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Localization and Characterization of Hypothetical Protein CT358 in The *Chlamydia trachomatis*-Infected Cells*

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Abstract To localize and characterize the hypothetical protein CT358 in the chlamydial infected cells. CT358 gene from the *Chlamydia trachomatis* (*C. trachomatis*) serovar D genome was amplified and cloned into the pGEX and pDSRedC1 vectors. The recombinant plasmid pGEX-CT358 was constructed and expressed as GST fusion proteins. The GST-CT358 fusion protein was used to immunize mice to raise the antibodies, which specifically recognized CT358 without cross-reacting with other unrelated proteins. The antibodies were then used to localize the endogenous CT358 protein and determine the expression pattern in Chlamydial infected cells using an indirect immunofluorescence assay (IFA). Meanwhile, pDSRedC1-CT358 recombinant plasmid was transfected to HeLa cells to evaluate the effect of CT358 expression on the subsequent chlamydial infection. The hypothetical protein CT358 was identified in the inclusion membrane of *C. trachomatis*-infected cells for the first time,and it was detected as early as 12 h after *C. trachomatis* infection and remained in the inclusion membrane throughout the rest of the infection cycle. Cytosolic expression of CT358 *via* a transgene failed to affect the subsequent chlamydial infection. These observations together have demonstrated that CT358 is a newly identified chlamydial inclusion membrane protein, giving the potentially importance for further understanding the mechanisms of chlamydial intracellular parasitism.

Key words *Chlamydia trachomatis*, CT358, inclusion membrane protein
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C. trachomatis is the most significant human pathogen consisting of many different serovars, serovars A to C mainly infect human eyes, potentially leading to preventable blindness worldwide, and serovars D to K infect human urogenital tracts, which can cause severe complications such as ectopic pregnancy and infertility [1], serovars L1-3 can cause lymphogranuloma venereum. Despite the apparent differences in tissue tropism, all chlamydia possess a common intracellular growth cycle within the inclusion [2]. In order to establish and maintain a successful intravacuolar growth, *Chlamydia* has to exchange both materials and signals with host cells *via* the inclusion membrane. Consequently, the chlamydial proteins localized in the inclusion membrane (designated as Inc) are thought to play important roles. As a result, searching for new Incs for understanding the intricate interactions between chlamydial organisms and host cells has become a hot topic under intensive investigation[3~5].

In the present study, using an anti-fusion protein antibody, we have systematically evaluated the localization of the hypothetical protein CT358 in the *C. trachomatis*-infected cells, distribution patterns and primary functions.We found that the CT358 protein is localized in the inclusion membrane in the *C. trachomatis* infected cells, and it became detectable at 12 h after chlamydial infection. Further characterization of the protein revealed that the pre-existing cytosolic CT358 fusion protein did not affect the subsequent Chlamydial infection. Taken togther, the observations maybe lay a good foundation for understanding the

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mechanisms of chlamydial intracellular pathogen.

1 Materials and methods

1.1 Cell culture and infection

HeLa 229 cells were grown on glass coverslips overnight prior to chlamydial inoculation. Sera D diluted in DMEM medium with 10% fetal calf serum (GIBCO BRL)and 2 mg/L of cycloheximide (Sigma) were directly inoculated onto the cell monolayers. The cell samples were cultured at 37℃ in a CO_2 incubator and processed at various time points after infection for immunostainings.

1.2 Gene cloning, fusion protein expression

The hypothetical ORF CT358 was amplified with 5′ CGCGGATCCATGGCTACACCGATTACTGTA 3′ and 5′ TTTTCCTTTTGCGGCCGCTTAGCAGTGC-TCTTCGAGGC 3′, then the PCR products were cloned into pGEX vector and expressed as fusion protein with GST fused to the N-terminus of the chlamydial protein. Expression of the fusion protein was induced with IPTG and the fusion protein was extracted by lysing the bacteria *via* sonication in a Triton X-100 lysis (1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 75 U/ml of aprotinin, 20 μmol/L leupeptin, and 1.6 μmol/L pepstatin). After high-speed centrifugation to remove debris, the fusion proteins were further purified using glutathione-conjugated agarose beads (Pharmacia) for the subsequent experiments.

