Conversion of a Self Peptide Sequence into a K<sup>d</sup>-restricted Neo-Antigen by a Tyr Substitution

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Summary

We have previously found that a Tyr residue was critical for the interaction of peptides with the K<sup>d</sup> molecule, and therefore may be acting as an anchor residue. In the present report we show that it is possible to convert a self peptide sequence into a K<sup>d</sup>-restricted neo-antigen by a single Tyr substitution at position 2 of the peptide. This supports the idea that Tyr is a critical element in the binding motif of K<sup>d</sup>-restricted peptides and is a finding that could also prove useful for vaccine development.

T lymphocytes recognize antigen in the form of peptides bound to MHC class I or class II molecules (reviewed in references 1 and 2). X-ray crystallographic analysis of the class I molecules HLA-A2 and HLA-Aw68 (3–5) showed a cleft on the MHC molecule that was proposed to be the peptide binding site. By using a functional competition assay we have recently tried to define common structural features of peptides recognized in the context of the H-2K<sup>a</sup> (K<sup>a</sup>) MHC class I molecule (6). For the antigenic peptide HLA-A24 170-182, it was initially shown that three residues (Tyr 171, Thr 178, and Leu 179) were important in the interaction with K<sup>d</sup>. Subsequently, an analogue (AYPsTLA) containing these residues was demonstrated to be an efficient competitor for K<sup>d</sup> binding. A predominant influence on binding was found for the Tyr residue and we have demonstrated that four different K<sup>d</sup>-restricted epitopes all contain a common Tyr residue that appears to be critical for competitor activity (6, 7).

We reasoned that if Tyr was a critical residue for K<sup>d</sup> binding, it should be possible to introduce a Tyr residue into a protein sequence and create a neo-epitope restricted by K<sup>d</sup>. To test this, we took advantage of the well characterized antigenic mutant system developed by Boon et al. (reviewed in reference 8). They have shown that a high frequency of mutagenized P815 tumor cells express neo-antigens that can be recognized by CTL. The mutants are rejected by syngeneic mice, and hence they have been named tum<sup>−</sup> ("tumor-minus") mutants. For three different tum<sup>−</sup> mutants, the genes encoding the neo-antigens have been cloned and sequenced. The three genes, which are unrelated to each other and whose functions are as yet unknown, each contain a single point mutant that leads to the creation of a neo-epitope (8).

One example is tum<sup>−</sup> antigen P91A<sup>−</sup>, in which the point mutation resulted in a single amino acid change that created an L<sup>d</sup>-restricted CTL epitope (9).

To initiate the present study, we synthesized a series of six overlapping 11-mer peptides whose sequences correspond to the wild-type (i.e., self) P91A<sup>+</sup> (tum<sup>+</sup>) sequence, except for the replacement of a single residue in each peptide with Tyr. The Tyr residue was introduced at the second position from the NH<sub>2</sub> terminus in each peptide, since this position was found to be optimal for the four different K<sup>d</sup>-restricted epitopes that we have analyzed previously (10). After testing these six peptides in a competition assay, we selected one that appeared to be optimal for K<sup>d</sup> binding and immunized mice of the H-2K<sup>d</sup> MHC haplotype. From these mice we could isolate CTL specific for the immunizing peptide, and as predicted, all of the CTL clones thus obtained were H-2K<sup>d</sup> restricted.

Materials and Methods

Cells. The isolation and characterization of K<sup>d</sup>-restricted CTL clone CS.H2 (P. Romero et al., manuscript in preparation) and of L<sup>d</sup>-restricted CTL clone P91.6 (11) is described elsewhere. The CTL lines and clones specific for the peptide P91A 12-22/Y<sub>13</sub> were isolated from BALB/c or (BALB/c × C57BL/6)F<sub>1</sub> mice immunized with the peptide in Freund's adjuvant, as described elsewhere (P. Romero et al., manuscript in preparation).

Peptides. Peptides were synthesized and purified as described elsewhere (6), and amino acid composition was confirmed by amino acid analysis.

Cytolytic Assay. Experiments to assess peptide recognition or competition were performed as described elsewhere (6) in a 4-h ⁵¹Cr release assay. The percent specific lysis was calculated as: 100 × [(experimental − spontaneous release)/(total − spontaneous release − spontaneous release)].
### Table 1. Peptides Used in this Study

| Peptide    | Sequence          |
|------------|-------------------|
| P91A+12-24 | I STQNRLD LVA    |
| P91A-12-24 | I STQNHRALDLVA   |
| 11-21/Y12  | KYSTQNRLD        |
| 12-22/Y13  | IYTQNRLD         |
| 13-23/Y14  | SYQNRLD LVV      |
| 14-24/Y15  | TYNRLD LVA       |
| 15-25/Y16  | QYRRALDLVAA      |
| 16-26/Y17  | NYRALDLVAAK      |

The percent control lysis was calculated as: $100 \times \frac{[\text{percent specific lysis with competitor} - \text{background lysis}]}{[\text{percent specific lysis without competitor} - \text{background lysis}]}$. Clone 444/A1.1 (12) was used for P815 (H-2d) target cells. The derivation of L cell (H-2r) transfectants that express H-2K^d and/or the mouse inter-cellular adhesion molecule 1 (ICAM-1) is described elsewhere (C. Jaulin et al., manuscript in preparation).

