The Chlamydial Plasmid-Encoded Protein pgp3 Is Secreted into the Cytosol of Chlamydia-Infected Cells

Zhongyu Li,1,2 Ding Chen,1 Youmin Zhong,1 Shiping Wang,2 and Guangming Zhong1*

Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229,1 and Department of Parasitology, Xiangya Medical School, The Central South University, 168 Tongzipo Rd., Changsha, Hunan 410078, The People’s Republic of China2

Received 12 October 2007/Returned for modification 1 February 2008/Accepted 7 May 2008

The chlamydial cryptic plasmid encodes eight putative open reading frames (ORFs), designated pORF1 to -8. Antibodies raised against these ORF proteins were used to localize the endogenous proteins during chlamydial infection. We found that the pORF5 protein (also known as pgp3) was detected mainly in the cytosol of Chlamydia-infected cells, while the remaining seven proteins were found inside the chlamydial inclusions only. The pgp3 distribution pattern in the host cell cytosol is similar to but not overlapping with that of chlamydial protease/proteasome-like activity factor (CPAF), a chlamydial genome-encoded protein known to be secreted from chlamydial inclusions into the host cell cytosol. The anti-pgp3 labeling was removed by preabsorption with pgp3 but not CPAF fusion proteins and vice versa, demonstrating that pgp3 is a unique secretion protein. This conclusion is further supported by the observation that pgp3 was highly enriched in cytosolic fractions and had a minimal presence in the inclusion-containing nuclear fractions prepared from Chlamydia-infected cells. The pgp3 protein was detected as early as 12 h after infection and was secreted by all chlamydial species that carry the cryptic plasmid, suggesting that there is a selection pressure for maintaining pgp3 secretion during chlamydial infection. Although expression of pgp3 in the host cell cytosol via a transgene did not alter the susceptibility of the transfected cells to the subsequent chlamydial infection, purified pgp3 protein stimulated macrophages to release inflammatory cytokines, suggesting that pgp3 may contribute to chlamydial pathogenesis.

Chlamydia represents a group of obligate intracellular bacterial pathogens consisting of many different species and causing various health problems in both humans and animals. The species C. trachomatis is composed of more than 15 different serovars (including A to L serovars); some infect the human ocular epithelium, potentially leading to preventable blindness (64), while others infect human urogenital tract tissue, which can potentially cause severe complications such as ectopic pregnancy and infertility (56). C. muridarum, formerly known as the murine biovar of C. trachomatis (designated MoPn, with the single strain Nigg), has been extensively used to study C. trachomatis pathogenesis and immunology in mouse models (32, 37, 38, 42, 43). Although most isolates of C. pneumoniae organisms often infect the human respiratory system, causing respiratory pathologies and exacerbating lesions in the vascular wall (3, 28), the C. pneumoniae N16 strain has been isolated from equines (60). Other chlamydial species that mainly infect animals include C. caviae (50), C. psittaci (47), C. abortus (66), and C. felis (1). Despite the apparent differences in tissue tropism, all chlamydial species share similar genome sequences (1, 29, 49, 50, 66) and possess a common intracellular growth cycle with distinct biphasic stages (24). The chlamydial intracellular infection-induced inflammation is considered a major cause of Chlamydia-induced diseases (58).

A typical chlamydial infection cycle starts with endocytosis of an infectious elementary body (EB) into a host cell, followed by rapid differentiation of the EB into a noninfectious 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 the adjacent cells. Chlamydial organisms accomplish all their biosynthesis, replication, and differentiation within the cytoplasmic vacuole (designated an inclusion). The chlamydial inclusions not only support chlamydial replication but also protect the replicating organisms from host defense mechanisms such as lysosomal fusion (17). At the same time, Chlamydia must communicate with the host cells across the inclusion membrane barriers (53, 62). It is known that Chlamydia both imports nutrients and metabolic intermediates from host cells into the inclusions (25, 62) for maintaining intravacuolar growth and secretes chlamydial genome-encoded factors into either the inclusion membrane (22, 26, 35, 52, 54) or the host cell cytosol (14, 67, 72) for potentially interacting with and/or manipulating host cell signaling pathways. The chlamydial ability to manipulate host cells for promoting chlamydial intracellular survival and intercellular transmission plays a significant role in chlamydial pathogenesis. Therefore, searching for Chlamydia-secreted proteins has been an active area under intensive investigation, which has provided and will continue to provide much-needed information for advancing our understanding of the intricate interplay between chlamydial organisms and host cells.

Interestingly, many chlamydial species/strains contain a 7.5-kb cryptic plasmid (65). For example, the plasmids pCTA, pCTT1, pCHL1, pLVG440, and pLGV2 were identified and sequenced from C. trachomatis serovars A (5), B (57), D (10), L1 (27), and L2 (11), respectively; pMoPn from C. muridarum strain Nigg (49, 65); pCpnE1 from C. pneumoniae strain N16

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, Phone: (210) 567-1169. Fax: (210) 567-0293. E-mail: Zhongyu@UTHSCSA.EDU.

