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# Diphosphorylation of the Myosin Regulatory Light Chain Enhances the Tension Acting on Stress Fibers in Fibroblasts

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## **ABSTRACT**

Regulation of the contractile force is crucial for cell migration, cell proliferation, and maintenance of cell morphology. Phosphorylation of the myosin II regulatory light chain (MRLC) is involved in these processes. To show whether the diphosphorylation of MRLC increases the tension acting on stress fibers, changes in the stiffness of fibroblasts expressing wild-type MRLC and a mutant type, which cannot be diphosphorylated, on treatment with lysophosphatidic acid (LPA) were examined by a mechanical-scanning probe microscope (M-SPM). The LPA treatment increased cellular stiffness in the wild-type MRLC expressing cells, while it had no effect on the mutated cells. Immunostaining showed that LPA stimulation induced the diphosphorylation of MRLC. These results suggest that the diphosphorylation of MRLC enhances the tension acting on stress fibers.

## INTRODUCTION

In eukaryotic cells, contractile force is important for the performance of a large variety of physiological functions such as cell movement, cytokinesis, and maintenance of cell morphology. One of the main generators of the contractile force is the interaction between actin filaments and myosin II, in which the phosphorylation of the myosin II regulatory light chain (MRLC) is essential (Ikebe et al., 1986; Somlyo and Somlyo, 2003). Two phosphorylatable sites—threonine 18 and serine 19—are responsible for the activation of the myosin motor activity of MRLC. Serine 19 is more easily phosphorylated than threonine 18, allowing MRLC to be in either the de-, mono-, or diphosphorylated state (Ikebe et al., 1986). Earlier reports showed that myosin II in diphosphorylated MRLC (PP-MRLC) has a higher actin-activated  $\text{Mg}^{2+}$ -ATPase activity than that in monophosphorylated MRLC (P-MRLC) (Ikebe et al., 1988; Umemoto et al., 1989). In addition, several biochemical differences between the *in vitro* phosphorylated MRLCs were reported (Colburn et al., 1988; Trybus, 1989); however, a clear distinction between the phosphorylated MRLCs in the generation of contractile force has not been elucidated.

To measure the contractile force in a single cell, we used a mechanical-scanning probe microscope (M-SPM), which can visualize the spatial distribution of stiffness reflected by the tension in the stress fibers in a cell (Nagayama et al., 2004). By using the M-SPM, we were

successful in observing a dramatic change in cellular stiffness during cell migration (Nagayama et al., 2001) and in discovering tensional homeostasis against external deformation (Mizutani et al., 2004a). Our previous work showed that cellular stiffness increased on stimulation with lysophosphatidic acid (LPA) (Nagayama et al., 2004) that is a RhoA activator. Taking these findings into consideration, we concluded that the phosphorylation of MRLC increases cellular stiffness. However, the manner in which the mono- and diphosphorylation of MRLC contribute to the contractile force is unclear.

The purpose of this study was to examine whether PP-MRLC induces a higher *in vivo* contractile force than P-MRLC. We measured the stiffness of cells expressing green fluorescent protein (GFP)-tagged wild-type MRLC (MRLC<sup>WT</sup>-GFP) and a mutant type, which cannot be diphosphorylated (MRLC<sup>T18A</sup>-GFP), before and after LPA stimulation.

## **MATERIALS AND METHODS**

### **Cell culture and sample preparations**

Fibroblasts (NIH-3T3) were purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated bovine serum and 1% antibiotics (Invitrogen, Carlsbad, California) in 5% CO<sub>2</sub> at 37 °C. The cells were trypsinized and plated onto a glass dish coated with fibronectin (Boehringer Mannheim, Mannheim, Germany). Prior to M-SPM or immunofluorescence measurements, the culture medium in the dishes was replaced with DMEM buffered with serum-free HEPES (Sigma-Aldrich, St. Louis, Missouri) for 12 h. The cells were treated with 10 μM LPA (Sigma-Aldrich), 40 μM of the myosin light chain kinase (MLCK) inhibitor ML-7 (CALBIOCHEM, Fullerton, California), or 10 μM LPA + 40 μM ML-7 in HEPES buffer at suitable timings during the measurements. Although such an excess dose of ML-7 may cause the inhibition of both protein kinase A and protein kinase C (Krärup et al., 1998), the aim of the excess treatment performed here was to completely abolish the activity of the MLCK.

