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Critical contribution of β chain residue 57 in peptide binding ability of both HLA-DR and -DQ molecules

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ABSTRACT **Position 57 in the β chain of HLA class II molecules maintains an Asp/non-Asp dimorphism that has been conserved through evolution and is implicated in susceptibility to some autoimmune diseases. The latter effect may be due to the influence of this residue on the ability of class II alleles to bind specific pathogenic peptides. We utilized highly homologous pairs of both DR and DQ alleles that varied at residue 57 to investigate the impact of this dimorphism on binding of model peptides. Using a direct binding assay of biotinylated peptides on whole cells expressing the desired alleles, we report several peptides that bind differentially to the allele pairs depending on the presence or absence of Asp at position 57. Peptides with negatively charged residues at anchor position 9 bind well to alleles not containing Asp at position 57 in the β chain but cannot bind well to homologous Asp-positive alleles. By changing the peptides at the single residue predicted to interact with this position 57, we demonstrate a drastically altered or reversed pattern of binding. Ala analog peptides confirm these interactions and identify a limited set of interaction sites between the bound peptides and the class II molecules. Clarification of the impact of specific class II polymorphisms on generating unique allele-specific peptide binding "repertoires" will aid in our understanding of the development of specific immune responses and HLA-associated diseases.**

HLA class II molecules participate directly in the initiation of a specific immune response by binding peptides and presenting them for recognition by T cells. The extreme degree of polymorphism among class II alleles leads to differential peptide binding, with disparate peptides preferentially bound by separate alleles. Recently, sequence motifs have been identified for peptides bound to HLA-DR molecules, elucidating in particular the importance of the first, fourth, and sixth amino acid residues in the peptide that contact "pockets" formed by the DR molecule's peptide binding groove (1–8). Less is known about DQ binding requirements (9–13).

Some parameters in these sequence motifs seem to be characteristic of all DR molecules, while others are specific for particular alleles (3, 4, 7). For example, the first pocket in all DR molecules encompasses a hydrophobic site suitable for binding uncharged amino acids; thus, most peptides that bind DR molecules possess such a residue as their primary "anchor" in relative position 1 (14, 15). In contrast, polymorphisms among DR alleles create variation in both size and charge characteristics for pockets four and six, such that these sites tend to form the structural basis for discrimination in binding specificity that distinguishes different DR alleles (3, 6, 7). In addition to these three key interaction regions, class II molecules have an evolutionarily conserved dimorphic structure distal to pocket six, which is formed by the presence or absence of Asp at residue 57 of the DR or DQ β chain. In the HLA-DR1 crystallographic-derived structure, the Asp-57 residue forms a salt bridge with Arg-76 of the DR α chain, located at one end of the peptide binding groove (16). To evaluate the contribution of this site to specific peptide binding and to establish peptide motifs that are influenced by the codon 57 polymorphism, we have analyzed antigenic peptide motifs discriminated by highly homologous class II alleles that differ at codon 57. We report herein that the Asp/non-Asp dimorphism in both DR and DQ alleles plays a major role in determining preferences for binding particular peptides. By substituting a single residue at the appropriate location in the peptide that binds to the pocket influenced by this dimorphism, we can drastically alter or reverse the relative binding preferences of the alleles. In spite of general similarities in requirements for peptide binding motifs, each allele binds an overlapping but distinct set of peptides, with a resultant unique potential immune repertoire. This may provide a structural basis for some cases where very similar alleles are discordant for conferring susceptibility to autoimmune disease.

MATERIALS AND METHODS

Cell Lines and Antibodies. Homozygous B-lymphoblastoid cell lines used include Sweig (DRB11101), Madura (DRB1*0801), Priess (DRB1*0401, DQB1*0302), BSM (DRB1*0401, DQB1*0302), and HID (DQB1*0303), all described in the VI–XI International Histocompatibility Workshops. The class II-deficient BLS-1 cell line was a gift from Janet Lee (Memorial Sloan–Kettering Cancer Center, New York). Monoclonal antibodies include the anti-DR L243 (purchased from the American Type Culture Collection) and the anti-DQ SPVL3 (provided by Hans Yssel, DNAX).

Peptides. Peptides were synthesized using an Applied Biosystems model 432 peptide synthesizer and biotinylated as described (13).

