

# Structural Basis for T Cell Recognition of Altered Peptide Ligands: A Single T Cell Receptor Can Productively Recognize a Large Continuum of Related Ligands

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## Summary

T cells recognize short linear peptides bound to major histocompatibility complex (MHC)-encoded molecules. Subtle molecular changes in peptide antigens produce altered peptide ligands (APLs), which induce different T cell responses from those induced by the antigenic ligand. A molecular basis for how these slight molecular variations lead to such different consequences for the T cell has not been described. To address this issue, we have made amino acid substitutions at the primary T cell receptor (TCR) contact residue of the murine hemoglobin determinant, Hb(64-76)/I-E<sup>k</sup> and produced 12 peptides that interact with the TCR of the T cell clone 3.L2. The 3.L2 T cell responds to these peptides, which vary 1 million-fold in their activity, and enables them to be ranked according to their relative ability to signal through the 3.L2 TCR. Such a ranking reveals that the ability of the 3.L2 T cell to respond to these peptides depends on how well the structure of the side chain at the primary TCR contact site mimics that of the Asn residue present in the antigenic ligand. The reactivity of the 3.L2 T cell also depends on an MHC contact residue that is next to the primary TCR contact residue, suggesting that conformation of the Asn side chain is also important. By using nonnatural amino acids at a TCR contact residue, we have demonstrated that APLs can be rationally designed based on structure. These data are consistent with a model in which the affinity of a peptide-MHC complex for the TCR determines how the T cell will respond.

Cell-mediated immunity is dependent on the activation of antigen-specific  $\alpha/\beta$  T cells. This activation depends ultimately on the ability of a TCR to recognize a complex composed of an 8–20-amino acid peptide bound to a protein encoded by the MHC (1, 2). A molecular understanding of this interaction is required in order to understand antigen-specific immunity. Although this interaction is highly specific, there is a surprising amount of flexibility in this recognition event. A TCR can recognize ligands that are slightly altered, and recognition of these altered ligands can lead to dramatic functional consequences for the T cell (3). A knowledge of the structural basis for the interaction between the TCR and altered ligands is therefore important if we are to understand T cell recognition and antigen-specific immunity.

Flexibility in TCR recognition of ligand was initially demonstrated using a T cell clone that produced IL-4 and proliferated in response to its wild-type ligand but produced IL-4 in the absence of proliferation in response to a single-amino acid variant of the antigenic peptide (4). This finding showed that a subtle change in the ligand did not completely eliminate TCR recognition, but the TCR could

still recognize the altered peptide ligand (APL)<sup>1</sup>. Subsequently, it has been shown that TCR recognition of APLs can lead to a variety of responses from the T cell, many of which are fundamentally different from that induced by the wild-type ligand (5). These altered responses include cytokine production in the absence of proliferation, differential cytokine production, anergy, and antagonism of the response to the wild-type antigen (4, 6–8).

Evidence suggests that flexibility in recognition of ligand is an essential feature of the life and function of T cells. In particular, thymic development of T cells may depend entirely on the ability of the TCR to interact with a suboptimal ligand (9). Fetal thymic organ culture experiments using thymi from mice expressing a transgenic TCR have shown that positive and negative selection of T cells can be mediated by peptides closely related to the cognate antigenic peptide (10–13). Because of the flexibility in TCR ligand recognition, a few self peptides may be able to select

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<sup>1</sup>Abbreviations used in this paper: Abu,  $\alpha$ -aminobutyric acid; APL, altered peptide ligand; MCC, moth cytochrome C; Nva, norvaline; Pra, propyl-argylglycine.

an entire repertoire of T cells that is capable of recognizing a vast array of foreign antigens. Additionally, numerous *in vitro* studies of cloned T cell lines have clearly demonstrated that peripheral T cells have retained the capacity to recognize suboptimal ligands. The two main effects that APLs have on peripheral T cells are to act as antagonists and inhibit the T cell response (8, 14–16), or to act by forcing production of a certain pattern of cytokines (6, 17). These effects can potentially lead to subversion of the immune response by a virus, and also raise the possibility of treatment of autoimmune diseases with APLs.

Although it is clear that APLs play a major role in the life of T cells, a structural basis for how APLs interact with TCR, and how this relates to a change in function, has not been established. Our aim is to understand the structural basis for the interaction of the TCR with APLs. Such an understanding would help to answer two main questions. First, we would like to know the molecular basis for how recognition of an APL is different from recognition of the wild-type ligand. This understanding would lead to knowledge of how APL recognition leads to a different T cell response and better understanding of the general mechanisms underlying T cell recognition of MHC and T cell activation. Second, we would like to identify principles that would allow a rational design of APLs. The potential usefulness of APLs makes it important to be able to easily produce them once the sequence of a peptide antigen has been determined. Guiding principles for APL design would therefore be of great benefit.

The differences between peptides that act as APLs and their wild-type counterparts have so far been difficult to categorize. Crystallographic studies of antigenic peptide–MHC complexes have revealed that the peptide fits into a binding site that leaves much of the peptide inaccessible for recognition (18–21). Some amino acid side chains of the peptide do, however, protrude from the surface of the MHC molecule and are available for interaction with the TCR. In general, peptides have three or four residues that are available to contact the TCR and, together with amino acid side chains from the MHC molecule, make up the surface thought to interact with the TCR directly. Therefore, the surface made up of both peptide and MHC is the ligand recognized by a TCR and will change if the peptide sequence is changed.

