A potential mechanism for regulating myosin I binding to membranes in vivo

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Abstract Myosin Is are associated with specific membranes, however, the mechanism for regulating their intracellular localization is unclear. As a first step towards understanding this mechanism, membrane rebinding assays using *Dictyostelium* myoB were performed. Crude, cytosolic myoB bound to intact, but not to NaOH-treated plasma membranes. In contrast, partially purified myoB binds to both intact and NaOH-treated plasma membranes. Chemical cross-linking of cytosolic myoB yielded several products, whereas none were found with the partially purified myoB. These results suggest a model where proteins regulating the specific binding of myoB to the plasma membrane may exist both in the cytosol and on the plasma membrane.

Key words: Unconventional myosin; *Dictyostelium discoideum*; Plasma membrane; Membrane association; Myosin I

1. Introduction

The class I myosins are ubiquitous and many cells express more than one of these actin-based motor proteins. In lower eukaryotes and fungi, these myosins play important roles in endocytosis, secretion, polarization of the actin cytoskeleton, and cell migration [1–4]. They have also been implicated in endocytosis in mammalian cells where they have been found to participate in various steps along the endocytic pathway [5–7]. Class I myosins all share a conserved N-terminal motor domain, a neck region that binds one–six low molecular weight light chains, and a C-terminal tail region that has a polybasic region that binds to membranes and actin filaments with high affinity [8,9]. There are several subclasses of myosin I, as defined by phylogenetic analyses [8]. The amoeboid subclass is the most widely expressed and it is distinguished from other class I myosins by the presence of two additional C-terminal tail domains – a region rich in glycine, proline, and alanine (or serine or glutamate; GPA) that binds actin in an ATP-independent manner and a *src* homology 3 domain (SH3).

The existence of high affinity binding sites for both actin (10 μM) and membranes (100 nM) in the tail of amoeboid myosin Is suggests that the majority of this type of myosin I might be associated with either the actin cytoskeleton or intracellular membranes. Immunolocalization results are largely consistent with this view, the vast majority of *Acanthamoeba* myosin IB and IC are found in association with membranes [10], yeast Myo3p is co-localized with actin patches and caps adjacent to the plasma membrane [3], *Aspergillus* MYOA is enriched at growing hyphal tips where membrane deposition occurs [1], and *Dictyostelium* myoB and D are localized to the actin-rich leading edge of migrating cells [11]. In contrast to these findings, a recent analysis of the membrane association of several *Dictyostelium* class I myosins (myoA, B, C and D) using a cellular fractionation approach revealed that a substantial amount of these, $>90\%$, are soluble (Senda et al., manuscript submitted). Interestingly, this also appears to be the case with *Acanthamoeba* myosin IA [12]. Additionally, the *Dictyostelium* myosin Is (Senda et al., manuscript submitted), as well as *Acanthamoeba* myosin IA [12] are exclusively associated with the plasma membrane, in spite of apparently having the capability to bind to any membrane that exhibits a net negative charge [13,14]. These results indicate that there must be a mechanism for regulating the specific membrane association of a given myosin I, however, that mechanism remains to be identified. An initial investigation into the membrane binding abilities of cytosolic myosin I was undertaken in an effort to begin defining the means by which its membrane association is controlled.

2. Materials and methods

2.1. Preparation of membrane fractions

Dictyostelium plasma membranes (containing bound myoB), contractile vacuoles, and lysosomal membrane were prepared as described by others [15]. The TKMC buffer (20 mM TES (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$) included 10 mM MgATP and a protease inhibitor cocktail (1 mM PMSF, 100 μM TPCK, 100 μM TLCK, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin and 10 μM 3,4-dichloroisocoumarin; Sigma Chemical Co., St. Louis, MO, USA). This buffer is referred to throughout as TKMC-ATP buffer. First, the cells were lysed and separated on a sucrose step gradient into cytosolic and total membrane fractions (Fig. 1). The total membrane was applied to a 30–70% sucrose gradient in TKMC-ATP buffer and centrifuged at $100\,000\times g$ in a linear gradient to separate the various membrane compartments. Equal volume fractions were collected and an equal amount of each analyzed for contractile vacuole marker enzyme activity (alkaline phosphatase) and lysosomal marker enzyme activity (acid phosphatase) [16]. The fractions were also analyzed by immunoblotting using chemiluminescent detection (Super Signal, Pierce Chemical Co., Rockford, IL,

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USA) for the presence of myoB using a rabbit polyclonal antibody directed against *Dictyostelium* myoB tail region [17]. Previous results have demonstrated that myoB co-fractionates with the plasma membrane (Senda et al., manuscript submitted). The peak plasma membrane, contractile vacuole, and lysosomal fractions were pooled.

