Kb, Kd, and Ld Molecules Share Common Tapasin Dependencies as Determined Using a Novel Epitope Tag

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The endoplasmic reticulum protein tapasin is considered to be a class I-dedicated chaperone because it facilitates peptide loading by proposed mechanisms such as peptide editing, endoplasmic reticulum retention of nonpeptide-bound molecules, and/or localizing class I near the peptide source. Nonetheless, the primary functions of tapasin remain controversial as do the relative dependencies of different class I molecules on tapasin for optimal peptide loading and surface expression. Tapasin dependencies have been addressed in previous studies by transfecting different class I alleles into tapasin-deficient LCL721.220 cells and then monitoring surface expression and Ag presentation to T cells. Indeed, by these criteria, class I alleles have disparate tapasin-dependencies. In this study, we report a novel and more direct method of comparing tapasin dependency by monitoring the ratio of folded vs open forms of the different mouse class I heavy chains, Ld, Kd, and Kb. Furthermore, we determine the amount of de novo heavy chain synthesis required to attain comparable expression in the presence vs absence of tapasin. Our findings show that tapasin dramatically improves peptide loading of all three of these mouse molecules. The Journal of Immunology, 2000, 165: 5656–5663.

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recent studies have defined several molecular interactions that occur in the endoplasmic reticulum (ER) that potentially facilitate and may edit the peptide loading process (1). Furthermore, these molecular interactions combine to assure that only class I heavy (H) chains that are completely assembled with \( \beta_2 \)-microglobulin (\( \beta_2 \)-m) and peptide are allowed to egress from the ER and transit to the cell surface. Although several members of this class I peptide-loading complex have been identified, their selective roles and importance for different class I molecules remain controversial. Initially, nascent H chains are detected in association with calnexin, a lectin-like chaperone associated with various incompletely assembled oligomeric glycoproteins (2). After assembly with \( \beta_2 \)-m (3), class I/\( \beta_2 \)-m heterodimers then associate with calreticulin (4–6), another lectin-like, general chaperone for assembly of oligomeric glycoproteins (7). Subsequently, class I/\( \beta_2 \)-m heterodimers are detected in physical association with TAP (8–11), the class I-dedicated peptide transporter, as well as the MHC-encoded 48-kDa glycoprotein, tapasin (4, 12, 13). More recent studies have shown that the thiol-dependent reductase ERp57 is also a component of the peptide loading complex (14–16). Once the peptide binds, fully assembled class I molecules dissociate from the peptide-loading complex and transit to the cell surface.

Importance of tapasin in the expression of various human class I alleles is based largely on studies of tapasin-deficient LCL721.220 (.220) cells (4, 10, 12, 17, 18). Class I molecules in .220 cells are not detected in association with TAP. Furthermore, at least certain alleles were found to be more peptide accessible in cell lysates and had reduced surface expression. Although \( \beta_2 \)-m and TAP are expressed in .220 cells, no functional tapasin protein was detected. However, due to a single nucleotide change in the tapasin gene and a resulting frame shift, .220 cells express a small amount of a truncated tapasin protein missing the last 8 amino acids of the signal peptide and the first 49 amino acids of the N terminus (19). Expression of various human class I alleles in .220 cells suggested that different alleles may display different tapasin dependency for surface expression and Ag presentation (17). This result could imply that different alleles may bind to tapasin with different affinities. Alternatively, the available peptide pool capable of binding to each class I allele may influence its observed tapasin dependency. Thus, the nature of the reported differences in the expression of various human class I alleles in .220 cells and the implications of these differences on tapasin function are unclear.

The observation that the association of class I/\( \beta_2 \)-m complexes with TAP is dependent upon tapasin, suggested that tapasin might bridge class I with TAP (4). Thus one of the proposed functions of tapasin is to bring class I molecules into physical proximity with the peptide source, TAP (8–9). Although it remains to be proven, this physical association of class I with TAP could promote peptide binding, at least to certain class I alleles. Alternatively, tapasin appears to have chaperone functions that are independent of promoting physical association with TAP. For example, the removal of the transmembrane and cytoplasmic domains of tapasin resulted in a secreted molecule that no longer facilitated class I binding to TAP (20, 21). Interestingly, this truncated tapasin increased surface expression and Ag presentation of class I (20). From these findings it was concluded that the association of tapasin with class I was sufficient to facilitate class I folding. In another study, mouse
tapasin was shown in Drosophila cells to retain peptide-empty mouse Kb 

