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Regulation of Myosin II Dynamics by Phosphorylation and Dephosphorylation of Its Light Chain in Epithelial Cells[D] [V]

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Nonmuscle myosin II, an actin-based motor protein, plays an essential role in actin cytoskeleton organization and cellular motility. Although phosphorylation of its regulatory light chain (MRLC) is known to be involved in myosin II filament assembly and motor activity in vitro, it remains unclear exactly how MRLC phosphorylation regulates myosin II dynamics in vivo. We established clones of Madin Darby canine kidney II epithelial cells expressing MRLC-enhanced green fluorescent protein or its mutants. Time-lapse imaging revealed that both phosphorylation and dephosphorylation are required for proper dynamics of myosin II. Inhibitors affecting myosin phosphorylation and MRLC mutants indicated that monophosphorylation of MRLC is required and sufficient for maintenance of stress fibers. Diphosphorylated MRLC stabilized myosin II filaments and was distributed locally in regions of stress fibers where contraction occurs, suggesting that diphosphorylation is involved in the spatial regulation of myosin II assembly and contraction. We further found that myosin phosphatase or Zipper-interacting protein kinase localizes to stress fibers depending on the activity of myosin II ATPase.

INTRODUCTION

Nonmuscle myosin II (hereafter, myosin II) is an actin-based motor protein that plays a crucial role in a variety of cellular processes, including cell migration, polarity formation, and cytokinesis (Sellers, 2000). Among tissue culture cells attached to the substratum, stress fibers containing myosin II and actin filaments typically form near the basal membrane. Despite myosin II activity being well known as important in the organization of stress fibers (Chrzanowska-Wodnicka and Burridge, 1996), exactly how myosin II filament assembly is regulated within living cells remains relatively unknown. During chemotaxis, myosin II accumulates at the rear edge of migrating cells (Yumura and Fukui, 1985). At wound closure or cytokinesis, a purse string containing actomyosin transiently assembles and disassembles at the cell cortex facing the wound or at the equator of dividing cells, respectively, by mechanisms that remain poorly understood (Martin and Parkhurst, 2004).

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Abbreviations used: 0P-, nonphosphorylated; 1P-, monophosphorylated; 2P-, diphosphorylated; MLCK, myosin light chain kinase; MRLC, myosin regulatory light chain; MYPT1, myosin phosphatase targeting subunit 1; NMHC, nonmuscle myosin heavy chain; ZIP, zipper-interacting protein.

Vertebrates have three nonmuscle myosin II heavy chains (NMHC), NMHC-IIA, -IIB, and -IIC, and these NMHCs are expressed differently in a variety of tissues (Golomb *et al*., 2004). They also differ in the kinetics of ATPase activity (Kovács *et al*., 2003; Wang *et al*., 2003) and in subcellular localizations (Maupin *et al*., 1994; Kelly *et al*., 1996; Kolega, 1998). NMHC-IIA- or -IIB-null mice or cells revealed that they have distinct roles within cells (Tullio *et al*., 1997; Conti *et al*., 2004). Defects caused by depletion of NMHC-IIB in Cos7 cells that express almost only NMHC-IIB, however, were rescued to a certain degree by introducing NMHC-IIA or -IIC, suggesting that they also share common functions (Bao *et al*., 2005).

Myosin II consists of two NMHCs, two essential light chains and two regulatory light chains (MRLCs), and myosin II activity is stimulated by phosphorylation of MRLC (Somlyo and Somlyo, 2003). Monophosphorylation of MRLC at Ser19 increases both actin-activated Mg^{2+}-ATPase activity and the stability of myosin II filaments, whereas diphosphorylation at Thr18 and Ser19 promotes both myosin II activity and the stability of myosin II filaments significantly more than by monophosphorylation (Ikebe and Hartshorne, 1985; Ikebe *et al*., 1988). In *Drosophila*, *Spaghetti squash* gene mutants encoding MRLC display defects in cytokinesis (Karess *et al*., 1991). Such phenotypes were also not rescued by expression of the A20, A21 mutant of MRLC that cannot be phosphorylated, but they were rescued by expression of the wild-type or pseudophosphorylated form (E21) of MRLC, indicating the importance of phosphorylation of MRLC in vivo (Jordan and Karess, 1997). Antibodies specific for monophosphorylated MRLC (1P-MRLC) or diphosphorylated MRLC (2P-MRLC) have been generated previously (Bennet *et al*., 1988; Matsumura *et al*., 1998; Sakurada *et al*.,

1998; Uchimura *et al*., 2002; Komatsu and Ikebe, 2004). The distribution of 1P-MRLC seems to be similar to that of total myosin II in most cases, although the difference between them has been emphasized (Matsumura *et al*., 1998; Sakurada *et al*., 1998). 2P-MRLC was, however, localized to more restricted regions compared with 1P-MRLC (Sakurada *et al*., 1998; Uchimura *et al*., 2002; Komatsu and Ikebe, 2004). The amount of 2P-MRLC was found to change in greater quantities than that of 1P-MRLC at stimulation for contraction in smooth muscle cells in culture (Sakurada *et al*., 1998).

Kinases responsible for the phosphorylation involve myosin light chain kinase (MLCK) (Kamm and Stull, 1985), ROCK/Rho-kinase (Amano *et al*., 1996), citron kinase (Yamashiro *et al*., 2003), MRCK (Leung *et al*., 1998), or Zipper-interacting protein (ZIP) kinase (Murata-Hori *et al*., 1999). Experiments using either inhibitors for each kinase, knockdown by RNA interference, or knockout mice have suggested an important role for each kinase activity in myosin II function (Totsukawa *et al*., 2000, 2004; Thumkeo *et al*., 2003; Komatsu and Ikebe, 2004; Shimizu *et al*., 2005; Yoneda *et al*., 2005). Myosin phosphatase is responsible for MRLC dephosphorylation (Hartshorne *et al*., 2004), and this phosphatase contains the myosin phosphatase targeting subunit 1 (MYPT1) that binds directly to myosin II and is required for myosin phosphatase activity (Alessi *et al*., 1992). The activity of this phosphatase is known to be regulated by MYPT1 phosphorylation through ROCK (Kimura *et al*., 1996). Mutant analysis of myosin phosphatase (Wissmann *et al*., 1999; Mizuno *et al*., 2002) or inhibition of this activity by microinjection of an antibody against MYPT1 (Totsukawa *et al*., 2000) has revealed that myosin phosphatase activity is also essential for the proper function of myosin II.

Although these experiments with mutants, inhibitors or mutant protein expression revealed that these kinases and phosphatase are important, they were unsuccessful in revealing exactly how actomyosin structures are constructed and destructed locally within cells. Many previous studies have demonstrated and measured myosin II dynamics in several species of cells by introducing fluorescently-labeled myosin II or by expression of GFP-myosin II (DeBiasio *et al*., 1988; McKenna *et al*., 1989; Verkhovsky *et al*., 1995; Kolega, 1998; Clow and McNally, 1999; Yumura, 2001; Peterson *et al*., 2004). As part of any attempt to understand the regulatory mechanism for myosin II dynamics in more detail, it would be intriguing to see the effects of MRLC phosphorylation on myosin II dynamics.

In this study, we used highly polarized Madin Darby canine kidney (MDCK) II epithelial cells because these cells exhibit a number of events in which myosin II activity is involved, such as stress fiber formation, cell–cell adhesion, cytokinesis, purse string formation at wound closure, cell polarization, and cell migration. Endogenous myosin II, 1P-MRLC, or 2P-MRLC was initially localized in these cells to obtain information about the role of MRLC phosphorylation in the formation of actomyosin structure. We then generated MDCK II clones stably expressing enhanced green fluorescent protein (EGFP)-fused MRLC or its mutants in the hope of revealing the effect of MRLC phosphorylation on myosin II dynamics within living cells. Our findings suggest that both phosphorylation and dephosphorylation are required for proper rapid dynamics of myosin II.

MATERIALS AND METHODS

Cells

Polarized epithelial cell lines, dog MDCK strains II (MDCK II), and mouse MTD-1A and mouse EpH4 were donated by M. Murata (Tokyo University, Tokyo, Japan), M. Takeichi (RIKEN, Center for Developmental Biology, Kobe, Japan), and E. Reichmann (University Children's Hospital, Zurich, Switzerland), respectively. They were cultured in DMEM with 10% fetal bovine serum (FBS). For live imaging, cells were cultured in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine, and 20 mM HEPES, pH 7.4.

