A Polymorphic Pocket at the P10 Position Contributes to Peptide Binding Specificity in Class II MHC Proteins

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Summary
Peptides bind to class II major histocompatibility complex (MHC) proteins in an extended conformation. Pockets in the peptide binding site spaced to accommodate peptide side chains at the P1, P4, P6, and P9 positions have been previously characterized and help to explain the obtained peptide binding specificity. However, two peptides differing only at P10 have significantly different binding affinities for HLA-DR1. The structure of HLA-DR1 in complex with the tighter binding peptide shows that the peptide binds in the usual polyproline type II conformation, but with the P10 residue accommodated in a shallow pocket at the end of the binding groove. HLA-DR1 variants with polymorphic residues at these positions were produced and found to exhibit different side chain specificity at the P10 position. These results define a new specificity position in HLA-DR proteins.

Introduction
Major histocompatibility complex (MHC) proteins play an important role in the generation of immune responses by binding peptide antigens and presenting them at the cell surface for inspection by antigen receptors on T cells [1]. T cell recognition of MHC bound antigens is required for the generation of adaptive immune responses against pathogenic viruses, bacteria, and fungi, and for selection and maintenance of the large repertoire of circulating T cells. Class II MHC proteins are found in macrophages, B cells, dendritic cells, and other “antigen-presenting cells” of the immune system, where they bind peptides produced by endosomal proteolysis. The endosomal antigen-processing machinery results in a large variety of peptide antigens bound to class II MHC proteins, with an average length of 15–20 residues [2, 3]. Peptides bind to class II MHC proteins in an extended conformation, with up to 12 peptide residues in direct contact with the MHC peptide binding groove [4]. Pockets in the peptide binding groove are spaced to accommodate some of the peptide side chains, with the remainder held accessible for interaction with T cell receptors. In structures of human and mouse class II MHC proteins, pockets within the overall peptide binding site are found at the P1, P4, P6, and P9 positions, with smaller pockets at P3 and P7 (Figure 1A) [5–12]. Pockets are numbered along the peptide relative to a large hydrophobic pocket near the peptide binding site. These positions are consistent with experimentally determined peptide binding motifs, which indicate peptide sequence preferences at these positions [13, 14].

Class II MHC proteins are highly polymorphic in all species that express them. Over 400 alleles have been characterized for the β2 subunit of HLA-DRB, the most highly expressed of the three human class II MHC proteins [15]. (The α subunit is nonpolymorphic). Most of the polymorphisms can be found in positions that correspond to the residues lining the pockets within the peptide binding site. The allelic differences influence the peptide binding specificity of the various class II MHC alleles and thus determine allele-specific differences in the spectrum of peptides presented to the immune system. These differences have important consequences for susceptibility to autoimmune diseases, resistance to infection, vaccine efficacy, and rejection of transplanted organs and administered proteins [16]. In the common allelic variant HLA-DR1 (DRB1*0101), for example, the P10 pocket shows strong preferences for large hydrophobic residues (such as Tyr, Trp, Phe, Leu, and Ile), the P6 pocket prefers smaller residues (Gly, Ala, Ser, and Pro), and the P4 and P9 pockets tend to have weaker preferences for residues that have aliphatic character [14]. Algorithms designed to predict peptide binding affinities for HLA-DR1 and for other class II MHC allelic variants have been developed based on experimental peptide and phage display binding data together with the pattern of MHC-peptide side chain contacts observed in the crystal structures [17–20]. Generally, such approaches have considered only positions P1–P9 or a subset of these positions, although one report analyzed specificity at positions P1–P9 by using randomized peptide libraries [21].

A recent study of HLA-DR52a (HLA-DRB3*0101) bound to an integrin β3-derived peptide suggested a role for the P10 position in peptide binding [22], and a similar effect was observed in a systematic study of contributions of anchor and nonanchor positions to the overall peptide binding affinity of HLA-DR1 [23]. Here, we have investigated the structural basis for specificity at the P10 position of peptides bound to HLA-DR1. A newly described shallow pocket or shelf on the edge of the MHC peptide binding site interacts with peptide P10 side chains, and common MHC polymorphisms in this region alter the peptide side-chain specificity. These results suggest that the P10 position can play an important role in MHC peptide binding selectivity.
would appear to be due to an interaction between the peptide P10 residue and HLA-DR1. As noted above, a side-chain binding pocket at this position has not been described in HLA-DR1 or any other class II MHC protein, and peptide binding motifs generally do not include a side chain preference at this position. It is possible, however, that these peptides bind in an expected conformation, or that one (or both) of the peptides binds in an unexpected register or conformation; for example, the peptide might “bulge,” as observed in complexes of peptides bound to class I MHC proteins [24], shifting the peptide side chains that are accommodated in the MHC pockets. To elucidate the structural basis for the apparent specificity at the P10 position, we crystallized HLA-DR1 in complex with the AAYSDQATPLLSPR peptide and determined its structure by X-ray diffraction methods.

