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Two Regions of the Tail Are Necessary for the Isoform-specific Functions of Nonmuscle Myosin IIB

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To function in the cell, nonmuscle myosin II molecules assemble into filaments through their C-terminal tails. Because myosin II isoforms most likely assemble into homo-filaments in vivo, it seems that some self-recognition mechanisms of individual myosin II isoforms should exist. Exogenous expression of myosin IIB rod fragment is thus expected to prevent the function of myosin IIB specifically. We expected to reveal some self-recognition sites of myosin IIB from the phenotype by expressing appropriate myosin IIB rod fragments. We expressed the C-terminal 305-residue rod fragment of the myosin IIB heavy chain (BRF305) in MRC-5 SV1 TG1 cells. As a result, unstable morphology was observed like MHC-IIB/H11546. This phenotype was not observed in cells expressing BRF305 mutants: 1) with a defect in assembling, 2) lacking N-terminal 57 residues (N-57), or 3) lacking C-terminal 63 residues (C-63). A myosin IIA rod fragment ARF296 corresponding to BRF305 was not effective. However, the chimeric ARF296, in which the N-57 and C-63 of BRF305 were substituted for the corresponding regions of ARF296, acquired the ability to induce unstable morphology. We propose that the N-57 and C-63 of BRF305 are involved in self-recognition when myosin IIB molecules assemble into homo-filament.

INTRODUCTION

Myosins constitute a large superfamily of actin-based molecular motors (Sellers, 1999; Berg et al., 2001). Nonmuscle myosin II, one of the members of the superfamily, is involved in various cell functions such as cytokinesis (Robinson and Spudich, 2004; Matsumura, 2005) and migration (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). In mammalian cells, there are three isoforms of the nonmuscle myosin II heavy chain (MHC) referred to as MHC-IIA, MHC-IIB, and MHC-IIIC, which together with two pairs of light chains form myosin IIA, myosin IIB, and myosin IIIC, respectively (Berg et al., 2001; Golomb et al., 2004). Recently, it has been shown that each myosin II isoform has specific functions. Concerning two of the isoforms, myosin IIA and myosin IIB, the functional differences are suggested by the embryonic lethal phenotypes after their ablation in mice (Tullio et al., 1997; Conti et al., 2004). From the unstable morphology of MHC-IIB−/− cells, it was clearly demonstrated that myosin IIB is involved in the guidance of fibroblast migration by coordinating protrusive activities and stabilizing cell polarity (Lo et al., 2004). Because this defect was rescued by the exogenous expression of MHC-IIB but not MHC-IIA, it is probable that the specific functions of each myosin II isoform could not be complemented by the other completely.

Myosin II functions by assembling of monomers into filaments (Craig and Woodhead, 2006). Carboxyl terminal α-helical coiled-coil rod-like tails are involved in assembly through electrostatic interaction (McLachlan, 1984), and critical regions for the assembly are located in its carboxyl-terminus (Hodge et al., 1992; Sohn et al., 1997; Nakasawa et al., 2005). In nonmuscle cells, dynamic assembly/disassembly of myosin II molecules is organized spatiotemporally in response to various signals. To function effectively, it is reasonable to consider that they assemble in an isoform-specific mode to form homo-filaments (homogeneous with respect to their subunit composition) in the cell because of differences in their subcellular localization (Maupin et al., 1994; Rochlin et al., 1995; Kolega, 1998, 2003; Saitoh et al., 2001) as well as their ATPase and motor activities (Kelley et al., 1996; Golomb et al., 2004). However, hetero-filaments were formed when their carboxyl-terminal rod fragments were mixed in vitro (Murakami et al., 2000). These gave us an idea that some self-recognition mechanisms of individual myosin II isoforms might exist and result in myosin II isoforms assembling into homo-filaments in the cell.

Because filament formation is necessary for myosin II to function, exogenous expression of rod fragment containing the critical regions for assembly could exhibit a dominant negative effect to prevent the normal assembly of endogenous myosin II. In fact, it was shown that a 72-kDa rod fragment of MHC-IIB acts as a dominant-negative form and induced aberrant cell shape (Ben-Ya’acov and Ravid, 2003). This study induced us to see the effects of exogenous expression of the rod fragments of each isoform in the cell. If some sites responsible for self-recognition are found to reside in the expressed rod fragments, they would be expected to bind the corresponding myosin II isoform and inhibit the function in an isoform-specific manner. We expected to reveal the self-recognition sites of myosin IIB from the pheno-
type with aberrant cell shape by expressing appropriate myosin IIB rod fragment.