† Published ahead of print on 12 May 2008.
The chlamydial organisms were propagated, purified, aliquoted, and stored as muridarum plasmid in many different chlamydial species and strains such as designated pCT7 or pgp7 (18), and as a result, pgp3 or about the pORF annotation in the literature. For example, the protein encoding and copy number (65). There has been some confusion protecting immunity against chlamydial challenge in a mouse model (9, 13, 23). pORF6 encodes a protein of 101 to 102 amino acids with unknown function, while pORF7 and -8 likely encode proteins that may be involved in regulation of partitioning and copy number (65). There has been some confusion about the pORF annotation in the literature. For example, the pORF downstream of the plasmid origin of replication was also designated pCT7 or pgp7 (18), and as a result, pgp3 or pgCT3 corresponds to pORF5 (65). Regardless of the various pORF designations, the important question is what biological functions the plasmid-encoded proteins have in addition to maintaining plasmid replication. The wide distribution of the plasmid in many different chlamydial species and strains suggests that there is selection pressure for maintaining the plasmids. Do any of the plasmid-encoded proteins participate in chlamydial interactions with host cells? Since Chlamydia restricts its own biosynthetic activity within an inclusion, any chlamydial proteins that are secreted into either the inclusion membrane or the host cell cytosol might be relevant in chlamydial interactions with the host cells. In the current study, we used an anti-fusion protein antibody approach to analyze the locations of the eight plasmid-encoded proteins. We found that pgp3 (encoded by pORF5) was detected mainly in the cytosol of Chlamydia-infected cells, while the other seven proteins were detected inside the chlamydial inclusions only. The pgp3 protein distribution pattern in the host cell cytosol was similar to but not overlapping with that of chlamydial protease/proteasome-like activity factor (CPAF), a chlamydial genome-encoded protease factor known to be secreted from chlamydial inclusions into the host cell cytosol. We further confirmed that the anti-pgp3 labeling was specific to pgp3 produced during chlamydial infection by using both microscopic and protein immunoochemical approaches. Most importantly, purified pgp3 proteins stimulated macrophages to release inflammatory cytokines, suggesting that pgp3 may contribute to the Chlamydia-induced inflammatory pathologies.

MATERIALS AND METHODS

Cell culture and chlamydial infection. HeLa cells (human cervical carcinoma epithelial cells; ATCC catalog no. CCL2) and the following chlamydial organisms were used in the current study: Ch. trachomatis serovars A, D, and L2; C. muridarum strain Nigg; C. psittaci strain 6BC; and C. pneumoniae strain AR39. The chlamydial organisms were propagated, purified, aliquoted, and stored as described previously (6). For infection, HeLa cells grown in either 24-well plates with coverslips or tissue flasks containing Dulbecco modified Eagle medium (GIBCO BRL, Rockville, MD) with 10% fetal calf serum (GIBCO BRL) at 37°C in an incubator supplied with 5% CO2 were inoculated with chlamydial organisms at an multiplicity of infection of 0.5 (or as indicated for individual experiments) as described previously (6). The infected cultures were processed at different time points after infection for either immunofluorescence assays or Western blot analyses as described below.

Chlamydial gene cloning, fusion protein expression, and antibody production. The eight ORFs carried by the pCHL1 plasmid (10) from Ch. trachomatis serovar D organisms were cloned into pGEX vectors (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The forward primers used for cloning of each of the eight pORFs were as follows: pORF1, 5’-CCGGGAATTCT(restriction site)-(overlapping region)-ATGCGGCAGTGTTCCTCCCA-3’, pORF2, 5’-CCGGGATCTATGGTAAATTATAGTAACTGCCA-3’; pORF3, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’; pORF4, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’; pORF5, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’; pORF6, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’; pORF7, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’; and pORF8, 5’-CCGGGATCTATGGGAAATTTGCTTATAATTTTTGTTG-3’.

The cloned ORFs were expressed as fusion proteins with glutathione S-transferase (GST) fused to the N terminus of the chlamydial proteins as previously described (55). Expression of the fusion proteins was induced with isopropylβ-D-thiogalactoside (IPTG) (Invitrogen, Carlsbad, CA), and the fusion proteins were extracted by lysing the bacteria via sonication in Triton X-100 lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 150 mM NaCl). After a high-speed centrifugation to remove debris, the fusion protein-containing supernatants were purified using glutathione-conjugated agarose beads (Pharmacia, Piscataway, NJ) and the purified proteins were used to immobilize mice for producing both polyclonal antibodies (PAs) (72) and monoclonal antibodies (MAbs) (71, 75). The fusion protein-specific antibodies were then used to localize the endogenous proteins in chlamydial organisms-infected cells via an indirect immunofluorescence assay (35, 70, 75). To purify pgp3 for macrophage stimulation experiments, the GST-pgp3 fusion protein was absorbed onto the glutathione beads, and, after thorough washing, the pgp3 protein was cleaved off the beads with a precision protease from Pharmaacia following the manufacturer’s instruction. Since the precision protease was supplied in the form of a GST fusion protein, it bound to the glutathione beads while cleaving off the pgp3. Therefore, only the pgp3 protein was released into the supernatant and collecting using glutathione beads and Centricron (Millipore, Billerica, MA), the protein was ready to use in the macrophage stimulation experiments. As a negative control for the macrophage stimulation experiments, we also similarly cloned, expressed, and purified Cpn0324, a Chlamydia pneumoniae genome-encoded hypothetical protein with a molecular mass of 45 kDa designated LcrE and predicted to be a regulatory molecule for the type III pathway (http://stdgen.northwestern.edu/). In addition, the pCHL1 ORFs 5’- and 8’- and CPAFeI (CPAF from Ch. trachomatis serovar D) 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 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 manufacturer (Invitrogen, Carlsbad, CA). At 24 hours after transfection, the RFP chlamydial fusion proteins were visualized via either the fusion tag RFP or the mouse anti-chlamydial protein antibody labeling. In other experiments, some of the pDsRed-transfected cultures were subsequently infected with chlamydial organisms 12 h after transfection, and the effect of the RFP fusion protein expression on the chlamydial infection was evaluated 30 h after infection. All plasmid clones carrying chlamydial genes used in the current study were confirmed via DNA sequencing using a service from the DNA core at the University of Texas Health Science Center, San Antonio.

