Phosphorylation of myosin II regulatory light chain is necessary for migration of HeLa cells but not for localization of myosin II at the leading edge

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To investigate the role of phosphorylated myosin II regulatory light chain (MRLC) in living cell migration, these mutant MRLCs were engineered and introduced into HeLa cells. The mutant MRLCs include an unphosphorylatable form, in which both Thr-18 and Ser-19 were substituted with Ala (AA-MRLC), and pseudophosphorylated forms, in which Thr-18 and Ser-19 were replaced with Ala and Asp, respectively (AD-MRLC), and both Thr-18 and Ser-19 were replaced with Asp (DD-MRLC). Mutant MRLC-expressing cell monolayers were mechanically stimulated by scratching, and the cells were forced to migrate in a given direction. In this wound-healing assay, the AA-MRLC-expressing cells migrated much more slowly than the wild-type MRLC-expressing cells. In the case of DD-MRLC- and AD-MRLC-expressing cells, no significant differences compared with wild-type MRLC-expressing cells were observed in their migration speed. Indirect immunofluorescence staining showed that the accumulation of endogenous diphosphorylated MRLC at the leading edge was not observed in AA-MRLC-expressing cells, although AA-MRLC was incorporated into myosin heavy chain and localized at the leading edge. In conclusion, we propose that the phosphorylation of MRLC is required to generate the driving force in the migration of the cells but not necessary for localization of myosin II at the leading edge.

Key words: cell motility, diphosphorylation, monophosphorylation, wound healing.

INTRODUCTION

In non-muscle cells, phosphorylation of myosin II has been thought to be important to cell motility [1,2] and cytokinesis [3–5]. The activity of myosin II is stimulated by phosphorylation of the myosin II regulatory light chain (MRLC). Monophosphorylation of MRLC at Ser-19 increases both the actin-activated Mg-ATPase activity and the stability of myosin II filaments, while diphosphorylation at Thr-18 and Ser-19 promotes both the myosin II activity and the stability of myosin II filaments more significantly than does monophosphorylation [6,7].

Several protein kinases are known to catalyse the phosphorylation of MRLC [8–15]. Among them, p21-activated kinase, Rho kinase and myosin light-chain kinase (MLCK), have been shown to regulate cell motility [16–18]. It has been reported that p21-activated kinase phosphorylates MRLC at Ser-19 [13,16], and plays a key role in the determination of cell polarity in lamellipodium formation leading to directional movement [16]. Rho-kinase, which mono- and diphosphorylates MRLC [9,15], is essential for detachment of the rear of migrating leucocytes [17]. A fluorescence resonance energy transfer-based biosensor study showed that there is a strong correlation between activated MLCK and phosphorylated MRLC within lamellipodia in PTK-2 cells [18]. However, no evidence regarding how the phosphorylated MRLC acts during the motility of living cells has been provided to date, whereas there is much indirect evidence in support of the regulation of cell motility through MRLC kinases.

It is likely that myosin II is highly activated at the region where diphosphorylated MRLC localizes intensively. To a lesser extent, activation of myosin II may be observed at the region where monophosphorylated MRLC accumulates. A current study showed the distribution of both mono- and diphosphorylated form of MRLC in the migrating fibroblasts [2]. Accordingly the highly activated myosin II localizes in the restricted regions at both the leading edge and the posterior region of the cells, suggesting that activated myosin II plays some important roles in polarity and directionality in cell migration. However, to what extent the activated myosin II affects the migrating speed of living cells has not been assessed to date, since such analyses could not be carried out with the conventional approaches using fixed cells.

