Nonclassical Behavior of the Thymus Leukemia Antigen: Peptide Transporter–independent Expression of a Nonclassical Class I Molecule

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Summary

The thymus leukemia (TL) antigen is a major histocompatibility complex–encoded nonclassical class I molecule. Here we present data demonstrating that expression of the TL antigen, unlike other class I molecules, is completely independent of the function of the transporter associated with antigen processing (TAP). The TL antigen is expressed by transfected TAP-2–deficient RMA-S cells when these cells are grown at 37°C. In transfected RMA cells, the kinetics of arrival of TL antigen on the cell surface are similar to those of a classical class I molecule. The kinetics are not altered in TAP-deficient RMA-S cells, demonstrating that surface TL expression in TAP-deficient cells is not due to the stable expression of a few molecules that leak out by a TAP-independent pathway. Soluble TL molecules produced by Drosophila melanogaster cells are highly resistant to thermal denaturation, unlike peptide-free classical class I molecules synthesized by these insect cells. In addition, these soluble TL molecules are devoid of detectable bound peptides. The results demonstrate that the TL antigen is capable of reaching the surface without bound peptide, although acquisition of peptide or some other ligand through a TAP-independent pathway cannot be formally excluded. We speculate that the ability of the TL antigen to reach the cell surface, under conditions in which other class I molecules do not, may be related to a specialized function of the TL molecule in the mucosal immune system, and possibly in the stimulation of intestinal $\gamma\delta$ T cells.

G enes located in the MHC encode molecules that are involved in antigen presentation to T lymphocytes (for reviews see references 1, 2). Classical class I, or class Ia, H chains are encoded in the H-2 K and D subregions. They become noncovalently associated with a 12-kD non-MHC-encoded protein, β_2-microglobulin $(\beta_2m)^1$, in the endoplasmic reticulum (ER) before their transport to the cell surface (3). Class I molecules present endogenously derived antigenic peptides of eight to nine amino acids to cytotoxic T lymphocytes (4–6). A transporter associated with antigen processing (TAP) is encoded by two genes in the MHC called TAP-1 and TAP-2. TAP-1 and TAP-2 proteins are required for transport of cytosolic peptides into the ER for binding to classical class I molecules (7–10). In general, both β_2m and peptide are required for classical class I molecules to be expressed on the cell surface.

Nonclassical class I molecules, or class Ib molecules, are similar to the classical class I molecules in terms of their amino acid sequence, and most require β_2m for cell surface expression (11–13). In the mouse, the majority of these molecules are encoded in the Q, T, and M subregions of the MHC; some are encoded outside of the MHC (12–16). With a few exceptions, the function of these molecules remains unknown, although a role for these molecules in antigen presentation is most likely (for review see reference 14). Several examples of class Ib presentation of specific antigens to T cells have been documented (14, 17–20). In addition, nonameric peptides have been eluted and sequenced from at least one class Ib molecule, Qa-2 (21, 22).

The mouse thymus leukemia (TL) antigen is a class Ib molecule encoded in the T region of the MHC (for review see reference 23). In all strains of mice, it is expressed only by

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¹ Abbreviations used in this paper: β_2m, β_2-microglobulin; Endo H, endoglycosidase H; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; TL, thymus leukemia.

small intestinal epithelial cells in adults. In a number of inbred strains, however, it is expressed by additional cell types, including thymocytes (24–26). The TL antigen is >50% similar in sequence to K and D molecules for the $\alpha 1$ and $\alpha 2$ domains. Conserved amino acids in the peptide-binding groove of classical class I molecules that make contact with bound peptide also are present in the TL antigen, suggesting that TL antigen may bind nonameric peptides (23). Although peptide specific, TL antigen–restricted T cells have not been reported, allorecognition of TL antigen by CTL has recently been demonstrated, providing further evidence that this molecule may function similarly to classical class I molecules (27). Here we show that expression of the TL antigen is unique among MHC-encoded class I molecules in that it is expressed completely independently of TAP function. By kinetic criteria, the TL antigen appears to traffic via an intracellular pathway similar to the K and D molecules. We also provide evidence suggesting that TL antigen is thermostable at physiological temperatures in the absence of peptide, implying that it does not require the binding of specific peptides for its transport to and stable expression at the cell membrane.

Materials and Methods

Cell Lines. The RMA-S and RMA cell lines were obtained from Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA). They were maintained in 5% CO_2 at 37°C in RPMI medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum (JRH Laboratories, Lenexa, KS), 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin, 5×10^{-5} M 2-ME, 100 μg/ml nonessential amino acids, and 100 μg/ml sodium pyruvate unless otherwise indicated. Culture of *Drosophila melanogaster* embryonic SC cells was carried out in Schneider medium (Gibco Laboratories) supplemented with 7% FCS at 27°C. For the large-scale production of soluble TL antigen, *D. melanogaster* cells were grown in serum-free insect medium (Insect Xpress; Whittaker Bioproducts, Walkersville, MD) in a roller bottle apparatus at 27°C.

Gene Constructs and Transfectants. A $T18^d$ cDNA clone was obtained from Dr. F.-W. Shen (Tampa Bay Research Institute, St. Petersburg, FL). The TL antigen expression constructs, $pDT18^dhGH$ and $pH\beta AprT18^d$, and the chimeric molecule L^d/TL expression construct $pH\beta AprLT18^d$, have been previously described (28). $pH\beta AprT18^d$ encodes a full-length $T18^d$ cDNA, while $pH\beta$-$AprLT18^d$ encodes a genomic sequence containing the L^d $\alpha 1$ and $\alpha 2$ domains coupled to a $T18^d$ genomic sequence encoding the $\alpha 3$, transmembrane, and cytoplasmic domains. Both expression plasmids are under the control of the human β-actin promoter and contain the gene for neomycin resistance (29). $pDT18^dhGH$ contains the full-length $T18^d$ cDNA under the control of the H-$2D^d$ promoter (30). The plasmid pL^dneo, containing the H-$2L^d$ promoter and genomic coding sequences colinear with pSV2neo, was a gift of Dr. Martha Zuniga (University of California at Santa Cruz, Santa Cruz, CA). For expression of soluble TL molecules in *D. melanogaster* cells, the plasmid TL.s-pRM, which encodes a truncated form of the $T18^d$ cDNA containing the signal peptide and the $\alpha 1$–$\alpha 3$ domains, was generated by PCR. Oligonucleotides corresponding to the 5' end of the $T18^d$ leader sequence (CAAGAATTCACC-ATGGGGATGGGGACACCAGTGCCT) and the 3' end of exon 4 (TCATAGGTACCAGGATCCATCCCATCTCAGGGTGAGAGG-CTCAG) introduced restriction enzyme sites, which allowed the

cloning of the truncated cDNA into the pRMHa-3 expression vector (31). This vector has the *D. melanogaster metallothionein* gene promoter 5' to the inserted cDNA and a sequence of six histidine amino acids followed by a translational stop codon at the 3' end. β_2m-pRM was generated by cloning a mouse β_2m cDNA clone (32) into the insect cell expression vector.

