Species-specific Differences in Proteasomal Processing and Tapasin-mediated Loading Influence Peptide Presentation by HLA-B27 in Murine Cells*

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Expression of HLA-B27 in murine cells has been used to establish animal models for human spondyloarthritides and for antigen presentation studies, but the effects of xenogeneic HLA-B27 expression on peptide presentation are little known. The issue was addressed in this study. HLA-B27-bound peptide repertoires from human and murine cells overlapped by 75–85%, indicating that many endogenous HLA-B27 ligands are generated and presented in both species. Of 20 differentially presented peptides, 40% were sequenced, the only 40% were sequenced, and these were found to diffuse inter-species protein polymorphism, suggesting that differences in antigen processing-loading accounted for many species-specific ligands. Digestion of synthetic substrates with human and murine 20 S proteasomes revealed cleavage differences that accounted for or correlated with differential expression of particular peptides. One HLA-B27 ligand found only in human cells was similarly generated in vitro by human and murine proteasomes. Differential presentation correlated with significantly decreased amounts of this ligand in human tapasin-deficient cells reconstituted with murine tapasin, indicating that species-specific interactions between HLA-B27, tapasin, and/or other proteins in the peptide-loading complex influenced presentation of this peptide. Our results indicate that differences in proteasomal specificity and interactions involving tapasin determine differential processing and presentation of a significant number of HLA-B27 ligands in human and murine cells.

The xenogeneic expression of HLA-B27, an MHC I class I molecule, strongly associated with spondyloarthritides (1, 2), has been used to establish transgenic animal disease models and to study the antigen-presenting and other properties of this molecule. HLA-B27 transgenic rats develop a spontaneous disease with many similarities to human spondyloarthritis (3). Disease manifestations are dependent on the genetic background and transgene copy number (4) and are modulated by alterations of the HLA-B27-bound peptide repertoire (5). Transgenic mice have also been used as a possible model for human HLA-B27-associated disease. Development of spontaneous inflammatory arthritis in HLA-B27 transgenic mice lacking β2m (6) may be related to absence of this polypeptide rather than to presence of the HLA-B27 heavy chain (7). In contrast, HLA-B27 transgenic mice expressing β2m are being used in reactive arthritis studies (8, 9) and have increased susceptibility to develop ankylosing spondylitis (10, 11). Other HLA class I molecules have also been expressed on murine cells for antigen presentation, epitope identification, and T-cell recognition studies (12–16).

However, antigen presentation by HLA-B27 or any other HLA class I molecule expressed on murine cells implies some inherent differences, relative to human cells, that have not been sufficiently characterized at a molecular level. Thus, species-related differences in the proteome, in proteasome cleavage specificity, in the peptide specificity of the transporter associated with antigen processing (TAP), and in the interaction of the human class I molecule with human or murine tapasin or other proteins in the peptide-loading complex, all might influence the HLA-B27-bound peptide repertoire and antigen presentation upon expression on murine cells. Numerous studies have addressed the peptide-transporting preferences of human and murine TAP (reviewed in Refs. 17 and 18), but the actual influence of species-related differences in this and other steps of the processing-loading pathway on HLA class I-mediated antigen presentation in murine cells is little known. Knowledge of such differences is critical for assessing the validity of HLA class I transgenic models for antigen presentation and human disease.

In this study, we have comparatively analyzed the HLA-B27-bound peptide repertoires expressed on human and murine cells and have characterized the origin of the differential expression of multiple HLA-B27 ligands in only one cell type. The results indicate a substantial lack of overlap between both peptide repertoires, which is only partially explained by species- or cell type-related protein differences. Both proteasome specificity differences and heterologous interactions in the peptide-loading complex contribute to differential peptide presentation by HLA-B27 on either human or murine cells. Our results have general implications for human MHC class I-mediated antigen presentation in murine systems.

MATERIALS AND METHODS

Cell Lines and Monoclonal Antibodies—HMY2 C1R (C1R) is a human lymphoid cell line with low expression of its endogenous class I antigens (19, 20). B*2705-C1R transfected cells were described elsewhere (21). P815-HTR (P815) is a murine mastocyte cell line. B*2705-P815
diffuse transfectant cells were previously described (22). Both the human and mouse transfectants express high and similar HLA-B27 levels (23). These were periodically checked by flow cytometry to ensure stable expression of this molecule. The C1R and P815 cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal bovine serum (both from Invitrogen, Paisley, UK). 72.120 is a human lymphoblastoid cell line (a gift from Dr. James McCluskey, University of Melbourne, Australia) in which HLA-A and HLA-B genes have been deleted and a non-functional tapasin protein is expressed (24, 25). This cell line expresses low levels of endogenous HLA-Cw*0102. Transfectants of HLA-B*2705 and wild type human or murine tapasin into C1R or P815 cell line expresses low levels of endogenous HLA-Cw*0102. Transfectants of HLA-B*2705 and wild type human or murine tapasin into 72.120 have been previously described (26). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The murine B27 transfectant cells were W6/32 (IgG2a, specific for a monomorphic determinant) (27) and ME1 (IgG1, specific for HLA-B27, -B7, and -B22) (28).

