Polymorphisms in Pockets of Major Histocompatibility Complex Class I Molecules Influence Peptide Preference
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Summary
The set of peptides that is bound by a given major histocompatibility complex class I product can be described by one or two properly spaced anchor residues, and two properly spaced peptide termini, approximately 8–10 residues apart. Using radiolabeled peptide libraries, we examined whether mutations in those "pockets" in class I Kβ molecules that do not seem critically involved in the interaction with the peptide anchor residues, do exert an effect on the set of preferred peptides. We find that mutations in all the pockets found in the structure of Kβ have a significant effect on the peptide preference of the molecule, and their recognition by cytotoxic T cells. Alterations in substrate specificity are also observed for mutations involving residues that interact with main chain atoms in both peptide termini. These findings challenge a static view of the interaction of peptide termini with their respective pockets in the class I molecule, and imply a role for the minor pockets in peptide selectivity.

The isolation of naturally processed peptides as bound by different class I products has revealed that for each allele these peptides adhere to a "motif" (1, 2), characterized by the (almost) exclusive occurrence of one or a few amino acids at one or two positions within the sequence. For Kβ molecules, the prime anchor residue seems to be Tyr/Phe-5 (1) (for nonamer peptides Tyr/Phe-6 [3]). At other positions in these peptide motifs, a much greater variety of amino acids is observed, suggesting that either the side chains at these positions contact solvent, or that a much greater variety of side chain residues is tolerated by the regions in the class I molecule that combine with them.

Resolution of the crystal structure of Kβ molecules, complexed with either the Sendai-N- or vesicular stomatitis virus (VSV)-N-derived Kβ-restricted peptides (4–6) showed that the Tyr-5 side chain docks in one of the pockets found in class I molecules that has been implicated in peptide binding (7, 8). Apart from the interaction of the Tyr residue with pocket C, and the peptide termini with their respective pockets, other side- and mostly main chain atoms interact to some extent with amino acids forming the minor pocket D and other residues lining the peptide binding groove (4–6). (We use the prefix ‘major’ for pocket C in Kβ, since it seems the prime determinator of peptide specificity for this allele. Pockets B, D, and E, which have a less defined role in peptide preference of Kβ molecules are called ‘minor pockets’. Pockets A and F are considered separately, since they interact (at least in part) with invariant parts of the peptide chain, and were therefore thought to be of limited importance to class I specificity [4–7].) However, it is hard to assess the relative importance of the observed interactions based on structural data only.

Naturally occurring class I mutants (such as Kbmn), in which only amino acids in supposedly minor pockets, or the pockets that combine with the peptide termini, are different from those in Kβ, appear in some cases unable to present any of the known Kβ-restricted epitopes (9). A critical role for these pockets in the determination of peptide binding specificity is therefore likely, but direct proof is lacking.

To investigate more directly the role of these minor pockets, and those regions in the molecule that combine with the peptide termini in the determination of substrate specificity, we have confronted a series of Kβ mutants with a radiolabeled peptide library (10), and monitored peptide selection by two-dimensional display of class I captured peptides. We find that alterations throughout the class I structure have a major effect on the set of peptides bound, not only in those regions of the molecule that combine with variable parts of the peptide, but also in those parts that interact with main chain atoms of the peptide termini.
Materials and Methods

Generation of H-2 K\(^b\) Mutants and Cell Culture. Class I variant genes were prepared by site-directed mutagenesis of the K\(^b\) gene (11–16). The wild-type K\(^b\) molecule, and K\(^b\)163A are encoded by exons 1–8 of K\(^b\). All other variants are composed of exons 1–3 of K\(^b\) and exons 4–8 of the L\(^d\) gene, and can be immunoprecipitated using a mAb against the α\(^d\) domain of L\(^d\) (17, 18). Sequences of exons that were mutagenized were confirmed by sequencing. Class I genes were cotransfected with the herpes simplex thymidine kinase gene in L cells. Cells surviving HAT selection were screened for expression of the transfected genes by FACS\(^\text{®}\) (Becton Dickinson & Co., Mountain View, CA) using 28-14-85 or B8-24-3. All mutants are named after the residue involved, followed by the single letter code of this amino acid in the mutant. LPS blasts were obtained from spleen cells by a 4-d culture of cells in the presence of LPS (30 µg/ml LPS-B; Difco Laboratories, Inc., Detroit, MI).

