Chlamydia trachomatis secretion of hypothetical protein CT622 into host cell cytoplasm via a secretion pathway that can be inhibited by the type III secretion system inhibitor compound 1

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Using antibodies raised with *C. trachomatis* fusion proteins, we localized a hypothetical protein encoded by the ORF ct622 in the cytoplasm of *C. trachomatis*-infected mammalian cells. The detection was specific since the antibody labelling of CT622 protein was removed by preabsorption with CT622 but not other fusion proteins. We similarly confirmed that CT621, a known secretion protein encoded by a hypothetical ORF downstream of ct622, was secreted into host cell cytosol. Proteins CT622 and CT621 displayed a similar secretion pattern, with both intra-inclusion and host cell cytosol localization, that was distinct from that of CPAF (chlamydial protease/proteasome-like activity factor). However, the expression and secretion kinetics differed significantly between CT622 and CT621: CT622 mRNA was detected at 2 h, protein at 6 h and secretion of protein into host cell cytoplasm at 36 h post-infection, while CT621 mRNA was detected at 8 h, protein at 16 h and secretion at 24 h. The secretion of both CT622 and CT621 was blocked by *N'*-(3,5-dibromo-2-hydroxybenzylidene)-4-nitrobenzohydrazide (compound 1), an inhibitor known to target the type III secretion system of bacteria. These results suggest that CT621 and CT622 may fulfil different functions during chlamydial intracellular growth. Further characterization of these proteins may generate important information for understanding chlamydial pathogenesis.

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INTRODUCTION

Chlamydia trachomatis infects both human ocular and urogenital epithelial tissues, causing trachoma (Wright *et al.*, 2008) and sexually transmitted diseases (Sherman *et al.*, 1990). Although trachoma is disappearing in much of the developed world as sanitation and overall living conditions improve, sexually transmitted diseases caused by *C. trachomatis* are increasing worldwide (Mertz *et al.*, 1998; Peterman *et al.*, 2006). *C. trachomatis* is the most common cause of sexually transmitted bacterial diseases in the United States. It is estimated that more than 4 million people are infected each year, with ~10–15% of the untreated individuals developing pelvic inflammatory diseases. *C. trachomatis*-induced inflammatory damage in

the fallopian tubes can lead to ectopic pregnancy and infertility (den Hartog *et al.*, 2006; Toye *et al.*, 1993). Despite the severe health problems caused by *C. trachomatis* infection, the pathogenic mechanisms remain unclear and there is no licensed vaccine for this bacterium (Rockey *et al.*, 2009).

The obligate intracellular replication of *C. trachomatis* has been hypothesized to contribute significantly to the inflammatory pathologies (Stephens, 2003). Although *C. trachomatis* invades epithelial cells via its infectious form known as the elementary body (EB), for much of its intracellular life it exists as a non-infectious form known as the reticulate body (RB), with varying levels of metabolic activity. All chlamydial biosynthesis activities are restricted within a cytoplasmic vacuole known as an inclusion (Hackstadt *et al.*, 1997). After multiple rounds of replication, the resultant RBs can differentiate into EBs for exiting the infected cells and spreading to new cells. In order to generate sufficient progeny EBs, *C. trachomatis* has to both take up nutrients and energy from host cells

Abbreviations: C1, compound 1 [*N'*-(3,5-dibromo-2-hydroxybenzylidene)-4-nitrobenzohydrazide]; CPAF, chlamydial protease/proteasome-like activity factor; EB, elementary body; GST, glutathione *S*-transferase; MOMP, major outer-membrane protein; RB, reticulate body; T3SS, type III secretion system.

(Cocchiario *et al.*, 2008; Hackstadt *et al.*, 1995; McClarty, 1994; Su *et al.*, 2004) and maintain the integrity of the host cells by preventing the infected cells from undergoing apoptosis and host immune detection (Zhong, 2009). To achieve these goals, *C. trachomatis* has evolved the ability to secrete proteins into both the inclusion membrane (Li *et al.*, 2008a; Rockey *et al.*, 2002) and host cell cytoplasm (Fields *et al.*, 2003; Valdivia, 2008; Zhong, 2009; Zhong *et al.*, 2011). The first *C. trachomatis* protein identified as being secreted into host cell cytosol was CPAF, a chlamydial protease/proteasome-like activity factor (Zhong *et al.*, 2001). CPAF is a serine protease (Chen *et al.*, 2009; Huang *et al.*, 2008) that can degrade a wide array of host proteins, including cytokeratins for assisting chlamydial inclusion expansion (Dong *et al.*, 2004; Kumar & Valdivia, 2008; Scidmore, 2008), transcriptional factors required for major histocompatibility complex antigen expression for evading immune detection (Zhong *et al.*, 1999, 2000) and BH3-only domain proteins for inhibiting apoptosis (Fan *et al.*, 1998; Pirbhai *et al.*, 2006).

