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Tapasin Enhances Peptide-Induced Expression of H2-M3 Molecules, but Is Not Required for the Retention of Open Conformers

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H2-M3 is a class Ib MHC molecule that binds a highly restricted pool of peptides, resulting in its intracellular retention under normal conditions. However, addition of exogenous M3 ligands induces its escape from the endoplasmic reticulum (ER) and, ultimately, its expression at the cell surface. These features of M3 make it a powerful and novel model system to study the potentially interrelated functions of the ER-resident class I chaperone tapasin. The functions ascribed to tapasin include: 1) ER retention of peptide-empty class I molecules, 2) TAP stabilization resulting in increased peptide transport, 3) direct facilitation of peptide binding by class I, and 4) peptide editing. We report in this study that M3 is associated with the peptide-loading complex and that incubation of live cells with M3 ligands dramatically decreased this association. Furthermore, high levels of open conformers of M3 were efficiently retained intracellularly in tapasin-deficient cells, and addition of exogenous M3 ligands resulted in substantial surface induction that was enhanced by coexpression of either membrane-bound or soluble tapasin. Thus, in the case of M3, tapasin directly facilitates intracellular peptide binding, but is not required for intracellular retention of open conformers. As an alternative approach to define unique aspects of M3 biosynthesis, M3 was expressed in human cell lines that lack an M3 ortholog, but support expression of murine class Ia molecules. Unexpectedly, peptide-induced surface expression of M3 was observed in only one of two cell lines. These results demonstrate that M3 expression is dependent on a unique factor compared with class Ia molecules.


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these interrelated functions are primary vs secondary manifestations of tapasin’s interaction with class I.

In addition to the uncertainty regarding the precise functions of tapasin, significant differences have been noted in the relative levels of TAP/tapasin association between different class I molecules and allelic gene products (15). Although these differences in peptide-loading complex association may reflect structural differences between class I molecules, they may also reflect the fact that class I molecules have to compete for TAP/tapasin docking sites (16). Furthermore, there is clear evidence that the size of the peptide pool capable of binding to a given class I molecule is inversely correlated with the steady-state interaction of that molecule with the loading complex (17, 18). In addition to these apparent differences in the levels of class I association with TAP/tapasin, various class I molecules have been reported to differ in their dependency on tapasin for peptide binding. For example, certain HLA molecules were found to be relatively tapasin independent in their surface expression, whereas others were found to be relatively tapasin dependent (19). One confounding factor in determining the differences in the relative tapasin dependencies of various class I molecules is the differences between the respective peptide pools that are present for different molecules. Furthermore, as mentioned above, tapasin dependency could reflect either a direct role for tapasin in H chain folding, a requirement for tapasin-mediated ER retention of peptide-empty forms to achieve binding of high-affinity ligands, TAP stabilization, or a combination of these activities. In fact, different class I molecules could vary in their relative dependence on the different functions of tapasin.

To address some of these outstanding questions, we sought an experimental system that would permit a direct test of the putative tapasin functions independent of one another. In this regard, we considered it relevant to study the biosynthesis of the MHC class Ib molecule, H2-M3. M3 is a MHC-encoded (H2-M3) protein with its steady-state interactions with the mouse or human peptide-loading complex (17, 18). In addition to these apparent differences in the levels of class I association with TAP/tapasin, various class I molecules have been reported to differ in their dependency on tapasin for peptide binding. For example, certain HLA molecules were found to be relatively tapasin independent in their surface expression, whereas others were found to be relatively tapasin dependent (19). One confounding factor in determining the differences in the relative tapasin dependencies of various class I molecules is the differences between the respective peptide pools that are present for different molecules. Furthermore, as mentioned above, tapasin dependency could reflect either a direct role for tapasin in H chain folding, a requirement for tapasin-mediated ER retention of peptide-empty forms to achieve binding of high-affinity ligands, TAP stabilization, or a combination of these activities. In fact, different class I molecules could vary in their relative dependence on the different functions of tapasin.

To address some of these outstanding questions, we sought an experimental system that would permit a direct test of the putative tapasin functions independent of one another. In this regard, we considered it relevant to study the biosynthesis of the MHC class Ib molecule, H2-M3. M3 is a MHC-encoded (H2-M3) protein with the capacity to present relatively short peptide Ags (five to seven residues) that possess an N-terminal formyl moiety (20). Since the N termini of prokaryotic proteins initiate with formylmethionine residues, M3 molecules can function as restriction elements for CD8 T cell responses against intracellular bacteria. In contrast to class Ia molecules, M3 is expressed at very low levels on the surface of normal cells (21, 22) presumably due to the rarity of endogenous formylmethionine-containing peptides that could only arise from mitochondrial proteins. Despite the low level of constitutive surface expression of M3, Chiu et al. (22) demonstrated recently that there is an extensive pool of peptide-receptive M3 molecules in the ER that can be brought to the cell surface by culturing cells with exogenous M3 peptide ligands. This induction was largely TAP dependent, as are some (23, 24), but not all (25) T cell responses to M3. These properties of M3 present a unique opportunity to probe both 1) how a highly restricted pool of peptide ligands affects the steady-state association of a class I molecule with the peptide-loading complex, and 2) the role of tapasin in ER retention of an extensive pool of open class I molecules.

