Chlamydia trachomatis Outer Membrane Complex Protein B (OmcB) Is Processed by the Protease CPAF

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We previously reported that the *Chlamydia trachomatis* outer membrane complex protein B (OmcB) was partially processed in *Chlamydia*-infected cells. We have now confirmed that the OmcB processing occurred inside live cells during chlamydial infection and was not due to proteolysis during sample harvesting. OmcB processing was preceded by the generation of active CPAF, a serine protease known to be able to cross the inner membrane via a Sec-dependent pathway, suggesting that active CPAF is available for processing OmcB in the periplasm. In a cell-free system, CPAF activity is both necessary and sufficient for processing OmcB. Both depletion of CPAF from *Chlamydia*-infected cell lysates with a CPAF-specific antibody and blocking CPAF activity with a CPAF-specific inhibitory peptide removed the OmcB processing ability of the lysates. A highly purified wild-type CPAF but not a catalytic residue-substituted mutant CPAF was sufficient for processing OmcB. Most importantly, in chlamydial culture, inhibition of CPAF with a specific inhibitory peptide blocked OmcB processing and reduced the recovery of infectious organisms. Thus, we have identified OmcB as a novel authentic target for the putative chlamydial virulence factor CPAF, which should facilitate our understanding of the roles of CPAF in chlamydial biology and pathogenesis.

Chlamydia trachomatis is a major cause of bacterial sexually transmitted diseases in the United States (1), which, if untreated, can lead to long-term complications such as pelvic inflammatory diseases, ectopic pregnancy, and infertility (2). It is thought that the inflammatory responses induced by the obligate intracellular growth of *C. trachomatis* significantly contribute to the pathogenicity (3–5). The *C. trachomatis* organisms initiate their intracellular life by invading epithelial cells in the form of elementary bodies (EBs). The intracellular EBs rapidly differentiate into reticulate bodies (RBs) that are metabolically active and able to proliferate. The progeny RBs have to differentiate back into EBs to exit the infected cells and spread to new cells. The EB-to-RB and RB-to-EB conversions require ~5-fold volume changes in the bacteria's size. It is not known how these large volume alterations are achieved. Nevertheless, the intracellular life of *C. trachomatis* organisms occurs strictly inside cytoplasmic vacuoles termed inclusions and takes 48 to 72 h to complete in cell culture systems, during which the intrainclusion chlamydial organisms secrete numerous proteins including the serine protease CPAF via a Sec-dependent pathway and inclusion membrane proteins via a type III secretion system (6–9). Despite many advances in chlamydial biology, the precise molecular mechanisms of how chlamydial organisms invade epithelial cells, how the intracellular organism differentiation is regulated, and how the intrainclusion organisms communicate with host cells remain unknown.

The chlamydial outer membrane complex protein B (OmcB) is an abundant outer membrane protein (10, 11) and highly conserved among *Chlamydia* species (12), suggesting a significant role of OmcB in chlamydial biology. OmcB has been shown to function as an adhesin for chlamydial invasion into host cells (13, 14). Free heparin or heparan sulfate can block the infectivity of some *C. trachomatis* serovars by binding to a positively charged peptide in the N terminus of OmcB (15, 16). OmcB may also be involved in the conversion of RBs to EBs (11, 17) and contribute to cell wall rigidity and osmotic stability of the EBs (11). OmcB is an immunodominant antigen with both B-cell and T-cell epitopes (18–24)

and has been considered a candidate for developing both serodiagnostics (12, 25) and subunit vaccines (26–28). Nevertheless, there has been considerable debate regarding the precise location and role of OmcB during *C. trachomatis* infection. OmcB was detected at the inner surface of the outer membrane and only became surface accessible after treatment with reducing reagents and proteases (17). The identification of CD8 T-cell epitopes in the OmcB C terminus (21) suggests that the C-terminal region can enter the host cell cytosol. We have recently reported that OmcB is partially processed into C-terminal (OmcBc) and N-terminal (OmcBn) fragments, and the processed OmcBc is released into the host cell cytosol while the processed OmcBn and remaining full-length OmcB are retained within the chlamydial inclusions (29). The released OmcBc becomes highly immunogenic during chlamydial infection in humans.

