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Characterization of the *Chlamydia trachomatis* Vacuole and Its Interaction with the Host Endocytic Pathway in HeLa Cells

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***Chlamydia trachomatis*, an obligate intracellular parasite and a major human pathogen, invades eukaryotic host cells and replicates within a membrane-bound compartment (termed the vacuole or inclusion) in the cytoplasm of the host cell. In this report, we describe in detail the characteristics of the vacuole throughout the chlamydial life cycle in terms of the endocytic pathway, as determined by epifluorescent and confocal immunofluorescence microscopy. By indirect immunofluorescence, the transferrin receptor (TfR), a component of early endosomes, and the cation-independent mannose-6-phosphate receptor (CI-M6PR), a component of late endosomes, were found in close association with the chlamydial vacuole as early as 4 h postinfection (hpi) and as late as 20 hpi. Fluorescein isothiocyanate (FITC)-labeled Tf was also found to colocalize with the vacuole at 4, 12, and 20 hpi, indicating that exogenously added ligands can be transported to the region of the vacuole. Antibodies to several different lysosomal proteins failed to label the chlamydial vacuole at any time point during the life cycle. Indirect immunofluorescence of cells infected with chlamydiae stained with an antibody to the *trans*-Golgi network (TGN) protein TGN38 demonstrated that in infected cells, the integrity and structure of the TGN was altered. The rates of Tf recycling in infected and uninfected cells were compared by fluorescence microscopy and quantitated with ^{125}I-Tf. While the rate of FITC-Tf recycling from endocytic compartments in chlamydia-infected cells did not appear different from that of uninfected cells, a small pool of FITC-Tf that had accumulated adjacent to the chlamydial vacuole recycled at a slower rate. Quantitation of Tf recycling with ^{125}I-Tf showed that Tf was recycled more slowly in infected cells than in uninfected cells. The altered distribution of several endocytic pathway markers and the slowed Tf recycling are consistent with the hypothesis that the chlamydial vacuole interacts with the endocytic pathway of the host. These results furthermore suggest that the chlamydial vacuole does not correspond to a canonical endocytic compartment but that it is a unique and dynamic organelle that shares several characteristics with recycling endosomes of the host cell. Interactions with the early and/or late endosomal compartments, in addition to the Golgi apparatus, may provide a source of membrane or nutrients for the replicating organisms.**

Chlamydia trachomatis, a gram-negative bacterium and an obligate intracellular parasite, is a major human pathogen worldwide. It is the leading cause of sexually transmitted disease in the Western world and is the main cause of noncongenital blindness in developing nations (18). During its life cycle, *C. trachomatis* alternates between two distinct morphological forms: the replicative, intracellular reticulate body (RB), and the infectious, but metabolically inactive elementary body (EB) (14, 26). EBs bind to an unknown receptor on the host cell, potentially through an interaction with a bacterially derived heparan sulfate-like glycosaminoglycan present on the chlamydial surface (30). After binding, the bacteria are internalized, enveloped within membrane-bound compartments, and transported to a perinuclear location. The *C. trachomatis* compartments fuse with each other to form one or a few large vacuoles. Within the vacuole, EBs differentiate into RBs in 6 to 8 h; the RBs subsequently undergo binary fission to yield approximately 100 progeny. During replication, the vacuole enlarges, often reaching a size greater than that of the nucleus. Between 24 and 72 h postinfection (hpi), the RBs redifferentiate into EBs and are released into the extracellular space to start a new round of infection.

The compartment in which *C. trachomatis* resides is, like that of *Toxoplasma gondii* and *Mycobacterium tuberculosis*, unique and nonacidified (9, 19). While EBs enter through an endocytic mechanism (16, 21, 27), the vacuole fails to fuse with the terminal compartment of the endocytic pathway, the lysosome (3). The lysosomal fusion block is restricted to the vacuole, since phagosomes containing yeast particles fuse with lysosomes in chlamydia-infected cells (6). Despite the failure of the chlamydial vacuole to fuse with lysosomes, several lines of evidence indicate that the vacuole is fusogenic for at least some time after infection. Early in infection, *C. trachomatis* vacuoles fuse with each other to form one large vacuole. Vesicles containing newly entered EBs can fuse with mature vacuoles 24 h after the initial infection (17). Additionally, three members of the annexin family, annexins III, IV, and V, were shown to colocalize with the *C. trachomatis* vacuole (13). These calcium-dependent proteins have been postulated to be responsible for regulating the fusogenicity of the vacuole. Recently, it has been demonstrated that vesicles derived from the *trans*-Golgi network (TGN) can fuse with the chlamydial vacuole and that lipids contained in these vesicles could be incorporated into the bacteria themselves (7, 8). Additionally, several Golgi complex markers were found in close apposition to the vacuole. Based on this observation and the finding that the fluid-phase markers fluorescein isothiocyanate (FITC)-labeled dextran and Lucifer yellow and several lysosomal resident proteins did not colocalize with the vacuole (9), it has been proposed that the chlamydial vacuole receives lipids from exocytic vesicles derived from the TGN, while being disconnected from the

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endocytic pathway. Of note, these experiments were carried out only at later times during infection (after 20 hpi) and did not examine the association of early endosome markers with the chlamydial vacuole.

Even if chlamydiae eventually reside in a vacuole that is disconnected from the endocytic pathway, EBs are thought to enter eukaryotic cells through the endocytic pathway and therefore may interface with early or late endosomes at least early in their life cycle. To investigate this hypothesis, we have carried out epifluorescence and confocal microscopy of HeLa cells infected with *C. trachomatis* by using antibodies to several endocytic pathway markers at both early (1 and 4 hpi), intermediate (12 hpi), and late (20 hpi) times during the intracellular life cycle.

MATERIALS AND METHODS

Reagents and chemicals. The primary antibodies and the dilution used were H68.4 anti-transferrin receptor (anti-TfR) antibody (28) at 1:1,000, the monoclonal antibovine cation-independent mannose-6-phosphate receptor (CI-M6PR) antibody 2G11 at 1:5, the monoclonal anti-TGN38 antibody at 1:250 (12), the monoclonal antilysosome-associated membrane protein-1 (LAMP-1; 1D4B; American Type Culture Collection [ATCC], Rockville, Md.) and anti-LAMP-2 (ABL-93, ATCC) antibodies at 1:5, the anti-cathepsin D antibody at 1:100, and the antilysosomal antibody AC-17 at 1:100. The secondary antibody used was an FITC-labeled goat anti-mouse antibody diluted 1:200 (Zymed, South San Francisco, Calif.). Antibodies against mouse pneumonitis (MoPn) major outer membrane protein (MOMP [MO336]) and lymphogranuloma venereum (LGV) L2 serovar MOMP (L2-I45) were conjugated to Texas red (Molecular Probes, Eugene, Oreg.) according to the manufacturer's instructions. Both were used at a dilution of 1:200. Iron-loaded human Tf was purchased from Sigma (St. Louis, Mo.), and human FITC-Tf was obtained from Molecular Probes. All chemicals were obtained from Sigma unless otherwise stated.

