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Examination of the endosomal and lysosomal pathways in *Dictyostelium discoideum* myosin I mutants

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SUMMARY

The role of myosin Is in endosomal trafficking and the lysosomal system was investigated in a *Dictyostelium discoideum* myosin I double mutant *myoB*⁻/*C*⁻, that has been previously shown to exhibit defects in fluid-phase endocytosis during growth in suspension culture (Novak et al., 1995). Various properties of the endosomal pathway in the *myoB*⁻/*C*⁻ double mutant as well as in the *myoB*⁻ and *myoC*⁻ single mutants, including intravesicular pH, and intracellular retention time and exocytosis of a fluid phase marker, were found to be indistinguishable from wild-type parental cells. The intimate connection between the contractile vacuole complex and the endocytic pathway in *Dictyostelium*, and the localization of a myosin I to the contractile vacuole in *Acanthamoeba*, led us to also examine the structure and function of this organelle in the three myosin I mutants. No alteration in contractile vacuole structure or function was observed in the *myoB*⁻, *myoC*⁻ or *myoB*⁻/*C*⁻ cell lines. The transport, processing, and localization of a lysosomal enzyme, α -mannosidase, were also unaltered in all three mutants. However, the *myoB*⁻ and *myoB*⁻/*C*⁻ cell

lines, but not the *myoC*⁻ cell line, were found to oversecrete the lysosomal enzymes α -mannosidase and acid phosphatase, during growth and starvation. None of the mutants oversecreted proteins following the constitutive secretory pathway. Two additional myosin I mutants, *myoA*⁻ and *myoA*⁻/*B*⁻, were also found to oversecrete the lysosomally localized enzymes α -mannosidase and acid phosphatase. Taken together, these results suggest that these myosins do not play a role in the intracellular movement of vesicles, but that they may participate in controlling events that occur at the actin-rich cortical region of the cell. While no direct evidence has been found for the association of myosin Is with lysosomes, we predict that the integrity of the lysosomal system is tied to the fidelity of the actin cortex, and changes in cortical organization could influence lysosomal-related membrane events such as internalization or transit of vesicles to the cell surface.

Key words: Myosin(s), *Dictyostelium discoideum*, Endosome, Lysosome

INTRODUCTION

Lysosomes exist in virtually all eukaryotic cells and consist of membrane-delimited vesicles with acidic lumens containing hydrolytic enzymes (reviewed by Holtzman, 1989). These organelles function to degrade macromolecules brought into the cell by endocytosis (heterophagy) and to degrade and aid in the turnover of cellular components (autophagy). There are two routes by which macromolecules can reach lysosomes: (1) the biosynthetic pathway, which targets newly synthesized lysosomal hydrolases to lysosomes; and (2) the endocytic pathway, by which material from the cell exterior can be brought to lysosomes.

The biosynthetic pathway for lysosomal hydrolases has been extensively characterized in *Dictyostelium discoideum* (reviewed by Cardelli, 1993). Newly synthesized precursors of lysosomal enzymes are phosphorylated and sulfated on N-linked oligosaccharide side-chains in the Golgi, proteolytically cleaved in a late Golgi/endosomal compartment and then trans-

ported to dense secondary lysosomes where processing is completed. Subsequent enzyme secretion from lysosomes is regulated, with significant secretion of processed enzymes occurring following the onset of starvation, a process that also triggers *D. discoideum* development.

D. discoideum exhibits robust endocytosis in liquid culture and this pathway has also been characterized. During endocytosis, fluid phase markers are ingested by clathrin-coated vesicles (O'Halloran and Anderson, 1992; Ruscetti et al., 1994), which then enter larger acidic lysosome-like vesicles (Padh et al., 1993; Aubry et al., 1993). Finally, these markers enter a larger non-acidic post-lysosome prior to their egestion (Padh et al., 1993; Aubry et al., 1993). The contractile vacuole system of *D. discoideum* appears to be intimately tied to the endocytic pathway, as mutants lacking the clathrin heavy chain are endocytically defective and lack contractile vacuoles (O'Halloran and Anderson, 1992; Ruscetti et al., 1994). Also, *D. discoideum* cells lines overexpressing a dominant negative mutant form of Rab4, a small molecular mass GTPase residing

in the membranes of both the lysosomal and contractile vacuole systems, are altered in both endocytosis and osmoregulation (Bush et al., unpublished data).

The transport of material to and from lysosomes requires the proper movement and fusion of membrane vesicles, and the transit of these membrane-bound organelles is believed to be powered, in large part, by motor proteins. The association of the microtubule-based motors, cytoplasmic dynein and kinesin, with a variety of intracellular organelles has been well-documented, and these proteins are believed to provide organelles with a means of moving along polarized arrays of microtubules in either direction (Hollenbeck and Swanson, 1990; Aniento et al., 1993; Skoufias et al., 1994). Transport of endosomal or lysosomal organelles has been shown to involve cytoplasmic dynein and kinesin, respectively (Hollenbeck and Swanson, 1990; Aniento et al., 1993). The association of actin-based motors with membrane-bound organelles has been observed by immunolocalization, although less is known about their role in the transport of intracellular vesicles (Baines et al., 1992; Wagner et al., 1992; Yonemura and Pollard, 1992; Conrad et al., 1993; D'Andrea et al., 1994). Accumulating evidence suggests that vesicles may possess both actin-based and microtubule-based motors that would enable them to traverse both filament systems. Vesicles derived from squid axoplasm have been observed to move along microtubule tracks and then switch to microfilaments (Kuznetsov et al., 1992), and Golgi-derived vesicles from intestinal crypt cells have been shown to possess both myosin I and cytoplasmic dynein (Fath and Burgess, 1993; Fath et al., 1994).

Dictyostelium strains lacking one of two myosin Is, either myoA or myoB, were found to have decreased rates of intracellular particle movement (Wessels et al., 1991; Titus et al., 1993), suggesting that these motors may play a role in vesicle movement. The family of *Dictyostelium* myosin Is, myoA-F, are homologous to their well-studied *Acanthamoeba* counterparts in both their overall structure and biochemical properties (Pollard et al., 1992; Lee and Cote, 1993). They have a highly conserved N-terminal head domain that is followed by a polybasic region that has been shown to bind directly to anionic phospholipids (Adams and Pollard, 1989; Miyata et al., 1989). In myoB, C and D, the C-terminal polybasic region is followed by a domain rich in glycine, proline and alanine (GPA) and contains a src-homology 3 domain (SH3) (Jung et al., 1989, 1993; Peterson et al., 1995). The other myosin Is, myoA, E and F, do not possess GPA and SH3 domains (Titus et al., 1989, 1995; Urrutia et al., 1993). The presence of a membrane binding domain in all of these myosins suggests that they may be able to move membranes and vesicles along actin filaments. Additional support for the role of *Dictyostelium* myosin Is in intracellular transport comes from studies of two myosin I double mutants, *myoA⁻B⁻* and *myoB⁻C⁻* (Novak et al., 1995). These two mutant strains exhibited a defect in pinocytosis; the double mutants grown in suspension culture were significantly impaired in their ability to take up fluid from the medium. Moreover, the double mutants exhibited an abnormal intracellular vesicle profile, as determined by thin-section electron microscopy. The original analysis of these mutants did not reveal whether the defect observed was in the initial stages of pinocytosis or in later steps along the endocytic pathway on route to lysosomes. Here we describe the characterization of various aspects of the endosomal and lysosomal pathway in both *Dictyostelium* myosin I single and double mutants, *myoA⁻*, *myoB⁻*, *myoC⁻*, *myoA⁻B⁻*, and

myoB⁻C⁻, in an effort to determine the role of these myosin Is in intracellular vesicle movement.

