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Limited Diversity of Peptides Related to an Alloreactive T Cell Epitope in the HLA-B27-Bound Peptide Repertoire Results from Restrictions at Multiple Steps Along the Processing-Loading Pathway

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The influence of various factors along the processing-loading pathway in limiting the diversity of HLA-B27-bound peptides around a core protein sequence was analyzed. The C5 proteasome subunit-derived RRFPYYV and RRFPYYVY peptides are natural B*2705 ligands. The octamer is an allospecific CTL epitope. Digestion of a 27-mer fragment of C5 revealed that both ligands are generated from this precursor substrate with the 20S proteasome in vitro in a ratio comparable to that in the B*2705-bound peptide pool. The C5 sequence allowed to derive a nested set of six additional peptides with 8–11 residues containing the core octamer sequence and the Arg2 motif of HLA-B27, none of which was found in the B27-bound pool. Together, low proteasomal yield, disfavored TAP-binding motifs, and low affinity for B*2705 accounted for the absence of four of the six peptides. The two remaining differed from the natural octamer or nonamer ligands only by an additional N-terminal Ser residue. Their stability in complex with B*2705 was lower than the respective natural ligands, raising the possibility that N-terminal trimming might have favored a shift toward the more stable peptides. The results suggest that the B*2705-bound peptide repertoire has a highly restricted diversity around a core alloantigenic sequence. This is not explained by a single bottleneck feature, but by multiple factors, including proteasomal generation, TAP-binding motifs, MHC-binding efficiency, and perhaps optimized stability through N-terminal trimming. Tapasin-dependent restrictions, although not excluded, were not required to explain the absence in vivo of the particular peptide set in this study.


Maj or histocompatibility complex class I molecules constitute bind and present at the cell surface a large repertoire of endogenous peptides which can be recognized by CD8+ T cells. Class I-bound peptides generally range in size from 8- to 11-amino acid residues, with a majority of nonamers. Several factors, including endogenous Ag processing, peptide transport to the endoplasmic reticulum (ER), assisted loading, peptide affinity, and stability of the MHC-peptide complex determine the nature of the peptides bound to a given class I molecule (1). However, the precise contribution of these factors in shaping class I-bound peptide repertoires is insufficiently known.

Proteasomes are multicatalytic protease complexes that mediate most of the protein degradation in the cytosol. They are the main source of HLA class I-bound peptides and can directly generate natural MHC ligands in vitro. However, the contribution of additional proteases from the cytosol or other cell compartments is suggested by evidence that proteasome-specific inhibitors do not totally block class I expression or generation of particular class I ligands (2, 3). Recently, a subtilisin-like protease with the potential to substitute for some of the proteasome function has been reported (4). In addition, peptide trimming could occur in the cytoplasm and ER, generating peptides not produced directly by the proteasome (5–10). Furin, a protease in the trans-Golgi network may contribute to the production of class I-restricted viral Ags (11). However, the actual contribution of nonproteasomal peptidases to shaping class I-bound peptide repertoires is not known.

Peptides generated in the cytosol are transported into the ER by the TAP transporter, an heterodimeric protein located in the membrane of the ER. Human TAP deficiency causes a severe drop in cell surface expression of class I proteins, indicating that transport of most peptides is TAP dependent (12, 13). Human TAP is less restrictive for peptides than rat and murine counterparts but may exhibit large peptide-binding differences, as revealed by combinatorial peptide libraries (14, 15).

Once into the ER, peptide binding to class I molecules occurs in a process that requires the cooperative activity of at least four chaperones: calnexin, calreticulin, Erp57, and tapasin. The first three proteins have a role in assembling and correct folding of the class I protein and are not known to introduce sequence-dependent restrictions to peptide loading. Tapasin directly mediates the association of TAP and the class I molecule during peptide loading (16, 17). Allelic variation in MHC class I molecules influences their dependence on tapasin for peptide loading and Ag presentation. In
particular, HLA-B*2705 seemed able to efficiently form peptide complexes in the absence of tapasin (18).

Finally, peptide binding in the ER will be determined by MHC affinity. Peptides bind to class I molecules through conserved contacts involving the peptide main chain and the peptide ends. Additional interactions take place between peptideic anchor residues and side chain pockets in the peptide-binding site of the class I molecule (19). Class I polymorphism affects mainly residues located in or close to the pockets, modulating in this way the nature of class I-bound peptide repertoires.

