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A Peptide That Antagonizes TCR-Mediated Reactions with Both Syngeneic and Allogeneic Agonists: Functional and Structural Aspects

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We identify and consider some characteristics of a peptide antagonist for the Ag-specific receptor on 2C cells (the 2C TCR). The peptide, GNYSFYAL (called GNY), binds to H-2K\textsuperscript{b}, and a very high-resolution crystal structure of the GNY-K\textsuperscript{b} complex at 1.35 Å is described. Although the GNY peptide does not bind to L\textsuperscript{d}, the potency of GNY-K\textsuperscript{b} as an antagonist is evident from its ability to specifically inhibit 2C TCR-mediated reactions to an allogenic agonist complex (QLSPFPFDL-L\textsuperscript{d}), as well as to a syngeneic agonist complex (SIYRYYGL-K\textsuperscript{b}). The crystal structure and the activities of alanine-substituted peptide variants point to the properties of the peptide P4 side chain and the conformation of the Tyr-P6 side chain as the structural determinants of GNYSFYAL antagonist activity.


The general perception of how T cells respond to peptide MHC (pMHC) complexes was radically altered by the discovery that minor variations in the sequence of a bound peptide can profoundly alter the T cell response (reviewed in Ref. 1). Among the pMHC that interact with the TCR, some complexes, termed agonists, elicit the entire range of effector T cell responses from expression of activation markers to stimulation of cell division and production of cytokines. Other complexes, termed partial agonists, elicit some, but not all, of these responses. Still others, of particular interest in this study, are termed antagonists because they elicit no obvious response, except that they specifically block the T cell response to agonist complexes. The peptide components of these variously acting complexes are also referred to as agonists, partial agonists, and antagonists, respectively. Antagonist pMHC that are recognized by the TCR called 2C may be particularly useful in clarifying the basis for the striking functional differences between agonists and their antagonists, because much information about the properties of T cells expressing this receptor has been amassed (2, 3), and considerable insights have emerged from the x-ray crystallographic structure analyses of the 2C TCR bound to both strong and weak agonist pMHC complexes (4–6).

The CD8\textsuperscript{+} T cell that was first found to express the 2C TCR arose in an H-2\textsuperscript{b} mouse in response to injected H-2\textsuperscript{d} cells (7). Among the many agonist pMHC complexes that are recognized by this receptor, K\textsuperscript{b} is the restricting class I MHC protein for some complexes and L\textsuperscript{d} for others (8). In view of the origin of TCR in an H-2\textsuperscript{b} mouse, the peptide-K\textsuperscript{b} complexes are syngeneic, whereas the peptide-L\textsuperscript{d} complexes are allogeneic. This distinction is notable because all of the antagonists described to date for other TCRs inhibit only syngeneic reactions, i.e., the antagonist peptides are restricted by the same class I MHC protein as the agonists they antagonize. In this study, we describe a potent antagonist peptide for the 2C TCR and a very high-resolution structure at 1.35 Å of its complex with K\textsuperscript{b}. The effectiveness of this antagonist is strikingly evident from its ability to inhibit 2C CTL responses not only to syngeneic (K\textsuperscript{b}-restricted) agonists, but also to a strong allogeneic (L\textsuperscript{d}-restricted) agonist. Amino acid substitutions at key hot spot positions (P4, P6) (5) either eliminated the peptide’s antagonist activity or, more notably, converted it to an agonist. The significance of these changes is illuminated by x-ray crystallographic structures described in this work and previously (4–6, 9).

Materials and Methods

Pep tides

Peptides were synthesized by f-moc chemistry in the Massachusetts Institute of Technology Biopolymer Labs and purified by HPLC. Peptide concentrations were determined by amino acid analyses. The principal agonist peptides SIYRYYGL (called SIY) and SIINFEKL (called OVA) are both restricted by K\textsuperscript{b}, whereas QLSPFPFDL (called QL9) is restricted by L\textsuperscript{d} (10). Stock solutions of peptides were usually in 20 mM phosphate, pH 7.3, 150 mM NaCl (PBS) or, for poorly soluble peptides, in PBS containing 1% DMSO.

Cells

Two CTL clones expressing the 2C TCR were derived by limiting dilution from 2C TCR transgenic mice (11). Clones K and L3.100 were maintained...
in culture and stimulated weekly with irradiated P815 cells, as described (8). They were used in cytolytic assays 4–9 days after stimulation.

Unless otherwise specified, the 31Cr-labeled target cells in cytolytic assays were T2-Kb and EL-4 cells. The T2-Kb cells, a generous gift from P. Cresswell (Yale University, New Haven, CT), are TAP+/−T2 cells transfected with Kb. EL-4 cells likewise express Kb, but are TAP+. The principal antigen peptide studied (GNY, see below) binds to Kb, but not to Ld. Therefore, in assays that tested the ability of the GNY peptide to inhibit the activity of Kb-restricted agonist peptides, the target cells were either T2-Kb or spleen cells from TAP+/−B6 (H-2b) mice, in which the spleen cells were activated by incubating them with Con A at 2 μg/ml for 48 h. In assays that tested the ability of the GNY peptide to inhibit an agonist (QL9) restricted by Ld (an allogeneic reaction), the target cells were 31Cr cells that expressed both Kb and Ld. These cells were either Con A-stimulated spleen cells from KBL/TAP−/− mice (see below) or an immortalized cell line established by transfecting bone marrow cells from these mice with c-Abelson virus.

