H-2M3^a Violates the Paradigm for Major Histocompatibility Complex Class I Peptide Binding

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Summary

The major histocompatibility (MHC) class I-b molecule H-2M3^a binds and presents *N*-formylated peptides to cytotoxic T lymphocytes. This requirement potentially places severe constraints on the number of peptides that M3^a can present to the immune system. Consistent with this idea, the M3^a-L^d MHC class I chimera is expressed at very low levels on the cell surface, but can be induced significantly by the addition of specific peptides at 27°C. Using this assay, we show that M3^a binds many very short *N*-formyl peptides, including *N*-formyl chemotactic peptides and canonical octapeptides. This observation is in sharp contrast to the paradigmatic size range of peptides of 8–10 amino acids binding to most class I-a molecules and the class I-b molecule Qa-2. Stabilization by fMLF-benzyl amide could be detected at peptide concentrations as low as 100 nM. While *N*-formyl peptides as short as two amino acids in length stabilized expression of M3^a-L^d, increasing the length of these peptides added to the stability of peptide–MHC complexes as determined by 27–37°C temperature shift experiments. We propose that relaxation of the length rule may represent a compensatory adaptation to maximize the number of peptides that can be presented by H-2M3^a.

Most peptides eluted from MHC class I molecules are 8–10 amino acids long, depending on the allele (1, 2), as confirmed by crystallography of various mouse and human class I molecules (3–5). The charged amino and carboxyl peptide termini engage deep pockets in the antigen-binding cleft by forming hydrogen bonds with highly conserved heavy chain residues within pocket A (for the NH_2 terminus) and pocket F (COOH terminus). For H-2K^b, peptides that are nine amino acids in length buckle at positions P4 and P5 to accommodate the termini in these pockets (3). Terminal modifications such as N-formylation or C-amidation inhibit formation of hydrogen bonds and severely impair binding (6, 7). Similarly, heavy chain mutations disrupting H bonding in these pockets greatly destabilize the MHC complex (8).

However, the length rule of 8–10 amino acids is not absolute. Longer peptides (14–17 amino acids) bind a minor fraction of HLA-A2, HLA-B27, and H-2K^b molecules (9–11), and L^d-restricted peptides as short as four amino acids have been reported to be presented to CTL (12, 13). In addition, inhibition of some (14) but not all (15) M3^a-restricted CTLs by chemotactic peptides has been reported.

Allele-specific pockets impart peptide specificity to class I molecules. Peptide anchor residues are often located at the P2, P5, and COOH terminal residues (1). To bind a particular class I-a molecule, peptides must have the correct length and appropriate binding motif. This paradigm also applies to the class I-b molecule Qa-2 (16, 17).

The requirement for a fixed NH_2 terminus is reiterated in M3^a, in which replacements of conserved residues in pocket A may account for the nearly absolute demand for the *N*-formyl moiety (18, 19). The *N*-formyl terminus of NADH dehydrogenase subunit I (fND1)[1] binds M3^a to stimulate fND1-specific CTL (18, 19). The natural length of endogenous *N*-formyl peptides bound by M3^a is not known. To quantify cell surface expression of M3^a, we have constructed a class I chimera comprised of the α_1 and α_2 domains of M3^a and α_3 of L^d (M3^a-L^d) that is detected by the L^d α_3-specific mAb HB27 (20). Surface expression of the chimera is limited physiologically by the poor availability of *N*-formyl peptide ligands (21). Although the mechanisms differ in detail, this phenotype resembles that of surface expression of MHC class I molecules in RMA-S cells. These possess defective transporters associated with antigen processing (TAP) proteins and consequently fail to transport peptides into the endoplasmic reticulum (22). Addition of specific peptide or incubation at cold temperatures (≤31°C) restored surface expression of class I molecules in these mutant cells (23). Similarly, addition of appropriate exogenous ligands stabilized surface expression of M3^a-L^d (21).

By focusing on *N*-formyl peptides, M3^a binds a far nar-

[1] *Abbreviations used in this paper:* CI, confidence interval; fND1, *N*-formyl terminus of NADH dehydrogenase subunit 1; GAMIg, goat anti-mouse Ig; IBS, independent binding of side chains; SMLF, specific mean linear fluorescence; TAP, transporters associated with antigen processing.

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rower range of peptides than do other characterized class I molecules. Its specificity for *N*-formyl peptides potentially narrows the thymocyte-selecting (self)-repertoire of $M3^a$ to a small subset of mitochondrial-encoded peptides. This stringency of peptide binding allows $M3^a$ to ignore most self-peptides and focus on bacterial peptides. Since as prokaryotes initiate proteins with *N*-formylmethionine, $M3^a$ may be adapted for presenting microbial peptides to $CD8^+$ T cells (24, 25). Consistent with this hypothesis, $M3^a$ presents peptides from *Listeria monocytogenes* to $CD8^+$ $\alpha\beta$ T cells (14, 15).

Infecting bacteria release *N*-formyl peptides that bind the chemotactic peptide receptor on neutrophils and monocytes to trigger migration and cytokine release (26). The chemotactic peptide receptor recognizes peptides of three to six amino acids in length, shorter than typical MHC class I–binding peptides (27). We postulated that to expand the number and diversity of its bacterial ligands, $M3^a$ may also bind short *N*-formyl peptides.

