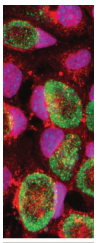

Pathogens and Disease ISSN 2049-632X

COMMENTARY

Analysis of CPAF mutants: new functions, new questions (The ins and outs of a chlamydial protease)

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Keywords
Chlamydia; pathogenesis; protease.

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Received 02 June 2014; accepted 06 June 2014. Final version published online August 2014.

doi:10.1111/2049-632X.12194

Editor: Peter Timms

Abstract

The role of the chlamydial protease CPAF, previously described as a secreted serine protease processing a wealth of host and chlamydial proteins to promote chlamydial intracellular growth, has recently been questioned by studies from the groups of Tan and Sütterlin, who demonstrated that the reported proteolysis of almost a dozen substrates by CPAF occurred during preparation of cell lysates rather than in intact cells. Valdivia *et al.* have now compared near-isogenic pairs of CPAF-deficient and secretion-deficient mutants of *Chlamydia trachomatis* and their wild-type parent. Their report, published in this issue of *Pathogens and Disease*, is a landmark study in the emerging era of *Chlamydia* genetics. The results of Tan and Sütterlin are confirmed with a few additions. While CPAF's role in pathogenesis is diminished considerably from these studies, CPAF remains an important factor in chlamydial biology as (1) CPAF mutants produce less infectious yield than wild type; and (2) CPAF is responsible for proteolytic cleavage of vimentin and LAP-1, but only after lysis of the inclusion membrane, not upon CPAF secretion to the cytosol. Here, we briefly review the evidence in support of CPAF's active secretion from the mid-to-late inclusion and conclude that new experimentation to establish whether or not CPAF is actively secreted should precede any new investigation of CPAF's cellular activities during mid-to-late development.

A major research goal in *Chlamydia* basic biology is the discovery of new virulence factors and how they contribute to disease pathogenesis. A prominent symbol of chlamydial virulence is the chlamydial protease- or proteasome-like activity factor, better known as CPAF, whose discovery by Zhong *et al.* (2001) instantly captured the imagination of the chlamydial research community. CPAF was characterized as a serine protease secreted into the cytosol of infected cells at mid-to-late development and, after self-processing to the active form (Shaw *et al.*, 2002; Dong *et al.*, 2004a; Huang *et al.*, 2008; Chen *et al.*, 2010a), was held responsible for the degradation of host transcription factors regulating MHC expression. Moreover, serine protease inhibitors that blocked activation also appeared to specifically block processing of the transcription factors, hence providing associative support for a role of CPAF in processing its eukaryotic substrates, and downstream immunologic consequences. In the years following the initial report, CPAF was shown to occur across *Chlamydia* spp. (Fan *et al.*, 2002; Shaw *et al.*, 2002; Heuer *et al.*, 2003; Dong *et al.*, 2005) and CPAF activity was experimentally linked to a number of host and chlamydial targets in ways that associated CPAF with a whole variety of chlamydial intracellular survival strategies and mechanisms of disease pathogenesis (Dong *et al.*, 2004b; Pirbhai *et al.*, 2006; Kawana *et al.*, 2007; Kumar & Valdivia, 2008; Paschen *et al.*, 2008; Sun & Schoborg, 2009; Christian *et al.*, 2011; Jorgensen *et al.*, 2011; Knowlton *et al.*, 2011; Yu *et al.*, 2011; Qi *et al.*, 2011b, reviewed in Zhong (2009). Among others, CPAF was reported to cleave pro-apoptotic factors, transcription factors important for NF-κB-mediated signaling along with cleavage of host cell junctional adherence proteins, and a variety of pro-inflammatory proteins and host cell cycle regulatory proteins.

As the list of CPAF targets continued to expand, Ming Tan, Christine Sütterlin *et al.* published an article in 2012 that demonstrated that proteolytic cleavage of 11 substrates attributed to CPAF might actually stem from the method used to lyse cells (Chen *et al.*, 2012). These investigators reported that when CPAF and its targets were obtained from a standard detergent lysate of infected cells, then CPAF clearly was the advertised master regulator of chlamydial

virulence. If, however, the risk of proteolysis was eliminated using 8 M urea for extraction or minimized using the CPAF inhibitor *clasto*-lactacystin just before lysis, at a time when CPAF activity was expected [e.g. 36 h postinfection (hpi)], the results were quite different. Although the amount of active (processed) CPAF was unchanged, the proteolysis of all 11 proteins was no longer detectable by immunoblotting. Most importantly, coincidental host cell alterations such as Golgi reorganization, resistance to apoptosis and remodeling of the cytoskeleton were still observed. This provided inescapable evidence that the reported degradation of these proteins was unlikely due to CPAF-mediated proteolysis as previously proposed.