The goal of the current study is to further investigate how OmcB is processed. Given a recent report that many proteins can be processed/cleaved during the lysis of *Chlamydia*-infected cell samples (30), we first confirmed that OmcB processing occurred inside cells during chlamydial infection since the processing was still detectable when cell samples were prepared using 8 M urea. CPAF is a serine protease that accesses the periplasmic space via an Sec-dependent pathway (31), suggesting that CPAF may be a candidate protease for cleaving the C terminus of OmcB. This hypothesis is consistent with the previous finding that OmcB is localized at the inner surface of the outer membrane (17). Thus, we also simultaneously monitored the expression of CPAF and found that active CPAF was detected before the detection of the pro-

Received 10 November 2012 Accepted 3 December 2012

Published ahead of print 7 December 2012

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doi:10.1128/JB.02087-12

cessed OmcBc, suggesting a possibility for OmcB processing by CPAF. Indeed, CPAF is not only necessary and sufficient for cleaving a recombinant OmcB to produce the typical OmcB species in a cell-free assay but also required for processing the endogenous OmcB in *Chlamydia*-infected cells during infection. Thus, we have identified the chlamydial virulence factor CPAF as the upstream protease responsible for processing OmcB, which makes OmcB a novel authentic target for CPAF.

MATERIALS AND METHODS

Cell culture and chlamydial infection. HeLa cells (human cervical carcinoma epithelial cells; ATCC catalog item CCL2) and *C. trachomatis* L2/LGV-434/Bu organisms were used in the current study. The chlamydial organisms were propagated, purified, aliquoted, and stored as described previously (32). For infection, HeLa cells grown in either multiple-well plates, tissue culture dishes, or flasks were inoculated with chlamydial organisms as described previously (32). The cultures were processed for assays as described below. In some experiments, the infected cultures were treated with a CPAF-specific inhibitory peptide (SLFYSPMVPHFWAELRNHYATSGLKRRRRRRRRR) or a scrambled peptide (NFALSHFRLPLSTYKEMPVYVSHWAGRRRRRRRRR). Both peptides were synthesized by Peptide 2.0, Inc. (Chantilly, VA). The CPAF-inhibitory peptide sequence is derived from a central region of CPAF that occupies the CPAF substrate binding groove and is sequentially processed during CPAF activation (33), as revealed in the CPAF crystal structure (34). The polyarginine sequence fused to both the CPAF-inhibitory and scrambled peptides is designed to enhance the delivery of the peptides into intracellular chlamydial organisms since it has been shown that clusters of positively charged residues can deliver candidate peptides to CPAF and chlamydial organisms inside cells (35–37). The peptides were added to the cell cultures 12 h after chlamydial infection for 1 h in 500 μ l of serum-free Dulbecco's modified Eagle's medium (DMEM). The culture medium was then supplemented with 500 μ l of DMEM containing 20% fetal calf serum (FCS). This treatment strategy was intended to enhance treatment efficacy with minimum cytotoxicity. The cultures were continued and harvested at 36 h after infection for further analyses.

