 758–1032 aa) containing the indicated amounts of GST-MBS-CT phosphorylated by GST-CAT and 200 fmol of GST-MBS-CT

Phosphorylation of MBS In Vivo

Rho-Kinase–dependent Phosphorylation of MBS in MDCK Cells

Rho/Rho-kinase are implicated in HGF- and phorbol ester–induced membrane ruffling in MDCK and KB epithelial cells (Nishiyama et al., 1994; Takaishi et al., 1995; Fukata et al., 1999). We examined whether MBS was phosphorylated at Ser-854 via the Rho/Rho-kinase pathway in vivo. When total cell lysate of nonstimulated MDCK cells was immunoblotted with anti-pS854 Ab, MBS phosphorylated at Ser-854 was weakly detected. The addition of TPA enhanced the phosphorylation of MBS at Ser-854 (Fig. 3 A). Similar results were obtained when the cells were stimulated with HGF. A minor band with apparent relative molecular mass of 90 kD was also detected. This minor band may be a degradation product of MBS, because the immunoreactive band detected by anti-MBS Ab was found at the same position (data not shown). After TPA stimulation, the phosphorylation level of MBS at Ser-854 elevated within 3 min, reached maximum at 30 min, and was sustained for at least 2 h (Fig. 3 B). The maximal phosphorylation level of MBS at Ser-854 was about fivefold the basal level. The stoichiometries of phosphorylation at Ser-854 were ~0.04 at the basal level and ~0.20 at the maximal level, respectively. Similar results were obtained when the cells were stimulated with HGF, although the level of MBS phosphorylation induced by HGF was slightly lower than that induced by TPA (Fig. 3 B). We also confirmed that the addition of dibutyryl cAMP did not induce the phosphorylation of MBS at Ser-854 (Fig. 3 A), whereas it induced the phosphorylation of cAMP-response element binding protein (CREB) at Ser-133 (data not shown). These results indicate that MBS is phosphorylated during the action of TPA and HGF in MDCK cells. We furthermore examined whether MBS was phosphorylated at Ser-854 via the Rho/Rho-kinase pathway during the action of TPA and HGF in MDCK cells (Fig. 3 C). Botulinum C3 ADP-ribosyltransferase (C3), which is thought to interfere with endogenous Rho functions, inhibited the focal adhesion formation in MDCK cells (~50% inhibition by incubation of 100 µg/ml of C3; data not shown). Under the conditions, the TPA-induced MBS phosphorylation was inhibited to a similar extent (Fig. 3 C). A similar inhibition was observed when the cells were pretreated with HA 1077 or Y-32885, both of which are inhibitors of Rho-kinase (Fig. 3 C; Uehata et al., 1997).

Distribution of Phosphorylated MBS in MDCK Cells

We investigated the subcellular distribution of MBS phosphorylated at Ser-854 in MDCK cells. In the nonstimulated MDCK cells, the immunoreactivity of phosphorylated MBS was strong in the nucleus and diffuse in the cytoplasm, but not detected in cell–cell contact sites and free end of plasma membrane (Fig. 4 A, a). The TPA-induced membrane ruffling was detectable within 5 min and reached maximum at 15 min of exposure to TPA in the outer cell edge colonies (Nishiyama et al., 1994). Under the conditions, the addition of TPA enhanced the immunoreactivity of phosphorylated MBS in the cytoplasm, and the immunoreactivity of phosphorylated MBS was weakly detected in the membrane ruffling area (Fig. 4 A, c, arrowhead), where F-actin (Fig. 4 A, d, arrowhead),
MBS (Fukata et al., 1998) and phosphorylated α-adducin accumulated (Fukata et al., 1999). Similar results were obtained when the cells were stimulated with HGF instead of TPA (Fig. 4, a, e, and f; arrowheads). The immunoreactivity of phosphorylated MBS in the nucleus, cytoplasm, and membrane ruffling area was abolished by preincubation of the antibody with the antigen phosphopeptide (Fig. 4, a, g, and h).

