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A Molecular Basis for How a Single TCR Interfaces Multiple Ligands

Alina Boesteanu,* Michael Brehm,* Lawrence M. Mylin,* Gregory J. Christianson,† Satvir S. Tevethia,* Derry C. Roopenian, † and Sebastian Joyce2*

CD8+ T cells respond to Ags when their clonotypic receptor, the TCR, recognizes nonself peptides displayed by MHC class I molecules. The TCR/ligand interactions are degenerate because, in its life time, the TCR interacts with self MHC class I-self peptide complexes during ontogeny and with self class I complexed with nonself peptides to initiate Ag-specific responses. Additionally, the same TCR has the potential to interact with nonself class I complexed with nonself peptides. How a single TCR interfaces multiple ligands remains unclear. Combinatorial synthetic peptide libraries provide a powerful tool to elucidate the rules that dictate how a single TCR engages multiple ligands. Such libraries were used to probe the requirements for TCR recognition by cloned CD8+ T cells directed against Ags presented by H-2Kb class I molecules. When H-2Kb contact residues were examined, position 3 of the peptides proved more critical than the dominant carboxyl-terminal anchor residue. Thus, secondary anchor residues can play a dominant role in determining the antigenicity of the epitope presented by class I molecules. When the four solvent-exposed potential TCR contact residues were examined, only one or two of these positions required structurally similar residues. Considerable structural variability was tolerated at the remaining two or three solvent-exposed residues of the Kb-binding peptides. The TCR, therefore, requires close physico-chemical complementarity with only a few amino acid residues, thus explaining why TCR/MHC interactions are of low affinity and degenerate. The Journal of Immunology, 1998, 161: 4719–4727.

The control of T cell responses by the classical MHC-encoded Ag-presenting molecules is termed MHC restriction (1). MHC restriction entails the presentation of Ag in the form of processed short peptides by the MHC molecules to T cells. MHC class I molecules control the development and function of CD8+ CTL. Class I molecules accomplish their function by chaperoning short intracellular peptides of 8 to 12 aa residues to the cell surface, thus displaying the class I-peptide complexes for an appraisal by circulating CD8+ T cells (reviewed in Ref. 2).

A molecular basis for MHC restriction has emerged from solving numerous three-dimensional structures of class I molecules (reviewed in Ref. 3). The heterotrimeric class I molecule consists of the highly diverse heavy chain, the invariant light chain β2-microglobulin (β2m),3 and a peptide. The α1 and α2 domains of the heavy chain fold in such a manner that they form an Ag-binding groove confined on the sides by two α-helices and at the bottom by two β-sheets of four antiparallel β-strands each. Peptide ligands bind within this groove. The α3 domain of the heavy chain and β2m attain an Ig-like fold; β2m is noncovalently associated with the α1, α2, and α3 domains (4, 5). Peptides bind the Ag-binding groove through hydrogen-bonding interactions between the side chain atoms of the conserved residues of pockets A and F of the heavy chain and the main chain atoms of the amino-terminal amine and the carbonyl oxygen of the carboxyl terminus of the peptide, respectively (6–10). Peptides bind class I molecules contain structurally conserved residues at the carboxyl terminus (P1) and at an additional internal position. A combination of these conserved residues within peptides constitutes the class I binding motif (11–13). Thus H-2Kb class I molecules bind peptides that predominantly contain phenylalanine (Phe) or tyrosine (Tyr) at position (P) 5 and a hydrophobic aliphatic residue (e.g., valine (Val), leucine (Leu), and isoleucine (Ile)) at P3 (11, 12, 14). Peptide binding studies using single amino acid variants of antigenic peptides have revealed secondary anchors required for effective binding (15–17). In the case of H-2Kb, residues at P2 and P3 serve as secondary anchors (15, 18).

