The Chlamydia-Secreted Protease CPAF Promotes Chlamydial Survival in the Mouse Lower Genital Tract

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Despite the extensive in vitro characterization of CPAF (chlamydial protease/proteasome-like activity factor), its role in chlamydial infection and pathogenesis remains unclear. We now report that a Chlamydia trachomatis strain deficient in expression of CPAF (L2-17) is no longer able to establish a successful infection in the mouse lower genital tract following an intravaginal inoculation. The L2-17 organisms were cleared from the mouse lower genital tract within a few days, while a CPAF-sufficient C. trachomatis strain (L2-5) survived in the lower genital tract for more than 3 weeks. However, both the L2-17 and L2-5 organisms maintained robust infection courses that lasted up to 4 weeks when they were directly delivered into the mouse upper genital tract. The CPAF-dependent chlamydial survival in the lower genital tract was confirmed in multiple strains of mice. Thus, we have demonstrated a critical role of CPAF in promoting C. trachomatis survival in the mouse lower genital tracts. It will be interesting to further investigate the mechanisms of the CPAF-dependent chlamydial pathogenicity.

Chlamydia trachomatis is a leading cause of sexually transmitted bacterial infection. Following the initial infection in the lower genital tract, the chlamydial organisms can ascend to the upper genital tracts. The upper genital tract infection may cause pathologies such as hydrosalpinx, leading to severe sequelae, including ectopic pregnancy and tubal factor infertility (1, 2). It remains unknown how C. trachomatis establishes a successful initial infection in the lower genital tract and achieves ascending infection. The mouse genital tract infection model has been used with either C. trachomatis (3–7) or C. muridarum (8–15) for studying chlamydial pathogenesis and immunity. C. trachomatis infection in the mouse genital tract, unlike C. muridarum infection, often fails to induce a long-lasting upper genital tract pathology such as hydrosalpinx. Furthermore, innate immunity alone appeared to be sufficient for controlling the infection (16). Nevertheless, following an intravaginal inoculation, the C. trachomatis organisms were found to survive in the lower genital tract for weeks and invasion of the uterine endometrial epithelia was also detected (3, 5). When C. trachomatis was directly introduced into the mouse endometrial epithelia via a transcervical or intravaginal inoculation (by-passing the cervical barrier), the organisms were able to induce more-robust inflammatory responses and immunity in the genital tract (7, 17). Thus, mouse genital tract infection with C. trachomatis via either intravaginal or intrauterine inoculation has been used for investigating C. trachomatis pathogenesis.

Using the C. trachomatis genital tract infection mouse model, Sturdevant et al. (3) correlated mutations in the hypothetical open reading frame (ORF) CT135 with the survival/infectivity of C. trachomatis serovar D in the mouse genital tracts. However, the mechanisms by which CT135 contributes to the pathogenesis of C. trachomatis serovar D remain unknown. Ramsey et al. (6) also used the mouse model for defining the role of the plasmid-encoded Pgp3 in the survival of C. trachomatis in the mouse genital tract. This finding was validated in a C. muridarum infection mouse model in which a Pgp3-deficient C. muridarum strain was no longer able to induce hydrosalpinx (18). Pgp3 is an immuno-dominant antigen that is secreted into the cytosol of the infected cells (19–21). Many other C. trachomatis proteins, including CT311 (22, 23), CT621/622 (24, 25), CT795 (26), and cHtrA (27) as well as GlgA (28), all encoded by hypothetical ORFs, have also been localized in the cytosol of the infected cells. However, the functions of these proteins are largely unknown.

Another well-characterized chlamydial secretion protein is the serine protease CPAF (chlamydial protease/proteasome-like activity factor). Although CPAF was initially discovered as a consequence of its ability to robustly degrade intracellular proteins of the infected cells (29–31), the extent to which these host targets are degraded during infection is a subject of debate (32–37). CPAF is a unique serine protease (38–42) that is secreted into the host cell cytosol via a secretion-dependent type II secretion pathway (34, 43). We have recently validated the secretion of CPAF into the host cell cytosol (44). To further investigate the role of CPAF in chlamydial pathogenesis, we took advantage of chemically derived C. trachomatis L2 mutants (34, 45, 46) and compared a CPAF-deficient C. trachomatis strain to a control strain derived by lateral gene transfer (34) for survival in the mouse genital tract. We found that the CPAF-deficient C. trachomatis strain lost its ability to establish a successful infection in the mouse lower genital tract following an intravaginal inoculation whereas its ability to infect the upper genital tract was not significantly affected. This result indicates that...
CPAF preferentially promotes *C. trachomatis* survival in the mouse lower genital tract.

