Cytopathicity of *Chlamydia* is largely reproduced by expression of a single chlamydial protease

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Chlamydiae replicate in a vacuole within epithelial cells and commonly induce cell damage and a deleterious inflammatory response of unknown molecular pathogenesis. The chlamydial protease-like activity factor (CPAF) translocates from the vacuole to the cytosol, where it cleaves several cellular proteins. CPAF is synthesized as an inactive precursor that is processed and activated during infection. Here, we show that CPAF can be activated in uninfected cells by experimentally induced oligomerization, reminiscent of the activation mode of initiator caspases. CPAF activity induces proteolysis of cellular substrates including two novel targets, cyclin B1 and PARP, and indirectly results in the processing of pro-apoptotic BH3-only proteins. CPAF activation induces striking morphological changes in the cell and, later, cell death. Biochemical and ultrastructural analysis of the cell death pathway identify the mechanism of cell death as nonapoptotic. Active CPAF in uninfected human cells thus mimics many features of chlamydial infection, implicating CPAF as a major factor of chlamydial pathogenicity, *Chlamydia*-associated cell damage, and inflammation.

Introduction

*Chlamydia trachomatis*, an obligate intracellular bacterium, is the leading cause of bacterially sexually transmitted disease and a main cause of preventable blindness. *C. trachomatis* has a biphasic developmental cycle. The infectious elementary body (EB) infects primarily epithelial cells where it develops within a membrane-bound vacuole (called an inclusion) into a replicating noninfectious reticulate body (RB). Within two days, the RB redifferentiates into an EB and is released from the infected cell. Chlamydiae thus develop in a compartment that is separate from the rest of the human or animal host cell. However, the bacteria impact on a number of signaling pathways in the host cell, and cause substantial changes to cellular transcription as well as cell damage (McClarty, 1994; Wyrick, 2000; Fields and Hackstadt, 2002). How *Chlamydia* achieves this is not known in great detail. Several chlamydial species have been found to possess the components of a functional type III secretion system, which likely enables the bacteria to inject effector proteins into the host cytosol, and a number of such candidate proteins have been identified (for review see Peters et al., 2007).

Infection with *Chlamydia* causes massive stress to the host cell, and cytolytic activity associated with *Chlamydia* infection has been described for more than 30 years (Friis, 1972; Todd and Storz, 1975; Chang and Moulder, 1978; Wyrick et al., 1978). By electron microscopy, massive changes to organelles were noticed at later stages of infection, such as dilation and vacuolation of ER, distortion of mitochondria, and nuclear condensation (Todd and Storz, 1975; Todd et al., 1976). Although some of these changes resemble features of apoptosis (Ojcius et al., 1998; Perfettini et al., 2002; Ying et al., 2006), further characterization of signaling pathways indicates that the apoptotic pathway is not activated by *Chlamydia* and the cytopathic changes observed are nonapoptotic (Ying et al., 2006). Cytopathicity and cell death may be a defense mechanism of the cell to block bacterial replication or may aid bacterial spreading and cause infection-associated inflammation.

It has been entirely unclear how *Chlamydia* induces cell death. The gene of a potential cytotoxin has been identified in the *C. trachomatis* genome (Belland et al., 2001), but was later found to be nonfunctional in many serovars (Carlson et al., 2004).
One chlamydial protein has been directly purified from the cytosol of infected cells. Named chlamydial protease-like activity factor (CPAF), this protein was isolated as a factor that can degrade two host transcription factors, RFx5 and USF-1 (Zhong et al., 2001). Because these transcription factors are involved in the expression of major histocompatibility complex (MHC) molecules, it has been speculated that CPAF may contribute to immune evasion of Chlamydia (Zhong et al., 2001). Similarly, it has been suggested that CPAF plays a role in the loss of expression of the MHC-like protein CD1d during infection, which may also enhance escape from host immune surveillance (Kawana et al., 2007). The cytoskeletal protein cytokeratin (CK) 8, a component of intermediate filaments, has also been identified as a substrate of CPAF proteolysis (Dong et al., 2004c), and recently it has been proposed that the pro-apoptotic BH3-only proteins, which are degraded during chlamydial infection (Fischer et al., 2004; Ying et al., 2005), are CPAF substrates (Pirbhai et al., 2006). Because Chlamydia cannot be genetically modified, direct proof of the role of CPAF has been difficult.