In this article, we demonstrated that the myosin IIB rod fragment, BRF305 (Phe 1672-Glu 1976), can inhibit the function of endogenous myosin IIB by inhibiting normal filament assembly. Moreover, we demonstrated that the self-recognition sites reside in the N-terminal 57 and the C-terminal 63 residues of BRF305.

MATERIALS AND METHODS

Cell Culture

MRC-5 SV1 TG1 cells, SV40-transformant of human embryonic lung fibroblast MRC-5, were obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in RITC 80-7 medium (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum (BioSource, Rockville, MD), 50 U/ml penicillin, and 50 µg/ml streptomycin under the condition of 37°C, humidified 5% CO2.

Antibodies and Reagents

Anti-MHC-IIA polyclonal antibody (pAb) was produced against a synthetic peptide, Cy5-Lys-Ala-Asp-Gly-Ala-Glu-Ala-Lys-Pro-Ala-Glu (carboxy terminus of MHC-IIB). Anti-MHC-IIB (C-term) pAb against carboxy terminus of MHC-IIB was described previously (Saitoh et al., 1994). Anti-MHC-IIA (C-term) pAb against carbohydrate terminus of MHC-IIA was described previously (Saithob et al., 2001). Anti-MHC-IIB (N-term) pAb against amino terminus of MHC-IIB was a kind gift from Dr. Robert S. Adelstein (National Heart, Lung, and Blood Institute). Anti-β-tubulin mAb and TRITC-labeled phalloidin were purchased from Sigma (St. Louis, MO), and Phe 1672-Glu 1976 of human nonmuscle myosin II (nonmuscle MHC-IIA with extra Cys at the N-terminus). Immunization and purification of the antibody were performed according to the protocol described previously (Takahashi et al., 1999). Anti-MHC-IIA (C-term) pAb against carbohydrate terminus of MHC-IIA was described previously (Saitoh et al., 2001). Anti-MHC-IIB (N-term) pAb against amino terminus of MHC-IIB was a kind gift from Dr. Robert S. Adelstein (National Heart, Lung, and Blood Institute). National Institutes of Health, Bethesda, MD). Anti-green fluorescent protein (GFP) mAb was purchased from Roche Applied Science (Indianapolis, IN). Anti-a-tubulin mAb and TRITC-labeled phalloidin were purchased from Sigma (St. Louis, MO). Cy3-conjugated anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Construction of Plasmid DNA

The DNA fragment encoding Asp 1729-Glu 1976 of human nonmuscle MHC-IIB (BRF305) was amplified from a human brain cDNA library (Clontech, Mountain View, CA) as a template by PCR. The fragment was subcloned into the HindIII-BamHI sites of pEGFP-C3 (Clontech) to generate the pEGFP-ARF296exN and pEGFP-ARF296exC, pEGFP-ARF296exN, and pEGFP-ARF296exC were constructed using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol using primer sets described in Table 1 and pEGFP-ARF296 as a template. Plasmids for expressing the chimeric rod fragments of ARF296 were constructed by using a two-step PCR according to the method of Geiser et al. (2001). In brief, first PCRs were performed using pEGFP-ARF296exN or pEGFP-ARF296exC as templates and the appropriate mutagenic primer sets described in Table 1. The mutagenic primers were designed to anneal cDNA of one isoform with additional sequence for another. Then second PCRs were performed using the products from the first PCR as megaprimer and either pEGFP-ARF296exN or pEGFP-ARF296exC as a template for an inverse PCR following the QuikChange protocol. To construct pEGFP-ARF296exN or pEGFP-ARF296exC, pEGFP-ARF296 was used as a template. To construct pEGFP-ARF296exN or pEGFP-ARF296exC, pEGFP-ARF296 was used as a template. To construct pEGFP-ARF296exNexC, pEGFP-ARF296exC was used as a template. The fragments produced by these plasmid DNA constructs are illustrated in Figure 1. DNA sequences were confirmed using a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Transfection

Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol with the following modifications. Cell suspension in Opti-MEM I Reduced Serum Medium (Invitrogen) was mixed with DNA-Lipofectamine 2000 complexes and then plated on a 24-well plate. After incubation for 5 h in culturing condition, cells were replated onto a 35-mm tissue culture dish (Iwaki, Tokyo, Japan) or a collagen type IV (Nitta Gelatin, Tokyo, Japan) coated coverslip. After 24 h, the transfected cells were subjected to subsequent experiments. Transfection efficiency was ~60% in all cases.

