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Distinct Functions of Tapasin Revealed by Polymorphism in MHC Class I Peptide Loading

Chen Au Peh,* Nihay Laham,† Scott R. Burrows,‡ Yong Zhu,§ and James McCluskey*¶

Peptide assembly with class I molecules is orchestrated by multiple chaperones including tapasin, which bridges class I molecules with the TAP and is critical for efficient Ag presentation. In this paper, we show that, although constitutive levels of endogenous murine tapasin apparently are sufficient to form stable and long-lived complexes between the human HLA-B*4402 (B*4402) and mouse TAP proteins, this does not result in normal peptide loading and surface expression of B*4402 molecules on mouse APC. However, increased expression of murine tapasin, but not of the human TAP proteins, does restore normal cell surface expression of B*4402 and efficient presentation of viral Ags to CTL. High levels of soluble murine tapasin, which do not bridge TAP and class I molecules, still restore normal surface expression of B*4402 in the tapasin-deficient human cell line 721.220. These findings indicate distinct roles for tapasin in class I peptide loading. First, tapasin-mediated bridging of TAP-class I complexes, which despite being conserved across the human-mouse species barrier, is not necessarily sufficient for peptide loading. Second, tapasin mediates a function which probably involves stabilization of empty class I molecules and which is sensitive to structural compatibility of components within the loading complex. These discrete functions of tapasin predict limitations to the study of HLA molecules across some polymorphic and species barriers. The Journal of Immunology, 2000, 164: 292–299.

The mechanisms involved in loading MHC class I molecules with antigenic peptides are far more complex than originally appreciated. It is now known that multiple molecular interactions collaborate to efficiently load peptides into the Ag-binding cleft of class I molecules. These interactions include association of empty class I/β2-microglobulin (β2m) complexes with the chaperones calnexin and calreticulin as well as the thiol oxidoreductase, ER-60 (1–5). Ultimately, efficient loading of most MHC class I molecules is critically dependent upon the endoplasmic reticulum (ER) glycoprotein tapasin (6). Tapasin is thought to augment peptide loading by several mechanisms including bridging the class I heavy chain complex to TAP, which enhances TAP expression, increases peptide translocation, and stabilizes class I complexes in a peptide-receptive conformation. These functions are believed to be highly conserved both within and across species such that murine tapasin restores normal surface expression of HLA-B8 in the human tapasin-defective cell line 721.220 (7). Indeed studies of HLA class I function in transgenic mice are widely used for evaluation of epitope selection in models of tumor immunity and vaccine development, and in murine models of human disease. These models generally assume conserved Ag presentation function and physiological peptide selection by human class I molecules in murine APC. Notwithstanding these assumptions, it is not clear whether all components of the Ag presentation machinery function normally across species. In particular, it is not known whether efficient tapasin function is influenced by structural polymorphisms in class I molecules either within or across the human/rodent species barriers.

In this paper, we show that constitutively expressed murine tapasin does not support normal surface expression of the human class I molecule HLA-B*4402 (B*4402) in mouse cells expressing normal levels of H-2Kk and H-2Dk. Although constitutive expression of murine tapasin in these mouse APC is sufficient to mediate a stable and prolonged association between TAP and B*4402 molecules, this alone is insufficient for efficient loading of B*4402 molecules. Expression of membrane-anchored murine tapasin at high levels fully restores the surface expression of B*4402 in mouse APC without augmenting the level of B*4402-TAP association. Furthermore, overexpression of soluble forms of either murine or human tapasin (sol-mTapasin or sol-hTapasin) also rescued surface expression of B*4402 molecules, revealing a critical role for tapasin in peptide loading of class I molecules independent of TAP-class I bridging, increased peptide translocation, or enhanced TAP expression. These data also reveal a degree of incompatibility between human B*4402 molecules and components of the murine Ag presentation machinery, and they suggest an important role for structural class I polymorphism in controlling the efficiency of interactions with components of the peptide-loading complex. The findings imply that the use of HLA class I transgenic mice as human models of specific immunity might be conceptually flawed if species incompatibility in tapasin interaction leads to presentation of an altered peptide repertoire.

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Received for publication August 11, 1999. Accepted for publication October 13, 1999.

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This work was supported by grants from the National Health and Medical Research Council (Australia) (NH&MRC), the Australian Kidney Foundation, and the Australian Research Council. C.A.P. is a recipient of an NH&MRC medical postgraduate scholarship.

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Abbreviations used in this paper: β2m, β2-microglobulin; B*4402, HLA-B*4402; hTAP, human TAP, ER, endoplasmic reticulum; sol-hTapasin, soluble human tapasin; sol-mTapasin, soluble murine tapasin; LCL, lymphoblastoid cell line.

