A Role for a Rab4-like GTPase in Endocytosis and in Regulation of Contractile Vacuole Structure and Function in *Dictyostelium discoideum*

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The small M_r Rab4-like GTPase, RabD, localizes to the endosomal pathway and the contractile vacuole membrane system in *Dictyostelium discoideum*. Stably transformed cell lines overexpressing a dominant negative functioning RabD internalized fluid phase marker at 50% of the rate of wild-type cells. Mutant cells were also slower at recycling internalized fluid. Microscopic and biochemical approaches indicated that the transport of fluid to large postlysosome vacuoles was delayed in mutant cells, resulting in an accumulation in acidic smaller vesicles, probably lysosomes. Also, RabD N121I-expressing cell lines missorted a small but significant percentage of newly synthesized lysosomal α -mannosidase precursor polypeptides. However, the majority of the newly synthesized α -mannosidase was transported with normal kinetics and correctly delivered to lysosomes. Subcellular fractionation and immunofluorescent microscopy indicated that in mutant cells contractile vacuole membrane proteins were associated with compartments morphologically distinct from the normal reticular network. Osmotic tests revealed that the contractile vacuole functioned inefficiently in mutant cells. Our results suggest that RabD regulates membrane traffic along the endosomal pathway, and that this GTPase may play a role in regulating the structure and function of the contractile vacuole system by facilitating communication with the endosomal pathway.

INTRODUCTION

Proteins and lipids can be transported from one intracellular organelle to another by the formation and consumption of vesicles. The regulation of vesicle traffic is therefore critical to ensure the biogenesis and structural integrity of endomembrane compartments. Not surprisingly, genetic and biochemical approaches have identified a large number of proteins that participate in and direct such membrane flow (Rothman, 1994). These proteins function at each of the steps of vesicle trafficking, including the production of vesicles from donor compartments, the transport and uncoating of these vesicles, and their fusion with acceptor

membranes. The Rab family of small M_r Ras-like GTPases plays an important role in these processes (Pfeffer, 1994) and includes more than 30 members. For example, Rab1 associates with endoplasmic reticulum (ER) and Golgi membranes and regulates vesicle transport between them (Tisdale *et al.*, 1992), while Sec4 associates with post-Golgi secretory vesicles and is required for the last step in exocytosis (Salminen and Novick, 1987). Rab4 (van der Sluijs *et al.*, 1991), Rab5 (Chavrier *et al.*, 1990), Rab7 (Chavrier *et al.*, 1990; Meresse *et al.*, 1995), and Rab9 (Lombardi *et al.*, 1993) associate with different subcompartments along the endocytic pathway. Rab4 (van der Sluijs *et al.*, 1992) and Rab5 (Bucci *et al.*, 1992) regulate early stages of endocytosis while Rab7 (Wichmann *et al.*, 1992; Schimmoller and Riezman, 1993; Feng *et al.*, 1995) and Rab9 (Lombardi *et al.*, 1993) function to regulate membrane flow to and from late endosomes.

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The simple eukaryote, *Dictyostelium discoideum*, has proven to be a useful system in which to investigate the molecular mechanisms regulating protein and vesicular trafficking along the secretory and endosomal pathways leading to lysosomes (reviewed in Cardelli, 1993). The biosynthetic targeting pathway in this organism to lysosomes has been well characterized. Lysosomal hydrolases are synthesized as glycosylated, membrane-associated precursor polypeptides (Mierendorf *et al.*, 1985; Cardelli *et al.*, 1986; Bush and Cardelli, 1989). The mannose-rich carbohydrate side chains on these proteins are sulfated and phosphorylated in the Golgi complex (Mierendorf *et al.*, 1985; Freeze, 1986), although these moieties are probably not required for their sorting to lysosomes (Cardelli *et al.*, 1990; Freeze *et al.*, 1990). Instead, N-terminal lysosomal enzyme propeptides have been found to contain lysosomal targeting information (Ruscetti and Cardelli, unpublished data), and proteolytic cleavage of these pro regions, which occurs in a late Golgi or endosomal compartment (Cardelli *et al.*, 1990b; Wood and Kaplan, 1988), may be required for proper sorting of the hydrolases (Richardson *et al.*, 1988). Acidic endosomal/lysosomal compartments are not required for efficient sorting of hydrolases but low pH is required for complete processing of the proteins (Cardelli *et al.*, 1989).

The endocytic pathway has also been characterized in *Dictyostelium*. Fluid phase markers rapidly enter the cell via clathrin-coated pinosomal vesicles (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994), although other mechanisms of internalization may exist (Temesvari and Cardelli, unpublished data). For instance, influx is regulated by myosin I proteins (Novak *et al.*, 1995; Temesvari, *et al.*, 1996a) and the enzyme phosphatidylinositol 3-kinase (Buczynski *et al.*, manuscript in preparation), proteins known to interact with and regulate the actin cytoskeleton (Luna and Condeelis, 1990). Pinosomes rapidly enter larger (0.5–1.0 μm), acidic, hydrolase-rich lysosomal vacuoles (Cardelli *et al.*, 1989; Nolte *et al.*, 1995; Aubry *et al.*, 1993; Padh *et al.*, 1993). Finally, the fluid phase material enters still larger (>2 μm in diameter) neutral pH postlysosomal vacuoles (Aubry *et al.*, 1993; Padh *et al.*, 1993). The fluid phase is then exocytosed from the postlysosomal vacuoles approximately 40 min after internalization and no efficient early endosomal recycling compartment has been identified. Although maintenance of acidic lysosomal compartments is not necessary for hydrolase sorting (Cardelli *et al.*, 1989), inactivation of the vacuolar proton translocating ATPase (V-H⁺-ATPase) pump with concanamycin A inhibits both fluid phase influx and efflux (Temesvari *et al.*, 1996b). Finally, the lysosomal biosynthetic and endocytic pathways described above are thought to merge in a prelysosomal compartment (Richardson *et al.*, 1988; Cardelli *et al.*, 1990b).

