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Current Opinion in Microbiology

Acquisition of nutrients by *Chlamydiae*: unique challenges of living in an intracellular compartment

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The *Chlamydiae* are obligate intracellular pathogens that replicate within a membrane-bound vacuole, termed the 'inclusion'. From this compartment, bacteria acquire essential nutrients by selectively redirecting transport vesicles and hijacking intracellular organelles. Rerouting is achieved by several mechanisms including proteolysis-mediated fragmentation of the Golgi apparatus, recruitment of Rab GTPases and SNAREs, and translocation of cytoplasmic organelles into the inclusion lumen. Given *Chlamydiae's* extended coevolution with eukaryotic cells, it is likely that co-option of multiple cellular pathways is a strategy to provide redundancy in the acquisition of essential nutrients from the host and has contributed to the success of these highly adapted pathogens.

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Current Opinion in Microbiology 2010, **13**:4–10

This review comes from a themed issue on
Host–microbe interaction: bacteria
Edited by C Erec Stebbins and David O'Callaghan

Available online 16th December 2009

1369-5274/$ – see front matter
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DOI 10.1016/j.mib.2009.11.002

Introduction

The *Chlamydiaceae* comprise a distinct family of closely related, obligate intracellular bacteria that diverged early in evolution and parasitize a wide range of hosts [1]. The human pathogens are widely distributed and include *Chlamydia trachomatis* (leading cause of genital infections and infectious blindness), *C. pneumoniae* (common cause of respiratory tract infections and community-acquired pneumonia), and *C. psittaci* (a zoonotic pathogen that can cause severe pneumonia in humans) [2–6].

All *Chlamydiaceae* undergo a biphasic developmental cycle involving the infectious and environmentally resistant form, or elementary body (EB), and the replicative, non-infectious form or reticulate body (RB). After entering their target eukaryotic cells, EBs differentiate into RBs, and replicate within a membrane-bound parasitophorous vacuole, termed the 'inclusion'. Finally, RBs differentiate back into EBs, which are released to the extracellular medium to infect neighboring cells [7].

Chlamydia species have undergone massive genome condensation and are lacking in several biosynthetic pathways [8–12], suggesting that they have acquired compensatory mechanisms to allow the import and incorporation of nucleotides, amino acids, lipids, and other nutrients from the host cell [13–18]. Though it is a crucial step in their pathogenesis, the molecular mechanisms used by *Chlamydia* species to acquire nutrients are poorly characterized largely because of the lack of a system for mutational analysis and cell-free cultivation methods.

In this review, we discuss our understanding of *Chlamydia* nutrient acquisition pathways, based on information from genome sequencing and new cell biological observations detailing the extensive interactions between the inclusion and host organelles.

Nutrient uptake systems

The current estimation of *Chlamydia*'s metabolic capacity is founded on hints from genomic sequencing. As for any living organism, *Chlamydia* needs pyrimidine and purine nucleotides for energy transduction and nucleic acid biosynthesis [19,20], but is unable to synthesize them *de novo*. *Chlamydia* encodes enzymes that can generate ATP via substrate level phosphorylation, and CTP from UTP through a CTP synthetase; however, these organisms still require host cell-derived ATP, GTP, and UTP [10,12,14,15,21,22]. The bacteria import these nucleotides by an unusual transport system that is found only in a small number of obligate intracellular bacteria and plant plastids [23–25]. *C. trachomatis* has at least two nucleotide transport proteins (Npts): Npt1 and Npt2 [26]. Npt1 mediates the import of ATP from the host cell into the bacteria coupled with the export of ADP. Npt2 catalyzes the uptake of GTP, UTP, CTP, and ATP, in a proton-dependent manner [26]. These transport systems are found in several *Chlamydia* species, *Chlamydia*-related amoeba symbionts, and *Rickettsia* [8,12,26,27•,28,29]. *Chlamydia* species differ in their ability to metabolize nucleotides. For instance, *C. muridarum* harbors *guaAB-add* and *upp* genes whose predicted products enable ATP to GTP conversion and the

