Cleavage of the NF-κB Family Protein p65/RelA by the Chlamydial Protease-like Activity Factor (CPAF) Impairs Proinflammatory Signaling in Cells Infected with Chlamydiae*

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Chlamydiae are obligate intracellular bacteria that frequently cause human disease. Chlamydiae replicate in a membranous vacuole in the cytoplasm termed inclusion but have the ability to transport proteins into the host cell cytosol. Chlamydial replication is associated with numerous changes of host cell functions, and these changes are often linked to pro-inflammatory events. It has been shown earlier that the member of the NF-κB family of inflammation-associated transcription factors, p65/RelA, is cleaved during chlamydial infection, and a chlamydial protease has been implicated. We here provide evidence that the chlamydial protease chlamydial protease-like activity factor (CPAF) is responsible for degradation of p65/RelA during infection. This degradation was seen in human and in mouse cells infected with either Chlamydia trachomatis or Chlamydia pneumoniae where it correlated with the expression of CPAF and CPAF activity. Isolated expression of active C. trachomatis or C. pneumoniae CPAF in human or mouse cells yielded a p65 fragment of indistinguishable size from the one generated during infection. Expression of active CPAF in human cells caused a mild reduction in 1xBo phosphorylation but a strong reduction in NF-κB reporter activity in response to interleukin-1β. Infection with C. trachomatis likewise reduced this responsiveness. IL-1β-dependent secretion of IL-8 was further reduced by CPAF expression. Secretion of CPAF is, thus, a mechanism that reduces host cell sensitivity to a proinflammatory stimulus, which may facilitate bacterial growth in vivo.

Chlamydia trachomatis causes eye infections on a large scale especially in the developing world (often leading to blindness) and is the most frequent bacterial agent of sexually transmitted disease (1–3). Chlamydia (Chlamydophila) pneumoniae is an extremely common agent of usually mild respiratory infection and may through infection of arteries play a role in the pathogenesis of atherosclerosis (4). One clinically relevant feature of chlamydial infections is the potential for chronicity. A considerable share of female infertility for instance is the result of chronic pelvic inflammatory disease caused by C. trachomatis (3).

Chlamydiae have a life style that is atypical for bacteria. Chlamydiae can only replicate inside human (or animal) cells where they reside in a membrane-bounded vacuole termed inclusion. From this site the bacteria are able to interact with metabolic and signaling pathways of the host cell. Chlamydial infection causes alterations in gene expression, redirects vesicle transport especially to achieve lipid acquisition by the vacuole, blocks apoptosis, and can induce non-apoptotic cell death in human cells (5–7). These results are probably in many cases linked to the ability of Chlamydia to transfer bacterial proteins from the chlamydial inclusion into the host cell; in many cases this may be achieved by a protein injection apparatus known as type III secretion system (8).

A number of the effects on the host cell are caused by proteolysis of host proteins through bacterial proteases that gain access to the cytosol. A prominent role has been shown here for the protease chlamydial protease-like activity factor (CPAF)2 (9), which cleaves, for instance, the cytoskeleton components vimentin and cytokeratin 8 and the nuclear proteins PARP (poly(ADP-ribose) polymerase) and cyclin B1 (10–12).

It has recently been shown that the transcription factor NF-κB p65/RelA is cleaved at a single site during infection of human but not mouse cells with C. trachomatis or C. pneumoniae (13). Expression of candidate chlamydial proteases showed that the tail-specific protease (Tsp) CT441 has the capacity of cleaving p65 (13, 14).

NF-κB is a family of transcription factors with important gene regulatory functions in inflammation (15), and the inhibition of NF-κB by Chlamydia may be a relevant mechanism by which the bacteria counter inflammation and clearance of the infection by the immune response. Indeed, a second mechanism has been proposed by which Chlamydia blocks NF-κB activation, namely the de-ubiquitination of IkBα (16).

The protease CT441 is not known to be secreted from the vacuole to the cytosol, and although a cytosolic function of the protease is not impossible, CT441 is likely to play a role in the processing of bacterial proteins within the inclusion, as

2 The abbreviations used are: CPAF, chlamydial protease-like activity factor; NF-κB, nuclear factor κB; MMP, matrix metalloproteinase; m.o.i., multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MEF, mouse embryonic fibroblast.