Antibodies and Reagents

Commercial antibody sources included Sigma-Aldrich (St. Louis, MO) (actin, NMHC-IIA, MLCK, ROCK-I, and ZIP-kinase), BD Biosciences (San Jose, CA) (MYPT1), Cell Signaling Technology (MRLC, #3672; 1P-MRLC, #3675; and 2P-MRLC, #3674, phospho-cofilin), Upstate Cell Signaling Solutions (Lake Placid, NY) (phospho-Thr850 MYPT1), Covance (Princeton, NJ) (NMHC-IIB), and Santa Cruz Biotechnology (Santa Cruz, CA) (2P-MRLC, ROCK-II). Rabbit anti NMHC-IIC polyclonal antibody was kindly provided by R. Adelstein (National Institutes of Health, Bethesda, MD). Mouse anti-cofilin monoclonal antibody was provided by T. Obinata (Chiba University, Chiba, Japan). We localized filamentous actin (F-actin) by using Alexa Fluor 488-phalloidin (Invitrogen). Fluorescein isothiocyanate-, Cy3-, or Cy5-conjugated secondary antibodies were from Jackson ImmnoResearch Laboratories (West Grove, PA). Y27632, Calyculin A, ML-7, ML-9, latrunculin A, and (±)-blebbistatin were purchased from Calbiochem (San Diego, CA). Fibronectin was purchased from Sigma-Aldrich.

Fixation Protocols

We chose the following protocols for appropriate fixation of cells depending on antibodies used.

Formaldehyde (FA). Fixation was performed with 1 or 4% paraformaldehyde in 0.1 M HEPES buffer, pH 7.5, at room temperature or on ice for 15 min, followed by washing with PBS containing 30 mM glycine (G-PBS).

Methanol. Cells cultured on coverslips were immersed in methanol at −20°C for 10 min, followed by washing with G-PBS.

Trichloroacetic Acid (TCA). TCA (10%) in distilled water was made from 100% TCA (wt/vol) stock solution just before use (Hayashi *et al*., 1999). Cells were immersed in ice-cold 10% TCA for 15 min and then washed with G-PBS.

Fluorescence Microscopy

Fixed specimens were stained by conventional immunofluorescence microscopy (Yonemura *et al*., 2004) and observed using a Nikon Eclipse E600 microscope. Images were recorded with a cooled charge-coupled device (CCD) camera (ORCA ER, 1344 × 1024 pixels; Hamamatsu Photonics, Hamamatsu, Japan) controlled by a Power Macintosh G4 and the software package IPLab Spectrum version 3.5.4 (Scanalytics, Fairfax, VA), by using a Plan APO 60×/1.40 numerical aperture (NA) oil Ph3 lens. Specimens also were analyzed using a confocal microscope, LSM510 (Carl Zeiss, Jena, Germany) with a PlanAPO 63×/1.40 NA oil differential interference contrast lens.

For live imaging, cells were cultured on glass-based dishes (Iwaki or MatTek, Ashland, MA) for 3 d and observed under a Leica DMIRE2 microscope equipped with incubator at 37°C. Images were recorded with a CCD camera (SensiCam QE; Cooke, Auburn Hills, MI) controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA), by using an HCX PL APO 63×/1.40 NA oil Ph3 lens. For wound closure analysis, an Olympus IX81 equipped with a CCD camera (ORCA ER AG; Hamamatsu Photonics) controlled by MetaMorph software was used.

Gel Electrophoresis and Immunoblotting

Cells were treated with ice-cold 10% TCA for 10 min and then washed with phosphate-buffered saline (PBS) and harvested in SDS-sample buffer without 2-mercaptethanol. Protein concentration was determined with a BCA protein assay kit (Pierce Chemical, Rockford, IL). After adding 2-mercaptoethanol and bromphenol blue (Nacalai Tesque, Koyoto, Japan) and boiling, cell lysates containing 5 μg of proteins were resolved on polyacrylamide gel (7.5 or 15%) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline and incubated with antibody against NMHC-IIA (1:1000), NMHC-IIB (1:1000), NMHC-IIC (1:500), MRLC (1:100), 2P-MRLC (1:1000), phospho-MYPT1 (The850, 1:500), phospho-cofilin (Ser3, 1:500), 1P-MRLC (1:1000), MYPT1 (1:1000), or cofilin (1:100). Enhanced chemiluminescence (Immobilon Western; Millipore) was used to image labeled proteins.

Plasmids and Transfection

Four types of mutant human MRLC (termed AA-, AD-, DD-, or AS-MRLC) and wild type MRLC (WT-MRLC) tagged with EGFP at the C terminus had been generated previously (Uchimura *et al*., 2002). MDCK II or EpH4 cells were transfected with DNA in Ca^{2+}-free DMEM (Sigma-Aldrich) by using Lipofectamine Plus (Invitrogen). Stable transfectants were selected by treat-

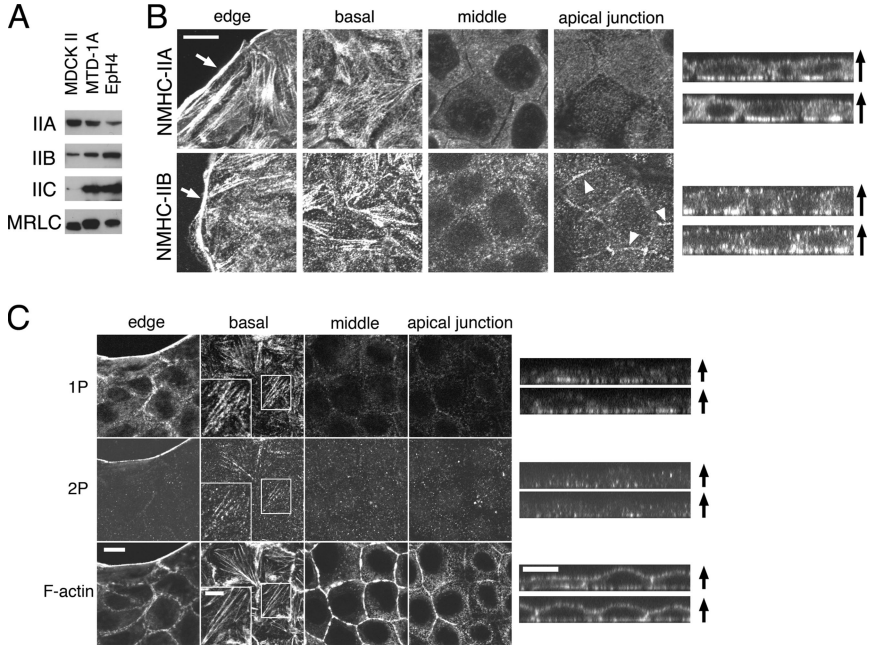

Figure 1. Localization of NMHC isoforms and phosphorylated myosin II. (A) Expression level of NMHC-IIA, -IIB or -IIC was compared among MDCK II, MTD-1A and EpH4 cells by immunoblotting. The amount of MRLC was used as a loading control. (B) Localization of NMHC-IIA and -IIB in MDCK II cells fixed with methanol and processed for confocal microscopy. At free edges of cells, both isoforms make thick bundles (white arrows). NMHC-IIB accumulates at apical junction regions (arrowheads). Black arrows indicate apical direction along apicobasal axis in vertical sections of cells constructed from serial optical sections. (C) Localizations of 1P-, 2P-myosin II, and F-actin of MDCK II cell fixed with 1% FA and processed for confocal microscopy. Arrows indicate apical direction along apicobasal axis in vertical sections of cells constructed from serial optical sections. Insets indicate magnified views of boxed areas. Bars, 10 and 5 μm (insets).

ment with 400 μg/ml G-418 for 2 wk. Resistant colonies were cloned by a limiting dilution method and then screened by fluorescence microscopy.