1.3 Production of anti-fusion protein antibody

For antibody production, the purified fusion proteins were used to immunize mice. Briefly, two female BALB/c mice (4 weeks old) were immunized with purified fusion protein mixed with an equal amount of complete Freund′s adjuvant by intraperitoneal injection for three times. One week after the final injection, mouse sera were screened using IFA, after the titer of the specific antibody reached 1∶2 000 or higher, the mice were sacrificed. Mouse sera were collected and stored in 50% glycerol at –20℃ until use.

1.4 Analysis of specific anti-fusion protein antibody

To check the specificity of anti-fusion protein antibody, Western blot was carried out as described previously [6~9]. Briefly, GST-CT358 was loaded to SDS-polyacrylamide gel well, as a control the same amount of GST-IncA, GST-CPAF, GST-HSP60 were also loaded in the same gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane and the blots were detected with anti-CT358 fusion protein antibody. Anti-fusion protein antibody binding was probed with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz, CA). Meanwhile, anti-fusion protein antibody was preabsorbed with either the corresponding or heterologous fusion proteins immobilized onto agarose beads prior to the staining of cell samples. The preabsorption approach was carried out by incubating the antibodies with bead-immobilized antigens for overnight at 4℃, followed by pelleting of the beads, the remaining supernatants were used for immunostaining.

1.5 Immunofluorescence staining

HeLa cells infected with sera D were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by permeabilization with 0.1% Triton X-100 for an additional 4 min. After being washed and blocked, the cell samples were subjected to various combinations of antibody and chemical staining. Hoechst (Sigma) was used to visualize nuclear DNA, a rabbit anti-chlamydia antibody plus a goat anti-rabbit IgG conjugated with Cy2 (Jackson ImmunoResearch) were used to visualize chlamydial inclusions, the mouse antibody against the CT358 protein, IncA, HSP60, CPAF plus goat anti-mouse IgG conjugated with Cy3 (Jackson ImmunoResearch) were used to visualize the corresponding antigens. The cell samples with immunolabeling were subjected to image analysis and acquisition using an AX-70 fluorescence microscope (Olympus, Melville, NY) as described previously[10, 11].

1.6 Transient transfection of mammalian cells

CT358 gene from the *C. trachomatis* serovar D genome was cloned into the pDsRed-Monomer-C1 mammalian expression vector with a red fluorescence protein (RFP) fused to the 5′ end of CT358. The recombinant plasmid was transfected into HeLa cells using Lipofectamine 2000 following the protocol recommended by the manufacturer (Invitrogen). 12 h after transfection, the transfected cells were subsequently infected with the *C. trachomats* serovar D, 24 h after infection, CT358 protein expression and inclusions were visualized *via* either the fusion tag RFP or rabbit anti-chlamydial organism antibody labeling.

2 Results

2.1 Expression and purification of the CT358 fusion protein

CT358 gene was successfully amplified from the *C. trachomatis* serovar D genome (Figure 1). The sequence of CT358 gene was found to be 100% homologous to the GenBank accession number nucleotide sequence. After induction with IPTG, the bacteria harboring the recombinant plasmid expressed GST fusion protein with molecular mass of about 46 ku (Figure 2).

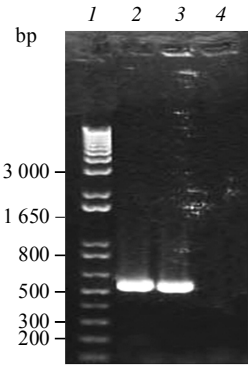

Fig. 1　Amplification of the CT358 gene from *C. trachomatis* serovar D

1: DNA marker; *2*, *3* : PCR product of CT358 gene; *4*: Negative control.

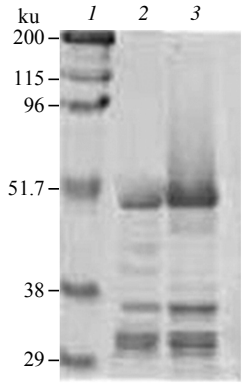

Fig. 2　SDS-PAGE analysis of purified GST-CT358 fusion protein

1: Protein marker; *2*: 2 μl of purified GST-CT358; *3*: 12 μl of purified GST-CT358.

