Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11032–11036, December 1991
Immunology

The structure of the antigen-binding groove of major histocompatibility complex class I molecules determines specific selection of self-peptides

(K^b mutants/binding motif)

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Contributed by Stanley G. Nathenson, September 5, 1991

ABSTRACT **We have examined the effect of diversity in the antigen-binding groove of the K^b, D^b, K^{bm1}, and K^{bm8} major histocompatibility complex (MHC) class I molecules on the set of self-peptides they present on the cell surface, by using a procedure we recently developed in our laboratory to isolate endogenously processed peptides bound to MHC class I molecules. We found that such naturally processed peptides are 7–10 amino acids long. A major motif of tyrosine and phenylalanine residues at positions three and five was found for peptides binding to K^b. The availability of K^b mutant molecules K^{bm1} and K^{bm8}, each with localized clustered changes in the antigen-binding cleft, allowed us to probe the effect of such small alterations on peptide selection. We found that such changes in different regions in the antigen-binding groove exert an absolute effect by changing subsets of self-peptides bound to these MHC molecules. In the K^{bm1} mutant, the binding of the characteristic major set of K^b-associated peptides with tyrosine at position three or both positions three and five is abrogated, although this MHC molecule still binds peptides with tyrosine at position seven; the latter peptides also bind to K^b. K^{bm8} shares the major Tyr-3, Tyr-5 peptide set that binds to K^b but does not bind the peptides with tyrosine at position seven. Thus differences in binding selectivity in K^{bm1} and K^{bm8} appear to be the major determinant for the observed alterations in *in vivo* immune responses.**

Cytotoxic T lymphocytes recognize short peptide fragments of intracellularly processed proteins, which are presented on the cell surface by major histocompatibility complex (MHC) class I molecules (1–5). By sampling an intracellular peptide pool, MHC molecules continuously communicate to cytotoxic T lymphocytes the internal status of the cell. T cells are tolerant to MHC molecules carrying peptides derived from cellular proteins and under normal circumstances will only react against cells that present unusual peptides, for instance those derived from viral proteins after infection.

Crystallographic studies have shown that the two membrane distal domains, α_1 and α_2, of MHC class I molecules form a superdomain comprised of two α-helices supported on a platform of eight β-pleated sheets (6, 7). A space bounded by the α-helices and the β-strands has been postulated to be the antigen-binding groove. The enormous polymorphism among the class I molecules is found primarily in amino acid residues that line this antigen-binding groove, and these residues are therefore likely to be involved in the selection of specific sets of peptides that are presented by specific MHC molecules. This would provide a basis for the known diversity in immune responsiveness between animals expressing different MHC alleles.

We have examined this idea by determining the features of the peptides that are actually presented *in vivo*, by using a procedure we recently developed for the isolation of naturally processed peptides from MHC class I molecules (2). In the present study we compared a number of individual major self-peptides derived from cellular proteins naturally processed by a cell and associated with the murine class I molecules K^b and D^b and two K^b mutant molecules: K^{bm1} and K^{bm8}. These mutant molecules were used in this study because they differ from K^b in just a few amino acids in the antigen-binding groove, making it possible to directly probe the effect of such small changes in peptide selection. K^{bm1} carries mutations in three amino acid residues (E152A, R155Y, and L156Y) (8, 9) located in the α-helix of the α_2 domain, which forms one wall of the antigen-binding groove (Fig. 1*A*). Analysis of a K^b model constructed by computer graphic techniques from the coordinates of the human MHC molecule HLA-A2 predicted that the side chains of two of the altered amino acid residues, 152 and 156, project into the groove (10). K^{bm8} has four amino acid changes (Y22F, M23I, E24S, and D30N) (12) of which residues 22, 23, and 24 are located in the β-sheet (Fig. 1*B*). Residue 24 and, to a somewhat lesser extent, residue 22 appear to be located such that they could contact peptides that are bound in the groove, whereas the side chain of residue 23 points away from the groove towards β_2-microglobulin (10, 13).

