Immunity, Vol. 9, 475–483, October, 1998, Copyright ©1998 by Cell Press

Peptide Antagonism and T Cell Receptor Interactions with Peptide-MHC Complexes

Yuri Sykulev,*§ Yulia Vugmeyster,* Anders Brunmark,† Hidde L. Ploegh,* and Herman N. Eisen*‡

*Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

†Department of Immunology
The Scripps Research Institute
La Jolla, California 92037

Summary

We describe antagonist peptides that specifically inhibit cytolytic activity of T cell clones and lines that express the antigen-specific receptor of $CD8^+$ T lymphocyte clone 2C, which recognizes peptides in association with syngeneic (K^b) and allogeneic (L^d) MHC proteins. Addition of an antagonist peptide that can bind to K^b on 2C cells decreased the tyrosine phosphorylation of CD3 ζ chains elicited by prior exposure of the cells to an agonist peptide-K^b complex. Contrary to previous agonist-antagonist comparisons, the 2C T cell receptor had higher affinity for an antagonist peptide-K^b complex than for a weak agonist peptide-K^b complex. This difference is considered in light of evidence that antigen-specific receptor affinity values can be substantially higher when determined with the receptor on live cells than with the receptor in cell-free systems.

Introduction

The nature of a T cell's response to complexes formed by peptides with major histocompatibility (MHC) proteins (pMHC complexes) can be greatly affected by variations in the peptide's amino acid sequence (Kersh and Allen, 1996). Some peptides (agonists) elicit the full range of known responses; others (partial agonists), often differing from agonists by only one or a few amino acid residues, elicit partial responses (i.e., effector but not proliferative responses); and the principal effect of still others (antagonist peptides) is to inhibit the activity of agonists. Although a role for antagonist peptides in normal immune responses is not entirely clear, these peptides are of considerable interest because they provide the tools for exploring previously unappreciated complexities in the responses of T cells to ligation of their antigen-specific receptor (T cell receptor or TCR). We have accordingly sought to identify antagonist peptides for the CD8 cytotoxic T cell known as 2C, a clone whose TCR recognizes defined allogenic and syngeneic pMHC complexes.

‡To whom correspondence should be addressed (e-mail: hneisen@mit.edu).

§Present address: Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107.

Clone 2C arose in an H-2^b mouse (BALB/b strain) immunized with H-2^d cells (from DBA/2 and BALB/c mice). It responds to the class I MHC protein L^d (an alloreaction) in association with naturally processed peptides (e.g., LSPFPFDL, termed p2Ca; Udaka et al., 1992) that derive from a ubiquitous intracellular protein (α-ketoglutarate dehydrogenase; Udaka et al., 1993). Using the alloreactive cytolytic responses to L^{d+} target cells loaded with diverse variants of p2Ca, we were unable to identify antagonist peptides. Subsequently, Udaka et al. (1996) discovered that 2C CTL can respond vigorously to a synthetic peptide, SIYRYYGL (here termed SYRGL), in association with K^b, which is syngeneic for 2C cells. Using highly sensitive cytolytic assays based on SYRGL-pulsed K^{b+} target cells, we describe here several naturally occurring peptides that act as antagonists for the 2C TCR.

One of the antagonists (RGYVYQEL, termed EVSV) is of interest because of two findings. First, in contrast to the few previous comparisons between antagonist and agonist pMHC complexes, we found that the EVSV-K^b complex is bound with about 30 times higher affinity by the 2C TCR than the weak agonist p2Ca-K^b complex. Second, the addition of the EVSV antagonist peptide to 2C cells after they had been briefly in contact with an immobilized agonist-MHC complex (SYRGL-K^b) brought about a rapid and profound decrease in tyrosine phosphorylation of the cells' CD3 ζ chains. In considering the significance of the affinity difference between the antagonist and agonist complexes, we note that there can be substantial differences between TCR affinity values determined with the receptor on intact cells and in cell-free systems. The sequences of all the peptides discussed below and the terms used to designate them are listed in Table 1 and Figure 2 (see below).

Results

The Cytolytic Assay for Antagonist Peptides

As shown in Figure 1A, half-maximal lysis of an optimal K^{b+} target cell (T2-K^b) was achieved with the SYRGL peptide at the extraordinarily low concentration of around 5×10^{-14} M (SD_{50} value). Since SYRGL and a peptide RGYVYQG (VSV) from vesicular stomatatis virus (VSV) were about equally effective in blocking the K^b-dependent cytolytic activity of an unrelated CTL clone (4G3, specific for K^b plus the ovalbumin peptide SIINFEKL [pOV8]) (Figure 1C; Table 1), it appears that the affinity of K^b for SYRGL is close to its previously measured affinity for VSV (1×10^8 M^{-1}; Matsumura et al., 1992). In accord with the low SD_{50} value (Sykulev et al., 1996), we found that soluble SYRGL-K^b complexes bound to the TCR on live 2C cells with the relatively high affinity of 1×10^7 M^{-1} (Figure 1D; Tables 1 and 2).

To identify antagonist peptides for the 2C TCR, we took advantage of the SYRGL peptide's potency as an agonist to pulse T2-K^b target cells (^{51}Cr labeled) with this peptide at 5×10^{-14} M, leaving a large number of empty K^b molecules that could be loaded with other peptides.

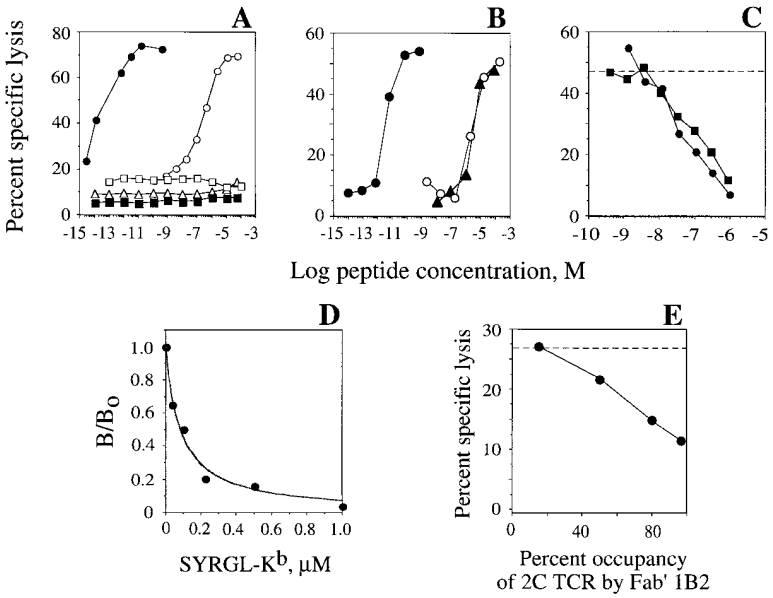

Figure 1. Recognition of Synthetic Peptide SIYRYYGL by 2C CTL

(A) Specific lysis of ^{51}Cr-labeled T2-K^b target cells by 2C CTL in the presence of SYRGL and other K^b-binding peptides at various concentrations. Peptides were SIYRYYGL (SYRGL, filled circles), SIINFEKL (pOV8, open triangles), LSPFPFDL (p2Ca, open circles), LSPYPFDL (p2Ca-Y4, open squares), and RGYVYQGL (VSV, filled squares).

