Characterization of the hypothetical protein Cpn1027, a newly identified inclusion membrane protein unique to *Chlamydia pneumoniae*

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The hypothetical protein Cpn1027 was detected in the inclusion membrane of *Chlamydia pneumoniae*-infected cells with antibodies raised with Cpn1027 fusion proteins in an indirect immunofluorescence assay. The inclusion membrane staining by the anti-Cpn1027 antibodies co-localized with the staining of an antibody recognizing a known inclusion membrane protein designated IncA and these membrane stainings were blocked by the corresponding but not irrelevant fusion proteins. Although Cpn1027 was not predicted to be an inclusion membrane protein, it contained a bi-lobed hydrophobic domain region at its N-terminus, a signature secondary structural motif possessed by most chlamydial inclusion membrane proteins. The Cpn1027 protein was detected as early as 12 h after *C. pneumoniae* infection and remained in the inclusion membrane throughout the rest of the infection cycle. Cytosolic expression of Cpn1027 via a transgene failed to affect the subsequent chlamydial infection. The anti-Cpn1027 polyclonal antisera failed to detect any significant signals in cells infected with chlamydial species other than *C. pneumoniae*, which is consistent with the sequence analysis result that no significant homologues of Cpn1027 were found in any other species. These experiments together have demonstrated that Cpn1027 is a newly identified inclusion membrane protein unique to *C. pneumoniae*.

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INTRODUCTION

Although *Chlamydia pneumoniae* mainly causes asymptomatic infections in the respiratory tracts of immunocompetent individuals (Grayston, 1992; Kuo *et al.*, 1995b), respiratory infection with *C. pneumoniae* is also associated with non-respiratory pathologies such as atherosclerosis (Campbell & Kuo, 2004; Campbell *et al.*, 2005; Hu *et al.*, 1999; Kuo *et al.*, 1995a, 2002; Liu *et al.*, 2000; Sharma *et al.*, 2004). Like other chlamydial species, *C. pneumoniae* has an obligate intravacuolar biphasic life cycle (Hackstadt, 1998; Hackstadt *et al.*, 1997; Kuo *et al.*, 1995b). A typical *C. pneumoniae* infection starts with the entry of an infectious elementary body (EB). The endocytosed EB can rapidly differentiate into a non-infectious but metabolically active reticulate body (RB). After the RB undergoes numerous rounds of replication, the progeny RBs can differentiate back into EBs before exiting to infect adjacent cells. Although *C. pneumoniae* organisms can accomplish all their biosynthesis within the cytoplasmic vacuole (designated the inclusion), they have to interact with host cells via

the inclusion membrane in order to establish and maintain successful intravacuolar growth.

Chlamydial organisms have evolved the ability both to acquire nutrients and metabolic intermediates from host cells (Carabeo *et al.*, 2003; Hackstadt *et al.*, 1995, 1996; Scidmore *et al.*, 1996; Su *et al.*, 2004) and to secrete chlamydial products into host cell cytoplasm (Fan *et al.*, 2002; Shaw *et al.*, 2002; Vandahl *et al.*, 2005; Zhong *et al.*, 2001). However, the mechanisms of these two-way interactions are not clear. The chlamydial proteins localized in the inclusion membrane (the chlamydial inclusion membrane proteins are designated Incs) are thought to play important roles in chlamydial interactions with host cells (Hackstadt *et al.*, 1999; Rockey *et al.*, 2002). Therefore, hunting for new Incs has been an area of intensive investigation. Many different approaches including both computer program-based prediction and experimental methods have been employed to search for new Incs. We have recently used an anti-fusion protein antibody approach for localizing chlamydial proteins in *C. pneumoniae*-infected cells and found that the hypothetical protein Cpn1027 is localized in the *C. pneumoniae* inclusion membrane although it was not predicted to be so.

Abbreviations: GST, glutathione S-transferase; RFP, red fluorescent protein.

METHODS

Cell culture and chlamydial infection. Monolayers of HeLa 229 cells (ATCC, Manassas, VA, USA) were infected with *Chlamydia pneumoniae* AR39, *C. caviae* GPIC, *C. psittaci* 6BC, *C. muridarum* MoPn, and *C. trachomatis* serovar D and L2 organisms at an m.o.i. of 0.5 in the presence of 2 µg cycloheximide ml⁻¹ for various periods of time as indicated in individual experiments. The chlamydial organisms and infection procedures were as described elsewhere (Chen *et al.*, 2006; Dong *et al.*, 2005). The cultures grown on coverslips were processed for immunostaining.

