

## H-2K<sup>d</sup>-restricted Antigenic Peptides Share a Simple Binding Motif

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### Summary

We have defined structural features that are apparently important for the binding of four different, unrelated antigenic epitopes to the same major histocompatibility complex (MHC) class I molecule, H-2K<sup>d</sup>. The four epitopes are recognized in the form of synthetic peptides by cytotoxic T lymphocytes of the appropriate specificity. By analysis of the relative potency of truncated peptides, we demonstrated that for each of the four epitopes, optimal antigenic activity was present in a peptide of 9 or 10 amino acid residues. A comparison of the relative competitor activity of the different-length peptides in a functional competition assay, as well as in a direct binding assay based on photoaffinity labeling of the K<sup>d</sup> molecule, indicated that the enhanced potency of the peptides upon reduction in length was most likely due to a higher affinity of the shorter peptides for the K<sup>d</sup> molecule. A remarkably simple motif that appears to be important for the specific binding of K<sup>d</sup>-restricted peptides was identified by the analysis of peptides containing amino acid substitutions or deletions. The motif consists of two elements, a Tyr in the second position relative to the NH<sub>2</sub> terminus and a hydrophobic residue with a large aliphatic side chain (Leu, Ile, or Val) at the COOH-terminal end of the optimal 9- or 10-mer peptides. We demonstrated that a simple peptide analogue (AYP<sub>6</sub>L) that incorporates the motif can effectively and specifically interact with the K<sup>d</sup> molecule. Moreover, all of the additional K<sup>d</sup>-restricted epitopes defined thus far in the literature contain the motif, and it may thus be useful for the prediction of new epitopes recognized by T cells in the context of this MHC class I molecule.

T lymphocytes recognize antigen in the form of complexes with polymorphic cell surface proteins encoded by the MHC (reviewed in references 1 and 2). The two major subpopulations of T lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, recognize antigen in the context of MHC class II or class I molecules, respectively. In many instances, it has been demonstrated that specific T cells can recognize antigen in the form of synthetic peptides that correspond to linear sequences of the native antigen (reviewed in references 3 and 4), provided the peptides are presented by cells expressing the appropriate MHC molecules. In recent reports, naturally processed antigens recognized by virus-specific CD8<sup>+</sup> CTL were isolated from infected-cell extracts by chromatographic techniques. In each case the naturally occurring antigen was identified as a short peptide composed of eight or nine amino acid residues (5, 6).

The molecular details of the binding of antigenic peptides to MHC molecules are not completely understood. From an X-ray crystallographic analysis of the HLA-A2 class I MHC molecule, a region for the binding of antigenic peptides has been proposed (7, 8). In that model, the peptide binding site

consists of a groove that contains most of the polymorphic MHC residues. A similar model has been proposed for MHC class II molecules (9). More recently, a comparison of the three-dimensional structure of HLA-A2 with that of a second human MHC class I molecule, HLA-Aw68 (10), revealed that the antigen binding site contains pockets shaped by polymorphic residues. It was suggested that the binding of amino acid side chains from antigenic peptides in these pockets could provide a structural basis for the allele specificity of peptide binding.

One approach for identifying structural features that confer on antigenic peptides their ability to interact with particular MHC molecules has been to compare the relative binding capacity of different peptides to purified MHC molecules. Based on the analysis of a series of MHC class II-restricted antigenic peptides and closely related variants, allele-specific motifs have been proposed for the MHC class II molecules, IA<sup>d</sup> and IE<sup>d</sup>, in the mouse and HLA-DR in man (11-13). Thus far, no allele-specific motifs have been identified for MHC class I-restricted peptides.

We have developed a functional competition assay (14, 15)

to analyze peptides recognized in the context of MHC class I molecules. For the antigenic peptide HLA-A24 (A24) 170–182, we identified three residues (Tyr<sub>171</sub>, Thr<sub>178</sub>, Leu<sub>179</sub>) that appear to allow the peptide to interact with the H-2K<sup>d</sup> class I molecule (16). We demonstrated that a peptide analogue (AYP<sub>5</sub>TLA) of the A24 peptide that incorporates the Tyr<sub>171</sub> (Y) residue and the Thr<sub>178</sub>-Leu<sub>179</sub> (TL) pair of residues separated by a pentaproline spacer was an efficient competitor for at least four different K<sup>d</sup>-restricted peptides (16, 16a).

Of the three residues in the A24 motif, Tyr<sub>171</sub> appeared to be the most critical for efficient competitor activity. In a recent study (16a), we noted that all of the K<sup>d</sup>-restricted CTL epitopes that had been defined in the literature had in common the presence of at least one Tyr residue. In an analysis of variant peptides in which Tyr residues were replaced with Ala, we demonstrated that Tyr was likewise crucial for the optimal competitor activity of three additional K<sup>d</sup>-restricted peptides, namely nucleoprotein (NP)<sup>1</sup> 147-158 (R<sup>-156</sup>), P198<sup>-14-24</sup>, and *Plasmodium berghei* circumsporozoite protein (*P.b.* CS) 249-260. Based on these results, we proposed that Tyr may function as a strong “anchor” for binding peptides to the K<sup>d</sup> molecule.