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Chlamydial CPAF Cleaves NF-κB p65

Frank Hänel, Leibniz-Institute for Nature Products and Infection Biology, Hans-Knöll-Institute, Jena, Germany) were performed using FuGENE HD (Roche Applied Science) following the manufacturer’s instructions. In T-REx-293 cells, CPAF expression was induced by the addition of AHT/CM. Both compounds were added 1 h after transfection. In MEF cells lacking the tetr repressor, where induction of the CPAF was not necessary, 1 μM coumermycin was added 1 h after transfection to cluster gyrB-CPAF (12).

**Nuclear Fractionation**—T-REx-293 CPAF K6 cells (12) were stimulated with 10 ng/ml IL-1β (Peprotech, Hamburg, Germany) for various times. Cells were harvested and washed with PBS. Cells were pelleted (7 min, 1500 rpm, 4 °C) and incubated in ice-cold hypotonic buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, pH 7.9) supplemented with a protease inhibitor mixture on ice for 15 min at 4 °C. Cells were lysed by passage through a 26-gauge needle attached to a 1-ml syringe. The nuclei were pelleted by centrifugation for 5 min at 6,800 rpm at 4 °C. The cytosolic fraction was subjected to a second centrifugation step for 15 min at 13,000 rpm at 4 °C, frozen in liquid nitrogen, and stored at −80 °C. The nuclear fraction was washed 8 times in the hypotonic buffer and then incubated in cold buffer B (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 7.9) supplemented with protease inhibitor mixture and incubated for 45 min at 4 °C and 300 rpm. Finally the nuclear fractions were centrifuged for 15 min at 13,000 rpm, 4 °C. The supernatants were frozen in liquid nitrogen and stored at −80 °C.

**Immunoblotting**—Cells were harvested after infection or CPAF expression and lysed in radioimmune precipitation assay buffer. Cell extracts were separated using EZ-Run Gels (Fisher), and proteins were transferred onto nitrocellulose membranes. Equivalent amounts of protein were loaded, and equal loading was confirmed by detection of β-actin using a specific antibody (Sigma). Membranes were probed with anticaspase 8, anti-κB, anti-phospho-κB, anti-p42/p44 Erk, anti-phospho-p42/p44 Erk, anti-IKK-α (all from Cell Signaling Technology, Beverly, MA), anti-IKK-β (Upstate Biotechnology, Lake Placid, CA), anti-histone deacetylase 1 (Millipore, Schwalbach, Germany), anti-GAPDH (Chemicon, Temecula, CA), anti-vimentin (Acris, Herford, Germany), anti-p65/RelA (Santa Cruz, Heidelberg, Germany), and anti-CPAF (generated by immunization of rabbits with a peptide from the C-terminal fragment of *C. trachomatis* CPAF, Pineda, Berlin, Germany) antibodies. Proteins were visualized using peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (GE Healthcare).

**NF-κB Reporter Assay**—Cells stably expressing CPAF were lentivirally transduced with an NF-κB-luciferase reporter construct obtained from Dr. Hans Häcker (St. Jude’s Children’s Research Hospital, Memphis, TN). 2 × 10^5 cells were seeded in a 96-well plate and either infected with *C. trachomatis* or treated with AHT/CM to induce and activate CPAF. For the times indicated, 10 ng/ml IL-1β was added. After various periods of time cells were lysed using luciferase cell culture lysis reagent (Promega, Mannheim, Germany), and firefly luciferase activity was assayed with a luminometer (Berthold Detection Systems, Pforzheim, Germany).
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cell viability was tested by the MTT (Sigma) assay. After treatment of the cells with either *C. trachomatis*, AHT/CM, and/or IL-1/H9252, MTT was added to cells at a concentration of 0.5 mg/l and incubated at 37 °C for 1 h. The formazan crystals generated in viable cells were dissolved in DMSO, and the optical density at a wavelength of 570 nm was measured using a photometer (Tecan, Crailsheim, Germany).

IL-8 ELISA—T-REx-293 CPAF K6 cells were stimulated with IL-1β (10 ng/ml) to activate NF-κB signaling. Simultaneously, AHT/CM was added to induce and activate CPAF. Supernatants were collected after 8 h, and commercially available ELISA (R&D Systems, Wiesbaden-Nordenstedt, Germany, OptEIA™ set from BD Biosciences) was used to determine the concentration of secreted IL-8. The assays were performed as instructed by the manufacturer.

