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# Structural analysis of two HLA-DR-presented autoantigenic epitopes: crucial role of peripheral but not central peptide residues for T-cell receptor recognition

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**Abstract**

Specific and major histocompatibility complex (MHC)-restricted T-cell recognition of antigenic peptides is based on interactions of the T-cell receptor (TCR) with the MHC alpha helices and solvent exposed peptide residues termed TCR contacts. In the case of MHC class II-presented peptides, the latter are located in the positions p2/3, p5 and p7/8 between MHC anchor residues. For numerous epitopes, peptide substitution studies have identified the central residue p5 as primary TCR contact characterized by very low permissiveness for peptide substitution, while the more peripheral positions generally represent auxiliary TCR contacts. In structural studies of TCR/peptide/MHC complexes, this has been shown to be due to intimate contact between the TCR complementarity determining region (CDR) three loops and the central peptide residue. We asked whether this model also applied to two HLA-DR presented epitopes derived from an antigen targeted in type 1 diabetes. Large panels of epitope variants with mainly conservative single substitutions were tested for human leukocyte antigen (HLA) class II binding affinity and T cell stimulation. Both epitopes bind with high affinity to the presenting HLA-DR molecules. However, in striking contrast to the standard distribution of TCR contacts, recognition of the central p5 residue displayed high permissiveness even for non-conservative substitutions, while the more peripheral p2 and p8 TCR contacts showed very low permissiveness for substitution. This suggests that intimate TCR interaction with the central peptide residue is not always required for specific antigen recognition and can be compensated by interactions with positions normally acting as auxiliary contacts. 

*Keywords:* Antigen presentation; Peptides/epitopes; Autoimmunity; Major histocompatibility complex; T-cell receptors

## 1. Introduction

T-cell mediated specific immunity is based on recognition of peptide-complexed major histocompatibility complex (MHC) molecules by specific T-cell receptors (TCRs). TCR recognition of MHC-presented peptides displays flexibility, allowing for thymic positive selection by low-affinity interactions with self peptide/MHC complexes, high frequency responses to foreign MHC, as well as productive T cell responses to non-self peptides displayed by self MHC in the periphery (Kersh and Allen, 1996a). Molecular understanding of these events has been advanced greatly by elucidation of the

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three-dimensional structure of MHC/peptide complexes and TCR molecules (Garboczi and Biddison, 1999).

MHC class II molecules accommodate peptide ligands in a groove-like binding site formed by two parallel alpha helices on a cushion of beta sheets (Brown et al., 1993). The peptide, whose ends project out of the binding site, is twisted in an extended polyproline type II-like conformation so that successive side chains point toward pockets in the binding site, or solvent (Jardetzky et al., 1996; Stern et al., 1994). Typically, five main pockets whose size, hydrophobicity and charge varies in an allele-specific manner, accommodate the so-called human leukocyte antigen (HLA)-anchor residues. Peptide positions p1 and p9 are completely buried while positions p4, p6 and p7 fit in their respective pockets, yet are to varying extents exposed to solvent and thus also potential TCR contacts (Dessen et al., 1997; Fremont et al., 1998; Ghosh et al., 1995; Murthy and Stern, 1997; Scott et al., 1998; Stern et al., 1994). The fitting of these residues into the respective pockets provides the necessary specificity for binding, yielding affinities in nM range. The other peptide residues, i.e. p2, p3, p5, p8 and residues beyond the binding core formed by residues p1–p9, are solvent-, and therefore, TCR-accessible. Additional affinity for peptide binding to murine and human MHC class II molecules is provided by multiple hydrogen bonds between the peptide backbone and strategically placed polar conserved MHC residues.

Crystallographic studies have demonstrated a conserved mode of TCR recognition of MHC class I/peptide complexes (Garboczi et al., 1996; Ding et al., 1998; Garcia et al., 1996; Teng et al., 1998) which has been proposed to be generally applicable to TCR recognition of MHC class I and class II/peptide complexes (Ding et al., 1998; Garboczi and Biddison, 1999). This view is supported by indirect experimental evidence (Sant'Angelo et al., 1996) but challenged by other studies (Sim et al., 1997). The relatively flat TCR interface contacts the MHC class I/peptide complex in a diagonal fashion dictated by elevations on opposed ends of the MHC alpha helices. This interface is formed by three loops from each of the TCR alpha and beta chains which correspond to the three highly variable complementarity-determining regions (CDR). While the CDR1 loops are located over the peptide N-terminus (CDRlα) and C-terminus (CDR1β), respectively, and the CDR2 loops contact the MHC alpha helices, the two CDR3 loops form a pocket that can accommodate and interact with the side chain projecting upward from the central peptide residue (Ding et al., 1998; Garboczi et al., 1996; Garcia et al., 1996). Recently, the structure of a TCR/peptide/MHC class II complex has been published (Reinherz et al., 1999). Somewhat surprisingly, this study revealed an almost orthogonal arrangement between TCR and MHC/peptide axes, and a largely dominant role of the TCR alpha chain in recognition of MHC/peptide ligand.

Based on the extensive interactions between the CDR3 loops and the central exposed residue in a non-self peptide antigen, the latter residue has been postulated to act as a lynchpin in the peptide/TCR interface, ensuring specific T-cell activation by increasing binding affinity (Garboczi and Biddison, 1999). This hypothesis, which is compatible with recent data on TCR/peptide/MHC class II structure (Reinherz et al., 1999), assigns the strongest contribution to the binding energy in the TCR/peptide interface to the most variable moiety of the TCR and is thereby consistent with the requirement of highly specific antigen recognition by T cells. The hypothesis is also consistent with the finding that substitution of the corresponding central residue in MHC class II-presented peptides tends to have the most deleterious effects on T-cell recognition (Kersh and Allen, 1996a). Taken together, these observations suggest that specific peptide recognition relies to a significant extent on the highly variable CDR3 sequences (Garcia and Teyton, 1998). In contrast, the germline-encoded CDR1 and 2 sequences are thought to play a dominant role in the recognition of conserved and allelic MHC features, thereby ensuring correct orientation of the TCR and MHC restriction of antigen-specific responses, but possibly also allo-responses and positive selection (Jones et al., 1998; Speir et al., 1998). In the case of a TCR specific for a peptide/allo-MHC class I complex, CDR1 and 2 loops have recently been shown to contribute more than 65% of binding energy (Manning et al., 1998). The energetic contribution of individual CDRs to recognition of non-self-peptide/self-MHC complexes remains to be established.

Given the limited amount of crystallographic data on TCR/peptide/MHC complexes, it is important to subject the issue of TCR recognition of MHC class II-presented peptides to further examination. Although definite answers can only be expected from further crystallographic studies, analysis of T-cell recognition of substituted peptides can be highly instrumental in their absence. In such studies, individual peptide positions are identified as HLA anchors when substitution results in decreased binding affinity, while loss of T-cell stimulation by substitutions without much of an effect on binding affinity characterizes TCR contacts (Kersh and Allen, 1996a). However, cases have been recorded where conservative substitutions at anchor positions that did not affect binding, caused a considerable shift in the dose-response curve of the cognate T-cell clone, and sometimes an abolition of the response altogether (Evavold et al., 1993; Kersh and Allen, 1996b; Reay et al., 1994). Among TCR contacts, complete lack of permissiveness for conservative substitution is a hallmark of principal contacts, while moderate permissiveness identifies auxiliary TCR contacts.

