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# Characterization of Two Conformational Epitopes of the *Chlamydia trachomatis* Serovar L2 DnaK Immunogen

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***Chlamydia trachomatis* DnaK is an important immunogen in chlamydial infections. DnaK is composed of a conserved N-terminal ATP-binding domain and a variable C-terminal peptide-binding domain. To locate the immunogenic part of *C. trachomatis* DnaK, we generated monoclonal antibodies (MAbs) against this protein. By use of recombinant DNA techniques, we located the epitopes for two MAbs in the C-terminal variable part. Although the antibodies reacted in an immunoblot assay, it was not possible to map the epitopes completely by use of 16-mer synthetic peptides displaced by one amino acid corresponding to the C-terminal part of *C. trachomatis* DnaK. To determine the limits of the epitopes, *C. trachomatis* DnaK and glutatione *S*-transferase fusion proteins were constructed and affinity purified. The purified DnaK fusion proteins were used for a fluid-phase inhibition enzyme-linked immunosorbent assay with the two antibodies. The epitopes were found not to overlap. To obtain DnaK fragments recognized by the antibodies with the same affinity as native *C. trachomatis* DnaK, it was necessary to express, respectively, regions of 127 and 77 amino acids. The MAbs described in this study thus recognized conformational epitopes of *C. trachomatis* DnaK.**

Chlamydiae are obligate intracellular bacteria with a unique biphasic life cycle. The genus *Chlamydia* is divided into four species: *Chlamydia trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum*. *C. trachomatis* and *C. pneumoniae* have humans as their natural host, whereas *C. psittaci* is a common animal pathogen that occasionally can infect humans, causing ornithosis, and *C. pecorum* is a pathogen of sheep and calves (12). *C. trachomatis* is divided into trachoma inclusion conjunctivitis strains and lymphogranuloma venereum strains. Lymphogranuloma venereum is a sexually transmitted disease in the Third World. The trachoma inclusion conjunctivitis serotypes cause trachoma in the Third World, and serotypes D to K cause sexually transmitted disease worldwide. While primary infections with the *C. trachomatis* trachoma inclusion conjunctivitis serotypes usually are mild, severe problems are caused by immunological sequelae such as blinding trachoma, tubal infertility, ectopic pregnancy, and sexually acquired reactive arthritis (4).

Chlamydial infection is mediated by the extracellular metabolically inactive elementary body (EB), which adheres to host cells and induces its own uptake. After internalization, the EB is transformed into the metabolically active reticulate body. The reticulate body starts to divide by binary fission, and 24 to 48 h after infection, reticulate bodies transform into EBs which are liberated by burst of the inclusion 48 to 72 h after infection. Among the chlamydial genes that are highly transcribed during the transformation process are the heat shock genes *dnaK* and *groEL* (24).

The humoral immune response to a *C. trachomatis* infection is directed against a small number of chlamydial components: the major outer membrane protein, cysteine-rich outer membrane protein 2, the lipopolysaccharide (9, 22, 31), and cytoplasmic proteins chlamydial GroEL and DnaK (22, 40). Thus, Larsen et al. (22) found that 30% of patients with reactive arthritis and antibodies to *C. trachomatis* lipopolysaccharide and outer membrane protein 2 also had antibodies to DnaK.

Functional analysis of *Escherichia coli* DnaK has been performed. DnaK is a molecular chaperone with two domains: the N-terminal ATP-binding domain and a C-terminal substrate recognition domain (44). Thermodynamic and structural analysis of the folding-unfolding transitions of *E. coli* DnaK revealed this molecule to exhibit well-defined transitions at pH 7.6 at both the N- and C-terminal parts (29). The sequence of the ATP-binding domains is extremely well conserved, even between prokaryotic DnaK and eukaryotic HSP70 (4), whereas the substrate recognition domain is variable. The peptide-binding domain binds peptides with internal hydrophobic residues (13). The peptide-binding affinity of DnaK can be increased by phosphorylation of the threonine at position 199 in the ATP-binding domain when no peptide is bound to the peptide-binding domain (25, 32). Upon heat shock, the number of phosphorylated DnaK molecules increases and thus the affinity for binding to denatured protein does also (36). DnaK functions together with GrpE and DnaJ as shown by the fact that DnaK-DnaJ-GrpE can reactivate heat-denatured enzymes such as RNA polymerase and luciferase better than DnaK alone can (35, 43). In this complex, GrpE can bind to the ATP-binding domain of DnaK and inhibit the autophosphorylation of DnaK, mediating regulation of the refolding process (32). The structure of the ATP-binding domain of bovine heat shock cognate 70 has been determined by X-ray crystallography (11). The structure of the substrate recognition domain is unknown, but it has been proposed to be similar to that of the human major histocompatibility complex class I antigen (34).

Because of the evolutionary conservation of DnaK, it is important to determine whether the humoral immune response is directed against the conserved ATP-binding domain or the variable peptide-binding domain. Despite the high homology in the ATP-binding domain between prokaryotic

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DnaK and eukaryotic HSP70, this part of the molecule is immunogenic, and epitopes for two monoclonal antibodies (MAbs) have been described for this part of the molecule. The epitope for one MAb against *E. coli* DnaK was mapped to residues 288 to 310 and was specific for DnaK of gram-negative bacteria (21). The other epitope for a MAb against *C. trachomatis* DnaK was mapped to residues 360 to 367. This epitope was present in several gram-positive bacteria but not in other gram-negative bacteria, as revealed by a database search and experimental procedures (5).

