Decrypting the Structure of Major Histocompatibility Complex Class I-Restricted Cytotoxic T Lymphocyte Epitopes with Complex Peptide Libraries

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Summary

Complex synthetic peptide libraries with defined amino acids in one or more positions of the H-2K^b–restricted cytotoxic T lymphocyte (CTL) epitopes SIINFEKL and RGYVYQGL and mixtures of 19 amino acids in the remaining positions were used to analyze the structural requirements of peptide binding to MHC class I molecules and antigen recognition by CTLs. This approach provides means to assess semiquantitatively the contribution of every amino acid to the binding of peptides to major histocompatibility complex (MHC) molecules without biases introduced by naturally processed peptides. Primary and secondary anchor residues were defined for their major contribution to the binding efficiency of the peptides. In contrast to primary anchors, secondary anchor amino acids vary greatly in their side chains and position in the sequences. All amino acids in the octapeptide sequences were found to exhibit positive or negative influences on binding to the MHC molecules and on recognition of the resulting complexes by CTLs. Strong interdependence of the effects of the individual residues in the epitope sequences was demonstrated. CTL responses to peptide libraries were suppressed when residues were introduced; however, they were augmented when the critical residues for T cell recognition were fixed, suggesting a potential use of the peptide libraries for defining epitope sequences in general.

C TLs recognize peptide antigens bound to cell surface glycoproteins that are encoded by the highly polymorphic class I MHC (MHC-I)[1] genes (1). Thereby, the specificity of T cell responses is governed by two selective binding events. The first step is selection and binding of suitable peptides by MHC molecules; the second is recognition of the unique conformation of MHC–peptide complexes by complementary TCRs. The current understanding of the rules for peptide selection by MHC molecules is derived from sequencing of peptides and natural peptide libraries extracted from MHC proteins (2, 3), from analyses of the effects of mutations in sequences of unknown CTL epitopes on peptide binding to MHC molecules and on T cell responses (4, 5), as well as from crystal structure analyses (6–8) and molecular dynamic studies (9) of defined MHC–peptide complexes.

The MHC-I ligands are mostly octa- or nonapeptides and show MHC allele–specific sequence motifs (2), as determined by pool sequencing (10) of natural isolates. The motif-determining amino acids were interpreted as anchor residues. Crystal structure analysis has confirmed this concept. A peptide-binding cleft was identified that is framed by two α-helices and a β-pleated sheet and is stabilized from beneath by the noncovalently associated β2-microglobulin (6, 7, 11). Specific pockets in the binding groove accommodate the anchor residues of the peptide. The orientation of the peptides is determined by conserved side chains of the MHC-I protein that compensate the NH$_2$- and COOH-terminal charges. Peptide-free MHC-I molecules are not stable at physiological temperature of 37°C (12) but are expressed when cells are incubated at 26°C (13, 14).

The ternary complex of α chain, β2-microglobulin, and peptide is assembled during biosynthesis of the MHC-I protein in the endoplasmic reticulum (15). Most of the peptides are generated in the cytosol by proteolytic enzyme systems and are delivered into the endoplasmic reticulum by specialized ATP-binding casette-type transporter proteins (16). However, the sequences of the source proteins are not random (17),

and the processing enzymes of the proteasomes (18, 19), the peptide transporters (20, 21), as well as potentially involved molecular chaperons (22, 23) exhibit sequence and size preferences. Antigen processing creates a preselected peptide repertoire available for association with MHC molecules. But peptides either from natural sources or chemically synthesized can also be loaded into MHC-I molecules when applied externally, provided they fulfill the sequence motif requirements of the particular MHC-I allomorph (12, 24). By this approach, selection of peptides by MHC molecules and formation of the specific ligand structures for recognition by complementary TCRs can be studied independently from biases imposed by the processing machinery.

We have used complex synthetic peptide libraries (25, 26) to investigate the detailed structure of MHC–peptide complexes, the contribution of primary anchor, secondary anchor, and nonanchor residues to peptide–MHC interaction and to the response of T cells, and the interdependence of the effects of individual amino acids. To meet the quality criteria required for reliable biological applications, the synthesis of these complex mixtures was optimized to yield reproducibly close to equimolar representation of individual peptides (27). The qualities of the libraries were controlled by amino acid analysis, pool sequencing (10), and mass spectrometry (28). Deviation from equimolar representation of the peptides were within the error limits of these analytical procedures and estimated to be 3%.

The basic design of the peptide libraries used in this study follows the sequences of two known CTL epitopes: SIINFEKL (derived from ovalbumin; 29, 30) and RGYVYQGL (derived from the nucleoprotein of vesicular stomatitis virus [VSV]; 31). Both epitopes are presented by the mouse MHC-I allomorph H-2K^b, which requires amino acids with aromatic side chains (phenylalanine or tyrosine) in position 5 and with aliphatic side chains (leucine, methionine, isoleucine, or valine) in position 8 of octapeptides (2). Single or combinations of amino acids in these sequences were replaced by equimolar mixtures of 19 amino acids (all protein amino acids except for cysteine). With such highly complex peptide libraries, the impact of individual residues could be specified by randomizing the remaining positions and studied by using MHC stabilization assays (12, 24) and cell-mediated lympholysis (CML) assays. The described approaches and results should support the analysis of T cell epitopes as well as the design of MHC blockers (32) and antagonists of natural epitopes (33, 34). On the other hand, the results show the limits of exact prediction of the properties of CTL epitopes.

Materials and Methods

Cell Lines and Cell Culture Conditions. The mutated thymoma cell line RMA-S (35) was obtained from Dr. Rammensee (DKFZ, Heidelberg) and was cultured in DMEM (Gibco Laboratories, Eggenstein, FRG) that was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), Hepes (5 mM), β-mercaptoethanol (30 μM), and 5% heat-inactivated (45 min, 56°C) FCS. For stabilization assays, RMA-S cells were kept overnight at 26°C to allow accumulation of peptide-free MHC-I molecules. The CTL clones 4G3 (36) and 269/3 (37) were grown

in the same medium but with 10% FCS and, as a source of growth factors, with 2% conditioned medium from rat spleen cell cultures that had been stimulated with Con A (5 μg/ml) for 24 h. The CTLs were restimulated every other week with γ-irradiated (3,300 rads) stimulator cells (ovalbumin-expressing EG7.OVA cells (29) for 4G3 and peptide-pulsed spleen cells from C57BL/10 mice for 269/3) as described elsewhere (30, 37). Hybridoma cell lines producing mAbs B8.24.3, Y3, AF6-88.5.3, or 28-13-3S were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM with 5% FCS.