Western blot assay. A Western blot assay was carried out as described elsewhere (29, 38, 39). HeLa cells infected with *C. trachomatis* organisms were harvested at different time points after infection with either 8 M urea supplemented with 325 U/ml of Benzonase nuclease (both urea and nuclease were from Sigma-Aldrich, St. Louis, MO) or radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 mM sodium orthovanadate [all from Sigma-Aldrich]). To harvest culture samples grown in 35-mm dishes with 8 M urea, after medium was removed and the monolayer was washed with warmed phosphate-buffered saline (PBS) once, 700 μ l of 8 M urea was added to each dish, and samples were incubated for 10 min on ice, followed by collection of the lysates into Eppendorf tubes. The lysate samples were measured for protein concentrations (Bio-Rad). The addition of 100 μ l of an 8 $\times$ SDS sample buffer to each 700 μ l of cell lysates, the samples were stored at -80°C in aliquots. To harvest culture samples with RIPA buffer, cells were collected from each dish via trypsinization and pelleted at 1,500 rpm. Each pellet was resuspended in 700 μ l of ice-cold RIPA buffer for 30 min, followed by a brief sonication. After protein concentrations were measured, 100 μ l of 8 $\times$ SDS sample buffer was added to each 700 μ l of RIPA buffer lysates, and the samples were stored at -80°C in aliquots. The samples were resolved in an SDS-polyacrylamide gel after each lane was loaded with 2 μ g of total proteins. The resolved protein bands were transferred onto nitrocellulose membrane for detection with various primary antibodies. Primary antibody binding was probed with a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immunochemicals, West Grove, PA) and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology).

The following primary antibodies were used for Western blot assays in the current study: mouse monoclonal antibody (MAb) against OmcB (clone 3H2; IgG2a) (29), MAb clone 100a against CPAF (100a recognizes the C-terminal 35-kDa fragment of CPAF) (32), clone MC22 against chlamydial major outer membrane protein (MOMP) (32), clone W27 against host cell HSP70 (catalog number Sc-24; Santa Cruz Biotechnology, CA), clone AC-40 against beta-actin (catalog number A3853; Sigma-Aldrich), clone 26 against golgin-84 (catalog number 611382; BD Biosciences, San Jose, CA), clone V9 against vimentin (catalog number V6389; Sigma-Aldrich), and clone M20 against cytokeratin 8 (catalog number C5301; Sigma-Aldrich).

Cell-free degradation assay. A cell-free degradation assay was carried out as described previously (32). The following materials were used as the source of enzymes: fusion proteins with glutathione-S-transferase (GST) fused to the N terminus of chlamydial proteins, including GST-CPAF, GST-cHtrA (where cHtrA is chlamydial high-temperature requirement protein A), and GST-CT441 (19, 40, 41), or with a His tag fused to the C terminus of chlamydial proteins, including wild-type CPAF (designated CPAFwt) and the CPAF mutant with the 558 catalytic residue glutamic acid (E) replaced with alanine (A) [designated CPAF(E558A)] as described previously (34, 42, 43). The cell lysates included lysates of uninfected and *C. trachomatis*-infected HeLa cells. The lysates were prepared by resuspending HeLa cell samples at 1×10^7 cells per ml in 1 $\times$ MLB buffer (1% [wt/vol] Igepal, 10 mM MgCl₂, 1 mM EDTA, 150 mM NaCl, 10% [vol/vol] glycerol, 1 mM Na₃VO₄, 25 mM HEPES, pH 7.4, with a protease inhibitor cocktail [1 mM PMSF (catalog number P7626), 20 μ M leupeptin (catalog number L2884), 1.6 μ M pepstatin A (catalog number P5318), 1.7 μ g of aprotinin/ml (catalog number A6279)]; all from Sigma). The resuspension mixtures were incubated on ice for 30 min. After centrifugation at $21,000 \times g$ for 15 min at 4°C , the remaining supernatant was collected as cell lysates for degradation assay. In some experiments, *C. trachomatis*-infected HeLa cell lysates were separated into pellet and supernatant fractions by using either anti-CPAF (clone 100a) or anti-cHtrA (MAb clone 6A2) antibody-mediated precipitation as described previously (32, 41). Both the pellet and remaining supernatant were used as sources of enzyme in cell-free degradation assays. The substrate was the highly purified recombinant OmcB (reOmcB) with a His tag fused to the N terminus of OmcB (29). The reOmcB protein was pretreated with 20 mM dithiothreitol (DTT) for 30 min at 50°C in 50 mM Tris-HCl (pH 7.0) plus 20 mM MgCl₂ to expose the cleavage site for CPAF prior to the cell-free cleavage assays. Either a given fusion protein or lysate with or without precipitation was mixed with 0.1 μ g of reOmcB in 50 mM Tris buffer as described above. For some experiments, either CPAFwt or *C. trachomatis*-infected HeLa cell lysate was preincubated with the CPAF-specific inhibitory peptide as described above (SLFYSPMVPHFWAELRNHYATSGLKRRRRRRRRR) for 30 min at 37°C before being mixed with the substrate. After the cleavage was allowed for 2 h at 37°C , the mixture was dissolved in an 8 $\times$ SDS sample buffer for Western blot detection of the remaining substrate as described above.