Materials and Methods

Antibodies. Supernatants of hybridomas were used for immunofluorescence. mAbs were specific for the α_3 domain of the L^d molecule, HB27 (American Type Culture Collection [ATCC], Rockville, MD); a conformationally sensitive epitope on K^b, TIB139 (ATCC); another conformationally sensitive epitope on K^b, HB176 (ATCC; 6); and a conformationally sensitive epitope on D^b, HB19 (ATCC; 28). IgG fractions of culture supernatants were separated using a protein G column in an FPLC™ system (Pharmacia Fine Chemicals, Piscataway, NJ) and used for all immunoprecipitations.

Peptide Synthesis and Purification. The fND1 peptide corresponds to the amino terminus of $ND1^\alpha$. The sequence of the dodecameric peptide is fMFFINILTLLVP. f-Bla-$z_{1\text{-}8}$ peptide (fMFVLNKFF), from *Bacillus cereus* β-lactamase, binds to $M3^a$ as determined by CTL competition assays (24) and $M3^a$-L^d stabilization (21). Ac-Bla-z and Bla-z are the N-acetylated and unsubstituted forms of this peptide, respectively. The SIINFEKL peptide refers to a peptide from chicken ovalbumin that has been shown to bind K^b (29). The Sendai virus nucleocapsid peptide FAPGNYPAL binds both to K^b and D^b (30). Indicated lengths of these peptides were synthesized and HPLC purified as described previously (19). All other short *N*-formyl peptides were obtained from Sigma Chemical Co. (St. Louis, MO) or Bachem Biochemicals (King of Prussia, PA). Several non–N-formylated short peptides (Sigma Chemical Co.) were used as negative controls. These include GRGDSPK, GGFM, GGFL-NH_2, AGSE, GQ, GLM-NH_2, and *N*-carboxymethyl-FL.

Cytotoxic T Lymphocyte Generation. The fND1$^\alpha$-specific, $M3^a$-restricted mCTL clones 1D8 and 3D5 were described elsewhere (18). These mCTLs are specific for a peptide from the mitochondrial gene product ND1 (NADH dehydrogenase subunit 1) in the context of $M3^a$.

Cell Culture. SVCAS2.F6 tail cell fibroblasts ($H\text{-}2^{w17}$ $M3^b$) were transfected with pM3^a-L^d to create the 13S2 cell line (21). Cells were passaged in DMEM plus 10% FCS, 1% gentamicin reagent solution, and 1 mg/ml G418. WEHI 105.7 thymoma cells were described elsewhere (18). All cell culture reagents were from GIBCO BRL (Gaithersburg, MD).

Immunofluorescence Staining and Flow Cytometry Analysis. Immunofluorescence analysis was done as described (21, 31). Briefly, subconfluent transfected cells were incubated with the designated peptide for 12–16 h at 37 or 27°C in culture media. Cells were trypsinized, collected, and washed three times in ice-cold PBS before resuspension in PBS at a final concentration of 10^7 cells/ml. 100 μl of cell suspension was incubated with 100 μl of mAb in the appropriate dilution for 30 min at 4°C. After two washes with cold PBS, the cells were stained with a 1:50 dilution of FITC-conjugated goat anti-mouse (GAMIg) antibodies (Baxter Healthcare Corp., Mundelein, IL) for 30 min at 4°C. Samples were then fixed with 1% paraformaldehyde (vol/vol in PBS) and analyzed on a flow cytometer (EPICS Profile; Coulter Corp., Hialeah, FL). Specific mean linear fluorescence (SMLF) of a population on a four-decade scale (0.1–1,000) was calculated by subtracting the mean linear fluorescence of cells stained with FITC-GAMIg alone from the mean linear fluorescence of cells stained with a particular mAb. Voltages were adjusted to ensure that control staining with the secondary antibody alone (FITC-GAMIg) was $\sim 1 \pm 0.2$ U. Additionally, gates were set around narrow windows of forward light scatter to minimize cell size–dependent artifacts (31).

Peptide Competition Assay. This assay was described elsewhere (18). WEHI 105.7 cells were washed and resuspended to 6×10^6 cells/ml in growth medium. 15×10^6 cells were incubated with 250 mCi of $Na_2[^{51}Cr]O_4$ in a 15-ml graduated conical tube (Falcon 2099; Becton Dickinson & Co., Lincoln Park, NJ) on a rocker platform at intermediate speed at 37°C for 90 min. These cells were washed once and placed over an isolymph gradient to enrich for viable cells. After two washes, cells were incubated in prewarmed growth medium at a concentration of 1.33×10^6 cells/ml. 3 ml of cells was added to 12-well tissue culture plates (Costar Corp., Cambridge, MA). Increasing concentrations of putative competitor peptide were dissolved in 1 ml of prewarmed media in the presence of 50 nM fND1$^\alpha_{1\text{-}12}$ and added to the cells. The cells were incubated at 37°C in 5% CO_2 for 90 min. Target cells were washed twice before incubation with either CTL clone 1D8 or 3D5 in a 4-h $Na_2[^{51}Cr]O_4$ release assay using an E/T ratio of 20:1. Percentage of specific lysis was calculated as follows: percent specific lysis = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100%. Unless otherwise indicated, standard errors of the measurements, calculated by propagation of errors (32), were <5%.

