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Autoprocessing and self-activation of the secreted protease CPAF in Chlamydia-infected cells

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Abstract

The Chlamydia-secreted protease/proteasome-like activity factor (CPAF) is synthesized as a proenzyme (proCPAF) and requires processing for proteolytic activity. Recent structural studies have further demonstrated that CPAF is a serine protease that can undergo autoprocessing and self-activation in a concentration-dependent manner *in vitro*. However, it is not known how CPAF is processed and activated during chlamydial infection. In the current study, we used a mutant CPAF designated as CPAF(E558A) that is deficient in processing by itself as a substrate to search for putative CPAF activation factor(s) in Chlamydia-infected cells. CPAF(E558A) was processed by the lysates made from Chlamydia-infected cells and the processing activity correlated with the presence of endogenous active CPAF in the fractionated lysate samples. CPAF produced in the Chlamydia-infected cells is required for processing the mutant CPAF(E558A) since the processing activity was removed by depletion with anti-CPAF but not control antibodies. Furthermore, a purified and activated wild type CPAF alone was sufficient for processing CPAF(E558A) and no other chlamydial proteases are required. Finally, fusion tag-induced oligomerization can lead to autoprocessing and self-activation of the wild type CPAF in mammalian cells. These observations together have demonstrated that CPAF undergoes autoprocessing and self-activation during chlamydial infection.

Keywords

Chlamydial infection; serine protease; CPAF; autoprocessing; self-activation

1. Introduction

Chlamydiae represent a large family of obligate intracellular Gram-negative bacteria that can cause severe health problems in both humans and animals [1-5]. Chlamydial intracellular replication and survival significantly contributes to the Chlamydia-induced inflammatory pathologies [6-8]. A Chlamydia-secreted protease designated as CPAF

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(chlamydial protease/proteasome-like activity factor; ref: [9]) may benefit chlamydial intracellular replication and survival by cleaving cytoskeleton and junction proteins to promote chlamydial inclusion expansion and to aid chlamydial organism dissemination [10-13], degrading BH3-only domain proteins to block intracellular stress-triggered apoptosis [14-16], and proteolyzing transcriptional factors required for MHC antigen expression to evade immune detection [17,18]. CPAF is both unique to and highly conserved among Chlamydia species [19], suggesting that CPAF may play an indispensable role in chlamydial infection. The fact that CPAF targets host proteins for manipulating host cell signaling pathways to both benefit chlamydial survival and allow chlamydial infection to inflict inflammatory pathologies to the infected hosts certainly qualifies CPAF as a virulence factor for Chlamydia.

CPAF is synthesized as a proform with a MW of ~70 kDa. To acquire proteolytic activity, the proCPAF has to be processed into a C-terminal fragment of ~35 KDa (CPAFc) and N-terminal fragment of ~29 KDa (CPAFn) [20] and the two fragments have to form intramolecular dimers (CPAFc/n) [21]. Recent crystal structure and mutagenesis studies [22] have further demonstrated that CPAF is a serine protease with a water-mediated catalytic triad consisting of histidine on the 105th position (H105), S (serine) 499 and E (glutamic acid) 558. A fully active CPAF forms an intermolecular homodimer consisting of two identical intramolecular dimers (CPAFc/n:CPAFc/n). CPAF is activated by 3 sequential cleavages of an internal inhibitory segment (covering residues R243 to S283) that binds to the active site of CPAF. The cleavage of the inhibitory peptide is initiated by a *trans*-autocatalytic activity when CPAF is maintained at high concentrations in a cell-free system. The trans-cleavage site is between residues M242 and R243. Transient dimerization is required for this cleavage, suggesting that one CPAF molecule cleaves the site in another CPAF molecule. This trans-cleavage further promotes homodimerization and conformational changes that lead to the assembly of the catalytic triad within a CPAF molecule. The assembled catalytic triad carries out two additional cleavages (between M264 and V265, S283 and G284) of the inhibitory peptide in *cis* (the same CPAF molecule cleaves its own inhibitory peptide), leading to complete removal of the inhibitory peptide, stabilizing homodimers and enabling full CPAF activity. Although the structural studies have provided convincing evidence for an autoprocessing and self-activation mechanism of CPAF in a cell-free environment, it is still unknown how CPAF is processed and activated in Chlamydia-infected cells during chlamydial infection. The finding that the first cleavage of the internal inhibitory peptide was carried out in *trans* suggests that CPAF processing can be initiated by other enzymes in Chlamydia-infected cells. The fact that the *C. trachomatis* genome does encode 28 putative proteases in addition to CPAF is consistent with the hypothesis. The goal of the current study is to understand how CPAF is processed during chlamydial infection and to test whether there are any additional upstream proteases/factors that can initiate CPAF processing in Chlamydia-infected cells. We generated a mutant CPAF with the catalytic residue E558 replaced with an alanine designated as CPAF(E558A). This mutant CPAF(E558A) can no longer undergo autoprocessing and self-activation but still susceptible to processing by an activated wild type CPAF in a cell-free system [22]. In the current study, we used the CPAF(E558A) as a bait substrate to search for factors that can initiate CPAF processing in Chlamydia-infected cells. We found that CPAF(E558A) was processed by the lysates made from Chlamydia-infected cells and the processing activity was removed by depletion with anti-CPAF but not control antibodies. A purified wild type CPAF that was activated was sufficient for processing CPAF(E558A) and no other chlamydial proteases were required for or able to carry out the processing activity. CPAF were induced to undergo autoprocessing and self-activation inside mammalian cells. Thus, we have provided evidence that CPAF is autoprocessed during chlamydial infection and no additional factors are required for CPAF autoprocessing in the Chlamydia-infected cells.

2. Results

2.1. The mutant CPAF(E558A) is processed by fractions of Chlamydia-infected cell lysates that contain endogenous CPAF

CPAF is a serine protease with a catalytic triad consisting of H105, S499 & E558. A mutant CPAF with E558 replaced with an alanine [designated as CPAF(E558A)] is no longer able to undergo autoprocessing and self-activation [22]. We expressed the mutant CPAF(E558A) with a His tag fused to the C-terminus and used the CPAF(E558A)-His fusion protein as a bait substrate to search for factors that can initiate CPAF processing in Chlamydia-infected cells. When different amounts of total lysates made from Chlamydia-infected cells were mixed with CPAF(E558A) fusion protein (Fig. 1A), a shorter fragment was detected with an anti-His tag antibody (panel a). We designated this fragment as CPAF(E558A)c-His since it contained a His-tagged C-terminus of CPAF, which was confirmed by the anti-CPAF C-terminus antibody 100a (panel b). It is worth noting that the antibody 100a also detected the matured C-terminal fragment of the endogenous CPAF in the Chlamydia-infected cell lysates (CPAFc, covering residues 284-609). Based on the migration patterns of CPAF(E558A)c-His and CPAFc and the previously determined trans-cleavage site of CPAF [between M242 and R243; ref: [22]], it is clear that the CPAF(E558A)c-His fragment consists of residues 243 to 609 plus the His tag. The processed N-terminal fragments from both the mutant CPAF(E558A) and Chlamydia-encoded wild type CPAF were identical in length and detected with the anti-CPAFn antibody 54b (panel c). These observations suggest that Chlamydia-infected cells contain a processing activity that can carry out the trans-cleavage between residues M242 and R243 of CPAF(E558A). This trans-cleavage is known to be both necessary and sufficient for initiating CPAF activation *in vitro* [22]. The control lysates made from normal HeLa cells contained no endogenous CPAF and failed to carry out the trans-cleavage of CPAF(E558A) into CPAFn and CPAF(E558)c-His fragments, suggesting that the trans-cleavage or processing activity is dependent on chlamydial infection. More importantly, the above observations have demonstrated that the fusion protein CPAF(E558A)-His as a substrate combined with the anti-His tag antibody detection can be used to search for putative upstream factors that can carry out the trans-cleavage of CPAF in Chlamydia-infected cells. To facilitate the identification of the putative processing factor(s), the whole cell lysates were subject to a high speed centrifugation, which separated the lysate samples into supernatant and pellet fractions and both were monitored for their ability to process CPAF(E558A) (Fig. 1B). All processing activity was restricted to the supernatant fraction. When the supernatant was further fractionated via column chromatography, the processing activity co-migrated with the endogenous active/mature CPAF but not proCPAF (Fig. 1C), suggesting that the Chlamydia-produced endogenous CPAF activity may participate in the processing of CPAF(E558A).