CPAF is synthesized in the chlamydial inclusion as one polypeptide, but is rapidly processed into two subunits that assemble into heterodimers and are proteolytically active in the host cytosol (Dong et al., 2004a,b). Expression of the CPAF precursor in human cells did not induce CPAF processing and yielded no proteolytic activity (Dong et al., 2004a).

Although potential functions had thus been assigned to CPAF, we reasoned that an active protease, free in the cytosol of a human cell, may be expected to damage the cell, and that CPAF therefore should be a candidate factor for cytopathic activity. However, until now it has not been possible to express active CPAF in human cells in the absence of infection, and meaningful analysis of CPAF effects in infected cells is very difficult due to the presence of numerous bacterial components in the cell.

We here show that CPAF can be activated by "induced proximity" in the absence of infection. The induced proximity model was proposed to explain the activation of initiator caspases during induction of apoptosis (Salvesen and Dixit, 1999; Pop et al., 2006; Bao and Shi, 2007). According to this model, the dimerization and activation of initiator caspases requires their adaptor-mediated clustering, followed by caspase processing. Similarly, we describe that forced clustering of CPAF leads to its processing and activation in human cells. Activation of CPAF caused massive morphological changes and nonapoptotic death of human host-cells, strongly resembling the changes and the form of cell death induced by chlamydial infection. CPAF should therefore be regarded as a major factor of chlamydial pathogenicity. CPAF activity may help Chlamydia at earlier stages to establish the growing inclusion in the cell and at later stages may facilitate release of newly replicated bacteria.

**Results**

**Activation of CPAF by induced proximity**

To study the role of CPAF in cytopathicity, a model had to be developed to express active CPAF. We had noticed certain parallels of caspase-9 and CPAF. Both are synthesized as inactive precursors, both are proteolytically processed into two subunits during their physiological activation, and in both cases the active enzyme is made up of a complex of both subunits. We therefore aimed at achieving experimental oligomerization of CPAF to model the physiological clustering of caspase-9 after its recruitment into the apoptosome during apoptosis (Bao and Shi, 2007). The open reading frame of C. trachomatis CPAF was fused to an N-terminal partner consisting of the FLAG antibody epitope and a triple repeat of a fragment of bacterial gyrase (gyrB). GyrB binds the cell-permeable synthetic ligand coumermycin (CM). Because CM has two gyrB binding sites, it can bind two gyrB proteins simultaneously, inducing dimerization of the fusion partner of gyrB (Fig. 1A). A triple repeat rather than an individual gyrB molecule was chosen to enhance complex formation upon CM addition. We and others have used this system in the past for conditional complex formation of intracellular signaling proteins (Farrar et al., 1996; Hacker et al., 2006). Because transient transfections indicated that the protein was toxic to human cells (unpublished data), the fusion construct was placed under the control of a tetracycline (TET)-inducible promoter, where expression was silenced in cells carrying the tet-repressor but induced upon addition of tetracycline ("tet-on").

When the gyrB-CPAF construct was transfected into 293T cells stably carrying the tet-repressor (T-REx-293), the addition of tetracycline or its analogue anhydrotetracycline (AHT) induced the appearance of a protein of the expected size of the full-length protein as well as a smaller product, which ran at the expected size of a protein containing the N-terminal tag fused to the N-terminal CPAF-fragment, indicating processing of the protein (Fig. 1B). T-REx-293 cells were then transfected and clones were selected that stably carried the TET-inducible gyrB-CPAF construct. Five clones were identified, in which addition of TET/AHT induced expression of a protein of the same size as the smaller product during transient transfection, again very likely corresponding to the fragment of gyrB and the N-terminal fragment of CPAF (Fig. 1B and unpublished data). No band corresponding to the intact protein was detected in these clones, suggesting more efficient processing. All of the clones showed the same phenotype upon induction of gyrB-CPAF (see following paragraph). Expression of gyrB-CPAF thus leads to spontaneous processing of the protein.