In the present study, several green fluorescent protein (GFP)-tagged recombinant MRLCs were constructed and introduced into HeLa cells to determine the effect of highly activated form of myosin II produced via diphosphorylation of MRLC, on the motility of living cells. The mutant MRLCs include phosphorylatable wild-type MRLC (Wt-MRLC), an unphosphorylatable form in which both Thr-18 and Ser-19 were substituted with Ala (AA-MRLC) and pseudophosphorylated forms in which Thr-18 and Ser-19 were replaced with Ala and Asp, respectively (AD-MRLC), and both Thr-18 and Ser-19 were replaced with Asp (DD-MRLC). As previously reported, AA-MRLC and AD-/DD-MRLC mimic the unphosphorylated and phosphorylated forms of MRLC, respectively [19,20]. The present study describes the role of myosin II activity at the leading edge in the motility of migrating cells.

EXPERIMENTAL

Chemicals

PP1, a specific antibody against diphosphorylated MRLC (recognizing Lys^{12}-Arg-Pro-Gln-Arg-Ala-phosphoThr-phosphoSer-Asn-Val-Phe^{20} in MRLC), was used as described previously [21,21a]. Secondary antibody of Alexa Fluor 568 anti-rabbit IgG (H + L) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Anti-GFP and anti-MRLC antibodies were purchased

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from Clontech (Palo Alto, CA, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Plasmid construction

Three recombinant MRLC cDNAs were generated as described previously [5]. The wild-type and mutated cDNAs of MRLC fragments were amplified by PCR and subcloned into the pEGFP-N1 vector (Clontech).

Cell culture and transfection

HeLa cells were grown in Eagle's minimal essential medium (MEM; Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY, U.S.A.) in 10 cm culture dishes. Before 24 h transfection, HeLa cells were plated for indirect immunofluorescence analysis and time-lapse recording at 10^4 cells/3.5 cm dish with normal coverslips and CELLocate coverslips (Eppendorf, Hamburg, Germany), respectively.

For transfection, HeLa cells were incubated for 4 h in 1 ml of Opti-MEM containing 1 μg of DNA and 6 μl of lipofectamine (Gibco-BRL, Rockville, MD, U.S.A.). Then, the medium was replaced with MEM containing 10% fetal bovine serum. After culturing for 24 h, cells were treated as described below.

Immunoblotting and indirect immunofluorescence

Transfected cells were collected with $5 \times$ SDS sample buffer [22] and cell pellets were homogenized. Each sample was separated by SDS/PAGE and then blotted on to PVDF membranes (Immobilon, Millipore, Tokyo, Japan). SDS/PAGE, immunoblotting and an indirect immunofluorescence analysis were performed as described elsewhere [3,21]. Images of immunostained cells 30 min after wounding were captured under a FLUOVIEW FV300 confocal microscope (Olympus, Tokyo, Japan) and all images were processed with custom software.

Immunoprecipitation

For immunoprecipitation, the transfected cells were washed with PBS containing 0.2 mM EGTA, 5 mM $MgCl_2$ 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, 10% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μM leupeptin, 1 μM pepstatin A and 1 mM PMSF]. The cell lysates were preincubated with Protein G–Sepharose beads (Amersham Biosciences, Little Chalfont, Bucks, U.K.) for 1 h at 4 °C. After centrifugation, the cell lysates were incubated with absence or presence (1 μg) of anti-GFP antibody for 1 h at 4 °C. Protein G–Sepharose beads were added to the cell lysates and incubated for 1 h at 4 °C. The beads were washed three times with the ice-cold RIPA buffer, washed with PBS and stored at 0 °C.

Time-lapse video microscopy

The wild-type and mutant MRLC-expressing HeLa cells were prepared as described above and incubated at 37 °C in 5% CO_2. A constant pH of 7.0–7.4 and temperature of 37 °C were maintained throughout the experiments. Phase-contrast images were captured sequentially at 1 h intervals for 5 h using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) with the 10 $\times$

objective. The transfected cells were identified by the green fluorescent signals of GFP.

Wound-healing assay

HeLa cells on CELLocate coverslips were transfected with MRLC-encoding constructs as described above, when 90% confluent. The coverslips, which had a microgrid (55 μm), were used to identify the cells in a given frame so that cell migration could be traced in different frames. The cells were treated with a serum-free medium containing 0.1% BSA 24 h after transfection and incubated for a further 24 h. After serum starvation, a scratch, mimicking wounding, was made in the cell monolayer using a rubber policeman. Afterwards, the cells were washed twice with PBS and incubated in MEM at 37 °C in 5% CO_2, and phase-contrast images of migrating cells were captured as described above.

