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Cleavage of Host Keratin 8 by a Chlamydia-Secreted Protease

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Chlamydiae have to replicate within a cytoplasmic vacuole in eukaryotic cells. Expansion of the chlamydia-laden vacuole is essential for chlamydial intravacuolar replication, which inevitably causes host cell cytoskeleton rearrangements. A cleavage fragment of keratin 8 corresponding to the central rod region was detected in the soluble fraction of chlamydia-infected cells. Since keratin 8 is a major component of the intermediate filaments in simple epithelial cells, cleavage of keratin 8 may increase the solubility of the host cell cytoskeleton and thus permit vacuole expansion in chlamydia-infected cells. A chlamydia-secreted protease designated CPAF (chlamydial protease/proteasome-like activity factor) was both necessary and sufficient for keratin 8 cleavage in chlamydia-infected cells, suggesting that chlamydiae have evolved specific mechanisms for modifying the host cell cytoskeleton.

Chlamydiae are obligate intracellular bacterial pathogens that invade mammalian cells via endocytosis. Infection by chlamydiae can cause many severe diseases in humans. For example, *Chlamydia trachomatis*, which invades epithelial cells in the urogenital tract, has become a leading cause of sexually transmitted bacterial diseases with complications that include ectopic pregnancy and infertility (22). Although how chlamydial infections cause diseases is still not fully understood, the hypothesis is that the long-term survival of chlamydiae within infected cells may be mainly responsible for the chlamydia-induced pathologies (1, 2). Therefore, uncovering the molecular basis of chlamydial interactions with host cellular components is essential for understanding the chlamydial pathogenic mechanisms.

Chlamydial infection starts with endocytosis of an infectious particle called the elementary body (EB), which triggers various downstream processes, including differentiation of the EB into a metabolically active particle termed the reticulate body (RB), multiplication of RBs, and differentiation of RBs back to EBs (7, 26). The complete developmental cycle takes place within a cytoplasmic vacuole termed an inclusion, which expands as the number of RBs increases (26).

To complete the intracellular growth cycle, chlamydiae and the infected host cell must avoid attack by host defense systems. Various chlamydial strategies for protecting the infected cells from host immune detection (30, 31) and effector mechanisms (6) have been identified previously. A chlamydia-secreted protein with proteolytic activity designated CPAF (chlamydial protease/proteasome-like activity factor) is responsible for degrading host transcription factors required for major histocompatibility complex antigen expression in chlamydia-infected cells (29). This process may prevent the infected cells from immune detection. We hypothesize that chlamydiae may have also evolved specific mechanisms for facilitating vacuole expansion in chlamydia-infected cells and intravacuolar growth.

The cytoskeleton of cells consists of three major filamentous systems, and each system participates in distinct cellular processes; microfilaments polymerized by actin subunits are mainly involved in cellular motility, microtubules polymerized by tubulins are involved in cell division, and the intermediate filaments polymerized by keratins are involved in cellular integrity. Keratins, the subunits of the intermediate filaments, have three distinct structural domains; the N-terminal head and C-terminal tail domains flank the central helical rod region (12, 25). Although the keratin rod domain can still oligomerize (15), it often fails to form complete intermediate filaments (20). The keratin 8 gene is one of the most common and characteristic members of the intermediate filament gene family expressed in simple epithelial cells (3). Various viral proteases have been shown to cleave host cell keratins (4, 23), which may cause cytoskeleton collapse and facilitate viral release (27). On the other hand, proteolytic fragments of keratin 8 have been detected in carcinoma cells but not in normal epithelial cells (5, 14), suggesting that there is a correlation between keratin cleavage and cellular malignancy. We hypothesize that proteolytic modification of keratin 8 may benefit chlamydial intracellular growth and survival by increasing the fluidity of the host cell cytoskeleton and promoting the survival of the infected cells. We identified a cleavage fragment of keratin 8 in the soluble fraction of chlamydia-infected cells but not in the soluble fraction of uninfected cells. Sequence analysis revealed that the fragment identified represents the central helical rod region of keratin 8. We determined that CPAF, a secreted protease/proteasome factor that was identified previously, is both necessary and sufficient for cleavage of keratin 8 in the rod region.

