Deviant Expression of Rab5 on Phagosomes Containing the Intracellular Pathogens Mycobacterium tuberculosis and Legionella pneumophila Is Associated with Altered Phagosomal Fate

DANIEL L. CLEMENS,* BAI-YU LEE, AND MARCUS A. HORWITZ

Division of Infectious Diseases, Department of Medicine, UCLA School of Medicine, Center for Health Sciences, Los Angeles, California 90095

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The intracellular human pathogens Legionella pneumophila and Mycobacterium tuberculosis reside in altered phagosomes that do not fuse with lysosomes and are only mildly acidified. The L. pneumophila phagosome exists completely outside the endolysosomal pathway, and the M. tuberculosis phagosome displays a maturation arrest at an early endosomal stage along this pathway. Rab5 plays a critical role in regulating membrane trafficking involving endosomes and phagosomes. To determine whether an alteration in the function or delivery of Rab5 could play a role in the aberrant development of L. pneumophila and M. tuberculosis phagosomes, we have examined the distribution of the small GTPase, Rab5c, in infected HeLa cells overexpressing Rab5c. Both pathogens formed phagosomes in HeLa cells with molecular characteristics similar to their phagosomes in human macrophages and multiplied in these host cells. Phagosomes containing virulent wild-type L. pneumophila never acquired immunogold staining for Rab5c, whereas phagosomes containing an avirulent mutant L. pneumophila (which ultimately fused with lysosomes) transiently acquired staining for Rab5c after phagocytosis. In contrast, M. tuberculosis phagosomes exhibited abundant staining for Rab5c throughout its life cycle. To verify that the overexpressed, recombinant Rab5c observed on the bacterial phagosomes was biologically active, we examined the phagosomes in HeLa cells expressing Rab5c Q79L, a fusion-promoting mutant. Such HeLa cells formed giant vacuoles, and after incubation with various particles, the giant vacuoles acquired large numbers of latex beads, M. tuberculosis, and avirulent L. pneumophila but not wild-type L. pneumophila, which consistently remained in tight phagosomes that did not fuse with the giant vacuoles. These results indicate that whereas Rab5 is absent from wild-type L. pneumophila phagosomes, functional Rab5 persists on M. tuberculosis phagosomes. The absence of Rab5 on the L. pneumophila phagosome may underlie its lack of interaction with endocytic compartments. The persistence of functional Rab5 on the M. tuberculosis phagosomes may enable the phagosome to retard its own maturation at an early endosomal stage.

Following phagocytosis, phagosomes containing inert particles follow an intracellular pathway that mirrors the stages of the endosomal-lysosomal pathway (16, 17, 34, 35). At early time points after phagocytosis there is a rapid sorting of membrane proteins and recycling of many plasma membrane proteins to the plasma membrane (34, 35). The early phagosomes of inert particles rapidly acquire markers of early endosomes, including the mannose receptor and Rab5 (16, 17, 35). Subsequently, the phagosome loses Rab5 and the markers of early endosomes and acquires Rab7 and markers associated with late endosomes, such as lysosome-associated membrane glycoproteins (LAMPs), and cathepsin D (16, 17, 35). With still more time and maturation, the phagosome fuses with secondary lysosomes, acquires higher concentrations of acid hydrolases and LAMPs, and loses the Rab7-GTPass but acquires other, as-yet-undefined, small GTPasses (17, 27).

The pathways of the intracellular parasites Legionella pneumophila and Mycobacterium tuberculosis deviate from the above pathway of inert particles in that they reside and multiply in phagosomes that resist acidification and fusion with lysosomes (4, 12, 15, 25, 26, 44). The pathways of L. pneumophila and M. tuberculosis also differ from one another (12, 13). Whereas phagosomes containing wild-type L. pneumophila do not acquire the transferrin receptor or any other markers of the endosomal-lysosomal pathway studied to date (13), phagosomes containing virulent M. tuberculosis show a persistent interaction with early endosomes, as evidenced by the presence of transferrin receptor (13) and acquisition of exogenously added transferrin (14). The mechanisms underlying the altered maturation of L. pneumophila and M. tuberculosis phagosomes have not been determined.

Since Rab-GTPass play a pivotal role in the regulation of membrane trafficking within eukaryotic cells, we considered the possibility that a disruption in the function or delivery of Rab-GTPass to L. pneumophila or M. tuberculosis phagosomes could play a role in the altered development of these phagosomes. Rab-GTPasses are low-molecular-weight members of the Ras superfamily that regulate docking and fusion between different subcellular organelles (21, 31, 33). Over 30 different Rab-GTPasses have been identified, and it is likely that each compartment of the endocytic and secretory pathways in eukaryotic cells has a unique subset of Rab-GTPasses. The Rab-GTPass cycle between GTP-bound and GDP-bound forms. In the GTP-bound form, the molecule is in the “on” configuration and permits fusion between vesicles bearing homologous Rab-GTPasses. The GDP-bound form is the “off” configuration, which does not permit fusion. In addition to
cycling between on and off configurations, the Rab-GTPases also cycle between membrane-bound and soluble forms. The GDP-bound, but not the GTP-bound, form of a Rab protein can be extracted in a reversible fashion from a membrane-bound form to a soluble cytosolic form by the Rab chaperon protein, Rab-GDP dissociation inhibitor (GDI). Acquisition of a particular Rab-GTPase by a membrane requires that the membrane have specific receptor mechanisms for the Rab-GTPase (5, 42). In addition, functional activity of a Rab-GTPase on the membrane in promoting membrane fusion events requires specific effector machinery (39). The specific receptor and effector mechanisms for Rab-GTPases have not been completely elucidated.