In this study, we used a novel epitope tag strategy that permitted a direct comparison of the chaperone interactions of M3 vs class Ia molecules, using the same anti-H chain mAb. We present three lines of evidence that M3 assembly is regulated differently than class Ia molecules. First, intrinsic properties of M3, independent of its restricted pool of endogenous ligands, determine its steady-state interactions with the mouse or human peptide-loading complex. Second, a novel factor absent in certain human cell lines is required for peptide-induced folding of M3, but not class Ia molecules. Third, peptide-empty forms of M3 are not present at the cell surface in tapasin-deficient cells, in contrast to class Ia molecules. However, tapasin can facilitate peptide binding in the ER by a mechanism independent of TAP stabilization and ER retention.

Materials and Methods

DNA clones and mutagenesis

To introduce the 64-3-7 epitope (which is specific for open forms of Ld and L1), a single substitution (R48Q) was required to convert the sequence of M3 surrounding the epitope to the sequence found in Ld (26). Site-directed mutagenesis of the wild-type M3 cDNA was performed using the Quik Change mutagenesis kit (Stratagene, La Jolla, CA) essentially as described (26). The following primers were used to introduce the R48Q mutation: forward primer, CCG AGG ATG GAA CTA GAA GCA CCA TGG ATG GAG AAG GAA AGA CC; antisense primer, GGT CTT TTC TTC ATC CAT GGT GCT TGA GGT TCC ATC TCT GG. DNA sequence analysis was used to confirm the presence of the mutation on the resulting epitope-tagged M3 cDNA (etM3). The etM3 cDNA was inserted into the mammalian expression vector pIRENeo (CLONTECH Laboratories, Palo Alto, CA) for all transfections. To construct the etM3/flagyellow fluorescent protein (YFP) chimera, the flag coding sequence (from pEYFP-C1; CLONTECH Laboratories) was appended to the cytoplasmic tail of etM3. The last residue in M3 was modied to alter the first residue of YFP by an ala-ser spacer. DNA sequence analysis veriied the correct sequence of the chimeric construct. This construct, as well as the full-length murine tapasin cDNA and the soluble tapasin cDNA were expressed from pIRENeo. A soluble murine tapasin construct was generated that includes residues 1–384 and, thus, lacks the transmembrane segment and cytoplasmic tail (10).

Cell lines and transfections

Mouse L cell transfectants of Ld (Ld4) have been described elsewhere (27). An etM3-expressing DAP-3 fibroblast line was generated by transfection of the parental line with the etM3 cDNA using Lipofectin (Life Technologies, Gaithersburg, MD). Stable expressers were selected with 0.6 mg/ml genetin (Life Technologies), identified by intracellular staining/flow cytometry (see below), and cloned by limiting dilution. etM3 transfectants of the human cervical carcinoma line, HeLa, were also generated using Lipofectin, followed by selection in 0.6 mg/ml genetin. The human B lymphoblastoid 721.221 cell line (28) was a gift from T. Spies (University of Washington, Seattle, WA). Transfection of these cells with etM3 was accomplished via electroporation using the Gene Pulser II System (Bio-Rad, Hercules, CA). Briefly, 8 X 10⁵ cells (at 10⁵ cells/ml in PBS) were mixed with 10 μg plasmid DNA and pulsed with 400 V at 950 μF. Stably expressing cells were selected with 0.6 mg/ml genetin and cloned by limiting dilution. Ld-expressing 721.221 lines have been described previously (12). The tapasin-deficient mouse ear fibroblast line (Tpn⁻/⁻ MEF) was derived from tapasin knockout mice (6), and a clonal line was obtained by limiting dilution for use in these studies (called Tpn⁻/⁻/MEF). Transient transfections were performed using FuGene 6 (Boehringer Mannheim, Indianapolis, IN). All cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% bovine calf serum or 10% FCS (for the Tpn⁻/⁻/MEF line; HyClone Laboratories, Logan, UT), 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1.25 mM HEPES, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (all from the Tissue Culture Support Center, Washington University School of Medicine). Where appropriate, genetin was added to a final concentration of 0.6 mg/ml.

Peptides

The M3-binding peptides used in this study were: LemA (MIHGWII) and F38 (FIMVIL) from Listeria monocytogenes (25, 29); ND1 (IMFFINIL) from murine NADH dehydrogenase (30); and T6 (IMFFLDA; T. Chun and C.-R. Wang, manuscript in preparation). The Ld peptide p29 (YPN VNIHNF) was used (31). All peptides were synthesized using F-moc solid-phase chemistry (32) on an Applied Biosystems (Foster City, CA) model 432A peptide synthesizer. Purity was assessed by reverse-phase HPLC and mass spectrometry. For all experiments described in this study, peptides were dissolved in DMSO at a concentration of 10–20 mM to create stock solutions from which appropriate dilutions were obtained. The M3 peptides require DMSO solubilization, and the class Ia peptides were also dissolved in DMSO for consistency.