The immunogenicity of chlamydial DnaK was studied by Zhong and Brunham (42). They used 10-mer peptides and hyperimmune rabbit serum to scan the *C. trachomatis* DnaK amino acid sequence for antigenic epitopes and found that the conserved ATPase domain is the most immunogenic region. To determine whether the variable peptide-binding domain is antigenic, we generated MAbs specific for DnaK and used recombinant DNA technology to screen for MAbs that recognize this part of the molecule. In this report, we describe two MAbs (18.1 and 22.2) recognizing the peptide-binding domain of *C. trachomatis* DnaK. These epitopes could not be mapped by use of 16-mer peptides but needed 77 and 127 amino acids, respectively, for high-affinity binding. MAb 18.1 was genus specific, whereas MAb 22.2 was species specific. Our data demonstrate the limitation of the use of synthetic peptides for mapping of immunogenic regions of the variable peptide-binding domain of DnaK.

## MATERIALS AND METHODS

**Production and isotyping of MAbs.** BALB/c mice were immunized as described by Birkelund et al. (5). At day 93, $5 \times 10^8$ spleen cells were fused with $2.5 \times 10^8$ NS-1 azoguanine-resistant cells (American Type Culture Collection, Rockville, Md.) in the presence of 50% polyethylene glycol. Fusion and subcloning were done as described by Birkelund et al. (6).

**Isotyping of antibodies.** Isotyping of the MAbs was done by using the Ouchterlony double-diffusion test. For subtype determination, rabbit anti-mouse antibodies specific to immunoglobulin G1 (IgG1), IgG2A, IgG2B (MELOY, Springfield, Va.), and IgG3 (Zymed) were used.

**Cultivation of *C. trachomatis*.** *C. trachomatis* LGV-II/434/Bu, *C. psittaci* cal10, and *C. pneumoniae* VR-1310 were cultivated as previously described (5), except that HeLa cells were used. The strains were obtained from the American Type Culture Collection and the Statens Serum Institut (Copenhagen, Denmark).

**Recombinant clones.** Recombinant *E. coli* K-12 strain pl248 harboring plasmid pCtX2-43 was used for subcloning of *dnaK*. pCtX2-43 has a 4-kb insert of *C. trachomatis* L2 DNA containing *grpE*- and *dnaK*-like genes. The complete *Chlamydia* protein was expressed by recombinant *E. coli* pl248(pCtX2-43) (7, 8).

**Immunoblotting (Western blotting) and immunofluorescence (IMF) assays.** Immunoblotting and IMF assays were done as previously described (5). For the IMF assay, *C. trachomatis* serotypes A, B, C, and L2 and *C. psittaci* cal10 were cultivated in McCoy medium and *C. pneumoniae* VR-1310 was cultivated in HeLa cells.

**Subcloning of digested pCtX2-43 DNA into vector pEX2.** Subcloning of digested pCtX2-43 DNA into vector pEX2 was done as previously described (5). Briefly, pCtX2-43 DNA was digested with DNase I in a buffer containing 20 mM Tris-HCl (pH 7.5), 4 mM $MnCl_2$, and 100 μg of bovine serum albumin per ml (27). The DNA was digested at (24°C) for 10 to 60 min to produce short, random fragments. The DNA fragments were size fractionated by agarose gel electrophoresis, and end repaired by treatment with bacteriophage T4 DNA polymerase (Boehringer GmbH, Mannheim, Germany) in the presence of deoxynucleoside triphosphates. The DNA fragments were ligated into expression vector pEX2 (Boehringer), and the hybrid DNA molecules were transformed into *E. coli* pl248. Recombinant bacteria were screened by colony blotting with MAbs 18.1 and 22.2 as previously described (7, 38).

**DNA sequence analysis of recombinant clones.** The plasmid DNA was sequenced as described by Hattori and Sakaki (16), with [α-$^{32}$P]dATP (Amersham International, Buckinghamshire, United Kingdom) and T7 DNA polymerase (Pharmacia, Uppsala, Sweden).

**Construction of specific fragments of DnaK by cloning of a PCR fragment into pGEX-3X.** For expression and purification of fragments of DnaK, vector pGEX-3X was used. pGEX-3X is an expression vector encoding a truncated glutathione *S*-transferase (GST) gene with a downstream multiple cloning site. A foreign DNA fragment encoding protein can be expressed as a fusion protein with GST and purified on glutathione beads. Primers specific for selected regions of the *dnaK* gene were produced (DNA-technology, Aarhus, Denmark). A *Bam*HI site was introduced into the upstream primers, and a stop codon was introduced into the downstream primer (Table 1). The primers were mixed with *C. trachomatis* L2 DNA and subjected to PCR (94°C for 1 min, 45°C for 1 min, 72°C for 1 min). The PCR products were ligated into pCR-II (Invitrogen Corp., San Diego, Calif.). Recombinant clones were control sequenced, the inserts were cut out by *Bam*HI and *Eco*RI digestion, and the fragments were ligated into pGEX-3X digested with the same combination of enzymes. The ligation mixture was transformed into *E. coli* XL1-blue (Stratagene, La Jolla, Calif.). Colonies were transferred to Luria-Bertani medium containing ampicillin (100 μg/ml), and when the cultures had reached an optical density at 600 nm of 0.1, they were induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) for 2 h. After harvesting of bacteria by centrifugation, proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris [pH 6.8], 10% [wt/vol] glycerol, 2.3% [wt/vol] SDS, 5% [wt/vol] β-mercaptoethanol) and analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

**Purification of GST-DnaK fusion proteins.** For purification of GST-DnaK fusion proteins, 250 ml of an induced culture was harvested, washed with phosphate-buffered saline (PBS), resuspended in 5 ml of PBS, and frozen and sonicated to disrupt the cells. Triton X-100 was added to 1%, and the supernatant was cleared by centrifugation. The GST-DnaK fusion proteins were purified on glutathione beads as previously described (37) and used for immunoblotting analysis or in an inhibitory enzyme-linked immunosorbent assay (ELISA) at concentrations of 1.25 to 50 nM. The purity of the fusion proteins was >95% as judged by SDS-PAGE and Coomassie blue staining. Removal of GST was done by cleavage with factor Xa (Boehringer).