2.2 Specificity of antibody raised with GST-CT358 fusion protein

Using Western blot assay to evaluate whether inclusion membrane labeling by the anti-CT358 antibody is specific to the CT358 protein (Figure 3), as a control, we also loaded the same amount of GST-IncA, GST-CPAF, GST-HSP60, it shows the anti-CT358 antibody reacted positively with the GST- CT358 fusion protein of molecular mass 46 ku, but not other fusion proteins. The specificity was further verified by a preabsorption experiment, the anti-CT358 protein signals were removed by pre-absorption of the antibodies with the GST-CT358 fusion protein, but not other heterologous fusion protein (Figure 4), which suggested that the antibody is specific to the CT358 protein.

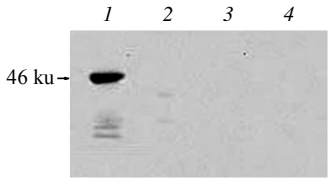

Fig. 3　Immunoblot analysis of purified GST fusion proteins

1: GST-CT358 fusion protein; *2*: GST-IncA; *3*: GST-CPAF; *4*: GST-HSP60.

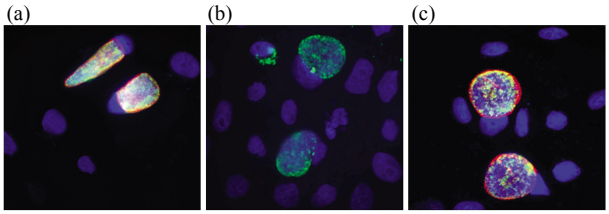

Fig. 4　Specificity of anti-CT358 antibody by IFA after preabsorption

The mouse anti-CT358 was preabsorbed with or without either the corresponding or heterologous fusion proteins. (a) Chlamydial infected HeLa cells were stained with antibody without preabsorption; (b) Stained with the antibody after preabsorption with GST-CT358; (c) Stained with the antibody after preabsorption with heterologous fusion protein. Red: the endogenous CT358 protein; Green: chlamydial organisms; Blue: DNA.

2.3 CT358 is detected in the inclusion membrane of *C. trachomatis*-infected cells

Immunofluorescence microscopy with anti-CT358 demonstrated that CT358 is localized in the *C. trachomatis* inclusion membrane (Figure 5). Serovar D-infected HeLa cells were stained with anti-GST-CT358 (a～d), anti-IncA (e～h), anti- (CPAF) (i～l) and anti-Hsp60 (m～p). The anti-CT358 raised with GST-CT358 fusion protein detected a dominant inclusion membrane signal similar to the signal revealed by anti-IncA, but not anti-CPAF, anti-HSP60 antibodies under a conventional fluorescence

microscope. Since IncA is a known Inc protein in *C. trachomatis*- infected cells[12], the above observations have demonstrated that CT358 is also an Inc protein.

