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Use of Monoclonal Antibodies To Facilitate Identification, Cloning, and Purification of *Chlamydia trachomatis* hsp10

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As a requisite for a physiological and immunological investigation, reagents were developed that facilitated the identification and purification of *Chlamydia trachomatis* hsp10 (chsp10). Monoclonal antibodies that specifically recognize chsp10 were generated with multiple-antigen peptides (MAPs) to promote recognition of *Chlamydia*-specific epitopes. MAP2, containing amino acids 54 to 69 of the hsp10 sequence, elicited strong antibody responses after immunization of BALB/c mice. Monoclonal antibodies from several cloned hybridomas reacted on immunoblots with an approximately 15-kDa chlamydial protein and recombinant chsp10. Because of its strict specificity for chsp10, monoclonal antibody M1.2 was selected for routine use. M1.2 reacted by immunoblot with the hsp10s of several *C. trachomatis* strains but not with *Chlamydia psittaci* hsp10 or *Escherichia coli* homolog GroES, suggesting that M1.2 recognizes a species-specific epitope. Recombinant chsp10 was purified by immunoaffinity chromatography with M1.2. For large-scale purification, chsp10 was appended with a C-terminal six-histidine tag for purification by nickel chelate affinity chromatography. The *hypA* gene encoding the chsp10 of *C. trachomatis* serovar E/Bour was cloned into the pQE-60 vector (QIAGEN, Inc.) following PCR amplification from genomic DNA. *E. coli* DH5 transformants were screened for chsp10 expression by colony immunoblotting with M1.2, were tested for nickel matrix binding, and were sequenced. The sequence of serovar E/Bour chsp10 was found to be closely homologous to those of hsp10s of other chlamydiae. Purified chsp10 and specific anti-chsp10 monoclonal antibodies will be useful for investigating the biological and immunological roles of hsp10 in chlamydial infections.

Heat shock proteins are ubiquitous in nature and perform necessary cellular tasks such as protein assembly and folding and prevention of protein aggregation (for reviews, see references 18 and 24). They are also prominent antigens of several microorganisms and have been associated with both protective and immunopathological responses (29, 32). Following *Chlamydia trachomatis* infection, prominent immune responses to the 60-kDa heat shock protein (chsp60) and 70-kDa heat shock protein (chsp70) have been documented (4, 12, 13, 16). Furthermore, chsp60 has been implicated as an inducer of delayed hypersensitivity responses (34, 35, 38), and exposure to chsp60 has been associated with conditions of immunopathological sequelae (2, 41, 43, 50–53). Thus, an analysis of heat shock proteins of *Chlamydia* is of physiological and immunological importance.

Chlamydia reticulate bodies show induction of several proteins, including chsp60 and chsp70, when exposed to heat shock (20). It has also been shown that chlamydiae undergo a stress response under conditions of nutrient starvation (7, 17). When the essential amino acid tryptophan is omitted from in vitro growth media, chlamydiae respond with an induction of heat shock proteins (6–8). On stimulation with gamma interferon, human cells catabolize tryptophan via the inducible enzyme indoleamine 2,3-dioxygenase (49). In an in vitro model of persistent infection (8), chlamydiae within gamma interferon-stimulated host cells undergo morphologic and biochemical changes characteristic of a stress response (20). Several proteins are induced, including chsp60 and chsp70, while levels of outer membrane components such as the major outer membrane protein and lipopolysaccharide are reduced (8, 10). This stress response is believed to interrupt the normal progression of reticulate bodies to infectious elementary bodies (EBs), thus resulting in a longer-term persistent infection. Furthermore, persistent infections may serve as antigenic reservoirs for potentially immunopathogenic anti-hsp immune responses (9).

Homologs of *Escherichia coli* GroES make up a closely related family of heat shock proteins of approximately 10 kDa (hsp10s) that are vital to cellular function (24, 37). Known as molecular cochaperones, hsp10s interact with members of the hsp60 family (GroEL in *E. coli*) to promote the proper folding of nascent polypeptides. Typically found arranged in operons with hsp60s, these proteins are superinduced by stressful stimuli and are thought to protect cellular proteins from the effects of stress.

The difficulty of obtaining specific reagents to identify chlamydial hsp10 (chsp10) has precluded an extensive investigation of its role in the chlamydial stress response. Still, much interest in the study of chsp10 has arisen from its genetic linkage (34, 36) and potential to associate with chsp60, a reported immunopathological antigen (34, 35, 38). Although Morrison et al. (34, 36) determined that chsp10 and chsp60 are coexpressed, it is unknown what contribution chsp10 may make to the immune response. As a requisite for a physiological and immunological investigation of chsp10, reagents were developed to facilitate the identification and purification of this protein.