Chlamydial gene cloning, fusion protein expression and antibody production. The hypothetical ORFs, including the ORF Cpn1027 (designated Cp0825 in the AR39 genome), encoded in the *C. pneumoniae* AR39 genome (<http://www.stdgen.lanl.gov/>) were cloned into pGEX vectors (Amersham Pharmacia Biotech) and expressed as fusion proteins with glutathione S-transferase (GST) fused to the N-terminus of the chlamydial proteins as previously described (Chen *et al.*, 2006; 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 a Triton X-100 lysis buffer (1% Triton X-100, 1 mM PMSF, 75 units aprotinin ml⁻¹, 20 µM leupeptin and 1.6 µM pepstatin). The GST fusion proteins were purified using agarose beads conjugated with glutathione (Pharmacia) and used to immunize mice for producing both polyclonal antisera [pAb (Zhong *et al.*, 1993)] and monoclonal antibodies [mAb (Zhong *et al.*, 1994, 1997)]. The fusion protein-specific antibodies were then used to localize the endogenous proteins in *C. pneumoniae*-infected cells via an indirect immunofluorescence assay (Xiao *et al.*, 2005; Zhong *et al.*, 2001). Some chlamydial ORFs were also cloned into the pDsRed Monomer C1 mammalian expression vector (BD Biosciences Clontech) and expressed as fusion proteins with a red fluorescent protein (RFP) fused to the N-terminus. The recombinant plasmids were transfected into HeLa cells using the lipofectamine 2000 transfection reagent following the protocol recommended by the manufacture (Invitrogen). Twenty-four hours after transfection, the RFP-chlamydial fusion proteins were visualized via either the fusion tag RFP or the mouse anti-chlamydial protein antibody labelling.

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 1% saponin (Sigma) for an additional 30 min. After washing and

blocking, the cell samples were subjected to antibody and chemical staining. Hoechst (blue; Sigma) was used to visualize nuclear DNA. A rabbit anti-chlamydial organism antibody (R12AR39, raised with *C. pneumoniae* AR39 organisms; unpublished data) or anti-CT395 (raised with the CT395 fusion protein: CT395 is a GrpE-related chaperonin with >70% amino acid sequence identity among all chlamydial species; unpublished data) plus a goat anti-rabbit IgG secondary antibody conjugated with Cy2 (green; Jackson ImmunoResearch Laboratories) was used to visualize chlamydial inclusions. The mouse antibodies including both pAbs and mAbs raised against various reference proteins and *C. pneumoniae* GST fusion proteins plus a goat anti-mouse IgG conjugated with Cy3 (red; Jackson ImmunoResearch) were used to visualize the corresponding antigens. In some cases, the primary antibodies were pre-absorbed with either the corresponding or heterologous fusion proteins immobilized onto agarose beads (Pharmacia) prior to staining cell samples. The pre-absorption approach 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. For the transfected cell samples, the RFP chlamydial fusion proteins were visualized via the fusion tag RFP (red) or by co-staining with a mouse antibody.

The immunofluorescence images were acquired with an Olympus AX-70 fluorescence microscope equipped with multiple filter sets (Olympus) as described previously (Fan *et al.*, 1998; Greene *et al.*, 2004; Xiao *et al.*, 2004). Briefly, the multi-colour-labelled samples were exposed under a given filter set at a time and single colour images were acquired using a Hamamatsu camera. The single colour images were then superimposed with the software SimplePCI. An Olympus FluoView laser confocal microscope was used to further analyse the co-stained samples (service kindly provided by the UTHSCSA institutional core facility). All microscopic images were processed using Adobe Photoshop (Adobe Systems).

Western blot assay. This assay was carried out as described elsewhere (Dong *et al.*, 2005; Sharma *et al.*, 2005; Xiao *et al.*, 2005; Zhong *et al.*, 1997). Briefly, the chlamydial GST fusion proteins were solubilized in 2% SDS sample buffer and loaded to SDS-polyacrylamide gel wells. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the blots were detected with primary antibodies. The primary antibody binding was probed with an HRP (horseradish peroxidase)-conjugated secondary antibody and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology).