Our earlier experiments suggested that the relative position of Tyr within the antigenic peptide might also be important. For a series of homologous K<sup>d</sup>-restricted A24 and CW3 antigenic peptides having in common a COOH terminus at position 182, the most potent peptides both as antigens and competitors were those with the Tyr<sub>171</sub> residue at the second position relative to the NH<sub>2</sub> terminus (17). For peptide P198<sup>-14-24</sup>, the Tyr residue is also situated in the second position, and truncation of the NH<sub>2</sub>-terminal residue rendered the peptide 3–10-fold less active as a competitor (Maryanski, J.L., and G. Corradin, unpublished observations). Moreover, the latter peptide (P198<sup>-15-24</sup>) was no longer recognized by P198-specific CTL (18). For the influenza NP peptide NP 147-158 (and variant peptide NP 147-158 [R<sup>-156</sup>] [19]), the Tyr residue is also located in the second position, but in this case it is not known whether a truncated NP peptide having Tyr at the NH<sub>2</sub> terminus would retain antigenic or competitor activity. However, according to a recent report, the naturally presented NP peptide recovered from virus-infected cells appears to correspond to the sequence TYQRTRALV that contains Tyr in the second position (6).

For the synthetic peptides that were used to define the K<sup>d</sup>-restricted CTL epitopes of the *P. berghei* and *P. yoelii* CS proteins (peptides *P.b.* CS 249-260 and *P.y.* CS 277-288), the critical Tyr residues are instead located in the fifth position (20, 21). In the present report, we have assessed whether the position of Tyr within these two homologous malaria CTL epitopes was likewise important. We found that indeed the most active CS peptides tested were 9-mer species with Tyr in the second position. The enhanced activity of the shortened CS peptides prompted us to synthesize COOH-terminally truncated versions of the A24, NP, and P198 peptides. For

each of these peptides, we found a shortened sequence that was considerably more potent, both as an antigen and as a competitor, than the peptides that had originally been defined. Furthermore, a comparison of the optimal sequences for the four antigenic peptides analyzed in this study allows us to suggest the existence of a structural motif that may govern the binding of peptides to the K<sup>d</sup> molecule and hence may be useful for the prediction of new K<sup>d</sup>-restricted antigenic epitopes.

## Materials and Methods

**Cells.** The isolation and characterization of CTL clones specific for the CS protein of *P. berghei* (20), of CTL clones specific for HLA-CW3 (CTL-CW3/1.1 and CTL-CW3/701.1) or HLA-A24 (CTL-A24/10.1) (22), and of CTL clone P198.6 specific for antigenic mutant P198 of the mouse mastocytoma P815 (23) is described elsewhere. CTL clones specific for the CS protein of *P. yoelii* (Y.A8 and Y.B15) were isolated from sporozoite-immune spleen cells of BALB/c mice (21) following procedures described elsewhere (20). CTL-NP-B25 was derived from a BALB/c mouse immunized with influenza peptide NP 147-158 (Romero et al., manuscript in preparation). The latter CTL clones are K<sup>d</sup> restricted. CTL clone P91.6 (23), which recognizes the P91 mutant of P815, is L<sup>d</sup> restricted.

**Peptide Synthesis and Purification.** The Fmoc strategy for solid phase peptide synthesis was used as described by Merrifield (24) and by Atherton et al. (25). 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 (26), 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-bottomed microtiter plates containing 100-μl volumes of the appropriate peptide diluted in DME supplemented with 5% FCS and 10 mM Hepes. CTL (6 × 10<sup>3</sup>) were added in 50-μl volumes. After a 4-h incubation at 37°C, the supernatants (100 μl) were harvested for counting. The percent lysis was calculated as: 100 × [(experimental – spontaneous release)/(total – spontaneous release)]. The relative antigenic activity of the peptides was calculated for the peptide concentration required to obtain 50% of the maximal lysis. For competition experiments, the target cells were incubated for 15 min with the competitor peptide (100-μl volume) before addition of a suboptimal concentration of the antigenic peptide (50-μl volume). CTL (50-μl volume) were added after 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 × [(percent lysis with competitor – background lysis)/(percent lysis without competitor – background lysis)]. Background lysis represents the percent 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.

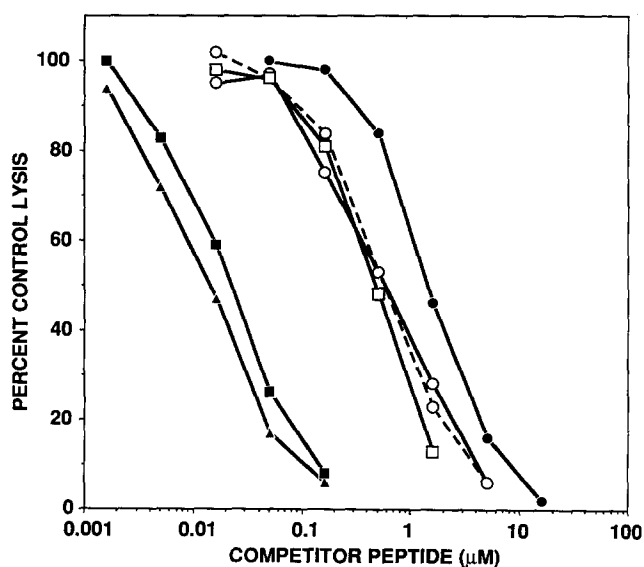
**Competition Assay by Photoaffinity Labeling.** The H-2K<sup>d</sup> photo-reactive probe (27) used in this study was (<sup>125</sup>I)iodo-4-azido salicyloyl (IASA)-YIPSAEK(Biotin)I, a derivative of the peptide *P.b.* CS 253-260. The compound YIPSAEK(Biotin)I was synthesized by reacting Fmoc-YIPSAEKI with *N*-hydroxysuccinimidyl-biotin (Biotin-ONSu) in DMSO in the presence of 1-hydroxybenzotriazole. The reaction mixture was treated with piperidine and the resulting YIPSAEK(Biotin)I derivative was purified by RP-HPLC. Freshly

<sup>1</sup>Abbreviations used in this paper: CS, circumsporozoite; NP, nucleoprotein.

