Identification of a Chlamydial Protease–like Activity Factor Responsible for the Degradation of Host Transcription Factors

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Abstract

Microbial pathogens have been selected for the capacity to evade or manipulate host responses in order to survive after infection. Chlamydia, an obligate intracellular pathogen and the causative agent for many human diseases, can escape T lymphocyte immune recognition by degrading host transcription factors required for major histocompatibility complex (MHC) antigen expression. We have now identified a chlamydial protease– or proteasome–like activity factor (CPAF) that is secreted into the host cell cytosol and that is both necessary and sufficient for the degradation of host transcription factors RFX5 and upstream stimulation factor 1 (USF-1). The CPAF gene is highly conserved among chlamydial strains, but has no significant overall homology with other known genes. Thus, CPAF represents a unique secreted protein produced by an obligate intracellular bacterial pathogen to interfere with effective host adaptive immunity.

Key words: chlamydial enzyme • degradation • transcription factors • antigen presentation • immune evasion

Introduction

Chlamydia has to replicate within a cytoplasmic vacuole of eukaryotic cells and has adapted so well that it can persist in its host for a long period of time. Although chlamydial infection is known to be responsible for many severe human diseases ranging from trachoma after ocular infections to life-threatening complications after urogenital infections, the mechanism of chlamydial pathogenesis is still not clear. It is thought that the persistent infection is a major cause of chlamydia-induced diseases in humans (1, 2). Chlamydia-infected cells are able to continuously release inflammatory cytokines (3). Unfortunately, the tissue-damaging inflammatory responses induced by chlamydial infection often fail to efficiently eliminate the chlamydial organisms that are hidden inside the persistently infected host cells. We have hypothesized that chlamydia may have evolved strategies for evading host defense mechanisms in order to establish and to maintain a persistent infection.

Multicellular organisms have evolved various responses for controlling microbial infections, which, in turn, select for microbial pathogens with the ability to evade these same host defenses. Our previous studies have shown that chlamydia possesses varied strategies for interfering with or preventing both immune recognition and immune effector activity (4–6). Chlamydial infection suppressed both MHC class I and class II antigen expression in the infected cells. The inhibition of MHC antigen expression was correlated with the degradation of host transcription factors RFX5 and upstream stimulation factor 1 (USF-1). Furthermore, degradation of both RFX5 and USF-1 was caused by a chlamydial protease– or proteasome–like activity (CPA) in the cytosol of chlamydia-infected cells and the CPA was inhibited only by an irreversible proteasomal inhibitor lactacystin among all the inhibitors tested (4, 5). A logical extension of these previous studies is to identify the factor(s) responsible for the chlamydial proteasome–like activity, which is the focus of this study. Using a column chromatography approach, we have purified a chlamydial protein that correlates with the CPA. We designated this chlamydial protein as CPA factor (CPAF).
Materials and Methods

Column Chromatography. For purification and sequence identification of CPAF, cytosolic protein preparation from ~200 large flasks (150 cm²) of chlamydia-infected HeLa cells (HeLa L2 S100; reference 6) was subjected to the following three consecutive column separations: DEAE, heparin, and Mono Q (all columns were from Amersham Pharmacia Biotech). An AKTA purifier 10 (Amerham Pharmacia Biotech) was used to run the columns. Fractions collected from each column were monitored for both degradation activity in a cell-free assay as we have established previously (4), and total protein profiles on SDS-polyacrylamide gels. Fractions with degradation activity were pooled and both the protein concentration and degradation activity of the pooled fractions were carefully titrated. These measurements were used to calculate the purification efficiency. The pooled fractions were loaded to the next column for further fractionation. However, the pooled fractions collected from DEAE column were diluted 1:2 with H₂O to reduce the salt concentration before the heparin column purification. Two dominant protein bands that correlated with the degradation activity from the final Mono Q column were excised for protein sequence identification using both mass spectrometry analysis (Biorealis Biosciences Inc.) and NH₂-terminal sequence determination (University of Victoria, British Columbia, Canada). For chromatographic analysis of CPA, a size exclusion column was loaded with HeLa L2 S100 and eluted with PBS as we described previously (4). The eluted fractions were subjected to both the RFX5 degradation activity measurement and Western blot detection of host 20S proteasome and CPAF components.