Fig. 1. Detection of Cpn1027 in *C. pneumoniae* inclusion membrane. HeLa cells were infected with *C. pneumoniae* AR39 organisms at an m.o.i. of 0.5 in the presence of 2 µg cycloheximide ml⁻¹ for 72 h. The infected cultures grown on coverslips were processed for the following immunostainings. (A) Cpn1027 was probed with mouse antisera against the C-terminus (αCpn1027c, panel a) and N-terminus (αCpn1027n, panel b) and mAbs AE1 (IgG1, c), BF12.3 (IgG2b, d), EH9.2 (IgG1, e) and IE6 (IgG2a, f), and visualized with a Cy3-conjugated goat anti-mouse IgG (red). A rabbit anti-AR39 antiserum (R12AR39) together with a Cy2-conjugated goat anti-rabbit IgG (green) was used to visualize the *C. pneumoniae* organisms and Hoechst to visualize DNA. (B) The infected cell samples were co-stained with the anti-Cpn1027 mAb IE6 (green) and DNA Hoechst dye (blue) together with one of the following antibodies recognizing *C. pneumoniae* reference proteins, including CPAFcp (mAb EB3.1, IgG3), IncA (2B12.1, IgG1), and HSP60 (BC7.1, IgG1; all in red). Images of the immunostainings were obtained using an AX70 fluorescence microscope equipped with a CCD camera as described previously (Greene *et al.*, 2004). (C) The samples were co-stained with the anti-Cpn1027 mAb IE6 plus a Cy2-conjugated goat anti-mouse IgG2a (green), the anti-IncA mAb 2B12.1 plus a Cy3-conjugated goat anti-mouse IgG1 (red) and the rabbit anti-AR39 antiserum R12AR39 plus a Cy5-conjugated goat anti-rabbit IgG (blue). The images were acquired sequentially one colour at a time and overlaid in tri-colour and each image was obtained from a different z-axis depth using a confocal microscope (Olympus, provided by the UTHSCSA imaging core). Note that the anti-Cpn1027 antibodies detected strong inclusion membrane signals similar to and overlapping with the labelling by the anti-IncA but not the other antibodies.

RESULTS

Detection of Cpn1027 in the inclusion membrane of *C. pneumoniae*-infected cells

Since most of the already identified Incs are encoded by hypothetical ORFs (Fling *et al.*, 2001; Rockey *et al.*, 1995, 2002), we expressed proteins encoded by the hypothetical ORFs in the *C. pneumoniae* AR39 genome as fusion proteins (Sharma *et al.*, 2006). The antibodies raised with the fusion proteins were used to localize the endogenous proteins in *C. pneumoniae*-infected cells via an indirect immunofluorescence assay (Chen *et al.*, 2006; Xiao *et al.*, 2005; Zhong *et al.*, 1997). An anti-Cpn1027 C-terminal fragment (Cpn1027c) fusion protein antibody labelled the *C. pneumoniae* inclusion membrane (Fig. 1A, panel a). This staining was further confirmed with mAbs made from the same immunized animals (panels c–f) and a pAb raised with the Cpn1027 N-terminal fragment (Cpn1027n) fusion protein (panel b). The anti-Cpn1027 antibodies detected a dominant inclusion membrane signal overlapping with the signal revealed by the anti-IncA (inclusion membrane protein A), but not the anti-CPAF [chlamydial proteasome/protease-like activity factor known to be secreted into host cell cytosol; (Fan *et al.*, 2002; Zhong *et al.*, 2001)], or anti-HSP60 (heat-shock protein 60; mAb clone BC7.1) antibodies (Fig. 1B). We further verified the inclusion membrane localization of Cpn1027 using confocal microscopy (Fig. 1C). The anti-Cpn1027 antibody labelling co-localized with the anti-IncA labelling at different focal levels along the z-axis. IncA, encoded by the *C. pneumoniae* ORF *cpn0186*, is a known inclusion membrane protein in *C. pneumoniae*-infected cells (Bannantine *et al.*, 2000; Kalman *et al.*, 1999; Read *et al.*, 2000). The above observations demonstrated that Cpn1027 is an inclusion membrane protein similar to IncA.

The antibody binding specificities were further verified using various approaches. The anti-Cpn1027n or c antibodies only reacted with the GST-Cpn1027n or c but not the GST-Cpn0186 (IncA) or GST-CPAFcp fusion proteins although all fusion proteins were detectable by their corresponding homologous antibodies in a Western blot assay (Fig. 2A). Furthermore, the anti-Cpn1027 antibodies only detected the RFP-Cpn1027 but not the RFP-IncA or RFP-MOMP fusion proteins while the anti-IncA and MOMP antibodies only recognized the RFP-IncA and RFP-MOMP fusion proteins, respectively, in transfected HeLa cells (Fig. 2B). Finally, the detection of the endogenous antigens in the *C. pneumoniae*-infected cells by the anti-Cpn1027 and anti-CPAFcp antibodies was blocked by the corresponding homologous but not the heterologous GST fusion proteins (Fig. 2C). Together, the above experiments demonstrated that the anti-Cpn1027 antibodies specifically detected the Cpn1027 antigen in the inclusion membrane of the *C. pneumoniae*-infected cells.

