Effect of host fatty acid-binding protein and fatty acid uptake on growth of Chlamydia trachomatis L2

Guqi Wang,1 Frank Burczynski,2 Judy Anderson3 and Guangming Zhong1

1Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
2Faculty of Pharmacy, University of Manitoba, Winnipeg, MB R3T 2N2, Canada
3Faculty of Medicine, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

Chlamydia trachomatis is an obligate intracellular bacterium and acquires both building blocks and energy from host cells for growth. The fatty acid-binding protein (FABP) plays an important role in uptake of long-chain fatty acids (LCFA) and energy metabolism by eukaryotic cells. The roles of FABP and LCFA in chlamydial infection were evaluated. Infection of liver cells with chlamydial organisms promoted fatty acid uptake by the infected cells, suggesting that LCFA may benefit chlamydial growth. Introduction of FABP into the liver cells not only enhanced fatty acid uptake, but also increased chlamydial intravacuolar replication and maturation. The FABP-enhanced chlamydial intracellular growth was dependent on the host cell uptake of fatty acids. These results have demonstrated that C. trachomatis can productively infect liver cells and utilize FABP-transported LCFA for its own biosynthesis.

INTRODUCTION

Chlamydiae consist of multiple species, including two major species causing various pathologies in humans. Chlamydia trachomatis consists of many serovars, including serovars A to K and L1 to L3. Serovars A to C mainly infect human ocular tissues, which is a leading cause of preventable blindness in developing countries (Frajz & Jones, 1988; Schachter & Dawson, 2002). Serovars D to K mainly affect human urogenital tracts, causing complications such as pelvic inflammatory disease, ectopic pregnancy and infertility in women (Frajz & Jones, 1988; Stephens et al., 1998). Serovars L1 to L3 can infect both urogenital and rectal tissues, causing lymphogranuloma venereum (LGV) (Bauwens et al., 2002). It has been recently reported that the LGV serovars can cause occasional outbreaks of LGV among men having sex with men in industrialized nations (Farhi & Dupin, 2005). Chlamydia pneumoniae is a major cause of various respiratory illnesses, and respiratory infection with C. pneumoniae is also linked to cardiovascular pathologies such as atherosclerosis (Campbell & Kuo, 2002, 2004). There are other chlamydial species that can cause various animal diseases (Azuma et al., 2006; Everett & Hatch, 1995; Read et al., 2003). Regardless of the diverse host tropism and disease phenotypes, all members of the genus Chlamydia have evolved a common biphasic intracellular life cycle (Hackstadt et al., 1997). A typical chlamydial infection cycle starts with the entry of an infectious elementary body (EB) into mammalian cells via endocytosis. Once inside the endosome, the EB differentiates to a reticulate body (RB). The RB is metabolically active and able to replicate but no longer infectious. RBs replicate within the endosomal vacuoles, referred to as the chlamydial inclusions. RBs divide by binary fission at approximately 2–3 h per generation. After an incubation period of 16–20 h in the host, while some RBs are still replicating, others have begun to differentiate back to the infectious EBs. The RB growth and differentiation continues up to approximately 48–72 h after infection. The matured EBs can finally exit the infected cells and infect the adjacent cells.

During their obligate intravacuolar growth, chlamydiae have developed an intricate relationship with host cells. Chlamydial organisms secrete factors both into the inclusion membrane (Fields & Hackstadt, 2000) and into the host cell cytosol (Zhong et al., 2001), and import nutrients (Hackstadt et al., 1995; Su et al., 2004) and energy (Tipples & McClarty, 1993) from host cells. It is known that chlamydial organisms can take up host-cell-derived lipids including sphingomyelin (Hackstadt et al., 1995), cholesterol (Carabeo et al., 2003) and triglycerol phospholipids (Su et al., 2004; Wylie et al., 1997). Chlamydiae encode an ATP/ADP translocase (Schmitz-Esser et al., 2004; Stephens et al., 1998), allowing for exchange of host lipids (Su et al., 1997). Chlamydiae have evolved a common biphasic intracellular life cycle (Hackstadt et al., 1997). A typical chlamydial infection cycle starts with the entry of an infectious elementary body (EB) into mammalian cells via endocytosis. Once inside the endosome, the EB differentiates to a reticulate body (RB). The RB is metabolically active and able to replicate but no longer infectious. RBs replicate within the endosomal vacuoles, referred to as the chlamydial inclusions. RBs divide by binary fission at approximately 2–3 h per generation. After an incubation period of 16–20 h in the host, while some RBs are still replicating, others have begun to differentiate back to the infectious EBs. The RB growth and differentiation continues up to approximately 48–72 h after infection. The matured EBs can finally exit the infected cells and infect the adjacent cells.