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Materials and Methods

Cell lines and Abs

J26 (ATCC CRL-1802) of C3H/HeN background is derived from murine L cells by transfection with the human β2m gene; Jurkat (ATCC TIB-152) is a human T cell line; 721.220 is derived from mutagenesis of a human lymphoblastoid cell line (LCL) in which the HLA-A, HLA-B, and tapasin genes have been deleted but HLA-C remains intact on one chromosome (8); and EL4 (ATCC TIB-39) of C57BL/6 background is a mouse T lymphoma. 50125 is an EBV-transformed B*4402 homozygous human B cell line (as a gift from Sue Lester, Red Cross Blood Bank, Adelaide, Australia). Cells were grown in either RPMI 1640 or DMEM supplemented with 10% FCS (CSL, Melbourne, Australia), 2 mM glutamine, and antibiotics.

B LCL (a gift from Sue Lester, Red Cross Blood Bank, Adelaide, Australia) and EL4 (ATCC TIB-39) of C57BL/6 background is a mouse T cell leukemic line; 721.220 is derived from mutagenesis of a human T cell line using EMS and a human lymphoblastoid cell line (LCL) in which the HLA-A, HLA-B, and tapasin genes have been deleted but HLA-C remains intact on one chromosome (8); and EL4 (ATCC TIB-39) of C57BL/6 background is a mouse T lymphoma. Cells were generated from EBV-sero-positive donor SE15. Cells were grown in either RPMI 1640 or DMEM supplemented with 10% FCS (CSL, Melbourne, Australia), 2 mM glutamine, and antibiotics.

Gene constructs and DNA-mediated gene transfer

Genomic B*4402 was subcloned from pSV2neo.B*4402 (15) into the pUC13 vector. Genomic B*2705 was also contained in pUC13 vector (16). cDNAs encoding hTAP1 and hTAP2 were contained in pH2Apr-1-res vector (17). The cDNAs encoding the transmembrane-anchored (6) and soluble forms (18) of human tapasin were cloned into pMCFR.puromycin (6). The wild-type murine tapasin cDNA (courtesy of Bodo Ortman, Universität zu Koeln, Cologne, Germany) was also subcloned into pMCFR.puromycin. A cDNA (pMCFR.sol-mTapasin.puromycin) was constructed to encode soluble mTapasin using standard PCR methods analogous to those described for generating sol-hTapasin (18). Mouse J26 cells were transfected with the calcium phosphate-DNA precipitation method (19) with either pUC13.B*4402 or pUC13.B*2705 and the selectable marker gene pSV2neo. In other transfections, pH2Apr-1-neohTAP1 and/or pH2Apr-1-neohTAP2 were cotransfected with a genomic B*4402. A clone of J26.B*4402 was subsequently cotransfected with either pMCFR.Htapasin.puromycin or pMCFR.mTAPasin.puromycin. J26.B*2705 transfectedants were also co-transfected with pmCFR.Htapatasin.puromycin. Jurkat was transfected with linearized pSV2neo.B*4402 plasmid by electroporation at 220 V and 960 μF. The same electroporation settings were used to transfect EL4 with either pUC13.B*4402 or pUC13.B*2705 and pSV2neo, and 721.220 with pUC13.B*4402 or pSV2neo. 721.220.B*4402 transfectants were subsequently cotransfected with either pMCFR.puromycin vector alone, pMCFR.Htapatasin.puromycin, pMCFR.sol-mTAPasin.puromycin, pMCFR.mTAPasin.puromycin, or pMCFR.sol-mTAPasin.puromycin. J26 transfectants were selected with 0.5 mg/ml G418 ± 4 μg/ml puromycin. Jurkat transfectants were selected with 2.0 mg/ml G418 initially. EL4 transfectants were selected with 0.4 mg/ml G418. 721.220 transfectants were selected with 0.6 mg/ml G418 ± 0.5 μg/ml puromycin. Where possible, positive transfectants were sorted for peak channel expression of HLA molecules using magnetic beads that had been coated with an appropriate mAb.

Flow cytometry

Cell surface staining for HLA expression was performed by indirect immunofluorescence on cells cultured overnight at 37°C, unless indicated otherwise. mAbs W6/32, 116.5.28, 28.14.8, and 16.1.2 were used as primary Abs followed by goat anti-mouse Ig conjugated to FITC (Silenus, Melbourne, Australia). 104 cells were analyzed for each histogram using a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA) with logarithmic gain.

