Myo1c facilitates G-actin transport to the leading edge of migrating endothelial cells

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addition of actin monomer (G-actin) to growing actin filaments (F-actin) at the leading edge generates force for cell locomotion. The polymerization reaction and its regulation have been studied in depth. However, the mechanism responsible for transport of G-actin substrate to the cell front is largely unknown; random diffusion, facilitated transport via myosin II contraction, local synthesis as a result of messenger ribonucleic acid localization, or F-actin turnover all might contribute. By tracking a photoactivatable, nonpolymerizable actin mutant, we show vectorial transport of G-actin in live migrating endothelial cells (ECs). Mass spectrometric analysis identified Myo1c, an unconventional F-actin–binding motor protein, as a major G-actin–interacting protein. The cargo-binding tail domain of Myo1c interacted with G-actin, and the motor domain was required for the transport. Local microinjection of Myo1c promoted G-actin accumulation and plasma membrane ruffling, and Myo1c knockdown confirmed its contribution to G-actin delivery to the leading edge and for cell motility. In addition, there is no obvious requirement for myosin II contractile–based transport of G-actin in ECs. Thus, Myo1c-facilitated G-actin transport might be a critical node for control of cell polarity and motility.

Introduction

Actin polymerization drives cell locomotion, proceeding by addition of monomeric actin (G-actin) to the barbed end of actin filaments (F-actin; Pollard and Borisy, 2003). Actin polymerization is highly polarized and spatially restricted in lamellipodia within a band ~1–3 µm in width along the leading edge of a moving cell (Watanabe and Mitchison, 2002; Ponti et al., 2004; Lai et al., 2008). A high amount of lamellipodial G-actin is consumed to drive movement—for example, ~3.6 million actin molecules per minute in a crawling breast cancer cell (Chan et al., 1998). Passive diffusion has been suggested to be the major pathway for providing G-actin to the cell leading edge (Koestler et al., 2009). However, diffusion might be insufficient for entering and traversing the viscous, dense, and highly structured lamellipodial space. Recent experimental and theoretical studies are consistent with diffusion-limited actin polymerization (Noireaux et al., 2000; Mogilner and Edelstein-Keshet, 2002; Plastino et al., 2004). Other mechanisms might contribute to delivery of G-actin to lamellipodia, including local synthesis as a result of mRNA relocalization (Lawrence and Singer, 1986; Shestakova et al., 2001), facilitated transport via myosin II contraction (Peckham et al., 2001; Zicha et al., 2003), or actin treadmilling by rapid F-actin turnover (Cramer, 1999). Forward actin flow reported in the protrusion region suggests active transport of G-actin to the leading edge (Zicha et al., 2003). However, little is known about molecular mechanisms regulating G-actin delivery to the leading edge. Here, we reveal an important contributory role of Myo1c in G-actin transport during endothelial cell (EC) migration.

Results and discussion

Vectorial transport of G-actin to the EC leading edge during migration

To examine G-actin localization during cell migration, bovine aortic ECs were induced to move by razor wound (Ghosh et al., 2002) and stained with fluorescence-labeled DNase I. Confocal microscopy showed uniform distribution in quiescent cells but pronounced G-actin accumulation at the leading edge of migrating cells (Fig. 1 A), consistent with a previous study in fibroblasts (Cao et al., 1993). To determine the contribution of F-actin
introduced exogenously to permeabilized cells, also accumulated at the cell leading edge, directly showing mRNA-independent G-actin translocation (unpublished data). To investigate the role of vectorial transport, directed movement of G-actin was measured by photoactivation of a chimera of nonpolymerizable actinG13R and photoactivatable GFP (paGFP; Patterson and Lippincott-Schwartz, 2002). The reporter was photoactivated near the leading edge of live migrating cells, and time-lapse fluorescence intensity was measured in three regions. (top) Representative fluorescent images of paGFP before (left) and after (center) photoactivation; brightness of preactivated paGFP was enhanced to show cell and regions of photoactivation and signal acquisition (right). (bottom) Fluorescence intensity in each of three regions was measured for 120 s. The data shown are representative from multiple experiments (n = 5–7 cells).

