A Rho-dependent signaling pathway operating through myosin localizes β-actin mRNA in fibroblasts

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Background: The sorting of mRNA is a determinant of cell asymmetry. The cellular signals that direct specific RNA sequences to a particular cellular compartment are unknown. In fibroblasts, β-actin mRNA has been shown to be localized toward the leading edge, where it plays a role in cell motility and asymmetry.

Results: We demonstrate that a signaling pathway initiated by extracellular receptors acting through Rho GTPase and Rho-kinase regulates this spatial aspect of gene expression in fibroblasts by localizing β-actin mRNA via actomyosin interactions. Consistent with the role of Rho as an activator of myosin, we found that inhibition of myosin ATPase, myosin light chain kinase (MLCK), and the knockout of myosin II-B in mouse embryonic fibroblasts all inhibited β-actin mRNA from localizing in response to growth factors.

Conclusions: We therefore conclude that the sorting of β-actin mRNA in fibroblasts requires a Rho mediated pathway operating through a myosin II-B-dependent step and postulate that polarized actin bundles direct the mRNA to the leading edge of the cell.

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Background

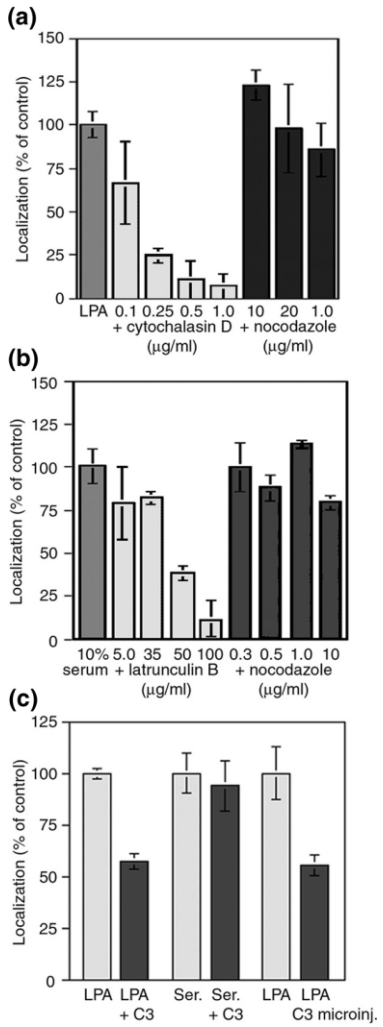
The leading edge of fibroblasts is a structure specialized for cell motility by the polymerization of actin filaments [1]. Just proximal to the leading edge, we have shown, is the site of β-actin mRNA localization in the lamella [2]. A specific sequence in the 3′ untranslated region (UTR) of the β-actin mRNA, termed the "zipcode" [3], is necessary for the localization of the mRNA and specific *trans*-acting factors, termed "zipcode binding proteins" [4], recognize this zipcode and are likewise part of the mechanism that transduces the nucleic acid information into cellular spatial information. The spatial distribution of the β-actin mRNA is important for cellular asymmetry and polarity; when β-actin mRNA is missorted in fibroblasts by the use of antisense oligonucleotides directed against the zipcode sequence, it leads to diminished cell polarity [2] and decreased motility of the fibroblasts [5, 6]. How does the mRNA know where to go? Growth factors can affect the site of β-actin synthesis in the cytoplasm by inducing rapid localization of β-actin mRNA toward the leading edge in chicken embryo fibroblasts (CEFs) [7]. This movement of RNA is signaled through a signal transduction cascade involving the G proteins, but it is currently unknown how this mechanism impinges on the RNA movement or what the players are in this process.

Results

In order to understand the cellular mechanisms effecting this sorting, we investigated how signals are transduced from cellular receptors into RNA movement. Localization of β-actin mRNA is dependent on the actin cytoskeleton since CEFs treated with cytochalasin D at concentrations that disrupt microfilaments inhibit the induction of localization by the growth factor LPA [7], while treatment with nocodazole at concentrations that disrupt microtubules had no effect (Figure 1a). Similarly, introduction of latrunculin B at concentrations disrupting microfilaments also strongly inhibited localization (Figure 1b).

