

Diphosphorylated MRLC is Required for Organization of Stress Fibers in Interphase Cells and the Contractile Ring in Dividing Cells

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ABSTRACT. Activity of nonmuscle myosin II is regulated by phosphorylation of its regulatory light chain (MRLC). Phosphorylation of MRLC at both Thr18 and Ser19 (diphosphorylation) results in higher MgATPase activity and in promotion of the assembly of myosin II filaments than does that of MRLC at Ser19 (monophosphorylation) *in vitro*. To determine the roles of the diphosphorylated MRLC *in vivo*, we transfected three kinds of MRLC mutants, unphosphorylated, monophosphorylated and diphosphorylated forms (MRLC2^{T18AS19A}, substitution of both Ser19 and Thr18 by Ala; MRLC2^{T18AS19D}, Ser19 by Asp and Thr18 by Ala; and MRLC2^{T18DS19D}, both Ser19 and Thr18 by Asp, respectively), into HeLa cells. Cells overexpressing the mutant MRLC2^{T18DS19D} contained a larger number of actin filament bundles than did those overexpressing the mutant MRLC2^{T18AS19D}. Moreover, cells overexpressing the nonphosphorylatable mutant MRLC2^{T18AS19A} showed a decrease in the number of actin filament bundles. Taken together, our data suggest that diphosphorylation of MRLC plays an important role in regulating actin filament assembly and reorganization in nonmuscle cells.

Key words: phosphorylation/myosin II filament/myosin II regulatory light chain/cytokinesis

Myosin II is an essential protein to generate force for cell locomotion (Berlot *et al.*, 1985, 1987) and cytokinesis (Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987). In smooth muscle and vertebrate nonmuscle cells, myosin II activity is both negatively and positively regulated by phosphorylation of its regulatory light chain (MRLC). The phosphorylation sites of MRLC by Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK) are mainly Ser19 (monophosphorylation) and, under some conditions, both Ser19 and Thr18 (diphosphorylation) *in vitro* (Ikebe and Hartshorne, 1985; Ikebe *et al.*, 1986). Phosphorylation of MRLC by MLCK enhances the actin activated Mg ATPase activity (Sellers *et al.*, 1981; Trybus, 1989) and promotes the assembly of myosin II filaments (Suzuki *et al.*, 1978). These effects are more effective when MRLC is diphosphorylated (Ikebe *et al.*, 1988). On the other hand, protein kinase C (PKC) phosphorylates MRLC at Ser1/2

and Thr9. Phosphorylation of MRLC by PKC down-regulate the MgATPase activity of myosin II phosphorylated by MLCK (Nishikawa *et al.*, 1984; Ikebe *et al.*, 1987).

It has been reported that substitution of amino acid residues on phosphorylation sites of MRLC affects the biochemical properties of myosin II *in vitro*. Substitution of both Ser19 and Thr18 to Asp, which mimics the diphosphorylated MRLC, exerted the actin activated MgATPase activity of myosin II and formation of myosin II filaments (Kamisoyma *et al.*, 1994; Sweeney *et al.*, 1994).

In this study, we attempted to determine the roles of diphosphorylated MRLC *in vivo*. To address this, we transfected three kinds of MRLC mutants, which mimic non-phosphorylated, monophosphorylated and diphosphorylated MRLC, into HeLa cells and compared their localization in both interphase and mitotic cells.

Materials and Methods

Materials

MLCK was purified from chicken gizzard (Adelstein and Klee, 1981). Calmodulin from bovine brain, calyculin A, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA) and coumarin phenylisothiocyanate (CPITC)-conjugated phalloidin were

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Abbreviations: MRLC, myosin II regulatory light chain; MLCK, myosin light chain kinase; REF, rat embryo fibroblast; CPITC, coumarin phenylisothiocyanate.

purchased from Sigma Chemical (St. Louis, MO, USA), leupeptin and pepstatin A from the Peptide Institute (Osaka, Japan) and the mouse anti-myc monoclonal antibody from Invitrogen (Groningen, The Netherlands). The rabbit anti-myosin II heavy chain polyclonal antibody (Fujiwara and Pollard, 1976) was kindly provided by Drs K. Fujiwara (University of Rochester, NY, USA) and K. Katoh (National Cardiovascular Center Research Institute, Tokyo, Japan). Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Fc) was purchased from TAGO Inc. (Burlingame, CA, USA). Rhodamine-conjugated phalloidin, Alexa Fluor 488 conjugated goat anti-mouse IgG and Alexa Fluor 568 conjugated goat anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA). HeLa cells (RCB0007; just like ATCC CCL2, HeLa) were obtained from the Riken Cell Bank (Tsukuba, Japan) and cultured as described previously (Murata-Hori *et al.*, 2000). Rat embryo fibroblast (REF) cells (RCB0165) were also obtained from the Riken Cell Bank, and cultured in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum in 10 cm plastic culture dishes (Sumitomo Bakelite, Tokyo, Japan).