Flow Cytometry—About 6 × 10⁷ cells were washed twice in 200 μl of PBS and resuspended in 50 μl of undiluted mAb supernatant. After incubating 30 min, cells were washed twice in 200 μl of PBS and resuspended in 50 μl of fluorescein isothiocyanate-conjugated antimouse IgG rabbit antiserum (Calbiochem-Novabiochem GmbH, Schwalbach, Germany), incubated for 30 min, and washed two times in 200 μl of PBS. All operations were done at 4 °C. Flow cytometry was carried out on a BD Biosciences FACSCalibur instrument using CellQuest software.

Isolation of B*2705-bound Peptides—This was carried out from 10⁷ C1R or P815 transfectant cells lysed in 1% Nonidet P-40 in the presence of a mixture of protease inhibitors, after immunopurification of HLA-B27 with the W6/32 mAb and acid extraction, exactly as described elsewhere (29). HLA-B27-bound peptide pools were fractionated by HPLC at a flow rate of 100 μl/min as previously described (30), and 50-μl fractions were collected.

Mass Spectrometry Analysis and Sequencing—The peptide composition of HPLC fractions was analyzed by matrix-assisted desorption ionization time-of-flight (MALDI-TOP) MS using a calibrated Kompacta Probe instrument (Kratos- Shiimadzu) operating in the positive linear mode, as previously described (30). Alternatively, a Bruker Reflex III MALDI-TOF mass spectrometer (Buerkner-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT™ source in positive ion reflector mode was also used, as described elsewhere (31). Peptide sequencing was carried out by quadrupole ion trap nanoelectrospray MS/MS in an LCQ instrument (Finnigan ThermoQuest, San Jose, CA), as previously described (32, 33). In a few cases microelectrospray MS/MS in an LCQ instrument (Finnigan ThermoQuest, San Jose, CA), as previously described (32, 33). In a few cases microelectrospray MS/MS was used, using the same procedure, except that samples were injected, through an HPLC equipped with a C18 capillary column (150 × 0.18 mm) connected online, at a flow rate of 1.5 μl/min. In some cases, peptide sequencing was also done by post-source decay (PSD)-MALDI-TOF MS, as previously described (31).

In all cases peptide-containing HPLC fractions were dried and resuspended in 5 μl methanol/water (1:1) containing 0.1% formic acid. Aliquots of 0.5 or 1 μl were used for MALDI-TOF or nanoelectrospray MS analyses, respectively. For microelectrospray MS/MS-dried samples were resuspended in 0.5% acetic acid.

Synthetic Peptides—Peptides were synthesized using the standard solid-phase Fmoc (N-α-fluorenylmethoxy carbonyl) chemistry and were purified by HPLC. The correct composition and molecular mass of purified peptides were confirmed by amino acid analysis using a 6300 Amino Acid Analyzer (Beckman Coulter, Palo Alto, CA), which also allowed their quantification, and MALDI-TOF MS, respectively.

Purification of 20 S Proteasome—The 20 S proteasome was purified from 3 × 10⁷ B*2705-C1R or B*2705-P815 cell lysates by ion-exchange chromatography in an immobilized protein A column (previously described (30) with the following modifications. Protease-containing fractions from the previous purification step were identified by 12% SDS-PAGE and further subjected to anion-exchange chromatography by fast protein liquid chromatography using a Mono-Q HR5/5 column (Amersham Biosciences, Upsala, Sweden), at a flow rate of 1 ml/min, at a constant gradient of 0 to 100% buffer B (50 mM Tris/HCl, pH 8) for 1 h, followed by a linear gradient of 0–100% buffer B (50 mM Tris/HCl, 0.5 M KCl, pH 8) for 1 h. Purity of the fractions was assessed by SDS-PAGE. Aliquots of purified proteasome were stored at −80 °C. Absence of contaminant proteins in the 20 S proteasome samples was assessed by inhibition of proteolytic cleavage of a synthetic peptide substrate, histone 2A-(77–105), by protease inhibitors lactacystine (50 μg/ml) and epoxomicin (1 μg/ml).

Two-dimensional Gel Electrophoresis of 20 S Proteasomes—Samples of purified 20 S proteasomes were loaded by hydration of immobilized pH gradient (IPG) strips, non-linear pH 3–10, of 18-cm length (Amersham Biosciences), previously diluted to a total volume of 350 μl in 6 M urea, 2 M thiourea, 2% CHAPS, IPG non-linear pH 3–10, 1 mM Tris-(2-carboxymethyl)phosphine-HCl, and bromphenol blue. In the first dimension, IEF was performed in a IPGphor (Amersham Biosciences) under the following conditions: 30 V for 6 h, 60 V for 6 h, 500 V for 30 min, 1,000 V for 30 min, a gradient of 1,000–8,000 V for 30 min, and 8,000 V up to 32,000 Vh. After IEF, strips were equilibrated in 8 M urea, 30% glycerol in which HLA-A, -B, and -C were resolved and equilibrated for 20 min. Dithiothreitol (2%) and 4% iodoacetamide were added in the second and equilibration steps, respectively. The second dimension was performed using 12.5% SDS-PAGE. Gels were stained with silver nitrate and analyzed using the software ImageMaster (Amersham Biosciences). Spots were assigned by tryptic digestion followed by MS fingerprinting. The peptide composition of Synthetic Substrate—Peptide substrate (125 μg/ml) were incubated at 37 °C with purified 20 S proteasome at an enzyme/substrate ratio of 1:10 (w/v) in 20 mM Hepes buffer, pH 7.6. Digestion was stopped by adding 1/5 volume of 0.4% aqueous trifluoroacetic acid. Digestion mixtures were dried down to 100 μl in a Speed-Vac and fractionated by HPLC using the same conditions as for HLA-B27-bound peptides. Individual digestion products were identified on the basis of their molecular mass by MALDI-TOF MS and, when necessary for unambiguous assignment, by PSD-MALDI-TOF or electrospray MS/MS sequencing.