Next, we compared the distribution of phosphorylated MBS and total MBS. In nonstimulated MDCK cells, the immunoreactivity of total MBS was strong in the cytoplasm (Fig. 4, b, i; Inagaki et al., 1997). In TPA-stimulated MDCK cells, the immunoreactivity of total MBS was detected in the cytoplasm and the TPA-induced membrane ruffling area (Fig. 4, b, f; Fukata et al., 1998). The merged image of anti-pS854 A b (green) and anti-MBS A b (red) immunofluorescence enabled us to roughly estimate the phosphorylation state of MBS. Green, yellow, and red images...
indicate high, intermediate, and low levels of phosphorylation of MBS at Ser-854, respectively. The merged image of phosphorylated and total MBS of the cytoplasm and free end of plasma membrane in the TPA-stimulated cells was more greenish than that of the cytoplasm in the nonstimulated cells (Fig. 4 B, c and g). Consistently, the ratio (phosphorylated MBS/MBS) was high in cytoplasm and membrane ruffling area of TPA-stimulated MDCK cells as compared with that of nonstimulated MDCK cells (Fig. 4 B, d and h). The ratio in nucleus was also increased by TPA stimulation. We found that phosphorylated MBS was more enriched in the membrane ruffling area as compared with Rho GDI, which is one of the cytoplasmic proteins (data not shown; Ueda et al., 1990). Similar results were obtained when the cells were stimulated with HGF instead of TPA (Fig. 4 B, k and l).

To further determine the subcellular distribution of phosphorylated MBS, we carried out the subcellular fractionation analysis (Fig. 4 C). Total MBS was detected mainly in cytoplasmic fractions in non- and TPA-stimulated MDCK cells. The amount of total MBS in each fraction of subcellular fractionation is consistent with the result of immunofluorescence study (Fig. 4 B and C). In nonstimulated MDCK cells, phosphorylated MBS was weakly detected in nuclear and cytoplasmic fractions. The addition of TPA increased phosphorylated MBS mainly in the cytoplasmic fraction and modestly in the nuclear fraction. In addition, phosphorylated MBS was weakly detected in membrane fraction. It should be also noted that although the immunoreactivity of phosphorylated MBS in immunofluorescence study was strong in the nucleus in the presence or absence of TPA (Fig. 4 A and B), the phosphorylation level of MBS in the nuclear fraction in the cells stimulated with TPA was lower than that in the cytoplasmic fraction during subcellular fractionation (Fig. 4 C). This might be due to the rapid dephosphorylation of MBS during nuclear isolation. Severe dephosphorylation of MBS was not prevented during the subcellular fractionation under the present conditions. Therefore, the amount of phosphorylated MBS after subcellular fractionation may be less than that of phosphorylated MBS in intact cells. We found the additional band above phosphorylated MBS in nuclear fraction. We do not know exactly whether the additional band is an isoform of MBS or a nonspecific band. In fact, Shimizu et al. identified two isoforms of MBS (M130 and M133) from chicken gizzard (Shimizu et al., 1994). The similar isoforms may exist in MDCK cells. However, we could not rule out the possibility that pS854 A b cross-reacted with other insoluble nuclear component in immunofluorescence study.

**Distribution of Phosphorylated MBS in Migrating MDCK Cells**

We compared the subcellular distribution of phosphorylated MBS with that of F-actin and phosphorylated MLC at Ser-19 in migrating MDCK cells. Between 2 and 16 h after the addition of TPA, the cells dissociated from each other and migrated, with polarized morphology and membrane ruffling in the leading edge. In the TPA-induced migrating cells, phosphorylated MBS was localized in the leading edge, where F-actin accumulated, and the posterior region containing the nucleus (Fig. 5 A, a and b). Similar results were obtained as to the distribution of phosphorylated MLC in migrating MDCK cells (Fig. 5 A, c and d) as described (Matsuura et al., 1998). The merged image of phosphorylated and total MBS in the leading edge and the posterior region containing the nucleus in migrating MDCK cells was more greenish than that in the cytoplasm in the nonstimulated MDCK cells (Fig. 5 B, c and Fig. 4 B, c). Consistently, the ratio (phosphorylated MBS/MBS) was high in the leading edge and the posterior region containing the nucleus in migrating MDCK cells as compared with that in nonstimulated MDCK cells (Fig. 4 B, d and Fig. 5 B, d).