Fluorescence Recovery after Photobleaching (FRAP) Analysis

MDCK II cells expressing MRLC-EGFP or its mutants were plated on glass-based 35-mm culture dishes (Asahi Techno Glass, Funabashi, Japan) coated with 50 μg/ml fibronectin and cultured for 3 d until they contained well-developed stress fibers. For FRAP measurements, cells were observed using a Delta Vision microcopy system (Applied Precision, Seattle, WA) equipped with an Olympus IX71 microscope situated in a room maintained at 37°C. Time-lapse images were recorded using a cooled CCD camera (Series300 CSNAP; Photometrics, Tucson, MA). Photobleaching was achieved with a 488-nm laser beam at 20 mW for 1 s focused by a PlanApo60×/1.40 NA oil Ph3 lens by using laser optics module (Applied Precision). Data analysis was performed using Meta Imaging version 6.1 (Molecular Devices).

Wound Closure Experiments

Single or several cells in cell sheets cultured on glass-based dishes were killed using a MicroPoint laser ablation system (Photonic Instruments, St. Charles, IL) attached to an Olympus IX81 microscope using a UplanApo 40×/1.00 Oil Iris Ph3 lens as described previously (Miyake *et al.*, 2006).

RESULTS

Throughout this study, 1P-MRLC refers to phospho-Ser19 MRLC and 2P-MRLC to diphospho-Thr18/Ser19 MRLC. 1P- or 2P-myosin II refers myosin II containing 1P- or 2P-MRLC, respectively. As we describe below, we used several mutant MRLCs (e.g., AA, AD, and so on). In these mutants, Thr18 and Ser19 were converted to Ala or Asp to examine the role of phosphorylation (Figure 2A).

Localization of Endogenous Myosin II in Epithelial Cells

We used three polarized epithelial cell lines, MDCK II, MTD-1A, and EpH4. Immunoblot data subsequently showed the differences of each NMHC isoform in its expression among these cells (Figure 1A). Because each NMHC isoform seemed to bind to common light chains, the amount of myosin II was normalized with the amount of MRLC. Although we found that MTD-1A and EpH4 cells expressed all three NMHCs, MDCK II mainly expressed NMHC-IIA and -IIB, but not -IIC. In addition, MDCK II cells expressed a relatively higher amount of NMHC-IIA compared with other cells. We chose MDCK II cells for further analysis because the exact characteristics of NMHC-IIC were relatively unclear, and we found that it was easy to obtain stable clones expressing myosin II-EGFP fusion proteins from these MDCK II cells.

MDCK II cells found in small colonies have thick actomyosin bundles at their free edges near the basal membrane. These bundles contain both NMHC-IIA and -IIB (Figure 1B). Although distributions of both NMHC isoforms are roughly similar in highly polarlized confluent cells, they differ somewhat depending on their level along the apicobasal axis. NMHC-IIA accumulates highly at stress fibers just above basal membranes. At the middle level, NMHC-IIA distributes almost evenly throughout the cytoplasm. At the level of apical junctions, NMHC-IIA distributes throughout the cytoplasm with occasional weak accumulation at the junction regions. NMHC-IIB also localizes at stress fibers near the basal membrane and distributes throughout the cytoplasm. As one study has reported (Hildebrand, 2005), a considerable amount of NMHC-IIB localizes at the apical junction region under normal conditions in MDCK II cells. Vertical sections constructed from serial optical sections showed that stress fibers are major structures containing myosin II, especially NMHC-IIA. Although myosin II does not accumulate as highly to cell adhesion areas, we have already shown that myosin II transiently accumulates at the wound edges of MDCK II cells to form purse string structures that pull adjacent cells through adherens junctions and help in wound closure (Miyake *et al.*, 2006). We also examined localization of 1P- or 2P-myosin II using antibodies against 1P- or 2P-MRLC, respectively (Figure 1C and Supplemental Figure S1). We were able to find that in MDCK II cells, 1P-myosin II occasionally localizes near apical junction regions, although 2P-myosin II is hardly detected. Although both these proteins strongly accumulated at thick actomyosin bundles at the cell's free edges, their distribution was very different in other regions. 1P-myosin II localized fairly evenly along stress fibers with actin filaments and was distributed throughout the cytoplasm (Figure 1C). In contrast,

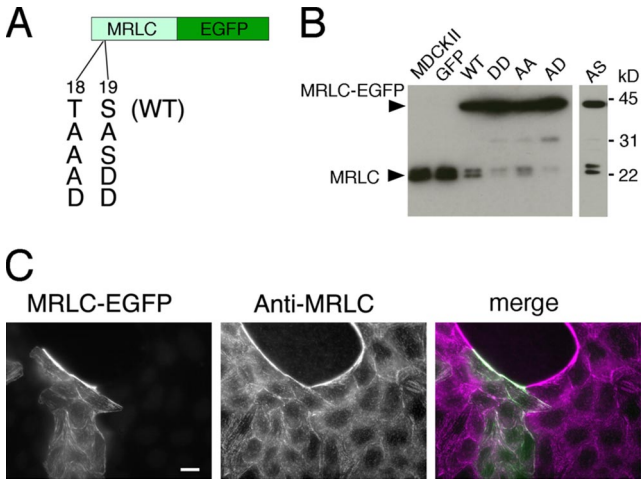

Figure 2. Establishment of MDCK II clones stably expressing MRLC-EGFP and its mutants. (A) Scheme of recombinant MRLCs; WT-MRLC, AA-MRLC, AS-MRLC, AD-MRLC, and DD-MRLC. EGFP-tag was added to the C-terminal of each recombinant MRLC. (B) Expression of endogenous and introduced EGFP-tagged MRLCs in each clone. Parental, GFP-expressing or MRLC-EGFP or its mutant-expressing MDCK II cells were processed for immunoblotting using anti-MRLC antibody. Equivalent amounts of proteins were loaded. (C) Mosaic cultures consisting of parental MDCK II cells and WT-MRLC-EGFP (green in merge)-expressing cells were fixed with 1% FA and stained for MRLC (magenta in merge). Bar, 20 μm.

2P-myosin II accumulated at relatively limited regions within cells (Figure 1C and Supplemental Figure S1) as reported with SM-3 cells (Sakurada *et al*., 1998). These results suggest that 2P-myosin II is more deeply involved in spatial regulation of myosin II activity within cells than 1P-myosin II.

Establishment of MDCK II Clones Stably Expressing MRLC-EGFP or Its Mutants

In an attempt to test the involvement of MRLC phosphorylation in myosin II dynamics and actin cytoskeleton organization, we introduced MRLC-EGFP or its mutants into MDCK II cells and obtained stable clones. They included WT- (diphosphorylatable), AA- (nonphosphorylatable), AS- (monophosphorylatable), AD- (mimicking monophosphorylation), and DD (mimicking diphosphorylation)-MRLC (Uchimura *et al*., 2002; Fumoto *et al*., 2003) (Figure 2A). We had already established WT-MRLC–expressing MDCK II cells previously (Miyake *et al*., 2006). All clones expressed recombinant EGFP-fusion proteins with expected molecular weight (Figure 2B). Interestingly, the expression of endogenous MRLC was detectable but highly suppressed in each clone. The amount of endogenous MRLC to total MRLC in each clone was 14.6% (WT), 10.7% (AA), 3.3% (AD), and 3.5% (DD). After staining mosaic cultures consisting of parental MDCK II cells and WT-MRLC-expressing MDCK II cells with anti-MRLC antibodies (Figure 2C), we found that fluorescence intensity with MRLC was almost the same between the two groups of cells, suggesting that the total amount of MRLC was maintained at a similar level after the