Screening of HeLa MRLC cDNAs

Three kinds of MRLC cDNAs (termed as HeLa MRLC1, HeLa MRLC2 and HeLa MRLC3, respectively) were amplified from the HeLa cell cDNA library (Murata-Hori *et al.*, 1999) using a primer set encoding a partial sequence of chicken MRLC (MFLTMF-GEK; amino acid 84–92), and then subcloned into pBluescript SK(-). Screening of HeLa cDNA library using the obtained cDNA fragments was performed as described previously (Murata-Hori *et al.*, 1999). Sequencing of the cloned cDNA was done with a DNA sequencer (ALF Express II; Amersham Pharmacia Biotech, Uppsala, Sweden). Sequences were analyzed with the software DNASIS (Hitachi Software Engineering, Kanagawa, Japan).

Construction of recombinant MRLC expressing vectors

Three types of mutant HeLa MRLC2 (termed as MRLC2^{T18AS19A}, MRLC2^{T18AS19D} and MRLC2^{T18DS19D}, respectively) and wild type MRLC2 were generated by site-directed mutagenesis using PCR. In the MRLC2^{T18AS19A} mutant, both Ser19 and Thr18 were substituted by Ala. In the MRLC2^{T18AS19D} mutant, Ser19 was substituted by Asp and Thr18 by Ala, respectively. In the MRLC2^{T18DS19D} mutant, both Ser19 and Thr18 were substituted by Asp. PCR products were subcloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced. Wild type and three mutant MRLC2s were inserted into the frame with the C-terminal myc peptide of pcDNA3.1/Myc-HisA vector (Invitrogen).

Expression of recombinant MRLC2 in HeLa cells

HeLa cells were plated at a density of 10^4 cells per 3.5 cm dish for immunoblotting and immunofluorescence, and at a density of 10^5 cells per 10 cm dish for immunoprecipitation. The cells were incubated with the LipofectAMINE (Gibco BRL, Rockville, MD, USA)-DNA complex at 37°C for 5 h in OPTI-MEM I Reduced Se-

rum Medium (Gibco BRL). After removing the medium, Minimum Essential Medium (Nissui Pharmaceutical) containing 10% fetal bovine serum (Intergen, NY, USA) was added and the cells were cultured for an additional 24 h at 37°C.

Immunoprecipitation and immunoblot analysis

The transfected cells were washed with PBS containing 0.2 mM EGTA, 5 mM MgCl₂ and 1 mM PMSF, and then lysed with ice cold RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitors (10 μ M leupeptin, 1 μ M pepstatin A and 1 mM PMSF). The cell lysates were preincubated with protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. After centrifugation, the cell lysates were incubated with 3 μ g of anti-myc antibody or mouse IgG for 1 h at 4°C. Protein G-Sepharose beads were added into the cell lysates and incubated for 1 h at 4°C. The beads were washed three times with ice cold RIPA buffer and then washed with PBS. Immunoblotting was performed according to Murata-Hori *et al.* (1998).

Protein kinase assay

Phosphorylation of immunoprecipitants by MLCK was carried out in 20 μ l of the kinase mixture containing 25 mM Tris-HCl (pH 7.2), 125 mM NaCl, 8 mM MgCl₂, 1.3 mM CaCl₂, 1.2 mM EGTA, 1.7 mM DTT, 0.1 mM PMSF, 0.5 μ M calyculin A, 0.9 mM [γ -³²P] ATP (0.1 mCi/ml) and 50 μ g/ml calmodulin. MLCK was added at a final concentration of 1 or 100 μ g/ml, and incubated for 30 min or 90 min at 25°C, respectively. After incubation, the reaction was stopped by the addition of 5 μ l of 5 \times SDS sample buffer (Okubo *et al.*, 1999). The samples were separated by SDS-PAGE. Radiolabeled bands were visualized with an imaging analyzer (Bas 2000; Fuji Photo Film, Tokyo, Japan).

Indirect immunofluorescence staining

Indirect immunofluorescence was carried out by the method of Murata-Hori *et al.* (2001). Imaging was performed under a Nikon ECLIPSE TE300 microscope. All images were obtained with a digital CCD camera (ORCA, C4742-95-12; Hamamatsu Photonics, Shizuoka, Japan) and processed with a custom software.

Results and Discussion

We first isolated three isoforms of MRLC cDNAs (termed as HeLa MRLC1, HeLa MRLC2 and HeLa MRLC3, respectively) from the HeLa cell cDNA library (Fig. 1). All MRLCs were highly homologous with each other, and showed more than 90% amino acid identity to that from chicken smooth muscle (Pearson *et al.*, 1984). Moreover, the phosphorylation sites of MRLC by MLCK were well conserved among these three MRLC isoforms (Fig. 1, underlined portions). This result suggests that nonmuscle MRLC regulates myosin II activity in the same manner as that of smooth muscle myosin II (Umemoto *et al.*, 1989).