**MATERIALS AND METHODS**

**Cell culture and chlamydial organisms.** HeLa cells (human cervical epithelial carcinoma cells) (ATCC CCL2; ATCC, Manassas, VA) were grown and maintained in Dulbecco modified Eagle medium (DMEM; Sigma, Saint Louis, MO) containing 10% fetal calf serum (FBS; Gemini Bio-Products, West Sacramento, CA) in a humidified incubator in the presence of 5% CO₂. The *C. trachomatis* L2 wild-type organisms (strain L2/LGV-434/Bu [designated L2wt]) were purchased from ATCC. The CPAF-deficient *C. trachomatis* L2 organisms (designated L2-17) and the corresponding control CPAF-sufficient strain of L2-5 were provided by R. H. Valdivia (34). The pGFP:SW2 plasmid expressing a wild-type allele of CPAF or mCherry was transformed into L2-17 to produced L2-17/CPAF or L2-17/mCherry, respectively (44). All chlamydial organisms were propagated, purified, divided into aliquots, and stored as described elsewhere (47).

**Mouse infection and live-organism shedding.** Female C57BL/6J (Jackson stock number 000664), C3H/HeJ (000659), and BALB/cJ (000651) mice were all purchased from Jackson Laboratories (Bar Harbor, ME). Mice were subjected to intravaginal or intrauterine inoculation with either 1.0 × 10⁵ or 5.0 × 10⁶ inclusion-forming units (IFUs) of the corresponding chlamydial organisms as indicated in individual experiments. Five days prior to infection, each mouse was injected subcutaneously with 2.5 mg medroxyprogesterone (Depo-Provera; Pharmacia Upjohn, Kalamazoo, MI). To monitor live-organism shedding from the lower genital tract, vaginal/cervical swabs were taken every 2 to 4 days for the first 3 weeks and weekly thereafter until negative shedding results were observed for 2 consecutive time points. The live chlamydial organisms in each swab were quantitated using an immunofluorescence assay as described previously (48). The calculated total number of IFUs per swab was converted into a log₁₀ value and used to calculate the mean and standard deviation or median.

**Genital tract pathology.** To evaluate the genital tract pathology, mice were sacrificed on days 21 to 60 postinfection for collecting genital tract tissues for histological scoring of inflammatory infiltration as described elsewhere (47). The hematoxylin-and-eosin (H&E)-stained sections were scored for inflammatory cell infiltrations by researchers who were blind to the mouse group designations, and the entire genital tract, including vaginal tissue, vaginal lumen, uterine tissue, uterine lumen, oviduct tissue, and oviduct lumen, was evaluated using scoring criteria described previously (10, 50) as follows: 0, no significant infiltration; 1, infiltration at a single focus; 2, infiltration at two to four foci; 3, infiltration at more than four foci; 4, confluent infiltration. Scores from left and right uterine horns or oviducts of the same mouse were added to represent the scores for a given mouse.

**Statistical analyses.** The Wilcoxon rank sum test was used for analyzing both the log₁₀ IFU shedding titers and the pathology scores.

**RESULTS**

CPAF is required for chlamydial survival in the mouse lower genital tract. In the comparisons of L2-17 to L2-5 (34) or L2wt for determinations of survival rates in the lower genital tract following an intravaginal inoculation (Fig. 1), the L2-17 organisms were shown to have rapidly cleared from the lower genital tract with a minimal level of recoverable live organisms on days 3 and 7 (a) whereas L2-5 (b) and L2wt (c) continued to shed significant levels of live organisms for at least 14 days after inoculation. These results suggest that CPAF plays a significant role in promoting chlamydial survival in the mouse lower genital tracts of the mouse. When the genital tract tissues from the infected mice were examined for inflammatory infiltration (Fig. 2), mice intravaginally with L2-17 displayed a minimal level of inflammatory infiltration in the uterine tissues with an inflammatory score significantly lower than those displayed by mice infected with L2-5 or L2wt, indicating that the L2-17 organisms failed to stimulate inflammation in the uterine tissues, which is consistent with the observation that the L2-17 organisms were rapidly cleared in the mouse lower genital tract upon inoculation. It is worth noting that all mice developed an inflammatory infiltration score of ~2 (Fig. 2b) in the oviduct tissue regardless of the organisms used for infection. This universal level may reflect a general background in these mice and does not necessarily indicate tubal infection. It is not clear exactly how all of the tubal tissue sections came to display the elevated levels of inflammatory infiltrates. The similar distribution characteristics of the infiltrates in all mice suggest that the inflammation may not have been caused by the tubal infection since it is very difficult to obtain consistent tubal infection using intravaginal inoculation even with the wild-type L2 organisms.