uracil-phosphoribosyl-transferase-mediated biosynthesis of UTP from uracil, respectively. *C. pneumoniae* encodes *udk*, which may mediate uridine-kinase-dependent synthesis of UTP from uracil, a UMP synthetase (PyrE), and a nonfunctional *guaAB-add* operon [11,12]. *C. caviae* encodes an intact *guaAB-add* operon and *pyrE* [11]. *Chlamydia* also lacks the components for NAD^+ synthesis, indicating that this essential molecule must be scavenged from the host as has been demonstrated in the amoeba symbiont *Parachlamydia* UWE25 [23].

Genomic comparisons reveal that *Chlamydia* has several incomplete amino acid biosynthesis pathways [8,10–12,30]. Not surprisingly, *Chlamydia* also contains several amino acid transporters including *aat* (neutral amino acid transporter), *xasA* (amino acid antiporter), *brnQ*-like (branched amino acid transporter), and a substantial number of ABC transporters (at least 13 in *C. trachomatis*) that are likely associated with amino acid and oligopeptide transport [8,10–12,31]. The regulation of tryptophan biosynthesis is of particular interest for *Chlamydia* infections. Interferon-gamma (IFN-γ) secreted by immune cells activates indoleamine 2,3-dioxygenase, which inhibits chlamydial replication by depleting intracellular pools of tryptophan [32]. The ability of different *Chlamydia* species to synthesize or to acquire tryptophan precursors correlates with their susceptibility to IFN-γ-mediated killing and is linked to tissue tropism [33–35]. IFN-γ inhibits the replication of *C. pneumoniae*, *C. muridarum*, and ocular strains of *C. trachomatis*, but has limited effect on genital strains that contain genes for converting scavenged indole tryptophan [33]. Indeed, the antichlamydial activity of IFN-γ in human cells can be abrogated *in vitro* by the addition of tryptophan to the culture media [35].

Although *Chlamydia* has several efficient and specialized uptake systems, these systems are confined to bacterial membranes. How nutrients cross from the host cytoplasm through the inclusion membrane, which is not permissive to the diffusion of molecules >520 Da [36], is largely unknown.

Co-option of host organelles and trafficking pathways promote delivery of nutrients to the inclusion

One potential mechanism for nutrient acquisition may involve the interception of vesicular transport intermediates. Even though the chlamydial inclusion is predominantly segregated from classical endo/lysosomal transport pathways, it can exploit membrane trafficking events [37–39,40•,41–44] and host lipases [45] to acquire lipids. The molecular basis for these events is unclear, but a subset of Rab GTPases and SNARE proteins, which regulate membrane trafficking, are recruited to the inclusion membrane [46–48,49••,50•]. Indeed, recent findings indicate that interaction with multivesicular bodies (MVB), the Golgi apparatus, mitochondria, and lipid droplets (LD), are required for optimal chlamydial replication [51,52,53••,54••,55,56•,57•] and suggest that membrane fusion events between host-derived vesicles and the inclusion may deliver nutrients to *Chlamydia*.

