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 multi-subunit 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 β_2-microglobulin ($\beta2m$). 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 $\beta2m$ 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 trans-membrane 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 immuno-precipitated complexes. By using the heterodimer-specific antibody Y3, Kb heterodimers could only be immuno-precipitated 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 precipita-tion (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-transfec-tion 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

Code[a]	Transfected gene					
	Tapasin[b] (N)	TAP[c] (T)	Ova8[d] (M)	SS-Ova8[e] (S)	Ovalbumin[f] (O)	ICAM-1/B7.1[g] (I/7)
KB						
KBN	o					
KBS				o		
KBNS	o			o		
KBTO-B1		o			o	
KBTNO-D4	o	o			o	
KBTM		o	o			
KBTNM	o	o	o			
KBTOI7		o			o	o
KBTNOI7	o	o			o	o

[a]All cell lines were co-transfected with Kb heavy chain (K) and murine β2 (B) microglobulin.
[b]Murine tapasin (N; Grandea *et al.* 1998).
[c]Murine TAP1 and TAP2 (T) were co-transfected in these cell lines.
[d]The minigene (M) sequence is MSIINFEKL.
[e]The signal sequence (S)-bearing Ova8 sequence is MALPVTALLLPLALLLHAARPSIINFEKL.
[f]Chicken ovalbumin (O).
[g]Murine co-stimulatory molecules ICAM-1 (I) and B7.1 (7) were co-transfected in these cell lines.

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.
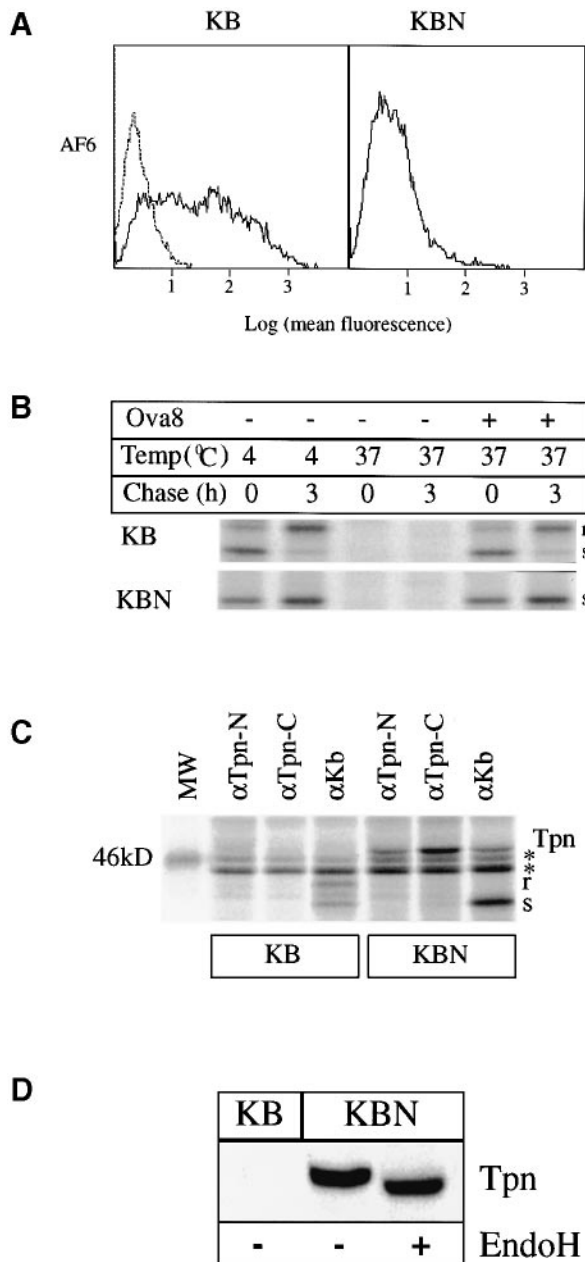

