Retention of empty MHC class I molecules by tapasin is essential to reconstitute antigen presentation in invertebrate cells

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Presentation of antigen-derived peptides by major histocompatibility complex (MHC) class I molecules is dependent on an endoplasmic reticulum (ER) resident glycoprotein, tapasin, which mediates their interaction with the transporter associated with antigen processing (TAP). Independently of TAP, tapasin was required for the presentation of peptides targeted to the ER by signal sequences in MHC class I-transfected insect cells. Tapasin increased MHC class I peptide loading by retaining empty but not peptide-containing MHC class I molecules in the ER. Upon co-expression of TAP, this retention/release function of tapasin was sufficient to reconstitute MHC class I antigen presentation in insect cells, thus defining the minimal non-housekeeping functions required for MHC class I antigen presentation.

Keywords: antigen presentation/chaperone/MHC/proteasome/transporter associated with antigen processing

Introduction

Cytotoxic T lymphocytes recognize complexes of polymorphic major histocompatibility complex (MHC) class I heterodimers and short peptides of 8–10 amino acids, which are derived from intracellular antigens (Rammensee et al., 1993). The different subsets of peptides bound by individual MHC class I alleles are produced in the cytosol through proteolytic breakdown of antigens by the multisubunit proteasomal complex present in all eukaryotic cells (Goldberg and Rock, 1992). From the total pool of proteolytic breakdown intermediates, peptides of at least seven amino acids are translocated selectively across the membrane of the endoplasmic reticulum (ER) by the dedicated peptide transporter TAP (transporter associated with antigen processing) (Heemels and Ploegh, 1995). The transmembrane-spanning TAP consists of the two MHC-encoded subunits TAP1 and TAP2, which belong to the ATP-binding cassette (ABC) superfamily of transporters (Townsend and Trowsdale, 1993). In the lumen of the ER, peptides are loaded onto newly synthesized MHC class I heterodimers formed by class I heavy chains (HCs) and β2-microglobulin (β2m). Only properly assembled trimeric complexes are transported along the secretory pathway to the cell surface. Each step of MHC class I assembly is assisted by ER-resident chaperones. Following translocation, folding of HCs is controlled by calnexin (Vassilakos et al., 1996). Free HCs have also been found in association with Bip (Noessner and Parham, 1995). Upon β2m association, they associate with calreticulin (Sadasivan et al., 1996) and subsequently with TAP, from which they dissociate upon peptide binding (Ortmann et al., 1994; Suh et al., 1994). In addition, the thiol-dependent reductase ER-60 recently has been shown to be part of this peptide loading complex (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998).

Essential for the formation of TAP–MHC class I complexes is the 48 kDa glycoprotein tapasin which was identified initially by co-precipitation with TAP and MHC class I (Sadasivan et al., 1996). Tapasin restores the defect in the mutant human cell line .220, in which MHC class I molecules do not interact with TAP and fail to present antigen to T cells (Grandea et al., 1995; Ortmann et al., 1997). The stoichiometry of affinity-purified TAP–MHC class I complexes suggests that each TAP heterodimer associates with four MHC class I–tapasin–calreticulin complexes (Ortmann et al., 1997). The primary structure of tapasin revealed a type I transmembrane protein belonging to the immunoglobulin superfamily with no close relatives (Li et al., 1997; Ortmann et al., 1997). The tapasin gene is located within 500 kb of the TAP genes at the centromeric end of the MHC (Grandea et al., 1998; Herberg et al., 1998). The putative cytoplasmic portion of tapasin contains a functional ER-retention motif (Jackson et al., 1990), and removal of the transmembrane and cytoplasmic domains results in a secreted molecule (Lehner et al., 1998). Interestingly, this truncated version of tapasin restored antigen presentation in .220 cells, although the class I–TAP association was no longer detectable (Lehner et al., 1998). These results question whether the primary function of tapasin is to form a bridge between MHC class I molecules and TAP as previously hypothesized. We now demonstrate that tapasin functions as a molecular chaperone, which retains empty MHC class I heterodimers in the ER until they acquire peptides.

Results

Tapasin retains empty MHC class I molecules

To dissect the role of tapasin in antigen processing, we chose Drosophila melanogaster cells which, like all other invertebrate cells, lack the MHC. This provided a system
for reconstitution of MHC class I peptide loading via transfection with various combinations of cDNA clones encoding the individual molecules. We have shown previously that, in contrast to mammalian cells, empty MHC class I molecules are transported to the cell surface in *D. melanogaster* SC2 cells (Jackson et al., 1992). Thus, when the murine MHC class I heavy chain Kb was co-transfected with murine β2m into SC2 cells, a high percentage of cells from the resulting cell line KB (for nomenclature of cell lines, see Table I) expressed Kb heterodimers at their surface, as shown by cytofluorometry with the monoclonal antibody (mAb) AF6 (Figure 1A, left panel). Surprisingly, however, almost no surface expression of Kb was detected upon co-transfection with murine tapasin in KBN cells (Figure 1A, right panel), despite the fact that both cell lines synthesized similar amounts of Kb and β2m, as shown by metabolic labeling and immunoprecipitation (Figure 1B). Since Kb heavy chains failed to acquire EndoH resistance in KBN cells, we conclude that tapasin impaired the intracellular transport of Kb (Figure 1B and C).