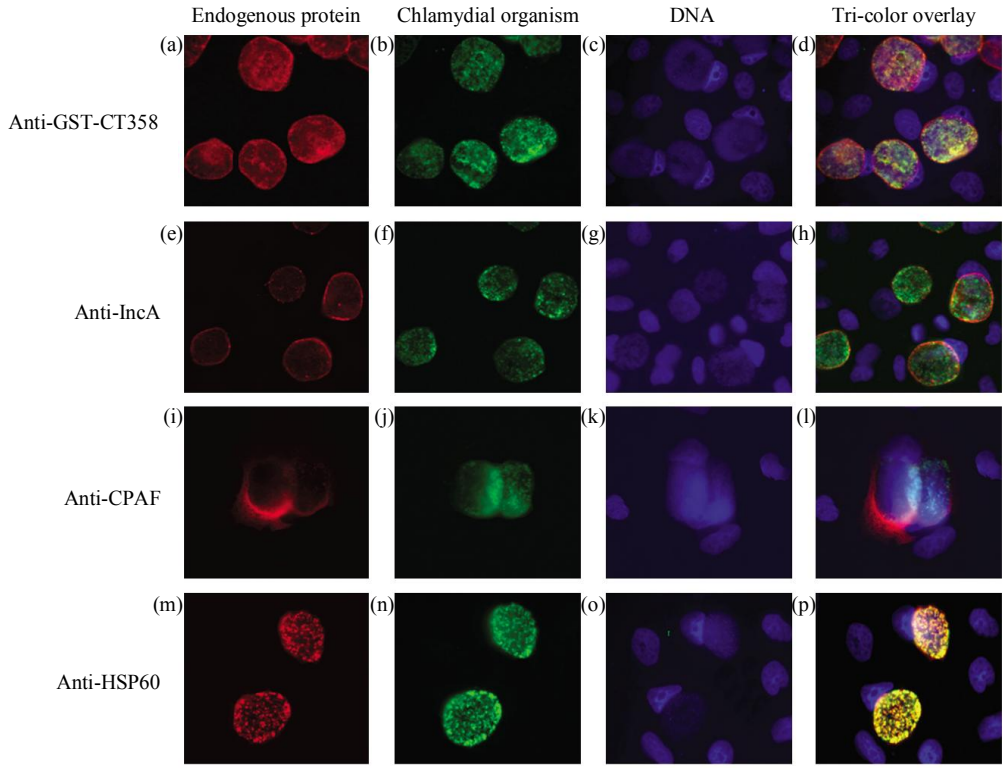

Fig. 5 Localization of CT358 in *C. trachomatis*-infected cells

HeLa cells were infected with *C. trachomatis* for 40 h. The infected HeLa cells were probed with anti-GST-CT358 (a～d), anti-IncA (e～h), anti-CPAF (i～l) and anti-HSP60 (m～p), then visualized with a Cy3-conjugated goat anti-mouse IgG (red). A rabbit anti-chlamydial antiserum together with a Cy2-conjugated goat anti-rabbit IgG (green) was used to visualize the *C. trachomatis*, and Hoechst was used to visualize DNA.

2.4 Time course expression of CT358 during chlamydial infection

Using the specific antibody raised with GST-CT358 fusion protein, we evaluated the expression pattern of the inclusion membrane protein CT358 during chlamydial infection. In IFA assay, CT358 protein was detected as early as 12 h after *C. trachomatis* infection and was obvious by 24 h after infection (Figure 6). When the protein expression was examined using Western blot, we found that CT358 was first detected at 24 h after infection, the protein increased its levels as infection progressed (Figure 7). These results indicated that CT358 protein may play essential role in chlamydial interactions with host cells either by supporting the inclusion membrane structure and/or mediating/regulating the interactions.

2.5 Expression of CT358 in host cell cytosol does not affect the subsequent chlamydial infection

To evaluate the effect of CT358 expression on the subsequent chlamydial infection, pDSRed-CT358 recombinant plasmid was transfected to HeLa cells (Figure 8), the rates of inclusion-forming units were compared between the transfected and untransfected cells 24 h after infection. We found that HeLa cells were equally susceptible to the chlamydial infection regardless of the pre-existing cytosolic CT358 fusion protein, the cells expressing RFP-CT358 fusion protein displayed an infection rate of 70% while the adjacent untransfected cells in the same coverslip displayed 66% in the *C. trachomatis*-infected culture (Figure 9). Transfection with the RFP vector alone did not affect the subsequent infection (84%, 79% seperately). These observations suggested that pre-existing cytosolic CT358 did not result in inhibition of the subsequent *C. trachomatis* infection.

