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Structural Basis of Specificity and Degeneracy of T Cell Recognition: Pluriallelic Restriction of T Cell Responses to a Peptide Antigen Involves Both Specific and Promiscuous Interactions Between the T Cell Receptor, Peptide, and HLA-DR

Derek G. Doherty,† Julie E. Penzotti,‡ David M. Koelle,‡ William W. Kwok,* Terry P. Lybrand,† Susan Masewicz,* and Gerald T. Nepom†‡

TCR engagement of peptide-MHC class II ligands involves specific contacts between the TCR and residues on both the MHC and peptide molecules. We have used molecular modeling and assays of peptide binding and T cell function to characterize these interactions for a CD4+ Th1 cell clone, ESL4.34, which recognizes a peptide epitope of the herpes simplex type 2 virus virion protein, VP16 393–405, in the context of several HLA-DR alleles. This clone responded to VP16 393–405 in proliferation and cytotoxicity assays when presented by DRB1*0402, DRB1*1102, and DRB1*1301, which share a common amino acid sequence, ILEDE, at residues 67–71 in the α-helical portion of the DRβ polypeptide, but not when presented by other DR4, DR11, and DR13 alleles that are negative for this sequence. Using a panel of APCs expressing DR4 molecules that were mutagenized in vitro at individual residues within this shared epitope and using peptide analogues with single amino acid substitutions of predicted MHC alleles that are negative for this sequence. Using a panel of APCs expressing DR4 molecules that were mutagenized in vitro at individual residues within this shared epitope and using peptide analogues with single amino acid substitutions of predicted MHC and TCR contact residues, a unit of recognition was identified dependent on DRβ residues 67–71 and relative position 4 (P4) of the VP16 393–405 peptide. The interactions of this portion of the peptide-DR ligand with the ESL4.34 TCR support a structural model for MHC-biased recognition in some Ag-specific and alloreactive T cell responses and suggest a possible mechanism for autoreactive T cell selection in rheumatoid arthritis. The Journal of Immunology, 1998, 161: 3527–3535.

The ligand recognized by TCR on CD4+ T cells consists of a class II MHC molecule complexed with a peptide fragment of a protein Ag on the surface of an APC. The structure of MHC molecules imposes constraints on the nature of peptides that they can bind, and therefore the selection of antigenic determinants that can be presented to T cells (1). Each MHC class II molecule can bind and present an extremely large number of structurally diverse peptides based on motif residues which are anchored by complementary pockets within the MHC molecule (2–7). Similarly, a given peptide can be bound and presented by many class II molecules with varying efficiencies for recognition by T cells (2, 7, 8).

The interaction between αβ TCRs and peptide-MHC class II ligands involves direct contact between the TCR and residues on both the MHC molecule and the bound peptide (9, 10). Analyses of the crystal structures of MHC class II molecules complexed with antigenic peptides (11–15) and studies using substituted peptides and mutated MHC molecules (6, 16, 17) have identified putative TCR contact residues on both the antigenic peptides and the α-helical regions of the MHC class II molecules. Two recent crystal structures of class I MHC-peptide-TCR complexes (18–20) have demonstrated that the αβ TCR recognizes its ligand through three variable loops (complementarity-determining regions [CDRs]) on each polypeptide that form a relatively flat surface that fits diagonally over the MHC-peptide complex. All three CDRs of the TCR α-chain and CDR3 of the β-chain contact the MHC α-helices, while the CDR1 and CDR3 loops of both the TCR α- and β-chains contact the bound peptide. Based on sequence and structure comparisons between peptides complexed with MHC class I and class II molecules, a similar mode of TCR interaction with peptide-MHC class II ligands has been predicted (19, 21).

While the overall conformation of the peptide-MHC-TCR ternary complex is thought to be similar for all MHC class I and class II molecules and all αβ TCRs (19, 21), the nature of the signal generated upon TCR ligation can vary greatly, leading to activation, apoptosis, or nonresponsiveness of the T cell and positive or negative selection in the thymus (22, 23). TCR activation can, in turn, result in a range of different effector functions, including T cell proliferation, cytokysis, and cytokine secretion of the Th0, Th1, or Th2 profiles. Factors that contribute to the outcome of TCR ligation include the nature and cell surface density of the peptide-MHC ligand, the avidity of its interaction with the TCR, the presence or absence of other costimulatory molecules, and the local cytokine milieu (22, 24–26). Specific contacts between amino acid residues on the peptide, MHC, and TCR molecules have profound effects on the nature of T cell stimulation. Single

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substitutions of MHC or peptide residues predicted to contact the TCR can enhance, antagonize, or energize a T cell response or selectively inhibit some but not all effector functions (27–32).

