J. Biochem. 128, 435–440 (2000)

Activation of Actin-Activated MgATPase Activity of Myosin II by Phosphorylation with MAPK-Activated Protein Kinase-1b (RSK-2)[1]

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Received May 12, 2000; accepted June 20, 2000

Regulatory light chain of myosin II (MRLC) was identified as a novel substrate of p90 ribosomal S6 kinase (RSK)-2, a Ser/Thr protein kinase which is phosphorylated and activated by mitogen-activated protein kinase (MAPK) *in vitro* and *in vivo*. Phosphopeptide map of MRLC phosphorylated by RSK-2 was identical to that by myosin light chain kinase (MLCK). Phosphoserine was recovered by the phosphoamino acid analysis of MRLC phosphorylated by RSK-2. Further, phosphorylation using recombinant glutathione *S*-transferase (GST) fusion proteins of HeLa MRLC2 revealed that RSK-2 phosphorylated wild-type MRLC2 (GST-wtMRLC2) but not its mutants GST-MRLC2^{S19A} or GST-MRLC2^{T18AS19A} (alanine substituted for Ser19 or both Ser19 and Thr18). These results revealed that RSK-2 phosphorylates MRLC at Ser19 as did MLCK. Phosphorylation of myosin II by RSK-2 resulted in activation of actin-activated MgATPase activity of myosin II. Interestingly, RSK-2 activity to phosphorylate MRLC was suppressed by phosphorylation with MAPK. RSK-2 might be a mediator that regulates myosin II activity through the MAPK cascade.

Key words: actin-activated MgATPase activity, MAPK, MRLC, myosin II, RSK.

Mitogen-activated protein kinase (MAPK) is believed to play an important role in signal transduction through the network of intracellular protein kinases in response to many growth factors, polypeptide hormones, and neurotransmitters (*1*). Several lines of evidence indicate that p90 ribosomal S6 kinase (RSK)-1, -2, and -3, also known as MAPK-activated protein kinase (MAPKAPK)-1a, -1b, and 1c, are *in vivo* substrates for MAPK (*2*, *3*). RSKs activated by phosphorylation with MAPK have been found to phosphorylate several proteins containing ribosomal S6 protein, transcription factor CREB, core histone H3, and ABP-280, *in vitro* (*4–7*).

Growth factors/cytokine receptors promote MAPK signaling and the immediate induction of cell migration (*8*), suggesting that MAPK can lead to direct activation of the cell motility machinery. Ultimately, the motogenic signals generated by cytokine receptors impact the actin-myosin cytoskeleton, which is critical for cell movement (*8*, *9*). Myosins are actin-activated ATPases capable of generating force by promoting translational movement along actin cables. While several classes of myosins have been identified, myosin II is the best characterized as a motor protein to promote smooth muscle contraction, or locomotion and cytokinesis of non-muscle cells (*10*, *11*). The phosphorylation of regulatory light chain of myosin II (MRLC) is an important factor for the regulation of myosin II activity (*12*, *13*). Our previous study demonstrated that MAPKAPK-2 and MAPKAPK-4, a subfamily of RSK, phosphorylated MRLC *in vitro* (*14*, *15*). These observations suggest that the phosphorylation of MRLC by downstream kinases of MAPK might participate in regulation of cell migration through MAPK signal transduction in response to an extracellular signal.

In this study, we show that RSK-2 phosphorylated MRLC at Ser19, which resulted in activation of actin-activated MgATPase activity of myosin II. Further experiments demonstrated that the kinase activity of RSK-2 is not only positively but also negatively regulated by the phosphorylation with MAPK, suggesting that RSK-2 may function as a mediator in the regulation of myosin II activity through the MAPK signaling pathway.