Identifying *C. trachomatis* proteins secreted into the host cell cytoplasm can provide essential tools for further understanding the pathogenic mechanisms of the bacterium. Thus, searching for *C. trachomatis*-secreted proteins has become a hot topic (Chellas-Géry *et al.*, 2007; Clifton *et al.*, 2004; Dong *et al.*, 2006; Hobolt-Pedersen *et al.*, 2009; Hower *et al.*, 2009; Li *et al.*, 2008b; Misaghi *et al.*, 2006; Subtil *et al.*, 2005; Valdivia, 2008; Vandahl *et al.*, 2005; Zhong *et al.*, 2001). We have used an anti-fusion protein antibody approach to identify *C. trachomatis*-secreted proteins. In the current study, we not only confirmed that protein CT621 was localized in the infected host cytoplasm but also found that protein CT622 was secreted into the cytosol of *C. trachomatis*-infected host cells. However, CT622 and CT621 displayed significantly different kinetics in expression and secretion, suggesting that they may play different functions during the chlamydial intracellular developmental cycle. Nevertheless, both CT622 and CT621 appeared in organism-free vesicles inside inclusions and in the host cell cytosol. More importantly, the secretion of both proteins was blocked by an inhibitor known to target the bacterial type III secretion system. These findings provide important information for further investigating the mechanisms of *C. trachomatis* pathogenesis.

METHODS

Cell culture and chlamydial infection. HeLa cells (human cervical carcinoma epithelial cells, ATCC cat. no. CCL2) and the *C. trachomatis* serovars LGV2 (L2/434/Bu) and D (UW-3/Cx) organisms were used. The chlamydial organisms were propagated, purified, aliquoted and stored as described previously (Zhong *et al.*, 2001). For infection, HeLa cells, grown in either 24-well plates with coverslips or tissue flasks containing DMEM (Gibco-BRL) with 10% fetal calf serum (FCS; Gibco-BRL) at 37 °C in an incubator supplied with 5% CO₂, were inoculated with chlamydial organisms as described previously (Zhong *et al.*, 2001). The infected cultures were processed

at different time points after infection for either immunofluorescence assays or Western blot analyses as described below. In some experiments, at different time points after infection, the cultures were treated with *N'*-(3,5-dibromo-2-hydroxybenzylidene)-4-nitrobenzohydrazide (ChemBridge, cat. no. 5113023) ('compound 1', C1), a small molecule known to inhibit the bacterial type III secretion system (T3SS) (Kauppi *et al.*, 2003; Nordfelth *et al.*, 2005) and block chlamydial growth (Wolf *et al.*, 2006). The treated cultures were processed for immunofluorescence microscopy analyses at 40 h after infection. C1 was dissolved in DMSO (Sigma) at a stock concentration of 50 mM and diluted into culture medium to a final concentration of 10–50 µM with 0.1% DMSO.

Chlamydial gene cloning, fusion protein expression and antibody production. The ORFs CT621 and CT622 from *C. trachomatis* were cloned into pGEX vectors (Amersham Pharmacia Biotech). The following primers were used for cloning the two ORFs: CT621 forward primer, 5'-CGCGGATCC (restriction site)-(overlapping region) ATGAACCGTATTCATCGTAC-3'; CT621 reverse primer, 5'-AAAGGAAAAGCGGCCGC-CTATCTTAAGAGATTACGCG-3'; CT622 forward primer, 5'-CGCGGATCC-ATGGAATCAGGACCAGAAATCA-3'; and CT622 reverse primer, 5'-AAAGGAAAAGCGGCCGC-TTAA-GAAAGATAACCAGAGAATA-3'. Both ORFs were expressed as fusion proteins with glutathione *S*-transferase (GST) fused to the N-terminus of the chlamydial proteins as previously described (Sharma *et al.*, 2006). Expression of the fusion proteins was induced with IPTG (Invitrogen) and the fusion proteins were extracted by lysing the bacteria via sonication in lysis buffer (1% Triton X-100, 1 mM PMSF, 75 units aprotinin ml⁻¹, 20 µM leupeptin and 1.6 µM pepstatin). After a high-speed centrifugation to remove debris, the fusion-protein-containing supernatants were purified using glutathione-conjugated agarose beads (Pharmacia) and the purified proteins were used to immunize mice for producing antibodies as previously described (Sharma *et al.*, 2004, 2006; Zhong *et al.*, 1997). The fusion-protein-specific antibodies were used to localize endogenous proteins in *C. trachomatis*-infected cells via an indirect immunofluorescence assay and to detect endogenous proteins using a Western blot assay. In some experiments, the GST fusion proteins bound onto the glutathione-agarose beads, after thorough washing, were also used to pre-absorb the mouse antibodies.

