Novel HLA-B27-restricted Epitopes from *Chlamydia trachomatis* Generated upon Endogenous Processing of Bacterial Proteins Suggest a Role of Molecular Mimicry in Reactive Arthritis*

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**Background:** Reactive arthritis is an HLA-B27-associated disease triggered by *Chlamydia trachomatis*, a frequent intracellular parasite. HLA-B27-restricted T-cell responses are elicited against this bacterium in ReA patients, but their pathogenetic significance, autoimmune potential, and relevant epitopes are unknown. High resolution and sensitivity mass spectrometry was used to identify HLA-B27 ligands endogenously processed and presented by HLA-B27 from three chlamydial proteins for which T-cell epitopes were predicted. Fusion protein constructs of ClpC, Na⁺-translocating NADH-quinone reductase subunit A, and DNA primase were expressed in HLA-B27⁺ cells, and their HLA-B27-bound peptidomes were searched for endogenous bacterial ligands. A non-predicted peptide, distinct from the predicted T-cell epitope, was identified from ClpC. A peptide recognized by T-cells in vitro, NQRA(330–338), was detected from the reductase subunit. This is the second HLA-B27-restricted T-cell epitope from *C. trachomatis* with relevance in ReA demonstrated to be processed and presented in live cells. A novel peptide from the DNA primase, DNAP(211–223), was also found. This was a larger variant of a known epitope and was highly homologous to a self-derived natural ligand of HLA-B27. All three bacterial peptides showed high homology with human sequences containing the binding motif of HLA-B27. Molecular dynamics simulations further showed a striking conformational similarity between DNAP(211–223) and its homologous and much more flexible human-derived HLA-B27 ligand. The results suggest that molecular mimicry between HLA-B27-restricted bacterial and self-derived epitopes is frequent and may play a role in ReA.

**Molecular Mimicry**

MHC class I (MHC-I) molecules present endogenous peptides derived from self-proteins or intracellular pathogens at the cell surface for recognition by cytotoxic T lymphocytes (CTL). HLA-B27, an allotype that is present worldwide, shows one of the strongest associations between MHC-I and a human disease (1–3). This association concerns a group of inflammatory rheumatic diseases termed spondyloarthropathies, which include ankylosing spondylitis (AS), where this allele occurs in about 90% of patients, and reactive arthritis (ReA), where the prevalence of HLA-B27 is less well defined but probably around 30–50% (4). This latter disorder is triggered by various Gram-negative bacteria (5). Although it is frequently a self-limited disease, ReA evolves sometimes toward AS, particularly among HLA-B27⁺ individuals. In contrast to AS, where HLA-B27 is probably a true pathogenic factor, epidemiologic and other studies suggest that in ReA, it may influence the severity of clinical manifestations rather than being a truly causative allele (4, 6, 7).

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6 The abbreviations used are: CTL, cytolytic T lymphocyte(s); AS, ankylosing spondylitis; β₂m, β₂-microglobulin; DNAP, DNA primase; EGFP, enhanced GFP; C1R, Hmy2.C1R; MD, molecular dynamics; NQRA, Na⁺-translocating NADH-quinone reductase subunit A; PqqC, pyrroloquinoline-quinone synthase-like protein; ReA, reactive arthritis; rep, representative structure; RMSD, root mean square deviation; RMSF, root mean square fluctuation; RT, retention time.
Chlamydia trachomatis is a major agent in sexually transmitted infections (8). It is often asymptomatic, highly persistent, and difficult to detect by conventional diagnostic tests. It is an obligate intracellular pathogen, which infects mucosal epithelial cells, vascular endothelial cells, and other cells, such as monocytes and macrophages (9), and is one of the main pathogenic agents in ReA.

C. trachomatis has developed multiple strategies to evade the immune system, including modulation of host cell apoptosis (10–14) and replication inside a specialized vacuole, called the inclusion, which limits its exposure to antibodies and to the antigen-processing machinery (15). A third mechanism is associated with secretion of IFN-γ by immune cells. This cytokine inhibits bacterial growth through deprivation of the tryptophan pool, which leads to bacterial persistence under subinhibitory IFN-γ concentrations (16, 17). Finally, C. trachomatis secretes a protease into the cytosol of the infected cell, the chlamydial protease-like activating factor, that degrades transcription factors for MHC, inhibiting the expression of MHC-I and -II at the cell surface shortly after infection (18–21). Despite this, both CD4+ and CD8+–mediated immune responses are activated upon infection (22).

The pathogenetic role of HLA-B27 in spondyloarthropathies remains ill defined. Among the various proposed mechanisms (23), the arthritogenic peptide hypothesis (24) claims that a bacterial peptide presented by HLA-B27 would elicit a CTL response cross-reactive with a self-derived B27 ligand showing antigenic mimicry, thus breaking the self-tolerance and triggering an autoimmune attack (25). Although this mechanism does not satisfactorily explain AS pathogenesis, because the HLA-B27-associated spondyloarthropathy in transgenic rats does not require CD8+ T-cells (26), it may well play a role in exacerbating the proinflammatory nature of HLA-B27, particularly in ReA. Indeed, splenocytes from rats immunized with HLA-B27 and stimulated in vitro with Chlamydia-treated cells from HLA-B27 transgenic rats resulted in the generation of Chlamydia-specific CD8+ T-cells (27). Moreover, splenocytes from HLA-B27 transgenic rats immunized with HLA-B27 developed HLA-B27-directed autoreactivity upon exposure to C. trachomatis in vitro (28). The immunological relationship between Chlamydia and HLA-B27 revealed by these studies was suggestive of molecular mimicry between bacterial and self-derived HLA-B27-restricted epitopes. Despite difficulties in substantiating molecular mimicry as a mechanism of autoimmunity (29), it played a key role in the pathogenesis of Chlamydia-induced autoimmune myocarditis in mice (30). Thus, there is a sound basis to search for HLA-B27-restricted chlamydial T-cell epitopes and their possible relationship to self-derived HLA-B27 ligands (31).