Cpn1027 is an inclusion membrane unique to *C. pneumoniae*

Cpn1027 is listed as a *C. pneumoniae* species-specific

hypothetical protein (<http://www.stdgen.lanl.gov/>). Indeed, BLAST searching has revealed no significant homologues of Cpn1027 in any other species (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). We further assessed whether the polyclonal antisera raised with the GST-Cpn1027 fusion proteins could pick up any signals in cells infected with other chlamydial species (Fig. 3A). The two antisera raised with the Cpn1027 C- and N-terminal fragments detected an obvious inclusion membrane signal in *C. pneumoniae* AR39-infected cells (panels a and g) but failed to detect any significant signals in cells infected with *C. caviae* GPIC (b, h), *C. psittaci* 6BC (c, i), *C. muridarum* MoPn (d, j), and *C. trachomatis* serovar D (e, k) and serovar L2 (f, l). Previous studies have shown that although chlamydial Incs share very limited primary sequence homology, they contain a highly conserved bi-lobed hydrophobic domain (Bannantine *et al.*, 2000). Although Cpn1027 was not predicted to be a putative Inc by various computer programs based on the conserved structure features (Bannantine *et al.*, 2000; Toh *et al.*, 2003), we still analysed the Cpn1027 primary sequence with the Kyte–Doolittle hydropathy plot program (Kyte & Doolittle, 1982; http://occawlonline.pearsoned.com/bookbind/pubbooks/bc_mccampbell_genomics_1/medialib/activities/kd/kyte-doolittle.htm). Under this program, the hydrophobic transmembrane regions are identified by peaks with hydropathy scores greater than 1.8 when using a window size of 19 (http://occawlonline.pearsoned.com/bookbind/pubbooks/bc_mccampbell_genomics_1/medialib/activities/kd/kyte-doolittle-background.htm). As shown in Fig. 3(B), IncA proteins from three different chlamydial species displayed two consecutive peaks with a hydropathy score above 1.8 in their N-terminal regions (panels a–c). Interestingly, Cpn1027 also contained two hydrophobic peaks in the N-terminal region (panel d). Although Cpn1027 was not predicted to be an Inc protein by previous computer prediction methods (Bannantine *et al.*, 2000; Toh *et al.*, 2003), the similar secondary structural features currently revealed between Cpn1027 and the IncA proteins support the conclusion that Cpn1027 is an inclusion membrane protein.

Cpn1027 is expressed in the *C. pneumoniae* inclusion membrane as early as 12 h post-infection

Using the Cpn1027-specific antibodies, we compared the expression pattern of the Inc Cpn1027 with that of IncA during *C. pneumoniae* infection (Fig. 4). Both Cpn1027 and IncA proteins were detected as early as 12 h after infection (panels c and k). Cpn1027 and IncA were likely secreted to the inclusion membrane once they became detectable since these anti-Inc protein antibody labellings appeared to surround the staining of the organisms (panels c1 and k1, white arrows). Both Cpn1027 and IncA proteins remained in the inclusion membranes of the infected cells throughout the rest of the infection cycles (panels c–h and k–p), suggesting that Cpn1027 may be as important as IncA in chlamydial biology.

Fig. 2. Specificity of the anti-Cpn1027 antibody detection of *C. pneumoniae* inclusion membrane. (A) Reactivity in a Western blot assay of the antibodies against Cpn1027c (IE6), Cpn1027n (antiserum), Cpn0186 (IncA, 2B12.1) and CPAFcp (EB3.1) with the GST fusion proteins as listed at the top of the figure. Protein bands representing the corresponding GST fusion proteins are marked on the right. Degradation fragments of the fusion proteins are indicated with an asterisk (*). Note that each antibody only reacted with the corresponding fusion protein, without cross-reactivity with the unrelated fusion proteins. (B) HeLa cells transfected with the recombinant plasmids pDsRed-C1 monomer/Cpn1027, Cpn0186 (IncA) or Cpn0695 (MOMP; all expressed as RFP fusion proteins; red) for 24 h were processed for immunostaining with various antibodies listed along the left side of the figure (green) plus Hoechst (blue). It is clear that the antibodies only labelled the corresponding homologous gene-transfected cells without cross-reacting with the unrelated gene-transfected cells. (C) The anti-Cpn1027c polyclonal antiserum and mAb IE6 and anti-CPAFcp mAb EB3.1 were pre-absorbed with or without the GST fusion proteins listed at the top of the figure prior to the immunostainings. The immunofluorescence staining was carried as described in the legend of Fig. 1(A). Note that antibody staining was only blocked by pre-absorption with the corresponding homologous GST fusion proteins.