Immunofluorescence assay. HeLa cells grown on coverslips were fixed with 2% paraformaldehyde (Sigma) dissolved in PBS for 30 min at room temperature, followed by permeabilization with 2% saponin (Sigma) for an additional 30 min. After washing and blocking, the cell samples were subjected to antibody and chemical staining. Hoechst 33258 (blue, Sigma) was used to visualize DNA. A rabbit anti-chlamydial organism antibody (R1L2, raised with *C. trachomatis* L2 organisms, unpublished data) or anti-IncA (kindly provided by Ted Hackstadt, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana, USA; Hackstadt *et al.*, 1999) plus a goat anti-rabbit IgG secondary antibody conjugated with Cy2 (green; Jackson ImmunoResearch) was used to visualize chlamydial organisms or inclusion membrane. The various mouse antibodies plus a goat anti-mouse IgG conjugated with Cy3 (red; Jackson ImmunoResearch) were used to visualize the corresponding antigens. The mouse antibodies included polyclonal antibodies (pAbs) made against GST-CT622, GST-CT621 (both current study) and GST-CT813 (Chen *et al.*, 2006) fusion proteins, and mAbs 4F4 against CT621 (IgG1, current study), 100a against CPAF (Zhong *et al.*, 2001) and 1L11C3 against chlamydial HSP60 (IgG2a, unpublished data). In some cases, the primary antibodies were pre-absorbed with either the corresponding or heterologous fusion proteins immobilized onto glutathione-conjugated agarose beads (Pharmacia) prior to staining. The pre-absorption was carried out by incubating the antibodies with bead-immobilized antigens for 1 h at room temperature or overnight at 4 °C followed by pelleting the beads. The remaining supernatants were used for immunostaining. The immunofluorescence images were

acquired using an Olympus AX-70 fluorescence microscope equipped with multiple filter sets and Simple PCI imaging software (Olympus) as described previously (Fan *et al.*, 1998). The images were processed using Adobe Photoshop (Adobe Systems).

Western blot assay. The Western blot assay was carried out as described elsewhere (Zhong *et al.*, 1997, 2000). Briefly, HeLa cells with or without *C. trachomatis* infection and with or without fractionation (into pellet and S100 fractions), purified chlamydial RB and EB organisms and GST fusion protein samples were resolved in SDS-polyacrylamide gels. The resolved protein bands were transferred to nitrocellulose membranes and the membrane was probed with primary antibodies, including mouse pAbs against GST-CT622, GST-CT621 and GST-CT813 fusion proteins as described above and mouse mAb clone MC22 against chlamydial major outer-membrane protein (MOMP; Zhong *et al.*, 2001), clone AC-40 against human β -actin (Sigma, cat. no. A4700, IgG2a), 100a against CPAF (Zhong *et al.*, 2001) and W27 against host cell HSP70 (Santa Cruz Biotechnology, cat. no. Sc-24). The primary antibody binding was probed with a horseradish-peroxidase-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch) and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotech).

RT-PCR. The reverse transcriptase-PCR (RT-PCR) assay was carried out as described previously (Belland *et al.*, 2003). The *C. trachomatis*-infected cells were harvested at various times after infection for RNA extraction using the TRIzol reagent (Invitrogen). After treatment with DNase (Invitrogen, 24 units per 10 μ g RNA) at 37 °C for 3h (preliminary data showed that this treatment condition was necessary to completely eliminate DNA contamination), the RNA samples from each time point were subjected to the reverse transcription reactions (first-strand cDNA synthesis) with or without reverse transcriptase. The first-strand cDNA was synthesized using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase and random hexamer primers (Invitrogen). The following gene-specific primers were used to amplify the cDNA templates: 5'-TTAGTATCCTCTCTTATGCACT-3' (forward) and 5'-ATCGCCTAGAGCGATCTCAT-3' (reverse) for amplifying a 453 bp fragment of gene *ct621* (453 bp); 5'-CAGCAAAATAATAGCCAGATA-3' (forward) and 5'-TGAGCCTCCATAGCTTGTA-3' (reverse) for *ct622* (401 bp); 5'-GAGATATTTTGTATCCGACGT-3' (forward) and 5'-CATTAACCATGGCATAGCTG-3' (reverse) for *ct623* (351 bp); 5'-TATCCATGGGAATCTTTCAAGT-3' (forward) and 5'-CATAACCAACATAACAGCAAC-3' (reverse) for *ct624* (251 bp); 5'-TTTGTTATGGGCTGTGACAGATT-3' (forward) and 5'-GGACTCTTTGGTCTCCGAAT-3' (reverse) for a 335 bp fragment between *ct622* and *ct623* (335 bp). All PCRs were carried out under identical conditions in the same thermal cycler so that the amounts of PCR products would reflect the amounts of cDNA templates. Preliminary data showed that all the gene-specific primers generated PCR products with the expected numbers of nucleotides when chlamydial DNA was used as template. The pair of primers designed to amplify cDNA covering a portion of *ct622* and *ct623* was used to test whether *ct622* and *ct623* were co-transcribed into a single transcript since preliminary data showed that these two adjacent genes displayed a similar expression pattern.