The experiments described in this study arose from the identification of two closely related natural ligands of B*2705: the RRFFPYVV octamer and the C-terminally extended nonamer RRFPPPVYV. These peptides derive from the C5 subunit of the proteasome. The octamer was the specific epitope of an alloreactive CTL clone raised against B*2705, which recognized the nonamer much less efficiently (20). Starting from these observations, we have now addressed the following issues: 1) Are both B*2705 ligands directly generated by the proteasome or require further processing steps? 2) Does the proteasome generate additional peptides from the same region of the parental protein with the capacity to bind HLA-B27? 3) If so, do they bind B*2705 in vivo? 4) What factors impair HLA-B27-binding peptides generated by the proteasome from becoming natural ligands? Together these questions attempt to define the influence of several structural and functional factors in shaping the B27-bound peptide repertoire.

Materials and Methods

Cell lines

The anti-B*2705 alloreactive CTL 27S69 clone and its culture conditions have been described (20, 21). Hmy2.CIR (CIR) is a human lymphoid cell line with low expression of its endogenous class I Ags. The transfectant expressing B*2705 was cultured in DMEM (Life Technologies, Paisley, U.K.) with 7.5% heat-inactivated FCS. T2 is a TAP-deficient human cell line of lymphoid origin (12). The B*2705-T2 transfectant was a kind gift from Dr. David Yu (University of California, Los Angeles, CA). It was cultured in DMEM supplemented with 5% FCS. RMA-S is a TAP-deficient murine cell line (22, 23). The B*2705-RMA-S transfectant was cultured in RPMI 1640 supplemented with 10% FCS. When cultured at 26°C, T2 and RMA-S transfectants express class I molecules presumably devoid of peptides. These molecules are unstable at 37°C, but their surface expression at this temperature can be stabilized by exogenous peptide ligands.

Isolation of HLA-B27-bound peptides

This was done as described previously (20), with minor modifications. Briefly, 1–1.5 × 10^10 B*2705-C1R cells were lysed at 4°C in 20 mM Tris/HCl buffer, 150 mM NaCl, and 1% Nonidet P-40 (pH 7.5) with a mixture of protease inhibitors. Cell lysates were subjected to affinity chromatography using the W6/32 mAb (IgG2a, specific for a monomorphic HLA-A, HLA-B, and HLA-C determinant; see Ref. 24). HLA-B27-bound peptides were eluted from the column with 0.1% trifluoroacetic acid (TFA) in water at room temperature, filtered through Centricron 3 (Amicon, Beverly, MA), and concentrated to 100 μl for HPLC fractionation. This was conducted in a Waters alliance system (Waters, Milford, MA), using a Vydac C18 (0.21 × 25 cm) 5-μm particle size column (Vydac, Hopersia, CA), at a flow rate of 100 μl/min, as follows: isocratic conditions with buffer A (0.08% TFA in water) for 15 min, followed by a linear gradient of 0–44% buffer B (80% acetonitrile and 0.075% TFA in water) for 90 min, and a linear gradient of 44–100% buffer B for another 35 min. Peptide fractionation was simultaneously monitored at 210 and 280 nm. Fractions of 50 μl were collected and stored at −20°C.

Mass spectrometry (MS)

The peptide composition of individual HPLC fractions was determined by matrix-assisted laser desorption/ionization time of flight (MALDI/TOF) MS. A calibrated Kompact Probe instrument (Kratos-Sharpadzlu, Manches- ter, U.K.) operating in the positive linear mode was used. Dried fractions were resuspended in 5 μl methanol/water (1:1) containing 0.1% formic acid, and 0.5 μl was applied onto target and dried out. A total of 0.5 μl of saturated α-ciano-4-hydroxycinnamic acid matrix in water:acetonitrile (1:1) containing 0.1% TFA was then added and dried out. Sometimes, 1 μl of these samples was subjected to peptide sequencing in a LCQ electrospray/ion trap mass spectrometer (Finnigan Thermoquest, San Jose, CA), exactly as previously described (20).

Peptide synthesis and purification

Peptides were synthesized using standard F-moc chemistry and purified by HPLC to a purity >95%. 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 by MALDI-TOF and electrospray ion/trap MS.