Cell lines. The human epithelial cell line HeLa 229 (ATCC CCL2) was maintained in Dulbecco's modified essential medium (DME) H-16 (Cell Culture Facility, University of California, San Francisco [UCSF], San Francisco, Calif.) supplemented with 5% fetal bovine serum (FBS; Gibco, Bethesda, Md.) at 37°C in 5% CO_2. The mouse fibroblast cell line L929 (ATCC CCL1) was maintained in RPMI 1640 (Cell Culture Facility, UCSF) supplemented with 5% FBS at 37°C in 5% CO_2. Madin-Darby canine kidney (MDCK) cells were grown in modified Eagle's medium (MEM; Cell Culture Facility, UCSF) supplemented with 5% FBS at 37°C in 5% CO_2. Rat-1 cells were maintained in DME H-21 (Cell Culture Facility, UCSF) supplemented with 10% FBS at 37°C in 5% CO_2.

Bacteria. The LGV L2 serovar and the MoPn biovar of *C. trachomatis* were propagated and purified as previously described (11). Briefly, L929 cells in spinner flasks maintained in RPMI 1640 supplemented with 5% FBS, vancomycin hydrochloride (60 μg/ml; Abbott Laboratories, N. Chicago, Ill.), and gentamicin sulfate (10 μg/ml) were infected for approximately 44 h. Cells were pelleted and sonicated in 35 ml of sterile phosphate-buffered saline (PBS). The lysate was centrifuged at 1,928 × *g* in a Sorvall RT6000B tabletop centrifuge for 10 min, and the pellet was resuspended in 2 ml of sucrose phosphate glutamate (SPG) buffer. The resuspended pellet was loaded on a 30% discontinuous Renografin (Squibb Diagnostics, BMS Pharmaceutical Group, Irvine, Calif.) gradient and centrifuged at 37,250 × *g* for 1 h in a Beckman SW55 Ti rotor. The pellet was resuspended in 1 ml of SPG, loaded on a 40% discontinuous Renografin gradient, and centrifuged at 64,250 × *g* for 1 h in a Beckman SW55 Ti rotor. The resulting pellet was resuspended in 5 ml of SPG, and aliquots were stored at −70°C. The titer of the *C. trachomatis* stock was determined with the Merifluor Chlamydia Detection Kit (Meridian, Cincinnati, Ohio).

Infection of tissue culture cells for microscopy. Tissue culture cells were plated onto 12-mm-diameter glass coverslips that had been treated with 1 N HCl. After 24 to 44 h, the medium was aspirated, and EBs diluted in 200 μl of DME-H16 supplemented with 5% FBS were added and incubated at 37°C in 5% CO_2. The multiplicity of infection (MOI) was approximately 50 to 300. After 1 h, the inoculum was removed, and fresh DME H-16 with 5% FBS was added. Cells were incubated at 37°C in 5% CO_2 for the remainder of the time course.

Immunofluorescence. HeLa cells were fixed 1, 4, 12, or 20 hpi according to the protocol of Bacallao and Stelzer (2) with the following modifications. Cells were washed five times with PBS and fixed with low-pH fixative (4% paraformaldehyde in 80 mM PIPES [piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)], 5 mM $MgCl_2$, 2 mM EGTA [pH 6.0 to 6.5]) for 5 min. Cells were subsequently fixed with high-pH fixative (4% paraformaldehyde in 100 mM sodium borate [pH 11.0]) for 10 min. The fixation process was quenched with 20 mM glycine–75 mM NH_4Cl for 15 min. Cells were blocked by incubation at 37°C in the presence of 0.7% (wt/vol) fish skin gelatin (FSG) in PBS with 0.005% saponin (Sap) (PBS-FSG-Sap) for 15 min. Cells were then incubated in the presence of primary antibody diluted in PBS-FSG-Sap for 1 h at 37°C, washed four times with PBS-FSG-Sap, and again incubated at 37°C in the presence of an FITC-labeled antimouse antibody (Zymed) for 1 h. Cells were washed four times with PBS-FSG-Sap and incubated at 37°C for 1 h in the presence of an LGV- or MoPn-specific anti-MOMP antibody (L2-I45 or MO336, respectively) conjugated to Texas red (Molecular Probes). Cells were washed three times with PBS-FSG-Sap and twice with PBS and postfixed with 4% paraformaldehyde in 100 mM sodium cacodylate (Eastman Kodak, Rochester, N.Y.) at pH 7.4 for 15 min. Coverslips were mounted with 2% *p*-phenylene diamine in 90% glycerol (Fisher Scientific, Fair Lawn, N.J.) buffered with 10 mM Tris (pH 8.2). Slides were viewed under a Bio-Rad MRC600 confocal microscope attached to a Nikon Optiphot II microscope with a Pan Apo ×60 1.4 NA objective lens. Samples were scanned simultaneously for the FITC and Texas red emissions with the K1 and K2 filter blocks. Data were analyzed with COMOS software. Images were converted to the tagged-information file format, and contrast levels were optimized and colors were added in the Adobe Photoshop 3.0 program (Adobe Co., Mountain View, Calif.). No immunofluorescence was detectable in samples stained with secondary antibody alone.

FITC-dextran labeling. L2-infected and uninfected HeLa cells were incubated in the presence of 10 mg of FITC-dextran (molecular weight of 10,000, lysine fixable; Molecular Probes) per ml in DME H-16 supplemented with 5% FBS for 2 h. Cells were fixed and stained as described above. For 1-h infections, EBs were added to the sample 1 h after the addition of the FITC-dextran.

FITC-Tf recycling. Uninfected cells or cells infected with MoPn for 2, 10, or 18 h were deprived of Tf by incubation in the presence of serum-free DME H-16 for 1 h at 37°C at 5% CO_2. The cells were loaded with FITC-Tf (Molecular Probes) by incubation in the presence of 30 μg of FITC-Tf per ml in serum-free DME H-16 for 1 h at 37°C. The cells were washed twice with PBS and allowed to recycle the FITC-Tf for 0, 5, 10, 20, 30, or 60 min. The cells were fixed, and chlamydiae were visualized by confocal microscopy with the Texas red-labeled MO-336 antibody as described above.

^{125}I-Tf recycling. To measure the rate of Tf recycling, we followed the procedure described by Apodaca et al. (1), with the following modifications. Uninfected HeLa cells or HeLa cells infected with LGV L2 for 2, 10, or 18 h were incubated for 1 h in a 37°C waterbath in the presence of MEM containing Hanks' balanced salts, 0.6% (wt/vol) bovine serum albumin (BSA), and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.4]) (MEM/BSA) for 1 h. Cells were incubated in a 37°C waterbath in the presence of 350 ng of ^{125}I-Tf per ml for 1 h. Uptake was terminated by washing the cells four times with cold PBS. Recycling was started by washing the cells three times rapidly with prewarmed MEM/BSA containing 35 μg of unlabeled holo-Tf per ml and incubating the cells in a 37°C waterbath. After 5, 10, 30, and 60 min, the medium was transferred to a microcentrifuge tube and replaced with MEM/BSA containing unlabeled Tf. After 120 min, the medium was transferred to a microcentrifuge tube, and the cells were lysed with 0.5 ml of a 0.5 N NaOH–1% sodium dodecyl sulfate solution. This solution was transferred to a microcentrifuge tube, and the lysis step was repeated. Radioactivity in the tubes was counted with a Beckman Gamma 4000 gamma counter.