RESULTS

Localization of the hypothetical proteins CT622 and CT621 in both inclusions and host cell cytosol

When a mouse antiserum raised with a GST-CT622 fusion protein was used to localize the endogenous protein in *C. trachomatis*-infected HeLa cells in an immunofluorescence assay, we found that CT622 was localized both inside and

outside the chlamydial inclusions, although the amount of intra-inclusion labelling was greater since the labelling outside the inclusions disappeared first as the dilution of the antiserum increased (Fig. 1A). A similar staining pattern was found with the anti-CT621 antibodies (data not shown). Interestingly, the intra-inclusion granular staining with either anti-CT622 or anti-CT621 antibodies did not always overlap with the anti-*C. trachomatis* organism staining, suggesting that some CT622 and CT621 may be secreted from the organisms in organism-free vesicles but still trapped inside the inclusions (Fig. 1B). In contrast, most CPAF molecules were secreted outside the inclusions, without obvious intra-inclusion accumulation, while most of the chlamydial heat-shock protein molecules co-localized with the chlamydial organisms (Fig. 1B). The secretion of CT622 into host cell cytosol was further confirmed using the inclusion membrane as a reference (Fig. 1C). These results together demonstrated that CT622 and CT621 were secreted into both the intra-inclusion space and the host cell cytosol.

We next confirmed the antibody labelling specificity by using a preabsorption procedure (Fig. 2A). Both the granular staining inside the inclusions and the diffused staining in the host cell cytosol labelled by the anti-CT622 antiserum were removed by preabsorption with GST-CT622 but not GST-CT621 fusion proteins. The same was true for the anti-CT621 stainings, demonstrating that both anti-CT622 and anti-CT621 antibodies specifically labelled the corresponding endogenous proteins without cross-reacting with each other. On a Western blot (Fig. 2B), these antibodies only recognized protein bands migrating at the molecular masses that matched the corresponding proteins from the *C. trachomatis*-infected whole-cell lysate samples. These results demonstrated that the anti-CT622 and anti-CT621 antibodies can specifically detect the corresponding endogenous proteins in both immunofluorescence and Western blot assays without cross-reacting with any other chlamydial or host proteins.

The secretion of CT622 and CT621 was also demonstrated in an independent assay, in which the *C. trachomatis*-infected cells were fractionated into cytosolic (S100) and nuclear/inclusion (pellet) fractions. The distribution of CT622 and CT621 in the different fractions was compared in a Western blot (Fig. 3). CPAF was only detected in either the *Chlamydia*-infected whole-cell lysate (Ct-HeLa) or cytosolic fraction (Ct-HeLa S100) but not in other samples, which is consistent with previous reports (Fan *et al.*, 2002; Zhong *et al.*, 2001). Like CPAF, both CT622 and CT621 were detected in the cytosolic fraction (Fig. 3, panels a and b, lane 4), suggesting secretion of these two proteins into the host cell cytosol. Unlike CPAF, CT622 and CT621 were also detected in the pellet fraction and in purified organisms (lanes 3, 5 and 6), suggesting that portions of the proteins are retained within the inclusions and associated with the organisms. Degradation fragments of CT622 were noted, especially, in the RB preparation (lane 6); the significance of these fragments is not clear. It is

Fig. 1. Immunofluorescence localization of CT622 and CT621 in the cytoplasm of *C. trachomatis*-infected cells. HeLa cells infected with *C. trachomatis* L2 organisms were processed 40 h post-infection for co-staining with mouse antibodies visualized with a goat anti-mouse IgG conjugated with Cy3 (red), rabbit antibodies visualized with a Cy2-conjugated goat anti-rabbit IgG (green) and the DNA dye Hoechst 33258 (blue). The mouse antibodies were an anti-CT622 antiserum (raised with GST-CT622 fusion protein) at various dilutions (A, panels a–c), the anti-CT622 antiserum at 1 : 1000 dilution (B, panel a), an antiserum (b) and a mAb (clone 4F4; c) raised with GST-CT621 fusion protein, a mAb (100a) against CPAF (d), an antiserum raised with GST-CT813 (e), and a mAb (1L11C3) against chlamydial HSP60 (f). Note that the anti-CT622 and CT621 antibodies detected signals both inside the chlamydial inclusions, with (yellow arrowheads) or without (red arrowheads) overlap with the chlamydial organisms, and in the host cell cytosol (red arrows) while the anti-CPAF antibody mainly detected signals in the host cell cytoplasm. Abbreviation: α , anti- (also in Figs 2, 4 and 6). (C) Co-staining of the mouse anti-CT622 (red) with the rabbit anti-IncA antibodies (green) was also carried out.