**Peptide synthesis.** Solid-phase peptide synthesis of 16-residue peptides was performed by the fluorenylmethyloxycarbonyl strategy (1) with fluorenylmethoxycarbonyl amino acid pfp esters (Milligen) and a PepSyn KA resin as the solid support with the first amino acid attached (S = 0.1 mmol/g; Milligen). The synthesis were performed on a parallel multiple-column peptide synthesizer developed in our laboratory (18, 19, 28). Amino acid analyses were performed by the PICOTAG method (Waters). High-performance liquid chromatography was performed on a Waters high-performance liquid chromatography system by using a $C_{18}$ reversed-phase column (Hamilton PRP-3; flow rate, 1.5 ml/min for analytical preparations) with a buffer consisting of 0.1% trifluoroacetic acid and a buffer consisting of 0.1% trifluoroacetic acid and 10% water in acetonitrile.

**Measurement of binding of MAbs to covalently linked peptides by ELISA.** Synthetic peptides were covalently linked to CovaLink plates (Nunc, Roskilde, Denmark) as described by the manufacturer. The peptide carboxy terminals were covalently attached to secondary amino groups on the CovaLink spacer arms. Fifty microliters of double-distilled $H_2O$ was added to each CovaLink well. One microliter of a synthetic peptide (2 mg/ml) was mixed with 25 μl (185 μg/ml) of sulfo-*N*-hydroxysuccinimide and added to the well (Pierce). The coupling reaction was started by addition of 50 μl (1.23 mg/ml) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma, St. Louis, Mo.). The coupling reaction was carried out for 2 h at room temperature. The wells were washed in PBS with 0.05% Tween, and excess binding capacity (adsorption sites) was blocked with PBS containing 1% gelatin (15).

**Inhibitory ELISA.** For an inhibitory ELISA, purified, recombinant, nondenatured pGEX/DnaK$^{16–660}$ (0.5 μg/ml) or recombinant clone pCtX2-43 expressing *C. trachomatis* DnaK lysed in 2% SDS, diluted 1:500 in carbonate buffer (pH 9.6) to a final concentration of 2 μg of protein per ml was used as the antigen. Microtiter plates (Nunc) were coated for 1 h at 37°C with the antigen suspension. Excess binding capacity was blocked with 1% gelatin in PBS. A MAb (1.5 μg/ml) and peptides (from 0 to 10 μM) or fusion proteins (from 0 to 60 μM) were preincubated for 1 or 24 h in a microtiter plate before the mixtures were transferred to the antigen-coated microtiter plate. Antibody binding was measured with a goat anti-mouse immunoglobulin-horseradish peroxidase conjugate and *o*-phenylenediamine dihydrochloride (15).

**Sequence analysis.** For sequence analysis, PILEUP from the Wisconsin sequence analysis software package was used (10). The DnaK sequences used for alignment were from *E. coli* (2), *C. trachomatis* L2 (8), *C. pneumoniae* (20), *Bacillus subtilis* (17), *Mycobacterium leprae* (26), *Halobacterium marismortui* (14), *Clostridium acetobutylicum* (30), *Borrelia burgdorferi* (39), and *Streptomyces griseus* (accession number D14499).

## RESULTS

**Characterization of MAbs.** MAbs against purified *C. trachomatis* L2 EBs were generated and then screened by ELISA (6). Two MAbs, 18.1 (IgG2a) and 22.2 (IgG1), that reacted in an immunoblot assay both with a 75-kDa *C. trachomatis* L2 antigen and with a 75-kDa antigen expressed by recombinant clone pCtX2-43 were obtained. pCtX2-43 contains the *C. trachomatis* L2 *dnaK* and *grpE* genes, and in this recombinant clone *C. trachomatis* L2 DnaK is expressed in its native form (7, 8). The reactivity of MAbs 18.1 and 22.2 with different microorganisms was analyzed with immunoblotting and IMF assays as shown in Table 2. MAb 18.1 reacted with *C. trachomatis*, *C. pneumoniae*,

TABLE 1. Primers used in this study

| pGEX clone | Primers used | Size,kDa |
|---|---|---|
| pGEX/DnaK$_{16-660}$ | -[a] | 97.3 |
| pGEX/DnaK$_{384-503}$ | 5'-AGG ATC CAA GTG AAA GAC GTT CTG TTG-3' (*BamHI*) | 39.5 |
| | 5'-A CTA AGA GCT TGC TTC AAT ACG G-3' (*Stop*) | |
| pGEX/DnaK$_{462-503}$ | 5'-GGG ATC CCT CCT GCT CCT CGC GGC C-3' (*BamHI*) | 31 |
| | 5'-A CTA AGA GCT TGC TTC AAT ACG G-3' (*Stop*) | |
| pGEX/DnaK$_{462-588}$ | 5'-GGG ATC CCT CCT GCT CCT CGC GGC C-3' (*BamHI*) | 40.3 |
| | 5'-CTA ATC AGA AGC TGC TTT GAT AG-3' (*Stop*) | |
| pGEX/DnaK$_{462-588}\alpha$ | 5'-GGG ATC CCT CCT GCT CCT CGC GGC C-3' (*BamHI*) | 40.8 |
| | 5'-TCA CTT TTT CTT GGC CAT TTC TTC CGC (*Stop*) | |
| | GGC CGC TTT GAT AGC TGT TGT GGAAGC-3' (*NotI*) | |
| pEX/DnaK$_{541-617}$ | -[a] | 34.8 |
| pGEX/DnaK$_{549-588}$ | 5'-AGG ATC CTG AAA GAT TAC CAC GAC-3' (*BamHI*) | 30.7 |
| | 5'-CTA ATC AGA AGC TGC TTT GAT AG-3' (*Stop*) | |
| pGEX/DnaK$_{549-588}\alpha$ | 5'-AGG ATC CTG AAA GAT TAC CAC GAC-3' (*BamHI*) | 31.2 |
| | 5'-TCA CTT TTT CTT GGC CAT TTC TTC CGC (*Stop*) | |
| | GGC CGC TTT GAT AGC TGT TGT GGAAGC-3' (*NotI*) | |
| pGEX/DnaK$_{549-660}$ | 5'-AGG ATC CTG AAA GAT TAC CAC GAC-3' (*BamHI*) | 38.7 |
| | 5'-TTA CTC AGG TTT ATC AAC AAT TTC-3' (*Stop*) | |
| pGEX | -[b] | 26 |

