

## CHLAMYDIAL DISEASE PATHOGENESIS The 57-kD Chlamydial Hypersensitivity Antigen Is a Stress Response Protein

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Members of the genus *Chlamydia* are obligate intracellular bacteria that are differentiated from all other prokaryotes by their unique intracellular growth cycle. Two species of *Chlamydia* exist; *C. trachomatis*, strictly a human pathogen, and *C. psittaci*, a pathogen of lower mammals. Chlamydiae primarily infect mucosal epithelia, and in humans *C. trachomatis* causes a formidable group of infections, some of which can progress to severe complications including blindness, infertility, and perhaps arthritis. The most significant of these in the numbers of people afflicted is trachoma, the leading cause of preventable blindness in the world (1).

Although the pathogenic events that lead to development of severe and often debilitating, postinfection sequelae are not known, an immunological mechanism has been suggested (2-5). That hypothesis was based on studies of human trachoma and nonhuman primate models of ocular chlamydial infection. Early studies in humans and in nonhuman primates indicate that prior vaccination with killed chlamydiae frequently results in more severe trachoma upon reinfection (6-10). Moreover, in some individuals with trachoma, chlamydial antigens and DNA are detected in conjunctival tissue in the absence of cultivatable chlamydiae (11, 12). These data support the hypothesis of an immunologically mediated pathogenesis.

*C. trachomatis* infection of nonhuman primates and *C. psittaci* infection of guinea pigs are good model systems for studying chlamydial pathogenesis. Previous studies using those models show that repeated ocular exposure to infectious chlamydiae is necessary to establish the chronic inflammation characteristic of trachoma (13, 14). Interestingly, repeated challenge with infectious chlamydiae results in an atypical infection of shortened duration in which chlamydiae are difficult to reisolate, and severe ocular disease results; thus suggesting that immune responses are partly protective, but also deleterious. Repeated infection produces a submucosal cellular infiltrate of lymphocytes and macrophages (13-15), like that observed in individuals

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with trachoma (16). Collectively, the human and animal studies argue for a pathogenic role of delayed hypersensitivity (DH)<sup>1</sup> in chlamydial disease.

The most direct evidence for DH in pathogenesis of chlamydial disease comes from the observations that a crude extract of viable chlamydiae elicits severe ocular inflammation in immune animals (17, 18). In immune guinea pigs, this extract produces an ocular inflammatory response whose histopathology is consistent with human trachoma and chlamydial-induced tubal infertility (16, 17, 19). Subsequently, we identified and isolated the DH-evoking component, a 57-kD chlamydial protein (20). Those results support the hypothesis that the host's immune response to chlamydial infection is, in part, deleterious. In this study, the chlamydial gene that encodes the 57-kD protein was cloned, and the recombinant protein elicited an ocular DH response in immune guinea pigs. The sequence reveals a close relatedness to the heat-shock or stress proteins GroEL of *Escherichia coli*, HtpB of *Coxiella burnetii*, 65k of *Mycobacterium tuberculosis*, and Hsp60 of *Saccharomyces cerevisiae*.

### Materials and Methods

**Organisms.** The *C. trachomatis* serovars B/TW-5 and L2/LGV-434, and *C. psittaci* strain guinea pig inclusion conjunctivitis (GPIC) were grown in HeLa 229 cells, and elementary bodies were purified by discontinuous density centrifugation in Renografin (E. R. Squibb and Sons, Princeton, NJ) (21). Inclusion-forming units (IFU) were determined by methods described previously (22). *E. coli* strain JM109, and pUC8 and pTZ18R plasmids have been described previously (23, 24; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