Mice

C57BL/6 (B6) mice were from The Jackson Laboratory (Bar Harbor, ME) or bred in the Massachusetts Institute of Technology animal care facility. TAP−/−B6 mice that expressed Ld as a transgene were generated as follows. A 12-kb linearized DNA encoding H-2Ld was isolated from a HindIII digest of the genomic clone 27.5.27 (8). The transgenic founders were produced by pronuclear injection of the linearized DNA into TAP1−/−embryos.

Peripheral blood from all offspring was initially analyzed with a FITC-labeled α2-domain-specific Ab (30-5-7) to test for expression of the Ld transgene. Founder males were crossed to C57BL/6 females, and the offspring was intercrossed to generate homozygous TAP1−/−, H-2b, Ld+/+ animals. The genotypes of offspring were analyzed by Southern blot and PCR, using tail DNA digested with EcoRI. The PCR products from primers specific for the Ld transgene (Ld forward, 5′-GAT TCC CCA AAG GCA CAT GTG ACC-3′; Ld reverse, 5′-ACA ATC TCG GAG AGA CAT CCT AGA GCT-3′) was random primed, labeled, and used as a hybridization probe for Southern analyses. Primers specific for the neo resistance gene (neo forward, 5′-CTT GGG TGG AGA GGC TAT TC-3′, neo reverse, 5′-CTG GAG GAC AGT AGA GTG GA-3′) were used to detect the disrupted TAP1 allele by PCR. TAP1 homozygous nulls were confirmed by absence of detectable cell surface MHC class I expression by FACS. Mice were confirmed to be homozygous for Ld by production of 20 Ld progeny consecutively when outcrossed to B6 mice.

Cytolytic assays

Assays were performed in round-bottom 96-well plates in a total volume of 200 μl, with 5000 31Cr-labeled target cells and 4–5× more CTL in the presence of peptides at various concentrations. After 4 h, the extent of target cell lysis was determined from supernatant 51Cr cpm.

To measure antigen activity, the target cells were 31Cr labeled and peptide pulsed by incubating them with 31CrO4− and an agonist peptide (SIY, QL9, or OVA) in PBS for 1.5 h and then washed with PBS to yield agonist-pulsed target cells. When SIY or OVA was used to pulse target cells (Kb+), the cells were washed three times; when the pulsing peptide was QL9, the cells (Ld+) were subjected to a single wash with a large volume (12 ml) of cold PBS to minimize loss of bound peptide. For peptide stock solutions in PBS, 1% DMSO, the final DMSO concentration (0.25%), had no appreciable effect in 4-h cytolytic or Kd stabilization assays.

Specific lysis was calculated as 100(a − b)/(c − b), where a denotes cpm in supernatant of experimental wells, b is cpm released spontaneously from target cells in absence of CTL, and c is total cpm in target cells (in 1% Nonidet P-40). Inhibition of lysis of pulsed target cells was calculated as 100(x − y)/x, where x and y are, respectively, specific lysis in the absence and presence of the putative antagonist peptide. Quadruplicate wells were used to measure 31Cr release values for b and c in assays for agonist activity, and for x in assays for antagonist activity; all other wells were in duplicate.

The extent of specific target cell lysis in the absence of added peptides (background values) ranged from 0 to 5%, when the target cells were T2-Kb or B6 spleen cells. When, however, the target cells expressed Ld, background lysis values were higher (~20%) because some naturally occurring (endogenous) cellular peptides associate with Ld to form pMHC agonists for 2C cells (10, 12). Background lysis values were not subtracted from the reported results.

Variability

Duplicate wells in any given cytolytic assay agreed to within 5% and results shown in figures omit error bars (SD), as they generally fall within the size of the symbols. Each of the assays was repeated at least three times, and representative assays are shown.

Antibodies

mAb Y3 was used to stain Kd and 30-5-7 to stain Ld for flow cytometry.

Crystallization data collection, structure determination, and model analysis

Crystals of the Kd/GNY complex were grown, as described previously (13), and cryo-cooled after soaking in mother liquor (2.1 M K2HPO4/NaH2PO4, pH 6.5 and 2% 2-methyl-2,4-pentanediol) supplemented with 20% glycerol. Data were collected on a single, flash-cooled crystal at the Stanford Synchrotron Radiation Laboratory (Stanford, CA) beamline 9-1 on a MAR345 image plate using a monochromatic wavelength of 0.97 Å. Data reduction was performed with the DENZO/SCALEPACK suite (14) (Table I). The Kd/GNY crystals belong to the orthorhombic space group P212121 and are isomorphous with the previously reported Kd crystals (13, 15). The structure was determined by direct refinement, without rigid body protocols, using the coordinates of the Kd/melittin complex (16) as the initial model (Brookhaven Protein Data Bank code 1FZK) with the peptide removed and variant side chains truncated to alanine. A random set of 5%