Fig. 1. Tapasin prevents intracellular transport of empty MHC class I molecules. (A) Immunofluorescence and flow cytometry of *D.melanogaster* SC2 cells transfected with Kb, β2m (KB; left panel) and tapasin (KBN; right panel). Kb molecules were detected by phyocerythrin-conjugated mAb AF6 (Pharmingen). 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. All samples were 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 were 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 KBTNO cells were also co-transfected with tapasin cDNA (Table I). After G418 selection and single cell cloning, two clones (KBTO-B1 and KBTNO-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 KBTNO-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
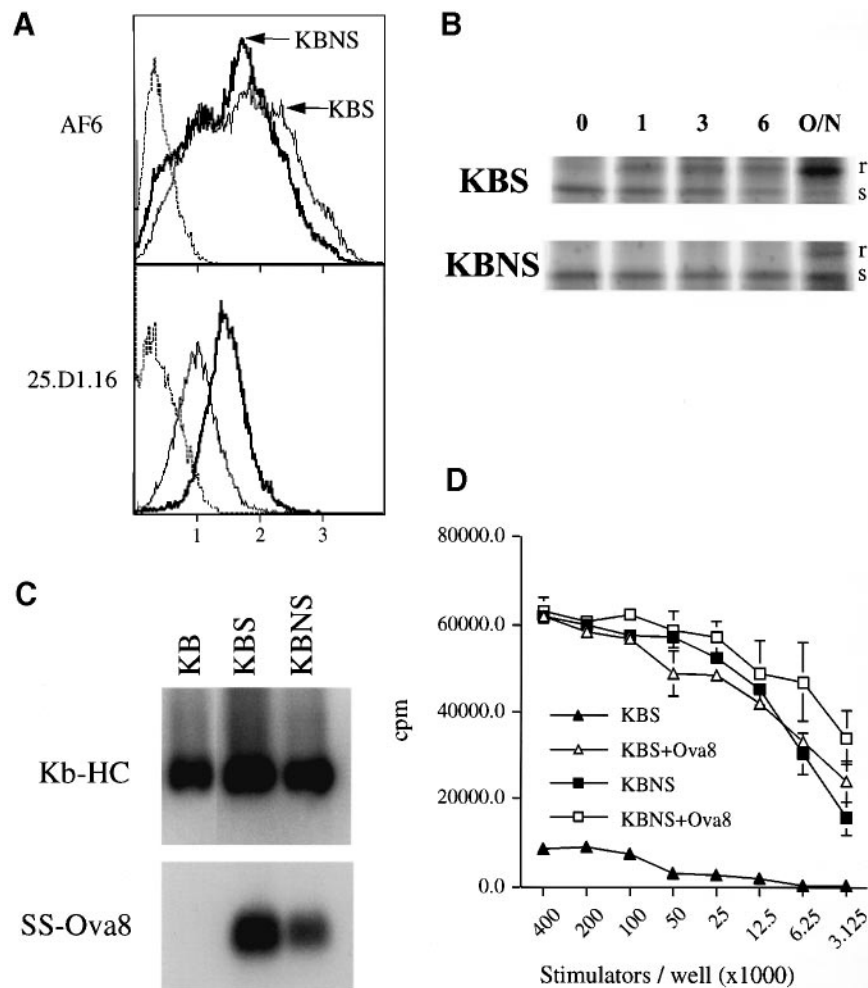
Fig. 2. Tapasin does not retain peptide-loaded MHC class I molecules and increases the efficiency of complex formation independently of TAP. (**A**) Immunofluorescence and flow cytometry of SC2 cells transfected with Kb, β2m without tapasin (KBS cells, thin line) or with tapasin (KBNS cells, thick line) 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 ($2{\times}10^5$/well) for 20 h. Addition of Ova8 prior to fixation is indicated by open symbols. Interleukin-2 production by B3 was measured by [^{3}H]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
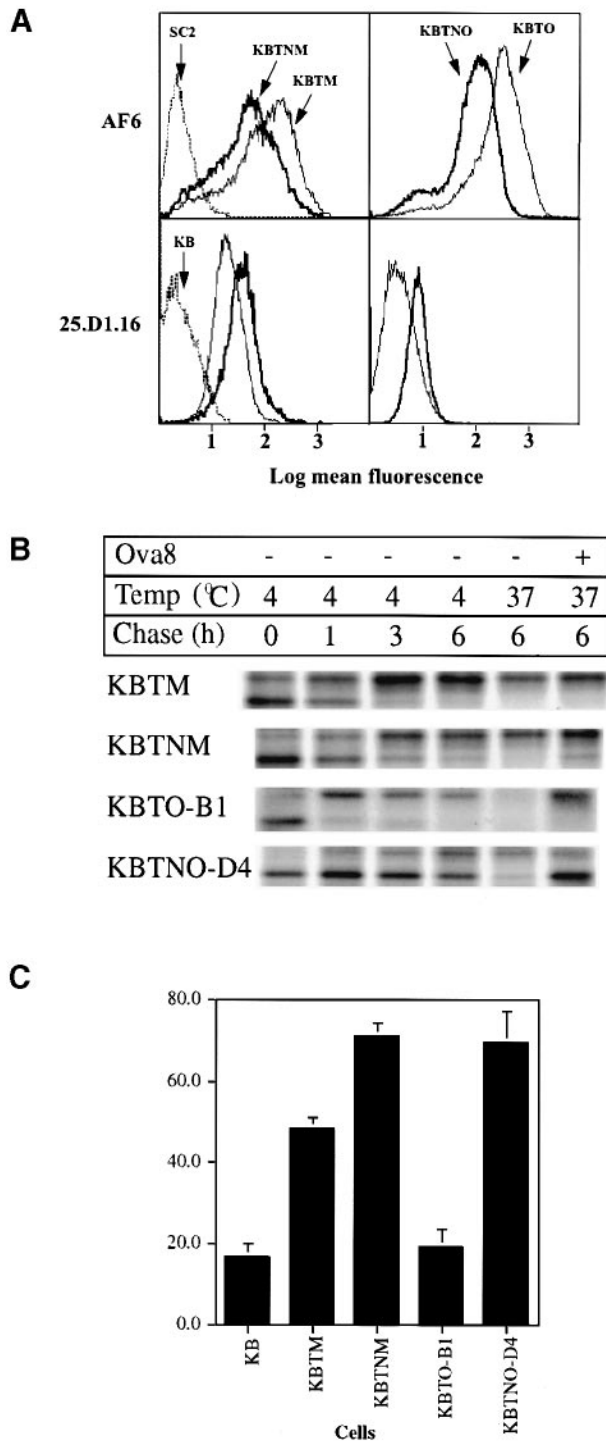
