MHC Class I Molecules Form Ternary Complexes with Calnexin and TAP and Undergo Peptide-regulated Interaction with TAP via Their Extracellular Domains

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Summary

Newly assembled heavy chain-β_2m heterodimers of class I histocompatibility molecules associate with the endoplasmic reticulum (ER) peptide transporter, TAP, and subsequently dissociate from TAP in parallel with their transport from the ER to the Golgi apparatus. It appears that TAP-associated class I molecules are waiting to bind appropriate peptides before they dissociate from TAP and leave the ER since binding of high affinity peptides to class I molecules in vitro leads to dissociation of TAP–class I complexes. In further support of this notion, we report that limiting peptide supply through inhibition of proteasome activities prolongs the association of mouse class I molecules with TAP and concomitantly slows their transport to the Golgi apparatus. By using a series of deletion mutants and hybrid class I molecules we demonstrate that the extracellular domains of class I molecules are sufficient for their peptide-regulated interaction with TAP. Furthermore, based on the inability of an α_3 domain-specific mAb to recognize TAP–class I complexes and the fact that a point mutant of the D^d molecule at residue 222 is unable to bind to TAP, it is likely that a major site of interaction with TAP resides in the membrane-proximal region of the heavy chain α_3 domain. Finally, we examined the relationship between the interaction of mouse heavy chain-β_2m heterodimers with TAP and with the resident ER chaperone, calnexin. Most heterodimers that bound to TAP were found to associate simultaneously with calnexin. Upon delivery of peptide to class I molecules in permeabilized cells, dissociation from TAP was observed but the interaction with calnexin was largely maintained. Therefore, both TAP and calnexin may participate in the ER retention of peptide-deficient class I molecules. However, since release from calnexin occurs after dissociation from TAP, it appears that calnexin ultimately determines if a class I molecule is to be exported from the ER.

Class I molecules of the major histocompatibility complex (MHC) bind peptides derived from endogenously synthesized proteins and present them at the cell surface to $CD8^+$ cytotoxic T lymphocytes. Functional class I molecules consist of three components: an MHC-encoded transmembrane heavy chain (H chain), a soluble protein termed β_2-microglobulin (β_2m)[1], and a peptide ligand that is typically 8–10 residues in length. The majority of peptides found associated with class I molecules are derived from nuclear and cytosolic proteins and they are generated largely through the proteolytic action of the cytosolic proteasome complex. Peptides are delivered from the cytosol into the lumen of the ER and possibly the *cis*-Golgi by the transporter associated with antigen processing (TAP) and subsequently those peptides of appropriate length and sequence bind to class I molecules (1, 2).

The TAP transporter functions as a heterodimer of two MHC-encoded polypeptides, TAP1 and TAP2. Each subunit is predicted to span the ER membrane six to eight times with only small loops penetrating the ER lumen and with a large domain containing an ATP binding cassette residing in the cytosol (1, 3, 4). TAP1 and TAP2 proteins are located in the ER and *cis*-Golgi as determined by immuno-

[1]*Abbreviations used in this paper:* β_2m, β_2-microglobulin; DSP, dithiobis(succinimidyl propionate); endo H, endoglycosidase H; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; H chain, class I heavy chain; LLnL, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; PMSF, phenylmethylsulfonylfluoride; TAP, transporter-associated with antigen processing.

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electron microscopy or immunofluorescence although they lack known sequences that would confer this localization (5, 6). Experiments with either isolated microsomes or permeabilized cells have shown that TAP preferentially transports peptides of 8–15 residues in an ATP-dependent fashion. Furthermore, in rat and mouse there is some sequence selectivity for the carboxy terminus of peptides which may favor peptides with higher binding affinity for class I molecules (1, 3, 4, 7, 8).

We and others have demonstrated that newly assembled mouse and human H chain-β_2m heterodimers, but not H chains alone, associate with TAP and that subsequent dissociation from the transporter occurs in parallel with class I movement from the ER to the Golgi (9, 10). Moreover, delivery of known peptide ligands to class I molecules in permeabilized cells leads to the dissociation of TAP–class I complexes. On the basis of these data we proposed a model wherein newly assembled class I heterodimers associate with TAP, capture appropriate peptide ligands in a process facilitated by their proximity to the transporter, and then dissociate from TAP for subsequent rapid transport to the cell surface (9). In addition to enhancing access to peptides, the association with TAP may help to retain class I heterodimers within the ER until peptides are acquired.