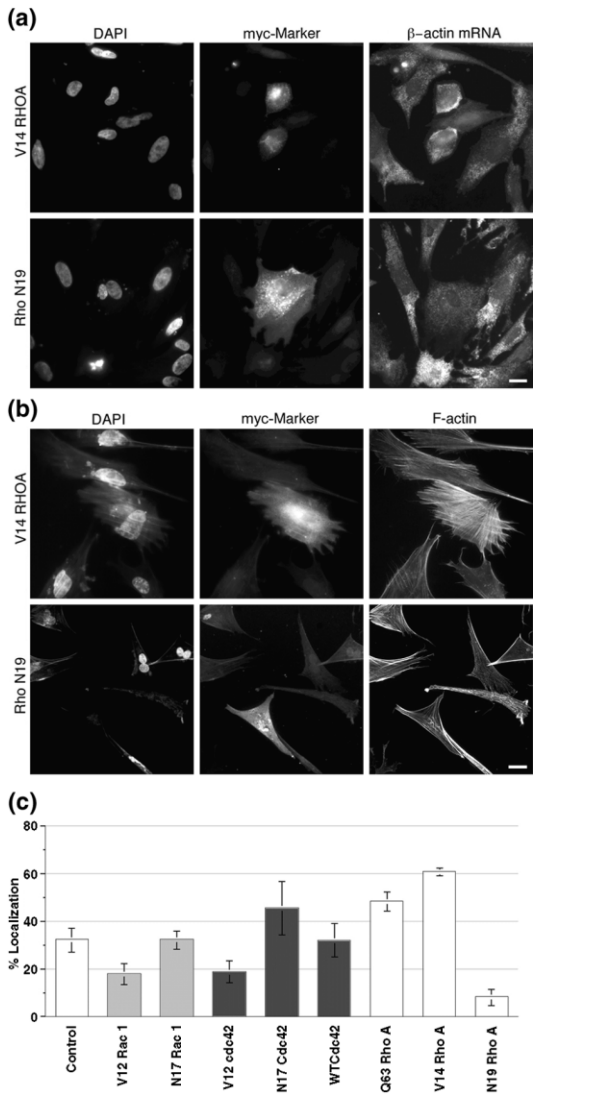
Since Rho family GTPases regulate actin cytoskeleton organization [8], one or more of these signaling proteins may also regulate β-actin mRNA localization. C3 transferase inactivates Rho [9] when it is added to the culture media [10] or microinjected [11]. In the present study, we found that when fibroblasts were exposed to C3 transferase, the localization of β-actin mRNA was inhibited when it was induced by LPA but not by serum (Figure 1c). This suggests that β-actin mRNA localization can be induced through a Rho dependent pathway after LPA stimulation for 10 min. It may also be induced via alternative stimulatory pathways, presumably by other factors

Figure 1



Actin cytoskeleton and activated Rho GTPase are required for the localization of β-actin mRNA. CEFs were serum starved for 18 hr and pretreated with cytoskeletal disruptors. **(a)** CEFs were treated with cytochalasin D and nocodazole before a 10 min induction with LPA. **(b)** Serum-starved CEFs were pretreated with latrunculin for 30 min followed by stimulation with 10% serum for 30 min. **(c)** CEFs were pretreated with Rho inhibitor C3 transferase for 3 hr or microinjected with either C3 or buffer before induction with LPA for 10 min (200 ng/ml) or 10% serum induction for 1 hr (n = 800). The percentages were normalized, with 100% equal to LPA or serum induction.

Figure 2



Activated Rho induces β-actin mRNA localization. **(a)** β-actin mRNA localization of CEFs transfected with V14RhoA and N19RhoA growing in 10% serum. Coverslips were fixed and processed for in situ hybridization with Cy 3-labeled β-actin probes and with antibodies to detect the Myc marker epitope for transfected cells (upper, n = 354 transfected cells; lower, n = 206 transfected cells). Cells were stained with DAPI for nuclei. The scale bar represents 20 μm. **(b)** Phalloidin staining of CEFs transfected with V14RhoA and N19RhoA. The scale bar represents 20 μm. **(c)** Cells were transfected with Rho, Rac, and Cdc42 constructs, processed with in situ hybridization for β-actin mRNA and with immunofluorescence for the Myc-marker, and scored according to their localization of β-actin mRNA (sampling population [n] = 204–415 cells). The control bar represents the mean percent localization of untransfected cells on each GTPase-transfected coverslip. The percent localization of untransfected cells was comparable to transfections with the vector alone (not shown).

that are present in the serum and can induce localization [7, 12] and by the longer stimulation time with serum (1h) versus the early maximal (10 min) stimulation seen with LPA [7].