Predictive binding and proteasomal cleavage algorithms were used to localize putative chlamydial epitopes. The candidates were tested for recognition by specific CTL from transgenic mice or HLA-B27+ ReA patients (32) or for generating B27 tetramers to detect peptide-specific T-cells (33). These studies identified some HLA-B27-restricted epitopes for which specific CTL could be found in Chlamydia-infected ReA patients. However, due to the intrinsic cross-reactivity of T-cells (34), recognition of a synthetic peptide in vitro does not guarantee that this peptide is the actual immunogenic epitope in vivo.

The direct biochemical identification of endogenous chlamydial T-cell epitopes from infected cells has been accomplished only in the mouse system (35, 36). It is hardly feasible in humans, due to the very low amounts of bacterial epitopes on infected cells, the difficulties associated with working with large amounts of Chlamydia-infected human cells, and, especially, the down-regulation of MHC-I expression and induction of apoptosis by C. trachomatis (19, 37). Thus, we developed an alternative strategy involving the stable expression of chlamydial fusion proteins on HLA-B27+ human cells. Endogenously processed chlamydial peptides, including a predicted T-cell epitope, were identified by comparing the HLA-B27-bound peptidomes from transfected and untransfected cells. These studies (38, 39) were based on comparative MALDI-TOF MS and concerned three chlamydial proteins containing sequences highly homologous to known human-derived HLA-B27 ligands or from which synthetic peptides were recognized by CTL from ReA patients: DNA primase (DNAP) (CT794), Na+-translocating NADH-quinone reductase subunit A (NQRA) (CT634), and pyrroloquinoline-quinone synthase-like protein (PqqC) (CT610).

In two different studies, based on a predictive search for HLA-B27-restricted chlamydial ligands in ReA patients (32, 33), a sequence from ClpC protein, spanning residues 7–15, was recognized as a synthetic peptide by CD8+ T-cells from multiple individuals, suggesting that this epitope could be immunodominant. Here we used MS techniques of high sensitivity and accuracy to investigate the endogenous processing and presentation of this and other HLA-B27-restricted peptides from ClpC and other chlamydial proteins. Molecular dynamics simulations were also carried out to analyze the relationship between chlamydial and homologous human-derived B27 ligands at the conformational level.

EXPERIMENTAL PROCEDURES

ClpC Gene Constructs—Enhanced GFP (EGFP)-ClpC fusion proteins were generated by fusing the cDNA of the clpC gene (CT286) of C. trachomatis serovar L2 (Advanced Biotechnologies, Columbia, MD) or truncated forms of it in frame to the 3’-end of the EGFP gene. Full-length cDNA of ClpC was amplified by PCR using the following primers: 5’-CTCTCTCTAGATCTATGTGGAGATTTACATCG and 3’-CTCTCTCTAGATCTATGTGGAGATTTACATCG. The PCR products were cloned into the pEGFP-C1 vector (BD Biosciences, Columbia, MD) or truncated forms of it in frame to the 3’-end of the EGFP gene. Full-length cDNA of ClpC was amplified by PCR using the following primers: 5’-CTCTCTCTAGATCTATGTGGAGATTTACATCG and 3’-CTCTCTCTAGATCTATGTGGAGATTTACATCG. The PCR products were cloned into the pEGFP-C1 vector (BD Biosciences, Clontech) using 5’ BglII and 3’ Sall restriction sites. Two constructs were made based on the EGFP-CT286 plasmid sequence and the internal restriction sites BglII at 5’ and ApaI at 3’, respectively.

Cell Culture and Transfections—Stable transfectants were generated as described previously (38). Briefly, The EGFP-ClpC constructs were co-transfected by electroporation in C1R-B+B27:05 cells (40), with the RSV5 vector (41) containing the hygromycin resistance gene. The transfected cells were selected with 250 μg/ml hygromycin (Invitrogen). All cell lines were cultured in RPMI 1640 medium, supplemented
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with 10% FBS, 200 mM l-Gln, 25 mM HEPES, streptomycin, and penicillin.

Flow Cytometry—The C1R transfectants were analyzed by measuring their EGFP-associated fluorescence. Briefly, 1 × 10⁶ cells were washed twice with 200 µl of PBS and centrifuged at 1500 rpm for 5 min. The detection was carried out in a flow cytometer FACSCalibur (BD Biosciences). All data were acquired using CellQuest™ Pro version 4.0.2 software (BD Biosciences) and analyzed using FlowJo version 7.5 (Tree Star, Inc.).

Immunoprecipitation and Western Blot—About 2 × 10⁶ cells were lysed in 0.5% Igepal CA-630 (Sigma), 5 mM MgCl₂, 50 mM Tris-HCl, pH 7.4, containing protease inhibitors (Complete Mini, Roche Applied Science) for 30 min. After centrifugation, the lyse supernatants were pre cleared with anti-rabbit IgG beads (TrueBlot, eBioscience, San Diego, CA) and immunoprecipitated for 3 h with the rabbit anti-GFP polyclonal antibody (A6455) (Invitrogen) coupled to anti-rabbit IgG beads, at 4 °C and continuous shaking. Immunoprecipitates were washed three times, denatured for 5 min in sample buffer, subjected to 10% SDS-PAGE, and transferred overnight to a nitrocellulose membrane (Amersham Biosciences Hybond-ECL, GE Healthcare) at 20 V and 4 °C. The immunodetection was carried out using the A6455 antibody and horseradish peroxidase-conjugated anti-rabbit IgG (TrueBlot, eBioscience, San Diego, CA) at 1:1000 and 1:5000 dilutions, respectively. Antibodies were diluted in blocking buffer containing 5% nonfat dry milk, 0.1% Tween 20, PBS, pH 7.4. The immunoblots were developed using the ECL immunodetection system (Amer sham Biosciences).

