

# RabD, a *Dictyostelium* Rab14-related GTPase, regulates phagocytosis and homotypic phagosome and lysosome fusion

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## Summary

RabD, a *Dictyostelium* Rab14-related GTPase, localizes in the endo-lysosomal pathway and contractile vacuole system of membranes. Cell lines expressing dominant-negative RabD were defective in endocytosis, endosomal membrane flow and homotypic lysosome fusion. In support of a role for RabD in fusion, cells overexpressing constitutively active RabD<sup>Q67L</sup> accumulated enlarged hydrolase-rich acidic vesicles ringed with GFP-RabD, consistent with RabD directly regulating lysosome fusion. To determine whether RabD also regulated phagocytosis and/or homotypic phagosome fusion (a process stimulated by many intracellular pathogens), cells overexpressing dominant-active (RabD<sup>Q67L</sup>) or dominant-negative (Rab<sup>N121I</sup>) RabD were analyzed microscopically and biochemically. The rate of phagocytosis was increased two-fold in RabD<sup>Q67L</sup>-expressing cells and reduced by 50% in RabD<sup>N121I</sup>-expressing cells compared with control cells. To examine the role of RabD in the formation of multiparticle

phagosomes, we performed a series of pulse-chase experiments using fluorescently labeled bacteria and fluorescent latex beads. The rate of fusion of newly formed phagosomes was five times higher in the RabD<sup>Q67L</sup>-expressing cells and reduced by over 50% in RabD<sup>N121I</sup>-expressing cells as compared with control cells. GFP-RabD<sup>Q67L</sup> was found to ring multiparticle spacious phagosomes, which supports a direct role for this protein in regulating fusion. Inhibition of PI 3-kinase activity, which is known to regulate phagosome fusion in the wild-type cells, reduced the rate of phagosome fusion in RabD<sup>Q67L+</sup> cells, indicating that RabD acted upstream of or parallel with PI 3-kinase. We hypothesize that RabD and, possibly, Rab14, a related GTPase that associates with phagosomes in mammalian cells, are important regulators of homotypic phagosome and endo-lysosome fusion.

Key words: Phagocytes, Phagosome fusion, Rab14, *Dictyostelium*

## Introduction

Phagocytosis, a process involving the internalization of bacteria and particles by professional phagocytes, is a critically important first line of defense employed by multicellular organisms in response to invading pathogens. Phagosomal maturation, involving fission/fusion events with the endosomal pathway is a process that usually results in the destruction of intracellular pathogens in both mammalian and simple eukaryotic cells (Tjelle et al., 2000). Rab GTPases, members of the Ras superfamily, play an important role in endocytic and exocytic membrane trafficking (Takai et al., 2001), and these proteins have been implicated in phagosome formation and maturation (Duclos and Desjardins, 2000; Rupper et al., 2001). It has been proposed that intracellular bacterial pathogens manipulate membrane trafficking to prevent phagosome-lysosome fusion in part by regulating the localization or activity of Rab GTPases. For example, both *Leishmania* and *Salmonella* bacterium reside in phagosomes that are devoid of Rab7, a Rab GTPase important in regulating fusion of late endosomes and lysosomes with phagosomes (Hashim et al., 2000; Scianimanico et al., 1999). Selective accumulation of Rab5 and lack of retention of Rab7 have been hypothesized to be important in blocking maturation of phagosomes containing *Mycobacterium bovis* (Deretic et al., 1997). Rab11, when localized to recycling endosomes, has also

been implicated in the internalization of bacteria by macrophages, perhaps by regulating membrane transport from recycling endosomes to the forming phagocytic cup (Cox et al., 2000). These data support an important role for Rab GTPases in the formation and maturation of the phagosome and the clearance of pathogens in mammalian cells.

Phagosome-phagosome fusion is an important but poorly characterized maturation process occurring in mammalian cells infected with certain bacteria. As an example, phagosomes containing virulent strains of *Helicobacter pylori*, a human pathogen thought to cause gastritis, gastric ulcers and gastric adenocarcinomas, fuse to form multiparticle phagosomes in macrophages. Phagosome fusion requires bacterial protein synthesis and may facilitate survival of intracellular bacteria. Less virulent strains (type II) lacking the *cag* pathogenicity island are killed and do not stimulate phagosome fusion (Allen et al., 2000). Phagosomes containing *Chlamydia trachomatis* elementary bodies fuse with each other to form an intracellular inclusion necessary for replication of the bacteria (Majeed et al., 1999). Finally, *Coxiella burnetii*, the causative agent of Q fever, and *Leishmania amazonensis*, the agent of leishmaniasis, can undergo heterotypic fusion with particle-containing phagosomes as well as undergoing homotypic fusion (Dermine et al., 2001; Howe and Mallavia, 2000).

*Dictyostelium discoideum*, a single-celled eukaryotic organism, is a professional phagocyte and is a useful genetically tractable system in which to study macropinosome and phagosome formation and maturation (Cardelli, 2001). For instance, *Legionella* replicates and prevents phagosome-lysosome fusion in *Dictyostelium* (Hagele et al., 2000; Solomon et al., 2000), mimicking the situation observed in mammalian cells (Roy et al., 1998). Furthermore, many of the steps involved in particle internalization and processing appear to be similar to those found in mammalian cells, including homotypic phagosome fusion (reviewed in Rupper and Cardelli, 2001a). Phagosomes mature through at least three different stages in *Dictyostelium*. First, newly formed phagosomes contain primarily plasma-membrane-derived proteins and F-actin and associated proteins (Rezabek et al., 1997). Next, many of these early proteins are recycled from phagosomes, which then fuse sequentially with hydrolase- and glycosidase-rich lysosomes, and the phagosomal lumens become acidic (Rezabek et al., 1997; Rupper et al., 2001a). After about 60 minutes, the intra-phagosomal pH rises to near neutral levels and phagosomes begin to fuse together in a PI 3-kinase- and protein kinase B (PKB)/Akt-dependent process, creating spacious multiple particle phagosomes (Rupper et al., 2001b). PI 3-kinase and PKB also regulate macropinocytosis and endosome fusion but not phagocytosis (Buczynski et al., 1997b; Rupper et al., 2001c). The increase in pH, regulated by PI 3-kinase, is important in regulating homotypic fusion, although the mechanisms required for this are still unclear.

Rab GTPases have also been implicated in phagosome formation and maturation in *Dictyostelium*. For instance, Rab7 and RabB appear to act in a positive fashion to regulate particle internalization (Rupper et al., 2001a) (E.H., unpublished). Rab7 is delivered to the phagosome within one minute of formation and is retained on the phagosome during the entire maturation process. Cells overexpressing dominant-negative Rab7 are not defective in the formation of spacious phagosomes, but these phagosomes do not contain normal levels of glycosidases or the lysosomal membrane protein, LmpA, suggesting that Rab7 regulates phagosome-lysosome membrane traffic (Rupper et al., 2001a). As observed in mammalian cells, Rab11 is also implicated in the internalization of particles (Harris et al., 2001), although in *Dictyostelium* this Rab may act in a negative fashion.

RabD, a *Dictyostelium* GTPase related to mammalian Rab14, appears to regulate macropinocytosis and endo-lysosomal fusion (Bush et al., 1996). RabD is primarily found on the contractile vacuole network of membranes, an organelle important in osmotic regulation and, in lesser amounts, in the endo-lysosomal pathway (Bush et al., 1994). Cells expressing dominant-negative RabD (RabD<sup>N121I</sup>) contained a morphologically altered contractile vacuole, were osmotically sensitive and exhibited a reduced rate of endocytosis (Bush et al., 1996). Furthermore, lysosomal fusion to generate post-lysosomes was also delayed (Bush et al., 1996). These studies did not examine the role of RabD in regulating phagocytic processes. To begin to determine the role of RabD in phagocytosis and phagosomal maturation, we have biochemically analyzed cells expressing dominant-negative and constitutively active forms of this GTPase. We demonstrate that RabD (1) regulates phagocytosis, (2) plays a prominent role in phagosome-phagosome and endo-lysosome fusion and

(3) and acts upstream or in a parallel pathway with respect to PI 3-kinase to regulate fusion.

