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*J Immunol 2006; 177:3884-3892; doi: 10.4049/jimmunol.177.6.3884
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Crystallographic Structure of a Rheumatoid Arthritis MHC Susceptibility Allele, HLA-DR1 (DRB1*0101), Complexed with the Immunodominant Determinant of Human Type II Collagen

Edward F. Rosloniec, Robert A. Ivey III, Karen B. Whittington, Andrew H. Kang, and Hee-Won Park

The expression of HLA-DR1 (DRB1*0101) is associated with an enhanced risk for developing rheumatoid arthritis (RA). To study its function, we have solved the three-dimensional structure of HLA-DR1 complexed with a candidate RA autoantigen, the human type II collagen peptide CII (259–273). Based on these structural data, the CII peptide is anchored by Phe263 at the P1 position and Glu266 at P4. Surprisingly, the Lys at the P2 position appears to play a dual role by participating in peptide binding via interactions with DRB1-His81 and Asn82, and TCR interaction, based on functional assays. The CII peptide is also anchored by the P4 Glu266 residue through an ionic interaction with DRB1-Arg71 and Glu28. Participation of DRB1-Arg71 is significant because it is part of the shared epitope expressed by DR alleles associated with RA susceptibility. Potential anchor residues at P6 and P9 of the CII peptide are both Gly, and the lack of side chains at these positions appears to result in both a narrower binding groove with the peptide protruding out of the groove at this end of the DR1 molecule. From the TCR perspective, the P2-Lys264, P5-Arg267, and P8-Lys270 residues are all oriented away from the binding groove and collectively represent a positive charged interface for CII-specific TCR binding. Comparison of the DR1-CII structure to a DR1-hemagglutinin peptide structure revealed that the binding of these two peptides generates significantly different interfaces for the interaction with their respective Ag-specific TCRs.

The Journal of Immunology, 2006, 177: 3884–3892.

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1 This work was supported in part by grants from the Department of Veterans Affairs, Memphis, TN (to E.F.R. and A.H.K.), by U.S. Public Health Service Grants AR39166 and AR47379 (to E.F.R. and A.H.K.) from the National Institute for Arthritis and Musculoskeletal Diseases, and the American Lebanese Syrian Associated Charities.

2 E.F.R. and R.A.I. contributed equally to this work.

3 Abbreviations used in this paper: RA, rheumatoid arthritis; CII, type II collagen; CIA, collagen-induced arthritis; OeG, octyl glucoside; PB, phosphate buffer; HA, hemagglutinin.

Received for publication January 20, 2006. Accepted for publication June 16, 2006.
chains of the amino acids located at these positions within the antigenic peptide insert into binding pockets formed by the first domains of the class II molecule. In our previous analysis of the binding of the CII (259–273) peptide to HLA-DR1, a number of variations from the P1-P4-P6-P9-binding motif were observed (20). The CII peptide binds with a low affinity and binding appears to be dependent primarily on the P1 and P2 residues with some contribution of the P4 residue. Because P6 and P9 are Gly residues in the CII peptide, there are no side chains to interact with their corresponding DR1-binding partners. Because these interactions differ significantly from those of other structural studies of peptide binding to DR1 (21), and based on the potential role of the DR1-CII complex in the pathogenesis of RA, we solved the crystallographic structure of CII (259–273) bound to HLA-DR1 (DRB1*0101) to identify the molecular interactions between this peptide and the DR1 molecule. Combined with functional data from peptide binding and T cell stimulation, this structure provides essential insights into the presentation of an autotigent with a low affinity for the DR molecule.

