HLA-DP4, the Most Frequent HLA II Molecule, Defines a New Supertype of Peptide-Binding Specificity

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Among HLA-DP specificities, HLA-DP4 specificity involves at least two molecules, HLA-DPA1*0103/DPB1*0401 (DP401) and HLA-DPA1*0103/DPB1*0402 (DP402), which differ from each other by only three residues. Together, they are present worldwide at an allelic frequency of 20 – 60% and are the most abundant human HLA II alleles. Strikingly, the peptide-binding specificities of these molecules have never been investigated. Hence, in this study, we report the peptide-binding motifs of both molecules. We first set up a binding assay specific for the immunopurified HLA-DP4 molecules. Using multiple sets of synthetic peptides, we successfully defined the amino acid preferences of the anchor residues. With these assays, we were also able to identify new peptide ligands from allergens and viral and tumor Ags. DP401 and DP402 exhibit very similar patterns of recognition in agreement with molecular modeling of the complexes. Pockets P1 and P6 accommodate the main anchor residues and interestingly contain only two polymorphic residues, β86 and β11, respectively. Both positions are almost dimorphic and thus produce a limited number of pocket combinations. Taken together, our results support the existence of three main binding supertypes among HLA-DP molecules and should significantly contribute to the identification of universal epitopes to be used in peptide-based vaccines for cancer, as well as for allergic or infectious diseases. The Journal of Immunology, 2002, 169: 6928 – 6934.

In humans, the three molecules HLA-DR, HLA-DQ, and HLA-DP present antigenic peptides to CD4+ T lymphocytes. They are each composed of two separate α- and β-chains, which assemble into a very similar structure (1, 2). Assembly of the N-terminal domain of both chains forms the peptide-binding site, which is composed of two α helices standing above an eight-stranded β sheet floor. Conserved residues present in the binding site compose a hydrogen bond network with the peptide backbone. They impose on the peptide an extended conformation and sustain the broad peptide-binding specificity of the HLA II molecules. Nevertheless, these molecules possess their own peptide-binding selectivity (3, 4). This is due to the polymorphic residues, which mainly lie within the binding site. They have been distributed into five specificity pockets, which accommodate the peptide amino acids numbered P1, P4, P6, P7, and P9 (1). Analyses of a wide spectrum of naturally bound peptides and of synthetic analogs have identified peptide-binding motifs and amino acid preferences for multiple HLA II molecules (3, 4). These motifs generally correlate with the content of the specificity pockets, allowing a detailed documentation of HLA II molecules. Mainly HLA-DR and HLA-DQ have been investigated and appear to be drastically different from each other. HLA-DR molecules are well known to accommodate aromatic and hydrophobic residues, especially in the P1 pocket (4 – 7), while HLA-DQ molecules generally accept relatively short side chains (8). The differences appear less important between molecules encoded by the same locus (9). Comparison of the repertoire of binding peptides shows that HLA-DR molecules share common binding properties and hence can be assembled into HLA II supertypes (6, 7). This degenerated specificity allows the discovery of promiscuous peptides, which bind to multiple HLA II molecules, and greatly enhances the potential for the use of epitope-based vaccines (10 – 15). Supertypes were initially described for the class I molecules (16 – 18) and only attributed to HLA-DP molecules for the class II molecules (6, 7), probably because they are the most investigated molecules.

In fact, the HLA-DP molecules have scarcely been studied. Initially, they appeared less important in the immune response than HLA-DR and HLA-DQ molecules, because HLA-DP incompatibility did not seem to contribute to the risk of graft-vs-host disease (GVHD)3 (19, 20). However, a single mismatch is now known to suffice in triggering a specific T cell response after bone marrow transfer, confirming the whole functionality of these molecules (21). Both the α- and β-chains of HLA-DP molecules are polymorphic molecules allowing multiple combinations of the 17 HLA-DPA1 and 86 DPB1 allelic forms (22). However, only a limited number of HLA-DP molecules are abundant in the worldwide population, the molecule DPA1*0103/DPB1*0401 (DP401) being overrepresented (22). This molecule differs by only three aa from the DPA1*0103/DPB1*0402 (DP402) molecule, which is also frequently encountered (Table I). Together, both molecules have a gene frequency of 50% in Europe, 60% in South America, 80% in North America, 60% in India, 25% in Africa, and only 15% in

