Recruitment of a Specific Amoeboid Myosin I Isoform to the Plasma Membrane in Chemotactic Dictyostelium Cells*

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The Dictyostelium class I myosins, MyoA, -B, -C, and -D, participate in plasma membrane-based cellular processes such as pseudopod extension and macropinocytosis. Given the existence of a high affinity membrane-binding site in the C-terminal tail domain of these motor proteins and their localized site of action at the cortical membrane-cytoskeleton, it was of interest to determine whether each myosin I was directly associated with the plasma membrane. The membrane association of a myosin I heavy chain kinase that regulates the activity of one of the class I myosins, MyoD was also examined. Cellular fractionation experiments revealed that the majority of the Dictyostelium MyoA, -B, -C and -D heavy chains and the kinase are cytosolic. However, a small, but significant, fraction (appr. 7–15%) of each myosin I and the kinase was associated with the plasma membrane. The level of plasma membrane-associated MyoB, but neither that of MyoC nor MyoD, increases up to 2-fold in highly motile, streaming cells. These results indicate that Dictyostelium specifically recruits myoB to the plasma membrane during directed cell migration, consistent with its known role in pseudopod formation.

The class I myosins are expressed in a wide range of organisms and cell types where they have roles in moving membranes along actin filaments (1). They possess a conserved N-terminal motor domain, 1–6 light chain-binding sites, and a C-terminal tail that has a region rich in basic residues (the polybasic domain). The polybasic domain binds directly to either pure anionic phospholipid vesicles or stripped native plasma membranes with high affinity, in the 100 nM range, in vitro (2–4). Phylogenetic analyses reveal that there are at least four Myo subclasses. The amoeboid subclass is the most widely expressed and its members are distinguished by the presence of two additional C-terminal tail domains (5, 6). The first is a region rich in glycine, proline, and alanine (or serine or glutamate), referred to as the GPA domain, that binds to actin in an ATP-insensitive manner, and the second is a Src homology domain 3 (SH3) domain either at the extreme C terminus or within the GPA domain (4, 7–9). The SH3 domain is essential for myosin I function (10–12), however, in the case of a Dictyostelium and a mammalian myosin I, it does not play a role in localization (12, 13) but does in the case of a yeast myosin I (11).

Molecular genetic analysis of myosin I function in yeast, Aspergillus, and Dictyostelium reveals that the individual members of this family have distinct, yet overlapping roles in mediating the functions of the cortical actin cytoskeleton (14–18). These include nonreceptor mediated fluid-phase uptake (i.e. macropinocytosis (14–18)), exocytosis (14, 16, 19), the orderly extension of pseudopodia during cell migration (20–22) and regulation of the distribution of cortical F-actin (15, 17). The nature of the roles played by myosin Is suggests that they may specifically interact with the plasma membrane and intracellular transport vesicles such as early endosomes or lysosomes.

The mechanism by which myosin I interacts with membranes in vivo and how that interaction is regulated remains unclear. The ability of the polybasic domain to mediate the binding of myosin I to membranes via electrostatic interactions (2–4) indicates that this motor protein could interact nonspecifically with any membrane that contains negative phospholipids such as phosphatidylserine. This suggests that in Dictyostelium, for example, myosin I could be associated with the contractile vacuole, the plasma membrane, and lysosomes, all compartments that contain least 15–20% acidic phospholipids (23). However, the amoeboid myosin Is are discretely localized to particular membrane compartments as well as in regions enriched for actin (3, 17, 24–28). Therefore, there must be a mechanism to direct the specific membrane association of myosin I in vivo, such as a receptor.

A complete understanding of the mechanism(s) by which myosin I may be localized within the cell requires the isolation of a myosin I-containing membrane fraction and identification of factors that may interact with myosin I either in the cytosol or on specific membranes to determine its localization in vivo. Dictyostelium has emerged as an excellent system for such studies as several of the amoeboid myosin Is have been purified and analyzed (29) and information regarding their in vivo roles has been obtained by molecular genetic methods (30). Therefore, an analysis of the membrane association of several Dictyostelium myosin Is, MyoA, -B, -C, and -D, and a myosin I heavy chain kinase (MIHCK) that regulates the activity of

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1 The abbreviations used are: SH3, Src homology domain 3; MIHCK, myosin I heavy chain kinase; MES, 4-morpholineethanesulfonic acid; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein.
MyoD (31) has been performed as the first step in elucidating how their subcellular distribution and, by extension, their function, may be controlled.