Materials and Methods

Protein production

Soluble DR1 was purified from culture supernatants of S2 Drosophila cells transfected with DRB1*0101 and DRA1*0101 as described previously (22). Both the α- and β-chains of the DR1 constructs contain murine I-E leader sequences followed by DRα or DRβ first domains and murine I-Eα and I-Eβ second domains. The immunodominant peptide, residues 259–273, was inserted following the third residue of the β-chain, and a flexible [Gly]4-Ser linker was added that allowed the peptide to fold into the binding groove of the DR molecule (22, 23). The sequences encoding the cytoplasmic and transmembrane portions of these molecules were deleted from the cDNA using PCR, and for the α-chain, a new stop codon was inserted at the 3' end of the second domain. The β-chains were altered by adding a linker and a biotinylation site 3' to the Eβ second domain as described by Crawford et al. (24). The resulting cDNA was cloned into the Drosophila expression vector pRmHA-3 (gift from Dr. D. Zaller, Merck Research Laboratories, Rahway, NJ). S2 cells were transfected with a 10:10:1 ratio of chimeric DRB1:DRA1:pUChSNeo using calcium phosphate precipitation. Soluble DR1-CII production was induced by 1 mM CuSO4, and 5 days later the culture supernatant was collected and infected with a 10:10:1 ratio of chimeric DRB1:DRA1:pUChSNeo using calcium phosphate precipitation. Soluble DR1-CII production was induced by 1 mM CuSO4, and 5 days later the culture supernatant was collected and adjusted to 0.05% octyl glucoside (OgG). DR1-CII was purified by passage of the culture supernatant over an affinity column coupled with the anti-DR Ab LB3. The column was washed with 0.05% octyl glucoside and 0.15 M NaCl in phosphate buffer (PB) (pH 7.5), followed by 0.05% OgG and 0.5 M NaCl in PB (pH 7.5), and finally washed with 10 mM Tris in 0.5 M NaCl (pH 7.5). DR1-CII was eluted with 100 mM Tris, 0.5 M NaCl (pH 11.2), and the fractions were immediately neutralized with acetic acid. Recovered DR1-CII was concentrated using an Amicon Stirred Cell, quantified by OD280 absorption and ELISA, and evaluated by SDS-PAGE for purity before use in functional assays.

For crystallization experiments, the DR1-CII fusion protein was prepared as described above with an additional ion exchange chromatographic step to increase purity to >95%. The protein sample was loaded onto a Q-Sepharose column (GE Healthcare) equilibrated with 30 mM Tris-HCl (pH 7.5), 30 mM NaCl and eluted with a linear gradient of increasing NaCl. DR1-CII eluted at ~140 mM NaCl. Finally, the protein was concentrated to 20 mg/ml and stored at 4°C.

Crystalization

Crystals of the fusion protein DR1-CII were grown by the hanging drop vapor diffusion method. Before crystallization, the protein was dialyzed into 10 mM Tris-HCl (pH 7.0)/1 mM EDTA. Hanging drops containing equal volumes of the protein and reservoir solutions were equilibrated at 18°C against the reservoir solution (20% polyethylene glycol 3350 and 200 mM ammonium chloride). After 4 mo, orthorhombic crystals appeared. The crystals were transferred to 50/50 Paratone-N/Mineral Oil for cryocarta collection.

X-ray diffraction data collection and structure determination

Single wavelength native data were measured from a single crystal of DR1-CII with a MAR225 CCD detector operating at 100 K at the SER-CAT beam line 22-ID at the Advanced Photon Source of the Argonne National Laboratory. The data set was indexed, integrated, and scaled with the program mosflm (25). The crystal belonged to space group P212121 with unit cell dimensions a = 60.7 Å, b = 112.4 Å, and c = 170.8 Å. Data collection statistics are summarized in Table I.

The structure of DR1-CII was determined by the molecular replacement method using one protein heterodimer from the HLA-DR1-bacterial superantigen staphylococcal enterotoxin B (PDB ID ISEB; Ref. 26) as a search model. Cross-rotation and translation functions and Patterson correlation refinement were calculated using the program CCP4 (27). Data between 10.0 and 4.0 Å were used with the rotation function and generated two outstanding solutions corresponding to two DR1-CII heterodimers in the asymmetric unit. Two iterations of applying the translation function to each solution allowed determination of the orientation and position of the second heterodimer. The stacked dimer of dimers in the asymmetric unit is similar to other published DR1 structures (21, 28). The model of two heterodimers was subjected to rigid body refinement using data between 6.0 and 4.0 Å in the program CNS (29) and Refmac5 (27) further lowered both R factors. The electron density map of the CII peptide is shown in Fig. 1B. The final refinement statistics are shown in Table I. Because of very slight differences between the two heterodimers, noncrystallographic symmetry restraints were applied to only 92% of the total residues for each subunit.