MATERIALS AND METHODS

Strains and culture conditions

The generation of stable *Dictyostelium discoideum* myosin I single (*myoA⁻*, *myoB⁻*, *myoC⁻*) and double null mutants (*myoA⁻B⁻*, *myoB⁻C⁻*) cell lines is described elsewhere (Novack et al., 1995). The parental strain, Ax3, and the myosin I single mutants were grown in suspension in HL5 medium with constant shaking at room temperature. The myosin I double mutants were maintained in HL5 medium supplemented with 10 µg/ml G418 (Sigma Chemical Co., St Louis, MO). All assays described below were performed with suspension grown log phase cells.

Measurement of fluid phase traffic

Fluid phase endocytosis and exocytosis were measured according to the methods of Aubry et al. (1993). Log phase cells were harvested by centrifugation (500 g, for 5 minutes), and resuspended at a concentration of 5×10^6 cells/ml in fresh HL5 supplemented with 2 mg/ml FITC-dextran (70,000 M_r; Sigma Chemical Co., St Louis, MO). Endocytosis was allowed to proceed for 2 hours, after which time the cells were harvested, washed twice with HL5 medium, and finally resuspended in fresh HL5 medium to allow release of the fluid phase marker. Exocytosis of FITC-dextran was allowed to proceed for 2 hours. At the times indicated during endocytosis or exocytosis, 1 ml samples (5×10^6 cells) were harvested by centrifugation, washed twice with HL5 medium, once with wash buffer (5 mM glycine, 100 mM sucrose, pH 8.5) and then stored on ice prior to fluorescence measurements.

The cells were lysed by the addition of 0.1 ml of 10% (v/v) Triton X-100 to the pellets and diluted 20× in wash buffer for fluorescence measurements. Fluorescence was measured on a Hitachi (model F-4010) fluorimeter using excitation and emission wavelengths of 492 nm and 525 nm, respectively. The FITC-dextran was diluted to generate a standard curve, and the fluid phase volume taken up by 10^6 cells was calculated. All values were corrected for cellular autofluorescence and surface adhesion of FITC-dextran by subtracting the time zero value.

Cells were exposed to FITC-dextran (10 mg/ml) for 10 minutes (pulse) as described above, and chased in HL5 medium for 60 minutes. The cells were sampled, and fluorescence was measured, at the times indicated as described above to measure intracellular transit of FITC-dextran.

Measurement of endosomal pH

Endosomal pH in living *D. discoideum* amoebae was measured by the dual excitation ratio method with FITC-dextran as a pH probe (Cardelli et al., 1988). Amoebae were loaded with FITC-dextran for 3 hours as described above for measurement of endocytosis. The cells were collected by centrifugation after 3 hours of incubation, washed and resuspended in 50 mM MES buffer (pH 6.5) at a concentration of 5×10^6 cells/ml. The fluorescent excitation intensities (*I*) at 450 nm and 495 nm were determined. The emission wavelength was 520 nm in both cases. The fluorescence excitation ratio at 495 nm and 450 nm ($I_{495\text{nm}}/I_{450\text{nm}}$) was calculated, and endosomal pH determined from an in vitro standard curve of FITC-dextran in a pH range of 4-7.

Standard secretion assays

Log phase cells were harvested by centrifugation and resuspended at a concentration of 5×10^6 cells/ml in either HL5 medium or starvation buffer (10 mM phosphate buffer, pH 6.5). A 1 ml sample (5×10^6 cells) was harvested by centrifugation at the times indicated. Enzyme assays were performed on the supernatant (extracellular) or on the pellets of cells lysed by the addition of 1 ml of 0.5% (v/v) Triton X-100 (intracellular). The α-mannosidase and acid phos-

phatase activity was measured as previously described (Bennet and Dimond, 1986; Free and Loomis, 1974).

Pulse chase analysis and immunoprecipitation

Exponentially growing cells were harvested by centrifugation (500g for 5 minutes), resuspended at a concentration of 10^7 cells/ml in fresh HL5 medium supplemented with 500–800 μ Ci of [35 S]methionine (NEN) to label proteins. Cells were harvested by centrifugation after incubation at 21°C, for 40 minutes (pulse), resuspended in unlabeled medium, and then incubated at room temperature for 4 hours (chase). A total of 10^7 cells were harvested by centrifugation at the times indicated during the chase, and resuspended in 0.5% Triton X-100. The supernatants (media) over the cell pellets were also preserved for analysis. Total protein from cellular and supernatant samples were separated by SDS-PAGE (Laemmli, 1970) and visualized by fluorography or immunoprecipitated (see below) with a specific monoclonal antibody, 2H9 (Mierendorf and Dimond, 1983), that recognized α -mannosidase.

For immunoprecipitation, the cell and media samples were adjusted to a final concentration of 1 \times C buffer (5 mM Na₂EDTA, 150 mM NaCl, 50 mM Tris-base, 0.5% NP-40, 2 mM methionine, 1 mM NaN₃) and incubated on ice for 30 minutes with 0.1 volume of Pansorbin (Calbiochem, San Diego, CA). Samples were centrifuged at 12,000 g for 3 minutes to pellet the Pansorbin and remove non-specifically bound proteins, and the resulting supernatants were incubated on ice for 1.5–2 hours with an excess of monoclonal antibody against α -mannosidase (Mierendorf and Dimond, 1983). Excess Pansorbin was then added and incubation continued for an additional 60–90 minutes, after which time the immunoprecipitated proteins were collected by centrifugation, washed three times with 1 \times C buffer, and resuspended in SDS-PAGE sample buffer (Laemmli, 1970). The proteins were separated by SDS-PAGE and visualized by fluorography.

Immunofluorescence cell staining

Immunofluorescence cell staining was performed using specific monoclonal antibodies recognizing α -mannosidase (Mierendorf and Dimond, 1983), the 100 kDa V-H⁺-ATPase subunit (kind gift from Dr K. Nolte), calmodulin (kind gift from Dr M. Clarke) or polyclonal antibodies reactive to *D. discoideum* RabD (Bush et al., 1994; Bush and Cardelli, 1995). Polyclonal antibodies recognizing RabD were generated by immunizing New Zealand male rabbits (Cocalico Biologicals, Inc.) with bacterially expressed (recombinant) antigen (Bush et al., 1994; Bush and Cardelli, 1995). Specific antibodies used in this study were affinity purified using a column containing the recombinant RabD protein coupled to CNBr-activated Sepharose (Pharmacia). The antibodies were specific for RabD and cross-reacted negligibly with other Rab proteins.

Visualization of lysosomally localized α -mannosidase was performed on cells fixed onto glass coverslips in a solution of 3.7% formaldehyde in phosphate buffered saline (PBS). The 100 kDa V-H⁺-ATPase subunit, RabD, and calmodulin were visualized in cells fixed onto glass coverslips in 2% formaldehyde in PBS containing one-third strength HL5 growth medium and 0.1% DMSO for 5 minutes at room temperature followed by incubation for 5 minutes at –20°C in 1% formaldehyde in 100% methanol. The cells were washed with PBS and then stained with primary antibody followed by secondary antibody as described previously in all cases following fixation (Bush and Cardelli, 1989; Bush et al., 1994).

Statistical analysis

ANOVA were performed using the computer program GraphPAD InStat® (Version 1.12a, IBM®).

