

# The identification of tyrosine as a common key residue in unrelated H-2K<sup>d</sup> restricted antigenic peptides

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## Abstract

We have compared the activity of several K<sup>d</sup>- or L<sup>d</sup>-restricted antigenic peptides as competitors in a functional competition assay using cytolytic T lymphocyte (CTL) clones. All of four unrelated K<sup>d</sup>-restricted peptides tested could compete with each other but not with the L<sup>d</sup>-restricted peptide P91A<sup>1</sup>.12–24 (P91A). Moreover, the P91A peptide failed to compete with the four K<sup>d</sup>-restricted peptides. In contrast, another L<sup>d</sup>-restricted peptide [mouse cytomegalovirus (MCMV) pp89 167–176] could clearly compete with both K<sup>d</sup>- and L<sup>d</sup>-restricted peptides. The comparison of a series of modified MCMV pp89 peptides suggested that distinct structural features allow the interaction of the peptide with the two different MHC class I molecules. We showed previously that the competitor activity of two different K<sup>d</sup>-restricted antigenic peptides was reduced substantially upon Ala substitution of the single Tyr residues present in these peptides. We now show a similar effect for two additional K<sup>d</sup>-restricted peptides. Our results thus suggest that Tyr may function as an 'anchor' residue for many antigenic peptides that bind to the K<sup>d</sup> molecule. Molecular modeling of the presumed antigen-binding site of the K<sup>d</sup> molecule revealed the presence of two deep cavities that may be involved in binding peptide amino acid side chains. A model illustrating one possible interaction of a Tyr-containing peptide with the K<sup>d</sup> molecule is presented.

## Introduction

T lymphocytes recognize antigen in the context of specific cell-surface molecules encoded by the major histocompatibility complex (MHC) (1,2). In many cases, T cell antigens can be mimicked by defined natural or synthetic peptides (3,4). Analysis of the structure of the MHC class I molecule HLA-A2 by X-ray crystallography showed that most of the polymorphic residues are located within a groove formed by two  $\alpha$ -helices overlying an eight-strand  $\beta$ -sheet (5,6). This region was therefore proposed as the binding site for antigenic peptides. Based on sequence similarities, an analogous structure and antigen-binding site was proposed for MHC class II molecules (7).

Analysis of the structural requirements for the specific interaction of antigenic peptides with MHC class II molecules has been facilitated by the use of both direct binding assays and

functional competition assays (8–13). Recent studies (14–17) suggest that peptides that are strong binders to the I-A<sup>d</sup> class II molecule share a similar six-residue amino acid sequence 'motif', while those that bind to the I-E<sup>d</sup> allele share a different three- to four-residue motif.

We have described recently a functional competition assay that appears to be useful for the analysis of peptides recognized by MHC class I-restricted T cells (18,19). Cytolytic T lymphocyte (CTL) clones were isolated from DBA/2 mice immunized with syngeneic (H-2<sup>d</sup>) P815 cells transfected with genes encoding the human MHC class I molecules HLA-CW3 or HLA-A24 (20). The HLA-specific CTL clones are H-2K<sup>d</sup>-restricted and recognize untransfected P815 cells incubated with synthetic peptides that correspond to region 170–182 of the CW3 or A24

**Table 1.** Peptides recognized by H-2K<sup>d</sup>- or H-2L<sup>d</sup>-restricted CTL

| Antigen                   | Peptide                           | Sequence <sup>a</sup>   | MHC restriction | Reference |
|---------------------------|-----------------------------------|-------------------------|-----------------|-----------|
| HLA-CW3                   | CW3 170 – 182                     | RYLKNGETLQRA            | K <sup>d</sup>  | 18        |
| HLA-A24                   | A23 170 – 182                     | RYLENGKETLQRA           | K <sup>d</sup>  | 18        |
| Influenza NP              | NP 147 – 158 (R <sup>-156</sup> ) | TYQRTRALVTG             | K <sup>d</sup>  | 23        |
| <i>P. berghei</i> CS      | CS 249 – 260                      | NDDSYIPSAEKI            | K <sup>d</sup>  | 24        |
| P198                      | P198 <sup>-</sup> .14 – 24        | KYQAVTTTLEE             | K <sup>d</sup>  | 45        |
| Influenza HA (A/NT/60/68) | HA 202 – 212                      | RTLYQNVGTYV             | K <sup>d</sup>  | 46        |
|                           | HA 211 – 221                      | YVSVGTSTLNK             | K <sup>d</sup>  | 46        |
| Influenza HA (A/JAP/57)   | HA 523 – 545                      | VYQILAIYATVAGSLSLIAMMAG | K <sup>d</sup>  | 47        |
| P91A                      | P91A <sup>-</sup> .12 – 24        | ISTQNHRALDLVA           | L <sup>d</sup>  | 27        |
| MCMV pp89                 | pp89 168 – 176                    | YPHFMPNTNL              | L <sup>d</sup>  | 37        |

<sup>a</sup>Peptide sequences are given in the one-letter amino acid code: A, alanine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

molecules (18,20). The two HLA peptides could clearly compete with each other for recognition by the appropriate CTL clones. Moreover, reciprocal competition also occurred between the HLA peptides and an unrelated peptide [NP 147 – 158 (R<sup>-156</sup>)] that is recognized by H-2K<sup>d</sup>-restricted CTL specific for the influenza nucleoprotein (NP) (19).