Bacteria. M. tuberculosis Erdman (ATCC 35801), a highly virulent strain, was obtained from the American Type Culture Collection (Manassas, Va.). The organism was passaged through guinea pig lung to maintain virulence as described previously (13). Before experimentation, a vial of pneumon- enate was rapidly thawed at 37°C, and the bacteria were cultured on 7H11 agar at 37°C with 5% CO2 and 100% humidity. Seven to 8 days later, bacteria were scraped from 20 to 40 plates into 10 to 20 ml of DMEM containing 10% fetal bovine serum (FBS) and 5% heat-inactivated horse serum (HIS). Bacteria containing predominantly single bacilli was prepared by sonicating the bacteria in a water bath (model 9; Astrason, Plainview, N.Y.) for 60 s, sedimenting any remaining clumps of organisms by centrifugation at 20 × g for 10 min and resuspending the pellet single bacilli-suspending drop of the tube. The concentration of organisms was determined by the 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 previously described (26). The egg口语 grown L. pneumophila was cultured one time only on 1/10 Columbia chocolate agar (Difco) incubated 7 days at 37°C, and the material was used immediately. The avirulent mutant L. pneumophila 2S5 was prepared and maintained as described previously (24). This strain has been shown to bear a mutation in the dot-tem virulence locus (30).

Rab-GTPases. Immunization, expression, and purification of recombinant Rab5 and preparation of antisera. To clone the human rab5 genes, we screened a human fetal lung cdNA library (Invitrogen) by colony hybridization with a cDNA probe (ATCC 84765) encoding the 3’-third of a rab5-like gene. The probe was labeled with [α-32P] dCTP (Amersham) by the random-priming method. Purified polyribosomes were incubated at 42°C in a solution containing 2× PIPES, 50% deionized formamide, 0.5% (wt/vol) sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml. Positive clones were selected after three rounds of colony hybridization and analyzed by restriction enzyme digestion. The identities of the positive clones were confirmed by sequencing both strands of DNA in opposite directions. The rab5 sequence obtained (GenBank accession no. AF141304) was identical to the previously published sequences for human rab5a except for two nucleotide changes in the eight codon region corresponding to alanine rather than an arginine. This single amino acid change was confirmed by nucleotide-sequence analyses of four independently derived rab5c clones. The human rab5c gene is highly homologous to the canine rab5c sequence as well as to rab5a and rab5b sequences (8, 10). The cDNA for the complete rab5c gene was amplified by PCR and cloned into expression vectors for Escherichia coli. The rab5c gene was cloned into PET15 between NdeI and XhoI cutting sites. The construct was under the control of the T7lac promoter with an amino-terminal sequence coding for a thrombin-cleavable His6 tag. High-level expression of Rab5c in E. coli BL21(DE3) was induced with 1 mM IPTG (isopropyl- β-D-thigalactopyranoside), and the recombinant proteins were purified to homogeneity from sonicated cell pellet extracts by a combination of nickel affinity and gel filtration chromatography. The His tag was removed by thrombin cleavage (T7 Tag protease kit; Novagen). Recombinant Rab5c was removed by a second round of nickel affinity chromatography. The resulting material was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to exhibit a single 25-kDa band by Coomassie blue staining. Rabbit polyclonal antibodies were raised in 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-acetyl muramidyl-β-alanyl-β-asparaginyl (Sigma Chemical Co.). Rabbits immunized with the recombinant proteins yielded high-titer-specific polyclonal antiserum to human Rab5c. The resulting polyclonal antibodies were affinity purified by binding to recombinant E. coli Rab5c, eluted with glycine-HCl (pH 2.5) containing 0.1% bovine serum albumin (BSA) carrier protein, and immediately neutralized with Tris-HCl (pH 8.0). The purified antibodies reacted equally well with geranlated and nongeranlated Rab5c and did not cross-react with L. pneumophila or M. tuberculosis antigens. Antisera to Rab5c did not cross-react with Rab7.

Stable transfection of human cell lines with Rab5c and a GTPase-deficient, constitutively active Rab5c mutant. To facilitate the immunolocalization of Rab5 in infected cells, we developed a stably transduced human HeLa cell line that overexpresses the Rab5c isoform of Rab5 (HeLa-Rab5c). We cloned the human rab5c cDNA into pTR vector for transient expression, a vial of ampicillin, and used gold conjugates (5, 10, and 15 nm) were provided by G. Posthuma (Utrecht University, Utrecht, The Netherlands).

MATERIALS AND METHODS

Reagents and antibodies. Glutaraldehyde was purchased from Polysciences (Warrington, Pa.); PIPES [piperazine-N,N’-bis(2-ethanesulfonic acid)] (Sigma-Cellulose, polyvinylpyrrolidone, and paraformaldehyde were purchased from Sigma Chemical Co. (St. Louis, Mo.); and Dulbecco’s phosphate-buffered saline (Dulbecco’s phosphate-buffered saline) was provided by R. Reiss (University of California, Los Angeles). G. Posthuma (Utrecht University, Utrecht, The Netherlands).

Mouse monoclonal antibody to the human transferrin receptor (immunoglobulin G1 [IgG1]) was purchased from AMAC (Westbrook, Maine). Mouse monoclonal antibody to Lamp-1 (HA3, IgG3) was obtained from the Hybridoma Bank of the University of Iowa, Iowa City. Isotypic mouse myeloma control proteins were obtained from Cappel Organon Teknica (West Chester, Pa.). Rabbit antibody to mycobacterial lipoarabinomannan (LAM) was prepared as described previously (13). Rabbit antibody to L. pneumophila lipopolysaccharide (LPS) was prepared by immunizing rabbits with LPS purified from L. pneumophila Ph1 in Freund’s adjuvant (18). Purified rabbit anti-mouse IgG antibody was purchased from Sigma Chemical Co. Rat anti-human serum albumin (IgG1 isotype) was obtained from Sigma Chemical Co. Reactive IgG was purified by affinity chromatography on Protein A/G (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Cell culture. Human HeLa cells were cultured in 7H9 liquid medium with 1% (wt/vol) bovine serum albumin (BSA) and 10% (vol/vol) FBS (Gibco). The media were supplemented with 10 μg/ml of amphotericin B. HeLa cells were grown in T-25 flasks (Corning) at 37°C in a humidified atmosphere containing 5% CO2.