RESULTS

In order to investigate whether components of the host endocytic pathway associate with the chlamydial vacuole, HeLa cells infected with either the MoPn biovar or LGV biovar (serovar L2) of *C. trachomatis* were fixed, stained with antibodies to markers of several endocytic pathway compartments, and visualized by immunofluorescence microscopy. The markers used in this study were TfR for early endosomes; CI-M6PR for late endosomes; TGN38 for the TGN; LAMP-1 and -2, AC-17, and cathepsin D for lysosomes; and FITC-dextran for fluid-phase endocytosis. HeLa cells were infected with *C. trachomatis* for 1 h and fixed at four different time points postinfection, each representing different stages of the chlamydial life cycle: 1 hpi, at which time the bacteria have adhered to the host cells, but only few have entered; 4 hpi, when EBs have entered into vacuoles and begun to aggregate in the perinuclear region, although only few vacuoles have fused with each other at this time; 12 hpi, at which time the vacuoles containing individual EBs have fused to form a large vacuole, EBs have transformed to RBs, and replication has commenced; and 20 hpi, at which time the vacuole has expanded to occupy a large volume of the cytoplasm and many of the RBs have begun to redifferentiate into EBs. Later time points were not assessed because of the increasing asynchrony of development and the onset of host cell lysis. It was necessary to use an MOI of 50 to 300 in order to ensure that a majority of the cells were infected and to examine cells at early times postinfection. While this inoculum is superphysiologic, no detectable damage to the host

cell was observed after 24 h of infection. Similar results were obtained when cells were infected at a lower MOI.

The TfR, an early endosome protein, is closely associated with the chlamydial vacuole. To visualize early endosomes, HeLa cells infected with L2 were stained with the anti-TfR antibody H68.4 (28). The TfR is the host cell receptor for the Fe^{3+}-transporting protein Tf. After Tf and its receptor are taken up into the cell by clathrin-mediated endocytosis, they are transported to an early endosome compartment. In this slightly acidified environment, the Fe^{3+} dissociates from Tf. Tf remains bound to its receptor and is recycled to the plasma membrane through a recycling endosome, where it dissociates from its receptor. Since it is transported to the early endosome and then recycled to the plasma membrane, Tf and TfR are frequently used as markers for early endosomes.

When HeLa cells were infected with L2, the TfR did not localize with the entering chlamydiae at 1 hpi (data not shown). Only a few EBs had entered at this time, and most had not yet aggregated in the perinuclear region where mature vacuoles reside. At 4 hpi, many EBs had entered the host cell, and most were concentrated near the nucleus. At this time, antibody staining of the TfR and chlamydiae coincided (Fig. 1A and B). This overlap was only observed for EBs in the perinuclear region; very few of the EBs located at the periphery of the cell colocalized with TfR staining. The TfR also appeared to be more concentrated in the perinuclear region of infected cells than in that of uninfected cells (compare Fig. 1B with E). At 12 hpi (data not shown) and 20 hpi (Fig. 1C and D) antibodies specific for the TfR stained part or all of the circumference of the vacuole in almost every infected cell. In the cells in which only a portion of the circumference was stained, the labeling was usually heaviest in the region between the vacuole and the nucleus and weakest on the side farthest away from the nucleus. It was not possible at this level of microscopy to distinguish whether the TfR resided on the vacuolar membrane or in endosomes directly adjacent to the vacuole. Especially at the early time point of 4 h, the confluent staining of the chlamydiae suggests that the TfR is localized within the vacuole. However, electron microscopy studies (unpublished data) suggest that at 4 hpi, the EBs are located within multiple nonfused vacuoles in the perinuclear region; thus, it is not possible to distinguish whether the staining observed represents vacuolar membrane or perivacuolar staining. In any case, these results indicate that the chlamydial vacuole is closely associated with the TfR, potentially pointing to a direct interaction of TfR-containing endosomes and the chlamydial vacuole.

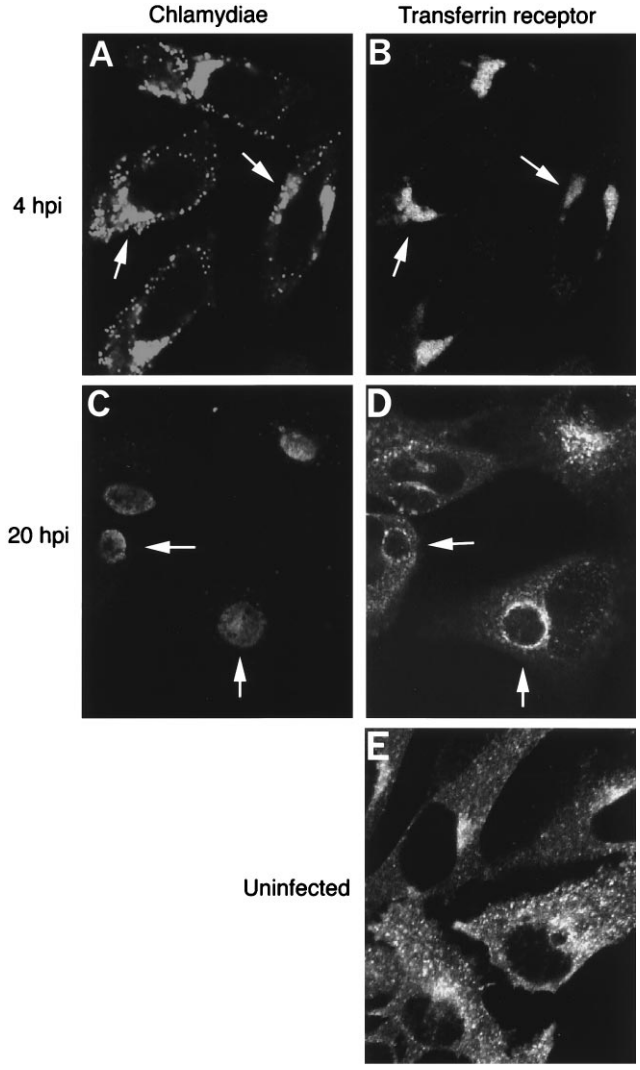

FIG. 1. Colocalization of the TfR and the chlamydial vacuole. Confocal microscopy was carried out with L2-infected HeLa cells stained with an anti-*Chlamydia* antibody (A and C) and an anti-TfR antibody (B and D). Cells were fixed 4 (A and B) and 20 (C and D) hpi. The distribution of the TfR in uninfected HeLa cells is shown in panel E. Uninfected cells were seeded and fixed at the same time as the infected cells. Images were collected by confocal microscopy and further processed with Adobe Photoshop. Arrows point to vacuolar staining (A and C) and to the corresponding region in the panel showing TfR staining. At 4 hpi, the TfR staining overlaps with the chlamydial staining, while at 20 hpi, the TfR staining surrounds the vacuole.

