Differential localization of non-muscle myosin II isoforms and phosphorylated regulatory light chains in human MRC-5 fibroblasts

Takayuki Saitoh[a], Shuhei Takemura[a], Kozue Ueda[b], Hiroshi Hosoya[b], Masafumi Nagayama[c], Hisashi Haga[c], Kazushige Kawabata[c], Akihiko Yamagishi[a], Masayuki Takahashi[d,*]

[a] *Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[b] *Department of Biological Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan*
[c] *Division of Physics, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[d] *Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*

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Abstract We investigated the localization of non-muscle myosin II isoforms and mono- (at serine 19) and diphosphorylated (at serine 19 and threonine 18) regulatory light chains (RLCs) in motile and non-motile MRC-5 fibroblasts. In migrating cells, myosin IIA localized to the lamella and throughout the posterior region. Myosin IIB colocalized with myosin IIA to the posterior region except at the very end. Diphosphorylated RLCs were detected in the restricted region where myosin IIA was enriched. In non-motile cells, myosin IIA was enriched in peripheral stress fibers with diphosphorylated RLCs, but myosin IIB was not. Our results suggest that myosin IIA may be highly activated by diphosphorylation of RLCs and primarily involved in cell migration. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Myosin II heavy chain isoform; Regulatory light chain; Phosphorylation; Localization; Cell migration; Stress fiber

1. Introduction

Myosin II, one of the best-studied members of the myosin superfamily, plays an important role in cell migration [1,2]. Myosin II-based contraction has been implicated in the generation of forces to maintain a polarized cell shape, and the direction of migration. In migrating *Dictyostelium*, myosin II is concentrated in the posterior region where it plays a role in uropodal retraction [3]. On the other hand, in migrating vertebrate cultured cells it has been demonstrated that myosin II is present in the lamella, as well as in the posterior region, using various methods such as immunofluorescence in fixed cells [4], microinjection of fluorescent analogs [5–7] and electron microscopy [8,9]. The contractility of an actin–myosin II complex both in the lamella and the posterior region is involved in cell migration.

In vertebrate cells, there are at least two different isoforms of the non-muscle myosin heavy chain (MHC) referred to as MHC-IIA and MHC-IIB, which together with two pairs of light chains forms myosin IIA and myosin IIB, respectively [10–13]. To date, subcellular localization of these isoforms has been investigated in order to understand their unique functions at a cellular level [14–20]. These observations suggested that there are differences in localization between myosin IIA and IIB. However, no consensus as to these differences has emerged, including their localization in migrating cells.

The activities of non-muscle myosin II are regulated by phosphorylation of the regulatory light chains (RLCs) at serine 19 in a manner similar to smooth muscle myosin II [21,22]. Phosphorylation at this site increases the actin-activated Mg^{2+}-ATPase activity and the rate of movement of actin filament. Under some conditions, threonine 18 of the RLCs is also phosphorylated in addition to serine 19 [23]. This diphosphorylation results in increasing the actin-activated Mg^{2+}-ATPase activity compared with monophosphorylated myosin at serine 19 [24–26]. It has also been demonstrated that the cytoskeletal fraction of cultured endothelial cells contains monophosphorylated and diphosphorylated myosin II [27]. The localization of monophosphorylated myosin II was demonstrated in motile and non-motile cells [28–31], but that of diphosphorylated myosin II was only demonstrated in cultured smooth muscle cells [32].

In this study, we investigated the immunolocalization of non-muscle myosin II isoforms and their phosphorylated RLCs, in motile and non-motile fibroblasts. Our results indicate that myosin IIA may largely function in order to generate force in migrating cells as well as in stationary cells.