Epitope stabilization assays

The quantitative epitope stabilization assay used to measure binding to B*2705 has been described (25). Briefly, B*2705-RMA-S cells were incubated for 24 h at 26°C. They were then incubated without FCS for 1 h at 26°C, with 10^-4–10^-9 M peptide, transferred to 37°C for 4 h, and collected for flow cytometry analysis with the ME1 mAb (IgG1, specific for HLA-B27, HLA-B8, HLA-B22; see Ref. 26). Binding was expressed as the C50, which is the molar concentration of the peptide at 50% of the maximum fluorescence obtained with that peptide in the concentration range used. When multiple peptides were compared, the C50 value of a reference peptide was first obtained. Binding of other peptides was expressed as EC50, which is the molar concentration of a given peptide required to obtain the fluorescence value at the C50 of the reference peptide. EC50 values were calculated as described previously (25). Peptides with EC50 values <10 μM were considered to bind with high affinity. EC50 values between 10 and 50 μM were considered to reflect intermediate affinity, and EC50 > 50 μM indicated low affinity.

A previously described (27) cell surface MHC-peptide complex stability assay was used. Briefly, T2-B*2705 cells (10^7 cells/well) were incubated overnight at 37°C, in serum-free cell culture medium (Serotec, Oxford, U.K.), in the presence of 100 μM peptide and 100 nM β2-microglobulin. After intensive washing, cells were incubated for 1 h at 37°C in RPMI 1640 containing 10% FCS and brefeldin A (10 μg/ml) to block egress of newly synthesized class I molecules. Cells were washed and incubation continued in the presence of 0.5 μg/ml of brefeldin A at 37°C. Cells were removed at various times and stained with the ME1 mAb as described above. The decay of B*2705-peptide complexes was determined as follows: % mean fluorescent intensity (MFI) remaining = [(MFI_bref/pep) - MFI_bref(0)]/(MFI_0 - MFI_bref(0)) (MFI_bref(0) = MFI_bref(0-τ) + MFI_0 - ατ).
to anion-exchange chromatography in a MonoQ SR5/5 column (Pharmacia, Uppsala, Sweden), at a flow rate of 0.5 ml/min, as follows: isocratic conditions with buffer A [50 mM Tris/HCl and 50 mM KCl (pH 8)] for 10 min, followed by a linear gradient of 0–30% buffer B [50 mM Tris/HCl and 0.5 M KCl (pH 8)] for 5 min and a linear gradient of 30–100% buffer B for another 30 min. Purity of the fractions was assessed by denaturing SDS-PAGE as above. Aliquots of purified proteasome were stored at 2°C.

Proteasome digestions were performed at 37°C in 20 mM HEPES buffer (pH 7.4). The peptide substrate was incubated with 4 × 10^6 cell-equivalents of 20S proteasome/mg at a final substrate concentration of 250 μg/ml. Digestions were stopped by adding 1 vol of 0.1% aqueous TFA and stored at −80°C.

HPLC fractionation of the proteasomal digestions was conducted in a Waters 625LC system using the same column and flow rate as for B*2705-bound peptides (see above) and the following chromatographic gradient: isocratic conditions with buffer A (0.1% TFA in water) for 20 min, followed by a linear gradient of 0–44% buffer B (80% acetonitrile and 0.1% TFA in water) for 80 min, and a linear gradient of 44–100% buffer B for another 40 min.

Results

The epitope recognized by CTL 27S69 is proteasome dependent

In preliminary experiments, we tested whether lysis of B*2705-C1R targets could be affected by the proteasome inhibitors LLnL and the more specific LCT. As shown in Fig. 1, cells previously treated with LLnL were not lysed by CTL 27S69 upon adding brefeldin A to prevent egress of newly synthesized molecules. The effect was not due to brefeldin A, and lysis was restored upon addition of the synthetic RRFFPYVV peptide. The same result was obtained with LCT. This indicates that expression of the RRFFPYVV epitope is critically dependent on proteasome activity.

FIGURE 1. Inhibition of CTL 27S69-mediated lysis of B*2705-C1R target cells by LLnL or LCT. Cells were treated with the indicated concentrations of proteasome inhibitor and subsequently with brefeldin A as described in Materials and Methods. Percent specific lysis of target cells either untreated, incubated with 4 μg/ml brefeldin A, or brefeldin A plus proteasome inhibitor. Lysis of the same targets in the presence of brefeldin A, proteasome inhibitor, and 10 μM RRFFPYVV peptide. Specific lysis of untransfected C1R cells at the E:T ratio used (2:1) was 0%. Data are means of two (for LLnL) or three (for LCT) independent experiments.

