Evidence for a Recycling Role for Rab7 in Regulating a Late Step in Endocytosis and in Retention of Lysosomal Enzymes in *Dictyostelium discoideum*

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The mammalian small molecular weight GTPase Rab7 (Ypt7 in yeast) has been implicated in regulating membrane traffic at postinternalization steps along the endosomal pathway. A cDNA encoding a protein 85% identical at the amino acid level to mammalian Rab7 has been cloned from *Dictyostelium discoideum*. Subcellular fractionation and immunofluorescence microscopy indicated that Rab7 was enriched in lysosomes, postlysosomes, and maturing phagosomes. Cell lines were generated that overexpressed Rab7 wild-type (WT), Rab7 Q67L (constitutively active form), and Rab7 T22N (dominant negative form) proteins. The Rab7 T22N cell line internalized fluid phase markers and latex beads (phagocytosis) at one-third the rate of control cells, whereas Rab7 WT and Rab7 Q67L cell lines were normal in uptake rates but exocytosed fluid phase faster than control cells. In contrast, fluid phase markers resided in acidic compartments for longer periods of time and were more slowly exocytosed from Rab7 T22N cells as compared with control cells. Light microscopy indicated that Rab7-expressing cell lines contained morphologically altered endosomal compartments. Compared with control cells, Rab7 WT- and Rab7 Q67L-expressing cells contained a reduced number of vesicles the size of postlysosomes ($>2.5\ \mu\text{m}$) and an increased number of smaller vesicles, many of which were nonacidic; in control cells, $>90\%$ of the smaller vesicles were acidic. In contrast, Rab7 T22N cells contained an increased proportion of large acidic vesicles relative to nonacidic vesicles. Radiolabel pulse-chase experiments indicated that all of the cell lines processed and targeted lysosomal α -mannosidase normally, indicating the lack of a significant role for Rab7 in the targeting pathway; however, retention of mature lysosomal hydrolases was affected in Rab7 WT and Rab7 T22N cell lines. Contrary to the results observed for the fluid phase efflux experiments, Rab7 T22N cells oversecreted α -mannosidase, whereas Rab7 WT cells retained this hydrolase as compared with control cells. These data support a model that Rab7 may regulate retrograde transport of lysosomal enzymes and the V-type H^+ -ATPase from postlysosomes to lysosomes coupled with the efficient release of fluid phase from cells.

INTRODUCTION

The Rab family of small molecular weight Ras-like GTPases plays an important role in the regulation of vesicle trafficking between intracellular organelles (Pfeffer, 1994), a process necessary for the biogenesis of organelles and important in maintaining the

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uniqueness of endomembrane compartments (Rothman, 1996). More than 30 members of the Rab family have been described, and each protein associates with a defined intracellular compartment. For instance, Rab4 and Rab5 associate with early endosomes (Bucci *et al.*, 1992; van der Sluijs *et al.*, 1991), and Rab7 and Rab9 associate with late endosomes (Chavrier *et al.*, 1990; Lombardi *et al.*, 1993). Rab4 and Rab5 regulate early steps in endocytosis (van der Sluijs *et al.*, 1992), and Rab9 regulates transport of material from late endosomes to the Golgi complex (Lombardi *et al.*, 1993).

The exact step in the endosomal pathway that is regulated by Rab7 remains controversial. Some investigators have proposed that Rab7 is involved in the regulation of forward membrane flow from late endosomes to lysosomes (Schimmoller and Riezman, 1993; Vitelli *et al.*, 1997), whereas others have proposed that Rab7 regulates an earlier step in the endosomal pathway (Wichmann *et al.*, 1992; Feng *et al.*, 1995). Interestingly, Rab7 appears to be essential for the formation of large endosomal vacuoles induced in cells treated with the *Helicobacter pylori* cytotoxin VacA (Papini *et al.*, 1997). There is also some dispute about which intracellular compartment Rab7 is associated with: earlier studies suggested that Rab7 associated with late endosomes (Chavrier *et al.*, 1990), but a more recent study concluded that Rab7 was localized to a vesicular compartment that only partially colocalized with the cation-independent mannose-6-phosphate receptor (a marker for late endosomes) and that a (GTPase) defective form of Rab7 was associated with lysosomes (Meresse *et al.*, 1995).

The unicellular organism *Dictyostelium discoideum* has proven to be a useful system in which to investigate the molecular mechanisms regulating endosomal and lysosomal membrane trafficking. Lysosomes in *Dictyostelium* form a functional hub that connects to at least three membrane trafficking pathways: 1) the endocytic pathway, 2) the biosynthetic hydrolase targeting pathway, and 3) the phagocytic pathway (reviewed in Cardelli, 1993). Recent evidence also suggests a connection exists between the endosomal pathway and the contractile vacuole complex of membranes (CV) responsible for osmotic regulation (Padh and Tanjore, 1995; Bush *et al.*, 1996; Temesvari *et al.*, 1996a).

The *Dictyostelium* biosynthetic and endocytic pathways have been the most extensively studied. Concerning the biosynthetic pathway, lysosomal enzymes are synthesized as precursor polypeptides (Mierendorf *et al.*, 1985; Wood and Kaplan, 1985; Cardelli, 1986; Bush and Cardelli, 1989), and short N-terminal "pro" regions, proteolytic processing, and the clathrin heavy chain are important in regulating targeting of hydrolases (Richardson *et al.*, 1988; Ruscetti *et al.*, 1994; Ruscetti and Cardelli, unpublished results). N-linked

mannose-rich oligosaccharide side chains and sulfate and probably phosphate sugar modifications only influence the rate of transport of the enzymes to lysosomes and not the fidelity of the targeting process (Cardelli *et al.*, 1990; Freeze *et al.*, 1990).

Pinocytosis occurs at a high rate in *Dictyostelium* (Thilo and Vogel, 1980). A major pathway, after internalization of fluid, involves pinosomes rapidly fusing to form acidic lysosomes, followed by the delivery of fluid phase to larger less acidic postlysosomal vacuoles (Cardelli, 1989; Aubry *et al.*, 1993; Padh *et al.*, 1993; Nolte *et al.*, 1995). Essentially all of the internalized fluid phase is exocytosed from cells from postlysosomes, beginning approximately 45 min after internalization; no early endosomal recycling compartment has been identified (Aubry *et al.*, 1993; Padh *et al.*, 1993). Endocytosis is regulated by a number of proteins in *Dictyostelium* including the clathrin heavy chain (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994), the actin cytoskeleton (Temesvari and Cardelli, unpublished results), the vacuolar H⁺-ATPase (Temesvari *et al.*, 1996a), select myosin I molecules (Novak *et al.*, 1995; Jung *et al.*, 1996; Temesvari *et al.*, 1996b), and phosphatidylinositol 3-kinases (Buczynski *et al.*, 1997). It is currently not known how much the clathrin-dependent and -independent pathways individually contribute to pinocytosis.

Although *Dictyostelium* amoebae actively phagocytose bacteria, yeast, and inert particles, relatively little is known about the signal transduction pathways regulating this process or how the newly formed phagosome is modified by contact with endolysosomal membranes (Vogel *et al.*, 1980; Cohen *et al.*, 1994; Rodriguez-Paris *et al.*, 1996; Rezabek *et al.*, 1997). Recent data, however, supports a positive role for coronin (Maniak *et al.*, 1995), myosin I molecules (Jung *et al.*, 1996), the β subunit of G proteins (Wu *et al.*, 1995; Buczynski and Cardelli, unpublished results), and the small Ras-like GTPase Rap1 (Seastone and Cardelli, unpublished results) in regulating the internalization step.

