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 \(\alpha_1\) and \(\alpha_2\) domains of M3\(^a\) and \(\alpha_3\) of L\(^a\) 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\(^\circ\)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\(^\circ\)C (but not at 37\(^\circ\)C) greatly enhanced expression of M3\(^a\) on cell surface in a dose-dependent manner, suggesting stabilization of unoccupied molecules. Stable association of \(\beta_2\)-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)\(^i\) (5, 6). In the endoplasmic reticulum, the nascent MHC-encoded transmembrane H chain (44,000 \(M_1\)) forms a ternary complex with \(\beta_2\)-microglobulin (\(\beta_2m\)) (12,500 \(M_1\)) (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 \(\beta_2m\)-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 \(\beta_2m\) 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
of class I mRNA transcripts does not necessarily equate to abundant cell surface expression. For instance, Qa-1b 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\textsuperscript{d} (31, 32). Moreover, the cell surface expression of L\textsubscript{d} can be induced by peptide, suggesting that some H chain is retained intracellularly (33).

In contrast, we found that M3\textsuperscript{a}-L\textsubscript{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\textsuperscript{a}-L\textsubscript{d} chimera.

Materials and Methods

Construction of the MHC Class I Chimera Vector. The M3\textsuperscript{a}-L\textsubscript{d} expression vector was derived from pKb-L\textsubscript{d}, a gift from Dr. Larry Pease (Mayo Clinic, Rochester, MN). The exons that encode \(\alpha\)\textsuperscript{1} and \(\alpha\)\textsuperscript{2} domains of M3\textsuperscript{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\textsuperscript{a} were generated independently by genomic PCR. Specifically, intronic oligonucleotides that flank each exon were used to amplify these segments from NZB/B1NJ genomic DNA. For exon 2, the 5\textsuperscript{\prime} oligonucleotide (5\textsuperscript{\prime}-CAATGCTTGTCAGCTGGC) possessed a SalI site and the 3\textsuperscript{\prime} oligonucleotide (5\textsuperscript{\prime}-AGAGCTCTGAAGCCCGAC) a HindIII site. The gel-purified DNA was separated from the agarose using Magic Mini-prep (Promega) and sequenced using a protein G column in an FPLC system (Pharmacia, Piscataway, NJ) and used for all immunoprecipitations.

Cytotoxic T Lymphocyte Generation. The M3\textsuperscript{a}-specific, M3\textsuperscript{a}-restricted monoclonal CTL (mCTL) clones ID28 and D35 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\textsuperscript{a}. OVA-specific polyclonal CTL were produced by immunizing C57BL/6J (B6) mice with 5 \times 10\textsuperscript{6} 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 (\(\gamma\)-irradiated 20,000 rad) and syngeneic cells (\(\gamma\)-irradiated 3,000 rad). OVA-specific CTL clone 2GI2 was obtained by limiting dilution and was shown to be restricted by H-2K\textsuperscript{b} (25).

Peptide Synthesis and Purification. The f-ND1\textsubscript{52-59} peptide corresponding to the NH\textsubscript{2}-terminus of ND1, f-Bla\textsubscript{25-31} peptide, from Bacillus cereus \(\beta\)-lactamase binds to M3\textsuperscript{a} as determined by CTL competition assays (26). Ac-Bla\textsubscript{25-31} and Bla\textsubscript{25-31} 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\textsuperscript{a}. VSVNPS2-59 is the vesicular stomatitis virus nucleocapsid peptide that binds to K\textsuperscript{b}, but not to M3\textsuperscript{a} (25). All peptides were synthesized and HPLC purified as described previously (38).

Cell Culture. SVCA52.F6 tail cell fibroblasts were generated from B10.CAS2 mice (H-2\textsuperscript{b}, M3\textsuperscript{a}) using SV40-mediated transformation. These cells were transfected with EcoRI linearized pM3\textsuperscript{a}-L\textsubscript{d} and KpnI linearized ppol2 (vector encoding G418 resistance) (39) at a molar ratio of 20:1, by CaPO\textsubscript{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\textsubscript{25-31} 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\textsubscript{2}. E.G7 cells were passaged 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).