MATERIALS AND METHODS

Cell culture and chlamydial infection. HeLa cells (American Type Culture Collection, Manassas, Va.) were grown in a medium consisting of Dulbecco modified Eagle medium (Invitrogen, Grand Island, N.Y.) and 10% fetal calf serum (Novo-Tech, Grand Island, N.Y.) in a humidified incubator in the presence of 5% CO_2. *C. trachomatis* LGV2 (serovar L2) or serovar D was grown and

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purified as previously described (33) and was used to infect HeLa cells at a multiplicity of infection of 5 or a multiplicity of infection indicated below. Infection was carried out by directly addding the stock organisms to the growth medium. The infected cultures were harvested ~40 h after infection or at other times to obtain measurements as described below.

Gel electrophoresis and amino acid sequence determination. Two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and preparation of S100 samples (cytosolic fractions prepared by Dounce homogenization and centrifugation at 100,000 × *g*) were carried out as previously described (6, 31). Our initial goal was to identify chlamydia-secreted proteins. The protein spots obtained for the infected and uninfected S100 samples separated on the two-dimensional gels were compared after visualization by Coomassie blue staining. A unique protein spot with a molecular mass of ~38 kDa and pI of ~4.8 was identified in the gel loaded with the chlamydia-infected S100 samples but not in the gel loaded with the uninfected S100 samples. This protein spot was designated L2p38 since generation of the spot was dependent on chlamydial serovar L2 infection. The L2p38 spot was excised from multiple replicates of gels in order to raise antibodies in mice. One copy of a gel replicate was transferred to a polyvinylidene difluoride membrane in order to determine the sequence of L2p38 by an N-terminal amino acid sequencing procedure (29), which was done commercially by the DNA/protein core facility at the University of Victoria, Victoria, British Columbia, Canada. A total of 28 cycles were successfully completed. To further characterize L2p38, the L2p38 tryptic peptide profile was compared with the profile of full-length keratin 8. L2p38 was purified from an S100 sample from an L2-infected cell cytosol (L2S100) with a mouse antibody raised with L2p38, while full-length keratin 8 was purified from a detergent-soluble fraction of normal HeLa cells with the anti-keratin 8 antibody M20. After the precipitates were resolved in an SDS-polyacrylamide gel, the corresponding protein bands were excised and subjected to in-gel digestion with trypsin. The molecular sizes of the tryptic peptides were determined by using a mass spectrum approach. Both the in-gel digestion and mass spectrum analyses were carried out by the proteomics core facilities at the University of Oklahoma and the University of Texas Health Sciences Center at San Antonio. The tryptic peptide profile of L2p38 was compared with that of keratin 8.

Immunoprecipitation assays. The immunoprecipitation assays were carried out as previously described (28). For antibody depletion experiments, either S100 or detergent-extracted cytosol samples were mixed with protein A/G agarose-immobilized antibody complexes (29, 31). After 1 h of incubation at room temperature, the agarose pellets were centrifuged, and the remaining supernatants were reprecipitated with a fresh set of the corresponding antibody complexes in order to completely remove the corresponding antigens in the supernatants. The final remaining supernatants and/or precipitates were analyzed to determine the presence of corresponding antigens in a Western blot or autoradiograph (for radioisotope-labeled samples) and for the keratin 8 cleavage activity in a cell-free assay. Monoclonal antibody M20 (Sigma, St. Louis, Mo.) was used for depleting keratin 8, CK5 (Sigma) was used for depleting keratin 18, LDS-68 (Sigma) was used for depleting keratin 7, MC22 (29) was used for depleting the chlamydial major outer membrane protein (MOMP), and 54b was used for depleting the N terminus of CPAF (29).

Cell-free cleavage assays. The cell-free assays were carried out as previously described (29). The enzyme was mixed with the substrate in the presence or absence of inhibitors. All reactions were carried out in phosphate-buffered saline and at 37°C for 1 h or as indicated below. The enzyme sources included the cytosol S100 samples obtained from HeLa cells with or without a chlamydial infection, the supernatants remaining after antibody depletion, the antibody-precipitated pellets, and the recombinant glutathione *S*-transferase (GST)-CPAF (29). The substrate preparations included both crude cytosol extracts made with an NP-40 buffer (CE) and a purified recombinant GST-keratin 8. A CE was prepared by extracting 1×10^7 to 2×10^7 HeLa cells with 1 ml of NP-40 buffer consisting of 1% NP-40, 0.5% Triton X-100, and 150 mM NaCl in 50 mM Tris (pH 8.0) supplemented with a protease inhibitor cocktail (phenylmethylsulfonyl fluoride at a final concentration of 1 mM, 20 μM leupeptin, 1.6 μM pepstatin A, and 1.7 μg of aprotinin per ml). The GST-keratin 8 fusion was generated by cloning the human keratin 8 cDNA (catalog no. 61514; clone ID p8.1.1; American Type Culture Collection) into a pGEX-6p vector (Amersham Pharmacia Biotech, Piscataway, N.J.), and the fusion protein expressed in bacteria was purified to homogeneity with glutathione-conjugated agarose beads (Amersham Pharmacia Biotech).