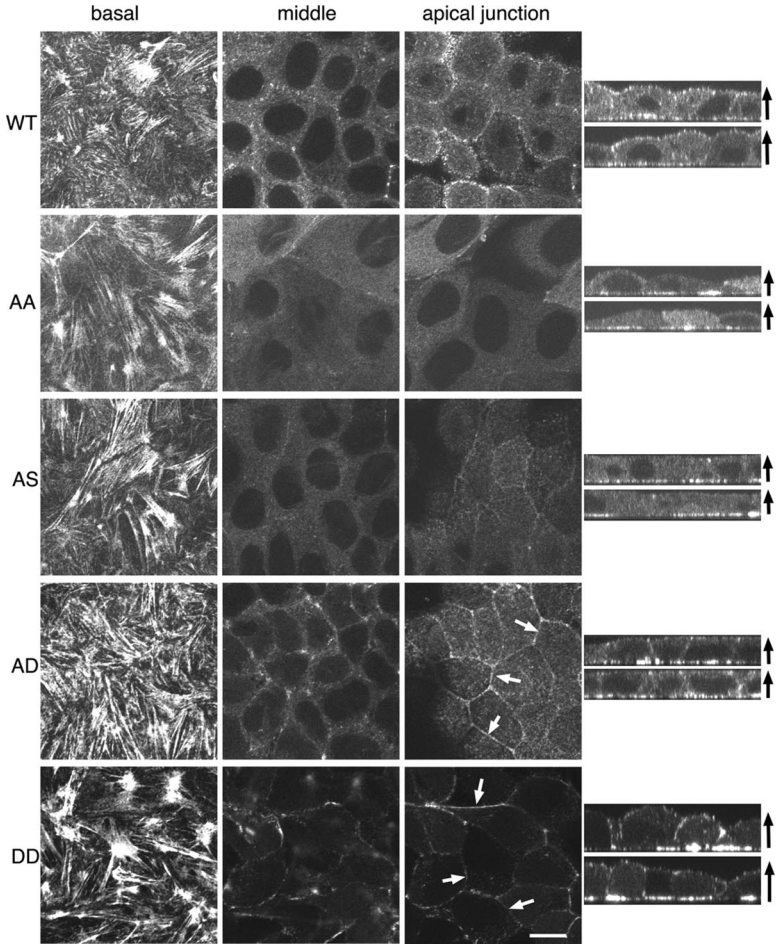

Figure 3. Localizations of introduced MRLC-EGFP and its mutants. Each clone was fixed with 4% FA and processed for confocal microscopy to detect EGFP fluorescence. White arrows indicate apical junction regions. Black arrows indicate apical direction along apicobasal axis in vertical sections of cells constructed from serial optical sections. Bar, 10 μm.

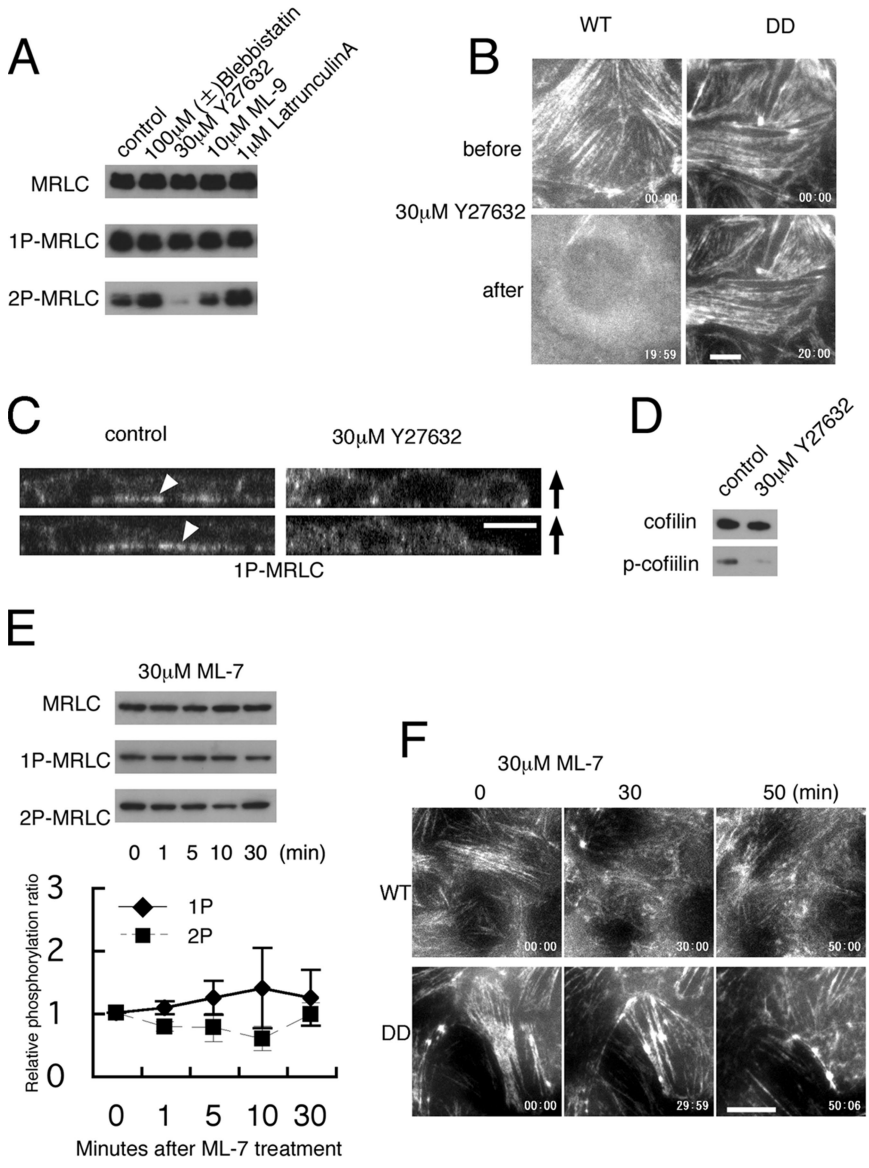

Figure 4. MRLC dephosphorylation with inhibitors and myosin II organization. (A) Effects of several reagents for cytoskeletons on MRLC phosphorylation. MDCK II cells were treated with each reagent for 30 min at indicated concentration and processed for immunoblotting to detect total, 1P- and 2P-MRLC. (B) MDCK II cells expressing WT-MRLC or DD-MRLC were treated with 30 μM Y27632 for 20 min. Live images before and after treatment show that stress fibers in DD-mutants are resistant for Y27632. AD-mutant showed similar results. (C) MDCK II cells treated with 30 μM Y27632 for 30 min and control cells were fixed with 1% FA, stained for 1P-MRLC, and processed for confocal microscopy. Vertical sections constructed from serial optical sections are shown. Arrowheads indicate stress fibers. Arrows indicate apical direction along apicobasal axis. (D) MDCK II cells treated with 30 μM Y27632 and control cells were processed for immunoblotting to detect total cofilin and phosphorylated cofilin. (E) MDCK II cells treated with 30 μM ML-7 were processed for immunoblotting at indicated time to detect total, 1P-, and 2P-MRLC. The relative amounts of 1P- and 2P-MRLC to total MRLC are shown (n = 3, error bars indicate SD). (F) Effects of ML-7 on myosin II organization in WT- or DD-MRLC–expressing cells. Live images at indicated time after ML-7 treatment are shown. Bars, 10 μm.

introduction of exogenous MRLC. Furthermore, as anti-MRLC antibodies stained similar actomyosin structures, such as stress fibers in both cells, the introduction of exogenous MRLC did not seem to affect the organization of actin cytoskeleton. WT-MRLC distribution also reflected the distributions of both NMHC isoforms (data not shown). Together, we therefore concluded that WT-MRLC tagged with EGFP reflects the myosin II localization in MDCK II cells. Transient expression of the MRLC–EGFP fusion protein has already been used in other cells in monitoring the myosin II localization within cells (Komatsu *et al*., 2000; Uchimura *et al*., 2002; Peterson *et al*., 2004). The functional authenticity of WT-MRLC-GFP has been confirmed previously (Komatsu *et al*., 2000).

Although this study also looked at other cell lines such as L or EpH4, we were unable to obtain clones stably expressing MRLC mutants, suggesting that MRLC mutants affect cell function. All MDCK II clones that we obtained had a similar normal appearance and a comparable growth rate, suggesting that even AA-MRLC or DD-MRLC did not severely affect cell function in this cell line. MRLC mutant distribution within cells, however, differed considerably between each clone (Figure 3). AA-MRLC did not show a clear localization at apical and lateral membranes, and it was found to be distributed at stress fibers and throughout the cytoplasm. In contrast, DD-MRLC localized at the cell cortex, including stress fibers, and lateral and apical membranes, with less distribution throughout the cytoplasm. We observed a noticeable accumulation of AD- or DD-MRLC at apical junction regions and several large aggregates of myosin II connected to stress fibers were often found in DD-MRLC-expressing cells. AD-MRLC was distributed at stress fibers, the cytoplasm, and at apical junction regions. AS-MRLC was distributed in a similar manner to WT-MRLC.