Peptide Binding Assay. Whole cell peptide binding assays were performed essentially as described (13). Briefly, 10^6 B-lymphoblastoid cells per sample were fixed in 0.5% paraformaldehyde, washed extensively, and incubated with 10 μM of the appropriate biotinylated peptide for 18–24 h at pH 5.4. After cells were lysed in a buffer containing 0.5% Nonidet P-40, 0.15 M NaCl, 50 mM Tris·HCl (pH 8.0), and protease inhibitors, the lysates were cleared by centrifugation, transferred to 96-well plates precoated with the appropriate monoclonal antibody (L243 for capture of DR, SPVL3 for DQ) at 10–12.5 μg/ml and incubated overnight at 4°C. In DQ experiments, the buffer used in the plates contained 0.02% dodecyl β-D-maltoside (13); for DR binding experiments, the buffer was modified to 0.75% *n*-octyl β-D-glucopyranoside in 50 mM Tris·HCl (pH 7.15). Europium-labeled streptavidin (Wallac, Gaithersburg, MD) was added for 4 h, followed by enhancement buffer (Wallac) for 1 h at room temperature, and fluorescence was measured in a Delfia 1232 fluorometer.

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RESULTS

Differential Binding of Two Peptides to DRB1*1101 and *0801. HLA-DRB1*1101 (DR5) and *0801 (DR8) are two highly homologous alleles that vary at only six positions (Fig. 1). Juxtaposition of these polymorphic positions on the HLA-DR1 class II three-dimensional structure indicates that the most radical changes likely to affect binding pockets occur at residues 57 and 58, where Asp-Glu in *1101 becomes Ser-Ala in *0801; these changes would be expected to alter pocket 9. Testing synthetic peptides for binding to both alleles based on published motifs for binding to *1101 (3), we found examples of peptides that bind to one of these alleles preferentially: peptide SK-273 (EKYYVLKKGEEP), derived from residues 273–284 of the streptokinase protein from *Streptococcus pyogenes*, binds strongly to the *0801 allele but poorly to *1101, while peptide Parvo-58 (SLFDLVARIKNNL), derived from residues 58–70 of a human parvovirus B19 product, binds much better to *1101 than to *0801 (Fig. 2). Binding to the more distantly related *0401 (DR4) allele (10 amino acid differences compared with *1101) is included as a negative control. As described by others (3), there are major differences in pockets 4 and 6 for the *0401 binding motif compared with that of *1101.

Both peptides SK-273 and Parvo-58 contain residues compatible with binding *1101 and *0801 in an extended conformation, analogous to the binding of the HA peptide to DR1 determined by crystallography (14). Predicted anchor residues Tyr, Leu, and Lys for SK-273 and Phe, Val, and Arg for Parvo-58 correspond to pockets 1, 4, and 6, respectively, in the class II molecule. Distal to these homologous residues, however, the two peptides carry differences that correlate with their patterns of binding: the peptide with Glu at the ninth position (relative to Tyr as the first anchor position) (SK-273) bound *0801 better than *1101; the peptide with Asn in that position (Parvo-58) bound *1101 better than *0801. This pattern correlates with the structural difference in the class II molecules at positions 57 and 58. A negatively charged Asp at position 57 forms a salt bridge with the positively charged Arg at position 76 in the DR α chain (16). In contrast, a non-Asp at position 57, such as the Ser in *0801, leaves an unopposed positive charge from the Arg; alleles in this category may facilitate the binding of negatively charged residues in the peptide at position 9 relative to anchor position 1. This is consistent with the findings shown in Fig. 2, where the SK-273 peptide, which binds well to the *0801 allele, has a negatively charged Glu at position 9, and the Parvo-58 peptide, which binds better to *1101, has an uncharged Asn in that position.

Anchor Position 9 Is Responsible for Discrimination of These Peptides by *1101 and *0801. We next synthesized variant peptides altering only the ninth residue: SK-273-9A, which substitutes an Ala for the Glu at position 9 (EKYYV-LKKGE*A*P), and Parvo-58-9D, which substitutes an Asp for the Asn at position 9 (SLFDLVARIK*D*NL). These were tested on the same cell lines, and, as predicted, in both cases the changes now allow strong binding of the altered peptides by the alleles previously not binding well (Fig. 3). Fig. 3 *Upper* demonstrates that the original Parvo-58 peptide (with an Asn at position 9) binds to the *1101 allele dramatically better than to *0801, while Parvo-58-9D exactly reverses the pattern. In Fig. 3 *Lower*, peptide SK-273 binds poorly to allele *1101 and

DRB1 allele:	Polymorphic amino acid position: 13 16 47 57 58 74
*1101	S H D D E A
*0801	G Y Y S A L

FIG. 1. Amino acid polymorphism differences between DRB1*1101 and *0801.

