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Allelic Variation in Key Peptide-Binding Pockets Discriminates between Closely Related Diabetes-Protective and Diabetes-Susceptible HLA-DQB1*06 Alleles

Ruth A. Ettinger,2* † George K. Papadopoulos, ‡ Antonis K. Moustakas, § Gerald T. Nepom,* † and William W. Kwok* †

HLA-DQA1*0102-DQB1*0602 is associated with protection against type 1 diabetes (T1D). A similar allele, HLA-DQA1*0102-DQB1*0604, contributes to T1D susceptibility in certain populations but differs only at seven amino acids from HLA-DQA1*0102-DQB1*0602. Five of these polymorphisms are found within the peptide-binding groove, suggesting that differences in peptide binding contribute to the mechanism of their association with T1D. In this study, we determine the peptide-binding motif for HLA-DQA1*0102-DQB1*0604 allelic protein (DQ6004) in comparison to the established HLA-DQA1*0102-DQB1*0602 (DQ6002) motif using binding assays with model peptides from T1D autoantigens and homology modeling using the coordinates of the DQ6002-hypocretin 1–13 crystal structure. The peptide binding preferences were deduced with a peptide from insulin that bound both with a 2- to 3-fold difference in avidity using the same amino acids in the peptide as anchors. Peptide binding differences directly influenced by the polymorphisms in or nearby pockets 1, 6, and 9 were observed. In pocket 1, DQ6004 was better able to accommodate aromatic residues due to the β86 and β87 polymorphisms. A negatively charged amino acid was preferred by DQ6004 in pocket 6 due to the positively charged β30HIs. In pocket 9, DQ6004 preferred aromatic amino acids due to the β9 and β30 polymorphisms and had low tolerance of acidic residues. β57Val in DQ6004 functions differently than β57Ala, in that it pushes ε76Arg outside of the pocket, preventing the formation of a salt bridge with an acidic amino acid in the peptide. This study furthers our understanding of the structure-function relationships of MHC class II polymorphisms. The Journal of Immunology, 2006, 176: 1988–1998.

Received for publication October 18, 2005. Accepted for publication November 15, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported by National Institute of Health Grant AI-44443 (to W.W.K.), a Juvenile Diabetes Research Foundation International Grant (to W.W.K.), and a grant from the European Union (EPEAEK II scheme, Third European Union Regional Development Framework for Greece, Program “Archeides”) (to G.K.P.). Address correspondence and reprint requests to Dr. Ruth A. Ettinger at her current address, University of Washington, R. H. Williams Laboratory, Box 357710, HSB K-165, 1959 NE Pacific Street, Seattle, WA 98195-7710. E-mail address: ettinger@uwashington.edu

ABBREVIATIONS: T1D, type 1 diabetes; B-LCL, B lymphocyte cell line; BLS, bare lymphocyte syndrome; p, position; GAD, glutamic acid decarboxylase.

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0022-1767/06/S02.00
DQB1*0603 and DQA1*0501-DQB1*0604; thus, the same α-chain complexes with either DQB1*0602 or DQB1*0604 polypeptide and the antigenic peptide, functioning as a ligand for the TCR.

Recently, a crystal structure for DQ0602 bound to a hypocretin peptide was determined (18). Hypocretin is an Ag that is absent in narcolepsy, a disease which is positively associated with DQB1*0602. The hypocretin 1–13 peptide side chains at amino acids 3, 6, 8, and 11 occupy the peptide-binding pockets 1, 4, 6, and 9, respectively. These amino acids correspond to a Leu in pocket 1, Thr in pocket 4, Val in pocket 6, and Ala in pocket 9, residues which are preferred by DQ0602 for peptide binding in these pockets (19). Two key structural features were hypothesized to be important for dominant protection against T1D. First, it was determined from the crystallography data that pocket 6 of the DQ0602-hypocretin 1–13 structure was ~42 Å̃ bigger than the corresponding pocket in the DQ8 (DQ0302)-insulin B 9–23 structure (DQ6 is encoded by DQA1*0301-DQB1*0302 (20)). Thus, it was suggested that the volume of pocket 6 in DQ0604 would fall in between the large volume of DQ0602 and the small volume of DQ0302. Furthermore, the large volume in pocket 6 of DQ0602 would allow more peptides to bind to DQ0602, implying that presentation of an expanded peptide repertoire is important for dominant protection. Second, crystallography of DQ0602 and DQ0302 showed that the noncovalent interactions between the β57 polymorphism, invariant α76Arg, and the peptide residue in pocket 9 differed, as seen in all MHC class II-β2m peptides thus far. It was predicted that DQ0302 and DQ0604 would have similar peptide specificities due to the presence of a β57-non-Asp amino acid in both.

In this study, the peptide binding preferences for DQ0604 are determined using the insulin B 5–15 peptide that was used to determine the peptide-binding motif for DQ0602 (19). Structural interactions that control these preferences are proposed using homology molecular modeling based on the DQ0602 crystal structure. These experiments further the hypotheses set forth regarding the peptide-binding interactions that distinguish DQ0602 and DQ0604 and therefore lead to dominant protection and susceptibility in T1D. Our results demonstrate a role for the polymorphism at β9, β30, β57, β86, and β87 in controlling the peptide binding preferences in pockets 1, 6, and 9. These data further our understanding of DQ0602 and DQ0604, in conjunction with our previous studies identifying β57 as a key determinant of αβ heterodimer stability and β70 in TCR recognition (21, 22).