**Colocalization of Phosphorylated MBS, F-Actin, and Phosphorylated MLC in REF52 Fibroblasts**

We further investigated the subcellular distribution of phosphorylated MBS, F-actin, and phosphorylated MLC in REF52 fibroblasts. REF52 cells grown in 10% fetal bovine serum have thick actin stress fibers and vinculin-containing focal adhesions, and show a filamentous periodical pattern of myosin on stress fibers. We have previously shown that total MBS is localized on stress fibers (Inagaki, N., et al., 1997). Phosphorylated MBS was localized on stress fibers, cortical actin filaments, and in the nucleus in REF52 cells (Fig. 6 A, a). The double-immunostaining by TRITC-phalloidin and anti-pS854 A b (Fig. 6 A, a and b) or anti-pp2b Ab (Fig. 6 A, a and d) demonstrated coexistence of phosphorylated MBS, F-actin and phosphorylated MLC on stress fibers and cortical actin filaments. The intracellular localization of phosphorylated MBS is consistent with in vitro binding of MBS and myosin (Alessi et al., 1992; Shimizu et al., 1994). There was the difference in localization of phosphorylated MBS between REF52 and MDCK cells. REF52 cells highly developed thick stress fibers (Fig. 6 A, b), whereas serum-starved MDCK cells had a few thin stress fibers (Fig. 4 A, b). Thus, phosphorylated MBS might not be detected in stress fibers in MDCK cells. Next, we compared the distribution of phosphorylated and total MBS. The distribution of phosphorylated MBS was similar to that of total MBS (Fig. 6 B, c). The ratio (phosphorylated MBS/MBS) image showed that the phosphorylation level of MBS was high on stress fiber and in nucleus (Fig. 6 B, d).

We examined the effects of C3 and dominant negative Rho-kinase (RHo/PH TT)) on the localization of phosphorylated MBS in REF52 cells (Fig. 6 C). C3 A D P-ribosylates RHo at A sn-41 and inactivates it, whereas RHo A 141 is not ADP-ribosylated by C3 and is insensitive to C3. RHo/PH TT (941–1386 aa) is composed of RHo-binding (RB) and pleckstrin-homology (PH) domains of RHo-kinase (Matsu et al., 1996). RHo/PH TT, which has point mutations in the RB domain and does not bind to RHo, interacts with the kinase domain of RHo-kinase and thereby inhibits the RHo-kinase activity without titrating out RHo in vitro (Amano et al., 1999). RHo/PH TT functions as the dominant negative form of RHo-kinase in vivo (Amano et al., 1998). The microinjection of C3 into REF52 cells disrupted the stress fibers and decreased the phosphorylated MLC staining (Fig. 6 C, e and h) as described (Chihara et al., 1997). The filamentous pattern of phosphorylated
Figure 4. Distribution of Ser-854–phosphorylated MBS in the TPA- or HGF-stimulated MDCK cells. (A) Distribution of Ser-854–phosphorylated MBS. Serum-deprived MDCK cells were stimulated with 200 nM TPA (c, d, and h) or 50 pM HGF (e and f) for 15 min. MDCK cells were stained with anti-pS854 Ab (a, c, and e) or TRITC-phalloidin (b, d, and f). MDCK cells were also stained with anti-pS854 Ab absorbed with a 100-fold amount of antigen phosphopeptide (g and h). Arrowheads indicate the induced membrane ruffling. (B) Distribution of phosphorylated and total MBS. Serum-deprived MDCK cells were stimulated with 200 nM TPA (e-h) or 50 pM HGF (i-l) for 15 min. MDCK cells were doubly stained with anti-pS854 Ab (a, e, and i) and anti-mMBS Ab (b, f, and j). The merged (c, g, and k) and ratio (phosphorylated MBS/total MBS; d, h, and l) images are shown. Arrowheads indicate the induced membrane ruffling. (C) Subcellular distribution of phosphorylated MBS. Non- (−) and TPA- (+) stimulated MDCK cells were separated into nuclear (nucleus), cytoplasmic (cytoplasm), and membrane (membrane) fractions, and the fractions were immunoblotted with anti-pS854 Ab (upper panel) and anti-pMBS Ab (lower panel). These results are representative of three independent experiments. Bars, 10 μm.
MBS was also perturbed by the microinjection of C3 (Fig. 6 C, f). Under these conditions, total MBS and MLC were scattered in the cytoplasm (Fig. 6 C, g; Chihara et al., 1997). The coinjection of GTPγS-RhoA^I41 with C3 reversed the effects of C3 (Fig. 6 B, i–l). The microinjection of RB/PH(TT) also disrupted the stress fibers (Fig. 6 C, m) and decreased the staining of phosphorylated MBS and MLC in REF52 cells (Fig. 6 C, n and p). Similar results were obtained when cells were treated with Rho-kinase inhibitors (HA1077 and Y-32885) (data not shown). It should be noted that the microinjection of C3 into REF52 decreased the staining of phalloidin in most cells, whereas that of RB/PH(TT) induced the disorganization of actin filaments (Fig. 6 C, m). C3 may induce depolymerization of F-actin through the inhibition of other Rho-targets such as p140mDia.