Table I. Data collection and refinement statistics

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<th>Parameter</th>
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<th>Number of reflections</th>
<th>Measured</th>
<th>Unique</th>
<th>Completeness</th>
<th>Overall</th>
<th>Last shell*</th>
<th>Rmerge (%)</th>
<th>Average</th>
<th>Refinement</th>
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<td>20,455</td>
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<td>97.1</td>
<td>10.0 (36.3)</td>
<td>10.2</td>
<td>2.6 (2.6)</td>
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Rmerge = Σhkl,i | Fobs,i | - | Fcalc,i | / | Σhkl,i | Fobs,i | * Icutoff = 0.234 (0.307) Rwork = 0.279 (0.394)

Number of reflections (F > 3σ(F)) 19,334 Number of protein atoms 6,187 Number of water molecules 41 Stereochemistry Rmsd bond length (Å) 0.008 Rmsd bond angles (°) 1.19 Average B factors (Å²) 41.9/42.9

Residues from Ramachandran plot

Most favored regions (%) 90.8 Additional allowed regions (%) 8.6 Generously allowed regions (%) 0.6

a Rmerge = Σhkl,i | Fobs,i | - | Fcalc,i | / | Σhkl,i | Fobs,i | * Icutoff = 0.234 (0.307) Rwork = 0.279 (0.394)

Resolution range: 3.18–3.10 Å Resolution (Å) 30.0–3.1

Rmerge = Σhkl,i | Fobs,i | - | Fcalc,i | / | Σhkl,i | Fobs,i | * Icutoff = 0.234 (0.307) Rwork = 0.279 (0.394)
Ag presentation assay

Ag presentation experiments were performed in 96-well microtiter plates in a total volume of 0.3 ml containing 4 x 10^5 syngeneic spleen cells as APCs, 10^5 T-hybridoma cells, and 100 µl of the CII (259–273) peptide at various concentrations in DMEM complete (DMEM supplemented with 10% FBS, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine). Cell cultures were maintained at 37°C in 5% humidified CO2 for 24 h, after which 80% 10% FBS, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine). Cell cultures were maintained at 37°C in 5% humidified CO2 for 24 h, after which 80% were removed from each well and 2-fold serial dilutions were made through each row of the plate. IL-2-dependent HT-2 cells (6 x 10^5) were then added to each well of the 96-well plate and following an 18-h incubation, HT-2 cell viability was assessed by cleavage of MTT and quantitation of the OD_{690} with background absorption at 560 nm subtracted (31, 32). IL-2 titers were quantified by the reciprocal of the highest 2-fold serial dilution maintaining HT-2 cell viability >2-fold over control cultures. Results are presented as units of IL-2 per milliliter of undiluted supernatant as described by Kappler et al. (33).

DR1-binding assay

Soluble DR1 and DR4 were purified from culture supernatants of S2 Drosophila cells transfected with DRB1*0101 or DRB1*0401 and DRA1*0101 as described previously (22). The cytoplasmic and transmembrane portions of these molecules were deleted from the cDNA using PCR, a new stop codon was inserted immediately before the transmembrane domain, and the resulting cDNA was cloned into the Drosophila expression vector pRmHA-3. S2 cells were transfected with a 10:10:1 ratio of DRB1:DR4:Drosophila using calcium phosphate precipitation. Soluble DR production was induced by 1 mM CuSO_4, and 5 days later the culture supernatant was collected and adjusted to 0.05% OEG. Soluble DR was purified by passage of the supernatant over an affinity column coupled with the anti-DR Ab L227. The column was washed with 0.05% OEG and 0.15 M NaCl in PB (pH 7.5), followed by 0.05% OEG and 0.5 M NaCl in PB (pH 7.5). DR was eluted with 100 mM Tris, 0.5 M NaCl (pH 11.2), and the fractions were immediately neutralized with acetic acid. Recovered DR was concentrated using an Amicon Stirred Cell and quantitated by OD_{280} absorption and SDS-PAGE before use.