turnover to G-actin localization, two nonpolymerizable actin mutants, G13R and R62D, mutated at the nucleotide-binding pocket and the salt bridge that joins actin subdomains, respectively (Posern et al., 2002), were expressed as GFP chimeras. Both mutant proteins accumulated at the leading edge (Figs. 1 B and S1), suggesting an F-actin turnover-independent mechanism for G-actin polarization. To determine the potential contribution of actin mRNA relocalization (Lawrence and Singer, 1986; Shestakova et al., 2001), cells were pretreated with cycloheximide to block de novo actin synthesis. The protein synthesis inhibitor did not alter G-actin accumulation in the lamellipodia (unpublished data), consistent with a previous study showing that de novo synthesis contributes only ~7% of the G-actin required for polymerization in migrating cells (Condeelis and Singer, 2005). Fluorescent Alexa Fluor 488–labeled actin, introduced exogenously to permeabilized cells, also accumulated at the cell leading edge, directly showing mRNA-independent G-actin translocation (unpublished data). To investigate the role of vectorial transport, directed movement of G-actin was measured by photoactivation of a chimera of nonpolymerizable actinG13R and photoactivatable GFP (paGFP; Patterson and Lippincott-Schwartz, 2002). The reporter was photoactivated near the leading edge of live migrating cells, and time-lapse fluorescence intensity was measured in front of and behind the photoactivation region. The initial rate of forward movement of paGFP-actinG13R was about twice that of the rearward rate (Fig. 1 C). No difference was detected between forward and rearward rates of movement of the paGFP control protein, which is likely a result of random diffusion. Furthermore, FRAP for GFP-actinG13R at the leading edge of migrating ECs is about
cargo-binding tail domain of Myo1c interacts with G-actin. (A) Identification of Myo1c as a G-actin–interacting protein. Lysates from migrating cells transfected with pcDNA-actinWT-myc were immunoprecipitated (IP) with anti-myc antibody (Ab) and resolved by SDS-PAGE. Proteins in gel-eluted bands were identified by MS. (B) Domain structure of Myo1c including head, IQ-containing neck, and tail domains. (C) Binding of Myo1c tail domain to G-actin. G-actin was immobilized on a sensor chip, purified Myo1c was injected, and the interaction was measured by SPR. (Inset) Kd calculated from three experiments. RU, relative unit. (D) Tail domain interacts with G-actin in migrating cells. ECs were cotransfected with pDream-Flag-actinWT and pcDNA-Myo1c-myc. Cell lysates were immunoprecipitated with anti-myc antibody and immunoblotted (IB) with anti-myc, Flag, and actin antibodies. (E) Myo1c tail domain preferentially binds G-actin. ECs were cotransfected with pcDNA-Myo1c tail-myc and pDream-Flag-actinWT or pDream-Flag-actinG13R and treated with 1 µM jasplakinolide for 1 h. Cell lysates were immunoprecipitated with anti-myc antibody and immunoblotted with anti-myc and Flag antibodies. Lysates were immunoblotted with anti-Flag and anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies.
study showing that Myo1b localization in the protrusive edge of spreading epithelial cells requires the motor domain (Tang and Ostap, 2001). Remarkably, overexpression of headless Myo1c prevented G-actin accumulation at the cell leading edge, possibly by competing with endogenous Myo1c for G-actin binding and transport. The interaction of Myo1c with G-actin and its spatial distribution during cell migration were investigated by Förster resonance energy transfer (FRET) coupled to confocal microscopy. An elevated, but nonuniform, interaction was observed in the lamellipodial region (Fig. S2 A), suggesting that a principal function of Myo1c might be local G-actin transport in lamellipodia, compatible with structural and biochemical studies identifying myosin I isoforms as low–duty ratio motor proteins (De La Cruz and Ostap, 2004; O’Connell et al., 2007).

We determined the effect of Myo1c on leading edge dynamics by microinjecting purified Myo1c into lamellipodia.

