

## PGP3 is a Chlamydial Plasmid-Encoded Virulence Factor

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### Abstract

Sexually transmitted infections with Chlamydia can lead ectopic pregnancy and tubal infertility. However, the precise pathogenic mechanisms remain unclear. The chlamydial cryptic plasmid is known to play a critical role in chlamydial pathogenesis in the genital tract. *C. muridarum* depleted of plasmid or deficient in the plasmid-encoded pGP3 protein was highly attenuated in ascending infection and inducing upper genital tract pathology, demonstrating that the plasmid-encoded pGP3 is a key virulence factor. pGP3 was found to neutralize the antichlamydial activity of the host antimicrobial peptides particularly the cathelicidin peptide LL-37 (human) or CRAMP (mice). This pGP3-mediated neutralization was dependent on Pgp3 to form stable complexes with LL-37. The complex formation was further mapped to the middle domain of pGP3. These observations suggest that Chlamydia may secrete Pgp3 for trapping antimicrobial peptides during chlamydial spreading from cell to cell in the genital tract mucosal tissue. The tertiary structure of pGP3 reveals that pGP3 may also directly participate in the induction of tubal inflammation by activating TNF signaling pathway. Finally, pGP3 was recently reported to be essential for chlamydial colonization in the gastrointestinal tract. Thus, careful analyses of pGP3 functionality may reveal novel information on how Chlamydia interacts with its host and induces pathologies in the upper genital tract.

**Keywords:** Chlamydia muridarum; Virulence factor; pGP3; Antimicrobial peptides; LL-37

### Introduction

*Chlamydia trachomatis* is the most common cause of sexually transmitted bacterial infections worldwide, and women carry the major burden of the infection. Although the symptoms of the sexually transmitted *C. trachomatis* infection can be mild or absent, the infection, if not treated appropriately, can ascend to the upper genital tract to induce inflammatory pathologies such as tubal fibrosis and hydrosalpinx, leading to severe complications like ectopic pregnancy or infertility [1]. Despite the extensive research on chlamydial biology, the pathogenic mechanism of *C. trachomatis* has not yet been elucidated. The species *C. muridarum*, which naturally infects mice, has been extensively used to study the pathogenic mechanisms and immune responses of *C. trachomatis* [2-8]. This is because genital tract infection of mice with *C. muridarum* induces hydrosalpinx [3,9], closely mimicking the tubal pathology induced by *C. trachomatis* in humans.

Most chlamydial organisms contain a highly conserved cryptic plasmid that encodes 8 open reading frames (ORFs), designated pORF1-8 or pGP1-8 [10,11]. The plasmid is thought to play an important role in chlamydial pathogenesis since plasmid-free trachoma serovar A organisms failed to cause pathology in primate ocular tissues [12] and plasmid-free *C. muridarum* organisms were highly attenuated in ascending infection and pathogenicity in mouse upper genital tract [7]. All eight plasmid transcripts [13] are translated into proteins during chlamydial infection [10] and extensive efforts

have been made to define the functions of these proteins. By using deletion and introducing premature termination mutation analyses, it is confirmed that pGP1, 2 and 6 are plasmid maintenance factors [14-16], pGP4 is a positive regulator of genes that are dependent on plasmid for expression [14,15], pGP5 acts as a negative regulator of the same plasmid-dependent genes [16]. Importantly, pGP3 represents a major virulence factor in *C. muridarum* pathogenesis since pGP3 deficiency largely phenocopied plasmid deficiency when infecting animals [17,18].