**Construction of Genomic Library, Selection, Subcloning, and Sequencing.** *C. psittaci* (GPIC) genomic DNA isolated from  $6 \times 10^{10}$  IFU (25), was partially digested with Sau3A, and sized by electrophoresis on a 0.7% agarose gel. 5-10-kb fragments were electroeluted and ligated into Bam HI-digested, alkaline phosphatase-treated pUC8 (Boehringer Mannheim Biochemicals, Indianapolis, IN) (23). *E. coli* strain JM109 was transformed with the recombinant plasmids (26), grown in Luria broth supplemented with 250  $\mu$ g/ml of carbenicillin, and screened by colony blot (27) using hyperimmune anti-GPIC rabbit serum. An immunoreactive clone, JM109[pGP57], was isolated and analyzed by SDS-PAGE and immunoblotting. Two highly expressed recombinant products, a 57-kD immunoreactive polypeptide and a 20-kD nonimmunoreactive polypeptide, were visualized in Coomassie blue-stained gels of whole cell lysates (see below). The 7.2-kb GPIC insert of pGP57 was restriction mapped (Fig. 1) and shown to hybridize with GPIC DNA by Southern blot analysis. An internal 2.0-kb Eco RI fragment (E1) was subcloned into pTZ18R and shown to produce an immunoreactive polypeptide of 50 kD, presumably a truncated version of the 57-kD protein found in pGP57. A partial sequence of the 2.0-kb chlamydial DNA fragment from the E1 subclone was obtained by the dideoxy-chain termination method using pUC forward and reverse universal primers following the manufacturer's suggested procedures (Sequenase; United States Biochemical Corp., Cleveland, OH). After obtaining a partial DNA sequence from the E1 subclone, sequencing was continued using synthetic oligonucleotide primers (SAM1; Milligen-Bioscience, Inc., San Rafael, CA), and cesium chloride purified pGP57 plasmid DNA.

**SDS-PAGE, Electrophoretic Transfer, and Immunoblotting.** SDS-PAGE was performed using 12.5% polyacrylamide gels as described by Dreyfuss et al. (28), except samples were prepared with Laemmli sample buffer (29). Electrophoretic transfer and processing of immunoblots were done as described previously (30).

**In Vitro Detection of Recombinant Polypeptides.** In vitro transcription-translation of pGP57 was performed according to the manufacturers instructions (Amersham Corp., Arlington Heights, IL). [<sup>35</sup>S]Methionine-labeled products were analyzed by SDS-PAGE and fluorog-

<sup>1</sup> Abbreviations used in this paper: DH, delayed hypersensitivity; IFU, inclusion-forming units; GPIC, guinea pig inclusion conjunctivitis; ORF, open reading frame; TX-100, Triton X-100.

raphy as described previously (30, 31). The *in vitro* transcription-translation mixtures were also subjected to immunoprecipitation with a monospecific anti-57-kD rabbit serum (20) or normal rabbit serum, and analyzed by SDS-PAGE and fluorography (30, 31).

**Antigen Preparation, Purification, and Ocular DH.**  $10^9$  JM109 or JM109[pGP57] were washed three times with saline, resuspended in 10 ml of PBS containing 0.5% Triton X-100 (TX-100) (17), incubated at 37°C for 30 min, and sonicated for 3–5 min. Insoluble material was removed by centrifugation at 100,000 *g* and the 57-kD protein was purified from the soluble extract by immunoaffinity chromatography (20). Briefly, the soluble extract of JM109[pGP57] was passed over an affinity column prepared with monospecific anti-57-kD rabbit serum (20). The column was washed with 10 vol of PBS containing 0.5% Triton X-100 and 0.5 M NaCl. Absorbed antigen was eluted with 3.0 M potassium thiocyanate, dialyzed against PBS, and analyzed by SDS-PAGE and immunoblotting. A single 57-kD polypeptide was seen by Coomassie blue staining, and it reacted with monospecific anti-57-kD serum by immunoblot analysis (data not shown). The ability of these antigen preparations to elicit an ocular DH response was assessed by placing 25  $\mu$ l of antigen preparation ( $\sim$ 2–6  $\mu$ g of protein) onto the lower conjunctival sac of ocular immune guinea pigs (20). The hypersensitivity response was assessed clinically at 24 h, and scored using a scale of 0–4 (20): 0, negative; 1, slight hyperemia and edema of the lower palpebral conjunctivae; 2, hyperemia and edema of the lower palpebral conjunctiva with slight hyperemia of the bulbar conjunctivae; 3, overt hyperemia and edema of the lower palpebral and bulbar conjunctivae; 4, same as 3 with the addition of mucopurulent exudate.

