Availability of Endogenous Peptides Limits Expression of an M3^a-L^d Major Histocompatibility Complex Class I Chimera

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Summary

Taking advantage of our understanding of the peptide specificity of the major histocompatibility complex class I-b molecule M3^a, we sought to determine why these molecules are poorly represented on the cell surface. To this end we constructed a chimeric molecule with the α_1 and α_2 domains of M3^a and α_3 of L^d thereby allowing use of available monoclonal antibodies to quantify surface expression. Transfected, but not control, B10.CAS2 (H-2M3^b) cells were lysed readily by M3^a-restricted monoclonal cytotoxic T lymphocytes. Thus, the chimera bound, trafficked, and presented endogenous mitochondrial peptides. However, despite high levels of M3^a-L^d mRNA, transfectants were negative by surface staining. This finding was consistent with inefficient trafficking to the cell surface. Incubation at 26°C, thought to permit trafficking of unoccupied heavy (H) chains, resulted in detectable cell surface expression of chimeric molecules. Incubation with exogenous peptide at 26°C (but not at 37°C) greatly enhanced expression of M3^a-L^d molecules in a dose-dependent manner, suggesting stabilization of unoccupied molecules. Stable association of β_2-microglobulin with the chimeric H chain was observed in labeled cell lysates only in the presence of exogenous specific peptide, indicating that peptide is required for the formation of a ternary complex. These results indicate that surface expression of M3^a-L^d is limited largely by the steady-state availability of endogenous peptides. Since most known M3^a-binding peptides are N-formylated, native M3^a may normally be expressed at high levels only during infection by intracellular bacteria.

MHC class I molecules monitor the interior of cells for the presence of peptides derived from pathogens such as viruses and intracellular bacteria (1, 2). Proteins are thought to be degraded into peptides by proteasomes (3, 4) and delivered to the endoplasmic reticulum through the ATP-dependent transporter associated with antigen processing (TAP)[1] (5, 6). In the endoplasmic reticulum, the nascent MHC-encoded transmembrane H chain (44,000 M_r) forms a ternary complex with β_2-microglobulin (β_2m) (12,500 M_r) (7) and a peptide of 8–10 amino acids in length (8). In the absence of infection, cellular proteins serve as peptide donors. Studies using a variety of mutant cell lines, such as the TAP-mutant RMA-S (9, 10) and the β_2m-deficient R1E (11, 12), have clearly demonstrated the need for all three components of the MHC molecule to be in the endoplasmic reticulum for proper cell surface expression. At the cell surface, the MHC class I molecule presents peptides to CD8$^+$ T cells (13, 14).

In the mouse, MHC class I H chain genes can be classified into the highly polymorphic, class I-a (classical) K, D, and L and the relatively invariant, class I-b (nonclassical) Q, T, and M (15). Mounting evidence indicates that class I-b gene products can bind and present peptides to CD8$^+$ T cells (16–19). Similar to other MHC class I molecules, M3^a associates with β_2m and peptide and can present peptides to T cells (20–23). The biochemical specificity of M3^a is striking in that it binds *N*-formylated peptides (24, 25). Because bacteria and mitochondria both initiate protein synthesis with *N*-formyl methionine, we hypothesized that M3^a binds *N*-formylated peptides of bacterial origin for presentation to T cells (26). Consistent with this hypothesis, recent data have shown that M3^a presents peptides from *Listeria monocytogenes* (27, 28).

To study the intracellular requirements for peptide binding and cell-surface expression of M3^a, we assessed synthetic levels of H chain. At the mRNA level, M3^a expression has been detected in a variety of cell types and tissues, and is especially high in the thymus (29). However, an abundant level

We have chosen to hyphenate I-a and I-b to avoid confusion of I-a with Ia as an original designation for MHC class II molecules.

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; GAM, goat anti-mouse; mCTL, monoclonal CTL; RT, reverse transcriptase; TAP, transporter associated with antigen processing.

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of class I mRNA transcripts does not necessarily equate to abundant cell surface expression. For instance, Qa-1^b transcripts are present at levels comparable to those of *H-2K* and *D* genes, but the amount of surface product is quite low (30). The same holds true for H-$2L^d$ (31, 32). Moreover, the cell surface expression of L^d can be induced by peptide, suggesting that some H chain is retained intracellularly (33).

As in other systems, a specific antiserum or mAb is critical for tracking the H chain through the cell. To date, however, efforts by ourselves and others to generate a specific antiserum to $M3^a$ have been unsuccessful. To circumvent this problem, we developed an MHC class I chimeric molecule in which the α_1 and α_2 domains are derived from $M3^a$ and the α_3 domain from L^d. Moreover, this construct is driven by the K^b promoter, which has strong constitutive expression and is readily inducible by IFN-γ. Similar chimeric K^{bm}-L^d molecules were recognized by K^{bm}-specific CTLs and found at abundant levels on the cell surface of transfected cells (34). In contrast, we found that $M3^a$-L^d chimeras were not expressed at the cell surface constitutively in quantities adequate for serological detection. In these studies, we determined the intracellular requirements for expression of the $M3^a$-L^d chimera.

Materials and Methods

Construction of the MHC Class I Chimera Vector. The $M3^a$-L^d expression vector was derived from pK^b-L^d, a gift from Dr. Larry Pease (Mayo Clinic, Rochester, MN). The exons that encode α_1 and α_2 domains of $M3^a$ replaced the corresponding exons in the original vector utilizing restriction endonuclease sites that were introduced in the original vector. Exons 2 and 3 of $M3^a$ were generated independently by genomic PCR. Specifically, intronic oligonucleotides that flank each exon were used to amplify these gene segments from NZB/B1NJ genomic DNA. For exon 2, the 5′ oligonucleotide (5′-CAATGCTTGTCGACTGGCCC) possessed a SalI site and the 3′ oligonucleotide (5′-AGAGCTCTGAAGCCC-AACTCCAAACATCTAAGCTTGCACCCA) had a HindIII site. The gel-purified DNA was separated from the agarose using Magic PCR preps (Promega, Madison, WI). The DNA was cut with SalI and HindIII and cloned into pBluescript II SK$^-$ (Stratagene, La Jolla, CA). The resultant plasmid (termed pM3^a-α_1) was transformed into XL-1 Blue *Escherichia coli* (Stratagene). Plasmid DNA was prepared using Magic Mini-prep (Promega) and sequenced using Sequenase Version 2.0 (US Biochemical, Cleveland, OH) to ensure that the clone was free of PCR-induced mutations. The sequenced insert was cut with SalI and HindIII and ligated into the similarly cut pK^b-L^d vector, creating pM3$^a_{\alpha 1}$K$^b_{\alpha 2}$-L^d. The process was repeated to obtain exon 3 of $M3^a$. In this case the 5′ (5′-GGA-GTTGGGCTTCAGAGCTCTCAGAAAGCTTTAACC) and the 3′ oligonucleotides (5′-TTCCTCCCTCTCGAGACATCA) had a HindIII and XhoI site, respectively. The resultant plasmid (termed pM3^a-α_2) was transformed into XL-1 Blue *E. coli* (Stratagene). After sequencing, the pM3^a-α_2 insert was cut with HindIII and XhoI and ligated into the pM3$^a_{\alpha 1}$K$^b_{\alpha 2}$-L^d vector cut with HindIII and XhoI, creating pM3^a-L^d.

Antibodies. Supernatants of hybridomas were used for immunofluorescence. mAbs were specific for the α_3 domain of the L^d molecule, 28-14-8S (ATCC HB27; American Type Culture Collection, Rockville, MD) (35); β_2m^b, S19.8 (a gift from Dr. U. Hämmerling, Sloan-Kettering Memorial Institute, New York) (36); or an MHC class I molecule from the H-2^{w17} haplotype, Tü232 (a gift of Dr. Jan Klein, Max Planck Institute, Tübingen, Germany) (37). IgG fractions of culture supernatants were separated using a protein G column in an FPLC™ system (Pharmacia, Piscataway, NJ) and used for all immunoprecipitations.

Cytotoxic T Lymphocyte Generation. The fND1$^\alpha$-specific, $M3^a$-restricted monoclonal CTL (mCTL) clones 1D8 and 3D5 were described elsewhere (24). These mCTLs are specific for a peptide from the mitochondrial gene product ND1 (NADH dehydrogenase subunit 1) in the context of $M3^a$. OVA-specific polyclonal CTL were produced by immunizing C57BL/6J (B6) mice with 5×10^3 E.G7-OVA cells (EL-4 cells transfected with chicken OVA gene [13]). After 2–3 wk, spleen cells of immunized B6 mice were cultured for 3–6 d with E.G7-OVA (γ-irradiated 20,000 rad) and syngeneic cells (γ-irradiated 3,000 rad). OVA-specific CTL clone 2G12 was obtained by limiting dilution and was shown to be restricted by H-2K^b (25).