Antibodies

mAb 64-3-7 (27) is speciﬁc for open forms of Ld and L1, and was used for immunoprecipitation, immunoblot, and flow cytometry. mAb 30-5-7 recognizes fully assembled, βm-associated Ld (33) and was used for immunoprecipitations. mAb130 (22) is speciﬁc for M3.
and was used for flow cytometry and immunoprecipitation. Marine TAP prec epitopes and blots were performed with rabbit antiserum 502 (34). The following Abs were used for immunoblot: rabbit anti-human TAP (35); rabbit anti-human tapasin (12); mAb BBM1 (anti-human β2m) (36); rabbit anti-murine tapasin (2668; M. R. Harris, L. Lybarger, Y. Y. L. Yu, N. B. Myers, and T. H. Hansen, unpublished observations); rabbit anti-murine β2m (1419; M. R. Harris, L. Lybarger, Y. Y. L. Yu, N. B. Myers, and T. H. Hansen, unpublished data); chicken anticalreticulin serum (Affinity BioReagents, Golden, CO); and rabbit anticalnexin (StressGen, Victoria, BC, Canada).

Flow cytometry

All flow cytometric analyses were performed using a FACSCalibur (BD Biosciences, San Jose, CA). Dead cells and debris were excluded from analysis on the basis of forward angle and side scatter light gating. A minimum of 10,000 gated events was collected for analysis. Data were analyzed using CellQuest software (BD Biosciences). For surface staining, ~5 x 10^5 cells per sample were incubated on ice in microtiter plates with culture supernatant from the appropriate hybridoma. After washing, fluorochrome-conjugated secondary Ab was added. To visualize mAb130 staining, FITC-labeled goat anti-Armenian hamster IgG was used (Jackson Immunoresearch, West Grove, PA) in most cases. For two-color analyses involving YFP fluorescence, mAb130 staining was visualized using PE-conjugated mouse anti-hamster IgG (BD PharMingen, San Diego, CA). YFP fluorescence was collected in the FITC channel, and electronic compensation, based on single-color controls, was applied to segregate the two signals. For 64-3-7 staining, FITC-conjugated goat anti-mouse IgG was used (ICN Pharmaceuticals, Costa Mesa, CA). For intracellular staining of 64-3-7, cells were fixed and permeabilized in PBS containing 1% paraformaldehyde, 1% BSA, and 0.5% saponin (all from Sigma, St. Louis, MO) for 20 min on ice. Permeabilized cells were stained in PBS containing 1% BSA and 0.5% saponin into which FITC-conjugated mouse anti-hamster IgG (BD PharMingen, San Diego, CA). After washing, fluorescein-conjugated secondary Ab was added. To visualize mAb130 staining, FITC-labeled goat anti-Armenian hamster IgG was used (Jackson Immunoresearch, West Grove, PA) in most cases. For two-color analyses involving YFP fluorescence, mAb130 staining was visualized using PE-conjugated mouse anti-hamster IgG (BD PharMingen, San Diego, CA). YFP fluorescence was collected in the FITC channel, and electronic compensation, based on single-color controls, was applied to segregate the two signals. For 64-3-7 staining, FITC-conjugated goat anti-mouse IgG was used (ICN Pharmaceuticals, Costa Mesa, CA). For intracellular staining of 64-3-7, cells were fixed and permeabilized in PBS containing 1% paraformaldehyde, 1% BSA, and 0.5% saponin (all from Sigma, St. Louis, MO) for 20 min on ice. Permeabilized cells were stained in PBS containing 1% BSA and 0.5% saponin into which FITC-conjugated 64-3-7 was diluted.

Immunoprecipitations and immunoblot

For communoprecipitations, cells, lysed in TBS plus 1% digitonin (Wako, Richmond, VA) that contained a saturating concentration of precipitating Ab. After lysis for 30 min on ice, postnuclear lysates were incubated with protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h. Beads were washed four times in TBS plus 0.1% digitonin, and bound proteins were eluted by boiling in SDS sample buffer. Immunoprecipitation was performed following SDS-PAGE separation of precipitated proteins and transfer to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked (1 h to overnight) with PBS plus 10% dried milk plus 0.05% Tween 20. Primary Abs were added and incubated for 1 h, followed by washing in PBS plus 0.05% Tween 20. As a second step, membranes were incubated for 1 h with biotin-conjugated anti-mouse or anti-rabbit IgG (Caltag Laboratories, South San Francisco, CA). After washing, HRP-conjugated streptavidin (Zymed, San Francisco, CA) was added for 1 h, followed by three washes. Specific proteins were visualized by chemiluminescence using the ECL system (Amersham, Boston, MA).