Peptides. The synthetic octapeptides and octapeptide mixtures were prepared by solid-phase peptide synthesis using Fmoc L-amino acid-*p*-benzyloxybenzylalcohol-polystyrene resins loaded with a single amino acid, or equimolar mixtures of 19 resins for all protein amino acids without cysteine (26). A robot system (RSP 5032; Tecan, Hombrechticon, Switzerland) equipped for multiple peptide synthesis and supported by an optimized software for multiple peptide synthesis (MultiSyn Tech, Bochum, FRG) was used. Couplings were done either with prepared equimolar mixtures of Fmoc amino acids equimolar with the coupling sites on the resins for the X positions or with a fivefold molar excess with respect to the coupling sites of single Fmoc amino acids for the O positions. The dicyclohexylcarbodiimide/1-hydroxybenzotriazole method was used (27). The procedure was optimized to generate equimolar representation of the peptides in the libraries and is characterized by extended coupling times, double coupling, an initially high content of the solvent dichloromethane, and open vessels to allow evaporation of dichloromethane and thereby concentration of the reagents during the course of the coupling cycles. The peptides were cleaved off the resins, and side chain were deprotected with TFA/phenol/ethanedithiol/thioanisole (96:2:1:1 [vol/vol/vol/vol]). The resins were filtered off, and peptides were precipitated by adding cold *n*-heptane/diethyl ether (1:1 [vol/vol]), washed, and lyophilized from acetic acid/water/*tert*-butyl alcohol (1:10:50 [vol/vol/vol]). The peptide libraries were subjected to amino acid analysis, pool sequence analysis (10), electrospray mass spectrometry (28), and HPLC–mass spectrometry analysis to establish their sequences, to define side products, and to evaluate the amino acid compositions in the X positions. Equimolarity could be validated for the peptide libraries within the error limits of the analytical methods (38).

Randomized positions were designated X; defined positions, O. Indices denote the stoichiometry. Peptides and peptide mixtures were dissolved in DMSO at a concentration of 20 mg/ml and diluted in water to 200 μg/ml. The concentrations were determined by micro BCA assays and are given in grams per liter in order to account for the complexities of the mixtures.

MHC Stabilization Assay. The efficiency of peptide binding to K^b was assessed in stabilization assays (24): 100,000 RMA-S cells that had been cultured overnight at 26°C were incubated for 30 min at room temperature in a total volume of 100 μl of DMEM plus 0.25% BSA with serial threefold dilutions of the peptides. BSA was used instead of FCS for cell suspensions in order to avoid potential degradation of peptides by serum proteases (39). Temperature was then shifted to 37°C and held for 45 min, after which cells were collected and stained with FITC-labeled mAb B8.24.3, which detects conformationally intact K^b. The level of stabilized K^b was analyzed by flow cytometry with a FACScan® (Becton Dickinson, Heidelberg, FRG). Samples were gated according to forward and side scattering, and fluorescence values were measured with a logarithmic mode setting to obtain better resolution. List mode data were transferred to a personal computer with the FAST488 system (JTES BioTec, Freienwill, FRG), transformed to linear fluorescence values, and averaged to obtain mean fluorescence

intensities (MFI) using the MFI software (E. Martz, University of Massachusetts, Amherst, MA).

Fig. 1 shows titration curves for SIINFEKL and X_8. From such titration curves, the concentrations required for half-maximal effect were calculated by using the formula of the occupancy concepts and linear regression over plots with $\log (p) = \ln[p/(1 - p)]$ versus $\log$ (concentration) with $p = (MFI_{exp} - MFI_{min})/(MFI_{max} - MFI_{min})$ (40). In figures in which data from different experiments are compared, results are standardized and expressed as stabilization index (SI) in order to compensate for variations among the different experiments. SI values were calculated by dividing the concentrations of the X_8 peptide library required to achieve half-maximal effect by the corresponding concentration needed for the indicated test peptide. All tests were done in duplicate and were reproducible in two independent titrations.

CML Assay. The efficiency of peptide recognition by CTLs was determined in ^{51}Cr release assays (30): ^{51}Cr-labeled (New England Nuclear, Dreieich, FRG) target cells (RMA-S cells that had been cultured overnight at 26°C) were incubated for 30 min at room temperature with peptides that were serially diluted in DMEM plus 0.25% BSA. Subsequently, effector cells were added to yield a volume of 200 μl, a final BSA concentration of 0.5%, and an effector to target ratio of 4:1. After 5 h at 37°C, 150 μl of the supernatants was removed, and radioactivity released from the cells was measured. Percent specific ^{51}Cr release was calculated as (experimental release - spontaneous release)/(total release - spontaneous release) × 100.

Results

Synthetic Peptide Libraries Stabilize Conformationally Intact K^b. Since MHC-I proteins expressed on cell surfaces are normally occupied by endogenous peptides, only very few peptide receptors are free for binding measurements. However, some tumor cells like RMA-S (35) lack peptide transporters that deliver cytosolic peptides to the endoplasmic retic-
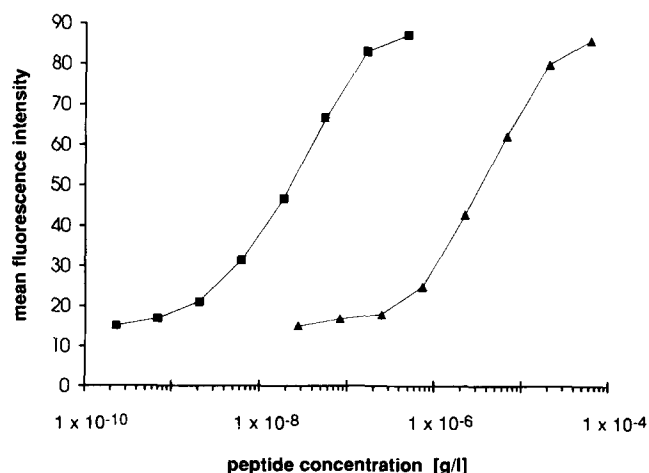
Figure 1. Stabilization of K^b by SIINFEKL and X_8 peptide library. RMA-S cells cultured overnight at 26°C were incubated for 30 min with different concentrations of SIINFEKL (■) or X_8 (▲). After incubation for 45 min at 37°C, conformationally intact K^b molecules were detected by flow cytometry using the FITC-labeled mAb B8.24.3. The means of linearized fluorescence intensity values are shown as a function of peptide concentration.

ulum and, as a consequence, show drastically reduced cell surface MHC-I expression (12). Mutant cells, cultured at 26°C, regain normal expression levels of MHC-I molecules, which are mostly free of peptides and denature when the temperature is increased to 37°C (13). These "empty" MHC-I molecules can be stabilized by adding synthetic peptides to the cells (24). With mAbs that specifically detect conformationally intact and therefore peptide-loaded MHC-I protein, the efficiency of peptide binding to these receptors can be assessed. Analysis by confocal microscopy of the binding of a fluorochrome-labeled peptide to transporter-deficient RMA-S cells demonstrated allele-specific accumulation of the antigen at the cell surfaces before its internalization (14). From these experiments, we concluded that MHC stabilization assays are direct measurements of peptide binding to MHC-I molecules on cell surfaces.