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ATP for parasite ADP. The net result is that chlamydiae utilize host ATP for their energy metabolic reactions and dramatically reduce host ATP levels (Wang et al., 2002), which may trigger the elevated mitochondrial metabolism in the infected host cells (Hatch & McClarty, 1998; Wylie et al., 1997).

Long-chain fatty acids (LCFA) play essential roles in many aspects of the cellular processes, including as a major energy substrate, building blocks for membrane biosynthesis and precursors for producing signal molecules. However, the role of LCFA in chlamydial infection and development is unknown. The fatty acid-binding protein (FABP) belongs to a large group of distinct intracellular lipid-binding proteins, each with a tissue-specific distribution (Glatz & van der Vusse, 1996). FABP has been reported to bind and transport a wide variety of ligands such as LCFA, haem, bilirubin, bile acids, thyroid hormones, retinoids and certain hydrophobic reactants. In the current study, we evaluated the effects of chlamydial infection on host cell fatty acid uptake and the dependence of chlamydial growth on host cell LCFA uptake and FABP expression. We found that C. trachomatis infection induced a significant increase in fatty acid uptake by host cells, and that overexpression of FABP promoted the chlamydial intracellular growth without affecting chlamydial infection rate.

METHODS

**Chang liver cells and their transfection with pcDNA-FABP.** Chang liver cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (GIBCO). Chang liver cells stably expressing L-FABP were generated as follows (Wang et al., 2005). FABP cDNA from liver tissues (L-FABP) was produced by PCR using a Marathon-Ready cDNA kit (Clontech Laboratories). The primers used in the PCR were 5'-CTATGGCCACCATGAGTT-3' (forward) and 5'-AATAATGAAATGCAGACTTG-3' (backward). The PCR product was cloned into pc-Blunt II TOPO vector (Invitrogen). A 530 bp fragment containing the L-FABP cDNA was excised with HindIII and Xhol from the TOPO plasmid and ligated into the mammalian expression vector pcdNA3.1/V5-His B (Invitrogen). The recombinant plasmid, designated pcDNA-FABP, was transfected into Chang liver cells using the transfection reagent Lipofectamine (Invitrogen). The pcDNA vector plasmid was used as a control. Stable transfectants were selected and established in the presence of G418 (800 μg ml⁻¹; Invitrogen). Clones were analysed individually by RT-PCR and Western blot for levels of FABP expression as previously described (Wang et al., 2005). The Chang liver cells stably expressing L-FABP were named Chang L-FABP, while the cells stably transfected with the vector plasmid alone were named Chang pcDNA or vector cells.

**Chlamydial infection.** C. trachomatis LGV (serovar L2) was propagated and purified as described previously (Zhong et al., 2001; Zhong & Brunham, 1991). Aliquots of the organism were frozen at −80 °C before being used for infection. Both vector alone and L-FABP-transfected Chang liver cells were maintained in 10% fetal bovine serum (FBS) DMEM containing G418 (400μg ml⁻¹). Prior to chlamydial infection experiments, the G418 was removed by growing the cells in G418-free medium for three generations. For chlamydial infection, cells were grown on coverslips in a 24-well plate at 50 000 cells per well for 24 h in 10% FBS/DMEM without G418. The L2 organisms were directly diluted in growth medium and added to each well at an m.o.i. of 2. The organisms were allowed to grow for various periods of time as indicated in the experiments, before terminating for measurements. For the fatty acid supplementation experiment, cells were starved in glucose-free medium (10% FBS/DMEM without glucose; GIBCO catalogue no. 11866-025) for 24 h. A fatty acid supplement (Sigma) was added to the culture at 0.1 ml per 100 ml of medium. For determining chlamydial inclusion burst size, total cell lysates harvested from the infected cultures were serially diluted and inoculated onto HeLa cell monolayers grown on coverslips. Two days after infection, the coverslips were fixed for immunofluorescence staining as described below, and the number of chlamydial inclusions (also designated inclusion-forming units, IFU) per view was counted for 15 random views from duplicate coverslips. The number of IFU generated from a given cell lysate sample was calculated based on dilution factors and number of inclusions counted.