MATERIALS AND METHODS

Cell Growth and Maintenance—The Dictyostelium Ax3 strain was used for these studies and standard methods for the maintenance of Dictyostelium cells were employed (32). Cells were grown in suspension in HL5 medium at 220 rpm. Transformants expressing green fluorescent protein (GFP)-MyoA or -MyoB tail fragments were maintained in HL5 supplemented with 10 μg/ml G418 (Genetixc, Life Technologies, Inc., Gaithersburg, MD).

Preparation of Membrane Fractions—A slight modification of the procedures described by Cardelli et al. (33) and Zhu and Clark (34) was employed for the preparation of total cellular membranes. Briefly, cells were collected by centrifugation, washed once with 20 mM MES (pH 6.8), 2 mM MgSO4, and 0.2 mM CaCl2, resuspended with 20 mM TES (pH 7.5), 25 mM KC1, 5 mM MgCl2, 0.1 mM CaCl2, and 10 mM MgATP (TKMC-ATP buffer) containing 0.25 μM sucrose and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM 1-tosyl-2-phenyl-ethyl chloromethyl ketone, 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, and 10 μg 3,4-dichloro-1-2-aminobenzoic acid) to a final density of 4 × 107 cells/ml. The cells were lysed by passage through 5-μm polycarbonate filters (Poretics Corp., Livermore, CA). In the initial experiments, biotinylation of the cell surface proteins was carried out by incubating cells with sulfo-NHS-biotin at 4 °C (Pierce Chemical Co., Rockford, IL) prior to cell lysis (35). The lysate was spun at 1,300 × g for 5 min to remove unlysed cells and nuclei. The post-nuclear supernatant was loaded on a 12% gel and transferred to either PVDF membranes by electroblotting (see above) for quantification of MyoB levels. Bacteria expressing the fusion protein were lysed using an N2 nebulizer (Glas-Col, Terre Haute, IN) and the high-speed supernatant of the lysate incubated with glutathione-agarose beads (Sigma). After washing the beads with phosphate-buffered saline and phosphate-buffered saline, 1 mM NaCl, the fusion protein was extracted from the beads by incubation with 3 mM NaSCN. The fusion protein was then dissolved with USB, separated on 10% SDS-polyacrylamide gel, and purified to homogeneity by electroelution (Eppendorf, Photodyne, Bi-Rad). The purified fusion protein was stored at –80 °C adjusting the protein concentration, as determined using the Bio-Rad DC protein assay, to 1 ng/μl with USB. A Dictyostelium total cell lysate was prepared by suspending a known number of cells with USB, and immunoblotting increasing amounts of sample together with known amounts (0.1–10 ng range) of the fusion protein on the same gel to generate a standard curve. After detection and exposure, the intensities of each protein band were measured as described above. Only intensities in the linear range of the standard curve were used to determine the absolute amount of MyoB per number of cells. Given that the same fusion protein used for generation of the polyclonal antibody against MyoB was used for the quantification (15), immunoactivity to the polyclonal antibody was assumed to be the same as that to intact MyoB.

Quantitative transfer of proteins in the range of the full-length MyoB heavy chain and the fusion protein to polyvinylidene difluoride membranes was confirmed.

RESULTS

Association of the Myosin I with Cellular Membranes—The fraction of Dictyostelium myosin Is and an MIHCK (31) associated with membranes was first determined using quantitative immunoblotting with antibodies specific for the Dictyostelium amoeboid myosin Is, MyoB, -C, and -D (15, 29) and MIHCK (31). The membrane association of a Dictyostelium mutant strain (Dictyostelium myosin I that lacks the C-terminal GPA and SH3 domains, MyoA (42), was also analyzed using a strain of cells that express GFP-MyoA. The GFP-MyoA strain was generated as all attempts to generate a strain expressing GFP-MyoB or -MyoC failed.

Total cell lysates were prepared from either wild type or GFP-MyoA cells and experiments were performed using buffers containing 10 mM MgATP to prevent the precipitation of myosin I with its own motor domain. Lysates were applied to a sucrose step gradient to separate the cytosol from total cellular membranes. Quantitative immunoblotting revealed that, surprisingly, only a relatively small proportion (6.8–8.7%) of each of the three amoeboid myosin Is (MyoB, -C, and -D) was
present in the total membrane fraction (Fig. 1). In contrast, a significant proportion of GFP-MyoA (32%) was present in the total membrane fraction. However, since it is not known if the GFP-MyoA levels are significantly higher than endogenous MyoA levels, this value might be an overestimate. Indeed, cells expressing excess MyoB using the same extrachromosomal vector (10) have a 2-fold greater amount of membrane-associated MyoB than wild type cells (14 versus 6.8%). This would suggest that the amount of membrane-associated MyoA in wild type cells is approximately half that observed in the GFP-MyoA cells, or 16%.