Results and Discussion

Experimental strategy

Studies of class Ia biosynthesis have been aided greatly by the availability of serological reagents that recognize H chain conformations that change during assembly (27, 37). For example, mAb 64-3-7 defines a unique conformation of Ld H chains that exists before peptide binding and after peptide dissociation, which we term the open conformation (26). Recently, we have demonstrated that this open conformation is defined primarily by the orientation of amino acid side chains at H chain residues 48 and 50 that undergo a secondary structure alteration coincident with high-affinity peptide binding. Class I H chain is in the open conformation when associated with the peptide-loading complex and, accordingly, 64-3-7 can be used to coprecipitate all known members of the loading complex. Our laboratory has developed a strategy by which the 64-3-7 epitope can be transfected with limited mutagenesis to other class I molecules and, thus, can be used to tag their respective open conformers (12, 26). We considered this a powerful tool to study the biosynthesis of M3 and how it compares with the biosynthesis of various class Ia molecules naturally expressing (e.g., Ld) or tagged with the same epitope. Using this approach, mAb 64-3-7 could be used to compare peptide-induced conformational changes in the ER and at the cell surface, of M3 vs class Ia molecules, and determine how these differences correlate with observed differences in their respective molecular chaperone interactions. In regard to studies of M3, this strategy was particularly efficacious owing to the comparisons that can be made with another mAb (130) described recently that recognizes M3 irrespective of its conformation (22).

Expression and detection of etM3

A point mutation was introduced into the M3 cDNA (R48Q) that converted its sequence to the 64-3-7 epitope sequence found in Ld. etM3 was transfected into a murine fibroblast line (DAP-3), and stable etM3-expressing clones were identified by intracellular staining with 64-3-7. Intracellular staining revealed a sizable pool of etM3 (Fig. 1A) and demonstrated that the epitope transfer was successful. This pool of 64-3-7+ etM3 molecules was markedly decreased when DAP-3.etM3 cells were cultured with an M3-binding peptide ligand, Fr38. Thus, intracellular 64-3-7+ etM3 molecules are highly peptide accessible. Either before or after peptide treatment, no 64-3-7+ etM3 molecules were detected at the cell surface. However, peptide treatment did result in the expression of high levels of etM3 detected with mAb130. It should be noted that mAb130 also detected a low level of endogenous, wild-type M3 molecules expressed on the surface of DAP-3 cells following induction (Fig. 1B, right panel). The fact that mAb130 staining was ~1.5 logs higher on peptide-treated DAP-3.etM3 vs DAP-3 demonstrates that the transfected etM3 gene is overexpressed relative to the endogenous M3 gene in this cell line. These findings indicate that open forms of etM3, as accurately defined by mAb 64-3-7, can be detected intracellulrly, but not at the cell surface either before...
or after peptide treatment. In support of this conclusion, pulse-chase experiments demonstrated that 64-3-7-reactive etM3 remained endo H sensitive (hence, ER resident) after peptide treatment (data not shown). By contrast, mAb130 reacts with M3 or etM3 surface molecules after peptide treatment. The fact that surface etM3 molecules were only detected with mAb130 and not mAb 64-3-7 provides compelling evidence that they are indeed peptide occupied.

**Peptide binding and dissociation from etM3 molecules**

The detection by mAb130 of high levels of peptide-induced etM3 on DAP-3.etM3 cells allowed us to compare the peptide-binding specificity of etM3 molecules with that previously determined with wild-type M3 molecules. Two high-affinity M3 ligands (LemA and Fr38), one intermediate binder (ND1), and one weak binder (TB6) were tested for their ability to induce surface expression of etM3. As shown in Fig. 2, the relative binding affinities of the different peptides for etM3 matched those that were reported for wild-type M3 using the same assay (22) (T. Chun and C.-R. Wang, manuscript in preparation). Therefore, transfer of the 64-3-7 epitope tag into M3 did not alter its peptide-binding specificity, a conclusion supported by our extensive studies of peptide binding to epitope-tagged forms of Kd, Kb, and HLA-B27 (Refs. 12 and 26, and data not shown).

Previous analyses of Ld, etKd, and etKb surface expression revealed that both open and folded conformers of each molecule are present under normal conditions (12). Such open forms of class Ia molecules most likely arise from peptide dissociation after ER egress, and thus reflect the relative peptide-binding ability of different class Ia alleles. However, some open forms may also arise by lack of complete ER retention of peptide-empty class I molecules (13). To determine whether peptide dissociation also results in conversion of folded molecules to the open form with M3, DAP-3.etM3 cells (after peptide induction) were treated briefly with low pH (38). As shown in Fig. 3, acid stripping resulted in a severe reduction in the detection of mAb130-reactive etM3 molecules and a sharp increase in the detection of 64-3-7-reactive etM3. These findings provide additional evidence that the 64-3-7 epitope tag can be used to identify etM3 molecules that are truly peptide empty. What is less clear is why such peptide-empty forms of etM3 were not also reactive with mAb130, since mAb130 detects M3 molecules by immunoblot, and can immunoprecipitate nascent M3 molecules derived from the ER that are capable of binding exogenous peptide (22). Perhaps the mAb130 epitope is especially acid sensitive, whereas the 64-3-7 epitope is not. In any case, the obvious differences in the reactivity of mAb 64-3-7 vs 130 with etM3 molecules before and after acid stripping demonstrated the utility on using both of these mAb to identify distinct conformational intermediates during M3 biosynthesis.