Previously it was shown that insect cell-expressed MHC molecules are empty (Jackson et al., 1992). To confirm that the ER-retained Kb molecules were also devoid of peptides, we tested the thermostability of the immunoprecipitated complexes. By using the heterodimer-specific antibody Y3, Kb heterodimers could only be immunoprecipitated from detergent lysates incubated at 37°C when the Kb-specific epitope Ova8 corresponding to the amino acid sequence 257–264 of ovalbumin (Carbone and Bevan, 1989) was added as synthetic peptide to lysates (Figure 1B). We next examined whether tapasin associated with Kb by immunoprecipitation from metabolically labeled KB and KBN cells lysed in 1% digitonin. As shown in Figure 1C, antisera against Kb co-precipitated a 48 kDa molecule from tapasin-expressing KBN cells but not from KB cells. This 48 kDa molecule was identified as tapasin by using two different antisera (anti-Tpn-N and anti-Tpn-C) raised against murine tapasin in parallel precipitation (Figure 1C) and reprecipitation experiments (Figure 5B). Anti-Tpn-N did not co-precipitate Kb molecules, whereas anti-Tpn-C co-precipitated small amounts of Kb, suggesting that the complexes were disrupted or not recognized by anti-Tpn-N antibodies. All tapasin molecules synthesized in the insect cells remained EndoH sensitive as shown by immunoblotting (Figure 1D). Thus, we conclude that tapasin associated with and retained empty MHC class I molecules in a pre-Golgi compartment. ER retention by tapasin is not specific to Kb molecules, since the intracellular transport of other murine MHC class I molecules is also impaired (data not shown).

### TAP-independent import of peptides releases MHC class I from tapasin retention

We hypothesized that by transfecting tapasin we had reconstituted the retention of empty MHC class I molecules observed in TAP-deficient mammalian cells (Townsend et al., 1989). Since in TAP mutants class I molecules can be loaded with peptides directed into the ER via fusion to a signal sequence (Anderson et al., 1991), we examined the retention of MHC class I molecules upon co-transfection of the construct SS-Ova8 encoding the signal sequence of CD8 followed by Ova8. Indeed, Kb molecules loaded with peptides via this TAP-independent pathway were released from tapasin retention, as shown by comparing the fluorescence-activated cell sorting (FACS) profiles of KBNs cells with KBS cells which do not express tapasin (Table I, Figure 2A). Release from tapasin retention was also demonstrated by the finding that some of the HCs became EndoH resistant during overnight labeling in pulse–chase experiments (Figure 2B) which was not observed in the absence of SS-Ova8 (Figure 1C). To visualize peptide-loaded MHC complexes directly, cells were co-stained with mAb 25.D1.16, which specifically recognizes class I–Ova8 complexes (Porgador et al., 1997). KBS and KBNs cells were gated for Kb-positive cells and analyzed for binding of 25.D1.16. Interestingly, a significantly higher 25.D1.16 fluorescence was observed for KBNs compared with KBS (Figure 2A, lower panel). To ensure that this result was not caused by lower SS-Ova8

### Table I. Cell line nomenclature

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aAll cell lines were co-transfected with Kb heavy chain (K) and murine β2 (B) microglobulin.
bMurine tapasin (N; Grandea et al. 1998).
cMurine TAP1 and TAP2 (T) were co-transfected in these cell lines.
dThe minigene (M) sequence is MSIINFEK.
eThe signal sequence (S)-bearing Ova8 sequence is MALPVTALLPLALLLHAARPSIINFEKL.
fChicken ovalbumin (O).
gMurine co-stimulatory molecules ICAM-1 (I) and B7.1 (7) were co-transfected in these cell lines.
Tapasin controls MHC class I peptide loading

expression in KBS cells, we hybridized RNA isolated from KB, KBS and KBNS cells with a radiolabeled SS-Ova8 DNA probe. The latter two cell lines clearly expressed Ova8, with KBS giving the stronger signal (Figure 2C, lower panel). Kb heavy chain mRNA used for control was expressed in all three cell lines (Figure 2C, upper panel). Therefore, it seemed that the presence of tapasin was responsible for the increased formation of peptide-class I complexes. Moreover, when the two cell lines were used to stimulate the Ova8-specific, Kb-restricted T cell hybridoma B3, only the tapasin-expressing KBNS cell line was able to stimulate B3 as a result of endogenously processed SS-Ova8 (Figure 2D). Upon exogenous Ova8 peptide addition, both cell lines stimulated B3 equally well. The lack of T cell stimulation by KBS cells (Figure 2D) despite the presence of Ova8 complexes at the cell surface (Figure 2A) suggests that the number of Ova8 complexes is below the threshold required to activate B3 cells. We conclude that tapasin retains empty Kb molecules until they acquire peptides. By this mechanism, which occurred independently of TAP, tapasin facilitated an increase in the number of peptide-MHC complexes.