Western blot assays. Western blot assays were carried out as previously described (32). All samples used for Western blot assays were subjected to SDS-polyacrylamide gel separation, and the separated proteins were transferred onto a nitrocellulose membrane for immunostaining. The first antibodies used for Western blotting included a mouse antibody raised with L2p38 protein, monoclonal antibody M20 (Sigma) for detecting keratin 8, a mouse monoclonal antibody against a host 20S proteasome subunit (Affiniti Research Prodcuts Ltd.), a rabbit antibody against RFX5 (Rockland Immunologicals for Research, Gilbertsville, Pa.) (31), and a mouse antibody recognizing the CPAF N-terminal region (unpublished data). A goat anti-rabbit or anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase was used to probe the first antibody binding, and the results were visualized with an ECL-based detection system.

RESULTS

Unique protein band identified in the cytosol of chlamydia-infected cells represented a cleavage product of host cell keratin 8. In our efforts to use a proteomics approach to identify chlamydia-secreted proteins, we detected a unique protein band with an apparent molecular mass of ~38 kDa in the cytosol of chlamydia-infected cells but not in the cytosol of uninfected cells (data not shown). This unique protein, designated L2p38, was isolated and used to raise antibodies in mice. Western blotting confirmed that L2p38 was present in the cytosolic fraction of infected cells (L2S100) but not in either the normal HeLa cell cytosol (S100 fraction of HeLa cells [HeLaS100]) or purified EBs or RBs (Fig. 1A), supporting the notion that L2p38 is secreted rather than retained by chlamydia. Production of L2p38 was blocked by chloramphenicol and rifampin, which are inhibitors of prokaryotic translation and transcription, but not by cycloheximide, which is an inhibitor of eukaryotic translation, or penicillin, which prevents RB cell division and reorganization of RBs to EBs (Fig. 1B). These observations indicate that the production of L2p38 is dependent on chlamydial protein synthesis and that L2p38 is not generated by new host protein synthesis in response to infection.

We performed an N-terminal amino acid analysis of the 38-kDa L2p38 peptide to aid in identification of the peptide. To our surprise, the sequence of the first 28 amino acids matched the sequence of human keratin 8 residues 73 to 100 rather than the sequence of a protein encoded by the chlamydial genome (p05787) (http://www.ncbi.nlm.nih.gov/Entrez/protein.html). The predicted molecular mass of human keratin 8 from residue 72 to the C-terminal residue 483 is 46.5 kDa; therefore, L2p38 most likely represents a fragment of keratin 8 cleaved at both the N and C termini. The predicted molecular mass of the central rod region of keratin 8 from residue 72 to residue 483 is 38.3 kDa, and the calculated pI of this peptide is 4.83; these values are close to the observed apparent molecular mass (38 kDa) and pI (~4.8) of L2p38. A mass spectrum analysis of tryptic peptides derived from L2p38 and full-length keratin 8 was used to further characterize L2p38. The L2p38 sample generated 26 tryptic peptides, all of which matched peptides in the sequence of the human keratin 8 central rod region, while the full-length keratin 8 sample generated a total of 33 tryptic peptides that matched peptides in the sequence of the entire length of keratin 8, including four peptides matching the N-terminal head peptides and three peptides matching the C-terminal tail region peptides, which confirmed that L2p38 represents the central rod region of keratin 8. We carried out antibody depletion experiments to further confirm that the L2p38 detected in the cytosol of chlamydia-infected cells represents the central region of human keratin 8. We found that monoclonal antibody M20, which is known to recognize an

