Identification of Endogenously Presented Peptides from *Chlamydia trachomatis* with High Homology to Human Proteins and to a Natural Self-ligand of HLA-B27*©*

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A strategy for the stable expression of proteins, or large protein fragments, from *Chlamydia trachomatis* into human cells was designed to identify bacterial epitopes endogenously processed and presented by HLA-B27. Fusion protein constructs in which the green fluorescent protein gene was placed at the 5′-end of the bacterial DNA primase gene or some of its fragments were transfected into B*2705-C1R cells. One of these constructs, including residues 90–450 of the bacterial protein, was stably and efficiently expressed. Mass spectrometry-based comparative analysis of HLA-B27-bound peptide pools led to identification of three HLA-B27 ligands differentially presented in the transfectant cells. Sequencing of these peptides confirmed that they were derived from the bacterial DNA primase. One of them, spanning residues 211–221, showed 55% sequence identity with a known self-ligand of HLA-B27 derived from its own molecule. The other two bacterial ligands, P-(112–121) and P-(112–122), were derived from the same region and differed in length by one residue at the C terminus. Both peptides showed >50% identity with multiple human protein sequences that possessed the optimal peptide motifs for HLA-B27 binding. Thus, expression of proteins from arthritogenic bacteria in HLA-B27-positive human cells allows identifying bacterial peptides that are endogenously processed and presented by HLA-B27 and show molecular mimicry with known self-ligands of this molecule and human proteins. *Molecular & Cellular Proteomics* 7:170–180, 2008.

*Chlamydia trachomatis*, an obligate intracellular parasite that primarily infects the urogenital epithelium, is one of the most common infectious agents in humans. *Chlamydia*-induced arthritis is the most frequent form of reactive arthritis (ReA) in Western countries (1). Persistent forms of the bacteria, rather than actively growing forms, are found in chronically infected tissues and, presumably because they sustain chronic infection, are critical in the development of ReA. Persistent forms of *Chlamydia* are produced by differentiation of the metabolically active reticulate bodies in response to tryptophan starvation induced by interferon-γ (2). Several studies have established that proteins involved in DNA replication are expressed during persistence and that the bacterial chromosome is continuously replicated. Despite the multiple mechanisms used for *C. trachomatis* to evade the immune system, which include down-regulation of the major histocompatibility complex (MHC) class I and class II molecules (5–7) and persistence, the occurrence of CD4+ and CD8+ T cell responses is well established (8), and HLA-B27-restricted cytotoxic T lymphocytes (CTLs) are found in patients with *Chlamydia*-induced ReA (9, 10). The joint role of HLA-B27 and *Chlamydia* infection in determining susceptibility to ReA, especially in its chronic form, suggests that molecular mimicry between bacterial and self-antigens presented by HLA-B27 may provide an autoimmune pathogenetic mechanism for this disease (11, 12). This is supported by studies in rats showing that immunization with HLA-B27 rendered the animals capable to mount a *Chlamydia*-specific CD8+ T cell response after *in vitro* stimulation with this bacteria (13). Moreover HLA-B27 transgenic rats activated an autoreactive B27-directed CTL response upon exposure to *C. trachomatis* (14). Although these studies show an immunological interplay between HLA-B27 and *Chlamydia*, it must be noted that molecular mimicry is only one of the possible mechanisms of autoimmunity. Actually the elusive nature of the concept of antigenic mimicry at a molecular level, because the T cell receptor may cross-react against structurally disparate epitopes (15, 16), and the difficulty of actually establishing a causative link between molecular mimicry and autoimmunity cast doubts on the actual relevance of this mechanism (17). Nevertheless cross-reactivity between chlamydial peptides and homologous peptides from the heart muscle-specific protein α-myosin was shown to be involved in the pathogenesis of autoimmune myocarditis in mice (18).