More than 25 members of the Ras superfamily have been described in *Dictyostelium* (reviewed in Bush and Cardelli, 1995). At least 10 of these proteins belong to the Rab family and include proteins very related to Rab1 (Bush *et al.*, 1993b), Rab2 (Bush *et al.*, 1994), Rab4 (Bush *et al.*, 1994), Rab8 (Saxe and Kimmel, 1990), and Rab11 (Bush and Cardelli, 1995), as well as three novel Rab proteins named RabA, RabB, and RabC (Bush *et al.*, 1993b). Rab4 (also named RabD) plays a role in endocytosis and in the regulation of the structure and function of the CV complex (Bush *et al.*, 1996). Consistent with these roles, the CV complex contains the majority of the cellular RabD antigen (Bush *et al.*, 1994) and the V H⁺-ATPase (Heuser *et al.*, 1993; Nolte *et al.*, 1993), whereas the remainder of the two proteins associate with endolysosomal membranes (Rodriguez-

Paris *et al.*, 1993; Temesvari *et al.*, 1994). In addition, Rab11 associates with elements of the CV (Bush and Cardelli, 1995) and may also regulate the morphology and perhaps the function of the CV complex (Bush and Cardelli, unpublished results).

As described in this report, we have cloned a cDNA that encodes a protein very related in amino acid sequence to mammalian Rab7 and have determined that it localizes to lysosomes and postlysosomes. We have approached the general problem of understanding the function of Rab7 by generating cell lines that modestly overexpress wild-type Rab7 (Rab7 WT) and mutant proteins that are either constitutively active (Rab7 Q67L; locked in the GTP-bound conformation) or dominant negative (Rab7 T22N; locked in the GDP-bound state). These mutations (single amino acid substitutions) were first described for the Ras protein (Valencia *et al.*, 1991) and have been demonstrated to produce the appropriate active or inactive mutants for a variety of Ras-like proteins. Biochemical and microscopic examination of cell lines overexpressing the various Rab7 proteins indicated that Rab7 played an important role at a late step in the endosomal pathway and regulated fluid phase exocytosis and retention of lysosomal hydrolases. We discuss two models to account for the function of Rab7. One model is similar to one proposed by Schimmoller and Riezman, (1993), and the other new model proposes that Rab7 operates in a retrograde manner.

MATERIALS AND METHODS

Organism and Materials

Rab7-expressing *D. discoideum* strains (all derived from the AX4 strain; see below) were grown axenically in HL5 medium in shaking water baths at 150 rpm at 21°C.

Cloning of Rab7 cDNA

A ³²P end-labeled oligonucleotide [GT(A/T/C)AA(T/C)AAA-AAATT(T/C)TC(A/T)AA(T/C)CAATC(A/T)AAAGC(A/T/C)AC] that codes for the amino acid sequence ²⁹VNKKFSNQSKA³⁹ was used as a probe to screen a cDNA library constructed in λgt11 (Sambrook *et al.* [1989] was the source for most of the molecular biology techniques). This stretch of amino acids overlaps with the putative effector region and is unique to Rab7. Hybridizations, washing conditions, and polymerase chain reaction (PCR) sequencing of DNA were as described (Bush *et al.*, 1993).

Construction of Rab7 Overexpressing Cell Lines

Nucleotide changes in the Rab7 cDNA were engineered by site-directed mutagenesis using a PCR procedure similar to that described by Mikaelian and Sergeant (1992). The mutant primers used to generate Rab7 Q67L and Rab7 T22N were 5'-ACGTTCTAAAC-CAGCTGTATCCCATTTTG-3' and 5'-GAGAGAAATTTTACCA-ACACCTGAATC-3', respectively (changed bases are underlined). The outer primers flanking the cDNA were 5'-GCCACAAAGAAG-GTTTTATTAAG-3' and 5'-AATTAAACAACAACCTGATT-TAGCTGG-3'. The Rab7 WT PCR product was generated with only the outer primers. PCR products were subcloned into the TA vector, digested with *Hind*III, and subcloned into the pDA80-HA vector (a

kind gift from Dr. A. DeLozanne) behind and in-frame with the flu hemagglutinin (HA) epitope. Expression of the chimeric protein was driven by the constitutively active actin 15 promoter. CsCl-purified DNA was then transformed into *D. discoideum* by using the calcium phosphate method (Sadeghi *et al.*, 1988), and G418-resistant colonies were screened for overexpression by Western blot analysis.

Antibody Production

The Rab7 cDNA was subcloned in-frame from nucleotide 54 (first nucleotide of Ala-2 codon) to nucleotide 612 (end of TAA stop codon) into the RSET bacterial fusion protein expression vector at the *Eco*RI site (Invitrogen, San Diego, CA). Inserts were screened for correct orientation and frame by restriction digests and PCR sequencing. The plasmid was transformed into *Escherichia coli* strain JM109, followed by the induction of the Rab7 fusion protein. Bacterially expressed Rab7 was purified by the manufacturer's instructions (Invitrogen). The purified protein was subjected to SDS-PAGE and gel slices containing the Rab7 protein were excised and injected into New Zealand White rabbits (Cocalico Biologicals, Reamstown, PA). Specific antibodies raised against the Rab7 protein were purified with a column containing the recombinant Rab7 protein coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ).

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol (blotting buffer) for 1 h at 100 V in a Bio-Rad transblotter. Blots were incubated with primary antibodies (1:200 dilution of polyclonal antibodies to Rab7) in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20, and 0.1% gelatin. The blots were washed and incubated with a 1:30,000 dilution of goat anti-rabbit secondary antibodies coupled to alkaline phosphatase (Sigma, St. Louis, MO). Proteins decorated with antibodies were visualized by using 5-bromo-4-chloro-indoyl phosphate (Amresco, Solon, OH) and nitro blue tetrazolium (Amresco) as substrates in 0.1 M Tris (pH 8.0), 100 mM NaCl, and 5 mM MgCl₂.

Endocytosis and Phagocytosis Assays

Fluid phase uptake, exocytosis, and flux were measured using fluorescein isothiocyanate (FITC)-dextran (FD) as described previously (Temesvari *et al.*, 1996b). Measurement of luminal pH was performed as described (Padh *et al.*, 1993) using FD as a pH-sensitive probe. For phagocytosis assays, fluorescently labeled (crimson) carboxylate-modified latex beads (1 μm in diameter; Molecular Probes, Eugene, OR) were added to cells in growth medium at a concentration of 50 beads/cell. At the times indicated, 1 ml of cells were removed and processed after centrifugation as described (Temesvari *et al.*, 1996a). Fluorescence was measured with a fluorimeter set at an excitation wavelength of 625 nm and an emission wavelength of 645 nm. The number of beads internalized was calculated from a standard curve obtained by measuring the fluorescence of a known number of beads.

Pulse-Chase Radiolabeling and Immunoprecipitation

Radiolabel pulse-chase analysis was performed as follows. Exponentially growing cells were collected by centrifugation (1000 × g for 3 min) and resuspended to a titer of 1 × 10⁷ cells/ml in fresh growth medium containing 650–750 μCi/ml [³⁵S]methionine (New England Nuclear, Boston, MA). The cells were pulse labeled for 20 to 30 min while shaking at 150 rpm at 20°C, harvested, and resuspended in fresh medium. At the times indicated, 5–7 × 10⁶ cells were removed and separated into cell and extracellular medium fractions by centrifugation (1000 × g for 30 s). The pellets were solubilized with 0.5% Triton X-100, and α-mannosidase was immu-

nonprecipitated from the cell pellet and medium samples with monoclonal antibodies as described (Mierendorf *et al.*, 1985). The immunoprecipitated proteins were separated on 7.5% polyacrylamide gels according to the procedure of Laemmli (1970). The gels were placed in 10% trichloroacetic acid overnight to precipitate the proteins in the gels, treated with Enhance by the manufacturer's instructions (New England Nuclear), dried under vacuum, and exposed to Kodak XAR-5 film at -70°C .