Isolation of HLA-B27-bound Peptides—B*27:05-bound peptides were isolated from about 1 × 10¹⁰ cells or, for some analyses, 1 × 10⁹ C1R-B*27:05 cells, as described previously (42). Briefly, cells were lysed in the presence of a mixture of protease inhibitors (Complete, Roche Applied Science). The soluble fraction was subjected to affinity chromatography using the W6/32 mAb (IgG2a; specific for a monomorphic HLA class I determinant) (43). HLA-B27-bound peptides were eluted with 0.1% aqueous TFA at room temperature, filtered through CentriTIP devices (Amicon, Beverly, MA), concentrated, and desalted and concentrated with Micro-Tip reverse-phase columns (Millipore) and analyzed by MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA) as described previously (45), with minor modifications. Briefly, the peptides were eluted at flow rates of 0.25 µl/min, with linear gradients of 7–40% acetonitrile in 0.1% formic acid, for 90 min, followed by 17 min at 95% acetonitrile in 0.1% formic acid. In some cases, the same gradient was used during 214 min, with a final isocratic elution for 29 min. The spectra were collected in the Orbitrap mass analyzer using full ion scan mode over the mass-to-charge (m/z) range 400–2000, which was set to 60,000 resolutions. The most intense seven masses from each full mass spectrum, with single, double, and triple charge states, were selected for fragmentation by collision-induced disintegration in the linear ion trap.

Electrospray-LTQ-Velos MS/MS—Particular peptides were searched in 10 µl of individual HPLC fractions by MS/MS in a dual mode, using selected multiple ion monitoring and dynamic exclusion mode in an LTQ-Velos instrument. Briefly, each particular fraction was dried down and resuspended in 9 µl of 0.1% formic acid and analyzed in an Agilent 1100 system coupled to a linear ion trap LTQ-Velos mass spectrometer (Thermo Fisher Scientific). The peptides were separated by reverse phase chromatography using a 0.18 × 150-mm BioBasic C18 RP column (Thermo Fisher Scientific) and eluted using an 80-min gradient from 5 to 40% solvent B (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid, 80% acetonitrile in water) at 1.8 µl/min. Peptides were detected in selected multiple ion monitoring mode at single, double, and triple charged states. In parallel to the selected multiple ion monitoring mode, a full ion scan over the m/z range 400–2000 (1–µs scans) was also performed, followed by data-dependent MS/MS scans, using an isolation width of 2 m/z units and normalized collision energy of 35%, and dynamic exclusion was applied for 30 s. Alternatively, 10–µl aliquots of various consecutive HPLC fractions were pooled together and analyzed in the same way. The synthetic peptides were detected using only the selected multiple ion monitoring mode as above, except that a 35-min elution gradient was used.

Database Searches—The Mascot server 2.2 (Matrix Science Inc., Boston, MA) (46) was used as the main search engine. The search parameters were 0.5 Da mass tolerance for both precursor and fragment ions for MS/MS spectra from LTQ-Velos and 0.01 and 0.5 Da for precursor and fragment ions, respectively, for data from LTQ-Orbitrap. Met oxidation and Asn and Gln deamidation were selected as variable modifications. A small sequence database consisting of the chlamydial ClpC (Swiss-Prot accession B0B7K2), DNAP (B0B920), and NQRA (O84639) sequences as well as HLA-B27 (P03989), HLA-B35 (P30685), HLA-C04 (P30504), and EGFP (GenBank™ accession AA02576.1) was used for the specific search of chlamydial peptides. In addition, all raw files were run against the human subset of the Uniprot database (release 57.6, 07/2009, with 20,331 entries), using the same parameters described above. Those sequences showing the highest scores in these preliminary searches were analyzed manually and validated by comparison with the experimental MS/MS spectrum of the corresponding synthetic peptide.

The search for homology between chlamydial peptides and human proteins was carried out using the UniProtKB/Swiss-Prot database (release 07/2012, with 20,231 entries) and the BLASTP 2.2.26+ software.
Proteasome Cleavage Predictions—Proteasome/immuno-proteasome cleavage was predicted with previously described algorithms (47) available on the Proteasome Cleavage Prediction Server.

Homology Modeling—Three-dimensional models for the complexes between B*27:05/β2m and DNAP(211–221), DNAP(211–223), or B27(309–320) were built by homology modeling. A total of 23 x-ray structures of HLA-B27-peptide complexes were aligned using the MAFFT software (48). Because all of the x-ray complexes contained bound 9-mers, the alignments of these peptides with the longer ones in our study was done by introducing gaps at internal peptide positions. The four N-terminal and two C-terminal positions on each peptide were constrained, whereas certain flexibility was allowed for their central parts. B*27:05 in complex with the pVIPR(400–408) peptide in its canonical conformation (Protein Data Bank code 1OGT) (49) was finally selected as template, due to its high resolution (1.47 Å), and the alignment was subjected to homology modeling using the MODELLER program.