Immunofluorescence assay. HeLa cells grown on coverslips were fixed with 2% paraformaldehyde (Sigma, St. Louis, MO) dissolved in phosphate-buffered saline 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 stain (blue; Sigma)
was used to visualize nuclear DNA. A rabbit anti-chlamydial organism antibody (R1L2, raised with C. trachomatis L2 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 immunoglobulin G (IgG) secondary antibody conjugated with Cy2 (green; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to visualize chlamydial inclusions. 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 PAbS made against the pCHL1 ORF GST fusion proteins (current study) and against GST-CT119 (InCa) (data not shown) and MAbS 2H4 (IgG2a) and 4E6 (IgG1) against GST-pORF5 (pgp3) (current study) and 100a against the CPAF C terminus (72). In some cases, the primary antibodies were preabsorbed with either the corresponding or heterologous fusion proteins immobilized onto glutathione-conjugated agarose beads (Phar- macia) prior to staining. The preabsorption 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 containing between pgp3 (MAb2H4, IgG2a) and CPAF (MAb100a, IgG1), a goat anti-mouse IgG2a-Cy3 conjugate (red) in combination with a goat anti-mouse IgG-Cy2 conjugate (green) was used. For the transfected cell samples, the RFP chlamydial fusion proteins were visualized via the fusion tag RFP (red) or by costaining with a mouse antibody. Some RFP-pgp3- and RFP-IncA-transfected cell samples were also subjected to sub- sequent chlamydial infection in order to evaluate the effect of the RFP fusion protein expression on chlamydial infection (22). In these experiments, the fusion proteins were visualized by RFP (red) and the chlamydial organisms were la- beled with a combination of rabbit anti-chlamydial organism antibody and goat anti-rabbit IgG-Cy2 conjugate (green). The chlamydial infection rates were calculated for RFP-positive and -negative cell populations (from the same cov- erslips, respectively).

Cell samples after the appropriate immunolabeling were used for image analysis and acquisition with an Olympus AX-70 fluorescence microscope equipped with various filter sets (Olympus, Melville, NY) as described previously (69, 72). Briefly, the multi-color-labeled samples were subjected to sub- sequent chlamydial infection in order to evaluate the effect of the RFP fusion protein expression on chlamydial infection (22). In these experiments, the fusion proteins were visualized by RFP (red) and the chlamydial organisms were la- beled with a combination of rabbit anti-chlamydial organism antibody and goat anti-rabbit IgG-Cy2 conjugate (green). The chlamydial infection rates were calculated for RFP-positive and -negative cell populations (from the same cov- erslips, respectively).

RESULTS

Production of antibodies against chlamydial plasmid-encoded proteins. To determine the location of the plasmid-encoded proteins during chlamydial infection, we used a fusion protein approach to raise antibodies for all eight plasmid ORFs. Two different annotations of the pORFs are compared in Table 1, and the annotation described by Thomas et al. (65) was used in the current study. The eight pORFs were success- fully expressed as GST fusion proteins (Fig. 1A). Although degradation fragments were always detectable, a significant amount of full-length fusion protein was produced for all eight ORFs. These fusion proteins were used to immunize mice and generate PAbS. The anti-fusion protein antibodies recognized the corresponding fusion proteins without obvious cross-reactivity with heterologous fusion proteins at the antibody concentra- tions used in the Western blot assay (Fig. 1B). Despite the fact that all fusion proteins contained a common GST fusion tag, the anti-fusion protein antibodies failed to recog- nize GST in unrelated fusion proteins when the antibodies were properly diluted. These observations suggest that the fusion protein immunogens predominantly induced antibodies specifically recognizing the corresponding chlamydial plasmid proteins, possibly due to the relatively high immunogenicity of

| TABLE 1. Comparison of two different annotation systems for the putative chlamydial pORFs
| Annotation by Thomas et al. (65) | Annotation by Fahr et al. (18)
| pORF1 | pCT7 or pgp7 |
| pORF2 | pCT8 or pgp8 |
| pORF3 | pCT1 or pgp1 |
| pORF4 | pCT2 or pgp2 |
| pORF5 | pCT3 or pgp3 |
| pORF6 | pCT4 or pgp4 |
| pORF7 | pCT5 or pgp5 |
| pORF8 | pCT6 or pgp6 |