## Results

**Identification and Characterization of Recombinant Clones.** Recombinant colonies were screened by blotting with a polyclonal antiserum to GPIC. One recombinant, JM109[pGP57], expressed two products with apparent molecular masses of 57 kD and 20 kD (Fig. 2 A); the 57-kD species reacted with a polyclonal monospecific anti-57-kD serum by immunoblotting (Fig. 2 B). An *E. coli* polypeptide of the same size was also recognized by this antiserum. To verify that the 20- and 57-kD polypeptides were encoded by the plasmid and were not due to an increased expression of the *E. coli* proteins, *in vitro* transcription-translation was done. The recombinant plasmid, pGP57, encoded two polypeptides of 57 and 20 kD (Fig. 3), and the *in vitro* synthesized 57-kD polypeptide immunoprecipitated with anti-57-kD serum. A recombinant clone expressing polypeptides similar to those of pGP57 has been briefly described elsewhere (32).

**Ocular DH Elicited by the Recombinant 57-kD Protein.** Immune guinea pigs, previously infected with GPIC and recovered, were challenged with a soluble extract of JM109, JM109[pGP57] or the immunoaffinity-purified recombinant 57-kD antigen. Both the soluble extract of JM109[pGP57] and the purified recombinant 57-kD protein elicited an ocular DH response when administered topically to the conjunctivae of immune but not naive guinea pigs (Fig. 4). Severity of inflammation resembled

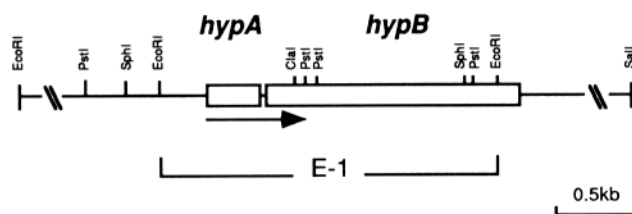


FIGURE 1. Restriction endonuclease map of the 7.2-kb chlamydial DNA insert of pGP57. The internal 2.0-kb Eco RI fragment (E1) is indicated. Open boxes represent the *hypA* and *hypB* ORFs. The direction of transcription is indicated by an arrow.

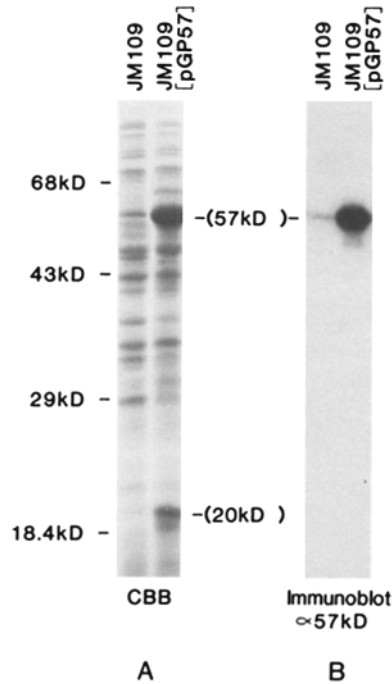


FIGURE 2. SDS-PAGE and immunoblot analysis of *E. coli* strain JM109[pUC12] and recombinant strain JM109[pGP57]. (A) Coomassie brilliant blue-stained gel of whole cell lysates. (B) Immunoblot probed with monospecific polyclonal anti-57-kD serum. Recombinant polypeptides are indicated by approximate  $M_r$  values ( $57 \times 10^3$  and  $20 \times 10^3$ ). Immunological reagents specific for the 20-kD polypeptide were not available, and it was not reactive with hyperimmune anti-GPIC serum.