Temperature Shift Assay. 13S2 cells were incubated with the indicated peptide at 27°C for 16 h. Cells were transferred to 37°C for graded periods of time, rapidly placed on ice, and stained using the protocol described above. Fold increase was calculated as the SMLF of cells incubated with peptide divided by the SMLF of cells incubated in medium containing DMSO alone. In some experiments, cells induced with peptide at 27°C overnight were shifted to 37°C without changing the medium to monitor the loss of surface expression. The rate of loss was estimated by nonlinear regression using an approach-to-steady-state model described by the following equation (33):

$$C_t = \frac{k_0}{k_1} - \left(\frac{k_0}{k_1} - C_0\right) * e^{-k_1 t}$$

Data were analyzed using an adaptive nonlinear least-squares algorithm (34) in the program Nonlin (Phillip H. Sherrod, 4410 Gerald Place, Nashville, TN 37205-3806. 76166.2640@compuserve.com). The program was instructed to minimize the error of three parameters: C_0, the steady-state value at 27°C; k_0, the zero-order rate constant of appearance of epitopes at the surface; and k_1, the first-order rate constant of loss (where $t_{1/2} = \ln 2/k_1$). Data supplied were the time (t) and SMLF (C_t) after the return to 37°C.

Results are reported as $t_{1/2}$ with 95% confidence intervals estimated as $\ln 2/(k_1 \pm 2 * \text{std}(k_1))$.

Results

N-Formyl ND1 Peptides As Short As Two Amino Acids Stabilized M3^a-L^d Expression on the Cell Surface. To test the hypothesis that M3^a might bind very short peptides, a series of *N*-formyl peptides corresponding to truncated NH_2-terminal fragments of the mitochondrially encoded NADH dehydrogenase subunit 1 (35) were incubated with M3^a-L^d chimera-transfected cells (13S2) at 27°C overnight. *N*-formyl peptides as short as two amino acids significantly increased SMLF (4.2-fold; Fig. 1 *C*) compared with the solvent-treated cells (Fig. 1 *A*) or cells treated with *N*-formyl methionine (Fig. 1 *B*). The COOH-terminal addition of the third residue (phenylalanine) increased SMLF 6.5-fold. Moreover, the surface expression induced by the tetrameric (fMFFI; Fig. 1 *E*), pentameric (fMFFIN; Fig. 1 *F*), hexameric (fMFFINI), and octameric (Fig. 1 *H*) peptides increased only slightly over this range (fold increases of 7.9, 8.2, 8.3, and 8.0, respectively). β_2-microglobulin was shown to be associated with these MHC complexes by immunoprecipitation (data not shown). Untransfected cells remained negative for staining (data not shown).

Short N-Formyl Peptides Other Than ND1 Stabilized M3^a-L^d. To test whether such binding was sequence specific, we assayed an expanded panel of *N*-formyl peptides for their ability to induce expression of M3^a-L^d. The *N*-formyl dipeptide fMW induced the SMLF sixfold over controls at 27°C (Table 1 and Fig. 2). However, not all *N*-formyl peptides stabilized expression of the M3^a-L^d chimera. Two dipeptides, fMK and fMA, failed to induce M3^a-L^d (Table 1). The classical chemotactic tripeptide fMLF induced M3^a-L^d sevenfold. As with most other peptides that bind to M3^a, N-acetylated and unsubstituted versions of the chemotactic peptide failed to stabilize the chimera. All *N*-formyl tripeptides tested except fMAS were active. Only one (fAGSE) of five *N*-formyl tetrapeptides tested failed to stabilize M3^a-L^d. All short peptides tested that lacked an *N*-formyl group (n = 11) failed to stabilize the chimera (listed in Materials and Methods; data not shown), indicating that the *N*-formyl moiety is required for the binding of such short peptides.

fMLF-Benzyl Amide Stabilized the M3^a-L^d Chimera at a Peptide Concentration As Low As 100 nM. In crystal structures of class I molecules, the COOH terminus of the peptide forms H bonds and salt bridges with the heavy chain (3), suggesting specific interactions between peptide and heavy chain at this subsite. Peptides shorter than the canonical length cannot make significant interactions in both pockets A and F. To determine if the terminal carboxylate moiety is required for binding of very short peptides to M3^a, the fMLF-benzyl amide compound was tested. This peptide analogue resembles the tetrameric peptide fMLFF with the terminal carboxyl group re-