To further confirm the possible roles for each of the GTPases in the LPA pathway, we transfected and expressed constitutively active and dominant-negative mutated Rho, Rac, and Cdc42 [13]. CEFs transfected with activated V14RhoA localized β-actin mRNA in the lamella or near the leading edge (Figure 2a, top) and significantly increased the percentage of cells with localized β-actin by 2-fold ($p = 0.0030$, 354 transfected cells; Figure 2c)

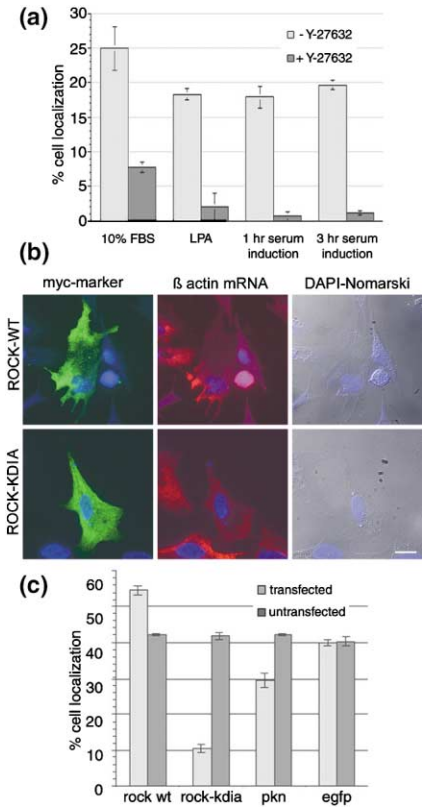
over control cells (untransfected). Another activated Rho construct, Q63LRhoA, also showed a significant increase in localization ($p = 0.0197$, 275 transfected cells). In contrast, when cells were transfected with dominant-negative N19RhoA, β-actin mRNA was delocalized (Figure 2a, bottom), and the percentage of cells localizing β-actin mRNA decreased by 3-fold over control cells ($p = 0.0042$, 206 transfected cells; Figure 2c). V14RhoA transfections increased stress fibers throughout the cell (Figure 2b, top), while transfection with N19RhoA resulted in a decrease in stress fibers (Figure 2b, bottom). Neither activated nor inactive Rac (V12Rac1 and N17Rac1, respectively) showed highly significant differences in the percentage of transfected cells with β-actin mRNA at the leading edge ($p = 0.0665$, 204 transfected cells and $p = 0.3414$, 376 transfected cells, respectively; Figure 2c). Activated and dominant-negative Cdc42 did not demonstrate as highly significant differences in localization compared to RhoA ($p = 0.0896$, 264 transfected cells and $p = 0.9758$, 415 transfected cells, respectively). These data provide further evidence supporting the significance of activated Rho GTPase in the induction of β-actin mRNA localization.

Since transfection with activated RhoA increased β-actin mRNA localization, downstream effectors of Rho may mediate this induction of β-actin mRNA localization. Downstream effectors ROCK (Rho-associated coil coiled-forming protein kinase) I and ROCK II, have been shown to induce the formation of stress fibers and focal adhesions [14]. A pyridine derivative,Y-27632, inhibits smooth muscle contraction and selectively inhibits ROCK kinases [15]. It binds ATP binding sites of these kinases with a 10-fold higher affinity than protein kinase N (PKN) and citron kinase and with a 100-fold higher affinity than protein kinase A (PKA), protein kinase C (PKC), or MLCK [16]. Treatment with Y-27632 resulted in delocalization of β-actin mRNA in cells grown in serum (Figure 3a). This delocalization was accompanied by a decrease in stress fiber formation (not shown). The correlation between stress fiber formation and β-actin mRNA localization suggested the possibility that sorting could occur thereon. Inhibition of β-actin mRNA localization with Y-27632 was even more apparent in cells induced by serum or LPA (Figure 3a), and this finding indicated that cells actively localizing mRNA were more affected by the inhibitor than cells in steady state. Serum-induced localization was inhibited by Y-27632, but not with C3 transferase. This may be due to the lower permeability of the cells to C3 relative to Y-27632, and this lower permeability may result in less effective inhibition of stress fiber formation.

