Introduction

Chlamydiae are aetiological agents of several human diseases of medical significance. *C. trachomatis* is the most commonly encountered sexually transmitted pathogen in the United States and developed countries, and worldwide is the leading cause of infectious blindness (Schachter, 1988). The spectrum of disease manifestations caused by *Chlamydiae* also encompasses *C. pneumoniae*, a causative agent of acute respiratory infections and implicated as a potential risk factor of cardiovascular disease (Grayston, 2000).

Chlamydiae are obligate intracellular pathogens that reside exclusively in a membrane-bound vacuole termed an inclusion. Within this protected organelle, chlamydiae acquire host-cell-derived biosynthetic precursors necessary for intracellular subsistence, yet the mechanisms and pathways responsible for this acquisition remain elusive. The present study identifies an interaction between the chlamydial inclusion and multivesicular bodies, complex organelles pivotal in protein and lipid transport that are positioned along the endosome-lysosome pathway, and intersect the exocytic pathway in various cell types. Resident protein and lipid constituents of multivesicular bodies colocalized with intracellular chlamydiae, with direct delivery of the resident protein CD63 to the chlamydial inclusion. Interruption of trafficking from multivesicular bodies by pharmacological inhibitors and exogenous antibodies subsequently disrupted sphingolipid delivery to the maturing chlamydial inclusion and intracellular bacterial growth. This study identifies a trafficking pathway from CD63-positive multivesicular bodies to the bacterial inclusion, a novel interaction that provides essential lipids necessary for maintenance of a productive intracellular infection.

Key words: *Chlamydia trachomatis*, Multivesicular bodies, CD63, Trafficking

Summary

Chlamydiae are obligate intracellular bacterial pathogens that replicate solely within the confines of a membrane-bound vacuole termed an inclusion. Within this protected organelle, chlamydiae acquire host-cell-derived biosynthetic precursors necessary for intracellular subsistence, yet the mechanisms and pathways responsible for this acquisition remain elusive. The present study identifies an interaction between the chlamydial inclusion and multivesicular bodies, complex organelles pivotal in protein and lipid transport that are positioned along the endosome-lysosome pathway, and intersect the exocytic pathway in various cell types. Resident protein and lipid constituents of multivesicular bodies colocalized with intracellular chlamydiae, with direct delivery of the resident protein CD63 to the chlamydial inclusion. Interruption of trafficking from multivesicular bodies by pharmacological inhibitors and exogenous antibodies subsequently disrupted sphingolipid delivery to the maturing chlamydial inclusion and intracellular bacterial growth. This study identifies a trafficking pathway from CD63-positive multivesicular bodies to the bacterial inclusion, a novel interaction that provides essential lipids necessary for maintenance of a productive intracellular infection.

Key words: *Chlamydia trachomatis*, Multivesicular bodies, CD63, Trafficking
phospholipid with variable membrane compositions defining a diverse population (Gillooly et al., 2000; Kobayashi et al., 1998). LBPA-rich membranes are thought to play a role in sphingolipid hydrolysis and regulation of intracellular cholesterol transport (Kobayashi et al., 1999; Kolter et al., 1999). The tetraspanin protein CD63 is abundant in MVBs and cycles between these compartments and the exocytic pathway (Kobayashi et al., 2000). Restricted to the limiting membrane of CD63- and LBPA-positive late endosomes is the transmembrane protein metastatic lymph node 64 (MLN64) (Alpy et al., 2001). MLN64 shares homology with the StAR-related lipid transfer (START) domain of the steroidogenic acute regulatory protein (StAR) (Watari et al., 1997). The MLN64 START domain binds cholesterol and stimulates the mobilization of free cholesterol from sterol-rich donor vesicles to acceptor membranes (Kallen et al., 1998).

In this study, candidate MVB-related proteins and lipids were evaluated for their association with intracellular chlamydial infection. Demonstration of an intersection between the chlamydial inclusion and MVB-related pathways, identifies these dynamic host cell compartments as a novel source for factors essential for intracellular bacterial growth and inclusion biogenesis.

Results

MVB constituents localize to the chlamydial inclusion in infected HEp-2 cells.

To evaluate an association between the chlamydial inclusion and MVBs, the distribution of MVB markers was analyzed in C. trachomatis-infected cells by confocal microscopy. CD63 and MLN64, protein constituents of MVBs, and LBPA (a lipid highly enriched in internal vesicles of MVBs) all showed specific localization at the site of the chlamydial inclusion (Fig. 1). This association was demonstrated in HEp-2 cells infected with C. trachomatis E and labeled by indirect immunofluorescence with antibodies specific to these MVB markers. Confocal analysis of midplane Z-sections through the center of the inclusion confirmed the internal location of all MVB markers. Labeling with antibodies specific to CD63, MLN64 and LBPA all revealed a fine punctate pattern at the site of the chlamydial inclusion (Fig. 1), which colocalized with TOPRO-3-labeled bacteria within the inclusion. Immunolabeling with anti-LAMP-1 antibody revealed that LAMP-1 was excluded from the inclusion, therefore serving as a negative control. In addition, TOPRO-3-labeled host cell nuclei did not specifically label with antibodies to MVBs and provided in each image an internal control for the level of background label.