Fig. 2. The anti-CT622 and anti-CT621 fusion protein antibodies specifically detect the corresponding endogenous proteins without cross-reactivity. The anti-CT622 and anti-CT621 antibodies with or without preabsorption with GST fusion proteins were used to detect the endogenous proteins in *C. trachomatis*-infected cells and on nitrocellulose membranes. (A) *C. trachomatis*-infected cells were processed for immunostaining as described in the legend of Fig. 1. Note that the antibody labellings of endogenous antigens were blocked by absorption with the corresponding fusion protein but not other fusion proteins. (B) In a Western blot assay, HeLa cells with (+) or without (-) *C. trachomatis* infection were used as antigens and the antigens blotted on nitrocellulose membrane were detected with corresponding antibodies as indicated at the top. Note that each antibody only detected a major protein band migrating at the molecular mass that matched the corresponding chlamydial or host proteins as indicated on the right.

worth noting that a dominant cleavage fragment (~45 kDa) of CT621 was produced from the S100 (cytosolic fraction) sample, as reported previously (Hobolt-Pedersen *et al.*, 2009). Since this cleavage fragment was not detectable in the *C. trachomatis*-infected HeLa cell sample that was rapidly dissolved in 2% SDS sample buffer (lane 2), we concluded that it was artificially generated during the process of preparing the S100 fraction. Apparently, the cleavage fragment was soluble and probably generated from the secreted portion since it was only detected in the S100 cytosolic but not the pellet fractions (Fig. 3, panel b, lane 4 vs 3) nor the purified organisms (lanes 5 and 6). To monitor the quality of the fractionation, the anti-MOMP antibody was used to indicate the pellet fraction that contained the chlamydial inclusions while an anti-human HSP70 antibody was used to indicate the host cell cytosolic fraction that contained the *Chlamydia*-secreted proteins. Detection with these antibodies revealed no cross-contamination between the pellet and cytosolic fractions. Detection with the anti-MOMP antibody also showed that the amounts of

chlamydial organisms in the infected HeLa whole-cell lysate, the pellet fraction and purified EB and RB samples were equivalent.

Differential expression and secretion kinetics of CT622 and CT621

We further used the specific antibodies to monitor the biosynthesis and secretion of both CT622 and CT621 at the single-cell level (Fig. 4). CT622 was first detected at 6 h post-infection. However, clear secretion into host cell cytosol was only detected 36 h after infection. Even the intra-inclusion granular labelling that did not overlap with the organisms was only first detected 24 h after infection. Apparently, CT622 displayed a long delay in secretion after biosynthesis. In contrast, CT621 was synthesized at 16 h and secreted into host cell cytosol at 24 h post-infection. The intra-inclusion non-overlapping granules indicative of secretion outside the organisms were detected as early as 16 h, suggesting that CT621 was rapidly secreted after synthesis. As a control, CPAF was synthesized as early as

Fig. 3. Both CT621 and CT622 are detected in the cytosolic fraction of *C. trachomatis*-infected cells. The *C. trachomatis*-infected cells were dounced and fractionated into cytosolic (Ct-HeLa S100) and pellet (containing both chlamydial inclusions and host nuclei) fractions. The cytosolic (lane 4) and pellet (lane 3) fractions together with the whole-cell lysates from uninfected (lane 1) and infected (lane 2) HeLa cells as well as purified chlamydial EB (lane 5) or RB (lane 6) organisms were loaded onto an SDS-polyacrylamide gel. The resolved protein bands were transferred onto nitrocellulose membrane and probed with antibodies against CT622 (panel a), CT621 (b), CPAF (c), CT813 (d), MOMP (e) and mammalian HSP70 (f). Note that both CT621 and CT622 were detected in the cytosolic fraction, similar to the detection of CPAF, a known *Chlamydia*-secreted protein. However, unlike CPAF, CT621 and CT622 were also detected in the pellet and purified organisms.

12 h after infection, and the secretion was immediate and became very obvious at 16 h after infection. The long delay in secretion of CT622 after biosynthesis may imply that it fulfils a different function than CT621 although they are encoded by two adjacent ORFs.

