JOURNAL OF VIROLOGY, Apr. 1995, p. 2297–2305 Vol. 69, No. 4
0022-538X/95/$04.00+0


# Optimal Lymphocytic Choriomeningitis Virus Sequences Restricted by H-2D$^b$ Major Histocompatibility Complex Class I Molecules and Presented to Cytotoxic T Lymphocytes†

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Received 14 September 1994/Accepted 22 December 1994

**Infection with lymphocytic choriomeningitis virus induces the generation of CD8$^+$ cytotoxic T lymphocytes (CTL). In the *H-2$^b$* mouse, this cellular immune response is directed against three viral structural epitopes (GP1, GP2, and NP) presented by the major histocompatibility complex (MHC) class I H-2D$^b$ molecules. This study was undertaken to delineate which sequence of each of these three epitopes is optimal for MHC binding and CTL recognition. The first step was to synthesize the relevant peptides truncated at the N or C terminus and flanking the crucial H-2D$^b$-anchoring Asn residue in position 5. These peptides were then tested (i) for their binding properties in two H-2D$^b$-specific assays with viable cells (upregulation of H-2D$^b$ expression on the surface of RMA-S cells and competition against the D$^b$-restricted peptide $^{125}$I-gp276-286 on T2-D$^b$ cells) and (ii) for their abilities to sensitize *H-2$^b$* target cells for CTL lysis in vitro. For optimal antigenic presentation, all three epitopes required the MHC-anchoring Asn residue at position 5 of their sequences. The results clearly and unambiguously delineated optimal lengths for two of the epitopes and two options for the third. NP appeared as a conventional 9-amino-acid (aa)-long peptide, np396-404 (FQPQNGQFI). GP2 was defined as a longer peptide (11 aa), gp276-286 (SGVENPGGYCL). Characterization of the GP1 epitope was more complex: the 9-aa-long peptide gp33-41 (KAVYNFATC) and the carboxyl-extended 11-aa-long peptide gp33-43 (KAVYN FATCGI) were both established as possible optimal sequences depending on the cell line used to test binding and lysis.**

Viral antigens bind to major histocompatibility complex (MHC) class I molecules and are then transported to the surfaces of infected cells, where they are presented to virus-specific CD8$^+$ cytotoxic T lymphocytes (CTL). Recognition of the viral antigen-MHC class I complex by the T-cell receptor occurs in the context of H-2 (murine) or HLA (human) restriction (31, 36). The spectacular progress in knowledge about antigen presentation in the last several years has come largely from physicochemical and/or structural spectrometric studies. Endogenously processed peptides, including viral antigens, were eluted from different murine or human MHCs, analyzed, and sequenced (5–8, 26, 35). The results showed that naturally presented peptides exhibited allele-specific binding motifs with a remarkable homogeneity in length. Most were of 8 to 9 amino acids (aa), although longer peptides (10 to 11 aa long) occurred and were confirmed by crystallographic studies. The three-dimensional structures of crystallized MHC molecules in complex with endogenous peptides (12, 20, 21) or viral antigens (9, 19, 30, 34, 35) showed an extended conformation of the peptide bound to the MHC. Clearly, MHC molecules can accept and bind peptides of different lengths, since the peptides can adopt whatever conformation enables their anchoring positions to fit into the MHC binding groove. This flexibility was nicely demonstrated in the case of H-2K$^b$ crystallized in complex with two viral peptides of different lengths: the octapeptide np52-59 derived from the vesicular stomatitis virus nucleoprotein (NP) and the nonapeptide np324-332 derived from the Sendai virus NP (35). Further, the rules of ligand-receptor interactions were successfully applied to peptide-MHC interactions, and the development of binding studies with synthetic, labeled peptides allowed precise analysis of peptide sequences interacting with MHC molecules (3, 10, 14, 23).

Lymphocytic choriomeningitis virus (LCMV) encodes a 558-aa NP and a 498-aa glycoprotein (GP) posttranslationally cleaved into GP1 (aa 1 to 262) and GP2 (aa 263 to 498). In the *H-2$^b$* mouse, the major cellular immune response induced after infection with LCMV is CD8$^+$ CTL directed against three epitopes presented by H-2D$^b$ molecules. The CTL recognition sites of these viral proteins have been mapped, and their minimal sequences in NP (28), GP1 (16), and GP2 (24) have been delineated. However, none of the endogenously processed LCMV viral peptides presented by the H-2D$^b$ MHC molecules has been isolated and characterized to date. This study was therefore undertaken, first, to define the sequence of each of the three epitopes optimal for MHC binding and CTL recognition and, second, to uncover new information on the presentation of viral antigens by H-2D$^b$.

## MATERIALS AND METHODS

**Cell lines.** The murine *H-2$^b$* mutant lymphoma cell line RMA-S (18) was used in peptide-induced MHC upregulation experiments. The mutant human hybrid (B×T) lymphoblastoid cell lines T2 (4, 27) and T2 transfected with the mouse MHC gene *H-2D$^b$* (T2-D$^b$) (1) were used in binding experiments. The murine

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† Publication 8811-NP from the Department of Neuropharmacology, The Scripps Research Institute, La Jolla, Calif.

TABLE 1. H-2D$^b$-restricted epitopes of LCMV: synthetic peptides used for determination of the optimal sequences for MHC presentation to LCMV-specific CTL[a]

| Protein | C-terminus-truncated peptide | | | N-terminus-truncated peptide | | |
|---|---|---|---|---|---|---|
| | Epitope | Sequence | Length (aa) | Epitope | Sequence | Asn (N) anchor position |
| NP | np396-403 | FQPQNGQF | 8 | np397-404 | QPQNGQFI | 4 |
| | np396-404 | FQPQNGQFI | 9 | np396-404 | FQPQNGQFI | 5 |
| | np396-405 | FQPQNGQFIH | 10 | np395-404 | IFQPQNGQFI | 6 |
| | np396-406 | FQPQNGQFIHF | 11 | | | |
| | np396-407 | FQPQNGQFIHFY | 12 | | | |
| GP1 | gp33-40 | KAVYNFAT | 8 | gp34-41 | AVYNFATC | 4 |
| | gp33-41 | KAVYNFATC | 9 | gp33-41 | KAVYNFATC | 5 |
| | gp33-42 | KAVYNFATCG | 10 | gp32-41 | IKAVYNFATC | 6 |
| | gp33-43 | KAVYNFATCGI | 11 | | | |
| | gp33-44 | KAVYNFATCGIF | 12 | | | |
| GP2 | gp276-283 | SGVENPGG | 8 | gp278-286 | VENPGGYCL | 3 |
| | gp276-284 | SGVENPGGY | 9 | gp277-286 | GVENPGGYCL | 4 |
| | gp276-285 | SGVENPGGYC | 10 | gp276-286 | SGVENPGGYCL | 5 |
| | gp276-286 | SGVENPGGYCL | 11 | gp275-286 | SSGVENPGGYCL | 6 |
| | gp276-287 | SGVENPGGYCLT | 12 | | | |
| | gp276-288 | SGVENPGGYCLTK | 13 | | | |

[a] Peptides were synthesized by the solid-phase method on automated peptide synthesizers and purified by HPLC. Purity of the peptides used was >98%. Identity of the purified peptides was confirmed by fast atom bombardment-mass spectrum analysis.