LDH assay. To monitor cellular integrity, the culture supernatants were collected (before the cell samples were processed for other assays) to measure the level of lactate dehydrogenase (LDH) using a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO). The results were expressed in optical density (OD) values at 490 nm.

Immunofluorescence assay. An immunofluorescence assay was carried out as described previously (44) for titrating the infectious organisms from *Chlamydia*-infected cultures. HeLa cells infected with chlamydial organisms with or without a CPAF-inhibitory or scrambled peptide treatment were harvested into a sucrose-phosphate-glutamate (SPG) buffer via sonication, and the sonicated lysates after serial dilutions were titrated on fresh HeLa cell monolayers grown on coverslips in 24-well plates, as described previously (45). The infected HeLa cells were fixed with 2% paraformaldehyde (Sigma, St. Louis, MO) dissolved in PBS for 1 h at room temperature, followed by permeabilization with 2% saponin (Sigma) for an additional 1 h. After cell samples were washed and blocked,

they were subjected to antibody and chemical staining. Hoechst (blue; Sigma) was used to visualize DNA. A rabbit antichlamydial organism antibody (raised with *C. trachomatis* D organisms [unpublished data]), plus a goat anti-rabbit IgG secondary antibody conjugated with Cy2 (green; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), was used to visualize chlamydial inclusions under a fluorescence microscope. Five random views were counted from each coverslip, and the final number of inclusions per sample was calculated based on the number of inclusions per view, number of views per coverslip, and dilution factors.

RESULTS

Processing of OmcB occurs inside cells during chlamydial infection and correlates with CPAF activity. We have previously shown that OmcB is processed into N and C termini (designated OmcBn and OmcBc, respectively) (29). However, a recent study has revealed that many proteins can be cleaved during cell lysis of *Chlamydia*-infected cells in low-stringency buffer such as RIPA buffer and that this artificial processing can be stopped when cell samples are prepared under high-stringency conditions, such as 8 M urea (30). We compared OmcB cleavage under both 8 M urea and RIPA conditions and found that OmcB was cleaved into OmcBc regardless of the sample-processing buffer used (Fig. 1A). However, the various proteins previously reported to be cleaved in *Chlamydia*-infected cells samples, including golgin-84 (46), vimentin (36), and keratin-8 (47), were processed only when the mild RIPA buffer but not 8 M urea was used for sample preparation. These observations have demonstrated that processing of OmcB is not an artifact generated during sample preparation but an intracellular event occurring during infection. We also monitored host cell integrity along with the infection time course and found no obvious cell membrane permeability change (Fig. 1B), suggesting that OmcB processing did occur in live cells and was not due to dying cells. Next is the question of what upstream protease is responsible for processing OmcB. Since the serine protease CPAF is secreted via a Sec-dependent secretion pathway and can access the periplasmic space (31) where OmcB is exposed (17), we monitored the protein level of CPAF and found that the detection of processed OmcBc was preceded by the detection of the C-terminal fragment of CPAF, or CPAFc, an indicator of active CPAF (32, 34). Specifically, the full-length OmcB was detected at 24 h, and the processed OmcBc was detected at 28 h after infection (Fig. 1A, panel a). CPAFc was detected as early as 24 h after infection (Fig. 1A, panel b), suggesting that active CPAF is available in time for processing of OmcB. Together, the above observations show that OmcB processing occurred inside cells during chlamydial infection and is not an artifact of the sample preparation process and that the processing correlates with the production of active CPAF.

