Distinct Roles of Nonmuscle Myosin II Isoforms in the Regulation of MDA-MB-231 Breast Cancer Cell Spreading and Migration

Venkaiah Betapudi, Lucila S. Licate, and Thomas T. Egelhoff

Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio

Abstract

Initial stages of tumor cell metastasis involve an epithelial-mesenchyme transition that involves activation of amoeboid migration and loss of cell-cell adhesion. The actomyosin cytoskeleton has fundamental but poorly understood roles in these events. Myosin II, an abundant force-producing protein, has roles in cell body translocation and retraction of the posterior of the cell during migration. Recent studies have suggested that this protein may also have roles in leading edge protrusive events. The metastasis-promoting protein metastasin-1, a regulator of myosin II assembly, colocalizes with myosin IIA at the leading edge of cancer cells, suggesting direct roles for myosin II in metastatic behavior. We have assessed the roles of specific myosin II isoforms during lamellar spreading of MDA-MB-231 breast cancer cells on extracellular matrix. We find that the two major myosin II isoforms IIA and IIB are both expressed in these cells, and both are recruited dramatically to the lamellum margin during active spreading on fibronectin. There is also a transient increase in regulatory light chain phosphorylation that correlates the recruitment of myosin IIA and myosin IIB into this spreading margin. Pharmacologic inhibition of myosin II or myosin light chain kinase dramatically reduced spreading. Depletion of myosin IIA via small interfering RNA impaired migration but enhanced lamellar spreading, whereas depletion of myosin IIB impaired not only migration but also impaired initial rates of lamellar spreading. These results indicate that both isoforms are critical for the mechanics of cell migration, with myosin IIB seeming to have a preferential role in the mechanics of lamellar protrusion. (Cancer Res 2006; 66(9): 4725-33)

Introduction

Metastasis is thought to initiate with tumor cells undergoing a transition from a nonmotile epithelial-like state to a more amoeboid or mesenchymal-like migratory state, often referred to as epithelial-mesenchyme transition (EMT; refs. 1, 2). Local microenvironment, including proximity to blood and lymph vessels, seems to stimulate directed migration of tumor cells at the earliest stages of metastasis (3, 4). Although chemotactic migration clearly contributes to metastatic behavior at this stage and later stages of metastasis, the cellular mechanics of cancer cell migration remain poorly understood. Single cell migration is an integrated cellular process that requires remodeling of cytoskeletal elements as well as extracellular matrix contacts. Migration occurs as a result of propulsive forces generated at the leading edge of the cell, cell body translocation, and posterior retraction forces that contribute to moving the bulk of the cell forward. Nonmuscle myosin II is a major component of the actomyosin cytoskeleton and is generally accepted as contributing to contraction of the posterior of the cell during migration (5). Protrusion events are widely regarded as being driven by filamentous-actin polymerization, independent of myosin II (5–7). Recent studies in noncancer cell types, however, have begun to suggest roles for myosin II in anterior protrusive events related to cell migration (8). However, the exact mechanical roles of myosin II during cell migration remain remarkably poorly understood.

Although myosin II is clearly involved in cell migration, there have been few studies to date assessing either protein expression profiles or behaviors of the multiple myosin II isoforms during cancer cell migration. One recent clinical study of non–small cell lung cancer patients found a significant positive correlation between expression levels of myosin light chain kinase (which activates myosin II) and likelihood of disease recurrence and metastasis (9), indicating that excessive myosin II activation could be a contributing factor to metastasis. A key role for myosin II in metastatic cancer cell behavior is further suggested, indirectly, by a number of published studies focused on the small calcium-binding protein metastasin-1 (mts1). This protein is up-regulated in many metastatic cell lines and when overexpressed artificially mts1 enhances metastatic behavior (10). A major cellular target of mts1 seems to be myosin IIA (reviewed in ref. 11). Recent studies have shown that mts1 colocalizes with myosin IIA at the leading edge of migrating cancer cells (12), and that mts1 influences both the assembly behavior of myosin IIA and its phosphorylation by protein kinases thought to modulate filament assembly (13, 14). Although studies on mts1 suggest critical roles for myosin II in metastasis, exactly how myosin II contributes to metastasis, and which isoforms are important, remain completely unknown. To develop a better understanding of possible roles for myosin II isoforms during migration, we have used lamellar spreading on extracellular matrix as a model.