(A)

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CCCAGTTCCACGACCCAGCGAGCCCAAGCGCTTCTCCGACACAGGGAAGCCCAACCA 60
CCAGAAAGCCCAAGATGTCCAGCAAGCGGCCCCAAGCCCAAGACCAAGAAAGCGGCCACAG 120
      M S S K R A K A K T T K K R P Q 16
CGGGCCACATCCAAATGCTCTTCCGAATGTTTACAGTCCAGATCCAGGAGTTTAAGGAG 180
R A T S N V F A M F D Q S Q I Q E F K E 36
GCTTTCAACATGATTGACAGAACCGTGTATGGCTTCATTGACAAGGAGGACCTGCACGAC 240
A F N M I D Q N R D G F I D K E D L H D 56
ATGCTGGCTCGCTGGGGAAGAACCCACAGACGAATACCTGGAGGGCATGATGAGCGAG 300
M L A S L G K N P T D E Y L E G M M S E 76
GCCCGGGGCCCATCAACTTCACCATGTCTCTACCATGTTTGGGAGAAGCTGAACGGC 360
A P G P I N F T M F L T M F G E K L N G 96
ACAGACCCCGAGGATGTGATTTCGCAACGCCCTTGGCTGCTTCGACGAGGAAGCCCTCAGGT 420
T D P E D V I R N A F A C F D E E A S G 116
TTTATCCATGAGGACCACTCCGGGAGCTGCTACACCATGGTGACCGCTTCACAGAT 480
F I H E D H L R E L L T T M G D R F T D 136
GAGGAAGTGAGCAGATGTACCGGAGGACCACTTGATAAGAAAGGCAACTTCAACTAC 540
E E V D E M Y R E A P I D K K G N F N Y 156
GTGGAGTTACCGCATCTCTCAAAACATGGCGCAAGGATAAAGACGACTAGGCCACCCCA 600
V E F T R I L K H G A K D K D D * 173
GCCCTTGACACCCAGCCCGCGCAGTACCCCTCCCGCAGACACCGCTCCATACACAG 660
CTCCCTGCCCCATGACCTCTGCTCAGAGGATCCCCCTTGGAGGGTTAGGTCCTCCAGTTCCC 720
AGTGGAGAAGAACAGCGCAGGAGAAGTGCCTGCCGAGCTGAGGCAGATGTTCCACAGTG 780
ACCCGAGACCCCTGGGCTTAGTGTCTTACCCCTTCAAGGAAGACCACTTCTGGGGAG 840
ATGGGCTGGAGGCGAGGACCTAGAGGACCAAGGAGGAGGCCCATTCGGGGCTGTTCCTC 900
CGAGAGGAAGGGAAGGGGCTGTGTGTGCCCGCAGGAGGAAGGCGCTGAGTCTGGG 960
ATCAGACACCCCTTACAGTGTATCCCCACACAAATGCAAGCTACCAAGGTCCCTCTCA 1020
GTCCCTTCCCTTACACCTTGACGCGCCACTGCGCGACACCCACCCAGAGCACGCCACCG 1080
CCATGGAGTGTGCTCAGGAGTGCAGGCGAGCTGGACATCTGTGCCAGAGGGGCGAGAA 1140
TCTCAATAGAGGACTGAGCACTGAAAAAAGAAAAAAGAAAAAAGAAAAA 1189

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(B)

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CTTCGCTGTCTGTTTGGTCTCGCGAGGGCGGCCCCGGTTCTGGTGTGGCGTCGAA 60
TTAAACAACCATGTGCGAGCAAAAGGCAAGACCAAGACCAAGAAAGCGCCCTCA 120
      M S S K K A K T K T T K K R P Q 16
CGGTGCAACATCCAATGTGTTTGGCATGTTTGACGAGTCACAGATTCAGGAGTTCAAGA 180
R A T S N V F A M F D Q S Q I Q E F K E 36
GGCCTTCAACATGATTGATCAGAACAGAGATGGCTTCATCGACAAGGAGATTTCATGA 240
A F N M I D Q N R D G F I D K E D L H D 56
TATGCTTGTCTCTAGGGAAGAAATCCACTGATGCATACCTTGTATGCCATGATGAATGA 300
M L A S L G K N P T D A Y L D A M M N E 76
GGCCCCAGGGCCATCAATTTACCATGTTCTGACCATGTTTGGTGAAGATTAAATGG 360
A P G P I N F T M F L T M F G E K L N G 96
CACAGATCTGAAGATGTCTACAGAAACGCCCTTGGCTTGTGATGAAGAACACAGG 420
T D P E D V I R N A F A C F D E E A T G 116
CACCATTCAGGAAGATTACCTAAGAGAGCTGTGACAACCATGGGGGATCGGTTTACAGA 480
T I Q E D Y L R E L L T T M G D R F T D 136
TGAGGAAGTGGATGAGCTGTACAGAAACACCTTATGCAAAAAGGGAATTCAATTA 540
E E V D E L Y R E A P I D K K G N F N Y 156
CATCGAGTTACACCGCATCTTCAACATGGAGCCAAAGACAAGATGACTGAAAGAACTT 600
I E F T R I L K H G A K D K D D * 173
TAGCTAAAAATCTCCAGTTACATTGTCTTACTCTCTTTTACTTCTCAGACACTTCCCCCA 660
CCCTCATAGAACCTGTGTGATGCAACTTAGTTTTCACAGCTTGGCTCTCTCTTTTATGATG 720
ATTTATTCAGACCTTTTGGCCACTTAGCACTTGTATAATCAGACTGGAATGGGATGA 780
GGGTGTAATTTGATTGAAAAAGATCGCGAATAAAATCAACAAATGTGAAAGCCCAAAA 840
AAAAAAAAAAAAAAAAAAAAAAAAA 864