Western Blot. The Western blot assay was carried out as we described previously (7). Mouse antibodies were used to detect the NH₂-terminal portion of CPAF (CPAFn) fragment (CPAFn54b; mlgG1; data not shown), chlamydial major outer membrane protein (MOMP) (MC22; mlgG3; data not shown), eukaryotic HSP70 (IgG2a; Santa Cruz Biotechnology, Inc.), and 20S proteasome subunits (AFFINITI Research Products Limited). Rabbit antibodies were used to detect RFX5 (Rockland Immunochemicals), USF-1, and USF-2 (Santa Cruz Biotechnology, Inc.).

Cell-free Degradation Assay. Western blot assay was performed as described previously (4). To generate fusion proteins for the cell-free assay, chlamydial DNA sequences coding for CPAF or CPAF fragments were cloned into a pGEX vector (Amersham Pharmacia Biotech) and expressed as fusion proteins with the glutathione S-transferase (GST) as fusion partner. The fusion proteins were purified with glutathione-conjugated agarose beads as described in the manufaturer’s manual (Amersham Pharmacia Biotech). The following procedure was used to prepare nuclear extracts (NEs) as substrate (containing RFX5 and USF-1 and USF-2) for the cell-free assay. Normal HeLa cells were dounced to break cytoplasmic membranes and the pellets were repeatedly washed with a buffer consisting of 1% NP-40 and 150 mM NaCl in 50 mM Tris, pH 8.0, plus a protease inhibitor cocktail to remove cytosol/membrane proteins as much as possible. The final washed nuclear pellets were extracted with a buffer consisting of 0.5 M NaCl and 1% Triton X-100 in 20 mM Tris, pH 8.0. The NEs thus prepared are essentially free of 20S proteasome components as detected on Western blot (data not shown). The human RFX5 gene from pREP-4/RFX5 plasmid (provided by Dr. P.J. van den Elen, Leiden University Medical Center, Leiden, The Netherlands) was cloned into the pGEX vector and RFX5 was expressed in the JM109 Escherichia coli strain as a GST fusion protein. The GST–RFX5 fusion protein was purified to homogeneity using glutathione-conjugated agarose beads as described above. The purified GST–RFX5 was used as substrate in the cell-free degradation assays.

Immunofluorescence Staining Assay. Immunofluorescence detection of CPAF in chlamydia-infected cells was carried out as described previously (6, 7). In brief, HeLa cell monolayer was infected with Chlamydia trachomatis L2 for 30 h. The monolayer, after it was fixed with paraformaldehyde (Sigma-Aldrich) and permeabilized with Saponin (Sigma-Aldrich), was stained with Hoechst 32258 (blue), anti-MOMP antibody MC22 (probed with an FITC-conjugated, mouse IgG3-specific secondary antibody), and anti-CPAFn antibody 54b (probed with a Cy3-conjugated, mouse IgG1-specific secondary antibody). Images were acquired individually for each stain in gray using a Cooker digital camera connected to an AX70 Olympus microscope, and the single-color images were merged in frame into the triple-color image using the software Image Pro.

Immunoprecipitation Assay. The immunoprecipitation assays were carried out as described previously (4, 8). For antibody depletion of CPAF and the MOMP, both the anti-CPAFn and anti-MOMP antibodies were used to precipitate proteins in lysates of chlamydia-infected HeLa cells without radiolabeling (4). The intact lysate, the supernatant after antibody precipitation, and the proteins precipitated by the antibodies were all examined for their ability to degrade RFX5 in a cell-free assay. To visualize the proteins precipitated by the above antibodies, a radioimmunoprecipitation assay was performed (8). In brief, HeLa cells infected with chlamydia were metabolically labeled with [S³⁵]methionine/cysteine (ICN Biomedicals) and the proteins in the labeled cell lysate were precipitated with the corresponding antibodies. The supernatant after the first precipitation (I¹) was subjected to a second immunoprecipitation (I²) with the same antibodies. Both the anti-CPAFn and anti-MOMP antibodies completely removed the corresponding molecules from the lysates during I¹ precipitation.