MATERIALS AND METHODS

Cell lines, bacteria, and growth media. The HeLa cell line and Sp2/0-Ag14 myeloma cells were obtained from the American Type Culture Collection. HeLa cells were maintained in minimal essential medium with 10% fetal bovine serum (FBS) containing 50 μg of vancomycin/ml and 10 μg of gentamicin/ml. The A57-B9 hybridoma (55), which produces anti-chsp60 monoclonal antibodies, was a kind gift from Richard P. Morrison (University of Alabama-Birmingham). Sp2/0-Ag14 cells and hybridomas were grown in Dulbecco's modified Eagle

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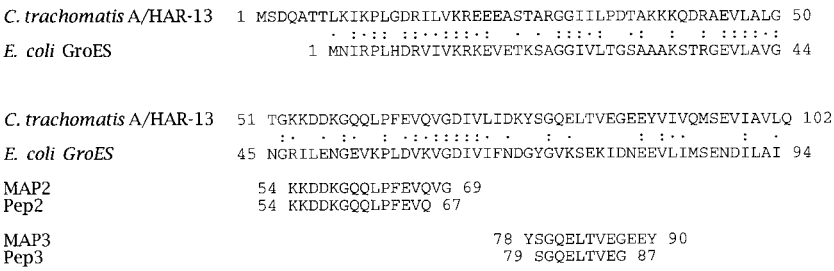

FIG. 1. Peptides used in this study. To promote monoclonal antibody recognition of *Chlamydia*-specific epitopes within chsp10, four-branch MAPs containing divergent regions of chsp10 were synthesized and used as immunogens. The amino acid sequence of *C. trachomatis* serovar A/HAR-13 hsp10 (36) is shown, indicating positions of MAPs and linear peptides used in monoclonal antibody development. The amino acid sequence of *E. coli* GroES (25) has been aligned to chsp10 with the GenBank BLAST program to illustrate regions of sequence diversity (1). Identical residues are indicated with a colon, and conservative substitutions are indicated with a period.

medium with 10% FBS containing 50 μg of vancomycin/ml, 50 μg of gentamicin/ml, 50 μM 2-mercaptoethanol, and 1× oxaloacetate-pyruvate-insulin (OPI) supplement (23). During the initial cloning and expansion of the hybridomas, Dulbecco's modified Eagle medium was supplemented with 20% FBS and 100 to 200 U of recombinant human interleukin 6 (IL-6) (BioScience International, Camarillo, Calif., and Collaborative Biomedical Products, Bedford, Mass.) per ml in lieu of feeder cells.

E. coli JM109 and DH5 were routinely grown on Luria-Bertani (LB) agar plates or in LB broth cultures (42). Recombinant *E. coli* JM109/pTA571 (36), a strain that expresses chsp10 and chsp60, was a kind gift from Richard P. Morrison. Recombinant chsp10 (rhsp10) expressed by *E. coli* JM109/pTA571 is a fusion protein of chsp10 with the 12-amino-acid vector-derived alpha peptide and lacks the first two amino acids of the chsp10 sequence. It has an observed molecular mass of approximately 18 kDa. All recombinant strains used in this study were maintained on LB media supplemented with 100 to 200 μg of ampicillin per ml.

C. trachomatis serovars A/HAR-13, B/TW-5, and E/Bour and mouse pneumonitis (strain MoPn) were received from the Rocky Mountain Laboratories of the National Institutes of Health, Hamilton, Mont.; serovar C/TW-3 was received from Peter Rapoza (University of Wisconsin); serovar E/UW-5 was received from Priscilla Wyrick (University of North Carolina); and serovar F/UW-6 was received from Indiana University. *C. trachomatis* serovar L2/434 and *Chlamydia psittaci* GPIC and 6BC were originally obtained from the University of Chicago.

Peptides and MAPs. To promote monoclonal antibody recognition of *Chlamydia*-specific epitopes within chsp10, four-branch multiple-antigen peptides (MAPs) (46, 47) were used as immunogens (Fig. 1). Two MAPs were synthesized by the University of Wisconsin Peptide Synthesis Center, each incorporating a unique peptide region of chsp10. MAP2 contained peptide KKDDKGQQLPF EVQVG corresponding to amino acids 54 to 69 of the serovar A/HAR-13 chsp10 sequence (36). MAP3 contained peptide YSGQELTVEGEEY corresponding to amino acids 78 to 90 of chsp10. Slightly shorter linear peptide forms of MAP2 (Pep2; KKDDKGQQLPFEVQ) and MAP3 (Pep3; SGQELTVEG) were synthesized for use in enzyme-linked immunosorbent assays (ELISAs).

Hybridoma development. BALB/c mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) and maintained in the University of Wisconsin Animal Care Facility. Mice were immunized intraperitoneally with 50 μg of MAP per 200-μl injection, with or without RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, Mont.) in sterile phosphate-buffered saline (PBS). Immunized mice received a total of six immunizations with a single MAP at regular intervals. Control mice were immunized with PBS only. Sera were taken by retro-orbital bleeding to evaluate anti-chsp10 responses by ELISA against the immunizing MAP and by immunoblot reactivity to chlamydiae.

Three days after final boosting without adjuvant, mice were sacrificed and spleens were removed. Splenocytes were fused with Sp2/0-Ag14 myelomas and expanded in the presence of IL-6 (54). Supernatants from resulting hybridomas were screened for potential anti-chsp10 activity by ELISA against the immunizing MAP and formalin-fixed chlamydial EBs. Hybridomas were cloned at least twice by limiting dilution (54). Final candidate hybridomas were evaluated by immunoblotting for anti-chsp10 and anti-MAP reactivity and by immunofluorescent and immunochemical staining of chlamydial inclusions within infected HeLa cells.