*H-2$^b$* cell line MC57 was used for in vitro cytotoxicity assays. The cell lines RMA-S and MC57 were grown in RPMI 1640 containing 7% inactivated fetal bovine serum and antibiotics. The cell lines T2 and T2-D$^b$ were grown in Iscove's modified Dulbecco's medium supplemented with 10% inactivated fetal bovine serum and antibiotics, with (T2-D$^b$) or without (T2) 500 μg of geneticin (G418 sulfate) per ml.

**Synthetic peptides.** Peptides were synthesized by the solid-phase method on Applied Biosystems 430A automated peptide synthesizers with either the *t*-BOC or the FMOC chemistry (Table 1). Peptides were purified by high-pressure liquid chromatography (HPLC) on reverse-phase columns (Vydac C18 and a Waters apparatus or Brownlee Lab RP300-C8 and an ABI 130 apparatus). The purity of the peptides used was >98%. Identity of the purified peptides was confirmed by fast atom bombardment-mass spectrum analysis. Peptide stock solutions were made at a peptide concentration of $3 \times 10^{-3}$ M in a solution of 5% dimethyl sulfoxide and 1% bovine serum albumin–phosphate-buffered saline (BSA-PBS) and stored at −20°C.

**Peptide-induced upregulation of H-2D$^b$ surface expression.** The upregulation of MHC expression by peptides added exogenously was measured as previously described (10, 11). Briefly, RMA-S cells were grown at 25°C for 40 h before the assay to enhance the amount of empty H-2D$^b$ molecules available on the cell surface (18). Cells were then incubated at 37°C in the absence or presence of increasing concentrations ($10^{-10}$ to $10^{-5}$ M) of peptide, and the amount of stabilized H-2D$^b$ was measured after a 4-h incubation period. Expression of H-2D$^b$ was studied by fluorescence-activated cell sorter (FACS) analysis on a Becton Dickinson FACScan analyzer. Cells ($5 \times 10^5$ per sample) were incubated on ice for 1 h with 0.1 ml of the mouse monoclonal antibody 28-14-8s (anti-α3 H-2D$^b$ [25]) or, as negative controls, with medium alone or an irrelevant primary antibody. After two washes with ice-cold 1% BSA-PBS, cells were incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G secondary antibody. Cells were then washed three times and fixed in 1% paraformaldehyde in PBS and analyzed.

**Competition binding experiments.** Binding experiments were performed at 37°C with T2-D$^b$ cells, with a Millipore MultiScreen assay system. The H-2D$^b$ LCMV antigen gp276-286 (SGVENPGGYCL) was radioiodinated, and the radiolabeled peptide was purified as previously described (11). Cells ($2 \times 10^5$ per well) were incubated in MultiScreen-HV 96-well filtration plates (pore size, 0.45 μm) with $^{125}$I-gp276-286 (10 nM [final concentration]) for 90 min at 37°C. Cells were washed three times with ice-cold 1% BSA-PBS and by filtration under vacuum. The radioactivity bound to the cells retained on the filter was counted with a gamma counter. Direct binding was measured in the absence (total binding) or the presence (nonspecific binding) of a 1,000-fold excess (10 μM) of unlabeled gp276-286. Specific binding to H-2D$^b$ was defined as the difference between total binding and nonspecific binding. Nontransfected T2 cells were used as a negative control under the same experimental conditions. Competition assays were performed with increasing concentrations ($10^{-10}$ to $10^{-5}$ M) of unlabeled peptides competing against a fixed concentration ($10^{-8}$ M) of $^{125}$I-gp276-286. The percent inhibition of binding was calculated as $100 \times [1 -$ (counts per minute in the presence of competitor − counts per minute of nonspecific binding)/counts per minute of specific binding].

**In vitro cytotoxicity assays.** A standard 5-h $^{51}$Cr-release assay (23) was used to measure CTL lysis. Target cells were incubated for 1 h with $^{51}$Cr, washed, and placed in flat-bottom 96-well plates. Uninfected target cells were subjected to lysis by LCMV-specific *H-2$^b$*-restricted CTL clones in the presence of increasing concentrations (0 to $10^{-4}$ M) of synthetic peptides. Bulk splenocytes obtained from C57 (*H-2$^b$*) mice primed 7 days before the assay with LCMV Armstrong were used at an effector/target (E/T) ratio of 50:1 against cells coated with the GP1 peptides. The CTL clones 228 (25) and NP18 (28) were used at an E/T ratio of 5:1 against cells coated with the GP2 and NP peptides, respectively. Cells infected 48 h before the assay with the LCMV Armstrong strain (multiplicity of infection = 2) were used as positive controls. Uninfected cells in the absence of peptides (50 μl of culture medium) were used as negative controls. Target and effector cells were incubated at 37°C in a final volume of 200 μl. After a 5-h incubation period, fractions (100 μl per well) were removed and counted for $^{51}$Cr activity. The percent specific lysis was calculated as $100 \times$ [(counts per minute of experimental release − counts per minute of spontaneous release)/(counts per minute of total release − counts per minute of spontaneous release)]. Total release and spontaneous release were determined by incubating the labeled cells with 1% Nonidet P-40 and culture medium, respectively. In all experiments, samples were run in triplicate, and for the results, the mean values are given. Variance among the samples was less than 10%.