[a] —, Transferred from pEX clones.
[b] —, Outlined sequences are of *dnaK* origin.

and *C. psittaci* but not with any of the other microorganisms tested. MAb 22.2 reacted only with *C. trachomatis* and was thus species specific (Table 2).

**Limitations of epitopes by recombinant fusion protein analysis.** To confine the epitopes of MAbs 18.1 and 22.2, random fragments of the *C. trachomatis* L2 *dnaK* gene generated from pCtX2-43 were cloned into expression vector pEX2 downstream of the *cro*–β-galactosidase hybrid gene and the colonies were screened with MAbs 18.1 and 22.2 by colony blotting. Ten positive colonies were found. In SDS-PAGE, they showed in-

TABLE 2. Reactions of MAbs 18.1 and 22.2 to different microorganisms[a]

| Microorganism, cell line, or medium | MAb 18.1 | | MAb 22.2 | |
|---|---|---|---|---|
| | Immunoblot assay | IMF assay | Immunoblot assay | IMF assay |
| *C. trachomatis* L2 | + | + | + | + |
| *C. trachomatis* A | ND | + | ND | + |
| *C. trachomatis* B | ND | + | ND | + |
| *C. trachomatis* C | ND | + | ND | + |
| *C. pneumoniae* | ND | + | ND | − |
| *C. psittaci* | ND | + | ND | − |
| *E. coli* K-12 | − | ND | − | ND |
| *E. coli*(pCtX2-43) | + | ND | + | ND |
| *Y. enterocolitica* | − | ND | − | ND |
| *N. gonorrhoeae* | − | ND | − | ND |
| *S. typhimurium* | − | ND | − | ND |
| *L. pneumophila* | − | ND | − | ND |
| *Bacillus subtilis* | − | ND | − | ND |
| HeLa cells | − | − | − | − |
| McCoy medium | ND | − | ND | − |

[a] ND, not determined; +, positive reaction; −, negative reaction.

creases of 5 to 15 kDa in the molecular sizes of the *cro*–β-galactosidase fusion proteins. Three clones were recognized by MAb 18.1, and seven clones were recognized by MAb 22.2. None of the clones were recognized by both antibodies. The clones were sequenced to determine the localizations of the cloned DNA fragments within the *dnaK* gene, and the amino acid sequences expressed by the fusion proteins were deduced (Fig. 1). By these experiments, the epitope for MAb 18.1 was limited to amino acids 462 to 503 and the epitope for MAb 22.2 was limited to amino acids 549 to 588. Thus, both MAbs react with the peptide-binding domain of DnaK.

**Analysis of the epitope for MAbs 18.1 and 22.2 by use of synthetic peptides.** We have previously defined an epitope in *C. trachomatis* L2 DnaK by use of synthetic peptides (5). A similar strategy was used to characterize the epitopes for MAbs 18.1 and 22.2. We synthesized 16-mer peptides displaced by one amino acid with start points at amino acids 453 to 473 and 531 to 575 and tested them by an inhibitory ELISA in concentrations of up to 10 μM for inhibition of binding of the MAbs to native *C. trachomatis* DnaK. No inhibition was observed (data not shown), indicating that a conformationally correct epitope was not present in the peptides. To look for low-affinity binding of the MAbs to the peptides, they were covalently linked to an ELISA tray. Weak binding of MAb 18.1 to the peptides starting at amino acids 453 to 461 of the DnaK sequence was observed (Fig. 2A).

More specific binding by MAb 22.2 was seen. This MAb bound to the peptides starting at amino acids 549 to 556 covering the sequence from 549 to 571 of *C. trachomatis* DnaK, with the highest binding found in the peptide starting at amino acid 555 (Fig. 2B). Since none of the peptides could confer

