Exhibit Arrested Maturation despite Acquisition of Rab7

Rab7 is a small GTPase that regulates vesicular traffic from early to late endosomal stages of the endocytic pathway. Phagosomes containing inert particles have also been shown to transiently acquire Rab7 as they mature. Disruption in the pathway prior to the acquisition of Rab7 has been suggested as playing a role in the altered maturation of Mycobacterium bovis BCG phagosomes. As a first step to determine whether disruption in the delivery or function of Rab7 could play a role in the altered maturation of Legionella pneumophila and M. tuberculosis phagosomes, we have examined the distribution of wild-type Rab7 and the GTPase-deficient, constitutively active mutant form of Rab7 in HeLa cells infected with L. pneumophila or M. tuberculosis. We have found that the majority of L. pneumophila and M. tuberculosis phagosomes acquire relatively abundant staining for Rab7 and for the constitutively active mutant Rab7 in HeLa cells that overexpress these proteins. Nevertheless, despite acquisition of wild-type or constitutively active Rab7, both the L. pneumophila and the M. tuberculosis phagosomes continue to exhibit altered maturation as manifested by a failure to acquire lysosome-associated membrane glycoprotein 1. These results demonstrate that L. pneumophila and M. tuberculosis phagosomes have receptors for Rab7 and that the altered maturation of these phagosomes is not due to a failure to acquire Rab7.

Following phagocytosis, phagosomes containing inert particles undergo a series of maturational steps that mirror the stages of the endocytic pathway (16, 17, 36, 37). Immediately following phagocytosis, the early phagosomes acquire markers of early endosomes, such as mannose receptor and Rab5 (17, 36, 37). With maturation, the phagosomes lose these early endocytic markers and acquire markers of late endosomes, such as mannose-6-phosphate receptor and Rab7 (17, 36, 37). With further maturation, the phagosomes lose Rab7 but acquire other, as yet unidentified, GTPases (17, 30), fuse with lysosomes, and acquire increasing amounts of lysosomal markers such as lysosome-associated membrane glycoproteins (LAMP-1, LAMP-2, and CD63) and acid hydrolases (such as cathepsin D and acid phosphatase) (17, 36, 37). In addition, phagosomes containing inert particles acquire the vacuolar proton pump and, with maturation, become highly acidified (20).

The pathways of phagosomes containing the intracellular parasites Legionella pneumophila and Mycobacterium tuberculosis deviate markedly from the pathway followed by phagosomes containing inert particles (3, 10, 11, 14, 25, 26, 28, 47). Both L. pneumophila and M. tuberculosis phagosomes resist acidification (14, 28), inhibit phagosome-lysosome fusion (3, 26), and acquire little or none of the markers of lysosomes (10, 11, 26). Despite these similarities, the L. pneumophila and M. tuberculosis phagosomes also exhibit important differences from one another. The L. pneumophila phagosome, but not the M. tuberculosis phagosome, exhibits unique interactions with other host cell organelles (25). Within minutes of phagocytosis, smooth vesicles appear to be fusing with or budding off of the nascent L. pneumophila phagosome. Subsequently, the L. pneumophila phagosome develops interactions with mitochondria, ribosomes, and endoplasmic reticulum (ER), ultimately forming a ribosome-lined replicative vacuole (25). Whereas the L. pneumophila phagosome excludes markers of early endosomes (11, 13), the M. tuberculosis phagosome shows a persistent staining for the transferrin receptor (11) and Rab5 (13) and demonstrates a persistent capacity to acquire exogenously added transferrin from early endosomes (12). A related mycobacterium, Mycobacterium bovis BCG, has also been shown to exclude LAMP-1 from its phagosome and to demonstrate a persistence of the transferrin receptor and Rab5 on its phagosome (45). Thus, in different ways, both L. pneumophila and M. tuberculosis alter the maturation of their phagosomes and produce a phagosomal environment that is more hospitable to their growth and multiplication. However, the mechanisms by which they do so remain to be elucidated.

Rab-GTPases are members of the Ras superfamily that have been shown to play a pivotal role in regulating docking and fusion events between different compartments of eukaryotic cells (23, 34, 35). Because Rab-GTPases play a crucial role in the regulation of membrane trafficking within eukaryotic cells, disruption of their distribution or function by an intracellular parasite could also play an important role in altering the maturation path of the phagosome containing the intracellular parasite.

Over 30 different Rab-GTPases have been identified thus far, and it is thought that every compartment of the secretory and endocytic pathway has a unique set of Rab-GTPases. For example, Rab5 is present on early endosomes (9) and on phagosomes immediately after phagocytosis (16, 17, 30) and regulates membrane trafficking events involving these compartments (2, 5, 7, 42). Rab7 has been shown to overlap with mannose-6-phosphate-receptor-positive late-endocytic compartments (9), and the constitutively active Rab7 also shows a partial colocalization with lysosomal compartments (33). Green fluorescence protein-tagged canine Rab7 overexpressed in HeLa cells has also been observed to overlap with lysosomal compartments (8). The functional importance of Rab7 in regulating membrane trafficking in the endocytic pathway has
been established by the demonstration that expression of a dominant-negative Rab7 mutant interrupts the normal endocytic flow from early to late endosomes and causes an accumulation of endocytosed vesicular stomatitis virus G protein (18) and cathepsin D and mannos-6-phosphate receptor (38) in early endocytic compartments. In addition, overexpression of the green fluorescence protein-tagged Rab7 dominant-negative mutant has been shown to lead to dispersal of the lysosomal compartment and impairment in the capacity of the lysosomes to acidify and to acquire endocytosed material (8). The role of Rab7 in the formation of L. pneumophila or M. tuberculosis phagosomes in human cells has not previously been reported. However, the acquisition of various Rab-GTPases by L. pneumophila phagosomes (39) or M. bovis BCG phagosomes (45) has been studied in mouse bone marrow macrophages. In the case of L. pneumophila, Roy et al. (39) recently employed immunofluorescence microscopy to examine the acquisition of Rab7 and LAMP-1 by wild-type and dotA mutant L. pneumophila phagosomes at early times after phagocytosis. These authors observed that approximately 35% of wild-type L. pneumophila acquired Rab7 by 5 min after phagocytosis, and this percentage of L. pneumophila phagosomes bearing Rab7 declined to approximately 10% by 30 min after phagocytosis. A similar percentage (approximately 45%) of the dotA mutant L. pneumophila phagosomes were observed to acquire Rab7 by 5 min, but essentially all of the dotA mutant L. pneumophila phagosomes lost the Rab7 staining by 30 min in these experiments. In the case of M. bovis BCG, an avirulent form of a mycobacterial species related to M. tuberculosis, Via et al. (45) used biochemical techniques to examine Rab-GTPases on a population of phagosomes isolated from mouse bone marrow macrophages infected with M. bovis BCG. These investigators reported that M. bovis BCG phagosomes acquire Rab5 but not Rab7 or LAMP-1.