Distribution of Phosphorylated MBS and ERM Family Proteins during Cytokinesis

It has been shown that ERM family proteins and MLC phosphorylated at Ser-19 highly accumulate at the cleavage furrow during cytokinesis (Sato et al., 1991; Matsumura et al., 1998). In a recent study, we have found that Rho-kinase also highly and circumferentially accumulates at the cleavage furrow in various cell lines (Kosako et al., 1999), and that dominant negative Rho-kinase inhibits the progress of cytokinesis (Yasui et al., 1998). Here we examined the distribution of phosphorylated MBS during the different mitotic stages of MDCK cells. Phosphorylated MBS was enriched at the mid zone between the daughter chromosomes in late anaphase and at the cleavage furrow in telophase (Fig. 7 A, a and b). Phosphorylated MBS persisted at the mid body until the end of cytokinesis (Fig. 7 A, c). Next, we compared the distribution of phosphorylated MLC and ERM family proteins to that of phosphorylated MBS during different mitotic stages of MDCK cells. The staining patterns of phosphorylated MLC were spatially and temporally similar to that of phosphorylated MBS in dividing cells (Fig. 7 A, g–i). Phosphorylated ERM family proteins accumulated in the microvilli-like structures in the cell body at all stages as described (Oshiro et al., 1998), and highly and circumferentially accumulated around the mid zone in late anaphase, and the cleavage furrow in telophase. The staining patterns of phosphorylated ERM family proteins were also similar to that of phosphorylated MBS, but phosphorylated ERM family proteins did not persist at the mid body until the end of cytokinesis (Fig. 7 A, m–o). Vimentin is the most widely expressed intermediate filament protein, which is phosphorylated by Rho-kinase at Ser-71 (Goto et al., 1998). Using TM71, which recognizes the phosphorylation of vimentin at Ser-71, vimentin is shown to be specifically phosphorylated at the cleavage furrow whereas total vimentin is diffusely localized throughout the cytoplasm (Yasui et al., 1998). Although phosphorylated vimentin, MBS and ERM family proteins accumulated around the cleavage furrow, they were not completely colocalized (Fig. 7 B, f and l). Phosphorylated adducin, which is one of the Rho-kinase substrates, was diffusely localized throughout the cytoplasm (Fig. 7 B, m). It should be noted that total MBS was diffusely localized throughout the cytoplasm, but not accumulated at the cleavage furrow (Fig. 7 B, a). In contrast, phosphorylated MBS strongly accumulated at the cleavage furrow (Fig. 7 B, d), indicating that MBS was phosphorylated specifically at the cleavage furrow. Total ERM family proteins was diffusely localized throughout the cytoplasm, and at the microvilli and cleavage furrow.