Gene transfer of 30 μg of $pH\beta AprLT18^d$ or $pH\beta AprT18^d$ into 10^7 RMA or RMA-S cells was accomplished by standard electroporation procedures (gene transfector 300; BTX Inc., San Diego, CA). Alternatively, 20 μg of $pDT18^dhGH$ was coelectroporated with 10 μg of pSV2neo (33) into 10^7 RMA-S cells. G418 (Gibco Laboratories) selection at 700 μg/ml (RMA-S) and 300 μg/ml (RMA) active drug began 48 h after electroporation, and stable transfectants were analyzed for expression of the gene product 3–4 wk later. SC2 *D. melanogaster* tissue culture cells were cotransfected with 12 μg of TL.s-pRM, 12 μg of mouse β_2m-pRM, and 1 μg of pUChsneo (34) by the calcium phosphate method and selected with 500 μg/ml of G418 for 2 mo.

Antibodies. Antibodies used for flow cytometric analysis were 28-8-6S for detection of K^b/D^b (35; a gift of Dr. Rita Effros, Department of Pathology, University of California at Los Angeles, Los Angeles, CA), 30-5-7 for the detection of L^d and the L^d/TL molecule (36; a gift of Dr. Iwona Stroynowski, Southwestern Medical Center, Dallas, Texas), AF6-88.5.3 for detection of K^b (37; American Type Culture Collection, Rockville, MD), and HD168 (38; a gift from Drs. Elisabeth Stockert and Lloyd Old, Memorial Sloan Kettering, New York), 18/20 (39; a gift of Dr. Kirsten Fischer Lindahl, Southwestern Medical Center), and M4 (40; a gift from Drs. S. Kimura and E. Boyse, Memorial Sloan Kettering) for the detection of the TL antigen. All of the antibodies are of the IgG2a isotype and were raised in mice, with the exception of HD168, which is a rat IgG1 mAb. PE-conjugated goat anti–mouse Ig (CALTAG Labs, South San Francisco, CA) and goat anti–rat Ig (CALTAG Labs) were used as secondary reagents. For biochemical analyses, 28-14-8S, a mouse IgG2a antibody, was used to detect the D^b molecule (36; American Type Culture Collection).

Flow Cytometry. For the flow cytometric analyses, cells were cultured at 37°C or at room temperature overnight before the addition of saturating amounts of mAb. Temperature shift assays were performed by incubating cells overnight at room temperature and then at 37°C for 3–5 h. Where indicated, peptides were added to the transfectants before shifting the cells to 37°C. Cells were incubated for 30 min, washed three times in PBS, and incubated in PE-conjugated secondary antibodies. Labeled cells were analyzed by use of a FACScan® (Becton Dickinson & Co., Mountain View, CA) at the UCLA Flow Cytometry Core Facility. Dead cells were excluded by incubating the cells with 7-amino actinomycin-D (41; Calbiochem Corp., La Jolla, CA) before analysis.

Pulse–Chase Labeling. Cells were preincubated in RPMI without methionine (Gibco Laboratories) supplemented with 5% dialyzed fetal bovine serum (Gibco Laboratories) for 1 h. They were then metabolically labeled with 500 μCi [^{35}S]methionine (Tran^{35}S-label; ICN, Irvine, CA) per 10^7 cells for 30 min, washed, and chased in RPMI containing two times the normal methionine concentration. Cells were lysed in 1 ml PLB (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.1 M NaCl, 0.01 M Na phosphate, 1 mM PMSF) on ice for 1 h and cellular debris removed by centrifugation. For immunoprecipitation of the TL antigen, the lysates were incubated with HD168 for 2 h at 4°C with constant rotation. Protein G–Sepharose beads (Pharmacia Inc., Piscataway, NJ) were added, and the mixture was rotated for an additional 2 h at 4°C. Beads were collected, washed three times in KLB (150 mM NaCl, 10

mM Na phosphate [pH 7.0], 0.05% SDS, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml aprotinin), and once in wash buffer 2 (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5], 5 mM EDTA, 1 mM PMSF). The immunoprecipitated protein samples were dissolved by boiling the Sepharose beads in 60 μl of elution buffer (0.02% SDS, 0.01 M Tris HCl [pH 7.5], 0.1 M Na citrate, 1 mM PMSF) for 5 min. 1 μl of endoglycosidase H (Endo H; Boehringer Mannheim Corp., Indianapolis, IN) was added to half of the sample, and the other half remained untreated. Both treated and untreated samples were incubated overnight at 37°C in 5% CO_2. After immunoprecipitation for TL antigen, lysates were incubated with 28-8-6S or 28-14-8S for the immunoprecipitation of K^b/D^b or D^b, respectively. These samples were treated as described for the immunoprecipitation of TL antigen. Samples were analyzed by electrophoresis on SDS–12% polyacrylamide gels.