CPAF can process a recombinant OmcB to generate OmcBc. We next tested whether CPAF can process OmcB into OmcBc in a cell-free assay (Fig. 2). We compared a GST-CPAF fusion protein with two other serine protease fusion proteins including the periplasmic cHtrA encoded by the open reading frame (ORF) CT823 and the tail-specific serine protease encoded by CT441 for their ability to cleave a recombinant OmcB (reOmcB). To our surprise, only the CPAF fusion protein cleaved reOmcB into OmcBc (Fig. 2A, lane 3). A *Chlamydia trachomatis*-infected HeLa cell lysate known to contain CPAF activity also processed the reOmcB to produce OmcBc. When a highly purified recombinant CPAF with a short His tag fused to the C terminus was used to cleave the reOmcB (Fig. 2B), only the wild-type CPAF and not the CPAF

FIG 1 OmcB is processed during *C. trachomatis* infection. (A) HeLa cells either uninfected or infected with *C. trachomatis* were harvested at various time points after infection, as indicated on the figure, for Western blot detection of OmcB (a), CPAF (b), golgin-84 (c), vimentin (d), keratin-8 (e), the major outer membrane protein (MOMP) (f), and human HSP70 (g). The CPAFc fragment was first detected at 24 h, and processed OmcBc was detected at 28 h after infection. OmcB processing was detected regardless of the cell lysis methods while the degradation fragments (df) of previously reported CPAF substrates golgin-84, vimentin, and keratin-8 were detectable only in samples harvested with RIPA buffer and not 8 M urea. The question mark indicates a protein that may represent OmcB with incomplete reduction or an OmcB fragment. (B) The supernatants of the above cultures were harvested to measure the levels of LDH. No significant LDH was detected in any of the culture supernatants. The whole-cell lysate of a 36-h chlamydia-infected cell sample was used as a positive control. The data were from three independent experiments. P.I., postinfection; α , anti; MW, molecular weight (in thousands).

mutant with a catalytic residue substitution (E558A) cleaved reOmcB into OmcBc. Importantly, the cleavage of reOmcB by the wild-type CPAF was inhibited by a CPAF-specific inhibitory peptide (Fig. 2C). These results together have demonstrated that CPAF alone is sufficient for processing OmcB into OmcBc.

CPAF in *Chlamydia*-infected cells is necessary for processing a recombinant OmcB. Although we have demonstrated above that a highly purified recombinant CPAF could process reOmcB into OmcBc, we still do not know whether the endogenous CPAF activity from *Chlamydia*-infected cells is necessary for processing reOmcB. We used a similar cell-free system to measure reOmcB processing by the endogenous CPAF in *Chlamydia*-infected cells (Fig. 3). Lysates of HeLa cells infected with *Chlamydia trachomatis* was used as the source of the endogenous CPAF with the HeLa lysate as a control. It was obvious that the *C. trachomatis*-infected

FIG 2 CPAF is sufficient for processing OmcB into OmcBc. (A) In a cell-free cleavage assay, a recombinant OmcB (reOmcB) was used as the substrate for cleavage by GST fusion proteins CPAF (lane 3), cHtrA (lane 4), or CT441 (lane 5). A lysate made from *Chlamydia trachomatis*-infected HeLa cells (Ct-HeLa lysate) (lane 2) was used as a positive control. Both *C. trachomatis*-infected HeLa cell lysate and GST-CPAF but not other GST fusion proteins cleaved reOmcB into OmcBc. (B) The recombinant His-CPAF wild-type or His-CPAF with an alanine substitution of the catalytic residue glutamic acid at position 558 (E558A) was used to cleave reOmcB. The wild-type CPAF (lane 4) but not the E558A mutant (lane 5) cleaved reOmcB to produce OmcBc. The positive-control *C. trachomatis*-infected HeLa cell lysate also cleaved reOmcB into OmcBc (lane 3). (C) The processing of reOmcB into OmcBc by CPAF was inhibited by a CPAF-specific inhibitory peptide. The inhibition was noticed at 7 μ M and peaked at 700 μ M. The question mark indicates a protein that may represent OmcB with incomplete reduction or OmcB fragments; the asterisk indicates preexisting OmcBc from the *C. trachomatis*-infected HeLa cell lysates.