Orthogonal analysis confirms the presence of CD63 within the chlamydial inclusion

Immunolabling studies indicated that protein and lipid constituents of MVBs colocalize with chlamydial inclusions. Potential trafficking of MVBs or MVB-related compartments to the chlamydial inclusion was further evaluated using CD63 as a surrogate marker for this interaction. HEp-2 cells were infected with C. trachomatis E for 48 hours and analyzed by indirect immunofluorescence. To precisely identify the boundary of the chlamydial inclusion, cells were labeled with antibody specific to the cytosolic C-terminal domain of the inclusion membrane protein IncA, using conditions that result in labeling of the inclusion membrane exclusively (see Materials and Methods). The inclusion membrane was then detergent-permeabilized and cells were labeled with mouse antibody specific to CD63. Confocal microscopy clearly identified the presence of CD63 within the inclusion (Fig. 2, upper panel) and this colocalized with TOPRO-3-labeled Chlamydia. Orthogonal views unequivocally demonstrated localization of CD63 with TOPRO-3-labeled Chlamydia within the confines of the IncA-labeled inclusion membrane (Fig. 2, lower panel).

Immunoelectron microscopy localizes CD63 to the chlamydial inclusion

The presence of CD63 in the chlamydial inclusion was further investigated at the ultrastructural level using immunoelectron microscopy. Cryo sections of cells that had been infected with Chlamydia for 48 hours and labeled with anti-CD63 antibody revealed the presence of CD63 in multivesicular organelles adjacent to the chlamydial inclusion (Fig. 3). In addition, immunolabeling clearly showed CD63 within the chlamydial inclusion and associated with bacterial membranes. Discrete patches of CD63 were evident in the inclusion membrane itself, suggesting a direct fusion of CD63-positive vesicles with the inclusion (Fig. 3, upper panel). In addition, CD63-positive vesicles were present within the inclusion (Fig. 3, lower panel), further implicating a direct fusion of MVBs with the chlamydial inclusion.

Fig. 1. Constituents of late endocytic multivesicular compartments localized to the chlamydial inclusion by indirect immunofluorescence. HEp-2 cells were infected with C. trachomatis E for 36 hours, immunolabeled with the indicated MVB-specific antibodies and analyzed by confocal microscopy. (Upper panels) 0.5-μm-thick optical sections of infected cells, immunolabeled with antibodies against CD63, MLN64, LBPA and LAMP-1, followed by the appropriate secondary antibody conjugated to Alexa Fluor 594. (Middle panels) TOPRO-3 labeling of the equivalent confocal slice to reveal intracellular bacteria and host-cell nuclei. (Lower panels) Merged images. Arrows indicate representative chlamydial inclusions, arrowheads indicate representative nuclei. CD63, MLN64 and LBPA localized to the TOPRO-3-positive chlamydial inclusions. Bar, 20 μm.
Chlamydial inclusion with release of their internal vesicles into the lumen of the inclusion. Quantitative analysis by immunoelectron microscopy revealed that the cross-sectional labeling-density of anti-CD63 was more than tenfold and eightyfold per area of the inclusion and MVBs, respectively, over background (over labeling of mitochondria and nuclei).

**CD63-HA localizes to the chlamydial inclusion in transfected cells**

Localization of HA-tagged CD63 in transfected cells confirmed the distribution of CD63 and localization to the chlamydial inclusion. HEp-2 cells were transfected with CD63-HA for 24 hours, infected with *C. trachomatis* E for 48 hours, and subsequently labeled by indirect immunofluorescence using antibodies specific against HA. In transfected cells, anti-CD63 and anti-HA colocalized perfectly, confirming the correct targeting of the HA-fusion protein to CD63-positive compartments (data not shown). Confocal analysis of midplane Z-sections through the center of the inclusion revealed a fine punctate pattern of CD63-HA in transfected cells (Fig. 4, left panel), that colocalized with TOPRO-3-labeled bacteria within the inclusion. No labeling was evident in the inclusion of untransfected cells, which served as a negative control for anti-HA labeling.

Immunoelectron microscopy analysis of CD63-HA-transfected cells further confirmed the presence of CD63 within the chlamydial inclusion. Labeling of cryo sections of *Chlamydia*-infected cells with anti-HA antibody revealed the presence of CD63-HA in multivesicular organelles adjacent to the chlamydial inclusion (Fig. 4, right panel). In addition, immunolabeling clearly showed CD63-HA within the lumen of the inclusion and often associated with vesicles (Fig. 4, right lower panel insert), further implicating a direct fusion of MVBs with the chlamydial inclusion.

**Internalized exogenous anti-CD63 antibody traffics to the chlamydial inclusion and perturbs intracellular chlamydial development**

MVBs lie within the endocytic pathway providing a direct route for exogenously added tracers. This route can be exploited by following the fate of endocytosed antibodies that will selectively accumulate after binding to an antigen.

![Fig. 2. CD63 accumulated within the chlamydial inclusion. HEp-2 cells were infected with *C. trachomatis* E for 48 hours and analyzed by indirect immunofluorescence. The cytosolic face of the inclusion membrane was immunolabeled with anti-IncA antibody (anti-rabbit Alexa Fluor 488, see Results for details) and infected cells were then labeled with anti-CD63 antibody (anti-mouse Alexa Fluor 594). TOPRO-3-labeling was used to identify both intracellular bacteria and the host cell nuclei. (Upper panels) A 0.5-μm-thick optical section shows labeled CD63 localized to TOPRO-3-positive intracellular bacteria within the confines of the IncA-positive inclusion membrane. The white line in the merged image indicates the position of orthogonal analysis. Bar, 20 μm. (Lower panels) An orthogonal view of the infected cell (height of combined Z-sections 9.2 μm.) confirmed CD63 localization to TOPRO-3-labeled intracellular bacteria within the depths of the chlamydial inclusion as defined by IncA-labeling.](image1)