We set out to identify HLA anchor and TCR contact residues and determine their permissiveness for substitution for HLA-DR-presented epitopes contained in a type 1 diabetes autoantigen. Using T-cell proliferation and HLA-binding assays with a large number of substituted epitope analogues, we find an – in one case striking – absence of the dominant role of the p5 residue as principal TCR contact. The potential significance of this finding with respect to the selection of the autoantigen-specific T-cell repertoire is discussed.

## 2. Materials and methods

### 2.1. T cells

T cells were derived by repeated stimulation of PBMC from two type 1 diabetes patients with baculovirus-expressed purified 65 kDa glutamic acid decarboxylase (GAD65) and have been described previously (Bach et al., 1997). Line A recognized peptide GAD88-99 in the context of HLA-DRA/DRB1*0101 complexes, while line B recognized epitope GAD248-57 in the context of DRA/DRB5*0101 (Bach et al., 1997). Both lines were $CD3^+CD4^+$, secreted IFN-$\gamma$ upon antigenic stimulation and started showing proliferative responses to antigen at concentrations of $\approx 1$ nM.

For the purpose of this study, both lines were cloned by limiting dilution. Clonality of obtained T cells was analyzed by "immunoscope analysis" as described previously (Manfras et al., 1997). Briefly, first strand T-cell cDNA was amplified using a panel of TCRAV and TCRBV-family specific primer pairs, followed by high resolution "spectratyping" for CDR3 length of each obtained PCR product. In the case of line A, all T-cell populations (including Al used for the experiments) responding to antigen that were obtained by limiting dilution appeared to be biclonal. Line A was cloned three times, using seeding densities of 0.3–10 cells/well. Cloning efficiency was very low (below 5%), and all tested populations were found to be at least biclonal. In a representative experiment, 70% of TCR transcripts were TCRBV1.12, 60% TCRAV2, 36% TCRAV20, and 23% TCRVB6.1-9; similar proportions of TCRBV transcripts were found in another experiment with Al cells and in two independent line A-derived subpopulations. T cell B18 derived from line B was found to be monoclonal and expressed TCRBV2.123 and TCRVA22 (in addition to a second out-of-frame TCRVA6 transcript).

### 2.2. Proliferation assays

T-cell stimulation by epitope variants was tested in standard proliferation assays. Briefly, 5000 T cells were cultured in the presence of peptide antigen and 5000 irradiated (8000 rad) APCs in round bottom 96 well plates and in RPMI 1640 medium supplemented with non-essential amino acids, $5 \times 10^{-5}$ M 2-ME and 5% delipidated pooled human AB serum. After 48 h, 1 μCi/well of $^3$H-thymidine was added, and cells were incubated for another 16 h before harvesting and counting. Homozygous B cell lines Jesthom (DRA/B1*0101-DQA1*0101/B1*0501), and EA (DRA/B1*1501-DRA/B5*0101-DQA1*0102/B1*0602) were used as APCs. All peptides (obtained in $\geqslant 95\%$ purity and verified by mass spectrometry (Bach et al., 1998)) were tested in triplicates in four concentrations: 5000, 1000, 200 and 40 nM.

### 2.3. HLA-DR-binding assays

Peptide binding affinity for restricting HLA-DR molecules was measured in two competitive binding assays: a cellular assay recently developed in our laboratory for the DRB5-binding epitope and its variants (Harfouch-Hammoud et al., 1999), and an in vitro assay (30) using purified empty DR1 molecules for the other epitope. The former assay measures the binding of aminoterminally biotinylated reporter peptides to HLA-DR molecules on paraformaldehyde-fixed B cells or L cell transfectants in the presence of competitor test peptides. A transfected murine LTK fibroblast line (DAP3-DR2a, obtained from NCBR, Genova, Italy) was used to measure peptide binding to DRA/B5*0101. Peptide 83-97 from myelin basic protein (MBP), used as reporter peptide, was biotinylated after synthesis of the sequence on the resin by first building up an εLys-βAla-εAha-βAla spacer and then coupling in five-fold D-biotin molar excess. We have previously shown that untransfected fibroblasts do not bind this reporter peptide, and that DRB5*0101-transfected fibroblasts do not bind other reporter peptides with high binding affinity for unrelated HLA class II molecules (Harfouch-Hammoud et al., 1999).

In cellular binding assays, cells ($2 \times 10^6$ per sample) were fixed in 0.5% paraformaldehyde, washed and incubated for 2 h in 150 mM citrate–phosphate buffer (pH 4.4) at 37°C in the presence of 3 μM reporter peptide MBP83-97 and increasing concentrations (0.1–300 μM) of test competitor peptides. Then cells were washed to remove unbound peptides, lysed in 100 μl of a Tris/NaCl buffer (pH 7.4) containing 0.5% Nonidet-P40 and protease inhibitors. Cleared lysates were transferred to ELISA plates coated with HLA-DR-specific mAb L243 (hybridoma obtained from H. Coppin, INSERM U100, Toulouse, France) and blocked with PBS/2% BSA. After addition of 100 μl of 50 mM Tris (pH 7.15) containing 0.02% dodecyl β-D-maltoside, lysates were incubated overnight at 4°C. Plates were washed extensively with PBS-0.02% Tween 20, incubated 4 h at 4°C with europium-streptavidin (Wallac, Gaithersburg, MD), washed again, and incubated for 5 min with

enhancement buffer (Wallac). Finally, fluorescence was measured in a Delfia 1232 fluorometer (Wallac). The concentration corresponding to 50% inhibition of reporter peptide binding ($IC_{50}$) was determined graphically by plotting fluorescent signals in the presence of increasing competitor concentrations. All experimental measurements were performed in duplicates, and values represent the mean of at least two, and generally three independent experiments.

Dissociation constants for the DR1 binding peptide and its variants were estimated using empty HLA-DR1 produced in insect cells (Stern and Wiley, 1992) in an in vitro competition assay essentially as described (Sato et al., 2000). Peptide concentration was measured by absorbance using HPLC-UV detection (at 300 nm for peptides containing Trp, 280 nm for Tyr, 254 nm for Phe). Serial dilutions of peptide ($10^{-12}$–$10^{-5}$ M) were incubated with 10 nM biotinylated reporter peptide influenza hemagglutinin (HA) 306-318 and 10 nM empty HLA-DR1 for 3 days at 37°C. Peptide complexes were recovered in microtiter wells coated with anti-HLA-DR1 antibody LB3.1, and quantified using europium-streptavidin as described above. $IC_{50}$ values were determined by fitting to an hyperbolic equation describing simple competitive binding, and were converted to $K_d$ values using the equation $K_d = IC_{50}/[1 + [BioHA]/Kd,bioHA)]$, with the dissociation constant of the biotinylated probe peptide Kd,bioHA previously determined to be 14 nM.

