Binding of Longer Peptides to the H-2K\textsuperscript{b} Heterodimer Is Restricted to Peptides Extended at Their C Terminus: Refinement of the Inherent MHC Class I Peptide Binding Criteria

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Binding of Longer Peptides to the H-2K\textsuperscript{b} Heterodimer Is Restricted to Peptides Extended at Their C Terminus: Refinement of the Inherent MHC Class I Peptide Binding Criteria

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MHC class I molecules usually bind short peptides of 8–10 amino acids, and binding is dependent on allele-specific anchor residues. However, in a number of cellular systems, class I molecules have been found containing peptides longer than the canonical size. To understand the structural requirements for MHC binding of longer peptides, we used an in vitro class I MHC folding assay to examine peptide variants of the antigenic VSV 8 mer core peptide containing length extensions at either their N or C terminus. This approach allowed us to determine the ability of each peptide to productively form K\textsuperscript{b}/β\textsubscript{2}-microglobulin/peptide complexes. We found that H-2K\textsuperscript{b} molecules can accommodate extended peptides, but only if the extension occurs at the C-terminal peptide end, and that hydrophobic flanking regions are preferred. Peptides extended at their N terminus did not promote productive formation of the trimolecular complex. A structural basis for such findings comes from molecular modeling of a H-2K\textsuperscript{b}/12 mer complex and comparative analysis of MHC class I structures. These analyses revealed that structural constraints in the A pocket of the class I peptide binding groove hinder the binding of N-terminal-extended peptides, whereas structural features at the C-terminal peptide residue pocket allow C-terminal peptide extensions to reach out of the cleft. These findings broaden our understanding of the inherent peptide binding and epitope selection criteria of the MHC class I molecule. Core peptides extended at their N terminus cannot bind, but peptide extensions at the C terminus are tolerated. The Journal of Immunology, 1999, 163: 4434–4441.
accommodated. Therefore, structural features of the class I cleft might limit the binding of peptides with extensions at their amino-terminal end.

Within the cell, the endogenous peptides available for class I binding are generally limited to those generated by Ag processing and transported into the endoplasmic reticulum (ER) by the TAP. The TAP preferentially transports peptides of 8–12 aa in length; however, longer peptides (<24 aa) can also be transported, although at a reduced efficiency (14–16). The present evidence suggests that final processing of class I ligands may occur in the ER, but details of such processing are still controversial. Although the presence of various ER resident proteases has been suggested (8, 17–19), some longer peptides might be protected through binding to the MHC molecule itself (20) or to chaperones (21–24). Others have suggested that peptides are directly handed off from the TAP into the class I binding cleft without ever being free in solution (25, 26). In this model, the newly synthesized class I heterodimers physically associate with the TAP until the peptide is properly positioned in the binding cleft, which then leads to the release of the ternary class I complex (27, 28). Thus, longer peptides would only be found associated with class I molecules if they fulfilled the structural requirements for peptide transport and final delivery to the class I heterodimer.

In the in vitro study described here, we examined the inherent ability of MHC class I molecules to bind extended peptides without the limitations of a cellular system. We employed a detergent-free in vitro folding system (29) using highly purified recombinant murine heavy chain (Kb), light chain (β2m), and synthetic variants of the vesicular stomatitis virus (VSV) 8 mer core peptide, the immunodominant epitope of the VSV nucleocapsid protein. Synthetic variants of the VSV peptide used in this study included those with amino acid extensions at the N or C terminus as well as extended peptides containing various substitutions. Using stoichiometric analysis based on the molar extinction coefficients of the individual class I components at 280 nm, we have examined whether peptides extended at either their N or C terminus can physically associate with the TAP until the peptide is properly positioned in the binding cleft, which then leads to the release of the ternary class I complex. We have also examined whether the chemical nature of the extension affects the efficiency of complex formation. The availability of the three-dimensional structure of the H-2Kb/VSV 8 mer complex (30) allowed us to use energy minimization studies and three-dimensional modeling to provide a structural explanation for our in vitro folding results. We propose a revised model of the inherent peptide binding capabilities of the MHC class I and discuss the possible implications for peptide delivery to the class I.