Results

Purification and Sequence Identification of CPAF. A chlamydial protease–like activity (designated as CPA) has been detected in the cytosol of chlamydia-infected cells and its presence correlated with degradation of host transcription factors (4). To better understand the molecular mechanisms underlying chlamydial evasion of host immune recognition, we used a column chromatography approach to identify the factor(s) responsible for CPA (Fig. 1A). Although it was not possible to correlate any obvious protein bands with degradation activity in fractions eluted from either DEAE or heparin column, these two purification steps allowed us to remove most of the unwanted proteins and to dramatically enrich the degradation activity (Table I). The final Mono Q column purification resulted in ~1,000-fold increase in specific activity (Table I). Indeed, two predominant protein bands in fractions eluted from a final step Mono Q column correlated with the nuclear protein degradation activity (Fig. 1A). Both protein bands were excised for sequence identification using mass spectrometry (Fig. 1B). The sequences of a total of eight tryptic fragments derived from the bottom band matched...
the sequence of the NH₂-terminal portion of a chlamydial protein encoded by open reading frame (ORF) CT858 (sequence data are available from GenBank/EMBL/DDBJ under accession no. AE001359; http://violet.berkeley.edu:4231/ident.html; reference 9), whereas the sequences of 17 tryptic fragments from the top band matched the sequence of the COOH-terminal portion of the same chlamydial protein (Fig. 1 B). These observations indicate that the two purified protein bands are encoded by a single ORF in the chlamydial genome. We have thus designated the bottom band protein as CPAFn and the top as CPAFc (Fig. 1). The sequence identity of CPAF was further confirmed using conventional NH₂-terminal sequencing (data not shown). The CPAF gene has no significant overall homology to any other known genes despite its high conservation among chlamydial strains (9–11; data not shown). Although a short CPAF sequence (488 PICVLINEQDFSCADFFPVVLKDNLAR1IVGTRT 525) was found to share a significant homology with the COOH-terminal sequences of tail-specific proteinases from various species (data not shown), the function of the conserved sequence domain is not yet known. Because of the limited homology, the CPAF gene was designated as either hypothetical ORF or “predicted protease contain-
To monitor the purification efficiency, the pooled fractions from each column purification were titrated for determining both protein concentration and degradation activity. The activity was measured by serial dilution of the pooled fractions and expressed as the highest dilution factors per mg of protein, under which the degradation activity was still detectable. These measurements were further used for calculating the recovery and enrichment of CPAF activity.

*Final volumes of pooled fractions as indicated in Fig. 1 A (prior to dilution).

**CPAF activity was estimated based on the highest dilution of the pooled fractions when the CPAF degradation activity was still detectable and expressed as the highest dilution factors per mg of protein, highest dilution factors.

†The recovery of CPAF activity was calculated based on the total activity recovered after each purification step using the total activity in L2 S100 as 100%.

‡The enrichment was calculated based on CPAF activity per mg of protein (as described for Estimated CPAF activity above) using the activity in L2 S100 as the base.

| Purification step | Volume* | Total CPAF protein | Estimated CPAF activity ‡ | Estimated total CPAF activity | Estimated CPAF recovery † | Estimated CPAF enrichment ‡ |
|-------------------|---------|--------------------|---------------------------|------------------------------|---------------------------|------------------------------|
| L2 S100           | 100     | 5,310              | 0.094                     | 499                          | 100                       | 1                            |
| DEAE              | 70      | 214                | 0.97                      | 208                          | 42                        | 10                           |
| Heparin           | 150     | 13                 | 11.49                     | 149                          | 30                        | 122                          |
| Mono Q            | 5       | 0.5                | 9                         | 45                           | 9                         | 957                          |