Setup of the Systems and Molecular Dynamics (MD) Simulations—For each HLA-B27-peptide complex, the setup entailed the following steps: (a) adding missing heavy and hydrogen atoms (50) to assign atom types and charges according to AMBER ff10 force field (51) and to determine the protonation state of ionizable residues at pH 7; (b) employing the treep module from the AmberTools package (52) to immerse each system within a 10-Å box of TIP3P (53) explicit water molecules and to add Na+ counterions; (c) energy-minimizing the positions of water molecules and ions using the conjugated gradient method for 3000 steps while the atomic coordinates in the complexes were kept constrained, followed by equilibration at 298 K for 10 ps, maintaining the constraints; (d) transforming the constraints into progressively lower restraints and energy-minimizing the whole complexes, including the water molecules and the ions, as above.

MD simulations were carried out starting from the energy-minimized structures. All calculations were performed with the NAMD version 2.8 program (54) using constant temperature (298 K) and pressure (1 atm). Short and long range forces were calculated every one and two time steps, respectively (each time step = 2.0 fs), constraining the covalent bonds involving hydrogen atoms to their equilibrium values. Long range electrostatic interactions were accounted for using the particle mesh Ewald approach (55). The systems were heated up to 298 K and then equilibrated at this temperature for 200 ps. The equilibration was performed under harmonic restraint conditions on all of the heavy atoms. These restraints were gradually reduced until they were almost removed. Finally, these equilibrated structures were further simulated for an additional 50 ps with a minimal restraint. These were the starting points for a 30-ns MD production period during which the system coordinates were collected every 2 ps for further analysis.

Analysis of MD Trajectories—The stability of a given complex was evaluated by calculating the root mean square deviation (RMSD) of the Cα atoms along the trajectories, using as reference their starting structures. Additionally, the root mean square fluctuation (RMSF) of each residue, relative to the corresponding average value, was calculated once each snapshot had been fitted to its initial structure. Further analysis was carried out by clustering the sampled conformational space during the trajectory production period (last 10 ns), using the ptraj module from the AmberTools package, the snapshots sampled as described above, and the average linkage algorithm based on the peptide backbone atoms. Adaptive Poisson-Boltzmann Solver (56, 57) was used to perform the Poisson-Boltzmann electrostatic calculations for the most representative structures in each cluster. Dielectric constants were set to 4 and 80 for protein and solvent, respectively. Other parameters were set as default. The free energy of binding between each peptide and the B*27:05 molecule was calculated by the MM-ISMSA approach (58). We also calculated the pairwise decomposition of the free energy of binding following the scheme developed in MM-ISMSA to determine the main residues responsible for the interaction of the peptides with B*27:05. Mean and S.D. for the free energy of binding was calculated for the MD trajectories fit to a normal frequency distribution using R (59). Contacts between residues were analyzed following the MM-ISMSA methodology.

RESULTS

Expression of Chlamydial ClpC Fusion Proteins—ClpC is an ATP-dependent protein-unfolding subunit of the bacterial ClpCP protease complex (60, 61). In C. trachomatis, it has 854 amino acid residues and binds ATP through two nucleotide-binding domains, AAA+ (Fig. 1A). EGFP-ClpC fusion proteins were expressed in C1R-B*27:05 cells in order to detect endogenously processed HLA-B27 ligands from this protein, including a predicted T-cell epitope, ClpC(7–15). Our initial attempts to express the whole ClpC protein using full-length cDNA failed to generate stable C1R transfectants. To avoid functional interference of the ClpC protein in human cells, two fusion protein constructs, ClpC(1–570) and ClpC(1–512), with partial or total deletions of the C-terminal AAA+ domain, were made in which residues 1–570 or 1–512, respectively, were fused at the C-terminal end of EGFP (Fig. 1A). Stable transfectants in C1R-B*27:05 cells were obtained for both constructs, whose expression levels and correct size were determined by flow cytometry (Fig. 1B) and Western blot (Fig. 1C), respectively. The ClpC(1–512) transfectant in C1R-B*27:05 was used for further experiments, due to its higher expression compared with ClpC(1–570).

One ClpC-derived Ligand Distinct from the Predicted T-cell Epitope Is Endogenously Presented by HLA-B*27:05 on C1R Cells—A first approach to search for endogenously processed ClpC-derived HLA-B27 ligands was the comparative analysis of HLA-B27-bound peptides from untransfected C1R-B*27:05 cells and the ClpC(1–512) transfectant, based on identity of chromatographic retention time (RT) and molecular weight, through systematic comparison of the MALDI-TOF MS spectra from correlated HPLC fractions. Although this strategy was successful in previous studies with other fusion proteins (38, 39), it failed to identify any ClpC-derived peptides. Thus, two further approaches were undertaken (Fig. 1D). The first one involved high throughputs sequencing, using LTQ-Orbitrap MS/MS, performed on the unfraccionated B27-bound peptide pool from ClpC(1–512)-transfected C1R-B*27:05 cells.
The second one involved a targeted search for specific candidates in the fractionated B27-bound peptide pool performed on HPLC fractions at the RT ± 3 min of each of the corresponding synthetic peptides. The relevant HPLC fractions, either individually or pooled together, were subjected to MS/MS fragmentation of all ions corresponding to the m/z ratios of the candidate peptide, using a LTQ-Velos mass spectrometer.

The MS/MS spectra from the unfractionated B27 peptidome from the ClpC(1–512) transfectant obtained in the LTQ-Orbitrap were searched against a small database including ClpC and a few other chlamydial proteins. Two putatively significant matches with sequences containing the canonic B27 binding motif R2 from ClpC were obtained. Manual inspection of the corresponding MS/MS spectra showed a good match with the theoretical fragmentation of only one of these sequences, SRLDPVIGR, spanning ClpC residues 203–211 (Fig. 2A). A search against the human proteome database did not show a match of this MS/MS spectrum with any human peptide. SRLDPVIGR did not match any human sequence upon BLAST analysis, confirming the bacterial origin of this peptide.