Immunofluorescence (IF) Microscopy

Approximately 1×10^5 cells were pipetted onto glass coverslips and allowed to attach for 30 min. Cells were then fixed in 2% formaldehyde in phosphate-buffered saline (PBS = 137 mM NaCl, 25 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) containing one-third strength HL5 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. Immunostaining of the 100-kDa V-H^+ -ATPase subunit used a 1:20 dilution of a monoclonal antibody generated against the 100-kDa subunit of the vacuolar proton pump protein. The Rab7 antigen was immunostained with a 1:20 dilution of the affinity-purified Rab7 polyclonal antibodies. Antibodies were diluted in PBS containing 2.5 mg/ml bovine serum albumin and 0.1% saponin. After a 2-h incubation, the cells were washed three to five times in PBS and secondary antibodies (Molecular Probes) coupled to FITC or tetramethyl rhodamine isothiocyanate (TRITC) were added by following the manufacturer's instructions. Cells were photographed with an Olympus BX50 fluorescence microscope using T-MAX 400 speed film.

Purification of Lysosomes and Phagosomes

Lysosomes were purified using a previously described magnetic isolation technique (Rodriguez-Paris *et al.*, 1993). Phagosomes were purified on discontinuous sucrose gradients as described (Desjardin *et al.*, 1994; Rezabek *et al.*, 1997). CV membranes were purified as described (Nolta *et al.*, 1993).

RESULTS

Cloning of the Rab7 cDNA Encoding a Rab7-like Protein

With an oligonucleotide probe synthesized based on a highly conserved region found in all Ras-like GTPases (WDTAGQE), we have cloned 19 cDNAs encoding proteins belonging to the Ras, Ran, Rab, and Rho families (Bush and Cardelli, 1992; Bush *et al.*, 1993a,b, 1994; Daniels *et al.*, 1993). None of these cDNAs encoded proteins highly related to Rab7; therefore, we rescreened the cDNA library with an oligonucleotide encoding the amino acids $^{29}\text{VNKKFSNQSKA}^{39}$, representing a region of Rab7 that overlaps with the putative effector region based on analogy to the Ras protein. The nucleotide sequence of one of the isolated cDNAs clones (710 nucleotides long) contained an open reading frame beginning with an ATG start codon at nucleotide position 51 and ending with a TAA stop codon at nucleotide position 610 (Figure 1). The 203-amino acid sequence predicted by the open reading frame contained all four of the highly conserved nucleotide binding domains including GDS-GVGKT (amino acid positions 15–22), WDTAGQE (amino acid positions 62–68), GNKID (amino acid

Figure 1. Alignment of *D. discoideum* Rab7 sequence with the human protein Rab7 and the yeast protein YPT7. Top, nucleotide and amino acid sequence of the *D. discoideum* Rab7 cDNA. The cDNA was cloned from a λ gt11 library and sequenced using the dideoxynucleotide sequencing method (femtomolar kit; Promega). Bottom, the nucleotide sequence was analyzed with the MacVector computer software of IBI (New Haven, CT). The putative amino acid sequence was used to search the National Biomedical Research Foundation data base. Identical and similar amino acids are denoted by the symbols $:$ and $;$, respectively.

positions 124–128), and ETSK (amino acid positions 154–158) that are found in all members of the ras-like GTPase family. A search of the National Biomedical Research Foundation protein data base program (release 31.0) indicated the *D. discoideum* cDNA encoded a protein highly related to the human Rab7 and yeast YPT7 proteins with 88.5% and 71.5% identity at the amino acid level, respectively, from amino acid positions 10 to 175 (Figure 1). Also, the putative effector region ($^{37}\text{YKATGADF}^{45}$) was identical in all the proteins. Southern blot and Northern blot analyses indicated *Dictyostelium* Rab7 was encoded by a single copy gene that was expressed at a relatively constant level during growth and development (our unpublished results).

Rab7 Localizes to Lysosomes and Postlysosomes

To begin to determine the intracellular location of Rab7, a His-tagged Rab7 fusion protein was expressed in *E. coli*, and the purified protein was injected into rabbits to generate polyclonal antibodies. The antibodies were then purified with a Rab7 antigen column. Lysosomes were also isolated by a magnetic fractionation procedure from cells pulse labeled for 15 min with iron-coated dextran followed by a 15-min chase (Rodriguez-Paris *et al.*, 1993; Nolte *et al.*, 1995). Membranes of the CV were purified by sucrose density gradient fractionation (Nolte *et al.*, 1993). Ten micrograms of protein from each fraction were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis using affinity-purified antibodies indicated that Rab7 and RabD were enriched at least fivefold in magnetically purified lysosomes compared with a postnuclear supernatant (Figure 2A). In contrast, unlike RabD, a Rab4-like GTPase (Bush *et al.*, 1994), Rab7 was not enriched in the contractile vacuole system of membranes relative to total membranes (Figure 2A). Figure 2A also demonstrates that the Rab7 antibody does not cross-react with RabD (note the absence of a significant signal in the CV lane with Rab7 antibody). Dot blot analysis using purified bacterially expressed Rab7, Rab11, RabD, and RabB also demonstrated that the antibody was specific for Rab7 (our unpublished results).

Western blot analysis of samples recovered after Percoll gradient fractionation of *Dictyostelium* organelles indicated that Rab7 distributed bimodally across the gradient (Figure 2B). One peak of Rab7 corresponded in position with dense lysosomes (α -mannosidase activity; fractions 2–8), and a second peak of Rab7 partially overlapped with more buoyant membranes (Figure 2B). The position of the second peak of Rab7 corresponds to the position of endoplasmic reticulum membranes (α -glucosidase II activity), early endosomes (Cardelli *et al.*, 1990), and postlysosomes (Richardson *et al.*, 1988). Therefore, to more accurately determine the location of Rab7 along the endosomal pathway, pinosomes (cells were pulse labeled with iron dextran for 5 min with no chase), lysosomes (15-min pulse and 15-min chase), and postlysosomes (15-min pulse and 90-min chase) were isolated by magnetic fractionation, and 10 μ g of protein per sample were separated by SDS-PAGE. Western blots were probed with antibodies to Rab7 and the 70-kDa and 100-kDa subunits of the proton pump. As indicated in Figure 2C, three- to fivefold higher levels of Rab7 were observed in lysosomes and postlysosomes compared with pinosomes. The proton pump was also preferentially enriched in lysosomes and postlysosomes compared with pinosomes, and in contrast to Rab7, the pump was also abundant in the CV system of membranes (Figure 2C).

After internalization of bacteria or latex beads, the *D. discoideum* phagosome is rapidly modified by fusion with membranes of the endolysosomal pathway (Rodriguez-Paris *et al.* 1996; Rezabek *et al.*, 1997). To determine whether Rab7 was delivered to phagosomes, we fed cells 1- μ m latex beads for 2 min or for 10 min (pulse time) and then chased cells in the absence of beads for 0, 15, 60, and 240 min. Phagosomes were purified on sucrose gradients as described (Desjardin *et al.*, 1994; Rezabek *et al.*, 1997), and samples containing 10 μ g of protein were separated by SDS-PAGE. Western blot analysis indicated that Rab7 was enriched at least fivefold in phagosome preparations from cells pulsed for 10 min and chased for various periods of time (Figure 2D, lanes 3–6); in contrast, phagosomes purified from cells pulse labeled for 2 min with beads were not highly enriched in Rab7 relative to total membranes (Figure 2D, lane 2). These data suggest that soon after formation, phagosomes fuse with Rab7-containing membranes (probably lysosomes and/or postlysosomes) and that Rab7 remains associated with phagosomes for at least 4 h.