Native nonmuscle myosin II is comprised of a complex of two nonmuscle myosin II heavy chains (NMHC II), two essential light chains and two regulatory light chains (RLC). In humans and mice, three NMHC II isoforms have been identified (NMHC IIA, NMHC IIB, and NMHC IIC), which have distinct tissue and cell type expression patterns (15). Recent NMHC II gene disruption studies in mice have revealed dramatically different embryonic lethal phenotypes upon disruption of NMHC IIA versus NMHC IIB, with early embryonic cell-cell adhesion failing in the former (16), and lethal cardiac and neuronal developmental defects in the latter (17, 18). Cell line studies have further suggested possible isoform specific roles in a range of processes, including vesicle trafficking during repair of damaged plasma membranes (19), coordination of neurite outgrowth and retraction (20, 21),...
regulation of fibroblast polarity (22), and collagen fiber retraction by fibroblasts (23).

However, despite the likely importance of myosin II functions during EMT and during other stages of metastasis, there have been few studies characterizing myosin II functions and isoform behaviors in the context of cancer cell migration. Although many models in the literature emphasize myosin II localization in the posterior of migrating cells (5), both activated myosin II and its activator myosin light chain kinase (MLCK) have been found to be enriched in lamellar protrusive structures in several cell types (24–26). To begin addressing the roles of myosin II during breast cancer cell migration, we have evaluated localization behavior and functional requirements for myosin IIA and IIB for fibronectin-induced cell spreading in MDA-MB-231 human breast cancer cells.

The MDA-MB-231 breast cancer cell line is derived from a metastatic pleural effusion (27) and is widely used as a model for metastatic cell migration. Based upon microarray gene expression studies and strongly invasive behavior in vitro, this line is thought to represent a highly dedifferentiated mesenchymal-like line, which has lost most epithelial markers and character (reviewed in ref. 28). MDA-MB-231 cells also overexpress mts1 (also known as CAPL or S100A4), consistent with their strong metastatic behavior (29). This array of features renders MDA-MB-231 cells ideal for investigation of the roles of myosin II in metastatic cell migration.

Our studies reveal adhesion-stimulated increases in RLC phosphorylation temporally correlated with dramatic recruitment of myosin IIA and IIB isoforms to expanding lamellipodium of the spreading cells. This strong lamellar recruitment argues for a role for these isoforms in the mechanics of lamellar protrusion. Furthermore, pharmacologic or small interfering RNA (siRNA)–mediated inhibition of myosin II and MLCK indicate that both myosin IIA and IIB contribute to overall cell migration, but that the IIB isoform specifically contributes to the mechanics of lamellipodium spreading on extracellular matrix.

Materials and Methods

Cell culture and reagents. The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD) and was grown as a monolayer culture in MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin, and 1% t-glutamine (Life Technologies, Gaithersburg, MD) at 37°C with 6% CO2. The cells were used not more than 15 to 20 passages after the initiation of cultures. Specific inhibitors used were ML-7, blebbistatin, Y-27632, and SB-202190 purchased from Calbiochem (La Jolla, CA). Antibodies purchased from Sigma (St. Louis, MO) include those for nonmuscle myosin IIA, myosin IIB, and actin. Antibodies purchased from Cell Signaling, Inc. (Beverly, MA) include those for phosphorylated (S19)-RLC [phospho(S19)-RLC] and phospho(T18/S19)-RLC. Antiserum against myosin IIC (15) were a gift from Dr. R.S. Adelstein (Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, NIH).

siRNAs transfections. Cells (1 × 106 to 1 × 107) growing in tissue culture plates (60-70% confluent) were collected by using cell dissociation reagent (Sigma, USA), washed with PBS (phosphate buffer saline) at room temperature, resuspended in 90 μL of transfection solution (Ammax Biosystems, USA, kit “T”), then mixed with 5-20 pmol of Smart Pool siRNA oligonucleotides specific to NMHC IIA or NMHC IIB (Dharmacon RNA Technologies, USA). This suspension was transferred to a cuvette and electroporated by using program A-23 (Ammax Biosystems). Cells were then diluted in growth medium, seeded in tissue culture plates (60-mm), and incubated for 72 hours before collection for migration or spreading assays.

