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Nonmuscle Myosin IIB Is Involved in the Guidance of Fibroblast Migration

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Although myosin II is known to play an important role in cell migration, little is known about its specific functions. We have addressed the function of one of the isoforms of myosin II, myosin IIB, by analyzing the movement and mechanical characteristics of fibroblasts where this protein has been ablated by gene disruption. Myosin IIB null cells displayed multiple unstable and disorganized protrusions, although they were still able to generate a large fraction of traction forces when cultured on flexible polyacrylamide substrates. However, the traction forces were highly disorganized relative to the direction of cell migration. Analysis of cell migration patterns indicated an increase in speed and decrease in persistence, which were likely responsible for the defects in directional movements as demonstrated with Boyden chambers. In addition, unlike control cells, mutant cells failed to respond to mechanical signals such as compressing forces and changes in substrate rigidity. Immunofluorescence staining indicated that myosin IIB was localized preferentially along stress fibers in the interior region of the cell. Our results suggest that myosin IIB is involved not in propelling but in directing the cell movement, by coordinating protrusive activities and stabilizing the cell polarity.

INTRODUCTION

The functional roles of myosin II in nonmuscle cells have been an important topic of investigation. Although its involvement in cytokinesis has been investigated in detail (Robinson and Spudich, 2000), there is also strong evidence that myosin II plays a role in cell migration. For example, although myosin II null mutants of Dictyostelium are capable of migration, they display a lower migration speed and a loss of forward bias in protrusion compared with wild-type cells (Wessels et al., 1988), particularly on surfaces of increased adhesiveness (Jay et al., 1995).

Fibroblast migration involves a number of controlled and coordinated processes, including protrusion, adhesion, translocation, and detachment (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Sheetz et al., 1998). It is commonly accepted that translocation of the cell body and detachment from the substrate require contractile forces (Jay et al., 1995; Wolenski, 1995; Anderson et al., 1996; Svitkina et al., 1997). Consistent with the idea, treatment of cells with myosin II inhibitors causes relaxation of traction forces and impairment of cell migration (Pelham and Wang, 1999). Equally important is a guidance mechanism in response to environmental cues. In addition to chemotaxis, fibroblasts show profound responses in morphology, traction forces, and motility rates, to physical signals (Pelham and Wang, 1997; Lo et al., 2000). They are also able to steer their migration toward substrates of high rigidity (Lo et al., 2000). Because the detection of such physical characteristics as rigidity cannot be achieved through purely chemical means, the cell must invoke a contractile mechanism that probes the environment. Myosin II may be involved in such a sensing structure at focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996) and/or in the transduction of extracellular physical cues into intracellular chemical signals.

There are three known nonmuscle myosin II isoforms, referred to as myosin IIA, myosin IIB, and myosin IIC, respectively, in vertebrates (Katsuragawa et al., 1989; Simons et al., 1991; Berg et al., 2001; Golomb et al., 2004). Myosin IIA and IIB are expressed in a variety of cultured cells, whereas myosin IIC may be induced during hemopoietic differentiation (Buxton et al., 2003). The differential localization of myosins IIA and IIB suggests that they are involved in distinct functions (Maupin et al., 1994; Rochlin et al., 1995; Kelley et al., 1996; Kolega, 1998). In migrating endothelial cells, myosin IIB was enriched at the trailing edge, whereas myosin IIA was found preferentially toward the leading edge (Kolega, 1998). In contrast, myosin IIB was localized at the leading edge of Xenopus A6 cells, whereas myosin IIA was present along fibrillar structures in the more interior cytoplasm (Kelley et al., 1996).