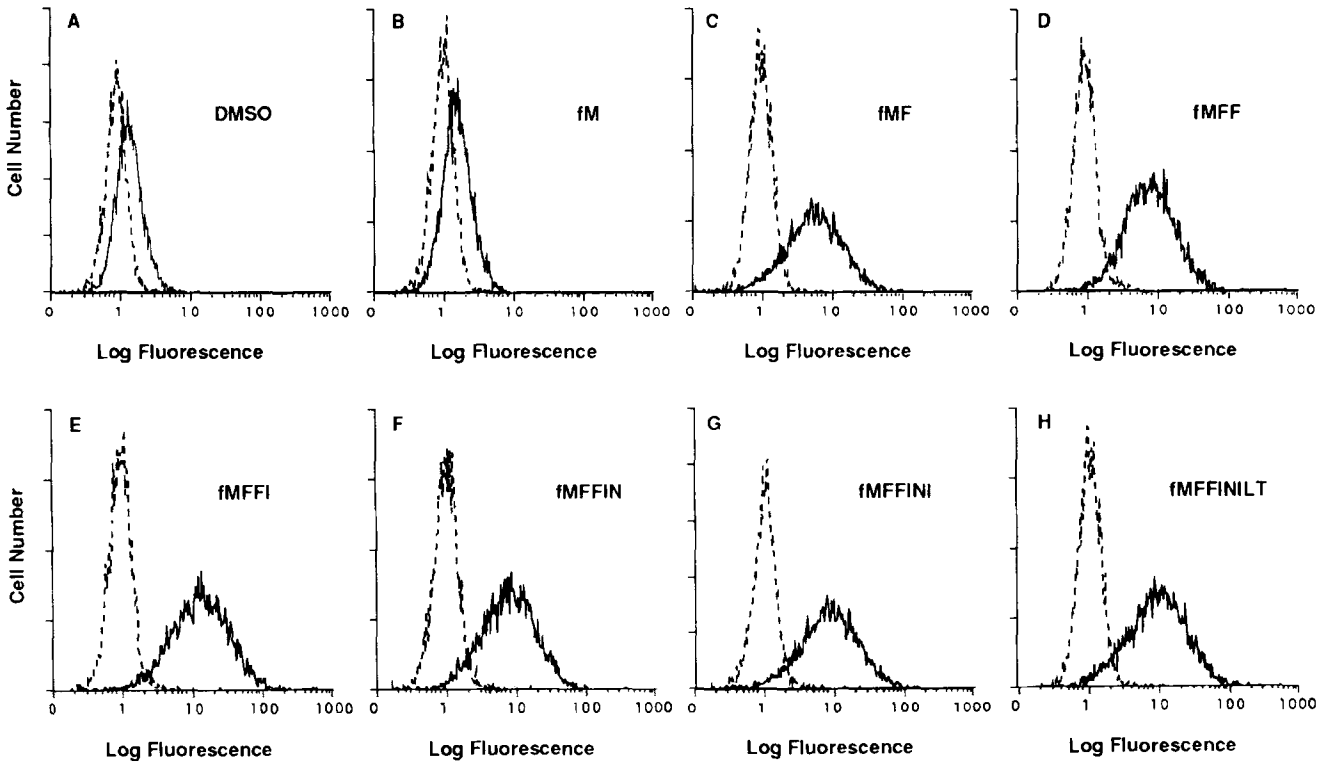

Figure 1. (*A–H*) Very short *N*-formyl peptides derived from the NH_2 terminus of fND1 stabilized the expression of the MHC class I chimera M3^a-L^d on transfected fibroblasts. Fluorescence of cells incubated with secondary antibody alone (- - -) or HB27 (———) [anti-L^d α_3]. Cells were incubated in 20 μM of designated peptide overnight at 27°C.

Table 1. *Stabilization of H-2M3^{a}L^d Surface Expression by Short N-Formyl Peptides*

Sequence	Fold increase ± SD	Sequence	Fold increase ± SD
fM	1.0 ± 0.02		
		fMLY	3.8 ± 0.2
fMW	5.9 ± 0.3	fMAS	0.9 ± 0.1
fMF	4.1 ± 0.5	fZLF	3.6 ± 0.2
fMV	3.7 ± 0.3		
fMK	1.0 ± 0.1	fMLFF	10.2 ± 0.4
fMA	1.2 ± 0.2	fMFVL	8.7 ± 0.4
		fMFFI	8.1 ± 0.4
fMLF	7.3 ± 0.4	fZLFK	3.3 ± 0.1
AcMLF	1.3 ± 0.1	fMLFK	2.9 ± 0.1
MLF	1.0 ± 0.2	fAGSE	1.3 ± 0.3
t-Boc-MLF	1.0 ± 0.1		
		fMFVLN	8.1 ± 0.5
fMLF-oME	6.5 ± 0.2	fMFFIN	8.4 ± 0.5
fMLF-oBE	5.6 ± 0.2		
fMLF-BeA	13.6 ± 0.7	fMFFINI	8.6 ± 0.4
fMMM	5.3 ± 0.2	fMFFINILT	8.9 ± 1.0
fMFF	6.5 ± 0.2	fMFVLNKFF	12.4 ± 0.6
fMFM	5.4 ± 0.3	AcMFVLNKFF	1.0 ± 0.02

* 13S2 cells were incubated in 20 μM of the indicted peptide overnight at 27°C. Numbers indicate the mean fold increase of three experiments ± 1 SD. Fold increase was calculated as the SMLF of cells incubated with peptide divided by the SMLF of cells incubated in medium containing DMSO alone. SMLF in DMSO was 2.5, 2.5, and 2.45 U in the three experiments.
f, *N*-formyl; *Ac*, *N*-acetyl; *t-Boc*, t-Butyloxycarbonyl; *Z*, norleucine; *MeE*, methyl ester; *BeE*, benzyl ester; *BeA*, benzyl amide.

moved. Interestingly, the fMLF-benzyl amide compound was very active in its ability to upregulate cell surface expression of the M3^a-L^d chimera. Increased surface expression was detected with this compound at concentrations as low as 100 nM (Fig. 3). Moreover, the dose curves of fMLFF, fMLF-benzyl amide, and fBla-z_{1-8} (fMFVLNKFF) virtually overlapped, indicating that the components required for stable peptide binding are present in these short peptides.