For binding assays, a 10 nM solution of purified DR1 was incubated for 18 h at 37°C with hemagglutinin (HA) (307–319) peptide (0.5 nM) that had been labeled at the NH2 terminus with biotin as previously described (20). Various concentrations of collagen peptides were added as competitors to HA peptide binding. Bound peptides were separated from free peptides by immobilizing the DR molecules on microtiter plates coated with the mAb L227 and subsequent washing. The Ab was adhered to the plate by an overnight incubation of a 10 µg/ml solution at 4°C. Bound biotinylated peptides were detected by incubations with streptavidin-europium followed by a chelating enhancement solution. Fluorescence was quantitated using a microplate fluorometer (FluoroMark; Bio-Rad), and data are expressed as relative fluorescence units measured. Each binding assay was performed in duplicate, and data are representative of three experiments.

Results

Structure of the CII peptide

Based on the electron density, the CII peptide was bound by the DR1 molecule in an extended conformation typical of most class II bound peptides, although some differences in comparison to other peptides bound by HLA-DR1 were evident (21). As shown in Fig. 1, the CII peptide has only two side chains capable of interacting with the binding pockets in the DR1 molecule, with the Phe263 side chain occupying the P1 pocket, and Gln267 occupying the P4 pocket. CII amino acid side residues that would normally interact the P6- and P9-binding pockets of the DR1 molecule are both Gly and therefore have no side chain to interact with DR1. The net effect appeared to be that, although the N-terminal and central portions of the CII peptide resided fully in the DR1-binding groove, the C-terminal residues emerged almost completely from the binding cleft (Fig. 1A). Yet, based on the electron density maps, the overall conformation of the entire CII peptide was rigid within the DR1 structure (Fig. 1B). As viewed from the perspective of the TCR, the DR1-CII complex had three prominent side chains from the CII peptide pointing toward the TCR interface. The side chains on residues Lys264 (P2), Gln267 (P5), and Lys270 (P8) were all oriented away from the binding cleft and toward the aqueous environment, indicating likely interaction sites with CII-specific TCRs (Fig. 1). The positions of the CII-peptide side chains extending out of the binding groove from the N terminus of the peptide (residues 259, 260, and 261), as well as those forming the covalent linker, a repeating Gly4Ser sequence, could not be determined because they are disordered in the structure.

FIGURE 1. Crystal structure of the CII (259–273) peptide bound to HLA-DR1 (*0101). A. Side view of the DR1-CII complex with the DRB1 chain in the front (light gray ribbon) and the DRA1 chain in the back (dark gray ribbon). The CII peptide (yellow backbone) is oriented as in B, and is represented by residues 261 through 273. B. The Fo–Fc (magenta) and 2Fo–Fc (blue) electron density maps of the structure of the CII peptide indicate that the overall structure of the CII peptide is rigid when bound to the DR1 molecule. The maps were contoured at 2.5 and 1.0 σ, respectively.

FIGURE 2. Interaction of the CII Phe263 with the P1 pocket of DR1. The side chain of the Phe263 residue of the CII peptide (yellow) inserts deep into the highly hydrophobic P1 pocket. Side chains shown in green indicate the hydrophobic residues of both the DRB1 and DRA1 chain that form the P1 pocket.
Peptide interactions with the DR1 molecule

Despite using only two of four conventional binding pockets of the DR1 molecule, the CII peptide bound efficiently to DR1 through three sets of interactions along the peptide’s N-terminal half. First, the P1 residue of the CII peptide, Phe, was anchored in the hydrophobic pocket of HLA-DR1 formed primarily by residues Phe\textsuperscript{24}, Ile\textsuperscript{31}, Phe\textsuperscript{32}, and Phe\textsuperscript{48} on the DR1 α-chain, and residues Val\textsuperscript{85} and Phe\textsuperscript{99} of the DR1 β-chain (Fig. 2). The structure of this P1-binding pocket, with its hydrophobic side chain interactions, was similar to that of other DR1 structures previously described (21), and its role as a primary anchor in the binding of the peptide was supported by our functional data (Fig. 3A). Replacement of P1\textsubscript{Phe} in the CII peptide with an Ala resulted in a peptide whose relative affinity for DR1 was below the detection limits of the assay. However, based on the Ag presentation assays in Fig. 4, it was clear that this analog peptide had limited ability to bind to DR1, as the T cells with the highest affinity receptors for the mutant complex were stimulated with very high concentrations of the Ala-substituted peptide (Fig. 4, A and B).

