Activation of Raf/MEK/ERK/cPLA2 Signaling Pathway Is Essential for Chlamydial Acquisition of Host Glycerophospholipids*

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Heng Su‡, Grant McClarty§, Feng Dong‡, Grant M. Hatch¶, Zhixing K. Pan†, and Guangming Zhong***

From the §Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229; Departments of §Medical Microbiology and §Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada, and ¶Department of Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio 43614

Chlamydiae, a diverse group of obligate intracellular pathogens replicating within cytoplasmic vacuoles of eukaryotic cells, are able to acquire lipids from host cells. Here we report that activation of the host Raf/MEK-cPLA2 signaling cascade is required for the chlamydial uptake of host glycerophospholipids. Both the MAP kinase pathway (Ras/Raf/MEK/ERK) and Ca2+-dependent cytosolic phospholipase A2 (cPLA2) were activated in chlamydia-infected cells. The inhibition of cPLA2 activity resulted in the blockade of the chlamydial uptake of host glycerophospholipids and impairment in chlamydial growth. Blocking either c-Raf-1 or MEK1/2 activity prevented the chlamydial activation of ERK1/2, leading to the suppression of both chlamydial activation of the host cPLA2 and uptake of glycerophospholipids from the host cells. The chlamydia-induced phosphorylation of cPLA2 was also blocked by a dominant negative ERK2. Furthermore, activation of both ERK1/2 and cPLA2 was dependent on chlamydial growth and restricted within chlamydia-infected cells, suggesting an active manipulation of the host ERK-cPLA2 signaling pathway by chlamydiae.

Infection with chlamydiae has been recognized as a major cause and/or exacerbating factor for various diseases in both humans and animals (1–7). Despite the wide spectrum of tissue tropisms exhibited by different strains of chlamydiae, a common denominator in chlamydial pathogenesis is the ability of the parasite to invade and then manipulate host cells. For example, a chlamydia-infected host cell has altered metabolic (8–10), immunological (11–13), and cell biological (14–17) characteristics. Chlamydiae have adapted to replicate within cytoplasmic vacuoles of eukaryotic cells (18, 19), which not only allows chlamydiae to evade host immune detection but also provides the opportunity for chlamydiae to exploit the nutrient-rich host cell cytosol. The availability, from the mammalian cell cytoplasm, of numerous metabolites not normally found in the external environment has provided selective pressure for chlamydiae to evolve a unique capacity to utilize host metabolic intermediates (20–22). Indeed, analysis of chlamydial genome sequences (23–27) has revealed that chlamydiae not only short-circuit biosynthetic pathways (22, 28) but also possess many novel genes for potentially manipulating host cells (27, 29–31).

The analysis of glycerophospholipids isolated from purified chlamydial organisms has revealed that membranes contain cardiolipin (CL)1, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phospholipids typically found in prokaryotes, as well as phosphatidylcholine (PC) and phosphatidylinositol (PI), phospholipids more commonly associated with eukaryotic membranes. We have shown that these eukaryotic glycerophospholipids are trafficked from the host cell to chlamydiae (33). Unlike mammalian cell glycerophospholipids, which contain straight chain fatty acids, chlamydial phospholipids possess branched chain fatty acids. Isoleucine is the precursor of the α-keto acid primer needed to initiate branched chain fatty acid biosynthesis in prokaryotes. Chlamydia-specific glycerophospholipids contain straight carbon chain fatty acids at their S1 but branched carbon chain fatty acids at the S2 position, indicating that chlamydiae take up and modify host glycerophospholipids. Obviously, a prerequisite of such modification is liberation of the free fatty acid at the S2 position of the host phospholipids, which is achieved through hydrolysis as catalyzed by phospholipase A2 (PLA2). However, no phospholipase A2 homologues have been identified in chlamydiae (34, 35), including both Ca2+- and Ca2+-independent PLA2 (iPLA2). The PLA2 activity can be inhibited by either chemical inhibitors (e.g. A2COCF3 or Ca2+ depletion. The activation of cPLA2 requires phosphorylation at multiple sites, which can be carried out by various upstream kinases including the MAP kinase ERK1/2 (36–38). Here we demonstrate that both MAP kinase ERK1/2 and cPLA2 are activated during chlamydial intracellular replication, and inhibition of either leads to suppression of chlamy-

