LOCALIZATION OF MYOSIN 1b TO ACTIN PROTRUSIONS REQUIRES PHOSPHOINOSITIDE BINDING
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The class I myosin, Myosin 1b (Myo1b), is a widely-expressed, single-headed, actin-associated molecular motor. Transient kinetic and single-molecule studies indicate that it is kinetically slow and responds to tension. Localization and subcellular fractionation studies indicate that Myo1b associates with the plasma membrane and certain subcellular organelles such as endosomes and lysosomes. Whether Myo1b directly associates with membranes is unknown. We demonstrate here that full-length rat Myo1b binds specifically and with high affinity to phosphatidylinositol-4,5-bisphosphate (PIP$_2$) and phosphatidylinositol-3,4,5-triphosphate (PIP$_3$), two phosphoinositides that play important roles in cell signaling. Binding is not Ca$^{2+}$-dependent and does not involve the calmodulin-binding IQ region in the neck domain of Myo1b. Furthermore, the binding site is contained entirely within the C-terminal tail region, which contains a putative PH domain. Single mutations in the putative PH domain abolish binding of the tail domain of Myo1b to PIP$_2$ and PIP$_3$ in vitro. These same mutations alter the distribution of myc-tagged Myo1b at membrane protrusions in HeLa cells where PIP$_2$ localizes. In addition, we found that motor activity is required for Myo1b localization in filopodia. These results suggest that binding of Myo1b to phosphoinositides plays an important role in vivo by regulating localization to actin-enriched membrane projections.

Class I myosins are single-headed members of the myosin superfamily that bind actin filaments and produce mechanical force by hydrolyzing ATP. Class I myosins consist of an amino terminal head or motor domain containing the ATP- and actin-binding sites; a neck region containing repeats of a light chain-binding region known as an IQ domain; and a C-terminal tail domain. Class I myosins are widely expressed in protozoans and metazoans. In mammals, there are 8 class I myosins, Myo1a-h (1), which play roles in diverse cellular events such as membrane trafficking, formation of membrane protrusions, cell migration and transcription in the nucleus (2).

The myosin I tail domain, a basic region referred to as the TH1 domain, is involved in membrane binding. Acanthamoeba myosin IC binds PS (phosphatidylinerine) and PIP$_2$ and colocalizes with PIP$_2$ in dynamic regions of the plasma membrane including pseudopods, endocytic cups and the base of filopodia (3). Vertebrate Myo1a, abundant in the brush border of the small intestine, also binds PS and PIP$_2$ (4), suggesting that Myo1a tethers the core bundles of actin filaments in the microvilli directly to the membrane (5). The mammalian myosin I, Myo1c, which mediates GLUT 4 transport in adipocytes (6,7), and adaptation in the specialized hair cells of the inner ear (8,9), associates with phosphoinositides having phosphates at the 4- and 5-positions of the inositol ring (10).

Vertebrate Myo1b is widely expressed in tissues including brain, heart, lung, kidney and liver (11). Myo1b is kinetically slow and the interaction of actin-Myo1b with ATP is biphasic consisting of a fast phase followed by a slow phase (12,13). In single-molecule studies the interaction of Myo1b with actin can be separated into two mechanical phases, the first phase is thought to be associated with Pi release and the second phase is presumably associated with ADP release (14). Moreover, like other class I myosins (15-18) Myo1b exhibits an ADP-induced conformational change (Arthur, C., Lin, A., Coluccio, L. and Milligan, R. A., personal communication). The results from the kinetic, single-molecule and structural studies suggest that Myo1b undergoes a conformational change before ADP release and predict that this step is load dependent (12,13). Single-molecule studies subsequently showed that the rate of Myo1b...
dissociation from actin is force-dependent (19). The results implicate Myo1b as a force-sensing motor protein that can crosslink load-bearing actin filaments. Such a protein is better able to maintain and control cortical tension rather than to transport cargo (12).

In fractionation studies of rat liver, Myo1b associates predominantly with the plasma membrane and endoplasmic reticulum (20). In NRK cells, Myo1b is concentrated in actin-enriched protrusions of the membrane such as ruffles and lamellipodia (21). When expressed the tail domain localizes to the plasma membrane and associates with membrane fractions similar to full-length Myo1b suggesting that the tail domain primarily determines the cellular localization (21). Myo1b is also associated with endosomes and lysosomes whose distribution and morphology are affected by Myo1b overexpression (22,23).

