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To better understand TCR discrimination of multiple ligands, we have analyzed the crystal structures of two Hb peptide/I-E\(^k\) complexes that differ by only a single amino acid substitution at the P6 anchor position within the peptide (E73D). Detailed comparison of multiple independently determined structures at 1.9 Å resolution reveals that removal of a single buried methylene group can alter a critical portion of the TCR recognition surface. Significant variance was observed in the peptide P5-P8 main chain as well as a rotamer difference at LeuP8, ~10 Å distal from the substitution. No significant variations were observed in the conformation of the two MHC class II molecules. The ligand alteration results in two peptide/MHC complexes that generate bulk T cell responses that are distinct and essentially nonoverlapping. For the Hb-specific T cell 3.L2, substitution reduces the potency of the ligand 1000-fold. Soluble 3.L2 TCR binds the two peptide/MHC complexes with similar affinity, although with faster kinetics. These results highlight the role of subtle variations in MHC Ag presentation on T cell activation and signaling. *The Journal of Immunology, 2001, 166: 3345–3354.*

Cell development and effector function are controlled by signaling through the TCR, a cell surface receptor that specifically recognizes peptide/MHC complexes (pMHC)\(^a\) on the surface of APCs (reviewed in Ref. 1). TCR \(\alpha\) - and \(\beta\)-chains are assembled from separate gene segments by somatic DNA rearrangement. In theory, this mechanism of gene assembly can produce an enormous number of TCR specificities, with sufficient diversity to bind almost any structure. However, only a fraction of the TCRs produced by this method have useful specificities (2); therefore, a selection process takes place during T cell development to ensure a functional T cell repertoire (3). Developing T cells undergo positive selection in the thymus to ensure that they are able to, in some manner, recognize pMHC. Furthermore, T cells also undergo negative selection in the thymus to ensure that each TCR will not be triggered by self peptides presented on self MHC molecules. TCRs that survive selection represent a balance between high specificity to avoid autoreactivity, and some degree of flexibility to recognize self pMHC (4). It becomes apparent then, that to understand how the TCR functions, it is necessary to decipher in detail how this receptor is able to recognize multiple ligands, and how multiple ligands can induce different biological responses.

Altered peptide ligands (APLs) represent a useful tool for studying differential recognition by the TCR. Numerous APLs have been identified by introducing single amino acid substitutions into the peptide sequences recognized by individual T cell clones (5, 6). The majority involve conservative amino acid substitutions at positions known to be accessible to the TCR (7). Comparison of the available crystal structures for a single TCR bound to different pMHC class I complexes shows that the TCR is able to accommodate different ligands by making only minor structural adjustments to the TCR-pMHC interface (8, 9). This leaves open the question, how do structural changes in the pMHC result in differential TCR recognition? The major parameter that the TCR uses to distinguish between ligands is now believed to be the dissociation rate of the TCR-ligand complex (10, 11). Several studies taken together show that for a given TCR, the interactions leading to a partial agonist response have a shorter \(t_{1/2}\) than the interactions leading to a full agonist response (1). Although the equilibrium-binding affinity of partial agonist ligands is often lower, it seems not to be a determining factor in T cell response (12). The current model is that small changes in the off-rate of the receptor-ligand interaction can lead to differences in the signals transduced by the TCR, resulting in differences in the biological response.

In this study, we investigate the basis by which a TCR can discriminate between two peptides differing at only a single MHC anchor residue. While this substitution does not significantly alter binding of the peptide to the class II molecule, it reduces the 3.L2 T cell response approximately 1000-fold. In this study, we show that the bulk T cell response for the two pMHCs are essentially distinct and nonoverlapping. To examine the structural basis for this differential T cell response, we have determined and compared multiple 1.9-Å crystal structures of each pMHC. In contrast to

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\(^{2}\) G.J.K. and M.J.M. contributed equally to the work.