**FIGURE 3.** Functional analysis of the role of the P1, P2, and P4 residues in the binding of the CII (259–273) peptide to DR1. The binding of analog CII peptides containing single Ala substitutions to the DR1 molecule was measured using a competitive binding assay using biotinylated HA (307–319) and avidin-europium time resolved fluorescence. Data shown are relative fluorescent units of HA peptide binding to DR1 in the presence of increasing concentrations of the CII analog peptides. A, Ala replacement of Phe\textsuperscript{263} (F263A) or Lys\textsuperscript{264} (K264A) significantly reduced the affinity of the peptide by 100-fold. B, Role of E266 in binding of the CII peptide to DR1. Ala replacement of E266 (E266A) increased the affinity of the peptide by nearly 10-fold, where as replacement of the Glu\textsuperscript{266} with Asp, the naturally occurring residue in murine CII, decreased the affinity of the peptide by 100-fold. Data are based on duplicate samples for each point and a minimum of two experiments.

Surprisingly, the Lys\textsuperscript{264} at the P2 position also appeared to be involved in binding the CII peptide to the DR1 molecule. As shown in Fig. 3, replacement of this residue with an Ala greatly decreased the affinity of this peptide for the DR1 molecule. In Fig. 5, the structure indicated that the side chain of P2\textsubscript{Lys} was not oriented toward any of the putative binding pockets within the DR1 molecule but was stacked against His\textsuperscript{81} and also appeared to interact with Asn\textsuperscript{82}, both located on a α-helical portion of the DR1 β-chain. Based on the predicted binding motif of P1-P4-P6-P9 for the DR1-CII complex, with the highest affinity clone in A and the lowest in D, Relative affinities of the T cell hybridomas were defined on the basis of peptide titration curves using wild-type CII peptide. Weak responses were detected to the F263A peptide (Ala substituted for Phe) by the two highest affinity T cells, indicating that this peptide can bind although, as shown in Fig. 3, at very low affinity. The T cell recognition of the K264A peptide (Ala substituted for Lys) was highly clonotypic, only the DR1–16 clone responded (C), and this clonotypic recognition appeared to be independent of the affinity of the TCR. Data are representative of a minimum of three experiments.

**FIGURE 4.** Functional analysis of the role of the P1 and P2 residues in the presentation of the CII (259–273) peptide to DR1-restricted, CII-specific T cells. The CII analog peptides from Fig. 3A were used in Ag presentation experiments to determine the role of these residues in T cell recognition of the DR1-CII complex. A–D, The response of four different T cell hybridoma clones that are specific for CII (259–273) and restricted by the DR1 molecule. T cells are arranged in order of their affinity for the DR1-CII complex, with the highest affinity clone in A and the lowest in D. Relative affinities of the T cell hybridomas were defined on the basis of peptide titration curves using wild-type CII peptide. Weak responses were detected to the F263A peptide (Ala substituted for Phe) by the two highest affinity T cells, indicating that this peptide can bind although, as shown in Fig. 3, at very low affinity. The T cell recognition of the K264A peptide (Ala substituted for Lys) was highly clonotypic, only the DR1–16 clone responded (C), and this clonotypic recognition appeared to be independent of the affinity of the TCR. Data are representative of a minimum of three experiments.
β-chain, which in turn was in contact with another DR1 β-side chain residue, Glu28. Peptide-binding studies focused on this residue, in combination with the structural data, indicated a complex relationship between side chains preferred in this binding pocket and the overall binding affinity of the peptide (Fig. 3B). Conservative replacement of the P4 Glu with an Asp resulted in a peptide that had nearly a 100-fold lower affinity for the DR1 molecule, likely due to the shorter side chain of the Asp that placed the negatively charged side chain in an unfavorable position near the residues of the DR1 β-chain. However, insertion of an Ala at this position resulted in a peptide that had a 10-fold higher affinity for binding to DR1 in comparison to the wild-type peptide, and these data were in agreement with other studies indicating that a negative charge at the P4 position was not favored by DR1 (21, 34). The ability of the DR1-CII-P4Ala complex to be recognized by T cells was also greatly affected. Only the T cells with the highest affinity for DR1 were able to respond (Fig. 7). Conversely, T cells was arranged in order of their affinity for the DR1-CII complex, with the highest affinity clone in A and the lowest in D. Despite the fact that the CII peptide containing the Ala substitution (E266A) had a higher affinity for DR1 than the WT peptide, only the highest affinity T cell was able to recognize this complex. In contrast, the low affinity Asp substituted peptide (E266D) complexed with DR1 was able to stimulate three of the four T cells and in a manner that appears to be related to the affinity of the TCR.