Diphosphorylation of MRLC Is an Important Regulatory Step for Spatial Stress Fiber Assembly and Contraction

In the hope of understanding the role of MRLC phosphorylation in more detail, we examined the relationship between the amount of 1P- or 2P-myosin II and the myosin II-containing cytoskeleton organization by using several reagents that could change this organization. We identified

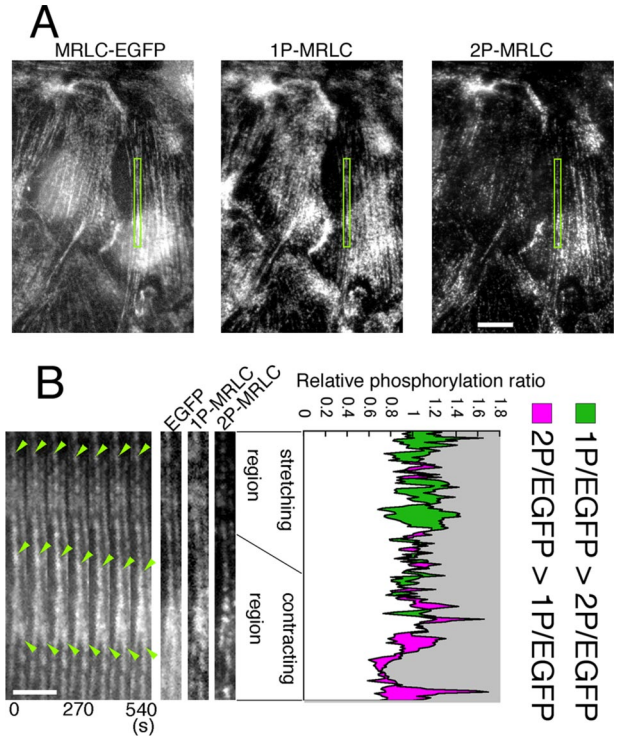

Figure 5. MRLC-diphosphorylation and stress fiber contraction. (A) MDCK II cells expressing WT-MRLC-EGFP were fixed with 1% FA immediately after time-lapse imaging and stained for 1P- and 2P-MRLC. Bar, 5 μm. (B) Kymograph of the boxed area in (A) constructed from time-lapse images of MRLC-EGFP showing stress fiber contraction and stretching during 540 s immediately before fixation and images of the fixed sample showing MRLC-EGFP, 1P-MRLC, and 2P-MRLC localizations. Arrowheads indicate positions of several bright points along the stress fiber. Fluorescence intensity of every point along the length of the stress fiber of each image of fixed cells was measured and ratios of the intensity, 1P-MRLC/EGFP and 2P-MRLC/EGFP were plotted along the length of the stress fiber. Areas where 1P-MRLC/EGFP > 2P-MRLC/EGFP are shown in green. Areas where 2P-MRLC/EGFP > 1P-MRLC/EGFP are shown in magenta. Bar, 2 μm.

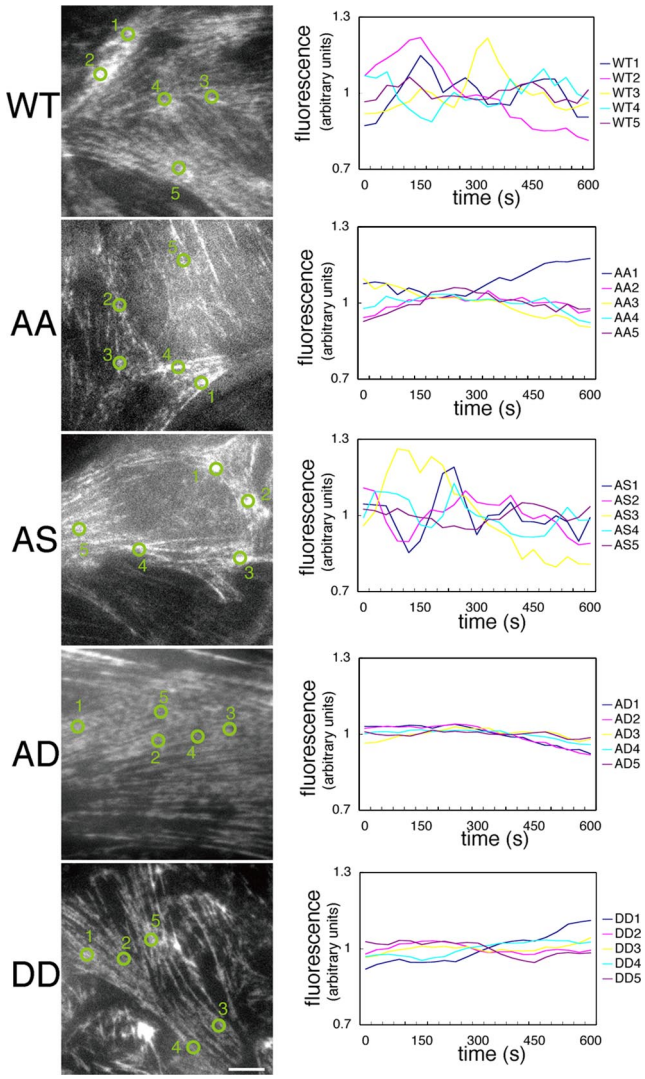

Figure 6. Changes in local fluorescence intensity of time-lapse images of cells expressing MRLC-EGFP or its mutant. Average fluorescence intensity of the same areas indicated by numbered circles of each frame (left) at every 30 s was measured for 10 min and average intensity of the same area of all frames was calculated. Fluorescence intensity of the same area at each frame relative to the average intensity was plotted as a function of time (right). Bar, 5 μm.

stress fibers as being of such an organization for this purpose because they are major actin- and myosin II-containing structures in MDCK II cells, and it proved relatively easy to record their whole structure within a single focal plane. A ROCK/Rho-kinase inhibitor, Y27632, is known to suppress MRLC phosphorylation and disrupts stress fibers (Uehata *et al*., 1997; Hirose *et al*., 1998). It also induces cofilin dephosphorylation, leading to activation of its actin filament-depolymerizing activity (Maekawa *et al*., 1999). To our surprise, we found that diphosphorylation, but not monophosphorylation, of MRLC is mainly affected by Y27632 in MDCK II cells (Figure 4A). 1P-MRLC and 2P-MRLC were reduced by 22 ± 2 and 57 ± 24%, respectively (n = 3), indicating that ROCK has a major role in diphosphorylating MRLC. Stress fibers in WT- or AA- (Figure 4B; data not shown) MRLC-expressing cells were disrupted by Y27632. Vertical sections reconstructed from serial optical sections showed that the amount of 1P-myosin II near the basal membrane, where stress fibers are located under normal conditions, was specifically reduced by Y27632 treatment (Figure 4C). In DD- or AD-MRLC–expressing cells, however, stress fibers were resistant to Y27632 treatment (Figure 4B; data not shown), although this treatment also induced cofilin dephosphorylation (Figure 4D), indicating that Y27632 affects stress fiber formation mainly through MRLC dephosphorylation but not through actin depolymerization by cofilin activation. An actin filament-depolymerizing drug, latrunculin A, disrupted stress fibers, and blebbistatin, a myosin II ATPase inhibitor, affected stress fiber organization as reported previously (Straight *et al*., 2003; Kolega, 2004). These actually did not affect but rather enhanced MRLC phosphorylation (Figure 4A).