MATERIAL AND METHODS

Animals. C57BL/6 (H-2^b), B6.C-H-2^{bm1}, and B6-H-2^{bm8} mice were bred in the animal facilities of Albert Einstein College of Medicine.

Cells. EL4 cells are a C57BL/6 T-cell tumor. Con A-stimulated blast cells were prepared from spleen cells after lysis (in 170 mM NH_4Cl, 10 mM Tris·HCl, pH 7.4) of erythrocytes, by culturing for 3 days in RPMI containing 10% fetal calf serum, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 20 μM 2-mercaptoethanol, 2 mM L-glutamine, and Con A at 4 μg/ml at 37°C.

Radiolabeling and Immunoprecipitations. EL4 cells (13×10^8; Fig. 2) or Con A blast cells (5×10^8) harvested on day 3 (Fig. 3) were washed in phosphate-buffered saline, resuspended, and cultured for 4 hr (EL4 cells) or 5 hr (Con A blast cells) at 37°C in Dulbecco's modified Eagle medium (without the amino acid used for labeling) containing 5% dialyzed fetal calf serum, 20 μM 2-mercaptoethanol, penicillin, streptomycin, (Con A at 2 μg/ml, only for Con A blast cells), and the appropriate ^3H amino acid ([^3H]tyrosine, TRK.530; [^3H]phenylalanine, TRK.648; [^3H]leucine, TRK.683, all from Amersham) at 0.1 mCi/ml (1 Ci = 37 GBq). The total incubation

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Abbreviations: ACN, acetonitrile; MHC, major histocompatibility complex; VSV, vesicular stomatitis virus.

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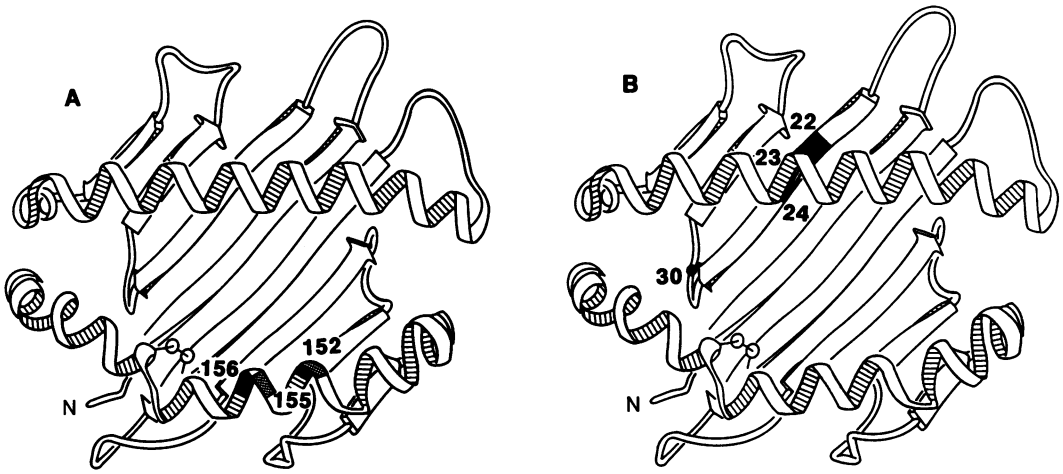

FIG. 1. Top view of the antigenic groove of K^{bm1} (*A*) and K^{bm8} (*B*). The locations of amino acid residues in the mutant molecules that are different from wild type are indicated. Their locations are based on a model of K^b, built on the coordinates of the α-carbon backbone and side chain orientations of HLA-A2 (10), using computer graphics program FRODO (11).

volume was 120 ml. After incorporation of labeled amino acids into the cells, K^b, K^b mutant, and D^b molecules were immunoprecipitated from Nonidet P-40 lysates that were precleared with normal mouse serum essentially as described (2), using monoclonal antibodies B22.249 (D^b specific) and B8.24.3 and Y3 (K^b specific) successively in that order. Immune complexes coupled to protein A-Sepharose beads were washed three times in 10 mM Tris Cl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40, twice in the same buffer without Nonidet P-40, and once in Tris buffer without NaCl.