Most APLs have been generated empirically, by making changes in the few TCR contact residues of the peptide. A study of the DR1-restricted 307–319 hemagglutinin peptide epitope suggested that only conservative substitutions at TCR contact residues would yield APLs (22). However, a study of an H-2K<sup>b</sup>-restricted ovalbumin peptide was unable to draw any correlation between the type of substitution made and how the TCR responded (23). Our own experience with the I-E<sup>k</sup>-restricted hemoglobin 64–76 epitope has been that substitutions at TCR contact residues are the most likely places to find APLs, but that the limited number of side chains that we have been able to find at any one position that interact with a single TCR have made it impossible to draw any conclusions about what makes an ef-

fective substitution. The lack of a paradigm for the rational design and function of APLs makes a systematic study of the recognition of a single TCR contact residue necessary.

We have carefully examined recognition by the 3.L2 T cell of the Hb(64–76) peptide bound to I-E<sup>k</sup> (24). By making amino acid changes in the P5 residue of this peptide that is known to point toward and interact directly with the TCR, we have identified peptides that can act as weak agonists or as APLs for a particular TCR over an extensive activity range. These amino acid substitutions have allowed us to identify properties of this TCR contact residue that allow it to induce a T cell response. Based on this knowledge, we have produced APLs containing nonnatural amino acid substitutions to which the T cell responds in a predictable manner. Such peptides are potentially valuable tools in the manipulation of immune responses and show that recognition of APLs has a structural basis.

## Materials and Methods

**Cells.** The 3.L2 Th1 clone and the 3.L2.12 T cell hybridoma were grown in RPMI 1640 supplemented with 10% FCS (HyClone Laboratories Inc., Logan, UT),  $2 \times 10^{-5}$  M  $\beta$ -ME, 2 mM Glutamax (GIBCO BRL, Gaithersburg, MD), 50  $\mu$ g/ml gentamicin, and 10 mM Hepes. The 3.L2 Th1 clone was derived as described previously (24) and is specific for the Hb(64–76) peptide as presented by the I-E<sup>k</sup> MHC class II molecule. The 3.L2.12 hybridoma was derived by polyethylene glycol fusion of the 3.L2 Th1 clone to the BW5147  $\alpha$ <sup>-</sup> $\beta$ <sup>-</sup> thymoma (25). Both the 3.L2 clone and the 3.L2.12 hybridoma are CD4<sup>+</sup>, and the role of CD4 in the response of these cells is currently under investigation. The 3.L2 clone and the 3.L2.12 hybridoma use the same TCR based on the fact that we can account for all of the TCR on the surface of these cells by staining with an anti-TCR clonotype antibody. Furthermore, we have found that the TCR recognized by the anticlonotype antibody is responsible for the observed activity by transfection of the proper TCR chains into the TCR-negative 58 $\alpha$ <sup>-</sup> $\beta$ <sup>-</sup> hybridoma (data not shown). The CH27 B cell lymphoma expresses the I-E<sup>k</sup>/I-A<sup>k</sup> MHC class II molecules and was grown in RPMI media supplemented as described (26).

**Peptides.** Peptides were synthesized using standard Fmoc chemistry on a multiple peptide synthesizer (Symphony/Multiplex; Protein Technologies Inc., Tuscon, AZ). Fmoc-Nva was purchased from Calbiochem-Novabiochem (La Jolla, CA), and the remaining Fmoc-protected amino acids as well as Lys-coupled resin were purchased from Advanced Chemtech (Louisville, KY). Peptides were purified on a C<sub>18</sub> reverse-phase HPLC column. The C72 peptide was resuspended at 2 mM in an aqueous solution of 100 mM  $\beta$ -ME before HPLC purification, and the purified, reduced C72 peptide was stored under nitrogen. Amino acid content and accurate concentrations of all peptides were determined by analysis on an amino acid analyzer (model 6300; Beckman Instruments, Inc., Fullerton, CA) and comparison with a standard of known concentration. The purity and accuracy of each of the peptides were confirmed by mass spectrometry at the Washington University Mass Spectrometry Resource. Altered peptides of Hb(64–76) are referred to using the one-letter amino acid code for the substituted amino acid, followed by its position. For example, A72 refers to an Hb(64–76) peptide that has Ala substituted for Asn at position 72. The amino acid residues themselves are referred to by their three-letter codes. Therefore, Asn72

refers to the asparagine residue at position 72. For the nonnatural amino acids, the three-letter codes are used to refer both to the peptides and to the amino acid residues. The names and sequences of the peptides used in this study are as follows: Hb(64-76) = GKKVITAFNEGLK; A72 = GKKVITAFAEGLK; C72 = GKKVITAFCEGLK; D72 = GKKVITAFDEGLK; E72 = GKKVITAFEEGLK; F72 = GKKVITAFFEGLK; G72 = GKKVITAFGEGLK; H72 = GKKVITAFHEGLK; I72 = GKKVITAFIEGLK; K72 = GKKVITAFKEGLK; L72 = GKKVITAFLEGLK; M72 = GKKVITAFMEGLK; P72 = GKKVITAFPEGLK; Q72 = GKKVITAFQEGLK; R72 = GKKVITAFREGLK; S72 = GKKVITAFSEGLK; T72 = GKKVITAFTEGLK; V72 = GKKVITAFVEGLK; W72 = GKKVITAFWEGLK; Y72 = GKKVITAFYEGLK; Abu72 = GKKVITAF(Abu)EGLK; Pra72 = GKKVITAF(Pra)EGLK; Nva72 = GKKVITAF(Nva)EGLK; D73 = GKKVITAFN-DGLK; C72D73 = GKKVITAFCDGLK; T72D73 = GKKVIT-AFTDGLK; and V72D73 = GKKVITAFVDGLK.

