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Localization of the hypothetical protein Cpn0585 in the inclusion membrane of *Chlamydia pneumoniae*-infected cells

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Abstract

Cpn0585, encoded by a hypothetical open reading frame in *Chlamydia pneumoniae* genome, was detected in the inclusion membrane during *C. pneumoniae* infection using both polyclonal and monoclonal antibodies raised with Cpn0585 fusion protein. The anti-Cpn0585 antibodies specifically recognized the endogenous Cpn0585 without cross-reacting with IncA (a known inclusion membrane protein of *C. pneumoniae*) or other control antigens. A homologue of Cpn0585 in the *C. caviae* species (encoded by the ORF CCA00156) was also localized in the inclusion membrane of the *C. caviae*-infected cells. The Cpn0585 protein became detectable 24 hours while CCA00156 as early as 8 hours after infection. Once expressed, both proteins remained in the inclusion membrane throughout the rest of infection course.

Keywords

Chlamydia pneumoniae; Cpn0585; inclusion membrane protein

1. Introduction

Chlamydiae represent a diverse group of obligate intracellular bacterial pathogens that are classified into multiple species, including the human pathogens *Chlamydia trachomatis* (*C. trachomatis*) [1] and *C. pneumoniae* [2] and animal pathogens *C. muridarum* (formerly known as *C. trachomatis* mouse pneumonitis agent, designated as MoPn, ref: [2]), *C. caviae*, *C. psittaci*, *C. abortus* and *C. felis*. The species *C. pneumoniae*, *C. caviae*, *C. psittaci*, *C. abortus* & *C. felis* are also grouped as an independent genus termed Chlamydophilae based on their genetic relatedness [3]. The *C. pneumoniae* organisms mainly infect the human respiratory system, not only causing various respiratory diseases but also exacerbating pathologies in the cardiovascular system [4]. The *C. caviae* GPIC organisms can infect both the ocular and urogenital tissues in guinea pig, which has been used as a model system for studying chlamydial pathogenic mechanisms. Despite the differences in host range, tissue tropism, disease process,

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all chlamydial species share similar genome sequences [1,2] and possess a common intracellular growth cycle with distinct biphasic stages [5].

Chlamydial organisms must replicate within a cytoplasmic vacuole of eukaryotic cells [5]. A typical chlamydial infection starts with entry of the infectious form of the organisms, elementary body (EB), into host cells via endocytosis. The endocytosed EB can differentiate into reticulate body (RB) that is no longer infectious but metabolically active and able to multiply. After many rounds of replication, RBs can differentiate back into EBs that can exit the infected cells to infect neighboring cells. The entire process of chlamydial biosynthesis and differentiation occurs within the cytoplasmic vacuoles (also termed inclusions). The inclusions not only support chlamydial replication but also protect the replicating organisms from host defense responses such as lysosomal fusion [6,7]. Chlamydia has developed an intimate relationship with host cells by both secreting factors into host cell cytosol [8,9] and taking in nutrients and metabolic intermediates from the host cells [10,11]. However, the mechanisms by which chlamydial organisms interact with host cells are largely unknown.

The fact that Chlamydia-encoded proteins are found in the inclusion membrane (designated as Inc proteins; [12]) suggests that Inc proteins may participate in the chlamydial interactions with host cells [13]. Searching for novel Inc proteins may help unravel the molecular basis of chlamydial interactions with host cells and has thus become a hot topic in the chlamydial research field. Using an antibody labeling approach for identifying new Inc proteins in *C. pneumoniae*-infected cells, we have found that the hypothetical protein Cpn0585 is localized in the *C. pneumoniae* inclusion membrane.

2. Results

2.1. Cpn0585 is detected in the inclusion membrane of *C. pneumoniae*-infected cells by antibodies raised with Cpn0585 fusion protein

After screening more than 100 antibodies raised with *C. pneumoniae* fusion proteins, we found that antibodies raised with glutathione S-transferase (GST)-Cpn0585 fusion protein labeled the *C. pneumoniae* inclusion membrane (Fig. 1). Both the anti-Cpn0585 polyclonal (pAb) and monoclonal (mAb; clone 3D11 with an isotype of IgM; 12F2, IgM) antibodies obtained from the same immunized mouse consistently detected a dominant inclusion membrane signal similar to (Fig. 1A) and partially overlapping with (Fig. 1B) the signal revealed by anti-IncA (clone 2B12.1 raised with GST-Cpn0186 fusion protein), but not anti-CPAFcp (EB3.1), anti-MOMP (GZD1E8) or anti-HSP60 (BC7.1, although raised with GST-CT110 but cross-reacting with Cpn0134) antibodies under a conventional fluorescence microscope. The inclusion membrane localization of Cpn0585 was further confirmed using confocal microscopy. The anti-Cpn0585 antibody revealed an antigen pattern distinct from those of CPAFcp and MOMP but partially overlapping with that of IncA even at different focal points along the Z-axis (data not shown). Since IncA, encoded by *C. pneumoniae* ORF *cpn0186*, is a known Inc protein in *C. pneumoniae*-infected cells [14], the above observations have demonstrated that Cpn0585 is also an Inc protein. It is worth noting that although Cpn0585 and IncA were both localized in the inclusion membrane, they did not completely overlap with each other under confocal microscopy (data not shown), suggesting that Cpn0585 and IncA may differ from each other in distribution in the membrane. Resolving the precise topological relationships between Cpn0585 and IncA will require higher resolution methods such as immuno-EM, which will be carried in the future.