An analysis of variant HLA-A24 peptides as competitors identified Tyr171, Thr178 and Leu179 as critical peptide residues for the interaction of the A24 peptide with the H-2K<sup>d</sup> molecule (21,22). The demonstration that peptide analogs containing the Tyr...Thr-Leu motif with polyproline or polyglycine spacers functioned as efficient competitors for the HLA and NP peptides confirmed the importance of the motif (22). Of the three residues, Tyr171 appeared to be the most crucial for the competitor activity of the HLA-A24 and CW3 peptides (21,22). It was therefore intriguing to find that the Tyr residue of the NP peptide was likewise critical for its competitor activity (22).

As shown in Table 1, all of the peptides thus far defined as antigens for H-2K<sup>d</sup>-restricted CTL contain at least one Tyr residue. In the present study we present a comparison of the competitor activity and specificity of four of these unrelated K<sup>d</sup>-restricted peptides. In addition, we compare two peptides recognized by H-2L<sup>d</sup>-restricted CTL, and analyze the structural basis for the activity of one of them as a potent competitor for both K<sup>d</sup>- and L<sup>d</sup>-restricted peptides. Our results indicate that the amino acid Tyr may function as a strong 'anchor' residue for many peptides that bind to the H-2K<sup>d</sup> molecule.

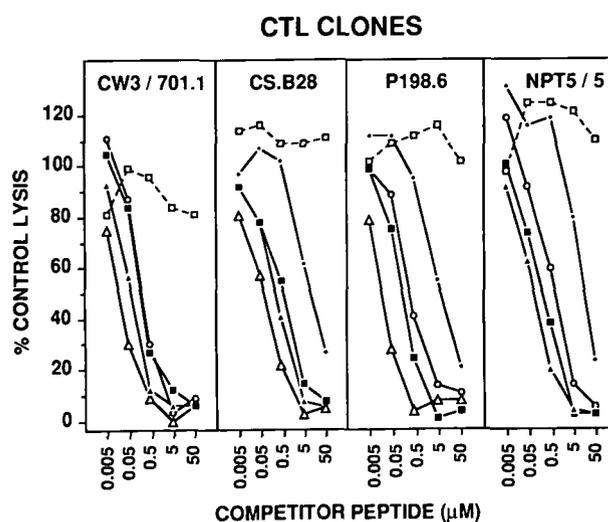
## Methods

### Cells

The isolation and characterization of the CTL clones specific for HLA-CW3 or HLA-A24 (18,20), of CTL clone NP T5/5 specific for influenza NP (23), of CTL clone CS.B28 specific for the *Plasmodium berghei* circumsporozoite (CS) protein (24), and of CTL clones P198.6 and P91.6 (25) specific for different antigenic mutants of the mouse mastocytoma P815 is described elsewhere. The CTL clones are H-2K<sup>d</sup> restricted, except for CTL-P91.6 which is H-2L<sup>d</sup> restricted (26,27).

### Peptide synthesis and purification

The F-moc, t-Bu strategy for solid phase peptide synthesis was used as described by Merrifield (28) and by Atherton *et al.*



**Fig. 1.** Peptides recognized by H-2K<sup>d</sup>-restricted CTL cross-compete with each other. P815 target cells were incubated with the indicated concentrations (final) of competitor peptides corresponding to CW3 170 – 182 (●), CS P.b. 249 – 250 (○), P198<sup>-</sup>.14 – 24 (▲), NP 147 – 158 (R<sup>-156</sup>) (△), AYP<sub>5</sub>TLA (■), and AAP<sub>5</sub>TLA (□). The antigenic peptides were CW3 170 – 182 (0.1 μM) for CTL-CW3/701.1, CS P.b. 249 – 260 (0.01 μM) for CTL-CS.B28, P198<sup>-</sup>.14 – 24 (0.005 μM) for CTL-P198.6, and NP 147 – 158 (R<sup>-156</sup>) (0.002 μM) for CTL-NP T5/5. Control lysis in the absence of competitor peptides was between 30 and 43% for the four CTL clones, and lysis in the absence of peptides was <3%.