Although one of the proposed roles of the endocytic pathway in *Dictyostelium* may be to expel excess water entering cells during osmotic stress, the contractile vacuole (CV) system of membranes is the organelle that is primarily responsible for removing excess water. The CV system in *Dictyostelium* consists of a reticular labyrinth of water-collecting tubules and one or more large translucent vacuoles. These large vacuoles represent either swollen reticular elements (Heuser *et al.*, 1993) or a distinct compartment of the CV (bipartite structure model; Nolte and Steck, 1994). The reticular membranes of the CV are enriched in V-H⁺-ATPase (Heuser *et al.*, 1993; Bush *et al.*, 1994; Fok *et al.*, 1994; Nolte and Steck, 1994), calmodulin (Zhu *et al.*, 1993), a P-type ATPase (Moniakakis *et al.*, 1995), and at least one member of the Rab GTPase family related to Rab4 named RabD (Bush *et al.*, 1994). In contrast, the bladder component of this organelle is also enriched in alkaline phosphatase (Nolte and Steck, 1994). Very little information is available concerning the molecular mechanisms regulating the biogenesis and function of the CV or the intracellular organelles with which the CV communicates. An active V H⁺-ATPase is required, however, for the CV to function normally in response to osmotic stress. Inactivation of the proton pump with concanamycin A results in cells that swell and burst when placed in distilled water (Temesvari *et al.*, 1996b).

At least 10 members of the small M_r Rab GTPase family have been identified in *Dictyostelium*. These include potential homologues of mammalian Rabs including Rab1 (Bush *et al.*, 1993), Rab2 (Bush *et al.*, 1994), Rab4 (Bush *et al.*, 1994), Rab7 (Bush and Cardelli, 1995), Rab8 (Saxe and Kimmel, 1990; Kimmel *et al.*, 1993), and Rab11 (Bush and Cardelli, 1995) as well as three novel Rabs named RabA, RabB, and RabC (Bush *et al.*, 1993). Rab7 has been localized to lysosomal and postlysosomal compartments (Temesvari *et al.*, 1994) and this GTPase may regulate fluid phase recycling (Buczynski and Cardelli, unpublished results). RabD colocalizes with the V-H⁺-ATPase in endosomal/lysosomal membranes in addition to colocalization in the CV system (Bush *et al.*, 1994). In this report, we demonstrate that RabD may function to regulate both the endosomal pathway and the CV system, and we discuss the evidence that suggests that the CV and the endosomal pathway are physically linked via membrane traffic.

MATERIALS AND METHODS

Organism

The *D. discoideum* wild-type strain Ax4 and RabD N121I-expressing strains were grown axenically in TM broth in shaking water baths at 120 rpm at 21°C.

Molecular Techniques

RabD was mutagenized to change N to I at position 121 using an oligonucleotide-mediated site-directed technique based on the Kunkel method as described by Sambrook *et al.* (1989). The mutated cDNA was completely sequenced to confirm that only this one mutation was introduced. RabD was subcloned into the pHA 80 expression vector (a kind gift from Dr. Arturo DeLozanne) and wild-type cells were transformed as described (Bush *et al.*, 1994). Proteins expressed from this vector are tagged at the N-terminus with the HA epitope recognized by the monoclonal antibody 12CA5.

Endocytosis Assays

Fluid phase uptake, exocytosis, and flux were measured using fluorescein isothiocyanate-dextran (FD) as described previously (Temesvari *et al.*, 1994). Measurement of pH flux was performed as described previously (Padh *et al.*, 1993).

Western Blot Analysis

Proteins separated by SDS-PAGE (Laemmli *et al.*, 1975) were transferred to nitrocellulose using a Hoefer Transfor Unit as described (Bush *et al.*, 1994). Proteins were transferred for 3 h at 600 mA in (25 mM Tris-HCl, 192 mM glycine, 20% methanol). Blots were incubated with primary antibodies (either a 1:100 dilution of affinity-purified rabbit anti-RabD or anti-Rab7 antibodies or a 1:100 dilution of a mouse monoclonal anti-70- or anti-100-kDa ATPase subunit antibody) in 10 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20, and 0.1% gelatin (TBSTG), washed, and incubated in TBSTG with goat anti-rabbit or anti-mouse antibodies coupled to alkaline phosphatase (Bio-Rad). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates according to the manufacturers' instructions (Amresco). Relative amounts of RabD were estimated by comparison to a standard curve generated using known amounts (0.01–1.0 μ g) of the recombinant RabD antigen subjected to Western blot analysis. Densitometric scans of photographic negatives of the blots indicated that the signal was linear over a 50-fold range of antigen concentration.

Subcellular Fractionation

Mutant and wild-type intracellular organelles were fractionated on linear sucrose gradients according to the method of Nolte *et al.* (1991). In this procedure, low-speed pellets (10^4 g) contain 75% of the proton pump (remnants of the reticular CV network). The high-speed pellet (6×10^5 g-min) contains 75% of the alkaline phosphatase activity, a marker for the CV bladder. Following centrifugation, fractions were collected from the bottom of the gradient and characterized using enzyme assays according to previously described methods (Cardelli *et al.*, 1987) or subjected to Western blot analysis as described above.

Acidification Assays

Osmotically shocked lysosomes were prepared as described by Rodriguez-Paris *et al.* (1993) from cells allowed to internalize iron dextran and FITC-dextran for 2 h. In vitro acidification was measured according to the method of Rodriguez-Paris *et al.* (1993). Following acidification, nigericin was added to a final concentration of 3 mM to demonstrate that the change in fluorescence was due to the influx of protons.

Immunofluorescence Microscopy

Cells were pipetted onto coverslips and allowed to settle for 15 min. Cells were then fixed in 2% formaldehyde in phosphate-buffered saline containing one-third strength TM growth medium and 0.1% dimethyl sulfoxide for 5 min at room temperature followed by

incubation for 5 min at -20°C in 100% methanol containing 1% formaldehyde. Staining with antibodies was also done as described previously (Bush and Cardelli, 1989). Anti-RabD rabbit antibodies were added at a 1:20 dilution, anti-V-H⁺-ATPase mouse antibodies were added at a 1:50 dilution, and anti-calmodulin rabbit antibodies were added at a 1:100 dilution. Secondary antibodies (either goat anti-mouse conjugated with fluorescein or goat anti-rabbit conjugated with rhodamine) were used at a 1:100 dilution. Cells were photographed using an Olympus model BH-2 fluorescence microscope and Kodak T-MAX 400 speed film.