Peptide Synthesis and Purification. The f-ND1$^\alpha_{1-12}$ peptide corresponds to the NH_2-terminus of ND1. f-Bla-z_{1-8} peptide, from *Bacillus cereus* β-lactamase binds to $M3^a$ as determined by CTL competition assays (26). Ac-Bla-z and Bla-z are the *N*-acetylated and unsubstituted forms of this peptide, respectively. f-L25 is from *E. coli* ribosomal protein L25 and also can bind to $M3^a$. $VSVNP_{52-59}$ is the vesicular stomatitis virus nucleocapsid peptide that binds to K^b, but not to $M3^a$ (25). All peptides were synthesized and HPLC purified as described previously (38).

Cell Culture. SVCAS2.F6 tail cell fibroblasts were generated from B10.CAS2 mice (H-2^{w17}, $M3^b$) using SV-40-mediated transformation. These cells were transfected with EcoRI linearized pM3^a-L^d and KpnI linearized ppo12 (vector encoding G418 resistance) (39) at a molar ratio of 20:1, by $CaPO_4$ precipitation. Cells resistant to 1 mg/ml G418 were isolated by ring cloning and grown in separate wells. 35 cell lines were generated from two independent transfections. To generate subline 13S2, cell line "13" was incubated with 500 nM f-Bla-z peptide overnight at 26°C. The cells were stained as described below, omitting the fixing step. Cells were selected using a cell sorter (EPICS model 753; Coulter Electronics, Hialeah, FL) for high expression of the 28-14-8S epitope, and returned to culture media. After 1 wk, this process was repeated to generate subline 13S2 (cell line 13 sorted twice, hence 13S2). Cells were then passaged in DMEM plus 10% FCS, 1% gentamicin reagent solution, 1 mg/ml total G418 at 37°C in 5% CO_2. E.G7 cells were passaged using the same medium. 24SV cells were generated from (B10.CAS2 × NZB/B1NJ) F_1 tail cell fibroblasts (40) using SV40-mediated transformation. 24SV and SVCAS2.F6 cells were passaged in DMEM plus 10% FCS (40). All cell culture reagents were from Gibco BRL (Gaithersburg, MD).

Cell-mediated Cytolysis. A colorimetric assay was used as described (41) with some modifications. Briefly, 15,000 irradiated (1,500 rad) fibroblasts were plated overnight in flat-bottom 96-well microtiter plates in 100 μl of supplemented Mishell-Dutton medium (SMDM) (42). Triplicate dilutions of mCTL in 100 μl of SMDM were added to targets for 12 h. Surviving cells were stained for 3 min with 5% ethanol containing 0.5% crystal violet (wt/vol) and destained in cold water. The dye was solubilized in acidified ethanol (50% ethanol, 0.4% glacial acetic acid [vol/vol]). Absorbances were read at 600 nm on a microplate reader (model 2550; Bio-Rad Laboratories, Hercules, CA). Standard errors of the measurements, calculated by propagation of errors (41), were <5% unless otherwise indicated.

Reverse Transcriptase (RT) PCR Assay. RNA was extracted from 13S2 cells with a standard protocol (43) and reverse transcribed using Moloney murine leukemia virus (Mo-MLV) RT (Perkin-Elmer

Corp., Norwalk, CT). The RT product served as a template for PCR using oligonucleotide pairs that are specific for β-actin (5'-GTGGGCCGCTCTAGGCACCAA) and (5'-CTCTTTGATGTCACGCACGATTTC); *H-2K* (5'-CTCCAGTGACTATTGCAG) and (5'-ACCTGGAGGGCGAGTGCGTGGAGTG), or *M3*^{a}-*L*^{d} (5'-GAGCTGCTCCACAGATACCTACGGAATGGC) and (5'-CACAGCTCCAATGATGGCCATAGCTCCAAG). PCR products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, recorded with Polaroid type 55 film, and quantified densitometrically on an Ultrascan XL Enhanced Laser Densitometer (Pharmacia).

Immunofluorescence Staining and Flow Cytometry Analysis. Immunofluorescence analysis was done as described (40). Briefly, subconfluent transfected cells were incubated with the designated peptide for 12–16 h at 37°C or 26°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 were 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, 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 Electronics). Data are expressed as mean log fluorescence of a population on a four decade scale (0.1–1,000) Voltages were adjusted to ensure that control staining with the secondary antibody alone (FITC-GAMIg) was in the range of 1 ± 0.2 U. Additionally, gates were set around narrow windows of forward light scatter to minimize cell size–dependent artifacts (40).

Preparation of Radiolabeled Proteins. For metabolic labeling, subconfluent monolayers of 13S2 or parental SVCAS2.F6 cells ($\sim$3 × 10^7 cells) were preincubated for 1 h in methionine-free RPMI 1640 medium supplemented with 5% (vol/vol) FCS and 1% (vol/vol) 200 mM L-glutamine (Gibco BRL). Cells were labeled with 50 μCi/ml [^{35}S]methionine (>1,000 Ci/mmol; NEN Research Products, Wilmington, DE) for 20 min at 37°C. Labeled cells were briefly trypsinized, washed three times in ice-cold PBS, and lysed in a lysis buffer containing 1% NP-40 (Calbiochem-Novabiochem Corp., La Jolla, CA), 1 mM PMSF, 33 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 20 μg/ml leupeptin, and 40 μg/ml bestatin (all from Sigma Chemical Co., St. Louis, MO) in PBS.

Iodination of cell surface molecules was catalyzed by lactoperoxidase (44). Briefly, 3 × 10^7 cells cultured at either 37°C or 26°C for 12 h in the presence or absence of specific peptide were washed three times in ice-cold PBS and labeled with 180 μl of 1 mg/ml lactoperoxidase, 125 μl of 0.045% H_2O_2 in PBS, and 2.5 mCi ^{125}I (17–20 Ci/mM on analysis date; NEN Research Products) for 15 min. The reaction was terminated by the addition of 10 ml of ice-cold PBS.

Immunoprecipitation and SDS-PAGE. For in vitro stabilization of the MHC complex, lysates were centrifuged at 15,000 *g* for 15 min at 4°C. An aliquot of the supernatant was precipitated with TCA to assess total radiolabel incorporation. Aliquots of supernatants were equalized on the basis of total radioactivity and incubated with specific peptide at 4°C for 16 h. The lysates were precleared using 150 μl of BSA-Sepharose, goat anti-mouse-Sepharose (GAMIg from Pel-Freez Biologicals, Rogers, AR), and protein A-Sepharose (Pierce, Rockford, IL) sequentially for 5 min each at room temperature. Precleared lysates were divided into three tubes and incubated with specific or control antibodies at 4°C overnight. The immune complexes were collected on protein-A-Sepharose at 4°C for 1 h and washed four times with 0.5% NP-40, 0.25% sodium deoxycholate, 0.1% SDS in Tris-buffered saline, and two times with water. Immunoprecipitates were eluted from protein A-Sepharose in SDS-solubilizing buffer (pH 6.8) containing 62.5 mM Tris, 10% (vol/vol) glycerol, 3% (wt/vol) SDS, and 5% (vol/vol) 2-ME, and analyzed by SDS-PAGE on a 12.5% gel as described by Laemmli (45). SDS-PAGE low range molecular weight standards (Bio-Rad Laboratories) were used. The gels were treated with Amplify (Amersham Corp., Arlington Heights, IL), dried, and fluorographed at −70°C. Gels were also analyzed on a Betascope 603 Blot Analyzer (Betagen, Mountain View, CA). The specific activity of the H chain and β_2m bands was determined by subtracting the mean background count from the actual values. For ^{125}I-labeled proteins, the procedure was identical except that cell lysates were directly immunoprecipitated after preclearing, and after samples were analyzed by SDS-PAGE, the gels were dried and autoradiographed at −70°C.

Immunoblots. Subconfluent 13S2 cells were harvested, washed, and lysed in lysis buffer. Lysates were electrophoresed through a 12% SDS-PAGE gel. For convenience, Rainbow™ protein molecular weight markers (Amersham Corp.) were used. This gel was electroblotted (Trans-Blot Cell; Bio-Rad Laboratories) to Hybond C (Amersham Corp.) at 30 V for 16 h in Towbin transfer buffer (46). The blots were blocked with 5% (wt/vol) non-fat dry milk in PBS-T (PBS plus 0.05% Tween-20) for 1 h and washed three times with 1% non-fat dry milk in PBS-T. The blots were probed with 28-14-8S in 1% non-fat dry milk in PBS-T for 1 h at room temperature. After three extensive washes, GAMIgG conjugated to horseradish peroxidase (Amersham Corp.) was added at a dilution of 1:3,000 and incubated for 1 h. The blots were washed as before and developed using the enhanced chemiluminescent detection system (Amersham Corp.) and exposed to film.

