Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4877–4881, May 1995
Microbiology

Lipid metabolism in *Chlamydia trachomatis*-infected cells: Directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion

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Communicated by Stanley Falkow, Stanford University School of Medicine, Stanford, CA, January 30, 1995

ABSTRACT **_Chlamydia trachomatis_ undergoes its entire life cycle within an uncharacterized intracellular vesicle that does not fuse with lysosomes. We used a fluorescent Golgi-specific probe, {*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]}aminocaproylsphingosine (C_6-NBD-Cer), in conjunction with conventional fluorescence or confocal microscopy to identify interactions between the Golgi apparatus and the chlamydial inclusion. We observed not only a close physical association between the Golgi apparatus and the chlamydial inclusion but the eventual presence of a metabolite of this fluorescent probe associated with the chlamydiae themselves. Sphingomyelin, endogenously synthesized from C_6-NBD-Cer, was specifically transported to the inclusion and incorporated into the cell wall of the intracellular chlamydiae. Incorporation of the fluorescent sphingolipid by chlamydiae was inhibited by brefeldin A. Chlamydiae therefore occupy a vesicle distal to the Golgi apparatus that receives anterograde vesicular traffic from the Golgi normally bound for the plasma membrane. Collectively, the data suggest that the chlamydial inclusion may represent a unique compartment within the trans-Golgi network.**

Chlamydia trachomatis is the causative agent of several significant human diseases including trachoma, the leading cause of infectious blindness worldwide, and is the most common cause of sexually transmitted disease in the United States and in developed countries (1). Chlamydiae are obligate intracellular bacteria with a biphasic life cycle characterized by functionally and morphologically distinct cell types adapted for extracellular survival and intracellular multiplication. This developmental cycle takes place entirely within an intracellular vesicle (inclusion) that is not believed to be acidified and does not fuse with lysosomes (2). Infection is initiated by a small, metabolically dormant cell type called the elementary body (EB). After endocytosis, an EB differentiates into a larger, pleomorphic, and metabolically active cell type called the reticulate body. The reticulate bodies divide by binary fission throughout the remainder of the infection until the cell lyses at 40–44 hr after infection. However, at ≈18 hr after infection the developmental cycle becomes asynchronous, as increasing numbers of reticulate bodies differentiate back to EBs that accumulate within the inclusion until cell lysis occurs. The environmental signals that regulate this developmental cycle are unknown. There are many fundamental questions regarding the nature of the chlamydial inclusion—including its composition, permeability properties, biosynthetic origin, and lumenal contents. The chlamydial inclusion is isolated from established routes of intracellular trafficking; with the exception of vacuoles containing other chlamydiae, no cellular vesicles are known to fuse with the chlamydial inclusion (3). Although chlamydiae obviously acquire essential nutrients from the host cell, the mechanisms for obtaining these across the inclusion membrane have not been identified.

We have used a variety of specific probes for cellular organelles in conjunction with conventional fluorescence and confocal microscopy in an attempt to identify cellular organelles that may interact with the chlamydial inclusion. Our results indicate a direct involvement of the Golgi apparatus in trafficking of sphingolipids to the chlamydial inclusion and imply a close interaction between the chlamydial inclusion and the Golgi network.

MATERIALS AND METHODS

Organisms. *C. trachomatis*, LGV-434, serotype L2, was grown in HeLa 229 cells as described (6). Infectivity of *C. trachomatis* EBs was titrated by determination of inclusion-forming units on HeLa 229 cells, as described by Furness *et al.* (4), except that inclusions were visualized by indirect immunofluorescence using polyclonal antisera against formalin-killed *C. trachomatis* L2 EBs and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobin serum (Zymed).

{*N*-[7-(4-Nitrobenzo-2-oxa-1,3-diazole)]}aminocaproylsphingosine (C_6-NBD-Cer) Labeling. Monolayer cultures of HeLa 229 cells (CCL 2.1; American Type Culture Collection) were grown on 12-mm-diameter glass coverslips (no. 1 thickness) in 24-well plates. The cells were infected with *C. trachomatis* LGV-434, serotype L2, at a multiplicity of infection of ≈0.5 and incubated for 18 hr in minimum essential medium (MEM)/10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2/95% humidified air. Fluorescent C_6-NBD-Cer (Molecular Probes) was complexed with 0.034% defatted bovine serum albumin (dfBSA) in MEM as described (5) to yield complexes ≈5 μM in both dfBSA and C_6-NBD-Cer. *C. trachomatis* (L2)-infected HeLa cells were incubated with the dfBSA/NBD-Cer complex at 4°C for 30 min, washed with 10 mM Hepes-buffered calcium- and magnesium-free Puck's saline, pH 7.4 (HCMF), and incubated for various times in MEM/0.34% dfBSA to "back-exchange" excess probe from the plasma membrane. Cultures on coverslips were rinsed in HCMF solution before mounting for fluorescent microscopy.