At this juncture in the history of CPAF, the previous prevailing notion that CPAF was THE central player of chlamydiae-induced host cell modulation was compromised and a more careful evaluation of CPAF was necessary. Fortunately, the ongoing mini CPAF scientific revolution has coincided with a more general technological revolution in *Chlamydia* research. At last, we have the means to genetically manipulate these important human pathogens, and we now stand at the doorstep of evaluating putative chlamydial virulence factors in targeted mutants that are otherwise isogenic with their 'wild-type' parents. The article published by Raphael Valdivia *et al.* in this issue of *Pathogens and Disease* (Snavely *et al.*, 2014) addresses the question of the function of CPAF in this way by exploiting the analytical power of well-defined near-isogenic mutant–parent pairs of *Chlamydia trachomatis*, following the classic molecular Koch postulates approach for the identification of virulence genes described by Stanley Falkow (1988) many years ago. Thus in addition to dealing with one of the most important issues in *Chlamydia* research, this article represents a pioneering effort in what is likely to become a prototype study for the field.

The report is justifiably economical in discussing its results as the analysis of two loss-of-function CPAF mutants and one type II secretion mutant makes a compelling enough statement in and of its own. First and foremost, this analysis of CPAF-deficient mutants provides unequivocal support to the results produced by the Tan and Sütterlin groups that raised significant doubts about the ability of CPAF to cause specific host cell phenotypes via proteolysis of reported substrates (Chen *et al.*, 2012). In short, CPAF is not responsible for golgin-84 cleavage-mediated Golgi reorganization into mini stacks as previously thought (Christian *et al.*, 2011), although Golgi reorganization does indeed occur (Heuer *et al.*, 2009). Likewise, CPAF does not protect against staurosporine-inducible programmed cell death by degradation of the proapoptotic BH3-only proteins Bim, Bik and Puma as previously reported (Pirbhai *et al.*, 2006). Valdivia *et al.* further demonstrate that CPAF activity is not required for *Chlamydia*-infected cells to inhibit expression of NF-kB-dependent genes (Christian *et al.*, 2010). CPAF activity is also not needed to protect infected cells from super-infection as initially reported (Jorgensen *et al.*, 2011). Tan, Sütterlin *et al.* had extensively investigated a number of previously described CPAF substrates [listed in Table 1 of Chen *et al.*, 2012) that also remained intact when artificial proteolysis was eliminated upon extraction with urea. Although most of these were not tested by Valdivia *et al.*, the demonstrated noninvolvement of CPAF, using both biochemical (Chen *et al.*, 2012) and mutant (Snavely *et al.*, 2014) analyses for several of these, warrants that the role of CPAF processing be questioned for the others. In our view, it is likely that the processing of many of the previously described CPAF cellular targets will require search for alternative effectors. Conversely, many of the phenotypes previously attributed to CPAF activity (e.g. Golgi fragmentation) still occur during chlamydial infection, irrespective of CPAF. Strong candidates for initial screening of the chlamydial factors responsible for these phenotypes are the type III secreted effectors whose numbers continue to rise (Subtil *et al.*, 2000, 2001, 2005; Clifton *et al.*, 2004; Fields *et al.*, 2005; Hobolt-Pedersen *et al.*, 2009; Hower *et al.*, 2009; Pennini *et al.*, 2010; Stone *et al.*, 2012; Hovis *et al.*, 2013; Pais *et al.*, 2013).

The silver lining of the findings by the Valdivia group is that not all CPAF targets are artifactual. CPAF may still play an important role in chlamydial pathogenesis as revealed by two distinct findings: the demonstration of a reduced infectious yield in the mutants compared to their isogenic parent, and the demonstration of the very late CPAF-mediated processing of vimentin and the lamin-associated protein LAP-1. While the former potentially opens a new exciting avenue of research on the late stages of chlamydial development, during RB differentiation to infectious EB, the latter is an equally intriguing finding that likely illustrates a genuine function of CPAF at the inclusion lysis stage that occurs within the still unlysed infected cell. Using infected cells expressing the membrane-impermeant red-fluorescent tdTomato protein as a test of inclusion and plasma membrane integrity, the authors were able to show that vimentin and LAP-1, two previously characterized substrates of CPAF (Bednar *et al.*, 2011), were actually processed in a CPAF-dependent manner, but only after lytic disruption of the inclusion membrane and prior to that of the plasma membrane. Based on these results, the authors suggest '*that the bulk of active CPAF is sequestered within the inclusion lumen*'.