The crystal structure of class I molecules also revealed that the majority of the amino acid residues that vary among class I allotropic products are those that contact the bound peptide by virtue of their location in the Ag-binding groove. Thus the physico-chemical nature of the Ag-binding groove dictates the anchor motif required for specific peptide binding to a class I molecule (5, 19, 20). In the three crystal structures of H-2Kb molecules complexed with different octameric peptides, the dominant anchors, P5 Phe or Tyr, and the P2 Leu are completely buried in the groove (8, 9, 21). Of the secondary anchors, those at P2 are tucked into pocket B, and those at P3 are almost completely buried within pocket D of the Ag-binding groove (8, 9, 21), consistent with the view that they can influence peptide binding to H-2Kb. Thus, in octameric peptides displayed by H-2Kb, residues at P2, P3, P5, and P2 are in intimate contact with the class I molecule; the side chains at the

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3Abbreviations used in this paper: β2m, β2-microglobulin; CDR, complementarity determining region; gB, glycoprotein B; HSV-1, herpes simplex virus type 1; RR-1, ribonucleotide reductase-1.
The remainder of the peptide, i.e., at P1, P4, P6, and P7, are solvent exposed and potentially accessible to the TCR (8, 9, 21).

Functional studies have suggested that the TCR interacts with a composite structure of the class I-peptide complex in an orientation diagonal to the α-helices (22). In addition to confirming these aspects of the TCR/class I-peptide interaction, the two recently solved crystal structures of the TCR cocomplexed with its cognate ligand, H-2Kb-self peptide in one and HLA-A2-HIV tax peptide in another, revealed that the TCR interfaces Ag through its complementarity determining regions (CDR) 1, 2, and 3 of the Tcrα- and Tcrβ-chains (23, 24). Of these six CDRs, CDR3 makes the majority of the contacts with the bound peptide (23), consistent with the fact that CDR3α, CDR3β, and the peptide are the most variable parts of the interacting surfaces. X-ray crystallographic analyses of TCR/class I-peptide complexes have provided an in-depth view of how these macromolecules interact with each other. However, it provides a static snapshot of the most thermodynamically favored structure of the interacting molecules. Therefore, further structure function analyses are required to understand how the TCR interacts with its ligand.

Combinatorial libraries provide a powerful tool for probing the interfaces of two interacting macromolecules (25–31). In this study, combinatorial peptide libraries were used to gain insight into the role of H-2Kβ contact and TCR accessible residues in Ag presentation and recognition. The results revealed that the display of Ag to the TCR critically depended on the class I-contact residue at P3 of the peptide. In several instances the amino acid residue at P3 was more critical than the dominant PΩ anchor. Additionally, the TCR requires only one or two TCR-contact residues of the peptide to achieve specificity, while it can interact with multiple structurally dissimilar amino acids of the remaining solvent-exposed residues. Although the critical TCR contact residues seemed predisposed to the carboxyl terminus of the peptide, the position of these residues was T cell, therefore TCR, dependent. The requirement for only a few specific TCR contact residues within the peptide explains why TCR/MHC-peptide interactions are of low affinity and degenerate.

### Materials and Methods

#### Cells and CTL clones

RMA cells (32) were used as targets in cell-mediated lysis. These cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 5% heat-inactivated FCS, 100 U/ml penicillin, and 100 μM streptomycin. The H-2Kβ-restricted, H-4β-specific CTL line M9 was isolated from C57BL/10 mouse, primed, and restimulated with H-4-congenic B10.129-H-4β (21 M) splenocytes. A cloned line was established and maintained with weekly stimulation using irradiated 21 M-derived spleen cells in the presence of 30 μM recombinant IL-2, as described (33).

The H-2Kβ-restricted, gB-specific CTL clone 2D5 and the RR1-specific CTL clone 1D11 were derived from splenocyte populations of HSV-1 immunized C57BL/6 mice (34, 35). Both the 2D5 and 1D11 CTL clones were maintained with 10% Rat T-Stim culture supplement (Collaborative Biomedical Research Products, Bedford, MA) along with 0.05 M streptomycin and 100 μM L-phenylalanine, leucine, methionine, valine, and tyrosine. A mixture of all naturally occurring L-amino acids, except cysteine and tryptophan was used in experiments involving libraries 1–6 and an E:T of about 10 in assays using libraries 7–11.