Tapasin-independent loading of minigene products imported by TAP

Next we examined whether TAP-dependent import of a peptide product expressed from a minigene would result in intracellular transport of Kb molecules in the presence of tapasin. Two cell lines were generated expressing murine TAP1 and TAP2 together with the minigene for Ova8 lacking the signal sequence. Cells were also transfected with Kb, β2m (KBTM) or, in addition, tapasin (KBTNM, Table I). In agreement with our previous demonstration that murine TAP1 and TAP2 form a functional peptide transporter in *D.melanogaster* cells (Ahn *et al.*, 1996), labeled reporter peptides were imported into the ER in both cell lines expressing TAP, as shown in a standard transport assay (data not shown; Neefjes *et al.*, 1993). Both cell lines expressed Kb on the cell surface as observed by FACS analysis (Figure 3A), showing that TAP-dependent import of the Ova8 minigene product released class I molecules from tapasin retention. Binding of TAP-imported Ova8 to Kb was confirmed by co-staining with mAb 25.D1.16 (Figure 3A, lower panel). In the presence of tapasin, we observed an increase of Kb–Ova8 complexes. However, the difference between cells with or without tapasin was less pronounced than the difference observed for the SS-Ova8-containing cell lines. A significant number of Kb–Ova8 complexes were formed independently of tapasin (compare Figures 2A and 3A). Together with the pulse–chase analysis and T-cell assay shown below, these results suggest that import by TAP of a pre-processed high affinity peptide can override the dependence on tapasin for efficient class I peptide loading.

**Fig. 1.** Tapasin prevents intracellular transport of empty MHC class I molecules. (A) Immunofluorescence and flow cytometry of *D.melanogaster* SC2 cells transfected with Kb, β2m (KB; left panel) and tapasin (KBN; right panel). Kb molecules were detected by phyocerythrin-conjugated mAb AF6 (Pharmingen). Non-transfected cells were used for the control (stippled line). (B) Immunoprecipitation of Kb molecules from cells lysed in 1% NP-40 after metabolic labeling for 30 min or additional chase for 3 h. Prior to immunoprecipitation with mAb Y3, lysates were either kept at 4°C or incubated at 37°C for 1 h in the absence or presence of 50 μg/ml of synthetic Ova8 peptide, as indicated. All samples were treated with EndoH before electrophoretic separation. EndoH-resistant or -sensitive Kb heavy chains are denoted with r or s, respectively. The protein band corresponding to β2m is not shown. (C) Co-precipitation of tapasin and Kb molecules from KB or KBN cells lysed in 1% digitonin after metabolic labeling for 16 h. Rabbit antisera anti-Tpn-N or anti-Tpn-C were raised against the N- or C-terminal end of tapasin, respectively. The anti-Kb antiserum recognizes EndoH-resistant and -sensitive Kb heavy chains denoted with r or s, respectively. The protein band corresponding to β2m is not shown. (D) Immunoblot of tapasin. KB or KBN cells were lysed in 1% NP-40 and digested with EndoH prior to electrophoretic separation. The anti-Kb antiserum recognizes EndoH-resistant and -sensitive HC (r and s) as well as β2m (not shown). Proteins which are bound non-specifically to protein A–Sepharose are indicated by an asterisk. (D) Immunoblot of tapasin. KB or KBN cells were lysed in 1% NP-40 and digested with EndoH where indicated. Cell lysates were separated by SDS–PAGE transferred to Immobilon membranes and probed with anti-Tpn-N antiserum (1:1000).
TAP and tapasin are sufficient for class I antigen presentation in insect cells

To determine whether processing and presentation of antigens by MHC class I could be reconstituted in insect cells, we co-transfected cDNAs for Kb, β2m, mTAP1, mTAP2 and ovalbumin into KBTO cells, whereas KBTN cells were also co-transfected with tapasin cDNA (Table I). After G418 selection and single cell cloning, two clones (KBTO-B1 and KBTN-D4) were selected for further analysis. With both clones, ovalbumin expression was verified by immunoprecipitation (data not shown). Expression of all other polypeptides is shown in Figure 5. As shown in the upper right panel of Figure 3A, Kb molecules were transported to the cell surface even in the presence of tapasin, thus demonstrating that insect cells generated TAP-dependent peptides capable of releasing Kb from tapasin retention. To determine whether ovalbumin-derived Ova8 was among those peptides, we co-stained with mAb 25.D1.16. A low but significant 25.D1.16 reactivity was observed for KBTN-O4 whereas no significant staining was observed with KBTO-B1 cells which lack tapasin (Figure 3A). These results suggested that Ova8 was produced from ovalbumin, translocated into the ER by TAP, and bound to Kb with the help of tapasin.