Kinetics of Arrival of Class I Molecules at the Cell Surface. Cells were labeled with [^{35}S]methionine for 30 min and chased in medium containing excess methionine as described above. Before cell lysis, cell surface proteins were biotinylated twice for 20 min with 1 μg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) in PBS containing Ca^{2+} and Mg^{2+} (PBS$^+$). The reaction was quenched by washing the cells with PBS$^+$ containing 50 mM lysine. The cells were then lysed in RIPA buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM PMSF) without SDS and centrifuged to remove cellular debris. Before immunoprecipitation, SDS was added to a final concentration of 0.1%. Lysates were serially immunoprecipitated as described above first for the TL antigen and then for K^b/D^b. Protein bound to protein G beads was recovered by boiling twice in 100 μl of elution buffer (1% SDS, 0.2 M Tris-HCl [pH 8.5], 5 mM ethylene glycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid) for 2 min and then washing in 300 μl RIPA buffer. The eluates and wash were pooled and incubated with 50 μl of slurry containing Streptavidin–agarose beads (Pierce Chemical Co.) overnight at 4°C. The beads were washed four times in RIPA buffer and boiled in 30 μl sample buffer. Samples were analyzed by SDS–12% polyacrylamide gels.

Isolation and Purification of Soluble TL Antigen. 5 liters of insect cells cotransfected with TL.s-pRM and β_2m-pRM was induced with 1 mM $CuSO_4$ and incubated for 3 d. Cells were centrifuged at 3,000 g for 10 min and the supernatant concentrated 20 times by use of a tangential flow concentrator with a 30-kD cutoff membrane (Filtron Technology Corp., Northborough, MA) before dialysis against PBS. Isolation of the soluble TL/β_2m complex was done by Ni-agarose (Ni-NTA; Qiagen, Inc., Chatsworth, CA) affinity chromatography. The nickel metal binds the six-histidine tag at the COOH terminus of the TL antigen H chain. The material was eluted with 100 mM imidazole and was further purified by fast protein liquid chromatography using an anion exchange Mono Q column (Pharmacia Inc.) in a buffer (50 mM Tris-HCl pH 8, 1 mM EDTA) with a salt gradient.

Thermal Stability Assay for Soluble Molecules. 5 μg of either soluble TL/β_2m or soluble K^b/β_2m complex was incubated in 100 μl of PBS, 1% Triton X-100, for 2 h at the indicated temperatures. TL or K^b H chain was recovered by absorption to 5 ml of Ni-NTA agarose beads. Beads were then washed with PBS, 1% Triton X-100, 10 mM imidazole, and TL antigen or K^b was eluted either with PBS, 1% Triton, 100 mM imidazole, or by boiling in SDS-PAGE sample buffer. The isolated material was analyzed by SDS–15% polyacrylamide gels and silver staining (Bio-Rad Laboratories, Richmond, CA).

Amino Acid Sequencing and Biochemical Analysis. 4 nmol of

soluble TL/β_2m complex purified as described above by Ni affinity chromatography was washed twice with a 50-mM NH₄Ac buffer using a Centricon 10 (10 kD cutoff; Amicon, Beverly, MA) and concentrated to 200 μl. Bound peptides were extracted with 0.1% TFA for 30 min and filtered through a Centricon-10 membrane as previously described (42). The extract was dried and subjected to peptide sequencing or laser desorption mass spectrometry. In addition, 100 pmol of the whole complex were directly subjected to Edman degradation to compare the level of amino acid signal from the putative TL-bound peptides versus those corresponding to the NH_2-terminal positions of β_2m and TL H chain.

Results

Expression of the TL Antigen is TAP Independent. RMA-S is a T lymphoma cell line that lacks the TAP-2 subunit of the peptide antigen transporter (9, 10, 43). Class I molecules are poorly expressed on the cell surface of RMA-S cells cultured at 37°C, but empty molecules are expressed on the surface when the cells are incubated at lower temperatures (44). Expression is stabilized if peptide capable of binding to the class I molecule is added before incubation at 37°C. We therefore intended to use RMA-S cells that express the TL antigen to assay for TL-binding peptides. A cDNA clone encoding the TL antigen expressed under the control of the human β-actin promoter was transfected into both mutant RMA-S and parental RMA cells. Stable transfectants were incubated overnight at either 37°C or 22°C and analyzed by flow cytometry for surface expression of the TL antigen and K^b/D^b. We were surprised to find that high levels of TL antigen could be equally well detected at 37°C and at room temperature (Fig. 1, *a* and *b*). Similar results were ob-
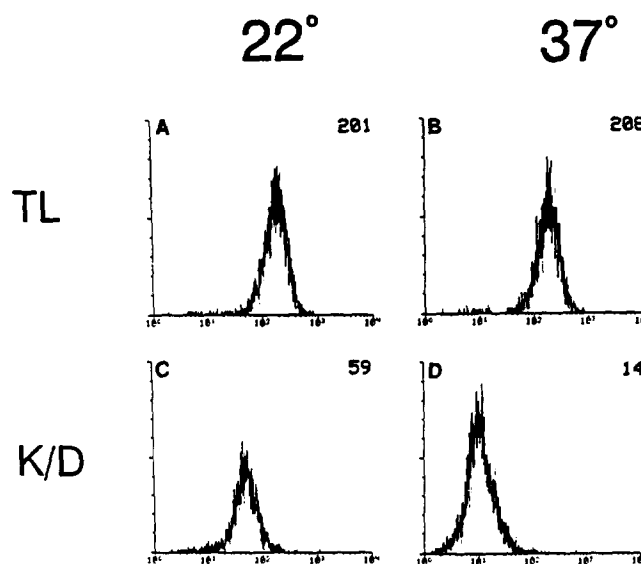
Figure 1. High levels of the TL antigen are expressed at 37°C on RMA-S cells. RMA-S transfectants were incubated at 22°C (*a* and *c*) or at 37°C (*b* and *d*) and analyzed by flow cytometry for cell surface expression of the TL antigen (*a* and *b*) or K^b/D^b molecules encoded by endogenous genes in the transfectants (*c* and *d*). The mean fluorescence is indicated in the upper right corner of each panel.

tained with 8 different, stable transfectants, including those expressing $T18^d$ under the control of the D^d promoter as well as those in which TL antigen expression is driven by the β-actin promoter (data not shown). In contrast, high levels of K^b/D^b were expressed only at 22°C (Fig. 1, c and d). Similar results on TL antigen expression were obtained when the RMA-S transfectants were analyzed in serum-free medium (data not shown), indicating that antigen processing and binding were not occurring in the tissue culture medium.