2.2. The endogenous CPAF in Chlamydia-infected cells is required for the CPAF(E558A) processing activity

To test whether the endogenous CPAF present in the Chlamydia-infected cell samples is necessary for processing CPAF(E558A), lactacystin, a proteasomal inhibitor known to irreversibly inhibit CPAF activity [9,22], was used to treat the Chlamydia-infected cell lysates prior to digestion of CPAF(E558A). Lactacystin but not the solvent DMSO alone blocked the processing activity (Fig. 2A), suggesting that a lactacystin-susceptible proteolytic activity is responsible for processing CPAF(E558A). Although lactacystin can inhibit both host proteasome and chlamydial CPAF, processing of CPAF(E558A) correlated well with the presence of Chlamydia-secreted CPAF as shown in Fig. 1C. We further tested whether the endogenous CPAF produced in Chlamydia-infected cells is required for the processing activity using an antibody depletion assay (Fig. 2B). We found that both the anti-CPAFc (mAb 100a) and CPAFn (mAb 54b) antibodies completely removed the processing

activity from the Chlamydia-infected cell lysates while a rabbit anti-MOMP antibody failed to do so (panel a). Since the anti-CPAF antibodies specifically depleted all CPAF but not MOMP (panel b) while the anti-MOMP antibody depleted MOMP but not CPAF from the Chlamydia-infected cell lysates (panel c), we can conclude that the endogenous CPAF is necessary for processing the mutant CPAF(E558A).

2.3. CPAF is sufficient for processing CPAF(E558A)

We further demonstrated that a purified and activated wild type CPAF alone was sufficient for processing CPAF(E558A) (Fig. 3). In a cell-free cleavage assay, the GST-CPAF fusion protein purified from a bacterial expression system processed the CPAF(E558A)-His fusion protein to generate the characteristic CPAF(E558A)c-His fragment while a similarly purified mutant GST-CPAF(L281G) known to lack CPAF activity [20] failed to do so. As a positive control, the Chlamydia-infected but not normal HeLa cell lysate samples cleaved CPAF(E558A). The GST-CT775 fusion protein as an irrelevant control failed to process CPAF(E558A). The *C. trachomatis* genome encodes a total of 29 putative proteases including CPAF [as defined in the STDGEN database (http://stdgen.northwestern.edu); ref: [23]]. To assess the potential role of these proteases in CPAF processing, we also expressed the remaining 28 putative proteases as GST fusion proteins in addition to CPAF and evaluated their ability to process the CPAF(E558A)-His fusion protein (Fig. 4). Although all 29 putative proteases were successfully expressed and purified (panel a), only GST-CPAF was able to cleave CPAF(E558A) and no other chlamydial proteases were able to do so (panel b), suggesting that CPAF is the sole source of enzyme encoded by Chlamydia for autoprocessing CPAF. Since the chlamydial protease fusion proteins were extracted and purified from bacteria using buffers that contained protease inhibitors and some of the irreversible protease inhibitors interfere with the measurements of CPAF(E558A) cleavage activity of the purified chlamydial protease fusion proteins, all chlamydial protease fusion proteins were re-extracted and re-purified from bacteria using buffers without any protease inhibitors. The protease fusion proteins purified with or without protease inhibitors were compared for their ability to digest the mutant CPAF(E558A) and no difference in CPAF(E558A) cleavage was found between the two different preps of chlamydial protease fusion proteins (Data not shown).

2.4. Induction of CPAF autoprocessing and self-activation in mammalian cells

To test whether wild type CPAF can undergo autoprocessing and self-activation in mammalian cells in the absence of chlamydial infection, we expressed CPAF as fusion proteins with different fusion tags and evaluated the processing and activity of the CPAF fusion proteins (Fig. 5A & B). When CPAF was expressed as a small Myc epitope-tagged fusion protein, no CPAFc fragment (that is indicative of CPAF activation) was detected, which is consistent with a previous observation [20]. However, when CPAF was fused to a large RFP fusion tag, the CPAFc fragment appeared in the cell samples (Fig. 5A, panel a), indicating CPAF processing and activation. More importantly, the RFP tag encoded by the pDsRed Express C1 vector (that is known to allow RFP to oligomerize) induced more pronounced CPAFc fragment than the RFP tag encoded in the pDsRed-monomer C1 vector (that is engineered to minimize RFP oligomerization) [24, 25]. These observations suggest that the fusion tag-forced oligomerization can trigger CPAF autoprocessing, which is consistent with a previous observation [26]. The autoprocessing also led to CPAF activation since Puma, a BH3-only domain protein known to be targeted by CPAF during chlamydial infection [14, 15], was cleaved in the transfected cells carrying the CPAFc fragment (panel b). Furthermore, the fusion tag-induced CPAF autoprocessing appears to depend on the CPAF's own catalytic activity since the mutant CPAF(E558A) failed to undergo autoprocessing and activation by itself even when it was induced to undergo oligomerization

by the fusion tag RFP (Fig. 5B), which is consistent with the observation made in cell-free systems [22].

2.5. The chlamydial organism-secreted CPAF fails to access to the plasmid-expressed CPAF(E558A) in the same mammalian cells

To further test whether chlamydial organism-secreted CPAF can process the mutant CPAF(E558A) expressed in the same cells, we expressed the mutant CPAF in HeLa cells via a transgene and infected the transfected cells with live chlamydial organisms. The processing of the transgene-expressed CPAF(E558A) was then monitored using a Western blot (Fig. 6A). To our surprise, no C-terminal fragment was detected from the transgene-expressed CPAF(E558A) although the chlamydial organisms produced active CPAF that was able to cleave the same CPAF(E558A) in a cell-free system, suggesting that chlamydial organism-secreted CPAF was unable to access to the transgene-expressed CPAF(E558A). Indeed, under an immunofluorescence microscope, we found that the chlamydial organism-secreted CPAF did not overlap with the transgene-expressed CPAF(E558A) (Fig. 6B). We tested many different types of tags and various versions of CPAF mutants (data not shown) and all the experimental results led us to the same conclusion, that's the chlamydial organism-secreted endogenous CPAF is unable to access to the transgene-expressed CPAF(E558A) in the same cells. Although this technical difficulty prevented us from visualizing the intracellular processing of CPAF(E558A), the observations have demonstrated that the Chlamydia-secreted CPAF is highly regulated in the host cell cytosol via compartmentalization. Chlamydial organisms may use compartmentalization to regulate the access of CPAF to its substrates in the host cell cytosol, which may partially explain why only a selected subset of host cell proteins are cleaved by CPAF although CPAF is known to possess a broad range of substrate specificity [22]. Efforts are underway to understand how the secreted CPAF is compartmentalized and regulated in the host cell cytosol.

3. Discussion

CPAF was identified as a Chlamydia-secreted protein with a proteolytic activity and recent crystal structure studies revealed CPAF as a serine protease with some unique characteristics [22]. Particularly, the mode of activation via sequential cleavage of an internal inhibitory peptide (consisting of residues 243 and 283) is intriguing. The first cleavage between M242 and R243 requires a trans processing activity, which can be triggered at high concentrations in cell-free systems [22]. The goal of the current study was to determine whether this trans-cleavage could be accomplished by upstream enzymes in Chlamydia-infected cells. To address this question, we generated a mutant CPAF(E558A) that remained susceptible to the trans-cleavage between residues M242 and R243 by the activated wild type CPAF but failed to continue onto the subsequent cleavages due to lack of a *cis* catalytic activity in the same molecule [22]. This mutant can be a perfect substrate for searching for upstream factors that can carry out the initial transcleavage. Using this mutant CPAF(E558A) His-tagged at the C-terminus as a substrate, we have acquired compelling evidence that CPAF, like its mode of activation in cell-free environment, is also activated via an auto-processing mechanism in Chlamydia-infected cells. First, the trans-cleavage of CPAF(E558A) was carried out by Chlamydia-infected but not normal HeLa cell lysates and the trans-cleavage (processing) activity correlated well with the Chlamydia-secreted CPAF in the infected cells (Fig1); Second, an intermediate fragment was detected in Chlamydia-infected cells by the anti-CPAFc but not anti-CPAFn antibody (Fig.1C), suggesting the Chlamydia-secreted CPAF is also sequentially processed during infection; Third, depletion of the endogenous CPAF from or lactacystin treatment of the Chlamydia-infected cell lysate samples blocked the processing activity; Fourth, purified wild type CPAF is sufficient for the processing activity and no other putative proteases encoded by *C. trachomatis* can process CPAF; Finally,

CPAF can undergo autoprocessing and self activation upon oligomerization induced by fusion tags in mammalian cells [26].