We next determined whether this peptide was just overlooked in our previous MALDI-TOF comparison or hidden by a co-eluting human HLA-B27 ligand. For this purpose, the RT of the synthetic peptide in the same chromatographic condition was determined.
FIGURE 2. Identification of the chlamydial B*27:05 ligand SRLDPVIGR from ClpC(1–512) transfec tant cells. A, MS/MS spectra of the [M + 2H]^{2+} ion peaks at m/z 506.80 detected in the LTQ-Orbitrap from the unfractionated HLA-B27 peptidome (top) or in the LTQ-Velos from fraction 142 of the HPLC-fractionated HLA-B27 peptidome (middle) and the synthetic SRLDPVIGR peptide, corresponding to residues 203–211 of the ClpC protein (bottom). B, MS/MS spectrum of the [M + 3H]^{3+} ion peak at m/z 338.20 detected in a pool of HPLC fractions at the RT ± 3 min of the synthetic peptide, using an LTQ-Velos mass spectrometer (top) and of the synthetic peptide corresponding to residues 203–211 of the ClpC protein (bottom).

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ions was determined, and the fractions corresponding to its RT ± 3 min were fragmented in a LTQ-Velos mass spectrometer. Parental ions with m/z 506.80 and 338.20, compatible with the [M + 2H]^{2+} and [M + 3H]^{3+} forms of the chlamydial SRLDPVIGR peptide, respectively, were detected in fraction 142. The MS/MS spectrum of the former ion showed virtual identity with those from the LTQ-Orbitrap and the synthetic peptide (Fig. 2A). This assignment was further confirmed by the identity of the MS/MS spectrum of the ion with m/z 338.20 with that of the [M + 3H]^{3+} ion of the synthetic SRLDPVIGR (Fig. 2B). Comparative MALDI-TOF analysis of fraction 142 and adjacent ones confirmed the presence of a co-eluting self-derived B*27:05 ligand, as revealed by an ion peak with m/z 1012.53, identical to the [M + H]^{+} of SRLDPVIGR, in cells lacking the chlamydial fusion protein (data not shown). This explains our failure to detect this bacterial peptide by MALDI-TOF.
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TABLE 1
Chlamydial HLA-B27 ligands processed in vivo from endogenous fusion proteins

| Peptide       | Source protein | Residues | Source/Reference | Predicted |
|---------------|----------------|----------|------------------|-----------|
| KRALETVI      | NQRA           | 86–94    | Ref. 39          | No        |
| MRDHITL       | NQRA           | 330–338  | This study       | Yes       |
| RFRNREAQBF    | DNA primase    | 112–121  | Ref. 38          | NA        |
| RFRNREAQFRP   | DNA primase    | 112–122  | Ref. 38          | NA        |
| RRFKEGGGRGK   | DNA primase    | 211–221  | Ref. 38          | NA        |
| RRFKEGGGRGKYI | DNA primase    | 211–223  | This study       | NA        |
| SELDPYTOR     | ClpC           | 283–211  | This study       | No        |
| ARKLLDLNL     | PqqC-like protein | 70–78   | Ref. 39          | Yes       |

* A combination of predictive binding and proteasome cleavage algorithms was used in a previous study to scan the proteome of *C. trachomatis* for potential HLA-B27-restricted nonameric epitopes, followed by antigen recognition assays in vitro. Only nonamers were searched (32). NA, not applicable.

Because the Orbitrap-based sequencing described above failed to detect the predicted T-cell epitope ClpC(7–15), NRAKQVIKL, an alternative approach was used for the specific search of this and the related peptide ClpC(7–17), NRAKVVIKL, which also has the B*27:05 binding motif, in the HPLC-fractionated HLA-B27-bound peptide pool from the ClpC(1–512) transfectant. Both peptides were synthesized and used for a targeted search (Fig. 1D), monitoring the m/z ratios corresponding to [M + 2H]^{2+} and [M + 3H]^{3+} ions of both peptides. These analyses failed to show any reliable fragmentation compatible with ClpC(7–15) or ClpC(7–17).

**Novel Chlamydial Peptides from Other Proteins Processed and Presented by HLA-B27 in Live Cells**—Several chlamydial peptides endogenously processed and presented by HLA-B27 were identified in previous studies from our laboratory (38, 39) by comparative MALDI-TOF MS of HPLC-fractionated B27-bound peptide pools from C1R-B*27:05 transfectants expressing chlamydial NQRA, PqqC, or DNAP fusion proteins (Table 1). Due to the limitations of this approach, revealed by our results on ClpC, a search for novel peptides from NQRA and DNAP was undertaken, using more sensitive MS techniques.

**NQRA**—The NQRA(330–338) peptide, MRDHITL, was recognized in vitro, as a synthetic peptide, by CD8+ T-cells from a ReA patient (32), but it was not found in C1R-B*27:05 cells expressing the EGFP-NQRA(1–465) fusion protein in a MALDI-TOF-based study (39). Thus, the most intense ions in the full MS spectrum of the pooled fractions corresponding to the RT ± 3 min of the synthetic peptide in the fractionated HLA-B27-bound peptide pool from the EGFP-NQRA(1–465) transfectant were subjected to MS/MS fragmentation. The MS/MS spectrum of one of the main ion peaks in the full MS scan, with m/z 558.33, was compatible with the [M + 2H]^{2+} species of the oxidized form of MRDHITL. Its correct assignment was confirmed by comparison with the MS/MS spectrum of the corresponding synthetic peptide in its oxidized form (Fig. 3). This result demonstrates the endogenous processing and presentation by HLA-B27 of the predicted chlamydial epitope NQRA(330–338) in NQRA transfecant cells. This is the second HLA-B27-restricted T-cell epitope with demonstrated relevance in Chlamydia-infected ReA patients that has been shown to be generated in live cells.