Total plasma membrane lipids were extracted from using the procedure described by Bligh and Dyer [18]. Organic solvents were evaporated by leaving samples in a chemical hood overnight. The dried lipids were solubilized with 10% sodium cholate and the solution dialyzed against several changes of 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM EGTA until turbid. The dialysate was sonicated by Micro Ultrasonic Cell Disruptor (Kontes, Vineland, NJ, USA) on ice until the solution became clear. The lipid vesicles were collected by centrifugation at 200 000 × *g* for 10 min, resuspended with the rebinding buffer and immediately used for the rebinding assay (described above). Quantification of phopholipids was performed as described by Ames [19].

2.2. Partial purification of myoB

The progress of myoB purification was monitored throughout by immunoblotting. *Dictyostelium* Ax-3 cells were developed in starvation buffer for 4 h to increase the levels of membrane-associated myoB (Senda et al., submitted). The cells were collected by centrifugation, washed once with 50 mM HEPES (pH 7.5) and suspended with 50 mM HEPES (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 5 mM MgATP and 15% sucrose (final concentrations). The cells were lysed by passage through an N_2 nebulizer (BioNeb Cell Disruptor, GLAS-COL, Terre Haute, IN, USA) in the presence of protease inhibitor cocktail. The lysate was spun at 125 000 × *g* for 2 h and the supernatant proteins were precipitated by the addition of solid ammonium sulfate (AS) to 40%. The precipitant collected by centrifugation was resuspended to 25% AS (final concentration) with 50 mM HEPES (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$ and 5 mM MgATP. The soluble fraction was obtained by ultracentrifugation at 200 000 × *g* for 2 h, loaded on Phenyl-650M column (TosoHaas, Montgomeryville, PA, USA) and eluted with a step gradient of AS in 10 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 0.1 mM EGTA and 0.1 mM MgATP. The eluted myoB was pooled, precipitated by the addition of solid AS to a final concentration of 80% and collected by centrifugation. The myoB pellet was dissolved in and dialyzed against 25 mM MES (pH 6.1), 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM MgATP (CM buffer) and 1 mM DTT for 3 h at 4°C. The dialysate was spun at 200 000 × *g* for 2 h in the presence of 5 mM MgATP and the supernatant was loaded on Toyopearl CM-650M column (TosoHaas) equilibrated with CM buffer. Proteins were eluted with a linear gradient of NaCl in CM buffer and myoB-containing fractions were pooled. The pooled fraction is referred to as partially purified myoB.

2.3. Rebinding assay

The cytosolic fraction and partially purified myoB were dialyzed against 20 mM PIPES (pH 7.0), 100 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (rebinding buffer) for 6 h at 4°C. The dialysate was spun at 14 000 × *g* for 20 min after adjusting to 5 mM MgATP, 100 μg/ml BSA and 1 mM DTT. The supernatants were carefully collected and immediately used in rebinding studies. The plasma membrane fraction was diluted with TKMC-ATP buffer and collected by centrifugation at 14 000 × *g* for 20 min. The plasma membranes were then resuspended with TKMC-ATP buffer and the protein concentration adjusted to 1 mg/ml. Half of the plasma membrane suspension was treated with 100 mM NaOH (pH ~ 11.0) for 1 min on ice to remove peripheral proteins and the other half was untreated. Prior to the rebinding assay, the various membrane fractions were pelleted by centrifugation at 14 000 × *g* for 20 min and washed once with TKMC-ATP buffer and once with the rebinding buffer. The membranes were finally resuspended with the rebinding buffer containing 5 mM MgATP, 100 μg/ml BSA and 1 mM DTT. The total cytosol or partially purified myoB were incubated with intact membrane or NaOH-stripped membrane for 16 h at 4°C with gentle agitation. The mixtures were spun at 14 000 × *g* for 20 min. For the rebinding assay with *Dictyostelium* lipids, partially purified myoB was incubated with the lipids for 1 h at 22°C and spun at 200 000 × *g* for 10 min. The supernatants were carefully collected and precipitated by adding trichloroacetic acid (TCA) to a final concentration of 5%. The TCA precipitate and pelleted membranes were dissolved with an equal volume of SDS–PAGE sample buffer containing 6 M urea (USB) following neutralization with ammonium bicarbonate. Equal volumes of the supernatant and membrane pellet were analyzed by immunoblotting for the presence of myoB.