To investigate the role of molecular mimicry in *Chlamydia*-induced HLA-B27-associated ReA, two experimental strategies have been undertaken. In the first one, chlamydial pep-
tides recognized by HLA-B27-restricted CTLs from both transgenic mice and patients were identified by screening a panel of synthetic peptides with binding motifs and proteasome cleavage features compatible with presentation with HLA-B27 (9, 10). However, given the large cross-reactive potential of CTLs, the relationship of these peptides to naturally processed chlamydial epitopes is unknown. In a second approach a peptide derived from the cytoplasmic tail of HLA-B27 and other class I molecules was shown to be presented as an endogenously processed natural ligand of three HLA-B27 subtypes, B*2702, B*2704, and B*2705, associated with spondyloarthritides and not by two subtypes, B*2706 and B*2709, weakly or not disease-associated. This peptide showed high homology with a sequence encompassing residues 211–222 of the DNA primase of *C. trachomatis*, and the corresponding chlamydial peptide was directly generated in vitro from a synthetic precursor by the 20 S proteasome (11). This study was the first to directly address the molecular mimicry between natural ligands of HLA-B27 arising from human self-proteins and chlamydial protein sequences. However, a critical test for the relevance of the DNA primase-derived peptide was to determine whether this peptide, or a closely related one, was actually processed and presented in vivo by HLA-B27. A direct molecular approach aiming at directly mapping chlamydial peptides presented by HLA-B27 in infected cells by biochemical methods is hardly feasible due to the exceedingly low expression of bacterial antigens on these cells as reported, for instance, with *Salmonella* (19, 20). In the case of *Chlamydia*, this approach is further complicated by the down-regulation of MHC class I expression induced by the bacteria shortly after infection (6, 7). Thus, we adopted an alternative strategy to ask the specific question of whether the DNA primase-(211–222) peptide, or closely related ones, could be generated and presented by HLA-B27 when the bacterial protein was endogenously expressed in the cell. To this end, we devised a method to endogenously and stably express a viable construct derived from the DNA primase of *C. trachomatis* and demonstrated the endogenous presentation of three peptides from this protein by HLA-B27 in vivo, including one spanning residues 211–221.

**EXPERIMENTAL PROCEDURES**

**DNA Primase Gene Constructs, Transfectants, and Monoclonal Antibodies—** Green fluorescent protein (GFP)-DNA primase fusion proteins were generated by fusing the cDNA of the DNA primase gene of *C. trachomatis* serovar L2 (CT794) (Advanced Biotechnologies, Columbia, MD), or truncated forms of this gene, in frame to the 3’-end of the GFP gene. All the DNA primase gene constructs were cloned into the pEGFP-C1 vector (BD Biosciences Clontech) in 5’ EcoRI and 3′ BamHI sites. Three gene constructs were made (see Fig. 1A): the complete DNA primase sequence, P-(1–595), a truncated form lacking the first 89 codons, P-(90–595), and a second truncated form lacking codons 1–89 and 451–595, P-(90–450). The chlamydial DNA primase cDNA was amplified by PCR using the following primers: for P-(1–595), 5′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC and 3′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC; for P-(90–595), 5′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC and 3′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC; and for P-(90–450), 5′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC and 3′-TCTCTCTCGGATCTTATGGATCTGTTTGAGGAC. Hmy2.C1R (C1R) is a human lymphoid cell line with low expression of its endogenous HLA class I molecules (21, 22). The GFP-DNA primase constructs were cotransfected with the RSV vector (23) carrying the hygromycin resistance gene (a kind gift of Dr. D. Jaraquemada, Autonomous University, Barcelona, Spain) at a 20:1 ratio in B*2705-C1R transfectant cells (24) by electroporation at 300 mV and 960 microfarads. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS (both from Invitrogen). Stable GFP or GFP-DNA primase transfectants were selected with 250 µg/ml hygromycin (Invitrogen). The monoclonal antibody (mAb) W6/32 (IgG2a, specific for a monomorphic HLA-A,-B,-C determinant) (25) was used.

**Flow Cytometry—** Approximately 10^6 cells were washed twice in 200 µl of PBS. The GFP-associated fluorescence was directly measured in a FACSCalibur instrument, and data were analyzed using CellQuest software (both from BD Biosciences).

**Immunoprecipitation and Western Blot—** About 2 x 10^6 cells were lysed in Igepal CA-630 (Sigma) lysis buffer (0.5% Igepal, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2) containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science). After centrifugation to remove insoluble material, lysates were preclurred with anti-rabbit IgG beads (TrueBlot, eBioscience, San Diego, CA) for 2 h. Immunoprecipitation was done by overnight incubation with the GFP-specific polyclonal Ab A6455 (Invitrogen) followed by incubation with anti-rabbit IgG beads for 1 h. All procedures were carried out at 4 °C with continuous shaking. Immunoprecipitates were washed three times with lysis buffer, boiled for 5 min in SDS sample buffer, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad). Immunoblotting was done with the A6455 Ab and horse-radish peroxidase-conjugated anti-rabbit IgG (TrueBlot, eBioscience) at 1:10,000 and 1:5000 dilution, respectively. Antibodies were diluted in PBS–milk buffer (PBS, 5% milk, 0.1% Tween 20). The immunoblots were developed with the ECL immunodetection system (Amersham Biosciences).