Although it is now clear that CPAF is activated via an autoprocessing mechanism both in cell-free systems and in Chlamydia-infected cells, it is still not known how the CPAF autoprocessing is regulated during chlamydial infection. Many mammalian procaspases can also undergo autoprocessing [27], which is often triggered by death adaptor molecule-induced proximity [28]. However, it is unclear what brings the CPAF zymogen together during chlamydial infection. Nevertheless, it is always possible that passive accumulation of CPAF zymogen molecules in Chlamydia-infected cells along the infection cycle may be sufficient for triggering CPAF autoprocessing and activation. CPAF protein becomes detectable inside the inclusions 8 to 12 hours and starts to accumulate in the host cell cytosol 18 to 24 hours after chlamydial infection. As infection progresses to late stages of the growth cycle, the concentration of CPAF can reach very high in the cytoplasm of the infected cells although amount of CPAF inside inclusions is always maintained at a minimal level. Indeed, most CPAF activity and function have been detected at and after middle stages when CPAF becomes very abundant in the host cells. The concentration-dependent activation of CPAF may maximally benefit the chlamydial intracellular survival and growth. CPAF does not accumulate inside chlamydial inclusion to any large amount, which allows Chlamydia to keep CPAF dormant before CPAF is secreted out of the inclusions. This may represent an important strategy for Chlamydia to protect its own proteins from degradation by CPAF. However, once CPAF is secreted into host cell cytosol, CPAF can accumulate and gradually reach the critical concentration needed for activating CPAF. Interestingly, immunofluorescence detection has revealed that CPAF is highly concentrated around the cytosolic face of chlamydial inclusions [9,29], suggesting that the concentration-dependant activation of CPAF can take place around chlamydial inclusions. Since CPAF is known to solublize host cell cytoskeleton proteins [10,11] and other constitutively expressed proteins [17,18] to make way for chlamydial inclusion expansion, this confined activation of CPAF may allow Chlamydia to cleave host proteins based on Chlamydia's needs. This need-based degradation of host proteins can, on one hand, meet the needs of varied chlamydial growth modes, and on the other, avoid rapid destruction of host cells before Chlamydia complete their own replication. The controlled CPAF activation is especially important during persistent infection, when chlamydial organisms hide inside host cells in a nonproductive and noninfectious form for long periods of time. Indeed, under persistent infection conditions, CPAF activity was maintained at a minimal level [9,30].

The concentration-dependent activation of CPAF may also affect the types of cellular proteins CPAF can target or access to. The crystal structural data suggests that CPAF may have a broad substrate specificity [22]. However, only selective groups of host cell proteins are cleaved by Chlamydia during chlamydial infection, suggesting that CPAF activation and activity are controlled. The concentration-dependent CPAF activation mechanism may allow Chlamydia to limit CPAF's ability to act on target proteins before CPAF reaches a critical concentration required for triggering autoprocessing and self-activation. Obviously, biologically relevant substrates of CPAF should always be defined in the context of chlamydial infection. It is worth noting that although autoprocessing is both necessary and sufficient for CPAF activation and no other chlamydial proteases are required for CPAF activation, other chlamydial proteases such as CT441 (Tsp) may directly cleave host proteins [31,32]. Some studies even suggested that host cell proteases activated during chlamydial infection might also contribute to host cell protein degradation [33]. The participation by multiple proteases together with the concentration-controlled activation of CPAF may enable Chlamydia to specifically target multiple host proteins for degradation during their intracellular replication.

4. Materials and Methods

4.1. 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 bovine serum (Atlanta Biologicals, Lawrenceville, GA) in a humidified incubator in the presence of 5% CO_2. *C. trachomatis* serovar D organisms were grown and purified as previously described [34]. For making Chlamydia-infected HeLa cell lysates, HeLa cells grown in 175cm^2 flasks were infected with serovar D organisms at an MOI of 5. The infected cells were harvested 44 hours after infection for making whole cell lysates as described below.

4.2. Prokaryotic expression and purification of chlamydial fusion proteins

The ORFs coding for 29 putative proteases from *C. trachomatis* serovar D genome (ref: [23]; http://stdgen.northwestern.edu) were cloned into pGEX6p-1 or 2 vectors (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and expressed as fusion proteins with glutathione-s-transferase (GST) fused to the N terminus of the chlamydial proteins according to a previously established protocol [35]. The following primers were used: For cloning CT020, 5′-CGC*GGATCC*.ATG.ACG.AGC.AGT.TAC.ATG.AGT-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AGG.GAA.TAG.CCT.ATG.GTT -3′ (reverse); For cloning CT045, 5′-CGC*GGATCC*.ATG.AAG.AAT.TCT.AAA.GCT.CAA.GAA-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.TTTAGATAGGAATTTCTCCAT-3′ (reverse); For cloning CT072, 5′-CCG*GAATTC*.ATG.ACA.ATA.ATA.TAT.TTT.GTT.CTT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TCA.CCC.AAC.GAA.AAC.TCT.AGA-3′(reverse); For cloning CT082, 5′-CGC*GGATCC*.ATG.TCA.ATT.TCT.GGA.AGT.GGT-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TCA.TGA.ATC.GCC.TCC.TGC-3′(reverse); For cloning CT112, 5′-TCCC*CCCGGG*.ATG.ACC.ACT.GCT.ACT.ACT.TCA-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TAG.CAA.GCT.AGC.TAG.TTC-3′(reverse); For cloning CT113, 5′-CGC*GGATCC*.ATG.CAA.ATA.GGA.AGT.TTA.CCT-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.CAC.AGC.TGG.ATT.TGT.TTT-3′(reverse); For cloning CT138, 5′-CGC*GGATCC*.ATG.ATA.GTG.GAT.ATG.CAT.TGT-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.GAA.CGC.AAT.GTG.ATT.GAC-3′(reverse); For cloning CT197, 5′-CGC*GGATCC*.ATG.TTG.ACA.CTA.GGC.TTA.GAA-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.GGA.GCA.AGC.AGA.TTC-3′(reverse); For cloning CT286, 5′-TCCC*CCCGGG*.ATG.GGA.CAA.CCT.TCG.GAC-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.TGA.TTC.ATC.AGC.TGT.AAT-3′(reverse); For cloning CT343, 5′-CGC*GAATTC*.ATG.GCC.GTG.TAC.AAA.TAT.TTT.ATT.GTA-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.AAA.AAA.GGA.AGA.GAT.GCT-3′(reverse); For cloning CT344, 5′-CGC*GGATCC*.ATG.CGT.GAT.GAA.GCA.GAA.ATC-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AAC.CCC.AGG.GAA.AGC.AAT-3′(reverse); For cloning CT408, 5′-CGC*GGATCC*.ATG.CCG.ACC.CGT.TCT.CTC-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AAA.GTA.TTT.TTT.ACT.TTC.TTT-3′ (reverse); For cloning CT422, 5′-CGC*GGATCC*.ATG.CTT.ATT.TTG.GAT.CGA.TCT.TCT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TAC.TGC.AGG.ACG.TAA.TAA-3′ (reverse); For cloning CT431, 5′-CGC*GGATCC*.ATG.CCT.GAA.GGG.GAA.ATG.ATG-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.CAA.GTC.GTT.AAA.AGA.GAA-3′ (reverse); For cloning CT441, 5′-CCG*GAATTC*.ATG.ATG.AGA.TTC.GCT.CGC.TTT-3′(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TGA.TAT.AGA.TTT.TAG.AAG.GAT-3′ (reverse); For cloning CT494, 5′-CGC*GGATCC*.ATG.CTA.AAA.GCG.ATT.AAT.AGA.TTT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TCA.ACC.CAT.GTA.CCA.CAA.AGA-3′ (reverse); For cloning CT574, 5′-TCCC*CCCGGG*.ATG.AAT.CAA.AAT.CCG.ATC.AAA.CGA-3′

(forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AAT.GAT.AAT.TAT.CTC.GGA.AGA-3′ (reverse); For cloning CT705,5′-TCCC*CCCGGG*.ATG.ACA.AAA.AAA.AAT.CTT.GCG.GTC-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AGC.AAT.CGC.CTC.TGG-3′(reverse); For cloning CT706, 5′-TCCC*CCCGGG*.ATG.ACG.TTA.GTA.CCA.TAC.GTT.GTT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.AGA.CGC.AAT.ACT.CTT.ATC-3′ (reverse); For cloning CT779, 5′-CGC*GGATCC*.ATG.CAT.TCA.CTT.GCT.GTT.TTT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.TGA.CTT.CTG.CAT.AGA.GGC.GGA-3′ (reverse); For cloning CT806,5′-TCCC*CCCGGG*.ATG.GAC.AAC.CAC.CCT.CCT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TTT.TTC.CTG.AGA.CGA.GTT-3′ (reverse); For cloning CT823, 5′-CG*GGATCC*.ATG.ATG.AAA.AGA.TTA.TTA.TGT.GTG-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.CTC.GTC.TGA.TTT.CAA.GAC-3′ (reverse); For cloning CT824, 5′-TCCC*CCCGGG*.ATG.AAA.ACT.GGG.GAT.ACC.TAT.AGA-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AAA.TGC.AGC.CTC.TAT.TTG-3′ (reverse); For cloning CT841, 5′-TCCC*CCCGGG*.ATG.GCT.AAA.GAT.AAA.AAA.ACA.AAT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TGT.GCT.AGT.ATT.AAA.CTT.CAA-3′ (reverse); For cloning CT851,5′-CGC*GGATCC*.ATG.AAA.AGA.AAC.GAT.CCT.TGC-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.CTT.ATC.TAG.AAG.AGT.TAG.TAC-3′ (reverse); For cloning CT859, 5′-CGC*GGATCC*.ATG.CGT.AAA.ATT.ATA.CTC.TGT.TCG-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.TAA.TTC.CTT.AGG.CAA.CTG-3′ (reverse); For cloning CT867, 5′-CG*GGATCC*.ATG.GAA.CCA.ATT.CAT.AAT.CCT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.ATC.CGT.AGT.TGG.CCA-3′ (reverse); For cloning CT868, 5′-CG*GAATTC*.ATG.TTG.TCT.CCC.ACC.AAC.TCA-3′ (forward) and 5-TTTCCTTTT*GCGGCCGC*.TTA.GAA.AAG.AGC.TTT.TGC.TTC-3′ (reverse); For CT775 (as an irrelevant control), 5′-CGC*GGATCC*.ATG.AAG.ATA.GGG.TTT.TGG.CGT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.CTA.TGC.GTA.GGG.GAC.CTC-3′(reverse); For CT858 (CPAF), 5′-CGC*GGATCC*.ATG.GGT.TTT.TGG.AGA.ACA.TCG.ATT-3′ (forward) and 5′-TTTCCTTTT*GCGGCCGC*.TTA.AAA.ACT.ACC.ATC.TTC.CGC-3′ (reverse).