(B) Specific lysis of ^{51}Cr-labeled Con A–activated splenocytes (Con A blasts) from TAP-deficient mice (H-2^b) by 2C CTL in the presence of various concentrations of the strong agonist peptide SYRGL (filled circles) and two weak agonist peptides, naturally processed p2Ca (open circles) and dEV8 (filled triangles). Note the difference in peptide concentration required for half-maximal lysis of these suboptimal target cells and the optimal target cells in (A).

(C) Relative affinity of K^b for two peptides, SYRGL and VSV, estimated from effectiveness of blocking a K^b-dependent cytolytic reaction. Shown is the specific lysis of T2-K^b target cells sensitized with pOV8 by pOV8-K^b-specific CTL (clone 4G3) in the absence (dotted) or presence (solid curves) of various concentrations of SYRGL (filled circles) or VSV (filled squares). SYRGL and VSV alone did not elicit a cytolytic reaction with these CTL. For peptide sequences, see legend for (A).

(D) Affinity of the TCR on live 2C cells for the SYRGL-K^b complex.

Amount of radiolabeled Fab′ fragments of the clonotypic anti-2C TCR antibody (1B2) specifically bound to the TCR on live 2C cells (clone L3.100) in the absence (B_o) or presence (B) of various concentrations of the SYRGL-K^b complex.

(E) Ligation of TCR molecules on 2C CTL by a clonotypic TCR-specific antibody (1B2; Kranz et al., 1984) decreases specific lysis of SYRGL-pulsed T2-K^b target cells. The extent of target cell lysis in the absence of the 1B2 antibody is indicated by a dotted line. About 80% of 2C TCR had to be blocked by the antibodies to decrease specific lysis of the target cells by half. Percent occupancy (α) of the TCR was calculated as $\alpha = (K{\cdot}C/(1 + K{\cdot}C)) \times 100$, where C is free concentration of 1B2 Fab′ fragment (assumed to be the same as the total concentration) and K is the equilibrium binding constant (2×10^8 M^{-1}) for the reaction between 2C TCR and 1B2 Fab′. The concentration of free SYRGL was 4×10^{-14} M.

These pulsed target cells were then incubated with diverse test peptides and various CD8$^+$ CTL that expressed the 2C TCR, including the original 2C clone and several 2C TCR$^+$ T cell clones from 2C TCR transgenic mice. When added alone to T2-K^b cells, the tested peptides behaved either as weak agonists (high SD_{50} values) or elicited no cytolytic activity (Figure 1A).

Antagonist Peptides

As evident from Figure 2, lysis of the pulsed target cells was enhanced by two peptides (agonists), unaffected by two others (null peptides), and inhibited by six others (antagonists). The antagonist effects were not always observed: it appeared that they were more consistently seen with recently derived clones than with those that had been in culture for many months, and they were not observed with the original 2C clone. Representative results are shown in Figures 3A, 3C, and 3D for three newly derived clones (L3.100, K2.30, and G3.1). We also found that polyclonal 2C TCR$^+$ cell lines (derived as described below) were indistinguishable from clones L, K, and G in responding strongly to the SYRGL agonist and in their susceptibility to inhibition by the antagonist peptides (Figure 3B).

Affinity of the 2C TCR for an Antagonist Peptide-MHC Complex

The few comparisons that have been made between TCR binding of agonist and antagonist pMHC complexes suggest that TCR have lower affinity and form less stable complexes with antagonist than with agonist ligands (Alam et al., 1996; Lyons et al., 1996). We previously found the 2C TCR affinity for the weak agonist p2Ca-K^b complex to be about 3×10^{-3} M^{-1}, at the lower limit of what can be measured on live cells (Sykulev et al., 1994a). Accordingly, we expected the 2C TCR affinity for the antagonist EVSV-K^b complex to be still lower, i.e., to be essentially immeasurable on 2C cells. Surprisingly, however, the affinity proved to be about 9×10^4 M^{-1}, or about 30-fold higher (Figure 4; Table 1).

Comparison of Different Target Cells

We attribute the detection of antagonist activity by a high proportion of the peptides tested to the sensitivity of the assay. It is likely that the use of T2-K^b as target cells was responsible for this sensitivity, as we did not observe antagonism when the peptides were tested in the same way with two other K^{b+} target cells, EL4 and RMA-S. The advantage of T2-K^b cells was not that they were of human origin (the others are mouse cells) but rather stems from two considerations: (1) for inhibition to be observed, antagonist peptide concentrations had to greatly exceed the agonist peptide concentration used to pulse target cells (see Figure 3), and (2) much higher agonist peptide concentrations were required to pulse EL4 and RMA-S than T2-K^b target cells. As a result, the antagonist concentrations needed to observe inhibition could be readily achieved with T2-K^b but not with the other target cells. The concentrations of agonist

Table 1. 2C TCR Recognition of Diverse Peptides Associated with Syngeneic and Allogeneic MHC Class I Proteins and Various Responses of 2C Cells to These Peptides[a]

Peptide[b]				Affinity (M^{-1})[c]	
Name	Sequence	MHC	Activity	MHC for Peptide	TCR for pepMHC
SYRGL	SIYRYYGL	K^b	agonist	1×10^8	1×10^7
EVSV	RGYVYQEL		antagonist	1×10^8	9×10^4
p2Ca	LSPFPFDL		agonist	4×10^{4d}	3×10^{3d}
VSV	RGYVYQGL		antagonist	1×10^{8d}	$<10^{3d}$
pOV8	SIINFEKL		null	1×10^{8d}	$<10^{3d}$
p2Cb	VAITRIEQLSPFP**FDL**	L^d	agonist	1×10^7	4×10^6
RL12	RIEQLSPFP**FDL**		agonist	1×10^7	4×10^6
QL9	QLSPFP**FDL**		agonist	2×10^{8d}	2×10^{7d}
p2Ca	LSPFP**FDL**		agonist	4×10^{6d}	2×10^{6d}
SL7	SPFP**FDL**		agonist	6×10^6	1×10^6
PL4	P**FDL**		agonist	6×10^3	1×10^5
p2Ca-Y4	LSPYP**FDL**		agonist	2×10^7	4×10^6
QL9-Y5	QLSPYP**FDL**		agonist	2×10^9	7×10^6
I_1-QL9-Y5	QLSPY(I_1)P**FDL**		agonist	2×10^7	6×10^6
	. . . VAITRIEQLSPFPFDLLLKE . . .		α-ketoglutarate dehydrogenase (α-KGDH)		

[a] 2C $CD8^+$ T cells are positively selected by K^b syngeneic class I MHC but also recognize an allogeneic MHC class I protein L^d in association with peptides from α-ketoglutarate dehydrogenase (α-KGDH [Udaka et al., 1993]).
[b] Naturally processed peptides p2Ca, p2Cb, and p2Ca-Y4 are underlined (Udaka et al., 1992, 1993; Wu et al., 1995) and their analogs are aligned to match the sequence of α-KGDH. FDL motif for L^d-binding peptides (Al-Ramadi et al., 1995; Robinson and Lee, 1996) is depicted in bold. Naturally processed K^b restricted peptides are also underlined.
[c] Intrinsic affinities of soluble L^d and K^b proteins for the peptides shown were determined as previously described (Matsumura et al., 1992); equilibrium binding constants of the TCR-pMHC reactions were measured from competition binding of the soluble pMHC complexes and radiolabeled clonotypic anti-TCR (1B2) antibodies (Fab′ fragments) to TCR on live 2C cells (Sykulev and Eisen, 1997).
[d] Values reported previously (Matsumura et al., 1992; Sykulev et al., 1994a, 1994b).
Note: A previous reference to unpublished results showing that the DL dipeptide could sensitize target cells for lysis by 2C CTL (cited as an addendum to Eisen et al., 1997) was mistaken, probably because the dipeptide preparations used were contaminated with traces of a strong peptide agonist.

