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Immunobiological Analysis of TCR Single-Chain Transgenic Mice Reveals New Possibilities for Interaction between CDR3α and an Antigenic Peptide Bound to MHC Class I[1]

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The interaction between TCRs and peptides presented by MHC molecules determines the specificity of the T cell-mediated immune response. To elucidate the biologically important structural features of this interaction, we generated TCR β-chain transgenic mice using a TCR derived from a T cell clone specific for the immunodominant peptide of vesicular stomatitis virus (RGYVYQGL, VSV8) presented by H-2K^b. We immunized these mice with VSV8 or analogs substituted at TCR contact residues (positions 1, 4, and 6) and analyzed the CDR3α sequences of the elicited T cells. In VSV8-specific CTLs, we observed a highly conserved residue at position 93 of CDR3α and preferred Jα usage, indicating that multiple residues of CDR3α are critical for recognition of the peptide. Certain substitutions at peptide position 4 induced changes at position 93 and in Jα usage, suggesting a potential interaction between CDR3α and position 4. Cross-reactivity data revealed the foremost importance of the Jα region in determining Ag specificity. Surprisingly, substitution at position 6 of VSV8 to a negatively charged residue induced a change at position 93 of CDR3α to a positively charged residue, suggesting that CDR3α may interact with position 6 in certain circumstances. Analogous interactions between the TCR α-chain and residues in the C-terminal half of the peptide have not yet been revealed by the limited number of TCR/peptide-MHC crystal structures reported to date. The transgenic mouse approach allows hundreds of TCR/peptide-MHC interactions to be examined comparatively easily, thus permitting a wide-ranging analysis of the possibilities for Ag recognition in vivo. *The Journal of Immunology,* **2001, 167: 4396–4404.**

The specificity of the T cell-mediated immune response is dependent on the recognition by TCRs of antigenic peptides presented by MHC molecules. The $\alpha\beta$ TCR is a disulfide-linked heterodimer. The α- and β-chains each contain a constant region, assumed to be associated with CD3 accessory molecules that trigger downstream signaling, and a variable region involved in specific Ag recognition. The variable region of the α- and β-chains includes three Ag-binding loops or complementarity-determining regions (CDRs),[7] referred to as CDR1, CDR2, and CDR3, which are analogous to those found in Igs. CDR1 and CDR2 are encoded by Vα or Vβ gene segments present in the germline. For the α-chain, the CDR3 loop is encoded following the joining of a V gene to one of a number of J genes, whereas for the β-chain, V-D-J joining encodes CDR3 (1, 2). This gene recombination is partly responsible for creating the diversity in CDR3 loop sequences, and further diversity is generated by deletion or addition of nucleotides at the junctions. It was recently demonstrated for Abs that diversity only in the H chain CDR3 is sufficient to develop responses to a variety of protein and hapten Ags (3).

Extensive structural and functional studies exploring the specificity of TCR recognition for peptide-MHC (pMHC) have been conducted. Recently reported crystal structures of TCR/pMHC complexes have revealed that TCRs bind to pMHC complexes with a generally diagonal footprint in which the α-chain is over the N-terminal half of the peptide and the β-chain over the C-terminal half (4–11). The crystal structures, along with functional analyses using TCR single-chain transgenic mice (12–14) or site-directed mutagenesis of CDR3 loops (15, 16), have all demonstrated the existence of interaction sites between the CDR3s and the antigenic peptide, thus identifying the CDR3 loops as important determinants for peptide recognition.

Although the overall diagonal orientation of the TCR over pMHC class I appears to be general, the particular contact sites vary for different TCRs bound to the same ligand (7), as well as for the same TCR complexed with different ligands (8, 10). For a fuller understanding of the general rules for TCR/pMHC interactions, a much more extensive functional and structural database is needed. Over the years, we have used a variety of structural and functional strategies to elucidate the interactions that occur between TCRs and the immunodominant peptide of vesicular stomatitis virus (RGYVYQGL, VSV8) presented by H-2K^b (17). In

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[7] Abbreviations used in this paper: CDR, complementarity-determining region; pMHC, peptide-MHC; Tgα, TCR α-chain transgenic; Tgβ, TCR β-chain transgenic; VSV, vesicular stomatitis virus.

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early studies, we identified the residues at positions 1, 4, and 6 of VSV8 as potential TCR contacting sites by using x-ray crystallography (18) and a biological T cell recognition assay (19). Next we used a series of H-2K^b mutants to map the TCR binding surface on the MHC, work that allowed us to first propose the diagonal orientation of TCR over pMHC (20). Structural information regarding the interaction of TCRs with VSV8/H-2K^b is limited to a single low-resolution structure that is nonetheless sufficient to confirm the overall diagonal orientation observed in the TCR/pMHC structures that have been reported in other systems (21).

To better "visualize" the interactions that occur between TCRs and VSV8, and to observe a large number of such interactions comparatively easily, we have used the technique of variant peptide immunization of TCR single-chain (α or β) transgenic mice. In our previous efforts to identify the residues in CDR3β that are important for the recognition of VSV8 and its variants (14), we introduced the Vα2 TCR α-chain of a VSV8-specific CTL clone (N30.7) into TCRα-deficient mice, so that all the T cells would express an identical α-chain. These TCR α-chain transgenic (Tgα) mice were immunized with VSV8 peptide analogs with a substitution at position 6, and the CDR3β sequences of peptide-specific CTL were analyzed. We found that a substitution at position 6 of VSV8 can induce a compensatory change at position 98 of CDR3β as well as a change in CDR3β loop length. It was then concluded that position 98 of CDR3β is a key residue determining the specificity of TCR recognition of position 6 of the peptide and that an optimal length of CDR3β is required to facilitate interaction. Site-directed mutagenesis also confirmed the importance of position 98 in VSV8/H-2K^b recognition (22, 23).