The CII peptide backbone formed 13 hydrogen bonds with HLA-DR1 (data not shown). In comparison, 15 hydrogen bonds were observed between the HA peptide backbone and DR1 in the DR1-HA structure (21). The same DR1 residues are involved in the hydrogen bonds in the DR1-HA structure (21). The exception is His81 of the DR1 β subunit. The side chain of His81 forms a hydrogen bond with the HA peptide backbone in the DR1-HA structure whereas this hydrogen bond is not observed in the DR1-CII structure. Instead, the side chain of His81 interacts with the hydrophobic portion of P2lys of the CII peptide, helping DR1 bind to the CII peptide (Fig. 5). The multiple hydrogen bonds of the HA peptide backbone were suggested to contribute substantially to the binding of HA peptide to HLA-DR1 (21). Similarly, the hydrogen bonds involving the CII peptide backbone may play a significant role in mediating DR1-CII interactions.

**Peptide-specific changes in DR structure**

Because the CII peptide used an unusual binding motif for DR1, we sought to determine whether there were any significant structural differences between the CII peptide or DR1-CII molecule and other DR1-peptide structures that have been studied. As shown in Fig. 8A, an alignment of the α-carbon backbones of the CII and HA (308–316) peptides indicated there were differences in their overall shape when held within corresponding, prealigned DR1-binding clefts. There were clear differences in the rotation of the backbone at the N terminus, despite the fact that both peptides used side chains with large hydrophobic rings for binding to the P1 pocket (Phe for CII, Tyr for HA). These differences also include the shape of the complexed DR1 molecule. As can be seen in Fig. 8B, the first domains of the α- and β-chain of the DR1 molecule with a bound CII peptide showed a significant longitudinal shift compared with DR1 bound to the HA peptide. This was especially true of the α-helical portion of the N-terminal domains, and more
than the DR1-CII structure. DR1-HA structure being more closed across the binding groove differences in the compactness of these surfaces, with the variations in the structure of the α-carbon backbones of these two peptides are evident, despite using the same binding pockets in the DR1 molecule. A significant shift in the location of the α-helical portions of the DRA1 and DRB1 chains is clearly evident with the DR1-CII α-helical structure (yellow) elongated in comparison to the DR1-HA structure (blue).

So of the β-chain than the α-, although changes in both are clearly evident (average shifts are 0.51 and 1.00 Å for the α- and β-chains, respectively). These changes did not appear to be artifacts of crystallization, since differences in the β-pleated sheets of the first domains were minor, as were the C-terminal domains of both chains, despite that fact that one was HLA-DR and the second was derived from mouse I-E (see Materials and Methods). These differences in the conformations of the N-terminal domain of the DR1 molecule could also be seen in the surface potential maps in Fig. 9. As viewed from the perspective of the TCR, a number of the DR1 side chains had different orientations when comparing the HA and CII models. For example, the βHis<sup>81</sup> residue rotated inward toward the Lys<sup>264</sup> residue in the peptide, while in the DR1-HA molecule it was rotated away from the P4 Gln residue (Fig. 9, red arrows). Similar differences were evident for other surface residues including αGln<sup>57</sup> (white arrows) and βAsp<sup>66</sup> (green arrow). In addition, differences between these two DR1 molecules were also evident along the binding groove, especially in the region of the P6 and P9 pockets. Changes in the orientation of the side chains of residues αAsn<sup>62</sup>, βTrp<sup>61</sup>, and βTyr<sup>60</sup> were obvious around these pockets, resulting in clear differences in the compactness of these surfaces, with the DR1-HA structure being more closed across the binding groove than the DR1-CII structure.