ML7 or ML9 is known as an MLCK inhibitor and has also been used to suppress MRLC phosphorylation (Saitoh *et al*., 1987). Although by 10 min of ML7 (30 μM) treatment, the amount of 2P-MRLC had been reduced by 42 ± 20%, it recovered to the initial level by 30 min of the treatment (Figure 4E). 1P-MRLC, in contrast, had actually increased by 10 min of ML7 treatment. Stress fiber organization in MDCK II cells was somewhat affected, however, by 30–50 min of the treatment when the phosphorylation level had already

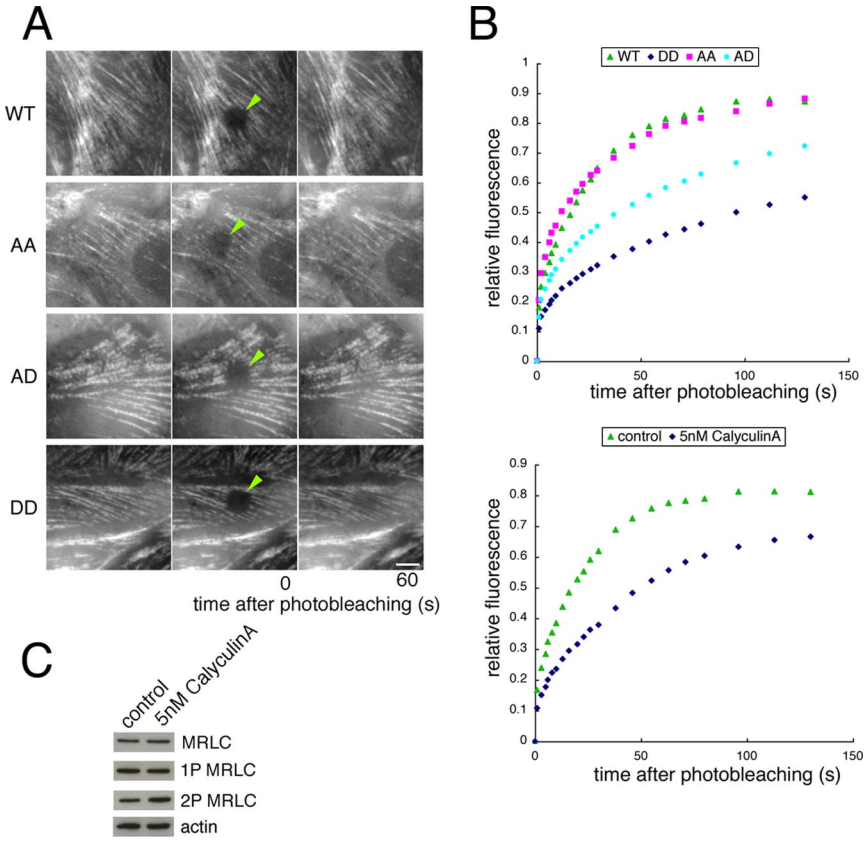

Figure 7. FRAP analysis of MRLC-EGFP or its mutants. (A) Time-lapse images of WT-, AA-, AD-, and DD-MRLC before and during FRAP analysis. Arrowheads indicate bleached areas in stress fibers. Bar, 5 μm. (B) Top graph shows FRAP of each MRLC. Bottom graph shows FRAP of WT-MRLC in control cells and cells treated with 5 nM calyculin A for 1 h. (C) Effects of calyculin A on MRLC phosphorylation of MDCK II cells. Control cells and cells treated with 5 nM calyculin A for 1 h were immunoblotted for detection of total, 1P-, and 2P-MRLC. The amount of actin was used as a loading control.

recovered (Figure 4F). Furthermore, even stress fibers in DD-MRLC–expressing cells that were resistant to MRLC dephosphorylation by Y27632 were also affected at ~50 min of ML7 treatment, indicating that although ML7 is a potent inhibitor of MRLC diphosphorylation at least transiently in MDCK II cells, it can affect stress fiber formation not through inhibition of MRLC phosphorylation but probably through its side effects on other molecules.

To see how 2P-myosin II is involved in the regulation of spatial actomyosin organization or activity, we fixed WT-MRLC-EGFP–expressing cells immediately after time-lapse imaging and processed them for immunofluorescence microscopy to reveal 1P- or 2P-myosin II localization. Single stress fiber exhibited a contracting portion and a stretching portion (Peterson *et al*., 2004). 1P-myosin II almost colocalized with WT-MRLC-EGFP that shows total myosin II distribution (Figure 5A). We found accumulation of 2P-myosin II, however, in regions where stress fibers were contracting just before fixation (Figure 5B). We did not see significant 2P-myosin II accumulation, however, in regions where stress fibers were stretching (Figure 5B) or disassembling (Figure 5A and Supplemental Video 1). These results indicate the importance of 2P-myosin II in spatial regulation of stress fiber contraction and assembly.

Both Phosphorylation and Dephosphorylation Are Required for Proper Dynamics of Myosin II

Using MRLC-EGFP–expressing cells (Supplemental Videos 2–6), we further measured fluorescence intensities in several regions of stress fibers in time-lapse images of each clone (Figure 6, left images) for 10 min (Figure 6, right graphs). During this period, stress fibers were maintained within the same focal plane of our conventional fluorescence microscope. WT-MRLC–containing myosin II showed rapid and sharp changes to its fluorescence intensity, indicating the occurrence of rapid myosin II filament assembly and disassembly. In contrast, AA-, AD-, or DD-MRLC–containing myosin II showed much slower changes. AS-MRLC behaved similarly to WT-MRLC. These results indicate that both phosphorylation and dephosphorylation are required for rapid myosin II assembly and disassembly.

Attempting to analyze myosin II dynamics in more detail, we used each stable clone and measured FRAP. A 4- to 6-μm-diameter spot was photobleached, and we acquired a time-lapse series of images (Figure 7A). The relative fluorescence intensity within the bleached area was measured during recovery (Figure 7B, top graph). WT- or AA-MRLC-myosin II showed quick recovery (>80% complete after 130 s). Although AD- or DD-MRLC, in contrast, showed considerably immobile fractions (30 or 50%, respectively) during the period (130 s) of measurement. Measuring over longer period proved difficult due to the stress fibers moving significantly and often escaping the small area used for measurement during this time. Assuming that mobile fractions for all MRLCs were 100% as the recovery did not reach a plateau at 130 s, $t_{1/2}$ ±SD for each mutant myosin II was 17.5 ± 4.5 s (WT; n = 9), 12.8 ± 4.2 s (AA; n = 10), 45.7 ± 9.6 s (AD; n = 9), or 99.4 ± 35.9 s (DD; n = 10). These results indicate that phosphorylated myosin II exchanges more slowly than nonphosphorylated myosin II. To confirm whether endogenous phosphorylated myosin II also show slow recovery, we treated WT-MRLC–expressing cells with calyculin A, a phosphatase inhibitor that is known to cause stress fiber formation and contraction (Peterson *et al*., 2004) and examined these cells by FRAP measurement. Calyculin A at 5 nM mainly enhanced diphosphorylation of MRLC

(Figure 7C) and slowed $t_{1/2}$ (74.5 ± 56.6 s, n = 14) as expected (Figure 7B, bottom graph). Dephosphorylation at both Thr18 and Ser19 of MRLC can therefore be seen as being important for rapid exchange of myosin II.

At the wound closure of polarized epithelial cell sheets, an actomyosin purse string rapidly forms at the wound edges of cells encircling the wound (Martin and Lewis, 1992; Bement *et al*., 1993). Our previous study also showed that in MDCK II cells, wounds of single or several cells killed by laser irradiation causes rapid myosin II accumulation to wound edges (Miyake *et al*., 2006). To examine the importance of MRLC phosphorylation in the formation of a new actomyosin structure as a physiological response, we analyzed the recruitment of myosin II to wound edges at wound closure using each stable clone. All clones finally completed the closure probably due to endogenous MRLC being sufficient to perform the correct closure. In WT-MRLC-expressing cells, myosin II began to accumulate at the wound edges to form a purse string typically within 5 min of wounding. AA- or AS-MRLC began to accumulate similarly to WT-MRLC, although the degree of accumulation was weaker than WT-MRLC. In contrast, DD-MRLC showed only weak accumulation during the recording period (10 min) (Figure 8 and Supplemental Videos 7–9). The degree of accumulation of each myosin II was judged to be as follows, WT > AA = AS > AD > DD (n = 4). This clearly shows that nonphosphorylatable myosin II can participate similarly to phosphorylatable myosin II in the formation of actomyosin structures consistent with our FRAP analysis. Phosphorylated myosin II, in turn, proved hard to rapidly form a new filament, indicating that a dephosphorylation step is required for rapid filament assembly through rapid disassembly of preexisting filaments. In the presence of Y27632 at 30 μM, WT-MRLC was recruited to the wound edges to form the purse string (Supplemental Video 10). The fluorescence intensity of the purse string, however, did not increase and the purse string did not contract significantly throughout the 60-min period. Together, these results suggest that dephosphorylation is essential for rapid recruitment or assembly of myosin II filaments and that phosphorylation is required for maintenance and contractility of actomyosin structures.