2. Materials and methods

2.1. Antibodies

Anti-human MHC-IIB polyclonal antibody (pAb) was produced against a synthetic peptide, S-D-V-N-E-T-Q-P-P-Q-S-E (carboxyl-terminus of human non-muscle MHC-IIB). Immunization and purification of the antibody were performed according to a published protocol [33]. Anti-MHC-IIA pAb was a kind gift of Dr. Robert S. Adelstein (NHLBI, NIH). Anti-MHC-IIB monoclonal antibody (mAb) was a kind gift of Dr. Tomoaki Shirao (Gunma University). Anti-vinculin mAb was purchased from Sigma-Aldrich. fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody and Cy3-conjugated anti-mouse IgG antibody were purchased from Jackson ImmunoResearch Lab. The antibody P1 was characterized as described previously [30]. The antibody PP1 was produced against a diphosphorylated peptide, K-R-P-Q-R-A-phospho T-phospho S-N-V-F, and the detailed characterization was described in another paper [34].

2.2. Preparation of total cell extracts and immunoprecipitation

MRC-5 cells were obtained from the Riken Cell Bank (Tsukuba,

*Corresponding author. Fax: (81)-11-706 4924.
E-mail address: takahash@sci.hokudai.ac.jp (M. Takahashi).

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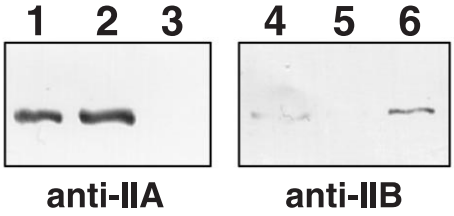

Fig. 1. Expression of non-muscle myosin II isoforms in human MRC-5 fibroblast cells. Lanes 1 and 4 are total cell extracts. Lanes 2 and 5 are immunoprecipitates using anti-MHC-IIA pAb. Lanes 3 and 6 are immunoprecipitates using anti-MHC-IIB pAb. Immunoblot was performed with anti-MHC-IIA (lanes 1–3) or anti-MHC-IIB (lanes 4–6) anti-sera.

Japan) and were grown in MEM1 medium (Nissui) with 10% fetal bovine serum (ICN Biomedicals). Total cell extracts were prepared as follows: cells were homogenized in an extraction buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630 (Sigma-Aldrich), 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM ATP, 0.1 mM PMSF and 5 μg/ml leupeptin) via several passages through a 26-gauge needle. A portion of the homogenate was used as a total cell extract for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). Immunoprecipitation was performed as follows. In brief, the homogenates were centrifuged at 15 000×*g* and the resulting supernatants were immunoprecipitated with anti-MHC-IIA or anti-MHC-IIB pAbs crosslinked to Protein A-Sepharose beads (Amersham Pharmacia Biotech) by rotating overnight at 4°C. The immunocomplexes were washed four times in a wash buffer (20 mM Tris–HCl at pH 7.5, 0.5 M NaCl, 1% IGEPAL CA-630, 5 mM EDTA, 5 mM EGTA and 1 mg/ml BSA) and then rinsed with phosphate-buffered saline (PBS) three times. For SDS–PAGE, the immunoprecipitates were eluted in 2×SDS sample buffer with boiling for 3 min.

2.3. SDS–PAGE and immunoblotting

Total cell extracts and immunoprecipitates were separated on SDS–5% polyacrylamide gels with 0.065% bisacrylamide, using the buffer system of Laemmli [35]. The gel was electro-transferred onto Immobilon transfer membranes (Millipore). The membranes were incubated with 1% non-fat skim milk in PBS at room temperature for 30 min followed by immunostaining with specific anti-sera against MHC-IIA (1:1000) or IIB (1:500) overnight at 4°C. Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibody (1:500) (ICN Biomedicals) and 4-chloro-1-naphthol as a substrate.

2.4. Indirect immunofluorescence

MRC-5 cells grown on coverslips were fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 10 min and rinsed with PBS three times. The fixed cells were incubated with primary antibodies for 1 h at the following concentrations: anti-MHC-IIA pAb (1:1500), anti-MHC-IIB pAb (1:1500), anti-MHC-IIB mAb (1:2), P1 (1:20), PP1 (1:10) and anti-vinculin mAb (1:500). Indirect immunolabeling was performed by incubation with FITC-conjugated anti-rabbit IgG antibody (1:200) and Cy3-conjugated anti-mouse IgG antibody (1:500) as secondary antibodies for 1 h. The cells were examined using a conventional fluorescence microscope (BX50WI, Olympus), equipped with a color chilled 3CCD camera (M3204-C, Olympus). A confocal laser scanning microscope (PCM2000, Nikon) was used to visualize the localization of vinculin.