Binding of substituted variants of epitope GAD88-99 to DR1 was also studied with the cellular binding assay described above, using the $DR1^+$ B-cell line Jesthom and biotinylated reporter peptide HA306-318 (not shown). For almost all test peptides, results obtained with the cellular assay corresponded to those observed with purified DR1. However, one peptide with high stimulatory capacity for T cells did not have measurable binding affinity in the cellular assay. As this assay is prone to interference from cellular proteases and also requires high reporter peptide concentrations, suggesting partial peptide consumption by processes other than binding to HLA class II, results obtained with purified HLA-DR are likely to be more reliable and therefore shown for DR1.

### 2.4. Modeling

Modeling of the GAD88-99 peptide as well as of some of its variants into the antigen-binding groove of DRA/B1*0101 was accomplished using the QUANTA-CHARMm simulation program and force field (Molecular Simulations, San Diego, CA). Briefly the structure of the complex of DR1 and HA306-318 (Stern et al., 1994) was subjected to energy minimization, where very little deviation was observed between the crystal and the minimized structures of DR1 and the HA306-318 peptide. This indicated that the crystal structure reported had no strained conformational parameters and that the force field used is adjusted to the observed protein conformation, introducing no shifts to observed crystallographic 3D models. Thus it is an excellent starting point for minimizations with other antigenic peptides. In all our simulations, a layer of water 10 Å thick was incorporated into the DR1-HA306-18 complex around the binding site, as it is known from other published structures of MHC molecules that water mediates several peptide–MHC interactions (Fremont et al., 1992; Murthy and Stern, 1997; Fremont et al., 1998). Omission of this layer of water molecules gave very different results, that were not in agreement with binding experiments (Papandreou, van Endert et al., unpublished results). Thereafter, the proper residues corresponding to the GAD88-99 peptide and its variants were substituted at specific positions, keeping the antigenic backbone immobile, and subsequently the new complexes were subjected to energy minimization. In order to take account of the fact that the force field used in the calculations of the minimized energy is an empirical one, and thus the values for various physical parameters would not be absolutely related to the actual values, we used a constant numerical factor $f$; which when multiplied by the calculated value of the free energy of peptide–DR1 interaction would yield the true value for this process. This factor was obtained by minimizing the root-mean-square differences between the calculated and the experimentally obtained values for the free energy of interaction (Papandreou, Bliopoulos, Stem, van Endert, and Papadopoulos, unpublished results). Graphical representations of the resulting molecules were performed on a Silicon Graphics Indigo workstation using the QUANTA-CHARMm program and force field.

## 3. Results

We chose two epitopes from GAD65 (GAD88-99 and 248-57) as models to study structural features of type 1 diabetes autoantigen recognition by HLA-DR-restricted human T cells. T cells recognizing these epitopes (Bach et al., 1997) had been derived by stimulation with recombinant GAD65 protein of PBMC from two diabetes patients. For this study, T cells were cloned and subjected to "immunoscope" analysis of Vα and Vβ element usage (Manfras et al., 1997). This showed monoclonality for clone B18 recognizing epitope GAD248-57. However, we have been unable to generate monoclonal T cells recognizing GAD88-99; several independently cloned populations, including Al used for this study, were found to contain the same two Vα and two Vβ elements in a ratio of approximately 7:3 (major Vα/β to minor Vα/β).

In a previous study, we had determined HLA restriction, minimal peptide length and crucial positions for efficient T-cell stimulation by the two epitopes (Bach et al., 1997). GAD88-99 is restricted by HLA-DRA/B1*0101 and contains nine positions whose substitution by Ala or Glu affects T-cell stimulation, while GAD248-57 is restricted by HLA-DRA/B5*0101 and comprises eight positions crucial for T-cell stimulation (Fig. 1). Both epitope sequences can easily be aligned to published binding preferences for the restricting HLA-DR molecules (Rammensee et al., 1995), as shown in Fig. 1. GAD88-99 contains optimal HLA anchor residues in each of the positions p1, p4, p6 and p9. Indeed, in competitive binding assays (see below), this peptide binds to DRB1*0101 with slightly higher affinity ($K_d = 7$ nM) than HA306-318, a peptide frequently used as experimental DR1 ligand and also crystallized with it (De Magistris et al., 1992; Stern et al., 1994). Although the requirements for peptide binding to DRB5*010l have not been studied extensively, peptide GAD248-57 matches the known criteria fully (Fig. 1) and has a significantly higher binding affinity than reference peptide MBP84-102 (Wucherpfennig et al., 1994; Bach et al., 1997). Moreover, in a comprehensive analysis of HLA class II-binding peptides covering the entire sequences of the diabetes autoantigens GAD65 and IA-2, GAD248-57 was found to possess the highest affinity for DRB5*0101 (Harfouch-Hammoud et al., 1999).

To study characteristics of autoantigen recognition by T cells, large panels of peptides with single, mainly conservative substitutions in all presumable HLA anchor or T-cell contact positions were generated and tested for T-cell stimulation capacity and HLA binding affinity. Figs. 2 and 5 show raw data of selected proliferation assays and HLA-DR binding assays with substituted peptides, while Figs. 3 and 6 summarize T-cell stimulation and binding data for the complete panel of epitope variants. HLA-DR binding was measured in two assays. For peptide GAD88-99, we measured competition between reporter peptide HA 306-318 and test peptides for binding to soluble DR1 purified from insect cells. As an insect cell expression system for DRB5*010l was unavailable, binding of peptide GAD248-57 and its variants was measured in a competitive cellular binding assay (Harfouch-Hammoud et al., 1999), using paraformaldehyde-fixed DRB5*0101-transfected mouse fibroblast cells and biotinylated reporter peptide MBP83-97.

### 3.1. Epitope GAD88-99: HLA-anchor residues

Specificity of peptide binding to DRB1*0101 is based on side chain interaction with pockets accommodating residues p1, p4, p6, p7 and p9 (Stern et al., 1994). The GAD epitope residue Tyr89 is predicted to be accommodated in the large hydrophobic p1 pocket. Out of four conservative/semiconservative substitutions of p1 Tyr by aliphatic or aromatic hydrophobic amino acids, none decreased HLA-DR binding or T-cell stimulation significantly (Figs. 2 and 3). All accepted substitutions are among the amino acids preferred by DRB1*0101 in p1 (Fig. 1). Conservative substitution of p4Lcu by amino acids preferred by DRB1*0101, including aliphatic hydrophobic amino acids, Ala and Gln, had little or no effect on binding affinity; only non-conservative replacement by Asn reduced affinity significantly (factor 25). However, all non-conservative substitutions, including those without effect on binding, abrogated T-cell stimulation. Thus, non-conservative substitutions of p4Leu are likely to affect T-cell recognition. Partial exposure of the p4 residue to solvent, and therefore, possibly to the TCR has been reported for DR1/HA306-318 complexes (4) and may contribute to the effects of substitutions in this position.