molecules stabilize solubilized MHC heterodimers at 37°C (Townsend *et al.*, 1990). Furthermore, acquisition of such thermostability correlates with intracellular transport of MHC molecules in mammalian cells. To examine whether Kb molecules had acquired stabilizing peptides in insect cells upon co-transfection 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, KBTO-B1 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, KBTO-B1 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. (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: KBTO-B1 cells co-express TAP with ovalbumin (light line). 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).
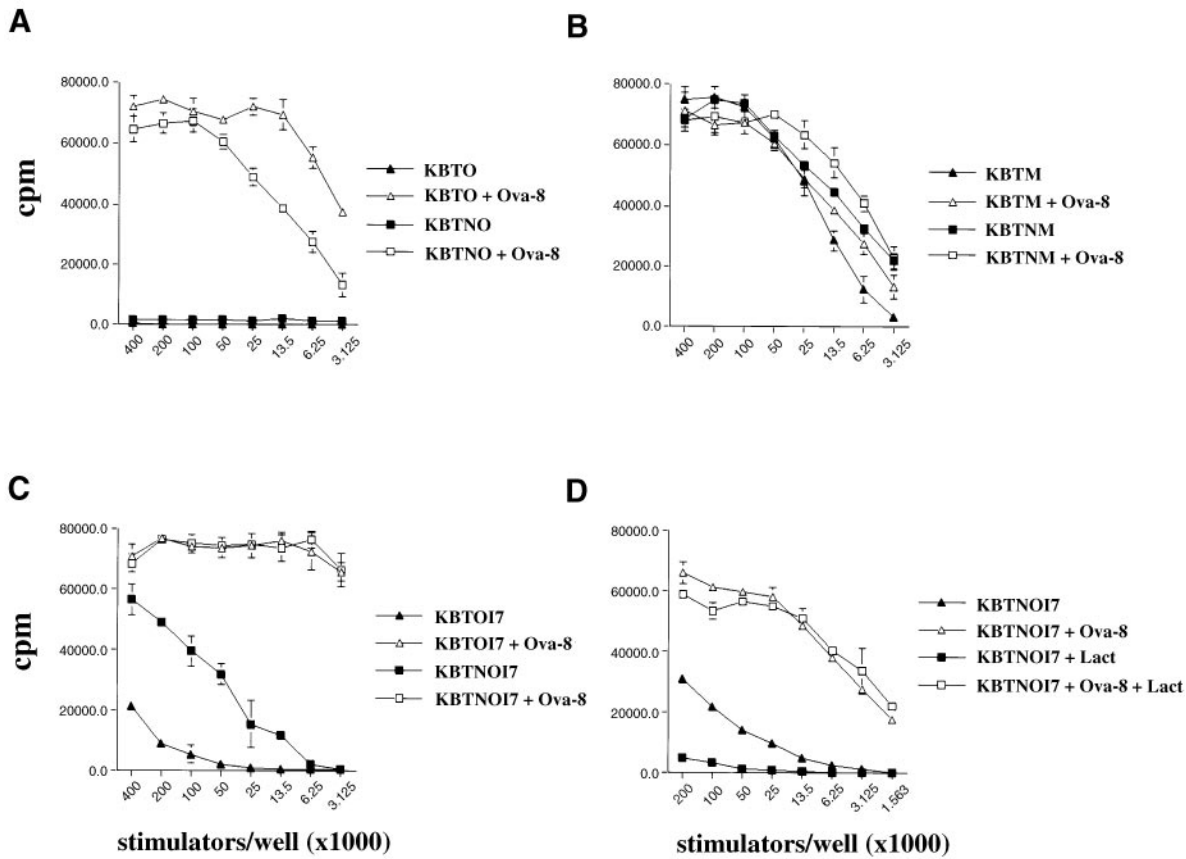
Fig. 4. T cell stimulation by ovalbumin-containing SC2 transfectants requires tapasin and the proteasome. (**A**) Stimulation of B3 hybridomas with KBTO-B1 cells expressing ovalbumin (triangles) or KBTNO-D4 cells expressing ovalbumin and tapasin (squares). Prior addition of Ova8 peptide is indicated by open symbols. Results are the mean of duplicate measurements ($\pm$SD). (**B**) B3 assay with the minigene-expressing cell lines KBTM (triangles) or KBTNM (squares). T cells were stimulated by both cell lines in the presence (open symbols) or absence (filled symbols) of Ova8 peptide. (**C**) T cell stimulation with SC2 cells co-transfected with Kb, TAP, ovalbumin, B7.1 and ICAM-1 (KBTOI7, triangles) and tapasin (KBTNOI7, squares). Prior addition of Ova8 peptide is indicated by open symbols. (**D**) KBTNOI7 cells induced for 40 h and either untreated (triangles) or treated with 10 µM lactacystin (Calbiochem; squares) for 16 h prior to the T cell assay. Note that less stimulators were used in this experiment than in (B).