Although Myo1b associates with membrane, whether it binds to membrane directly or indirectly, i.e., through a binding partner that binds to both Myo1b and membrane, is unknown. The specificity of Myo1b binding to membranes, and whether it resembles that of the other mammalian myosins I that have been studied to date, remains unclear. Thus, in this study, we investigated the interaction of Myo1b with lipids and its specificity. In addition, we examined the roles of Myo1b-lipid binding in determination of its cellular distribution.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant cDNAs**—For expression in Sf9 insect cells, full-length Myo1b, Myo1b IQ domain and tail domain (Myo1b IQ-tail; D706-P1107) or the tail domain only (tail; V824-P1107) was amplified by PCR to contain a C-terminal FLAG tag using rat Myo1b cDNA as a template (the kind gift of Dr. Martin Bähler, University of Muenster). As a control the IQ and tail domain (Myo1c IQ-tail; A690-R1028) of mouse Myo1c was amplified by PCR to contain a C-terminal FLAG tag using EGFP-mouse Myo1c (a kind gift from Dr. Thomas Friedman, National Institutes of Deafness and Communication Diseases, NIH). The PCR products were then cloned into a pFastBac Dual vector (Invitrogen, Carlsbad, CA) containing a calmodulin expression cassette (24). For expression in mammalian cells, Myo1b cDNAs in pFastBac Dual were amplified by PCR and ligated into pMyc (25) followed by verification of DNA by automatic sequencing. Point mutations were introduced by site-directed mutagenesis and verified by DNA sequencing. Espin/pCDNA3 construct (untagged) was kindly provided by Dr. James R. Bartles (Northwestern University, IL).

**Protein Expression and Purification**—The constructs were expressed in insect cells according to the manual provided for the Bac-to-Bac Baculovirus Expression System (Invitrogen). The infected insect cells were pelleted and homogenized in 50 mM Tris-HCl (pH 7.5), 300 mM KCl, 2 mM MgCl2, 1 mM EGTA, 2 mM ATP and protease inhibitors. After centrifugation at 200,000 x g for 30 min, anti-FLAG M2 agarose (Sigma Chem. Co., St. Louis, MO) was added to the supernatant and incubated for 1 h at 4 °C. The expressed protein was eluted with 100 µg/ml FLAG peptide in 30 mM HEPES (pH 7.5), 100 mM KCl, 2 mM MgCl2 and 1 mM EGTA. Purified protein containing 10% sucrose and 1 mM DTT was stored at –80 °C.

**Lipid-Bead-Protein Pull-Down Assay**—PIP Bead Sample Pack (Echelon Biosciences Inc., Salt Lake City, UT) consisted of 50% slurries of 8 different phosphoinositide-coated beads and control beads containing no phosphoinositide. In each case 20 µl of the 50% slurry was washed by adding to 1 ml of binding buffer (30 mM HEPES, pH 7.5; 100 mM KCl, 2 mM MgCl2 and 0.25% Igepal CA630) with 0.1 mM free calcium or 1 mM EGTA then centrifuging at 100 x g for 5 min at 4°C. Purified Myo1b in 250 µl binding buffer (final concentration: 40 µg/ml) was incubated with the washed beads on a Labquake (Thermo Scientific, Asheville, NC) with gentle mixing for 90 min at 4°C. The beads were collected at 100 x g for 5 min at 4°C followed by washing three times with 500 µl binding buffer at 100 x g for 3 min each. The supernatant was removed and the bound protein was eluted with 20 µl 2X Laemmli sample buffer followed by analysis by SDS-PAGE and Coomassie-blue staining.

**Liposome Pull-Down Assay**—Myo1b IQ-tail (4 µM) or Myo1c IQ-tail (3.7 µM) was prespun at 355000 x g (Beckman TL100 centrifuge, TLA 100 rotor; Beckman Instruments, Palo Alto, CA) for 30 min at 25°C in polycarbonate tubes (7 X 20 mm; Beckman Centrifuge Tubes #343775) to eliminate
any aggregated protein. Then, Myo1b IQ-tail (final concentration: 25 nM) or Myo1c IQ-tail (final concentration: 50 nM) was incubated with various amounts (0-50 μM total lipid for Myo1b and 0-200 μM total lipid for Myo1c) of PolyPIPosomes (65% phosphatidylincholine, 30% phosphatidyl-ethanolamine, 5% PI, PIP₂ or PIP₃; Echelon Biosciences Inc.) in 200 μl of 30 mM HEPES (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA and 0.1 mg/ml BSA for 20 min at room temperature in the same polycarbonate centrifuge tubes only pretreated for 1 hr with 300 μl of 100 μM phosphatidylcholine to lessen the possibility of absorption of the liposomes to the tubes. The protein-liposome complexes were centrifuged at 353000 x g for 30 min at 25 °C. The pellets were resuspended in 12.5 μl 2X Laemmlı sample buffer followed by analysis by SDS-PAGE and staining with Coomassie Brilliant Blue. The gels were scanned and analyzed using an Odyssey Infrared Imaging System (Licor, Lincoln, NE). The data are expressed as percentage of bound Myo1b IQ-tail or Myo1c IQ-tail as a function of total lipid concentration and the data were fit to hyperbolae with Prism. Kₐₙ₉₆₆ refers to the concentration of total lipid at which 50% of the protein is bound, whereas KPIP and KPIP₃ are the concentrations of accessible PIP₂ and PIP₃, respectively [(total lipid x 0.05)/2] at which 50% of the protein is bound.