Localization of G-actin in lamellipodia by motor activity of Myo1c
To determine Myo1c subcellular localization during EC movement, migrating cells were visualized with anti-Myo1c antibody. Myo1c was enriched and colocalized with F-actin at the leading edge of migrating ECs (Fig. 3 A). To determine whether Myo1c specifically colocalizes with G-actin, monomeric DsRed fusion protein of full-length Myo1c or proteins lacking the head or tail domains were expressed in migrating ECs. Full-length Myo1c colocalized with G-actin at the protrusive leading edge (Fig. 3 B). Myo1c lacking the tail region also accumulated in the leading edge. However, deletion of the head markedly blocked polarization of Myo1c localization, consistent with a previous study showing that Myo1b localization in the protrusive edge of spreading epithelial cells requires the motor domain (Tang and Ostap, 2001). Remarkably, overexpression of headless Myo1c prevented G-actin accumulation at the cell leading edge, possibly by competing with endogenous Myo1c for G-actin binding and transport. The interaction of Myo1c with G-actin and its spatial distribution during cell migration were investigated by Förster resonance energy transfer (FRET) coupled to confocal microscopy. An elevated, but nonuniform, interaction was observed in the lamellipodial region (Fig. S2 A), suggesting that a principal function of Myo1c might be local G-actin transport in lamellipodia, compatible with structural and biochemical studies identifying myosin I isoforms as low–duty ratio motor proteins (De La Cruz and Ostap, 2004; O’Connell et al., 2007).

We determined the effect of Myo1c on leading edge dynamics by microinjecting purified Myo1c into lamellipodia.
Myo1c and G-actin transport in cell migration

The role of Myo1c in G-actin transport during cell migration was investigated by knockdown experiments. Transfected, U6 promoter–driven short hairpin RNA (shRNA) and siRNA targeting identical Myo1c sequences reduced Myo1c expression by 70 and 90%, respectively, after 72 h (Fig. 5 A). Myo1c knockdown markedly reduced G-actin localization at the leading edge compared with cells transfected with control shRNA–targeting luciferase (Luc) mRNA (Fig. 5 B). Lamellipodial F-actin was also reduced. To investigate the role of Myo1c in G-actin translocation, cells were cotransfected with plasmids expressing Myo1c shRNA and the photoactivatable, nonpolymerizable G-actin chimera paGFP-actinG13R. Myo1c knockdown markedly reduced the rate of forward G-actin transport (Fig. 5 C). Finally, we examined the role of Myo1c in EC movement. Transfection of ECs with siRNA-targeting Myo1c reduced wound-induced planar migration and VEGF-A–induced chemotaxis by 70% and 60%, respectively, after 72 h (Fig. 5 D). Myo1c knockdown markedly reduced G-actin localization at the leading edge compared with cells transfected with control shRNA–targeting luciferase (Luc) mRNA (Fig. 5 B).

Local injection of full-length Myo1c protein, but not motor domain, increased G-actin localization and F-actin content at the leading edge, rapidly inducing plasma membrane ruffling and lamellipodia extension (Fig. 4). Microinjection of Myo1c tail domain reduced G-actin accumulation and induced retraction of lamellipodia. Microinjection of anti-Myo1c antibody targeting the head domain responsible for motor activity rapidly induced G-actin delocalization at the leading edge and lamellipodial retraction (unpublished data). Collectively, these results indicate that Myo1c dynamically traffics to the leading edge and mediates G-actin localization to the leading edge, membrane ruffling, and lamellipodial extension.

Yeast Myo1 directly stimulates F-actin polymerization (Lee et al., 2000; Lechler et al., 2001; Sirotkin et al., 2005), and mammalian Myo1b promotes assembly of F-actin foci (Almeida et al., 2011), suggesting that Myo1c might increase G-actin at the cell leading edge by facilitating F-actin polymerization. To test this possibility, the effect of purified Myo1c on in vitro actin polymerization was investigated and found to be without effect (Fig. S2 B). Furthermore, inhibition of actin turnover with jasplakinolide did not influence G-actin localization at the cell leading edge following overexpression of Myo1c, suggesting an F-actin turnover–independent mechanism for Myo1c-induced G-actin localization (Fig. S3 A).