#### Peptides

All combinatorial peptide libraries were produced by automated solid phase F-moc synthesis (Chiron Technologies, Emeryville, CA). The peptide libraries were synthesized with Phe at P5 in all cases and either a single amino acid except Cys at defined positions (solid circles in the Figures) or with equimolar mixtures of all naturally occurring l-amino acids except for Cys for randomized positions (x). The peptide preparations were controlled for purity by reverse-phase chromatography, and electrospray-ionization mass spectrometry (Chiron Technologies). Stock solutions of the peptide were prepared in DMSO at ~20 mg/ml. All peptides were diluted in RPMI 1640 containing 5% FCS for the CTL assay. Note that heat-inactivated FCS has low concentrations of proteases, hence suggested for use in cell cultures where peptide stability is important (37). For libraries 1 through 6, 5 μl of peptide library stock were resuspended in 1.3 ml RPMI 1640 (~76.9 μg/ml) whereas 5 μl of stock of libraries 7 through 11 were resuspended in 1 ml RPMI 1640 (100 μg/ml) and used to sensitize 31Cr-labeled RMA cells. The number of peptides in each library and the concentration of individual peptides in each library in the assay are presented in Table I.

### Table I. Concentration of individual peptides in each library

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<thead>
<tr>
<th>Library No.</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>Peptides/Set</th>
<th>Concentration (pM)</th>
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<td>x</td>
<td>x</td>
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<td>x</td>
<td>x</td>
<td>a</td>
<td>x</td>
<td>F</td>
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<td>x</td>
<td>x</td>
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<td>10,000,000</td>
<td>11.17</td>
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<tr>
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<td>x</td>
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<td>F</td>
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<td>x</td>
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<td>F</td>
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<td>809.96</td>
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<td>L</td>
<td>123,462</td>
<td>809.96</td>
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*The concentration of individual peptides in each library was calculated based on an average yield of peptide synthesis of ~1 mg (estimated at Chiron). Each library was dissolved in 50 μl of DMSO of which 5 μl was diluted into 1.0 or 1.3 ml of CTL medium as described in Materials and Methods. The resulting solution has an estimated concentration indicated in the last column of the Table. Also note that an E:T ratio of at least 25 was used in experiments involving libraries 1–6 and an E:T of about 10 in assays using libraries 7–11.

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A mixture of the 20 natural L-amino acids except Cys. z at P8 indicates a H-4 b Ag presentation. Thus, library 1 consisting of octameric peptides at the carboxyl terminus. The E:T ratio used for testing libraries 1, in library 3. Library 3 is identical to library 1 but is extended by a Gly CTL clone did not recognize RMA cells pulsed with peptides represented release. The residue at P3 is critical for the presentation of the H-4 b Ag-derived epitope by H-2K b to specific CTL clones. A, Identification of the P1 anchor with the 20 natural L-amino acids except Cys and each set of 47,045,881 possible peptides is distinguished by the amino acid residue represented at P1. RMA cells do not express the H-4 b minor H Ag and hence were used as the APCs. RMA cells pulsed individually with each of the six sets of peptides were used in a standard 3Cr release assay to determine the P1 anchor for the unknown H-4 b-specific CTL epitope. None of the peptide sets were recognized by the H-4 b-specific CTL clone. B, Second to the dominant PSF anchor, the residue at P3 is more critical than the P1 anchor in the presentation of H-4 Ag to specific CTL. RMA cells pulsed individually with each of the 11 sets of peptides of library 2 were probed with H-4 b-specific peptide library 2. Library 2 is nonameric with a carboxyl-terminal Gly extension. Each set of 14,856,594 possible peptides is distinguished by the indicated amino acid residue at P3. x denotes a mixture of the 20 natural L-amino acids except Cys. z at P8 indicates a mixture of Phe, Ile, Leu, Met, Val, and Tyr. The peptide set with an Ile residue at P3 was recognized by H-4 b-specific CTL. C. The H-4 b-specific CTL clone did not recognize RMA cells pulsed with peptides represented in library 3. Library 3 is identical to library 1 but is extended by a Gly residue at the carboxyl terminus. The E/T ratio used for testing libraries 1, 2, and 3 was 25:1. The data represented in the three panels are results of a single experiment that was repeated at least twice.

Results

The approach taken to define the rules for peptide Ag presentation by class I molecules to specific CTL was to probe synthetic combinatorial peptide libraries displayed by H-2K b with a CTL clone whose epitope is unknown. Thus, an H-2K b-restricted minor histocompatibility (H) H-4 Ag-specific CTL clone (38) was used to avoid bias from the knowledge of its epitope. The only bias laid in the synthesis of the combinatorial library was the use of an invariant Phe at P5 that is essential for the binding of peptides to H-2K b molecules (11, 14). The rules thus gleaned were further tested using two viral Ag-specific CTL clones whose epitopes have been already established (34, 35).