Immunofluorescence Microscopy—HeLa or COS-7 cells were transfected using Fugene 6 (Roche Applied Science, Palo Alto, CA) then replated on poly-L-lysine-coated glass cover slips. The cells were fixed in 4% formaldehyde and 0.05% glutaraldehyde 24 h after transfection and were permeabilized and blocked in 0.5% saponin, 5% goat serum and 2.5% BSA in PBS for 1h, followed by overnight incubation with the appropriate primary antibodies in 0.1% saponin, 5% goat serum and 2.5% BSA in PBS. Primary antibodies used in this study were Myc-Tag polyclonal antibody (Cell Signaling Technology, Danvers, MA), and purified PI(4,5)P₂ mouse monoclonal antibody (Echelon Biosciences Inc.). The cells were then incubated with secondary antibodies (Alexa Fluor 488 and/or Alexa Fluor 594 goat anti-mouse or anti-rabbit) and/or rhodamine phalloidin (Invitrogen). The cells were viewed with a Leica TCS SP5 AOBS 405 UV Spectral confocal microscopy system. The localization patterns of the Myo1b mutants did not appear different over a wide range of expression levels; nevertheless, cells expressing similar amounts of protein were compared. Images were processed with Leica Application Suite Advanced Fluorescence imaging software and Adobe Photoshop.

RESULTS

Specific Binding of Myo1b to PIP₂ and PIP₃—To determine whether Myo1b interacts with phosphoinositides directly, we used a pull-down assay involving beads coated with various phosphoinositides and purified expressed full-length Myo1b (Fig. 1B). As shown in Fig. 2, full-length Myo1b bound specifically to PIP₂ and PIP₃ in the presence and absence of Ca²⁺. This Ca²⁺-independent binding suggests that the calmodulin-binding IQ motifs in the neck domain are not involved in the interaction between Myo1b and phosphoinositides. To determine the phosphoinositide-binding domain of Myo1b, tail domain without the IQ motifs was used in pull-down assays (Fig. 1B, 2B). The tail domain also showed specific binding to PIP₂ and PIP₃; binding was not dependent on Ca²⁺. Taken together, these results indicate that Myo1b interacts with specific lipids via its tail domain in a Ca²⁺-independent manner.

A putative PH domain is the binding site of phosphoinositides—Hokanson et al. (26) reported that Myo1c interacts with phosphoinositides through a PH domain and proposed that most mammalian class I myosins have a putative PH domain in the tail. Certain PH domains have a common secondary structure, β₁-loop-β₂, in which there are two conserved basic residues that are important for interacting with phosphoinositides (26,27). Myo1b tail domain has a putative PH domain containing a β₁-loop-β₂ structure and the corresponding two basic residues are conserved (K966 and R977) (Fig. 1A). To determine whether these residues are involved in binding of Myo1b to specific phosphoinositides, we constructed two Myo1b tail mutants, K966A and R977A, (Fig. 1B) and examined the effect of these mutations on phospholipid binding using PIP-bead pull-down assays. Fig. 2B shows that a single alanine mutation at K966 or R977 completely abolished binding to PIP₂ and PIP₃, suggesting that the K966 and R977 residues within
the putative PH domain play an critical role in the interaction of Myo1b with PIP2 and PIP3.

To test whether the IQ domain contributes to lipid binding, full-length Myo1b K966A (Fig. 1B) was used in pull-down assays with lipid-coated beads (Fig. 2C). Since the tail mutation eliminates lipid binding via the putative PH domain in the tail, any observed lipid binding could potentially result from the IQ domain, which was initially thought to contribute to lipid binding in the case of Myo1c (8,28). Full-length Myo1b K966A does not bind PI(4,5)P2 or PI(3,4,5)P3 in the presence or absence of Ca2+ demonstrating that the putative PH domain is the sole lipid-binding domain and that neither the motor nor the IQ domain is involved in lipid binding.

