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Mycobacterium tuberculosis and *Legionella pneumophila* Phagosomes Exhibit Arrested Maturation despite Acquisition of Rab7

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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 maturational pathway 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

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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 mannose-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.); piperazine-*N,N*′-bis(2-ethanesulfonic acid) (PIPES), methylcellulose, polyvinylpyrrolidone, paraformaldehyde were purchased from Sigma Chemical Company (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 G_1 [IgG$_1$]) was purchased from AMAC (Westbrook, Maine). Mouse MAb to LAMP-1 (H4A3, IgG$_1$) was obtained from the Hybridoma Bank of the University of Iowa, Iowa City. Mouse MAb to the bovine cation-dependent mannose-6-phosphate receptor was provided by Stuart Kornfeld (Washington University, St. Louis, Mo.). Isotypic mouse myeloma control proteins were obtained from Cappel Organon-Technica (Westchester, Pa.). Rabbit antibody to

mycobacterial lipoarabinonmannan (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 rabbit anti-mouse IgG antibody was obtained from Sigma Chemical Company. Reactivity of this commercial antibody to mycobacterial antigens was eliminated by three consecutive overnight adsorptions to an excess of heat-killed *M. tuberculosis*. Protein A colloidal gold conjugates (5, 10, and 15 nm) were provided by G. Posthuma (Utrecht University, The Netherlands).

Bacteria. *M. tuberculosis* Erdman (ATCC 35801), a highly virulent strain, was obtained from the American Type Culture Collection (Rockville, Md.). The organism was passaged through guinea pig lung to maintain virulence, and infecting 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 bear a mutation in the *dot-icm* 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′ one-third of a human *rab7*-like gene on a 240-bp *Eco*RI-*Pst*I fragment excised from IMAGE Consortium Clone 108659 (ATCC 330444). The probe was labeled with [α-³²P]dCTP (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 (15). 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 *Nco*I and *Bam*HI cleavage sites. The construct was under the control of the T7 promoter with an amino-terminal sequence coding for a His$_6$ tag. High-level expression of Rab7 in *Escherichia coli* BL21(DE3) was induced with 0.4 mMisopropyl-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 (purified from *E. coli*) in Syntex adjuvant (1). This adjuvant was used to avoid the production of antibodies to mycobacterial antigens present in Freund's adjuvant. The first immunization was supplemented with 100 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma Chemical Co.). Rabbits immunized with the recombinant proteins yielded high-titer specific polyclonal antisera to human 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 Rab5 or Rab4.

Stable transfection of human cell lines with Rab7 and a constitutively active Rab7 mutant. To facilitate the immunolocalization 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, transfected 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).

GTPase-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′-GGAACCGTTC<u>AA</u>GTCCTGCTGTGTCCCATAT-3′ (mutated nucleotides underlined) and pTRE forward and reverse sequences (5′-TCCAGCCTCCGCGGCCCC-3′) and 5′-TCATCAATGTATCTTATCAT GTCT-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 the wild-type *rab7*.

Transient transfection of HeLa cells with the bovine cation-dependent mannose-6-phosphate receptor. A 1.1-kb *Eco*RI-*Pst*I fragment containing the bovine
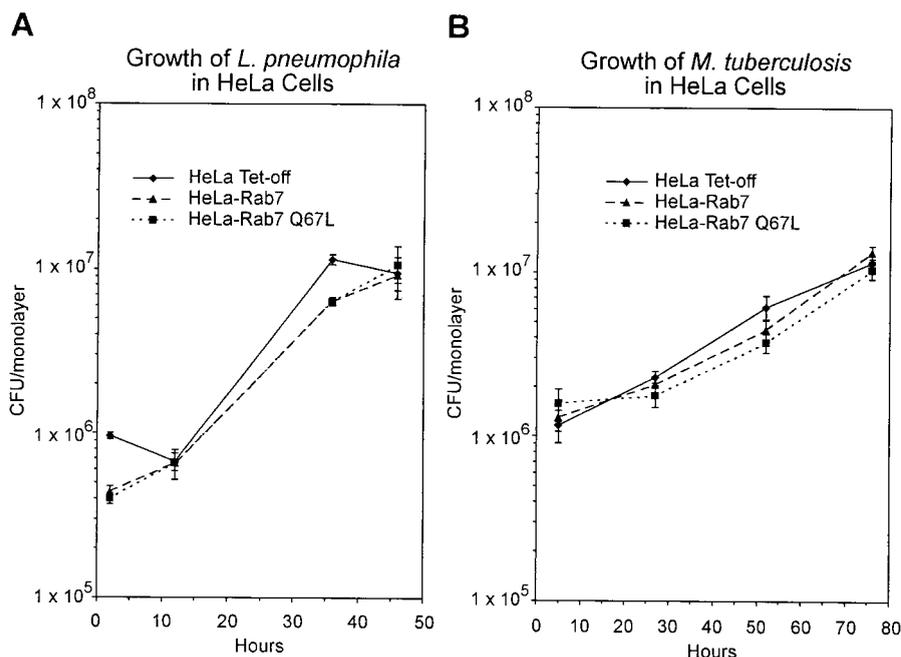
FIG. 1. Growth of *L. pneumophila* and *M. tuberculosis* in HeLa–Tet-off cells or in Hela-Rab7 or HeLa-Rab7 Q67L cells overexpressing Rab7 or Rab7 Q67L. Monolayers of HeLa–Tet-off, HeLa-Rab7, and HeLa-Rab7 Q67L cells in 2-cm² wells were grown for 2 days (A) or 1 day (B) in the absence of tetracycline to induce expression of Rab7 or Rab7 Q67L. The cells were incubated with *L. pneumophila* (2×10^7/ml [A]) or *M. tuberculosis* (10^6/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.