The existence of an apparently nonspecific electrostatic membrane-binding site in all myosin I tail regions (5) suggested that myosins could potentially be associated with any membrane compartment that contains sufficient amounts of anionic phospholipids. The intracellular compartment that contains bound myosin Is was identified by subfractionation of the total cellular membranes on a linear 30–70% sucrose gradient (33, 34). The resulting fractions were analyzed for the presence of endoplasmic reticulum, lysosomal, contractile vacuole, and mitochondrial enzymes by a series of enzymatic assays (36–38). Several distinct peaks of enzyme activity were reproducibly found for endoplasmic reticulum, lysosomes, contractile vacuole, and mitochondria (Fig. 2A). A broad peak of biotin (that marks the plasma membrane) was routinely found between the peaks of lysosome and contractile vacuole activity (Fig. 2A and C). Analysis of the protein composition of the fractions (Fig. 2B) revealed the presence of a 45-kDa band in fractions 3–10 that was identified as actin by immunoblotting (data not shown). Immunoblotting with myosin I-specific antibodies was used to detect the position of MyoB, -C, and -D in the gradient. A peak of MyoB immunoreactivity consistently coincided with that of the plasma membrane marker (Fig. 2C). The MyoB peak was somewhat broader than that of the plasma membrane peak, suggesting that a fraction of the MyoB was associated with actin present in the heavier fractions (numbers 8–10; Fig. 2B). The MyoC and MyoD heavy chains were in the same position as MyoB (Fig. 2D) indicating that these myosin Is were also associated with the plasma membrane. Analysis of fractionated membranes from GFP-MyoA expressing cells revealed that MyoA was also associated with the plasma mem-

![Fig. 1. Membrane association of myosin Is and MIHCK. Immunoblots of Dictyostelium cytosolic (C) and membrane (M, concentrated 10-fold relative to the cytosol) fractions with antisera specific for MyoB, MyoC, MyoD, and MIHCK are shown. The proportion of each protein found in the two fractions as a percentage of the total myosin I isoform is shown below each panel. The position of the 116-kDa molecular mass standard is indicated on the right.](http://www.jbc.org/)

![Fig. 2. MyoB, -C, and -D are associated with the plasma membrane. A, distribution of marker enzymes in each fraction of Dictyostelium total membranes separated by a 30–70% linear sucrose-density gradient. The following enzyme assays were performed: alkaline phosphatase (▵, contractile vacuole marker), acid phosphatase (□, lysosome marker), α-glucosidase-2 (●, endoplasmic reticulum marker), and succinic dehydrogenase (×, mitochondria marker). The relative activities of marker enzymes were plotted against the sucrose percentage of each fraction. The asterisk indicates the position of the peak of MyoB immunoreactivity. B, SDS-PAGE of fractionated membranes. Equal volumes of fractions 1–10 from a 30–70% sucrose density gradient were electrophoresed on 7.5% SDS-PAGE gel and stained by Coomassie Blue. The position of known molecular weight standards, in kDa, are indicated on the right. C, co-fractionation of MyoB with the plasma membrane as determined by quantitative immunoblotting. Biotinylated cell surface proteins (plasma membrane marker) were detected by horseradish peroxidase-labeled streptavidin. The relative intensities of MyoB (+) and biotinylated proteins (+) were plotted against the sucrose percentage of each fraction. The results shown are representative of four independent experiments. D, MyoC and MyoD are also associated with the plasma membrane. Equal amounts of each fraction of the sucrose density gradient were analyzed for the presence of myosin I heavy chains by immunoblotting. Shown is the co-fractionation of the 125-kDa MyoB heavy chain (top panel) with the 135-kDa MyoC and 125-kDa MyoD heavy chains (bottom panel). The position of the 116-kDa molecular mass standard is indicated on the right.)
membrane, based on its co-fractionation with MyoB (Fig. 3A). Therefore, despite differences in their C-terminal tail regions, the amoeboid myosin Is and MyoA are all associated specifically with the plasma membrane.