To test for CM-mediated oligomerization, cell extracts from the stable clone K6 expressing gyrB-CPAF were analyzed by size exclusion chromatography. GyrB-N-CPAF was detectable with a peak around the expected molecular weight of the protein (120 kD). Addition of CM caused a shift in the protein to higher molecular weight fractions (Fig. 1C). Because of the low expression of wild-type gyrB-CPAF, we performed additional experiments with a cleavage-deficient mutant of gyrB-CPAF (CPAFmut1 [see following paragraph], which is expressed at considerably higher levels), expressed by transient transfection and induction in T-REX-293 cells. As shown in Fig. 1C, most of the protein eluted at about the predicted size of the unprocessed monomer (150 kD), although easily detectable amounts appeared in fractions containing higher molecular weight proteins. Addition of CM caused a shift of the protein peak, which then eluted...
CPAF activity requires its processing site and an intact protease motif

CPAF activity in lysates from infected cells was inhibited by an inhibitor of the cellular proteasome, lactacystin, but not another one, MG-132 (Zhong et al., 2001). We used CPAF K6 cell lysate as a source of CPAF and lysate from cells transfected with a construct encoding myc-tagged CK8 as a substrate, to test for the effects of standard protease inhibitors on CPAF. Lactacystin but not MG-132, nor any other tested inhibitor, prevented degradation of CK8 (Fig. 2A and unpublished data). (The following inhibitors were tested: E64, pepstatin A, PMSF, TPCK, and a standard mix of protease inhibitors [Roche].) These results confirm the inhibitory activity of lactacystin previously reported in lysates from infected cells and identify an unusual inhibitor profile for CPAF.

It has been shown that a small amount of CPAF is cleaved when expressed in *Escherichia coli*, and the processing site has been mapped; a cleavage site mutant was not processed and was inactive when expressed in *E. coli* (Dong et al., 2004a). It has further been noted that CPAF contains a domain characteristic of bacterial Tail-specific proteases (Tsp) (Shaw et al., 2002), a class of serine proteases originally described in *E. coli* (Silber et al., 1992). We therefore generated gyrB-CPAF constructs with

Around 500 kD and might correspond to a trimer or tetramer of the protein. A substantial fraction of the protein appeared to be engaged in formation of even higher molecular weight complexes. Most of these CM-induced complexes could be disrupted by addition of an excess of the monomeric ligand of gyrase B, novobiocin (NB) (Fig. 1C). These results show the expected oligomerization of gyrB-CPAF by CM, as well as substantial spontaneous complex formation. The spontaneous oligomerization was probably due to the gyrase B domains because novobiocin could reduce the extent of complex formation (compare lanes AHT and AHT/CM/NB in gyrB-CPAFmut1; Fig. 1C).

We next measured the proteolytic activity of gyrB-CPAF, using cleavage of CK8, one of the reported cellular CPAF substrates, as a read-out. As shown in Fig. 1D, titration of AHT on the CPAF K6 clone caused increasing cleavage of CK8 even in the absence of CM-induced oligomerization, suggesting that spontaneous aggregation was sufficient for activation of gyrB-CPAF. Addition of CM to cells induced with a lower concentration of AHT caused a higher level of CK8 degradation, which could be partly blocked by increasing concentrations of novobiocin (Fig. 1D). GyrB-CPAF thus shows some spontaneous aggregation and activity, which can be enhanced by CM; the effect of CM can be prevented by NB.
A number of control proteins (Bak, Bcl-2, actin) were not degraded (Fig. 3; see following paragraph regarding cleavage of BH3-only proteins). The expression of active CPAF thus recapitulates the known proteolytic activities observed during infection with whole chlamydiae. Based on the levels of substrate cleavage, the amount of active gyrB-CPAF produced corresponds to the levels of CPAF expressed relatively early in the developmental cycle, at least in T-REx-293 cells. The amounts generated during later stages of infection are probably substantially higher and cause more complete degradation of cellular substrates (Fig. 3).

Degradation of anti-apoptotic BH3-only proteins is likely an indirect consequence of CPAF expression