Kb surface expression was lower in KBTN-D4 cells compared with KBTO-B1 (Figure 3A). This was also observed for other clones as well as the parental cell line (not shown). To examine whether this lower surface expression was due to tapasin retention, we studied the intracellular transport of Kb by pulse–chase analysis and immunoprecipitation. Kb heavy chains rapidly acquired EndoH resistance in KBTO-B1 cells so that some EndoH resistance was already observed at the end of the 30 min labeling period (Figure 3B, time point 0). By contrast, in

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**Fig. 2.** Tapasin does not retain peptide-loaded MHC class I molecules and increases the efficiency of complex formation independently of TAP. (A) Immunofluorescence and flow cytometry of SC2 cells transfected with Kb, β2m without tapasin (KBS cells, thin line) or with tapasin (KBSNS cells, thick line) as well as the Ova8 epitope expressed as the signal sequence conjugate. Cells were co-stained with phycoerythrin-conjugated mAb AF6 specific for Kb (upper panel) and mAb 25.D1.16 (lower panel) which recognizes Kb–Ova8 complexes (Porgador et al., 1997). 25.D1.16 staining is shown for cells gated for AF6-positive immunofluorescence. Negative controls (stippled lines) for AF6 reactivity were non-transfected cells, whereas 25.D1.16 staining was controlled with KB cells. (B) Pulse–chase analysis and immunoprecipitation of KBS and KBNS. Lanes 1–4: cells were labeled for 30 min and chased for 0, 1, 3 and 6 h, respectively. Lane 5: cells were labeled overnight (16 h) prior to solubilization in 1% NP-40 and immunoprecipitation with mAb Y3. Lane 5 was pasted from a shorter exposure of the same gel. (C) Expression of SS-Ova8 mRNA and Kb mRNA. Northern blots of RNA isolated from KB, KBS and KBNS were probed with labeled DNA fragments corresponding to Kb heavy chain (upper panel) or SS-Ova8 (lower panel). (D) Stimulation of T cell hybridoma B3 by SS-Ova8-expressing cells. KBs (triangles) and KBNS (squares) cells were fixed and serially diluted before co-incubating with B3 hybridoma cells (2×10^3/well) for 20 h. Addition of Ova8 prior to fixation is indicated by open symbols. Interleukin-2 production by B3 was measured by [3H]thymidine incorporation into CTLL2 cells. Results are the mean of duplicate measurements (±SD).
KBTNO-D4 cells, most Kb molecules remained Endo H sensitive even after 6 h of chase, indicating continuous tapasin retention. Since overexpression of the minigene Ova8 induced a more rapid release from tapasin retention (compare KBTNM with KBTNO-D4), it seems that insect cells were unable to provide sufficient peptides to release all of the Kb molecules from tapasin retention. However, TAP-dependent peptide transport seemed to provide more peptides than signal sequence-dependent translocation since intracellular transport of Kb in KBTNO-D4 cells was faster compared with KBNS cells (Figure 2B).

Peptides which bind with high affinity to MHC class I molecules stabilize solubilized MHC heterodimers at 37°C (Townsend et al., 1990). Furthermore, acquisition of such thermostability correlates with intracellular transport of MHC molecules in mammalian cells. To examine whether Kb molecules had acquired stabilizing peptides in insect cells upon co-transfection with tapasin, we incubated the respective lysates obtained after 6 h of chase for 1 h at 37°C with or without addition of Ova8 before immunoprecipitation (Figure 3B). A high proportion of Kb molecules were thermostable in both minigene-containing cell lines (Figure 3B), consistent with the mAb 25.D1.16 staining shown in Figure 3A. By contrast, KBTNO-D4 cells expressed mainly empty Kb molecules, similar to results obtained in the absence of TAP (Figure 1B). Thus it seems that, despite the presence of a functional transporter in these cells, no stabilizing peptides were acquired by the Kb molecules in the absence of tapasin. By contrast, the transported EndoH-resistant fraction of Kb expressed by the tapasin-containing clone KBTNO-D4 was thermostable while the majority of the EndoH-sensitive heterodimers dissociated at 37°C. Thus, the Kb molecules which were exported to the cell surface of KBTNO-D4 cells contained peptides which were of sufficient affinity to confer thermostability. The tapasin-retained fraction, however, was unstable, clearly demonstrating that tapasin binds specifically to MHC class I molecules which are either empty or do not contain stabilizing peptides. These data also show that Kb molecules were released from tapasin retention during the chase period after they acquired stabilizing peptides.

In an independent experimental approach, we tested the thermostability of surface-expressed Kb molecules by subjecting whole cells to temperature challenge prior to analyzing the presence of Kb heterodimers by cytofluorometry (Figure 3C). The mean fluorescence of Kb cells incubated at 37°C was ~20% of the mean fluorescence of cells incubated at 37°C in the presence of synthetic Ova8, indicating that most molecules did not contain stabilizing peptides. Also, KBTNO-D4 cells displayed predominantly empty Kb molecules at their cell surface. By contrast, 50–70% of Kb molecules at the cell surface of the two minigene-expressing cells were thermostable.