Statistical Analysis

Analysis of variance was performed using the computer program GraphPAD Instat (Version 1.12a, IBM). Exponential curve fitting and determination of the efflux *K* value of the fluid phase was performed using CA-Cricket Graph III (Version 1.5.1, Macintosh).

RESULTS

Generation of Stable Cell lines Overexpressing RabD N121I

The cDNA encoding the full-length *Dictyostelium* RabD protein was mutagenized in vitro to change an encoded asparagine to an isoleucine at amino acid position 121. This amino acid plays a critical role in the binding of GTP and GDP, and comparable mutations in other Rab proteins have resulted in the formation of proteins that function in a dominant negative manner (Walworth *et al.*, 1989; Tisdale *et al.*, 1992). The mutated cDNA was subcloned behind and in-frame with a DNA element encoding the HA flu epitope; N-terminal epitope tagging has been shown not to affect the location or function of small *M_r* GTPases (Chen *et al.*, 1993; Bush *et al.*, 1994). Expression of the RabD cDNA was under the control of the Act15 promoter that is constitutively active in axenically growing cells. Wild-type cells were transformed with the plasmid and several G418-resistant clones were isolated. On Western blots of extracts prepared from three independent clones, an anti-RabD antibody recognized two forms of the RabD protein in transformed cell lines (Figure 1; top panel): a 23-kDa protein representing the endogenous RabD (also observed in cells transformed with the empty vector; Figure 1, lane 1) and two closely spaced 24–25-kDa species (Figure 1, lanes 2–4) representing the HA-tagged protein (confirmed by decoration with HA-specific antibodies; unpublished results). Differences in prenylation or proteolytic processing may account for the differences in molecular weight. Densitometric scanning indicated that the HA-tagged RabD N121I protein was expressed at 5–10-fold higher levels as compared with the endogenous RabD. Two of the mutant clones, clones 6 and 25, were chosen for further study.

Phase-contrast microscopy and protein analysis indicated that the mutant and wild-type cells were

Figure 1. Overexpression of RabD N121I reduces endocytic influx and efflux. Cell extracts of wild-type (top panel, lane 1) and three clonal isolates of cells (lane 2, clone 6; lane 3, clone 25; lane 4, clone 35) stably transformed with an RabD N121I-expressing plasmid were fractionated by SDS-PAGE and the proteins were blotted onto nitrocellulose. Blots were decorated with affinity-purified antibodies to RabD. (A) Influx rates of FD were measured three separate times for wild type, clone 6, and clone 25 as described in MATERIALS AND METHODS. Analysis of variance indicated that the reduction in influx rates in the mutants was significant. (B) Efflux rates were measured three separate times using cells loaded with FD for 3 h. Cells were washed and resuspended in FD-free medium to initiate the efflux chase period. The percentage of FD remaining intracellular was measured over time. Statistical analysis indicated that efflux of the fluid phase from the cell lines followed exponential kinetics. Analysis of variance of the exponential K value indicated that efflux of FD from the mutant cell lines was significantly slower than that of the parent. Clone 6, $K = -0.007 \pm 0.0008$ ($n = 4$, $p < 0.05$); clone 25, $K = -0.007 \pm 0.0015$ ($n = 4$, $p < 0.05$); wild type, $K = -0.011 \pm 0.0015$. (C) Recycling (flux) times for FD were measured with pulsing cells for 10 min with FD. Cells were washed to remove free FD and resuspended in fresh medium. The percentage of FD remaining was measured over time. (D) The rate of phagocytosis was measured by incubating cells with fluorescent 1- μ m latex beads and measuring uptake over time. Analysis of variance demonstrated that there was no significant difference among the rates of phagocytosis for any of the cell lines examined.

of comparable size. Furthermore, mutant cells completed normal development at the same rate as wild-type cells. Finally, the doubling time for mutant cultures was slightly longer than that for wild-type (11 h versus 9 h, respectively).

RabD Regulates Fluid Phase Influx and Efflux

RabD is enriched in both endosomes and lysosomes (Bush *et al.*, 1994) and conceivably could regulate endocytosis; therefore, the influx rate of a fluid phase

marker fluorescein isothiocyanate-dextran (FD) was determined for the parental and the two mutant cell lines growing in HL5 medium. Figure 1A indicates that the influx rate of FD was linear over 60 min for all of the strains examined, and that the influx rates for the mutants were approximately 50% of that observed for the parental strain ($0.06 \mu\text{l}/\text{min}/\mu\text{g}$ protein for wild-type versus $0.02\text{--}0.03 \mu\text{l}/\text{min}/\mu\text{g}$ protein for the mutant clones). Analysis of variance indicated that the differences in the influx rates were significantly different.

The efflux rates for the internalized fluid phase was also measured. Amoebas were loaded with FD for 3 h, at this time the influx and efflux rates are equal. The cells were then washed and placed in fresh medium. Efflux of FD began immediately during the chase, and 20–25% of the fluid phase was released from all strains after 10 min (Figure 1B). This probably represents release of FD from postlysosomal vacuoles, which suggests that RabD may not regulate this step in the endosomal pathway. Fifty percent of the FD was released from the parental cells after about 30 min of chase (32.6 ± 5.9 min), and <25% of the marker remained intracellular after 60 min. In contrast, 50 min of chase was required before 50% of the FD was released from the two mutant strains (47.4 ± 11.8 min, clone 6; 50.0 ± 3.6 min, clone 25). Efflux of the fluid phase markers followed exponential kinetics, and analysis of variance of the K values demonstrated that the efflux rate of FD from mutant cell lines was significantly slower than that of the parent. These data suggest that both the influx and efflux rates of the fluid phase endocytosis were reduced in cells overexpressing RabD N121I.