Cell spreading and migration assays. Quantitative cell spreading assays were carried out in 96-well microtiter plates (Nunc, Naperville, IL) using cells harvested from subconfluent tissue culture plates. Wells were coated with fibronectin (20 μg/mL; Sigma) for 1 hour at room temperature and rinsed with PBS just before seeding the cells. Briefly, cells were collected from the culture plates using trypsin and then pelleted by microcentrifugation at 4°C for 10 minutes. The cell pellet was resuspended in 100-200 μL of MEM solution with or without inhibitors) were added to each well and placed in 37°C incubator for 60 minutes. Samples were then fixed with 20% paraformaldehyde, rinsed with PBS, and stained with Alexa 595-conjugated wheat germ agglutinin (WGA; Molecular Probes, Eugene, OR) and with 4,6-diamidino-2-phenylindole (DAPI). Fluorescent images were collected from six independent fields of view for each condition, and per cell area (Alexa 595/WGA stain) was quantified using the ImageJ software package (NIH). Areas representing aggregates or clusters of cells were excluded from the data set based upon evaluation of DAPI nuclear fluorescence, so that all scored data was derived from individual spreading cells.

Transwell migration was done using Costar #3422 8-μm transwell chambers. Chambers were pretreated with 20 μg/mL fibronectin for 1 hour and then rinsed with PBS before use. MDA-MB-231 cells (2 × 104) were applied to the upper chamber in cell migration medium [MEM, 1% glucose, penicillin/streptomycin, 0.1% bovine serum albumin (BSA), FCS (5%) was added to lower chamber. After 6 hours of migration at 37°C, samples were fixed with 4% paraformaldehyde, and nuclei were stained with DAPI. The upper chambers were wiped with cotton swabs to remove nonmigrating cells, and nuclei on the lower surface were photographed using fluorescence and a ×4 objective. Four photographs were collected from each chamber, representing ~70% of the entire membrane filter surface. ImageJ software (NIH) was used for automated counting of nuclei present on the lower surface of each membrane, with each nuclei scored as one migration event.

For analysis of myosin II localization during spreading, samples were processed as described above for WGA staining, except that spreading, fixation, and immunostaining was done on fibronectin-coated glass coverslip surfaces in Lab-Tek II Chambered Coverglass (Nalge Nunc International, Naperville, IL).

Stock cell migration assays, cells were grown in tissue culture dishes, and scratches were made using pipette tip and incubated in the growth chamber. Six different circles were made using a marker pen on the bottom of the glass chamber, and images of these spots were collected at time intervals. Migration of the cell front was measured using the ImageJ software package (NIH).

Immunostaining. Cells growing or actively spreading on Lab-Tek II Chambered Coverglass were washed twice with PBS. Cells were fixed by incubating with 1 mL of fixing buffer I [1.1 mmol/L Na2HPO4, 0.4 mmol/L KH2PO4, 4 mmol/L NaCl, 5 mmol/L MgCl2, 2 mmol/L EGTA, 0.1% glutaraldehyde, 1% paraformaldehyde, and 0.3% Triton X-100] for 1 minute at 37°C followed by post-fixing with 1 mL of fixing buffer II (fixing buffer I carrying 0.5% glutaraldehyde) and no paraformaldehyde and Triton X-100) at 37°C for 15 minutes. Cells were washed with PBS and blocked with 1 mL of blocking buffer (1× TBS with 0.1% Tween and 1% BSA) at 37°C for 10 minutes followed by incubating with antibodies (diluted in blocking buffer) at 37°C for 35 to 60 minutes. After washing with 1 mL of washing buffer (blocking buffer with no BSA), cells were incubated with Alexa-conjugated secondary antibodies (Molecular Probes) diluted to 1,000-fold in blocking buffer for 1 hour at 37°C. Cells were washed with washing buffer and incubated with 1 mL of DAPI (20 μg/mL) solution at room temperature. Cells were washed with PBS several times before imaging with a Zeiss LSM510 confocal microscope.