Western blot analysis

For anti-hTAP immunoblots, cells were lysed in SDS buffer and then boiled for 5 min. Proteins were separated by SDS-PAGE in a 10% gel, transferred onto nitrocellulose paper, blocked with 5% skim milk in PBS, probed with either anti-hTAP1 mAb 148.3 or anti-hTAP2 mAb 429.3 for 1 h, washed extensively with 5% skim milk in PBS, and incubated with secondary anti-mouse Ig conjugated to HRP for 1 h (Silenus). For anti-tapasnin immunoblots, cells were lysed in 1% Triton X-100 in TBS (10 mM Tris and 150 mM NaCl (pH 7.4)) and then centrifuged to remove nuclei. Extracts from 50,000 cells per lane were boiled in SDS sample buffer and separated by SDS-PAGE in a 10% gel. After transfer onto nitrocellulose paper, proteins were probed with either anti-human tapasin Rgp84N Ab (courtesy of P. Cresswell, Yale University Medical School, New Haven, CT) or an anti-mouse tapasin Ra#2223 Ab (courtesy of T. Hansen, Washington University School of Medicine, St. Louis, MO), and subsequently sheep anti-rabbit Ig conjugated to HRP (Silenus). For anti-tapasnin immunoblots, the entire procedure was conducted in the presence of 0.2% Tween 20. The immunoblots were developed with chemiluminescence agent (NEN, Boston, MA) and exposed to radiographic film.

Immunoprecipitation

Cells (4 x 106) were starved of methionine and then labeled with [35S]methionine for 15 min and lysed in 0.5% digitonin buffer in TBS containing PMSF, leupeptin, and aprotinin for 30 min at 4°C. After centrifugation to remove cell nuclei, samples were precleared overnight with normal rabbit serum and protein A-Sepharose. Primary immunoprecipitation was conducted with anti-HAM1 (anti-murine TAP1) Ab before protein A-Sepharose beads were added. Beads were washed twice in 0.1% digitonin buffer containing 450 mM NaCl and 10 mM Tris (pH 7.4), and once in 10 mM Tris buffer. Immune complexes were dissociated by incubation in 50 μl of 0.2% SDS in TNE (50 mM Tris, 150 mM NaCl, and 5 mM EDTA (pH 7.4)) for 1 h at 37°C. Supernatants were equilibrated with nine volumes of 1% Nonidet P-40 and 0.5% nonanoyl-N-methyl-glucamide (Sigma, St. Louis, MO) in TNE. Secondary immunoprecipitation was conducted with mAb HC-10; protein A-Sepharose beads were added, and the beads were washed twice with 0.5% Nonidet P-40 in TNE. Beads were boiled in SDS sample buffer, proteins were separated by SDS-PAGE in a 10% gel, and the gel was then fixed, amplified, dried, and exposed to radiographic film for 5 days. For pulse-chase immunoprecipitation, aliquots of 4 x 106 cells were radiolabeled for 15 min and chased in medium containing excess methionine (0.5 mM) and cystine (0.5 mM). At the indicated time points, cells were collected, washed in cold PBS, and then treated as outlined above.

Cytotoxicity assay

Target cells were tested for lysis in duplicate in a standard 5-h chromium release assay. Synthetic peptide was added to some target cells during chromium labeling (10 μM for 1 h), and unbound peptide was washed off target cells before effector CTL was added. A topcount microplate β scintillation counter (Packard Instrument, Meriden, CT) was used to measure 51Cr levels in assay supernatant samples. The mean spontaneous lysis for target cells in culture medium was < 20%, and the variation from the mean specific lysis was < 10%. Peptides were synthesized by Chiron Mimotopes (Chiron, Emeryville, CA) using pin technology (20).

Results

Poor cell surface expression of B*4402 in murine cells suggests a species-incompatible interaction with the murine Ag presentation machinery

We have previously observed poor surface expression of B*4402 in murine J26 cells (L cells expressing human β2m) after transfection with either B*4402 cDNA or a genomic construct (21). Poor surface expression was evident with mAbs recognizing both the W6/32 and Bw4 epitopes and was associated with high levels of intracellular B*4402 heavy chains (21). Moreover, the low level surface expression of B*4402 could be greatly improved by overnight incubation of these transfectants at 26°C, indicating that B*4402 complexes could not load efficiently with peptides in murine cells (Fig. 1A). Low surface expression of B*4402 was also evident in another commonly studied murine cell line, EL4 (Fig. 1B). After gene transfection, the surface expression of B*4402 was low in murine cells, whereas the surface expression of other HLA

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Overexpression of murine tapasin restores surface expression and Ag presentation function of B*4402 in a tapasin-defective cell line