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3 Abbreviations used in this paper: GVHD, graft-vs-host disease; DP401, DPA1*0103/DPB1*0401; DP402, DPA1*0103/DPB1*0402; bOxy, biotinylated Oxy; TT, tetanus toxoid.
Japan (22). In the Caucasian population, they are carried by ~76% of individuals and hence are as frequent as the well-known HLA-A2 molecule. In comparison, approximately six molecules of HLA-DR are required to cover the same percentage of people. Therefore, we could expect that peptides that efficiently bind to DP401 and DP402 will have a large impact as epitope-based vaccines. However, although multiple clones or T cell lines of various peptide specificities are restricted to HLA-DP4 (23–30), the binding specificity of HLA-DP4 molecules remains unknown. To our knowledge, only the peptide specificities of HLA-DP2 and HLA-DP9 molecules have been investigated (31, 32), and only preliminary data of naturally processed peptides eluted from a DP4 molecule have been reported (33). Therefore, we used specific binding assays to describe the peptide-binding motif of the HLA-DP4 molecules and to demonstrate that they define a new peptide specificity supertype.

Materials and Methods

Peptides

Peptides were synthesized as previously described (7) and were purified by reversed-phase HPLC on a C18 Vydac column (Interchim, Montluçon, France). Mutated peptides were used without purification, because they did not exhibit >10% contaminants in analytical HPLC. The NY-ESO-1-derived peptides (119–143 and 158–166) and MART-1/Melan-A peptides (1–20, 41–60, 51–73, 62–72, and 103–118) were synthesized using standard F-moc chemistry by the University of Pittsburgh Peptide Synthesis Laboratory (shared resource) as described previously (34). The biotinylated Oxy (bOxy) peptide 271–287 was biotinylated with biotinyl-6-amino-caproic acid (Fluka Chimie, St. Quentin Fallavier, France) on the N terminus before cleavage from the resin and HPLC purification. Quality of the peptides was assessed by electrospray mass spectrometry. The peptides encompassing the sequence of the major b venom are the following: 1–18, 5–22, 9–26, 13–30, 17–34, 21–38, 25–42, 29–46, 33–50, 37–54, 41–58, 45–62, 49–66, 53–70, 57–74, 61–78, 65–82, 69–86, 73–90, 77–94, 81–98, 85–102, 89–106, 93–107, 98–114, 105–118, 107–116, 112–128, 117–118, 119–128, 122–147, 137–168, 155–165, 175–190, and 182–198.

Cell cultures and purification

EBV homozygous cell lines PITOUT (DP4A1*0103, DPB1*0401), HHKB (DP4A1*0103, DPB1*0401), HOM2 (DP4A1*0103, DPB1*0401), and SCHU (DP4A1*0103, DPB1*0402) were used as sources of human HLA-DP4 molecules. They were cultured up to 5 × 10^6 cells in RPMI 1640 medium supplemented by 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 500 µg/ml gentamicin, and 1% nonessential amino acids (Sigma-Aldrich, St. Quentin Fallavier, France). B7/21 hybridoma was a kind gift from Dr. Y. van de Wal (Department of Immunohematology and Blood Bank, Leiden University Medical Center, Leiden, The Netherlands). It was cultured in DMEM supplemented by 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 500 µg/ml gentamicin, 1% nonessential amino acids, 10 mM HEPES, and 50 µM 2-ME (Sigma-Aldrich). Abs were purified by immunoaffinity on protein A-Sepharose as recommended by the manufacturer (Pharmacia, Orsay, France). HLA-DP4 molecules were purified by affinity chromatography using B7/21 mAb (35) coupled to protein A-Sepharose CL 4B gel (Pharmacia) as described previously for L243 mAb (7).

HLA-DP4-specific binding assays

Binding assays were based on previously published protocols (8, 32, 36). Briefly, they were performed in 10 mM phosphate, 150 mM NaCl, 1 mM n-dodecyl β-d-maltoside, 10 mM citrate, and 0.003% thimerosal (pH 5) buffer with 10 mM of bOxy271–287, an appropriate dilution of HLA-DP4 molecules (~0.1 µg/ml), and serial mid-dilutions of competitor peptides. After 24-h incubation at 37°C, samples were neutralized and applied to B7/21-coated plates for 2 h. Bound biotinylated peptide was detected by means of streptavidin-alkaline phosphatase conjugate (Amersham, Little Chalfont, U.K.), and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm in a Victor II spectrophotometer (PerkinElmer Instruments, Les Ulis, France). Data were expressed as the peptide concentration that prevented binding of 50% of the labeled peptide (IC50). IC50 values of the bOxy271–287 peptides served as reference in each experiment.