**Isolation of HLA-B27-bound Peptides—** B*2705-bound peptides were isolated from about 10^10 or, in one case, 2 x 10^10 C1R-B*2705 transfectant cells as described previously (26). Briefly cells were lysed in 1% Igepal CA-630 (Sigma), 20 mM Tris/HCl, 150 mM NaCl, pH 7.5, in the presence of a mixture of protease inhibitors. After ultracentrifugation, the soluble fraction was subjected to affinity chromatography using the W6/32 mAb. HLA-B27-bound peptides were eluted with 0.1% aqueous TFA at room temperature, filtered through Centricon 3 devices (Amicon, Beverly, MA), concentrated, and subjected to reverse phase HPLC fractionation in a Waters Alliance system (Waters, Milford, MA) using a Vydac 218TP52-C18 column (Vydac, Hesperia, CA) at a flow rate of 100 µl/min as previously described (27). Fractions of 50 µl were collected and stored at −20 °C.

**Mass Spectrometry Analysis and Sequencing—** HPLC fractions were analyzed by MALDI-TOF MS using a Bruker Reflex™ mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with the SCOUT™ source operating in positive ion reflector mode as described previously (28). Briefly dried HPLC fractions were resuspended in 0.5 µl of TA (33% aqueous acetonitrile, 0.1% TFA), deposited onto the MALDI plate, and allowed to dry at room temperature. Then 0.5 µl of matrix solution (m-cyano-4-hydroxycinnamic acid in TA) was added and allowed to dry again. MS spectra were processed using the MoverZ software (version 2001.02.03) (Genomic Solutions).

Alternatively a MALDI-TOF/TOF Instrument (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA) was used in this case.
fractions were reconstituted with 0.6 μl of TA, loaded onto an
Opti-TOF™ 384-well MALDI insert (Applied Biosystems), and
allowed to dry at room temperature. Then 0.6 μl of the matrix solution
(3 mg/ml) was added. These mass spectra were acquired in reflector
positive mode and processed using the 4000 Series Explorer
software version 3.5.

Peptide sequencing was carried out by quadrupole ion trap nano-
ESI MS/MS in an LCQ Classic or LCQ DECA-XP instrument (Finnigan
Thermoquest, San Jose, CA) using the Xcalibur 2.0 software after
on-line chromatographic separation of samples as described previously
(28). Alternatively sequencing was performed by MALDI-TOF/
TOF. The acquisition method was MS/MS at 1 kV with collision-
induced dissociation where the collision gas was atmospheric air and
the precursor mass window was set as ±10 Daltons. The parameter
used for processing data was a signal-to-noise ratio of 10.

Interpretation of mass spectra was done manually but assisted by
various software tools as follows. Manual inspection of the spectrum
allowed us to determine a tentative sequence. This was used to
screen the chlamydial DNA primase protein sequence (UniProtKB/
Swiss-Prot accession number O84799). When a match was obtained,
a list of theoretical fragment ions of the corresponding peptide se-
quence was generated using the MS-product tool available at the
Protein Prospector tools web site: prospector.ucsf.edu/prospector/
4.0.8/html/msprod.htm (University of California, San Francisco, CA)
as an assistance to match the putative candidate sequences to our
experimental MS/MS spectrum. In addition, the corresponding syn-
thetic peptide was made, and its MS/MS spectrum was used to
confirm the manually assigned sequence of the chlamydial B27
ligand.

Homology Searches in Human Databases—The search for homol-
gies between chlamydial peptides and human proteins was carried
out in the UniProtKB database (release 54.0; July 24, 2007) at www.ebi.ac.uk/fasta.

Stable Isotope Tagging and Proteasome Inhibition—This was done
essentially as described previously (29) with minor modifications.
Briefly three batches of 6 × 10^8 B2705-C1R transfectant cells ex-
pressing the chlamydyal P-(90–450) fusion protein were separately
cultured for 4 h in Dulbecco’s modified Eagle’s medium without Arg
supplemented with 10% FCS. Then one flask was supplemented with
standard [14N]Arg (100 μg/ml), the second flask was supplemented with
100 μg/ml L-[guanido-15N]Arg-HCl (Cambridge Isotope Laboratories,
Andover, MA) in which two nitrogen atoms of the guanidinium
group have been replaced with 15N, and the third flask was treated
with a 20 μM concentration of the irreversible proteasome inhibitor
MG132 (Calbiochem) for 30 min prior to the addition of 100 μg/ml
15N-tagged Arg to ensure that the proteasome was inhibited from
the start of the labeling; the inhibitor was left for the complete labeling
period. After 5 h, cells were washed twice in 20 mM Tris/HCl, 150 mM
NaCl, pH 7.5, and stored at −70 °C for further processing. This
labeling time was used because the much longer times required for
quantitative protein labeling are not feasible due to the limited viability
of cells in the presence of proteasome inhibitors.

Synthetic Peptides—These were obtained using standard N-(9-
fluorenyl)methoxycarbonyl chemistry and purified by HPLC. The correct
molecular mass of purified peptides was verified by MALDI-TOF
MS.