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 unless synthetic Ova8 was added to the cells (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 KBTOI7 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 (Craiu *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 KBTO-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 KBTO-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 antiserum 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 antiserum (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 KBTO 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,
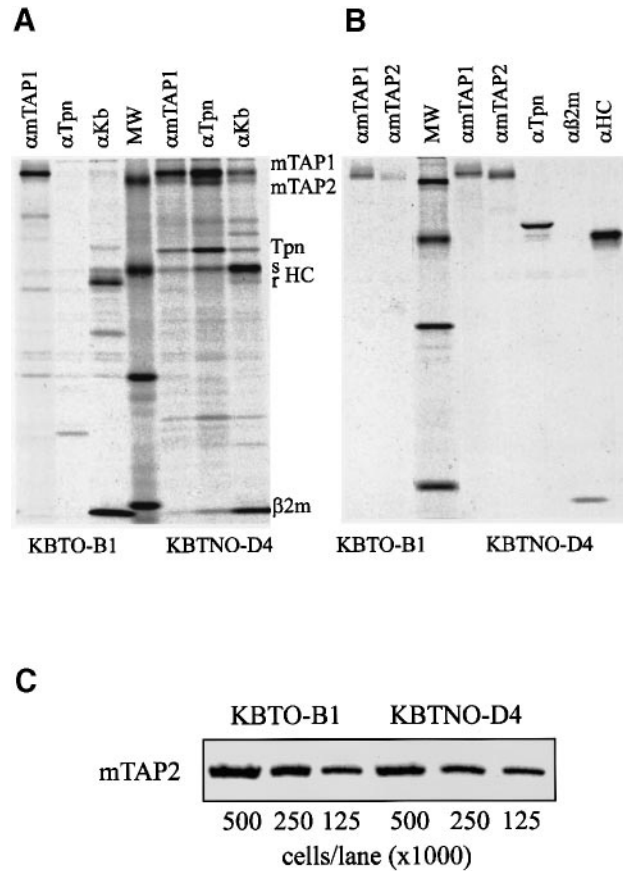
Fig. 5. Tapasin is sufficient for MHC class I binding to TAP and increases the stability of TAP heterodimers. (**A**) KBTO-B1 and KBTNO-D4 cells were metabolically labeled with [^{35}S]methionine for 16 h and lysed in 1% digitonin. To reduce non-specific precipitations, lysates were mixed with a 10-fold excess of unlabeled, non-transfected SC2 cell lysate. Immunoprecipitations obtained with the indicated antibodies were separated on a 8–15% SDS–gel without EndoH treatment. EndoH-resistant HCs show a faster electrophoretic mobility due to the absence of sialic acid addition in insect cells. Molecular weight markers (MW, Bio-Rad) correspond to 69, 46, 30 and 14.3 kDa. (**B**) Complexes of TAP, Kb and tapasin precipitated from KBTNO lysates by anti-Kb antibodies as well as the anti-TAP1 immunoprecipitate of KBTO-B1 cells were denatured in 1% SDS. After 10-fold dilution in 1% NP-40, the respective polypeptides were reprecipitated using the indicated antibodies. Kb heavy chain was precipitated with antiserum 193, β2m was reacted with antiserum K355. Samples were separated on a 10–15% gradient gel. Molecular weight markers were as in (A). (**C**) Comparison of mTAP2 expression in KBTO-B1 and KBTNO-D4 cells in immunoblot. Serial dilutions of lysates corresponding to the indicated cell numbers were probed with anti-mTAP2 antiserum