3. Results

3.1. Subcellular localization of non-muscle myosin IIA and IIB in motile and non-motile fibroblasts

In order to understand the function of non-muscle myosin II isoforms during cell migration, we investigated the localization of myosin IIA and IIB in motile and non-motile fibroblasts. Immunoblot analyses of total cell extracts demonstrated that both isoforms of non-muscle myosin II were expressed in human MRC-5 fibroblast cells (Fig. 1). Fig. 1 also shows that MHC-IIA and MHC-IIB were immunoprecipitated separately with isoform-specific antibodies, indicating that MHC-IIA and MHC-IIB do not form heterodimers in MRC-5 cells.

The localization of these isoforms was visualized by indirect immunofluorescence using anti-MHC-IIA pAb and anti-MHC-IIB mAb. In migrating cells, the fluorescent signal of myosin IIA was strongly detected close to the leading edge, as well as throughout the posterior region (Fig. 2A). It was noted that the signal was detected along transverse fiber structures in the lamella. Myosin IIB colocalized with myosin IIA in the posterior region except at the very end, but it was low or absent in the anterior region (Fig. 2B,C). That is, the isoform preferentially located in the anterior region of migrating fibroblasts was myosin IIA (Fig. 2C). In non-motile cells, both isoforms were detected in stress fibers (Fig. 2D,E). Myosin IIA was found throughout stress fibers, but myosin IIB was low or absent in the peripheral fibers (Fig. 2F).

3.2. Distribution of mono- and diphosphorylated myosin RLCs in motile and non-motile fibroblasts

The antibodies referred to as P1 and PP1 specifically react

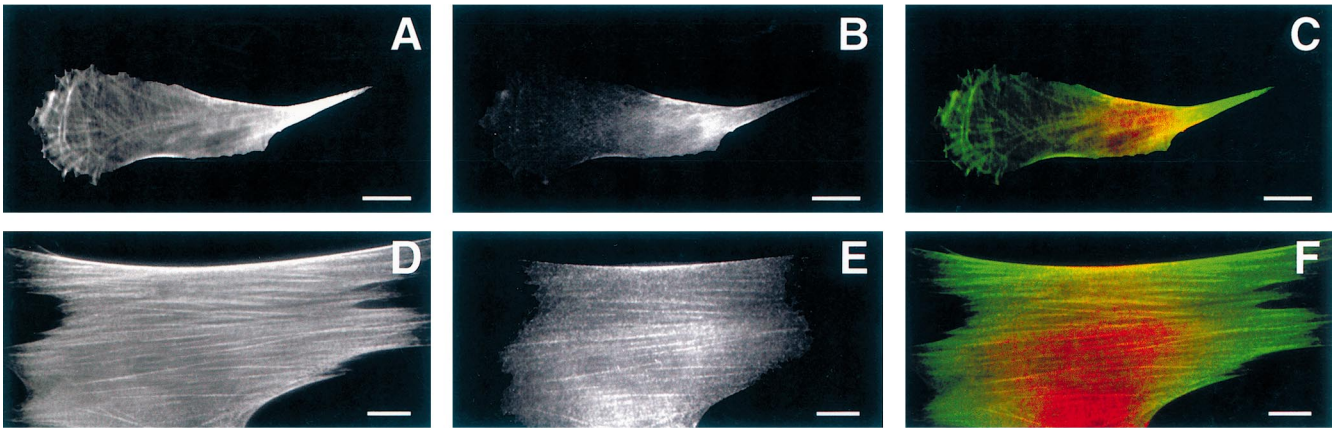

Fig. 2. Subcellular localization of non-muscle myosin II isoforms in MRC-5 cells. Myosin IIA (A,D) was detected using anti-MHC-IIA pAb and an FITC-conjugated secondary antibody. Myosin IIB (B,E) was detected using anti-MHC-IIB mAb and a Cy3-conjugated secondary antibody. A–C: Motile cells. D–F: Non-motile cells. C,F: Merged images. The green and red colors indicate myosin IIA and IIB, respectively. Yellow indicates area of colocalization. Bars: A–C: 20 μm; D–F: 10 μm.