Regarding the interactions between TCRs and VSV8, several questions remained to be answered, including: 1) Which positions of the peptide interact with CDR3α? 2) How does CDR3α loop length and composition change to recognize different peptides? 3) Are these changes qualitatively different from those observed in CDR3β? and 4) Does position 4 of VSV8 contact CDR3α and/or CDR3β? To approach these questions, we recently derived TCR β-chain transgenic (Tgβ) mice using the Vβ13 TCR β-chain from N30.7, the same VSV8-specific CTL clone whose α-chain was used to generate the Tgα mice used in our previous work (14). In these Tgβ mice, the presence of the transgene inhibits the rearrangement and expression of endogenous TCR β-chain genes due to allelic exclusion. After immunization of the mice with VSV8 and variants bearing substitutions at potential TCR contact residues, the CDR3α loop sequences of peptide-specific T cells were analyzed to see which, if any, amino acid substitutions could induce compensatory changes in CDR3α composition and/or length. Here we show that this approach, in which hundreds of TCR/pMHC interactions could be examined, has revealed previously undescribed interactions between TCRs and VSV8 and has highlighted the general importance of the TCR Jα region in determining Ag specificity.

Materials and Methods

N30.7 Tgα mice

Derivation of the Tgα mice has been described in detail previously (14). These mice are transgenic for the TCR α-chain of the VSV8-specific CTL clone N30.7 (24). They also carry a targeted disruption of both alleles of the endogenous *TCR Cα* locus and are therefore unable to express their endogenous TCR α-chain genes.

Generation of N30.7 Tgβ mice

The VDJ segment of the TCR β-chain cDNA (Vβ13) of the VSV8-specific CTL clone N30.7 was amplified by PCR (using primers containing appropriate intronic sequences, splice acceptor/donor sites, and restriction sites) and cloned into the *Cla*I/*Not*I sites of the TCR β shuttle vector (Ref. 25; provided by M. Davis, Stanford University, Stanford, CA). After removal of prokaryotic sequences by digestion with *Pvu*I/*Sal*I, the transgene construct was injected into (CBA $\times$ C57BL/6)F_2 zygotes. Mice carrying the Vβ13 transgene were identified by PCR analysis of tail DNA using the primer set 5′-CTTTGTCTCCTGGGTGCAGGT-3′ and 5′-TTCACCAC CCACCCAGTGCAT-3′. Transgene carriers were crossed for several generations with C57BL/6 mice to obtain transgenic mice homozygous for H-2^b. MHC haplotype was monitored by PCR using primer sets specific for H-2K^b and H-2K^k.

Generation of peptide-specific CTLs

Peptides (Table I) were synthesized and purified to >98% homogeneity by HPLC before being used for immunization. Tgα or Tgβ mice were immunized in their hind footpads with 20 μg of peptide emulsified in CFA and boosted 1 wk later with 20 μg of peptide emulsified in IFA. One week after the boost, spleen cells were cultured at 5×10^6/ml with 1 μM immunizing peptide in IMDM supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). Cells were restimulated once in vitro, and cell sorting was performed after 13 days of culture.

Cytolytic assay

CTL activity was measured in a standard 4-h ^{51}Cr-release assay as described (31). The percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release)] $\times$ 100%.

FACS analysis and cell sorting

To detect surface expression of the Vβ13 transgene, dissociated lymph nodes of 6- to 8-wk-old mice were stained with an anti-Vβ13 (MR12-3; BD PharMingen, San Diego, CA) Ab. To sort Tg$^+$ CTLs from in vitro cultures, cells were stained with anti-CD8 (53-6.7; BD PharMingen) and anti-Vβ13 Abs and CD8$^+$ Vβ13$^+$ cells were collected. FACS analysis and cell sorting of stained cells were performed on a FACScan and FACStar (BD Biosciences, Mountain View, CA), respectively.

Preparation of mRNA and cDNA and Vα family usage typing

mRNA was extracted from 5×10^5 to 2×10^6 sorted Tg$^+$ CTLs, and single-stranded cDNA was made as described (14). Vα family usage was determined by PCR using a TCR Cα primer paired with 1 of 20 Vα primers, each specific for a particular Vα gene family and designed to recognize all known members of that family (32). Each culture was found to express a limited number (i.e., 2–4) of Vα families (data not shown).

Analysis of TCR CDR3α sequences

Double-stranded TCR α-chain cDNA was prepared by PCR as described above from sorted CD8$^+$Vβ13$^+$ cells. PCR products were cloned into

Table I. *Peptides used for immunization of Tgα and/or Tgβ mice*[a]

Peptide	Notation	Peptide Sequence
Wild type	VSV8	**R** G Y **V** Y **Q** G L
Position 1 variants	K1	**K** – – – – – – –
	E1	**E** – – – – – – –
	I1	**I** – – – – – – –
Position 4 variants	A4	– – – **A** – – – –
	I4	– – – **I** – – – –
	D4	– – – **D** – – – –
	E4	– – – **E** – – – –
	K4	– – – **K** – – – –
	R4	– – – **R** – – – –
	Y4	– – – **Y** – – – –
Position 6 variants	K6	– – – – – **K** – –
	R6	– – – – – **R** – –
	D6	– – – – – **D** – –
	E6	– – – – – **E** – –

[a] Positions 1, 4, and 6 of VSV8 are the potential TCR contacting residues (18, 26) and are underlined and bolded. These residues were substituted in the peptide variants as indicated, and the notation for each variant indicates the substituting residue and its position. In the variant peptide sequences, a dash indicates that the same residue as in VSV8 is present at that position. In general, single amino acid changes in the TCR contacting residues of VSV8 do not dramatically alter the affinity of the peptide for H-2K^b (Refs. 27–29, and data not shown) or its conformation within the peptide-binding groove (28, 30).

pCR2.1 (Invitrogen, San Diego, CA) and the TCR CDR3α sequences in the resulting plasmids were determined. At least two Tgβ mice were immunized with each peptide variant and the sequencing data pooled.