Next, we used IF microscopy to further examine the intracellular location of Rab7. Detergent-permeabilized control cells grown in HL5 were incubated with the affinity-purified Rab7 antibodies for 2 h, followed by the addition of goat anti-rabbit secondary antibodies coupled to FITC. Figure 3A shows two typical *D. discoideum* WT Ax4 cells visualized by phase-contrast microscopy. These cells appeared amoeboid in shape and contained numerous small vesicles (<1 μ m), as well as several large vesicles (>2.0 μ m; two are marked with an arrowhead and an arrow). Some of these large vesicles are postlysosomes and the others are contractile vacuole bladder membranes. These former structures are novel nonacidic endocytically terminal organelles from which fluid phase material is egested (Padh *et al.*, 1993). IF microscopy indicated that Rab7 was localized to numerous small (<0.5 μ m) punctate structures distributed throughout the cell and to larger vesicles (>2 μ m in diameter) that were ringed with punctate fluorescing structures (Figure 3B, arrow). These large vesicles are not CVs and are most likely postlysosomes. The more transparent vesicles (Figure 2A, arrowhead) did not contain Rab7 and may represent CV “bladder” membranes.

Next, growing cells were recovered by centrifugation and resuspended for 1 h in growth medium containing 100 μ M sucrose; endocytosis of sucrose results in the formation of large endocytically derived vacuoles termed sucrosomes (Jahraus *et al.*, 1994). IF microscopy indicated that Rab7 ringed a greater number of large vesicles in cells that had internalized sucrose compared with control cells (Figure 4, compare B with D). These swollen vesicles were derived from endosomes [i.e., Rab7 ringed large vesicles that also contained FD (Figure 4, E and F, vesicles marked with

Figure 2. Rab7 localizes to lysosomes, postlysosomes, and maturing phagosomes. (A) Ten micrograms of protein from total membranes (Tot), purified contractile vacuoles (CV), and lysosomes (Lys) were subjected to SDS-PAGE and Western blotting. The blots were labeled with affinity-purified antibodies to Rab7 and RabD, followed by goat anti-rabbit antibodies coupled with alkaline phosphatase. (B) Intracellular organelles were separated by centrifugation on self-forming Percoll gradients and equal volumes from even fractions were collected and subjected to SDS-PAGE and Western blot analysis using Rab7 antibodies. The developed blot was scanned with a laser densitometer and the distribution of Rab7 was plotted along with the distribution of α -mannosidase (α -Mann, a lysosomal marker) and α -glucosidase II (α -Glu II, a marker for the endoplasmic reticulum) activity. (C) Pinosomes (Pino), lysosomes (Lys), and postlysosomes (P.Lys) were purified by magnetic fractionation as described (Nolta *et al.*, 1995), and along with CV membranes, equal amounts of protein were subjected to SDS-PAGE and Western blot analysis using Rab7 antibodies and a combination of monoclonal antibodies to the 70-kDa and 100-kDa proton pump subunit proteins. (D) Phagosomes of different ages containing latex beads were purified on sucrose gradients and subjected to SDS-PAGE and Western blot analysis. Blots were labeled with affinity-purified Rab7 antibodies. Lane 1, 10 μ g total membrane protein; lane 2, 10 μ g of protein from phagosomes prepared by pulsing cells for 2 min with latex beads followed by a 0-min chase; lane 3, 10-min pulse/0-min chase phagosomes; lane 4, 10-min pulse/15-min chase phagosomes; lane 5, 10-min pulse/60-min chase phagosomes; lane 6, 10-min pulse/240-min chase phagosomes.

open arrowheads)]. Interestingly, the 100-kDa membrane-associated proton pump subunit, normally localized to a vesicular-reticular network in control cells (Bush *et al.*, 1994; Fok *et al.*, 1994; Figure 4A), was associated with numerous small vesicles in cells that had internalized sucrose (Figure 4C, arrow). For the most part, these vesicles did not colocalize with the Rab7-positive vesicles (Figure 4, compare C and D). Thus, the data in this section indicate that Rab7 is

probably associated with lysosomes, postlysosomes, and smaller vesicles of unknown origin.

Rab7 Regulates Multiple Steps in the Endosomal Pathway

As one approach to determine the function of Rab7 in *D. discoideum*, we generated cell lines that overexpressed a WT, a constitutively active, and a dominant

Figure 3. Rab7 is localized to punctate structures and large vesicles. Growing *D. discoideum* control cells were pipetted onto glass coverslips, incubated in growth medium for 30 min, fixed with formaldehyde, and permeabilized as described in MATERIALS AND METHODS. Cells were incubated with a 1:20 dilution of affinity-purified Rab7 polyclonal antibodies for 2 h. Coverslips were washed and incubated with a 1:40 dilution of secondary goat anti-rabbit antibodies coupled to FITC for an additional 2 h. After washing, the coverslips were mounted onto slides and viewed with an Olympus fluorescence microscope. (A) Phase-contrast micrograph (100 $\times$ magnification). (B) IF micrograph (100 $\times$ magnification). Bar, 1.5 μ m. Arrowhead, phase-translucent (A) and Rab7-negative vesicle (B); arrow, phase-opaque (A) and Rab7-positive (B) vesicle.

negative form of Rab7. The point mutations made in the Rab7 protein to generate the constitutively active and dominant negative overexpressing cell lines were designed to mimic the effect of similar mutations made in the guanine nucleotide binding regions of the Ras protein. The constitutively active form of Rab7 (Rab7 Q67L) was generated by changing a glutamine residue at position 67 to a leucine, in the WDTAGQE region. This mutation has been shown to cause Ras to preferentially bind GTP and decreases the intrinsic

GTPase activity of the protein (John *et al.*, 1993; Frech, *et al.*, 1994). The dominant negative form of Rab7 (Rab7 T22N) was generated by substituting a threonine residue at position 22 for an asparagine in the GKTS region. Ras mutant proteins containing this substitution preferentially bind GDP (Feig and Cooper, 1988; Farnsworth and Feig, 1991; John *et al.*, 1993). The WT and the mutated forms of the Rab7 cDNAs were subcloned into the pAD80-HA vector containing the flu HA epitope so that the overexpressed forms of

Figure 4. Rab7 localizes to sucrosomes containing fluid phase markers. Control cells growing in HL5 medium were seeded onto glass coverslips, fixed with formaldehyde, and labeled with affinity-purified Rab7 antibodies (B, D, and F) or monoclonal antibodies to the 100-kDa proton pump subunit (A and C). Cells in A and B were not treated, but cells in C–F were exposed to sucrose to form sucrosomes. FITC-labeled goat secondary antibodies were used for cells in A and C, and goat anti-rabbit rhodamine isothiocyanate (RITC)-labeled antibodies were used for cells in B, D, and F. Cells that had internalized FD (E) were visualized with the FITC filter. Bar, 2 μ m. Open arrows in C and D, Rab7-positive ATPase-negative vesicles; arrows in C and D, ATPase-positive Rab7-negative vesicles; open arrows in E and F, Rab7-positive FD-positive vesicles.

Rab7 were epitope-tagged at the N terminus and expressed constitutively from the act15 promoter.