Affinity of Myo1b IQ-tail for phosphoinositide—Binding of Myo1b IQ-tail to liposomes containing 5% PIP2 or PIP3 as a function of total lipid concentration is shown in Fig. 3. Although binding of Myo1b IQ-tail to PI was limited (open triangles), Myo1b IQ-tail bound PIP2 (open circles) tightly and PIP3 (closed circles) less tightly. An accurate KpIP2, the concentration of PIP2 at which 50% of the protein is bound, could not be determined, but is estimated at 6 nM. The KpIP3 of Myo1b IQ-tail was determined to be 32 nM. More Myo1b (80%) associated with PolyPIPosomes containing 5% PIP2 than with PolyPIPosomes containing 5% PIP3 (50%). As the same Myo1b was used in each case this difference in behavior cannot be attributed to the use of different batches of protein. Furthermore, the protein was prespun to eliminate any aggregates and in the absence of PolyPIPosomes, little, if any, (2-3%) Myo1b pelleted indicating that the difference is also not due to the quality of the protein. One possibility is that the off rate is higher for PIP3 vs. PIP2 and that during centrifugation, more Myo1b dissociates from PIP3 than from PIP2.

KpIP2 of Myo1c was also determined and is estimated to be 0.8 μM, which is close to previously reported values of 0.23 μM (29) and 0.53 μM (10). These results show that Myo1b has a higher affinity for PIP2 than PIP3 and a much higher affinity (>100-fold) for PIP2 than does Myo1c.

Localization of Myo1b in filopodia requires phosphoinositide binding—To examine the effect of a mutation in the putative PH domain that abolishes the interaction of Myo1b with phosphoinositides on the cellular distribution of Myo1b, we transfected HeLa cells with myc-tagged full-length wild-type Myo1b or Myo1b K966A mutant (Fig. 1C) and stained with anti-myc. Myc-tagged wild-type Myo1b was found at the cell periphery and in filopodia (Fig. 4A) as previously reported for endogenous and myc-tagged Myo1b (21) and GFP-tagged Myo1b (23,30) in other cell types. Myo1b K966A mutant localized diffusely throughout the cytoplasm and was not concentrated at the periphery or in filopodia (Fig. 4D). Transfected cells were also stained with PIP2 antibodies (Fig. 4, B and E) in addition to anti-myc. PIP2 antibodies strongly stained filopodia and the cell periphery where wild-type Myo1b (Fig. 4C), but not Myo1b K966A mutant (Fig. 4F), localized. These results suggest that phosphoinositide binding is required for localization of Myo1b in PIP2-enriched regions such as filopodia. Surprisingly, the R977A mutant localized diffusely in the cytoplasm like K966A, but also localized to filopodia like wild type (Fig. 4, G-I).

Myo1b and PLCδ1-PH colocalized at the cell periphery and in filopodia—To monitor the Myo1b-PIP2 interaction in cells, HeLa cells were transfected with PLCδ1-PH-GFP (31), a PIP2-specific binding protein, and myc-tagged full-length Myo1b constructs (Fig. 5). PLCδ1-PH-GFP was found at the cell periphery and in filopodia (Fig. 5, B and F) and wild-type Myo1b colocalized with PLCδ1-PH-GFP (Fig. 5, C and D). On the other hand, Myo1b K966A mutant did not colocalize with PLCδ1-PH-GFP (Fig. 5, E-H). Myo1b R977A did not concentrate at the periphery and hence did not colocalize well with PLCδ1-PH (Fig. 5, I-L).

Motor activity is required for localization in filopodia—We also examined whether motor activity is required for localization of Myo1b to filopodia, which contain an actin core. Amino acid residues R165 and G390 of Myo1b are located in the switch I and switch II regions, respectively. These sites are strictly conserved among myosins and replacement with alanine blocks ATP hydrolysis and isomerization, respectively. These myosins are held in the weak-binding state in ATP (32,33). We made myc-tagged Myo1b constructs in which R165 and G390 were mutated to alanine to render the Myo1b
motors inactive. These constructs were transfected into HeLa cells and stained with antimitc and anti-PIP2. Both mutants, R165A (Fig. 6, A) and G390A (Fig. 6, D), were concentrated in the cell periphery, but not in filopodia where PIP2 is enriched (Fig. 6, B and E). These mutants were also cotransfected into HeLa cells with PLCδ1-PH-GFP (Fig. 7). Both the R165A and G390A mutants colocalized with PLCδ1-PH-GFP at the cell periphery. These results indicate that although these mutants should be freely diffusing and can associate with the cell periphery, motor activity is required for Myo1b to enter and/or remain in filopodia.

Localization of Myo1b relative to PIP2 in espin-induced filopodia of COS-7 cells—Because filopodia of HeLa cells are very thin making it difficult to see the localization of Myo1b and PIP2 in detail, COS-7 cells were transfected with the actin-bundling protein, espin, which induces actin/membrane protrusions (34). PIP2 localized at the membrane of espin-induced filopodia (Fig. 8, C; Fig. S1, B and D), whereas Myo1b localized within the core (Fig. 8, A and D, Fig. S1, A, C, G and I). Actin filaments, stained with phallolidin, colocalized with Myo1b (Fig. 8, B and D; Fig. S1, E, F, H and I).