HeLa cell lysate already contained OmcBc, probably processed from the endogenous OmcB (Fig. 3A, lane 2). After the *C. trachomatis*-infected HeLa cell lysate was further mixed with reOmcB, additional OmcBc was produced (lane 3) while mixing HeLa lysate with reOmcB produced no OmcBc (lane 5), indicating that the *C. trachomatis*-infected HeLa cell lysate contains an activity that can process reOmcB into OmcBc. We then used an antibody-mediated depletion approach to test whether CPAF in the lysate contributed to the reOmcB processing activity (Fig. 3B). When 20 μ l of *C. trachomatis*-infected HeLa cell lysate was precipitated with 20 μ l of agarose-immobilized anti-CPAF antibody into a pellet (P) and both the pellet and remaining supernatant (S) were used to cleave reOmcB, we found that 15 μ l of the pellet successfully

FIG 3 CPAF activity is necessary for processing reOmcB. (A) Both *C. trachomatis*-infected (Ct-HeLa) and uninfected HeLa cell lysates were used to digest reOmcB in a cell-free system. The *C. trachomatis*-infected but not the uninfected HeLa cell lysate cleaved reOmcB to produce OmcBc. (B) *C. trachomatis*-infected HeLa cell lysate with or without precipitation into pellet and supernatant fractions with anti-CPAF or anti-cHtrA antibody-conjugated protein G agarose beads, respectively, was used to digest reOmcB. The anti-CPAF-precipitated pellet at 15 μ l (out of the total of 20 μ l) significantly cleaved reOmcB to produce OmcBc (lane 4) while 15 μ l (out of the total of 20 μ l) supernatant failed to do so (lane 6). In contrast, a similarly precipitated pellet with the anti-cHtrA antibody failed to process reOmcB (lane 7) while the supernatant retained reOmcB processing ability (lane 8). (C) The processing of reOmcB by *C. trachomatis*-infected HeLa cell lysate was significantly inhibited by a CPAF-inhibitory peptide. The question mark indicates a protein that may represent OmcB with incomplete reduction or OmcB fragments; the asterisk indicates preexisting OmcBc from the *C. trachomatis*-infected HeLa cell lysates.

processed a portion of reOmcB into OmcBc (lane 4) while the remaining supernatant failed to do so (lane 6). In contrast, a pellet similarly precipitated with an anti-cHtrA antibody failed to produce any OmcBc (lane 7) while the remaining supernatant maintained the OmcB processing ability (lane 8). These observations have demonstrated that CPAF in the *C. trachomatis*-infected HeLa cell lysate is necessary for processing reOmcB into OmcBc. This conclusion is further confirmed by the inhibition of *C. trachomatis*-infected HeLa cell lysate-mediated processing of reOmcB with a CPAF-specific inhibitory peptide (Fig. 3C).