Table I. Data processing and refinement statistics

<table>
<thead>
<tr>
<th>Data Processing</th>
<th>Refinement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2 2 2</td>
</tr>
<tr>
<td>Cell (a, b, c) (Å)</td>
<td>135.5, 87.7, 45.1</td>
</tr>
<tr>
<td>Resolution range (outer shell) (Å)</td>
<td>30–1.35 (1.37–1.35)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.9 (95.3)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>5.3 (81.3)</td>
</tr>
<tr>
<td>Average I/σ(I)</td>
<td>23.9 (1.1)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.4 (2.5)</td>
</tr>
<tr>
<td>Mosacity (%)</td>
<td>0.6</td>
</tr>
<tr>
<td>Wilson B (Å2)</td>
<td>16.5</td>
</tr>
<tr>
<td>Without TLS refinement</td>
<td>9.69/7.34/63.3/2</td>
</tr>
</tbody>
</table>

a Numbers in parentheses refer to the highest resolution shell.

b Rmerge = 100 × ΣhΣi|I(h,i)−<I(h)>)/ΣhΣi|I(h,i)|, where I(h,i) is the i th measurement of reflection h of average reflection intensity <I(h)>.

c Rmerge = 100 × ΣhΣi[I(h,i)−<I(h)>]/ΣhΣi[I(h,i)], where I(h,i) and I(h) are the structure factor amplitudes from the data and the model, respectively. Rmerge in Rcryst with 5% of test set structure factors.

d Other three sugar residues, three MPD molecules, and one phosphate ion.

e Cross-validated (CV) coordinate error based on maximum likelihood.

f Calculated using PROCHECK (24).
of reflections was excluded from refinement to monitor Rsyn (17). Rigid body, conjugate gradient minimization, simulated annealing with iterative cycles of torsion angle dynamics, and restrained individual temperature factor refinement using the maximum likelihood target (18) were performed in CNS (17). Bulk solvent and anisotropic corrections were applied throughout the refinement, except for rigid body protocols. The model was further improved by anisotropic temperature factor refinement in SHELXL (21), yielding Rsyn and Rfree values of 16.1 and 19.9%, respectively. Further improvement of the model was achieved by translation, libration, screw rotation (TLS) refinement using REFMAC5 (22). Forty-nine TLS groups consisting of the secondary structure elements of the MHC H chain, β2-microglobulin, and the peptide were used to model group anisotropy. The large number of TLS domains was justified by the high resolution of the diffraction data and by monitoring Rfree. The model was further rebuilt into σA-weighted 2Fo−Fc and Fo−Fc difference electron density maps. Water molecules were assigned for >3σ peaks in Fc−Fc difference maps, and retained if they obeyed hydrogen-bonding criteria according to HBPLUS (23) and retained >1.2σ density after refinement. In the last rounds of refinement, the TLS refinement step was followed by restrained individual anisotropic temperature factor refinement, and hydrogen atoms were included at their riding positions. The final refinement statistics are summarized in Table I. The stereochemical quality of the model was assessed using the programs PROCHECK (24) (Table I) and WHATCHECK (25).

The motif used for a database search was based upon sequences of some Kα-binding peptides found previously to have strong or weak agonist or weak antagonist activity for the 2C TCR. The motif for use in a database search was based upon sequences of some Kα-binding peptides and several previously identified weak antagonists for the 2C TCR are shown in Fig. 1A. From these sequences, a consensus motif was chosen for a BLAST search of a protein database (Genetics Computer Group, University of Wisconsin, Madison, WI). Several thousand peptides conforming to this motif were identified, and the number was reduced to ~900 by limiting the search to proteins of mammalian origin. Of these, five were selected for further study (Fig. 1B). One of these peptides (GNYSFYAL, hereafter referred to as GNY) behaved as a strong antagonist for the 2C TCR (Fig. 1B). SwissProt and TrEMBL database searches revealed that it corresponds to a sequence in the mouse insulin receptor precursor.

The inhibitory activity of GNY peptide is illustrated in Fig. 1C. In the absence of added GNY, T2-Kβ target cells pulsed with SIY (at 0.5 μM) were 66% lysed (specific lysis) by a cultured 2C CTL clone (K), but had no effect on the response of another TCR (4G3) to its equally potent agonist (OVA-Kβ). Numbers are percent inhibition of lysis of the pulsed target cells in absence of the putative antagonists. C. Specificity of antagonism. GNY inhibited lysis of SIY-pulsed 51Cr-labeled T2-Kβ target cells by a 2C CTL clone (K), but not OVA-pulsed 51Cr-labeled T2-Kβ cells by a CTL clone (4G3) that is specific for the OVA-Kβ complex. T2-Kβ cells were pulsed with SIY at 0.5 μM or with OVA at 25 pM.