BH3-only proteins are essential mediators of mitochondrial apoptosis (Hacker and Weber, 2007). These proteins are degraded during chlamydial infection (Fischer et al., 2004), which can account for the protection against apoptosis of infected cells. Re-expression of active BH3-only proteins overcomes the Chlamydia-imposed block of apoptosis (Fischer et al., 2004), indicating that this loss is functionally relevant. It has recently been reported that CPAF can degrade BH3-only proteins in cell lysates (Pirbhai et al., 2006). In our initial analyses, we failed to see degradation of the BH3-only proteins Bim and Puma (unpublished data), which were easily detectable in CPAF K6 cells, CPAF: A number of control proteins (Bak, Bcl-2, actin) were not degraded (Fig. 3; see following paragraph regarding cleavage of BH3-only proteins). The expression of active CPAF thus recapitulates the known proteolytic activities observed during infection with whole chlamydiae. Based on the levels of substrate cleavage, the amount of active gyrB-CPAF produced corresponds to the levels of CPAF expressed relatively early in the developmental cycle, at least in T-REx-293 cells. The amounts generated during later stages of infection are probably substantially higher and cause more complete degradation of cellular substrates (Fig. 3).
although both proteins were degraded during infection with
C. trachomatis (Fig. 4 A). However, longer periods of CPAF in-
duction in K6 cells did lead to the degradation of Bim and Puma
(Fig. 4 A). More extensive time-course studies revealed that
Bim degradation occurred later than cleavage of the other sub-
strates (Fig. 4 B). Although vimentin was already cleaved at 10 h
after CPAF induction/activation with AHT/CM, the Bim levels
were unchanged or even increased up to 15 h of CPAF activa-
tion, after which point they began to decrease. At 18 h after acti-
vation, Bim levels were clearly reduced. No smaller fragments
of Bim were detected (Fig. 4 B; vimentin cleavage starts around
4 h under this protocol; unpublished data). This suggested that
the degradation of Bim was not mediated directly by CPAF but
by subsequent proteolytic events that had been initiated by
CPAF. This interpretation is supported by another finding: the
degradation of Bim by prolonged activation of CPAF was
blocked by lactacystin but not MG-132 (Zhong et al., 2001; Fig.
2 A). It is thus unlikely that CPAF directly degrades BH3-only proteins. Nevertheless, the CPAF-induced degradation of BH3-only proteins is the main reason for apoptosis inhibition in infected cells, and CPAF is therefore the main anti-apoptotic factor of Chlamydia.

CPAF expression leads to nonapoptotic cell death
These results showed that gyrB-CPAF could reproduce the known proteolytic events of chlamydial infection. We then turned to the
question of the cellular consequences of CPAF activity. On the
one hand, CPAF induced the degradation of BH3-only proteins
and is therefore an anti-apoptotic effector. On the other hand, free
proteases in the cytosol have the potential to cause cell death.
This has been shown not only for the specialized caspases, but also,
for example, for lysosomal peptidases (Lockshin and Zakeri,
2004) and even the promiscuous protease proteinase-K (Wilhelm
and Hacker, 1999). Chlamydial infection causes massive mor-
phological changes to the host cell as well as nonapoptotic cell
death (Ojcius et al., 1998; Belland et al., 2001; Perfettini et al.,
2002; Ying et al., 2006). We therefore asked whether CPAF might
contribute to this cytopathicity.

The expression and activation of CPAF in K6 cells (Fig. 5 A)
causedit striking morphological changes in the cells. The cells
rounded up and began to detach from the culture dish. They
formed clusters and at later stages, smaller vesicular fragments
appeared (Fig. 5 A). Similar albeit less pronounced changes
were observed upon transient transfection and activation of gyrB-
CPAF in T-REx-293 or T-REx-HeLa cells (Fig. S1, available at
http://www.jcb.org/cgi/content/full/jcb.200804023/DC1). Indeed,
the morphology resembled the changes observed during chla-
mydial infection of T-REx-293 cells (Fig. S2). Although not
conclusive, this similarity suggests that CPAF is involved in at
least some of the morphological changes induced during chla-
mydial infection. AHT on its own caused less dramatic changes
in CPAF K6 cells than when CM was included, and an excess of
novobiocin could reduce the phenotypical changes (Fig. 5 B).
Cleavage of cellular proteins by CPAF may therefore be one
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The dramatic changes as observed by microscopy sug-
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prominent cleavage band was different from the one generated during apoptosis; a very light band corresponding to apoptotic cleavage appears to be visible on the blot, although the appearance of this band was not sensitive to caspase inhibition (Fig. 6E). Induction of apoptosis by treatment of the cells with TNF/CHX yielded the typical caspase-dependent PARP fragment (Fig. 6E). Although PARP cleavage is thus seen upon CPAF activation, it is not mediated by caspase-3 but probably by CPAF itself. The relevance of the cleavage of this new CPAF substrate for the infection remains to be seen.