In the standard stabilization assays used here, RMA-S cells were cultured overnight at 26°C to allow accumulation of peptide-free MHC-I molecules at the cell surfaces. Then test peptides were added, and cells were incubated for 30 min at room temperature. During an additional incubation period of 45 min at 37°C, empty MHC-I proteins denatured. The remaining conformationally intact K^b molecules were detected with mAb B8.24.3. This assay depends on the specificity of the antibody and the quality of the peptide libraries.

Several MHC-I–specific mAbs that are sensitive to the particular peptide bound to the MHC molecule have been described (41). We therefore analyzed the effect of three K^b-restricted peptides (SIINFEKL, RGYVYQGL, and FAPG-NYPAL) on K^b recognition by B8.24.3 and three additional antobodies (Y-3, AF6-88.5.3, and 28-13-3S). K^b recognition by B8.24.3 was not influenced by the particular peptide sequence (data not shown). Similar results were obtained with Y-3 and AF6-88.5.3. On the other hand, 28-13-3S detected K^b only as a complex with SIINFEKL, as it was described by Hogquist et al. (41). Antibody binding to D^b, the second MHC-I molecule expressed by RMA-S cells, could potentially interfere with our analyses. But no cross-recognition of D^b by B8.24.3 was detected when defined D^b-binding peptides were tested in stabilization assays (data not shown). In the case of complex peptide libraries, cross-recognition of D^b cannot be excluded that easily. However, K^b and D^b require different anchor amino acids in the fourth position from the COOH terminus of the peptide (K^b, tyrosine; D^b, asparagine). The OX_7 peptide library with asparagine in position 5 (XXXXNXXX) therefore would be most suitable for binding to D^b. In spite of this fact, XXXXNXXX is very inefficient in MHC stabilization assays with B8.24.3 (data not shown).

To investigate the representation of individual peptides, random peptide mixtures were characterized by amino acid analysis, by pool sequencing (10, 38), and in the case of less complex mixtures, by HPLC–mass spectrometry (28, 38). Deviations from the expected representation of single amino acids in randomized positions were within the error limits of the analytical procedures. Therefore, we estimated deviations from equimolar representation of single peptide species in complex peptide mixtures to be within these limits and

maximally 3% (38). Moreover, mass spectrometry analyses of numerous octapeptides synthesized by the same procedure used in this study revealed only minor amounts of truncated peptides (data not shown). Pentapeptides investigated in our laboratory (42) and by Reddehase et al. (43) were effective only at very high concentrations (millimolar range), which were not achieved in the assays presented here.

Using the highly complex peptide X_8 library, which consists of 19^8 = 16,983,563,041 octapeptides, the expression of K^b could be titrated to the same saturation level obtained with the well-defined CTL epitope SIINFEKL (Fig. 1). This result indicates that a sufficiently high fraction of the peptides in X_8 can bind to and stabilize K^b. However, 180 times more X_8 than SIINFEKL was required to obtain half-maximal stabilization effect. This shift of the X_8 titration curve with respect to the SIINFEKL titration curve was larger than calculated from the proportion of peptides with the correct combination of K^b anchor amino acids in this library. Pool sequence analysis of natural peptide libraries extracted from K^b revealed two anchor positions in the octapeptides. In the fifth position 2 amino acid residues (phenylalanine and tyrosine) and in the eighth position 4 amino acid residues (leucine, methionine, isoleucine, and valine) were found (2). Thus, the $19^6 \times 2 \times 4$ = 376,367,048 peptides in X_8 with the sequence XXXX(F,Y)XX(L,M,I,V) conform to the K^b-binding motif. If these peptides all bound equally well to this MHC-I molecule, 45 but not 180 times more peptide would be required for the half-maximal stabilization effect. Such apparent deviations were also found for sublibraries with one defined amino acid and seven X positions (OX_7; Fig. 2 a). These observations suggest an influence of amino acids in nonanchor positions on the MHC-binding properties of peptides. Analysis of the OX_7 sublibraries that were designed to scan the SIINFEKL sequence reveals enhanced binding promoted by all peptide libraries except for XXX-NXXXX and XXXXXEXX. These two sets act in a destabilizing manner when compared with X_8, which means that more OX_7 than X_8 is required for half-maximal stabilization of K^b. Sublibraries with phenylalanine in position 5 and leucine in position 8, the known motif residues, most efficiently promoted binding to K^b. The sublibraries SXXXXXXX, XIXXXXXX, XXIXXXXX, and XXXXXXXKX revealed additional anchor functions of the defined amino acids.

In a reciprocal experiment, single amino acids in SIINFEKL were replaced by a mixture of 19 different amino acid residues. The inverse picture was obtained (Fig. 2 b). Randomizing defined positions in SIINFEKL caused reduced stabilization efficiencies in all cases, which is indicated by the higher concentration of peptides required for half-maximal K^b expression. This loss of binding capacity was most pronounced for SIINXEKL and SIINFEKX, in which the motif amino acids were randomized. The two sets of results shown in Fig. 2 provide a synthetic proof for the anchor residue concepts originally derived from pool sequencing of natural peptide libraries extracted from MHC-I molecules (2). Moreover, additional anchors are apparent. OX_7 sublibraries with serine, isoleucine, isoleucine, and lysine in positions 1, 2, 3, and 7, respec-
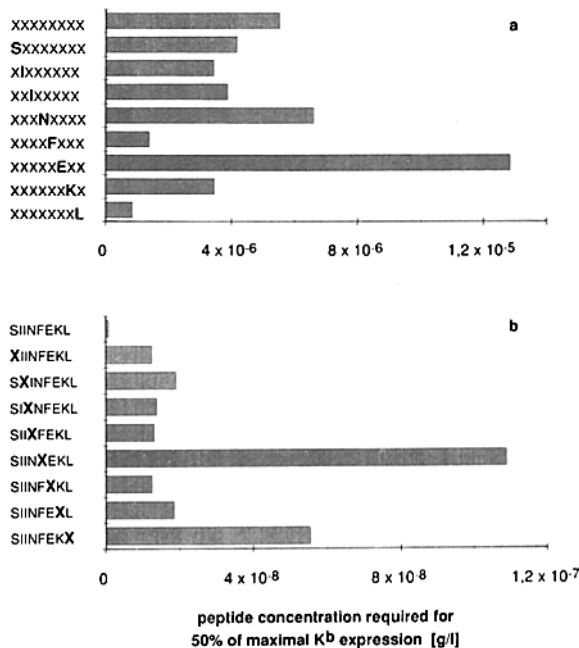
Figure 2. Impact of individual amino acids of the CTL epitope SIIN-FEKL on peptide binding to K^b. Concentrations of peptides were titrated, and the amount required for half-maximal K^b expression was determined. (a) One defined amino acid is introduced into the positions of the X_8 octapeptide sublibraries OX_7. (b) Individual positions in the defined SIIN-FEKL sequence were randomized in OX_7 sublibraries by replacing the defined amino acids with a mixture of 19 different amino acids.

