Cutting Edge: HLA-B27 Acquires Many N-Terminal Dibasic Peptides: Coupling Cytosolic Peptide Stability to Antigen Presentation


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Cutting Edge: HLA-B27 Acquires Many N-Terminal Dibasic Peptides: Coupling Cytosolic Peptide Stability to Antigen Presentation

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Ag presentation by MHC class I is a highly inefficient process because cytosolic peptidases destroy most peptides after proteasomal generation. Various mechanisms shape the MHC class I peptidome. We define a new one: intracellular peptide stability. Peptides with two N-terminal basic amino acids are more stable than other peptides. Such peptides should be overrepresented in the peptidome of MHC class I-associated peptides. HLA-B27 binding peptides use anchor residue R at P2 and, although most amino acids are allowed, particular amino acids are overrepresented at P1, including R and K. We show that such N-terminal dibasic peptides are indeed more efficiently presented by HLA-B27. This suggests that HLA-B27 can present peptides from Ags present in fewer copies than required for successful peptide generation for other MHC class I molecules. The Journal of Immunology, 2006, 176: 2697–2701.

Almost all patients with ankylosis spondylitis express HLA-B27. Also other spondyloarthropathies like reactive arthritis and psoriatic arthritis have strong linkage disequilibria with HLA-B27 (1). The linkage is so strong that HLA-B27 should be causally involved in these rheumatic diseases. HLA-B27 is certainly not the only factor involved but the other factors are unknown, although bacterial infections may contribute (2).

Why HLA-B27 is essential for the development of these rheumatic diseases is unknown. The gene is sequenced (3), the cell biology is studied (4), the protein structure is solved (5), and many peptides presented by HLA-B27 are identified (6). HLA-B27 does not differ from other HLA alleles, perhaps with two exceptions. First, HLA-B27 contains a free cysteine (C)4 in the peptide-binding groove, and a small fraction of HLA-B27 H chains are disulfide-linked homodimers at the plasma membrane (7). Second, sequencing of HLA-B27-associated peptides identified a dominant basic anchor residue R at P2 (8). The HLA-B27 structure revealed a corresponding deep pocket in the peptide-binding groove filled by the R anchor residue (9). Of all other HLA alleles, only HLA-B39 binds peptides with a R or H anchor residue at P2, and HLA-B*1402 uses this anchor residue at P2 facultatively (10). HLA-B39 and HLA-B*1403 have overlapping peptide specificity with HLA-B27 (9) and may replace HLA-B27 in patients with ankylosis spondylitis (11, 12). Gorillas express an MHC class I molecule (Gogo-B*0101) that also links to a form of ankylosis spondylitis (13). The common denominator of these alleles is not the core sequence (which is not strongly related to HLA-B27) but the associated peptides that use the anchor residue R at P2. Still, how could Ag presentation of such class I alleles relate to rheumatic diseases?

Ag presentation by MHC class I is the result of a series of biochemical processes. Ags are degraded by the proteasome and further trimmed by peptidases like TPPII, TOP and others (14). After transport into the endoplasmic reticulum (ER) by the peptide transporter TAP, peptides can associate to MHC class I molecules before or after further trimming by ER peptidases (15) before transport to the plasma membrane (16). The collective specificities of all individual steps in the Ag presentation pathway should determine the MHC class I peptidome. This process is extraordinary inefficient, because ~2000 protein copies have to be degraded for each peptide presented by a MHC class I molecule (16, 17). A major cause of this inefficiency is the high cytosolic peptidase activity, and the specificities of these peptidases are poorly defined. Analysis of the combined cytosolic activities revealed that 1) cytosolic peptidases are exclusively aminopeptidases, 2) N-terminal amino acid extensions increase the half-life of peptides, and 3) N-terminal amino acids in the substrate peptide have a small effect on peptide stability in the cytosol (at best a factor 2–3) (14).

In this study, we show that peptides with the HLA-B27 anchor residue R at P2 are considerably more stable in the cytosol, when preceded by a K or R. Consequently, amino acid R and K are overrepresented at P1 in HLA-B27 when produced from
equal amounts of substrate. This is the first study showing a direct effect of cytosolic peptidase activity on the HLA-bound peptide. This new parameter should be included in peptide prediction programs, and may provide another rationale for the link between HLA-B27 and related rheumatic diseases.