FIGURE 1. Identification of a peptide antagonist for the 2C TCR. A. The motif used for a database search was based upon sequences of some Kα-binding peptides found previously to have strong or weak agonist or weak antagonist activity for the 2C TCR. B, Antagonist activity in cytolytic assays. Five candidate peptides from the BLAST search were synthesized and tested for ability to inhibit lysis of 51Cr-labeled T2-Kβ target cells, which had been pulsed with a strong agonist peptide, SIY, at 2.5 μM. After washing the pulsed, labeled cells, they were incubated for 4 h with a 2C CTL clone in the absence or presence (at ~10 μM) of the peptides shown. Numbers are percent inhibition of lysis of the pulsed target cells in absence of the putative antagonists. C. Specificity of antagonism. GNY inhibited lysis of SIY-pulsed 51Cr-labeled T2-Kβ cells by a 2C CTL clone (K), but not OVA-pulsed 51Cr-labeled T2-Kβ cells by a CTL clone (4G3) that is specific for the OVA-Kβ complex. T2-Kβ cells were pulsed with SIY at 0.5 μM or with OVA at 25 pM.

The inhibitory activity of GNY peptide is illustrated in Fig. 1C. In the absence of added GNY, T2-Kβ target cells pulsed with SIY (at 0.5 μM) were 66% lysed (specific lysis) by a cultured 2C CTL clone. In the presence of increasing concentrations of GNY, progressively less lysis was observed; 10 μM of GNY inhibited specific lysis almost completely. Even with GNY at 1 nM, some inhibition was evident (10–15%).

Much higher pulsing concentrations of the SIY agonist were needed for robust lysis of those target cells, such as EL-4, whose Kα molecules are less readily loaded with exogenous peptides than Kβ molecules on the TAP-deficient T2-Kβ cells. The antagonistic effect of GNY was correspondingly more pronounced when the agonist-pulsed targets were T2-Kβ cells than EL-4 cells. For instance, GNY at 8 μM caused >90% inhibition of T2-Kβ cells pulsed with 0.2 pM of SIY, whereas the same GNY concentration (8 μM) caused only ~30% inhibition of EL-4 cells pulsed with 6 pM of SIY (titrations not shown).

The specificity of the antagonist activity of the GNY peptide was evident from its failure to affect lysis of T2-Kβ target cells by a different CTL clone, 4G3, in response to that clone's cognate peptide (SIINFEKL, called OVA), which is also restricted by Kβ
Ld molecules with peptides). Lysis of these target cells by cytolytic 2C cells was inhibited by the GNY peptide when the agonist used to pulse these 51 Cr-labeled H-2 b /Ld target cells was either SIY, which binds to K b , or QL9, which binds to Ld (Fig. 2B). In some experiments, the GNY peptide was more effective in inhibiting the allogenic response than the syngeneic response (Fig. 2) and, in others, the syngeneic response was antagonized better than the allogeneic response. The variability seemed to depend on the extent of lysis elicited by the pulsing agonist in absence of the GNY peptide. In all assays, however, using 51 Cr-labeled K b /H11001/Ld target cells, GNY consistently inhibited the cytolytic response of 2C CTL, regardless of whether the agonist peptide was associated with Ld (QL9) or K b (SIY).

Substitution of serine at P4 by arginine or lysine converts GNY from an antagonist to an agonist

Because SIY, at sub-pM concentrations, can mediate substantial lysis of some K b + target cells, this peptide has been referred to as a superagonist (5). The x-ray crystallographic studies of SIY-K b in complex with the 2C TCR attributed the unusual agonist potency of the peptide to the Arg side chain at P4 (5). Hence, we examined variants of GNY in which Arg or Lys replaced Ser at P4. The variant with Arg at P4 exhibited distinct, although slight, agonist activity (Fig. 3). The peptide with Lys at P4 also had very slight agonist activity, but only at the highest concentration (>10 μM).
**FIGURE 5.** Structural implications of the K\(^{b}\)/GNY complex. **A,** Ribbon diagram of the K\(^{b}\)/GNY complex. The MHC backbone is colored in gray, and the peptide is shown as a red tube. **B,** Electron density of the GNY peptide and overlay of the GNY peptide with other K\(^{b}\) ligands. **Upper panel,** The \(\alpha_1\)-weighted \(F_o-F_c\) difference electron density map, contoured at 3\(\sigma\), was obtained after molecular replacement using a peptide-free K\(^{b}\) model (Brookhaven Protein Data Bank-ID 1FZK) and shows clear density for all peptide atoms. The final peptide coordinates are superimposed on the map and are color coded according to atom type. **Middle panel,** Comparison of the GNY (red) and dEV8 (blue) peptide conformations bound to unliganded K\(^{b}\). The \(\beta\)-sheet floor of the MHC was used to align the peptides. The orientation of the P6 side chains differs substantially in different structures. **Lower panel,** Comparison of the dEV8 and SIY peptides in the liganded (TCR 2C-bound) complexes. The orientation of the P6 side chains is very similar, resembling the conformation of the GNY P6 side chain in the unliganded complex. **C,** Comparison of the unliganded K\(^{b}\)/GNY and the liganded K\(^{b}\)/dEV8/2C TCR complex. The dEV8 and GNY peptides are colored blue and red, respectively. TCR (top) and MHC (bottom) backbones are shown as gray tubes (light gray for the liganded and dark gray for the unliganded complexes). The peptide side chains are drawn as stick models. The CDR loops of the 2C TCR are color coded, as follows: CDR1\(\alpha\) (24–31), dark blue; CDR2\(\alpha\) (48–55), magenta; CDR3\(\alpha\) (93–104), green; CDR1\(\beta\) (26–31), cyan; CDR2\(\beta\) (48–55), pink; CDR3\(\beta\) (95–107), yellow; and HV4 (69–74), orange.
Tyr, or diiodo-Tyr in place of Tyr-P6 and are referred to as Y6E, at P6 were tested. The variants examined had Glu, Lys, monoiodo-nity for Kb (see Fig. 4).