radiiodinated IASA-ONSu (28, 29) was reacted with YIPSAEK-(Biotin)I and the (<sup>125</sup>I) IASA-YIPSAEK(Biotin)I derivative was purified by RP-HPLC. All procedures involving photoreactive reagents were performed under dimmed light. For photoaffinity labeling, cells were washed two times in PBS and resuspended in DME supplemented with Hepes (20 mM), gentamycin (40 μg/ml), and FCS (0.5%). P815 cells (2 × 10<sup>6</sup>) were incubated with the (<sup>125</sup>I) IASA-YIPSAEK(Biotin)I photoprobe (10<sup>7</sup> cpm, sp act, 1,700–2,300 Ci/mmol) at a concentration of 3 × 10<sup>-9</sup> M in the absence or presence of various concentrations of competitor peptides in six-well plates (Costar, Cambridge, MA) at 37°C. After a 5-h incubation at 37°C, the mixtures were irradiated for 10 min at 4°C with UV light (15-W lamp with a maximum emission at 312 nm; Bio-block Scientific, Illkirch, France). The labeled cells were solubilized by adding NP-40 (0.7%) in the presence of iodoacetamide (10 mM), PMSF (1 mM), and leupeptin (10 μg/ml) on ice. The samples were then subjected to immunoprecipitation with the anti-K<sup>d</sup> mAb SF1-1.1.1 as described (27). The samples were analyzed by SDS-PAGE on 10% linear polyacrylamide gels under reducing conditions.

## Results

**Enhanced Potency of *P. yoelii* and *P. berghei* Antigenic Peptides by Truncation at the NH<sub>2</sub> Terminus.** To determine the optimal length of the *P. yoelii* CTL epitope CS 277-288, several NH<sub>2</sub>-terminally truncated peptides were synthesized and tested for their ability to interact with the K<sup>d</sup> molecule in an indirect functional competition assay. The 9-mer and 8-mer peptides, SYVPSAEQI and YVPSAEQI, respectively, were at least 30-fold more potent competitors than the originally defined 12-mers (Fig. 1 and Table 1). Moreover, when tested for recognition by two independent *P. yoelii*-specific CTL clones, the 9-mer peptide was ~100-fold more active than the 12-mer, and the 8-mer was at least 10-fold more active. In contrast, the activity of the intermediate-length



**Figure 1.** Comparison of the competitor activity of NH<sub>2</sub>-terminal truncated *P. yoelii* CS peptides. <sup>51</sup>Cr-labeled P815 target cells were incubated with the indicated concentrations (final) of competitor peptides corresponding to CS *P.y.* residues 277-288 (○), 278-288 (●), 279-288 (□), 280-288 (▲), 281-288 (■), or a peptide from a separate synthesis of CS *P.y.* 277-288 (○-○). The antigenic peptide CW3 170-182 was added at a final concentration of 10<sup>-7</sup> M, and cells from CTL clone CW3/1.1 were added at an E/T ratio of 5:1. Lysis in the absence of competitor peptides was 66%, and lysis in the absence of the antigenic peptide was 2%.

peptides was not significantly improved compared to the original 12-mer (Table 1).

A similar panel of *P. berghei* NH<sub>2</sub>-terminally truncated peptides was also prepared. As had been found for the *P. yoelii*

**Table 1.** Relative Activity of Truncated *P. yoelii* CS Peptides as Competitors and as Antigens

<i>P. yoelii</i> CS Peptide	Sequence	Relative activity as:			
		Competitor*		Antigen†	
		Exp. A	Exp. B	Y.A8	Y.B15
277-288	N E D S Y V P S A E Q I	(1)	(1)	(1)	(1)
278-288	E D S Y V P S A E Q I	0.4	0.4	0.3	0.4
279-288	D S Y V P S A E Q I	0.7	1.2	3.6	3.5
280-288	S Y V P S A E Q I	52	56	68	100
281-288	Y V P S A E Q I	38	33	10	27

\* Competitor activity for the CS *P. yoelii* panel of truncated peptides was assessed with CTL clone CW3/1.1 and antigenic peptide CW3 170-182 (1.5 × 10<sup>-7</sup> M for Exp. A and 10<sup>-7</sup> M for Exp. B). Control lysis in the absence of competitor peptides was 43% (Exp. A) and 66% (Exp. B), and lysis in the absence of antigenic peptide was <4%. 50% inhibition of control lysis with reference competitor peptide CS *P.y.* 277-288 was obtained at 1.2 × 10<sup>-6</sup> M for Exp. A and 5.6 × 10<sup>-7</sup> M for Exp. B. The competitor activity is expressed as the relative concentration of peptide required to obtain 50% inhibition of control lysis, compared to the CS *P.y.* 277-288 peptide.

† Antigenic activity of the panel of CS *P. yoelii*-truncated peptides was assayed with two independent *P. yoelii*-specific CTL clones. For reference peptide CS *P.y.* 277-288, 50% maximal lysis was obtained with 1.5 × 10<sup>-10</sup> M for clone Y.A8, and with 8 × 10<sup>-10</sup> M for clone Y.B15. The antigenic activity is expressed as the relative concentration of peptide required to obtain 50% maximal lysis compared to the CS *P.y.* 277-288 peptide.

**Table 2.** Relative Activity of Truncated *P. berghei* CS Peptides as Competitors and as Antigens

<i>P. berghei</i> CS Peptide	Sequence	Relative activity as:	
		Competitor	Antigen
249-260	N D D S Y I P S A E K I	(1)*	(1)†
250-260	D D S Y I P S A E K I	2	1
251-260	D S Y I P S A E K I	7	3
252-260	S Y I P S A E K I	87	104
253-260	Y I P S A E K I	40	25
249-260	N D D S Y I P S A E K I	(1) <sup>§</sup>	(1) <sup>  </sup>
252-262	S Y I P S A E K I L E	11	15
252-261	S Y I P S A E K I L	24	11
252-260	S Y I P S A E K I	55	85
252-259	S Y I P S A E K	0.3	<10 <sup>-5</sup>
252-258	S Y I P S A E	ND <sup>¶</sup>	<10 <sup>-5</sup>

\* Competitor activity of the panel of NH<sub>2</sub>-terminally truncated CS *P.b.* peptides was assessed with CTL clone CW3/1.1 and antigenic peptide CW3 170-182 (10<sup>-7</sup> M). Control lysis in the absence of competitor was 84% and lysis in the absence of antigenic peptide was 10%. 50% inhibition of control lysis with reference competitor peptide CS *P.b.* 249-260 was obtained at 2 × 10<sup>-5</sup> M.