To further investigate whether M. tuberculosis or L. pneumophila disrupts the maturation of their phagosome by altering the function or distribution of Rab-GTPases, we have examined the distribution of Rab7 in human HeLa cells infected with L. pneumophila or M. tuberculosis. We have employed immunoelectron microscopy, a technique that provides considerably more ultrastructural detail than immunofluorescence and allows one to distinguish whether a phagosome is truly decorated with a marker or simply surrounded with vesicles that bear the marker. The technique allows a determination of whether or not individual phagosomes that stain for Rab7 do or do not also stain for other markers, such as LAMP-1, a question that is not easily addressed with studies of populations of isolated phagosomes. In addition to studying the wild-type Rab7, we have also examined the distribution of a constitutively active mutant of Rab7 and the effect of this mutant on the phenotype of the L. pneumophila and M. tuberculosis phagosomes.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Glutaraldehyde was purchased from Polysciences (Warrington, Pa.); piperazone-N,N-bis(2-ethanesulfonic acid) (PIPES), methylcellulose, polyvinylpyrrolidone, paraformaldehyde were purchased from Sigma (St. Louis, Mo.); Dulbecco’s phosphate-buffered saline was purchased from Gibco Laboratories (Santa Clara, Calif.); and DMEM (Dulbecco’s modified Eagle’s Medium) was purchased from Irvine Scientific Co. (Santa Ana, Calif.).

Mouse monoclonal antibody (MAb) to the human transferrin receptor (immunoglobulin G1, IgG1) was purchased from AMAC (Westbrook, Maine). Mouse MAb to LAMP-1 (IIA3, IgG1) was obtained from the Hybridioma Bank of the University of Iowa, Iowa City. Mouse MAb to the bovine cation-dependent mannose-6-phosphate receptor was obtained from Cappel Organon-Technica (West Chester, Pa.). Rabbit antibody to mycobacterial liposarcoaminomannan (LAM) was prepared as described previously (11). Rabbit antibody to L. pneumophila lipopolysaccharide (LPS) was prepared by immunizing rabbits with LPS purified from L. pneumophila Philadelphia 1 in Freund’s adjuvant (19). Purified recombinant anti-mouse IgG antibody was obtained from Sigma Chemical Company. Reactivity of this commercial antibody to mycobacterial antigens was eliminated by three consecutive overnight absorptions to an excess of heat-killed M. tuberculosis. Protein A colloidal gold conjugates (5, 10, and 15 nm) were provided by G. Postuma (University of British Columbia, Vancouver, B.C.).

**Bacteria.** M. tuberculosis Erdman (ATCC 35801), a highly virulent strain, was obtained from the American Type Culture Collection (Rockville, Md.). The organisms were passaged through one passage in mouse macrophages infected inocula were prepared as described previously (13). The concentration of organisms was determined by measurement of optical density at 540 nm and by counting in a Petroff-Hauser chamber. Viability of the organisms was determined by plating serial dilutions of the infecting inoculum on 7H11 agar. Viability ranged from 67 to 86% in these experiments.

L. pneumophila Philadelphia 1 was grown in embryonated hens’ eggs, harvested, tested for viability and contaminants, and stored at ~70°C, as described (29). The egg yolk-grown L. pneumophila was cultured one time only on charcoal yeast extract (CYE) agar, harvested after four days of growth, and used immediately. The avirulent mutant L. pneumophila 25D was prepared and maintained as described previously (27). This mutant has been shown to be a mutant in the virG3 virulence locus (32, 40).

**Cloning, expression, and purification of recombinant Rab7 and preparation of antisera.** To clone the human rab7 gene, we screened a human fetal lung cDNA library (Invitrogen) by colony hybridization by using a cDNA probe encoding the 3’-untranslated region of a human rab7 clone on a 240-bp EcoRI fragment excised from IMAGE Consortium Clone 108659 (ATCC 330444). The probe was labeled with [α-32P]CTP (Amersham) by the random priming method. Prehybridization and hybridization were carried out at 42°C in a solution containing 2× PIPES, 50% deionized formamide, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 100 μg of denatured, sonicated salmon sperm DNA per ml. Positive clones were selected after three rounds of colony hybridization and were analyzed by restriction enzyme digestions. The identities of the positive clones were confirmed by sequencing both strands of DNA in opposite directions. The nucleotide sequences of our clones were identical at the nucleotide level to the sequence of a rab7 gene isolated from a human placenta cDNA library (46), and a similar sequence has also been found by PCR amplification of total mRNA of human U937 cells (13). The human rab7 gene is highly homologous to the canine rab7 sequence (9). The cDNA for the complete rab7 gene was amplified by PCR and cloned into pET3a between Ncol and BamH1 cleavage sites. The construct was under the control of the T7 promoter with an amino-terminal sequence coding for a His6 tag. High-level expression of Rab7 in E. coli was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the recombinant proteins were purified to homogeneity from sonicated cell pellet extracts by a combination of nickel-affinity, gel filtration, and ion-exchange chromatography. The resulting material was found by SDS-polyacrylamide gel electrophoresis to exhibit a single band of 25 KDa by Coomassie blue staining. Rabbit polyclonal antibodies to recombinant human rab7 were raised by immunizing New Zealand White rabbits three times, 3 weeks apart, with 1 mg of recombinant protein coupled to keyhole limpet hemocyanin (1). The antibody was affinity purified to avoid the production of antibodies to mycobacterial antigens present in Freund’s adjuvant. The first immunization was supplemented with 100 μg of N-acetylmyr- amyl-L-α-naphthylamide (Sigma Chemical Co.). Rabbits immunized with the myr-hemocyanin proteins yielded a specific rabbit polyclonal antibody to recombinant Rab7. The resulting polyclonal antibodies were affinity purified by binding to recombinant E. coli Rab7, eluted with glycine-HCl, pH 2.5, containing 0.1% bovine serum albumin carrier protein, and immediately neutralized with Tris-HCl, pH 8.0. The purified antibodies reacted equally well with geranylated and nongeranylated Rab7 and did not cross-react with L. pneumophila or M. tuberculosis antigens. Antisera to Rab7 did not cross-react with Rab3 or Rab4.