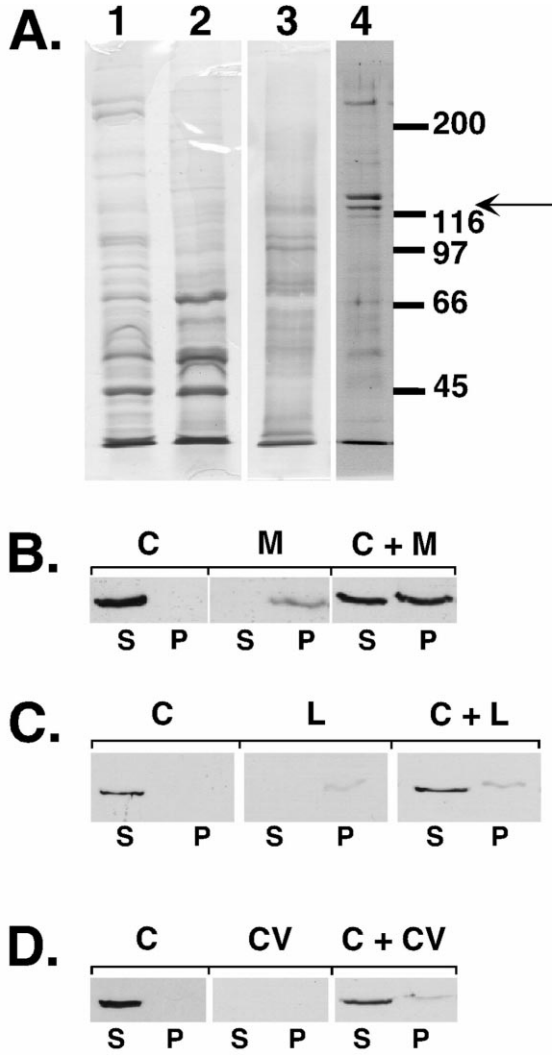

Fig. 1. Crude myoB binds selectively to plasma membranes. A: SDS–PAGE of total cytosol, enriched myoB and membranes. Proteins present in the major fractions used in the rebinding experiments are shown. Lane 1, total cytosol; lane 2, intact plasma membrane; lane 3, NaOH-stripped plasma membrane; and lane 4, partially purified myoB. The samples were electrophoresed on a 7.5% SDS–polyacrylamide gel stained with Coomassie blue (1, 2, 4) or silver nitrate (3). The positions of known molecular standards (in kDa) are indicated on the right. The presumptive myoB band is indicated by an arrow in lane 4. The other prominent band in that lane (at ~135 kDa) is likely to be the myoC, a closely related *Dictyostelium* amoeboid myosin I. B: Crude myoB binds to the plasma membrane. Shown are the supernatants (S) and pellets (P) of rebinding samples following overnight incubation, centrifugation and immunoblotting of equal loadings of each sample for the myoB heavy chain. Note that the myoB antibody only recognizes a single band of 125 kDa. Total cytosol (C), intact plasma membrane (M) and total cytosol plus intact plasma membranes (C+M). C: Crude myoB does not bind to lysosomal membranes. Total cytosol (C), lysosomal membranes (L) and total cytosol plus lysosomal membranes (C+L). Note that because of the overlap between the plasma membrane and lysosomal enzyme peaks, a small amount of myoB is present in the lysosome fractions. D: Crude myoB does not bind to contractile vacuole membranes. Total cytosol (C), contractile vacuole membranes (CV) and total cytosol plus contractile vacuole membranes (C+CV).

2.4. Chemical cross-linking of myoB

The total cytosol and partially purified myoB were treated with 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, Sigma, St. Louis, MO, USA) up to 20 min at room temperature. The reaction was stopped by the addition of an equal volume of USB.

3. Results and discussion

The ability of cytosolic myoB (i.e. myoB present in the total cytosol fraction) to bind to intact plasma membranes was first examined. It should be noted that all rebinding experiments were performed in the presence of MgATP to prevent the association of myosin with membrane-associated actin via the head domain. A total cytosol fraction (Fig. 1A, lane 1) was incubated with purified membrane fractions (plasma membrane, contractile vacuole, or lysosomes) and the samples spun to separate the bound from unbound myoB following overnight incubation in rebinding buffer. In the absence of added membranes, all of the myoB present in the cytosol fraction is in the supernatant (Fig. 1B). However, a small amount of myoB was found in the pellet of the control membrane sample as the plasma membrane contains bound myoB (Fig. 1B; Senda et al., submitted). Incubation of the cytosol with the intact plasma membrane (Fig. 1A, lane 2) fraction resulted in a significant increase in the amount of membrane-associated myoB (Fig. 1B). In contrast, no significant rebinding of myoB to either contractile vacuole or lysosomal membranes was observed when cytosol was incubated with either of these fractions (Fig. 1C, D). These results demonstrate that myoB present in the cytosol is capable of binding only to isolated, intact plasma membrane. The myoB present in the cytosol binds neither to contractile vacuole nor lysosomal membranes, in spite of the relatively high content of acidic phospholipids in these membranes [16]. Additionally, the increase in the amount of membrane-associated myoB in the plasma membrane fraction suggests that the isolated plasma membranes have the capacity to bind more myosin I (i.e. their myoB binding sites are not saturated). These results indicate that there is specificity of membrane binding in vitro, as has been observed in vivo.