Materials and Methods

Cell lines

Homozygous EBV-transformed B lymphocyte cell lines (B-LCLs) from the Tenth International Histocompatibility Workshop include MGAR (DQA1*0102-DQB1*0602), AMAI (DQA1*0102-DQB1*0602), EMJ (DQA1*0102-DQB1*0604), WHK-HOM-2 (DQA1*0101-DQB1*0501), HTS (DQA1*0301-DQB1*0401), AMALA (DQA1*0501-DQB1*0301), JIMV (DQA1*0501-DQB1*0301), EK (DQA1*0101-DQB1*0503), TEM (DQA1*0101-DQB1*0503), BSM (DQA1*0301-DQB1*0302), DEU (DQA1*0301-DQB1*0301), COX (DQA1*0501-DQB1*0201), OMW (DQA1*0303-DQB1*0603), and CB66 (DQA1*0103-DQB1*0603) (23). Other EBV-transformed B-LCLs used in this study include LG2 (DQA1*0101-DQB1*0501), HAS-15 (DQA1*0301-DQB1*0401), KASA11 (DQA1*0102-DQB1*0502), AZH (DQA1*0102-DQB1*0502), PRIESS (DQA1*0301-DQB1*0302), PF97387 (DQA1*0301-DQB1*0301), and MAT (DQA1*0501-DQB1*0201), and they were HLA typed by high-resolution oligonucleotide typing (Pugnet Sound Blood Center, Seattle, WA). BLS-1 is a HLA class II-null EBV-transformed B-LCL generated from the cells of a patient with bare lymphocyte syndrome (BLS). Cells were grown in IMDM with l-glutamine and 25 mM HEPES buffer (Invitrogen Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Peptides

Peptides were synthesized at Benaroya Research Institute at Virginia Mason with an Applied Biosystems 432 Peptide Synthesizer (PerkinElmer) or synthesized by Genemed Synthesis. Peptides were biotinylated as described previously (25). The molecular mass of each peptide was determined by mass spectrometry (Protein and Carbohydrate Structure Facility, University of Michigan, Ann Arbor, MI and Genemed Synthesis). The sequence of all peptides is included in the tables and figures.

Peptide-binding assays

Measurement of binding of 10 μM biotinylated peptide to HLA-DQ on paraformaldehyde-fixed EBV-transformed B-LCLs was performed as described elsewhere (19). DQ0602 and DQ0604 were affinity purified from GMAR and EMJ EBV-transformed B-LCLs, respectively, as described for DQ0602 (19). Protein concentration was determined by Bradford microassay with BSA as the standard. Measurement of binding of 0.001–10 μM biotinylated peptide to purified DQ0602 and DQ0604 at pH 5.4 was essentially as described for DQ0602, except that, in the present report, BSA was added to the reaction mixture to determine nonspecific binding (19). In some experiments, the pH of the reaction mixture was varied from pH 4.0 to 8.0 using different 150 mM citrate-phosphate buffers for the pH range of 4.0–7.0, and 200 mM phosphate buffers for the pH range of 7.1–8.0, prepared as described previously (26). The binding of peptides to purified DQ0602 and DQ0604 was also evaluated in a competition assay with non-biotinylated peptides competing for binding with 1.0 μM biotinylated insulin B 5–15 as described elsewhere (19). The nonbiotinylated peptides were tested at 0.1, 0.3, 1.0, 3.0, 10, 30, and 100 μM final concentrations in triplicate. The fluorescence values were plotted vs peptide concentration and the peptide concentration at which 50% inhibition in binding of the biotinylated peptide (IC50) occurred was extrapolated from the curve. Relative binding values were calculated by dividing the IC50 for insulin B 5–15 by the IC50 for the analog peptide. Binding of each peptide to DQ0602 and DQ0604 was compared within the same assay.

Molecular modeling

Models of DQ0602 and DQ0604 with the insulin B 5–15 peptide and its variants were prepared on a Silicon Graphics Indigo 2 work station using the program Insight II, version 2000 (Accelrys), essentially as previously described (22, 27). The crystal structure of DQ0602 complexed to the insulin B 1–15 peptide was used for the molecular modeling studies (18). The numbering scheme developed by Fremont et al. (28) for mouse H-A MHC class II molecules was used here for HLA-DQ alleles. Essentially, it yields identical numbering in equivalent structural locations in all MHC class II alleles.

Results

Insulin B 5–15 peptide binds to DQ0604

The binding of insulin peptides, covering the primary structure of insulin, to several HLA-DQ alleles was previously described (19). The HLA-DQ alleles examined consisted of a panel representing at least one allele from each HLA-DQ serological specificity except DQ9. Insulin B 1–15 was identified as the insulin peptide that bound with highest avidity to DQ0602. In addition, insulin B 1–15 bound poorly to most HLA-DQ allelic proteins tested. The minimal binding epitope was insulin B 6–14, with insulin B 5–15 conferring maximal binding. The binding of insulin B 5–15 to DQ0602 led to the question of whether it would bind other HLA-DQ molecules within the DQ6 serological specificity. DQ0604 and the DQA1*0103-DQB1*0603 allelic protein expressed on EBV-transformed B-LCLs were examined in comparison with DQ0602 and eight other HLA-DQ alleles (Fig. 1). For each HLA-DQ genotype, two different EBV-transformed B-LCLs were included in the assay. Fig. 1 shows that DQ0604 bound insulin B 5–15 at a level 1.7- to 3.9-fold reduced compared with DQ0602, whereas the DQA1*0103-DQB1*0603 allelic protein bound insulin B 5–15 very poorly, at a level slightly above background. In agreement with our previous data (19), there is some
binding to the DQA1*0101-DQB1*0501 allelic protein, which is 4.9- to 5.7-fold reduced compared with DQ0602. This binding was reduced to almost background for the DQ5 allelic proteins DQA1*0102-DQB1*0502 and DQA1*0101-DQB1*0503. Since DQB1*0502 complexes with the same H9251 chain as DQB1*0602 and DQB1*0604, the inability of the DQA1*0102-DQB1*0502 allelic protein to bind insulin B 5–15 is conferred by the polymorphisms in the H9252 chain.

The binding avidity of insulin B 5–15 to DQ0604 in comparison to DQ0602 was further characterized in binding assays with affinity-purified MHC proteins. Binding of biotinylated insulin B 5–15 increased in a linear fashion from 0.001 to almost 1 M (Fig. 2A). Within the linear range, binding of insulin B 5–15 to DQ0604 was 1.8- to 2.9-fold lower at each concentration (0.001, 0.01, 0.1, and 1 M) compared with DQ0602. A similar difference in avidity of the insulin B 5–15 peptide for DQ0602 and DQ0604 was seen in a competition assay where different concentrations of nonbiotinylated insulin B 5–15 are competing for binding with 1.0 M biotinylated insulin B 5–15 (Fig. 2B). The IC50 of insulin B 5–15 was always 2- to 3-fold lower for DQ0602 compared with DQ0604 and ranged from 1.5 to 3.4 μM for DQ0602 and 3.3 to 13 μM for DQ0604 (n = 10).