In *Dictyostelium* cells, newly internalized fluid is retained in intracellular compartments for 40 min before recycling to the cell surface (flux time). We also measured the transit or flux time for newly internalized fluid (Figure 1C). Cells were pulsed for 10 min with FD and immediately washed and resuspended in fresh growth medium to initiate the chase. Approximately 35 min after the end of the pulse period, the FD began to be released from parental cells and by 60 min 50% of the FD remained intracellular. In contrast, for both RabD N121I-overexpressing cell lines examined, the beginning of recycling of the fluid phase began after 45 min (a 10-min delay relative to wild type), and 90–100 min were required before 50% of the fluid exited the cells. This result was reproducibly seen in four separate experiments.

In addition, RabD is also enriched in purified *Dictyostelium* phagosomes (Rodriguez-Paris and Cardelli, unpublished results) and conceivably could also regulate phagocytosis; therefore, phagocytosis rates were determined by measuring uptake rates of $1\text{-}\mu\text{m}$ fluorescent latex beads. Figure 1D indicates that beads were internalized at equivalent rates for all of the

strains examined for the first 20–30 min, suggesting that the dominant negative form of RabD did not affect this aspect of the endosomal pathway. Analysis of variance demonstrated that there was no significant differences among the rates of phagocytosis for any of the cell lines examined. On average, mutant cells internalized 10% less beads than wild-type cells but this was not statistically significant. Together, these results indicate that RabD may regulate pinocytosis but not phagocytosis.

Internalized Fluid Phase Resides in Acidic Lysosome-like Compartments Longer in RabD N121I Mutants

We next initiated a series of experiments to determine what steps along the endocytic pathway were altered in RabD N121I-expressing cells, accounting for the observed reduction in fluid phase efflux rates. Cells were pulsed with FD as described above; however, in this instance the intraendosomal pH was measured with fluorometry over the chase period (Figure 2A). As previously observed for wild-type cells (Aubry *et al.*, 1993; Padh *et al.*, 1993), newly internalized FD rapidly entered an acidic compartment (pH 5.0). Ten minutes into the chase period, FD began to be transported from the most acidic compartments to less acidic (postlysosomal) compartments (Figure 2A). After 50 min of chase, FD resided in postlysosomal vacuoles, with an average luminal pH of 6.0. In contrast, FD entered acidic compartments in the mutant cells with nearly the same kinetics as observed for the parental strain; however, the rate of increase in the intravesicular pH, as measured by FD fluorescence, was much slower. In fact, after 60 min of chase, the FD still resided in endosomal compartments whose intraluminal pH averaged 5.6.

This result suggests that newly internalized FD resided in acidic lysosomal compartments longer in RabD N121I-expressing cells and perhaps this GTPase regulated transport of FD from lysosomes to postlysosomes. A prediction of this hypothesis is that mutant cells loaded to steady state with FD should contain a greater number of small fluorescent acidic vesicles ($<1 \mu\text{m}$ in diameter) relative to the number of postlysosomal vacuoles ($>2.0 \mu\text{m}$). In fact, RabD N121I-expressing cells (representative cell shown in Figure 2C) contained a significantly greater number of FD-containing vesicles that were smaller than $1 \mu\text{m}$ as compared with wild-type cells (Figure 2B). On average, RabD mutant cells contained 160 vesicles that were $0.5\text{--}1 \mu\text{m}$ ($n = 30$), whereas parental cells contained 70 vesicles in this size range ($n = 25$) and, in addition, contained at least one and as many as five FD-loaded postlysosomes ($>2 \mu\text{m}$ in diameter). In contrast, 50% of the RabD N121I cells contained no large vacuoles, and the remainder of the cells con-

Figure 2. Internalized FD remains in acidic pH compartments longer in mutant cells compared with wild-type cells. Cells were pulsed for 10 min with FD and chased in fresh FD-free growth medium. At the times indicated in A, cells were harvested and intravacuolar pH was measured fluorometrically. The arrival time of FD to acidic compartments in clone 25 was not significantly different from that of wild-type cells. Cells labeled to steady state with FD were visualized in a fluorescent microscope; B represents parent cells and C represents clone 6 mutant cells. Bar, 3 μm .

tained on average only one to two postlysosomes. Furthermore, all of the small vesicles accumulated acridine orange, indicating that they were acidic, whereas postlysosomes $>2.0 \mu\text{m}$ did not accumulate acridine orange, suggesting they contained more alkaline interiors (unpublished results).

These data further support the model that RabD regulates fluid phase transport from lysosomes to postlysosomes. However, since RabD and the V-H^+ -ATPase colocalize in endosomes and lysosomes and the CV system, RabD N121I may also alter the distribution and/or functional activity of the proton pump. Endosomes and lysosomes (and potentially postlysosomes) may be more acidic in mutant cells than wild-type cells because these compartments contain a greater number of functional proton pumps that are responsible for acidification. In fact, we observed that the steady-state intravesicular pH in the two mutant

clones was significantly lower than the pH in wild-type endosomal compartments (Figure 3A). To examine the possibility that mutant lysosomes contained more proton pumps, wild-type and mutant cells were loaded for 120 min with FD and iron dextran, and the endosomes and lysosomes were purified by using magnetic fractionation (Rodriguez-Paris *et al.*, 1993). Western blot analysis (Figure 3B) of endosomes and lysosomes purified from parental cells and clone 6 mutant cells indicated that these organelles contained identical amounts of two of the 70- and 100-kDa proton pump subunits. Coomassie blue staining of preblotted gels and decoration of blotted glycoproteins with wheat germ agglutinin or antibodies to lysosomally localized Rab7 confirmed equal protein loads (Figure 3B). Although identical amounts of proton pumps were found, the V-H^+ -ATPase in mutant cells may function more efficiently to pump protons into the lumen of endosomes/lysosomes. Therefore, the acidification rates were measured in purified lysosomes *in vitro* by measuring decreases in FD fluorescence over time, after the addition of ATP. Figure 3C indicates that the rate of acidification of wild-type and mutant lysosomes was identical. Similar results were also observed when acidification rates were measured for lysosomes in crude extracts, thus eliminating the possibility that purification of mutant lysosomes removed a loosely associated acidification "enhancing" factor (Figure 3C). The addition of nigericin resulted in a return of the fluorescence reading to slightly higher than the initial reading, indicating that the reduction in fluorescence was due to acidification.