## RESULTS

**Peptide-induced upregulation of H-2D$^b$ molecules on the RMA-S cell surface.** Empty H-2D$^b$ molecules are expressed on the surfaces of the mouse mutant lymphoma cell line RMA-S but become rapidly unstable at 37°C (18). However, surface expression of H-2D$^b$ or H-2K$^b$ MHC molecules can be upregulated (stabilized) by growing the cells at reduced temperatures (22 to 26°C) for 24 to 48 h (20, 34) or at 37°C by adding peptides exogenously (10, 11, 13, 29). We previously showed that the ability of a peptide to stabilize MHC molecules on the cell surface correlated with the peptide's affinity for the MHC (10, 11). Upregulation experiments are illustrated in Fig. 1, and summarized in Tables 2 and 3. H-2D$^b$ surface expression was measured with a FACS and the mouse monoclonal antibody 28-14-8, which recognizes the α3 domain of H-2D$^b$ (25). As expected, the amount of H-2D$^b$ molecules at the surfaces of cells cultured at 37°C was barely detectable (mean fluores-

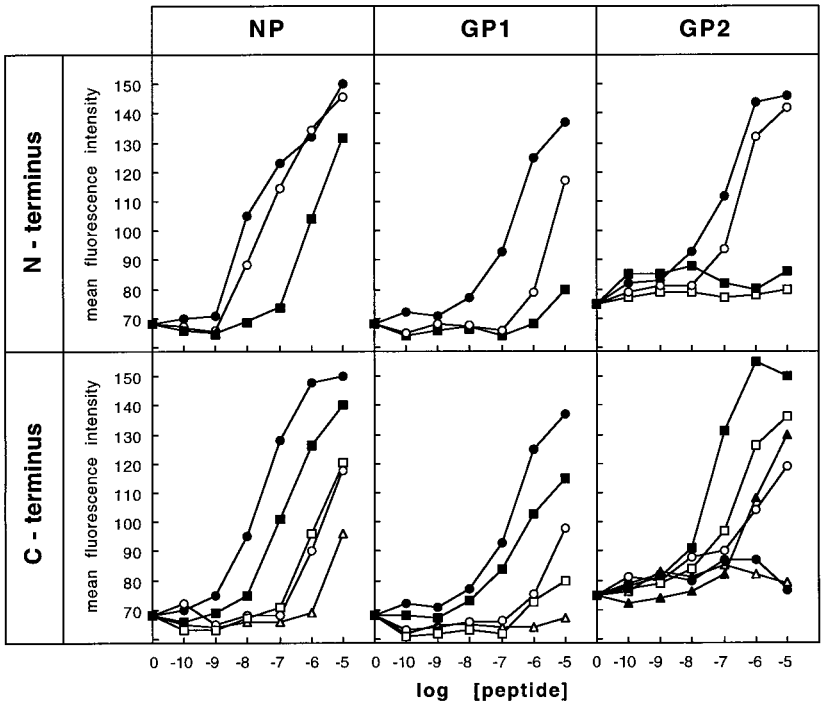


FIG. 1. Upregulation of H-2D$^b$ on the surfaces of RMA-S cells by N- and C-terminus-truncated analogs of the three H-2D$^b$-restricted epitopes, NP, GP1, and GP2, of LCMV. RMA-S cells were grown at 25°C for 40 h before the assay to induce empty, stable H-2D$^b$ molecules on the cell surfaces. Stability of MHC was then measured after a 4-h incubation at 37°C in the presence of increasing peptide concentrations (0 to $10^{-5}$ M). (Upper panels) In the N-terminus-truncated peptide series, the Asn anchoring position varied from position 4 (NP and GP1) or 3 (GP2) to position 6. □, Asn-3; ■, Asn-4; ●, Asn-5; ○, Asn-6. (Lower panels) In the C-terminus-truncated peptides series, the anchoring Asn residue was at position 5 of the sequence. △, 8 aa; ●, 9 aa; ○, 10 aa; ■, 11 aa; □, 12 aa; ▲, 13 aa. Peptide lengths varied from 8 to 12 aa (NP and GP1) or 13 aa (GP2). Expression of H-2D$^b$ was studied by FACS analysis. Cells ($5 \times 10^5$ per sample) were stained with the mouse monoclonal antibody 28-14-8s (anti-α3 H-2D$^b$ [25]) and then with fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G secondary antibody.

cence intensity [mfi] = 18 ± 2, and negative control mfi = 8 ± 2) but increased over fivefold (mfi = 110 ± 7) on cells given a preincubation period of 48 h at 25°C (data not shown). The decrease of thermostabilized H-2D$^b$ molecules observed after 4 h at 37°C in the absence of peptide (mfi = 72 ± 6) was then prevented in a concentration-dependent manner when peptides were added (Fig. 1). The stabilization effect reached a plateau (mfi, ~150) for the highest concentrations of peptides

TABLE 2. Determination of the N terminus peptide sequences for optimal MHC binding and CTL recognition of the three H-2D$^b$-restricted LCMV epitopes

| Viral protein | Peptide | | H-2D$^b$ binding affinity | | Biological activity: CTL sensitization[c] ($ED_{50}$ [nM]) |
|---|---|---|---|---|---|
| | Sequence | Asn (N) anchor position | Upregulation[a] ($SC_{50}$ [nM]) | Competition[b] ($IC_{50}$ [nM]) | |
| NP | QPQNGQFI | 4 | 1,323 ± 286 (3) | 245 ± 87 (3) | >100,000 (3) |
| | FQPQNGQFI | 5 | 50 ± 23 (6) | 20 ± 1 (3) | 0.0002 ± 0.00005 (3) |
| | IFQPQNGQFI | 6 | 467 ± 298 (2) | 54 ± 12 (3) | 0.0006 ± 0.0003 (3) |
| GP1 | AVYNFATC | 4 | >100,000 (3) | >100,000 (3) | 312 ± 138 (2) |
| | KAVYNFATC | 5 | 344 ± 30 (4) | 615 ± 74 (3) | 0.26 ± 0.06 (2) |
| | IKAVYNFATC | 6 | 6,177 ± 951 (3) | 30,600 ± 4,250 (3) | 1.8 ± 0.4 (2) |
| GP2 | VENPGGYCL | 3 | >100,000 (2) | 33,500 ± 6,200 (3) | 2,733 ± 592 (3) |
| | GVENPGGYCL | 4 | >100,000 (2) | 4,722 ± 155 (3) | 122 ± 40 (3) |
| | SGVENPGGYCL | 5 | 42 ± 7 (6) | 37 ± 6 (3) | 1.9 ± 0.9 (4) |
| | SSGVENPGGYCL | 6 | 758 ± 313 (2) | 360 ± 27 (3) | 3.3 ± 0.4 (3) |

[a] RMA-S cells previously grown at 25°C for 40 h were incubated at 37°C in the presence of increasing concentrations of peptide. Stabilization (upregulation) of H-2D$^b$ expression was measured after 4 h by FACS analysis. $SC_{50}$ is the concentration that induces half of the maximal stabilizing effect (values are the means ± standard errors of the mean of *n* [indicated in parentheses] independent experiments).

