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**Author:** Heemst, Jurgen van  
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CHAPTER 4

HLA-DQA1*01:03/DQB1*06:03, an HLA-DQ molecule linked to autoimmune resistance, presents a unique peptide repertoire.

Jurgen van Heemst, Arnoud H. de Ru, Marieke Bax, George K. Papadopoulos, Peter van Veelen and René E.M. Toes.

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ABSTRACT

HLA-DQ0603 (DQA1*01:03;DQB1*06:03) is associated with resistance to multiple autoimmune diseases including rheumatoid arthritis, type 1 diabetes, narcolepsy and myasthenia gravis. We aimed to elucidate the peptide-binding repertoire of HLA-DQ0603 by isolating HLA-DQ0603 molecules from B-LCL lines and subsequent characterization of HLA-DQ0603-bound ligands. Characteristics of the identified ligands were comparable to what was previously shown for other HLA-class II molecules (e.g. ligand length and localization of protein-sources). After peptide-alignment we obtained the peptide-binding motif of HLA-DQ0603. We found that HLA-DQ0603 presented a unique peptide-binding repertoire that differed from previously identified peptide-binding motifs of closely related HLA-DQ6 molecules. Differences were mainly in pocket 1 and pocket 6 and can be translated to structural differences between different HLA-DQ molecules. This motif allows for more precision in the identification of disease-linked epitopes.
INTRODUCTION

The Human Leukocyte Antigen (HLA) class II locus is the primary risk factor for many different autoimmune diseases (AID), including multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes (T1D), narcolepsy and Myasthenia Gravis (MG) [1-5]. These HLA associations with autoimmunity suggest an important role for antigen-specific T cells during pathogenesis. For most of these autoimmune diseases the antigens recognized by such autoimmunogenic T cells are unclear. The HLA class II locus contains genes that are in strong linkage disequilibrium [6]. As a result, these genes inherit together in haplotypes making it difficult to genetically pinpoint the causative gene. In addition, this locus can play a dual role. Certain haplotypes can be associated with susceptibility, while others are neutral or even associated with resistance. Most haplotypes are differentially associated with multiple autoimmune diseases. For example, the HLA-DRB1*04-DQB1*03:01-DQA1*03:02 haplotype is strongly associated with risk for RA, yet protective in T1D [7-9]. However, other haplotypes display a similar effect throughout different autoimmune diseases. The haplotype HLA-DRB1*13:01-DQB1*06:03-DQA1*01:03 is of particular interest as it is strongly associated with resistance to different autoimmune diseases including RA [7, 10], T1D [9, 11-13], MG [14] and narcolepsy [15]. The underlying immunological pathways are currently elusive. The ligandome of HLA-DRB1*13:01 was previously characterized [16], but the peptide-binding repertoire of HLA-DQ0603 (HLA-DQB1*06:03/DQA1*01:03) is unknown. We therefore aimed to elute naturally processed ligands and to characterize the peptide-binding repertoire for this HLA-DQ molecule.

MATERIALS AND METHODS

Ligand isolation from affinity-purified HLA class II molecules. HLA-DQ0603-molecules were isolated from DRB1*13:01-DQB1*06:03-DQA1*01:03 homozygous EBV-transformed B lymphoblastoid cell line APD. Approximately 2x10^9 cells were grown in IMDM supplemented with L-glutamine and 10% FCS. Subsequently, the cells were harvested, washed with PBS, and the cell pellet was stored at -80°C. The cells were lysed with 50 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors (Complete inhibitor mix; Roche)) to remove the nuclei and insoluble material. The lysate was centrifuged for 60 min at 10,000g. It was subsequently precleared for 60 min with Sepharose beads and mixed with SPV-L3 coupled sepharose beads. After 60 min of gentle mixing, the beads were washed in 10 bead volumes of lysis buffer followed by washing with 20 mM Tris-HCl, 120 mM NaCl (pH 8.0), followed by washing with 20 mM Tris-HCl, 1 M NaCl (pH 8.0), 20 mM Tris-HCl (pH 8.0), and finally with 10 mM Tris-HCl (pH 8.0). Subsequently, the HLA-peptide complexes were eluted with 25 ml of 10% acetic
acid in water. All purification steps were performed at 4°C. High molecular mass material (HLA molecules) was removed by filtration through Centriprep filtration units with a cutoff value of 10 kDa. Ligands were identified as previously described. Briefly, ligands were fractionated on a C18 reverse phase HPLC system and analyzed by tandem mass spectrometry.

