Effects of Peptide Length and Composition on Binding to an Empty Class I MHC Heterodimer†

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ABSTRACT: Class I major histocompatibility complex (MHC) proteins present peptide antigens to T cells during the immune response against viruses. Peptides are loaded into newly synthesized class I heterodimers in the endoplasmic reticulum such that most or all cell surface class I molecules contain peptides derived from endogenous or foreign proteins. We previously reported the assembly of empty heterodimers of the murine class I MHC molecule H-2K^d, from denatured heavy and light chains from which endogenous peptides had been removed [Fahnestock *et al.* (1992) *Science 258*, 1658–1662]. Here we measure thermal stability profiles of empty versus peptide-filled molecules and compare the effects of human versus murine light chains on the overall stability of the K^d heterodimer. The majority of empty heterodimers are stable at 37 °C regardless of the species of light chain, indicating that our previous report of the unexpectedly high thermal stability was an intrinsic property of the K^d molecule and not due to use of a murine/human chimeric protein. Binding constants are derived for a series of peptides interacting with empty K^d heterodimers. The dissociation constants of four known K^d-restricted peptides range from 2.3×10^{-7} to 3.4×10^{-8} M. Using a series of 24 analog peptides, the effects of length and peptide composition on binding affinity of one K^d-restricted peptide are explored, and the results are interpreted with reference to the known three-dimensional structures of class I MHC protein/peptide complexes.

Virally infected cells present antigenic peptides embedded in class I major histocompatibility complex (MHC)[1] molecules to cytotoxic T lymphocytes (Townsend & Bodmer, 1989). Recognition by a T cell receptor on a cytotoxic T cell is specific for the particular combination of class I molecule and peptide on the surface of the infected cell. Crystallographic analyses of class I molecules have shown that peptides bind in a groove located between two α-helices on the top surface of the molecule (reviewed in Bjorkman & Parham, 1990). Pockets at each end of the peptide binding site contact main-chain atoms of the N- and C-termini of octamer and nonamer peptides (Garrett *et al.*, 1989; Saper *et al.*, 1991; Fremont *et al.*, 1992; Madden *et al.*, 1992; Matsumura *et al.*, 1992a; Zhang *et al.*, 1992). These pockets (A and F) are lined with highly conserved residues, while the intermediate pockets (B–E) contain residues that vary in class I sequences. Elution and sequencing of endogenous peptides bound to class I molecules revealed a preference for peptides that are eight or nine amino acids in length and elucidated allele-specific sequence motifs and the presence of "anchor" residues important for allele-specific binding (Van Bleek & Nathenson, 1990; Falk & Rammensee, 1990; Rötzschke *et al.*, 1990a,b; Falk *et al.*, 1991; Jardetzky *et al.*, 1991). For example, peptides that bind to the murine class I molecule H-2K^d are nonamers that most commonly have a tyrosine at position 2 and leucine or isoleucine at position 9 (the anchor residues) (Falk *et al.*, 1991; Romero *et al.*, 1991; Rammensee *et al.*, 1993).

Class I molecules bind peptides in the endoplasmic reticulum during the assembly of the heavy chain with β2-microglobulin (β2m), the class I light chain (Townsend & Bodmer, 1989). Under normal circumstances, the majority of all class I molecules that reach the cell surface are occupied with a peptide, either derived from a self protein to which the immune system is tolerant, or derived from a foreign pathogen, to which the immune system will react. Class I molecules purified from wild type antigen presenting cells or transfected eukaryotic cells contain a mixture of endogenous peptides, and typically less than 1% of the purified protein will bind exogenous peptide (Chen & Parham, 1989). Assembly of class I heavy and light chains in the absence of peptide to make empty class I heterodimers was once thought to be a structural impossibility (Townsend *et al.*, 1989). However, recent experiments using mutant cell lines have established that empty class I molecules can assemble and reach the surface of cells grown at 26 °C (Ljunggren *et al.*, 1990; Schumacher *et al.*, 1990; Townsend *et al.*, 1990). The resulting empty class I heterodimers are less stable than their peptide-filled counterparts and rapidly become undetectable by conformationally sensitive antibodies at physiological temperature unless stabilized by addition of exogenous peptide (Ljunggren *et al.*,

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[1] Abbreviations: β2m, β2-microglobulin; CHO, Chinese hamster ovary; CD, circular dichroism; K_D, dissociation constant; K^d/haβ2m, K^d/hamster β2m; K^d/huβ2m, K^d/human β2m; K^d/mβ2m, K^d/murine β2m; MHC, major histocompatibility complex; T_m, transition midpoint.

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1990; Schumacher *et al.*, 1990; Townsend *et al.*, 1990; Ortiz & Hämmerling, 1991).

An empty form of a class I molecule would be the ideal reagent to use for peptide affinity measurements without the complication of displacing endogenous peptide. We previously reported the efficient *in vitro* reassembly of separated and denatured class I heavy and light chains in the absence of peptide to make an empty form of H-2K^d, which was shown to bind equimolar amounts of offered peptide (Fahnestock *et al.*, 1992). Thermal stability profiles of empty K^d molecules were compared to profiles of their peptide-filled counterparts to quantitate the increase in thermal stability provided by occupation of a peptide. The transition midpoint (T_m) of the melting curve of peptide-filled K^d was substantially higher than the T_m of empty K^d (57 °C compared to 45 °C), and a thermodynamic analysis indicated that the free energy of stabilization due to peptide binding was 4.4 kcal/mol (Fahnestock *et al.*, 1992). Further analysis of the data indicated that 75% of empty K^d molecules were in a native configuration at 37 °C, which was unexpected due to the reported instability of empty class I molecules at physiological temperature. However, because the reassembled class I heterodimers consisted of the murine K^d heavy chain complexed to human β2m (K^d/huβ2m), it was possible that the higher than expected stability of the empty molecules could reflect the greater stability reported for murine class I heavy chains that are complexed with human rather than with murine β2m (Hochman *et al.*, 1988).

In this report, we extend our original studies in several ways: first, we report a method for purifying completely murine heterodimers (K^d heavy chain complexed with murine β2m; K^d/mβ2m) from transfected cells grown in serum free medium and record thermal stability profiles of K^d/mβ2m for comparison to profiles of K^d/huβ2m. Second, we explore the effects of varying peptide length and composition on the ability of a K^d-restricted peptide to bind empty K^d heterodimers. During preparation of the K^d/mβ2m protein, we noted that the majority or all of the K^d/mβ2m heterodimers secreted from transfected CHO cells were occupied with endogenous peptides, whereas ~70% of secreted K^d/huβ2m appeared to be empty (Fahnestock *et al.*, 1992). However, the thermal stability profiles of the empty and peptide-filled forms of K^d/mβ2m differ little from the profiles of their K^d/huβ2m counterparts (the T_m of empty K^d/mβ2m is 42 °C, the T_m of peptide-filled K^d/mβ2m is 56 °C), suggesting that the higher than expected thermal stability originally reported for K^d (Fahnestock *et al.*, 1992) was an intrinsic property of this class I molecule and not due to the use of a nonphysiological chimeric protein. Finally, we use equilibrium dialysis to derive an affinity constant for the interaction between a radiolabeled K^d-restricted peptide and empty K^d and measure the affinities of other K^d-restricted peptides and peptides of altered length and amino acid sequence to evaluate the effects of altering the anchor residues and extending or deleting residues at both ends of the peptide. Many comparative studies on sets of related peptides use T cell assays to access effects of alterations in the peptide sequence. Such assays cannot distinguish between the effects of peptide alteration on binding to the MHC molecule and the effects on T cell recognition of the resulting peptide/MHC complex. In order to separate these two binding events, a direct measurement of peptide/MHC binding affinities is required, as is reported here. The peptide binding studies are interpreted and rationalized using the three-dimensional structure of the class I peptide binding site.