Given the ability of the amoeboid myosin Is to bind to F-actin via their GPA domains (4, 7–9), it was important to establish whether myosin I was truly associated with membranes. The myosin I-containing membrane fractions were pooled, incubated with 1% Triton X-100 to solubilize all membranes, and then subjected to centrifugation. The majority of the MyoB and -C heavy chains were released into the supernatant (Fig. 4), confirming that these myosins were indeed membrane-associated and not simply present in the membrane fraction via association with F-actin. While a substantial amount of the MyoD heavy chain was also solubilized (Fig. 4), a larger proportion than observed for MyoB and -C remained in the pellet. This could be due to association of MyoD with actin via its GPA domain. GFP-MyoA behaved in a similar manner to MyoB and -C, with the majority of this myosin I being released into the supernatant following Triton treatment (Fig. 4).

The binding of Acanthamoeba myosin I to membranes in vitro is salt-sensitive (2, 3). The total membrane fraction was incubated with 150 mM or 1 M NaCl to test if each myosin I associated with membranes via an electrostatic interaction. Only a small amount of the MyoB heavy chain was solubilized following salt treatment (Fig. 4) and, surprisingly, neither the MyoC nor the MyoD heavy chains were released into the supernatant (Fig. 4). Similarly, GFP-MyoA was not released from the membrane following salt treatment (Fig. 4). These results suggest that the interaction of the Dictyostelium myosin I with isolated cellular membranes does not occur through electrostatic interactions.

The Polybasic Domain Is Responsible for Myosin I Association with Membranes in Vivo—Total membranes from Dictyostelium transformants expressing either the whole MyoB tail region or the GPQ/SH3 domain (Fig. 5, A and B) were fractionated using the same method described above, to identify the domain responsible for the association of MyoB with membranes. Immunoblotting revealed that ~25% of the MyoB tail was present in the total membrane fraction. No significant diminution of the endogenous MyoB heavy chain from the plasma membrane was observed, suggesting that the excess tail does not successfully compete for binding sites on the membrane with the native MyoB. The GPQ/SH3 fragment was not detected in the total membrane fraction (Fig. 5B), suggesting that the polybasic domain alone is required for association of MyoB with membranes. Attempts to test this by overexpression of the isolated polybasic domain were unsuccessful as this domain appears to be unstable.2 The MyoB tail fragment was not released from the membrane fraction by treatment with 1 M NaCl, but was partially solubilized by detergent treatment (Fig. 4). Fractionation of the total membranes from MyoB tail expressing cells revealed that the tail was associated with the plasma membrane (Fig. 5C). Phenotypic analysis of cells expressing either the full-length MyoB tail or the GPQ/SH3 domain revealed that despite the expression of a 2–5-fold excess of either tail fragment, no alteration in behavior was observed. Growth in suspension, pinocytosis, development, and streaming were normal.3

Membrane Association of MIHCK—The physiological activity of the lower eukaryotic amoeboid myosin I is tightly regulated by phosphorylation of a single serine or threonine residue in the motor domain. A Dictyostelium MyoD-specific MIHCK has been identified (31) and found to be a member of the PAK family of G-protein-regulated kinases (46). Recent experiments revealed that it interacts with acidic phospholipids and a fraction of this kinase cosediments with the membrane-cytoskeleton in a salt-dependent manner (47). The presence of MIHCK in either the cytosolic or total membrane fractions was examined. Similar to what was found for the myosin Is, the majority of MIHCK was found in the cytosolic fraction with a small percentage of the total MIHCK (8.6%) present in the total membrane fraction (Fig. 1). The identity of the membranes containing the tightly bound MIHCK was determined by subcellular fractionation and immunoblotting (Fig. 3B). The MIHCK fractions largely overlapped with MyoB fractions (that mark the plasma membrane), however, the peak of MIHCK immunoreactivity seems to be shifted toward the denser fractions (Fig. 3B), suggesting that the kinase may be associated with more than one membrane compartment. Further analysis of the membrane-bound MIHCK revealed that 1 M salt treatment was not effective at releasing the kinase from the membranes, but incubation with Triton X-100 was effective (Fig. 4).