[b] T2-D$^b$ cells were incubated at 37°C in the presence of $^{125}$I-gp276-286 (10 nM) and increasing concentrations of cold peptides. After 90 min, cells were washed, and the radioactivity bound to the cells was counted on a gamma counter. $IC_{50}$ represents the concentration of competitor that inhibits 50% of the specific binding of the radioactive peptide to H-2D$^b$ (values are the means ± standard errors of the mean of *n* [indicated in parentheses] independent experiments).

[c] $^{51}$Cr-labeled MC57 cells were incubated with increasing concentrations of peptides and subjected to lysis by CTL clones NP18 and 228 specific for the epitope studied (NP and GP2, respectively) at an E/T ratio of 5:1, or by LCMV-specific *H-2$^b$* bulk splenocytes for GP1 at an E/T ratio of 50:1 in a standard 5-h $^{51}$Cr-release assay. $ED_{50}$ is the peptide concentration inducing half of the maximal lysis effect (values are the means ± standard errors of the mean of *n* [indicated in parentheses] independent experiments).

TABLE 3. Determination of the C terminus peptide sequences for optimal MHC binding and CTL recognition of the three H-2D$^b$-restricted LCMV epitopes

| Viral protein | Peptide | | H-2D$^b$ binding affinity | | Biological activity: CTL sensitization[c] ($ED_{50}$ [nM]) |
|---|---|---|---|---|---|
| | Sequence | Length (aa) | Upregulation[a] ($SC_{50}$ [nM]) | Competition[b] ($IC_{50}$ [nM]) | |
| NP | FQPQNGQF | 8 | 21,710 ± 3,892 (3) | 12,025 ± 1,680 (3) | 0.32 ± 0.02 (3) |
| | FQPQNGQFI | 9 | 50 ± 23 (6) | 20 ± 1 (3) | 0.0002 ± 0.00005 (3) |
| | FQPQNGQFIH | 10 | 2,583 ± 499 (3) | 189 ± 25 (3) | 0.003 ± 0.001 (3) |
| | FQPQNGQFIHF | 11 | 985 ± 563 (3) | 60 ± 32 (3) | 0.0012 ± 0.0004 (3) |
| | FQPQNGQFIHFY | 12 | 6,056 ± 1,884 (4) | 114 ± 21 (3) | 0.018 ± 0.004 (3) |
| GP1 | KAVYNFAT | 8 | >100,000 (3) | 45,300 ± 3,800 (3) | 3,270 ± 120 (2) |
| | KAVYNFATC | 9 | 344 ± 30 (4) | 615 ± 74 (3) | 0.26 ± 0.06 (2) |
| | KAVYNFATCG | 10 | 10,630 ± 5,015 (3) | 585 ± 56 (4) | 239 ± 24 (2) |
| | KAVYNFATCGI | 11 | 1,989 ± 412 (4) | 109 ± 25 (4) | 2.0 ± 0.1 (2) |
| | KAVYNFATCGIF | 12 | 7,417 ± 2,117 (3) | 1,088 ± 347 (3) | 3.4 ± 0.1 (2) |
| GP2 | SGVENPGG | 8 | >100,000 (3) | 44,050 ± 3,325 (3) | >100,000 (4) |
| | SGVENPGGY | 9 | >100,000 (3) | 13,155 ± 2,600 (3) | 23,500 ± 6,000 (3) |
| | SGVENPGGYC | 10 | 6,481 ± 1,041 (2) | 464 ± 44 (3) | 1,150 ± 677 (3) |
| | SGVENPGGYCL | 11 | 42 ± 7 (6) | 28 ± 3 (3) | 1.9 ± 0.9 (4) |
| | SGVENPGGYCLT | 12 | 553 ± 75 (2) | 142 ± 26 (3) | 637 ± 343 (4) |
| | SGVENPGGYCLTK | 13 | 1,919 ± 226 (2) | 533 ± 7 (3) | 8.1 ± 3.4 (4) |

[a] See footnote *a* to Table 2.
[b] See footnote *b* to Table 2.
[c] See footnote *c* to Table 2.

tested. Allele-mismatched ($K^b$ or $L^d$) peptides were used as negative controls and tested at concentrations of up to $10^{-4}$ M but induced no significant (less than 10%) upregulation of H-2D$^b$ molecules (not shown).

**Competition binding experiments on living T2-D$^b$ cells.** Human T2 cells transfected with the *H-2D$^b$* gene (T2-D$^b$) express empty H-2D$^b$ molecules on their surfaces (1). Interestingly, we found (12a) that such empty MHC molecules were stable at 37°C and that lowering the temperature to 25°C did not enhance expression, in contrast to what is observed for RMA-S cells. We thus took advantage of the availability of the MHC molecules to perform binding experiments directly at the surfaces of living cells. The H-2D$^b$ LCMV epitope SGVENPG GYCL (gp276-286) was first radioiodinated as previously described (11). As shown in Fig. 2a, $^{125}$I-gp276-286 (10 nM) bound efficiently to T2-D$^b$ cells (total binding, 3,300 ± 150 cpm). Binding of the radiolabeled peptide was exquisitely specific for H-2D$^b$, since it was completely inhibited by the presence of $10^{-4}$ M cold peptide (nonspecific binding, 210 ± 10 cpm) and no binding was observed on T2 cells (background level, ~200 to 300 cpm). Thus, the high level of D$^b$-specific binding on T-2D$^b$ cells allowed the use of competition experiments for the measurement of binding affinities for H-2D$^b$. As shown in Fig. 2b, inhibition of binding was concentration dependent. Complete (100%) inhibition was reached at the highest concentrations of peptides able to bind to H-2D$^b$. When the allele-mismatched peptides used previously as a negative control in stabilization studies were tested for competition, no significant inhibition (less than 10%) of the binding of the radiolabeled H-2D$^b$ peptide occurred at concentrations of up to $10^{-4}$ M (not shown).

Within a series, the relative affinities of the analogs (i.e., ranked from the strongest to the weakest binder) determined in the stabilization studies corresponded to those found in competition experiments, except for the C-terminus-truncated analogs of GP1 (see Table 2 and Results).

**Peptide sensitization of *H-2$^b$* target cells to lysis by epitope-specific CTL clones.** After determination of their MHC binding properties, the analogs were tested for their abilities to sensitize MC57 target cells for recognition and lysis by LCMV-specific CTL clones (Fig. 3). A general observation was that the concentrations of peptides needed to sensitize target cells for lysis were lower than those used either to stabilize H-2D$^b$ molecules or to compete with the radiolabeled peptide in binding experiments. Each of these effects has a different threshold. Triggering of CTL lysis is observed when as little as 0.1% of the total amount of MHC molecules on surfaces of target cells is in complex with the viral antigen (3), whereas in contrast, MHC binding is significantly observed when at least 10% variation of the amount of antigen-MHC complex occurs. Also, the CTL clone's avidity for the antigen-MHC complex must be taken into account. As observed in stabilization and competition experiments, a maximal effect was reached with the active peptides in cytotoxicity assays, allowing the calculation of 50% effective dose ($ED_{50}$) values. Maximal lysis was sometimes followed by a plateau or even by a slight decrease for the most efficiently lytic peptides (see, for example, the N-terminus-truncated Asn-5 and Asn-6 NP peptides in Fig. 3).