RESULTS

Cleavage of p65 during Infection of Human and Mouse Cells with *C. trachomatis* or *C. pneumoniae*—Human 293 cells (we used the variant T-REx-293 for better comparison with CPAF expressing cells; below) or MEFs were infected with *C. trachomatis* or *C. pneumoniae*, and p65 levels were followed over time by Western blotting. As reported earlier, p65 fragmentation was observed (13). A fragment of ~25 kDa appeared in human T-REx-293 cells between 16 and 18 h upon infection with *C. trachomatis* (Fig. 1A). As for the cleavage of other cellular substrates, the time course of cleavage varied with infectious dose (higher bacterial numbers caused earlier and stronger degradation; not shown). The time course of p65 cleavage correlated well with the appearance of active CPAF and with the cleavage of cellular vimentin, a well known CPAF substrate (Fig. 1A). Similar results were obtained when MEFs were infected with *C. pneumoniae* (m.o.i. of 5 for T-REx-293 or 10 for MEF) for the time periods indicated. Cell lysates were subjected to Western blot analysis for p65, CPAF, or the CPAF substrate vimentin. Shown is a representative result of three independent experiments.

**FIGURE 1. Infection with *C. trachomatis* causes cleavage of p65.** A. *C. trachomatis* infection leads to p65 cleavage in T-REx-293 cells. Cells were infected with *C. trachomatis* with an m.o.i. of 3 for the times indicated. Cell lysates were subjected to Western blot analysis for p65, CPAF, or the CPAF substrate vimentin. Shown is a representative result of five independent experiments. F, cleavage products of p65; *, unspecific background bands. CPAFc, C-terminal autoprocessing product of CPAF. B. in MEF cells *C. trachomatis* infection leads to p65 cleavage. Mouse embryonic fibroblasts were infected with *C. trachomatis* with an m.o.i. of 3 as indicated. Radioimmune precipitation assay buffer extracts were prepared and subjected to Western blot analysis for p65, CPAF, or vimentin. Shown is a representative result of four independent experiments. C. *C. pneumoniae* infection causes p65 cleavage in both in T-REx-293 and MEF cells. Cells were infected with *C. pneumoniae* (m.o.i. of 5 for T-REx-293 or 10 for MEF) for the time periods indicated. Cell lysates were subjected to Western blot analysis for p65, CPAF, or the CPAF substrate vimentin. Shown is a representative result of three independent experiments.
Chlamydial CPAF Cleaves NF-κB p65

CPAF Can Cause the Degradation of p65 in the Absence of Infection—CPAF is in bacteria synthesized as an inactive precursor, and its activation requires its proteolytic autoprocessing. When expressed in human cells, CPAF is inactive, but its activation can be induced by clustering of the precursor (12, 18). We used a system of CPAF activation by induced dimerization in human cells. In this system a fusion protein of bacterial gyrase B and the CPAF precursor is expressed in T-REx-293 cells under the control of a tetracycline-inducible promoter ("tet-on"). Dimerization of the fusion protein is induced by the compound coumermycin, which can bind two gyrase B-molecules and thereby dimerize gyrase B-CPAF; this process leads to autoprocessing and activation of CPAF (12).

We expressed and activated CPAF in this way and again monitored p65 levels by Western blotting. As shown in Fig. 2A, active CPAF (visible as the C-terminal autoprocessing product) was detectable after 6–8 h of CPAF-induction/dimerization. This correlated with the known cleavage of vimentin but also with the appearance of a p65 fragment of the same size as during infection. Disappearance of p65 was blocked by lactacystin (an inhibitor of CPAF and the cellular proteasome) but not by epoxomicin (a specific proteasome inhibitor) (Fig. 2B).