A secondary anchor residue at P3 plays a key role in Ag presentation to class I-restricted CTL clones

The initial goal was to determine the role of P1 anchor residue in H-4 Ag presentation. Thus, library 1 consisting of octameric peptides with an invariant Phe at P5 (PSF), all naturally occurring L-amino acids except cysteine (Cys) at P1, P2, P3, P4, P6, and P7, and either Phe, Ile, Leu, methionine (Met), Tyr, or Val at P3 (Fig. 1A), was used to determine the P2 anchor. Cys was omitted to avoid complications that could arise from covalent modification of the free sulfhydryl group of this residue. The resulting library consisted of six sets of peptides distinguished by the amino acid residue at P2 and contained 47,045,881 peptides per set. Peptides were presented to the H-4 b-specific CTL clone by RMA cells (described in Materials and Methods); RMA cells express H-2K b molecules of C57BL/6 origin but do not express the BALB.B-derived H-4 b minor H Ag (data not shown). The H-4 b-specific CTL recognizes H-2K b overexpressed by a BALB/c-derived plasmacytoma, K-b high (39) (data not shown). Surprisingly, none of the six peptide sets within this library were recognized by the H-4 b-specific CTL clone (Fig. 1A).

Peptides in library 1 may not have sensitized H-4 b-specific CTL targets for the following reasons. First, the peptides in the library may have been insoluble, hence not presented to the CTL. However, all six peptide sets in library 1 were presented equally well to a C57BL/6-derived polyclonal population of CTL directed against at least four distinct H-2K b-restricted minor H Ags, including H-4 b, of BALB.B origin (data not shown). Thus, solubility is less likely to be the reason why this CTL clone did not recognize peptides of library 1. Second, concentration of individual peptides in library 1 might have been too low to sensitize target cells for lysis by the H-4 b-specific CTL clone. However, the low concentration of peptides in library 1 may not be the reason for the lack of recognition. This conclusion is based on the fact that the polyclonal CTLs raised in C57BL/6 mice against BALB.B splenocytes recognized peptides in library 1 very efficiently (40% specific lysis; data not shown). Moreover, peptide library 1 is recognized with a high efficiency by a CTL clone directed against HSV-I ribonucleotide reductase (RR-I)-derived epitope (described below; see Figure 5). Thus, neither concentration nor solubility could account for the lack of recognition of peptides in library 1 by the H-4 b-specific CTL clone.

The third plausible reason why the H-4 b-specific CTL clone did not recognize library 1 is that a critical amino acid residue at another position, presumably a secondary anchor residue, is essential for the proper presentation of the epitope to CTL. To test this hypothesis, library 2 was synthesized, which consisted of eleven sets of peptides extended by a glycine (Gly) residue at the carboxyl terminus and PSF as the conserved anchor. Note that the carboxyl terminus of peptide library 2 was extended to facilitate the synthesis of peptides with multiple residues at the original P1 anchor residue but was not meant to replace the P1 anchor, which is at P8 of K b-binding peptides. That the Gly-extended libraries did not replace the P1 anchor is substantiated by the crystal structure of HLA-A2 complexed with a decamer peptide that has an additional Gly residue at P10 of a naturally processed nonamer peptide (40). In this structure, Gly at P10 extended out of the Ag-binding groove without replacing the original P1 anchor at P9 (40). The naturally processed peptides longer than nonamers bind HLA-A2 with high affinity (41) and are stable at the physiologic temperature (40). Thus, we predicted that the extension of a normally octameric peptide epitope(s) by a Gly at the carboxyl terminus could provide a combinatorial library to test the role of a hitherto unknown secondary anchor.