(29). HPLC-purified peptides were >90% pure by analytical HPLC. Lyophilized peptides were dissolved in 0.7% sodium bicarbonate buffer or water and further diluted in DME containing 5% FCS. Amino acid analysis confirmed the expected peptide composition.

### Cytolytic assay

P815 cells (10<sup>6</sup>) were labeled with 150 μCi sodium [<sup>51</sup>Cr]-chromate, as described (30), for 1 h at 37°C and washed three times. Labeled targets (2 × 10<sup>3</sup>) in 50 μl volumes were added to wells of V-bottom microtiter plates containing 100 μl volumes of the appropriate peptide diluted in DME supplemented with 5% FCS and HEPES. CTL (6 × 10<sup>3</sup> cells) were added in 50 μl volumes. After a 4 h incubation at 37°C, the supernatants (100 μl)

were harvested for counting. The percentage lysis was calculated as:  $100 \times [\text{experimental} - \text{spontaneous release}] / [\text{total} - \text{spontaneous release}]$ . For competition experiments, the target cells were incubated for 15 min with the competitor peptide (100  $\mu\text{l}$  volume) before addition of a suboptimal concentration of the antigenic peptide (50  $\mu\text{l}$  volume). CTL (50  $\mu\text{l}$  volume) were added for a further 15 min incubation at room temperature. The plates were then incubated for 4 h at 37°C. The percent control lysis was calculated as:  $100 \times [(\% \text{ lysis with competitor} - \text{background lysis}) / (\% \text{ lysis without competitor} - \text{background lysis})]$ . Background lysis represents the percentage lysis of the target cells in the absence of peptides. The relative competitor efficiency of the peptides was calculated for the peptide concentration required to obtain 50% control lysis.

#### Modeling studies

The H-2K<sup>d</sup> model was generated by extension from the coordinates of the HLA-A2 MHC crystal structure (5), assuming that the backbone structures, as well as the coordinates of homologous amino acid side chains, were in essentially identical positions in the HLA-A2 and H-2K<sup>d</sup> molecules. The positions of the remaining side chains of the H-2K<sup>d</sup> molecule were determined using a statistical protocol that finds the most probable side-chain orientation of an amino acid residue in a particular structural context, as determined from observed distributions in a library of highly refined peptide structures (31). The resulting structure was minimized for 100 steps of steepest descent minimization, followed by 1000 steps of conjugate gradient minimization, using the Discover 2.5 program package from Biosym Technologies Inc. (La Jolla, CA).

## Results

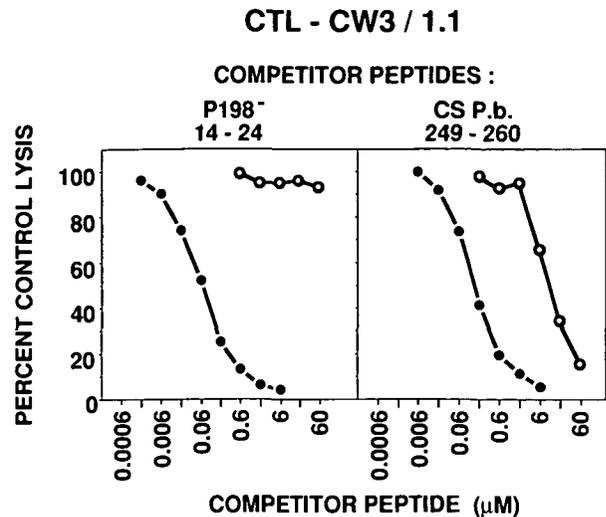
#### Four different K<sup>d</sup>-restricted peptides cross-compete with each other and share a critical Tyr residue

We have demonstrated previously that the unrelated antigenic peptides CW3 170–182 and NP 147–158 (R<sup>-156</sup>) recognized in the context of H-2K<sup>d</sup> could compete with each other for recognition by the appropriate CTL clones (19). We now extend this analysis to two additional K<sup>d</sup>-restricted peptides, CS P.b. 249–260 and P198<sup>-</sup>.14–24. As shown in Fig. 1, recognition of each of the four antigenic peptides could clearly be inhibited by each of the others, as well as by the pentaprolin peptide analog AYP<sub>5</sub>TLA. In contrast, minimal or no inhibition was obtained with the closely related peptide AAP<sub>5</sub>TLA that contains Ala (A) in place of the Tyr (Y) residue.

In view of our previous finding that the activity of both the HLA and NP peptides was decreased by at least 100-fold upon replacement of Tyr residues with Ala, we performed a similar analysis on the P198<sup>-</sup>.14–24 and CS P.b. 249–260 peptides. Both of the latter two peptides also contain single Tyr residues (Table 1). Upon replacement of Tyr with Ala the competitor activity of the CS peptide was reduced by ~80-fold, whereas that of the P198 peptide was reduced by >1000-fold (Fig. 2).