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\(^{5}\) Abbreviations used in this paper: pMHC, peptide/MHC complex; Abu, 2-aminobutyric acid; APL, altered peptide ligand; Nle, norleucine; Nva, norvaline; RMSD, root mean square deviation.
Table I. Crystallographic data collection

<table>
<thead>
<tr>
<th>Data Set</th>
<th>a (Å)</th>
<th>b (Å)</th>
<th>c (Å)</th>
<th>β (°)</th>
<th>Total</th>
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<td>66.294</td>
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<tr>
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<td>117.03</td>
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<td>134.812</td>
<td>63.993</td>
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<td>91.97</td>
<td>445.426</td>
<td>76.496</td>
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<tr>
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<td>134.812</td>
<td>63.993</td>
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</tr>
<tr>
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<td>58.85</td>
<td>116.73</td>
<td>91.76</td>
<td>364.002</td>
<td>72.521</td>
</tr>
</tbody>
</table>

Glutammin and 50 µg/ml gentamicin in 200 µl total volume in a 96-well tissue culture plate with the indicated concentration of peptide. Culture wells were pulsed at 48 h with 0.4 µCi [3H]thymidine and harvested 18–24 h later.

Stability of the peptide/IEk complexes

The 1E6-positive B cell lymphoma CH27 was prepulsed with either 31.6 µM Hb(D73) or 0.316 µM Hb for 2 h at 37°C and at a concentration of 1.5 × 10^6 cells/ml. The cells were then washed and incubated at 37°C in a single well of a 24-well plate. At 0, 1, 2, 4, 6, or 10 h later, the cells were washed and added at 2 × 10^4 per well to a 96-well plate containing the cell hybridoma YO1.6 at 1 × 10^5 per well. Supernatants were removed at 22 h, and the U/ml of IL-2 was determined using the indicator line CTLL-2. For the experiment shown in Fig. 2B, the IL-2 produced when prepulsed CH27 were added to the YO1.6 cells immediately after washing (time 0) was as follows: Hb, 893 U/ml; Hb(D73), 2010 U/ml.

Peptide-binding assay

Relative binding strength of Hb and Hb(D73) peptides to 1E6 was determined essentially as described (14). Briefly, a soluble form of 1E6 was made by cleavage from the surface of Chinese hamster ovary cells transfected with an expression plasmid encoding a glycopeptidolipid-linked form of 1E6 (a generous gift from Mark Davis (Stanford University, Palo Alto, CA)). Purified 1E6 was then incubated for 72 h with a 125I-labeled standard peptide (AAAYGKXATAFNEGLKL) in the presence or absence of various concentrations of the test peptide, pMHC were separated from unbound peptide using Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA). The relative amount of bound standard peptide was determined using a gamma counter. The data are expressed as a percentage of the counts obtained in the absence of competitor peptide.

Preparation of soluble pMHCs

The method used for production and purification of the soluble ecto-domains of 1E6 with covalently bound peptides has been described previously (15). Briefly, a baculovirus transfer vector was prepared that contained the genes for the I-Ed molecule as well as the CLIP transgene. The 1E6-β chain was modified to contain an N-terminal covalent linkage that includes a flexible linker and either the Hb or Hb(D73) peptide. The transfer vectors were used to produce high titer stocks of baculovirus from S9 cells. The

Table II. Crystallographic refinement statistics

<table>
<thead>
<tr>
<th>Data Set</th>
<th>Rcryst (%)</th>
<th>Rfree (%)</th>
<th>RMSD (Å)</th>
<th>Coordinate Error</th>
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<td></td>
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<td>Last shell (1.96–1.90 Å)</td>
<td>Total (100–1.90 Å)</td>
<td>Last shell (1.96–1.90 Å)</td>
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<td>38.7</td>
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<td>26.0</td>
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<td>29.6</td>
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<td>31.8</td>
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<tr>
<td>Hb(D73)IEk 1</td>
<td>22.9</td>
<td>34.5</td>
<td>26.4</td>
<td>36.3</td>
</tr>
<tr>
<td>Hb(D73)IEk 2</td>
<td>22.7</td>
<td>32.0</td>
<td>27.9</td>
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</tr>
<tr>
<td>Hb(D73)IEk 3</td>
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<td>23.2</td>
<td>36.0</td>
<td>27.0</td>
<td>40.5</td>
</tr>
</tbody>
</table>

* Experimental coordinate error is calculated as the RMSD of a given molecule in the asymmetric unit (M1 and M2) compared to its equivalent in the merged structure. The coordinate error for each merged structure is the average of the calculations.
large scale infection of S19 cells produced the desired molecules as soluble secreted proteins in the medium. These proteins were purified from the S19 supernatants using immunoaffinity columns made from the I-Eα-specific Ab 14.4.4.4s. This was followed by gel filtration over a HiPrep 16/60 Sephacryl S-200 column (Pharmacia). Fractions were analyzed by ELISA, and those containing I-Ek heterodimers were pooled, concentrated, and stored in 10 mM HEPES, pH 7.5. The identities of the purified proteins were confirmed by N-terminal sequencing (Midwest Analytical, St. Louis, MO).