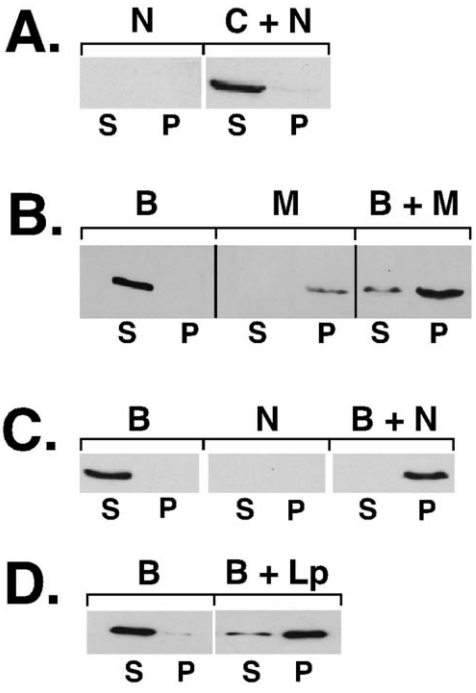

Fig. 2. Binding of myoB to the plasma membrane and extracted plasma membrane lipids. Shown are the supernatants (S) and pellets (P) of rebinding samples following overnight incubation, centrifugation and immunoblotting of equal loadings of each sample for the myoB heavy chain. A: Crude myoB incubated with NaOH-stripped plasma membranes. NaOH-stripped plasma membranes (N) do not contain myoB and myoB present in the total cytosol does not bind to the NaOH-stripped membrane (C+N). B: Partially purified myoB incubated with intact plasma membranes. Partially purified myoB (B) is soluble while myoB is present in the pellet of sedimented intact plasma membrane (M) fraction. There is increased membrane-associated myoB when partially purified myoB is incubated with intact plasma membrane (B+M). C: Partially purified myoB incubated with NaOH-stripped plasma membranes. Partially purified myoB (B) is soluble and myoB is not present in NaOH-stripped plasma membranes (N). Membrane-associated myoB results when partially purified myoB is incubated with NaOH-stripped plasma membranes (B+N). D: Partially purified myoB incubated with extracted plasma membrane lipids. Partially purified myoB (B) is soluble but can bind to extracted plasma membrane lipids (B+Lp).

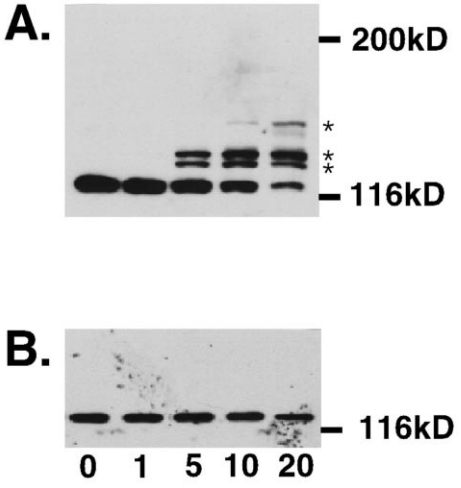

Fig. 3. Chemical cross-linking of myoB-containing fractions. The total cytosolic fraction (A) and the enriched myoB fraction (B) were incubated with EDAC and the reaction terminated at the times indicated in min. The samples were analyzed by immunoblotting for the myoB heavy chain. The positions of known molecular weight standards are indicated on the left.