We further used three independent approaches to confirm the antibody binding specificities (data not shown). First, in a Western blot assay, the anti-Cpn0585 antibodies reacted with the GST-Cpn0585 but not the GST-IncA, GST-CPAFcp, GST-HSP60 or GST-Cpn0285 fusion proteins although all fusion proteins were loaded at an equivalent amount and detected by their

corresponding homologous antibodies. Second, when the RFP (red fluorescence protein)-*C. pneumoniae* fusion proteins expressed in transfected cells were used as antigens, the anti-Cpn0585 antibodies only detected RFP-Cpn0585 but not RFP-IncA, RFP-Cpn0285 or RFP-CPAFcp fusion proteins. Finally, in a pre-absorption experiment, the binding to the endogenous antigens in the *C. pneumoniae*-infected cells by anti-Cpn0585, anti-IncA & anti-Cpn0285 antibodies was blocked only by the corresponding homologous but not the unrelated heterologous GST fusion proteins.

2.2. The GPIC homologue of Cpn0585, CCA00156, is also localized in the inclusion membrane of GPIC-infected cells

Sequence analysis revealed that Cpn0585 [with a full length sequence of 651 amino acids (AA); accession# NP_224781, <http://www.ncbi.nlm.nih.gov/entrez>] shared ~23% amino acid sequence homologies with the N-terminal regions of the hypothetical protein CCA00156 (1006AA; NP_829093) in *C. caviae* GPIC, the hypothetical protein CF0851 (1012AA; YP_515768) in *C. felis* Fe/C-56 and the hypothetical protein CAB154 (1012AA; CAH63612) in *C. abortus* S26/3 organisms. No other significant homologue was found. It appears that Cpn0585 and its homologues are uniquely acquired by the chlamydial species classified as the genus Chlamydophilae although Cpn0585 from the human pathogen *C. pneumoniae* has the shortest sequence presumably by lacking the C-terminal half when compared to the homologues from the other Chlamydophilae species that use animals as natural hosts. To test whether the homologues from the animal chlamydial species are also localized in the inclusion membrane, we used a pAb raised with the GST-CCA00156 fusion protein to localize the endogenous CCA00156 in *C. caviae* GPIC-infected cells (Fig. 3). The anti-CCA00156 antibody revealed an antigen pattern (panels a & d) similar to that of IncA (detected with pAb raised against GST-CCA00550; e & h) but different from those of CPAF (against GST-CCA00745; i & l) and HSP60 (mAb clone BC7.1; m & p), suggesting that CCA00156 is localized in the inclusion membrane of *C. caviae* GPIC-infected cells. The anti-CCA00156 antibody binding specificity was confirmed using a pre-absorption experiment (data not shown). The recognition of endogenous CCA00156 in GPIC-infected cells by the anti-CCA00156 antibody was removed by pre-absorption with GST-CCA00156 but not GST-IncA or GST-CPAF fusion proteins.

2.3. Time course expression of Cpn0585 and CCA00156 during chlamydial infections

Using specific antibodies described above, we monitored expression patterns of the inclusion membrane proteins Cpn0585 and CCA00156 during chlamydial infection (Fig. 3). Cpn0585 became detectable 24 hours (Fig. 3A, panel d) while IncA as early as 12 hours after *C. pneumoniae* infection (k & k1), suggesting that Cpn0585 is a late protein, which is consistent with a previous observation that Cpn0585 transcript was only detected at a late stage of *C. pneumoniae* infection [15]. Both Cpn0585 and IncA proteins were likely secreted to the inclusion membrane once they became detectable since these Inc proteins always surrounded *C. pneumoniae* organisms (white arrows in panels d & k1 of Fig.3A). The GPIC-encoded CCA00156 and GPIC-IncA proteins were both detected in the inclusion membrane (Fig. 3B, panels d1 & k1) as early as 8 hours after *C. caviae* GPIC infection (Fig. 3B, panels d & k). Despite the differences in time when the *C. pneumoniae* and *C. caviae* Inc proteins first became detectable, all Inc proteins remained in the inclusion membranes throughout the rest of infection courses (Fig. 3A & B), suggesting that these Inc proteins may play essential roles in chlamydial interactions with host cells either by supporting the inclusion membrane structure and/or mediating/regulating the interactions between Chlamydia and host cells.