§ Competitor activity of the panel of COOH-terminally truncated CS *P.b.* peptides was assessed with CTL clone CW3/1.1 and antigenic peptide CW3 170-182 (1.5 × 10<sup>-7</sup> M). Control lysis in the absence of competitor peptide was 66% and lysis in the absence of antigenic peptide was 12%. 50% inhibition of control lysis with reference competitor peptide CS *P.b.* 249-260 was obtained at 1.1 × 10<sup>-5</sup> M.

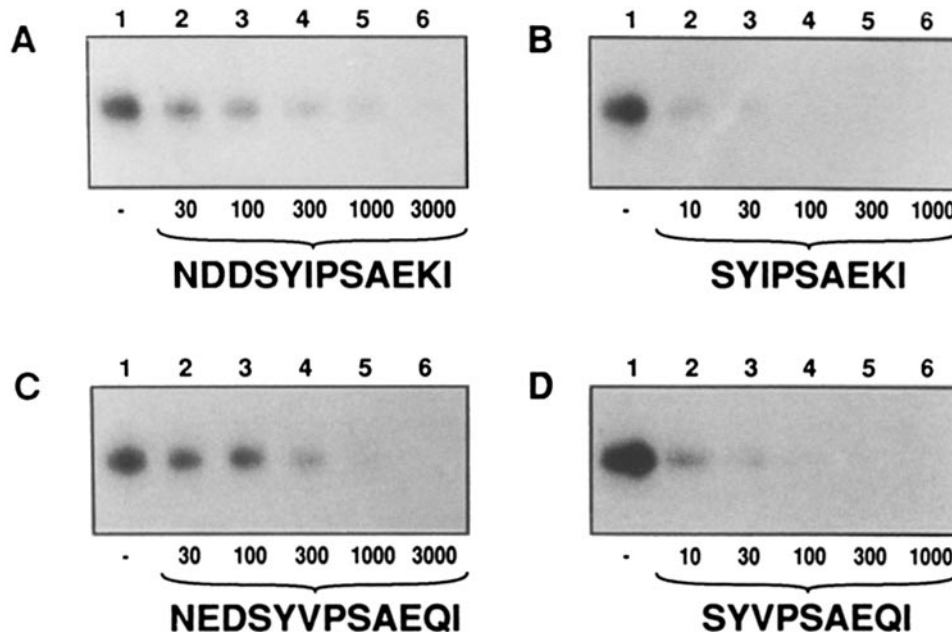
¶ Competitor activity for the CS *P.b.* 252-258 peptide was not defined in this experiment. It was similar to the competitor activity of peptide CS *P.b.* 252-259 in an independent experiment.

† Antigenic activity of the panel of NH<sub>2</sub>-terminally truncated CS *P.b.* peptides was assayed with CTL clone CS.B28. For reference peptide CS *P.b.* 249-260, 50% maximal lysis was obtained with 5 × 10<sup>-10</sup> M.

|| Antigenic activity of the panel of COOH-terminally truncated CS *P.b.* peptides was likewise assessed with CTL clone CS.B28. 50% maximal lysis for reference peptide CS *P.b.* 249-260 was obtained with 2 × 10<sup>-9</sup> M.

sequence, both the 9-mer (SYIPSAEKI) and 8-mer (YIPSAEKI) *P. berghei* peptides displayed a considerably higher competitor activity than the original 12-mer (Table 2). In this case, the increase in potency was at least 40-fold for both

peptides. In terms of recognition, the relative activity of the different length *P. berghei* peptides was similar to the relative competitor activity. However, for CTL clone CS.C1, the increase in antigenic activity of the 8-mer peptide YIPSAEKI



**Figure 2.** Comparison of the competitor activity of 9-mer and 12-mer peptides in a K<sup>d</sup> peptide binding assay based on photoaffinity labeling. P815 cells were incubated with the photoprobe (<sup>125</sup>I)IASA-YIPSAEK(Biotin)I in the absence or presence of the indicated molar-fold excess of peptides CS *P.b.* 249-260 (A), CS *P.b.* 252-260 (B), CS *P.y.* 277-288 (C), and CS *P.y.* 280-288 (D), for 5 h at 37°C. After UV irradiation, the material immunoprecipitated by mAb SF1-1.1.1 (anti-K<sup>d</sup>) was analyzed by SDS-PAGE as described in Materials and Methods.

was only 20-fold (not shown). The two intermediate-length *P. berghei* peptides, DDSYIPSAEKI and DSYIPSAEKI, had intermediate activities both as antigens and as competitors (Table 2).

*No Further Improvement in Potency of the Highly Active Peptide P. berghei CS 252-260 by Extension or Deletion at the COOH Terminus.* The activity of the highly efficient 9-mer peptide *P.b.* CS 252-260 (SYIPSAEKI) could not be improved by adding or deleting one or two residues at the COOH terminus. In fact, as shown in Table 2, the 11-mer peptide SYIPSAEKILE was at least fivefold less active both as an antigen and as a competitor than the 9-mer, and the activity of the 10-mer, SYIPSAEKIL, was decreased by several-fold. Moreover, deletion of the COOH-terminal residue Ile<sub>260</sub> resulted in a 100-fold reduction in competitor activity and the peptide was no longer recognized by the CTL clones tested (Table 2, and data not shown). A similar reduction in activity was observed for the shortest of the peptides tested, SYIPSAE (Table 2).