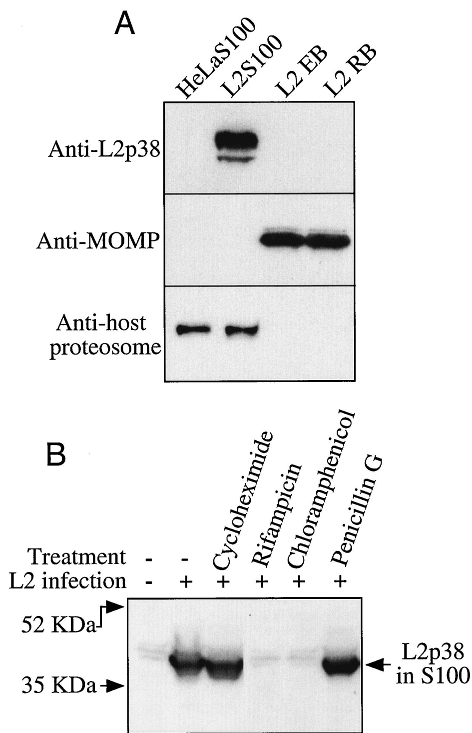

FIG. 1. Identification of a unique protein designated L2p38 in the cytosol of chlamydia-infected cells. (A) Anti-L2p38 antibody was used to detect L2p38 in the cytosol of HeLa cells that were not infected (HeLaS100) or were infected with *C. trachomatis* serovar L2 (L2S100) and in the EBs (L2 EB) or RBs (L2 RB) of purified L2 organisms on a Western blot. L2p38 was detected only in L2S100 (top panel). Parallel samples were also used for detection of MOMP (monoclonal antibody MC22) (middle panel) and a host 20S proteosomal subunit (bottom panel). (B) L2-infected HeLa cells were treated with the antibiotics indicated at the top, and the cytosol fractions (S100) derived from the culture samples were subjected to a Western blot analysis with the anti-L2p38 antibody. Both rifampin (final concentration, 1 μg/ml) and chloramphenicol (60 μg/ml), but not penicillin G (100 U/ml) or cycloheximide (2 μg/ml), blocked generation of L2p38. All antibiotics were added at the beginning of the infection, and the concentrations were maintained throughout the infection.

epitope in the human keratin 8 central rod region, was able to specifically remove L2p38 from cytosol samples of chlamydia-infected cells, while antibodies that recognized either keratin 18 or keratin 7 epitopes did not remove L2p38 (Fig. 2).

CPAF is required for the cleavage of keratin 8 in chlamydia-infected cells. Suspecting that the secreted chlamydial protease CPAF may be responsible for cleaving keratin 8 to form L2p38, we correlated the breakdown of keratin 8 with the degradation of RFX5, a transcription factor which has been shown to be a target of CPAF in infected cells (29). Full-length, 52-kDa soluble keratin 8 was detected with monoclonal antibody M20 in the detergent-soluble cytosolic extracts of uninfected cells, as well as in infected cells, up to 10 h after infection (Fig. 3). However, as chlamydial infection progressed, the amount of full-length keratin 8 decreased, and keratin 8 completely disappeared 16 h after infection, about the time when expansion of the chlamydial vacuole became obvious. The decrease in the amount of keratin 8 was accompanied by accumulation of degradation fragments that were larger than 38 kDa through

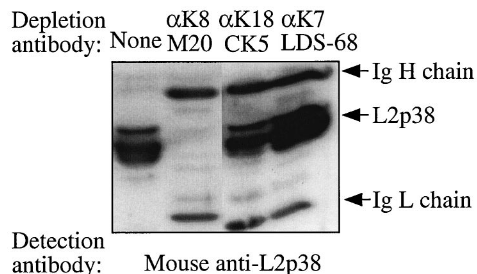

FIG. 2. L2p38 represents the central rod domain of host cell keratin 8. Mouse monoclonal antibodies against human keratin 8 (αK8 M20), keratin 18 (αK18 CK5), and keratin 7 (αK7 LDS-68) were used to precipitate L2p38 in the cytosol of chlamydia-infected HeLa cells (L2S100). The precipitation procedure was repeated once in order to completely deplete the corresponding antigens in the supernatants. The final remaining supernatants were subjected to Western blot analysis with the anti-L2p38 antibody. Note that only the M20 antibody recognizing an epitope in the keratin 8 rod region depleted L2p38 from the L2S100 cytosol sample, confirming that L2p38 contains the keratin 8 rod region. Ig H chain, immunoglobulin heavy chain; Ig L chain, immunoglobulin light chain.

20 h postinfection. By 30 h, all detergent-soluble keratin 8 in the infected cells was cleaved to products with molecular masses of about 38 kDa, the apparent molecular mass of L2p38. The infection time-dependent cleavage of keratin 8 (Fig. 3, top panel) correlated well with the degradation of RFX5 (Fig. 3, middle panel) and the expression of CPAF (Fig. 3, bottom panel). These observations support the conclusion that CPAF is responsible for cleaving keratin 8 to form a 38-kDa peptide.