Peptide Isolations. Antigen–antibody complexes were dissociated by denaturation by boiling for 3 min in 10% acetic acid. The low molecular weight fraction was separated from higher molecular weight material by Centricon-10 (Amicon) centrifugation, which retains <5–10% of peptides of 20–25 amino acids.

Sephadex G-50 Gel Filtration. The low molecular weight peptide fraction, after Centricon centrifugation of the peptides eluted from K^b molecules of EL4 cells, was lyophilized to a volume of 500 μl and size fractionated on a Sephadex G-50 (fine) gel-filtration column in 10% acetic acid. One-milliliter fractions were collected, and the radioactivity was determined in 0.1-ml aliquots.

HPLC. Radiolabeled peptide fractions, after Centricon centrifugation, were analyzed by HPLC on a mini reverse-phase column (RP.8, 100 × 2.1 mm, 5 μm, Brownlee) using Hewlett–Packard equipment. The gradient was made from solution A (0.1% trifluoroacetic acid in doubly distilled H_2O) and solution B (0.1% trifluoroacetic acid in acetonitrile). The flow rate was 250 $\mu l \cdot min^{-1}$, and two-drop fractions were collected. Radioactivity was measured in one-fourth of every fraction.

Partial Radiolabel Sequence Analysis. Peptide peaks from HPLC fractionation were pooled and concentrated by Speed-Vac centrifugation to 30 μl. Peptides were sequenced on an Applied Biosystems 477A pulsed liquid sequencer. Anilinothiazolinone derivatives of amino acid fractions were collected, and the radioactivity was measured in these fractions. In the cases where multiple positions were positive within the run of 10 cycles, these amino acid residues were considered to be located in the same peptide when the signals were of similar yields. When positions showed different yields, the sample was considered to be a mixture of peptides, each with a labeled amino acid residue at a different position.

RESULTS

In a previous study we determined that the length of a naturally processed viral peptide that associated with K^b was 8 amino acids (2). To explore whether, as a rule, peptides that are physiologically processed and bound to MHC molecules are also highly restricted in length, we metabolically labeled cells with [^{3}H]leucine, immunoprecipitated the K^b molecules, and size fractionated the K^b-associated peptides on a Sephadex G-50 gel-filtration column. The radioactively labeled peptides eluted as a discrete peak ranging in molecular size from 0.8 to 1.1 kDa, which corresponds to a peptide length of 7–10 amino acids (Fig. 2). Similar results were obtained with [^{3}H]tyrosine-labeled peptides (data not shown). Because no material of higher or lower molecular weight was recovered, it appears that the antigenic groove of these class I molecules sets a size limit for peptides of 7–10 amino acid residues.

To probe the role of diversity in MHC molecules in peptide selection, we determined the characteristics of the sets of self-peptides bound to K^b and to *in vivo* K^b variants K^{bm1} and K^{bm8}, as well as those bound to the paralogous H-2^b class I product D^b. We analyzed MHC molecules from Con A blast cells that had been metabolically labeled with ^{3}H amino acids to increase the sensitivity of detection of the peptides and to assure that peptides isolated were the result of intracellular protein synthesis. K^{bm1} peptides labeled with [^{3}H]tyrosine, [^{3}H]leucine, or [^{3}H]phenylalanine, when analyzed on reverse-phase HPLC, showed profiles extremely different from