Peptide Length Affected the Rate of Disappearance of M3^a-L^d Chimera from the Cell Surface. The maximum surface expression potential achieved by very short *N*-formyl ND1 peptides appeared to vary as a function of length. To determine if the length of the peptide bound affected the stability of HB27-reactive epitopes at the cell surface, 13S2 cells were incubated with DMSO (solvent with no peptide), fND1$_{1-4}$, fND1$_{1-5}$, or fND1$_{1-8}$ at 27°C for 16 h to achieve surface expression. Cells were then shifted to 37°C and the disappearance of epitopes was monitored at given time points (Fig. 4). The half-life of the approach to a new steady state was estimated by nonlinear regression. The M3^a-L^d chimera induced with the octameric peptide exhibited a half-life of 105 min. Complexes induced with the shorter peptides were substantially less stable in this assay. The complex with the pentapeptide was less stable at 37°C ($t_{1/2}$ = 29 min) than the complex with the tetrapeptide ($t_{1/2}$ = 62 min). These observations were somewhat surprising because at 27°C, the pentapeptide routinely induced more surface expression of M3^a-L^d than did the tetrapeptide. Chimeric molecules induced by cold temperature alone rapidly decayed from the cell surface at 37°C, with a half-life of <15 min (Fig. 4).

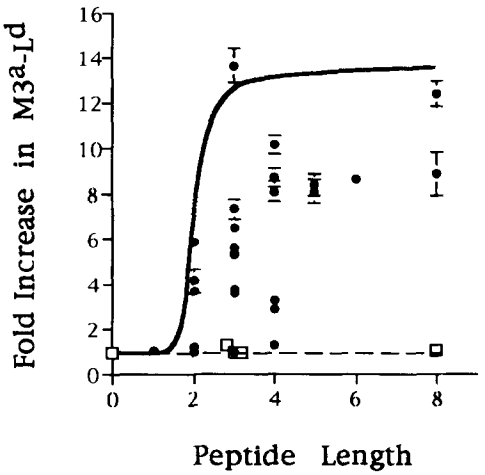

Figure 2. Average fold increase in M3^a-L^d expression for different peptides as a function of peptide length. Data are from Table 1. ●, *N*-formyl peptides; □, nonformylated peptides. The dark line indicates range of probable maximum induction for each length.

Short N-Formyl Peptide Binding Is Not an Artifact of the M3^a-L^d chimera. To rule out the possibility that induction of

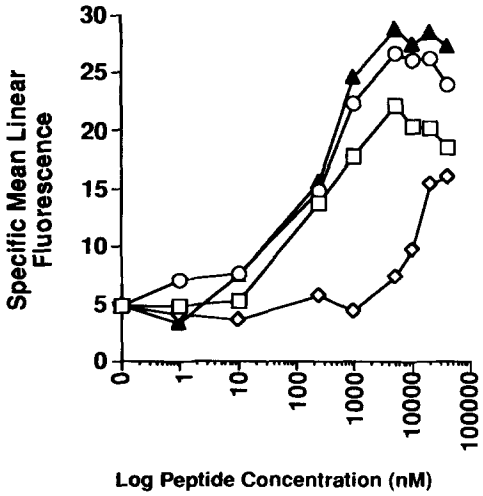

Figure 3. Dose curve of selected short *N*-formyl peptides. Graded concentrations of fMLF (◇), fMLFF (□), fMLF-benzyl amide (O), and fBla-z_{1-8} (▲) were incubated with 13S2 cells at 27°C overnight.

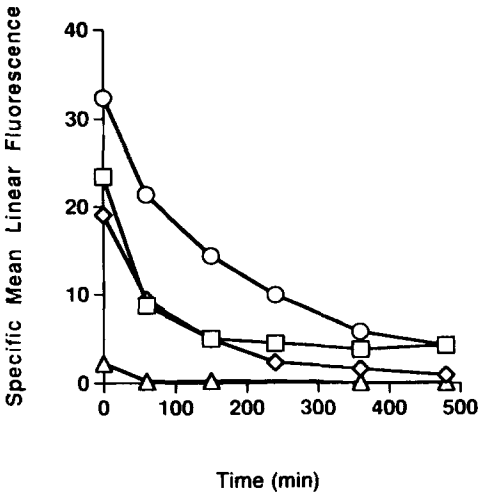

Figure 4. Rate of M3^a-L^d chimera decay was dependent on the length of *N*-formyl peptide bound. 13S2 cells were incubated with fND1$_{1-8}$ (○), fND1$_{1-5}$ (□), fND1$_{1-4}$ (◇), and DMSO (solvent) (△) at 27°C overnight, shifted to 37°C for the indicated period of time, and stained with HB27. Nonlinear regression analyses yielded estimates of $t_{1/2}$ for complexes with each peptide: fND1$_{1-4}$, 62 min, 95% confidence interval (CI), 56–69 min, r^2 = 0.99; fND1$_{1-5}$, 29 min, 95% CI, 28–31 min, r^2 = 0.99; fND1$_{1-8}$, 105 min, 95% CI, 94–120 min, r^2 = 0.99.