(SYRGL) chosen to pulse target cells were based on the agonist concentration required for half-maximal lysis of these cells (SD_{50} value); with EL4 and RMA-S target cells, the SYRGL SD_{50} values were about 100 times higher than for T2-K^b target cells (data not shown). All of these differences reflect the greater number of empty K^b molecules available for peptide loading on T2-K^b than on EL4 and RMA-S cells. Although the total number of K^b molecules on EL4 and T2-K^b cells are approximately the same (about 5×10^5 per cell; unpublished data), the peptide transporter TAP is defective in T2-K^b cells but not in EL4. While RMA-S is also transport defective, there are far fewer K^b molecules on these cells than on T2-K^b, which expresses a transfected K^b gene. Figure 1B provides another example that shows the advantage of T2-K^b over another K^{b+} target cell (compare Figure 1A).

Allogeneic versus Syngeneic Reactions

Before Udaka et al. (1996) described the SYRGL peptide, our efforts to identify peptides that inhibit the reaction of 2C cells with allogeneic (L^{d+}) target cells (T2-L^d), using several L^d-binding peptides, were fruitless (Tsomides et al., unpublished data). As shown in Table 1, these allogeneic targets were used to examine a series of peptides having sequences that are found in murine α-ketoglutarate dehydrogenase (shown at bottom of Table 1; see Udaka et al., 1993). Although these L^d-binding peptides varied from 4 to 16 amino acids in length, they were all agonists for 2C cells, albeit with different degrees of efficacy; e.g., peptide concentrations for half-maximal lysis ranged from about 10^{-5} to 10^{-12} M (Sykulev et al., 1994a, 1994b; unpublished data).

In Table 1, the L^d-binding peptides are aligned from the C-terminal leucine. This alignment was used because systematic alanine substitutions of the p2Ca octapeptide and the QL9 nonapeptide had shown that the C-terminal sequence FDL is critical for binding these peptides to L^d (Al-Ramadi et al., 1995; Robinson and Lee, 1996). Stronger justification for viewing the peptides in register from the C terminus has emerged with the recent description of the crystal structure of L^d (Balendiran et al., 1997; Speir et al., 1998). Although 2C TCR affinity spans a 200-fold range from the weakest (PL4-L^d) to the strongest binding L^d-peptide complex (QL9-L^d), it is striking how little the affinity values vary otherwise between complexes that differ considerably in peptide length (e.g., compare p2Cb, RL12, p2Ca, SL7, etc., in Table 1).

How Do Antagonist Peptides Exert Their Effect?

In the cytolytic assay for antagonism, the target cells were pulsed with the strong agonist peptide at about 10^{-13} M before the test antagonist peptide was added. In view of this extremely low concentration, the number of MHC-binding sites on target cells occupied by the agonist is likely to be $<0.01\%$ of total MHC sites. Hence it is most unlikely that antagonists act by competing with agonists for target cell MHC. Previous studies have led to the same conclusion (Bertoletti et al., 1994; Evavold et al., 1994; Klenerman et al., 1994).

Table 2. Differences in Equilibrium Constants for 2C TCR Interactions with Soluble Peptide-MHC Complexes Measured with the TCR on Live 2C Cells or in a Cell-Free System[a,h]

Peptide-MHC Complexes[b]	Equilibrium Binding Constant (K_a) of pepMHC Complexes to 2C TCR, M^{-1}			
	TCR in a Cell-Free System[c]	TCR on Live 2C Cells[d]	R[e]	SD_{50}, nM[f]
p2Ca-L^d	3×10^{5}[g]	2×10^{6}	7	0.5
QL9-L^d	3×10^{5}	2×10^{7}	68	0.005
p2Ca-K^b	not measured	3×10^{3}	—	1,000
dEV8-K^b	1.2×10^{4}	not measured	—	≥1,000
SYRGL-K^b	3×10^{4}	1×10^{7}	330	0.00004

[a] All values of equilibrium (affinity) constants shown in this table have been measured using recombinant MHC class I proteins K^b and L^d expressed in a *Drosophila melanogaster*-based expression system (Jackson et al., 1992). Soluble 2C TCR was produced in the same system (Garcia et al., 1996a, 1996b).

[b] For peptide sequences see Table 1 and Figure 2.

[c] K_a values calculated from association and dissociation rate constants measured from the binding of soluble pMHC to soluble immobilized α,β-TCR by surface plasmon resonance (Garcia et al., 1996a).

[d] K_a values measured from direct binding of ^{125}I-pMHC complexes or competition binding with the Fab′ fragments of the 1B2 clonotypic antibody to the TCR on live 2C cells (see Table 1).

[e] R is a ratio of equilibrium binding constants for TCR-pMHC reaction measured with live cells and in a cell-free system.

[f] SD_{50} is a concentration of cognate peptide required to sensitize target cells (T2-K^b or T2-L^d, respectively) for half-maximal lysis by 2C CTL; the value of SD_{50} inversely correlates with the efficacy of cytolysis.

[g] The reaction of soluble p2Ca-L^d complex with soluble immobilized 2C TCR was also analyzed by surface plasmon resonance by Corr et al. (1994). Since the authors used the recombinant proteins produced in a different expression system, their data are not given here.

[h] In contrast to the cell free–live cell differences shown in this table, essentially no difference was found in the affinity values measured with the TCR on live hybridoma cells or in a cell-free system for the reaction between the TCR of the 2B4 $CD4^+$ T cell clone and its pMHC ligand, a moth cytochrome peptide (MCC) in association with the I-E^k MHC class II molecule (Matsui et al., 1991, 1994). Whether the results with the latter system and the consistent disparities shown in the table are due to differences between CD4 and CD8, MHC I and MHC II molecules, T cells and hybridomas, or other features remains to be determined.