1 The abbreviations used are: CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; FS, phosphatidylserine; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; pMEK1/2, phosphorylated MEK1/2; ERK, extracellular signal-regulated kinase; pERK1/2, phosphorylated ERK1/2; ELK-1, a component of the ternary complex that binds the serum response element; pERK1, phosphorylated ELK-1; MAP, mitogen-activated protein; IFU, inclusion-forming units; cPLA2, Ca2+-dependent cytosolic phospholipase A2; p-cPLA2, phosphorylated cPLA2; iPLA2, Ca2+-independent PLA2; A2COCF3, arachidonyltrifluoromethyl ketone; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid; BAPTA/AM, BAPTA tetraakis (acetoxymethyl) ester.

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dial uptake of the host glycerophospholipids and chlamydial replication.

MATERIALS AND METHODS

Cell Culture and Chlamydial Infection—HeLa, 293T (ATCC, Manassas, VA), and MS-74 (endometrial epithelial primary cells kindly provided by Dr. John Alderete from the University of Texas Health Science Center at San Antonio) were maintained in Dulbecco’s modified Eagle’s medium (catalog number 11995–065, Invitrogen) with 10% fetal calf serum (Invitrogen) at 37 °C in an incubator supplied with 5% CO2. The Chlamydia trachomatis serovar L2 (434/Bu) organisms obtained from Dr. C. Kuo at the University of Washington (Seattle, WA) were grown, purified, and titrated as described previously (39). Aliquots of chlamydial organisms were stored at −80 °C. The infection was carried out by diluting the chlamydial organisms in growth medium and applying the inocula onto the cell monolayers grown either in 35-mm tissue culture dishes or on glass coverslips in 24-well plates. The infectious dose was used at a multiplicity of infection of 5 or as indicated in the individual experiments. The infected cells were cultured at 37 °C in a CO2 incubator for various times as indicated in the individual experiments.

For treatments with inhibitors, all of the chemical inhibitors were added to the cultures 2 h after infection and maintained throughout the experiments. The inhibitors GW5074 (for inhibiting Raf-1, Tocris Cookson Ltd., Bristol, UK), U0126 (for MEK1/2, Cell Signaling Technology, Inc., Beverly, MA), AAOCCF3 (for pCL2A, Calbiochem, San Diego, CA), BEL (for pCL2A, Calbiochem), BAPTA/AM (cell membrane-permeable Ca2+ chelator, Sigma), and BAPTA (extracellular Ca2+ chelator, Sigma) were used at the concentrations indicated in individual experiments. For transfection, plasmids encoding either wild type ERK2 or dominant negative ERK2 were used to transfect 293T cells (ATCC) 24 h prior to chlamydial infection as described previously (40, 41). The epidermal growth factor (EGF) (Sigma) was used at 100 ng/ml for 5 min as a positive control for activation of the MAP kinase signaling pathway. At the end of the incubation period, the cultures were processed or harvested for analysis as described below.

For Western blot analysis, the cells were harvested with a SDS sample buffer (2% SDS, Sigma), 50 mM dithiothreitol (Sigma) in 62.5 mM Tris-HCl (Sigma) at pH 6.8. For titrating inclusion-forming units (IFUs), the cultures were sonicated in sucrose:phosphate-glutamine buffer (pH 7.2), and the cell lysates, after serial dilution, were inoculated onto fresh HeLa monolayers.