#### Ala-substitutions of peptide P198<sup>-</sup>.14–24 identify Tyr15 as the most critical residue for competitor activity

As described in detail elsewhere (21,22), the three residues Tyr171, Thr178 and Leu179 appear to constitute a binding motif



**Fig. 2.** Comparison of the competitor activity of P198 and CS peptides containing Ala substitutions for Tyr residues. P815 target cells were incubated with the indicated concentrations (final) of competitor peptides corresponding to P198<sup>-</sup>.14–24 or CS P.b. 249–260 containing either Tyr (●) or Ala (○) at positions 15 or 253 in the P198 or CS sequences respectively. The antigenic peptide CW3 170–182 was added at a final concentration of 0.03  $\mu\text{M}$ . Control lysis by CTL-CW3/1.1 in the absence of competitor peptides was 68%, and lysis in the absence of peptides was <3%.

allowing interaction of the A24 peptide with the K<sup>d</sup> restriction molecule. Since the same residues occur, albeit with different spacing, in the P198 sequence, it was of interest to determine whether they might likewise be involved in binding. Peptides that correspond to the sequence of P198<sup>-</sup>.14–24, except for single Ala substitutions, were tested as competitors for the antigenic peptide CW3 170–182 (Table 2). By this analysis, the most critical residue for competitor activity appeared to be Tyr15. The only other residue that upon substitution with Ala decreased competitor activity significantly was Leu22, but the effect was only 4-fold compared with several hundred-fold for Tyr. The Ala-substituted P198 peptides were also tested as antigen for CTL clone P198.6 (Table 2). Ala replacement of the P198 peptide residues Lys14, Tyr15, Gln16, Thr19 and Thr20 reduced antigenic activity by at least 100-fold.

#### The Tyr-containing MCMV peptide pp89 168–176 recognized in the context of H-2L<sup>d</sup> competes with K<sup>d</sup>-restricted peptides

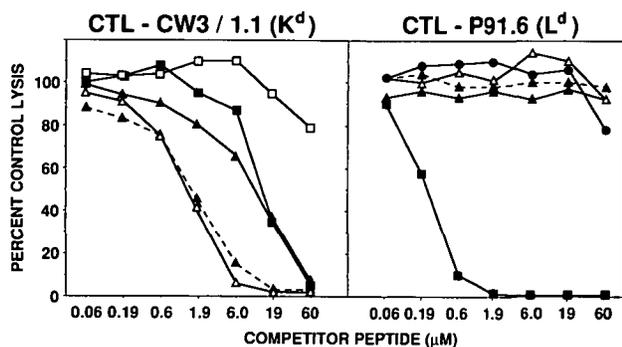
None of the four synthetic peptides including CW3 170–182, NP 147–158 (R<sup>-156</sup>), P198<sup>-</sup>.14–24 and CS P.b. 249–260, that are known to be recognized by K<sup>d</sup>-restricted CTL could efficiently inhibit recognition of the L<sup>d</sup>-restricted peptide P91A<sup>-</sup>.12–24 by the CTL clone P91.6 (Fig. 3). In reciprocal experiments, the P91A peptide was likewise found to be relatively inefficient as a competitor for the four K<sup>d</sup>-restricted peptides (Fig. 3 and data not shown). As a control, we confirmed previously published results (27) demonstrating that a peptide recognized by L<sup>d</sup>-restricted CTL specific for the pp89 protein of the mouse MCMV was a very efficient competitor for the P91A peptide. However, unlike the P91A peptide, the L<sup>d</sup>-restricted MCMV pp89 peptide was also an efficient competitor for the K<sup>d</sup>-

**Table 2.** Relative activity of Ala-substituted P198 peptides as antigens and competitors

| Peptide           | Sequence              | Relative activity <sup>a</sup> as: |         |
|-------------------|-----------------------|------------------------------------|---------|
|                   |                       | Competitor                         | Antigen |
| P198-.14-24       | K Y Q A V T T T L E E | (1)                                | (1)     |
| K14A              | A - - - - -           | 1.0                                | <0.001  |
| Y15A              | - A - - - - -         | <0.001                             | <0.001  |
| Q16A              | - - A - - - -         | 1.4                                | <0.001  |
| V18A              | - - - - A - - - -     | 1.5                                | 1.7     |
| T19A              | - - - - - A - - - -   | 1.9                                | <0.001  |
| T20A              | - - - - - A - - - -   | 1.0                                | 0.01    |
| T21A              | - - - - - A - - - -   | 1.8                                | 1.5     |
| L22A              | - - - - - - A - - -   | 0.27                               | 0.28    |
| E23A              | - - - - - - - A - -   | 2.6                                | 2.7     |
| E24A              | - - - - - - - - A -   | 2.5                                | 4.4     |
| TB6A <sup>b</sup> | K Y Q A V T T T L E E | 1.6                                | 1.4     |

<sup>a</sup>Competitor activity for the K<sup>d</sup>-restricted antigenic peptide CW3 170-182 was assayed with clone CTL-CW3/1.1. Antigenic activity was assayed with the P198-specific CTL clone P198.6. The results are presented as the average values of two separate experiments and are expressed relative to the activity of peptide P198-.14-24 as described in Methods.