**Stable transfection of human cell lines with Rab7 and a constitutively active Rab7 mutant.** To facilitate the immuno localization of Rab7 in infected cells, we used the “Tet-off” tetracycline-suppressible expression system (21, 33) to develop a stably transfected human HeLa cell line with regulated expression of recombinant human Rab7 (HeLa-Rab7). We cloned the human rab7 gene into pTRE, transduced the recombinant plasmids into HeLa-Tet-off cells (Clontech) by calcium phosphate precipitation, and selected stably transfected clones with hygromycin (200 μg/ml) in the presence of tetracycline (5 μg/ml). GTase-deficient, fusion-promoting mutant forms of Rab7 and other Rab-GTPases have been described (7, 31). We prepared the corresponding rab7 Q67L mutant with PCR-based mutagenesis by published methods (30, 41) by using the mutant primer 5′-GGAACGTTCAAGTCCTGCTGTGTCCCATA-3′ (mutated nucleotides underlined) and pTRE forward and reverse sequences (5′-GAGACCTTGACTCTTCCTCCTCCTCGCACTGCTGCT3′ and 5′-CAATGGAGCGTTTCATGTGAGGTTGCAGCCTGCT-3′, respectively) as outer primers in the amplifications. The mutant construct was confirmed by DNA sequencing. HeLa cells stably transfected with the constitutively active rab7 (HeLa-Rab7 Q67L) were selected as described above for HeLa-Rab7 cells.

**Transient transfection of HeLa cells with the bovine cation-dependent mannose-6-phosphate receptor.** A 1.1 kb EcoRI-Pfrl fragment containing the bovine

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This text contains information about the roles of Rab7 and L. pneumophila in phagosome formation, the use of immunofluorescence microscopy to study the acquisition of Rab7 and LAMP-1, and the use of immunoelectron microscopy to examine the distribution of Rab7 in infected cells. It also describes the methods used to clone and purify recombinant Rab7 and to prepare antisera against it. The text discusses the development of stable cell lines with regulated expression of Rab7 and the use of transient transfection to study the localization of the bovine cation-dependent mannose-6-phosphate receptor in HeLa cells.
cation-dependent mannose-6-phosphate receptor (CD-M6PR) was released from pSV-neo-cdmpR (provided by Stuart Kornfeld, Washington University) and subcloned into pcDNA3.1/Zeo(+) (Invitrogen). Coexpression of Rab7 and CD-M6PR was obtained by transfecting HeLa-Rab7 or HeLa-Rab7 Q67L cells with pcDNA3.1/Zeo(+)-vcdmpR. Transfected cells were kept in the absence of antibiotics for 2 days before infection with M. tuberculosis and L. pneumophila, consistent with published observations of other investigators (9).

Assessment of intracellular growth of M. tuberculosis and L. pneumophila in monolayers of THP-1 cells or HeLa cells. Monolayers of HeLa cells (10^6 cells/well) or THP-1 cells (4 x 10^5 cells/well) were cultured to confluency in 2-cm^2 tissue culture wells for 2 days in RPMI 1640 (THP-1) or DMEM (HeLa) with 10% fetal bovine serum without antibiotics in DMEM (low glucose) with 10% fetal calf serum (certified antibiotic- and mycoplasma-free) (Invitrogen). Coexpression of Rab7 and CD-M6PR, consistent with published observations of other investigators (9).

FIG. 1. Growth of L. pneumophila and M. tuberculosis in HeLa–Tet-off cells or in HeLa-Rab7 or HeLa-Rab7 Q67L cells overexpressing Rab7 or Rab7 Q67L. Monolayers of HeLa–Tet-off, HeLa-Rab7, and HeLa-Rab7 Q67L cells in 2-cm^2 wells were grown for 2 days (A) or 1 day (B) in the absence of tetracycline to induce expression of Rab7 or Rab7 Q67L. The cells were incubated with L. pneumophila (2 x 10^7/ml) [A] or M. tuberculosis (10^9/ml) [B] for 2 h at 37°C, were washed, and were incubated in fresh medium at 37°C. At sequential times thereafter, the monolayers were lysed and combined with the culture supernatant, and CFU were determined by plating serial dilutions on CYE (A) and 7H11 (B) agar plates. Data shown represent the means ± the standard deviations of triplicate determinations.

RESULTS

Establishment of a model human cell system suitable for evaluating Rab7 expression on phagosomes. We have found that endogenous levels of Rab5 and Rab7 in normal human monocytes, monocyte-derived macrophages, and cell lines are too low to be detected reliably by immunofluorescence or...
immunoelectron microscopy. Therefore, to study the distribution and function of these Rab-GTPases in host cells infected with intracellular pathogens, we cloned the rab5 (13) and rab7 genes from a human fetal lung library and sought to overexpress the genes in a variety of different cell types. Since macrophages are the natural host cells of L. pneumophila and M. tuberculosis, we initially sought to overexpress the rab genes in macrophage-like cell lines (U937, THP-1, and HL60) but were unable to achieve stable high-level expression compatible with immunofluorescence or immunoelectron microscopy studies. We therefore prepared a HeLa cell line capable of inducible expression of the human rab5 (13) and rab7 genes. Because long-term overexpression of Rab-GTPases is often associated with toxicity and loss of expression by the cell line, we used a tetracycline-regulated expression system (21). Expression of Rab7 and the constitutively active Q67L form of Rab7 by the isolated clones was tightly regulated by tetracycline and was stable for at least 3 days after removal of tetracycline from the culture medium (data not shown).