To verify the importance of these Rho-associated kinases in the induction pathway, we transfected CEFs with a wild-type construct for p160ROCK and with a mutant containing a defective kinase and defective Rho binding domains (KD-IA [17]). Wild-type p160ROCK significantly increased localization, whereas the KD-IA construct suppressed localization compared to untransfected cells ($p = 0.0425$, 447 transfected cells and $p = 0.0001$, 439 transfected cells, respectively; Figure 3b,c). PKN, which is another downstream effector of Rho and is not involved in Rho-kinase activation [18], did not induce localization; in this way it is similar to the EGFP vector alone (Figure 3c). These data indicate that p160ROCK is a downstream effector involved in Rho-mediated induction of β-actin mRNA localization, specifically in the movement of β-actin mRNA in response to growth factors.

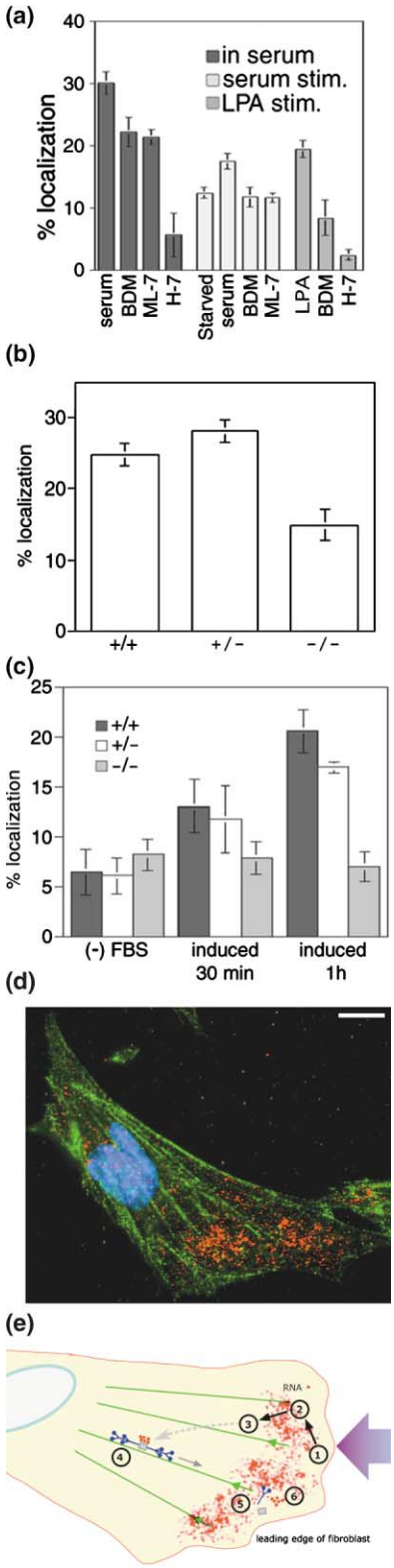
How might RNA localization occur on an actin-based struc-

Figure 3



Rho-kinase involvement in β-actin mRNA localization. **(a)** CEFs in serum alone and with 10 μm Y-27632 as indicated. β-actin mRNA localization was scored as the percent of cells showing localization ($n = 800$). **(b)** Upper panels show CEFs transfected with the p160ROCK wild-type expression vector. Lower panels show CEFs transfected with kinase-defective mutant ROCK-KD-IA. Transfected cells were detected with Myc antibody (left), processed with in situ hybridization for β-actin mRNA (center), and imaged for transmitted light (right). All panels were stained with DAPI (blue). The scale bar represents 25 μm. **(c)** CEFs transfected with ROCK and ROCK-KD-IA from (b) were scored for β-actin mRNA localization ($n = 447$ and 439 respectively). PKN represents protein kinase N. The EGFP vector alone represents the transfection control ($n = 491$).