The chlamydial conserved pORFs have been annotated in two different ways. Thomas et al. (65) designated the pORF immediately downstream of the plasmid origin of replication pgp6, while Fahr et al. (18) designated the same pORF pgp7. As a result, pORF5 encodes the pgp3 protein.

http://stdgen.northwestern.edu/.

http://www.chlamydiac.org/docs/biology/genome_plasmid.asp.

http://stdgen.northwestern.edu/.
FIG. 1. Production of antibodies against chlamydial plasmid-encoded proteins. (A) The eight ORFs encoded by pCHL1 from *C. trachomatis* serovar D organisms were expressed as GST fusion proteins, designated GST-pORF1 to 8. The fusion proteins were resolved in an SDS gel and protein bands visualized using Coomassie brilliant blue staining. Although degradation bands were always detected, all samples contained obvious full-length fusion proteins as indicated on the right. The pORF5-encoded protein is also called pgp3. (B) The SDS gel-resolved protein bands (as described for panel A) were blotted onto a nitrocellulose membrane for reaction with mouse sera (each raised with the corresponding GST-pORF fusion protein). The primary-antibody-recognized protein bands were visualized using an enzyme-conjugated secondary antibody and enhanced chemiluminescence. All eight anti-fusion protein antibodies recognized the corresponding full-length fusion proteins without significant cross-reactivity with heterologous fusion proteins. (C) The eight mouse anti-fusion protein antibodies were also reacted with HeLa cells infected with *C. trachomatis* L2 organisms. The mouse antibody bindings were visualized with a Cy3-conjugated goat anti-mouse secondary antibody (red), the chlamydial organisms were visualized with a combination of rabbit anti-chlamydial organisms and a goat anti-rabbit–Cy2 conjugate (green), and the nuclear DNA was visualized with Hoechst DNA dye (blue). It is clear that the anti-GST-pORF5 (pgp3) detected a strong cytosolic signal (panels i and m, arrows), while the other seven antibodies recognized the chlamydial organisms inside the inclusions. (D) The immunostaining was carried out as described for panel C. Both the anti-GST-pORF5 (pgp3) PAb (panels a and f) and MAb (clones 2H4 [panels b and g] and 4E6 [c and h]) detected strong signals in the chlamydia-infected cell cytosol (red) in HeLa cells. The transfected cells were stained with antibodies against pgp3, CPAF, and pORF8 as indicated on the left. All antibodies recognized only HeLa cells expressing the corresponding antigens (yellow) (panels a, d, g, k, and o) without cross-reacting with the heterologous antigens. (F). Antibodies against pgp3, CPAF, or pORF8 as listed on the left were reacted with chlamydia-infected cells as described for panel C with or without preabsorption using GST-pgp3, GST-CPAFct, and GST-pORF8 fusion proteins. The antibody binding to the endogenous chlamydial proteins was removed by preabsorption with the corresponding (panels b, f, j, o, and t) but not the heterologous (panels c, d, g, h, k, l, n, p, r, and s) fusion proteins.
the prokaryotic proteins compared to the common GST tag. We then used these anti-fusion protein antibodies to label the corresponding endogenous proteins in *Chlamydia*-infected cells (Fig. 1C). All antibodies detected specific signals, suggesting that all ORFs carried by the chlamydial cryptic plasmid were expressed during chlamydial infection. More importantly, the anti-GST-ORF5 (also designated pgp3) fusion protein antibody detected a dominant signal in the cytosol of the chlamydia-infected cells (Fig. 1C, panels i and m). We further raised MAbs against the GST-pgp3 fusion protein. Both the anti-GST-pgp3 PAbs and MAbs detected strong signals in the cytosol with a pattern similar to that of anti-CPAF but not anti-IncA antibodies (Fig. 1D). These PAbs and MAbs were further evaluated for their binding specificities. When these and other control antibodies were used to react with cells transfected with the recombinant pDsRed plasmids coding for RFP-pgp3, RFP-CPAFct, or RFP-pORF8, we found that the antibodies recognized only cells expressing the corresponding antigens and not those expressing heterologous antigens, although all antigens were successfully expressed as RFP fusion proteins (Fig. 1E). Furthermore, the ability of these antibodies to recognize endogenous proteins can be blocked only with the corresponding and not with the unrelated GST fusion proteins (Fig. 1F). The above-described experiments together have convincingly demonstrated that the anti-pgp3 antibodies specifically detected cytosolic signals in *Chlamydia*-infected cells.
pgp3 is a unique secretion protein during chlamydial infection. Having verified the antibody binding specificity, we used these antibodies to further confirm the cytosolic localization of pgp3 and to probe the relationship between the chlamydial plasmid-encoded pgp3 and genome-encoded CPAF. The chlamydia-infected cells were fractionated into cytosolic and nuclear fractions and probed for the presence of pgp3 and other control proteins in a Western blot assay (Fig. 2A). Most pgp3 proteins were detected in the cytosolic fraction (HeLa-L2S100) and only a small amount of pgp3 in the nuclear fraction (HeLa-L2pellet) or purified RBs or EBs. The cytosolic fraction was not contaminated by either chlamydial inclusions or chlamydial organisms, since neither CT813 (an inclusion membrane protein) nor MOMP (an abundant organism outer membrane protein) was detected in the cytosol fraction. CT813 was detected only in the whole infected cell lysate and infected cell nuclear fraction, while MOMP was detected in the purified organisms in addition to the inclusion-containing samples. The cell fractionation and Western blot detection results clearly supported the microscopic observation that most pgp3-specific fluorescent signals were in the cytosol of the chlamydia-infected cells, with a small amount present inside the inclusions (Fig. 1C, panels i & m; Fig. 1D, panels a to c and f to h; and Fig. 1F, panels a to l). Interestingly, CPAF was detected only in the cytosolic fraction (Fig. 2A, panel b), which was also consistent with the microscopic observation that the CPAF signal was only found in the cytoplasm of cells containing mature inclusions (Fig. 1D, panels d and i, and 1F, panels m to p). The difference between the distribution patterns of pgp3 and CPAF in chlamydial organism-infected cells may suggest different secretion pathways or functions of the two despite both being in the host cell cytosol. We further analyzed the relationship between cytosolic pgp3 and CPAF using a coimmunoprecipitation assay (Fig. 2B). Although pgp3 appeared to partially overlap with CPAF under a conventional fluorescence microscope (panels a to d), the two rarely overlapped with each other when being carefully examined using confocal microscopy (panels e to h and h1). The lack of association between pgp3 and CPAF was further confirmed using a coimmunoprecipitation assay (data not shown), in which neither pgp3 nor CPAF was coprecipitated by antibodies against the other although both were pulled down by the corresponding antibodies. Together these results have demonstrated that pgp3 is a newly identified secretion protein that is different from the known secretion protein CPAF, suggesting that pgp3 may fulfill a different function from CPAF during chlamydial infection.