Western blot analysis. Western blot analyses were carried out as described earlier (30) with some modifications. Total cell lysates were prepared in two different ways for performing Western blot analyses. For probing with phospho-specific antibodies, lysates from quiescent adherent cells or spreading cells were collected by completely removing growth medium and lysing directly by the addition of 10% ice-cold trichloroacetic acid to the dish. Cell lysates were then collected by scraping with a rubber policeman. Samples were then transferred to microfuge tubes and protein pellet by microcentrifugation at high speed at 4°C. Pellets were washed.
with cold acetone, air-dried briefly, and resuspended in SDS sample buffer. Protein samples were heated at 95°C for 1 minute and stored frozen. To ensure equal sample loading for SDS-PAGE and Western blotting, aliquots of samples from each experiment were subjected to SDS-PAGE and Coomassie staining. These gels were then used for total lane densitometry, allowing normalization of sample loading for subsequent Western blot analysis.

Cell lysates for total NMHC IIA and NMHC IIB Western blots were prepared using 1× lysis buffer (Cell Signaling). SDS samples were resolved on 4% to 20% acrylamide gels and then transferred to polyvinylidene difluoride membrane. Membranes were incubated with the primary antibodies and then with horseradish peroxidase–conjugated secondary antibodies, and the immunoblotted proteins were visualized using Super-Femto Western blot reagents (Pierce, Rockford, IL).

**Results**

Fibronectin-stimulated spreading and myosin II isoforms expression. During migration, cells undergo morphologic changes involving extension of lamellipodia along the extracellular matrix, formation of focal adhesion complexes, translocation of the cell body, and rear-end detachment from the extracellular matrix. Integrin-mediated adhesion and signaling processes are widely established as having critical roles in these events in mammalian cells (31). Adhesion to fibronectin dramatically stimulated lamellar protrusion and spreading in MDA-MB-231 breast cancer cells (Fig. 1A), leading us to explore the behavior of myosin II during this process. We did Western blotting to assess myosin II isoforms.
expression in MDA-MB-231 cells, using control cell lines or tissue known to express each of the three NMHC II isoforms. This analysis revealed robust expression of NMHC IIA and NMHC IIB but no substantial expression of NMHC IIC (Fig. 1B). We cannot completely rule out low level expression of NMHC IIC in the MDA-MB-231 line, as low level expression of this isoform clearly occurs in COS-7 cells (32), which was not detected in our Western blot conditions. However, NMHC IIA and NMHC IIB are clearly the major isoforms present in the MDA-MB-231 line. Further studies described in this work, therefore, were focused on characterizing the dynamics and functions of myosin IIA and myosin IIB isoforms during spreading and migration in these cells.

**Immunolocalization of myosin II isoforms in spreading cells.** Immunostaining of subconfluent cell cultures growing in standard medium revealed dispersed localizations, with some enrichment in basal stress fibers and minor enrichment to the cell cortex (data not shown). However, when cells were actively engaged in lamellar spreading on fibronectin, a dramatic enrichment of both isoforms was observed at the marginal spreading lamellar region of the cells (Fig. 1C, arrowheads). Stress fiber-like structures were also visible with both isoforms in the more central regions of the surface-attached cortical surface, but during this active spreading phase localization to the spreading margin was dramatic for both isoforms. Confocal Z sections were done to determine the three-dimensional organization of the cortical myosin II in cells that were fixed while actively spreading. This analysis revealed detectable but modest levels of myosin II in the cortical regions of the cell that were not adjacent to the fibronectin-coated surface. Thus, for both myosin IIA and IIB, the actively advancing lamellar margin was the major site of accumulation (Fig. 1C and D). This strong recruitment of both isoforms to the spreading margin of the cells is consistent with the hypothesis that myosin II isoforms may play mechanical roles either in driving this protrusive spreading event or formation of focal adhesion complexes with matrix or both.