Results

Rationale for Construction of an M3^a*-L*^{d} *Chimeric MHC Class I Gene.* We designed a chimeric MHC class I protein with two critical features: (*a*) the peptide binding specificity of M3^a and (*b*) an epitope for a mAb that would be independent of the α_1 and α_2 domains. To this end, we joined the α_1 and α_2 domain of M3^a with the α_3 domain of L^d (Fig. 1). Previous experiments of others established that the TCR interacts with peptide and residues from α_1 and α_2

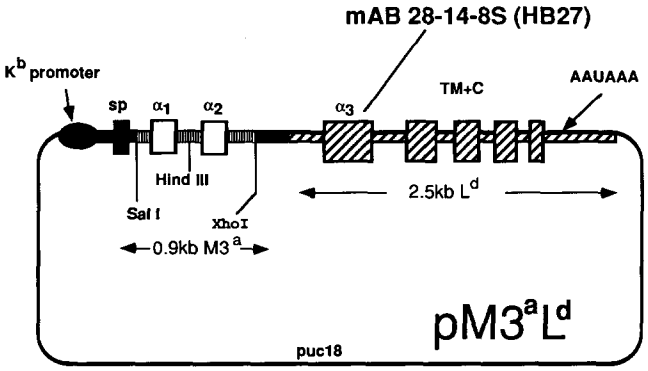

Figure 1. Diagram of vector construct. To attach a conformationally insensitive antibody epitope to the antigen binding domain of M3^a, exons 2 and 3 from M3^a replaced the corresponding exons in the K^b-L^d vector. This chimera possesses the promoter and signal peptide of K^b, and the α_3 transmembrane domain and cytoplasmic tail of L^d.

with little, if any, involvement of the α_3 region (47). In particular, Allen et al. (48) showed that L cells expressing a K^b (α_1, α_2)-L^d (α_3) hybrid molecule were lysed by K^b-restricted CTLs equivalently to fibroblasts expressing the native K^b molecule. A useful feature of the L^d α_3 domain is that a mAb (28-14-8S) exists that binds to a conformationally-insensitive epitope independently of the antigen-binding domain (35). Concerns that the α_3 domain of the L^d molecule might not bind β_2m well (49) were alleviated by two studies demonstrating that the inability of native L^d to associate stably with β_2m is determined by the α_1 domain (50, 51). Therefore, the L^d α_3 domain itself possesses the capacity to bind functionally with β_2m.

The $M3^a$-L^d Chimera Renders SVCAS2.F6 ($M3^b$) Fibroblasts Sensitive to Lysis by $M3^a$-restricted mCTLs. The chimera was transfected along with a drug-selecting marker into SVCAS2.F6 fibroblasts derived from the $H\text{-}2^{w17}$ mouse strain B10.CAS2. This mouse strain carries a different allele of the H chain ($M3^b$), rendering these fibroblasts resistant to lysis by $M3^a$-restricted mCTLs (52). To determine whether the chimera could bind the endogenous mitochondrial peptide $ND1^\alpha$ and traffic to the cell surface, G418-resistant fibroblasts were incubated with $M3^a$-restricted, $ND1^\alpha$-specific mCTL (Fig. 2 *a*). Transfected cells were lysed readily by the mCTL, whereas the untransfected control cells were not lysed. These data suggested that the MHC molecule appropriately bound, trafficked, and presented endogenous peptides. Moreover, this lysis was dose-dependent on mCTL (Fig. 2 *b*). To demonstrate that DNA transfection did not cause these fibroblasts to be nonspecific targets of CTL-mediated lysis, transfectants were incubated with OVA-specific mCTLs that recognize the OVA peptide bound to K^b (25). As expected, OVA-specific mCTLs failed to lyse transfected targets (Fig. 2 *b*).

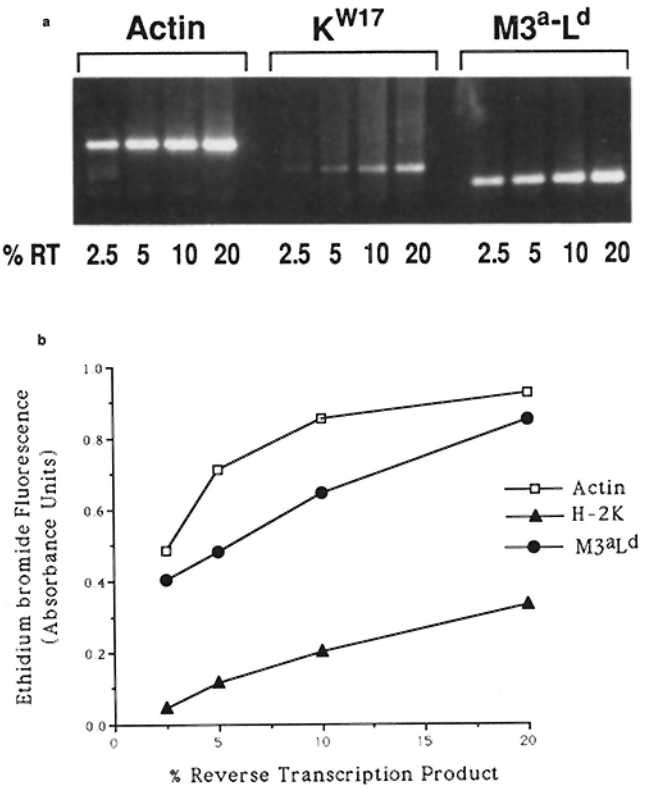

Figure 3. The amount of chimeric mRNA was relatively equivalent to another endogenous MHC class I molecule. (*a*) mRNA from 13S2 transfectants was extracted, reverse transcribed, and used as template for PCR. Using oligonucleotide specific to the $M3^a$-L^d chimera, K^{w17}, and β-actin, the relative level of mRNA could be established. (*b*) The ethidium bromide fluorescence (*absorbance units*) was plotted against the percent reverse transcription product used in PCR.

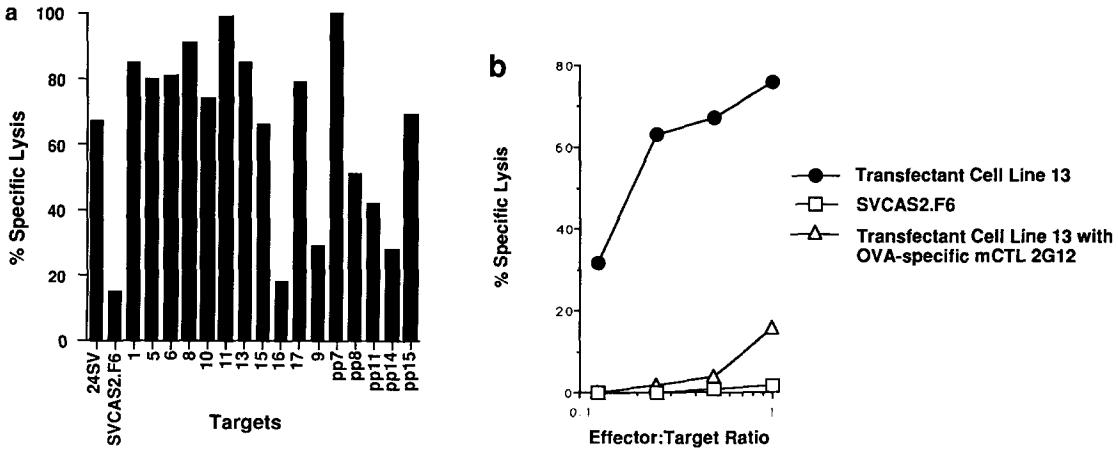

Figure 2. The $M3^a$-L^d chimera rendered SVCAS2.F6 fibroblasts ($M3^b$) sensitive to $M3^a$-restricted cytolysis. (*a*) A panel of G418-resistant transfectants were tested in a cell-mediated cytolysis assay for their sensitivity to $ND1^\alpha$-specific, $M3^a$-restricted killing at an E/T ratio of 1:1 with clone 1D8. The cell line 24SV, which is $ND1^\alpha$ and $M3^a$, served as the positive control for cytolysis. SVCAS2.F6 (untransfected control) showed only background lysis. All but three clones were positive by this assay. It is possible that cell lines 16, 9, and pp14 acquired only the drug-resistance gene, rendering them G418 resistant but negative for the chimera. (*b*) The cell line 13 could be killed in a dose-dependent manner by graded concentrations of mCTL clone 3D5, whereas the untransfected control was not lysed. The OVA-specific mCTL clone 2G12 was included as a specificity control.