Time course expression of pgp3 during chlamydial infection. We first monitored the expression of pgp3 in comparison with that of CPAF, CT813 (Inc), and MOMP in a Western blot assay (Fig. 3A). The pgp3 protein was first detected at 24 h after infection, and the anti-pgp3 PAb detected only pgp3 without cross-reacting with any other proteins from chlamydia-infected whole cell lysates (Fig. 3A, panel a). The three chlamydial genome-encoded proteins CPAF, CT813, and MOMP were also first detected at 24 h postinfection. All chlamydial
proteins, whether encoded by a plasmid or the genome, increased their levels as infection progressed to 36 h and stayed high up to 48 h. When the protein expression was examined using the immunofluorescence assay under the microscope, we found that both pgp3 and CPAF were expressed as early as 12 h after infection (Fig. 3B, panels c [c1 and c2] and h [h1 and h2]), and cytosolic secretion of these two proteins was obvious by 24 h after infection (panels d and i). Once secreted, both proteins stayed in the cytosol for up to 48 h after infection, and some pgp3, but not CPAF, signals remained in the inclusions during the late stages of infection (panels e, f, k, and l).

The pgp3 protein is secreted by all chlamydial species that carry the cryptic plasmid. We first checked whether pgp3 is encoded in all chlamydial plasmids and analyzed homology of the pgp3 amino acid sequences from different plasmids (Table 2). The sequences of pgp3 encoded by plasmids isolated from different strains of *C. trachomatis* are highly conserved, with more than 95% identity. However, the pgp3 sequence identity varies between 50 and 80% among different chlamydial species, with the highest identity (83%) between *C. caviae* and *C. psittaci* and the lowest (43%) between *C. pneumoniae* N16 and *C. suis*. The pgp3 amino acid sequence variation largely parallels with the genome sequence variation between the different chlamydial species, suggesting that the plasmid and pgp3 have adapted to their corresponding chlamydial host strain environments. We then used the antibody against pgp3 from *C. trachomatis* serovar D to probe the expression of pgp3 in various chlamydial strains and species (Fig. 4). In a Western blot,
the anti-pgp3 antibody recognized pgp3 in cells infected with all *C. trachomatis*, *C. muridarum*, and *C. psittaci* organisms, which are known to carry the cryptic plasmid, but failed to detect anything in cells without infection or in cells infected with *C. pneumoniae* human isolate AR39, which is known to lack the cryptic plasmid (Fig. 4A). We then used this antibody to localize pgp3 in cells infected with these chlamydial organisms. pgp3 was detected in the cytosol of HeLa cells infected with the chlamydial serovars and species that express pgp3 (Fig. 4B), suggesting that pgp3 secretion may be maintained by selection pressure imposed by the chlamydial organisms that carry the cryptic plasmid.

**Prior expression of pgp3 in host cell cytoplasm does not affect the subsequent chlamydial infection.** We next evaluated...
the potential effect of pgp3 on chlamydial infection by using a transfection approach (Fig. 5). At 12 hours after transfection with plasmids coding for RFP alone or various RFP fusion proteins, the HeLa cell cultures were infected with chlamydial organisms. At 30 hours after infection, the infection rates for RFP-positive cells (that were positively transfected) and RFP-negative cells (that were not transfected with pDsRed plasmids) from the same cultures were calculated separately. We found no significant difference in the chlamydial infection rates of these two cell populations in either the cultures transfected with the pDsRedC1-pgp3 plasmid or those transfected with the pDsRedC1 vector alone or those transfected with plasmids coding for RFP alone or various RFP fusion proteins, the HeLa cell cultures were infected with chlamydial organisms. However, it is not known whether the chlamydial proteins that are secreted either onto inclusion membranes or into host cell cytosol may participate in the chlamydial interactions with host cells during chlamydial infection.