RMA-S cells also were transfected with a construct encoding either the L^d molecule or a chimeric molecule containing the $\alpha 1$ and $\alpha 2$ regions of the L^d molecule and the $\alpha 3$, transmembrane, and cytoplasmic domains of the TL antigen from $T18^d$ (L^d/TL). MAb 30-5-7, which recognizes the $\alpha 2$ domain of L^d, was used to detect cell surface expression of molecules encoded by either construct. None of three anti-TL antigen mAbs that were tested reacted with the chimeric class I molecule (data not shown), indicating that the epitope(s) recognized by these antibodies requires the $\alpha 1$ and/or $\alpha 2$ domain. Only low levels of L^d or L^d/TL were expressed by RMA-S transfectants incubated at 37°C; the levels increased substantially when the cells were incubated at room temperature (Table 1 and data not shown). It is possible, however, that the chimeric L^d/TL class I molecule is unable to bind $\beta 2m$, and that this could result in the low levels of surface expression in RMA-S cells cultured at 37°C. We consider this unlikely, because L cell transfectants that express the L^d/TL chimera are recognized as effectively as L^d transfectants by anti-L^d alloreactive T cells (28). To further assess the ability of the L^d/TL molecule to bind $\beta 2m$, RMA-S cell transfectants that express the L^d/TL chimeric molecule were incubated overnight at room temperature, and two peptides known to bind specifically to the L^d molecule were then added to the transfectants before shifting the cells to 37°C for 3–5 h. Transfectants not incubated in the presence of peptide rapidly lost cell surface expression of L^d/TL. In contrast, the addition of either one of the two L^d-specific peptides stabilized cell surface expression of the transfected chimeric molecule, but not of endogenous K^b (Table 1). The level of L^d/TL expression is even higher than that in transfectants cultured at low temperature. Because there is in general only a minimal amount of peptide antigen binding to free class I H chains, the data suggest that the L^d/TL chimera is not defective for $\beta 2m$ association. Furthermore, because the β-actin promoter was used for the L^d/TL and TL antigen transfectants, this result also demonstrates that escape

to the cell surface in the absence of TAP is not likely to be due to the high levels of class I expression in the transfectants.

The TL Antigen Traffics Normally from the ER to the Golgi Apparatus in RMA-S Cells. Flow cytometric analysis of the TL antigen on the surface of RMA-S transfectants only shows the steady state level of TL antigen surface expression. If the TL molecule had a very long half-life on the cell surface, high levels of the TL antigen eventually could accumulate on the cell surface, even in TAP-deficient cells, due to possible escape from the ER. To test this possibility, a series of pulse–chase labeling studies was carried out to measure intracellular trafficking of the TL antigen. We first determined the rate at which the TL molecule reaches the Golgi apparatus by measuring the acquisition of Endo H resistance. Cells were pulse labeled with [^{35}S]methionine as described and chased with cold medium for the indicated times. At each time point, an aliquot of cells was lysed and immunoprecipitated for the TL antigen or D^b. Samples were then incubated in the presence or absence of Endo H, which cleaves the mannose-rich carbohydrates that are modified in the Golgi apparatus. Fig. 2 compares the acquisition of Endo H resistance by TL antigen immunoprecipitated from RMA-S (Fig. 2 a) and RMA (Fig. 2 b) transfectants. In both cell lines, the transport of TL antigen from the ER to the Golgi apparatus is complete in ~ 2.5–3 h, indicating that the TAP defect in RMA-S cells does not grossly affect the transport of the TL antigen. $\beta 2m$ is coprecipitated with TL antigen H chain in the mutant cell line as well as in the normal cell line. The D^b molecule arrived at the Golgi apparatus in RMA cells with similar or slightly more rapid kinetics than the TL antigen (data not shown). The difference may be attributed to the highly increased levels of intrinsic expression in the transfectants, which might overload the Golgi transport system, or to intrinsic differences in rate of transport of different class I molecules. In contrast to the results in RMA cells, no detectable D^b molecules became Endo H resistant in RMA-S cells (Fig. 3).

TL Antigen Traffics to the Cell Surface with Kinetics Similar to Classical Class I Molecules. TL antigen may traffic differently from classical class I molecules and acquire peptide in a site other than the ER, thus resulting in the TAP-independent expression of the TL antigen. Classical class I molecules are transported to the cell surface within 1–2 h, while class II molecules generally require up to 8 h for transport (45). The additional time required for class II molecules is due in part to transport through endosomes. To determine if TL antigen traffics more like classical class I or class II molecules, we

Table 1. *L^d-specific Peptides Stabilize Cell Surface Expression of L^d/TL on RMA-S Transfectants*

	26°C	37°C	26°C → 37°C*	Control peptide	QLSPFPFDL	YPHFMPTNL
L^d/TL	24	6	8	10	41	38
K^b	22	7	8	6	7	6