Transient transfection and activation of C. trachomatis CPAF caused the appearance of the same p65 fragment. In these experiments we also transfected CT441 for comparison. As shown in Fig. 3A, CT441 likewise induced degradation of p65 as reported. However, the fragment produced by CT441 was, although similar in size, appreciably bigger than the fragment generated by either CPAF transfection or by infection with C. trachomatis, suggesting that during infection it is CPAF that degrades p65. When transfected into MEFs, C. trachomatis CPAF also caused the appearance of a p65 fragment that ran slightly higher in the SDS-PAGE than the one in human T-REx-293 cells (Fig. 3B). Transfection of CPAF from C. pneumoniae (Cpn CPAF) produced a fragment of indistinguishable size (Fig. 3B). Cleavage of p65, therefore, appears to be conserved between by Ct and Cpn CPAF as well as between human and mouse cells.

CPAF Inhibits the Activation of NF-κB by IL-1β—To test for the functional relevance of CPAF-mediated p65-cleavage, we analyzed key steps in the activation of NF-κB in cells ex-
pressing active CPAF. T-REx-293-CPAF cells were stimulated with IL-1β, which activates the classical NF-κB-pathway through the IL-1-receptor, its adapter MyD88, and the activation of the IκB kinase IKK-β. Phosphorylated IκB is then degraded, releasing active p65, which translocates to the nucleus and activates NF-κB-dependent promoters, typically together with the NF-κB family member NF-κB1/p50 (15).

In cells expressing active CPAF, phosphorylation and consecutive degradation of IκBα upon stimulation with IL-1β were reduced although still clearly detectable, whereas the IL-1β-dependent phosphorylation of the mitogen-activated protein kinases Erk1/2 was normal (Fig. 4A). This may reflect a destabilization of the IκBα-p65 complex due to the detectable degradation of p65 (Fig. 4A). There was no reduction in the levels of the IκBκα/β either upon expression of active CPAF or during infection with C. trachomatis (Fig. 4, B and C). This suggests that the upstream pathway of MyD88-dependent signaling to the activation of the IKK-complex is unaffected. This is in accordance with the interpretation that it is specifically p65 and p65-dependent transcriptional activity that is targeted by CPAF during infection.

To test for this activity directly, we used T-REx-293-CPAF cells that had been transduced with a lentivirus carrying an NF-κB-luciferase reporter plasmid. In these cells the expression of active CPAF was induced as above for 8 h, and IL-1β was added for various periods of time. As shown in Fig. 5A, in the absence of CPAF, IL-1β induced a strong NF-κB-response as expected. When active CPAF was present, IL-1β-dependent induction was substantially reduced. As prolonged expression of active CPAF can induce cell death under these conditions, we controlled viability of the cells in parallel and found only very minor effects on viability under these conditions (Fig. 5A, right panel). A similar reduction in IL-1β-dependent NF-κB activation was detectable at later time points (i.e. upon prolonged stimulation with IL-1β) (Fig. 5A). Treatment of T-REx-293 NF-κB-luciferase cells which do not carry the CPAF construct, with AHT and CM had no effect on reporter activity (Fig. 5C).

When the T-REx-293-CPAF NF-κB-luciferase cells were infected with C. trachomatis simultaneously to the addition of IL-1β, there was no effect of Chlamydia at 6 h post-infection (Fig. 5B). However, when luciferase activity was measured 24 h after infection, the activation of NF-κB was again reduced (active CPAF is detectable after 16–18 h under these conditions; Fig. 1). Although it is difficult to gauge the possible effect of bacterial factors other than CPAF during infection (especially as the viability of the cells is also affected), the magnitude of the reduction during infection was not higher than during activation of CPAF in uninfected cells (compare Fig. 5, A and B). This is in accordance with the interpretation that CPAF is the main factor that Chlamydia uses to down-regulate the response to NF-κB-inducing stimuli.

The p65 fragment generated by active CPAF (the antibody detects the C-terminal fragment) remained exclusively cytosolic during stimulation with IL-1β (Fig. 6). This fragment contains the NF-κB-activating domain but lacks the nuclear localization domain (19). The observed effects (no nuclear translocation and lack of reporter activity) are in accordance with these structural features.
As a functional test of the relevance of this reduction in NF-κB activation by CPAF, we determined its effect on the NF-κB-dependent secretion of IL-8 upon IL-1β stimulation (20). We treated T-REx-293-CPAF cells with IL-1β for 8 h and induced and activated CPAF at the same time. Supernatants were analyzed for secreted IL-8. Activation of CPAF led to a clear reduction in the amounts of secreted IL-8 (Fig. 7). Taken together, these data suggest that the CPAF-dependent degradation of p65 and the consecutive reduction in NF-κB activity during infection with human or mouse cells with *Chlamydia* inhibits a proinflammatory response of infected host cells.