We finally used electron microscopy for the analysis of structural changes in cells containing active CPAF. At earlier stages (after 7 h of CPAF expression/activity), although the cells had already rounded up and detached from the plate, no ultrastructural changes were observed (unpublished data). This is perhaps somewhat surprising given the strong morphological changes, but is in accordance with the unaffected metabolic activity at this time. At 20 h, however, only relatively few cells were still morphologically intact while the majority were in a process of disintegration (Fig. 7A, B). Only few apoptotic cells were detected in this analysis but many necrotic cells, confirming that CPAF induced nonapoptotic (necrotic) cell death in CPAF K6 cells. At higher magnification, dying cells showed numerous lamellar structures of unknown composition as well as many mitochondria that still seemed only slightly affected (Fig. 7C). Ectopic expression and activation of CPAF thus causes a nonapoptotic form of cell death that appears indistinguishable from cell death induced during infection with *C. trachomatis*.
dying cells, identifying this cell death as nonapoptotic or necrotic. All the observed features recapitulated the changes seen in cells infected by C. trachomatis. Infections with Chlamydia commonly induce strong inflammatory responses. These reactions may thus be the result of the CPAF-mediated release of not only bacterial but also pro-inflammatory cellular molecules.

We were prompted to test for the possibility of activation by induced proximity by similarities between the known characteristics of CPAF and the well-established process of activation

Discussion

This study shows that C. trachomatis CPAF can be activated by oligomerization to induce degradation of host cell proteins. On the one hand, CPAF caused the degradation of pro-apoptotic BH3-only proteins and is therefore a mediator of chlamydial anti-apoptotic activity. On the other hand, CPAF induced cell death in the absence of hallmarks of apoptosis. Activation of the apoptotic pathway was observed only in a small minority of

Figure 5. CPAF expression leads to changes in cellular morphology. (A) Changes in cell morphology during CPAF expression. CPAF K6 cells were incubated either with 6 ng/ml AHT, CM, or both for 16 h. Cells were analyzed by light microscopy (left). Arrows indicate smaller vesicular fragments. Black bar, 10 μm. The right panel shows an enlarged section of either a control sample or a sample treated with AHT and CM. White bar, 3 μm. (B) Inhibition of CPAF oligomerization by novobiocin. CPAF expression was induced in CPAF K6 cells by adding AHT, or cells were left untreated. 30 min before addition of CM, the cells were incubated with indicated amounts of NB. Cells were analyzed by light microscopy. Bar, 10 μm.
or suspected (CPAF) to be autocatalysis, and both form, during physiological activation, complexes of the two subunits derived from intramolecular cleavage. Our analysis shows that they of initiator caspases, especially caspase-9. Both CPAF and caspase-9 are synthesized as zymogens that have low proteolytic activity. Both are activated by what has been shown (caspase-9) or suspected (CPAF) to be autocatalysis, and both form, during physiological activation, complexes of the two subunits derived from intramolecular cleavage. Our analysis shows that they

Figure 6. CPAF expression causes nonapoptotic cell death. (A) CPAF reduces cell viability. CPAF K6 or T-REx-293 cells were treated with the indicated combinations of TET, CM, and the caspase inhibitor zVAD-fmk (zVAD). As a positive control, cells were treated with TNF-α (TNF) and cycloheximide (CHX). After indicated time points, cell viabilities were measured by MTT assay. Relative cell viability was calculated (untreated cells were set to 100%). Data are normalized means/SEM of three independent experiments. (B) Analysis of nuclear morphology after CPAF expression by Hoechst staining. CPAF K6 cells were treated with 10 ng/ml AHT, CM, or zVAD-fmk as indicated. As a positive control of apoptosis, cells were treated with TNF/CHX (as described in A). After 16 h, cells were stained with the Hoechst 33342 dye and analyzed by fluorescence microscopy. Bar, 15 μm. (C) Caspase-3 activation during CPAF-expression. CPAF K6 cells were treated as described in B and analyzed by flow cytometry using an antibody specific for active caspase-3. (D) Analysis of caspase-3 activation by Western blotting. CPAF K6 cells were treated as described in B, and cell extracts were analyzed by Western blotting using an antibody specific for active caspase-3. Arrowheads indicate specific cleavage products of activated caspase-3. Detection of actin served as loading control. (E) Cleavage of PARP by infection with C. trachomatis or expression of active CPAF. (Left) CPAF K6 cells were infected with C. trachomatis for the indicated periods of time. (Right) CPAF K6 cells were treated as described in B. Cell extracts were analyzed by Western blotting using a PARP antibody. The arrowheads indicate cleavage products resulting from chlamydial infection or CPAF expression. The asterisk indicates a specific PARP cleavage product due to caspase activation by TNF/CHX.
mammals, and has been worked out in great detail (Bao and Shi, 2007). Our results suggest that this principle of protease activation is even much older in evolutionary terms, as it is already found in bacteria. CPAF is efficiently translocated from the bacteria-harboring vacuole into the cytosol, although the mechanism of this translocation is unclear. Our data indicate that oligomerization of CPAF is an essential step in its maturation. This is supported by the observation that CPAF secreted from the vacuole into the host cytosol during infection with C. trachomatis was also found in a complex of \( \sim 200 \) kD, which probably corresponds to 3–4 subunits (unpublished data). When and how CPAF oligomerizes is unclear. One possibility is that a bacterial chaperone binds and translocates CPAF into the host cytosol, during which process the spatial requirements for CPAF activation might be met. It is also conceivable that CPAF may transiently associate with the inclusion membrane at a sufficiently high local concentration to cause its own activation. Initiator caspases are activated by clustering induced by specific adaptors. In the best characterized example, caspase-9 is clustered after heptamerization of its adaptor, Apaf-1 (Bao and Shi, 2007). The existence of a specific bacterial adaptor for CPAF is therefore also possible.