Results

TCR α-chains of VSV8-specific CTLs from Tgβ mice exhibit conserved Jα usage and a conserved residue at position 93 of the CDR3 loop

Mice transgenic for the TCR β-chain (Vβ13) of the VSV8-specific CTL clone N30.7 were derived as described in *Materials and Methods*. FACS analysis revealed that virtually all peripheral $CD8^+$ T cells in these Tgβ mice expressed the Vβ13 transgene on their surface, and Vβ family usage typing by RT-PCR (32) indicated that the presence of the rearranged Vβ13 transgene eliminated the rearrangement and expression of endogenous TCR β-chain genes (data not shown). To investigate the features of CDR3α that are important for interaction with VSV8/H-$2K^b$, Tgβ mice were immunized with VSV8, and the CDR3α sequences of VSV8-specific CTLs were determined.

Cytotoxicity assays showed that VSV8-specific CTLs could be induced in Tgβ mice (Fig. 1), indicating that the transgenic TCR β-chain could pair with endogenous TCR α-chains to allow recognition of VSV8/H-$2K^b$. TCR Vα family usage typing indicated that the Vα1, Vα2, Vα5, and Vα15 families were used in VSV8-specific CTLs. As the N30.7 TCR α-chain is a member of the Vα2 family (24), we first compared the CDR3α loop sequences of the Vα2 TCRs with that of the N30.7 TCR (Table II). We found that the CDR3α loop sequences from VSV8-specific CTLs elicited from Tgβ mice showed striking similarity to that of the N30.7 TCR. Among the CDR3α sequences from the Vα2 family, position 93 (Pro) and the Jα15 region (YQGGRALI) are highly conserved. (Note that position 93 occurs at the V-J junction and is generally not germline-encoded.) These data suggest that the residue at position 93 and the Jα region of the CDR3α loop are important elements for interaction with VSV8. We also found that the predominant CDR3α length was 10-aa long (Table III), but that the CDR3α length was less restricted as compared with that previously observed for CDR3β (14).

To examine whether the conserved residues at position 93 and in Jα were also associated with usage of a specific Vα family, CDR3α loop sequences from VSV8-specific CTLs expressing different Vα families were examined and compared (Table II). We found that for three other Vα families (Vα1, Vα5, or Vα15), the residue at position 93 and the Jα region of CDR3α were highly conserved. However, for these Vα families, the residue at position 93 was Ser/Thr instead of Pro, which was found in Vα2 CTLs. Yet, Jα15 was again the predominant Jα family among sequenced CDR3α loops for all of the Vα families examined. Our data suggest that in VSV8-specific CTLs, the specific Jα usage is induced by the peptide independently of the Vα family, whereas the identity of the conserved residue at position 93 is determined by both the antigenic peptide and the Vα family.

In the crystal structure of the 2C TCR (4, 6), a portion of the Jα region lies adjacent to the CDR3β loop. This observation brought up a question: do Jα15 TCR α-chains preferentially pair with the transgenic TCR β-chain, resulting in predominant Jα15 usage even in naive Tgβ mice? To address this issue, CDR3α loops of TCRs from naive (i.e., unimmunized) Tgβ mice were sequenced (data not shown). We found that in naive mice, there is no predominant Jα usage and the occurrence of Jα15 among all CDR3 sequences is extremely low (1/22). Thus, the predominant Jα15 usage observed in VSV8-specific CTLs is

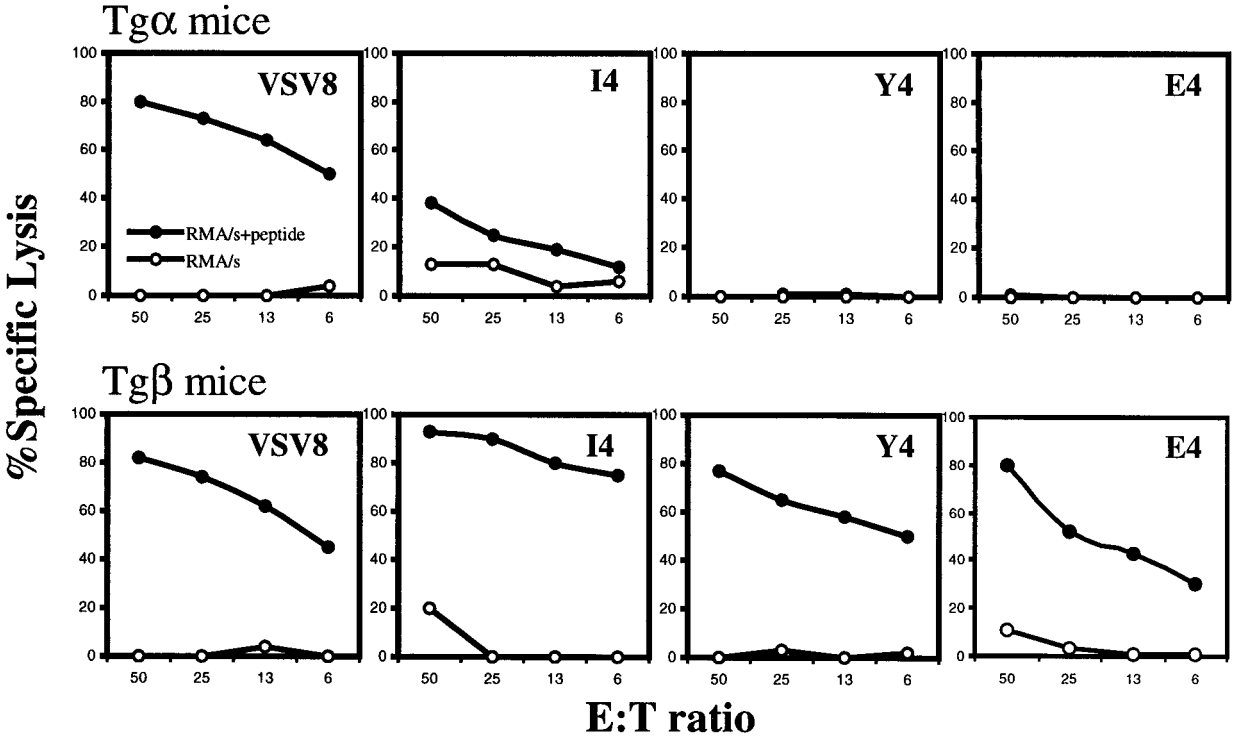

FIGURE 1. Induction of position 4 variant-specific CTLs in Tgα and Tgβ mice. Tgα and Tgβ mice were immunized with VSV8 or the indicated position 4 variants. After 1 wk in culture, cytotoxic activity was measured in a 4-h ^{51}Cr-release assay using RMA/S cells incubated with (●) or without (○) the immunizing peptide (0.1 μM) as targets.