Interception of exocytic vesicles

Chlamydia acquires eukaryote-specific lipids [58] from the host cell. Sphingomyelin and cholesterol are delivered to the inclusion, and subsequently incorporated into chlamydial membranes [43–45,59,60]. Cholesterol and sphingomyelin transport to *C. trachomatis* are brefeldin A-sensitive and microtubule-dependent. Since brefeldin A inhibits anterograde vesicular traffic from the Golgi, Golgi-derived exocytic vesicles are likely involved in the delivery of these lipids to the bacteria. Furthermore, chlamydial protein synthesis is required for sphingomyelin uptake, suggesting that co-option of these vesicles is a bacteria-driven process [59]. The utilization of exocytic pathways is more pronounced when analyzing *Chlamydia* infection of polarized epithelial cells [40•]. In these cells sphingomyelin, but not glucosylceramide, is retained by the *Chlamydia* inclusion and EBs because of preferential interception of basolaterally trafficked exocytic vesicles. However, the bulk protein cargo normally present within Golgi-derived secretory vesicles does not appear to accumulate in the inclusion, suggesting that a subset of vesicles may be preferentially targeted. The requirement of Golgi-derived vesicles for chlamydial replication is controversial because brefeldin A does not inhibit chlamydial replication [43]. However, RNAi screens identified COPI coat proteins as important for chlamydial growth [57•,61•]. The exact role of COPI in bacterial survival, remains to be determined. It is possible that a subpopulation of brefeldin A-insensitive COPI vesicles deliver nutrients to the inclusion, or that other COPI-dependent membrane trafficking events (endosomal sorting, LD biogenesis) are required by *Chlamydia*. Recently, the GPI-anchored plasma membrane protein CD59 was found to traffic to the inclusion in a brefeldin A-insensitive manner, which suggests that *Chlamydia* also exploits Golgi-independent pathways to acquire nutrients [62].

Chlamydia-mediated reprogramming of the Golgi apparatus

The Golgi apparatus processes and sorts newly synthesized proteins and lipids to their subcellular destinations [63]. Recent findings by Heuer *et al.* detail the importance of Golgi architecture in *C. trachomatis* replication [53••]. At early stages of infection, Golgi ribbon-like structures of normal morphology are found closely associated with the chlamydial inclusion. These structures progressively fragment into ministacks during the course of infection and fragmentation correlates with the cleavage of the Golgi protein golgin-84. The processing of golgin-84 is sensitive to caspase and calpain inhibitors, but it is unclear if secreted bacterial proteases also contribute to this process. Nonetheless, disruption of golgin-84 processing with the caspase

inhibitor, Z-WEHD-FMK, impaired both bacterial replication and sphingolipid transport into the inclusion. Moreover, when Golgi fragmentation was induced by knockdown of different Golgi matrix proteins, bacterial replication was increased. These results demonstrate that Golgi fragmentation enhances chlamydial replication and is required for the efficient transport of sphingolipids into the inclusion.

Recruitment of Rab GTPases and SNARE proteins: subversion of vesicular trafficking

Rab GTPases are pivotal regulators of membrane trafficking processes and organelle identity [64] and SNARE proteins are key components of the intracellular membrane fusion machinery [65]. Scidmore and colleagues were the first to show that a subset of Rab GTPases are recruited to the inclusion [46]. Rab1, Rab4, and Rab11 are recruited to inclusions of all chlamydial species tested, while recruitment of Rab6 is species-specific for *C. trachomatis* and Rab10 associates with both *C. pneumoniae* and *C. muridarum*. In addition, in RNAi screens several Rabs have been identified as important factors required for *Chlamydia* replication and development [57•,61•]. Recent observations indicate that this is partially because of the role of Rab GTPases in remodeling the Golgi apparatus [54••]. Depletion of Rab6 or Rab11 by RNAi inhibited *Chlamydia*-induced Golgi fragmentation despite processing of golgin-84, suggesting that these Rabs act downstream of golgin-84 cleavage. Interestingly, the impairment of chlamydial replication in Rab6 or Rab11 silenced cells is reversed when Golgi fragmentation is induced through disruption of the Golgi-tethering protein p115. These findings provide additional evidence for a functional link between Golgi fragmentation and chlamydial replication.

How *Chlamydia* mediates recruitment of Rab proteins is not fully understood. A family of integral inclusion membrane proteins (Incs) are attractive candidates for bacterial factors that may facilitate interaction between Rabs and the inclusion membrane [66]. The *C. trachomatis* Inc CT229 interacts with Rab4 [47], and the *C. pneumoniae* inclusion membrane protein Cpn0585 interacts with Rab1, Rab10, and Rab11 [48]. These interactions are important for bacterial development as ectopic overexpression of Cpn0585 in *C. pneumoniae*-infected cells [48] or the microinjection of anti-CT229 in *C. trachomatis*-infected cells [47] negatively impact bacterial replication.