**Immunofluorescence staining.** The immunofluorescence staining was carried out as described elsewhere (Wang et al., 2002). The cell monolayers were fixed in 2% paraformaldehyde (Sigma) for 30 min at room temperature, followed by permeabilization with 2% saponin (Sigma) for 30 min at room temperature. After blocking with 10% FBS/PBS for 1 h at room temperature, a mouse monoclonal antibody raised against chlamydial HSP60 (clone BC7,1, unpublished data) was used to label chlamydial inclusions. The first antibody staining was visualized with a goat anti-mouse IgG conjugated with Cy2 (green). A Hoechst DNA dye (blue; Sigma) was used to simultaneously reveal host cell nuclei. Images were acquired individually for each staining in grey and superimposed in colour images using an IX70 inverted fluorescence microscope equipped with a CCD camera (Olympus America) and SimplePCI imaging software.

**Cellular uptake of long-chain fatty acid.** Cells were plated at 10⁶ cells per 35 mm dish in 10% FBS/DMEM. The adherent monolayer cells were infected with C. trachomatis L2 organisms and 24 h after infection, the cells were washed with pre-warmed (37 °C) PBS and incubated with 100 μM fatty acid-free BSA (A8806; Sigma) in the presence of [³H]palmitate [2 μCi (74 kBq) per dish; Perkin-Elmer] at 37 °C. At 60 s intervals, the BSA solution was aspirated and the uptake was stopped by washing the dish wells with ice-cold and ligand-free PBS for 10 s. The washed cell samples were dissolved overnight in 0.2 M KOH and harvested as cell lysates. The cell lysates were counted for radioactivity using a Beckman LS6500TA liquid scintillation counter. Cellular LCFA ([³H]palmitate) uptake was measured as the rate of [³H]incorporation into cells from medium and expressed as μl min⁻¹ per 10⁶ cells. The calculation is as follows: cell-associated radioactivity divided by radioactivity in the medium vs time (uptake interval) per 10⁶ cells (Burczynski et al., 1997).

**RESULTS**

**Chlamydial infection significantly increased host cell fatty acid uptake.** The effect of chlamydial infection on the fatty acid uptake by Chang liver cells was assessed by comparing the fatty acid uptake between Chang liver cells with or without chlamydial infection using a [³H]palmitate clearance assay. The control Chang liver cells maintained a total palmitate clearance rate of 2.64 ± 0.07 μl min⁻¹ per 10⁶ cells, while chlamydial infection increased the rate to 3.24 ± 0.12 μl min⁻¹ per 10⁶ cells (mean ± SEM; n=4), which represents a 23% increase (P<0.01). Thus, chlamydial infection significantly increased the host cell LCFA uptake.
L-FABP expression promoted fatty acid uptake in Chang liver cells

Chang liver cells stably transfected with pcDNA-L-FABP plasmid expressed both L-FABP mRNA and protein, while cells transfected with the pcDNA vector plasmid alone failed to do so (Fig. 1). More importantly, the FABP expression increased fatty acid uptake from 2.64 ± 0.074 μl min⁻¹ per 10⁶ cells in vector alone-transfected cells to 3.69 ± 0.097 μl min⁻¹ per 10⁶ cells in FABP-transfected cells (mean ± SEM; n=4), which represents a 36% increase (P<0.01).