**Purified pgp3 protein induces inflammatory cytokines in mouse macrophages.** Since the Chlamydia-induced host inflammatory responses chlamydial greatly contribute to pathogenesis, we purified pgp3 protein and assessed the ability of the purified pgp3 protein to induce cytokine production in mouse Raw macrophages (Fig. 6). The pgp3 protein was purified by cleaving off the pgp3 portion from the GST-pgp3 fusion proteins immobilized onto the glutathione-agarose beads (Fig. 6A). We found that the purified pgp3 induced both TNF-α and MIP-2 (interleukin-8) production in the Raw macrophage cultures in a dose-dependent manner (Fig. 6B). Since the pgp3 was purified by cleavage from the agarose bead-immobilized GST-pgp3 fusion proteins, we expected very little contamination by bacterial LPS. This is because LPS that did not stick to the beads was washed away prior to the cleavage, while LPS that did stick to the beads remained on the beads after the cleavage. However, to further exclude the potential contribution of LPS to the pgp3-induced cytokines, we pretreated a parallel set of pgp3 preparations with 10 μg/ml polymixin B, an antibiotic known to bind to LPS and block LPS function, and the polymycin B was maintained in the macrophage culture throughout the experiments. These treatments effectively prevented LPS from inducing cytokines (P < 0.01) but failed to affect the pgp3-induced production of cytokines, including TNF-α (Fig. 6B, panel a) and MIP-2 (Fig. 6B, panel b). A negative control chlamydial protein (prepared in the same way as pgp3) showed no obvious stimulation of the macrophages. We further used heat treatment to exclude the potential LPS contribution. The fact that LPS is heat stable and proteins are generally heat labile has been utilized to distinguish the roles of proteins and LPS in cytokine induction (2). Both pgp3 and LPS samples were boiled for 10 min before being added to the cultures. As expected, the heat treatment completely blocked the TNF-α induction by pgp3 protein (P < 0.01) but had little effect on LPS stimulation of TNF-α. More importantly, the ability of pgp3 to stimulate TNF-α production was removed by depletion with the pgp3-specific MAb 2H4 but not the chlamydial MOMP-specific MAb MC22. The observations described above together have convincingly demonstrated that the purified pgp3 protein specifically induced cytokine production.

**DISCUSSION**

Many chlamydial species carry the 7.5-kb plasmid, suggesting a selection pressure for maintaining the plasmid in the chlamydial organisms. However, it is not known whether the plasmid-encoded proteins contribute to chlamydial pathogenesis. Since chlamydiae restrict their own biosynthesis/replication with plasmid-encoded proteins, we expected very little contamination by bacterial LPS. This is because LPS that did not stick to the beads was washed away prior to the cleavage, while LPS that did stick to the beads remained on the beads after the cleavage.
protein antibody approach, we have analyzed the intracellular locations of the eight ORFs carried by the chlamydial plasmid. We have convincingly demonstrated that the pORF5-encoded pgp3 protein represents a novel secreted protein during chlamydial infection. First, both PAb and MAb raised with the GST-pgp3 fusion protein detected strong fluorescent signals in the cytoplasm of cells containing chlamydial inclusions in an immunofluorescence assay. Second, pgp3 was found highly enriched in the cytosolic fraction when the chlamydia-infected cells were fractionated into cytosol and nuclear/inclusion fractions, which independently confirmed the above immunofluorescence staining results. Third, the anti-pgp3 fusion protein antibody specificity was confirmed using various approaches.

FIG. 4. pgp3 is secreted into host cell cytosol by chlamydial species that carry the chlamydial plasmid. HeLa cells grown on coverslips in 24-well plates were transfected with pDsRed vector alone or the recombinant plasmid pDsRed-IncA or -pgp3, and at 12 h after transfection the cultures were infected with C. trachomatis serovars D, L2, and A (40 h postinfection); C. muridarum (24 h); C. psittaci (30 h); and C. pneumoniae (72 h) were processed for Western blotting (A) or immunofluorescence staining (B) as described in Fig. 3A and 1C, respectively. The anti-GST-pgp3 fusion protein antibody specifically detected the pgp3 protein band in cells infected with the plasmid-containing chlamydial organisms (A, panel a), and this antibody detected significant signals (B, red) in the cytosol of cells infected with chlamydial organisms that express pgp3 (panels a to h, j, and k) but not the C. pneumoniae organisms deficient in pgp3 expression (panels i and l).