To assess the distance of cell migration within 5 h, cells showing strong GFP signals were selected, but dividing cells were excluded. An outline of the cell was traced and the co-ordinates (x, y) of the geometrical cellular centroids in each of the sequential images were determined using NIH image software. Then, the migration distance was calculated as follows:

$$\text{Migration distance} = [(x_t - x_0)^2 + (y_t - y_0)^2]^{1/2}$$

where $t = 5$ h. The means $\pm$ S.D. were determined for at least 14 cells in two independent experiments.
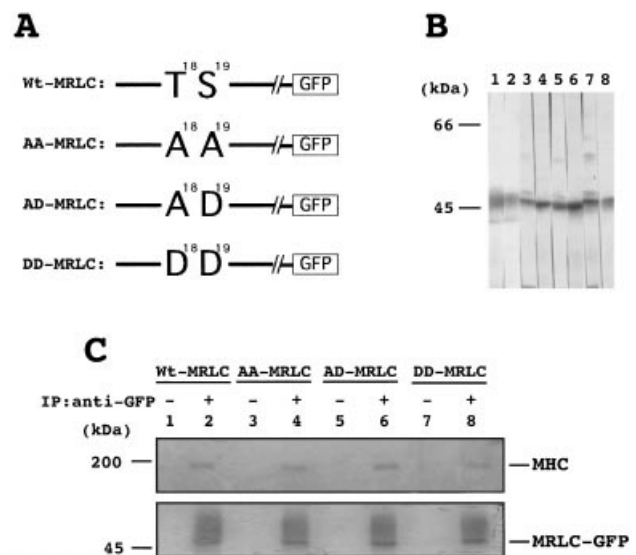