Materials and Methods

Reagents

Peptides were synthesized using standard 9-fluorenlymethoxycarbonyl chemistry. Internally quenched peptides were synthesized as described previously (18). GFP-ubiquitin-peptide constructs cloned in pEGFP-C1 (Clontech) were made by introducing BamHI-EcoRI restriction sites following the C-terminal G-residue of ubiquitin. The sequence for the various peptides was introduced by primers. The peptide sequence was HRC(QAIRKK) (with H replaced by R, K, A, Q, F, T). G replaced amino acid C in the control construct. The cDNA of HLA-B*2705 was cloned in pcDNA3.

Peptide dataset

All peptides included in the dataset of this study are naturally occurring HLA-B27 binding peptides that are published or found in public databases (see supplementary data). All peptides were found in at least one HLA-B27 subtype; those found in several HLA-B27 subtypes were included once. Partial sequences and peptides longer than 12 aas were excluded.

Peptide degradation

Internally quenched peptides were microinjected in MelJuSo cells, and peptide degradation was monitored as described previously (14, 18).

Peptide competition for TAP translocation

Various concentrations of nonradioactive peptides were used to compete for translocation of radioiodinated peptide number 417 as described previously (18).

Peptide binding studies

The TAP-deficient T2 cell line stably transfected with HLA-B*2705 was cultured in the absence or presence of 50 nM peptide (EBNA-3B17 –19, –17, and –15, sequence variants: XRCQAIRKK with X = A, D, E, F, H, K, L, R, Q, P, and T) for 18 h followed by analysis of HLA-B27 surface expression by FACS using anti-HLA-B Ab B1.23.2 as described previously (19).

Efficiency of peptide generation and HLA-B27 loading

The constructs for the various GFP-ubiquitin-peptide constructs were introduced by DNA transfection in a MelJuSo cell line stably expressing HLA-B*2705. Two days after transfection, cells were biosynthetically labeled with [35S]methionine (Met)/Cys for 17 h. After lysis (Nonidet P-40 containing lysis buffer), 10% of the volume was used to recover GFP-ubiquitin by immunoprecipitation with Ab B1.23.2 as described previously (18).

Results

We have determined the half-life of intracellular peptides by microinjecting internally quenched 9-mer peptides in living MelJuSo cells. Peptide degradation (by the total pool of cytosolic peptidases) was determined by the appearance of fluorescence when the quencher is spatially separated from the fluorophore and quenching is lost (14). Systematic amino acid variations at P1, 2, 3, and 9 did not reveal major differences in the half-life of intracellular peptides (14). Further analysis showed one exception that was not tested in a previous study (14): peptides with K at P1 and P2 were poorly degraded when compared with peptides with N-terminal K and D (Fig. 1A). The double-K peptide can be efficiently degraded in whole cell lysates (data not shown) but apparently only slowly in the cytosol of living cells. We tested whether this stabilization was a consequence of a double K or, more generally, two consecutive basic amino acids. We synthesized a set of internally quenched peptides varying only in amino acids at P1 and P2, and their half-life was determined. Only peptides with KK, KR, or RR sequences were stable (Fig. 1B). Surprisingly, the peptide with the RR sequence was normally degraded. To test whether a stabilizing KK sequence could act over long distances in a peptide, it was placed before or within the dabcyl–fluorescein box (Fig. 1C). The peptide with KK in the internally quenched box was efficiently degraded. This suggests that cytosolic amino-peptidases “eat their way in” until they reach the KK sequence, thus releasing the dabcyl quencher and inducing fluorescence.