**MHC binding and CTL sensitization properties of N-terminus-truncated peptides: the presence of the Asn anchor residue at position 5 is a common requirement for optimal presentation of the three LCMV H-2D$^b$ epitopes.** The importance of the Asn anchoring position was analyzed by studying the effect of N terminus truncation on MHC binding and CTL sensitization by the epitopes. The properties of peptide analogs with Asn at position 4, 5, or 6 (for NP and GP1) or 3, 4, 5, or 6 (GP2) tested comparatively (illustrated on the upper parts of Fig. 1, 2b, and 3) are summarized in Table 2. For all three epitopes, the analog bearing the Asn residue at position 5 was the most efficient in binding to H-2D$^b$ (in both binding assays) and in sensitizing MC57 target cells for CTL lysis. Lengthening the sequence (Asn at position 6) resulted in peptides with decreased functional ability. This effect was barely significant for NP and weak for GP2 but was more important for GP1, particularly for MHC binding. Shortening the sequence led to peptides with Asn at position 4 (NP, GP1, and GP2) and 3

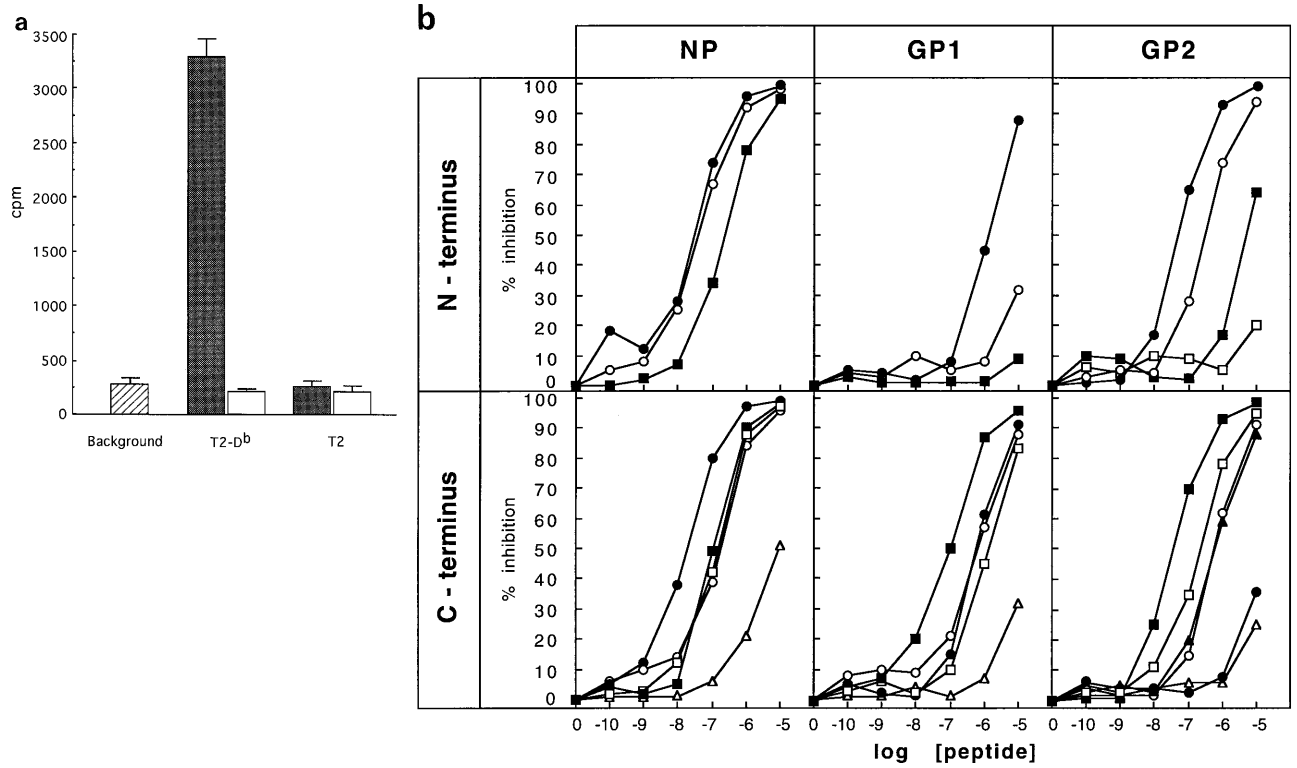


FIG. 2. Binding of the N- and C-terminus-truncated analogs of the three H-2D$^b$-restricted epitopes, NP, GP1, and GP2, of LCMV to H-2D$^b$ on viable T2-D$^b$ cells. LCMV gp276-286 was radioiodinated by the chloramine T method, and the radioactive peptide was separated from free $^{125}$I by gel permeation chromatography. Cells ($2 \times 10^5$ per sample) were incubated with $^{125}$I-gp276-286 (10 nM) for 90 min at 37°C. (a) Direct binding was measured in the absence (total binding [shaded column]) or presence (nonspecific binding [open column]) of a 1,000-fold excess of unlabeled gp276-286 (10 μM) on T2-D$^b$ and on untransfected T2 (negative control) cells. The background level (hatched column) was measured by counting the radioactivity retained on the filter in the absence of cells. (b) In competition assays, increasing concentrations (0 to $10^{-5}$ M) of unlabeled N-terminus (upper panels)- or C-terminus (lower panels)-truncated peptides (symbols are as in Fig. 1) were used for competition against a fixed concentration (10 nM) of $^{125}$I-gp276-286. Specific binding to H-2D$^b$ was defined as the difference between total binding and nonspecific binding. The percent inhibition of binding was calculated as described in Materials and Methods and is represented as a function of the concentration of competitor peptide.

(GP2) and produced a much more dramatic lytic effect. The Asn-4 NP peptide had a reduced, but still reasonably good, binding affinity (50% sensitization concentration [$SC_{50}$] = 1,323 nM; 50% inhibitory concentration [$IC_{50}$] = 245 nM) but was completely unable to sensitize *H-2$^b$* target cells for CTL lysis ($ED_{50}$ > 100,000 nM), indicating that the residue NP Phe-396 participated to some extend in MHC interaction but was mainly implicated in epitope recognition by the T-cell receptor. In contrast, the Asn-4 GP1 peptide exhibited a dramatically reduced binding affinity ($SC_{50}$ and [$IC_{50}$] values, > 100,000 nM) but was still able to sensitize target cells for CTL lysis ($ED_{50}$ = 312 nM). For GP2, the successive deletions from Asn-5 to Asn-3 led to a loss of the initial peptide properties either complete and immediate in upregulation experiments ($SC_{50}$ > 100,000 nM) or strong and gradual (about 1 log order of magnitude after each truncation) in competition and sensitization experiments.