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 \(\mu\)l of supplemented Mishell-Dutton medium (SMDM) (42). Triplicate dilutions of mCTL in 100 \(\mu\)l 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 acidic 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...

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The RT product served as a template for 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 10^6 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 (GAM Ig) antibodies (Baxter, Mundelein, IL) for 30 min at 4°C. Samples were then fixed with 1% cold PBS. The cells were stained with a 1:50 dilution of FITC-conjugated goat anti-mouse (GAM Ig) antibodies (Baxter, Mundelein, IL) for 30 min at 4°C. Samples were then fixed with 1% cold PBS.

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 10^6 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 (GAM Ig) antibodies (Baxter, Mundelein, IL) for 30 min at 4°C. Samples were then fixed with 1% cold PBS. The cells were stained with a 1:50 dilution of FITC-conjugated goat anti-mouse (GAM Ig) antibodies (Baxter, Mundelein, IL) for 30 min at 4°C. Samples were then fixed with 1% cold PBS.

Preparation of Radiolabeled Proteins. For metabolic labeling, subconfluent monolayers of 1322 or parental SV4027.6 cells (1 x 10^6 cells) were preincubated for 1 h in methionine-free RPMI 1640 medium supplemented with 5% (vol/vol) FCS and 1% (vol/vol) 200 mM r-glutamine (Gibco BRL). Cells were labeled with 50 μCi/ml [35S]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 x 10^6 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% H2O2 in PBS, and 2.5 mCi 125I (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 1 h. The lysates were precleared using 150 μl of BSA-Sepharose, goat anti-mouse-Sepharose (GAM Ig 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 Bllot Analyzer (Betagen, Mountain View, CA). The specific activity of the H chain and βm bands was determined by subtracting the mean background count from the actual values. For 125I-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 1322 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 PBST (PBS plus 0.05% Tween-20) for 1 h and washed three times with 1% non-fat dry milk in PBST. The blots were probed with 28-14-8S in 1% non-fat dry milk in PBST for 1 h at room temperature. After three extensive washes, GAM IgG 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 M3a-L4 Chimeric MHC Class I Gene. We designed a chimeric MHC class I protein with two critical features: (a) the peptide binding specificity of M3 and (b) an epitope for a mAb that would be independent of the α1 and α2 domains. To this end, we joined the α1 and α2 domain of M3 with the α3 domain of L4 (Fig. 1). Previous experiments of others established that the TCR interacts with peptide and residues from α1 and α2.

Figure 1. Diagram of vector construct. To attach a conformationally insensitive antibody epitope to the antigen binding domain of M3, exons 2 and 3 from M3 replaced the corresponding exons in the Kβ-L4 vector. This chimera possesses the promoter and signal peptide of Kβ, and the α3 transmembrane domain and cytoplasmic tail of L4.
with little, if any, involvement of the α3 region (47). In particular, Allen et al. (48) showed that L cells expressing a Kb (α1, α2)-Ld (α3) hybrid molecule were lysed by Kα-restricted CTLs equivalently to fibroblasts expressing the native Kb molecule. A useful feature of the Ld α3 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 α3 domain of the Lα molecule might not bind β2m well (49) were alleviated by two studies demonstrating that the inability of native Ld to associate stably with β2m is determined by the α1 domain (50, 51). Therefore, the Ld α3 domain itself possesses the capacity to bind functionally with β2m.