Peptide Labeling and Binding to Class I Molecules. The peptide mixture “poly8” was synthesized by standard t-boc chemistry, and has been described previously (10). Peptides were labeled by chloramine-T catalyzed iodination (19), and used within 24 h after iodination. Before peptide binding experiments, cells were cultured at 26°C for 2 d in Hepes-buffered DMEM/10% FCS.

Cells were incubated with 1 µM of the radiolabeled poly8 mixture at 22°C, for 2.5 h in Hepes-buffered DMEM without serum (total vol 5 ml). Subsequently, cells were washed, and lysed by Triton X-100 lysis buffer (10). After removal of cell debris, and preclearing of lysates, class I molecules were immunoprecipitated, using anti-exon-8 (20) for proteins containing the H-2 K\(^b\) α\(^b\) domain, and 28-14-85 (17, 18), for proteins containing the H-2 L\(^d\) α\(^d\) domain. Immunoprecipitates were washed three times. Class I-associated peptides were liberated by two cycles of TFA extraction (200 µl of 1% TFA for 20 min at room temperature). Extracts were filtered over a 0.22-µm filter and lyophilized. Peptides were dissolved in 0.05% TFA and analyzed by reverse phase HPLC on a column (model C18; Millipore Corp. Waters Chromatography, Milford, MA). Buffers used were: A, H\(_2\)O/0.05% TFA; B, acetonitrile/0.05% TFA. Elution profiles were performed for: 0–3 min, 95–95% A; 3–5 min, 95–85% A; 5–23 min, 85–60% A; 23–25 min, 60–35% A; 25–27 min, 35–35% A; and 27–33 min, 35–95% A, at a flow rate of 1.7 ml/min. 0.3-min fractions were collected, and fractions 41–65 were lyophilized. Fractions were dissolved in 6 µl H\(_2\)O and 3 µl of each sample was applied to silica gel 60 plates (EM Science, Gibbstown, NJ). TLC was performed in freshly made N-butanol/H\(_2\)O/pyridine/acetic acid 2:1:0.75:0.25.

Plates were exposed to Kodak X-AR5 films at –70°C. Note that TLC were exposed until the central part of the autoradiogram was of approximately equal intensity (with the exception of Kb97R, for which there are no peptides to be found in this region), therefore only alterations in the relative intensity of spots are relevant.

The set of peptides selected by all mutants was displayed by two-dimensional HPLC/TLC at least once and by one-dimensional HPLC in an independent experiment for confirmation. CTL Assay. The CTL clone 33(21) was provided by Jim Sheil (University of West Virginia, Morgantown, WV) and was maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO)/10% FCS/5% Con A supernatant. The CTL clone bm8 anti-B10.4-2.23 was a kind gift of Jeffrey A. Bluestone (University of Chicago, Chicago, IL) and was maintained in IMDM (Sigma Chemical Co.)/10% FCS. The C3H anti-C3H.SW T cell line was prepared by mixing 5 × 10\(^6\) irradiated C3H.SW splenocytes with 5–7 × 10\(^6\) C3H splenocytes. Responder cells were tested on day 5 for activity, then restimulated with irradiated splenocytes on day 7. Thereafter, cells were maintained in IMDM (Sigma Chemical Co.)/10% FCS containing 10–20 U/ml IL-2, and restimulated weekly. L cells expressing transfected class I molecules were used in a standard cytotoxicity assay. Briefly, target cells were labeled with 1.0 mCi Na\(^25\)Cr per 10\(^5\) cells for 45 min at 37°C. 5 × 10\(^3\) target cells were subsequently incubated with CTL, in a total vol of 200 µl (in the presence of 5 µM VSV-8 for clone 33). Plates were incubated at 37°C for 8 h, centrifuged, and 150 µl samples were removed for γ-spectroscopy. Maximal release was determined by the addition of 10% Triton X-100 (Sigma Chemical Co.). Spontaneous release was determined by incubating target cells for 8 h at 37°C in the absence of CTL. Specific lysis was calculated as: (Experimental release – spontaneous release)/(maximum release – spontaneous release).