FIGURE 2. Amino acid sequence of the region of the C5 proteasome subunit spanning residues 120–146, which includes the CTL 27S69 epitope sequence (underlined). The natural octamer and nonamer ligands containing this sequence and the nested set of related peptides with Arg2 and 8–11 residues in length are indicated.

FIGURE 3. MALDI-TOF MS spectra of unfractionated proteasome digests of the C5 (120–146) peptide at 4 h (A) and 24 h (B). About 8 μg of peptide was incubated with 24 × 10^6 cell-equivalents of 20S proteasome purified from B*2705-C1R cells. The octamer epitope, its related nonamer, the detected peptides from the J1-J6 set, and the undigested 27-mer are indicated. Peaks at the lowest mass/charge (m/z) values are due to the matrix. A control showing the absence of proteasome-derived peptide peaks after incubation of the 20S proteasome for 24 h in the absence of the C5 (120–146) substrate is included (B, inset).
The RRFFPYVV epitope and a nested set of related peptides are directly generated by the 20S proteasome

An examination of the primary structure of the C5 protein around the core sequence of the CTL 27S69 epitope revealed a nested set of sequences ranging from 8 to 11 residues with the HLA-B27 peptide motif R2 (Fig. 2). Thus, we asked whether the known natural ligands RRFFPYVV and its C-terminally extended nonamer RRFFPYVYV as well as the other peptides of the nested set (designated J1-J6) were directly generated in vitro by the 20S proteasome from B*2705-C1R cells. A precursor peptide spanning residues 120/146 of the C5 protein was digested for 4, 8, and 24 h at the same enzyme:substrate ratio. Approximately 30, 82, and 97%, respectively, of the substrate was digested at these three times on the basis of the absorbance of the HPLC peak corresponding to the undigested 27-mer at 210 nm. Unfractionated digests at both 4 and 24 h were analyzed by MALDI-TOF MS (Fig. 3). At both time points, the maximal signal among the digestion products corresponded to the RRFFPYVYV nonamer, and two other major peaks corresponded to RRFFPYVV and to J3. J2 and J5 yielded minor signals at 4 h, the former of which became more significant at 24 h. Other peptides, unrelated to the nested set, were also detectable. As a control for the absence of proteasomal autolysis in vitro, the 20S proteasome was incubated for 24 h in the absence of any substrate and, as a control for the absence of proteasomal autolysis in vitro, the 20S proteasome was incubated for 24 h in the absence of the 27-mer substrate. No peptide peaks were observed upon MALDI-TOF analysis (Fig. 3B).

A more complete screening of the proteasomal digest was conducted through HPLC fractionation of the digest at 8 h and MS analysis of the corresponding fractions (Fig. 4). This revealed a very complex digestion pattern and the direct generation of the two natural HLA-B27 ligands plus J1-J5, but not J6.

An estimate of individual peptide yields in this digestion was conducted by computing the peak area of the HPLC fractions in which each peptide eluted. These fractions usually contained peptide mixtures when analyzed by MS. Thus, each peptide was assigned a percentage of its corresponding HPLC fraction area that was its corresponding percentage of the added intensity of all of the MALDI-TOF peptide signals in that HPLC fraction. This procedure is not strictly quantitative, since the relationship between peptide amount and signal intensity in MALDI-TOF spectra is not the same for all of the peptides and may be influenced by multiple factors. In addition, it could not be applied to some peptides that were detected by electrospray, but not by MALDI-TOF MS. However, synthetic J1-J6 were all easily detectable by MALDI-TOF, and, in control measurements in which peptides coeluting in this experiment (nonamer/J3; octamer/J2, J4/J5) were mixed at known amounts, their molar ratio and the ratio of the peak intensities in MALDI-TOF spectra were similar (data not shown).

A total of 55 molecular species were detected in the HPLC-fractionated proteasomal digest (Fig. 4A), 34 of which were recovered with a yield $\geq 0.5\%$ (Fig. 4B). The most prominent peptide was RRFFPYVV, accounting for 21.4% of the total digest. The second most abundant peptide was J3 (4.4%), followed by the RRFFPYV octamer (2.5%) and J5 (2.3%). J2 and J4 were below 1% (0.5 and 0.8%, respectively). J1 was obtained with very low yield (0.05%), and J6 was not detected (Table I). The identity of the octamer, nonamer, and J1-J5 was confirmed by fragmentation analysis using electrospray MS/MS.