A more direct way to test the function of myosin II isoforms is to disrupt the expression or function of the proteins through targeted gene ablation. Ablation of nonmuscle myosin IIB gene in mice causes defects in cardiac and neuronal development (Tullio et al., 1997). Although the defects are possibly associated with impaired cell migration, the exact cellular basis is unclear. To understand how myosin IIB
Role of Myosin IIB in Fibroblast Migration

participates in cell migration, we have investigated the movement and mechanical characteristics of mouse embryonic fibroblasts lacking nonmuscle myosin IIB. The response of these cells to mechanical stimuli was further probed using polyacrylamide flexible substrates. Our results indicate that the main defect of myosin IIB null cells was not the rate of migration or the generation of traction forces, but the directional stability of migration and the ability to respond to mechanical stimulation. We suggest that nonmuscle myosin IIB plays an important role in steering and stabilizing the polarity of cell migration.

MATERIALS AND METHODS

Polyacrylamide Substrates and Cell Culture

Myosin IIB null and control mouse embryonic fibroblasts were derived by explanting and culturing day 13 embryos after removing the head and internal organs. Cells were maintained at 37°C and 5% CO2 in DMEM (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 2 mM l-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, and 250 ng/ml amphotericin B (Invitrogen, Carlsbad, CA).

The general method for preparing polyacrylamide substrate has been described previously (Wang and Pelham, 1998). The substrates contain 5% total acrylamide and either 0.1% (for stiff substrates) or 0.06% bioacrylamide (for soft substrates). The flexibility of polymerized sheets was determined as described previously (Lo et al., 2000). The Young’s modulus of substrates with 0.1 and 0.06% bioacrylamide was estimated as 28 and 15 kN/m2, respectively. Measurements of traction forces were limited to isolated, well spread/ extended cells (e.g., excluding mitotic cells) that fit within the central region of the imaging field (60 μm). To apply local mechanical strain to the flexible substrate, the tip of a blunted microneedle was gently lowered onto the surface with a micromanipulator, and the substrate was deformed by pushing the needle toward the leading edge of a migrating cell (Lo et al., 2000). Cells that showed immediate shortening in response to the substrate deformation were included in the analysis. Positive response was defined as the retraction of protrusions proximal to the needle and the expansion of protrusions distal to the needle. This caused the cell to change its direction of migration.

Rescue of myosin IIB knockout cells were performed by expressing green fluorescent protein (GFP)-tagged constructs of myosin IIB or IIA (constructs kindly supplied by Qize Wei, National Institutes of Health, Bethesda, MD). Transfection was carried out with a Nucleofector machine (Amaxa, Gaithersburg, MD) and 4.5 μg of DNA, by using program T20 and kit V.

Immunofluorescence Staining

For immunofluorescence staining of vinculin, cells were washed with 37°C phosphate-buffered saline (PBS) and fixed with 4% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), in PBS containing 0.1% Triton X-100 (Roche Diagnostics, Mannheim, Germany) at 37°C for 10 min as described previously (Pelham and Wang, 1997). Monoclonal anti-vinculin antibody conjugated to fluorescein isothiocyanate was obtained from Sigma-Aldrich (clone VIN-11-5) and used at a dilution of 1:100. Alexa-546-conjugated goat anti-mouse secondary antibodies were obtained from Molecular Probes (Eugene, OR) and used at a dilution of 1:100.

For immunofluorescence staining of myosin IIA and myosin IIB, cells were washed with 37°C PBS and fixed with a mixture of 0.1% glutaraldehyde (Polysciences, Warrington, PA) and 1.0% formaldehyde in cytoskeleton buffer containing 0.3% Triton X-100 (Small, 1981) at 37°C for 1 min and then postfixed with 0.5% glutaraldehyde in 37°C cytoskeleton buffer for 15 min (O’Connell et al., 1999). Staining was performed using polyclonal antibodies specific for the C-terminal region of MHC-A or MHC-B, at a dilution of 1:100. Alexa-546–conjugated anti-rabbit IgG secondary antibodies were purchased from Molecular Probes and used at a dilution of 1:100. Actin filaments were stained with Alexa-488–phalloidin (Molecular Probes) following manufacturer’s protocol.