Results and Discussion

A synthetic peptide library comprising 432 different peptides, based largely on the sequences of the K\(^b\)-restricted Sendai-N and VSV-N epitopes, was produced by the simultaneous coupling of different amino acids and has been described ([10], Fig. 1). For peptide selection experiments, peptides in this library were labeled by iodination of Tyr residues. Although iodination of the Tyr anchor residue in these peptides could conceivably alter their binding properties, such an effect would likely be similar for all mutant class I products, and should not hamper a comparison of their peptide preference. To examine the effect of naturally occurring mutations in the different pockets, we generated a series of mutants of the K\(^b\) molecule (Table 1, [11, 22]). L cells were transfected with genes encoding K\(^b\) molecules and the various mutants, and expression of the transfected products was determined by FACS\(^\text{®}\) analysis (see legends to Fig. 3). To ascertain that the binding properties of K\(^b\) molecules were independent of the cell type in which they were expressed, we first established that the sets of peptides captured by K\(^b\) molecules as present in B6 LPS blasts, and when expressed in L cells are indeed identical (Fig. 2). The reproducibility of this assay has been shown previously by a comparison of the set of peptides captured by K\(^b\) molecules on LPS blasts of three different mouse strains (10).

Most of the class I molecules we examined are hybrids between the α\(^b\) and α\(^d\) domains of K\(^b\) and the α\(^d\), transmembrane and cytoplasmic domains of L\(^d\). However, comparison

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**Figure 1.** Sequence of the poly8 peptide library. Sequences are given in single letter code. Note that both the octamer version of the Sendai-N epitope (for which a nonamer with a 1-amino acid extension at the NH\(_2\) terminus is optimal for binding to K\(^b\)), and the VSV-N epitope are represented once in this mixture.
Table 1. Amino Acid Substitutions in the Various K^b Mutants

| Mutant | Position and nature of substitution | Natural occurrence | Pocket |
|--------|-------------------------------------|--------------------|--------|
| 5A.44  | wt K^b plus α3 tm^* and ct of L^d | NA                 | NA     |
| K^b163A | 163: T-A                            | K^b^m90, K^a1, D^e, D^f, and others | A      |
| K^b5M  | 5: L-M                              | K^a1, D^e, D^f, L^d, and others | A      |
| K^b45V | 45: Y-V                             | D^e and D^f        | B      |
| K^b24S | 24: E-S                             | K^b^m9, K^a1, D^e, and many others | B      |
| K^b73W | 73: S-W                             | K^a1, K^a1, D^e, and others | C      |
| K^b74S | 74: F-S                             | K^a1, K^a1, and K^a1 | C      |
| K^b155Y| 155: R-Y                            | K^b^m9, K^a1, and L^d | D      |
| K^b152A| 152: E-A                            | K^b^m9, K^a1, D^e, D^f, and others | E      |
| K^b97R | 97: V-R                             | K^a1, K^a1, K^a1, and others | C and E|
| K^b77S | 77: D-S                             | K^b^m9, K^a1, D^e, and others | F      |

Amino acid substitutions in K^b mutants. Alleles in which substitutions at the positions indicated occur are only given for mouse class I. Assignment of the different amino acids to the various pockets in K^b was based on references 4, 5, and 24.