Fig. 3. Detection of Cpn1027 in cells infected with various chlamydial species and identification of hydrophathy regions in Cpn1027. (A) HeLa cells infected with *C. pneumoniae* AR39 (panels a and g), *C. caviae* GPIC (b, h), *C. psittaci* 6BC (c, i), *C. muridarum* MoPn (d, j), and *C. trachomatis* serovar D (e, k) and serovar L2 (f, l) for 30 h (GPIC, 6BC, MoPn, D and L2) or 72 h (AR39) were processed for immunostaining. Both the mouse anti-Cpn1027C (a–f) and N (g–l) polyclonal antisera were used for visualizing the inclusion membrane protein Cpn1027 (red) and the rest of the staining was as described in the legend of Fig. 1(A). Note that the anti-Cpn1027 antisera only labelled the inclusion membrane in *C. pneumoniae*-infected cells without picking up any significant signals in cells infected with other *Chlamydia* species. (B) The amino acid sequences of IncA from *C. trachomatis* (CT119, panel a), *C. caviae* (CCA00550, b) and *C. pneumoniae* (Cpn0186, c) and Cpn1027 were subjected to Kyte–Doolittle hydrophathy plot analysis. A hydrophathy score of 1.8 is indicated on the right of each panel and the bi-lobed hydrophobic regions in each sequence are highlighted with dashed vertical lines.

Expression of Cpn1027 in host cell cytosol does not affect the subsequent chlamydial infection

Finally, we studied the effect of Cpn1027 expression on the subsequent chlamydial infection (Fig. 5). The Cpn1027 was

expressed as a fusion protein with RFP as the N-terminal fusion tag in HeLa cells (Chen *et al.*, 2006). Twenty-four hours after transfection, the transfected cells were infected with the *C. pneumoniae* AR39 (panels a and e), *C. caviae* GPIC (b, f), *C. trachomatis* L2 (c, g) or D (d, h). Both the

Fig. 4. Expression of Cpn1027 protein during *C. pneumoniae* infection. HeLa cells were infected with *C. pneumoniae* AR39 for various periods of time as indicated above the panels and the culture samples were immunostained with anti-Cpn1027 (panels a–h, red) or anti-IncA (i–p, red). Rabbit antibodies against AR39 were used to visualize the organisms (green) and Hoechst dye for DNA (blue). The images were acquired using a conventional fluorescence microscope as described in the legend of Fig. 1. Note that both Cpn1027 and IncA proteins were first detected 12 h (panels c and k) after infection with *C. pneumoniae*. White arrows indicate that the inclusion membrane protein labelling (red) appears to surround the organism labelling (green) in panels c1 and k1.

Fig. 5. Effect of cytosolic expression of Cpn1027 on chlamydial infection. Cpn1027 was expressed as an RFP fusion protein (red) as described in the legend of Fig. 2(B). Twenty-four hours after transfection, the transfected cells were infected with the *C. pneumoniae* AR39 (panels a and e), *C. caviae* GPIC (b, f), *C. trachomatis* L2 (c, g) or D (d, h) organisms. The chlamydial organisms were visualized with a rabbit antiserum raised with GST-CT395 fusion protein (which cross-reacted with all chlamydial species) and a goat anti-rabbit IgG conjugated with Cy2 (green). Both the number and size of inclusions were compared between the transfected and untransfected cell populations 24 h (GPIC, 6BC, MoPn, D and L2) or 48 h (AR39) after infection. The experiment was repeated twice with duplicates. The images shown represent average counting on each coverslip. Note that HeLa cells were similarly susceptible to the chlamydial infection and intracellular growth regardless of the pre-existing cytosolic Cpn1027 fusion proteins.

rates of inclusion-forming units and the size of inclusions were compared between the transfected and untransfected cell populations 24 to 48 h after infection. We found that HeLa cells were equally susceptible to the chlamydial infection regardless of the pre-existing cytosolic Cpn1027 fusion protein. For example, when ~100 cells were counted from 5 to 10 random views of each coverslip, the cells expressing RFP-Cpn1027 fusion protein displayed an infection rate of 48% while the adjacent untransfected cells in the same coverslip displayed 56% in the *C. pneumoniae*-infected culture (Fig. 5, panel a). The infection rates were 48% (among RFP-Cpn1027-transfected cells) and 38% (among the adjacent untransfected cells) in GPIC (panel b), 86% and 82% in L2 (panel c), and 38% and 35% in serovar D (panel d)-infected cultures. Transfection with the RFP vector alone did not affect the subsequent infection either (panels e–h).