Computer modeling of the HLA-DP4/Oxy complex

HLA-DP4/Oxy complex was built by amino replacement from the DR1-HA complex (Brookhaven Protein Data Bank accession code 1dlh), most (~100) of the residues to be replaced being surface residues. The water molecules buried in the DR1-HA complex were first removed because of obvious clashes when left during residue replacements. Great care was taken to minimize perturbation of residues and backbone in the surroundings of the replaced residues. This was done by gently and progressively propagating the perturbation using the following procedure: 1) the replaced residue was accommodated by alternating energy minimization and low temperature (10–50 K) dynamics with the rest of the protein held fixed; 2) the neighboring residues in contact with the replaced residue were subjected to the same treatment; and 3) both the replaced residue and its neighbors were minimized again with the rest of the protein held fixed. When all the required replacements were completed, the whole DP4 protein obtained was thoroughly minimized (1000 steps) to anneal the small remaining strains. Except for the region where two residues were deleted (deletion of residues 23–24 in the β-chain), the DP4 structure was found to be very similar to the original DR1 structure. The Sybyl program (Tripos Associates, St. Louis, MO) was used for this work.

Results

HLA-DP4 molecules share a very similar peptide-binding motif

After a round of screening of biotinylated peptides, we selected the bOxy271–287 peptide to establish binding conditions for both DP401

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Table 1. Polymorphism of α- and β-chains of HLA-DP molecules

| Alleles | Frequency (%) | 8  | 9  | 11 | 37 | 38 | 57 | 58 | 59 | 67 | 71 | 78 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 |
|---------|---------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| β-Chain |               |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 401     | 40.1          | L  | F  | G  | F  | A  | A  | A  | E  | I  | K  | M  | G  | P  | M  |     |     |     |     |     |     |     |     |     |     |     |
| 402     | 11.0          | V  | D  | E  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 201     | 11.9          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 501     | 1.3           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 101     | 7.1           | Y  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 301     | 9.1           | Y  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 901     | 1.1           | H  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| α-Chain |               |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 103     | 78.2          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
| 201     | 21.2          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |

*Allelic frequencies are from the French population (22). Sequences were retrieved from http://www.ebi.ac.uk/imgt/hla. Positions are numbered based on HLA-DRB1 sequences as suggested by Stern et al. (1). As residues 23 and 24 are missing in the DPB1 sequences, listed residues from position 37 to the end correspond to positions 35, 36, 55, 56, 57, 65, 76, 84, 85, 86, and 87 in the DPB sequence, respectively. Numbers in parentheses correspond to the specificity pocket.
and DP402 molecules. This peptide has been sequenced from naturally processed peptides eluted from DPA1*0201/DPB1*0401 molecules (33) and presents 100% identity with a fragment of the oxytocinase protein (GenPept accession code U62769). As shown in Fig. 1, a concentration range of the nonbiotinylated Oxy271–287 peptide totally inhibits the binding of its biotinylated form to both molecules, thus demonstrating the peptide interaction specificity. The mid-inhibition occurred at ~10 nM concentration. In this assay, the binding specificity for the HLA-DP4 molecules was ensured by the known specificity of the B7/21 Ab (35). It was used to immunopurify the HLA-DP4 molecules and to trap them onto ELISA plates. Nevertheless, we also assessed that other peptides known to bind HLA-DP4 molecules were equally good binders in our assays. The HLA-DP4 eluted peptide IL3R127–140 (33) and the T cell epitopes MAGE3245–264 (28), HSV283–303 (29), NSP2 (24), and tetanus toxoid (TT) peptide 947–963 (26) inhibited the binding at different levels of efficiency. In sharp contrast, the HA306–318 peptide, which is known to bind efficiently to multiple HLA-DR molecules (5, 37), and the peptides HCl45–63 and DQB43–57, which bind efficiently to HLA-DQ molecules (38), were totally inactive.

Using these assays, a set of overlapping and truncated peptides was used to delineate more precisely the interacting region of the Oxy271–287 peptide (Table II). Three 13-mer peptides (271–283, 272–284, 273–285) were as active as the native peptide on both molecules, suggesting that the core region extended from K273 to L283. Moreover, removal of F275 was associated with a large decrease in the binding, suggesting that this position played a major role in the interaction. Accordingly, alanine (Oxy F275A) and lysine (Oxy F275K) substitution of this position altered the peptide’s ability to bind to DP401 (Table III). The same dramatic effect was seen toward DP402 with F5K but not with F5A. Interestingly, substitutions by either alanine or lysine of a second phenylalanine (F280) strongly affected the binding for both molecules. This residue appeared as the main anchoring residue. Finally, substitution of L283 by a lysine diminished the binding to both DP401 and DP402, while substitution of T278 by a lysine only slightly altered the binding to DP401. Three residues, F275, F280 (1 + 5), and L283 (1 + 8), therefore appeared as anchor residues and this pattern suggested strongly that they were accommodated by both HLA-DP4 molecules in the P1, P6, and P9 pockets, respectively (1). This was in agreement with the molecular modeling of the peptide Oxy271–285 bound to DP401 and DP402 (Fig. 2 and see below). We thus synthesized a novel set of peptides substituted at these positions to investigate the amino acids preferences of each pocket (Table IV). In the P1 and P6 pockets, conservative substitutions were well tolerated in sharp contrast to substitutions by E, N, and T. In the P4 pocket, only small effects were observed, while significant but moderate binding loss was found in the P9 pocket. Slight differences were observed between DP401 and DP402 as exemplified by substitutions with basic residues in the P4 pockets, N in the P1 and Y in the P9. However, as summarized in Table V, DP401 and DP402 exhibit a similar binding motif mainly characterized by the existence of two main anchor residues in the P1 and P6 pockets.