Crystal Structure of HLA-DR1 Reveals a P10 Shallow Pocket or Shelf
The X-ray crystal structure of HLA-DR1 bound to the AAYSDQATPLLSPR peptide was determined in two crystal forms, one with two molecules in the asymmetric unit, and another with one molecule in the asymmetric unit in complex with the bacterial superantigen SEC3-3B2 that has been used previously to facilitate crystallization of HLA-DR variants (Table 1). SEC3-3B2 makes contacts with the α/H9251 chain of HLA-DR1 outside the binding site and does not contact the peptide [25, 26]. In each of the structures, clear and continuous density was observed for essentially all of the MHC protein, excluding a disordered loop away from the peptide binding site, and also for all of the bound peptide from the Figure 1. The P10 Position of Peptides Can Affect Binding to HLA-DR1
(A) Surface of the HLA-DR1 peptide binding site with bound AAYSDQATPLLSPR peptide shown as a stick model. In the peptide, carbon atoms are yellow, nitrogen atoms are blue, and oxygen atoms are red. Peptide side chain binding pockets are labeled. (B) Competitive binding analysis of two peptides that differ only in the P10 position. A fixed concentration of peptide-free HLA-DR1 and biotinylated Ha peptide was incubated with increasing concentrations of either AAYSDQATPLLSPR or AAYSDQATPLLGSPR peptide. After binding for 3 days at 37°C, the amount of biotinylated Ha peptide/HLA-DR1 complexes was measured with alkaline phosphatase-labeled streptavidin in an antibody-capture assay.

Results and Discussion
The P10 Position Can Influence Binding of Peptides to HLA-DR1
Two peptides with sequences differing only by a single amino acid predicted to occupy the P10 pocket (AAYSDQATPLLSPR and AAYSDQATPLLGSPR) bind to HLA-DR1 with 15-fold different apparent affinity as measured by a competition assay (Figure 1B). The peptide sequences derive from the integrin β3 peptide previously implicated in P10 effects in the context of HLA-DR52a [22], but with the “anchor” P1, P4, P6, and P9 positions changed to the corresponding residues from the influenza haemagglutinin [306–318] peptide (HA), a well-characterized viral antigen that binds tightly to HLA-DR1 (Kd of ~10 nM) [12]. The difference in peptide binding affinity
Table 1. Data Collection and Refinement Statistics

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<th>HLA-DR1/AAYSDQATPLLSSPR/SEC3-3B2</th>
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*Rsym = |I − <I> |/|I − <I>|, where I is the observed intensity and <I> is the average intensity of multiple observations of symmetry-related reflections.

b Relationship on structure factors for reflection omitted from the refinement and used as a test set (10% of total).

c Relationship between the MHC or peptide components of the two NCS-related molecules in the asymmetric unit.

d Relationship between the MHC or peptide components of the R3 crystal form as compared to molecule 1 of the other crystal form. Values for molecule 2 are essentially identical by the P10 residue is hydrophobic. The fraction buried for the residue at P10 is 0.63, which is comparable to the fraction buried for residues in the P3 (0.65), P4 (0.69), and P7 (0.77) pockets. Crystal structures are currently available for six other HLA-DR molecules in complex with peptides that extend to the P10 position. In each of these, the residues that line the P10 pocket are organized essentially identically to the complex reported here (Figure S2A; see the Supplemental Data). In each case, the P10 residue is substantially buried in the pocket (with the exception of DRB5*0101 in complex with a peptide from Epstein-Barr virus, where the peptide binds in an unusually extended conformation with the P9 residue contacting the P10 pocket) (Figure S2B).