L-FABP-mediated fatty acid uptake promoted chlamydial intracellular growth

The observation that chlamydial infection increased fatty acid uptake suggests that additional fatty acid supply may benefit chlamydial growth. Since FABP expression also increased fatty acid uptake by Chang liver cells, we then evaluated the effects of L-FABP expression on chlamydial growth by comparing the chlamydial growth in Chang liver cells with or without FABP expression (Table 1). The number of IFU per 100 host cells was similar between cells with or without FABP expression (35 ± 18% vs 33 ± 12%, P>0.05), suggesting that FABP expression did not affect chlamydial attachment and entry to the host cells. However, the size of the inclusions was significantly larger in FABP-transfected cells compared with vector-alone-transfected cells (13 507 ± 5270 vs 9030 ± 3323 pixels, P<0.001). Since inclusion size is often in proportion to the number of the chlamydial organisms inside the inclusions, the increased inclusion size may suggest a faster chlamydial growth rate. Indeed, the density of the chlamydial inclusions was also higher in the FABP-transfected cells, which is consistent with the assumption that the increased inclusion size in Chang-FABP cells may represent more intra-vacuolar organisms. To test whether the intra-vacuolar organisms are infectious, we determined the inclusion burst size by comparing the number of infectious particles recovered from Chang-FABP vs the number recovered from Chang-vector cells. We found that Chang-FABP samples generated fourfold more IFU than Chang-vector cell samples (Table 1). The above observations demonstrated that FABP can enhance both the chlamydial intra-vacuolar replication and maturation.

The next question is whether the FABP-enhanced chlamydial growth is dependent on fatty acid uptake. Glucose and LCFA are two major carbon sources for energy and biosynthesis. It is known that under glucose-restriction conditions, chlamydial organisms grow poorly, which may provide an experimental condition for amplifying the effects of fatty acids and FABP on chlamydial growth. We therefore used the glucose-restriction model to assess the effects of fatty acid supplementation and FABP expression on chlamydial intracellular growth. We found that without

Table 1. Effects of FABP expression on chlamydial growth

Chang liver cells transfected with pcDNA-L-FABP or vector alone were infected with chlamydial organisms (L2), and at various time points after infection as indicated, chlamydial infection and growth were monitored in terms of infection rate (number of infected cells over total number of cells viewed under a microscope), inclusion breakage rate (number of broken inclusions over total inclusions counted), inclusion size and inclusion burst size (IFU, titrated in HeLa cells and expressed as number of inclusions per 100 cells). An average of 15 views was randomly selected from duplicate coverslips. Over 10⁵ cells were counted. Data are presented as mean ± sd. **P<0.01 ***P<0.001.

<table>
<thead>
<tr>
<th>Chang liver cells</th>
<th>Vector</th>
<th>FABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection rate at 24 h</td>
<td>33 ± 12%</td>
<td>35 ± 18%</td>
</tr>
<tr>
<td>Broken inclusion rate at 48 h</td>
<td>22 ± 11%</td>
<td>33 ± 13%</td>
</tr>
<tr>
<td>Broken inclusion rate at 96 h</td>
<td>68 ± 10%</td>
<td>83 ± 7%**</td>
</tr>
<tr>
<td>Inclusion size (pixels) at 24 h</td>
<td>9030 ± 3323</td>
<td>13507 ± 5270***</td>
</tr>
<tr>
<td>Inclusion burst size at 48 h (IFU) when lysates diluted 1 : 10 000</td>
<td>14 ± 7</td>
<td>28 ± 6***</td>
</tr>
<tr>
<td>1 : 100 000</td>
<td>3 ± 2</td>
<td>12 ± 3***</td>
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any fatty acid supplement, there was no significant difference in inclusion burst size between samples harvested from the FABP- or vector-alone-transfected cells. However, when a fatty acid supplement was introduced to the cultures, the FABP-transfected cells produced twofold more IFU than the vector-alone-transfected cells (Table 2), suggesting that the FABP-enhanced chlamydial intracellular replication is dependent on fatty acid.