3. Discussion

We have provided compelling evidence demonstrating that the hypothetical protein Cpn0585 is an Inc protein. First, both pAbs and mAbs raised with GST-Cpn0585 fusion protein labeled the inclusion membrane and the anti-Cpn0585 mAb labeling partially overlapped with that of the anti-IncA. Second, the anti-Cpn0585 fusion protein antibodies specifically recognized the endogenous Cpn0585 in the inclusion membrane of *C. pneumoniae*-infected cells. Third, the homologue CCA00156 encoded by *C. caviae* GPIC was also localized in the inclusion membrane of GPIC-infected cells. Finally, our finding on Cpn0585 localization in the inclusion membrane is consistent with a previous observation made by Dr. Ben Wizel's group that the Cpn0585 was accessible to host cell cytoplasm immune proteasomal processing [16].

Due to the potential importance of chlamydial inclusion membrane proteins in chlamydial pathogenesis, tremendous amounts of efforts have been made to search for new inclusion membrane proteins since the first Inc protein (IncA) was identified in GPIC-infected cells over a decade ago [12]. The approaches employed include antibody detection [12,17], accessibility to host cell cytoplasm immune proteasome processing [16,18], secretion by heterologous type III secretion systems [19] and common structural feature-based computer predictions [14,20]. Although both the computer prediction and heterologous secretion system approaches have provided important information on chlamydial Inc proteins, not all Inc proteins can be identified by these approaches and not all predicted Inc proteins are localized in the inclusion membrane of chlamydial organism-infected cells [14,18]. For example, the known Inc protein Cap1 (CT529) identified via its accessibility to host immune proteasomal processing [18] was not predicted to be in the inclusion membrane [14,20] while CT484 was predicted to be an Inc protein but it was detected only inside the inclusion [14]. Similarly in the current study, although both Cpn0585 and Cpn0285 were predicted to be Inc proteins [14,20], we found that only Cpn0585 but not Cpn0285 was in the inclusion membrane. Although a total of 104 hypothetical proteins encoded by *C. pneumoniae* genome were predicted to be Inc proteins [14,20], only IncA (Cpn0186) was proven to be in the inclusion membrane of the *C. pneumoniae*-infected cells by antibody labeling [14] and none of the rest of the putative Inc proteins was evaluated using antibody probing. Apparently, there is a serious lack of experimental evidence for localizing the *C. pneumoniae* endogenous proteins. Since not all Inc proteins can be predicted or secretable in heterologous secretion systems and not all predicted or heterologously secretable proteins are actually localized in the *C. pneumoniae* inclusion membrane, there is an urgent need to directly identify/confirm new Inc proteins in the *C. pneumoniae*-infected cells using chlamydial protein-specific reagents. Although this approach requires hard work, it is unfortunately unavoidable if one is serious in advancing the field. Using both pAbs and mAbs in the current study, we have not only provided the first experimental evidence for demonstrating the inclusion membrane localization of Cpn0585 and its GPIC homologue CCA00156, but also revealed the expression patterns of these Inc proteins during infection. As more *C. pneumoniae* Inc proteins are experimentally identified, we will be able to more precisely determine their common structural features (tertiary or linear) and to further derive information on their potential roles in *C. pneumoniae* pathogenesis.

4. Materials and Methods

4.1. Cell culture and chlamydial infection

HeLa 229 cell (ATCC, Manassas, VA 20108) monolayers were infected with *C. pneumoniae* AR39 or *C. caviae* GPIC organisms at an MOI of 0.5 in the presence of 2 µg/ml of cycloheximide for various periods of time as indicated in individual experiments. The infected cultures grown on coverslips were processed for immunostainings.

4.2. Chlamydial gene cloning, fusion protein expression and antibody production

The hypothetical ORFs, including the ORF *Cpn0585*, encoded in the *C. pneumoniae* genome (<http://www.stdgen.lanl.gov>) and the ORFs CCA00156 & CCA00550 in *C. caviae* GPIC genome (<http://www.ncbi.nlm.nih.gov/entrez>) were cloned into pGEX vectors (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and expressed as fusion proteins with GST fused to the N-terminus of the chlamydial proteins as previously described [17]. Expression of the fusion proteins was induced with isopropyl-beta-D-thiogalactoside (IPTG; Invitrogen, Carlsbad, CA) and the fusion proteins were extracted by lysing the bacteria via sonication in a Triton-X100 lysis buffer (1% TritonX-100, 1mM PMSF, 75 units/ml of Aprotinin, 20 μ M Leupeptin and 1.6 μ M Pepstatin). After a high-speed centrifugation to remove debris, the fusion proteins were purified using glutathione-conjugated agarose beads (Pharmacia) and the purified proteins were used to immunize mice for producing antibodies. The anti-fusion protein antibodies were used to localize the endogenous proteins in *C. pneumoniae*-infected cells [9]. Some chlamydial ORFs were also cloned into the pDsRed Monomer C1 mammalian expression vector (BD Biosciences Clontech, San Jose, CA) and expressed as fusion proteins with a Red fluorescence protein (RFP) fused to the N-terminus [17]. The recombinant plasmids were transfected into HeLa cells using the Lipofectamine 2000 transfection reagent following the protocol recommended by the manufacture (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the fusion proteins were visualized via either the fusion tag RFP or anti-chlamydial protein antibody labeling.