To determine if antagonist pMHC complexes are inhibitory because they compete with agonist complexes for binding to the TCR, we first determined the fraction of 2C cells' TCR molecules that would have to be blocked by the clonotypic anti-TCR antibody 1B2 (Fab′ fragments) in order to inhibit by 50% the specific lysis of SYRGL-pulsed T2-K^b cells by 2C CTL (in the standard 4 hr cytolytic assay) (Schodin et al., 1996). As shown in Figure 1E, 50% inhibition of lysis required occupancy

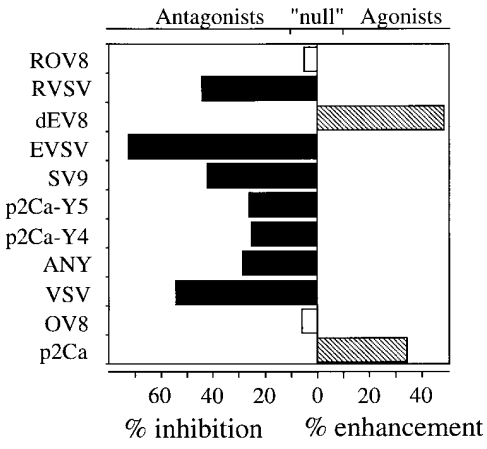

Figure 2. Effect of Diverse K^b-Binding Peptides on Specific Lysis of SYRGL-Pulsed T2-K^b Target Cells by 2C TCR^+ Clone L3.100

Shown is the percent inhibition (filled bars) or enhancement (striped bars) of specific target cell lysis in the presence of 1 μM of the indicated peptides. The naturally processed peptides tested were LSPFPFDL (p2Ca [Udaka et al., 1992]), LSPYPFDL (p2Ca-Y4 [Wu et al., 1995]), SIINFEKL (pOV8 [Carbone et al., 1992]), ANYDFICV (ANY [Malarkannan et al., 1996]), EQYKFYSV (dEV8 [Tallquist and Pease, 1995]), vesicular stomatitis peptide RGYVYQGL (VSV [van Bleek and Nathenson, 1990]), and Sendai virus peptide FAPGNYPAL (SV9 [Schumacher et al., 1991]). Other peptides were LSPFYFDL (p2Ca-Y5), SIIRFEKL (R-OV8), RGYRYQGL (R-VSV), and RGYVYQEL (EVSV).

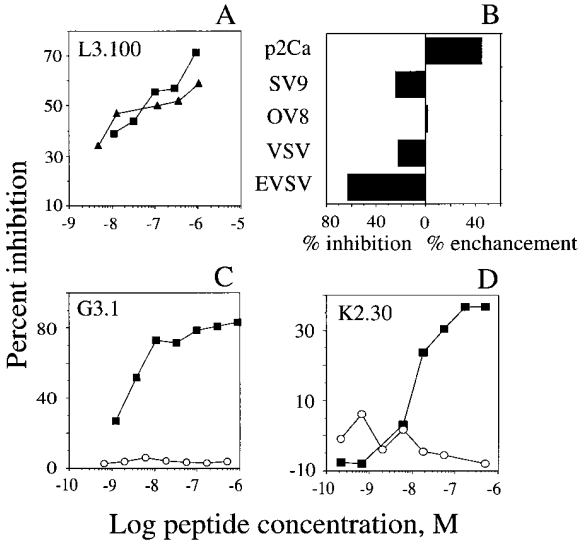

Figure 3. Inhibitory Effect of Various Peptides on Specific Lysis of SYRGL-Pulsed T2-K^b Target Cells by Various CTL Clones and a Polyclonal $CD8^+$ Cell Line that All Express Transgenic 2C TCR

2C clones ([A], L3.100; [C], G3.1; and [D], K2.30) and the cell line (B) were derived from the spleens of 2C TCR transgenic mice (H-2^b). Peptides in (A), (C), and (D) were pOV8 (open circles), VSV (filled squares), and p2Ca-Y4 (filled triangles). Inhibition of lysis of SYRGL-pulsed T2-K^b target cells by a polyclonal 2C CTL cell line is shown in the presence of 1 μM of the peptides indicated (B).

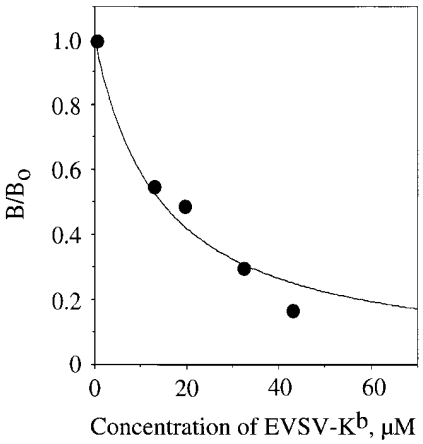

Figure 4. Soluble EVSV-K^b Complex Binds Specifically to the TCR on Live 2C Cells

The ratio (B/B$_o$) is the amount of the ^{125}I-Fab′ fragment of antibody 1B2 bound to 2C cells in the absence (B$_o$) or presence (B) of various concentrations of the EVSV-K^b complex. The equilibrium (affinity) constant for the reaction between 2C TCR and the EVSV-K^b complex was derived from the best fit of the experimental points (filled circles) to the theoretical curve (solid curve) as previously described (Sykulev et al., 1994a).

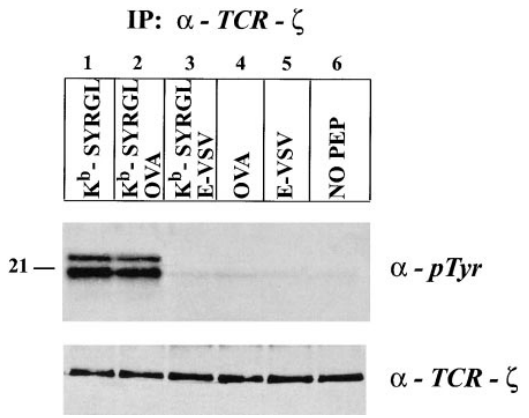

Figure 5. The Antagonist Peptide EVSV but Not the Null Peptide pOV8 Reduced the TCR ζ Chain Tyrosine Phosphorylation Induced in Cloned 2C Cells that Had Been Exposed to the Strong Agonist Complex SYRGL-K^b

2C cells were exposed first to the SYRGL-K^b complex in lanes 1–3 and then exposed to PBS in lane 1, to the null OVA peptide (SIINFEKL) in lane 2, or to the antagonist EVSV peptide (RGYVYQEL) in lane 3. In lanes 4–6 the cells were incubated only with OVA peptide (lane 4), EVSV peptide (lane 5), or PBS (lane 6). TCR ζ chain was immunoprecipitated from all six samples by anti-TCR ζ antisera (α-TCR ζ) and subjected to SDS-PAGE. The anti-phosphotyrosine antibody (α-pTyr) was used for immunoblotting to detect phosphorylated tyrosines of TCR ζ chains (top panel). The same six samples were run in additional lanes of the same gel and immunoblotted with anti-TCR ζ antisera to demonstrate that approximately equal amounts of TCR ζ chain were analyzed in the six samples (bottom panel).

of about 80% of TCR molecules on 2C cells. Can antagonist pMHC complexes on T2-K^b target cells occupy such a large fraction of the TCR on 2C cells under our assay conditions? The 2C TCR affinity for the EVSV-K^b antagonist complex was measured to be about 9×10^4 L/M (Figure 4; Table 1) or 1.5×10^{-19} L/complex. To approach 80% occupancy of the TCR molecules in a reaction mixture [1.5×10^9; i.e., (10^5 TCR per cell) x (1.5×10^4 T cells per well)] with this reaction's affinity would require the density of the antagonist pMHC complexes to be about one trillion (1×10^{12}) per cell. However, the total number of K^b molecules is only about a half million (5×10^5) per target cell. Even if each pMHC complex engaged as many as 200 TCR molecules (Valitutti et al., 1995), competition with agonist complexes for the TCR is a most unlikely explanation for the inhibitory effect of the antagonist complexes.