Table I. Names and amino acid sequences of peptides used in this study

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV 8merP52-59</td>
<td>RGYYQGL</td>
</tr>
<tr>
<td>C-terminal extended peptidesa</td>
<td></td>
</tr>
<tr>
<td>C-12 mer</td>
<td>RGYYQGL</td>
</tr>
<tr>
<td>C-12 mer-P9 mix</td>
<td>RGYYQGL</td>
</tr>
<tr>
<td>C-12 mer-P10 mix</td>
<td>RGYYQGL</td>
</tr>
<tr>
<td>N-terminal extended peptidesa</td>
<td></td>
</tr>
<tr>
<td>N-9 mer</td>
<td>LRGYYQGL</td>
</tr>
<tr>
<td>N-10 mer</td>
<td>DLRGYYQGL</td>
</tr>
<tr>
<td>N-11 mer</td>
<td>SDLRGYYQGL</td>
</tr>
<tr>
<td>N-12 mer</td>
<td>LSLDRGYYQGL</td>
</tr>
<tr>
<td>N-9 mer-P1 mix</td>
<td>XRGYYQGL</td>
</tr>
</tbody>
</table>

a The C- and N-terminal amino acid extensions are according to the natural sequence of the VSV nucleoprotein.
 b C-terminal extended peptides
 c The C-terminal amino acid extension is according to the natural sequence of the VSV nucleoprotein.
 " The N-terminal amino acid extension is according to the natural sequence of the VSV nucleoprotein.

Materials and Methods

Peptide synthesis and purification

VSV peptide and its variants were synthesized by the solid phase method using F-moc chemistry on an Applied Biosystems 433A peptide synthesizer (Foster City, CA). All peptides were purified by RP-HPLC to >95% purity with a Vyduac C18 column (2.1 or 4.6 mm × 25 cm; 300 Å) on a Hewlett Packard HP-1090 M instrument (Palo Alto, CA). Peptides were analyzed by electrospray ionization mass spectrometry on a PE-Sciex API-III instrument (PE Biosystems, Foster City, CA) to confirm their identity.

The peptide variants synthesized are shown in Table I. These include the C-12 mer peptide, consisting of the core VSV peptide with the Lys-Ser-Gly-Asn (KSGN) extension at the C-terminal end: the C-12 mer-P9 mix, in which the P9 position of the C-12 mer consists of a mixture of the amino acids Glu, Asp, Thr, Met, Leu, and Phe; and the C-12 mer-P10 mix in which the P10 amino acid position of the C-12 mer peptide is substituted by the same mixture of amino acids. N-terminal-extended peptides are the N-9 mer, N-10 mer, N-11 mer, and N-12 mer in which amino acids Arg, Thr, Met, Ala, and Phe at the P1 position, amino terminal to Arg at P1 of the core VSV peptide, consist of a mixture of the amino acids Glu, Lys, Arg, Asp, Thr, Met, and Phe. In each peptide mixture, equimolar amounts of the different peptide variants are present.

Purification of H-2Kb heavy chain and β2m light chain

Details of the cloning and expression of recombinant murine Kb heavy chain and β2m light chain have been reported previously (30). Briefly, Kb and β2m were produced as inclusion bodies in Escherichia coli and solubilized in 8.0 M urea, 10 mM Tris (pH 8.5), and 50 mM reduced glutathione. β2m was dialyzed (500 molecular weight cutoff) against 10 mM Tris (pH 8.5) for 24 h and purified by gel exclusion chromatography (Superose-75, Pharmacia, Piscataway, NJ) in 10 mM potassium phosphate, pH 7.0. Kb heavy chain was purified by ion exchange chromatography in 4 M urea and 20 mM Tris-HCl (pH 8.5) on a MonoQ column (Waters, Milford, MA). The protein purity was verified by SDS-PAGE and silver staining.