4.3. Immunofluorescence and Western blot assays

HeLa cells grown on coverslips were fixed with 2% paraformaldehyde (Sigma, St. Luis, MO) for 30 min at room temperature (RT), 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 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, Inc., West Grove, PA) was used to visualize chlamydial organisms. The mouse antibodies against various reference proteins and the *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 fusion proteins immobilized onto agarose beads (Pharmacia) prior to staining. The pre-absorption was carried by incubating the antibodies with bead-immobilized antigens for 1h at room temperature (RT) 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 cell samples with immuno-labeling were subjected to image analysis and acquisition using an AX-70 fluorescence microscope (Olympus, Melville, NY) as described previously [9,11]. An Olympus FluoView™ Laser Confocal Microscope was used to analyze some of the co-stained samples. The service was kindly provided by Dr. Victoria Centonze Frohlich at the UTHSCSA core optical imaging facility.

The Western blot assay was carried out as described elsewhere [9,11]. 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 (horse radish peroxidase)-conjugated secondary antibody and

visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

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Fig. 1. Detection of Cpn0585 in the inclusion membrane of *C. pneumoniae*-infected cells
 HeLa cells were infected with *C. pneumoniae* AR39 organisms at an MOI of 0.5 in the presence of 2 μ g/ml of cycloheximide for 72 hours. The infected cultures grown on coverslips were processed for the following immunostainings: (A) Cpn0585 was probed with a mouse antiserum (pAb, panel a) and monoclonal antibodies (mAb clone 3D11 with a heavy chain isotype of IgM, panel b; 12F2, IgM, panel c), all of which were raised with the GST-Cpn0585 fusion protein 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 AR39 organism-infected cell samples were co-stained with the anti-Cpn0585 mAb 3D11 (green) and DNA Hoechst dye (blue) in combination of antibodies recognizing other *C. pneumoniae* reference proteins, including CPAFcp (EB3.1, IgG1), IncA (2B12.1, IgG1), MOMP (GZD1E8, IgG1) and HSP60 (BC7.1, IgG1; all in red). The co-stained samples were also observed under a confocal microscope (images not shown) and the antibody specificities were confirmed using various approaches (data not shown).

Fig. 2. Localization of CCA00156 in the inclusion membrane of GPIC-infected cells

HeLa cells were infected with *C. caviae* GPIC organisms at an MOI of 0.5 in the presence of 2 µg/ml of cycloheximide for 40 hours. The infected cultures grown on coverslips were processed for immunostainings with mouse antibodies against CCA00156 (pAb, panels a–d), GPIC-IncA (pAb, e–h), GPIC-CPAF (pAb, i–l) and GPIC HSP60 (BC7.1, m–p), all of which were visualized with a Cy3-conjugated goat anti-mouse IgG (red). A rabbit anti-GPIC antiserum together with a Cy2-conjugated goat anti-rabbit IgG (green) was used to visualize the *C. caviae* organisms and Hoechst to visualize DNA. The images were acquired using a conventional fluorescence microscope. The antibody specificities were further confirmed using pre-absorption (data not shown).

Fig. 3. Monitoring the expression of Cpn0585 (A) and CCA00156 (B) proteins during chlamydial infections

HeLa cells infected with *C. pneumoniae* AR39 (A) or *C. caviae* GPIC (B) organisms for various periods of time as indicated on top of the figures and the culture samples were subjected to immunostaining with anti-Cpn0585 or anti-Cpn IncA (A, red) and anti-CCA00156 or anti-GPIC IncA (B, red). The rabbit antibodies against AR39 (A) or GPIC (B) were used to visualize the organisms (green) and Hoechst dye for DNA (blue). The images were acquired using the conventional fluorescence microscope. Note that Cpn0585 protein was first detected 24 hours (A, panel d) and Cpn IncA 12 hours (A, panels k & k1) after infection with *C. pneumoniae* while both CCA00156 and GPIC IncA were detected at 8 hours after infection with *C. caviae* (B; panels d, d1, k, k1). White arrows indicate that the inclusion membrane protein labeling (red) appears to surround the organism labeling (green) in panels d & k1 (A) and d1 & k1 (B).