Increased Motility of Dictyostelium Coincides with an Increase in the Membrane Association of Myosin I—The onset of development by starvation signals Dictyostelium cells to

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2 M. D. Peterson and M. A. Titus, unpublished observations.
3 M. D. Peterson, S. Senda, and M. A. Titus, unpublished observations.
The percentage of MyoB, -C, and -D associated with membranes from either growth phase or streaming cells was determined. The proportion of the MyoB heavy chain associated with membrane at the onset of streaming was increased, 1.9-fold, as determined by quantitative immunoblotting (Table I). A smaller, but still statistically significant, increase in the amount of membrane-associated MyoD, 1.4-fold, was observed in streaming cells, but no significant increase of MyoC was observed (Table I). Interestingly, the levels of membrane-associated MIHCK also increased by 1.5-fold (Table I).

The phenotype of Dictyostelium myosin I single null mutants suggested that these motor proteins may have overlapping functions and that the partial phenotypes observed in the myoA− and myoB− cells could be due to compensation for their loss by other members of the myosin I family (30). Given that highly motile Dictyostelium have an increased level of membrane-associated MyoB (Table I), the effect of deletion of MyoA or MyoB on the membrane association of MyoC and -D, as well as MIHCK, was examined. Following the onset of streaming in the myoA− or myoB− null mutants, which is delayed and occurs at 8 h after the initiation of starvation (15, 50, 52), the amount of both the MyoC and MyoD heavy chains in the total membrane fraction increases in both the myoA− and myoB− cells by ~2-fold (Table II). These increases in the amount of myosin I heavy chains on the plasma membrane are not due to a simple increase of MyoC and MyoD expression in these cells during streaming (Table II). Interestingly, there is also an almost 2-fold increase of MIHCK in the myoA− and myoB− total membrane fractions during the onset of streaming (Table II). The membrane-associated MyoB is increased in the myoA− cells by 2-fold (Table II), not significantly different from what is observed for wild type cells (Table I). Thus, loss of either MyoA or MyoB only results in an increase in the amount of the MyoC and MyoD heavy chains with the plasma membrane.

**Calculation of the Amount of Membrane-associated MyoB—**
Quantitative immunoblotting revealed that the growth phase cells have 32 ± 4.6 ng of MyoB/10⁶ cells (1.56 × 10⁵ MyoB molecules/cell; n = 3). This amount is 4-fold greater than the previously reported, 8 ng of MyoB/10⁶ cells (18), but the differences could be due to slightly different conditions for the determination of MyoB levels. If one assumes that the average diameter of Dictyostelium is 10.25 μm and the average volume of the cell is 565 μm³ (53), then the cellular concentration of MyoB is 460 nM. The 6.8% of MyoB present on the plasma membrane (assuming an internal surface area of 331 μm² at maximum) in growth phase cells would then be equivalent to 32 molecules/μm² of plasma membrane.

**DISCUSSION**

The membrane association of four Dictyostelium myosin Is, MyoA, -B, -C, and -D, has been investigated using a fractionation approach. Despite the presence of a high affinity membrane-binding domain in their C termini, only a small fraction, 7–15%, of each is membrane-associated (Fig. 1). The association membranes appears to be mediated by the polybasic domain, as a MyoB tail fragment lacking this domain is not membrane-associated (Fig. 5B). The finding that the majority of each myosin I is found in the cytosolic fraction is consistent with earlier observations that 80% of the total high salt K⁺ ATPase activity (presumably contributed largely by the myosin I) in a low salt Dictyostelium lysate is soluble (29). Acanthamoeba myosin IA is also largely cytosolic, as determined by quantitative immunoelectron microscopy (26). However, a large proportion of the closely related Acanthamoeba myosin Is, myosin IB and -IC, are membrane associated (26). Additionally, it has been reported that two different forms of mammalian myosin I, Myr1 and Myr2 (nonamoeboid type myosin Is), are

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4 S. Senda and M. A. Titus, unpublished data.
Membrane Recruitment of Myosin I

The percent of each of the Dictyostelium myosin I heavy chains and the MIHCK associated with the total membrane fraction in either growth or streaming (following 6 h of starvation on non-nutrient agar) conditions is shown \( \pm \) S.D. The number of trials for each sample is shown in parentheses. The asterisk (*) indicates a statistically significant increase \( (p < 0.05) \).