RabD N121I-expressing Mutants Are Less Efficient in Sorting of Newly Synthesized Lysosomal Hydrolases

To determine whether RabD N121I-expressing cells were altered in the processing and targeting of newly synthesized lysosomal hydrolases, growing cultures were pulsed for 15 min with [^{35}S]methionine, and the cells were washed and resuspended in nonradioactive growth medium. At the times indicated (Figure 4), samples were collected, and intracellular and secreted α -mannosidase were immunoprecipitated and then subjected to SDS-PAGE followed by fluorography. The half-time for processing of the newly synthesized 140-kDa precursor to the 58- and 60-kDa mature subunits was 25 min for both parental and mutant strains. The generation of mature subunits occurs in lysosomes, suggesting that transport rates from the ER through the Golgi and to the lysosomes was not influenced by the RabD N121I protein. Notably, RabD N121I-expressing cells missorted and rapidly secreted 15–20% of the newly radiolabeled α -mannosidase precursor; in contrast, $<5\%$ of the precursor was mis-sorted and secreted from wild-type cells. However, in

Figure 3. The steady-state intravacuolar pH is lower in mutant cells but lysosomal membranes in mutant and parent cells contain the same level of functional proton pumps. (A) Cells were loaded to steady state with FD and the intravacuolar pH was measured.

both strains the newly synthesized precursors that were missorted reached the cell surface by 10 min, suggesting that RabD N121I did not alter the kinetics of transport of proteins along the constitutive secretory pathway leading from the ER to the cell surface. Lysosomally and postlysosomally localized mature hydrolases are rapidly secreted when growing cells are starved (Cardelli, 1993). As indicated (Figure 5B), neither the secretion rates nor the extent of secretion of α -mannosidase activity was different among the various strains, suggesting that RabD N121I does not influence the trafficking of mature enzymes to the cell surface in cultures suspended in starvation buffer. Together, these results suggest that RabD functions at select steps in the biosynthetic and endocytotic pathways to lysosomes.

Morphology and Function of the Contractile Vacuole System of Membranes Is Altered in RabD N121I-expressing Strains

Previous studies have indicated that 10–15% of RabD and the vacuolar proton pump are enriched in membranes of endosomal/lysosomal compartments (Rodriguez-Paris *et al.*, 1993; Bush *et al.*, 1994); however, the majority (85–90%) of these two proteins colocalize in the reticular network of the CV system (Bush *et al.*, 1994). Therefore, to determine whether RabD N121I mutant cell lines were altered in contractile vacuole function, exponentially growing shaking suspension cultures of parent and mutant cells were allowed to attach to a plastic surface for 15 min and then the growth medium was removed and replaced with water or fresh growth medium. Phase-contrast micros-

(Figure 3 cont.) Analysis of variance demonstrated that the average endosomal/lysosomal pH of the mutants was significantly lower than that of the parent cell line. Wild-type, pH = 5.67 ± 0.05 ($n = 3$); clone 6, pH = 5.32 ± 0.06 ($n = 3$, $p < 0.01$); clone 25, pH = 5.25 ± 0.06 ($n = 3$, $p < 0.01$). (B) Lysosomes were purified by using magnetic fractionation from cells loaded for 120 min with iron dextran and FD. Fifty micrograms of protein were separated by SDS-PAGE from wild-type postnuclear supernatant (lane 1); mutant postnuclear supernatant (lane 2), wild-type lysosomes (lane 3), and mutant lysosomes (lane 4). Gels were stained with Coomassie blue and proteins were blotted onto nitrocellulose. Blots were decorated with antibodies to the 70- and 100-kDa proton pump subunits and to Rab7. Glycoproteins were detected with the lectin wheat germ agglutinin. (C) Homogenates and magnetically purified lysosomes were prepared from cells loaded for 2 h with FD and iron dextran. Acidification in vitro was measured after the addition of ATP (time of addition marked with arrowheads). Treatment of reaction mixtures with nigericin (final concentration of $3 \mu\text{M}$) completely reversed the decrease in fluorescence, demonstrating it was caused by an influx of protons (unpublished results). The half-time for acidification was 1.5 s for mutant and wild-type lysosomes and 1.25 s for mutant and wild-type homogenates. The final fluorescent reading for acidified lysosomes in mutant homogenates is higher than control readings because we increased the gain of the fluorometer to more closely match the curves (mutant cells internalized only one-half the amount of FD).

Figure 4. Processing, targeting, and secretion of lysosomal hydrolases in mutant and wild-type cells. Parent and clone 6 mutant cells were pulse labeled for 20 min with [35 S]methionine and chased in label-free medium. At the times indicated in A, cells and medium samples were prepared by centrifugation, and α -mannosidase was immunoprecipitated. Pellets were resuspended in Laemmli buffer and proteins were subjected to SDS-PAGE followed by fluorography. To measure secretion rates (B) of mature enzymes, cells were harvested from growth medium and resuspended in 10 mM phosphate buffer. At the indicated times, cells and medium were separated, and α -mannosidase enzyme activity was measured.

copy (Figure 5) indicated that most of the parental cells were attached to the plastic and appeared to be amoeboid in shape when exposed to water or growth medium. Mutant cells were attached and appeared to be morphologically normal in growth medium; however, in water the cells swelled rapidly and detached from the plastic surface. After 2–3 h, 75% of the cells

had lysed, suggesting that RabD may regulate water homeostasis.

To determine whether RabD N121I-expressing cell lines demonstrated alterations in the structure of the CV system, consistent with altered function, parental and mutant cells were collected by centrifugation and the postnuclear supernatants were fractionated into low- and high-speed membrane pellets by differential centrifugation (Nolta *et al.*, 1991). The low-speed pellets (enriched in the reticular membranes of the CV system and V-H⁺-ATPase activity) and the high-speed pellets (enriched in the large bladder like CV membranes and alkaline phosphatase activity) were fractionated on linear sucrose gradients. Following centrifugation, gradient fractions were collected and subjected to SDS-PAGE and Western blot analysis or analyzed biochemically to determine the distribution of lysosomal and CV marker enzymes. Table 1 lists the percentage of recoverable marker enzymes and antigens found in the low-speed versus high-speed membrane fractions of mutant and wild-type cells.