RESULTS

The role of *myoB* and *myoC* gene products in endocytosis

The role of members of the myosin I family of actin-based

motors in the endosomal-lysosomal system of *D. discoideum* was examined in stable single (*myoB*[–], *myoC*[–]) and double null mutant (*myoB*^{–/C}[–]) cell lines established by targeted gene disruption (Peterson *et al.*, 1995; Novak *et al.*, 1995). It was observed here, and in a previous study (Novak *et al.*, 1995), that the double mutant cell line *myoB*^{–/C}[–], but not the *myoB*[–] or *myoC*[–] single mutants, grew more slowly in axenic shaking culture than the parental wild-type cell line. Axenic cell lines have been shown to grow as a result of active fluid phase endocytosis, so it was likely that the slowed growth rate in the myosin I double mutants was due to a defect in this process. This possibility was examined using fluorescein-labeled dextran to measure the ability of the wild-type parental, and single and double myosin I mutants, to take up fluid from the surrounding media.

Consistent with previous findings, cell lines in which both the *myoB* and *myoC* genes were disrupted demonstrated an endocytosis rate in suspension that was approximately 50% slower than either the parental wild-type or single mutant cell lines (Fig. 1A). For instance, the initial rate of fluid phase uptake measured over 40 minutes was 0.009 μ l of medium/ 10^6 cells per minute for the wild-type parental, *myoB*[–], and *myoC*[–] cell lines and 0.004 μ l of medium/ 10^6 cells per minute for the *myoB*^{–/C}[–] cell line. Analysis of variance demonstrated that the reduction in the rate of uptake by the *myoB*^{–/C}[–] cell line, was statistically significant ($P < 0.01$, $n = 6$). Similar results have been found for another *Dictyostelium* myosin I double mutant, *myoA*^{–/B}[–] (Novak *et al.*, 1995). These results suggest that these myosin Is participate in a common process that is required for internalization of fluid.

Other stages of fluid phase movement, such as intracellular transit time or exocytosis rates of fluid phase markers, were also examined in the myosin I single and double mutants. Exocytosis was examined in wild-type parental and mutant cells that had been allowed to take up FITC-dextran for 2 hours, washed free of the marker, and resuspended in fresh medium. The release of the fluid phase marker was measured by monitoring the decrease in intracellular fluorescence. It was observed that the rate of fluid phase exocytosis was identical for all cell lines (Fig. 1B).

The intracellular fluid phase transit times were measured in cells that were allowed to take up FITC-dextran for 10 minutes (pulse) and then chased in fresh medium for 60 minutes. Cellular retention times for the fluid phase marker were monitored by measuring intracellular fluorescence. The average intracellular transit time (t_{lag}) of a fluid phase marker, prior to its release from the cell into the extracellular medium, was 33.3 minutes, 32.5 minutes, 35.0 minutes, and 30.0 minutes for the wild-type parental, *myoB*[–], *myoC*[–] and *myoB*^{–/C}[–] cell lines, respectively (Fig. 1C). Analysis of variance demonstrated that there was no significant difference among t_{lag} for any of the cell lines. Taken together these results suggest that in *Dictyostelium* *myoB* and *myoC* are not required for intracellular movement or release of fluid phase, and may influence only early stages of endocytosis.

Mutants defective in acidification of their endosomes may also display endocytic defects (Bof *et al.*, 1992); therefore, the average pH along the endocytic pathway of the parental, single and double myosin I mutant cell lines was measured using the dual-excitation ratio method with FITC-dextran as a pH probe (see Materials and Methods). The average endosomal pH was

approximately 5.5 (data not shown) in all cell lines examined, suggesting that *myoB* and *myoC* do not play a role in the maintenance of intra-endosomal acidity.

The role of the *myoB* and *myoC* gene products in sorting of lysosomal enzymes

The role of the *myoB* or *myoC* gene products in the processing, sorting, and secretion of lysosomal enzyme, α -mannosidase, in *Dictyostelium* was examined by carrying out radiolabel pulse-chase experiments with the wild-type parental, myosin I single and double mutant cell lines. The biosynthetic pathway for α -mannosidase has been elucidated (Cardelli et al., 1990a,b). α -Mannosidase is synthesized as a 140 kDa precursor and a small fraction of this precursor escapes further processing and is secreted constitutively (Cardelli et al., 1986;

Mierendorf et al., 1985). The remainder of this protein is proteolytically processed to an 80 kDa intermediate form and a 58 kDa mature subunit in a late Golgi compartment or early endosomes. We and others have shown that the 80 kDa intermediate is then further processed to a 60 kDa mature subunit in the lysosomes where, together with the 58 kDa subunit, it forms a soluble multimeric holoenzyme (Wood and Kaplan, 1985; Mierendorf et al., 1985; Richardson et al., 1988).

Log phase cultures of wild-type Ax3, *myoB*⁻, *myoC*⁻ or *myoB*⁻/*C*⁻ strains were metabolically labeled for 40 minutes with [³⁵S]methionine (pulse), and chased in unlabeled growth medium for 4 hours. Samples were taken at 0, 10, 30, 60, 120, 240 minutes of chase, and separated by centrifugation into cellular and supernatant (extracellular) fractions. α -Mannosidase was immunoprecipitated from each sample and analyzed by SDS-PAGE and fluorography (Fig. 2). A radiolabeled 140 kDa precursor of α -mannosidase was detected in wild-type cells, as well as in the single and double mutants of *myoB* and *myoC*. Negligible levels of this precursor were found extracellularly, at late chase time points, in the wild-type parental strain and in either the *myoB* or *myoC* single or double mutants, suggesting that *myoB* and *myoC* did not influence lysosomal enzyme sorting.

The kinetics of proteolytic processing of this protein was similar in all strains examined. Correct processing of the precursor to its 58 kDa and 60 kDa mature subunits was evident by 10 minutes of the chase period, and was essentially complete by 60 minutes of the chase. The half time of processing for all strains was approximately 15-20 minutes. The cleavage of the 80 kDa intermediate polypeptide into the 60 kDa subunit occurs in lysosomes (Wood and Kaplan, 1985; Mierendorf et al., 1985; Richardson et al., 1988) and, since the myosin mutants were capable of this final processing event, we suggest that α -mannosidase was correctly localized to lysosomes in these cell lines.