Like Rab GTPases, a group of SNARE proteins is targeted to the inclusion. Subtil and coworkers determined that the R-SNAREs Vamp3, Vamp7, and Vamp8 preferentially localize around the inclusion [49••]. Through bioinformatics and structural modeling, SNARE-like motifs were identified in at least three *C. trachomatis* Incs (IncA, CT813, and CT223) suggesting the potential for interaction with host SNAREs. IncA interacts with Vamp3, Vamp7, and Vamp8, and SNARE recruitment to the inclusion is reduced in *C. trachomatis* isolates lacking IncA. Furthermore, ectopically expressed CT813 interacts with Vamp7 and Vamp8 in coimmunoprecipitation assays. Importantly, the SNARE-like motif of these proteins was essential for these interactions. More recently, recombinant IncA was shown to block SNARE-mediated membrane fusion in an *in vitro* liposome fusion assay [50•]. These findings lead to the speculation that *Chlamydia* subverts host SNARE-mediated fusion events by expressing inhibitory SNARE-like proteins [50•].

Modulation of signaling pathways

Chlamydial membranes contain lipid species that are normally associated with eukaryotic membranes [67]. Using CHO cell lines deficient in various phospholipid components, the lipid composition of chlamydial membranes was found to closely mimic that of the host [67]. The bacteria modify host-derived glycerophospholipids by replacing the straight chain fatty acid at the *sn2* position with a bacteria-derived branched chain fatty acid [17]. The *sn2* deacylation of phospholipids requires phospholipase A2 (PLA2) activity. Because chlamydial genomes do not encode any obvious PLA2 homologs, it is speculated that *Chlamydia* relies on a host PLA2 [45]. In support of this, pharmacological inhibition of the Ca^{2+}-dependent cytosolic PLA2 (cPLA2) prevents *Chlamydia* uptake of host-derived phospholipids and severely limits replication [45]. Furthermore, cPLA2 is activated by the extracellular-signal-regulated map kinase (ERK1/2), which is fully activated during chlamydial infections [45]. These results suggest that *Chlamydia* manipulates ERK/cPLA2 signaling pathways to facilitate the acquisition of glycerophospholipids. However, this story is likely more complex than originally proposed, as components of these signaling pathways have not been recognized as necessary for infection by RNAi-based screens. Indeed, we have found through genetic ablation experiments that cPLA2 has an innate immune signaling function in *Chlamydia*-infected cells, and that these processes vary between host cell species (Vignola M. and Valdivia R., submitted).