A visual examination of the gradient tubes following centrifugation of low-speed pellets revealed that the overall membrane banding pattern was similar between mutant and wild-type extracts, except that there was an almost complete absence in mutant gradients of a tight, nearly white band sedimenting near the top of the gradient (unpublished results). This band of membranes consists primarily of acidosomes (Padh *et al.*, 1991a; Nolta *et al.*, 1991), an earlier name used for the V-H⁺-ATPase-rich reticular elements of the CV system (Heuser *et al.*, 1993; Bush *et al.*, 1994). The distribution of two of the 70- and 100-kDa proton pump subunits as determined by Western blot analysis supported this observation (Figure 6). A large percentage of the proton pump subunits (41% of the total) recovered from wild-type gradients were observed in fractions 10 and 11 corresponding to the position of the white membrane band. Although the proton pump subunits peaked in fractions 10 and 11 from gradients of fractionated mutant cell extracts, the distribution was broader and weighted more toward the bottom of the gradient. Furthermore, only 35% of the recoverable proton pump resided in the low-speed membrane pellets from mutant cells compared with 65% enzyme recovered from low-speed pellets from wild-type cells. The remainder was recovered in the high-speed pellets (Table 1). This may account for the absence of the white membrane band on mutant gradients of the low-speed pellet.

Alkaline phosphatase activity is primarily associated with the CV bladder-like vacuole (enriched in the speed pellet), and <50% of the enzyme was reported to be in the low-speed pellet fraction of wild-type cells (Nolta *et al.*, 1991; Nolta and Steck, 1994). On average, we found that 54% of the recoverable alkaline phosphatase activity was in the high-speed pellet fraction

Figure 5. Cells overexpressing RabD N121I are inefficient at handling osmotic stress. Parent and mutant cells were recovered by centrifugation from growth medium and resuspended in growth medium or water. Cells were allowed to settle in 12-well plastic plates and then photographed using an inverted phase-contrast microscope.

from wild-type cells and 61% of the enzyme was recovered in this fraction from mutant cells (Table 1). Alkaline phosphatase activity distributed into three peaks of activity when low-speed pellets were fractionated; one-half of the activity distributed between fractions 8–12 in gradients of wild-type extracts (Figure 6). Although three peaks of alkaline phosphatase activity (CV marker enzyme) were also observed following fractionation of RabD N121I extracts, a greater percentage of the enzyme was found in the bottom two peaks (60% in the mutant versus 40% in wild type).

RabD protein distributed as two peaks when low-speed pellets were fractionated, and as observed for alkaline phosphatase, a greater percentage of this GTPase sedimented in the bottom half of the mutant gradient. In addition, as observed for the proton pump

proteins, a greater percentage of the total RabD was recovered in the low-speed pellet from wild-type cells compared with mutant cells (Table 1). The RabD antigen observed in fraction 2 cofractionated with lysosomal acid phosphatase activity, and the RabD in the more buoyant fractions cofractionated with the proton pump in the reticular (acidosomal) CV membranes. Also, the HA-tagged RabD N121I showed the same pattern of distribution as the endogenous RabD. Finally, in mutant cells, 35–40% of Rab7 (an endosomal/lysosomal GTPase) sedimented to fractions 2–4 whereas, in contrast, only 10% of the Rab7 in wild-type cells sedimented to this position of the gradient (Figure 6).

The high-speed membrane pellets from wild-type and mutant cells were also fractionated by centrifugation on sucrose gradients (Figure 7). Most of the lyso-

Table 1. Distribution of protein antigens and enzyme activity in high-speed vs. low-speed pellets

	Percentage of protein recovered in each fraction				
	Rab7	RabD	ATPase	AlkPh	AcPh
AC WT	23	54	65	46	30
AC MUT	20	30	35	39	31
CV WT	77	46	35	54	70
CV MUT	80	70	65	61	69

The low-speed pellet (AC) from wild-type (WT) and mutant cells (MUT) and the high-speed pellet (CV) were prepared and analyzed for alkaline phosphatase activity (AlkPh), acid phosphatase activity (AcPh), or subjected to Western blot analysis. The relative levels of Rab7, RabD, and the 70 and 100 kDa ATPase antigens were determined as described in the MATERIALS AND METHODS section, using specific antibodies.

somal hydrolase activity (70%) and alkaline phosphatase activity (54–61%) was found in this membrane fraction prepared from both wild-type and mutant cells. Rab7 and the proton pump antigens distributed in a broad pattern on sucrose gradients in contrast to their sharper distribution on gradients when low-speed pellets were fractionated. However, as observed for low-speed pellets, fractionated mutant extracts contained a greater percentage of these antigens in the bottom half of the gradient compared with wild-type gradients.

To visually determine whether the morphology of the CV system was altered in RabD N121I cells, detergent-permeabilized parent and mutant cells were decorated with antibodies to CV antigens and visualized by using fluorescent microscopy. In control cells, RabD and the 100-kDa membrane-associated proton pump subunit colocalized in the reticular network of the CV system (Figure 8) as reported previously (Bush *et al.*, 1994). In contrast, RabD in mutant cells, distributed in a less reticular manner, and the 100-kDa subunit accumulated primarily in a patch-like structure positioned near the plasma membrane (Figure 8). The remainder of the 100-kDa antigens distributed in a hazy pattern throughout the cell. Furthermore, both the 70-kDa proton pump protein and calmodulin (one of the first identified CV marker antigens; Zhu *et al.*, 1993) also accumulated as a single patch in >90% of the mutant cells in contrast to the reticular distribution consistent with their localization to the CV system in wild-type cells. The patchy distribution of CV antigens was observed in <10% of the wild-type cells. This result also supports the possibility that the entire proton pump may be mislocalized cells overexpressing RabD N121I.