SDS-PAGE and Immunoblotting

Transfected cells were washed three times with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4) and were treated with SDS lysis buffer (1% SDS, 6.25 mM Tris-HCL pH 6.8, 10% glycerol, 10% 2-mercaptoethanol). The lysate was collected and boiled for 5 min. SDS-PAGE was performed using the buffer system of Laemmli (1970). The separated polypeptides were electrotransferred onto Immobilon-P membrane (Millipore, Billerica, MA). The membrane was preincubated with 5% skim milk and 0.1% Tween-20 in PBS for 60 min. The membrane was incubated with primary antibodies for 60 min and secondary antibody for 30 min at 23°C. The antibodies were diluted as follows: anti-GFP mAb (1:1000), anti-MHC-IIA pAb (1:10,000), anti-MHC-IIA (C-term) pAb (1:10,000), anti-a-tubulin mAb (1:10,000), horse-radish peroxidase–labeled anti-mouse IgG antibody (1:20,000), and horseradish peroxidase–labeled anti-rabbit IgG antibody (1:20,000). The chemiluminescent signals were produced using ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ). The signals were detected using

### Table 1. Primers used for mutagenic PCR

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<tr>
<td>BRF305-m3</td>
<td>5'-GACGACCTTAAAGAACACGCTGAGAGAAGC-3' 5'-CAGCCGCTTCCTCCATCTC-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CATCGGACGGTGGCCTCCATCTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CAGATCTGAGCTCAAGCTTGGCCAGCAGGCA-3'</td>
</tr>
<tr>
<td>BRF305ΔC63</td>
<td>5'-TGGAGGCGCATGAGACGACGACGAGCAGC-3' 5'-CTAGATCCGGTGGATCTACCTTG-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGGAGGCGCATGAGACGACGACGAGCAGC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTAGATCCGGTGGATCTACCTTG-3'</td>
</tr>
<tr>
<td>ARF296exN</td>
<td>5'-GAGCAGCTTAAAGAACACGCTGAGAGAAGC-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GAGCAGCTTAAAGAACACGCTGAGAGAAGC-3'</td>
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<tr>
<td>Reverse</td>
<td>5'-CAGATCTGAGCTCAAGCTTGGCCAGCAGGCA-3'</td>
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<tr>
<td>ARF296exC</td>
<td>5'-TGGAGGCGCATGAGACGACGACGAGCAGC-3'</td>
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<tr>
<td>Forward</td>
<td>5'-TGGAGGCGCATGAGACGACGACGAGCAGC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTAGATCCGGTGGATCTACCTTG-3'</td>
</tr>
<tr>
<td>BRF305exN</td>
<td>5'-GAGCAGCTTAAAGAACACGCTGAGAGAAGC-3'</td>
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<tr>
<td>Reverse</td>
<td>5'-CAGATCTGAGCTCAAGCTTGGCCAGCAGGCA-3'</td>
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* Letters with an underscore and boldface letters with an underscore indicate sequences annealing to pEGFP-BRF305 and pEGFP-ARF296 in first-step PCR, respectively.
Figure 1. Schematic diagrams of myosin II rod fragments and a full-length myosin II. The myosin II rod fragments were expressed as N-terminal GFP-fused proteins. Numbers indicate amino acid residues of MHC-IIA (for ARF296) and MHC-IIB (for other fragments). Dark and light grays represent the portions derived from myosin IIB and myosin IIA, respectively.

Immunoprecipitation
The transfected cells were lysed with lysis buffer (30 mM NaCl, 0.1% IGEPAL CA-630, 20 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 5 μg/ml aprotinin, 20 mM NaF, 1 mM Na3VO4). After centrifugation (22,000 g) for 15 min at 4°C, anti-MHC-IIB (N-term) pAb was added to the supernatant, which was followed by incubation for 60 min at 4°C. The immunocomplexes were captured by protein G Sepharose beads (GE Healthcare) and then collected by centrifugation (14,000 g) for 5 s. The beads were washed three times in a wash buffer (50 mM NaCl, 0.1% IGEPAL CA-630, 20 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 5 μg/ml aprotinin, 20 mM NaF, 1 mM Na3VO4). After centrifugation (22,000 g) for 5 s. The beads were washed three times in a wash buffer (50 mM Tris-HCl, pH 7.5, 0.1% IGEPAL CA-630, 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2). For SDS-PAGE, the immunoprecipitates were eluted in 2× SDS lysis buffer with boiling for 5 min.