To further confirm that CPAF alone is sufficient for the degradation activity, we expressed the human transcription factor RXF5 in a prokaryotic system as a GST fusion protein. The GST–RXF5 was purified to homogeneity (Fig. 2 E) and the purified protein was used as the substrate for measuring the degradation activity of the cloned CPAF in a cell-free assay (Fig. 2 F). The cloned CPAF completely degraded the endogenous RXF5 in HeLa cell NEs and the degradation activity was inhibited by lactacytin, which is consistent with the observations described above in Fig. 2 D. More importantly, the cloned CPAF also effectively degrading the MOMP failed to precipitate this activity from the lysates. To monitor the efficiency of the antibody precipitation, radiolabeled lysate from chlamydia-infected HeLa cells was similarly precipitated with anti-CPAFn (‡ precipitation) and the supernatant after the first precipitation was reprecipitated (II precipitation) with the same reagent (Fig. 2 C). Autoradiography after SDS-PAGE of the material in both the I and II immunoprecipitates revealed that the anti-CPAFn antibody effectively removed both the CPAFn and CPAFc fragments from the lysates during the II precipitation, because the II immunoprecipitate showed minimal amounts of CPAF fragments. The control anti-MOMP antibody efficiently precipitated MOMD but not CPAF molecules from the lysates (Fig. 2 C). Together, these experiments demonstrate that CPAF or CPAF-associated material is necessary for RFX5 degradation activity in chlamydia-infected cell lysates.

Table I. Summary of CPAF Purification

| Purification step | Volume* | Total CPAF protein | Estimated CPAF activity ‡ | Estimated total CPAF activity | Estimated CPAF recovery † | Estimated CPAF enrichment ‡ |
|-------------------|---------|--------------------|---------------------------|------------------------------|---------------------------|------------------------------|
| L2 S100           | 100     | 5,310              | 0.094                     | 499                          | 100                       | 1                            |
| DEAE              | 70      | 214                | 0.97                      | 208                          | 42                        | 10                           |
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To evaluate whether CPAF is sufficient for degrading host transcription factors, the protein encoded by the full-length CPAF gene was expressed as a fusion with GST, using a bacterial system. The purified GST–CPAF was evaluated for its ability to degrade RXF5 in a cell-free assay in comparison to material extracted from infected cells (HeLa L2 S100; Fig. 2 D). The HeLa L2 S100 degraded both the RXF5 and USF–1 transcription factors in NEs, whereas the material from uninfected HeLa cells (HeLa S100) failed to do so. Furthermore, even though the chlamydia proteolytic activity could be fractionated away from host cell 20S proteasomes (Fig. 2 A), the degradation activity of the HeLa L2 S100 was completely inhibited by lactacytin, an irreversible inhibitor of proteasomal proteases. These observations suggest that a chlamydia proteasome–like activity was being measured in this assay, as reported previously (4). More significantly, the purified GST–CPAF was able to degrade both RXF5 and USF–1 and the degradation was inhabitable by lactacytin. A GST fusion protein containing only the COOH-terminal portion of CPAF (GST–CPAf) failed to degrade either of the host nuclear proteins even at a concentration 20 times higher than that of GST–CPAF (Fig. 2 D). These observations demonstrated that the purified CPAF is sufficient to mediate the degradation of host transcription factors in HeLa cell NEs. However, this set of experiments failed to address whether other components in the NEs also contributed to the degradation activity.