The TCRs of human CD4+ T cells primarily recognize antigenic peptides presented by MHC class II (HLA-DR, DQ, or DP) molecules. HLA-DR molecules are heterodimeric glycoproteins composed of a nonpolymorphic DRα and a polymorphic DRβ chain. The polymorphic amino acid residues in DRβ polypeptides are mostly clustered in three hypervariable regions (HVRs) which line the peptide binding groove (33). HVRI (residues 9–14) and HVRII (residues 67–74) is located on the α-helical portion of the DRβ molecule and can affect both peptide binding and TCR interaction through direct contacts with both molecules (11, 13, 14). HVRIII amino acid sequences can vary greatly within serologically related DR types but are conserved among serologically distinct alleles. For example, the ILEDE amino acid position 67 is present on some but not all allelic variants of the DR1, DR4, DR11, and DR13 specificities. Another sequence at position 71, LLEQRRAA, which is encoded by certain DR1, DR4, and DR14 alleles, constitutes the “shared epitope” thought to play a role in the development of rheumatoid arthritis (RA) (34, 35).

In this study, we examined the molecular basis of the interactions between the TCR, MHC class II, and peptide molecules, using a herpes simplex type 2 virus (HSV-2)-specific human CD4+ T cell clone, ESL4.34, which recognizes a peptide determinant corresponding to residues 393–405 of the virion protein VP16, in the context of several HLA-DR alleles. This T cell, which elicits strong proliferative, cytolytic, and cytokine secretion responses, recognizes the VP16 393–405 peptide with a pattern of restriction that correlates with a specific amino acid sequence at positions 67, 70, and 71 of HVRIII of the DRβ polypeptide. By a combination of molecular modeling and assays of peptide binding and T cell stimulation using in vitro mutagenized DR molecules and substituted peptide analogues, we have dissected the structural interactions between DRβ HVRIII, the peptide, and the TCR. The DRβ HVRIII was found to have a direct effect on both peptide binding and TCR recognition, suggesting an important role for this epitope in alloreactivity, in T cell repertoire selection, and in autoimmune disease.

Materials and Methods

**Viruses and viral antigens**

HSV-1 strain E115, HSV-2 strain 333, and a recombinant virus consisting of VP16 of HSV-2 in an HSV-1 background, RP-2, were grown in human diploid cells and titered by plaque assay on Vero cells as previously described (36). Crude viral Ags were prepared from virus stocks by exposure to UV light for 3 min at 10 cm from a G7038 bulb (General Electric, Cleveland, OH), eliminating infectious virus, and used at a final dilution of 1:100 which corresponds to 104 to 105 plaque-forming units per ml before UV treatment. Peptides corresponding to amino acids 1–409 of VP16 of HSV-2, 13 amino acids long and overlapping by 9 amino acids, were obtained from Chiron Mimotopes, Clayton, Australia. The VP16 393–405 peptide (LVAPRMSFLSAGQ) and its analogues (see legend to figures and tables) were synthesized with an Applied Biosystems 432 Peptide Synthesizer (Foster City, CA). Peptides were biotinylated by amino-terminal amidohydrolase (Foster City, CA). Peptides were biotinylated by amino-terminal amidohydrolase (Foster City, CA). Peptides were biotinylated by amino-terminal amidohydrolase (Foster City, CA).

**T cell cloning**

Mononuclear cells were prepared from the herpetic vesicle fluid from a DRB1*0402/1*01-positive patient with culture-proved recurrent HSV-2 infection by Ficoll-Hypaque density gradient centrifugation. The cells were stimulated with PHA (0.4 μg/ml PHA-P; Murex Diagnostics, Dartford, UK) and an equal number of irradiated (3300 rads γ-irradiation) allogeneic PBMC in T cell medium (RPMI 1640 containing 25 μM HEPES, 2 μM l-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% heat-inactivated human male serum). Acyclovir (50 μM) was added for the first 2 weeks of culture to prevent viral replication (38). Human natural IL-2 (50 U/ml; Schiaperelli Biosystems, Columbia, MD) was added on day 3, and the cells were subsequently fed with IL-2 every 2 to 3 days. After 16 days of growth, 5 × 10^10 cells were restimulated with crude HSV-2 Ags and 5 × 10^10 autologous irradiated PBMC as APC and trinitramine as above. After 12 days, the cells were cloned at 1 cell/well using PHA, IL-2, and allogeneic feeders and subsequently restimulated every 12 to 14 days.