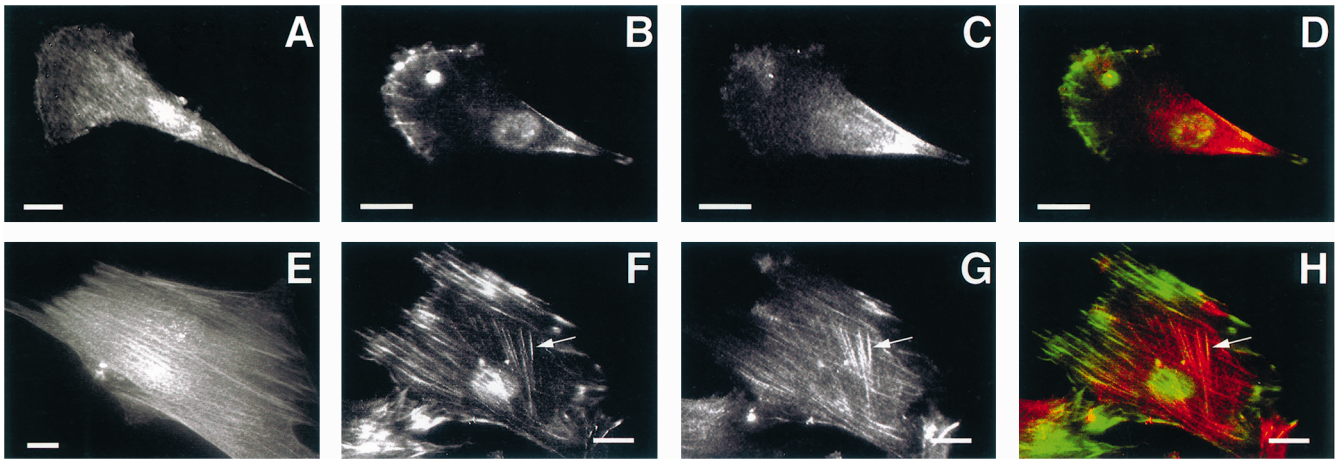

Fig. 3. Distribution of phosphorylated RLCs and myosin IIB in MRC-5 cells. Monophosphorylated RLCs (A,E) were detected with P1 and an FITC-conjugated secondary antibody. Diphosphorylated RLCs (B,F) were detected using PP1 and an FITC-conjugated secondary antibody. Myosin IIB (C,G) was detected using anti-MHC-IIB mAb and a Cy3-conjugated secondary antibody. A–D: Motile cells. E–H: Non-motile cells. D: Merged image of B and C. H: Merged image of F and G. The green and red colors indicate diphosphorylated RLCs and myosin IIB, respectively. Yellow indicates area of colocalization. Arrows indicate shortened stress fibers (F–H). Bars: A–D, F–H: 20 μm; E: 10 μm.

with monophosphorylated (at serine 19) and diphosphorylated (at serine 19 and threonine 18) RLCs, respectively. In migrating cells, P1 stained throughout the posterior region strongly, and the anterior region close to the leading edge stained slightly (Fig. 3A). PP1 stained in limited regions compared to P1, i.e. some fiber structures including transverse fiber-like structures close to the leading edge and also a portion of the cell cortex in the posterior region (Fig. 3B). Merged images of PP1 and anti-MHC-IIB mAbs revealed that the myosin II isoform which was highly activated in the anterior region was not myosin IIB (Fig. 3D). In non-motile cells, P1 stained throughout stress fibers (Fig. 3E). PP1 stained the peripheral stress fibers in which myosin IIA was enriched but myosin IIB was absent (Fig. 3F–H). These results suggest that only myosin IIA is especially activated because the distribution of diphosphorylated RLCs was similar to that of the myosin IIA heavy chains. Although colocalization of myosin IIB and diphosphorylated RLCs was observed in shortened stress fibers (Fig. 3H), myosin IIA was also present (Fig. 4A). Unfortunately, we could not perform double-staining of MHC-IIA and diphosphorylated RLCs, because the anti-MHC-IIA pAb and PP1 were both rabbit polyclonal antibodies. P1 and PP1 also stained nuclei, but the significance of this is unclear.