### **Construction of recombinant plasmids and transfection procedure**

The cDNA of wild-type nonmuscle MRLC (GenBank accession no. *BC004994*) was obtained from a human brain cDNA library (BD Biosciences Clontech, Mountain View,

California) using PCR, as described elsewhere (Yuasa et al., 2002). The cDNA fragment of the wild-type nonmuscle MRLC was subcloned into a pEGFP-N3 (BD Biosciences Clontech) vector. To generate two mutant MRLCs (termed as MRLC<sup>T18A</sup>-GFP and MRLC<sup>T18D, S19D</sup>-GFP), a part of the MRLC sequence was substituted using PCR. MRLC<sup>T18A</sup>-GFP in which the diphosphorylation of MRLC was prevented was constructed by the substitution of threonine 18 to alanine 18. MRLC<sup>T18D, S19D</sup>-GFP in which MRLC was constitutively diphosphorylated was constructed by the following substitutions: threonine 18 to aspartic acid 18 and serine 19 to aspartic acid 19. To construct GFP-not-tagged MRLC, the cDNA fragment of wild-type MRLC or MRLC<sup>T18A</sup> was substituted for the GFP fragment in the pEGFP-N3 vector. A plasmid coding for both dominant-negative RhoA and the DsRed expression marker (pIRM21-Flag-Rho<sup>N19</sup>) was kindly provided by Dr. M. Matsuda (Osaka University) (Aoki et al., 2004). All the plasmids were purified by cesium chloride density gradient centrifugation in order to obtain high protein expression. Cells were transfected using the Effectene transfection reagent (QIAGEN, Valencia, California) according to the manufacturer's instructions. Briefly, cells cultured at a concentration of 30 cells/mm<sup>2</sup> in a plastic flask were treated with 1 µg/mL of cDNA complexed with the Effectene reagent in the culture medium. The maximum efficiency of expression was achieved more than 1 day after the transfection.



## Stiffness measurements with M-SPM

The M-SPM used in this study, known as a wide-range SPM, is a modification of the commercial SPM (SPA400; Seiko Instruments Inc., Chiba, Japan) and is equipped with a tandem piezo scanner with a maximal scan range of 500  $\mu\text{m}$  ( $xy$  axis) and 26  $\mu\text{m}$  ( $z$  axis) (Mizutani et al., 2004b). A silicon-nitride cantilever with a spring constant of 0.01 N/m (MLCT-AUNM; Veeco, Woodbury, New York) was used. The spatial distribution of cellular stiffness was imaged in the force mapping mode, as described previously (Haga et al., 2000; Radmacher et al., 1996). In brief, this mode can simultaneously provide both the height and stiffness images of a sample by generating a force curve at each pixel point in a scan area consisting of 64 pixels  $\times$  64 lines. The local stiffness (Young's modulus) of the sample was evaluated quantitatively from a force curve that was fitted to the Hertz model by using a nonlinear least-squares method. During the measurements, a petri dish filled with HEPES buffer was maintained at physiological temperature. The dish was placed on a metal plate maintained at 37  $^{\circ}\text{C}$  by a temperature controller that consisted of a heater and a thermocouple.

To obtain the representative stiffness of a cell in each experiment, one set of height and stiffness images was used. The highest point in a height image was defined as the center of the cell. The stiffness values within a circular area of 100  $\mu\text{m}^2$  from this point were averaged. Statistical changes in the averaged stiffness were examined using Student's  $t$ -test. These data

were presented as mean  $\pm$  SEM.

### **Immunofluorescence analysis**

Cells were rinsed with phosphate-buffered saline (PBS, Invitrogen), followed by fixation with 4% formaldehyde/PBS for 10 min. They were then washed twice with PBS and permeabilized with 0.5% Triton X-100/PBS for 10 min. After blocking the nonspecific binding sites with 0.5% bovine serum albumin (BSA, Invitrogen) in PBS, the cells were stained with specific primary antibodies/PBS by incubation for 1 h on a mild shaker. Subsequently, the cells were rinsed three times with PBS containing 0.5% BSA and stained with secondary antibodies/PBS by incubation for 1 h on the same shaker. To avoid loss of fluorescence intensity, the cells were covered with 1,4-diazobicyclo[2.2.2]octane (DABCO; Wako Pure Chemicals, Osaka, Japan) diluted in glycerol. Fluorescence images were obtained with a confocal laser scanning microscope (C1 confocal imaging system; NIKON Instech Co., Ltd., Tokyo, Japan). Sectional images were collected and processed using the EZ-C1 software (NIKON Instech).