Figure 4



The localization of β-actin mRNA is dependent on myosin II-B. **(a)** The effects of actomyosin interaction inhibitors on β-actin mRNA transport and localization. The percentage of cells exhibiting localized β-actin mRNA is shown (n = 800). CEFs in 10% serum (dark bars), serum-starved cells (light bars) that were then serum-stimulated, and cells stimulated with LPA (medium-gray bars) are indicated. The inhibitors used are also indicated. **(b)** β-actin mRNA localization was scored in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) myosin II-B knockout fibroblasts cultured in serum (n = 800). **(c)** Wild-type, heterozygous, and homozygous myosin II-B knockout MEFs were serum starved, serum induced for 30 min or 1 hr, and then scored for β-actin mRNA localization (n = 800). **(d)** In situ hybridization for β-actin mRNA (red) and immunofluorescence for phosphorylated myosin (green) were performed on CEFs after 15 min serum induction . DAPI staining is shown in blue. The scale bar represents 10 μm. **(e)** Model of (d) for how mRNA may move on polarized actin bundles associated with myosin II-B. (1) Incoming signals (arrow) binding receptors (2) activate the RhoA cascade (3) through Rho-kinase, (4) which activates myosin II-B light chains and filament assembly, which forms an mRNA-complex, (5) which then moves over polarized actin bundles to disassemble toward the leading edge as a result of heavy chain phosphorylation, (6) and the RNA then anchors. The speed of myosin movement predicts that the mRNA would only be transported for 20 s before it becomes anchored. Hence, the steady-state distribution of the mRNA population would be at the lamella.

ture? Because of the known relationship of β-actin mRNA localization to the actin cytoskeleton (Figure 1a,b) and the corresponding relationship of Rho-kinase-mediated signaling with mRNA movement (Figure 3), actin stress fiber formation, and myosin activation, we inferred that actomyosin interactions were likely involved in the observed RNA movement. Rho is known to induce actomyosin interactions leading to cell contraction [19]. Rho-kinase can activate myosin by directly phosphorylating the myosin light chains. In addition, it can also increase myosin light chain phosphorylation by phosphorylating and inhibiting the myosin phosphatase in the presence of ongoing MLCK activity [20, 21]. We therefore used pharmacological agents that inhibit myosin activity: myosin ATPase inhibitor BDM [22, 23], MLCK and nonspecific protein kinase inhibitor H-7 [24], and specific MLCK inhibitor ML-7 [25]. Treatment with each of these inhibitors resulted in delocalized β-actin mRNA (Figure 4a).

In order to evaluate the specific myosin required for localization, we investigated RNA movement versus anchoring in mouse embryonic fibroblasts (MEFs) from myosin II-B knockout mice [26]. We focused on this specific knockout because isoforms of myosin II have been shown to localize differentially in some non-muscle cell types. For instance, in *Xenopus* A6 cells, myosin II-B localizes at the leading edge [27] and can be found in lamellar regions of other cell types [28]. Wild-type (NMHC-IIB$^{+/+}$) and heterozygous (NMHC-IIB$^{+/-}$) MEFs localized β-actin mRNA to the leading edge in a manner similar to that of CEFs when they were cultured in serum (not shown). MEFs from myosin II-B knockout mice (NMHC-IIB$^{-/-}$) did not localize β-actin mRNA as well as cells from heterozygous and wild-type mice (Figure 4b). However, an even more striking disparity was seen when we serum stimulated myosin II-B knockout cells in order to induce RNA movement.

In contrast to wild-type and heterozygous cells, these cells showed no increase in the localization of β-actin mRNA after serum stimulation (Figure 4c). These data suggest that myosin II-B plays a role in the *movement* of β-actin mRNA to the leading edge.

Myosin II-B could act as a motor in β-actin mRNA transport or play a structural role that is necessary for movement. This role in mRNA movement is additionally supported by the poly $(A)^+$ RNA movement and localization that are associated with F-actin in halos around fibronectin-coated beads and which are also prevented by myosin inhibitor BDM [29].

We characterized the distribution of myosin II-B in wild-type fibroblasts in order to determine its coincidence with β-actin mRNA during serum stimulation. Myosin II-B was not specifically localized to the leading edge but was dispersed throughout the cytoplasm (data not shown). However, since Rho-kinase regulates myosin light chain phosphorylation [20, 21] and consequent motor activity, we investigated phosphorylated myosin II distribution by using a specific antibody ([30]; Figure 4d). After serum stimulation, the antibody to phosphorylated myosin decorated stress fibers protruding into the leading edge, where the bulk of mRNA became localized.

Discussion

How could myosin II direct mRNA movement to the leading edge? We postulate that the two-headed myosin filaments can translocate on polarized bundles of actin filaments (a.k.a., "stress fibers" [31]) toward the leading edge associated with an mRNA complex that can bind the myosin (Figure 4e). This hypothesis is supported by work demonstrating that, in moving fibroblasts, these actin bundles have a polarity with barbed ends increasingly directed toward the lamellipodium and thus could constrain activated myosin II-B movement only toward the leading edge [31, 32]. There is evidence that myosin activation and deactivation can be spatially regulated [33]. Rho-kinase can lead to phosphorylation of the light chains internally near the nucleus, where myosin filament assembly, stress fiber formation, and motility occurs [33]. PKC in the periphery of the cell can phosphorylate myosin II-B heavy chains and promote disassembly [34, 35] at the leading edge. This could transport the mRNA bound to the myosin toward the leading edge, where it would anchor to actin filaments when the myosin filament disassembles (Figure 4e).