## Materials and Methods

### Cell lines

All cell lines were generated using the *Dictyostelium discoideum* axenic strain Ax4. A single site mutation in RabD, replacing the glutamine with leucine at position 67 to generate a constitutively active protein product and replacing the asparagine for isoleucine at position 121 to generate a dominant-negative protein product, was achieved by a two-step PCR reaction. To make the overexpressing constitutively active and dominant-negative expressing cell lines, the resulting PCR-modified cDNAs were inserted into the MCS of pHA80, tagging the N-terminus with the HA epitope, then excised from the plasmid and inserted in the pVEII-ATG vector behind the inducible discoidin  $\gamma$ 1 promoter. Cells were transformed with this plasmid using the CaPO<sub>4</sub> precipitation method (Loomis, 1987). Expression of the HA-tagged mutant proteins relative to endogenous RabD levels was analyzed by western blotting. The GFP-RabD-fusion-expressing cell lines were made by introducing the HA-tagged RabD<sup>wt</sup> and mutant cDNAs into pDS6 (Seastone et al., 1998), which is a variant of the pVEII-ATG. Expression of the fusion protein in Ax4 cell lines was assessed by both fluorescence microscopy and western blot analysis.

### Phagocytosis assay

To measure the rate of phagocytosis, cells in log phase of growth in T-175 flasks were harvested and resuspended to 3.0×10<sup>6</sup> cells/ml in HL-5. After a recovery period of 15 minutes in shaking suspension at 21°C, cells were incubated with an equal volume of HL-5-containing red fluorescent beads (Molecular Probes, FluoSpheres carboxylate-modified microspheres 1.0  $\mu$ m, crimson fluorescent) at a final concentration of 100 beads/cell. At each time point, 3.0×10<sup>6</sup> cells were harvested, washed twice in cold HL-5, washed once in cold sucrose buffer (5 mM Glycine, 100 mM sucrose, pH 8.5) and lysed in 1 ml of sucrose buffer with 0.5% Triton X-100. Bead fluorescence was measured with a Hitachi F-4010 spectrofluorimeter at an excitation of 625 nm and emission of 645 nm. The total amount of particles internalized was calculated on the basis of fluorescence per microgram of protein to correct for differences in cell size or titer.

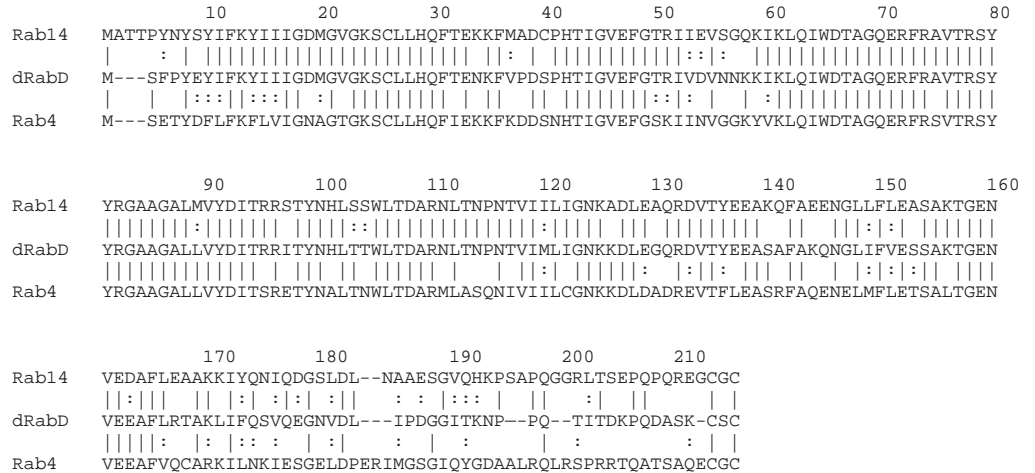
### Phagosome and endo-lysosome fusion assay

To measure rates of phagosome fusion, we used FITC-labeled bacteria (fl-bacteria) or deep blue latex beads (Sigma, L-1398) to visualize individual phagosomes. Cells were placed in shaking suspension and allowed to phagocytose fl-bacteria for 10 minutes followed by three washes with fresh HL-5. Next, cells were allowed to settle down on coverslips in HL-5 to initiate the 30 minute chase period. Coverslips were gently dipped in fresh HL-5 three times to wash the remaining fl-bacteria off followed by fixation of cells in 1% formaldehyde in HL-5. Coverslips were dipped again in HL-5 (10 times) to wash off any remaining fl-bacteria and mounted on slides to be examined with the fluorescence microscope. Cells with ~10 fl-bacteria per cell were examined, and the number of fl-bacteria in each phagosome was assessed using both phase-contrast and fluorescence optics. Control experiments indicated that particles were internalized individually.

## Results

### RabD is homologous to Rab14

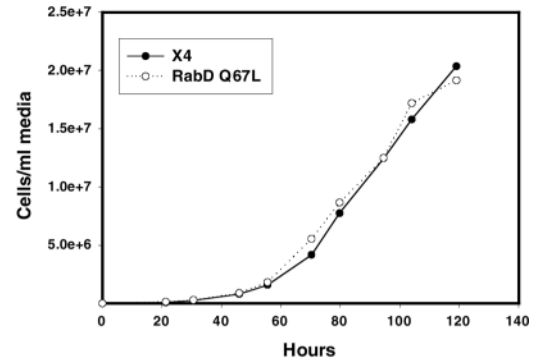
A previous search of an earlier version of the existing GenBank protein database revealed that RabD was closest in amino-acid sequence homology to mammalian Rab4, which is consistent



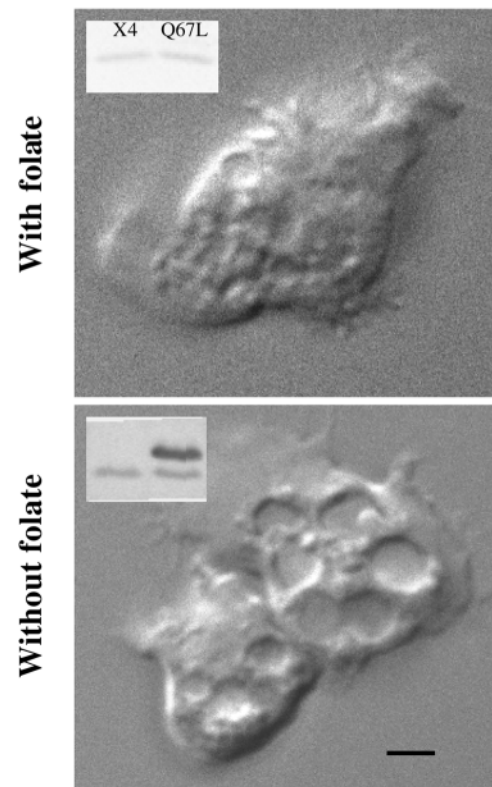
**Fig. 1.** RabD has a higher homology to Rab14 than to Rab4. The amino-acid sequence of human Rab14, *Dictyostelium* RabD and human Rab4 were compared using the BLAST program available on the NCBI database. Vertical lines represent identity and dotted lines represent similarity.

with the localization of both of these Rab GTPases to early endosomal compartments (Bush et al., 1994). A more current search of the newest protein database indicated that RabD is actually more homologous to Rab14 than to Rab4 (Fig. 1). In fact, from amino acid 3 to 163, RabD is 85% identical and 94% positive to Rab14 as compared with 70% identical and 84% positive to Rab4 over this same stretch of amino acids. A recent publication indicates that Rab14 is associated with phagosomal membranes (Garin et al., 2001), but nothing is known concerning the function of this protein. Given the high degree of amino-acid sequence homology between RabD and Rab14, we hypothesize that RabD may be a functional homologue of Rab14, and we began to explore the role of RabD in the phagocytic process.