A late endosome protein, CI-M6PR, also associates with the vacuole. To investigate if the vacuole interacts with the late endosomal part of the endocytic pathway, HeLa cells infected with L2 were stained with an antibody to the late endosome marker CI-M6PR. CI-M6PR binds mannose-6-phosphate residues on proteins in the TGN and transports these glycoproteins to the late endosome/prelysosome compartment. This protein is commonly used as a marker for late endosomal compartments.

At 1 hpi, no overlap of the anti-CI-M6PR staining and antichlamydia staining was detected (data not shown). At 4 hpi, CI-M6PR staining was found to overlap with the staining of chlamydiae (Fig. 2A and B). Interestingly, the overlap was seen only in the perinuclear region; individual organisms at the periphery of the cell did not appear to colocalize with the CI-M6PR. The distribution of the CI-M6PR was also altered in infected cells. In uninfected cells, the CI-M6PR was found in late endosomes distributed throughout the cytoplasm (Fig. 2E). In contrast, in infected cells 4 hpi, the bulk of the CI-M6PR staining was found primarily in the region of the vacuole (compare Fig. 2B and E). At later time points of infection (12 hpi [data not shown] and 20 hpi [Fig. 2C and D]), anti-CI-M6PR staining partially surrounded the vacuoles, although it did not usually completely encircle the entire vacuole. The majority of the staining was found in the region between the vacuole and the nucleus (for example, see the cell on the right half of Fig. 2C and D; the nucleus was identified by phase microscopy as well as by its slight faint red staining).

Cross-reactivity of the anti-CI-M6PR antibody with chlamydial proteins from both purified EB preparations and chlamydia-infected L929 cells was tested by immunoblot analysis (25). In neither preparation was any cross-reactivity other than the native CI-M6PR signal detected (data not shown). To control for the possibility that the staining of the chlamydial vacuole was nonspecific, these experiments were repeated with

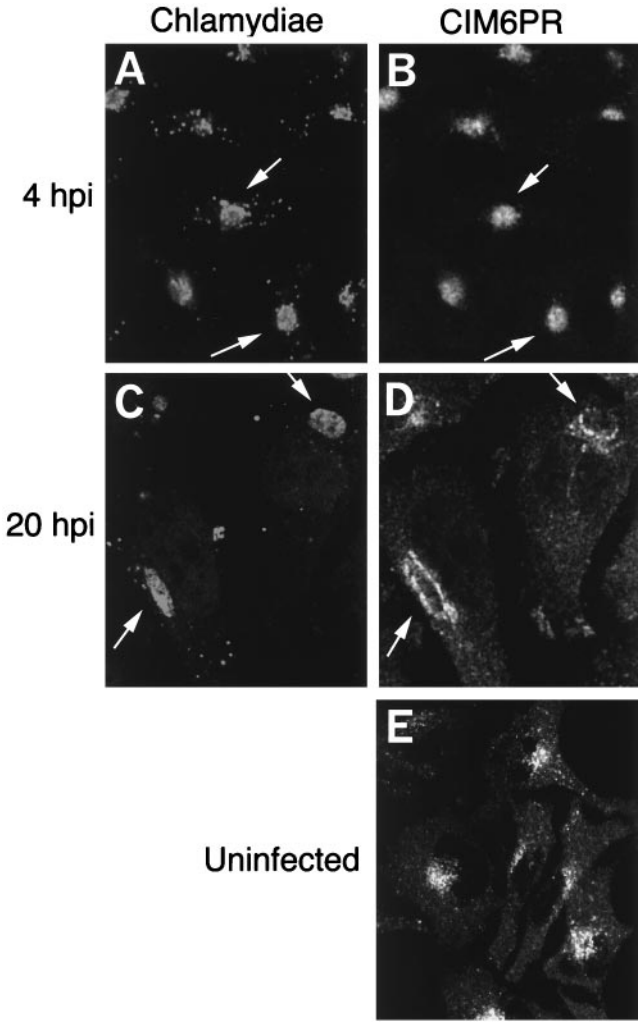

FIG. 2. Colocalization of the CI-M6PR and the chlamydial vacuole. Confocal microscopy was carried out on L2-infected HeLa cells stained with anti-chlamydia antibody (A and C) and an anti-CI-M6PR antibody (B and D). Cells were fixed 4 (A and B) and 20 (C and D) hpi. The distribution of the CI-M6PR in uninfected HeLa cells is shown in panel E. Arrows indicate vacuolar staining (A and C) and the corresponding region in the panel showing CI-M6PR staining.

Rat-1 cells. The monoclonal anti-CI-M6PR antibody used in these experiments does not recognize the rat homolog of the CI-M6PR. No staining of uninfected or infected Rat-1 cells was observed (data not shown). These findings suggest that besides the early endosome marker TfR, at least one marker of late endosomes is also found in association with the chlamydial vacuole. This finding could indicate that the vacuole interacts with late endosomes as well.

Two different *C. trachomatis* biovars interact identically with the endocytic pathway. The experiments described above were performed with the L2 serovar of the LGV biovar of *C. trachomatis*. The experiments were repeated with the MoPn biovar of *C. trachomatis*. No differences in the results were detected between the two biovars (data not shown), suggesting that at least two different biovars of *C. trachomatis* may employ similar strategies for interacting with the host endocytic pathway and for intracellular survival.

The redistribution of endocytic markers is specific to the *C. trachomatis* vacuole. To investigate the specificity of the redistribution of the endocytic pathway markers detected in infected cells, the distribution of both the TfR and the CI-M6PR in cells that have taken up heparin-coated beads was investigated. Heparin-coated beads may provide a model for the entry of *C. trachomatis*, although, as described below, their ultimate destination in the cell is different. Outside the context of other chlamydial components, latex beads coated with heparin, heparan sulfate, or the native chlamydial attachment ligand are efficiently endocytosed by epithelial cells, whereas latex beads coated with chondroitin sulfate are not. The entry process is trypsin sensitive and can be competitively inhibited by chlamydial organisms, mimicking several of the features associated with entry of chlamydiae (22).

HeLa cells were incubated in the presence of either heparin-coated beads, L2 EBs, or both for 2 h and incubated at 37°C for an additional 18 h. In addition, to some samples incubated only with heparin-coated beads, EBs were added to the cells 4 h before fixation, such that the cells contained both bacteria and beads. Likewise, to some samples infected with *C. trachomatis* initially, heparin-coated beads were added to the cells 4 h prior to fixation. In cells containing only beads, no redistribution of either the TfR or CI-M6PR was seen (compare Fig. 3A with 1E and 3F with 2E), nor was any colocalization of these markers with the bead-containing compartments observed (Fig. 3A and B and F and G). The beads in the cell in the center of panel 3B show some overlap with the TfR staining. However, this was seen only rarely, and the majority of the beads did not show this staining pattern. In cells containing both beads and bacteria, the marker staining around the chlamydial vacuoles was identical to the staining in chlamydia-infected cells that did not contain beads (compare Fig. 3C and H with 1D and 2D, respectively). Similar patterns were detected in cells infected with chlamydiae for 20 h and incubated with beads for 4 h and cells incubated with beads for 20 h and EBs for 4 h (not shown). The redistribution of the markers to the vacuole was thus not a general response to the entry of large particles, but a chlamydia-specific phenomenon.