The preceding work demonstrates how extracellular signals can act through the signaling mediators Rho and Rho-kinase in the regulation of β-actin mRNA distribution via actomyosin interactions. In this way, spatially localized protein synthesis is a component of gene expression that can respond rapidly (2 min) to extracellular signals and immediately effect physiological changes within specific cellular compartments.

Materials and methods

Materials

Lysophosphatidic acid (LPA: Oleoy-*sn*-glycero-3-phosphate) and BDM (2,3-butanedione 2-monoxime) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Cytochalasin D, nocodazole, H7, and ML-7 were purchased from CalBiochem (La Jolla, California). Mouse monoclonal anti-Myc antibody was purchased from Boehringer Mannheim (Indianapolis, Indiana). Cy 2(fab)- and Cy 5-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (Westgrove, Pennsylvania), and rhodamine phalloidin was purchased from Molecular Probes (Eugene, Oregon). pEXV-MycV14Rho A, pEXV-MycV12Rac1, pEXV-MycN17Rac1, pEXV-MycN17Cdc42, pEXV-MycV12Cdc42, and pEXV-MycWTCdc42 were provided by A. Hall (University College London). pEXV-MycN19RhoA was provided by Richard Pestell (Albert Einstein College of Medicine, Bronx, NewYork). Q63LrhoA was provided by Gary Bokoch (Scripps Research Institute, La Jolla, California). Y-27632 was provided by Yoshitomi Pharmaceuticals Japan. p160ROCK constructs were provided by Toshimasa Ishizaki and Shuh Narumiya, Kyoto University, Faculty of Medicine, Kyoto, Japan). Serine 19 phosphorylated myosin II antibody (pp2b) was provided by Fumio Matsumura (Rutgers University, Piscataway, New Jersey).

Cell culture and inhibitor treatments

CEFs were seeded on coverslips and grown for 24 hr, followed by treatment with the indicated concentrations of cytochalasin D or nocodazole for 30 min. They were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA). CEFs were also serum-starved for 18–20 hr, then pretreated with cytochalasin D or nocodazole for 30 min. This was followed either by induction with LPA (200 ng/ml) for 10 min or by induction with 10% serum for the indicated time. For the detection of β-actin mRNA, the CEFs were then fixed and processed for in situ hybridization and detected by the use of alkaline phosphatase [7]. Cells were treated with 10 μm Y-27632 for 30 min, then induced with LPA (200 ng/ml) for 10 min or induced with 15% serum for the indicated time. Next, the cells were fixed and processed for in situ hybridization as above. For C3 inhibition, cells were pretreated with C3 transferase (Upstate Biotechnology Inc. [UBI], Lake Placid, New York) at 30 μg/ml for 3 hr. For microinjection**,** CEFs were grown on 10 mm plates for 24 hr in Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 10% FCS. Cells were then seeded on 175 μm CELLocate coverslips (Eppendorf, Hamburg, Germany) and serum starved for 18 hr. Next, coverslips were placed in 10 mm dishes in DMEM 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; Gibco BRL, Grand Island, NY) buffer and injected at 50 psi via an Eppendorf microinjector in the manual mode. Cells were injected with 160 μg/ml C3 transferase (UBI) diluted in microinjection buffer (0.048 M K_2PO_4, 0.014 M NaH_2PO_4 [pH 7.2], 0.0045 M KH_2PO_4). Cells (25–50) were injected on each coverslip, incubated for 15 min at 37°C, then fixed and processed for in situ hybridization with alkaline-phosphatase detection. BDM (20 mM) and H7 (300 μM) were added to the cultures for 1 hr, and ML-7 (1 μM) was added for 30 min. Cells were fixed in PFA immediately after all treatments.