![Fig. 3. Tapasin requirement for TAP-dependent peptide loading in D. melanogaster cells.](image-url)

(A) Immunofluorescence and flow cytometry of SC2 cells transfected with Kb, β2m without tapasin (light line) or with tapasin (thick line) as well as the Ova8 epitope expressed as a minigene together with TAP (KBTM and KBTNM, left panels). Right panels: KBTNO-D4 cells also express tapasin (thick line). Co-staining with AF6 specific for Kb and mAb 25.D1.16, as well as negative controls (stippled lines) were as in Figure 2A. (B) Pulse–chase analysis and temperature stability of Kb molecules immunoprecipitated with Y3 antibody from NP-40 lysates of the indicated cell lines. Cells were metabolically labeled with [35S]methionine for 30 min prior to chase with unlabeled methionine for the indicated times. Lysates obtained after 6 h chase were incubated for 1 h at 37°C with or without Ova8 peptide. Prior to SDS-PAGE, all samples were digested with EndoH. Protein bands corresponding to co-precipitated β2m are not shown. (C) The stability of heterodimeric Kb molecules at the cell surface was analyzed by flow cytometry using mAb AF6 after incubating the indicated cell lines at 37°C for 1 h. The mean fluorescence is shown as a percentage relative to the mean fluorescence measured in the presence of 50 μg/ml Ova8 peptide. Results are the means of two independent experiments, (error bars = standard deviation).
Moreover, co-expression of tapasin rendered the majority of surface-expressed Kb molecules thermostable in KBTNO-D4 cells, thus confirming the thermostability observed by pulse–chase experiments. These results confirm that in the presence of tapasin, the surface-expressed fraction of the Kb molecules obtained stabilizing insect cell peptides imported by TAP.

In addition to the 25.D1.16-staining, we wanted to use specific T cells to prove that the peptide Ova8 was excised correctly from ovalbumin and loaded onto Kb molecules. However, both KBTNO-D4 and KBTO-B1 cells were unable to stimulate the Ova8–specific T cell hybridoma B3 (Figure 4A). By contrast, the Ova8 minigene-expressing cell lines KBTNM and KBTM stimulated B3 hybridomas in the absence of the synthetic peptide (Figure 4B). A possible explanation for this lack of T cell reactivity was that the numbers of Ova8–Kb complexes on the surface of KBTNO-D4 cells were insufficient to trigger T cell stimulation, as observed above for KBS cells (Figure 2D). To decrease the threshold for T cell triggering, we introduced ICAM-1 and B7.1, since it has been shown that expression of these adhesion and co-stimulatory molecules in Ld-transfected SC2 cells strongly increased T cell stimulation (Cai et al., 1996). Expression of the transfected molecules was verified by FACS (Kb, β2m, ICAM-1 and B7.1), immunoprecipitation (TAP1, TAP2, tapasin and ovalbumin) and TAP peptide transport assay (data not shown). The resulting cell lines KBTNOI7 and KBTNOI7 (Table I) were tested for stimulation of B3 cells in the presence or absence of exogenous Ova8. As shown in Figure 4C, both cell lines were equally effective in presenting the synthetic peptide. Without synthetic peptide added, however, the tapasin-containing cell line KBTNOI7 elicited a much stronger T cell response as compared with KBTOI7 cells. Thus, tapasin increased the efficiency of peptide presentation to T cells. These data are consistent with the 25.D1.16 staining and support the above notion that insect cells had faithfully generated the predominant epitope from ovalbumin.

**Insect cells generate peptides by proteasomal cleavage**

In mammalian cells, it has been shown that ovalbumin is cleaved by proteasomes to yield Ova8 (Crai et al., 1997). However, other proteases have been implicated in the generation of MHC class I-binding peptides as well (Beninga et al., 1998; Glas et al., 1998). To analyze whether proteasomal degradation was involved in the generation of Ova8 in the SC2 transfectants, we treated...
KBTNOI7 cells with the proteasome inhibitor lactacystin (Fenteany et al., 1995). In order to compare the effect of lactacystin on exogenous versus endogenous presentation of Ova8, we induced expression of the transfected genes 24 h prior to co-treatment of the cells with lactacystin, which was carried out for an additional 16 h. During the initial 24 h, sufficient Kb molecules were expressed to allow efficient T cell stimulation by exogenous peptide loading but not enough to trigger a T cell response from endogenously processed Ova8 (not shown). Therefore, presentation of synthetic Ova8 was not inhibited by additional incubation with lactacystin (Figure 4D). In contrast, presentation of SIINFEKL peptide from ovalbumin was markedly reduced by lactacystin treatment. We conclude that, as in mammalian cells, proteasomes are involved in ovalbumin degradation in D.melanogaster cells. The production of Kb-binding peptides therefore seems a result of the constitutive cytosolic protein turnover which is largely mediated by proteasomes.