MATERIALS AND METHODS

Cell Lines. Stable CHO cell lines expressing secreted K^d/huβ2m or K^d/mβ2m were generated using a glutamine synthetase-based amplification system (Bebbington & Hentschel, 1987) as described (Fahnestock *et al.*, 1992, 1994). In this system, amplification of transfected genes depends upon increasing concentrations of the drug methionine sulfoximine. In order to produce a secreted K^d heterodimer, a stop codon was introduced into the K^d heavy chain gene after the codon for amino acid 284 (Fahnestock *et al.*, 1992). An expression plasmid containing the truncated heavy chain gene was cotransfected with an expression plasmid containing the complete cDNA sequence of murine (*a* allele; Daniel *et al.*, 1983) or chimpanzee β2m. The protein sequence of mature chimpanzee β2m is identical to human β2m (Lawlor *et al.*, 1990); thus the protein product from the chimpanzee gene is referred to throughout this paper as human β2m. Transfected CHO cells secreting K^d/huβ2m were maintained in glutamine-free αMEM (Irvine Scientific) supplemented with 10% dialyzed fetal bovine serum (Gibco/BRL) and 100 μM methionine sulfoximine (Sigma). Transfected CHO cells secreting K^d/mβ2m were maintained in serum-free conditions as modified from Hamilton and Hamm (1977) (glutamine-free αMEM supplemented with bovine serum albumin, insulin, vitamins, trace metals, and 100 μM methionine sulfoximine). Penicillin (100 units/mL) and streptomycin (100 μg/mL) were included in all media used.

Protein Purification. Protein was purified from cells secreting K^d heterodimers by immunoaffinity chromatography as described (Fahnestock *et al.*, 1992). A column constructed with the monoclonal antibody 34-1-2 (Ozato *et al.*, 1982) was used for purification of K^d/huβ2m from supernatants of confluent cells grown in 10-cm plates. Typical yields were 9–10 mg of K^d/huβ2m/L of supernatant, with no detectable exchange of human β2m for endogenous hamster β2m or bovine β2m in the medium (Fahnestock *et al.*, 1992). For purification of K^d/mβ2m from serum free medium, an immunoaffinity column constructed with the monoclonal antibody M1/42 (Stallcup *et al.*, 1981) was used. This antibody recognizes murine class I MHC heavy chains only when associated with murine β2m (M.L.F., unpublished observations), allowing separation of K^d/mβ2m from K^d heavy chains associated with hamster β2m (K^d/haβ2m). A mobility difference on SDS–PAGE gels between hamster and murine β2m (Fahnestock *et al.*, 1994) allowed K^d/mβ2m and K^d/haβ2m to be easily distinguished. Protein eluted from the M1/42 column was verified to be free of hamster β2m by N-terminal sequence analysis (M.L.F. and P.J.B., unpublished data) and gel migration behavior. The flow-through material was saved and purifed by passage over the 34-1-2 immunoaffinity column as a source of K^d/haβ2m. To obtain sufficient quantities of K^d/mβ2m, cells expressing secreted K^d/mβ2m were introduced into a hollow fiber bioreactor device (Cell Pharm I; Unisyn Fibertec, San Diego, CA) in serum free medium, and supernatants were collected daily. The final yield of purified K^d/mβ2m protein was 2.5–3.0 mg/L of supernatant. An additional 1–2 mg/L was purified as K^d/haβ2m.

Acid Elutions of K^d Heterodimers. Purified K^d/huβ2m, K^d/mβ2m, or K^d/haβ2m was analyzed for the presence of bound peptides using established methods (Van Bleek & Nathenson, 1990; Jardetzky *et. al.*, 1991). Briefly, 0.25 mg of protein (quantitated by a BCA assay, Pierce Chemical Co.) was concentrated to 100 μL in a Centricon 10 (molecular weight cutoff of 10 000) ultrafiltration device (Amicon;

Beverly, MA). After dilution with 1.0 mL of 50 mM ammonium acetate, pH 7.5, the proteins were again concentrated to 100 μL, and this procedure was repeated. The washed protein was then treated with 1.0 mL of 12% acetic acid and concentrated again to 100 μL in the ultrafiltration unit, and this elution step was repeated. Using this procedure, the acid filtrate should contain any eluted peptide material. The acid eluates were lyophilized and analyzed by automated Edman degradation using an Applied Biosystems Model 477A protein sequencer.

Reassembly of Empty Heterodimers from Separated Heavy and Light Chains. Empty K^d/huβ2m or K^d/mβ2m heterodimers were prepared from denatured protein as described (Fahnestock *et al.*, 1992). Briefly, heavy and light chains were first denatured in 6.0 M guanidine hydrochloride and separated from endogenous peptides by gel filtration chromatography on a Superose 12 FPLC column (Pharmacia). Heavy and light chain peaks were pooled and renatured by dialysis against 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride containing 8 M urea and then twice against the same buffer without urea. Renatured material was concentrated 8–10-fold in a Centricon-10 (molecular weight cutoff 10 000) ultrafiltration device (Amicon; Beverly, MA) and passed over a Superdex FPLC size exclusion column (Pharmacia). Fractions corresponding to heterodimer were pooled and concentrated.

Circular Dichroism Spectra and Thermal Stability Analyses. A JASCO J-720 spectropolarimeter equipped with a Peltier thermal control unit and a rectangular cuvette with a 1-mm pathlength was used for CD measurements. Spectra were recorded from protein samples (0.25–0.4 mg/mL) in 5 mM phosphate buffer, pH 7.0. For measuring melting curves, the CD signal was monitored at 223 nm while the sample temperature was raised from 25 to 75 °C at a rate of 20 °C/h. T_m's were calculated by estimating the half-point of the ellipticity change between the pure native and pure denatured states.