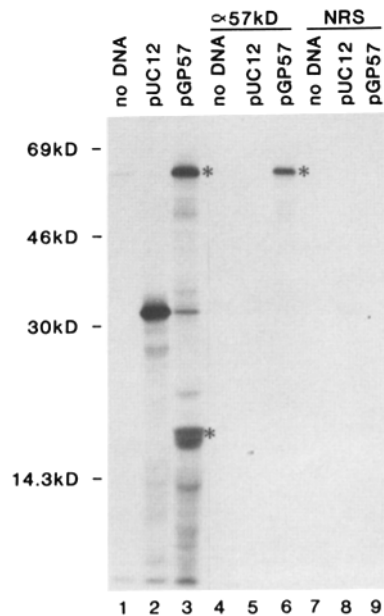


FIGURE 3. In vitro transcription-translation analysis of purified plasmid DNAs and immunoprecipitation of in vitro translated polypeptide. 1  $\mu$ g of purified plasmid DNA was used as suggested by the manufacturer of the commercial in vitro translation kit (Amersham Corp.). Reactions proceeded at 37°C for 45 min, followed by a 5-min chase with nonradiolabeled methionine. Reaction mixtures were subjected to SDS-PAGE and analyzed directly by fluorography (lanes 1, 2, and 3), or immunoprecipitated with polyclonal monospecific anti-57-kD serum (lanes 4, 5, and 6) or normal rabbit serum (lanes 7, 8, and 9), then analyzed. (Lanes 1, 4, and 7) No DNA added to reaction mixture; (lanes 2, 5, and 8) pUC12 DNA; (lanes 3, 6, and 9) pGP57 DNA. Asterisk indicates 57- and 20-kD recombinant polypeptides.

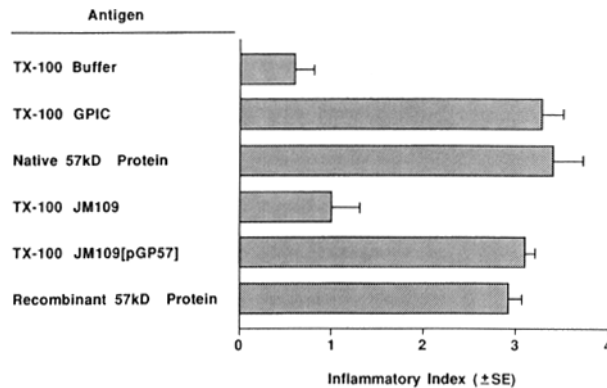


FIGURE 4. Ocular DH response elicited by chlamydial antigen preparations. Ocular hypersensitivity was assessed at 24 h after antigen challenge and scored as described in Materials and Methods. TX-100 buffer, TX-100 GPIC, and native 57-kD protein were prepared as described previously (20). The native and recombinant 57-kD proteins were mixed 1:1 with 2× TX-100 buffer before testing for hypersensitivity. Inflammatory index is the mean response from eight guinea pigs per group. Naive animals were challenged with antigen preparations, and in all instances, the inflammatory index was  $\leq 1$ .

that elicited by a crude extract of *C. psittaci* elementary bodies and immunoaffinity purified native 57-kD protein.

**Sequence Analysis.** A 2.4-kb DNA insert of pGP57 carries two open reading frames (ORF) whose deduced amino acid sequences are presented in Fig. 5. Sequences consistent with Shine-Dalgarno ribosomal binding sites (AGGA) preceded the ATG initiation codons of both ORFs. One ORF spanned 306 nucleotides and encoded a polypeptide of 102 amino acids ( $M_r$  11,202), and the other spanned 1,632 nucleotides to encode a polypeptide of 544 amino acids ( $M_r$  58,088). Because the 57-kD protein has a single known function, its ability to elicit an immunopathological response in primed animals (a DH response), we have termed the whole operon *hyp* (for hypersensitivity); *hypA* encodes the 11.2-kD protein and *hypB* encodes the 58.1-kD protein. The apparent molecular masses of HypA and HypB proteins on denaturing polyacrylamide gels are 20 kD and 57 kD, respectively. The presumptive TAA translational terminator sequence of *hypA* was followed by an intergenic region of 50 bases. The larger ORF, *hypB*, terminated at a TAA stop codon followed by sequences resembling a rho-independent terminator (33).

At nucleotide position -231, sequence like a heat shock promoter (-35 region, T-C-C-CTTGAA, -10 region, CCCCAT-T-) (34) was found. There was considerable sequence agreement for the -10 region, with only a single G for C substitution. The 3' end of the -35 region was in complete agreement, but the 5' half was not conserved. No other upstream consensus promoter regions were found. Although this inferred promoter region has similarities with promoters of genes for other heat-shock proteins, we have been unable to demonstrate a temperature dependent expression of the polypeptides encoded by this recombinant operon in *E. coli*. Expression of the two proteins in bacteria grown at 22°C is high, and may result from the high copy number of pGP57.