Because coexpression of human tapasin fully restored the intracellular maturation and surface expression of B*4402 complexes in murine cells (21), we reasoned that B*4402 molecules may not interact properly with endogenous murine tapasin to achieve assembly with peptides in murine cells. This possibility was consistent with the observation that human and murine tapasin protein sequences shared only 75% identity (7). Therefore, the genes encoding B*4402, and either human or murine tapasin were transfected into tapasin-defective human 721.220 cells. 721.220 is a HLA-A and -B negative, EBV-transformed human cell line which is defective in tapasin expression as a result of a mRNA splicing error (8, 22). B*4402 molecules are critically dependent upon human tapasin for surface expression and Ag presentation by these cells (21). Surprisingly, introduction of a murine tapasin cDNA restored the surface expression of B*4402 in 721.220 transfectants to the same degree as did a human tapasin cDNA (Fig. 2A). Furthermore, B*4402 complexes assembled in the murine or human tapasin transfectants were equally efficient at presenting endogenous EBV peptides to an EBV-specific, HLA-B44-restricted CTL clone (Fig. 2B). Together, these findings suggest that after gene transfection, murine tapasin could facilitate peptide loading of B*4402 complexes, but also raises the question of why B*4402 was poorly expressed on the surface of murine cells expressing endogenous tapasin.

Restoration of peptide loading and surface expression of B*4402 depends upon the quantitative level of murine tapasin

The poor surface expression of B*4402 in murine cells might have been due to low or absent expression of endogenous murine tapasin in these cells even though assembly of murine MHC class I complexes was normal in both J26 and EL4 cells. Therefore, we co-transfected cloned human J26.B*4402 cells with either murine or human tapasin cDNAs and correlated the surface expression of B*4402 with the level of tapasin expression (Fig. 3). Western blots of cell lysates showed that before tapasin transfection, J26.B*4402 cells expressed readily detectable endogenous murine tapasin (Fig. 3B, lower panel, lane 4). Therefore, the poor surface expression of B*4402 in J26 cells was not due to an absolute lack of murine tapasin. Co-transfection of the murine tapasin cDNA into J26.B*4402 cells (J26.B*4402.mTapasin) increased the total level of peptide loading and surface expression of B*4402 complexes. Notably, the constitutive level of human tapasin expressed in the human B LCL 50125 was less than that observed in human tapasin-transfected J26.B*4402 cells (Fig. 3A, filled grey and broken-line histograms).

Thus, the constitutive level of murine tapasin expressed in murine cells was inadequate for efficient assembly and transport of B*4402 peptide complexes. Notably, the constitutive level of human tapasin expressed in the human B LCL 50125 was less than that observed in human tapasin-transfected J26.B*4402 cells (Fig.

FIGURE 1. Poor cell surface expression of B*4402 in murine cells. FACS analyses were performed. A, J26 (mouse L cells expressing human β2m) transfectants expressing either B*4402 (open histogram) or B*2705 (grey-filled histogram) that have been stained with mAb W6/32 (anti-HLA class I). A cohort of J26.B*4402 transfectants was cultured overnight at 26°C before analysis (broken-line histogram). B, EL4 transfectants expressing either B*4402 (open histogram) or B*2705 (grey-filled histogram) that have been stained with mAb 116.5.28 (anti-Bw4). Black-filled histograms in A and B represent Ab staining of untransfected J26 and EL4 cells, respectively. C, Jurkat transfectants expressing B*4402 (open histogram) and 50125 cells (B*4402 human B LCL; grey-filled histogram) that have been stained with mAb 116.5.28. Black-filled and broken-line histograms represent background staining of 50125 (no primary Ab) and untransfected Jurkat cells (primary and secondary Ab), respectively. D, J26 transfectants (H-2k origin) expressing B*4402 that have been stained with either mAb 16.1.2 (anti-H-2Kk and anti-H-2Dk; open histogram) or mAb 28.14.8 (anti-H-2Dk; grey-filled histogram). E, EL4 transfectants (H-2b origin) expressing B*4402 that have been stained with either mAb 28.14.8 (grey-filled histogram) or mAb 16.1.2 (open histogram). Black-filled histograms in D and E represent background staining of these cells.
3B, upper panel, lanes 2 and 5) even though both cells expressed high surface amounts of B*4402 complexes. A, Tapasin-defective human 721.220 transfectants expressing B*4402 (721.220.B*4402; broken-line histogram) were cotransfected with cDNA encoding either human tapasin (721.220.B*4402.hTapasin; grey-filled histogram) or murine tapasin (721.220.B*4402.mTapasin; open histogram) and stained by indirect immunofluorescence with mAb 116.5.28 (anti-Bw4). Black-filled histogram represents background staining of untransfected 721.220 cells. B, Cytotoxicity assays were conducted on target cells incubated with CTL clone SE15 at E:T ratio of 2:1. SE15 is specific for the EBNA4-derived peptide VEITPYKPTW presented by either B*4403 or B*4402. Killing of the target cells was measured by specific 51Cr release. Targets included untransfected 721.220 cells, 721.220.B*4402 (721.220 transfectants expressing B*4402), 721.220.B*4402.hTapasin (721.220 transfectants expressing B*4402 plus human tapasin), and 721.220.B*4402.mTapasin (721.220 transfectants expressing B*4402 plus murine tapasin). Targets were either untreated or loaded with VEITPYKPTW peptide.