* tm, transmembrane domain; ct, cytoplasmic tail; NA, not applicable.

Amino acid substitutions in K^b and K^b/L^d molecules (which have identical peptide binding regions and differ only in the membrane proximal regions) both with respect to CTL recognition (data not shown) and peptide preference, demonstrated that these molecules behave identically (compare Fig. 2 bottom with Fig. 3 A). Fig. 3 shows a comparison of the set of peptides captured by wild-type K^b molecules (A) and variants thereof. To facilitate a comparison of the effect of the various mutations on substrate preference, we have tabulated the occurrence of all peptide spots observed in the two-dimensional separations in Fig. 4. The transfectant (K^b97R) was included as an illustration of the dramatic effect seen with some of the substitutions. Residue 97 contributes to both pockets C and E, and the amino acid substitution is major: V to R. This mutation (K^b97R, Fig. 3 B) leads to a sharp reduction in the number of peptides from our peptide library with which the molecule can combine. A similar mutation in the K^d molecule also results in a class I molecule with a greatly impaired peptide binding repertoire (Abastado, J.-P. et al., unpublished observations). A comparison of the more complex mutants bm8 45V, and bm8 45V-24A shows that not all mutations in the peptide binding groove exert an effect on peptide preference, and underscores the reproducibility of the assay (see Fig. 6).

The Minor Pockets. Single amino acid substitutions in pockets D (155 R to Y, Fig. 3 C) and E (152 E to A, Fig. 3 D) respectively, both give a slight but distinct alteration in the peptide preference of the class I molecule. These two substitutions are both present in the natural variant K^b^m9.
and are the main determinants of the Kbm1 phenotype with respect to CTL recognition (Van Bleek, G. M., and S. G. Nathenson, manuscript in preparation).

Molecular modelling, and more recently, the elucidation of the crystal structure of K\textsuperscript{b}, predict that in K\textsuperscript{b} the 45 side chain is hardly accessible to peptide because of steric hindrance by the large side chain of E24, and so is not predicted to play a role in peptide selection (4). Indeed, modification of residue 45 in pocket B (Y45V, Fig. 3 E) does not lead to a clearcut alteration in the set of peptides selected (see legend to Fig. 3). However, a single change at position 24 in pocket B (K\textsuperscript{24S}, Fig. 3 F) shifts peptide preference, and suffices to impose a Kbm\textsuperscript{b} phenotype on the K\textsuperscript{b} molecule (Kbm\textsuperscript{b}, Fig. 3 G). The importance of this amino acid substitution with regard to allo-CTL recognition has been described earlier (22). The parallel change in peptide binding capacities observed here underscores the importance of peptide recognition in class I–restricted allogeneic T cell responses.

Thus, naturally occurring amino acid substitutions in all the three minor pockets exert an effect on the set of peptides with which the class I molecule combines, indicating that the structure of pockets that do not interact with the pro-
posed anchor residues does influence the spectrum of peptides presented. Such an effect could either be due to steric hindrance or to an interaction between peptide and class I molecule in the minor pockets. In some cases, a reduction in size of the side chain (e.g., 152 E to A) leads to a reduced capacity to combine with certain peptides. At least in these cases, amino acid residues in the minor pockets may actually contribute to the affinity of the class I molecule for the peptide.
The Major Pocket. Pocket C is predicted to combine with the Tyr anchor in our peptide library. Of the two amino acid substitutions in pocket C, one (Kb74S) has only a moderate effect, whereas the other (Kb73W) leads to binding of a distinctly different set of peptides (Fig. 3, H and I, respectively). Because the Tyr-5 that docks in this pocket is fully conserved in our peptide mixture, this effect cannot be due to the preferential interaction of the altered pocket with different amino acids at this position. Rather, the way an anchor residue (or other invariant part of the peptide chain, see below) interacts with a given region of the class I molecule need not always be the same for different peptides. The elucidation of the crystal structure of K\textsuperscript{b} molecules complexed with either the Sendai-N or the VSV-N K\textsuperscript{b} restricted epitopes (4, 5) (the two of which form the basis of the peptide library employed here [10]), showed that for these two peptides, the way in which the Tyr side chains are positioned in pocket C is indeed measurably different.