Because of the tandem *hypA* and *hypB* ORFs and their striking resemblance to the *E. coli groE* and the *C. burnetii htp* operons, Northern hybridizations were done to determine whether both *hypA* and *hypB* sequences were contained in a single transcript. Oligonucleotide probes complementary to the 5' end of *hypA*, and the 3' end

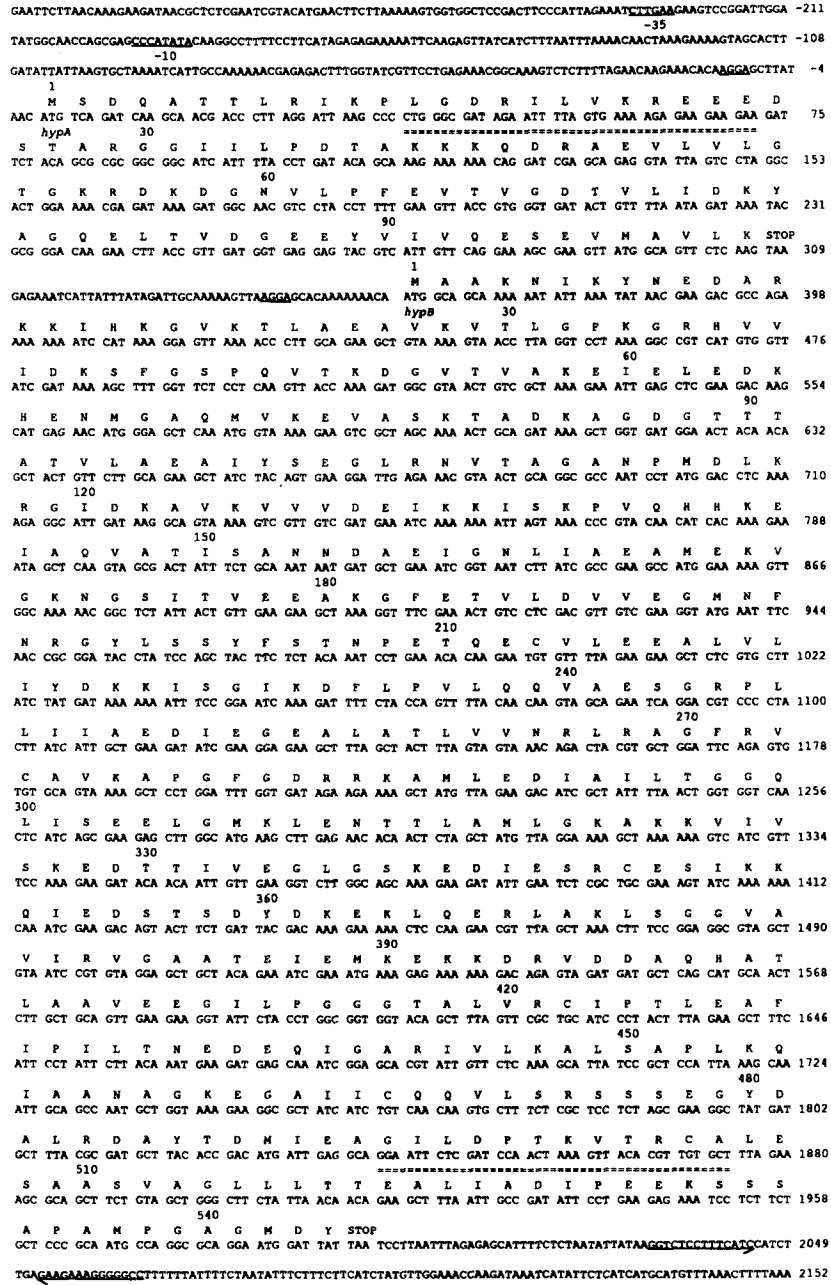


FIGURE 5. Nucleotide sequence of the *C. psittaci* strain GPIC *hyp* operon. The deduced amino acid sequences of the *hypA* and *hypB* ORFs are indicated above the nucleotide sequence. The inferred promoter region (-35 region and -10 region), ribosomal binding sites (single underscore), and the dyad symmetry (arrows) of the proposed transcription terminator are indicated. The sequence corresponding to the oligonucleotides used for Northern blot analysis are indicated (===). Numbers to the right of the figure refer to nucleotide position, and amino acid numbering is above the deduced amino acid sequence. The nucleotide sequence data reported here have been submitted to GenBank and assigned the accession number M25101.

of *hypB* (Fig. 5) revealed that *hypA* and *hypB* are expressed as a single mRNA transcript of ~2,300 nucleotides (data not shown).