**FIGURE 2.** Murine tapasin can potentially facilitate peptide loading of B*4402 complexes. A, Tapasin-defective human 721.220 transfectants expressing B*4402 (721.220.B*4402; broken-line histogram) were cotransfected with cDNA encoding either human tapasin (721.220.B*4402.hTapasin; grey-filled histogram) or murine tapasin (721.220.B*4402.mTapasin; open histogram) and stained by indirect immunofluorescence with mAb 116.5.28 (anti-Bw4). Black-filled histogram represents background staining of untransfected 721.220 cells. B, Cytotoxicity assays were conducted on target cells incubated with CTL clone SE15 at E:T ratio of 2:1. SE15 is specific for the EBNA4-derived peptide VEITPYKPTW presented by either B*4403 or B*4402. Killing of the target cells was measured by specific 51Cr release. Targets included untransfected 721.220 cells, 721.220.B*4402 (721.220 transfectants expressing B*4402), 721.220.B*4402.hTapasin (721.220 transfectants expressing B*4402 plus human tapasin), and 721.220.B*4402.mTapasin (721.220 transfectants expressing B*4402 plus murine tapasin). Targets were either untreated or loaded with VEITPYKPTW peptide.

**FIGURE 3.** Surface expression of B*4402 in murine cells is determined by the level of murine tapasin. A, Cloned murine J26 cells expressing B*4402 (J26.B*4402) were supertransfected with cDNA encoding for either human tapasin (J26.B*4402.hTapasin) or murine tapasin (J26.B*4402.mTapasin) and stained by indirect immunofluorescence with mAb W6/32 (anti-HLA class I). The black-filled histogram represents background staining of untransfected J26 cells. B, Expression of human tapasin (upper panel) and murine tapasin (lower panel) was determined by immunoblotting the indicated cell lysates with Rgp48N and Ra#2223 rabbit Abs, respectively. Cell lines studied were 721.220 (parental tapasin-defective LCL), 50125 (B*4402 human B LCL), J26.B*2705 (J26 transfectants expressing B*2705), J26.B*4402 (J26 transfectants expressing B*4402), J26.B*4402.hTapasin (J26 transfectants expressing human tapasin) and J26.B*4402.mTapasin (J26 transfectants expressing murine tapasin). C, Expression of endogenous H-2Kk and H-2Dk in J26.B*4402 (grey-filled histogram), J26.B*4402.hTapasin (open histogram), and J26.B*4402.mTapasin (broken-line histogram) was examined by indirect immunofluorescence with mAb 16.1.2 (anti-H-2Kk and anti-H-2Dk). Black-filled histogram represents background staining of untransfected J26 cells.
To examine the effect of increased tapasin expression upon the surface levels of murine MHC class I molecules, the expression of endogenous H-2K<sup>b</sup> and H-2D<sup>b</sup> in J26.B<sup>4402</sup>, J26.B<sup>4402.hTapsasin</sup>, and J26.B<sup>4402.mTapsasin</sup> transfectants was examined by staining with mAb 16.1.2 (Fig. 3C). Interestingly, the surface expression of endogenous MHC class I molecules was significantly increased after the overexpression of either murine or human tapasin. These findings indicate that the constitutive surface expression of MHC class I molecules in mouse APCs is modulated by the limiting expression of murine tapasin, implying a role for tapasin in the quantitative regulation of MHC class I ligands expressed at the cell surface.

Constitutively expressed murine tapasin mediates a prolonged and stable association between B<sup>4402</sup> and TAP without augmenting peptide loading

Besides class I-TAP bridging, other mechanisms have been proposed to account for the role of tapasin in class I Ag presentation. For instance, tapasin increases the expression of the TAP complex and hence may increase the rate of peptide translocation (18), perhaps by stabilizing the TAP1/TAP2 heterodimer (23). To test whether increased TAP expression alone could restore surface expression of B<sup>4402</sup> in J26 cells, human TAP1 and TAP2 cDNAs were co-transfected into J26.B<sup>4402</sup>. Expression of these proteins was verified by immunoblots of transfected cell lysates. As shown in Fig. 4, A and B, overexpression of human TAP in murine cells did not improve the poor surface expression of B<sup>4402</sup>, suggesting that increased TAP expression by itself is insufficient to restore B<sup>4402</sup> expression at the cell surface. The data also suggest that poor B<sup>4402</sup> surface expression was not due to either functional incompatibility with the murine TAP protein or delivery of peptide ligands unsuitable for binding to the B<sup>4402</sup> molecule.