Numbers indicate mean intensity of fluorescence.
* 26°C → 37°C means cells cultured overnight at 26°C and then shifted to 37°C for 3–5 h.
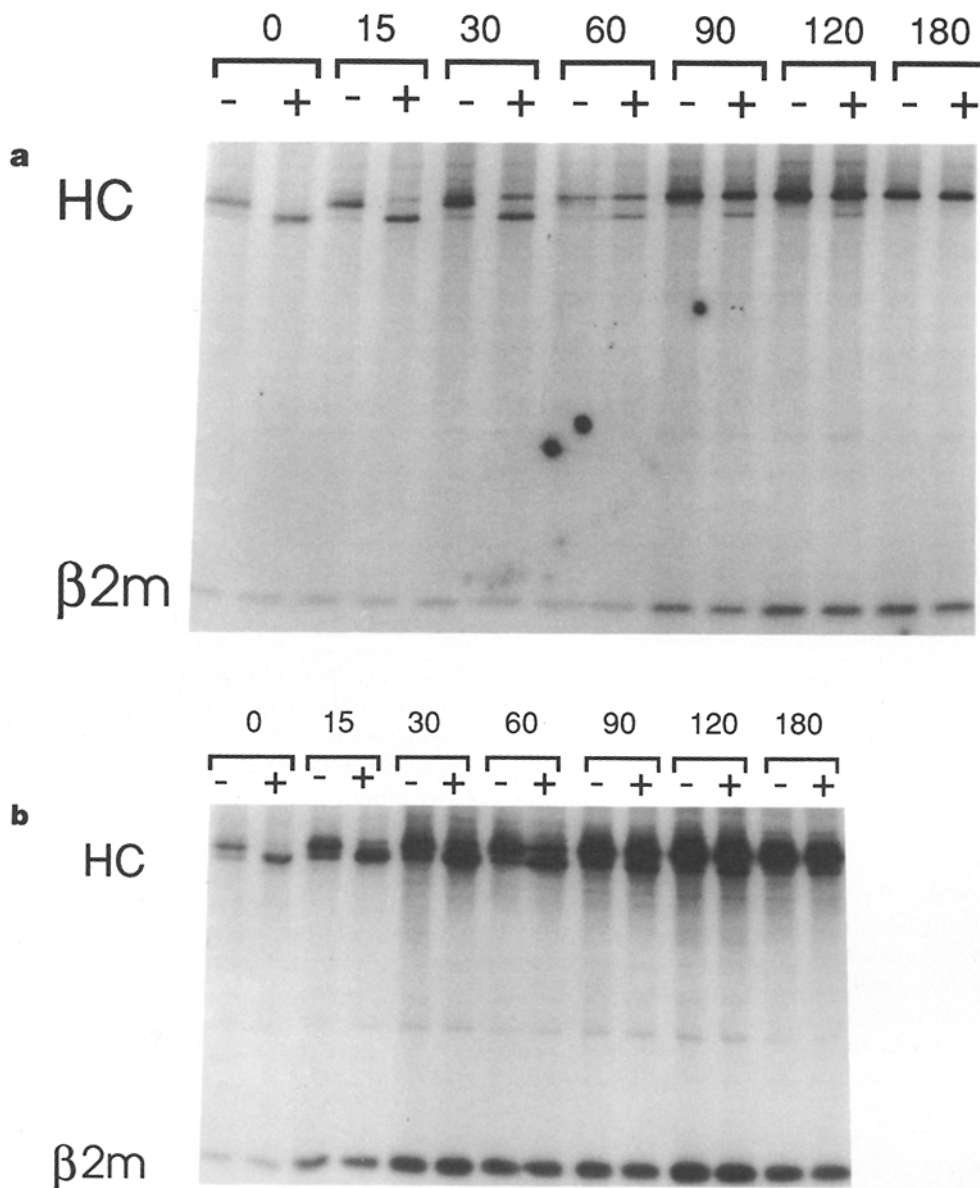
Figure 2. The TL antigen traffics normally from the ER to the Golgi apparatus in the absence of TAP. RMA-S (a) or control RMA (b) transfectants were pulse labeled with [^{35}S]methionine and chased in RPMI with excess cold methionine for various times. Cells were lysed at the times indicated, and TL antigen was immunoprecipitated from each sample. Half of the sample taken at each time point was incubated overnight in the presence of Endo H (+); the other half remained untreated (−). Samples were analyzed by gel electrophoresis. HC, heavy chain of the TL molecule.

metabolically labeled RMA-S and RMA transfectants with [^{35}S]methionine and chased for the indicated times. Surface proteins were biotinylated before cell lysis, and the TL molecule or K^b/D^b molecules were immunoprecipitated. After immunoprecipitation, molecules that had arrived at the cell surface were specifically reprecipitated using Streptavidin–agarose beads. The results are shown in Fig. 4. At time 0, no Streptavidin–agarose precipitated band corresponding either to the TL antigen or to classical class I molecules was evident on SDS-PAGE. This demonstrates that the biotinylation reagent did not leak into the cells, and therefore that internal proteins were not biotinylated. Low levels of TL antigen were present on the cell surface of RMA-S transfectants within 1 h, while maximal levels were expressed at 2 and 3 h after labeling. There was no significant difference in the kinetics of surface expression for the TL antigen iso-

lated from normal RMA or mutant RMA-S transfectants. Kinetics of arrival at the cell surface similar to those for the TL antigen were seen when classical class I molecules were immunoprecipitated from RMA cells, while no detectable classical class I molecules reached the cell surface of RMA-S cells (Fig. 4).

Soluble TL Antigen Is Resistant to Thermal Denaturation. To determine if TL antigen expression is dependent upon a TAP-independent peptide-loading pathway, we transfected SC2 embryo cells with a TL antigen expression construct. *D. melanogaster* is assumed to have a much broader defect in peptide loading of MHC molecules than the RMA-S cells. Consistent with this assumption, it has been shown that transfected insect cells are deficient for both classical class I and class II peptide loading (46, 47). Empty class I antigens in the native conformation produced by fly cells are unstable at 37°C, while
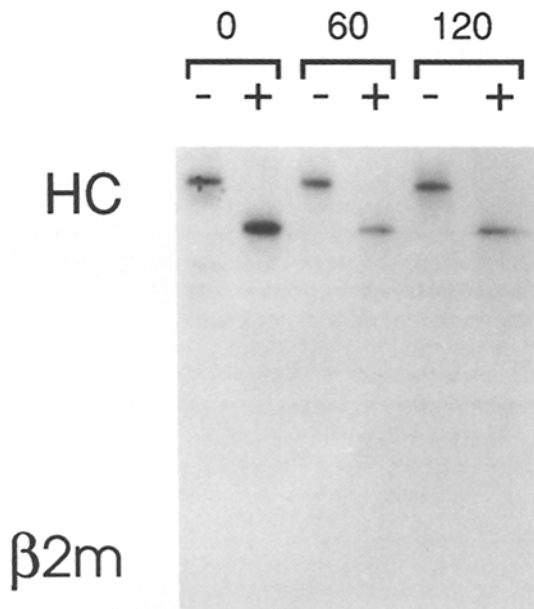
Figure 3. H-2D^b does not become Endo H resistant in RMA-S cells. RMA-S cell TL antigen transfectants were labeled with [^{35}S]methionine as described in Fig. 2, and H-2D^b was immunoprecipitated from each sample. Time points are indicated above the lanes. (+) indicates treatment with Endo H; (−) indicates that the samples remained untreated. *HC*, heavy chain of the D^b molecule.

binding of allele-specific peptides renders them thermostable. The loss of the native conformation upon heating is associated with the loss of β_2m. We therefore tested the thermal stability of TL antigen produced by insect cells by measuring its association with β_2m after incubation at various temperatures; K^b molecules from insect cells were tested in parallel.