RESULTS

HLA-B27 Presents Distinct Peptide Repertoires on Human and Murine Cells—Peptide pools were isolated by acid extraction from HLA-B*2705 immunopurified from C1R and P815 transfectant cells and fractionated by HPLC under identical conditions and consecutive runs. The peptide composition of correlative HPLC fractions from both peptide pools were systematically compared by MALDI-TOF MS, using the same strategy as previously used to compare HLA-B27 subtype-bound peptide repertoires (34, 35). In short, the MS spectrum of any given HPLC fraction from one peptide pool was compared with the MS spectra of the correlative, previous, and following HPLC fractions from the other peptide pool. This was done to account for slight shifts in retention time that may occur between consecutive chromatographic runs. Ion peaks of the same (±1 mass-to-charge (m/z) ratio and retention time were considered to reflect shared peptides on human and murine cells. Identity of retention time and m/z does not necessarily indicate peptide identity, because in very complex mixtures unrelated peptides sharing these features might eventually co-elute. However, it is reasonable to assume that the overwhelming majority of identical peptide masses compared correspond to identical peptides. Indeed, in four of four cases in which ion peaks of the same m/z and retention time were sequenced from both the human and murine peptide pools they corresponded to identical peptides (Fig. 1). In nine additional cases a peptide sequenced from the murine peptide pool showed an identical counterpart in the human pool known from previous sequencing studies of HLA-B27-bound peptides from C1R cells to be the same peptide (Fig. 1). Ion peaks found in only one cell type in two independent experiments were considered as differentially expressed peptides. A total of 1372 and 1551 molecular species were compared from C1R and P815, respectively (Table I). Of these, 211 (15%) and 390 (25%) peptides were found only in human or in murine cells, respectively. In addition, of 351 shared peptides that showed particularly strong intensity signals in the MALDI-TOP spectra of at least one cell line, 54 (15%) and 82 (23%) showed 10-fold or higher intensity in the human or murine cells in two independent experiments, respectively. This is consistent with substantially higher expression of the shared ligand in the corresponding cell line. No significant differences in the average size of peptides expressed on either cell type were found (Table I).

The reproducibility of the MALDI-TOP spectra was assessed in two ways: by obtaining independent spectra from the same sample and by performing two independent comparisons with...
different peptide preparations. In both cases, the MS spectra of the same, or equivalent, HPLC fraction were in general very reproducible both in the nature of the ion peaks detected and in their relative intensities, although occasionally some variation was found. For this reason, assignment of both qualitative and quantitative differences was always done on the basis of reproducibility in two independent experiments. These results indicate that the HLA-B27-bound peptide repertoires on human and murine cells, although highly overlapping, contain a significant number of differentially bound ligands, as well as shared ones presented at substantially different amounts.

### Murine TAP Does Not Impair Presentation of B*2705 Ligands with C-terminal Basic Residues

A total of 27 shared ligands, including 3 octamers, 13 nonamers, 7 decamers, 3 undecamers, and 1 dodecamer, were sequenced by MS (Fig. 1). In addition, the sequence of 20 B*2705 ligands found only in human (9 peptides) or murine cells (11 peptides) was also determined (Fig. 2). All shared peptides corresponded to conserved sequences between both species. All the peptides sequenced contained the canonical anchor motif of HLA-B27, Arg2. Shared ligands also presented the same variety of C-terminal peptide residues previously defined for HLA-B*2705 ligands from human cells, including aliphatic, aromatic, and basic residues.