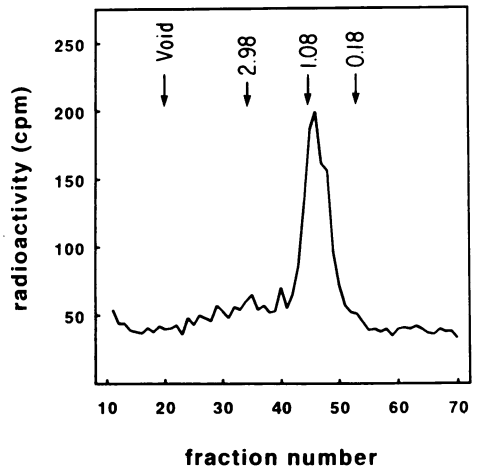

FIG. 2. Size distribution (in kDa) of the [^{3}H]tyrosine-containing peptide fractions isolated from K^b molecules derived from metabolically labeled EL4 cells, determined by Sephadex G-50 gel filtration.

those of K^b (Fig. 3 *A*, *D*, and *G*). In fact, K^{bm1}-bound peptides were as different from K^b-bound peptides as were the peptides associated with D^b (Fig. 3 *C*, *F*, and *I*). The HPLC profiles of K^{bm8}-derived peptides showed the most overall similarities with the K^b peptide profiles, but there were clear differences (Fig. 3 *B*, *E*, and *H*). The K^{bm8} molecule does not bind some of the peptides shown in the K^b profiles (for example, material eluting at retention times of 33.6, 45.1, and 46.5 min.; Fig. 3*B*), but does bind some peptides that are not complexed to K^b (material with a retention time of 35.1 min. in the phenylalanine profile; Fig. 3*H*). It should be noted that the extent of differences observed is somewhat underestimated, because of the resolution of the separation technique. Peptides that coelute on reverse-phase HPLC may or may not necessarily be the same—i.e., they can have different amino acid compositions and lengths. For example, a major [^{3}H]tyrosine-labeled peak at a retention time of 19.3 min and at an acetonitrile (ACN) concentration of 23.5% associated with D^b (Fig. 3*C*) appears to coelute with a K^b-derived peptide, but as shown in Table 1 it has a different primary sequence.

To further characterize the eluted peptides and to explore the existence of possible MHC-specific motifs, we determined the partial sequence of some of the major peaks in the HPLC profiles. When [^{3}H]tyrosine was used in K^b-eluted peptides, we repeatedly found a signal at position three and in some cases also at position five (Table 1). Only two peptides of the major set of [^{3}H]tyrosine-labeled peptides (at retention times of 45.1 and 46.5 min, Fig. 3; and, respectively, 46.0% ACN and 47.3% ACN, Table 1) did not have those tyrosine residues at position three but instead shared a tyrosine at position seven. The D^b peptide profile showed no strict positioning of tyrosine (Table 1). Strikingly, in K^{bm1}, which has changes in two amino acids that are presumed to be pointing into the antigen-binding cleft from the α_2-helix (E152A and L156Y), the binding of the peptide set with a tyrosine at position three was completely abrogated. However, it shares with K^b the two peptides that have tyrosine at position seven (Table 1 and Fig. 3*A*). On the other hand, K^{bm8}, with two amino acid changes in the antigen-binding groove diagonally across from the bm1 site (under the α_1-helix), bound a set of peptides like K^b with tyrosine at positions three and five (Table 1) but did not present the peptides with tyrosine at position seven (Fig. 3*B*).

Analysis of [^{3}H]leucine-labeled peptides revealed that this residue was more frequently present at positions three and eight in K^b. The peptide that was shared by K^b, K^{bm8}, and probably K^{bm1}, eluting at a retention time of 12.3 min (Fig. 3 *D* and *E*) and 17.3% ACN (Table 1), but that was not found in association with D^b (Fig. 3*F*) had the leucine signal at position four. Analysis of the peptides labeled with phenylalanine revealed that among this group positions one, three, and five were most frequently occupied.