DISCUSSION

Because of the potentially important roles of chlamydial Incs in chlamydial biology and pathogenesis, various approaches have been developed for searching for new Incs. The first Inc (IncA) was identified by using antisera from animals infected with live chlamydial organisms to screen a chlamydial expression library (Bannantine *et al.*, 1998; Rockey *et al.*, 1995). This wise approach has proven to be productive. However, not all Inc proteins are as immunogenic during chlamydial infection and high titres of antibodies against non-Inc proteins can reduce the efficacy of this approach. Based on the hypothesis that chlamydial Incs are secreted via a type III secretion pathway, various versions of heterologous type III secretion systems were used to evaluate the secretability of chlamydial proteins (Subtil *et al.*, 2001, 2005). Although this approach has provided confirmatory evidence that some known Incs can be secreted by the heterologous type III systems, not all Incs are secretable by the heterologous systems and not all secretable chlamydial proteins identified in the heterologous systems can be localized in the inclusion membrane of chlamydia-infected cells. Based on the hydropathy profiles identified in the known Incs, computer programs have been developed for predicting new Incs (Bannantine *et al.*, 2000; Toh *et al.*, 2003). Although this is potentially an effective approach in the long run, due to lack of sufficient/accurate information on and extreme heterogeneity in the chlamydial Incs, not all predicted proteins are localized in the inclusion membranes [<http://www.stdgen.lanl.gov/>] (Bannantine *et al.*, 2000)] and not all known Incs are predictable (Bannantine *et al.*, 2000; Fling *et al.*, 2001; Rockey *et al.*, 2002; Toh *et al.*, 2003). Therefore, each approach has its own advantages and limitations. We have used an anti-fusion protein antibody approach to localize the endogenous proteins in *C. pneumoniae*-infected cells and found that the hypothetical protein Cpn1027 is localized in the *C. pneumoniae* inclusion membrane. The fact that Cpn1027 has never been considered an Inc by any of the above three methods suggests that our anti-fusion protein antibody approach can

at least complement other approaches for uncovering novel Incs. It is worth noting that although a total of 104 hypothetical proteins encoded by the *C. pneumoniae* genome were predicted to be in the inclusion membrane by computer programs (Bannantine *et al.*, 2000; Toh *et al.*, 2003), only IncA (Cpn0186) was proven to be in the inclusion membrane of the *C. pneumoniae*-infected cells by antibody labelling (Bannantine *et al.*, 2000) and none of the rest of the putative Inc proteins from *C. pneumoniae* has ever been evaluated using antibody probing. Apparently, there is a lack of experimental evidence for directly localizing the *C. pneumoniae* endogenous proteins. Only by experimentally identifying more *C. pneumoniae* Inc proteins, as reported here, can we more precisely determine the common structural features of Inc proteins and possibly derive information on the potential roles of these proteins in *C. pneumoniae* pathogenesis.

With the identification of Cpn1027 as an Inc, the next obvious questions are how the Inc Cpn1027 is secreted and what functions it may have. Due to lack of genetic tools for manipulating chlamydial genomes, approaches are limited for analysing chlamydial protein functions. Using specific antibodies recognizing Cpn1027, we have characterized this protein in terms of its expression pattern during *C. pneumoniae* infection and its distribution in other chlamydial species. We also studied the effect of Cpn1027 expressed via a transgene on subsequent chlamydial infection since it was previously shown that both *C. trachomatis* and *C. caviae* IncAs when expressed as fusion proteins in the host cell cytosol inhibited subsequent chlamydial development (Alzhanov *et al.*, 2004; Delevoye *et al.*, 2004). Although Cpn1027 shared a similar expression pattern with IncA during *C. pneumoniae* infection, it lacked homologues in other chlamydial species and failed to affect the subsequent chlamydial infection. These observations suggest that Cpn1027 may play a role different from that of IncA in *C. pneumoniae* biology. Further characterization of Cpn1027 is under way.

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REFERENCES

- Alzhanov, D., Barnes, J., Hruby, D. E. & Rockey, D. D. (2004). Chlamydial development is blocked in host cells transfected with *Chlamydomonas caviae* incA. *BMC Microbiol* 4, 24.
- Bannantine, J. P., Rockey, D. D. & Hackstadt, T. (1998). Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane. *Mol Microbiol* 28, 1017–1026.
- Bannantine, J. P., Griffiths, R. S., Viratyosin, W., Brown, W. J. & Rockey, D. D. (2000). A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell Microbiol* 2, 35–47.