Table II. *CDR3α sequences of N30.7 and VSV8-specific CTLs of the Vα2 family from a representative Tgβ mouse*[a]

T cells	Vα	CDR3 Sequence 93	CDR3 Length	Jα Usage	Frequency
N30.7	2	PAYQGGRALI	10	15	
Tgβ CTLs	2	**PA<u>YQGGRALI</u>**	10	15	1/12
		P<u>YQGGRALI</u>	9	15	2/12
		PSV<u>YQGGRALI</u>	11	15	1/12
		PH<u>YQGGRALI</u>	10	15	2/12
		PT<u>YQGGRALI</u>	10	15	2/12
		PG<u>QGGRALI</u>	9	15	1/12

[a] Tgβ mice were immunized with VSV8, and TCR CDR3α sequences were determined after 2 wk of culture in the presence of VSV8. The single-letter amino acid code is used. CDR3α is taken to be from position 93 to just before the Jα-encoded "FG" (with the conserved C taken to be at position 90). Conserved CDR3 residues are shown in boldface and the Jα region is underlined. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family. The N30.7 sequence was previously reported (24).

specifically induced by the immunizing peptide and is not caused by preferential pairing with the transgenic TCR β-chain.

Position 4 variants elicit CTLs in Tgβ mice, but not in Tgα mice

Although immunization of N30.7 Tgα mice with VSV8 permits induction of peptide-specific CTLs (Ref. 14, and Fig. 1), immunization with the position 4 variants D4 (Val→Asp), E4 (Val→Glu), K4 (Val→Lys), R4 (Val→Arg), or Y4 (Val→Tyr) was unable to elicit a CTL response, whereas I4 (Val→Ile) induced only a weak response (Fig. 1 and data not shown). A possible explanation for these results is that position 4 of VSV8 interacts with the TCR α-chain, and that the fixed Tgα chain cannot tolerate any change at this position of the peptide. To determine whether position 4 might also interact with the β-chain, N30.7 Tgβ mice were immunized with these same peptide variants. A cytotoxic response was obtained in all cases (Figs. 1 and 2), supporting the idea that position 4 of the peptide interacts principally with the TCR α-chain and not with the β-chain.

Certain substitutions at peptide position 4 induce changes in CDR3α at position 93 and in Jα usage

To map potential interaction sites between the TCR α-chain and position 4 of the peptide, CDR3α sequence analysis was performed on CTLs from Tgβ mice responding to VSV8 variants in which Val was substituted by a positively charged (Lys in K4 or Arg in R4) or bulky (Tyr in Y4) residue. Comparison of CDR3α sequences within a specific Vα family showed that nonconservative substitution of Val to Lys, Arg, or Tyr induced compensatory changes in the CDR3α sequence (Table IV), suggesting a direct interaction between CDR3α and position 4 of the peptide. Consistent with the results for VSV8-specific CTLs, TCRs of K4-, R4-, or Y4-specific CTLs had a highly conserved residue at position 93 and a conserved Jα segment when a specific Vα family was considered. When position 4 was changed to Lys, the residue at position 93 of CDR3α was changed to Arg or Glu, the Jα15 gene segment used by VSV8-specific CTLs was replaced by Jα18 or Jα22, and changes in the CDR3α length were also observed. For the Val→Arg substitution, we observed changes in the residue at position 93 and Jα usage, but not in CDR3 length, whereas the Val→Tyr substitution induced compensatory changes in both the CDR3α motif and length. These CDR3α sequence data indicate that certain position 4 substitutions can induce changes both at position 93 and in the Jα region, leading to alterations in the nature of the CDR3α loop that have functional consequences, as revealed by the finding that K4-, R4-, and Y4-specific CTLs were unable to cross-react with VSV8 (Fig. 2).

VSV8 peptide variants with more conservative substitutions for Val at position 4 to similar hydrophobic residues (Ala in A4 or Ile in I4) could elicit strong CTL responses in Tgβ mice (Fig. 1 and data not shown), and A4- or I4-specific CTLs could cross-react with VSV8 (Fig. 2 and data not shown). The predominant CDR3α sequence used by Vα15 A4-specific CTLs (SXYQGGRALI; Table IV), with Ser at position 93 and usage of Jα15, was the same as that of VSV8-specific CTLs using the Vα15 family. For I4-specific CTLs using the Vα5 family, a substitution of Ile for Val also did not induce changes of the residue at position 93 or in the Jα15 usage, although the N-terminal residue (Y) of the Jα15 portion of CDR3 (YQGGRALI) was replaced with a random residue added at the VJ junction, thus maintaining a predominant CDR3α length of 10 (Table IV). These findings suggest that both the CDR3α sequence and the CDR3α length are important in recognizing the antigenic peptide. Overall, VSV8 peptide variants with a position 4 substitution of Val to Ala or Ile induced a similar CDR3α motif in the responding T cell population, which is consistent with the finding that the A4- and I4-specific CTLs were able to cross-react with VSV8.