Relative Position

| | 0 | **1** | 2 | 3 | **4** | 5 | **6** | 7 | 8 | **9** | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GAD 88-99 | N | **Y** | A | F | **L** | H | **A** | T | D | **L** | L | P |
| DRB1*0101 | - | **YVLFI** | - | - | **LAIV** | - | **AGST** | - | - | **LAIV** | - | - |
| Motif | | **MW** | | | **MNQ** | | **P** | | | **NFY** | | |
| GAD 248-57 | M | **Y** | A | M | **M** | I | A | R | F | **K** | | |
| DRB5*0101 | - | **FY**LM | - | - | **QVLM** | - | - | - | - | **RK** | | |
| Motif | | | | | | | | | | | | |

Fig. 1. Sequences of GAD epitopes and alignment with HLA-binding motifs: In the epitope sequences, residues in bold type represent HLA anchors, and underlined residues had been found to be essential for T-cell stimulation by an Ala/Glu replacement scan in a previous study (Bach et al., 1997). In the peptide binding motifs (as described by Rammensee et al. (1995), and confirmed and extended by Fleckenstein et al. (1996)), bold amino acids are dominant anchor residues and other amino acids secondary anchors. Ala is not included in p1 in the DRB1*0101 motif described by Rammensee et al. (1995) since a subsequent study showed its deleterious effect on binding affinity (Natarajan et al., 1999).

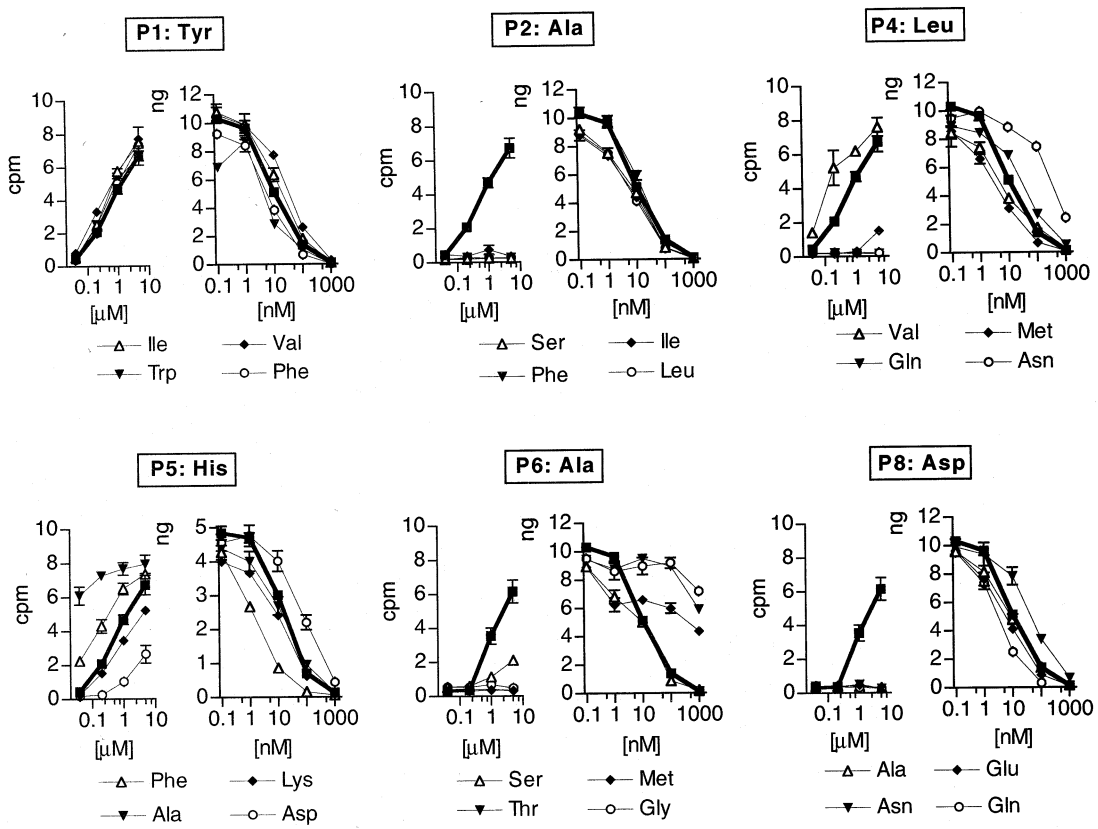


Fig. 2. Stimulation of A1 T cells, and HLA-DRA/B1*0101 binding by selected variants of epitope GAD88-99. Representative proliferation and binding data for variants in six positions from several experiments are shown. Four substitutions, represented by symbols shown under the panels, are shown for each of the selected six positions. Native residues in each position are indicated above the graphs, and data for the native epitope are shown in the graphs as filled boxes and bold lines. For each position, T-cell stimulation is shown in the left-hand panel, and HLA-DR binding data in the right-hand panel. Proliferation data is shown as thymidine incorporation (mean cpm $\pm$ S.D.; $y$-axis) in the presence of increasing peptide concentration ([μM]; $x$-axis). Binding affinity of variants was measured using empty DR1 molecules. Data are shown as ng bound reporter peptide (mean $\pm$ S.D.; $y$-axis) in the presence of increasing competitor concentration ([nM]; $x$-axis).

Positions p6 and p9, which both are accommodated in small to medium-size pockets (Stern et al., 1994), were the most sensitive to unfavorable effects of replacements on binding affinity. Substitution analysis of p6Ala provided a striking example for the permissiveness of peptide/HLA interaction for "motif-compatible" substitutions in anchor positions. All (4/4) amino acids comprised in the motif for p6 (Fig. 1) conferred unchanged high binding affinity, while all (6/6) motif-incompatible amino acids (hydrophobic and large residues) reduced it, in most cases dramatically. This pattern provides strong evidence for correct identification of residue GAD 94Ala as p6 anchor. However, most (5/6) replacements without effect on binding abrogated T-cell stimulation entirely (Fig. 3). This suggests that even substitutions of residues in peptide GAD88-99 which are likely to be buried in the HLA-binding site frequently affect peptide and/or HLA conformation, as reported in other examples (Reid et al., 1996); alternatively, the p6 residue of peptide GAD88-99 may be accessible to the TCR. Replacement of p9Leu by large residues and Asn reduced peptide binding and T-cell proliferation while more conservative substitutions by smaller aliphatic but still hydrophobic residues had little effect on binding or proliferation. This pattern is likely to be due to the small hydrophobic nature of the pocket receiving p9 and is in accord with the published DR1 motif for this position. Finally, conservative substitution of p7Thr by Ser or a non-conservative but nearly isochoric one by Ala had no effect on binding but abolished T-cell stimulation, suggesting that this residue is directly recognized by the TCR. Residue p7 is accommodated in a shallow pocket burying it only partially (Stern et al., 1994; Murthy and Stern, 1997).

