The Majority of H2-M3 Is Retained Intracellularly in a Peptide-receptive State and Traffics to the Cell Surface in the Presence of *N*-formylated Peptides

By Nancy M. Chiu, Taehoon Chun, Miriam Fay, Manas Mandal, and Chyung-Ru Wang

From the Gwen Knapp Center for Lupus and Immunology Research, Committee on Immunology and Department of Pathology, University of Chicago, Chicago, Illinois 60637

Summary

We used a new monoclonal antibody (mAb 130) to analyze the intracellular trafficking and surface expression of H2-M3, the major histocompatibility complex class Ib molecule that presents *N*-formylated peptides to cytotoxic T cells. M3 surface expression is undetectable in most cell types due to the paucity of endogenous antigen. M3 is induced on the cell surface by addition of high-affinity *N*-formylated peptides from mitochondria and listeria. Peptide-induced M3 expression is most efficient on antigen presenting cells. Basal and inducible expression of M3 is transporter associated with antigen processing (TAP)-dependent, distinguishing M3 from the class Ib molecules TL and CD1. Unlike the expression of class Ia molecules and a previously described M3/L^d chimera, surface expression of M3 cannot be rescued by lowered temperature, suggesting that the α3 domain and transmembrane region of M3 may control trafficking. Pulse–chase analysis and use of trafficking inhibitors revealed a pool of empty M3 in the endoplasmic reticulum or early Golgi apparatus. Addition of exogenous peptide allows maturation with kinetics matching those of D^d. The lack of endogenous *N*-formylated peptide allows discovery of novel pathogen-derived peptides in normal antigen presenting cells. The nonpolymorphic nature of M3 and its ability to present bacterial antigens rapidly and dominantly make it an attractive target for peptide vaccination strategies.

Key words: major histocompatibility complex class I • antigen presentation • cytotoxic T cells • *N*-formylated peptides • rodent

The H2-M3 MHC class Ib molecule differs from class Ia molecules in expression patterns, levels, and polymorphism (1, 2). H2-M3 was first identified as a component of a minor histocompatibility antigen, maternally transmitted antigen (Mta; 3, 4).[1] Mta was found to consist of an *N*-formylated mitochondrial peptide (maternally transmitted factor [MTF]) from NADH dehydrogenase subunit I(ND1) bound to M3 (5). Further studies have shown that the binding specificity of M3 is for *N*-formylated peptides with hydrophobic residues (6, 7), whereas the affinity of M3 for nonformylated peptides is 100–1,000-fold lower (8). As the class Ia molecules K and D do not bind *N*-formylated peptides appreciably (9), the binding specificity of M3 for *N*-formylated peptides may have been selected in evolution for the specialized presentation of a conserved structure of bacterial peptides. Because only mitochondria and prokaryotes initiate protein synthesis with *N*-formylated methionine, CTLs specific for *N*-formylated bacterial peptides would have little risk of cross-reacting to self-peptides. Protection afforded by adoptive transfer of M3-restricted CTLs specific for *Listeria monocytogenes* peptide LemA (fMIGWII) has shown the importance of M3 in the host defense against intracellular pathogens (10–13). Additional listerial antigens presented by M3 have also been identified, such as FR38 (fMIVIL; 14) and fMIVTLF (15).

The *H2-M3* gene is located in the telomeric end of the mouse *H-2* complex and shares many conserved features with class Ia MHC molecules (4, 16). By Northern blot analysis, it has been shown to be expressed in most tissues of all strains of mice at about one-twentieth of the level of class Ia molecules (4). *H2-M3* message can be detected by RT-PCR as early as day eight in embryonic development, and surface expression (17) has been detected by CTL assay in a thymic epithelial cell (TEC) line, which suggests that it may be active in shaping the TCR repertoire (Wang, C.-R., unpublished data). The crystal structure of M3 with bound ND1 reveals that the *N*-formylated peptide is shifted

[1]*Abbreviations used in this paper:* β2m, β2-microglobulin; ER, endoplasmic reticulum; LCMV, lymphocytic choriomeningitis virus; Mta, maternally transmitted antigen; MTF, maternally transmitted factor; TAP, transporter associated with antigen processing; TEC, thymic epithelial cell.

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one residue along the groove relative to peptides in other class I structures (18), and the optimal length of M3-binding peptide would be a heptamer. Additionally, the residues surrounding the M3 antigen binding groove are predominately hydrophobic, which may explain why most M3-bound peptides to date are hydrophobic.

Because of the unusual antigens presented by M3, it is of interest to see if M3 peptide loading and antigen processing follows the pathway outlined for class Ia molecules or whether it displays some nonclassical behaviors as reported for other class Ib molecules, i.e., TL and CD1 (19, 20). MHC class I molecules generally derive antigens from intracellular sources. The pathway involves cytosolic degradation of polypeptides by the proteasome complex (21) and transport to the endoplasmic reticulum (ER) lumen by the transporter associated with antigen processing (TAP) (22, 23). Nascent MHC class Ia molecules dimerize with β2-microglobulin (β2m) with the aid of the chaperone calnexin (24–26). Subsequently, a complex involving a second chaperone, calreticulin, and tapasin is formed (27). Tapasin may aid in both the final folding of MHC class I molecules and forming the peptide loading complex with TAP (28). Upon stable formation of the ternary complex of MHC class I heavy chain, β2m, and peptide (usually a nonamer), the molecule is transported through the Golgi apparatus to the cell surface. Variations in the pathway have been outlined in mutant cell lines, particularly those deficient in TAP (29), calnexin (30, 31), β2m (32), and tapasin (28, 33, 34). Although surface expression of high levels of MHC class Ia molecules is peptide dependent, analysis at reduced temperature has shown that it is possible for an empty heavy chain–β2m dimer to reach the cell surface and be stabilized by the addition of peptide; however, this is not the major route of maturation in normal cells (35).

Due to the lack of an appropriate serological reagent, the antigen presenting pathway and surface expression of M3 have not been visualized for the native molecule. Attempts to produce anti-M3 alloantibodies have failed (1), most likely because of the minimal divergence between different M3 alleles (2) and low level of M3 expression as predicted from its transcript levels. In this study, we have developed a hamster mAb against purified recombinant M3 that allows us to detect M3 expression directly and compare its behavior with that of class Ia molecules. Similar to class Ia, M3 expression is TAP-dependent; however, M3 differs from class Ia molecules in several respects. M3 behaves in a normal cell as class Ia molecules behave in a TAP-deficient background. This results in virtually undetectable expression of M3 on all cell types except B cells. M3 does not move to the cell surface without peptide at reduced temperatures as do classical class I molecules (35). The majority of M3 is retained in an immature peptide-receptive state and traffics rapidly to the cell surface upon addition of exogenous peptide. This resulting increase in M3 on the cell surface shows long-term stability. This property, combined with the lack of competition from self-antigens, assures potent display of even small amounts of bacterial peptide. The ability to analyze M3 surface expression allows determination of the binding specificity of M3 and may prove useful in predicting antigenic peptides.