Lipid Extraction and Thin-Layer Chromatography. HeLa cell cultures in 150-cm^2 culture flasks were infected with *C. trachomatis* L2 EBs at a multiplicity of infection of ≈2. At 18 hr after infection, the culture medium was removed, and the cells were rinsed once with MEM. The cultures were labeled with 5 μM C_6-NBD-Cer in MEM/0.034% dfBSA for 30 min at 37°C and rinsed three times with MEM; the medium was then replaced with MEM/10% fetal bovine serum and incubated for an additional 18 hr. EBs were purified by Renografin (Squibb) density gradient centrifugation (6). Total lipids from

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Abbreviations: EB, elementary body; dfBSA, defatted bovine serum albumin; C_6-NBD-Cer, {*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]}aminocaproylsphingosine.

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C_6-NBD-Cer-labeled EBs were extracted by the method of Bligh and Dyer (7). Lipid extracts were applied to silica gel G thin-layer plates (Analtech) and developed in $CHCl_3$/CH_3OH/H_2O (65:24:4). Fluorescent lipids were visualized and photographed under ultraviolet illumination.

Back-Exchange. Monolayer cultures of HeLa 229 cells were grown to confluency on 12-mm coverslips, infected with *C. trachomatis* L2 EBs at a multiplicity of infection of ≈2 or mock-infected and incubated at 37°C for 24 hr. The coverslips were rinsed once with MEM and incubated with 5 μM C_6-NBD-Cer in MEM/5% dfBSA for 30 min at 4°C. The cultures were rinsed three times with MEM, and the medium was replaced with prewarmed MEM/0.34% dfBSA. The cultures were incubated at 37°C, and at various times, coverslips were