### PGP3 is essential for chlamydial pathogenicity

The *C. muridarum* murine model-based studies have revealed that both ascending infection and tubal inflammation are required for chlamydial induction of hydrosalpinx in mice [19]. When *C. muridarum* transformants are deficient in pGP3, -4, or -7, they were evaluated for pathogenicity in mice, the transformants deficient in either pGP3 or -4 failed to induce hydrosalpinx in mice following intravaginal infection. The pGP3-deficient *C. muridarum* reduced survival in the mouse lower genital tract and decreased ascending to the mouse upper genital tract, suggesting that pGP3 was essential for *C. muridarum* ascension. Furthermore, pGP3-deficient *C. muridarum*, after directly delivered to the oviduct, was highly attenuated in inducing hydrosalpinx, suggesting that pGP3 may promote chlamydial induction of tubal inflammation [17]. Thus, the plasmid-encoded pGP3 may enhance chlamydial pathogenicity by both promoting ascending infection and inducing tubal inflammation. This conclusion is consistent with the following observations: First, of the plasmid-encoded proteins [10,20], pGP3 was the only secreted protein that accumulated in the host cell cytosol at late stages of chlamydial

infection [10]. Since Chlamydiae complete their biosynthesis inside the inclusion body, any chlamydial proteins that are secreted outside the inclusion may be used by chlamydial organisms as virulence factors for interacting with the infected host. Second, pGP3 is immunogenic in women who have been infected with *C. trachomatis* via either the genital tract [21,22] or the ocular route [23,24], suggesting that Pgp3 is highly accessible to the host immune system. Third, pGP3 formed a stable trimer [25] with a C-terminal trimerization domain similar to the receptor binding domain of tumor necrosis factor alpha (TNF- $\alpha$ ) [26], which suggests that pGP3 may be able to induce tubal inflammatory responses by activating TNFR1 pathway. Finally, pGP3-deficient *C. trachomatis* serovar L2 was also attenuated in infecting the mouse genital tract and inducing inflammatory responses [18]. The question is how pGP3 promotes chlamydial ascension and tubal pathology.

### **Pgp3 may promote chlamydial ascending infection by targeting antimicrobial peptides**

Cationic peptides that possess antimicrobial activities are defined as antimicrobial peptides (AMPs), including human alpha-defensins (HADs) or human neutrophil peptides (HNPs), human beta-defensins (HBDs), and cathelicidin LL-37 [27-30]. LL-37 is a C-terminal 37-amino-acid peptide starting with double leucines (LL) cleaved from human cationic antimicrobial protein 18 (hCAP18) that contains a highly conserved amino-terminal cathelin-like domain and a variable carboxy-terminal domain [27, 29]. The mouse orthologue of LL-37 is called cathelin-related antimicrobial peptide (CRAMP) [31]. HNPs are produced mainly by neutrophils, while HBDs are produced by epithelial cells. Interestingly, LL-37 or CRAMP is produced by both epithelial cells and neutrophils. These extracellular AMPs possess a broad spectrum of antimicrobial activity by inducing pore formation in the bacterial membrane and represent a potent first line of host defense [29,32].

Chlamydial intracellular infection starts with the entry of an infectious elementary body (EB) into an epithelial cell by endocytosis. EB differentiates to a noninfectious but metabolically active reticulate body (RB) in the cytoplasmic inclusion body. After replication, the progeny RBs differentiate back to EBs that exit the infected cells to invade other cells [33]. The spreading process inevitably exposes the progeny EBs to the extracellular mucosal environment where numerous host mucosal effectors, such as AMPs, are available for attacking EBs. The antichlamydial activity of AMPs was supported by two observations. One is in vitro study in which the antichlamydial activity of 9 AMPs was evaluated. The cathelicidin LL-37 or the mouse homologue CRAMP displayed the highest antichlamydial activity, followed by human beta defensin 3 (HBD3) and human neutrophil peptide 2 (HNP2) [34]. Another observation is that MyD88 deficiency significantly increased mouse susceptibility to *C. muridarum* ascending infection [35,36] indicating that the MyD88-dependent AMPs may contribute to the prevention of chlamydial ascension. To ascend to the upper genital tract, the *C. trachomatis* EB organisms must overcome these host defense mechanisms. The chlamydial chromosome-encoded serine protease CPAF that is secreted into host cell cytosol during infection has been shown to degrade LL-37 and neutralize its antichlamydial activity [37].