| Condition | MyoB | MyoC | MyoD | MIHCK |
|-----------|------|------|------|-------|
| Growth    | 6.8 \( \pm \) 1.0 (6) | 8.7 \( \pm \) 2.5 (7) | 7.7 \( \pm \) 1.3 (6) | 8.6 \( \pm \) 1.9 (3) |
| Streaming | 13.2 \( \pm \) 1.9 (7) | 10.8 \( \pm \) 1.1 (7) | 10.4 \( \pm \) 1.7 (7) | 12.9 \( \pm \) 2.1 (3) |
| Fold increase | 1.9\* | 1.2 | 1.4\* | 1.5\* |

**Table II**

Relative levels and membrane association of myosin is and MIHCK in myosin I null mutants

The relative levels of each myosin I heavy chain and MIHCK in streaming versus growing myosin I mutant cells are shown \( \pm \) S.D. Note that the overall levels (relative to those in wild type cells) of each protein are not significantly increased during streaming in the mutant cells. The percent of each of the Dictyostelium myosin I heavy chains and the MIHCK associated with the total membrane during either growth or streaming (following 8 h of starvation on non-nutrient agar) in the myoA\(^{-}\) or myoB\(^{-}\) single mutants is shown \( \pm \) S.D. The number of trials for each sample is shown in parentheses. The fold increase in the levels of membrane association during streaming is shown and the asterisk (*) indicates a statistically significant increase \( (p < 0.05) \).

|            | MyoB | MyoC | MyoD | MIHCK |
|------------|------|------|------|-------|
| MyoB       |      |      |      |       |
| myoA\(^{-}\) | 5.6 \( \pm \) 1.0 (3) | 9.4 \( \pm \) 1.7 (4) | 7.5 \( \pm \) 0.8 (4) | 6.0 \( \pm \) 0.7 (4) |
| myoB\(^{-}\) | 12.7 \( \pm \) 3.3 (3) | 16.5 \( \pm \) 2.5 (4) | 15.1 \( \pm \) 3.7 (4) | 12.2 \( \pm \) 0.7 (4) |
| MyoC       |      |      |      |       |
| myoA\(^{-}\) | 1.1 \( \pm \) 1.1 | 1.1 \( \pm \) 0.1 | 1.1 \( \pm \) 0.1 | 1.1 \( \pm \) 0.1 |
| myoB\(^{-}\) | 6.0 \( \pm \) 0.7 (4) | 5.7 \( \pm \) 0.9 (4) | 8.3 \( \pm \) 0.9 (3) | 10.4 \( \pm \) 2.3 (3) |
| MyoD       |      |      |      |       |
| myoA\(^{-}\) | 1.7 \( \pm \) 4.0 (4) | 18.1 \( \pm \) 3.9 (3) | 17.9 \( \pm \) 4.0 (3) |
| myoB\(^{-}\) | 2.0 \( \times \) | 2.3 \( \times \) | 2.2 \( \times \) | 1.7 \( \times \) |

Membrane associated in rat liver and smooth muscle cells (54, 55). These myosins also appear to be associated with more than one membrane compartment, although they are predominantly found in Golgi (Myr2 in rat liver) or plasma membrane (Myr1 in rat liver) fractions. This indicates that there must be specific sequences in each myosin I that directs a particular myosin to the appropriate membrane compartment and also regulates the proportion of that myosin that is cytosolic or membrane-bound. These unidentified distinctive tail sequences that most likely reside in the polybasic domain could directly play a role in targeting these myosins to a receptor on the appropriate membrane. Alternatively, they could be responsible for associating with a cytosolic protein that prevents binding to membranes or plays a role in mediating myosin I-myosin I interactions in the lipid bilayer.

The MIHCK that regulates MyoD activity was also found to be largely cytosolic under our fractionation conditions. Only a small proportion was found in the total membrane fraction following lysis of the cells (Fig. 1). In contrast to this result, a more significant amount of MIHCK was found in association with a high speed pellet that contains total membranes and the cytoskeleton when cells are directly lysed in the presence of 20 mM salt (47). This binding was significantly reduced, but not abolished, when the salt concentration was raised to 100 mM, consistent with mutant analyses that implicate MyoA and MyoB

in cell migration (20, 21) and all of these myosin I in macropinocytosis (15, 18), functions that require the activity of the actin-rich membrane cortex of cells.