Limited homology between CPAF and Tsp has been observed. Our results show that the Tsp-like active site in C. trachomatis CPAF is required for its activity. Chlamydia has a protein, CT441 in C. trachomatis, that shows higher similarity to known bacterial Tsp and that has recently been characterized (Lad et al., 2007). The role of Tsp in bacteria is not well understood, but these enzymes appear to be involved with proteolytic modifications and degradation of various bacterial proteins (Paetzel and Dalbey, 1997). The conservation of the active site in CPAF might be an indication of evolutionary origin, but the low overall homology suggests that it has assumed other functions. The translocation of CPAF into the host cytosol also indicates that its physiological targets may not be bacterial but host cell proteins.

It is interesting to note that CPAF both has anti-apoptotic activity and induces nonapoptotic cell death, reproducing two salient features of chlamydial infection. Although the relevance of cell death modulation for chlamydial pathogenesis is not known, it is likely that apoptosis inhibition may contribute to the ability of chlamydiae to complete their developmental cycle and perhaps to maintain persistent human infections. During viral infection, the host cell defense often includes the induction of apoptosis through the mitochondrial, BH3-only protein controlled pathway (Everett and McFadden, 2002). Although Chlamydia differs from viruses in important aspects, it shares their dependency on cellular integrity for its replication. The cell’s response to chlamydial infection may include the attempt to undergo apoptosis through activation of BH3-only proteins. These proteins are general sensors of cell stress and can be activated to induce apoptosis in many different situations (Strasser, 2005). It is therefore even conceivable that they are activated in response to CPAF activity. CPAF might thus induce apoptosis but at the same time counter it by causing the degradation of BH3-only proteins. In this scenario, as BH3-only proteins are mostly degraded, the net result of the activity of CPAF would be the observed nonapoptotic cell death. CPAF-induced cell death could therefore be a masked form of apoptosis that serves as a cellular defense reaction.

**Figure 7.** CPAF expression leads to changes in cellular ultrastructure. CPAF K6 cells were treated with 5 ng/ml AHT and CM for 20 h (middle and bottom), or left untreated (control; top). Cells were harvested, pelleted, and analyzed by electron microscopy. The bottom panel shows a 10-fold magnification. Scale bars are indicated. M, mitochondria.
The molecular mechanism for the cell death–inducing activity of CPAF is still unknown. The appearance of lamellar structures in the cell might suggest changes to organelles and intracellular membranes, such as lysosomes and the ER, and their membranes. The ultrastructural changes to these organelles have been described during chlamydial infection (Todd and Storz, 1975; Todd et al., 1976) are thus likely connected to CPAF activity, and it could be the release of, for instance, lysosomal peptides, that causes the damage and eventual death of the cell.