Materials and Methods

Cell Lines and Reagents. The following mAbs were purchased from PharMingen: FITC–anti-CD3 (2C11), FITC–anti–TCR-β (H57-597), FITC–anti-K^b (AF6-88.5), mouse anti–hamster IgG–FITC, PE–anti-B220, and PE–anti-CD11c (HL3). PE–F4/80, an antibody specific to macrophage, was purchased from Caltag Labs. Synthetic peptides were purchased from Research Genetics. All peptides were >90% pure as determined by mass spectrometry. Peptides were dissolved in DMSO at concentrations of 1–2 mg/ml. B cell lines A20 and SP2/0, macrophage cell lines P388 and J774, and thymoma BW5147 cells were purchased from American Type Culture Collection. RMA and RMA-S cells were provided by Dr. John Monaco (University of Cincinnati, Cincinnati, OH). TECs and thymic nurse cells (TNC.R3.1) were provided by Dr. Jim Miller (University of Chicago). Chemotactic peptide (fMLFF), normal hamster sera, human β2m, brefeldin A, phenylarsine oxide, chloroquine, cytochalasin B, and cycloheximide were purchased from Sigma Chemical Co.

Immunization and Production of H2-M3–specific Antibodies. Soluble M3 protein was purified from the culture supernatant of a *Drosophila melanogaster* cell line (SC2) cotransfected with the truncated *M3* and murine *B2m* cDNAs as described by Castaño et al. (36). 100 μg of purified M3 was emulsified in complete Freund's adjuvant and injected subcutaneously into 8-wk-old Armenian hamsters. Two to three additional immunizations were administered subcutaneously in incomplete Freund's adjuvant at 2-wk intervals. 4 d after the last immunization, lymphocytes isolated from immunized hamster were used to produce hybridoma cell lines by fusion with murine myeloma cell line SP2/0 using PEG1500. Hybridoma supernatants were screened in ELISA plates coated with 100 ng of purified M3. Positive wells were then tested for the ability to block the recognition of M3-restricted CTLs.

T Cell Clones and Cytotoxicity Assays. MTF^{α}-specific, M3-restricted CTLs (4E3, B6, and 5G5) (37, 38) were provided by Dr. Kirsten Fischer Lindahl (UT Southwestern Medical Center, Dallas, TX). P14, a lymphocytic choriomeningitis virus (LCMV) peptide–specific D^b-restricted CTL line, was provided by Dr. Philip Ashton-Rickardt (University of Chicago). RMA cells (MTF^{α}, $M3^{wt}$) and LCMV peptide-pulsed RMA-S cells were used as targets in a standard ^{51}Cr-release assay for M3-restricted CTLs and P14 CTLs, respectively. Target cells (10^6 cells) were labeled with 100 μCi [^{51}Cr]sodium chromate for 1 h at 37°C. Target cells (10^4 cells) were added to round-bottom microtiter wells containing effector cells. Supernatants containing anti-M3 or nonrelevant antibody were added to the wells at a final dilution of 1:4. After 4 h incubation at 37°C, 100 μl of supernatant from each well was assayed for ^{51}Cr release. Results are given as percentage of specific lysis = (experimental − spontaneous release) × 100/(maximal release − spontaneous release).

Cell Preparations. Single-cell suspensions from thymus, spleen, Peyer's patch, and lymph node were prepared by pressing the organs between the frosted ends of two microscope slides. Peritoneal macrophages were obtained by peritoneal lavage with DMEM (GIBCO BRL). Red blood cells were removed when necessary by hypotonic lysis. Intestinal epithelial cells were prepared and purified through discontinuous 40/70% Percoll gradient centrifugation as described by Tagliabue et al. (39). LPS blasts and ConA blasts were prepared by culturing splenocytes with 5 μg/ml of LPS and 3 μg/ml of ConA, respectively, in RPMI

1640 (GIBCO BRL) with 10% fetal bovine serum, 2 mM l-glutamine, 20 mM Hepes, 50 μM 2-ME, penicillin, and streptomycin (RPMI 10 media) for 48 h at 37°C.

Flow Cytometric Analysis of M3 Upregulation. 10^6 cells were incubated in RPMI 10 media with or without peptides for 18–20 h at 37 or 26°C. Cells were harvested and washed three times with PBS before cell surface staining experiments. M3 staining was detected by adding 100 μl hybridoma supernatants followed by mouse anti–hamster IgG FITC. Staining with each reagent was performed for 30 min on ice in immunofluorescence buffer (HBSS containing 2% fetal bovine serum and 0.1% NaN_3), followed by washing with the same buffer. The stained cells were analyzed by flow cytometry using a FACSCalibur™ with Cellquest™ software (Becton Dickinson). When inhibitors were present, they were added 3 h before the addition of peptide and remained during the overnight incubation with or without peptide at 37°C.

Cell Labeling, Immunoprecipitation, and SDS-PAGE Analysis. LPS blasts from C57BL/6 mice were surface labeled by lactoperoxidase-catalyzed iodination (40). Labeled cells were lysed in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 20 mM iodoacetamide, 1 mM PMSF, and 10 mg/ml aprotinin. Radiolabeled lysates were precleared successively with protein A–Sepharose (Pharmacia) and normal hamster sera bound to protein A–Sepharose at 4°C for 4 h. 1 ml of various mAb supernatants coupled to protein A–Sepharose were used for immunoprecipitation with precleared cell lysate at 4°C overnight. Immune complexes were washed with a buffer containing 0.25% NP-40, 5 mM PMSF, 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM KI, and 5 mM EDTA. After extensive washing, the immunoprecipitates were eluted by boiling for 5 min in SDS sample buffer and analyzed on 12.5% polyacrylamide gel.

For pulse–chase experiments, 5×10^6 P388 cells were used for each time point. After starvation in 3 ml of methionine/cysteine–free medium for 2 h, cells were pulsed with 0.5 mCi/ml of ^{35}S Translabel (ICN Biomedicals, Inc.) for 20 min and then chased in complete medium for various periods of time in the presence or absence of 10 μM of LemA peptide. Aliquots of cells for each chase point were lysed in lysis buffer. The lysates were precleared and M3 molecules were immunopurified as described. Immune complexes were eluted from the protein A–Sepharose beads by boiling with SDS-PAGE sample buffer containing 0.6% SDS and 1% 2-ME for 5 min. Eluates were diluted 1:5 with distilled water and split into two equal aliquots, one of which received 2 mU of endoglycosidase (EndoH) at pH 5.5, followed by overnight incubation at 37°C. Samples were analyzed by 12.5% SDS-PAGE and fluorography.