tapasin 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-

location released 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.*, 1997; Lehner *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 modulatory 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). Phycoerythrin-conjugated Kb-specific antibody AF6-88.5 was purchased from Pharmingen. Polyclonal anti-Kb antiserum 270 as well as β2m-specific antiserum K355 was described in Andersson *et al.* (1985). Anti-Kb HC antiserum 193 was generated against a synthetic peptide corresponding to the cytoplasmic tail of Kb (Song *et al.*, 1994). Antisera against recombinant ATP-binding domains of murine TAP1 and murine TAP2 were described previously (Früh *et al.*, 1992; Wang *et al.*, 1994).

DNA constructs
All cDNAs were inserted into expression plasmid pRMHa.3 under control of the Cu^{2+}-inducible metallothionein promoter (Jackson *et al.*, 1992). Mouse tapasin cDNA (Grandea *et al.*, 1998) was subcloned into pRMHa3 using *Sma*I and *Bam*HI restriction sites. Construction of vectors carrying Kb, β2m and ovalbumin was published previously (Jackson *et al.*, 1992); murine TAP1 and TAP2 constructs were described in Ahn *et al.* (1996). ICAM-1 as well as B7 expression plasmids were described in Cai *et al.* (1996). Oligonucleotides encoding SS-Ova8 (sequence: MALPVTALLLPLALLLHAARPSIINFEKL) or M-Ova8 (sequence: MSIINFEKL) were inserted into the *Sac*I and *Bam*HI restriction sites of pRMHa3.

Cell lines and cloned derivatives
SC2 cells were grown at 28°C in Schneider's medium with 10% fetal calf serum (FCS). A total of 1.2×10^7 cells were transfected with 24 µg of DNA by the $CaPO_4$ method using phshsneo as a marker selectable by 500 µg/ml of G418 (Jackson *et al.*, 1992). Individual cell lines were transfected with the respective DNA constructs. Stable cell lines were obtained after 4 weeks of selection in G418. Single cell clones were generated by limiting dilution using irradiated SC2 cells as feeders. Clones were grown initially in Schneider's medium containing 20% FCS. G418 was added as soon as individual colonies became visible.