Because substitution of Ala for Tyr at peptide position P6 led to almost as tightly as GNY (Fig. 4).

C C.

Only GNY and its variants with Ala at C.

P3 variant to Kb, with Tyr-P3 as a potential secondary anchor, others were less effective. The exceptionally poor binding of the small Ser residue at the P4 position

Ar

somewhat lower value for the SIY/Kb/TCR 2C complex (0.67) (5) may result from its lower resolution and, hence, its larger mean coordinate error compared with the other complexes. However, no correlation between the surface-complementarity coefficient values of TCR/pMHC complexes and the degree of peptide agonism was also noted in another TCR/pMHC system of the A6-TCR, HLA-A2, and variants of the Tax peptide (30). In the GNY/Kb complex, a large number of hydrogen bonds (29) between the peptide and Kb is observed, 13 of which are water mediated. The number of hydrogen bonds in the dEV8/Kb and SIY/Kb/TCR 2C complexes is markedly smaller (19 and 13, respectively), but, because the dEV8/Kb/TCR 2C complex displays a similarly large number of peptide-MHC hydrogen bonds as the GNY/Kb complex, the number of hydrogen bonds does not correlate with the degree of peptide agonism or antagonism. It is noteworthy, however, that the number of water-mediated hydrogen bonds is highest in the 1.35 Å GNY/Kb complex, confirming the earlier notion that water is an integral part of peptide-MHC-binding interactions (16, 31).

Because substitution of Ala for Tyr at peptide position P6 led to loss of antagonist activity, but retention of wild-type peptide affinity for Kb (see Fig. 4), several other amino acid substitutions at P6 were tested. The variants examined had Glu, Lys, monoiodo-Tyr, or diido-Tyr in place of Tyr-P6 and are referred to as Y6E, Y6K, Y6Y1, and Y6Y2, respectively. Compared with unmodified GNY, antagonist activity was reduced in the Y6E variant, lost in the Y6K peptide (which had minimal agonist activity), and preserved in Y6Y1 and Y6Y2 (Table II).

### Table II. Antagonist activity of GNY and its iodo tyrosine derivatives at position P6

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration (µM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNY</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>GNY-Y6Y1</td>
<td>5</td>
<td>57</td>
</tr>
<tr>
<td>GNY-Y6Y2</td>
<td>4</td>
<td>48</td>
</tr>
</tbody>
</table>

* The 2C T cells from clone L3.100 caused 41.9% specific lysis of 51Cr-labeled T2-Kb cells that had been pulsed with SIY at 3 pM in the absence of the added peptides shown. E:T ratio 5:1. Peptide stock solutions were in PBS, 1% DMSO.

Alanine scanning of the GNY sequence

To identify the contributions of various amino acid residues to the antagonist activity of GNY, each amino acid in this octapeptide was replaced by Ala, except at P5 and P8, in which Phe and Leu were retained because of their importance as anchor residues for binding to Kb. Each of the alanine analogues was evaluated for antagonist activity, using various Kb* target cells pulsed with the SIY agonist, as in Fig. 1C. Only GNY and its variants with Ala at P1 or P2 behaved as antagonists (Fig. 4A). Neither GNY nor any of the Ala analogues had agonist activity when present as the only peptide in assays with 2C CTL plus 51Cr-labeled T2-Kb cells.

Peptide binding to Kb on target cells

The binding of GNY and the Ala-substituted analogues to Kb was estimated from their ability to stabilize the cell surface expression of Kb on TAP-deficient Kb* cells (RMA-S). GNY was less effective in stabilizing Kb on these cells than the potent agonist peptides, SIY and OVA, but more effective than the weak agonist p2Ca (Fig. 4B). Of the Ala-substituted GNY variants, the one with Ala-P6 was at least as effective as GNY in stabilizing Kb, while the others were less effective. The exceptionally poor binding of the P3 variant to Kb, with Tyr-P3 as a potential secondary anchor, could account for its lack of antagonist (or agonist) activity (Fig. 4A). Although antagonist activity was lost in the peptide with Ala at P4 (GNY-A4) and preserved in the one with Ala at P2 (GNY-A2), GNY-A4 bound at least as tightly to Kb as GNY-A2, and almost as tightly as GNY (Fig. 4C).