Results

Development of Monoclonal Reagents for the Detection of M3 Expression. We generated mAbs against M3 by immunization of hamsters with recombinant soluble M3 protein. Recombinant M3 was purified from the culture supernatant of SC2 cells transfected with murine β2m and truncated M3 cDNAs. Three clones (mAb 32, 38, and 130) reacting positively with purified M3 by ELISA were screened for the ability to block the recognition of M3 by M3-restricted CTLs. One of the clones, mAb 130, significantly blocked the killing of all MTF-specific, M3-restricted CTL clones tested but had no effect on H-2D^b–restricted LCMV peptide–specific killing by the P14 CTL line (Fig. 1). Western

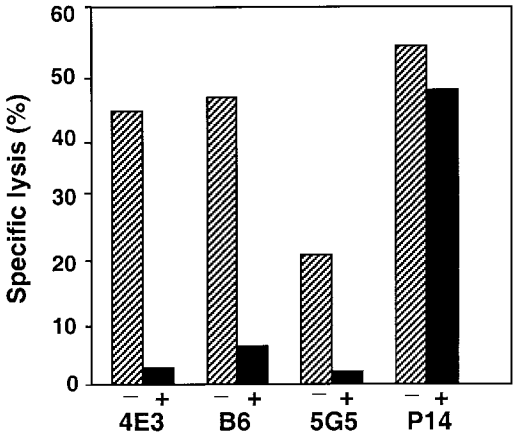

Figure 1. M3-restricted CTL killing is inhibited by mAb 130. Anti-MTF$^\alpha$ CTL clones 4E3, B6, and 5G5 were incubated with RMA cells (M3^{wt}, MTF$^\alpha$) in the presence of either 100 μl mAb 130 hybridoma supernatant (solid bars) or 100 μl of RPMI 10 media (hatched bars) during a Cr-release assay. Clone P14 recognizes an LCMV peptide in the context of D^b and is not affected by mAb 130. The E/T ratio was 9:1 for 4E3, 12:1 for B6, 4:1 for 5G5, and 10:1 for P14.

blot analysis of purified recombinant protein showed that mAb 130 reacts with the M3 heavy chain and not with the β2m light chain (data not shown).

M3 Surface Expression on a Range of Cell Types. Flow cytometric analysis was performed to detect M3 surface expression from various lymphoid organs. mAb 130 detected low levels of M3 on cells from the spleen, Peyer's patch, and lymph node (Fig. 2), consistent with the finding that *H2-M3* message is much less abundant than that of class Ia genes (4). M3 is not detectable on the surfaces of thymocytes, al-

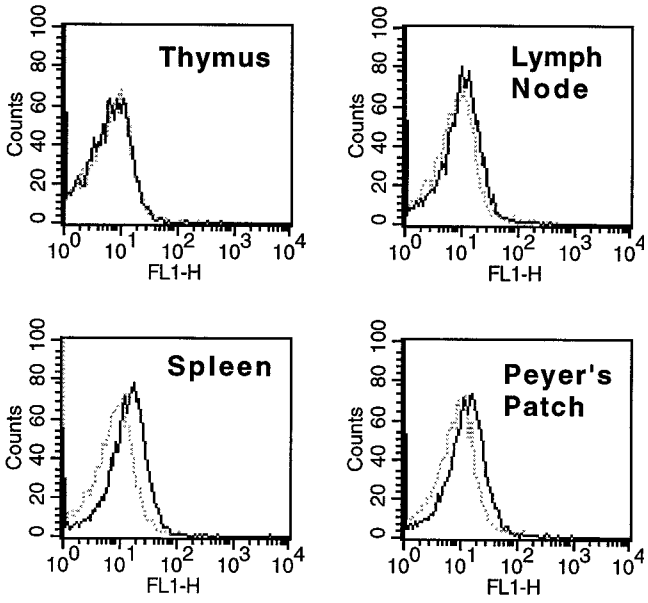

Figure 2. Low-level expression of M3 in lymphoid tissues. Single-cell suspensions from C57BL/6 thymus, spleen, lymph node, and Peyer's patch were stained with mAb 130 followed by secondary detection by FITC-conjugated mouse anti–hamster IgG. Dotted lines represent staining with a control hamster antibody; black lines are mAb 130 specific.

though M3 message is readily detectable in thymus RNA. Expression of M3 on the cell surface was also found on LPS blasts but not on cells activated by ConA (Table I). Low or undetectable M3 surface staining was observed on various cell types known to be targets for M3-specific CTLs (Table I). Although *H2-M3* message can be upregulated by IFN-γ (17), this treatment has no effect on M3 surface expression in cell lines (data not shown). Furthermore, an M3 transfectant (TR8.4a; reference 4) of a fibroblast cell line (B10.CAS2) that expresses high levels of *H2-M3* mRNA does not show detectable surface expression. The level of *H2-M3* message does not appear to correlate with the level of M3 surface expression, suggesting that surface expression of M3 may be controlled posttranscriptionally.

Induction of M3 Surface Expression by Increased Peptide Supply. Because the supply of endogenous *N*-formylated peptides is limited to 13 potential peptides from mitochondria, it remained possible that peptide supply had an influence on M3 surface expression. To examine whether increased peptide supply can induce M3 surface expression, LPS blast cells from C57BL/6 mice were incubated overnight with 10 μM of Fr38 peptide (fMIVIL), an antigenic peptide for a listeria-specific, M3-restricted CTL. Surface iodination was followed by immunoprecipitation with anti-M3 antibodies. Both mAb 38 and mAb 130 immunoprecipitated significant amounts of M3 heavy chain (41 kD) and β2m (12 kD) from Fr38-treated cells (Fig. 3). The amount of surface iodinated M3 is substantially less in untreated cells. In contrast, the amount of another class Ib molecule, CD1, is not affected by the peptide treatment.

Enhanced surface expression of M3 by incubation with Fr38 peptide was further confirmed by immunofluorescence assay. M3 surface expression in splenocytes is found to increase approximately fivefold after incubation with 10 μM of Fr38, whereas K^b expression is not affected (Fig. 4 A). Two-color immunofluorescence staining was performed to see whether the levels of M3 induction differ among cell types in the spleen (Fig. 4 B). Whereas $CD3^+$ T cells are only induced twofold (as assessed by change in fluorescence intensity), B cells are able to show a ninefold induction of M3. Macrophage and dendritic cell populations both display M3 at three to four times the level of uninduced cells. Unlike splenic T cells, thymocytes do not show significant induction of M3. Peritoneal macrophages also induce M3 to fourfold above background levels (Table I). Additionally, M3 induction can be detected on the following cell lines: B cell A20, SP2/0, macrophage J774, P388, T cell line RMA, TEC, and thymic nurse cell line TNC.R3.1. However, unlike other class Ib molecules (i.e., TL and CD1; reference 41–43), M3 expression and induction is not detected on the surface of intestinal epithelial cells (Table I). It is worth noting that peptide treatment has only a minimal effect on an M3-transfected fibroblast cell line, further suggesting the upregulation of M3 surface expression by exogenous peptide is cell type specific.