Immunoblot
Cell pellets were resuspended either directly in SDS sample buffer or solubilized in 1% NP-40 prior to adding sample buffer. Samples were separated on a 10–20% minigel (Bio-Rad) and electrophoretically transferred onto Hybond membranes (Amersham). Non-specific binding sites were blocked using 5% skim milk powder in Tris-buffered saline, 0.3% Tween-20 (TBST). Bound antibodies were detected using horse-radish peroxidase-conjugated secondary antibody and chemiluminescence (Amersham).

RNA blot
Labeled probes for SS-Ova8 or Kb-HC were prepared by PCR amplification followed by ^{32}P-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 ~3×10^6 c.p.m./ml.

FastTrack 2.0 mRNA isolation kit (Invitrogen) was used according to the manufacturer's instructions to isolate mRNA from ~10^8 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×100. 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 transfectants induced for 48 h with 1 mM $CuSO_4$ 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 NH_4Cl, 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 2×10^5 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 [^{3}H]-thymidine (NEN) was added. After 16 h, cells were harvested and counted by scintillation.

Metabolic labeling and immunoprecipitation
Approximately 10^7 transfected cells induced for 24 h in 1 mM $CuSO_4$ 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 mCi/ml of [^{35}S]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 at 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.

Acknowledgements

References

Ahn,K., Meyer,T.H., Uebel,S., Sempé,P., Djaballah,H., Yang,Y., Peterson,P.A., Früh,K. and Tampé,R. (1996) Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47. *EMBO J.*, **15**, 3247–3255.

Alexander,J., Payne,J.A., Murray,R., Frelinger,J.A. and Cresswell,P. (1989) Differential transport requirements of HLA and H-2 class I glycoproteins. *Immunogenetics*, **29**, 380–388.

Anderson,K., Cresswell,P., Gammon,M., Hermes,J., Williamson,A. and Zweerink,H. (1991) Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. *J. Exp. Med.*, **174**, 489–492.

Andersson,M., Pääbo,S., Nilsson,T. and Peterson,P.A. (1985) Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell*, **43**, 215–222.

Beninga,J., Rock,K.L. and Goldberg,A.L. (1998) Interferon-γ can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.*, **273**, 18734–18742.

Bouvier,M. and Wiley,D.C. (1998) Structural characterization of a soluble and partially folded class I major histocompatibility heavy chain/β2m heterodimer. *Nature Struct. Biol.*, **5**, 377–384.

Cai,Z., Brunmark,A., Jackson,M.R., Loh,D., Peterson,P.A. and Sprent,J. (1996) Transfected *Drosophila* cells as a probe for defining the minimal requirements for stimulating unprimed CD8+ T cells. *Proc. Natl Acad. Sci. USA*, **93**, 14736–14741.

Carbone,F.R. and Bevan,M.J. (1989) Induction of ovalbumin-specific cytotoxic T cells by *in vivo* peptide immunization. *J. Exp. Med.*, **169**, 603–612.

Carbone,F.R., Sterry,S.J., Butler,J., Rodda,S. and Moore,M.W. (1992) T cell receptor alpha-chain pairing determines the specificity of residue 262 within the Kb-restricted, ovalbumin 257–264 determinant. *Int. Immunol.*, **4**, 861–867.

Carreno,B.M., Solheim,J.C., Harris,M., Stroynowski,I., Connally,J.M. and Hansen,T.H. (1995) TAP associates with a unique class I conformation, whereas calnexin associates with multiple class I forms in mouse and man. *J. Immunol.*, **155**, 4726–4733.

Christodoulou,S., Lockyer,A.E., Foster,J.M., Hoheisel,J.D. and Roberts,D.B. (1997) Nucleotide sequence of a *Drosophila melanogaster* cDNA encoding a calnexin homolog. *Gene*, **191**, 143–148.

Craiu,A., Akopian,T., Goldberg,A. and Rock,K.L. (1997) Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc. Natl Acad. Sci. USA*, **94**, 10850–10855.

Deng,Y. *et al.* (1998) Assembly of MHC class I molecules with biosynthesized endoplasmic reticulum-targeted peptides is inefficient in insect cells and can be enhanced by protease inhibitors. *J. Immunol.*, **161**, 1677–1685.

Denzin,L.K. and Cresswell,P. (1995) HLA-DM induces CLIP dissociation from MHC class II ab dimers and facilitates peptide loading. *Cell*, **82**, 155–165.