To quantify the degree of MRLC diphosphorylation, the fluorescent intensity of PP-MRLC was measured using a commercial software (Image-Pro Plus; Media Cybernetics, Silver Spring, Maryland). In the fluorescent image, the contour of a cell was traced manually. The intensities of PP-MRLC in a cell were averaged, in which unlikely signals from the nucleus

were eliminated. Statistical changes in the averaged intensity were examined using Student's *t*-test. These data were presented as mean  $\pm$  SEM.

### **Electrophoresis and western blotting**

Cells cultured in flasks were quickly washed with cold PBS and were then frozen at  $-20^{\circ}\text{C}$ . Trichloroacetic acid (10% w/v) (Wako Pure Chemicals) was added to the flask. The cells were scraped 10 minutes after the application. To demineralize the cell pellet, acetone (Wako Pure Chemicals) was added to it and the mixture was centrifuged. We applied two types of sample buffers to the pellet depending on the electrophoresis method followed. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the sample buffer consisted of 125 mM Tris-HCl, pH 6.8; 2.3% (w/v) SDS; 10% (w/v) glycerol; 5% (w/v) dithiothreitol; and 10  $\mu\text{g/mL}$  bromophenol blue. For urea-PAGE, the sample buffer consisted of 20 mM Tris-glycine, pH 8.6; 8 M urea; 5% (w/v) dithiothreitol; and 10  $\mu\text{g/mL}$  bromophenol blue (Daniel and Sellers, 1992). The above agents were purchased from Wako Pure Chemicals. These samples were sonicated and then stored at  $-20^{\circ}\text{C}$ .

SDS-PAGE was performed following standard procedures. Urea-PAGE was performed as described previously (Daniel and Sellers, 1992). In brief, the samples were applied to a 16 cm  $\times$  16 cm urea gel and were electrophoresed for 5 h at 10 W, 500 V. Proteins were transferred to a

PVDF membrane by semidry blotting carried out for 1 h at 1 mA/cm<sup>2</sup> with a transfer buffer comprising 20 mM Tris-glycine, pH 8.6 and 6 M urea. After the SDS- and urea-PAGE, the membranes were washed with 0.05% polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemicals) in PBS and blocked with 1% BSA in PBS for 30 min. Immunoreactive proteins were detected using an enhanced chemiluminescent detection system according to the manufacturer's protocol (Amersham, Little Chalfont, UK). Images of protein blots were processed using Image-Pro Plus (Media Cybernetics).

To show the change in P-MRLC in urea-PAGE, the increase was calculated from

$$I = \frac{A_{P-MRLC} / A_{total-MRLC}}{B_{P-MRLC} / B_{total-MRLC}}, \quad (1)$$

where the notations are as follows:  $I$ , the increase in P-MRLC;  $A_{P-MRLC}$ , intensity of P-MRLC with LPA treatment;  $A_{total-MRLC}$ , intensity of total MRLC with LPA treatment;  $B_{P-MRLC}$ , intensity of P-MRLC without LPA treatment; and  $B_{total-MRLC}$ , intensities of total MRLC without LPA treatment.

## Antibodies

Phosphorylated MRLCs were detected by the following antibodies: anti-P-MRLC (#3671; Cell Signaling Technology, Beverly, Massachusetts) that reacted with P-MRLC<sup>T18A</sup>,

P-MRLC and PP-MRLC; anti-PP-MRLC (#3674; Cell Signaling Technology) that reacted with only PP-MRLC. An antibody that reacted with MRLC and MRLC-GFP was purchased from Cell Signaling Technology. Anti- $\alpha$ -tubulin specific for tubulin was purchased from Sigma-Aldrich. Fluorescently conjugated secondary antibodies as well as fluorescently tagged phalloidin were purchased from Invitrogen. Horseradish peroxidase (HRP)-conjugated secondary antibodies used for western blotting were purchased from Bio-Rad Laboratories (Hercules, California).

## RESULTS

To confirm whether the overexpression of  $\text{MRLC}^{\text{T18A}}$ -GFP was able to prevent the diphosphorylation of MRLC, cells transfected with  $\text{MRLC}^{\text{T18A}}$ -GFP were incubated for 1 h in HEPES buffer containing LPA, following which immunofluorescence staining for PP-MRLC was performed (Fig. 1A). Although the cells expressing  $\text{MRLC}^{\text{T18A}}$ -GFP showed fibrous MRLC structures, these structures were not stained with the anti-PP-MRLC antibody in both with and without LPA. The expression ratio of exogenous to endogenous MRLC was confirmed by western blotting (Fig. 1B). The expression level of exogenous MRLC was greater than that of endogenous MRLC. These results indicate that overexpressed  $\text{MRLC}^{\text{T18A}}$ -GFP cannot be diphosphorylated.