A recombinant pGEX6p plasmid coding for the mutant GST-CPAF (L281G) was previously created [20]. The constructs in pET30a vector (Novagen, Madison, WI) coding for the wild type (Wt) CPAF and mutant CPAF designated as CPAF(E558A) were described elsewhere [22]. All GST fusion proteins were induced at 30°C for 3 hours (to minimize denaturation). The GST-fusion proteins were released from bacteria by sonication on ice in PBS (phosphate-buffered saline solution, pH7.0) containing 1% Triton-X100 plus various protease inhibitors including 1mM PMSF (cat# P7626), 20uM leupeptin (L2884), 1.6uM pepstatin A (P5318) and 1.7 ug/ml of aprotinin (A6279) (all from Sigma, St. Louis, MO). The fusion proteins were purified using glutathione-conjugated beads (Amersham Biosciences Corp) following the instruction provided by the manufacturer. After thorough wash, the fusion proteins immobilized onto the beads were aliquoted and stored at −80°C. The amount of the bead-bound fusion proteins was quantified based on the Coomassie blue-staining intensity on SDS PAGE gel and the fusion proteins were adjusted to equivalent amounts for cell-free digestion experiments as described previously [20,21]. The expression and purification of the His-tagged mutant CPAF(E558A)-His fusion protein were carried out as previously described [22] and the fusion protein was used as the substrate in various cell-free degradation assays as described below. To minimize the interference with the measurements of CPAF(E558A) cleavage activity of the purified chlamydial protease fusion proteins, all chlamydial protease fusion proteins were re-extracted and re-purified from bacteria using buffers without any protease inhibitors. The protease fusion proteins purified

with or without protease inhibitors were compared for their ability to digest the mutant CPAF(E558A).

4.3. Cell-free cleavage/degradation/digestion assay

The cell-free assays were carried out as previously described [9]. The mutant CPAF(E558A)-His fusion protein was mixed with various sources of enzymes with or without the inhibitor lactacystin (Calbiochem, San Diego, CA) or DMSO alone (Dimethyl sulfoxide, Sigma, St. Luis, MO). All reactions were carried out in PBS (pH7.4) and at 37°C for 2h or as indicated in individual experiments. The types of enzyme sources include the whole HeLa cell lysate with or without chlamydial infection, the supernatant and pellet factions of the lysates, column-fractionated lysate supernatants and the purified GST fusion proteins. To prepare whole cell lysates, HeLa cells with or without Infection-infection from one 175cm^2 flask were harvested and lysed in 1ml PBS via sonication on ice. The lysates were centrifuged at 100,000g for 30 min. The supernatant was harvested while the pellet was resuspended in 0.5ml of PBS via sonication on ice and centrifuged again. After the remaining pellet was sonicated for a third time in another 0.5ml PBS, the three supernatants were combined and used for further fractionation or other applications. The final pellet was sonicated in 1ml PBS and used in the cell-free degradation assays as a source of enzyme. The procedure for the repeated washing of the pellet was designed for extracting all potential CPAF processing activity into the supernatant fraction. In some experiments, the supernatant sample was further fractionated using a mono-Q column as previously described [9]. The eluated fractions from the mono-Q column were both tested for the presence of the endogenous CPAF and used as a source of enzyme in the cell-free degradation assay for monitoring CPAF(E558A) processing activity. In other experiments, the supernatant sample was subjected to antibody depletion prior to mixing with the CPAF(E558A) substrate as previously described[9,14]. To deplete CPAF from the supernatant sample, antibodies against CPAF C-terminus (mAb100a, ref: [9,21] and N-terminus (mAb54b, ref: [9,21] were immobilized onto protein G-agarose beads and the bead/antibody complexes were used to absorb the supernatant sample for 2h at RT. After spinning down the agarose beads, the remaining supernatants were used as sources of enzyme in the cell-free cleavage assays. The antibody depletion efficacy was monitored by detecting the residual antigens in the remaining supernatants. A rabbit antiserum against the major outer membrane protein of *C. trachomatis* serovar D (unpublished data) was used as a control antibody in the depletion experiment. The final degradation mixtures were detected for the presence of both the remaining full length CPAF(E558A)-His fusion protein and the generated CPAF(E558A)c-His fragment in Western blot as described below. In some cell-free degradation experiments, a cytosolic extract (CE) from normal HeLa cells was used as the source of keratin 8 for measuring CPAF activity as described previously [10].

4.4. Mammalian cell expression of CPAF

The CPAF genes from *C. trachomatis* serovar D (CPAF or CPAF-Ct) or *C. muridarum* Nigg (CPAF-Cm) were cloned into mammalian expression vectors including pEF/Myc/Cyto (Invitrogen, Carlsbad, CA), pDsRed Monomer C1 and pDsRed Express C1 (Clontech, San Jose, CA). CPAF was expressed as fusion proteins with a Myc tag fused to the C-terminus (pEF) or red fluorescence protein (RFP, pDsRed) fused to the N-terminus of CPAF. To create the mutant CPAF(E558A) construct in pEF/Myc/Cyto or pDsRed Express C1, a site-directed mutagenesis kit (cat# 200518, Stratagene, La Jolla, CA) was used with the wild type (Wt) full length CPAF as template. Briefly, complementary primers incorporating the desired nucleotide substitutions were used to introduce the corresponding mutations. After primer extension and amplification using PfuUltra DNA polymerase, the parental methylated and hemimethylated DNA was digested with the nuclease DpnI. The mutated molecules were then transformed into competent bacterial cells for nick repair and gene

expression. The complementary primers 5′-GCC.TTC.ATT.GCC.AAC.ATC.GGA (forward, GAG coding for E in Wt CPAF was mutated to GCC coding for A) and 5′-TCC.GAT.GTT.GGC.AAT.GAA.GGC (reverse) were used. The recombinant plasmids were transfected into 293T cells using Lipofectamine 2000 transfection reagent (Invitrogen). Twenty-4 hours after transfection, the transfected cells were harvested with 2% SDS sample buffer and subjected to Western blot analysis for monitoring expression/activation/activity of the plasmid-encoded CPAF.

4.5. Western blot

The western blot assay was carried out as described elsewhere [36]. Samples such as cell lysate or its fractions, cell-free digestion mixtures, fusion proteins and transfected cell samples were heat-treated in 2% SDS sample buffer and loaded onto SDS-PAGE. After electrophoresis, the gel-separated protein bands were transferred to nitrocellulose membrane for antibody detection. The primary antibodies used include a mouse monoclonal antibody (mAb) recognizing His tag (Cat#34670, Qiagen, Valencia, CA), a mAb specific for an epitope in the CPAF C-terminal fragment (100a) and N-terminal fragment (54b), a mAb recognizing the rod region of cytokeratin 8 (clone M20, Sigma), a rabbit monoclonal antibody against human Puma (EP512Y, Abcam, Cambridge, MA) and a rabbit antiserum raised with *C. trachomatis* serovar D major outer membrane protein (MOMP). The primary antibody binding was probed with a horseradish peroxidase (HRP)-conjugated secondary antibody specific for mouse or rabbit IgGs and visualized using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

4.6. Immunofluorescence assay

The immunofluorescence assay was carried out as described elsewhere [36]. HeLa cells transfected with the mammalian expression vectors pDsRed Monomer C1 coding for CPAF(E558A)-Cm [pDsRedMono-CPAF(E558A)-Cm] for 8h were infected with *C. trachomatis* serovar D organisms. 40h after infection, the cell samples were processed for immunofluorescence assay. The plasmid-expressed CPAF(E558A)-Cm was visualized via the fusion tag RFP (red), the *C. trachomatis*-secreted CPAF-Ct was labeled with mAb100a and visualized with a goat anti-mouse IgG conjugated with Cy2 (green) while DAPI was used to visualized DNA (blue). After immuno-labeling, the cell samples were used for image analysis and acquisition with an Olympus AX-70 fluorescence microscope equipped with multiple filter sets (Olympus, Melville, NY) as described previously [36]. Briefly, the multi-color-labeled samples were exposed under a given filter set at a time and the single color images were acquired using a Hamamatsu digital camera. The single color images were then superimposed with the software SimplePCI to display multi-colors. All microscopic images were processed using the Adobe Photoshop program (Adobe Systems, San Jose, CA).