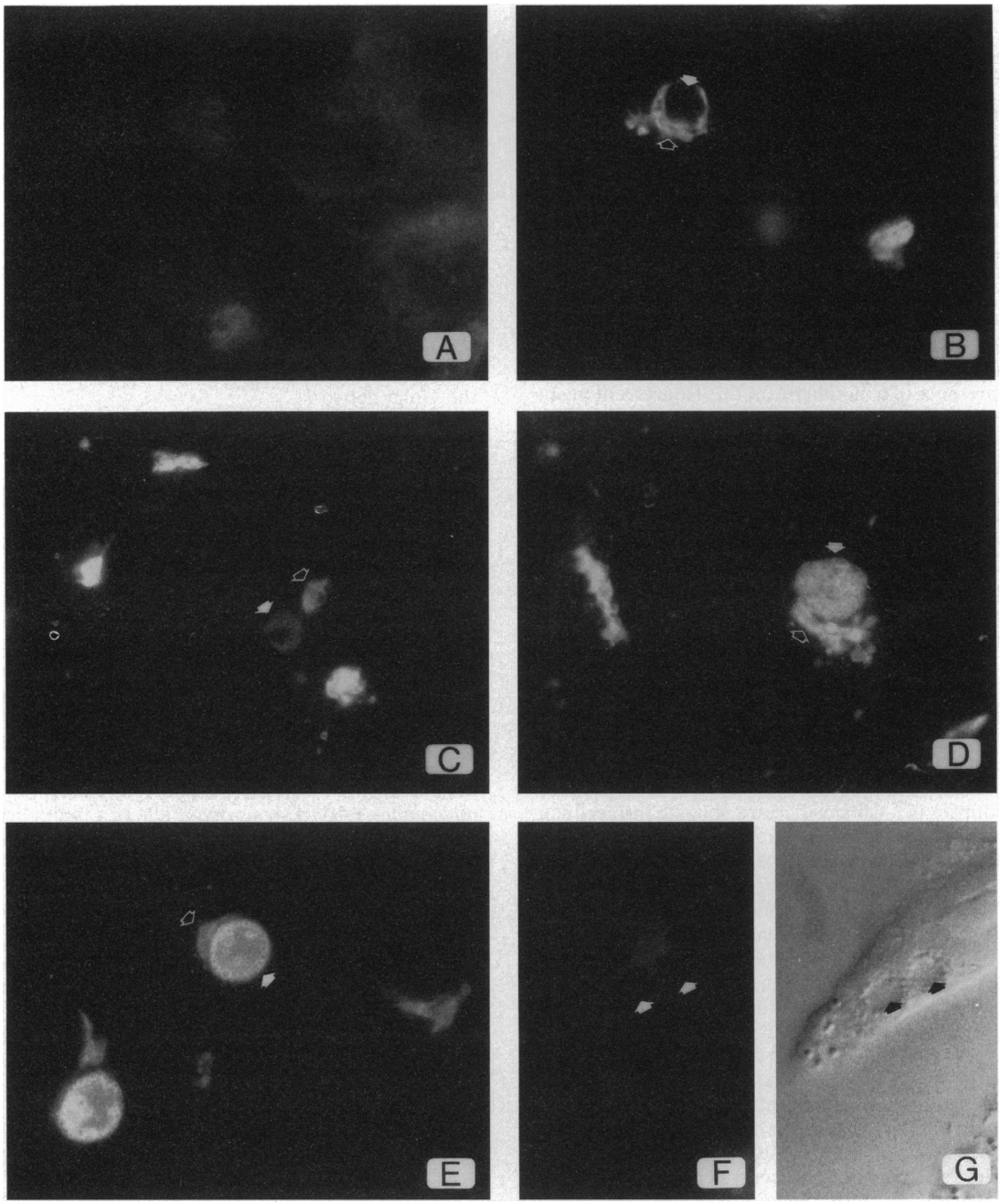

FIG. 1. C_6-NBD-Cer staining of *C. trachomatis* (L2)-infected HeLa cells at 18 hr after infection. Cells were labeled with fluorescent C_6-NBD-Cer at 4°C, warmed to 37°C, and photographed at selected intervals. Open arrows indicate the Golgi apparatus, and filled arrows indicate chlamydial inclusions. (*A*) Immediately after labeling at 4°C, there is diffuse staining of virtually all cellular membranes. (*B*) By 10 min of incubation at 37°C, the Golgi apparatus stains intensely. In this particular cell, the Golgi appears to essentially surround a chlamydial inclusion. (*C*) By 30 min, faint labeling of the chlamydiae within the inclusions is visible. (*D*) After 60 min at 37°C, both the Golgi apparatus and the intracellular chlamydiae stain with approximately equal intensity. (*E*) By 2 hr much of the probe has been exported from the Golgi apparatus of either infected or uninfected cells, whereas the intracellular bacteria remain intensely fluorescent for the duration of their intracellular growth. Continued incubation results in virtually complete loss of Golgi staining. Treatment of the cultures with brefeldin A (1 μg/ml) inhibits translocation of the fluorescent probe to the inclusion. Parallel fluorescence (*F*) and Nomarski differential interference contrast (*G*) images of the same field demonstrate the lack of transfer of the probe to the chlamydia by 60 min in the presence of brefeldin A. In those cultures treated with brefeldin A, the inhibitor was added 15 min before NBD-Cer labeling, and the cultures were kept in the continuous presence of brefeldin A throughout the labeling and back-exchange incubations. This treatment disrupted typical Golgi morphology (data not shown). All fluorescent micrographs were taken with the same exposure and printed under identical conditions. (×1000.)

rinsed three times with HCMF solution. Coverslips were inverted onto glass microscope slides, and areas for quantitation of retained fluorescence were identified by visualization using Nomarski differential interference contrast optics on a Nikon FXA photomicroscope with a Nikon ×20 Planapochromat objective. The selected area was demarcated using the photomicrographic spotmeter, and the transmitted light was extinguished. The photometric function of the microscope was used to quantify (in lux) the fluorescence intensity of the specimens under epifluorescent illumination using the fluorescein filter set. Six readings from each time point were averaged, and the SEMs were calculated.

