Binding of Viral Antigens to Major Histocompatibility Complex Class I H-2Db Molecules Is Controlled by Dominant Negative Elements at Peptide Non-anchor Residues

IMPLICATIONS FOR PEPTIDE SELECTION AND PRESENTATION*

(Received for publication, February 23, 1996, and in revised form, April 4, 1996)

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Binding of viral antigens to major histocompatibility complex (MHC) class I molecules is a critical step in the activation process of CD8+ cytotoxic T lymphocytes. In this study, we investigated the impact of structural factors at non-anchor residues in peptide-MHC interaction using the model of lymphocytic choriomeningitis virus (LCMV) infection of its natural host, the mouse. Altering viral genes by making reassortants, recombinants, and using synthetic peptides, CD8+ cytotoxic T lymphocytes were shown to recognize only three H-2Db-restricted epitopes, GP amino acids 33-41/43, GP 276-286, and NP 396-404. However, LCMV NP and GP proteins contain 31 other peptides bearing the H-2Db motif. These 34 LCMV peptides and 11 other known H2-Db-restricted peptides were synthesized and examined for MHC binding properties. Despite the presence of the H-2Db binding motif, the majority of LCMV peptides showed weak or no affinity for H-2Db. We observed that dominant negative structural elements located at non-analog positions played a crucial role in peptide-MHC interaction. By comparative sequence analysis of strong versus non-binders and using molecular modeling, we delineated these negative elements and evaluated their impact on peptide-MHC interaction. Our findings were validated by showing that a single mutation of a favorable non-anchor residue in the sequence of known viral epitopes for a negative element resulted in dramatic reduction of antigen presentation properties, while conversely, substitution of one negative for a positive element in the sequence of a non-binder conferred to the peptide an ability to now bind to MHC molecules.

Virus-specific, CD8+ cytotoxic T lymphocytes (CTL)1 recognize viral antigens at the surface of infected cells in the context of major histocompatibility complex (MHC) class I molecules (1, 2). Association of the intracellularly processed peptide to MHC molecules is a critical step of the antigen presentation pathway leading to CTL activation. The molecular and structural details of peptide-MHC interactions were critically delineated with the X-ray crystallographic elucidation of MHC structures in complex with viral antigens (reviewed in Ref. 3) and with the biochemical characterization of naturally presented peptides (reviewed in Refs. 4–6). Sequencing studies of peptides eluted from MHC class I molecules led to the identification of allele-specific anchor residues within the peptide sequence (7, 8). To date, however, the success of prediction of CTL epitopes or of identifying new or unknown antigens from various pathogens based solely on these anchor residues has been disappointing and at best limited to a few cases (9, 10). Indeed, the immunodominant CTL epitopes identified within a viral protein are still few despite the large number of peptides theoretically expected on the basis of the presence of the appropriate MHC binding motif in the sequence. Studies based on the extensive analysis of a HLA-A2-restricted peptide library or on the fine dissection of a H-2Kb-restricted OVA antigen showed that immunodominance of a CTL functional epitope was correlated with its high binding affinity for MHC (11, 12) and that the presence of the anchoring motif was necessary for binding but was not sufficient for high affinity (13). Furthermore, the critical importance of the minor pockets of the MHC binding cleft in peptide selectivity and CTL reactivity was demonstrated by mutational analysis of either murine H-2Ld (14) and H-2Kb (15) or human HLA-A2.1 molecules (16, 17). In toto, these observations support the concept that additional structural parameters play a role in peptide-MHC interactions and are likely responsible for the strong selection observed. Solving these allele-specific structural requirements for most human and murine MHCs would be a crucial step toward understanding and consequently manipulating peptide-MHC interaction.