Substitutions of GNYSFYAL at P6

Because substitution of Ala for Tyr at peptide position P6 led to loss of antagonist activity, but retention of wild-type peptide affinity for Kb (see Fig. 4), several other amino acid substitutions at P6 were tested. The variants examined had Glu, Lys, monoiodo-Tyr, or diido-Tyr in place of Tyr-P6 and are referred to as Y6E, Y6K, Y6Y1, and Y6Y2, respectively. Compared with unmodified GNY, antagonist activity was reduced in the Y6E variant, lost in the Y6K peptide (which had minimal agonist activity), and preserved in Y6Y1 and Y6Y2 (Table II).

Crystal structure of the GNY-Kb complex

To derive a structural basis for the antagonist activity of the GNY peptide and possibly explain the biological characteristics of the GNY variants, we determined the crystal structure of the GNY/Kb complex to a very high resolution of 1.35 Å (Fig. 5A) and compared it with other peptide/Kb and peptide/Kb/TCR complexes. The unbiased Fo-Fc difference electron density for the GNY peptide after molecular replacement with a Kb* model devoid of peptide coordinates is shown in Fig. 5B. The high quality of the electron density allows direct assignment of the GNY peptide sequence (Fig. 5B, upper panel). A comparison of the solvent-exposed peptide surface areas, surface-complementarity coefficients (27), number of hydrogen bonds, and van der Waals interactions in several related Kb and Kb/TCR complexes is shown in Table III.

Interestingly, the solvent-exposed area of the peptide in complex with Kb is smallest in the GNY/Kb complex (779 Å²) probably owing to the presence of a small Ser residue at the P4 position compared with a Lys or an Arg in the dev8 (880 Å²) and SIY (875 Å²) complexes, respectively. The surface-complementarity coefficients, a measure of the shape complementarity of two interacting surfaces, in which values of 1 and 0 denote perfect and no complementarity, respectively, are very similar to each other (~0.73). The somewhat lower value for the SIY/Kb/TCR 2C complex (0.67) (5) may result from its lower resolution and, hence, its larger mean coordinate error compared with the other complexes. However, no correlation between the surface-complementarity coefficient values of TCR/pMHC complexes and the degree of peptide agonism was also noted in another TCR/pMHC system of the A6-TCR, HLA-A2, and variants of the Tax peptide (30). In the GNY/Kb complex, a large number of hydrogen bonds (29) between the peptide and Kb is observed, 13 of which are water mediated. The number of hydrogen bonds in the dEV8/Kb and SIY/Kb/TCR 2C complexes is markedly smaller (19 and 13, respectively), but, because the dEV8/Kb/TCR 2C complex displays a similarly large number of peptide-MHC hydrogen bonds as the GNY/Kb complex, the number of hydrogen bonds does not correlate with the degree of peptide agonism or antagonism. It is noteworthy, however, that the number of water-mediated hydrogen bonds is highest in the 1.35 Å GNY/Kb complex, confirming the earlier notion that water is an integral part of peptide-MHC-binding interactions (16, 31).

The number of peptide-MHC van der Waals interactions in the GNY/Kb complex compared with the dEV8/Kb and SIY/Kb complexes may stem from the substitution of Lys-P4 and Arg-P4 for Ser-P4 in GNY. Thus, the interactions of the peptide with the MHC class I do not establish a structural basis for the strong antagonism of the

### Table III. Analysis of agonist and antagonist peptide interaction with the MHC class I in pMHC and pMHC/TCR complex structures

<table>
<thead>
<tr>
<th>Complexes</th>
<th>SA (Å²)</th>
<th>S_c</th>
<th>No. Hydrogen Bonds</th>
<th>No. vdW Contacts</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>GNY/Kb</td>
<td>779</td>
<td>0.72</td>
<td>29, 13 via H₂O</td>
<td>159</td>
<td>This work</td>
</tr>
<tr>
<td>dEV8/Kb</td>
<td>895</td>
<td>0.73</td>
<td>19, 7 via H₂O</td>
<td>186</td>
<td>6</td>
</tr>
<tr>
<td>dEV8/Kb/TCR 2C</td>
<td>885</td>
<td>0.73</td>
<td>27, 8 via H₂O</td>
<td>163</td>
<td>6</td>
</tr>
<tr>
<td>SIY/Kb/TCR 2C</td>
<td>875</td>
<td>0.67</td>
<td>13, 0 via H₂O</td>
<td>188</td>
<td>5</td>
</tr>
</tbody>
</table>

*Solvent-exposed surface areas (SA) and surface-complementarity coefficients (S_c) were calculated with MS (51) and SC (27), respectively, using a 1.7 Å probe radius.

* The number of hydrogen bonds between the peptide and Kb or Kb* was calculated using HBPLUS (23).