Immunofluorescence
In the case of staining by anti-MHC-IIB (C-term) pAb or TRITC-phalloidin, the cells cultured on a collagen (type IC)-coated coverglass were fixed with 3.7% formaldehyde in PBS for 20 min followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. The fixed cells were preincubated with 3% bovine serum albumin in PBS for 30 min. Incubation with the anti-MHC-IIB (C-term) pAb (1:1500) was carried out for 60 min. In the case of staining by anti-MHC-IIB (N-term) pAb, the cells were permeabilized in 0.5% Triton X-100 with 5% sucrose/PBS and 4% paraformaldehyde for 4 min and then fixed in 4% paraformaldehyde with 5% sucrose/PBS for 25 min. The fixed cells were preincubated with 1% bovine serum albumin in PBS for 30 min. Incubation with the anti-MHC-IIB (N-term) pAb (1:1500) was carried out for 2 h. Indirect immunolabeling was performed by incubation with Cy3-labeled anti-rabbit IgG antibody (1:500) containing TRITC-labeled phalloidin (67 ng/ml) and DAPI (10 ng/ml) for 60 min. The images were captured using a conventional fluorescence microscope (BX51WI; Olympus, Tokyo, Japan), equipped with a color chilled 3CCD camera (DP70; Olympus) and an objective lens (UPlanApo 20×/0.70; Olympus). All procedures were performed at 23°C.

Cell Imaging
Twenty-four hours after transfection, the images were captured using an inverted phase-contrast microscope (IX71; Olympus), equipped with a color chilled 3CCD camera (DP70; Olympus) and an objective lens (LCPPlanFl 20×/0.40; Olympus). For time-lapse microscopy, the images were captured every 1 min and analyzed by using Lumina Vision version 2.4.2 software (Fujifilm, Tokyo, Japan). During observation, cells were warmed at 37°C on a thermostate (MAT-SU8R30; Tokai Hit, Shizuoka, Japan).

RESULTS
Myosin IIB Rod Fragment, BRF305, Induces the Phenotype of Unstable Cell Shape Similar to MHC-IIB-/- Fibroblasts
To confirm whether the exogenous expression of myosin IIB rod fragment could inhibit the function of endogenous myosin IIB in the cell, the C-terminal rod fragment of MHC-IIB consisting of the C-terminal 305 amino acid residues (BRF305) was expressed as an N-terminal GFP-fusion protein (GFP-BRF305) in MRC-5 SV1 TG1 cells. In contrast to control cells expressing GFP (Figure 2, A and B), the cells expressing GFP-BRF305 displayed aberrant shape with multiple protrusions.
Isoform-specific Dominant Negative Effect

To verify the importance of the N-57 and C-63 of BRF305 for the isoform-specific dominant negative effect, we constructed five kinds of BRF305-ARF296 chimeric fragments (Figure 1) and analyzed their ability to induce the aberrant phenotype. All chimeric fragments showed an ability to act as dominant negative forms (Figure 4, A–J), although the degree of the effect was different from one another (Figure 4K). The abilities of both BRF305exC and BRF305exN were decreased from that of original BRF305. BRF305exC showed a more notable decrease than BRF305exN did. In contrast, the abilities of both N-57 and C-63 of BRF305 are essential for the isoform-specific dominant negative effect.
ARF296exN and ARF296exC were increased from that of original ARF296. In this case also, C-63 substitution was more effective. Interestingly, ARF296exNC, in which both regions were substituted, gained higher ability than the chimeras with substitution of only one side. Because the expression levels were almost the same among these fragments and significantly higher than that of endogenous MHC-IIB (Figure 4L), the difference of the effect would reflect the properties of each fragment. Time-lapse analysis revealed that the behaviors of GFP-ARF296exNC–expressing cells were quite similar to the GFP-BRF305–expressing cells (Supplementary Movie 4). These results indicate that N-57 and C-63 of BRF305 are both essential for the induction of aberrant cell shape; however, C-63 appears to be more important for this effect.