Peptide identification by mass spectrometry (MS) Ligands were characterized by an online nano-HPLC-MS system. The nano-HPLC column was connected to the needle of the electrospray source of the mass spectrometer. The mass spectrometer was run in data-dependent MS/MS mode during ligand elution. All tandem mass spectra produced in this way were searched against the human IPI database with the database search program MASCOT version 2.4 (Matrixscience). All reported hits were assessed manually, and ligands with best MASCOT ionscores <50 were discarded.

Peptide binding motif. The peptide binding motif of HLA-DQ0603 was constructed by aligning all the identified ligands and subsequent identification of the most likely nonamers. Aa frequency at each of the positions in the nonamer were compared to the frequency of these aa residues in the human proteome.

RESULTS

Characterization of the peptide-binding repertoire of HLA-DQ0603

The presence of HLA-DQ0603 is tightly associated with autoimmune resistance. Figure 1A shows the sequence of the HLA-DQB1*06:03 chain and a comparison with HLA-DQB1*06:02 and *06:04. Figure 1B shows a schematic representation
of the residues involved in shaping the various peptide-binding pockets of HLA-DQB1*06:03. Most notable is the β57D residue. The presence of β57D is strongly associated with T1D risk and it was described that this residue forms a salt-bridge with α76, thereby enhancing the stability of the peptide-MHC complex [17].

To examine the peptide-binding repertoire of HLA-DQ0603, we isolated HLA-DQ molecules from an EBV-transformed HLA-DQ0603 homozygous B cell line. Ligands derived from these HLA-DQ molecules were characterized. In total we identified 698 unique ligands. Most of the identified ligands were derived from HLA molecules (23%) as was previously described for ligands derived from other HLA class II molecules [18].

The ligands ranged in length between 7 and 20 amino acids. 94% of the ligands ranged between 12 and 18 aa in length as was previously shown for other HLA class II molecules (Figure 2A) [18, 19]. As HLA class II molecules are flexible in the size of ligands that can be accommodated, nested peptide-sets can be frequently found. These sets include different ligands with a shared core-sequence but of a different length. When we discard these length variants, 303 core sequences were identified. For 71% of the core sequences we found only a single length variant. For the remaining 29% we found up to 37 different length variants (Figure 2B). We also studied the localization of the proteins from which the identified ligands were derived. About 60% of the ligands were derived from the endocytic pathway, the remainder

![Figure 2: Characteristics of identified ligands. (A) Graph depicting the distribution of peptide length in the identified ligands. (B) Graph depicting the number of length variants identified for the different core ligand-sequences. (C) Graph depicting the percentage of peptides derived from a cytosolic or an endocytic source. (D) Graph depicting the different sources from which the identified peptides were derived. PM = plasma membrane, Lyso/Endo = lysosome or endosome, ER = endoplasmatic reticulum.]
from the cytosolic pathway (Figure 2C). Most ligands were derived from the plasma membrane, which includes all the HLA-derived ligands. The second most abundant source is the cytosol (Figure 2D. Peptide-localizations were comparable to what was previously shown for other HLA class II molecules [20, 21].