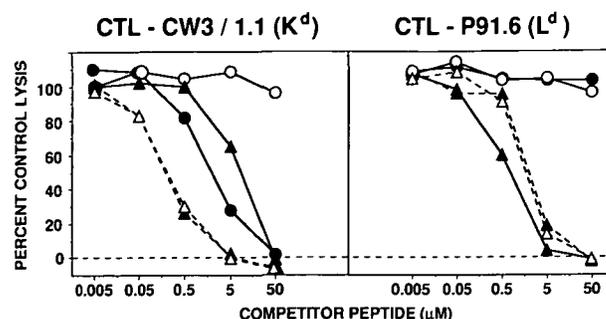
<sup>b</sup>Peptide TB6A represents an independent synthesis of peptide P198-.14-24.



**Fig. 3.** Comparison of peptides recognized by K<sup>d</sup>-restricted or L<sup>d</sup>-restricted CTL as competitors for each other. P815 target cells were incubated with the indicated concentrations (final) of competitor peptides CS P.b. 249-260 (▲-▲), P198-.14-24 (▲-▲), NP 147-158 (R-<sub>156</sub>) (△), CW3 170-182 (●), MCMV pp89 168-176 (■), and P91A-.12-24 (□). The antigenic peptides were CW3 170-182 (0.03 μM) and P91A-.12-24 (0.005 μM) for CTL clones CW3/1.1 and P91.6 respectively. Control lysis by CTL-CW3/1.1 and CTL-P91.6 in the absence of competitors was 78 and 76% respectively. Lysis in the absence of peptides was <3%.

restricted CW3, P198, NP and CS peptides (Fig. 3 and data not shown).

The pp89 peptide 168-176 contains a Tyr residue at the N-terminal end. In view of the importance of Tyr residues in the four different K<sup>d</sup>-restricted peptides that we have analyzed (22; Fig. 2), we considered the possibility that the Tyr residue of the pp89 peptide might be likewise involved in the interaction of the peptide with the K<sup>d</sup> molecule. For the HLA-A24 and -CW3 peptides we had found that the competitor activity improves ~5- to 10-fold upon addition of a single residue (Arg) N-terminal to the Tyr171 residue (21,22). To test for a similar effect in the



**Fig. 4.** The addition of an N-terminal residue to peptide pp89 168-176 improves its competitor activity for the CW3 (K<sup>d</sup>-restricted) peptide, but not for the P91A (L<sup>d</sup>-restricted) peptide. P815 target cells were incubated with the indicated concentrations (final) of competitor peptides AYP<sub>5</sub>TLA (●), AAP<sub>5</sub>TLA (○), pp89 168-176 (▲-▲), pp89 167-176 (▲-▲), and pp89 167-176 (167R) (△). The antigenic peptides were CW3 170-182 (0.05 μM) and P91A-.12-24 (0.01 μM) for CTL clones CW3/1.1 and P91.6 respectively. Control lysis in the absence of competitors was 76 and 80% for CTL-CW3/1.1 and CTL-P91.6 respectively. Lysis in the absence of peptides was <4%.

pp89 peptide, we synthesized pp89 peptides with an additional residue at the N-terminal end. The residue was either Met to correspond to the natural MCMV sequence, or Arg which is the residue preceding Tyr in the HLA 170-182 sequence. Both peptides were at least 20 times more active as competitors for the CW3 peptide recognized by K<sup>d</sup>-restricted CTL, but were several-fold less active as competitors for the P91A peptide (Fig. 4 and Table 3). The experiment presented in Fig. 4 also demonstrates that the polyproline-containing peptide analog AYP<sub>5</sub>TLA that was shown (Fig. 1) to compete with four different peptides recognized by K<sup>d</sup>-restricted CTL, fails to compete with the P91A peptide.

In a further analysis, a series of Ala-substituted pp89 peptides corresponding to region 168-176 was synthesized, and the peptides were compared as competitors for antigenic peptides recognized by either L<sup>d</sup>-restricted or K<sup>d</sup>-restricted CTL clones (Table 3). When tested as competitors for the CW3 peptide recognized by a K<sup>d</sup>-restricted CTL clone, the activity of peptides with Ala substitutions at Tyr168 or Leu176 was reduced by at least 10-fold (Table 3). Substitutions at Pro173 and Thr174 also reduced the competitor activity, but by <5-fold. Similar results were obtained with K<sup>d</sup>-restricted CTL that recognized the P198 antigenic peptide (Table 3).