The distribution of a TGN-specific marker is altered late during *C. trachomatis* infection. The TGN is the terminal region of the Golgi complex where secreted and lysosomal proteins are sorted. Many proteins are transported from the TGN to the late endosomes for further transport to the lysosome. To determine if TGN resident proteins are also found on or near the vacuole, HeLa cells infected with L2 were stained with an antibody to the TGN-specific marker TGN38, a TGN resident protein of unknown function (12). In uninfected cells, anti-TGN38 staining was restricted to a small perinuclear region corresponding to the TGN (data not shown). At 1 hpi (data not shown) and 4 hpi (Fig. 4A and B), no changes in the TGN staining pattern were detected. At 4 hpi, some EBs were seen in the region near the TGN, but no overlap with TGN38 staining was detected. Remarkably, at 12 hpi (data not shown) and 20 hpi (Fig. 4C and D), two different staining patterns were detected. Most prevalent was a pattern where the anti-TGN38 staining was dispersed into several regions, usually surrounding the vacuole (see the cell in the lower right-hand corner of Fig. 4C and D). In a minority of cells, the TGN38 staining had disappeared completely (not shown). An uncommon pattern is exemplified by the cell in the upper portion of Fig. 4D, where several regions of the TGN surrounded the nucleus and not the vacuole. These data indicate that the vacuole interfaces with the TGN, although it does not appear to fuse with it.

Lysosomal markers and a fluid-phase tracer are not found associated with the chlamydial vacuole at any time during the intracellular life cycle. To determine if lysosomal proteins were associated with the chlamydial vacuole at early times

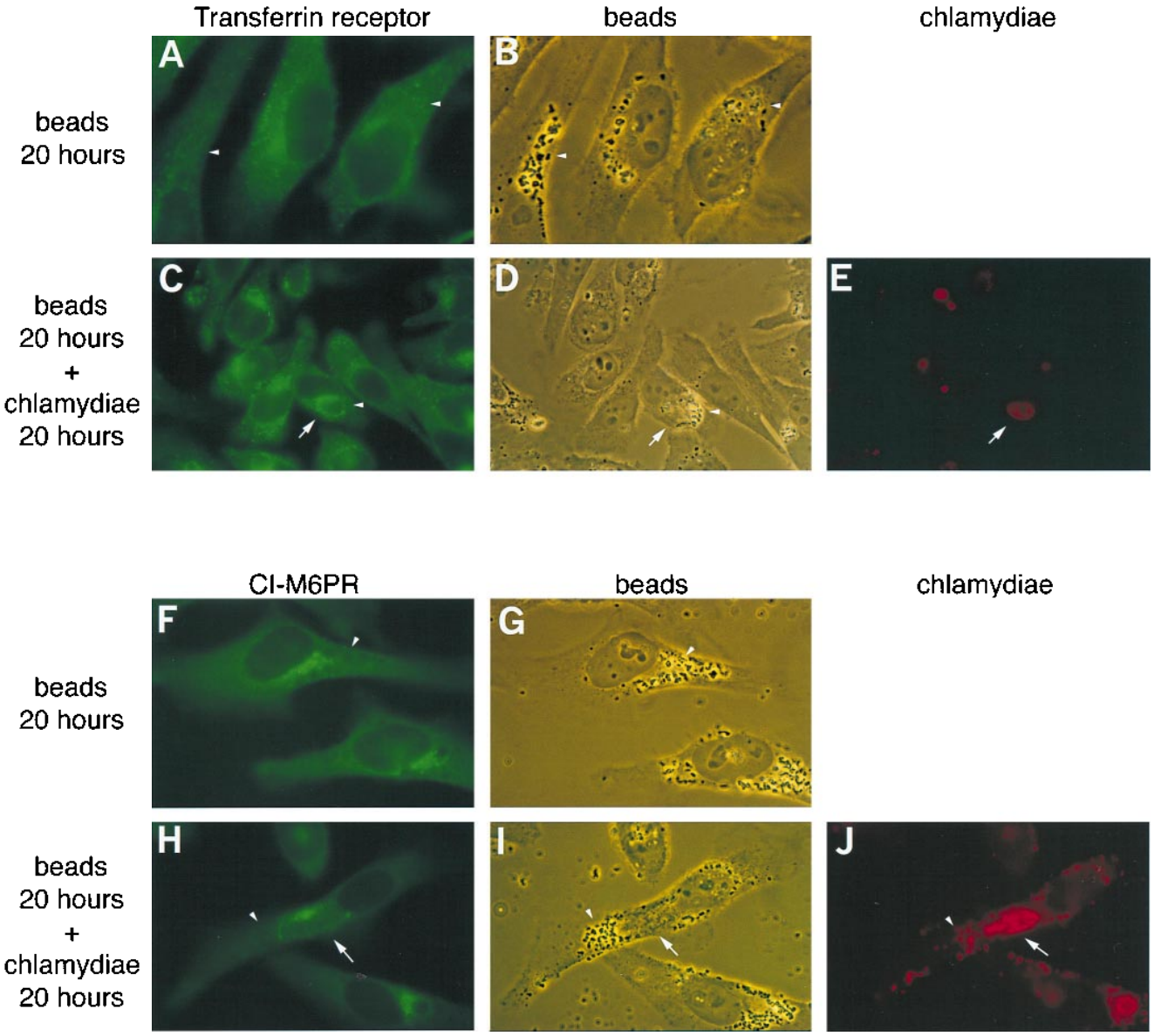

FIG. 3. Both the TfR and CI-M6PR staining selectively associate with the chlamydial vacuole but not with compartments containing heparin-coated beads. Results of epifluorescent microscopy of HeLa cells incubated in the presence of heparin-coated beads alone (A and B and F and G) or heparin-coated beads and L2 (C to E and H to J) for 2 h are shown. After 20 h, the cells were fixed and stained for TfR or CI-M6PR as described above. Cells containing only beads show no redistribution of either TfR or CI-M6PR, nor is there any apposition of the marker staining with the bead-containing compartment. In cells containing both chlamydiae and beads, the marker staining is again closely appositioned to the chlamydial vacuole; however, no overlap is seen between the marker staining and the bead-containing compartment. Arrowheads point to selected beads; arrows indicate vacuoles.

postinfection, MoPn-infected HeLa cells were stained with an antibody to cathepsin D, a lysosomal protease; MoPn-infected MDCK cells were stained with an antibody to the lysosomal antigen, AC-17; and L2-infected L cells were stained with an antibody to the lysosomal membrane proteins LAMP-1 and -2. These different cell lines were utilized because of the species specificity of the antibody. At no time during the infection (1, 4, 12, or 20 hpi) was any lysosomal marker found to preferentially associate with the chlamydial vacuole (not shown), confirming previous data that vacuoles do not fuse with lysosomes (3, 9, 15).