**Association of M3 with the peptide-loading complex**

Although expression of M3 is largely TAP dependent, it was not known whether M3 associates physically with the loading complex. To examine the potential interaction of M3 with members of the loading complex, coimmunoprecipitations were performed. etM3 was precipitated with 64-3-7, and the resulting precipitates were blotted to detect the presence of ER chaperones. As a control, the same precipitations were performed with Ld, which does associate with the loading complex. The results demonstrated that M3 readily associates with members of the loading complex, including TAP, tapasin, calreticulin, and B2m (Fig. 4). The levels of each of these molecules that coprecipitated were comparable with those obtained with Ld. These chaperones were not detected in precipitates of 30-5-7, an Ab that recognizes folded Ld only, as expected. In addition, the overall levels of open H chain were similar in Ld- and etM3-expressing cells. Therefore, comparable levels of Ld and etM3 H chains resulted in comparable levels of steady-state association of each of these molecules with the peptide-loading complex. It is noteworthy that these relative levels of loading complex association appear to be typical of class I molecules in transfected cells, as was shown by comparison of Ld with etKb and etKd (12). These results demonstrate that M3 is capable of association with the peptide-loading complex, and that the levels of association are similar between M3 and mouse class Ia molecules. The fact that the restricted peptide pool of M3 does not

![FIGURE 2.](http://www.jimmunol.org/) Surface induction of etM3 with various peptides. DAP-3.etM3 cells were incubated overnight with the indicated concentrations of four M3-binding peptides or with diluent only (no peptide). Surface staining with mAb130 was performed, and the mean fluorescence intensity of each sample is given.
bands. Two clonal lines of DAP-3.etM3 were analyzed in this experiment. Precipitations were performed with etM3- or Ld-expressing cells cultured with mAb 30-5-7 (specific for folded Ld) or 64-3-7 (open H chain). Precipitates were separated by SDS-PAGE and blotted using Abs specific for the indicated chaperone molecules. Chemiluminescence was used to visualize protein bands. Two clonal lines of DAP-3.etM3 were analyzed in this experiment.

result in significantly higher steady-state association of M3 with the loading complex, compared with class Ia molecules, suggests that intrinsic properties of M3 may influence its ability to bind TAP/tapasin complexes. This conclusion is strongly supported by studies of M3 expression in human cell lines (see below).

TAP release of M3 caused by exogenous peptide

M3 molecules are unique among TAP-associated molecules, since they are only expressed at a high level in the presence of exogenous peptides (21, 22) (Fig. 1). Binding of peptide to M3 appears to occur almost exclusively in the ER, as suggested by the loss of endo Hs forms of M3 H chains after peptide induction (Refs. 22 and 39, and data not shown). In contrast, class Ia molecules can undergo substantial peptide exchange at the cell surface (40, 41), and peptide binding to both endo Hs and endo Hf forms of class Ia molecules such as Ld or Kb has been observed when cells are incubated with exogenous ligands (40–42). This unique aspect of M3 raised the possibility that we could assay for peptide-induced release of M3 from TAP using live cells cultured with peptide. Initial demonstrations of peptide-induced release of class Ia molecules from TAP used permeabilized cells to better deliver peptides to the cytosol (35, 43). However, with M3, peptide uptake by live cells is clearly sufficient to promote a significant drop in the intracellular pool of peptide-accessible M3 molecules (22, 39, and Fig. 1). Thus, it was of interest to determine how peptide treatment of nonpermeabilized cells affected the steady-state levels of TAP-M3 association and how these findings correlate with values determined using fluorescence measurements of class I molecule (Ld and Kb) diffusion in membranes following peptide addition (47, 48). Thus, these findings suggest that peptide-binding affinity alone does not explain the incomplete dissociation of Ld from the loading complex, compared with M3. The disparity between Ld and M3 could be due to a unique uptake/delivery pathway for M3 ligands.

M3 expression in human cell lines

M3 appears to be unique to rodents, as no ortholog has been identified in humans, nor has a functional homologue been described. Therefore, if M3 requires any specialized biosynthetic processes not required for class Ia expression, then human cells may be incapable of supporting M3 expression. To test this, etM3 was expressed in two human cell lines, B lymphoblastoid cell line 721.221 (.221; 28) and HeLa (cervical carcinoma). Transfectants of each line were selected that contained sizable pools of intracellular open (64-3-7-positive) etM3, and neither line expressed M3 at the surface (Fig. 6). However, incubation of these cells with formylated peptide revealed a surprising difference between the two transfectants. Although HeLa.etM3 cells exhibited robust surface induction of M3 at levels similar to mouse L cells, .221.etM3 failed to express etM3 at the surface subsequent to peptide addition. Thus, human cells appear to possess all of the components necessary to support M3 expression, although the .221 line is defective in this process. It is noteworthy that murine class Ia genes can be expressed at high levels in .221 cells (12, 14), indicating an M3-specific defect with this cell line.