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(+)-*cdmpr*. Transfected cells were kept in the absence of antibiotics for 2 days before infection with *M. tuberculosis* and *L. pneumophila*. Immunofluorescence microscopy demonstrated partial colocalization of the Rab7 and the CD-M6PR, 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 (10^5 cells/well) or THP-1 cells (4×10^5 cells/well) were cultured to confluency in 2-cm² tissue culture wells for 2 days in RPMI 1640 (THP-1) or DMEM (HeLa) with 10% fetal bovine serum without tetracycline. The THP-1 cells were differentiated with phorbol myristic acid (0.16 nM). Monolayers were coincubated with either *M. tuberculosis* (10^6/ml) or *L. pneumophila* (2×10^7/ml or 2×10^8/ml). Monolayers were washed with culture medium and incubated in fresh medium at 37°C. CFU of *M. tuberculosis* were determined at sequential time points using the method described by Hirsch et al. (24). Briefly, the supernatant fluid was removed and retained, and the monocyte monolayer was lysed by adding 0.1% SDS in sterile distilled water. The tissue culture supernatant fluid and the lysate of the cell monolayer were combined, and serial dilutions were plated on 7H11 agar for 2 weeks at 37°C in 5% CO₂, and CFU were enumerated. In the case of *L. pneumophila*, CFU were determined by a modification of the method of Horwitz and Silverstein (29). Briefly, culture media in the wells were removed, saved, and replaced with sterile distilled water, and the monolayers of infected cells were lysed with a 2-mm probe tip sonicator (Heat Systems-Ultrasonics, Plainview, N.Y.) at setting 4 for 10 s. This amount of sonic energy lysed the HeLa cells completely, as determined by phase-contrast microscopy, but did not reduce *L. pneumophila* CFU. The culture supernate and the hypotonic sonicate were combined, serially diluted, and plated on CYE agar plates.

Infection of monolayers of HeLa cells with *M. tuberculosis* and *L. pneumophila*. Stably transfected HeLa cells were plated at a density of 2.5×10^6 per 75-cm² culture flask or at a density of 10^6 per 10-cm-diameter tissue culture plate. Optimal Rab7 expression was obtained by omitting tetracycline from the culture medium 1 to 3 days before the cells were to be fixed. Monolayers were cultured without antibiotics in DMEM (low glucose) with 10% fetal calf serum (certified tetracycline negative) (Clontech). To examine the interaction of HeLa cells with *L. pneumophila*, we plated stably transfected HeLa cells in 10-cm-diameter petri plates in DMEM containing 10% heat-inactivated fetal bovine serum without tetracycline. Two days later, the plates were chilled on ice, and suspensions of wild-type or avirulent *L. pneumophila* (2×10^9/ml) were added to the plates at 0°C. The plates were centrifuged for 20 min at $1,160 \times g$ in a biohazard-safe

rotor, were incubated at 37°C for 30 min (*L. pneumophila*), and were either fixed immediately or washed extensively and incubated for an additional 15 min to 8 h prior to fixation. To examine the interaction of HeLa cells with *M. tuberculosis*, we coincubated the HeLa cells with live or heat-killed *M. tuberculosis* (4×10^8/ml) for 2 h at 37°C. Subsequently, the monolayers were washed extensively with culture medium to remove noningested bacteria and beads, the medium was replaced with fresh DMEM containing 10% fetal calf serum, and the monolayers were incubated for 1 to 3 days prior to fixation. In some experiments, 1-μm-diameter latex beads (1:500 dilution of a 2.5% solid suspension) were added during coincubation with *L. pneumophila* or *M. tuberculosis*.

Immunoelectron microscopy. Monolayers were fixed with 2% paraformaldehyde in 0.1 M PIPES, pH 7.3, containing 6% sucrose for 2 h at 4°C and processed for cryoimmunoelectron microscopy as described previously (13). Cryosections were collected on formvar-coated nickel grids, blocked with 1% bovine serum albumin, 0.1% fish skin gelatin in 0.05 M HEPES, pH 7.5, containing 0.3 M NaCl for 1 h at 4°C. Immunogold double and triple labelings were performed by a modification of the sequential protein A gold technique as described by Slot et al. (41). Briefly, sections were stained first for Rab7 by using 15-nm-diameter protein A gold particles and were washed, and free protein A sites were destroyed by incubation with 2% glutaraldehyde for 10 min. Aldehydes were quenched by floating grids face down for 5 min on each of two consecutive drops of 50 mM glycine in phosphate-buffered saline. The sections were then incubated first with rabbit anti-LAM or anti-LPS and then with 5-nm-diameter protein A gold particles. Staining for the cation-dependent mannose-6-phosphate receptor, LAMP-1, or LAMP-2 was accomplished by incubating sections with primary mouse MAb (10 μg/ml) in blocking buffer for 1 h (22°C), washing, and incubating with rabbit anti-mouse IgG, (45 min at 22°C) followed by incubation with 10-nm-diameter protein A gold particles (30 min at 22°C). Sections were embedded in 1.8% methylcellulose–0.4% uranyl acetate (22). Consecutive phagosomes were photomicrographed with a JEOL 100 CX II electron microscope. Measurements of gold particles per micrometer of membrane or per square micrometer of cytoplasm were made with a Numonics 2210 digitizer tablet and SigmaScan software (Jandel Scientific Co., Corte Madera, Calif.).