This study focuses on the selection of viral peptides by H-2Db molecules. H-2Db belongs to a MHC subgroup characterized by an hydrophobic ridge in the binding cleft (18). This peculiar feature, which occurs in about 40% of the murine D and L alleles, imposes structural constraints to the bound peptide (18). The H-2Db binding motif is characterized by a peptide sequence of 9–11 amino acids (aa) with two anchors: Asnβ and an hydrophobic C-terminal residue (Met, Ile, Leu) (6, 7). Lymphocytic choriomeningitis virus (LCMV) infection of normal H-2b mice generates a predominant CD8+ CTL response (19) that recognizes three H-2Db-restricted immunodominant epitopes (20, 21, 22, 23): NP 396–404 (FQPQNGQFI), GP 33–41/43 (KAVYNFATC/GI), and GP 276–286 (SGVENPGGYCl). The LCMV NP and GP proteins contain 31 other peptides bearing the H-2Db motif, although no CTL response against these peptides has yet been reported (24). The 34 LCMV pep-
ties and 11 other known H-2D<sup>d</sup>-selective peptides were synthesized and quantitated for their MHC binding affinities. Most of the LCMV peptides did not bind to H-2D<sup>d</sup>, reflecting a strong negative control by non-anchor residues. The negative elements inhibiting MHC binding were then evaluated by: (i) comparative analysis of the sequences from strong and weak or non-binders, (ii) computerized molecular modeling, and (iii) analysis of mutation at single non-anchor residues to either change a positive into a negative binding element and conversely.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**Murine H-2<sup>b</sup> mutant RMA-S cells (25) and human T2 cells transfected with H-2D<sup>d</sup> (T2-D<sup>d</sup>) (26) were used in binding experiments. The murine H-2<sup>b</sup> cell line MC57 was used in in vitro cytotoxicity assays. Cells were grown in RPMI 1640 (RMA-S, MC57) or Iso-Soave’s modified Dulbecco’s medium (T2-D<sup>d</sup>) containing 8% bovine serum, L-glutamine (2 mM), and antibiotics (10 units/ml penicillin and 10 µg/ml streptomycin). Genetin (400 µg/ml) was added to Iso-Soave’s modified Dulbecco’s medium to maintain selection of T2-D<sup>d</sup> cells.

**Peptides—**Peptides were synthesized on an automated peptide synthesizer (Applied Biosystems 430A) by the solid-phase method using Fmoc-chemistry, purified by high pressure liquid chromatography on a RP300-C8 reversed-phase column (Brownlee Lab) and identified by fast atom bombardment or electrospray mass spectrometry. The H-2<sup>d</sup>-selective radioactive probe YAIENAEAL (specific activity: 40–80 TBq/mmol) was prepared and purified as described (27).

**Binding Studies—**For stabilization assays, RMA-S cells were grown at 25°C for 24 h prior to the assay to induce stable H-2D<sup>d</sup> expression at the cell surface (25, 28). Cells (5 × 10<sup>5</sup> cells/ml) were then incubated at 37°C in microtiter plates with increasing peptide concentrations (10–10<sup>−10</sup> M). The stability of MHC molecules was analyzed after a 4-h incubation period. Cells were incubated on ice for 1 h with 0.1 M glycerol (glycerol control) and 0.01 M EDTA to quantify the specific binding. The stability of MHC molecules was then analyzed after a 4-h incubation period. The samples were incubated at 37°C for 30 min, centrifuged at 1,000 g for 2 min, and the supernatant was removed and counted for radioactivity. Total and nonspecific binding was measured in the absence or presence of 1 µM unlabeled competitor. Specific binding to H-2D<sup>d</sup> was defined as the difference between total and nonspecific binding. Percent (%) inhibition of binding was calculated as

\[
(100 - \frac{cpm}{cpm \times 100})
\]

where cpm = counts per minute. Only a limited number of the LCMV peptides bound to H-2D<sup>d</sup> with significant binding affinities. Only a limited number of the LCMV peptides bound to H-2D<sup>d</sup> with significant binding affinities. Only a limited number of the LCMV peptides bound to H-2D<sup>d</sup> with significant binding affinities.