![Fig. 3. Immunoelectron microscopy analysis of CD63 association with the chlamydial inclusion. HEp-2 cells were infected with *C. trachomatis* E for 48 hours and analyzed by cryo immunoelectron microscopy. Infected cells immunolabeled with anti-CD63 antibody (anti-mouse-18 nm colloidal gold) revealed CD63 within the chlamydial inclusion and in compartments adjacent to the inclusion that morphologically resembled MVBs (arrows). CD63-labeling was also evident along the inclusion membrane (upper panel, arrowhead; enlarged in right panel) and in small vesicles within the inclusion lumen (lower panel arrowheads; enlarged in right panel). C, *Chlamydia*; m, mitochondria. Bar, 0.5 μm. For quantification of anti-CD63-labeling, the density of colloidal-gold particles was determined per cross-sectional area of the compartments indicated (particles/μm²). The density was also expressed as fold over background, based on the average background-labeling in mitochondria and nuclei.](image2)
Chlamydia intersects multivesicular bodies

Previous studies have demonstrated the accumulation of internalized anti-CD63 antibody in late endocytic compartments, with subsequent trafficking to secretory compartments (Kobayashi et al., 2000). Exogenously internalized anti-LBPA antibody traffics to late endocytic compartments, blocking subsequent transport (Kobayashi et al., 1999). In this study, the same approach was used to follow

**Fig. 4.** CD63-HA localizes to the chlamydial inclusion in transfected cells. HEp-2 cells were transfected with CD63-HA for 24 hours, then infected with *C. trachomatis* E for 48 hours and analyzed by indirect immunofluorescence and cryo immunoelectron microscopy. (Left panels) 0.5-μm-thick optical sections of infected cells immunolabeled with anti-HA antibody (anti-rabbit Alexa Fluor 488). TOPRO-3-labeling of the equivalent confocal slice was used to reveal intracellular bacteria and host-cell nuclei. Anti-HA antibody localized CD63-HA to TOPRO-3-positive intracellular bacteria in transfected cells (arrow) with no anti-HA antibody labeling associated with inclusions of adjacent untransfected cells (arrowhead). Bar, 20 μm. (Right panels) Transfected cells immunolabeled with anti-HA antibody (anti-rabbit-18 nm colloidal gold) revealed CD63-HA within the chlamydial inclusion and in compartments adjacent to the inclusion that morphologically resembled MVBs (arrows). Labeled CD63 was also evident in small vesicles within the inclusion lumen (lower panel, arrowhead; enlarged in insert). *C. Chlamydia*. The lack of label in mitochondria (m) served as an internal control for background labeling. Bar, 0.5 μm.

**Fig. 5.** Internalized exogenous anti-CD63 antibody localized to the chlamydial inclusion. HEp-2 cells were infected with *C. trachomatis* E and cultured in the presence of exogenous anti-CD63 and anti-LAMP-1 antibodies (left panel), or anti-CD63 antibody and FITC-labeled dextran (right panel) from 24 hours to 48 hours post infection. Infected cells were then fixed and immunolabeled with anti-mouse Alexa Fluor 594 and anti-rabbit Alexa Fluor 488 (left panel), or anti-mouse Alexa Fluor 594 only (right panel). TOPRO-3-labeling was used to identify intracellular bacteria and the host-cell nuclei. 0.5-μm-thick optical sections revealed that exogenously added anti-CD63 antibody trafficked to and accumulated in TOPRO-3-positive chlamydial inclusions (arrowheads). Concurrent addition of exogenous anti-LAMP-1 antibody and dextran-FITC to the culture medium resulted in their uptake into endocytic compartments of the host cell, but exclusion from the chlamydial inclusion. The arrow in the merged image indicates the position of the profile line used for analysis of intensity distribution. Bar, 20 μm. (Lower panels) Intensity distribution profiles confirmed the presence of anti-CD63 antibody (red line) within the TOPRO-3-positive inclusion (blue line). Anti-LAMP-1 antibody (green line, upper profile) and dextran-FITC (green line, lower profile) had high-intensity values peripheral to the inclusion and host-cell nuclei, but displayed background-intensity levels at the site of the chlamydial inclusion.
the fate of anti-CD63 antibodies in the *Chlamydia*-infected cells. Exogenously internalized anti-CD63 antibody accumulated in perinuclear vesicular compartments and in the chlamydial inclusion (Fig. 5). When anti-CD63 was added simultaneously with anti-LAMP-1 antibody or the fluid-phase marker dextran-FITC, anti-CD63 localized to the chlamydial inclusion, while anti-LAMP-1 and dextran were clearly excluded (Fig. 5). The distribution of these markers was shown quantitatively by generating a profile of the intensity distribution of each constituent along a line traversing the chlamydial inclusion (Fig. 5, lower panels). Anti-CD63 antibody clearly colocalized with TOPRO-3-labeled chlamydiae within the profile of the inclusion, while being excluded from the TOPRO-3-labeled host cell nuclei (Fig. 5, lower panels). Anti-LAMP-1 antibody and dextran-FITC displayed high-intensity values peripherally to the inclusion and the host cell nuclei but were clearly excluded from the inclusion itself, as revealed by background-intensity levels across this region of the profile (Fig. 5, lower panels). Orthogonal analysis of the entire Z-height of infected cells confirmed the presence of exogenous anti-CD63 within the inclusion (data not shown).