Antipeptide ELISA screening. ELISA protocols were based on standard methods (27). Amine-binding *n*-oxysuccinimide plates (Corning Costar Corp., Cambridge, Mass.) were coated with 1 μg of either MAP, linear-form peptide, or formalin-fixed serovar A/HAR-13 EBs per well in carbonate buffer, pH 9.6. Wells were blocked overnight with PBS–2% bovine serum albumin (BSA)–0.05% Tween 20 and then incubated for 1 to 2 h at 37°C with 100 μl of hybridoma supernatants per well. Plates were washed with PBS–0.05% Tween 20 and incubated with alkaline phosphatase-conjugated antimouse immunoglobulin (Ig) secondary antiserum. Plates were washed with Tris-buffered saline, pH 7.2. *p*-Nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, Mo.) was added to the wells, and the ensuing color reaction was read at 410 nm in a Dynatech 9000 ELISA plate reader. A positive reaction was required to be >500% of background values.

Isotyping ELISAs were similarly conducted with plates coated with MAPs and were confirmed with plates coated with formalin-fixed EBs. Isotype-specific alkaline phosphatase-conjugated secondary antisera were obtained from Southern Biotechnology, Inc.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with a Hoefer Scientific SE600 apparatus, as previously described (8). For gels to be silver stained, the Silver Plus silver staining kit (Bio-Rad Laboratories, Hercules, Calif.) was used according to the manufacturer's instructions. Gels to be Coomassie blue stained were fixed in 50% methanol–10% acetic acid, stained with 0.005% Coomassie brilliant blue G-250 in 10% acetic acid, and destained in 10% acetic acid. After staining, gels were transferred to a gel-drying solution (20% ethanol–8% glycerol) and dried between two sheets of cellophane with a gel-drying frame (NOVEX, San Diego, Calif.).

Immunoblot procedures were based on those previously described (8, 22), with the following modifications. Gels were transferred to 0.2-μm-pore-size nitrocellulose (Micron Separations, Inc., Westborough, Mass.) in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) with a Bio-Rad Trans-Blot electrophoretic transfer apparatus, according to the manufacturer's instructions. Blots were blocked before probing with 5% nonfat dried milk in PBS–0.05% Tween 20 for 1 h, and antibodies were diluted into PBS–2% BSA–0.05% Tween 20 for use. Goat anti-mouse IgG (γ-chain specific)–horseradish peroxidase-conjugated antiserum (Sigma), or sheep anti-rabbit IgG–horseradish peroxidase-conjugated antiserum (Amersham Corp., Arlington Heights, Ill.) was used as a secondary reagent where appropriate. Immunoblots were developed by enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations and exposed to film (HyperFilm; Amersham). As controls for purity analysis immunoblots, purified *E. coli* GroES and rabbit anti-GroES antiserum were purchased from Sigma.

Dried gels and immunoblot films were scanned for publication with an Apple Power Macintosh computer equipped with an Apple Color One scanner 600/27. Images were processed with Photoshop 3.0 software (Adobe Systems, Inc., Mountain View, Calif.) and further arranged with Canvas 3.5 software (Deneba Software, Miami, Fla.). Dye sublimation prints of images were printed with a Seiko dye sublimation printer.

Immunoaffinity purification of rhsp10. An immunoaffinity column was constructed for chsp10 purification (44). Briefly, anti-chsp10 monoclonal antibody M1.2 (see below) was chemically cross-linked to a protein A-Sepharose 6B support with dimethyl pimelimidate (Sigma). A PBS–1% *n*-octyl-β-D-glucopyranoside (OGP) extract of *E. coli* JM109/pTA571 (36) was applied to the column, washed with PBS–1% OGP, and eluted with 0.1 M glycine, pH 2.5. Eluted fractions were analyzed by SDS-PAGE and immunoblotting. Peak fractions were pooled and dialyzed into PBS with SpectroPor#1 6 to 8 kDa MWCO dialysis tubing (Spectrum Medical Industries, Inc., Houston, Tex.).

Vector and cloning strategy. The pQE-60 vector (QIAGEN, Inc., Chatsworth, Calif.) was chosen to append a tag of six histidines (His_6) to the C terminus of chsp10 for large-scale purification via nickel chelate affinity chromatography (40). Since it was desirable to clone the gene encoding chsp10, *hypA*, with no alterations to the open reading frame sequence, a strategy of cloning into the pQE-60 *Eco*RI and *Bgl*II sites was chosen (Fig. 2). While maintaining the vector's T5 promoter/Lac operator regulatory region, this strategy allowed ribosome binding and translation initiation at the native chlamydial sites. At the C terminus, the native stop codon was replaced in frame with the *Bgl*II site, and this allowed splicing of the His_6 tag directly to the tail of the protein with minimal extra amino acids.

Although the gene encoding chsp10 has been sequenced as part of the *hypAB* operon from other chlamydial strains (13, 20, 26, 30, 34, 36), there are no examples of hsp10 from serovars associated with genital tract infections (e.g.,

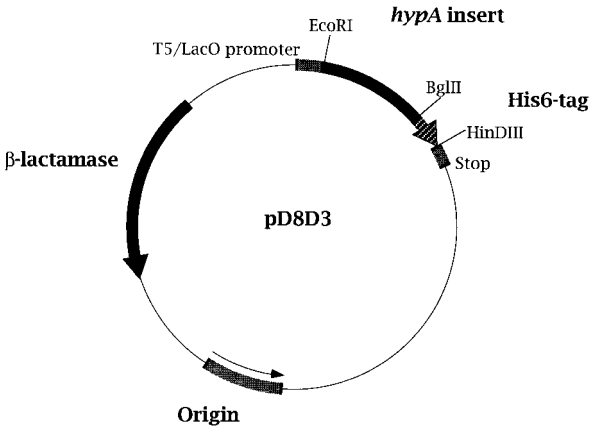

FIG. 2. Features of the recombinant plasmid pD8D3 encoding rhsp10-His6 from serovar E/Bour. A PCR-amplified insert containing the *hypA* gene from *C. trachomatis* serovar E/Bour was spliced into the pQE-60 vector between the *Eco*RI and *Bgl*II sites such that a His_6 tag was appended in frame with the C terminus of chsp10. Arrows indicate the direction of gene transcription.

cervicitis and pelvic inflammatory disease). Because an eventual use of the purified protein is to evaluate immune responses to chsp10 in patients with sexually transmitted diseases, the *hypA* gene was cloned from the reference strain of serovar E, strain Bour since serovar E infections are highly prevalent in the population.