Since ORFs *ct621*–*ct624* are oriented in the same direction in the chlamydial genome (Fig. 5A), we monitored the mRNA expression patterns of these genes using RT-PCR (Fig. 5B). We found that the *ct621* and *ct624* transcripts were detected at 8 h while the *ct622* and *ct623* transcripts were detected at 2 h after infection. Since ORFs *ct622* and *ct623* are adjacent to each other and shared a similar expression pattern, we further tested whether these two genes were co-transcribed into a single transcript. When a forward primer complementary to *ct623* and a reverse

primer complementary to *ct622* were used to amplify the intergenic region with the same cDNA templates as used for gene-specific primer amplification, we found no products, although an expected product was detected when the genomic DNA was used as template. These results suggest that these four genes, encoded in the same genomic region, were transcribed independently of each other. The pattern of gene activation correlates well with that of protein expression, with *ct622* mRNA detected at 2 h, protein at 6 h and secretion of protein into host cell cytoplasm at 36 h post-infection, and CT621 mRNA detected at 8 h, protein at 16 h and secretion at 24 h.

The secretion of both CT622 and CT621 is inhibited by the T3SS inhibitor C1

To determine the secretion pathway of CT622, we carefully analysed the amino acid sequence for secretion signal sequences, but we did not find any. CT621 does not contain any obvious secretion signal sequences either, as reported previously (Hobolt-Pedersen *et al.*, 2009). Since the secretion of CT621 was inhibited by the T3SS inhibitor C1 (Hobolt-Pedersen *et al.*, 2009), we tested whether CT622 secretion could be also affected by C1 (Table 1, Fig. 6). As a negative control treatment, 0.1 % DMSO alone was added to the cultures at 6 h after infection; this did not affect either inclusion development or protein secretion (Fig. 6, panels a, f and k). When C1 was added (at a final concentration of 50 μ M) at 6 h, the secretion of CT622, CT621 and IncA was completely inhibited (panels b and g). However, CPAF secretion was not affected (panel l), which is consistent with a previous report that CPAF secretion required a Sec-dependent but not T3SS pathway (Chen *et al.*, 2010). Unfortunately, the C1 treatment also severely affected chlamydial intracellular growth (panels b, g and l). To minimize the non-specific toxic effects, we introduced C1 into the cultures at 12 h or later, which no longer affected IncA secretion and normal inclusion development but still inhibited the secretion of CT622 and CT621. For example, secretion of CT622 was inhibited by 50 μ M C1 when added at 34 h after infection while secretion of CT621 was similarly inhibited when C1 was added at 20 h after infection. Thus, we can conclude that the C1 inhibition of CT621 and CT622 secretion was specific. The differential susceptibility of CT622, CT621 and IncA secretion to C1 inhibition may reflect the varied time-courses of secretion by these molecules, with IncA secretion the earliest (<6 h), followed by CT621 (24 h) and CT622 (36 h). It appears that inhibition of protein secretion by C1 requires the inhibitor to be added before the start of the protein secretion.

DISCUSSION

C. trachomatis can accomplish all its biosynthetic processes within a cytoplasmic inclusion by importing nutrients and energy from host cells. To successfully complete the intracellular replication/developmental cycle, the *C. tra*-

Fig. 4. Expression of CT622 and CT621 during *C. trachomatis* infection. The *C. trachomatis*-infected culture samples were processed at various times after infection (as indicated at the top) for immunofluorescence staining as described in the legend of Fig. 1. The mouse anti-CT622 (panels a to h), anti-CT621 (i to p) and anti-CPAF (mAb 100a; panels q to x) were visualized with a goat anti-mouse IgG conjugated with Cy3 (red) while the chlamydial organisms were visualized with a rabbit anti-chlamydia antibody and a goat anti-rabbit IgG-Cy2 conjugate (green). Note that CT622 was first detected inside the chlamydial inclusions at 6 h after infection (panel c, yellow arrowhead), CT621 at 16 h (m, yellow and red arrowheads) and CPAF at 12 h (t, red arrowhead). However, the host cell cytosolic signal was first detected at 36 h for CT622 (g, red arrows), 24 h for CT621 (n, red arrows) and 16 h for CPAF (u, red arrows) post-infection.