**MHC binding and CTL sensitization properties of C-terminus-truncated peptides.** The importance of peptide length was analyzed further by studying the effect of C terminus truncation on the MHC-binding and CTL sensitization properties of the epitopes. These experiments were performed with peptides bearing the Asn anchoring residue at their optimal position, position 5, and with lengths varying from 8 to 12 aa (NP and GP1) or 13 aa (GP2). The results of these experiments are illustrated on the lower part of Fig. 1, 2b, and 3 and are summarized in Table 3.

**(i) The 9-mer peptide np396-404 (FQPQNGQFI) is the optimal sequence of the NP epitope.** Unambiguous results, with well-correlated data between the three assays, were obtained when the effect of peptide length on the NP epitope was examined; whatever the assay, the nonameric sequence np396-404 was the most efficient. FQPQNGQFI both showed a good affinity for H-2D$^b$ (in the 20 to 50 nM range) and, as discussed above, was very efficient in sensitizing MC57 target cells ($ED_{50}$ = 0.0002 nM). Shortening the 9-mer sequence to an 8-mer dramatically decreased (about 3 logs) but did not completely abolish the binding or sensitization properties of the NP epitope. On the other hand, a significant but less pronounced decrease (of about 1 to 2 logs) was measured when the 9-mer sequence was extended to a 12-mer. Interestingly, with lengthening, CTL sensitization dropped gradually and binding fluctuated, with the affinity of the 11-mer being higher than those of the 10- and 12-mers.

**(ii) The 11-mer peptide gp276-286 (SGVENPGGYCL) is the optimal sequence of the GP2 epitope.** As for the NP epitope, a clear pattern also emerged for the GP2 epitope. However, in this case, the sequence displaying both optimal binding to MHC and optimal sensitization of target cells to CTL lysis was the 11-mer peptide. This GP2 fragment, gp276-286 (SGVEN PGGYCL), bound to H-2D$^b$ with a high affinity ($SC_{50}$ and $IC_{50}$ values were in the 30 to 40 nM range) and sensitized target cells efficiently ($ED_{50}$ = 0.26 nM). Affinity and activity of the shorter (10- to 8-mer) and longer (12- and 13-mer) C-termi-

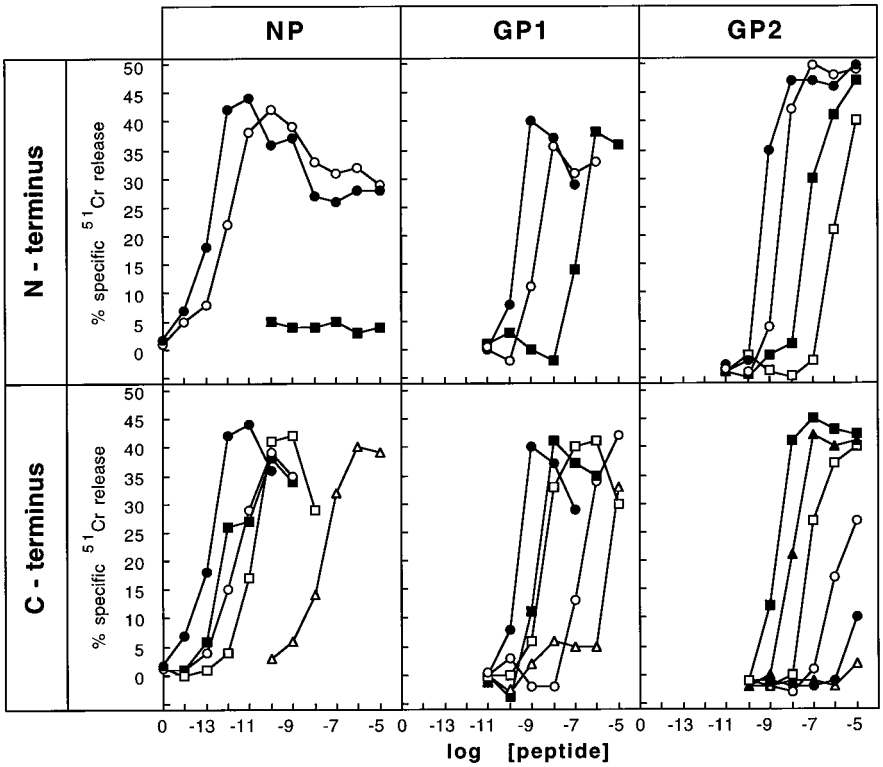


FIG. 3. Sensitization of *H-2$^b$*-restricted target cells to *H-2$^b$*-restricted LCMV-specific CTL lysis by N- and C-terminus-truncated analogs of the three H-2D$^b$-restricted epitopes, NP, GP1, and GP2, of LCMV. Uninfected MC57 (*H-2$^b$*) target cells were combined with *H-2$^b$*-restricted LCMV-specific CTL in the absence or presence of increasing concentrations of N-terminus (upper panels)- or C-terminus (lower panels)-truncated analogs of the NP ($10^{-14}$ to $10^{-5}$ M), GP1 ($10^{-11}$ to $10^{-5}$ M), or GP2 ($10^{-10}$ to $10^{-5}$ M) epitopes (symbols are as in Fig. 1). The NP-specific CTL clone NP18 (28) and GP2-specific CTL clone 228 (24) were used at an E/T ratio of 5:1 against cells coated with the NP and GP2 peptides, respectively. Bulk splenocytes from C57 mice primed 7 days before the assay with LCMV Armstrong were used at an E/T ratio of 50:1 against cells coated with the GP1 peptides. A standard 5-h $^{51}$Cr-release assay (24) was used to measure CTL lysis. Percentage of specific $^{51}$Cr release was calculated as described in Materials and Methods.

nus-truncated sequences were affected in a stepwise manner. The first shortening step (reducing the sequence to 10 aa) had a much stronger effect on CTL sensitization than on MHC binding. Further, C terminus deletions led to binding or sensitization properties which were either very weak (9-mer) or barely measurable (8-mer). The first lengthening step (extension of the 11-mer sequence to the 12-mer) resulted in an overall drop of the GP2 epitope's properties. Interestingly, an additional extension (13-mer), which decreased further the binding affinity (20 to 50 times less than the 11-mer), significantly restored CTL sensitization ($ED_{50}$ = 8.1 nM).