molecules in the absence of TAP (22). These authors concluded that tapasin increases the expression of fully assembled class I molecules by retaining empty class I molecules until they bind peptide. Furthermore, using another insect expression system (Lepidoptera), Lauvau et al. (23) concluded that tapasin facilitates assembly of peptide with class I independently of mediating their retention in the ER. Finally, other recent studies have suggested that tapasin may be involved in peptide editing of class I in a manner analogous to the role of DM with MHC class II molecules (24-26). Thus the potential functions of tapasin include: 1) localizing class I near the peptide supply, 2) facilitating peptide binding to class I and perhaps peptide editing, and 3) ER retention/release of class I upon peptide binding. Although there is little doubt that tapasin is a class I-dedicated chaperone, the relative importance of each of these alleged tapasin functions for different class I alleles remains to be elucidated.

In this study we compare the expression of three different mouse class I molecules Ld, Kb, and Kk, in tapasin-deficient .220 cells to that in tapasin-positive .221 cells. A novel strategy was employed whereby a serological epitope (64-3-7) specific for open forms of Ld was introduced into Kk and Kk molecules (27). This 64-3-7 epitope is located on a loop in the e1 domain that connects 6 strand with helical structure. Furthermore, it has been speculated that the region defining the 64-3-7 epitope constitutes a hinge region that changes conformation when peptide binds (27). The transfer of the 64-3-7 epitope to other class I molecules allows their respective open forms to be compared using the same mAb, thus facilitating analytical comparisons between expression of these three mouse class I molecules in the presence or absence of functional tapasin. Our findings clearly show that tapasin greatly facilitates intracellular peptide binding to Ld, Kk and Kk molecules, since new synthesis of 8- to 12-fold more class I was required in the absence of tapasin to attain comparable surface expression of each of these alleles. In addition a higher percentage of each of these class I molecules was detected in the open conformation at the cell surface when expressed in tapasin-deficient .220 cells compared with tapasin-positive .221 cells.

Materials and Methods

Cell lines, mutagenesis, and transfection

The .220 cell line is a human B lymphoblastoid cell line that does not express tapasin (10) and was kindly provided by Dr. Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA). The LCL721.221 (.221) cell line is a closely related cell line that does express tapasin (28, 29). L-Ld cells were made by introducing the Ld gene into Lk- DAP-3 fibroblast (30). To produce a site-directed mutant of Kk expressing the 64-3-7 epitope, a Kk cDNA was kindly provided by Dr. Larry Pease (Mayo Clinic, Rochester, MN). It was subcloned into the mammalian expression vector RSV5.neo (31). Site-directed mutagenesis was performed using the Quik Change mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. The synthetic oligodeoxyleucinolactones used for the reaction were as the forward primer: 5'-GAAGAAAGTTCGAGATATGCAACGGCAGGCTGGATGGAAGGGGCCGC-3' and the reverse primer: 5'-GCCCTCTGCTCTCCAACGCCGCACGGCTGGATGGAAGGGGCCGC-3'. The mutagenic oligonucleotide was transcribed with the cDNAs using LipoFectin and selected in 0.6-1.0 mg/ml geneticin, both from Life Technologies (Gaithersburg, MD), and transfected into HEK293 cells. After incubation for 30 min on ice, nuclei were removed by centrifugation, and lysates were incubated with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) for 2 h, washed three times with 0.5 M Tris (pH 8.0) in 0.1% Triton X-100, and 0.5 M NaCl. The bead suspension was then mixed with 10-20 mg/ml of antibody, washed three times in PBS containing 0.1% Triton X-100, and lysed with 0.1% Triton X-100. The lysates were transferred to Immobilon membranes (Millipore, Bedford, MA). After overnight block, membranes were incubated in a dilution of Ab for 2 h, washed three times with PBS, 0.05% Tween 20, and incubated for 1 h with biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag Laboratories, San Francisco, CA). Following three washes with PBS/0.05% Tween 20, membranes were incubated in 1 h with streptavidin-conjugated HRP (Zymed, San Francisco, CA), washed three times with PBS/0.3% Tween 20, and incubated with enhanced chemiluminescent reagents (Amersham).