RESULTS

Expression of Bacterial DNA Primase Constructs in B*2705-
C1R Cells—Stable transfectants expressing DNA primase
protein sequences from C. trachomatis in HLA-B27-positive
cells were required to analyze bacterial peptide presentation.

Three fusion proteins were constructed in which GFP was
fused to the N terminus of the complete DNA primase protein,
P-(1–595), to a fragment lacking the N-terminal 89 residues,
P-(90–595), and to a fragment lacking both the N-terminal 89
residues and the C-terminal 144 residues, P-(90–450). The
deleted N-terminal and C-terminal regions included the DNA
binding and helicase interaction domains of the DNA primase,
respectively (Fig. 1A). The three constructs included the
P-(211–222) sequence that was previously reported as ho-
logous to a natural ligand of HLA-B27 derived from its own
molecule (11). In preliminary experiments the three fusion
proteins were efficiently expressed in transient transfectants.
of COS and HeLa cells (data not shown). In contrast, stable transfectants in B*2705-C1R cells were obtained with P-(90–595) and P-(90–450) but not with the complete bacterial protein P-(1–595).

The expression levels of the fusion proteins as assessed by flow cytometry were higher for P-(90–450) than for P-(90–595), although in both cases the fluorescence was much lower than in the transfectant expressing only GFP used as a control (Fig. 1B). The expression of the fusion proteins with the correct size in the corresponding transfectants was determined by immunoprecipitation with anti-GFP Ab and Western blot (Fig. 1C).

Three Peptides Are Selectively Presented by HLA-B27 on C1R Cells Expressing the Chlamydial P-(90–450) Fusion Protein—The search for HLA-B27 ligands derived from the bacterial DNA primase was carried out by comparative analysis of the HLA-B27-bound peptide pools isolated from B*2705-C1R cells or transfectants of these cells expressing either GFP alone or P-(90–450). This transfectant was used, instead of the one expressing P-(90–595), due to its higher expression level of the fusion protein (Fig. 1). The strategy used was the same as that used previously for comparing HLA-B27 subtype-bound peptide repertoires (30, 31). HLA-B27-bound peptide pools were isolated by immunopurification of HLA-B27 with the W6/32 mAb followed by acid extraction. The peptide pools were fractionated by HPLC under identical conditions and consecutive runs, and the peptide composition of individual fractions was analyzed by MALDI-TOF MS.

To look for peptides specifically presented by HLA-B27 in cells expressing the chlamydial construct, the MS spectrum of each HPLC fraction of the B27-bound peptide pool from these cells was compared with the MS spectra of the corresponding HPLC fraction of untransfected and GFP-transfected B*2705-C1R cells as well as with the spectra of the previous and following fractions. This was done to account for small shifts in the retention times of individual peptides that might occur among distinct chromatographic runs.

In a survey of 1570 ion peaks from 150 HPLC fractions from each chromatography three ion peaks were detected in the P-(90–450) transfectant but not in the controls of untransfected and GFP-transfected B*2705-C1R: in HPLC fraction number 105 an ion peak with $m/z$ 1247.3, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools either in the same or in adjacent (labeled with an asterisk) fractions. B, comparison of the MALDI-TOF MS spectra of HPLC fraction number 134 of the B*2705-bound peptide pool from P-(90–450) transfectants and from untransfected B*2705-C1R cells. Only the 1270–1400 $m/z$ range is shown. The ion peak at $m/z$ 1346.3, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools either in the same or in adjacent (labeled with an asterisk) fractions. C, comparison of the MALDI-TOF MS spectra of HPLC fraction number 162 of the B*2705-bound peptide pool from P-(90–450) transfectants and from untransfected B*2705-C1R cells. Only the 1400–1600 $m/z$ range is shown. The ion peak at $m/z$ 1493.8, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools.

Fig. 2. Identification of peptides specifically presented by HLA-B27 in P-(90–450) transfectant cells. A, comparison of the MALDI-TOF MS spectra of HPLC fraction number 105 of the B*2705-bound peptide pool from P-(90–450) transfectants and from B*2705-C1R cells (B*2705). All spectra were recorded and compared in the 800–2000 $m/z$ range, but for simplicity, only the 1180–1390 range is shown. The ion peak at $m/z$ 1247.3, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools either in the same or in adjacent (labeled with an asterisk) fractions. B, comparison of the MALDI-TOF MS spectra of HPLC fraction number 134 of the B*2705-bound peptide pool from P-(90–450) transfectants and from untransfected B*2705-C1R cells. Only the 1270–1400 $m/z$ range is shown. The ion peak at $m/z$ 1346.3, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools either in the same or in adjacent (labeled with an asterisk) fractions. C, comparison of the MALDI-TOF MS spectra of HPLC fraction number 162 of the B*2705-bound peptide pool from P-(90–450) transfectants and from untransfected B*2705-C1R cells. Only the 1400–1600 $m/z$ range is shown. The ion peak at $m/z$ 1493.8, labeled with an arrow, was not detected in the corresponding HPLC fraction of untransfected B*2705-C1R cells or in the adjacent fractions (not shown). All other ion peaks were found in both peptide pools.
each of these peptides was detected. These results suggest that the direct comparison of HLA-B27-bound peptide repertoires by MS is sensitive enough to detect endogenously processed bacterial ligands of HLA-B27 upon transfection of the chlamydial protein construct and that at least three such ligands from the DNA primase are presented by HLA-B27 in vivo.