Likewise, when L2-infected HeLa cells were incubated in the presence of the fluid-phase marker FITC-dextran (molecular weight, 10,000), no FITC-dextran could be detected within the chlamydial vacuole at 1, 4, 12, or 20 hpi. Cells were incubated in the presence of FITC-dextran for 2 h, at which time endocytic compartments throughout the entire cell were labeled. Thus, fluid-phase vesicles (pinosomes) do not fuse with the chlamydial vacuole at any time during infection.

Effect of *C. trachomatis* infection on the recycling of Tf. The experiments described so far measure steady-state distribution of specific proteins. To determine how infection with *C. trachomatis* affects the rate and recycling of a specific endocytosed protein, the rates of recycling of Tf in MoPn-infected and uninfected HeLa cells were compared by two methods to determine the location of Tf in these cells and quantitate the rate of Tf recycling. At 3, 11, and 19 hpi, FITC-Tf was added to infected cells for 1 h, followed by incubation in the presence of

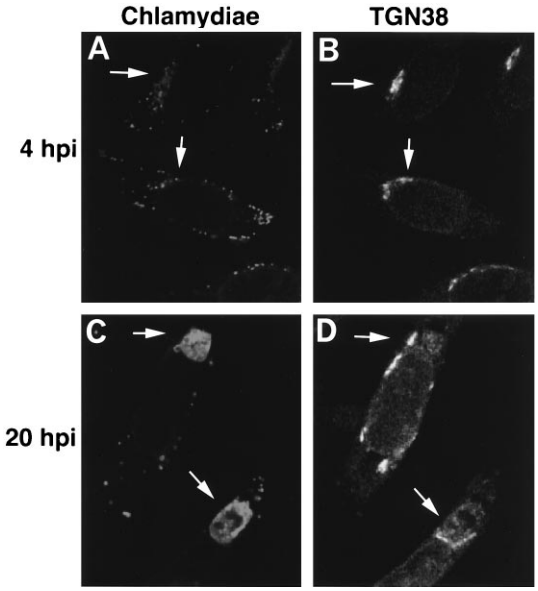

FIG. 4. TGN38 staining of L2-infected HeLa cells. Cells were infected for 4 (A and B) or 20 (C and D) h, fixed, and stained with antibodies recognizing *C. trachomatis* and TGN38. The images were collected and colored as described in the legend to Fig. 1. After 4 h, the staining of chlamydiae (A) does not overlap with the TGN staining (B) (compare arrows pointing to identical regions in the cells). After 20 h, the TGN staining (C) surrounds the staining of chlamydiae (D) but no overlap is detected.

unlabeled Tf for various times to allow the cells to recycle the FITC-Tf. When the cells were fixed immediately after the 1-h loading period, the staining pattern of FITC-Tf was similar to that of TfR (compare Fig. 5A and G to 1B and D, respectively). At all time points postinfection, FITC-Tf appeared concentrated next to the vacuole, but it was also present in vesicles in the cytoplasm (Fig. 5A, D, and G). The accumulation of FITC-Tf in the perinuclear region was not as marked as the accumulation of TfR shown in Fig. 1, presumably because of a difference in the MOI used in the different experiments. After 30 min of recycling (Fig. 5B, E, and H), uninfected cells (marked with an arrowhead) had recycled nearly all of the FITC-Tf, while infected cells (marked with an arrow) had retained a small amount of FITC-Tf. The retained FITC-Tf was localized adjacent to the vacuole; nearly all of the endosomal FITC-Tf appeared to have been recycled. The same retention of FITC-Tf was seen after 60 min (Fig. 5C, F, and I) and 120 min (data not shown) of recycling, although the levels of FITC-Tf at the latter time point had decreased substantially, suggesting that recycling of this FITC-Tf was still occurring, albeit slowly. The amount of FITC-Tf retained differed from cell to cell, and a small number of infected cells had been depleted of FITC-Tf entirely after 60 min. Since the retention of FITC-Tf was detected at all three time points postinfection, it is unlikely to be associated with the entry process of the EBs and is more likely to be a property of the vacuole. The rates of FITC-Tf uptake, as judged by the accumulation of FITC-Tf within the cell, did not appear to differ between infected and uninfected cells (data not shown).

The difference in Tf-recycling rates between infected and uninfected cells was quantitated with ^{125}I-Tf. To ensure that any potential effect was due to infection with chlamydiae and was not due to a contaminant in the EB preparation, uninfected cells were mock infected with an extract from uninfected HeLa cells that was prepared identically to the EB stock. Before each experiment, one coverslip was fixed and stained for chlamydiae to determine the percentage of the cells infected. In all cases, this was higher than 80%. L2-infected HeLa cells were incubated in the presence of ^{125}I-Tf for the last hour of infection, after which time the ^{125}I-Tf was removed, and the cells were washed extensively at 4°C with cold PBS. Recycling was started by washing the cells several times with prewarmed medium containing a 100-fold excess of unlabeled Tf. After 5 min, 10 min, 30 min, 1 h, and 2 h, the supernatant was transferred to a microcentrifuge tube and replaced with medium containing unlabeled Tf. After 2 h, the cells were lysed, and the amount of ^{125}I-Tf in the cell lysates and supernatants was counted. The accumulation of ^{125}I-Tf in the medium expressed as a percentage of the total amount of ^{125}I-Tf within the cells at the start of the recycling phase (the total amount released plus the amount in the cell lysate) is shown in Fig. 6. Consistent with the results of the fluorescence microscopy studies, ^{125}I-Tf accumulated in the supernatant of infected cells at a slower rate than was the case for mock-infected cells. The slower rate of Tf recycling was most pronounced between 10 and 60 min of recycling and was reduced to a statistically insignificant difference by 2 h of recycling, by which time nearly 95% of the ^{125}I-Tf had been recycled. The difference between infected and mock-infected cells was small, but the difference was reproducible over four independent experiments each done in either triplicate or quadruplicate. For each time point before 2 h, the difference in the amount of ^{125}I-Tf recycled was statistically significant (Student's two-tailed t test; $P < 0.02$). The inhibition of Tf recycling from the chlamydial vacuole was independent of the length of time of infection; a similar inhibition of Tf recycling was observed at 4, 12 (data not shown), and 20 hpi. These results are consistent with the fluorescence studies of FITC-Tf recycling, which showed that most of the FITC-Tf was recycled, and only a small fraction was retained adjacent to the vacuole. Presumably, it is this fraction of ^{125}I-Tf that is released into the medium at a slower rate in infected cells.

Together, these experiments demonstrate that infection with chlamydiae alters the recycling of Tf. The rate of recycling of Tf was slower in infected cells, and some Tf was retained adjacent to the vacuole. Together, these data support the idea that the vacuole interacts with the endocytic pathway.

