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Myosin II Regulatory Light Chain as a Novel Substrate for AIM-1, an Aurora/Ipl1p-related Kinase from Rat[1]

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Previous studies demonstrated that the phosphorylated myosin II regulatory light chain (MRLC) is localized at the cleavage furrow of dividing cells, suggesting that phosphorylation of MRLC plays an important role in cytokinesis. However, it remains unclear which kinase(s) phosphorylate MRLC during cytokinesis. AIM-1, an Aurora/Ipl1p-related kinase from rat, is known as a serine/threonine kinase that is required for cytokinesis. Here we examined the possibility that AIM-1 is a candidate for a kinase that phosphorylates MRLC during cytokinesis. As a result, we showed that AIM-1 monophosphorylated MRLC at Ser19 using two-dimensional phosphopeptide mapping analysis and several MRLC mutants. Furthermore, AIM-1 was colocalized with monophosphorylated MRLC at the cleavage furrow of dividing cells. We propose here that AIM-1 may participate in monophosphorylation of MRLC during cytokinesis.

Key words: AIM-1, cytokinesis, myosin II regulatory light chain (MRLC), myosin light chain kinase (MLCK), phosphorylation.

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Phosphorylation of the regulatory light chain of myosin II (MRLC) is thought to be important in generating force in nonmuscle cells as well as smooth muscle cells. It is well known that phosphorylation of MRLC at Ser19 and Thr18 results in enhancement of the actin-activated Mg-ATPase activity of myosin II and promotion of assembly of myosin II filaments *in vitro* (*1*). It is generally believed that phosphorylation of MRLC at Ser19 is enough to activate the ATPase activity of myosin II. We and other groups have demonstrated that MRLC phosphorylated at Ser19 is localized to the cleavage furrow of dividing cells, suggesting that phosphorylated MRLC plays a crucial role during cell division (*2*, *3*). Even though several kinases found in nonmuscle cells including MLCK (*4*), Ca^{2+}/calmodulin dependent protein kinase II (*5*), Rho-kinase (*6*), RSK-2 (*7*), MAPKAPK-2 (*8*), MAPKAPK-4 (*9*), and PAK (*10*) have been reported to phosphorylate MRLC at Ser19 *in vitro*, it has been unknown which kinase(s) phosphorylate MRLC during cell division.

AIM-1, identified in rat (*11*) and human (*12*) cells, belongs to the Aurora/Ipl1p-related protein kinase family including *Drosophila* aurora and *S. serevisiae* Ipl1, both of which have been shown to play an important role in mitotic events (see reviews *13* and *14*). AIM-1 is expressed in the G2/M phase of the cell cycle, and localized at the midzone of the cell during the late anaphase and the midbody during the telophase and cytokinesis (*11*). It is believed that AIM-1 plays a key role in the onset of cytokinesis, although its endogenous substrate remains unclear (*15–17*).

In this paper, we demonstrate that AIM-1 monophosphorylates MRLC at Ser19 using both two-dimensional phosphopeptide mapping of phosphorylated MRLC and unphosphorylatable mutants of MRLC. We also show that AIM-1 is colocalized with monophosphorylated MRLC at the cleavage furrow of dividing cells. These data suggest that AIM-1 may monophosphorylate MRLC during cytokinesis.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Myosin II and smooth muscle myosin light chain kinase (MLCK) were purified from chicken gizzard (*18*). Light chains from myosin II (MLCs) were also obtained as described (*18*). Calmodulin from bovine brain and anti–FLAG M5 antibodies were purchased from Sigma. Mouse monoclonal antibodies were generated from rat AIM-1 (124 aa from N-terminal). The latter antibodies cross-reacted to human, rat, and mouse AIM-1. The second antibodies, Alexa Flour 568 anti–rabbit IgG and FITC-conjugated anti–mouse Ig's, were purchased from Molecular Probes and Tago, respectively.

Cell Culture—COS-7 and HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba) and grown in MEM (Gibco BRL or Nissui Pharmaceuticals) supplemented with 10% fetal calf serum in 10 cm culture dishes, respectively. For indirect immunofluorescence staining, HeLa cells were plated at a density of 10^4 cells per 3.5 cm dish.