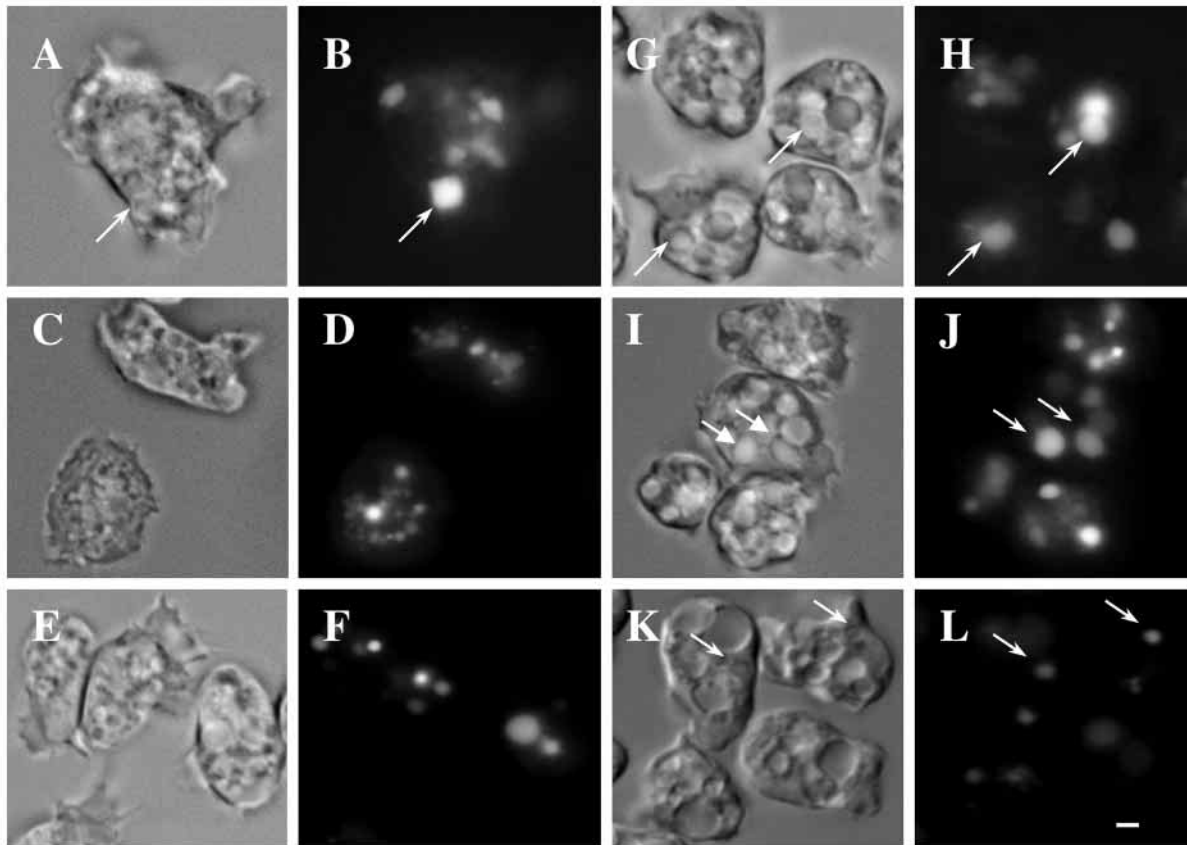
**Growth Curve of X4 and RabD Q67L in shaking suspension**



Cells expressing constitutively active RabD<sup>Q67L</sup> accumulate large endo-lysosome-like vesicles supporting a role for RabD in lysosome fusion. Cells expressing RabD<sup>N121I</sup> (a dominant-negative form of this GTPase) contain a larger number of acidic lysosomes and correspondingly a smaller number of larger less acidic post-lysosomes, as compared with control cells (Bush et al., 1994; Bush et al., 1996), suggesting a defect in lysosome fusion. To further examine the role of RabD in lysosomal fusion, a stable mutant strain of *Dictyostelium discoideum* was constructed that inducibly expresses HA-tagged RabD<sup>Q67L</sup> (a constitutively active mutant that preferentially binds to GTP). Removal of folate from the medium induces transcription of the discoidin promoter, which leads to an accumulation of activated RabD. Western blot analysis of a selected strain indicated that the level of the exogenously expressed mutant protein was two- to three-fold higher than the level of the endogenous form (Fig. 2) when cells were grown for two days without folate. Microscopic



**Fig. 2.** RabD<sup>Q67L</sup> expression results in the formation of large vacuolar structures. Ax4 cells were transformed with a plasmid containing the HA-tagged rabD constitutively active mutant cDNA driven by an inducible promoter that is turned off in the presence of folate or turned on in the absence of folate. The level of RabD expression was analyzed by western blot using anti-RabD antibodies (inset). The top panel indicates that the growth rate for wild-type and mutant cells are identical. Bar, 3 μm.



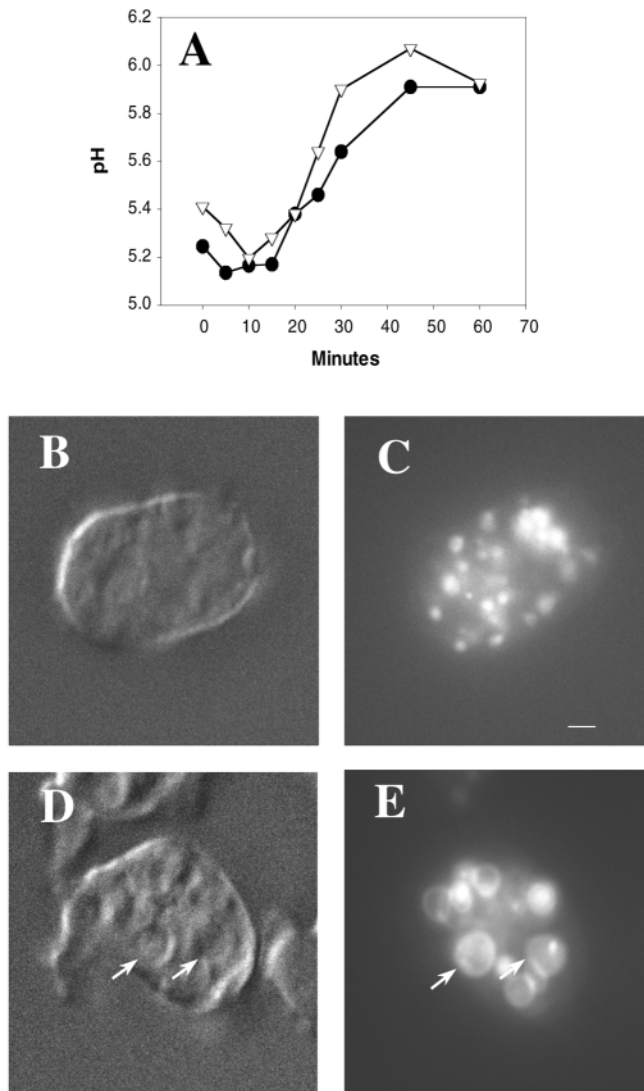
**Fig. 3.** The enlarged endocytic vesicles in cells expressing RabD<sup>Q67L</sup> are primarily lysosomes. Both wild-type cells (A-F) and RabD<sup>Q67L</sup>-expressing cells (G-L) were pulsed with FITC-dextran for 5 minutes and chased for 0 minutes to view macropinosomes (A,B,G,H); chased for 15 minutes to view lysosomes (C,D,I,J); and 60 minutes to view post-lysosomes (E,F,K,L). Cells were gently fixed and viewed using a fluorescence microscope. Bar, 2  $\mu$ m. The arrows reveal structures described in the text.

examination of cells expressing RabD<sup>Q67L</sup> (bottom panel; Fig. 2) indicated that they accumulated a greater number of enlarged vesicular structures as compared with the same clone of cells growing in the presence of folate (middle panel; Fig. 2), conditions that prevent expression of exogenous RabD. Control cells growing in the presence or absence of folate contained vesicles comparable in size to those observed in RabD<sup>Q67L</sup>-expressing cells grown in the presence of folate (data not shown for wildtype). Despite the accumulation of five or more vesicles  $>1.5 \mu$ m in diameter per cell, RabD<sup>Q67L</sup>-expressing cells increase in number at the same rate as control cells in the absence of folate (Fig. 2, top panel), suggesting that the presence of these enlarged vesicles did not adversely affect growth rate.