Fenteany,G., Standaert,R.F., Lane,W.S., Choi,S., Corey,E.J. and Schreiber,S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*, **268**, 726–731.

Früh,K. and Yang,Y. (1999) Regulation of MHC class I antigen presentation by interferon gamma. *Curr. Opin. Immunol.*, in press.

Früh,K., Yang,Y., Arnold,D., Chambers,J., Wu,L., Waters,J.B., Spies,T. and Peterson,P.A. (1992) Alternative exon usage and processing of the major histocompatibility complex-encoded proteasome subunits. *J. Biol. Chem.*, **267**, 22131–22140.

Glas,R., Bogyo,M., McMaster,J.S., Gaczynska,M. and Ploegh,H.L. (1998) A proteolytic system that compensates for loss of proteasome function. *Nature*, **392**, 618–622.

Goldberg,A.L. and Rock,K.L. (1992) Proteolysis, proteasomes and antigen presentation. *Nature*, **357**, 376–378.

Grandea,A.,III, Androlewicz,M.J., Athwal,R.S., Geraghty,D.E. and Spies,T. (1995) Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science*, **270**, 105–108.

Grandea,A.G.,III, Comber,P.G., Wenderfer,S.E., Schoenhals,G.J., Früh,K., Monaco,J.J. and Spies,T. (1998) Sequence, linkage to H2K and function in MHC class I assembly of mouse tapasin. *Immunogenetics*, **48**, 260–265.

Hämmerling,G.J., Rusch,E., Tada,N., Kimura,S. and Hämmerling,U. (1982) Localization of allodeterminants on H-2Kb antigens determined with monoclonal antibodies and H-2 mutant mice. *Proc. Natl Acad. Sci. USA*, **79**, 4737–4741.

Harris,M.R., Yu,Y.Y.L., Kindle,C.S., Hansen,T.H. and Solheim,J.C. (1998) Calreticulin and calnexin interact with different protein and glycan determinants during the assembly of MHC class I. *J. Immunol.*, **160**, 5404–5409.

Heemels,M.-T. and Ploegh,H. (1995) Generation, translocation and presentation of MHC class I-restricted peptides. *Annu. Rev. Biochem.*, **64**, 463–491.

Herberg,J.A., Sgouros,J., Jones,T., Copeman,J., Humphray,S.J., Sheer,D., Cresswell,P., Beck,S. and Trowsdale,J. (1998) Genomic analysis of the tapasin gene, located close to the TAP loci in the MHC. *Eur. J. Immunol.*, **28**, 459–467.

Hughes,E. and Cresswell,P. (1998) The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. *Curr. Biol.*, **8**, 709–712.

Jackson,M.R., Nilsson,T. and Peterson,P.A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.*, **9**, 3153–3162.

Jackson,M.R., Song,E.S., Young,Y. and Peterson,P.A. (1992) Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in *Drosophila melanogaster* cells. *Proc. Natl Acad. Sci. USA*, **89**, 12117–12121.

Jackson,M.R., Cohen-Doyle,M.F., Peterson,P.A. and Williams,D.B. (1994) Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science*, **263**, 384–387.

Kulig,K., Nandi,D., Bacik,I., Monaco,J.J. and Vukmanovic,S. (1998) Physical and functional association of the major histocompatibility complex class I heavy chain α3 domain with the transporter associated with antigen processing. *J. Exp. Med.*, **187**, 865–874.

Lehner,P.J., Surman,M.J. and Cresswell,P. (1998) Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220. *Immunity*, **8**, 221–231.

Lewis,J.W. and Elliot,T. (1998) Evidence for successive peptide binding and quality control stages during MHC class I assembly. *Curr. Biol.*, **8**, 717–720.

Lewis,J.W., Neisig,A., Neefjes,J. and Elliot,T. (1996) Point mutations in the alpha 2 domain of HLA-A2.1 define a functionally relevant interaction with TAP. *Curr. Biol.*, **6**, 873–883.

Li,S., Sjoegren,H.-O., Hellman,U., Pettersson,R.F. and Wang,P. (1997) Cloning and functional characterization of a subunit of the transporter associated with antigen processing. *Proc. Natl Acad. Sci. USA*, **94**, 8708–8713.

Lindquist,J.A., Jensen,O.N., Mann,M. and Hammerling,G.J. (1998) ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. *EMBO J.*, **17**, 2186–2195.