Taken together, analysis of positions in GAD88-99 likely to represent HLA anchors suggested the following conclusions: (i) almost all amino acids comprising in the DRB1*0101 binding motif can be introduced into the peptide at anchor positions without any effect on binding affinity; (ii) significant reduction of HLA-binding affinity ($\geqslant$20-fold) affects T-cell stimulation; (iii) substitution in several anchor positions (p4, p6 and p7) frequently affect T-cell recognition without chang-

ing binding affinity. Thus, indirect effects of substitution on peptide and/or MHC conformation, or direct TCR contact with anchor residues (p4, p7) may be frequent events.

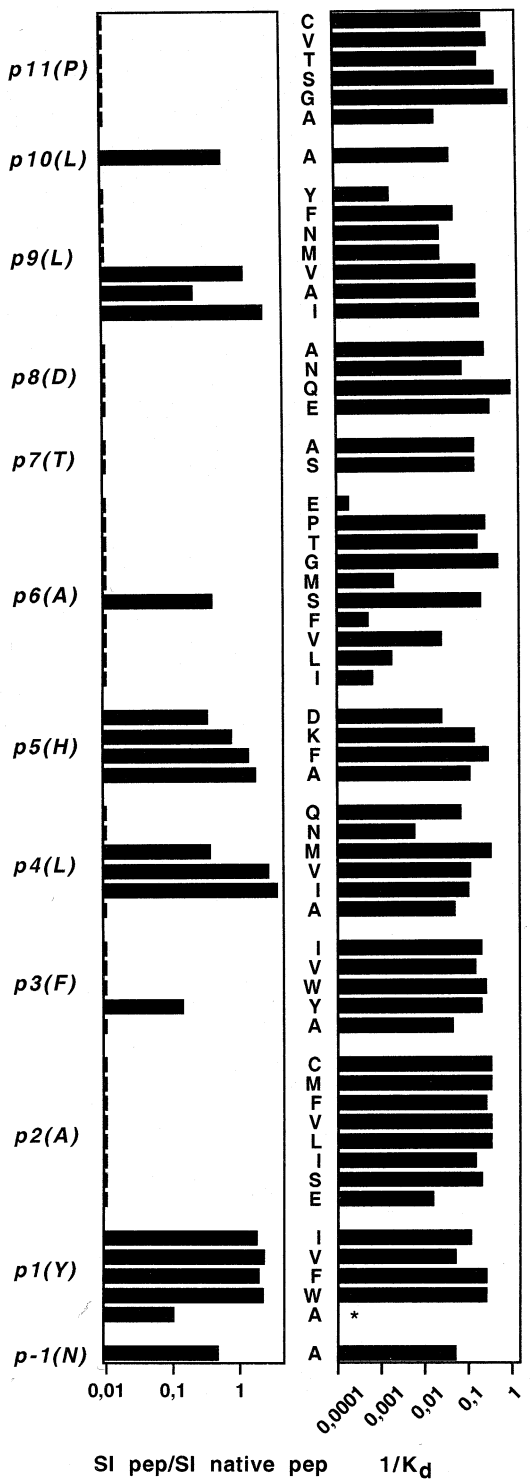


Fig. 3.

### 3.2. Epitope GAD 88-99: solvent exposed positions

Residues p-1, p2, p3, p5, p8 and p11 of a DRB1*0101-binding peptide have been found to project away from the binding site and can engage in TCR contacts (Stern et al., 1994). While semi- or non-conservative replacement of p-1 in GAD88-99 had no effect on T-cell recognition, replacement of p2Ala by small residues or aliphatic or aromatic hydrophobic residues completely abrogated T-cell proliferation but had no effect on binding affinity. Complete lack of permissiveness for semi-conservative substitution therefore qualifies this residue as a primary TCR contact (Kersh and Allen, 1996a), while identical binding affinity of variants with non-conservative substitutions rules out a role as HLA anchor. Conservative substitution of p3Phe by Tyr but not by other hydrophobic amino acids was compatible with (reduced) T-cell stimulation without affecting binding affinity, providing strong evidence for additional direct TCR contact with this residue. Similarly, all substitutions conservative or not, of the residues p7Thr (see above) and p8Asp had no effect on HLA binding but abolished T-cell stimulation completely, qualifying these residues as a second primary TCR contact region.

In contrast to the highly restricted recognition of p2, p3, p7 and p8, T-cell stimulation by the centrally positioned p5His, expected to be the principal TCR contact, surprisingly was highly permissive for non-conservative substitutions. Ala or Phe substitution in p5 increased efficiency of T-cell stimulation, Lys substitution reduced stimulation slightly, and even introduction of the negative charged Asp did not abrogate stimulation completely (Fig. 2). While the p5His→Asp variant

Fig. 3. Summary of stimulation of A1 T cells, and HLA-DRA/B1*0101 binding by variants of epitope GAD88-99. Residue positions are shown to the left of the two panels, with the amino acids in the native epitope in brackets; p1 refers to the first HLA anchor position. Substitutions are indicated between the two panels in one letter code. T-cell stimulation is shown in the left-hand panel, and HLA binding in the right-hand panel. T-cell stimulation values were calculated as follows: cpm incorporated by T cells at 200 nM and 5 μM peptide antigen was divided by cpm in medium only, resulting in two stimulatory indices (SI). The mean of these SI was then divided by the mean of the two SI of native peptide determined in the same assay, resulting in the normalized SI values shown. $IC_{50}$ values obtained for epitope variants were normalized by dividing through the $IC_{50}$ for native epitope peptide measured in the same assay. Normalized $IC_{50}$ values are represented as inverted values so that bar length is proportional to affinity. Binding affinity was determined by measuring the molar excess required for inhibition of 50% specific binding ($IC_{50}$) of biotinylated reporter peptide HA306-318 to empty soluble DR1 molecules. Binding data are expressed as inverse of the $K_d$ calculated as described in Section 2. Native peptide GAD88-99 and reporter peptide HA306-318 have a $K_d$ of 7 and 14 nM, respectively. The available amount of variant p11Tyr→Ala (marked by an asterisk) was insufficient for performing binding assays. Shortest bars in the left-hand panel indicate absence of T-cell stimulation.

had slightly reduced binding affinity, all other variants had unchanged or even higher (p5His → Phe) affinity (Fig. 2). Analysis of peptides with substitution of p11Pro uncovered an additional unusual feature of peptide GAD88-99 (Fig. 3). Six variants with p11Pro replacements by small amino acids or Val could not stimulate T cells, although all but p11Pro → Ala bound DRB1*0101 with unchanged affinity. Thus, p11 may be contacted by the TCR, as suggested for another epitope (Carson et al., 1997), although the single TCR/peptide/MHC class II structure published so far argues against this hypothesis (Reinherz et al., 1999).