Peptide Synthesis and Labeling. All peptides were synthesized by automated solid phase methodology on an Applied Biosystems Model 432A synthesizer using the manufacturer's standard Fmoc protocol. Preloaded resins were purchased from Bachem Bioscience Inc. (Philadelphia, PA), and Fmoc-protected amino acids and additional reagents were from Applied Biosystems (Foster City, CA). Cleavage from the resin and simultaneous side chain deprotection was accomplished by treatment with 90% trifluoroacetic acid, 2.5% thioanisole, 2.5% mercaptoethanol and 5% phenol for 2 h at room temperature. The precipitated, crude peptides were purified to homogeneity using a preparative C-4 Vydac column (Hesperia, CA) and a 0.1% aqueous trifluoroacetic acid/acetonitrile gradient. Peptide composition was confirmed by amino acid analysis. Lyophilized peptide samples were resuspended in water. The concentrations of peptides were estimated spectrophotometrically using the extinction coefficient of tyrosine at 274 nm (1420 M^{-1} cm^{-1}) (Wetlaufer, 1962) or by a BCA assay (Pierce Chemical Co.). A K^d-restricted peptide from influenza virus nucleoprotein (amino acids 147–155; sequence TYQRTRALV; Rötzschke *et al.*, 1990a) was tritiated by catalytic exchange (Amersham TR7 Tritium Labeling Service) to a specific activity of 1.61×10^9 counts per minute/μmol. Aliquots were purifed by reverse phase chromatography on an FPLC PepRPC column (Pharmacia) prior to use. A portion of the K^d-restricted peptide was iodinated with ^{127}I on the anchor tyrosine residue, using a technique that makes a stoichiometrically iodinated and thus chemically homogeneous peptide (Tsomides & Eisen, 1993). Mono- and di-iodinated species were separated using a C4 reverse phase HPLC column (Vydac) and analyzed by Edman degradation.

Equilibrium Dialysis. Equilibrium dialysis was performed in phosphate-buffered saline containing 0.5% gelatin and 0.02% NaN_3 using a Hoefer Microdialyzer EMD 101 (Hoefer; San Francisco, CA). For Scatchard analyses, eight inside compartments [each filled with 100 μL of K^d/huβ2m (2 μM)] were separated from eight outside compartments [each filled with 100 μL of varying concentrations of ^3H-labeled peptide (0.25–5.0 μM)] by a membrane with a molecular weight cutoff of 14 000. Dialysis was performed at room temperature for 24 h. Equilibrium was established during this time period, as no change of binding values was observed with longer incubations. Samples (50 μL) from each inside and outside compartment were then collected and transferred to a vial containing 10.0 mL of Safety-Solve (Research Products International Corp.) for scintillation counting on a Beckman LS 5000TD scintillation counter. The concentration of free peptide after reaching equilibrium was calculated using a specific activity of 1.61×10^9 counts per minute/μmol. The concentration of peptide bound to protein was estimated after subtracting the counts per minute measured in the chamber without protein from the counts per minute measured in the chamber that included protein. The data were plotted as [bound]/[free] versus [bound] and the K_D calculated as $-(1/\text{slope})$. For inhibition studies using unlabeled peptide, the inside compartments were filled with 100 μL of K^d/huβ2m (2 μM), and the outside compartments were filled with 100 μL of a solution containing ^3H-labeled peptide (2 μM) and varying concentrations of unlabeled inhibitor peptide (0.0–2 mM). For each concentration of inhibitor, the percent inhibition of peptide binding was calculated by comparing results to a pair of chambers that did not include inhibitor, and the percent inhibition was plotted versus the concentration of inhibitor on a logarithmic scale. The 50% inhibition value was determined from this graph and used to calculate the K_D of the inhibitor using the following equation (Müller, 1983): $K_D = (I_{50}-[^3H\text{-}P]) \times (1-1.5b + 0.5b^2)$, where b = percent peptide binding in the absence of inhibitor (75.2% ± 10.7%), I_{50} is the concentration of inhibitor required for 50% inhibition, and $[^3H\text{-}P]$ is the total concentration of ^3H-labeled peptide (1.0 μM after equilibrium). For each inhibition curve, seven concentrations of inhibitor were tested in duplicate or triplicate (although unpredictable leakage of the samples in the chambers sometimes prevented all samples from being analyzed), with the eighth chambers of the dialysis blocks tested under identical conditions in the absence of inhibitor. For the two peptides that bound with a higher affinity than the ^3H-labeled peptide, the K_D was estimated using the following relationship (Cheng & Prusoff, 1973): $K_D = I_{50}/(1 + [^3H\text{-}P]/K_{D(\text{labeled peptide})})$, where the $K_{D(\text{labeled peptide})}$ refers to the dissociation constant determined for the labeled peptide by Scatchard analysis.

RESULTS

The Majority of K^d/mβ2m, but Not K^d/huβ2m, Heterodimers Secreted from Transfected CHO Cells Are Occupied with Endogenous Peptides. K^d heterodimers purified from CHO cells transfected with the K^d and the murine β2m genes contain a mixture of murine and bovine β2m (Fahnestock *et al.*, 1992), which suggests that a large portion of murine β2m was replaced in an exchange reaction by serum-derived bovine β2m present in the medium (Bernabeu *et al.*, 1984). When K^d protein was purified from transfected CHO cells

Table 1: Picomoles of Amino Acids Recovered from Acid Elutions[a]

cycle number	K^d/huβ2m	K^d/mβ2m	K^d/haβ2m
1	406	872	947
2	280 (Y = 178)	766 (Y = 559)	937 (Y = 757)
3	190	630	666
4	172	412	554
5	134	576	328
6	94	843	375
7	87	263	224
8	55	530	91
9	42	223	45

[a] For peptides eluted from equivalent amounts of each protein, the total yield of amino acids from each sequenching cycle is presented. Only those amino acid residues that showed an increase in the absolute recovered compared to the previous cycle were considered significant.

maintained under serum free conditions, N-terminal sequence analysis showed that a portion of the purified heterodimers had incorporated endogenous hamster β2m as their light chain (data not shown). By contrast, K^d heterodimers purified from cells transfected with the K^d and human β2m genes did not contain detectable amounts of bovine or hamster β2m (Fahnestock *et al.*, 1992).

Peptides were eluted (Van Bleek & Nathenson, 1990; Jardetzky *et al.*, 1991) from equivalent amounts of purified K^d/mβ2m, K^d/haβ2m, and K^d/huβ2m heterodimers and analyzed by N-terminal sequencing (Table 1). To obtain homogeneous heterodimeric species of K^d/mβ2m and K^d/haβ2m for these analyses, K^d/mβ2m was separated from K^d/haβ2m on an immunoaffinity column, using a monoclonal antibody (M1/42; Stallcup *et al.*, 1981) that binds to K^d heavy chains only when complexed to murine β2m (M.L.F., unpublished results). Sequences of peptides isolated from all three forms of K^d showed a predominance of tyrosine and proline in the second and fourth positions, consistent with previously reported characteristics of K^d-restricted peptides (Falk *et al.*, 1991; Romero *et al.*, 1991; Rammensee *et al.*, 1993). However, on a molar basis, considerably less peptide material is found in the acid eluate of K^d/huβ2m as compared to the eluates of K^d/mβ2m or K^d/haβ2m. Using thermal stability profiles and analyses of near UV CD spectra, we had previously shown that ~70% of the K^d/huβ2m heterodimers secreted from transfected CHO cells behave as empty molecules, and the sequencing data in Table 1 and a previous sequencing analysis (Raghavan *et al.*, 1993) confirm the estimate that less than half of the purified K^d/huβ2m protein contains bound peptides. Three to four times more peptide material was eluted from the K^d/mβ2m and K^d/haβ2m proteins (based upon the picomoles of tyrosine in cycle 2 in the acid elutes from the three respective proteins). Assuming that the amount of peptide material eluted from K^d/huβ2m represents a typical recoverable yield from the 30% of the protein containing peptides, and that the recoverable yields of peptides from eluates from K^d/mβ2m and K^d/haβ2m are comparable, the 3–4-fold increase in peptide material suggests that the majority of those species of heterodimers are occupied with endogenous peptides.