FIG. 5. Effect of RFP-pgp3 fusion protein expression on chlamydial infection. HeLa cells grown on coverslips in 24-well plates were transfected with pDsRed vector alone or the recombinant plasmid pDsRed-IncA or -pgp3, and at 30 h after transfection the cell samples were processed for immunostaining with a rabbit anti-chlamydial organism antibody plus a goat anti-rabbit IgG conjugated with Cy2 (green) and a DNA dye (blue). The RFP fusion proteins were visualized via RFP (red). (A) Representative images of the cultures transfected with pDsRed (panel a), pDsRed-IncA (b), or pDsRed-pgp3 (c). (B) Quantitative results, for which 100 RFP-positive cells and 100 RFP-negative cells were counted from each coverslip culture that was transfected with the corresponding plasmids as listed along the x axis. The rates of chlamydial infection in these two different cell populations from the same cultures were calculated separately and displayed along the y axis. The data come from three independent experiments. A statistically significant difference in infection rate ($P < 0.05$, two-tailed t test) between the RFP-positive and -negative cells in the cultures transfected with pDsRedC1-CT119 (IncA) was observed. No other cultures showed any significant differences. Error bars indicate standard deviations.
including cross-staining and preabsorption approaches. For example, although both the anti-pgp3 and anti-CPAF antibodies detected cytosolic signals, they did not cross-react with each other. The recognition of endogenous antigens by the anti-pgp3 and anti-CPAF antibodies was blocked only by the corresponding and not by the heterologous GST fusion proteins. Finally, the intracellular distribution patterns of pgp3 and CPAF were different from each other. At late stages of infection, CPAF was exclusively in the host cell cytosol, while pgp3, although mostly in host cell cytosol, could always be found in the chlamydial inclusions. Even the secreted portion of pgp3 did not overlap with CPAF, although both were detected in the cytoplasm of the infected cells. The lack of overlap between cytosolic pgp3 and CPAF and the intrainclusion retention of pgp3 suggest that pgp3 may follow a different secretion pathway and/or fulfill a unique function in the chlamydial organisms that carry the cryptic plasmid.

The discovery of pgp3 in the infected host cell cytosol was a surprise to us, which is why we went to the extreme in proving the specificity of the pgp3 detection in the current study. pgp3 has been studied for decades. How did the previous investigations fail to detect the secretion of pgp3? There were several differences between the current study and previous studies. First, we have used both mouse antiserum and MAbs, while previous investigators only used PAbs from rabbits to visualize pgp3 in chlamydia-infected cells (8, 20). Second, we used a preabsorption approach to prove the antibody staining specificity, while previous studies failed to do so. Third, we used a much more gentle approach to process samples (paraformaldehyde plus saponin), while previous studies generally used dye staining, while panel b shows the Western blot results probed with a mouse anti-pgp3 polyclonal antibody. The amount of protein per lane for panel b was scaled down to 1/1,000 in order to be able to visualize clear bands in the Western blot. It is worth noting that the Coomassie blue dye visualized the GST precision protease bound to the glutathione on the beads (panel a, lane 6). However, no signal was detected in the same sample with the anti-pgp3 antibody (panel b, lane 6) despite the large amount of GST remaining bound to the beads (panel a, lane 6). (B) The cleaved pgp3 protein fractions were harvested and pooled together for stimulating mouse Raw 246.7 macrophages at final concentrations ranging from 1 to 10 μg/ml as indicated. As a negative control, the chlamydial protein Cpn0324 was similarly prepared and used at 1 and 10 μg/ml. LPS at a final concentration of 10 ng/ml was used as a positive control. A parallel set of pgp3, LPS, and Cpn0324 samples were pretreated with 10 μg/ml of polymyxin B for 30 min at 37°C, and the polymyxin B was maintained in the cultures throughout the incubation. At 24 hours after stimulation, the culture supernatants were harvested for cytokine measurements using ELISA and the concentrations of cytokines were determined. It is clear that both pgp3 and LPS induced significant amounts of TNF-α (panel a) and MIP-2 (panel b). However, polymyxin B inhibited only LPS-induced TNF-α production without affecting the cytokine production induced by pgp3. The data are from three independent experiments. Error bars indicate standard deviations. (C) Parallel samples of pgp3 and LPS were boiled for 10 min (bars 1 to 4) or depleted with the MOMP-specific MAb MC22 (bar 5) or the pgp3-specific MAb 2H4 (bar 6) prior to being added to the macrophage cultures. Note that the heat treatment almost completely blocked TNF-α induction by pgp3 without affecting LPS-induced TNF-α production, and the pgp3-specific MAb significantly depleted the TNF-α stimulation ability of pgp3. The data are from three independent experiments. Error bars indicate standard deviations.
more harsh conditions (methanol). It is likely that both antibody quality and sample processing techniques can contribute to the discrepancy between the current study and previous studies. What is important is that we have convincingly demonstrated that pgp3 is secreted into host cell cytosol by using high-quality reagents and careful technique as well as complementary approaches (microscopy and cell fractionation).