We next investigated whether a difference in chaperone associations between the HeLa.etM3 and .221.etM3 cells might account for their disparate M3 surface induction phenotypes. Coimmunoprecipitation experiments were conducted with these cell lines transient TAP-release effect on Ld. Multiple Ld-binding peptides have been tested at high concentrations, and all failed to induce near total release of Ld from TAP, including murine CMV, QL9, and p29, reported to be high-affinity ligands (Refs. 31, 44, and 45, and data not shown). Indeed, the experiment shown in this study is the highest level of TAP release of Ld that we have observed (~2-fold), and was obtained using a peptide, p29 (31), which has the highest affinity of any Ld ligands that we have tested (46). In addition, our biochemical data on the extent of Ld release from TAP correlate well with values determined using fluorescence measurements of class I molecule (Ld and Kb) diffusion in membranes following peptide addition (47, 48). Thus, these findings suggest that peptide-binding affinity alone does not explain the incomplete dissociation of Ld from the loading complex, compared with M3. The disparity between Ld and M3 could be due to a unique uptake/delivery pathway for M3 ligands.
wherein open etM3 was precipitated with 64-3-7 and the precipitates were subjected to immunoblot analysis to detect the presence of specific chaperones. For comparison, the same precipitations were performed with .221-Ld cells, and the results are shown in Fig. 7A. Although the 64-3-7-positive form of the mouse class Ia molecule (Ld) associated well with members of the human loading complex, M3 was associated relatively weakly with each chaperone, despite the similar levels of open etM3 present in all of the lines. This was the case with either the HeLa cells (which can express M3 at the surface) or .221 cells (which cannot express M3 at the surface). For example, the ratio of open H chain signal:TAP signal (determined by quantitation of the image shown in Fig. 7A) was 0.67 for Ld in .221 cells, 3.8 for etM3 in HeLa cells, and 2.6 for etM3 in .221 cells. Given the similar chaperone association profiles for M3 in the HeLa and .221 cells, the lack of induction in .221 cells cannot be explained by a failed association with the human loading complex. The finding that open forms of etM3 showed similar /H association levels to open Ld in .221 cells indicates that etM3 is not grossly misfolded in the .221 line.

An alternative explanation for the M3 inducibility difference between the two human cell lines is that the .221 cells, which are heavily mutagenized, are defective in the uptake/delivery of M3 peptide ligands. To examine this possibility, peptide-binding experiments were performed using cell lysates derived from HeLa-etM3 and .221.etM3. In this assay, peptide binding was assessed by conversion of etM3 to the folded (64-3-7-negative) form. Since peptide was added directly to lysates, it should have more direct access to etM3 and bypass at least certain steps in the normal uptake/delivery pathway. M3-binding peptide was added to digitonin lysates of HeLa-etM3, .221.etM3, and DAP-3.etM3. etM3 was then precipitated from the lysates with either 64-3-7 or mAb130 (Fig. 7B). Under these assay conditions, Fr38 peptide was able to induce substantial folding (loss of 64-3-7 reactivity) of etM3 in a concentration-dependent manner in lysates from HeLa and DAP-3. The levels of etM3 precipitated with mAb130 remained constant, indicating that this mAb130 reacts with M3 irrespective of its folding status, and provides clear evidence that peptide converts etM3 to a 64-3-7-negative form. By contrast, peptide had no effect on etM3 from the .221 lysate at the highest concentration tested. This finding predicts a defect in peptide acquisition by M3 in these cells that lies downstream of peptide entry/uptake from the culture medium. Although the nature of this defect is at present unknown, it is intriguing to speculate that it might involve a heat shock protein (HSP). HSPs have been recently implicated in peptide stabilization and delivery to MHC class I molecules (49). Perhaps a chemical property unique to M3 ligands, such as their shorter length or high degree of insolubility, might render them more dependent upon a particular HSP than class Ia peptide ligands. This may also help explain the dramatic TAP release of M3 caused by exogenous peptides, if a HSP capable of interacting with the loading complex was involved in the delivery of peptides to M3. Regardless of the exact nature of the defect in .221 cells, these observations revealed that a factor(s) dispensable for class Ia expression is required for M3 folding.

**FIGURE 6.** Expression of etM3 in human cell lines. etM3 transfectants of HeLa and lymphoblastoid cell line 721.221 were generated. Upper panels, Intracellular 64-3-7 staining of the parental cell lines (thin line) or etM3 transfectants (thick line) without any peptide addition. Lower panels, Surface mAb130 staining of etM3-expressing cells to which no peptide was added (thin line) or cells that were incubated overnight with 20 µM Fr38 peptide (thick line). Note: the HeLa transfectant is not clonal.