If this is true, then the postinclusion lysis timing of vimentin and LAP-1 processing is temporally dissociated from the autoprocessing of the CPAF zymogen to the active protease and its presumed translocation from the intact late cycle inclusion to the cytosol of the infected cell. The immediate question this raises is whether CPAF is actually secreted at all from an intact inclusion or whether active CPAF is simply released very late in development upon and after lysis of the inclusion. Here, we scrutinize anew the evidence that has led to the conclusion that CPAF is secreted to the cytosol of the infected cell from a mid-to-late developmental cycle inclusion.

The early conclusion that CPAF was secreted to the cytosol is primarily based on immunofluorescence data using CPAF-specific antibodies (e.g. Zhong *et al.*, 2001; Shaw *et al.*, 2002; Heuer *et al.*, 2003; Chen *et al.*, 2010a, b) or anti-FLAG tag antibodies with CPAF expressed ectopically as a FLAG tag fusion (Bauler & Hackstadt, 2014), that

is, a single methodology, albeit repeated multiple times by multiple groups, and with different antibodies. In these experiments, the timing of CPAF secretion varies [e.g. 10 hpi (Kawana *et al*., 2007) vs. 24 hpi (Shaw *et al*., 2002) for *C. trachomatis*], and CPAF-specific signal varies in intensity and appearance from punctate dispersed staining, possibly suggesting association with small vesicles (e.g. Zhong *et al*., 2001; Fan *et al*., 2002) to almost halo-like diffuse staining (e.g. Gong *et al*., 2011; Bauler & Hackstadt, 2014), suggesting a freely diffusible antigen. Further review of published reports documenting the presence of CPAF in the cytosol (Zhong *et al*., 2001; Fan *et al*., 2002; Shaw *et al*., 2002; Heuer *et al*., 2003; Kawana *et al*., 2007; Sun & Schoborg, 2009; Chen *et al*., 2010b; Gong *et al*., 2011; Qi *et al*., 2011a; Wang *et al*., 2011) indicates that paraformaldehyde (0.5–4%) and saponin (0.2–4%) or Tween-20 (0.1%) were almost uniformly used in these experiments, respectively, for fixation and permeabilization. Although widely used in cellular microbiology, immunofluorescence has previously been documented to produce a variety of often-spectacular artifacts (Schnell *et al*., 2012) that directly relate to the methods used for fixation and permeabilization. Thus, confirmation of the findings using alternative fixation and permeabilization methods or via another approach is usually preferable and is widely recommended. However, the notion that CPAF was in fact secreted was reinforced upon the immediate identification of highly significant cytosolic targets, indeed starting with the very first report on CPAF (Zhong *et al*., 2001). Each subsequent identification of a new cytosolic target served to reinforce that CPAF was indeed translocated to the cytosol at mid-to-late developmental cycle times. Using a different approach, Kleba & Stephens (2008) showed that CPAF was secreted to the cytosol of perfringolysin O (PFO)-permeabilized fibroblasts at mid-cycle. To demonstrate that CPAF was not 'leaking' transiently from PFO-treated inclusions, these authors showed that 10-kDa fluorescein-conjugated dextran was unable to diffuse into the inclusion from the cytosolic compartment. Although superficially resembling the approach of Valdivia *et al.* (Snavely *et al*., 2014), these two methods are fundamentally different in that Kleba and Stephens did not test for inclusion leakage at very late times. Moreover, systematic differences (e.g. sensitivity) or differential properties of the impermeant reagents used (10-kDa dextran vs. tdTomato protein) could account for the different results. In another attempt to produce evidence of CPAF translocation, Heuer *et al.* used immunoelectron microscopy (IEM) to assess CPAF cellular distribution at mid (2 days) and late (4 days) developmental times in *C. pneumoniae*-infected cells (Heuer *et al*., 2003). Unsurprisingly, CPAF signal was abundant in the day 2 inclusion. However, CPAF signal was absent from the cytosol at day 4 (D. Heuer, pers. commun.). Whether this owes to the poor sensitivity of IEM or other factors is not known.