**FIGURE 1.** Inhibition of peptide binding to DP401 and DP402. bOxy271–287 (10 nM) was incubated with a dilution of DP401 (upper panel) or DP402 (lower panel) and various dilutions of peptides to be tested. Complexes were revealed on ELISA plates previously coated with B7/21 Ab. Staining was performed using streptavidin-phosphatase and a fluorescent substrate. Data presented are representative of two independent experiments.

**Table II.** Binding to DP401 and DP402 of overlapping peptides from the Oxy271–285 peptide

| Peptides         | Sequences                | DP401 IC50 (nM) | DP402 IC50 (nM) |
|------------------|--------------------------|-----------------|-----------------|
| Oxy271–287       | EKKYFAATQFELAARL         | 9 (±6)          | 13 (±6)         |
| Oxy271–284       | EKKYFAATQFELAARL         | 50 (±35)        | 100 (±78)       |
| Oxy271–283       | EKKYFAATQFELAARL         | 90 (±46)        | 100 (±71)       |
| Oxy271–282       | EKKYFAATQFELAARL         | 10 (±12)        | 20 (±25)        |
| Oxy271–281       | EKKYFAATQFELAARL         | 10 (±12)        | 20 (±25)        |
| Oxy271–280       | KYKFAATQFELAARL          | 20 (±5)         | 20 (±4)         |
| Oxy271–279       | KYKFAATQFELAARL          | 50 (±35)        | 20 (±7)         |
| Oxy271–278       | KYKFAATQFELAARL          | 60 (±42)        | 30 (±32)        |
| Oxy270–277       | AAQFAATQFELAARL          | 15,000 (±0)     | 6,000 (±0)      |
| Oxy271–275       | KYKFAATQFELAARL          | 15,000 (±0)     | 13,000 (±0)     |
| Oxy271–274       | KYKFAATQFELAARL          | 20 (±11)        | 20 (±15)        |

* Results are IC50 expressed in nanomoles. Geometric means and SD were calculated in two independent experiments.
are only two major replacements between HLA-DR1 and HLA-DP4, which are in the exterior lateral side of the pocket: DR1-αAla\(^{22}\) to DP4-αF52 and DR1-βPro\(^{89}\) to DP4-βMet\(^{89}\). These mutations do not modify the size or hydrophobic character of this pocket, but the Oxy-Ph\(^{275}\) seems to be more deeply buried in HLA-DP4 than the longer HA-Tyr\(^{308}\) in HLA-DR1. In sharp contrast, the P6 pocket looks very different in the two models HLA-DP41 and DP42/Oxy as compared with the complex HLA-DR1/

HA. This pocket is quite shallow in HLA-DR1 because of the large side-chain floor residue βL11, while it is deep in the models of DP41 and DP42 because of the small floor residue βG11. The sole difference between DP41 and DP42 resides in the P9 and stays principally in the mutations DP41-A38 to DP42-V38 and DP41-A57 to DP42-D57, the position 58 being outside the binding groove. The mutation DP41-A57 to DP42-D57 seems to create a similar hydrogen bond network to that found in the P9 pocket of HLA-DR1. In these models, we also observed the presence of buried water molecules and a lower number of hydrogen bonds with the peptide as compared with the HLA-DR1 molecule (data not shown). As a result, the HLA-DP4/Oxy model strongly suggests the existence of two large aromatic pockets (P1 and P6),

![FIGURE 2. Specificity pockets of HLA-DR1/HA (A) and HLA-DP4/Oxy (B) complexes. The side views of the HLA molecules show the size disparity of the pockets. Surface HLA molecules are in green and brown to represent hydrophilic or hydrophobic areas, respectively. Peptides are in space filling representation (C in white, O in red, and N in blue).](Image 2)