Library 2 is distinct from library 1 in that it consists of a mixture of Phe, Ile, Leu, Met, Tyr, and Val at P8 and has one of the following eleven amino acids, alanine (Ala), Phe, Gly, Ile, Leu, Met, asparagine (Asn), proline (Pro), glutamine (Gln), Val, or Tyr, at P3. The residues at P3 distinguish each peptide set in library 2 (Fig. 1B). The choice of residues at P3 was based on the already known anchor motif of H-2K b-binding peptides (11, 14). The remaining positions, P1, P2, P4, P6, and P7, of the peptide contained all naturally occurring L-amino acids except Cys (Fig. 1B). One
peptide set, distinguished by the presence of Ile at P3 (P3I), among the eleven sets of peptides in library 2 was recognized by the H-2Kb-restricted, H-4b-specific CTL clone (Fig. 1B). To determine whether the Gly extension at the carboxyl terminus contributed to the CTL activity of the peptides in library 2, library 3 was synthesized. It is essentially similar to library 1 but extended by a Gly residue at the carboxyl terminus (Fig. 1C). Although this library was presented to the polyclonal CTL against minor H Ags described above (data not shown), none of the six sets of peptides in library 3 were recognized by the H-2Kb-specific CTL clone (Fig. 1C). Hence, the Gly extension does not contribute to the recognition of peptides in library 2 by H-4b-specific CTL clone. Together the data suggest that the amino acid residue at P3 can critically affect the presentation of peptide Ags by class I molecules to specific CTL.

Role of secondary anchor residue at P2 in Ag presentation to class I-restricted CTL clones

PΦI anchor of H-4b-derived CTL epitope. To determine the amino acid residue(s) at P2 in the H-4b-derived epitope, the PΦI anchor of H-4b Ag had to be defined. Therefore, library 4 of octameric peptides was constructed. It consists of five sets of peptides related to each other by virtue of containing P3I and P5F. The peptide sets are distinct from each other because each set of 2,476,099 peptides contains Phe, Ile, Leu, Met, or Val at P4 (Fig. 2A). P1, P2, P4, P6, and P7 are degenerate, containing all the natural L-amino acids except Cys and tryptophan (Trp; Figure 3) at P2. Trp was omitted because none of the H-2Kb-restricted peptides reported to date contain this residue at P2 (13). Additionally, peptides in library 7 consisted of invariant P3I, P5F, and PΦI, and all natural L-amino acids except Cys at P1, P4, P6, and P7 (Fig. 3). Note that, although Ile, Leu, and Val could be used as the Φ anchor, PΦI was chosen because Leu is most frequently used as the carboxyl terminus amino acid residue in peptide epitopes presented by H-2Kb molecules (11, 13). The results revealed that the H-4b-derived epitope accommodates only Gly at P2, without affecting CTL recognition (Fig. 3). Thus, the secondary anchor at P2 of H-4b-specific CTL epitope is invariant.

Role of the solvent-exposed residues of the displayed peptide in Ag recognition by the TCR

Both functional and crystal structure studies of three H-2Kb-restricted antigenic peptides have revealed that amino acid residues at P1, P4, P6, and P7 are solvent exposed and oriented in space in a manner accessible by specific TCR (8, 9, 21). The amino acid structure requirements of the four putative TCR-accessible residues for H-4b Ag recognition were determined using libraries 8, 9, 10, and 11. These libraries were similar to library 7 in that the 123,462 peptides in each library contained P3I, P5F, and PΦI. In addition, three of the four putative TCR contact positions and P2 contained a mixture of all the natural L-amino acids except Cys while the fourth position contained one of the 20 natural L-amino acids except Cys (Fig. 4). Thus library 8 has one of the 20 natural L-amino acids except Cys at P1 and a mixture of all of the natural L-amino acids except Cys at P2, P4, P6, and P7, and so on (see Figure 4).
The stringent requirement of an invariant residue at P3 observed with H-4\(^b\)-specific CTL clone was tested using the RR-1-specific CTL clone. Unlike H-4\(^b\)-specific CTL clone, the RR-1-specific CTL clone recognized library 1 (Fig. 5A) but did not recognize libraries 2 and 3 (data not shown). In fact, P3F of the RR-1-specific CTL clone recognized library 1 (Fig. 5). The H-2K\(^b\)-restricted, gB-specific CTL clone was further tested using two CTL clones whose epitopes are known. One CTL clone is specific for an immunodominant herpes-simplex virus (HSV)-I’s glycoprotein B (gB)-derived epitope, SSIEFARL (35). The second CTL clone is directed against an HSV-I’s ribonucleotide reductase-1 (RR-1)-derived epitope, QTFDFGRL (34). These two CTL clones were chosen because both their epitopes contain P5F and P7L, while one contains P3I (gB) and the other contains P3F (RR-1) (34, 35). The gB- and RR-1-specific CTL clones do not recognize RMA cells unless pulsed with their respective epitopes (data not shown); they interact with H-2K\(^b\) are below the line. The amino acid residue (single-letter code) at each position in the peptide recognized by the gB-derived epitope to specific CTL.