Figure 1   Expression of recombinant MRLCs in HeLa cells

(**A**) Recombinant MRLCs: Wt-MRLC, AA-MRLC, AD-MRLC and DD-MRLC. A GFP-tag was added to the C-terminal of each recombinant MRLC. (**B**) Extracts from HeLa cells transiently expressing GFP-tagged wild-type and mutant MRLCs were immunoblotted with anti-GFP (lanes 1, 3, 5 and 7) or anti-MRLC (lanes 2, 4, 6 and 8) antibodies. Lanes 1 and 2, lysates of Wt-MRLC-transfected cells; lanes 3 and 4, 5 and 6, and 7 and 8, lysates of AA-MRLC-, AD-MRLC- and DD-MRLC-transfected cells, respectively. (**C**) The cell lysates of HeLa cells transfected with recombinant MRLCs were immunoprecipitated with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) anti-GFP antibody and subjected to SDS/PAGE. Lanes 1 and 2, lysates of Wt-MRLC-transfected cells; lanes 3 and 4, 5 and 6, and 7 and 8, lysates of AA-MRLC-, AD-MRLC- and DD-MRLC-transfected cells, respectively. Upper panel: Coomassie Brilliant Blue staining. Lower panel: immunoblotting for anti-GFP antibody. The positions of molecular-mass markers (kDa) are indicated.
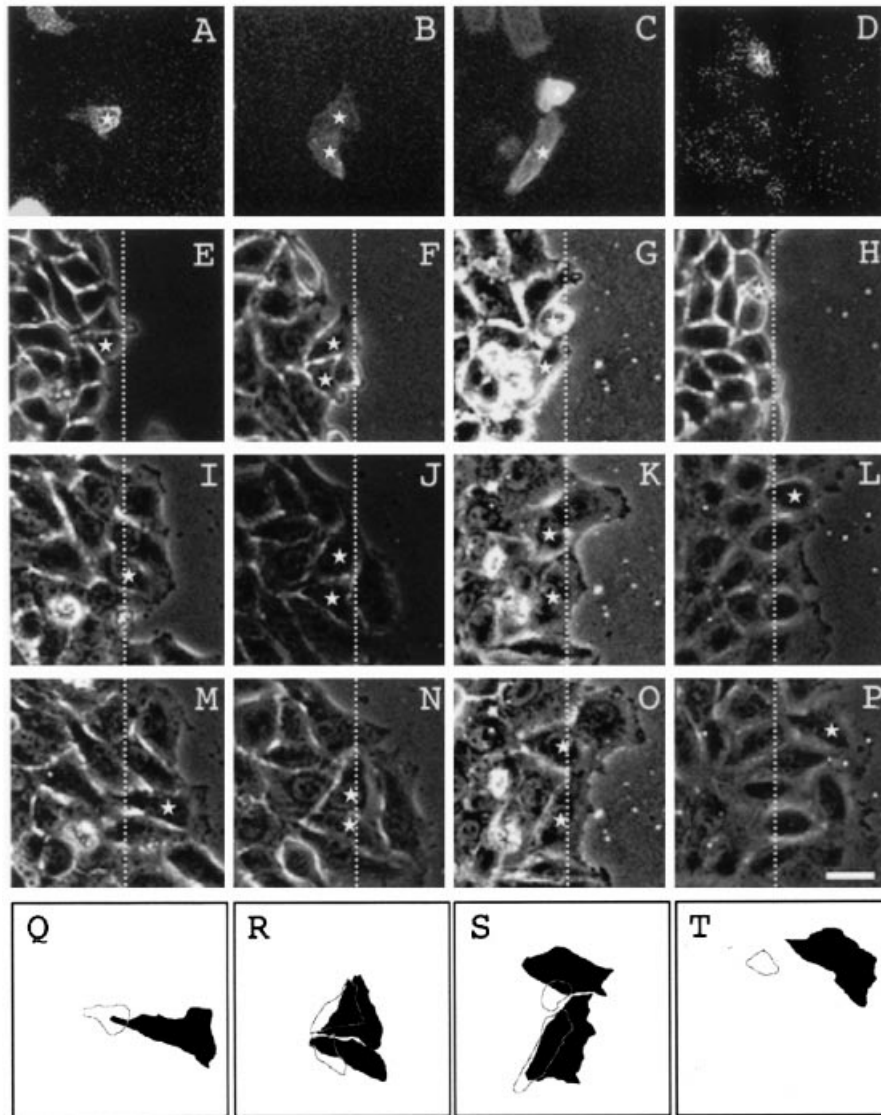
Figure 2   Effect of recombinant MRLC expression on the unidirectional cell migration stimulated by wounding

Wt-MRLC- (**A**, **E**, **I**, **M** and **Q**), AA-MRLC- (**B**, **F**, **J**, **N** and **R**), AD-MRLC- (**C**, **G**, **K**, **O** and **S**) and DD-MRLC- (**D**, **H**, **L**, **P** and **T**) transfected HeLa cell monolayers were scratched, and sequential images were taken as described in the Experimental section. GFP signals were used to identify the transfected cells 5 min after wounding (**A–D**). Phase-contrast images of HeLa cells at the wounded edge (dotted lines) 5 min, 3 h and 5 h after wounding are shown in **E–H**, **I–L** and **M–P**, respectively. **Q–T** indicate wild-type or mutant MRLC-expressing cells migrating toward the wounded space; the black and white images in these panels represent migrating cells 5 min and 5 h after wounding, respectively. Stars indicate the transfected cells. Scale bar, 50 μm.

Table 1   Quantitative analysis of wound-stimulated unidirectional migration of HeLa cells

The 5 h migration distance of mock (untransfected), Wt- and mutant MRLC-expressing cells was calculated as described in the Experimental section. *, $P < 0.01$ compared with AA-MRLC; †, $P < 0.001$ compared with DD-MRLC (Student's t test). Values represent means $\pm$ S.D. from two independent experiments; at least 14 cells were scored for each experiment.