Table I. *Reactivity of Anti-M3 mAb*

Cell type	Untreated	Treated
Thymocyte	−	±
Splenocyte	+	+++
LPS blast	+	+++
ConA blast	−	+
Intestinal epithelial cell	−	−
Peritoneal macrophage	−	++
B cell line		
A20	+	++
SP 2/0	−	+
Macrophage cell line		
P388	−	++
J774	−	+
T cell line		
BW5147	−	−
RMA	−	+
RMA-S	−	±
TEC	−	+
Thymic nurse cell line TNC.R3.1	−	+
Fibroblast cell line		
L929	−	±
B10.CAS2	−	−
TR8.4a	−	±

Reactivity of anti-M3 antibody was determined on cells incubated overnight with 10 μM of LemA (treated) or without peptide (untreated) by flow cytometric analysis. The reactivity is indicated as follows: −, negative staining; ±, <50% reactivity above background; the number of '+' (+, ++, +++) correlates with the staining intensity of cells.

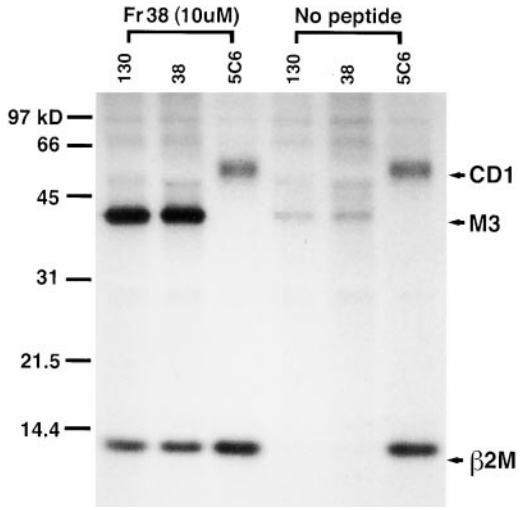

Figure 3. mAb 130 and mAb 38 immunoprecipitate M3 on the cell surface. LPS blasts from C57BL/6 spleen were incubated overnight with or without 10 μM Fr38, surface labeled with ^{125}I, lysed, and precipitated with either M3-specific antibodies (mAb 130 and mAb 38) or a control antibody to mouse CD1 (mAb 5C6). Eluted proteins were separated by SDS-PAGE through a 12.5% gel.

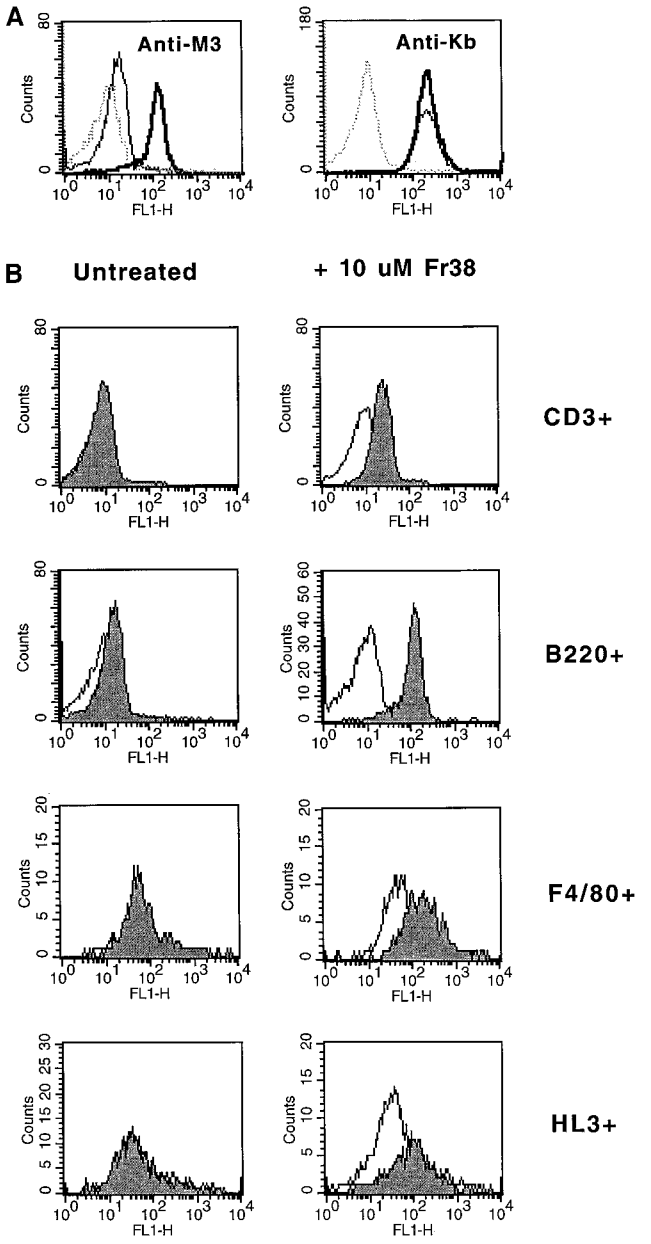

Figure 4. Cell type–specific induction of M3 on splenocytes. Splenocytes from a C57BL/6 adult mouse were incubated overnight with or without 10 μM Fr38 (fMIVIL). Cells were stained with mAb 130 followed by FITC-conjugated anti–hamster IgG and double staining with PE-conjugated markers for T cells (CD3), B cells (B220), macrophages (F4/80), or dendritic cells (HL3). (A) Histograms represent fluorescence profile from total splenocytes. Dotted lines, background staining with isotype control antibodies; thin black lines, mAb 130 or Y3 (anti–H-2K^b) staining of untreated cells; bold black lines, mAb 130 or Y3 staining of peptide-treated cells. (B) Histograms represent populations gated for expression of the indicated markers. Unfilled peaks, background staining with an isotype control hamster antibody specific for TNP; filled peaks, mAb 130-specific staining.

The Range of Antigens Presented by M3. Flow cytometric analysis was used to test a panel of *N*-formylated peptides for their ability to induce increased surface expression of M3. Fig. 5 A shows the extent to which each peptide enhances M3 expression after overnight incubation with 10 μM peptide. Not all *N*-formylated peptides or even all mitochondrially derived *N*-formylated peptides increase the surface level of M3 significantly. The listerial peptides LemA and Fr38 have the highest affinity for M3, followed by ND1 and COI, the only two mitochondrial peptides that cause significant induction. ND4 and COII enhance M3 expression only slightly, and the remaining mitochondrial sequences show no detectable binding. A nonformylated variant of ND1 cannot stabilize the surface expression of M3, confirming the requirement of an *N*-formyl group for high-affinity binding to M3. The relative efficiency of M3 induction by each sequence was further compared by titration of peptide concentration. Fig. 5 B shows the induction of M3 with respect to peptide concentration for high- (LemA, Fr38, ND1, COI) and low-affinity (COII, ND4) peptides. Maximal binding was approached with 10–20-μM concentrations with all tested peptides. Increased peptide concentration for lower affinity peptides cannot induce high levels of M3 on the surface. The relative ability of peptides to induce surface M3 correlates with affinities determined previously by competitive inhibition CTL assays (38). The peptides that bind to M3 with highest affinity are also those against which immunized animals are able to develop a CTL response, namely, ND1 (5, 38), COI (44), LemA (12), and Fr38 (14). ND4 and COII induce slight increases in M3 surface expression, although no CTL clones have been developed against these peptides. The peptides that bind to M3 show little common motif other than the *N*-formylated methionine and some hydrophobic residues. However, the peptides that do not bind M3 frequently contain charged residues at positions two and three. This is compatible with the predictions based on the crystal structure of the M3–ND1 complex (18), in which the interacting surface between ND1 and M3 is predominantly hydrophobic. The chemotactic peptide fMLFF also binds with high affinity to M3 (Fig. 5 A), confirming results obtained by competitive inhibition of Fr38-specific M3-restricted CTL lysis (45). Although this chemotactic peptide is short in length, its hydrophobicity allows significant binding to M3.