**The P3 rule.** The stringent requirement of an invariant residue at P3 observed with H-4\(^b\)-specific CTL clone was tested using the RR-1-specific CTL clone. Unlike H-4\(^b\)-specific CTL clone, the RR-1-specific CTL clone recognized library 1 (Fig. 5A) but did not recognize libraries 2 and 3 (data not shown). In fact, P3F of the native epitope could be substituted by Gly or Ile without affecting recognition by RR-1-specific CTL clone (Fig. 5, B, C, and D). Although RR-1-specific CTL clone recognized peptide library 6 containing P3I, it did not significantly recognize any of the peptides represented in libraries 7 and 8; the peptides in these two libraries contained invariant P3I, P5F, and P7L (Fig. 5, E and F). Note that the background in the assay presented in Figure 5 was high; in a repeat of the experiment using libraries 7 and 8, none of the peptide sets in these two libraries were recognized (data not shown). Additionally, multiple structurally distinct residues, including those not represented in the native epitope, were recognized by the RR-1-specific CTL clone when used to probe libraries 9, 10, and 11, which also contained P3I, P5F, and P7L (Fig. 5, G, H, and J). A summary of the RR-1-specific CTL recognition pattern is represented in Figure 5K. Together with the H-4\(^b\)-specific CTL recognition pattern, these data suggest that the residue at P3 critically affects Ag presentation by H-2K\(^b\) to specific CTL.

Unlike the H-4\(^b\)- and RR-1-specific CTL clones, none of the peptides within libraries 1 through 6 were recognized by the gB-specific CTL clone (data not shown); note that these libraries have fixed anchors only at P5 and P7 or at P3 and P5. However, peptides within libraries 7 through 11, containing P3I, P5F, and P7L as invariant residues, sensitized targets for recognition by this CTL clone (described in detail below; Figure 6). This suggests that the three anchors P3I, P5F, and P7L together are essential in presenting the gB-derived epitope to specific CTL.

**The P2 rule.** The H-2K\(^b\)-restricted, gB-specific CTL clone was used to determine the structural requirements at P2 for Ag recognition. Unlike the H-4\(^b\)-specific CTL, the gB-specific CTL clone tolerated multiple amino acids at P2. Asp, Pro, serine (Ser), and threonine (Thr) and, to a lesser extent, Ala, Ile, Leu, and Val at P2 did not affect recognition (Fig. 6A); note that the natural gB-derived epitope contains Ser at P2. These data are consistent with the finding that OVA-derived epitope, SIINFEKL, can accommodate Ala, Gly, Leu, Ser, Thr, and Val at P2 without affecting recognition by certain H-2K\(^b\)-restricted, OVA-specific CTL clones (18). Thus multiple structurally distinct amino acid residues can be accommodated at P2 of some H-2K\(^b\)-restricted CTL epitopes.

**The TCR contact rule.** This rule was tested using the H-2K\(^b\)-restricted, gB-specific CTL clone. Akin to H-4\(^b\)-specific CTL clone, gB-specific CTL clone also recognized peptides containing multiple structurally distinct amino acid residues at P1 (Phe, Gln, Ser, and Trp), as well as Asp and Thr to a lesser extent; Fig. 6B) and P6 (Ala, Phe, and Tyr, as well as Trp to a lesser degree; Fig. 6D).
At the remaining positions, the gB-specific CTL clone recognized only the residues represented in the native epitope, i.e., Glu at P4 and Arg at P7 (Fig. 6, C and E). These data are summarized in Figure 6F. Thus, although degenerate, the specific TCR interfaces Ag only when presented with common structurally conserved amino acid residues at one or two positions in the CTL epitope among the four putative solvent-exposed residues that may be accessible to the TCR.