**Analysis of TCR genes**

mRNA was prepared from T cell clones that were restimulated with lymphoblastoid cell lines (LCLs) as APC instead of PBMC using the Quick-Prep Micro RNA Purification Kit (Pharmacia Biotech, Alameda, CA) and used for first strand cDNA synthesis using the Superscript Preamplification system (Gibco BRL, Grand Island, NY). The 5′-end PCR segment usage was determined by PCR amplification of first strand cDNA (RT-PCR) using a set of subfamily specific 5′-primers (Vα1–29 and Vβ1–23) and constant region 3′-primers (kindly provided by Dr. Christine Vissinga, Virginia Mason Research Center, Seattle, WA) essentially as described by Choi et al. (39). Vβ typing was confirmed using the Clontech (Palo Alto, CA) TCR Amplimer Kit. Nucleotide sequences of the V-(D)-J junctions were determined by cycle sequencing of both strands of the PCR products obtained as above using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an Applied Biosystems 373 DNA Sequencer. Two different Vα7 genes were detected by sequence analysis; therefore, the Vα7 PCR products were cloned into the pCRII vector (Invitrogen, San Diego, CA), used to transfected into E. coli (Stratagene, La Jolla, CA), and plasmid DNA was prepared from overnight cultures of isolated colonies using the QiAprep Spin Miniprep Kit (Qiagen, Santa Clarita, CA) and amplified as above by PCR. The nucleotide sequence of both strands of the PCR products were determined by cycle sequencing as above.

**Lymphoblastoid cell lines and Abs**

The EBV-transformed LCLs YAR (DRB1*0402), MT (*0404), HHKB (*1301), JVM (*1102), TISI (*1103), and MAT (*0301) were obtained from the VIIth and Xth International Histocompatibility Workshop Panels. The LCL 8854 (*1303) was generated by EBV transformation of the locally characterized PBMC. The MHC class II-deficient BLS-1 line was kindly donated by Dr. Janet Lee, Sloan-Kettering Memorial, New York, NY. LCLs were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT) and sodium pyruvate. HEPES, l-glutamine, streptomycin, and penicillin as above. mAbs used were L243 (anti-DR; American Type Culture Collection, Rockville, MD), SV-13 (anti-DQ; kindly provided by Dr. Hans Yssel, DNAx, Palo Alto, CA), B7/21 (anti-DP; Becton Dickinson, San Jose, CA), anti-CD4 TCR and anti-Vβ8 (PharMingen, San Diego, CA), NFLD.D1, and NFLD.D11 (anti-DR4 and -DR544; kindly provided by Dr. Sheila Drover, University of St. John’s, St. Johns, Canada). Cell surface expression of HLA-DR and TCR was assayed by mAb staining as described previously (40) and analyzed on a Becton Dickinson FACSort flow cytometer.

**Retroviral gene transfer**

HLA-DR genes and mutagenized constructs were introduced into MAT LCLs by retroviral infection using the methods of Kwok et al. (41). The construction of the retroviral vectors containing wild-type and mutagenized DRβ genes which were altered at codons 67 (L to I), 70 (Q to D), 71 (R to E), and both 67 and 71 (L67 to I67 and R71 to E71) has been described previously (40). For the purpose of clarity, these mutants are named according to their differences from the DRB1*0402 amino acid sequence in this report, i.e., DRB1*0402-Q70/R71, DRB1*0402-L67,R71, DRB1*0402-L67,Q70, and DRB1*0402-Q70,R71, respectively. Surface expression of the transgene was monitored by flow cytometry using the NFLD.D1 and NFLD.D11 mAbs.

**T cell proliferation assays**

Stimulator cells (PBMC irradiated with 3,300 rads or LCL irradiated with 20,000 rads γ-irradiation) were pulsed with Ag (peptide concentrations shown in figure legends) for 2 to 4 h at 37°C, 5% CO2, followed by three washings with supplemented RPMI medium. PBMC (10^4) or LCL (2.5 × 10^10) were plated in 96-well U-bottom plates in T cell medium containing 10 μg/ml p/TF cells or bulk lesion-derived cells as responders in a final volume of 150 μl. All assays were performed in triplicate. After incubation for 48 h, [3H]thymidine (1 μCi/well; NEN, Boston, MA) was added for 12
to 16 h, the cells were harvested using a Tomtec 96 Mach III Harvester (Hamden, CT), and \(^{3}H\) incorporation was measured on a 1450 Microbeta Plus Liquid Scintillation Counter (Wallac, Gaithersburg, MD). Stimulation indices were calculated as \((\text{cpm of sample} - \text{cpm of spontaneous release})/\text{cpm in absence of stimulators}\).