Transfection, Preparation of a Cell Lysate and Immunoprecipitation—COS-7 cells were transfected with FLAG-AIM-1 (*11*) by application of Lipofectamine (Gibco BRL)-DNA complex. After 5 h incubation, MEM containing 10%

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FBS was added and the cells were cultured for an additional 24 h. Then the cells were treated for 18 h with 0.4 μg/ml nocodazole. After the cells had been washed with PBS, they were lysed in TBSN buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 10 μg/ml pepstatin A. The cell lysate was frozen with liquid nitrogen, thawed at 37°C and then centrifuged at 10,000 ×*g* for 20 min at 4°C. The supernatant was used for immunoprecipitation. Ten microliters of Protein G–Sepharose beads was added to 100 μl of the lysate for pre-cleaning, followed by incubation for 30 min at 4°C. After centrifugation, the cell lysate was incubated with 2 μl of mouse anti–FLAG M5 monoclonal antibodies (4.9 mg/ml) for 30 min at 4°C. Fresh protein G–Sepharose beads were added to the mixture, followed by incubation for an additional 30 min at 4°C. The mixture was then centrifuged at 10,000 ×*g* for 1 min at 4°C and the pellet was washed three times with lysis buffer. The protein immune complexes were dissolved in a buffer containing 50 mM Tris-HCl , pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 2 mM EGTA, and 0.5 mM Na_3VO_4, and then subjected to SDS-PAGE for the protein kinase assay.

Protein Kinase Assay—Plasmid construction and expression of recombinant MRLCs (termed MRLC2S19A and MRLC2S19AT18A) were carried out as described previously (*7*). Phosphorylation of isolated MLCs, MLCs of intact myosin II or recombinant MRLCs with immunoprecipitated AIM-1 was carried out in a buffer containing 40 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1.7 mM EGTA, 0.4 mM Na_3VO_4, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 8 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 0.15 mM PMSF, and 0.18 mM [γ-^{32}P]ATP with 0.17 mg/ml MLCs and immunoprecipitated AIM-1 for 30 min at 25°C. Then, the reaction mixtures were subjected to SDS-PAGE.

Two-Dimensional Phosphopeptide Mapping Analysis—Phosphorylation of isolated MLCs with immunoprecipitated AIM-1 was carried out in the same reaction mixture as described above containing 0.17 mg/ml MLCs and immunoprecipitated AIM-1 for 30 min at 25°C. Phosphorylation of MLCs by MLCK was performed in a buffer (30 mM Tris-HCl, pH 7.5, 35 mM NaCl, 1.3 mM EGTA, 0.25 mM Na_3VO_4, 0.45 μg/ml pepstatin A, 0.45 μg/ml leupeptin, 5.6 mM $MgCl_2$, 2.6 mM DTT, 0.2 mM PMSF, 2 mM $CaCl_2$, and 0.05 mg/ml calmodulin) containing 0.17 mg/ml MLCs and 1 μg/ml MLCK for 30 min at 25°C, followed by SDS-PAGE and then excision from the gel. The MRLC gel slices were washed three times with 25% (v/v) isopropanol and then three times with 10% (v/v) methanol, and finally lyophilized. To digest proteins, the gel slices were incubated in 50 mM NH_4HCO_3 and 0.1 mg/ml trypsin for 20 h at 37°C. Further trypsin was added up to 0.2 mg/ml at 10 h during the digesion. Tryptic phosphopeptides were lyophilized and dissolved in 10–20 μl of the electrophoresis solution (acetic acid : formic acid : H_2O = 150 : 50 : 800), and then spotted onto a cellulose plate [TLC precoated plates (glass) cellulose; Merck]. Electrophoresis was performed at 1 kV for 150 min in the electrophoresis solution. After drying each cellulose plate, ascending chromatography in the second dimension was performed in 1-butanol : pyridine : acetic acid : H_2O (195 : 150 : 30 : 120). The plate was dried and phosphopeptides were detected by autoradiography.

Phosphoamino Acid Analysis—Phosphopeptide mapping was carried out as described above. Each phosphopeptide was excised from the cellulose plate and then dissolved in the electrophoresis solution as described above. The phosphopeptides were lyophilized and then dissolved in 6 N HCl. Acid hydrolysis was performed for 120 min at 110°C. After removing HCl by lyophilization, the hydrolysates were dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine. The samples were processed for electrophoresis on cellulose plates at 1 V for 70 min in acetic acid : pyridine: H_2O (50 : 5 : 945). The markers were stained with ninhydrin and then the phosphoamino acids were detected by autoradiography.