Figure 5. Distribution of phosphorylated MBS in migrating MDCK cells. (A) Serum-deprived MDCK cells were stimulated with 200 nM TPA for 2 h. Migrating MDCK cells were doubly stained with TRITC-phalloidin (b and d) and anti-pS854 Ab (a) or anti-pp2b Ab (c). (B) Localization of phosphorylated and total MBS in migrating MDCK cells. Migrating cells were doubly stained with anti-pS854 Ab (a) and anti-mMBS Ab (b). The merged (c) and ratio (phosphorylated MBS/MBS; d) images are shown. Bars, 10 μm.
Figure 6. Colocalization of phosphorylated MBS, F-actin and phosphorylated MLC in REF52 fibroblasts. (A) Localization of phosphorylated MBS. REF52 cells were doubly stained with TRITC-phalloidin (b and d) and anti-pS854 Ab (a) or anti-pp2b Ab (c). (B) Distribution of phosphorylated and total MBS in REF52 cells. REF52 cells were doubly stained with anti-pS854 Ab (a) and anti-mMBS Ab (b). The merged (c) and ratio (phosphorylated MBS/MBS; d) images are shown. (C) Inhibition of phosphorylation of MBS by C3 or dominant negative Rho-kinase. REF52 cells were microinjected with MBP (5.0 mg/ml) (a–d), C3 (0.1 mg/ml) (e–h), C3 plus GTPγS-RhôA141 (0.4 mg/ml) (i–l), or MBP-RB/PH(TT) (5.0 mg/ml) (m–p). REF52 cells were stained with TRITC-phalloidin (a, e, i, and m), anti-pS854 Ab (b, f, j, and n), anti-pMBS Ab (c, g, k, and o), and anti-pp2b Ab (d, h, l, and p). The arrowheads indicate the injected cells. Bars, 10 μm.
Figure 7. Distribution of phosphorylated MBS and ERM family proteins during cytokinesis in MDCK cells. (A) Accumulation of phosphorylated MBS and ERM family proteins at the cleavage furrow. MDCK cells in late anaphase (a, d, g, j, m, and p), telophase (b, e, h, k, n, and q) or cytokinesis (c, f, i, l, o, and r; indicated by arrowheads) stained with anti-pS854 Ab (a–c), anti-pp2b Ab (g–i), or anti-pT558 Ab (m–o) are shown. DNAs were stained with bisbenzimide Hoechst (d–f, j–l, and p–r). (B) Specific localization of phosphorylated Rho-kinase substrates during cytokinesis. MDCK cells in cytokinesis doubly stained with TM71 (b, e, h, k, and n) and anti-pnMBS Ab (a), anti-pS854 Ab (d), anti-ERM Ab (g), anti-pT558 Ab (j), and anti-pT445 Ab (m). Merged images are shown (c, f, i, l, and o). (C) Elevation of phosphorylation of MBS at Ser-854 during cytokinesis. Total cell lysates were prepared from interphase cells (I), early mitotic cells (metaphase, M) and cells at different stages of cell division (time in minutes after the release of mitotic arrest), and immunoblotted with anti-pS854 Ab (upper panel) or anti-pnMBS Ab (lower panel). These results are representative of three independent experiments. Bars, 10 μm.
(Fig. 7 B, g), whereas phosphorylated ERM family proteins accumulated at the microvilli and cleavage furrow preferentially (Fig. 7 B, j). To further confirm that the phosphorylation level of MBS at Ser-854 elevates during the cytokinesis, immunoblot analysis of synchronized MDCK cells lysates was carried out (Fig. 7 C). By release from mitotic arrest by nocodazole, early mitotic cells synchronistically entered into later stages of cell division, and at 30 min after release of mitotic arrest, ~40% of the cells were in late anaphase, telophase or cytokinesis. At later phases, the cells were in post mitotic spreading (60–180 min) as described (data not shown; Matsumura et al., 1998). The immunoreactivity of anti-pS854 Ab was increased at 30 min after release of mitotic arrest as compared with that in interphase and early mitotic cells (Fig. 7 C). This increment of phosphorylation level of MBS was reversed at 60 min.

Discussion

Phosphorylation of MBS by Rho-Kinase In Vitro

Here we identified Ser-854 as one of the major sites of phosphorylation of MBS by Rho-kinase in vitro, and prepared a rabbit polyclonal antibody (anti-pS854 A b), raised against the synthetic phosphopeptide. A nti-pS854 A b specifically recognized MBS phosphorylated by Rho-kinase, but not by PKC in vitro. Since Ser-854 in MBS is the phosphorylation site specific to Rho-kinase among known MBS kinases, the phosphorylation of MBS at Ser-854 appears to be a useful indicator of the Rho/Rho-kinase activation in vivo. We found that Thr-697 was also one of the major sites of phosphorylation of MBS by Rho-kinase in vitro. We have previously reported that the phosphatase activity toward MLC is inhibited when MBS is phosphorylated by Rho-kinase (Kimura et al., 1996). The endogenous kinase that is copurified with MBS from chicken gizzard (Ichikawa et al., 1996), phosphorylates MBS at Thr-695 (M133), which corresponds to Thr-697 of Rat3 MBS, and thereby inactivates the phosphatase activity (Ichikawa et al., 1996). The phosphorylation of MBS at Thr-697 by Rho-kinase may result in inhibition of myosin phosphatase. The endogenous kinase appears to be distinct from Rho-kinase because the endogenous kinase is not inhibited by H7, which is one of the PKC inhibitors, whereas Rho-kinase is inhibited by H7 (unpublished data), and because the endogenous kinase but not Rho-kinase phosphorylates MLC at PKC sites (Ichikawa et al., 1996). Since several sites of MBS including Thr-697 and Ser-854 were phosphorylated by Rho-kinase in vitro, further studies are necessary to determine which sites are the major sites of phosphorylation of MBS by Rho-kinase in vivo, and which phosphorylation sites are responsible for the inhibition of the phosphatase activity by Rho-kinase in vitro and vivo.