Taken together, a re-analysis of published results suggests that the evidence for CPAF secretion is not nearly as compelling as previously thought and that it could represent yet another artifact. The apparent discordance in results and their interpretations we have summarized above epitomizes the need to be cautious in the interpretation of data obtained from a single experimental approach and, conversely, the need for experimental validation of any result using unrelated approaches. We therefore suggest that before any more flawed conclusions are reached on CPAF's role in chlamydial biology, a critical reexamination of the kinetics of CPAF secretion should be performed to unequivocally determine whether and when this protease is secreted into the host cell cytoplasm from an actively growing, uncompromised inclusion, and by what mechanism. This should be achieved using multiple methods of fixation and permeabilization in immunofluorescence or IEM, as well as nonimaging methods such as subcellular fractionation. With the advent of genetic methods for chlamydiae, imaging analysis of CPAF fused to a reporter such as GFP should also be possible in live cells, which would do away with the need to fix and permeabilize. Another toolset that has been critical in assigning function to CPAF consists of a number of CPAF inhibitors that have permitted to correlate CPAF activity to the processing of its presumed targets. These inhibitors were known from the onset to potentially have broad ranging effects on other proteases and on the host cell. However, this potential red flag went unheeded when faced with all the other data that cumulatively pointed to CPAF as a central regulator of chlamydial pathogenesis. With the benefit of hindsight, the pleiotropic activities of any CPAF inhibitor used in the context of cellular or subcellular studies of chlamydial pathogenesis should be better documented in any future investigation.

From the moment CPAF was described as a self-processing potent protease that was secreted by the actively developing inclusion to the host cell cytosol where it could 'hit' biologically relevant targets, any new investigation of CPAF as a cellular effector of chlamydial pathogenesis was 'doomed to succeed'. While each new target raised the role of CPAF as a master regulator of chlamydial pathogenesis to new heights, it also raised a few eyebrows when the number of targets put functional multitasking on a level rarely heard of for any enzyme in biology. Although the recent studies by the groups of Tan and Sütterlin (Chen *et al*., 2012) and Valdivia (Snavely *et al*., 2014) have brought CPAF down a notch, it remains that this protease is still likely to be a critical factor in the developmental biology of *Chlamydia* spp. with direct consequences on the pathogenesis of these organisms. The exploitation of CPAF mutants and the rigorous application of experimental standards that remove the possibility of artifacts is, however, a 'must' in any future study.

A recent review article by Conrad *et al.* (2013) is a forceful plaidoyer advocating for renewed investigation of CPAF, particularly its role in virulence. For the benefit of interested readers and new researchers entering the field, we believe that it is important to think carefully about how to develop testable hypotheses involving CPAF localization and activities. First and foremost, any new investigation that is focused on CPAF substrates at mid-to-late developmental stages should ensure that artifactual CPAF-mediated proteolysis owing to the extraction method does not confound the analysis. Studies using newly available technology and

multiple approaches should examine whether CPAF is indeed able to reach its presumed cytosolic target via secretion or some other mechanism that allows it to escape from the inclusion. If CPAF is found to not constitutively translocate, then CPAF substrate studies must take this important limitation into consideration. Likewise, the potential activities of CPAF-'specific' inhibitors need to be carefully re-evaluated before any additional conclusions are reached. The review by Conrad *et al.* (2013) correctly points out that we are entering a new age of *Chlamydia* research as the converging impact of emerging genetic analysis, next-gen 'omic' and system-based analyses together with the wealth of cell biology and immunology already acquired, is auguring quantum leaps in our understanding of *Chlamydia* pathogenesis and translational applications thereof. The 'CPAF treasure box' (Conrad *et al.*, 2013) thus still likely exists as strongly argued in a recent commentary by Häcker (2014) and highlighted by the results of Valdivia *et al.* (Snavely *et al.*, 2014), but we believe it will be critical to reopen it ever so cautiously so as to not repeat similar mistakes as we enter a new era of *Chlamydia* research.

Acknowledgements

The authors are grateful to Drs Ming Tan, Christine Sütterlin, Georg Häcker and Priscilla Wyrick for their critical reading of earlier drafts of the manuscript. This work was supported by NIH NIAID STI CRC U19 AI084044 to P.M.B. and by NIH NIAID RO1 AI019782 to G.I.B.

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