Therefore, we next determined whether the restoration of surface B<sup>4402</sup> expression in murine tapasin transfectants correlated with formation of intracellular B<sup>4402</sup>-TAP complexes. Equal numbers of J26.B<sup>4402</sup> cells expressing either constitutive murine tapasin alone, supertransfected murine tapasin, or human tapasin were radiolabeled and lysed in digitonin buffer. Sequential immunoprecipitation was performed with anti-HAM1 (14), an Ab reactive with murine TAP1. The next step was dissociation of bound immune complexes in the presence of SDS and reprecipitation by the mAb HC-10 that recognizes free HLA-B heavy chains (12). As formed with anti-HAM1 Ab (anti-murine TAP1) bound to protein A-Sepharose beads and washed. The immune complexes were separated by 10% SDS-PAGE and autoradiographed after fluorography (upper panel). In a separate experiment, immune complexes obtained from primary immunoprecipitation with anti-HAM1 Ab were sequentially dissociated in SDS buffer, and eluted proteins were then reacted with mAb HC-10 (anti-HLA free heavy chains, lower panel). The position of class I heavy chains is indicated by an arrow. The cell lines studied were J26 parental cells, J26.B<sup>2705</sup> (J26 transfectants expressing B<sup>2705</sup>), J26.B<sup>2705.hTapsasin</sup> (J26 transfectants expressing B<sup>2705</sup> plus human tapasin), J26.B<sup>3402.hTapsasin</sup> (J26 transfectants expressing B<sup>4402</sup> plus human tapasin), J26.B<sup>4402.hTapsasin</sup> (J26 transfectants expressing B<sup>4402</sup> plus human tapasin), and J26.B<sup>4402.mTapsasin</sup> (J26 transfectants expressing B<sup>4402</sup> plus murine tapasin).

The kinetic association of B<sup>4402</sup> with mouse TAP in J26.B<sup>4402</sup> vs J26.B<sup>4402.hTapsasin</sup> transfectants was examined by pulse-chase immunoprecipitation. Equal numbers of cells were labeled with [<sup>35</sup>S]methionine for 15 min, chased in excess methionine and cystine for the indicated time intervals, and lysed in digitonin buffer. Primary immunoprecipitates were formed with anti-HAM1 Ab (anti-murine TAP1), sequentially dissociated in SDS buffer, and secondary immunoprecipitation was performed with mAb HC-10 (anti-HLA free heavy chains). Eluted proteins were separated by 10% SDS-PAGE and autoradiographed after fluorography.
expected, in murine J26 cells, bands corresponding to endogenous TAP-associated MHC class I H-2K\(^k\) and H-2D\(^k\) heavy chains were detected (Fig. 4C, upper panel), but proteins reactive with mAb HC-10 were not detected in wild-type murine J26 cells (Fig. 4C, lower panel). In transfected cells, bands comprising HLA class I heavy chains were detected after dissociation of bound immune complexes and sequential reprecipitation with mAb HC-10 (Fig. 4C, lower panel, lanes 2–6). Significantly, the recovery of B*4402 from the murine TAP complex was indistinguishable in cells expressing only constitutive endogenous tapasin vs transfected cells expressing much higher levels of murine or human tapasin (Fig. 4C, lower panel, lanes 4–6). Thus, endogenous murine tapasin expressed at constitutive levels facilitates the binding of B*4402 to murine TAP but does not facilitate its normal surface expression. Notably, B*2705 molecules were also associated with constitutively expressed murine TAP, and this association was not significantly different from that observed with B*4402 or in human tapasin transfectants (Fig. 4C). The findings suggest that TAP-class I bridging alone does not correlate with full tapasin function.

Notwithstanding the apparently normal association between B*4402 and murine TAP, it was possible that complexes of endogenous mouse tapasin, B*4402, and TAP were unstable or too short-lived to permit proper peptide loading of B*4402. Therefore, we examined the stability of these complexes in a pulse-chase experiment using the same approach as in Fig. 4C. TAP complexes were recovered by immunoprecipitation at various time intervals after a 15-min pulse labeling, and the presence of B*4402 molecules in these complexes was ascertained by a second immunoprecipitation with HC-10 mAb. As shown in Fig. 4D, B*4402 heavy chains remained stably associated with TAP proteins for at least 2 h in J26 cells expressing only endogenous mouse tapasin. However, the introduction of human tapasin into these cells (J26.B*4402.hTapasin) resulted in a more rapid dissociation of B*4402-TAP complexes consistent with more efficient peptide loading of B*4402 molecules and their transport to the cell surface (21). These results confirm that the physical interaction between TAP and HLA class I complexes in itself does not necessarily augment peptide loading and indicates that tapasin has an additional function in the loading of peptides into peptide-receptive HLA class I complexes.