PGP3 is the only secreted protein among all the 8 chlamydial plasmid encoded ORFs, which is accumulated in the infected cell cytosol at late stages of infection [10]. Upon the lysis of the infected cells, the pre-stored cytosolic Pgp3 may be released into the

extracellular environment prior to the EB releasing, which is consistent with the observation that pGP3 is immunogenic during chlamydial infection in humans and animals [20,21]. The released pGP3 is able to confront the extracellular AMPs and neutralize their antichlamydial activity so that the progeny EBs subsequently released can safely infect the next target cell. Indeed, the antichlamydial activity of the cathelicidin LL-37 or CRAMP was prevented by preincubating LL-37 or CRAMP with pGP3 [34]. The inhibition of the LL-37 antichlamydial activity by pGP3 was confirmed by both monitoring the chlamydial protein synthesis and titrating the live chlamydial organism recovery [34].

### **PGP3 Neutralizes Antichlamydial Activity of Cathelicidin LL-37 by Forming Stable Complexes**

Many bacterial species have been selected to evolve strategies for evading AMPs via releasing soluble factors to trap [38,39] or degrade the antimicrobial peptides [40-43]. CPAF may cleave AMPs while pGP3 may trap AMPs by forming stable complexes. Indeed, in a GST pull-down assay, GST-pGP3 fusion protein precipitated all LL-37 into the pellet fraction while GST alone failed to do so [34]. Most interestingly, the pGP3-LL-37 complexes were highly stable since most of the complexes remained intact after boiling in 2% SDS lysis sample buffer [34]. This stable complex formation was validated in a Biocore-based affinity measurement assay, which showed stable binding even during elution phase [34]. However, the chemical basis of the stable complexes between pGP3 and LL-37 remains unknown. Determining whether the complex formation involves hydrophobic or covalent interactions or both will provide useful information for developing pGP3 into medically useful reagents.

Furthermore, the stable interactions of pGP3 with LL-37 were mapped to the middle domain of pGP3. The pGP3 middle domain (pGP3m) but not pGP3c or pGP3n not only formed SDS/boiling-resistant complexes with LL-37 but also neutralized antichlamydial activity of LL-37 [34]. This finding is consistent with the structural observation that pGP3m forms triple helices that are flexible for interacting with other molecules [26]. The above in vitro observations have led to the conclusion that pGP3 may promote chlamydial ascending infection via pGP3m-mediated neutralization of LL-37/CRAMP.

### **Future Study**

Many innate immune effectors, including the expression of AMPs, are regulated by MyD88-mediated signaling pathways [44]. Chlamydial ascension occurs very rapidly [45,46], it is likely that the major host obstacles for inhibiting chlamydial ascension are regulated by innate pathways such as the MyD88-dependent pathway. This hypothesis is consistent with the observation that mice deficient in MyD88 allowed more severe ascending infection [35,36]. The question is whether MyD88-deficiency can rescue the ascending infection by *C. muridarum*-deficient in pGP3 or pGP3m. If the answer is yes, the next step is to define the MyD88-regulated effectors responsible for preventing *C. muridarum* ascension. The most likely candidate may be CRAMP. If true, mice deficient in CRAMP should also be able to rescue the ascending infection by *C. muridarum*-deficient in pGP3 or pGP3m.

Besides promoting chlamydial ascension in the genital tract, pGP3 is also required for chlamydial colonization in the gastrointestinal (GI) tract [47]. Since chlamydial spreading from the genital to the GI tract

correlates with chlamydial induction of hydrosalpinx in the upper genital tract, it is reasonable to hypothesize that pGP3 may promote chlamydial pathogenicity in the upper genital tract by promoting chlamydial colonization in the GI tract. Thus, investigating how Pgp3 promotes chlamydial colonization in the GI tract is medically relevant although Chlamydia is not a GI tract pathogen.

Since chlamydial colonization in the GI tract alone is not pathogenic in animals [48] and not associated with any pathologies in humans, the question whether chlamydia is a commensal species in the GI tract has been raised. Thus, it is possible that Chlamydia may have acquired the plasmid or pGP3 for improving its fitness in the GI tract. It will be both interesting and medically significant to investigate how pGP3 improves chlamydial colonization in the GI tract.

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