### 3.3. Epitope GAD88-99: modeling

To visualize the structural correlates of substitutions in peptide GAD88-99, we modeled complexes of DRB1*0101 with the peptide, based on the published structure of DR1 with peptide HA306-318. As shown in Fig. 4A, models of DRl/HA306-318 and DR1/GAD88-99 complexes subjected to energy minimization displayed almost identical peptide backbone structures; common backbone configurations for DR1-bound peptides have been reported previously (Jardetzky et al., 1996; Murthy and Stern, 1997). Modification of GAD88-99 by substitutions tested in binding and proliferation assays also resulted in minimal movement of the antigenic peptide backbone in the groove; modeling was performed on conservative substitutions for anchor positions and conservative as well as non-conservative substitutions at TCR contact residues. As shown in Fig. 4B, substitutions at positions p2, p5 and p8 all gave residues that were pointing in the same general direction as the native residues at the respective positions. None of the substitutions resulted in any undue strained conformations. Thus, loss of T-cell stimulation by conservative substitution in these positions was unlikely to be caused by gross conformational changes in peptide GAD88-99. Fig. 4B also illustrates the significant differences in bulk of p5 side chains that were compatible with efficient T-cell stimulation only in case p5 is not a principal TCR contact residue.

### 3.4. Epitope GAD248-57: HLA-anchor residues

The published motif for DRA/B5*0101 which summarizes previous studies has aromatic residues at p1, mid-sized aliphatic residues or Gln at p4, and basic residues (Arg, Lys) at p9 (Rammensee et al., 1995). Like DRA/B1*0101, DRA/B5*0101 carries a Gly at 1386 and is, therefore, likely to possess a large pocket for p1. Substitution of Tyr249 (p1) by other aromatic or aliphatic amino acids had no effect on T-cell stimulation and little effect on HLA binding, although substitution by Ala reduced affinity 30-fold (Figs. 5 and 6), defining this significantly reduced affinity thereby as sufficient for efficient T-cell stimulation. Replacement of p2Ala by amino acids with large side chains (hydrophobic or charged) reduced binding affinity and abolished T-cell responses. However, since equivalent reductions in binding affinity by substitutions in other positions (e.g. p1Tyr → Ala, p4Met → Tyr) had no effect on T-cell recognition, p2 is likely to be contacted by the TCR.

Consequences of p4Met replacement resembled those of p1, in that most conservative or semi-conservative substitutions had little to moderate effects on affinity

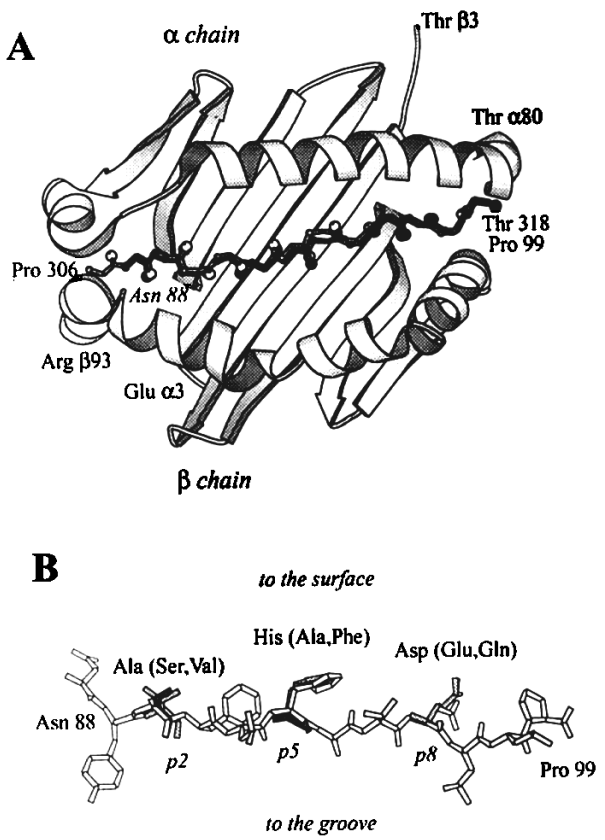


Fig. 4. Modeled structures of the DR1 complexed with GAD88-99 and some of its variants: (A) Top view of the α1β1 (peptide binding) domain of HLA-DR1 in complex with HA306-318 and GAD88-99, after energy minimization. The α1β1 domain is depicted in ribbon form (α chain from α3Glu to α80Thr, and β chain from β3Thr to β93Arg). The antigenic peptide backbones are shown in ball and stick representation, with the HA peptide in light gray and the GAD peptide in dark gray-black. The respective conventions for the HA and GAD peptide heavy atoms are: nitrogen, small white or black ball; oxygen, large white or black ball; carbon, not shown. Drawn using the program MOLSCRIPT. (B) Depiction of the complex of DR1 with GAD88-99 and some of its variants as obtained by energy minimization, viewed from the side of the β1 helix at the level of the beta-sheet floor. The native GAD peptide residues are shown in black stick representation. The substituted residues at p2, p5, and p8 (in parentheses) are represented either in white ball and white stick form (Ser, Ala and Glu, respectively), or in gray stick form (Val, Phe and Gln, respectively). At position 8 the minimization process yields identical orientations for the side chain heavy atoms of Glu and Gln, with overlapping stick representations shown in shaded gray.