tively, showed higher efficiency for K^b stabilization than X_8 (Fig. 2 a). A comparison of the results shown in Fig. 2, a and b, reveals an interesting discrepancy: asparagine in position 4 and glutamic acid in position 6 are clearly classified as destabilizing when OX_7 sublibraries are compared with X_8 (Fig. 2 a). In more defined peptide libraries, their replacement by a mixture of 19 amino acids resulting in SIIXFEKL and SIINFXKL caused reduced binding efficiency when compared with SIINFEKL (Fig. 2 b). Obviously, these two amino acids are destabilizing when they are isolated in OX_7 peptide libraries but have stabilizing effects in the sequence context of SIINFEKL.

Interrelationship of Amino Acids in the CTL Epitope SIIN-FEKL. The analysis was extended, and increasing numbers and combinations of fixed positions in the sequences of the peptide libraries were tested. The results obtained with sublibraries designed for different numbers of defined amino acids — O_2X_6, O_3X_5, and O_4X_4 — are shown in Figs. 3 and 4. To eliminate experimental variations, the data were standardized by dividing the concentrations of the sublibraries required for half-maximal stabilization of K^b by the corresponding concentration of X_8 determined in the same experiment. This ratio is expressed in reciprocal form as the SI. Obviously, increasing numbers of fixed positions result in increasing binding efficiencies. Besides the strongly stabilizing motif amino acids in positions 5 and 8, other side chains also enhanced the efficiency of peptide binding (Fig.
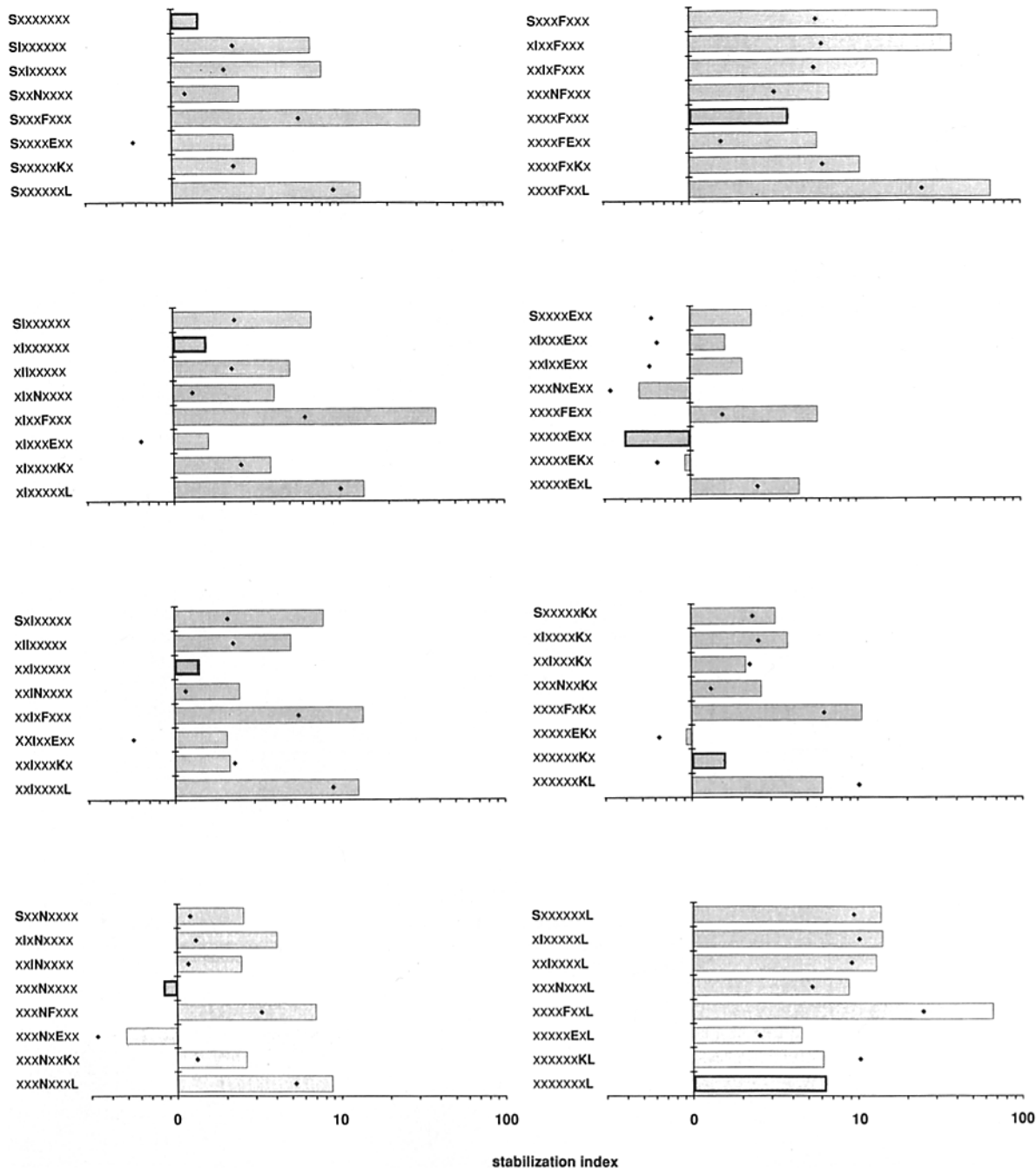
Figure 3. Interdependence of different side chain effects on MHC-I stabilization by peptide sublibraries. The SIINFEKL sequence was scanned with combinations of two defined residues in O_2X_6 peptide sublibraries. Each panel shows the results obtained with a group of sublibraries with one fixed amino acid kept constant throughout the entire group and a second fixed amino acid varied in the sequence positions. The relative efficiencies of peptide binding are illustrated as SI values (*bars*), which were calculated as follows: the concentration of X_8 required for half-maximal K^b expression was divided by the corresponding concentrations in the case of the test peptides. SI values calculated from those for the OX_7 peptide sublibraries (*thickly framed bars*) are shown as diamonds.