MATERIALS AND METHODS

Materials and Chemicals—Smooth muscle myosin light chain kinase (MLCK) and myosin II were purified from chicken gizzard as described previously (*16*). Light chains of myosin II (MLCs) were prepared as described (*17*) with slight modifications (*18*). Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (*19*). G-actin was further purified by gel filtration. Activated murine glutathione *S*-transferase (GST)-p42 mitogen–activated protein kinase (MAPK), ribosomal S6 protein kinase (RSK)-2 partially purified from rabbit skeletal muscle (Lot# 13479), anti–RSK-2 polyclonal antibody (Lot# 18111), and C-terminal peptide (RRRLSSLRA) corresponding to amino acids 231–239 of human 40S ribosomal protein S6 (S6 peptide) were purchased from Upstate Biotechnology (Lake Placid, NY). Calmodulin of bovine brain was purchased

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to H.H.).

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Abbreviations: MAPK, mitogen-activated protein kinase; MRLC, regulatory light chain of myosin II; MLCK, myosin light chain kinase; RSK, ribosomal S6 kinase; MAPKAPK, MAPK-activated protein kinase.

from Sigma Chemical (St. Louis, MO). Calyculin A was obtained from Wako Pure Chemical Industries (Osaka). Other materials and chemicals were obtained from commercial sources. HeLa cells (RCB0007; similar to ATCC CCL2, HeLa) were obtained from Riken Cell Bank (Tsukuba) and were grown as described previously (*20*).

Protein Kinase Assay—Myosin II (0.32 mg/ml) from chicken gizzard or MLCs (0.1 mg/ml) from myosin II were used as the substrates of RSK-2 (5 U/ml) in the presence or absence of GST-p42 MAPK (5 μg/ml). The assays were carried out in the kinase mixture containing 50 mM Tris-HCl (pH 7.2), 0.18 mM [γ-^{32}P]ATP (0.1 mCi/ml), 60 mM KCl, 0.1 mM dithiothreitol (DTT), 1 mM EGTA, 5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.2 mM NaF, 0.3 μg/ml leupeptin, 0.3 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 μM calyculin A. The reaction solution (total volume of 20 μl) was incubated for 1 h at 30°C, and the reaction was stopped by addition of 5 μl of 5× sample buffer (*21*). The proteins of the samples were separated by SDS-PAGE. ^{32}P-labeled proteins were detected with a Bio Imaging Analyzer (BAS2000, Fuji Photo Film, Tokyo). Phosphorylation of S6 peptide was carried out as described previously (*15*). Phosphorylation of S6 peptide (0.5 mg/ml) by RSK-2 (5 U/ml) was assayed in the reaction solution (final volume of 20 μl) containing 6 mM Hepes (pH 7.5), 0.18 mM [γ-^{32}P]ATP (0.1 mCi/ml), 1 mM EGTA, 5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.2 mM NaF, 1.4 mM PMSF, 0.1 μM calyculin A, and 1 mg/ml BSA in the presence or absence of GST-p42 MAPK (5 μg/ml). Each reaction was carried out for 40 min at 30°C and terminated by the addition of acetic acid [final 30% (v/v)]. The mixtures were applied to P81 phosphocellulose ion exchange paper (Whatman, Hillsboro, OR), then washed twice for 5 min in 30% acetic acid, and for 15 min in 15% acetic acid. The radioactivity was determined by Cerenkov counting of the papers with a liquid scintillation counter (LSC 4100; Aloka, Tokyo).

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Phosphorylation of MRLC by RSK-2 was performed as described above. Phosphorylation of MRLC by MLCK (1 μg/ml) was carried out in the kinase mixture containing 56 mM Tris-HCl (pH 7.5), 0.18 mM [γ-^{32}P]ATP (0.1 mCi/ml), 30 mM KCl, 0.1 mM DTT, 1 mM EGTA, 1.2 mM $CaCl_2$, 50 μg/ml calmodulin, 8 mM $MgCl_2$, 0.3 μg/ml leupeptin, 0.3 μg/ml pepstatin A, 0.1 mM PMSF, 0.1 μM calyculin A, and 0.1 μg/ml MLCs. ^{32}P-labeled MRLC was separated by SDS-PAGE and excised from the polyacrylamide gel. Two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described previously (*14*). Autoradiography was performed using a Bio Imaging Analyzer.

