



# *Chlamydia trachomatis* secretion of proteases for manipulating host signaling pathways

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The human pathogen *Chlamydia trachomatis* secretes numerous effectors into host cells in order to successfully establish and complete the intracellular growth cycle. Three *C. trachomatis* proteases [chlamydial proteasome/protease-like activity factor (CPAF), tail-specific protease (Tsp), and chlamydial high temperature requirement protein A (cHtrA)] have been localized in the cytosol of the infected cells either by direct immunofluorescence visualization or functional implication. Both CPAF and Tsp have been found to play important roles in *C. trachomatis* interactions with host cells although the cellular targets of cHtrA have not been identified. All three proteases contain a putative N-terminal signal sequence, suggesting that they may be secreted via a sec-dependent pathway. However, these proteases are also found in chlamydial organism-free vesicles in the lumen of the chlamydial inclusions before they are secreted into host cell cytosol, suggesting that these proteases may first be translocated into the periplasmic region via a sec-dependent pathway and then exported outside of the organisms via an outer membrane vesicles (OMVs) budding mechanism. The vesiculated proteases in the inclusion lumen can finally enter host cell cytosol via vesicle fusing with or passing through the inclusion membrane. Continuing identification and characterization of the *C. trachomatis*-secreted proteins (CtSPs) will not only promote our understanding of *C. trachomatis* pathogenic mechanisms but also allow us to gain novel insights into the OMV pathway, a well-known mechanism used by bacteria to export virulence factors although its mechanism remains elusive.

**Keywords:** *Chlamydia trachomatis*, proteases, secretion, pathogenesis

## INTRODUCTION

*Chlamydia trachomatis* is the most frequently reported bacterial sexually transmitted infection in the US (Centers for Disease Control Prevention, 2009), which, if untreated, can lead to severe complications characterized with inflammatory pathologies, including pelvic inflammatory diseases, ectopic pregnancy, and infertility (Land et al., 2010). The chlamydial intracellular replication is thought to significantly contribute to the *C. trachomatis*-induced inflammatory pathologies (Stephens, 2003; Wyrick, 2010). A typical chlamydial replication cycle starts with the invasion of an epithelial cell with a chlamydial infectious elementary body (EB), which is facilitated by chlamydial injection of preexisting effectors into the epithelial cell to induce and modulate endocytosis (Clifton et al., 2004, 2005; Engel, 2004; Hower et al., 2009). Once internalized, an EB differentiates into a non-infectious but metabolically active reticulate body (RB). The RB makes new proteins not only for multiplication but also for secretion into the inclusion lumen and membrane (Rockey et al., 1995, 2002; Luo et al., 2007a,b,c; Li et al., 2008) as well as host cell cytosol (Valdivia, 2008; Zhong et al., 2009, 2011; Betts-Hampikian and Fields, 2010) via a type III secretion (T3S, Fields and Hackstadt, 2000; Valdivia, 2008; Betts-Hampikian and Fields, 2010), sec-dependent secretion (Chen et al., 2010b), or an autotransporter (Henderson and Lam, 2001; Wehrle et al., 2004; Carlson et al., 2005; Vandahl et al., 2005; Kiselev et al., 2009; Byrne, 2010) pathways. After replication, the progeny RBs differentiate back into EBs for spreading to near-by cells. It is thought that the *C. trachomatis*-secreted proteins (CtSPs; ref:

Valdivia, 2008; Zhong, 2009, 2011; Betts-Hampikian and Fields, 2010) are not only necessary for completing the existing developmental cycle but also essential for ensuring a successful start of subsequent infection cycles. Identification and characterization of CtSPs may provide important knowledge for understanding chlamydial pathogenic mechanisms and improving diagnosis, treatment, and prevention of *C. trachomatis* infection. Thus, identification of CtSPs has become an intensively investigated topic for chlamydiaologists.