Crystallization and x-ray data collection

Protein solutions of Hb/I-Ek and Hb(D73)/I-Ek were concentrated to an OD280 of approximately 10 in 10 mM HEPES, pH 7, and 5 mM sodium azide. Crystals were produced in hanging drops by vapor diffusion at 20°C against wells filled with 15% polyethylene glycol 4000 (Fluka, Buchs, Switzerland), 15% 2-propanol, 300–500 mM ammonium acetate, and 100 mM citrate, pH 4.8. Diffraction quality crystals appeared within 48 h and were cryoprotected just before flash cooling through the addition of a mixture of 20% polyethylene glycol 4000, 20% ethylene glycol, 10% glycerol, 100 mM sodium citrate, and 100 mM ammonium acetate. All crystals belonged to the centered monoclinic space group C2 and had similar cell dimensions (Table I). Three data sets were collected at the Advanced Photon Source (APS) beamline 19-ID on a charged coupled device detector for each of the two proteins of interest (Hb/I-Ek and Hb(D73)/I-Ek). Data were indexed and processed using Denzo and Scalepack (16).

Model building and refinement

The coordinates of the 2.3-Å refined Hb/I-Ek complex (RCSB code 1IEA) (17) were used as the initial model for the refinement of the high resolution Hb/I-Ek structures. Rigid body refinements were conducted with CNS (18) using the platform domains and the membrane-proximal Ig domains as separate objects. Additionally, CNS was used to execute multiple rounds of refinement on the Hb/I-Ek models, which included temperature factor refinement, conjugate gradient minimization, and electron density map generation. The models were rebuilt in O (19) using 2Fo–Fc, Fo–Fc, and simulated annealing omit maps. A total of six independent Hb/I-Ek models were generated from three unique data sets, each with two molecules in the asymmetric unit. Similarly, six Hb(D73)/I-Ek models were built starting with a modified Hb/I-Ek model in which the appropriate mutation in the peptide, E73D, was made. Refinement was conducted as above with the additional of phased difference Fourier maps between Hb(D73)/I-Ek and Hb/I-Ek data sets. These maps were used to probe for shifts in atomic positions that result from the E73D substitution. Final coordinates for both pMHCs were generated using merged wild-type and mutant data sets (Table II). Coordinates have been submitted to the Protein Data Bank (Hb/I-Ek and Hb(D73)/I-Ek PDB codes 1FNG and 1FNE, respectively).

Each peptide/I-Ek structure contains 182 residues of I-Ek (α1-α182), 12 mouse Hb(65–76) residues (P-4 to P9), 16 linker residues (P + 1 to P + 16) connecting to 185 of the natural I-Ek (β4-β188), and three carbohydrates N linked to Asn118, Asn111, and Asn117.

For all models, no attempt was made to build the peptide NH2-terminal regions (P-8 to P-4) or the COOH terminal 10 residues of either the α- or β-chains, as these regions appear highly disordered in the electron density maps. A sequencing error in His177 was also corrected from the original I-Ek structure, which was built as Thr177.

Results

Hemoglobin is a model Ag for TCR cross-reactivity

The murine hemoglobin molecule is composed of two αβ heterodimers. Allelic forms of both α- and β-chains exist in mice and allow for use of the hemoglobin protein as a foreign Ag (20). For example, C57BL/6 mice (I-Ek, Hbb-) will produce a strong T cell response when immunized with hemoglobin protein prepared from CBA/J mice (I-Ek, Hbb+). This response was shown to be dependent on the presentation by I-Ek of a peptide derived from residues 64 to 76 of the minor form of the d allele of the hemoglobin β-chain (hereafter referred to as Hb) (5). Numerous APLs have been described for Hb/I-Ek-specific T cells, and this system is ideally suited for biophysical studies of recognition of multiple ligands by the TCR.