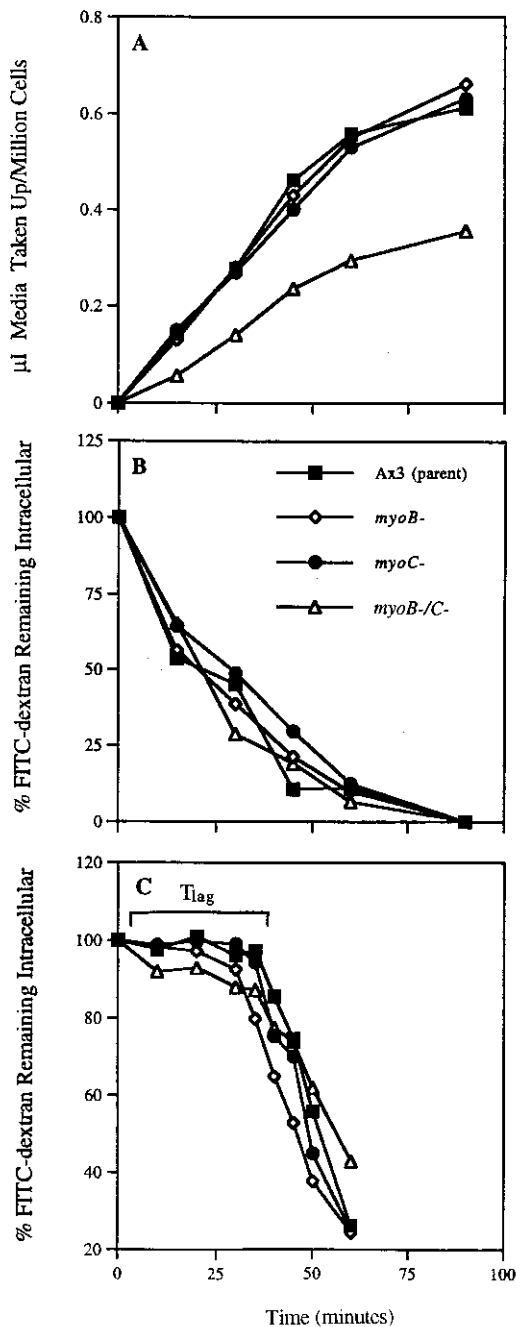


Fig. 1. The role of the *myoB* and *myoC* gene products in fluid phase traffic. (A) To examine endocytosis, wild-type parental (Ax3) (■), *myoB*⁻ (◇), *myoC*⁻ (●) and *myoB*⁻/*C*⁻ (△) cell lines were incubated with 2 mg/ml FITC-dextran. Intracellular fluorescence was measured at the time points indicated, and the volume of fluid phase internalized per 10⁶ cells was plotted. The initial rate of fluid phase uptake measured over 40 minutes was 0.009±0.003 μ l of medium/10⁶ cells per minute (mean \pm s.d., $n=10$), 0.009±0.002 μ l of medium/10⁶ cells per minute (mean \pm s.d., $n=2$), 0.009±0.003 μ l of medium/10⁶ cells per minute (mean \pm s.d., $n=2$), and 0.004±0.001 μ l of medium/10⁶ cells per minute (mean \pm s.d., $n=6$) for the wild-type parental, *myoB*⁻, *myoC*⁻ and *myoB*⁻/*C*⁻ cell lines, respectively. (B) To examine exocytosis, wild-type and mutant cells were incubated with 2 mg/ml FITC-dextran for 2 hours, washed twice with fresh HL5, and incubated in fresh HL5 for the time points indicated. Intracellular fluorescence was measured and the percentage of the total FITC-dextran remaining in the cells was plotted. The data correspond to the mean \pm s.d. ($n=3$) for all cell lines. (C) To examine intracellular retention time, wild-type and mutant cells were pulsed with 10 mg/ml FITC-dextran for 10 minutes, washed twice with fresh HL5, and chased in fresh HL5 for the time points indicated. Intracellular fluorescence was measured and the percentage of the total FITC-dextran remaining in the cells was plotted. The average intracellular transit time (t_{lag}) was 33.3±2.9 minutes (mean \pm s.d., $n=3$), 32.5±3.5 minutes (mean \pm s.d., $n=2$), 35.0±7.0 minutes (mean \pm s.d., $n=2$), and 30.0±7.0 minutes (mean \pm s.d., $n=4$) for the wild-type parental, *myoB*⁻, *myoC*⁻ and *myoB*⁻/*C*⁻ cell lines, respectively.

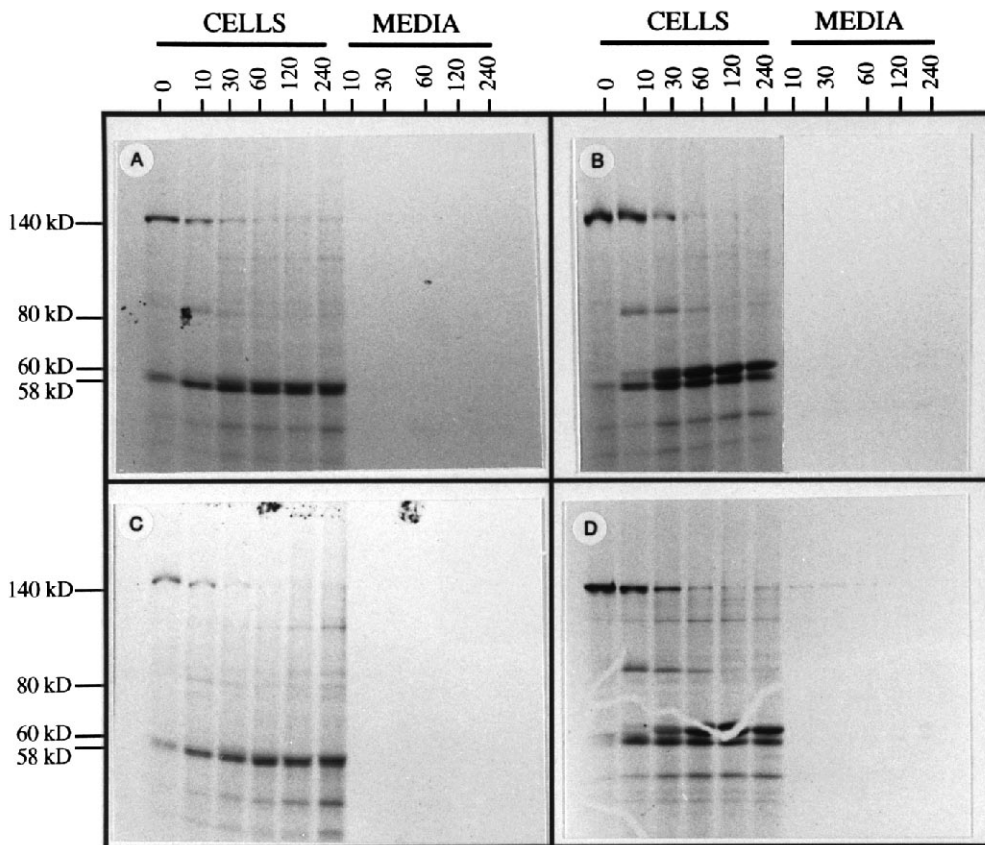


Fig. 2. Pulse-chase analysis of and immunoprecipitation of lysosomal α -mannosidase from wild-type parental (Ax3) (A), $myoB^{-}$ (B), $myoC^{-}$ (C) and $myoB^{-}/C^{-}$ (D) cell lines. Growing cells were pulsed with [^{35}S]methionine in HL5 medium for 40 minutes and chased for the times indicated in unlabeled medium. Each sample was incubated with monoclonal antibodies specific for α -mannosidase. SDS-PAGE and fluorography were used to separate and visualize the immunoprecipitated proteins.

Indirect immunofluorescence cell staining using α -mannosidase reactive antibodies was performed to examine further the subcellular localization of intracellularly retained α -mannosidase. In all cell lines examined, α -mannosidase was localized to punctate structures, reminiscent of lysosomally localized antigens (Fig. 3). This observation is consistent with the proper processing of α -mannosidase observed in the pulse-chases analysis described above (Fig. 2).

The role of myosin Is in the secretion of lysosomal enzymes

The role of myosin Is in the secretion of mature lysosomal enzymes was also examined by determining the steady state intracellular and extracellular distribution of two lysosomal enzymes, α -mannosidase and acid phosphatase. Log phase cells were collected by centrifugation and the enzyme activity was measured in the cells as well as in the supernatants (extracellular). The extracellular (secreted) enzyme activity was expressed as a percent of total enzyme activity. Less than 10% of the total α -mannosidase or acid phosphatase activity was found extracellularly in the Ax3 and $myoC^{-}$ cell lines (Fig. 4A). In contrast, approximately 30% of the total α -mannosidase and 35% of the total acid phosphatase activity accumulated in the media of the $myoB^{-}$ single or double mutants (Fig. 4A). Analysis of variance demonstrated that the accumulation of lysosomal enzymes in the media of the $myoB^{-}$ single or double mutants was significantly higher than that of the wild-type parental and $myoC^{-}$ cell lines ($P < 0.05$, $n = 3$). These results suggest that myoB plays a role in secretion of lysosomal enzymes from *D. discoideum* amoebae.