3). Combinations of one of the motif residues, phenylalanine in position 5 or leucine in position 8, with a secondary anchor (serine in position 1 or isoleucine in position 2 or 3) yielded efficiencies of K^b stabilization comparable to that of XXXXFXXL, which has both primary anchor residues fixed. The destabilizing effects of asparagine and glutamic acid were compensated by anchor amino acids and by serine in position 1 and isoleucine in position 2 or 3 (Fig. 3).

Fig. 4 summarizes the influence of additionally defined residues on peptides that conform to the K^b-binding motif.
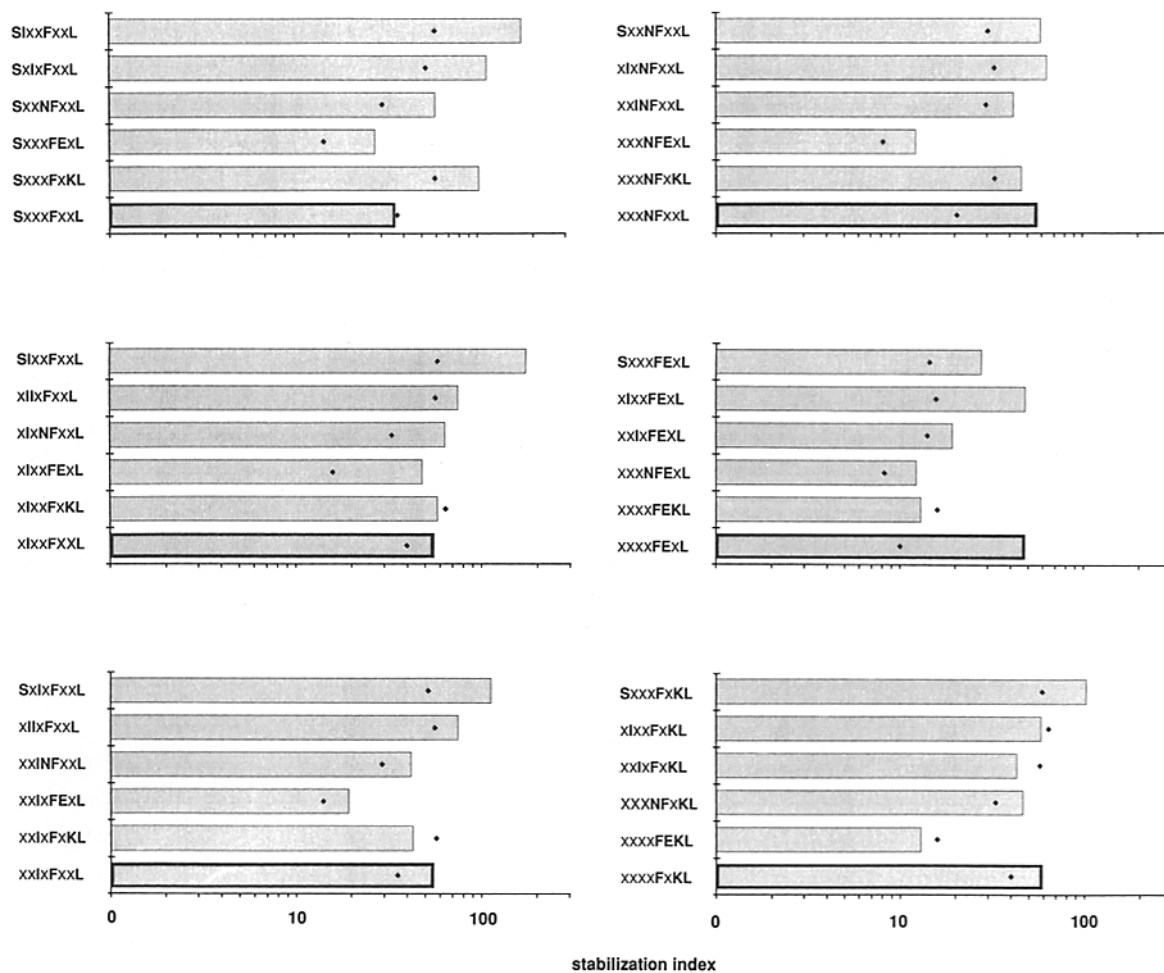
Figure 4. Additional influences of non-anchor amino acids on peptide binding to MHC-I. In peptide sublibraries with the two dominant anchor residues fixed (results are shown in Fig. 3), a third and fourth defined amino acid were introduced. Each panel shows the results obtained with sublibraries in which the two anchor residues plus one additional amino acid were kept constant (*thickly framed bars*) and one fourth fixed amino acid was varied in its sequence position (*bars*). The SI values for the individual peptide sublibraries are shown. For each of these libraries, SI values calculated from the SI of the OX_7 sublibraries shown in Fig. 3 are presented as diamonds.

Even with both anchors fixed, considerable influences by these residues can be observed. The SI values for SIINFEKL in these experiments were found to be between 450 and 500. Peptide libraries with up to five defined positions yielded SI values of maximally 180. A sublibrary with all primary and secondary anchor positions occupied by stabilizing amino acids, SIIXFXXL, was still 2.6 times less efficient (SI = 170) than SIINFEKL (SI = 450) when tested in the same experiment. Obviously, the efficiency of peptide binding to MHC-I molecules is determined by the entire sequence and not only by amino acids in the anchor positions.

The SI values of OX_7 peptide sublibraries reflect the relative contribution of the corresponding defined amino acids to the mean stabilization energy of the peptide–MHC complexes. According to the equation that relates energy (Gibbs free energy) changes with the concentrations of the reactants, these individual energy contributions are roughly proportional to the logarithms of the SI values. If the stabilizing or destabilizing effects of the individual amino acids were indepen-

dent of the other amino acids in the peptide, summation of the log SI values for the OX_7 sublibraries would give the SI for the entire peptide. We have presented data that illustrate mutual dependence of the impact of the individual amino acids on the MHC binding properties of octapeptides. Using the O_2X_6, O_3X_5, and O_4X_4 sublibraries, we investigated this interdependence of the effect of the amino acids in further details and studied potency and limitation of predicting peptide binding efficiencies on the basis of SI values. In Figs. 3 and 4, the measured SI values (*bars*) are compared with those calculated from the SI values of the OX_7 peptide sublibraries (*diamonds*). These reference SI data are shown as thickly framed bars in Fig. 3. The conclusion from the comparison of calculated and measured SI values for O_2X_6 peptide sublibraries is that the two sets of values do not match. The degree of correlation, however, improves with distance between the defined amino acids in the sequence. The best correlations are found for sublibraries with combinations of defined amino acids near the NH_2 terminus (serine in position 1 and the

two isoleucines in positions 2 and 3) and defined amino acids near the COOH terminus (lysine in position 7 and leucine in position 8) of the octapeptides. The lowest degree of correlation is seen for combinations of the centrally located amino acids: asparagine, phenylalanine, glutamic acid, and any other amino acid (Fig. 3). Calculated and measured SI values deviate strongly especially in the case of O_2X_6 sublibraries carrying glutamic acid in position 6. With one exception (XXXX-XXKL), the predicted SI values are underestimated.