To determine if the large vesicles represented lysosomes, the following experiments were performed. First, control cells and RabD<sup>Q67L</sup>-expressing cells were exposed to FITC-dextran in growth medium for 5 minutes followed by a chase period in dextran-free growth medium. A portion of the cells was quickly fixed in formaldehyde immediately following the pulse, and at 10 and 60 minutes following the initiation of the chase period. Representative DIC and fluorescent images of these cells are shown in Fig. 3. The 5 minute pulse period is sufficient to load primarily macropinosomes with fluid phase (Seastone et al., 1998), whereas the 10 minute and 60 minute chase period have been demonstrated to load lysosomes and

post-lysosomes, respectively (Padh et al., 1993). Following a 5 minute pulse with FITC-dextran, control cells (Fig. 3A,B) and RabD<sup>Q67L</sup>-expressing cells (Fig. 3G,H) contained one to three fluorescently labeled vesicles  $>1.0 \mu$ m in diameter, indicating that only a small percentage of the large vesicles in RabD<sup>Q67L</sup>-expressing cells were newly formed macropinosomes. Following a chase of 10 minutes, control cells (Fig. 3C,D) contained primarily fluorescent vesicles the size of lysosomes as consistent with previous studies (Bush et al., 1996). In addition, most of the large vesicles in RabD<sup>Q67L</sup>-expressing cells (Fig. 3I,J) contained FITC-dextran, suggesting most of these enlarged vesicles might be lysosomes on the basis of their kinetics of loading with internalized fluid. Finally, following a 60-minute chase period, control (Fig. 3E,F) and RabD<sup>Q67L</sup>-expressing cells (Fig. 3K,L) contained a few fluorescent post-lysosomes 1-2  $\mu$ m in diameter. Few of these fluorescently labeled post-lysosomes corresponded to the enlarged vesicles.

Next, control and RabD<sup>Q67L</sup>-expressing cells were pulsed with FITC-dextran for 5 minutes, followed by a chase period of up to 60 minutes in growth medium lacking this fluorescent marker. At the time points indicated in Fig. 4A, cells were collected by centrifugation, and the intra-endosomal pH was measured as a function of FITC fluorescence as described in Materials and Methods. Consistent with the large vesicles being acidic lysosomes, the kinetics of movement of internalized fluid to the most acidic compartments were



**Fig. 4.** Most of the enlarged vesicles are acidic lysosomes in RabD<sup>Q67L</sup>-expressing cells. In A, both wild-type (○) and RabD<sup>Q67L</sup> (▽) cells were pulsed with FITC-dextran, washed and chased in fresh HL-5. At the indicated time points, cells were harvested, washed and resuspended in cold 50 mM MES buffer, pH 6.5. The ratio of fluorescence of FITC-dextran at 525 nm emission upon excitation at 495 nm/450 nm were measured against an *in vitro* standard curve from which the pH was extrapolated ( $n=3$ ). Normal size lysosomes in the wild-type Ax4 cells (B,C) and the enlarged lysosomes in the RabD<sup>Q67L</sup> cells (D,E) were observed when live cells were incubated with the acidophilic dye LysoSensor DND-189 (Molecular Probes). Bar, 2  $\mu$ m. Arrows reveal enlarged vesicles in D and E.

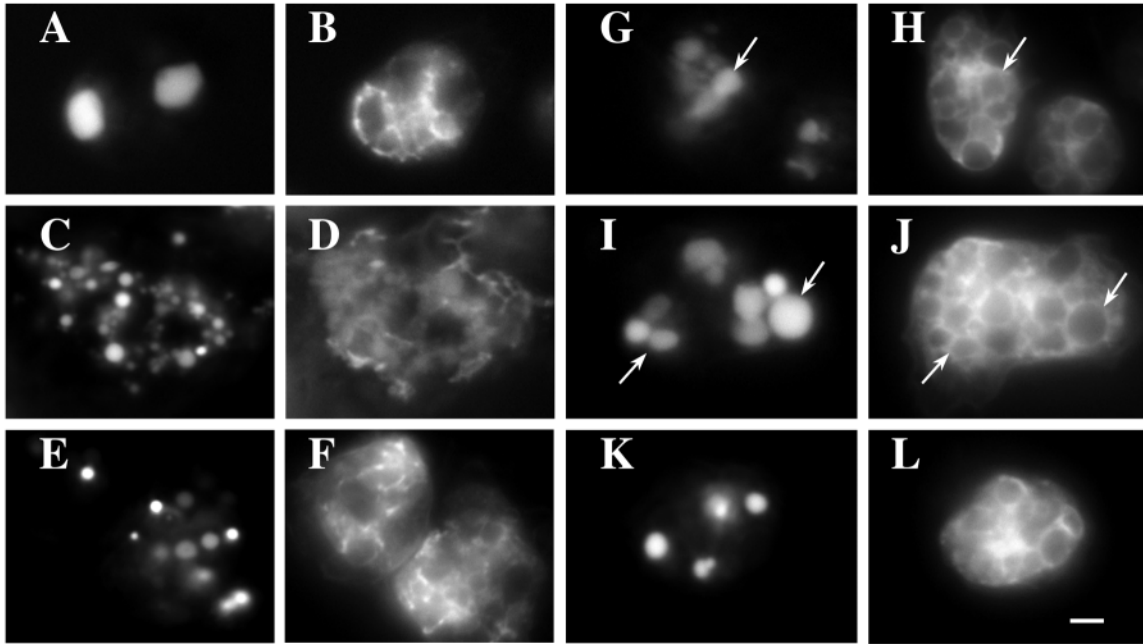
identical for control and RabD<sup>Q67L</sup>-expressing cells (Fig. 4A). Internalized fluid reached the most acidic compartments 15 minutes after internalization for both cell lines and accumulated in less acidic compartments over the next 45 minutes with the same kinetics. Together, these data suggest that most of the large endocytic vesicles in RabD<sup>Q67L</sup>-expressing cells are acidic, and they acquire fluid with the kinetics observed for the loading of smaller lysosomes in control cells.

Finally, live cells were incubated with LysoSensor Green DND-189 (Molecular Probes) for 2 minutes and immediately visualized by fluorescence microscopy. LysoSensor is only fluorescent under acidic conditions ( $\text{pH} < 5.0$ ). Fig. 4 indicates that most of the large vesicles in RabD<sup>Q67L</sup>-expressing cells were acidic (D,E), suggesting these vesicles had acquired the V-H<sup>+</sup>-ATPase proton pump. In the endosomal pathway the proton pump is most enriched in lysosomes, suggesting that the large vesicles in RabD<sup>Q67L</sup>-expressing cells may be lysosomes. As expected, control cells contained a greater number of smaller acidic vesicles (Fig. 5C). The large acidic vesicles were also purified using a previously published magnetic fractionation approach (Temesvari et al., 1996a) and demonstrated by western blot analysis to be enriched in lysosomal enzymes and the proton pump (results not shown).