3Cr release assay

The Ld-alloreactive, pCa-specific clone 2C was a generous gift from Herman Eisen (Massachusetts Institute of Technology, Cambridge, MA). 2C was maintained in 24-well plates at 5 × 105 cells/well and stimulated weekly with 5 × 105 irradiated (2000 rad) BALB/c spleen cells in 0.2 ml culture supernatant containing mAb 64-3-7 or control mAbs. L-Ld cells were transferred to Immobilon membranes (Millipore, Bedford, MA). After overnight block, membranes were incubated in a dilution of Ab for 2 h, washed three times with PBS, 0.05% Tween 20, and incubated for 1 h with biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag Laboratories, San Francisco, CA). Following three washes with PBS/0.05% Tween 20, membranes were incubated in 1 h with streptavidin-conjugated HRP (Zymed, San Francisco, CA), washed three times with PBS/0.3% Tween 20, and incubated with enhanced chemiluminescent reagents (Amersham).

Results and Discussion

To better quantify the effect that tapasin has on the expression of different mouse class I molecules, we introduced an epitope tag (et) that is specific for open forms of Ld into Kk and Kk molecules (27). We then expressed Ld or epitope-tagged Kk and Kk in tapasin-deficient .220 cells and tapasin-positive .221 cells. This approach provided an analytical determination of the quality of class I folding and thus a more direct assessment of the role of tapasin in expression of different class I molecules.
Ld molecules expressed at high levels in the absence of tapasin have a higher percentage of peptide-free forms at the cell surface

Studies of the synthesis and chaperone association of the Ld allele have been greatly facilitated by the use of mAb 64-3-7 that is specific for open forms (33, 34). For example, addition of specific peptide ligands to cell lysates showed that 64-3-7+ Ld conformers are precursors of folded Ld molecules as detected by a mAb such as 30-5-7. Furthermore, folded Ld molecules at the cell surface were found to transit through a 64-3-7+ conformation after peptide dissociation (34), and acid stripping results in the emergence of 64-3-7+ Ld molecules (Y.Y.L.Y., unpublished observations). Thus, mAb 64-3-7 is clearly capable of detecting Ld molecules that are truly empty, although it might also detect Ld molecules with ligands incapable of inducing a completely folded, native conformation. In any case, 64-3-7 can specifically detect both open forms of nascent Ld molecules awaiting peptide as well as open forms of Ld at the surface arising after peptide dissociation (34). Given these findings, we considered the ability to discriminate open from folded Ld molecules a novel approach to assess the role of tapasin in the expression of this allele.

Stable transfectants of Ld expressed in .220 and .221 cells were selected that showed comparable surface expression as determined using mAb 30-5-7 that detects folded/assembled Ld molecules. The fact the we were able to detect matched .220/.221 cell lines demonstrated that tapasin was not an absolute requirement for Ld surface expression. Furthermore, these matched cell lines were found to be comparable in their presentation of the endogenous peptide p2Ca to Ld-reactive 2C T cells (Fig. 1). Thus, based on criteria used in previous studies, namely surface expression and Ag presentation, Ld would be considered tapasin-independent. However, when the amount of open Ld (64-3-7+) was compared on .220 vs .221 cells, a striking difference was observed. As shown in Fig. 2A, .220-Ld cells express more open Ld than folded Ld at the cell surface, whereas .221-Ld cells expressed more folded Ld than open Ld. Indeed, the percentage of open Ld molecules is about 2-fold higher on .220-Ld cells compared with .221-Ld cells. We have compared the ratio of open to folded forms of Ld in independent assays of the same cell lines and found them to be remarkably constant. Furthermore, the ratios of independently derived .220-Ld or .221-Ld cell lines were found to be very similar (data not shown). When the ratios of open to folded forms on .220-Ld vs .221-Ld were compared, they were found to be significantly different (% open/total 0.5, Tukey’s multiple comparison test).

**FIGURE 1.** Tapasin-deficient .220-Ld cells and tapasin-positive .221-Ld cells are comparably lysed by Ld alloreactive 2C T cells. 2C T cells detect an endogenously processed peptide derived from either mouse or human α-keto glutarate dehydrogenase when bound to mouse Ld (48). Thus the middle two lines in the figure represent endogenous Ag presentation by .220-Ld and .221-Ld to 2C T cells. As a positive control for lysis, target cells were incubated with the 1 μM QL9 peptide (QLSPFPFDL), a strong agonist (upper two lines). As a negative control nontransfected, peptide-fed .220 and .221 cells were tested as targets (lower two lines).