Analysis of the results obtained with less complex sublibraries, O_3X_5 and O_4X_4, that are characterized by fixed primary anchor amino acids (phenylalanine in position 5 and leucine in position 8) confirms the aforementioned conclusions (Fig. 4). The best match of calculated and measured SI values for O_3X_5 libraries (Fig. 4, *thickly framed bars*) was obtained with SXXXFXXL, in which the defined amino acids are maximally spaced. The degree of correlation of the two SI values declined when the third defined amino acid was placed closer to the primary anchors. Introducing defined secondary anchor amino acids into the sublibraries resulted not only in improved binding efficiency, but also in a higher degree of correlation of measured and calculated SI values (Fig. 4). In contrast, introducing destabilizing amino acids led to a strong deviation between the predicted and observed binding efficiencies. Further reduction in the complexity of the peptide libraries by introducing more defined amino acids improved peptide binding to K^b. However, the reliability of the prediction of binding efficiencies declined. In the case of SIIXFXXL (all stabilizing amino acids defined), an SI value of 83 is predicted but in fact an SI of 170 was measured. The complete CTL epitope SIINFEKL reproducibly yielded SI values of 450–500. The predicted SI is 43. The comparison of calculated and experimental SI values supports the previous conclusion that the impacts of individual amino acids of an epitope sequence on peptide binding to MHC molecules are mutually dependent. This interdependence impedes predictability of the MHC binding properties of CTL epitopes.

Recognition of Complex Peptide Libraries by CTLs. The CTL clone 4G3 is specific for the ovalbumin epitope SIINFEKL bound to K^b (30). To analyze the efficiency of peptide recognition by 4G3, we used cytolysis of RMA-S target cells that were cultured for one night at 26°C to allow accumulation of empty K^b on their surfaces. Toxic effects of the peptides or peptide libraries were not detected when they were incubated with RMA-S cells alone (data not shown). SIIN-FEKL concentrations of ∼400 fM were needed for induction of the half-maximal cytolytic effect of the CTLs (Fig. 5). The same degree of specific cytolysis required a 1.8 × 10^6 times higher concentration of X_8 than of SIINFEKL. Since SIINFEKL itself is only 1 of 16,983,563,041 peptides in the X_8 library, 4G3 responds to nominally ∼10,000 different peptides. This number is not exactly defined because neither the range of affinities of K^b for the different peptides in the library nor the efficiencies of recognition of the MHC–peptide adducts by the TCR are known. A few peptides that bind to K^b much more efficiently than SIINFEKL or that produce an MHC–peptide complex that is bound with a
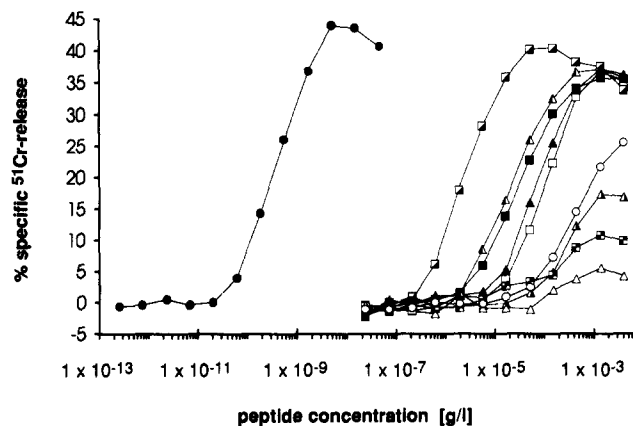

Figure 5. CTL responses to complex peptide libraries. SIINFEKL (●), X_8 (○), SXXXXXXX (◪), XIXXXXXX (■), XXIXXXXX (▲), XXXNXXXX (▲), XXXXFXXX (△), XXXXXEXX (□), XXXXXXKX (▲), and XXXXXXXL (◨) at different concentrations were incubated with RMA-S cells at room temperature. Subsequently, 4G3 CTLs were added, and after a 5-h incubation, specific ^{51}Cr release was determined.

higher affinity by the TCR could account for this enhanced CTL response.

The impact of individual residues on CTL responses is revealed by the analysis of target cell lysis induced by the eight OX_7 sublibraries (Fig. 5). The most striking observation in these experiments is the strong suppression of efficiency of cytolysis caused by the dominant anchor amino acids: phenylalanine in position 5 and leucine in position 8. Also, the secondary anchor isoleucine in position 3 shows this effect. This decrease of cytolysis is most likely caused by increased competition among peptides that can bind to K^b efficiently. OX_7 sublibraries with other defined residues (serine in position 1, isoleucine in 2, asparagine in 4, glutamic acid in 6, or lysine in 7) induce enhanced target cell lysis by 4G3 when compared with the effect of X_8 (Fig. 5). The concentrations of library peptides required for half-maximal cytolysis, however, were lower than those calculated from the frequency of SIINFEKL present in these OX_7 mixtures consisting of 893,871,739 different peptides. The sublibrary SXXXXXXX was ∼100,000 times, the sublibraries XXX-NXXXX and XXXXXEXX were ∼11,000 times, and the sublibraries XIXXXXXX and XXXXXXKX were ∼3,000 times more efficient than calculated from the complexity of these libraries. These results again indicate strong degeneracy of antigen recognition by 4G3.