**FIGURE 7.** etM3 chaperone associations and peptide binding in human cell lines. A, Coimmunoprecipitations: digitonin lysates from the indicated cell lines were precipitated with mAb 30-5-7 (specific for folded Ld) or 64-3-7. Precipitates were separated by SDS-PAGE and blotted for the indicated ER chaperones. B, Peptide-induced folding of etM3 in vitro. Digitonin lysates from the indicated cell lines were incubated for 4 h on ice with various concentrations of Fr38. Each sample was then split into two equal aliquots, and one was precipitated with mAb130 and the other with 64-3-7. Precipitates were separated by SDS-PAGE and blotted for H chain using 64-3-7.
M3 expression in tapasin-deficient cells

The finding that etM3 was poorly associated with the loading complex in HeLa cells, yet was retained within the cell and could be surface induced with exogenous peptide, raised the possibility that association with the loading complex was not required for these processes. To test this hypothesis more rigorously, we examined M3 expression in a fibroblast cell line derived from tapasin-deficient mice (6). M3 represented a uniquely powerful system to test the ER retention function that has been ascribed to tapasin (10, 13, 14), since the available pool of endogenous ligands is so limited, resulting in an extensive intracellular pool of open M3 molecules. In the tapasin-deficient background, we could test whether tapasin was required for folding of M3 leading to surface induction, and whether open forms were efficiently retained within the cell.

To perform these analyses, we constructed an etM3/YFP chimera, with YFP linked to the cytoplasmic tail of etM3. Such fluorescent protein fusions have been made with class Ia molecules without adverse effects on their processing, expression, and association with the loading complex (47, 48). Indeed, the etM3/YFP chimeric molecule associates with the peptide-loading complex (data not shown), and its expression is strictly regulated by peptide availability, identical to native M3 (Fig. 8 and data not shown). The YFP tag permits the unequivocal identification of etM3-transfected cells by flow cytometry, and the surface expression of etM3 can be monitored simultaneously using mAb130 staining. The tapasin-deficient cell line (Tpn−/− 3.5) was transfected transiently with the etM3/YFP construct alone or cotransfected with a mouse tapasin (Tpn) expression vector, and the transfectants were subsequently incubated overnight with Fr38 peptide. After peptide incubation, cells were analyzed for YFP expression and mAb130 reactivity. Fig. 8 depicts the results from one representative experiment (of four), and Table I summarizes the data from all experiments. Since a fraction of the transfectants expressed the etM3/YFP chimera, the surface expression pattern of both the endogenous M3 and the transfected etM3 could be evaluated, by gating on the YFP− or YFP+ fractions, respectively. Furthermore, the intensity of the YFP fluorescence signal could be used to normalize the samples for transfection efficiency.

Table I. Effects of tapasin and soluble tapasin on peptide-induced M3 expression

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Expt.</th>
<th>M3-YFP only</th>
<th>M3-YFP + soluble tapasin</th>
<th>Mean Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>602</td>
<td>435</td>
<td>ND</td>
<td>YFP</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>610</td>
<td>842 (270%)</td>
<td>Surface M3</td>
</tr>
<tr>
<td>2</td>
<td>507</td>
<td>593</td>
<td>505</td>
<td>YFP</td>
</tr>
<tr>
<td></td>
<td>243</td>
<td>505</td>
<td>390</td>
<td>Surface M3</td>
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<td>3</td>
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<td>529</td>
<td>582</td>
<td>YFP</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>927</td>
<td>736</td>
<td>Surface M3</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>1041 (190%)</td>
<td>751 (140%)</td>
<td>Surface normalized</td>
</tr>
<tr>
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<td>512</td>
<td>559</td>
<td>YFP</td>
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<td>413</td>
<td>429</td>
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</tr>
<tr>
<td></td>
<td>208</td>
<td>570 (270%)</td>
<td>543 (260%)</td>
<td>Surface normalized</td>
</tr>
</tbody>
</table>

Data from four independent transient transfections of Tpn−/− cells. Data given are from peptide-induced samples as described in Fig. 8 legend. Experiment 4 corresponds to plots shown in Fig. 8.

Indicates the expression constructs used for transfection.

Mean fluorescence intensity values were obtained from flow cytometric analyses of transfectants.

Mean fluorescence intensity of YFP fluorescence, taken only from the YFP−positive fraction of the transfectants (see Fig. 8).

Surface M3 expression values normalized for YFP expression levels, a measure of transfection efficiency. YFP mean fluorescence intensity values for the tapasin cotransfections were normalized to YFP signals from the M3-YFP-only transfections in each experiment. This correction factor was then applied to the surface M3 expression values for the tapasin transfectants. The corrected surface M3 mean fluorescence intensity values are given, as the percent change (in parentheses) relative to the M3-YFP-only samples.