Because many peptides are lost for Ag presentation by MHC class I molecules due to cytosolic peptidase activity (18), more stable peptides should be presented more efficiently. HLA-B27 uses a R as anchor residue at P2. We analyzed 338 different HLA-B27-associated naturally occurring peptide sequences (representing all sequences publicly available) and plotted the frequency of amino acids found at P1 and P2 (Fig. 2A). The anchor residue R is obviously very dominant at P2. However, P1 also shows a strong overrepresentation of a few amino acids: G, R, K, and to a lesser extent A and S, as observed before (6). Analysis of the HLA-B27 peptide structure does not explain the preference for these amino acids at P1, although it has been suggested that

The online version of this article contains supplemental material.
FIGURE 2. Dibasic peptides as dominant HLA-B27-presented peptides. A, The amino acid distribution at P1 and P2 was determined from 338 different HLA-B27 binding peptides representing all peptides available in the public domain. The anchor residue R at position 2 is dominant. At P1, mainly R, G, K, A, and S are present. B, Influence of N-terminal amino acids on HLA-B*2705 and TAP binding. Peptides with N-terminal amino acid variations (as indicated) of EBNA-3B_{149–157} peptide were tested for stabilization of surface HLA-B*2705 expressed in T2 cells as measured by FACS. The fluorescence index (FI) from two independent experiments with SE is indicated. The same peptides (except D and P; n.d., not done) were tested in a competition experiment for inhibition of TAP translocation of a radioiodinated peptide. The concentration to inhibit TAP translocation by half (IC_{50}) is shown.

R at P1 is preferred by hydrogen bonding with HLA-B27 amino acids (20). To test the effects of amino acids variations at P1, TAP-deficient T2 cells expressing HLA-B*2705 were incubated with the CTL epitope EBNA-3B_{149–157} peptide (HRCQAIRKK) (21) or with the same peptide varying at P1 (Fig. 2B). Stabilization and up-regulation of surface HLA-B27 was used as readout for peptide binding. Only peptides with P or D at P1 failed to stabilize HLA-B27, indicating that the majority of P1 amino acids in EBNA-3B_{149–157} can efficiently bind to HLA-B27. Peptides with R or A at P1 were binding more efficiently; the other amino acids did not differ in binding efficiency. This does not explain the preference for K, G, or S at P1 in the HLA-B27-associated peptide pool. Alternatively, there may be a difference in the efficiency of peptide transport by TAP. A classical TAP competition assay for translocation of radioiodinated peptide number 417 was performed (18). Number 417 contains an N-glycosylation site and is glycosylated upon TAP translocation in the ER. The glycosylated peptides are isolated with Con A-Sepharose and quantified. The concentration of nonradioactive competing peptides to reduce translocation of number 417 by half (IC_{50}) was determined (Fig. 2A).

TABLE 1. Amino acids contributing to HLA-B27 binding

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC_{50} (µM)</th>
<th>N-term</th>
<th>TAP competition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCOAIRKK</td>
<td>0.82 ± 0.05</td>
<td>0.1</td>
<td>n.d.</td>
<td>7</td>
</tr>
<tr>
<td>DROAIRKK</td>
<td>0.01 ± 0.14</td>
<td>0.1</td>
<td>n.d.</td>
<td>1</td>
</tr>
<tr>
<td>FRCQAIRKK</td>
<td>0.50 ± 0.28</td>
<td>0.1</td>
<td>n.d.</td>
<td>7</td>
</tr>
<tr>
<td>GRCQAIRKK</td>
<td>0.49 ± 0.02</td>
<td>0.1</td>
<td>n.d.</td>
<td>7</td>
</tr>
<tr>
<td>HRCQAIRKK</td>
<td>0.68 ± 0.03</td>
<td>0.1</td>
<td>n.d.</td>
<td>5</td>
</tr>
<tr>
<td>KRCQAIRKK</td>
<td>0.57 ± 0.03</td>
<td>0.1</td>
<td>n.d.</td>
<td>5</td>
</tr>
<tr>
<td>LRCQAIRKK</td>
<td>0.45 ± 0.11</td>
<td>0.1</td>
<td>n.d.</td>
<td>2.5</td>
</tr>
<tr>
<td>PRCQAIRKK</td>
<td>0.38 ± 0.11</td>
<td>0.1</td>
<td>n.d.</td>
<td>7</td>
</tr>
<tr>
<td>RRCQAIRKK</td>
<td>0.74 ± 0.05</td>
<td>0.1</td>
<td>n.d.</td>
<td>10</td>
</tr>
<tr>
<td>TRCQAIRKK</td>
<td>0.63 ± 0.11</td>
<td>0.1</td>
<td>n.d.</td>
<td>6</td>
</tr>
</tbody>
</table>