Production of recombinant Rab5c. A recombinant plasmid (pET5b) designed to express a histidine tagged Rab5c was constructed (31). The sequence coding for a thrombin-cleavable His6 tag. High-level expression of Rab5c in E. coli BL21(DE3) was induced with 1 mM IPTG (isopropyl- β-D-thigalactopyranoside), and the recombinant proteins were purified to homogeneity from sonicated cell pellet extracts by a combination of nickel affinity and gel filtration chromatography. The His tag was removed by thrombin cleavage (T7 Tag protease kit; Novagen). Recombinant Rab5c was removed by a second round of nickel affinity chromatography. The resulting material was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to exhibit a single 25-kDa band by Coomassie blue staining. Rabbit polyclonal antibodies were raised in 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-acetyl muramidyl-β-alanyl-β-asparaginyl (Sigma Chemical Co.). Rabbits immunized with the recombinant proteins yielded high-titer-specific polyclonal antiserum to human Rab5c. The resulting polyclonal antibodies were affinity purified by binding to recombinant E. coli Rab5c, eluted with glycine-HCl (pH 2.5) containing 0.1% bovine serum albumin (BSA) carrier protein, and immediately neutralized with Tris-HCl (pH 8.0). The purified antibodies reacted equally well with geranlated and nongeranlated Rab5c and did not cross-react with L. pneumophila or M. tuberculosis antigens. Antisera to Rab5c did not cross-react with Rab7.
Preparation of a HeLa cell line stably transfected with transferrin receptor. A 2.4-kb EcoRI fragment containing the human transferrin receptor gene was released from pGEM1-TR (provided by Marino Zerial, Heidelberg, Germany) and subcloned into pcDNA3.1/Zeo(1) (Invitrogen). HeLa-Rab5c cells were transfected with pcDNA3.1/Zeo(1)-TR by calcium phosphate precipitation. Transfected cells were maintained in complete DMEM containing hygromycin (100 μg/ml) and tetracycline (5 μg/ml) and were selected with zeomycin (200 μg/ml) for 3 to 4 weeks. Zeomycin-resistant clones were isolated and screened for coexpression of Rab5c and the transferrin receptor. Immunofluorescence microscopy demonstrated colocalization of Rab5c and the transferrin receptor, consistent with published observations of other investigators (11).

Assessment of intracellular growth of M. tuberculosis and L. pneumophila in monolayers of THP-1 or HeLa cells. 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 Rab5c 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 bovine serum (certified tetracycline negative; Clontech). In experiments designed to examine early time points after infection, stably transfected HeLa cells were plated in 10-cm-diameter petri plates in DMEM containing 10% heat-inactivated FBS without tetracycline. Two days later, the plates were chilled on ice, and L. pneumophila (2 × 10^8/ml) or M. tuberculosis (4 × 10^8/ml) was added to the plates at 0°C. The plates were centrifuged for 20 min at 1,160 × g in a biohazard-safe rotor, incubated at 37°C for 15 or 30 min (L. pneumophila) or 2 h (M. tuberculosis), and either fixed immediately or washed extensively and incubated for an additional 15 min to 4 h prior to fixation. In experiments designed to examine later time points, the plates were centrifuged for 20 min at 1,160 × g in a biohazard-safe rotor, incubated at 37°C for 24 h, fixed, and stained with rhodamine-conjugated phalloidin to visualize filaments of the bacteria.

Infection of monolayers of HeLa cells with M. tuberculosis and L. pneumophila. Stably transfected HeLa cells were plated at a density of 10^6 cells/well in 24-well tissue culture plates. Optimal Rab5c 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 bovine serum (certified tetracycline negative; Clontech). In experiments designed to examine early time points after infection, stably transfected HeLa cells were plated in 10-cm-diameter petri plates in DMEM containing 10% heat-inactivated FBS without tetracycline. Two days later, the plates were chilled on ice, and L. pneumophila (2 × 10^8/ml) or M. tuberculosis (4 × 10^8/ml) was added to the plates at 0°C. The plates were centrifuged for 20 min at 1,160 × g in a biohazard-safe rotor, incubated at 37°C for 15 or 30 min (L. pneumophila) or 2 h (M. tuberculosis), and either fixed immediately or washed extensively and incubated for an additional 15 min to 4 h prior to fixation. In experiments designed to examine later time points, the plates were centrifuged for 20 min at 1,160 × g in a biohazard-safe rotor, incubated at 37°C for 24 h, fixed, and stained with rhodamine-conjugated phalloidin to visualize filaments of the bacteria.
points after infection, the HeLa cells were coincubated with L. pneumophila (2 × 10⁸/ml) or M. tuberculosis (4 × 10⁹/ml) together with 1-µm-diameter latex beads (1:500 dilution of a 2.5% solid suspension) at 37°C. After coincubation at 37°C for 1 to 2 h, the monolayers were washed extensively with culture medium to remove noningested bacteria and beads, the medium was replaced with fresh DMEM with 10% fetal calf serum, and the monolayers were incubated for 6 h to 2 days prior to fixation.

**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. Aldehydes were quenched with 10 mM glycine in PBS, and the cells were scraped into PBS with 0.1% BSA, pelleted by centrifugation, embedded in 10% gelatin at 37°C, cryoprotected with 20% polyvinylpyrrolidone in 2.3 M sucrose, and frozen in liquid nitrogen. Cryosections were collected on drops of 2.3 M sucrose and 2% methylcellulose (1:1) (W. Liou and J. Slot, 13th International Congress on Electron Microscopy, p. 253–254, 1994), transferred to Formvar-coated nickel grids, and blocked with 1% BSA and 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 labelling were performed as described by Slot et al. (38). Sections were embedded in 1.8% methylcellulose–0.4% uranyl acetate (20). Consecutive phagosomes were photographed at a magnification of ×14,000 using a JEOL 100 CX II electron microscope. Measurements of the number of gold particles per micron of membrane and per square micron of cytoplasm were determined from the negatives with a Numonics 2220 digitizer tablet and Sigma Scan software (Jandel Scientific).