It is not clear how pgp3 is secreted. The chlamydial genome encodes both secretion apparatus components involved in many different secretion pathways and secretion effector proteins with destinations of the organism outer membrane, inclusion lumen, inclusion membrane, and host cell cytosol (59, 72). However, no functional chlamydial secretion pathways have been mapped out due to the lack of genetic tools for manipulating the chlamydial genome. For example, it is not known whether the various type III secretion pathway components encoded by genes scattered along the chlamydial genome (59) can form a functional apparatus. Nevertheless, indirect evidence has shown that the actin polymerization protein Tarp (encoded by C. trachomatis ORF CT456 [7]) and many inclusion membrane proteins (21, 39, 63) may be secreted via the type III secretion pathway. It has also been proposed that an autotransporter mechanism may be used to secrete chlamydial proteins either to the organism outer membrane (12, 30, 68) or into the host cell cytosol (67). Both transporter and passenger domains can be identified in some chlamydial proteins, and the passenger domains can be localized in the predicted secretion destination sites. Finally, the Chlamydia-secreted proteins CPAF (72) and Cpn0797 (14) may use a Sec-dependent pathway to exit the inner membrane, since they both contain a putative gram-negative bacterial signal peptide at their N termini. When the pgp3 amino acid sequence was analyzed for potential functional motifs, no signal peptide was found (http://www.cbs.dtu.dk/services/SignalP/). pgp3, with a molecular mass of 27.8 kDa and a pI of 4.54, is not likely to be secreted via the autotransporter mechanism, since it is too short to bear both the transporter and passenger domains. Efforts to determine whether pgp3 can be secreted via the type III secretion system are under way.

The chlamydial cryptic plasmid was first described a few decades ago (31). A tremendous amount of effort has been made to understand the function of the chlamydial plasmid and plasmid-carried ORFs since then. However, it is still unclear what role the plasmid may play during chlamydial infection. It seems that the contribution of the plasmid to chlamydial infection varies between different species and strains. For example, a plasmid-free C. trachomatis L2 strain behaved the same as the wild type (44), while a C. muridarum strain cured of plasmid displayed significantly reduced infectivity (41). This species/strain variation is also reflected by the fact that none of the human C. pneumoniae (4, 45, 65) isolates analyzed so far have the 7.5-kb cryptic plasmid, but an equine C. pneumoniae strain has. The fact that various chlamydial strains/isolates that are either deficient in the plasmid or carry mutated plasmids have been identified (20, 36, 41, 44, 51, 61) suggests that the plasmid-encoded function can be compensated for by genes/proteins encoded elsewhere. Nevertheless, the plasmid is widely distributed among many different chlamydial species/strains, suggesting that the cryptic plasmid offers certain advantages to the chlamydial organisms that carry the plasmid. This conclusion is further supported by the following observations. First, the plasmid sequence homology largely parallels the chlamydial genome sequence homology (65) (Table 2), suggesting that the plasmid has been selected to adapt to the many different chlamydial hosts and has been successfully maintained by the host organisms. Second, all eight putative ORFs are expressed as protein during chlamydial infection, and pgp3 is expressed as early as CPAF at 12 h postinfection, suggesting a functional need for the plasmid-encoded proteins. Finally, we have found that the pORF5-encoded protein pgp3 is localized in the cytoplasm of the chlamydia-infected cells. Secretion of chlamydial proteins into host cell cytosol may increase the chance for host cells to capture and present chlamydial antigenic information, which may lead to T-cell recognition of the infected cells, potentially endangering chlamydial intracellular survival. Therefore, there must be a functional need for pgp3 to be secreted into the host cell cytosol, which may be to participate in chlamydial manipulation of host cells. The secreted CPAF has been known to degrade a series of host proteins for facilitating chlamydial inclusion expansions (16), blockade of host cell apoptosis (15, 46), and evasion of immune recognition and detection (72–74). Although pgp3 is also secreted into the host cell cytoplasm as early as CPAF at 12 h postinfection, it has a different intracellular distribution pattern, suggesting that it may possess a unique function in the interplay between chlamydial organisms and mammalian host cells.

In our efforts to determine the potential biological functions of pgp3, we first assessed the effect of pgp3 cytoplasmic expression on the subsequent chlamydial infection. We found that pgp3 expression did not affect chlamydial infection, although similar expression of IncA significantly inhibited chlamydial infection under the same experimental conditions. We then evaluated the potential contributions of pgp3 to chlamydial pathogenesis by assessing the ability of the purified pgp3 proteins to stimulate host cells to secrete inflammatory cytokines. The purified pgp3 proteins induced macrophages to secrete inflammatory cytokines in a dose-dependent manner, and LPS did not contribute to the pgp3-induced cytokine production. The above observations together suggest that the secreted pgp3 may not directly alter chlamydial intracellular replication but may participate in chlamydial interactions with host cell signaling pathways, including the activation of host inflammatory genes. This hypothesis is further supported by a recent study that plasmid-free C. muridarum organisms induced significantly less inflammatory pathologies than the wild-type organisms, although both infected mice equally (40). In addition, several chlamydial genome-encoded proteins, including HSP60 (2) and PmpD (68), have also been shown to activate host cells to produce inflammatory cytokines. The finding that multiple Chlamydia-derived proteins, whether encoded by the plasmid or in the genome, can activate host inflammatory genes further supports the generally accepted concept that Chlamydia-induced diseases are largely due to host inflammatory responses triggered and sustained by chlamydial infection (58). We are in the process of further delineating the molecular mechanisms of chlamydial protein-induced inflammation and its roles in chlamydial pathogenesis.
ACKNOWLEDGMENT

This work was supported in part by grants (to G. Zhong) from the National Institutes of Health.

REFERENCES


Editor: J. F. Urban, Jr.