The possibility that CPAF participated in the cleavage of

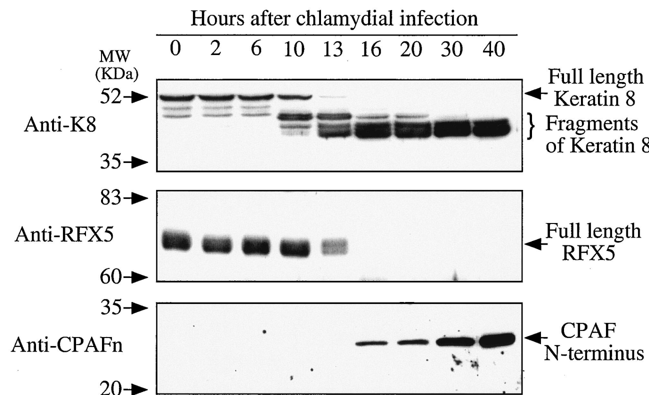

FIG. 3. Correlation of keratin 8 cleavage with degradation of RFX5 (as a measure of CPAF activity) and expression of CPAF during chlamydial infection. At various times after chlamydial infection, culture samples were extracted with an NP-40 buffer and used for Western blot analysis with antibody against keratin 8 (M20) to monitor keratin 8 cleavage (top panel), with antibody against RFX5 (rabbit anti-RFX5) to assess CPAF activity (middle panel), and with antibody against CPAF (mouse anti-CPAF N-terminal region [Anti-CPAFn]) to evaluate CPAF expression (bottom panel). Note that keratin 8 cleavage correlated well with both RFX5 degradation and CPAF expression. Also note that when HeLa cells were extracted with the NP-40 buffer, full-length keratin 8 with a molecular mass of 52 kDa appeared in the soluble fraction (top panel). However, when HeLa cells were subjected to Dounce homogenization to make S100, no full-length keratin 8 was detected in the supernatant (Fig. 1A, top panel). MW, molecular mass.

host keratin 8 was further evaluated by performing a cell-free cleavage assay (Fig. 4A). The cytosol samples from the infected HeLa cells (L2S100), but not the cytosol samples from the normal HeLa cells (HeLaS100), cleaved keratin 8. Not only was the cleavage dependent on the dose of L2S100, but it was also blocked by lactacystin, an irreversible proteosomal inhibitor known to inhibit CPAF activity (31). We used an immunoprecipitation technique to determine whether CPAF was the enzyme in L2S100 responsible for cleaving keratin 8. Immobilized antibodies specific for CPAF, but not antibodies specific for MOMP, depleted keratin 8 cleavage activity from L2S100 after two precipitations (Fig. 4B). Conversely, the pellets pulled down by the anti-CPAF antibody, but not the pellets pulled down by the anti-MOMP antibody, cleaved keratin 8. For quality control of the depletion assay, radiolabeled lysates of chlamydia-infected cells were consecutively precipitated three times with either CPAF- or MOMP-specific antibodies, and the precipitates resulting from each precipitation were assayed for the presence of the antigens (Fig. 4C). As previously demonstrated (29), the anti-CPAF antibody pulled down both the C- and N-terminal fragments of CPAF, whereas anti-MOMP antibody pulled down MOMP. The pellets from the third precipitation did not produce more radioactive bands, indicating that the first two precipitations completely removed the corresponding antigens from the supernatants.

CPAF is sufficient for cleaving keratin 8. To determine whether CPAF alone is sufficient for keratin 8 cleavage, we used a bacterially expressed GST-CPAF fusion protein to cleave keratin 8 in cell-free assays. In our first experiment, keratin 8 extracted from uninfected cells served as the substrate (CE). The S100 fraction of infected cells (L2S100) and low (0.2 μM) and high (0.6 μM) concentrations of GST-CPAF cleaved keratin 8 into a fragment corresponding to the rod region; lactacystin inhibited the cleavage in all cases, whereas dimethyl sulfoxide (DMSO), the solvent used to dissolve lactacystin, did not inhibit cleavage (Fig. 5A). In the second experiment, we used GST-keratin 8 expressed in bacteria as the substrate (Fig. 5B). L2S100 cleaved the recombinant GST-keratin 8, and the cleavage was inhibited by lactacystin, indicating that the GST-keratin 8 fusion protein was still accessible for cleavage by a CPAF activity in L2S100. Most importantly, the recombinant GST-CPAF also successfully cleaved the recombinant GST-keratin 8, and lactacystin but not DMSO blocked the cleavage. Since both GST-CPAF (9) and GST-keratin (Fig. 5C) were purified from a bacterial expression system (without any contamination from mammalian cells), the observations described above unambiguously demonstrate that CPAF is sufficient for cleavage of keratin 8.