Of the many amino acid substitutions possible in the Hb sequence, one of the most interesting is the substitution of Glu73 with an Asp. This substitution (hereafter referred to as Hb(D73)) not only changes the peptide from a good to a poor stimulator of many Hb-specific T cells, but in many cases causes the peptide to act as a TCR antagonist (5, 6). In fact, for mice immunized with Hb peptide, the recall proliferative response of bulk lymph node T cells in vitro requires 100- to 1000-fold more peptide if Hb(D73) is used as the recall Ag than if the Hb is used (Fig. 1A). The difference in biological response initially suggested that the Glu73 residue might be a TCR contact residue, or alternatively that the Hb(D73) substitution hinders binding of the peptide to the I-Ek molecule. As demonstrated below, neither of these initial possibilities has proven true, and a more elegant molecular mechanism accounts for the ability of TCRs to discriminate between the two ligands.

Two different methods indicate that the Hb(D73) peptide binds to I-Ek equally as well as the Hb peptide. First, Hb and Hb(D73) are equally effective at competing off a radiolabeled index peptide from a soluble form of the I-Ek molecule (Fig. 2A). This result suggests that the two peptides have a similar equilibrium-binding affinity for I-Ek. Second, we have compared the persistence of complexes at the cell surface formed from either Hb/I-Ek or Hb(D73)/I-Ek. This was done by pulsing APCs with a dose of Hb or Hb(D73) sufficient to induce an intermediate response from a T cell hybridoma and then incubating the APCs at 37°C for different time periods. After washing, the amount of pMHC remaining was assayed using a T cell hybridoma. Although not a direct measure of the off-rate of the two peptides, it is expected that the rate of decrease in the response of the T cell hybridoma is in direct proportion to the of-rate of the peptide from the I-Ek molecule. The rate of disappearance of the two complexes was similar when measured by the loss of T cell recognition (Fig. 2B), suggesting that these ligands have similar stability on the cell surface.

The Hb(D73) peptide can form an effective ligand when presented on I-Ek+. In addition, most T cells specific for Hb(D73) are weakly responsive to Hb. For example, in animals immunized with the Hb(D73) peptide, the recall proliferative response of bulk lymph node T cells in vitro requires 100- to 1000-fold more Hb.
from the PmHC surface, the side chain of Glu73 is not expected to

peptide than Hb(D73) peptide (Fig. 1B). The converse of this ex-

FIGURE 2. Hb and Hb(D73) peptides bind the I-E\(^{k}\) molecule similarly. A, Purified, detergent-solubilized I-E\(^{k}\) molecules (2.5 nmol) were loaded with a radiolabeled index peptide (25 pmol) in the presence of a titration of Hb peptide (■), or Hb(D73) peptide (○). The 100% value indicates the maximum amount of recovered radioactivity in the absence of competitor peptide. B, The I-E\(^{k}\)-positive B cell lymphoma CH27 was prepulsed with either Hb peptide (■), or Hb(D73) peptide (○), followed by washing to remove excess peptide. The prepulsed APCs were then incubated at 37°C for the indicated time periods before being used to stimulate the T cell hybridoma YO1.6. IL-2 was measured in the YO1.6 supernatants as an indicator of the cellular response. The graph shows the decrease in response over time given as a percentage of the initial response. ■, Hb peptide; ○, Hb(D73) peptide. Although this hybridoma recognizes both Hb and Hb(D73), it requires ~100 times more Hb(D73) peptide for full activation. The APCs were prepulsed with slightly more than the minimum amount of each peptide required for a maximal response. The concentra-

tions used for prepulse were: Hb, 0.316 \(\mu M\); Hb(D73), 31.6 \(\mu M\). Although different concentrations of peptide were used, the rate that the responses decayed was found to be independent of the initial concentration. Using the responses obtained at 4, 6, and 10 h, the \(t_{1/2}\) of the complexes were calculated to be: Hb/I-E\(^{k}\), 6 h; Hb(D73)/I-E\(^{k}\), 5.1 h. It is possible that the decay of the response represents internalization of the PmHC, and not peptide release. If this is true, then both peptides have very slow dissociation from I-E\(^{k}\), with \(t_{1/2}\) of greater than 5 h. We conclude that both the Hb and Hb(D73) complexes are sufficiently stable on the cell surface to induce productive T cell responses.