The effect of direct delivery of internalized anti-CD63 antibody on intracellular bacterial development was analyzed. *Chlamydia*-infected cells, in the absence of exogenous antibody, developed large inclusions containing primarily electron-dense elementary bodies at 48 hours post infection (Fig. 6, upper left panels). In the presence of exogenous anti-CD63 antibody, smaller, less dense inclusions were evidenced by fluorescence using TOPRO-3 labeling. Electron microscopy revealed that these inclusions contained predominantly RBs (0.6-1 μm), indicating an interruption in normal chlamydial maturation (Fig. 6, lower panels). In addition, the treatment of cells with exogenous antibody to CD63 resulted in a marked reduction in infectious progeny when analyzed at 48 hours post infection (Fig. 6, right panel). Equivalent concentrations of anti-LAMP-1 antibodies served as a negative control, with no effect on inclusion development or infectivity (data not shown). The inhibition of chlamydial development by exogenous CD63-specific antibody implies a role for the host protein CD63 or CD63-positive compartments in inclusion biogenesis and maturation.

**Inhibitors of MVB biogenesis disrupt chlamydial growth and development**

Inhibitors of phosphoinositide 3-kinase (PI 3-kinase) disrupt the biogenesis of MVBs, resulting in swollen organelles that are impaired in membrane trafficking out of this compartment (Fernandez-Borja et al., 1999). The pharmacological agent U18666A inhibits cholesterol transport from late endocytic compartments (Liscum and Faust, 1992) and alters the trafficking of MVB-associated membrane proteins (Higgins et al., 2001; Kobayashi et al., 1999; Kobayashi et al., 2000). In the presence of U18666A and the PI 3-kinase inhibitors 3-methyladenine and LY-294002, maturation of the chlamydial inclusion was disrupted with a marked reduction in inclusion size and yield of infectious progeny (Fig. 7). Electron microscopy of the intracellular bacteria revealed primarily EBs in the untreated control cells, but predominantly RBs delayed in re-differentiation to infectious EBs in infected cells treated with exogeneous anti-CD63. Arrowheads indicate representative EBs.

**Fig. 6.** Internalized exogenous anti-CD63 antibody disrupted intracellular chlamydial development. HEp-2 cells were infected with *C. trachomatis* E and cultured in the presence of exogenous anti-CD63 from 24 hours to 48 hours post infection. (Left panels) TOPRO-3-labeling was used to analyze morphological changes in inclusion development. TOPRO-3 labeled intracellular bacteria and the host-cell nuclei, with the chlamydial inclusions colorized blue (Photoshop 7.0) in obtained black and white images. 0.5-μm-thick optical sections revealed that the addition of exogenous anti-CD63 antibody resulted in reduced inclusion-size relative to untreated controls. (Center panels) Electron micrographs of the intracellular bacteria revealed primarily EBs in the untreated control cells, but predominantly RBs delayed in re-differentiation to infectious EBs in infected cells treated with exogeneous anti-CD63. Arrowheads indicate representative EBs. (Right panel) A reduction in recovery of infectious *Chlamydia* was observed in cells cultured in the presence of anti-CD63 antibody. Data are presented as the mean infectious-forming units of triplicate cultures ± s.e.m.
Chlamydia intersects multivesicular bodies

Golgi complex to the inclusion and incorporation into the cell wall of chlamydiae (Hackstadt et al., 1995). Because MVBs are pivotal in intracellular sphingolipid transport, the role of these compartments in sphingomyelin delivery to the chlamydial inclusion was analyzed. HEp-2 cells, infected with C. trachomatis for 18 or 48 hours, incorporated the fluorescent probe into both the Golgi complex and the chlamydial inclusion (Fig. 8, left panels). Culturing in the presence of the MVB inhibitors 3-methyladenine and U18666A for 48 hours resulted in smaller inclusions, which failed to incorporate the fluorescent probe (Fig. 8, right panels). However, these cells displayed intense accumulation of the fluorescent probe in swollen perinuclear CD63-positive compartments. The disruption of sphingomyelin transport to the chlamydial inclusion by inhibition of trafficking from MVBs, identifies CD63-positive MVBs within a novel transport pathway of lipid delivery to intracellular chlamydiae.

Discussion

MVBs are dynamic, heterogeneous, intermediate endocytic organelles important in segregating host-cell protein and lipid constituents (for reviews, see Denzer et al., 2000; Piper and Luzio, 2001). Two important features of MVBs make these compartments compelling candidates for an interaction with the chlamydial inclusion. First, MVBs are central to the intracellular trafficking of sphingolipids and cholesterol, two host-derived constituents acquired by the chlamydial inclusion (Carabeo et al., 2002; Hackstadt et al., 1995; Wylie et al., 1997). Second, MVBs are an active component of the exocytic pathway in various cell types (for reviews, see Denzer et al., 2000; Stoovogel et al., 2002), the only intracellular trafficking pathway that has been shown to intersect the chlamydial inclusion (Hackstadt et al., 1996; Hackstadt et al., 1995). The present study demonstrates a dynamic interaction between MVB trafficking pathways and the chlamydial inclusion, essential for intracellular bacterial development. Rerouting of MVB-derived exocytic compartments to the chlamydial inclusion might provide an essential source of biosynthetic macromolecules vital to intracellular chlamydial development.

A hallmark of MVBs is the presence of lipid-enriched internal membranes, which have been proposed to be important in protein sorting and transport of cholesterol and sphingolipids (Denzer et al., 2000; Kobayashi et al., 1999; Piper and Luzio, 2001). Constituents of MVBs include LBPA, a lipid abundant in internal membranes of MVBs (Kobayashi et al., 1998), which is thought to play a role in sphingolipid hydrolysis and regulation of cholesterol transport (Kobayashi et al., 1999). The tetraspanin protein CD63 is present in internal membranes and at the limiting membrane of MVBs (Escola et al., 1998; Kobayashi et al., 2002). MLN64 is a cholesterol-binding protein restricted to the limiting membrane of CD63- and LBPA-positive late endosomes (Alpy et al., 2001).