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Discussion

Efforts to date in elucidating the rules for T cell Ag recognition have relied on determining the effects of single amino acid changes within a peptide epitope on this process. Largely, such studies have utilized alanine-scanning mutagenesis, which provided key information on residues in the epitope that contact the class I molecule or the TCR (42, 43). In another approach, an epitope residue individually altered to several other natural L-amino acids has been used to determine the role of class I contact residues in binding the MHC molecule. This has led to the definition of secondary anchor positions (15, 17, 44). Very few studies, however, have attempted to characterize the role of peptide anchor residues in Ag presentation to, and recognition by, CD8+ T cells (18); most such studies have focused on the dominant anchor residues. To attempt a thorough analysis would require screening of ~1.7 × 10^10 individual peptide combinations for CTL recognition. Thus the combinatorial peptide library approach as reported here and by others provides a solution to massive screening for CTL recognition.

The combinatorial library approach is not without limitations. First, it is difficult to ascertain the solubility of individual peptides in a complex library. The solubility problem was addressed in this study using a polyclonal population of CTL against BALB.B-derived multiple minor H Ags, which also includes the anti-H-4b reactivities, to probe the libraries. The results revealed that a reasonable proportion of the peptides in the libraries tested were in solution. Second, competition for binding class I could exclude peptide(s) in a library from being presented. However, fewer than 100 class I peptide complexes are typically necessary for CTL recognition (45, 46); hence, this is unlikely to be a problem. Third, each peptide library would be expected to contain an admixture of agonist and antagonistic peptides for a specific TCR (27). The recognition of the targets sensitized by peptides in a complex library by CTL, then, represents the effect of those agonistic peptides not inhibited by the antagonistic peptides in the library. Whether a set of antagonist peptides in library I inhibited the recognition of agonist peptides in this library by the H-4b-specific CTL clone could not be tested because the H-4b-derived epitope hitherto remains elusive. Notwithstanding, the combinatorial peptide library approach can provide useful information regarding TCR/class I-peptide interactions.

Thus, synthetic combinatorial peptide libraries were used as an approach to define the rules for class I-restricted peptide Ag presentation and recognition. To avoid bias from the knowledge of a CTL epitope, the physico-chemical features of the unknown H-2Kb-restricted, H-4b Ag-specific CTL epitope was first determined using the combinatorial libraries. As expected, several mimotopes were identified. The physico-chemical features of the H-4b-derived mimotopes were compared with those of similarly derived mimotopes of two other H-2Kb-restricted, viral Ag (HSV-1 gB and RR-1)-specific CTL clones whose epitopes are already known. This allowed the definition of two important structural features for class I-restricted peptide Ag presentation and recognition: 1) The display of Ag to H-2Kb-restricted TCR critically depended on the class I-contact residue at P3 of the peptide. In several instances the amino acid residue at P3 was more critical than the dominant PΩ anchor. 2) The TCR requires only one or two TCR-contact residues of the peptide to achieve specificity, while it can interact with multiple structurally dissimilar amino acids at the remaining solvent-exposed positions.

Role of secondary anchor residues in Ag presentation

The role of dominant anchor residues, P5 and P9 in the case of H-2Kb-binding peptides, is thoroughly appreciated in numerous previous studies. Although the secondary anchor residues play an important role in peptide binding to class I molecules, their role in presentation of antigenic peptides to CTL is less clearly understood. A recent study demonstrated that the recognition of OVA epitope SIINFEKL by H-2Kb-restricted, OVA-specific CTL clones was altered by the structural features of the residues at P2. Nonetheless, although clone specific, these CTL clones tolerated structurally distinct amino acids (Ala, Asn, Gly, Ser, and Thr) at P2 in the SIINFEKL epitope (18). This is consistent with the finding presented here, in that the gB-specific CTL clone tolerates structurally distinct amino acid residues at P2 in its epitope. In contrast to the degeneracy at P2 in gB-specific CTL epitope, recognition by H-4b-specific CTL requires an invariant Gly residue at P2. On the other hand, appropriate presentation of H-4b and RR-1 Ags by H-2Kb critically depended on the nature of the residue at P3. Thus, residues at P3 serve as an important secondary anchor at least in appropriate presentation of Ag to CD8+ T cells studied here. The side chain of the residue at P3 is oriented toward the α2-helix and almost completely buried into pocket D in the three crystal structures of H-2Kb individually complexed with different peptides (8, 9, 21). Thus, residues at P3 probably assume the role of an important secondary anchor.