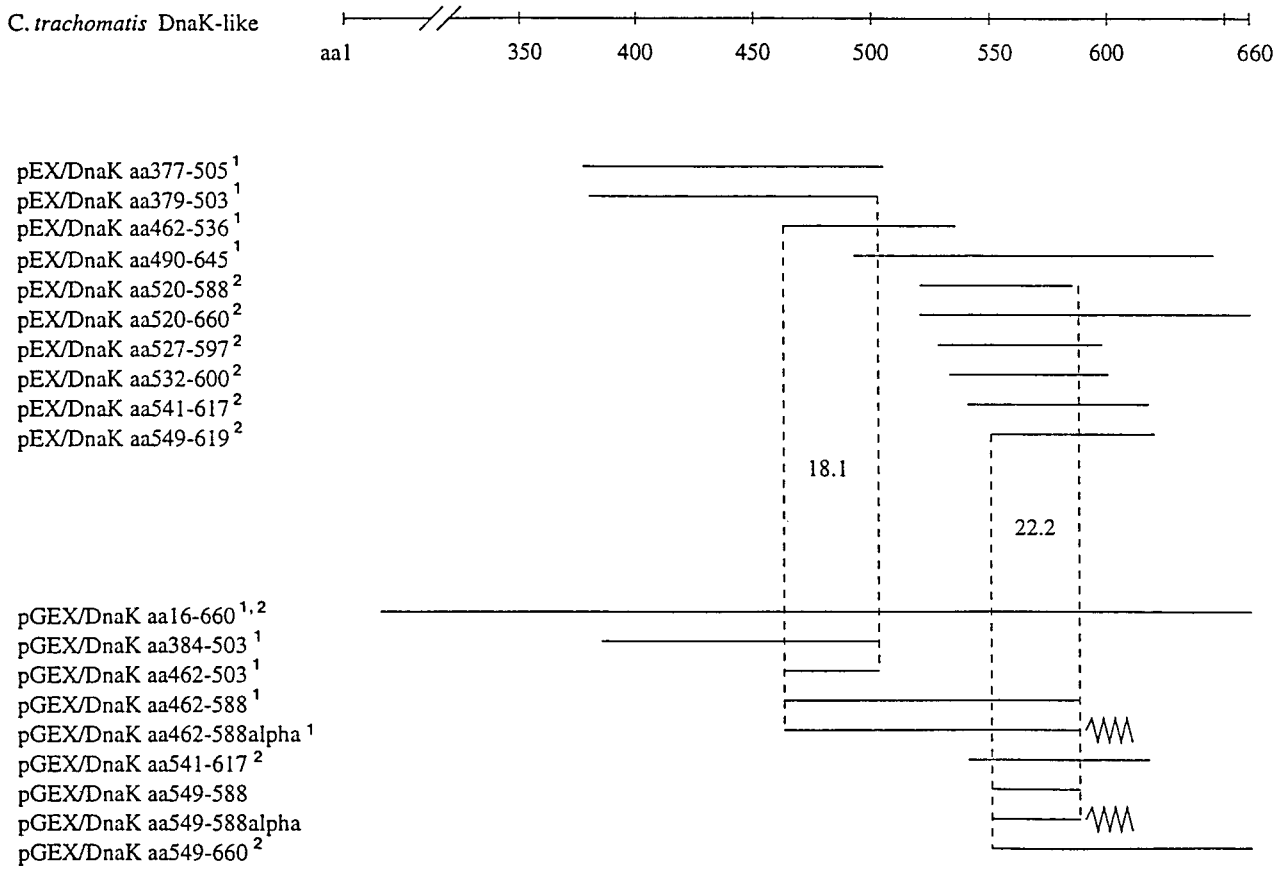


FIG. 1. Diagram showing the positions of the recombinant clones expressing part of the *C. trachomatis* L2 DnaK homolog. Amino acid numbers of the *C. trachomatis* DnaK homolog are shown on the top line. 18.1 and 22.2 indicate the limits of epitopes for MAbs 18.1 and 22.2, respectively. A superscript one indicates reactivity of MAb 18.1 with the clone, and a superscript two indicates reactivity of MAb 22.2 with the clone.

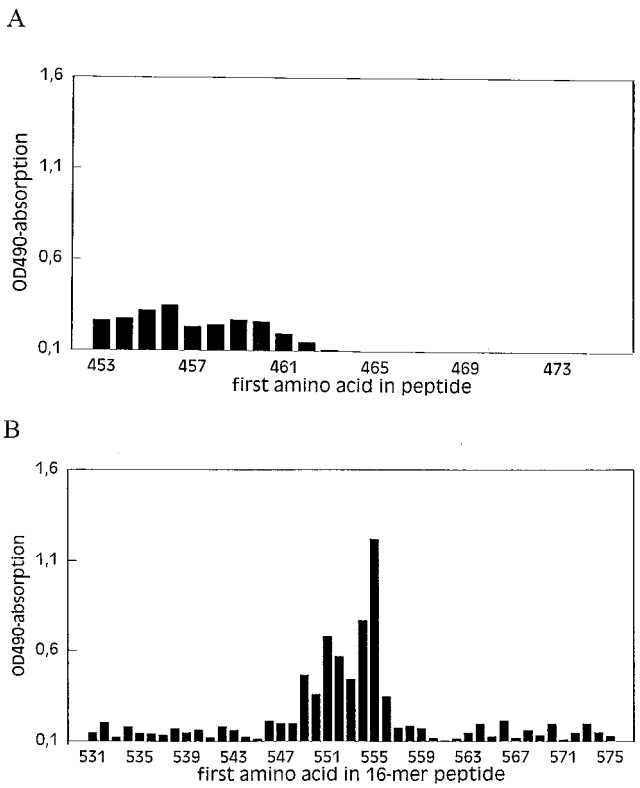


FIG. 2. Binding of MAbs 18.1 (A) and 22.2 (B) to synthetic 16-mer oligopeptides as measured by ELISA. Each bar is placed at the position of the first amino acid of the peptide. OD490, optical density at 490 nm.