To confirm that the study of L. pneumophila and M. tuberculosis phagosomes in infected HeLa cells is relevant to understanding the pathogenesis of L. pneumophila and M. tuber-

FIG. 2. Quantitation of Rab7 and LAMP-1 immunogold staining in HeLa-Rab7 cells infected with wild-type or avirulent L. pneumophila. Wild-type or avirulent L. pneumophila cells were spun down onto monolayers of HeLa-Rab7 cells at 4°C. The monolayers were incubated for 30 min at 37°C and were then fixed immediately (0 min) or washed to remove nonadherent bacteria and were incubated at 37°C for an additional 15 min, 1 h, 3 h, or 8 h, as indicated in the figure, and were then fixed. All monolayers were processed for cryoimmunoelectron microscopy. Rab7 (A) and LAMP-1 (B) immunogold particles were enumerated on phagosomal membranes, nuclear membranes, and plasma membranes, and the total number of immunogold particles per square micrometer of cytoplasm (including all cytoplasmic vesicles, phagosomes, and other organelles) was also determined. Data shown represent the means and standard errors of the means of gold particle counts for each compartment. (A, left panel) Rab7 was present on wild-type L. pneumophila phagosomes for the first 3 h after infection, but was scarce at 8 h after infection. Rab7 was also present on avirulent L. pneumophila phagosomes, but at somewhat lower overall levels than found on the wild-type L. pneumophila phagosomes. Avirulent L. pneumophila phagosomes were not examined at the 8-h time point. Rab7 was scarce on nuclear membranes and plasma membranes at all time points examined. Right panel, as a control, Rab7 staining in the cytoplasm of the HeLa cells was quantitated and found to be comparable in the cells containing wild-type or avirulent L. pneumophila. (B, left panel) LAMP-1 was scarce on wild-type L. pneumophila phagosomes at all time points examined but was abundant on phagosomes containing the avirulent L. pneumophila. Plasma membranes and nuclear membranes served as internal negative controls and had negligible staining for LAMP-1. Right panel, LAMP-1 staining per unit area of cytoplasm was similar between HeLa cells infected with wild-type and avirulent L. pneumophila. Total numbers of phagosomes evaluated were as follows: 29, 72, 44, 63, and 42 for wild-type L. pneumophila at 0 min, 15 min, 1 h, 3 h, and 8 h, respectively, and 29, 54, and 63 for avirulent L. pneumophila at 0, 1, and 3 h, respectively.
The cells (13). These results confirmed that LAMP-1 on phagosomes containing wild-type M. tuberculosis infected with the transferrin receptor gene stained positive for LAMP-1 on phagosomes containing the avirulent mutant M. tuberculosis cyte-derived macrophages (11), we found that the majority of the two pathogens in the HeLa cells.

(i) Intracellular growth of L. pneumophila and M. tuberculosis in HeLa–Tet-off, HeLa-Rab7, and HeLa-Rab7 Q67L cells. We found that although HeLa cells are poorly phagocytic, adequate uptake of L. pneumophila and M. tuberculosis could be obtained by increasing the multiplicity of infection relative to that used when infecting more phagocytic cells. Once taken up by the HeLa–Tet-off or HeLa-Rab5 cells, L. pneumophila and M. tuberculosis grow at rates comparable to that in THP-1 cells as described (13).

To determine whether overexpression of Rab7 or Rab7 Q67L alters the growth of L. pneumophila or M. tuberculosis in HeLa cells, we examined the growth of L. pneumophila and M. tuberculosis in HeLa–Tet-off, HeLa-Rab7, and HeLa-Rab7 Q67L cells 1 day after withdrawal of tetracycline (Fig. 1A and B). Overexpression of Rab7 or Rab7 Q67L did not alter the growth of L. pneumophila or M. tuberculosis in these host cells.

(ii) Distribution of endocytic markers on L. pneumophila or M. tuberculosis phagosomes in HeLa cells. To determine if phagosomes in infected HeLa cells have molecular characteristics similar to phagosomes in infected human macrophages, we have previously examined transferrin receptor expression on M. tuberculosis phagosomes and LAMP-1 expression on both L. pneumophila and M. tuberculosis phagosomes (13). Consistent with our published observations with human monocyte-derived macrophages (11), we found that the majority of M. tuberculosis phagosomes in HeLa-Rab5 cells stably transfected with the transferrin receptor gene stained positive for the transferrin receptor (13). Also consistent with our previous observations in human macrophages (11), we found little or no LAMP-1 on phagosomes containing wild-type L. pneumophila or live M. tuberculosis in HeLa-Rab5 cells, but intense staining for LAMP-1 on phagosomes containing the avirulent mutant L. pneumophila, heat-killed M. tuberculosis, or latex beads in these cells (13). These results confirmed that L. pneumophila and M. tuberculosis phagosomes in HeLa-Rab5 cells do not fuse with lysosomes and that overexpression of the Rab5 in HeLa cells does not fundamentally alter the membrane-trafficking properties of the L. pneumophila or M. tuberculosis phagosomes.

We concluded from these sets of studies that, while uptake of L. pneumophila and M. tuberculosis into HeLa cells is much less efficient than uptake into macrophages, the intracellular rates of bacterial growth and the interaction of the phagosomes with the endolysosomal pathway in these host cells are very similar. This implied that lessons learned from studying L. pneumophila and M. tuberculosis phagosomes in HeLa cells were likely to apply to phagosomes of these pathogens in macrophages.