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
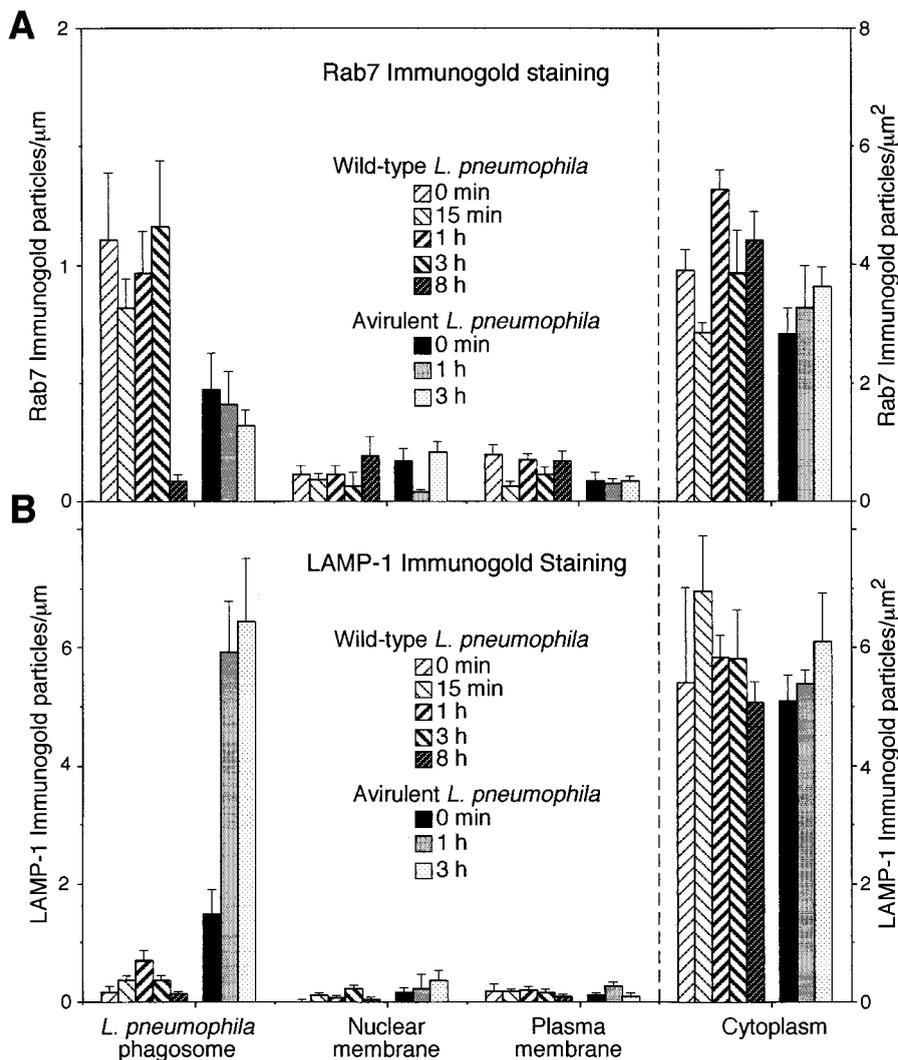
FIG. 2. Quantitation of Rab7 and LAMP-1 immunogold staining in HeLa-Rab7 cells infected with wild-type or avirulent *L. pneumophila*. Wild-type or avirulent *L. pneumophila* cells were spun down onto monolayers of HeLa-Rab7 cells at 4°C. The monolayers were incubated for 30 min at 37°C and were either 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.

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-*
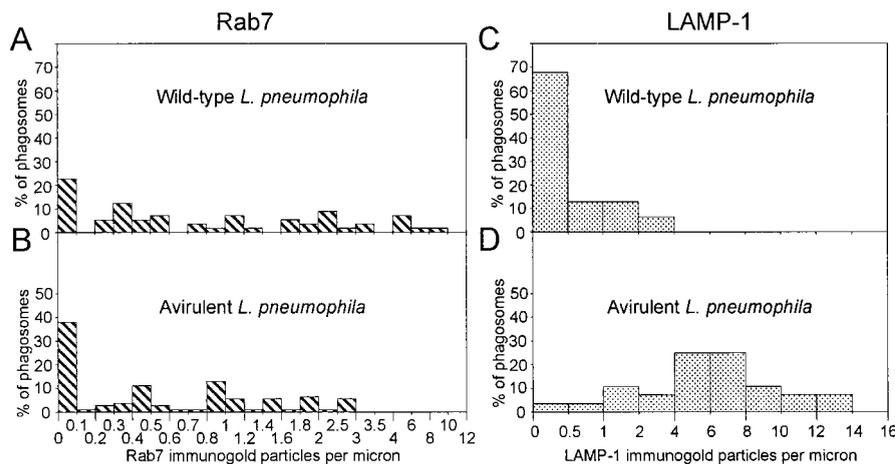
FIG. 3. Distribution of staining for Rab7 and LAMP-1 in HeLa-Rab7 cells fixed 3 h after infection with either wild-type or avirulent *L. pneumophila*. As described for Fig. 2, wild-type (A and C) or avirulent (B and D) *L. pneumophila* cells were spun down onto monolayers of HeLa-Rab7 cells at 4°C. The monolayers were incubated for 30 min at 37°C, were washed extensively, were incubated 2 h at 37°C, were fixed, and were processed for cryoimmunoelectron microcopy. Gold particles per micrometer of membrane on phagosomal membranes, plasma membranes, and nuclear membranes were enumerated for Rab7 (A and B) and LAMP-1 (C and D).