**Myosin RLC phosphorylation during spreading on fibronectin-coated surface.** If myosin II is actively involved in the mechanics of lamellar protrusion, fibronectin-induced increase in RLC phosphorylation might be predicted during active spreading. Western blot analysis was done with phospho-RLC-specific antibodies to test this possibility (Fig. 2A and B). MDA-MB-231 cell lysates were collected from attached undisturbed cells growing in tissue culture plates (attached), cells resuspended and held in suspension for ~60 minutes (Susp), or from cells resuspended, washed, and replated on fibronectin-coated surfaces for the indicated periods of time. This analysis revealed that a significant level of phospho-RLC exists in attached cells in culture, and this level decreased when cells were detached from culture dishes. Upon reattachment, a progressive increase in RLC phosphorylation occurred throughout the time course of spreading on fibronectin. Immunocytochemical analysis done during spreading with phospho-RLC-specific antibodies furthermore revealed the major phospho-RLC staining to be in the marginal spreading zone (Fig. 2C). These results further support the hypothesis that myosin II activation in the spreading margin may be important for lamellar protrusion. These localization and activation patterns are also consistent with earlier studies that revealed anterior localization of phospho-RLC in migrating fibroblasts and in extending margins of epithelial sheets (24), supporting the concept that lamellar localization and activation of myosin II during isotropic spreading of these breast cancer cells may reflect roles that are conserved with leading edge myosin II roles during polarized migration in other settings.

**Inhibition of MLCK potently blocks cell spreading.** To further assess the functional role of lamellar myosin II localization and activation during spreading, we tested the effects on spreading of inhibitors of myosin II ATPase and inhibitors of upstream enzymes capable of activating myosin II. We have observed that blockade of p38/mitogen-activated protein kinase (p38/MAPK) potently blocks cell spreading, and the p38/MAPK inhibitor SB202190 was therefore used as a control for these experiments. Cells collected from tissue culture plates were treated in suspension culture with vehicle or each inhibitor for 20 minutes. Cells were then seeded on fibronectin-coated tissue culture plates and incubated at 37°C for 60 minutes and fixed, and spread cell area was quantified for ~250 to 400 cells from each treatment condition. Cells treated with vehicle display robust lamellar spreading relative to the SB202190 treatment condition (Fig. 3A and B). The MLCK inhibitor ML-7 also blocked all detectable spreading at the 60-minute time point, implicating MLCK and presumably RLC phosphorylation as critical for efficient spreading on fibronectin. The myosin II motor activity inhibitor blebbistatin also significantly inhibited spreading, although this inhibition of spreading was not as
dramatic as was the effect of ML-7. Inhibition of Rho kinase with Y-27632 partially reduced spreading in this assay, but the effect was modest compared with the dramatic inhibition by ML-7. The strong inhibitory effect of ML-7 and minimal effect of Y-27632 treatment suggest that MLCK has a critical role in driving spreading on fibronectin, and that Rho kinase may be less directly involved in lamellar spreading mechanics. This observation is consistent with studies from Totsukawa et al., who have presented evidence that in fibroblasts MLCK seems to be predominantly responsible for RLC phosphorylation at the cell margin and in anterior protrusions, whereas Rho kinase is predominantly responsible for RLC phosphorylation in interior stress fibers and central regions of the cell (33, 34).