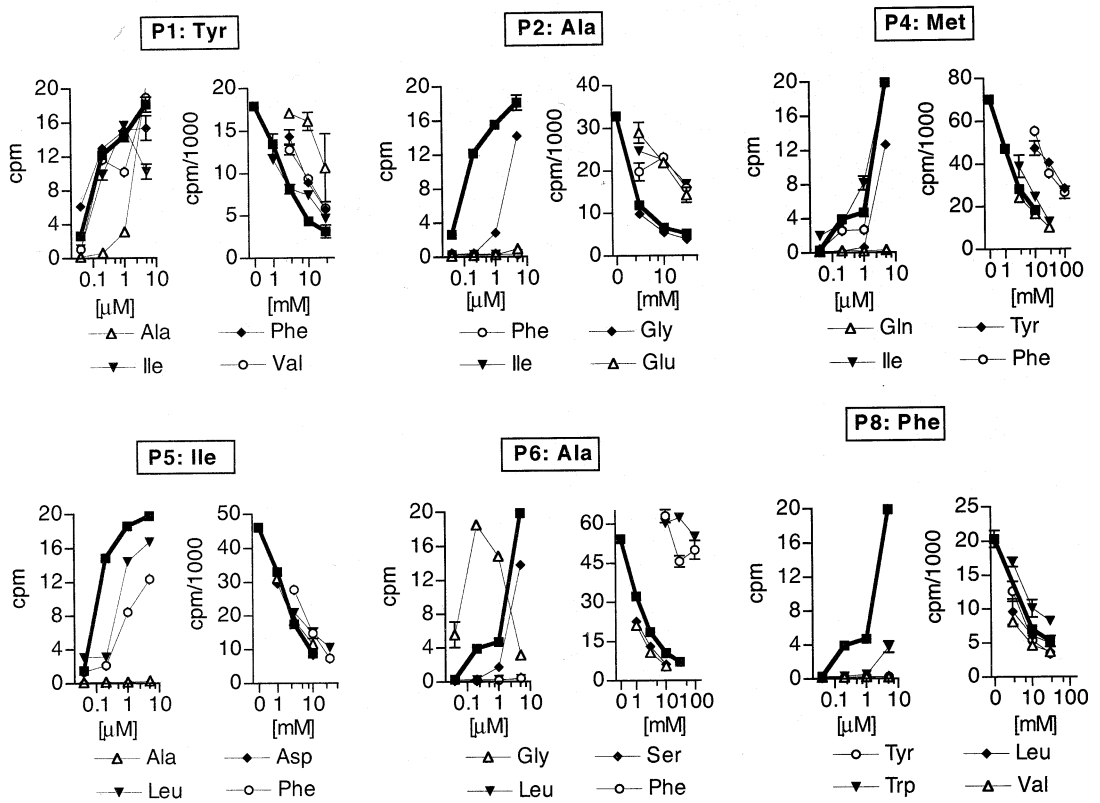


Fig. 5. Stimulation of B18 T cells, and HLA-DRA/B5*0101 binding by selected variants of epitope GAD248-5. Representative proliferation and binding data resulting from substitutions in six positions are shown. For proliferation assays (left-hand panels), data representation, symbol use, *y*-axis and *x*-axis scales are as described in the legend to Fig. 2. Binding data (right-hand panels) were obtained in the cellular assay, using DRA/B5*0101-transfected mouse fibroblasts and biotinylated reporter peptide MBP83-97. Mean fluorescent cpm ± S.D. (*y*-axis), corresponding to bound reporter peptide, in the presence of increasing concentrations of competitor test peptides ([mM]; *x*-axis) are shown.

and little to no effect on T-cell responses; only the non-conservative substitution p4Met→Gln abrogated T-cell stimulation. Similarly, while non-conservative substitution of p9Lys reduced binding affinity, all p9 variants were recognized efficiently by T cells, compatible with classification of p9 as HLA anchor. However, although p6 has not previously been shown to be important for binding to DRA/B5*0101, our results suggest that it contributes to binding of GAD248-57. Replacement of p6Ala by amino acids with longer hydrophobic side chains abolished peptide binding while Gly, Ser, Thr and Glu were tolerated, with Ser and Gly increasing affinity significantly. Thus, p6 may be accommodated in a small and possibly polar pocket in DRBS. However, p6Ala substitution by Thr abrogated T-cell responses, suggesting TCR contact with residues aligning p6 and perhaps p6 itself. Taken together, results for GAD248-57 are compatible with a classification of p1, p4 and p9 as HLA anchors. In addition, they suggest an important and selective contribution of p6 to binding affinity, and TCR contacts with p6 and p2.

### 3.5. Epitope GAD248-57: solvent exposed positions

Several other positions were also likely to be in direct contact with the TCR. Thus, Ala or Tyr but not other conservative substitutions of p3Met abolished T-cell stimulation without affecting binding. Surprisingly, most conservative replacements of central p5Ile did not affect T-cell recognition; only non-conservative replacements by Ala, Asp, Lys or Trp reduced or abolished T-cell responses. In contrast, positions 7 and especially 8 were less permissive for substitution; most conservative substitutions of p8Phe eliminated T-cell responses. Thus, epitope GAD248-57 resembled GAD88-99 with respect to relatively higher permissiveness of the central

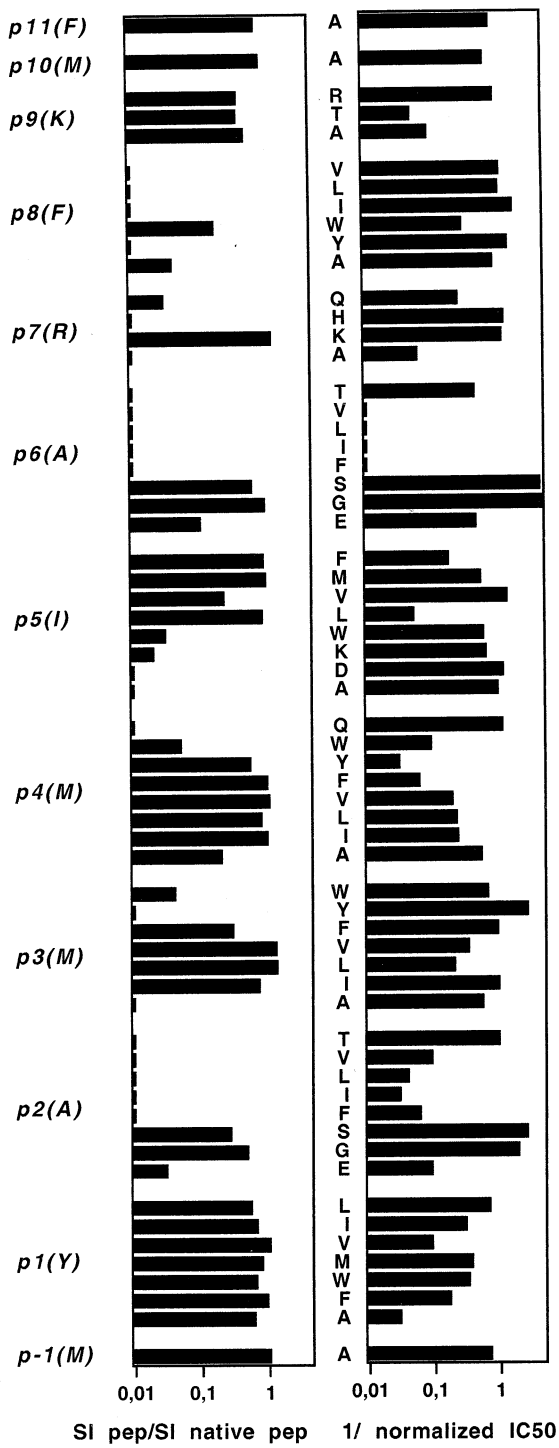


Fig. 6. Summary of stimulation of B18 T cells, and HLA-DRA/B5*0101 binding by variants of epitope GAD248-57. T-cell stimulation and HLA binding were measured as described in the legend to Fig. 5. T-cell SIs were calculated as described in the legend to Fig. 3. $IC_{50}$ values obtained in binding and T-cell assays for epitope variants were normalized with respect to the $IC_{50}$ of the native epitope measured in the same assay; native peptide had a mean binding $IC_{50}$ of 2.5 μM. Normalized $IC_{50}$ values are represented as inverted values (bar length proportional to affinity).

peptide residue for substitution compared to the C-terminal portion of the peptide. Ala substitution of p10Met and p11Phe had no effect on peptide binding or T-cell stimulation so that these positions were not analyzed by conservative replacements.

## 4. Discussion

We have performed an in-depth structural analysis of two HLA-DR-presented epitopes derived from a type 1 diabetes autoantigen. Having produced very large sets of single-substituted epitope variants, we were able to assign HLA anchor and TCR contact positions and determine their permissiveness for substitution. We find high-binding affinities, standard binding registers and TCR contact distributions. However, we observe a strikingly high permissiveness of the central peptide residues for substitution, suggesting that interactions between the central peptide residue and the TCR do not necessarily make an important energetic contribution in recognition of HLA-DR-presented antigens.