Levels of M3^a-L^d mRNA Were Not Limiting in Transfected Cells. The measurement of transcript levels required probes distinguishing the chimeric mRNA from all endogenous MHC class I transcripts. Preliminary experiments using oligonucleotides designed to react specifically with the hybrid joint of the chimeric mRNA were unsuccessful, perhaps because the juxtaposed termini of exons 3 and 4 are highly conserved. In contrast, by using regions of low homology, RT-PCR primers could be designed that react exclusively with the M3^a-L^d chimera. Graded concentrations of RT product served as template in PCR reactions using oligonucleotides specific for H-2K, β-actin, and the chimera (Fig. 3 *a*). Densitometry readings of each band were then plotted against input RT template. Lines were generated from values representing ethidium bromide fluorescence of bands versus RT product used as template for PCR reaction (Fig. 3 *b*). Although this assay is not a measure of absolute mRNA concentrations, these results suggest mRNA itself was not limiting surface expression of the M3^a-L^d chimera. Southern blot hybridization revealed that the transfectants had approximately one to five copies of the transgene (data not shown).

Very Low Cell Surface Expression of M3^a-L^d Chimera at 37°C. We used the mAb 28-14-8S to determine the level of cell surface expression. As seen in Fig. 4 *a*, no signal was detected over the nonspecific staining of the secondary antibody alone. This pattern of nonexpression was seen in all 35 lines from two transfections, suggesting that low expression was not linked to integration of the transfected gene in a transcriptionally inactive area. The expression of endogenous K^{w17} molecules could be detected at wild-type levels by the antibody Tü232 at 37°C (Fig. 4 *e*) indicating the absence of any general defect in the transport of MHC molecules to the cell surface. Staining with anti-β_2m also revealed normal levels of β_2m (Fig. 4 *f*). However, 13S2 cells did have some M3^a-L^d product on the cell surface as previously demonstrated in Fig. 2 by specific CTL reactivity. CTLs require only 50–200 peptide–class I complexes for efficient lysis (53). In contrast, flow cytometric analysis requires at least 1,000 molecules for detection (54). Therefore, the amount of M3^a-L^d on the cell surface was <1% of the number of K^{w17} molecules in these transfectants.

Surface Expression of M3^a-L^d Chimera Could Be Induced with Cold Temperature and further Stabilized with Peptide. Normal cell surface staining of other MHC class I molecules sug-

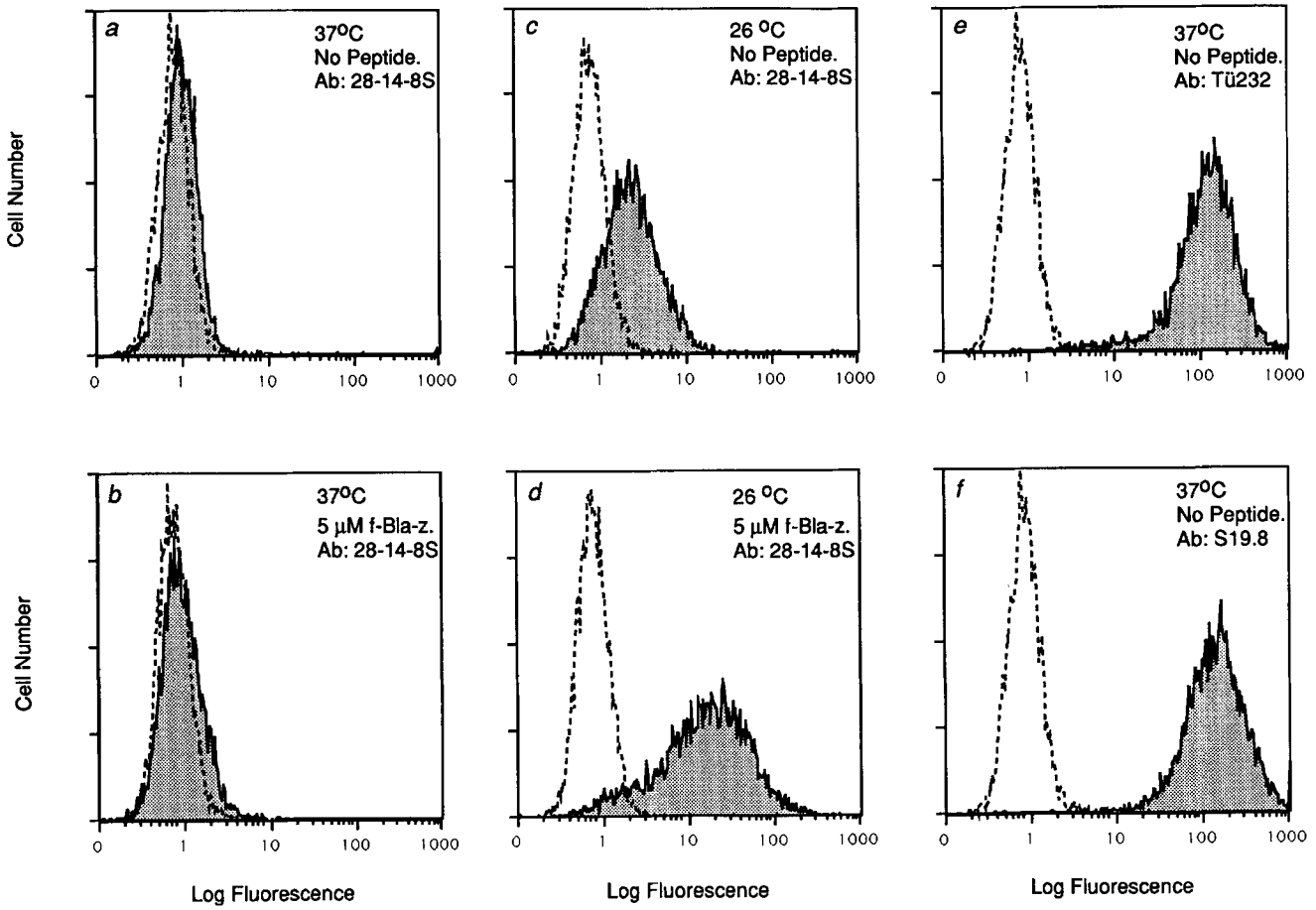

Figure 4. Cold temperature and specific peptide synergistically induced cell surface expression of M3^a-L^d. 13S2 transfectants were incubated at 37°C in the absence (*a*) or presence (*b*) of 5 μM f-Bla-z peptide and stained with the antibody 28-14-8S. Cells were also incubated at 26°C for 16 h and incubated in the absence (*c*) or presence (*d*) of 5 μM f-Bla-z peptide and stained with 28-14-8S. 13S2 cells at 37°C with no peptide were also stained with Tü232 (anti-K^{w17}) (*e*) and S19.8 (anti-β_2m^b) (*f*).

gested that the assembly pathway and its related proteins were functioning properly. We therefore evaluated the role of intracellular peptides specific for $M3^a$ in surface expression. Data from other laboratories using the RMA-S mutant cell line have shown that incubation with peptide could stabilize empty H chains on the cell surface (55). Additionally, cold temperature is thought to allow the egress of H chain from the endoplasmic reticulum through the Golgi apparatus and to the cell surface (56). Either cold temperature (26°C) or specific peptide restored MHC class I expression on RMA-S cells to near wild-type levels (57). To determine the effect of cold temperature and peptide on the expression of the chimera, we incubated 13S2 cells at 26°C or 37°C for 16 h in the presence or absence of 5 μM f-Bla-z peptide. As seen in Fig. 4 *c*, the chimera could be detected at the surface of cells incubated at 26°C using the anti-L^d antibody. This indicated that the chimera retained the epitope for this antibody, which thus could be used to track the molecule through the cell. Peptide incubation at 26°C resulted in significantly more cell surface expression than either condition alone (Fig. 4, *b–d*). Additionally, the kinetics of appearance of the chimera at the cell surface using cold temperature and specific peptide were similar to the kinetics of induction of $H\text{-}2K^b$ in RMA-S cells (data not shown).