Type of MRLC	Migration after 5 h (μm)
Mock	83.9 ± 33.3*
Wt-MRLC	85.9 ± 30.5*
AA-MRLC	64.8 ± 25.3†
AD-MRLC	74.4 ± 29.7
DD-MRLC	93.0 ± 35.4

RESULTS

Expression of GFP-tagged MRLCs in HeLa cells

To clarify the role of phosphorylated MRLC in cell movement, GFP-tagged recombinant MRLCs (AA-, AD- and DD-MRLC) were produced as shown in Figure 1(A) and introduced into HeLa cells. Since these constructs were introduced and expressed only transiently, the level of expression changed drastically with time. It was not possible to assess the relative amounts of native and engineered MRLCs present in the cells by Western blotting analysis or indirect immunofluorescence. Immunoblotting with anti-GFP antibody of lysates prepared from transfected cells expressing either wild-type or mutant MRLCs detected a single 46 kDa band (Figure 1B), indicating that the MRLC-GFP
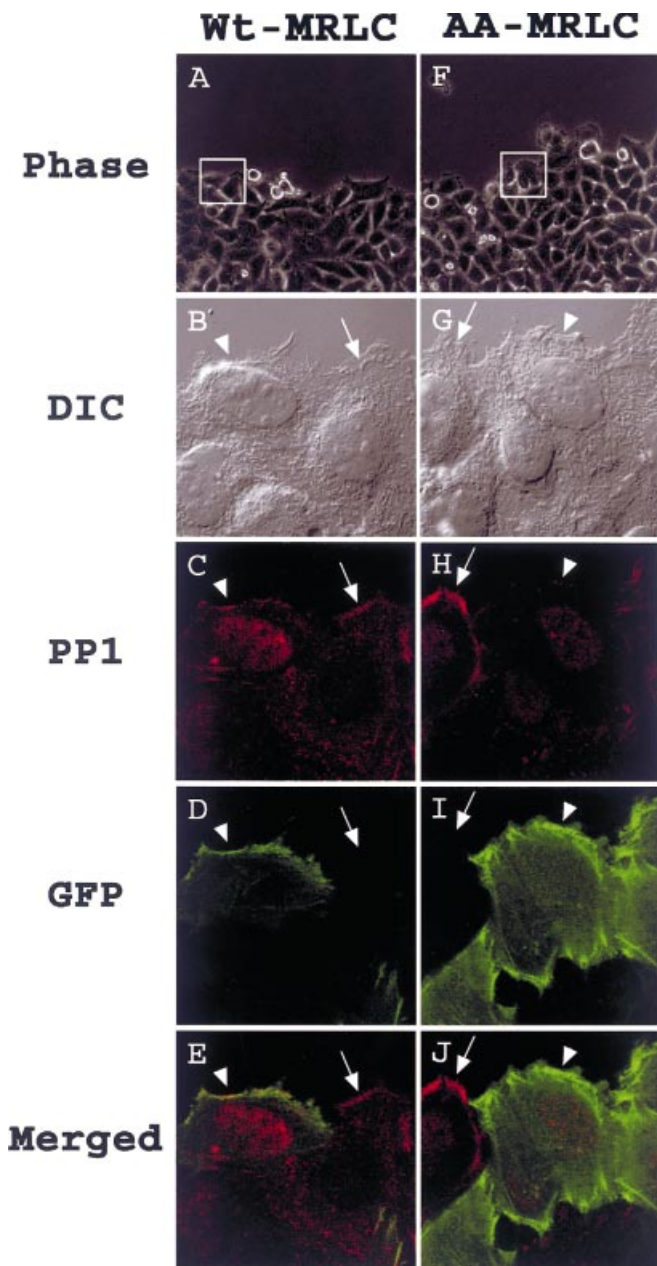
Wt-MRLC   AA-MRLC

Figure 3   Localization of diphosphorylated MRLC in migrating wild-type and AA-MRLC-expressing cells