* The number of van der Waals (vdW) contacts was calculated using CONTACTSYM (52).
The main differences between the peptide sequences of dEV8 and GNY are found at positions P1, P4, and P7. It is apparent that the P1 position does not contribute to the agonistic or antagonistic properties of the peptide (Fig. 4A). In contrast, peptide positions P3, P4, and P6, when mutated to Ala, display reduced antagonistic activity relative to the GNY peptide. The P4 position in the weak agonist dEV8 and the superagonist SIY is Lys and Arg, respectively, and was ascribed to a hot spot for contacting and modulating the activity of the 2C TCR (5). In GNY, an antagonist, the P4 position is Ser. Thus, a small side chain at the P4 position appears to contribute to the antagonistic activity of the GNY peptide. With Ala in place of Ser at this position, however, antagonist activity was lost. Because the side chain of Ala is smaller than that of Ser, it could be argued that the GNY-A4 peptide bound less tightly to K\* than GNY, but GNY-A4 bound to K\* at least as tightly as GNY-A2, which exhibited antagonist activity. This appears to suggest that the Ser hydroxyl contributes to antagonism. The results emphasize the significance of the P4 hot spot in 2C TCR/pMHC interactions. Construction of a hypothetical GNY/K\*2C TCR model (Fig. 5C) reveals that in this complex, the majority of contacts between the P4 peptide side chain and the 2C TCR are lost. Without substantial structural rearrangements of the TCR complementarity-determining region (CDR) loops, this would have a potentially destabilizing cavity in the GNY/K\*2C TCR complex, which may well contribute to the antagonistic signaling outcome induced by the GNY peptide. Avoiding the potential cavity by substituting Arg for Ser at P4 in GNY produces a weak agonist (Fig. 3), confirming the key role of this position for the agonistic activity of peptide-K\* complexes that bind to the 2C TCR (5).

An even more drastic effect of the GNY peptide on its ability to inhibit lysis of SIY-pulsed T2-K\* target cells is observed upon mutation of position P6, the other key hot spot residue (5). Although the GNY peptide is a potent antagonist (Fig. 1, B and C), mutation of Tyr-P6 to Ala results in loss of antagonist activity (Fig. 4A). This result seems surprising at first, given the fact that the Tyr-P6 residue is found in both agonistic (dEV8, SIY) and antagonistic (GNY) peptides (Fig. 1A). However, it may result from the specific P4/P6 residue combination of the peptide: neither dEV8 nor SIY has a small residue at the P4 position (Fig. 1A). A comparison of the P6 side chain conformations of the GNY, dEV8, and SIY peptides in all available crystal structures (4–6) reveals that the Tyr-P6 side chain conformation is similar in the various peptide/K\*2C/TCR and peptide/K\*2C/TCR complexes (Fig. 5B, lower panel). By contrast, in the TCR-unbound form, the Tyr-P6 conformation can be very different (Fig. 5B, middle panel). In the dEV8/K\* complex, the $\chi_1$ and $\chi_2$ angles of the Tyr-P6 side chain are $-70^\circ$ and $-10^\circ$, respectively, whereas in the GNY/K\* complex they have very different values, of $\chi_1 = -159^\circ$ and $\chi_2 = -90^\circ$. Thus, the phenol rings of the Tyr-P6 side chains in the two complexes are almost perpendicular to each other, and the side chain hydroxyl groups are $\sim 8 \pm$ Å apart. These Tyr-P6 conformations are specific for the respective pMHC complexes, as no residual electron density, which would point to alternative conformations of this side chain in the crystal, was visible in the electron density maps. It appears that in solution, the Tyr-P6 side chain is flexible and might adopt both conformations because the conformation of the Tyr-P6 side chain is similar in the dEV8/K\*2C complex, and in the TCR-bound complex (6). The binding of the 2C TCR to the GNY/K\* complex is most likely, then, accompanied by a large conformational change of the Tyr-P6 side chain and a reduction in its flexibility, leading to a decrease of entropy. Substituting Ala for Tyr-P6 eliminates such restrictions, and additionally may reduce the number of productive interactions between 2C and peptide-K\*. The lack of 2C binding to the pMHC would, thus, eliminate the antagonistic effect of the peptide.

Inclusion of bulky iodine atoms into the Tyr-P6 side chain leaves the antagonistic activity of the peptide intact (see above). Modeling of these iodine atoms into the peptide/K\*/TCR crystal structures reveals that, in case of a doubly iodinated peptide, one of the iodine atoms becomes sandwiched between the CDR\(\beta\) and CDR\(\beta\) loops of the TCR, while the other iodine atom contacts the region K164-W147 in the MHC $\alpha_\beta$-helix. Even without any changes in the side chain conformation of Tyr-P6, surprisingly little steric clashes with the TCR are observed. Asn\(^{31}\) in the CDR\(\beta\) loop would be too close to the iodinated peptide in all three TCR/pMHC complexes, while an additional steric clash is apparent between the iodines and Gly\(^{92}\) of the CDR3\(\beta\) in the dEV8/K\*3/2C complex only. All these close contacts amount to distance violations of $<1$ Å, and thus, should be avoidable by structural plasticity of the TCR CDR loops, which has been correlated with agonism and presumably antagonism, as in the SIY/ K\*/2C complex (5). The close contacts between the iodine atoms and the MHC molecules suggest that the backbone of the bound peptide would have to adjust slightly to establish sensible stereochemistry in the complex in this region. These required conformational changes may contribute to the observed antagonism of the iodinated peptides by increasing the number of hydrophobic interactions of the iodine atoms with the 2C TCR, in accord with the kinetic proofreading model for antagonism (see below).