Microscopy. Fluorescent and Nomarski differential interference contrast micrographs were taken on a Nikon FXA photomicroscope using a ×60 Planapochromat objective. Photomicrographs were obtained using T-Max ASA 400 film (Kodak). A Bio-Rad MRC-1000 confocal imaging system equipped with a krypton-argon laser (Bio-Rad) on a Zeiss Axiovert 135 inverted microscope with a ×63 Planapochromat objective was used for laser scanning confocal microscopy. Confocal images were processed by using Adobe PHOTOSHOP 2.5.1 (Adobe Systems, Mountain View, CA).

RESULTS

C_6-NBD-Cer Labeling of *C. trachomatis*-Infected Cells. After endocytosis, chlamydiae are found within a closely associated membrane vesicle that is characteristically located in the perinuclear region (8). To investigate any relationship between the Golgi apparatus and the chlamydial inclusion, *C. trachomatis*-infected cells were incubated with C_6-NBD-Cer, a vital stain for the Golgi apparatus (9). Control or infected cells incubated with C_6-NBD-Cer at 4°C displayed diffuse fluorescent labeling of virtually all cellular membranes. Upon shifting the temperature to 37°C, the fluorescence rapidly redistributed first to the Golgi apparatus and, with continued incubation, to the intracellular chlamydiae within the inclusion (Fig. 1). Incubation for longer periods resulted in virtually complete loss of fluorescence from the Golgi apparatus of either normal or infected cells. The probe accumulated, however, and remained associated with intracellular chlamydiae throughout the remainder of the growth cycle. Transport of the fluorescent lipid to the chlamydial inclusion was inhibited by brefeldin A, an inhibitor of Golgi function (10, 11). Transposition of the fluorescent probe from the Golgi apparatus to the intracellular chlamydiae could therefore be observed temporally, and this process was inhibited by disruption of anterograde Golgi transport.

A confocal image of an isolated chlamydial inclusion after ceramide labeling is depicted in Fig. 2. The chlamydiae within the inclusion displayed a distinct rim-like fluorescence pattern, suggesting incorporation of the probe into the cell wall of the parasite. No fluorescent labeling of the inclusion membrane *per se* could be documented by either confocal or conventional fluorescence microscopy. The probe appears to be rapidly incorporated by the intracellular chlamydiae once it is delivered to the inclusion. Indeed, purified chlamydiae, *in vitro*, rapidly incorporate NBD-Cer or NBD-sphingomyelin directly from the medium (data not shown) by what is likely an energy-independent mechanism.

To control for the possibility of nonspecific incorporation of ceramide into the cell wall of an intracellular bacterium, parallel experiments were carried out with *Coxiella burnetii*, a Gram-negative bacterium that multiplies within an intracellular vacuole with characteristics of lysosomes (12, 13). C_6-NBD-Cer was not accumulated by *Coxiella burnetii* (data not shown). Incorporation of the fluorescent probe, therefore, required specific transport to the chlamydial inclusion and was not incorporated by bacteria inhabiting an intracellular vesicle distinct from the chlamydial inclusion.

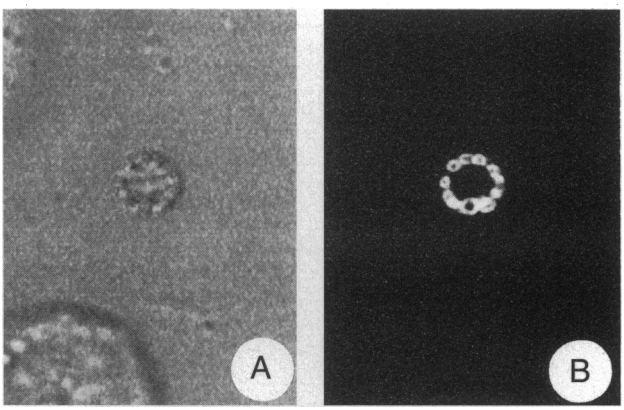

FIG. 2. Confocal micrograph of a single, cell-free, *C. trachomatis* inclusion. This inclusion was isolated from HeLa 229 cells infected with *C. trachomatis* L2 for 18 hr and labeled with C_6-NBD-Cer as described in the legend for Fig. 1. The cells were lysed by scraping the infected monolayers into HCMF solution. Simultaneous epifluorescent (*B*) and transmitted (*A*) light images were obtained using a Bio-Rad MRC-1000 confocal imaging system. (×1600.)