surface expression by short peptides might be an artifact of the M3^a-L^d chimera, fMLF-benzyl amide was tested for its ability to bind competitively to native M3^a (Fig. 5). Target cells expressing M3^a and an alternative allele of fND1 (fND1$^\beta$) cannot be lysed by M3^a-restricted, ND1$^\alpha$-specific CTLs. However, incubation of the fND1$^\alpha_{1-12}$ peptide renders these cells susceptible to lysis by these CTLs. When graded amounts of the putative competitor peptide fMLF-benzyl amide were added to sensitized targets, marked reduction of lysis was noted, indicating that the short competitor peptide bound to M3^a. The fBla-z$_{1-8}$ peptide also inhibited specific lysis and served as a positive control, whereas the AcBla-z$_{1-8}$

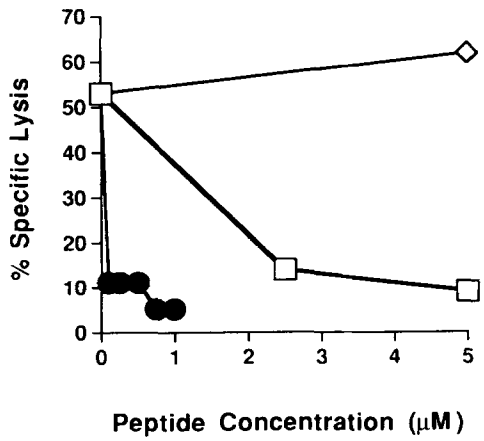

Figure 5. *N*-formyl MLF-benzyl amide competitively binds native M3^a. Competition by fMLF-benzyl amide (●), fBla-z$_{1-8}$ (□), and AcBla-z$_{1-8}$ (◇) of lysis by M3^a-restricted ND1$^\alpha$-specific CTLs using WEHI 105.7 NZB thymoma cells (H-2^d, M3^a, ND1$^\beta$) as target cells.

peptide failed to inhibit specific lysis and served as a negative control. These data demonstrate that short peptide binding to M3^a-L^d emulates that of the native M3^a.

Short Peptide Binding Is Not a Common Property of MHC Class I-a Molecules. To test whether MHC class I-a molecules can bind segments of active peptides, NH_2-terminal (SIIN and SIINF) and COOH-terminal fragments (NFEKL) of the K^b-restricted ovalbumin peptide (SIINFEKL) (29) were incubated with RMA-S cells overnight. The COOH-terminal fragment was included in this panel because it retained both major anchor residues (N, L) for peptide binding to K^b. As expected, SIINFEKL but not the short peptides stabilized K^b, as detected by mAbs TIB 139 and HB176 (Y3) (Fig. 6). Similarly, the Sendai virus nucleoprotein nonapeptide (FAPGNYPAL) (30) but not the NH_2-terminal (FAPGN) or COOH-terminal (NYPAL) fragments stabilized K^b and D^b. These data suggest that NH_2 and COOH termini for these peptides must be near pockets A and F for stable binding. These results confirm the concept that stable binding of short peptides is not a general feature of class I molecules.

Discussion

Graphical representation of the data shown in Table 1 suggest that maximal surface expression can be induced by peptides in the range of three to four amino acids (Fig. 2). We cannot so far rule out the possibility that an *N*-formyl monopeptide or dipeptide might induce surface expression

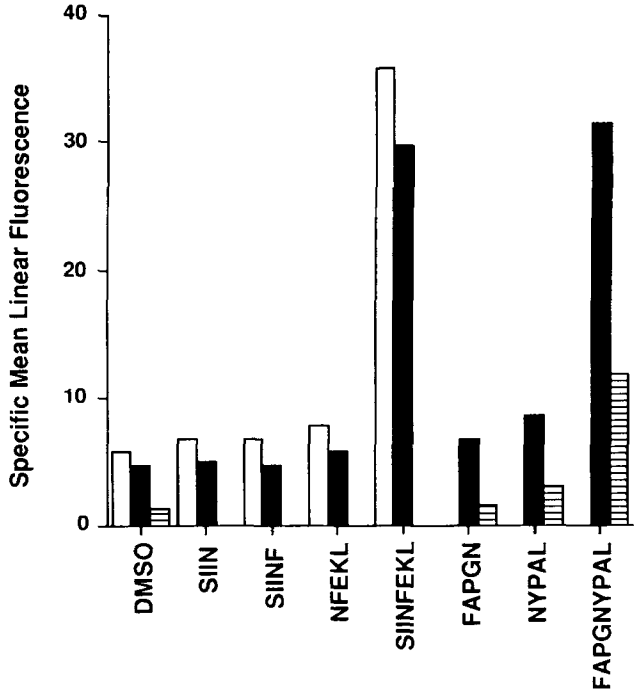

Figure 6. H-2K^b and H-2D^b molecules do not bind short peptides. RMA-S cells were incubated in 20 μM peptide overnight at 37°C and analyzed with mAb TIB 139 (anti-K^b, *white*), mAb HB 176 (Y3) (anti-K^b, *black*), and mAb HB19 (anti-D^b, *hatched*).