culosis infection of macrophages, the natural host cells of these pathogens in humans, we investigated the extent to which the interaction of the pathogens with HeLa cells resembles their interaction with human macrophages (13). We examined the capacity of the two pathogens to multiply within HeLa–Tet-off cells and HeLa–Tet-off cells expressing Rab5 (HeLa-Rab5 cells) and the expression of endolysosomal markers on the phagosomes 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 the HeLa 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/μm^2), with an average level of staining of 4.8 ± 0.6 gold particles/μm^2 (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/μm^2) (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.
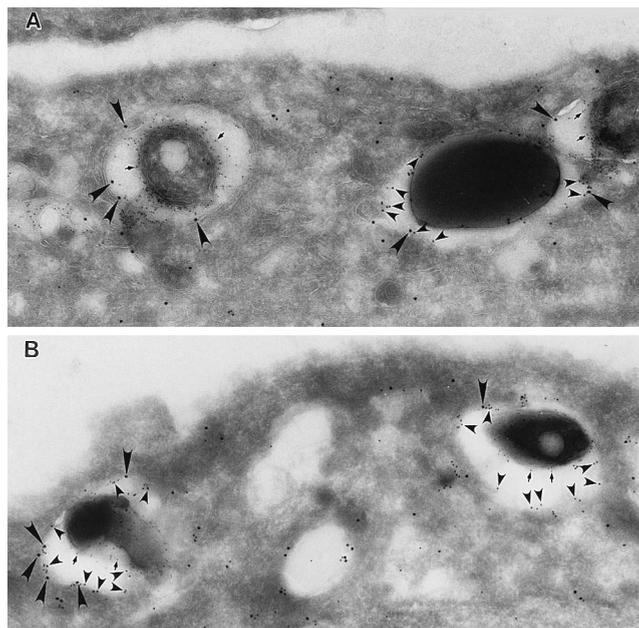
FIG. 4. Phagosomes containing wild-type *L. pneumophila*, but not avirulent *L. pneumophila*, lack LAMP-1 despite recruitment of Rab7. Monolayers of HeLa-Rab7 cells were coincubated with latex beads and wild-type (A) or avirulent (B) *L. pneumophila*, fixed, and processed for immunoelectron microscopy 3 h after infection. Rab7 was stained with 15-nm gold particles (large arrowheads), LAMP-1 was stained with 10-nm gold particles (small arrowheads), and *L. pneumophila* LPS was stained with 5-nm gold particles (small arrows). (A) Two wild-type *L. pneumophila* phagosomes and one latex bead phagosome are shown in this micrograph. The wild-type *L. pneumophila* phagosome on the left has four 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 (Fig. 5B, 6C, and 7). 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 10D 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*
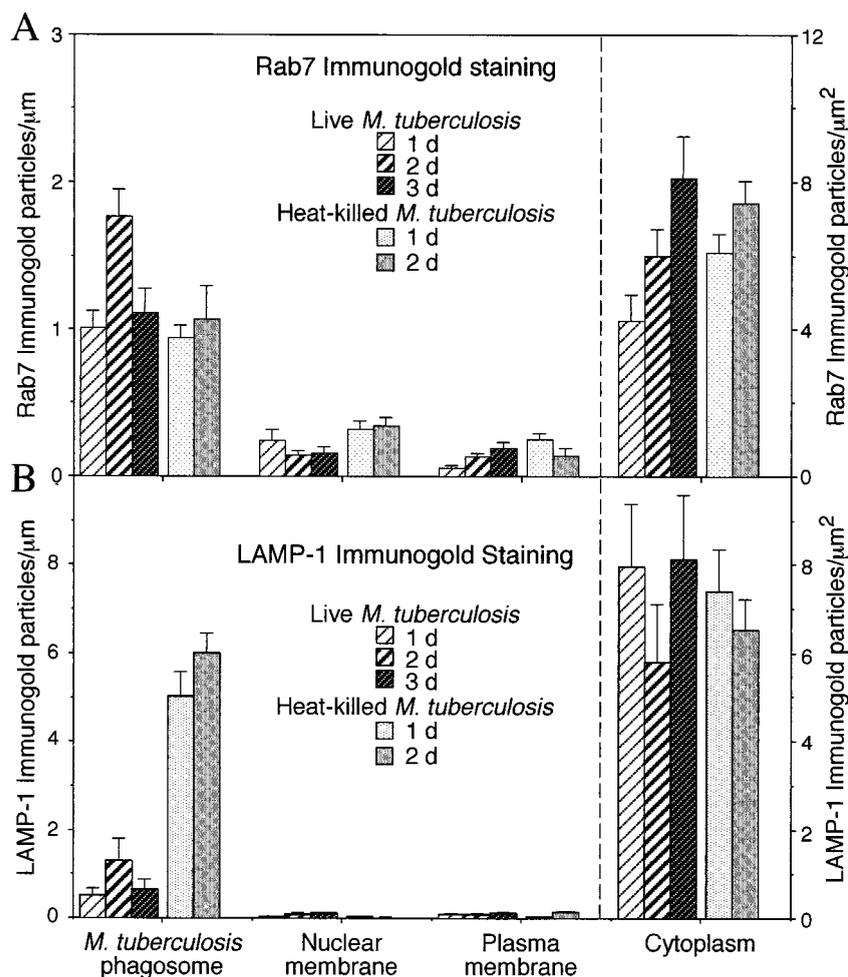
FIG. 5. Quantitation of Rab7 and LAMP-1 immunogold staining in HeLa-Rab7 cells infected with live or heat-killed *M. tuberculosis*. Monolayers of HeLa-Rab7 cells were incubated with suspensions of live or heat-killed *M. tuberculosis* for 2 h at 37°C, were washed to remove nonadherent bacteria, were incubated for an additional 1 to 3 days, were fixed, and were prepared for cryoimmunoelectron microscopy. Rab7 (A) and LAMP-1 (B) immunogold particles were enumerated on phagosomal membranes, nuclear membranes, and plasma membranes. 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 phagosomes containing live *M. tuberculosis* at 1 to 3 days and was also present on phagosomes containing heat-killed *M. tuberculosis*, but at a somewhat lower level. Rab7 staining was scarce on nuclear membranes and plasma membranes. (A, right panel) As a control, Rab7 staining per unit area of cytoplasm of the HeLa cells was quantitated and found to be comparable in cells containing live or heat-killed *M. tuberculosis*. The level of staining in the cytoplasm increased somewhat from 1 to 3 days due to longer tetracycline-free induction. (B, left panel) Live *M. tuberculosis* phagosomes had low levels of staining for LAMP-1. In contrast, heat-killed *M. tuberculosis* phagosomes stained richly for LAMP-1. Plasma membranes and nuclear membranes lacked LAMP-1 staining. (B, right panel) LAMP-1 per unit area of cytoplasm was comparable in HeLa cells containing live or heat-killed *M. tuberculosis*. The total number of phagosomes evaluated were as follows: 41, 112, and 61 for live *M. tuberculosis* at 1, 2, and 3 days, respectively, and 85 and 38 for dead *M. tuberculosis* at 1 and 2 days, respectively.