- Campbell, L. A. & Kuo, C. C. (2004). *Chlamydia pneumoniae* – an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* 2, 23–32.
- Campbell, L. A., Nosaka, T., Rosenfeld, M. E., Yaraei, K. & Kuo, C. C. (2005). Tumor necrosis factor alpha plays a role in the acceleration of atherosclerosis by *Chlamydia pneumoniae* in mice. *Infect Immun* 73, 3164–3165.
- Carabeo, R. A., Mead, D. J. & Hackstadt, T. (2003). Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc Natl Acad Sci U S A* 100, 6771–6776.
- Chen, C., Chen, D., Sharma, J., Cheng, W., Zhong, Y., Liu, K., Jensen, J., Shain, R., Arulanandam, B. & Zhong, G. (2006). The hypothetical protein CT813 is localized in the *Chlamydia trachomatis* inclusion membrane and is immunogenic in women urogenitally infected with *C. trachomatis*. *Infect Immun* 74, 4826–4840.
- Delevoye, C., Nilges, M., Dautry-Varsat, A. & Subtil, A. (2004). Conservation of the biochemical properties of IncA from *Chlamydia trachomatis* and *Chlamydia caviae*: oligomerization of IncA mediates interaction between facing membranes. *J Biol Chem* 279, 46896–46906.
- Dong, F., Pirbhai, M., Xiao, Y., Zhong, Y., Wu, Y. & Zhong, G. (2005). Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in *Chlamydia trachomatis*-infected cells. *Infect Immun* 73, 1861–1864.
- Dong, F., Zhong, Y., Arulanandam, B. & Zhong, G. (2005). Production of a proteolytically active protein, chlamydial protease/ proteasome-like activity factor, by five different *Chlamydia* species. *Infect Immun* 73, 1868–1872.
- Fan, P., Dong, F., Huang, Y. & Zhong, G. (2002). *Chlamydia pneumoniae* secretion of a protease-like activity factor for degrading host cell transcription factors required for [correction of factors is required for] major histocompatibility complex antigen expression. *Infect Immun* 70, 345–349.
- Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G. A., Nance, D. M., Greenberg, A. H. & Zhong, G. (1998). Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med* 187, 487–496.
- Fling, S. P., Sutherland, R. A., Steele, L. N., Hess, B., D'Orazio, S. E., Maisonneuve, J., Lampe, M. F., Probst, P. & Starnbach, M. N. (2001). CD8⁺ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A* 98, 1160–1165.
- Grayston, J. T. (1992). *Chlamydia pneumoniae*, strain TWAR pneumonia. *Annu Rev Med* 43, 317–323.
- Greene, W., Xiao, Y., Huang, Y., McClarty, G. & Zhong, G. (2004). Chlamydia-infected cells continue to undergo mitosis and resist induction of apoptosis. *Infect Immun* 72, 451–460.
- Hackstadt, T. (1998). The diverse habitats of obligate intracellular parasites. *Curr Opin Microbiol* 1, 82–87.
- Hackstadt, T., Scidmore, M. A. & Rockey, D. D. (1995). Lipid metabolism in *Chlamydia trachomatis*-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. *Proc Natl Acad Sci U S A* 92, 4877–4881.
- Hackstadt, T., Rockey, D. D., Heinzen, R. A. & Scidmore, M. A. (1996). *Chlamydia trachomatis* interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. *EMBO J* 15, 964–977.
- Hackstadt, T., Fischer, E. R., Scidmore, M. A., Rockey, D. D. & Heinzen, R. A. (1997). Origins and functions of the chlamydial inclusion. *Trends Microbiol* 5, 288–293.
- Hackstadt, T., Scidmore-Carlson, M. A., Shaw, E. I. & Fischer, E. R. (1999). The *Chlamydia trachomatis* IncA protein is required for homotypic vesicle fusion. *Cell Microbiol* 1, 119–130.
- Hu, H., Pierce, G. N. & Zhong, G. (1999). The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. *J Clin Invest* 103, 747–753.
- Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R. W., Olinger, L., Grimwood, J., Davis, R. W. & Stephens, R. S. (1999). Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* 21, 385–389.
- Kuo, C. C., Grayston, J. T., Campbell, L. A., Goo, Y. A., Wissler, R. W. & Benditt, E. P. (1995a). *Chlamydia pneumoniae* (TWAR) in coronary arteries of young adults (15–34 years old). *Proc Natl Acad Sci U S A* 92, 6911–6914.
- Kuo, C. C., Jackson, L. A., Campbell, L. A. & Grayston, J. T. (1995b). *Chlamydia pneumoniae* (TWAR). *Clin Microbiol Rev* 8, 451–461.
- Kuo, C. C., Campbell, L. A. & Rosenfeld, M. E. (2002). *Chlamydia pneumoniae* infection and atherosclerosis: methodological considerations. *Circulation* 105, E34.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157, 105–132.
- Liu, L., Hu, H., Ji, H., Murdin, A. D., Pierce, G. N. & Zhong, G. (2000). *Chlamydia pneumoniae* infection significantly exacerbates aortic atherosclerosis in an LDLR^{-/-} mouse model within six months. *Mol Cell Biochem* 215, 123–128.
- Read, T. D., Brunham, R. C., Shen, C., Gill, S. R., Heidelberg, J. F., White, O., Hickey, E. K., Peterson, J., Utterback, T. & other authors (2000). Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* 28, 1397–1406.
- Rockey, D. D., Heinzen, R. A. & Hackstadt, T. (1995). Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol Microbiol* 15, 617–626.
- Rockey, D. D., Scidmore, M. A., Bannantine, J. P. & Brown, W. J. (2002). Proteins in the chlamydial inclusion membrane. *Microbes Infect* 4, 333–340.
- Scidmore, M. A., Fischer, E. R. & Hackstadt, T. (1996). Sphingolipids and glycoproteins are differentially trafficked to the *Chlamydia trachomatis* inclusion. *J Cell Biol* 134, 363–374.
- Sharma, J., Niu, Y., Ge, J., Pierce, G. N. & Zhong, G. (2004). Heat-inactivated *C. pneumoniae* organisms are not atherogenic. *Mol Cell Biochem* 260, 147–152.
- Sharma, J., Dong, F., Pirbhai, M. & Zhong, G. (2005). Inhibition of proteolytic activity of a chlamydial proteasome/protease-like activity factor by antibodies from humans infected with *Chlamydia trachomatis*. *Infect Immun* 73, 4414–4419.
- Sharma, J., Zhong, Y., Dong, F., Piper, J. M., Wang, G. & Zhong, G. (2006). Profiling of human antibody responses to *Chlamydia trachomatis* urogenital tract infection using microplates arrayed with 156 chlamydial fusion proteins. *Infect Immun* 74, 1490–1499.
- Shaw, A. C., Vandahl, B. B., Larsen, M. R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G. & Birkelund, S. (2002). Characterization of a secreted *Chlamydia* protease. *Cell Microbiol* 4, 411–424.
- Su, H., McClarty, G., Dong, F., Hatch, G. M., Pan, Z. K. & Zhong, G. (2004). Activation of Raf/MEK/ERK/cPLA2 signaling pathway is essential for chlamydial acquisition of host glycerophospholipids. *J Biol Chem* 279, 9409–9416.
- Subtil, A., Parsot, C. & Dautry-Varsat, A. (2001). Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. *Mol Microbiol* 39, 792–800.
- Subtil, A., Delevoye, C., Balana, M. E., Tastevin, L., Perrinet, S. & Dautry-Varsat, A. (2005). A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* 56, 1636–1647.