Targeting of endosomal compartments

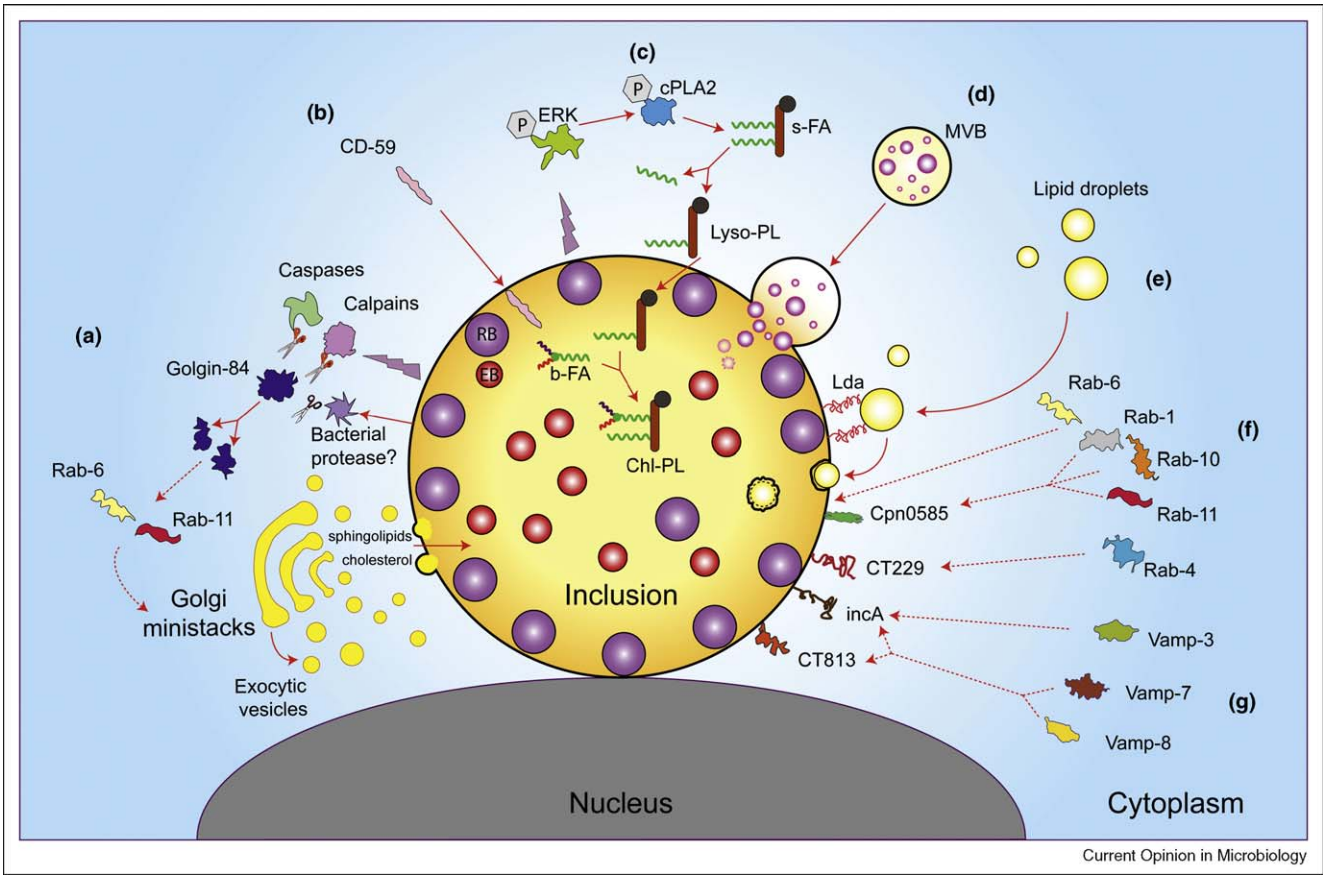
MVB are a specialized subset of late endosomes involved in the sorting of cargo proteins and lipids to lysosomes for degradation [68,69]. By immunofluorescence and immunoelectron microscopy, two different MVB markers, CD63 and MLN64, and the lipid LBPA (lysobisphosphatidic acid, highly enriched in intraluminal vesicles of MVBs) were found in the inclusion lumen. Inhibition of MVB biogenesis with pharmacological inhibitors or by exogenous addition of anti-CD63 antibodies impairs chlamydial replication and disrupts the traffic of sphingomyelin and cholesterol into the inclusion [51,52]. However, RNAi-mediated silencing of CD63 synthesis does not

prevent MVB interactions with the inclusion [52], and MVB components have not been identified in the host genome RNAi screens [57•,61•]. It is likely that Golgi and MVBs partially overlap in their ability to deliver essential nutrients to replicating *Chlamydia*. This would explain why disruption of individual components of either pathway is not sufficient to inhibit chlamydial replication.

Co-option of lipid droplets

LD are endoplasmic-reticulum-derived organelles composed of a neutral lipid core surrounded by a phospholipid monolayer [70]. These lipid storage compartments are being increasingly recognized as dynamic organelles involved in multiple biological processes [71]. These organelles proliferate and are recruited to the periphery of the inclusion during *Chlamydia* infection [55,56•]. Three chlamydial proteins (Lda1 to Lda3) were identified as potentially involved in targeting LDs based on their ability to localize to LDs when expressed exogenously in mammalian cells [55]. Strikingly, LDs enter into the inclusion lumen [56•]. These observations are significant because they demonstrate that intact organelles can be translocated into the inclusion and that vesicle fusion is not the only means to deliver potential nutrients into the inclusion lumen. What role these organelles play in *Chlamydia* biology is less clear, although pharmacological inhibition of neutral lipid biosynthesis, and thus LD biogenesis, impairs chlamydial development, suggesting