DISCUSSION

Chlamydial survival and replication in cytoplasmic vacuoles of host cells inevitably require that chlamydiae interact with the host cellular machinery through chlamydia-secreted proteins either on the vacuolar membrane or in the host cell cytosol. CPAF was previously identified as a chlamydia-secreted protein (29) that can degrade host transcription factors required for major histocompatibility complex antigen expression (30, 31), which may allow the chlamydia-infected cells to evade host immune detection. We report here that CPAF can

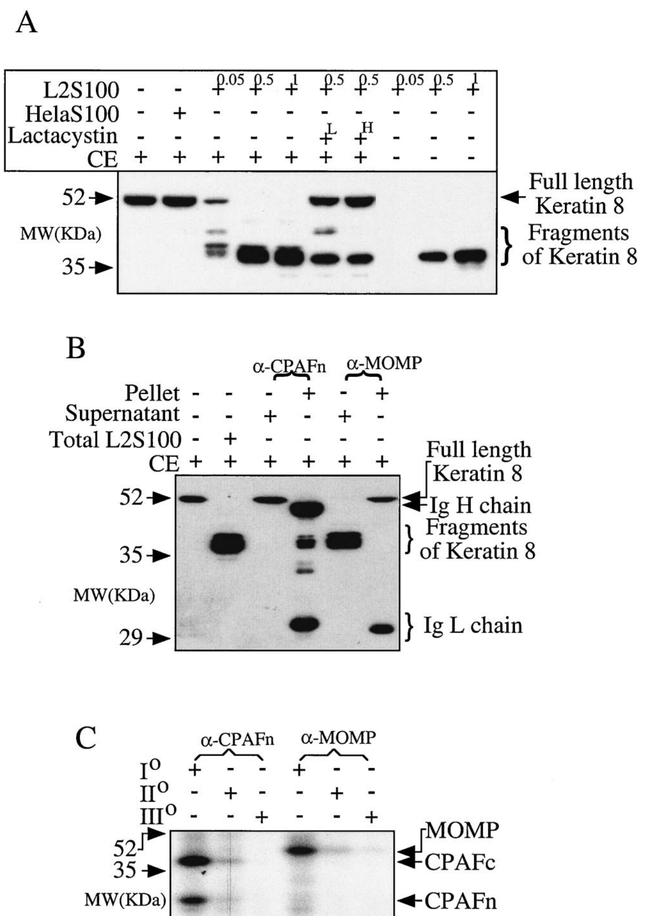

FIG. 4. CPAF is necessary for keratin 8 cleavage in the cytosol of chlamydia-infected cells. (A) A cell-free cleavage assay was used to evaluate the ability of L2S100 or HeLaS100 to cleave keratin 8 in CE. L2S100 was used at three different concentrations (equivalent to an L2S100 stock volume of 0.05, 0.5, or 1 μl). HeLaS100 was similarly used at a concentration of 5 μl per reaction mixture. Lactacystin was used at a final concentration of either 20 μM or 100 μM. Note that the keratin 8 fragment in L2S100 alone was detectable in 0.5- and 1-μl samples but not in 0.05-μl samples. (B) An antibody depletion approach was used to evaluate whether CPAF is necessary for cleavage of keratin 8. Monoclonal antibodies against either MOMP or CPAF were used to precipitate corresponding antigens in the cytosol extracts of chlamydia-infected cells (L2S100). To completely deplete the corresponding antigens from L2S100, the precipitation procedure was repeated once. The pellets from the first precipitation and the final supernatants were both evaluated for the ability to cleave keratin 8 in the cell-free cleavage assay, as described above. Ig H chain, immunoglobulin heavy chain; Ig L chain, immunoglobulin light chain. (C) The antibody depletion efficiency was monitored by using radiolabeled cell lysates. Both anti-CPAFn antibody (monoclonal antibody 54b) and anti-MOMP antibody (monoclonal antibody MC22) successfully precipitated the corresponding antigens during the first precipitation (I°). However, in the second precipitation (II°) minimal amounts of the antigens were precipitated, and after the third precipitation (III°) the preparation did not contain detectable amounts of the antigens, indicating that the first two precipitations completely removed the antigens from the cell lysates. The anti-CPAFn antibody 54b is known to precipitate both the N-terminal (CPAFn) and C-terminal (CPAFc) fragments of CPAF (29). MW, molecular mass.