Primers and PCR cloning. PCR primers were designed to amplify the *hypA* gene by using the Amplify 1.2 software program for the Macintosh computer developed by William Engels (University of Wisconsin-Madison). Primers were based on sequences flanking the serovar A/HAR-13 *hypA* sequence (36) and contained modifications to add appropriate internal restriction enzyme sites for cloning. Primers used were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). The 5′ end primer, incorporating an *Eco*RI site, was GGCC TCGTAGAATTCAAAAATAGGAGGAGC. The 3′ end primer, incorporating a *Bgl*II site, was CTCTCTTAGAGATCTTTGCAGAACTGCG.

Genomic DNA was isolated and purified from *C. trachomatis* serovar E/Bour EBs with the QIAamp DNA isolation kit (QIAGEN), according to the manufacturer's instructions. PCR was conducted with AmpliWax PCR-GEMS (Perkin-Elmer, Foster City, Calif.) under conditions specified by the manufacturer (39). Forty rounds of amplification were conducted with a Techne PHC-3 thermocycler. Products were separated on agarose gels, stained with ethidium bromide, and photographed (42). An amplified product of the expected size (359 bp) was excised from a preparatory agarose gel and purified with the QIAquick spin column purification protocol (QIAGEN).

Both amplified insert and vector were cut with *Eco*RI and *Bgl*II enzymes (Promega Corp., Madison, Wis.), ligated with T4 ligase (Promega), and transformed into competent *E. coli* DH5 by $CaCl_2$ treatment and heat shock (42). Transformants were selected with ampicillin, and recombinant His_6-tagged chsp10 (rhsp10-His6) producers were screened by colony immunoblotting. Briefly, nitrocellulose disks were soaked in PBS–50 mM isopropylthio-β-D-galactoside (IPTG), overlaid on the transforming plate, and allowed to incubate for 5 h. The positions of the disks were marked, and the disks were removed from the plates and washed in Towbin transfer buffer. Disks were then blocked with 5% nonfat dried milk–PBS–0.05% Tween 20 and incubated with monoclonal antibody M1.2 (see below). Disks were processed further as immunoblots and developed by enhanced chemiluminescence. Plasmid DNA was purified from several reactive colonies with a QIAquick miniprep kit (QIAGEN), was cut with the appropriate restriction enzymes, and was confirmed to carry an insert of the predicted size (343 bp).

Transformant finalists were screened by immunoblotting for rhsp10-His6 expression. In addition, cell extracts were tested for rhsp10-His6 binding to nickel-nitrilotriacetic acid (Ni-NTA) agarose matrix (QIAGEN) to confirm in-frame splicing of the His_6 tag and to evaluate suitability for purifications. Briefly, transformants were dissolved in 8 M urea–0.01 M Tris–0.1 M sodium phosphate, pH 8 (buffer B), and a small amount of rhsp10-His6 was purified using Ni-NTA spin columns (QIAGEN) according to the manufacturer's instructions (40). The product was evaluated by SDS-PAGE and immunoblotting.

Sequencing. Plasmids were isolated and purified with a QIAquick miniprep kit (QIAGEN). The insert region was sequenced on both strands from outside the restriction sites. Sequencing reaction primers used were GTGAGCGGATAAC AATTTCACAC and CATTACTGGATCTATCAACAGG. DNA sequencing was performed at the University of Wisconsin Biotechnology Center's Nucleic Acid and Protein Facility. Cycle sequencing reactions were performed with the Applied Biosystems, Inc. (Foster City, Calif.) AmpliTaq DNA polymerase, an FS Dye Terminator Ready Reactions kit and a Perkin-Elmer thermocycler model 9600. The reaction products were cleaned up with MicroSpin G-50 columns (Pharmacia Biotech) and analyzed on an Applied Biosystems, Inc. automated DNA sequencer, model 373.

Sequences were analyzed with the basic local alignment search tool (BLAST) and ENTREZ network programs at GenBank, National Center for Biotechnology Information (1).

Purification of rhsp10-His6. Transformant *E. coli* DH5/pD8D3 was chosen for purifications. For harvest, 1- to 2-liter cultures of *E. coli* DH5/pD8D3 were pelleted by using a Sorvall RC5-B centrifuge with a GS-3 rotor at 10,000 × *g* for 30 min. Pellets were washed in PBS and frozen at −80°C until used. Pellets were thawed and resuspended in buffer B, briefly sonicated to ensure complete resuspension, and then incubated at 4°C overnight. Cell debris was pelleted at 10,000 × *g* for 30 min, and the lysate was applied to a 5-ml bed volume of preequilibrated Ni-NTA agarose matrix (QIAGEN) in a 50-ml centrifuge tube. During incubation, the tube was agitated on a rotary platform to keep the matrix in suspension. The matrix was washed twice in the centrifuge tube with buffer B, resuspended in 8 M urea–0.01 M Tris–0.1 M sodium phosphate, pH 6.3 (buffer C), and then transferred to a glass flex-column (1.0 by 20 cm; Kontes Glass Co., Vineland, N.J.). The column was washed with several column volumes of buffer C and then washed with a more stringent buffer, 8 M urea–0.01 M Tris–0.1 M sodium phosphate, pH 5.9 (buffer D). Protein was eluted from the column with 8 M urea–0.01 M Tris–0.1 M sodium phosphate, pH 4.5 (buffer E).