**Tapasin-mediated association of MHC class I with TAP increases TAP heterodimer stability**

Tapasin is essential for the formation of a complex between MHC class I molecules and TAP (Ortmann et al., 1997). In addition, the chaperones calnexin, calreticulin and ER-60 are found in this complex (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). To investigate whether tapasin alone would be sufficient for MHC class I association with TAP in the heterologous ER folding compartment of D.melanogaster cells, we immunoprecipitated the complexes from digitonin lysates of KBTTO-B1 and KBTNO-D4 cells. As shown in Figure 5A, the TAP heterodimer was co-precipitated with Kb and tapasin in KBTNO-D4 cells. Either TAP-, tapasin- or Kb-specific antibodies co-precipitated the other molecules, although the amounts varied depending on the antibodies used. The individual proteins were identified by re-precipitation (Figure 5B). By contrast, TAP and Kb did not co-precipitate in the absence of tapasin in KBTTO-B1 cells. These data show that tapasin-mediated association of empty MHC class I molecules with TAP does not depend on the co-expression of mammalian calreticulin, calnexin or ER-60. Whether or not tapasin recruits the Drosophila homologs of these molecules (Christodoulou et al., 1997) to the complex remains to be investigated.

Interestingly, the anti-mTAP1 antisera co-precipitated less mTAP2 in the absence of tapasin (Figure 5A and B) despite the fact that both clones expressed similar amounts of mTAP2, as determined by immunoblotting with anti-mTAP2 antisera (Figure 5C). This suggested that tapasin influenced the stability of the TAP1–TAP2 heterodimer. This increased stability might also account for our observation that most KBTNO clones showed a higher TAP peptide transport activity than the KBTTO clones (data not shown), consistent with previous observations that peptide transport activity of TAP is higher in the presence of tapasin (Lehner et al., 1998). Increased heterodimer stability might also be responsible for the higher steady-state level of TAP observed upon tapasin transfection of .220 cells (Lehner et al., 1998). Thus, tapasin has several functions in the peptide loading process. In addition to controlling class I peptide loading, tapasin is responsible for facilitating formation of the Kb–TAP complex. Furthermore, tapeasin stabilized heterodimeric complexes of TAP1 and TAP2.

**Discussion**

By using D.melanogaster cells as an experimental system to rebuild the MHC class I pathway, we demonstrated that tapasin retains empty MHC class I molecules in the ER. Unlike the slower exit observed upon co-transfection of calnexin (Jackson et al., 1994) or calreticulin (D.Williams, personal communication) into D.melanogaster cells, intracellular transport of empty class I molecules was completely inhibited by tapasin. Peptide import into the ER either by TAP or by signal sequence-dependent trans-
A fraction of MHC class I molecules from tapasin retention, suggesting that tapasin is predominantly responsible for the ER retention of MHC class I molecules in TAP-deficient cells (Townsend et al., 1989). Thus, tapasin seems to be able to discriminate between the peptide-receptive and the peptide-bound conformation of MHC class I molecules. Furthermore, tapasin seems to monitor the quality of bound peptides because release from tapasin retention was dependent on the availability of peptides binding with high affinity to Kb, as shown by the fact that overexpression of the minigene Ova8 resulted in a higher proportion of transported Kb molecules when compared with peptides produced in the cytosol. We conclude that tapasin chaperones the peptide binding process by retaining MHC class I molecules until they acquire stabilizing peptides. Such a chaperone function for tapasin had been hypothesized earlier (Li et al., 1998; Alexander et al., 1998), but experimental support was missing until now. Since this quality control feature of tapasin also operates in the absence of TAP, we further conclude that the retention/release function of tapasin represents a distinct step in the antigen processing pathway which accounts for the observation that MHC class I molecules dissociate from TAP upon import of specific peptides (Ortmann et al., 1994; Suh et al., 1994) as well as for the finding that Ld molecules do not co-precipitate with calreticulin and tapasin after addition of specific peptides to lysates of TAP-deficient cells (Harris et al., 1998). Furthermore, the observation that HLA-B27 molecules transported to the cell surface in the absence of tapasin contained a different set of peptides rendering them unstable (Peh et al., 1998) is consistent with a quality control function of tapasin.

It might be that tapasin acts in a similar fashion to the HLA-DM molecule which monitors the peptide loading of MHC class II molecules in the endosomal compartment. HLA-DM (or H2-M in mouse) stabilizes MHC class II in a peptide-receptive state, thereby catalyzing the replacement of the invariant chain-derived low affinity peptide CLIP by peptides of higher affinity (Denzin and Cresswell, 1995; Weber et al., 1996). Similarly, tapasin might stabilize MHC class I during exchange of low affinity with high affinity peptides.