The effect of pH on the binding of insulin B 5–15 to DQ0602 and DQ0604 was examined. Interestingly, the pH optimum and shape of the profiles are different for DQ0602 and DQ0604 (Fig. 2C). Binding of insulin B 5–15 to DQ0602 was less affected by pH, with a 2.1-fold difference in pH over the range of 4.0–8.0 and a pH optimum of 5.5. In contrast, DQ0604 bound insulin B 5–15 poorly at pH 4.0, binding increased 19.4-fold in a linear fashion to pH 6.0, and then reached a plateau at an optimal level from pH 6.0 to pH 8.0. It should be noted that all other peptide binding experiments in this article were done at one acidic pH, that of pH 5.4, due to the acidic characteristics of the endosomal/lysosomal compartments where peptide-MHC class II binding generally occurs (29). However, other pH values may be physiologically relevant in the Ag processing and presentation pathway and as a result will regulate peptide binding levels.

Peptide-binding motif derivation for DQ0604 in comparison to DQ0602

The anchor residues for binding of insulin B 5–15 to DQ0602 and DQ0604 were defined by examining the effect of radical substitutions on binding. Arginine substitutions were used to map the primary anchors because of the effectiveness of using positively charged substitutions in identifying the amino acids in a peptide that are required for binding to HLA-DQ (19, 30, 31).
FIGURE 3. Effect of single Arg substitutions in insulin B 5–15 on binding to DQ0602 and DQ0604. Biotinylated insulin B 5–15 (wild-type (WT)- and insulin B 5–15 Arg (R)-substituted peptides (10 μM) were incubated with 1.5 x 10^6 paraformaldehyde-fixed B-LCLs in pH 5.4 buffer for 18 h at 37°C. Cells were washed to remove unbound peptide. HLA-DQ-bound biotinylated peptide was measured as described in figure legend 1. The HLA-DQ genotype of the homozygous B-LCLs is BLS-1 (none), MGAR (A1*0102-B1*0602), and EMJ (A1*0102-B1*0604). Data are the means ± SD of triplicate determinations.

and DQ0604 expressed on EBV-transformed B-LCLs. The Arg-substituted peptides that showed dramatically reduced binding compared with the unsubstituted (wild-type) peptide were insulin B 5–15 6R, 8R, 9R, 11R, and 14R peptides. The pattern of binding of the insulin B 5–15 Arg-substituted peptides was very similar for DQ0602 and DQ0604, with one exception; insulin B 5–15 7R bound DQ0602 2.0-fold better while binding DQ0604 1.8-fold less well than wild type. The sequence of insulin B 5–15 is shown in Fig. 4 and the anchor positions at amino acids 6, 8, 9, 11, and 14 are designated as relative positions (p) 1 (p1), 3 (p3), 4 (p4), 6 (p6), and 9 (p9).

The amino acid requirements in each of the primary anchor positions for binding to DQ0602 and DQ0604 were investigated. Single amino acid substitutions in insulin B 5–15 were made to represent all of the general classes of amino acid side chains: glycine (Gly), aliphatic (Ala, Val, Leu, Ile), hydroxy (Ser, Thr), amide (Asn, Gln), aromatic (Phe, Tyr, Trp), sulfur-containing (Cys, Met), cyclic imino acid (Pro), acidic (Asp, Glu), and basic (Lys, Arg, His). The effect of these substitutions in insulin B 5–15 at p1, p3, p4, p6, and p9 on relative binding to DQ0602 and DQ0604 is shown in Fig. 5. Fig. 4 summarizes the peptide-binding motif for DQ0602 and DQ0604. The amino acids are listed in order of preference from best tolerated to least well tolerated, with only those with a relative binding capacity >0.2 included.

The general motif derivation analysis indicated that DQ0602 is more tolerant of peptide substitutions than DQ0604. Fourteen insulin B 5–15-substituted peptides bind better to DQ0602 in pockets 1, 3, 4, and 6 (indicated by a star symbol in Fig. 5), while only the p1Phe and the p6Asp peptides were preferred by DQ0604 (indicated by an accent symbol). Pocket 9 has a more even distribution in number of amino acids preferred, with DQ0602 preferring Gly- and Ser-substituted insulin B 5–15 and DQ0604 preferring Leu- and aromatic (Tyr, Phe, Trp)-substituted insulin B 5–15 peptides.

Molecular modeling of insulin B 5–15 with DQ0602 and DQ0604

Models of DQ0602 and DQ0604 with insulin B 5–15 were created based on the crystal coordinates of DQ0602 (18). The structural simulation of the binding of insulin B 5–15 peptide in the groove of DQ0602 and DQ0604 revealed the means by which the different anchoring pocket residues are responsible for the different peptide residue preferences at these positions. The simulation of these complexes shows that this particular peptide fits well into the groove of either DQ allele (Fig. 6). There is no observed structural distortion, in comparison to the crystal structure of DQ0602, and the interactions of the antigenic peptide backbone with specific invariant residues (e.g., α62Asn, α68His, α69Asn, β81His, β82Asn) of both the DQ0602 and DQ0604 allele are present and unaltered, just as in all other MHC class II crystal structures recorded to date. There is a slight difference in the orientation of insulin B 5–15 peptide residues p1 to p5 between DQ0602 and DQ0604, and the surface of the proteins differ due to the β70 polymorphism (Table I). The surface exposure of residue β70Arg of DQ0604 is considerable (Fig. 6A), which most likely makes its contact with any cognate TCR unavoidable.