Western Blot Assay—The Western blot assays were carried out as described previously (42). Briefly, cell culture samples were lysed with a SDS sample buffer, and the lysates were subjected to SDS-polyacrylamide gel separation. After the proteins were blotted onto nitrocellulose membranes, primary antibodies were applied. These include mouse monoclonal antibodies to pCL2A and ERK1 (both from Cell Signaling Technology, Inc.), Ras (Upstate USA, Inc., Lake Placid, NY), chlamydial major outer membrane protein (clone MCC2), a FLAG tag (M2, Sigma) and rabbit IgGs to phosphorylated pCL2A (p-pCL2A), pERK1/2, pGSK3β, pERK1/2 (all from Cell Signaling Technology, Inc.), and total Raf-1 and Tyr-340-phosphorylated Raf-1 (both from Santa Cruz Biotechnology). The primary antibody binding was probed with corresponding secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by standard ECL (Amer sham Biosciences). The kinase activity of ERK1/2 in chlamydia-infected cells was measured with a recombinant ELK-1 as the substrate in a cell-free assay system following the instruction described in the manufacturer’s manual (Cell Signaling Technology, Inc.). The level of GTP-bound Ras in chlamydia-infected samples (Fig. 1A) was determined by immunoprecipitation with glutathione-agarose beads bound with the Ras-binding domain of Raf-1 glutathione S-transferase fusion protein following the manufacturer’s instructions (Upstate USA, Inc.).

Immunofluorescence Assay—For immunofluorescence staining (29), cells grown on coverslips were fixed with 2% paraformaldehyde in phosphate-buffered saline solution for 30 min at room temperature followed by permeabilization with 1% saponin for an additional 30 min. After the nuclei were stained with 4′,6-diamidino-2-phenylindole dye, the rabbit antiserum recognizing chlamydial organisms (RIL2)2 or an anti-chlamydial lipopolysaccharide monoclonal antibody (clone MBSH52)2 was used to detect chlamydial inclu sions. For localizing pERK1/2 or p-pCL2A, the cell samples were co-stained with a mouse monoclonal antibody to pERK1/2 or a rabbit antiserum to p-pCL2A (Cell Signaling Technology, Inc.). After washing away the unbound first antibodies, the appropriate secondary antibody conjugate combinations (consisting of a goat anti-rabbit IgG-conjugated with Cy2 (green), goat anti-mouse IgG-conjugated with Cy3 (red) (Jackson ImmunoResearch Laboratories, Inc.), and Hoechst dye (blue for DNA, Sigma)) were used to visualize the first antibody staining. The triply stained cell samples were used for image acquisition with an Olympus AX-70 fluorescence microscope. The images were acquired in a single color at a time using a Hamamatsu digital camera and superimposed with the software SimplePCI. For titrating IFUs, the chlamydia-infected cultures were singly stained for chlamydial inclusion as described above. The number of inclusions was counted (five random fields/coverslip) under a fluorescence microscope and expressed as IFU/ml of the sonicated cell lysates.

Localization of Radiolabeled Adenine into C. trachomatis DNA—C. trachomatis growth was assessed by measuring the incorporation of radiolabeled [2,8-3H]adenine (23 Ci/mmol, PerkinElmer Life Sciences) into parasite-specific DNA. Labeling conditions, cell harvesting, and quantitation of radiolabel incorporated into the DNA were conducted as described previously (21). Radiolabel was added at 20 h postinfection when C. trachomatis DNA synthesis is at a maximum, and cultures were harvested 3 h later. All results were normalized to 106 cells.

Extraction and Purification of Lipids—For radiolabeling of phospholipids, C. trachomatis-infected cells were incubated with 3 µCi/5 × 106 cells radiolabeled [1-14C]inositol (342 mCi/mmol, PerkinElmer Life Sciences) beginning at 20 h postinfection. After 6 h of incubation, the cell monolayer was washed once with ice-cold phosphate-buffered saline and scraped into 2 ml of methanol:water (1:1, v/v). The lipids were extracted and separated by a two-dimensional TLC procedure, which gave good separation of all the major phospholipids (32, 43). The organic phase containing the extracted lipids was dried under a stream of nitrogen and resuspended in 100 ml of chloroform:methanol:ammonium hydroxide (70:30:3) and in the second dimension with chloroform:methanol:water (65:35:5). Individual lipids were visualized with iodine vapor. Areas corresponding to individual lipids were excised and eluted in 2 ml of methanol containing Universal scintillation fluid (ICN Biomedicals, Inc.) for quantitation by scintillation counting (Beckman LS5000). The results were standardized based on the number of milligrams of protein/dish. Protein was assayed (44) with bovine serum albumin as the standard.