These results indicate that the natural octamer and nonamer ligands are both directly generated by the 20S proteasome in vitro. They also suggest that proteasome cleavage limits the number of putative HLA-B27 ligands within a nested set of peptides by failing to generate some of them and generating others in very low amounts. Thus, although cleavage occurred at many positions under the experimental conditions used, a few peptide bonds were strongly preferred (Fig. 4B).

Most peptides from the RRFFPYVV-related set bind B*2705 in vitro

Binding of J1-J6 to B*2705 in vitro was compared with that of the octamer and nonamer ligands using a peptide stabilization assay (Fig. 5). The RRFFPYVV nonamer, J2, J5, and J6 bound with the highest affinity (EC$_{50}$, 1–2 $\mu$M), J3 and the RRFFPYVV octamer bound well but somewhat less efficiently (EC$_{50}$, 6–7 $\mu$M). J1 and J4 bound weakly (EC$_{50}$ >50 $\mu$M). Thus, four of the six peptides in the J1-J6 set bound B*2705 in vitro similarly as the two natural ligands.

The J1-J6 peptides are not found among natural B*2705 ligands

On the basis of their efficient binding in vitro (Fig. 5), J2, J3, J5, and J6 had the potential of being natural B*2705 ligands. To search for their putative presence in the B*2705-bound peptide pool, we first determined the retention time of the corresponding synthetic peptides in HPLC. Then the B*2705-bound peptide pool from B*2705-C1R cells was fractionated in exactly the same chromatographic conditions, and the HPLC fractions at the retention time of each peptide, as well as neighbor ones, were analyzed by MALDI-TOF and, in some cases, also by electrospray ion trap MS (Figs. 6–8). The natural octamer and nonamer ligands provided a control for the reproducibility of retention times between the synthetic peptides and the peptide pool.

J3 showed almost the same retention time as the natural nonamer ligand (Fig. 6A). However, whereas the nonamer was clearly detected in HPLC fractions 194–195 of the B*2705-bound peptide pool, no signal corresponding to J3 was obtained in these fractions or in the following one (Fig. 6B). This was not due to deficient detection of J3 by MALDI-TOF MS, since in a mixture of the synthetic J3 and nonamer both peptides were detected with similar sensitivity by this technique (data not shown).

Similarly, J2 showed the same retention time as the natural octamer ligand (Fig. 7A) and both peptides generated similarly intense signals in MALDI-TOF spectra (data not shown). The octamer was detected in HPLC fractions 189–193 of the B*2705-bound peptide pool corresponding to retention times of 93.5–95.5 min. A prominent peak similar to the molecular mass of J2 (M+H" : 1235.4 Da) was seen in HPLC fraction 192 (Fig. 7B) and, in smaller amounts, in adjacent fractions (data not shown). When subjected to electrospray MS/MS fragmentation analysis, the fragmentation pattern was unrelated to J2, as none of the fragmentation ions of this peptide were observed, but was compatible with RRFFVVVPTF. This peptide was originally sequenced from B*2702 (35) and subsequently from B*2705 (36). It has the same molecular mass as J2 and coelutes with the octamer in the HPLC conditions used (our unpublished observations). Thus, J2 was not found in this analysis in the B*2705-bound peptide pool.

J4 and J5 also showed very similar retention time in HPLC (Fig. 8A) and similar sensitivity by MALDI-TOF (data not shown), but they were not present in the corresponding HPLC fractions of the B*2705-bound peptide pool (Fig. 8B). By the same analysis, we also failed to find J6 in this pool (data not shown).

The sensitivity of the method used to analyze the putative presence of the J1-J6 peptides in the HLA-B27-bound pool is determined by the features of the MALDI-TOF analysis. In the MS spectra of Figs. 6B and 8B, the intensity of the signal for each individual peptide in a given HPLC fraction is relative to the maximal signal from that fraction. For instance, in Fig. 6B, the maximal signal (100%) corresponded to the RRFFPYVV nonamer and background noise was about 1%. Thus, absence of a signal corresponding to J3 suggests that, if present, J3 should be <1% of
the nonamer. This is considerably lower than the proteasomal yield of J3, relative to the nonamer, in vitro (see above). Similarly, J4 and J5 would probably be <1% the amount of the peptides that give the maximal signals in Fig. 8B. Thus, we cannot exclude that J1-J6 might be present in amounts <100-fold lower than the reference nonamer or the most abundant peptides in the corresponding HPLC fractions.