A different pattern of relative competitor activity was observed when the Ala-substituted pp89 peptides were tested as competitors for the P91A peptide that is recognized by L<sup>d</sup>-restricted CTL (Table 3). In that case the competitor activity of peptides with Ala replacements for the residues Tyr168, Pro169, Pro173, Asn175 or Leu176 was reduced by at least 30-fold. In addition, Ala substitutions for His170 and Thr174 resulted in a 5- to 10-fold reduction in competitor activity (Table 3). Only for residues Phe171 and Met172 was the activity of the substituted peptides comparable to that of the control pp89 binding. The different patterns of relative competitor activity clearly suggest that the apparent capacity of the pp89 peptide to interact with both L<sup>d</sup> and K<sup>d</sup> molecules depends on distinct, but possibly also on partly shared structural features of the peptide.

**Table 3.** Comparison of Ala-substituted MCMV pp89 peptides as competitors for L<sup>d</sup>-restricted and K<sup>d</sup>-restricted peptides

| Peptide | Sequence          | Relative efficiency as a competitor for antigenic peptides <sup>a</sup> |                      |                      |                       |
|---------|-------------------|---|----------------------|----------------------|-----------------------|
|         |                   | P91A(L <sup>d</sup> )   | CW3(K <sup>d</sup> ) | CW3(K <sup>d</sup> ) | P198(K <sup>d</sup> ) |
| N1      | Y P H F M P T N L | ExpA<br>(1)   | ExpA<br>(1)          | ExpB<br>(1)          | ExpC<br>(1)           |
| N2      | -----A            | 0.011   | 0.08 <sup>b</sup>    | 0.06                 | 0.15                  |
| N3      | -----A-           | 0.033   | 0.64                 | 0.70                 | 1.6                   |
| N4      | -----A--          | 0.12  | 0.22                 | 0.23                 | 0.25                  |
| N5      | -----A---         | 0.034   | 0.28                 | 0.25                 | 0.79                  |
| N6      | -----A----        | 0.91  | 0.64                 | 0.58                 | 0.88                  |
| N7      | -----A-----       | 1.0   | 0.5                  | 0.54                 | 0.71                  |
| N8      | ---A-----         | 0.18  | 1.3                  | 1.8                  | 1.9                   |
| N9      | -A-----           | 0.009   | 0.58                 | 0.58                 | 0.6                   |
| O12     | A-----            | 0.015   | 0.06 <sup>b</sup>    | 0.04                 | 0.12                  |
| O11     | -----             | 1.82  | 1.4                  | 2.6                  | 2.3                   |
| N11     | M-----            | 0.32  | 29                   | 108                  | 107                   |
| N12     | R-----            | 0.41  | 22                   | 61                   | 56                    |

<sup>a</sup>Competition for antigenic peptides P91A<sup>-</sup>.12–24 (0.005 μM), CW3 170–182 [0.03 μM (ExpA) and 0.05 μM (ExpB)], and P198<sup>-</sup>.14–24 (0.005 μM) was assayed with CTL clones P91.6, CW3/1.1, and P198.6 respectively. Fifty percent inhibition of lysis for reference peptide N1 as a competitor was obtained at 0.2 μM for the P91A peptide, at 7 μM (ExpA) and 1.4 μM (ExpB) for the CW3 peptide, and at 15 μM for the P198 peptide. The values in the table represent the relative concentration of peptide required to obtain 50% inhibition of lysis, compared with the N1 peptide. Peptide O11 represents an independent synthesis of MCMV pp89 168–176 (N1).

<sup>b</sup>Inhibition was <50%, so the values were calculated at 25% inhibition of lysis.

## Discussion

Our study identifies the amino acid Tyr as a common critical residue for several unrelated antigenic peptides that are recognized in the context of H-2K<sup>d</sup>. The presumed binding of peptides to MHC class I molecules was assessed indirectly in a previously described (18,19) functional competition assay. Complete dose–response curves were obtained for each individual competitor peptide in order to compare the inhibitory capacity of the peptides quantitatively. We would also like to emphasize the advantage of testing competitor peptides in heterologous antigenic systems. By using CTL clones that recognize unrelated antigenic peptides, all variants of a peptide sequence can be compared to the original peptide as competitors, and the analysis is not limited to those that are no longer recognized as antigen. In the present study, we demonstrate that the four unrelated K<sup>d</sup>-restricted peptides CW3 170–182, NP 147–158 (R<sup>-</sup><sub>156</sub>), CS P.b. 249–260 and P198<sup>-</sup>.14–24 all compete reciprocally with each other (Fig. 1) and yet none competes with the L<sup>d</sup>-restricted peptide P91A<sup>-</sup>.12–24 (Fig. 3). Moreover, for all four peptides the competitor activity is reduced by at least 100-fold on substitution of Tyr residues with Ala (22; Fig. 2).