In conclusion, we found that the antigenic groove of MHC class I molecules strongly selects peptides that are of restricted lengths and have specific binding motifs. The finding that the profiles of K^b-, D^b-, and K^{bm1}-eluted peptides are so profoundly different demonstrates that *in vivo* presentation is highly specific and that the structure of the antigen-binding groove is a major component in the selection of peptides that are bound to MHC class I molecules. This is also illustrated by the fact that discrete local changes in opposite sides of the antigen-binding groove in K^{bm1} and K^{bm8} abrogate the binding of a different set of K^b-bound peptides.

DISCUSSION

Early *in vitro* studies on peptide binding to isolated MHC class I molecules showed not only an overall low binding efficiency but also a remarkable lack of allelic specificity (14–17). One major reason for this finding may have been that the synthetic peptides used, though containing known antigenic motifs, had arbitrarily chosen lengths that were not optimal, generally >8–10 residues. Recently it was observed that relatively short peptides, of the order of 8–9 amino acids long, bind to class I molecules with much higher affinities

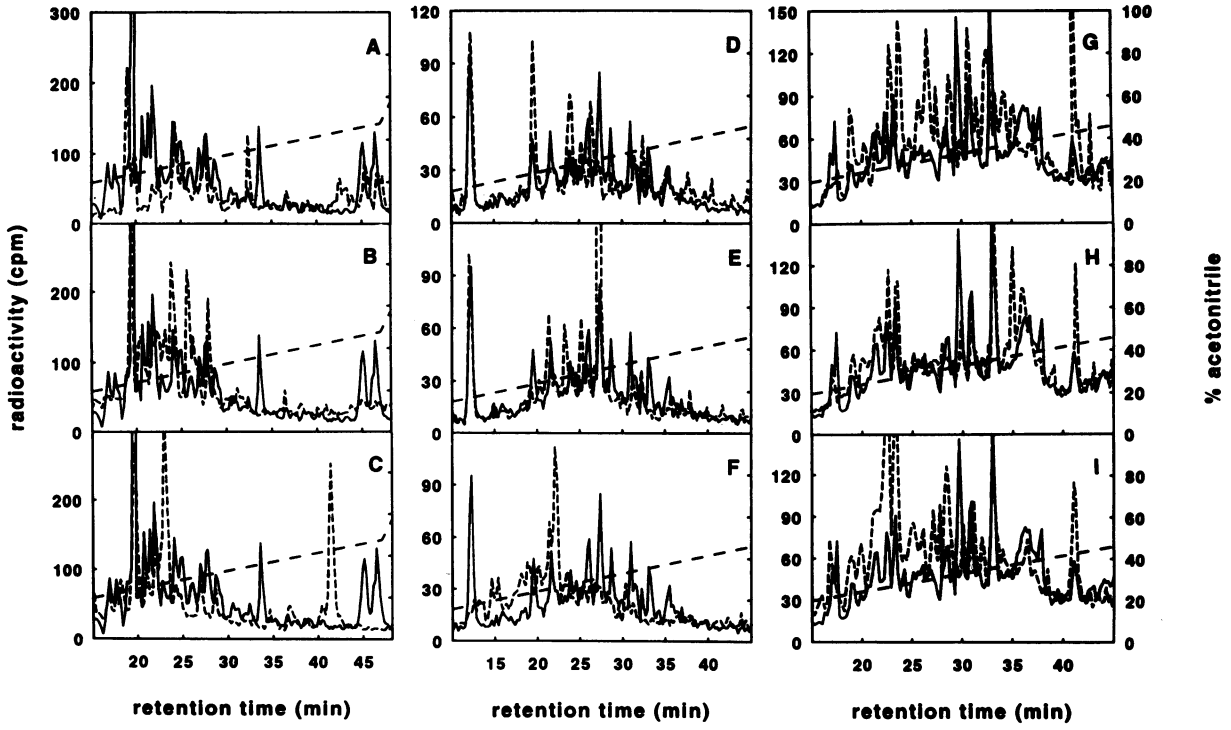

FIG. 3. HPLC profiles of peptide fractions eluted from [^{3}H]tyrosine (*A–C*), [^{3}H]leucine (*D–F*), and [^{3}H]phenylalanine (*G–I*) metabolically labeled Con A blasts. K^b peptides, solid lines; K^{bm1} peptides, dashed lines in *A*, *D*, and *G*; K^{bm8} peptides, dashed lines in *B*, *E*, and *H*; D^b peptides, dashed lines in *C*, *F*, and *I*.