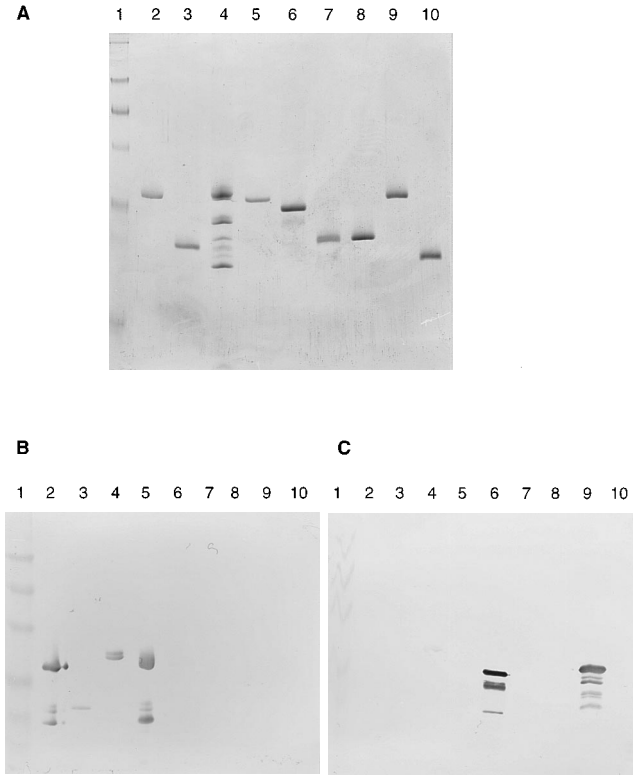


FIG. 3. SDS-PAGE of purified recombinant peptides expressed from fragments of the *C. trachomatis* L2 *dnaK* gene cloned into expression vector pGEX. A, Coomassie blue staining; B, immunoblotting with MAb 18.1; C, immunoblotting with MAb 22.2. Lanes: 1, molecular size markers; 2, pGEX/DnaK$_{384–503}$; 3, pGEX/DnaK$_{462–503}$; 4, pGEX/DnaK$_{462–588}$; 5, pGEX/DnaK$_{462–588}\alpha$; 6, pGEX/DnaK$_{541–617}$; 7, pGEX/DnaK$_{549–588}$; 8, pGEX/DnaK$_{549–588}\alpha$; 9, pGEX/DnaK$_{549–660}$; 10, pGEX-3X. Molecular size markers (from the top): A, 200, 96, 66, 36, and 31 kDa; B and C, 98, 64, 50, 36, and 30 kDa.

inhibition of the binding of MAb 22.2 to the native protein, additional amino acids are required for full affinity.

**Analysis of MAbs 18.1 and 22.2 with pGEX/DnaK fusion proteins.** Since the MAb 18.1 epitope limited by pEX/DnaK fusion proteins was not confined by 16-mer peptides, we used a PCR-based recombinant fusion protein strategy to describe its full extension. Fusion protein vector pGEX-3X was used for analysis of the epitope. The pGEX/DnaK clones used are shown in Fig. 1 and Table 1. A pGEX/DnaK clone spanning most of DnaK from amino acids 16 to 660 (pGEX/DnaK$_{16–660}$) was previously constructed (22). A pGEX clone was constructed covering amino acids 462 to 503 (pGEX/DnaK$_{462–503}$), which are the limits of the epitope for MAb 18.1 as determined by pEX/DnaK fusion protein analysis. Further clones that extended beyond either end of pGEX/DnaK$_{462–503}$ were constructed. pGEX/DnaK$_{384–503}$ started at the beginning of the peptide-binding domain, at amino acid 384, while pGEX/DnaK$_{462–588}$ started at amino acid 462 and terminated at amino acid 588. Coomassie blue-stained SDS-PAGE of purified fusion proteins is shown in Fig. 3A. A gel run in parallel was transferred to nitrocellulose and reacted with MAb 18.1. In addition to pGEX/DnaK$_{16–660}$, MAb 18.1 reacted in an immunoblot assay with the four clones shown in Fig. 3B (lanes 2 to 5).

In contrast to MAb 18.1, MAb 22.2 showed binding to 16-mer peptides starting at amino acids 549 to 556. To determine the sequence required for high-affinity antibody binding, we also constructed pGEX/DnaK fusion proteins for this epitope. In addition to the fusion proteins used for mapping of the MAb 18.1 epitope (pGEX/DnaK$_{462–588}$ and pGEX/DnaK$_{16–660}$), a fusion protein covering the epitope limited by the pEX clone (pGEX/DnaK$_{549–588}$) and a fusion protein with its start at the beginning of the epitope and extending to the end of DnaK (pGEX/DnaK$_{549–660}$) were constructed. Furthermore, the insert of pEX/DnaK$_{541–617}$ was moved to pGEX (pGEX/DnaK$_{541–617}$). The fusion proteins were expressed in *E. coli*, purified, and analyzed by immunoblotting. Fusion protein pGEX/DnaK$_{549–588}$, covering the epitope limited by the pEX clones, was not detected in an immunoblot assay with MAb 22.2, but constructs pGEX/DnaK$_{541–617}$ and pGEX/DnaK$_{549–660}$ did react in an immunoblot assay (Fig. 3C).

**Confinement of the epitopes for MAbs 18.1 and 22.2 by competition ELISA.** To determine whether fusion protein pGEX/DnaK$_{462–503}$ represents the full epitope for MAb 18.1, the binding affinity of MAb 18.1 for the clones was compared with its binding to native *C. trachomatis* DnaK or to purified recombinant pGEX/DnaK$_{16–660}$. pGEX/DnaK fusion proteins were purified on glutathione beads and used to inhibit the binding of MAb 18.1 to DnaK as measured by ELISA (Fig. 4A). Clone pGEX/DnaK$_{462–503}$, with or without cleavage by factor Xa, did not significantly inhibit the binding of MAb 18.1 to DnaK compared with constructs without the epitope. However, when constructs that contained additional N-terminal (pGEX/DnaK$_{384–503}$) or C-terminal (pGEX/DnaK$_{462–588}$, pGEX/DnaK$_{462–660}$) DnaK sequences were used, the inhibitions were identical to that obtained with nearly full-length DnaK (pGEX/DnaK$_{16–660}$). Even though a weak reaction to pGEX/DnaK$_{462–503}$ with MAb 18.1 in an immunoblot assay