FIGURE 3. The structures of I-E\(^{k}\) with covalently bound Hb or Hb(D73) differ significantly at peptide positions 73 (P6), 74 (P7), and 75 (P8). For each residue, the root mean square value of the residue deviations (RMSD) from a Hb/I-E\(^{k}\) molecule is shown plotted vs their positional deviation (\(\text{Sqrt}(B/8\pi^2)\)). RMSD values from the Hb/I-E\(^{k}\) structure are shown as filled squares in contrast to values from the Hb(D73)/I-E\(^{k}\) structure, which are shown as open circles. Three data sets were compared for each with two independent molecules per asymmetric unit.

To understand how the change from Glu\(^{73}\) to Asp\(^{73}\) could have such a profound effect on T cell response, we have determined the high resolution crystal structures of I-E\(^{k}\) complexed with covalently bound Hb and Hb(D73) peptides. Soluble forms of PmHCs were purified from baculovirus-infected insect cells. To increase the accuracy of the comparison, we set out to analyze three individual crystals of both pMHCs. A total of six independent data sets were collected for refinement at 1.9 Å resolution (Tables I and II): three from Hb/I-E\(^{k}\) and three from Hb(D73)/I-E\(^{k}\), each with two independent molecules per asymmetric unit.

The resulting coordinates were used to compare the two PmHCs. This was done by superimposing the independent structures to one of the Hb/I-E\(^{k}\) complexes. The root mean square deviations (RMSD) of peptide and binding platform residues for each independent model are shown plotted vs the root mean squared positional displacements as calculated from the thermal parameter B (\(\text{Sqrt}(B/8\pi^2)\)) (Fig. 3). Refined atomic B values represent a measure of the displacement of an atom due to thermal motion or conformational disorder. The deviations of residues with low thermal displacements can be considered to be more significant (24). Two identical structures would have a linear relationship between RMSD and \(\text{Sqrt}(B/8\pi^2)\), as is observed for the Hb/I-E\(^{k}\) structures. The analysis clearly reveals that the most significant statistical deviations between the two pMHCs occur in peptide residues. Peptide positions P6 (Glu\(^{73}\)) and Asp\(^{73}\) and P8 (Leu\(^{75}\)) have deviations far more significant than any other residue, either in the rest of the peptide positions P6 (Glu\(^{73}\)) and Asp\(^{73}\) and P8 (Leu\(^{75}\)) have deviations far more significant than any other residue, either in the rest of the peptide or in the I-E\(^{k}\) binding groove. A direct change introduced upon substitution of the buried side chain.

Hb(D73)/I-E\(^{k}\) differs from Hb/I-E\(^{k}\) in peptide but not MHC conformation

face (8, 9, 21–23). It seems likely then that the uniqueness of the Hb(D73)/I-E\(^{k}\) determinant does not result from a direct interaction of the Hb(D73) side chain with the TCR, but rather from an indirect change introduced upon substitution of the buried side chain.

Hb(D73)/I-E\(^{k}\) differs from Hb/I-E\(^{k}\) in peptide but not MHC conformation

Prior to the crystallization experiment, we have described the 2.3 Å crystal structure of a soluble form of the I-E\(^{k}\) molecule with a covalently attached Hb peptide (17). This structure revealed that Glu\(^{73}\) fits into the P6 pocket of the I-E\(^{k}\) molecule, adopting the role of a traditional MHC anchor residue. In contrast to the P5 (Asn\(^{72}\)) and P8 (Leu\(^{75}\)) residues, which have solvent accessible side chains that point away from the PmHC surface, the side chain of Glu\(^{73}\) is not expected to directly interact with the TCR. Nevertheless, based on the available crystal structures of TCR/PmHC complexes, residues flanking the Hb(D73) substitution should be integral to the recognition sur-