**(iii) The 9-mer gp33-41 (KAVYNFATC) and 11-mer gp33-43 (KAVYNFATCGI) peptides are the optimal sequences of the GP1 epitope.** In stabilization experiments performed on RMA-S (*D$^b$ K$^b$*) cells (lower part of Fig. 1; Table 3), the most efficient peptides to induce upregulation of H-2D$^b$ molecules at the cell surface were the 9-mer sequence KAVYNFATC ($SC_{50}$ = 344 nM) and then the 11-mer sequence KAVYN FATCGI, which was six times less effective ($SC_{50}$ = 1,989 nM). The longer (12-mer) and intermediate (10-mer) peptides were weak inducers ($SC_{50}$ values were greater than 5,000 nM). The shorter peptide (8-mer) was unable to stabilize H-2D$^b$ molecules ($SC_{50}$ > 100,000 nM). In competition experiments performed on T-2D$^b$ (*D$^b$*) cells (lower part of Fig. 2b; Table 3), a different profile was observed. The three contiguous sequences, 9-, 10-, and 11-mers, competed well with the radiolabeled peptide. Among them, the 11-mer peptide was the most efficient ($SC_{50}$ = 109 nM), with the 10- and 9-mer peptides being about six times less effective ($SC_{50}$ values were about 600 nM). The 12- and the 8-mer peptides exhibited weak (12-mer) or barely measurable (8-mer) affinity, in accordance with stabilization data. In cytotoxicity assays performed on MC57 (*D$^b$ K$^b$*) cells (lower part of Fig. 3; Table 3), three peptides induced effective CTL sensitization: the 9-, 11-, and 12-mers. The 9-mer was the most active ($ED_{50}$ = 0.26 nM), with the 11- and 12-mer peptides being about 10 times less efficient. Although of intermediate length between the two, the 10-mer sequence was a poor inducer of CTL activity. Finally, high concentrations of the 8-mer peptide (4 logs higher than those of the 9-mer) were needed to sensitize target cells ($ED_{50}$ = 3,270 nM).

To understand the apparent discrepancy between the 9-mer and the 11-mer as the optimal sequence of GP1, these two peptides were tested for their abilities to induce CTL sensitization of T-2D$^b$ target cells (expressing only D$^b$) compared with MC57 target cells (expressing both D$^b$ and K$^b$). Interestingly, the two peptides exhibited different profiles depending on the target cells tested (Table 4). As previously found, the 9-mer was about eight times more potent than the 11-mer in sensitizing MC57 cells. In this assay, their relative biological activities correlated with their relative binding properties determined in upregulation experiments using RMA-S (*D$^b$ K$^b$*) cells. In contrast, the two peptides were equipotent in sensitizing T-2D$^b$ cells ($ED_{50}$ = 2 to 3 nM), more closely matching their binding abilities in competition experiments.

TABLE 4. Comparative analysis of the abilities of GP1 9- and 11-mer peptides to sensitize *H-2$^b$* target cells to lysis by LCMV-specific CTLs

| GP1 peptide | | CTL sensitization ($ED_{50}$ [nM])[a] | |
|---|---|---|---|
| Sequence | Length (aa) | MC57 cells | T2-D$^b$ cells |
| KAVYNFATC | 9 | 0.9 ± 0.6 | 2.5 ± 0.7 |
| KAVYNFATCGI | 11 | 6.3 ± 3.8 | 2.0 ± 1.4 |

[a] $^{51}$Cr-labeled MC57 and T-2D$^b$ cells were incubated with increasing concentrations of the GP1 9-mer or 11-mer peptide and subjected to lysis by LCMV-specific *H-2$^b$* bulk splenocytes at an E/T ratio of 50:1 in a standard 5-h $^{51}$Cr-release assay. $ED_{50}$ is the peptide concentration inducing half of the maximal lysis effect (values are the means ± standard errors of the mean of two independent experiments).

## DISCUSSION

Our study established the three LCMV H-2D$^b$ epitopes that are optimal for MHC binding and CTL recognition. The optimal NP and GP2 sequences were determined very precisely as the 9-mer np396-404 and 11-mer gp276-286, respectively, although some ambiguousness persisted for GP1 between the 9-mer gp33-41 and the C-terminal-extended 11-mer gp33-43. The H-2D$^b$ motif determined for the natural peptides eluted from H-2D$^b$ molecules (8) is mainly characterized by (i) a usual length of 9 residues and (ii) the presence of two anchoring residues, one at position 5 occupied by an Asn and one at the C terminus occupied by a hydrophobic residue (Met, Ile, or Leu). The optimal sequences of the LCMV epitopes fit this motif very closely (NP) or with some variation (GP2 and GP1).

Analysis of the N-terminus-truncated peptides allowed identification of the residue at position 1 and consequently the position of the Asn residue. Previously, we have shown that alanine substitution of Asn-400, Asn-280, and Asn-37 dramatically reduced binding of the NP and GP2 (10) and GP1 (unpublished observation) epitopes, respectively, thereby confirming the crucial role of Asn in anchoring the peptide to H-2D$^b$. In this study we demonstrated that the Asn anchor is optimally placed at position 5 of the sequences of all three LCMV epitopes. The first five N-terminal residues of the sequence of the naturally processed LCMV H-2D$^b$ epitopes are likely to be FQPQN, KAVYN, and SGVEN for NP, GP1, and GP2, respectively. Additionally, although extension of the epitopes' N termini was tolerated, in contrast, their truncation resulted in a much more dramatic effect. This indicates that position 1 plays a positive role in the interaction of the peptides with H-2D$^b$ molecules, even though the N-terminal residue is not commonly considered to be an anchor residue. Given the heterogeneous character of the LCMV epitopes at position 1, it seems unlikely that the side chain of the residue at the N terminus is involved in MHC binding, in agreement with the crystallographic data obtained for the structure of MHC molecules in complex with a peptide (9, 19, 34). The LCMV NP epitope is a good example to illustrate the orientation of the peptide side chain at position 1 outside the peptide-MHC complex, since deletion of NP Phe somewhat diminishes MHC binding but also, and more importantly, completely abrogates recognition of the peptide by the CTL clone NP18, indicating that the side chain is oriented towards the T-cell receptor. This possibility may not be unique, and conformational effects exerted by the residue at position 1 may be transmitted through the peptide structure and result in reduced T-cell receptor recognition of the bound antigen. However, this is not observed for LCMV GP1 or GP2, suggesting that GP1 Lys-1 and GP2 Ser-1 side chains do not serve as recognition sites for their respective CTL.