Finally, when Tgβ mice were immunized with VSV8 variants with a change at position 4 from Val to negatively charged residues (Glu in E4 or Asp in D4), CTL responses could be elicited

Table III. *Summary of the CDR3α sequences of VSV8-specific CTLs of different Vα families*[a]

Vα	CDR3 Sequence 93	CDR3 Length	Jα Usage	Frequency
1	**SX<u>YQGGRALI</u>**	10	15	6/7
2	**PX<u>YQGGRALI</u>**	10	15	18/24
5	**(S/T)X<u>YQGGRALI</u>**	10	15	6/7
15	**SX<u>YQGGRALI</u>**	10	15	21/29

[a] Tgβ mice were immunized with VSV8, and TCR CDR3α sequences were determined after 2 wk of culture in the presence of VSV8. The single-letter amino acid code is used. CDR3α is taken to be from position 93 to just before the Jα-encoded "FG" (with the conserved C taken to be at position 90). Conserved CDR3 residues are shown in boldface and the Jα region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

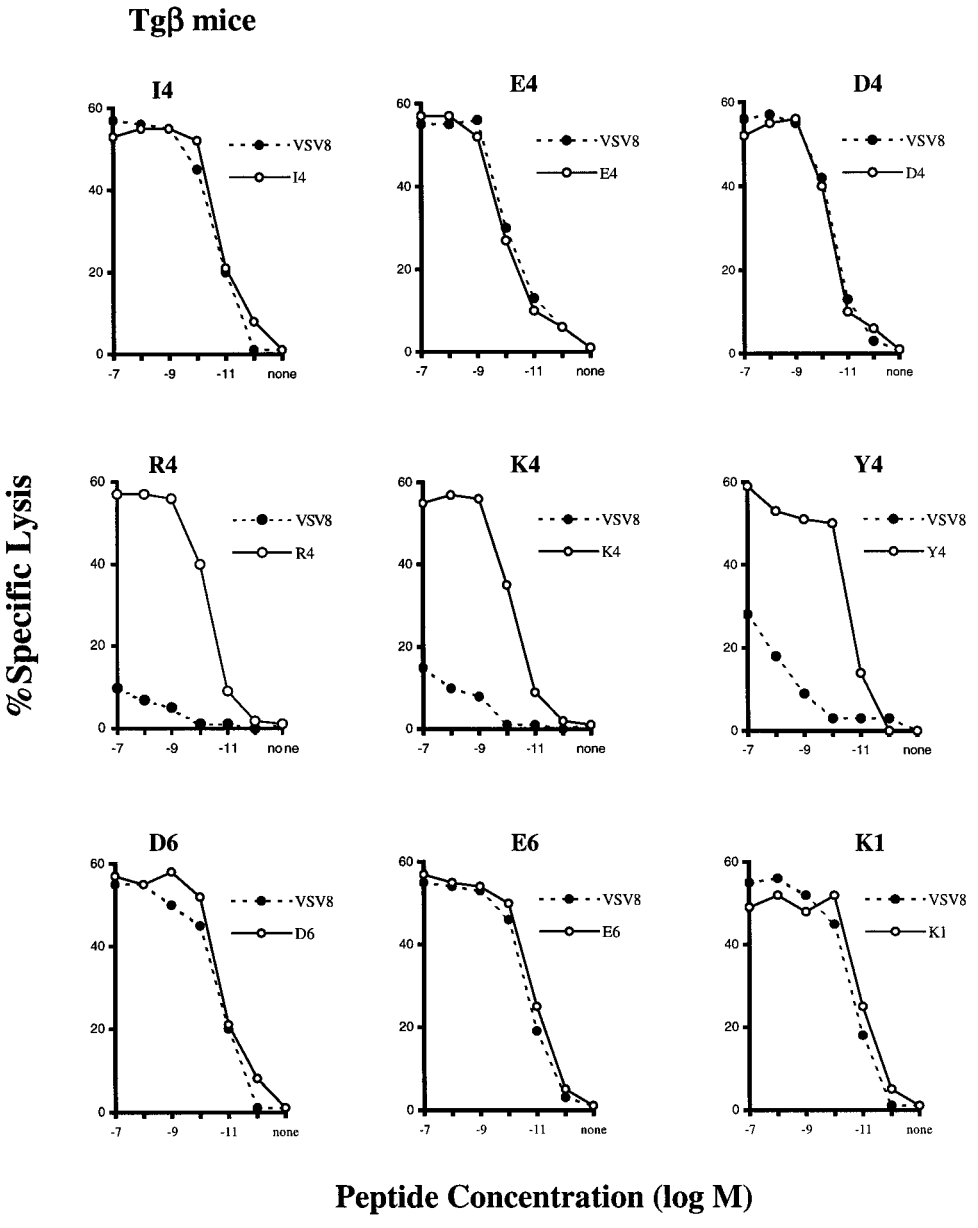

FIGURE 2. Cross-reactivity of peptide variant-induced CTLs with VSV8. CTLs were elicited from Tgβ mice by immunization with the indicated peptide. After 2 wk in culture with the immunizing peptide, CTL recognition of varying concentrations of the immunizing peptide (○) or VSV8 (●) was measured in a 4-h ^{51}Cr-release assay using an E:T ratio of 2.

and, surprisingly, E4- or D4-specific CTLs showed strong cross-reactivity with VSV8 (Fig. 2). Although we expected that a Val→Glu or Asp substitution at position 4 of VSV8 would change the CDR3α motif (because Glu and Asp have very different electrostatic properties from Val), we found instead that TCRs of E4- or D4-specific CTLs had similar CDR3α motifs to those of VSV8-specific CTLs (Table IV) in that Jα15 usage was maintained. However, although position 93 of CDR3α was conserved for a specific Vα family, for Vα5 E4-specific CTLs, Thr was found at position 93 rather than the Ser seen in Vα5 VSV8-specific CTLs. Also, although the Jα15 usage was maintained, two residues (YQ) at the N terminus of the Jα region were not present, and the predominant CDR3α loop length was 9 residues rather than 10. Similarly, when Val at position 4 of VSV8 was substituted with Asp, Vα1 D4-specific CTLs exhibited Leu at position 93 instead of the Ser seen there in Vα1 VSV8-specific CTLs, one residue (Y) was absent from the N terminus of Jα15, and the CDR3α length was changed from 10 to 9 residues. Taken together, the data showed that the Val→Asp/Glu substitution can induce CTLs having compensatory changes of the CDR3α sequence at position 93 and deletions at the N terminus of the Jα region, again suggesting that position 93 and the N-terminal region of Jα may interact with position 4 of the peptide. Moreover, the CDR3α length was altered, presumably to allow interaction between CDR3α and the substituting residue at position 4 of the peptide.