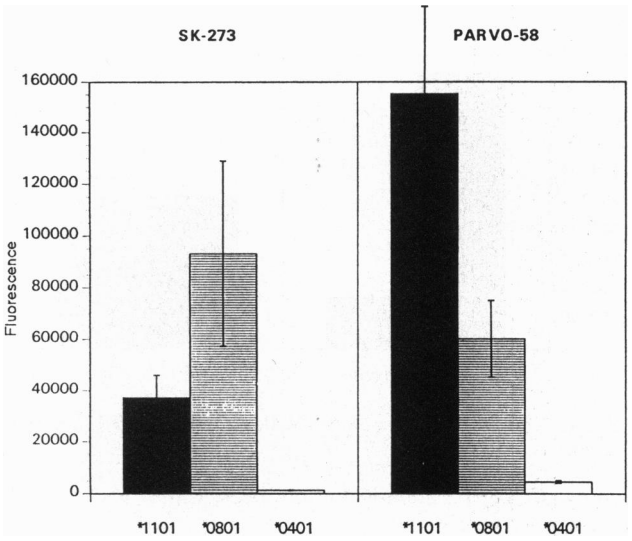

FIG. 2. Binding of 10 μM peptides SK-273 (EKYYVLKKGEEP) and Parvo-58 (SLFDLVARIKNNL) (presumed anchor positions 1, 4, 6, and 9 are in boldface type) to DR molecules from cell lines expressing DRB1*1101 (Sweig), *0801 (Madura), and *0401 (Priess) at pH 5.4. Monoclonal antibody L243 was used to capture DR molecules.

well to *0801. Mutating position 9 from Glu to Ala results in a striking increase in binding to *1101 and continued good binding to *0801.

Analog Peptides Confirm Predicted Anchor Positions. To confirm our interpretation of which residues are acting as anchors, we tested Ala analogs corresponding to these peptides. We synthesized peptides repeating the predicted anchors at positions 1, 4, 6, and 9, with all other residues being Ala. Peptide FVRN represents the Parvo-58 peptide with the sequence AAFAAVARAANAA, and peptide FVRD corresponds to Parvo-58-9D (AAFAAVARAADAA). These were tested on the *1101 and *0801 cell lines, and the results are shown in Fig. 4. Binding of FVRN replicates that of Parvo-58, with much better binding by the *1101 allele compared with *0801. When the ninth residue is changed to Asp in FVRD,

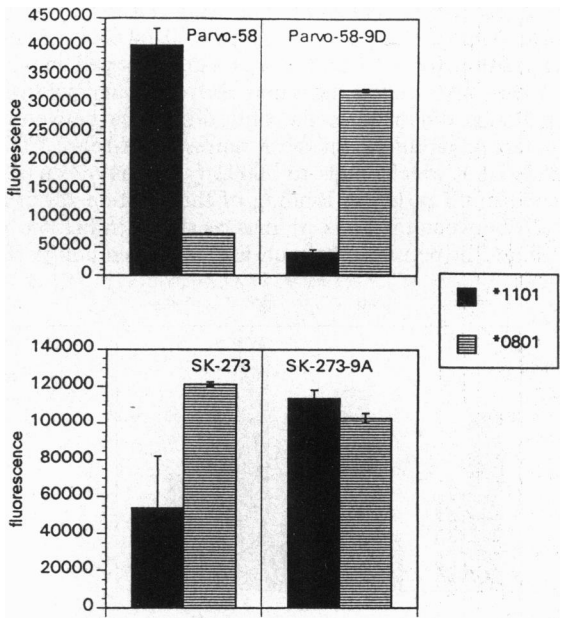

FIG. 3. Peptide binding of substituted peptides to DR molecules as described in Fig. 2.