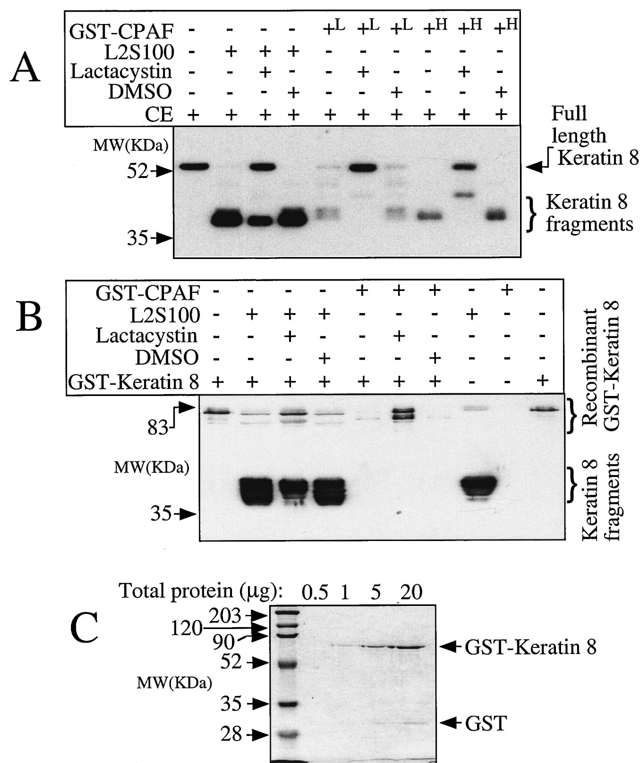

FIG. 5. CPAF is sufficient for cleavage of keratin 8. (A) Purified recombinant GST-CPAF was used to cleave keratin 8 in CE in a cell-free assay, and L2S100 was used as a positive control for monitoring the quality of the assay. Keratin 8 and its cleavage fragments were monitored by using the M20 antibody in a Western blot assay. GST-CPAF successfully cleaved keratin 8 at a final concentration of either 0.2 μM (L) or 0.6 μM (H), and the cleavage was inhibited by 50 μM lactacystin but not by the solvent DMSO alone. (B) Cell-free assay similar to the assay whose results are shown in panel A, except that purified recombinant GST-keratin 8 (free of other mammalian component contamination) was used as the substrate. Both GST-CPAF and L2S100 cleaved the recombinant keratin 8 and lactacystin, and DMSO did not inhibit the cleavage. GST-CPAF was used at a concentration of 0.2 μM, L2S100 was used at a concentration of at 0.5 μl/reaction mixture, lactacystin was used at a concentration of 50 μM, and GST-keratin 8 was used at a concentration of 1 μg/reaction mixture. It is not clear why no degradation fragment was detected in the sample in which GST-keratin 8 was cleaved by GST-CPAF. (C) Purified recombinant GST-keratin 8 with a molecular mass of 78 kDa was analyzed on an SDS-polyacrylamide gel stained with Coomassie blue dye. Although free GST was detected in lanes loaded with 5 to 20 μg of protein, we used 1 μg of protein as the substrate in panel B. MW, molecular mass.

also cleave keratin 8, a key component of the intermediate filaments in simple epithelial cells, into a fragment corresponding to the rod region alone, which may facilitate chlamydial intravacuolar replication and survival. Interestingly, various viral enzymes have been shown to cleave either the head or tail of host cytokeratins in infected cells (4, 23, 24), suggesting that proteolytic modification of host cytoskeleton proteins may be an important strategy exploited by intracellular pathogens for manipulating host cells.