#### GFP-RabD<sup>Q67L</sup> is enriched in large acidic lysosomes that arise by fusion

If RabD<sup>Q67L</sup> is directly involved in the formation of enlarged acidic lysosomes, one would expect to find this activated Rab associated with these compartments as observed for phagosomes. To test this hypothesis, stable cell lines inducibly expressing GFP-RabD and GFP-RabD<sup>Q67L</sup> were subjected to the same pulse-chase regiment using FITC-dextran as described in Fig. 3. Panels B, D and F of Fig. 5 indicate that the GFP-RabD wild-type protein distributed in control cells in the reticular network of the CV, a result consistent with previous published data on the basis of immunofluorescence microscopy (Bush et al., 1994). GFP-RabD only infrequently ringed macropinosomes (compare panel A with B), lysosomes (compare C with D) or post-lysosomes (compare E with F). By contrast, a small percentage of GFP-RabD<sup>Q67L</sup> localized to membranes of macropinosomes (G,H) and predominantly with membranes of large acidic lysosomes (I,J), consistent with a direct role for this activated Rab protein in the formation of these large vesicles. Only rarely was GFP-RabD<sup>Q67L</sup> found to associate with post-lysosomes (K,L).

To determine if RabD<sup>Q67L</sup> increased fusion between endo-lysosomes, as observed for phagosomes, cells were pulsed with RITC-dextran for 5 minutes, washed with growth medium for 2 minutes and then exposed to FITC-dextran for an additional 5 minutes. Cells were gently fixed after a chase period of 5 minutes, and images were collected using a digital camera attached to a fluorescence microscope. Fig. 6 indicates that a larger percentage of the endocytic vesicles in RabD<sup>Q67L</sup>-expressing cells (Fig. 6H) contained both RITC and FITC-dextran as compared with control cells (panel G), indicating an increased rate of fusion between different temporal classes of endo-lysosomes. Images, from over 100 cells for each strain were analyzed, and the number of vesicles that contained both fluorescent markers was calculated. The results indicate that at each chase point following the sequential internalization of two different fluid phase markers, the rate of fusion of temporally distinct early endosomes in RabD<sup>Q67L</sup>-expressing cells was twice that observed for control cells (data not shown). Interestingly, efflux rates of fluid were identical in mutant and wild-type cells, indicating that the accumulation of enlarged lysosomes does not significantly influence endosomal membrane trafficking (data not shown).



**Fig. 5.** GFP-RabD<sup>Q67L</sup> rings primarily enlarged lysosomes but not post-lysosomes. Both wild-type cells expressing GFP-RabD<sup>wt</sup> and cells expressing GFP-RabD<sup>Q67L</sup> (A-F) and RabD<sup>Q67L</sup> (G-L) cells were pulsed with Texas Red dextran for 5 minutes and chased for 0 minutes to view macropinosomes (A,B,G,H); chased for 15 minutes to view lysosomes (C,D,I,J); and 60 minutes to view post-lysosomes (E,F,K,L). Cells were gently fixed and viewed using a fluorescence microscope using GFP and rhodamine filter sets. GFP-RabD(WT) is normally predominately localized to the contractile vacuole (B,D,F), whereas GFP-RabD<sup>Q67L</sup> rings large vesicles (H,J,L) that accumulate fluid with the kinetics of lysosomes. Bar, 2  $\mu$ m. The arrows in G and H reveals a macropinosome, whereas the arrows in I and J reveal enlarged lysosomes.

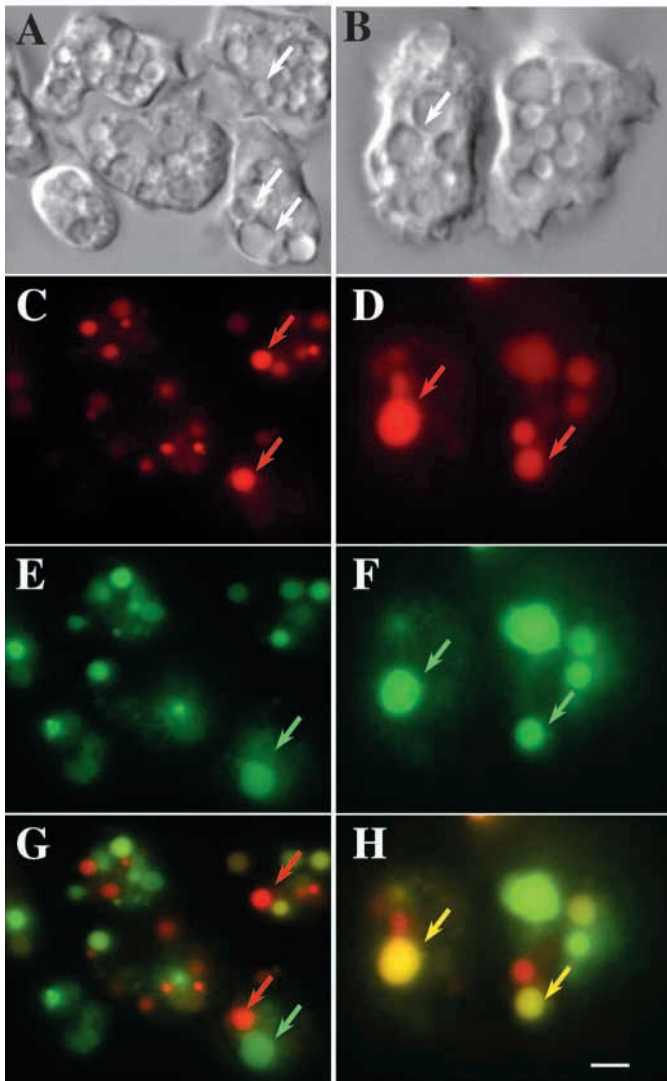
### RabD regulates phagocytosis

The following series of experiments was done to determine the role of RabD in the phagocytic pathway. To measure the rate of phagocytosis, both mutant-expressing cell lines and the parent strain, Ax4, were assayed for their ability to internalize latex beads. A representative experiment shown in Fig. 7A indicates that the rate of phagocytosis of latex beads in cells expressing RabD<sup>Q67L</sup> was more than twice the rate observed for the Ax4 parent strain. Conversely, cells expressing the dominant-negative mutant RabD<sup>N121I</sup> internalized beads at approximately 50% of the rate of the parent strain. A more complete quantitative analysis of particle uptake was performed and subjected to statistical analysis. Fig. 7B reveals that at the 30 minute time point, RabD<sup>Q67L</sup> cells had internalized more than twice as many beads as the control ( $n=5$ ,  $P<0.0001$ ) and over five times the number of beads as the dominant-negative cell lines ( $n=5$ ,  $P<0.001$ ). These data suggest that RabD may be involved in regulating the rate of internalization of beads. Comparable results were observed using *E. coli*, suggesting that RabD also regulates internalization of a physiologically relevant particle (data not shown).

### RabD regulates phagosomal fusion

Phagosome-phagosome fusion occurs in *Dictyostelium* and in mammalian cells containing a variety of intracellular pathogens, including species of *Helicobacter* and *Chlamydia*. Homotypic phagosome fusion in *Dictyostelium* depends on the activities of PI 3-kinase and PKB and is dependent on increases in pH beginning 45-60 minutes following internalization of

particles (Rupper et al., 2001b). In preliminary experiments, we observed that bacterially infected cells expressing RabD<sup>Q67L</sup> contained more large multiparticle phagosomes than control cells, suggesting RabD might regulate homotypic phagosome fusion. To quantify the rate of phagosome fusion, we pulsed cells in shaking suspension for 10 minutes with FITC-bacteria and allowed the cells to chase for 60 minutes while they settled on coverslips. Under these conditions bacteria enter as single particles (Harris et al., 2002), and 60 minutes of chase corresponds to the linear portion of the phagosome fusion curve. A representative fluorescence and phase contrast micrograph is shown in Fig. 8 and reveals that cells expressing activated RabD (middle panel) contain a greater number of multiparticle phagosomes as compared with control cells (left panel). At this time point, a smaller fraction of wild-type cells have multiparticle phagosomes containing two to three bacteria, whereas, by contrast, most of the cells expressing RabD<sup>Q67L</sup> contained multiple particle phagosomes each with more than three particles per phagosome. To confirm that phagosomes were fusing with each other as opposed to particles being internalized as aggregates, a fusion assay was employed in which cells were sequentially pulsed with both FITC-labeled bacteria and deep blue latex beads (fluorescing weakly in the rhodamine channel). Cells were first pulsed with the FITC-bacteria for 10 minutes, washed for 2 minutes in cold HL-5, pulsed with latex beads for 10 minutes and then chased in fresh HL-5 for 30 minutes. Most of the phagosomes in control cells (Fig. 9A,B) contained only one particle, whereas in RabD<sup>Q67L</sup> cells (C,D), most phagosomes contained both FITC-bacteria and latex beads, proving that phagosomes formed at different times and fused together at a higher rate.