Total RLC phosphorylation levels were assessed during spreading in the presence of the same set of inhibitors to correlate spreading with overall myosin II activation levels (Fig. 3C and D). ML-7, blebbistatin, and Y-27632 all potently reduced RLC phosphorylation. It is noteworthy that Rho kinase blockade with Y-27632 potently reduced RLC phosphorylation, although this compound only modestly reduced spreading. We suggest that this behavior is consistent with the model proposed by Totsukawa et al., discussed above, that Rho kinase may activate RLC phosphorylation in central regions of cells rather than at the cell margin. In this context, Y-27632 treatment during spreading would be predicted to reduce RLC phosphorylation in central regions and in stress fibers but have less effect or no effect at the cell margin. In contrast, ML-7 treatment, inhibiting MLCK, would be predicted to preferentially inhibit RLC phosphorylation at the cell margin in our experiments. The potent inhibition of lamellar spreading that we observe in the current work upon MLCK inhibition, taken together with the model of Totsukawa et al., argues that phosphorylation on marginally localized myosin II is critical for lamellar spreading, but RLC phosphorylation in more central regions of the cell are not critical for lamellar spreading.

Figure 3C and D also indicates that p38-MAPK blockade with SB202190 potently blocks spreading. We suggest that inhibition of spreading indirectly reduces matrix-induced RLC phosphorylation, but that the primary positive role of p38-MAPK during spreading is mediated via targets other than activation of RLC phosphorylation or myosin II contractility, possibly related to paxillin (35, 36) or
Hsp27 (37), both of which have roles in leading edge protrusion and adhesion.

**Inhibition of Rho kinase alters myosin II isoform localization during spreading.** MLCK inhibition via ML-7 potently blocked spreading and reduced RLC phosphorylation, whereas Rho kinase inhibition with Y-27632 reduced RLC phosphorylation with only a modest inhibition of spreading. To determine whether Y-27632 treatment resulted in spatially restricted effects on myosin II assembly and phosphorylation, we did immunostaining on cells spreading in the presence of Y-27632. This analysis showed a substantial reduction of cortical staining for both myosin IIA and IIB isoforms (Fig. 4, top and middle). However, immunostaining for myosin IIA isoform consistently revealed a greater depletion in the marginal lamellar zones than did immunostaining for myosin IIB. Many cells still displayed weak but detectable myosin IIB in the marginal zones of extended lamellae in the presence of Y-27632. These results suggest that myosin IIB in the cell margin may be preferentially more resistant than myosin IIA to disassembly in response to Y-27632 treatment and thus might still be contributing to mechanics of lamellar extension despite the blockade of the Rho kinase pathway. As a further test of this possibility, we did immunocytochemistry on Y-27632-treated spreading cells to determine whether RLC phosphorylation was still occurring in the extending lamellipodia in the presence of this inhibitor. This analysis revealed clear enrichment of phospho-RLC at the cell margin, despite the presence of Y-27632 (Fig. 4, bottom). Although the overall staining intensity was reduced compared with untreated spreading cells, phospho-RLC was clearly detectable in the spreading margin of most cells treated with Y-27632. We suggest that although Y-27632 significantly reduces overall RLC phosphorylation levels in spreading cells, this reduction may occur predominantly in the central regions of the cell and less at the margins of the spreading cells.

**Isoform-specific siRNA depletion of myosin II results in defects in cell migration and lamellar spreading.** To gain further insights into isoform-specific roles of myosin IIA and IIB during spreading and migration, we established conditions for siRNA oligonucleotide-based depletion of myosin IIA and myosin IIB isoforms (Fig. 5A). Importantly, substantial depletion of myosin IIA was obtained with these conditions with no effect on levels of myosin IIB in the same cell population, and conversely, depletion of myosin IIB occurred with no alteration in levels of myosin IIA.

These NMHC IIA– and NMHC IIB–depleted cell populations were assessed for competence to initiate migration in a scratch
wound assay (Fig. 5B and C). siRNA-treated cells were grown for 72 hours to near confluence and then subjected to scratch wounding with a pipette tip. Migration was scored at 2, 4, and 6 hours. This analysis revealed a significant defect in initiation of polarized cell migration in each siRNA-depleted cell population, indicating that both myosin IIA and myosin IIB isoforms have important roles in some aspect of migration, such as extension of lamellipodia, formation of focal adhesion complexes, generation of cell body translocation force, or posterior detachment from the extracellular matrix. These siRNA-treated cell populations also displayed defects in serum-stimulated transwell migration (Fig. 5D). These results argue that both isoforms contribute to migration during migration across flat fibronectin-coated surfaces and during chemotactic migration through transwell pores.