Myo1c contributes to G-actin delivery to leading edge and optimal cell migration

The role of Myo1c in G-actin transport during cell migration was investigated by knockdown experiments. Transfected, U6 promoter–driven short hairpin RNA (shRNA) and siRNA targeting identical Myo1c sequences reduced Myo1c expression by 70 and 90%, respectively, after 72 h (Fig. 5 A). Myo1c knockdown markedly reduced G-actin localization at the leading edge compared with cells transfected with control shRNA–targeting luciferase (Luc) mRNA (Fig. 5 B). Lamellipodial F-actin was also reduced. To investigate the role of Myo1c in G-actin translocation, cells were cotransfected with plasmids expressing Myo1c shRNA and the photoactivatable, nonpolymerizable G-actin chimera paGFP-actinG13R. Myo1c knockdown markedly reduced the rate of forward G-actin transport (Fig. 5 C). Finally, we examined the role of Myo1c in EC movement. Transfection of ECs with siRNA-targeting Myo1c reduced wound-induced planar migration and VEGF-A–induced chemotaxis by 70% and 60%, respectively, compared with scrambled siRNA (Fig. 5 D). Transient overexpression of the Myo1c tail domain reduced planar EC migration by 40% (P < 0.01), but overexpression of the head domain was ineffective (unpublished data). Together, these experiments establish the requirement for Myo1c in vectorial G-actin trafficking and EC motility.
filaments. However, single-headed Myo9b is a processive motor (Inoue et al., 2002; Post et al., 2002), and a cryptic insertion in the motor domain constitutes a second F-actin–interacting site in addition to the classical ATP-sensitive actin-binding domain and is likely responsible for processive movement. It is unclear whether Myo1c has a similar domain or adaptor proteins that facilitate pairwise interaction with F-actin in vivo or whether there is cargo-mediated dimerization, by which processive activity is generated. We suggest two possible molecular mechanisms underlying Myo1c-mediated G-actin transport: processive movement of the Myo1c–G-actin complex and a ballista-in-relay–like mechanism in which Myo1c does not translocate but rather drives G-actin forward by repeated cycle of conformational switch-driven forward passes of G-actin along F-actin, involving binding and release during each cycle. Previous biophysical studies with recombinant Myo1c suggest that it is an inefficient motor for movement, as indicated by an ATP hydrolysis–induced force of 2 pN acting against thermodynamic fluctuations ($kT = 4\text{ pN/nm}$ at 37°C) and a velocity of ~80 nm/s in vitro (Lin et al., 2011), which is much lower than the speed of forward actin flow at the cell front, ~5 µm/s (Zicha et al., 2003). However, single-headed Myo9b is a processive motor (Inoue et al., 2002; Post et al., 2002), and a cryptic insertion in the motor domain constitutes a second F-actin–interacting site in addition to the classical ATP-sensitive actin-binding domain and is likely responsible for processive movement. It is unclear whether Myo1c has a similar domain or adaptor proteins that facilitate pairwise interaction with F-actin in vivo or whether there is cargo-mediated dimerization, by which processive activity is generated. We suggest two possible molecular mechanisms underlying Myo1c-mediated G-actin transport: processive movement of the Myo1c–G-actin complex and a ballista-in-relay–like mechanism in which Myo1c does not translocate but rather drives G-actin forward by repeated cycle of conformational switch-driven forward passes of G-actin along F-actin, involving binding and release during each cycle. Previous biophysical studies with recombinant Myo1c suggest that it is an inefficient motor for movement, as indicated by an ATP hydrolysis–induced force of 2 pN acting against thermodynamic fluctuations ($kT = 4\text{ pN/nm}$ at 37°C) and a velocity of ~80 nm/s in vitro (Lin et al., 2011), which is much lower than the speed of forward actin flow at the cell front, ~5 µm/s (Zicha et al., 2003). In contrast, Myo1c efficiently transports nuclear factor κB essential modulator, Neph1, and Glut4-containing...
vesicles in cells (Bose et al., 2002; Nakamori et al., 2006), suggesting that unknown mechanisms might facilitate Myo1c movement in vivo. Given the G-actin diffusion rate of 2–6 µm²/s in lamellipodia measured in ECs and other cells (McGrath et al., 1998; Zicha et al., 2003; Plastino et al., 2004), active transport by Myo1c and passive diffusion may both contribute to the forward actin flow.