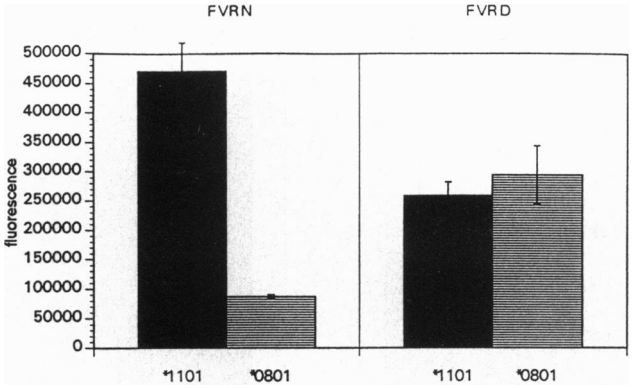

FIG. 4. Binding of Parvo-58 Ala analog peptides to DR molecules as in Fig. 2.

binding of *0801 dramatically improves, while that of *1101 decreases by nearly half.

Extension of these studies using related Ala analog peptides is summarized in Fig. 5. We had available a series of peptides similar to those described above, except that Ile rather than Val was the anchor at position 4 (a conservative change). Into this backbone amino acids of differing charge characteristics were substituted at the ninth anchor position. Thus we have peptides FIRS (AAFAAIARAASAA), FIRE (AAFAAIARAAE-AA), FIRD (AAFAAIAARAADAA), and FIRK (AAF-AAIARAAKAA); all were tested for binding to the *1101 and *0801 alleles. FIRS, with an uncharged Ser at position 9, binds better to *1101 than to *0801, analogous to FVRN above. When the Ser is replaced by either residue with a negative charge, Glu or Asp, the binding pattern is reversed, with *0801 binding extremely well. Finally, substitution of the ninth residue with the positively charged Lys again dramatically reduces binding to *0801, while allowing binding to *1101.

The Asp/Non-Asp Dimorphism Similarly Influences Peptide Binding to Homologous DQ Alleles. A parallel Asp/non-Asp dimorphism at position 57 occurs in HLA-DQB alleles as well as in DRB1 alleles. We investigated the hypothesis that peptide characteristics similar to those shown for DR would also hold for differential binding to DQ molecules varying at this position. To test this, we utilized two highly homologous DQB1 alleles, *0302 (DQ3.2) and *0303 (DQ3.3), that have identical sequences except for codon 57: *0302 encodes an Ala at that position, while *0303 encodes Asp. In the cell lines used, both alleles also utilize the same DQ α chain, making the Asp/non-Asp dimorphism the only difference between the molecules. A peptide from the λ-repressor (λ-rep, LEDAR-RLKAIYEK), which binds to *0302 (13), has a Glu at the predicted ninth position. Binding of this peptide varies dramatically between the two cell lines, as shown in Fig. 6 *Upper*: *0302 binds the peptide extremely well, while binding by *0303

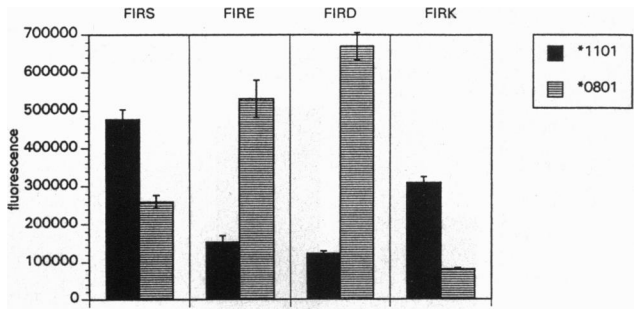

FIG. 5. Influence of variation at anchor position 9 for binding of a series of anchored Ala analog peptides to DR molecules, as in Fig. 2.

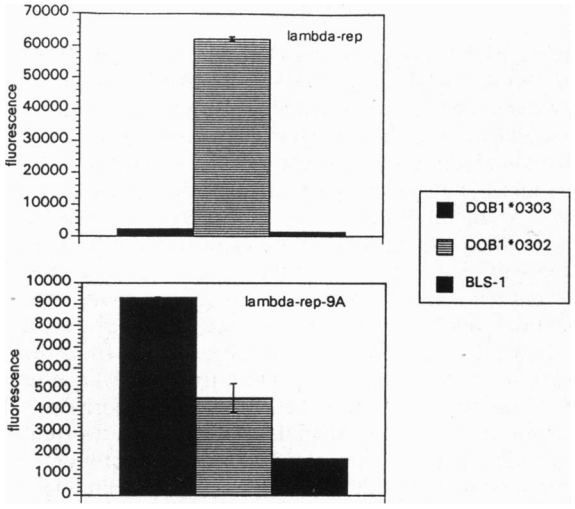

FIG. 6. Binding of wild-type and substituted λ-repressor peptides (10 μM) to cell lines expressing DQB1*0303 (HID),*0302 (Priess), or no class II (BLS-1) at pH 5.5. Monoclonal antibody SPVL3 was used to capture DQ molecules.

is barely detectable. Both of the cell lines used are also homozygous for DRB1*0401, which does not bind the λ-rep peptide (data not shown). BLS-1 is a class II-negative control cell line (17).