*The Highly Active 9-mer Malaria Antigenic Peptides Are Also Potent Competitors for a K<sup>d</sup>-specific Photoprobe.* We have recently demonstrated that photoreactive derivatives of the *P. berghei* CTL epitope bind specifically to the K<sup>d</sup> molecule in living cells (27). Moreover, binding of the radiolabeled photoprobe, IASA-YIPSAEK(Biotin)I, to the K<sup>d</sup> molecule could be inhibited by different peptides known to be presented by

K<sup>d</sup>, but not by peptides presented by other class I MHC molecules. It was thus possible to compare the competitor activity of the different length malaria peptides in this direct peptide binding assay. In this experiment, P815 cells (H-2<sup>d</sup>) were incubated with the 12-mer or 9-mer peptides corresponding to the *P. berghei* (NDDSYIPSAEKI and SYIPSAEKI) or *P. yoelii* (NEDSYVPSAEQI and SYVPSAEQI) sequences and the radiolabeled photoprobe at various molar ratios. As shown in Fig. 2, both 9-mer peptides inhibited the photoaffinity labeling of the K<sup>d</sup> molecule at ~100-fold lower concentrations than did the corresponding 12-mers. These results are thus in agreement with those obtained in functional competition experiments.

*Other K<sup>d</sup>-restricted Antigenic Peptides with Tyr Residues in the Second Position Also Become More Potent Antigens and Competitors upon Truncation at the COOH Terminus.* The significant increase in antigenic and competitor activity for the malaria CTL epitopes upon truncation prompted us to ask whether a similar improvement would be observed for other K<sup>d</sup>-restricted antigenic peptides. For this analysis, we chose the previously defined epitopes HLA-A24 170-182, influenza NP 147-158, and P198<sup>-</sup>.14-24. For the reasons outlined in the introduction, the peptide Tyr residues were kept in the second position, and the sequences were truncated at the COOH-terminal ends.

As shown in Table 3, truncated peptides that showed a

**Table 3.** Relative Competitor Activity of Truncated Peptides Corresponding to Three Different K<sup>d</sup>-restricted CTL Epitopes

Antigen	Peptide	Sequence	Relative competitor activity for antigenic peptide:	
			CS <i>P.b.</i> 252-260	CW3 170-182
HLA-A24	A24 170-182	R Y L E N G K E T L Q R A	(1)*	(1)†
	A24 170-179	R Y L E N G K E T L	39	31
	A24 170-178	R Y L E N G K E T	2	2
	A24 171-179	Y L E N G K E T L	2	2
Influenza NP	NP 147-158	T Y Q R T R A L V R T G	(1)*	(1)†
	NP 147-155	T Y Q R T R A L V	45	43
	NP 147-154	T Y Q R T R A L	0.4	0.4
	NP 148-155	Y Q R T R A L V	5.6	2.8
P198	P198 <sup>-</sup> .14-24	K Y Q A V T T T L E E	(1)§	(1)§
	P198 <sup>-</sup> .14-23	K Y Q A V T T T L E	0.65	0.8
	P198 <sup>-</sup> .14-22	K Y Q A V T T T L	4.6	10
	P198 <sup>-</sup> .14-21	K Y Q A V T T T	0.14	0.31

\* Competitor activity for antigenic peptide CS *P.b.* 252-260 (10<sup>-10</sup> M) was assayed with CTL clone CS.B28. The percent specific lysis in the absence of competitor was 79%. Percent lysis in the absence of antigenic peptide was 0%. 50% inhibition of lysis for reference A24 peptide, A24 170-182, as a competitor was obtained at 3.5 × 10<sup>-6</sup> M, and that for reference NP peptide, NP 147-158, was obtained at 5 × 10<sup>-6</sup> M.

† Competitor activity for antigenic peptide CW3 170-182 (3 × 10<sup>-8</sup> M) was assayed with CTL clone CW3/701.1. The percent specific lysis in the absence of competitor was 79% and percent lysis in the absence of antigenic peptide was 4%. 50% inhibition of lysis for reference A24 peptide, A24 170-182, as a competitor was obtained at 5 × 10<sup>-7</sup> M, and that for reference NP peptide, NP 147-158, was obtained at 6 × 10<sup>-7</sup> M.

§ Competitor activity for antigenic peptides CS *P.b.* 252-260 (10<sup>-10</sup> M) and CW3 170-182 (3 × 10<sup>-8</sup> M) was assayed with CTL clones CS.B28 and CW3/701.1, respectively. The percent specific lysis in the absence of competitor was 66% for CTL CS.B28 and 82% for CTL CW3/701.1. Lysis in the absence of antigenic peptides was 3% and 7%, respectively. 50% inhibition of lysis for reference peptide P198<sup>-</sup>.14-24 as competitor was obtained at 8 × 10<sup>-8</sup> M for CTL CS.B28, and at 8.8 × 10<sup>-8</sup> M for CTL CW3/701.1.