DISCUSSION

Ca2+-independent Myo1b-lipid binding is through the tail domain—In this study, we show direct binding of Myo1b through the putative PH domain in the tail domain to PIP2 and PIP3, two phosphoinositides that play major roles in cell signaling cascades that lead to important cellular events such as reorganization of the actin cytoskeleton. The results can be compared to those with the related mammalian myosin I, Myo1c. Hirono et al. reported that Myo1c binds phosphoinositide through the IQ motif upon dissociation of calmodulin by calcium (9). On the other hand, Hokanson et al. demonstrated that the Myo1c tail without the IQ domain is the major binding domain to lipid and that binding is not calcium/calmodulin dependent (29). In the presence of calcium, the motor activity of Myo1b is inhibited and this inhibition is reversed in the presence of exogenous calmodulin suggesting that calcium induces dissociation of one or more calmodulins from the neck region of Myo1b (35); however, binding of full-length Mo1b to phosphoinositides is not Ca2+-dependent and the tail domain without the IQ motifs binds both PIP2 and PIP3 (Fig. 2A). That the IQ domain plays no role in lipid binding in the case of Myo1b was further confirmed using the full-length Myo1b K966A tail mutant, which showed no binding to PIP2 or PIP3 in the presence or absence of Ca2+ (Fig. 2C).

Acanthamoeba myoC (3) and chicken Myo1a (4) bind PS and PIP2; specificity to other phosphoinositides has not been investigated. Mouse Myo1c also binds to PIP2; however, binding is not specific for this particular phosphoinositide because Myo1c also binds to other phosphoinositides having phosphates on position 4 and 5 of the inositol headgroup with a similar affinity (29). Mutation of the conserved basic residues K872A and R903A in the b1-loop-β2 motif of the putative PH domain of mouse Myo1c decreased its affinity for phosphoinositides by more than 8 fold, while the equivalent mutation in Acanthamoeba myoC, R779A, resulted in only a 2-4 fold decrease in affinity for PS and PIP2.

Both of the homologous mutations in the b1-loop-β2 motif of Myo1b, K966A and R977A, completely abolished binding to PIP2 and PIP3 in vitro (Fig. 2B for K966A and R977A); moreover, the K966A mutant localized diffusely in the cytoplasm and lost the specific distribution at the cell periphery and filopodia found for wild type (Fig. 4, 5). Although the R977A mutant also localized diffusely in the cytoplasm, it was found in filopodia (Fig. 4G, 5I). The difference is localization between the two tail mutants, neither of which bind PIP2 or PIP3 in vitro, can be explained by small differences in affinity for lipids between the two mutants with the affinity of R977A for PIP2 and/or PIP3 exceeding that of K966A. In the cell these slightly different affinities for phosphoinositides could lead to differential localization.

Specificity and affinity of Myo1b-phosphoinositide binding—Although Myo1b binds both PIP2 and PIP3 in vitro, its affinity for PIP2 exceeds that of PIP3 (Fig. 3). In addition, the cellular concentration of PIP3 is much less than PIP2 (36). Expression in HeLa cells of PLCδ1-PH-GFP, a specific PIP2-binding protein, demonstrates that PIP2 is concentrated at the cell...
periphery and in filopodia in which Myo1b colocalizes with PIP₂ (Fig. 5), while Btk-PH-GFP (37), which binds specifically to PIP₃, is found throughout the cytoplasm (Fig. S2). Together, the evidence from in vitro and in vivo studies indicates that Myo1b is more likely to associate with PIP₂ than PIP₃ in HeLa cells. Upon stimulation, PIP₂ and PIP₃ levels dramatically increase in cells (36). Sharma et al. reported that in stimulated neutrophils, PIP₂ and PIP₃ localize to the leading edge of cells (38). Myo1b localization may also change upon stimulation involving an increase in PIP₂ and PIP₃.

The affinity of Myo1b for PIP₂ is substantially higher than that determined for Myo1c with the same method (this report; Fig. 3) or similar methods (10,29). Myo1b’s high affinity for PIP₂ might explain why Myo1b is more concentrated at the cell periphery than is Myo1c (21).

**Physiological role of Myo1b-lipid binding**—PIP₂ plays an important role in actin reorganization in cellular protrusions. Many actin-binding proteins that regulate actin dynamics by capping, severing, branching and bundling actin interact with and are activated by PIP₂ (39). These actin-binding proteins are thought to function in membrane-cytoskeleton interactions (40). Reduction of cellular PIP₂ by expression of PLCδ1-PH, which sequesters PIP₂, or by targeting 5'-PIP₂-phosphatase to the plasma membrane results in a decrease in plasma membrane-cytoskeletal adhesion energy (41). Activation of phospholipase-C by ionomycin and calcium that results in a decrease in the cellular PIP₂ level leads to translocation of Myo1c from the plasma membrane to the cytoplasm (29). Myo1b may also be regulated by PIP₂ given its localization at the membrane in regions exhibiting dynamic rearrangements of the actin cytoskeleton.