**FIGURE 2.** Comparison of open vs folded conformers of Ld, epitope-tagged (et) Kd and Kb on the cell surface of tapasin+ and tapasin-deficient cells. For these comparisons each mouse class I molecule was expressed in tapasin+ (mouse L cells and human .221 cells (28) as well as tapasin-deficient human .220 cells (10)). Open forms (Ⅰ) of Ld, etKd, and etKb were detected using mAb 64-3-7 (33, 34), whereas folded forms (Ⅲ) were detected using mAb 30-5-7 (49), SF1-1.1.1 (40), and B8-24-3 (41) respectively. Surface expression was quantified by FACS, and relative linear fluorescence is indicated by the height of the bar. The percentile below each pair of bars depicting folded vs open forms for a given cell line is the percent open forms (open/open + folded × 100). Surface class I expression by each of these cell lines has been tested numerous times and the ratio of open to folded forms for each cell line has remained constant.

**TABLE 1.** Tapasin modulates peptide-free forms of Ld at the cell surface. The percentage of peptide-free forms at the cell surface was determined by addition of specific peptide ligands to cell lysates. Tapasin+ .220-Ld cells express more peptide-free Ld than tapasin-deficient .220-Ld, whereas .221-Ld cells express more peptide-free Ld than .221-Ld. Tapasin modulates peptide-free forms of Ld at the cell surface.
Thus measurements of the amount of open to folded class I molecules on the cell surface is a reliable method to analytically compare the quality of class I expression.

The empty forms of Ld at the surface could have arisen by either ER escape before peptide loading or melting due to dissociation of weakly bound peptides (34–36). Therefore, the finding of more open forms of Ld on the surface of .220 vs .221 cells implies that Ld molecules expressed in the absence of functional tapasin have impaired ER retention and/or peptide loading. Importantly, the ratio of folded to open Ld expressed by human .221 cells was comparable to Ld expressed in mouse L cells (Fig. 2). Thus Ld appears to productively interact with either human or mouse tapasin to increase the fraction of Ld molecules expressed in the folded conformation.

Surface expression of open forms of Ld on both .221 and .220 cells is ablated by culture with exogenous peptides

To compare the peptide accessibility of open forms of Ld molecule expressed in the presence or absence of tapasin, .220-Ld and .221-Ld cells were cultured overnight with the Ld-binding CMV peptide, YPH FMPTNL (37). As shown in the top panels of Fig. 3A, the level of open Ld as detected by 64-3-7 was sharply reduced on both .221-Ld and .220-Ld cells. Published studies suggest that exogenous peptide can either bind peptide-empty surface class I molecules (33) or, alternatively, peptide can be transported into cells and bind nascent class I molecules in the ER (38). However, regardless of mechanism, the reduction of the expression of 64-3-7+ Ld molecules in the presence of peptide provides further evidence that 6-3-7+ molecules are indeed peptide empty. Furthermore, these findings show that open Ld forms expressed in the presence or absence of tapasin can be eliminated by incubation with exogenous peptide. In summary, these comparisons of .220-Ld and .221-Ld demonstrate that high levels of surface Ld expression can be achieved in the absence of tapasin, but such molecules display a higher percentage of open forms than Ld molecules expressed in the presence of mouse or human tapasin.

Epitope-tagging peptide open forms of Kd and Kb

To determine whether our findings regarding the tapasin dependencies of Ld could be extended to additional mouse class I alleles, we introduced the 64-3-7 epitope into Kd and Kb molecules. In a previous study we determined that 64-3-7 recognition of Ld was determined largely by glutamine at position 48 and proline at position 50 (27). Furthermore, peptide inhibition of 64-3-7 binding to Ld showed that the epitope was contained within the 21 amino acid sequence corresponding to residues 35–55. To further define the 64-3-7 epitope, length variants were tested and a 10-mer peptide (residues 46–55) was found to be as potent as the 21-mer (data not shown). We next truncated the 10-mer peptide at both termini and determined that the 7-mer peptide (EPQAPWM) was the minimal length peptide that gives maximal inhibition of 64-3-7 binding to Ld (Fig. 4A). To define critical residues within this 7-mer peptide, peptides with alanine substitutions were tested. As shown in Fig. 4B, residues at positions 48, 50, and 51 were critical for 64-3-7 inhibition. Within the 7-mer sequence comprising the 64-3-7 epitope, Kd only differs from Ld by the single amino acid R vs P at residue 48, and Kb only differs by the two amino acids R vs Q at 48 and R vs P at 50 (Fig. 5, top left panel). Thus, to transfer the 64-3-7 epitope to Kb, the double substitution R48Q,R50P was introduced (previously shown in Ref. 27, and shown in this study for comparison) and, to transfer the epitope to Kb, the double substitution of R48Q,R50P was introduced (Fig. 5, bottom panels). As shown in Fig. 5, these respective substitutions rendered a subset of the Kd and Kb molecules positive with mAb 64-3-7 when expressed in Ld (Fig. 4).