Preparation of Cell Extracts—HeLa cells were grown to confluent levels on 14 large culture dishes (25 × 25 cm, SUMILON) in Eagle's Minimal Essential Medium (MEM, Nissui Pharmacologh) containing 10% FBS (INTERGEN). The cells were collected and washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.3) containing 5 mM $MgCl_2$, 0.2 mM EGTA, and 1 mM PMSF. Cell pellets were homogenized with an equal volume of Triton/glycerol solution (0.1 M Pipes, pH 6.9, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.05% Triton X-100, and 4 M glycerol). The homogenates were then centrifuged at 100,000 ×*g* for 1 h at 4°C, and the supernatants were used as cell extracts.

Immunoprecipitation and Kinase Assay for Immunoprecipitates—Immunoprecipitation was performed using HeLa cell extracts which were prepared as described above. The cell extracts was precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h, then incubated with 2 μg of the anti–RSK-2 antibody for 1 h at 4°C by rotation. Protein G-Sepharose beads were added to the extracts and incubated for an additional 1 h at 4°C by rotation. The beads were washed three times with cold PBS. Immunoprecipitated complex was washed twice with the reaction mixture (50 mM Tris-HCl, pH 7.2, 60 mM KCl, 0.1 mM DTT, 1 mM EGTA, 5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.2 mM NaF, 0.3 μg/ml leupeptin, 0.3 μg/ml pepstatin A, 0.1 mM PMSF, and 0.1 μM calyculin A). Phosphorylation of myosin II (0.32 mg/ml) by immunoprecipitates was performed for 1 h at 30°C in the reaction mixture (total volume of 20 μl) containing 0.18 mM [γ-^{32}P]ATP with or without GST-p42 MAPK (5 μg/ml), and the reaction was stopped by addition of 5 μl of 5× sample buffer (*21*). The proteins of the samples were separated by SDS-PAGE. ^{32}P-labeled proteins were detected with a Bio Imaging Analyzer.

Plasmid Construction and Expression of Recombinant MRLC—Two mutants of MRLC were generated by site-directed mutagenesis using one of HeLa MRLC cDNAs (termed as HeLa MRLC2) as a template. We named them MRLC2^{S19A} and MRLC2^{T18AS19A}, respectively. In MRLC2^{S19A} and MRLC2^{T18AS19A}, the phosphorylation sites by MLCK, Ser19 and both Ser19 and Thr18, were replaced by alanine, respectively. Wild-type (wt) MRLC2 and its two mutant cDNAs were in-frame inserted into pGEX-5X-3 vector (Amersham Pharmacia Biotech) in order to express the GST fusion protein. *E. coli* cells (BL21) were cultured in 2× YT medium containing 10 mg/ml Bacto yeast extract (Difco), 16 mg/ml Bacto tryptone (Difco), 5 mg/ml NaCl, and 100 μg/ml ampicillin at 37°C with vigorous agitation. After 2.5 h of culture, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM. After an additional 2 h at 15°C, cells were harvested and lysed by sonication in cold PBS containing 1 mM PMSF, 10 μg/ml leupeptin, and 1 mM DTT; and GST-MRLC2 fusion protein was purified with Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as described in the manufacturer's instructions.

Assay of the MgATPase Activity of Myosin II—The MgATPase activity of myosin II was assayed as described previously (*14*) with slight modification. Phosphorylation of myosin II was carried out by incubating 0.6 mg/ml of myosin II with RSK-2 (5 U/ml) in a buffer containing 30 mM PIPES (pH 7.2), 0.2 M KCl, 1 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.2 mM NaF, 1 mM EGTA, 0.1 mM DTT, 0.2 μM calyculin A, and 1 mM ATP for 1 h at 30°C. The MgATPase activity of myosin II unphosphorylated or phosphorylated by RSK-2 was assayed in a reaction mixture containing 30 mM Tris-HCl (pH 7.5), 85 mM KCl, 1.2 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM EGTA, 1 mM ATP, and 0.1 mg/ml of myosin II in the presence or absence of 1 mg/ml F-actin at 25°C. The MgATPase reaction was started by addition of F-actin to the reaction mixture. Aliquots of the reaction mixture were removed and mixed with an equal volume of 0.6 M perchloric acid and chilled on ice. After centrifugation of the reaction mixture at 14,000 ×*g* for 10

min, the supernatant was neutralized with 2.5 M Tris-HCl (pH 8.8). The amount of inorganic phosphate was determined by using Iatro-Chrom Pi-S (IATRON Labs, Tokyo) according to the method of Sugiura *et al.* (*22*). MgATPase activity was calculated by analysis of the initial linear phase of the time course data.