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Prior to the crystallization experiment, we have described the 2.3 Å crystal structure of a soluble form of the I-E\(^{k}\) molecule with a covalently attached Hb peptide (17). This structure revealed that Glu\(^{73}\) fits into the P6 pocket of the I-E\(^{k}\) molecule, adopting the role of a traditional MHC anchor residue. In contrast to the P5 (Asn\(^{72}\)) and P8 (Leu\(^{75}\)) residues, which have solvent accessible side chains that point away from the PmHC surface, the side chain of Glu\(^{73}\) is not expected to directly interact with the TCR. Nevertheless, based on the available crystal structures of TCR/PmHC complexes, residues flanking the Hb(D73) substitution should be integral to the recognition sur-
peptide, or in the α1 and β1 domains of I-Ek (Fig. 3). A smaller yet significant movement is also seen for the P7 position (Gly 74).

The structural differences between the two pMHCs can be directly visualized by phased difference Fourier maps. For nearly isomorphous crystals, these maps reveal both positive and negative differences in electron density, which result from structural alterations. While no notable differences in electron density were observed between atoms of the I-Ek molecules, significant differences were observed for the P6 substituted residue and its neighbors. A close-up view of one of these phased difference Fourier maps is displayed in Fig. 4. Superimposed on the electron density map are models of the two peptide structures. The \( F_{o,hb} - F_{o,d73} \) difference electron density map (yellow) contoured at 4σ shows positive density (yellow), defined as density that is present in the Hb/I-Ek structure, but not in Hb(D73)/I-Ek. Conversely, the bottom panel displays negative density (green), which is present in the Hb(D73)/I-Ek structure, but not in Hb/I-Ek. This difference map clearly shows the changes resulting from the Asp \(^{73}\) substitution: the side chain of residue 73 (P6) has moved, the main chain between residues 73 (P6) and 75 (P8) has shifted, and a water molecule has migrated in the P6 pocket. This traveling water is colored yellow in the top panel and green in the bottom panel. All the density differences between the two peptides were localized to a small region between residues 72 (P5) and 76 (P9). Similar analysis of difference electron density between the identical complexes yielded virtually featureless maps.

A summary and quantitation of the differences between the two peptides are presented in Fig. 5, with RMSD values plotted for both side chain and main chain atoms. The side chains of residues at P6 and P8 are displaced by approximately 1.5–2 Å. The difference at P6 is due to the introduction of the new side chain at this position, and the difference at P8 is due partly to the shift in the main chain of the peptide, and partly to the new rotamer conformation adopted by the side chain of Leu \(^{75}\) in the Hb(D73)/I-Ek structure. Significant main chain displacement can also be observed for the P6, P7, and P8 residues. These movements (RMSD \( ~0.4–0.6 \) Å) are clearly larger than those observed upon comparison of the Hb/I-Ek structures with themselves (Fig. 5, gray bars).

The difference maps also show how the I-Ek-binding groove is able to accommodate the Asp \(^{73}\) side chain substitution. We previously observed an unusual cluster of carboxylate groups in the P6 pocket of the Hb/I-Ek structure (17) and proposed that this cluster of acidic residues would interact only at the acidic pH of the endosomal peptide-loading compartment. Once assembled, this structure would remain stable even after a shift to higher pH due to the solvent inaccessibility of the pocket. The cluster in the P6 pocket of Hb/I-Ek involves interaction of the Glu \(^{11}\) carboxylate group with Asp \(^{a}\). However, when Asp \(^{73}\) is present, there is still an acidic cluster, but the Asp \(^{73}\) carboxylate group now lies within 2.5 Å of Glu \(^{11}\) from I-Ek (Fig. 6). A preference for Asp side chains to interact closely with Glu side chains has been previously shown (25), but it is not known whether this is the driving force...
The structural changes alter the kinetics of the TCR-ligand interaction