**Stability of the J1-J6 peptides in complex with B*2705**

In a final set of experiments, we analyzed peptide stability in complex with B*2705. The natural octamer and nonamer ligands and J1-J6 were separately incubated in the presence of human β2-microglobulin with B*2705-T2 transfectant cells. After loading the exogenous peptides on cell surface-expressed B*2705, the cells were treated with brefeldin A, to prevent B*2705 egress, and the decay of HLA-B27-associated fluorescence at the cell surface was measured as a function of time. The stability of each peptide in complex with B*2705 was expressed as the time required to obtain 50% of the maximal fluorescence (DT50), measured just before adding brefeldin A. The results are shown in Table I. The natural octamer and nonamer ligands had DT50 values of 13.2 and 17.9 h, respectively, indicating that DT50 values in this range or higher are appropriate for natural ligands. Thus, absence of J5 and J6 (DT50, 15.2 and 17.4 h, respectively) in vivo was not explained by low stability. The DT50 value of J4 (9.9 h) was somewhat lower than for the octamer. However, its binding in vitro was weak (EC50, 61 mM), suggesting that J4 associates inefficiently with B*2705. The lowest DT50 value corresponded to J1 (7.6 h), a peptide that bound very weakly in vitro (EC50 >100 μM), J2 (8.5 h), which nevertheless bound efficiently in vitro (EC50, 2 μM), and J3 (9.1 h). It is unclear whether any of the DT50 values of J1-J6 are outside the range allowed for natural ligands. However, J2 and J3, which differ from the octamer and nonamer, respectively, by one additional N-terminal Ser residue, showed lower stability than the respective natural ligands. This might be related to the absence of J2 and J3 in vivo (see Discussion).

**Discussion**

We have attempted to dissect the factors limiting the peptides naturally presented by HLA-B27 out of the potential ligands derived from the same region of a protein. The HLA-B27-allospecific epitope RFFPYYV and its C-terminally extended nonamer RFFPYYVYN were both found in the B*2705-bound peptide pool. Presence of the nonamer, but not the octamer, in B*2701, despite efficient binding of this peptide to B*2701 in vitro, raised

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**Table I. Relationship between processing, transport, and binding features of HLA-B27 ligands and their presence in vivo**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Name</th>
<th>Proteasome Yield (%)</th>
<th>TAP P1 Motif</th>
<th>Affinity</th>
<th>Stability* DT50 (h)</th>
<th>Found in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFFPYYV</td>
<td>J1</td>
<td>2.5</td>
<td>+</td>
<td>High</td>
<td>13.2 ± 1.8</td>
<td>Yes</td>
</tr>
<tr>
<td>RFFPYYYYV</td>
<td>J2</td>
<td>21.4</td>
<td>+</td>
<td>High</td>
<td>17.9 ± 4.4</td>
<td>Yes</td>
</tr>
<tr>
<td>SRRFPYV</td>
<td>J3</td>
<td>0.05</td>
<td>+</td>
<td>Low</td>
<td>7.6 ± 0.8</td>
<td>No</td>
</tr>
<tr>
<td>SRRFPYYV</td>
<td>J4</td>
<td>0.5</td>
<td>+</td>
<td>High</td>
<td>8.5 ± 1.3</td>
<td>No</td>
</tr>
<tr>
<td>SRRFPYYVY</td>
<td>J5</td>
<td>4.4</td>
<td>+</td>
<td>High</td>
<td>9.1 ± 2.2</td>
<td>No</td>
</tr>
<tr>
<td>SRRFPYYYV</td>
<td>J6</td>
<td>0.8</td>
<td>−</td>
<td>Low</td>
<td>9.9 ± 3.3</td>
<td>No</td>
</tr>
<tr>
<td>RFFPYYNYV</td>
<td>J7</td>
<td>2.3</td>
<td>−</td>
<td>High</td>
<td>15.2 ± 4.1</td>
<td>No</td>
</tr>
</tbody>
</table>

* Features with the potential to impair the presence of the peptide in the B*2705-bound pool in vivo are underlined.