**Peptide Binding to I-E<sup>k</sup>.** The relative ability of all of these peptides to bind I-E<sup>k</sup> was determined by assaying the ability of the peptides to compete with a peptide composed of amino acids 92-103 of moth cytochrome C (MCC) for binding and thereby inhibit the response of the T cell hybridoma 2B4. To measure this competition for binding to available cell surface I-E<sup>k</sup> molecules, 10<sup>5</sup> 2B4 cells were incubated with 2 × 10<sup>4</sup> CH27 in the presence of 0.2 μM MCC peptide plus a titration of the Hb(64-76) peptide or one of its analogs. Supernatants were collected 20 h later and assayed for IL-2 production using the CTLL-2 cell line. The Hb(64-76) peptides all gave a 50% inhibition of the 2B4 response at a concentration between 10 and 100 μM.

**T Cell Hybridoma Assay.** Stimulation of T cell hybridomas was determined by measuring IL-2 production using the IL-2-dependent cell line CTLL-2 (24).

**Antagonist Assay.** A previously described assay for TCR antagonism of a T cell hybridoma was used (8, 27). CH27 cells were prepulsed with the Hb(64-76) peptide by incubation of 3 × 10<sup>6</sup> CH27 cells in 2 ml RPMI media with 0.1 μM Hb(64-76) peptide for 2 h at 37°C. After washing three times, 2 × 10<sup>4</sup> prepulsed CH27 cells were added to wells of a 96-well plate containing 10<sup>5</sup> T cell hybridoma cells in 200 μl of RPMI media and a titration of potential antagonist peptides. After 20 h of incubation at 37°C, supernatant was assayed for IL-2 content using CTLL-2.

**Apoptosis Assay.** Apoptosis of the CH27 cells was assayed as previously described (26). Briefly, 3 × 10<sup>6</sup> CH27 cells were radiolabeled by incubation for 5 h in 1 ml of RPMI 1640 media containing 4 mCi [3H]thymidine. After washing three times, 3 × 10<sup>4</sup> radiolabeled CH27 cells were cultured with 10<sup>5</sup> 3.L2 T cells in the presence of the indicated peptides. 24 h later, cells were harvested, and apoptosis was quantitated by liquid scintillation counting. Apoptosis results in a decrease in the number of recovered counts due to fragmentation of the labeled DNA. The EC<sub>40</sub> of these peptides was calculated as the concentration of peptide required to reduce the amount of recovered counts by 40%. This percentage was selected in order to include all of the peptides that were positive in the assay.

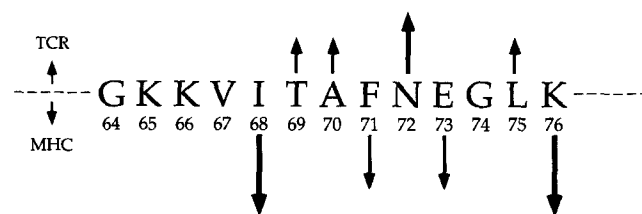
## Results

**The Hb(64-76) Epitope.** Previous studies have characterized the binding of the Hb(64-76) peptide to the I-E<sup>k</sup> class II MHC molecule and recognition of that peptide-MHC complex by TCRs (Fig. 1) (4, 24, 25). Amino acids Ile68 and Lys76 of Hb(64-76) are the main MHC anchor

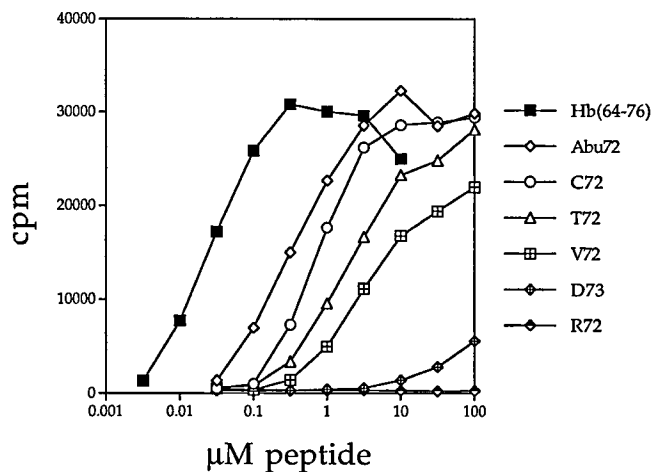
residues, although Phe71 and Glu73 also interact with the MHC. Comprehensive studies of a panel of 11 Hb(64-76)-specific T cells have shown that residues Thr69, Ala70, Asn72, and Leu75 are important for interaction with the TCR (4, 24, 25). Importantly, position 72 has been designated the primary T cell contact residue because almost all T cells that respond to the Hb(64-76) epitope focus their recognition intensely on this position. In fact, all T cells isolated so far that respond to Hb(64-76) will not respond to a variant of Hb(64-76) that has the Asn at position 72 substituted with a Gln (Q72), a change that adds one methylene group. Recently, the crystal structure for the Hb(64-76) peptide complexed to I-E<sup>k</sup> has been solved, and it shows that Asn72 is positioned in the center of the peptide-MHC complex and points directly up from the surface of the MHC molecule in an ideal position to interact with the TCR (21). The structure also reveals that Glu73 is an MHC-binding residue, and its side chain is inaccessible to the TCR. This finding was unanticipated because a peptide with a Glu-to-Asp mutation at position 73 (D73) binds to the MHC equally as well as the wild-type peptide but interacts with the TCR in a different manner (4, 24, 25).

Although most T cells that are reactive with the Hb(64-76) peptide only recognize Asn at position 72, we found that the CD4<sup>+</sup> T cell clone 3.L2 will tolerate some changes at the 72 position. Because we knew that side chains at this position would interact directly with the TCR, our goal was to identify APLs for the 3.L2 T cell that were created by single-amino acid changes at position 72 of Hb(64-76). We reasoned that the identification of the side chain structures that are able to interact with the 3.L2 TCR would show how subtle variations in the structure of a ligand can influence T cell response. We have therefore characterized the ability of the 3.L2 T cell to respond to Hb(64-76) peptides that bear mutations at position 72, encompassing all 19 other naturally occurring amino acids.