To assess the physical state of the chimeric protein, 13S2 cells and SVCAS2.F6 cells (untransfected controls) were incubated at either 37°C or 26°C in the presence or absence of 500 nM f-Bla-z peptide for 16 h. Cell surface proteins were labeled with ^{125}I. Transfected cells treated with cold temperature and peptide showed the greatest amount of material at 50 kD, the predicted size of the glycosylated chimeric H chain (Fig. 5 *a*). The H chain was also immunoprecipitated from cells that were treated with 37°C plus peptide or with cold temperature alone, but in lesser amounts. Two additional bands (23 and 28 kD) could be seen in the lanes with peptide. Immunoreactivity with 28-14-8S in immunoblots suggested that the 28-kD band was a degradation product of the H chain (Fig. 5 *b*). Control immunoprecipitations with K^{w17} under identical conditions did not yield any degradation products (data not shown).

The $M3^a$-L^d Chimera Retained the Peptide Binding Characteristics of Native $M3^a$. The fine specificity of native $M3^a$ for peptide binding was established earlier by CTL-peptide competition assays. These studies established that $M3^a$ could bind certain synthetic mitochondrial and bacterial peptides (26). The ability to monitor peptide binding by cell surface stabilization of the H chain in the presence of specific peptide and cold temperature allowed us to determine whether $M3^a$-L^d possessed the peptide binding specificity of the native molecule by testing a panel of $M3^a$-binding and nonbinding peptides. As seen in Fig. 6, the *N*-formyl $ND1^{\alpha}{}_{1-12}$ mitochondrial peptide, the *N*-formyl ribosomal L25 peptide

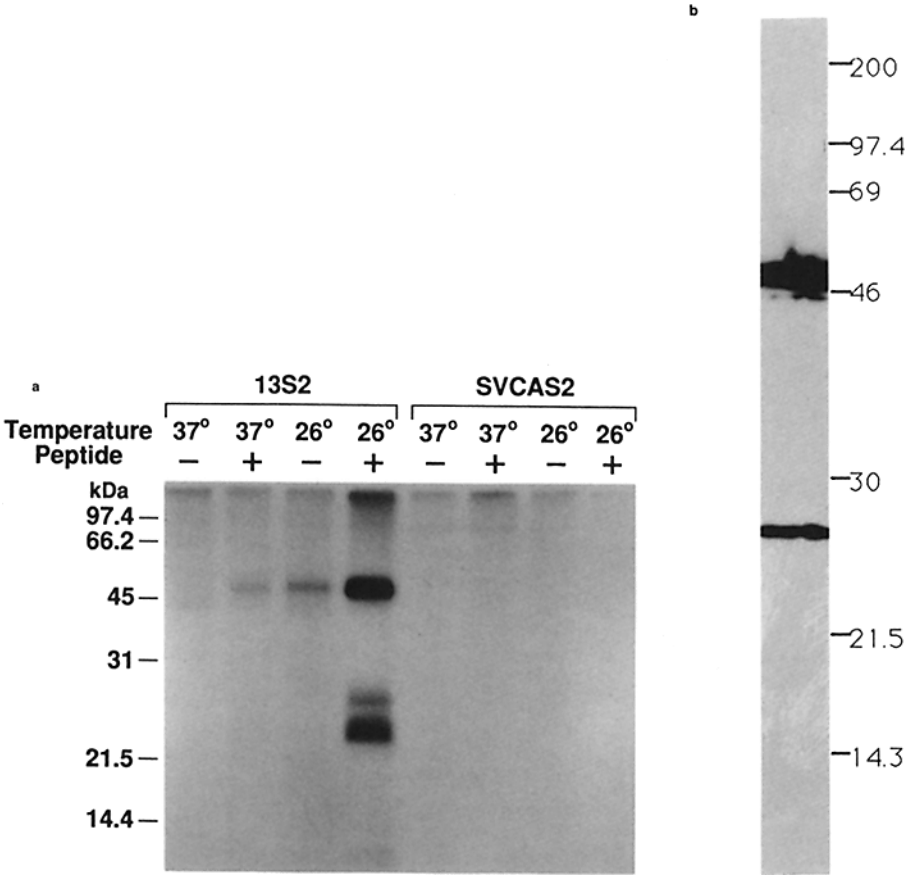

Figure 5. Highest level of cell surface chimera could be detected on 13S2 cells incubated at 26°C with f-Bla-z peptide. (*a*) 13S2 cells were incubated at 26°C or 37°C in the presence or absence of 500 nM f-Bla-z peptide for 16 h. Lysates from surface-iodinated cells were immunoprecipitated with 28-14-8S and electrophoresed on a SDS-PAGE gel. (*b*) 13S2 cells were incubated at 26°C in the presence of 500 nM f-Bla-z peptide for 16 h. Lysates were electrophoresed on a SDS-PAGE gel and transferred to nitrocellulose. The blot was then probed with 28-14-8S.

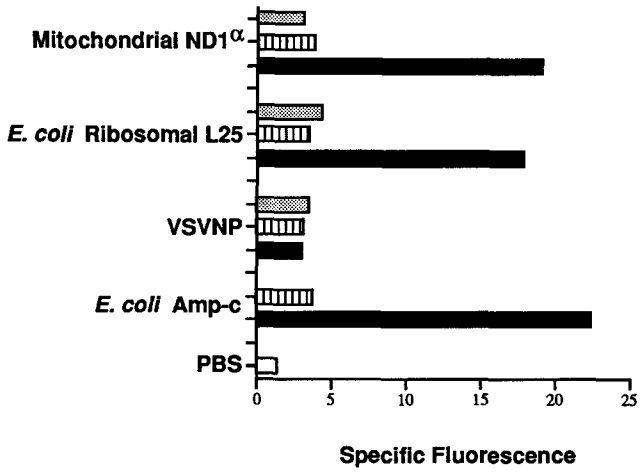

Figure 6. Synthetic peptide binding profile of the M3ª-Lᵈ chimera mimicked native M3ª. Synthetic peptides were incubated with 13S2 cells at 26°C for 16 h at a concentration of 20 μM and stained with the mAb 28-14-8S. Three known M3ª-binding peptides (mitochondrial ND1α_{1-12}, *E. coli* ribosomal L25, and *E. coli* Amp-c) induced *N*-formyl dependent cell surface expression of the chimera. (▦) Acetylated; (▥) unmodified; (■) formylated.

from *E. coli*, the *N*-formyl β-lactamase peptide from *E. coli* all caused significant cell surface stabilization of M3ª-Lᵈ. Similarly, the upregulation of cell surface expression by peptide and cold temperature was peptide specific; the acetylated and nonsubstituted Bla-z peptides caused no specific increase of staining over cold temperature alone (data not shown). These peptides did not affect surface expression of K^{w17}, a control MHC class I molecule (data not shown). Moreover, the peptides bound in an *N*-formyl–dependent manner, which resembled the peptide-binding profile of the native molecule. Finally, an *N*-formylated version of the vesicular stomatitis virus nucleoprotein (fVSVNP$_{52-59}$), which has been shown not to bind M3ª (25), also did not stabilize the H chain above cold temperature background.

In Vitro Stabilization of the MHC–Peptide Ternary Complex by Addition of Exogenous Peptide. The collective data suggest that peptide was the limiting component in the formation of the ternary MHC complex. In this respect, 13S2 cells resemble RMA-S cells, as well as other mutant cells (58, 59) in which the availability of peptide is limited by a defect in the putative peptide transporter protein. Whereas the defect in RMA-S cells and others affect all MHC class I proteins, low surface expression was limited to M3ª-Lᵈ in these transfectants. Other investigators have shown that if specific peptide were added to RMA-S cell lysates, formation of the MHC H chain-β_2m–peptide complex could be seen as determined by the appearance of peptide-dependent, conformationally sensitive epitopes, including the association with β_2m (60–62). In contrast, no such dependence on peptide was seen in lysates from RMA cells, implying that endogenously bound peptides do not dissociate from H chains in this assay (60).