**Predicted Amino Acid Sequence Homology.** The amino acid sequence encoded by *hypA* showed identity with HtpA (42%) and GroES (38%) proteins (Fig. 6 A) (35, 36). The HypB protein showed more identity to the HtpB protein of *C. burnetii* (61%) (36), the GroEL protein of *E. coli* (60%) (35), the 65-kD protein of *M. tuberculosis* (58%) (37), and the mature Hsp60 protein of *S. cerevisiae* (53%) (38) (Fig. 6 B). Regions of identity were scattered throughout the sequence. However the NH<sub>2</sub>- and COOH-terminal sequences, and sequences 318 to 361 and 421 to 481 exhibited more divergence and may be determinants of the polypeptide that specify chlamydial-specific epitopes.

Because the 57-kD chlamydial protein showed considerable amino acid identity with the common GroEL antigen of *E. coli*, we examined other prokaryotic organisms by immunoblotting with anti-57-kD serum (Fig. 7). This antiserum reacted with polypeptides of similar *M<sub>r</sub>* in all bacteria examined. These results along with the

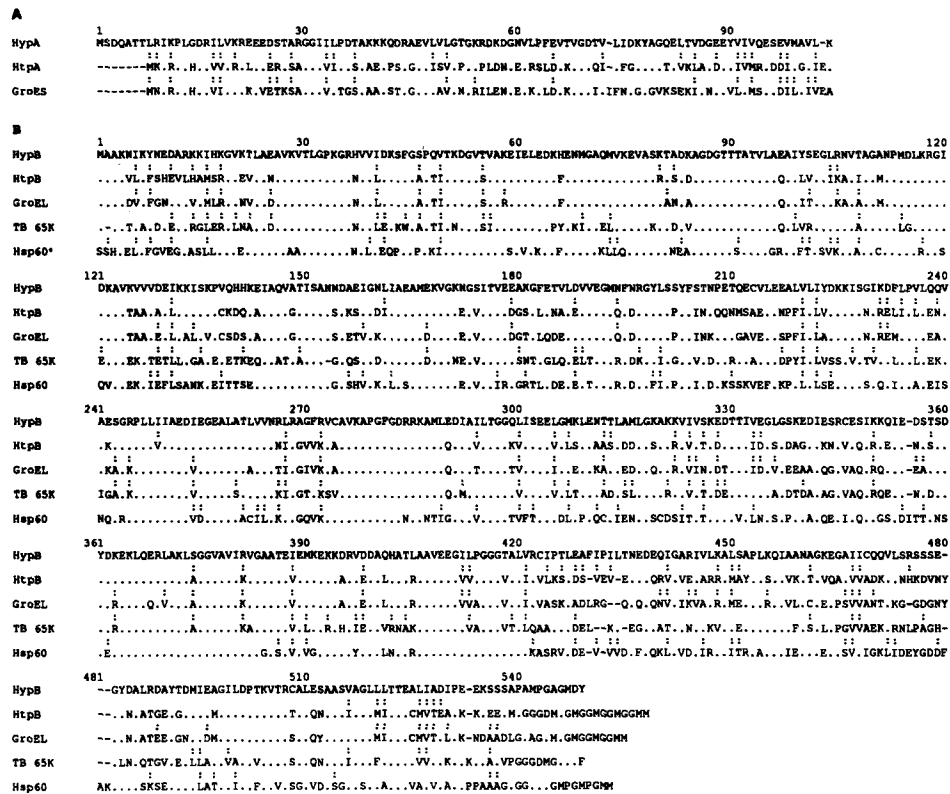


FIGURE 6. Comparison of the deduced amino acid sequences of (A) the *C. psittaci* HypA protein and the *C. burnetii* HtpA and *E. coli* GroES proteins, and (B) the *C. psittaci* HypB protein and the *C. burnetii* HtpB, *E. coli* GroEL, *M. tuberculosis* 65k and *S. cerevisiae* Hsp60 proteins. Identity is represented by a period, conserved amino acid changes by a colon above the amino acid, and gaps introduced for alignment purposes by a hyphen. The Hsp 60 sequence is aligned beginning at amino acid number 22. Amino acid position number does not directly correspond with the amino acid numbers in Fig. 5 because of the inserted gaps needed for alignment.