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/μm^2). 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/μm, compared with nuclear membrane staining of 0.13 ± 0.11 gold particles/μm; $P = 0.007$, 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/μm^2) 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).
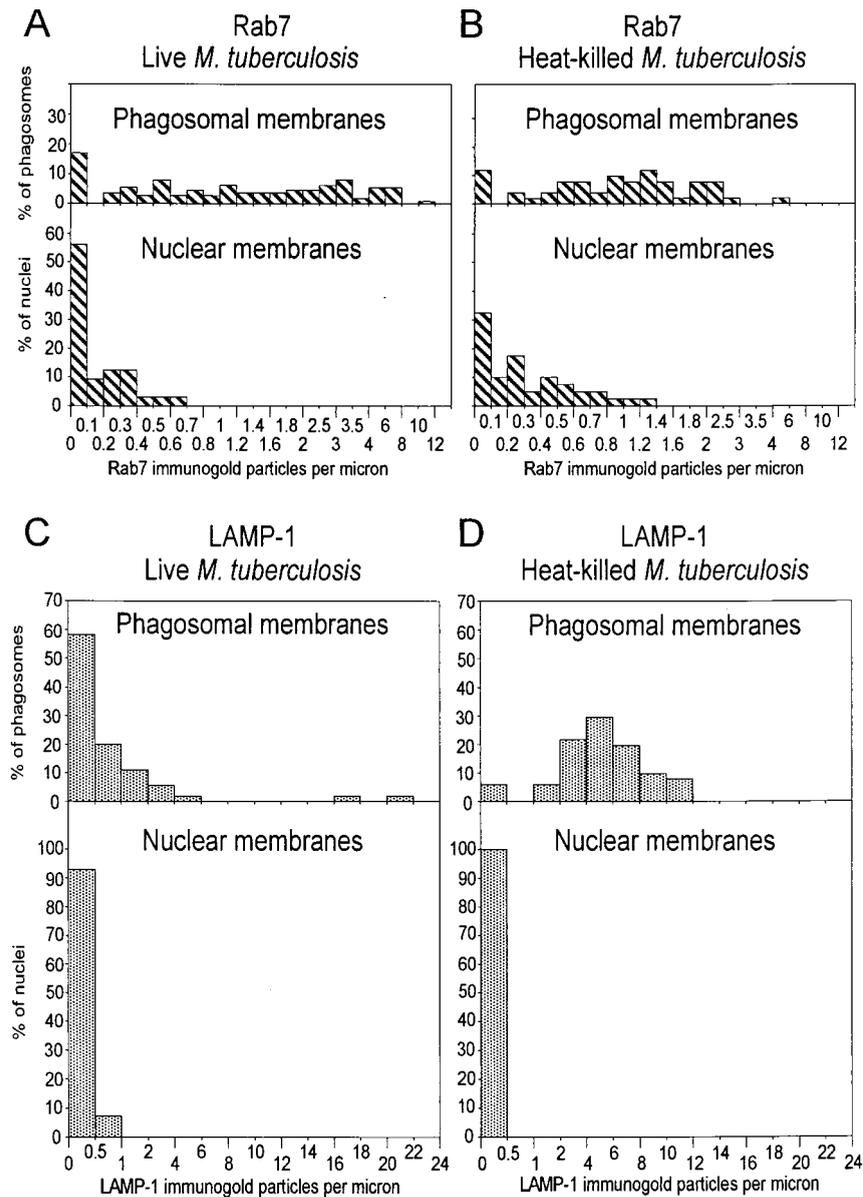
FIG. 6. Distribution of staining for Rab7 and LAMP-1 in HeLa-Rab7 cells 2 days after infection with live or heat-killed *M. tuberculosis*. HeLa-Rab7 cells expressing Rab7 were incubated with live (A and C) or heat-killed (B and D) *M. tuberculosis* for 2 h, were washed extensively, were incubated at 37°C for 2 days, were fixed, and were processed for cryoimmunoelectron microscopy. Gold particles per micrometer of membrane on phagosomal membranes and nuclear membranes are shown for Rab7 (A and B) and for LAMP-1 (C and D).