### Table III. Alanine and lysine scanning of the Oxy\(^{273-285}\) peptide on DP41 and DP42 molecules

| Peptides   | Sequences             | DP401 | DP402 |
|------------|-----------------------|-------|-------|
| Oxy K273A  | KYFAATQFEPDELA        | 1 (±0) | 1 (±0) |
| Oxy Y274A  | KYFAATQFPEEPDELA      | 2 (±0.2) | 2 (±0.5) |
| Oxy F275A  | KYAAATQFPEEPDELA      | 40 (±25) | 7 (±2.1) |
| Oxy T278A  | KYAAATQFPEEPDELA      | 1 (±0.2) | 1 (±0.1) |
| Oxy Q279A  | KYFAATQFPEEPDELA      | 1 (±0.7) | 1 (±0.3) |
| Oxy F280A  | KYAAATQFPEEPDELA      | 3.7 (±0) | 3.7 (±0) |
| Oxy E281A  | KYAAATQFPEEPDELA      | 0.5 (±0.3) | 0.5 (±0) |
| Oxy P282A  | KYAAATQFPEEPDELA      | 2 (±0.2) | 2 (±0.1) |
| Oxy L283A  | KYAAATQFPEEPDELA      | 4 (±1.3) | 4 (±1.3) |
| Oxy Y274K  | KYAAATQFPEEPDELA      | 2 (±0.2) | 2 (±0.1) |
| Oxy F275K  | KYAAATQFPEEPDELA      | 400 (±70) | 190 (±60) |
| Oxy A276K  | KYAAATQFPEEPDELA      | 2 (±0.7) | 2 (±0.2) |
| Oxy A277K  | KYAAATQFPEEPDELA      | 3 (±0.9) | 2 (±0.5) |
| Oxy T278K  | KYAAATQFPEEPDELA      | 30 (±17) | 5 (±0.4) |
| Oxy Q279K  | KYAAATQFPEEPDELA      | 2 (±0.1) | 1 (±0.1) |
| Oxy F280K  | KYAAATQFPEEPDELA      | 270 (±180) | 190 (±135) |
| Oxy E281K  | KYAAATQFPEEPDELA      | 2 (±0.5) | 1 (±0.2) |
| Oxy P283K  | KYAAATQFPEEPDELA      | 3 (±1.2) | 2 (±1.5) |
| Oxy L283K  | KYAAATQFPEEPDELA      | 70 (±45) | 80 (±37) |
| Oxy A284K  | KYAAATQFPEEPDELA      | 2 (±0.9) | 2 (±0.5) |
| Oxy A285K  | KYAAATQFPEEPDELA      | 2 (±0.9) | 2 (±0.7) |

*Capacity of the substituted peptides to bind HLA-DP401 and HLA-DP402 was investigated. Results are presented as relative activity (ratio between native and substituted peptide IC\(_{50}\)). Geometric means and SD were calculated in two to three independent experiments. Relative activities superior to a factor of 10 are in bold.

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### Table IV. Effect of substitutions at the P1, P4, P6, and P9 positions on the capacity of the Oxy\(^{273-285}\) peptide to bind to DP401 and DP402