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(C)

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CAGCGAGGGGCTCGGAGAGGTGCTCGGATTCTCGTAGCTGTGCGGAGCTTAACACCAC 60
CATGTCGAGCAAAAGAACAAAGACCAAGACCGCCCTCAGCGTGCACATCCAA 120
      M S S K R T K T K T K K R P Q R A T S N 20
TGTTTGTCTATGTTTACGAGTCACAGATTGAGGAGTTCAAGAGGCTTCAACATGAT 180
V F A M F D Q S Q I Q E F K E A F N M I 40
TGTCAGAACAGAGATGGTTTCATCGACAAGGAAGATTGTCATGATGCTTGTCTTATT 240
D Q N R D G F I D K E D L H D M L A S L 60
GGGGAAGAAATCAACTGATGATATCTAGATGCCATGATGAATGAGGCTCCAGGCCCAT 300
G K N P T D E Y L D A M M N E A P G P I 80
CAATTCACCATGTTCTTCCATGATGTTTGGTGAAGTTAAATGGCAGATCTTGAAGA 360
N F T M F L T M F G E K L N G T D P E D 100
TGTCATCAGAAATGCCCTTGTCTTGTGATGAAGAACCACTGGCACCATACAGGAAGA 420
V I R N A F A C F D E E A T G T I Q E D 120
TTACTTGAAGAGAGTGTGACAAACATGGGGATCGGTTTACAGATGAGGAAGTGATGA 480
Y L R E L L T T M G D R F T D E E V D E 140
GCTGTACAGAGAACCACTTATGATAAAAGGGAATTCAATTACATGAGTTTACACAG 540
L Y R E A P I D K K G N F N Y I E F T R 160
CATCTGAACATGGAGCCAAAGACAAGATGACTGAAATACTCAAAATTCAGCCAAA 600
I L K H G A K D K D D * 172
CGTTCTCTGTGCGCACTTGGGTATCTGAGATTTTCTCTGTCATGCCCTTAGCTTTACA 660
GCTTTTGCATTTCTGTGTTATTTATCTCAGCATTGTTGGCATATGTATCTTTAAT 720
CAGACTGGAACGGGACTTTCTATTAATATCAATTTTCAAGATAAAATAGGATAATTA 780
ACCTACAGCCCTTCTCCCAATAACTGTGGCTATACAGAGTCAATAATTTTTTCAG 840
AGAAAGTTATTCGCTGATTTTTCTGAAATCAATTTAACTTTATGATAAAATAAAAA 900
AAAAAAAAAAAAAAAAAAAAAAAAA 929

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Fig. 1. Determined nucleotides and the deduced amino acid sequences of MRLCs from HeLa cell. (A), (B) and (C) correspond to HeLa MRLC1, HeLa MRLC2 and HeLa MRLC3, respectively. Underlined amino acids indicate the phosphorylation sites of MRLC by MLCK. Nucleotide sequence data of HeLa MRLC1, HeLa MRLC2 and HeLa MRLC3 will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases with the accession numbers D82057, D82058 and D82059, respectively.

In cultured mammalian cells, monophosphorylated MRLC was localized on stress fibers or cell periphery of interphase cells (Matsumura *et al.*, 1998) and along the contractile ring in dividing cells (Matsumura *et al.*, 1998; Murata-Hori *et al.*, 1998). Recently, we showed that the diphosphorylated MRLC was colocalized with actin stress fibers in interphase rat embryo fibroblast (REF) cells (Murata-Hori *et al.*, 2001). To determine the localization of the diphosphorylated MRLC in dividing cells, we doubly stained REF cells with rhodamine-phalloidin and the antibody PP1, which specifically recognizes the diphosphorylated but not the non- or monophosphorylated MRLC (Murata-Hori *et al.*, 2001). The diphosphorylated MRLC was well colocalized along the contractile ring as well as actin stress fibers in interphase cells (Fig. 2). These results indi-

cate that the diphosphorylated MRLC is colocalized with actin filaments in mitotic cells as well as interphase cells, as described previously (Murata-Hori *et al.*, 2001).