Other Procedures—Protein concentration was determined using bovine γ-globulin as a standard as described previously (*14*). SDS-PAGE was carried out as described previously (*21*).

RESULTS AND DISCUSSION

Figure 1 shows a SDS–polyacrylamide gel following phosphorylation of myosin II by RSK-2 using [γ-^{32}P]ATP. Autoradiography shows that MRLC of intact myosin II serves as a substrate for RSK-2. Unlike MLCK, RSK-2 phosphorylated MRLC in the absence of Ca^{2+}/calmodulin. A component with high molecular weight (arrowhead in lane 4) is probably an autophosphorylated RSK-2, because autophosphorylation of RSK-2 has been reported (*23*). MRLC is phosphorylated at the PKC sites (Ser1 and/or Ser2 and Thr9), and this phosphorylation inhibits actin-activated MgATPase activity and reduces the stability of myosin filaments (*12*). On the other hand, MLCK phosphorylates MRLC (Thr18 and/or Ser19, MLCK site) and activates actin-activated MgATPase activity of myosin II (*13*). To determine the phosphorylation site of MRLC phosphorylated by RSK-2, two-dimensional phosphopeptide mapping of MRLC phosphorylated by RSK-2 was performed (Fig. 2). The mobility of the phosphopeptide spots recovered from MRLC phosphorylated by RSK-2 was identical to that by MLCK. Phosphoamino acid analysis revealed that phosphorylation of MRLC by RSK-2 was at a serine residue (panel D). These results were obtained using partially purified RSK-2. We examined the kinase activity of immunoprecipitated RSK-2 from HeLa cell extracts with anti–RSK-2 antibody. The immunoprecipitated RSK-2 also phosphorylated MRLC at the serine residue of the MLCK site as well as did the partially purified RSK-2 (data not shown). Furthermore, RSK-2 phosphorylated the isolated MRLC from chicken gizzard and GST-wtMRLC2 but not its two mutants, GST-MRLC2^{S19A} and GST-MRLC2^{T18AS19A} (panel E). Taken together, these results indicate that RSK-2 phosphorylated MRLC at Ser19 of the MLCK site. Furthermore, phosphorylation of myosin II by RSK-2 enhanced its actin-activated MgATPase activity (Fig. 3).

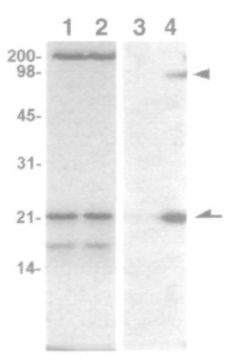

Fig. 1. **Phosphorylation of MRLC by RSK-2.** Myosin II from chicken gizzard was incubated with (lanes 2 and 4) or without (lanes 1 and 3) RSK-2 as described under "MATERIALS AND METHODS." Samples were analyzed by SDS-PAGE with Coomassie Brilliant Blue (left panel) and by autoradiography (right panel). As shown in lane 4, MRLC is phosphorylated by RSK-2 (arrow). A component with high molecular weight in the right panel (arrowhead) is probably an autophosphorylated RSK-2. The positions of molecular mass markers in kilodaltons are indicated.