G418 antibiotic-resistant cell lines were isolated, and the levels of overexpression of Rab7 were determined by Western blot analysis. As shown in Figure 5, the Rab7 antibody recognizes two major forms of the Rab7 protein, the endogenous form (22 kDa; found in all lanes) and a higher molecular weight form (23 kDa; found only in lanes WT, Q67L, and T22N). This higher molecular weight form is tagged with HA as determined by decoration of blots with antibodies recognizing the HA epitope (Bush *et al.*, 1994). The faint bands below 22 kDa represent nonspecific proteins recognized by the crude antiserum used in these experiments. Rab7 proteins were estimated to be overexpressed only two to threefold over the endogenous protein, thus minimizing the potential that these proteins would nonspecifically modulate the function of other Rabs in other pathways. Multiple clonal cell lines were isolated expressing all forms of Rab7, and three independent pools of transformed clones (10–20 colonies per plate) were isolated for Rab7 T22N. After

the completion of preliminary experiments, representative clones were chosen for Rab7 WT and Rab7 Q67L, and a representative “pool” was chosen for Rab7 T22N. The control cell line used was the Ax4 strain transformed with the pAD80-HA plasmid without a cDNA insert. All strains were maintained under selection with G418 at 20 μ g/ml.

D. discoideum cells rapidly internalize fluid phase material by pinocytosis. We therefore measured the rates of fluid phase uptake in control cells and cell lines overexpressing Rab7 WT, Rab7 Q67L, or Rab7 T22N by feeding cells FD. At the times indicated in Figure 6A (a representative experiment), cells were removed, centrifuged, washed, and lysed with detergent; and the intracellular fluorescence was measured as described in MATERIALS AND METHODS. To correct for any variation in the volume of different cell lines, internalization of FD was normalized to protein levels. As shown in Figure 6A, control cells and the overexpressing Rab7 WT and Rab7 Q67L cell lines internalized FD in a linear manner and at comparable rates for 60 min (see Figure 7A for statistical analysis);

Figure 5. Generation of cell lines overexpressing Rab7 WT, Rab7 Q67L, and Rab7 T22N. Cell lines growing in HL5 medium and overexpressing Rab7 proteins were solubilized with SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose (5×10^5 cell equivalents). Rab7 was detected as described in Figure 2. Lanes correspond to the following: Control, empty vector cells; WT, Rab7 wild-type cell line; Q67L, Rab7 Q67L cell lines; and T22N, a Rab7 T22N-expressing pool of 10 clones.

in contrast, cells overexpressing Rab7 T22N internalized FD at 25–30% of the rate of control cells (Figures 6A and 7A). Rab7 WT and Rab7 Q67L cells internalized FD at a slower rate than control cells after 60 min.

Although observed in mammalian cells, no major rapid recycling early endosomal compartment has been identified in *D. discoideum*; it takes 45–60 min for internalized fluid phase material to traverse the entire endocytic pathway (called the flux time) and then be exocytosed (effluxed) from cells. To measure the timing and the rate of efflux of FD from control and Rab7-overexpressing cell lines, logarithmically growing cells were fed FD for 10 min, centrifuged, washed, and resuspended in fresh growth medium. At the indicated times, samples were removed, washed, and lysed with detergent, and the intracellular fluorescence was measured and plotted as the percentage of FD remaining in the cells over time. As shown in Figure 6B (a representative experiment), FD began to be effluxed from the Rab7 WT cells by 30 min as compared with 45–60 min of chase for the control cells (the symbols used in Figure 6A apply to Figure 6, B–D). In addition, less than 20% of the FD remained internally in Rab7 WT cells by 60 min of chase; in contrast, only 20–25% of the FD had been effluxed from control cells at this time. This result (summarized in Figure 7B) indicates the efflux rate for FD was higher in the Rab7 WT cells than in the control cells. Comparable results were also seen for the Rab7 Q67L cell line, although the efflux rates were intermediate between control cells and the Rab7 WT cell line (Figures 6B and 7B). In contrast, a cell line overexpressing Rab7 T22N effluxed fluid phase material at a significantly reduced rate compared with control cells (Figures 6B and 7B). For instance, 50% of the FD remained intracellular after 120 min of chase for Rab7 T22N versus 20% for control cells at this time (Figure 6B). Thus, these results indicate Rab7 may also regulate postinternalization steps along the endocytic pathway leading to lysosomes and postlysosomes.

Lysosomes are small ($<1 \mu\text{m}$) acidic (average pH 4.8–5.0) vesicles, whereas postlysosomes are larger ($>2 \mu\text{m}$) and less acidic vesicles (Cardelli *et al.*, 1989;

Figure 6. Rab7 regulates endocytosis and exocytosis of the fluid phase. Fluid phase influx rates (A), efflux rates (B and C), and phagocytosis rates (D) were measured as described in MATERIALS AND METHODS. Cells were fed FD (A and B, 2 mg/ml; C, 5 mg/ml) or crimson-labeled 1- μm latex beads (D), and at the times indicated, 1 ml of cells was removed, and washed twice with growth medium and once with 5 mM glycine (pH 8.5) containing 100 mM sucrose (A, B, and D). The cells were lysed with Triton X-100 and fluorescence measured as described in MATERIALS AND METHODS. The percentage of FD remaining intracellular was calculated by dividing the intracellular fluorescence measurement at the time indicated by the fluorescence reading at the start of the chase. For pH measurements (C), samples were washed twice with growth medium and once with 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5. The intravesicular pH was determined by calculating the fluorescence ratio (excitation wavelengths, 495 nm/450 nm) at an emission wavelength of 520 nm. $\square$, Control, empty vector; $\diamond$, HA7, Rab7 WT cell line clone 7; $\circ$, QL5, Rab7 Q67L clone 5; $\triangle$, TNP5, Rab7 T22N pool 5.

Aubry *et al.*, 1993; Padh *et al.*, 1993). To begin determine the postinternalization step along the endosomal pathway regulated by Rab7, we used a similar pulse-chase format as discussed in Figure 6B using FD as a pH-sensitive probe to measure the intravacuolar pH. At various times, cells were isolated and the intravacuolar pH was measured as described in MATERIALS AND METHODS. As indicated in Figure 6C, in control cells, FD rapidly (within 10 min) entered a compartment with a luminal pH < 5.0 . After 10 min, FD entered compartments of a more alkaline pH, reaching vesicles (postlysosomes) with a luminal pH of 5.6 by 60 min of chase. Furthermore, internalized FD moved from vesicles with a low intraluminal pH to vesicles with a higher pH more rapidly in Rab7 WT

and Rab7 Q67L cell lines. In contrast, after internalization in Rab7 T22N cell lines, FD entered an acidic compartment with a pH of 5.1 by 20 min of chase and remained in vesicles with this lower pH for the remainder of the chase period (Figure 6C). Thus, these results support the hypothesis that Rab7 regulates trafficking at a late step along the endocytic pathway, perhaps between the lysosomal and postlysosomal compartments.

Since Rab7 was enriched in purified maturing phagosomes (Figure 2D), we determined whether Rab7 played a role in phagocytosis. The different Rab7-expressing cell lines were fed crimson carboxylate-modified latex beads (1 μ m in diameter; 50 beads/cell) for up to 120 min. At the times indicated in Figure 6D, cells were removed, centrifuged, washed, and lysed with detergent, and the fluorescence was measured. Fluorescence was normalized to micrograms of protein to correct for any size variation between the cell lines. The rates of phagocytosis of beads for the control, Rab7 WT, and Rab7 Q67L cell lines were not significantly different (Figure 7C). In contrast, the Rab7 T22N cells phagocytosed beads at a decreased rate (one-third the rate of control cells), indicating the Rab7 T22N cells might be defective in phagocytosis (Figures 6D and 7C).