Thermal Stability Profiles of Empty and Peptide-Filled K^d/mβ2m and K^d/huβ2m Heterodimers Are Similar. Empty K^d heterodimers were prepared using previously established methods (Fahnestock *et al.*, 1992) by reassembling denatured heavy and light chains in the absence of endogenous peptides. Heat-induced unfolding of empty K^d/mβ2m was monitored by recording the CD signal at 223 nm while the sample temperature was increased from 25 to 80 °C, and the resulting melting curve was compared to that obtained for K^d/huβ2m

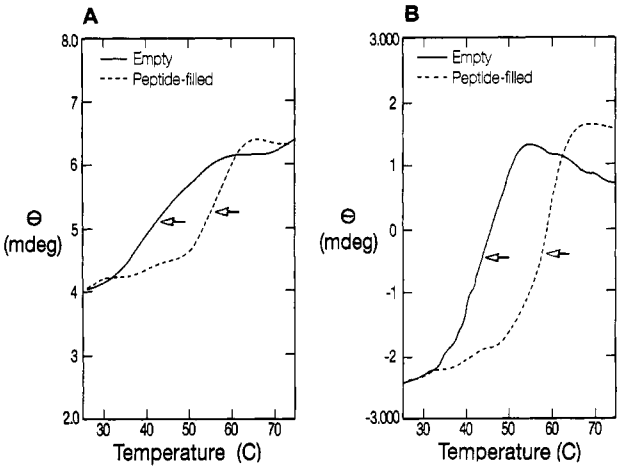

FIGURE 1: Thermal denaturation profiles of empty and peptide-filled K^d/mβ2m (panel A) compared to empty and peptide-filled K^d/huβ2m (panel B). The CD signal at 223 nm of each protein in 5 mM phosphate buffer at pH 7 was monitored using a 1-mm rectangular cell and a Peltier thermal control unit while the temperature was raised from 25 to 75 °C at a rate of 20 °C/h. The data were smoothed using the accompanying J720 software. T_m's for the melting of the heavy chain (marked with arrows) were derived by estimating the half-point of the ellipticity change between the beginning and end of each transition. The melting curves for K^d/mβ2m and K^d/huβ2m were collected using two different cuvettes and are presented as obtained (θ, mdeg). Differences in the absolute numbers for ellipticity shown reflect differences in the offset caused by different characteristics of the cuvettes. These differences are irrelevant for the determination of the T_m.

(Figure 1A,B). As previously reported, empty K^d/huβ2m shows two unfolding transitions, the first (T_m = 45 °C) due to dissociation and denaturation of the heavy chain and the second (T_m = 64 °C) from denaturation of the light chain (Fahnestock *et al.*, 1992). The empty K^d/mβ2m melting curve shows very similar behavior, with the heavy chain melting transition centered at 42 °C. The β2m melting transition is not apparent for empty K^d/mβ2m in Figure 1A, perhaps being obscured by the CD signal from the melted K^d heavy chain. Upon addition of a 2-fold molar excess of a K^d-restricted peptide from influenza virus nucleoprotein (sequence: TYQRTRALV; Rötzschke *et al.*, 1990a), the heavy chain denaturation transitions for both K^d/mβ2m and K^d/huβ2m are shifted to higher temperatures (T_m = 56 °C for K^d/mβ2m; T_m = 57 °C for K^d/huβ2m).

Equilibrium Dialysis Measurements of Binding Affinities of a Series of K^d-Restricted Peptide Analogs. Empty K^d heterodimers were used in equilibrium dialysis experiments to obtain dissociation constants for peptide binding. Because K^d/mβ2m and K^d/huβ2m showed similar thermal stability profiles to each other in both their empty and peptide-filled forms and because of the greater ease in obtaining starting material and reassembling empty heterodimers, we used K^d/huβ2m for affinity measurements. The influenza nucleoprotein K^d-restricted peptide (Rötzschke *et al.*, 1990a) used in the thermal stability studies was labeled to high specific activity with ^3H by catalytic exchange. Equilibrium dialysis was then performed with empty K^d molecules and the ^3H-labeled peptide, and the dissociation constant (K_D) was obtained by a Scatchard analysis of the data (Figure 2). Data from six separate experiments were analyzed in a similar way, obtaining a value for the K_D of (6.9 ± 2.3) × 10^{-8} M.

A series of unlabeled peptides were next analyzed for their ability to compete for binding of the ^3H-labeled K^d-restricted peptide. The concentrations of inhibitor peptides that produced 50% inhibition of labeled peptide binding were

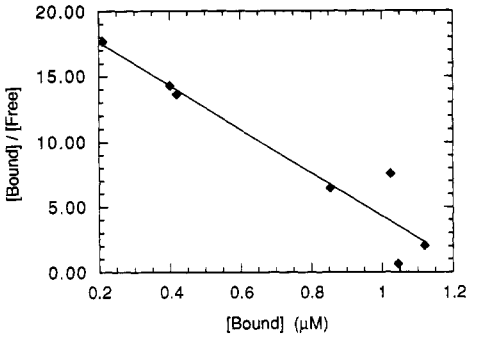

FIGURE 2: Representative Scatchard analysis of ^{3}H-labeled peptide binding to empty K^d/huβ2m heterodimers. The dissociation constant, K_D, is calculated as the negative of the inverse of the slope of the line. For this experiment, $K_D = 6.0 \times 10^{-8}$ M. Data from six separate experiments were analyzed in a similar way, obtaining a value for the K_D of $(6.9 \pm 2.3) \times 10^{-8}$ M.

determined by plotting the percent inhibition of binding versus inhibitor concentration (Figure 3). The concentrations resulting in 50% inhibition were then used to calculate dissociation constants for the inhibitor peptides as described in Materials and Methods. As a test of the validity of the K_D values derived from the inhibition studies, an unlabeled version of the ^{3}H-labeled peptide was used to compete with labeled peptide for binding. The K_D derived from the inhibition analysis was 1.0×10^{-7} M as compared to the value of $(6.9 \pm 2.3) \times 10^{-8}$ M derived from the Scatchard analysis (Figure 2), demonstrating that the two methods of affinity constant derivation give comparable values.