Assembling class I molecules also associate with calnexin, a molecular chaperone that resides within the ER membrane (11). In the mouse, newly synthesized H chains bind rapidly and quantitatively to calnexin and remain bound during assembly with β_2m (12, 13). Dissociation from calnexin, like TAP, occurs in parallel with the transport of assembled class I molecules from the ER to the Golgi (12). During this interaction, calnexin promotes folding of class I H chains and their assembly with β_2m (14). Furthermore, calnexin retains class I assembly intermediates in the ER so that for the most part only fully assembled, peptide-containing class I molecules are transported to the cell surface (13, 15). Calnexin also retains human class I H chains and facilitates their folding (14, 16, 17). However, there have been several reports suggesting that in humans calnexin binds only to free H chains and dissociates upon H chain assembly with β_2m (10, 18, 19). In contrast, Carreno et al. recently reported that calnexin does indeed associate with human H chain-β_2m heterodimers (20). Additional studies are required to determine if the apparent disparity between the mouse and human systems is correct or if it has arisen through technical problems in detecting calnexin complexes with human class I heterodimers.

There are a number of unresolved issues surrounding the interaction of assembling class I molecules with both TAP and calnexin. For example, what is the molecular nature of these associations? Calnexin has been shown to recognize class I H chains via a carbohydrate binding site that has affinity for the $Glc_1Man_9GlcNAc_2$ oligosaccharide processing intermediate (14, 21, 22) and also through interactions in the vicinity of the H chain transmembrane segment (23, 24). However, the site(s) on class I molecules that interact with TAP have not been identified. This issue is of particular interest since the association seems to be dependent on the assembly state of class I. Furthermore, given that substantial populations of class I heterodimers have been shown to bind to calnexin and TAP (9, 12), do ternary complexes of the proteins exist? Finally, the nature of the folding or assembly event leading to calnexin dissociation has remained elusive. Does peptide binding to class I influence this process in a manner analogous to its effects on the interaction with TAP? These issues are addressed in the present study.

Materials and Methods

Cells and Antibodies. The murine T cell lymphoma EL4 (H-2^b), the lymphoreticular tumor line MDAY-D2 (H-2^d), and the murine thymoma R1.1 (H-2^k) were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, and antibiotics. Transfected R1.1 cells expressing either the D^b molecule, D^b lacking all but three amino acids of its cytoplasmic domain (D^b–cyt), the glycosyl phosphatidylinositol (GPI)-anchored $Q7^b$ molecule, or the GPI-anchored $D^b/Q7^b$ hybrid molecule (D^bcp/cp$Q7^b$) have been described elsewhere (23). R1.1 transfectants were grown in the above RPMI 1640 medium containing 0.5 mg/ml Geneticin (GIBCO BRL, Gaithersburg, MD). Mouse Ltk^- cells expressing soluble D^d (D^d/Q10) were a gift of Dr. David Margulies (NIAID, Bethesda, MD; 25). L cells expressing wild-type D^d or D^d molecules having single Glu to Lys point mutations at residues 222, 223, or 232 were provided by Dr. Terry A. Potter (National Jewish Center for Immunology and Respiratory Diseases, Denver, CO) and have been described previously (26). Ltk^- cells and L cell transfectants were maintained in DME supplemented with 2 mM glutamine, 10% FCS and antibiotics.

Rabbit antisera recognizing TAP1, TAP2, and calnexin have been described elsewhere (9, 15). For the selective isolation of unassembled class I H chains, a rabbit antiserum raised against denatured mouse H chains (anti-HC) was provided by Dr. H. Ploegh and R. Machold (MIT, Cambridge, MA; 27). mAb Y3, which recognizes the α_2 domain of K^b, was used for the selective isolation of β_2m-associated K^b molecules (28, 29). For the isolation of K^b H chain regardless of assembly state, a rabbit antiserum (designated anti-8) directed against the cytoplasmic peptide encoded by exon 8 of the K^b gene was used (29, 30). Anti-8 antiserum was provided by Dr. Brian Barber (University of Toronto). mAb B22.249.R1 that recognizes the α_1 domain of D^b H chain was used for selective isolation of β_2m-associated D^b molecules (31, 32) whereas the α_3 domain-specific mAb 28-14-8s was used for isolation of both free and β_2m-associated D^b or L^d molecules (31–33). The α_1 domain-specific mAb 20-8-4s was used for isolation of β_2m-associated K^b and $Q7^b$ molecules (29, 31). mAb 30-5-7s recognizes the α_2 domain of β_2m-associated L^d molecules (33) and mAb 34-2-12 recognizes the α_3 domain of D^d molecules (33) focusing on the polymorphism at residue 227 (26). D^d molecules associated with β_2m were isolated with the α_2-domain specific mAb 34-5-8s (34).