To determine restricting HLA molecules, mAbs L243, SPV-L3, and B7/21, which recognize HLA-DR, DQ, and DP, respectively, were used as previously described (38).

**T cell cytotoxicity assays**

Target LCLs \((5 \times 10^5)\) were pulsed with peptide (concentrations shown in figure legends) and simultaneously loaded with \(^{51}\text{Cr}\) (25 \(\mu\text{Ci};\) NEN) for 8 to 12 h at 37°C, 5% \(\text{CO}_2\), followed by gentle washing three times with supplemented RPMI medium. Labeled targets \((1–2 \times 10^5)\) were incubated with effector T cells at various E:T ratios in a final volume of 150 ml in T cell medium in 96-well U-bottom plates. After 4 h, cell supernatants \((50 \mu\text{l})\) were assayed for \(^{51}\text{Cr}\) release in a 1450 Microbeta Plus Liquid Scintillation Counter. Percent specific lysis was expressed as \((\text{cpm of sample} - \text{cpm of spontaneous release})/\text{cpm of maximum release} - \text{cpm of spontaneous release}\).

**Peptide binding assays**

Binding of biotinylated peptides to HLA-DR on whole cells, with subsequent capture of the peptide-class II complex, was performed using a europium-labeled streptavidin assay as described previously (37), except that the L243 Ab was used to immobilize the HLA-DR molecules. A 10 \(\mu\text{M}\) peptide concentration was used in all experiments, this concentration having been previously determined to be submaximal for binding. The class II-deficient LCL, BLS-1, served as a negative control for nonspecific binding.

**Modeling of peptide-HLA-DR complexes**

Molecular models of DRB1*0402, *1102, and *1301 were constructed from the crystal structure coordinates of the HLA-DR1 molecule complexed with the influenza hemagglutinin peptide 306–318 (11) as previously described (42). Using the interactive graphics program PSSHOW (43), amino acid side chains were replaced and assigned to the preferred conformer from the Ponder and Richards rotamer database (44) that was closest to the side chain conformer in the crystal structure template. The HCV VP16 393–405 peptide was constructed from the hemagglutinin peptide coordinates (11), and several different alignments of the anchor residues with the VP16 393–405 sequence were examined to predict the binding motif. Peptide binding experiments were designed to test the predicted binding motif.

**Results**

**Characterization of T cell clone ESL4.34**

Recovery of cells from herpetic vesicle fluid and subsequent stimulation with whole HSV-2 Ag followed by cloning yielded a T cell clone, ESL4.34, that responded to HSV-2 and RP-2 but not HSV-1 in proliferation assays (Fig. 1), indicating a specificity for VP16 of HSV-2 but not HSV-1. The fine specificity of ESL4.34 was determined by testing its proliferative response to a set of 10 pools of overlapping peptides corresponding to amino acids 1–409 of VP16 of HSV-2. Only a pool spanning amino acids 361–409 (pool 10) gave a positive response (Fig. 1). Assay with individual peptides of this pool identified the epitope as one contained in overlapping peptides 389–401 and 393–405 (Fig. 1). Peptide VP16 393–405 was used in subsequent studies to characterize the residues required for MHC binding and T cell recognition.

The HLA locus that serves as the restriction element for ESL4.34 was determined in proliferation assays using HSV-2 and VP16 393–405, presented by autologous PBMC, in the presence and absence of mAbs to HLA-DR, -DQ, and -DP. Inhibition of proliferation was found in the presence of the anti-DR mAb only (data not shown), indicating that ESL4.34 is DR restricted. Oligonucleotide typing of autologous PBMC revealed positivity for DRB1*0402 and DRB1*1301. To determine which allele is the restriction element for ESL4.34 recognition of VP16 393–405, DR-homozygous LCLs expressing DRB1*0402 and DRB1*1301 (HHKB) were tested as APCs for the peptide. Figure 2 shows that both DRB1*0402 and DRB1*1301 presented VP16 393–405 to ESL4.34, eliciting both proliferative and cytolytic responses.
ESL4.34 TCR α- and β-chain gene usage was determined by PCR amplification of cDNA using primers specific for Vα1–29 and Vβ1–23. PCR products corresponding to Vα7 and Vβ8 only were obtained. Flow cytometric analysis of ESL4.34 using anti-αβ TCR and anti-Vβ8 mAbs confirmed that all αβ TCR+ cells expressed Vβ8. Nucleotide sequence analysis of the Vβ8 PCR product in both directions revealed a unique V-α-J junctional sequence that encodes a TCR β-chain CDR3 (residues 92–117) amino acid sequence CASSERGDTDTQYFGPGLRTVLEDL, which corresponds to Vβ8, DJ β (ERGD), Jβ2.3 (TDTQYFGPGLRTV LEDL), and Cβ2. Sequencing of the Vα7 PCR product revealed two Vα7 TCR gene sequences, and cloning and sequencing of these two products identified distinct Vα7αα junctional sequences. One of these contained a G to C nucleotide substitution which, if translated, would result in the substitution of the conserved cysteine residue at position 90 with a serine residue, resulting in a nonfunctional TCR α-chain (45). The viable TCR α-chain CDR3 region was encoded by Vα7.2, Jβ2.3, and a V-J junctional sequence (residues 90–106), which code for the amino acid sequence CAPRGAGRRALTFGSGT.