Using a cell-biological approach, MVB markers, in addition to tracers and inhibitors, were used to analyze the interaction of these dynamic compartments with the chlamydial inclusion. This study demonstrates that CD63, MLN64 and LBPA localize to the chlamydial inclusion, as shown by indirect immunofluorescence (Fig. 1). Because the chlamydial inclusion has been proposed to intersect an exocytic pathway (Hackstadt et al., 1996; Hackstadt et al., 1995), CD63 – a host protein associated with both MVBs and the exocytic pathway (Kobayashi et al., 2000) – was used as a surrogate marker for additional characterization. Electron microscopy (Fig. 3) and orthogonal analyses obtained from confocal 3D-reconstruction (Fig. 2) substantiated the accumulation of CD63 within the
chlamydial inclusion. Localization of HA-tagged CD63 in infected cells confirmed the distribution of CD63 to the chlamydial inclusion (Fig. 4).

The potential importance of MVB-associated CD63 was further explored in experiments based on internalization of exogenous CD63-specific antibodies. Previous studies have shown that endosomal CD63 protein is accessible to antibodies internalized by fluid-phase endocytosis. Exogenously added CD63-specific antibodies partition preferentially to and accumulate in LBPA-positive late endocytic compartments, with subsequent transport to secretory compartments (Kobayashi et al., 2000). Internalized anti-LBPA antibodies traffic to late endocytic compartments, resulting in the impairment of trafficking out of these compartments, as characterized by a massive accumulation of cholesterol (Kobayashi et al., 1999). Here, this approach was used to determine the fate and potential effect of anti-CD63 antibodies on *Chlamydia*-infected cells. Anti-CD63 antibodies that trafficked to and were incorporated into the chlamydial inclusion demonstrate a direct interaction between a CD63-positive compartment and intracellular chlamydiae (Fig. 5). The exclusion of anti-LAMP-1 antibody and dextran from the inclusion indicated that anti-CD63 acquisition was the result of selective vesicular-sorting and -transport from the endocytic pathway or subsequent MVB-related compartments. The disruption in chlamydial development by CD63-specific antibody further implies that this MVB constituent is important for inclusion-biogenesis and -maturation (Fig. 6).

Disruption of MVBs leads to detrimental accumulation of cholesterol and sphingolipids as shown experimentally and in disease states (Kobayashi et al., 1999; Vanier and Millat, 2003). Inhibitors of PI 3-kinases, key regulators of protein sorting, disrupt the biogenesis of MVBs, which results in swollen vesicles that are impaired in membrane trafficking out of these compartments (Fernandez-Borja et al., 1999; Kundra and Kornfeld, 1998). The pharmacological agent U18666A inhibits cholesterol transport from late endocytic compartments (Liscum and Faust, 1992) and alters the trafficking of CD63 (Higgins et al., 2001; Kobayashi et al., 1999; Kobayashi et al., 2000). Because inhibitors can pleiotropically effect cellular function, their potential effect on MVBs was initially confirmed in the HEp-2 cells used in this study. Culturing in the presence of defined concentrations of 3-methyladenine, LY-294002 or U18666A resulted in swollen CD63-positive compartments but was excluded from TOPRO-3-positive chlamydial inclusions. Arrowheads indicate representative swollen NBD- and CD63-positive compartments. Arrows indicate chlamydial inclusions. Insert in lower left panel shows NBD-labeled *Chlamydia* (untreated, 18 hours post infection) in inclusions of equivalent size to 3-methyladenine- and U18666A-treated inclusions (48 hours post infection) that lacked NBD incorporation. Bar, 20 μm.

![Fig. 8. Inhibitors of MVBs disrupted trafficking of sphingomyelin to the chlamydial inclusion. HEp-2 cells infected with *C. trachomatis* were cultured in the absence or presence of 5 mM 3-methyladenine or 10 μM U18666A. Post infection, at the times indicated, cells were labeled with NBD-ceramide and analyzed 1 hour after back-exchange. Infected cells were fixed, immunolabeled with anti-golgin-97 or anti-CD63 antibody (anti-mouse Alexa Fluor 594), and 0.5-μm-thick optical sections were acquired by confocal microscopy. (Left panels) Control cells, 18 and 48 hours post infection. The NBD probe was incorporated in the Golgi complex and TOPRO-3-positive chlamydial inclusions. (Right panels) Treatment of infected cells with 3-methyladenine or U18666A resulted in the incorporation of fluorescent probe into swollen CD63-positive compartments but was excluded from TOPRO-3-positive chlamydial inclusions. Arrowheads indicate representative swollen NBD- and CD63-positive compartments. Arrows indicate chlamydial inclusions. Insert in lower left panel shows NBD-labeled *Chlamydia* (untreated, 18 hours post infection) in inclusions of equivalent size to 3-methyladenine- and U18666A-treated inclusions (48 hours post infection) that lacked NBD incorporation. Bar, 20 μm.](Journal_of_Cell_Science_119_2)
the effects of inhibitors of autophagy on chlamydial growth are potentially a result of downstream effects, subsequent to autophagosome-MVB interception.