Peak fractions were pooled and extensively dialyzed against PBS with SpectroPor#7 3.5-kDa MWCO dialysis tubing (Spectrum). Purified protein solution was filter sterilized, separated into aliquots, and frozen at −80°C. Protein yield was determined with Coomassie protein assay reagent (Pierce, Rockford, Ill.) with a BSA protein standard (Pierce).

Nucleotide sequence accession number. The sequence of *C. trachomatis* serovar E/Bour hsp10, as cloned with a His_6 tag, has been submitted to GenBank under accession number CTU69255.

RESULTS

Development of anti-chsp10 monoclonal antibodies. To promote recognition of *Chlamydia*-specific epitopes, segments of the chsp10 sequence corresponding to regions of relative diversity were chosen for incorporation into four-branch MAPs (Fig. 1). One MAP, MAP3, containing amino acids 78 to 90 of the hsp10 sequence (YSGQELTVEGEEY), did not elicit significant anti-MAP3 or anti-chsp10 serum responses by inoculated mice even after an extended immunization regimen. The other MAP, MAP2, containing amino acids 54 to 69 of the hsp10 sequence (KKDDKGQQLPFEVQ), elicited strong antibody responses after immunization of BALB/c mice. Spleen cells from MAP2-hyperimmunized mice were fused with Sp2/0-Ag14 myelomas and expanded in the presence of IL-6. Hybridoma supernatants were screened by ELISA for reactivity to the immunizing peptide and to formalin-fixed EBs.

Monoclonal antibodies generated in this study are reported in Table 1. All monoclonal antibodies were found to react with MAP2, its linear form Pep2, and formalin-fixed EBs by ELISA. In addition, all identified an approximately 15-kDa chlamydial protein, MAP2, and rhsp10 expressed by *E. coli* JM109/pTA571 on immunoblots (Fig. 3). Monoclonal antibodies M1.2 (Fig. 3A), M1.3, and M1.4 (data not shown) showed no cross-reactivity with *E. coli* or HeLa cell proteins. Other monoclonal antibodies cross-reacted with additional chlamydial and/or host

TABLE 1. Characterization of anti-chsp10 monoclonal antibodies

Monoclonal antibody	Isotype	ELISA reactivity		Immunoblot reactivity			
		MAP2	EBs	MAP2	chsp10	*E. coli* GroES	Cross-reactivity[a]
M1.2	IgG2a	+	+	+	+	−	−
M1.3	IgG1	+	+	+	+	−	−
M1.4	IgG1	+	+	+	+	−	−
M1.7	IgG2b	+	+	+	+	−	+
M1.9	IgG1	+	+	+	+	−	+
M1.14	IgG1	+	+	+	+	−	+
M4.1	IgG1	+	+	+	+	−	+

[a] Reactivity to additional host and/or chlamydial proteins.

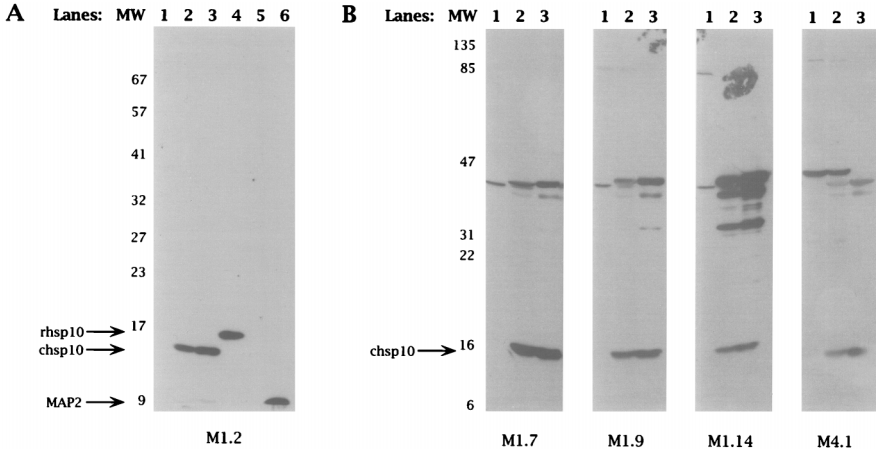

FIG. 3. Demonstration of monoclonal antibody reactivity against chsp10. (A) Specific recognition of native chsp10, rhsp10, and MAP2 antigens by monoclonal antibody M1.2. Monoclonal antibodies M1.3 and M1.4 had similar recognition profiles (data not shown). (B) Reactivities of anti-chsp10 monoclonal antibodies to additional host and chlamydial proteins. Lanes: 1, uninfected HeLa cells; 2, *C. trachomatis* A/HAR-13-infected HeLa cells; 3, purified *C. trachomatis* A/HAR-13 EBs; 4, recombinant *E. coli* JM109/pTA571 expressing the chlamydial *hypAB* operon; 5, *E. coli* JM109 parent; 6, MAP2 immunogen.

cell proteins (Fig. 3B). Recognition of an unidentified 45-kDa protein in both HeLa cells and chlamydiae was common among several of the cross-reactive antibodies. A BLAST search of GenBank with the MAP2 immunogen sequence did not suggest any candidates for identification of cross-reactive proteins.