Indirect Immunofluorescence—Cells grown on coverslips were fixed for 15 min in 3.7% formaldehyde in PBS at room temperature. After washing with PBS, the fixed cells were permeabilized by incubation in PBS containing 0.2% Triton X-100 for 15 min, and then washed with PBS. After blocking with 1% BSA dissolved in PBS, antibodies were applied directly to the coverslips. The coverslips were incubated for 1 h in a moist chamber and then washed for 15 min with several changes of PBS. The cells were then stained with second antibodies in a manner identical to that for the first staining. After washing with PBS, the coverslips were mounted on a microscope slide with a drop of Perma Flour Aqueous Mounting Medium for preserving fluorescence (Immunon), containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Nacalai Tesque, Kyoto). Imaging was performed under a Nikon ECLIPSE TE300 microscope. All images were acquired with a digital CCD camera (ORCA, C4742-95-12; Hamamatsu Photonics) and processed with custom software.

Other Procedures—SDS-PAGE was carried out as described previously (*18*). Affinity purification of antibody P1 was described elsewhere (*2*).

RESULTS AND DISCUSSION

As AIM-1 was expressed at the G2/M phase of the cell cycle (*11*), we transfected FLAG-AIM-1 into COS-7 cells and then treated the cells with nocodazole for harvesting in the M phase. The cell lysate from these cells was used for immunoprecipitation of FLAG-AIM-1. To examine the possibility that AIM-1 phosphorylates MRLC, immunoprecipitated FLAG-AIM-1 was added to the reaction mixture containing the isolated MRLC or intact myosin II, followed by incubation. As shown in Fig. 1, a radiolabeled band corresponding to a molecular mass of 21 kDa appeared for the reaction mixture containing MLCs (Fig. 1, left panel) and intact myosin II (Fig. 1, right panel), respectively. This indicates that FLAG-AIM-1 phosphorylated both isolated MRLC and MRLC of intact myosin II.

Next, we determined the phosphorylation site(s) of MRLC for AIM-1 (Fig. 2). Two-dimensional phosphopeptide mapping revealed that the mapping pattern of MRLC phosphorylated by FLAG-AIM-1 coincided with that in the case of MLCK, which phosphorylates MRLC at Ser19 (Fig. 2A). To determine the phosphorylated amino acids, we performed phosphoamino acid analysis. It was shown that the phosphorylated amino acid was serine (Fig. 2B). This suggested that AIM-1 phosphorylates MRLC at Ser19. To further confirm this, FLAG-AIM-1 was incubated with glutathione-S-transferase (GST)–fused HeLa MRLC2 and its

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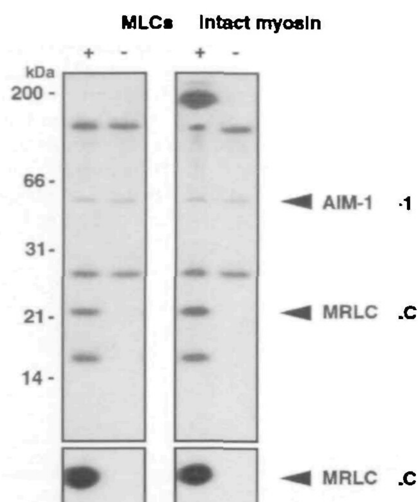

Fig. 1. **Phosphorylation of isolated MLCs and MLCs of intact myosin II by AIM-1.** FLAG-AIM-1 was immunoprecipitated from COS-7 cells with anti-FLAG M5 antibodies. In the left panel, immunoprecipitated FLAG-AIM-1 was incubated with (+) or without (−) isolated MLCs as indicated. In the right panel, immunoprecipitated FLAG-AIM-1 was also incubated with (+) or without (−) MLCs of intact myosin II as indicated. Samples were analyzed by SDS-PAGE with Coomassie Brilliant Blue (CBB) G-250 staining (upper panel), and by autoradiography (lower panel). The positions of molecular mass markers, in kDa, are indicated.

mutants (*7*) in the presence of radiolabelled ATP (Fig. 2C). Ser19 of GST-MRLC2S19A was mutated to Ala, and both Ser19 and Thr18 of GST-MRLC2S19AT18A were mutated to Ala. As shown in Fig. 2C (lane 2), no band was detected when GST-MRLC2S19AT18A was incubated with AIM-1. On the contrary, a highly radiolabelled band appeared when wild type GST-MRLC2 was incubated with AIM-1 (Fig. 2C, lane 6). When GST-MRLC2S19A was incubated with AIM-1, a faintly radiolabelled band was observed (Fig. 2C, lane 4). A previous study revealed that the MRLC-2S19A mutant incorporates a phosphate with a high concentration of MLCK (*19*). We also observed that the MRLC2S19A mutant was faintly phosphorylated by MLCK (data not shown), although it is well known that MLCK phosphorylates MRLC predominantly at Ser19 not Thr18 (*20*). Thus, we suppose that AIM-1 faintly phosphorylates Thr18 of MRLC as well as MLCK does. Overall, we concluded that AIM-1 precisely monophosphorylated MRLC at Ser19.