All three of the additional peptides defined (to date) in the literature as antigens for K<sup>d</sup>-restricted T cells also contain at least one Tyr residue (Table 1). The occurrence of Tyr in peptide sequences, however, is not unusual. For example, 44% (25 out of 57) of the peptides listed in one study as reported T cell epitopes (32) contain at least one Tyr residue. In this context, it is interesting to recall that the Tyr-containing peptides NP 50–63 and Y(NANP)<sub>3</sub>NA that are recognized by H-2K<sup>k</sup>- and IA<sup>b</sup>-restricted T cells respectively (33,34), could also compete, although only inefficiently, with K<sup>d</sup>-restricted peptides (18,19). Moreover, the peptides HA 307–318 and pp89 168–176 that are recognized by DR1- and L<sup>d</sup>-restricted T cells respectively

(35–37), clearly compete with the K<sup>d</sup>-restricted peptide CW3 170–182 (Fig. 3; unpublished results). For both of the latter peptides the competitor activity was found to be sensitive to Ala substitution of the Tyr residues (Table 3; unpublished results). It appears, however, that the presence of a Tyr residue within a peptide sequence is not sufficient for K<sup>d</sup> binding activity since, for example, the Tyr-containing peptide horse cyt C 39–53 failed to compete (18). The analysis of a larger panel of peptides will clearly be required to assess the general importance of Tyr residues for K<sup>d</sup> binding activity, and to identify additional features such as peptide length, the relative position of Tyr, and the nature of other amino acid side chains in the peptide sequence that might also influence peptide binding.

For the HLA peptide A24 170–182, the residues Thr178 and Leu179 together with Tyr171 appear to be important for optimal peptide binding. Initially, the putative binding motif Tyr171...Thr178-Leu179 was identified by amino acid substitution of the A24 peptide. Subsequently, we showed that peptide analogs containing the motif and polyproline or polyglycine spacer residues to separate Tyr from the Thr-Leu pair could also function as competitors (22). For example, a pentaproline analog with the sequence AYP<sub>5</sub>TLA was found to be a potent competitor for the K<sup>d</sup>-restricted HLA and NP peptides (22). We now show that the same analog also competes efficiently with two other K<sup>d</sup>-restricted peptides (CS P.b. 249–260 and P198<sup>-</sup>.14–24) (Fig. 1), but fails to compete with the L<sup>d</sup>-restricted peptide P91A<sup>-</sup>.12–14 (Fig. 4).

Another K<sup>d</sup>-restricted peptide, P198<sup>-</sup>.14–24, also contains a Thr-Leu pair of residues, but the spacing with respect to the critical Tyr residue differs from that of the HLA peptides by one residue (Table 1). With the exception of Tyr15, no individual residue in the P198 peptide could be clearly identified as critical for peptide binding in an analysis of Ala-substituted peptides (Table 3). At most, replacement of Leu22 with Ala reduced

competitor activity by ~4-fold. In contrast, Ala substitutions at five of the 11 positions reduced by at least 100-fold the activity of the peptides as antigen for CTL clone P198.6. Similar observations in analyses of peptides recognized in the context of MHC class II molecules have been interpreted as indications of the stringency of the specificity of the T cell antigen receptor for peptide – MHC antigenic complexes compared with that of the MHC molecule for peptides (13).