In HeLa and COS-7 cells Myo1b localizes preferentially in filopodia, dynamic structures believed to sense the microenvironment and to determine the direction of cell movement. Filopodia are actin-filled membrane protrusions that function in diverse cellular processes such as cell migration, neurite outgrowth, and wound healing (42). The length of actin filaments in the core bundle is a consequence of actin polymerization and depolymerization, mediated by actin-binding proteins, which sever and cap filaments or bundle actin filaments. During the dynamic process of filopodia formation the interaction of actin filaments with the membrane must be coordinated. The ability of Myo1b to sense tension between the membrane and the actin cytoskeleton (43) makes it an excellent candidate for regulating such actin-membrane interactions.

HeLa cells expressing motor-dead Myo1b or Myo1b unable to bind lipids have many filopodia similar to that of untransfected cells and cells expressing wild-type Myo1b. Raposo and colleagues demonstrated that overexpression of GFP-labeled wild-type Myo1b or Myo1b mutant in the motor domain affects the distribution of transferrin receptors in the hepatoma cell line, BWTG3 (22). Together, these results suggest that Myo1b mutants have a dominant negative effect on endocytosis, but not on filopodia.

Other class I myosins that bind phosphoinositides also localize to actin-enriched cell protrusions. Myo1a localizes to the lateral links found between the core bundle of actin filaments and the membrane in microvilli of the small intestine (5,44,45). Microvilli shed small vesicles into the intestinal lumen and this activity is perturbed in Myo1a-deficient mice suggesting a role for Myo1a in vesicle production (46). Myo1c is found in the brush border of the proximal tubules in kidney cells (47), in the stereocilia of the hair cells in the inner ear (48), and in membrane ruffles where it supports vesicle fusion with the plasma membrane (7,49,50). In Myo1a knock-out mice, other myosins I redistribute to the microvilli and may compensate for the lack of Myo1a (5,51). Similarly, other myosins I may also compensate for Myo1b in cells expressing mutant Myo1b.

In addition to class I myosins, other myosins are involved in actin-membrane interactions. Myosin VI, a minus-ended-directed motor (52), binds PIP₂ and dimerizes upon binding (53). In the renal brush border, myosin VI is required for parathyroid hormone-induced internalization of the sodium phosphate cotransporter and moves down along the actin bundle toward the base of microvilli (minus end of actin filament) together with the membrane-integrated transporter (54).

We show here that Myo1b localizes within filopodia adjacent to both PIP₂ and actin filaments within filopodia (Fig. 8) and hypothesize that Myo1b associates with actin bundles and the plasma membrane and plays a role similar to the
role of Myo1a in microvilli. The requirement of motor activity for Myo1b for proper localization to filopodia (Fig. 6, 7) supports this idea and suggests that whereas a motor-independent mechanism is responsible for Myo1b’s location at the plasma membrane, Myo1b must actively move into filopodia. Myo1b’s ability to sense mechanical force and to change its motor properties depending on load (13,19) presumably allows Myo1b to regulate actin/membrane dynamics in addition to anchoring actin filaments to the plasma membrane.

While this paper was under revision, a report appeared in which it was predicted based on a computer program designed to recognize unstructured membrane-binding sites in protein sequences that the tail of Myo1b binds lipids through an unstructured basic and hydrophobic (BH) region, rather than through a highly defined tertiary structure such as a PH domain (55). No such BH region was identified in the tail of Myo1c, consistent with the previous identification of a PH domain in its tail (10). However, our studies provide experimental evidence clearly demonstrating that a PH domain exists in Myo1b and is responsible for lipid binding of Myo1b both in vitro and in vivo.

During this same time, two other reports showed that a PH-domain-like motif in the tail (and the motor domain) contribute to the localization of mammalian Myo1g to the plasma membrane (56,57). Although not yet rigorously tested, the specificity of Myo1g for lipids appears distinct from that of Myo1c suggesting, as our studies with Myo1b show, that different class I myosins have different affinities for phosphoinositides.

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REFERENCES
FOOTNOTES

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The abbreviations used are: Myo1b, Myosin 1b; Myo1c, Myosin 1c; PS, phosphatidylserine; PI, phosphatidylinositol; PIP$_2$, PI(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate; PIP$_3$, PI(3,4,5)P$_3$, phosphatidylinositol-3,4,5-triphosphate.