**Fig. 6.** The enlarged vesicles in the RabD<sup>Q67L</sup>-expressing cells are the result of an increase in the rate of fusion between newly formed endo-lysosomes. Wild-type (A,C,E,G) and RabD<sup>Q67L</sup>-expressing (B,D,F,H) cells were first pulsed with RITC-dextran for 5 minutes (C,D), washed, pulsed with FITC-dextran for 5 minutes (E,F), washed, chased for 5 minutes and gently fixed with formaldehyde. Captured images of cells were viewed in the fluorescein, rhodamine and merged channels. The extent of fusion between endo-lysosomes containing both fluoros was measured in the wild-type (G) and RabD<sup>Q67L</sup>-expressing (H) cells by detecting colocalization of both FITC in the same vesicle. BAR, 2.5  $\mu$ m. The colored arrows indicate FITC-dextran-positive, RITC-dextran-positive and FITC/RITC-dextran-positive vesicles.

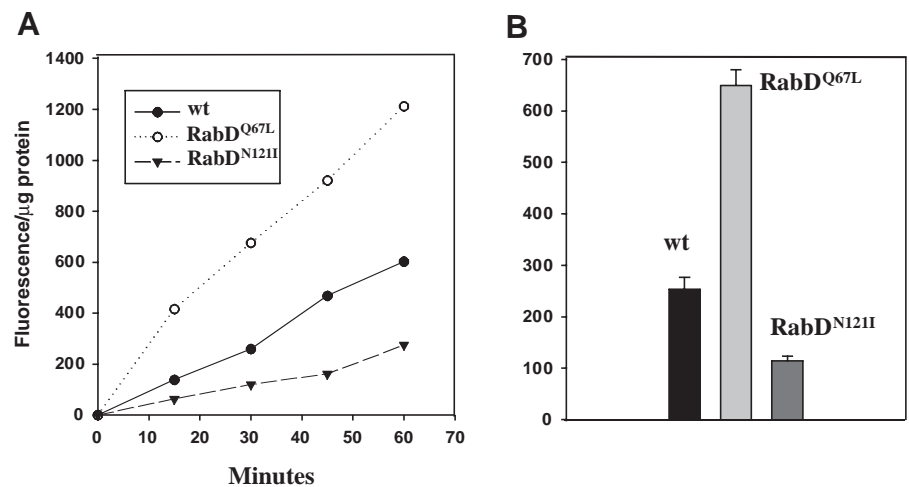
in the RabD<sup>Q67L</sup> cells versus control cells (Fig. 10A). We performed separate phagosomal fusion experiments with RabD<sup>N121I</sup>-expressing cells and found that the rate of fusion in this mutant was less than half the rate found in the control cells (Fig. 10B).

PI 3-kinase and PKB play important roles in the regulation of phagosome-phagosome fusion during the late stages of phagosome maturation (Rupper et al., 2001a). To determine if PI 3-kinase was involved in the RabD-mediated fusion pathway, we treated RabD<sup>Q67L</sup>-expressing cells with LY294002, a specific inhibitor for PI 3-kinase, and repeated the phagosome fusion experiments. Figs 8 and 10 indicate that the rate of phagosome fusion in RabD<sup>Q67L</sup> cells treated with LY294002 was significantly reduced compared with the untreated RabD<sup>Q67L</sup> cells. Specifically, drug-treated RabD<sup>Q67L</sup>-expressing cells treated with PI 3-kinase inhibitors contained phagosomes that were similar in size and contained the same number of bacteria as the control cell line (Fig. 8, far right panel). These results confirm that PI 3-kinase regulates homotypic fusion of phagosomes and that PI 3-kinase apparently acts downstream of or in parallel with RabD to regulate fusion.

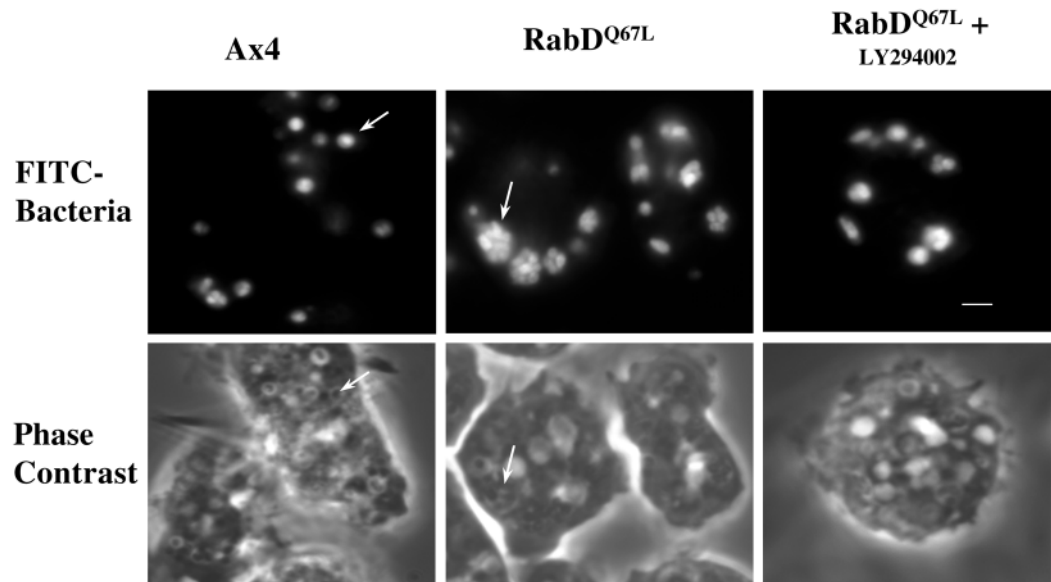
To determine if multiparticle phagosomes contained RabD, consistent with this GTPase playing a direct role in fusion, we pulsed GFP-RabD<sup>Q67L</sup>-expressing cells with RITC-labeled bacteria for 10 minutes, followed by a chase in fresh HL-5 for 30 minutes. Live cells were examined using a fluorescence microscope. Fig. 11 indicates that GFP-RabD<sup>Q67L</sup> ringed multiparticle phagosomes in nearly all of the cells observed,

The average number of particles per cell and particles per phagosome was enumerated, and the rate of fusion was calculated as described in the Materials and Methods. Phagosome-phagosome fusion occurred at five times the rate

**Fig. 7.** RabD regulates phagocytosis in *Dictyostelium* Ax4 cells. In A, the rate of phagocytosis was measured using fluorescent latex beads (F-8816) as a phagocytic marker. Cells were shaken in suspension, and for each time point,  $3 \times 10^6$  cells were harvested, washed and lysed. Bead fluorescence was measured at an excitation of 625 nm and emission of 645 nm. B depicts the number of beads that were internalized at the 30 minute time point of the phagocytosis assay. Both mutants had statistically significant differences in the number of beads internalized compared with the control. (RabD<sup>Q67L</sup>:  $P < 0.0001$ ; RabD<sup>N121I</sup>:  $P < 0.001$ ).