Products of C_6-NBD-Cer Metabolism Associated with Chlamydiae. Ceramide is the immediate biosynthetic precursor of sphingomyelin and glucocerebrosides. This enzymatic modification occurs within the cis and medial cisternae of the Golgi apparatus (14). The major fluorescent product isolated from purified chlamydiae after ceramide labeling is sphingomyelin (Fig. 3). This finding further indicates that NBD-Cer is processed through the Golgi apparatus before delivery to the chlamydial inclusion and is consistent with a report that chlamydial cell walls contain ≈3% sphingomyelin, an otherwise rare lipid in prokaryotes (15).

Export of C_6-NBD-Cer Metabolites. C_6-NBD-sphingomyelin and C_6-NBD-glucocerebroside synthesized endogenously by metabolism of C_6-NBD-Cer are translocated to the plasma

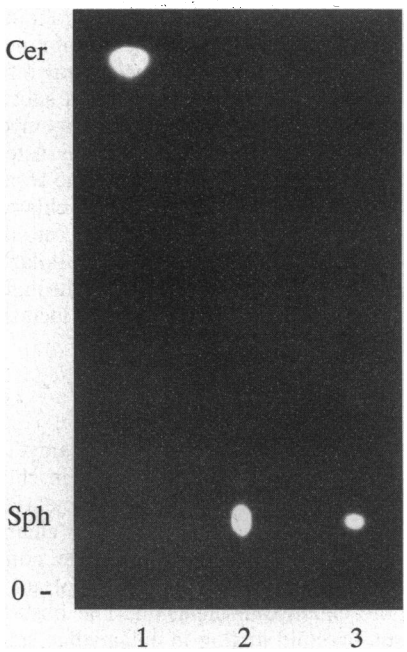

FIG. 3. Identification of sphingomyelin in purified *C. trachomatis* elementary bodies. Shown here is a thin-layer chromatogram of C_6-NBD-Cer as used to label the cultures (lane 1); C_6-NBD-sphingomyelin standard (lane 2); and a total lipid extract of purified *C. trachomatis* EBs (lane 3). Mobilities of C_6-NBD-Cer (Cer), and C_6-NBD-sphingomyelin (Sph) relative to the origin (O) are indicated.

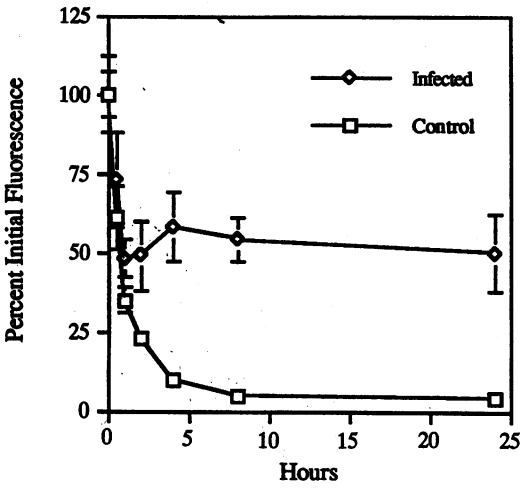

FIG. 4. Retention of fluorescent sphingolipids by mock- or *C. trachomatis* L2-infected HeLa cells. Fluorescent lipids translocated to the plasma membrane were back-exchanged from the cell surface membrane by incubation in MEM/0.34% dfBSA as described (17), and at various times the retained fluorescent lipid was quantified by photometry and expressed as percentage of initial values (control = 0.110 ± 0.013 lux; infected = 0.084 ± 0.006 lux).

membranes of eukaryotic cells (16). The fluorescent lipids may be extracted from the plasma membrane by incubation in the presence of an appropriate acceptor by a process referred to as back-exchange. To obtain a quantitative estimate of the rates of translocation of the probe to the plasma membrane versus the chlamydial inclusion, this procedure was applied to study lipid export in *C. trachomatis*-infected cells. Fig. 4 shows that there was an initial rapid loss of fluorescent lipid from both control and *C. trachomatis*-infected cells. This loss of fluorescence continued in mock-infected cells to levels of <4% of the initial observation. In contrast, the retained fluorescence in infected cells stabilized at a level of ≈50% of the initial values. This observation agrees with the results of the fluorescence microscopy. Typically, sphingolipids, or their analogs, are transported to the plasma membrane via Golgi-derived vesicles (18). Thus in *C. trachomatis*-infected cells, normal Golgi trafficking is disrupted and a substantial proportion of endogenously synthesized sphingomyelin that is normally destined for the plasma membrane is instead directed to the chlamydial inclusion. Furthermore, the results indicate that transport of NBD-sphingomyelin to the chlamydial inclusion is unidirectional and, once incorporated into the bacteria, is no longer exchanged or transported intracellularly. Whether cellular processing and export of proteins through the Golgi are disrupted by the presence of a chlamydial inclusion remains to be determined.