**Identification of Peptides That Induce a Response through the 3.L2 TCR.** For the initial characterization of these peptides, we have used the 3.L2.12 hybridoma, a CD4<sup>+</sup> cell line that was derived from the 3.L2 T cell clone. This hybridoma uses the same TCR as the 3.L2 clone based on



**Figure 1.** A representation of the Hb(64-76) epitope. The sequence of this peptide is shown using the standard one letter amino acid code. Based on a combination of mutational analyses and the crystal structure of Hb(64-76) complexed to I-E<sup>k</sup>, residues of the peptide can be designated to interact with the MHC molecule or primarily with the TCR. Arrows pointing down indicate the residues that interact with the MHC molecule, and the upward pointing arrows indicate the TCR contact residues. The primary MHC anchor residues are Ile68 and Lys76, and the Asn at position 72 interacts exclusively with the TCR.

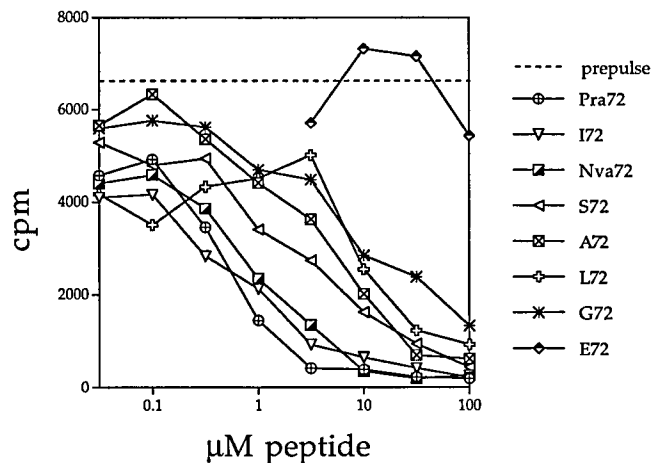


**Figure 2.** Identification of agonist peptides for the 3.L2 TCR. Hb(64-76) peptides bearing all 20 natural amino acids at position 72 were tested for their ability to stimulate IL-2 production from the 3.L2.12 hybridoma. The nonnatural amino acids Abu, Nva, and Pra were also substituted at position 72 and tested for their ability to stimulate 3.L2.12. In addition, a peptide with a Glu-to-Asp mutation at position 73 was also tested. The following peptides are not displayed on the graph but behaved identically to the R72 peptide and gave no stimulation at concentrations as high as 100  $\mu\text{M}$ : A72, D72, E72, F72, G72, H72, I72, K72, L72, M72, P72, Q72, S72, W72, Y72, Pra72, and Nva72. IL-2 production was determined by proliferation of CTLL-2 cells. The values represent the mean of triplicate wells, and the SDs were <15% of the mean. Positive peptides were tested at least four times, and negative peptides were tested at least two times.

staining with an anticonotypic antibody and gene transfer studies. Therefore, the 3.L2.12 T cell hybridoma gives us a convenient method to analyze the panel of peptides for the ability to act as agonists or antagonists for the 3.L2 TCR. All of the substituted peptides used in this study bind the I-E<sup>k</sup> MHC molecule with a similar affinity (see Materials and Methods), and the crystal structure of the wild-type Hb(64-76) peptide shows that the Asn at position 72 has minimal interactions with the MHC molecule (28). Therefore we presume that the peptides used in this study that are substituted at position 72 bind to the MHC molecule in the same manner and that all of the position 72 side chains point toward the TCR and are available for interaction.

We first tested these peptides for their ability to stimulate the 3.L2.12 hybridoma to produce IL-2 and thereby act as agonists. This resulted in the identification of three peptides substituted at the 72 position that are weak agonists for the 3.L2 TCR (Fig. 2). The peptides C72, T72, and V72 recapitulate the activity of the Hb(64-76) peptide at higher concentrations. Hb(64-76) stimulates IL-2 production at a dose as low as 0.01  $\mu\text{M}$ , whereas the peptides C72, T72, and V72 require a 10–100-fold higher concentration of peptide to achieve the same effect. All other naturally occurring amino acids at position 72 failed to stimulate any IL-2 production from 3.L2.12 at doses up to 100  $\mu\text{M}$  (Fig. 2 and data not shown).

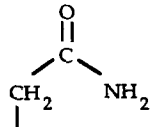


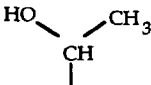
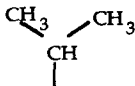
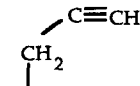
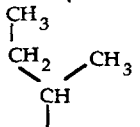
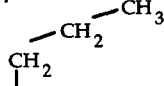

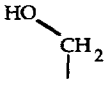
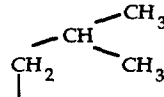

We next wanted to determine if among the peptides substituted at position 72 that were negative in the agonist



**Figure 3.** Identification of antagonist peptides for the 3.L2 TCR. All of the Hb(64-76) peptides substituted at position 72 that failed to act as agonists for the 3.L2 TCR were tested for the ability to act as antagonists. APCs were prepulsed with 0.1  $\mu\text{M}$  of the wild-type Hb(64-76) peptide and stimulated the 3.L2.12 hybridoma to the level indicated by the dashed line. The graph shows those peptides that gave a dose-dependent inhibition of this response, as well as one peptide (E72) that did not act as an antagonist. The peptides W72, P72, Q72, E72, R72, K72, F72, and H72 all failed to antagonize and were tested two times at concentrations up to 316  $\mu\text{M}$ . The peptides M72, D72, and Y72 gave a slight inhibition at concentrations >100  $\mu\text{M}$ . The values represent the mean of triplicate wells, and the SDs were <15% of the mean.