Binding Specificity to the P10 “Shelf” on HLA-DR1
In order to explore peptide binding specificity for the P10 side chain, we synthesized a set of peptides that contained the AAYSDQATPLLXSPR sequence, where X is one of the 20 natural amino acids, and measured their relative affinity for HLA-DR1 in a competition binding assay (Figure 3A). The peptide sequence is similar to that used in the structural work described above, except that (P7) was changed to the more favorable Leu (P7), eliminating possible backbone conformational and entropic effects unique to proline (I.S., Z.Z.-R., and L.J.S., unpublished data). Peptide binding competition IC₅₀ values are shown in Table S1 (see the Supplemental Data) and are plotted in Figure 3B relative to the average of all peptides in the series. Clearly there is peptide side chain specificity at the P10 position, with phenylalanine and tyrosine strongly preferred and asparagine, proline, and serine disfavored.

Allelic Polymorphism at the P10 Region of HLA-DR
Inspection of the HLA-DR1 structure in the vicinity of the P10 shelf shows that the residues Arg₇₆, Pro₅₆, Asp₅₇, and Tyr₆₀ play the major roles in formation of the shelf and interaction with the peptide (Figures 2C and 2D). Arg₇₆ is part of the nonpolymorphic α subunit, and Pro₅₆ is conserved within all the HLA-DR alleles,
Figure 2. Crystal Structure of HLA-DR1 Bound to AAYSDQATPLLSPR
(A) 2Fo-Fc electron density map contoured at 1σ using data in the resolution range of 30–2.4 Å, with all peptide atoms omitted from the map calculation. The peptide carbon atoms are yellow, and nitrogen and oxygen atoms are blue and red, respectively.
(B) The AAYSDQATPLLSPR peptide from the HLA-DR1/SEC3-3B2 complex (carbon atoms colored in yellow) was superimposed with the peptides present in the structure of HLA-DR1 without the superantigen (carbon atoms colored in green or magenta for the two molecules in the asymmetric unit).
(C) 2Fo-Fc electron density maps contoured at 1σ for the P10 region of HLA-DR1/AAYSDQATPLLSPR. (All the labeled residues were omitted from the map calculation). The carbon atoms for the peptide are yellow, and the ones for HLA-DR1 are green. The view is from the right side of (A), with the α subunit helix to the right and the β subunit helix to the left.
(D) Surface of the HLA-DR1 P10 region shown with the same view as (C). Residues lining the pocket are labeled. Residues β57 and β60 are polymorphic among HLA-DR proteins. Figures were generated with PyMol [43].

but Aspβ57 and Tyrβ60 are highly polymorphic (Table S2). In the context of other class II MHC proteins, polymorphism at Aspβ57 has been linked to human (HLA-DQ) and murine (I-Aγ) diabetes [5, 8].

We attempted to evaluate the effect of these polymorphisms on peptide side chain specificity at the P10 position by using published studies. The influence of β57 and β60 on HLA-DR peptide binding specificity has been studied systematically as part of a pocket-based matrix approach to epitope prediction [17], but the P10 pocket was not included in the analysis. For I-Aγ, a mouse class II MHC protein, it has been suggested that polymorphism at Aspβ57 can effect side chain preference at both the P9 and P10 positions [27]. Lists of peptides known to bind to particular human MHC alleles, and predicted allele-specific peptide binding motifs, have been collected in several databases, although quantitative data on the MHC-peptide binding interaction are not included [18, 19]. We scanned one such database, SYFPEITHI, for sets of similar HLA-DR1 alleles that differ in the P10 pocket residues β57 and β60 and for which reliable peptide binding motifs were available. DRB1*0401, DRB1*0404, and DRB1*0405 form such a set. These alleles differ only at positions β57 (Asp or Ser), β71 (Arg or Lys), and β86 (Gly or Val) (Figure 4). The polymorphism at β71 is not expected to significantly influence peptide side chain specificity, as both Argβ71 and Lysβ71 form a similar hydrogen bond with the peptide main chain carbonyl at position 5, between the P4 and P6 pockets [7, 12]. Residue β86 is found on the wall of the P1 pocket, and this polymorphism is expected to influence only the specificity at the P1 position [12, 17, 28]. Thus, the pattern of substitutions at these positions allows the effects of the Asp/Ser polymorphism at β57 to be evaluated: preferences observed for DRB1*0405 that are not shared by DRB1*0401 and DRB1*0404 can be attributed to the presence of Serβ57. The peptide binding motif
Figure 4. Analysis of Peptides that Bind to Different MHC Alleles

The frequencies of different groups of amino acids at the P10 position were calculated by using a list of peptides known to bind to DRB1*0401, DRB1*0404, and DRB1*0405 from the SYFPEITHI database [18]. The aromatic group includes Trp, Tyr, and Phe; the hydrophobic group includes Ile, Leu, Val, and Met; the small group includes Ala, Pro, and Gly; the polar group includes Thr, Ser, Cys, Asn, and Glu; and the charged group includes His, Glu, Arg, Lys, and Asp. Sequence differences between the three alleles are indicated; only β57 is in the P10 regions (each of the alleles has Tyr560).