Distribution of Rab7 on phagosomes containing wild-type and avirulent L. pneumophila in HeLa-Rab7 cells. Two days after removal of tetracycline from the culture medium, 90% of HeLa-Rab7 cells had abundant immunogold staining for Rab7 on cytoplasmic vesicles (>1 gold particle/μm²), with an average level of staining of 4.8 ± 0.6 gold particles/μm² (Fig. 2A, right side of panel). In contrast, parental HeLa–Tet-off cells had only a low level of staining for endogenous Rab7 (average level of cytoplasmic immunogold staining = 0.21 gold particle/μm²) (data not shown). In HeLa-Rab7 cells infected with wild-type L. pneumophila, the majority of L. pneumophila phagosomes stained positive for Rab7 for at least the first 3 h after infection (Fig. 2A and 3A). Although there was heterogeneity in the levels of staining for Rab7 (Fig. 3), during the first 3 h, 60% of the phagosomes had more immunogold staining than 90% of the nuclei in the same cells. However, by 8 h after infection, the Rab7 immunogold staining dropped to very low levels, with no significant difference between the low levels of phagosomal or nuclear staining (Fig. 2). Avirulent L. pneumophila phagosomes had lower levels of staining for Rab7 than the wild-type phagosomes (Fig. 2A), but a majority of both types of phagosomes stained positive. While wild-type and avirulent phagosomes displayed a similar distribution of staining during the first 3 h after phagocytosis (Fig. 3A and B), none of the avirulent phagosomes exhibited the strikingly high levels of Rab7 staining (>3 gold particles/μm) found on 15 to 20% of the wild-type phagosomes.
avirulent L. pneumophila does not immunogold staining for LAMP-1 (Fig. 4A). In contrast, L. pneumophila type I pneumophila lack LAMP-1 immunogold staining on the wild-type phagosome on the left has five Rab7 immunogold particles, and the phagosome on the right has one Rab7 immunogold particle. Neither L. pneumophila phagosome has any LAMP-1 immunogold staining. The latex bead phagosome located between the two L. pneumophila phagosomes has two Rab7 immunogold particles, and approximately 20 LAMP-1 immunogold particles. (B) Two avirulent L. pneumophila phagosomes are shown in this micrograph. The avirulent L. pneumophila phagosome on the left has five Rab7 immunogold particles, and the phagosome on the right has one Rab7 immunogold particle. Both avirulent L. pneumophila phagosomes stain intensely for LAMP-1. Magnifications are (A) ×26,220 and (B) ×24,681.

Distribution of LAMP-1 on phagosomes containing wild-type or avirulent L. pneumophila in HeLa-Rab7 cells. Little or no LAMP-1 immunogold staining was observed on the wild-type L. pneumophila phagosomes at all time points examined, from 0 min to 8 h (Fig. 2B and 3C). Even L. pneumophila phagosomes that stained positively for Rab7 showed little or no immunogold staining for LAMP-1 (Fig. 4A). In contrast, avirulent L. pneumophila phagosomes showed high levels of immunogold staining for LAMP-1 (Fig. 2B, 3D, and 4B).

Distribution of Rab7 on phagosomes containing live and heat-killed M. tuberculosis cells in HeLa-Rab7 cells. In HeLa-Rab7 cells infected with M. tuberculosis, a substantial proportion of M. tuberculosis phagosomes stained positive for Rab7 at all time points examined, from 1 to 3 days after phagocytosis (Fig. 5A, 6A, and 7). Although there was heterogeneity in the intensity of staining, 65% of the M. tuberculosis phagosomes had a higher level of Rab7 immunogold staining than the nuclei within the same cells (Fig. 6A). The majority of phagosomes containing heat-killed M. tuberculosis also stained positive for Rab7, but tended to have a lower level of staining than the phagosomes containing live M. tuberculosis (Fig. 5A and 6B).

Distribution of LAMP-1 on phagosomes containing live or heat-killed M. tuberculosis in HeLa-Rab7 cells. Despite the presence of relatively high levels of Rab7 on the phagosomes containing live M. tuberculosis, the majority of such phagosomes acquired only low levels of LAMP-1. Phagosomes containing heat-killed M. tuberculosis, on the other hand, acquired abundant staining for LAMP-1 (Fig. 5B and 6D).

It is possible that phagosomes containing heat-killed M. tuberculosis would have exhibited higher levels of staining for Rab7 at earlier time points, when phagosomes containing inert particles might be expected to have more Rab7. However, we were unable to examine levels at earlier time points because, at the multiplicities of infection used, heat-killed M. tuberculosis was taken up inefficiently by HeLa cells.

Effect of overexpression of the Rab7 constitutively active mutant on L. pneumophila and M. tuberculosis phagosomes. Whereas wild-type Rab7 exhibits only a limited colocalization with LAMP-1, the GTPase deficient, constitutively active Rab7 Q67L mutant exhibits a much greater degree of colocalization with lysosomal compartments (33). Therefore, we examined the distribution of the constitutively active Rab7 Q67L in L. pneumophila- and M. tuberculosis-infected cells and the effect of expression of the constitutively active Rab7 on the phagosomal phenotype. We found that the constitutively active Rab7 is recruited to L. pneumophila (Fig. 8A and see Fig. 10B and C) and M. tuberculosis phagosomes (Fig. 9A and 10B and E), just as was the case with the wild-type Rab7. At 2 h after infection, the level of Rab7 Q67L present on the avirulent L. pneumophila phagosomes was similar to the level found on wild-type L. pneumophila phagosomes (Fig. 8A). Despite similar levels of Rab7 Q67L on wild-type and avirulent L. pneumophila phagosomes, LAMP-1 was scarce on the wild-type L. pneumophila phagosomes but abundant on the avirulent L. pneumophila phagosomes (Fig. 8B and 10B and C). As expected, Rab7 Q67L colocalized extensively with LAMP-1 in cytoplasmic vesicles of the cells infected with wild-type L. pneumophila; many of these vesicles appeared to be autophagosomes or multivesicular bodies (Fig. 10A). Nevertheless, wild-type L. pneumophila phagosomes that stained richly for Rab7 Q67L did not acquire LAMP-1 (10B). Similarly, the levels of Rab7 Q67L found on live M. tuberculosis phagosomes were similar to or greater than the levels found on latex bead phagosomes in the same cells (Fig. 9). Nevertheless, M. tuberculosis phagosomes that stained positive for Rab7 Q67L exhibited little or no immunogold staining for LAMP-1, whereas latex bead phagosomes acquired abundant amounts of LAMP-1 (Fig. 9 and 10C and D).