DISCUSSION

The nature and biosynthetic origin of the chlamydial inclusion membrane are major unresolved questions in chlamydial biology. Although amino acids (19) and nucleotides (20–22) from host pools are known to be used by chlamydiae, the incorporation of ceramide, to our knowledge, constitutes the only demonstration of direct trafficking of an exogenous substrate to the chlamydial inclusion. The observation of a fluorescent sphingolipid analog in chlamydiae subsequent to its accumulation in the Golgi apparatus and the inhibition of trafficking of the probe to the chlamydial inclusion by brefeldin A indicate a direct link between the Golgi apparatus and the chlamydial inclusion. This effect is specific to chlamydiae because the intracellular parasite *Coxiella burnetti*, which inhabits a lysosomal vesicle, does not display similar accumulation of fluorescent sphingolipids. Delivery of Golgi-derived vesicles to the chlamydial inclusion suggests a potential biosynthetic source of the inclusion membrane. Sphingolipids in transit to the plasma membrane are believed to be localized to the lumenal surface of Golgi-derived vesicles. Fusion of these vesicles with the plasma membrane delivers the fluorescent sphingolipids to the outer leaflet of the plasma membrane (16). Presumably, the topology of sphingolipids in the inclusion membrane is analogous to that of the outer leaflet of the plasma membrane. Fusion of Golgi-derived membranous vesicles with a chlamydial inclusion would then be expected to deposit fluorescent sphingomyelin on the inner surface of the inclusion membrane, where it is rapidly incorporated by chlamydial developmental forms. The chlamydial inclusion appears to occupy a site distal to the Golgi apparatus, situated such that it receives a substantial proportion of vesicular traffic otherwise destined for the plasma membrane. We propose that the incorporation of Golgi-derived lipids provides for the growth of the inclusion membrane.

Establishing routes of intracellular communication between the host cell and the chlamydial inclusion has implications not only for the study of chlamydial biology but also for other obligate and facultative intracellular parasites which inhabit intracellular vesicles that do not fuse with lysosomes (23, 24). Although the mechanism is unclear, the avoidance of lysosomal fusion by chlamydiae is not due to a general failure of lysosome function in infected cells but is limited to the chlamydial inclusion (25). One possibility is that chlamydiae modify the inclusion membrane by insertion of parasite-specified polypeptides (26) to prevent lysosomal fusion. Another possibility is that the chlamydial inclusion, by occupying a compartment within the trans-Golgi network, may evade a lysosomal response by appearing (to the cell) as a cytoplasmic organelle not destined to fuse with lysosomes. These two possibilities are not mutually exclusive; together they would imply that the chlamydial inclusion represents an aberrant compartment within the trans-Golgi network modified by the insertion of chlamydial polypeptides.

This proposed relationship between the chlamydial inclusion and the Golgi apparatus may suggest additional approaches to address many of the unanswered questions critical to understanding chlamydial biology. For example, glycosaminoglycans have been proposed as mediators of chlamydial attachment and entry (27). As the Golgi apparatus is the site of glycosaminoglycan synthesis (28), it is conceivable the chlamydiae might enlist host enzymatic machinery to synthesize these compounds not typically produced by bacteria. Although glycosylation of chlamydial proteins remains somewhat controversial (15), the proximity of the inclusion to the Golgi complex allows for the possibility of host involvement in posttranslational modification. In addition to advancing our understanding of chlamydial–host interactions, the specific trafficking of ceramide to the chlamydial inclusion may have practical applications in the design of ceramide derivatives, such as therapeutic agents or probes of physical parameters, that are targeted to and accumulate within the inclusion.

We thank Harlan Caldwell, John Swanson, Vito Cieplak, and Clif Barry for critical review of the manuscript; Janet Sager and Jim Simmons for technical assistance; Pam Small for use of the confocal microscope; and Gary Hettrick and Bob Evans for graphic arts.

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