### TABLE I

Comparison of HLA-B*2705-bound peptides from human and murine cells

| Peptide        | Protein (Accession N.) | Sequenced from | Other cells | Ref. |
|----------------|------------------------|----------------|-------------|------|
| RRYNIPVL       | Vacular proton pump subunit (Q921X8) | P815-B*2705 |         |      |
| VRAAKPWK       | Hypothetical protein FLJ20311 (Q9NXD3) | P815-B*2705 |         |      |
| YRPQWVAL       | H3B histone (Q8VDJ2) | CIR/P815-B*2705 |      |      |
| ARDLERK       | Vimentin (P20152) | P815-B*2705 |         |      |
| ARLEKLYE       | Farnesyl pyrophosphate synthase (Q14329) | CIR-B*2705 |         |      |
| ARVSVNQY       | XPM2C protein (Q9GZ2) | P815-B*2705 |         |      |
| GRIGVITNH      | Ribosomal protein S4 (P47961) | CIR/P815-B*2705 | (60) |      |
| GRTLKHTKF      | 60 Ribosomal protein L36 (Q9Y3U8) | CIR/P815-B*2705 |         |      |
| GVRPSGETL      | Fatty acid synthase (P49327) | P815-B*2705 | CIR-B*2704 | (34) |
| GRYGGETRV      | Similar to splicing factor Arg/Ser-Rich 7 (Q9Y51) | P815-B*2705 | CIR-B*2705 | (61) |
| IRLPSQYP        | KIAA0906 protein (O94980) | CIR-B*2705 |         |      |
| KRPEGLTRG      | Serine/Threonine kinase (Q8TBX7) | CIR-B*2705 |         | (43) |
| KRPEKANNF      | 60S Ribosomal protein L7 (P14148) | P815-B*2705 | CIR-B*2705 | (61) |
| LRVDPFK      | Hmoglobin alpha 2 (Q96746) | CIR-B*2705 |         | (61) |
| SRFQQLRL       | KIAA1750 protein (Q9CUB3) | CIR-B*2705 |         |      |
| SRLNQSVP       | Farnesyl-diphosphate farnesyltransferase (P57978) | P815-B*2705 | CIR-B*2705 | (61) |
| ARDLTEVSAK     | Hypothetical protein (XP_174846) | CIR/P815-B*2705 |         |      |
| ARNPQLQQL      | ATP synthase protein (P48202) | P815-B*2705 |         |      |
| GPPNQFQTKT     | 40S Ribosomal protein (Q9CQR2) | P815-B*2705 | CIR-B*2705 | (45) |
| HRFYGGNNSY     | Ras-GTPase-activating protein (P97855) | P815-B*2705 |         |      |
| NRFAFQGIGL     | TBI (Q04197) | P815-B*2705 | CIR-B*2705 | (36) |
| RRALSCTPI       | Hypothetical protein XP_06852 | P815-B*2705 |         |      |
| RRKDAKSVKI      | 60S Ribosomal protein L38 (P23411) | P815-B*2705 |         |      |
| GRLPGQIVREL    | Transcription Initiation Protein (Q16550) | P815-B*2705 | CIR-B*2705 | (35) |
| RRYLENGKETL     | HLA-B27 (Q9TNS9) | CIR-B*2705 |         | (62) |
| SRSVAVLAL      | Beta-2-microglobulin precursor (P01884) | P815-B*2705 | CIR-B*2706 | (60) |
| RRRSGGQGGSY     | HLA-B27 (Q9TNS9) | P815-B*2705 | CIR-B*2705 | (63) |

**Fig. 1.** Amino acid sequence of HLA-B*2705 ligands present in both human (C1R) and murine (P815) cells. All sequences were determined by quadrupole/ion trap electrospray MS/MS. Isobaric residues (Ile/Leu and Lys/Gln) were assigned on the basis of unambiguous matching with sequences in the protein data base. The putative parental protein, with which full match was obtained, and the corresponding SwissProt accession number (www.ebi.ac.uk/swissprot/access.htm), is indicated. The one or more cell lines from which the sequence was determined are also indicated. When a peptide was sequenced from only one cell line, its presence in the other one was deduced from the finding of an ion peak of equal m/z and retention time. References are given for those peptides sequenced in this study that were previously reported as HLA-B27 ligands.

**TABLE I**

Comparison of HLA-B*2705-bound peptides from human and murine cells

| Peptide | Protein (Accession N.) | Sequenced from | Other cells | Ref. |
|---------|------------------------|----------------|-------------|------|
| C1R-B*2705 | 1372 | 1551 | 860-1667 Da | 860-1667 Da |
| Average mass | 1154 Da | 1154 Da | 1154 Da | 1154 Da |
| Shared peptides | 1161 (85%) | 1161 (75%) | 1161 Da | 1161 Da |
| Specific peptides | 211 (15%) | 390 (20%) | 390 Da | 390 Da |
| Average mass of shared peptides | 1144 Da | 1144 Da | 1144 Da | 1144 Da |
| Average mass of specific peptides | 1205 Da | 1193 Da | 1193 Da | 1193 Da |
| Major peaks counted<sup>a</sup> | 351 | 351 | | |
| Quantitative differences<sup>b</sup> | 54 (15%) | 82 (23%) | | |

<sup>a</sup> Ion peaks that showed particularly strong intensity in the MALDI-TOF spectrum from one or both cell lines.

<sup>b</sup> Ion peaks that showed 10-fold or more intensity in the MALDI-TOF spectrum from one cell line.
residues was 6 of 27. Among differential peptides (Fig. 2), 5 of 11 found only in C1R cells and 2 of 11 found only in P815 cells showed a C-terminal basic motif. Thus, of a total of 36 peptides from human cells and 38 peptides from murine cells, 11 (31%) and 8 (21%), respectively, contained C-terminal basic residues. The percentage from human cells is nearly the same as that (32%) previously reported in an independent compilation (36) and is only moderately higher than the percentage of B*2705 ligands with C-terminal basic residues found in murine cells. These results strongly suggest that the low preference of murine TAP for C-terminal basic residues reported from in vitro transport studies (37) introduces little bias against presentation of peptides with these motifs by HLA-B27 on murine cells.

Differential Presentation of HLA-B27 Ligands Is Only Partially Due to Protein Polymorphism—The 20 B*2705 ligands found only in either human or murine cells that were sequenced could be classified in three subsets (Fig. 2). Group 1, which included two peptides (10%), consisted of ligands arising from species- or cell type-specific proteins, with no counterpart in the other cell. Group 2 includes peptides arising from proteins present in both cells but differing in one or more amino acid residues within the sequence of the ligand. Group 3 includes peptides arising from proteins that are identical totally (i.e. the HLA-B27 heavy chain and human β2m transgene products) or within and around the sequence of the ligand.