For HLA-DR4 has been studied extensively [14, 17], allowing us to align a moderately sized set of known binders comprising 42 DRB1*0401 binding peptides, 14 DRB1*0404 binding peptides, and 17 DRB1*0405 binding peptides, according to their expected peptide binding frame. In this analysis, we included only peptides for which a single dominant binding register was apparent (Table S3). While the databases do not contain quantitative binding data on these peptides, it is evident that the alleles have selected sets of peptides that differ in the residue predicted in the P10 position, with the HLA-DRB1*0405 binding set enriched in charged amino acid residues at the expense of hydrophobic and aromatic residues (Figure 4). Thus, the β57 polymorphism appears to influence the P10 peptide binding specificity.

**HLA-DR1 with Polymorphic Substitutions in the P10 Region Exhibits Altered P10 Specificity**

To evaluate in a more quantitative way the influence of HLA-DR polymorphic residues on P10 specificity, we constructed a series of P10 pocket variants of HLA-DR1 (DRB1*0101) and evaluated their binding to the P10 peptide libraries as described above. Single and double amino acid replacements of polymorphic residues in the P10 pocket (β57A, β57A/Y60H, β57V, and β57V/Y60S) were introduced into HLA-DR1 so that their effects on P10 binding specificity could be isolated (naturally polymorphic variants at these positions also have substitutions in other pockets, like the HLA-DRB1*04 set described above). The resulting proteins were produced by expression in E. coli inclusion bodies and in vitro folding in the presence of peptide. The mutants were characterized to ensure that the substitutions did not result in gross alterations to the structure or to the peptide binding activity. First, each of the mutants exhibited a peptide-dependent decrease in gel filtration elution volume (Figure S3A), which previously has been described for HLA-DR1. The change results from a conformational alteration that accompanies the peptide as the binding site converts from a more open empty form to a more tightly packed closed form in the presence of peptide [29]. Second, each of the mutants bound the HA peptide (P10 Ala) with essentially identical apparent affinity (Figure S3B). Each of the mutants was also able to bind the CLIP peptide, a fragment of the class II-associated invariant chain that associates with class II MHC proteins during biosynthesis and intracellular trafficking. Apparent affinities for CLIP (P10 Gln) were similar but not identical, with D57V and D57V/Y60S binding more weakly (~3 fold). Thus, the mutations do not appear to have grossly altered the fold or activity of HLA-DR1.

To assess the effects of introduction of polymorphic residues in the P10 pocket on the peptide binding affinity and specificity at the P10 position, peptide binding competition assays were done for each mutant by using the AAYSDQATLLLXSPR series (Figure 5, Table S4). To facilitate comparison with the HLA-DR1 preferences, the bar graphs in Figure 5 are shown in the same order as in Figure 3D. Clear differences were observed between the P10 side chain specificities of the HLA-DR1 variants. For example, asparagine at the P10 position promotes binding to the β57A/Y60H and β57V/Y60S variants but significantly decreases binding in HLA-DR1 β57A and β57V variants. Some general preferences are apparent for the entire set; for example, Phe, Leu, Trp, and Met bind well, while Ile and His bind poorly to each of the variants.

**Significance**

MHC genes are the most polymorphic in the human genome. This polymorphism results in different peptide binding specificities for the corresponding allelic variant proteins. Thus, different individuals will react differently to a set of peptides from a particular antigen, with implications for vaccine design and susceptibility to autoimmune and infectious diseases. Algorithms that predict peptide binding to common human MHC protein therapeutics, and in development of subunit-based vaccines. For HLA-DR proteins, the most prevalent type of human class II MHC protein, such approaches generally account only for specificity at the P1–P9 positions of peptide antigen. Here, we have investigated an interaction outside this region, at the P10 position, which contributes significantly to the overall peptide binding affinity. In a crystal structure of HLA-DR1 bound to a
peptide antigen, we observed a shallow pocket that accommodates the P10 side chain. The residues that line this pocket are polymorphic among human HLA variants. We prepared HLA-DR1 variants carrying the polymorphic residues and analyzed their peptide binding specificity. Amino acid changes at the P10 position resulted in changes up to 50-fold in the apparent peptide binding affinity as measured by a competition assay. The P10 side chain binding specificity was significantly different for HLA-DR1 and for each of the variants. These results provide new data on peptide selection by human class II MHC proteins and should improve our ability to predict MHC allele-specific peptide binding patterns and individual differences in immunogenicity of protein therapeutics, autoimmune antigens, and vaccines.