To elucidate whether the intracellular tensional force in the cells expressing  $\text{MRLC}^{\text{T18A}}$ -GFP changes after LPA stimulation, we measured cellular stiffness before and 1 h after the LPA treatment. Figure 2A shows the change in the stiffness of the cells expressing  $\text{MRLC}^{\text{T18A}}$ -GFP or  $\text{MRLC}^{\text{WT}}$ -GFP as measured by the M-SPM. Stiffer fibrous structures corresponding to the stress fibers were observed in both the images. In the cells expressing  $\text{MRLC}^{\text{T18A}}$ , LPA stimulation did not alter cellular stiffness, whereas the cells expressing  $\text{MRLC}^{\text{WT}}$  had stiffened after LPA stimulation, as previously reported (Nagayama et al., 2004). In particular, the regions indicated by the arrows in Fig. 2A had stiffened after LPA stimulation.

The cellular stiffness in each image was averaged over the central area (see materials and methods) of the cellular surface to quantify the degree of change in stiffness due to the LPA treatment. The averaged stiffness before and after the LPA treatment was plotted (Fig. 2B). There was a significant difference in cellular stiffness between  $\text{MRLC}^{\text{T18A}}$ - and  $\text{MRLC}^{\text{WT}}$ -expressing cells after LPA treatment, although there was little difference between these cells in this regard before LPA stimulation. Statistical analysis showed that the stiffness of cells expressing  $\text{MRLC}^{\text{T18A}}$  did not change after LPA stimulation. To strengthen the effect of the diphosphorylation of MRLC on stiffness, we measured the stiffness of  $\text{MRLC}^{\text{T18D, S19D}}$ ,  $\text{S19D}$ -GFP-expressing cells in a serum-free condition. The stiffness of  $\text{MRLC}^{\text{T18D, S19D}}$ -expressing cells was found to be greater than that of  $\text{MRLC}^{\text{WT}}$ -expressing cells (data not shown). Therefore, the diphosphorylation of MRLC should enhance the intracellular tension due to stress fiber contraction.

To confirm whether LPA stimulation induces the diphosphorylation of endogenous MRLC, normal cells were treated with 10  $\mu\text{M}$  LPA and stained with anti-PP-MRLC and phalloidin (Fig. 3A). All the experiments were performed under starved conditions by using a serum-free medium. Cells stimulated by LPA showed filamentous structures of PP-MRLC, whereas the control cells showed a low amount of PP-MRLC. The structure of the actin filament was observed in LPA-treated or un-treated cells. The intensity of PP-MRLC was quantified (Fig.

3B). Since the antibody for PP-MRLC recognized nuclei (Ueda et al., 2002), immunofluorescence in the nuclear area was excluded from the calculation; however, it was used as a reference level for the immunofluorescence intensity. A significant difference exists between the LPA-stimulated cells and control cells in terms of PP-MRLC. These results indicate that the diphosphorylation of MRLC in stress fibers is involved in the increase in the stiffness change.

There is a possibility that the difference in increase in P-MRLC may contribute to the stiffness change in MRLC<sup>T18A</sup>- and MRLC<sup>WT</sup>-expressing cells. To confirm the change in the amount of P-MRLC by the LPA treatment, we performed a combination of urea-PAGE and SDS-PAGE along with western blotting (Fig. 4A). We used the same amount of samples in urea- and SDS-PAGE in order to compare the relative change in the amount of phosphorylated MRLCs. The amount of PP-MRLC in MRLC<sup>WT</sup>-expressing cells increased with LPA, whereas it did not increase in MRLC<sup>T18A</sup>-expressing cells. The relative change in the amount of P-MRLC was analyzed and is shown in Fig. 4B. There was no significant difference between the MRLC<sup>T18A</sup>- and MRLC<sup>WT</sup>-expressing cells. These results suggest that the diphosphorylation but not the monophosphorylation of MRLC increases the intracellular tension by LPA treatment.