To elucidate whether the diphosphorylation of MRLC is involved in actin filament assembly in both interphase and mitotic cells, we constructed three recombinants of HeLa MRLC2 using pcDNA3.1/Myc-HisA vector as shown in Fig. 3A. The first mutant of MRLC, MRLC2^{T18AS19A} (substitution of Thr18 and Ser19 by Ala), mimics the unphosphorylated MRLC. The second one, MRLC2^{T18AS19D} (substitution of Thr18 with Ala and Ser19 with Asp), mimics the monophosphorylated form of MRLC. The third one mimics the diphosphorylated form of MRLC (MRLC2^{T18DS19D}), by substituting both Thr18 and Ser19 with Asp.

Each recombinant MRLC2 was transfected into HeLa

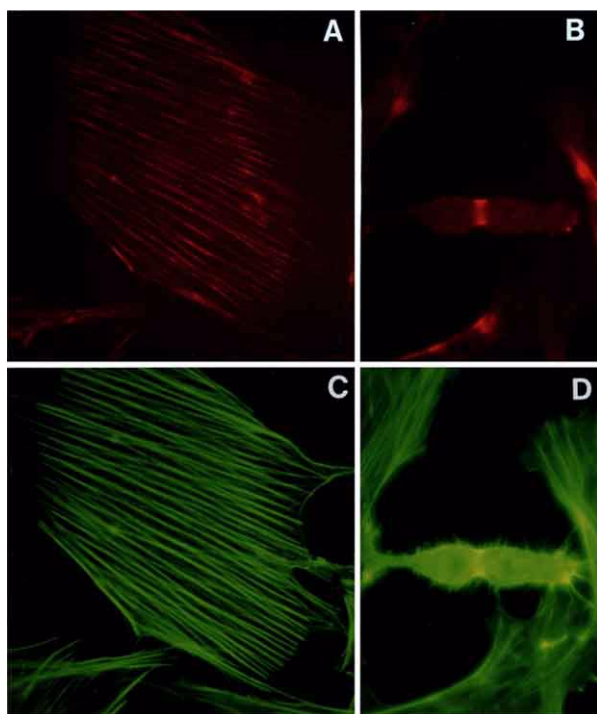


Fig. 2. Localization of endogenous diphosphorylated MRLC in REF cells. REF cells were doubly stained with the antibody against diphosphorylated MRLC (PP1) (rhodamine; A and B) and phalloidin (FITC; C and D). (A and C) Interphase cell. (B and D) Mitotic cell.

cells and subjected to immunoprecipitation with anti-myc antibody. Fig. 3B showed that the reacted band corresponding to the recombinant MRLC2 was obtained in each immunoprecipitant (lower panel; lanes 1, 3, 5 and 7). A 200-kDa myosin II heavy chain was also co-immunoprecipitated with all MRLC2 mutants by anti-myc antibody (Fig. 3B, upper panel). This result indicates that each of recombinant MRLC2 could be incorporated into myosin II molecules *in vivo*. Immunoprecipitated MRLC2 mutants were subjected to *in vitro* kinase assay (Fig. 3C). The immunoprecipitants were incubated with [γ - 32 P] ATP and 1 μ g/ml or 100 μ g/ml MLCK for monophosphorylation or diphosphorylation of MRLC, respectively. Phosphorylation of the recombinant MRLC2s was detected by autoradiography. Expressed wild type MRLC2 was phosphorylated by MLCK (Fig. 3C, WT), whereas other recombinants were not phosphorylated by any concentrations of MLCK (Fig. 3C, AA, AD and DD). These findings suggest that each recombinant MRLC2 has the ability to mimic endogenous nonphosphorylated, monophosphorylated and diphosphorylated MRLC in the cells, respectively.

When the unphosphorylated form of MRLC, MRLC2^{T18AS19A}, was transfected into HeLa cells, most of the MRLC2^{T18AS19A} became diffusely distributed in the cytoplasm (Fig. 4A, d). In most MRLC2^{T18AS19D}-expressing cells, the monophosphorylated form of MRLC colocalized with actin filaments containing myosin II heavy chain (Fig.