As a test of whether either myosin IIA and IIB or both isoforms contribute to protrusive activity during migration, we assessed the efficiency of siRNA-depleted cell populations for spreading on fibronectin. This analysis revealed that depletion of myosin IIA enhanced spreading at 60 minutes, whereas depletion of myosin IIB decreased spreading at 60 minutes (Fig. 6). These differences were statistically significant, with both the IIA- and IIB-treated populations differing from the control (P < 0.01, paired t test). Taken together, these siRNA results show that both myosin IIA and IIB isoforms are critical for cell migration, but that myosin IIB specifically contributes to lamellar protrusion events in MDA-MB-231 cells. Although the myosin IIA isoform is clearly necessary for normal migration rates in scratch wound assays or transwell migration, it does not seem to contribute to spreading on fibronectin, and its presence is perhaps even inhibitory to spreading. These results suggest that myosin IIA may have other roles, perhaps in formation of focal adhesion complexes, cell body translocation, or in rear end detachment from extracellular matrix during migration.

Discussion

We found that fibronectin dramatically stimulates MDA-MB-231 cell spreading, and that this spreading is coupled to dramatic recruitment of both myosin IIA and IIB to the marginal lamellar zone. This result supports the model that myosin II may contribute to generation of protrusive forces in these cells. Gupton et al. recently reported that introduction of excess tropomyosin into epithelial cells to stabilize and elevate lamellar acto-myosin II structures enhanced rates of lamellar extension and migration (8). Their results support the argument that marginal actomyosin II bundles are related to protrusive force generation.

A series of earlier studies also indirectly support the concept that myosin II may have regulated roles during lamellar extension. A FRET biosensor for MLCK reported enrichment and activation of MLCK in extending lamellipodia (25), and MLCK inhibition has been shown to block lamellipodial extension through transwell chamber pores (38). In mouse embryonic fibroblasts (MEF), spreading on fibronectin involves periodic contraction and protrusion of the cell margin that correlate with waves of assembly and inward flow of actin and MLCK-containing cytoskeletal bundles (39), consistent with our observation of marginal enrichment of myosin II. Tension generation within extending lamellipodia has been strongly implicated as critical for normal focal adhesion maturation (40, 41), and MLCK activity is involved in focal contact dynamics (42). These observations, together with our localization data support the concept that myosin II isoforms play an important role or roles during lamellar extension.

During active spreading on fibronectin, we observed a concurrent increase in RLC phosphorylation (Fig. 2), supporting the model that myosin II–based force generation is involved in the mechanics of lamellar protrusion. Inhibition of RLC phosphorylation by ML-7 severely blocked lamellar protrusion, further supporting this hypothesis. Our results and this model are consistent with observations of Giannone et al. who found that MLCK inhibition reduced spreading by MEFs (39). Interestingly, these results are in sharp contrast to conclusions drawn by Wakatsuki et al. These workers found that kinase inhibitors intended to block MLCK actually enhanced spreading of chicken embryonic fibroblasts and therefore proposed that myosin II acts in cells to drive inward contraction only and is thus antagonistic to lamellar spreading (7). We suggest two reasons that their experiments yielded such dramatically contrasting results. First, these workers assessed lamellar spreading on tissue culture plastic in the absence of any extracellular matrix coating. As presented in Fig. 1A of our work, the presence of fibronectin profoundly enhances the rate of lamellar spreading. We suggest that integrin engagement and outside-in signaling may be a direct trigger for the dramatic myosin II recruitment that we observe in our assays, and that the recruited myosin II is a direct factor in the enhanced rates of spreading in this setting. Such a mechanism would also explain the fact that Wakatsuki et al. did not observe lamellar accumulation of myosin II...
in their spreading assays done on uncoated plastic dishes. Second, neither inhibitor used in that study (KT5926 and staurosporine) is specific to MLCK. KT5926, described in the work as an MLCK inhibitor, has also been used in published studies as an inhibitor of unrelated protein kinases, such as CaM kinase II and heat shock protein kinase HSP25 (43, 44). Inhibitors used in the Wakatsuki work likely inhibited many cellular processes unrelated to MLCK.