Cell Migration Assays

Experiments were performed 15 h after plating the cells on coverslip or polyacrylamide substrates at a low density. Phase contrast images were recorded using a cooled charge-coupled device camera (TE/CCD-576EM; Princeton Instruments, Trenton, NJ), attached to an IM-35 microscope (Carl Zeiss, Jena, Germany) equipped with a 40×, numerical aperture (NA) 0.65 Achromat phase objective lens and a stage incubator. The position of the cell was determined every 2 min for a period of 50–80 min, based on the center of the nucleus. Migration speed (S) and directional persistence (P) were calculated based on the persistent random walk equation dθ(t) = 2πP[t − P(1 − e^−t)], where dθ(t) is the mean squared displacement (Dunn, 1983). Migration speed was estimated from double reciprocal plots of the square root of dθ(t) against t (as 1/slope), and directional persistence was esti-
protrusive activities (Figure 2, B and C). In contrast, 8 of 11 cells transfected with GFP-myosin IIA showed an irregular morphology as for untransfected mutant cells (Figure 2, D and E).

The migration of control and myosin IIB null cells was analyzed quantitatively based on mean squared displacement of 11 control and 17 mutant cells (Dunn, 1983). As seen in Figure 3, double reciprocal plots of mean square displacement against elapsed time indicated that mutant cells migrated with a higher speed but a reduced directional persistence than did control cells (Table 1), i.e., myosin IIB null cells showed a higher frequency of turning. The combination of higher speed and lower persistence resulted in a similar persistent distance between mutant and control cells, indicating that myosin IIB null and control cells migrated for a similar distance before turning. However, the faster pace made the mutant cells seem much more unstable.

**Myosin IIB Null Cells Are Defective in Directional Movement**

The reduced directional persistence suggested that myosin IIB cells might not be able to maintain a stable direction during targeted migration such as haptotaxis. We therefore

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**Figure 1.** Morphology and migratory behavior of control and myosin IIB null cells. Although most control cells maintained their morphology over a 60-min period (A), mutant cells showed rapid changes in cell shape during the same period of time (B). In addition, mutant cells migrate with a highly unstable polarity (B, arrows), compared with control cells (A, arrows). Images of high magnification show protrusion, elongation (C, long arrows), and rapid retraction of processes (C, short arrows). The latter creates small pieces of motile cytoplasm that litter the glass surface (C, arrowheads). Time in hours and minutes is indicated in each image. Bar, 50 μm.

**Figure 2.** Restoration of normal morphology of myosin IIB mutant cells by the reexpression of GFP-myosin IIB. Myosin IIB nulls were transfected with a vector carrying the sequence of GFP-myosin IIB. GFP signals are found along stress fibers (A). Cells expressing GFP-myosin IIB show a normal morphology and no random protrusive activities (B and C). In contrast, cells expressing GFP-myosin IIA maintained the irregular, unstable morphology as for the original myosin IIB null cells (D and E). Time in hours and minutes is indicated. Bar, 50 μm.

**Figure 3.** Quantitative analysis of cell migration. Plotting of mean squared distance against time (from 11 control cells and 17 mutant cells; each data point represents a mean ± SEM) indicates that mutant cells (A, open square) are able to migrate over a longer distance than do control cells (A, solid square), irrespective of the direction. However, double reciprocal plot of root mean squared distance against time, where migration speed is calculated as 1/slope and directional persistence is calculated as slope/6×y-intercept, indicates that mutant cells (B, open square) migrate at a higher speed but with a decreased persistence compared with control cells (B, solid square; Table 1).
tested the cell behavior with a modified Boyden chamber assay. In this test, cells migrated from an uncoated surface of a porous membrane toward the opposite surface coated with extracellular matrix (haptotaxis). Membranes coated on both sides served as the reference (random migration). As seen in Figure 4, control cells showed a ratio of 5–6 between haptotaxis and random migration on collagen-coated membrane and 6 on fibronectin-coated membrane. In contrast, this ratio for myosin IIB null cells was close to 2 on collagen- and 1 on fibronectin-coated membrane. These results indicate that myosin IIB is required for haptotaxis.