M3 Is Not Inducible at 27°C. The response of M3 to antigen supply is similar to that of class Ia molecules in the absence of TAP. In TAP-deficient cells, class Ia expression is increased at reduced temperature and can be stabilized by the addition of peptide, suggesting that empty class I molecules are transported to the cell surface but are not stable. To examine whether surface expression of M3 can be induced by lowered temperature, we compared M3 surface expression on B6 splenocytes at 37°C and 27°C by FACS™ analysis. Fig. 6 A shows that surface expression of M3 cannot be induced by incubation at low temperature, suggesting that the empty M3 molecule is not efficiently transported to the cell surface. In contrast to class Ia, M3 induction is at least 50% less efficient at 27°C, even after overnight incubation with peptide (Fig. 6 B). This reduction may be due to the effect of temperature reduction on endocytosis and intracellular transport, which could cause a reduction in peptide delivery to the ER.

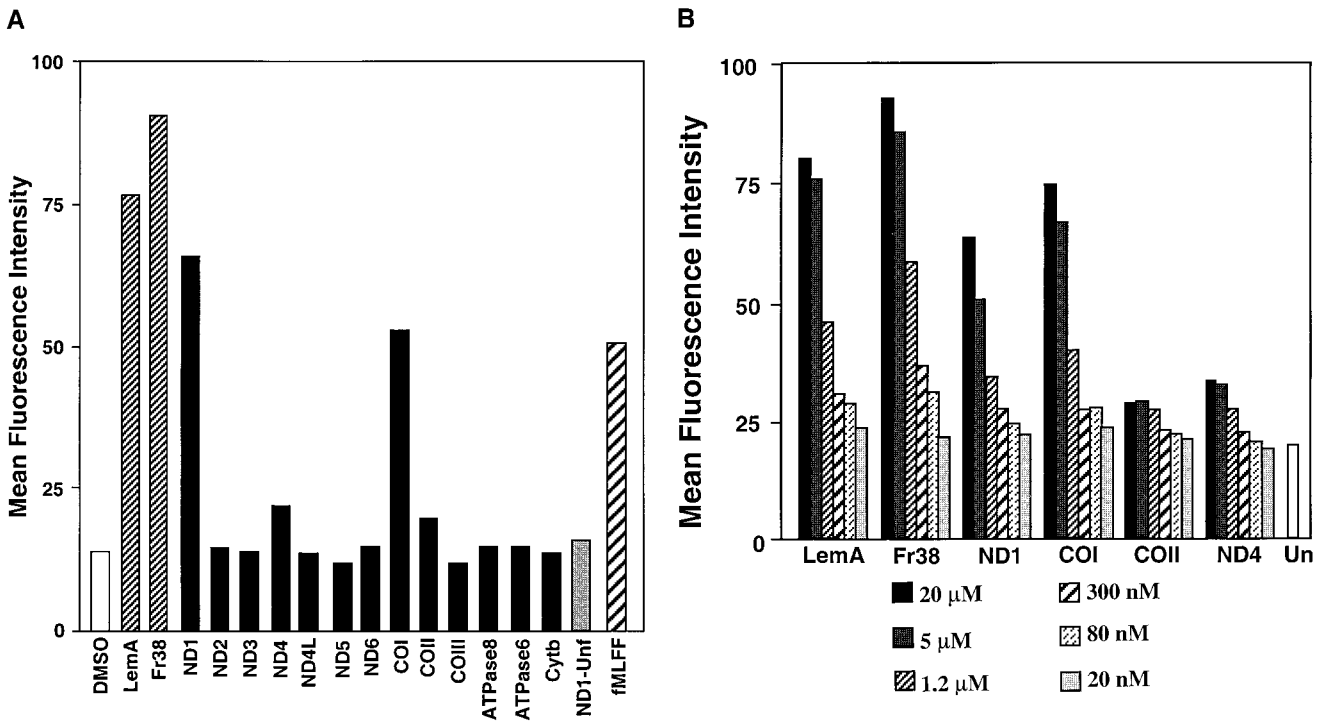

Figure 5. *N*-formylated peptides increase surface expression of M3. (A) C57BL/6 splenocytes were incubated with synthetic listerial peptides (LemA-fMIGWII; Fr38-fMIVIL), mitochondrial peptides (ND1-fMFFINIL; ND2-fMNPITLA; ND3-fMNLYTVI; ND4-fMLKIILP; ND4L-fMPSTFFN; ND5-fMINIFTTS; ND6-fMNNYIFV; COI-fMFINRW; COII-fMAYPFQL; COIII-fMTHQTHA; ATPase6-fMNENLFA; ATPase8-fMPQLDTS; Cytb-fMTNMRKT; and ND1-Unf-MFFINILTL) and chemotactic peptide (fMFLL) at a concentration of 10 μM overnight. Bars represent mean fluorescence intensity after staining with mAb 130 as described. Unf-ND1 shares the same sequence as ND1 but is not formylated. (B) Splenocytes were incubated overnight with varying concentrations of M3-binding peptides. The range of concentrations and the corresponding hatchmarks are shown. DMSO and Un represent background M3 expression on splenocytes either treated with solvent alone or untreated.

Effect of Antigen Trafficking Inhibitors and TAP on M3 Induction. To determine whether the induction of M3 surface expression by peptide requires new protein synthesis, transport from ER, or acidification of the endosomal compartment, we analyzed the effects of various inhibitors on M3 induction by peptide (Fig. 7). M3 surface expression is not significantly affected by the protein translation inhibitor cycloheximide, suggesting that there is an existing intracellular pool of M3 that remains available for binding increased antigen supply. Expression is not increased in the presence of exogenous human β2m, which suggests that there is little trafficking of free M3 heavy chain to the cell surface. M3 induction is inhibited by brefeldin A, which blocks cis-Golgi apparatus transport, indicating that the intracellular pool may be located in the ER or early Golgi compartment. Inhibitors of endocytosis (phenylarsine oxide and cytochalasin B) also affect M3 expression, presumably due to a requirement for antigen to be endocytosed and eventually reach the cytoplasm for transport to the ER. Chloroquine, an inhibitor of the class II exogenous antigen presentation pathway, had little effect on the expression level of M3, which may reflect the lack of a lysosomal processing requirement for short peptides.