* Peptide yields (see text) are the means of two digestion experiments at 8 h carried out under the same conditions and with similar results.

* TAP-binding motifs are from Refs. (14 and 15).

* DT50 ± SD. Data are the means of five to seven independent experiments. See text for an assessment of the DT50 values for J2 and J3.
the possibility that the octamer might be generated by trimming of the nonamer in the ER and that this might be impaired by B*2701 polymorphism (20). However, because both peptides are generated by the 20S proteasome, trimming is not required to explain the presence of the octamer in the B*2705-bound peptide pool. This is in agreement with previous observations that the proteasome directly generates the C-terminal ends of most presented peptides (2). There are several outstanding aspects of our proteasome digestion experiments. First, the nonamer and the octamer were generated in a 8.5:1 ratio. This is not very different from the estimated 4:1 ratio of these peptides in the B*2705-bound pool (20). It has been pointed out that in vitro digestions of relatively short peptides with the 20S proteasome do not resemble the situation in vivo where ubiquitinated proteins are digested by the 26S proteasome (2). However, generation of the octamer and nonamer in vitro in a ratio comparable to that found in the B*2705-bound peptide pool suggests that their generation in vivo may be similar and a major determinant of their presence in that pool, with transport and tapasin-mediated loading not having a major influence on their relative abundance.

Second, that the nonamer was the major digestion product after 4 h, when only 30% of the precursor peptide was digested, and the

FIGURE 5. Peptide binding to B*2705. Peptides were incubated at various concentrations with B*2705-RMA-S cells at 26°C. HLA-B27-associated fluorescence was measured 4 h after transfer at 37°C. Binding efficiencies were expressed as EC50 values (see Materials and Methods) and are given for each peptide. The KTGGPIYKR peptide was used as negative control. Data are means of two independent experiments.

FIGURE 6. A, Retention times of synthetic RRFPYYVY and J3. Both peptides were separately subjected to HPLC under the same chromatographic conditions, and their chromatograms were superimposed. B, MALDI-TOF spectra of HPLC fractions 194–196 (retention times, 96–97.5 min) of the B*2705-bound peptide pool from B*2705-CIR cells. The intensity peak corresponding to RRFPYYVY in fractions 194 and 195 is marked. The prominent peak in fraction 196 corresponded to another known B*2705 ligand. Arrows close to mass/charge (m/z) 1400 indicate the expected place for the J3 signal (M+H+: 1398.6 Da), which was absent.
octamer was also in significant amounts indicates that proteasomal generation of these peptides is strongly favored. That their yields were maintained after 24 h indicates that their kinetics of generation and stability with the 20S proteasome is similar. Although the proteasome can destroy class I epitopes (37) and despite cleavage within the core octamer sequence, the stability of both peptides in the presence of the proteasome might favor their abundance in vivo.

The J1-J6 set provided the opportunity to follow, in a defined set of related peptides, the influence of different factors on shaping the HLA-B27-bound peptide repertoire. Although our MS analysis does not rule out that the J1-J6 peptides could be present in very

**FIGURE 7.** A, Retention times of synthetic RRFFPYVV and J2. Both peptides were separately subjected to HPLC under the same chromatographic conditions, and their chromatograms were superimposed. B, MALDI-TOF spectrum of HPLC fraction 192 (retention times, 95–95.5 min) of the B*2705-bound peptide pool from B*2705-C1R cells. The intensity peak corresponding to RRFFPYVV is marked. The peak at mass/charge (m/z) 1237.4 was analyzed by quadrupole ion trap electrospray MS/MS. Its fragmentation pattern was compatible with RRFFNVVPTF, a previously reported B*2705 ligand (see text).

**FIGURE 8.** A, Retention times of synthetic J4 and J5. Both peptides were separately subjected to HPLC under the same chromatographic conditions, and their chromatograms were superimposed. B, MALDI-TOF spectra of HPLC fractions 184–186 (retention times, 91–92.5 min) of the B*2705-bound peptide pool from B*2705-C1R cells. Arrows indicate the expected place for the J4 (M+H⁺: 1512.7 Da) and J5 (M+H⁺: 1425.6 Da) signals.
low amounts, failure to detect them in the B*2705-bound pool suggested a significant restriction on the number of putative ligands arising around a core sequence from a given protein, a situation quite different from the nested peptide sets frequent among class II Ags (38–40).