**CPAF is not important for chlamydial survival in the mouse upper genital tract.** When the L2-17 or L2-5 organisms were directly delivered into the upper genital tract via an intrauterine inoculation (bypassing the cervical barrier), all mice continuously shed live organisms for at least 4 weeks (Fig. 3). Although the infection represented by the overall shedding time course of L2-17 was less robust than that seen with L2-5 following the intrauterine inoculation, the reduction was not significant. Thus, we can conclude that CPAF is a more significant contributor to chlamydial survival in the lower than in the upper genital tract. Consistently, after an intrauterine inoculation, both L2-17 and L2-5 induced robust inflammation in the endometrial tissue (Fig. 4), confirming that CPAF was not required for chlamydial survival and stimulation of inflammation after the organisms were delivered to the endometrial tissue.

The CPAF-dependent chlamydial survival in the lower genital tract is reproduced in multiple mouse strains, and the CPAF deficiency is rescued with a plasmid-encoded CPAF. C57BL/6J
mice are known to be more resistant to chlamydial infection than C3H/HeJ mice (51). C57BL/6J mice tend to develop Th1-dominant adaptive immunity upon chlamydial infection, while BALB/c mice develop Th2-dominant adaptive immunity (52). We then compared the survival rates seen with L2-17 and L2-5 in the lower genital tracts of C57BL/6J, C3H/HeJ, and BALB/c mice (Fig. 5). The L2-17 organisms failed to survive in the lower genital tracts regardless of the mouse strains tested. The organisms were cleared from the mouse lower genital tract within a few days of the inoculation. These observations suggest that the lower genital tract factors responsible for clearing L2-17 infection are shared among different mouse strains. To further validate whether the failure of L2-17 to survive in the mouse lower genital tract is mainly due to lack of CPAF, we complemented L2-17 with a plasmid-encoded wild-type CPAF to produce the transformant L2-17/CPAF. We found that the L2-17/CPAF organisms showed significantly increased survival in the mouse lower genital tract (Fig. 6). As a control, the L2-17 organisms were similarly transformed with a mCherry-expressing plasmid. The L2-17/mCherry organisms remained defective in survival in the mouse lower genital tract.

DISCUSSION

In the current report, we have presented the first experimental evidence demonstrating that CPAF promotes chlamydial survival in the mouse lower genital tract but not in the upper. First, the CPAF-deficient C. trachomatis L2-17 strain failed to infect the mouse lower genital tract whereas the CPAF-sufficient L2-5 control strain was able to cause a 2-week-long infection, indicating that CPAF plays a critical role in promoting C. trachomatis survival in the mouse lower genital tract tissues. Second, the uterine tissue of mice intravaginally inoculated with L2-17 lacked significant inflammatory infiltration whereas the uterine tissue of mice similarly inoculated with L2-5 developed significant inflammatory infiltration, suggesting that L2-17 either failed to reach the uterine tissue or was unable to provoke uterine inflammation. Third, both L2-17 and L2-5 developed robust infections in the mouse genital tracts and induced significant inflammatory infiltration in the uterine tissue when the organisms were directly delivered into the upper genital tract. These observations have confirmed that CPAF is not essential for the C. trachomatis survival and induction of inflammation in the upper genital tracts. The result described above further suggests that lack of uterine inflammation in mice intravaginally infected with L2-17 is probably due to the failure of L2-17 to reach the uterine tissue, since L2-17 is able to activate uterine inflammation when directly delivered to the uterine tissue. Fourth, the requirement for CPAF to aid in chlamydial survival in the mouse lower genital tract was reproduced in 3 different strains of mice despite their different local cytokine profiles and varied adaptive immunity phenotypes (52, 53). Finally, complementation of the L2-17 organisms with a CPAF-expressing plasmid partially restored their
sponding to each mouse were plotted individually along the oviducts (Ov), were semiquantitatively scored for inflammatory infiltration lumen (Lum) and tissue (Tis) of vagina (Va), uterine/uterine horns (Ut), and functions of the genital tract and whether CPAF aids in chlamydial infectivity in the mouse lower genital tract. This mouse model may also allow us to further investigate the mechanisms by which CPAF promotes chlamydial survival in the mouse lower genital tract whereas a similar transformation performed with a mCherry-expressing control plasmid failed to do so. The significant but partial rescue of the chlamydial survival in the mouse lower genital tract was at least partially due to the lack of CPAF.