Recently, we and other groups showed that MRLC phosphorylated at Ser19 was localized at the cleavage furrow of dividing mammalian cells, suggesting that phosphorylation of MRLC plays an important role during cytokinesis (*2*, *3*). AIM-1 is also localized at the midbody during cytokinesis (*11*). Thus, we propose that AIM-1 participates in phosphorylation of MRLC during cytokinesis. In order to clarify this, we conducted immunolocalization of monophosphorylated MRLC and AIM-1 during cytokinesis by double staining (Fig. 3). HeLa cells were doubly stained with P1, which specifically recognizes MRLC phosphorylated at Ser19 (*2*), and a monoclonal antibody against AIM-1. Monophosphorylated MRLC was found to be localized at the cleavage furrow of dividing HeLa cells (Fig. 3A). AIM-1 was also localized at the cleavage furrow of dividing cells as the

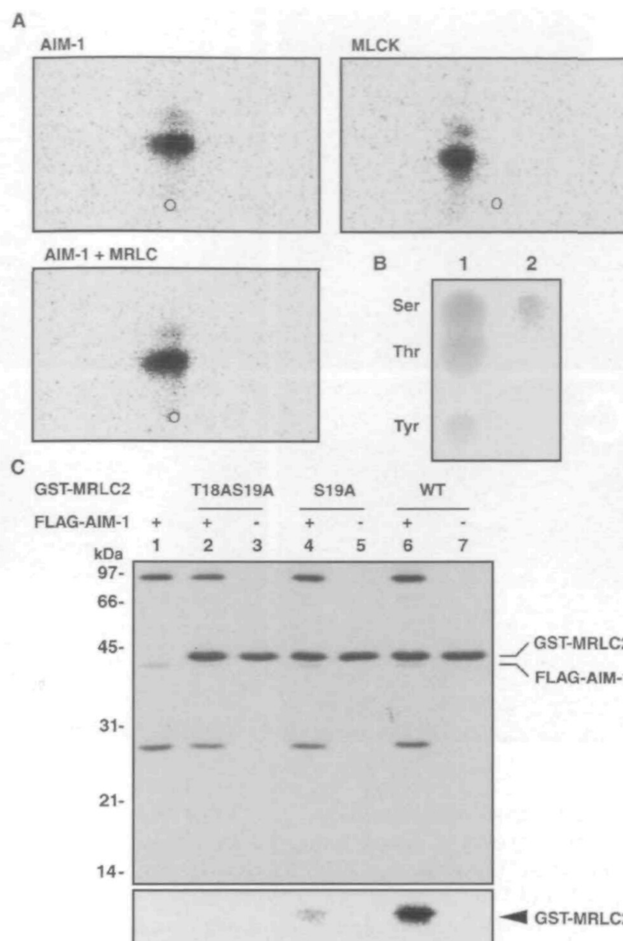

Fig. 2. **Identification of phosphorylation sites of MRLC for AIM-1.** A: Two-dimensional tryptic peptide maps of MRLC phosphorylated with AIM-1 and MLCK, respectively. MRLC phosphorylated by AIM-1 or MLCK was digested with trypsin and then processed on a cellulose plate for electrophoresis (horizontal dimension) followed by chromatography (vertical dimension). Open circles indicate the origin. B: Phosphoamino acid analysis of MRLC phosphorylated by AIM-1. Phosphoamino acid markers were detected with ninhydrin (lane 1). Phosphorylation of MRLC by AIM-1 was detected with autoradiography (lane 2). Ser, phosphoserine; Thr, phosphothreonine; Tyr, phosphotyrosine. C: Phosphorylation of recombinant GST fused MRLC by AIM-1. GST-MRLC2T18AS19A (lanes 2 and 3), GST-MRLC2S19A (lanes 4 and 5), or GST-WTMRLC2 (lanes 6 and 7) was incubated with (lanes 2, 4, and 6) or without (lanes 3, 5, and 7) AIM-1 in the presence of radiolabelled ATP. AIM-1 was also incubated without any substrates (lane 1). After incubation, samples were analyzed by SDS-PAGE with CBB staining (upper panel), and by autoradiography (lower panel).