Rab7 Regulates the Morphology of the Endolysosomal System

The data described above suggest that Rab7 regulates a late endosomal trafficking step, perhaps the step regulating transport from acidic lysosomes to less acidic postlysosomes. A prediction of this hypothesis is that because Rab7 WT-expressing cells efflux fluid phase faster than control cells (efflux occurs from postlysosomes), the former cells might have a proportionally greater number of large less-acidic postlysosomes and a small number of acidic lysosomes compared with the control cells after a pulse with FD. In contrast, Rab7 T22N cells might contain a proportionally greater number of small acidic lysosomes as compared with control cells, because the fluid phase appears to reside in acidic compartments longer during a chase and is more slowly effluxed (Figure 6C). To test this hypothesis, cells were pulse labeled with FD for 3 h, washed, placed on glass coverslips, and examined by fluorescence microscopy. Control cell lines contained on average 50–100 small FD-positive (weakly fluorescent) vesicles (<1 μ m in diameter;

Figure 7 (cont). Statistical representation of data used for endocytosis, efflux, and phagocytosis. Selected time points for endocytosis of FD (A, 30 min), efflux of FD (B, 30, 60, and 90 min), and phagocytosis (C, 30 min) were averaged and plotted. Data are the average $\pm$ SD ($n = 3$).

marked with an arrowhead) as well as three or four large FD-positive (strongly fluorescent) vesicles ($>2 \mu\text{m}$ in diameter; marked with a bar; Figure 8A). Cells were also exposed to acridine orange (AO), a substance that accumulates in high concentrations in acidic compartments and as a result fluoresces red-orange. As indicated in Figure 8B, $>90\%$ of the small faint FD-positive vacuoles were also AO-positive, indicating that these vesicles were acidic, but in contrast, the large vesicles (postlysosomes), being less acidic than lysosomes, did not take up as much AO and were only weakly fluorescent (Figure 8B). Interestingly, and in contrast to the hypothesis presented above, Rab7 Q67L (Figure 8, C and D) and Rab7 WT (Figure 8, G and H) contained a nearly equal mixture of small FD-positive/AO-positive acidic vesicles and "clusters" of small FD-positive/AO-negative (less-acidic) vesicles. In contrast, Rab7 T22N cells contained on average >5 large FD-positive/AO-positive vesicles and a relatively small number of vesicles $< 0.5 \mu\text{m}$. These larger vesicles were similar in size to the postlysosomes found in the control cells except that they were acidic (Figure 8, compare E and F with A and B), in agreement with the pH data described in Figure 6C. These large vesicles might represent enlarged lysosomes or acidic postlysosomes.

Rab 7 Does Not Regulate the Processing and Sorting of Lysosomal Enzymes but Regulates Intracellular Retention of Hydrolases

To determine whether Rab7 regulated the lysosomal enzyme biosynthetic targeting pathway to lysosomes, logarithmically growing cells were pulse labeled with [^{35}S]methionine for 20 min, and the cells were centrifuged and resuspended in label-free medium to begin the chase. At various times, samples were removed and separated into cell pellets and extracellular medium fractions. α -Mannosidase was immunoprecipitated from both cell fractions and medium supernatants with the monoclonal antibody 2H9, and radiolabeled proteins were separated by SDS-PAGE and detected by fluorography. α -Mannosidase is synthesized as a 140-kDa precursor that is processed to an 80-kDa intermediate and an 58-kDa mature subunit (Mierendorf *et al.*, 1985; Wood and Kaplan, 1985). The 80-kDa intermediate is then processed in lysosomes to a 60-kDa mature subunit. As shown in Figure 9, 90–95% of the 140-kDa α -mannosidase precursor was processed to the mature 58- and 60-kDa subunits with a half time of 20–30 min in all cell lines tested. In addition, $<5\%$ of the labeled precursors were missorted and secreted. Because the final proteolytic events occurs in lysosomes, it is apparent that Rab7 does not function to significantly regulate the transport of hydrolases from the endoplasmic reticulum to lysosomes. In control, as well as in cells overexpressing

Figure 8. Overexpression of Rab7 alters the morphology of FD-containing compartments. Control cells and cells overexpressing Rab7 were fed FD (2 mg/ml) for 3 h, washed, and placed on ice. AO was then added for 10 min, and the cells were washed and viewed with a fluorescence microscope using the appropriate filters to visualize only fluorescein (A, C, E, and G) or AO (B, D, F, and H). Vesicles that are FD positive and AO negative are marked with a bar and vesicles that are FD positive and AO positive are marked with arrowhead. (A and B) control cells; (C and D) Rab7 Q67L cells; (E and F) Rab7 T22N cells; and (G and H) Rab7 WT cells. Bar, 2.5 μm .

Rab7 T22N, approximately 50% of the radiolabeled mature α -mannosidase subunits were detected in the

extracellular medium fractions by 240 min (Figure 9). In contrast, at 240 min of chase, <5% and <20% of the radiolabeled α -mannosidase was detected in the extracellular medium fractions recovered from Rab7 WT cells and Rab7 Q67L cells, respectively.

We also measured the levels of intracellular and extracellular α -mannosidase activity in logarithmically growing cultures (Figure 10A). Sixty-five percent of the total α -mannosidase activity was extracellular in control cell cultures and Rab7 Q67L cultures, but only 10% of α -mannosidase activity was extracellular in cultures of Rab7 WT cells. In contrast, >95% of the α -mannosidase activity was secreted from cells expressing Rab7 T22N.

Rates of Secretion of Lysosomal Enzymes Were Altered in Cell Lines Overexpressing Rab7

To quantitate the rates of secretion of lysosomal enzyme activity from the different cell lines, standard secretion assays were performed with cells suspended in growth medium and starvation buffer. *D. discoideum* can be induced to enter the developmental cycle by removal of its food source, and an early response to starvation is the rapid secretion of lysosomal hydrolases (Cardelli, 1993). Logarithmically growing cells were harvested and resuspended in either fresh growth medium or 10 mM sodium phosphate buffer (starvation buffer). At the times indicated in Figure 10, B and C, samples were removed and washed, and α -mannosidase activity was measured in both the cell pellets and the extracellular supernatants and expressed as the percentage of the total enzyme activity (intracellular and extracellular). α -Mannosidase activity was secreted from control and Rab7 Q67L cell lines suspended in HL5 growth medium at approximately the same rate (5–6% of the total enzyme activity was released per hour; Figure 10B). In contrast, α -mannosidase was released at twice this rate from Rab7 T22N cells (12% released per hour), whereas Rab7 WT cells released <1.2% of the total enzyme activity per hour. These results are in relatively good agreement with the steady-state enzyme distribution results summarized in Figure 10A. Finally, starvation resulted in a 5- to 10-fold increase in the rate of secretion of α -mannosidase from all cell lines (Figure 10C), suggesting Rab7 did not regulate induced secretion.

Figure 9 (cont). Rab7 does not effect the processing and sorting of the lysosomal hydrolase α -mannosidase. Cells were pulse labeled with [35 S]methionine for 20 min, centrifuged, and resuspended in methionine-free medium. At the times indicated, samples were removed and α -mannosidase was immunoprecipitated from the cell pellets (Cells) and the extracellular supernatants (Medium) with the monoclonal antibody 2H9 and processed as described in MATERIALS AND METHODS. Numbers on the left correspond to the α -mannosidase precursor (140 kDa) and the mature subunits (58 and 60 kDa).

Figure 10. Secretion of lysosomal enzymes from Rab7-expressing cells. (A) Logarithmically growing cells were harvested by centrifugation and the percentage of total of α -mannosidase activity that was extracellular was measured. Logarithmically growing cells were also placed in growth medium (B) or 10 mM phosphate buffer (C) to induce secretion. At the times indicated, samples were removed and the percentage of α -mannosidase secreted was calculated from the amount of enzyme secreted into the extracellular medium vs. total enzyme activity in the culture. Symbols representing secretion kinetics for α -mannosidase for each strain are indicated in B.