**RESULTS**

**Establishment of a model human cell system suitable for evaluating Rab5 expression on phagosomes.** We have found that the endogenous levels of Rab5 in normal human monocytes, monocyte-derived macrophages, and cell lines are too low to be detected reliably by immunofluorescence or immunoelectron microscopy. Therefore, to undertake studies of the distribution and function of Rab5 on phagosomes containing intracellular pathogens, we cloned the rab5 gene from a human fetal lung library. Although we found four independent clones with identical Rab5c sequences, we found no clones corresponding to either Rab5a or Rab5b in the human fetal lung cDNA library, despite our use of probes that would have detected such clones. We first sought to overexpress the gene in macrophage-like cell lines (U937, THP-1, and HL60), since macrophages are the natural host cells of L. pneumophila and M. tuberculosis. However, we were unable to achieve stable high-level expression compatible with immunofluorescence or immunoelectron microscopy studies in these cell lines. We therefore prepared a HeLa cell line capable of inducible expression of the human rab5c gene. Long-term overexpression of Rab-GTPases is often associated with toxicity and loss of function in such cell lines. Therefore, we used a tetracycline-regulated expression system (7, 19).

**Expression of Rab5c by the isolated clones.** Expression of Rab5c by the isolated clones was found to be tightly regulated by tetracycline, with strong expression evident upon addition of the repressor. However, we were unable to maintain the clones in the absence of tetracycline for extended periods, leading to a decrease in expression levels. We therefore established a stable expression system (7, 19).

**Quantitation of LAMP-1 immunogold staining in HeLa-Rab5c cells infected with L. pneumophila or M. tuberculosis.** (A) HeLa-Rab5c cells were coincubated with wild-type or avirulent L. pneumophila or M. tuberculosis for 15 min at 37°C and fixed immediately or coincubated at 37°C for 30 min, washed, and incubated for 6 or 8 h and then fixed. (B) HeLa-Rab5c cells expressing Rab5c were coincubated with latex beads and either live or heat-killed M. tuberculosis for 2 h and either fixed immediately or washed, incubated for 2 days at 37°C, and then fixed. After fixation, all cells were processed for cryoimmunoelectron microscopy and stained for LAMP-1. LAMP-1-bound immunogold particles were enumerated on phagosomal, nuclear, and plasma membranes. Data shown represent the mean and standard deviation of gold particle counts on at least 20 cells (each with at least one phagosome) on each of at least three electron microscopy grids. Wild-type L. pneumophila lacks LAMP-1 at both 15 min and 8 h (A). In contrast, avirulent L. pneumophila phagosomes have a modest level of LAMP-1 at 15 min and stain intensely for LAMP-1 at 6 h. Phagosomes containing live M. tuberculosis have very little LAMP-1, whereas phagosomes containing heat-killed M. tuberculosis and latex beads stain intensely for LAMP-1 at both 2 h and 2 days (B). The nuclear membrane and plasma membrane have negligible staining for LAMP-1 and serve as internal negative controls (A and B).

**FIG. 3.** Quantitation of LAMP-1 immunogold staining in HeLa-Rab5c cells infected with L. pneumophila or M. tuberculosis. (A) HeLa-Rab5c cells were coincubated with wild-type or avirulent L. pneumophila or M. tuberculosis for 15 min at 37°C and fixed immediately or coincubated at 37°C for 30 min, washed, and incubated for 6 or 8 h and then fixed. (B) HeLa-Rab5c cells expressing Rab5c were coincubated with latex beads and either live or heat-killed M. tuberculosis for 2 h and either fixed immediately or washed, incubated for 2 days at 37°C, and then fixed. After fixation, all cells were processed for cryoimmunoelectron microscopy and stained for LAMP-1. LAMP-1-bound immunogold particles were enumerated on phagosomal, nuclear, and plasma membranes. Data shown represent the mean and standard deviation of gold particle counts on at least 20 cells (each with at least one phagosome) on each of at least three electron microscopy grids. Wild-type L. pneumophila lacks LAMP-1 at both 15 min and 8 h (A). In contrast, avirulent L. pneumophila phagosomes have a modest level of LAMP-1 at 15 min and stain intensely for LAMP-1 at 6 h. Phagosomes containing live M. tuberculosis have very little LAMP-1, whereas phagosomes containing heat-killed M. tuberculosis and latex beads stain intensely for LAMP-1 at both 2 h and 2 days (B). The nuclear membrane and plasma membrane have negligible staining for LAMP-1 and serve as internal negative controls (A and B).
L. pneumophila scarce on wild-type and avirulent somes containing avirulent L. pneumophila. Rab5c is scarce on wild-type phagosome) on each of at least three electron microscopy grids. (Left) At 15 min, standard deviations of gold counts on at least 20 cells (each with at least one on phagosomal, nuclear, and plasma membranes. Data shown are the means and

from 24 to 72 h 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-Rab5c cells is relevant to understanding the pathogenesis of L. pneumophila and M. tuberculosis 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-Rab5c cells resembles their interaction with human macrophages. We specifically examined (i) the capacity of the two pathogens to multiply within HeLa-Rab5c cells and (ii) the pattern of expression of endosomal markers on the phagosomes of the two pathogens in HeLa-Rab5c cells.