The accumulation of endogenous diphosphorylated MRLC at the leading edge was suppressed in AA-MRLC-expressing but not Wt-MRLC-expressing cells. Wt-MRLC-expressing cells (**A–E**) and AA-MRLC-expressing cells (**F–J**) were fixed and stained 30 min after wounding with PP1. Phase-contrast images are shown in **A** and **F**. Nomarski differential interference contrast (DIC) images (**B** and **G**) and fluorescent images (**C–E** and **H–J**) correspond to the boxed areas in **A** and **F**, respectively. The cells stained with PP1 are shown in **C** and **H**. The cells expressing GFP-tagged MRLC are shown in **D** and **I**. **E** and **J** are superimposed images of **C** and **D**, and **H** and **I**, respectively. Arrowheads indicate recombinant MRLC-expressing cells; arrows indicate untransfected cells.

chimaeric proteins were successfully expressed in HeLa cells. Also, wild-type and mutant MRLCs were all co-immunoprecipitated with myosin heavy chain, indicating that the exogenously expressed MRLC is exchangeable with endogenous MRLC (Figure 1C).

Effect of mutant MRLCs on cell migration in physically stimulated cells

To investigate the role of phosphorylated MRLC in cell migration, a scrape was made across the mutant and Wt-MRLC-transfected cell monolayers and the cells were forced to migrate unidirectionally. Images of the migrating cells were captured at 1 h intervals for 5 h. Each of the MRLC-expressing cells was detected by GFP signal (Figures 2A–2D). Sequential images of cell migration taken 5 min (Figures 2E–2H), 3 h (Figures 2I–2L) and 5 h (Figures 2M–2P) after wounding are shown in Figure 2. Compared with Wt-MRLC-expressing cells (Figure 2Q), the AA-MRLC-expressing cells migrated only a short distance during 5 h of incubation after wounding (Figure 2R), whereas DD-MRLC-expressing cells migrated to the same extent with Wt-MRLC (Figure 2T). AD-MRLC-expressing cells seemed to migrate only a short distance during 5 h of incubation after wounding as shown in Figure 2(S).

To quantify the effect of mutant MRLCs on cell migration stimulated by wounding as shown in Figure 2, migration distances were calculated as described in the Experimental section. Table 1 shows the comparison of 5 h migration distance among untransfected cells, and wild-type and mutant MRLCs-expressing cells in migration. Wt-MRLC-expressing cells migrated as fast as untransfected cells, indicating that the overexpression of Wt-MRLC did not cause any change in the cell migration. Overexpression of AA-MRLC resulted in lowered migration by 20–30% compared with wild-type and DD-MRLC-expressing cells. In the case of DD-MRLC- and AD-MRLC-expressing cells, no significant differences compared with Wt-MRLC-expressing cells were observed in their migration speed. These results suggest that phosphorylation of MRLC efficiently enhances the cell migration through regulation of myosin II activity.

Suppressed accumulation of diphosphorylated MRLC at the leading edge in AA-MRLC-expressing cells

To understand why AA-MRLC-expressing cells migrated much more slowly than the cells expressing other types of MRLC, the AA-MRLC-expressing cells were stained 30 min after wounding with a specific antibody, PP1, raised against diphosphorylated MRLC [21], and the effect of AA-MRLC expression on the endogenous diphosphorylated MRLC was examined. As shown in Figure 3, PP1 stained the leading edge of Wt-MRLC-expressing cells (Figure 3C, arrowhead) and non-transfected cells (Figures 3C and 3H, arrows) but not AA-MRLC-expressing cells (Figure 3H, arrowhead). Interestingly, AA-MRLC localized at the leading edge of cells as did Wt-MRLC. The results suggest that the expression of AA-MRLC suppresses the accumulation of diphosphorylated MRLC at the leading edge but does not affect the localization of myosin II.