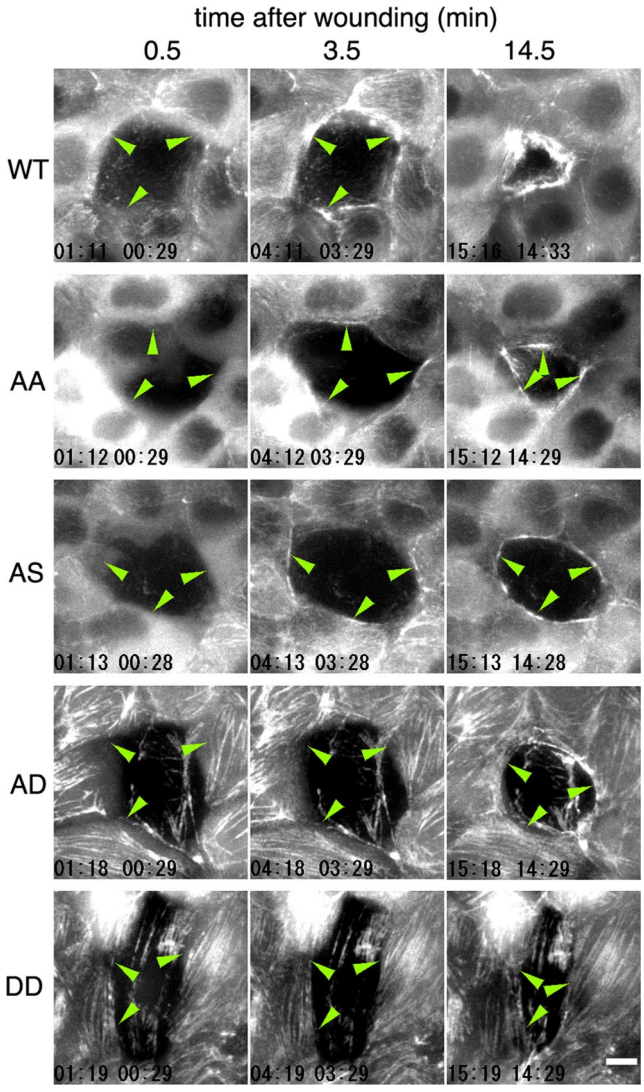

Figure 8. Recruitment of myosin II to the purse string at wound closure and MRLC phosphorylation. A wound was made by laser irradiation in WT-MRLC-EGFP- or its mutant-expressing cell sheets during time-lapse imaging. Arrows indicate edges of the wounds. Bar, 10 μm.

Myosin ATPase Activity-dependent Accumulation of MYPT1 and ZIP Kinase on Stress Fibers

Our data suggest that localization or localized activities of kinases and phosphatases for MRLC regulate the localized phosphorylation of MRLC. Moreover, MLCK (Poperechnaya *et al*., 2000), ROCK/Rho-kinase (Katoh *et al*., 2001), ZIP kinase (Komatsu and Ikebe, 2004), and MYPT1, a subunit of myosin phosphatase (Katoh *et al*., 2001) are known to localize to stress fibers. Furthermore, it has been shown that ZIP kinase induces diphosphorylation of MRLC and reorganization of actin filaments in HeLa cells (Murata-Hori *et al*., 2001). Although we could not detect MLCK or ROCK in stress fibers in MDCK II cells, we confirmed the localization of ZIP kinase and MYPT1 in stress fibers (data not shown; Figure 9A for AD-MRLC–expressing cells). Compared with ZIP kinase, the accumulation of MYPT1 to stress fibers was weak. MYPT1 is phosphorylated at Thr850 by ROCK, and inhibition of ROCK activity shows the enhanced affinity of MYPT1 to myosin II (Velasco *et al*., 2002). We therefore first examined whether ROCK activity regulates the localization of MYPT1. We decided to use Y27632-resistant stress fibers in AD- or DD-MRLC-expressing cells as stress fibers are readily disrupted by Y27632 in normal cells. Although phosphorylation at Thr850 was efficiently suppressed in the presence of Y27632 in AD-MRLC–expressing cells (Figure 9B), MYPT1 localization to stress fibers in AD-MRLC expressing cells did not significantly change (Figure 9A), indicating that ROCK activity or MYPT1 phosphorylation at Thr850 does not regulate MYPT1 localization. We then inhibited tension generation itself in stress fibers by using blebbistatin. Myosin II in blebbistatin-treated cells did not disperse into the cytoplasm but was still associated with actin filament bundles that are not straight but wavy, probably due to the loss of tension generated by myosin II (Figure 9A; data not shown). Blebbistatin abolished localization of MYPT1 on stress fibers: Even when MYPT1/myosin II interaction was activated by Y27632, blebbistatin removed MYPT1 from stress fibers. Similarly, we found that ZIP kinase localization on stress fibers was not dependent on ROCK activity, but rather on myosin II ATPase activity. We obtained similar results from DD-MRLC–expressing cells (data not shown).

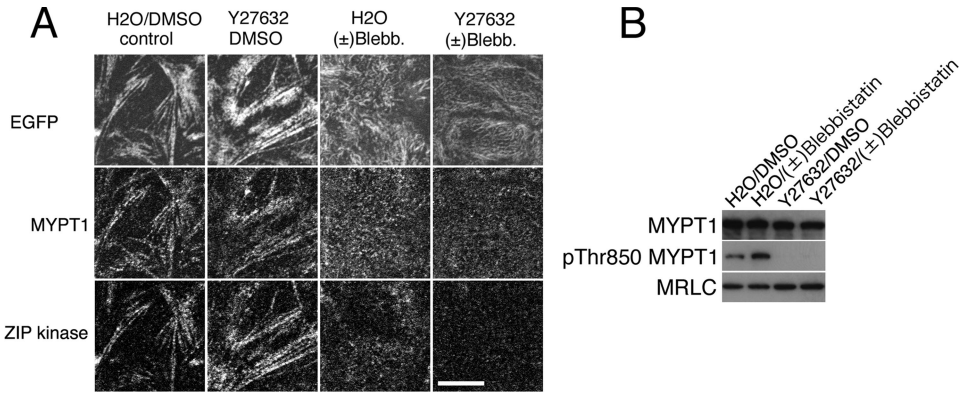

Figure 9. Myosin II-ATPase activity is involved in stress fiber-localization of MYPT1 and ZIP kinase. MDCK II cells expressing AD-MRLC-EGFP were treated with control medium (H2O/DMSO), 30 μM Y27632 (Y27632 DMSO), 100 μM blebbistatin [H2O (±)Blebb.], or both [Y27632 (±) Blebb.] for 30 min. (A) Each sample was fixed with 1% FA on ice and stained for MYPT1 and ZIP kinase. Bar, 10 μm. (B) Each sample was processed for immunoblotting to detect MYPT1, MYPT1 phosphorylated at Thr850, and MRLC.

DISCUSSION

Although in vitro experiments have revealed that myosin II motor activity and filament assembly are regulated by MRLC phosphorylation, the importance of the phosphorylation on myosin II dynamics in vivo has not yet been fully examined. In the present study, we addressed this issue by using MDCK II cells expressing MRLC-EGFP or its mutants mimicking various states of phosphorylation. The role of nonsphosphorylated (0P-), 1P-, or 2P-myosin II in myosin II dynamics in vivo was revealed.