The phenotype of another myosin I mutant, $myoA^{-}$, is

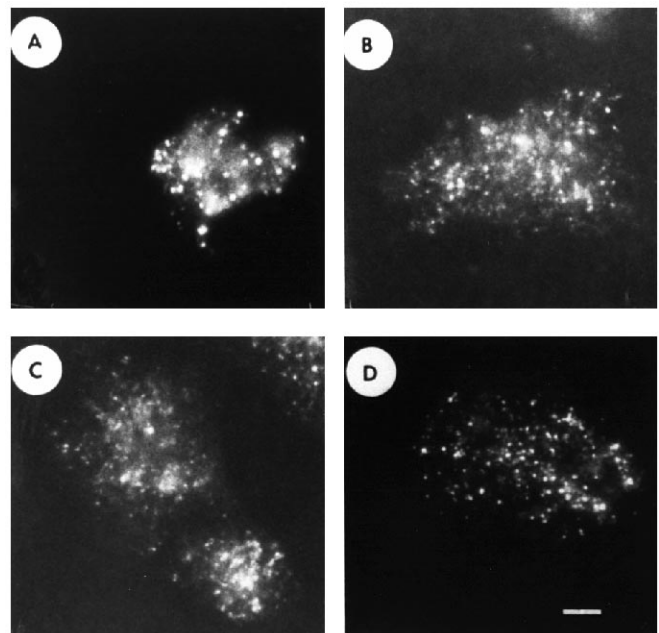


Fig. 3. Subcellular localization of α -mannosidase in wild-type parental (Ax3) (A), $myoB^{-}$ (B), $myoC^{-}$ (C) and $myoB^{-}/C^{-}$ (D) cell lines by immunofluorescence cell staining. Growing cells were allowed to adhere to coverslips, fixed with 3.7% formaldehyde in PBS, and permeabilized with saponin. Murine monoclonal antibodies specific for α -mannosidase were incubated with the fixed cells and the bound antibodies were visualized using FITC-conjugated anti-mouse antibodies. The antibodies predominantly decorated distinct punctate structures in all cells lines examined, indicating that α -mannosidase correctly localized to lysosomes. Bar, 5 μm .

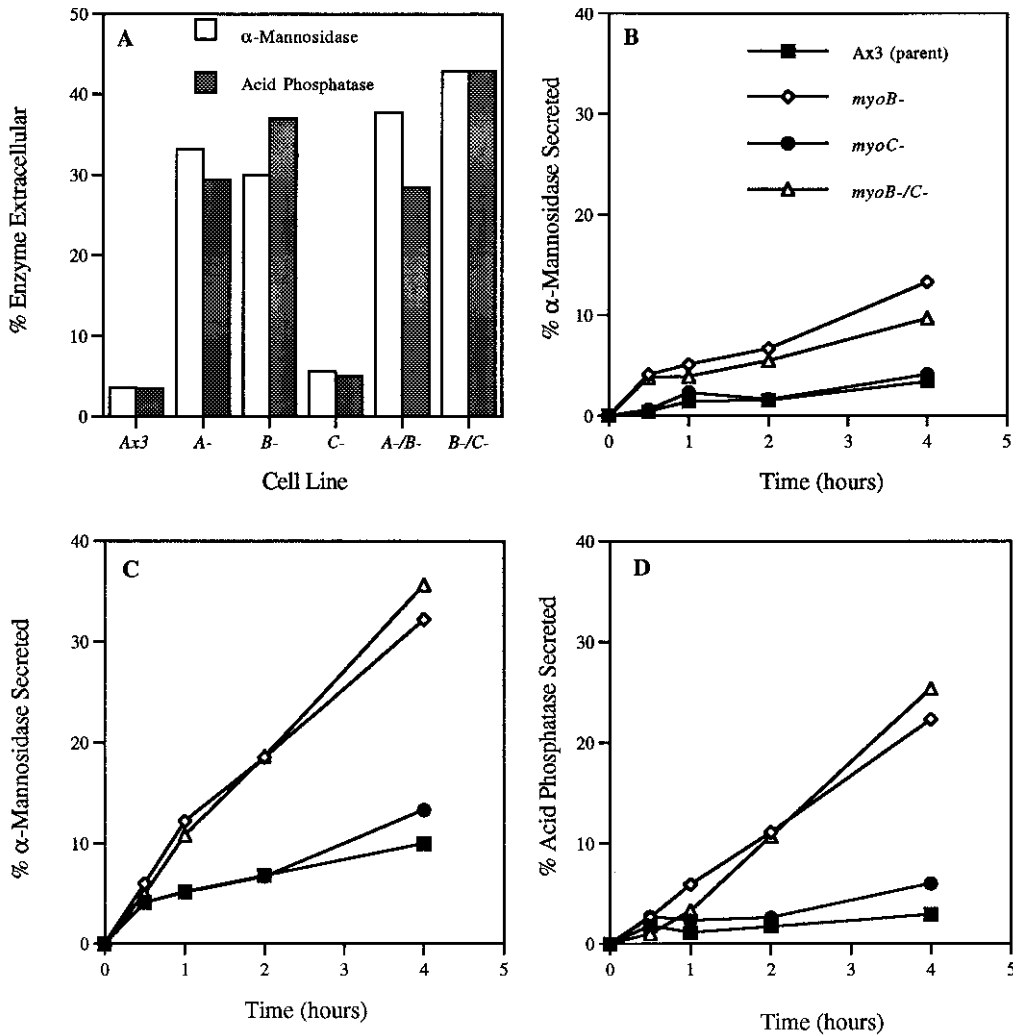


Fig. 4. Secretion of lysosomal enzymes from wild-type parental (Ax3) (\blacksquare), *myoB*⁻ (\diamond), *myoC*⁻ (\bullet), *myoB*⁻/*C*⁻ (\triangle), *myoA*⁻ and *myoA*⁻/*B*⁻ cell lines. The data correspond to the average of at least 2 independent experiments for all cell lines. (A) Logarithmically growing cells were separated into cellular and media fractions by centrifugation, lysed with Triton X-100, and each fraction was assayed for α -mannosidase or acid phosphatase activity. (B) Medium was removed from growing cells and replaced with fresh medium (growth conditions). Samples were removed at the times indicated, the cells were pelleted and solubilized, and both the cell samples and supernatants were assayed for α -mannosidase activity. (C,D) Medium was removed from growing cells and replaced with 10 mM phosphate buffer (starvation conditions). Samples were removed at the times indicated, the cells were pelleted and solubilized, and both the cell samples and supernatants were assayed for α -mannosidase (C) or acid phosphatase (D) activities. The *myoA*⁻, *myoB*⁻, *myoA*⁻/*B*⁻, and *myoB*⁻/*C*⁻ cell lines were found to oversecrete the lysosomal enzymes α -mannosidase and acid phosphatase, indicating that *myoB* and *myoA* may participate in controlling the secretion of mature lysosomal enzymes.

virtually indistinguishable from that of *myoB*⁻ cells (Titus et al., 1993; Novak et al., 1995). Therefore, we measured secretion in two additional myosin I mutant cell lines, *myoA*⁻ and *myoA*⁻/*B*⁻. Like the *myoB*⁻ cell line, *myoA*⁻ mutants were found to oversecrete α -mannosidase and acid phosphatase as compared to control cells (Fig. 4A). The extent of the over-secretion defect in *myoA*⁻ cells was similar to that in the *myoB*⁻ cells; however, this defect was not additive in a *myoA*⁻/*B*⁻ double mutant (Fig. 4A). Pulse-chase analysis demonstrated normal biosynthesis, processing and transport of α -mannosidase in the *myoA*⁻ and *myoA*⁻/*B*⁻ cell lines (data not shown).