Experimental Procedures

Peptide Synthesis

Peptides were synthesized by using solid-phase Fmoc (N-[9-fluorenyl]methoxycarbonyl) chemistry on a Symphony instrument (Protein Technology, Inc.). All peptides were amidated at the C terminus. N-biotinylated peptides were prepared by coupling aminocaproyl-(LC)-biotin (Anaspec) to the N terminus of the resin bound, side-chain-protected peptide by using a standard amino acid coupling procedure. The peptides were deprotected and cleaved from the resin by a 2 hr treatment at room temperature with a mixture of trifluoroacetic acid/H₂O/thioanisole/phenol/dithiothreitol (82.5:5:5:5:2.5). The solution Blue supercompetent cells (Stratagene). Sequencing of five randomly chosen clones revealed only the desired mutations. To make HLA-DR1-inclusion bodies, followed by refolding in vitro as described previously [31]. Refolded HLA-DR1 was purified by immunoaffinity chromatography by using the conformation-specific monoclonal antibody LB3.1, followed by gel filtration chromatography in phosphate-buffered saline (pH 6.8). The protein concentration was measured by UV absorbance at 280 nm by using ε₂₈₀ of 54,375 M⁻¹ cm⁻¹ for empty HLA-DR1. SEC-3B2 superantigen was expressed as a soluble protein in E. coli and isolated from the periplasmic fraction as described previously [32].

Construction and Analysis of HLA-DR1 Variants Carrying Single or Double Amino Acid Substitutions in the P10 Shelf

We used in vitro site-directed mutagenesis to make single or double point mutations on the β chain of HLA-DR1 that corresponded to polymorphic changes in the P10 pocket (QuikChange mutagenesis system, Stratagene). For each reaction, a pLM1 [31] double-stranded vector carrying an insert of the β chain of HLA-DR1 was hybridized to two synthetic oligonucleotides primers, both containing the desired mutations. The oligonucleotides, each complementary to opposite polymorphic residues and analyzed their peptide binding patterns and individual differences in immunogenicity of protein therapeutics, autoimmune antigens, and vaccines.

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sense) were used. The HLA-DR1 mutants were expressed in E. coli inclusion bodies and refolded and isolated as described above for wild-type HLA-DR1.

Peptide Binding Assays

A standard peptide binding competition assay [26, 33–35] was used to determine binding affinities of peptides to the HLA-DR1 molecule and the mutants. Peptide-free wild-type HLA-DR1 produced in insect cells or a mutated form of HLA-DR1 produced in E. coli (25 nM) was mixed together with biotinylated Hα[306–318] peptide probe (Hαaq, 25 nM) and varying concentrations of unlabelled competitor peptide (10⁻¹⁰–10⁻⁶ M). The mixtures were incubated for 3 days at 37°C in 100 mM sodium phosphate buffer at pH 5.5, containing 50 mM NaCl, 1 mg/ml PMSF, 37 μg/ml iodoacetamide, 10 mM EDTA, 0.02% NaN₃, and 0.5 mg/ml octylglucoside, followed by detection of bound, biotinylated peptide using an immunoprecipitate that employed anti-DR1 capture antibody LB3.1 and alkaline phosphatase-labeled streptavidin. IC₅₀ values were obtained by fitting a binding curve to the plots of fluorescence versus concentration of competitor peptide. The IC₅₀ value for the control Ha peptide included in each assay was 29 ± 14 nM (n = 6).

A direct binding assay was used to determine binding abilities of HLA-DR1 mutants. The assay was performed for 3 days at 37°C in the same buffer as described above; constant concentration of the proteins (25 nM) were used, and the range of concentrations of biotinylated peptides depended on the binding affinity of the particular peptide (10⁻¹–10⁻⁶ M for tight binders and 10⁻⁶–10⁻³ M for weak binders). The incubation was followed by immunoprecipitation as described above.