DISCUSSION

In this article, we report that overexpression of the mutant GTPase RabD N121I altered the function of two membrane systems, the endosomal pathway and

the CV. RabD N121I-expressing cell lines were reduced in the rate of influx and efflux of fluid phase markers. The influx step affected was early in the pathway, whereas at least one of the efflux steps affected involved transport of the fluid phase from lysosomes to postlysosomes. The effects of RabD N121I were specific, since the constitutive pathway, phagocytosis, and regulated secretion of lysosomal enzymes were not altered in mutant cells. In addition, subcellular fractionation, light microscopy, and functional tests indicated that the CV system was altered in morphology and its ability to regulate water homeostasis. Together, these results suggest that RabD may regulate membrane traffic between the endosomal pathway and the CV complex.

The endosomal pathway in *Dictyostelium* is distinct from that in mammalian cells in a number of respects (Aubry *et al.*, 1993; Padh *et al.*, 1993). First, no early endosomal sorting or recycling compartment has been identified in *Dictyostelium*. Pinosomes rapidly form from the cell surface and fuse to generate acidic lysosomes (Nolta *et al.*, 1995). Second, fluid phase markers are transported from lysosomes to larger (>2.0 μm), nearly neutral postlysosomal vacuoles from which they are egested (Aubry *et al.*, 1993; Padh *et al.*, 1993). There is no known precedent for postlysosomes in mammalian cells, although similar large nonacidic endosomal vacuoles exist in protists including *Entamoeba histolytica* (Aley *et al.*, 1984). A current model proposes that endosomal vacuoles in *Dictyostelium* increase in size and arise along the entire pathway by the process of fusion of smaller upstream vacuoles (Nolta *et al.*, 1995). However, vesicle traffic occurs between the endosomal pathway and organelles like the Golgi to deliver membrane proteins and hydrolases (reviewed in Cardelli, 1993).

Dictyostelium RabD is 70% identical to human Rab4 in amino acid sequence and is highly enriched in acidic lysosomes (Bush *et al.*, 1994); pinosomes and postlysosomes contain less than one-third of the amount of this GTPase in the cell. In contrast,

Figure 6. Subcellular fractionation of membranes recovered by using low-speed centrifugation from parent and mutant cells. Growing cells were harvested by using centrifugation and resuspended in homogenization buffer containing 100 mM sucrose. Cells were disrupted by passage through filters (5- μ m pores), and low-speed pellets (10^4 g-min) were prepared after the removal of cell debris. Pellets were resuspended in homogenization buffer and layered onto linear 25–45% sucrose gradients. Following centrifugation, fractions were collected and analyzed for acid phosphatase activity (lysosomal marker enzyme) and alkaline phosphatase (marker for the CV “bladder”). Fractions were also subjected to SDS-PAGE and proteins were blotted onto nitrocellulose. Blots were decorated with antibodies to RabD, Rab7, and the 70- and 100-kDa proton pump proteins. The middle panel represents densitometric scans of the blots indicated in the bottom panels.

human Rab4 is enriched in early endosomes and absent from lysosomes (van der Sluijs *et al.*, 1991). Although these two GTPases are very similar in amino acid sequence, differences in the morphology and function of the *Dictyostelium* endosomal pathway compared with mammalian endosomal compartments preclude any simple predictions concerning the functional role of RabD versus Rab4.

Overexpression of RabD N121I reduced the rate of fluid phase influx by 50%. It appeared that the earliest step in internalization (perhaps formation of pinosomes) may have been affected because the decreased rate of influx was evident as early as 3 min after the addition of the fluid phase marker (unpublished results). In contrast, overexpression of mammalian Rab4 N121I had no effect on initial internalization rates for

Figure 7. Subcellular fractionation of membranes recovered from the low-speed pellet supernatant. The supernatant was recovered after centrifugation of the homogenates as described in the legend to Figure 6. Membranes were pelleted by centrifugation (6×10^5 g-min) and resuspended in homogenization buffer. Following centrifugation, fractions were collected and analyzed as described in the legend to Figure 6.

the fluid phase (van der Sluijs *et al.*, 1992). Instead, the Rab5 GTPase has been shown to regulate pinocytosis in mammalian cells (Bucci *et al.*, 1992).

It is presently unclear how RabD may regulate pinocytosis. Based on the examination of clathrin heavy chain minus cells (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994), it has been proposed that at least 75% of pinocytosis in *Dictyostelium* is clath-

rin dependent. However, the absence of clathrin in null cells may disrupt the normal targeting of components required for pinocytosis and clathrin may only play an indirect role in pinocytosis. In fact, we have recently observed that disruption of the actin cytoskeleton with cytochalasin A results in >95% inhibition of fluid phase uptake (Temesvari and Cardelli, unpublished results). It has also been dem-

Figure 8. Immunofluorescent microscopy indicates that CV antigens in mutant cells are abnormal in intracellular distribution. Wild-type and mutant cells were permeabilized and decorated with antibodies to RabD, the 70- and 100-kDa proton pump subunits, and calmodulin. Cells were then incubated with secondary antibodies coupled with rhodamine isothiocyanate (goat anti-rabbit) or FITC (goat anti-mouse) and visualized in an Olympus fluorescent microscope. Control experiments indicated that there was no fluorescent bleedover between rhodamine isothiocyanate and FITC channels.

onstrated that a functional proton pump (Temesvari *et al.*, 1996b) and myosin I proteins regulate influx (Novack *et al.*, 1995; Temesvari *et al.*, 1996a). Conceivably, RabD could alter the distribution or activity of the proton pump in the endosomal pathway, thus accounting for a reduction in influx of the fluid phase in mutant cells. However, endosomal/lysosomal membranes prepared from mutant cells contained the same amount of proton pump as wild-type membranes, and the acidification rates for both

purified lysosomes and lysosomes in homogenates from wild-type and mutant cells were identical. We cannot eliminate the possibility that RabD regulates the activity of the proton pump in vivo. Until more is known about the components regulating pinocytosis, we cannot propose a molecular mechanism to account for a direct role of RabD in pinocytosis. It is also possible that RabD N121I indirectly affects pinocytosis by blocking steps later in the pathway.