The Chlamydial P-(211–221) Peptide Is Endogenously Processed and Presented by HLA-B27—The peptide corresponding to the ion peak at \( m/z \) 1247.3 in HPLC fraction 105 of the B*2705-bound peptide pool from the P-(90–450) transfectant (Fig. 2A) was sequenced by MALDI-TOF/TOF from 2 \( \times \) 10^9 cell equivalents (Fig. 3). The sequence of this peptide was identical to that of residues 211–221 from the DNA primase of C. trachomatis. The assignment was validated by identity with the MALDI-TOF/TOF MS spectrum of the corresponding synthetic peptide (Fig. 3). A search in the human genome database failed to reveal identity with human proteins, confirming the bacterial origin of this peptide. P-(211–221) is just a residue shorter at the C terminus than the predicted bacterial homolog, P-(211–222), of the natural self-ligand of HLA-B27 derived from the cytoplasmic tail of its own molecule, B27-(309–320) (11). Thus, these results indicate that a chlamydial peptide with high sequence homology to a natural ligand of HLA-B27 derived from its own molecule is endogenously processed and presented by HLA-B27 in cells expressing the P-(90–450) fusion protein.

To rule out the possibility that a small amount of P-(211–222) might have escaped detection by MALDI-TOF MS, a specific search for this peptide was carried out. The elution position of the synthetic P-(211–222) peptide in the same conditions used to fractionate the B*2705-bound peptide pool was determined to correspond to fraction number 119 or 121 in two independent chromatographic runs. The MALDI-TOF MS spectra of fractions 115–123 from the P-(90–450) peptide pool were examined at high resolution in the region corresponding to the \( m/z \) of P-(211–222) to look for small ion peaks consistent with this peptide, but they were not observed. The same HPLC fractions were also subjected to MS/MS fragmentation in the \( m/z \) range corresponding to the \([M + 2H]^2+, [M + 3H]^3+, \) and \([M + 4H]^4+\) parent ions of P-(211–222). Again no fragmentation compatible with this peptide was observed. A similar strategy was used to search for the related P-(211–218) and P-(211–223) peptides, whose structural motifs were also compatible with HLA-B27 binding, but they were not detected in the B*2705-bound peptide pool from P-(90–450). These results suggest that, within the detection limits of the techniques used in this study, the P-(211–218), P-(211–222), and P-(211–223) are not presented endogenously by HLA-B27 in cells expressing the P-(90–450) fusion protein.

Two Additional Peptides from the Chlamydial DNA Primase Are Presented in Vivo by HLA-B27—The peptides corresponding to ion peaks 1346.3 from HPLC fraction number 134 and 1493.8 from fraction number 162 were sequenced by
quadrupole ion trap nano-ESI MS/MS, and their assigned sequences were confirmed with the MS/MS spectra of the corresponding synthetic peptides (Fig. 4). The two peptides corresponded to residues 112–121 and 112–122 of the chlamydial DNA primase. A search against the human genome failed to reveal identity of these peptides with human proteins, further supporting their bacterial origin.

Because a putative peptide with the bacterial sequence spanning residues 112–120 would also have the appropriate size and motifs for HLA-B27 binding, we looked for this peptide in the B*2705-bound peptide pool from the P-(90–450) transfectant using the strategy described in the previous paragraph. We first determined that the retention time of the corresponding synthetic peptide in the same chromatographic conditions used for fractionation of the peptide pool corresponded to fraction number 104. Then we looked for an ion peak with the corresponding m/z (M + H⁺, 1199.7) in the MALDI-TOF MS spectra of HPLC fractions 102–106 of the P-(90–450) trans-
fectant and subjected to MS/MS fragmentation the same chromatographic fractions in the m/z range corresponding to the [M + 2H]2⁺ and [M + 3H]3⁺ parent ions. This was done to account for the possibility that the peptide would not show up in the MALDI-TOF MS spectra but might still be detectable by electrospray MS. Using this approach the P-(112–120) was not found in the B*2705-bound pool.