The detailed examination of the simulated structures identifies polymorphisms that distinguish DQ0604 and DQ0602 in pockets 1, 6, and 9. In pocket 1 of DQ0604, the presence of B86Gly renders this pocket more spacious and able to accommodate aromatic residues (Phe) even better than large aliphatic ones (Leu) (data not shown). In addition, the β87 polymorphism, Tyr in DQ0604 vs Phe in

![Graph showing peptide binding](image)

**Table 1.** Relative Position and Amino Acid Substitutions

<table>
<thead>
<tr>
<th>Insulin B 5-15 5 6 7 8 9 10 11 12 13 14 15</th>
<th>Relative Position</th>
<th>H</th>
<th>L</th>
<th>C</th>
<th>G</th>
<th>S</th>
<th>H</th>
<th>L</th>
<th>V</th>
<th>E</th>
<th>A</th>
<th>L</th>
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<tr>
<td>DQ0602 Motif 1 allophatic (L) amin (N)</td>
<td>3 glycine (G)</td>
<td>4 hydroxy (S &gt; T)</td>
<td>6 aliphatic (L &gt; A)</td>
<td>9 alanine (A)</td>
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</tr>
<tr>
<td>DQ0604 Motif 1 hydrophobic (F)</td>
<td>3 glycine (G)</td>
<td>4 hydroxy (S &gt; T)</td>
<td>6 aliphatic (L &gt; A)</td>
<td>9 alanine (A)</td>
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**Figure 4.** DQ0602 and DQ0604 peptide-binding motifs. Motifs were determined within the context of the insulin B 5–15 peptide. Anchor positions are designated with arrows. Amino acids accepted for binding (relative binding capacity: >0.2) in each position are listed in order of preference from the best binder to the least well-accepted binder. In parentheses is the one letter code for the specific amino acid tested.
FIGURE 5. Relative binding capacity of insulin B 5–15 analog peptides for DQ0602 and DQ0604. A, Amino acid 6 (p1)-substituted insulin B 5–15 peptides. B, Amino acid 8 (p3)-substituted insulin B 5–15 peptides. C, Amino acid 9 (p4)-substituted insulin B 5–15 peptides. D, Amino acid 11 (p6)-substituted insulin B 5–15 peptides. E, Amino acid 14 (p9)-substituted insulin B 5–15 peptides. Insulin B 5–15 analog peptides (0.1, 0.3, 1.0, 3.0, 10, 30, 100 μM) were incubated with 25 nM purified DQ0602 or DQ0604 and biotinylated insulin B 5–15 (1.0 μM) in pH 5.4 buffer for 48 h at 37°C. HLA-DQ-bound biotinylated peptide was measured as described in figure legend 2. Relative binding values were calculated by dividing the IC50 for insulin B 5–15 by the IC50 for the analog peptide. Inequality sign (≤) indicates peptides that had an IC50 > 100 μM and thus a relative binding capacity less than the value indicated on the graph. Two-fold or greater differences in relative binding capacity between DQ0602 and DQ0604 were designated with a star symbol (★) for peptides that prefer DQ0602 and an accent symbol (∧) for peptides that prefer DQ0604.

DQ0602, allows for stronger aromatic-aromatic interactions because of the substantially larger permanent dipole moment of Tyr compared with Phe. Hence, DQ0604 was observed to better accommodate aromatic residues at p1 (Fig. 5A). By the same argument, smaller residues that are only slightly hydrophilic (Ala, Thr, Cys) are not well accommodated in p1 of DQ0604 compared with the same pocket in DQ0602, because water molecules are not energetically favored to occupy the space between the MHC class II residues lining the pocket and such a p1 residue of the antigenic peptide.

In pocket 6, while both alleles accept aliphatic residues, the DQ0604 allele also binds a p6Asp-substituted insulin B 5–15 peptide (Fig. 5D). This preference in all probability is due to the presence of β30His at the “mouth” of pocket 6, which is positively charged at endosomal pH (Fig. 7). Furthermore, this negatively charged Asp at p6 would keep β30His positively charged by induction, even at the slightly alkaline extracellular pH, once the DQ0604-peptide complex is on the cell membrane and exposed to the extracellular environment. The volume of this pocket in DQ0604 appears to be similar to that in DQ0602 (data not shown) and is sufficient in both for accommodating a bulky residue such as Leu, but not an inflexible aromatic hydrophobic residue such as Phe (Fig. 5D). The hydrophobicity of both pockets is a result of α63Val and α66Ala in addition to the methylene groups of the three Asn residues (α11, α62, and α69). The latter two form hydrogen bonds with the peptide backbone so their terminal amides do not determine the character of the pocket. The lower tolerance for substitutions at p6 by DQ0604 compared with DQ0602 is attributed to the polymorphisms at β30 and β9, which decrease the hydrophobic character of pocket 6 in DQ0604.

Pocket 9 of DQ0604 accepts well aromatic and aliphatic residues, yet has low tolerance of acidic residues (Glu > Asp) (Fig. 5E). This ability is apparently due to the presence of three polymorphic residues at positions β9, β30, and β57 (Table I), the combination of which shapes such an environment in pocket 9, that the binding of the said residues is promoted. Of the three aromatic amino acids, Tyr is best-suited as an anchor because its hydroxyl group interacts favorably with the hydroxyl groups of β9Tyr and β37Tyr and the imidazole group of β30His via hydrogen bonding and aromatic-aromatic interactions (Fig. 8). The “walls” of the pocket are hydrophobic, hence the preference for hydrophobic residues of shorter length (Ala > Tyr > Leu > Phe > Trp). The low preference given to acidic residues in this pocket stems from the presence of β57Val that pushes α76Arg outside the pocket, so that the formation of an energy-stabilizing salt bridge between this Arg residue and p9Asp is no longer tenable. By contrast, p9Glu, being one methylene group longer, can interact weakly with α76Arg, but the distance between the interacting group is longer because of the interfering β57Val.