**Fig. 8.** Phagosome-phagosome fusion rates are increased in cells expressing RabD<sup>Q67L</sup>. Control (left panels), RabD<sup>Q67L</sup> (middle panels) and RabD<sup>Q67L</sup> treated with 20  $\mu$ M LY294002 (right panels) cells were incubated with FITC bacteria for 10 minutes, washed and chased for 60 minutes. Cells were gently fixed and phase contrast and fluorescent images were taken using a fluorescence microscope. Bar, 3  $\mu$ m.



supporting a direct role for RabD in regulating phagosome fusion. In addition, most of the phagosomes in the GFP-RabD<sup>Q67L</sup> cells contained multiple bacteria when the chase period was extended beyond 30 minutes, consistent with the results presented earlier.

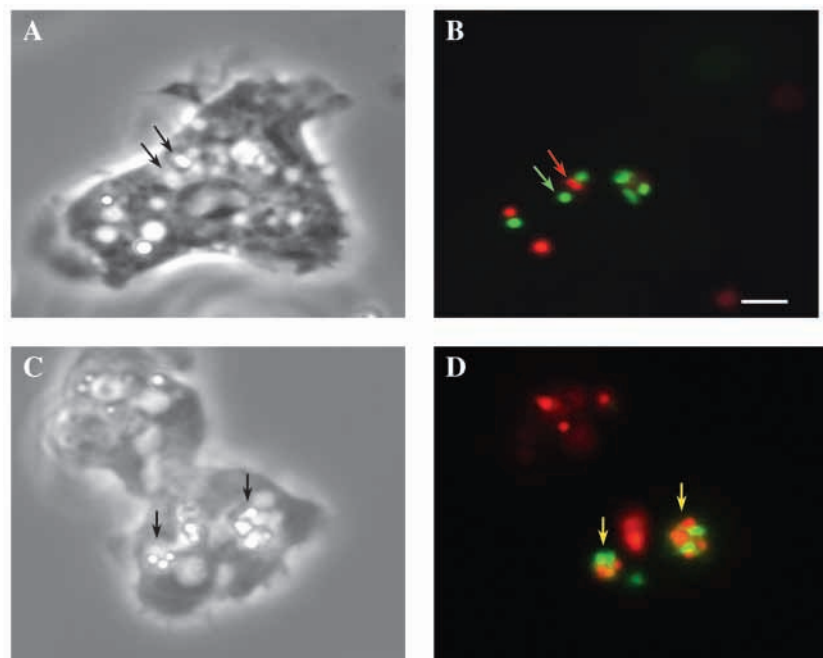
### Discussion

Pulse chase experiments confirmed that the enlarged vesicles observed in cells overexpressing RabD Q67L were acidic lysosomes that arise because of an increase in fusion. In this report, we also present data derived from a variety of assays that demonstrate that the Rab14-like GTPase RabD regulates phagocytosis and the homotypic fusion of phagosomes. The latter process required PI 3-kinase activity, suggesting this lipid kinase acts in parallel with or downstream of RabD to facilitate fusion.

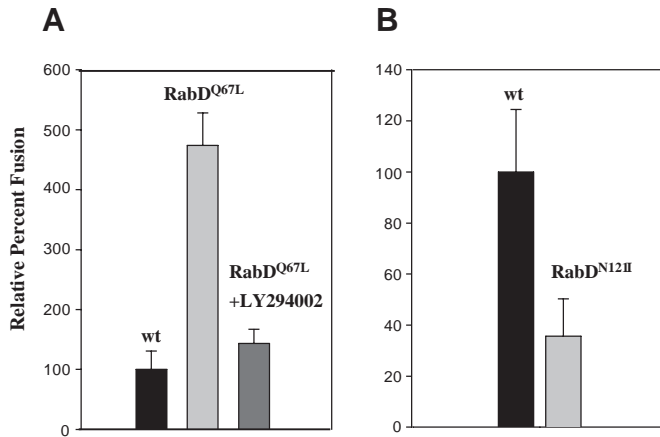
This study provides additional evidence supporting a role proposed a few years ago for RabD in regulating lysosome fusion (Bush et al., 1996). Cells expressing RabD<sup>Q67L</sup> contained enlarged endocytic vacuoles, which appeared by multiple criteria to be enlarged lysosomes. In

support of this observation, these large vesicles were acidic, they received internalized fluid with the kinetics of normal lysosomes and they contained the proton pump and hydrolytic lysosomal enzymes. The data presented here, as well as that previously reported (Bush et al., 1996), are most consistent with a RabD-mediated increase in fusion of lysosomes. For instance, RabD<sup>N121I</sup>-expressing cells contained fewer post-lysosomes (formed by fusion of lysosomes) than control cells, whereas cells expressing RabD<sup>Q67L</sup> contained a greater number of large lysosomes. Furthermore, GFP-RabD<sup>Q67L</sup> ringed the large lysosomes consistent with a direct role in regulating fusion. Finally, sequential pulse-chase periods, using two different fluorescent fluid phase markers, indicated an increase in the rate of mixing between endo-lysosomal compartments in cells expressing RabD<sup>Q67L</sup>.

**Fig. 9.** RabD<sup>Q67L</sup> promotes fusion of separate phagosomes containing bacteria and beads. Both Ax4 and RabD<sup>Q67L</sup>-expressing cells were pulsed with FITC-bacteria for 10 minutes, washed and pulsed with deep blue latex beads (L-1398) for 10 minutes in shaking suspension. After the sequential pulse periods, the cells were washed and allowed to chase in fresh HL-5 while settling on coverslips for 30 minutes. The cells were gently fixed and images were taken of control cells (A,B) and RabD<sup>Q67L</sup> cells (C,D) in the GFP and rhodamine channels of a fluorescent microscope. The scale bar represents 3  $\mu$ m. The red and green arrows reveal separate latex bead and bacterial phagosomes. The yellow arrow marks a phagosome that has beads and bacteria.







**Fig. 10.** Phagosomes fuse at a higher rate in cells expressing RabD<sup>Q67L</sup>, and this is dependent on PI 3-kinase activity. In A, control and RabD<sup>Q67L</sup> cells incubated with or without the PI 3-kinase inhibitor, 20  $\mu$ M LY294002 (Calbiochem), were pulsed for 10 minutes with FITC-bacteria in shaking suspension, washed and allowed to chase for 60 minutes in fresh HL-5 on coverslips. After a gentle fixation, phase contrast and fluorescent images were taken at 1000 $\times$  magnification. The number of phagosomes within each cell and the number of bacteria within each phagosome were counted in a pool of at least 20 cells. A fusion event is defined as  $FE=n-1$ , where FE is a fusion event and  $n$  equals the number of bacteria within the phagosome. Differences in FEs between control and RabD<sup>Q67L</sup> cells were very significant ( $P<0.0001$ ). FEs between control and RabD<sup>Q67L</sup> cells + LY294002 were not significantly different ( $P=0.2623$ ). B depicts the comparison of fusion events between control and RabD<sup>N121I</sup>-expressing cells in the same conditions described above. The difference in the number of fusion events between the two strains is significant ( $P=0.0232$ ).

Lysosome fusion, like phagosome fusion, is also not a process unique to *Dictyostelium*. Earlier studies involving cell-fusion assays demonstrated that lysosomes could exchange membrane proteins and content proteins in mammalian cells (Ferris et al., 1987; Deng and Storrie, 1988). More recently, it has been demonstrated that lysosomes can undergo heterotypic fusion with late endosomes as well as homotypic fusion (Ward et al., 2000a). It has been proposed that Rab7 may regulate fusion of mammalian lysosomes, although this remains controversial.