We immunoprecipitated with the conformationally insensitive antibody 28-14-8S and monitored the appearance of β_2m as a marker of peptide binding. Lysates from [^{35}S]methionine-labeled cells were divided into tubes containing 200 μM of f-Bla-z peptide or an equivalent volume of DMSO (peptide solvent control). Immunoprecipitation of control lysates produced the H chain alone, even on an

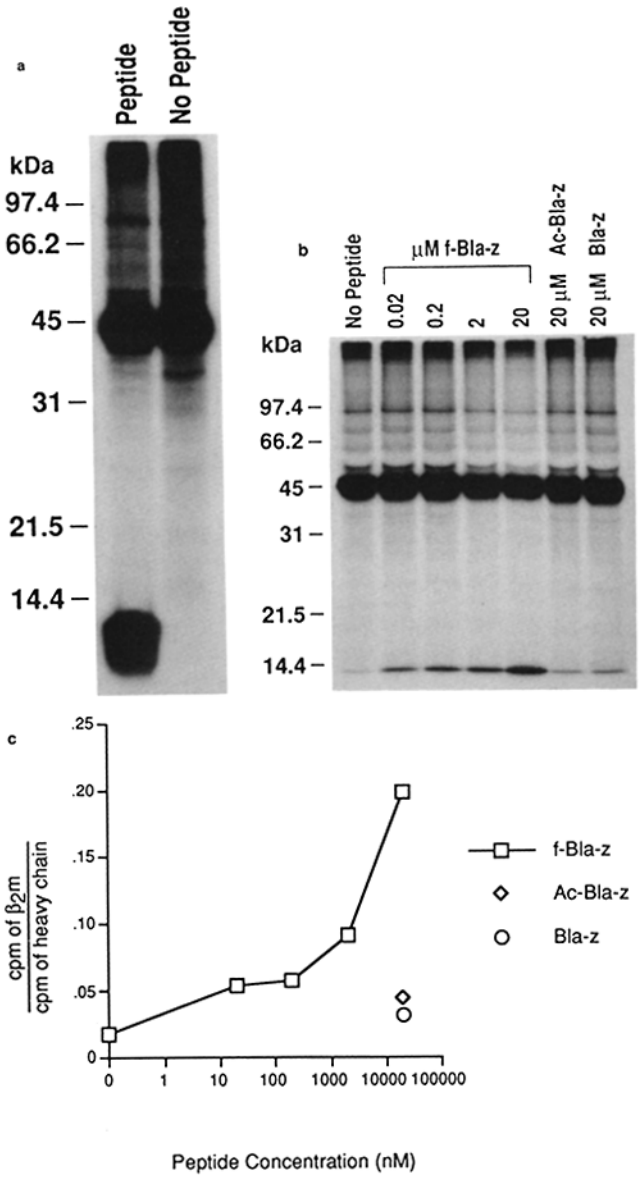

Figure 7. Specific peptide allowed formation of ternary complexes in cell lysates. (*a*) Cells labeled with [^{35}S]methionine for 20 min were lysed in the presence or absence of 200 μM f-Bla-z peptide and incubated at 4°C overnight. After immunoprecipitation with 28-14-8S, the sample was electrophoresed on a SDS-PAGE gel. (*b*) Labeled lysates were divided into tubes containing 0.02, 0.2, 2, 20, and 200 μM of f-Bla-z peptide, 200 μM Ac-Bla-z, or 200 μM Bla-z, and incubated overnight at 4°C. After immunoprecipitation with 28-14-8S, the sample was electrophoresed on a SDS-PAGE gel. Mouse β_2m migrates slightly faster than the 14.4-kD marker. (*c*) The dried gel was analyzed on a betascope and counts for β_2m and H chain were recorded. The ratio of the β_2m cpm to the H chain cpm was plotted against the peptide concentration.

overexposed gel (Fig. 7 *a*, lane *2*). In contrast, immunoprecipitation with peptide-treated lysates yielded both heavy chain and β_2m. Hence, although H chain can associate with β_2m quite well, it did so only in the presence of sufficient cognate peptide. β_2m could be coprecipitated with the H chain in cell lysates in a manner dependent on the concentration of f-Bla-z (Fig. 7, *b* and *c*). Finally, β_2m did not associate with the H chain if lysates were treated with 200 μM Ac-Bla-z or Bla-z peptide, indicating that this ternary complex formation occurred only in the presence of specific peptide (Fig. 7, *b* and *c*).

Discussion

In this report, we have demonstrated that cell surface expression of an M3^a-L^d chimera is governed by the availability of appropriate peptides. Crystal structure analysis of MHC class I-a molecules has defined pockets within the antigen-binding groove that are thought to determine the specificity of peptide binding (63–65). One feature common to all crystallized MHC class I molecules is the presence of highly conserved tyrosines at 7, 59, 159, and 171 in pocket A which may establish hydrogen bonds with the positively charged peptide NH_2 terminus (66–68). In contrast, phenylalanine occupies position 171 in M3^a (22) and may allow both the uncharged *N*-formyl moiety to bind as well as prevent the binding of most charged NH_2 termini. Thus, the ultrastructure of the antigen-binding domain may allow only a narrow range of peptides to bind. This level of selectivity translates into fewer peptide-containing molecules of M3^a at the cell surface.

A model for the fate of this H chain can be proposed from these data. M3^a-L^d mRNA was made in the transfectants at levels comparable to other endogenous MHC class I molecules and the protein was normally translated and translocated into the endoplasmic reticulum. Here, most of the H chain was retained because it presumably had not adopted the correct conformation. Williams et al. (69) have shown that calnexin (p88), a Ca^{2+}-sensitive, endoplasmic reticulum–resident protein, can bind MHC class I H chains. Thus, it is possible that this chimera was retained by calnexin. However, some endogenous (mitochondrial) peptide was accessible to and was bound by the chimera in the endoplasmic reticulum. β_2m also bound stably, forming a ternary complex that was transported to the cell surface rendering these transfectants sensitive to M3^a-restricted mCTLs. However, the number of cell surface complexes was quite small as the cells were negative by surface staining. Cold temperature may allow empty H chains to adopt the correct conformation, to release from the quality control machinery in the endoplasmic reticulum and to egress to the cell surface via the Golgi complex. Alternatively, but not mutually exclusively, reduced temperatures may inhibit proper function of the endoplasmic reticulum–resident proteins, causing empty H chains not to be retained. In any case, empty molecules are allowed to come to the cell surface. These molecules are still relatively unstable. Addition of specific peptide stabilizes the M3^a-L^d chimeras and increases their steady-state surface expression.

The chimeric M3^a-L^d molecule resembles that of MHC class I molecules in RMA-S cells. In both systems, one sees low or absent surface expression of MHC class I molecules. Both systems can be described as a peptide deprivation phenotype (70). Whereas the defect in processing in RMA-S cells is at the level of the peptide pump, in the M3^a-L^d transfectants, it is at the level of H chain specificity for peptide. For both RMA-S cells and the chimera transfectants, the incubation at 26°C greatly increases relevant class I expression at the cell surface. However, there is one major difference. Whereas peptide was sufficient to stabilize empty molecules in RMA-S cells at 37°C, it did not cause an appreciable increase in cell surface expression in the 13S2 cells. Hence, the level of empty chimeric molecules at 37°C at the cell surface is quite small in contrast to RMA-S cells.

Low level constitutive expression is a general feature of class I-b molecules. Since peptide is the limiting factor for expression of the M3^a-L^d chimera, other class I molecules with low cell surface expression may be limited by endogenous peptides. In this respect, the L^d molecule resembles a class I-b molecule. The L^d molecule has been shown to have low constitutive cell-surface expression, but is induced fourfold by the addition of specific peptide (33, 50), suggesting that L^d may possess an unusual biochemical specificity for peptides. Additionally, HLA-C and HLA-E in humans are found at low levels at the cell surface (71, 72). We suggest that for all of these molecules, extraordinary selectivity of peptide binding intracellularly may result in lower surface expression.

As with other MHC class I molecules (73, 74), the M3^a-L^d chimera conformationally changed upon peptide binding. This was most clearly identified when peptide was added to labeled cell lysates and induced the formation of the ternary complex. Also, when ^{125}I-labeled material was immunoprecipitated from cells incubated at either 37 or 26°C in the presence of peptide, two additional bands were detected. Western blot analysis showed that 28-14-8S reacted with one of the lower molecular weight bands, indicating that it retained the antibody epitope. The fact that this degradation product was seen only when peptide was added to cells suggests that peptide binding to the chimera may cause a conformational change in the H chain, allowing a trypsin-sensitive site to be exposed. Conformational effects on the H chain by peptide have been seen with other MHC class I molecules (75, 76).

The conclusion that the cell surface expression of M3^a is strictly regulated by the availability of endogenous peptides has important implications for peptide elution from MHC molecules. Strategies employed to increase the amount of H chain by the use of strong heterologous promoters may not yield increased expression of relevant molecules on the cell surface. The data from our studies suggest that surface expression of MHC molecules is regulated posttranslationally. For most MHC class I-a molecules, the peptide pool is not limiting and thus overexpression of these molecules at the cell surface can be achieved. However, for M3^a and possibly

other class I molecules that possess a narrow biochemical specificity for their peptide ligands, overexpression of the H chain may result in little change at the cell surface, making peptide elution from surface molecules more difficult.