Stable transfectants expressing a truncated form of the TL

antigen H chain, consisting of the $\alpha1$–$\alpha3$ domains and ending with a six-histidine tag, and mouse β_2m were established in order to produce soluble TL/β_2m complexes. We produced and analyzed soluble molecules for three reasons. First, the purified molecules can be treated at a variety of temperatures, while the insect cells are not viable for long periods above 33°C. Second, the levels of cell surface expression of class I molecules in *D. melanogaster* cells tend to be quite variable, even after CuSO$_4$ induction of molecules expressed under the control of the metallothionein promoter. In contrast, the level of production of the soluble class I–expressing cell lines is ∼0.5 mg/liter of culture (Castaño, A. R., data not shown). Third, for classical class I molecules, there is no difference in the thermal properties of soluble versus cell surface peptide-free molecules.

To test the thermal stability, class I molecules were incubated for 2 h at different temperatures, precipitated with Ni beads, and electrophoresed on SDS-PAGE. The class I H chain and β_2m bands were detected by silver staining. As can be seen from Fig. 5 *a*, similar amounts of both TL antigen H chain and β_2m are precipitated at 37°C and room temperature, indicating that the TL antigen heterodimer is stable at physiological temperatures. Consistent with previous experiments (46), the level of β_2m associated with the K^b H chain at 37°C is much lower than that detected at room temperature (data not shown). Scanning and integration analysis of the signal allowed quantification of the gel data for both the TL antigen and K^b. As shown in Fig. 5 *b*, which averages data from several different experiments, almost 90% of the TL complex remains stable, or β_2m associated, at 37°C, compared with only 15% of K^b/β_2m. From the bar graph shown in Fig. 5 *b*, we calculated the temperature at which 50% of the molecule is still folded, $t_{1/2}$, to be 44°C for the TL antigen, compared with 26°C determined for K^b in these and previous experiments (46). These data suggest that
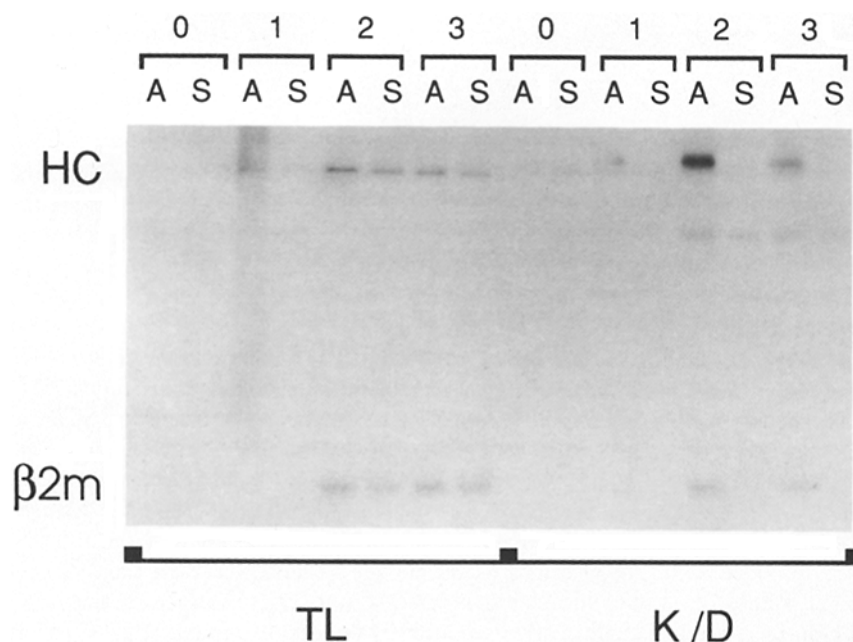
Figure 4. TL antigen traffics to the cell surface within 2 h in both normal and TAP-mutant cells. Cells were pulse labeled with [^{35}S]methionine and chased for the indicated times as described. Surface proteins were biotinylated before cell lysis. Lysates were incubated with HD168 for the immunoprecipitation of TL antigen (lanes *1–8*) or with 28-8-6S for the immunoprecipitation of H-2K^b/D^b (lanes *9–16*). Samples were eluted from protein G beads, and Streptavidin-agarose beads were added to precipitate the biotinylated surface proteins. *A*, molecules immunoprecipitated from RMA transfectants; *S*, molecules immunoprecipitated from RMA-S transfectants.
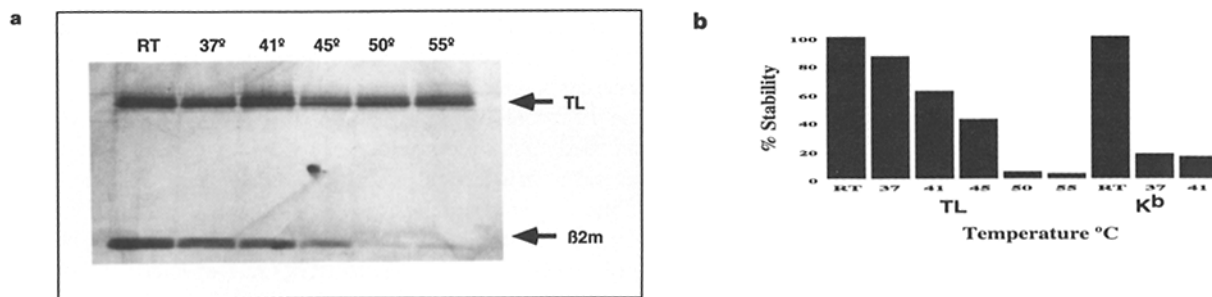
Figure 5. Thermal stability of β_2m association with TL and K^b molecules from insect cells. (*a*) SDS-PAGE of soluble TL antigen after incubating at the indicated temperatures. The upper band of the silver-stained gel corresponds to the TL H chain and the lower band to β_2m. (*b*) Quantification of thermostability assay. Scanning and analysis of the silver-stained gels was done with a densitometer to quantify the amount of protein in each band. Stability was determined as the ratio of β_2m to TL antigen, or β_2m to K^b, and is expressed as a percentage of the ratio of β_2m/class I H chain at room temperature. Histograms represent the average of four experiments for the TL antigen and two for K^b.
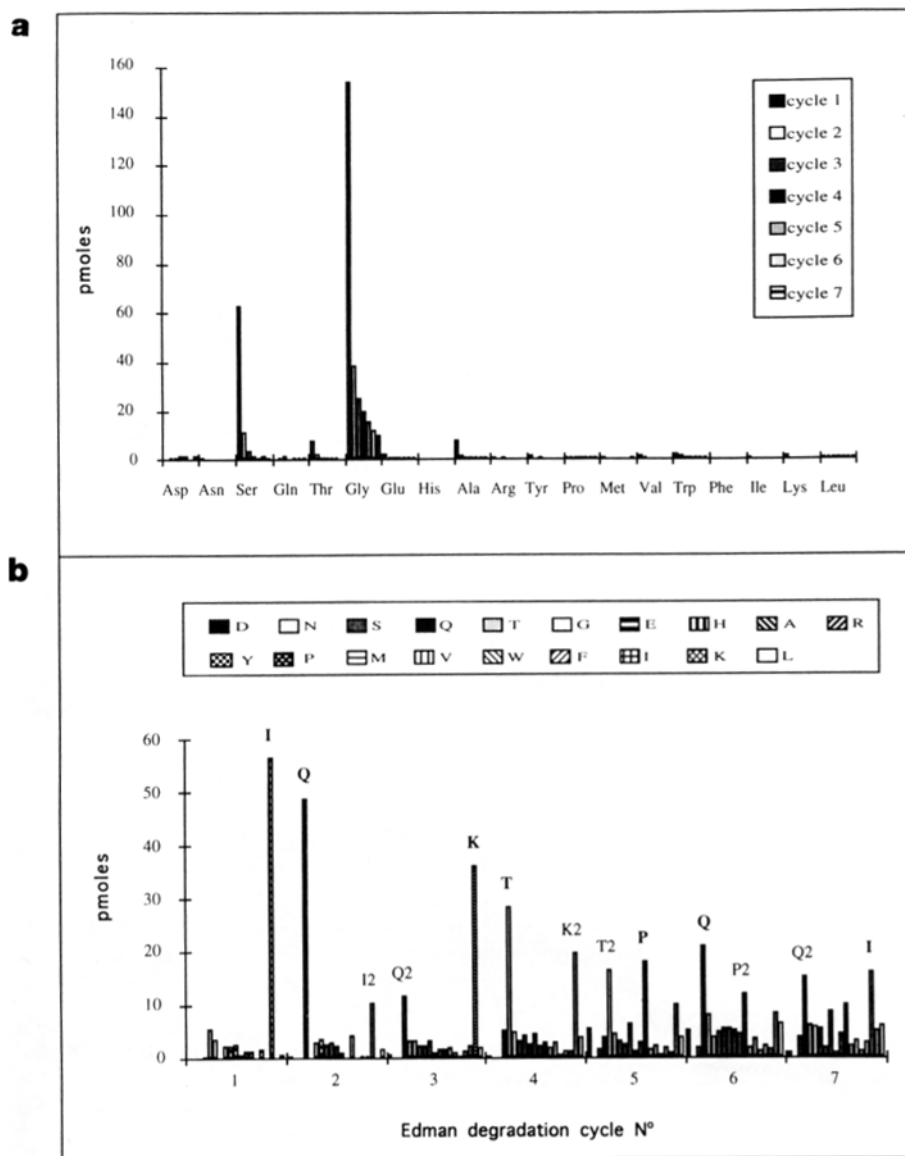
Figure 6. Amino acid sequences analysis. (*a*) Analysis of the acid extract from 4 nmol of soluble TL from insect cells. The total amount detected at each cycle of the Edman degradation is represented, in pmoles, for each amino acid. Scale represents 4% of the starting material. (*b*) Amino acid sequence of ~100 pmol of the TL/β_2m complex. Total amount of residues detected at each cycle of the Edman degradation are represented, in pmol, versus cycle number. Amino acid signals corresponding to the β_2m NH$_2$ terminus, IQKTPQI, are indicated in bold letters. The one-letter code followed by 2 denotes amino acid signals corresponding to carry over from the previous cycle.