FIGURE LEGENDS

FIGURE 1. Schematic diagram of rat Myo1b and constructs used in this study. A, Rat Myo1b structure and alignment of the β-loop-β motif of the PH domain of class I myosins. The rat Myo1b isoform used in this study consists of a motor domain, neck domain with 5 IQ domains and a tail domain. The tail domain of Myo1b contains the β-loop-β motif of a putative PH domain in which conserved basic residues are shown in red. The red band in the amino acid alignment indicates conserved basic residues in other class I myosins. Accession numbers of the myosin isoforms: Myo1b (CAA48287), Myo1a (EDM16453), Myo1c (CAA52807), Myo1d (CAA50871), Myo1e (CAA52815), Myo1f (NP_001101546), Myo1g (NP_001128315) and Myo1h (NP_001158045). B, Myo1b constructs used in vitro experiments. A FLAG tag was fused to the C-terminus of full-length Myo1b, Myo1b IQ-tail (Asp706-Pro1107) or Myo1b tail fragment only (Val824-Pro1107). Lysine 966, a conserved basic residue in the β-loop-β motif, was replaced with alanine in full-length Myo1b K966A-FLAG and Myo1b tail K966A-FLAG. Also, arginine 977 was replaced with alanine in Myo1b tail R977A-FLAG. C, Constructs used for expression in mammalian cells. A myc tag was fused at the N-terminus to full-length wild-type Myo1b, full-length Myo1b K966A tail mutant or full-length Myo1b R977A tail mutant. The mutations, R165A and G390A, reside in switch I and switch II, respectively, critical regions of the myosin motor domain.

FIGURE 2. Lipid-binding assays with epitope-tagged full-length wild-type Myo1b, Myo1b tail or Myo1b tail mutants. Pull-down assays with lipid-coated beads were carried out in the presence of 0.1 mM free calcium or absence (EGTA). A, Coomassie blue-stained SDS polyacrylamide gels show the amount of full-length Myo1b or Myo1b tail associating with various phosphoinositide (PI)-coated beads. 4% of the total protein used in the pull-down assays is shown as input. 21% of bound protein was loaded in the other lanes. Control lanes contain uncoated beads. B, FLAG-tagged Myo1b tail, Myo1b tail
mutant K966A, or Myo1b tail mutant R977A was used in pull-down assays in the absence of calcium with beads coated with PI, PI(4,5)P2 or PI(3,4,5)P3. Myo1b tail, but not the tail mutant, associates with PI(4,5)P2 and PI(3,4,5)P3. C, Full-length Myo1b associates with PI(4,5)P2 and PI(3,4,5)P3 in a Ca2+-independent manner. Full-length Myo1b tail mutant, K966A, did not associate with PI(4,5)P2 or PI(3,4,5)P3 regardless of the Ca2+ concentration.

FIGURE 3. Myo1b IQ-tail binding to liposomes containing PI, PIP2 or PIP3 as a function of lipid concentration. Pull-down assays of liposomes and Myo1b IQ-tail (25 nM) were performed with various concentrations of liposomes composed of 5% PI, PIP2 or PIP3. Data are representative of three experiments for PIP2 and two experiments for PI and PIP3. The concentration at which 50% Myo1b IQ-tail binds PIP2, K_{PIp2}, is 6 nM and the concentration at which 50% Myo1b IQ-tail binds PIP3, K_{PIP3}, is 32 nM. Open triangles, 5% PI; open circles, 5% PIP2; closed circles, 5% PIP3. Inset, Binding of 50 nM Myo1c IQ-tail to liposomes containing 5% PIP2 at various concentrations. The solid line is the best fit to a hyperbola yielding \( K_{lipid} = 31.8 \mu \text{M} \) and \( K_{PIP2} = 0.8 \mu \text{M} \).

FIGURE 4. Localization of Myo1b, Myo1b mutants and PIP2 in HeLa cells. HeLa cells were transfected with full-length myc-tagged wild-type Myo1b (A, B and C), full-length myc-tagged Myo1b K966A mutant (D, E and F) or full-length myc-tagged Myo1b R977A (G, H, and I) and stained with antimyc (A, D and G; green) and anti-PIP2 (B, E and H; red). Panels C, F and I are merged images of A and B, D and E, and G and H, respectively. PIP2 staining was observed at the cell periphery and in filopodia. Wild-type Myo1b, but neither of the mutants, colocalized with PIP2 at the periphery; however, like the wild type some staining of filopodia was observed for R977A. Bars, 10 \( \mu \text{m} \).

FIGURE 5. Cotransfection of Myo1b or Myo1b mutants with PLC81-PH-GFP in HeLa cells. HeLa cells were transfected with myc-tagged wild-type Myo1b (A), Myo1b mutant K966A (E) or Myo1b mutant R977A (I) together with PLC81-PH-GFP (B, F and J, green), then stained with myc antibody (red). Merged images are shown in C, G and K for A and B, E and F, and I and J, respectively. D, H and L show fluorescence intensity along the white lines indicated in C, G and K, respectively. PLC81-PH-GFP, a PIP2-specific binding protein, localized at the cellular periphery and in filopodia (B, F and J) and colocalized with wild-type Myo1b (C and D). On the other hand, Myo1b K966A (E) did not colocalize with PLC81-PH-GFP in either the periphery or filopodia (F, G and H). Myo1b R977A did not colocalize with PLC81-PH-GFP at the periphery. Bars, 10 \( \mu \text{m} \).