RESULTS
cPL2A Is Activated during Chlamydial Infection and Required for Chlamydial Uptake of Host Glycerophospholipids—To evaluate whether host cPL2A is activated following chlamydial infection, we monitored the extent of phosphorylation of cPL2A during the entire course of chlamydial infection (Fig. 1A). There was a basal level of cPL2A phosphorylation in HeLa cells in the absence of chlamydial infection (Fig. 1A, 0 h, top panel). However, the level of phosphorylated cPL2A was significantly higher in HeLa cells infected with chlamydiae for 12 h and continued to increase as the infection progressed. As the sample loading control, the total amount of cPL2A was monitored and showed no significant difference between cell samples (Fig. 1A, bottom panel). We next used a cPL2A-specific inhibitor, AAOCCF3, to test whether activation is required for the chlamydial uptake of host glycerophospholipids. As shown in Fig. 1B, AAOCCF3 dramatically reduced chlamydial uptake of host PE, PG, PC, PS, PI, and CL. Furthermore, AAOCCF3, but not BEL (an iPL2A-specific inhibitor), significantly reduced chlamydial growth as assessed by the incorporation of radiolabeled adenine into parasite DNA (Fig. 1C) and production of infectious elementary bodies as measured by quantitating the number of infectious particles recovered (Fig. 1D). Because the activity of cPL2A requires Ca2+, we next determined the effects of Ca2+ depletion on the chlamydial uptake of host glycerophospholipids. As shown in Fig. 1E, the cell-permeable calcium chelator BAPTA/AM was as efficient as AAOCCF3 (see Fig. 1B) in reducing the amount of host lipids trafficked to chlamydiae. The intracellular calcium depletion also inhibited chlamydial growth (Fig. 1F). In contrast, chelating extracellular Ca2+ with...
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Figure 1. The role of cPLA2 activation in chlamydial uptake of host phospholipids. A, detection of cPLA2 phosphorylation during chlamydial infection. HeLa cells with or without chlamydial infection were harvested at the times postinfection indicated at the top of the figure for detection of both phosphorylated (top panel) and total cPLA2 (bottom panel) in a Western blot assay. *ns* denotes nonspecific binding. B, analysis of chlamydial glycerophospholipids harvested from HeLa cell cultures infected for 26 h in the presence (solid bars) or absence (open bars) of the cPLA2 inhibitor AACOCF3 at a final concentration of 50 μM. The amount of individual phospholipids (CL, PE, PG, PC, PS, and PI) was expressed as lipid-associated radioactivity (dpm) per mg of protein. Due to the differences in the total amount of radioactivity incorporated into the various glycerophospholipids, the data were plotted in different scales with PE, PG, and PC along the left and PS, PI, and CL along the right vertical axes. No radioactivity was detected in phospholipids isolated from cells without chlamydial infection (also see Ref. 33). C, the effect of cPLA2 inhibition on the chlamydial uptake of radioactive adenine into parasite-specific DNA. HeLa cells infected with chlamydiae in the absence or presence of either AACOCF3 (*solid circles*) or BEL (*open circles*) at various concentrations (as indicated along the x axis) were harvested to measure the radioactivity associated with chlamydial organisms. The results were expressed as percent of radioactivity incorporated, using the radioactivity from samples without inhibitors as 100%. The inhibitors were kept in culture throughout the experiment, and BEL was highly toxic to host cells at concentrations higher than 30 μM, whereas there was no obvious toxicity with AACOCF3 up to 100 μM (data not shown). D, HeLa cells were treated exactly the same as described in the legend to Fig. 1C but were harvested for titrating infectious chlamydial particles. The results were expressed as IFU/ml (in log scale). E, the experiment was carried out similar to that described in B, except that the Ca<sup>2+</sup> chelator BAPTA/AM was used for treating the cultures at a final concentration of 10 μM, F, the experiment was carried out similar to that described in C, except that the cells were treated with either the cell-permeable Ca<sup>2+</sup> chelator BAPTA/AM (*solid circle*) or the cell-impermeable BAPTA at concentrations as shown along the horizontal axis. All quantitative experiments were carried out three times, and similar results were obtained in all three experiments. Shown in the figures are the representatives of the three experiments.
FIG. 2. The role of the MAP kinase pathway in chlamydial activation of cPLA2 and uptake of host phospholipids. A, activation of ERK1/2 during chlamydial infection. Cell samples were processed as described in the Fig. 1A legend for Western blot detection of phosphorylated ERK1/2 (pERK1/2, top panel), total ERK1/2 (middle panel), and phosphorylated ELK-1 (pELK-1, bottom panel). A recombinant ELK-1 was used as the substrate in an in vitro kinase assay for measuring pERK1/2 activity. B, the effect of ERK1/2 inhibition on cPLA2 activation. HeLa cells with