Peptides were synthesized that correspond to extensions of the K^d-restricted influenza nucleoprotein derived peptide, with additions at either the N- or C-terminus of the peptide. Sequences of the peptides and their calculated K_D values are presented in Table 2. Additions to either end had a deleterious effect on the binding affinity, although additions to the N-terminus appeared to be tolerated slightly better. The K_D values derived for peptides with one or two amino acid extensions at the N-terminus (peptides NP2 and NP3; Table 2) indicated that the binding was 750- and 2000-fold weaker than the binding of the labeled peptide, while the binding of a peptide with a single addition at the C-terminus (peptide NP4) showed a weaker affinity than that of the peptide with two additions at the N-terminus. Lengthening the peptide by insertion of a single alanine residue in the middle of the sequence (peptide NP9) had a less deleterious effect, lowering the affinity by ~130 fold. The effects of deletion at the N- or C-terminus followed an opposite trend, with a single deletion at the C-terminus (peptide NP7) having a less deleterious effect on binding than a single deletion at the N-terminus (peptide NP6), while a 7-mer peptide with an amino acid deleted at both ends (peptide NP8) was not observed to inhibit labeled peptide binding at concentrations up to 1 mM, suggesting that the K_D is $\geq$1 mM. Substitutions of the anchor tyrosine residue at position 2 of the peptide also affected peptide binding: the conservative substitution of phenylalanine for tyrosine (peptide NP10) reduced the binding affinity by 65-fold, whereas no binding was observed if the tyrosine was replaced by threonine (peptide NP11). An alanine-substituted peptide (peptide NP12) also failed to bind, in agreement with results of others (Ojcius *et al.*, 1993). Altering the anchor residue by iodination diminished the ability of the peptide to bind: a stoichiometrically labeled monoiodinated derivative (peptide NP13) reduced the binding affinity by ~700 fold, while the di-iodinated derivative (peptide NP14) showed even weaker affinity. When the C-terminal valine of the peptide was replaced by polar and/or large residues, the effects varied: replacement by charged residues interfered with binding more than replacement by uncharged residues. Replacements with both the small polar residue serine (peptide NP15) and the large nonpolar residue tryptophan (NP16) reduced the affinity by ~400 fold, whereas replacement by a positively charged (lysine; NP17) or negatively charged (aspartic acid; NP18) residue reduced the affinity by 5 orders of magnitude or to the point that no inhibition was observed with 1 mM peptide. By contrast to the effects of substitutions at positions 2 or 9, single substitutions of residues at positions 3–7 with cysteines (peptides NP19 through NP23) had very little effect on binding. However, simultaneous replacement of the same five internal residues with prolines or glycines was detrimental to binding, resulting in a 1000-fold reduction in the binding affinity (five prolines; NP24) or a 2000-fold reduction in binding affinity (five glycines; NP25). None of the altered peptides in the influenza nucleoprotein analog series were found to have an increased binding affinity. However, two other K^d-restricted peptides of unrelated sequence (KD1, Falk *et al.*, 1991; KD2, Romero *et al.*, 1991) were found to bind with slightly higher affinities than the influenza nucleoprotein peptide to empty K^d heterodimers.

DISCUSSION

Class I MHC molecules bind peptides during their assembly in the endoplasmic reticulum (Townsend & Bodmer, 1989); thus purified preparations of class I molecules contain mixtures of endogenous peptides. The presence of endogenous peptides complicates binding measurements of defined peptides, largely preventing binding, so that the percent of class I molecules that can accept exogenous peptide is low (Chen & Parham, 1989). The ideal reagent for the comparison of binding affinities of different peptides is a class I protein devoid of endogenous peptides, *i.e.* an empty molecule. One strategy to produce empty molecules has been to express the protein in *Drosophila* cells (Jackson *et al.*, 1992; Matsumura *et al.*, 1992b), with the assumption that invertebrate cells do not have the proper mechanism for intracellular peptide loading and that the resulting empty molecules will be stable at the reduced temperature of insect cell culture (27 °C). Indeed, class I molecules produced in *Drosophila* cells are capable of quantitative binding of exogenous peptide (Matsumura *et al.*, 1992b), allowing the solution of several defined peptide/MHC crystal structures (Fremont *et al.*, 1992; Matsumura *et al.*, 1992a).

We previously reported an alternative method for producing an empty form of the murine class I K^d heavy chain complexed with human β2m, referred to as K^d/huβ2m (Fahnestock *et al.*, 1992). The method relies upon a biochemical separation of endogenous peptides from denatured class I heavy and light chains, followed by reassembly in the absence of added peptide. Initial attempts to produce the completely murine heterodimer (K^d/mβ2m) were complicated by exchange of murine β2m for endogenous hamster β2m and bovine β2m in the medium (Fahnestock *et al.*, 1992). For this study, we developed a method to purify K^d/mβ2m from CHO cells transfected with the K^d and murine β2m genes and compared the percent of purified K^d/mβ2m molecules that are occupied with peptide to the percent of K^d/huβ2m and K^d/haβ2m that contain peptide. While only ~30% of purified K^d/huβ2m molecules contain peptides (Fahnestock *et al.*, 1992), the majority or all of the K^d/mβ2m and K^d/haβ2m molecules were occupied (Table 1). A possible explanation for this difference is related to our observation that CHO cells transfected with the K^d and