Fundamental questions, regarding intracellular mediators and signals essential for inclusion formation, maturation and sequestration, remain unknown. In contrast to other intracellular pathogens that reside in membrane-bound vacuoles, an association between chlamydiae and endocytic pathways of its host cell has not been identified. However, common among all Chlamydiae species is the fusigencity of the inclusion with an apparent exocytic pathway (Hackett et al., 1996; Rockey et al., 1996; Wolf and Hackstadt, 2001). Fluorescent analogs of ceramide demonstrate the direct trafficking of an exogenous substrate to the chlamydial inclusion. Ceramide is synthesized in the endoplasmic reticulum and enzymatically converted to sphingomyelin or glucosylceramide at the cis- or medial-Golgi before being transported to the plasma membrane (Furterman et al., 1990; Lipsky and Pagano, 1985a). Labeling infected cells with NBD-ceramide led to the diversion of fluorescent label to the chlamydial inclusion and the incorporation of NBD-sphingomyelin into the chlamydial cell wall (Hackett et al., 1995). The acquisition of endogenously synthesized sphingomyelin was disrupted by brefeldin A, an inhibitor of anterograde transport from the Golgi complex. These studies were expanded to analyze cholesterol, a lipid identified in purified EBs and the chlamydial inclusion membrane (Caraboe et al., 2003; Wylie et al., 1997). Acquisition of de novo-synthesized and low-density lipoprotein-derived cholesterol occurs through this brefeldin-A-sensitive, Golgi-dependent pathway (Caraboe et al., 2003). However, brefeldin A inhibition of sphingomyelin and cholesterol delivery failed to interrupt bacterial multiplication (Caraboe et al., 2003; Hackstadt et al., 1996), suggesting that, in addition to direct Golgi transport, chlamydiae possess an alternative pathway for lipid acquisition.

The phospholipid composition of purified Chlamydia trachomatis includes both typical prokaryotic lipids (phosphatidylserine, phosphatidylethanolamine and phosphatidylglycerol) and lipids generally associated with eukaryotes (sphinogmyelin, cholesterol, phosphatidylcholine, and phosphatidylinositol) (Newhall, 1988; Wylie et al., 1997). In addition to de novo phospholipid synthesis, studies utilizing radiolabeled isoleucine demonstrated that host cell phospholipids are trafficked to and modified by chlamydiae, a process that occurs independently of Golgi transport (Wylie et al., 1997). Eukaryotic sphingolipids are required for intracellular bacterial growth and production of infectious progeny (Van Ooij et al., 2000), however, the transfer mechanism of host-derived lipids has not been identified. In the present study, the effect of MVB disruption on the direct delivery of sphingomyelin to the chlamydial inclusion was analyzed using the NBD-ceramide as previously described by Hackstadt (Hackett et al., 1995). Loading cells with the fluorescent probe resulted in the labeling of the Golgi complex and also of vesicles that occupied a larger perinuclear area beyond the Golgi that were subsequently identified as CD63-positive compartments. Culturing infected cells in the presence of 3-methyladenine and U18666A, the pharmacological inhibitors that prevent trafficking out of MVBS, resulted in loaded probe accumulating in enlarged CD63-positive MVBS.

This accumulation was accompanied by the inhibition of delivery of synthesized sphingomyelin to the chlamydial inclusion (Fig. 8). These observations indicate that host lipids are trafficked to the chlamydial inclusion by a pathway that intercepts MVBS. Disruption of this pathway blocks both delivery and bacterial growth, hence, trafficking from MVBS is essential to productive intracellular development of the chlamydial inclusion.

Intersecting the endosome-lysosome- (Van Deurs et al., 1993), autophagy- (Luceq and Walker, 1997) and exocytic-pathways (Denzer et al., 2000; Stoorvogel et al., 2002), MVBS are ideally positioned to interact with the chlamydial inclusion. In addition, the functional attributes of MVBS make these and related organelles an intriguing source of biosynthetic factors that might be essential for inclusion biogenesis and maturation. This study demonstrates a dynamic interaction between MVBS-derived constituents and the chlamydial inclusion. It is not clear whether a direct interaction exists, or whether MVB-related or downstream compartments intersect the inclusion.

Identification and characterization of chlamydial inclusion-flux pathways and corresponding requisite host factors, will have important implications on the general biology of Chlamydia and the potential development of therapeutic intervention targeting nutrient-acquisition pathways essential for Chlamydia propagation.

Materials and Methods

Reagents

HSCB, a mouse monoclonal antibody against human CD63, and HA3A, a mouse monoclonal antibody against human LAMP-1, were developed by Thomas August and James E. K. Hildreth (John Hopkins University School of Medicine, Baltimore, MD) and obtained from the Developmental Studies Hybridoma Bank/NICHD (University of Iowa, Iowa City, IA). MX-49,259,5, mouse monoclonal antibody against human CD63, and H-228, a rabbit polyclonal antibody against human CD63, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PA1-562, a rabbit antibody against recombinant rat MLN64 protein, was purchased from Affinity Bioreagents (Golden, CO). The mouse monoclonal antibody 6C4, generated to lysobisphosphatidic acid (LBPA), was kindly provided by Toshihide Kobayashi and Jean Gruenberg (University of Geneva, Geneva, Switzerland). AS789, a mouse monoclonal antibody against the chlamydial heat shock protein-60 (hsp60), was generously provided by Richard Morrison (University of Alabama Birmingham, AL). The rabbit polyclonal antibody against IncA was kindly provided by Ted Hackstadt (Rocky Mountain Laboratories, NIH, NIAID, Hamilton, MT). Anti-golgin-97 antibody (mouse monoclonal CD143), dextran-FITC (10,000 kDa), TOPRO-3, and secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594 were purchased from Molecular Probes (Eugene, OR). 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)hexanoyl)sphingosine (NBD-C6-ceramide) was also obtained from Molecular Probes. Rabbit anti-HA antibody (H6908), 3-methyladenine, LY-294002 hydrochloride, and 3-beta-(2-diethylaminoethoxy)-androstenone HCl (U18666A) were obtained from Sigma (St Louis, MO).