BRF305 Interacts with Endogenous Myosin IIB through Its N-57 and C-63

Next, we investigated whether the dominant negative effect of BRF305 is caused by the isoform-specific interaction of the exogenous rod fragment and endogenous myosin IIB. Immunoprecipitation of endogenous myosin IIB followed by immunoblotting with the anti-GFP antibodies revealed that GFP-BRF305 but not GFP-ARF296 coprecipitated with endogenous myosin IIB from extracts of the cells expressing each fragment (Figure 5). GFP-ARF296exNC also coprecipitated with endogenous myosin IIB (Figure 5). These results demonstrate that BRF305 interacts with endogenous myosin IIB in an isoform-specific manner and that the N-57 and C-63 of BRF305 are essential for this interaction. The mutant BRF305-m3 with defect in assembly did not coprecipitate with endogenous myosin IIB (Figure 5), indicating that the assembling ability of the rod fragment is necessary for the interaction as expected. These results support the idea that the induction of unstable cell shape is caused by the isoform-specific interaction of BRF305 with endogenous myosin IIB, and the N-57 and C-63 of BRF305, as well as its assembling ability, are essential for the induction.

Cortical Actin Cytoskeleton Including Myosin IIB Disappeared during Expression of BRF305 But Not ARF296

To explore the effect of exogenous expression of GFP-BRF305 on the actin cytoskeleton, we performed TRITC-phalloidin staining. In the control cells expressing GFP, F-actin appeared at the cell cortex as thick fiber structures and also in the cytoplasm as thin fiber structures (Figure 6, A–C). However, in GFP-BRF305–expressing cells, the well-defined actin fiber structures were not observed (Figure 6, D–F). On the other hand, in GFP-ARF296–expressing cells, the cortical actin cytoskeleton including myosin IIB was not significantly affected (Figure 6, G–J). These results indicate that the expression of BRF305 disrupts the cortical actin cytoskeleton in a manner similar to the effect of the dominant negative myosin IIB, while ARF296 does not have this effect.
the control cells, myosin IIB localized strongly at the cell membrane in the rods expressing cells. We performed immunostaining of MHC-IIB to see endogenous myosin IIB localization in the rods expressing cells. GFP and endogenous myosin IIB, respectively. Bar, 20 μm.

Figure 7. Myosin IIB remained at the cell cortex in GFP-ARF296-expressing cells. The cells expressing GFP as control (A–C), GFP-BRF305 (D–F), and GFP-ARF296 (G–I) were fixed and stained with DAPI staining (blue), respectively. The green and red colors indicate GFP and F-actin, respectively. Bar, 20 μm.

DISCUSSION

To clarify the role of vertebrate nonmuscle myosin II in cell motile processes, functional knockdown assays have been carried out, such as microinjection of antibodies against myosin II to a cell (Höner et al., 1988; Zurek et al., 1990). These studies demonstrated that myosin II molecules are involved in maintaining the cell shape in addition to changing cell shape such as cytokinesis. Recently, studies on the treatment of cells with blebbistatin, a specific inhibitor of nonmuscle myosin II ATPase activity, confirmed the function of myosin II (Straight et al., 2003; Nigglì et al., 2006) and also revealed new aspects of the function (Rosenblatt et al., 2004; Ryu et al., 2006). However, these techniques cannot distinguish the function of each myosin II isoform.

Recently, information concerning the specific functions of myosin IIB have been increased by the studies of cells isolated from MHC-IIB knockout mice or small interfering RNA (siRNA)-treated cells. MHC-IIB−/− neurons appeared to have abnormal shapes and motile growth cones (Brown and Bridgman, 2003). MHC-IIB−/− fibroblasts demonstrated instability of cell shape and direction of migration (Lo et al., 2004) and decreased ability to contract 3D collagen gel (Meshel et al., 2005). A defect in cytokinesis was observed in MHC-IIB−/− cardiac myocyte (Takeda et al., 2003) and siRNA-treated COS-7 cells (Bao et al., 2005), possibly because myosin IIA is absent in these cells. Abnormal morphology of dendritic spines was also observed in siRNA-treated neurons (Ryu et al., 2006).