For both epitopes (and especially GAD88-99), the roles of either HLA anchors or TCR contacts could be assigned unambiguously to almost all of the nine residues forming the core of DR-presented peptides. While both epitopes contained binding registers that perfectly matched the known motifs, analysis of GAD248-57 strongly suggests that p6 functions as an anchor position for binding to DRB5*0101, so that this molecule falls in line with the HLA anchor distribution known for other MHC class II molecules (Rammensee, 1995). In the case of peptide GAD88-99, anchor and TCR contact assignment is based on the following arguments: (i) identification of a single register for binding to DRB1*0101 which fully matches the published motif; (ii) complete concordance of the effect of substitutions in the proposed p6 position with the published motif; (iii) modeling of GAD88-99 bound without undue strain in the DR1-binding site; (iv) absence of effects on binding of non-conservative substitutions in non-anchor positions (p2, p3, p5, p8, p11). Taken together, these findings provide overwhelming evidence for correct register assignment.

Several residues (p2 and p6 in GAD248-57, and p4, p6 and p7 in GAD88-99) may function both as HLA anchors and TCR contacts. Alternatively, substitutions in these residues may have indirect effects on peptide and/or HLA conformation, as is well documented in other cases (Fremont et al., 1992; Reid et al., 1996; Ghendler et al., 1998). Overall substitutions in the vast majority of positions in epitope GAD88-99 could abolish T-cell stimulation without interfering with peptide binding; these positions include p2, p3, p4, p6, p7, p8 and p11, but significantly not p5. Thus, almost every residue in this peptide can affect T-cell recognition, presumably by a variety of mechanisms including direct TCR contact and changes in peptide and/or HLA conformation. Strong effects of p11 substitutions in this epitope are highly

unusual and reminiscent of one previous report (Carson et al., 1997). As was proposed in that case, p11 may be contacted by the TCR; alternatively, p11 substitution may alter peptide conformation.

It is interesting to compare the two GAD epitopes to two other autoantigenic epitopes previously studied in detail. These are derived from MBP and presented to autoreactive T cells by MHC class II molecules associated with multiple sclerosis or the corresponding murine model disease. Both epitopes display poor fit to their respective class II binding sites (Lee et al., 1998; Smith et al., 1998), and are recognized by T cells displaying highly promiscuous peptide recognition (Wucherpfennig and Strominger, 1995; Hemmer et al., 1997; Hausmann et al., 1999b). In the case of the murine epitope, low MHC-binding affinity due to suboptimal anchor residues has been proposed to result in inefficient thymic deletion of auto-reactive T cells (Liu et al., 1995). Clearly, with their high HLA binding affinities, full binding registers and highly efficient T-cell recognition, both GAD epitopes studied herein are distinct from the MBP epitopes. T-cell recognition of the epitopes also displays limited (for GAD248-57) promiscuity or none at all (for GAD88-99), as shown recently (Bach et al., 1998). Thus, not all autoantigenic epitopes display low HLA-binding affinity and highly promiscuous T-cell recognition, as already demonstrated in the case of an encephalitogenic epitope derived from proteolipid protein (Greer et al., 1996; Carrizosa et al., 1998).

For both epitopes, principal T-cell contacts could be assigned to peptide residues p7/8 and p2(/3), while recognition of residue p5 was highly (albeit not completely) permissive, thereby qualifying the position at best as auxiliary T-cell contact. While identification of these three contact regions is in accordance with previous studies (Stern and Wiley, 1994; Sant'Angelo et al., 1996) and also direct structural data on MHC/peptide/TCR complexes (Garboczi and Biddison, 1999; Reinherz et al., 1999), the relative permissiveness of these three regions (for GAD88-99: $p5 \gg p2 = p8$) is highly unusual for antigen-specific T cells. In detailed studies of murine and human MHC class II-restricted antigen-specific T cells, the central residue has regularly been identified as principal TCR contact characterized by extremely low permissiveness for conservative substitution, while one or both of the p8 and p2 regions typically act as auxiliary anchors (Jorgensen et al., 1992; Wucherpfennig et al., 1994; Hsu et al., 1996; Sant'Angelo et al., 1996). Thus, particularly in the case of the DR1-restricted T cells, we find an exchange of the roles of auxiliary and principal TCR contacts. Two other examples of relatively permissive recognition of the central p5 residue have been described recently. Two human CD4 clones recognizing the autoantigenic peptide MBP84-102 displayed more restricted recognition of p2/p3 than of p5 (Hausmann et al., 1999b). Another example of more restricted recognition of p8 associated with higher permissiveness for conservative substitution of p5 recognition has recently been demonstrated for one of two HLA-A2/tax11-19 specific TCRs (Hausmann et al., 1999a). This shift was due to a larger, and therefore, more permissive pocket formed by the CDR3 loops and a longer CDR3β loop able to contact p8 more closely (Ding et al., 1998). However, epitope of DR1/GAD88-99 appears unique with respect both to extremely restricted recognition of p2 and p8, and to high permissiveness even for non-conservative substitutions in p5.

We can only speculate on the reasons for the high level of permissiveness of the central p5 residue in epitope GAD88-99. Highly efficient T-cell stimulation by non-conservative substitutions including Ala renders interactions between p5His side chains and the TCR CDR3 loops, as found for other peptides (Garboczi and Biddison, 1999; Reinherz et al., 1999), unlikely. The ensuing loss of binding energy between the TCR and peptide may either be compensated by additional contacts between the TCR and p2 and/or p8, or by a greater relative role of interactions between the TCR and the MHC alpha helices. While p2 and p8 of MHC class I-presented peptides interact directly with the CDR1α, and with the CDR1β and CDR3β loops, respectively (Garboczi et al., 1996; Ding et al., 1998; Garboczi and Biddison, 1999), these residues are contacted by CDR1α, and CDR3α/β, respectively, for a MHC class II-presented peptide (Reinherz et al., 1999). Contacts between the CDR3 loops and p8 and/or p2 may be facilitated by the known capacity of these loops to move upon contact with peptide/MHC (Garcia and Teyton, 1998; Reinherz et al., 1999).

Poor interaction between the central peptide residue and the TCR CDR3 domains has been demonstrated for the 2C TCR co-crystallized with a positively selecting weak agonist peptide (Garcia et al., 1996) and with an allo-MHC class I/peptide complex (Speir et al., 1998). In this case, TCR mutagenesis analysis suggested that the CDR3 loops contribute less binding energy to the TCR/MHC/peptide interaction than the CDR1 and 2 loops (Manning et al., 1998). Moreover, Daniel and associates recently found that TCR recognition of an allo-MHC class II/peptide complex was much more permissive for p5 substitutions than recognition of nominal antigen plus self MHC class II by the same TCR (Daniel et al., 1998). In both cases, it has been proposed that a shift in the relative contribution of MHC and peptide residues toward the overall strength of binding between the TCR and MHC/peptide ligand characterizes allo-recognition. It cannot be ruled out that TCR recognition of the GAD88-99/DR1 complex, and possibly other MHC class II-presented autoantigens, by T cells escaping thymic deletion resembles recognition of allo-MHC/peptide with respect to a greater contribution of MHC/TCR interactions.

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