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
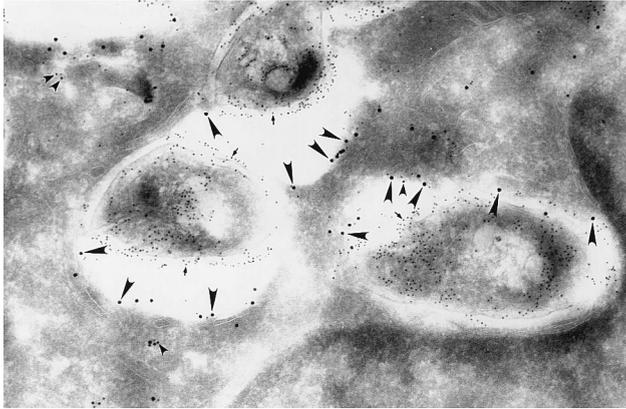
FIG. 7. *M. tuberculosis* phagosomes in HeLa-Rab7 cells stain positively for Rab7 but exclude LAMP-1. HeLa-Rab7 cells were maintained and expanded in the presence of tetracycline (5 μg/ml). One day prior to infection with *M. tuberculosis*, tetracycline was removed from the culture medium to induce Rab7 expression. The HeLa cells were incubated for 2 h with *M. tuberculosis* using a multiplicity of infection of 50:1. Nonadherent bacteria were washed away, and the monolayers were incubated for 2 additional days. Monolayers were fixed and processed for cryoimmunoelectron microscopy. Rab7 was stained with 15-nm immunogold particles (large arrowheads) and is abundant on the *M. tuberculosis* phagosomal membranes. Mycobacterial lipoarabinomannan was stained with 5-nm gold particles and is present on the mycobacterial cell wall (small arrows). LAMP-1 immunogold staining (10-nm gold particles) is scarce on the *M. tuberculosis* phagosomal membrane but is present on adjacent cytoplasmic vesicles (small arrowheads). Magnification, ×33,930.
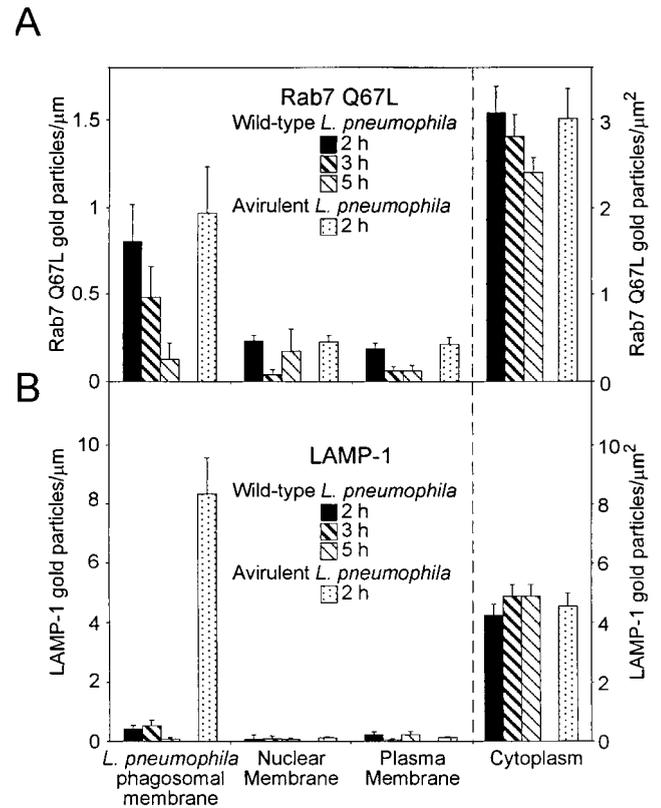

FIG. 8. Quantitation of Rab7 Q67L and LAMP-1 immunogold staining in HeLa-Rab7 Q67L cells after infection with wild-type or avirulent *L. pneumophila*. Monolayers of HeLa cells expressing Rab7 Q67L were infected with wild-type or avirulent *L. pneumophila* as described in the legend of Fig. 2. Cells were fixed at 2 to 5 h after infection and were processed for immunoelectron microscopy. Immunogold staining for Rab7 Q67L (A) and LAMP-1 (B) was quantitated on phagosomal membranes, plasma membranes, and nuclear membranes. (A, left panel) Wild-type *L. pneumophila* phagosomes stained positive for Rab7 Q67L at 2 to 3 h, with an overall level of staining similar to that found on avirulent *L. pneumophila* phagosomes at 2 h. However, Rab7 Q67L levels on wild-type *L. pneumophila* phagosomes fell to background levels at 5 h after infection. Nuclear membranes and plasma membranes served as negative controls and had relatively low levels of staining for Rab7 Q67L. (B, left panel) LAMP-1 staining was excluded from wild-type *L. pneumophila* phagosomes but was abundant on avirulent *L. pneumophila* phagosomes. Plasma membrane and nuclear membrane staining served as internal negative controls. (A and B, right panels) Immunogold staining per unit area of cytoplasm was counted as a positive control and found to be comparable under the two infection conditions both for Rab7 Q67L (A) and for LAMP-1 (B). Data shown represent the means and standard errors of the means of gold particle counts for each compartment. Total numbers of phagosomes evaluated were as follows: 32, 19, and 38 for wild-type *L. pneumophila* at 2, 3, and 5 h, respectively, and 45 for avirulent *L. pneumophila* at 2 h.