Substitution at peptide position 6 to Asp or Glu induces a compensatory change at position 93 of CDR3α

Previous results from Tgα mice showed that changes at position 6 of VSV8 could induce compensatory changes at position 98 of CDR3β, suggesting that position 6 of the peptide specifically interacts with position 98 (14). From the crystal structures of TCR/pMHC class I complexes (4–8, 10, 11), residues close to the C terminus of the peptide interact with the TCR β-chain and not with the TCR α-chain. We would hypothesize that: 1) a nonconservative substitution for Gln at position 6 of VSV8 would not induce a CTL response in Tgβ mice; and 2) even if substitutions of Gln to certain residues could induce a CTL response, these substitutions would not induce compensatory changes in the CDR3α loop.

Table IV. *Summary of the CDR3α sequences of TCRs of position 4 variant-specific CTLs elicited from Tgβ mice*[a]

Peptide	Recognition of VSV8	Vα	CDR3 Sequence 93	CDR3 Length	Jα Usage	Frequency
K4	No	2	**RGSALGRLH**	9	18	13/13
	No	4	**EXSGSWQLI**	9	22	21/26
R4	No	2	**EXSSGSWQLI**	10	22	21/28
Y4	No	15	**LSNYNVLY**	8	21	31/38
A4	Yes	15	**SXYQGGRALI**	10	15	8/10
I4	Yes	5	**SXXQGGRALI**	10	15	13/19
E4	Yes	5	**TXXGGRALI**	9	15	13/15
D4	Yes	1	**LXQGGRALI**	9	15	16/25

[a] Mice were immunized with the indicated peptides, and CDR3α sequences were determined after 2 wk of culture in the presence of the immunizing peptide. The single-letter amino acid code is used. CDR3α is taken to be from position 93 to just before the Jα-encoded "FG." Conserved CDR3 residues are shown in boldface, and the Jα region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. "Recognition of VSV8" indicates whether the position 4 variant-specific CTLs can cross-react with VSV8. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

To test these hypotheses, Tgβ mice were immunized with position 6 variants of VSV8. As expected, K6 (Gln→Lys) and R6 (Gln→Arg) peptide variants could not elicit CTL responses (data not shown). However, we found that D6 (Gln→Asp) or E6 (Gln→Glu) could induce very strong CTL responses in Tgβ mice, and that D6- or E6-induced CTLs could cross-react with VSV8 (Fig. 2). Like position 4 variant-specific CTLs, we found that in the TCR α-chains of D6- or E6-specific CTLs, position 93 and Jα usage were highly conserved (Table V). Surprisingly, we found that position 93 is compensatorily changed to a positively charged residue when Gln at position 6 is changed to the negatively charged Asp (D6) or Glu (E6). These data suggest the possibility of a direct interaction between CDR3α and position 6 of the peptide. Such an interaction has not yet been revealed by the limited number of TCR/pMHC class I crystal structures reported to date (4–8, 10, 11).

Substitution at position 1 of VSV8 does not induce compensatory changes in CDR3α

Our previous data from Tgα mice, as well as the crystal structures of TCR/pMHC complexes, suggested that position 1 of VSV8 might interact with the TCR α-chain (14, 33). To determine whether position 1 of VSV8 interacts with the CDR3α loop, Tgβ mice were immunized with VSV8 peptide variants bearing a substitution at position 1, and the CDR3α loop sequences of these peptide variant-specific CTLs were examined (Table V). It was found that all position 1 peptide variants examined (i.e., K1, I1, and E1) elicited CTLs, all of which cross-reacted with VSV8 (Fig. 2, and data not shown). CDR3α sequence analysis showed that position 1 substitution of Arg to Lys (K1), Ile (I1), or Glu (E1) did not induce changes at position 93 or in Jα usage. Consistent with the reported crystal structures of TCR/pMHC class I complexes (4–8, 10, 11), these data indicated that position 1 of the peptide is not specifically contacted by CDR3α.

Discussion

The TCR α-chain, rather than the β-chain, interacts with position 4 of VSV8 in vivo

In our previous (14, 31, 33) and present studies, we used Tgα and Tgβ mice as tools to define TCR interactions with VSV8/H-2K^b. Although VSV8 elicits strong CTL responses in both Tgα and Tgβ mice, a wide variety of position 4 VSV8 variants elicit strong responses only in Tgβ mice (Figs. 1 and 2), suggesting that position 4 of VSV8 interacts with the TCR α-chain. Alteration of position 4 of the peptide apparently disrupts interaction with the Tgα chain, rendering position 4 variants unable to induce CTL responses in Tgα mice. However, in Tgβ mice, the TCR α-chain can vary, so a position 4 peptide variant can induce T cells expressing compatible α-chains. Our finding provides biological evidence that the central residue of the antigenic peptide primarily contacts the TCR α-chain rather than the β-chain. Crystal structures of TCR/pMHC class I complexes are consistent with this idea, despite the significant differences in details among the different structures (4–8, 10).