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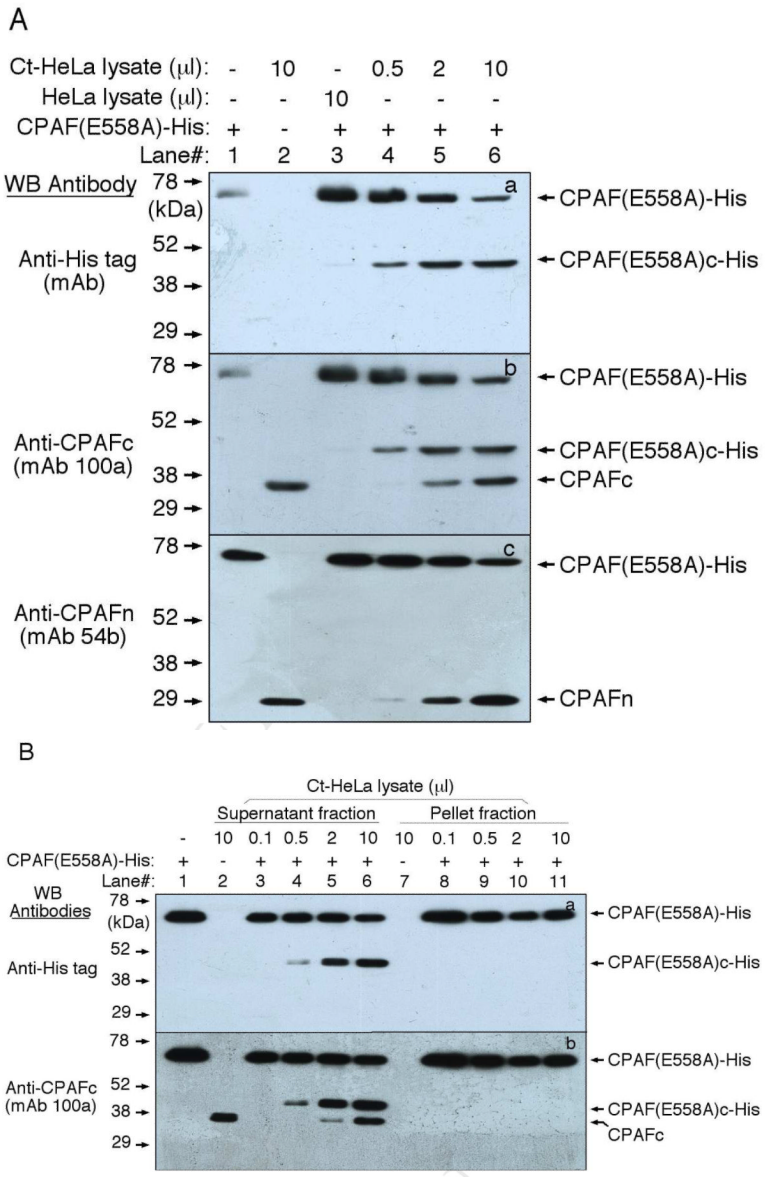


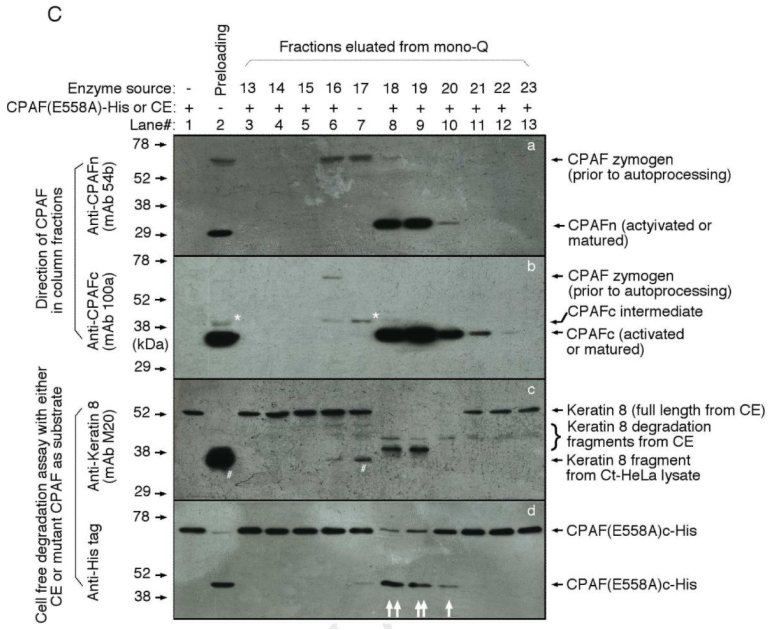


Fig. 1. Correlating the endogenous CPAF activity in Chlamydia-infected cells with the processing of the mutant CPAF(E558A)

(A) Different amounts of lysates made from HeLa cells with (Ct-HeLa) or without (HeLa) chlamydial infection were used to cleave the fusion protein CPAF(E558A)-His in a cell-free degradation assay. The degradation mixtures were detected with antibodies against His tag (anti-His tag, panel a) and CPAF C-terminus (mAb 100a, panel b) and N-terminus (mAb 54b, panel c) in a Western blot. Note that Ct-HeLa lysate contained endogenous CPAFc fragments (indicative of activated CPAF) and processed the CPAF(E558A)-His fusion protein into its C-terminal fragment that contains His tag, designated as CPAF(E558A)c-His. The N-terminal fragment derived from both the endogenous CPAF and mutant CPAF(E558A)-His co-migrate in a single band as CPAFn (panel c). (B) Both the supernatant and pellet fractions prepared from the Chlamydia-infected HeLa cells were used to cleave the CPAF(E558A)-His fusion protein and the cleavage products were detected with the anti-His tag (panel a) and CPAFc (panel b) antibodies. Note that only the supernatant fraction contained the activated endogenous CPAF and cleaved the mutant CPAF(E558A)-His. (C) The supernatant fraction was subjected to further fractionation via a mono-Q column and the eluted fractions (labeled from 13 to 23 on top of the figure) were both detected for the presence of CPAFn (panel a)/CPAFc (panel b) fragments and measured for their ability to cleave Keratin 8 [from a cytosolic extract (CE)], a known CPAF substrate (panel c) and to process the mutant CPAF(E558A) (panel d). Note that fractions# 18,19 and 20 (marked with white arrows in panel d) contained the endogenous active CPAF (panels a & b) with relatively high CPAF activity (panel c) and processed the mutant CPAF fusion protein, correlating the endogenous CPAF activity with the processing of CPAF(E558A)-His. Both 100a and 54b mAbs also detected the endogenous CPAF zymogen and 100a further detected the C-terminal processing intermediate (marked with a white star, panel b). The shortest keratin 8 degradation fragment detected in fraction 16 & 17 likely came from the Q-column-fractionated Chlamydia-infected HeLa lysate samples (marked with white # in panel c).