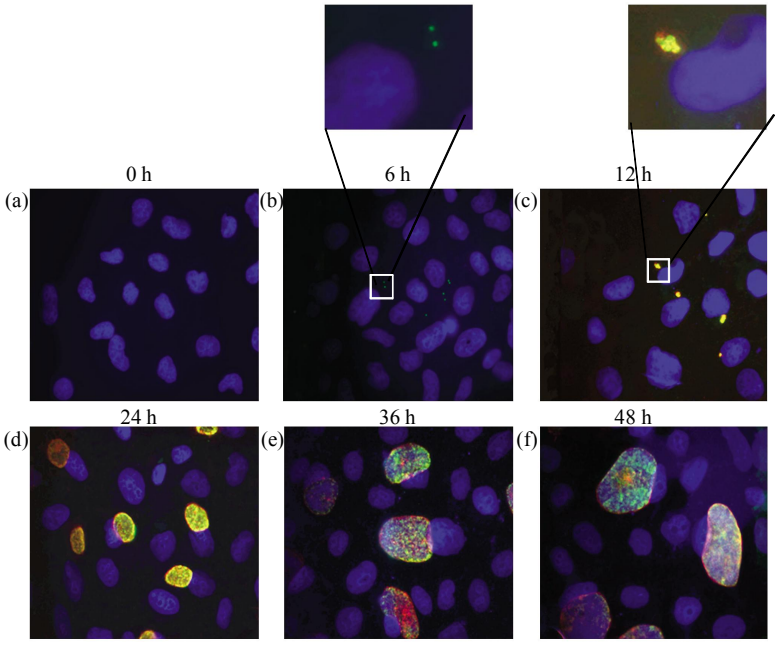

Fig. 6　IFA assay of CT358 protein expression in chlamydial infected cells

The *C. trachomatis*-infected cells were processed at various times after infection for immunofluorescence staining with the primary mouse anti-CT358. The mouse antibody stains were visualized with a goat anti-mouse immunoglobulin G (IgG) conjugated with Cy3 (red). The chlamydial organisms were visualized with anti-chlamydial organism plus a Cy2-conjugated goat anti-mouse IgG (green), DNA was stained with Hoechst (blue).

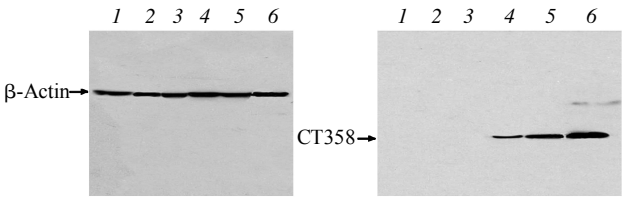

Fig. 7　Western blot assay of CT358 protein expression in chlamydial infected cells

HeLa cells infected with *C. trachomatis* were processed for detecting the endogenous CT358 by Western blot at different time. CT358 antibody specifically detected the endogenous proteins at 24 h postinfection, but the mammalian β-actin was detected in all sample. *1*: 0 h; *2*: 6 h; *3*: 12 h; *4*: 24 h; *5*: 36 h; *6*: 48 h.

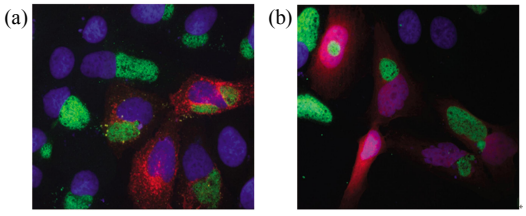

Fig. 8　Expression of CT358 protein during chlamydial infections

(a) HeLa cells were transfected with the recombinant pDsRedC1-CT358 plasmids. (b) HeLa cells were transfected with pDsRedC1 vector. Red: RFP fusion protein(a) or RFP(b); Green: *C. trachomatis* serovar D; Blue: DNA.

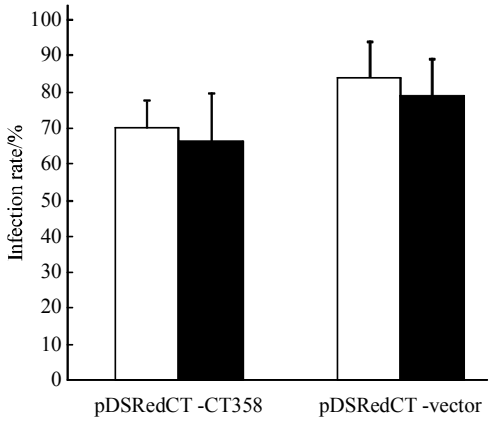

Fig. 9　Effect of RFP-chlamydial fusion protein expression on chlamydial infection

No significant difference between the transfected and untransfected cells ($P < 0.05$). Each bar represents the $\bar{x} \pm s$ obtained from three different experiments. □: RFP+cells; ■: RFP−cells.