The M3α-Ld Chimera Renders SVCAS2.F6 (M3b) Fibroblasts Sensitive to Lysis by M3α-restricted mCTLs. The chimera was transfected along with a drug-selecting marker into SVCAS2.F6 fibroblasts derived from the H-2w17 mouse strain B10.CAS2. This mouse strain carries a different allele of the H chain (M3b), rendering these fibroblasts resistant to lysis by M3α-restricted mCTLs (52). To determine whether the chimera could bind the endogenous mitochondrial peptide ND1α and traffic to the cell surface, G418-resistant fibroblasts were incubated with M3α-restricted, ND1α-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, transfected cells were incubated with OVA-specific mCTLs that recognize the OVA peptide bound to Kb (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 M3α-Ld chimera, Kb, 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.
Levels of M3a-Lα 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 M3a-Lα chimera. Graded concentrations of RT product served as template in PCR reactions using oligonucleotides specific for H-2Kβ, β-actin, and the chimera (Fig. 3a). Denitometry 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. 3b). Although this assay is not a measure of absolute mRNA concentrations, these results suggest mRNA itself was not limiting surface expression of the M3a-Lα 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 M3a-Lα Chimera at 37°C. We used the mAb 28-14-8S to determine the level of cell surface expression. As seen in Fig. 4a, 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 Kw17 molecules could be detected at wild-type levels by the antibody Tü232 at 37°C (Fig. 4e) indicating the absence of any general defect in the transport of MHC molecules to the cell surface. Staining with anti-β2m also revealed normal levels of β2m (Fig. 4f). However, 13S2 cells did have some M3a-Lα 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 M3a-Lα on the cell surface was <1% of the number of Kw17 molecules in these transfectants.

Surface Expression of M3a-Lα 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 M3a-Lα. 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-Kw17) (e) and S19.8 (anti-β2m) (f).
gested that the assembly pathway and its related proteins were functioning properly. We therefore evaluated the role of intracellular peptides specific for M3α 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. 4c, the chimera could be detected at the surface of cells incubated at 26°C using the anti-Ld 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. 4b-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-2Kb 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 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 [125I]. 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. 5a). 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. 5b). Control immunoprecipitations with K wit under identical conditions did not yield any degradation products (data not shown).

The M3α-Ld Chimera Retained the Peptide Binding Characteristics of Native M3α. The fine specificity of native M3α for peptide binding was established earlier by CTL-peptide competition assays. These studies established that M3α 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 M3α-Ld possessed the peptide binding specificity of the native molecule by testing a panel of M3α-binding and non-binding peptides. As seen in Fig. 6, the N-formyl ND1-1-12 mitochondrial peptide, the N-formyl ribosomal L25 peptide...
from *E. coli*, the N-formyl β-lactamase peptide from *E. coli* all caused significant cell surface stabilization of M3-L4. 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\textsuperscript{wt7}, 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 (VSVNP Nas, 69), which has been shown not to bind M3\textsuperscript{a} (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 M3-L4 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\textsubscript{2}m–peptide complex could be seen as determined by the appearance of peptide-dependent, conformationally sensitive epitopes, including the association with β\textsubscript{2}m (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\textsubscript{2}m as a marker of peptide binding. Lysates from [\textsuperscript{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
overexposed gel (Fig. 7 a, lane 2). In contrast, immunoprecipitation with peptide-treated lysates yielded both heavy chain and β2m. Hence, although H chain can associate with β2m quite well, it did so only in the presence of sufficient cognate peptide. β2m 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, β2m 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 M3a-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 NH2 terminus (66-68). In contrast, phenylalanine occupies position 171 in M3a (22) and may allow both the uncharged N-formyl moiety to bind as well as prevent the binding of most charged NH2 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 M3a at the cell surface.

A model for the fate of this H chain can be proposed from these data. M3a-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 Ca2+-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. β2m also bound stably, forming a ternary complex that was transported to the cell surface rendering these transfectants sensitive to M3a 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 M3a-L d chimeras and increases their steady-state surface expression.

The chimeric M3a-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 M3a-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 M3a-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 M3a-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 125I-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 M3a 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 M3a 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-L4 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-L4 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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