How tapasin senses the conformational changes occurring in the MHC class I molecule upon binding of stabilizing peptides currently is not known. However, some conclusions about residues involved in the class I–tapasin interaction can be drawn from studies investigating class I–TAP association. Co-immunoprecipitation experiments have indicated that residues within the peptide-binding groove may be involved in TAP association (Neisig et al., 1996). Moreover, a point mutation in the α2 domain of HLA-A2.1 molecules abrogates their ability to associate with TAP and accelerates their transport to the cell surface (Lewis et al., 1996; Peace-Brewer et al., 1996). Although the intracellular transport of these mutant molecules was dependent on TAP, they did not contain stabilizing peptides at the cell surface (Lewis and Elliot, 1998). Upon inhibition of intracellular transport by brefeldin A, however, stabilizing peptides were acquired, thus indicating a missing interaction with a retention molecule. Our demonstration that tapasin retains MHC class I implicates tapasin for this role. The observed lack of association of HLA-A2.1 T134K with calreticulin (Lewis and Elliot, 1998) could be secondary to a missing tapasin interaction. The peptide-binding site of MHC class I molecules is in a molten globule state in the absence of peptides (Bouvier and Wiley, 1998), undergoing a conformational change upon peptide insertion (Rigney et al., 1998). By interacting with the peptide-binding domain, tapasin might monitor these changes during peptide loading.

For murine MHC class I alleles, the α3 domain has been implicated in the association with tapasin–TAP (Carreno et al., 1995; Suh et al., 1996; Harris et al., 1998; Kulig et al., 1998). Whether or not human class I molecules also interact with tapasin via the α3 domain remains to be investigated. Since murine tapasin failed to promote peptide loading of some human class I alleles, it seems that there are species-specific differences in this interaction (Peh et al., 1998). Similarly, human tapasin binds less efficiently to the murine molecule Kb in insect cell transfectants (our unpublished observations). Moreover, Yewdell and co-workers did not observe an effect of human tapasin on murine MHC class I molecules expressed by vaccinia virus in a mosquito cell line (Deng et al., 1998). Failure of human tapasin to retain murine MHC class I might cause empty murine MHC molecules to be transported to the cell surface of TAP-deficient human cells (Alexander et al., 1989). In addition to species differences, it seems that different alleles within one species (human) show a distinctive dependence on tapasin for surface expression and antigen presentation (Peh et al., 1998). Thus, it could be that tapasin binds with different affinity to various alleles. However, murine MHC alleles did not differ markedly in their retention by tapasin in insect cells (our unpublished observations). Alternatively, the availability of peptides binding to the various MHC alleles could determine their individual dependence on tapasin for obtaining stabilizing peptides. The latter assumption is supported by our observation that high levels of Ova8 expression obliterated the need for tapasin to promote assembly with peptides.

This observation also shows that house-keeping chaperones such as calnexin and calreticulin, which have Drosophila homologs (Christodoulou et al., 1997), are sufficient for MHC class I molecules to fold into a peptide-receptive conformation even in the absence of tapasin. By contrast, invertebrates lack the MHC class I-specific retention/release function of tapasin. Insect cells also lack the ability to promote MHC class I association with TAP, similarly to tapasin-deficient mammalian cells (Ortmann et al., 1997). The finding that tapasin can perform this function in the invertebrate folding environment suggests that additional chaperones found in the TAP–tapasin complex (Sadasivan et al., 1996; Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998) are either not necessary for complex formation or can be replaced by eukaryotic house-keeping chaperones. By contrast, tapasin was not only necessary but sufficient for complex formation. In addition, tapasin enhanced TAP1–TAP2 association in insect cells, which is consistent with the previous finding that tapasin association with TAP increased the peptide transport activity in .220 cells (Lehner et al., 1998). The multiple functions of tapasin seem to be localized to separate parts of the tapasin.
molecule. Whereas the interaction with MHC class I molecules involves the luminal domain of tapasin, it seems that the interaction with TAP localizes in the transmembrane domain of tapasin, since a truncated version of tapasin bound to MHC class I but not to TAP (Lehner et al., 1998). Interestingly, tail-deleted tapasin was still able to promote peptide loading despite its secretion due to the missing ER retention signal (Lehner et al., 1998). It seems likely that tail-deleted tapasin resides long enough in the ER to chaperone peptide loading, particularly since transfection ensured a constant supply of highly expressed truncated tapasin in these experiments. Thus, the molecular chaperone activity of tapasin seems to be less dispensable than the tapasin-dependent promotion of TAP association and peptide transport.

The importance for tapasin-mediated editing of peptide loading is also stressed by the finding that tapasin was required to reconstitute antigen processing in invertebrate cells. Retention/release by tapasin was as crucial for antigen processing as peptide transport by TAP and peptide display by MHC class I. In contrast to these dedicated molecules which were developed during the evolution of the MHC (Herberg et al., 1998), the ability to generate peptides from antigens seems to be already present in lower eukaryotes. The observation that Ova8 presentation could be inhibited by lactacystin implicates the proteasome in the processing of ovalbumin in D. melanogaster cells, as observed in mammalian cells (Craiu et al., 1997). The ability of invertebrate proteasomes to generate the correct epitopes from precursor peptides has also been demonstrated in vitro (Niedermann et al., 1997). Taken together, these data support the hypothesis that the MHC class I system has taken advantage of the pre-existing ubiquitin–proteasome system to display the by-products of protein turnover at the cell surface. Peptide generation for MHC class I was optimized during vertebrate evolution due to the development of interferon-γ-induced subunits, some encoded in the MHC, as well as modulators of the proteasome (Früh and Yang, 1999). However, our data suggest that these modular subunits are not absolutely required for antigen processing. Thus, the minimal specific components which are needed for MHC class I antigen presentation are MHC class I, TAP and tapasin.