DISCUSSION

To date, there have been only a few published studies investigating the function of the small molecular weight GTPase Rab7. Two studies, one in yeast (the GTPase is called Ypt7 in this organism) and the other in mammalian cells, support a role for Rab7 in the transport of proteins from early endosomes to late endosomes (Wichmann *et al.*, 1992; Feng *et al.*, 1995), whereas other studies (Schimmoller and Riezman, 1993; Vitelli *et al.*, 1997) implicate this GTPase in the regulation of traffic between late endosomes and the yeast vacuole (a lysosome-like organelle). Our study, presented herein, demonstrates that a *Dictyostelium* Rab7-like protein localized to lysosomes and postlysosomes may regulate retrograde transport of material late in the endosomal pathway.

Dictyostelium Contains a Single-Copy Gene Encoding a Protein Closely Related to Mammalian Rab7 that Localizes to Lysosomes and Postlysosomes

The *Dictyostelium* Rab7 protein is 88.5% identical to the mammalian Rab7 from amino acid positions 10 to 175 and completely homologous throughout the putative effector region (³⁷YKATIGADF⁴⁵; Chavrier *et al.*, 1990). This high degree of homology suggests that the *Dictyostelium* Rab7 may be a functional homologue of the mammalian Rab7. In addition, the *Dictyostelium* Rab7 protein is 71.5% identical in amino acid sequence to the yeast Ypt7 protein (Wichmann *et al.*, 1992) throughout this same region. Southern and Northern blot analyses indicated that *Dictyostelium* probably contained only one Rab7-like gene that was expressed at a fairly constant level during both growth and the

24-h developmental cycle that leads to the formation of a fruiting body structure (our unpublished results). Lysosomes in *Dictyostelium* are functionally and morphologically similar to mammalian lysosomes (Cardelli, 1993). These organelles are acidic, hydrolase rich, and are between 0.3 and 1.0 μ m in diameter. In mammalian cells, lysosomes represent a terminal endocytic compartment, but in *Dictyostelium*, lysosomes are a preterminal compartment. The terminal endocytic compartment in *Dictyostelium*, termed postlysosomes (Aubry *et al.*, 1993; Padh *et al.*, 1993), are relatively novel vesicles that are >2.0 μ m in diameter and contain a lesser amount of V-H⁺-ATPase and hydrolases than lysosomes (Rodriguez-Paris *et al.*, 1993; Nolte *et al.*, 1995). Magnetic fractionation of endocytic compartments containing colloidal iron coupled with Western blot analysis indicated Rab7 was highly enriched in lysosomes and postlysosomes, but the protein was not significantly enriched in pinosomes. Consistent with this observation, IF localization studies indicated that Rab7 was associated with both small punctate structures and membranes of vesicles the size of postlysosomes. In addition, large sucrosomes containing FD were also positive for Rab7. Interestingly, the Rab7 protein was associated with these large vesicles in a discontinuous "punctate" manner. One interpretation of this result is that Rab7 associates with small vesicles carrying proteins from lysosomes to postlysosomes, and the discontinuous staining pattern on large vacuoles represented vesicles that have docked but not yet fused. Magnetic fractionation studies of late endosomal compartments have, however, failed to detect the presence of iron-containing vesicles of the small size seen in Figure 2 (Nolte *et al.*, 1995).

Mammalian Rab7 associates with late endosomes and perhaps lysosomes (Chavrier *et al.*, 1990; Meresse *et al.*, 1995). Our Rab7 localization studies are consistent with this finding if one considers late endosomes in mammalian cells and lysosomes in *Dictyostelium* as preterminal endocytic compartments and mammalian lysosomes and *Dictyostelium* postlysosomes as terminal endocytic compartments. Finally, Rab7 was not enriched in very early phagosomes, but the protein was delivered along with other lysosomal components soon after particles were internalized (Rodriguez-Paris *et al.*, 1996; Rezabek *et al.*, 1997).

Dictyostelium Rab7 May Regulate an Early Step in Pinocytosis and Phagocytosis

A cell line overexpressing Rab7 T22N (the putative dominant negative form of Rab7) internalized fluid phase markers and latex beads at approximately one-third of the rate of control cells. In contrast, cell lines overexpressing Rab7 WT and Rab7 Q67L (constitutively active form) internalized material at rates similar to those observed for control cells. One interpretation of this result is that Rab7 directly regulates the internalization of vesicles budding from the plasma membrane. However, a number of lines of evidence argue against this hypothesis. First, Rab7 primarily associates with preterminal or terminal endocytic compartments in *Dictyostelium* and mammalian cells (Chavrier *et al.*, 1990; Meresse *et al.*, 1995). Second, newly formed phagosomes contain very little Rab7; this GTPase is delivered to phagosomes approximately 5 min after particles are internalized. Finally, previous studies in other systems have demonstrated that Rab7 probably does not regulate endocytosis (Wichmann *et al.*, 1992; Feng *et al.*, 1995), but other Rab proteins such as Rab5 and Rab4 have been suggested to play functional roles early in the endosomal pathway (Bucci *et al.*, 1992; van der Sluijs *et al.*, 1992; Bush *et al.*, 1996).

Therefore, we favor the possibility that Rab7 T22N may only indirectly regulate the earliest steps of pinocytosis and phagocytosis in *Dictyostelium*. For instance, as described below Rab7 T22N regulates a late step in the endosomal pathway, and in these mutant cell lines a rate-limiting endocytic component normally localized to the plasma membrane may be mislocalized. Alternatively, a block in transport late in the endosomal pathway may "jam" or freeze movement along early portions of the pathway. We cannot, however, rule out the possibility that the Rab7 T22N protein, because it is overexpressed, nonspecifically binds a protein required for the activation of another Rab protein such as Rab5 that directly regulates pinocytosis. However, the Rab7 T22N protein was only overexpressed two to threefold as compared with the en-

dogenous Rab7, making it unlikely that Rab7 T22N interferes with the function of other Rabs.

Rab7 also Regulates a Late Stage in Endocytosis

Internalized fluid phase markers were exocytosed at a significantly faster rate from cells overexpressing Rab7 WT compared with control cells. This suggests that Rab7 may function to positively regulate endocytosis at a postinternalization step and that this GTPase may normally be rate limiting in this process. In support of this latter possibility, fluid phase was exocytosed significantly slower from Rab7 T22N cell lines. Finally, the rate of fluid phase exocytosis was higher in Rab7 Q67L cell lines compared with that of control cells, but this difference was not significant, a result that is more difficult to interpret. One possibility is that Rab7 has to cycle between the GTP-bound and GDP-bound states to positively affect fluid phase recycling, consistent with the mechanism proposed for a variety of other Rab GTPases.

We therefore speculate that Rab7 may directly regulate the transport of protein and lipid between acidic lysosomes and less acidic postlysosomes. This hypothesis is supported by the following observations: 1) Rab7 was associated with lysosomes and postlysosomes in *Dictyostelium*; 2) fluid phase remained in acidic compartments (lysosomes?) for longer periods of time in Rab7 T22N cells compared with control cells; 3) FD exited acidic vesicles and reached less-acidic vesicles faster in Rab7 WT cells compared with control cells. Furthermore, the biosynthetic and endocytic pathways merge in a prelysosomal compartment (Richardson *et al.*, 1988; Cardelli, *et al.*, 1990), and as reported herein, lysosomal α -mannosidase was proteolytically processed to mature subunits at identical rates in all the strains examined. Because the final processing event occurs in lysosomes (Richardson *et al.*, 1988; Cardelli, *et al.*, 1989), this result suggests that Rab7 probably acts downstream of lysosomes to regulate endocytic flow. Finally, 95% of the α -mannosidase precursor polypeptides were processed and retained intracellularly, suggesting that Rab7 does not regulate the sorting event that delivers newly synthesized hydrolases to the endosomal pathway.