Pluriallelic restriction of ESL4.34 responses to VP16 393–405

The above experiments indicate that ESL4.34 is a Vα7+ Vβ8+ T cell clone specific for VP16 393–405 presented by both autologous DR alleles, DRB1*0402 and DRB1*1301, in both proliferation and cytotoxicity assays (Fig. 2). This clone, however, does not respond to the peptide presented by the allogeneic DRB1*0404 allele, which differs from *0402 only at three amino acid positions (residues 67, 70, 71) in the DRβ α helix (Fig. 2). To further investigate the pluriallelic restriction of ESL4.34, a set of LCLs expressing DRB1 allelic variants of three serologically defined DR types, DR4, DR11, and DR13, which differ in their α-helical amino acid sequences, were used as APCs for VP16 393–405 in proliferation and cytotoxicity assays. Figure 3, A and B, shows that VP16 393–405 was recognized by ESL4.34 in proliferation and cytotoxicity assays, when presented on LCL expressing DRB1*0402, DRB1*1301, and DRB1*1102. Each of these DRB1 alleles encode the same sequence at DRβ residues 67–71 (HVR3), namely ILEDE, whereas the other DR4, DR13, and DR11 alleles that did not support ESL4.34 responses had different HVR3 sequences, as summarized in Table I. Even the single isoencein to phenylalanine substitution at DRβ position 67, that distinguishes DRB1*1102 and DRB1*1103, is sufficient to abolish ESL4.34 responses. Further analyses of ESL4.34 responses VP16 393–405-pulsed LCLs expressing other DRβ 67–71 ILEDE-negative DR4 (DRB1*0401 and *0405) and DR11 (DRB1*1101) alleles (data not shown) were consistent with the hypothesis that this motif at HVR3 is required for ESL4.34 recognition.

The qualitative differences in the proliferative and cytolytic responses of ESL4.34 to VP16 393–405 restricted by the different DRB1 alleles might be explained either by differences in the avidity of the TCR for the peptide-MHC complexes or by structural interactions between the peptide and MHC molecule alone. Figure 3C shows that the relative binding of VP16 393–405 to DR expressed on the various APCs varied greatly for the different DRB1 alleles and showed no correlation with the proliferative and cytolytic responses of ESL4.34 to VP16 393–405. While DRB1*1303 and DRB1*1103 bound the peptide as well as, or better than, their stimulatory DRB1*1301 and DRB1*1102 counterparts, no proliferative or cytolytic responses were observed using these ligands. This suggests that the ability of ESL4.34 to respond to its peptide-MHC ligand is controlled in part by the presenting MHC molecule itself, through specific interactions between residues on the TCR and the DR molecule. This hypothesis, however, does not exclude the involvement of the peptide in ESL4.34 responses, since no proliferation or cytotoxicity was observed in the absence of VP16 393–405, and the responses increased proportionately with peptide concentration (Fig. 2).

Specific HVR3 residues on HLA-DRB1 control ESL4.34 responses

The three DRB1 alleles (DRB1*0402, DRB1*1102, and DRB1*1301) that could present VP16 393–405 to ESL4.34 in

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Table 1. DRβ specificities of LCLs used as APC for VP16 393–405, their position 67 to 71 amino acid sequences, and responses of T cell clone ESL4.34 in proliferation and cytotoxicity assays