![FIGURE 3.](http://www.jimmunol.org/) Open forms of Ld, etKd, and etKb are reduced or eliminated on the surface of .221 cells and .220 cells. Cells were incubated overnight with the indicated concentration of peptide and open forms were monitored using mAb 64-3-7. A, Cells expressing Ld (top panels) were incubated with the CMV peptide (a known Ld ligand; Ref. 37) or control peptide CW3 that does not bind to Ld. Cells expressing etKd (middle panels) were incubated the CW3 peptide (a known Kd ligand; Ref. 43) or control peptide NP that does not bind to Kd. Cells expressing etKb (bottom panels) were incubated with the OVA peptide (Ref. 44; a known Kb ligand) or control peptide CW3 that does not bind to Kb. To facilitate comparisons the scale of the panels is different and reflective of more open forms being expressed by .220 cells than .221 cells. These findings were replicated in three or more similar experiments. B, .221.etKb (upper panel) and .220.etKb (lower panel) were incubated with the indicated concentration of SIINFEKL. Data is shown as the percent of open forms (open/open + folded × 100).
cells. These epitope-tagged forms, subsequently referred to as etK\textsubscript{d} and etK\textsubscript{b}, were tested extensively for peptide binding and T cell recognition and were found to be indistinguishable from wild type molecules (Ref. 27 and our unpublished data). Thus epitope transfer did not interfere with peptide binding or T cell interaction, a finding consistent with its location on the 3D structure of the folded class I molecule (27). Indeed, residues 48 and 50 are on a loop in the \(\alpha_1\) domain that connects the last \(\beta\) strand with the beginning of the \(\alpha\) helix. These residues point out and away from the peptide binding groove of the folded class I molecule, and it has been proposed that this region displays conformational flexibility when peptide binds (27, 39). In any case, epitope tagging offers a unique opportunity to identify open forms of K\textsubscript{d} and K\textsubscript{b} molecules, and better determine the role of tapasin in their expression.

Surface K\textsubscript{d} and K\textsubscript{b} molecules expressed at high levels on .220 cells have a higher percentage of peptide-free forms

To determine the role of tapasin in the expression of K\textsubscript{d} and K\textsubscript{b} molecules, epitope-tagged forms of these class I alleles were expressed in .221 and .220 cells lines. As shown in the middle panel of Fig. 2, a matched pair of .221-etK\textsubscript{d} and .220-etK\textsubscript{d} cells that have about the same level of expression of folded K\textsubscript{d} as detected by mAb SF1–1.1.1 (40) were selected for comparison. Similarly, a matched pair of .221-etK\textsubscript{b} and .220-etK\textsubscript{b} cells that have about the same level of expression of folded K\textsubscript{b} as detected with mAb B8-24-3 (41) were selected for comparison (Fig. 2, right panel). Thus, tapasin is clearly not an absolute requirement for the expression of folded K\textsubscript{d} or K\textsubscript{b} molecules. It is important to note that K\textsubscript{d} and K\textsubscript{b} molecules expressed on L cells have about the same ratio of folded to open forms as K\textsubscript{d} and K\textsubscript{b} molecules expressed respectively on .221 cells (Fig. 2). This result suggests that K\textsubscript{d} and K\textsubscript{b} alleles can functionally interact with either mouse or human peptide-loading complex (i.e., tapasin, calreticulin, and Erp57). By contrast K\textsubscript{d} and K\textsubscript{b} molecules expressed in the .220 cells showed about 2- to 3-fold more empty forms, compared with K\textsubscript{d} and K\textsubscript{b} respectively expressed in .221 cells. Therefore, the expression of open forms of L\textsubscript{d}, K\textsubscript{d}, and K\textsubscript{b} is increased about two to three times in the absence of functional tapasin, thus defining a common tapasin dependency of these three mouse class I alleles. However, interestingly, there were significantly fewer open forms of K\textsubscript{d} and K\textsubscript{b} than L\textsubscript{d} in either the presence or absence of tapasin (Fig. 2). This higher level of open L\textsubscript{d} forms is consistent with the relatively weak peptide binding characteristic of this class I molecule (42).