Table 1. Partial radiolabel sequence analysis of peptides eluted from K^b, K^{bm1}, K^{bm8}, and D^b molecules

MHC	% ACN	Sequence cycle number 1	2	3	4	5	6	7	8	9	10	
K^b	23.5	—	—	Y	—	Y	—	—	—	—	—	
	24.2	—	—	Y	—	Y	—	—	—			
	25.0	—	—	Y	—	Y	—	—	—			
	25.3	—	—	Y	—	—	—	—	—	—	—	
	26.4	—	—	Y	—	—	—	—	Y	—	—	
	27.3	—	—	Y	—	Y	—	Y	—	—	—	Mix?
	30.8	—	—	Y	—	—	—	—	—	—	—	
	35.9	—	—	Y	—	—	—	—	—	—	—	
	46.0	—	—	—	—	—	—	Y	—	—	—	
	47.3	—	—	—	—	—	—	Y	—	—	—	
K^{bm1}	23.1	—	—	—	—	—	Y	—	—	—	—	
	23.8	—	—	—	—	Y	—	—	—	—	—	
	27.4	—	—	—	—	—	—	Y	—	—	—	
	28.6	—	—	—	—	Y	—	Y	—	—	—	
	35.0	—	—	—	—	—	Y	—	—	—	—	
	46.1	—	—	—	—	—	—	Y	—	—	—	
	47.4	—	—	—	—	—	—	Y	—	—	—	
K^{bm8}	23.5	—	—	Y	—	Y	—	—	—	—	—	
	25.5	—	—	Y	—	Y	—	—	—	—	—	
	27.5	—	—	Y	—	Y	—	—	—	—	—	
	28.5	—	—	Y	—	Y	Y	—	—	—	—	Mix?
	30.9	—	—	Y	—	—	—	—	—	—	—	
D^b	23.1	—	—	—	—	—	—	—	—	Y	—	
	23.5	—	—	—	Y	—	Y	—	—	—	—	
	25.9	—	—	—	—	—	—	—	—	Y	—	
	26.2	Y	—	—	—	—	—	—	—	—	—	
	26.3	—	—	Y	—	—	—	—	Y	—	—	
	42.7	—	—	—	—	—	—	Y	—	—	—	
K^b	26.3	F	—	—	—	—	—	—	—	—	—	
	27.0	—	—	—	—	F	—	—	—	—	—	
	32.7	—	F	F	—	—	—	—	—	—	—	Mix?
	33.0	—	—	F	—	F	—	—	—	—	—	
	33.5	F	—	—	—	F	—	—	—	—	—	
	35.6	—	—	F	—	—	—	—	—	—	—	
K^b	17.3	—	—	—	L	—	—	—	—	—	—	
	23.5	—	—	—	—	—	—	—	L	—	—	
	25.3	—	—	L	—	—	—	—	—	—	—	
	27.2	—	—	—	—	—	—	—	L	—	—	
	28.7	—	L	L	—	—	—	—	L	—	—	Mix?
	32.0	—	—	—	—	—	L	—	—	—	—	
	33.9	—	—	L	—	—	—	—	—	—	—	
K^{bm8}	17.3	—	—	—	L	—	—	—	—	—	—	
	30.5	—	—	—	L	—	—	—	—	—	—	

Amino acid residues are indicated by the single-letter code. When multiple signals of similar yield were found within the run of 10 cycles, the amino acid residues were considered to be located in the same peptide. When they were of different yields, the sample was considered to be a mixture and where this was likely it is indicated in the table as "Mix?".

than longer peptides (18, 19). In this paper and in previous work, it has been demonstrated that this size coincides with the length of endogenously produced peptides (2, 20).