One interpretation of our results would be that the aromatic side chain of the amino acid Tyr acts as an 'anchor' to secure the peptide within the MHC binding site, and that one or more additional residues provide further stabilization. In this context, it is noteworthy that the putative peptide binding site of the MHC class I molecule contains numerous aromatic residues, some of which are polymorphic (5,6). Moreover, the involvement of a disproportionate number of aromatic residues has been observed in the contact region of antigen – antibody complexes (38,39). According to the model for peptide – MHC interaction proposed by Garrett *et al.* (40) our results suggest that the peptide-binding cleft of the K<sup>d</sup> molecule contains at least one 'pocket' that accommodates Tyr residues of antigenic peptides. In order to assess this possibility, a model of the H-2K<sup>d</sup> MHC molecule was constructed by extension from the coordinates of the HLA-A2 crystal structure (5). Figure 5(a) shows the accessible contact surface of the H-2K<sup>d</sup> model in the region of the presumed antigenic peptide binding site, comprising two sections of  $\alpha$ -helix (residues G56 to N86 of the  $\alpha$ -1 domain, and residues N139 to E173 of the  $\alpha$ -2 domain) superimposed on the anti-parallel  $\beta$ -sheet formed by the N-terminal stretches of the  $\alpha$ -1 and  $\alpha$ -2 domains. As illustrated, there are two pronounced cavities in the molecular surface that offer potential tight binding sites for amino acid side chains of antigenic peptides. Both 'pockets' could conceivably accommodate the aromatic side chain of Tyr residues. Figure 5(b and c) illustrates how the conformationally constrained peptide AYP<sub>5</sub>TLA that is an effective competitor for K<sup>d</sup>-restricted peptides (Fig. 1 and ref. 22) might interact with the H-2K<sup>d</sup> molecule. The model was obtained by docking the peptide to the H-2K<sup>d</sup> model, followed by energy minimization. In the model shown (Fig. 5b and c), the peptide Tyr side chain fits into a deep and elongated pocket and makes contact with MHC Tyr residues 155, 156 and 159, while the peptide Tyr OH group accepts an H-bond from the MHC Gln114 side chain NH<sub>2</sub> group. In agreement with this latter proposed interaction, we have found recently that a variant analog, AFP<sub>5</sub>TLA, that contains a Phe replacement of Tyr was at least 50-fold less active as a competitor than the original AYP<sub>5</sub>TLA analog (J. L. Maryanski and G. Corradin, unpublished results). Interactions with the peptide Leu side chain includes MHC residues W73, S77, T80, Y123, Y84, T143 and W147. It should, however, be emphasized that many alternative binding interactions could also occur. For instance, the peptide might bind in the reverse orientation such that the peptide Tyr side chain could fit into the smaller pocket that is defined in part by K<sup>d</sup> residues V76 and K146 (see Fig. 5a). Other possible interactions might involve changes in the peptide Tyr orientation or even shifts of residues within the binding groove, such as the side chain of MHC Arg97. Nevertheless, the model shown does allow a simultaneous fit of the peptide Tyr and Leu residues into pockets on the MHC surface.

As mentioned above, the MCMV peptides pp89 168 – 176 and



**Fig. 5.** A model for the interaction of peptide AYP<sub>5</sub>TLA with the K<sup>d</sup> molecule. (a) The molecular surface of a model of the H-2K<sup>d</sup> class I molecule in the region of the putative antigen binding site. Note the presence of two pockets on the molecular surface. (b) A van der Waals model of the conformationally restricted peptide AYP<sub>5</sub>TLA docked onto the H-2K<sup>d</sup> surface. The model is one of the many potential alternatives (see Discussion). (c) A side-view section of the bound peptide model illustrating how the peptide Tyr and Leu side chains might fit into the MHC surface pockets.

167–176 appear to bind to both L<sup>d</sup> and K<sup>d</sup> MHC molecules as assessed by our functional competition assay. In BALB/c (H-2<sup>d</sup>) mice this region has been defined as an epitope for L<sup>d</sup>-restricted, but not K<sup>d</sup>- or D<sup>d</sup>-restricted T cells (36). In view of our present results, it might be possible to reveal a K<sup>d</sup>-restricted T cell response by stimulating immune lymphocytes with the longer peptide that was found to be optimal as a competitor for K<sup>d</sup>-restricted peptides. Alternatively, the use of BALB/c-H-2<sup>dm2</sup> mice that lack expression of L<sup>d</sup> (41) might preferentially select for K<sup>d</sup>-restricted T cells. A number of antigenic peptides have been shown to bind to more than one MHC class II molecule, but in some combinations no T cell response can be detected (11,42). The lack of responsiveness in combinations where *in vitro* binding could be demonstrated has been interpreted as evidence for the existence of 'holes in the T cell repertoire' (42).

In agreement with analyses of peptides that bind to more than one MHC class II molecule (15,43,44), our results suggest that both distinct and partially overlapping structural features allow the interaction of MCMV pp89 peptides with both L<sup>d</sup> and K<sup>d</sup> class I MHC molecules. The addition of either Met or Arg N-terminal to the Tyr168 residue improved K<sup>d</sup> competitor activity by at least 20-fold, whereas that for L<sup>d</sup> decreased several-fold. Ala substitutions at two positions (Tyr168 and Leu176) reduced K<sup>d</sup> competitor activity by ~10-fold and those at other positions showed at most only marginal effects. In contrast, L<sup>d</sup> competitor activity was sensitive to Ala substitution of both Pro residues as well as at least four other residues, and for five of these replacements the activity was reduced by >30-fold. One possible explanation for these findings might be that a particular peptide conformation determined in part by the Pro residues is necessary for optimal L<sup>d</sup> binding, whereas a less rigid conformation is required for K<sup>d</sup> binding. We are currently attempting to design structural analogs (22) of the pp89 sequence that might allow a further discrimination between the K<sup>d</sup> and L<sup>d</sup> binding activities.

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## Abbreviations

|      |                           |
|------|---------------------------|
| CTL  | cytolytic T lymphocyte(s) |
| CS   | circumsporozoite protein  |
| MCMV | mouse cytomegalovirus     |
| NP   | nucleoprotein             |

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