Analysis of the C-terminus-truncated peptides allowed determination of the optimal length of the epitopes and consequently identification of the anchor residue at the C terminus. The optimal NP sequence matches perfectly the H-2D$^b$ endogenous peptide motif (8). Interestingly, in addition to Asn at position 5 and Ile at position 9, the amino acids located at all the other positions of the NP peptide (i.e., Phe at positions 1 and 8; Gln at positions 2, 4, and 7; and Pro at position 3; Gly at position 6 is excepted) are among those identified in the pool of the natural peptides (8). Shortening or lengthening of the 9-mer sequence led to peptides with lower affinities and lower CTL sensitization activities. We can thus assume that the sequence of np396-404 (FQPQNGQFI) may correspond to that of the naturally presented NP epitope at the surfaces of LCMV-infected *H-2$^b$* cells.

The optimally active sequence of the GP2 epitope does not correspond to the predictable 9-aa standard length but bears at position 11 the expected hydrophobic residue, Leu-286, as the C-terminal anchor. The Pro-Gly-Gly motif in the C-terminal part of the GP2 peptide may account for this added length. Proline residues are known to be strong conformational inducers (17), and we previously showed that replacing LCMV GP Pro-281 with its D-isomer reduced by at least 100-fold the MHC-binding properties of the peptide by changing its secondary structure (11). The local constraints imposed by the Pro-Gly-Gly sequence might confer to the GP2 backbone the correctly arched structure, which would allow the peptide to rise over the peculiar H-2D$^b$ hydrophobic ridge (34) and direct the leucine side chain at position 11 toward the F pocket of the MHC molecule. Furthermore, Pro and Gly are considered to be small amino acids, and this likely influences (shortens) the linear distance between the two anchors, as already noted (8). In fact, the H-2D$^b$-restricted adenovirus E1A epitope, a 10-aa peptide with a Pro-Pro motif in the same region (SGPSNTPPEI) (16), shares sequence similarity with the LCMV GP2 optimal epitope. Thus, the predictable sequence of the GP2 epitope to be naturally processed is likely to be gp276-286 (SGVENPGGYCL).

The GP1 epitope had two possible optimal sequences. One, nonamer gp33-41 (KAVYNFATC), corresponded to the usual optimal length. Strikingly, the C-terminal anchoring position was not occupied by an expected Met, Ile, or Leu residue. This is a unique observation, since all the viral epitopes (naturally processed or putative) characterized so far have had the hydrophobic anchor residue at their C termini (5, 6). In fact, the nature of the MHC F pocket determines the identity of the C-terminal peptide residue. In the H-2D$^b$ molecule, the presence of Phe-74 and Phe-116 imposes the hydrophobic character of the side chain of the residue. However, the fact that the GP1 epitope possesses a strong residue (Phe) at position 6 (although NP and GP2 do not) may favor the required alignment of the C-terminal cysteinyl residue with the F pocket of the MHC. However, the overall interactions of the H-2D$^b$ molecule with such a peptide should be weaker than with a peptide bearing the correct anchors. In confirmation, the GP1 epitope exhibited significantly less affinity for H-2D$^b$ than did NP and GP2. The other GP1 sequence also found to be optimal was the 11-mer gp33-43 (KAVYNFATCGI). In this case, the C-terminal anchor position was correctly occupied by the hydrophobic residue Ile-43. However, unlike the GP2 epitope, the C-terminal half of this GP1 peptide bore no evidence of a peculiar motif that might impose a specific conformation to, or influence the linearity of, the peptide sequence. Other peptides longer than 9-mers have been shown to bind to HLA-Aw68 via

their terminal and anchor residues but to bulge out in the middle (12). Such a scheme might also apply to the GP1 11-mer. As stated above and previously (34), contacts with the MHC are likely to be weak or fail to occur at the location of the bulge, thus lowering the peptide's binding affinity. In accord, the GP1 11-mer had a significantly lower affinity for H-2D$^b$ than did the NP and GP2 epitopes. The fact that the 11-mer prevailed with T2-D$^b$ cells, which express only the transfected murine allele H-2D$^b$ and that, conversely, the 9-mer prevailed in experiments using RMA-S and MC57 cells, which constitutively express both D$^b$ and K$^b$, led us to examine carefully the sequence of the GP1 epitope. We found that the 11-mer could actually be superimposed onto the H-2K$^b$ binding motif (5, 8). If so, the hydrophobic Ile-43 as the C-terminal anchor could promote binding not only to D$^b$ but also to K$^b$ when present. The possible interference in binding between D$^b$ and K$^b$ is supported by the fact that a single peptide can bind to two different MHC alleles by reorientation of the side chains of some residues (22). This scenario would account for the apparent discrepancy in our results and is currently under investigation. Further, the possibility that an H-2K$^b$-restricted epitope is contained in the GP1 sequence cannot be ruled out (2, 16). In contrast to the NP and GP2 epitopes, the dual optimal sequences for GP1 hamper the unambiguous prediction of a potential unique peptide and further prevent ruling out a possible natural copresentation of the two sequences.

In this study, the properties of the peptides were analyzed, and the components in their linear sequences were compared. The relative simplicity of delineating a unique and clearcut profile of the N terminus contrasted sharply with the greater difficulty of identifying (unambiguously or not) the C terminus. This clearly suggests a predominant role of the peptide primary structure (length) at the N-terminal half and, in contrast, of the secondary structure (conformation) at the C-terminal half of the epitopes. This is in perfect accord with the crystallographic model of H-2D$^b$ (34) showing that residues located between position 5 and the C terminus are excluded from the inside of the MHC cleft by the presence of the hydrophobic ridge and are, therefore, likely to interact with the T-cell receptor. The identification of LCMV variants with a mutation in this region and escaping CTL recognition support this hypothesis. Sequence analysis of these CTL escape mutants revealed the presence of a single mutation within the epitope occurring at position 6 (Phe to Leu) of GP1, position 7 (Gly to Asp) of GP2, or position 8 (Phe to Leu) of NP without affecting MHC binding (17a).

Among the endogenously presented viral or tumoral antigens characterized so far, the sequence of the natural peptide corresponds to the optimal sequence for both MHC binding and CTL sensitization. Obtaining and using defined optimal sequences will allow approaches to prime or upregulate the immune response during suppression or downregulate it during virus-induced immune injury. Such studies are currently in progress in our laboratories.

## ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service grants AI09484 and NS12428 and Conseil Régional Midi-Pyrénées contract 9300254 to Jean Gairin.

We acknowledge the excellent technical assistance of H. Lewicki and A. Tishon and manuscript preparation by Gay Schilling and Phyllis Minick.

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