While we have found that overexpression of constitutively active Rab5 resulted in dramatic changes in morphology and membrane markers of M. tuberculosis phagosomes (12), overexpression of the constitutively active form of Rab7 caused no change in the phenotype of L. pneumophila or M. tuberculosis phagosomes, despite the presence of extremely rich staining for the constitutively active Rab7 on the phagosomes (Fig. 10B, D, and E).

Distribution of the cation-dependent mannose-6-phosphate receptor in HeLa cells infected with L. pneumophila or M. tuberculosis. Both Rab7 and the cation-dependent mannose-6-phosphate receptor (CD-M6PR) are present on late endosomes. To determine if L. pneumophila and M. tuberculosis arrest the maturation of their phagosomes at a CD-M6PR+/LAMP− late endosomal stage, we examined whether L. pneumophila or M. tuberculosis phagosomes that stain intensely for Rab7 or Rab7 Q67L would also stain for the CD-M6PR. HeLa Rab7 and HeLa Rab7 Q67L cells were transiently transfected with the gene for the CD-M6PR and were infected with L. pneumophila or M. tuberculosis. In the case of L. pneumophila
phagosomes within HeLa-Rab7 cells expressing CD-M6PR, we found no significant colocalization of the CD-M6PR with the L. pneumophila phagosomes, despite intense staining of other cytoplasmic vesicles within the cells for the CD-M6PR (Fig. 11A; mean level of phagosomal membrane staining = 0.08 ± 0.06 CD-M6PR gold particles/μm; mean level of nuclear staining = 0.13 ± 0.04 CD-M6PR gold particles/μm; mean level of cytoplasmic staining = 13.1 ± 3.6 M6PR gold particles/μm²). In the case of M. tuberculosis phagosomes within HeLa-Rab7 cells expressing the CD-M6PR, M. tuberculosis phagosomal membranes displayed a low level of staining for CD-M6PR (Fig. 11B; 0.88 ± 0.18 gold particles/μm², compared with nuclear membrane staining of 0.13 ± 0.11 gold particles/μm²; P = 0.007, unpaired t test). This low level of staining for the CD-M6PR is comparable to the relatively low level seen for LAMP-1 on these phagosomes and was unimpressive compared with the overall level of cytoplasmic staining (10.3 ± 1.9 gold particles/μm²) and the abundant staining for CD-M6PR seen on adjacent cytoplasmic vesicles (Fig. 11B). Similarly, in HeLa Rab7-Q67L cells, CD-M6PR was absent from L. pneumophila phagosomes and scarce on M. tuberculosis phagosomes (data not shown). We have also examined immunogold staining for endogenous CD-M6PR and the cation-independent M6PR in human monocyte-derived macrophages and found that staining for these markers is scarce on the M. tuberculosis phagosome (data not shown), in agreement with the findings of this study and that of Xu et al. (47).

**DISCUSSION**

L. pneumophila and M. tuberculosis both prevent the maturation of their phagosomes to phagolysosomes. Part of the normal maturation of phagosomes containing inert particles to phagolysosomes involves the acquisition of Rab7 (16, 17, 30).
To determine if *L. pneumophila* or *M. tuberculosis* phagosomes block this stage of maturation, we examined whether or not their phagosomes had the capacity to acquire Rab7. We found that the majority of *L. pneumophila* and *M. tuberculosis* phagosomes did acquire staining both for Rab7 and for the constitutively active mutant form of Rab7 (Rab7 Q67L) in HeLa cells overexpressing these proteins. Moreover, phagosomes containing wild-type *L. pneumophila* or live *M. tuberculosis* tended to have more Rab7 than phagosomes containing avirulent *L. pneumophila* or heat-killed *M. tuberculosis* cells, respectively. Despite their relatively abundant acquisition of Rab7 and Rab7 Q67L, phagosomes containing wild-type *L. pneumophila* and live *M. tuberculosis* acquired little or no staining for LAMP-1. In contrast, phagosomes containing avirulent *L. pneumophila*, heat-killed *M. tuberculosis*, and latex beads acquired intense staining for LAMP-1.

Prior to our studies, a plausible hypothesis for the capacity of *L. pneumophila* and *M. tuberculosis* to alter the maturation of their phagosomes was that these pathogens either prevented their phagosomes from acquiring Rab7 or prevented the Rab7 from binding to GTP. However, our results render these hypotheses untenable. While it is likely that the remarkably high levels of staining for Rab7 on *L. pneumophila* and *M. tuberculosis* phagosomes are unique to host cells that overexpress Rab7, our results demonstrate that (i) the *L. pneumophila* and *M. tuberculosis* phagosomes have receptors that allow the phagosomes to recruit Rab7 and (ii) the altered maturation of the *L. pneumophila* and *M. tuberculosis* phagosomes is not due
to a failure to recruit Rab7 to the phagosomes. Clearly, both L. pneumophila and M. tuberculosis phagosomes exhibit altered maturation despite the acquisition of either the wild-type Rab7 or the constitutively active mutant (GTP-bound) form of Rab7. The recruitment of Rab7 to the phagosomes in our system is specific and is not due simply to abundance of the Rab7 in the cytoplasm, since nuclear membranes and plasma membranes in these cells showed only low levels of staining for Rab7.