A second test was to determine the ability of myosin IIB cells to cover a wound on the monolayer. Six independent observations were performed for both mutant and control cells. A typical example is shown in Figure 5A, where the cell-free area was covered completely by control cells in 6 h. In contrast, only 60–70% of the open area was covered by mutant cells over the same period (Figure 5, B and C). However, at such high cell densities, mutant cells showed no apparent increase in unstable protrusions as seen for cells at a low density (Figure 1). The reduced rate of wound closure seemed to result from an increase in the resistance to forward migration due to cell-cell adhesion.

**Myosin IIB Null Cells Are Defective in the Organization of Traction Forces and in Sensing Mechanical Signals**

We have previously proposed a force-dependent mechanism for cells to respond to external physical signals (Lo et al., 2000). To investigate the possible involvement of myosin IIB in generating traction forces and in detecting substrate rigidity, cells were cultured on flexible polyacrylamide substrates coated with type 1 collagen. Despite the ablation of myosin IIB, null cells maintained 75% of the total mechanical output compared with control cells. As for control cells (Figure 6A) and 3T3 fibroblasts (Dembo and Wang, 1999),
strong inward traction forces were concentrated at protrusive or ruffling regions of myosin IIB null cells (Figure 6C).

We showed previously that for 3T3 cells, the direction of maximal traction forces remained antiparallel to the direction of cell migration (Munevar et al., 2001). This was confirmed with 52 measurements made during >2 h of two migrating control cells (Figure 6, A and B). In most cases, the magnitude of traction forces in control cells exhibited an apparently sinusoidal dependence on the angle, i.e., with symmetric peaks and valleys separated by 90° (observed in 52 of 72 measurements made with 22 cells; Figure 6B; see also Munevar et al., 2001). The exceptions were always associated with the reorientation of cell migration.

In contrast, the direction of maximal traction forces in mutant null cells was unstable, showing no consistent relationship to the direction of cell migration (based on 97 measurements made during >2 h of three cells; Figure 6, C and D). Less than 50% of the measurements showed an apparently sinusoidal relationship between traction forces and the angle (57 of 121 measurements made with 27 cells). In addition, the angular dependence curve showed no well-defined peak or valley (Figure 6D), and the shape was highly variable.

As shown previously, normal fibroblasts responded to increasing substrate rigidity by increasing their spreading area and mechanical output (Lo et al., 2000; Table 2). In contrast, myosin IIB null cells showed no apparent response to substrate rigidity (Table 2). Furthermore, both 3T3 fibroblasts and control cells reoriented their migration away from pushing forces (Lo et al., 2000; observed with all of the eight 3T3 cells and eight control cells tested; Figure 7A and Video 4), whereas none of the seven myosin IIB null cells tested showed an apparent turning response to pushing forces (Figure 7B and Video 5).

**Organization of Myosin and Vinculin in Control and Myosin IIB Null Cells**

Additional insights into the function of myosin IIB may be gained from the localization of IIA and IIB. Phalloidin staining of actin filaments showed the presence of similar stress fibers in myosin IIB null cells and control cells (Figure 8, B, E, and H). Staining of myosin IIA showed localization along stress fibers and the lateral cortex in both control and myosin IIB null cells (Figure 8, A and G). The stress fibers looked like arrays of discrete, punctate structures, and stress fibers in different regions showed a similar extent of myosin IIA localization relative to actin filaments (Figure 8, C and I).

In control cells, staining of myosin IIB seemed more continuous along stress fibers than did myosin IIA (Figure 8, D, E, and F). In contrast, myosin IIB null cells showed an unstable, poorly defined direction of maximal traction forces (Figure 8, D, G, and H), which bears no apparent relationship to either the direction of cell migration (C, long hollow arrow) or the direction of minimal traction force (D).