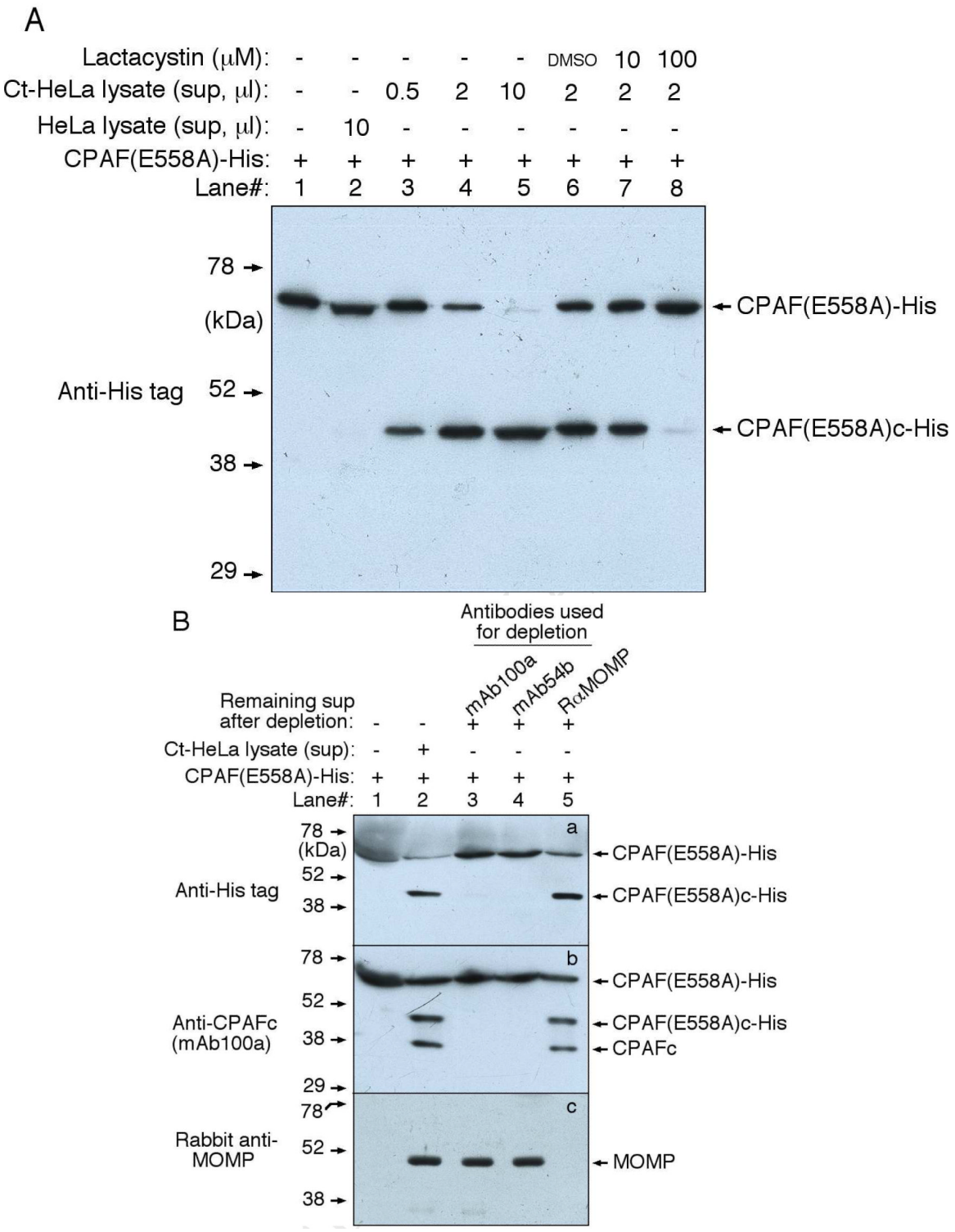


Fig. 2. The endogenous CPAF in Chlamydia-infected HeLa cells is required for processing the CPAF(E558A)-His fusion protein

(A) The lysate supernatant (sup) made from Chlamydia-infected HeLa cells (Ct-HeLa) with or without treatment with lactacystin at the indicated concentrations shown on top of the figure were used to digest the CPAF(E558A)-His fusion protein and the digestion products

were detected with the anti-His tag antibody as described in Fig.1A legend. Note that lactacystin at the final concentration of 100uM completely blocked the processing CPAF(E558A)-His (lane 8). (B) Aliquots of the Chlamydia-infected HeLa (Ct-HeLa) lysate supernatants (sup) were depleted with or without antibodies as indicated on top of the figure before mixing with the substrate CPAF(E558A)-His fusion protein. The antibodies used for depletion include mAb 100a (recognizing CPAFc), mAb 54b (recognizing CPAFn) and rabbit antiserum raised with *C. trachomatis* serovar D MOMP (RαMOMP). The digestion products were monitored for both the residual His-tagged fusion protein/fragment (panel a) and the endogenous CPAFc (panel b) and MOMP (panel c) fragments in Western blot. Note that these antibodies efficiently depleted the corresponding specific antigens without affecting the irrelevant antigens (panel b & c) but only the anti-CPAF antibody depletion removed the processing activity from Chlamydia-infected cell samples.

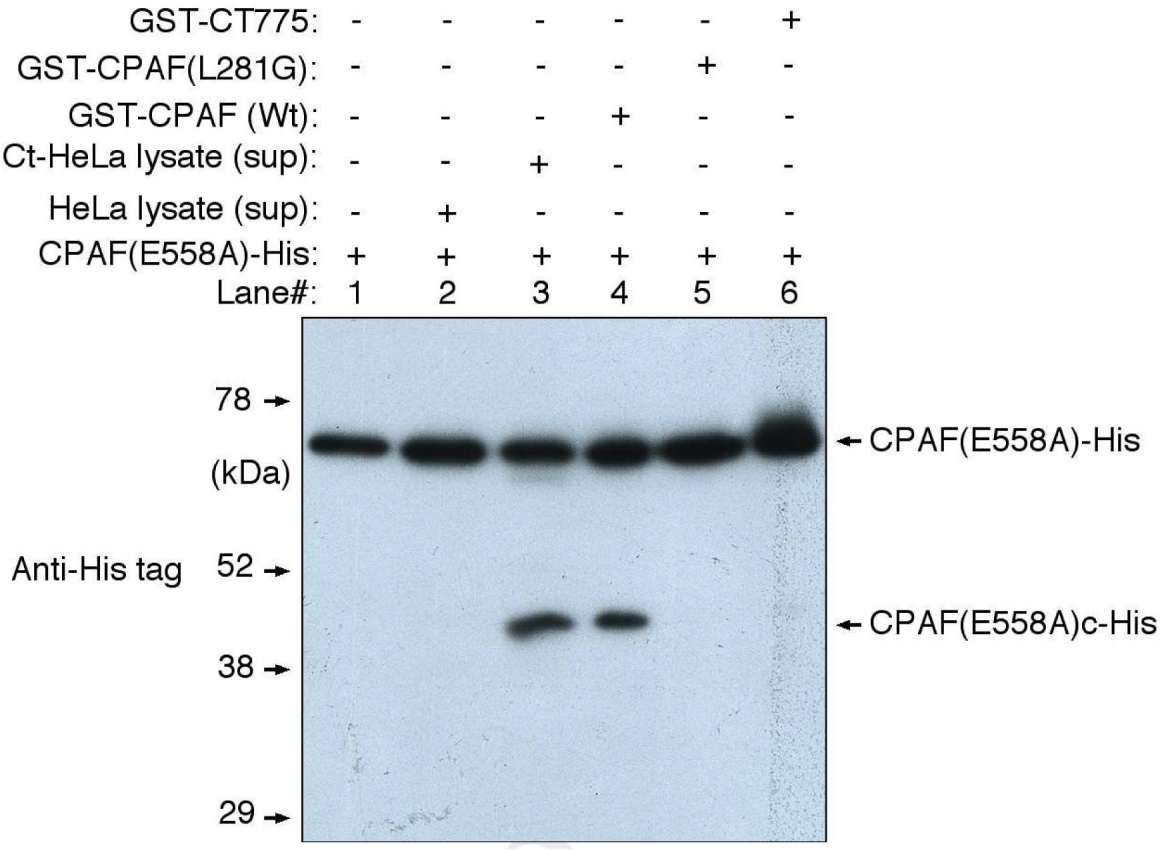


Fig. 3. Processing of the mutant CPAF(E558A) by a purified wild type CPAF

The CPAF(E558A)-His fusion protein was digested with a wild type GST-CPAF(Wt), a lack of function mutant GST-CPAF(L281G) and an irrelevant GST fusion protein as indicated on top of the figure. Lysates from HeLa cells with (Ct-HeLa) or without chlamydial infection were used as positive and negative controls. The digestion products were subjected to Western blot detection with an anti-His tag mAb. Note that only the wild type GST-CPAF (known to contain activated CPAF fragments) and the Chlamydia-infected HeLa lysates processed the mutant CPAF(E558A)-His fusion protein into the His-tagged C-terminus of CPAF designated as CPAF(E558A)c-His as indicated on the right side of the figure.