Further, to examine the manner in which LPA stimulation induces the diphosphorylation of MRLC, the involvement of the small GTPase RhoA that regulates the



phosphorylation of MRLC, was tested by using dominant-negative RhoA (Rho<sup>N19</sup>). The cells expressing Rho<sup>N19</sup> were examined in the LPA stimulation experiment. The immunofluorescence micrograph of PP-MRLC in a cell expressing Rho<sup>N19</sup> that was stained after LPA stimulation is shown in Fig. 5A. The diphosphorylation of MRLC by LPA stimulation was inhibited in cells expressing Rho<sup>N19</sup> (Fig. 5A, B). RhoA is known to be an activator of the Rho-associated kinase (ROCK). ROCK is able to effect myosin II motor activity by both inhibiting MRLC phosphatase (MLCP) *in vivo* (Kimura et al., 1996) and phosphorylating MRLC directly *in vitro* (Amano et al., 1996). In such a case, there are two possibilities for the signal pathways in which MRLC was diphosphorylated by LPA stimulation. One is that MLCK diphosphorylates MRLC because of the inhibition of MLCP activity by ROCK activation and the other is that ROCK diphosphorylates MRLC directly (Matsumura et al., 2001). In order to determine which mechanism is involved, the cells were treated with both ML-7, a known inhibitor of MLCK, and LPA. The cells showed sufficient amounts of PP-MRLC in the stress fibers (Fig. 5C, D). To confirm the effect of ML-7, cells treated only with ML-7 showed a few actin fibers and no phosphorylated MRLC (Fig. 5E, F). Finally, we measured the stiffness of Rho<sup>N19</sup>-expressing or ML-7 pretreated cells in LPA stimulation. The Rho<sup>N19</sup> expressing cells did not increase their stiffness, however, ML-7 pretreated cells increased stiffness with LPA (data not shown). These results indicate that ROCK directly induces the diphosphorylation of MRLC via the LPA-RhoA

signaling cascade and enhances the tension of stress fiber.

## DISCUSSION

We demonstrated that the diphosphorylation of MRLC enhances the intracellular tension via a LPA-RhoA-ROCK signaling cascade. The stiffness of cells expressing MRLC<sup>T18A</sup> did not change on LPA stimulation. In contrast, the stiffness of cells expressing MRLC<sup>WT</sup> had increased. Immunofluorescence staining for PP-MRLC indicated that endogenous MRLC was diphosphorylated by LPA stimulation. The combination of urea- and SDS-PAGE showed that there was no difference in the change of P-MRLC between the MRLC<sup>T18A</sup>- and MRLC<sup>WT</sup>-expressing cells by LPA stimulation. The endogenous MRLC in cells expressing dominant-negative RhoA was not diphosphorylated despite LPA stimulation. Treatment with both LPA and ML-7 revealed that ROCK diphosphorylated MRLC directly. These results suggest that the diphosphorylation of MRLC enhances the intracellular tension due to actomyosin contraction. A recent study showed that LPA treatment induced the phosphorylation of MRLC (both mono- and diphosphorylation of MRLC) in hepatic myofibroblasts, thereby increasing the contractile force and migration velocity (Tangkijvanich et al., 2003). In addition, ROCK is involved in the phosphorylation of MRLC and the regulation of cellular contractile force (Yee et al., 2001). The major difference between this study and ours lies in the revelation of the distinct role played by the diphosphorylation of MRLC.

There is a possibility that LPA stimulation increases the number of stress fibers; the

transition from the dephosphorylation to monophosphorylation of MRLC might induce the formation of new stress fibers. Our results indicate that in cells expressing MRLC<sup>T18A</sup>, the contractile force at the cell center did not change despite the cell's capability of constructing additional stress fibers; moreover, in cells expressing MRLC<sup>WT</sup>, the tension in the central area increased. Furthermore, a previous report showed that in cells stimulated by calyculin A—a serine/threonine phosphatase inhibitor—the amount of P-MRLC increased at the cell periphery but was unchanged at the center (Peterson et al., 2004). Taken together, it appears that stress fibers are not formed at the cell center by LPA stimulation; instead, these results strongly suggest that the transition from mono- to diphosphorylated MRLC enhances the tension acting on stress fibers.