monophosphroylated MRLC was (Fig. 3B). A superimposed image of panels A and B revealed the colocalization of monophosphorylated MRLC and AIM-1 at the cleavage furrow (yellow, indicated by an arrowhead in Fig. 3C). This suggests that AIM-1 participates in monophosphorylation of MRLC during cytokinesis. Phosphorylation of MRLC at Ser19 by MLCK and other kinases is known to be responsible for the increase in the actin-activated Mg-ATPase activity of myosin II (*6*–*8*). Thus, AIM-1 may play an important role in the activation of myosin II activity during cytokinesis.

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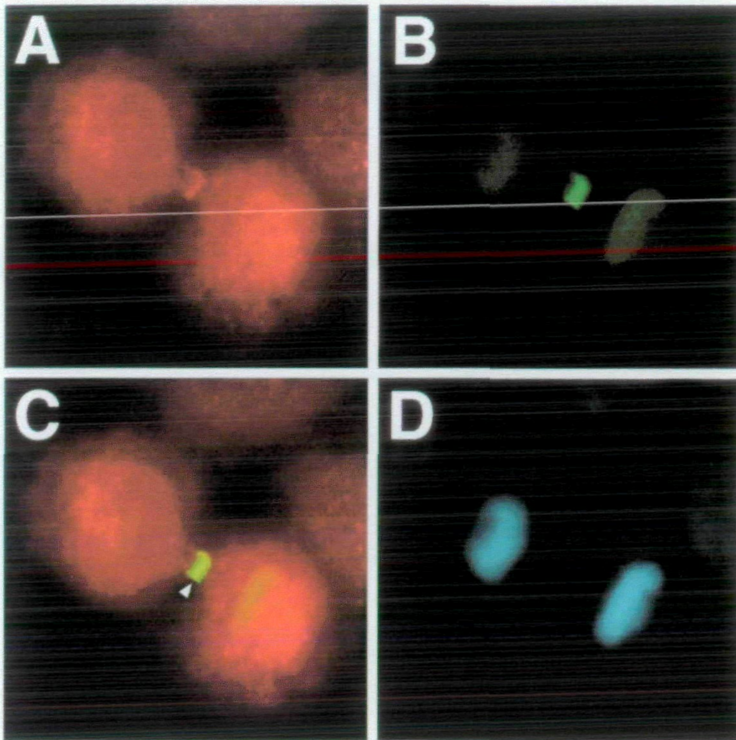

Fig. 3. **Localization of monophosphorylated MRLC and AIM-1 in a dividing HeLa cell.** Monophosphorylated MRLC was colocalized with AIM-1 at the cleavage furrow of the dividing cell. HeLa cells were triply stained with P1 (red in panel A), AIM-1 monoclonal antibodies (green in panel B), and DAPI (blue in panel D). C is a superimposed image of A and B. Monophosphorylated MRLC localized in the cleavage furrow was stained by AIM-1 monoclonal antibodies in the dividing cell (yellow, shown by an arrowhead in panel C).

MRLC monophosphorylated at Ser19 was colocalized with the myosin II heavy chain in the cleavage furrow of dividing cultured cells, suggesting that the phosphorylated MRLC could be incorporated into the heavy chain during cytokinesis (*3*). Several reports have suggested that MRLC might have another regulation or function independently of the myosin II heavy chain. Because microinjection of MRLC into living cells resulted in incorporation of the injected MRLC into the endogenous heavy chain of myosin II, the heavy and light chains of myosin II might be able to exist in an independent manner in the cell (*21*). Olsson *et al.* reported that an ERM family protein, termed MIR, interacts with MRLC (*22*). It is known that ERM family proteins are highly concentrated at the cleavage furrow during cytokinesis (*23*). Thus, some of the phosphorylated MRLC might participate in cytokinesis through its association with ERM family proteins but not the myosin II heavy chain.

In conclusion, we showed here that AIM-1 monophosphorylates MRLC at Ser19 and is colocalized with monophosphorylated MRLC at the cleavage furrow of dividing cells. This is the first report of the identification of a substrate for AIM-1.

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