A number of specific hypotheses can be proposed to explain the limited availability of $M3^a$-binding peptides to the $M3^a$ antigen-binding cleft. The simplest explanation is that low rates of synthesis of *N*-formyl peptides limits peptide concentration at the site of loading. Alternatively, postsynthetic mechanisms may play a role in limiting peptides. These include proteolytic cleavage by proteasome, ubiquitination, and autophagy of organelles (3, 77, 78). The proteosome machinery, including the LMP2 and LMP7 subunits, has been put forth as the leading candidate for the process of peptide generation for MHC class I molecules (3, 79). Peptide transport by TAP might demonstrate selectively inefficient transport of *N*-formylated peptides. Finally, chaperones or other hypothesized loading proteins involved with MHC class I loading might limit the rate of formation of peptide–$M3^a$ complexes. Peptide limited high expression of the $M3^a$-L^d chimera. This leads us to propose that the pool of *N*-formylated peptides might rise substantially after invasion by intracellular bacteria. When *N*-formyl peptides derived from such bacteria interact with retained H chains, the complex will egress to the cell surface, signaling $CD8^+$ T cells of its infected state. By extension, we propose that other MHC H chains similar to $M3^a$ and L^d in displaying narrow biochemical specificities for their peptide ligands localize predominantly to the endoplasmic reticulum. Upon infection by organisms that generate appropriate antigens, these specialized MHC class I molecules will be mobilized to the cell surface. If such MHC class I molecules are poorly expressed in the thymus, this hypothesis further predicts that specialized molecules will uncommonly serve as selecting agents during thymic education, perhaps accounting for the low precursor frequency of class I-b restricted T cells in the peripheral lymphoid tissues.

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References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I–restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
2. Pfeifer, J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark, and C.V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature (Lond.).* 361:359.
3. Goldberg, A.L., and K.L. Rock. 1992. Proteolysis, proteasomes and antigen presentation. *Nature (Lond.).* 357:375.
4. Yang, Y., J.B. Waters, K. Fruh, and P.A. Peterson. 1992. Proteasomes are regulated by interferon-γ: implications for antigen processing. *Proc. Natl. Acad. Sci. USA.* 89:4928.
5. Monaco, J.J. 1992. A molecular model of MHC class-I-restricted antigen processing. *Immunol. Today.* 13:173.
6. Van, K.L., R.P.G. Ashton, H.L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^-8^+$ T cells. *Cell.* 71:1205.
7. Townsend, A., C. Öhlén, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Kärre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature (Lond.).* 340:443.
8. Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (Lond.).* 351:290.
9. Powis, S.J., A.R.M. Townsend, E.V. Deverson, J. Bastin, G.W. Butcher, and J.C. Howard. 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature (Lond.).* 354:528.
10. Attaya, M., S. Jameson, C.K. Martinez, E. Hermel, C. Aldrich, J. Forman, K. Fischer-Lindahl, M.J. Bevan, and J.J. Monaco. 1992. *Ham-2* corrects the class I antigen-processing defect in RMA-S cells. *Nature (Lond.).* 355:647.
11. Maloy, W.L., and J.E. Coligan. 1985. Is β_2-microglobulin required for MHC class I heavy chain expression? *Immunol. Today.* 6:263.
12. Williams, D.B., B.H. Barber, R.A. Flavell, and H. Allen. 1989. Role of β_2-microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules. *J. Immunol.* 142:2796.
13. Moore, M.W., F. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell.* 54:777.

14. Rothbard, J.B., and M.L. Gefter. 1991. Interactions between immunogenic peptides and MHC proteins. *Annu. Rev. Immunol.* 9:527.
15. Klein, J., C. Benoist, C.S. David, P. Demant, K. Fischer Lindahl, L. Flaherty, R.A. Flavell, U. Hämmerling, L.E. Hood, S.W. Hunt III, et al. 1990. Revised nomenclature of mouse *H-2* genes. *Immunogenetics.* 32:147.
16. Fischer Lindahl, K. 1993. Peptide antigen presentation by nonclassical MHC class I molecules. *Seminars in Immunology* 5:117.
17. Hedrick, S.M. 1992. Dawn of the hunt for nonclassical MHC function. *Cell.* 70:177.
18. Aldrich, C.J., R. Waltrip, E. Hermel, M. Attaya, K. Fischer Lindahl, J.J. Monaco, and J. Forman. 1992. T cell recognition of Qa-1^b antigens on cells lacking a functional *Tap-2* transporter. *J. Immunol.* 149:3773.
19. Forman, J., J. Trial, S. Tonkonogy, and L. Flaherty. 1982. The *QA2* subregion controls the expression of two antigens recognized by *H-2*-unrestricted cytotoxic T cells. *J. Exp. Med.* 155:749.
20. Fischer Lindahl, K., E. Hermel, B.E. Loveland, and C.-R. Wang. 1991. Maternally-transmitted antigen: a model transplantation antigen. *Annu. Rev. Immunol.* 9:351.
21. Loveland, B., C.-R. Wang, H. Yonekawa, E. Hermel, and K. Fischer Lindahl. 1990. Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. *Cell.* 60:971.
22. Wang, C.-R., B.E. Loveland, and K. Fischer Lindahl. 1991. *H-2M3* encodes the MHC class I molecule presenting the maternally transmitted antigen of the mouse. *Cell.* 66:335.
23. Shawar, S.M., J. Vyas, J.R. Rodgers, and R.R. Rich. 1994. Antigen presentation by major histocompatibility complex class I-b molecules. *Annu. Rev. Immunol.* In press.
24. Shawar, S.M., R.G. Cook, J.R. Rodgers, and R.R. Rich. 1990. Specialized functions of MHC class I molecules. I. An *N*-formyl peptide receptor is required for construction of the class I antigen Mta. *J. Exp. Med.* 171:897.
25. Shawar, S.M., J.M. Vyas, E. Shen, J.R. Rodgers, and R.R. Rich. 1993. Differential amino-terminal anchors for peptide binding to H-2M3^a or H-$2K^b$ and H-$2D^b$. *J. Immunol.* 151: 201.
26. Shawar, S.M., J.M. Vyas, J.R. Rodgers, R.G. Cook, and R.R. Rich. 1991. Specialized functions of major histocompatibility complex class I molecules. II. Hmt binds N-formylated peptides of mitochondrial and prokaryotic origin. *J. Exp. Med.* 174:941.
27. Kurlander, R.J., S.M. Shawar, M.L. Brown, and R.R. Rich. 1992. Specialized role for a murine class I-b MHC molecule in prokaryotic host defenses. *Science (Wash. DC).* 257:678.
28. Pamer, E.G., C.-R. Wang, L. Flaherty, K. Fischer Lindahl, and M.J. Bevan. 1992. H-2M3 presents a Listeria monocytogenes peptide to cytotoxic T lymphocytes. *Cell.* 70:215.
29. Wang, C.R., and K. Fischer-Lindahl. 1993. HMT, encoded by *H-2M3*, is a neoclassical major histocompatibility class I antigen. *Proc. Natl. Acad. Sci. USA.* 90:2784.
30. Wolf, P.R., and R.G. Cook. 1990. The *TL* region gene 37 encodes a Qa-1 antigen. *J. Exp. Med.* 172:1795.
31. Lie, W.-R., N.B. Myers, J.M. Connolly, J. Gorka, D.R. Lee, and T.H. Hansen. 1991. The specific binding of peptide ligand to L^d class I major histocompatibility complex molecules determines their antigenic structure. *J. Exp. Med.* 173:449.
32. Smith, J.D., W.-R. Lie, J. Gorka, C.S. Kindle, N.B. Myers, and T.H. Hansen. 1992. Disparate interaction of peptide ligand with nascent versus mature class I major histocompatibility complex molecules: comparisons of peptide binding to alternative forms of L^d in cell lysates and the cell surface. *J. Exp. Med.* 175:191.
33. Lie, W.-R., N.B. Myers, J. Gorka, R.J. Rubocki, J.M. Connolly, and T.H. Hansen. 1990. Peptide ligand-induced conformation and surface expression of the L^d class I MHC molecule. *Nature (Lond.).* 344:439.
34. Pullen, J.K., H.D. Hunt, R.M. Horton, and L.R. Pease. 1989. The functional significance of two amino acid polymorphisms in the antigen-presenting domain of class I MHC molecules. *J. Immunol.* 143:1674.
35. Ozato, K., G.A. Evans, G. Shykind, D.H. Margulies, and J.G. Seidman. 1983. Hybrid H-2 histocompatibility gene products assign domains recognized by alloreactive T cells. *Proc. Natl. Acad. Sci. USA.* 80:2040.
36. Tada, N., S. Kimura, A. Hatzfeld, and U. Hämmerling. 1980. Ly-m11: The *H-3* region of mouse chromosome 2 controls a new surface alloantigen. *Immunogenetics.* 11:441.
37. Klein, D., Z. Zaleska-Rutczynska, W.C. Davis, F. Figueroa, and J. Klein. 1987. Monoclonal antibodies specific for mouse class I and class II Mhc determinants. *Immunogenetics.* 25:351.
38. Vyas, J.M., S.M. Shawar, J.R. Rodgers, R.G. Cook, and R.R. Rich. 1992. Biochemical specificity of H-2M3^a: stereospecificity and space-filling requirement at position one maintains *N*-formyl peptide binding. *J. Immunol.* 149:3605.
39. Ahearn, J.M., Jr., M.S. Bartolomei, M.L. West, L.J. Cisek, and J.L. Corden. 1987. Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II. *J. Biol. Chem.* 262:10695.
40. Rodgers, J.R., P.R. Wyde, and R.R. Rich. 1991. Mutational analysis of regulation of MHC and anti-viral genes. *J. Immunol.* 146:1979.
41. Rodgers, J.R., S.M. Shawar, M.M. Guenther, and R.R. Rich. 1992. Kinetics of killing by monoclonal cytotoxic T lymphocytes: I. Colorimetric detection of cryptic CTL determinants on adherent target cells and survivorship analysis. *J. Immunol. Methods.* 152:159.
42. Smith, R., III, and R.R. Rich. 1985. Polymorphism and tissue distribution of maternally transmitted antigen defined by cytotoxic T lymphocyte lines. *J. Immunol.* 134:2191.
43. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
44. Cook, R.G., R.R. Rich, and L. Flaherty. 1981. The Qa-1 alloantigens. I. Identification and molecular weight characterization of glycoproteins controlled by *Qa-1^a* and *Qa-1^b* alleles. *J. Immunol.* 127:1894.
45. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature (Lond.).* 227:680.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
47. Reiss, C.S., G.A. Evans, D.H. Margulies, J.G. Seidman, and S.J. Burakoff. 1983. Allospecific and virus-specific cytolytic T lymphocytes are restricted to the N or C1 domain of H-2 antigens expressed on L cells after DNA-mediated gene transfer. *Proc. Natl. Acad. Sci. USA.* 80:2709.
48. Allen, H., D. Wraith, P. Pala, B. Askonas, and R.A. Flavell. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature (Lond.).* 309:279.
49. Hansen, T.H., N.B. Myers, and D.R. Lee. 1988. Studies of two antigenic forms of L^d with disparate β_2-microglobulin (β_2m) associations suggest that β_2m facilitate the folding of