As discussed above, mammalian Rab4 has been localized to endosomal compartments and has been demonstrated to regulate recycling of membrane receptors and fluid phase from early endosomal compartments back to the cell surface (van der Sluijs *et al.*, 1992). We also report here that expression of RabD N121I slows recycling of the fluid phase back to the cell surface. In *Dictyostelium*, unlike mammalian cells, recycling is a late event in the endosomal pathway and involves the release of material through postlysosomes (Padh *et al.*, 1993), large vacuoles downstream from mature acidic lysosomes. Notably, expression of RabD N121I delayed the transit of the fluid phase from the acidic compartments, resulting in an increase in the number of small FD-positive lysosomal vesicles (0.5–1.0 μm) and a reduction in the number of FD-positive postlysosomal vacuoles. This delay could account for the delay in efflux of the fluid phase. As one model, we propose that RabD might directly regulate transport of the fluid phase from lysosomes to postlysosomes, an endocytic step for which very little is known. Current evidence favors a model in which smaller lysosomes fuse to form larger postlysosomes, and RabD may regulate this fusion event in a manner similar to that proposed for Rab5, which regulates early endosome fusion (Bucci *et al.*, 1992). In fact, it has been demonstrated that phosphatidylinositol (PI) 3-kinase may couple functionally with Rab5 to regulate endosomal fusion (Li *et al.*, 1995). Interestingly, we have observed that *Dictyostelium* (PI) 3 kinase regulates traffic from lysosomes to postlysosomes and perhaps this occurs by modulation of the activity of RabD (Buczynski *et al.*, manuscript in preparation). Alternatively, lysosomes and postlysosomes may be stable compartments and RabD might regulate vesicle traffic between these compartments.

Although we favor the fusion model to account for the effects of RabD N121I, it is still a formal possibility that the mutant Rab GTPase altered the morphology and size of the endosomal compartments. For instance, postlysosomes may form but they remain small in RabD N121I-expressing cell lines. However, the fact that these organelles remain acidic based on fluorescence of accumulated acridine orange argues against this possibility.

The effects of RabD N121I were fairly specific to the endosomal pathway, most other membrane transport systems were not altered in mutant cells. For instance, the kinetics of transport of newly synthesized lysosomal enzyme precursors to the cell surface or to lysosomal processing compartments was identical in mutant and wild-type cells. Regulated secretion of lysosomal hydrolases and the phagocytosis rates of latex beads were also identical. Although the sorting efficiency of newly synthesized α -mannosidase precursors was slightly less in mutant cells compared with wild-type cells, 80% of the precursors were pro-

teolytically processed and correctly transported. *RabD* gene disruption experiments may provide further insight into the role of this GTPase in hydrolase sorting and phagocytosis.

Although enriched in lysosomes, >85% of RabD associates with reticular elements of the CV system (Bush *et al.*, 1994). Given the dual localization of this GTPase, it is postulated that the endosomal pathway and CV complex may communicate by membrane traffic (Bush *et al.*, 1994; Padh and Tanjore, 1995). The following lines of evidence support this possibility. First, clathrin heavy chain minus cells are defective in pinocytosis and contain no detectable CV (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994); these mutant cells also swell when placed in water. Second, Padh *et al.* (1991b) have demonstrated that elements of the CV and endosomes can fuse in vitro, and have proposed that proton pumps are delivered to the endosomal pathway from the CV system. Third, Rab GTPases usually associate with compartments and/or vesicles that are linked by membrane traffic (Pfeffer, 1994); RabD is localized in the CV complex and lysosomes (Bush *et al.*, 1994). Finally, RabD N121I-expressing cell lines contain inefficiently functioning CV, and multiple CV-associated antigens accumulate as a patch in mutant cells in contrast to the reticular pattern observed in wild-type cells. Subcellular fractionation studies also suggested that the CV system of membranes was altered in size and/or density in RabD N121I-expressing cells.

Therefore, RabD may directly regulate the trafficking of membranes to and organization of the CV membrane system and the altered intracellular distribution of CV antigens is an accurate reflection of the organelle. Alternatively, the reticular membrane system may still be present in mutant cells, but the proton pump has not been delivered properly. The absence of the V-H⁺-ATPase in the water-collecting tubules of the CV would account for the inability of the mutant cells to pump water out. In fact, direct inhibition of the proton pump inactivates the CV (Heuser *et al.*, 1993; Temesvari *et al.* 1996b). Conceivably, RabD may regulate the transport of vesicles rich in V-H⁺-ATPase that bud from lysosomes to the CV complex, and this process could be coupled to the formation of proton pump-depleted postlysosomes (Nolta *et al.*, 1995). A block at this point in the pathway (presumably caused by overexpression of RabD N121I) could result in a reduction in the rate of formation of postlysosomes and a disruption in the transport of proton pumps to the reticular network. This model is consistent with the known localization of RabD and the phenotypic changes observed in RabD N121I mutants. Our results involving acidification assays suggest that RabD does not directly regulate the function of the proton pump.

Recently, it was reported that the cyclic AMP receptor accumulated in the CV presumably after internal-

ization from the cell surface (Padh and Tanjore, 1995). This suggests that for at least one membrane protein, early recycling from the endosomal pathway may be possible and conceivably RabD might be involved in this process. In this primitive eukaryote, one could envision that RabD functions in a manner analogous to mammalian Rab4 and that the contractile vacuole may represent a primitive version of the mammalian "recycling endosome" with water efflux being an important additional function. RabD function may have been preserved in Rab4, although the CV was no longer required in mammalian cells exposed to constant isotonic conditions.

Recently, we have localized a Rab7-like GTPase (Temesvari *et al.*, 1994; Bush and Cardelli, 1995) and a novel GTPase, RabB (Buczynski and Cardelli, unpublished), to overlapping compartments of the *Dictyostelium* endosomal pathway. Efforts are underway to determine the function and interaction of these multiple GTPases, along with other proteins and enzymes, in regulating membrane flow along the endosomal pathway and from the endosomal pathway to other intracellular organelles such as the CV.

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