DISCUSSION

Intracellular pathogens have developed several different strategies for survival within the eukaryotic host cell, including escape from the vacuole into the cytoplasm, survival within lysosomes, and inhibition of lysosomal fusion. Survival of chlamydiae within the host is dependent upon the prevention of fusion of the chlamydia-laden vacuole with lysosomes. Here, we report that while avoiding lysosomal fusion, the vacuole interacts with or affects several host endocytic compartments as early as 4 hpi, including early and late endosomes and the TGN. After this paper was submitted, two other groups reported investigations of the localization of endocytic markers in chlamydia-infected cells at later times after infection (20, 24). While the overall results are similar, specific differences are discussed below.

We demonstrate that at several time points during the chlamydial life cycle, corresponding to different developmental forms of the bacterium, several endocytic markers were found in close apposition to the developing chlamydial vacuole. Early in infection (between 1 and 4 hpi), the uptake of bacteria into the perinuclear region was accompanied by an accumulation of both the TfR and CI-M6PR in the perinuclear region that

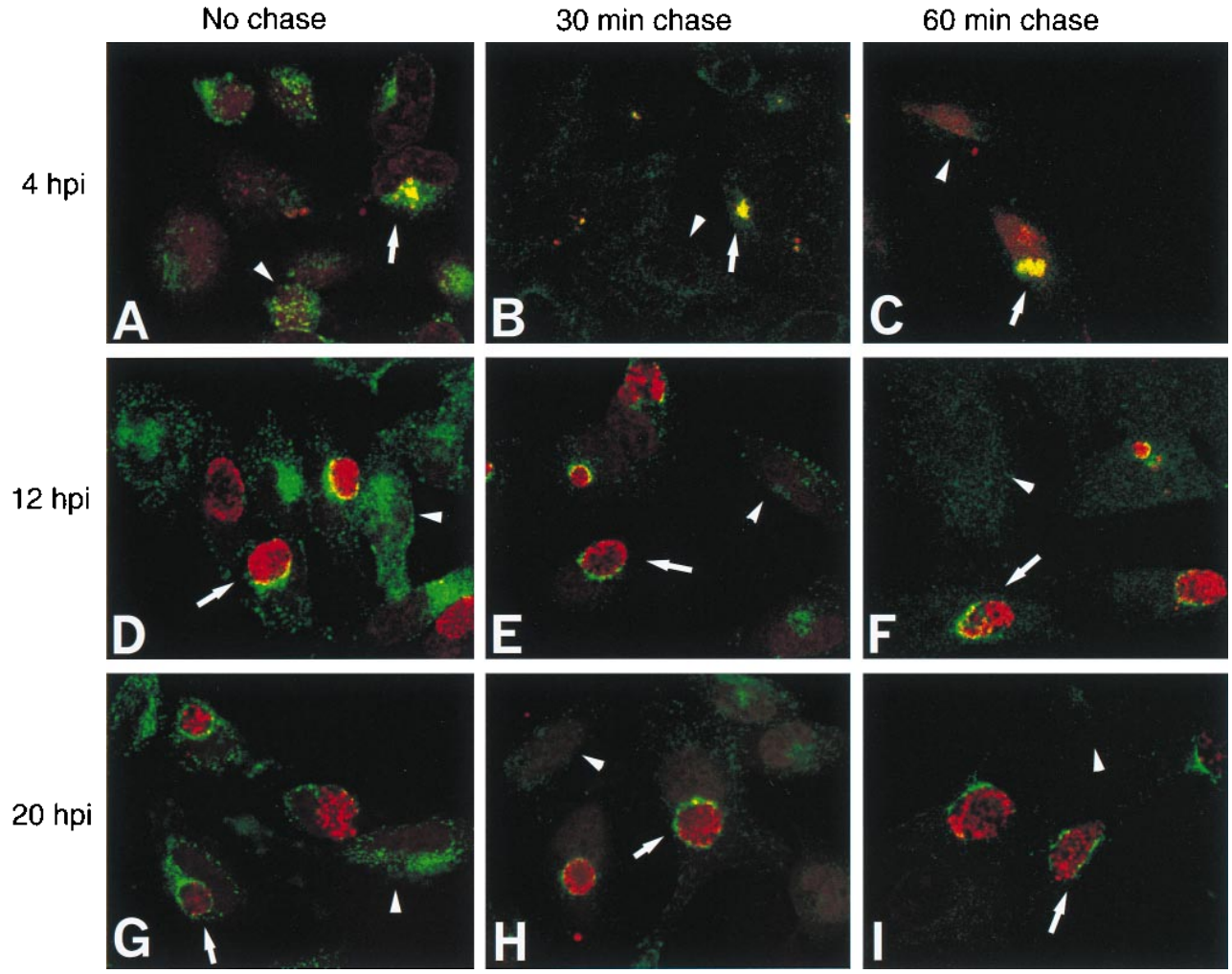

FIG. 5. FITC-Tf recycling is delayed in L2-infected HeLa cells. HeLa cells 4 (A to C), 12 (D to F), or 20 (G to I) hpi were incubated in the presence of FITC-Tf for 1 h. Cells were allowed to recycle the FITC-Tf for 0 (A, D, and G), 30 (B, E, and H), and 60 (C, F, and I) min in the presence of unlabeled Tf. Cells fixed immediately after the uptake of FITC-Tf showed staining throughout the cytoplasm, although it was concentrated adjacent to the vacuole. However, after 30 or 60 min of recycling (chase), only infected cells (arrows) retained FITC-Tf, whereas uninfected cells (arrowheads) had recycled most of the FITC-Tf.

overlapped with the EBs. Control experiments with heparin-coated beads suggest that the perinuclear localization of the EBs is likely to be active, since the beads, which are similar in size to EBs, do not accumulate in a perinuclear location. At later time points, the vacuole was circumscribed by TfR staining and to a lesser extent by CI-M6PR staining. At no time were lysosomal markers or fluorescently labeled low-density lipoprotein (unpublished results) detectable on or near the vacuole. Likewise, FITC-dextran, a fluid-phase marker, was not detected in the lumen of the vacuole, indicating that vesicles containing fluid-phase markers do not fuse with the vacuole. Additional evidence for an interaction of the vacuole with the host cell endocytic pathway comes from our observation that the rate of Tf recycling is decreased in chlamydia-infected cells. The close association of the TfR with the vacuole may be responsible for the decrease in the rate of Tf recycling.