Antagonist peptides on antigen-presenting cells induce altered patterns of tyrosine phosphorylation of CD3 ζ chains in T cells (Sloan-Lancaster et al., 1994, Madrenas et al., 1995). We therefore examined tyrosine phosphorylation of CD3 ζ chains in 2C cells that had been exposed first to an agonist and then to an antagonist. To separate the effects of agonist and antagonist complexes, we first confronted 2C cells for a few minutes with SYRGL-K^b complexes adsorbed on plastic (96-well plates). The cells were then removed from the immobilized agonist complexes and incubated with either an antagonist (EVSV) or null (pOV8) peptide. As shown previously, the binding of extracellular peptides to MHC molecules on T cells can result in effective formation and presentation of the corresponding pMHC complexes by the T cells to each other (Walden and Eisen, 1990; Su et al., 1993). Figure 5 shows that exposure to the SYRGL-K^b complex resulted in tyrosine phosphorylation of CD3 ζ chains (compare lanes 1 and 6) and subsequent exposure to the EVSV antagonist, but not to the null ligand, resulted in almost complete elimination of the agonist-induced ζ chain tyrosine phosphorylation without affecting the total amount of immunoprecipitated ζ chain (compare lanes 1, 2, and 3). The effect shown differs from that described by Reis e Sousa et al. (1996), who reported that there was less tyrosine phosphorylation of CD3 ζ chains in CTL exposed to antagonist peptide than in those exposed to agonist peptide, but more than in those exposed to null (or no) peptide.

Discussion

Two principal findings emerged from this study of peptide antagonism. First, the affinity of the 2C TCR for an antagonist pMHC (EVSV-K^b) was found to be higher than for a weak agonist (p2Ca-K^b), although much lower than for a strong agonist (SYRGL-K^b). Second, exposure of activated 2C cells to the EVSV antagonist resulted in a pronounced decrease in agonist-induced tyrosine phosphorylation of CD3 ζ chains. These findings are evaluated below with respect to various models proposed to account for the inhibitory effects of antagonist peptides. The models are largely focused on affinity and kinetics of TCR-pMHC reactions. Since affinity values measured for a given TCR under different conditions can, however, differ substantially (see Table 2, below) we evaluate these differences before considering our results in relation to the models.

In the present study and in a previous one (Sykulev et al., 1994a), the 2C TCR affinity for the agonists SYRGL-K^b and p2Ca-K^b and the antagonist EVSV-K^b

were measured with the TCR in its natural environment on live 2C cells and with soluble pMHC complexes formed from synthetic peptides and recombinant K^b and L^d molecules produced in *Drosophila* cells (Figures 1D and 4; Sykulev et al., 1994a). As shown in Table 2, TCR affinity values measured under these conditions are considerably higher than those determined in cell-free systems using soluble pMHC complexes formed in the same way. One possible reason for the differences shown in Table 2 is that TCR-pMHC complexes can be stabilized by CD8 molecules (Luescher et al., 1995; Garcia et al., 1996a), which are usually abundant on intact $CD8^+$ cells and generally not present in cell-free systems. Another possible reason is suggested by the convoluted nature of the cell surface membrane and the abundance of TCR molecules on the T cell surface: a pMHC complex that dissociates from a TCR molecule on the cell might thus have more opportunities than in a cell-free system to rebind to the same or neighboring TCR molecules (Valitutti et al., 1995).

Are the 2C TCR affinity values determined with intact cells ("cellular affinity") or in cell-free systems ("cell-free affinity") more useful for our present purposes? Measured on live T cells, the 2C TCR cellular affinity for SYRGL-K^b is about 3000 times higher than for p2Ca-K^b (Table 2; $1 \times 10^7\ M^{-1}$ versus $3 \times 10^3\ M^{-1}$). This difference parallels the great difference in efficacy of the corresponding peptides in cytolytic assays with K^b target cells (see Figures 1A and 1B). Similarly, the much greater effectiveness of the QL9 than the p2Ca peptide in cytolytic assays with L^{d+} target cells is in accord with the 2C TCR's having a 10 times higher cellular affinity for QL9-L^d than p2Ca-L^d, but it is not in accord with this receptor's having the same cell-free affinity for these peptide-L^d complexes (Table 2; Sykulev et al., 1994a, 1994b). Moreover, the 2C TCR's cell-free affinity for SYRGL-K^b and dEV8-K^b differed only around 2.5-fold (Garcia et al., 1996a; see Table 2); this small difference, which is essentially within experimental error, is also not in accord with the great difference in efficacy between the corresponding peptides in cytolytic assays (Figure 1B). Differences in the binding of extracellular peptides to K^b and L^d on target cells can certainly contribute to the disparate efficacies of these peptides in cytolytic assays, but they cannot account for all of it; e.g., the million-fold difference in peptide concentration required for half-maximal lysis shown in Figure 1B. Thus, cellular affinity values for agonist pMHC complexes generally correlate more closely than cell-free affinity values with the behavior of peptides in cytolytic assays (see also Sykulev et al., 1994a, 1994b). Therefore, in asking if affinity values fit any of the models proposed to explain agonist-antagonist differences, we think it reasonable to focus on cellular affinity values rather than on cell-free values for both antagonists and agonists.

According to some views, TCR have lower affinity and form less stable complexes with antagonist pMHC than with agonist pMHC complexes (Lyons et al., 1996). The suggestion with respect to affinity is clearly not universally the case, as shown by the finding that the 2C TCR has about 30 times higher affinity for an antagonist (EVSV-K^b) than a weak agonist (p2Ca-K^b) complex (Figure 4; Sykulev et al., 1994a). The kinetic issue is more difficult to deal with, as general procedures for determining the stability (i.e., dissociation rates) of TCR-pMHC complexes on intact T cells have not yet been developed. Nevertheless, it is unlikely that the higher affinity reaction with the antagonist EVSV-K^b has a faster k_{off} rate constant than the approximately 30-fold lower affinity reaction with the agonist p2Ca-K^b.

In an interesting kinetic discrimination model (Rabinowitz et al., 1996), the binding of pMHC to TCR is assumed to result in stepwise modifications (e.g., different levels of phosphorylation of the receptor-CD3 complex): incompletely modified receptors are assumed to elicit negative signals and more extensively modified receptors are assumed to elicit positive signals (cell activation), with the balance between the two determining the extent to which a pMHC ligand acts as an antagonist or a weak or strong agonist. In this model the steady state level of the extensively modified receptor is determined not by the rate at which the ligand dissociates from the receptor per se (k_{off}), but by the relative values of k_{off} and the rate of transition (k_2) from an incomplete state of the receptor to a complete state. The model is thus compatible with an antagonist ligand such as EVSV-K^b having a higher affinity and slower k_{off} than an agonist such as p2Ca-K^b—if, for example, the transition rate k_2 were much slower when the receptor is liganded with an antagonist pMHC than with an agonist pMHC.