most of the TL antigen produced by *D. melanogaster* cells is loaded with peptide or some other ligand, or that ligand-free molecules are thermally stable.

TL Antigen from Insect Cells is Devoid of Peptides. To determine if the soluble TL molecules produced by insect cells contain bound peptides, four nmol of the TL/β_2m complex were acid extracted in the presence of 0.1% TFA, and the low molecular weight fraction of the extract was subjected to peptide sequencing, as was previously done for class I molecules (42). Only the typical contaminants glycine and serine were found in the first cycle, and their carryover was found in the succeeding seven cycles (Fig. 6 *a*). Their signal represents <6% of the starting material. No other amino acids were found significantly above the detection limit of the apparatus (1 pmol), although the amount of protein used was in a 4,000-fold excess.

To corroborate the absence of material suitable for Edman degradation bound to the TL H chain, 100 pmol of the purified TL/β_2m complex before acid elution were subjected to peptide sequencing. In principle, this should reveal the presence of three sequences in molar equivalence: β_2m, TL H chain, and the anchor amino acids from the bound peptide. As shown in Fig. 6 *b*, only amino acids corresponding to the sequence of the β_2m were found, with a 60% yield in the first cycle respective to the 100 pmol of starting material. No other amino acids were found above 3 pmol in the first cycles, except threonine in cycle one. These data suggest that if peptides are bound to the TL antigen, they lack a dominant or anchor amino acid. In addition, excluding the β_2m carryover from the previous cycle, the sum of all of the amino acids detected was never >15% of the β_2m signal, suggesting that all or most of the molecules do not contain peptides, or that bound peptides are blocked in their NH$_2$ terminus. Similarly, the absence of any amino acid corresponding to the H chain of the TL antigen suggests that its NH$_2$ terminus is blocked. A subsequent attempt to sequence the H chain isolated from an SDS-PAGE gel of purified TL antigen also failed, supporting this interpretation (data not shown). The NH$_2$-terminal blocked TL H chain cannot be attributed to an artifact caused by the *D. melanogaster* expression system because the TL H chain isolated from mammalian cells also is blocked (Joyce, S., and M. Chorney, personal communication).