Standard secretion assays were also performed to measure the rate of secretion of hydrolases. Logarithmically growing cells were harvested and resuspended in growth medium, and at indicated time points the lysosomal enzyme activity was measured in both cells and the medium (Fig. 4B). In growth medium, α -mannosidase was secreted from wild-type and *myoC*⁻ cell lines at the same rate (approximately 4% of the total enzyme activity was found in the extracellular medium at 4 hours) (Fig. 4B). While the *myoB*⁻ single and double mutants exhibited similar rates of α -mannosidase secretion into growth medium relative to each other, these rates were significantly higher (3- to 5-fold) than those of either the Ax3 or *myoC*⁻ cells ($P < 0.05$, $n = 3$) (Fig. 4B).

Starvation initiates the developmental cycle in *Dic-*

tyostelium, which is, in part, characterized by induced secretion of lysosomal hydrolases relative to secretion during growth (Cardelli, 1993). This response was examined in the myosin I mutants by performing standard secretion assays, as described above, using cells that had been resuspended in starvation buffer. During starvation the wild-type and *myoC*⁻ cell lines secreted α -mannosidase at levels 2-fold greater than those observed during growth (compare Fig. 4B and C). Similarly, the *myoB*⁻ and the *myoB*⁻/*C*⁻ cell lines responded to starvation by the induced secretion of α -mannosidase and, as observed for growth conditions, these rates were significantly higher (3-fold) than those of either the Ax3 or *myoC*⁻ cell lines ($P < 0.05$, $n = 3$) (Fig. 4C). To determine if the oversecretion exhibited by cells with a disrupted *myoB* gene was specific for α -mannosidase, the secretion of another lysosomal enzyme, acid phosphatase, was examined during starvation. The *myoB*⁻ single and double mutants also secreted acid phosphatase at a significantly higher rate (3- to 4-fold) than either the Ax3 or *myoC*⁻ cell lines ($P < 0.05$, $n = 3$) (Fig. 4D), indicating that the oversecretion defect was more general and not specific for α -mannosidase. Finally, the *myoA*⁻ and *myoA*⁻/*B*⁻ cell lines also oversecreted hydrolases during starvation (data not shown).

The stability of lysosomal enzymes was not altered in the myosin mutants; the total amount of enzyme activity (intracellular and extracellular), as measured in the secretion assays

described above, was identical in all of the cell lines examined (data not shown). The lysosomal enzyme oversecretion phenotype for the *myoB* and *myoA* null cell lines was not the result of cell lysis as the cells remained viable as determined by dye exclusion assays. The oversecretion defect was dramatic, as measured in the secretion assays; however, it was not apparent in the radiolabel pulse-chase experiments described above (Fig. 2). This is not surprising; lysosomal enzymes are released continuously and immediately when the cells are placed in fresh unlabeled medium during a radiolabel pulse-chase experiment (and the activity of these enzymes can be detected); however, there is a delay in the appearance of 'labeled' lysosomal enzymes consistent with the notion that lysosomal enzymes must pass through an additional secretory compartment prior to their release (Wood et al., 1983). When longer chase times were examined, the oversecretion defect for the *myoB* and *myoA* cell lines was evident (data not shown).

To gain insight into the specificity of the secretion defect in *myoB*-deficient cell lines, radiolabel pulse-chase experiments were carried out as described above; however, in this instance, total extracellular protein was examined. Log phase cultures of wild-type Ax3, *myoB*⁻, *myoC*⁻ or *myoB*⁻/*C*⁻ strains were metabolically labeled for 40 minutes with [³⁵S]methionine (pulse), and chased in unlabeled growth medium for 4 hours. Samples were taken at 0, 10, 30, 60, 120, 240 minutes of chase, and separated by centrifugation into cellular and supernatant (extracellular) fractions. Total extracellular protein was analyzed by SDS-PAGE and fluorography (Fig. 5). It was observed that both the levels and the profile of the proteins, secreted into the extracellular milieu, were identical for the wild-type parental (Fig. 5), *myoB*⁻/*C*⁻ (Fig. 5), *myoB*⁻ (data not shown) and *myoC*⁻ (data not shown) cell lines. These data indicate that the oversecretion defect may be specific to the lysosomal compartment as no gross alterations in the constitutive secretion of proteins were observed.

Morphology and function of the contractile vacuole system in myosin mutants

In *Dictyostelium*, there is an intimate connection between the contractile vacuole and the endolysosomal systems. For example, clathrin heavy chain minus (CHC⁻) cells are defective in endocytosis and do not contain a functional contractile vacuole (O'Halloran and Anderson, 1992; Ruscetti et al., 1994). Also, *D. discoideum* cells lines overexpressing a mutant Rab4-like protein are altered in both endocytosis and contractile vacuole function (Bush et al., unpublished data). Finally, myosin I has been localized to the contractile vacuole in *Acanthamoeba* (Baines and Korn, 1990; Baines et al., 1992). Therefore, the morphology of the contractile vacuole system in the myosin mutants was characterized by performing immunofluorescence cell staining to determine the intracellular localization of several CV markers, RabD (Fig. 6), the 100 kDa subunit of the V-H⁺-ATPase (Fig. 6) and calmodulin (data not shown). *Dictyostelium* RabD, a Rab4-like GTPase, has been shown to be associated with lysosomal membranes (Temesvari et al., 1994; Bush et al., 1994), as well as with reticular membranes of the contractile vacuole system (Bush et al., 1994). Likewise, the 100 kDa subunit of the V-H⁺-ATPase and calmodulin have been shown to be associated predominantly with contractile vacuole membranes (Fok et al., 1993), and a smaller percentage of the 100 kDa subunit of the

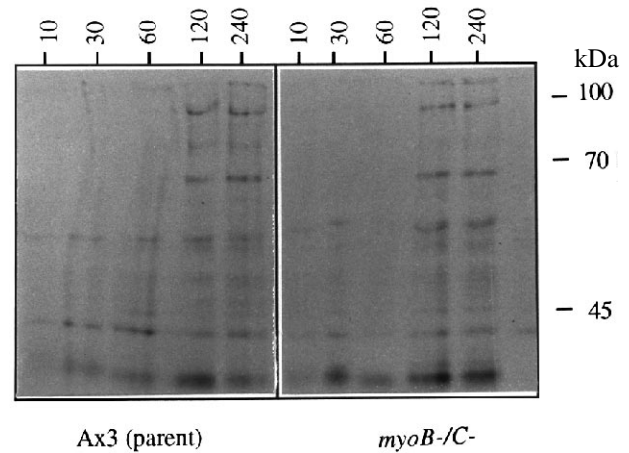


Fig. 5. Pulse-chase analysis of the constitutive secretory pathway of wild-type parental (Ax3) and *myoB*⁻/*C*⁻ cell lines. Growing cells were pulsed with [³⁵S]methionine in HL5 medium for 40 minutes and chased for the times indicated in unlabeled medium. SDS-PAGE and fluorography were used to separate and visualize total extracellular proteins.

V-H⁺-ATPase associates with lysosomal membranes (Temesvari et al., 1994). The V-H⁺-ATPase is likely to function in energy provision for water homeostasis (CV function) and in proton pumping for the acidification of lysosomes.

The cellular morphology of the CV membrane system and the subcellular localization of CV markers remained unchanged in the absence of *myoB* and/or *myoC*, since the RabD, the 100 kDa V-H⁺-ATPase subunit and the calmodulin antigens localized to a tubular-vesicular network in all of the wild-type, single and double mutants cell lines examined (Fig. 6, and data not shown). The staining pattern obtained with these antibodies was similar to that previously reported (Bush et al., 1994; Fok et al., 1993). Staining of the CV bladder membrane with FM-464, a styrene dye that preferentially labels the plasma membrane and CV of *D. discoideum* (Bacon et al., 1994), revealed that there were no structural differences in the CV among the cell lines (data not shown). Finally, the CV system of the myosin mutants appeared to function normally; the cells did not lyse when exposed to hypo-osmotic conditions (data not shown).