**RESULTS**

**Only a Limited Number of the LCMV Peptides Baring the H-2D<sup>d</sup> Motif Bind to H-2D<sup>b</sup> with High Affinity—**Scanning of the LCMV NP and GP proteins revealed 34 sequences that harbored the H-2D<sup>d</sup> anchoring motif (Asn at position 5 and Met, Ile, or Leu at the C terminus) (29). These 34 peptides (that included the 3 known H-2D<sup>b</sup> epitopes) were synthesized and tested for their H-2D<sup>b</sup> binding affinity. Two assays based either on stabilization of thermodynamically unstable empty MHC molecules at the surface of RMA-S cells (SC<sub>50</sub>, assay (25, 30) or on competition of binding against the H-2D<sup>d</sup>-selective radioactive probe YAIENAEAL on T2-D<sup>d</sup> cells (IC<sub>50</sub> assay) (27) were used. Results are presented in Table I and illustrated in Fig. 1. Peptides were classified as strong, weak, or non-binders according to their IC<sub>50</sub> and SC<sub>50</sub> values. By this means, we determined whether the presence of specific aa at each of the non-anchor positions could alter the binding affinity to H-2D<sup>d</sup>. We first classified the aa into nine groups (Tyr, Phe, and Trp; Val, Leu, Ile, and Met; Ala; Pro; Gly; Ser, Thr, and Cys; Gin and Asn; Asp and Glu; Arg, Lys, and His). The peptide sequences were then analyzed by the crystal structure of H-2D<sup>d</sup> complexed with the influenza virus NP 366–374 (ASNENMETM) as solved to 2.4 Å (18). The LCMV H-2D<sup>d</sup>-restricted peptide GP 16–24 (DEVINIVII) was built from the reference crystal structure using the program O (Biobyte) or Macromodel (MSI). The structures were then manually refined and then submitted to 100 cycles of energy minimization using the program Insight (Biosym Technologies, CA). Coordinates for docking were taken from the crystal structure of H-2D<sup>d</sup> complexed with the influenza virus NP 366–374 (ASNENMETM) and P. trypsin (2.8 Å) (19). The LCMV H-2D<sup>d</sup>-restricted peptide GP 16–24 (DEVINIVII) was built from the reference peptide structure using Macromodel (MSI). The structures were then manually refined and then submitted to 100 cycles of energy minimization using the program Insight (Biosym).

In vitro Cytotoxicity Assays—CTL lysis was measured in a standard 5-h 51Cr release assay (20). Target cells (uninfected MC57 cells cultured in the absence or presence of increasing concentrations (10–10<sup>−10</sup> M) of peptides or infected for 48 h with LCMV ARM 53b) were labeled with 51Cr and tested for specific binding to H-2D<sup>d</sup> (specific binding) (cpm for specific binding (cpm for specific binding) (cpm for specific binding (cpm for specific binding). Total and spontaneous releases were determined by incubating the labeled cells with 1% Non-ident P-40 and culture medium, respectively. In all experiments, samples were run in triplicate, and the mean values are given. Variance among the samples was less than 10%. The effector to target (E:T) ratio for CTL clones was 5:1 and for splenic CTL 50:1 and 25:1. Targets and effector cells were incubated at 37°C in a final volume of 200 µl. After 5 h, 100 µl of cell-free supernatant fluid was removed from each well and counted for 51Cr radioactivity. The percent specific lysis was calculated as 100 × (cpm for experimental release − cpm for spontaneous release)/(cpm for specific binding (cpm for specific binding (cpm for specific binding (cpm for specific binding). The percent specific lysis was calculated as 100 × (cpm for experimental release − cpm for spontaneous release)/(cpm for specific binding (cpm for specific binding (cpm for specific binding (cpm for specific binding).
determined groups of residues at each of the non-anchor positions associated with either strong or no binding and excluded LCMV GP 91–101 and SV40 Tag 205–215 from further studies. Table III and Fig. 2 show that negatively charged residues (Asp and Glu) were frequently found in weak viral binders or non-binders (at position P2 or P3) or strong (at P4 or P7) binders. Hydrophobic residues (Val, Leu, Ile, and Met) were associated with either strong (at P3) or weak (at P1 or P7) binding. Interestingly, two aa groups were found in only strong binders: residues with an OH- or SH-group on their side chain (Ser, Thr, and Cys) at P1 or P2 and residues with bulky side chains (Tyr, Phe, and Trp) at P8. In contrast, positively charged (Arg, His, and Lys), neutral (Gln and Asn) or small (Ala, Pro, and Gly) residues were not implicated significantly at any of the non-anchor positions. Further, no aa group was predominant at P6 of the strong binders or at P4 and P8 of the weak binders.