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Panel) protein level was significantly altered by the inhibitor. These data indicate that the chlamydial-activated ERK1/2 is responsible for the activation of cPLA2 during chlamydial infection. As expected, the inhibition of cPLA2 with U0126 also blocked the chlamydial uptake of host glycerophospholipids (Fig. 2C, top panel) and growth (bottom panel) in a dose-dependent manner.

We have also used transfection approach to demonstrate the role of ERK1/2 in chlamydial activation of cPLA2 in 293T cells (Fig. 2D). Cells transfected with a dominant negative (DN) ERK2, but not the wild type (WT) ERK2 construct, displayed a minimal level of cPLA2 phosphorylation regardless of chlamydial infection, which independently confirms that cPLA2 is activated in chlamydia-infected cells by the MAP kinase ERK1/2.

After demonstrating the chlamydial activation of MEK1/2-ERK1/2-cPLA2 in two different tumor cell lines, we next tested the chlamydial activity in the primary endometrial epithelial cells MS-74 (Fig. 2, E and F). Chlamydial infection induced significant phosphorylation of both ERK1 and cPLA2 during the entire infection course, although EGFR only activated ERK1/2 but not cPLA2 (Fig. 2E). More importantly, the chlamydia-induced phosphorylation of both ERK1/2 and cPLA2 was inhibited by U0126, an irreversible inhibitor of MEK1/2 (Fig. 2F), indicating that chlamydial activation of cPLA2 in the MS-74 primary cells is also dependent on chlamydial activation of ERK1/2.

We next evaluated whether the upstream steps of MEK1/2 are activated during chlamydial infection (Fig. 2, G and H). We found that the level of c-Raf-1 phosphorylated at Tyr-340 was increased as the infection progressed (Fig. 2G, third panel from top), whereas the total Raf-1 level remained similar regardless of infection (Fig. 2G, second panel from top). The Raf phosphorylation correlated with the phosphorylation of ERK1/2 (Fig. 2G, bottom panel), suggesting the involvement of Raf in chlamydial activation of MEK/ERK. Interestingly, the level of GTP-bound Ras, a small GTPase required for Raf-1 phospho-

activation during chlamydial infection (Fig. 2G, top panel). More importantly, a c-Raf-1 inhibitor (GW5074) significantly inhibited chlamydial-induced phosphorylation of MEK1/2 (Fig. 2H, second panel from top), ERK1/2 (fourth panel from top), and cPLA2 (Fig. 2H, bottom panel), whereas the total protein level of the corresponding molecules remained the same (Fig. 2H, top, third, and fifth panels), demonstrating the necessity of Raf-1 in the chlamydial activation of cPLA2.

Activation of ERK1/2 and cPLA2 Is Dependent on Chlamydial Growth—So far we have demonstrated that the MEK1/2-ERK1/2 signaling pathway is required for cPLA2 activation in chlamydia-infected cells. However, it is not known how MEK1/2-ERK1/2 itself is activated during chlamydial infection. We found that activation of both ERK1/2 and cPLA2 correlated well with chlamydial infection dose, and as infection doses are blocked, higher levels of second ERK1/2 and cPLA2 phosphorylation were detected (Fig. 3A), which suggests that chlamydial replication is involved in activating these host signaling molecules.