Peptides should assume a specific conformation in the antigen-binding groove, and the availability of space for main chain and side chains and the presence of charged, polar, and hydrophobic amino acids at certain positions would determine whether a given peptide can fit (21). The fact that positions three and five are frequently occupied by phenylalanine or tyrosine (and leucine at position three) in K^b-bound peptides suggests that these amino acid residues with their similar side chains (i.e., aromatic ring and a large hydrophobic group) are important for interaction of the peptide with the MHC antigen-binding groove and use the same "pockets" (21, 22) in the K^b groove. In contrast to the K^b motif, in peptides binding to D^b we found no dominant presence of tyrosine at any one particular position. Therefore tyrosine does not seem to play a role in peptide binding to this MHC molecule.

From amino acid replacement studies of a K^d-restricted peptide, Maryanski *et al.* (23) also found that a tyrosine residue, at position two, played an important role in peptide binding to K^d, another K allelic product. Furthermore, tyrosine could be replaced with phenylalanine and the peptide would retain binding capabilities. We have found in peptide competition assays that, in the K^b-restricted vesicular stomatitis virus (VSV) peptide (N52-59), Tyr-3, Tyr-5, and Leu-8 are the important anchor residues (K. Shibata, M. Imarai, G.M.v.B., S. Joyce, and S.G.N., personal communication), a finding correlating with the major binding motif in the natural self-peptides analyzed in the present study. From an alternate approach using sequence data of unfractionated peptide mixtures eluted from MHC molecules, Falk *et al.* (20) postulated that for different MHC alleles there were specific peptide-anchoring residues. This conclusion was based on the presumption that a significant signal for an amino acid residue at a certain sequence cycle should indicate the occurrence of that residue in a considerable subset of peptides. For K^b they concluded that tyrosine or phenylalanine at position five and leucine at position eight were major anchor residues and that a tyrosine at position three was a minor anchor. Also they found no major contribution of tyrosine amino acids as an anchor residue in D^b-binding peptides. Despite the subtle difference in the predicted contribution of tyrosine at position three, which in our K^b-isolated peptides seemed more abundant than tyrosine at position five, the results of the two approaches are clearly similar.

By separating the endogenous peptides before partial structural characterization, our procedure has allowed us to identify certain individual peptides that do not have the major binding motifs. Examples are the two late eluting peptides in the K^b profile with tyrosine at position seven (retention times of 45.1 and 46.5 min, Fig. 3*A*; 46.0% ACN and 47.3% ACN, Table 1) and the early eluting peak in the leucine profile (retention time of 12.3 min, Fig. 3*D*; 17.3% ACN, Table 1) of K^b, K^{bm1}, and K^{bm8} in which tyrosine is completely absent. Thus while for most self-peptides isolated from K^b our data have suggested an important role for tyrosine and phenylalanine at positions three and five, alternative motifs also exist. If only major binding motifs were considered and used for the prediction of antigenic peptides in proteins, certain peptides will be overlooked.

The HPLC profiles of MHC eluted peptides show that as few as two neighboring changes in the amino acid residues lining the antigen-binding groove in K^{bm1} and K^{bm8} profoundly alter the set of endogenously bound peptides. The altered binding of peptides containing a particular motif by the mutated K^{bm1} and K^{bm8} molecules is consistent with the idea that local steric or charge changes alter the geographical features of the cleft, which determine the sequence of the peptides that are bound. Of the two amino acid changes in the antigen-binding groove in K^{bm1}, the change E152A represents a change in charge at a position in the α_2-helical wall of the cleft, distal from the floor of the groove and more toward the region where the T-cell receptor may interact with the MHC molecule. The L156Y change is, based on our model, at a position deep in the groove close to the β-sheet and might alter the local architecture of the K^b groove by obstructing a small pocket defined as the 133 pocket (10).