| Peptides   | Sequence             | DP401 | DP402 |
|------------|----------------------|-------|-------|
| Oxy F275Y  | KYAAATQFPEEPDELA     | 3 (±1.3) | 3 (±2) |
| Oxy F275L  | KYAAATQFPEEPDELA     | 3 (±1.6) | 3 (±3.7) |
| Oxy F275E  | KYAAATQFPEEPDELA     | 70 (±20) | 70 (±65) |
| Oxy F275N  | KYAAATQFPEEPDELA     | 210 (±37) | 60 (±14) |
| Oxy F275T  | KYAAATQFPEEPDELA     | 140 (±40) | 70 (±65) |
| Oxy T278F  | KYAAATQFPEEPDELA     | 2 (±0.8) | 1 (±0.7) |
| Oxy T278L  | KYAAATQFPEEPDELA     | 2 (±0.8) | 2 (±1.4) |
| Oxy T278E  | KYAAATQFPEEPDELA     | 2 (±0.7) | 3 (±2.5) |
| Oxy T278D  | KYAAATQFPEEPDELA     | 2 (±0.1) | 2 (±0.1) |
| Oxy T278N  | KYAAATQFPEEPDELA     | 4 (±1.3) | 4 (±1.1) |
| Oxy T278Y  | KYAAATQFPEEPDELA     | 4 (±1.3) | 1 (±0.9) |
| Oxy T278R  | KYAAATQFPEEPDELA     | 14 (±2.5) | 5 (±0.9) |
| Oxy T278S  | KYAAATQFPEEPDELA     | 2 (±0.5) | 3 (±0.4) |
| Oxy F280Y  | KYAAATQFPEEPDELA     | 2 (±0.7) | 3 (±0.4) |
| Oxy F280L  | KYAAATQFPEEPDELA     | 6 (±1.0) | 5 (±2.0) |
| Oxy F280E  | KYAAATQFPEEPDELA     | 800 (±230) | 800 (±650) |
| Oxy F280N  | KYAAATQFPEEPDELA     | 1400 (±79) | 1200 (±530) |
| Oxy F280T  | KYAAATQFPEEPDELA     | 700 (±0.0) | 300 (±165) |
| Oxy F280W  | KYAAATQFPEEPDELA     | 2 (±0.6) | 2 (±0.5) |
| Oxy L283F  | KYAAATQFPEEPDELA     | 1 (±0.1) | 5 (±1.8) |
| Oxy L283Y  | KYAAATQFPEEPDELA     | 2 (±0.5) | 15 (±0.7) |
| Oxy L283E  | KYAAATQFPEEPDELA     | 11 (±3.0) | 8 (±6) |
| Oxy L283D  | KYAAATQFPEEPDELA     | 7 (±0.0) | 8 (±1.6) |
| Oxy L283N  | KYAAATQFPEEPDELA     | 14 (±3.0) | 7 (±8) |
| Oxy L283R  | KYAAATQFPEEPDELA     | 50 (±1.2) | 40 (±5) |
| Oxy L283M  | KYAAATQFPEEPDELA     | 1 (±0.3) | 2 (±0) |
| Oxy L283V  | KYAAATQFPEEPDELA     | 2 (±0.4) | 2 (±0.5) |

*Capacity of the substituted peptides to bind HLA-DP401 and HLA-DP402 was investigated. Results are presented as relative activity (ratio between native and substituted peptide IC\(_{50}\)). Geometric means and SD were calculated in two to three independent experiments. Relative activities superior to a factor of 10 are in bold.

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### Table V. Amino acid preferences of DP401 and DP402 molecules

| Residues | P1 | P4 | P6 | P9 |
|----------|----|----|----|----|
| Favorable| FY| FY| FYW| FY |
|          | L | L | L | LV |
|          | D | A | N | ED |
| Unfavorable| TA| KR| TA| N |
|           | N | E | N | N |
|           | E | E | KR | E |
|           | K | K | K | K |

*The data presented in Tables III and IV are summarized in this table. Favorable” refers to residues that do not provoke a loss of binding greater than a factor of 10 as compared to native amino acid. Differences between DP401 and DP402 are underscored.*
which may account for our binding observations that aromatic/hydrophobic amino acids at these positions serve as anchor residues.

**DP4-specific motif is present in most of the peptides that bind to DP401 and DP402**

We then assessed the relevance of the HLA-DP4 motifs in peptides different from the Oxy peptides in relation with their capacity to bind to HLA-DP4 molecules. We first aligned the sequences of the good DP4 binders that we described in Fig. 1: IL3Rα127–146 (33), MAGE3245–268 (28), HSV283–302 (29), NSP2 (24), and TT497–506 (26) (Table VI). Only the NSP2 peptide did not exhibit the HLA-DP4 motif although it bound efficiently to HLA-DP4 molecules.

The motif was also found in the active peptide NY-ESO-1158–1170, which we also retained for this study, because it stimulated a HLA-DP4-restricted T cell clone (30). We then looked for the presence of the binding motif in unselected sets of peptides. In the 30 peptides that encompassed the whole sequence of the major bee venom allergen, 4 peptides (API m1 73–90, 77–94, 81–98, and 117–134) had the motif. Two of them (77–94 and 81–98) were found active but with different levels of activity (Table VI). In the 13 peptides that entirely covered the HIV Nef protein, only the peptide Nef66–94 possessed the motif and bound to the HLA-DP4 molecules. However, a second peptide (Nef132–147) also bound to HLA-DP4 molecules but harbored a partial binding motif only. None of the 4 peptides from MART-1/Melan-A exhibited the motif and only 1 peptide (MART-151–73) displayed a weak activity. Finally, the motif was also found in the peptide NY-ESO-1119–1143 (34). It exhibited a good binding activity. As a result, from the 13 binders we identify, 10 contain the motif in their sequence. Reciprocally, 2 peptides contain the binding motif but did not bind to HLA-DP4 molecules. Forty peptides have no binding motif and no activity. We also found few discrepancies between peptides DP401 and DP402, strongly suggesting that both molecules share very similar anchor residues in the P1, P6, and P9 positions.