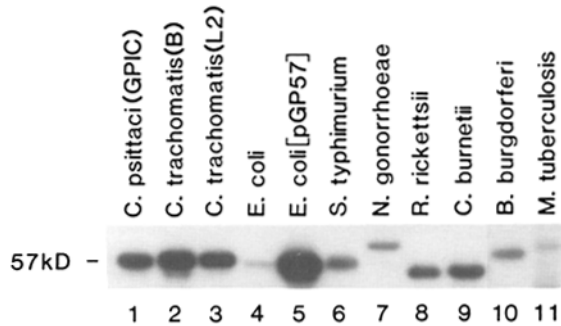


FIGURE 7. Immunoblot analysis of whole-cell lysates probed with monospecific anti-57-kD serum. Each sample consisted of 20  $\mu$ g of total protein. (Lane 1) *C. psittaci* strain GPIC; (lane 2) *C. trachomatis* serovar B; (lane 3) *C. trachomatis* serovar L2; (lane 4) *E. coli* strain JM109; (lane 5) *E. coli* strain JM109[pGP57]; (lane 6) *S. typhimurium* strain SL3261; (lane 7) *N. gonorrhoeae* strain MS11; (lane 8) *R. rickettsii* R strain; (lane 9) *C. burnetii* strain Nine Mile; (lane 10) *B. burgdorferi* strain B31; and (lane 11) *M. tuberculosis* strain H37RA.

amino acid homologies demonstrate that the 57-kD chlamydial protein is a member of the family of widely conserved stress-response proteins referred to as common antigen (39).

### Discussion

The 57-kD chlamydial protein, previously implicated in the pathogenesis of chlamydial disease, belongs to the family of stress-response proteins common to both prokaryotic and eukaryotic organisms. The arrangement of *hypA* and *hypB* in a *groE*-like operon, the identity between the inferred promoter region and known heat-shock promoter sites, and the striking amino acid identity (>50%) between HypB and other known stress response proteins imply that we have identified an analogous operon in *Chlamydia*.

Whether *hypA* and *hypB* expression in *Chlamydia* is responsive to heat-shock or other stresses is not known. The question is not easily tested because of the obligate intracellular growth of *Chlamydia*. The 57-kD protein is the second most abundant protein in chlamydial whole-cell lysates (20), and is expressed throughout the chlamydial growth cycle (our unpublished observations); clearly the conditions of intracellular growth promote its expression at high levels. The intracellular growth environment of *Chlamydia* is regarded as "hostile" (40), and presents both physiological and immunological conditions which may induce a stress response. For example, as obligate intracellular parasites chlamydiae grow in an environment in which they must compete continually with the host cell for nutrients and energy (40). Immunological conditions may exist during chlamydial infection that could also signal a stress-response. For example, IFN- $\gamma$  affects chlamydial growth (41, 42) and might provide a persistent stimulus for a stress response. Thus, conditions exist in the intracellular environment that could provide a persistent stimulus for a stress response and promote elevated expression of the 57-kD protein.

We suggested previously that the host's immune response to the 57-kD chlamydial antigen might be deleterious (20). Inflammation elicited by the 57-kD antigen may damage tissue, with progression to scarring of conjunctival and fallopian tube mucosae, which result in blindness and infertility, respectively. It has been proposed that T cells with specificity for the immunodominant 65-kD stress protein of mycobacteria contribute to protection (43), and that this antigen may have immunoprophylactic potential for a broad spectrum of human pathogens (44). The different effects of immuno-



logical responses (deleterious vs. protective) to highly related proteins from these intracellular prokaryotes may relate to their differences in host cell tropisms. Mycobacteria multiply within macrophages, and protection against mycobacterial disease involves cell-mediated immunity (45) that can be passively transferred with immunologically primed CD4<sup>+</sup> T cells (46). The 65-kD mycobacterial antigen elicits strong DH responses, and 20% of the CD4<sup>+</sup> T cells from mice immunized with *M. tuberculosis* proliferate when cultured with this antigen (43, 47). Proliferating CD4<sup>+</sup> T cells release a number of immunologically active cytokines, including IFN- $\gamma$ , which increases the bactericidal activity of macrophages, and thus might contribute to the control of mycobacterial disease (48, 49).