Because MTF presentation to CTLs has been shown to be TAP dependent (46), TAP deficiency may also affect M3 induction by exogenous peptide. We analyzed the induction of M3 on splenocytes from TAP-deficient animals (47) and found that maximum levels of expression could not be reached even after lengthy incubation times (Fig. 8). The decrease in efficiency of induction ranged from 50% reduction for LemA to 80% reduction for ND1. This suggests that there is a requirement for TAP translocation of exogenously added *N*-formylated peptides into the ER. Alternatively, TAP or a TAP-dependent complex may mediate peptide association with M3 before maturation and trafficking to the cell surface can be completed.

Stability of the M3–Peptide Complex. The kinetics of increased M3 surface expression were rapid, with increases detected at 30 min and levels approaching plateau at 4 h (Fig. 9 A). Culturing the cells in the presence of peptide for a longer period of time, i.e., 20 h, further increased M3 expression. This increase could be due to stabilization of newly synthesized M3, which could have an additive effect if M3–peptide complexes on the cell surface are long lived. To detect relative stability of M3–peptide complexes, we followed the loss of surface expression on splenocytes after peptide had been removed from the medium by thorough washing. At the 2 h time point, no significant change in fluorescence intensity could be detected for most of the peptides tested. The fluorescence intensity decreased signif-

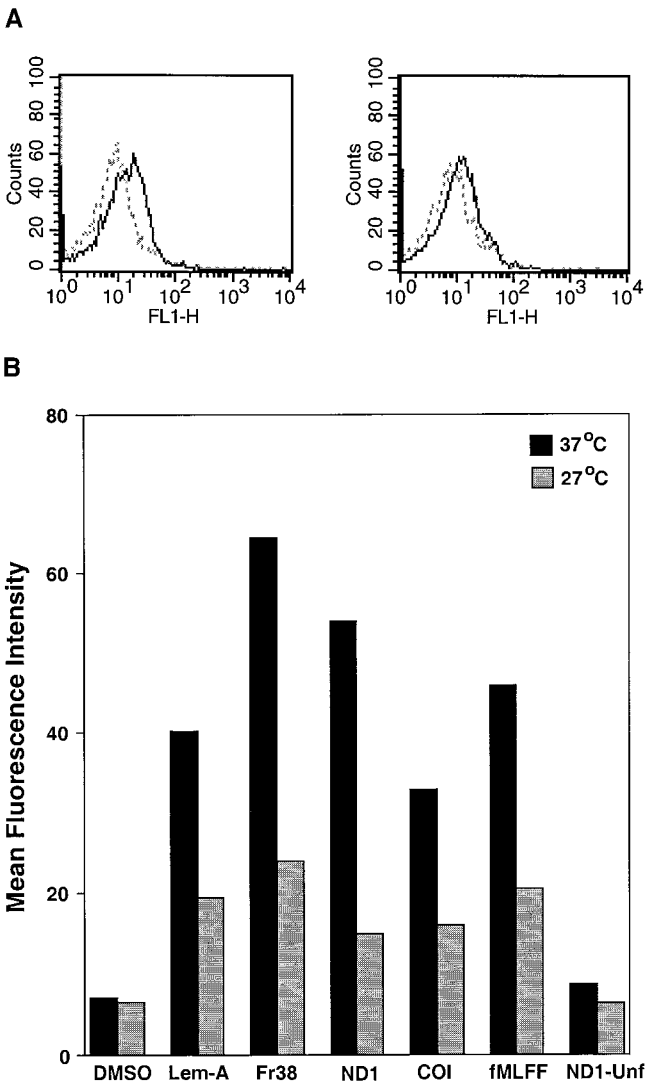

Figure 6. Cell surface expression of M3 cannot be rescued by lowered temperature. (A) C57BL/6 splenocytes were incubated overnight at the indicated temperatures and stained for FACS™ analysis. Surface staining with mAb 130 is shown (black line) superimposed on background staining of cells with control antibody (dotted line). Left, 37°C; right, 27°C. (B) B6 splenocytes were incubated overnight with 10 μM of various peptides. Bars represent staining with mAb 130 for treatment at either 27°C (gray bars) or 37°C (black bars).

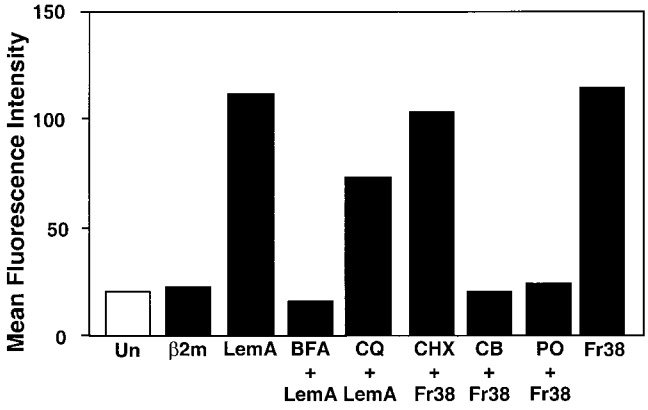

Figure 7. Effect of inhibitors on the peptide-induced expression of M3. C57BL/6 splenocytes preincubated with or without the inhibitor were cultured overnight with 10 μM peptide and stained for M3 expression. BFA, 5 μg/ml brefeldin A; CQ, 20 μM chloroquine; CHX, 20 μg/ml cycloheximide; CB, 10 μM cytochalasin B; PO, 10 μM phenylarsine oxide. The bar labeled β2m shows M3 expression after overnight incubation with 10 μg/ml human β2m in the absence of peptide.

icantly after 4 h for all peptides tested, with 50% reduction seen between 4 and 6 h after washing. After 12 h, increased levels of M3 still remained on the cell surface (Fig. 9 B).