Myo1c transport of G-actin is restricted primarily to the lamellipodia, where it can supplement diffusion in translocating G-actin from the region of treadmilling to the cell front (i.e., from ~1 µm behind to the leading edge). Myo1c has a work stroke of ~5 nm (Laakso et al., 2008), and, thus, it might require ~200 strokes to move G-actin the required distance, consuming ~200 ATP molecules. A typical mammalian cancer cell polymerizes ~6 × 10⁵ actin monomers per second during cell migration (Chan et al., 1998). Assuming all actin polymerization is derived from Myo1c delivery, then ~1 × 10⁸ ATPs per second are consumed. The mean cellular ATP concentration is about 5 mM (range is 2–10 mM), and our analysis indicates each cell has ~7 × 10¹⁰ ATP molecules. Finally, a typical cell has an ATP turnover rate of 1–2 min, i.e., ~1% of total ATP is consumed and produced per second (Alberts et al., 2004), or ~7 × 10⁸ ATP/s. Based on these estimates, translocation of G-actin by Myo1c utilizes ~15% of total ATP consumption of the cell. Given that ATP-independent diffusion is likely to account for a significant fraction of G-actin translocation to the cell and because ECs are relatively slow-moving cells (0.05–0.3 µm/min for breast cancer cells; Yuasa-Kawada et al., 2009), it is likely that ATP utilization by Myo1c-driven G-actin mobilization is considerably less than 15% of total ATP consumption.

An important role for Myo1c in cell movement is suggested by its abundance in actin-rich ruffles and by the induced ruffling activity after overexpression in 3T3-L1 adipocytes (Bose et al., 2002; Nakamori et al., 2006). Our analysis indicates Myo1c concentrations up to 10 µM in patches near the leading edge in motile ECs (Fig. S3 D). Reports of free G-actin in lamellipodia have been highly variable, ranging from 0.2 to 150 µM, depending on method, cell type, and G-actin status, i.e., interacting partners (Huber et al., 2008; Koestler et al., 2009; Kiuchi et al., 2011). At the low end of the range, there is sufficient Myo1c to transport a substantial fraction of the G-actin. At the high end, Myo1c is not sufficient to transport all G-actin at a 1:1 ratio; however, a cyclic throwing mechanism would allow each Myo1c molecule to facilitate forward movement of multiple G-actin molecules. In addition to delivery of G-actin to the cell leading edge by binding and transport, Myo1c may regulate actin-based cell motility through other mechanisms. Myo1c transports multiple signaling molecules to the plasma membrane, including rapamycin-insensitive companion of mammalian target of rapamycin (Hagan et al., 2008), nuclear factor κB essential modulator (Nakamori et al., 2006), Neph1 (Arif et al., 2009; Kiuchi et al., 2011). At the low end of the range, there is sufficient Myo1c to transport a substantial fraction of the G-actin. At the high end, Myo1c is not sufficient to transport all G-actin at a 1:1 ratio; however, a cyclic throwing mechanism would allow each Myo1c molecule to facilitate forward movement of multiple G-actin molecules. In addition to delivery of G-actin to the cell leading edge by binding and transport, Myo1c may regulate actin-based cell motility through other mechanisms. Myo1c transports multiple signaling molecules to the plasma membrane, including rapamycin-insensitive companion of mammalian target of rapamycin (Hagan et al., 2008), nuclear factor κB essential modulator (Nakamori et al., 2006), Neph1 (Arif et al., 2009; Kiuchi et al., 2011), and phosphatidylinositol 4,5-bisphosphate (Hokanson et al., 2006), which might indirectly contribute to actin dynamics. Alternatively, Myo1c-driven vesicle transport to the plasma membrane could influence actin polymerization. For example, interaction of Myo1c with RalA, a small GTPase residing in Glu4-containing vesicles, induces translocation of the glucose transporter to the cell surface, a process that may alter local bioenergetic processes (Bose et al., 2002; Chen et al., 2007; Yip et al., 2008). In summary, our results reveal an important role of Myo1c in G-actin transport to the leading edge of moving ECs and might present a critical node for control of cell polarity and motility.