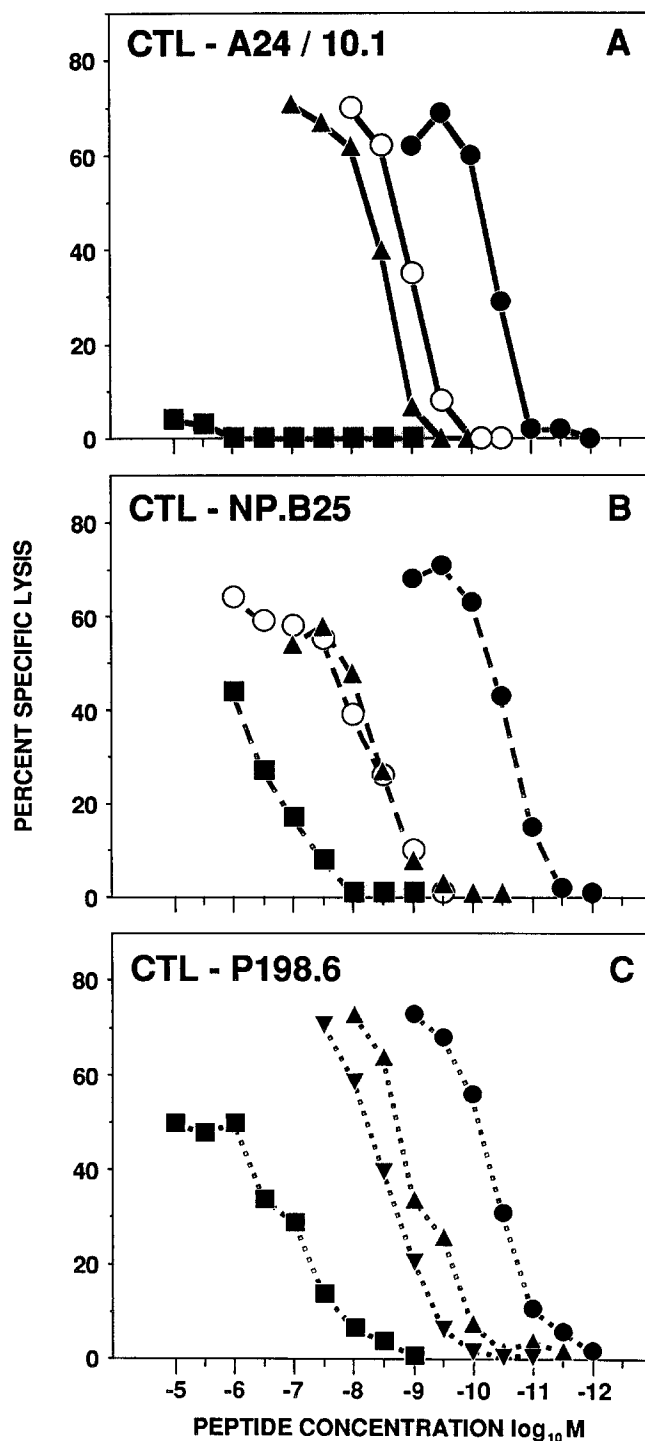
significant increase in competitor activity were found for each antigenic system. The HLA-A24 peptide A24 170-179 was >30-fold more active than the previously described peptide A24 170-182. Similarly, the influenza peptide NP 147-155 was >40-fold more active than the longer peptide NP 147-158, and peptide P198<sup>-</sup>.14-22 was 5–10-fold more active than the original 11-mer. Moreover, the most active truncated peptides in terms of competitor activity were likewise the most potent as antigens for CTL clones of the appropriate specificity (Fig. 3).

*A Peptide Analogue that Incorporates the Tyr...Leu Motif and a Pro<sub>6</sub> Spacer Is a Potent Competitor for K<sup>d</sup>-restricted Peptides.* In a previous study, we demonstrated that the peptide analogue AYP<sub>5</sub>TLA that contains the HLA-A24 residues Tyr<sub>171</sub>, Thr<sub>178</sub>, and Leu<sub>179</sub>, which were identified as possible contact residues for the K<sup>d</sup> molecule, could function as an efficient competitor for K<sup>d</sup>-restricted antigenic peptides (16). A comparison of the truncated peptides found to be optimal as antigens and competitors in this study suggested that important common features might be the presence of Tyr in the second position and the residues Leu, Ile, or Val at the COOH terminus (Table 3). We therefore analyzed a new, simplified series of polyproline analogues containing Tyr and Leu residues separated by a variable number of Pro residues. The peptides were synthesized as pairs, with either Tyr or Ala-Tyr residues at the NH<sub>2</sub> terminus.

Of this series, the most efficient competitor for K<sup>d</sup>-restricted antigenic peptides was peptide AYP<sub>6</sub>L (Table 4). The activity of the AYP<sub>6</sub>L analogue compares favorably with that of two different K<sup>d</sup>-restricted peptides, A24 170-179 and P198<sup>-</sup>.14-22, that contain the "Tyr...Leu" motif, and with the previously described analogue AYP<sub>5</sub>TLA (Table 4). The nearly identical analogues with P<sub>7</sub> or P<sub>5</sub> spacers, AYP<sub>7</sub>L and AYP<sub>5</sub>L, were less active than the AYP<sub>6</sub>L analogue by five fold and 170-fold, respectively. Analogues with P<sub>4</sub> spacers, YP<sub>4</sub>L and AYP<sub>4</sub>L, were completely inactive in this system (data not shown). For each pair of peptides containing a P<sub>5</sub>, P<sub>6</sub>, or P<sub>7</sub> spacer, the peptide with the Ala-Tyr NH<sub>2</sub> terminus was at least 20-fold more active than its counterpart with only Tyr. A very different pattern of activity was observed when the same series of analogues was tested in a competition assay with an L<sup>d</sup>-restricted CTL clone. In this case, none of the analogues was found to be an efficient competitor (Table 4). The analogue for which at least a low level competitor activity could be measured in the L<sup>d</sup>-restricted system, peptide YP<sub>7</sub>L, was ~30-fold less potent than the L<sup>d</sup>-restricted reference peptide, pp89 168-176. Moreover, in contrast to the K<sup>d</sup> system, the analogue AYP<sub>7</sub>L with an additional NH<sub>2</sub>-terminal Ala residue was less potent than its counterpart without Ala.