Validation of the Structural Rules Defined for Antigen Selection and Presentation by H-2D\(^b\)—We focused our analysis on the local structural constraints at P2 in which small aa (Ser, Thr, Cys, and, to a lesser extent, Gly or Ala) and negatively charged residues (Asp and Glu) were predominant in strong and in weak binders, respectively. The crystallographic data of H-2D\(^b\) in complex with influenza NP 366–374 (18) was used as a comparative molecular model of the interaction of H-2D\(^b\) with either a strong (influenza virus NP 366–374, AS\(\text{NENMETM}\)) or a weak (AS\(\text{NENM}^\text{a}\)) epitope.
or a non-binder (LCMV GP 16–24, DEVINIVII). As shown in Fig. 3A and previously by Young et al. (18), the carboxylic moiety of Glu63 of the MHC α1 helix and the side chain HO-group of Ser2 of the influenza NP 366–375 form a tight hydrogen bond that contributes to the high affinity binding properties of the epitope. In contrast, modeling DEVINIVII in the H-2Db binding groove with a conformation deduced from that of the influenza NP showed the peptide Glu2 side chain facing the MHC Glu63 at a 2.54-Å distance (Fig. 3B). The strong repulsive forces between the two negatively charged moieties result in unfavorable interaction of DEVINIVII with H-2Db and makes their association unlikely to occur.

These findings were further validated by testing the effect of substituting a natural residue of a strong binder for a negative
Table III

Relative importance of amino acid groups at non-anchor positions of peptides bearing the H-2D<sup>b</sup> binding motif

Peptides that contained the H-2D<sup>b</sup> anchors N at position 5 and M, I, or L at the C terminus (position 9, 10 or 11) derived from LCMV proteins or from other origins were synthesized and tested for their MHC binding affinity (see Table I). The frequency of occurrence of certain amino acid groups was determined for each position of strong and weak binders and their relative importance calculated as exemplified in Table II. For each position, the highest value of each peptide category is bolded. Significantly predominant amino acid groups are framed and shaded (a 5-fold ratio was the threshold level as criteria for significant predominance).

| amino acid group | P1 strong | P1 weak | P2 strong | P2 weak | P3 strong | P3 weak | P4 strong | P4 weak | P6 strong | P6 weak | P7a strong | P7a weak | P8 b strong | P8 b weak |
|-----------------|----------|---------|----------|---------|----------|---------|----------|---------|----------|---------|------------|---------|------------|---------|
| Y, F, W         | 13       | 3       | 4        | 3       | 5        | 5       | 11       | 5       | A        | 7       | 1          | 0       | 0          | 21      | 1          |
| V, L, I, M      | 1        | 4        | 16       | 5       | 50       | 4       | 7        | 6       | C        | 13      | 20         | 0       | 30         | 6       | 10         |
| A               | 2        | 2        | 15       | 0       | 2        | 6       | 0        | 0       | H        | 2       | 6          | 0       | 10         | 5       | 5          |
| P               | 0        | 0        | 4        | 9       | 4        | 2       | 2        | 6       | O        | 5       | 5          | 10      | 0          | 0       | 0          |
| G               | 0        | 4        | 12       | 6       | 2        | 5       | 2        | 6       | R        | 5       | 5          | 0       | 2          | 4       | R          |
| S, T, C         | 34       | 3        | 20       | 4       | 0        | 13      | 3        | 12      | 11       | 11      | 3          | 13      | 9          | 12      |
| Q, N            | 5        | 5        | 5        | 0       | 3        | 2       | 18       | 3       | 7        | 1       | 3          | 13      | 2          | 9       |
| D, E            | 0        | 9        | 0        | 35      | 0        | 22      | 25       | 0       | 0        | 17      | 61         | 1       | 11         | 4       |
| R, H, K         | 11       | 1        | 1        | 14      | 0        | 4       | 7        | 5       | 5        | 5       | 0          | 10      | 2          | 6       |

* Relative importance at P7 was calculated on the basis of 9-mer peptides only.
* P8 represents the position adjacent to the C-terminal residue (i.e. position 8, 9, or 10 of 9-, 10-, or 11-mer peptides, respectively).

Fig. 2. Non-anchor residues associated with strong or weak binding to H-2D<sup>b</sup>. Based on results obtained in Table III, residues found to be predominantly associated with strong (upper part) or weak (lower part) H-2D<sup>b</sup> binding. Anchor residues are Asn (N) at position 5 and Met (M), Ile (I), or Leu (L) at the C terminus. P8 represents the position adjacent to the C-terminal residue (i.e. position 8, 9, or 10 of 9-, 10-, or 11-mer peptides, respectively).