To further confirm that CPAF alone is sufficient for the degradation activity, we expressed the human transcription factor RXF5 in a prokaryotic system as a GST fusion protein. The GST–RXF5 was purified to homogeneity (Fig. 2 E) and the purified protein was used as the substrate for measuring the degradation activity of the cloned CPAF in a cell-free assay (Fig. 2 F). The cloned CPAF completely degraded the endogenous RXF5 in HeLa cell NEs and the degradation activity was inhibited by lactacytin, which is consistent with the observations described above in Fig. 2 D. More importantly, the cloned CPAF also effectively degrading the MOMP failed to precipitate this activity from the lysates. To monitor the efficiency of the antibody precipitation, radiolabeled lysate from chlamydia-infected HeLa cells was similarly precipitated with anti-CPAFn (‡ precipitation) and the supernatant after the first precipitation was reprecipitated (II precipitation) with the same reagent (Fig. 2 C). Autoradiography after SDS-PAGE of the material in both the I and II immunoprecipitates revealed that the anti-CPAFn antibody effectively removed both the CPAFn and CPAFc fragments from the lysates during the II precipitation, because the II immunoprecipitate showed minimal amounts of CPAF fragments. The control anti-MOMP antibody efficiently precipitated MOMD but not CPAF molecules from the lysates (Fig. 2 C). Together, these experiments demonstrate that CPAF or CPAF-associated material is necessary for RFX5 degradation activity in chlamydia-infected cell lysates.
Figure 2. CPAF is both necessary and sufficient for degradation of host transcription factors. (A) HeLa L2 S100 was subjected to Superdex 200 size exclusion column analysis as described previously (4). The fractions were assayed for both RFX5 degradation activity and the presence of either host 20S proteasome subunits or CPAFn fragment. The presence of CPAFn but not host 20S proteasomes correlated with RFX5 degradation activity. (B) The effect of antibody depletion of CPAF on RFX5 degradation activity in chlamydia-infected HeLa cell lysates. Both the anti-CPAFn (54b) and anti-MOMP (MC22) antibodies were used to precipitate proteins from chlamydia-infected HeLa cell lysate. The intact (total) lysate, the supernatant after antibody precipitation, and the proteins precipitated by the antibodies (pellet) were all examined for their ability to degrade RFX5 in a cell-free assay. The anti-CPAFn antibody precipitated RFX5 degradation activity from supernatants to pellets. (C) Radioimmunoprecipitation analysis of proteins precipitated by anti-CPAFn or anti-MOMP antibodies. HeLa cells infected with chlamydia were metabolically labeled and the proteins in the labeled cell lysate were precipitated with antibodies. The supernatant after the first precipitation (I) was subjected to a second immunoprecipitation (II) with the same antibodies. Both the anti-CPAFn and anti-MOMP antibodies completely removed the corresponding molecules from the lysates during I precipitation. The ratio of cell lysate vs. antibody was the same as in Fig. 2 B. (D) Degradation of transcription factors RFX5 and USF-1 by CPAF in a cell-free assay with or without the inhibitor lactacystin. HeLa L2 S100 (containing chlamydia-synthesized CPAF) was used as positive control, and HeLa S100 was used as a negative control. Bacterium-expressed GST-CPAF was used at a final concentration of either 0.2 (low; sufficient for RFX5 degradation) or 0.6 mM (high; sufficient for both RFX5 and USF-1 degradation). The degradation was inhibitable by the proteasome inhibitor lactacystin (100 mM). A fusion protein containing GST and the COOH-terminal portion of CPAF (GST-CPAf) failed to degrade any of the nuclear factors even at 4 mM. The anti-USF-2 antibody can detect both USF-1 and USF-2 as described previously (reference 4). Ns, nonspecific binding. (E). Purification of the recombinant human RFX5 from a bacterial expression system. The GST-RFX5 fusion protein was purified with glutathione-agarose beads as described in Materials and Methods. Different amounts of the purified protein were loaded to a 12% polyacrylamide SDS gel. After electrophoresis, the gel was stained with Coomassie blue dye. The GST-RFX5 fusion protein has a MW of ~100 kD. (F). Degradation of the purified recombinant RFX5 by CPAF in a cell-free assay. The cell-free assay was carried out in the exact same way as described in D, except that 0.5 mg of the purified GST-RFX5 instead of the NEs was used as substrate in some reactions as indicated in the figure. The digestion experiment with the purified GST-RFX5 as substrate was repeated four times and similar results were observed. RFX5 in NEs is defined as endogenous and the bacterium-expressed GST-RFX5 fusion protein defined as recombinant.
graded the recombinant human RFX5 purified from a bacterial expression system (Fig. 2 F). Degradation of the recombinant RFX5 was inhibited by lactacystin, suggesting that the same enzymatic activity was responsible for the degradation of both endogenous and recombinant RFX5. A similarly purified control GST-CPAFc fusion protein failed to degrade the recombinant RFX5, suggesting that degradation of RFX5 measured in the assay was not contributed by bacterial contaminants (if there was any) or the GST fusion partner. These observations clearly demonstrate that the chlamydial CPAF alone is sufficient for degrading host RFX5.