Crystallization

HLA-DR1 peptide bound complexes were prepared by incubating purified empty HLA-DR1 (1–5 μM) with at least 5-fold molar excess peptide for 3 days at 37°C in phosphate-buffered saline with 0.02% sodium azide. The complex was purified by gel filtration to remove aggregates and free peptide. Crystals of HLA-DR1/AAYSDQATPLLSPR were grown at room temperature by the vapor diffusion method in 10% polyethylene glycol 6000, 100 mM glycine (pH 4.0), with 1 μl precipitant solution mixed with 1 μl 10 mg/ml protein. For X-ray diffraction experiments, the crystal was soaked in mother liquor with 25% glycerol for 2 min and flashed cooled in liquid nitrogen. HLA-DR1 was isolated from insect cells.

For the HLA-DR1/AAYSDQATPLLSPR/SEC3-2B2 crystal structure, purified HLA-DR1/AAYSDQATPLLSPR/SEC3-2B2 complex was grown in equimolar ratio with purified SEC3-2B2. Crystals of HLA-DR1/AAYSDQATPLLSPR/SEC3-2B2 were grown by the vapor diffusion method in hanging drops at 4°C under the following conditions: 2%–6% ethylene glycol 4000, 5%–10% ethylene glycol 100 mM sodium acetate (pH 5.2–5.6), with 1 μl precipitant solution mixed with 1 μl 10 mg/ml protein complex. For X-ray diffraction experiments, the crystals were soaked for ~1 min in a cryoprotectant solution consisting of 25% ethylene glycol in the mother liquor and then flashed cooled in liquid nitrogen. The HLA-DR1 was purified from E. coli.

Data Collection and Processing

Diffraction data for HLA-DR1/AAYSDQATPLLSPR and SEC3-2B2 was collected at the National Synchrotron Light Source (NSLS) with X25 beamline (λ = 1.10 Å) to a resolution of 2.4 Å.

A high-resolution data set (2.40 Å) of the HLA-DR1/AAYSDQATPLLSPR/SEC3-2B2 complex was collected on a single crystal (300 μm × 200 μm × 200 μm) on an R-AXIS IV image plate detector with CuKα radiation. Collected data for both complexes were processed and scaled with Denzo, Scalepack, and the CCP4 package [36, 37].

Structure Determination

The structure of each complex was determined by molecular replacement. For the HLA-DR1/AAYSDQATPLLSPR structure, coordinates from another complex of HLA-DR1 (Protein Data Bank code 1QAG) were used as the search model. After an initial rigid body refinement, five rounds of refinement included minimization, B factor, and minimization. A 2-fold noncrystallographic symmetry (NCS) averaging improved the quality of the electron density map.

In the case of HLA-DR1/AAYSDQATPLLSPR/SEC3-2B2, coordinates for another complex of E. coli-derived HLA-DR1 and SEC3-2B, carrying an N-methylated-designed peptide (Protein Data Bank code 1PYY) [26] were used as the search model to find a molecular replacement solution. Waters and the peptide were removed from the search model before use. After an initial rigid body refinement, eight rounds of refinement included minimization, B factor, and minimization.

For both structures, refinement was carried out with CNS [38], and manual inspection and rebuilding were carried out with XtalView [39]. Waters were added to the refined molecule by using CNS. The final models were verified for distortions on the secondary structure features with Procheck [40]. LSQKAB [41] was used to determine RMSD values for aligned coordinate sets.

MHC-Peptide Database Analysis

The SYFPEITHI database of known MHC binding peptides [18] and the IMGT HLA sequence database [15] were examined to find sets of similar HLA-DR1 alleles that differed in the P10 pocket residues and for which reliable motifs and many known peptide binders were available. HLA-DRB1*0401, HLA-DRB1*0404, and HLA-DRB1*0405 HLA-DR allele-specific binding peptides from the SYFPEITHI databases generally were used without further alignment, although multiple-length variants of the same antigen and some ambiguous alignments were deleted. Residues predicted to bind into the P10 pocket were identified. A Fisher-Free

man-Halton Exact Test [42] revealed that the association between MHC alleles and residues at the P10 position approached significance (p = 0.056). To gain some insight into the nature of the allele specificity, residues were grouped by generic side chain properties: aromatic (Trp, Tyr, Phe), hydrophobic (Ile, Leu, Val, Met), small (Ala, Pro, Gly), polar (Thr, Ser, Cys, Asn, Gin), and a charged group (His, Glu, Arg, Lys, Asp), and the frequency of each group was calculated for each allele.

Supplemental Data

Supplemental Data including additional information regarding the crystal structures described and data/values used in the analysis of the P10 region of HLA-DR and the generation of figures are available at http://www.chembiol.org/cgi/content/full/11/10/1395/ DC1.

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References

4. McFarland, B.J., and Beeson, C. (2002). Binding interactions...