Fig. 3. Molecular modeling of positive versus negative H-2D<sup>b</sup>-peptide interaction. The three-dimensional models for the influenza NP 366–374 (AS-NENMETM) (A) and LCMV GP 16–24 (DEVINIVII) (B) interacting with H-2D<sup>b</sup> are shown from above the α1 and α2 domains. Favorable (influenza NP 366–374) or unfavorable (LCMV GP 16–24) interactions are illustrated at the level of the peptide side chain of residue at position 2. The distance between H-2D<sup>b</sup> Glu<sup>63</sup> and influenza Ser<sup>367</sup> (Ser<sup>2</sup>) (2.86 Å) or LCMV Glu<sup>17</sup> (Glu<sup>2</sup>) (2.54 Å) results, respectively, in either a strong hydrogen bond or a repulsive effect between the peptide and the MHC molecule. The model of LCMV GP 16–24 was built by HOMOLOGY starting from the coordinates of influenza NP 366–374 bound to H-2D<sup>b</sup> (18).

structural element. Substitution of Ser, Ala, or Gly for Glu at P2 of influenza NP 366–374, LCMV GP 33–43, and GP 276–286, respectively, abrogated the high affinity binding properties of the three epitopes (Table IV, upper part) and thus altered their ability to be presented by MHC to CTL. Indeed, the mutated LCMV peptides showed a dramatic decrease (at least 2-3 logs compared to the authentic peptides) in their ability to sensitize target cells to lysis by virus-specific MHC-restricted CTL (Fig. 4). Conversely, substitution of the negative element Glu<sup>2</sup> of the non-binder peptide DEVINIVII for the
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**TABLE IV**

Effect of mutation at non-anchor position P2 on the H-2D<sup>b</sup> binding properties of H-2D<sup>b</sup>-restricted viral peptides

Peptides were synthesized by solid-phase method, HPLC purified and identified by FAB mass spectrometry. Affinity for H-2D<sup>b</sup> was measured in two H-2D<sup>b</sup> specific binding assays as described under "Experimental Procedures" and in Table I.

| Peptide | Competition (IC<sub>50</sub>) | Stabilization (SC<sub>50</sub>) |
|---------|-------------------------------|-------------------------------|
| Influenza |                               |                               |
| NP366–374 | Ala Ser Asn Glu Asn Met Glu Thr Met | 7 ± 1 | 10 ± 1 |
| [Glu]<sup>2</sup>-NP366–374 | Ala Glu Asn Glu Asn Met Glu Thr Met | 1939 ± 109 | 12,350 ± 350 |
| LCMV |                               |                               |
| GP33–41 | Lys Ala Val Tyr Asn Phe Ala Thr Cys Gly Ile | 21 ± 4 | 470 ± 63 |
| [Glu]<sup>2</sup>-GP33–41 | Lys Glu Ala Val Tyr Asn Phe Ala Thr Cys Gly Ile | 13,000 ± 3790 | 53,000 ± 8460 |
| GP276–286 | Ser Gly Val Glu Asn Pro Gly Gly Tyr Cys Leu | 13 ± 2 | 23 ± 3 |
| [Glu]<sup>2</sup>-GP276–286 | Ser Glu Val Glu Asn Pro Gly Gly Tyr Cys Leu | 15,067 ± 5715 | 43,000 ± 5650 |

LCMV

| Peptide | Competition (IC<sub>50</sub>) | Stabilization (SC<sub>50</sub>) |
|---------|-------------------------------|-------------------------------|
| GP16–24 | Asp Gly Val Ile Asn Ile Val Ile Ile | >100,000 | >100,000 |
| [Ser]<sup>2</sup>-GP16–24 | Asp Ser Val Ile Asn Ile Val Ile Ile | 1400 ± 450 | 2050 ± 390 |
| [Gly]<sup>2</sup>-GP16–24 | Asp Gly Val Ile Asn Ile Val Ile Ile | 2033 ± 887 | 4930 ± 721 |

**FIG. 4.** CTL lysis of H-2<sup>b</sup> target cells presenting LCMV GP33–43 or GP276–286 is influenced by Glu-substitution at P2 residue. A standard 5-h <sup>51</sup>Cr release assay (20, 23) was used to measure lysis of uninfected MC57 (H-2<sup>b</sup>) target cells by H-2<sup>b</sup>-restricted LCMV-specific CTLs in the absence (filled squares) or presence of increasing concentrations of GP1 (GP 33–43) (10<sup>-10</sup> M to 10<sup>-7</sup> M) or GP2 (GP 276–286) (10<sup>-11</sup> M to 10<sup>-8</sup> M) analogues (open circles). The specific effect of negatively charged residues measured at P2 was calculated as: 100 × ([cpm for experimental release – cpm for spontaneous release]/cpm for total release – cpm for spontaneous release).