Motif prediction and testing with glutamic acid decarboxylase 65 (GAD65), IA-2, and proinsulin peptides

Previously, in deducing the peptide-binding motif for DQ0602, the predictive power of the motif was examined by scanning GAD65, IA-2 (also called ICA512), and proinsulin for the DQ0602 peptide-binding motif (19). Autoantibodies against these three β cell Ags
are highly predictive of T1D, with 90% of newly diagnosed subjects having one or more of these autoantibodies (32). This analysis identified a total of 24 peptides, which were synthesized with an additional amino acid flanking each side of the 9-mer core. Of these peptides, 79% (19 of 24) bound to DQ0602 with an IC50 <100 μM (range, 0.7–90 μM). The same approach was applied to the DQ0604 motif. Twenty peptides were identified in human GAD65 (33), IA-2 (34), and proinsulin (35) that contain the peptide-binding motif deduced for DQ0604. Table II shows the IC50 of each of these peptides for binding to DQ0604 by competition with biotinylated insulin B 5–15. Of these peptides, 65% (13 of 20) bound to DQ0604 (IC50 <100 μM). The IC50 value of the binders ranged from 1.9 to 86 μM, with insulin B 5–15 binding with an IC50 of 4.8 μM. When an entire Ag (HSV-2 VP16) was studied with a panel of 60 overlapping 20-mer, there was a 75% agreement between the presence and absence of the DQ0604 motif and peptide binding (data not shown). It is not known why certain peptides that contain the motif determined herein do not bind DQ0604 with an avidity comparable to insulin B 5–15. Factors that may play a role, but have not been investigated, are the presence of proline and glycine in the peptide and the effect of neighboring non-anchor amino acids on peptide binding.

The binding specificity of the human GAD65, IA-2, and proinsulin peptides selected with the DQ0604 (Table II) and DQ0602 (Table III) motifs were tested by binding to DQ0602 for the DQ0604 motif peptides and DQ0604 for the DQ0602 motif peptides. Because of the overlap in motif, many of the peptides bound to both DQ0602 and DQ0604. This was particularly evident when the DQ0604 motif was used to select the peptides, since all of the peptides except for two had an IC50 for binding that was 2-fold different for DQ0602 and DQ0604. One of these two peptides, IA-2 586–596 actually had a lower IC50 for DQ0602. This was not

**FIGURE 6.** Molecular simulation of the binding of insulin B 5–15 peptide at pH 5.4 to DQ0604 (A) and DQ0602 (B), based on the crystal structure of DQ0602. TCR view of the αβ1 domain in the same orientation and identical rendering pattern is shown for DQ0604 and DQ0602 in complex with the insulin B 5–15 peptide. The αβ1 domain is shown in a transparent van der Waals surface representation, color coded according to the electrostatic potential (positive, blue; negative, red; neutral, gray). The peptide is shown in space filling mode (atomic color code: carbon, green; oxygen, red; nitrogen, blue; hydrogen, white), with the two histidines at p1 and p5 as positively charged residues (pH 5.4). The six amino acids that are different in this domain between DQ0602 and DQ0604, except β30His, are shown in stick form with the same atomic color code as for the peptide, except that carbon atoms are in orange. The secondary structure of the domain is shown for orientation purposes, with a helix in red, β-pleated sheet in turquoise, and random coil in gray. The depiction of the structures was performed with the program WebLabViewer of Accelrys.

[Table I. The seven polymorphic amino acids that distinguish DQA1*0102-DQB1*0602 and DQA1*0102-DQB1*0604 and the corresponding amino acids at these positions in DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302]

<table>
<thead>
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<th>DQA1+/DQB1+</th>
<th>Polymorphic Amino Acids</th>
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<tr>
<td>0102–0602</td>
<td>Phe Tyr Asp Gly Ala Phe Arg</td>
</tr>
<tr>
<td>0102–0604</td>
<td>Tyr His Val Arg Gly Tyr Gln</td>
</tr>
<tr>
<td>0501–0201</td>
<td>Tyr Ser Ala Arg Glu Leu Arg</td>
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<tr>
<td>0301–0302</td>
<td>Tyr Tyr Ala Arg Glu Leu Arg</td>
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with biotinylated insulin B 5–15. Of these peptides, 65% (13 of 20) bound to DQ0604 (IC50 <100 μM). The IC50 value of the binders ranged from 1.9 to 86 μM, with insulin B 5–15 binding with an IC50 of 4.8 μM. When an entire Ag (HSV-2 VP16) was studied with a panel of 60 overlapping 20-mer, there was a 75% agreement between the presence and absence of the DQ0604 motif and peptide binding (data not shown). It is not known why certain peptides that contain the motif determined herein do not bind DQ0604 with an avidity comparable to insulin B 5–15. Factors that may play a role, but have not been investigated, are the presence of proline and glycine in the peptide and the effect of neighboring non-anchor amino acids on peptide binding.

The binding specificity of the human GAD65, IA-2, and proinsulin peptides selected with the DQ0604 (Table II) and DQ0602 (Table III) motifs were tested by binding to DQ0602 for the DQ0604 motif peptides and DQ0604 for the DQ0602 motif peptides. Because of the overlap in motif, many of the peptides bound to both DQ0602 and DQ0604. This was particularly evident when the DQ0604 motif was used to select the peptides, since all of the peptides except for two had an IC50 for binding that was <2-fold different for DQ0602 and DQ0604. One of these two peptides, IA-2 586–596 actually had a lower IC50 for DQ0602. This was not

**FIGURE 7.** Details of the modeled structure of pocket 6 of DQ0604 with the p6Asp (D) substituted insulin B 5–15 peptide. The orientation is rotated by 20° with respect to the y-axis running through the middle of the photograph (right-hand side coming up, left-hand side going into the plane of the paper), in comparison to the photograph in Fig. 6, so that the interaction of the charged imidazole ring of β30His with p6Asp can be shown in detail. Color code for surfaces and atoms of the antigenic peptide and the residues of the αβ1 domain of DQ0604 is identical to that of Fig. 6.
surprising because this peptide was selected with the DQ0602 motif and demonstrated the highest binding avidity for DQ0602 of all of the tested peptides (Table III). The lone peptide that bound specifically to DQ0604 was GAD65 339–349. In contrast, the peptide selected with the DQ0602 motif identified six peptides (IA2 586–596, IA2 499–509, GAD65 334–344, GAD65 396–406, IA2 335–345, and GAD65 86–96) with more than 7-fold lower IC₅₀ for DQ0602 compared with DQ0604.