To detect blocked peptides that may have been bound to the TL antigen, acid extract from 100 pmol of the TL/β_2m complex was subjected to MALDI-mass spectrometry. No detectable signal of <10 kD was detected. Only a peak of 12 kD, which most likely corresponds to a minimal amount of β_2m that passed through the Centricon-10 membrane, was detected. These results strongly suggest that most, if not all, of the soluble TL molecules produced in *D. melanogaster* are devoid of peptide.

Discussion

We have demonstrated that the TL molecule is stably expressed on the surface of mouse cells lacking TAP-2. Surface expression in the absence of transporter protein function results from normal transport to the cell surface rather than a slow

accumulation of molecules that leak from the ER, and the α1 and α2 domains of the TL antigen apparently are necessary, although they may not be sufficient. In this respect, the TL molecule behaves differently from all other MHC-encoded class I molecules that have been studied, including several nonclassical class I molecules (9, 48–50). The Qa-1 molecule, encoded by the *T23* gene, may be a partial exception to the TAP-dependent rule for MHC-encoded class I molecules. Only some of a panel of Qa-1–specific CTL clones required transporter function (51). Although this result suggests that Qa-1 expression is partially TAP independent, specific mAbs for quantitation of Qa-1 surface expression are not available, and it is not known to what degree surface expression of this molecule is affected by the absence of TAP. In contrast to these results on MHC-encoded nonclassical class I molecules, class I-like CD1 molecules that are encoded by genes unlinked to the MHC are expressed in TAP-2–deficient humans (52) and by TAP-deficient transfectants (Teitell, M., and M. Kronenberg, manuscript in preparation).

Although improperly folded classical class I molecules are in some cases able to reach the cell surface, they do so relatively inefficiently. We therefore consider it unlikely that the relatively high levels of surface TL expression in the absence of TAP function are due to the possible conformation-insensitive reactivity of the anti-TL antibodies. Consistent with this, after biosynthetic labeling, two anti-TL mAbs failed to immunoprecipitate TL antigen from R1E, a β_2m-deficient mutant (53, data not shown). TL molecules could be immunoprecipitated, however, from the β_2m$^+$ R1.1 parent cell line. Thus, these two mAbs require that TL antigen be associated with β_2m for detection, and therefore they are likely to require the native conformation.

Soluble TL antigen produced in *D. melanogaster* cells is associated with β_2m at temperatures up to 45°C, providing further conformation that class I molecules in the TAP-deficient cells are in the native confirmation. The thermostable, soluble TL molecules produced by insect cells behave similarly to classical class I molecules that have been loaded with peptide. Several attempts to identify peptides from the soluble TL/β_2m complexes indicate that these molecules are devoid of detectable peptide. It is possible that peptides bound by the TL antigen have a modified NH$_2$ terminus that precludes sequencing by Edman degradation. However, this hypothesis is not supported by results obtained from mass spectometry analysis (data not shown). These mass spectometry data further suggest that nonpeptide ligands also are not present, although very low molecular weight ligands would not have been detected. In summary, there are two possible interpretations of the data concerning TL antigen expression by insect cells. The first, which we consider most likely, is that TL molecules can reach the cell surface and be secreted without bound peptides. The alternative possibility, that peptides are present, is difficult to formally exclude. This would require that some pathway be present in *D. melanogaster* cells for loading peptides into the TL molecule. In addition, to elude detection, the peptides would have to behave very differently during purification and analysis from the peptides bound to either class I or class II molecules.

From a structural viewpoint, it is not obvious why peptide-free TL molecules might be stable while classical class I molecules clearly are not. Binding of β_2m can, however, contribute to the stabilization of both empty and peptide-containing class I molecules. It has been reported that a number of mouse class I molecules can be stably expressed in human T2 cells (54). Mouse class I H chains have a higher affinity for heterologous human β_2m than they do for mouse β_2m (55, 56). In fact, the mouse class I H chain/human β_2m complexes are more thermostable than their class I H chain/mouse β_2m counterparts, increasing the $t_{1/2}$ of the complexes by ~8° (46). Therefore, a plausible explanation for the TAP-independent expression of mouse class I molecules in human cells is that the high affinity for human β_2m permits stable expression of the mouse class I H chains even in the absence of bound peptide. By analogy with this case, it is possible that the TL antigen has a higher avidity for mouse β_2m than classical class I molecules, thus allowing stable transport of empty TL molecules out of the ER in mouse cells.

Although it remains to be determined whether the TL antigen is capable of binding peptide under any circumstances, its primary sequence is so similar to that of classical class I molecules that this seems most likely. If this were the case, then the ability of the TL antigen to efficiently leave the ER without peptide could free it to pick up peptide in some other site. Recent experiments suggest that under some circumstances post-Golgi classical class I molecules might acquire peptides (57). It is possible that what may be a relatively minor pathway for peptide acquisition by K and D molecules could be the major pathway for the TL antigen.

We have shown that the mouse CD1 molecule, which also is expressed by gut enterocytes, is expressed in a TAP-independent fashion similar to the TL antigen (Teitell, M., and M. Kronenberg, manuscript in preparation). It is not likely to be an accident that the two nonpolymorphic class I molecules expressed by mouse enterocytes show this characteristic and unusual behavior. Although the TAP-independent expression of these molecules is likely to relate to their specialized immune function in the mucosae, we can only speculate as to what this function might be. It is possible that empty class I molecules are recognized by mucosal lymphocytes that express $\gamma\delta$ TCRs. Recognition of MHC molecules by $\gamma\delta$ T cells may in some cases not be dependent on bound peptides (50). Alternatively, empty TL antigen, and possibly also CD1, may bind lumenal peptides by preferentially sorting to the apical surface of enterocytes, or they may bind lumenal-derived peptides by recycling from the surface to endosomes and back again. Peptide acquired in this way could subsequently be presented to intestinal intraepithelial lymphocytes or to lamina propria lymphocytes located in the submucosa. Soluble TL antigen produced in fly cells would not have the opportunity to recycle, and therefore would be expected to be free of peptide.

In summary, we have shown that the TL antigen traffics normally and is stably expressed on the surface of cells that lack a functional transporter. In addition, soluble TL antigen expressed in *D. melanogaster* cells appears to be peptide free. These properties give us insight into the possible specialized functions of the TL antigen, and perhaps also the functions of other nonclassical class I molecules, in gut mucosae.

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