assay, there were any peptides that could act as APLs for the 3.L2 TCR. We tested these remaining peptides by assaying for the ability to antagonize the 3.L2.12 hybridoma response to Hb(64-76) (Fig. 3). I72, S72, A72, L72, and G72 all showed a dose dependent antagonism of the response to wild-type Hb(64-76). The peptides W72, P72, Q72, E72, R72, K72, F72, and H72 all failed to yield any detectable antagonism even at concentrations as high as 316  $\mu\text{M}$ . The peptides M72, D72, and Y72 did inhibit the response of 3.L2.12 slightly, but only at concentrations >100  $\mu\text{M}$ . It is possible that such slight antagonism at very high concentrations may be significant, but in this study we did not consider these very weak antagonist peptides further.

The two assays described above enabled us to identify three agonists and five antagonists for the 3.L2 TCR. Based on our knowledge of the structure of the Asn at position 72 and the structures of the other side chains to which 3.L2.12 responded, we next attempted to rationally design APLs using peptides substituted at position 72 with nonnatural amino acids. Because G72 is a weak antagonist and A72 is a more active antagonist, we reasoned that side chains that extended a straight aliphatic side chain further than Ala would possibly more closely mimic the size of Asn and induce a more potent response through the 3.L2 TCR. Therefore, we tested peptides substituted at position 72 with  $\alpha$ -aminobutyric acid (Abu) and norvaline (Nva). Abu has a straight aliphatic side chain of two carbons, and Nva has a straight three-carbon side chain (Fig. 4). Abu72 is a weak agonist for the 3.L2 TCR and is in fact the peptide that comes closest to recapitulating the activity of the

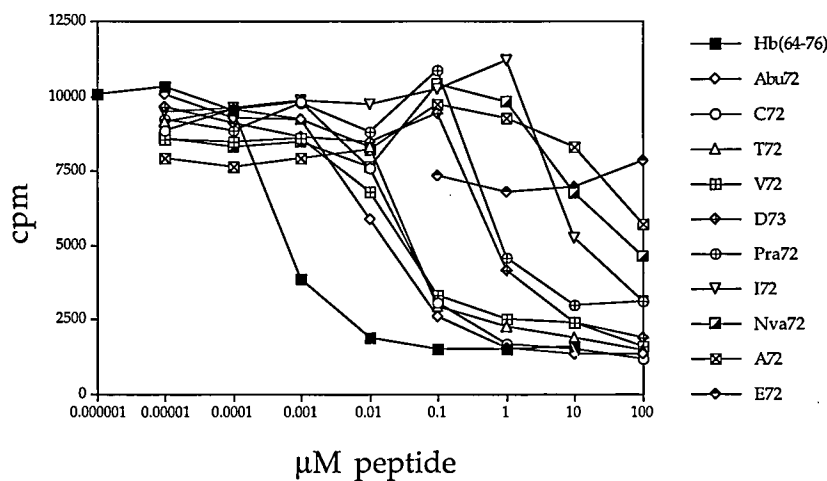
<u>Amino acid at position 72</u>	<u>EC<sub>40</sub>(<math>\mu</math>M)</u>	<u>Relative Activity</u>
Asn 	0.0004	100
Abu 	0.0085	5
Cys 	0.02	2
Thr 	0.023	2
Val 	0.018	2
Pra 	0.6	0.07
Ile 	7	0.006
Nva 	20	0.002
Ala 	60	0.0007
Ser 		(0.0007)
Leu 		(0.0003)
Gly 		(0.0001)

**Figure 4.** Relative activities of peptides that interact with the 3.L2 TCR. 12 peptides that differ only in the structure of the amino acid side chain at position 72 of Hb(64-76) were ranked according to their ability to stimulate through the 3.L2 TCR. A schematic diagram of the structures of the side chains of these amino acids at position 72 is shown. The wild-type Hb(64-76) peptide has an Asn at position 72 and has been arbitrarily assigned an activity of 100. The EC<sub>40</sub> of the peptides Abu72 through A72 was determined from the apoptosis assay (Fig. 5), and these numbers were used to assign an activity of these peptides relative to the wild-type peptide. Because S72, L72, and G72 had no activity in the apoptosis assay, a relative activity for these peptides was estimated by comparing the concentration of these peptides required for antagonist activity with that required for A72. Parentheses placed around the relative activities for these peptides indicate that these activities are derived by extrapolation.

Hb(64-76) peptide (Fig. 2). Nva72 is an antagonist for the 3.L2 TCR and is active at  $\sim$ 10-fold lower concentrations than the A72 peptide (Fig. 3). We next wanted to test an amino acid at position 72 that not only resembled Asn in size but also could possibly mimic some of the chemical nature of the amide group. We therefore synthesized a peptide that used propargylglycine (Pra) at position 72, an amino acid that is thought to be able to mimic an Asn side chain. Like Nva, Pra has a straight three-carbon side chain,

but in Pra the last two carbons are joined by a triple bond (Fig. 4) (29, 30). The Pra72 peptide was a very good antagonist for the 3.L2 TCR, even at lower concentrations than Nva72 (Fig. 3).