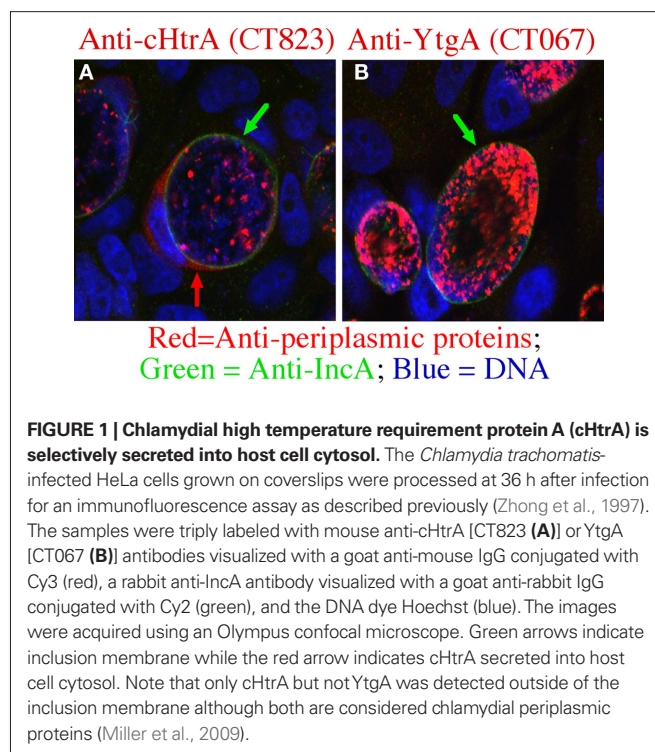
## CHLAMYDIA TRACHOMATIS-SECRETED PROTEASES IN PATHOGENESIS

A *C. trachomatis* genome typically encodes more than two dozens of proteins with proteolytic activity (Stephens et al., 1998). A function-driven approach has led to the identification of a novel serine protease, designated as chlamydial proteasome/protease-like activity factor (CPAF; Zhong et al., 2001). The chlamydial proteases are usually distributed in the chlamydial cytosol, periplasmic region and/or various membranes for fulfilling their respective roles in chlamydial biology. However, some, including CPAF (encoded by ORF CT858), the tail-specific protease (Tsp, CT441), and the chlamydial high temperature requirement protein A protease (cHtrA, CT823), are also found outside of the chlamydial organisms by direct visualization and/or functional implication, suggesting that these proteases may be used by *C. trachomatis* organisms to target host proteins for manipulating host signaling pathways.

Chlamydial proteasome/protease-like activity factor is an extensively studied *Chlamydia*-secreted serine protease with a water molecule-mediated catalytic triad consisting of residues H105, S499, and E558. CPAF and it can undergo autoprocessing for activation (Dong et al., 2004a,b; Huang et al., 2008; Chen et al., 2009, 2010a). CPAF has a broad substrate specificity and attacks a wide spectrum of host proteins, including the transcriptional factors USF-1 (Zhong et al., 1999) and RFX5 (Zhong et al., 2000) for potentially evading immune recognition, and HIF-1 (Rupp et al., 2007) for dealing with stress responses, the BH3-only proteins (proapoptotic members of the Bcl-2 family; Fischer et al., 2004; Dong et al., 2005; Pirbhai et al., 2006) for inhibiting apoptosis (Fan et al., 1998; Greene et al., 2004; Xiao et al., 2004, 2005; Zhong et al., 2006), the DNA repairing enzyme PARP [Poly (ADP-ribose) polymerase], and cell cycling proteins (Balsara et al., 2006; Paschen et al., 2008) for altering cell cycle, cytoskeleton proteins (keratins 8 and 18, vimentin Dong et al., 2004c; Kumar and Valdivia, 2008; Savijoki et al., 2008) for promoting inclusion expansion, and even cell surface proteins CD1d (Kawana et al., 2007) and nectin-1 (Sun and Schoborg, 2009). Although the functional consequences of some of the host protein degradation by CPAF remain unknown, the host protein degradation should benefit *C. trachomatis* intracellular growth (Zhong, 2009).

Although Tsp was not detected outside of inclusions using standard immunofluorescence assays (data not shown), it was found to cleave host NF- $\kappa$ B in *C. trachomatis*-infected cells (Lad et al., 2007a,b), suggesting that an undetectable amount of Tsp might be secreted into host cell cytosol for interrupting NF- $\kappa$ B function. During *C. trachomatis* infection, there were conflicting observations: On one hand, *C. trachomatis* infection activated a wide variety of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF $\alpha$  (Rasmussen et al., 1997; Cheng et al., 2008); On the other, no significant NF- $\kappa$ B activation was detected in the infected cells (Xiao et al., 2005; Lad et al., 2007a). It turned out that the *C. trachomatis* organisms used the MAP kinase pathway to promote chlamydial acquisition of host lipids, during which inflammatory responses were inevitably activated (Su et al., 2004). The lack of NF- $\kappa$ B activation was probably due to the chlamydial ability to actively silence the NF- $\kappa$ B inflammatory pathway. NF- $\kappa$ B p65 was cleaved into two major fragments (p40 and p20) by Tsp (Lad et al., 2007a) and CPAF (Christian et al., 2010). Since the N-terminal fragment p40 maintained the ability to interact with I- $\kappa$ B $\alpha$  (a cytoplasmic inhibitor of NF- $\kappa$ B) and to bind to DNA but lacked transactivation capability, the p40 might be able to block the residual full length p65-mediated response via a dominant negative effect (Lad et al., 2007a). Tsp was also reported to interact with the host SRAP1 co-activator of estrogen receptor  $\alpha$  (Borth et al., 2010). However, the biological significance of chlamydial Tsp-host SRAP-1 interaction remains unknown.