Included 12 peptides (60%) consisted of ligands arising from proteins that are either identical in both cell types (i.e. the HLA-B27 and human β2m transgene products) or identical in the region corresponding to the peptide ligand and its neighborhood. Thus, less than half of the species- or cell type-related differences in the HLA-B27-bound peptide repertoire are explained by obvious differences in the parental proteins (groups 1 and 2). These results imply that differential processing, transport, and/or loading have a significant influence on differential peptide presentation by HLA-B27 on human or murine cells.

Distinct Proteasomal Cleavage Contributes to Differential Presentation of HLA-B27 Ligands—To assess the contribution of proteasomal processing to differential expression of particular HLA-B27 ligands in one cell type, we used synthetic precursors of four differentially expressed ligands from group 3 (Fig. 2), including two peptides found only in C1R cells and two others found only in P815 cells, with the sequence of the parental proteins in and around the sequence of the ligand. Each of these substrates was digested in vitro, in parallel experiments, with 20 S proteasomes purified from C1R and from P815 cells. Two-dimensional gel electrophoresis analysis indi-
cated that both the human and murine proteasome preparations contained a mixture of proteasome and immunoproteasome. The proteasome/immunoproteasome ratio was similar in both cases, within the limits of the technique used, as judged from the relative intensities of the spots corresponding to \( \alpha_1/\beta_1 \) and \( \alpha_2/\beta_2i \) subunits (the murine \( \beta_5 \) subunit did not appear in the two-dimensional gel due to its very basic pI) in the human and murine samples (Fig. 3). This technique does not allow us to rule out putative small differences in proteasome composition between cell lines, due to inaccuracies inherent to silver staining, whose intensity is variable for different proteins. However, given the similarity in proteasome/immunoproteasome composition between both cell lines, it is very unlikely that the cleavage differences observed (described below: Figs. 4–7), in particular differential cleavage of specific peptide bonds by the 20 S proteasome of only one cell line, can be attributed to cell-dependent variation in proteasome composition.

Each digestion mixture was fractionated by HPLC, and peptide-containing fractions were analyzed by MALDI-TOF MS. The yield of individual products was estimated on the basis of the corresponding chromatographic absorbance peaks at 210 nm, normalized to take into account peptidic length differences. When several peptides co-eluted, the contribution of each one to the absorbance of the corresponding peak was estimated on the basis of their intensities in the MALDI-TOF MS spectra. This is only an approximation, because ion peak intensity may not strictly correlate with peptide abundance. This approach has been used in previous studies from our laboratory (30, 31, 35, 38).

Of the four ligands analyzed, three different situations were found. The first one corresponded to the C1R-specific peptide IRNDEELNK, arising from residues 87–95 of histone 2A. Digestion of a synthetic precursor spanning residues 77–105 from this protein, which is identical in this region between mouse and human subjects, with human and murine proteasomes showed that (Fig. 4): 1) cleavage of the same substrate by the human or murine proteasome was not identical, revealing reproducible quantitative and qualitative differences in the cleavage of certain peptide bonds, 2) cleavage occurred at the exact N and C termini of the IRNDEELNK peptide (after Ala-86 and after Lys-95) with human proteasome, leading to recovery of the peptide ligand in the digest, albeit with low yield (0.1% of the total digest), 3) the murine proteasome failed to cleave after K95; this fact alone can explain the absence of the IRNDEELNK ligand in P815 cells, 4) cleavage at the exact N terminus of the peptide, after Ala-86, was less efficient with the murine than with the human proteasome (6 and 30%, respectively), and 5) cleavage within the sequence of the peptide ligand was significantly higher with the murine than with the human proteasome (39 and 9%, respectively). This was mainly due to increased cleavage after Asn-89 and Asn-94 by the murine proteasome.

These results indicate that the 20 S proteasome from C1R and P815 cells have distinct cleavage specificities. For the particular peptide analyzed, this explains its presentation by HLA-B27 on the human but not in the murine cell line. Digestion of this substrate by the human or murine proteasomes was inhibited with lactacystine (50 \( \mu \)g/ml) and epoxomicin (1 \( \mu \)g/ml), indicating that the differential cleavage observed was not due to contaminant proteases in the 20 S proteasome samples (data not shown).

A second situation was observed with two other peptides found only in P815, but not in C1R cells: KRAYLQAR, corresponding to residues 1699–1706 of the fatty acid synthase, and QRTPKIQVY, corresponding to residues 2–10 of human \( \beta_2m \) (Figs. 5 and 6). As shown below, absence of these ligands in the human cells correlated with failure or lower efficiency of the human proteasome to cleave at the exact N terminus of the...
ligand. In the first example (Fig. 5) two slightly different precursor substrates, differing only by the Ser to Asp change at position 1712, were used, to reflect the polymorphism of the parental human and murine proteins in this region. The following cleavage differences were found: 1) the murine proteasome cleaved at the exact N and C termini of KRAYLQAR (after Glu-1698 and after Arg-1706) and directly generated this ligand in the digestion mixture (0.3% of the total digest), 2) the human proteasome failed to cleave after Glu-1698 and, therefore, to directly generate the peptide ligand, and 3) the human proteasome cleaved at the C terminus of the ligand, after Arg-1706, with somewhat lower efficiency than the murine proteasome (8 and 16%, respectively). N-terminal precursors of the natural ligand were generated by both proteasomes.