*Peptides That Induce a Response through the 3.L2 TCR Can Be Ranked Based on Their Relative Activity.* From these studies, we have identified 11 peptides that differ from the wild-type Hb(64-76) peptide at position 72 but can still interact with the 3.L2 TCR in some manner. We next at-



**Figure 5.** Induction of peptide-dependent B cell apoptosis by the 3.L2 T cell clone. The agonist and antagonist peptides that were identified using the 3.L2.12 hybridoma were tested for the ability to induce apoptosis of the CH27 B cell lymphoma. Shown are the peptides that were positive in this assay as well as the negative control peptide E72. Apoptosis is measured by a reduction in counts due to fragmentation of prelabeled CH27 DNA. The peptides S72, L72, and G72 did not induce any apoptosis even though these peptides were positive in the antagonist assay. The values represent the mean of triplicate wells, and the SDs were <15% of the mean. This experiment was performed four times.

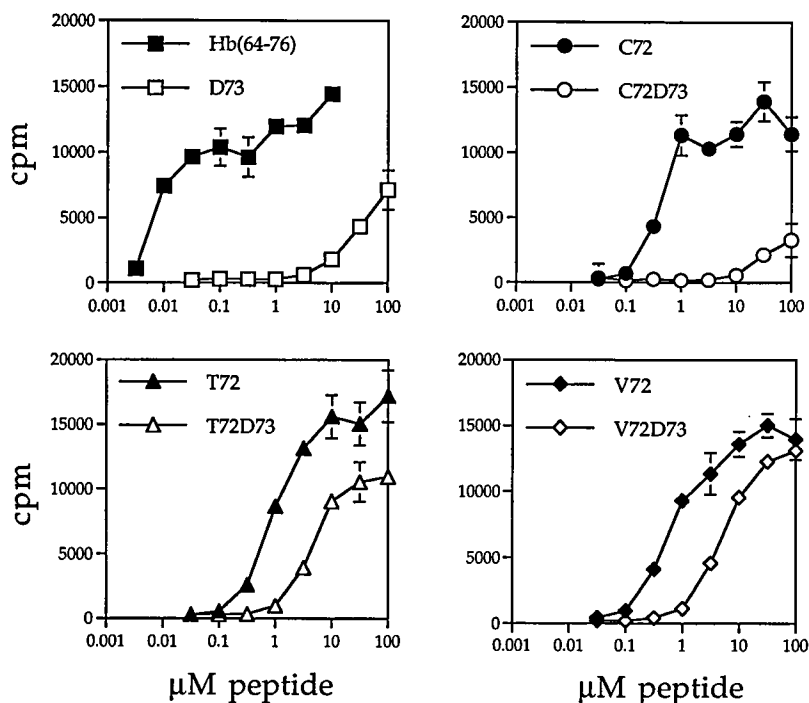
tempted to use a single assay to rank these peptides based on their stimulatory capacity. The assay that we have used is the induction of apoptosis in the B cell lymphoma CH27 by the 3.L2 clone. This rapid and sensitive apoptosis assay has previously been used to identify APLs for other T cell clones, and it allows agonists and partial agonists to be compared on the same scale (26). Apoptosis of CH27 can be mediated by the 3.L2 clone and is observed over a 100,000-fold concentration range of peptides (Fig. 5). The peptides that were identified as weak agonists require  $\sim 10$ –100-fold higher concentrations of peptide than wild-type Hb(64-76). Peptides that were antagonists in the hybridoma assay also induce apoptosis of CH27, but at concentrations that are 1,000–100,000-fold higher than wild-type Hb(64-76). However, this assay appears to be somewhat less sensitive than the antagonist assay, because S72, L72, and G72 did not induce any apoptosis of CH27. Significantly, the order that the peptides fall into using the apoptosis assay of the T cell clone is the same as that obtained using the two separate lymphokine assays of the T cell hybridoma. The advantage of using the apoptosis assay is that it allows us to quantitatively compare weak agonist and antagonist peptides in a single assay.

Based on the combination of assays outlined above, we could rank 12 peptides differing only in the structure of the side chain at position 72 for their ability to induce a response from the 3.L2 T cell. A schematic for the structure of each of these side chains and their relative activity in the 3.L2 assays is displayed in Fig. 4. The Hb(64-76) peptide was arbitrarily assigned an activity of 100, and the relative activity for the peptides Abu72 through A72 was determined by the relationship between the concentration of Hb(64-76) and the substituted peptide required for apoptosis of 40% of the CH27 cells in the assay depicted in Fig. 5. Because S72, L72, and G72 do not have any activity in the apoptosis assay, a relative activity was estimated based on the antagonist activity of these peptides compared with A72. In antagonist assays, S72 and A72 acted at essentially the same concentration, so these peptides have been assigned the same relative activity even though S72 does not have any activity in the apoptosis assay.

#### *An MHC Contact Residue Influences TCR Recognition.*

The substitution of the Glu at position 73 of the Hb peptide with Asp does not affect the affinity of peptide binding to MHC but does change T cell recognition of the peptide-MHC complex (4, 24, 25). Interestingly, this residue was shown to be an MHC binding residue in the crystal structure of the Hb(64-76)/I-E<sup>k</sup> complex (21). For the 3.L2 TCR, the D73 peptide is a weak agonist, requiring  $\sim 10,000$ -fold higher concentration than Hb(64-76) to induce IL-2 production from the 3.L2.12 hybridoma (Fig. 2). Because the position 73 residue does not contact the TCR directly, we wondered how a change at this position could affect TCR recognition. One hypothesis is that a change at this position could alter the conformation of other side chains either in the peptide or in the MHC molecule. Another possibility is that the TCR makes some contacts with atoms in the peptide backbone, and the accessibility of these atoms is changed by the D73 substitution. Of these suggestions, one of the most likely possibilities is that the D73 substitution causes the residue at position 72 to be in a different conformation. We reasoned that if an Asp at position 73 was influencing the side chain at position 72, then it should have different effects depending on how well the position 72 side chain is recognized. We tested this hypothesis by examining the influence of a D73 mutation on not only the peptide containing Asn at position 72, but also its effect on other position 72 amino acids.