3.3. Localization of diphosphorylated RLCs and focal adhesion (FA) protein

In order to understand the relation between the distribution of highly activated myosin II molecules in stress fibers and FAs, we observed non-motile cells double-stained with PP1 and anti-vinculin mAb by confocal microscopy. Myosin IIA was present in stress fibers whose ends linked to FAs (Fig. 4A). However, the tips of stress fibers immunostained with myosin IIB mAb did not link to FAs (Fig. 4B). PP1 stained the peripheral stress fibers whose ends linked to FAs as well as myosin IIA (Fig. 4C). It was noted that diphosphorylated RLCs were detected throughout shortened stress fibers whose ends linked to FAs (Fig. 4D).

4. Discussion

In polarized migrating cells, myosin IIA was distributed within lamellae and throughout the posterior regions of cells. On the other hand, myosin IIB was low or absent in the anterior region but was detected in a part of the posterior region. These results are similar to the observation in living endothelial cells [18], but the difference between myosin IIA and IIB was much more obvious in the fibroblast cells in this study. In addition, we show here that the diphosphorylated RLCs dramatically localized in some fibers including transverse fibers in lamella where myosin IIA was present. This suggests that myosin IIA within lamella is highly activated by phosphorylation at both serine 19 and threonine 18 of its RLCs. It is thought that contraction of an actin–myosin II network in lamella is coupled to forward translocation of the cell body [2]. In particular, the contraction of transverse fibers within lamellae could be necessary to maintain a polarized cell shape and the direction of migration [6]. In the posterior region of migrating cells, monophosphorylated RLCs were diffusely distributed in the cytoplasm, which was similar to earlier studies [28,29], whereas diphosphorylated RLCs were concentrated in a part of the cell cortex. At present, it is unclear which isoform of myosin II was diphosphorylated, because both isoforms colocalized there. Myosin II-mediated contraction is believed to generate force to retract the cells in the posterior region [1,2], and maintain a polarized cell shape and direction of migration by inhibiting extension of lamellipodia in the lateral and posterior region [6,36]. Our results indicate that highly activated myosin II could be related to the latter function. Our observations in polarized migrating cells suggest that myosin IIA would primarily be responsible for cell migration in general and myosin IIB would support the function of myosin IIA in the posterior region.

In non-motile fibroblasts, we observed that both isoforms of myosin II were located in stress fibers but that the periphery was enriched in myosin IIA. This distribution of non-muscle myosin II isoforms in non-motile cells is similar to