Assembly of Kb/β2m/peptide complexes

Heavy chain (Kb), light chain (β2m), and peptide were combined in a molar ratio of 1:3:5, as described in detail previously (29). Approximately 6 nmol of Kb were precipitated with 10% TCA, washed twice in ethanol, and air-dried. To this pellet, β2m and peptide were added in the presence of 17.5 mM reduced glutathione and enzymatic inhibitors (1 mM EDTA, 10 μM each of amastatin and leupeptin). The reaction mixture was buffered in 55 mM Tris-HCl (pH 8.5) and incubated at 4°C for 24 h on a rotating platform.

Analysis of the molar stoichiometry of the individual class I components

After 24 h, the Kb/β2m/peptide complex (Mz, 45 kDa) was separated by gel exclusion chromatography using a Superose-75 column (Pharmacia) equilibrated in 10 mM potassium phosphate (pH 7.0) and 150 mM NaCl operating at a flow rate of 0.75 ml/min. The peak containing the ternary class I complex was isolated and denatured in 800 mM GuHCl. The denatured
complex was then separated by RP-HPLC with a Vydac C₄ column (2.1 mm × 25 cm, 300 Å, 0.2 ml/min, 1%/min increase in acetonitrile (0.1% trifluoroacetic acid)) on a Hewlett Packard HP-1090 M instrument, and its individual components were assayed via their absorbance at 214 and 280 nm. Integration of the individual 280-nm peaks corresponding to heavy chain (MHC class I), light chain (β₃m), and peptide allowed for the calculation of the molar stoichiometry of the three components in each complex. The integration of the heavy chain peak area was difficult to assess, as Kₚ eluted in a tailing peak caused by its highly hydrophobic nature, as previously reported (29). The individual peaks corresponding to each component in the complex were collected and analyzed by mass spectrometry. The identity of the Kₚ peak was verified by mass spectrometry across the previously reported (29). The individual peaks corresponding to each component were located in allowed regions of the Ramachandran plot. For the peptide was created using PROCHECK (32). All peptide bonds in the extended peptide were located in allowed regions of the Ramachandran plot. diec to allow for the calculation of the molar stoichiometry of the three components in each complex. The integration of the heavy chain peak area was difficult to assess, as Kₚ eluted in a tailing peak caused by its highly hydrophobic nature, as previously reported (29). The individual peaks corresponding to each component were located in allowed regions of the Ramachandran plot. The C-12 mer peptide was modeled by building KSGN onto the end of the core VSV eight-residue peptide in the H-2Kₚ/VSV 8 mer structure (30) using the molecular modeling program TOM (31). The backbone torsion angles of KSGN were adjusted until all obvious steric overlaps with the MHC were removed, and the extension lay roughly flat along the surface of the protein. This model was then subjected to 1000 cycles of steepest descent energy minimization using INSIGHT (Biosym Technologies, San Diego, CA). All atoms of the MHC were fixed with the exception of those within 6 Å of peptide residues 7–12. Peptide residues 1–7 were also fixed, and peptide residue 8 (Leu) was tethered with a force constant of 100 Cal Å⁻². The final minimized structure was analyzed in INSIGHT for steric overlaps, of which there were none, and the Ramachandran plot for the peptide was created using PROCHECK (32). All peptide bonds in the extended peptide were located in allowed regions of the Ramachandran plot.

Reagents
Biochemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Sequanal grade urea and 8 M GnHCl were obtained from Pierce (Rockford, IL). All water used was passed through a MilliQ apparatus. HPLC solvents were obtained from Burdick & Jackson (Muskegon, MI).