We and others have demonstrated that weakly stimulatory ligands display faster dissociation rates from the TCR than do full agonist ligands. Previously, we determined the kinetics of a particular Hb-specific TCR (3.L2) binding to soluble, covalently linked forms of both Hb/I-E\(^k\) and Hb(D73)/I-E\(^k\) (12). The Hb/I-E\(^k\) complex bound the 3.L2 TCR with a \(t_{1/2}\) of 10.8 \(\pm\) 0.09 s. In comparison, the \(t_{1/2}\) of the interaction between Hb(D73)/I-E\(^k\) and the 3.L2 TCR was only 7.5 \(\pm\) 0.22 s. Interestingly, with the Hb(D73) peptide a 3-fold increase in the association rate was observed, resulting in a higher equilibrium-binding affinity for the Hb(D73)/I-E\(^k\) complex with the 3.L2 TCR than seen with the Hb/I-E\(^k\) complex. Results similar to these have been observed in other receptor/ligand systems in which conservative mutations were introduced to the binding interface (27, 28). What is remarkable about our kinetic results is that they are attributable to the loss of a single solvent inaccessible methylene group.

The P8 side chain is important for ligand recognition by the 3.L2 TCR

TCR-docking models suggest that the Leu\(^{75}\) side chain at P8 should make important contacts with the TCR, and that substitutions at this position should affect T cell responses. We have tested this directly by examining the 3.L2 T cell response to Hb peptides substituted at P8 (Fig. 8). By using a series of P8 substitutions that differ in side chain length (Nle > Nva > Abu > Ala), three conclusions can be drawn. First, this side chain is important for ligand recognition by the 3.L2 TCR. Ligands using Ala, Nle, or Abu at position 75(P8) stimulate weak responses (Fig. 8). Second, there is a preferred size for the side chain at this position: Ala is too small for good recognition, whereas Nle is too large, whereas Nva stimulates a strong response. Third, the position and conformation of the side chain in the unbound ligand seem to be important: the Leu in the Hb(D73) peptide has a different rotamer conformation and only weakly stimulates the 3.L2 T cells. The magnitude of the reduction is similar to having either a slightly smaller (Abu75) or larger (Nle75) side chain at P8. The results demonstrate the sensitivity of the TCR to subtle changes in size and orientation, and show that the P8 side chain is an important TCR contact for the 3.L2 T cell.

Discussion

In this study, we have examined TCR recognition of two pMHCs that differ only by a Glu to Asp substitution in the peptide sequence. The substituted side chain is directed into the binding groove of the MHC molecule and is unlikely to contact the TCR directly. Our goal was to understand the 1000-fold decrease in activity caused by this substitution. After ruling out the possibilities that the Hb(D73) peptide is a poor binder of I-E\(^k\), or that Hb(D73) does not form a stable ligand, it became of interest to compare the atomic structures of the two complexes. The results are quite remarkable, in that a significant structural variation results from the removal of a single methylene group. The position of the peptide main chain is reoriented as is the rotamer of a solvent-exposed residue nearly 10 Å distal from the altered P6 pocket side chain. However, there are no significant differences in the positions of any of the I-E\(^k\) Ag-binding platform residues that accommodate the APL. We will consider how these results might be important for T cell response, and discuss other factors that may also influence the recognition.
A significant change in position of the peptide main chain

The Asp<sub>73</sub> (P6) substitution causes an alteration in the peptide main chain between the P5 and P9 residues, which is dominantly shifted toward the peptide C terminus (Figs. 4 and 5). The root mean square displacement for these atoms is on the order of 0.4 Å to 0.6 Å. Although these differences are small, they are localized to a region of the pMHC that forms the TCR determinant (Fig. 7). These alterations are most likely due to the repacking of the P6 pocket. It is somewhat surprising that the 3.L2 T cell is so sensitive to this particular alteration, especially because substitutions at either of the nearby solvent-exposed side chains, P5 or P8, can have only modest effects. For example, a Thr for Asn<sub>72</sub> substitution at P5 results in only a 50-fold decrease in activity (7). Similarly, the substitution of Nva for Leu<sub>75</sub> (P8) results in only about a 10-fold decrease in activity, although Ala substitutions at either position result in ligands that do not induce any IL-2. The main chain adjustments in Hb(D73)/I-E<sup>k</sup> occur along an approximately 15 Å length of the peptide, in a region that is contacted by TCR in all known TCR/pMHC complexes. We suggest that these documented differences in the free pMHCs could easily account for their distinct biological potency.