DISCUSSION

The finding that *Dictyostelium* myosin I double mutants exhibit conditional defects in fluid phase endocytosis suggested that these motors may play a role in the normal functioning of endosomal and lysosomal trafficking in the cell. However, examination of the endosomal and lysosomal pathways in the *myoB*⁻/*C*⁻ myosin I double mutant, as well as in the *myoB*⁻ and *myoC*⁻ single mutants, reveals that these myosins are not required for the proper intracellular fluid phase retention, fluid phase exocytosis, or lysosomal enzyme processing and sorting. The same results have been found for the suspension-grown *myoA*⁻/*B*⁻ double mutant, another *Dictyostelium* strain that exhibits defects in pinocytosis. It is unlikely, therefore, that these myosin Is serve as motors to power the intracellular

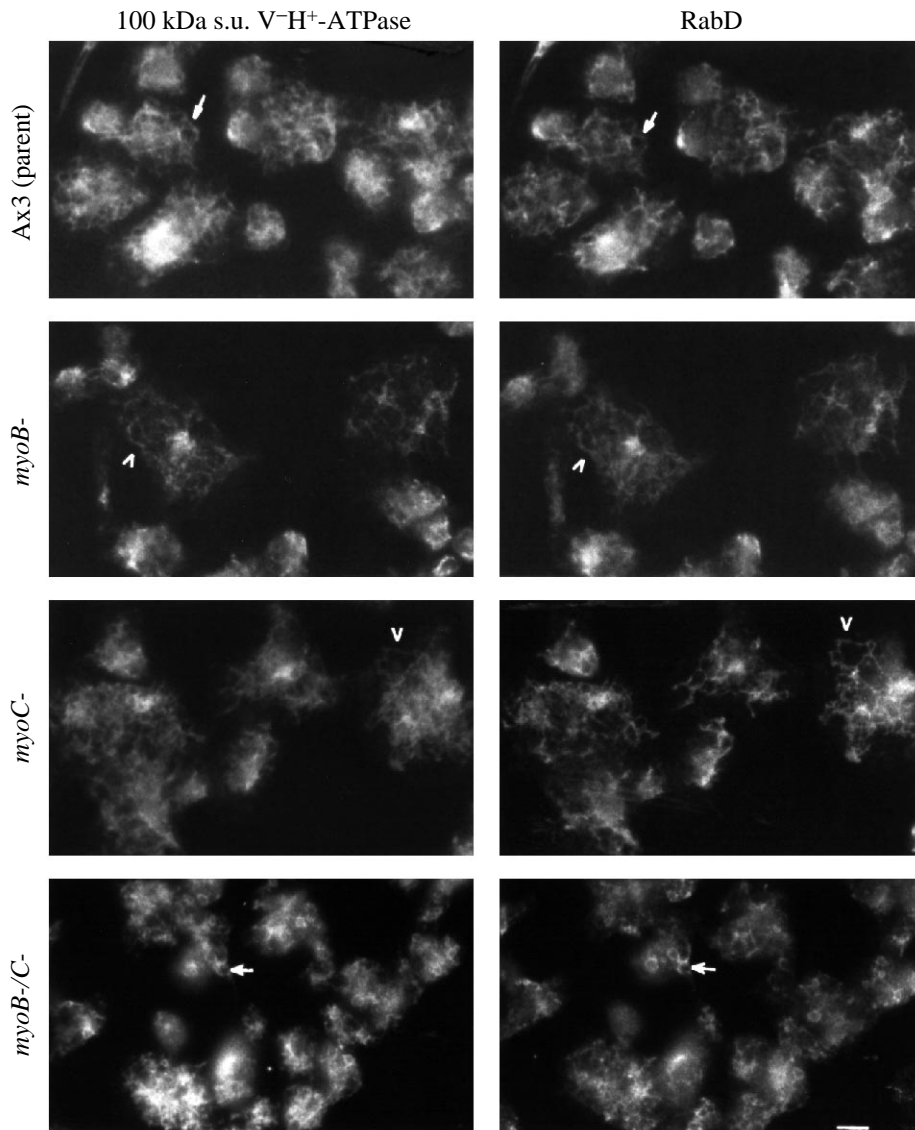


Fig. 6. Subcellular localization of the 100 kDa subunit of the vacuolar H^+ -ATPase ($V-H^+$ -ATPase) and RabD in wild-type parental (Ax3), *myoB*⁻, *myoC*⁻ and *myoB/C*⁻ cell lines by immunofluorescence cell staining. Growing cells were allowed to adhere to coverslips, fixed in 2% formaldehyde in PBS containing one-third strength HL5 growth medium and 0.1% DMSO for 5 minutes at room temperature, followed by incubation for 5 minutes at 20°C in 1% formaldehyde in 100% methanol. The cells were washed with PBS and then stained with primary antibody followed by secondary antibody as described (see Materials and Methods). In all cell lines examined, these antigens localized to a tubular-vesicular network, indicating that the morphology of the contractile vacuole system was unaffected in the myosin I mutants (see the text). Bar, 5 μ m. (→) corresponds to co-localization of the two antigens in vacuoles. (>) corresponds to co-localization of the two antigens in the reticular network of the contractile vacuole system.

movement of endosomes or lysosomes along actin filaments. These findings, coupled with the original analysis of the two myosin I double mutants (Novak et al., 1995), suggest that the endocytic defect is a result of a delay in the internalization of fluids. The internalization step is thought to require the proper interaction between the actin cytoskeleton and plasma membrane, and the engulfment of liquid that results from the retraction of actin-rich membrane extensions most likely requires the action of a motor protein, such as myosin I. Both *myoB* and *myoC* are located at or near the plasma membrane, making them good candidates for powering the actin-based retraction of the membrane.

These findings can be compared with findings on the mutant phenotype observed in the clathrin heavy chain mutant (*CHC*⁻). Both the myosin I double mutant and the clathrin heavy chain mutant have significant defects in pinocytosis, as measured by the uptake of FITC-dextran (O'Halloran and Anderson, 1992; Ruscetti et al., 1994; Novak et al., 1995). The *CHC*⁻ mutants, however, have a much broader spectrum of additional defects, including the mistargetting of the unprocessed form of α -mannosidase (Ruscetti et al., 1994). The clathrin heavy chain, *myoB*

and *myoC* proteins all participate in the early events required for endocytosis, but only clathrin is required for other processes that occur along the endocytic pathway. It is likely that any necessary intracellular trafficking of endosomal and lysosomal vesicles occurs either by microtubule-based motors, as has been found in mammalian cells, or via an uncharacterized myosin (Titus et al., 1994).

The *CHC*⁻ cell lines are also unable to produce a functional contractile vacuole (O'Halloran and Anderson, 1992). In contrast, the *myoA*⁻, *myoB*⁻, *myoC*⁻, and *myoD*⁻ single and double mutant cell lines contained a morphologically normal CV system. Furthermore, the *myoA*, *myoB* and *myoC* null cells did not lyse when suspended in distilled water, suggesting that the CV system is functioning normally to expel water. Myosin Is have been shown to play a role in the CV system of *Acanthamoeba* (Doberstein et al., 1993), and while our data suggest that *myoA*, *myoB* or *myoC* are not critical for the organization and function of the CV, our data cannot rule out the possibility that another one of the *Dictyostelium* myosin Is, such as *myoE* or *myoF*, may function in this system.