Toh, H., Miura, K., Shirai, M. & Hattori, M. (2003). In silico inference of inclusion membrane protein family in obligate intracellular parasites chlamydiae. *DNA Res* **10**, 9–17.

Vandahl, B. B., Stensballe, A., Roepstorff, P., Christiansen, G. & Birkelund, S. (2005). Secretion of Cpn0796 from *Chlamydia pneumoniae* into the host cell cytoplasm by an autotransporter mechanism. *Cell Microbiol* **7**, 825–836.

Xiao, Y., Zhong, Y., Greene, W., Dong, F. & Zhong, G. (2004). *Chlamydia trachomatis* infection inhibits both Bax and Bak activation induced by staurosporine. *Infect Immun* **72**, 5470–5474.

Xiao, Y., Zhong, Y., Su, H., Zhou, Z., Chiao, P. & Zhong, G. (2005). NF-kappa B activation is not required for *Chlamydia trachomatis* inhibition of host epithelial cell apoptosis. *J Immunol* **174**, 1701–1708.

Zhong, G., Toth, I., Reid, R. & Brunham, R. C. (1993). Immunogenicity evaluation of a lipidic amino acid-based synthetic

peptide vaccine for *Chlamydia trachomatis*. *J Immunol* **151**, 3728–3736.

Zhong, G., Berry, J. & Brunham, R. C. (1994). Antibody recognition of a neutralization epitope on the major outer membrane protein of *Chlamydia trachomatis*. *Infect Immun* **62**, 1576–1583.

Zhong, G., Reis e Sousa, C. & Germain, R. N. (1997). Production, specificity, and functionality of monoclonal antibodies to specific peptide-major histocompatibility complex class II complexes formed by processing of exogenous protein. *Proc Natl Acad Sci U S A* **94**, 13856–13861.

Zhong, G., Fan, P., Ji, H., Dong, F. & Huang, Y. (2001). Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors. *J Exp Med* **193**, 935–942.

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