FIGURE 3. HLA-B*2705 peptide presentation from defined cytosolic peptide input. A, Experimental system. GFP-ubiquitin-EBNA-3B_{149–157} constructs with variations at P1 are expressed in MelJuSo cells expressing HLA-B*2705. The peptides are cleaved from GFP-ubiquitin by DUBs. Cells are biosynthetically labeled to label peptide, GFP-ubiquitin, and endogenous proteins. The same construct without C was expressed (n.c.). HLA-B27 complexes containing radioiodinated peptides were purified. The input GFP-ubiquitin was immunosolated and quantified by phosphorimaging after separation by SDS-PAGE. Input GFP-ubiquitin and output-radioiodinated peptide are then related and plotted, representing the amount of radioiodinated HLA-B27 binding peptides produced per unit GFP-ubiquitin input. B, Analysis of input GFP-ubiquitin. GFP-ubiquitin was isolated, separated by SDS-PAGE, and analyzed by phosphorimaging. GFP-ubiquitin can be free, used for monoubiquitin modifications, or incorporated in polyubiquitin moieties. The position of polyubiquitin and free GFP-ubiquitin is indicated. The * shows a major monoubiquitin-modified protein. The corresponding P1 of the input peptide is indicated. n.c. is the isolation of GFP-ubiquitin with the control nonradioative peptide. For quantification, the signal of every lane was determined and background signal (i.e., nontransfected cells) subtracted. C, Relative efficiency of HLA-B27 loading with radioactive peptides varying in P1. The amount of HLA-B27-bound radioactive peptides varying at P1 only was determined and related to the amount of input-radioactive GFP-ubiquitin. The highest ratio was set at 100%, and the other peptides were related to this. n.c. defined the background as it reflects the recovery of endogenous peptides, and the nonradioactive EBNA-3B variant. The gray area indicates background signal. Shown is the mean (and variation) of two independent experiments.
Cys was quantified by phosphorimage analysis. Note that GFP-ubiquitin-peptide did not accumulate in any of the lanes. Full-length constructs (GFP-Ub-peptide) would not have been incorporated in polyubiquitin chains because this requires a free C terminus of ubiquitin. This implies that the peptide sequences were all efficiently released by intracellular DUBs.

Radiolabeled peptides bound to HLA-B27 were quantified by a standard procedure involving HLA-B27 purification, peptide elution, and peptide separation by HPLC. Chemically synthesized nonradioactive peptides corresponding to the intracellular-generated radiolabeled peptide were included in the HPLC separation and used to position the peptide. The correct fractions were sampled and [35S]Met/Cys incorporation quantified (Fig. 3A). The relative efficiency of radioactive peptide binding to HLA-B27 was determined by dividing the amount of radioactive peptides (dpm) by the input signal (GFP-Ub) as detected by phosphorimaging analysis. The highest value was set at 100% (Fig. 3C shows the result of two independent experiments with SE). Amino-terminal RR peptides were best presented by HLA-B27 followed by KR. TR was ~30% as efficient as RR (after subtraction of the background signal) and FR ~20%. The other peptides, including peptides having a T or A at P1 were only poorly selected despite proper peptide production. Stable peptides with R or K at P1 are apparently overrepresented in the HLA-B27-associated peptide pool, as confirmed in the analysis of HLA-B27 peptidome (Fig. 2A).