Cell culture and propagation of chlamydiae

C. trachomatis serovar E (obtained from Harlan Caldwell, Rocky Mountain Laboratories, NIH, NIAID) was propagated in HEP-2 cells (ATCC, Manassas, VA) and elementary bodies (EBs) were purified by Renografin gradient-centrifugation as previously described (Caldwell et al., 1981). HEP-2 cells were maintained at 37°C with 5.5% CO2 in Iscove's MDM medium supplemented with 12.5 mM HEPES, 10% (vol/vol) FBS, and 10 μg/ml gentamicin. HEP-2 cells were infected by incubating monolayers with Chlamydia EBs at a multiplicity of infection of 0.5 for 1 hour at 37°C on a platform rocker. Cells were washed twice with PBS and incubated in Iscove's medium for the times indicated.

Plasmids and transfection

Full length cDNA for CD63 (Gentbank accession no. BT007073) was obtained by PCR amplification from human cDNA with KlenTaq LA (Sigma). CD63 was cloned as a C-terminal HA9-epitope fusion in the pDNA 3.1/V5 His vector (Invitrogen, Carlsbad, CA). HEP-2 cells at subconfluence were transfected with plasmid using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) as described in the manufacturer's protocol. Cells transfected for 24 hours were then infected with Chlamydia as described above.
Immunofluorescence and confocal microscopy

HEp-2 cells were grown on glass coverslips and infected with Chlamydia as described above. For immunofluorescence analysis, cells were fixed for 20 minutes at room temperature in 1% formaldehyde (37% stock) containing 10-15% methanol, Fisher Scientific, Hanover Park, IL). Fixation with this concentration of formaldehyde and methanol, commonly used in the host cell cultures, did not permeabilize intracellular vesicular membranes or the chlamydial inclusion membrane. To subsequently permeabilize these intracellular membranes, cells were treated for 1 minute with 1 mg/ml Zwittergent®. After permeabilization, cells were incubated with the indicated primary antibodies used in these studies. Incubating with 0.2 μM TOMO-4917950 and the same concentration of rabbit anti-LAMP-1 antibody (1:220) or 100 μg/ml dextran-FITC. At 48 hours post infection, cells were harvested for analysis of antibody uptake by indirect immunofluorescence with anti-mouse Alexa Fluor 594 and anti-rabbit Alexa Fluor 488 antibodies. Effects of exogenous antibody on inclusion development and morphology were determined by infectivity assays and TOPRO-3 labeling, respectively.

Electron microscopy

For analysis of CD63 localization at the ultrastructural level, 48-hour Chlamydia-infected cells were prepared for cryo immuno electron microscopy. Infected cells were fixed in 4% paraformaldehyde/0.1% glutaraldehyde (Polysciences Inc.) in 100 mM phosphate buffer (Griffiths, 1993). After fixation, cells were incubated with 2 μg/ml goat serum/PBS, cells were incubated with the indicated primary antibodies for 20 minutes. Cells were washed three times in blocking buffer and subsequently incubated with the indicated secondary antibodies conjugated to 10 nm gold. Samples were then rinsed extensively in dH2O before en-bloc staining with 1% aqueous uranyl acetate. Following a 30 minute staining, sections were washed in PIPES buffer followed by an extensive water rinse and stained with 1% uranyl acetate/1.6% lead citrate and viewed by transmission electron microscopy.

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References

Al-Younes, H. M., Brinkmann, V. and Meyer, T. F. (2004). Interaction of Chlamydia trachomatis serovar L2 with the host autophagic pathway. Infect. Immun. 72, 4751-4762.

Al-Younes, H. M., Brinkmann, V. and Meyer, T. F. (2004). Interaction of Chlamydia trachomatis serovar L2 with the host autophagic pathway. Infect. Immun. 72, 4751-4762.

Al-Younes, H. M., Brinkmann, V. and Meyer, T. F. (2004). Interaction of Chlamydia trachomatis serovar L2 with the host autophagic pathway. Infect. Immun. 72, 4751-4762.

Al-Younes, H. M., Brinkmann, V. and Meyer, T. F. (2004). Interaction of Chlamydia trachomatis serovar L2 with the host autophagic pathway. Infect. Immun. 72, 4751-4762.
Chlamydia intersects multivesicular bodies

Higgins, M. E., Davies, J. P., Chen, F. W. and Ioannou, Y. A. (2001). Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. Mol. Genet. Metabol. 68, 1-13.

Kallen, C. B., Billheimer, J. T., Summers, S. A., Stayrook, S. E., Lewis, M. and Strauss, J. F., III (1998). Stereoidogenic acute regulatory protein (StAR) is a sterol transfer protein. J. Biol. Chem. 273, 26285-26288.

Klionsky, D. J. and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. Science 290, 1717-1721.

Kobayashi, T., Stang, E., Fang, K. S., de Moorloose, P., Parton, R. G. and Gruenberg, J. (1998). A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature 392, 193-197.