We next generated a substituted λ-rep peptide to confirm the hypothesized influence of the Asp/non-Asp polymorphism on binding. λ-rep-9A replaces the negatively charged Glu at position 9 with an uncharged Ala (LEDARRLKAIY*A*K). Those binding studies are shown in Fig. 6 *Lower*. As predicted, binding of the *0303 allele increases and the *0302 allele decreases, paralleling the situation with the DR alleles.

Finally, we created another pair of peptides, varying only at position 9, predicted to bind to *0302 or *0303. In this case we synthesized the peptide ILLE (AAIAALKLAAEAA) with Glu in the ninth position, and the peptide ILLA (AAIAALK-LAAAAA), identical except for an Ala substituted at position 9. As shown in Fig. 7, these too behave as predicted: the peptide with a negative charge at position 9 (ILLE) binds better to the allele lacking Asp-57, *0302, while the homologous peptide with a neutral residue at position 9 (ILLA) has the reverse preference and binds much better to the *0303 allele, which carries the Asp at position 57. These peptides with different anchors further substantiate the importance of this dimorphism in allele-specifc peptide binding preferences for DQ as well as DR molecules.

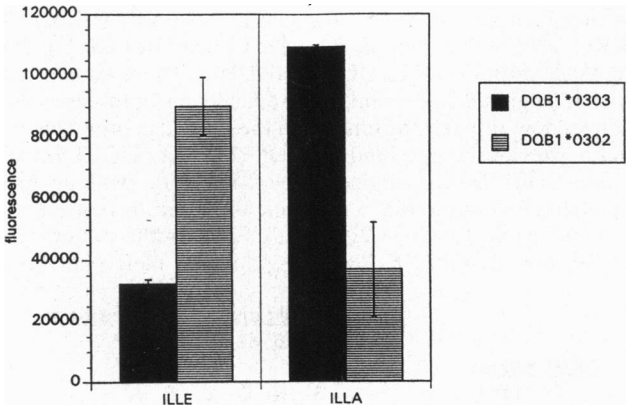

FIG. 7. Binding of 10 μM of synthetic peptides ILLE and ILLA to cell lines expressing DQB1*0303 (HID) and *0302 (BSM) at pH 5.5, performed as in Fig. 6.

DISCUSSION

Much interest has been focused recently on requirements for HLA class II and peptide interactions. A precise understanding of binding characteristics for specific class II alleles will have major implications for our ability to identify pathogenic antigens in HLA-associated autoimmune diseases, to design specific peptides for immunotherapy, and to design effective vaccines. The solution of the crystal structure of a DR molecule (16) and especially of a DR molecule binding a known peptide (14) have greatly enhanced our understanding of the factors governing how peptides are bound by class II molecules. In addition, a growing number of direct peptide binding studies have begun to define peptide characteristics that allow, enhance, or prevent binding (1–6). For DR molecules in particular, much has been learned. For example, an important role has been established for hydrogen bonding interactions between the class II amino acid residues and the peptide's backbone. The importance of the first binding pocket in the DR peptide binding groove, which is primarily contributed by the monomorphic DR chain and preferentially interacts with hydrophobic residues on the peptide, has also been demonstrated. These both represent major influences on peptide interactions with DR molecules in general and contribute to the ability of many peptides to bind promiscuously to many or all DR alleles.