the α1 and α2 domains during de novo synthesis. *J. Immunol.* 140:3522.
50. Beck, J.C., T.H. Hansen, S.E. Cullen, and D.R. Lee. 1986. Slower processing, weaker β_2-m association, and lower surface expression of H-2L^d are influenced by its amino terminus. *J. Immunol.* 137:916.
51. Ribaudo, R.K., and D.H. Margulies. 1993. Polymorphism at position nine of the MHC class I heavy chain controls the stability of association with β_2-microglobulin as evaluated in a cell free protein assembly system. *J. Cell. Biochem.* 17C:61.
52. Fischer Lindahl, K., B. Hausmann, and V.M. Chapman. 1983. A new *H-2* linked class I gene whose expression depends on a maternally inherited factor. *Nature (Lond.).* 306:383.
53. Christinck, E.R., M.A. Luscher, B.H. Barber, and D.B. Williams. 1991. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature (Lond.).* 352:67.
54. Vogt, R.F., Jr., D. Cross, D.L. Phillips, L.O. Henderson, and W.H. Hannon. 1991. Interlaboratory study of cellular fluorescence intensity measurements with fluorescein-labeled microbead standards. *Cytometry.* 12:525.
55. Schumacher, T.N.M., M.-T. Heemeis, J.J. Neefjes, W.M. Kast, C.J.M. Melief, and H.L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell.* 62:563.
56. Ljunggren, H.G., N.J. Stam, C. Öhlén, J.J. Neefjes, P. Hoglund, M.T. Heemels, J. Bastin, T.N. Schumacher, A. Townsend, K. Kärre, and H.L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature (Lond.).* 346:476.
57. Rock, K.L., C. Gramm, and B. Benacerraf. 1991. Low temperature and peptides favor the formation of class I heterodimers on RMA-S cells at the cell surface. *Proc. Natl. Acad. Sci. USA.* 88:4200.
58. Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (Lond.).* 345:449.
59. Spies, T., V. Cerundolo, M. Colonna, P. Cresswell, A. Townsend, and R. DeMars. 1992. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature (Lond.).* 355:644.
60. Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. *Cell.* 62:285.
61. Kvist, S., and U. Hamann. 1990. A nucleoprotein peptide of influenza A virus stimulates assembly of HLA-B27 class I heavy chains and β_2-microglobulin translated *in vitro*. *Nature (Lond.).* 348:446.
62. Elvin, J., V. Cerundolo, T. Elliott, and A. Townsend. 1991. A quantitative assay of peptide-dependent class I assembly. *Eur. J. Immunol.* 21:2025.
63. Saper, M.A., P.J. Bjorkman, and D.C. Wiley. 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol.* 219:277.
64. Fremont, D.H., M. Matsumura, E.A. Stura, P.A. Peterson, and I.A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science (Wash. DC).* 257:919.
65. Zhang, W., A.C.M. Young, M. Imarai, S.G. Nathenson, and J.C. Sacchettini. 1992. Crystal structure of the major histocompatibility complex class I H-2K^b molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. *Proc. Natl. Acad. Sci. USA.* 89:8403.
66. Matsumura, M., D.H. Fremont, P.A. Peterson, and I.A. Wilson. 1992. Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science (Wash. DC).* 257:927.
67. Madden, D.R., J.C. Gorga, J.L. Strominger, and D.C. Wiley. 1992. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight binding to MHC. *Cell.* 70:1035.
68. Guo, H.-C., T.S. Jardetsky, T.P.J. Garrett, W.S. Lane, J.L. Strominger, and D.C. Wiley. 1992. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature (Lond.).* 360:364.
69. Degen, E., M.F. Cohen-Doyle, and D.B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both β_2-microglobulin and peptide. *J. Exp. Med.* 175:1653.
70. Howard, J.C., E.V. Deverson, S. Powis, and G.W. Butcher. 1993. Peptide transporters and selectivity. *J. Cell Biochem.* 17C:51.
71. Ulbrecht, M., J. Kellermann, J.P. Johnson, and E.H. Weiss. 1992. Impaired intracellular transport and cell surface expression of nonpolymorphic HLA-E: evidence for inefficient peptide binding. *J. Exp. Med.* 176:1083.
72. Güssow, D., R.S. Rein, I. Meijer, W. de Hoog, G.H.A. Seemann, F.M. Hochstenbach, and H.L. Ploegh. 1987. Isolation, expression, and the primary structure of *HLA-Cw1* and *HLA-Cw2* genes: evolutionary aspects. *Immunogenetics.* 25:313.
73. Elliott, T., J. Elvin, V. Cerundolo, H. Allen, and A. Townsend. 1992. Structural requirements for the peptide-induced conformational change of free major histocompatibility complex class I heavy chains. *Eur. J. Immunol.* 22:2085.
74. Otten, G.R., E. Bikoff, R.K. Ribaudo, S. Kozlowski, D.H. Margulies, and R.N. Germain. 1992. Peptide and β_2-microglobulin regulation of cell surface MHC class I conformation and expression. *J. Immunol.* 148:3723.
75. Čatipović, B., P.J. Dal, M. Mage, T.E. Johansen, and J.P. Schneck. 1992. major histocompatibility complex conformational epitopes are peptide specific. *J. Exp. Med.* 176:1611.
76. Bluestone, J.A., S. Jameson, S. Miller, and R. Dick II. 1992. Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition. *J. Exp. Med.* 176:1757.
77. Ortiz-Navarrete, V., A. Seelig, M. Gernold, S. Frentzel, P.M. Kloetzel, and G.J. Hämmerling. 1991. Subunit of the '20S' proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex. *Nature (Lond.).* 353:662.
78. Seglen, P.O., and P. Bohley. 1992. Autophagy and other vacuolar protein degradation mechanisms. *Experientia.* 48:158.
79. Martinez, C.K., and J.J. Monaco. 1991. Homology of proteasome subunits to a major histocompatibility complex-linked LMP gene. *Nature (Lond.).* 353:664.