Figure 1



Schematic representation of host cell pathways exploited by *Chlamydiae* to acquire nutrients. **(a)** Golgin-84 is proteolytically processed during infection through the activation of host caspases, calpains, and potentially by bacterial protease(s). This leads to Golgi fragmentation into ribbon-like structures in a process mediated by Rab6 and Rab11. These Golgi ministacks are recruited around the inclusion. Formation of Golgi ministacks is necessary for chlamydial uptake of host sphingolipids and replication. Golgi-derived exocytic vesicles containing sphingolipids and cholesterol fuse with the inclusion. **(b)** GPI-anchored plasma membrane protein CD59 is trafficked to the inner face of the inclusion in a Golgi-independent manner. **(c)** Infection leads to increased phosphorylation of extracellular signal-regulated map kinase (ERK) which, in turn, activates calcium-dependent cytosolic phospholipase A2 (cPLA2). cPLA2 removes straight chain fatty acids from the *sn2* position of host glycerophospholipids to generate lyso-phospholipids (Lyso-PL). *Chlamydia*-derived branched fatty acids (b-FA) are incorporated into Lyso-PL to generate *Chlamydia*-modified phospholipids (Chl-PL). **(d)** *Chlamydia* targets multivesicular bodies (MVB) to sequester required nutrients like sphingolipids and cholesterol. **(e)** Lipid droplets, the main store of neutral lipids, are targeted by *Chlamydia* lipid droplet-associated proteins (Lda) and translocated into the inclusion. **(f)** Pan-species recruitment of Rab1, Rab4, and Rab11, whereas Rab6 and Rab10 are recruited to *C. trachomatis* and *C. pneumoniae* inclusions, respectively. Candidate bacterial recruitment factors have been identified (Cpn0585 and CT229). **(g)** SNARE proteins Vamp3, Vamp7, and Vamp8 are recruited to the inclusion, possibly by chlamydial proteins containing SNARE-like motifs (like IncA and CT813) in the inclusion membrane.

a function for these organelles in *Chlamydia* pathogenesis [55].

Conclusions

In contrast with the numerous trafficking pathways intercepted by *Chlamydiae* during infection, little is known about the bacterial factors, which mediate these processes. The molecular underpinnings of these complex host–pathogen interactions may be difficult to decipher without the development of a robust system for mutational analysis in *Chlamydiae*. Here we reviewed how some of the most recent cell biological findings present new potential mechanisms for *Chlamydia* acquisition of nutrients while sequestered in an intracellular vacuole (Figure 1). Like many highly adapted and successful pathogens we predict that *Chlamydia* has evolved to tap several redundant mechanisms to obtain nutrients. However, it should also be noted that the host cell is not a passive partner in this interaction and actively seeks to destroy the microbial invader. Therefore, the re-routing of membrane and protein transport events cannot be solely viewed not only as a 'feeding' mechanism but also as bacterial countermeasure against innate immune functions.

Note added in proof

A very recent study characterizing the interactions between *C. trachomatis* and host cell sphingolipids [72] provides evidence that sphingolipid biosynthesis is required for inclusion membrane stability, homotypic fusion of inclusions, and proper RB to EB developmental transition. Moreover, through pharmacological inhibition of sphingolipid traffic this study shows that both the Golgi apparatus and MVBs contribute to the delivery of host-derived lipids to the inclusion.

Acknowledgements

We thank J Cocchiaro and JD Dunn for comments and feedback on this manuscript. Because of space limitations, not all contributions to this field could be included in this review and we apologize to those investigators whose work was not cited. Work in our laboratory is supported by grants from the NIH and the Burroughs Wellcome Trust Program in Infectious Diseases. HA Saka is supported by a fellowship from the Pew Latin American Fellows Program.

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