### Results and Discussion

The Interaction of Tyr-substituted P91A^+ Peptides with H-2K^d and H-2L^d. The peptides shown in Table 1 were tested for their capacity to interact with K^d and L^d molecules by using a functional competition assay. The most active competitor for K^d binding was the peptide 12-22/Y13, which was as active as the peptide analogue AYPsTLA previously described (6) (Fig. 1). Peptides Y12, Y14, Y15, and Y16 also competed but were 10-100-fold less active, whereas Y17 was a poor competitor.

![Figure 1](image1.png)

**Figure 1.** Comparison of Tyr-substituted P91A^+ peptides as competitors for K^d or L^d-restricted peptides. 

| COMPETITOR PEPTIDE | pM |
|--------------------|----|
| P91A+12-24         | 100|
| Y12               | 100|
| Y13               | 100|
| Y14               | 100|
| Y15               | 100|
| Y16               | 100|
| MCMV              | 100|

CTL - CS . H-2 (K^d) and CTL - P91.6 (L^d).

![Figure 2](image2.png)

**Figure 2.** CTL specific for the P91A Y13 mutant peptide. 

| PEPTIDE CONCENTRATION (log M) |
|-------------------------------|
| 0.001 | 0.01 | 0.1  | 1.0  | 10  | 100 |

(closed symbols), the P91A^+ peptide (open symbols, solid line), or the P91A^- peptide (open symbols, broken line). The CTL clones were isolated from line F1-1 by limiting dilution after the fourth in vitro stimulation. They express V\_13, V\_14, or V\_10, respectively, and are thus clearly different clones. TCR expression was determined by flow cytometric analysis (not shown) using mAbs specific for V\_13 (VB-TCR-6B; Pharmingen, San Diego, CA) or V\_14 (14.2; reference 15) and/or by PCR (J.-L. Casanova, personal communication).
The series of Tyr-substituted P91A + peptides were also tested as competitors for Ld binding. Most of the peptides were relatively inefficient competitors in the Ld system compared with the Ld-restricted reference peptide MCMV pp89 168-176 (13) (Fig. 1). Moreover, the best competitor in the Ld system, peptide Yt3, was the least effective competitor in the Kd system, and inversely, peptide Yt1, which was the most efficient in the Kd system, did not compete at all in the Ld system.

(BALB/c x C57BI/6)F1 and BALB/c Mice Give a Kd-restricted CTL Response when Immunized with Peptide P91A * Y13. Since the Yt1 peptide was the optimal Kd binder and the weakest Ld binder, it was selected as an immunogen. A CTL line (F1-1) obtained from the spleen of a (BALB/c x C57BI/6)F1 mouse immunized with the Y13 peptide was highly active and specific for the immunizing peptide (Fig. 2 A). 11 CTL clones were isolated from the F1-1 line by limiting dilution and all were found to be specific for the Y13 peptide. As shown in Fig. 2 B, three of the CTL clones that express different TCR Vβ chains (and can thus be considered different clones) recognize the Y13 peptide but not peptides corresponding to the P91A + (self) sequence, or the P91A - (turn) sequence. The latter peptide is recognized by the Ld-restricted CTL clone P91.6 (reference 9 and Fig. 2 C). As expected, this control CTL clone did not recognize peptide Yt3 (Fig. 2 C).

All of the CTL clones were shown to be Kd restricted. The results obtained with two independent clones are shown in Fig. 3. The Kd-ICAM-1 double-transfectant target cells were specifically lysed in the presence of the Y13 peptide, whereas control Kd-negative transfectant target cells were not. We had found previously that the expression of ICAM-1 on L cells improved levels of lysis (C. Jaulin and J. Maryanski, data not shown).

We have extended these findings by showing that Y13-specific Kd-restricted CTL could be obtained from the spleens of three additional (BALB/c x C57BI/6)F1 mice, as well as from two BALB/c mice (Fig. 4).

We have recently defined a simple Kd binding motif for synthetic peptides corresponding to four different Kd-restricted antigens (10). The motif is characterized by a Tyr residue in the second position from the NH2 terminus, and a hydrophobic residue of Leu, Ile, or Val at position 9 or 10. The optimal peptide from this study (Y13) contains a Leu residue at position 9 and thus incorporates the complete Kd motif that includes the Tyr residue introduced at position 2 (Table 1).

Using chromatographic techniques, Falk et al. (14) have recently identified putative binding motifs in peptides isolated from several different MHC class I molecules. Consistent with our results defining a Kd binding motif, they have shown that naturally occurring peptides bound to Kd appear to be predominantly 9-mers that have a Tyr at position 2 and a COOH-terminal Leu or Ile residue. Our present study provides further confirmation of the validity of the Kd binding
motif and also suggests that it may be feasible to predict antigenic epitopes using structural motifs, a finding that could prove useful for vaccine development. Finally, the possibility of designing MHC-directed neo-antigens from self sequences (like P91A+) may be helpful for studies on self tolerance and the TCR repertoire.

We thank K. Muhlethaler and J. Guillou for excellent technical assistance, C. Knabenhans for FACS analysis, Drs. L. Luescher and G. Corradin for advice on peptide synthesis, Dr. J. Gagnon for peptide sequencing and for advice on amino acid analysis, and A. Zoppi for her secretarial expertise.

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Received for publication 16 August 1991.

Note Added in Proof: As would be predicted from our recent analysis of other Kd-restricted antigenic peptides (10), we have now found that CTL clones that recognize the 11-mer Y13 peptide also recognize the truncated nonamer sequence IYTQNRRAL, which contains the Leu anchor of the Kd binding motif at the COOH-terminus.

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