Phosphorylation of MBS by Rho-Kinase In Vivo

We have previously shown that expression of dominant active Rho in NIH 3T3 cells results in an increment of MBS phosphorylation (Kimura et al., 1996). MBS is phosphorylated and the myosin phosphatase activity is inactivated during the action of thromboxane A2 in platelets, and both reactions are inhibited by a prior treatment of platelets with C3 (Nakai et al., 1997). Similar observations are obtained in endothelial cells during the action of thrombin (Essler et al., 1998). Here we found by use of anti-pS854 Ab that the stimulation of MDCK cells with TPA or HGF induced the phosphorylation of MBS at Ser-854, and that pretreatment of the cells with C3 or Rho-kinase inhibitors inhibited the TPA- or HGF-induced MBS phosphorylation. It is possible that TPA induced the phosphorylation of MBS at Ser-854 through direct phosphorylation by PKC. However, this possibility is unlikely because anti-pS854 Ab did not recognize MBS phosphorylated by PKC in vitro. Phosphorylated MBS accumulated on stress fibers in REF52 cells. The microinjection of C3 or dominant negative Rho-kinase into REF52 cells weakened the accumulation of phosphorylated MBS. These results indicate that MBS is phosphorylated by Rho-kinase downstream of Rho in non-muscle cells. Myosin phosphatase binds to phosphorylated Rho-kinase substrates such as MLC via MBS and dephosphorylates them. Rho-kinase phosphorylates MBS, which leads to the inactivation of myosin phosphatase in vitro (Kimura et al., 1996, 1998; Fukata et al., 1998). Taken together, these observations suggest that the phosphorylation of MBS by Rho-kinase is involved in regulating the phosphorylation level of Rho-kinase substrates in non-muscle cells.

Evidence is accumulating that Rho regulates the phosphorylation level of MLC through Rho-kinase and myosin phosphatase in smooth muscle cells (Hirata et al., 1992; Noda et al., 1995; Gong et al., 1996; Kureishi et al., 1997). Because contraction of smooth muscle cells determines the size of lumen in blood vessels, airways, the gastrointestinal tract, uterus, and bladder, abnormal contraction can cause diseases such as hypertension and asthma (Somlyo, 1997). It has recently been shown that Y-27632 (one of the specific inhibitors for Rho-kinase) selectively inhibits smooth muscle contraction and corrects blood pressure in several hypertensive rat models (Uehata et al., 1997). Thus, the Rho-kinase–mediated pathway appears to be involved in the pathogenesis of hypertension. Phosphorylation of MBS by Rho-kinase may play an important role in generating a certain types of abnormal contraction of smooth muscle. In this regard, we have recently found that Rho-kinase phosphorylates MBS at Ser-854 during porcine coronary artery spasm (Kandabashi et al., 1999).

Phosphorylation of MBS in Motile Cells

Membrane ruffling is observed in the leading edges of motile cells and is thought to be essential for cell motility (Cooper, 1991). A force arising from actin polymerization appears to drive lamellipodial protrusion (Mitchison and Cramer, 1996), which is thought to be regulated by the small GTPase Rac (Ridley et al., 1992; Hall, 1998). A ctn in the membrane ruffling area is thought to be continuously depolymerized and then repolymerized during cell movement (Mitchison and Cramer, 1996). A force derived from myosin II driven by MLC phosphorylation, which is thought to be regulated by Rho (Amano et al., 1996a, 1998) in the membrane ruffling area and posterior region.
of motile cells may also contribute to cell movement (Lauffenburger et al., 1996; Mitchison and Cramer, 1996). Indeed, injection of anti-M LC-kinase A b diminishes the cell motility of macrophages (Wilson et al., 1991). Moreover, M atsumura et al. (1998) have recently shown that the phosphorylation level of M LC is high in the leading edge and posterior region containing the nucleus during the cell migration. It has been previously reported that the addition of TPA decreases force that the whole cell applies to the substrate in certain migrating fibroblasts (D anowski and H arris, 1988). The cycling between phosphorylated and nonphosphorylated states of M LC may be necessary for cell migration. In this regard, we have recently reported that microinjection of either dominant negative or constitutively active R ho-kinase inhibits cell migration of N R K cells (Fukata et al., 1999).