Results
C-terminal-extended peptides allow a higher H-2Kₚ folding efficiency than do N-terminal-extended peptides
Highly purified recombinant H-2Kₚ heavy chain (33 kDa) and β₃m (11 kDa) were assembled together with the VSV 8 mer peptide or its N- and C-terminal-extended peptides (~1 kDa; see Table I) using our previously described in vitro folding method (29). After incubation, the MHC class I complexes (~45 kDa) were purified by gel size exclusion chromatography (Fig. 1). For the VSV 8 mer and all the extended peptides, the formation of an ~45-kDa product was observed. A comparison of the integrated area of each complex peak at 280 nm allowed us to estimate the efficiency with which each N- and C-terminal-extended peptide promoted folding of a class I complex (Table II). Folding of Kₚ, β₃m, and the core VSV 8 mer peptide was used as the standard and was defined as 100%. Although it appears as though all the peptide variants promoted complex formation, none did so as efficiently as the VSV 8 mer. Complex formation with each of the carboxyl-terminal-extended peptides occurred with an efficiency of 30%, while the efficiency of complex formation using amino-terminal-extended peptides was even lower, ranging from 10–20%, depending on the length and composition of the N-terminal extension (Table II). As previously reported, folding of an H-2Dₚ-binding peptide (AS-NENMETM) from influenza virus did not lead to the formation of a 45-kDa product, presumably due to the lack of anchor residues that fulfill the Kₚ motif requirement (29). Similarly, a 45-kDa product was never observed in the absence of peptide.

Stoichiometry of H-2Kₚ/peptide complexes reveals ternary complex formation with C-terminal-extended peptides, but not with N-terminal-extended peptides
To determine the molar stoichiometry of the individual class I components (heavy chain, β₃m, and peptide) within each of the purified MHC class I complexes, the isolated 45-kDa products were denatured and subjected to RP-HPLC analysis, and the 280-nm peaks corresponding to the individual components were integrated. In Fig. 2, RP-HPLC chromatograms are shown for the denatured H-2Kₚ/VSV 8 mer (Fig. 2A) and H-2Kₚ/C-12 mer (Fig. 2B) complexes. The VSV 8 mer and C-12 mer peptides were identified by their unique retention times (Fig. 2), and these assignments were confirmed by mass spectrometric analysis and protein microsequencing (data not shown). In the case of trimolecular complex formation, the individual class I components should be present in equimolar ratio. To estimate the molar ratio of the class I components, we used their molar extinction coefficients at 280 nm: VSV 8 mer peptide, 2,560; β₃m, 17,900; and Kₚ, 73,270 (29). Based on
these molar extinction coefficients, the ratio of the integrated HPLC peak areas at 280 nm should be 1:7:29 for a trimolecular complex in which peptide, $\beta_2m$ and $K^b$ have a molar stoichiometry of 1:1:1. Experimentally, for the H-2K$b$/VSV 8 mer complex, although the integrated HPLC peak areas of the VSV 8 mer peptide and $\beta_2m$ did reveal the predicted 1:1 stoichiometry, the $K^b$ value was only 0.6. This is due to the highly hydrophobic nature of the heavy chain $K^b$ causing a tailing peak eluting throughout a 10-min period. As the VSV 8 mer peptide and $\beta_2m$ were in the predicted 1:1 ratio, we used the relative stoichiometry of peptide and $\beta_2m$ as a measure of trimolecular complex formation in our experiments with the extended peptides. Hence, the molar ratio obtained for the carboxyl-terminal-extended C-12 mer peptide complex was 1:1.6 (peptide:$\beta_2m$; Table II). This result indicates a less ideal ratio of peptide to $\beta_2m$, which may be explained by the lower affinity of the C-12 mer, leading to some peptide displacement or some empty peptide binding grooves.

Considerably less peptide was identified in those complexes formed by folding with peptides extended at their N termini (Table II). Complex formation with the N-terminal-extended peptides N-9 mer, N-10 mer, and N-11 mer resulted in an estimated molar stoichiometry of peptide to $\beta_2m$ of 1:3.9. With the N-12 mer peptide (Table II), not even trace amounts of peptide could be identified on denaturation, even though an ~45-kDa complex had been observed upon gel size exclusion chromatography. The ratio of one peptide molecule to nearly four $\beta_2m$ molecules for the N-9 mer, N-10 mer, and N-11 mer, along with the absence of peptide in the complex formed in the presence of N-12 mer peptide, suggests that N-terminal-extended peptides could only initiate the folding of empty class I heterodimers without the peptide being placed into the class I binding groove. These results support the idea that N-terminal-extended peptides are unable to productively form trimolecular H-2K$b$ complexes. As the isolated complexes were immediately subjected to denaturation and subsequent RP-HPLC analysis, the degradation of complex can be excluded.