**DISCUSSION**

Many bacteria have the ability to interfere with host signaling pathways. *Chlamydia* is well known for its capacity to af-
that is responsible for this cleavage during infection. Although CPAF was tested in the earlier study and was found not to cleave p65 (13), this was done by transient expression of CPAF. It is now clear that CPAF is not active when expressed in that way but requires intermolecular clustering for its activation (12, 18, 22). This peculiarity explains the lack of activity of CPAF in those experiments. CT441 was further shown not to cleave mouse p65 (13, 14), whereas CPAF did (this study). However, although the previous studies also failed to detect p65 cleavage during \textit{C. trachomatis} infection of mouse cells, we did detect this cleavage. Infection and growth of \textit{Chlamydia} in mammalian cells can vary strongly between host cells, and it is plausible that the lack of detectable p65 cleavage was the result of less efficient growth of \textit{C. trachomatis} in the NIH 3T3 cells used (13). We find clear, albeit somewhat weaker cleavage of mouse p65 compared with human p65, because recombinant CT441 can cleave human but not mouse p65, this profile, therefore, also argues for CPAF as the protease responsible. Furthermore, the p65 cleavage product generated during infection is indistinguishable on SDS-PAGE from the product generated by CPAF cleavage but was slightly smaller than the product generated by expression of CT441.

Last, although CPAF is clearly secreted into the cytosol, there is no reported evidence that CT441 is released from the vacuole into the host cell. Together, we believe that this is strong evidence that the cleavage of p65 during chlamydial infection is mediated by CPAF.

It is perhaps surprising that two chlamydial proteases, CPAF and CT441, have the ability to cleave at least human p65. However, both proteases have structural features of Tsp (although only CT441 has the typical PDZ-domain), and this may explain this related cleavage specificity. Tsp are a relatively little characterized group of bacterial proteases (23, 24) and are usually involved in processing steps of bacterial proteins (25, 26). CT441 may, therefore, have important functions in growth and development of \textit{Chlamydia} in the inclusion. Although this may also be the case for CPAF, a number of host cell substrates have been identified that are cleaved by active CPAF and that are cleaved during infection (21). The precise role of CPAF for chlamydial development is not clear. Effects of CPAF activity include impairment in antigen presentation (cleavage of transcription factors (9) and the major histocompatibility complex-like molecule CD1d (27)) and the cleavage of cytoskeletal proteins (vimentin, cytokeratin-8 (10, 28)) and the histocompatibility complex-like molecule CD1d (27)) and the cleavage of cytoskeletal proteins (vimentin, cytokeratin-8 (10, 28)).

Any of these events may contribute to the success of \textit{Chlamydia} in surviving and replicating in human cells.

However, the phylum Chlamydia does not only include the human-pathogenic chlamydiae such as \textit{C. trachomatis} and \textit{C. pneumoniae} but also a large group of bacteria that have been found as symbionts in free-living amoebae (28). Notably, these “environmental chlamydiae” can also contain genes coding for CPAF (29). It may, therefore, be speculated that CPAF is an enzyme that has already been required for replication of \textit{Chlamydia}-like organisms (the evolutionary precursor of today’s bacteria) in single-celled hosts. Some core CPAF functions may be conserved to all these \textit{Chlamydia}-like organisms, and some may have been acquired for survival in...
metazoan hosts, and p65 cleavage would be an example of the latter category.

NF-κB is an important transcription factor family in the immune response, and it is likely that impaired NF-κB signaling will be of benefit to Chlamydia. During the infection and the accompanying inflammation, a broad array of inflammatory mediators is produced that also signal to the infected cells. As in these cells, NF-κB activation will be impaired; this inhibition can be expected to limit the defense response of the infected cell and to enhance replication of Chlamydia. CPAF may, therefore, have adopted a new function in human pathogenic chlamydiae, which adds to the ability of this group of bacteria to survive for a sufficient time to replicate despite a functional immune system. This novel function may even contribute to the ability of the bacteria to survive the host response and to proceed into the chronic state of the infection.

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