**The peptide-binding motif of HLA-DQ0603**

To determine the peptide-binding motif of HLA-DQ0603 from the eluted ligands, we performed both manual and automated ligand alignment, both resulting in similar results. Figure 3 shows the results from automated ligands alignments. The presence of aa residues in positions interacting with the various peptide-binding pockets were compared to the frequency of that particular aa in the human proteome. In the aa residues predicted to interact with pocket 1, there are clear preferences for asparagine, serine, threonine, leucine and isoleucine residues, all small to medium sized amino acid residues (Figure 3A). In pocket 4, a serine, threonine, alanine and valine are enriched. These are all small amino acid residues (Figure 3B). Residues in p6 are enriched for acidic residues and
proline (Figure 3C). In pocket 7, ring-shaped aa histidine and proline are preferred (Figure 3D). Finally, pocket 9 prefers the small amino acid residues alanine and serine (Figure 3E). The corresponding binding motif is summarized in Figure 3F.

**DISCUSSION**

This is the first report characterizing the peptide-binding repertoire of HLA-DQ0603, an HLA-DQ molecule tightly associated with autoimmune resistance. Using naturally eluted ligand sequences we have also established its peptide-binding motif. This allows for a comparison with other previously characterized HLA-DQ molecules. HLA-DQ0602 (DQB1*06:02-DQA1*01:02) is of particular interest. This HLA-DQ molecules is tightly linked with susceptibility for narcolepsy [15, 22]. This HLA-DQ molecule is highly related to the HLA-DQ0603 (Figure 1A). Both HLA-DQ molecules differ only in two amino acid positions: β9 that shapes peptide-binding pocket 9 and β30 that shapes peptide-binding pocket 6 [23]. The difference in β9 is relatively a minor difference (phenylalanine in DQB1*06:02 and tyrosine in DQB1*06:03). In contrast, the difference in β30 (tyrosine in DQB1*06:02 and a histidine in DQB1*06:03) would result in a more positively charged p6 pocket. Likewise, if we compare the peptide-binding motif of HLA-DQ0602(24;25) and HLA-DQ0603, we

<table>
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<th>Molecule</th>
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<tr>
<td>Hypocretin (O43612)</td>
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<td>Hcrt 6-14</td>
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<td></td>
<td>Hcrt 83-91</td>
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<td>HcrtR1 125-133</td>
<td>LQAVSVSVA</td>
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<td>HcrtR1 300-308</td>
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**Table 1**: List of predicted epitopes that bind to HLA-DQB1*06:02 but not to HLA-DQB1*06:03.
cannot identify differences in the p1, p4, and p9 residues, but we do find large differences in the p6 residues. HLA-DQ0602 is reported to prefer leucine, isoleucine and valine residues, all medium-sized hydrophobic residues [24, 25]. In contrast, we showed a preference for acidic residues in the p6 pocket of HLA-DQ0602, which can be directly explained by an β30V→H substitution. An understanding of these differences allows for predicting candidate narcolepsy epitopes. In Table 1, we have summarized candidate epitopes from three narcolepsy candidate autoantigens (hypocretin and hypocretin receptor 1 and 2) that we predict to selectively bind to HLA-DQ0602 and not to DQ0603 and that can therefore be attractive candidate epitopes. Indeed, Hcrt3-11 was previously crystallized in the context of HLA-DQB1*06:02 and was presented in the predicted register [26].

The peptide-binding motif of HLA-DQ0604 (DQB1*06:04-DQA1*01:02) has also been established [25]. This allele is associated with T1D susceptibility. HLA-DQB1*06:03 differs from HLA-DQB1*06:04 in three different positions: β86 (A→G) that shapes the p1 pocket, β57 (D→V) that is involved in peptide-MHC stability and β70(G→R) that is involved in TCR recognition [17, 23, 27] (Figure 1A). β86 renders the p1 pocket more spacious allowing the accommodation of large residues [25]. Together, these comparison illustrates how small differences in the structure of HLA-DQ molecules are translated in large differences in the peptide-binding repertoire. This study shows that HLA-DQ0603 presents a unique peptide-binding repertoire, distinct from highly related HLA-DQ6 molecules, likely resulting in distinct T cell repertoires that could explain HLA-DQ0603-mediated autoimmune resistance.

REFERENCES


26. Siebold C, et al. Crystal structure of HLA-DQ0602 that protects against type 1 diabetes