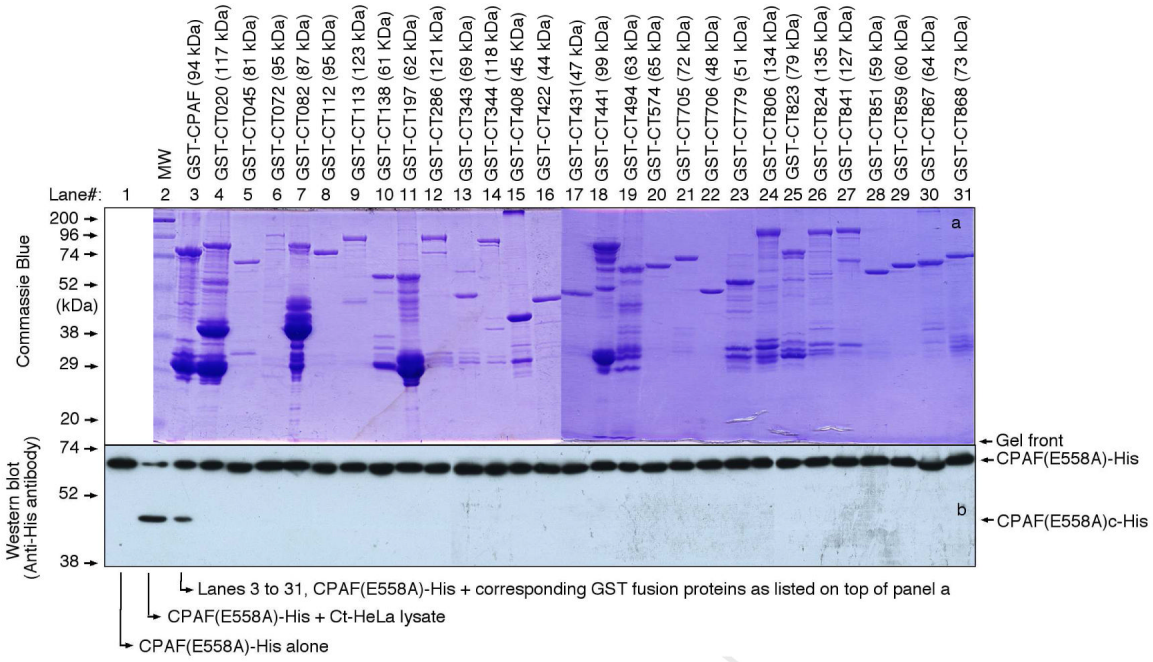
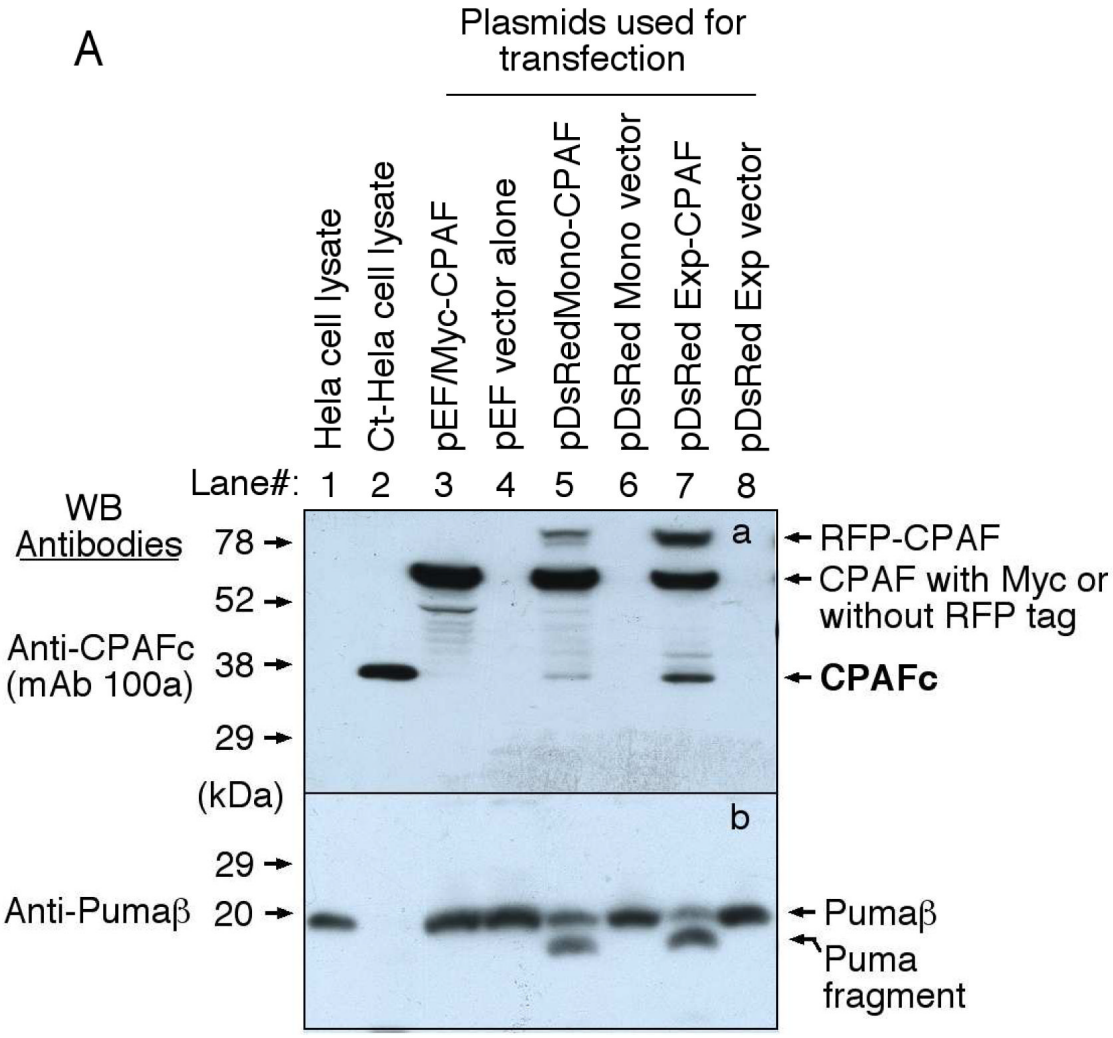


Fig. 4. Processing of the mutant CPAF(E558A) by 29 putative proteases encoded in *C. trachomatis* genome

All 29 putative chlamydial proteases encoded in *C. trachomatis* serovar D genome were produced as GST fusion proteins and the purified fusion proteins were checked for quantity and quality on a Coomassie blue-staining SDS-Page gel (panel a). Note that full-length GST chlamydial fusion proteins were purified for all 29 proteases including CPAF although certain levels of degradation fragments and free GST molecules were also detected. A parallel set of the same GST fusion proteins were further used to digest the CPAF(E558A)-His fusion protein in a cell-free assay and the digestion products were detected with an anti-His tag antibody on a Western blot as indicated on the left of the figure (panel b). Note that only the positive control Infected-infected HeLa (Ct-HeLa) lysate (lane 2) and the GST-CPAF fusion protein cleaved the CPAF(E558A)-His fusion protein into the CPAF(E558A)c-His fragment (see arrow pointed along the right side of the figure) and none of other chlamydial proteases was able to do so.



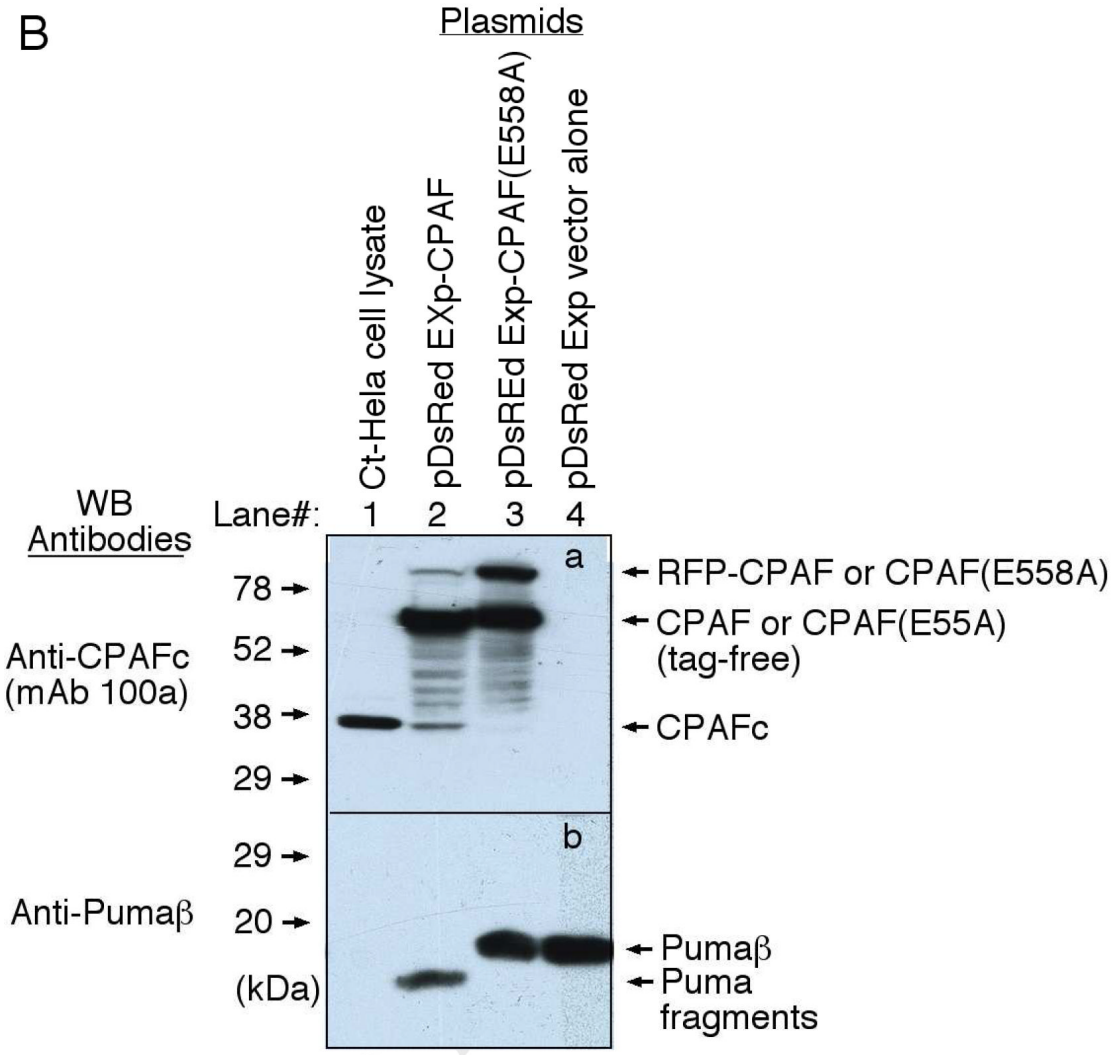
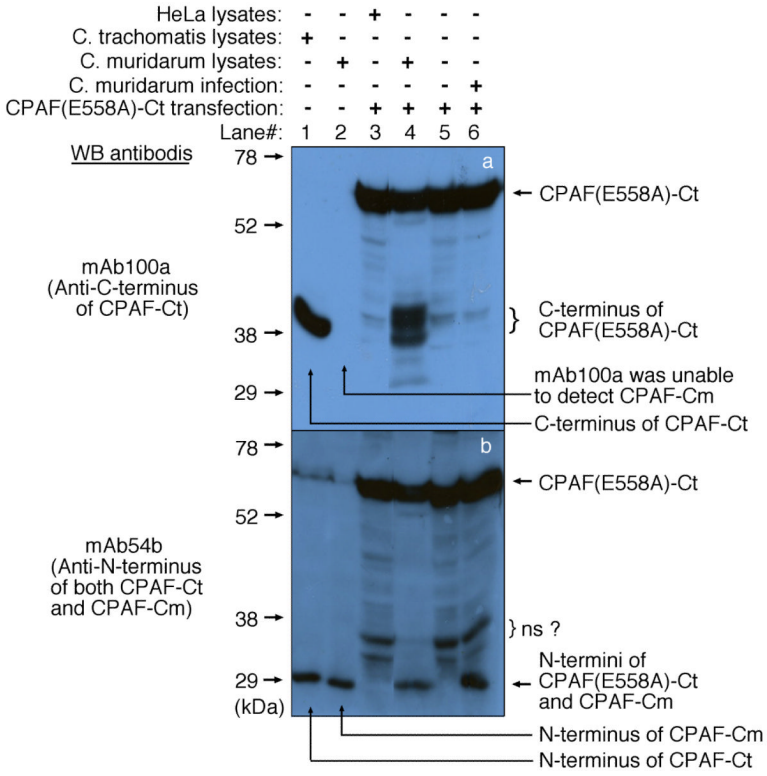