A thorough x-ray crystallographic analysis of HLA-A2 individually complexed with five different peptides revealed that the secondary anchors can influence the orientation of the solvent-exposed residues without altering the conformation of the dominant anchors. Importantly, none of the peptides induced any significant variation in the HLA-A2 structure. Because the solvent-exposed peptide residues are accessible to the TCR, it was concluded that interactions with such residues would determine the antigenicity of the class I-peptide complex (47). Thus the results presented herein provide functional evidence for the role of secondary anchor residues, such as those at P3, in altering the antigenicity of the class I-peptide complex.

A model for TCR/class I-peptide interactions

The two recently solved crystal structures of TCR/class I-peptide cocomplexes have provided detailed information on how the receptor interfaces its ligand (23, 24). Based on these structures, we would predict that the CDR3β loop interfaces the carboxyl half of the peptide Ags derived from H-4b, gB, and RR-1. In this regard, it is noteworthy that the majority of the TCR expressed by over 15 distinct CTL clones against H-4b (48) and gB (49) contain an invariant acidic residue in their CDR3β loop. Being of opposite charge, the acidic residue in CDR3β can make a productive interaction with the basic Arg (H-4b and gB) or His (H-4b) residues present in the respective epitopes. This interaction may be important but does not impart the recognition specificity between the receptor and the ligand because the two CTL clones do not have overlapping epitopes or mimotopes. Thus, P6l and P4E of the H-4b- and gB-specific CTL epitopes, respectively, may play a key role in imparting specificity of the recognition process. Thus, very few atoms of the epitopes are essential for the specificity in Ag recognition by the TCR.

Although much has been learned regarding how the TCR interfaces its Ag, the basis for the degenerate nature of the TCR/class I-peptide interactions remains to be established. Several studies have established that the interaction of the TCR with its ligand is of low affinity (50–52). These affinity values are similar to those for the interaction between IgM and its Ag and contrast that of IgG, which has a much higher affinity for its Ag (53–55). Consequently, the TCR/class I-peptide interactions occur with a slow on and fast off rates (50, 51). Calculations of the Gibbs free energy
change accompanying the formation of Ab and Ag complexes revealed that the higher the affinity of the interaction, the greater the binding energy. Additionally, both the affinity and the binding energy are directly proportional to the number of amino acid residues that are critical in the Ab-Ag interactions (56). Thus, by analogy, the binding energy accompanying the ternary complex formation would be minimal, based on the physical parameters of TCR/class I-peptide interactions. The low binding energy of this reaction would suggest that the specificity of the TCR/class I-peptide binding is imparted by interactions between few atoms of the interacting components with the remaining large (~600 Å²) interface area probably participating in physico-chemical complementarity.

In support of the above model is the finding that the TCR of the H-4p and the g-specific CTL clones require only one of the four solvent-exposed residues in their cognate epitopes for effective Ag recognition. Additionally, this model would also assume a strict spatial positioning of the critical TCR contact residue(s) in relation to the remaining interface area on the class I-peptide complex for effective TCR/class I-peptide interactions. Thus, alterations in the strict spatial order of the critical TCR contact residues by differences in the secondary anchors, as seen with the H-4p and RR-I-specific TCR, can affect TCR/class I-peptide interaction patterns. Finally, the above model for TCR/class I-peptide interaction also explains how a single TCR interfaces multiple ligands.

Our finding that the TCR contacts only one or two of the four solvent-exposed residues of the peptide to achieve specificity is supported by the recently described crystal structure of the murine 2C TCR complexed with H-2Kb bound to self-peptide dEV8 (52). The dEV8 peptide is buried deep in the H-2Kb groove, hence contributing very little to the overall TCR/MHC-peptide interactions. Moreover, the interface between the 2C TCR and the H-2Kb-dEV8 complex has poor three-dimensional complementarity and has large empty spaces, making very few contacts with the peptide possible (52). Similarly, the A6 TCR/HLA-A2-HIV tax complex also revealed poor structural complementarity at the interface between the receptor and its ligand (23). Thus, the poor complementarity observed in the above crystal structures explains why the TCRs studied here tolerate multiple structurally dissimilar amino acids at most of the TCR contact residues of the peptides presented by H-2Kb.

In conclusion, akin to the class I-peptide interactions whose specificity is determined by the structure of the residues at select positions in the peptide (anchor residues), the TCR/class I-peptide interaction also requires only an amino acid or two among at least four available TCR contact residues within the peptide for specificity. In this manner a single TCR can interface multiple ligands.

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References