At the biological level, it is relevant that the K^{bm1} mutant mouse, which only differs from C57BL/6 wild-type mice in this *H-2K* gene product, behaves very differently in the cytotoxic T lymphocyte response against K^b-restricted viruses (24). That the endogenous peptide set binding to K^{bm1}

is extremely different from peptides binding to K^b gives a plausible explanation of this observation—namely, that in general K^{bm1} will present different peptides than K^b. For instance, the major K^b-restricted peptide of VSV contains the most frequent binding motif that was found for K^b-binding peptides: tyrosine at positions three and five and leucine at position 8 (ref. 3; K. Shibata, M. Imarai, G.M.v.B, S. Joyce, and S.G.N., personal communication). This contains the motif (Tyr-3, Tyr-5) of the major set of peptides for which binding was abrogated in K^{bm1}. K^b-restricted VSV-specific cytotoxic T lymphocytes do recognize the peptide in the context of K^{bm8} (25), which is consistent with the fact that this mutant binds peptides with the Tyr-3, Tyr-5 motif. Also, in other class I molecules (HLA), changes in residues 152 and 156 (the bm1 positions) are found to profoundly alter peptide presentation to virus-specific as well as allospecific CTL, especially when combined changes of residues 152 and 156 occur (26–32).

Although the special characteristics of the antigen-binding groove exert a strong selection on the peptides presented, other possible factors might influence peptide presentation, such as the specificity of the cytosolic protein degradation mechanism and a peptide transport system that translocates cytosolic peptides into the endoplasmic reticulum (33–38) where binding to class I molecules is thought to occur (39). Further, the relative abundance of a certain peptide in the total available pool may also play a role in the selection of peptides that will eventually bind to MHC molecules (40). We found that in the VSV nucleoprotein there is a second amino acid stretch (residues 70–77; Asn-Ser-Tyr-Leu-Tyr-Gly-Ala-Leu) (41) containing the major Tyr-3, Tyr-5, Leu-8 motif. This peptide is not present as a major virus-specific peak in K^b peptide profiles of VSV-infected cells. Thus, besides a specific binding motif, other factors could influence whether a peptide is ultimately presented. The presence of amino acid residues elsewhere in the peptide could interfere with binding, or certain structural features in the regions flanking the peptide in the protein could interfere with processing and transport. Interestingly, in recent reports it was suggested that the presence of one antigenic peptide in a protein can affect the presentation by MHC class I molecules of a second antigenic peptide present in the same protein (42, 43).

In summary, we have described the isolation and characterization of endogenous peptides isolated from class I molecules and shown that they have a restricted length. Discrete clustered changes in the MHC antigenic groove can profoundly affect the binding of subsets of peptides that share certain amino acid motifs, suggesting that these residues are involved in anchoring the peptide to a specific MHC molecule. Understanding the rules for binding of peptides by identification of amino acid motifs in peptides bound to specific MHC molecules, as well as determination of binding affinities, will be crucial in the search for antigenic peptides in viruses and in the development of peptide vaccines. Our procedure of isolating individual peptides from MHC before sequencing also has the potential to contribute to the identification of the relevant peptides in autoimmune diseases or tissue graft rejection.

We thank R. Angeletti for her invaluable discussions; the Laboratory for Macromolecular Analysis for the use of their facility; J. Sacchettini, M. Scharff, S. Joyce, and M. Imarai for carefully reading the manuscript; and R. Spata for excellent typing. This work was supported by National Institutes of Health Grants 5R37 AI 07289, 2 PO1 AI-10702, 2P30CA13330, and 1R01 AI 27199 and the Irvington Institute for Medical Research.

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