### Discussion

The crystal structure of HLA-DR1 complexed with the CII (259–273) peptide reveals several structural characteristics that differ from other HLA-DR-β-peptide complexes. First, unlike the HA peptide that uses all four binding pockets (21), the CII peptide uses only two of the four binding pockets in the DR1 molecule, P1 and P4, the effects of which are reflected in its overall low binding affinity (20). Second, despite having its side chain oriented in a direction that would be considered favorable for TCR interaction, the P2 residue Lys<sup>264</sup> of the CII peptide interacts with the helical portion of the DRβ1 chain, providing significant binding energy for the peptide. The primary interaction of Lys<sup>264</sup> is with the βHis<sup>81</sup> residue, and a distinct change in the orientation of this His side chain is clear in the DR1-CII structure in comparison to its location in the DR1-HA structure, where the P2 residue is Val (21). In addition, replacement of Lys<sup>264</sup> with an Ala residue greatly reduces the binding affinity of the CII peptide. Finally, significant differences are observed between the crystal structures of the DR1-CII and the DR1-HA molecules. In addition to the peptide binding groove of the DR1-CII structure being more closed toward the C terminus of the CII peptide, the conformations of the surface of these two DR1 structures represent very different targets for the TCR. A number of differences in side chain orientations as well as compactness of the structures are obvious in comparing the DR1 molecules first domains in the CII and HA crystal structures.

A role for the P2 Lys<sup>264</sup> residue in the binding of the CII peptide to the DR1 molecule would not have been predicted based on a prototypical binding motif of P1-P4-P6-P9 for DR molecules. Functional studies indicated that replacing the Lys<sup>264</sup> with an Ala residue decreased the affinity of the CII peptide for DR1 by at least 100-fold, and the structural data indicates that this Lys residue interacts with βHis<sup>81</sup> and βAsn<sup>52</sup> of the DR molecule. Whether these interactions are solely responsible for the affinity changes...
been proposed to explain the function of the shared epitope in RA. Conversely, the Ala substitution at this position may have caused a change in the overall structure of the CII peptide that makes it more difficult for the peptide to use the P1 or P4 anchors, thus reducing the overall affinity. In addition to peptide binding, the orientation of the Lys264 side chain also places it in a position that would be highly favorable for TCR interaction. Ag presentation studies with CII-specific T cells indicated a clonotypic pattern of recognition of this CII residue, with most TCR requiring this residue be present in the DR1 complex. Collectively, CII residues Lys264 (P2), Gln267 (P5), and Lys270 (P8) represent the most likely targets for TCR interaction, and, as a group, represent a positively charged interface for the interaction with CII-specific TCR. The two Lys residues are of particular interest in that both can be hydroxylated in the native CII molecule, and the Lys264 can also be glycosylated (35). The expression system, we used for production of the rDR1-CII precluded our ability to analyze the potential role of these side chain modifications, although both of these modifications may play a role in CII-specific T cell stimulation (36–38).

Comparison of the structural and functional data concerning the interaction of the CII residue Glu266 with the P4 pocket indicated complex changes in the DR1 conformation may occur with the use of the analog peptides. Structurally, the Glu266 residue is clearly buried in the P4-binding pocket and is thus an anchor residue for the CII peptide. Although this residue would be predicted to occupy the P4 pocket based on the DR1-HA structure, the DR1 molecule does not prefer a negative charge at this location because the high-affinity HA peptide has a Gln at this position (21). When Glu266 of the CII peptide was replaced with an Ala, the binding affinity of the CII peptide increased significantly, yet the ability of the CII-specific T cells to recognize this complex was poor. In contrast to the Ala substitution, if the Glu266 is replaced by an Asp, the wild-type residue in murine CII, the binding affinity dropped 10-fold, yet the majority of the CII-specific T cells still recognizes the CII peptide. Thus, without the structural data, the Glu266 would have been considered a primary TCR interaction site, yet our structure clearly indicates that it serves as an anchor residue whose orientation precludes direct TCR interaction. These data raise several remarkable questions concerning the use of analog peptides in functional studies to identify MHC anchor or TCR interaction residues. Based on our data, these seemingly simple substitutions appear to have broad-ranging effects on the DR-peptide complex, and may cause alterations in the conformation of the peptide or even possibly the conformation of the DR molecule. For example, the Ala substitution increased the affinity of the peptide for the DR1 molecule, yet significantly altered its recognition by the TCR, indicating that more changes may have occurred to this structure than just a side chain substitution. In contrast, the Asp substitution at P4 appears to affect only the DR1-binding affinity of the peptide, as T cells generally recognized this analog in a manner that was directly related to the affinity of the TCR.