**DNAP**—The unfraccionated HLA-B27-bound peptide pool from C1R-B*27:05 transfected with the EGFP-DNAP(90–450) fusion protein (38) was subjected to MS/MS analysis in an LTQ-Orbitrap mass spectrometer and searched against a small database including the chlamydial DNAP fusion protein sequence. A parental ion of m/z 508.62, compatible with DNAP(211–223) (RRFKEGGRGKKGYI) was identified (Fig. 4A). This peptide was two residues longer than one previously found from this protein, DNAP(211–221) (Table 1). Both sequences show high homology with a natural ligand of HLA-B27, arising from the endogenous processing of the HLA-B27 heavy chain, B27(309–320) (RRKSSGGKGGSY) (62). To confirm the tentative assignment from the Orbitrap analysis, a targeted search for this peptide (Fig. 1D) was carried out in the HPLC-fractionated B27-bound peptide pool from the DNAP transfectant, focusing on the m/z values corresponding to the [M + H]^{+}, [M + 2H]^{2+}, and [M + 3H]^{3+} forms of DNAP(211–223). The analysis revealed the presence of this peptide as the charge variants [M + 3H]^{3+} (m/z 508.62) (Fig. 4A) and [M + 2H]^{2+} (m/z 762.43) (Fig. 4B), whose identity was confirmed by comparison with the MS/MS spectra of the synthetic peptide.

**High Homology between the ClpC and NQRA-derived HLA-B27 Ligands and Human Sequences**—To explore the possible molecular mimicry between the B27-restricted peptides from *C. trachomatis* found in this study and putative self-derived HLA-B27 ligands, we looked for human sequences showing high homology to ClpC(203–211) and NQRA(330–338). The search was performed against the human proteome, looking for sequences containing >50% amino acid identity with the bacterial peptides and the main binding motif of HLA-B27 ligands, R2. Only human sequences with residues present among known HLA-B27 ligands (63, 64) with a frequency of >1% at the anchor P1, P3, and PΩ positions were considered. Multiple human sequences homologous to the ClpC- and NQRA-derived peptides were found (Table 2). Most of the sequences showed predictive scores compatible with proteasome/immunoproteasome cleavage at their C-terminal residue (>0.5).

**MD Simulation of Chlamydial DNAP and Homologous Human-derived HLA-B27 Ligands**—To explore the similarity of DNAP(211–221) and DNAP(211–223) with B27(309–320) at the three-dimensional level, comparative MD simulation of their interaction in complex with B*27:05 was carried out. The initial, energy-minimized, three-dimensional structures of the complexes involving the three peptides, all built by homology modeling, and pVIPR(400–408) in its canonical conformation were subjected to MD simulations for 30 ns. After this time, the stability of the trajectories was analyzed. Both the mean Ca RMSD and the mean RMSF for the B*27:05 heavy chain and βm were similar among the three complexes (Fig. 5, A and B). In contrast, the mean RMSD and RMSF values for the peptides were more variable, spreading from 0.58 to 2.25 Å and from...
about 2.0 to 2.4 Å, respectively, in the different complexes (Fig. 6, A and B). Large RMSF values (above 3.0 Å) were observed for certain residues (Fig. 6B), such as Arg-8 in DNAP(211–221) and Gly-6, Gly-7, and Lys-8 in B27(309–320). The very low RMSD fluctuation of DNAP(211–223) after the first 5–10 ns of MD simulation and the smaller RMSF values, relative to DNAP(211–221) and B27(309–320), suggest a less flexible structure of the former peptide.

Clustering Analysis Reveals Distinct Peptide Flexibility and Conformations—A total of 5000 structures sampled during the last 10 ns of the MD simulation were subdivided in up to five clusters on the basis of similarity (RSMD) in the peptide backbone. Two predominant clusters were found for DNAP(211–221), one for DNAP(211–223), three for B27(309–320), and one for the x-ray template (Table 3). The distinct flexibility of the three peptides revealed by this analysis was further apparent upon considering the intracluster RMSD variability. This was calculated as the distance to the centroid, which is the average distance of all members of a cluster to its geometrical center. Smaller (0.43), intermediate (0.54), and larger values (0.7) were found for the major clusters of DNAP (211–223),
DNAp(211–221), and B27(309–320), respectively. These results indicate that, in complex with B*27:05, B27(309–320) is highly flexible, DNAp(211–221) has less flexibility, and DNAp(211–223) is significantly rigid. The overall structure of the peptide binding site showed no significant differences among the various complexes. A set of 100 unclustered structures homogeneously sampled at 100-ps intervals in each modeled complex from the last 10 ns of the trajectories is shown in Fig. 6C.