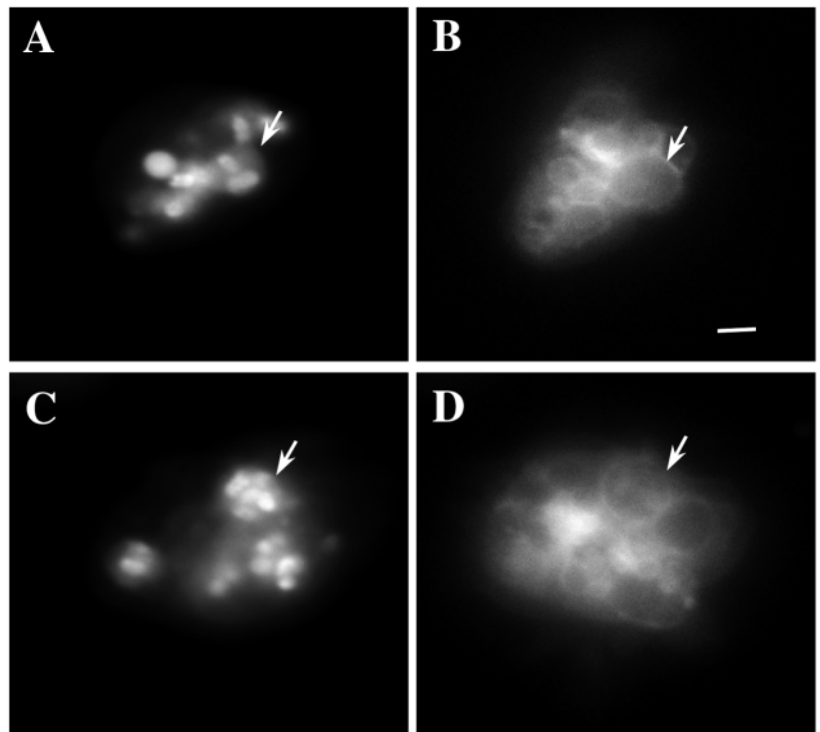
The enlarged lysosomes accumulating in RabD<sup>Q67L</sup>-expressing cells are reminiscent of the enlarged lysosomes observed in a variety of cells from patients with the inherited disorder Chediak-Higashi Syndrome (Ward et al., 2000a). The *CHS* gene encodes a large protein named Beige/Lyst (Barbosa et al., 1996) that may regulate fusion of lysosomes. Disruption of a related gene in *Dictyostelium*, *lvsB*, also led to the accumulation of large acidic lysosomes (Harris et al., 2002) and the formation of multiparticle phagosomes. These enlarged vesicles are most likely to be generated as a result of an increase in homotypic lysosome and phagosome fusion, suggesting that LvsB normally acts to negatively regulate membrane fusion. Thus, homotypic lysosome and phagosome fusion may be negatively and positively regulated by proteins like LvsB (Lyst-like) and RabD (Rab14), respectively, and the final size of lysosomes or number of particles in a phagosome will depend on the relative balance between the activities of these two opposing groups of protein.

Moderate overexpression of RabD<sup>Q67L</sup> increased the rate of phagocytosis, whereas expression of RabD<sup>N121I</sup> decreased the rate of

phagocytosis, results consistent with RabD acting directly to regulate internalization of particles. In fact, it has been hypothesized that the formation of phagosomal membrane cups may require directed exocytosis of internal membranes, a process most probably regulated by Rab GTPases (Cox et al., 2000). In support of this, mammalian and *Dictyostelium* Rab11 has been demonstrated to regulate phagocytosis, perhaps by directing recycling endosomal and contractile vacuole membrane, respectively, to the forming phagocytic cup (Cox et al., 2000). RabD, as well as Rab11, is highly enriched in the CV system of membranes and regulates the structure and function of this organelle (Bush et al., 1996) and conceivably could traffic membrane to the forming phagocytic cup.

This current study also revealed that RabD regulated the rate of homotypic phagosome fusion. Homotypic phagosome fusion also has been observed to occur in cells infected with a variety of intracellular pathogens, including *Helicobacter*,

**Fig. 11.** GFP-RabD<sup>Q67L</sup> rings multiparticle phagosomes. Cells expressing GFP-RabD<sup>Q67L</sup> were incubated with RITC-labeled bacteria for 10 minutes in shaking suspension, washed and chased in fresh HL-5 while settling on coverslips. Live cells were examined using a fluorescent microscope in which images were taken in the rhodamine channel (A,C) and GFP channel (B,D). Bar, 3  $\mu$ m. The arrows mark multiparticle phagosomes ringed with GFP-RabD.



*Coxiella* and *Chlamydia* (Allen et al., 2000; Dermine et al., 2001; Howe and Mallavia, 2000; Majeed et al., 1999). It has been proposed that formation of multiparticle phagosomes may aid the survival of bacteria in professional phagocytes. Essentially nothing is known concerning the molecular factors that regulate phagosome fusion in mammalian cells. Also, no published data is available concerning the function of Rab14, the human Rab most related to RabD, although a recent report demonstrates that this Rab is enriched in phagosomes containing latex beads (Garin et al., 2001). On the basis of amino-acid sequence homology, we hypothesize that Rab14 may function in mammalian cells such as RabD in *Dictyostelium* to regulate lysosomal-phagosomal fusion.

Most of what we currently understand about how Rab proteins regulate membrane fusion comes from studies analyzing the Rab5-mediated homotypic fusion of early endosomes (Mills et al., 1999) and ypt7-mediated fusion of yeast vacuoles (Eitzen et al., 2000). In the former case, Rab5 recruits a variety of effector proteins, including EEA1, rabaptin-5 and rabex-5, during the tethering stage of early endosomal homotypic fusion (Lippe et al., 2001; Stenmark and Gillooly, 2001). In addition, the action of PI 3-kinase is required to generate PI-(3)P, a phosphoinositide that facilitates attachment of EEA1 to early endosomes. Homotypic yeast vacuole fusion has been intensely studied (Wickner and Haas, 2000), and a large complex of proteins has been identified that regulates this process. Although ypt7 (a Rab7-related GTPase) has been demonstrated to be involved in the regulation of vacuole fusion in yeast (Eitzen et al., 2000), and Rab GTPases have been implicated in mammalian lysosome fusion (Ward et al., 2000b), the biochemical identity of the mammalian lysosomal fusion-regulating Rab protein remains unknown.

Interestingly, PI 3-kinases also play a role in regulating lysosome- and phagosome-homotypic fusion in *Dictyostelium* (Buczynski et al., 1997a; Rupper et al., 2001b), although in this latter case the product required appears to be phosphoinositide (3,4,5)-trisphosphate. PKB/Akt, in addition to the PI 3-kinases PIK1 and PIK2, regulates homotypic phagosome fusion in *Dictyostelium* (Rupper et al., 2001b). As demonstrated here, phagosome-phagosome fusion is reduced to control level in cells expressing RabD<sup>Q67L</sup> exposed to LY294002, an inhibitor of PI 3-kinases, suggesting that PI 3-kinase acts in parallel with RabD or as a downstream effector. RabD and PI 3-kinase may functionally interact in at least two ways. First, RabD may act upstream of PI 3-kinase in a similar manner to Rab5, which binds to two distinct PI 3-kinases, hVPS34 and p85-p110. It is proposed that Rab5 transiently interacts with these PI 3-kinases and associates with EEA1, Rabaptin-5, Rabenosyn-5 and Rabex-5 to form a complex that facilitates fusion of early endosomes (Christoforidis et al., 1999; Nielsen et al., 2000). Second, RabD may act in a parallel pathway with PI 3-kinase to act upon a common downstream effector that is primarily responsible for membrane fusion.

A search of the most current *Dictyostelium* protein database indicates that proteins homologous to many of the mammalian-tethering factors (described above) exist, and these proteins conceivably participate in lysosome-lysosome and phagosome-phagosome fusion (J.C., unpublished). Other proteins already implicated in lysosome fusion in *Dictyostelium* include profilin (Temesvari et al., 2000) and Scar (a WASp-related protein) (Seastone et al., 2001), the ATPase proton pump (Temesvari et

al., 1996b), LmpA (a lysosomal membrane protein) (Temesvari et al., 2000) and RtoA (Brazill et al., 2000). Finally, affinity purification approaches using GST-RabD have identified a potential number of effector proteins, including a calcium-binding protein and a dynein subunit that together with the other proteins named above regulates phagosome fusion. It remains to be determined if any of these proteins act as RabD effectors and/or activators and if they also regulate phagosome fusion.

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