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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 and stabilizing cell polarity (Lo et al., 1997; Conti et al., 2004). From the unstable morphology of MHC-II-/- 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 a-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; Saiioh 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) against amino terminus of MHC-IIA was purchased from ICN Pharmaceuticals (Aurora, OH). Horseradish peroxidase–labeled anti-mouse IgG was purchased from Bio-Rad Laboratories (Indianapolis, IN). Anti-β-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). Horseradish peroxidase–labeled anti-rabbit IgG (1:100) was purchased from Xilong Biotechnology Co. Ltd. (Hefei, China). Anti-GFP mAb (1:1000), anti-MHC-IIB (C-term) pAb (1:10,000) were purchased from Roche Applied Science (Indianapolis, IN). Anti-α-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). Horseradish peroxidase–labeled anti-rabbit IgG (1:100) was purchased from Xilong Biotechnology Co. Ltd. (Hefei, China).

**Construction of Plasmid DNA**

The DNA fragment encoding Asp 1729-Glu 1976 of human nonmuscle MHC-IIA with extra Cys at the N-terminus was cloned into the HindIII-BamHI sites of pEGFP-C3 (Clontech) to generate the pEGFP-ARF296 and pEGFP-BRF305, respectively. pEGFP-BRF305-m3 and pEGFP-BRF305AC63 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-BRF305 as a template. Plasmids for expressing the chimeric rod fragments of ARF296-BRF305 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-ARF296 or pEGFP-BRF305 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-ARF296 or pEGFP-BRF305 as a template for an inverse PCR following the QuikChange protocol. To construct pEGFP-ARF296exN and pEGFP-ARF296exC, pEGFP-ARF296 was used as a template. To construct pEGFP-ARF296exN and pEGFP-BRF305exC, pEGFP-BRF305 was used as a template. To construct pEGFP-ARF296exNC, 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 IC (Nitta Gelatin, Tokyo, Japan) coated coverglass. 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 polyptides 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-IA pAb (1:10,000), anti-MHC-IIA (C-term) pAb (1:10,000), anti-α-tubulin mAb (1:10,000), horseradish 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

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**Table 1. Primers used for mutagenic PCR**

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<tr>
<td>BRF305-m3</td>
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</tr>
<tr>
<td>Forward</td>
<td>5'-GAGCAGCTTAAAAAGAACACGTGAGGAAGCA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CATCCGAGCGTTCGCTCTCCATCTGCTC-3'</td>
</tr>
<tr>
<td>BRF305AC63</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGGATGTGACCCAGGCTAACGATCCACCGGATAGTCAG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTAGATCCGTTGATCCCTACTACGTC-3'</td>
</tr>
<tr>
<td>ARF296exN</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CTAGATCCGTTGATCCCTACTACGTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTAGATCCGTTGATCCCTACTACGTC-3'</td>
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<tr>
<td>ARF296exC</td>
<td></td>
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<tr>
<td>Forward</td>
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</tr>
<tr>
<td>Reverse</td>
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</tr>
<tr>
<td>Reverse</td>
<td>5'-CTAGATCCGTTGATCCCTACTACGTC-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 IA, 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 pepstatin A, 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 NaCl, 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 (BX50WI; 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 (LCPlanFl 20×/0.70; 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 thermoplate (MAT-S-200, Tokyo, 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.

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.
Aberrant cell shape (Figure 3, A, B, and G). The relative GFP-ARF296 in the cell. GFP-ARF296 did not induce the unstable phenotype, we exogenously expressed IIA rod fragment ARF296 corresponding to the BRF305 in- assembe is necessary for the induction of the unstable phenotype. The expression of BRF305 in the cell could inhibit the function of endogenous myosin IIB. The ability of the rod fragment to assemble is necessary for the induction of the unstable 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 BRF305exN and C63 (E and F) were taken from live cells expressing GFP-rod fragments (third panel) and endogenous MHC-IIB (fifth panel) were detected simultaneously on the same immunoblot. α-tubulin was used as a loading control.

Figure 3. Aberrant cell shape was not induced by the expression of ARF296 or the deletion fragments of BRF305. The images were taken from live cells expressing GFP-ARF296 (A and B), GFP-BRF248 (C and D), and GFP-BRF248C63 (E and F). Bar, 20 μm. (G) Percentage of cells with aberrant shapes seen 24 h after transfection were calculated from at least 70 cells expressing GFP fluorescence. Error bars, ±SD of three independent experiments. The effect of GFP-BRF305 was reconfirmed in each experiment. (H) Immunoblot analyses for checking the expression levels of each exogenous protein. Lysates from cells expressing GFP-ARF296 (lane 1), GFP-BRF248 (lane 2), GFP-BRF305C63 (lane 3), and GFP-BRF305C63 (lane 4) were analyzed using anti-GFP mAb (top panel), anti-MHC-IIA pAb (second and forth panels), anti-MHC-IIB (C-term) pAb (third and fifth panels), and anti-α-tubulin mAb (bottom panel). Because GFP-BRF305C63 lacks a C-terminus, it was not detected by anti-MHC-IIB (C-term) pAb. The signals of GFP-rod fragments (second panel) and endogenous MHC-IIA (fifth panel) were detected simultaneously on the same immunoblot. The signals of GFP-rod fragments (third panel) and endogenous MHC-IIB (fifth panel) were detected simultaneously on the same immunoblot. α-tubulin was used as a loading control.
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 remained intact (Figure 6, G–J). These results suggest that BRF305, but not ARF296, disrupts the actin cytoskeleton and myosin IIB, which are essential for maintaining cell shape.