Our siRNA depletion studies further suggest that although myosin IIA and IIB are both recruited to spreading lamellipodia, myosin IIB seems to make the more critical contribution to the mechanics of protrusion. Depletion of myosin IIA in fact resulted in a slight increase in spreading rates in our assays. These results are noteworthy in the context of the model of Totsukawa et al. that Rho kinase may predominantly activate myosin II contractility in central regions of cells with MLCK having a predominant role in myosin II activation at the cell margin (34). We suggest that isotropic spreading on extracellular matrix occurs via a shift in the balance of myosin II–based contractile forces, with activation of marginal myosin II contractile force favoring spreading, and with this force outweighing the “rounding-up” cell forces driven by more central myosin II–based contractile structures (such as stress fibers). Blockade of MLCK shifts the balance in the opposite direction, so that central contractile forces predominate and cells round up. Rho kinase blockade, in contrast, lowers overall RLC phosphorylation levels but does so predominantly by reducing RLC phosphorylation in central regions of the cell. The reduced but persistent RLC phosphorylation at the cell margin with Rho kinase blockade is thus still sufficient to allow significant rates of cell spreading (as in Fig. 3).

We suggest that the myosin IIB isoform may be most fundamentally involved in the mechanics of lamellar protrusion based upon our siRNA studies. Interestingly, Meshel et al. recently reported that myosin IIB null MEFs display defects in lamellipodial retraction of collagen fibers towards the cell body (23). That defect may represent a related function to the function for myosin IIB revealed in our spreading analysis, as both collagen bundling and lamellar extension could involve centripetally directed myosin II–based force generation. This pattern is also consistent with an earlier report that neurite outgrowth in cultured cells can be blocked by myosin IIB antisense treatment but not with myosin IIA antisense (21). Our results, taken together with these earlier observations, suggest that the myosin IIB isoform of mammalian cells has a role in the mechanics of lamellar protrusive events, whereas the myosin IIA isoform is critical for normal migration but seems to function in some process distinct from facilitation of protrusion.

In sum, the dramatic recruitment of myosin II to the margin of actively spreading lamellae, the activation of RLC phosphorylation in this zone during spreading, and the effects of isoform-specific siRNA depletion all argue for an important role for myosin II in lamellar extension, with myosin IIB seeming to have the predominant critical role in the process. We suggest that myosin IIB in particular may serve a role in direct mechanical force generation. However, myosin II proteins might conceivably also participate in membrane trafficking events related to leading edge functions. Myosin II has been implicated in vesicle trafficking in several settings (19, 45), which could be relevant to leading edge functions, such as delivery of adhesion proteins or signaling molecules during migration. Further studies are needed to clarify the exact mechanical role or roles of myosin IIA and IIB during lamellar protrusion and migration.

**Acknowledgments**

Received 11/28/2005; revised 2/13/2006; accepted 2/27/2006.

**Grant Support**: NIH grant GM0009 (T.E. Egelhoff) and Case Comprehensive Cancer Center seed funds from NIH grant CA5703.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Bob Adelstein for gifts of siRNA oligonucleotides and anti-MHC-IIC antibodies and for helpful discussions throughout the course of this work.

**References**

26. Kolega J. Asymmetric distribution of myosin IIIB in migrating endothelial cells is regulated by a...
rho-dependent kinase and contributes to tail retraction. Mol Biol Cell 2003;14:4745–57.
Distinct Roles of Nonmuscle Myosin II Isoforms in the Regulation of MDA-MB-231 Breast Cancer Cell Spreading and Migration

Venkaiah Betapudi, Lucila S. Licate and Thomas T. Egelhoff


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/66/9/4725

Cited articles  This article cites 44 articles, 28 of which you can access for free at: http://cancerres.aacrjournals.org/content/66/9/4725.full#ref-list-1

Citing articles  This article has been cited by 36 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/66/9/4725.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.