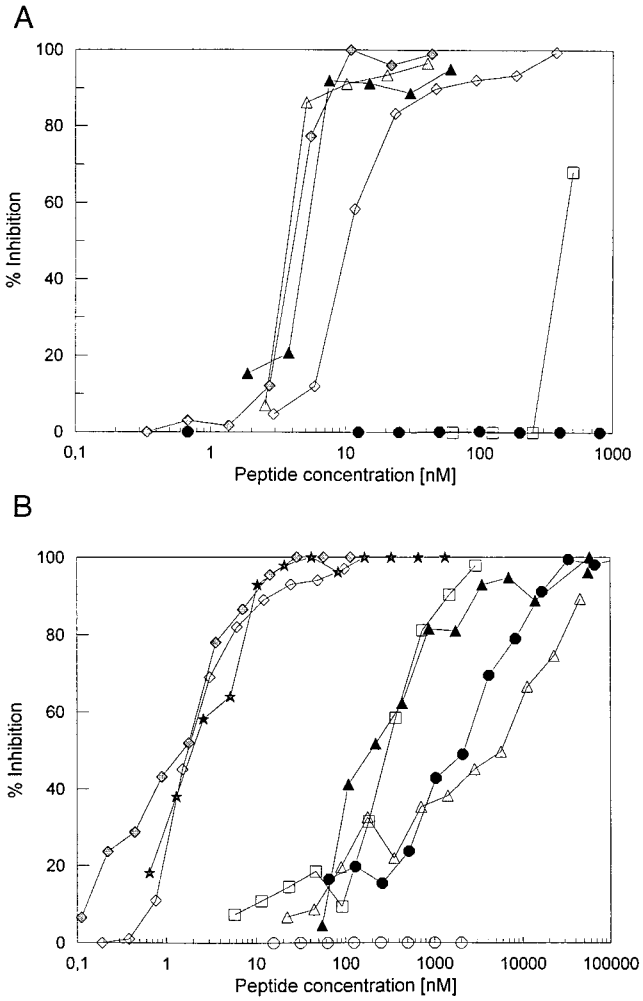


FIG. 4. Competition ELISA. Inhibition of binding of MAbs 18.1 (A) and 22.2 (B) to a full-length recombinant *C. trachomatis* L2 DnaK homolog by increasing amounts of recombinant fragments of the protein was measured by ELISA. Percent inhibition was calculated from optical density at 490 nm. Panel A symbols: ◈, pGEX/DnaK$_{16–660}$; ▲, pGEX/DnaK$_{384–503}$; □, pGEX/DnaK$_{462–503}$; ◇, pGEX/DnaK$_{462–588}$; △, pGEX/DnaK$_{462–588}\alpha$; ●, pGEX. Panel B symbols: ◈, pGEX/DnaK$_{16–660}$; ▲, pGEX/DnaK$_{462–588}$; □, pGEX/DnaK$_{462–588H}$; ◇, pGEX/DnaK$_{541–617}$; △, pGEX/DnaK$_{549–588}$; ●, pGEX/DnaK$_{549–588}\alpha$; ★, pGEX/DnaK$_{549–660}$; ○, pGEX.

was seen, inhibition of binding to native DnaK was obtained only when additional amino acids were present. Since the additional amino acids could be placed at either end of the fusion protein, it is likely that the additional amino acids are required for stabilization of the protein conformation.

Also, the fusion proteins reacting with MAb 22.2 were used for an inhibitory ELISA. Nearly full-length DnaK (pGEX/DnaK$_{16–660}$), construct pGEX/DnaK$_{549–660}$ with its start at the N-terminal limit of the epitope, and pGEX/DnaK$_{541–617}$ had identical inhibitions at a concentration of approximately 5 nM. In contrast, a construct starting at amino acid 462 and ending at the C-terminal limit of the epitope, pGEX/DnaK$_{462–588}$, as found by the pEX clones, had to be used at a 100-fold higher concentration than pGEX/DnaK$_{16–660}$ to obtain 50% inhibition (Fig. 4B).

By computer analysis, the secondary structure of the fragment covering amino acids 549 to 600 for *C. trachomatis* DnaK was predicted to be an α-helix. Furthermore, comparison of the secondary structure of all other known DnaK sequences homologous to amino acids 549 to 600 of *C. trachomatis* DnaK showed that this region, in general, was predicted to be an α-helix. To determine if an α-helix, in general, could stabilize the epitope for MAb 22.2, a DNA sequence encoding an amino acid sequence with high α-helix-forming potential was included in the primer for amplification of noninhibiting constructs pGEX/DnaK$_{462–588}$ and pGEX/DnaK$_{549–588}$. The clones in which an α-helix-encoding sequence was present were designated pGEX/DnaK$_{462–588}\alpha$ and pGEX/DnaK$_{549–588}\alpha$. By inhibitory ELISA, however, none of them were found to inhibit the binding of MAb 22.2 to native DnaK more than the original clones (Fig. 4B).

The concentrations of fusion proteins used for 50% inhibition of the binding of MAbs 22.2 and 18.1 to DnaK (Fig. 4) were nearly identical, but the slopes of the curves were lower for MAb 22.2, indicating that the binding affinity of MAb 22.2 for DnaK is lower than the corresponding binding affinity of MAb 18.1.

Nine DnaK sequences from the Swissprot protein database were aligned. Compared with the other DnaK sequences, the homology is high until amino acid 488, after which the sequences differ. The DnaK sequences of *C. trachomatis* and *C. pneumoniae* differ by only four amino acids in the region spanning amino acids 462 to 503. This finding is in agreement with the notion that MAb 18.1 is genus specific (Table 2). Despite the high homology seen from amino acids 462 to 488, none of the other microorganisms reacted with MAb 18.1 (Table 2). In contrast, the sequence from amino acids 549 to 588 had only 50% identity between *C. trachomatis* and *C. pneumoniae* and less to the other DnaK sequences. This is in agreement with the idea that MAb 22.2 is species specific (Table 2).