What are the possible biological consequences of the keratin 8 cleavage? First, cleavage of keratin 8 in the rod region may increase the solubility of the intermediate filaments and thus facilitate vacuole expansion. The keratin 8 central rod region, which has a molecular mass of 38 kDa, was always detected in the soluble fractions of chlamydia-infected cells (Fig. 1 and 3), suggesting that the rod region alone may not be able to participate in assembly of the complex intermediate filaments. It has been shown that keratins without a head domain cannot assemble into the 10-nm filaments (13) and that the isolated rod region alone cannot form the intermediate filaments (20). It has also been reported that cleavage of the keratin aminoterminal head domain by an adenovirus proteinase in HeLa cells can lead to reorganization of the intermediate filaments into spheroid globules (4). All these observations support the concept that keratin cleavage can effectively alter the intermediate filamentous network. Furthermore, because the rod domain is able to oligomerize (15, 25), it may act as a dominant negative mutant structure and interfere with filament formation by full-length keratins, although this possibility requires further experimental evaluation. Nevertheless, the CPAF-mediated cleavage of keratin 8 should reduce the amount of intermediate filaments available for restricting vacuole expansion in chlamydia-infected cells. We indeed found that inhibition of the CPAF activity required for keratin 8 cleavage led to a significant reduction in the vacuole size (data not shown), suggesting that keratin 8 cleavage may be necessary for vacuole expansion in chlamydia-infected cells. However, this observation requires confirmation with more specific inhibitors since the lactacystin used for blocking CPAF activity can also inhibit host 20S proteasomal activity. Second, the keratin 8 cleavage by CPAF may also be associated with the enhanced survival of infected cells. A keratin 8 fragment starting at residue 73 with a molecular mass of 38 kDa was detected in human breast adenocarcinoma cell line MCF-7 (14), indicating that keratin 8 can be similarly cleaved in tumor cells independent of chlamydial infection. The present study demonstrated that the keratin 8 fragment is present in chlamydia-infected HeLa cells but not in normal HeLa cells (Fig. 1 and 3). Since MCF-7 cells are considered to be more malignant and invasive than HeLa cells, the presence of keratin 8 fragments may correlate with cellular malignancy. Although increased expression of keratin 8 has been found at the invasive front of some tumors (11, 21), the degree of keratinization was inversely correlated with the metastasis potential of tumors (18), suggesting that the increased expression of keratins may lead to generation of soluble keratins and keratin fragments that are associated with cellular malignancy. This hypothesis is supported by the observation that both proteolytic fragments of keratin 8 and increased levels of keratins have been detected in cancer cells but not in normal epithelial cells (5). In addition, keratin 8 was detected on the external surfaces of some tumor cells (10), and carcinoma cell-released keratin 8 can act as a plasminogen receptor (8, 9). However, it is still not clear how the enhanced soluble keratins and keratin fragments positively contribute to cellular malignancy (11, 16, 19). Regardless of the mechanisms by which keratin proteolysis affects cellular malignancy, chlamydiae have somehow acquired a similar mechanism for enhancing the survival of the infected cells, which is consistent with the previous observation that chlamydiae can prevent infected cells from undergoing apoptosis (6). Finally, degradation of host proteins by CPAF may also provide nutrients for chlamdyial biosynthesis. So far, three host proteins targeted by CPAF, including the transcription factors USF-1 (30) and

RFX5 (31) and cytoskeleton keratin 8 (this study), have been clearly identified. Interestingly, all three of these proteins are abundant and are both constitutively and ubiquitously expressed in epithelial cells, the natural targets of chlamydial infection. Degradation of these proteins can provide a stable source of nutrients for chlamydial biosynthesis even when the levels of extracellular nutrients are low and host new protein synthesis is completely shut down. The chlamydial genome does encode various transporters for oligopeptides and amino acids, which may allow chlamydiae to exploit the nutrients generated in the host cell cytosol. However, more experiments are required to evaluate whether host protein degradation products are indeed preferentially used by chlamydiae.

The fact that CPAF can effectively cleave both keratin 8 (this study) and transcription factors USF-1 and RFX5 (30, 31) suggests that chlamydiae have evolved a single molecule for multiple purposes, which is also observed during viral infection. For example, the viral 2A proteinase can cleave both host keratin 8 and the eukaryotic initiation factor 4 G in order to alter cellular integrity and, at the same time, turn off host cell protein synthesis (17, 23). The next question is how a single enzyme targets multiple molecules. The viral 2A proteinase can degrade its multiple targets by recognizing a consensus cleavage sequence (23). It is likely that CPAF can also recognize a structural motif shared by all its target molecules. Efforts are under way to further characterize CPAF enzymatic activity and determine the structural basis of CPAF substrate specificity.

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