Detection of an Intracellular Pool of M3 Molecules. To further study the intracellular trafficking of M3 in response to increased peptide supply, we performed pulse–chase analysis on P388 cells incubated with and without peptide. [^{35}S]methionine and cysteine were incorporated during a brief metabolic labeling (20 min) and then chased with or without the addition of peptide for various periods of time (Fig. 10). The lysates were immunoprecipitated with mAb 130 or a control antibody (34-2-12S) for H-2D^{d}. Immunoprecipitates were digested with EndoH to measure the transport of class I molecules from the ER (EndoH sensitive) through the mid-Golgi compartment (EndoH resistant). At the zero time point, all molecules are EndoH sensitive, and there is a lack of β2m stably associated with M3 (data not shown). After 1-h chase in the presence of peptide, a significant portion of M3 acquires EndoH resistance and is associated with β2m. However, when no peptide is added, M3 remains in an immature state for at least 6 h and does not appreciably mature to a slower migrating, EndoH-resistant, β2m-associated form. This result suggests

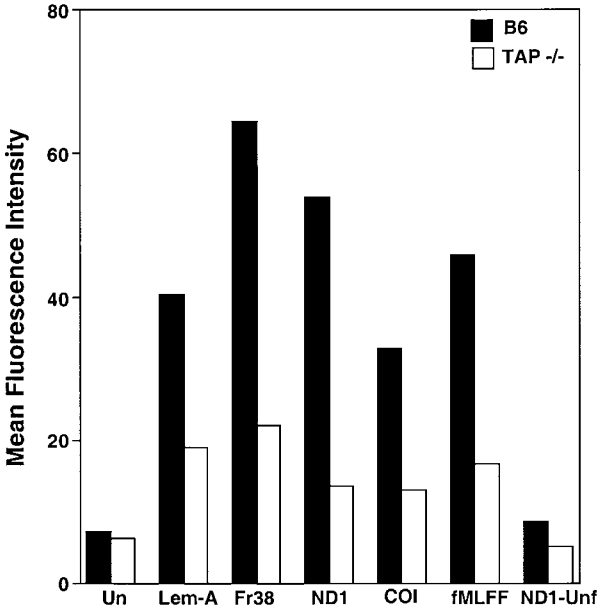

Figure 8. The loading of M3 with exogenously added peptide is largely TAP dependent. Splenocytes from TAP-deficient mice in 129×B6 background (white bars) or from C57BL/6 mice (black bars) were incubated with 10 μM peptide overnight at 37°C. Fluorescence intensity after staining with mAb 130 is shown for the peptides indicated; treatment with DMSO (Un) or with a nonformylated version of the ND1 peptide (ND1-Unf) are also shown.

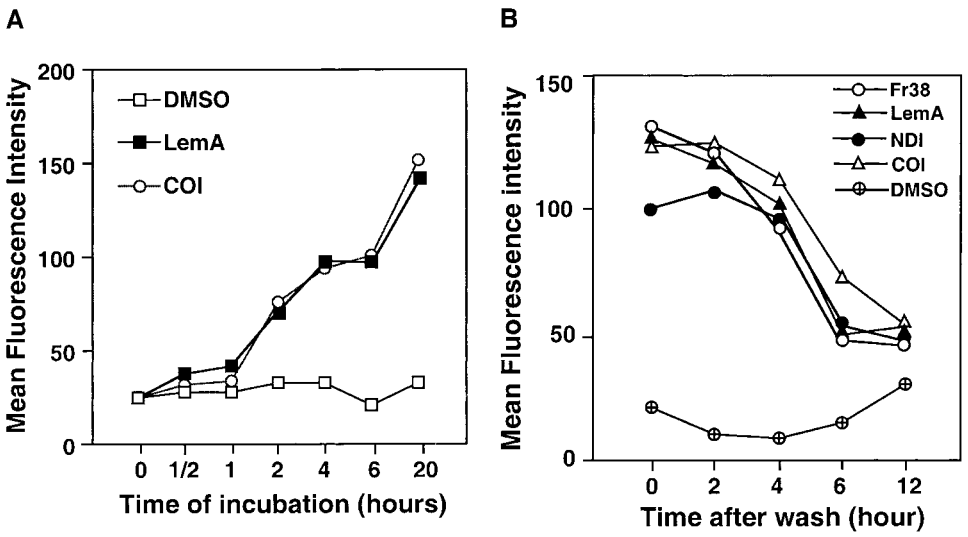

Figure 9. Time course study of peptide-induced M3 surface expression. (A) The peptides LemA and COI were added at 10 μM to splenocytes. At each time point, samples were washed at 4°C and stained for M3 expression. (B) Splenocytes were incubated with 10 μM of each peptide for 4 h. The cells were washed three times and cultured without peptide over the course of 12 h. At each time point, surface expression was assessed by staining with mAb 130.

that there is a steady-state intracellular pool of M3 and that free M3 heavy chain cannot egress from the ER/cis-Golgi compartment in the absence of antigen. At the 20 h time point, most of the M3 molecules were degraded in the absence of peptide, whereas a significant amount of mature M3 molecules remained in the peptide-treated cells, confirming the longevity of M3–peptide complexes detected by FACS™ analysis. The precipitation of comparable amounts of immature and mature forms of M3 over time shows that M3 is truly transported to the cell surface in response to peptide and that mAb 130 does not recognize a peptide-dependent conformation of M3. No difference in the maturation and stability of H-2D^d can be detected from peptide-treated and untreated cells. Furthermore, the kinetics of M3 trafficking are similar to those of H-2D^d except that the M3–peptide complex appears to be more stable than D^d.

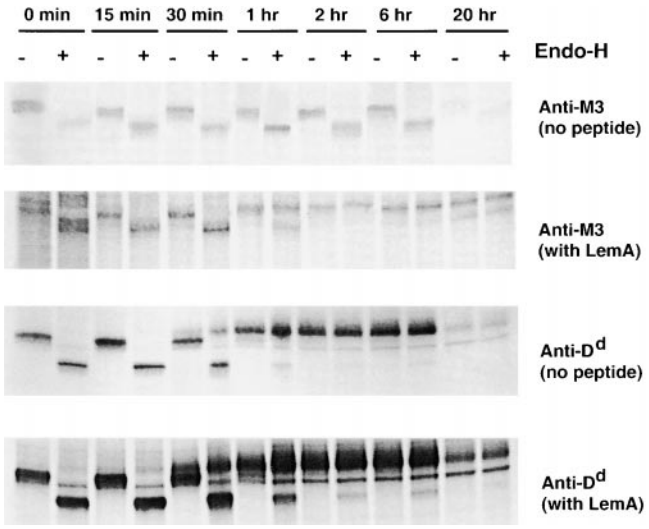

Figure 10. Pulse–chase analysis of ^{35}S metabolically labeled M3. P388 cells were pulsed with ^{35}S Translabel for 20 min and then harvested or chased up to 20 h either in the presence or absence of 10 μM LemA peptide. At the time points indicated, cells were lysed and subjected to immunoprecipitation with mAb 130 (for M3) and mAb 34-2-12S (for D^d). Immune complexes were digested with (+) or without (−) EndoH and separated by SDS-PAGE.