Peptides with hydrophobic residues in their C-terminal flanking regions bind preferentially to the H-2K$b$ heterodimer

We next investigated whether the amino acid composition of the peptide extensions might influence peptide binding and proper folding of MHC class I complexes. To do this, a peptide mixture (C-12 mer-P9 mix) containing various amino acids (Gln, Arg, Asp, Thr, Met, Leu, and Phe) at position 9 in the C-terminal extension of the C-12 mer peptide was synthesized (Table I). The substituted

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Efficiency of Complex Formation$^a$ (%)</th>
<th>Integrated HPLC Peak Area Ratio at 280 nm$^b$ (peptide:$\beta_2m$($K^b$))</th>
<th>Estimated Molar Ratio, (peptide:$\beta_2m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV 8 mer</td>
<td>100</td>
<td>1:7:(19)</td>
<td>1:1.0</td>
</tr>
<tr>
<td>C-terminal extended peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-12 mer</td>
<td>25–30</td>
<td>1:11:(39)</td>
<td>1:1.6</td>
</tr>
<tr>
<td>C-12 mer-P9 mix</td>
<td>25–30</td>
<td>1:10:(36)</td>
<td>1:1.4</td>
</tr>
<tr>
<td>C-12 mer-P10 mix</td>
<td>25–30</td>
<td>1:10:(37)</td>
<td>1:1.4</td>
</tr>
<tr>
<td>N-terminal extended peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-9 mer</td>
<td>15–20</td>
<td>1:27:(53)</td>
<td>1:3.9</td>
</tr>
<tr>
<td>N-10 mer</td>
<td>15–20</td>
<td>1:27:(73)</td>
<td>1:3.9</td>
</tr>
<tr>
<td>N-11 mer</td>
<td>15–20</td>
<td>1:27:(50)</td>
<td>1:3.9</td>
</tr>
<tr>
<td>N-12 mer</td>
<td>10–15</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>N-9 mer-P$^2$ 1 mix</td>
<td>10–15</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
</tr>
</tbody>
</table>

$^a$ Using the in vitro folding system, the efficiency of complex formation using the VSV 8 mer peptide was ~20–30%. To compare the efficiency of complex formation using VSV peptide analogs, the VSV 8 mer efficiency was set to 100%.

$^b$ Due to the tailing nature of the $K^b$ peak, its value could not be accurately determined and is therefore shown in parentheses.

$^c$ There was no peptide peak identified and, therefore, no ratio determined.

FIGURE 2. RP-HPLC separation of denatured MHC class I complexes. A, H-2K$b$/VSV 8 mer; B, H-2K$b$/C-12 mer. After assembly, putative MHC class I complexes were purified by gel exclusion chromatography, denatured as described in Materials and Methods, and the individual class I components were separated on a Vydac C18 column (2.1 mm × 25 cm) using a flow rate of 0.2 ml/min, and a gradient of 1%/min acetonitrile containing 0.1% trifluoroacetic acid. Absorbance at 280 nm is shown. The retention times of the individual components are: C-12 mer, 26.8 min; VSV 8 mer, 31.5 min; $\beta_2m$, 42.7 min; $K^b$, 46.2–56 min.
amino acids were representative in terms of their acidic (Asp), basic (Arg), polar (Gln, Thr), or hydrophobic (Met, Leu, Phe) properties. A second peptide mixture (C-12 mer-P10 mix) was also generated, with the amino acids mixture at position P10 of the C-12 mer peptide (Table I). Both peptide mixtures gave rise to the same folding efficiency for H-2Kb complex formation as the C-12 mer peptide (30% of that of the core VSV peptide; Table II). As before, the 45-kDa complexes were isolated by gel exclusion chromatography, then denatured and subjected to RP-HPLC analysis. The area under each peptide peak of the peptide stock (Fig. 3, A and B, upper panels) and of each peptide peak of the denatured class I complexes (Fig. 3, A and B, bottom panels) was measured. Then, the relative abundance of each peptide in the peptide stock and in the complex was compared, and this allowed us to establish whether certain peptide variants were selected for complex formation. The results show that for the C-12 mer-P9 mix, those peptides with Met, Leu, and especially Phe at P9 were clearly selected for binding to the MHC class I molecule over the more hydrophilic amino acids (Gln, Arg, Asp, or Thr; Fig. 3A). Similarly, for the C-12 mer-P10 mix, the peptide with the hydrophobic amino acid Leu at P10 was selected for class I binding (Fig. 3B). In a complex produced using either the C-12 mer-P9 mix or the C-12 mer-P10 mix, peptide and \( \beta_2 \)m were found in a molar ratio of 1:1.4 (Table II), slightly more ideal than the ratio of 1:1.6 obtained using the original C-12 mer peptide with Lys at P9 and Ser at P10. This again suggests preferential binding of peptides with hydrophobic residues in the C-terminal-flanking region.