The second example (Fig. 6) corresponded to the QRTPKIQVY peptide, arising from residues 2–10 of human /H9252. Its selective presence in murine cells correlated with the following: 1) significantly higher cleavage efficiency at the exact N terminus of the peptide (after Leu-1) by the murine proteasome, relative to the human one (14% versus 1.5%), 2) about 4-fold higher yield of the QRTPKIQVY ligand with the murine proteasome than with the human proteasome (8 and 16%, respectively), and 3) presumably, a higher intracellular expression of the human /H9252 in murine cells, because the corresponding gene was introduced by transfection. However, this higher expression does not, by itself, explain the absence of the ligand in human cells, because /H9252 is a very abundant protein also in the CIR cell line.

A third situation was observed with the RRYLENGKETLQR peptide, arising from residues 169–181 of the HLA-B27 heavy chain, and found only in CIR cells. In this case cleavage at the exact N and C termini (after Leu-168 and Arg-181) by the human and murine proteasomes occurred with comparable efficiency (Fig. 7). In addition, cleavage within the sequence of the ligand was globally similar, although differences in cleavage efficiency at particular peptide bonds (i.e.: after Arg-169 and Leu-179) were observed between both proteasomes. As a result, the peptide ligand was generated in vitro by the human and murine proteasomes with similar yields (0.5% and 0.3%, respectively). Thus, absence of the B27-(169–181) peptide in P815 transfectant cells cannot be explained by differences in proteasomal processing, as judged from in vitro digestions with 20 S proteasomes.

Species-specific Interactions with Tapasin Influence Presentation of a Natural Ligand by HLA-B27—We next examined whether absence of the B27-(169–181) ligand in the HLA-B27 heavy chain, and found only in CIR cells. In this case cleavage at the exact N and C termini (after Leu-168 and Arg-181) by the human and murine proteasomes occurred with comparable efficiency (Fig. 7). In addition, cleavage within the sequence of the ligand was globally similar, although differences in cleavage efficiency at particular peptide bonds (i.e.: after Arg-169 and Leu-179) were observed between both proteasomes. As a result, the peptide ligand was generated in vitro by the human and murine proteasomes with similar yields: 0.5% and 0.3%, respectively. Thus, absence of the B27-(169–181) peptide in P815 transfectant cells cannot be explained by differences in proteasomal processing, as judged from in vitro digestions with 20 S proteasomes.

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ion peak at m/z 1662.6/1662.8, eluted at HPLC fraction #129 from both transfectants.

The amount of this peptide in each pool was estimated as follows. First, the absorbance of HPLC fraction #129 relative to the total absorbance of each peptide pool was calculated. On this basis, the peptide amount in this fraction was 1.1% of the total peptide pool from each transfectant. Second, the per-
centage of the B27-(169–181) peptide, relative to the total peptide amount in fraction #129 from each peptide pool, was estimated on the basis of the intensity of the ion peaks in the corresponding MALDI-TOF MS spectra (Fig. 8, B and C).

This is only an approximate estimation, because MALDI-TOF MS is not quantitative. Nevertheless, the difference was reproducible both in two independent spectra obtained from the same sample, and in two independent preparations (data not shown). The intensity corresponding to the B27-(169–181) peptide in the MALDI-TOF MS spectrum of #129 from 721.220 cells transfected with human (m/z: 1662.8) or murine tapasin (m/z: 1662.6) was 25.9% and 4.5%, respectively, of the added intensity of all ion peaks in each fraction. Therefore, the estimated abundance of this peptide in the HLA-B27-bound peptide pools was: 1.1 × 0.259 = 0.28% and 1.1 × 0.045 = 0.05% in the human and murine tapasin transfectants, respectively. Thus, in the presence of human tapasin, loading of the B27-(169–181) peptide into HLA-B27 was 5.6-fold higher.

The reliability of this estimation was confirmed in a second independent experiment. In this one, the estimated abundance of the B27-(169–181) peptide in the 721.220 cell transfectants with human or murine tapasin was 0.4% and 0.04%, respectively, or a 10-fold increase in the presence of human tapasin. In turn, B27-(169–181) was previously estimated to represent 0.4% of the HLA-B27-bound peptide pool from B*2705-C1R transfectants cells, which are human lymphoid cells with fully functional tapasin (31).

These results indicate that the heterologous interaction between B*2705 and/or other components of the peptide-loading complex and murine tapasin significantly decreases the loading efficiency of the B27-(169–181) ligand, and strongly suggest that this influence contribute to impairing presentation of this ligand in murine cells.

Because 721.220 cells are of human origin, they do not fully reproduce the situation of an HLA-B27 transfectant in a murine cell. Thus, it is likely that additional species-related interactions in the peptide-loading complex, besides those involving HLA-B27 and tapasin, further contribute to impairing presentation of B27-(169–181) in murine cells. This is strongly suggested from a comparison of the estimated abundance of this ligand in various cell types, as summarized in Table II.