Surface expression of open forms of K\textsubscript{d} and K\textsubscript{b} on both .221 and .220 cells is reduced by culture with exogenous peptide

To compare the peptide accessibility of open forms of surface K\textsubscript{d} and K\textsubscript{b} molecules generated in the presence or absence of tapasin,
transfected cells were cultured overnight in the presence of known peptide ligands. For the K\textsuperscript{b} allele we used the CW3 peptide RYLKNGKETL (43) as a known K\textsuperscript{b} ligand, and a length matched control peptide YASNENMETM (NP) as a non-K\textsuperscript{b} binder. As shown in the middle panels of Fig. 3A, culture of either .221-etK\textsuperscript{b} or .220-etK\textsuperscript{b} cells overnight with exogenous CW3 peptide resulted in a peptide-specific, dose-dependent drop in 64-3-7 expression. For the K\textsuperscript{b} molecule we used the OVA peptide SIINFEKL (44) as a positive control and the CW3 peptide as a negative control. As shown in the bottom panels of Fig. 3A, overnight incubation with peptide resulted in the dose-dependent decrease in open forms of etK\textsuperscript{b} as detected with mAb 64-3-7. However, this decrease was complete with .221-etK\textsuperscript{b} cells, but less pronounced with .220-etK\textsuperscript{b} cells. To extend these findings, .220-etK\textsuperscript{b} and .221-etK\textsuperscript{b} cells were treated with a wider range of OVA peptide concentrations up to 500 μM. As shown in Fig. 3B treatment with exogenous SIINFEKL resulted in complete elimination of open forms of etK\textsuperscript{b} on .211 cells. By contrast about 1/3 of the open forms of etK\textsuperscript{b} on .220 cells remained after treatment with high concentrations of OVA peptide. Thus a fraction of the open forms of surface K\textsuperscript{b} molecules are more refractory to peptide binding when expressed in the absence vs presence of tapasin.

The relative refractory nature to exogenous peptide of etK\textsuperscript{b} molecules synthesized in the absence of tapasin is intriguing. We know epitope tagging K\textsuperscript{b} does not influence its ability to bind SIINFEKL or other known K\textsuperscript{b} ligands (e.g., Fig. 3B, upper panel and data not shown). Furthermore, etK\textsuperscript{b}-SIINFEKL complexes were found to stimulate a T cell hybridoma (not shown). Thus, the relative refractory nature of etK\textsuperscript{b} molecules synthesized in the absence of tapasin does not reflect aberrant peptide binding. Alternatively, this refractoriness may reflect a unique structural feature of K\textsuperscript{b} or the manner by which it interacts with the human proteins in LCL721-derived cell lines (i.e., .220 and .221). Indeed, the high level of expression of K\textsuperscript{b} in human TAP-deficient T2 cells and not mouse TAP-deficient RMA-S cells has been proposed to result from the high affinity of K\textsuperscript{b} for human vs mouse β\textsubscript{2}m (45). The proposed model was that a higher affinity interaction with human β\textsubscript{2}m could help K\textsuperscript{b} better form stable complexes with peptides and thus attain a higher level of surface expression. However, it should be mentioned that L\textsuperscript{d} and K\textsuperscript{d} have also been reported to bind human β\textsubscript{2}m better than mouse β\textsubscript{2}m (46). Thus, the refractoriness of open forms of K\textsuperscript{b} to bind exogenous peptide, relative to L\textsuperscript{d} and K\textsuperscript{d} (Fig. 3), cannot easily be explained by it having a higher affinity for human vs mouse β\textsubscript{2}m. Furthermore, it warrants noting that all three of these mouse class I molecules had the very similar percentage of open forms when each was respectively expressed on the surface of L cells (mouse β\textsubscript{2}m\textsuperscript{+}) vs .221 cells (human β\textsubscript{2}m\textsuperscript{+}; Fig. 2). Thus, using the approach reported in this study, we detected no differences in the expression of L\textsuperscript{d}, K\textsuperscript{d}, or K\textsuperscript{b} in the presence of mouse vs human β\textsubscript{2}m.