Table II. Binding of human GAD65, IA-2, and proinsulin peptides selected with the DQ0604 peptide-binding motif to DQ0604 and DQ0602

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC₅₀ for DQ0604 (µM)</th>
<th>IC₅₀ for DQ0602 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65 91–101</td>
<td>FLVATDLLPAC</td>
<td>1.9</td>
<td>2.6</td>
</tr>
<tr>
<td>GAD65 339–349</td>
<td>TVYGAEDPLLA</td>
<td>4.0 &gt;100</td>
<td></td>
</tr>
<tr>
<td>Insulin B 5–15</td>
<td>TLCSHIDVEAL</td>
<td>4.8</td>
<td>1.8</td>
</tr>
<tr>
<td>GAD65 113–123</td>
<td>DWXNLLQYVYV</td>
<td>4.9</td>
<td>5.2</td>
</tr>
<tr>
<td>IA-2 576–586</td>
<td>SVLLTVLAGV</td>
<td>7.4</td>
<td>8.0</td>
</tr>
<tr>
<td>GAD65 206–216</td>
<td>TSYIAAPVFVL</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>IA-2 581–591</td>
<td>LVALAGVAGLL</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>IA-2 504–514</td>
<td>FINISVQPLA</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>IA-2 694–704</td>
<td>MIALYREDHLR</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>IA-2 836–846</td>
<td>EIVLVLEHIC</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>IA-2 586–596</td>
<td>GVLGLVALV</td>
<td>26</td>
<td>1.1</td>
</tr>
<tr>
<td>IA-2 957–967</td>
<td>DQWPAALTAV</td>
<td>37</td>
<td>52</td>
</tr>
<tr>
<td>IA-2 831–841</td>
<td>LNYVSLEVLVS</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>GAD65 117–127</td>
<td>TLLOQVVKSPD</td>
<td>86</td>
<td>64</td>
</tr>
<tr>
<td>GAD65 378–388</td>
<td>FNLWLEVDERAN</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>Proinsulin 53–63</td>
<td>LQLPAGLEGLSQ</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>GAD65 138–148</td>
<td>SFNELLHYNYS</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>IA-2 60–70</td>
<td>QQVQCVQQR</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>IA-2 272–282</td>
<td>LFBDGLLLYLA</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>IA-2 278–288</td>
<td>LLVLAQELPAP</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
<tr>
<td>IA-2 329–339</td>
<td>VQDDAQLRLA</td>
<td>&gt;100 &gt;100</td>
<td>&gt;100 &gt;100</td>
</tr>
</tbody>
</table>

Testing of the pocket predictions with substituted GAD65 peptides

Two of the peptides in Tables II and III that demonstrated substantial binding specificity for either DQ0602 or DQ0604 were further analyzed. The objective was to test whether the deduced motifs could be used to turn a nonbinding peptide into a binder.

The first peptide examined was GAD65 339–349, which showed relatively strong binding to DQ0604 (IC₅₀ = 4 µM with a relative binding capacity of 1.2 compared with insulin B 5–15) and no detectable binding to DQ0602 (IC₅₀ > 100 µM). Direct binding of biotinylated GAD65 339–349 to DQ0602 and DQ0604 showed a binding differential similar to the competition assay (Fig. 9A). This peptide would be expected to bind DQ0604 well because of anchors that are well accepted at p1, p3, p4, p6, and p9 (relative binding capacity: 1.0, 1.0, 0.72, 0.28, 0.48, respectively) and not bind DQ0602 because of the Asp at p6 which is barely tolerated (relative binding capacity: 0.033). Also, the Leu at p9 is less well tolerated by DQ0602 (relative binding capacity: 0.15) relative to DQ0604 (relative binding capacity: 0.48). To test these predictions, amino acid substitutions were made in GAD65 339–349 at p6 and p9 and these peptides were tested for binding to DQ0602 (Fig. 9B). A dramatically increased binding to DQ0602, particularly at higher concentrations (>0.1 µM), was observed by changing p6 from an Asp to a Leu; changing p9 from a Leu to an Ala did not allow for binding, implying that the Asp at p6 may have a strong inhibitory effect on binding to DQ0602. Finally, changing both p6 and p9 to Leu and Ala resulted in increased binding compared with p6Leu alone at the low peptide concentrations of 0.001 µM (p6Leu, 1.939 ± 105; p6Leu9Ala, 5.366 ± 244) and 0.01 µM (p6Leu, 13.583 ± 527; p6Leu9Ala, 30.944 ± 980).

The second peptide examined was GAD65 334–344, which bound DQ0602 with an IC₅₀ of 2.6 µM (relative binding capacity...
of 0.73 compared with insulin B 5–15) and DQ0604 with an IC50 > 100 μM. The substantially better binding of biotinylated GAD65 334–344 to DQ0602 compared with DQ0604 is shown in Fig. 9C. GAD65 334–344 contains a motif well-suited to binding DQ0602 with strong anchors in all five positions (relative binding capacity: 0.49, 1.0, 0.44, 0.52, and 1.0, respectively). This peptide is not expected to bind DQ0604 because of the Thr in p1 that is not tolerated (relative binding capacity: <0.1), and the Val in p6 that is less well accepted by DQ0604 (relative binding capacity: 0.21) than DQ0602 (relative binding capacity: 0.52). The p1 and p6 amino acids were both substituted with Leu, an amino acid that is better suited for DQ0604 pockets 1 and 6. Fig. 9D shows the binding of single-substituted peptides, p1Leu and p6Leu, and the double-substituted peptide to DQ0604. Each of the single-substituted peptides substantially increased binding to DQ0604. The double-substituted peptide resulted in increased binding at each concentration relative to the single-substituted peptides; however, the increase was not additive. These results substantiate the value of the motifs in predicting the peptide side chains preferred in pockets 1, 6, and 9 for DQ0602 and DQ0604.