We recently detected the *C. trachomatis* periplasmic protease cHtrA in both the chlamydial inclusion lumen and host cell cytosol (Figure 1), suggesting that the chlamydial periplasmic protein cHtrA is also secreted into host cells. The secretion appeared to be specific since no other chlamydial periplasmic proteins including CT067 (Miller et al., 2009) were detected outside of the chlamydial inclusions (Figure 1 and data not shown). Normally, HtrA from eukaryotic and prokaryotic species exhibits both chaperone and



**FIGURE 1 | Chlamydial high temperature requirement protein A (cHtrA) is selectively secreted into host cell cytosol.** The *Chlamydia trachomatis*-infected HeLa cells grown on coverslips were processed at 36 h after infection for an immunofluorescence assay as described previously (Zhong et al., 1997). The samples were triply labeled with mouse anti-cHtrA [CT823 (A)] or YtgA [CT067 (B)] antibodies visualized with a goat anti-mouse IgG conjugated with Cy3 (red), a rabbit anti-IncA antibody visualized with a goat anti-rabbit IgG conjugated with Cy2 (green), and the DNA dye Hoechst (blue). The images were acquired using an Olympus confocal microscope. Green arrows indicate inclusion membrane while the red arrow indicates cHtrA secreted into host cell cytosol. Note that only cHtrA but not YtgA was detected outside of the inclusion membrane although both are considered chlamydial periplasmic proteins (Miller et al., 2009).

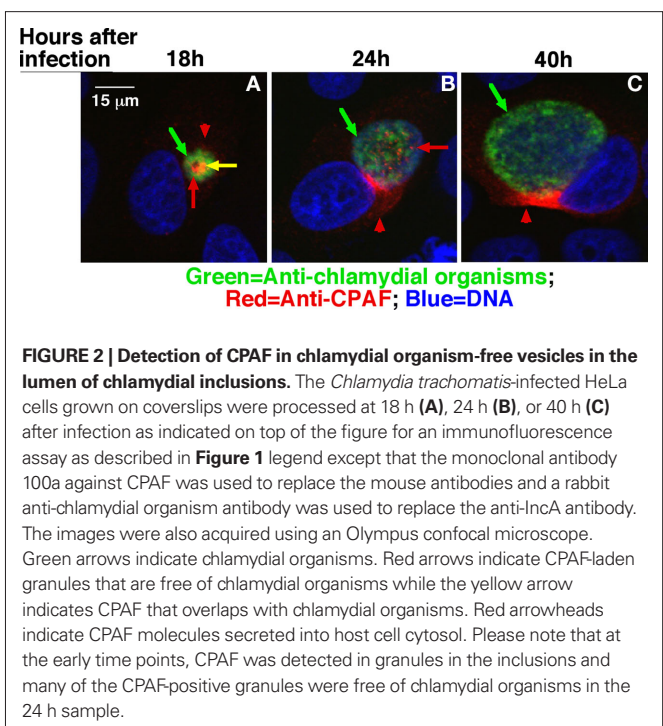
proteolytic activities with a broad proteolytic substrate specificity (Huston et al., 2007, 2008). HtrA is a hexamer formed by staggered association of trimeric rings and the access to the proteolytic sites in central cavity is controlled by 12 PDZ domains in the sidewall (Krojer et al., 2002, 2010). In eukaryotic cells, HtrA responds to unfolded proteins in the endoplasmic reticulum (ER) by cleaving and releasing the ER membrane-anchored transcription factors ATF6 and SREBP into nucleus to activate the expression of proteins required for the unfolded protein response and cholesterol biosynthesis (Brown and Goldstein, 1999; Ye et al., 2000). In bacteria, the periplasmic HtrA, in response to the binding of C-terminal peptides from unfolded/reduced outer membrane proteins, cleaves, and releases the  $\sigma^E$ -factor to activate stress response genes (Walsh et al., 2003). Since HtrA is required for bacterial survival under high temperature, it is called High temperature requirement (Htr) protein (Missiakas et al., 1997). Although both the tertiary structure and function of HtrA are well-known, the role of cHtrA in chlamydial pathogenesis remains unclear. The finding that cHtrA was localized both in the chlamydial inclusion lumenal space and the host cell cytosol suggests that the chlamydial periplasmic cHtrA may also contribute to the chlamydial proteolysis strategies for manipulating host cell signaling pathways. However, it is still unknown how the secreted cHtrA contributes to chlamydial pathogenesis. Can the secreted cHtrA gain access to host cell ER to regulate the host unfolded protein stress responses? What are the cellular targets of the secreted cHtrA during chlamydial infection? Interestingly, HtrA from the human gastric pathogen *Helicobacter pylori* is also secreted outside the bacteria (Lower et al., 2008). More importantly, it can cleave E-cadherin to disrupt epithelial tight junction, which may benefit the bacterial invasion of the gastric epithelial tissues (Hoy et al., 2010). Since HtrA and other conserved proteases are