Position 93 and the Jα region of CDR3α are important elements for interaction with position 4 of the peptide

Analysis of CDR3α sequences of TCRs of VSV8-specific CTLs from Tgβ mice (Tables II and III) showed that the residue at position 93 and the Jα region are highly conserved for one specific Vα family. Even for different Vα families, we discovered a predominant Jα15 usage. Taken together, we conclude that the residue at position 93 and the Jα region of the CDR3α loop are critical for recognition of VSV8. Because in VSV8-specific CTLs derived from Tgα mice, only two conserved residues were found in the

Table V. *Summary of the CDR3α sequences of TCRs of position 6 or position 1 variant-specific CTLs elicited from Tgβ mice*[a]

Peptide	Recognition of VSV8	Vα	CDR3 Sequence 93	CDR3 Length	Jα Usage	Frequency
E6	Yes	13	**(K/R)XYQGGRALI**	10	15	25/27
D6	Yes	5	**(K/R)XYQGGRALI**	10	15	28/28
K1	Yes	15	**SXYQGGRALI**	10	15	16/19
E1	Yes	15	**SXYQGGRALI**	10	15	15/21
I1	Yes	15	**(S/T)XYQGGRALI**	10	15	11/16

[a] Mice were immunized with the indicated peptides, and CDR3α sequences were determined after 2 wk of culture in the presence of the immunizing peptide. The single-letter amino acid code is used. CDR3α is taken to be from position 93 to just before the Jα-encoded "FG." Conserved CDR3 residues are shown in boldface, and the Jα region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. "Recognition of VSV8" indicates whether the variant-specific CTLs can cross-react with VSV8. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

CDR3β loop (14), our current data from Tgβ mice immunized with VSV8 suggest that a greater number of CDR3α residues may make critical contacts with the peptide in vivo, a conclusion not inconsistent with the crystal structures of certain TCR/peptide-H-2K^b complexes (6).

Nonconservative substitution of Val at position 4 of VSV8 to Lys, Arg, or Tyr, which are distinct residues from Val in terms of size and other characteristics such as hydrophobicity and electropolarity, induces dramatic conserved changes at position 93 of CDR3α and in Jα usage (Table IV), indicating that these elements are important for interaction with position 4 of the peptide. The negatively charged residue Glu is present at position 93 in most K4- and R4-specific CTLs, suggesting that this Glu may interact through a salt bridge with the positively charged side chain at position 4. In addition, the change of Jα usage indicates the importance of this region for interaction with position 4 of the peptide. Our data indicate that, unlike the CDR3β loop in which position 98 is mostly involved in the interaction with position 6 of VSV8, residues at position 93 and in the Jα segment of the CDR3α loop may contact peptide position 4, providing strong biological evidence for the complexity of the interaction of the CDR3α loop with position 4 of the peptide in vivo. Crystal structures of TCR/pMHC complexes (4–7) have revealed that position 93 and the Jα region of the CDR3α loop have extensive contacts with the central residue of the antigenic peptide. Based on the crystal structure of the 2C TCR complexed with its ligand (dEV8/H-2K^b), we have modeled the interaction between the VSV8-specific TCR N15 and the VSV8 peptide (Fig. 3). The model supports the likelihood of interaction between the Jα region of CDR3α and position 4 of VSV8.

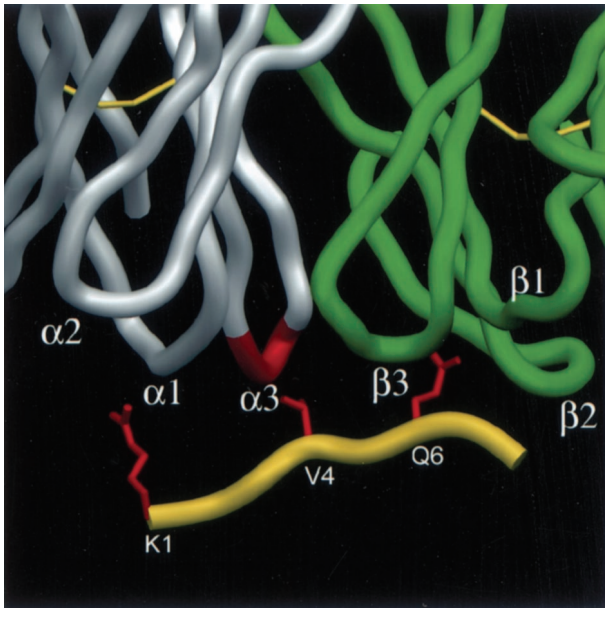

FIGURE 3. An interaction model between the VSV8-specific TCR N15 and the VSV8 peptide. This structural model was built, using the crystal structures of the unliganded N15 TCR (34) and VSV8/H-2K^b (18), to fit the overall structure of the 2C TCR complexed with dEV8/H-2K^b (6), and was presented using the program SETOR (35). TCRα, TCRβ, and the VSV8 peptide (*bottom*) are colored gray, green, and yellow, respectively. CDR loops of the N15 α-chain are labeled α1, α2, and α3, whereas those of the β-chain are labeled β1, β2, and β3. The Jα region of CDR3α is colored red. The side chains of the residues at positions 1, 4, and 6 of VSV8 are depicted as red sticks. The figure serves only to depict the likely overall placement of the N15 TCR relative to VSV8 based on the liganded 2C TCR structure. No attempt has been made to predict atomic contacts or the precise positioning of the CDRs after rearrangement upon ligand binding.

Having determined that CDR3α interacts with position 4 of VSV8 and its variants, we were initially surprised to find that immunization of Tgβ mice with the position 4 variants A4 and I4 induces CDR3α sequences that are quite similar to those elicited by VSV8 in terms of length, the residue at position 93, and the Jα sequence (Tables II–IV). However, cytotoxicity assays then revealed that A4- and I4-induced CTLs could cross-react with VSV8 and that VSV8-induced CTLs could cross-react with A4 and I4 (data not shown). Because Val, Ala, and Ile all have hydrophobic side chains, we propose that the same types of interaction (possibly van der Waals) may be involved between these hydrophobic residues and the CDR3α loop.