An analysis of the effect of D73 on Asn, Cys, Thr, and Val at position 72 is shown in Fig. 6. By analyzing the ability of these doubly substituted peptides to induce IL-2 production from 3.L2.12, we have found that stronger agonists at position 72 are more influenced by a change at position 73. Hb(64-76) is active at a concentration of 0.01  $\mu\text{M}$ , and the D73 mutation is active at a 3,000-fold higher concentration. The T72 and V72 peptides are not nearly as stimulatory as the Hb(64-76) peptide, and combining these mutations with the D73 mutation only shifts the response  $\sim 10$ -fold. These results are consistent with the hypothesis that the amino acid at position 73 influences the conformation of the residue at position 72. For a residue at 72 that interacts very well with the 3.L2 TCR, such as Asn, a slight



**Figure 6.** A mutation in the MHC contact residue at position 73 of Hb(64-76) influences recognition of the amino acid at position 72. Peptides with single substitutions at position 72 or double substitutions at positions 72 and 73 were tested for the ability to stimulate the 3.L2.12 hybridoma to produce IL-2. The four graphs represent data obtained from a single agonist assay that was performed in the same manner as the assay in Fig. 2. The data points represent the mean of triplicate wells, and this result is representative of at least three experiments.

change in conformation will significantly alter the effectiveness of the peptide. However, for residues at 72 that have a less-than-optimal interaction with the 3.L2 TCR, such as Thr, a slight change in conformation is not nearly as influential, because these residues already have a suboptimal fit with the TCR. We have tested other amino acids at position 72 that are not stimulatory for 3.L2.12, and in no case does addition of the D73 substitution cause conversion to a stimulatory peptide.

## Discussion

In these studies we have identified some of the structural parameters that determine how the 3.L2 TCR recognizes its ligand. By examination of Hb(64-76) peptides that use all 20 naturally occurring amino acids at position 72, we have determined that peptides using a side chain at position 72 that resembles Asn are the peptides that induce T cell responses. Based on this knowledge, we rationally designed APLs that used nonnatural amino acid side chains at position 72. In addition, we have found that even if the structure of the position 72 side chain is unaltered, an APL can be made by substitution of the neighboring MHC contact residue at position 73. These data suggest that a substitution at position 73 induces a conformational change in the position 72 side chain, thus producing an altered ligand. The data show that a single TCR can recognize a broad spectrum of ligands that can stimulate the T cell over a 1 million-fold range of activity.

Examination of the structures of the side chains that induce a response through the 3.L2 TCR reveals a correlation between structure and responsiveness. The response of the 3.L2 TCR to the Abu-substituted peptide is  $\sim 1/20$  of

the response to the peptide with an Asn side chain. The 3.L2 T cell also responds well to peptides with side chains that have a  $\beta$  and  $\gamma$  carbon like Abu but have additions to the  $\beta$  carbon. T72 and V72 have only  $\sim 2.5$ -fold lower activity than Abu72, and these side chains have an hydroxyl and a methyl group addition at the  $\beta$  carbon, respectively. Larger additions at the  $\beta$  carbon are much more influential, as I72 is  $\sim 1,000$ -fold lower in activity than Abu72. Although this TCR tolerates additions to the  $\beta$  carbon, additions to the  $\gamma$  carbon have greater consequences. Notably, the Nva side chain adds a methyl group to Abu and reduces the relative activity 2,500 times. This suggests that the 3.L2 TCR has very strict requirements for recognition of the amide structure that is present on the  $\gamma$  carbon of the Asn side chain. Although the addition of one methyl group on the  $\gamma$  carbon reduces activity 2,500-fold (Nva72) and addition of two methyl groups reduces activity 16,600-fold (L72), not all additions to the  $\gamma$  carbon have such an extreme effect; the Pra72 peptide has an activity that is only 71-fold less than Abu72. Thus, among the amino acids that are larger than Abu, Pra has the side chain that most closely resembles Asn structurally, and the Pra72 peptide has the activity that most closely resembles Hb(64-76).

The data suggest that for the type of signaling through the 3.L2 TCR that leads to antagonism, the side chain at position 72 must either be small and relatively nonpolar (Ala, Ser), or a little larger than Val and also nonpolar (Ile, Nva, Leu). Side chains that fall into an optimum size range and have limited polarity (Abu, Cys, Thr, Val) deliver the type of signal through the TCR that makes a weak agonist. We would like to presume that the TCR forms a binding surface that is able to accept a side chain with two carbons, and hydrophobic interactions between the TCR and the  $\beta$

and  $\gamma$  carbons of the P5 side chain are very important, because the wild-type Hb(64-76) peptide only stimulates 20 times better than the Abu72 peptide. The contribution to binding by TCR residues that contact the amide group may be responsible for the increased activity that Hb(64-76) has compared with Abu72. This part of the interaction would be highly specific because any addition made to the  $\gamma$  carbon of Abu reduced the activity of the peptide.