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
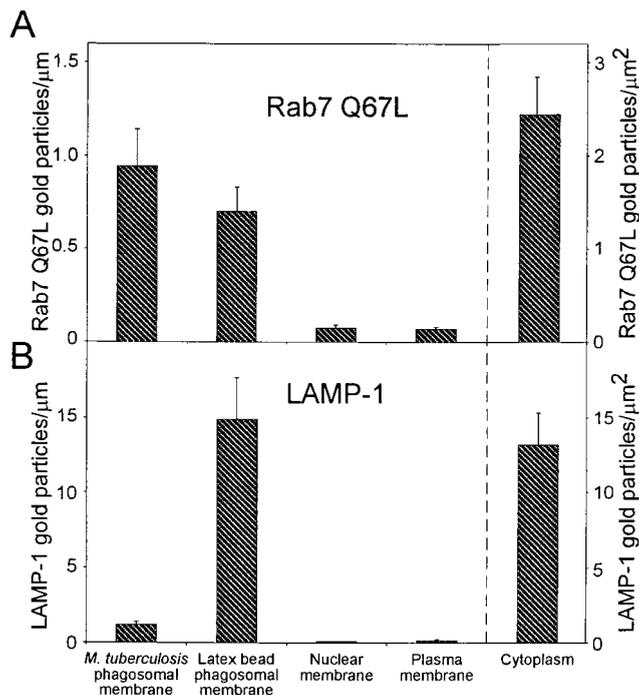
A

B

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 immuno-electron 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 means of gold particle counts for each compartment. A total of 59 *M. tuberculosis* phagosomes and 51 latex bead phagosomes were evaluated.