DISCUSSION

To extend our understanding of the role of phosphorylated MRLC in cell migration, we produced AA-MRLC and AD-/DD-MRLC (unphosphorylatable and pseudophosphorylated forms of MRLC, respectively), transfected each construct into HeLa cells and conducted a wound-healing assay to observe the unidirectional and synchronous movement of the cells.

We showed that the expression of AA-MRLC but not DD-MRLC suppressed the migration of HeLa cells, suggesting that the migration of the cells requires the phosphorylation of MRLC. *In vitro* studies showed that AA-MRLC, which is not phos-

phorylated by MLCK [5] or other MRLC kinases [10,14], is incorporated into myosin heavy chain (Figure 1). These data suggest that AA-MRLC functions as an inhibitor of myosin II activity, having dual actions causing the displacement of endogenous MRLC from myosin II and functioning as a direct inhibitor of MLCK or other MRLC-phosphorylatable kinases. Interestingly, myosin II associated with AA-MRLC can be incorporated into the leading edge (Figures 1 and 3), suggesting that phosphorylation of MRLC is not necessarily required for the localization of myosin II in cytoskeletal structures at the leading edge in migrating cells. However, in non-motile cells, previous studies have suggested that phosphorylation of MRLC is required for incorporation of myosin II into cytoskeletal structures [5,23]. In migrating cells, it is likely that the other mechanisms mediated by the regulation by several myosin-binding proteins, such as 38k protein [24] and telokin [25], rather than phosphorylation of MRLC, may regulate the incorporation of myosin II into cytoskeletal structures at the leading edge.

We also analysed AD-MRLC-expressing cells (Table 1). As past studies have shown, mutant MRLC in which Ser-19 is substituted with Asp partially activates myosin ATPase more than AA-MRLC, and less than DD-MRLC [19,20]. Likewise, it is possible that the myosin ATPase was activated partially in AD-MRLC-expressing cells. Therefore, the mean values shown in Table 1 indicate that AD-MRLC expression causes cell migration that between that of AA-MRLC and DD-MRLC, with no statistically significant differences.

Recent studies have shown that diphosphorylated MRLC is localized at the anterior and posterior regions of human fibroblasts [2], and similarly at the leading edge of HeLa cell (this study), suggesting the importance of diphosphorylation of MRLC in cell migration. The localization of diphosphorylated MRLC represents the distribution of the activated form of myosin II in the cells. Our present studies showed that, in AA-MRLC-expressing cells, the accumulation of endogenously diphosphorylated MRLC at the leading edge was not detectable and the migration speed in these cells was lowered by 20–30 % when compared with that in Wt-MRLC-expressing cells. These results suggest that activated myosin II is required for efficient cell migration. Since monophosphorylated MRLC also localizes at the leading edge of HeLa cells [26], the inhibition of cell migration by AA-MRLC expression might be due partly to the suppression of monophosphorylated MRLC. It will be necessary to elucidate and document the relative contribution of mono-phosphorylated and diphosphorylated MRLC in cell migration in the near future.

In epithelial cells, MLCK is highly active in the lamellipodia, suggesting that activated MLCK participates in the regulation of actin-based membrane activity at the leading edge through MRLC phosphorylation, resulting in integrated forward movement [18]. Based on the similarity between the localization of diphosphorylated MRLC observed in this study and the localization of activated MLCK in this other report [18], we speculate that the activated form of MLCK takes part in the diphosphorylation of MRLC at the leading edge in migrating HeLa cells. However, *in vitro* studies show that a non-physiological concentration (0.1 mg/ml) of MLCK is required for diphosphorylation of MRLC [6,7]. Recently, two novel kinases capable of MRLC diphosphorylation were identified as zipper-interacting protein kinase (ZIPK) in HeLa cells [21,27], which diphosphorylates MRLC at a lower concentration (1.25 μg/ml) than MLCK [27], and Rho kinase [15]. Although the localizations of these kinases in the migrating HeLa cells have not been clarified, they may be alternative candidates for causing the diphosphorylation of MRLC at the leading edge during cell migration.

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