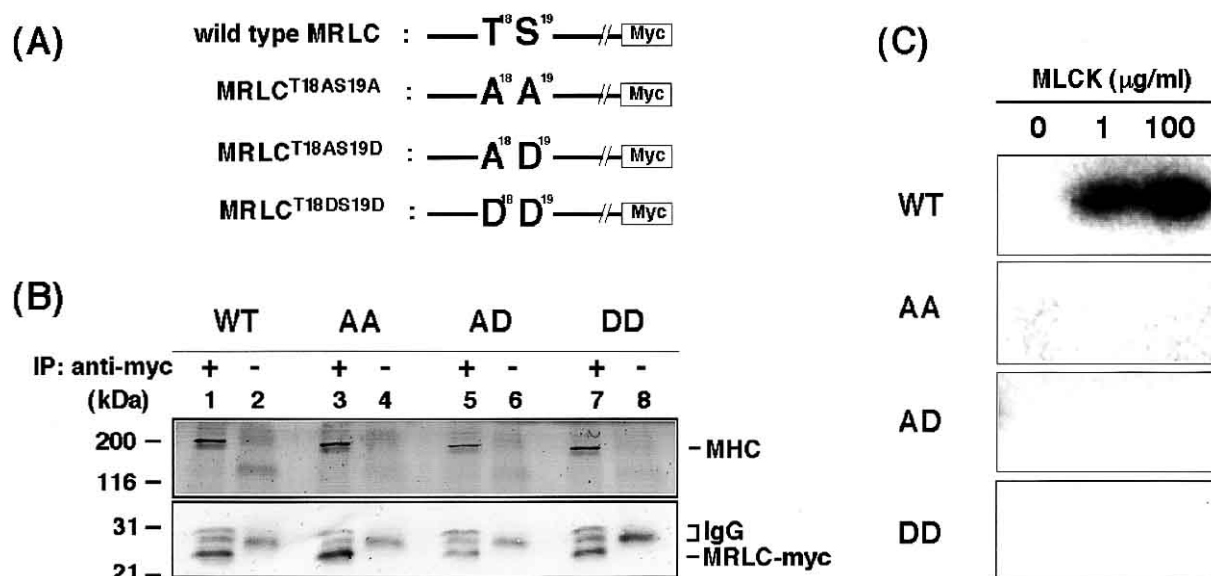


Fig. 3. Biochemical analysis of expressed recombinant MRLC2s in HeLa cells. (A) Scheme of recombinant MRLC2s, wild type MRLC2, MRLC2^{T18AS19A}, MRLC2^{T18AS19D}, and MRLC2^{T18DS19D}. Myc tag was added to the C-terminal of each recombinant MRLC2. (B) Western blot analysis of recombinant MRLC2s. The cell lysates of HeLa cells transfected with recombinant MRLC2s were immunoprecipitated by anti-myc antibody (lanes 1, 3, 5 and 7) or mouse IgG (lanes 2, 4, 6 and 8), and subjected to SDS-PAGE. Upper panel is the CBB staining. Lower panel indicates the immunoblotting for anti-myc antibody. The positions of molecular mass markers, in kDa, are indicated. (C) Phosphorylation of recombinant MRLC2s. Recombinant MRLC2s were immunoprecipitated with anti-myc antibody and incubated with absence (left) or 1 μ g/ml (center) or 100 μ g/ml (right) of MLCK. Recombinant MRLC2s were then subjected to SDS-PAGE followed by autoradiography. WT, AA, AD and DD indicate wild type MRLC2, MRLC2^{T18AS19A}, MRLC2^{T18AS19D} and MRLC2^{T18DS19D}, respectively.