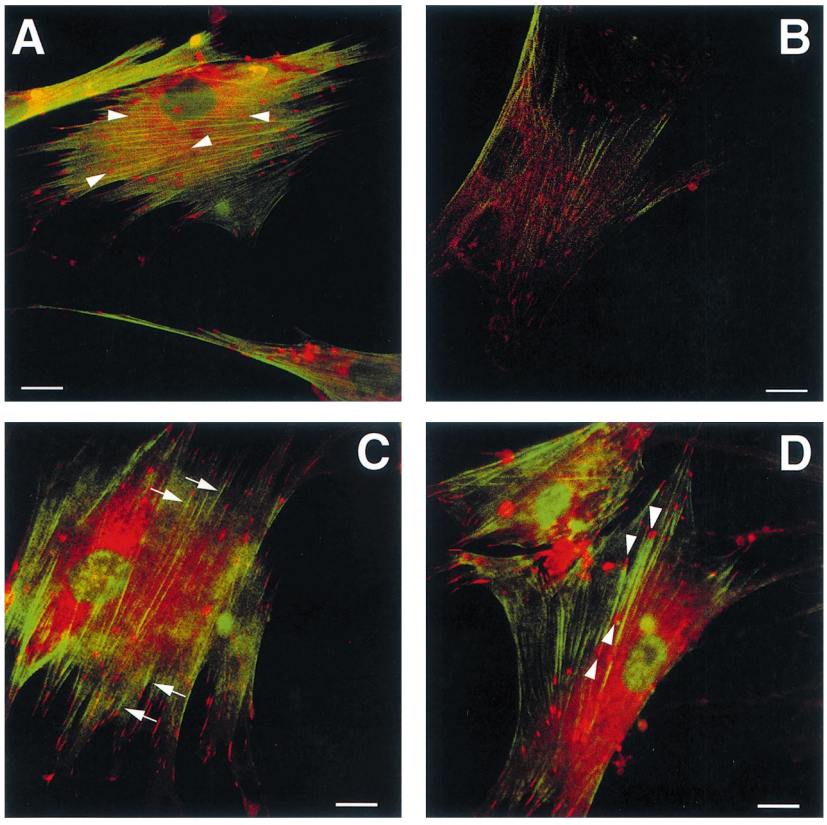

Fig. 4. Confocal images of the localization of vinculin and myosin II isoforms or diphosphorylated RLCs in non-motile MRC-5 cells. Myosin IIA (A) and IIB (B) were detected using anti-MHC-IIA and anti-MHC-IIB pAbs, respectively. Indirect immunolabeling was performed using FITC-conjugated secondary antibody (green). Diphosphorylated RLCs (C,D) were detected using PP1 and an FITC-conjugated secondary antibody (green). Vinculin was detected using anti-vinculin mAb and a Cy3-conjugated secondary antibody (red). Arrows indicate the peripheral stress fibers (C). Shortened stress fibers are indicated between the arrowheads (A,D). Bars: 20 μm.

observations in interphase melanoma cells [15]. The peripheries of stress fibers are linked to FAs. Smilenov et al. demonstrated that FAs are highly motile toward the cell center in non-motile cells and that the contractility of myosin II is necessary for this FA movement [37]. They suggest that cells are able to polarize rapidly toward migratory cues by maintaining FAs in a motile 'idling' state. It has been demonstrated that myosin IIA is required for the function of FAs in neuroblastoma cells [38] and in glial cells [39]. We showed that diphosphorylated RLCs were enriched in the peripheral stress fibers, where myosin IIB was absent but myosin IIA was present. Thus, myosin IIA might be preferentially diphosphorylated and highly activated in non-motile cells also, since it may be required for the idling state of FAs. We also showed that diphosphorylated RLCs were found throughout shortened stress fibers. Our data suggests that myosin IIA would be predominantly diphosphorylated, although both isoforms are present in the shortened stress fibers.

Which kinase(s) phosphorylate non-muscle myosin II at both serine 19 and threonine 18? It is known that myosin light chain kinase (MLCK) phosphorylates at both serine 19 and threonine 18 of RLCs. However, phosphorylation of threonine 18 by MLCK is much slower than that of serine 19 [23]. This indicates that threonine phosphorylation may not be due to MLCK. Recently, zipper-interacting protein kinase (ZIPK) was found to phosphorylate the RLC of non-muscle and smooth muscle myosin II at both serine 19 and threonine 18 [40,41]. Phosphorylation of the RLC at both serine 19 and threonine 18 occurs at the same rate in smooth muscle cells [41]. Consequently, it is possible that ZIPK could phosphorylate the RLC of non-muscle myosin II at both serine 19 and threonine 18 in motile and non-motile fibroblasts.

In conclusion, we have demonstrated that non-muscle myosin IIA and IIB revealed a distinct pattern of localization and, moreover, myosin IIA may be preferentially phosphorylated at both serine 19 and threonine 18. Interestingly, diphosphorylated RLCs showed more restricted distribution than monophosphorylated RLCs. These results raise the possibility that non-muscle myosin II isoforms may participate in cell migration in an isoform-dependent manner.

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