Despite the high resolution afforded by confocal microscopy, we cannot determine whether TfR and CI-M6PR are present on the vacuolar membrane or whether the vacuole is surrounded by TfR- and CI-M6PR-containing vesicles. Potentially, the vacuole, because of its size, merely displaces TfR- or CI-M6PR-containing endosomes from their usual perinuclear localization. We consider this unlikely for two reasons. First, the redistribution of the TfR and CI-M6PR was seen as early as 4 hpi, at which time the vacuoles are still relatively small. The concentration of the markers appeared to have increased in the region overlapping with the EBs, which is the opposite of what would be expected if the vacuoles were displacing the endosomes containing TfR or CI-M6PR. Second, no redistribution of these endocytic proteins was observed during the uptake of heparin-coated latex beads. These beads occupy a volume within the host cells roughly equivalent to that of the chlamydial vacuoles at 4 hpi. Cells containing both heparin-coated beads and chlamydiae showed a close apposition of TfR and CI-M6PR staining only around the chlamydial vacuole and not around the bead-containing compartments. Thus, the redistribution of the endocytic markers appears to be specific for the chlamydial vacuole and is not the consequence of entry of large particles into the cell. Using electron microscopy, Taraska et al. (24) and Scidmore et al. (20) showed that horseradish peroxidase-conjugated Tf was not delivered to the lumen of the vacuole, but instead resided in small compartments surrounding the vacuole. Because both of these studies were

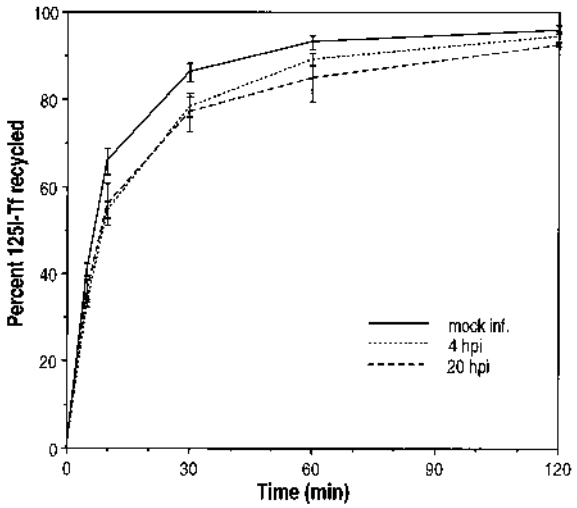

FIG. 6. ^{125}I-Tf is recycled at a slower rate in infected cells. Recycling of the ^{125}I-Tf was quantitated by measuring the amount of ^{125}I-Tf released into the supernatant. L2-infected HeLa cells were incubated with ^{125}I-Tf for 1 h. The total amount of ^{125}I-Tf in the cells at the start of the recycling was set equal to the total amount of ^{125}I-Tf released during the recycling phase plus the amount of ^{125}I-Tf remaining in the cells. The percentage of the total amount of ^{125}I-Tf released during the recycling phase is plotted. ^{125}I-Tf accumulates in the supernatant at a slower rate in infected cells than in uninfected (mock inf.) cells. Each time point represents analysis performed in quadruplicate, and the experiment was repeated twice with identical results.

carried out at intermediate stages of the life cycle (18 or 24 hpi), it is still possible that the vacuole is accessible to Tf at an earlier stage of the life cycle, but subsequently loses this fusogenicity. In any case, the function of the Tf-containing vesicles that surround the vacuole and their role in the chlamydial life cycle are unclear.

We note that our results concerning the colocalization of CI-M6PR differ from those reported by Heinzen and coworkers, who showed an absence of CI-M6PR staining near the chlamydial vacuole (9). Since both investigators utilized the same antibody, the same cell line, and the same serotype of *C. trachomatis* at equivalent time points postinfection, the reason for these disparate results is unclear. The disparity could relate to differences in the MOI used. Notably, our results are similar to those reported by Taraska et al. (24), who also utilized a high MOI.

In addition to these early and late endocytic markers, a TGN marker, TGN38, was also found in close proximity to the vacuole. These data are in agreement with recent reports by Hackstadt and coworkers that demonstrated that the vacuole and individual bacteria themselves can incorporate 6-{[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl} sphingomyelin (NBD-sphingomyelin) endogenously synthesized in the Golgi apparatus from C_6-NBD-ceramide (8). They also demonstrate that antibody probes for Golgi-specific proteins, including β-COP, α-mannosidase II, and the Golgi p58 protein and the Golgi-specific lectins *Helix pomatia* agglutinin, *Ricinus communis* agglutinin, and *Triticum vulgaris* agglutinin, showed staining that was closely associated with the chlamydial vacuole, but did not appear to stain the vacuolar membrane (9).

Based on their results, Hackstadt et al. proposed that *C. trachomatis* interrupts an exocytic pathway to intercept host sphingolipids in transit from the Golgi apparatus to the plasma membrane and that *C. trachomatis* replicates within a vacuole that is disconnected from endosome-lysosome trafficking (7). The finding that treatment with the Golgi-disrupting drug brefeldin A, while changing the morphology of the vacuoles, does not impede the ability of the chlamydiae to replicate and does not prevent the incorporation of fluorescently labeled lipids into the vacuolar membrane (7) hints that the vacuole may acquire lipids from a different source as well.

Our studies, which examine early and late endocytic markers in detail at both early and late time points during infection, are consistent with the hypothesis that *C. trachomatis* enters the host cell through an endocytic pathway into a compartment that continues to interact with the endocytic pathway and additionally interacts with the exocytic pathway. Since the patterns of TfR and FITC-Tf staining are similar, the TfR surrounding the vacuole is likely derived from the plasma membrane rather than from de novo synthesis. This result is consistent with the hypothesis that endocytic trafficking from the membrane to the vacuolar region occurs. The accumulation of Tf, TfR, and CI-M6PR in or near the vacuole may reflect the fact that these markers are trafficked efficiently to the chlamydial vacuole but recycle or depart from the vacuole slowly. The finding that iodinated Tf is recycled more slowly in infected cells than in uninfected cells is consistent with this model and differs from the findings of Scidmore et al. (20), who did not find such a difference between infected and uninfected cells. A major difference between the two experiments is the MOI used in these experiments. In our experiments, we ascertained that at least 80% of the cells were infected, and we could therefore detect a small difference in the recycling rate, which may not be detectable when a lower percentage of the cells is infected.

Collectively, the data described by Hackstadt and coworkers (20) and by Taraska et al. (24) along with the results described here are consistent with the hypothesis that the chlamydial vacuole has some similarity to recycling endosomes (29). Recycling endosomes are the endocytic compartments through which recycled receptors and substrates are trafficked before being transported to the plasma membrane. The *C. trachomatis* vacuole shares several characteristics with this compartment. Both the *C. trachomatis* vacuole and the recycling endosome contain receptors and substrates (such as Tf and TfR) that are recycled to the plasma membrane. Furthermore, both are inaccessible to ligands destined for the lysosome, such as low-density-lipoprotein and fluid-phase markers (5). Finally, they are both physically situated in a peri-Golgi region within the host cell (10, 29), and both can fuse with vesicles of the exocytic pathway (4). However, a major difference between the vacuole and the recycling endosome is the probable lack of Tf and TfR in the vacuole.

A common theme emerging from studies of intracellular pathogens is their ability to usurp host cell trafficking to enable their intracellular survival. Our results demonstrate that, like several other intracellular pathogens, *C. trachomatis* replicates in a vacuole that is unique and does not correspond to a canonical endocytic vesicle. It is selective with respect to the host endocytic compartments and components with which it interacts. The challenge now is to unravel the mechanisms whereby each intracellular pathogen uniquely perturbs the eukaryotic endocytic pathway to create a specialized niche that allows the continued intracellular persistence or replication of the organism.

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