Structure of the VSV Nucleoprotein Epitope RGYVYQGL. The results obtained with the ovalbumin epitope SIINFEKL were compared with the results from the analysis of the CTL epitope RGYVYQGL (31). The response of clone 269/3 to this epitope is restricted by K^b (37). Fig. 6 *a* shows results from stabilization assays done with OX_7 peptide libraries that scan the RGYVYQGL sequence. The contribution of the anchor amino acids — tyrosine in position 5 and leucine in position 8 — is prominent. Tyrosine in position 3 and valine in position 4 served as secondary anchors. The residues
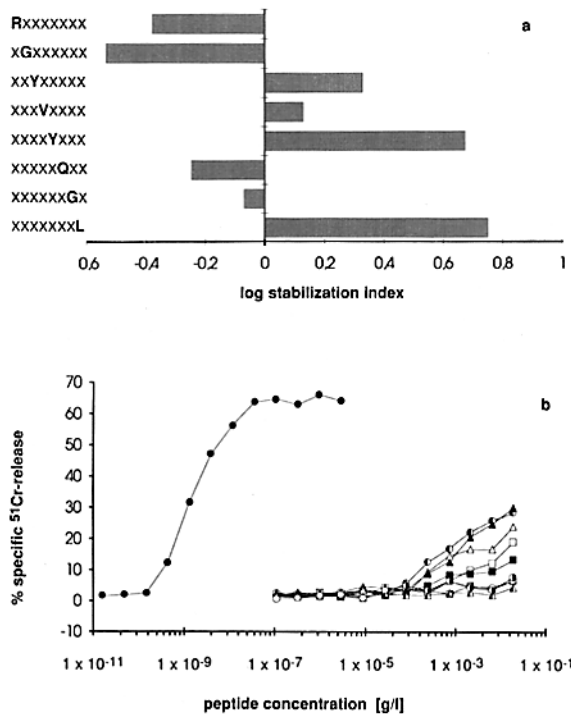
Figure 6. Epitope structure of the VSV antigen RGYVYQGL. (a) SI values for OX7 peptide libraries that scan the sequence of the CTL epitope RGYVYQGL were determined and are shown on logarithmic scale. (b) A CML assay was performed with the same peptide libraries and with CTL clone 269/3 as effector and RMA-S as target cells. The peptides were RGYVYQGL (●), X8 (○), RXXXXXXX (◐), XGXXXXXX (□), XXYXXXXX (■), XXXVXXXX (△), XXXXYXXX (◪), XXXX-XQXX (▲), XXXXXXGX (▲), and XXXXXXXL (◑).

arginine, glycine, glutamine, and glycine in the positions 1, 2, 6, and 7, respectively, are destabilizing; this was especially true for arginine in position 1 and glycine in position 2. Thus, only position 3 contained a secondary anchor amino acid for both CTL epitopes tested. The side chains in this position, however, showed a higher degree of heterogeneity than that found for the dominant anchor amino acids. Moreover, the positions of the secondary anchor residues in an epitope varied with the sequence of the epitope.

In CML assays with the CTL clone 269/3 (which recognizes RGYVYQGL and K^b) X8, the library with the highest complexity, did not induce significant lysis of RMA-S target cells (Fig. 6 b). Detectable cytolysis, however, was induced with sublibraries in which arginine in position 1, glycine in 2, tyrosine in 3, valine in 4, or glutamine in 6 was fixed. OX7 sublibaries with one of the dominant anchor amino acids defined (tyrosine in position 5 or leucine in position 8) were as inefficient as the X8 mixture, confirming the observations made with the ovalbumin-specific clone 4G3. The inability to detect X8-induced cytolysis of RMA-S cells by the CTL clone 269/3 is compatible with the complexity of this peptide library. RGYVYQGL is 1 of 16,983,563,041 peptides in this mixture and would not be detectable in the concentration range tested. Higher concentrations of X8 were not tested in order to avoid potential problems with limited

solubility of individual peptides in the mixture. RGYVYQGL is 1 of 893,871,739 peptides in the OX7 sublibraries. However, when the titration curves obtained with the two most efficient sublibraries (RXXXXXXX and XXXXXQXX) and RGYVYQGL are compared, 1.67×10^7 times more OX7 peptides were required for the same degree of cytolysis. This discrepancy indicates that additional peptides can be recognized by clone 269/3. A 50-fold degeneracy can be calculated when the average efficiencies of active peptides are assumed to be similar to that of RGYVYQGL. This degree of degeneracy appears to be >100 times less than the degeneracy observed with clone 4G3. As discussed earlier, an exact number of active peptides in the mixtures cannot be calculated, but it is obvious that the CTL clones cross-recognize different peptides to varying degrees.

269/3 cells were also tested with the eight OX7 peptide sublibraries that define the epitope recognized by clone 4G3. No cytolytic response was induced (data not shown). In the reverse experiment, recognition of sublibraries carrying the known epitope amino acids of clone 269/3 by 4G3 cells were analyzed. Two of these peptide mixtures (XXYXXXXX and XXXVXXXX) induced intermediate levels of cytolysis; the remaining six mixtures were not effective (data not shown). These observations confirm that degeneracy of antigen recognition by CTLs varies among different T cell clones.

Discussion

The analysis of natural peptide libraries extracted from MHC-I proteins revealed allele-specific peptide motifs (2). The amino acids that define these motifs serve as anchor residues for peptide binding to MHC-I and are the structural basis for MHC-restricted antigen recognition by T cells. The side chains of these amino acids were shown by crystal structure analysis of defined peptide–MHC-I complexes to fit into specific pockets in the peptide-binding groove of MHC-I proteins that are formed by some of their most polymorphic residues (6–8).

Results obtained with the synthetic peptide libraries used in the study presented here validate the prominent role of these motif amino acids. Our assays clearly show that they are crucial for peptide binding to MHC-I and for the stability of the entire complex. A similar conclusion was derived from binding measurements with soluble K^b and two CTL epitopes in which single amino acids were replaced by alanine (44). Besides the dominant anchors that define allele-specific binding motifs, additional residues that serve as secondary anchors were identified. The side chain residues of these amino acids and their position in the sequence vary with the epitope. In the K^b-restricted CTL epitope SIINFEKL, which carries phenylalanine and leucine as primary anchors, serine in position 1 and isoleucine in positions 2 and 3 contribute as secondary anchors to the stability of the K^b–peptide complex. In RGYVYQGL, which is presented by the same MHC allomorph and carries tyrosine in position 5 and leucine in position 8 as dominant anchors, tyrosine in position 3 and valine in position 4 are the secondary anchors. On the other hand, position 4 of SIINFEKL is occupied by

the destabilizing asparagine, and position 2 of RGYVYQGL is held by the strongly destabilizing glycine. Tyrosine in the third position is frequently present in K^b-binding octapeptides; it was also detected by pool sequencing of natural peptide libraries that were extracted from K^b isolates (2). An anchoring function of amino acids in position 3 of the octapeptides was also suggested by others who analyzed the effects of single amino acid substitutions in known CTL epitopes (4, 5, 45). This secondary anchor concept is supported by crystal structure analysis of defined MHC–peptide complexes. The peptide binding groove of K^b has an additional, but shallower pocket for peptide side chains that is located in a position suitable for amino acids in position 3 of octapeptides (6, 7). In case of other MHC-I proteins, among them HLA-A2.1, this pocket is deeper and accommodates the side chain of one of the dominant anchor amino acids (8, 11).