at levels higher than that induced by fMLF-benzylamide. Similarly, we cannot yet exclude the hypothesis that peptides other than those tested can induce surface expression more than suggested by the plateau of the dark line in Fig. 2. Despite these caveats, our working model is that elements present in short peptides (no longer than four amino acids) are sufficient for maximum induction of surface expression. In addition, longer peptides that deviate downward from the dark line in Fig. 2 may reflect the presence of destabilizing side chains rather than the absence of anchoring residues. Destabilizing or "dominant negative" (36) peptide side chains have been observed for other class I molecules, including H-2K^b (37), H-2K^d (38), HLA-A2 (39, 40), and for the class II molecule H-2E^k (36).

The ability of M3^a to bind short peptides further differentiates this I-b molecule functionally from the model established for other class I molecules. The most dramatic difference remains the requirement for the *N*-formyl moiety (18, 21). The results presented here suggest that the free energy of binding to M3^a derives mostly from interactions with the *N*-formyl group and the first several peptide bonds. We previously showed that glycine in P1 can relax the requirement for the *N*-formyl moiety (19). In addition, the inactivity of all tested dipeptides with glycine or alanine in P2 suggests that the R group of P2 interacts at least weakly with the MHC heavy chain (Fig. 7). In contrast, peptide binding to MHC class I-a molecules involves multiple side chains in addition to both termini and the backbone.

The remarkable ability of this MHC class I-b molecule to bind short peptides suggests that the antigen-binding cleft of M3^a may be quite different from those found in MHC class I-a molecules. A model comparing the peptide interaction with the prototypical MHC class I-a molecule K^b and the MHC class I-b molecule M3^a is depicted in Fig. 7. K^b-binding peptides must be eight or nine residues long for their termini to anchor into the conserved residues of the cleft. Additionally, K^b-binding peptides must possess particular amino acids at P5 (Tyr or Phe) and P9 (Leu) to bind stably in this cleft. Although the data indicate that K^b and D^b are incapable of binding short peptides, we do not imply that all other MHC class I molecules lack this ability. Indeed, it would be of interest to determine whether other MHC class I molecules, especially I-b molecules, may bind short peptides.

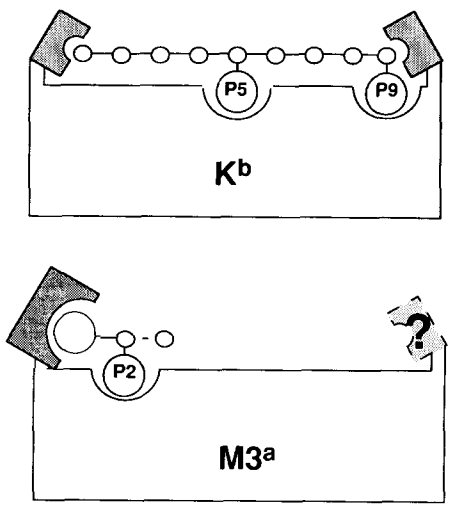

Figure 7. Model representing peptide binding to K^b (*top*) and M3^a (*bottom*). Peptides of eight to nine amino acids in length fit into the antigen-binding groove of K^b. These peptides possess the appropriate binding motif at P5 and P9. Moreover, the peptide termini engage deep pockets and anchor the peptide in this position. In contrast, M3^a binds *N*-formyl peptides of heterogenous lengths by interacting directly with the *N*-formyl moiety. For short peptides, the COOH terminus does not interact with heavy chain residues in pocket F. For M3^a, the contribution of P2 may be important.

H-2K^b appears prototypical among class I-a molecules to date. Saito et al. (37) have argued that optimal binding to H-2K^b actually involves proper fit of all side chains of the peptide in addition to the dominant anchors. Similarly, based on extensive kinetic analyses of peptide dissociation from HLA-A2, Parker et al. (39) have advanced a quantitative independent binding of sidechains (IBS) model of peptide binding in which each side chain contributes additively (and independently) to the free energy of binding, even though the magnitude of each contribution may vary. In addition, their model presupposes a constant additive contribution from the peptide backbone and the termini. In applying the IBS model to M3^a, it appears that the *N*-formyl moiety and perhaps the first several peptide linkages contribute the majority of binding energy. Additionally, data presented in this report support that notion that large hydrophobic amino acids such as phenylalanine or tryptophan are optimal residues for occupancy at P2. The additional residues of longer peptides may be restricted to hydrophobic amino acids because of the hydrophobic nature of the antigen-binding groove of M3^a.