**Discussion**

Using the techniques of peptide binding (direct and competitive) to allele-specific HLA-DQ-bearing B-LCLs and to purified HLA-DQ6 alleles, we have deciphered the peptide-binding motif of the DQ0604 allele (T1D susceptible/neutral). We in parallel compared this motif to that of the already established T1D-resistant allele DQ0602. The recently solved crystal structure of the latter molecule, in complex with the hypocretin 1–13 narcolepsy peptide (18), is not expected to bind DQ0604 because of the Thr in p1 that is not accepted by DQ0604 (relative binding capacity: 0.21) compared with insulin B 5–15) and DQ0604 with an IC50 > 100 μM. The substantially better binding of biotinylated GAD65 334–344 to DQ0602 compared with DQ0604 is shown in Fig. 9C. GAD65 334–344 contains a motif well-suited to binding DQ0602 with strong anchors in all five positions (relative binding capacity: 0.49, 1.0, 0.44, 0.52, and 1.0, respectively). This peptide is not expected to bind DQ0604 because of the Thr in p1 that is not tolerated (relative binding capacity: <0.1), and the Val in p6 that is less well accepted by DQ0604 (relative binding capacity: 0.21) than DQ0602 (relative binding capacity: 0.52). The p1 and p6 amino acids were both substituted with Leu, an amino acid that is better suited for DQ0604 pockets 1 and 6. Fig. 9D shows the binding of single-substituted peptides, p1Leu and p6Leu, and the double-substituted peptide to DQ0604. Each of the single-substituted peptides substantially increased binding to DQ0604. The double-substituted peptide resulted in increased binding at each concentration relative to the single-substituted peptides; however, the increase was not additive. These results substantiate the value of the motifs in predicting the peptide side chains preferred in pockets 1, 6, and 9 for DQ0602 and DQ0604.

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**Figure 9.** Testing of DQ0602 and DQ0604 pocket predictions with substituted GAD65 339–349 and 334–344 peptides. A. Binding of biotinylated GAD65 339–349 to DQ0602 and DQ0604. B. Binding of biotinylated GAD65 339–349 analog peptides to DQ0602. C. Binding of biotinylated GAD65 334–344 to DQ0602 and DQ0604. D. Binding of biotinylated GAD65 334–344 analog peptides to DQ0604. Biotinylated peptide (0.001, 0.01, 0.1, 1.0, 10 μM) was incubated with 25 nM purified DQ0602, DQ0604, or BSA in purified peptide binding buffer (pH 5.4) for 48 h at 37°C. HLA-DQ-bound biotinylated peptide was measured as described in figure legend 2. Nonspecific binding for each peptide was determined by incubation with BSA and was subtracted from the total binding with DQ0602 and DQ0604. Data are the means ± SD of triplicate determinations.
cannot easily overcome. The situation is slightly better with p9Glu
due to the extra methylene group that confers greater ability of the
terminal side chain carboxylate to reach the α76Arg guanidine side
chain. This same aliphatic barrier along with residues β37Tyr and
β61Trp promote van der Waals interactions between the side
chains of these DQ0604 amino acids above and bulk aliphatic and
aromatic p9 residues (Tyr > Leu > Phe > Trp). In particular, p9Tyr is further favored because in its presence the β9Tyr and
β37Tyr residues form hydrogen bonds via their hydroxyl groups to
the hydroxyl group p9Tyr. The presence of p9Phe in DQ0602
precludes the latter possibility. In addition, there are aromatic-ar-
omatic interactions among all of the above aromatic residues and
β30His, in pairs and extended partners. Thus far, the only other
HLA-DQ molecules to accommodate hydrophobic residues in the
p9 pocket, are the DQ2 allelic proteins, DQA1*0501-DQB1*0201
and DQA1*0201-DQB1*0202, which accept Tyr, Phe, and Trp
with equal propensity (31, 38, 39). This is because of the combi-
nation of several unique, smaller, and flexible residues (β30Ser,
β37Leu, β57Ala) at close proximity to and in the p9 pocket.
DQ0604 is the first, β57non-Asp HLA-DQ molecule that does not
tolerate well acidic residues at p9 (30, 38, 40–42). In the three
HLA-DQ molecules that have Ala at β57 and have established peptide-binding motifs, all accept an acidic residue at p9. Our data
suggest that β57Val results in a poor propensity for p9 acidic res-
ides and is supported by the observation that acidic residues are
absent at p9 for DRA*0101-DRB1*0701 and DRA*0101-
DRB1*1201 allelic proteins, two HLA-DR β57Val-positive al-
leles for which a p9 has been identified and preferences examined
(43, 44). The combination of β9Tyr, β30His, and β57Val, which
distinguish DQ0604 from DQ0602 in pocket 9, is found in only four
other HLA-DQ alleles (DQB1*0501, *0608, *0617, and
*0621) (37). It will be interesting to test whether the pocket 9
preferences in these alleles mimic DQ0604 or whether other poly-
morphisms will play a role producing different preferences.

In the first homology models of HLA-DQ alleles, it was hy-
pothesized that pocket 4 is prominent and widely accommodating
(45, 46). The large size has been verified since in the different DQ
allele crystal structures (18, 20, 47). In the DQ0602 crystal struc-
ture, pocket 4 was the largest with a volume of ~100 Å³ (18).
The experiments in this work show that both DQ0602 and DQ0604
can accommodate a number of substitutions well, including the bulky
hydrophobic amino acid Phe; however, there are clear preferences
in that Cys, Pro, Asp, and Arg are not tolerated by either allele.
The difference in residue preferences at p4 (Gly and Asn preferred
during pocket 4) between the two alleles is probably due to the pre-
ence of radically different residues in the vicinity of pocket 4
(β71Arg vs Gly and β30His vs Tyr).

Last, both alleles exhibit the peculiar selectivity at p3, first seen
in the complex of DR3-CLIP and termed “p3 shell” (48). In other
words, even though this position is exposed to solvent, there is
only a select group of residues that can occupy it in both DQ0602
and DQ0604 (Gly > Phe > Leu > Ala). Contrast, of the two
serological DQ2 molecules (DQA1*0501/B1*0201 and
DQA1*0201/B1*0202), only the latter exhibits the p3 shell in not
allowing Pro, His, Arg, and Lys at this position (38). No structural
explanation has been proposed thus far for this phenomenon,
which is worth taking into consideration in the design of allele-
specific HLA-DQ ligands.