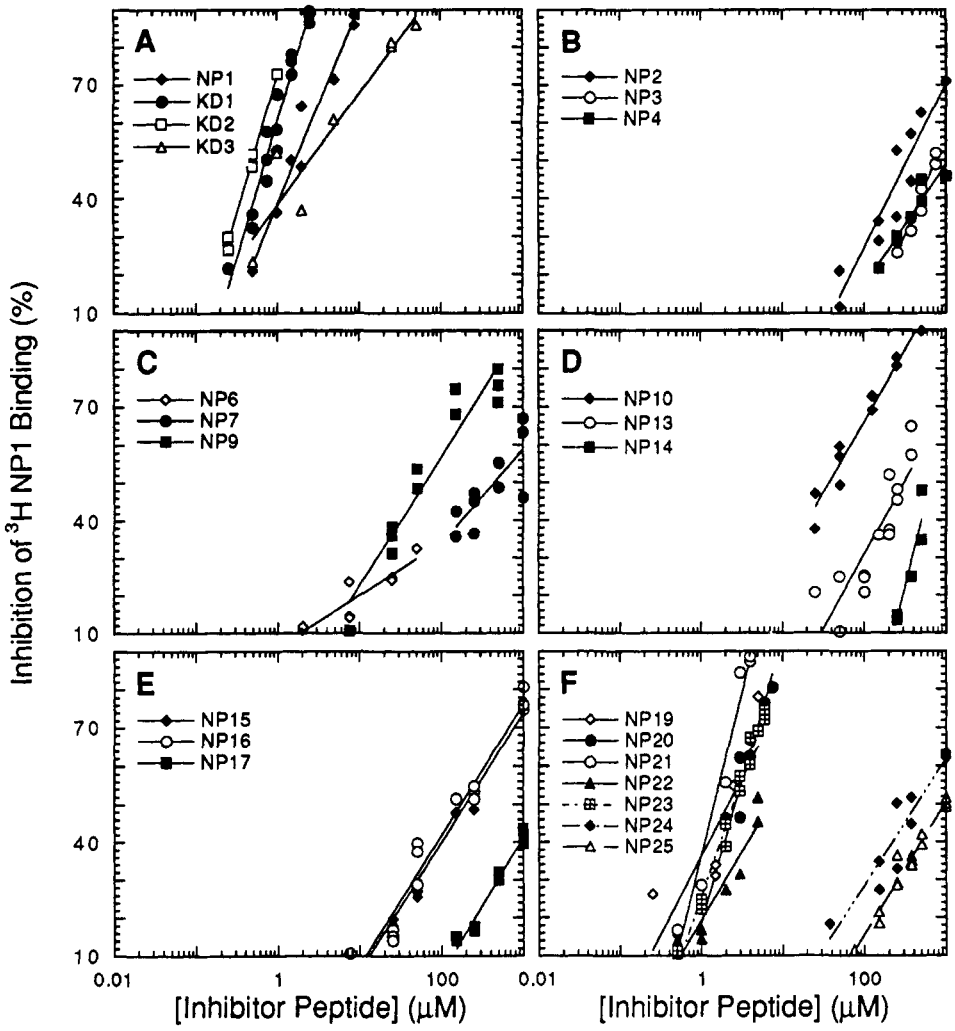

FIGURE 3: Determination of the 50% inhibition values for unlabeled peptides competing for binding of ^{3}H-labeled peptide NP1 to empty K^d/huβ2m heterodimers. Only the data points corresponding to between 10% and 90% inhibition are plotted, since this portion of the inhibition curve can be approximated by a straight line (Müller, 1983). See Table 2 for peptide names and sequences. (A) Comparison of binding of unaltered K^d-restricted peptides. (B) Comparison of binding of NP1 analog peptides with additions to the N- or C-terminus. (C) Comparison of binding of NP1 analog peptides with insertions or deletions. (D) Comparison of binding of NP1 analog peptides with altered residues at the position 2 anchor residue. (E) Comparison of binding of NP1 analog peptides with altered residues at position 9 (the C-terminus). (F) Comparison of binding of NP1 analog peptides with substitutions at positions 3–7. Data for peptides that did not compete for binding of ^{3}H-labeled NP1 are not shown on the graphs.

human β2m genes consistently produce a much higher yield of K^d than CHO cells transfected with the K^d and murine β2m genes: ~100 mg of K^d/huβ2m are recovered/L of supernatant harvested from cells grown in a hollow fiber bioreactor device (Fahnestock *et al.*, 1992) compared to ~2–3 mg of K^d/mβ2m or 1–2 mg of K^d/haβ2m. The high proportion of empty K^d/huβ2m molecules produced under these conditions may reflect a limiting peptide supply inside the transfected CHO cells under conditions of overproduction of a class I molecule.

In a previous study, we found a surprisingly high thermal stability for empty K^d/huβ2m (T_m of the heavy chain denaturation = 45 °C), which suggested that ~75% of these empty heterodimers are folded at 37 °C (Fahnestock *et al.*, 1992). These results contrast with studies of the behavior of empty murine class I molecules at the cell surface, which become undetectable at 37 °C by immunoprecipitation with conformationally dependent antibodies unless stabilized by appropriate peptides (Ljunggren *et al.*, 1990; Schumacher *et al.*, 1990; Townsend *et al.*, 1990). In order to ascertain if the unexpected stability observed at temperatures above 37 °C was due to increased stabilization caused by pairing of the murine heavy chain with the human β2m light chain (Hochman *et al.*, 1988), we compared the thermal stability of empty K^d/mβ2m to empty K^d/huβ2m. The stability profile of empty K^d/mβ2m (T_m of the heavy chain denaturation = 42 °C) revealed only a slight decrease in thermal stability compared to empty K^d/huβ2m (Figure 1A,B). The equilibrium constant derived at 37 °C (as described in Fahnestock *et al.*, 1992) suggests that ~70% of the empty K^d/mβ2m heterodimers are folded at the physiological temperature, as compared to ~75% of empty K^d/huβ2m heterodimers (Fahnestock *et al.*, 1992). These results are compatible with studies of a soluble form of the murine class I molecule K^b produced in *Drosophila* cells (Matsumura *et al.*, 1992b). The K^b protein (which behaves as if it is empty) was shown to be immunoreactive with a conformationally sensitive antibody at temperatures up to 47 °C, as long as the immunoprecipitation was carried out in the absence of detergent. Upon addition of 1% Triton X-100, the immunoreactivity was lost at 37 °C. If the presence of the detergent interferes with the association of the heavy and light chains, the empty heterodimers would dissociate at temperatures lower than 37 °C, reconciling our stability data and the K^b data (Matsumura *et al.*, 1992b) with observations of the instability of empty cell surface class I molecules in detergent lysates (Ljunggren *et al.*, 1990; Schumacher *et al.*,

Table 2: Analysis of Dissociation Constants of K^d-Binding Peptides[a]

name	length	sequence	category	I_{50} (μM)	K_D (M)	ratio (relative to NP1)
NP1	9	TYQRTRALV	viral		6.9×10^{-8}	1
NP2	10	ATYQRTRALV	NH_2 addition	338	5.2×10^{-5}	754
NP3	11	DATYQRTRALV	NH_2 addition	754	1.2×10^{-4}	1739
NP4	10	TYQRTRALVR	COOH addition	1091	1.7×10^{-4}	2464
NP5	13	DATYQRTRALVRT	both additions		no binding	
NP6	8	YQRTRALV	NH_2 deletion	1441	2.2×10^{-4}	3188
NP7	8	TYQRTRAL	COOH deletion	414	6.4×10^{-5}	928
NP8	7	YQRTRAL	both deleted		no binding	
NP9	10	TYQRATRALV	insertion	63	9.6×10^{-6}	139
NP10	9	TFQRTRALV	Y → F	37	5.6×10^{-6}	81
NP11	9	TTQRTRALV	Y → T		no binding	
NP12	9	TAQRTRALV	Y → A		no binding	
NP13	9	TXQRTRALV	monoiodinated	304	4.7×10^{-5}	681
NP14	9	TXQRTRALV	diiodinated	646	1.0×10^{-4}	1449
NP15	9	TYQRTRALS	V → S	197	3.0×10^{-5}	435
NP16	9	TYQRTRALW	V → W	172	2.7×10^{-5}	391
NP17	9	TYQRTRALK	V → K	1763	2.7×10^{-4}	3913
NP18	9	TYQRTRALD	V → D		no binding	
NP19	9	TYCRTRALV	Q → C	2.2	1.9×10^{-7}	2.8
NP20	9	TYQCTRALV	R → C	2.6	2.5×10^{-7}	3.6
NP21	9	TYQRCRALV	T → C	1.4	6.2×10^{-8}	0.90
NP22	9	TYQRTCALV	R → C	7.1	9.5×10^{-7}	14
NP23	9	TYQRTRCLV	A → C	2.5	2.3×10^{-7}	3.3
NP24	9	TYPPPPPLV	five changes	438	6.8×10^{-5}	986
NP25	9	TYGGGGGLV	five changes	956	1.5×10^{-4}	2174
KD1	9	SYFPEITHI	self	0.72	4.7×10^{-8}	0.68
KD2	9	SYIPSAEKI	parasitic	0.49	3.2×10^{-8}	0.46
KD3	8	YIPSAEKI	parasitic	2.5	2.3×10^{-7}	3.3

[a] Peptide name, length, sequence, and category are summarized with the concentration at which peptide inhibited 50% of the binding of a ^3H version of NP1 (I_{50}) (see Figure 3 for determination of I_{50} values). K_D values are calculated as described in the Materials and Methods using the I_{50} values and an average percent bound in the absence of inhibitor of 75.2% (average of 24 experiments with an error range of ±10.7%). Peptide NP1 is a known K^d-restricted epitope from influenza nucleoprotein (Rötzschke *et al.*, 1990a), peptides NP2 through NP25 are analogs of NP1, peptides NP13 and NP14 are stoichiometrically iodinated (Tsomides & Eisen, 1991) forms of NP1, peptide KD1 represents the sequence of a prominent self peptide eluted from K^d molecules on P815 cells (Falk *et al.*, 1991), and peptides KD2 and KD3 are nonamer and octamer versions of a K^d-restricted peptide from *P. berghei* circumsporozoite protein (Romero *et al.*, 1991). "No binding" indicates that a peptide did not inhibit binding of the ^3H-labeled NP1 peptide at concentrations up to 1 mM.