More detailed studies have begun to elucidate peptide characteristics that allow preferential binding to specific alleles. For example, using random peptide display libraries bound to purified class II molecules, Sinigaglia and colleagues (3) have described different binding motifs for three DR alleles, DRB1*0101, *0401, and *1101. Of note, residues in the peptide's sixth anchor position seem to determine specificity of binding among the alleles: *0101 prefers Ala or Gly in this position, *0401 prefers Thr or Ser, and *1101 prefers Arg or Lys. Others have eluted and sequenced peptides from purified class II alleles and attempted to align them to deduce allele-specific motifs (4, 18–20). These types of studies are leading to consensus motifs that differ among the major DR alleles (21). Consensus motifs for DQ alleles are also being investigated. Thus far, it appears that each dimer has unique binding characteristics, probably because DQ α as well as DQ β alleles are polymorphic (9–13).

Within DR and DQ products, differences in peptide binding "repertoire" occur even among highly homologous alleles and may play a functional role in conferring susceptibility to certain autoimmune diseases. For example, DRB1*0401 is highly associated with rheumatoid arthritis, while the homologous *0403 is not. Likewise, DQB1*0302 is strongly correlated with insulin-dependent diabetes mellitus, while *0301 is not. Relatively little is known about the more subtle differences between similar alleles that could lead to critical variation in the unique pattern of peptides preferred by each.

We focus herein on one such polymorphism, an Asp/non-Asp dimorphism found at position 57 of the class II β chain. This seemed likely to have functional implications, based on the facts that the dimorphism is implicated in susceptibility to insulin-dependent diabetes mellitus (22, 23) and that it has been conserved through evolution. According to the crystal structure studies (14, 16), residue 57 lies on the β chain's α-helix at one end of the antigen binding groove, where it forms an ion pair with an Arg present at position 76 on the DR α chain (homologous to position 79 on the DQ α chain), forming a salt bridge (16). As position 57 appears to contribute to the ninth peptide binding pocket, one can envision two effects that could be altered depending on which residue occurs there: (*i*) the presence of a salt bridge could conceivably narrow that end of the groove, favoring smaller amino acid side chains in the peptide at that pocket and (*ii*) the absence of an Asp at that position would leave an unopposed positively charged Arg from the α chain, such that complementary negatively charged residues in the peptide residue interacting at that position would be preferred.

We chose to evaluate two pairs of highly homologous alleles, DRB1*1101 and *0801 and DQB1*0302 and *0303. While little is known of the binding motif for *0801, superimposing its sequence on the DR1 three-dimensional structure suggests that the sixth pocket, the one suggested by Sinigaglia's group (3) to be important in major differences among DR alleles, is identical to that of *1101, thus allowing the Ser → Asp substitution at position 57 to be analyzed fairly independently. Similarly, the natural occurrence of DQB1 *0302 and *0303 allows analysis of two DQ molecules that vary only at position 57. We utilized model peptides that bound the two alleles of each pair differentially and reversed this binding pattern by altering only the residue in the peptide predicted to occupy the ninth pocket, in contact with β57. In fact, we could drastically alter the level of binding of each peptide solely by varying the charge at residue 9. Peptides containing a negative charge at that position bind well to class II molecules with a non-Asp residue at position 57 but not to those with Asp. Uncharged residues at peptide position 9 led to improved binding to Asp-57-positive class II molecules. These results are consistent with the hypothesized effect of a salt bridge at the end of the class II binding groove, as discussed above.

Others have noted the importance of this dimorphism. Wucherpfenning and Strominger (24) suggested that β57 is one of two key polymorphisms that may be important in determining peptide binding in representative autoimmune diseases. Marshall *et al.* (7) addressed the role of β57 as well as other polymorphic sites in peptide binding to a series of DR alleles using competition assays and concluded that the charge and size of the peptide's ninth anchor position were important factors in allele-specific binding. We now extend these studies using direct binding assays, including alleles, to our knowledge, not previously reported and describe an analogous site in DQ alleles as well.

The pivotal ability of the codon 57 dimorphism to determine whether or not a particular peptide will be bound can be influenced by other positions as well. This was evident in the different levels of binding seen with Ala analogs compared with the model peptides. For example, the Parvo-9D peptide (Fig. 3) binds to the DR*1101 allele very poorly, while its Ala analog FVRD (Fig. 4) binds significantly better, even though the overall patterns of binding relative to *0801 are retained. This is likely due to the combined effects of residues other than the major anchors, some of which may decrease affinity. In contrast, Ala residues appear to be permissive for binding in most positions (25).

Thus, the structural determinants of class II-peptide binding are a complex set of permissive and nonpermissive interactions in which a few key elements, notably the β57 dimorphism, dictate site-specific constraints.

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