The myosin I mutants were found to have an unexpected

defect in the secretion of lysosomal enzymes. The *myoA*⁻, *myoB*⁻, *myoA*⁻/*B*⁻ and *myoB*⁻/*C*⁻ cell lines, but not the *myoC*⁻ cell line, were found to oversecrete the lysosomal enzymes α -mannosidase and acid phosphatase, during growth and starvation (Fig. 4, and data not shown). The secretion of newly synthesized proteins following the constitutive pathway was not altered in the mutants, suggesting that the defect was confined primarily to the lysosomal system. The extent of oversecretion of lysosomal enzymes was similar in the *myoA*⁻ and *myoB*⁻ single mutants, yet not increased in the *myoA*⁻/*B*⁻ double mutant (Fig. 4A). The lack of an additive effect on lysosomal enzyme secretion when both of these myosin Is are removed suggests that these myosin Is may be participating in the same process that regulates lysosomal enzyme secretion. Once either *myoA* or *myoB* is removed, the pathway is disabled and removal of the other myosin I does not result in a further increase in secretion.

Deletion of *myoA*, a gene encoding an essential myosin I, from *Aspergillus nidulans*, results in a significant decrease in the secretion of acid phosphatase (McGoldrick et al., 1995). Coupled with the punctate localization of MYOA to the tips of growing hyphae, these results suggest that this class of amoeboid myosin I is required for the transport of vesicles to the extending hyphal tip (McGoldrick et al., 1995). The differences in the secretory defects observed in the *Dictyostelium* and *Aspergillus* myosin I mutants suggest that the myosin Is are playing a different role in *D. discoideum* than they play in the fungal cells. The greater complexity of cellular movements that are carried out by *Dictyostelium* may be the result of an elaboration of the roles that myosin Is play in these highly motile cells. Alternatively, while *myoA*, B and C are apparently not transporting endosomal or lysosomal vesicles (see below), it is possible that another one of the *Dictyostelium* myosin Is, such as *myoD-F*, might be fulfilling such a role.

There are at least two pathways by which lysosomal enzymes can be exported from *Dictyostelium* cells: the enzymes can be missorted as unprocessed precursors and secreted prior to reaching lysosomes through a default constitutive secretory pathway or lysosomally localized mature enzymes can be exported through post-lysosomes by a regulated mechanism. Disruption of the *myoA* or *myoB* genes had no effect on the constitutive secretory pathway as little or no missorting of unprocessed precursors was observed and the profile of total protein secretion was not altered when examined by radiolabel pulse-chase experiments (Fig. 2, and data not shown). Moreover, the kinetics of processing of the 140 kDa precursor of α -mannosidase to the 58 kDa and 60 kDa mature subunits and the subcellular localization of α -mannosidase also remained unchanged in the mutants. However, there was a significant increase in lysosomal enzyme secretion observed in the *myoB*⁻ cell lines, but not in the *myoC*⁻ cell line during growth and starvation (Fig. 3B, C, D). This increase in secretion of lysosomal enzymes did not result from an increase in efflux of fluid phase markers. A similar oversecretion defect was observed in two other myosin I mutant cell lines, *myoA*⁻ and *myoA*⁻/*B*⁻. These results are consistent with a role for *myoB* and perhaps *myoA*, but not *myoC*, in the secretion of lysosomal enzymes.

The mechanism of lysosomal enzyme secretion and retention is, at present, poorly understood. In one model for an increase in the rate of release of mature lysosomal enzymes but

not fluid phase, *myoB* and *myoA* may normally function to restrict the movement of lysosomal enzyme-containing vesicles towards the plasma membrane. This could be achieved either by tightly controlling the rate of transit of vesicles through the cell or by altering the actin network in such a way that the movement of the lysosomal vesicles through the cortical regions of the cell is impaired and they are prohibited from fusing with the plasma membrane. This model implies that lysosomal enzyme containing compartments are functionally separate from the endosomal system and that there are two populations of vesicles that reach the plasma membrane: vesicles that contain lysosomal enzymes (1-2 μ m in size) and vesicles that carry fluid phase (<1 μ m in size). The smaller vesicles carrying fluid phase are unrestricted (or less restricted) and are free to fuse with the plasma membrane under all conditions of growth or starvation. The movement of vesicles harboring lysosomal enzymes would, in contrast, be restricted; a restriction that is partially removed during starvation.

There are several lines of evidence, however, that support the hypothesis that almost all lysosomal enzymes reside with fluid phase markers in a secondary lysosomal compartment that is functionally connected to the endosomal system and that both lysosomal enzymes and fluid phase markers are released from this compartment. Firstly, biochemical characterization of magnetically purified post-lysosomes, a large neutral compartment from which fluid phase is released into the extracellular medium, demonstrated that this compartment also contained mature lysosomal enzymes (Nolta et al., 1994). Secondly, in density shift experiments, it was demonstrated that greater than 90% of lysosomal enzyme-containing compartments were functionally connected to the endosomal system by their ability to receive material from the extracellular milieu (Cardelli et al., 1990). Finally, Percoll gradient fractionation of organelles, prepared from cells pulse labeled with [³H]dextran and chased for various times indicated that, while fluid phase markers first appeared in low density vesicles, these vesicles increase rapidly in density reaching values equivalent to the density of lysosomal organelles (Ebert et al., 1989). While it is unlikely that two separate populations of vesicles (lysosomal enzyme and fluid phase carriers) exist, our data cannot exclude the possibility that larger portions of, or the entire post-lysosomal compartment, is (are) fusing with the plasma membrane in mutants lacking *myoA* or *myoB*. Fluid phase would continue to exit the cells at a normal rate, enclosed in small vesicles that had budded from the surface of post-lysosomes prior to their fusion with the plasma membrane.

It has been demonstrated here and in previous studies (reviewed by Cardelli, 1993) that the secretion of lysosomal enzymes from the cell is a highly regulated process. At 90 minutes of chase, there is almost complete turnover of fluid phase in the cell (100% of fluid phase marker has been released; Fig. 1B and C); however, less than 30% of total α -mannosidase (Fig. 4B; Ruscetti et al., 1994) or acid phosphatase (Ruscetti et al., 1994) can be found extracellularly in this time period. These results suggest that lysosomal enzymes are selectively retained and/or recycled in order to prevent their continuous release with fluid phase markers. While no direct evidence has been found for the association of *myoB* with lysosomes, either based on immunofluorescence localization (Fukui et al., 1989; J. Bush and J. Cardelli, data not shown) or immunoblotting analysis of sucrose density gradient fractions

(S. Senda and M. Titus, unpublished observation), the over-secretion defect observed in *myoB*⁻ cell lines suggests that *myoB* may play a role in the recycling or retention of lysosomal enzymes. *myoA* may also contribute to this function in *Dictyostelium* cells.

The results of our analyses of the myosin I mutants suggest that these motor proteins play a role in controlling events that occur at the actin-rich cortical regions of the cell. The *myoA* and *myoB* proteins, in particular, are important for controlling the extension of pseudopods (Titus et al., 1993) and the secretion of lysosomal enzymes (this work). The similarity of the *myoA*⁻ and *myoB*⁻ phenotypes suggests that these actin-based motors participate in a common pathway in a non-redundant fashion. The *myoC* protein may be playing a role in a similar pathway that is not uncovered in the *myoC*⁻ single mutant, but is revealed in the *myoB*⁻/*C*⁻ double mutant. The nature of the observed defects including the increased number of pseudopods (Wessels et al., 1991; Titus et al., 1993), over-secretion of lysosomal enzymes found in the single mutants (this work) and the pinocytosis defect found in the double mutants (Novak et al., 1995), provides support for a general role for the myosin is in controlling the state of the actin cortex. Changes in the organization of the cortex or the manner in which the actin is linked to the membrane can profoundly effect the internalization of membrane, the extension of actin-rich projections and the transit of vesicles from within the cell to the plasma membrane. The myosin Is, encoded by the *myoA*, *B* and *C*, genes are all required for *Dictyostelium* cells to properly execute these processes.

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