Because of its specificity, monoclonal antibody M1.2 was selected for routine use. M1.2 was found to identify the chsp10 of several *C. trachomatis* strains by immunoblotting, including representatives of ocular infection-associated serovars (A, B, and C), genital infection-associated serovars (E and F), the lymphogranuloma venereum biovar (L2), and the mouse biovar (MoPn) (Fig. 4). However, M1.2 did not show reactivity to *C. psittaci* GPIC or 6BC chsp10 (Fig. 4), suggesting recognition of a species-specific epitope. Interestingly, the chsp10 of serovar B/TW-5 had an approximately 2- to 3-kDa lower molecular mass than other *C. trachomatis* strains.

Immunoaffinity chromatography purification of rhsp10. An immunoaffinity column was constructed by chemically crosslinking M1.2 antibodies to a protein A-Sepharose matrix. rhsp10 was purified from OGP extracts of *E. coli* JM109/pTA571 (Fig. 5A) and was shown to be free of chsp60 and *E. coli* GroES by immunoblotting (data not shown). Yields were only a few micrograms per liter of starting culture, so an alternative approach to purify chsp10 was sought.

Cloning of chsp10 from serovar E/Bour for large-scale purifications. chsp10 was appended with a C-terminal His_6 tag for purification by nickel chelate affinity chromatography. Three transformants that were found to express rhsp10 by colony immunoblotting with M1.2 and that tested positive for nickel matrix binding were sequenced. The sequence encoding rhsp10-His6 was identical among the three transformants, and the sequence from a representative transformant, *E. coli* DH5/pD8D3, is shown in Fig. 6A.

The sequence of *C. trachomatis* serovar E/Bour hsp10 was found to be closely homologous to those of other chlamydiae (Fig. 6). The serovar E/Bour *hypA* nucleotide sequence differed from that of serovar A/HAR-13 (36) at only two positions, +246 (C to T) and +252 (A to C), and did not result in a change in amino acid sequence (Fig. 6B). Likewise, the serovar E/Bour *hypA* nucleotide sequence differed from that of serovar L2/434 (13) at only three positions, +142 (T to G), +194 (A to T), and +252 (A to C). In this case, however, two of these nucleotide substitutions (those at positions +142 and +194) resulted in changes to the chsp10 amino acid sequence (L to V at position 48 and E to V at position 65, respectively).

Nickel chelate affinity chromatography purification of rhsp10-His6. Nickel chelate affinity chromatography was employed to purify large quantities of rhsp10-His6. Sustained high-level expression of rhsp10-His6 by *E. coli* DH5/pD8D3 was achieved with levels of up to 40% total protein in freshly transformed cells. Induction from 1 to 12 h with 2 mM IPTG did not raise rhsp10-His6 levels significantly higher. For harvest, 1- to 2-liter cultures were grown at 27°C for 3 to 4 days. An average of 32 mg of purified rhsp10-His6 was recovered per liter of starting culture. It was found that 250 ng of purified rhsp10-His6 showed no contaminating proteins on silver-stained SDS-PAGE gels (Fig. 5B, lane 6). The rhsp10-His6 was analyzed by immunoblotting to be free from chsp60 and *E. coli* GroES (data not shown).

Expression of rhsp10-His6 by several transformants was found to vary with extended passage at 37°C. It was determined that growth of transformants at 27°C (room temperature) preserved high levels of rhsp10-His6 expression over passages, while expression tended to be reduced or lost following serial passage at 37°C (data not shown). The loss of rhsp10-His6 expression was probably not related to protease activity at 37°C since a shift to lower-temperature growth or the introduction of protease inhibitors (such as phenylmethylsulfonyl fluoride) had no effect on restoring lost expression. Perhaps the high level of rhsp10-His6 expression obtained with this system, especially at 37°C, was toxic to the cells.

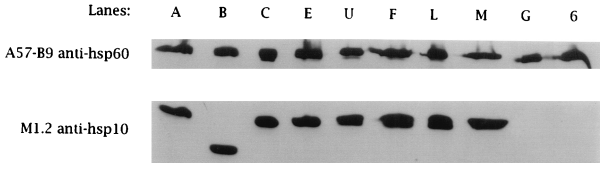

FIG. 4. A57-B9 anti-hsp60 and M1.2 anti-hsp10 reactivity to different chlamydial strains. M1.2 recognized an approximately 15-kDa protein in several strains of *C. trachomatis* but not in *C. psittaci* GPIC or 6BC. Serovar B/TW-5 hsp10 was observed to migrate at a lower relative molecular weight that is closer to the expected size of chsp10 (approximately 11 kDa) than other strains. Lanes: A, A/HAR-13; B, B/TW-5; C, C/TW-3; E, E/Bour; U, E/UW-5; F, F/UW-6; L, L2/434; M, MoPn; G, GPIC; 6, 6BC.