Proteasome-mediated Processing of the P-(90–450) Fusion Protein—To determine whether the chlamydial B*2705 ligands were generated in a proteasome-dependent way the B*2705-C1R transfectants expressing P-(90–450) were metabolically labeled with [15N2]Arg in the absence and presence of the proteasome inhibitor MG132. This approach was used previously to determine the proteasome dependence of B*2705 ligands (29). Because the isotopically labeled Arg contains two 15N atoms, its incorporation results in an increase of 2 Da per Arg residue in the molecular mass of any given Arg-containing peptide. Because cell viability is severely compromised upon long exposure to proteasome inhibitors the labeling time was only of 5 h so that the intracellular pool of unlabeled peptides was not depleted. In these conditions in the MALDI-TOF MS spectrum a labeled peptide will be similar to the unlabeled one except for a selective increase of the corresponding isotopic species. Of the three chlamydial peptides only the most abundant one, P-(112–121), was amenable to this analysis (Fig. 5) due to the low amounts of the other ligands, which precluded their reliable analysis with this method. Upon labeling, a significant increase in the intensity of the M + 8 ion peak (where M is the monoisotopic peak) was observed, corresponding to the presence of 4 Arg residues in the P-(112–121) peptide. This increased intensity was totally abolished in the presence of MG132, indicating that this ligand is generated from P-(90–450) in a proteasome-dependent way.

P-(112–121) and P-(112–122) Show High Sequence Homology with Human Proteins—To address the possible existence of self-HLA-B27 ligands showing molecular mimicry with these chlamydial peptides, we looked for homologous human protein sequences containing the binding motifs of HLA-B27. P-(112–121) and P-(112–122) were searched against the human proteome to look for sequences containing 50% amino acid identity with the respective bacterial sequence and the major binding motif for HLA-B27, R2. As many as 50 sequences filled these initial criteria. This does not reflect any particular feature of B27-binding peptides because a similar search carried out with sequences of the same size from the DNA primase lacking the B27 binding motif yielded a comparable number of matches with the human proteome (data not shown). In a second step sequences containing residues at a given position that have not been observed among natural HLA-B27 ligands (32) were selected out. The following filters were applied: 1) presence of an acidic residue at position (P)1 and 2) presence of an acidic or polar C-terminal residue. After applying these filters, 33 human sequences remained. A further selection focused on the subset of sequences containing optimal binding motifs at the anchor positions P1, P3, and P9. The optimal residues were considered to be those with the highest frequency at the corresponding position among natural B*2705 ligands (32): Gly or a basic residue at P1, an aliphatic/aromatic residue (except Ala) at P3, and a basic, aliphatic (except Ala) or aromatic residue at P9. A total of 16 human sequences fulfilled these criteria (Table I). These results indicate that the human proteome contains multiple
HLA-B27 Ligands from Chlamydia trachomatis

**TABLE I**

| Sequenceα | Identityβ | Protein | Accession no. |
|-----------|-----------|---------|--------------|
| GELKKEABYE | 60% | POZ domain-containing protein KCTD16 | Q68DU8 |
| PRAAQворь | 60% | Negative elongation factor C/D | Q8IXH7 |
| PRAИDEEKAФ | 60% | Cadherin-10 precursor | Q9Y6N8 |
| PRAИDEEKAФь | 55% | Cadherin-10 precursor | Q9Y6N8 |
| PRAИGKEQКБ | 60% | Threonyl-tRNA synthetase, mitochondrial precursor | Q9BW92 |
| PRAИGKEQКБф | 60% | Threonyl-tRNA synthetase, mitochondrial precursor | Q9BW92 |
| PRAИGКИф | 55% | Sperm 1 POU domain transcription factor | Q8N7G0 |
| PRAИнГКЕКФ | 60% | Cyclin G1-binding protein 1 | O43257 |
| PRAИDИЕККол | 64% | Guanine nucleotide-binding protein, q polypeptide | Q6NT27 |
| PRRTRENDРд | 55% | Hypothetical protein DKFZp547E087 | Q8ND77 |
| PRLDГЕБел | 60% | Hypothetical protein DKFZp667M2010 | Q8NCY6 |
| PRLГKЕГЕККФ | 58% | cDNA FLJ41781 fis, clone IMR322018117 | Q6ZW18 |
| PRLГKЕГЕККФф | 60% | cDNA FLJ41781 fis, clone IMR322018117 | Q6ZW18 |
| PРЛГЕКЕККф | 60% | Serine/threonine-protein kinase MRCK γ | Q6DT37 |
| PРЛГЕКЕККфп | 55% | DCP1 protein | Q15540 |
| PРГДГшф | 55% | Docking protein 4 | Q8TEW6 |

α Residues identical with the bacterial peptide are underlined.
β Relative to the bacterial peptide of equal length.
γ This sequence was aligned allowing for the introduction of a gap in the bacterial peptide.