Figure 4. Induction of aberrant cell shape by the expression of BRF305-ARF296 chimeric fragments. The images were taken from live cells expressing GFP-ARF296exN (A and B), GFP-BRF305exN (C and D), GFP-ARF296exC (E and F), GFP-BRF305exN (G and H), and GFP-ARF296exNC (I and J). Bar, 20 μm. (K) Percentage of cells with aberrant shapes seen 24 h after transfection were calculated from at least 70 cells expressing GFP fluorescence. Error bars, ± SD of three independent experiments. The morphological scoring of cells was performed in a double-blind manner. The effect of GFP-BRF305 was reconfirmed in each experiment. (L) Immunoblot analyses for checking the expression levels of each exogenous protein. Lysates from the cells expressing GFP-ARF296exN (lane 1), GFP-BRF305exN (lane 2), GFP-ARF296exC (lane 3), GFP-BRF305exN (lane 4), GFP-ARF296exNC (lane 5), and GFP-BRF305 (lane 6) were analyzed by anti-GFP mAb (top panel), anti-MHC-IIA pAb (second and forth panels), anti-MHC-IIB (C-term) pAb (third and fifth panels), and anti-α-tubulin mAb (bottom panel). The signals of GFP-rod fragments (second panel) and endogenous MHC-IIA (forth panel) were detected simultaneously on the same immunoblot. The signals of GFP-rod fragments (third panel) and endogenous MHC-IIB (fifth panel) were detected simultaneously on the same immunoblot. α-tubulin was used as a loading control.

Figure 5. Coimmunoprecipitation of BRF305 and ARF296exNC with endogenous myosin IIB. Lysates from the cells, each expressing GFP-ARF296, GFP-BRF305, GFP-ARF296exNC, and GFP-BRF305-m3 (indicated below the panels), were incubated with anti-MHC-IIA (N-term) pAb to immunoprecipitate endogenous myosin IIB. The immunocomplexes were collected and analyzed by immunoblotting using anti-MHC-IIA (C-term) pAb and anti-GFP mAb for detecting endogenous MHC-IIB and each GFP-rod fragments, respectively. Top and bottom panels indicate endogenous MHC-IIA and GFP-rod fragments, respectively. Left lanes (1, 4, 7, and 10) of each panel are total lysates before immunoprecipitation. Center lanes (2, 5, 8, and 11) are samples of the immunocomplex. Right lanes (3, 6, 9, and 12) are samples without antibodies as negative control of immunoprecipitation.
cortical localization of endogenous myosin IIB was reduced in GFP-BRF305–expressing cells (Figure 7, D–F), this still remained in GFP-ARF296–expressing cells (Figure 7, G–I). These results demonstrate that the cortical actin cytoskeleton including myosin IIB is reduced by the exogenous expression of BRF305 but not ARF296. This suggests that cells missing functional myosin IIB from the cell cortex could not maintain the actin cytoskeleton there and consequently induce the aberrant cell shape.

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, reconfirmed the function of myosin II (Straight et al., 2003; Niggli 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 (Leu1672-Leu1728) and C-63 (Asn1914-Glu1976) of BRF305, form-specific manner. Moreover, two regions, N-57 (Leu1672-Leu1728) and C-63 (Asn1914-Glu1976) of BRF305, were important for this effect.

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 Dicyostelium 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.
The next question is why the inhibition of endogenous myosin IIB function induces instability of cell shape. It has been demonstrated that myosin IIB tended to localize in restricted regions in a cell compared with myosin IIA (Maupin et al., 1994; Rochlin et al., 1995; Saitoh et al., 2001; Kolega, 2003; Lo et al., 2004). For example, in migrating endothelial cells, myosin IIA biased toward the front, whereas myosin IIB accumulated in the rear (Kolega, 2003).

In migrating fibroblasts, myosin IIA localized to the lamella whereas myosin IIB accumulated in the rear (Kolega, 2003). In migrating fibroblasts, myosin IIA localized to the lamella and throughout the posterior region, whereas myosin IIB was localized to the posterior region, especially at the lateral cell cortex (Saitoh et al., 2001). From these observations and the unstable phenotype of MHC-IIB−/− fibroblasts (Lo 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-Faifelson 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-Faifelson 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 Ca2+-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 (Kriaevska 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.
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-IIA 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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