Morrice,N.A. and Powis,S.A. (1998) A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. *Curr. Biol.*, **8**, 713–716.

Neefjes,J.J., Momburg,F. and Hämmerling,G.J. (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science*, **261**, 769–771.

Neisig,A., Wubbolts,R., Zang,X., Melief,C. and Neefjes,J. (1996) Allele-specific differences in the interaction of MHC class I molecules with transporters associated with antigen processing. *J. Immunol.*, **156**, 3196–3206.

Niedermann,G. *et al*. (1997) Potential immunocompetence of proteolytic fragments produced by proteasomes before evolution of the vertebrate immune system. *J. Exp. Med.*, **186**, 209–220.

Noessner,E. and Parham,P. (1995) Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J. Exp. Med.*, **181**, 327–337.

Ortmann,B., Androlewicz,M.J. and Cresswell,P. (1994) MHC class I/β2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature*, **368**, 864–867.

Ortmann,B. *et al*. (1997) A critical role for tapasin in the assembly and function of multimeric MHC class I–TAP complexes. *Science*, **277**, 1306–1309.

Peace-Brewer,A.L., Tussey,L.G., Matsui,M., Li,G., Quinn,D.G. and Frelinger,J.A. (1996) A point mutation in HLA-A*0201 results in failure to bind to the TAP complex and to present virus derived peptides to CTL. *Immunity*, **4**, 505–514.

Peh,C.A., Burrows,S.R., Barnden,M., Khanna,R., Cresswell,P., Moss,D.J. and McCluskey,J. (1998) HLA-B27-restricted antigen in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity*, **8**, 531–542.

Porgador,A., Yewdell,J.W., Deng,Y., Bennink,J.R. and Germain,R.N. (1997) Localization, quantitation and *in situ* detection of specific peptide–MHC class I complexes using a monoclonal antibody. *Immunity*, **6**, 715–726.

Rammensee,H.G., Falk,K. and Rötschke,O. (1993) Peptides naturally presented by MHC class I molecules. *Annu. Rev. Immunol.*, **11**, 213–244.

Rigney,E., Kojima,M., Glithero,A. and Elliott,T. (1998) A soluble major histocompatibility complex class I peptide-binding platform undergoes a conformational change in response to peptide epitopes. *J. Biol. Chem.*, **273**, 14200–14204.

Sadasivan,B., Lehner,P.J., Ortmann,B., Spies,T. and Cresswell,P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity*, **5**, 103–114.

Song,E.S., Yang,Y., Jackson,M.R. and Peterson,P.A. (1994) *In vivo* regulation of the assembly and intracellular transport of class I major histocompatibility complex molecules. *J. Biol. Chem.*, **269**, 7024–7029.

Suh,W.-K., Cohen-Doyle,M.F., Früh,K., Wang,K., Peterson,P.A. and Williams,D.B. (1994) Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science*, **264**, 1322–1326.

Suh,W.-K., Mitchell,E.K., Yang,Y., Peterson,P.A., Waneck,G.L. and Williams,D.B. (1996) MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J. Exp. Med.*, **184**, 337–348.

Townsend,A. and Trowsdale,J. (1993) The transporters associated with antigen presentation. *Semin. Cell Biol.*, **4**, 53–61.

Townsend,A., Öhlén,C., Bastin,J., Ljunggren,H.-G., Foster,L. and Kärre,K. (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature*, **340**, 443–448.

Townsend,A., Elliott,T., Cerundolo,V., Foster,L., Barber,B. and Tse,A. (1990) Assembly of MHC class I molecules analyzed *in vitro*. *Cell*, **62**, 285–295.

Vassilakos,A., Cohen-Doyle,M.F., Peterson,P.A., Jackson,M.R. and Williams,D.B. (1996) The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules. *EMBO J.*, **15**, 1495–506.

Wang,K., Früh,K., Peterson,P.A. and Yang,Y. (1994) Nucleotide binding of the C-terminal domains of the major histocompatibility complex-encoded transporter expressed in *Drosophila melanogaster* cells. *FEBS Lett.*, **350**, 337–341.

Weber,D.A., Evavold,B.D. and Jensen,P.E. (1996) Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science*, **274**, 618–620.