As described above, the N-9 mer peptide with Leu at its N terminus could not efficiently form trimolecular H-2Kb complexes. To determine whether the nature of the N-terminal residue might alter the ability of an N-terminal-extended peptide to form a trimolecular complex, we synthesized a peptide mixture (N-9 mer-P2\( ^{1} \) mix, Table I) containing various amino acids (Gln, Lys, Arg, Asp, Thr, Met, Phe) at P\( ^{1} \). Using this peptide mixture for folding, an ~45-kDa complex was observed by size exclusion (Table II); however, denaturation and subsequent RP-HPLC analysis yielded not even trace amounts of any peptide. Thus, altering the composition of the N-terminal flanking region did not improve the binding properties of the N-terminal-extended N-9 mer peptide. These results are consistent with the above-mentioned ideas that N-terminal-extended peptides fail to productively form H-2Kb complexes and that the composition of the N-terminal-flanking region cannot adjust for the binding of the extended N-terminal peptide end into the corresponding pocket of the binding groove.

Energy minimization studies provide a structural explanation for the formation of the H-2Kb/C-12 mer complex

To explain our findings at the three-dimensional structural level, we applied the molecular modeling program TOM to build the C-terminal-extended C-12 mer peptide and to model it into the H-2Kb cleft. The model containing the extended peptide was subjected to energy minimization as described in Materials and Methods. In this minimized structure (Fig. 4) the P8 to P9 peptide bond is accommodated at the end of the cleft, with no perturbation of the
Peptides longer than the canonical size of 8–10 aa have been eluted from cellular MHC class I molecules (5–8). In one case, however, these longer peptides were associated with the heavy chain alone (5), rather than being part of a ternary class I complex. Studies designed to measure peptide/MHC affinities have also suggested that extended peptides are able to bind to the class I heterodimer (33–35). However, in such studies, the stoichiometry of the resulting complexes is not determined, so one must make the assumption that proper folding of ternary class I complexes has occurred. It is perhaps due to this potential limitation that peptide affinity studies have yielded somewhat conflicting results regarding the ability of the class I molecule to accommodate N- and/or C-terminal peptide extensions. To avoid the possible limitations of cellular systems and peptide affinity studies, we used a cell-free, detergent-free in vitro class I MHC folding system to investigate the inherent ability of the class I MHC heterodimer to bind peptides longer than the canonical size. We specifically addressed whether core peptides could be extended at their carboxyl or amino termini. The data presented here provide both biochemical and stoichiometric evidence that extended peptides can productively form MHC class I complexes, but only if the peptides are C-terminally extended.