FIGURE 8. Transient expression of an etM3/YFP fusion protein in tapasin-deficient fibroblasts. Tapasin-deficient fibroblasts (Tpn−/− 3.5) were transfected transiently with an etM3/YFP chimeric construct alone, or cotransfected with this construct and either full-length or soluble murine tapasin constructs ∼48 h before flow cytometric analysis. A total of 20 μM Fr38 peptide or diluent alone (no peptide) was added to the appropriate samples ∼18 h before analysis. Cells were analyzed for surface etM3/YFP expression using mAb130 staining, and total etM3/YFP expression was monitored by YFP fluorescence. The DNA(s) used for transfection and the treatment of each sample is indicated. The numbers within the histograms represent the mean channel fluorescence of the mAb130 staining for the YFP− and YFP+ fractions of each sample. Note that the secondary Ab used to visualize mAb130 staining was PE conjugated, resulting in brighter staining of surface M3 than in Fig. 1, in which a FITC-conjugated Ab was used.
The analysis of M3 regulation in the TpnD cells revealed several striking findings. First, we observed that very low to undetectable levels of either endogenous M3 (YFP fraction) or transfected etM3 (YFP fraction) were present at the cell surface in the absence of exogenous peptide (Fig. 8). This was true even for the cells that expressed very high levels of transfected etM3/YFP (>1000-fold range of expression). These findings indicated that tapasin was not required for intracellular retention of M3, and are supported by studies of tapasin-deficient mice, in which M3 molecules remained endo H sensitive (hence, ER resident) in the absence of exogenous peptide.4 Another striking finding of this experiment was that both endogenous and transfected M3 could be induced at the cell surface by peptide in the TpnD cells. The level of induction of endogenous M3 was comparable (~5-fold) in these cells with that observed with wild-type fibroblasts under the same conditions (data not shown), and the transfected etM3 (YFP fraction) was induced to still higher levels. This demonstrated that tapasin was not required for intracellular retention or induced surface expression of M3 in this cell line. However, tapasin did enhance peptide-induced expression of M3, since cotransfection of tapasin with etM3 increased the surface induction of etM3 ~2 to ~3 fold (Fig. 8, middle panels, and Table I). Given the ER localization of tapasin, this provides further evidence that exogenous peptide binding by etM3 molecules occurs primarily within the cell, a finding strongly supported by data presented earlier (Figs. 1 and 5) and previous reports (22, 39).

Tapasin has been shown to stabilize the TAP heterodimer, leading to increased peptide transport (10), and fibroblasts from TpnD mice have a drastic TAP transport defect (6), presumably due to lower levels of TAP expression. To distinguish between the TAP-stabilizing effects of tapasin and a more direct role of tapasin in M3 expression, TpnD cells were transiently transfected with a soluble murine tapasin construct. Soluble tapasin (lacking the transmembrane and cytosolic portions) binds to class I H chain, but cannot bridge the H chain to TAP or stabilize the TAP heterodimer (10). Importantly, soluble tapasin also fails to retain class I molecules (slow the intracellular maturation kinetics) (10, 14). Analysis of cells that were cotransfected with the soluble tapasin and etM3/YFP constructs revealed that soluble tapasin was able to enhance M3 surface expression to levels similar to those observed with the full-length tapasin construct. Immunoblot analysis of the transfecteds revealed that both the full-length and soluble tapasin constructs were expressed at comparable levels (data not shown). These data support a direct role for tapasin in peptide-induced H chain folding independent of intracellular retention.

The efficient retention of M3 in the absence of tapasin contrasts with surface expression of Kb in insect cells. Using a serological approach similar to that used in this study, Schoenhaus et al. (13) showed that in the absence of tapasin, high levels of open Kb conformers were expressed at the cell surface. However, when tapasin and Kb were coexpressed, open Kb conformers were retained intracellularly. In addition, studies using mammalian cell lines have demonstrated that full-length tapasin decreases the maturation kinetics of class Ia molecules in tapasin-deficient .220 cells (10, 14). Thus, the lack of a requirement for tapasin in the ER retention of M3 appears to conflict with reported findings for class Ia molecules. Furthermore, using calnexin-deficient cells (51, 52), we have found that calnexin is also not required for efficient intracellular retention of M3 (data not shown). Collectively, our data provide compelling evidence that tapasin directly facilitates peptide binding to M3 by a process independent of the role of tapasin in ER retention and TAP stabilization. How might our findings with M3 relate to ER retention of class Ia molecules? It should be noted that evidence for the role of tapasin in ER retention is based on studies using invertebrate cells (13) or human .220 cells (which are heavily mutagenized) (10, 14). Thus, either of these cell types may lack additional quality control molecules that are normally present in mammalian cells. Furthermore, in tapasin-deficient mouse cells, class I molecules escape the ER in a peptide-accessible form, suggesting that they may contain suboptimal peptides (6). The apparent difference between M3 and class Ia molecules, in terms of a requirement for tapasin in the ER retention of open conformers, might be attributable to their respective peptide pools. More specifically, tapasin may not be required for ER retention of peptide-empty forms of either class Ia or M3 molecules. Rather, class Ia molecules that leave the ER in the absence of tapasin could be fully conformed with peptide, but these peptides are of generally lower quality/affinity, whereas endogenous ligands of sufficient affinity to induce folding and ER egress of M3 are lacking. In any case, our findings with soluble tapasin point to a direct role of tapasin in peptide binding to M3, and our findings with human .221 cells indicate that M3 expression requires a unique factor that is dispensable for the expression of class Ia molecules.

Acknowledgments
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References