## Discussion

Individual MHC molecules can bind a large number of different peptides, and yet peptide/MHC interactions appear to be largely MHC allele specific. Demonstrations that peptides recognized in the context of the same MHC molecule can compete specifically and reciprocally suggests that MHC



**Figure 3.** Recognition of HLA-A24, influenza NP, and P198-truncated peptides. <sup>51</sup>Cr-labeled P815 target cells were incubated with the indicated (final) concentrations of A24 peptides (A) 170-182 (—▲—), 170-179 (—●—), 170-178 (—■—), or 171-179 (—○—); NP peptides (B) 147-158 (—▲—), 147-155 (—●—), 147-154 (—■—), or 148-155 (—○—); or P198 peptides (C) 14-24 (—▲—), 14-23 (—▼—), and 14-22 (—●—), and 14-21 (—■—), and cells from CTL clones A24/10.1 (A), NP.B25 (B), or P198.6 (C) at a 3:1 E/T ratio. The assay was terminated after 4 h incubation at 37°C.

**Table 4.** Polyproline-containing Analogues that Incorporate the K<sup>d</sup>-binding Motif "TYR...LEU" Are Potent Competitors

Antigen or analogue	Sequence	Relative activity as competitor for antigenic peptides*	
		P.b. CS (K <sup>d</sup> )	P91A (L <sup>d</sup> )
A24 170-179	R Y L E N G K E T L	2.2	<0.007
P198 <sup>-</sup> .14-22	K Y Q A V T T T L	11.8	<0.007
MCMV pp89 168-176	Y P H F M P T N L	0.11	(1)
Y...TL analogue	A Y P <sub>5</sub> T L A	(1)	<0.007
Y...L analogues	Y P <sub>5</sub> L	0.001	<0.007
	A Y P <sub>5</sub> L	0.024	<0.007
	Y P <sub>6</sub> L	0.13	<0.007
	A Y P <sub>6</sub> L	4.0	<0.007
	Y P <sub>7</sub> L	0.011	0.029
	A Y P <sub>7</sub> L	0.84	<0.007

\* Competitor activity for antigenic peptides P.b. CS 249-260 ( $10^{-10}$  M) and P91A<sup>-</sup>.12-24 ( $5 \times 10^{-8}$  M) was assayed with CTL clones CS.B28 (K<sup>d</sup>-restricted) and P91.6 (L<sup>d</sup>-restricted), respectively. 50% inhibition of lysis for reference peptide AYP<sub>5</sub>TLA (in the experiment with CTL-CS-B28) as a competitor was obtained at  $2.6 \times 10^{-8}$  M, and that for reference peptide pp89 168-176 (in the experiment with CTL-P91.6) was obtained at  $3.5 \times 10^{-7}$  M. The percent specific lysis in the absence of competitor was 61% for CTL-CS.B28 and 76% for CTL-P91.6. Lysis in the absence of antigenic peptides was <4%.

molecules probably contain a single peptide binding site (15, 30–34). In that case, peptides that interact with the same MHC molecules might be expected to share structural features that allow binding to the same ligand (11–13). Our study identifies a simple motif for K<sup>d</sup>-restricted epitopes and also suggests that optimal K<sup>d</sup>-binding peptides are relatively short.

Our experiments document the marked increase in potency for four K<sup>d</sup>-restricted, but otherwise unrelated, CTL epitopes upon reduction in their length to 9 or 10 amino acid residues. The increased potency of the shortened peptides was apparent for their activity as antigens, in CTL recognition assays, and as competitors in functional competition experiments. To assess peptide/MHC binding more directly, we

**Table 5.** H-2K<sup>d</sup>-restricted Antigenic Peptides and Analogues Share a Simple Motif

Antigen*	Peptide	Sequence <sup>†</sup>	Reference
HLA-CW3	CW3 170-182	R <u>Y</u> LKNGKETLQRA	14
HLA-A24	A24 170-182	R <u>Y</u> LENGKET <u>T</u> QRA	14
Influenza NP	NP 147-158	T <u>Y</u> QRTRALV <u>R</u> TG	19
<i>P. berghei</i> CS	CS 249-260	NDD <u>S</u> <u>Y</u> IPSAEK <u>I</u>	20
<i>P. yoelii</i> CS	CS 277-288	NED <u>S</u> <u>Y</u> VPSAEQ <u>I</u>	21
P198	P198 <sup>-</sup> .14-24	K <u>Y</u> QAVTTT <u>L</u> EE	18
Influenza HA (A/NT/60/68)	HA 202-212	R <u>T</u> L <u>Y</u> QNVGTY <u>V</u>	37
	HA 211-221	<u>Y</u> VSVGTSTL <u>N</u> K	37
Influenza HA (A/JAP/57)	HA 523-545	VYQILAI <u>Y</u> ATVAGSL <u>S</u> LIAMMAG	38
MCMV pp89	pp89 167-176	M <u>Y</u> PHFMPTN <u>L</u>	35
Analogue	AYP <sub>5</sub> TLA	A <u>Y</u> PPPPPT <u>L</u> A	16
Analogue	AYP <sub>6</sub> L	A <u>Y</u> PPPPPP <u>L</u>	This study

\* Antigen from which the CTL epitope sequence has been defined. All listed antigenic peptides can be recognized by K<sup>d</sup>-restricted CTL with the possible exception of the peptide pp89 168-176, which has been described as an L<sup>d</sup>-restricted epitope (35), but that is also a potent K<sup>d</sup> competitor (16a), and the two peptide analogues which were designed by us as K<sup>d</sup> competitors.