Transfections and in situ hybridization

For transfections, CEFs were seeded on coverslips, grown for 24 hr in DMEM containing 10% FBS, washed in HBSS, and then placed in 6-well dishes containing DMEM. DMEM (2 ml) with 2.5 μg plasmid DNA and 11.25 μl TFX 50 (Promega) was then placed in each well and incubated for 2 hr. The medium was then gently poured off and replaced with DMEM +10% fetal bovine serum (FBS) for 24–48 hr. Alternatively, cells on coverslips in 6-well plates were transfected with Effectene (Qiagen) (5–25 μl Effectene, 0.4–1.0 μg DNA) in 10% FBS and 2 ml either DMEM or MEM from 4 hr to overnight, washed in Hanks Balanced Salt Solution (HBSS), and replaced with fresh 10% FBS and DMEM or MEM. Cells were then fixed in 4% PFA and stored in PBS or 70% EtOH.

Coverslips were processed for in situ hybridization by alkaline-phosphatase detection as described or by the use of Cy-3 fluorochrome-labeled β-actin oligonucleotide probes (total = 20 ng probe, 20 μg salmon sperm [SS] DNA [Sigma], 20 μg tRNA [Boehringer], and 10 mM vanadyl ribonuclease complex [VRC, Gibco]). In situ hybridization conditions were standard (50% formamide and 2X SSC [0.3M NaCl 0.03 sodium citrate]); incubation was for 3 hr at 37°C. Alternative conditions were 30% formamide and 1X SSC for 3 hr at 50°C, followed by 1X SSC washes and overnight protein renaturation in 1X PBS/5 mM $MgCl_2$. Myc or HA tags were detected with mouse monoclonal antibodies (Boehringer) and secondary Cy-2 fluorochrome-conjugated antibodies (Molecular Probes). Washes were in TBS (50 mM Tris [pH 7.4], 150 mM NaCl in DEPC ddH_2O) and/or 1X PBS/5 mM $MgCl_2$, and nuclei were stained with DAPI. Cells were viewed on an Olympus fluorescent microscope for the detection of Myc labeling and β-actin mRNA, and they were scored for localization. F-actin was visualized with rhodamine-labeled phalloidin (Molecular Probes).

Quantitation of β-actin mRNA localization

After in situ hybridization, coverslips were viewed by bright field microscopy for inhibitor studies or by fluorescence microscopy for transfections and scored for the percentage of CEFs showing β-actin mRNA localization. Cells were scored as having localized β-actin mRNA if at least 80% of the signal was peripherally concentrated over leading lamellae, an area comprising approximately 25% of the cell area. CEFs that were scored as nonlocalized had diffusely distributed β-actin mRNA throughout the cytoplasm. Cells with perinuclear staining also were scored as nonlocalized. Random fields of cells under low power were selected from at least two separate coverslips per condition, and all cells in the field were scored. Approximately 400 cells per coverslip were counted for inhibitor studies, and all transfected cells excluding adherent rounded cells were counted for GTPase studies. Counts were routinely performed by more than one investigator. As controls, untransfected cells from the same coverslip were also scored. Replicates were averaged, and data were either plotted as the percent of cells showing peripheral localized β-actin mRNA or converted to a ratio of the percent of treated cells showing localized mRNA to the percent of untreated controls. Statistical analysis was done with the unpaired t test and Statmost software.

Myosin knockout cells

MEFs were isolated from mouse embryos of myosin II-B knockout mice. MEFs of wild-type mice and mice heterozygous or homozygous for the II-B knockout were grown in DMEM and 10% FCS on coverslips. For immunofluorescence and imaging, Myosin II-B antibodies were incubated for 1 hr at 37°C at a 1:100 dilution in TBS with 2% donkey serum, 2% BSA, and 0.1% triton. Cells were then washed three times for 10 min in TBS. A secondary antibody (Cy 3, Cy 2, or fluorescein at 1:750 in 2% donkey serum and 2% bovine serum albumen [BSA]) was applied for 1 hr at 37°C. pp2B (phosphorylated myosin II antibody) was first blocked for 1 hr with 2% donkey serum and 2% BSA. After in situ hybridization and protein renaturation overnight, coverslips were treated with 1:200 dilution pp2B antibody in 1% BSA/10 mM VRC at 37°C for 1 hr. Antibodies were detected with conjugated secondary antibodies (1:750 dilution) in 1% BSA and 10 mM VRC at 37°C for 1 hr. Images were taken with an Olympus fluorescent microscope with an Olympix cooled CCD camera and Esprit software. Image intensity was scaled and formatted with Adobe Photoshop.

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