## DISCUSSION

In contrast to B-cell epitopes, T-cell epitopes correspond to the primary amino acid sequence of a protein, because short proteolytic fragments of the protein which present the epitopes to T cells are present in the groove of the HLA class II molecule. B-cell epitopes on proteins are more complex and can be either conformational or continuous. A continuous epitope is located within a continuous segment of amino acids in the primary sequence of the protein. A conformational epitope consists of residues from different parts of the primary sequence that are apart but are brought together upon folding of the protein into its tertiary structure (3). Since B cells recognize the surface of a molecule, most epitopes are conformational and the epitopes determined by cocrystallizing antigen and antibody appear to be conformational (23). Many MAbs react with proteins in immunoblot assays even though the protein samples used for immunoblotting have been boiled in SDS sample buffer and separated under denaturing conditions. However, by transfer of the denatured proteins to nitrocellulose, the SDS may be sufficiently removed to allow renaturation of the proteins on the nitrocellulose. Young et al. (41) showed that this was the case with a MAb against apolipoprotein B-100 that reacted in an immunoblot assay. In this protein, two amino acid sequences separated by 53 amino acids proved to be necessary to obtain strong binding of the antibody to the recombinant apolipoprotein B-100 fusion protein. This indicates that refolding of the antigen had occurred on the nitrocellulose sheet. In the present investigation, we analyzed the structures of two epitopes localized within the C-terminal peptide-binding part of *C. trachomatis* DnaK. We used synthetic peptides, immunoblotting of *C. trachomatis* DnaK fusion proteins, and a competition ELISA to find the minimal part of

DnaK to which the MAbs bound with the same specificity as full-length *C. trachomatis* DnaK.

The immunogenicity of bacterial DnaK has been investigated in several studies, and both polyclonal antibodies and MAbs against the N-terminal ATPase domain of DnaK have been described (5, 21, 42). These studies have indicated the presence of linear epitopes in this domain. In contrast to the N-terminal domain, only few antibodies to the C-terminal peptide-binding domain have been detected despite intensive investigations. Therefore, this domain has been considered to be less immunogenic than the ATPase domain. For the C-terminal peptide-binding domain of *M. leprae* DnaK, however, Peake et al. (33) obtained three MAbs. By recombinant DNA techniques, those investigators mapped the epitopes to 142 amino acids but only one of the MAbs was partially inhibited by an 18-mer peptide in a fluid-phase inhibition ELISA, similar to the results presented here.

Because of the pronounced conservation of the N-terminal part of DnaK (14), antibodies to this part of DnaK produced upon bacterial infection cross-react with the N-terminal part of DnaK from other bacterial species. Therefore, since only full-length DnaK has been used as an antigen in an immunoblot assay, it is not known whether a specific immune response against DnaK is induced upon bacterial infection. Consequently, the only way to measure a species-specific humoral antibody response to a given DnaK protein is to use the variable peptide-binding domain, or a part of it, as an antigen in an ELISA or an immunoblot assay. Therefore, we speculated whether anti-DnaK MAbs generated by immunization of mice with whole microorganisms could detect fragments of the peptide-binding domain with the same specificity as for detection of native DnaK. To address this issue, we used synthetic peptides and recombinant proteins corresponding to specific parts of the C-terminal part of DnaK from *C. trachomatis* as antigens in an ELISA and an immunoblotting assay with two different anti-DnaK MAbs.

By inhibitory ELISA, MAb 18.1 was found to bind pGEX/$DnaK_{383–503}$ and pGEX/$DnaK_{462–588}$ with 100% affinity relative to full-length DnaK, whereas the affinity for pGEX/$DnaK_{462–503}$ was only 1%. It is therefore suggested that the epitope recognized by MAb 18.1 is conformational. This is also supported by the observed lack of binding of MAb 18.1 to 16-mer synthetic peptides corresponding to fragments of the C-terminal part of DnaK.

The results obtained by immunoblotting were similar to those of the inhibitory ELISA, indicating that the fusion proteins on the nitrocellulose may have obtained a folded conformation.

Similar results were obtained by using MAb 22.2, demonstrating the presence of at least two conformational epitopes in the C-terminal part of *C. trachomatis* DnaK. We therefore suggest that the C-terminal part of DnaK is stably folded.

Analysis of the folding-unfolding of the *E. coli* DnaK domains by calorimetric scans has shown that the 44-kDa N-terminal and 23-kDa C-terminal proteolytic fragments of this protein are stable, folded structures (29). In the present study, we showed that MAbs 18.1 and 22.2 could detect fragments of approximately 8 kDa, indicating that these fragments are in a conformation similar to that in the native DnaK protein. It was, however, not possible to identify the specific amino acids involved in the antibody binding.

The conserved nature of bacterial heat shock proteins makes these proteins obvious candidates as triggering molecules in the development of immunological complications associated with infectious diseases. In acute reactive arthritis, a previous genital or gastrointestinal infection is thought to mediate the disease. In a recent study, we analyzed the immune response of patients with acute reactive arthritis by immunoblotting of patient sera by using purified EBs or recombinant DnaK as the antigen (22). Because of cross-reacting antibodies to the ATPase domain, in that study it was not possible to determine whether antibodies to DnaK were species specific. For detection of species-specific antibodies to DnaK, it is necessary to detect antibodies to the variable peptide-binding domain since only nonlinear epitopes seem to be present in this region (this study and reference 33). This might also explain why Zhong and Brunham (42), using hyperimmune rabbit antiserum and 10-mer synthetic peptides covering the complete *C. trachomatis* DnaK sequence, failed to detect any significant reaction to the peptides corresponding to the C-terminal part of the molecule. Therefore, serological detection of species-specific antibodies to DnaK requires the use of fusion proteins of sufficient size to cover the C-terminal part of DnaK. It is hoped that the fusion proteins described here will be valuable for serodiagnosis of patients with chlamydial infections.

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