**Discussion**

In this study, we report the peptide-binding motifs of HLA-DP4 molecules, which are the prevalent HLA II molecules in the worldwide population. These motifs have been deduced from binding activities of analogs and slightly differ from that previously proposed from sequence alignment of a few peptides eluted from the molecule DPA1*0201/DPB1*0401 (33). They mainly seem to depend on two large and hydrophobic pockets: P1 and P6. In the HLA-DP4 models, the P1 pocket accommodates aromatic and aliphatic residues in exactly the same manner as HLA-DR molecules (1, 3, 4). The P6 pocket in the models of HLA-DP4 molecules appears larger because of the small side-chain glycine residue at position β11 as numbered by alignment with DRB1. It also accepts large aromatic and aliphatic residues. As compared with HLA-DR molecules, this pocket might be more hydrophobic as a result of the replacement of αD66 and αE11 in DRB1*0101 by αL66 and αA11 residues. This is probably why a glycine at position β11 does not favor aromatic or hydrophobic residues in HLA-DR molecules as illustrated by the P6 specificity of DRB1*0701 (4).

Therefore, structural characteristics of the P1 and P6 pockets appear to account for amino acid preferences observed in the binding assays specific for HLA-DP4. Moreover, HLA-DP401 and -DP402 molecules differ by only three substitutions, which reside in the P9 pocket and which do not provoke major changes in the amino acid preferences of this pocket. Therefore, it is not surprising that they share very similar binding motifs. Based on the computer model of HLA-DP4 molecules, the P1 and P6 pockets contain only two polymorphic positions, β86 and β11, respectively. Both positions are almost dimorphic: β86 is either a glycine or an aspartate while β11 is either a glycine or a leucine. Therefore, amino acid preferences in the P1 and P6 pockets are expected to be controlled by a limited number of key amino acid combinations. Thus, we propose a supertype subdivision of the HLA-DP molecules based on the β11 and β86 positions.

DPB1*0402 differs from DPB1*0201 by only one aa at position β71 as numbered by alignment with DRB1 in the peptide-binding groove. As this position resides in the P4 pocket, it does not modify the amino preferences in the P1 and P6 pockets. Accordingly, the motif HLA-DP2 exhibits exactly the same amino acid preferences in these pockets as HLA-DP4 (32). It differs only slightly from HLA-DP4 by the residues accommodated by the P4 pocket. More precisely, a sequence alignment suggests that the amino acids preferred in this pocket act as hydrogen bond donors in HLA-DR molecules, this pocket might be more hydrophobic as a result of the replacement of αD66 and αE11 in DRB1*0101 by αL66 and αA11 residues. This is probably why a glycine at position β11 does not favor aromatic or hydrophobic residues in HLA-DR molecules as illustrated by the P6 specificity of DRB1*0701 (4).

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Table VI. Capacity to bind to DP401 and DP402 of various peptides derived from allergens, viruses, or tumor Ags

| Peptides | Sequences | IC₅₀ (nM) | DP401 | DP402 |
|----------|-----------|-----------|-------|-------|
| TT497–506 | FNNFTVSFELVFKVSASHLE | 5 (±0.5) | 7 (±2) |
| Oxy271–286 | EKKYFAAAGNPELAARL | 10 (±3) | 10 (±2) |
| HSV283–302 | RELAVPRFGAGALEEPHA | 20 (±6) | 20 (±7) |
| NY-ESO-1158–160 | LLKFFITPLFQPFGSRQR | 30 (±12) | 70 (±13) |
| IL3Rα127–146 | GPAPAVQYDLYLNAVRR | 30 (±13) | 30 (±2.4) |
| NSP2 | GVQYRVRGERSFLKRW | 40 (±12) | 40 (±2.0) |
| NY-ESO-1119–143 | PGVLLKEFTGSLTNILTADHR | 100 (±33) | 100 (±33) |
| MAGE3245–258 | LLQTPFQVNELEY | 250 (±150) | 400 (±100) |
| HIV Nef132–147 | GVRPPFQTPGWYCLDP | 250 (±150) | 350 (±180) |
| Api m177–94 | TISSYFQGKRFNLIDTK | 450 (±100) | 180 (±35) |
| HIV Nef66–94 | VGFYTPVPQPPRMTYKAKALHFPLKEK | 500 (±150) | 100 (±100) |
| Api m81–98 | VFGYRFPNLIDTKYK | 2300 (±350) | 1200 (±400) |
| MART-151–73 | RRRGNYRALMDKIVHTQCALTRR | 8000 (±1000) | 4000 (±1000) |