Representative structures (reps) from each of the main clusters observed in B27(309–320), DNAp(211–221), and DNAp(211–223) (Table 3) illustrate the three-dimensional
configuration preferences of the peptides in their bound states (Fig. 6D). For B27(309–320), rep1 and rep2 showed similar conformations and small differences in their molecular surface, but rep4 was significantly different. For DNAP(211–221), the representative conformers of its two main clusters were very similar and were different from those of B27(309–320). In contrast, the only major cluster in DNAP(211–223) showed a striking similarity to B27(309–320), looking like an intermediate form of rep2 and rep4 of this peptide. DNAP(211–223) also showed a surface charge distribution with similarities to both rep2 and rep4 of this peptide. DNAP(211–221), the conformations and small differences in their molecular surface, but rep4 was significantly different. For DNAP(211–221), the representative conformers of its two main clusters were very similar and were different from those of B27(309–320). In contrast, the only major cluster in DNAP(211–223) showed a striking similarity to B27(309–320), looking like an intermediate form of rep2 and rep4 of this peptide. DNAP(211–223) also showed a surface charge distribution with similarities to both rep2 and rep4 of B27(309–320) (Fig. 6E).

**Binding Energy**—MM-ISMSA was used to estimate the total free energy of binding of the peptides in the binding groove of B*27:05 and the contribution of each peptide residue to the total free energy of binding. The N- and C-terminal residues each contributed ~20 kcal/mol to the total binding of each peptide. Residue 2 showed the highest contribution, ~25 kcal/mol, whereas the central regions of the peptides showed greater variation and a smaller contribution (Fig. 5C). These results are in full agreement with the known canonical interactions governing binding of MHC-I ligands.

**DISCUSSION**

Two issues were addressed in this study: first, the endogenous processing and presentation of predicted T-cell epitopes, recognized as synthetic peptides by CTL from *Chlamydia*-infected ReA patients, and second, the structural similarity between chlamydial and human-derived HLA-B27 ligands. Our approach was the direct identification of endogenously processed chlamydial peptides using high sensitivity and accuracy MS. Although, ideally, this search should be performed on *Chlamydia*-infected cells, this approach is virtually unfeasible in humans, due to induction of MHC-I down-regulation and apoptosis (38). Some chlamydial proteins are injected into the cytosol through the type III secretion system (65–68). However, many others reach cytosolic cross-presentation pathways (69, 70) after uptake of bacterial debris from infected cells undergoing apoptosis and are subjected to proteasomal degradation, similarly to endogenous proteins. Thus, the endogenous processing of chlamydial fusion proteins is likely to mimic that in infected cells to a large degree, as confirmed by the direct identification of chlamydial T-cell epitopes using fusion proteins in this and a previous study (39). However, proteasome-independent pathways might also generate chlamydial MHC-I ligands after transfer of bacterial components following the fusion of inclusion-derived vesicles with the endoplasmic reticulum (71) and perhaps also through non-cytosolic cross-presentation pathways. Thus, some chlamydial antigens may not be revealed with our approach.

Although studies based on MALDI-TOF MS allowed us to identify several HLA-B27 ligands from *C. trachomatis*, the limitations of this approach justified a more in depth search using electrospray-based MS techniques to look for novel chlamydial epitopes. Despite the technical improvements, the direct iden-
Identification of immunologically relevant bacterial peptides by biochemical methods is less sensitive than CTL because these can recognize minute antigen amounts, down to a few copies, at the cell surface (72). Although the relatively high expression of bacterial fusion proteins results in the generation of many more copies of chlamydial peptides than on infected cells, partially compensating for the lower sensitivity of biochemical analyses, the different thresholds relative to CTL recognition must always be kept in mind.

Our study focused on three chlamydial proteins. For two of them, ClpC and NQRA, HLA-B27-restricted T-cell epitopes had been predicted (32, 33). For the third one, DNAP, an endogenous peptide, DNAP(211–221), with high homology to a natural human-derived B27 ligand, was previously reported (38). Both the transcriptional profile (73) and the proteomic characterization of the Chlamydia life cycle (74) indicate that ClpC is expressed in the infectious elementary body and, at a higher level, in the replicative but non-infectious reticulate body and is up-regulated by IFN-γ (75). The presence of ClpC in both developmental stages and its up-regulation in an inflammatory context is compatible with the possibility that HLA-B27-restricted T-cells, directed against epitopes from this protein, may be relevant in controlling both the bacterial infection and the development of ReA. Detection of NQRA in the

**FIGURE 5.** MD simulation of HLA-B*27:05 and β2m and contribution of individual peptide residues to B*27:05 binding. A, Cα RMSD (in Å) for each complex along the trajectories compared with their initial reference structures, HLA-B*27:05 heavy chain and β2m, are colored in blue and green, respectively. B, mass-weighted atomic positional fluctuations (RMSF, in Å) of the HLA-B27 heavy chain and β2m for each HLA-B27-peptide complex, DNAP(211–221) (orange), DNAP(211–223) (brown), B27(309–320) (purple), and pVIPR-A (black). About 32% of the residues along the B*27:05 heavy chain, mainly in α3, showed RMSF values above 3.0 Å. C, contribution of each single residue to the total free energy of binding of the corresponding peptide according to the MM-ISMSA energy decomposition scheme: DNAP(211–221) (orange), DNAP(211–223) (red), B27(309–320) (blue), and pVIPR-A (black).
elementary body, but not in the reticulate body, is likewise compatible with the possibility that peptides from this protein may trigger B27-restricted T-cell responses at early stages of the infection. The finding of HLA-B27-restricted T-cells against peptides from these proteins in ReA patients (32, 33) is consistent with both their expression patterns and possible pathological relevance.