**Comparable surface expression of L\textsuperscript{d}, K\textsuperscript{d}, or K\textsuperscript{b} in the absence of tapasin requires strikingly more newly synthesized H chains**

The above findings demonstrate that L\textsuperscript{d}, K\textsuperscript{d}, and K\textsuperscript{b} can be expressed at high levels on .220 cells, implying there is no strict requirement for tapasin for each of these alleles. However, to compare the efficiency of expression of these mouse class I molecules in the presence or absence of tapasin, we quantified the amount of newly synthesized class I molecules in each pair of cell lines matched to have comparable levels of surface expression, i.e., .221-L\textsuperscript{d} vs .220-L\textsuperscript{d}, .221-etK\textsuperscript{b} vs .220-etK\textsuperscript{b}, and .221-etK\textsuperscript{d} vs .220-etK\textsuperscript{d}. To compare the levels of newly synthesized class I molecules, cells were pulse labeled for 5 min and precipitated with mAb 64-3-7. Labeled H chain bands were resolved by SDS-PAGE and precipitin bands were then quantified by densitometry. For each mouse class I molecule studied, the .220 partner cell line had about 6–12 times the level of newly synthesized H chains compared with the .221 partner (Fig. 6, top panels). The implication of these findings is that comparable surface expression of L\textsuperscript{d}, K\textsuperscript{d}, or K\textsuperscript{b} requires 6- to 12-fold higher de novo H chain synthesis. This observation thus provided evidence using mammalian cells that tapasin greatly facilitates the assembly of these three mouse class I alleles. To estimate the steady-state levels of open H chains in each of the matched cell lines, mAb 64-3-7 was used to stain cells intracellularly and whole cell lysates were precipitated and Western blotted with mAb 64-3-7. In both assays we estimated the steady-state level of open forms of each allele was 2- to 3-fold higher in .220 vs .221 cells. Thus, the relative difference in the steady-state level of opens forms on the matched .220/221 appears to closely reflect what is on the cell surface (i.e., 2–3 to 1). These combined findings imply that to achieve comparable surface expression of L\textsuperscript{d}, K\textsuperscript{d}, or K\textsuperscript{b} in the absence of tapasin, more H chains need to be synthesized, and many of these are rapidly turned over.

**46-3-7** forms of L\textsuperscript{d}, K\textsuperscript{d}, and K\textsuperscript{b} are detected in association with TAP, but only in the presence of tapasin

Consistent with our previous findings with L\textsuperscript{d} (11) and etK\textsuperscript{d} (27), 64-3-7 etK\textsuperscript{b} molecules were detected in association with TAP (Fig. 6, middle panels). Furthermore, association of each of these mouse class I molecules with TAP is dependent upon tapasin. Indeed this tapasin-dependency is rather striking. Despite the higher levels of H chain synthesized by each .220-transfected cell line compared with its matched .221-transfected cell line, class I molecules only displayed prominent TAP association in the presence of functional tapasin (Fig. 6). The implication of the combined findings in this figure is that association of each of these mouse class I alleles with TAP/tapasin facilitates peptide binding and surface expression of fully assembled class I molecules. In support of this conclusion, each of these three mouse class I molecules displayed a higher rate of surface turnover, when expressed in the absence of tapasin (.220 cells) compared with their expression in the presence of tapasin (.221 cells) (data not shown).

Pepptide preferentially folds mouse class I molecules in association with TAP/tapasin in cell lysates

For this analysis we initially used L-etK\textsuperscript{b} cells. L-etK\textsuperscript{b} cells were metabolically labeled for 10 min and lysates were incubated with...
the OVA peptide or the non-K\textsuperscript{b} binding peptide, CW3. As shown in Fig. 7A, mAb 64-3-7 precipitated substantial levels of etK\textsuperscript{b} molecules, consistent with its detection of nascent class I molecules awaiting peptide. Furthermore, the addition of OVA peptide led to a modest (maximal 25% by densitometry) increase in the detection of folded etK\textsuperscript{b} molecules as detected with mAb B8-24-3. A commensurate loss of 64-3-7\textsuperscript{b} K\textsuperscript{b} was detected with addition of OVA peptide (as determined by densitometry of a significantly lighter exposure than the autoradiograph shown in Fig. 7A). Interestingly, the \(\beta\text{m}\)-associated 64-3-7\textsuperscript{b} etK\textsuperscript{b} molecules disappeared upon the addition of peptide, demonstrating that peptide was preferentially folding \(\beta\text{m}\)-associated K\textsuperscript{b} molecules. Immune precipitates of open and folded etK\textsuperscript{b} molecules were also tested for TAP association by Western blotting. OVA peptide eliminated etK\textsuperscript{b} molecules associated with TAP (Fig. 7A, lower panel) and tapasin (data not shown). This result implied that OVA peptide was preferentially binding to \(\beta\text{m}\)-assembled, peptide-empty forms of K\textsuperscript{b} in physical association with TAP/tapasin. Similar findings were also obtained with .221-etK\textsuperscript{b} cells (data not shown), demonstrating that mouse vs human tapasin functioned similarly in this assay. Using a reciprocal approach peptide was added to cell lysates, TAP was precipitated and etK\textsuperscript{b} molecules were blotted with 64-3-7. As shown in Fig. 7B, OVA peptide induced a dose-dependent release of K\textsuperscript{b} from TAP. Thus, in cell lysates, peptide preferentially binds K\textsuperscript{b} molecules assembled with \(\beta\text{m}\) and induces their release from association with TAP. Furthermore, as shown in Fig. 7C peptide-induced folding of etK\textsuperscript{b} was significantly less efficient in tapasin-deficient .220 cell lysates compared with tapasin-positive .221 cell lysates. These findings thus strongly support the direct involvement of tapasin/TAP in facilitating peptide binding to class I molecules as previously suggested using disparate approaches (47). Furthermore, these findings provide additional evidence that 64-3-7\textsuperscript{b} forms of class I are peptide receptive while associated with the peptide-loading complex, thus further highlighting the utility of the 64-3-7 epitope.