Materials and methods

Antibodies

Rabbit antisera to murine tapasin (Grandea et al., 1998) were raised against bovine serum albumin (BSA)-conjugated peptides corresponding to either the 20 amino acids (anti-Tpn-N) following the predicted signal sequence or the C-terminal 20 amino acids (anti-Tpn-C). mAb Y3 recognizes heterodimeric Kb molecules (Hämmerling et al., 1982). Phycocerythrin-conjugated Kb-specific antibody AF6-88.5 was purchased from Pharmingen. Polyclonal anti-Kb antiserum 270 as well as Phycoerythrin-conjugated Kb-specific antibody AF6-88.5 was purchased from Pharmingen. Polyclonal anti-Kb antiserum 270 as well as anti-Tpn-C. mAb Y3

Immunoblot

Labeled probes for SS-Ova8 or Kb-HC were prepared by PCR amplification followed by 32P-labeling using an oligonucleotide labeling kit (Pharmacia). Unincorporated nucleotides were removed by column purification (Qiagen). Probes were denatured and added to the hybridization solution at a concentration of ~3×106 c.p.m./m.l. FastTrack 2.0 mRNA isolation kit (Invitrogen) was used according to the manufacturer’s instructions to isolate mRNA from ~10⁶ cells. mRNA was separated on a 1.5% (w/v) agarose gel containing formaldehyde and transferred to a nylon membrane in 20× SSC. The mRNA was cross-linked to the nylon membrane by two cycles of irradiation at 1200 μjoules/ml. Pre-hybridization was performed for 4 h in Northern pre-hybridization buffer (5→3) containing 50% (v/v) formamide and denatured salmon sperm DNA at 42°C. Hybridization was performed overnight in Northern hybridization buffer (5→3) containing 50% (v/v) formamide and denatured salmon sperm and probe DNA. The blots were washed twice in 2× SSC, 0.1% SDS and twice in 0.1× SSC, 0.1% SDS at 50°C prior to autoradiography.

T cell assay

B3.1 hybridoma cells (Carbone et al., 1992) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with glutamine (Gibco-BRL), 10% FCS, 10% NCTC-109 (BioWhittaker) and 700 μg/ml G418 (Calbiochem). SC2 cell transfecants induced for 48 h with 1 mM CuSO₄ were washed once in defined Insect Xpress medium (BioWhittaker) and incubated for 4 h with or without synthetic peptide Ova8 (SIINFEKL). Cells were washed twice in phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde in PBS for 15 min at room temperature. Free aldehyde was quenched using NH₄Cl, 50 mM in PBS. After an additional two washes in PBS, cells were serially diluted in 96-well plates in a volume of 100 μl. A total of 2×10⁵ B3 cells/well were co-incubated for 20 h and the supernatant was harvested. CTLL-2 cells were washed five times in CTLL2 medium [RPMI (Gibco-BRL), 10% FCS, 50 μM β-mercaptoethanol, 10% NCTC-109] and adjusted to 5000 cells/well. At 24 h after adding 100 μl of B3 supernatant, 1 μCi/well of [3H]-thymidine (NEN) was added. After 16 h, cells were harvested and counted by scintillation.

Metabolic labeling and immunoprecipitation

Approximately 10⁶ transfected cells induced for 24 h in 1 mM CuSO₄ were used for each immunoprecipitation. Prior to labeling, cells were starved for 30 min in Grace’s insect medium without methionine (Gibco-BRL). Cells were labeled with 0.5 μCi/ml of [35S]methionine for 0.5–16 h. For chase experiments, labeled cells were washed once in PBS and incubated in complete Grace’s medium (Gibco-BRL) for the respective time periods. Cells were lysed by 4°C in PBS containing either 1% NP-40 (Sigma) or 1% digitonin (Calbiochem) for 30 min. Lysates were cleared of nuclei and debris by 30 min centrifugation.
at 14 000 r.p.m. in the microcentrifuge. For temperature challenge experiments, lysates were incubated for 1 h at 37°C. After pre-clearing with protein A-Sepharose beads (Pharmacia), the cleared lysate was incubated for 4 h with the respective antibodies which subsequently were bound to protein A-Sepharose beads. Bound antigen complexes were washed five times in PBS, 0.1% Triton X-100 (Sigma) or 0.1% digitonin and, when needed, digested with endoglycosidase H (Boehringer Mannheim) according to the manufacturer’s specifications.

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References


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