To evaluate whether chlamydial protein synthesis is required for the ERK1/2 and cPLA2 activation during chlamydial infection, we assessed the effects of antibiotics that selectively block either chlamydial protein synthesis or chlamydial particle assembly on host ERK1/2 and cPLA2 activation (Fig. 3B). Both rifampin (able to inhibit prokaryotic transcription) and chloramphenicol (able to prevent prokaryotic translation) successfully blocked chlamydial protein synthesis (Fig. 3B, bottom panel). Consequently, the levels of both phosphorylated ERK1/2 and cPLA2 were dramatically suppressed in chlamydia-infected cultures treated with either rifampin or chloramphenicol (Fig. 3B, top panel and third panel from the top). However, penicillin G, known to interfere with chlamydial reticulate body replication and reticulate body to elementary body differentiation without affecting protein synthesis, failed to block chlamydia-induced ERK1/2 and cPLA2 phosphorylation. These observations together have demonstrated that chlamydial protein synthesis is required for chlamydial activation of the ERK1/2-cPLA2 signaling pathway during chlamydial intravacuolar replication. Although ERK1/2 was also transiently activated as early as 10 min after infection (data not shown) (46), this immediate response is independent of chlamydial biosynthesis (data not shown).

We used immunofluorescence microscopy to localize the phosphorylated ERK1/2 (Fig. 3C, left panel) and cPLA2 (right panel) in chlamydia-infected cells. An enhanced level of both phosphorylated ERK1/2 and cPLA2 was detected only in the cells infected with chlamydia but not in the adjacent cells without chlamydial infection. The fact that the chlamydial-induced activation of ERK1/2 and cPLA2 is only restricted within the infected cells suggests that ERK1/2 and cPLA2 phosphorylation is a direct result of chlamydial intracellular infection but not an indirect effect from cytokines released by chlamydia-infected cells.

(+) or without (-) chlamydial infection and in the presence or absence of the various concentrations of the MEK1/2 inhibitor U0126 indicated at the top of the figure were harvested ~24 h after infection for Western blot detection of pERK1/2 (top panel), total ERK1/2 (second panel), p-cPLA2 (third panel) and total cPLA2 (bottom panel). ns stands for nonspecific binding. C, the effect of ERK1/2 inhibition on chlamydial lipid uptake and growth. HeLa cells infected with chlamydia in the absence or presence of the MEK1/2-specific inhibitor U0126 at the final concentrations, as indicated along the bottom of the figure, were harvested for Western blot detection of GTP-bound Ras (top panel), and 30 h after the transfection, the cells were infected with chlamydia. The cell samples were harvested 35 h after infection for Western blot detection of p-cPLA2 (top panel), total cPLA2 (middle panel), and the construct-encoded ERK2 (both the wild type and dominant negative ERK2s were detected with a mouse anti-FLAG tag antibody; bottom panel). E, the primary endometrial epithelial cells designated as MS-74 with or without chlamydial infection were harvested at the various times after infection indicated at the top of the figure for Western blot detection of total ERK1 (top panel), pERK1/2 (middle panel), or phosphorylated cPLA2 (bottom panel) as described under "Materials and Methods." The EGF was used at 100 ng/ml to stimulate MS-74 cells for 5 min as a positive control. Clearly, EGF induced a dramatic increase of ERK1/2 phosphorylation without causing any significant cPLA2 phosphorylation. F, MS-74 cells with (+) or without (-) chlamydial infection were treated with U0126 at the concentrations indicated on the top of the figure, and 30 h after infection, the culture samples were harvested for Western blot detection of total ERK1 (top panel), pERK1/2 (second panel), total cPLA2 (third panel) and phosphorylated cPLA2 (bottom panel). G, HeLa cells with or without chlamydial infection were harvested at the times postinfection indicated at the top of the figure for Western blot detection of GTP-bound Ras (top panel), total c-Raf-1 (second panel), phosphorylated c-Raf-1 at tyrosine residue 340 (third panel), total ERK1 (fourth panel), and pERK1/2 (bottom panel). GTP-bound Ras was precipitated from HeLa cell lysates with the agarose bead-immobilized GTP Ras-binding domain of Raf (RBD) and then subjected to Western blot detection with an anti-Ras antibody as described under "Materials and Methods." EGF was used for the positive control as described in the Fig. 2E legend. H, HeLa cells with (+) or without (-) chlamydial infection were treated with the Raf-1 inhibitor GW5074 at the concentrations indicated at the top of the figure, and 30 h after infection, the culture samples were harvested for Western blot detection of total MEK1/2 (top panel), phosphorylated MEK1/2 (second panel), total ERK1 (third panel), pERK1/2 (fourth panel), total cPLA2 (fifth panel), and phosphorylated cPLA2 (bottom panel).
Fig. 3. Chlamydial protein synthesis is required for chlamydial activation of ERK1/2 and cPLA2. A, correlation of the infection dose with ERK1/2 and cPLA2 phosphorylation. HeLa cells infected with chlamydiae, at the various multiplicities of infection indicated at the top of the figure, were harvested 30 h postinfection for Western blot detection of phosphorylated ERK1/2 (pERK1/2, top panel); total ERK1/2 (second panel); phosphorylated cPLA2 (p-cPLA2, third panel); total cPLA2 (fourth panel); and chlamydial major outer membrane protein (MOMP, bottom panel). B, the effect of antibiotics on chlamydial activation of ERK1/2 and cPLA2. HeLa cells with (+) or without (−) chlamydial infection and in the absence or presence of various antibiotics, as indicated at the top of the figure (PG, penicillin G at a final concentration of 100 μg/ml; RF, rifampin at 1 μg/ml, and CH, chloramphenicol at 60 μg/ml), were harvested 30 h postinfection for Western blot detection of phosphorylated ERK1/2 (pERK1/2, top panel); total ERK1/2 (second panel); phosphorylated cPLA2 (p-cPLA2, third panel); total cPLA2 (fourth panel); and chlamydial major outer membrane protein (MOMP, bottom panel). The antibiotics were present throughout the entire culture period. C, immunofluorescence detection of phosphorylated ERK1/2 (left panels) and cPLA2 (right panels) in chlamydia-infected cells. HeLa cells infected with chlamydiae for ~40 h were processed and triple stained with antibodies against phosphorylated ERK1/2 or cPLA2 (red) and chlamydial organisms (green) and DNA dye (blue). The images were acquired as a single color at a time and overlaid into triple color images using the software SimplePCI as described under “Materials and Methods.” The phosphorylated ERK1/2 (arrowheads) and cPLA2 (arrows) around chlamydial inclusions in the infected cells are indicated.