Pockets A and F. Pockets A and F bind the NH\textsubscript{2} and COOH termini of the peptide, respectively. We find that mutations in both pockets (pocket A: 163 T to A, and 5 L to M; pocket F: 77 D to S) affect peptide selection (Fig. 3, J, K, and L, respectively). The structure of K\textsuperscript{b} predicts that the side chain of P1 points upwards into solvent, and the main polar interactions in pocket A are between MHC side chains and the peptide backbone (5). The peptide side chain in pocket F is oriented towards the B-sheets. However, the interactions with peptide for the amino acid mutated in this pocket again seem to involve peptide backbone atoms (5). Furthermore, the amino acid position in our peptide library that docks in the F pocket is invariable (only Leu). We conclude that MHC side chains can influence preference of peptide binding even when combining with the carbon backbone of the peptide; polymorphism in those regions of the class I molecule that combine with the peptide termini might help determine allele specificity of peptide binding.

Consequences for CTL Recognition. Some of the amino acid substitutions whose effect on peptide preference has been examined here, are main determinants of phenotype in allo- and antiviral CTL responses. The amino acid substitutions in the minor pockets D and E as they occur in K\textsuperscript{b} and K\textsuperscript{a} phenotype to K\textsuperscript{b} molecules (Van Bleek, G. M., and
S. G. Nathenson, manuscript in preparation). The 24 E-S substitution in the minor pocket B imposes a K\textsuperscript{bm8} phenotype on K\textsuperscript{b} molecules (22); and substitution of residue 77 (pocket F), which interacts with the peptide α-carboxyl group, has been shown to influence recognition of both B27 (23) and K\textsuperscript{bm3} (11). To directly assess the effect of alterations in peptide preference as described here on CTL recognition, we have used this series of L cell transfectants as targets for two CTL clones, one antigen specific and one alloreactive, and one alloreactive CTL line.

All of the mutations that affect peptide preference result in changes in CTL recognition by one or more of the CTL clones used (Fig. 5). Different substitutions affect the recognition by these three clones in distinct ways, much as these substitutions all result in unique alterations in peptide preference.

We have compared the substrate preference of a large series of related class I molecules, using a previously described “fingerprinting” approach (10). Both the reproducibility of this assay (10, Fig. 6), and the observation that the mutation of amino acids in the peptide binding groove can lead to either drastic, (Fig. 3 B) or no detectable changes (Fig. 6), indicate that this is a highly sensitive method to prove the specificity of related class I molecules. The experiments described here indicate that substitutions within a pocket that combines with anchor residues can lead to an altered preference for subsets of a set of peptides all sharing this anchor residue.
Secondly, naturally occurring mutations in pockets of the class I molecule that do not interact with peptide anchor residues do influence the peptide binding characteristics of a class I product. Most of the interactions between H-2 K\textsuperscript{b} and both VSV-N and Sendai-N peptides involve main chain atoms of these peptides (4–6). The alteration in preference for peptide side chains in the K\textsuperscript{b} mutants might therefore be due to an effect of these peptide side chains on the conformation of the peptide, thereby influencing the availability of main chain atoms for hydrogen bonding.

Finally, mutations in regions of the class I molecule that are known to interact with constant portions of the peptide chain can exert a strong effect on the set of peptides selected for, presumably by influencing the positioning of other peptide residues in the peptide binding groove.

Although the pocket that combines with the proposed anchor residue side chain for a given allele still seems the prime determinant of peptide suitability, the relative importance of other regions of the class I molecule is greater than previously acknowledged.

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