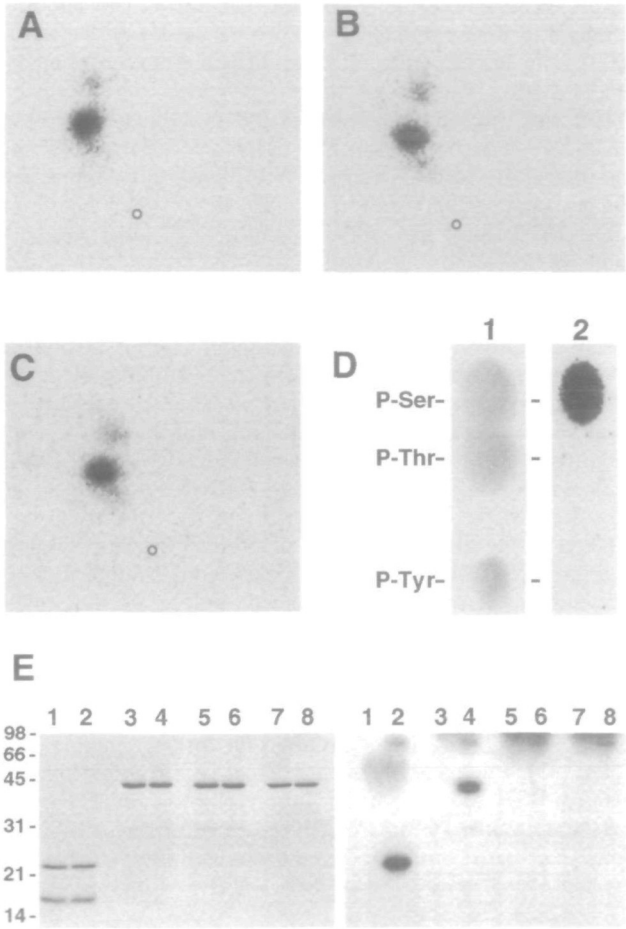

Fig. 2. **Identification of phosphorylation site of MRLC by RSK-2.** Two-dimensional tryptic peptide maps of MRLC phosphorylated by RSK-2 (panel A) and MLCK (panel B). The phosphorylated bands of MRLC excised from polyacrylamide gel were digested with trypsin and processed for electrophoresis (horizontal dimension) followed by chromatography (vertical dimension) as described under "MATERIALS AND METHODS." The circles indicate the origins. Panel C is a map of a 1:1 mixture of the samples mapped in panels A and B. Panel D shows phosphoamino acid analysis of MRLC phosphorylated by RSK-2. Phosphoamino acid analysis was performed as described under "MATERIALS AND METHODS." Lane 1, phosphoamino acid marker detected by ninhydrin; lane 2, autoradiography. Panel E indicated the phosphorylation of recombinant GST-MRLC fusion proteins by RSK-2. Isolated MLCs (0.1 mg/ml) from chicken gizzard (lanes 1 and 2), or 0.18 mg/ml of GST-wtMRLC2 (lanes 3 and 4), GST-MRLC2^{S19A} (lanes 5 and 6) and GST-MRLC2^{T18AS19A} (lanes 7 and 8) were incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) RSK-2 (5 U/ml) as described in "MATERIALS AND METHODS." After phosphorylation, samples were analyzed by SDS-PAGE with Coomassie Brilliant Blue (left panel) and by autoradiography (right panel). The positions of molecular mass markers in kilodaltons are indicated. The nucleotide sequence data of HeLa MRLC2 will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number D82058.

RSK-2 has been found to be activated by direct phosphorylation at serine and threonine residues with MAPK (*23*) or 3-phosphoinositide–dependent protein kinase-1 (PDK1) (*24*), or by autophosphorylation (*25*). Consequently, we examined whether RSK-2 activity to phosphorylate MRLC is enhanced by phosphorylation with MAPK (Fig. 4). This RSK-2 purified by the method of Sutherland *et al.* can autophosphorylate, and its kinase activity against a peptide related to C-terminus of ribosomal protein S6 (S6 peptide) is activated about twofold by phosphorylation with MAPK (*23*). Under our experimental conditions, autophosphorylation of RSK-2 was observed and the level of ^{32}P-labeled RSK-2 was apparently increased by phosphorylation with MAPK (filled arrowhead in Fig. 4A). However, the ability to phosphorylate MRLC which RSK-2 originally possessed was obviously suppressed in the presence of MAPK (arrow in Fig. 4A). In contrast, when S6 peptide was used as a substrate of RSK-2, similar results to those reported by Sutherland and coworkers were obtained (Fig. 4C). Further, like the partially purified RSK-2, an immunoprecipitated RSK-2 from HeLa cell extracts also underwent suppression by MAPK of its activity to phosphorylate MRLC (Fig. 4B).