chomatis organisms have to secrete effector molecules into host cells for manipulating host signalling pathways (Betts-Hampikian & Fields, 2010; Fields *et al.*, 2003; Valdivia, 2008; Zhong, 2009; Zhong *et al.*, 2011). Here we have presented convincing evidence that *C. trachomatis* secretes the hypothetical protein CT622 into host cell cytosol. First, using antibodies raised with GST-CT622 fusion protein, we localized CT622 in the cytoplasm of *C. trachomatis*-infected host cells in an immunofluorescence assay. Second, CT622 was not detected by antibodies pre-absorbed with CT622 but not control fusion proteins. The anti-CT622 antibody detected endogenous CT622 from *C. trachomatis*-infected cell samples without cross-reactivity with any other chlamydial or host proteins on a Western blot. Third, the secretion of CT622 along with CT621, a previously reported secretion protein (Hobolt-Pedersen *et al.*, 2009), was confirmed in a fractionation assay, showing that, like CPAF, both CT622 and CT621 were detected in the host cell cytosolic fraction. However, CT622 and CT621 displayed significantly different kinetics in expression and secretion, suggesting that these two proteins may fulfil different functions during the chlamydial intracellular replication cycle. Finally, the secretion of CT621 and CT622 was blocked by C1, a small-molecule inhibitor known to target bacterial (Nordfelth *et al.*, 2005) and chlamydial (Wolf *et al.*, 2006) type III secretion systems.

The precise secretion pathway of CT622 and CT621 remains unknown. Due to the lack of a reliable genetic tool for

manipulating the chlamydial genome, it has been difficult to define secretion pathways of chlamydial proteins in the context of chlamydial infection. Although bioinformatics analysis and heterologous systems can be helpful, these approaches can only provide indirect evidence. The next best approach for defining chlamydial protein secretion pathways in chlamydial cultures is the use of small-molecule inhibitors. Such inhibitors have been used to both study secretion and signalling pathways in prokaryotic (Aiello *et al.*, 2010; Harmon *et al.*, 2010) and eukaryotic (Xiao *et al.*, 2005) systems and to develop pathway-specific therapeutics (Andersen *et al.*, 2010). The C1 series compounds were found to block bacterial T3S (Harmon *et al.*, 2010; Aiello *et al.*, 2010; Kauppi *et al.*, 2003; Wolf *et al.*, 2006). However, the precise targets of C1 remain unclear despite the demonstration that the anti-chlamydial activity of this compound was reversed by iron supplementation (Slepenkin *et al.*, 2007). Indeed, when C1 was used at a high concentration and during early stages of chlamydial infection, chlamydial inclusion development was severely affected (Slepenkin *et al.*, 2007; Wolf *et al.*, 2006; Fig. 6 of the current study). Fortunately, the non-specific effects can be minimized by introducing C1 into chlamydial cultures at a later time after infection. Under such non-toxic conditions, chlamydial inclusions developed normally and the secretion of IncA was unaltered but the secretion of CT622 and CT621 was still inhibited (Table 1, Fig. 6), demonstrating that secretion of CT622 and CT621 is dependent on a pathway that is susceptible to C1 inhibition. Although C1 is considered to be a T3SS inhibitor, CT622 and

Fig. 5. (A) Genomic organization of genes ct621 to ct624. All four genes are oriented in the same direction. (B) Expression of ct622 and ct621 mRNAs during *C. trachomatis* infection. The *C. trachomatis*-infected cells were harvested at various times after infection (as indicated at the top). RNA samples extracted from cells at each time point were subjected to reverse transcription (first-strand cDNA synthesis) reactions with or without reverse transcriptase (RT). Primer-specific cDNA fragments were amplified via PCR and visualized in agarose gel. The gene-specific primers only amplified cDNA from samples treated with RT, not those without RT (panels a to d). However, a pair of primers designed to amplify cDNA covering portions of ct622 and ct623 failed to produce any products (panel e). All primers generated PCR products when chlamydial DNA was used as template (lane 2).

CT621 may not be secreted into the host cell cytosol via the conventional single-step T3S pathway since both proteins appear in the inclusion luminal vesicles before they reach the host cell cytosol (Fig. 4). It has been hypothesized that the intra-inclusion luminal vesicles may represent outer membrane vesicles (OMVs) (Chen *et al.*, 2010; Giles *et al.*, 2006). In other Gram-negative bacterial systems, the OMVs can engulf periplasmic and outer-membrane proteins and transport them out of bacterial organisms (Balsalobre *et al.*, 2006; Ellis & Kuehn, 2010; Galka *et al.*, 2008; Kim *et al.*, 2008; Kouokam *et al.*, 2006; Kuehn & Kesty, 2005; Mullaney *et al.*,

Table 1. Inhibition of CT621 and CT622 secretion by C1

At the indicated time points after chlamydial infection, C1 (at a final concentration of 10 or 50 mM), or 0.1% DMSO solvent alone, was added to the cultures. All culture samples were processed 40 h after infection for observation under a fluorescence microscope. '+' indicates obvious secretion in all infected cells; '+/-' indicates partial secretion or secretion in some infected cells; '-' indicates lack of secretion in any infected cells. Representative immunofluorescence images from the results highlighted in bold are shown in Fig. 6. It is worth noting that inhibition of IncA secretion required C1 to be added as early as 6 h, and complete inhibition of secretion was achieved when C1 was added at 20 h for CT621 and 34 h for CT622, consistent with their respective secretion time-courses. C1 at no time inhibited secretion of CPAF, a protease secreted via a Sec-dependent pathway.