known to play important roles in bacterial pathogenesis (Ingmer and Brondsted, 2009), identifying cellular targets of the Chlamydia-secreted cHtrA should provide novel insights into chlamydial pathogenic mechanisms.

### POTENTIAL PATHWAYS REQUIRED FOR *C. TRACHOMATIS* SECRETION OF PROTEASES INTO HOST CELL CYTOSOL

All three proteases localized in the host cell cytosol contain an N-terminal signal sequence, suggesting that they may be translocated into the periplasmic region via a sec-dependent secretion pathway. Among the many known secretion pathways, the sec-dependent or twin-arginine translocon (Tat) pathway is used by bacteria to deliver proteins into the periplasmic space. The Tat translocase, consisting of the TatA/E, B and C proteins, is responsible for transporting folded proteins across the inner membrane (Dilks et al., 2003; Lee et al., 2006). However, *C. trachomatis* genome does not encode any homolog of the Tat translocases (Stephens et al., 1998) but encodes all essential components required for a functional sec-dependent pathway (Stephens et al., 1998), suggesting that *C. trachomatis* organisms can use the universally conserved sec-dependent pathway to translocate proteins with an N-terminal signal sequence from cytoplasm into periplasmic regions. Indeed, we have previously demonstrated that a sec-dependent pathway is required for exporting CPAF to host cell cytosol (Chen et al., 2010b). The N-terminal signal sequence of CPAF (CPAFss) was cleaved from mature CPAF and CPAFss directed translocation of PhoA into bacterial periplasm.

However, the sec-dependent pathway alone can only deliver its cargoes into the periplasmic region. The periplasmic proteins are further exported outside of the bacterial organisms using the chaperone/usher, autotransporter, or Type II outer membrane GspD pore complex pathways. Although chlamydial genome encodes homologs of both autotransporters and GspD, these pathways deliver periplasmic proteins out of the organisms in free form. It is hard to imagine how free proteins secreted into the inclusion lumen can pass through the inclusion membrane and enter host cell cytosol in a regulated/controlled manner. Interestingly, both CPAF (Figure 2) and cHtrA (data not shown) were detected in organism-free granules in the lumen of inclusions before their secretion into host cell cytosol. It is thus hypothesized that the chlamydial proteases that are translocated into the periplasmic regions may be further exported to the lumen of inclusions via an outer membrane vesicles (OMVs) budding mechanism. This hypothesis is supported by various previous observations that the chlamydial RB outer



membrane was induced to undergo vesiculation (Matsumoto and Manire, 1970) and chlamydial organism-free vesicles were detected both inside (Jorgensen and Valdivia, 2008) and outside of inclusion membrane (Giles et al., 2006). The vesiculated proteases may further enter host cell cytosol by vesicle fusing with or passing through the inclusion membrane. Although OMVs have been recognized as an essential means for gram-negative bacteria to secrete virulence factors (Bomberger et al., 2009; Ellis and Kuehn, 2010; Parker et al., 2010; Unal et al., 2010), the precise mechanisms on how OMVs are regulated remain unknown (Haurat et al., 2011). That's why some remain skeptical about whether OMVs can represent a specific mechanism for protein secretion. The *C. trachomatis*-infected cells by allowing multiple vesiculated "effectors" to be studied simultaneously in a confined space may provide a unique opportunity for us to gain novel insights into the mechanisms of OMVs.

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