All sequenced chlamydial strains have a gene coding for CPAF. The genome of an endosymbiont of free-living amoeba, *candidatus protochloramydia amoebophila* (UWE25), has recently been sequenced, and even this distantly related bacterium carries a recognizable CPAF homologue (Horn et al., 2004; Collingro et al., 2005). This suggests that CPAF serves a function that is similar for the different species and perhaps even the very different requirements of infection of human cells and amoeba. When first discovered, CPAF’s activity to degrade transcription factors required for MHC-expression was noted. Although such a mechanism might contribute to immune evasion by *Chlamydia*, it appears more likely that this is not the evolutionarily selected function of CPAF, especially not in amoeba lacking MHC. Cytoskeletal structures like the intermediate filament components CK8 and vimentin are good candidates as essential targets of CPAF activity. The host cell cytosol has to accommodate the rapidly growing inclusion, and the disruption of intermediate filaments might facilitate expansion of the inclusion and perhaps eventually the release of the inclusion by lysis or extrusion (Hybiske and Stephens, 2007). Whether CPAF-induced cell death is beneficial for the host or for the bacteria is therefore uncertain at this stage. Either way, our results strongly suggest that CPAF is an important factor in chlamydial pathogenicity, and cellular alterations and responses induced by CPAF might be involved in causing protracted infections.

**Materials and methods**

**Cloning of expression vectors**

A fragment encoding for amino acid residues 2–221 of gyrB of *E. coli* was amplified by PCR and cloned into a pcDNA4/TO/mycHis vector (Invitrogen). Consecutively, two additional gyrB fragments, separated by linker sequences of 16 amino acid residues, were inserted 5′ of the first copy. A sequence coding for a FLAG tag was added 3′ of the gyrB fragments (gyrB construct). The coding sequence of CPAF of *C. trachomatis* (amino acid residues 18–601) was inserted behind the FLAG-gyrB construct (gyrB-CPAF). CPAF mutants were generated by point mutations using a Stratagene XL Mutagenesis kit. The open reading frame of human cytokeratin 8 (CK8; ATCC #61515) was amplified by PCR, thereby adding a myc-tag coding sequence of 570 nm was measured. As a positive control, cells treated with TNF-α 1 μg/ml and incubated at 37°C for 24 h were used. The caspase inhibitor zVAD-fmk was used at a concentration of 25 μM.

**MTT assay**

Cell viability was tested by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay. CPAF was added to cells at a concentration of 0.5 μg/ml and incubated at 37°C for 1 h. Generated formazane crystals were dissolved in DMSO, and the OD at a wavelength of 570 nm was measured. As a positive control, cells treated with TNF-α (R&D Systems)/cycloheximide (0.5 ng/ml each) for 16 h were used. The caspase inhibitor zVAD-fmk was used at a concentration of 25 μM.

**Analysis of cell morphology and detection of apoptosis**

Cell morphology was analyzed in culture media by light microscopy at RT (BX51 inverted microscope, 40x/0.5 lens, U-CMAD3 video adaptor, F-View II Camera, Cell-F Soft Imaging Solution; all from Olympus). For detection of apoptosis, cells were stained with 1 μg/ml Hoechst 33342 dye (Roche) and incubated for 30 min at 37°C. Cells were harvested, washed with PBS, suspended in PBS, and embedded in mounting fluid (Labotek). Nuclei were examined using an Epifluorescence microscope at RT (DMRBE microscope, 40x/0.70 lens, both from Leica; AxiosCam MRc camera with AxioVision software; Carl Zeiss, Inc.). Adobe Photoshop and Microsoft PhotoEditor were used to adjust image size and resolution, and to enhance contrast of the whole image for better visibility in some pictures.

**Flow cytometry**

Caspase-3 activation was detected by flow cytometry analysis. CPAF K6 cells were harvested, fixed in 2% neutral-buffered paraformaldehyde, and permeabilized with 0.5% saponin (Sigma-Aldrich). Active caspase-3 was detected with an anti-active caspase-3 antibody (Abcam) and FITC-conjugated...
goat anti-rabbit secondary antibody (Dianova). Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson).

Electron microscopy

Cells were harvested and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Electron Microscopy Sciences) and embedded in epoxy resin (epon 812; Electron Microscopy Sciences). Ultrathin sections were examined under a TEM 10 CR transmission electron microscope (Carl Zeiss, Inc.). For image acquisition, a MegaView III camera system (Olympus) was used.

Online supplemental material

Fig. S1 shows the morphological changes in TReX-293 and TReX-Hela cells, respectively, due to transient transfection of CPAF. Fig. S2 compares the changes in cellular morphology between infection with C. trachomatis and the expression of CPAF in CPAF K6 cells. Fig. S3 describes the cell viability of CPAF K6 cells after CPAF induction measured by propidium iodide uptake. Fig. S4 shows nuclear morphology after CPAF expression or infection with C. trachomatis at higher magnification. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804023/DC1.

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