(i) Uptake and growth of L. pneumophila and M. tuberculosis in parental HeLa Tet-off cells and HeLa cells overexpressing Rab5c. Although HeLa cells are poorly phagocytic, adequate uptake of L. pneumophila and M. tuberculosis can be obtained by increasing the multiplicity of infection (MOI) relative to that used when infecting more phagocytic cells. By immunofluorescence microscopy, 12% of HeLa Tet-off cells had associated bacteria after incubation with L. pneumophila for 2 h at an MOI of 2,000:1. By electron microscopy, approximately 1 in 15 HeLa Tet-off cells contained an L. pneumophila phagosome in the plane of the section. By immunofluorescence microscopy, 7% of HeLa Tet-off cells had associated bacteria after incubation for 2 h at an MOI of 400:1, and by electron microscopy, approximately 1 in 20 HeLa cells contained an M. tuberculosis phagosome in the plane of the section.

L. pneumophila adheres five times more avidly to THP-1 cells than to HeLa Tet-off cells (Fig. 1A). Similarly, M. tuberculosis adheres 15 times more avidly to monolayers of THP-1 cells than to comparable monolayers of HeLa Tet-off cells (Fig. 1B). However, once taken up by the HeLa Tet-off cells, L. pneumophila and M. tuberculosis grow at rates comparable to that in THP-1 cells (Fig. 1C and D). M. tuberculosis grows with a doubling time of approximately 19 h in HeLa Tet-off cells and 15 h in THP-1 cells (i.e., 1 log in 3 days, matching the growth rate that we have previously observed for M. tuberculosis in THP-1 cells and human monocyte-derived macrophages) (13, 29). L. pneumophila grows in HeLa Tet-off cells and THP-1 cells with an initial doubling time of approximately 3 h, which is also very similar to its previously published doubling time of 2 to 3 h in human monocyte-derived macrophages (26).

To determine whether overexpression of Rab5c alters the rate of intracellular growth of M. tuberculosis or L. pneumophila in HeLa Tet-off cells, we compared the intracellular growth of M. tuberculosis and L. pneumophila in HeLa Tet-off and HeLa-Rab5c 1 day after withdrawal of tetracycline (Fig. 1C and D). The growth rate of L. pneumophila and M. tuberculosis in cells overexpressing Rab5c was equal to the growth rate in parental nontransfected HeLa Tet-off cells. When separated from HeLa Tet-off cells by a 0.2-μm-pore-size filter in a parabiotic chamber (Transwells; Costar), neither L. pneumophila nor M. tuberculosis grew in the same culture medium. Thus, once inside parental HeLa Tet-off cells or HeLa-Rab5c

FIG. 4. Quantitation of Rab5c immunogold staining in HeLa-Rab5c cells

FIG. 5. Distribution of staining for Rab5c in HeLa-Rab5c cells fixed immediately after a 15-min coincubation with either wild-type or avirulent L. pneumophila. HeLa-Rab5c cells were coincubated with wild-type (A) or avirulent (B) L. pneumophila for 15 min, fixed, and processed for cryoimmunoelectron microscopy. The number of gold particles per micrometer of membrane on phagosomal, plasma, and nuclear membranes was enumerated. Data shown are the means ± standard deviations of the distributions from two separate experiments.
cells overexpressing Rab5c, *L. pneumophila* and *M. tuberculosis* multiply at a rate comparable to that in human macrophages.

(ii) Distribution of transferrin receptor and LAMP-1 on *L. pneumophila* or *M. tuberculosis* phagosomes in HeLa-Rab5c cells. To determine if phagosomes in infected HeLa-Rab5c cells have molecular characteristics similar to phagosomes in infected human macrophages, we studied transferrin receptor expression on *M. tuberculosis* phagosomes and LAMP-1 expression on both *L. pneumophila* and *M. tuberculosis* phagosomes. Consistent with our published observations with human monocyte-derived macrophages (13), we found that in HeLa-Rab5c cells the majority of *M. tuberculosis* phagosomes stably transfected with the transferrin receptor gene stain positively for the transferrin receptor (Fig. 2).

Also consistent with our previous observations of human macrophages (13), we found little or no LAMP-1 on phagosomes containing wild-type *L. pneumophila* (Fig. 3A) or live *M. tuberculosis* (Fig. 3B) in HeLa-Rab5c cells but intense staining on phagosomes containing either the avirulent mutant *L. pneumophila* (Fig. 3A) or heat-killed *M. tuberculosis* or latex beads (Fig. 3B) in these cells. These results confirmed that *L. pneumophila* and *M. tuberculosis* phagosomes in HeLa-Rab5c cells do not fuse with lysosomes and that overexpression of Rab5c in HeLa cells does not fundamentally alter the membrane-trafficking properties of *L. pneumophila* or *M. tuberculosis* phagosomes. We concluded from these sets of studies that, while uptake of *L. pneumophila* and *M. tuberculosis* into HeLa Tet-off cells is much less efficient than that into macrophages, both 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-Rab5c cells were likely to apply to phagosomes of these pathogens in macrophages.

**Distribution of Rab5c on phagosomes containing wild-type and avirulent *L. pneumophila* in HeLa-Rab5c cells.** Two days after removal of tetracycline from the culture medium, 90% of HeLa-Rab5c cells had abundant immunogold staining for Rab5c on cytoplasmic vesicles (>1 gold particle/μm²), with an average level of 4 gold particles/μm². As expected, we found that the Rab5c immunogold particles colocalized extensively with early endosomes labeled kinetically with 5-nm BSA-bound gold particles in the Rab5c-overexpressing cells (data not shown). Parental HeLa Tet-off cells, on the other hand, lacked significant staining for Rab5c (of 20 consecutive cells, none had more than 0.5 gold particle/μm²; mean level of immunogold staining, 0.14 gold particle/μm²). In HeLa-Rab5c cells infected with wild-type *L. pneumophila*, the majority of phagosomes had little or no detectable Rab5c at all time points examined, from 15 min to 18 h after phagocytosis. At 15 min, the earliest time point at which examination was feasible, 60% of the phagosomes had no detectable staining for Rab5c (Fig. 4 to 6A). In marked contrast, 90% of phagosomes containing the avirulent mutant *L. pneumophila* 25D did stain positive for Rab5c at 15 min (Fig. 4, 7B, and 8B). As typically occurs with phagosomes that mature to phagolysosomes, expression of Rab5c on mutant *L. pneumophila* phagosomes was transient, as staining was absent by 6 h of infection (Fig. 4 and 6C).