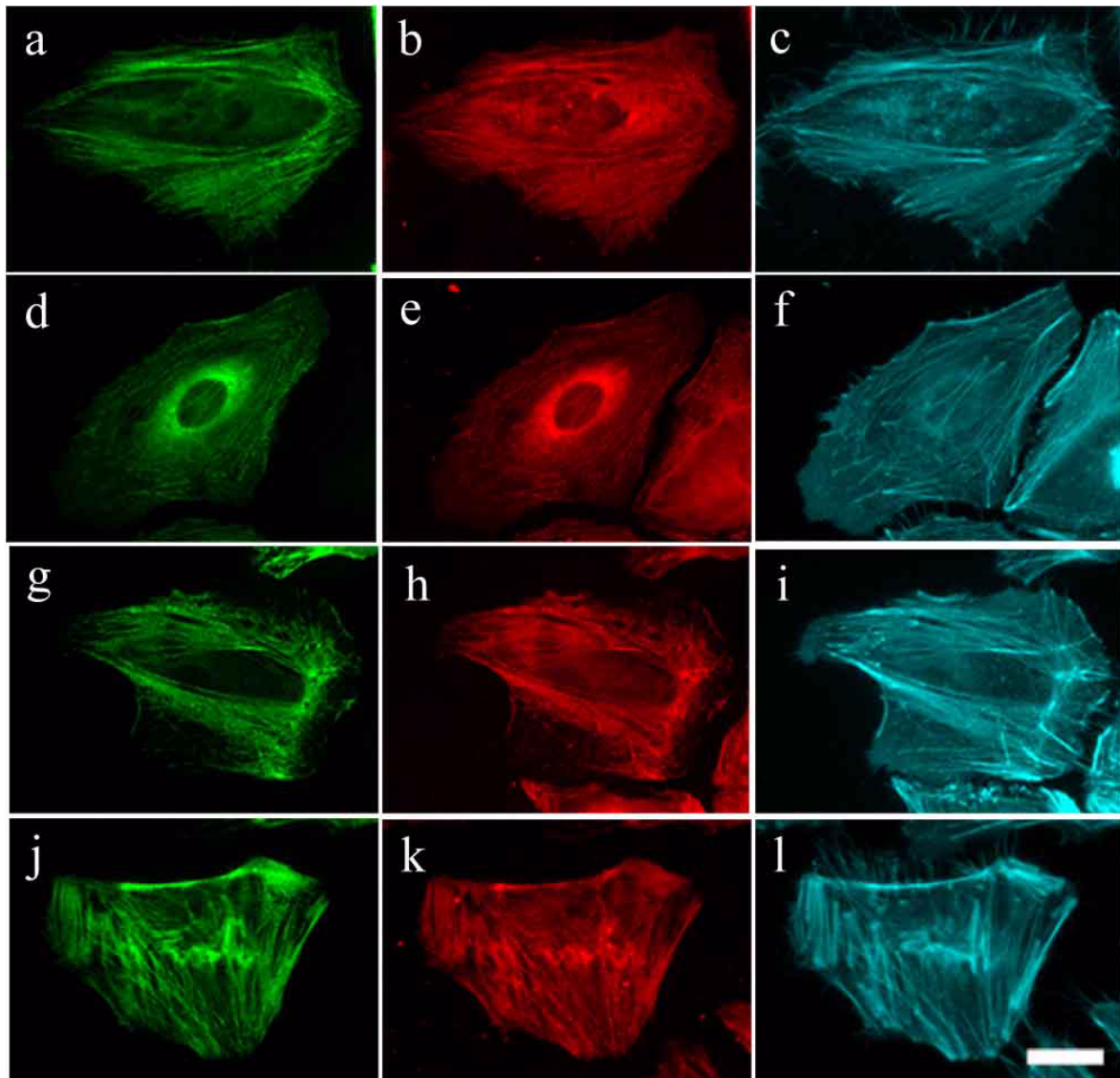


Fig. 4A. Effect of recombinant MRLC2s on organization of actin stress fibers in interphase HeLa cells. HeLa cells were transfected with wild type MRLC2 (a–c), MRLC2^{T18AS19A} (d–f), MRLC2^{T18AS19D} (g–i) and MRLC2^{T18DS19D} (j–l), respectively. After 24 h incubation, the cells were triply stained with anti-myc antibody (Alexa 488; a, d, g and j), anti-myosin II heavy chain antibody (Alexa 568; b, e, h and k) and phalloidin (CPITC; c, f, i and l). Bar, 30 μ m.

4A, g–i), as in wild type MRLC2 transfected cells (Fig. 4A, a–c). In the majority of MRLC2^{T18DS19D}-expressing cells, many thick fibers containing the diphosphorylated form of MRLC were observed throughout the cytoplasm (Fig. 4A, j). They were heavily stained with both anti-myosin II heavy chain antibody and CPITC-phalloidin in the cell (Fig. 4A, k and l), suggesting that diphosphorylation of MRLC induces the formation of thick actin bundles containing myosin II. Interestingly, in MRLC2^{T18AS19A}-expressing cells, less obvious thick fibers stained with both anti-myosin II heavy chain antibody and CPITC-phalloidin were observed throughout the cytoplasm. This suggests that the unphos-

phorylated form of MRLC dominant-negatively inhibits formation of actin bundles in the cytoplasm. Based on the localization of each mutant in the cell, we classified the transfected cells into three types (Fig. 4B). Fig. 4B revealed that MRLC2^{T18DS19D} can promote bundling of actin filaments much more effectively than does MRLC2^{T18AS19D} or MRLC2^{T18AS19A} in interphase HeLa cells, suggesting that diphosphorylated MRLC can promote actin bundle organization much more effectively than does mono- or unphosphorylated MRLC in interphase HeLa cells. Recently, we firstly reported the *in vivo* diphosphorylation of MRLC by ZIP kinase in HeLa cells (Murata-Hori *et al.*, 2001). In this