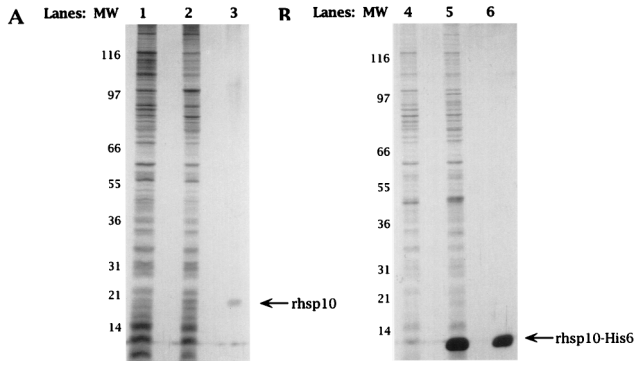

FIG. 5. Purification of rhsp10 assessed with silver-stained SDS-PAGE gel. (A) Purification of rhsp10 by immunoaffinity chromatography with M1.2. Lane 1, *E. coli* JM109 parent; lane 2, rhsp10 source strain *E. coli* JM109/pTA571; lane 3, immunoaffinity-purified rhsp10 product. (B) Purification of rhsp10-His6 by nickel chelate affinity chromatography. Lane 4, *E. coli* DH5/pQE-60 vector only; lane 5, rhsp10-His6 source strain *E. coli* DH5/pD8D3; lane 6, purified rhsp10-His6 product.

DISCUSSION

hsp10 family proteins are highly homologous and found ubiquitously in nature. As a result, cross-species reactivity has posed a challenge when the reagents to specifically identify the hsp10 of chlamydiae are developed. Since an unambiguous reagent was desired to identify and characterize chsp10, specific monoclonal antibodies were developed with MAPs. MAPs consist of a lysine core with several attached peptide branches and offer several advantages over carrier proteins for peptide immunizations (47). MAPs allowed incorporation of specific regions of the chsp10 sequence that tended to diverge from those of other hsp10s such as GroES (Table 1). Thus, the resulting monoclonal antibodies recognized *Chlamydia*-specific epitopes within the chsp10 sequence.

Mice immunized with MAP3 failed to elicit a significant anti-peptide or antichlamydial response as determined by ELISA or immunoblotting even after an extended immunization regimen. MAP2, however, elicited a rapid and strong antibody response, and hybridomas that produced chsp10-specific monoclonal antibodies were successfully developed. The difference in the reactivities of the two MAPs may reflect inherent differences in the abilities of their sequences to elicit responses in BALB/c mice. It has been reported that the presence of T-helper-cell epitopes within the MAP sequence has a significant influence on MAP antibody stimulation (14, 15, 48). It is possible that MAP2 contains a T-helper-cell epitope necessary for an antibody response to develop. Indeed, the sequence of *Mycobacterium leprae* hsp10 homologous to the MAP2 region of chsp10 overlaps with a proposed immunodominant T-cell epitope recognized by *M. leprae*-specific T cells (33). With the availability of highly purified rhsp10-His6, T-cell reactivity to chsp10 can now be addressed.

Monoclonal antibody M1.2 was chosen for routine experimental use since it displayed no immunoblot cross-reactivities (Fig. 3). It has also been used successfully in our laboratory for immunochemical staining of inclusions within infected cells and for immunoprecipitations (data not shown). Furthermore, M1.2 enabled immunoaffinity purification of rhsp10 (Fig. 5A)