For the C-terminal-extended peptides, a putative class I MHC complex (45 kDa) was formed during an in vitro folding reaction, as judged by gel exclusion chromatography. After isolation and denaturation, this was verified because we could identify each individual component of the ternary class I complex. The C-terminal-extended C-12 mer peptide and β2m were found at a molar ratio of 1:1.6, a ratio close to the model ratio of 1:1 (VSV 8 mer peptide:β2m; Table II). In all our experiments, the identity of the recovered peptide was always verified by mass spectrometry. This ensured that the input extended peptide was truly in the complex, rather than a shortened version that could have been generated by proteolytic clipping during the experimental period. Thus, a peptide extended at its carboxyl terminus is able to induce productive class I complex formation, although the extent of formation of such complexes is only 20–30% of that of the VSV 8 mer. The lower affinity of the C-terminal-extended peptides might cause some peptide displacement or some empty class I binding sites. To evaluate the importance of the chemical nature of the peptide extension, peptides with amino acid substitutions at positions P9 and P10 of the VSV C-12 mer were tested for binding. The analysis showed that peptides with hydrophobic amino acid residues at position P9 or P10 were preferentially selected for complex formation compared to peptides with hydrophilic residues at these positions.

In contrast, for the N-terminal-extended peptides, the putative class I complex was formed at an even lower efficiency than was found for the C-terminal-extended peptides (Table II). Further, after denaturing of those putative complexes, the N-terminal-extended peptides could be detected just over background. Thus, no substantial ternary complex formation occurred for the N-terminal-extended peptides (peptide:β2m:β2mβ2m = 1:3.9). Substitutions in the flanking region of the N-terminal-extended peptide (P-1 for the VSV N-9 mer) did not improve the folding efficiency.

A consideration of the three-dimensional structure of trimolecular MHC class I complexes, in conjunction with modeling of H-2Kβ with the C-12 mer peptide, allowed us to provide a structural basis for our findings concerning peptide binding. Three-dimensional structures of MHC class I complexes have shown that peptide binding in the class I cleft is dependent upon two or three anchor residues within pockets in the cleft, as well as an array of H-bond interactions between conserved residues of the cleft and mainly main chain atoms of the peptide (1, 3). As it has been demonstrated that both the anchor residues and the H-bond interactions are important for the stability of the complex, we have assumed that if any extended peptide can bind to an MHC class I molecule, it will do so while maintaining the binding pattern of the core peptide. Hence, in the modeling studies performed here, we have preserved the geometry of the core 8 residues of the VSV peptide and the surrounding MHC cleft residues (30); consequently, this preserved the interactions between peptide and cleft that have been seen to be critical for binding, while accommodating the extra four residues beyond the end of the cleft. The residues in the extension could either lie along the surface of the molecule, as shown in Fig. 4, maintaining contact with the surface of the H-2Kβ molecule or, alternatively, they could form a free and disordered tail. What is central to the binding of this extended peptide is how the peptide bond between the main chain O atom of P8 (PC in the core VSV 8 mer peptide) and the N atom of P9 is accommodated. In this minimized structure (Fig. 4) the P8 to P9 peptide

FIGURE 4. Final energy minimized model of the C-12 mer peptide superimposed on the solved H-2Kβ/VSV 8 mer structure. The VSV 8 mer (RGYYVQGL) is shown in yellow, with its N-terminal -NH₂ group (green) pointing down into the P1 pocket, and its C-terminal-COOH group (pink) pointing out of the PC pocket of the MHC class I molecule. The C-terminal extension of the C-12 mer (KSGN) is shown in blue.
bond can be accommodated at the end of the cleft, with no perturbation of the P8 anchor residue in the F pocket and no steric overlap with residues of the MHC. Obviously, there are no apparent constraints on the binding orientation of the peptide residues that are located outside the cleft itself, the peptide tail could assume a virtually infinite number of conformations, in any of which there would be no steric hindrance with the MHC.

We have chosen, for the purposes of the model, to fix the geometry of the core VSV peptide as well as the surrounding residues of the MHC cleft. However, it is likely that the existence of the four-residue C-terminal peptide tail induces subtle conformational changes in the precise three-dimensional structure of the remainder of the peptide and thereby in residues lining the cleft. Indeed, small shifts in the positions of the core anchor residues and/or alterations in the H-bonding pattern between the core peptide and the MHC cleft are probably the cause of the observation that the carboxy-terminal-extended peptides form complex with only 30% the efficiency of the core VSV peptide. Nonetheless, it remains the case that the core peptide anchor residues must be located in their appropriate cleft pockets for the extended peptide to bind. Thus, it can be assumed that the model presents a reasonable approximation of the actual complex.