Our observations regarding M. tuberculosis phagosomes in HeLa cells differ markedly from those of Via et al. (45) with avirulent M. bovis BCG phagosomes in mouse macrophages. Via and coworkers reported that M. bovis BCG phagosomes did not acquire Rab7 and concluded that maturation of the BCG phagosome was blocked at a stage between the acquisition of Rab5 and Rab7. However, in our system, using virulent M. tuberculosis in human HeLa cells, we found that M. tuberculosis can arrest the maturation of its phagosome at a stage subsequent to the acquisition of Rab7. These different observations could reflect different model systems (M. bovis BCG in mouse macrophages versus M. tuberculosis in human HeLa cells that overexpress Rab7) or the difference in detection method (subcellular fractionation versus immunoelectron microscopy). Failure to observe Rab7 on the M. bovis BCG phagosome could be due to loss of Rab7 during the lengthy procedure used to isolate the phagosomes (which includes a 15-h ultracentrifugation step) or to the sensitivity of the technique. In our system, the levels of Rab7 on the M. tuberculosis phagosome may be exaggerated by the use of transfected cells that overexpress the protein. In addition, cycling of an overexpressed Rab-GTPase theoretically could be impaired if the chaperon proteins, such as Rab-GDI and Rab escort protein, are stoichiometrically overwhelmed, thereby leading to the persistence of higher levels of the Rab protein on the phagosome. Nevertheless, recruitment of Rab-GTPases requires specific receptor machinery on the target membrane (4, 44), and the presence of Rab7 on the M. tuberculosis phagosome cannot be accounted for by either overexpression of the Rab protein or impaired cycling from the membrane by chaperon proteins. Thus, our data demonstrate that the M. tuberculosis phagosome has receptor machinery for Rab7 and that M. tuberculosis can block the maturation of its phagosome at a step subsequent to Rab7 acquisition.

Our observations on the recruitment of Rab7 to L. pneumophila phagosomes in HeLa cells by immunoelectron microscopy are generally consistent with the findings of Roy et al. (39) who also observed Rab7 immunofluorescence on a substantial percentage of L. pneumophila phagosomes in mouse bone marrow macrophages. Although we observed a larger percentage of L. pneumophila phagosomes to be Rab7+, this probably reflects differences in model systems and detection.
HeLa-Rab7 Q67L cells 2 days after coincubation with live M. tuberculosis phagosomes and 51 latex bead phagosomes were evaluated. (B, left panel) LAMP-1 was scarce on the latex bead phagosomal membranes stained positive for Rab7 Q67L. Nuclear membranes. (A, left panel) Both the was quantitated on phagosomal membranes, nuclear membranes, and plasma electron microscopy. Immunogold staining for Rab7 Q67L (A) and LAMP-1 (B) incubated for an additional 2 days, were fixed, and were processed for immunoelectron microscopy. Immunogold staining for Rab7 Q67L (A) and LAMP-1 (B) was quantitated on phagosomal membranes, nuclear membranes, and plasma membranes. (A, left panel) Both the M. tuberculosis phagosomal membranes and the latex bead phagosomal membranes stained positive for Rab7 Q67L. Nuclear membranes and plasma membranes had negligible levels of staining for Rab7 Q67L. (B, left panel) LAMP-1 was scarce on M. tuberculosis phagosomes but abundant on the latex bead phagosomes in these cells. (A and B, right panels) As a positive control, staining per unit area of cytoplasm was determined for both Rab7 Q67L and LAMP-1. Data shown represent the means and standard errors of the gold particle counts for each compartment. A total of 59 M. tuberculosis phagosomes and 51 latex bead phagosomes were evaluated.

FIG. 9. Quantitation of Rab7 Q67L and LAMP-1 immunogold staining in HeLa-Rab7 Q67L cells 2 days after coincubation with live M. tuberculosis and latex beads. Monolayers of HeLa cells expressing Rab7 Q67L were coincubated with M. tuberculosis and latex beads for 2 h, were washed extensively, were incubated for an additional 2 days, were fixed, and were processed for immunoelectron microscopy. Immunogold staining for Rab7 Q67L (A) and LAMP-1 (B) was quantitated on phagosomal membranes, nuclear membranes, and plasma membranes. (A, left panel) Both the M. tuberculosis phagosomal membranes and the latex bead phagosomal membranes stained positive for Rab7 Q67L. Nuclear membranes and plasma membranes had negligible levels of staining for Rab7 Q67L. (B, left panel) LAMP-1 was scarce on M. tuberculosis phagosomes but abundant on the latex bead phagosomes in these cells. (A and B, right panels) As a positive control, staining per unit area of cytoplasm was determined for both Rab7 Q67L and LAMP-1. Data shown represent the means and standard errors of the gold particle counts for each compartment. A total of 59 M. tuberculosis phagosomes and 51 latex bead phagosomes were evaluated.

In both our study and that of Roy et al., wild-type L. pneumophila phagosomes displayed a trend to lose Rab7 immunostaining with time, suggesting that the wild-type L. pneumophila phagosome loses receptors for the Rab7-GTPase by 5 to 8 h after infection, a time period corresponding to that in which L. pneumophila completes the formation of its ribosome-lined replicative vacuole (25). Our studies have expanded upon the observations made by Roy et al. (39) by examining the phenotype of L. pneumophila phagosomes in host cells expressing a GTPase-deficient, constitutively active Rab7 mutant.

Studies of populations of isolated phagosomes containing latex beads (16, 17, 30) have suggested a sequential acquisition and loss of Rab5 and subsequent acquisition and loss of Rab7. However, at early time points, the populations of phagosomes containing the inert particles exhibit both markers (17, 30). Because these studies examined populations of phagosomes (rather than individual phagosomes), it is unclear from them whether an individual phagosome can have both Rab5 and Rab7 simultaneously or whether acquisition of Rab5 is a prerequisite for acquisition of Rab7 by a phagosome. In prior work, we have found that M. tuberculosis phagosomes exhibit a persistence of Rab5 and that L. pneumophila phagosomes never acquire Rab5 (15). Combined with our present findings, these results suggest that in the case of M. tuberculosis phagosomes, both Rab5 and Rab7 can be present simultaneously on the phagosome and that in the case of L. pneumophila, acquisition of Rab5 is not a prerequisite for acquisition of Rab7. However, confirmation of these hypotheses will require examination of cells that simultaneously express both markers at detectable levels.