There is a specific recruitment of MyoB (2-fold, Table II) to the plasma membrane during chemotaxis (Table I). The net increase in membrane-associated MyoB is substantial if one takes into account the observation that there is an overall 5-fold increase in the total amount of this protein during streaming (18). Thus, streaming cells have 10 times more MyoB on the plasma membrane than do normal growth phase cells. This recruitment of MyoB may be required for controlling appropriate pseudopod formation as myoB\(^{-}\) mutants extend an increased number of pseudopodia (20). In contrast, significantly less MyoC and MyoD is present on the plasma membrane in streaming cells. The total amounts of these myosin I are lower than that of MyoB and they do not increase during streaming (18), and a much smaller increase in their association with the membrane is observed (Table I). The lower levels of plasma membrane-associated MyoC and MyoD in streaming cells are consistent with the finding that myoC\(^{-}\) and myoD\(^{-}\) mutants move at normal rates of speed and do not exhibit any delays in streaming (51, 52).

The finding that the deletion of a single amoeboid myosin I from either yeast or Dictyostelium does not result in profound phenotypes, suggests that these motors share overlapping roles (15–18). In addition, compensation for the loss of one myosin I by another could occur either up-regulation of the activity or amount of one or more remaining myosin I. Examination of the remaining amoeboid myosin I (MyoC and MyoD) in the myoA\(^{-}\) and myoB\(^{-}\) cells and MyoB in the myoA\(^{-}\) cells reveals that the overall levels of these myosin I are not increased in the mutants (Table II). However, there is a 2-fold increase in the amount of membrane-associated MyoC and -D in the myoA\(^{-}\) and myoB\(^{-}\) mutants (Table II). The availability of free myosin I-binding sites on the plasma membrane may allow an increased amount of MyoC and MyoD to bind to the plasma...
membrane. Alternatively, the mutant cells may have a mechanism for increasing the recruitment of the remaining myosin I to the plasma membrane during streaming in an effort to compensate for the loss of either MyoA or MyoB. The 2-fold increase in the levels of membrane-associated MyoC and MyoD in the myoA− or myoB− mutants (Table II) is clearly insufficient to compensate for the loss of either of these myosin I. This is most likely due to the fact that the levels of MyoC and -D expressed by the cell during streaming are significantly lower than those of MyoB (18). However, there is evidence that the relatively small increase in MyoC and -D on the plasma membrane could be playing a role in maintaining some level of normal function related to motility in the mutant cells. The motility of a myoB−/ID− double mutant does not appear to be worse than that of the myoB− single mutant, however, the myoB−/C−/D− triple mutant moves at a more slowly and is more severely delayed in streaming (18). Thus, MyoC may play a role in the efficient motility of Dictyostelium myosin I mutants but, despite increased recruitment to the plasma membrane, it is not sufficient to fully compensate for the loss of MyoB.

The role of membrane association in myosin I function remains unclear. A kinetic analysis of two Acanthamoeba myosin Is, IA and IB, demonstrates that this class of motor protein is not processive, and indicates that myosin I must tightly gather on membranes and/or the actin cytoskeleton to power membrane movement or contraction of the actin cortex (57). Ostap and Pollard (57) estimated that a cluster of at least 20 molecules of myosin I would be necessary for membrane-based myosin I function. The present results showing 32 molecules of MyoB present per μm² of plasma membrane would suggest that this amount is insufficient to move the plasma membrane along actin if it were uniformly dispersed across the inner plasma membrane surface. However, immunolocalization data indicate that all three of the Dictyostelium amoeboid myosins are specifically concentrated in one region of the cell (such as in an extending pseudopod or macropinocytic ruffle) (25, 56). A highly motile, motility-secretory cell must have a high local concentration of MyoB on the plasma membrane. This would result from a combination of a 5-fold increase in levels of MyoB (when compared with growth phase cells) and a 2-fold overall increase in MyoB on the plasma membrane during streaming (18) (Table II). The average amount of plasma-membrane bound MyoB in motility-secretory cells, now increased to 320 molecules per μm², could occupy 0.1 μm² (assuming that 20 molecules of myosin I occupy 25 × 25 nm on the plasma membrane; Jontes and Milligan as cited in Ref. 57) of the membrane surface. In other words, 10% of the unit membrane area would be covered by MyoB. Given that the average length of F-actin in Dictyostelium is 0.2 μm (58), this appears to be sufficient to generate force for motility along F-actin. Therefore, the membrane association of MyoB could potentially be important in allowing this myosin I to participate in the regulated formation of pseudopodia during directed migration, consistent with the finding that deletion of MyoB results in significant defects in pseudopod formation (20).

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Recruitment of a Specific Amoeboid Myosin I Isoform to the Plasma Membrane in Chemotactic Dictyostelium Cells

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