**Discussion**

Peptides presented by MHC class I can be predicted using anchor residues, as defined for most HLA alleles (22). An even more accurate prediction can be obtained by including proteasome and TAP specificity (23). The specificity of cystosolic aminopeptidases is not yet taken into account, possibly because these and their relative contribution to total peptidase activity are poorly defined. Still, cystosolic peptidases destroy most peptides before they can reach MHC class I molecules (18). Peptides with a higher stability should therefore be more successfully presented by MHC class I molecules. The combined cystosolic peptidase activities only show minor sequence specificity (14). In this study, we report an exception. Peptides with R or K at P1 and R at P2 are more stable than other sequences tested. This increased stability was not due to binding to other intracellular proteins or chromatin, as tested by biophysical techniques (data not shown) (18). Consequently, peptides binding to HLA-B27, which use anchor residue R at P2, may overrepresent R or K at P1.

Analysis of the naturally presented HLA-B27 peptide pool revealed R and K, but also A, G, and S as dominant amino acids at P1. Efficient presentation of peptides with R at P1 can be explained by a combination of peptide stability and efficient binding to HLA-B27 because R may be stabilized by E163 in the HLA-B27 structure (20). This does not apply to Gogo*0101, which contains a T at that position and seems to prefer RR sequences. In this study, we have used peptide EBNA-3B[149–157] to test the effect of P1 variations on HLA-B27 stability, TAP translocation, and the efficiency of presentation by MHC class I. We observed only small differences in TAP translocation and HLA-B27 binding, and these cannot explain the amino acids preferences at P1. For example, peptides with A at P1 were among the best binding and translocated peptides, but the efficiency of presentation from defined peptide input was relatively low. This indicates that other prepeptide processes, such as proteasome cleavage specificity, might account for the overrepresentation of peptides with A at P1 in HLA-B27 peptide pool. In addition, although peptides with K or L at P1 have similar HLA-B27 binding affinities and TAP binding, the more stable peptide with K on P1 is more efficiently presented. These data suggest a linkage between peptide stability and the preference of amino acids at P1 in HLA-B27. However, this linkage is not absolute, because the specificities of the proteasome, TAP, ERAP, and HLA-B27 contribute as well. This may explain why amino acids T and F are more dominant than A at P1 in the peptide delivery experiment (Fig. 3), whereas A, G, and S are found preferentially at this position in the naturally presented peptidome. We show that the prevalence of R and K at P1 in combination with R at P2 can be explained by increased cystosolic peptide stability. Consequently, peptidase specificity should be an additional component to the HLA-binding peptide prediction programs, but one selective for particular HLA alleles. Further analysis of amino acid distributions at P1 of peptides binding to other HLA alleles may reveal other combinations with different susceptibilities for cystosolic aminopeptidases for incorporation in MHC class I epitope prediction programs.

What is the consequence for Ag presentation by HLA-B27 molecules? MHC class I Ag presentation is very inefficient (17). More than 99.9% of peptides generated are destroyed by cystosolic peptidase activities (18). This sets a threshold, and Ags expressed in only few copies per cell are probably not presented by MHC class I and ignored by the immune system. We speculate that HLA-B27 selects more stable peptides with the anchor residue R at P2, which implies that fewer antigenic protein molecules are required to load one HLA-B27 molecule with antigenic peptides simply because fewer peptides are destroyed. This may have consequences for presentation of self-Ags, but also for presentation of pathogens that maintain antigenic protein expression below the Ag presentation threshold for CTL recognition. It could be an advantage for HLA-B27 individuals to better respond to such pathogens, as observed in, for example, HIV patients (24). Either or both items could be related to the onset of spondyloarthropathies. Importantly, the three MHC class I alleles associated to these diseases (HLA-B27, HLA-B39, and Gogo*0101) are not unique in sequence, structure, or biochemistry, but are unique in their associated peptide pool with anchor residue R at P2. Both HLA-B27 and Gogo*0101 overrepresent R and K at P1 (HLA-B39 is poorly defined). HLA-B14*02 and *03 have K/R anchor residues at P2 but no obvious enrichment for K/R at P1 (10) probably due to different amino acids around the P1 site. E163 in HLA-B27 differs from T163 in HLA-B14, illustrating a further contribution of HLA amino acids to peptide selection. Still, presentation of poorly expressed Ags through stable peptide products may be a unique feature of HLA-B27 and related molecules, which could provide a rationale to rheumatic diseases.

**Disclosures**

The authors have no financial conflict of interest.

**References**