**Distribution of Rab5c on phagosomes containing live and heat-killed *M. tuberculosis* in HeLa-Rab5c cells.** In HeLa-Rab5c cells infected with *M. tuberculosis*, the majority of *M. tuberculosis* phagosomes stained positively for Rab5c at all time points examined—from 2 h to 3 d after phagocytosis (Fig. 7 and 8). In contrast, phagosomes containing heat-killed *M. tuberculosis* in HeLa-Rab5c cells lacked significant staining for Rab5 at any of the time points examined, ranging from 2 h to 2 days (Fig. 7). At the MOIs used, heat-killed *M. tuberculosis*...
and latex beads were taken up inefficiently by HeLa cells. Because of this, we were unable to examine time points earlier than 2 h, times at which the phagosomes of inert particles or dead *M. tuberculosis* would be expected to have Rab5.

Also in contrast to phagosomes containing live *M. tuberculosis*, latex bead phagosomes in HeLa-Rab5c cells within the same monolayers usually lacked staining for Rab5c, even within cells that also contained Rab5c-immunopositive *M. tuberculosis* phagosomes (Fig. 7). Interestingly, however, latex bead phagosomes in HeLa-Rab5c cells that were very heavily infected with live *M. tuberculosis* did stain positively for Rab5c.

**Effect of overexpression of the Rab5c GTPase-deficient, constitutively active mutant on *L. pneumophila* and *M. tuberculosis* phagosomes.** Whereas the majority of *L. pneumophila* phagosomes do not acquire detectable Rab5c, a minority do acquire some of the overexpressed Rab5c. It is possible that this low level of association is an artifact of overexpression and that the Rab5c on the phagosomes is not truly intimately associated with the phagosome or not functional, due, for example, to an absence of downstream effectors. Likewise, although the *M. tuberculosis* phagosomes appeared to recruit Rab5c very avidly, it is possible that the recombinant Rab5c recruited to the *M. tuberculosis* phagosome is either not truly incorporated into the membrane or not biologically functional. It has been demonstrated that overexpression of constitutively active mutant forms of Rab5 leads to dramatic enlargement of early endosomes (9, 39, 40). To determine whether the overexpressed Rab5c is specifically associated with the bacterial phagosomes and whether it is biologically functional in these sites, we examined the effect of overexpression of the GTPase-deficient, constitutively active Rab5c Q79L mutant on *M. tuberculosis* and *L. pneumophila* phagosomes, reasoning that if the Rab5c is functional on the bacterial phagosomes, then there would be enlargement of the bacterial phagosomes. On the other hand, if Rab5c was not truly incorporated into the phagosomal membrane or was not biologically functional, then there would be no alteration in the phagosomal membrane.

Two days after withdrawal of tetracycline to induce expression of the mutant Rab5c, 77.5% of the HeLa-Rab5c Q79L cells had developed large vacuoles measuring over 2 μm in

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**FIG. 8.** *M. tuberculosis* phagosomes in HeLa-Rab5c cells stain positively for Rab5c. HeLa-Rab5c 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 Rab5c expression. The HeLa cells were coincubated for 2 h with *M. tuberculosis* using an MOI of 400:1. Nonadherent bacteria and beads were washed away, and the monolayers were incubated for 2 additional days. Monolayers were fixed and processed for cryoimmunoelectron microscopy. Rab5c was stained with 15-nm immunogold particles (arrowheads) and is abundant on the *M. tuberculosis* phagosomal membrane. Mycobacterial LAM was stained with 5-nm gold particles and is present on the mycobacterial cell wall (arrows). Nu, nucleus. Magnification, ×55,700.
diameter. Immunogold staining revealed that these large vacuoles stained positively for Rab5c. To our surprise, many of these vacuoles also stained moderately to intensely for LAMP-1; in contrast, little or no colocalization of Rab5c with LAMP-1 was observed in cells overexpressing wild-type Rab5c. The LAMP-1 in the large vacuoles was present both on the membranes of the vacuoles and also on membranes within the vacuoles. When wild-type or avirulent L. pneumophila was spun into cell monolayers, heat-killed M. tuberculosis phagosomes examined in HeLa cells overexpressing wild-type Rab5c, both L. pneumophila and M. tuberculosis multiplied in the HeLa-Rab5c Q79L cells. However, we were precluded from obtaining accurate measurements of their growth rates in these cells because during the long culture period required for this assessment there was significant loss of the HeLa cells from the monolayer.