In this article, we showed that exogenous expression of BRF305 in the MRC-5 SV1 TG1 cells induced an unstable cell shape similar to MHC-IIB−/− fibroblasts. We demonstrated this dominant negative effect was likely caused in an isoform-specific manner. Moreover, two regions, N-57 (Leu1988; Zurek et al., 1990). A defect in cytokinesis was observed in MHC-IIB−/− cardiac myocyte (Takeda et al., 2003) and siRNA-treated COS-7 cells (Bao et al., 2005), possibly because myosin IIA is absent in these cells. Abnormal morphology of dendritic spines was also observed in siRNA-treated neurons (Ryu et al., 2006).

How BRF305 can inhibit the endogenous myosin IIB function in the cell and then induce an abnormality in the cell motile processes? Formation of bipolar filament is necessary for myosin II to function, and the dynamic filament assembly-disassembly transition is particularly important in nonmuscle cells. In practice, dynamic exchange of myosin II in the cell cortex was observed in living Dictyostelium cells, and the mutants having a defect in the assembly-disassembly transition could not achieve this dynamic exchange process (Yumura, 2001). We showed here that overexpression of the fragments being able to interact with endogenous myosin IIB could induce the aberrant phenotype (Figures 2, 4, and 5). These results can be understood as follows: BRF305 could interact with a monomer of endogenous myosin IIB that dissociated from the filament during the dynamic assembly-disassembly process, and the resulting myosin IIB-BRF305 complex could not reassemble into normal filaments under the experimental conditions with high expression levels of exogenous BRF305. As a result, functional myosin IIB filaments were lost.
Kolega, 2003; Lo

The unstable phenotype of MHC-IIB localized to the posterior region, especially at the lateral cell and throughout the posterior region, whereas myosin IIB is in migrating fibroblasts, myosin IIA localized to the lamella whereas myosin IIB accumulated in the rear (Kolega, 2003).

endothelial cells, myosin IIA biased toward the front, phosphorylation within this region (Kriajevska et al., 1998; Murakami et al., 2004), it has been suggested that myosin IIB is involved in the maintenance of cell shape and polarity to prevent undesirable protrusion in posterior region. We showed here, that myosin IIB was preferentially localized at the cell cortex with actin fibers in MRC-5 SV1 TG1 cells. This localization still remained in the ARF296-expressing cells, but was not maintained in the BRF305-expressing cells (Figures 6 and 7). Taken together, we speculate that a cell losing functional myosin IIB at the cell cortex cannot maintain the actin network, resulting in the induction of an unstable phenotype.

The present results of the exogenous expression of the deletion mutants (Figure 3) and of the chimeric fragments (Figure 4) revealed that two regions, N-57 and C-63 of BRF305, were important for the induction of aberrant cell shape. In other words, these two regions would be involved in self-recognition of myosin IIB to form homo-filament in the cell. The results indicated that C-63 was more effective for this recognition than N-57 (Figure 4).

It should be noted that C-63 includes the nonhelical tail-piece where the differences in primary structures of isoforms are clustered (Figure 8). Moreover, a few phosphorylation sites have been identified within this C-terminal region (Murakami et al., 1990, 1998; Conti et al., 1991; Even-Faitelson and Ravid, 2006; Rosenberg and Ravid, 2006). Phosphorylation within this region by PKC isoforms reduced the assembling ability of myosin IIB (Murakami et al., 1995; Even-Faitelson and Ravid, 2006; Rosenberg and Ravid, 2006). On the other hand, it was demonstrated that the assembly of myosin IIA was also regulated through the C-terminal region. In vitro studies revealed that Mts1, a member of S100 family of Ca$^{2+}$-binding proteins, binds to this region of myosin IIA and keeps the myosin IIA in a monomer state (Murakami et al., 2000; Li et al., 2003) and also inhibits the phosphorylation within this region (Kriaievskaya et al., 1998; Dulyaninova et al., 2005). It has been suggested that the assembly of each isoform is regulated by a distinct mechanism through modification of the C-terminal end region, that is, phosphorylation by protein kinase C for myosin IIB and Mts1 binding for myosin IIA, and this distinction contributes to the homo-filament formation (Murakami et al., 2000). Because the 72-kDa rod fragment of myosin IIB was phosphorylated in the cell (Ben-Ya’acov and Ravid, 2003), it is possible that the rod fragments used in this study were modified by phosphorylation or by binding of other protein such as Mts1 in the cell. This modification could be involved in self-recognition of the rod fragments expressed in the cell.