Discussion

We have produced a mAb against the class Ib molecule H2-M3 that allows us to study the expression and intracellular trafficking of M3. M3 is expressed at low levels on the surfaces of B cells and can be induced by peptide on the surface of many cell types, most efficiently on APCs, i.e., B cells, macrophages, and dendritic cells. Despite high levels of *M3* RNA in the transfected fibroblast line (TR8.4a), M3 expression is not induced on the cell surface. This suggests that RNA levels are not ultimately the limiting factor in M3 surface expression and that use of increased ligand supply requires mechanisms specialized to APCs. We have demonstrated the existence of an intracellular pool of M3 in APCs that is rapidly transported to the cell surface when supplied with sufficient antigen. Due to a limited supply of endogenous peptides, M3 behaves in the wild-type background as a class Ia molecule does in a TAP-deficient cell. Under normal conditions, the majority of M3 is retained and degraded in the ER due to the lack of suitable antigens. Addition of exogenous peptides, or presumably infection by intracellular bacteria, allows M3 to rapidly mature with kinetics similar to those for class Ia molecules.

The antigen supply pathway for exogenously added peptide appears to be through endocytosis and then release to cytoplasm, followed by TAP transport into the ER. Although direct transport of pinocytosed peptide to the ER has been demonstrated for fluorescently labeled peptides (48), in the TAP-deficient background, M3 does not reach maximum cell surface levels. In an analogous experiment where RMA and RMA-S cells were pulsed with the K^b-binding peptide SIINFEKL, an antibody specific for K^b–SIINFEKL detected similar amounts of this complex on the surface of both cell types (49). In contrast, M3-binding peptides seem to require peptide translocation from the cytosol into the

ER. Alternatively, M3 peptide loading may be more strongly dependent on the chaperone-like function of the complex of tapasin, Erp57 (50–52), and calreticulin that is stabilized by TAP. It is unlikely that significant antigen loading takes place on empty molecules on the cell surface or through recycling of empty M3, given the inhibitory effect of phenylarsine oxide and brefeldin A and the complete lack of maturation of the M3 heavy chain during the pulse–chase in the absence of peptide. It is unclear why M3 surface expression is induced to greater levels on APCs. This differential expression may be due to differences in the chaperone environment in APCs or to differences in the endosome to cytosol pathway in APCs.

Unlike class Ia molecules, the surface expression of M3 cannot be induced by incubation at low temperature (27°C), suggesting that empty M3 is not efficiently transported to the cell surface. Two possible explanations may account for the lack of empty M3 on the cell surface. First, M3 may have lower affinity for β2m, and thus the M3/β2m heterodimer is less stable than empty class Ia heterodimers. Alternatively, M3 may be actively retained by an ER chaperone protein until acquiring a conformation that depends on peptide association. Active retention of empty class I molecules by tapasin has been demonstrated in insect cells (53). To determine the stability of M3 in vitro, we examined the dissociation of M3 heavy chain from β2m at a range of temperatures using a soluble "empty" M3–β2m complex produced in Drosophila cells. We found that the M3–β2m complex is more stable than that reported for H-2K^b and D^b (54; Chun, T. and C.-R. Wang, unpublished results). Unlike K^b and D^b, empty recombinant M3 molecules are stable at 37°C. Therefore, there may be selective pressure to retain empty M3 molecules intracellularly to prevent the expression of a pool of *N*-formylated peptide receptors at the cell surface. In contrast to our results, a prior study using an M3/L^d chimera has shown that M3/L^d can traffic to the cell surface at 27°C (55). The chimera was stabilized by addition of peptides at low temperature but not at 37°C, thus differing substantially from the behavior of the endogenous M3 molecule. M3 has a very short cytoplasmic tail (GER) that does not support a specific ER retention mechanism. Because the M3/L^d chimera contained the α3 domain and transmembrane region from L^d but was not retained like native M3, it is likely that the α3 domain and/or the transmembrane region of M3 may play an important role in M3 trafficking by mediating differential interaction with ER chaperone(s).

A given class Ia molecule may present 100–1,000 different peptide sequences on the surface of a cell (56); however, these peptides must share an allele-specific motif for that class I molecule. The range of peptides that bind M3 appears to be limited by *N*-formylation and hydrophobicity rather than specific sequence constraints. The endogenous supply of *N*-formylated peptide is limited by the amount gleaned from mitochondrial protein synthesis or supplied on degradation of mitochondria. The limited supply of mitochondrial peptide is compounded by the infrequent occurrence of sequences that bind M3, that is, only 2/13 possible peptides. It appears that M3 complexes with peptide are more stable than most class Ia–peptide complexes (57). This can counteract the extreme lack of peptide in maintaining some surface expression of the M3, which may be critical for the generation of T cell repertoire and/or maintenance of self-tolerance to M3.

The expression pattern of M3 has implications for its role in antigen presentation. In the uninfected condition, the basal surface level of M3 is minimal. Upon invasion by an intracellular pathogen such as *L. monocytogenes*, empty M3 is available for immediate transport to the cell surface in proportion to antigen supply, with little competition from endogenous peptides. It is possible that there may be as many or more M3–antigen complexes on the cell surface as there are class Ia molecules complexed with a specific peptide. Although the overall level of M3 is ∼20–100 times less than that of a class Ia molecule, the proportion of M3 molecules containing a particular antigenic peptide may be 100–1,000 times greater due to the lack of competition from self-peptides. In further experiments, the immature form of M3 could be induced to mature by addition of peptide at the 6 h time point of the chase (data not shown), demonstrating that the immature molecules are peptide receptive and reside in a compartment where peptide can be loaded. This allows M3 to be a potent antigen presentation moiety despite its low expression level. In support of this concept, M3-restricted T cells have been generated in vitro by stimulation with peptide-coated splenocytes (58), and we have successfully initiated M3-restricted T cell lines by immunization of mice with *N*-formyl peptide–coated APCs (Chun, T. and C.-R. Wang, unpublished results).

A significant aspect of our system is that the antigen-dependent behavior of M3 can be analyzed in the presence of intact antigen processing systems. High-affinity ligands for M3 can be easily screened by induction of surface expression; however, this induction can only be found on appropriate APCs. Had we relied on the frequently used RMA-S system, no induction of M3 would have been detected. As unconventional antigens and nonclassical molecules are increasingly found to play a role in specific immunity, it will become important not to assume that all cell types will give equal information about antigen presentation. The recent demonstration of the importance of exogenous antigen presentation by bone marrow–derived APCs in initiating the CTL response suggests that it is important to look for antigens accessible to MHC molecules in the most relevant cell type (59).

Due to the efficient presentation of exogenous peptide by M3 and its lack of polymorphism, an M3-based peptide vaccine may be a useful method for boosting specific immunity to intracellular pathogens in a broad range of recipients. Southern blot analysis has revealed no genetic human homologue of *M3* (4); however, lack of sequence homology does not preclude functional equivalence as seen in the case of the class Ib molecules Qa-1 and HLA-E, which bind leader peptides from class Ia molecules in mice and humans, respectively (60–62). The unusual cell biology of M3 described here may provide the basis for the search for functional homologues of M3 in humans.

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Address correspondence to Chyung-Ru Wang, Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, 924 E. 57th St., Chicago, IL 60637-5420. Phone: 773-702-4727; Fax: 773-702-1576; E-mail: cwang@midway.uchicago.edu

M. Mandal's current address is College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065.

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