Positive structural elements Ser and, to a lesser degree, Gly, led to a significant enhancement of the peptide's ability to bind to H-2D<sup>b</sup> (Table IV, lower part).

**DISCUSSION**

This study documents that, in addition to the anchors, the non-anchor residues play a major role in determining peptide selection by MHC molecules. Not only must their role be taken into account to define the rules governing peptide-MHC interactions, but understanding their influence on MHC binding is surely to be reflective in what viral mutation allows CTL escape variants to occur, how to better design a vaccine to elicit optimal CTL activity and the constraints viral peptides must have with host molecules to favor molecular mimicry and thus virus-induced autoimmunity.

The finding that many LCMV peptides (28/34, or >80%) are very weak or non-H-2D<sup>b</sup> binders despite the presence of the relevant MHC binding motif clearly indicates that dominant negative factors at non-anchor positions control MHC (H-2D<sup>b</sup>)-peptide interactions. The structural elements involved in these interactions were identified for most positions within the peptide. A well defined profile was observed at positions P1, P2, and P3, a finding consistent with the known tight fit of the N-terminal end of the peptide during its interaction with the H-2D<sup>b</sup> molecule (18, 23). In the P1-P2 domain, the strong binders contained residues with side chains favoring the formation of hydrogen bonds that insure the stability of the peptide-MHC complex (18, 31). For the non-binder peptides, the residues that counter-balanced the positive effect in P1, P2 differed with the position. Steric hindrance, elongated hydrophobic side chain (Val, Leu, Ile, and Met) and electrostatic repulsion of negatively charged side chains (Asp and Glu) were the important negative elements at P1 and P2, respectively. These observations complement the solved crystal structure of the H-2D<sup>b</sup> influenza NP 366–374 complex (18). The detrimental effect of negatively charged residues measured at P2 was still effective at P3 for which hydrophobic residues (Val, Leu, Ile, and Met) were the most favorable as observed previously (23, 27). We were unable to define the aa responsible for either positive or negative binding at P6. One apparent reason is that this position is minimally or not involved in H-2D<sup>b</sup>-peptide interaction. In addition, no negative elements were defined at P4 and P8, indicating that these positions accommodate residues of any nature without interfering with antigen presentation. Interestingly, both molecular modeling studies and measurements of CTL activity directed against peptides whose aa were mutated in P4, P6, and P8 indicated that these residues preferentially pointed away from the MHC groove, being directed toward the T cell receptor (18, 32). The absence of detrimental factors at these three potential CTL target positions is of strategic importance to the host in terms of immune response.
recognition. Because of the flexibility allowed in P4, P6, and P8, a large number of aa combinations (20^3 – 8000) enhances the possibility of generating a CTL response against a wider spectrum of peptides.

Comparative analysis of the impact of the structural elements at non-anchor residues indicated that negative rather than positive factors primarily influenced antigen selection by MHC molecules. In the strong binder category, we found no evidence of correlation between the number of positive factors in a peptide sequence and its MHC affinity. For example, peptides with multiple favorable residues (peptides g, j, and k) did not show higher affinities than most of the viral epitopes with only one favorable residue (peptides 30, a, d, and e). Furthermore, the optimally designed peptide SMIENLTVY (j) (30) did not gain in affinity compared to natural epitopes, and none of the peptide tested showed IC_{50} or SC_{50} values below the nanomolar range. This limitation in affinity likely reflects the adaptability of the MHC binding pocket to a wide range of peptide sequences. As peptide-MHC interactions follow the rules of ligand-receptor interaction, selection of peptides with higher affinities than those measured requires more stringent binding conditions (27). However, the result in vivo would be a considerable narrowed spectrum of peptides available for presentation by an MHC molecule, an option that is in conflict with the MHC function.