Fig. 5. Induction of CPAF autoprocessing and self-activation in mammalian cells

(A) Mammalian expression vectors pEF/Myc, pDsRed Monomer C1 or pDsRed Express C1 with (pEF/Myc-CPAF, pDsRedMono-CPAF, pDsRedExp-CPAF) or without (pEF/Myc, pDsRedMono or pDsRedExp alone) coding for CPAF as listed on top of the figure were used to transfect 293T cells and 24h after transfection, the whole cell samples were subjected to Western blot detection with the mouse anti-CPAFc mAb 100a (panel a) or rabbit anti-PUMA mAb (panel b). Note that a matured CPAFc fragment identical to that from the Chlamydia-infected HeLa (Ct-HeLa) lysate as indicated on the right side of the figure was detected in samples transfected with CPAF gene encoded in pDsRed but not pEF vectors and more obvious CPAFc appeared in the pDsRedExp-CPAF plasmid-transfected sample. The appearance of CPAFc correlated with PUMA cleavage in the cell samples. (B) The mutant CPAF(E558A) was cloned into the pDsRedExp vector and compared for processing in mammalian cells with the wild type CPAF. The transfection and Western blot detection were carried similarly as described above in legend for (A). Note that only the wild type CPAF sample produced the matured CPAFc fragment (panel a) and displayed Puma cleavage activity (panel b).



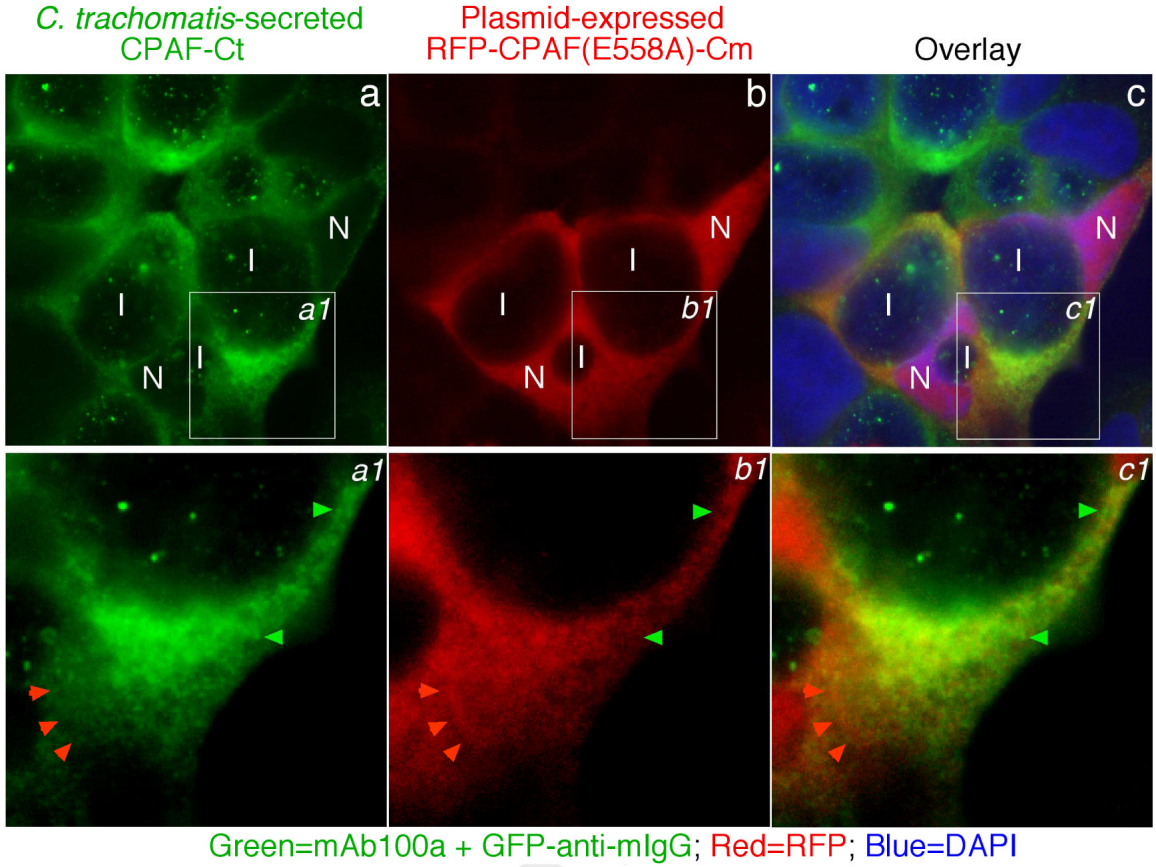


Fig. 6. Processing of CPAF(E558A) in Chlamydia-infected cells

(A) Mammalian expression vector pEF/Myc coding for the mutant CPAF(E558A) from *C. trachomatis* [pEF/Myc-CPAF(E558A)-Ct] was used to transfect HeLa cells and 8h after transfection, some transfected cells were infected with *C. muridarum* organisms. 30h after infection, all transfected cells with or without infection were harvested, some for direct Western blot detection (lanes 5 & 6) while others were incubated with lysates made from either HeLa alone (lane 3) or *C. muridarum*-infected cells (lane 4) prior to the Western blot detection with the mouse mAbs 100a (panel a) and 54b (panel b) as primary antibodies. Lysates made from either *C. trachomatis* serovar D- or *C. muridarum* organism-infected cells were directly loaded as controls (lanes 1 & 2). Note that there was no detection of C-terminal fragment in the CPAF(E558A)-transfected and *C. muridarum*-infected cell sample (lane 6, panel a) although the *C. muridarum*-infected cell lysates contained CPAF-Cm (lane 6, panel b) and cleaved CPAF(E558A)-Ct in a cell-free system (lane 4), suggesting that the Chlamydia-secreted CPAF-Cm was unable to access to the plasmid-expressed CPAF(E558A)-Ct in the same infected cells. (B) Mammalian expression vectors pDsRed Monomer C1 coding with CPAF(E558A)-Cm [pDsRedMono-CPAF(E558A)-Cm] were used to transfect HeLa cells and 8h after transfection, the transfectants were infected with *C. trachomatis* serovar D organisms. 40h after infection, the cell samples were processed for immunofluorescence assay. The plasmid-expressed CPAF(E558A)-Cm was visualized via the fusion tag RFP (red, panel b), the *C. trachomatis*-secreted CPAF-Ct was labeled with mAb100a and visualized with a goat anti-mouse IgG conjugated with Cy2 (green, panel a) while DAPI was used to visualized DNA (blue). “N” stands for nuclei while “I” for chlamydial inclusion. The selected areas from each panel were shown in the bottom row marked with a1, b1 & c1. Note that the Chlamydia-secreted CPAF-Ct (green or green arrowheads) did not overlap with the plasmid-expressed CPAF(E558)-Cm (red or red arrowheads).