CPAF is required for OmcB processing during *C. trachomatis* infection. We have shown above that both recombinant CPAF and endogenous CPAF can process OmcB *in vitro*. The remaining question is whether OmcB processing during chlamydial infection is dependent on CPAF. To address this question, we used a CPAF-specific inhibitory peptide known to be able to inhibit CPAF activity (34) and access to CPAF in *Chlamydia*-infected cells (35, 36) to treat *Chlamydia*-infected cultures and monitored the OmcB processing (Fig. 4). To maximize the specific inhibitory effect and

FIG 4 CPAF activity is required for processing the endogenous OmcB during *Chlamydia trachomatis* infection. (A) HeLa cells infected with *C. trachomatis* organisms were treated with an inhibitory peptide targeting CPAF or a scrambled peptide as a control at the concentrations listed at the top of the figure. At 36 h after infection, all samples were harvested in 8 M urea for Western blot detection with antibodies recognizing OmcB (a), MOMP (b), CPAFc (c), or host β -actin as indicated. Treatment with the CPAF-inhibitory peptide blocked processing of OmcB starting at 5 μ M and peaked at 9 μ M while the control peptide failed to do so even at 10 μ M. The total amounts of chlamydial proteins including the remaining OmcB, MOMP, and CPAFc were reduced by the CPAF-inhibitory peptide treatment while the host protein β -actin maintained equal amounts regardless of the treatments. The question mark indicates a protein that may represent OmcB with incomplete reduction or OmcB fragments. (B) HeLa cells infected with *C. trachomatis* organisms and treated with peptides as described above were harvested for titrating infectious organisms, and the results were expressed as infection-forming units (IFUs), as displayed along the y axis. The data were from three independent experiments. *, $P < 0.05$ (Student *t* test). MW, molecular weight in thousands.

to reduce cytotoxicity, the inhibitory or control peptide was added to the cell culture 12 h after infection for 1 h in 500 μ l of DMEM without serum. After the 1-h treatment, the medium was supplemented with 500 μ l of fresh DMEM containing 20% fetal calf serum, and the culture continued to 36 h after infection before being harvested for analyses. As the inhibitory peptide concentration increased, less OmcBc was detected, and the inhibition peaked at 8 to 9 μ M. However, the control peptide treatment at 10 μ M failed to significantly alter OmcBc processing (Fig. 4A). This observation has demonstrated that CPAF is required for OmcB processing during chlamydial infection. Interestingly, the amounts of full-length OmcB, MOMP, and CPAFc were also reduced by the CPAF-inhibitory peptide treatment, suggesting that the CPAF-mediated processing of OmcB may be necessary for optimal chlamydial intracellular growth. This hypothesis is further supported by the observation that the recovered live organisms were significantly reduced in the cultures treated with the CPAF-specific inhibitory but not control peptide (Fig. 4B).

DISCUSSION

We have confirmed that OmcB, a chlamydial outer membrane-associated, highly conserved, abundant and immunogenic protein (12, 18–21, 25–28), is processed during chlamydial infection since the processing was still detectable after the infected culture samples were harvested in 8 M urea, which has been shown to prevent proteolysis during cell lysis (30). Although we have previously reported that OmcB is partially cleaved (29), careful confirmation of OmcB processing in the current study is still necessary since a recent study has revealed that almost all previously reported protein cleavages in *Chlamydia*-infected cell samples are due to proteolysis during cell lysis (30). We indeed found that host golgin-84 (46), vimentin (36), and keratin-8 (47), all of which were previously reported to be processed in *Chlamydia*-infected cells, were cleaved only when the cell samples were harvested with a much less stringent RIPA buffer but not with the more powerful 8 M urea solution. Only the processing of OmcB into OmcBc was detectable under both lysis conditions, indicating that only the OmcB processing took place prior to cell sample harvesting. Furthermore, the integrity of host cells with or without chlamydial infection was maintained along the entire infection time course (prior to the sample harvesting), demonstrating that OmcB processing did occur in live cells. Thus, we can conclude that OmcB is processed inside live cells during chlamydial infection but not during sample harvesting.