Using cells expressing AD- or DD-MRLC with Y27632-resistant stress fibers, we were able to increase our existing level of knowledge of the role of ROCK in stress fiber formation. ROCK activity has been known to regulate MRLC phosphorylation by directly mono- (Amano *et al.*, 1996) or di- (Ueda *et al.*, 2002) phosphorylating MRLC and by indirectly dephosphorylating MRLC through activation of myosin phosphatase (Amano *et al.*, 1996; Kimura *et al.*, 1996). Inactivation of ROCK, in contrast, reportedly leads to cofilin dephosphorylation through inactivation of LIM kinase. This may also cause stress fiber disruption through actin depolymerization induced by activated cofilin (Maekawa *et al.*, 1999). Our experiments using AD- or DD-MRLC-expressing cells clearly showed that ROCK regulates stress fiber formation mainly through MRLC phosphorylation but not cofilin phosphorylation at least in MDCK II cells. Although ROCK mainly regulates the conversion between 1P- to 2P-MRLC in the whole MDCK II cells, its activity seems to be essential for maintaining 1P-MRLC in stress fibers with several reasons. First, MYPT1 localizes at stress fibers and can readily dephosphorylate MRLC when ROCK activity is lowered (Figure 9A). Second, reduction of the 1P-MRLC level by ROCK inhibition was limited to stress fiber regions and this inhibition caused stress fiber disruption (Figure 4). Third, as AD-MRLC mimicking 1P-MRLC is sufficient to maintain stress fibers during Y27632 treatment (Figures 4 and 9), production of 0P-MRLC must occur in stress fibers of normal cells treated with Y27632.

We also found problems in using MLCK inhibitors, such as ML-7. The effect of ML-7 or -9 on cytoskeletal organization or cell motility has been interpreted as being the effect of reduced myosin II activity through the inhibition of MLCK activity (Saitoh *et al.*, 1987). Using pseudophosphorylated MRLCs that are not sensitive for phosphorylation/dephosphorylation, we elucidated that ML-7 or -9 affects stress fiber organization by side effects other than MRLC phosphorylation. Because MLCK-knockout mice showed almost normal embryonic development (Somlyo *et al.*, 2004) and because ZIP kinase is also sensitive to ML-9 and responsible for MRLC phosphorylation at least in NIH3T3 fibroblasts (Komatsu and Ikebe, 2004), it is clear that the importance of MLCK in actomyosin structure and function is limited to a subset of organs. From these findings, we suggest that it is necessary to reevaluate reports claiming the importance of MLCK or myosin II activity based on results by using these inhibitors.

We demonstrate in this study that distribution of 1P-myosin II is similar to that of total myosin II in general whereas 2P-myosin II is concentrated locally in line with previous reports (Sakurada *et al.*, 1998; Uchimura *et al.*, 2002; Komatsu and Ikebe, 2004). Furthermore, 2P-myosin II accumulation coincided with the contracting regions in stress fibers (Figure 5). Diphosphorylation of MRLC was more sensitive than monophosphorylation to inhibitors for kinase/phosphatase and to mediators such as prostaglandin $F_{2\alpha}$ (Sakurada *et al.*, 1998; this study). Diphosphorylation caused a low turnover of myosin II (Figure 7). It is therefore reasonable to think that although monophosphorylation itself is essential for myosin II functions, diphosphorylation rather than monophosphorylation of MRLC locally regulates myosin II assembly or contraction in many cases within cells.

We analyzed myosin II dynamics by live imaging of EGFP-tagged MRLC. Fluorescence intensity changed rapidly with WT- or AS-MRLC-EGFP but not other mutant constructs (Figure 6), indicating that MRLC phosphorylation/dephosphorylation is important for rapid assembly and disassembly of myosin II. As shown in vitro (Ikebe *et al.*, 1988) and in vivo (Totsukawa *et al.*, 2004; this study) studies, we consider 1P-myosin II to be stable for maintaining filaments compared with 0P-myosin II, and that 2P-myosin II is more stable than 1P-myosin II. According to FRAP measurement (Figure 7) and purse string formation at wound closure (Figure 8), nonphosphorylatable AA-MRLC–containing myosin II can be incorporated into filaments and dephosphorylation of MRLC is required for rapid turnover of myosin II filaments. Slow recovery with AD-, DD-, or phosphorylated WT-MRLC in FRAP measurements may be due to the stability of myosin II filaments that inhibits fast release of myosin II molecules from the filaments into the cytoplasm. This raises the question of why stress fibers are maintained in AA-MRLC–expressing cells. Because these stress fibers are also sensitive to Y27632, endogenous MRLC-containing myosin II must therefore participate in stress fibers and its phosphorylation plays a crucial role in maintaining stress fibers. Moreover, we observed a considerable amount of 1P-myosin II in stress fibers in AA-MRLC–expressing cells (data not shown). We obtained a small value of $t_{1/2}$ with

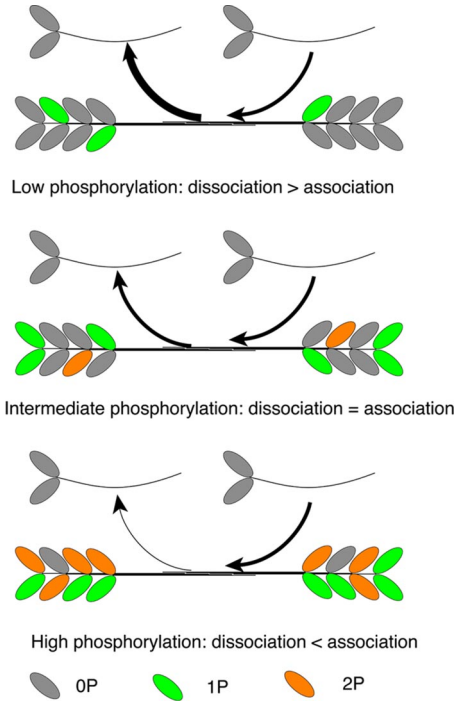

Figure 10. Model of myosin II dynamics within cells regulated by phosphorylation. For details, see text.

WT-MRLC (17.5 s) compared with that (8.5 min) in gerbil fibroma cells (Peterson *et al*., 2004). Another study, however, has shown fast recovery in perinuclear regions in Swiss 3T3 cells (DeBiasio *et al*., 1988). This discrepancy may be due to differences in the phosphorylation state of MRLC and/or the existence of proteins that might change myosin II filament stability among various cells.

We depict myosin II dynamics in stress fibers in MDCK II cells as in Figure 10. MRLC in stress fibers is frequently dephosphorylated to 0P-state and this is required for the rapid release of myosin II molecules from filaments. In stable stress fibers, release and incorporation of myosin II molecules are generally balanced (intermediate phosphorylation). When MRLC phosphorylation is reduced to under a certain level in restricted regions of stress fibers of normal cells or in stress fibers in cells treated with inhibitors for the phosphorylation, release becomes dominant and stress fibers are relaxed, stretched and disrupted (low phosphorylation). When MRLC phosphorylation is locally elevated, incorporation becomes dominant, resulting in myosin II filament assembly and higher contractile activity being shown (high phosphorylation). We think that this mechanism is essentially common to all myosin II-containing structures within cells other than stress fibers. Although AA-MRLC-containing myosin II in stress fibers showed rapid turnover ($t_{1/2}$ = 12.8 s), its fluorescence intensity was relatively stable over longer time (~10 min), indicating that release and incorporation are rapid but balanced. Phosphorylation level of MRLC in AA-MRLC–expressing cells may be relatively stable compared with that in WT-MRLC–expressing cells probably because AA-MRLC–expressing cells contain only a small amount of endogenous MRLC that can be phosphorylated or dephosphorylated.

In an attempt to understand the mechanism of spatial regulation of MRLC, we tested two factors that may regulate localizations of kinase or phosphatase for MRLC (Figure 9). One is ROCK activity. Although MYPT1, a myosin-binding subunit of myosin phosphatase is phosphorylated by ROCK, resulting in lower affinity for myosin II (Velasco *et al*., 2002), ROCK inhibition did not alter MYPT1 accumulation. The second factor is myosin II ATPase activity. When this activity was blocked by blebbistatin, both ZIP kinase and MYPT1 were released from myosin II filaments even in the presence of the ROCK inhibitor. Conformational changes of myosin II by force generation rather than by phosphorylation seem to regulate the association of the kinase/phosphatase to myosin II. We presume that this myosin II activity-dependent localization of kinase/phosphatase is involved in the balance of contractility within or between cells. Further details of the force-dependent mechanism of these associations should be elucidated using in vitro reconstitution systems.

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