Only the absence of J1 and J4 could be explained on the basis of low binding to B*2705 in vitro, as all other peptides bound similarly as natural ligands. Proteasome processing accounted only partially for further limitation, since only J6 was undetected, and J1 was generated with very low yield. The possibility that processing in vivo may yield a different peptide pattern is clearly open. Indeed, since proteasome activity is highly regulated in vivo, it is possible that the pattern of proteolytic products, especially for those reflecting minor cleavage specificities, may not be conserved in vivo. However, on the basis of the correlation between in vitro processing and in vivo occurrence of the octamer and nonamer ligands, our results suggest that at least J1-J5 might be generated in vivo and some (J3 and J5) perhaps in an amount comparable to the octamer ligand.

The efficiency with which these peptides bind TAP could influence their presence in the B*2705-bound repertoire. TAP-mediated transport was not addressed in this study. However, previous analyses using combinatorial peptide libraries have established the importance of the three N-terminal residues and, especially, the C-terminal one for human TAP binding, and defined the effect of different amino acid residues at these positions (14, 15). On that basis, J1-J3 and J6 have favored TAP-binding motifs and would presumably be transported in a similar way. In contrast, C-terminal Asn is a disfavored TAP-binding motif, and this might limit availability of J4 and J5 in the ER.

Thus, despite the limitations of in vitro assays, which cannot obviously reproduce the situation in vivo, it was possible to correlate low proteasomal digestion yields, disfavored TAP-binding motifs, and/or low B27-binding efficiency with the absence of J1, J3, J4, J5, and J6 in the B*2705-bound pool. However, the absence of J3, and perhaps also J2, was not satisfactorily explained by these criteria.

A possible explanation for the absence of J3 among natural ligands might be its low stability (about 50% in our assay), relative to the natural nonamer ligand, from which it differs only by an N-terminal Ser extension. There is evidence for non-proteasomal cytosolic and ER proteases capable of trimming the N-terminal residues of proteasomal peptide products (2, 5, 7, 9, 10). It is conceivable that trimming of J3 to generate the RRFFPYVYV nonamer might be favored by the higher stability of this peptide in complex with B*2705. A similar mechanism might explain the absence of J2, an N-terminal extension of the octamer, which also binds HLA-B27 in vitro with lower stability (64%) than the octamer ligand.

Thus, of a nested set of eight peptides with size and peptide motifs appropriate for binding to HLA-B27, only two become bound in vivo. This limitation cannot be explained by a single bottleneck feature, but rather by a combination of requirements including efficient proteasomal processing, appropriate TAP-binding motifs, high affinity for HLA-B27, and sufficient stability of the B27-peptide complex (Table I). In addition, N-terminal trimming might contribute to further reduce the number of closely related ligands toward those forming more stable complexes with HLA-B27. A role of tapasin in limiting loading of these peptides into HLA-B27, although not ruled out, does not need to be invoked.

In conclusion, in contrast to class II molecules, nested peptide sets are unlikely to exist in class I-bound repertoires. The more strict length requirements for class I-ligands do not fully account for this absence, since in the allowed size range there is room for nested peptide sets with the potential to bind the class I molecule, as in the case analyzed here. Rather, besides size and MHC affinity, the specificity features of the processing and transport systems for class I-bound peptides and the stability of the MHC-peptide complex are critical limiting factors.

A final word should be devoted to the experimental strategy used in this study. It was based on in vitro analysis of proteasome cleavage patterns, peptide binding to “empty” HLA-B27 on the cell surface, stability of such complexes, and a consideration of TAP-binding motifs as defined with peptide libraries. Obviously, in vitro assays do not reproduce the conditions of peptide-processing, transport and MHC binding in vivo. Therefore, our results do not exclude that processing and binding of the peptides analyzed in this study may be different in vivo. Despite these limitations, our analysis explains the presence of two natural ligands of HLA-B27 on the basis of their processing and binding features in vitro. Similarly, failure to detect various related peptides in the B27-bound pool correlated with one or more unfavorable features related to their processing, transport, binding, or stability in vivo. This close correlation cannot be ignored and strongly suggests that some major factors influencing the composition of class I-bound peptide repertoires can be dissected and characterized in vitro.

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