Steady-state and kinetic analyses of fND1 peptides of different lengths suggest that distal elements of the peptide can affect binding affinity and/or the conformation of the complex if upstream elements are not optimal. Because the natural length of fND1 is not known, a synthetic octamer was used as a surrogate. Compared with the shorter analogues, fND1$_{1-8}$ induced a slightly higher steady state level of M3^a-L^d at 27°C that was also significantly more stable after shifting to 37°C. Thus, the average fold induction increased uniformly for peptides of the fND1 series (octamer sequence fMFFINILT), from 4.1-fold for the dimer to 8.9-fold for the octamer (see Table 1). The tetramer and pentamer peptides exhibited intermediate fold inductions of 8.1- and 8.4-fold. A much larger relative effect on the rate of decay at 37°C was measured in the temperature shift experiment, in which the $t_{1/2}$ of the octamer-containing complex was 105 min compared with 62 and 29 min for complexes containing the tetramer and pentamer peptides, respectively. The apparent discrepancy that complexes with the tetrapeptide are longer lived than complexes with the pentapeptide may suggest that the asparagine in P5 either increases the dissociation rate constant for the peptide–heavy chain complex, or that it induces a conformational change in the complex that renders it less stable. Both of these models are consistent with the concept of "dominant negative" side chain interactions.

Stabilizing interactions along the backbone of the peptide may account for the increase in the apparent half-life of the $M3^a$-L^d heavy chain epitopes when these cells were shifted to 37°C. By occupying more of the heavy chain cleft, longer peptides might make a larger number of favorable contacts, resulting in a net decrease in the rate of dissociation of the peptide from the binding cleft. Alternatively (but not mutually exclusively), these data could mean that complexes of $M3^a$-L^d containing different peptides may exhibit different intrinsic decay rates. In this view, different peptides induce distinct conformations. Differentiating these models will require direct measurements of k_{off}.

Since short peptides might establish a reduced number of direct contacts with TCR residues, positive selection of developing thymocytes by short peptides might involve allosteric or conformational effects on the heavy chain. Thus, TCR of $M3^a$-restricted CTL may focus on the heavy chain rather than on peptide side chains. Support for this idea comes from crystallographic analyses of K^b and HLA-A2 molecules associated with peptides. These studies suggest that peptide binding induces small but discrete changes in the orientation of heavy chain residues (induced fit) (3, 41). Alternatively, TCR may interact with $M3^a$ asymmetrically such that most of the binding energy is provided by contacts close to pocket A, a feature seen in some T cells allospecific for HLA-B27 (42).

A conservative model of class Ib function assumes that the rules governing positive and negative selection of responsive T cells are similar to those governing T cells restricted by class Ia molecules. Assuming that $M3^a$ does play a protective role, one problem posed when this hypothesis is applied to $M3^a$ and other class I-b molecules that bind a narrow specificity of peptides is how a suitable repertoire of TCR may be positively selected. Thus, recent evidence suggests that self-peptides are required for positive selection of thymocytes (43, 44) and that peripheral T cells react with only those foreign peptides that closely resemble self-peptides. This model imposes an extraordinary limitation on $M3^a$ since mitochondria encode only 13 proteins (45). Violating the size rule may increase the repertoire of thymic peptides available for positive selection. On the other hand, it remains possible that positive selection of T cells restricted by $M3^a$ does not depend on engagement by the TCR of particular peptides, but rather on the mere presence of $M3^a$ at the cell surface. In this view, the limited availability of endogenous mitochondrial *N*-formyl peptides available for promoting surface expression of $M3^a$ (21) would limit the quantity but not the complexity of the repertoire of T cells restricted by $M3^a$.

Alternatively, one might surmise from the paucity of obvious thymic-selecting peptides that T cells restricted by $M3^a$ are positively selected in an extrathymic environment that has access to *N*-formyl peptides. One such environment could be Peyer's patches or similar intestinal sites in which *N*-formyl peptides from normal microbial flora might be available for positive selection. In this regard, it is interesting to note that the non-MHC–encoded class I-b molecule CD1 is expressed in the intestinal epithelia in the absence of either TAP transporter or β_2-microglobulin function (46, 47). Similarly, the MHC-linked class I-b molecule Tla is expressed at high levels by intestinal epithelial cells, where it may present ligands to resident $\gamma\delta$ T cells (48).

MHC class I-a molecules present a relatively broad repertoire of peptides to T cells during thymic education and antigen recognition. By analogy to species occupying broad ecological niches (49), we have termed such MHC molecules eurytopic (50). In contrast, $M3^a$ is stenotopic, not strictly because of the limited repertoire of peptides it can present, but because it apparently targets a specialized niche in the economy of the immune system. Stenotopic MHC genes may evolve rapidly by gene duplication but with minimal polymorphism (50). Relaxation of the length rule for $M3^a$ is consistent with the evolution of a gene under selection pressure to maximize restriction of bacterial peptides within the confines of the *N*-formyl niche. Alternatively, if self-peptides are required for positive selection of $M3^a$-restricted T cells, it is possible that the violation of the length rule by $M3^a$ was a necessary preadaptation required before evolution of an *N*-formyl pocket was possible.

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