In contrast, chlamydiae are typically pathogens of mucosal epithelium and do not grow within macrophages. The role of cell-mediated immunity in chlamydial disease is poorly understood. Chlamydial-specific cytotoxic T cells have not been demonstrated, though helper T cells are clearly needed for production of protective antibodies, and cytokines appear to inhibit chlamydial replication (50, 51). Components of the immune response to mycobacterial and chlamydial infection may function differently because of the differing cellular tropism of these intracellular pathogens. For example, IFN- $\gamma$  increases the bactericidal activity of macrophages which inhibits mycobacterial growth. In contrast, IFN- $\gamma$  inhibits chlamydial replication by depleting the essential amino acid tryptophan (52), not by increasing the bactericidal activity of epithelial cells. This inhibits differentiation of chlamydial reticulate bodies into infectious elementary bodies and can result in growth stasis (41, 42). Thus, a stressful intracellular environment may result to influence expression of the 57-kD protein by intracellular chlamydiae. The synthesis and release of the 57-kD antigen from such chronically infected cells might provide a prolonged antigenic stimulation that causes chronic inflammation and immune-mediated disease rather than protection. In this regard, intracellular growth of chlamydiae in the presence of penicillin induces a state of persistent infection, characterized by the development of large aberrant reticulate body inclusions and inhibition of the reticulate body to elementary body differentiation process. These cells release the 57-kD chlamydial antigen, which upon purification elicits an ocular DH response in immune guinea pigs (our unpublished observations). Since IFN- $\gamma$  can induce a similar infectious state (i.e., persistence), it may produce an analogous situation in vivo with persistent infection and release of the 57-kD hypersensitivity antigen.

In human disease, inflammatory damage to the conjunctival epithelium occasionally leads to conjunctival and corneal scarring, and blindness. Inflammatory damage and scarring of the fallopian tube mucosa results in tubal blockage and infertility. We believe that the inflammation observed in these disease conditions is a consequence of a chronic hypersensitivity response to the 57-kD protein. In support of this hypothesis, we have found that the cellular infiltrate of the local inflammatory response elicited by the 57-kD protein is consistent with that observed in the conjunctivae of individuals with trachoma and in the fallopian tubes of women rendered infertile as the result of chlamydial-induced salpingitis (16, 19, 20).

The conserved nature of the 57-kD protein among prokaryotic and eukaryotic organisms presents a situation in which serious consequences may develop as a result of the stimulation of an inappropriate immune response. Repeated stimulation of the immune response through re-exposure to highly conserved epitopes may pro-

voke an autoimmune response. It has been proposed that the 65-kD mycobacterial antigen is involved in the development of an autoimmune arthritis (53), and a similar mechanism may function in certain chlamydial disease conditions, such as Reiter's disease.

An intriguing relationship exists between the chlamydial homologue of the ~60-kD stress-response proteins and the pathogenesis of chlamydial disease. Indeed, more detailed studies of the host's immune response to antigenic determinants of the 57-kD protein are essential to further our understanding of the relationship of this protein to chlamydial disease pathogenesis and immunity.

### Summary

*Chlamydia trachomatis* infection of humans is commonly a localized inflammation that can result in infertility, blindness, and perhaps arthritis. The pathogenic process(es) that cause these sequelae are thought to be immunological. A 57-kD protein that is common among *Chlamydia* elicits ocular inflammation when introduced onto the conjunctivae of guinea pigs or nonhuman primates previously sensitized by chlamydial infection. This protein is thought to mediate the immunopathology that follows chlamydial infection. To more thoroughly characterize this chlamydial component, we cloned its gene from a *C. psittaci* strain and identified a particular recombinant that produced the 57-kD polypeptide. The recombinant gene product was immunoreactive with a monospecific anti-57-kD serum, and elicited an ocular inflammation similar to that produced by the 57-kD antigen isolated from chlamydiae. Sequencing identified two ORFs that encode polypeptides of 11.2 and 58.1 kD and are co-transcribed. These two polypeptides show homology with *Escherichia coli* *groE* and *Coxiella burnetii* *htp* heat-shock proteins. Striking homology (>50%) was found between the 57-kD protein and the HtpB, GroEL, 65-k *Mycobacterium tuberculosis* and Hsp60 proteins. Thus, the 57-kD chlamydial protein, previously implicated as mediating a deleterious immunologic response to chlamydial infections, is a stress-induced protein similar to those that occur universally in both prokaryotic and eukaryotic organisms.

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