These modeling studies are consistent with our data demonstrating the binding of C-terminal-extended peptides in the H-2Kb cleft. Such binding is possible because the side chain of the P8 residue of peptides binding to H-2Kb is buried within the F pocket, leaving an O atom of the terminal carboxyl group exposed on the surface of the complex. Because it appears to be universal that all MHC class I molecules bind peptides with their PC peptide residue buried as an anchor in the F pocket (4), it follows that all MHC class I molecules ought to be able to bind peptides extended at their C-terminal ends.

Similarly, the three-dimensional structures of MHC class I complexes reveal why N-terminal-extended peptides cannot bind to an MHC class I molecule, although we did not attempt to verify this through modeling. Because the N terminus of the VSV peptide is completely buried, with the P1 peptide residue side chain pointing out of the cleft, the formation of a peptide bond to the buried N atom is sterically prohibited. N-terminal-extended peptides could only bind in the MHC class I cleft via major rearrangements of the MHC residues at this end of the cleft or if the N terminus of the peptide were not bound within the cleft. Neither scenario is energetically likely, supporting our observation that H-2Kb cannot produce C-terminal-extended peptides at their N terminus. Again, we postulate that this finding should be universal for all MHC class I molecules, because they all appear to bind peptides whose P1 residue side chain is directed out of the cleft toward solvent, such that the amino-terminal group is buried in the A pocket. The importance of the proper positioning of the N-terminal peptide end had previously been suggested by Matsumura and colleagues (33) upon consideration of the affinities of extended peptides for purified class I molecules.

The processes involved in peptide delivery from the TAP to the MHC class I molecule are still being clarified. Recent findings revealed that the class I heterodimer physically associates with the TAP (25, 26, 36), and several residues on the α3 domain of the class I (219–233) have been identified to interact with the TAP (37). A model by Elliott and colleagues (27, 28) suggested that the anchoring of the C-terminal peptide residue (PC) in the F pocket of the class I binding groove causes a conformational switch of the short α3 domain (residues 139–149) from a peptide-receptive open position into a non-peptide-receptive closed position. This conformational change would be responsible for disrupting the interaction between the class I molecule and the TAP, leading to the release of loaded class I complexes (28, 38). Such a mobile region impinging upon the F pocket may also facilitate the binding of C-terminal-extended peptides, as long as the side chain of the PC anchor residue of the core peptide is sufficiently anchored into the F pocket of the binding groove, as was observed in the crystal structure of HLA-A2 with a C-terminal extended core peptide (13). Our modeling data of the Kb/C-12 mer peptide also suggest that the binding of a C-terminal-extended core peptide still preserves sufficient anchoring into the F pocket, and thus would allow the final release of the Kb/C-12 mer peptide complex by the TAP.

Class I complexes containing C-terminal-extended core peptides may serve a number of important functions in vivo. The extended peptides might subsequently be cleaved and may therefore serve as precursors for antigenic core peptides. Perhaps more importantly, however, class I complexes containing extended peptides, due to their relative instability, may be part of a pool of class I molecules having readily exchangeable peptides in their peptide binding grooves. For example, in the ER, high affinity peptides derived from pathogens can exchange with host-derived peptides during an infection (39). Peptide exchange may also occur at the cell surface and in the endocytic compartment (40–42). Therefore, some class I complexes containing extended peptides might be sufficiently stable to be transported to the cell surface, where the extended peptide could be exchanged with exogenous class I epitopes. Alternatively, endocytosis of such complexes might provide a source of class I molecules that can readily exchange their peptides in the endocytic compartment with peptides derived from exogenous Ags. Thus, the ability of MHC class I molecules to bind exchangeable C-terminal-extended peptides is likely to have important functional implications.

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References


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