1990; Townsend *et al.*, 1990). Our data predict that at least the extracellular portion of an empty class I heterodimer can exist as a native folded structure under physiological conditions. However, *in vivo* studies show that surface class I expression is greatly reduced in mutant mice in which the gene encoding the peptide transporter associated with antigen processing was disrupted (Ashton-Rickardt *et al.*, 1993). Thus, there may be other mechanisms in addition to the decreased thermal stability of empty class I molecules that keep large numbers of them from remaining at the surface of cells.

Most studies to detect peptide binding to MHC molecules have used peptides labeled by iodination of tyrosine residues (Benjamin *et al.*, 1991; Cerundolo *et al.*, 1991; Christinck *et al.*, 1991), either within the antigenic peptide sequence or as an N- or C-terminal addition. The iodine atom is large, similar in size to a benzene ring (Bolton & Hunter, 1973). Thus iodination would be expected to introduce steric hindrance as well as ionic effects that may interfere with the hydrogen bonding potential of the tyrosine hydroxyl. When a tyrosine for labeling is added to the beginning or end of the peptide, the peptide is no longer the optimal length for binding to class I molecules (Rötzschke & Falk, 1991), and the interactions of one of the termini with conserved pockets in the class I peptide binding site (Fremont *et al.*, 1992; Guo *et al.*, 1992; Madden *et al.*, 1992; Matsumura *et al.*, 1992a) are disrupted. In addition, traditional iodination procedures label only ~1% of the peptide molecules, leaving the remainder unlabeled, complicating the interpretation of inhibition or other experiments performed using a mixture. Other studies use peptides modified by biotinylation at their N-termini (Lévy *et al.*, 1991), which would also disrupt interactions with the conserved pocket for the N-terminal residue. To avoid these problems, measurements of binding affinity were done with a peptide labeled with ^3H to high specific activity by catalytic exchange.

The binding affinity of a ^3H-labeled K^d-restricted peptide (peptide NP1, Table 2) from influenza virus nucleoprotein (Rötzschke *et al.*, 1990a) for empty K^d/huβ2m heterodimers was measured using equilibrium dialysis. The K_D was determined from multiple experiments as $(6.9 \pm 2.3) \times 10^{-8}$ M, in close agreement with the binding affinity of this peptide reported by others (Ojcius *et al.*, 1993) and with the value ($K_D = 5.0 \times 10^{-8}$ M) derived from measuring the binding of the same ^3H-labeled peptide to lipid-linked K^d molecules expressed on the surface of CHO cells (Fahnestock *et al.*, 1994).

Binding affinities of a series of unlabeled analog peptides were determined by quantitation of inhibition of binding of the labeled peptide. Using the knowledge of the three-dimensional structures of class I molecules, we can attempt to interpret differences in peptide binding affinities caused by altering the K^d-restricted peptide on a structural basis. Although the three-dimensional structure of K^d has not yet been determined, the crystal structures of other class I molecules have revealed a common mode of peptide binding (Garrett *et al.*, 1989, Saper *et al.*, 1991; Fremont *et al.*, 1992; Madden *et al.*, 1992; Matsumura *et. al.*, 1992a; Zhang *et al.*, 1992), so that the structure of K^d and peptides bound to it can be modeled upon that of known structures of K^b/peptide complexes (Fremont *et al.*, 1992; Matsumura *et al.*, 1992a; Zhang *et al.*, 1992). To facilitate the following discussion, a view of the peptide binding site of K^b, including the location of pockets A–F, is shown in Figure 4.

Peptides with one or two extensions to the N- or C-terminus of the K^d-restricted peptide were tested for binding affinity. The particular residues introduced in the extended peptides were those amino acids on either side of the nonamer peptide, based upon the sequence of influenza nucleoprotein (Winter & Fields, 1981). Extending the sequence beyond nine residues significantly lowered the binding affinity, with additions at the C-terminus being more detrimental to binding. The specific residues added to the C-terminus of the peptide may have had a particularly deleterious effect upon binding. Peptides eluted from K^d proteins exhibit a preference for an aliphatic amino acid at their C-terminus (Falk *et al.*, 1991; Romero *et al.*, 1991). The side chain of the C-terminal peptide

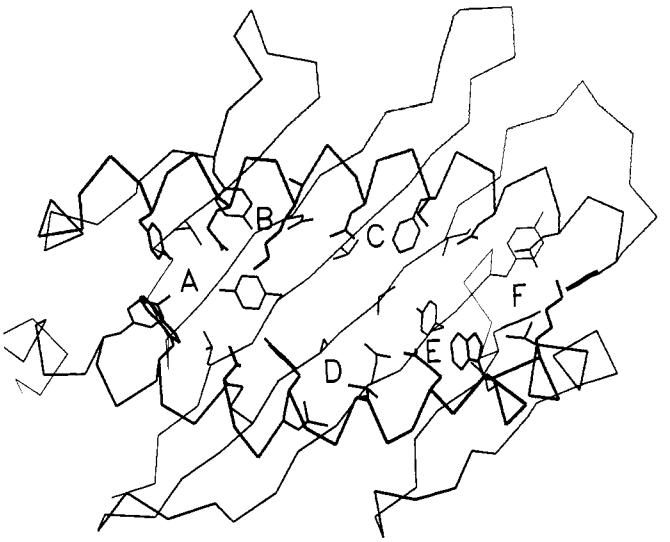

FIGURE 4: View of the peptide binding site of H-2K^b with location of pockets A–F labeled. K^d molecules, used in this study, are expected to have a similar structure, although the side chains within some of the pockets are different. This figure was generated with the program SETOR (Evans, 1993), using coordinates from the K^b structure (Fremont *et al.*, 1992; Zhang *et al.*, 1992).