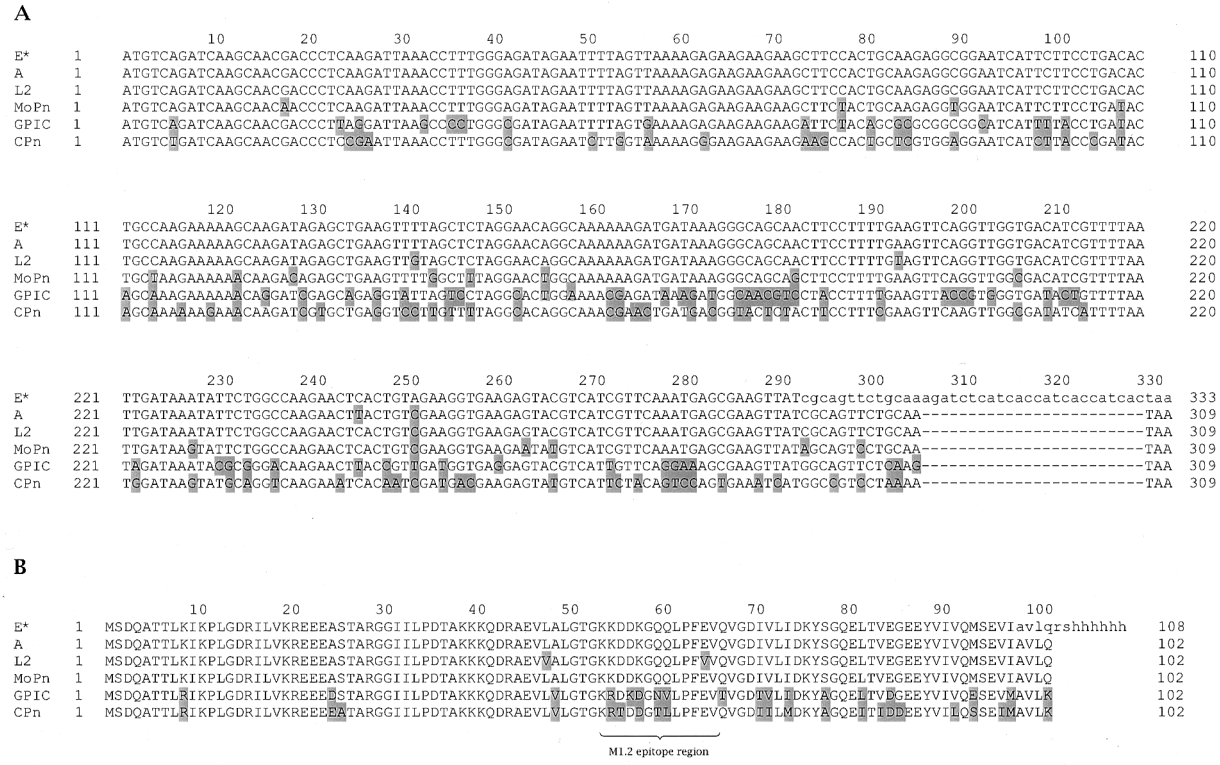

FIG. 6. Sequence of *C. trachomatis* E/Bour hsp10 as cloned with the His_6 tag and comparison with coding regions of other *Chlamydia hypA* sequences. (A) Nucleotide sequences; (B) corresponding deduced amino acid sequences. Shading indicates positions that differ from E*. Primer- and vector-derived sequences of E* are indicated in lowercase type. E*, E/Bour sequence as cloned with His_6 tag; A, A/HAR-13 (36); L2, L2/434 (13); MoPn, mouse pneumonitis (20, 26); GPIC, *C. psittaci* guinea pig inclusion conjunctivitis (34); CPn: *C. pneumoniae* AR-39 (30).

and provided a means of identifying transformants when cloning rhsp10-His6. Monoclonal antibodies developed in this study are currently being used to identify and measure chsp10 under normal and stressful growth conditions.

M1.2 reacted with several strains of *C. trachomatis* but did not react with *C. psittaci* strains (Fig. 4). This apparent species specificity is likely a result of differences within the amino acid sequences of the monoclonal antibody epitope regions in the chsp10s of the two species (Fig. 6B). The sequences spanning the M1.2 epitope regions are quite similar among the four known *C. trachomatis hypA* sequences but differ from that of *C. psittaci* GPIC. Of note, one of the serovar L2 amino acid differences, E to V at position 65, lies within the M1.2 epitope region but does not affect monoclonal antibody recognition of serovar L2 chsp10 (Fig. 4).

Despite specific recognition of chsp10, some monoclonal antibodies also showed cross-reactivity to other host cell and chlamydial proteins (Fig. 3B). The identities of these proteins are currently unknown. The presence of shared epitopes on other chlamydial proteins could potentially amplify immune responses to chsp10 or contribute to immunodominance of a particular region of chsp10. The observed shared monoclonal antibody epitopes on human proteins of 45 or 47 kDa are particularly interesting in light of a potential autoimmune response.

Some discrepancies in chsp10 SDS-PAGE mobility were noted. The molecular weight of rhsp10-His6 appeared to be lower than that of native serovar E/Bour chsp10 despite the fact that the recombinant protein migrated at the molecular mass of 12.3 kDa predicted from its gene sequence (Fig. 5B). It is possible that the His_6 tag may have affected the recombinant protein's secondary structure and/or charge resulting in altered gel mobility. Native chsp10 from EBs and *Chlamydia*-infected cells consistently migrated to 15 kDa (Fig. 3 and 4), which is larger than the size of about 11 kDa deduced from the *hypA* sequences of serovars A and L2 and strain MoPn (13, 20, 26, 36). An exception, serovar B hsp10, migrated to ~13 kDa (Fig. 4). The reason for these different mobilities is unknown. Interestingly, the mobility of serovar B hsp10 appeared to be similar to that of recombinant serovar E hsp10-His6, but this may be coincidental. Since the gene encoding serovar B hsp10 has not been conclusively sequenced, the difference in mobility may be accounted for by a difference in its primary amino acid sequence. Alternatively, it is possible that *C. trachomatis* post-translationally modifies hsp10 but the serovar B strain is incapable of this function.

While immunoaffinity chromatography was successful in purifying rhsp10, a vast improvement in yield was obtained by employing nickel chelate affinity chromatography. The relatively large quantity of rhsp10-His6 now available enables an investigation of the immunological properties of chsp10. Though the role of hsp10 in the immune response to chlamydiae is unknown, hsp10 homologs of several other species elicit prominent T-cell responses. Mycobacterial hsp10s induce strong TH1-type responses (3, 19, 28, 31), including delayed hypersensitivity responses in sensitized individuals. The hsp10 homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice, possibly via a TH2-specific response (21, 45). *Chlamydia*-reactive T cells that recognize cytoplasmic antigens smaller than 20 kDa have been identified by T-cell immunoblotting (5), but the antigenic specificities of these responders are unknown.

Interest in the immune response to chsp10 has also arisen from its coexpression with chsp60, a reported immunopathological antigen (34, 35, 38). Clinical investigations have revealed an association of serologic exposure to chsp60 with the sequelae of pelvic inflammatory disease (2, 11, 43, 50). Since chsp10 is coexpressed with chsp60 (34, 36), it can be hypothesized that exposure to chsp10 will parallel that of chsp60. Purified chsp10 can now be used to address serological correlation of chsp10 exposure with chsp60 exposure and immunopathology.

Purified chsp10 and specific anti-chsp10 monoclonal antibodies will stimulate research to better define the biological and immunological properties of chsp10. Furthermore, these reagents provide a means to evaluate the serologic and cellular immune responses to chsp10 in individuals with chlamydial disease.

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