Kobayashi, T., Beuchat, M. H., Lindsay, M., Frias, S., Palmiter, R. D., Sakuraba, H., Parton, R. G. and Gruenberg, J. (1999). Late endosomal membranes rich in lysothiposphatidic acid regulate cholesterol transport. Nat. Cell Biol. 1, 113-118.

Kobayashi, T., Vischer, U. M., Rosnoblet, C., Lebrand, C., Lindsay, M., Parton, R. G., Kruthoff, E. K. and Gruenberg, J. (2000). The tetrascipin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. Mol. Biol. Cell 11, 1829-1843.

Kobayashi, T., Beuchat, M. H., Chevallier, J., Makino, A., Mayran, N., Escola, J. M., Lebranch, C., Cosson, P. and Gruenberg, J. (2002). Separation and characterization of late endosomal membrane domains. J. Biol. Chem. 277, 32157-32164.

Kolter, T., Doering, T., Wilkening, G., Werth, N. and Sandhoff, K. (1999). Recent advances in the biochemistry of glycocephalosphingolipid metabolism. Biochem. Soc. Trans. 27, 409-415.

Kundra, R. and Kornfeld, S. (1998). Wortmannin retards the movement of the mannose 6-phosphate/insulin-like growth factor II receptor and its ligand out of endosomes. J. Biol. Chem. 273, 3848-3853.

Lipsky, N. G. and Pagano, R. E. (1983). Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. Proc. Natl. Acad. Sci. USA 80, 2608-2612.

Lipsky, N. G. and Pagano, R. E. (1985a). Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucocerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. J. Cell Biol. 100, 27-34.

Lipsky, N. G. and Pagano, R. E. (1985b). A vital stain for the Golgi apparatus. Science 228, 745-747.

Liscum, L. and Faust, J. R. (1992). The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-3H]-cholesteryl[diethyamino]ethoxy]-androst-5-en-17-one. J. Biol. Chem. 264, 11796-11806.

Lucey, J. and Walker, D. (1997). Evidence for fusion between multilamellar endosomes and autophagosomes in HeLa cells. Eur. J. Cell Biol. 72, 307-313.

McClarty, G. (1994). Chlamydiaceae and the biochemistry of intracellular parasitism. Trends Microbiol. 2, 157-164.

Newhall, W. J. (1988). Macromolecular and Antigenic Composition of Chlamydiaceae. Boca Raton: CRC Press.

Pan, B.-T., Teng, K., Wu, C., Adam, M. and Johnstone, R. M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J. Cell Biol. 101, 942-948.

Piper, R. C. and Luzio, J. P. (2001). Late endosomes: sorting and partitioning in multivesicular bodies. Traffic 2, 612-621.

Rockey, D. D., Fischer, E. R. and Hackstadt, T. (1996). Temporal analysis of the developing Chlamydia psittaci inclusion by use of fluorescence and electron microscopy. Infect. Immun. 64, 4269-4278.

Schachter, J. (1988). Overview of Human Disease. Boca Raton: CRC Press.

Scidmore, M. A., Fischer, E. R. and Hackstadt, T. (2003). Restricted fusion of Chlamydia trachomatis vesicles with endocytic compartments during the initial stages of infection. Infect. Immun. 71, 973-984.

Seglen, P. O. and Gordon, P. B. (1982). 3-Methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. USA 79, 1889-1892.

Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J. and Raposo, G. (2002). The biogenesis and functions of exosomes. Traffic 3, 321-330.

Tarska, T., Ward, D. M., Ajoka, R. S., Wyrick, P. B., Davis-Kaplan, S. R., Davis, C. H. and Kaplan, J. (1996). The late chlamydial inclusion membrane is not derived from the endocytic pathway and is relatively deficient in host proteins. Infect. Immun. 64, 3713-3727.

Van Deurs, B., Holm, P. K., Kayser, L., Sandvig, K. and Hansen, S. H. (1993). Multivesicular bodies in Hep-2 cells are maturing endosomes. Eur. J. Cell Biol. 61, 208-224.

Van Ooij, C., Kalman, L., Van Ijzendoorn, S., Nishijima, M., Hanada, K., Mostov, K. and Engel, J. N. (2000). Host cell-derived sphingolipids are required for the intracellular growth of Chlamydia trachomatis. Cell. Microbiol. 2, 627-637.

Vanier, M. T. and Millat, G. (2003). Niemann-Pick disease type C. Clin. Genet. 64, 269-281.

Vidal, M., Mangeat, P. and Hoeckstra, D. (1997). Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. J. Cell Sci. 110, 1867-1877.

Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., K. and Engel, J. N. (2000). Host cell-derived sphingolipids are required for the intracellular growth of Chlamydia trachomatis. Cell. Microbiol. 2, 627-637.

Wolin, C. H. and Kaplan, J. (1996). The late chlamydial inclusion membrane is not derived from the endocytic pathway and is relatively deficient in host proteins. Infect. Immun. 64, 3713-3727.

Vidul, M., Mangeat, P. and Hoeckstra, D. (1997). Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. J. Cell Sci. 110, 1867-1877.

Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., K. and Engel, J. N. (2000). Host cell-derived sphingolipids are required for the intracellular growth of Chlamydia trachomatis. Cell. Microbiol. 2, 627-637.

Wolf, K. and Hackstadt, T. (2001). Sphingomyelin trafficking in Chlamydia psittaci infected cells. Cell. Microbiol. 3, 145-152.

Wylde, J. L., Hatch, G. M. and McClarty, G. (1997). Host cell sphingolipids are trafficked to and then modified by Chlamydia trachomatis. J. Bacteriol. 179, 7233-7242.