**High levels of sol-mTapasin restore surface expression of B*4402 in human tapasin-deficient 721.220 cells**

The restoration of B*4402 surface expression in J26 mouse cells after overexpression of mouse tapasin did not appear to be the result of enhanced association between TAP and B*4402, which was already demonstrable with endogenously expressed mouse tapasin. However, improved B*4402 expression could still reflect enhanced peptide translocation associated with increased TAP expression or improved TAP-class I-tapasin oligomerization (18, 24). Soluble tapasin lacking the transmembrane and cytoplasmic domains still restores surface expression and Ag presentation by HLA class I molecules in the tapasin-deficient 721.220 cell line despite a lack of detectable TAP-class I association due to loss of the C-terminal region which mediates TAP association (25). Therefore, we tested whether sol-mTapasin and sol-hTapasin could correct the impaired surface expression of B*4402 in these tapasin-deficient human cells. As shown in Fig. 5A, the introduction of genes encoding either sol-hTapasin or sol-mTapasin into B*4402-deficient 721.220.B*4402 cells significantly restores surface expression of B*4402 by these cells. Western blots of detergent lysates from transfected cells confirmed the overexpression of sol-mTapasin in 721.220.B*4402 cells (Fig. 5B, lane 4) relative to the transmembrane-anchored form of mouse tapasin expressed endogenously by J26.B*4402 (Fig. 5B, lane 6) or after transfection of 721.220 cells (Fig. 5B, lane 5). Because soluble tapasin does not associate efficiently with TAP or enhance TAP expression or peptide translocation, the findings reveal a critical function of tapasin which can be separated from its other roles associated with TAP-class I bridging (18).

**Discussion**

The assembly and surface expression of B*4402 complexes in human cells is critically dependent upon the presence of tapasin (21). The efficiency of peptide loading and surface expression of B*4402 in murine cells is also critically dependent upon the level of expression of murine tapasin. Whereas constitutive levels of endogenous human tapasin are sufficient to achieve proper surface expression of B*4402 in human cells, similar expression of B*4402 in murine cells requires much more than constitutive amounts of murine tapasin. Transfection of the tapasin gene in our system frequently results in overexpression of tapasin that can easily mask quantitative dependence upon the tapasin molecule. This
The observation that overexpression of murine tapasin overcomes the poor surface expression of B*4402 in murine cells suggests an underlying structural incompatibility in the interaction between B*4402 and murine tapasin. Presumably, high concentrations of murine tapasin can compensate for suboptimal interactions between these molecules. However, the possibility of an incompatible interaction between B*4402 and some other component of the murine Ag processing and presentation pathway cannot be entirely discounted. For instance, species-specific generation of peptide epitopes has been implicated as an explanation of differential presentation of a HLA-A3-restricted antigenic epitope from influenza A nucleoprotein by mouse and human cells (28). There are several reasons why it is unlikely that differences in cytosolic processing of proteins between rodent and human APC account for the low expression of B*4402 in mouse cells. First, expression of peptide-loaded B*4402 molecules on the surface of mouse cells is partially restored by culture of cells at 26°C, which is known to stabilize class I molecules and facilitate the capture of peptides (21). Second, expression of both human and mouse tapasin restores intracellular assembly of B*4402 peptide complexes, which rescues normal maturation, transport, and surface expression of B*4402 in mouse cells (21). Together these observations suggest that appropriate ligands for binding to B*4402 are generated normally in mouse cells. Impaired translocation of suitable peptides into the ER lumen by mouse TAP also seems improbable because appropriate ligands for binding to B*4402 are generated normally in mouse cells. Failure of retention by tapasin evidently cannot solely account for the inefficient assembly of B*4402 in murine cells. Incompatibility with either murine calnexin or murine calreticulin is unlikely to contribute significantly to the poor surface expression of B*4402 in murine cells because class I molecules assemble properly even in calnexin-deficient cells (29–31), and murine calreticulin shares a high degree of identity with human calreticulin (>95%). More recently, the thioerdastase ER-60 has also been detected in the MHC class I peptide-loading complex (3–5). It remains to be seen whether species-specific differences in ER-60 may influence the assembly of human HLA class I molecules in murine cells.

Recently, it has been reported from studies of MHC class I assembly in Drosophila cells that tapasin may have a role in ER retention of empty MHC class I complexes until they have been loaded with peptides (23). Failure of retention of mutant HLA-A2 molecules (with a T134K substitution in the α2-domain) by molecular chaperones may also account for impaired association with TAP, inefficient peptide loading, and faster rates of egress from the ER compared with wild-type HLA-A2 complexes (32). However, failure of retention by tapasin evidently cannot solely account for the inefficient assembly of B*4402 in murine cells because these complexes are already retained for much longer than those molecules assembled in the presence of excess tapasin (Fig. 4 and Ref. 21).