More importantly, we have presented evidence that CPAF but not cHtrA or CT441 is responsible for OmcB processing. First, OmcB processing occurs after active CPAF is detectable although both events take place during the late phase of intracellular infection (Fig. 1), suggesting that CPAF is synthesized and activated early enough to contribute to OmcB processing. Second, CPAF activity in the *Chlamydia*-infected cell lysate is necessary for cleaving a recombinant OmcB into OmcBc (Fig. 3). Depletion of CPAF from the *C. trachomatis*-infected HeLa cell lysate with an anti-CPAF antibody but not an anti-cHtrA antibody removed the OmcB processing activity. Furthermore, a CPAF-specific inhibitory peptide also inhibited the OmcB processing ability of the *C. trachomatis*-infected HeLa cell lysate. Third, a highly purified recombinant wild-type CPAF but not a mutant CPAF with a catalytic residue substitution was sufficient for processing the recombinant OmcB into OmcBc (Fig. 2). The serine proteases cHtrA and CT441 failed to process OmcB. Finally, OmcB processing during chlamydial infection was inhibited by a CPAF-specific inhibitory peptide but not a scrambled peptide. Thus, together we can conclude that OmcB is processed by CPAF during chlamydial infection.

OmcB processing likely occurs in the chlamydial periplasmic region. Both OmcB and CPAF carry a typical Sec-dependent secretion leader sequence, and we have previously shown that the CPAF leader sequence is functional (31), suggesting that both OmcB and CPAF can cross the inner membrane and enter the periplasmic region via the Sec-dependent pathway. Nevertheless, colocalization in the same place does not necessarily guarantee a functional relationship. For example, HtrA is a known periplasmic serine protease that can process bacterial proteins in the periplasmic region (48). However, we found that cHtrA failed to process OmcB in a cell-free degradation assay. Thus, there must be a specific enzyme-substrate relationship between CPAF and OmcB in the chlamydial periplasm. OmcB is anchored in the

outer membrane although the precise orientation of OmcB in the outer membrane is disputable (17). It is possible that the C terminus of OmcB is exposed to the periplasmic region where active CPAF is available. The periplasmic CPAF is further secreted out of the organism and into host cells possibly via an outer membrane vesicle (OMV) budding mechanism (31). The processed OmcBc was also shown to release into the host cell cytosol (29). It is possible that OmcBc may gain access to the host cell cytosol by being copackaged into the OMVs with CPAF. The hypothesis that *Chlamydia* may use the OMV pathway to secrete factors into host cells is supported by the observations that the chlamydial outer membrane was induced to undergo vesiculation (49), and organism-free vesicles were detected both inside (50) and outside the inclusion membrane (51).

Regardless of how OmcB is processed by CPAF and how the processed OmcBc is released into host cells, the next question is the significance of CPAF-mediated OmcB processing in chlamydial biology and pathogenesis. It has been proposed that OmcB may participate in the differentiation from RBs to EBs (11, 17) in addition to its other functions (11, 13–16). Since RB-to-EB differentiation requires a 5-fold reduction in volume, processing a portion of OmcB and budding of outer membrane vesicles loaded with OmcBc fragments may promote RB-to-EB conversion, which is supported by the observation that inhibition of OmcB processing with a CPAF-specific inhibitory peptide also reduced the recovery of infectious organisms (Fig. 4B). Furthermore, the release of OmcBc into the host cell cytosol may allow OmcBc to have the opportunity to interact with host cell pathways. The localization of OmcBc in the host cell cytosol correlates well with its immunodominance in *C. trachomatis*-infected women (29), which is consistent with the concept that exposure of chlamydial proteins to the host cell cytosol is accompanied by increased immunogenicity (19, 52, 53). OmcBc is dominantly recognized by not only human antibodies (19) but also human CD4⁺ (54) and CD8⁺ T cells (21), indicating that OmcBc can access multiple immune processing compartments (55). These observations suggest that OmcB is a highly dynamic molecule and that release of OmcBc into the host cell cytosol may allow OmcB to participate in both the chlamydial intracellular interaction with host cells and chlamydial modulation of host immune responses.

ACKNOWLEDGMENT

This work was supported in part by grants (to G.Z.) from the U.S. National Institutes of Health.

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