Model I: Rab7 Regulates Membrane Traffic between Preterminal Lysosomes and Terminal Postlysosomes in a Forward Direction

On the basis of the results we have discussed thus far, a relatively simple model can be proposed to explain the actions of Rab7: this GTPase regulates transport of material in a forward direction between lysosomes and postlysosomes (Figure 11, model I). If one assumes that lysosomes and postlysosomes in *Dictyostelium* are preterminal and terminal endocytic compartments, respectively, then this model is nearly identical

Figure 11. Two models to account for the actions of Rab7. See DISCUSSION for a description of evidence supporting either model I or model II.

to the model proposed to explain the function of Rab7 in yeast (Schimmoller and Riezman, 1993). This model could apply regardless of the exact mechanisms involved in movement of material from lysosomes to postlysosomes in *Dictyostelium*. One likely mechanism, however, involves lysosomes fusing with each other to generate postlysosomes (Nolta *et al.*, 1995) that in turn either directly fuse with the plasma membrane or less likely release small vesicles to transport fluid phase into the extracellular medium. An alternative possibility is that vesicles bud from stable lysosomes and fuse with stable postlysosomes. Consistent with this second hypothesis, IF microscopy indicated that Rab7 was associated with small punctate structures that might represent transport vesicles. This "forward flow" regulation model accurately predicts that cell lines overexpressing Rab7 WT would transport fluid more quickly from lysosomes to postlysosomes, and the opposite would be observed for cell

lines overexpressing dominant negative Rab7 (Rab7 T22N).

Our data cannot eliminate the possibility that Rab7 regulates transport in a forward direction from a pre-lysosomal compartment. However, the subcellular fractionation data (suggesting Rab7 localized to lysosomes and postlysosomes) and the lack of an effect of expressed mutant Rab7 proteins on the processing rates of newly synthesized hydrolases that enter the endosomal pathway just upstream of lysosomes would argue against this possibility.

Model I also predicts that if Rab7 regulates traffic from lysosomes to postlysosomes in a forward direction, then FD might accumulate, after a pulse, in a proportionally greater number of acidic vesicles the size of lysosomes in Rab7 T22N cell lines compared with control cells. In addition, after a pulse there should be more FD-containing large postlysosomes in Rab7 WT cells compared with control cells. Finally, if model I is correct, then mature lysosomal enzymes might be released at a faster rate and to a greater extent into the medium from growing Rab7 WT cells compared with control cells, and the opposite should be observed for Rab7 T22N cell lines. As reported in RESULTS and described in the next section, these results were not observed.

Model II: Rab7 Acts in a Retrograde Manner to Regulate Traffic from Postlysosomes and Lysosomes

After a 2-h pulse, FD accumulated primarily in small vesicles (mostly $<1.0 \mu\text{m}$ in diameter) in Rab7 Q67L and Rab7 WT cell lines. Approximately one-half of these vesicles did not accumulate significant levels of AO, suggesting they were not acidic, and very few FD-containing large nonacidic postlysosomes were observed in these cell lines. In contrast, in control cells, FD accumulated primarily in small acidic vesicles and in 5 or 10 less acidic larger vesicles (postlysosomes) per cell. In support of these observations, transmission electron microscopy demonstrated that Rab7 Q67L and Rab7 WT cell lines contained proportionally more small iron dextran containing vesicles and a smaller number of large vacuoles than control cells (our unpublished results). Equally surprising, after a pulse, FD primarily accumulated in Rab7 T22N cells in acidic vesicles as large or larger than postlysosomes observed in control cells.

In addition, contrary to the release of fluid phase, only 5–10% of the total lysosomal α -mannosidase activity accumulated extracellularly in cultures of Rab7 WT cells, whereas 60–70% and >95% of the total hydrolase activity was found extracellular in control cultures and Rab7 T22N cultures, respectively. Experiments to measure the rate of release of hydrolases from the different cell lines during growth reflected the steady-state distribution data, suggesting that

Rab7 may act in a positive manner to retain hydrolases.

Therefore, when we take these data into account, we propose a second model (Figure 11) to explain the function of Rab7. We propose that Rab7 may normally function to regulate the retrograde transport (postlysosomes to lysosomes) of small vesicles containing hydrolases and proton pumps. This model accounts for the fact that fluid phase markers such as FD and mature hydrolases colocalize in postlysosomes but that FD is very rapidly released from growing cells whereas the hydrolases are preferentially retained (Padh *et al.*, 1993). This model also predicts that the vesicles that bud from postlysosomes would be rich in hydrolases but almost depleted of fluid phase markers. Because these vesicles may not contain high levels of fluid phase material (such as iron dextran), we predict that, as reported (Rodriguez-Paris *et al.*, 1993; Nolte *et al.*, 1995), they would not be recovered in the population of vesicles and vacuoles purified by magnetic fractionation. This model also is consistent with IF microscopy indicating Rab7 was associated with small punctate structures, some of which were associated with vacuoles the size of postlysosomes; these later structures might represent Rab7-rich vesicles budding from postlysosomes. Finally, unlike model I, model II describes a mechanism to account for the reduction of proton pumps in postlysosomes compared with lysosomes.

Model II also correctly predicts that overexpression of Rab7 WT should result in a greater retention of hydrolases because of a more efficient delivery of enzymes from secretory postlysosomes to lysosomes. More efficient vesicle budding and transport might also result in a steady-state decrease in the size of nonacidic postlysosomes. In fact, most of the nonacidic FD-containing vesicles were smaller than control postlysosomes in Rab7 WT and Rab7 Q67L cell lines. Model II also predicts that overexpression of dominant negative Rab7 T22N should result in the formation of large acidic vacuoles the size of postlysosomes and that a greater percentage of the total cellular α -mannosidase would reside, in steady state, in postlysosomes, resulting in a greater percentage being secreted.

The fluid phase recycling data used to formulate model I is not inconsistent with the predictions of model II. For instance, retrograde transport of proton pumps back to lysosomes may be coupled to or occur soon after the formation of postlysosomes, and this may be a necessary event for the fluid phase to be efficiently released from cells. Therefore, model II predicts that Rab7 WT cell lines would more efficiently recover proton pumps from postlysosomes and fluid would be released more quickly, Rab7 T22N cells would only inefficiently recycle the V-H⁺-ATPase,

and fluid would be retained longer in acidic compartments compared with control cells.

Rab7 would not be the first example of a GTPase regulating retrograde membrane transport. In fact, it has been postulated that Rab6 controls intra-Golgi transport by acting as a positive regulator of retrograde transport (Martinez *et al.*, 1994). In addition, Rab9 has been implicated in the transport of mannose-6-phosphate receptors from late endosomes back to the *trans*-Golgi network (Riederer *et al.*, 1994). Also, recent evidence has been reported that supports the possibility of lysosome to late endosome retrograde transport (Jahraus *et al.*, 1994), and others have speculated that Rab7 vesicles may bud from lysosomes and return to late endosomes via a microtubule-dependent route (Meresse *et al.*, 1995).

CONCLUSION

We have determined that Rab7 regulates membrane flow at a late step in the endosomal pathway possibly between lysosomes and postlysosomes. Rab7 also plays a role in regulating internalization of fluid and particles. The consequences of overexpressing a dominant negative form of Rab7 include a reduction in the rate of exocytosis of the fluid phase and surprisingly an oversecretion of mature lysosomal enzymes. In contrast, overexpression of WT Rab7 resulted in an increase in the rate of exocytosis of the fluid phase and a greater cellular retention of lysosomal enzymes. The biosynthetic targeting pathway was not apparently affected in any of the Rab7-expressing cell lines. Although a number of models can account for these data, we currently favor and are testing a model that proposes that Rab7 functions to regulate retrograde transport from postlysosomes to lysosomes.

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