That the postulated rate of transition of ligated receptor from one state to another could vary with different pMHC ligands raises the additional possibility that the TCR might assume a different conformation when bound to an agonist or antagonist complex. This possibility is in accord with recent evidence that soluble TCR molecules dimerize on binding to agonist pMHC but not on binding to an antagonist pMHC (P. J. Travers and N. R. Gascoigne, personal communication). That a TCR can assume different conformations resulting in various T cell responses has been suggested previously (e.g., from different responses of T cells to different anti-TCR antibodies) (Rojo and Janeway, 1988; Yoon et al., 1994). The recent crystallographic studies of the 2C TCR (Garcia et al., 1996b) provide a solid basis from which to search for significant conformational differences between agonist- and antagonist-liganded TCR.

The limited evidence now available indicates that the changes in tyrosine phosphorylation of CD3 ζ chains elicited by an antagonist pMHC complex are quantitatively reduced but qualitatively the same as those elicited by agonist complexes (Reis e Sousa et al., 1996), although actual sites of phosphorylation were not mapped. The evidence presented here based on a somewhat different experimental protocol (Figure 5) suggests, in contrast, that the antagonist complex stimulates removal of phosphoryl groups from tyrosine residues in CD3 ζ chains as though from the activation of a tyrosine phosphatase (e.g., Neel, 1997). Since cellular responses to different altered peptide ligands can vary considerably (anergy, weak agonism, antagonism at one concentration and agonism at another, or antagonism only), it is possible that TCR interactions with these diverse ligands can also elicit different patterns of protein tyrosine phosphorylation.

That peptide antagonism was more readily detected

in 2C reactions with syngeneic pMHC than allogeneic pMHC raises the possibility that allogeneic and syngeneic reactions might generally differ in susceptibility to antagonism. One reason for speculating about such a difference is that in comparison with syngeneic pMHC, in allogeneic pMHC the MHC moiety may generally contribute more, and the peptide less, to the binding energy of TCR-pMHC interactions (Eisen et al., 1997). Thus, as in Table 1, variations in peptide length and sequence can have relatively little effect on equilibrium constants when the involved MHC is allogeneic (L^d). Indeed, in some TCR reactions with allogeneic pMHC, the peptide's sequence appears not to matter at all, so long as it stabilizes the MHC moiety (Smith et al., 1997; Zhou et al., unpublished data). Even if they prove to be generally less susceptible than syngeneic reactions to peptide antagonism, it appears that some alloreactions can also be specifically inhibited by potent antagonist pMHC complexes (unpublished data).

Experimental Procedures

Cells

CTL clones and cell lines that express the rearranged α and β chain genes of the 2C TCR were derived from 2C transgenic mice (Sha et al., 1988). Isolated splenocytes were initially stimulated with irradiated P815 (H-2^d) cells in K medium (RPMI supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycine, and 50 μM β-mercaptoethanol) and restimulated 6 days later and thereafter at 1 week intervals with irradiated P815 cells (20×10^3 rad) in K medium containing supernatants from concanavalin A (Con A)-activated rat splenocytes. Established CTL lines was tested in cytotoxicity assays using ^{51}Cr-labeled P815 cells and/or T2-L^d or T2-K^b cells sensitized with appropriate peptides as target cells (see below). Clones L3.100, K2.30, and G3.1 were isolated from a 2C TCR^+ CTL line by limiting dilution in 96-well plates, initially with an average of 100, 30, and 1 cell per well, respectively. These clones were maintained and tested as described above.

The human mutant cell line T2 (Alexander et al., 1989), transfected with L^d (T2-L^d) or K^b (T2-K^b) genes, was a generous gift from Dr. P. Cresswell (New Haven, CT); it was maintained as described elsewhere (e.g., Sykulev et al., 1994a). EL4 thymoma cells (H-2^b), RMA-S cells (H-2^b TAP deficient), and P815 (H-2^d) mastocytoma cells were all cultured in K medium.

Peptides

The Biopolymers Laboratory of Massachusetts Institute of Technology synthesized the peptides used in this study (tBoc method) and purified many of them by HPLC. Peptide concentrations were based on amino acid analyses or BCA assays or weight/volume.

Soluble MHC Class I Molecules

Soluble K^b and L^d class I MHC molecules were expressed in *Drosophila melanogaster* cells (Jackson et al., 1992) and isolated from culture supernatants as described (Sykulev et al., 1994a). Soluble peptide-K^b complexes used to study protein tyrosine phosphorylation in 2C TCR^+ cells were produced in *E. coli* according to Nathenson and colleagues (Zhang et al., 1992).

Cytolytic Assay

In all experiments, ^{51}Cr-labeled H-$2K^{b+}$ target cells (T2-K^b, EL4, or RMA-S) and various 2C CTL clones or 2C cell lines (expressing transgenic 2C TCR) were used. Usually 5,000 target cells (in 100 μl K medium) and 25,000 CTL (in 50 μl K medium) were combined with 50 μl of PBS containing various peptides at different concentrations in round-bottom 96-well plates. The plates were centrifuged (for 1–2 min) at 500 g and then incubated at 37°C in a 5% CO_2/95% air atmosphere. After 4 hr, the plates were centrifuged for 5 min and 100 μl of supernatant was harvested from each well to determine the amount of ^{51}Cr released into extracellular medium. Percent specific lysis was calculated from the following: (^{51}Cr release into supernatant − spontaneous release)/(total release in detergent − spontaneous release) × 100. Wells containing peptide were in duplicate. Similarly treated control wells (in quadruplicate) provided values for spontaneous release (CTL omitted) and total release (1% NP-40 included in medium).

To evaluate the ability of various peptides to inhibit specific lysis of K^{b+} target cells by 2C CTL, the target cells were pulsed with the agonist peptide SYRGL by incubating them with this peptide while the cells were being labeled with ^{51}Cr (1 hr at 37°C). The peptide concentration was 4×10^{-14} M for T2-K^b and 5×10^{-12} M for the other K^{b+} target cells. Pulsed target cells were washed three times to remove unbound peptide and unincorporated ^{51}Cr and were mixed with various concentrations of the test peptides and CTL in round-bottom 96-well plates as described above. Percent inhibition of specific lysis was calculated as 100 × (A − B)/A, where A and B are percentages of specific lysis in the absence and presence of the tested peptide.

Peptide Binding to K^b and L^d

Equilibrium binding constant of SIYRYYGL (SYRGL) to K^b was estimated in a peptide competition assay using as a reference peptide RGYVYQGL (VSV), whose equilibrium (binding) constant for K^b protein (1×10^8 M^{-1}) was previously determined (Matsumura et al., 1992). ^{51}Cr-labeled T2-K^b target cells were incubated with SIINFEKL (pOV8) peptide (at about 10^{-11} M) and various dilutions of either SYRGL or VSV; all peptides were added at the same time (unlike the conditions used to pulse target cells in the antagonist assay; see above). 4G3 CTLs (Walden and Eisen, 1990), which recognize pOV8 in association with K^b, were then added at a CTL:target cell ratio of 3 to 1. Percent specific lysis, determined as described above after 4 hr incubation at 37°C under 5% CO_2, was plotted as a function of concentration of competing peptide. Equilibrium binding constant of K^b for RGYVYQEL (EVSV) was estimated in a peptide stabilization assay with the RMA-S cell line using reference peptides (VSV or pOV8); the affinities of K^b for the latter peptides were measured previously (Matsumura et al., 1992).