C1 concn (μM)	Time after infection (h)	CT621	CT622	IncA
0 (0.1% DMSO)	6	+	+	+
50	6	-	-	-
10	12	-	-	+
50	12	-	-	+
10	20	+	-	+
50	20	-	-	+
10	30	+	+/ -	+
50	30	+/ -	-	+
10	32	+	+/ -	+
50	32	+/ -	-	+
10	34	+	+/ -	+
50	34	+/ -	-	+

2009). To be vesiculated by OMVs, CT622 and CT621 must be first translocated into the chlamydial periplasmic region or outer membrane. The question is whether CT622 and CT621 are translocated across the inner membrane via a C1-susceptible pathway. Further characterization of CT622 and CT621 secretion may allow us to discover novel pathway(s) of chlamydial protein secretion.

Regardless of how CT622 and CT621 are secreted into host cell cytosol, searching for *C. trachomatis*-secreted effector molecules will continue to be a productive approach to understanding chlamydial pathogenic mechanisms. This is because as more effectors are identified, more tools will be available for dissecting the molecular pathways of chlamydial interactions with host cells. Various approaches have been used to identify chlamydial proteins that are secreted into host cell cytosol. Based on the characteristics of the T3S effector molecules from other bacterial systems, software and algorithms have been developed to predict chlamydial effectors (Arnold *et al.*, 2009; Samudrala *et al.*, 2009). Heterologous T3SSs from other bacteria have also been used to screen chlamydial ORFs for T3SS secretability (Chellas-Géry *et al.*, 2007; Hower *et al.*, 2009; Subtil *et al.*, 2005). In addition, various predicted chlamydial T3S chaperones have been used as baits to identify potential effectors via either co-precipitation or bacterial/yeast two-

Fig. 6. The secretion of both CT622 and CT621 is inhibited by the T3SS inhibitor C1. HeLa cells infected with *C. trachomatis* were treated with C1 at a final concentration of either 10 or 50 μM , or with 0.1 % (v/v) DMSO alone, at various time points after infection as indicated at the top. All culture samples were processed 40 h after infection for immunofluorescence microscopy analysis as described in the legend of Fig. 1. Note that secretion of both CT622 and CT621 but not CPAF was inhibited by C1 even when it was added at 20 h after infection, when chlamydial inclusions developed normally. Red arrows indicate chlamydial proteins secreted into host cell cytosol; white arrows indicate LncA protein inhibited from secretion.

hybrid assays (Slepenkin *et al.*, 2005; Spaeth *et al.*, 2009). Although these approaches have generated useful information and discovered functionally relevant/important effector proteins (Chellas-Géry *et al.*, 2007; Clifton *et al.*, 2004; Hower *et al.*, 2009), there are still more important effectors to be identified. We have been using an anti-fusion protein antibody approach to search for chlamydial secretion proteins in the host cell cytosol. Using this approach, we have visualized various secreted effector molecules, including CPAF, a chlamydial protease (Zhong *et al.*, 2001), Cpn0796 (Dong *et al.*, 2006) and Cpn0797 (Dong *et al.*, 2006; Vandahl *et al.*, 2005), both hypothetical proteins encoded in the *C. pneumoniae* genome, and Pgp3, a plasmid-encoded hypothetical protein (Li *et al.*, 2008b). Here we describe two more *Chlamydia*-secreted proteins, CT621 and CT622. Although CT621 was previously reported (Hobolt-Pedersen *et al.*, 2009), the discovery of CT622 as a secretion protein is new. Thus, we will continue to use the anti-fusion protein antibody approach to search for additional *Chlamydia*-secreted proteins. As more effectors are identified, characterization of the diverse effectors will speed up our understanding of chlamydial pathogenic mechanisms. Our characterization of

ct622 and ct621 expression has revealed that ct621 mRNA is expressed as early as 8 h, protein at 16 h and secretion of protein into host cell cytoplasm at 24 h, while ct622 mRNA is expressed at 2 h, protein at 6 h and secretion at 36 h post-infection. The distinct expression and secretion patterns suggest that CT622 and CT621 may fulfil different functions during the chlamydial intracellular development cycle. Further characterization of these effectors should allow us to gain novel insights into chlamydial pathogenic mechanisms.

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