The pockets in the peptide-binding groove of DQ0604 are dif-
ferring from those of the two main alleles conferring susceptibility
to T1D, DQA1*0301-B1*0302, and DQA1*0501-B1*0201. Just
among the amino acids that are polymorphic between DQ0602 and
DQ0604, there are obvious differences among DQ0604, DQ0302,
and the DQA1*0501-DQB1*0201 allelic protein (DQ0201) at β86
and β87 in pocket 1, β30 in pocket 6, and β57 in pocket 9 (Table
1). This is reflected in the observed preferences in the peptide-
binding motifs for the three allelic proteins (30, 31, 38–40). Some
of the more obvious differences regarding charged and bulky hy-
drophobic amino acids are that: 1) at p1, DQ0302 and DQ0201 can
tolerate a negatively charged amino acid while DQ0604 does not.
2) At p4 and p7, DQ0201 favors negatively charged amino acids
while DQ0302 and DQ0604 do not. 3) At p9, DQ0302 favors
negatively charged residues and does not tolerate hydrophobic
amino acids, while DQ0201 favors aromatic amino acids (Trp =
Tyr = Phe) and tolerates negatively charged amino acids
(Glu > Asp), and differently still, DQ0604 favors hydrophobic
amino acids (Tyr > Phe > Trp) with low tolerance for negatively
tolerate a negatively charged amino acids. Despite these differences,
overlap in peptide binding specificity between DQ0302 and DQ0201
has been suggested (40, 49). DQ0302 and DQ0201 can bind many
of the same peptide epitopes, although it has not been shown that
they use the same anchor residues. A common motif was proposed for
DQ0302 and DQ0201 based on pool sequencing with the following
preferences: p1, bulky hydrophobic, polar residues; p4, al-
phatic; p6/7, aliphatic, and p9, negatively charged. This common
motif is acceptable for DQ0604 except at p9 where there is a low
tolerance for negatively charged amino acids. Further study of ad-
ditional MHC class II alleles would be helpful in evaluating
whether indeed MHC class II T1D susceptibility alleles exhibit
greater similarity in peptide binding preferences.

The synergy exhibited in certain populations for susceptibility to
T1D by DQ0302 and DQ0604 most likely is not due to formation
of DQA1*0301-DQB1*0604 and DQA1*0102-DQB1*0302
trans-heterodimers. These trans-heterodimers are not expected to
form due to steric incompatibility resulting from structural differ-
ences at e45–53 and β85–90, which were noted when the crystal
structures of DQ0602 and DQ0302 were compared (18). Experi-
mentally, it has been demonstrated that there are allele-specific
restraints that prevent the cell surface expression of certain com-
binations of DQα and DQβ (50). DQA1*0101 does not pair with
DQB1*0201 and DQB1*0302. Similarly, the β-chain that pairs in
cis with DQA1*0101, i.e., DQB1*0501, does not pair with
DQA1*0301, DQA1*0401, and DQA1*0501.

The explanation that we can provide for the contrasting roles of
the DQ0602 and DQ0604 allelic proteins in the pathogenesis of
T1D are a consequence of the differences in antigenic peptide mo-
tifs, αβ heterodimer stability (21, 51), and TCR recognition (22).
The dominant protection associated with DQA1*0102-DQB1*0602
may originate from the unusual αβ heterodimer stability of
DQ0602 (51). This stability has been attributed to the salt bridge
between α76Arg and β57Asp plus an extra hydrogen bond that
forms between β57Asp and the main chain amide nitrogen at p9
(18, 21). In addition, the DQ0602 peptide-binding motif appears
more accommodating than DQ0604 in pockets 1, 4, and 6 (Figs.
4 and 5). As a result, DQ0602 can bind more peptides, as suggested
by the observation that all but one of the DQ0604 selected peptides
bound DQ0602 with almost equal avidity (Table II). This is con-
sistent with our previous study (19) in which DQ0602 was capable
of binding five overlapping insulin peptides better or with equal
avidity compared with seven other HLA-DQ allelic proteins. This
presents the possibility that in vivo, DQ0602 can present a more
diverse peptide repertoire leading to deletion of autoreactive T
cells, immune tolerance, and the denial of these epitopes to T1D-
susceptible HLA-DQ allelic proteins.

In the study presented here, there is one epitope from GAD65,
IA-2, and proinsulin, that of GAD65 339–349, that binds with
high avidity to DQ0604 and very low avidity to DQ0602. How-
ever, the overlapping epitope GAD65 334–344 is bound with high
avidity to DQ0602 and very low avidity to DQ0604. It is thus possible that the antigenic peptide generated during processing contains both overlapping epitopes, in which case DQ0602 probably binds to the majority of the free peptide, denying the epitope to DQ0604. It is particularly noteworthy that HLA-DQ allicic proteins conferring susceptibility to T1D have also overlapping epitopes in the insulin B chain (core nonamer 13–21 for DQ0302 and 6–14 for DQ0604), while the protective allicic protein DQ0602 binds with higher avidity to the same insulin B 6–14 epitope (Refs. 19 and 20; Fig. 3). These two cases support an earlier hypothesis that genetic protection is a result of the higher avidity of DQ0602 for the same peptide (52).

In conclusion, the T1D susceptibility associated with DQ0604 is likely to require an Ag-specific set of interactions to target the pancreatic β cell. Ag-specific interactions are controlled by MHC peptide-binding groove polymorphisms and furthermore by polymorphisms on the surface of the groove such as the β70Arg/Gly polymorphism, which dictates TCR recognition of the MHC class II peptide complexes (22). Human GAD65, IA-2, and proinsulin have been found to colocalize to cells in thymus, spleen, and lymph nodes that express MHC class II (53), suggesting that these Ags will be presented by MHC class II proteins and contribute to the shaping of the T cell repertoire. In this study, we determined a peptide-binding motif for DQ0604 that differs from the DQ0602 motif. Although clearly there is significant overlap, the consequence in vivo is likely a different pancreatic β cell peptide repertoire and subsequent T cell repertoire that is skewed toward recognition of the β cell when DQ0604 is present. It remains therefore to delve deeper to determine the molecular link between MHC class II alleles associated with T1D susceptibility and destruction of the pancreatic β cell by the immune system.

Acknowledgments

We thank Patsy Byers for peptide synthesis, Demetrios Kyriakos and Dr. George Bodinas for laboratory assistance, and Dr. Åke Lernmark for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1998 HLA-DQ6 PEPTIDE-BINDING MOTIFS AND MODELS