techniques. 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 (13). 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
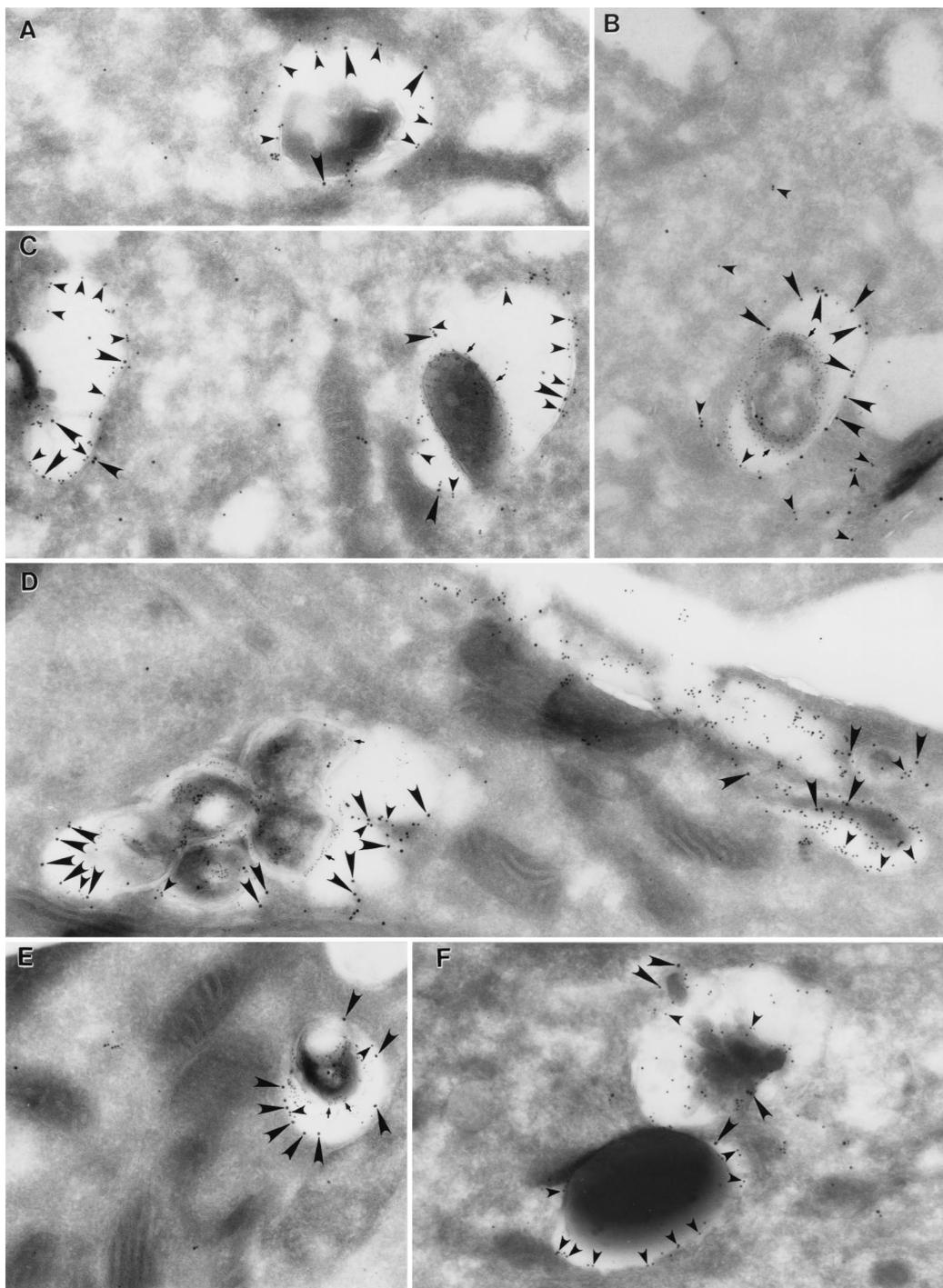
FIG. 10. In HeLa cells expressing Rab7 Q67L, Rab7 Q67L colocalizes with LAMP-1 on cytoplasmic vesicles, but LAMP-1 remains absent from *L. pneumophila* and *M. tuberculosis* phagosomes. HeLa-Rab7 Q67L cells were fixed 2 h after infection with wild-type (A and B) or avirulent *L. pneumophila* (C), as 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, frequently 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.
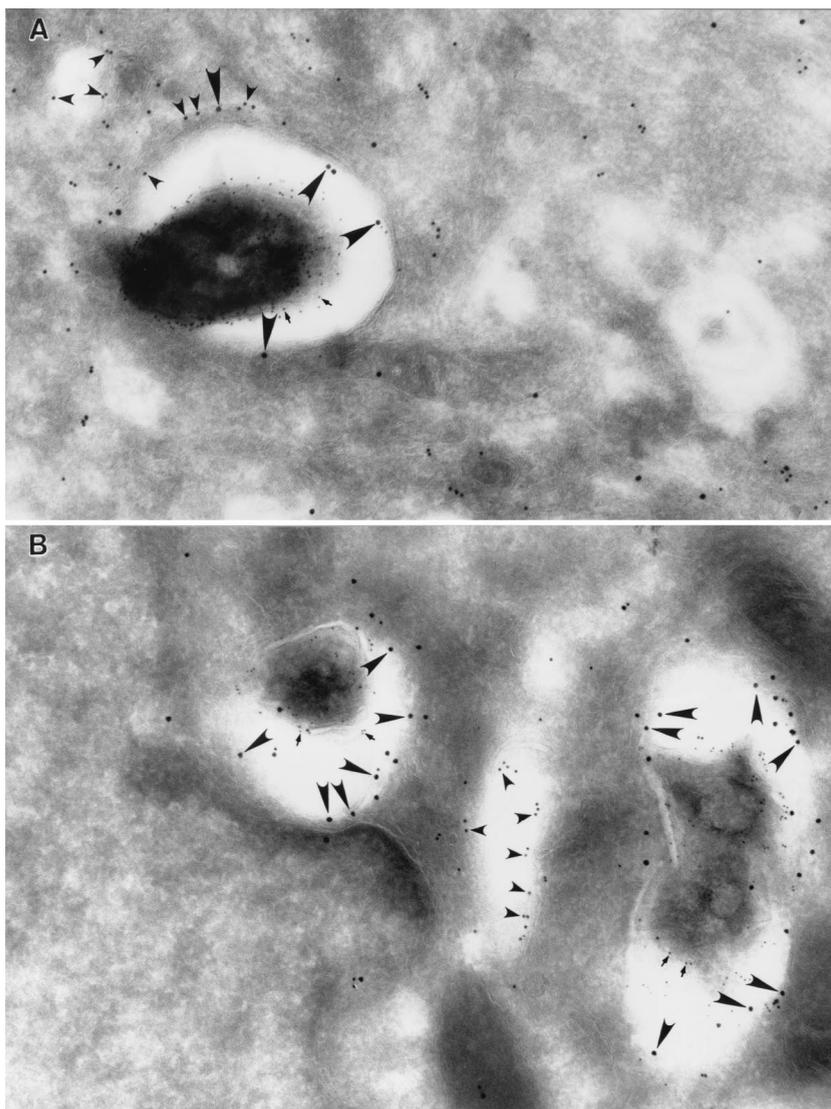
FIG. 11. *L. pneumophila* and *M. tuberculosis* phagosomes that stained positive for Rab7 had only low levels of staining for the late-endosomal marker, cation-dependent mannose-6-phosphate receptor. HeLa-Rab7 cells were transiently transfected with pZeo-*cd-m6pr* and incubated for 2 h with *L. pneumophila* (A) or for 2 days with *M. tuberculosis* (B) as described in the legends of Fig. 2 and 6, respectively. (A) The *L. pneumophila* phagosome shown has four Rab7 immunogold particles (15-nm gold particles; large arrowheads) but only one M6PR immunogold particle (10-nm gold particles; small arrowhead). M6PR is abundant on cytoplasmic vesicles outside of the *L. pneumophila* phagosome. (B) *M. tuberculosis* phagosomes stained positively for Rab7 (15-nm gold particles; large arrowheads) but had relatively few CD-M6PR immunogold particles (10-nm gold particles; small arrowheads). Although the CD-M6PR staining of the *M. tuberculosis* phagosomes was statistically significant, it was unimpressive when compared with the levels of Rab7 immunogold staining on the *M. tuberculosis* phagosomes and the level of M6PR staining on other cytoplasmic vesicles (note the vesicle staining intensely for M6PR between the two *M. tuberculosis* phagosomes). Magnifications are (A) ×33,120 and (B) ×47,520.

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 remain-

der of its life cycle in the host cell, is consistent with this hypothesis.

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