Susceptibility to RA is tightly associated with the expression of several HLA-DRβ chains that all share the same DRβ amino acid sequence at residues 70–74, Gln-Arg/Lys-Arg-Ala-Ala, termed the shared epitope (39, 40). Although several hypotheses have been proposed to explain the function of the shared epitope in RA (39, 41–44), its role in mediating the susceptibility to this disease remains unclear. In the DR1-CII structure described here, the DRβ Arg71 of the shared epitope plays a key role in anchoring the CII Glu266 residue in the P4-binding pocket. The side chain of DRβ Glu70 also points toward the binding groove but does not appear to directly interact with any of the side chains of the CII peptide. The rest of the shared epitope residues either points away from the binding groove, or their side chains are located too far from the CII peptide to make contact. Despite limited interaction between HLA-DR1 and the CII peptide, the overall molecular organization of these five shared epitope residues may still impart specific properties on the P4-binding pocket that enhance the pathogenic nature of the DR1 molecule as it relates to susceptibility to RA.

Comparison of the conformations of the bound peptides and the N-terminal domains of the DR1-CII and DR1-HA structure reported by Stern et al. (21) indicates some significant differences in the conformations of these molecular complexes. When bound by the DR1 molecule, the CII and HA peptides are in significantly different orientations in terms of the rotational periodicity of their α-helical backbone. The CII peptide is bound by the DR molecule in an extended helical form reminiscent of its origins as a collagen peptide, whereas the backbone of the HA peptide forms a more compact helix when bound to the DR1 molecule. In addition, the conformation of the DR-HA and DR1-CII structures are noticeably different in terms of the surface that would be perceived as the TCR contact region. Alignment of the α-carbon backbones of these two molecules indicated that the α-helical regions of the DR1-CII N-terminal domains were less compact than the DR1-HA structure, yet the β-pleated sheets forming the floor of the binding groove are almost structurally identical. Using a surface potential map of these structures, variations in the location of several of the DR1 amino acid side chains around the peptide binding grooves are evident, and this is reflected in the differences in the widths of the peptide-binding groove with the DR1-CII structure. Although not fully tested, it is likely that these differences in the conformation of the DR1 molecule reflect the variations in the affinity of these two peptides, with the HA peptide having at least 10-fold higher affinity and forming a more tightly packed DR1-peptide complex. These differences in DR1 conformation are most pronounced at the C terminus of the bound peptides where the CII peptide has no anchor residues for the P6 and P9 pockets. Because the CII peptide is a potential autoantigen, it is intriguing to speculate that there may be an association between the generation of autoimmune T cell responses and MHC-peptide complexes that assume alternate conformations or rely on nontraditional anchor residues to achieve sufficient binding affinity. Indeed, a number of similarities exist between the DR1-CII structure and other HLA-DR structures associated with the development of autoimmune disease (45, 46). Like the interaction between the CII peptide and the DR1 molecule that is focused on the N portion of the CII peptide, TCR recognition of myelin basic protein presented by two DR2 molecules, DRB1*1501 and DRB5*0101, occurs primarily through contact of the TCR with the N-terminal portion of the myelin basic protein autoantigen. This skewed structural interaction appears to be unique to these autoantigens, as TCR structures interacting with microbial Ags presented by class II molecules are primarily focused on the central portion of the peptide as it is bound by the class II molecule (47–50). Although the structure of a TCR interacting with the DR1-CII molecule is yet to be resolved, the fact that the CII peptide is held tightly in the DR-binding groove only at its N terminus in contrast to its weak interactions at the C terminus, supports the notion that the TCR interaction with this complex may also be focused on the N terminus of the CII peptide. The most intriguing aspect of this altered geometric interaction between TCR and MHC:autoantigens is that it may enhance the probability of molecular mimicry playing a role in the generation
of autoimmunity due to the reduced surface area involved in the interaction and resulting low affinity. Recent evidence to support this possibility has been demonstrated in both class II (45, 51) and class I (52–54) structural and functional studies.

Acknowledgments

We thank Dr. Shehab Ismail for assisting in structural refinement.

Disclosures

The authors have no financial conflict of interest.

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