* Sets of peptides entirely encompassing the sequences of the major bee venom Api m1 (30 peptides (7)) and the HIV Nef protein (13 peptides) were used, while limited numbers of peptides were used for the other Ags. Only the most active peptides are reported. Residues compatible with the amino acid preferences of DP401 and DP402 are in bold. Complete binding motifs are underscored.
and has been shown to account for most of the few binding discrepancies between these two HLA-DR4 alleles (39). Thus, as the major binding effects result from P1 and P6 substitutions in both HLA-DP2 and HLA-DP4, these molecules are expected to share a common repertoire of peptides and hence to form a supertype of HLA II molecules. In sharp contrast, the HLA-DP9 molecule seems to belong to another supertype (31). This motif is nicely associated with HLA-DP2 and HLA-DP4, respectively (31). This molecule is also dimorphic and is occupied by a valine or a glycine. It is known to control the P1 anchor residue (3, 4, 40) and to contribute to the dimer stability of HLA II molecules (41). It has also been identified as a key position that segregates the preponderant alleles upon different binding modes (4, 5, 7).

Moreover, as an unexpected consequence, our data may provide new insights into the allelic reactivity of HLA-DP and their debated role in GVHD. It is known that HLA-DP incompatibility only leads to low mixed lymphocyte reaction. Accordingly, the HLA-DPB1 disparity was not initially found to influence the risk of acute GVHD (19, 20). Recent studies performed on HLA-A, -B, -DR, and -DQ identical pairs demonstrated its role in the graft outcomes (34, 42) as agreed by isolation of allogeneic T-cell clones (21). However, the authors concluded that not all HLA-DP incompatibilities elicit a measurable MLR response (42). Therefore, HLA-DR appears to be of less importance in comparison with other HLA II molecules. It is tempting to discuss these observations in the light of our binding data. We suggest that the repertoire of natural peptides that bind to HLA-DP molecules is preferentially circumscribed by the amino acid combinations at positions β11 and β86. Although HLA-DP2 and HLA-DP4 did not exhibit evident common peptides (32, 33) and differ by the β71 which appeared to be involved in allorecognition (42), subsets of HLA-DP molecules from the same supertype may have in common a large number of naturally presented peptides. In this context, precursors of allogeneic T cells might be low in number as a result of negative thymic selection and hence rarely give rise to graft rejection. In contrast, HLA-DP from different supertypes would be fully allogeneic.

The binding assays presented in this paper constitute a promising way of selecting peptides for vaccination. Peptide-based strategies of vaccination generally use HLA-DR-restricted peptides as TT830–843 (43) or PADRE peptide (13) to trigger a specific immune response. Such peptides have been primarily selected, because they are able to bind multiple HLA-DR molecules and hence are expected to be active in most individuals. Therefore, much effort has been devoted to finding such peptides in pathogens (11, 15, 44, 45), allergens (7, 46), and tumor Ags (34, 47). As outlined previously (28, 30), HLA-DP4 are present in ~75% of Caucasians. Their frequency is equivalent to that of the well-known class I molecule HLA-A2 (22). Therefore, HLA-DP4-restricted peptides have a similar impact in the Caucasian population to HLA-DR peptides that bind approximately six different HLA-DR molecules. We can also notice that all the immunodominant HLA-DP4-restricted peptides that have been delineated by others using T-cell lines or clones are included in the best peptide binders found by our assays (24, 26, 28–30). Among them, NY-ESO-1 seems to promote a T-cell response that supports the humoral response against NY-ESO-1 (30). Therefore, HLA-DP4-restricted T cells appear as active as HLA-DR-restricted T cells to provide a helper activity. Together, these observations support the use of HLA-DP4-specific binding assays to delineate new sequences for vaccination. As the HLA-DP4-specific motifs clearly differ from that of HLA-DR molecules, these peptides could nicely complement the HLA-DR-restricted peptides in a multipotent strategy and hence diminish the risk of pathogen evasion. In particular, in this study, we propose new sequences from the major bee venom allergen, which could be used in specific immunotherapy of patients allergic to bee venom (7), from the HIV Nef protein and the melanoma Ag NY-ESO-1, which could be included in a peptide vaccine (34, 48). However, as compared with investigations of HLA-DR molecules, we identified fewer active peptides (7, 34, 49). This might result from the two constraints imposed on the P1 and P6 pockets and may suggest that HLA-DP4 epitopes are rarer than HLA-DR epitopes. Therefore, considering their frequency in the worldwide population, these epitopes are of great value for vaccination and constitute an alternative to the preparation of universal T-cell epitopes.

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