Equilibrium binding constants of α-ketoglutarate dehydrogenase peptides to soluble L^d were measured by competition binding of these peptides and radiolabeled peptide from mouse cytomegalovirus (MCMV [Reddehase et al., 1989]) to the soluble L^d. Equilibrium constant for the reaction between ^{125}I-MCMV and soluble L^d was determined in separate experiment. A detailed protocol for the assay was previously described (Matsumura et al., 1992).

Equilibrium Binding of Soluble Peptide-MHC Complexes to 2C TCR on Intact Cells

The equilibrium binding constant for the TCR-pMHC reaction was determined from the competition of soluble pMHC complexes with ^{125}I-labeled Fab′ fragments of the clonotypic anti-TCR antibody 1B2 for binding to the TCR on intact 2C cells, as previously described (Sykulev and Eisen, 1997). Soluble pMHC complexes were produced by loading empty recombinant K^b and L^d proteins (Jackson et al., 1992) with an appropriate peptide at a concentration (10^{-5} to 10^{-6} M) that was 100- to 1000-fold higher than the equilibrium dissociation constant for the peptide-MHC reaction (i.e., sufficient to saturate the MHC-binding sites for peptide).

Tyrosine Phosphorylation of TCR ζ Chains

The wells of 24-well plates were coated by incubating 1–20 μg of SYRGL-K^b complex per well at 4°C overnight. Wells were then washed with PBS, blocked with 1% BSA (in PBS) at 25°C for 30–45 min, and washed with RPMI 1640 medium. 2C cells ($3–10 \times 10^6$) in 500 μl of RPMI 1640 were added to each well, spun at 800 rpm for 2 min, and incubated at 37°C under 5% CO_2/95% air. After 20 min, the 2C cells were transferred to Eppendorf tubes containing 50 μl of 10^{-5} M test peptide (EVSV or OVA) in PBS or 50 μl of PBS without peptide and incubated for 20 min at 37°C under 5% CO_2/95% air. For further controls, cells were not exposed to SYRGL-K^b but were incubated under similar conditions with test peptide (EVSV or OVA) or no peptide at all. Equal volumes (≈500 μl) of ice-cold PBS containing 1 mM sodium orthovanadate were then added to all six

samples and the tubes were transferred to ice. Cells were pelleted at 14,000 rpm for 20 s, and pellets were lysed in 50–100 μl of lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Lysates were precleared with normal rabbit serum and formalin fixed Staphylococcus aureus and then immunoprecipitated with TCR ζ-specific rabbit antiserum (a generous gift of Dr. P. Allen, St. Louis, MO). The immunoprecipitated proteins were separated by 12.5% SDS-PAGE under reducing conditions and blotted onto nitrocellulose. The blots were probed with either the anti-phosphotyrosine monoclonal antibody (clone 4G10 [UBI, Lake Placid, NY]) or the TCR ζ-specific antisera; in both instances they were followed by peroxidase-conjugated goat antibodies to mouse or rabbit immunoglobulins (Pharmingen) and were developed by chemiluminescence.

Acknowledgments

We are grateful to Dr. Paul Allen for rabbit antibodies to CD3 ζ and for instructions in their use, to Joseph Delaney and Susumu Tonegawa for spleen cells from TAP-deficient mice, to Stanley Nathenson for the plasmid containing the K^b gene, to Carol McKinley for excellent technical support, to Mimi Rasmussen for deriving the 2C clones from 2C transgenic mice, and to Richard F. Cook and colleagues of the Massachusetts Institute of Technology Biopolymers Laboratory for peptides and amino acid analyses. This work was supported by National Institutes of Health research grants (CA60686 and AI34247), a training grant in immunology (CA09255), and a core grant to the Center for Cancer Research at the Massachusetts Institute of Technology (CA14051).

Received June 25, 1998; revised September 11, 1998.

References

Al-Ramadi, B.K., Jelonek, M.T., Boyd, L.F., Margulies, D.H., and Bothwell, A.L. (1995). Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR. J. Immunol. *155*, 662–673.

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. Nature *381*, 616–620.

Alexander, J., Payne, J.A., Murrey, J.A., Frelinger, J.A., and Cresswell, P. (1989). Differential transport requirements of HLA and H-2 class I glycoproteins. Immunogenetics *29*, 380–388.

Balendiran, G.K., Solheim, J.C., Young, A.C.M., Hansen, T.H., Nathenson, S.G., and Sacchettini, J.C. (1997). The three-dimensional structure of an H-2L^d-peptide complex explains the unique interaction of L^d with β_2m and peptide. Proc. Natl. Acad. Sci. USA *24*, 6880–6885.

Bertoletti, A., Sette, A., Chisari, F.V., Penna, A., Levrero, M., De Carli, M., Fiaccadori, F., and Ferrari, C. (1994). Natural variants of cytotoxic epitopes are T-cell receptor antagonists for anti-viral cytotoxic T cells. Nature *369*, 407–410.

Carbone, F.R., Sterry, S.J., Butler, J., Rodda, S., and Moore, M.W. (1992). T cell receptor α-chain pairing determines the specificity of residue 262 within the K^b-restricted, ovalbumin 257–264 determinant. Int. Immunol. *4*, 861–867.

Corr, M., Slanetz, A.E., Boyd, L.F., Jelonek, M.T., Khilko, S., Al-Ramadi, B.K., Kim, Y.S., Maher, S.E., Bothwell, A.L.M., and Margulies, D.H. (1994). T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. Science *265*, 946–949.

Eisen, H.N., Sykulev, Y., and Tsomides, T.J. (1997). The antigen-specific T-cell receptor and its reactions with peptide-MHC complexes. Adv. Prot. Chem. *49*, 1–56.

Evavold, B.D., Sloan-Lancaster, J., and Allen, P.M. (1994). Antagonism of superantigen-stimulated helper T-cell clones and hybridomas by altered peptide ligand. Proc. Natl. Acad. Sci. USA *91*, 2300–2304.

Garcia, K.C., Scott, C.A., Brunmark, A., Carbone, F.R., Peterson, P.A., Wilson, I.A., and Teyton, L. (1996a). CD8 enchances formation of stable T-cell recptor/MHC class I molecule complexes. Nature *384*, 577–581.

Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A., Teyton, L., and Wilson, I.A. (1996b). An αβ T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. Science *274*, 209–219.

Garcia, K.C., Tallquist, M.D., Pease, L.R., Brunmark, A., Scott, C.A., Degano, M., Stura, E.A., Peterson, P.A., Wilson, I.A., and Teyton, L. (1997). An αβ T cell receptor interactions with syngeneic and allogeneic ligands—affinity measurements and crystallization. Proc. Natl. Acad. Sci. USA *94*, 13838–13843.