Materials and methods

Cells and reagents

ECs were isolated from adult bovine aortas and cultured in DMEM/Ham’s F-12 medium (Invitrogen) containing 5% FBS. Cells were induced to migrate by a rabbit polyclonal anti-bovine Myo1c antibody and the number of migrating cells was determined with ImageJ software (National Institutes of Health; Ghosh et al., 2002). Rabbit polyclonal anti-bovine Myo1c antibody was raised against a synthetic peptide (REASELLEICRRKNMWKTY) and purified by peptide affinity chromatography (Thermo Fisher Scientific).

Plasmid construction and siRNA

Full-length Myo1c cDNA (NCBI Protein database accession no. NP_776821) was amplified from a bovine cDNA pool (BioChain) by PCR. Full-length and truncated fragments were subcloned into pcDNA 3.1/myc/His (Invitrogen) or pDxRed-N1 (Takara Bio Inc.). pGFP-actin (Takara Bio Inc.) was used as a template to generate G13R and R62D mutations by PCR using GeneTailor Mutagenesis System (Invitrogen). Actin cDNA was subcloned into pEGFP-C1 (Takara Bio Inc.), pPA-GFP-C1 (provided by J. Lippincott-Schwartz, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD), or pDream with a Flag tag (GenScript). siRNA duplexes (targeting sequences of bovine Myo1c, nucleotides 1,071–1,095 or 1,213–1,237, or scrambled sequence) were synthesized (Invitrogen). Duplex oligonucleotides encoding Myo1c shRNA targeting the same Myo1c sequence or control Luc shRNA (Invitrogen) were annealed and cloned into pRNA-U6-GFP/Neo (GenScript). Plasmids and siRNA were transfected into cells with Lipofectamine 2000 (Invitrogen), respectively.

Immunoprecipitation and MS

Lysates from migrating cells were subjected to immunoprecipitation using anti-myc antibody–immobilized agarose beads, and the precipitated proteins were detected by SDS-PAGE and immunoblot analysis. To identify actinG13R/myc–interacting proteins, gels were stained with Coomassie blue, and bound proteins were identified by HPLC-MS. In brief, proteins were alkylated with iodoacetamide before digestion with trypsin, and the peptides were analyzed by collisionally induced dissociation spectrometry in an LTQ ion trap MS system.

Protein expression and purification

Full-length or truncated Myo1c cDNA was subcloned into pET41-GST, and plasmids were transformed into Rosetta-2 (b) bacteria (EMD Millipore). Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at 30°C for 5 h with chloramphenicol, streptomycin, tetracycline, and kanamycin. Soluble protein was extracted with Cellytic B lysis reagent (Sigma-Aldrich) and purified with B-PER GST purification kit (Thermo Fisher Scientific). For protein expression in insect cells, Myo1c was in vitro synthesized from pcDNA-Myo1c-myc/His vector with insect EasyXpress kit (QIAGEN) and purified with MagenHis system (Promega).

Protein–protein interaction

Purified β-actin (Cytoskeleton) was centrifuged through a 50-kD filter (EMD Millipore) to remove any polymerized actin and immobilized on a CM5 sensor chip. Binding of purified bacterial Myo1c to G-actin was determined by SPR (BioCore 3000). Dissociation constants were calculated for a range of analyte concentrations using BIACore evaluation software (BioCore).

Confocal imaging

Migrating cells were fixed with 3.7% PFA and imaged with a 63× objective lens on an upright microscope (DMIRE2) equipped with a confocal scanning system (TCS SP2; Leica). Live cells were imaged in phenol red-free. 

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GST-tagged Myo1c was expressed in bacteria, purified, and dialyzed. Fluor 488–DNase I and Alexa Fluor 568–phalloidin, and G- and F-actin were imaged in a microscope (Leica) equipped with charge-coupled device camera imaging of cells seeded on glass coverslips. Morphological changes were monitored by FRET software (CircuSoft; Fan et al., 2009).

Fig. S1 shows GFP-actin G13R accumulation at the cell leading edge with temporal changes in G-actin concentration revealing its effect on stimulus-induced actin polymerization and ILK-Akt2 activity during endothelial cell migration. Dev. Cell. 16:661–674. http://dx.doi.org/10.1016/j.devcel.2009.03.009