Prior studies (33, 38) employing immunofluorescence examination of cells overexpressing Rab7 have found that Rab7 exhibits only a limited colocalization with late endosomal-lysosomal markers. For example, whereas CD-M6PR is typically restricted to a perinuclear area, Rab7 has been observed to extend to the cell periphery. In our studies, with the resolution afforded by electron microscopy, it is clear that many vesicles in the cell that stain richly for Rab7 completely lack LAMP-1 and CD-M6PR immunogold staining. Thus, while functional studies employing constitutively active and negative forms of Rab7 have indicated that Rab7 is important in regulating membrane trafficking between early and late endocytic compartments (18, 38), it is also clear that much of Rab7 is found on compartments that are neither late endosomes nor lysosomes. One possible explanation for these findings is that Rab7 is present on an intermediate compartment or on a shuttle vesicle compartment between the classical early and late endosomal compartments and that this intermediate or shuttle vesicle compartment is rich in Rab7 but has very little of the classical late endosomal and lysosomal markers. An alternative explanation is that Rab7 may also function in pathways other than the usual endocytic pathway. For example, it may function to promote autophagy of other organelles (e.g., ER and mitochondria) or to remodel subcellular organelles by regulating fission of membrane vesicles from the organelles and the subsequent fusion of these vesicles with late endosomes. In this model, phagosomes and organelles that do not themselves fuse with late endosomes and lysosomes may undergo remodeling such that vesicles derived from them are targeted to late endosomes. Either of these models would account for the limited colocalization observed between Rab7 and the classical late endosomal marker, CD-M6PR.

Our observations of relatively high levels of Rab7 on L. pneumophila and M. tuberculosis phagosomes could be explained by at least two different hypotheses. First, the bacterial pathogens may interrupt the maturation of their phagosomes at a Rab7"-LAMP-1" stage by blocking the action of Rab7. Failure of the L. pneumophila and M. tuberculosis phagosomes to mature beyond the Rab7"-LAMP-1" stage could be due to absence of downstream effectors of Rab7 or due to inactivation or inhibition of the function of downstream effectors of Rab7 that promote phagosomal maturation. In the case of M. tuberculosis, which exhibits a persistence of early endosomal markers, this would be at a stage between early and late endosomes. The exclusion of CD-M6PR from the M. tuberculosis phagosome in this study, as well as that of Xu et al. (47), confirms that the block in maturation occurs at a stage prior to interaction of the phagosome with late endosomes. In the case of L. pneumophila, which never acquires early endosomal markers, this stage might represent an early stage of an as-yet-unidentified pathway. Noting that L. pneumophila phagosomes and autophagic vacuoles are both surrounded by ER, Swanson and Isberg (43) have previously speculated that the L. pneumophila phagosomal pathway might reflect an aberrant autophagic pathway. If so, then L. pneumophila may be interrupting maturation of an early stage of autophagosome formation, prior to acquisition of late endosomal or lysosomal markers by the autophagic vacuole. While arrested maturation on an autophagosomal pathway could account for the presence of Rab7 and
FIG. 10. In HeLa cells expressing Rab7 Q67L, Rab7 Q67L colocalizes with LAMP-1 on cytoplasmic vesicles, but LAMP-1 remains absent from *L. pneumophila* and *M. tuberculosis* phagosomes. HeLa-Rab7 Q67L cells were fixed 2 h after infection with wild-type (A and B) or avirulent *L. pneumophila* (C), as described in the legend of Fig. 8, or 2 days after coinfection with live *M. tuberculosis* and latex beads (D, E, and F), as described in the legend of Fig. 9, and processed for immunoelectron microscopy. (A) Rab7 Q67L (15-nm immunogold particles; large arrowheads) colocalized extensively with LAMP-1 (10-nm immunogold particles; small arrowheads) on vesicles that appeared to be autophagosomes or multivesicular bodies. The absence of LPS excludes the possibility that the vesicle shown contains *L. pneumophila*. (B) *L. pneumophila* phagosomes often stained richly for Rab7 Q67L (15-nm gold particles; large arrowheads) but showed little or no staining for LAMP-1. LAMP-1 (10-nm gold particles; small arrowheads) was present on adjacent cytoplasmic vesicles. *L. pneumophila* LPS was stained with 5-nm gold particles (small arrows). (C) Avirulent *L. pneumophila* phagosomes frequently stained positive for Rab7 Q67L (15-nm gold particles; large arrowheads) and consistently stained intensely for LAMP-1 (10-nm gold particles; small arrowheads). *L. pneumophila* LPS was stained with 5-nm gold particles (small arrows). (D and E) *M. tuberculosis* phagosomes, like *L. pneumophila* phagosomes, often stained richly for Rab7 Q67L (15-nm gold particles; large arrowheads) yet acquired only low levels of LAMP-1 (10-nm gold particles; small arrowheads). Mycobacterial LAM was stained with 5-nm immunogold particles (small arrows). An autophagosome shown on the right side of panel D stains richly for both Rab7 Q67L and LAMP-1. (F) Latex bead phagosomes in these cells, on the other hand, had low to moderate levels of staining for Rab7 Q67L (15-nm gold particles; large arrowheads), but stained intensely for LAMP-1 (10 nm gold particles; small arrowheads). The latex bead phagosome shown in this panel has one Rab7 Q67L immunogold particle and approximately 20 LAMP-1 immunogold particles. An adjacent vacuole with multiple internal membranes stains positive for both Rab7 Q67L and LAMP-1. Magnifications are (A) ×41,300; (B) ×29,400; (C, D, and E) ×32,200; and (F) ×32,900.
recruitment of ER to the *L. pneumophila* phagosome, several features of the *L. pneumophila* phagosome are not adequately explained by this model. For example, the *L. pneumophila* phagosome develops intimate interactions with mitochondria and ribosomes, whereas autophagic vacuoles do not. A second hypothesis to account for the presence of Rab7 but a paucity of LAMP-1 on the *M. tuberculosis* and *L. pneumophila* phagosomes is that Rab7 may be involved in pathways other than the endocytic and phagocytic pathways, such as membrane remodeling. Hence, the presence of Rab7 on the *L. pneumophila* and *M. tuberculosis* phagosomes could reflect active remodeling of the phagosomes by Rab7 shuttle vesicles. That Rab7 expression on the *L. pneumophila* phagosome diminishes after the phagosome completes the formation of its ribosome-lined replicative vacuole, in which the organism resides for the remainder of its life cycle in the host cell, is consistent with this hypothesis.

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