Our results show that the diphosphorylation of MRLC by LPA stimulation was directly induced by the activation of ROCK, which agrees with previously reported results (Manning et al., 2000). ROCK can activate myosin II motor activity not only by inhibiting MRLC phosphatase (Kimura et al., 1996) but also by monophosphorylating (Amano et al., 1996) or diphosphorylating (Ueda et al., 2002) MRLC in a  $\text{Ca}^{2+}$ /calmodulin-independent manner. Although  $\text{Ca}^{2+}$ /calmodulin-dependent MLCK can diphosphorylate MRLC, the amount of calmodulin available for the phosphorylation of MRLC appears limited, even at high  $\text{Ca}^{2+}$  concentrations (Geguchadze et al., 2004). Therefore, we believe that the diphosphorylation of

MRLC mainly depends on  $\text{Ca}^{2+}$ /calmodulin-independent kinases such as ROCK, ZIP kinase (Murata-Hori et al., 2001), and citron kinase (Yamashiro et al., 2003). These kinases are localized at different sites in a cell. To control the contractile force in a cell spatiotemporally, the cells must utilize these kinases in different ways in order to phosphorylate MRLC depending on the physiological requirements. For example, citron kinase regulates cytokinesis (Madaule et al., 1998), and ZIP kinase is necessary for cell motility (Komatsu and Ikebe, 2004). These reports suggest that the diphosphorylation of MRLC is crucial in many physiological events, although the manner in which the functions of these kinases are coordinated in the cell is unclear. In future studies, we will focus on the spatiotemporal regulation of the phosphorylation of MRLC in eukaryotic cells.

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## FIGURE LEGENDS

### FIGURE 1

Inhibition of MRLC diphenosphorylation in cells overexpressing MRLC<sup>T18A</sup>-GFP. **A:** Cells expressing MRLC<sup>T18A</sup> were treated with or without 10  $\mu$ M LPA and stained with anti-PP-MRLC after fixation. The bar denotes a length of 50  $\mu$ m. **B:** The expression level of exogenous to endogenous MRLCs was analyzed by western blotting. Lysates from MRLC<sup>T18A</sup>-GFP (T18A), MRLC<sup>WT</sup>-GFP (WT), non-transfected (control) cells were subjected to SDS-PAGE. Proteins were monitored by anti-MRLC and anti-tubulin, respectively.

### FIGURE 2

Change in the stiffness of cells expressing MRLC<sup>T18A</sup>-GFP or MRLC<sup>WT</sup>-GFP after LPA stimulation. **A:** Stiffness images were measured by an M-SPM before and after LPA stimulation. A significant increase in stiffness was observed in cells expressing MRLC<sup>WT</sup> after LPA stimulation (arrows). On the other hand, MRLC<sup>T18A</sup> expression suppressed the increase in stiffness after LPA stimulation. The bar denotes a length of 20  $\mu$ m. **B:** The bar graph shows the averaged stiffness of the cells expressing MRLC<sup>T18A</sup>-GFP or MRLC<sup>WT</sup>-GFP before (–) and after (+) LPA stimulation. The details of the stiffness analysis have been described in the Materials

and Methods section. Data represent mean  $\pm$  SE from six experiments (\* $P < 0.01$ ).

### FIGURE 3

Diphosphorylation of endogenous MRLC by LPA stimulation. **A:** The immunofluorescence micrographs show PP-MRLC (green) and filamentous actin (red) in LPA stimulated and unstimulated cells. The bar denotes a length of 50  $\mu$ m. **B:** The intensity of PP-MRLC from the immunofluorescence micrographs (A) was averaged in each cell and is shown in the bar graph.

Data represent mean  $\pm$  SE from more than 30 cells (\* $P < 0.001$ ).

### FIGURE 4

Diphosphorylation and monophosphorylation of MRLC<sup>T18A</sup>- or MRLC<sup>WT</sup>-expressing cells by LPA stimulation. **A:** Cells transfected with MRLC<sup>T18A</sup> or MRLC<sup>WT</sup> were treated with LPA. The lysates from cells were subjected to urea-gel western blotting. Phosphorylated MRLCs were monitored by an antibody for P- and PP-MRLC (upper panel). The same amount of samples was subjected to SDS-PAGE and western blotting. The total MRLC was monitored by anti-MRLC (lower panel). **B:** The increase in P-MRLC in MRLC<sup>T18A</sup> or MRLC<sup>WT</sup> expressing cells was compared by statistical analysis (see materials and methods).