DISCUSSION

During chlamydial intravacuolar replication, the readily available nutrients and energy in host cell cytosols are transported into the chlamydial vacuoles (15, 20, 33, 47–49). We have presented previously evidence that chlamydiae salvage glycerophospholipids from host cells (32, 33). In addition, it is known that sphingomyelin (16, 47) and cholesterol (15) are also trafficked to chlamydiae from the host. Unlike sphingomyelin and cholesterol, which are trafficked unmodified, host cell glycerophospholipids are modified such that a host-synthesized straight chain fatty acid is replaced with a chlamydia-synthesized branched chain fatty acid. Most of the branched chain fatty acid is located in the S₂ position of the host-derived glycerophospholipid. Results presented in this study conclusively demonstrate that activation of the host cell cPLA2 is a prerequisite for glycerophospholipid trafficking from the host to the chlamydiae, and blocking the glycerophospholipid trafficking severely impairs chlamydial growth. Thus, despite the fact that chlamydiae contain the genes encoding all enzymes required for de novo synthesis of PG, PS, and PE (23), importing glycerophospholipids from the host cell is still indispensable for normal growth and development. The evolution of this sort of intimate metabolic association between chlamydiae and its host (Fig. 4) is likely a major contributing factor to chlamydiae being obligate to intracellular parasites. This intimate metabolic association may have the following consequences. First, being able to obtain glycerophospholipids from the host cell provides major energy savings for chlamydiae. Chlamydiae have an unusually high demand for lipids, because in addition to an inner and outer membrane typical of Gram-negative bacteria, they possess a unique inclusion membrane that is indispensable for chlamydial survival. Second, dependence on host cells for phospholipids may also serve as a sensing mechanism for chlamydiae to monitor the host cytoplasmic environment. Chlamydiae may be able to adjust their phospholipid metabolism in response to the cytosolic environment of the host cell as in the case of chlamydial persistence induced by tryptophan or iron depletion (50–52). It will be interesting to test whether inhibition of host cPLA2 activity indeed triggers the development of persistent chlamydial forms. Third, it may be advantageous for chlamydiae to incorporate host lipids into their inclusion membrane as this could give the membrane a “host-like” appearance, which may help prevent the chlamydial vacuole from being detected as foreign by the host cell defense mechanisms. Finally, chlamydial activation of cPLA2 may also contribute to chlamydia-induced local inflammation as arachidonic acid release, an inevitable consequence of cPLA2 activity, is greatly increased in chlamydia-infected cells (data not shown). Determination of cPLA2 involvement in inflammation at the site of chlamydial infection, although beyond the scope of this study, is under way.