Incubation of crude myoB with NaOH-stripped plasma membranes, surprisingly, did not result in membrane binding (Fig. 2A), in contrast to what is observed when intact membranes are used (compare with Fig. 1B). These results suggested that a peripheral membrane protein is required for the binding of myoB to the plasma membrane. One obvious candidate is a specific myosin I receptor. However, previous experiments employing purified *Acanthamoeba* myosin Is and brush border myosin I (BBMI), that both contain a polybasic domain adjacent to the motor domain, revealed that these myosins are capable of high affinity binding to NaOH-treated biological membranes in vitro [13,14,20,21]. Since myoB also contains a similar polybasic domain, the different membrane binding properties of crude myoB could instead be attributed to the association of some inhibitory protein. An enriched *Dictyostelium* myoB fraction (Fig. 1, lane 4) was tested for its ability to bind to the NaOH-stripped plasma membrane fraction. The myoB present in the enriched fraction was capable of binding to intact plasma membranes, NaOH-stripped plasma membranes, and extracted plasma membrane lipids

(Fig. 2B, C, D). These results indicate that the purification procedure employed to obtain an enriched fraction of myoB removes the factor present in the cytosol that prevents myoB from binding to NaOH-stripped membranes and that, as has been reported for other amoeboid class I myosins [13,14], myoB is capable of binding directly to lipids.

The potential association of cytosolic myoB with a protein that might inhibit membrane binding was examined by performing cross-linking of this fraction. The cytosol was incubated for increasing lengths of time with EDAC, a zero-length chemical cross-linker. Following a 20 min incubation with EDAC, several higher molecular weight bands of 135, 140 and 150 kDa were observed when the samples were immunoblotted for myoB (Fig. 3A). Interestingly, when the partially purified myoB was treated similarly, only a single 125 kDa band was observed (Fig. 3B). This suggests that a protein(s) is associated with cytosolic myoB, but not the partially purified form. Since EDAC cross-links an amino residue directly to a carboxyl residue, proteins that can be cross-linked by EDAC must have close, specific interactions. The cross-linking data suggest that cytosolic myoB may form a specific complex with at least two small proteins (10 kDa and 15 kDa) and these proteins may be readily detached from myoB during purification (Fig. 4). The size of the cross-linked products is consistent with the likely size of a myoB light chain. The myoB heavy chain does have a potential light chain binding site [4], as is typical of most myosins. However, purified myoB was not found to have an associated light chain [22]. If myoB does have an associated light chain, then it might have an extremely loose association with the myoB heavy chain as it appears to be readily removed using two different types of purification protocols [22]. This would be highly unusual for a non-calmodulin light chain, as those myosin light chains appear to be tightly associated.

The results presented in this paper allow us to propose a dual mechanism for regulating the membrane association of *Dictyostelium* myoB in vivo (Fig. 4). We speculate that myoB is prevented from binding to the stripped plasma membrane by association with a cytosolic factor (CF). This could be a low molecular weight protein molecule (Fig. 3A), possibly even the myoB light chain itself. Given the high affinity of the myosin I polybasic domain for the plasma membrane

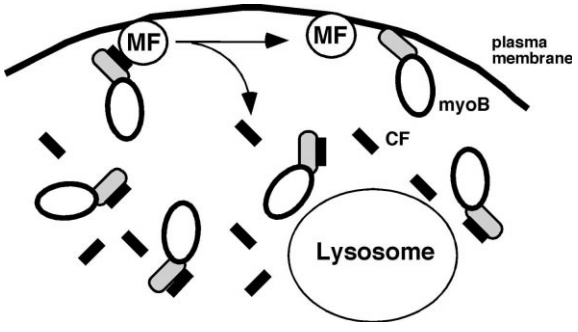

Fig. 4. A speculative model describing how myoB binding to the plasma membrane is regulated. Cytosolic myoB associated with a factor (CF) that inhibits its binding to membranes. Note that an excess of CF is postulated. Specific membrane binding occurs when the myoB/CF complex comes in contact with the plasma membrane that contains a regulatory membrane protein (referred to here as membrane factor, MF). Interaction of the CF with MF catalyzes the release of the CF and permits binding of myoB to the plasma membrane. If the myoB/CF complex comes into contact with membranes lacking MF, such as those of a lysosome, it would not be capable of binding to the membrane.

[13,14], it is likely that the CF binds to this region of myoB tail in order to prevent its interaction with the plasma membrane. Under appropriate conditions, myoB is capable of binding to the target membrane (i.e. the plasma membrane) only if a regulatory peripheral membrane protein is present. The regulatory peripheral protein is unlikely to be a receptor protein, based on the observation that myoB, as well as other myosin Is [13,14,20,21], can bind directly to stripped membranes and lipid vesicles (Fig. 2C, D). The regulatory protein is likely to play a role in the release of myoB from the CF, permitting the binding of the polybasic domain to the plasma membrane. Lack of this membrane-associated regulatory factor in other cellular membranes, such as the contractile vacuole or lysosomes, would prevent myosin I from binding to these membranes and becoming non-specifically distributed throughout the cell. Thus, the dual mechanism proposed here can account for the large proportion of observed soluble myosin I in *Dictyostelium* and the specificity of myosin I membrane binding in vivo.

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