## FIGURE 5

Functioning of ROCK as a kinase. The cells expressing Rho<sup>N19</sup> were treated with 10  $\mu$ M LPA and then stained with anti-PP-MRLC (A) after 30 min of LPA stimulation. The cells also express DsRed as an expression marker (B). Endogenous MRLC of cells expressing Rho<sup>N19</sup> were not diphosphorylated by the LPA treatment. However, cells that did not express Rho<sup>N19</sup> showed diphosphorylated MRLC (A). Cells stimulated with both LPA and ML-7 were stained with anti-PP-MRLC (C) and phalloidin (D). Each cell showed PP-MRLC in the stress fibers. Cells treated only with ML-7 were stained with anti-P-MRLC (E) and phalloidin (F). The bar denotes a length of 50  $\mu$ m.

Fig. 1

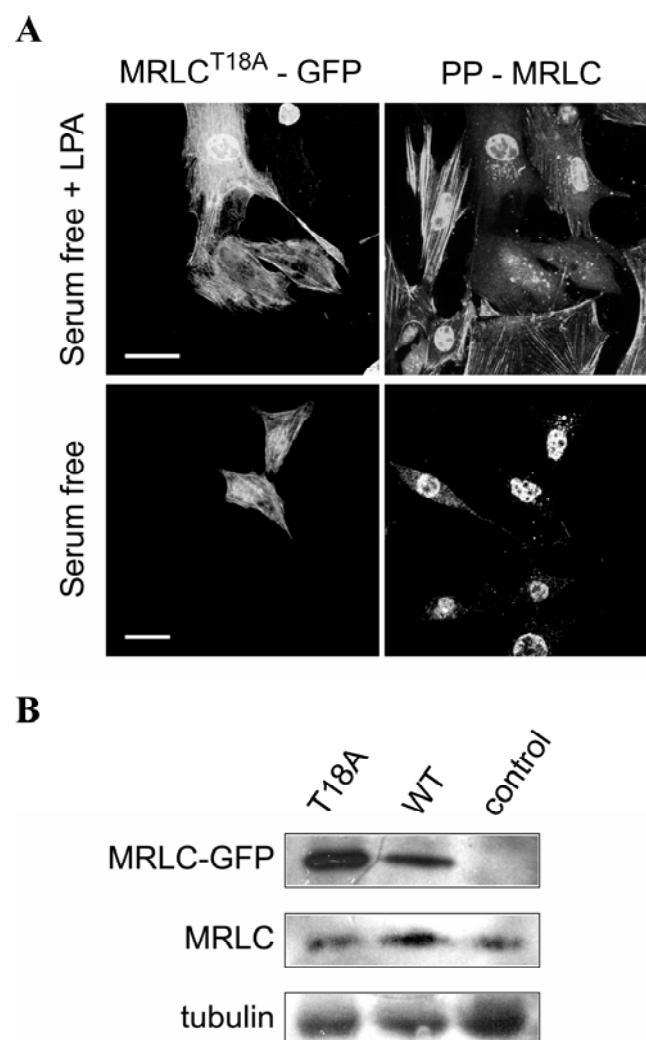


Fig. 2

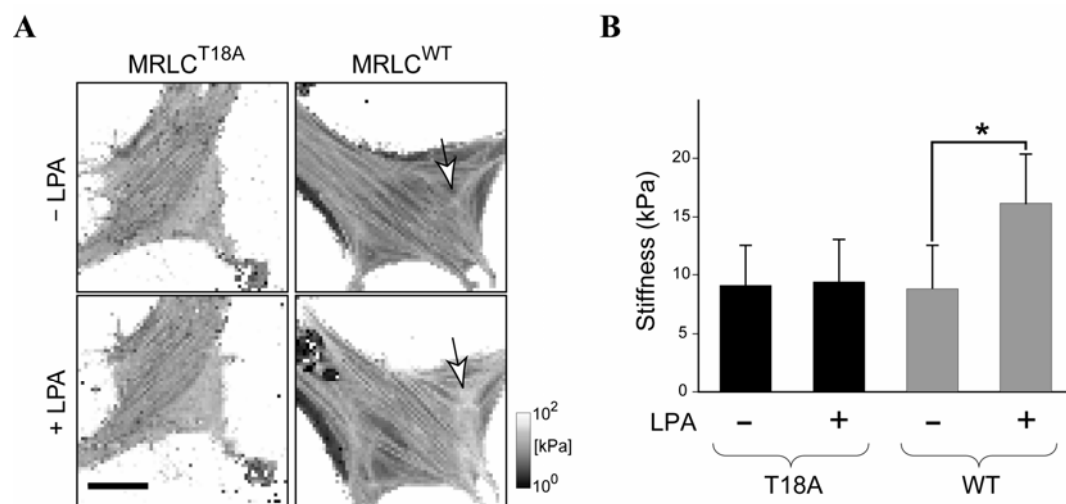




Fig. 3

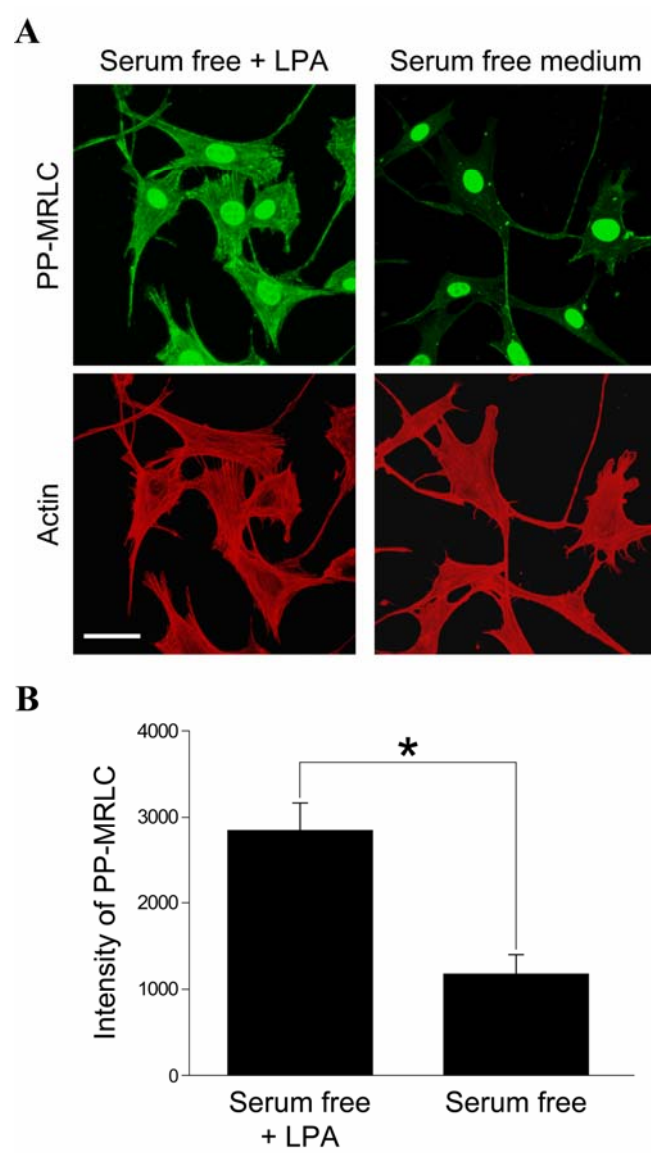


Fig. 4

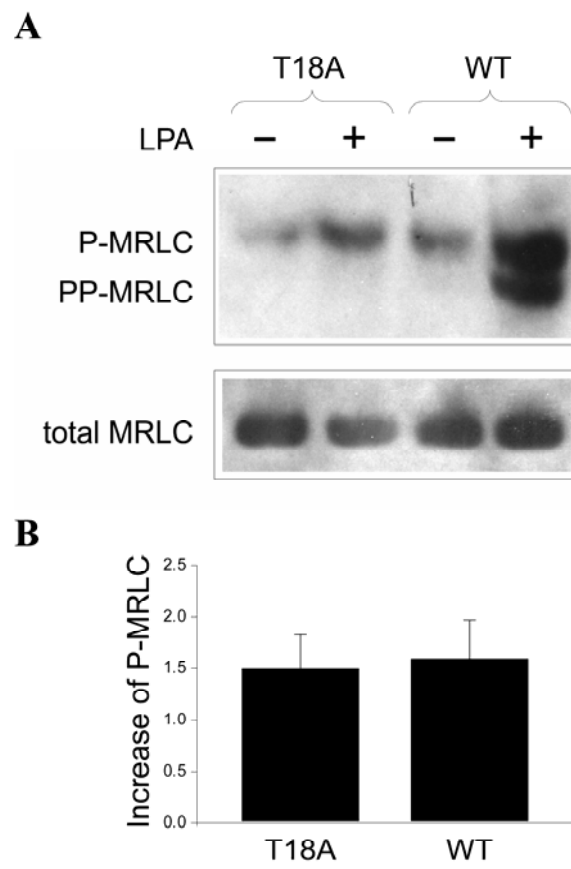


Fig. 5