<table>
<thead>
<tr>
<th>DRB1 Allele</th>
<th>Position 67–71 Amino Acids</th>
<th>Relative Peptide Binding</th>
<th>ESL4.34 Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>*0402</td>
<td>ILEDE</td>
<td>167</td>
<td>+</td>
</tr>
<tr>
<td>*0404</td>
<td>LQR</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td>*1301</td>
<td>–K</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>*1303</td>
<td>–K</td>
<td>39</td>
<td>–</td>
</tr>
<tr>
<td>*1102</td>
<td>–K</td>
<td>69</td>
<td>+</td>
</tr>
<tr>
<td>*1103</td>
<td>FQR</td>
<td>94</td>
<td>–</td>
</tr>
<tr>
<td>*0402-Q70</td>
<td>QQR</td>
<td>86</td>
<td>–</td>
</tr>
<tr>
<td>*0402-L7687</td>
<td>LQR</td>
<td>74</td>
<td>–</td>
</tr>
<tr>
<td>*0402-L7687</td>
<td>LQR</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>*0402-Q70-L7687</td>
<td>QQR</td>
<td>23</td>
<td>–</td>
</tr>
</tbody>
</table>

* Peptide concentrations of 10 μM were used in binding assays.
proliferation and cytotoxicity assays are serologically and structurally distinct but share a common amino acid sequence, ILEDE, at DRβ positions 67 to 71, whereas the other DRβ1 alleles tested in the present study encode different amino acid sequences at this region. This suggests a role for this “shared epitope” in association with VP16 393–405 in eliciting ESL4.34 responses. To test this hypothesis, we generated a panel of LCLs expressing mutagenized DRB1 molecules that distinguish the stimulatory DRB1*0402 allele from the nonstimulatory DRB1*0404 molecule, which differ only at the three polymorphic residues in this epitope, residues 67, 70, and 71. Surface expression of these mutant DR4 molecules was comparable with that of wild-type DRB1*0402 and DRB1*0404 in YAR and MT LCLs, respectively (Fig. 4). Figure 5 shows the proliferative and cytolytic responses of ESL4.34 to VP16 393–405 presented by these mutant APCs and the relative binding affinities of this peptide for the mutant DR molecules. Binding was drastically reduced by substitutions at 67 and 71 (DRB1*0402-L67, R71) and substitutions at 70 and 71 (DRB1*0402-Q70, R71), but changes at residues 67 and 70 (*0402-L67,Q70, and *0402-Q70) had less significant effects on VP16 393–405 binding (Fig. 5C). In contrast, substitution of any of the DRB1*0402 residues 67, 70, or 71 completely abrogated ESL4.34 proliferative and cytolytic responses to VP16 393–405. These data, together with the responses of ESL4.34 to the DR4, DR11, and DR13 allelic variants (Fig. 3), confirm that DRβ residues I67, D70, and E71 are essential (and possibly sufficient) for presentation of VP16 393–405 to ESL4.34. The involvement of HVR3 in peptide binding and ESL4.34 responses does not exclude less significant contributions by the first and second HVRs of DRβ and/or the V/G dimorphism at position 86. Significant influences of the DRB3 and DRB4 gene products, which are encoded on DR11, DR13, and DR4 haplotypes, however, are unlikely, because these molecules have distinct HVR3 sequences.

HSV-2 VP16 393–405 binding motif

Molecular modeling of the VP16 peptide 393–405 complexed with DRB1*0402, DRB1*1102, and DRB1*1103 suggested a binding motif such that V394 occupies pocket 1 and R397 occupies pocket 4. This predicted binding motif was tested by synthesizing peptides with nonconservative substitutions at these anchor positions and measuring their relative binding to LCLs expressing DRB1*0402, DRB1*0404, DRB1*1301, DRB1*1303, DRB1*1102, and DRB1*1103 (Fig. 6). Substitution of valine at position 394 with lysine (P1 [V→K]) dramatically decreased the binding of this peptide to DRB1*0402, DRB1*1102, and DRB1*1103, whereas replacing leucine at position 393 with lysine (P→L393K [L→K]) slightly increased the level of binding to these alleles (Fig. 6). This indicates that V394 is the first anchor residue. The increase in binding observed for L393K (P→L393K) may be due to favorable interactions between K393 and DRα E53. Substitution of arginine at VP16 393–405 position 397 with the negatively charged residue glutamic acid (P4 [R→E]) reduced peptide binding to the DRβ alleles containing negatively charged residues at positions 70 or 71, whereas binding to DRB1*0404 which has arginine at DRβ position 71 was increased by this substitution (Fig. 6). This clearly demonstrates the importance of the negatively charged residue glutamic acid (P4 [R→E]) reduced peptide binding to the DRβ alleles containing negatively charged residues at positions 70 or 71, whereas binding to DRB1*0404 which has arginine at DRβ position 71 was increased by this substitution (Fig. 6).