**DISCUSSION**

Our initial studies established the relevance of the HeLa-Rab5c model to understanding phagosome trafficking of the intracellular pathogens L. pneumophila and M. tuberculosis. First, once inside HeLa-Rab5c cells, these bacteria multiplied at rates equivalent to that in human macrophages. Second, just as in human macrophages, (i) the wild-type L. pneumophila phagosome in HeLa-Rab5c cells excluded markers of the endolysosomal pathway, (ii) the M. tuberculosis phagosome in HeLa-Rab5c cells readily incorporated transferrin receptors but generally excluded LAMP-1, and (iii) phagosomes in HeLa-Rab5c cells containing avirulent L. pneumophila, heat-killed M. tuberculosis, and latex beads stained intensely for LAMP-1. Additional studies of Rab5c distribution in HeLa-Rab5c cells lent further support to the use of this model for studies of phagosome-endosome interaction. Our finding that Rab5c was transiently displayed on phagosomes in HeLa-Rab5c cells containing avirulent L. pneumophila, which mature to phagolysosomes, lent further support to the relevance of this model, since other studies have similarly reported transient expression of Rab5 immediately after phagocytosis on phagosomes that mature to phagolysosomes (17, 27). Our subsequent studies showed that Rab5c is absent on L. pneumophila phagosomes but persistently expressed on M. tuberculosis phagosomes. The latter finding with the study by Alvarez-Dominguez et al. (2) exhibits a maturational arrest manifest by persistent expression of Rab5 on the phagosome and persistent interaction with early endosomes in an in vitro assay. Antibodies to Rab5 blocked the capacity of these L. monocytogenes phagosomes to interact with early en-
FIG. 10. The phenotype of *M. tuberculosis* phagosomes and avirulent *L. pneumophila* phagosomes but not wild-type *L. pneumophila* phagosomes is altered by expression of the constitutively active Rab5c Q79L mutant. Suspensions of wild-type *L. pneumophila* (A), avirulent *L. pneumophila* (B), or *M. tuberculosis* and latex beads (D) were added to monolayers of HeLa cells expressing Rab5c Q79L and centrifuged at 1,160 × g for 20 min at 4°C, incubated at 37°C for either 30 min (L. pneumophila [A and B]) or 2 h (*M. tuberculosis* [D]), fixed, and processed for cryoimmunoelectron microscopy. Rab5c was stained with 15-nm immunogold particles (large arrowheads), LAMP-1 was stained with 10-nm immunogold particles (small arrowheads), and *L. pneumophila* LPS (A and B) or mycobacterial LAM (C and D) was stained with 5-nm immunogold particles (arrows). (A) Wild-type *L. pneumophila* resides in a morphologically tight phagosome that lacks immunogold staining for Rab5c and LAMP-1, which are present on an adjacent large vacuole (+). (B) A large vacuole contains numerous avirulent *L. pneumophila* and stains positive for both Rab5c and LAMP-1. (C and D) *M. tuberculosis* resides in large vacuoles that stain positively for Rab5c and for LAMP-1. *M. tuberculosis* often shares the large vacuole with latex beads (D). Nu, nucleus. Magnifications, ×37,310 (A), ×37,310 (B), ×37,310 (C), and ×42,770 (D).
FIG. 10—Continued.
dosomes, underscoring the importance of Rab5 in mediating phagosome-endosome interaction.

Our study is the first to examine the role of Rab-GTPases in human cells infected with *L. pneumophila* and the first to examine Rab5 in *L. pneumophila*-host cell interaction. Roy et al. (36) recently reported persistence of low levels of Rab7 on a minority of phagosomes containing wild-type *L. pneumophila* and transient acquisition of Rab7 by phagosomes containing avirulent dotA *L. pneumophila* mutants in mouse bone marrow macrophages. However, Rab5 was not examined in this study. Our study is also the first to examine the role of Rab-GTPases in human cells infected with virulent *M. tuberculosis*. Via et al. (43) reported that isolated *M. bovis* BCG phagosomes from mouse J774 macrophages have persistent staining for Rab5 and do not acquire Rab4 or Rab7. These findings with *M. bovis* BCG in mouse macrophages are in agreement with our findings with the virulent Erdman strain of *M. tuberculosis* in the human HeLa cell line. Our two studies used very different but complementary methodologies. Via et al. used biochemical techniques to study a population of phagosomes isolated from infected cells. An advantage of this approach is that it allows the pooling of information from a very large number of phagosomes. However, the results can be distorted by contamination from other organelles, loss or gain of markers during the lengthy isolation procedure, and sample heterogeneity, in which case, high levels of staining on some phagosomes could skew the average level of staining. We used the cryosection immunogold technique to study the distribution of Rab-GTPases on individual phagosomes in fixed cells. This approach has the advantage of allowing the direct visualization of Rab5 on individual phagosomes and an assessment of the degree of heterogeneity of the phagosomes. This approach allowed us to conclude that the majority of *M. tuberculosis* phagosomes stain positive for Rab5, whereas the majority of *L. pneumophila* phagosomes do not.

Our study is the first to report on the subcellular distribution of human Rab5c. Previously, canine Rab5c overexpressed in BHK cells, as well as canine Rab5a and Rab5b, has been shown to localize to early endosomes (8, 11). We show here that human Rab5c localizes to early endosomes, early avirulent *L. pneumophila* phagosomes, and the *M. tuberculosis* phagosome, which has early endosomal properties (13, 14). We observe relatively little Rab5c on the plasma membrane. Therefore, it is unlikely that the Rab5c that we observe on the *M. tuberculosis* phagosome is derived from the plasma membrane. The Rab-GTPases cycle extensively between cytoplasm and membrane-bound forms, and it is likely that in these cells that overexpress Rab5c, the majority of the Rab5c observed on the *M. tuberculosis* phagosomes is recruited from the cytoplasmic pool, although some of it may also be derived from interaction with early endosomes.