In summary, the very different physicochemical properties of the P4 side chain and the conformational differences of the Tyr-P6 side chains in the GNY and dEV8/SIY peptides provide a structural basis for the antagonism exhibited by the GNY peptide and some of its derivatives, although the contribution of TCR CDR loop movements must await structure determination of the respective TCR/pMHC complexes.

### Discussion

The GNY peptide, identified in this work as an antagonist for the 2C TCR, inhibits cytolytic reactions of cultured 2C CTL clones. In most of these responses, the triggering agonist ligand was SIY-K\*, which binds with high affinity ($K_D = 1 \mu M$) to the 2C TCR on CD8\* T cells (32, 33). The efficacy of GNY as an antagonist for this strong syngeneic reaction was matched by its ability to inhibit, approximately equally well, the reaction of 2C TCR with the QL9 peptide on target cells that expressed L\*\(^\alpha\) and K\* (34). It is notable that the affinity of 2C TCR for the allogeneic QL9-L\* complex is at least 10-fold higher ($K_D = 0.05–0.1 \mu M$) than for SIY-K\* and is the highest to date measured for any naturally occurring TCR (34).

For antagonism to be observed, the TCR molecules on a reactive T cell have to be confronted at the same time by agonist and antagonist pMHC. That antagonist peptides do not act simply by competitively inhibiting the binding of agonist peptides to MHC molecules on APC (or target cells) is suggested by several considerations. One is the small number of agonist pMHC that can trigger a cytolytic T cell response (surely fewer than 10 and most likely even 1 per target cell) (35, 36), whereas the number of potentially available MHC molecules on the presenting target cell surface is orders of magnitude greater. It has been reported, moreover, that antagonism cannot be observed when the antagonist pMHC and the agonist pMHC are presented on separate cells to a reactive T cell (37). That the 2C TCR reacts strongly with agonists having K\* or L\* as the restricting MHC made it possible to determine with a target cell that expressed both K\* and L\* that GNY-K\* antagonized the cytolytic response elicited by QL9-L\* (36). Because GNY does not bind to L\*, this finding formally excludes the possibility
that, in this case, the antagonist peptide acts by competing with agonist peptides for binding to the same MHC molecules.

Amino acid substitutions at two positions in GNY were of interest. Replacing Ser-P4 with Arg converted the peptide from an antagonist to a weak agonist. Although the agonist activity was slight, the results are in agreement with evidence that Arg at P4 in the SIY peptide (see Fig. 1A) makes contact with a hot spot in the Ag-binding site of TCR (and Ab) molecules when engaged with a pMHC, perhaps because TCR/pMHC interactions, the transient nature of the complexes obtained. If antagonism indeed depends upon rapid dissociation of TCR/pMHC complexes, the transient nature of the complexes when the pMHC are antagonists may make it difficult to obtain the co-crystals needed to evaluate the conformation-difference hypothesis. The idea that Ag recognition by T cells is degenerative has arisen from many findings that diverse pMHC can serve as agonists for any particular TCR. That many other pMHC can also serve as antagonists expands considerably the number of pMHC that can be recognized by any particular TCR. In a previous study, for example, seven peptides, many differing from each other considerably in sequence, were found to weakly inhibit 2C TCR-mediated cytolytic activity of SIY-pulsed K\textsuperscript{b+} target cells (32). In this study, we have focused on GNY, a more potent antagonist of this receptor. We have also tentatively identified three additional peptides (of 14 tested) that antagonize the lysis of QL9-pulsed L\textsuperscript{d+} target cell by 2C cells (S. A. Lamontagne and H. N. Eisen, unpublished observations). Because T cells can respond to some extent to antagonist peptides (e.g., by limited phosphorylation of CD3\delta chains (45) and altered cytokine production (46)), it is likely that the total number of pMHC able to stimulate some response by any particular T cell is far greater than previously surmised by focusing exclusively on strong agonists.

Some antagonist peptides are able to exercise a role in positive selection during thymic development (47), presumably by virtue of the weak signals they deliver to thymocytes. Because the survival of naive T cells in the periphery is MHC dependent, it is likely that T cell homeostasis in the periphery is likewise dependent on weak signals that naive T cells receive from diverse, endogenous pMHC (48, 49). Not just one or a few endogenous (self) pMHC, but sets of diverse pMHC, including some that would act as antagonists in appropriate ex vivo assays, may deliver TCR-mediated signals to naive T cells. These signals, in conjunction with appropriate cytokines such as IL-7, are most likely essential for these cells to survive (50). The identity and characterization of these pMHC and their mode of action should eventually help illuminate the conditions required for the survival of naive T cells in vivo.

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