DISCUSSION

A specific aim of this study was to examine the possibility of molecular mimicry between HLA-B27 ligands from the DNA primase of *Chlamydia trachomatis* and from human self-proteins. The basis for this search was a previous finding from our laboratory that a peptide spanning residues 309–320 of the cytoplasmic tail of HLA-B27 and other class I molecules (RRKSSGKGGSY) was a natural ligand of three HLA-B27 subtypes associated to ankylosing spondylitis and that this peptide had a high homology with residues 211–222 of the chlamydial DNA primase (RRFKEGGRGGKY). The bacterial peptide encompassing these residues bound HLA-B27 in vitro and was directly generated by the 20 S proteasome from a synthetic precursor (11). Moreover because the persistent forms of *C. trachomatis*, which maintain the chronic infection and are present in the inflammation sites of patients with ReA induced by this bacteria, are active in DNA replication (3), the DNA primase should be actively induced in these forms and therefore a potential source of bacterial antigen in chronic disease. Although it is unclear how and what chlamydial proteins may reach the processing-loading pathway of MHC class I molecules and to what extent they may be processed in the cytosol or other cell compartments, we specifically addressed the question of whether intracellular processing of the endogenously synthesized DNA primase might lead to generation of the P-(211–222) peptide and/or other HLA-B27 ligands with homology to human protein sequences. This approach was also undertaken because a direct mapping of HLA-B27-restricted chlamydial epitopes from infected cells by MS techniques is exceedingly difficult due to very low expression of bacterial epitopes on infected cells (19, 20) and to down-regulation of MHC class I expression induced by *C. trachomatis* on infected cells (6, 7).

Two problems hampered our initial attempts to directly express the chlamydial DNA primase in human cells. First direct transfection of the bacterial gene resulted in active transcription but no evidence for significant translation of the protein. Presumably this was due to low compatibility of the bacterial RNA sequences with the eukaryotic translation machinery. To circumvent this problem and to easily monitor protein expression, a fusion protein was constructed by placing the GFP gene at the 5′-end of the DNA primase gene. The second problem was that this construction led to expression of the fusion protein in transient transfectants of human cells, indicating efficient translation, but stable transfectants could not be obtained. We speculated that expression of the bacterial protein might interfere with human DNA replication by competing for DNA binding with the human primase or by other blocking effects. Thus, alternative fusion proteins were made in which either the DNA binding domain or both this and the helicase interaction domain of the bacterial DNA primase were deleted because the P-(211–222) sequence was located in the central topoisomerase-primase domain of the molecule.

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J. J. Cragnolini and J. A. López de Castro, unpublished observations.
Both of these constructs could be expressed in stable transfectants of B*2705-C1R cells and were therefore useful for further biochemical analyses. Because the shorter construct was expressed at higher levels, this was used for the subsequent molecular characterization of chlamydial HLA-B27 ligands.

The main findings of our study are the following. First the P-(211–222) peptide, which is homologous to an MHC class I-derived HLA-B27 ligand, is not produced by endogenous processing of the bacterial DNA primase in C1R cells at least at the levels detected by the MS techniques used in this study. Instead the closely related P-(211–221) peptide was endogenously processed and presented by B*2705. Using synthetic peptides to assess the sensitivity of our MS protocols, we estimated that our detection level in MALDI-TOF MS for HLA-B27 ligands is around 150 molecules/cell equivalent (data not shown). We cannot rule out that smaller amounts of one or more of the P-(211–222) or other related peptides, such as P-(211–218) or P-(211–223), might be produced and presented by HLA-B27 in very low amounts. The endogenous presentation of P-(211–221) is consistent with the possibility that this sequence of the DNA primase might be a mediator of molecular mimicry between Chlamydia and the homologous HLA-B27 self-ligand and be relevant in HLA-B27-associated disease. This result validates the experimental approach used in this study as it demonstrates that a peptide from the endogenously produced bacterial protein can be efficiently processed, presented by HLA-B27, and detected by MS analysis of the HLA-B27-bound peptide pool.

The endogenous processing and presentation of P-(211–221), but not other related peptides, has two noteworthy aspects. First, in vitro digestions of the synthetic precursor P-(203–230) by purified 20 S proteasome revealed cleavage after Tyr-222 and, more efficiently, after Ile-223, but no cleavage after Lys-221. Thus, in contrast to other reported examples (33, 34) the proteasomal digestion pattern in vitro did not reflect the endogenous processing of the corresponding protein. Second, P-(211–221) is unlikely to be presented by other ankylosing spondylitis-associated subtypes such as B*2702, B*2704, and B*2707 because its C-terminal Lys residue is not a suitable anchor for these subtypes (32, 35). However, the possibility that related peptides with suitable anchors, particularly P-(211–223), which contains an aliphatic C-terminal residue, might be presented by these subtypes cannot be ruled out.