The contribution of individual amino acids in the epitope sequences to binding of the peptides to K^b is only exceptionally additive. In most cases the effects of individual amino acids are influenced by flanking amino acids and the whole sequence context of the epitope. This is most obvious for glutamic acid in position 6 of SIINFEKL. Strong mutual dependence of stabilizing and destabilizing effects exerted by the different residues of octapeptide CTL epitopes was found with sublibraries bearing two or more fixed positions. The stabilizing capacity of individual defined positions is dependent on residues in other sequence positions. From these results, it is conceivable that peptides which lack one or both anchor amino acids can also bind to and stabilize K^b efficiently and induce T cell responses. Thus, some cases of apparently inappropriate T cell epitopes (46–48) could be explained by MHC stabilization caused by an effective combination of amino acids in sequence positions other than anchor positions. On the other hand, peptides that conform to the K^b-binding motif can be rendered inefficient by unfavorable amino acids in nonanchor positions. The discrepancy between the calculated and measured efficiency of K^b stabilization by X$_8$ could be explained by the influence of additional residues in non-motif positions that either are essential for binding or are strongly destabilizing. The interdependence of the side chain residues observed here is reconcilable with the length of the peptides (2, 3), the extent of surface contact between peptide and MHC-I structures (6–9), and the strong contribution of the peptides to the thermal stabilization energy of MHC-I proteins (49). Our findings are also consistent with recent reports about crystal structure models for defined MHC-I–peptide complexes. The analysis of the conformation of different peptides bound to the same MHC-I protein, HLA-A2.1, reveals that single amino acid side chains influence the positioning of the peptide's main chain and the orientation of side chains of amino acids in other sequence positions, and thereby the extent of surface contact between peptide and MHC residues (8). Similar conclusions were derived from molecular dynamic studies applied to the same MHC allomorph (9).

Binding and CML assays with peptides carrying single amino acid substitutions in individual positions of known CTL epitopes have contributed to a more detailed knowledge of amino acids promoting binding to MHC-I molecules as dominant or secondary anchor residues (4, 5, 45). However, the entire sequence of the peptides and the stabilizing or destabilizing effects of single as well as of combinations of amino acids need to be analyzed for a precise prediction of the properties of MHC-binding peptides and their efficiency as T cell epitopes or as competitors for T cell epitopes. A simple summation in logarithmic form of the SI values for individual amino acids in OX$_7$ peptide sublibraries that are roughly proportional to their contribution to the average binding energy of the peptide–MHC interactions does not yield the SI for the entire epitope. Attempts have been made to predict the binding strength of peptides to MHC molecules from the summation of the energy contribution of individual amino acids in T cell epitopes (50). However, the assumed independence of the impact of single side chains on peptide binding to MHC molecules is questioned by the results shown in this report. Nevertheless, these approaches would allow one to use informations on primary as well as secondary anchors to search for CTL epitopes and could give an indication of relative binding efficiencies of different peptides for MHC-I molecules. It also might help to identify epitopes that do not conform to the canonical MHC-I–binding motifs (46–48). Extensive studies on peptide–MHC binding with a complete panel of OX$_7$ peptide sublibraries are in progress in our laboratory, and results will be used to test these different approaches to improved reliability of predictions of T cell epitopes.

While promoting efficient peptide binding to MHC, anchor residues in the fixed positions of peptide libraries strongly suppress CTL responses to the complex peptide mixtures. This is very likely due to increased competition among peptides with the correct combination of anchor amino acids. This interpretation is supported by results from earlier experiments in which peptides that fulfill the K^b-binding motif requirements but lack crucial residues for recognition by 4G3 were used as competitors of SIINFEKL (27). In these experiments, a 61,000-fold excess of foreign peptide did not reduce CTL activity. However, increasing the concentration of competitors by a factor of 9, yielding a 549,000-fold excess, abolished cytolysis almost completely. OX$_7$ sublibraries with one defined primary anchor amino acid originating from a CTL epitope contain 19 times more peptides with the correct combination of motif amino acids than X$_8$. This increases the frequencies of competitors for binding to K^b by this factor. Natural peptide mixtures isolated from MHC-I–expressing cells were repeatedly found to be more efficient than single peptides for positive selection of thymocytes during their development in fetal thymic organ cultures (51). The complexity of such peptide mixtures could have the same suppressive effect on T cell responses that we observed with synthetic peptide libraries. These natural libraries could reduce the efficiencies of active peptide species for binding to the MHC molecules and for recognition by cognate TCRs, thereby prevent induction of negative selection.

CTLs vary greatly in their tendency to cross-recognize antigenic peptides. Clone 269/3 seems to be more specific for

its cognate peptide than 4G3. In the case of humoral immune responses, high affinity antibodies tend to show more cross-reactions than low affinity antibodies (52). By analogy, it could be inferred that variations in the degree of degeneracy of T cell responses reflect differences in the affinities of the TCRs for cognate MHC-I–peptide complexes. If such considerations were valid, one would expect higher affinity of the TCRs of clone 4G3 than of clone 269/3 for their corresponding ligands. However, thorough affinity measurements have not been done. Recent reports demonstrate a crucial role for MHC-bound peptides in positive selection of T cells during thymic differentiation (51, 53, 54). This role necessitates degeneracy of peptide recognition. Recognition of peptide–MHC complexes and initiation of cellular reactions that lead to differentiation of T cells in the thymus are mediated by the same TCR that governs responses against foreign antigen. The peptides, and in some cases also the MHC molecules differ, in these two situations.

The observed degeneracy of peptide recognition by one TCR also implies that different synthetic peptides can be used for primary induction of CTLs with a desired specificity. This leads, in consequence, to new strategies for the generation of CTL-mediated immunity. Induction of effective CTLs against tumor or viral antigens does not require identification of the natural epitopes; any active peptide would suffice. Such artificial epitopes can be identified with the help of peptide libraries of the type used in the studies presented here (Udaka, K., K. H. Wiesmüller, S. Kienle, D. H. Jung, and P. Walden, manuscript submitted for publication) or with arrays of defined peptides.

Complex synthetic peptide libraries have proven to be a powerful tool for the investigation of the structural requirements for MHC-binding peptides and for CTL epitopes. Other types of peptide libraries consisting of sets of defined peptides were used to study the effects of subtle changes in the structure of the peptides or of the MHC molecules on the repertoire of MHC-bound peptides (55–57). In another case, a phage display library was used successfully to obtain structural information on peptide selection by MHC class II molecules (58). The major advance made with the present study is the reproducible synthesis of peptide libraries that consist of equimolar mixtures of the constituents, which made it possible to assess quantitatively the impact of individual amino acids on the formation of a stable MHC molecule and recognition of MHC–peptide complexes by T cells. The information obtained from experiments with such libraries allows identification of natural epitopes and the design of artificial epitopes, MHC blockers, and competitors or antagonists of natural epitopes.

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