† The residues corresponding to the putative K<sup>d</sup> binding motif are underlined.

have recently developed an assay based on photoaffinity labeling (27). In this assay, a photoreactive derivative of the H-2 K<sup>d</sup>-restricted peptide *P. berghei* CS 253-260 specifically labels K<sup>d</sup> molecules on living cells. We now show that the 9-mer *P. berghei* and *P. yoelii* peptides (SYIPSAEKI and SYVPSAEQI) inhibit the photoaffinity labeling more efficiently than the corresponding 12-mers. These results strongly suggest that the increased competitor activity of the shorter peptides is indeed due to their higher affinity for the H-2 K<sup>d</sup> molecule. Moreover, the parallel increases in antigenic and competitor activities upon truncation indicates that the different length peptides present similar epitopes to the TCR.

Our observation that the nonamer NP sequence TYQR-TRALV is the optimal antigenic peptide is in agreement with a recent report (6) on the characterization of the naturally processed NP peptide. Interestingly, the naturally processed antigens corresponding to a K<sup>b</sup>-restricted epitope from vesicular stomatitis virus or to a D<sup>b</sup>-restricted epitope from the influenza NP, were also found to be short peptides of eight or nine amino acid residues, respectively (5, 6). Moreover, truncation analysis of peptides corresponding to an L<sup>d</sup>-restricted epitope from the mouse cytomegalovirus identified a 9-mer as the optimal antigenic peptide (35). It is tempting to speculate that the optimal length for class I-restricted epitopes, either in synthetic or natural form, will generally be 8–10 residues.

Comparison of the short (9-mer and 10-mer) peptides that were found to be optimal antigens in the present study suggests that they share a simple structural motif that allows binding to the K<sup>d</sup> molecule. The motif is characterized by a Tyr residue in the second position from the NH<sub>2</sub> terminus, and a hydrophobic residue (Leu, Ile, or Val) at the COOH terminus (position 9 or 10). The latter three amino acids possess large aliphatic nonpolar side chains, and were found to be grouped together in an analysis of the frequencies of amino acid changes among homologous proteins of different species (36). Likewise, Tyr and Phe are included in another exchange group. In this context, we now have evidence that for all four K<sup>d</sup>-restricted epitopes analyzed in this study, the Tyr residue can be replaced with Phe without a major loss of antigenic or competitor activity (our unpublished observations). One might then predict the occurrence of other K<sup>d</sup>-restricted epitopes in which the Tyr residue of the motif would be replaced by Phe.

The actual involvement of the motif in K<sup>d</sup>-binding activity is clearly supported by several lines of experimental evidence. Our earlier results analyzing four unrelated K<sup>d</sup>-restricted antigenic peptides demonstrated that the Tyr residue was indeed critical for optimal interaction with K<sup>d</sup> in that substitution of Tyr with Ala residues resulted in at least a 50-fold reduction in competitor activity (16, 16a). The in-

clusion of a COOH-terminal hydrophobic residue in the K<sup>d</sup>-binding motif was suggested in part by truncation experiments, but its functional importance was demonstrated more directly by amino acid substitution. For instance, the activity of the 8-mer peptide *P.h* CS 253-260 (YIPSAEKI) and of the 11-mer peptide P198<sup>-14-24</sup> (KYQAVTTTLEE) was decreased by at least 100-fold and 10-fold, respectively, upon Ala substitution of the CS residue Ile<sub>260</sub> (Romero et al., manuscript in preparation) and the P198 residue Leu<sub>22</sub> (16a). Similarly, analysis of HLA-A24 170-182 synthetic peptides containing single amino acid substitutions indicated that Leu<sub>179</sub>, which is the COOH-terminal residue of the optimal 10-mer peptide, was important for competitor activity (16). In that analysis substitution of Thr<sub>178</sub> also reduced competitor activity, indicating that in this case Thr<sub>178</sub> may also form part of the K<sup>d</sup>-binding motif.

Evidence for the importance of the K<sup>d</sup> motif comes so far from experiments showing the loss of activity of peptides upon deletion or substitution. We have also developed a more positive approach to demonstrate the function of the motif. This involves the incorporation of the motif in a completely different molecular context. The peptide analogue AYP<sub>6</sub>L, which contains the simple K<sup>d</sup> motif "Tyr...Leu", was found in this study to be a highly active competitor. Its activity was over fivefold higher than the competitor activity of a similar analogue, AYP<sub>5</sub>TLA, which contains the presumed K<sup>d</sup>-binding residues (Y...TL) of the HLA-A24 peptide (16). The activity of both analogues appears to be allele specific and, interestingly, deletion of the NH<sub>2</sub>-terminal Ala within the analogues resulted in at least a 20-fold reduction in K<sup>d</sup> competitor activity (16 and Table 4).

An analysis of the sequences of each of the K<sup>d</sup>-restricted CTL epitopes reported to date reveals that the motif is contained within all of them (Table 5). Moreover, in a recent study (16a), we found that the L<sup>d</sup>-restricted antigenic peptide pp89 167-176 from a mouse cytomegalovirus (MCMV) protein (35), competes not only with L<sup>d</sup>-restricted peptides but also with K<sup>d</sup>-restricted peptides. This MCMV peptide (MYPHFMPTNL) also contains the proposed K<sup>d</sup> binding motif. The occurrence of a common motif suggests that K<sup>d</sup>-restricted peptides might bind in a similar manner to the MHC molecule. A model for the three-dimensional structure of the K<sup>d</sup> molecule (16a) was recently developed based on the atomic coordinates of the HLA-A2 crystal structure (7). Two pronounced cavities were apparent in the region of the K<sup>d</sup> model corresponding to the putative antigen-binding site. These two K<sup>d</sup> "pockets" could well accommodate the two K<sup>d</sup> motif residues (Tyr and hydrophobic residues like Leu, Ile, or Val) and thus provide a structural correlate for the motif.

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