Phosphorylation of MRLC controls the contractility of actomyosin in non-muscle and smooth muscle cells. Recent evidence shows that the phosphorylated MRLC at Ser19 is localized in the contractile ring of dividing mammalian cells, suggesting that phosphorylation of MRLC plays an important role during cytokinesis (*20*, *26*). While the phosphorylation of MRLC at mitosis has been studied, little is known about the kinases that phosphorylates MRLC in the mitotic phase. $p34^{cdc2}$ kinase and MAPK are known to be activated during mitosis (*27*). RSK-2 is found to be activated concomitantly with MAPK activation during meiosis and mitosis (*28*), suggesting that RSK-2 may have an important role in cell cycle progression during mitosis and meiosis. This suggestion is supported by three findings. (i) *Xenopus* RSK, closely related human RSK-1 and -2, participates in activation of $p34^{cdc2}$ kinase during meiosis by inactivation of $p34^{cdc2}$-inhibitory kinase Myt1 (*29*). (ii) Mitotic arrest is caused by microinjection of constitutively active RSK into cleaving *Xenopus* embryos (*30*). (iii) RSK-2 phosphorylates histone H3 and may contribute to the initiation of chromosome condensation at G_2/M transition (*6*, *31*). Furthermore, our present study demonstrated that RSK-2 phosphorylated MRLC at Ser19, as did MLCK, and enhanced the actin-activated MgATPase activity of myosin II. These findings suggest that RSK-2 may participate in signaling cytokinesis through the phosphorylation of MRLC.

In vivo phosphorylation at several serine and threonine residues on RSK-2 is found to be induced after stimulation by many growth factors and peptide hormone in mammalian cells (*5*, *24*, *25*). The phosphorylation of these amino acid residues is thought to be necessary for RSK-2 to possess full kinase activity (*24*). Our result also revealed that the phosphorylation by MAPK contributed to further enhancement of RSK-2 activity to phosphorylate S6 peptide. In contrast, the activity of RSK-2 to phosphorylate MRLC was suppressed by the phosphorylation with MAPK. Inactivation of RSK-2 activity is found to be carried out by *in vitro* dephosphorylation with a protein phosphatase PP2A at serine and threonine residues of the phosphorylation sites on RSK-2 (*23*). However, reduction of RSK-2 activity by phosphorylation with MAPK has not been reported previously. It is attractive to speculate that MAPK not only activates but also inactivates the kinase activity of RSK-2 through the phosphorylation. As the difference between

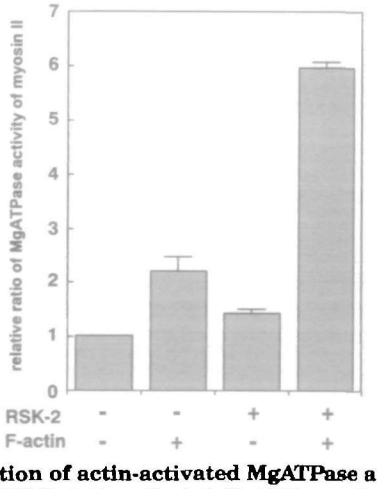

Fig. 3. **Activation of actin-activated MgATPase activity of myosin II by RSK-2.** Actin-activated MgATPase activity of unphosphorylated or phosphorylated myosin II by RSK-2 was assayed for MgATPase activity in the presence or absence of F-actin as described under "MATERIALS AND METHODS." The values shown are means the standard deviation of triplicates.