The leucine at P8 adopts a different rotamer conformation in Hb(D73) I-E<sup>k</sup>

Our results from six independently derived structures of both pMHCs clearly indicate that Leu<sup>75</sup> at P8 adopts a different preferred rotamer conformation in the Hb(D73)/I-E<sup>k</sup> complex compared with Hb/I-E<sup>k</sup>. In the Hb(D73)/I-E<sup>k</sup> complex, the alternative Leu<sup>75</sup> rotamer is adopted to maintain the same hydrophobic contacts with Val<sup>66</sup> and Ala<sup>68</sup>, which would otherwise be lost due to the repositioning of the peptide main chain. We have shown in this study that amino acid substitutions at P8 result in a modulation of T cell activity, with single methylene group alterations giving...
FIGURE 7.  A, A top view showing the Hb(D73)/I-Eκ-binding platform superimposed to that of the Hb/I-Eκ-binding platform. Shown are residues that lose solvent-accessible surface area when either structure is docked with the available TCR coordinates from TCR/pMHC complexes (8, 21–23). Hb residues are shown in yellow, while Hb(D73) residues are displayed in green. Note the shift in positions of peptide P5-P8 main chain atoms, and the flip in the Leu75 rotamer conformation at P8. B, Atomic RMSD differences normalized by positional deviation (\(\sqrt{B/8n^2}\)) between these two pMHCs are mapped to the molecular surface of Hb/I-Eκ, which was rendered with GRASP (33). Areas colored in red represent significant atomic variation that is solvent exposed. The majority of movement is localized to the P6-P8 region of the peptide, which is coincident with the TCR contact surface shown in the panel below. C, The colored regions represent the consensus solvent surface (34) predicted to be lost upon TCR ligation based on the docking of Hb/I-Eκ to multiple class I- and class II-restricted TCRs. The pMHC atoms that are predicted to be in direct contact (\(<4.2\ \text{Å}\)) with the class II TCR are colored blue. Atomic MHC class II TCR contacts are colored a lighter blue than the atomic peptide TCR contacts. The remaining peptide surface area is colored in yellow. Notice the majority of peptide contacts are localized to the P6-P8 peptide region.
We have shown by surface plasmon resonance studies that the Hb and Hb(D73)/I-Ek complexes bind soluble 3.12 TCR with similar affinities, and yet there are significant differences in both association and dissociation kinetics (12). The Hb(D73)/I-Ek complex associates with the TCR 3-fold faster than Hb/I-Ek, while its dissociation rate translates into a $\sim 3$-s decrease in $t_{1/2}$. Microscopy methods have allowed for the visualization of the immunological synapse formed between 3.12 T cells and Hb/I-Ek (29). Similar experiments utilizing Hb(D73)/I-Ek as the ligand failed to reveal a durable activation cluster, consistent with the interpretation that this pMHC is a weak agonist for the 3.12 TCR (unpublished results). In the context of results indicating the overall importance of dissociation rate in receptor-ligand interactions in general and TCR activation in particular (1, 11, 27), it seems entirely plausible that the $\sim 30\%$ change in $t_{1/2}$ could be the basis of the differential activities of the two pMHCs. The small kinetic deviations observed in this and related systems clearly illustrate the extraordinary sensitivity of the TCR in molecular discrimination. The positive and negative selection mechanisms of T cell development appear to work to maintain the low affinities of TCR/pMHC interactions to establish narrow kinetic thresholds. Indeed, stable high affinity TCR/pMHC complexes have been experimentally obtained by minor residue substitutions, indicating that there are no structural limitations of either the ligand or receptor for a more enduring embrace (9, 30). It may well be that TCRs are naturally selected in part for conformational flexibility to allow for promiscuous, low affinity engagement of multiple partners (31).

A wealth of information on the nature of receptor-ligand interactions has come from protein crystallography. However, a direct understanding of the energetic and kinetic roles of particular interfacial residues to the interaction is typically not revealed from the atomic coordinates alone (27, 32). Although the small structural variances in our two pMHCs are hard to relate to the large activity differences they elicit, their location, magnitude, and statistical significance are consistent with previously documented alterations of TCR/pMHC biophysics and activities (8, 9). Our study further highlights the extreme sensitivity TCRs possess by demonstrating the apparent discrimination of pMHCs that differ by a single methylene group located in the buried side chain of an MHC anchor residue.

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**References**