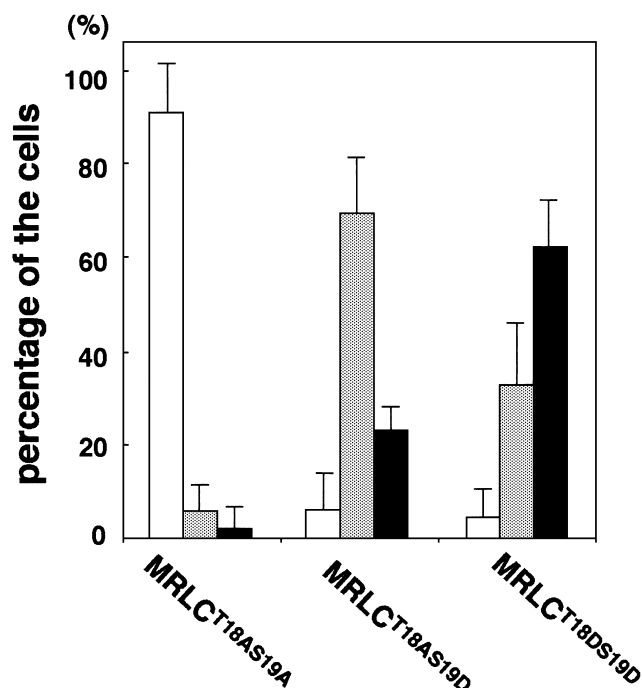


Fig. 4B. Cells transfected with each recombinant (MRLC2^{T18AS19A}, MRLC2^{T18AS19D} and MRLC2^{T18DS19D}) were classified into three distinctive patterns by the localization of MRLC as shown in d (open bars), g (gray bars) and j (closed bars) of Figure 4A. The means and standard deviation from three different coutings are indicated.

experiments, the diphosphorylated but not the monophosphorylated MRLC was localized prominently on aggregates of actin filaments induced by overexpression of ZIP kinase (Murata-Hori *et al.*, 2001). This strongly supports the results observed here.

In dividing HeLa cells, both MRLC2^{T18AS19D} and MRLC2^{T18DS19D} were also well colocalized with the actin filaments in the peripheral region (arrowheads) and the contractile ring (arrows) (Fig. 5c–f), while MRLC2^{T18AS19A} was localized diffusely throughout the cytoplasm in dividing cells (Fig. 5b) as well as in interphase cells (see Fig. 4A, d–f). It has been reported that both the monophosphorylated (Murata-Hori *et al.*, 1998; Matsumura *et al.*, 1998) and the diphosphorylated MRLC (Fig. 2) localized on the contractile ring. In this study, we could not find any obvious difference of myosin II localization on the actin bundles along the contractile ring between the cells transfected with MRLC2^{T18AS19D} (n=3) and MRLC2^{T18DS19D} (n=6). Taken together, these results indicate that phosphorylation of MRLC is essential for myosin II to localize at the contractile ring, suggesting that it is required for the contraction of the contractile ring.

In interphase cells, myosin II containing the diphosphorylated form of MRLC localized more densely along actin filaments and/or stress fibers rather than did that containing

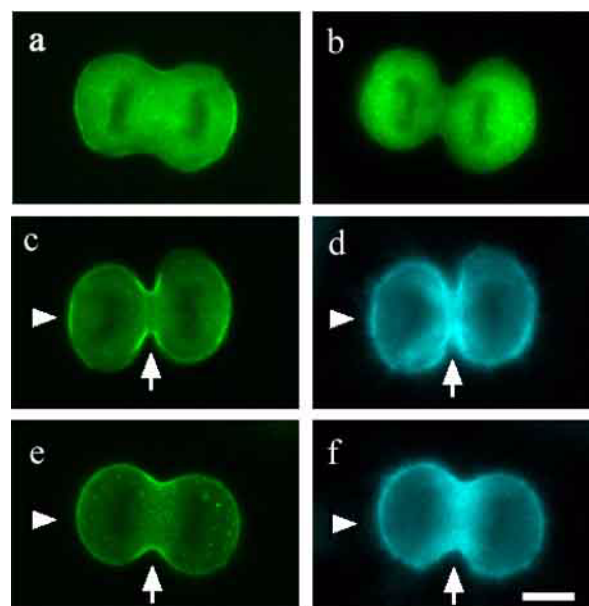


Fig. 5. Localization of recombinant MRLC2s in dividing HeLa cells. HeLa cells were transfected with wild type MRLC2 (a), MRLC2^{T18AS19A} (b), MRLC2^{T18AS19D} (c and d) and MRLC2^{T18DS19D} (e and f). The dividing cells were doubly stained with anti-myc antibody (Alexa 488; a, b, c and e) and phalloidin (CPITC; d and f). Both MRLC2^{T18AS19D} and MRLC2^{T18DS19D} were also well colocalized with the actin filaments in the peripheral region (arrowheads) and the contractile ring (arrows). Bar, 10µm.

the monophosphorylated form of MRLC. This suggests that the diphosphorylated myosin II plays more important roles, such as maintenance of stress fibers and regulation of cytoplasmic tension (Katoh *et al.*, 2001), than did the monophosphorylated myosin II.

In conclusion, the diphosphorylated form of MRLC, as well as monophosphorylated form of MRLC, is required for the organization of stress fibers in interphase cells and the contractile ring in dividing cells. Moreover, the diphosphorylated MRLC might be more effective in the organization of actin filaments than the monophosphorylated MRLC in interphase cells.

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