The involvement of HVR3 in peptide binding and ESL4.34 responses does not exclude less significant contributions by the first and second HVRs of DRβ and/or the V/G dimorphism at position 86. Significant influences of the DRB3 and DRB4 gene products, which are encoded on DR11, DR13, and DR4 haplotypes, however, are unlikely, because these molecules have distinct HVR3 sequences. The Journal of Immunology

FIGURE 4. Flow cytometric analysis of DR4 expression by lymphoblastoid cells YAR (DRB1*0402), MT (DRB1*0404), and MAT (DRB1*0301) expressing mutagenized DRB1*0402 genes, using DR4-specific mAb NFLD.D1, which recognizes all DR4 subtypes. NFLD.D1 staining of untransfected MAT cells was negative.
VP16 393–405 are not anchor residues but that they may further stabilize the association by interacting with residues on the DR molecule.

To further analyze this binding motif, VP16 393–405 analogues with amino acid substitutions of predicted TCR contact residues were synthesized and tested for their capacity to bind to DRB1*0402 and to stimulate ESL4.34 in proliferation and cytotoxicity assays. Table II compares the DRB1*0402-binding and ESL4.34-stimulatory capacities of the minimum VP16-binding epitope VP16 393–403 and two analogues of this peptide with substitutions at the predicted TCR contact positions 5 (P5 [M→NL]) and 7 (P7 [F→I]) of the truncated peptide. Although VP16 393–403 bound to DRB1*0402 more weakly than the full length VP16 393–405 peptide, the proliferation and cytolytic responses of ESL4.34 to the two peptides were similar, indicating that the two C-terminal residues of VP16 393–405 are not involved in T cell responses. The binding of VP16 393–403 analogues with a phenylalanine to isoleucine substitution at position 400 (P7 [F→I]), or a methionine to norleucine substitution at position 398 (P5 [M→NL]), was better or slightly reduced compared with that of the VP16 393–403 peptide. However, the responses of ESL4.34 to these APCs were greatly diminished or completely abrogated (Table II), indicating that these peptide residues are TCR contact residues that can modulate T cell recognition while playing only a minor role in binding to DR.

Discussion

Despite the high degree of specificity of ligand recognition by TCRs, several reports have described T cells that can recognize

Table II. Relative DRB1*0402 binding affinity of peptide VP16 393–405, the truncation form VP16 393–403, and VP16 393-403 analogs with a phenylalanine to isoleucine substitution at position 400 (P7 [F→I]) or a methionine to norleucine substitution at position 398 (P5 [M→NL]), and proliferative and cytolytic responses of ESL4.34 to these ligands

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide Binding (Fluorescence Units/1000)</th>
<th>Proliferation (Stimulation Index)</th>
<th>Cytotoxicity (% Specific Lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP16 393–405</td>
<td>157.7</td>
<td>186.4</td>
<td>88.4</td>
</tr>
<tr>
<td>VP16 393–403</td>
<td>39.8</td>
<td>196.4</td>
<td>89.2</td>
</tr>
<tr>
<td>P7 (F→I)</td>
<td>62.4</td>
<td>17.3</td>
<td>15.4</td>
</tr>
<tr>
<td>P5 (M→NL)</td>
<td>23.9</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Peptide concentrations used were 10 μM in binding assays and 1 μM in proliferation and cytotoxicity assays. E:T ratios in cytotoxicity assays were 20:1.
and respond to multiple peptide-MHC complexes. Such multiple ligands can be provided by the presentation of multiple peptides by a single MHC molecule (46), presentation of a single peptide by multiple MHC molecules (47, 48), or ligands that are distinct in both the peptide and MHC portions (21, 49). TCR recognition involves specific molecular contacts with residues on both the MHC molecule and the bound antigenic peptide. Although the overall conformation of the peptide-MHC-TCR ternary complex is thought to be similar for all MHC and αβ TCR molecules (19, 21), the relative contributions of peptide and MHC to T cell specificity can vary greatly. We therefore have investigated the structural interactions between the TCR, MHC, and peptide for a CD4+ T cell clone, ESL4.34, which recognizes the VP16 393–405 peptide epitope of HSV-2 in the context of multiple distinct HLA-DR alleles. This clone, which was isolated from a DRB1*0402*1301 heterozygous individual, responds to the peptide presented by both parental DR alleles, as well as the allogeneic DR11 allele, DRB1*1102, in proliferation and cytotoxicity assays. These three alleles are serologically and structurally distinct, but they share a common amino acid sequence motif, ILEDE, at positions 67–71 of the DRβ polypeptide. The role of this sequence motif in supporting ESL4.34 stimulation was confirmed by testing an extended panel of LCLs expressing DR4, DR13, and DR11 variants which were negative for this sequence and by site-directed mutagenesis experiments in which DRB1*0404-specific substitutions were introduced into the DRB1*0402 gene at codons 67, 70, and 71. We found that proliferative and cytoplastic responses of ESL4.34 to VP16 393–405 exhibited an absolute dependence on the presence of DRβ I67, D70, and E71 (summarized in Table I). This restriction of T cell recognition appears to be due to a direct interaction between these residues and the TCR, since no correlation was found between the relative binding affinities of VP16 393–405 for HLA-DR and ESL4.34 reactivity, and some DRβ 67–71 ILEDE-negative alleles strongly bound the peptide but failed to stimulate the T cells. Thus, amino acid substitutions that had little or no effect on relative peptide binding, such as the single I67 to F67 substitution that converts DRB1*1102 to *1103; mutations that change DRB1*0402 to DRB1*0402-Q70 and DRB1*0402-L67,Q70; and substitutions that distinguish DRB1*1301 and DRB1*1303, totally abolished ESL4.34 recognition. This suggests that the responses of ESL4.34 are solely directed against HVR3 of the DRβ molecule, similar to allospecific responses, but our observations that no T cell recognition occurred in the absence of VP16 393–405 and the responses increased proportionately with peptide concentration (Fig. 2) confirm the Ag-specific nature of ESL4.34 stimulation.