**DISCUSSION**

Expression of human MHC class I molecules, including HLA-B27, in murine cells and transgenic mice has been widely used to study antigen presentation to CTL (8, 9, 12, 13, 15, 16, 22, 23, 39–42) and to establish animal models for human disease (6, 10, 11). However, species-related differences both in the proteome and in the specificity of the antigen processing-loading pathway may influence HLA class I-mediated antigen pres-
presentation. Various studies have defined peptide specificity differences between human and murine TAP transporters (17, 18). However, to our knowledge, detailed analyses of the alterations of HLA class I-bound peptide repertoires upon expression on murine cells, and of the molecular basis for these alterations, have not been conducted in a systematic way. This analysis is of interest from the standpoint of the comparative biochemistry of MHC class I antigen processing in humans and mice and to assess the validity and putative limitations of HLA transfectant and transgenic mouse models for immunological and disease studies.

The present study was undertaken to determine: 1) the extent of the alterations in the HLA-B27-bound peptide repertoire upon expression of this allotype on murine cells, 2) to what degree these alterations are related to differences in the respective proteomes or in antigen processing, 3) the contribution of putative differences in cleavage specificity between human and murine proteasomes, and 4) the role of interactions involving tapasin in differential presentation of individual ligands of HLA-B27.

The large overlap observed between HLA-B27-bound peptide repertoires from human and murine cells indicates that antigen presentation is not dramatically altered upon expression of HLA-B27 on murine cells. This suggests that, globally, HLA class I expression in mice may be a good model for antigen presentation as it occurs in humans, as also suggested by others (15, 16). Yet, substantial differences between the endogenous peptide pools were observed, which were only partially explained by obvious species-related differences in the presence or structure of the parental proteins. This implies an influence of the antigen processing-loading pathway in altering the HLA class I-bound peptide repertoire on murine cells. Three steps along this route were considered in the present study: 1) differences in TAP specificity, 2) differences in proteasomal cleavage, and 3) influence of heterologous interactions in the peptide-loading complex.

The influence of differences in peptide specificity between human and murine TAP was not directly addressed, because the issue has been the subject of previous studies from other laboratories (17, 18). On the basis of the well-known differences between human and murine TAP in their preference for C-terminal peptide residues, namely the higher selectivity of murine TAP for C-terminal aliphatic and aromatic residues (37), a major influence of this feature on shaping the HLA-B27-bound peptide repertoire on murine cells was expected, because a substantial portion of the B*2705-bound peptide repertoire from human cells has C-terminal basic residues (35, 36, 43, 44), a motif that is favored by human, but not murine TAP. However, this was not the case. A C-terminal Arg motif was previously observed by pool sequencing of HLA-B27-bound peptides isolated from murine cells (45), in agreement with our results. Indeed, among the B*2705 ligands sequenced in the present study, the percentage of peptides with C-terminal basic residues found in murine cells was only moderately lower than on HLA-B*2705 ligands sequenced from human cells reported in previous studies (36). Thus, a role of C-terminal Arg motif was previously observed by pool sequencing of HLA-B27-bound peptides isolated from murine cells (45), in agreement with our results. Indeed, among the B*2705 ligands sequenced in the present study, the percentage of peptides with C-terminal basic residues found in murine cells was only moderately lower than on HLA-B*2705 ligands sequenced from human cells reported in previous studies (36). Thus, a role of C-terminal residue preferences by murine TAP seems to have limited influence on shaping the HLA-B27-bound peptide repertoire in murine cells. A likely explanation for this may be the important influence of the three N-terminal peptide positions on TAP specificity, as established for human TAP (46). An analogous influence of the N-terminal peptide region might also take place with murine TAP, but we are not aware of similar studies as those performed with human TAP using combinatorial chemistry. Conceivably, the influence of a combinatorial motif might compensate for the presence of a disfavored C-terminal one and allow transport of peptides with C-terminal basic residues by murine TAP.
High conservation of the proteasome in mammals would suggest that differences in proteasomal cleavage specificity between human and mouse subjects are unlikely to account for any significant variability in HLA class I-bound peptide repertoires. However, differential processing of an influenza nucleoprotein epitope (14) provided indirect evidence compatible with a role of the proteasome in generating this particular epitope only in human cells, although proteasomal cleavage was not analyzed in that study. Our results here demonstrate qualitative and quantitative differences in the cleavage patterns of synthetic peptide substrates between human and murine 20 S proteasomes. These differences accounted for differential presentation of one of four ligands analyzed, due to lack of cleavage at the C-terminal Lys residue of the peptide ligand by murine proteasomes. This result indirectly suggests that presentation of this ligand depends only on proteasomal cleavage at its exact C terminus and that the endopeptidase activity of the tripeptidyl peptidase II at Lys residues (47) is not involved. In two other instances there was correlation between lack of presentation of the peptide ligand and lack of cleavage at its exact N terminus or lower generation in vitro by the corresponding 20 S proteasome. MHC class I ligands can be produced after trimming of N-terminally extended precursors by ER aminopeptidases (48–52). The extent to which trimming accounts for generation of the constitutive MHC class I peptide repertoire is significant, but far from absolute, and it is conceivable that many ligands may require direct generation by the proteasome. Our data with KRAYLQAR and QRTPKIQVY are compatible with the possibility that, for these particular ligands, direct generation by the proteasome may determine presentation by HLA-B27, with little or no involvement of aminopeptidase-mediated trimming. The possibility that the observed differences in proteasome cleavage specificity might be due to a different proteasome/immunoproteasome ratio in the 20 S proteasomes isolated from C1R and P815 cells seems unlikely, because both cells showed a similar proteasome/immunoproteasome composition, within the limits of the analytical technique used. Moreover, because both cells contained a mixture of constitutive proteasome and immunoproteasome, small differences in the ratio of both forms would hardly explain that cleavage of certain peptide bonds occurred only with proteasomes from one cell type. Inhibition of proteolytic cleavage of a synthetic substrate by the proteasome inhibitors lactacystine and epoxomicin ruled out that the observed differences might be due to contaminant proteases in the 20 S proteasome samples. Thus, our results indicate that there are differences in the cleavage specificity of the 20 S proteasome between mouse and human subjects. In addition, although these experiments reflect in a rather crude and not quantitative way proteasomal processing in vivo (53), they strongly suggest that these differences have a significant influence on differential processing of particular peptides and, through this, on the shaping of HLA-B27-bound peptide repertoire differences between human and mouse subjects. Obviously this conclusion can be generalized to other HLA class I molecules expressed on murine cells.