**Table 2.** Force output and spreading area of control and myosin IIB null cells on substrates of different rigidity

<table>
<thead>
<tr>
<th>Young’s modulus (kN/m²)</th>
<th>Total force output (dyne)</th>
<th>Spreading area (10⁵ μm²)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Myosin II-B null</td>
</tr>
<tr>
<td>15</td>
<td>0.44 ± 0.03 (n = 18)</td>
<td>0.44 ± 0.04 (n = 20)</td>
</tr>
<tr>
<td>28</td>
<td>0.57 ± 0.06 (n = 18)</td>
<td>0.41 ± 0.03 (n = 20)</td>
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Total force output for each cell was calculated by multiplying the average traction stress with the cell area. For control cells, the force output on stiff substrates was higher than that on soft substrates (p = 0.02). No statistically significant difference was found for mutant cells on substrates of different rigidity. Values shown are mean ± standard error.
In addition, myosin IIB was localized preferentially along stress fibers in the central region of the cell (Figure 8, D and F). Neither myosin IIA nor IIB showed a concentration along the leading edge. As expected, immunofluorescence staining of the heavy chain of myosin IIB gave only background signals in null cells. Staining of vinculin showed qualitatively similar focal adhesions in both myosin IIB null and control cells (our unpublished data).

DISCUSSION

Recent studies have demonstrated strong, active mechanical interactions between migrating fibroblasts and the substrate, which likely serve not only to propel but also to guide cell migration (Munevar et al., 2001). Genetic and pharmacological manipulations indicated that myosin II plays an important role in these processes. For example, treatment of 3T3 fibroblasts with KT5926, an inhibitor of myosin light chain kinase, caused a dramatic reduction in traction forces and migration rate, without affecting the protrusion of the leading edge (Pelham and Wang, 1999). Similar treatments were found to inhibit uropod retraction, chemotaxis, and polarization of neutrophils (Eddy et al., 2000). Ablation of myosin II expression in Dictyostelium caused a similar defect in tail retraction and in the forward bias of pseudopod extension (Jay et al., 1995). Together, these observations suggested that myosin II may be required for regulating both frontal protrusion and tail retraction.

Defects of Myosin IIB Null Cells

In this study, we addressed the functional role of nonmuscle myosin IIB in fibroblast migration by using cells derived from mouse embryos where the gene of myosin IIB heavy chain has been ablated (Tullio et al., 1997). The ability of these animals to develop to the neonatal stage and of the cells to grow in culture suggests that myosin IIA alone is sufficient for cytokinesis, one of the key functions of myosin II (Robinson and Spudich, 2000). However, the defects in
cardiac and neuronal development indicate that myosin IIB is required for some other significant functions (Uren et al., 2000). Our initial speculation was that myosin IIB cells might be defective in their migration and/or mechanical output. To our surprise, myosin IIB null fibroblasts were not only capable of migration but also migrated at a higher raw speed than did control cells (Table 1 and Figure 3). In addition, myosin IIB null cells maintained most of the traction forces compared with control cells (Table 2 and Figure 6). Previous studies with myosin IIB-deficient neurons have also revealed substantial traction forces exerted by the filopodia (Bridgman et al., 2001).

From the present observations, the most striking defect of myosin IIB null cells was their multiple, disorganized, and transient lamellipodia (Figure 1). This leads to an unstable polarity of migration, manifested quantitatively as a decrease in migratory persistence (Table 1 and Figures 1 and 3). In addition, although normal migrating fibroblasts maintained a stable direction of maximal traction forces antiparallel to the direction of cell migration (Munevar et al., 2001), the multiple projections in myosin IIB null cells seemed to align to the direction of cell migration (Munevar et al., 2001), the multiple projections in myosin IIB null cells seemed to engage in a tug of war and could no longer define an unambiguous direction of cell migration (Figures 1C and 6).

The loss of stable polarity may explain the defects of myosin IIB null cells in haptotaxis as seen in the modified Boyden chamber assay (Carter, 1967; Figure 4). Myosin IIB null cells also showed a delay in an in vitro wound healing assay (Figure 5), although this seemed to be caused by an increased resistance to forward migration at high cell densities. In addition, null cells showed no apparent response in traction forces and spreading areas to substrate rigidity (Table 2, and no detectable directional guidance by mechanical stimulations (Figure 7), indicating that they are defective in responding to mechanical signals. Similar defects may affect the behavior of neuronal growth cones, including unstable lamellipodia and increased retrograde flow of the actin cytoskeleton (Brown and Bridgman, 2002).