Jackson, M.R., Song, E.S., Yang, Y., and Peterson, P. (1992). Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in Drosophila melanogaster cells. Proc. Natl. Acad. Sci. USA *89*, 12117–12121.

Kersh, G.J., and Allen, P.M. (1996). Essential flexibility in the T-cell recognition of antigen. Nature *380*, 495–498.

Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Köppe, B., Rosenberg, W., Boyd, D., Edwards, A., et al. (1994). Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature *369*, 403–407.

Kranz, D.M., Tonegawa, S., and Eisen, H.N. (1984). Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA *81,* 7922–7926.

Luescher, I.F., Vivier, E., Layer, A., Mahiou, J., Godeau, F., Malissen, B., and Romero, P. (1995). CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. Nature *373*, 353–356.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Chien, Y.-H., and Davis, M.M. (1996). A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity *5*, 53–61.

Madrenas, J., Wange, R.L., Wang, J.L., Isakov, N., Samelson, L.E., and Germain, R.N. (1995). ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science *267*, 515–518.

Malarkannan, S., Gonzalez, F., Nguyen, V., Adair, G., and Shastri, N. (1996). Alloreactive CD8+ T cells can recognize unusual, rare, and unique processed peptide/MHC complexes. J. Immunol. *157*, 4464–4473.

Matsui, K., Boniface, J.J., Reay, P.A., Schild, H., Fazekas de St. Groth, B., and Davis, M.M. (1991). Low affinity interaction of peptide-MHC complexes with T cell receptors. Science *254*, 1788–1791.

Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994). Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. Proc. Natl. Acad. Sci. USA *91*, 12862–12866.

Matsumura, M., Saito, Y., Jackson, M.R., Song, E.S., and Peterson, P.A. (1992). In vitro peptide binding to soluble empty class I major histocompatibility complex molecules isolated from transfected *Drosophila melanogaster* cells. J. Biol. Chem. *267*, 23589–23595.

Neel, B.G. (1997). Role of phosphatases in lymphocyte activation. Curr. Opin. Immunol. *9*, 405–20.

Rabinowitz, J.D., Beeson, C., Lyons, D.S., Davis, M.M., and McConnell, H.M. (1996). Kinetic discrimination in T-cell activation. Proc. Natl. Acad. Sci. USA *93*, 1401–1405.

Reddehase, M.J., Rothbard, J.B., and Koszinowski, U.H. (1989). A pentapeptide as minimal antigenic determinant for MHC class I-restricted T Lymphocytes. Nature *337*, 651–653.

Reis e Sousa, C., Levine, E.H., and Germain, R.N. (1996). Partial signaling by CD8+ T cells in response to antagonist ligands. J. Exp. Med. *184,* 149–157.

Robinson, R.A., and Lee, D.R. (1996). Studies of tum- peptide analogs define an alternative anchor that can be utilized by L^d ligands lacking the consensus P2 anchor. J. Immunol. *156*, 4266–4273.

Rojo, J.M., and Janeway, C.A., Jr. (1988). The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. J. Immunol. *140*, 1081–1088.

Schumacher, T.N.M., De Bruijn, M.L.H., Vernie, L.N., Kast, W.M.,

Melief, C.J.M., Neefjes, J.J., and Ploegh, H.L. (1991). Peptide selection by MHC class I molecules. Nature *350*, 703–706.

Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russell, J.H., and Loh, D.Y. (1988). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature *336*, 73–79.

Schodin, B.A., Tsomides, T.J., and Kranz, D.M. (1996). Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity. Immunity *5*, 137–146.

Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B., and Allen, P.M. (1994). Partial T cell signaling: altered phospho-ζ and lack of zap70 recruitment in APL-induced T cell anergy. Cell *79*, 913–922.

Speir, J.A., Garcia, K.C., Brunmark, A., Degano, M., Peterson, P.A., Teyton, L., and Wilson, I.A. (1998). Structural basis of 2C TCR allorecognition of H-2L^{d} peptide complexes. Immunity *8*, 553–562.

Su, M.W.-C., Walden, P.R., and Eisen, H.N. (1993). Cognate peptide-induced destruction of CD8+ cytotoxic T lymphocytes is due to fratricide. J. Immunol. *151*, 658–667.

Sykulev, Y., and Eisen, H.N. (1997). Measurements of affinity and kinetics of antigen-specific T cell receptor reactions with peptide-MHC complexes. In Immunology Methods Manual, I. Lefkovits, ed. (San Diego: Academic Press), pp. 489–496.

Sykulev, Y., Brunmark, A., Jackson, M., Cohen, R.J., Peterson, P.A., and Eisen, H.N. (1994a). Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. Immunity *1*, 15–22.

Sykulev, Y., Brunmark, A., Tsomides., T.J., Kageyama, S., Jackson, M., Peterson, P.A., and Eisen, H.N. (1994b). High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. Proc. Natl. Acad. Sci. USA *91*, 14487–14491.

Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J., and Eisen, H.N. (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity *4*, 565–571.

Tallquist, M.D., and Pease, L.R. (1995). Alloreactive 2C T cells recognize a self peptide in the context of mutant K^{bm3} molecule. J. Immunol. *155*, 2419–2426.

Udaka, K., Tsomides, T.J., and Eisen, H.N. (1992). A naturally occurring peptide recognized by alloreactive CD8^{+} cytotoxic T lymphocytes in association with a class I MHC protein. Cell *69*, 989–998.

Udaka, K., Tsomides, T.J., Walden, P., Fukusen, N., and Eisen, H.N. (1993). A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8^{+} T-cell clone. Proc. Natl. Acad. Sci. USA *90*, 11272–11276.

Udaka, K., Wiesmuller, K.-H., Kienle, S., Jung, G., and Walden, P. (1996). Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. J. Immunol. *157*, 670–678.

Valitutti, S., Muller, S., Cella, M., Padovan, E., and Lanzavecchia, A. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature *375*, 148–151.

van Bleek, G.M., and Nathenson, S.G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^{b} molecule. Nature *348*, 213–216.

Walden, P.R., and Eisen, H.N. (1990). Cognate peptides induce self-destruction of CD8+ cytolytic T lymphocytes. Proc. Natl. Acad. Sci. USA *87*, 9015–9019.

Wu, M.X., Tsomides, T.J., and Eisen, H.N. (1995). Tissue distribution of natural peptides derived from a ubiquitous dehydrogenase, including a novel liver-specific peptide that demonstrates the pronounced specificity of low affinity T cell reactions. J. Immunol. *154*, 4495–4502.

Yoon, S.T., Dianzani, U., Bottomly, K., and Janeway, C.A., Jr. (1994). Both high and low avidity antibodies to the T cell receptor can have agonist or antagonist activity. Immunity *1*, 563–569.

Zhang, W., Young, A.C.M., Imarai, M., Nathenson, S.G., and Sacchettini, J.C. (1992). Crystal structure of the major histocompatibility complex class I H-2K^{b} molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. Proc. Natl. Acad. Sci. USA *89*, 8403–8407.