The role of N-57 in self-recognition can be explained by the molecular packing model proposed previously (Nakasawa et al., 2005). The proposed antiparallel packing model of BRF248, though it lacks the N-57, allowed us to predict that N-57 in one fragment may be the interaction site for C-63 in the partner fragment. Although the primary structure of the N-57 region is not so different between myosin IIB and myosin IIA (Figure 8), our results (Figures 4 and 5) imply that the small difference could be important for self-recognition. The interaction between N-57 and C-63 may play a key role in self-recognition.

MHC-IIB-specific siRNA-treated MRC-5 SV1 TG1 cells showed aberrant cell shape like BRF305-expressing cells, but the percentage of the cells exhibiting the aberrant shape and their degrees of aberrancy were lower than those of BRF305-expressing cells (Supplementary Figure S2). Two different explanations could account for this result. 1) The few myosin IIB remaining in siRNA-treated cells (12.8 ± 3.3%) could work to maintain cell shape partially. 2) In BRF305-expressing cells, besides interacting with the endogenous myosin IIB (Figure 5), BRF305 may sequester some proteins maintaining cell shape. If such protein exists, it could interact with myosin IIB filament at N-57 and C-63, because ARF296exNC induced the aberrant cell shape but BRF305-m3 did not.

A part of endogenous myosin IIB existed in the aggregates observed in both BRF305- and ARF296-expressing cells (Figure 7, D–I). The exogenous expression of rod fragments could induce the collapse of actin cytoskeleton in isoform-specific manner, and then some dispersed components might aggregate randomly without distinction of isoforms. Because the immunoprecipitation assay revealed that endogenous myosin IIB did not interact with ARF296 (Figure 5), we assume that direct interaction between exogenous rod fragments and endogenous myosin II isoforms might not occur in these aggregates.

It was demonstrated that expression of a truncated fragment of myosin IIA, lacking N-terminal 592 amino acid residues, induced cell rounding in a HeLa cell line (Clontech Tet-off system: which does not express MHC-IIB) because of the disruption of focal adhesions (Wei and Adelstein, 2000). We could not detect a remarkable defect in cell shape of MRC-5 SV1 TG1 cells by the expression of ARF296 (Figure 3). However, we suppose that the expression of ARF296 showed some inhibitory effect, because we observed the disruption of actin thin-fiber structures in the cytoplasm (Figure 6). It is thus possible that the disruption exerts some cell motile processes, which we have not noticed yet. The lack of the clear effects of myosin IIA inhibition in MRC-5 SV1 TG1 cells might be explained as following reasons. 1) The relative expression level of GFP-ARF296 was approximately three times higher than that of endogenous MHC-IIA. On the other hand, the relative expression level of GFP-BRF305...
against the endogenous MHC-IIB was estimated ~90 times (Figure 3 and Supplementary Figure S1). 2) Differed from the HeLa cell line (Clontech Tet-off system), MRC-5 SV1 TG1 cells express MHC-IIB as well as MHC-IIA. We have treated MRC-5 SV1 TG1 cells with blebbistatin to inhibit myosin IIA besides myosin IIB and observed resulting morphological phenotypes. The blebbistatin-treated cells showed aberrant cell shape similar to the BRF305-expressing cells (Supplementary Figure S3). We cannot observe another defect on cell shape; it is thus speculated that myosin IIB makes a large contribution to maintain cell shape in this cell line. Recently by Betapudi et al. (2006), and during the revision of this manuscript (Sandquist et al., 2006; Cai et al., 2006), it was reported that depletion of myosin II isoforms by specific siRNA-treatments caused cells to alter their speed of migration and spreading. However, the effects of depletion of each isoform are not completely the same among the reports, possibly because of the difference of the used cell species. It is necessary to study the function of each isoform considering their expression levels in different cell types. We roughly estimated that the relative expression level of MHC-IIA was 15 times higher than that of MHC-IIB in MRC-5 SV1 TG1 cells by immunoprecipitation of each isoform with specific antibodies, followed by immunoblotting with a pan-myosin antibody (Supplementary Figure S1). Further investigation using this cell and also other cells, in which the relative expression level of MHC-IIB is lower than in this cell, might reveal an obvious dominant negative effect of ARF296.

In this work, we demonstrated that N-57 and C-63 regions of BRF305 are involved in self-recognition of myosin IIB by using the cytoplasm as a “living test tube” to study protein interactions and their effects on cell behavior. Further studies on the role of these regions would clarify the molecular mechanisms for homo-assembling processes and also for the isoform-specific cellular functions.

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1016 Molecular Biology of the Cell