Intracellular Distribution of CPAF during Chlamydial Infection. Although CPAF is encoded by the chlamydial genome, the host transcription factor degradation activity was detected in the cytosol of the infected cells (4), suggesting that CPAF is secreted into host cell cytosol. We next used cell fractionation followed by immunoblotting to further evaluate the subcellular distribution of CPAF during chlamydial infection (Fig. 3 A). The majority of RFX5 degradation activity was associated with the cytosolic fraction of HeLa cells infected with chlamydia (Fig. 3 A, top row; lane HeLa L2 S100), with a small amount of activity in the NE fraction (lane HeLa L2 NE). RFX5 degradation activity correlated very well with the presence of CPAFn fragment. The cytosolic fraction of uninfected HeLa cells had no protease activity (lane HeLa S100). Surprisingly, the purified chlamydial organisms themselves also had no capacity to degrade RFX5. These findings suggest that CPAF is mainly localized in the cytosolic fraction of the infected HeLa cells and does not accumulate to any appreciable extent in chlamydia organisms themselves.

![Figure 3](image-url)
An immunofluorescence assay was used to further confirm the intracellular distribution of CPAF (Fig. 3 B). CPAF was only detected in the cells containing chlamydial inclusion bodies but not the adjacent normal HeLa cells growing on the same coverslip, indicating that CPAF is restricted to chlamydia-infected cells. As expected from the cell fractionation studies, CPAF was mainly detected in the cytoplasmic portion of the infected cells, even though CPAF is encoded by the chlamydial genome. These observations suggest that the chlamydia-synthesized CPAF is secreted into the host cell cytoplasm, allowing it to access host proteins.

Discussion

Chlamydial growth occurs strictly within a modified cytoplasmic vacuole of eukaryotic cells (12), and the possibility of communication between chlamydial vacuoles and host cellular compartments has frequently been discussed (13–16). At least one purpose of such transfer of chlamydia-derived proteins to the invaded cell appears to be protection from host immune recognition (4, 5) and attack (6). Our previous studies suggested that chlamydia may secrete a CPAF into the host cell cytosol to degrade transcription factors required for MHC gene activation, thus limiting expression of these key proteins involved in T cell antigen recognition (4, 5). We have now identified the gene encoding CPAF that is secreted into host cell cytosol and demonstrated that CPAF is both necessary and sufficient for the degradation of the transcription factors RFX5 and USF-1. Interestingly, CPAF was identified as two separate polypeptides encoded by a single ORF in the chlamydial genome (Fig. 1 A). Both the CPAFn and CPAFc fragments are coprecipitated from chlamydia-infected cell lysates by an antibody that only recognizes the CPAFn fragment (Fig. 2 C). These observations suggest that CPAF may function in the form of intramolecular dimers. Whether CPAF cleavage into CPAFn and CPAFc is necessary for its degradation activity is not yet known. Although the details of the activation and secretion of CPAF remain to be fully determined, it is clear that by secreting a single factor into the cytosol, chlamydia can suppress both MHC class I and class II antigen expression. Many viruses are known to cause suppression or degradation of cellular proteins required for mounting host defense responses (17–19). However, these viral strategies are often dependent on the function of host proteasomes (20, 21). We have demonstrated that CPAF is both necessary and sufficient for degrading host transcription factors required for MHC antigen expression. Thus, this report identifies a novel molecular mechanism by which a nonviral intracellular pathogen interacts with its host and manipulates immune responses for its benefit. Further characterization of CPAF including its transportation, activation, and substrate accessibility and selectivity is underway.

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