In summary, 6- to 12-fold more de novo synthesis is required in the absence of tapasin to achieve comparable surface expression of Ld, K\textsuperscript{d}, or K\textsuperscript{b}. Based on this observation we would predict that in tapasin-deficient cells with normal haploid/diplod class I expression, all three of these class I alleles will exhibit severely reduced surface expression. Furthermore, we show in this study that all three of these mouse alleles displayed 2- to 3-fold more open forms at the cell surface in .220 vs .221 cell lines with matched surface expression. Thus it is tempting to generalize from these observations and conclude that many, if not most, class I molecules will be found to be comparably tapasin-dependent when the efficiency and quality of class I expression is evaluated in this manner.

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References


FIGURE 7. A. Peptide preferentially binds nascent K\textsuperscript{b} molecules in association with TAP. L-etK\textsuperscript{b} cells were labeled with [\(^{35}\)S]met for 5–10 min and were then lysed in digitonin. Aliquots of the lysate were treated with the indicated concentrations of known K\textsuperscript{b} ligand OVA (44) or negative control CW3 (43) or p29 (50) peptides for 2 h at 4°C. Samples were then precipitated with either B8-24-3 that detects folded etK\textsuperscript{b} molecules or mAb 64-3-7 that detects open etK\textsuperscript{b} molecules. Autoradiographs of the samples analyzed by 4–20% SDS-PAGE are shown in the upper panel. The lower panel shows a Western blot using an anti-TAP Ab. Arrows indicate lanes where the OVA peptide resulted in the complete loss of 64-3-7\textsuperscript{b} etK\textsuperscript{b} and the elimination of TAP association. B. Peptide induces the specific release of K\textsuperscript{b} molecules from TAP as demonstrated by Western blotting using mAb 64-3-7. Aliquots containing cell equivalents of a digitonin lysate of L-K\textsuperscript{d} (lane 1) or L-etK\textsuperscript{b} (lanes 2–8) were incubated with the indicated peptide or no peptide as a control at the indicated concentration for 2 h at 4°C. Samples were then treated with either B8-24-3 or mAb 64-3-7 that detects folded etK\textsuperscript{b} molecules or mAb 64-3-7 that detects open etK\textsuperscript{b} molecules. Autoradiographs of the samples analyzed by 4–20% SDS-PAGE are shown in the upper panel. The lower panel shows a Western blot using an anti-TAP Ab. Arrows indicate lanes where the OVA peptide resulted in the complete loss of 64-3-7\textsuperscript{b} etK\textsuperscript{b} and the elimination of TAP association. C. Peptide preferentially induces folding of K\textsuperscript{b} in lysates of tapasin-deficient .220 cells (lanes 1–5) compared with lysates of tapasin-positive .221 cells (lanes 6–10). Aliquots of each lysate were treated with the indicated concentration of the nonbinding control peptide CW3 (lanes 1 and 6), no peptide (lanes 2 and 7), or the K\textsuperscript{b}-binding OVA peptide (lanes 3–5 and 8–10). Samples were precipitated with mAb 64-3-7 to detect forms of K\textsuperscript{b} lacking peptide-induced folding.

A

B

C