The previously published controversial findings on CPAF degradation of cellular proteins in cell culture systems (32, 33) motivated us to use the L2-17 strain (34) for evaluating the potential functions of CPAF in the mouse genital tracts, which led us to discover an essential role of CPAF in promoting chlamydial survival in the mouse lower genital tract. This mouse model may also allow us to further investigate the mechanisms by which CPAF aids in chlamydial survival. For example, questions on whether CPAF promotes chlamydial ascension from the lower to the upper genital tract and whether CPAF aids in chlamydial infectivity in different genital tract tissues can now be addressed by simultaneously monitoring the numbers of infectious chlamydial organisms and the numbers of genome copies in multiple genital tract tissues. The next issue is that of how CPAF promotes chlamydial survival/ascension/infectivity. It has been hypothesized that CPAF accumulated in the infected host cell cytosol at the late stage of intracellular chlamydial growth (29) may be released to confront the extracellular mucosal effectors before the intrainclusion organisms are exposed to extracellular environments during host cell lysis and chlamydial spreading (33). The fact that L2-17 was cleared within a few days after inoculation suggests that the innate immunity effectors of the lower genital tract are effective for controlling the infection and that these same effectors may be targeted by CPAF. This hypothesis is consistent with the finding that innate immunity is sufficient for controlling C. trachomatis infection in the mouse genital tract (16). The CPAF-dependent chlamydial survival in the mouse lower genital tract was reproduced in 3 different strains of mice, suggesting that CPAF may target host...
factors in the lower genital tract shared by the different strains. It is unlikely that the different H-2 haplotypes (C57BL/6, H-2b; C3H/HeJ, H-2d; BALB/c, H-2d) and the other strain-specific factors (51–53) are targeted by CPAF. We recently reported that CPAF selectively degraded cathelicidin LL-37 and neutralized its antichlamydial activity (54). It will be interesting to use mice deficient in CRAMP (55), a cathelin-related antimicrobial peptide and the mouse homolog of human LL-37 that is produced in all mouse genital tracts (56), to test whether the CRAMP deficiency can rescue L2-17 in the mouse lower genital tract.

When CPAF was initially discovered, attention was mainly focused on its potential role in dealing with the intracellular proteins of the infected cells (57, 58). Recent studies suggest that CPAF and other chlamydial proteins secreted into the cytoplasm of the infected cells may target extracellular molecules (33, 54). However, caution should be taken in interpreting the interactions of chlamydial factors with host factors detected in in vitro systems. The biological relevance of the in vitro observations has to be validated using mutant chlamydial organisms in animal models as demonstrated in the current study. As generation of chlamydial mutants becomes easier and animal models continue to be optimized, it is expected that more biologically relevant chlamydial pathogenic mechanisms will be uncovered. A caveat is that some of these chlamydial mutants may be highly attenuated in their growth properties, including attachment, entry, intracellular replication, and exit. The reduced survival/infectivity of the mutants in the mouse genital tract may reflect only their slow/inefficient or defective growth abilities. Indeed, L2-17 produced 3-fold-fewer infectious progeny elementary bodies (EBs) in cultured cells than L2-5 (34), which may have contributed to the significantly shortened survival of L2-17 in the mouse lower genital tract. However, when L2-17 and L2-5 were directly delivered to the upper genital tract, both maintained robust infection courses. Although the infection course of L2-17 was less robust than that of L2-5, probably due to the reduced ability of L2-17 to produce EBs (34), the difference was not significant. Thus, CPAF deficiency preferentially reduced chlamydial survival in the mouse lower but not upper genital tract. We hypothesize that in addition to the growth defect, the L2-17 organisms may also be more susceptible to the lower genital tract effectors. A test of this hypothesis is under way.

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