We have recently found that the microinjection of dominant negative R ho-kinase inhibits the T P A - or H G F -induced membrane ruffling in M D C K cells, indicating that R ho-kinase is necessary for the cell motility (Fukata et al., 1999). Here we found that M BS phosphorylated at Ser-854 as well as M LC phosphorylated at Ser-19 were localized in the leading edge and posterior region in migrating M D C K cells. We have recently found that phosphorylated α-adducin accumulates in the leading edge in migrating M D C K cells (Fukata et al., 1999). Myosin phosphatase interacts with both M LC and adducin through M BS, and dephosphorylates the phosphorylated M LC and α-adducin (K i mura et al., 1996, 1998). Taken together, the above observations suggest that myosin phosphatase and R ho-kinase cooperatively regulate the M LC phosphorylation in the leading edge and posterior region in migrating M D C K cells, and the α-adducin phosphorylation in the leading edge.

Phosphorylation of MBS in Fibroblasts

R ho-kinase is thought to regulate the formation of actin stress fibers (Leung et al., 1996; A mano et al., 1997; I shizaki et al., 1997). We have recently found that the expression of mutant M LC T 18 D , S 19 D (substitution of residues by A sp), which is known to lead to the activation of myosin A T P ase and a conformational change of myosin II when reconstituted with myosin heavy chain in vitro (K a m i s o y a m a et al., 1994; Sweeney et al., 1994; B resnick et al., 1995), also enhances the formation of stress fiber (A mano et al., 1998). Thus, it is likely that the R ho/R ho-kinase pathway plays a critical role in the formation of stress fiber through myosin II activation. Here we found that phosphorylated M BS was localized on stress fibers in R E F 52 cells. The microinjection of C 3 or dominant negative R ho-kinase into R E F 52 cells disrupted stress fibers and weakened the accumulation of phosphorylated M BS. These observations suggest that myosin phosphatase and R ho-kinase cooperatively regulate the M LC phosphorylation in fibroblasts, which in turn induces the formation of stress fiber.

Phosphorylation of MBS during Cytokinesis

R ho, R ho-kinase, and E R M family proteins accumulate at the cleavage furrow (Sato et al., 1991; Takaishi et al., 1995; K osako et al., 1999), where M LC phosphorylation occurs (M atsumura et al., 1998). The expression of C 3 or dominant negative R ho-kinase inhibits cytokinesis, resulting in multiple nuclei (K i shi et al., 1993; M abuchi et al., 1993; Y asui et al., 1998). Thus, M LC phosphorylation by the R ho/R ho-kinase pathway appears to provide contractility to the contractile ring and to play a critical role in cytokinesis. R ho-kinase also phosphorylates intermediate filament proteins such as glial fibrillary acidic protein (G F A P ) and vimentin, exclusively at the cleavage furrow during cytokinesis (K osako et al., 1997; G o to et al., 1998). The expression of G F A P mutated at R ho-kinase phosphorylation sites induces impaired segregation of glial filament into postmitotic daughter cells (Y asui et al., 1998). Thus, R ho-kinase appears to be essential not only for cytokinesis but also for the segregation of G F A P filaments into daughter cells which in turn ensures efficient cellular separation. Here we found that phosphorylated M BS as well as phosphorylated E R M family proteins accumulated at the cleavage furrow, where phosphorylated M LC and vimentin accumulated. Indeed, the phosphorylation level of M BS elevated during cytokinesis. Taken together, these results suggest that myosin phosphatase spatiotemporally regulates the phosphorylation state of certain substrates including M LC and E R M family proteins during cytokinesis in cooperation with R ho-kinase under the control of R ho. Recently, it has been shown that citron kinase, another R ho-binding kinase with structural similarity in the kinase domain to R ho-kinase, accumulates at the cleavage furrow and may play an important role in the contractile process during cytokinesis (M adaule et al., 1998). Further analysis of different and redundant functions of both R ho-binding kinases will help us to elucidate the molecular mechanism underlying cytokinesis downstream of R ho.

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