We considered the possibility that the persistent recruitment of Rab5c to the *M. tuberculosis* phagosome might somehow be an artifact of the overexpression of Rab5c in the HeLa cells and that the Rab5c was not functionally integrated into the phagosomal membrane. Several findings argue against this possibility. First, the scarcity of Rab5c on latex bead phagosomes, wild-type *L. pneumophila* phagosomes, and late avirulent *L. pneumophila* phagosomes is consistent with a specific recruitment of recombinant Rab5c to the *M. tuberculosis* phagosome. Second, the presence of transferrin receptor (an early endosomal marker) on the *M. tuberculosis* phagosome 2 days after infection is consistent with persistence of functional Rab5c on the *M. tuberculosis* phagosome. Third, the dramatic changes in the *M. tuberculosis* phagosome resulting from overexpression of the fusion-promoting Rab5c Q79L mutant indicate that the Rab5c on the *M. tuberculosis* phagosome is functional. The *M. tuberculosis* phagosome in the Rab5c Q79L mutant is markedly different from that of a normal *M. tuberculosis* phagosome. Whereas a normal *M. tuberculosis* phagosome is tight, lacks LAMP-1, and does not fuse with latex bead compartments, the *M. tuberculosis* phagosome in the HeLa-Rab5c Q79L cell is spacious and LAMP-1 positive and often contains latex beads. In view of these phenotypic differences, it is very unlikely that *M. tuberculosis* disrupts the maturation of its phagosome by inhibiting GTP hydrolysis by the Rab5 on its phagosome. Although our studies using the GTPase-deficient, constitutively active Rab5 exclude inhibition of GTP hydrolysis as the mechanism by which *M. tuberculosis* disrupts the maturation of its phagosome, the persistence of wild-type Rab5 on the phagosome is likely to be an important mechanism in maintaining the capacity of the phagosome to interact with early endosomes and in maintaining early endosomal properties. Hence, these studies imply that *M. tuberculosis* blocks the maturation of its phagosome by disrupting events downstream of Rab5 acquisition and effector action, for example, by disrupting the acquisition or function of Rab7 effectors.

Whereas the *M. tuberculosis* phagosome undergoes dramatic phenotypic changes in HeLa cells expressing the GTPase-deficient, constitutively active Rab5c mutant, the wild-type *L. pneumophila* phagosome remains morphologically tight and continues to exclude Rab5 and LAMP-1. With regard to the mechanisms underlying the altered maturation of the *L. pneumophila* phagosome, our observations that the *L. pneumophila* phagosome (i) does not acquire wild-type Rab5c, (ii) does not acquire the constitutively active Rab5c Q79L mutant, and (iii) does not enter the large Rab5c Q79L-positive vacuoles strongly suggest that the *L. pneumophila* phagosome never acquires functional receptors for Rab5. The consequent exclusion of Rab5 from the *L. pneumophila* phagosome is likely to be an important aspect underlying the failure of the *L. pneumophila* phagosome to mature along the endocytic pathway.

The presence of LAMP-1 on the giant vacuoles in HeLa-Rab5c Q79L cells was unexpected, as the presence of LAMP-1 on the swollen endosomes of Rab5c Q79L mutant-expressing cells has not previously been reported. Possible explanations for this phenomenon are that (i) the high fusogenicity of the Rab5c Q79L endosomes promotes their fusion with late endosomal and lysosomal compartments or (ii) late endosomal-lysosomal proteins may normally traffic transiently through the early endosome and be present at very low levels in early endosomal compartments, but overexpression of the Rab5c Q79L mutant retards their trafficking and causes them to accumulate in these compartments.

Prior studies have found no difference in the subcellular distribution or functional role of Rab5a, Rab5b, or Rab5c in endocytosis (8, 11). Similarly, all three isoforms of Rab5 have been found on early latex bead phagosomes in mouse macrophages (17). Nevertheless, Alvarez-Dominguez and Stahl (3) recently examined the effect of antisense oligonucleotides to Rab5a and Rab5c on the maturation of phagosomes containing hemolysin-deficient *L. monocytogenes* in human monocyte-derived macrophages and found that antisense oligonucleotides to Rab5a, but not Rab5c, disrupted maturation of the bacterial phagosome. These data suggest that Rab5a plays a more important role than Rab5c in human macrophages under the conditions studied. However, it is possible that the greater role of Rab5a over Rab5c in the studies by Alvarez-Dominguez and Stahl reflect higher levels of endogenous expression in Rab5a than in Rab5c, rather than a fundamental biological difference in their functions. It is likely that various Rab5 isoforms are expressed at different levels in different cell types.
under various conditions, and the type of Rab5 isoform that is dominant in mediating a biological function may vary accordingly. We found no cDNA clone corresponding to Rab5a in our probe of a human fetal lung library but found four clones corresponding to Rab5c, suggesting that in human fetal lung, Rab5c may be expressed at higher levels than Rab5a.

When permeabilized cells are coincubated with a particular Rab-GTPase, the Rab-GTPase inserts into those membranes that have receptors for the particular Rab-GTPase (41). Likewise, when a particular Rab-GTPase is overexpressed in a cell, the Rab-GTPase will be delivered to the membranes that have receptors for that Rab-GTPase. When a constitutively active Rab-GTPase is overexpressed, it will lead to phenotypic changes in any membrane-bound compartments that have both the receptors for the Rab-GTPase and the appropriate effector machinery to allow downstream functions of the Rab-GTPase to proceed. Therefore, the fact that we observe recruitment of Rab5c to the phagosomes containing latex beads, M. tuberculosis, and avirulent L. pneumophila indicates that these phagosomes do have receptors that allow the recruitment of Rab5c. That we observe dramatic phenotypic changes in the morphology of these phagosomes (but not the wild-type L. pneumophila phagosome, which does not recruit Rab5c) in cells expressing the constitutively active Rab5c Q79L indicates that Rab5c can also have a major role in phagosomal development when it is overexpressed.

In conclusion, our study shows that the expression of Rab5 on L. pneumophila and M. tuberculosis phagosomes deviates from the typical pattern of expression on phagosomes containing inert particles. The latter phagosomes display transient expression of Rab5 as the phagosomes mature along the endosomal pathway, culminating in the formation of a phagolysosome. In contrast, the L. pneumophila phagosome does not display Rab5, and the M. tuberculosis phagosome displays Rab5 persistently. The absence of Rab5 on the L. pneumophila phagosome may underlie its lack of interaction with the endocytic pathway. The persistence of functional Rab5 on the M. tuberculosis phagosome is undoubtedly important in allowing the phagosome to maintain interaction with early endosomes and preserve early endosomal properties; it may be an important factor underlying the arrested maturation of the phagosome.

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