Although it seems clear that tapasin occupies a pivotal role in mediating the association of MHC class I peptide-loading complexes to TAP, it is unclear how tapasin functions to enhance the peptide-loading process. Lehner and colleagues (18) observed that soluble tapasin lacking the transmembrane and cytoplasmic domains restored peptide loading and surface expression of HLA-B8 without restoring HLA-B8-TAP binding. Our data confirm that sol-hTapasin and sol-mTapasin also restore surface expression of B*4402 in the 721.220 cell line. Thus, TAP-class I bridging is not essential for enhanced peptide loading. An important corollary to this observation is our finding that the stable association of class I molecules with TAP is not sufficient for enhanced peptide loading. This indicates that the most important function of tapasin can be exerted independently of bridging MHC class I to TAP.

We hypothesize that tapasin may play a vital role in stabilizing and transforming the conformation of the empty MHC class I complex into a peptide-receptive state. The impaired assembly of B*4402 in normal murine cells indicates that constitutive levels of endogenous murine tapasin are insufficient for this function. Indeed, it is unclear how well various HLA, or even murine H-2 class I, molecules interact with murine tapasin for assembly in murine cells. For example, in transgenic mice expressing HLA class I molecules, inefficient interactions between these molecules and components of the murine Ag presentation machinery (such as tapasin) may modify the spectrum of peptide Ags presented at the cell surface and may contribute to difficulties encountered in generating HLA-restricted CTL (33).

Little is known about the structural determinants controlling interactions between TAP, tapasin, and class I molecules (24, 34, 35). The α3 domain of class I molecules might be involved either directly or indirectly in binding to the TAP complex (24, 34), and the N-terminal domain of tapasin is required to stabilize the class I loading complex (25). Moreover, evidence also suggests that tapasin might interact directly with β2m (36). Thus, it is tempting to speculate that the N-terminal region of tapasin might interact directly with the class I heavy chain α3 domain/β2m complex. However, the relative species incompatibility of mouse tapasin for B*4402 does not seem to be controlled solely by the α3 domain of the MHC class I heavy chain because replacement of this region of B*4402 with a mouse equivalent does not improve expression of chimeric B*4402 molecules in mouse cells (data not shown). Nonetheless, it is still possible that in mice transgenic for other chimeric HLA class I heavy chain/murine α3-domain molecules (37) improved CTL activity may be partially due to improved interactions between these chimeric molecules and murine TAP/tapasin complex.

The C-terminal domain of tapasin mediates TAP-class I bridging (25). Because the cytosolic domain of mouse tapasin is 14 amino acids longer than the human analogue at the C terminus, it has been suggested this region might determine species specificity of interaction with class I or TAP1/2 (35). However, it does not seem likely that the C-terminus of tapasin alone controls species specificity of TAP-class I interactions (35) given that B*4402 associates stably with mouse TAP in J26 cells (Fig. 4, C and D) and that high levels of sol-mTapasin restore B*4402 expression in 721.220 transfectants (Fig. 5).

How essential is interaction with tapasin/TAP for peptide loading of MHC class I molecules? Whereas some MHC class I molecules such as B*4402 and HLA-B8 are highly dependent upon tapasin (6, 21), others like B*2705, HLA-A2, murine H-2Kb, and H-2Dd (E222K) may still retain residual capability to assemble and present endogenous peptide Ags in the absence of TAP bridging by tapasin (21, 24, 38, 39). The complexes which form under these circumstances may be suboptimal in quantity and in binding affinity, thus creating poor ligands for T cell recognition (Refs. 21 and 24; footnote 4). Even so, the existence of an active tapasin-independent loading mechanism for some class I alleles might be advantageous in the presence of viral immune evasion mechanisms.
which depend upon tapasin inhibition (40) or where there is competition between class I molecules in the ER for access to the TAP/tapasin complexes (41). Furthermore, the quantitative dependence of B*4402 surface expression upon the level of murine tapasin might indicate that constitutive expression of endogenous tapasin is normally finely tuned to exert sensitive control over the peptide supply to nascent class I molecules. This notion is consistent with the expression of some “empty” class I molecules on the surface of normal cells (42) and with the inducibility of tapasin by cytokines such as GM-CSF (43). Such sensitive tuning could effectively render peptide supply functionally limiting under basal conditions but would allow a rapid response of the Ag presentation machinery to new ligands generated upon infection or Ag uptake.

Acknowledgments

We thank the following scientists for their generous gifts of reagents: Veronique Braud, Frank Mombourg, Xiaoning Xu, Elisabeth Weiss, Robert DeMars, Robert Inman, Tom Novak, Kristin Lientert-Weidenbach, Greg Bennett, Sue Lester, Klaus Früh, Peter Cresswell, Gaby Vop-DeMars, Robert Inman, Tom Novak, Kristin Lienert-Weidenbach, Greg Bennett, Sue Lester, Klaus Früh, Peter Cresswell, Gaby Vop-

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