The H-2K^{b}-restricted epitope SEV NP 324–332 (peptide f) that also bears the H-2D^{b} motif but lacks positive elements at non-anchor positions binds tightly to H-2D^{b}, indicating again that presence of favorable elements at non-anchor positions is not necessary for high affinity MHC binding. This relative low impact of positive factors on MHC binding properties may explain why peptides bearing the MHC anchors can accept multiple alanine substitutions without dramatic changes in their binding properties (27, 33, 34). Thus, besides the primary sequence, conformational parameters strongly influence peptide-MHC interactions (35).

The role of negative peptide residues in MHC binding is clearly important. The presence of a single unfavorable residue at a non-anchor position is by itself sufficient to drastically hamper peptide-MHC interaction. For example, LCMV NP 45–53, despite two positive elements (Ser^2, Val^3), was unable to bind to H-2D^{b} due to the presence of the negative contact Glu^5. This observation further points to dominance of the negative effect of a peptide residue over the positive effect. Hence the absence of detrimental residues rather than presence of favorable residues is an important criteria for high affinity MHC binding.

From the four LCMV genes, only three peptides, two from the GP (GP 33–41/43, GP 276–286) and one from the NP (NP 396–404) but none from the L (polymerase) or Z proteins, are restricted by H-2D^{b}. Extending these findings, we note that NP 396–404 and GP 276–286 are strong binders while GP 33–41/43 is an intermediate binder to H-2D^{b}, confirming the correlation between the immunodominance of a viral epitope and its high MHC binding affinity (11, 12). Our studies shed light on why so few peptides within a viral protein are CTL epitopes. Besides the three known epitopes, three additional peptides (NP165–175, GP92–101, and GP392–400) bound with high affinity to H-2D^{b}, but none of them were able to sensitize H-2^{k} target cells to lysis by splenic CTL from LCMV-infected H-2^{k} mice. Furthermore, a LCMV variant virus in which epitopes GP 33–41/43, GP 276–286, and NP 396–404 were rendered useless by mutation also failed to generate CTL

to NP 165–175, GP 92–101, or GP 392–400 (24). What function these peptides with high affinity for MHC but devoid of CTL activation properties play or whether they are correctly processed in H-2^{k} cells for binding to H-2D^{b} is unknown at present. Studies looking at their processing from the NP and GP protein (36), the possibility they could act as T cell receptor antagonists (37) and/or play a role in T cell selection (38) is currently under evaluation.

Single mutations in a viral peptide sequence can have important consequence in vivo on antigen presentation (32, 39–43). In H-2^{k} mice infected with LCMV, CTL escape virus variants have been generated by point mutations that affect either antigen presentation by substitution of the crucial anchor N-5 (42) or CTL recognition by mutation of one residue oriented toward the T cell receptor (31, 40, 43). In addition, we demonstrate here that a mutation occurring at a non-anchor position could also lead to a dramatic decrease in the MHC binding properties of a viral antigen (see Table IV) and its consequent inability to trigger an efficient CTL response (see Fig. 4).

A peptide sequence with no affinity for MHC could be transformed by a single mutation to one able to bind, although weakly, to MHC (see Table IV). This finding is in accord with previous studies showing that alteration at non-anchor positions may improve presentation and immunogenicity of viral peptides (44, 45). The natural occurrence of such a phenomenon in a cell would allow an endogenous peptide that did not formerly could now associate to an MHC molecule and, once presented at the cell surface, behave as or mimic a non-self-antigen that triggers a CTL response leading to autoimmune response against self. Interestingly, such a mutated peptide would have a low MHC binding affinity and its immunogenicity may be weak (46), a typical profile of both tumor antigens that derive from mutated self-proteins as well as autoantigenic CTL implicated in autoimmunity (47, 48).

In summary, the approach provided here and elsewhere (12, 18, 31, 34, 44, 49) makes it possible to predict rules for peptide binding to MHC. The complete understanding of the structural requirements for optimal antigen presentation to CTL is necessary for an efficient prediction of CTL epitopes (50, 51) and/or of designing synthetic peptides to use as immunotherapeutic agents against viral infection or tumor progression (52, 53).

Acknowledgments—We thank Dr. S. Nathenson and J. Sacchettini for their contribution in molecular modeling studies and Dr. B. Monsarrat for mass spectrum analysis.

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