3　Discussion

Although about 50 *C. trachomatis* hypothetical proteins have been predicted to be inclusion membrane proteins, but not all predicted Inc proteins are localized in the inclusion membrane of chlamydial-infected cells[13], also not all known Incs are predictable[13~16].

And until now only a few have been experimentally demonstrated to be in the inclusion membrane. Due to the potential importance of chlamydial inclusion membrane proteins in chlamydial pathogenesis, tremendous amounts of efforts need to be made to search for new inclusion membrane proteins.

Using an anti-fusion protein antibody approach to localize the endogenous proteins in *C. trachomatis*-infected cells, we found that the hypothetical protein CT358 is localized in the *C. trachomatis* inclusion membrane for the first time. We also presented compelling evidence to show that the anti-CT358 staining of the inclusion membrane is specific to the CT358 protein. First, in a Western blot assay, the anti-CT358 antibody reacted positively with the GST-CT358 fusion protein but not the GST-IncA, GST-CPAF, GST-HSP60 fusion proteins although all fusion proteins were loaded at an equivalent amount and detected by their corresponding homologous antibodies. Second, the anti-CT358 fusion protein antibody specifically recognized the endogenous CT358 in the inclusion membrane of *C. trchomatis*-infected cells and also the antibody-detected endogenous protein signal was removed by pre-absorption of the antibodies with the corresponding GST fusion protein.

The research interested in Inc proteins is mainly from their potential roles in chlamydial pathogenesis, it has been shown that prior expression of IncA in host cell cytosol can inhibit the subsequent chlamydial infection[17, 18]. In our experiment, we found that HeLa cells were equally susceptible to the chlamydial infection regardless of the pre-existing cytosolic CT358 fusion protein, although IncA did so[17], suggesting that CT358 may play a role different from that of IncA in *C. trachomatis* biology. CT358 became detectable at 12 h after infection and once it became detectable it remains in the inclusion membranes throughout the rest of infection courses, suggesting that CT358 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.

In the current study, we have not only provided the first experimental evidence for demonstrating the inclusion membrane localization of CT358 but also revealed the expression pattern of CT358 protein during chlamydial infection. As more *C. trachomatis* Inc proteins are experimentally identified, the next obvious question is how the Inc CT358 is secreted and what functions it may have. We will be able to further derive information on its potential roles in *C. trachomatis* pathogenesis.

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CT358 蛋白在沙眼衣原体感染细胞中的定位及特性分析 *

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摘要 确定沙眼衣原体 CT358 蛋白在衣原体感染细胞中的位置并初步鉴定其生物学功能．采用 PCR 方法从 D 型沙眼衣原体的基因组中扩增 CT358 基因，并克隆入 pGEX 和 pDSRedC1 表达载体中．将重组质粒 pGEX-CT358 转化到 XL1-blue 宿主菌，并诱导表达融合蛋白 GST-CT358．纯化后的 CT358 融合蛋白免疫小鼠制备抗体，应用间接免疫荧光技术对 CT358 蛋白在衣原体感染细胞内的定位及表达模式进行分析．同时，pDSRedC1-CT358 重组质粒瞬时转染 HeLa 细胞，观察 CT358 蛋白对衣原体感染的影响．实验结果证明 CT358 蛋白为沙眼衣原体包涵体膜蛋白．该蛋白质在衣原体感染 12 h 后就表达定位于包涵体膜上，直至持续到整个感染周期，转基因在胞浆表达的 CT358 融合蛋白不影响其后的衣原体感染．该研究为深入研究衣原体与宿主细胞间相互作用提供了新的线索，并可为衣原体性的治疗、预防提供新方向．

关键词 沙眼衣原体，CT358，包涵体膜蛋白

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