The current study has presented compelling evidence that activation of the host MAP kinase ERK1/2-cPLA2 signaling pathway is required for chlamydial uptake of host glycerophos-
ERYK1/2-mediated signaling pathways that benefit microbial survival. For example, the inhibition of an ERK signaling pathway can significantly reduce Coxsackievirus B3 replication (61), and the Raf/MEK/ERK signaling cascade is required for the transportation of influenza viral ribonucleoprotein complexes from host cell nuclei into cytosol for viral particle assembly (62).

At the present time, we are unsure of how chlamydiae activate the MAP kinase-cPLA2 signaling pathway. In mammalian cells, MEK1/2 can be activated by c-Raf in response to stimulation of growth factors, and the Ras family small GTPases are required for transmitting the activation signals to c-Raf (53). The fact that both GW5074 (targeting c-Raf-1) and U0126 (targeting MEK1/2) blocked chlamydia-mediated activation of ERK1/2 and cPLA2 suggests that chlamydiae may interact with signaling pathways upstream of or at c-Raf-1 (Fig. 4). Although the level of GTP-bound Ras correlated with the activation of the Raf/MEK/ERK/cPLA2 pathway during chlamydial infection, it is not yet known whether Ras or other small GTPases are required for chlamydia-induced activation. Because chlamydia-induced ERK1/2 and cPLA2 activation was only found in the infected cells but not in the adjacent uninfected cells, it is likely that the interactions between chlamydiae and host cells occur intracellularly and are not dependent on the ectodomains of cell surface receptors. Efforts are under way to further pinpoint the cellular targets. Regardless of the precise intracellular molecules with which chlamydiae interact, the fact that chlamydia-mediated protein synthesis are required for the chlamydia-induced phosphorylation of ERK1/2 strongly suggests that chlamydiae secrete factors into host cell cytosols that activate the host MEK1/2-ERK1/2-cPLA2 signaling pathway. We are actively searching for those potential factors.

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Fig. 4. Model of the chlamydial activation of the MAP kinase/cPLA2 pathway and the chlamydial acquisition of host phospholipids. In cell host cytoplasm, the MAP kinase pathway is activated by chlamydiae at either of two steps: small GTPase Ras or c-Raf-1 kinase (which finally leads to phosphorylation of cPLA2). The activated cPLA2 hydrolyzes host phospholipids into lysophospholipids by removing the fatty acid at the S2 position. The lysophospholipids are then taken up by chlamydiae. In a chlamydia-organism inside the chlamydial inclusion, chlamydiae modify the host lysophospholipids by adding a chlamydia-synthesized fatty acid with a branched chain to the S2 position. Because only the prokaryotic chlamydia organisms can use the side chain of isoleucine to make the branched chain fatty acid, lipid-associated radioactivity isolated from chlamydia-infected cultures after [3H]isoleucine (ILE) pulsing can be used to measure the level of chlamydial lipid biosynthesis and the chlamydial uptake of host phospholipids. GLC, glucose; GLC6P, glucose-6-phosphate. The arrows stand for positive signaling/biochemical reactions, and the T shape stands for inhibitory reactions. All inhibitors are listed in italic and described under “Materials and Methods.”
Chlamydial Activation of MAP Kinase cPLA2 for Lipid Uptake

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