T-cell epitope assignments based on predictive algorithms have limitations that preclude a reliable identification of relevant antigens without their direct detection in vivo. These lim-

FIGURE 6. Structural analysis of modeled HLA-B*27:05 peptide complexes. A, RMSD (in Å) corresponding to the peptidic Cα atoms along the MD trajectories, compared with their initial reference structures, for DNAP(211–221) (orange), DNAP(211–223) (brown), B27(309–320) (purple), and pVIPR-A (black). B, mass-weighted atomic positional fluctuations (RMSF, in Å) per residue for the four peptides (color code as in A). C, overlay of 100 structures sampled along the last 10 ns of the MD trajectories. The peptide, HLA-B*27:05 heavy chain (blue), and β2m (green) backbones are shown. D, molecular surface of representative peptide conformations (rep) for each of the main clusters obtained during the last 10 ns of MD simulation. Oxygen, nitrogen, and other atoms are colored red, blue, and white, respectively. E, adaptive Poisson-Boltzmann solver analysis for the most similar structures found during clustering. The distribution of electrostatic potentials on the peptide surfaces is shown. Negative and positive electrostatic potentials are colored red and blue, respectively (range, ±5 kcal).
Chlamydial HLA-B27 Ligands

TABLE 3
Clustering analysis for the indicated peptides

| Cluster | DNAP(211–221) | DNAP(211–223) | B27(309–320) | pVIPR-A |
|---------|---------------|---------------|--------------|---------|
|         | NS | DC | NS | DC | NS | DC | NS | DC |
| 1       | 734 (14.7%) | 0.51 | 4987 (99.7%) | 0.43 | 2473 (49.5%) | 0.7 | 4984 (99.7%) | 0.35 |
| 2       | 4193 (83.9%) | 0.54 | 1 (0.0%) | 0 | 559 (11.2%) | 0.75 | 2 (0.0) | 0.26 |
| 3       | 30 (0.6%) | 0.43 | 1 (0.0%) | 0 | 190 (3.8%) | 0.67 | 3 (0.1%) | 0.3 |
| 4       | 41 (0.8%) | 0.4 | 3 (0.1%) | 0.29 | 1777 (35.5%) | 0.7 | 8 (0.2%) | 0.3 |
| 5       | 2 (0.0%) | 0.4 | 8 (0.2%) | 0.31 | 1 (0.0%) | 0 | 3 (0.1%) | 0.19 |

a Number of structures. The percentages of the predominant clusters (in parentheses) are highlighted in boldface type.

b Distance to centroid (Å).

The chlamydial DNAP shows a particularly interesting example of molecular mimicry between bacterial and self-derived HLA-B27 ligands. HLA-B27 presents an 11-mer from this protein, DNAP(211–221), with high homology to the human-derived HLA-B27 ligand B27(309–320), which is one residue longer than the chlamydial peptide (38, 62). The finding now of longer than the chlamydial peptide (38, 62). The finding now of increased the probability of molecular mimicry between peptides from DNAP and the human-derived ligand. MD simulations suggest that DNAP(211–221) and DNAP(211–223) adopt distinct conformations. Both peptides showed limited flexibility and a peptide-specific predominant conformation. In contrast, B27(309–320) was significantly more flexible. This is in agreement with x-ray data showing a single defined conformation of DNAP(211–221) and a diffuse electron density corresponding to the central region of B27(309–320) in complex with B27:05. The limited flexibility of the two chlamydial peptides, especially DNAP(211–223), observed in our MD simulations was apparently determined by intra-peptide hydrogen bonds established within their central regions, which are more frequent among long peptides, and by peptide-specific interactions of their central regions with HLA-B27 residues.

The higher flexibility of the human-derived peptide is likely to provide a wider spectrum of antigenically distinct conformations. The striking similarity of the conformation and surface charge distribution of DNAP(211–223) with some of the main conformational clusters of B27(309–320) could favor T-cell cross-reaction between both peptides. A peptide bound in a flexible and variable conformation in its middle part may be amenable to recognition by more T-cell clones, with preference for single conformations, than a peptide bound with lower flexibility. For instance, T-cell-mediated self-reactivity has been related to peptide antigens bound to HLA-B27 in dual conformation (76, 77). The antigenic similarity between the DNAP-derived peptides and the homologous self-derived B27 ligand must be confirmed in functional assays with peptide-specific T-cells. Although we recognize the importance of functional studies in this context, we were unable to perform them because it was extremely difficult to gain access to HLA-B27 patients with Chlamydia-induced ReA, a disease becoming increasingly rare or not unambiguously diagnosed (4) in Western countries. Attempts to stimulate peptide-specific, HLA-B27-restricted, CTL in vitro from a few individuals were unsuccessful. Due to the difficulties inherent to raising peptide-specific CTL in vitro, even from infected individuals, these studies must be performed with a sufficient number of patients, which was unfeasible because they were not available. In the absence of formal confirmation with T-cells, both the sequence homology and the predicted conformational features of DNAP(211–223) and B27(309–320) suggest a mechanism for increasing T-cell cross-reaction between endogenous chlamydial and self-derived HLA-B27 ligands through molecular mimicry.
presentation of related peptides of distinct length and conformation, homologous to self-peptides with high flexibility in their bound state.

In conclusion, the high accuracy and sensitivity of current MS technologies brought about a major improvement in the detection of naturally processed HLA-B27 ligands from *C. trachomatis*, allowing us to detect three novel peptides from distinct proteins, including the second known HLA-B27-restricted epitope recognized by T-cells from ReA patients. Both the homology of all of the reported peptides with human sequences carrying the binding motif of HLA-B27 and the finding of a peptide from DNAP with significant sequence and conformational similarity to a human-derived HLA-B27 ligand suggest that molecular mimicry between bacterial and self-derived HLA-B27 ligands may play a role in ReA. This mechanism could provide an autoimmune component that would exacerbate the proinflammatory role of HLA-B27, influencing disease severity and evolution toward chronicity.

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