In our study only one cell type from either humans or mice was compared, and it can be argued that cell- or organ-dependent variations in proteasome composition may influence proteasomal cleavage. Indeed, within a given species, proteasomal processing may vary depending on the exact proteasome/immunoproteasome balance in the cell or other factors, so that the whole spectrum of MHC class I ligands in vivo, and therefore inter-species differences, may be more complex than outlined in this study for HLA-B27. However, our data demonstrate that in two cell lines with similar 20 S proteasome/immunoproteasome composition there are distinct HLA-B27-bound peptide repertoires, and that some of the differences correlate with distinct cleavage patterns of the 20 S proteasomes from these cells. Therefore, our data indicate that human and murine 20 S proteasomes have differences in cleavage specificity and that

**Figure 7.** Digestion pattern of the HLA-B27-(165–194) synthetic substrate by purified 20 S proteasome from C1R (A) or P815 (B) cells. C, cleavage at individual peptide bonds of the HLA-B27-(165–194) substrate by 20 S proteasomes from C1R or P815 cells. Conventions are as in Fig. 4.

| Cleavage after | C1R    | P815   |
|---------------|--------|--------|
| W167          | 0.0    | 0.6-0.9|
| L168          | 9-12   | 8-4    |
| R169          | 2-1    | 10-15  |
| R170          | 8-6    | 7-8    |
| Y171          | 8-6    | 9-6    |
| L172          | 0.8-0.6| 0-0    |
| E173          | 0-0    | 3-2    |
| K176          | 0-0    | 0.6-0.2|
| T175          | 0-0    | 0.7-0.9|
| L179          | 7-5    | 1-2    |
| Q180          | 3-4    | 3-4    |
| R181          | 14-21  | 14-12  |
| D183          | 22-24  | 10-12  |
| P185          | 0-0    | 0.2-0.5|
| T187          | 5-6    | 8-5    |
| H188          | 13-16  | 12-14  |
| V189          | 3-2    | 14-18  |
| T190          | 5-4    | 3-1    |
| H191          | 7-6    | 6-5    |
| H192          | 5-4    | 6-7    |
| P193          | 0-0    | 2-1    |
these are reflected in specific differences in peptide presentation.

Differential cleavage did not account for failure of murine cells to present the HLA-B27-(169–181) peptide, suggesting that assisted peptide loading might be altered due to heterologous interactions between HLA-B27 and proteins in the loading complex of murine cells. Among these interactions, those involving tapasin may be particularly important due to the bridging role of this chaperone between TAP and the MHC molecule (54, 55), and to its influence on peptide loading (26, 56–58). Indeed, human cells expressing murine tapasin were significantly less efficient than those expressing human tapa-

Fig. 8. Recovery of the B27-(169–181) ligand from 721.220 cells expressing human or murine tapasin. A, HPLC fractionation of the HLA-B*2705-bound peptide pools isolated from 721.220 transfectant cells expressing human (solid line) or murine (dotted line) tapasin. Only the region of the chromatogram corresponding to retention times 61.5–68.5 min (fractions 125–139) is shown. The elution position of the B27-(169–181) ligand (retention time 63.5–64 min: fraction 129) is indicated. B, MALDI-TOF MS spectrum of fraction 129 from the 721.220 transfectant expressing human tapasin. C, MALDI-TOF MS spectrum of fraction 129 from the 721.220 transfectant expressing murine tapasin. The ion peaks corresponding to B27-(169–181); m/z: 1662.8/1662.6, indicated by arrows.
Differential Processing of HLA-B27 Ligands

The influence of differential processing/loading of HLA-B27 ligands in murine cells has a potential influence and should be taken into account, in cytolytic T lymphocytes and disease studies carried out in transgenic models, because presentation of relevant antigens might be drastically affected. Yet, a majority of the endogenous HLA-B27-bound peptide repertoire was conserved in the two cell types analyzed, suggesting that presentation of many HLA-B27 ligands is not significantly affected by expression of this molecule on murine cells.

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# Table II

|     | C1R    | P815 |
|-----|--------|------|
| HLA-B27 | Human  | Human |
| TAP    | Human  | Human |
| Tapasin | Human  | Human |
| B27-(169-181) | 0.4%<sup>a</sup> | 0.3–0.4%<sup>b</sup> |

<sup>a</sup> Data from Ref. 31.
<sup>b</sup> Results from two independent experiments.

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Species-specific Differences in Proteasomal Processing and Tapasin-mediated Loading Influence Peptide Presentation by HLA-B27 in Murine Cells
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