Numerous studies using allelic variants of class II and in vitro mutagenized class II molecules with substituted peptide analogues or using sequence analysis of naturally processed peptides presented by class II, have identified residues on MHC molecules that are critical for peptide binding (5, 17, 50). However, our observation that a nonpolar alanine residue at position P4 (V394 and Q405; P11 and P12) did not. Substitutions of M398 (P5) and F400 (P7) had small effects on peptide binding but substantially reduced ESL4.34 T cell responses, indicating that these changes influence TCR contact sites. The crystal structure of DRB1*0401 complexed with a human collagen II peptide (14) has confirmed previous predictions that the residue at DRβ position 71 influences the peptide that can be bound by forming a salt bridge with the side chain of the residue at peptide position P4 (5, 17, 50). Thus, while peptides with negatively charged P4 residues can bind to DRB1*0401 and other alleles with lysine or arginine at DRβ 71, peptides with positively charged P4 residues (lysine, arginine, or histidine) can bind to alleles expressing the ILEDE epitope at DRβ 67–71, such as DRB1*0402, DRB1*1102, and DRB1*1301. However, our observation that a nonpolar alanine residue at position P4 also supported good binding to DRB1*0404 suggests that size constraints are also important for this interaction.

In the diagonal binding mode observed in the class I MHC-peptide-TCR crystal structures, the CDR1 loops interact with the peptide termini, the CDR2 loops interact predominantly with the MHC α helices, and the CDR3 loops cover the central portion of the MHC-peptide ligand (18–20). In the absence of crystallographic data for class II MHC-peptide-T complex systems, a similar diagonal binding orientation has been predicted based on shared properties of the class I and class II MHC molecules (19, 21). It is interesting to note the presence of three arginine residues in the CDR3 sequence of the ESL4.34 TCR α chain and an arginine at position 97 in CDR3 of the TCR β chain. A binding configuration analogous to the crystal structures of the class I MHC-peptide-T complex would position the ESL4.34 CDR3 loops such that these arginines could potentially interact with residues of the negatively charged ILEDE epitope.

Our data indicate that although ESL4.34 displays promiscuous recognition of MHC class II molecules, its activation requirements are highly specific and depend on both the presence of the DRβ 67–71 ILEDE sequence and the VP16 393–405 peptide. Although multiple DR alleles expressing the ILEDE epitope were able to present this peptide to ESL4.34, conservative substitutions at this HVR eliminated T cell reactivity. Although the presence of the
specific recognition of particular DR reminiscent of recognition patterns of alloreactive T cells, in which this respect, the pluriallelic T cell recognition by clone ELS4.34 is nonconservative substitutions or unrelated peptides (27–32). In this respect, the pluriallelic T cell recognition by clone ELS4.34 is nonconservative substitutions or unrelated peptides (27–32). In this respect, the pluriallelic T cell recognition by clone ELS4.34 is nonconservative substitutions or unrelated peptides (27–32).

Instead T cell recognition appears to depend on specific interac-

the relative affinity of the peptide-DR interaction does not have a reflect the influence of the HVR3s of both parental alleles in biasing associated with possession of either HVR3 alone. This might reflect the influence of the HVR3s of both parental alleles in biasing the selection of T cells that exhibit promiscuous recognition of Ags in the context of both alleles, potentially contributing to the spec-

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