The second finding of our study was that two additional and closely related peptides of the chlamydial DNA primase are efficiently presented by HLA-B27 following endogenous processing. To our knowledge, P-(112–121) and P-(112–122) are, together with P-(211–221), the first peptides from C. trachomatis shown to be natural HLA-B27 ligands produced by endogenous processing of the bacterial proteins. Further studies specifically focused on presentation of these peptides on Chlamydia-infected cells and on looking for HLA-B27-restricted CTLs from ReA patients capable of recognizing these peptides will now be feasible.

The relatively high yield of P-(112–121) allowed us to establish the proteasome dependence of this peptide using stable isotope tagging and proteasome inhibition and to confirm that the P-(90–450) fusion protein is degraded through the proteasome pathway. The other chlamydial ligands were not amenable to this analysis due to their low yield. However, in a previous report in which this same approach was used to examine the proteasome dependence of B*2705 ligands, multiple peptides arising from the same parental protein consistently showed the same pattern of either sensitivity or insensitivity to proteasome inhibitors (29).

Of note, the P-(112–120) peptide, RRINREAER, was not found in the HLA-B27-bound pool, although the sequence of this peptide includes all the major HLA-B27 binding motifs. Indeed we are not aware of any natural HLA-B27 ligand with concomitant basic P1 and acidic P1′ – 1 residues, whereas the latter residues are relatively frequent among HLA-B27 ligands with C-terminal aliphatic/aromatic motifs (32). A possible explanation might be that the presence of Glu immediately before the C-terminal Arg residue might impair cleavage after Arg-120 by the trypsin-like activity of the proteasome. Adjacent acidic residues are well known to impair trypsin cleavage of the peptide bonds involving basic residues. The trypsin-like active site of the proteasome is different from that of trypsin in that the hydrolytic activity of both proteases is mediated by Thr and Ser, respectively. However, as in trypsin, acidic residues in the β2 and β2i subunits of the proteasome are presumably involved in directing the trypsin-like cleavage specificity toward basic residues (36). However, arguing against this explanation, multiple natural ligands with an acidic residue preceding a C-terminal basic residue have been reported for several HLA-A allotypes (37–39). Therefore, it is possible that absence of this motif among HLA-B27 ligands might be due to binding-related, rather than antigen processing, restrictions.

A limitation of the MALDI-TOF-based comparison of HLA-B27-bound peptide pools is that if a chlamydial peptide has the same molecular mass and retention time as an endogenous self-ligand it would not show up as a differential ion peak and might go undetected. For this reason, we cannot rule out the possibility that additional bacterial peptides, other than those specifically searched for with synthetic peptides and discussed above, might be presented by HLA-B27 in our experimental system and have gone unnoticed in our peptide pool comparisons.

That multiple human sequences showed high homology with P-(112–121) and P-(112–122) suggests that one or more self-ligands of HLA-B27 showing molecular mimicry with these bacterial peptides may actually exist. This issue can be directly addressed by specifically looking for the corresponding peptides in the constitutive HLA-B27-bound pool. The homology between P-(112–121) and P-(112–122) with human
sequences does not reflect any particular tendency of these or other B27-binding peptides to mimic human proteins because unrelated sequences from the bacterial DNA primase of the same length but lacking the B27 binding features showed comparable matching with the human proteome (data not shown).

In conclusion, the approach used in this study is a valid one to determine chlamydial peptides that are processed in vivo and presented by HLA-B27 in human cells. In its application to mapping HLA-B27-restricted epitopes of the DNA primase, it allowed us to identify three bacterial ligands, including one with high homology to a natural self-ligand of HLA-B27 previously suggested as a potential pathogenetic candidate in Chlamydia-induced ReA (11) and two peptides with high homology to human sequences containing HLA-B27 binding motifs, some of which might be potentially cross-reactive self-antigens. The experimental approach and results of this study now open the way to attempt a direct identification of these and other bacterial peptides from the HLA-B27-bound peptide pool of infected cells by directly searching such peptides on the basis of their molecular mass, chromatographic features, and MS fragmentation pattern. In addition, the transfectants generated in this study can be used as target cells for CTLs from patients with Chlamydia-induced ReA to test the putative pathogenetic relevance of the identified bacterial peptides. Both types of studies are of major importance to elucidate the role of C. trachomatis and HLA-B27 in the pathogenesis of this disease but hardly feasible at the present time in the absence of candidate peptide epitopes.

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