FIGURE 6. Motor activity is required for localization of Myo1b in filopodia. Localization of myc-Myo1b R165A (A) and myc-Myo1b G390A (D) in HeLa cells is shown. HeLa cells were stained with anti-PIP2 (B and E, red) in addition to anti-myc (A and D, green). C and F are merged images of A and B; and D and E, respectively. Both motor-dead Myo1b constructs were enriched at the cell periphery, but were not present in filopodia. Bars, 10 \( \mu \text{m} \).

FIGURE 7. Motor activity is required for colocalization of Myo1b with PLC81-PH-GFP in filopodia. Myo1b R165A (A, red) and Myo1b G390A (E, red) were enriched at the cell periphery in HeLa cells that were co-transfected with PLC81-PH-GFP (B and F, green). D and H show fluorescence intensity along the white lines in C and G, respectively. PLC81-PH-GFP localized at the cell periphery and in filopodia (B and F), but motor-dead Myo1b was not present in filopodia. C and G are merged images of A and B; and E and F, respectively. Bars, 10 \( \mu \text{m} \).

FIGURE 8. Localization of Myo1b, PIP2 and actin in COS-7 cells with filopodia induced by espin. COS-7 cells were co-transfected with myc-tagged wild-type Myo1b and untagged espin (upper panel). Transfected cells were stained with anti-myc (A), rhodamine phalloidin (B) and anti-PIP2 (C). Localization of Myo1b, F-actin and PIP2 are shown in green, red and blue, respectively in the merged
image (D). PIP<sub>2</sub> localized outside of Myo1b and actin in filopodia. Bar, 5 µm. Model of localization of Myo1b, actin and PIP<sub>2</sub> in filopodia (lower panel). Myo1b, green; actin, red; PIP<sub>2</sub>, blue. Myo1b binds to actin through its head domain and to PIP<sub>2</sub> through its tail domain.
FIGURE 1

A

Motor  |  IQ  |  Tail

β1    |  Loop  |  β2

Myo1b 958 IIAEVVNI  NRANGK  STrRIFLLT  982
Myo1a  VLVADTVKV  NRGNGK  TSSRILLLT
Myo1c  IQYAPVVKY  DRKGYK  PRSRQLLTT
Myo1d  VLFSCVHKV  NRF SK  VEDRAIFVT
Myo1e  VSIGGPLPN  ARPTRNNTVSSRGYSGTT
Myo1f  ISIGDGLPKS  TKPTRGCLAQQRPRRSAQ
Myo1g  VLFSHVKVK  NRF RK  SRDRALLLT
Myo1h  IQYGVPVIK  DRKGPK  ARQFQLLLT

B

Myo1b-FLAG

Myo1b K966A-FLAG  K966A

Myo1b IQ-tail-FLAG

Myo1b tail-FLAG

Myo1b tail K966A-FLAG  K966A

Myo1b tail R977A-FLAG  R977A

C

myc-Myo1b

myc-Myo1b K966A  K966A

myc-Myo1b R977A  R977A

myc-Myo1b R165A  R165A

myc-Myo1b G390A  G390A
FIGURE 2

A

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B

| Myo1b tail-FLAG | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |

| Myo1b tail K966A-FLAG | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |

| Myo1b tail R977A-FLAG | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |

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FIGURE 5

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Graphs D, H, and L show intensity profiles.
FIGURE S1. Localization of Myo1b, PIP2 and actin in COS-7 cells with espin-induced filopodia. COS-7 cells were co-transfected with myc-tagged full-length wild-type Myo1b and untagged espin. Transfected cells were stained with anti-myc (A and G, green), anti-PIP2 (B, red and D, green) and rhodamine phalloidin (E and H, red). Panels C, F and I are merged images of (A and B), (D and E), and (G and H), respectively. Myo1b colocalized with actin within the filopodial membrane containing PIP2. Bars, 5 µm.
FIGURE S2. Diffuse localization of Btk-PH-GFP in HeLa cells. HeLa cells were transfected with Btk-PH-GFP, a PIP$_3$-specific binding protein, and stained with anti-PIP$_2$. Btk-PH-GFP localized throughout the cytoplasm and nucleus, but not in filopodia (A), a pattern distinct from that of PIP$_2$ (B and C). Bar, 10 µm.
Localization of myosin 1b to actin protrusions requires phosphoinositide binding
Shigeru Komaba and Lynne M. Coluccio

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