residue fits into pocket F, consisting in K^d of residues Ser 77, Ala 81, Phe 95, and Phe 116, assuming a three-dimensional structure similar to that of K^b (Fremont *et al.*, 1992; Matsumura *et al.*, 1992a; Zhang *et al.*, 1992). The residue immediately following the end of the K^d-restricted nonamer peptide in influenza nucleoprotein is an arginine (Winter & Fields, 1981). If the additional length of the dodecamer peptide is accommodated by bulging in the middle (Guo *et al.*, 1992), the charged C-terminal peptide residue would have to fit into the F pocket, which is fairly hydrophobic in the K^d molecule and therefore not likely to tolerate charged hydrophilic groups. Indeed, in nonamer peptides, the substitution of the final amino acid for a charged amino acid reduces the K_D to millimolar values (see peptides NP17 and NP18, Table 2), demonstrating the poor complementarity in peptides of optimal length between a charged C-terminal residue in the peptide and pocket F in the K^d molecule. At the peptide N-terminus, the side chain is exposed to solvent, with contacts to pocket A made with main-chain peptide atoms (Fremont *et al.*, 1992; Matsumura *et al.*, 1992a). If the K^d-restricted peptide with a single extension at the N-terminus (peptide NP2) binds such that its N-terminal residue is positioned in pocket A and the extra length is accommodated with a bulge in the middle, there would be a threonine at position 2 instead of the required tyrosine. However, this mode of binding seems unlikely since analysis of a nonamer peptide with a threonine at position 2 (peptide NP11) reveals that the threonine substitution abrogates binding, even though both threonine and tyrosine are polar amino acids containing a hydroxyl group. These observations imply that peptides NP2 and NP3 retain the anchor tyrosine in pocket B and that their extra length is accomodated by extending the peptide beyond pocket A, resulting in significantly lower affinities.

For peptide binding to K^b, the opposite phenomenon was observed: peptides with extensions at the N-terminus bound more poorly than peptides extended at the C-terminus (Matsumura *et al.*, 1992b). Thus it appears that individual class I molecules will differ with regard to their tolerance for extensions of peptides at the N- or C-terminus, with the particular residues added being important determinants of the binding affinity. Our data suggest that versions of a

restricted peptide with extensions at either end bind with significantly lower affinity and imply that adding extra residues for the purpose of labeling a peptide should be avoided.

Although other class I molecules bind either octamer or nonamer peptides (e.g. K^b; Fremont *et al.*, 1992; Matsumura *et al.*, 1992a,b), deletion of either the N-terminal, C-terminal, or both residues of the viral nucleoprotein nonamer peptide greatly reduced the binding affinity. The N-terminal deletion had the greater effect, presumably because the resulting tyrosine at position 1 cannot simultaneously act as the first residue by fitting into pocket A and as the anchor residue by fitting into pocket B. Deletion of the C-terminal residue leaves a leucine as the final residue, and this side chain fulfills the K^d preference for aliphatic residues at this position to fit into pocket F, but the affinity is still reduced by ~1000-fold compared to the nonamer peptide. These observations imply that, *in vivo*, K^d molecules present the nonamer version of the influenza nucleoprotein peptide, with longer or shorter versions being significantly reduced in affinity such that a low percentage if any are presented. However, it is interesting to note that an octamer and nonamer version of a peptide from a *Plasmodium berghei* circumsporozoite protein (peptides KD2 and KD3; Romero *et al.*, 1991) do not show a large discrepancy in binding affinities, with the octamer binding with only 10-fold lower affinity than the nonamer. Thus for K^d-restricted peptides, the preference for a nonamer or octamer appears to be dependent on the particular peptide sequence.

As might be predicted, changing the tyrosine residue at position 2 affects binding affinity. A peptide with phenylalanine at this position (peptide NP10) still binds with fairly high affinity, as would be predicted by the finding that some of the peptides eluted from K^d molecules contained phenylalanine at position 2 (Falk *et al.*, 1991). However, no detectable binding is seen with an alanine-substituted peptide (peptide NP12) or a threonine-substituted peptide (peptide NP11). The poor binding of stoichiometric iodinated derivatives (Tsomides & Eisen, 1993) of the K^d-restricted peptide (peptides NP13 and NP14) demonstrates that the addition of the large iodine atom, as is commonly done for labeling purposes, has dramatic effects on the binding properties of the iodinated peptide compared to the peptide in its original state. These results suggest that great care must be used in labeling peptides for binding studies to make sure that the labeled peptide is not chemically altered in such a way as to change its binding properties.

The effects of single substitutions at the interior nonanchor positions were next explored using five peptides in which the natural residues at positions 3–7 were progressively altered to cysteine. On the basis of the structure of K^b, positions 3, 6, and 7 are predicted to fit into pockets D, C, and E respectively, while positions 4 and 5 are predicted to be solvent exposed (Matsumura *et al.*, 1992a). As expected, altering peptide residues 4 and 5 to cysteine has very little effect upon binding (peptides NP20 and NP21, Table 2). Changing residues 3 and 7 (peptides NP19 and NP23) also has only small effect upon binding, suggesting that pockets D and E in K^d can accommodate a number of different residues, in accordance with sequencing data of peptides eluted from purified K^d (Falk *et al.*, 1991). At position 6, a 14-fold reduction in affinity is seen upon substitution with cysteine (NP22), suggesting a slight preference in pocket C for residues other than cysteine. Indeed, in peptides eluted from K^d molecules, lysine and phenylalanine were frequently found at this position (Rammensee *et al.*, 1993), implying that pocket C prefers side chains larger than cysteine. Knowledge of the binding affinities of these cysteine-substituted peptides will allow greater ease of interpretation of future work involving covalent coupling of cysteine-substituted peptides to a solid support for kinetic and equilibrium analyses of peptide binding using a surface plasmon resonance assay similar to that reported by Khilko *et al.* (1993).

A previous report describes competitor analogs of K^d-restricted peptides synthesized using polyproline or polyglycine spacers (Maryanski *et al.*, 1990). Dodecamer pentaproline and pentaglycine analogs were found to compete for binding of a 13-mer K^d-restricted peptide, with the pentaproline analog being the more active. We synthesized comparable nonamer pentaproline and pentaglycine versions of the K^d-restricted influenza nucleoprotein peptide (peptide NP1) in order to measure their binding affinities for empty K^d. The pentaproline (peptide NP24) and pentaglycine (peptide NP25) analogs both showed reduced binding compared to the original peptide, possibly confirming the importance of secondary anchor residues in peptide binding, as was noted in the binding of peptides to the human class I molecule HLA-A2 (Ruppert *et al.*, 1993). By contrast, the dodecamer pentaproline analog of the 13-mer K^d-restricted peptide was as active as the original peptide in a functional competition assay (Maryanski *et al.*, 1990), perhaps because this pentaproline analog was closer to the optimal nonamer length than the original peptide. We and the previous workers (Maryanski *et al.*, 1990) both observed that the polyglycine analog was a less effective inhibitor, which may be due to the absence of a proline at position 4 (a frequently observed residue at this position in sequences of peptides eluted from purified K^d molecules; Rammensee *et al.*, 1993), or the greater flexibility allowed by a series of glycines that could adversely affect binding.

The four unaltered K^d-restricted peptides (NP1, KD1, KD2, and KD3) represent three different sequences with only the anchor positions in common, but all bind with fairly comparable affinities (K_D = ~10^{-7}–10^{-8} M). These values represent slightly lower affinities than have been reported for restricted peptides binding to K^b (Matsumura *et al.*, 1992b) but comparable or higher than affinities of HLA-A2 restricted peptides (Ruppert *et al.*, 1993). These results suggest that optimal peptides binding to K^d and other class I molecules have K_D values in the nanamolar range and that alteration of anchor residues or the length of the peptide drastically affect the binding affinity.

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