**DISCUSSION**

LCFA are a major carbon source for energy generation and biosynthesis. Chlamydial infection has previously been shown to stimulate both incorporation of fatty acids into phospholipids (Hatch & McClarty, 1998) and oxygen consumption (Gill & Stewart, 1970). However, the relative role of FABP, a major carrier protein for facilitating LCFA transportation, in *Chlamydia*-stimulated LCFA utilization is not clear. In the current study, we have demonstrated that chlamydial infection can stimulate host cell uptake of fatty acids. More importantly, we have shown that although FABP may not be absolutely required for chlamydial growth, FABP-facilitated fatty acid uptake can further promote chlamydial intracellular replication.

Chang liver cells do not express endogenous FABP (Fig. 1) and chlamydial infection can enhance LCFA uptake by Chang liver cells, suggesting that chlamydiae can either activate other host mechanisms or utilize their own mechanisms to allow the host cells to take up LCFA from the culture medium. Introduction of FABP into Chang liver cells can both enhance LCFA uptake and promote chlamydial growth (Fig. 1, Table 1), and the FABP-promoted chlamydial intracellular replication is dependent on host cell uptake of LCFA (Table 2), suggesting that chlamydiae can also utilize the LCFA transported by FABP. These observations have demonstrated that chlamydiae have evolved multiple pathways to acquire LCFA. Interestingly, chlamydiae can still productively (although not as robustly) grow in Chang liver cells under both glucose and fatty acid restriction (Table 2), suggesting that chlamydiae have evolved the ability to acquire carbon sources in forms other than glucose and fatty acids. Clearly, the interactions between chlamydial organisms and host cells are multi-faceted and the FABP- and LCFA-dependent pathways only represent the disposable portion of the complex process. These disposable pathways, although not essential for chlamydial growth in cell culture systems, may be necessary for chlamydial organisms to break tissue/host species barriers and to cope with the diverse intracellular environments.

Chlamydial organisms have adapted to an obligate intracellular growth niche by efficiently acquiring nutrients, metabolic intermediates, fuels and even energy in the form of ATP from the host cells. One of the chlamydial strategies to maintain adequate supplies of these materials from the host cells to chlamydial vacuoles is to enhance the uptake of these molecules by the infected host cells. Similarly to the chlamydial strategy for acquisition of fatty acids as demonstrated in the current study, chlamydiae maintain the supply of glucose, another major carbon source, by increasing both glucose transporter expression and glucose uptake in the infected cells (Ojcius et al., 1998). Both glucose transporters and FABPs are highly conserved tools for eukaryotic cells from different tissue types of all eukaryotic species to maintain the supply of carbon sources. Chlamydiae have learnt to manipulate both these mechanisms to create a nutrient-rich environment for their growth, which may enable the chlamydiae to infect multiple tissues of different species and to become a ubiquitous pathogen. However, it is far from clear how chlamydiae manage to acquire nutrients from and to survive in the hostile intracellular environment, despite their limited genome coding capacity and short-circuit metabolic pathways.

Chlamydiae are ubiquitous bacteria that can infect multiple types of tissues in many different animal species. In addition to their commonly known natural target cells such as epithelial/fibroblast cells and macrophages, chlamydiae can also infect other organ-specific cells, including astrocytes (Dreses-Werringloer et al., 2006; Levitt et al., 1986) and muscle cells (Dumrese et al., 2005; Rodel et al., 2003; Yang et al., 2005). We have recently shown that both *C. trachomatis* and *C. pneumoniae* can replicate in neonate heart muscle cells (Wang et al., 2002). In the current study, we have presented the first evidence that chlamydiae can also productively infect liver cells. However, the biological significance of this finding remains to be determined due to lack of information on the relationship between chlamydial infection and liver diseases.

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**Table 2. Effects of fatty acid supplement on chlamydial growth in the presence or absence of FABP and under glucose-restriction conditions**

<table>
<thead>
<tr>
<th>Chang liver cells transfected with:</th>
<th>Fatty acid supplement</th>
<th>Dilutions of Chang liver cell lysates</th>
<th>IFUs counted in HeLa cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>1:2000</td>
<td>5 ± 3</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Yes</td>
<td>1:8000</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>1:2000</td>
<td>10 ± 4</td>
<td>21 ± 3**</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>2 ± 1</td>
<td>5 ± 2**</td>
</tr>
</tbody>
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