**Functions of Myosin IIB in Cell Migration**

There are several possible ways that myosin IIB could contribute to cell migration. First, myosin IIB may be involved in the detection of mechanical signals. We have argued previously that responses to such physical parameters as substrate rigidity must involve the application of contractile forces at adhesion sites, coupled to the transduction of mechanical feedback into chemical events (Pelham and Wang, 1997; Lo et al., 2000). Myosin IIB may be responsible for generating such probing forces. Myosin was also suggested to mediate “inside-out” signaling, by promoting the assembly of stress fibers and focal adhesions from inside the cell in response to substrate adhesion and growth factors (Burridge and Chrzanowska-Wodnicka, 1996). Although we found no apparent defect in the distribution of vinculin, it is likely that focal adhesions are less stable in mutant cells than in control cells as suggested by their highly irregular behavior. The concentration of myosin IIB at the leading edge, as found in Xenopus A6 cells, is consistent with a role in membrane signaling (Kelley et al., 1996). However, because different regions of the cells are linked mechanically by the cytoskeleton network, even forces generated in the central region, as suggested by the distribution of myosin IIB (Figure 8), may propagate easily to the cell periphery.

Second, our observations suggest that myosin IIB is involved in the coordination of primary and secondary protrusions. In addition, normal fibroblast migration, secondary protrusions were generated continuously along the lateral edges, although most of them disappeared shortly and were unable to compete with the primary lamellipodium. Myosin IIB may be involved in such coordination by promoting the retraction of secondary protrusions and allowing the cell to maintain a regular shape and stable direction of migration. A defect in retraction may also lead to increased resistance and reduced rate of wound closure, as seen with myosin IIB null cells (Figure 5). Based on immunolocalization (Rochein et al., 1995), myosin IIB also has been speculated to play a role in the retraction of neuronal growth cone.

Third, myosin IIB may stabilize the direction of cell migration. Myosin IIB was found to mediate the anterior localization of β-actin mRNA (Latham et al., 2001), despite its preferential localization along stress fibers in the central region of the cell (Figure 8). The localization of β-actin mRNA may in turn play a role in the stabilization of protrusion (Shestakova et al., 2001). It is also possible that, through its preferential association with mature stress fibers in the central region, myosin IIB stabilizes the direction of cell migration. The distinct function of central stress fibers has also been suggested based on their different sensitivity to drugs against the Rho-dependent kinase and myosin light chain kinase from peripheral stress fibers (Totsukawa et al., 2000; Katoh et al., 2001).

**Differential Functions of Myosin IIA and Myosin IIB**

Previous studies with antisense technologies and dominant negative constructs suggested that myosin IIA may be involved in cell adhesion and cell shape (Wei and Adelstein, 2000; Wylie and Chantler, 2001), whereas myosin IIB is essential for maintaining a normal growth cone mobility (Wylie et al., 1998; Bridgman et al., 2001). We found that myosin IIB null cells are able to maintain ~75% of the traction forces as detected in control cells, which contained a similar amount of myosin IIA in addition to a roughly equal amount of myosin IIB (our unpublished observations). Therefore, the relative contribution of myosin IIA to total traction forces should be three times higher than that of IIB. Interestingly, myosin IIA also showed two- to threefold higher activities than myosin IIB in both the actin-activated ATPase assay and in vitro motility assay (Kelley et al., 1996).

A second prominent difference between the two myosin II isoforms is that myosin IIA is organized as sarcomere-like structures along stress fibers throughout the cell, whereas myosin IIB seemed to be more continuously distributed along stress fibers in the interior region (Figure 8). A similar differential distribution was described in previous studies with endothelial cells (Kolega, 1998), melanoma cells (Maupin et al., 1994), nonneuronal gangial cells (Rochein et al., 1995), and fibroblasts (Saitoh et al., 2001). Together, these observations support the idea that myosin IIA is involved in a contractile function as in muscles (Figure 8), whereas myosin IIB may play primarily a regulatory role.

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