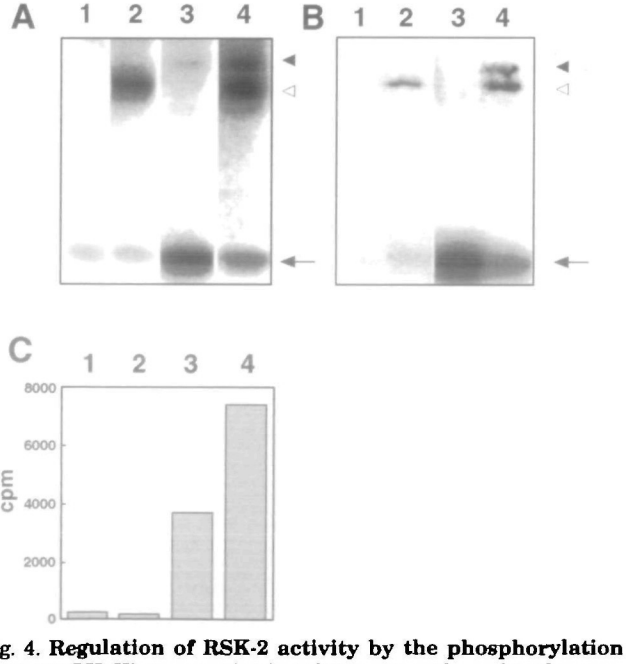

Fig. 4. **Regulation of RSK-2 activity by the phosphorylation with MAPK.** Kinase reaction by RSK-2 was performed in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of MAPK or in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of RSK-2 as described under "MATERIALS AND METHODS." As a substrate of RSK-2, intact myosin II (panels A and B) and S6 peptide (panel C) were used, respectively. Partially purified RSK-2 (panels A and C) and immunoprecipitated RSK-2 (panel B) were used for kinase assays, respectively. Filled arrowheads in lanes 3 and 4 of panels A and B are probably autophosphorylated or MAPK-phosphorylated RSK-2. Open arrowheads in lanes 2 and 4 of panels A and B indicate autophosphorylated GST-p42 MAPK. Arrows indicate MRLC phosphorylated by RSK-2.

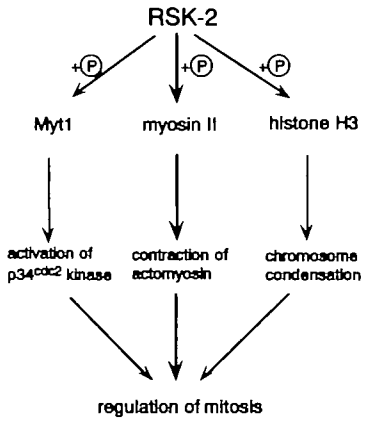

Fig. 5. **Model of possible signal transduction in the regulation of mitosis.** Myt1, $p34^{cdc2}$-inhibitory kinase.

these regulations seems to be dependent on the RSK-2 substrate, MAPK may render RSK-2 able to distinguish and select its own optimal substrate. Therefore, it is possible that the activation of RSK-2 activity to phosphorylate S6 protein and myosin II might respectively be concomitant with and not along with MAPK activation during the cell cycle. We have speculated that RSK-2 participates in the regulation of mitosis by phosphorylation of the downstream molecules including Myt1, histone H3, and myosin II, as the phosphorylation of these molecules is apparently observed during mitosis and seems to be required for activation of $p34^{cdc2}$ kinase, initiation of chromosome condensation, and contraction of contractile ring (Fig. 5). We are now investigating the localization of RSK-2 during the cell cycle in non-muscle cells. In future, it will be necessary to investigate how the phosphorylation of MRLC by RSK-2 is regulated through the phosphorylation with MAPK and participates in the cell function in response to MAPK signal transduction.

We thank Dr. Y. Yamamoto (Faculty of Applied Biological Science, University of Hiroshima) for providing chicken gizzard, Dr. K. Kohama (Department of Pharmacology, Gunma University School of Medicine) for skilled technical advice in preparing myosin II from chicken gizzard, A. Kikuchi for preparing MLCK from chicken gizzard, and colleagues in our laboratory for their advice.

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