The clear correlation between structure and activity of these different ligands allows us to conclude that the activity of the APLs depends on their ability to mimic the structure of the wild-type Asn residue. Similar structure/function relationships have been obtained in the study of interactions between antigen receptors and haptens. For B cells, the hapten TNP inhibited the effects of the hapten DNP on B cells specific for DNP (31). The TNP ligand was thought to have a lower affinity for the DNP-specific B cell receptors and could perhaps interact with the B cell receptor well enough to block interaction with DNP but not well enough to induce a response on its own. T cells specific for arsanlated ovalbumin have also been produced, and these can be thought of as a specialized case in which the TCR recognizes arsonate as if it were present at the P5 position of a peptide (32). The response of T cells specific for arsonate can be inhibited by arsonate derivatives that have substitutions on the aromatic ring (33). Again, it was suggested that the arsonate derivatives can interact well enough with the TCR to compete with arsonate for binding but not well enough to activate on their own, although some derivatives were able to activate at high concentrations (33). Therefore, we conclude that by making subtle changes in the structure of the P5 side chain of Hb(64-76), we have produced peptide-MHC complexes that interact well enough with the 3.L2 TCR to induce some T cell functions only at high concentrations (B cell apoptosis) and inhibit the ability of wild-type Hb(64-76) to induce other functions (antagonism).

The wide range of activities seen in this study and data from numerous previous studies show that APLs can trigger T cells in a fundamentally different way than the antigenic ligand (5). Although APLs need only be different from the antigenic ligand in a subtle manner, how these subtle differences are translated into the observed differences in biological response is not clear. Current theories about how these subtle differences lead to different responses can be grouped into the kinetic and conformational models (3). In a kinetic model, the TCR has a reduced affinity for the APL as compared to the wild-type ligand. This results in a reduction in the time that the TCR remains engaged with its ligand, that is, it has a faster off-rate. A short interaction with ligand then results in the transduction a different signal through the TCR than that induced by the longer interaction with the wild-type ligand (34, 35). In a conformational model, the transduction of signals through the TCR involves some kind of conformational change in the TCR upon recognition of ligand. This change is not the same when a TCR recognizes an APL versus recognition of the wild-type ligand (36).

Data acquired to date tend to favor the kinetic model. Direct measurements of the affinity of a TCR specific for I-E<sup>k</sup> presenting an MCC peptide and its analogs have shown a correlation between the off-rate of the interaction and the response of the T cell (37, 38). Peptides that are weak agonists or APLs have a faster off-rate for the mutant peptide-MHC complex as compared to the wild-type ligand (39). A study of the recognition of an ovalbumin peptide presented by K<sup>b</sup> has also shown a correlation between the affinity of the TCR-MHC interaction and the response of the T cell (28).

Although the measurements of these off-rates certainly favors the kinetic model, it is still possible that a conformational change is also occurring, and the reduced off-rates that are observed are only coincidental. The data presented in this study suggest that this is not the case, and that it is the kinetic aspect of TCR-ligand interaction that is important for APL function. The important observation is that there is an optimum size for the side chain at position 72. If we consider the Abu side chain to be the closest mimetic of Asn that we have, then it is clear that both the addition (Nva) and the subtraction (Ala) of a methyl group will have a similar effect. It is easy to see how both of these changes can have similar effects on the affinity of the TCR for its ligand. The extra methyl group would make the side chain too bulky and not allow it to fit as well into a binding pocket, and the loss of a methyl group would leave not enough side chain to go into the binding pocket. It is difficult, however, to see how these two different changes would lead to similar conformational changes in the TCR. Therefore, these data are consistent with a kinetic model that requires the affinity of the TCR for APLs to be reduced as compared to the cognate antigenic ligand.

The high potential for the use of APLs in the treatment of disease makes it desirable to be able to rationally design such antigen analogs once the antigenic peptide has been defined. Previous systematic studies have not produced a consistent strategy for making APLs (13, 22, 23). The strength of this study is that we have been able to find 12 different side chains that interact with a single TCR from the same contact position. This is in part due to our ability to produce some altered peptides that use nonnatural amino acids at this critical position. Based on these data, we suggest that APLs can be produced by substituting an amino acid that interacts directly with the TCR with an amino acid that structurally resembles that TCR contact residue. Although many TCRs will be so highly dependent on the interaction provided by the primary TCR contact residue that they will not tolerate any change at that position, evidence suggests that T cells other than 3.L2 can be manipulated at the primary TCR contact. For example, T cells that recognize the MCC peptide presented by I-E<sup>k</sup> will recognize changes at the P5 position of the peptide as APLs (13), and extensive analysis of the hemagglutinin 307-319 peptide presented by the DR1 class II molecule showed that conservative substitutions at TCR contact residues were most likely to produce antagonist peptides (22). Therefore, we conclude that APLs can be rationally designed by mak-



ing substitutions of TCR contact residues with amino acid structures that resemble the structure of the antigenic ligand.

By making such small changes in either size, hydrophobicity, or conformation of a TCR contact residue, we have identified 16 different ligands for a single TCR that have a 1 million-fold range in activity. Such an extreme flexibility in TCR recognition suggests that a large number of potential ligands may exist for each individual TCR. Conversely, for each peptide-MHC complex, a large number of TCRs

may exist that will recognize that ligand well enough to induce a biological response. This study has also shown that there is a strict relationship between the structure of an amino acid side chain that interacts directly with the TCR and how that T cell will perceive that ligand. These data are consistent with the idea that the affinity of the TCR-ligand interaction primarily determines the functional outcome of that interaction. Ongoing studies will determine the validity of this hypothesis.

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We thank Devraj Basu for help in the initial studies involving the doubly substituted peptides, Dave Doner-meyer and Dan Kopp for assistance in synthesis, purification, and characterization of the peptides, Ellen Neumeister for passage of the 3.L2 clone, the Washington University Mass Spectrometry Resource for mass spectrometry data, Jerri Smith for assistance in preparation of the manuscript, and Calvin Williams, Martina Reinhold, Christopher Nelson, and Andy Chan for insightful comments on the manuscript.

This work was supported by National Institutes of Health grant AI-24157. G.J. Kersh is the recipient of a Cancer Research Institute fellowship.

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Received for publication 25 April 1996 and in revised form 15 July 1996.

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