When the hydrophobic Val at position 4 of VSV8 was substituted with a negatively charged residue (Asp or Glu), we found that D4- or E4-induced CTLs also use the same Jα15 segment as VSV8-specific CTLs (Table IV), indicating that Glu or Asp can apparently form similar contacts with the Jα region as does VSV8. Thus, common structural features, rather than the charge, may be the determinant of the specificity of Glu or Asp interaction with the Jα region of the CDR3α loop. Consistent with this is our finding that D4- and E4-specific CTLs from Tgβ mice can cross-react with VSV8. Although the change of Val to Asp or Glu at position 4 of VSV8 does not alter Jα usage, it does induce changes at position 93. In previous studies with Tgα mice (14), a substitution of Gln to Glu or Asp at position 6 of VSV8 induced a change of Val or Thr at position 98 of CDR3β to the positively charged residue Lys, suggesting that there is a salt bridge between position 98 of the CDR3β loop and Glu at position 6 of VSV8. Here we observed that when Val at position 4 of VSV8 was changed to Glu or Asp, a positively charged residue was not induced at position 93, indicating the absence of a salt bridge with position 4 of the peptide variant. Instead, interaction between position 4 and Thr at position 93 might be mediated by a hydrogen bond.

The Jα region is an important determinant of TCR specificity

Our current data reveal the importance of the Jα region in recognizing a specific antigenic peptide in vivo. First, we found that VSV8 and its variants each induce a specific, predominant Jα usage (Tables II–V). Second, for particular peptide-specific CTLs, Jα usage is even conserved across different Vα families, whereas position 93 tends to be conserved only within a particular family (Tables II and III). Third, substitutions for Val at P4 can trigger compensatory changes not only at position 93 but also at the Jα region by deletion from the N terminus or replacement with a different Jα segment (Table IV). Fourth, we found a correlation of Jα usage with cross-reactivity of peptide variant-specific CTLs with VSV8: peptide variant-induced CTLs that could cross-react with VSV8 used Jα15. No cross-reactivity was seen when a different Jα was used (Tables IV and V). It is noteworthy that even among a panel of VSV8-specific CTL clones previously derived from standard C57BL/6 mice (24), a significant proportion (3 of 12) used Jα15 (referred to as A10 in that earlier report), a striking finding considering the fact that the murine germline contains >60 different Jα gene segments (36).

The crystal structures of TCR/pMHC class I complexes showed that the J region of CDR3β does not contact the antigenic peptide (4–7). Our previous studies of CDR3β loop sequences from position 6 variant-specific CTLs elicited from Tgα mice showed that Jβ usage is not highly conserved (14), suggesting that the Jβ region does not specifically interact with the peptide in vivo either. Instead, CDR3β residues that contact peptide are encoded by the D segment and N addition. In contrast, structural studies (4–7)

reveal that the N terminus of Jα is located at the apex of the CDR3α loop, enabling the Jα region to make multiple contacts with a central peptide residue. Our current data provide biological evidence for the importance of these Jα interactions in determining TCR specificity in vivo. Given this difference between the TCR α- and β-chains, it is relevant to note that the murine germline contains >60 Jα gene segments (36) but only 14 Jβ gene segments (37, 38).

Changes in CDR3α loop length are induced by substitutions at position 4 of VSV8

Our previous studies in Tgα mice showed that nearly all sequenced CDR3β loops for a particular position 6 variant-specific CTL population had an identical length, and that substitution at position 6 of VSV8 changed the CDR3β loop length (14). These results suggest that the loop length could be very important for interactions between CDR3β and position 6 of the peptide, and that loop length may facilitate the interaction between position 98 of the CDR3β loop and position 6 of VSV8 or its variants.

Our data in Tgβ mice now indicate that the length of the CDR3α loop could also be important for TCR recognition of VSV8 and its variants. In Tgβ mice, sequenced CDR3α loops of TCRs of peptide-specific CTLs have a favored length (Tables II–V). A substitution of Val at position 4 to the large residues Tyr or Lys not only changed the CDR3α loop sequence, but also shortened the CDR3α loop length. Recently, we have found that trinitrophenyl-labeled-K4-specific CTLs from Tgβ mice also have shorter CDR3α loops than do VSV8-specific TCR α-chains (39). One possible explanation is that a CDR3α loop of shorter length may create a bigger cavity at the TCR α and β interface, allowing bulky side chains or hapten groups to be accommodated.

The TCR α-chain may contact position 6 of VSV8 under certain circumstances

Using Tgα mice, we showed previously that interaction between position 98 of CDR3β and peptide position 6 is critical for recognition of VSV8 and its variants (14). Thus, we predicted that K6 and R6 peptides would be unable to induce CTL responses in Tgβ mice, as substitution of Gln at position 6 to Lys or Arg would likely abolish this critical contact with the Tgβ chain. We found that this was indeed the case, a result compatible with the finding that VSV8-specific CTLs, including N30.7, are generally unable to recognize position 6 variants of VSV8 (22, 31, 33). However, we were surprised to find that both D6 and E6 could elicit strong CTL responses in Tgβ mice, and that the residue at position 93 of CDR3α was changed from a Ser to a positively charged residue (Table V), suggesting the possibility of an interaction between position 6 of the peptide and the TCR α-chain in the special situation where the β-chain may be somewhat incompatible with the residue at position 6. One possible explanation is the following: Because Gln, Asp, and Glu have similar structures, Asp/Glu can still interact with the Tgβ chain CDR3, but with weaker affinity. Therefore, the residue at position 93 of CDR3α is reciprocally changed to Lys/Arg to enhance the binding of position 6 of the peptide to the TCR. This hypothesis is supported by the crystallographic finding that the VSV8/H-2K^b and E6/H-2K^b complexes are very similar in structure (30). Further support for the idea that the TCR α-chain can interact with position 6 in certain circumstances comes from our finding that immunization of Tgβ mice with a VSV8 variant in which Gln at position 6 is changed to trinitrophenyl-labeled-Lys can induce alterations both at position 93 and in Jα usage (39).

An analogous interaction between the TCR α-chain and a residue toward the C terminus of the peptide has not yet been seen in the limited number of TCR/pMHC class I crystal structures published to date (4–8, 10, 11). However, the extraordinary structure of the BM3.3 TCR complexed with pBM1/H-2K^b, in which the CDR3α loop bends away from the MHC peptide-binding groove and makes absolutely no contacts with the peptide (11), is an indication that much remains to be discerned, using a combination of x-ray crystallography and various biological strategies, regarding the possibilities for interaction between the TCR and its ligand.

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