Reagents. The peptide designated Flu NP Y367-374 (sequence YSNENMETM) corresponds to residues 367 to 374 of the influenza A/PR/8/1934 nucleoprotein with an additional tyrosine at the amino terminus. VSV G 52-59 is a peptide corresponding to residues 52-59 of the vesicular stomatitis virus G protein (sequence RGYVYQGL). Both peptides were synthesized by the Alberta Peptide Institute (Edmonton, Alberta) and judged to be more than 95% pure by RP-HPLC. The protease inhibitor, LLnL (*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; Sigma Chem.

Co., St. Louis, MO) was prepared as a 40 mM stock in DMSO and was added to cell culture medium to a final concentration of 100 μM. Digitonin was obtained from Sigma (~50% pure) and solution concentrations were expressed in terms of actual digitonin content. For chemical cross-linking experiments, dithiobis(succinimidyl propionate) (DSP; Pierce Chemical Co., Rockford, IL) was used. To enhance class I expression in certain transfected L cell lines, 400 U/ml recombinant mouse IFN-γ (PharMingen, San Diego, CA) was added to the culture medium for 24 h.

Metabolic Radiolabeling and Immunoprecipitation. Metabolic radiolabeling of EL4 cells and R1.1 transfectants and immunoprecipitation of class I molecules from digitonin cell lysates were performed as previously described (9). Immune complexes were eluted from the protein A–agarose beads with SDS-PAGE sample buffer either directly or after endo H treatment as described previously (15) and then analyzed by SDS-PAGE and fluorography (12.5% gel). For experiments that included the protease inhibitor LLnL, cells were preincubated in Met-free RPMI 1640 for 40 min in the absence or presence of 100 μM LLnL and the inhibitor was also present throughout the subsequent pulse labeling and chase incubations. Chemical cross-linking experiments were performed with DSP as previously described (12) except that digitonin was used instead of NP-40 in the lysis buffer.

For metabolic radiolabeling of L cell transfectants, a 60-mm dish of confluent cells was incubated in 3 ml of Met-free RPMI 1640 for 30 min and then radiolabeled in 0.1 mCi/ml [^{35}S]Met. Radiolabeled cells were lysed in 1 ml digitonin lysis buffer for 30 min at 4°C and the lysates were subjected to immunoprecipitation (9).

Sequential Immunoprecipitation. In some instances, a sequential immunoprecipitation technique was employed for the analysis of TAP-associated class I molecules (9). Briefly, TAP–class I complexes recovered in an initial round of immunoprecipitation with anti-TAP2 antiserum and protein A–agarose beads were dissociated by heating at 37°C for 1h in PBS, pH 7.4, containing 0.2% SDS. After centrifugation to remove the beads, the supernatant fraction was adjusted to contain 2% NP-40 and 5% skim milk powder and then was subjected to a second round of immunoprecipitation with mAb 28-14-8s (for D^b and its derivatives) or anti-8 antiserum (for K^b). Immune complexes were washed only once with 0.5% NP-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA before elution and analysis by SDS-PAGE. For analysis of calnexin-associated class I molecules, anti-calnexin antiserum was used in an initial immunoprecipitation to recover all calnexin-bound proteins. After dissociation of complexes in SDS as above, K^b and D^b molecules were recovered simultaneously in a second round of immunoprecipitation using a combination of anti-8 antiserum, mAb 28-14-8s, and anti-denatured H chain antiserum.

Cell Permeabilization and Peptide Incubations. Radiolabeled cells were permeabilized with 0.003% digitonin and incubated in the absence or presence of class I–binding peptides to assess dissociation of TAP–class I or calnexin–class I complexes as described before (9). After incubation, cells were collected by centrifugation and lysed in 0.5 ml of digitonin lysis buffer. TAP-associated class I molecules were recovered either by direct immunoprecipitation with anti-TAP2 antiserum or by sequential immunoprecipitation. Calnexin-associated class I molecules were recovered by sequential immunoprecipitation.

Western Blot Analysis. Proteins in anti-class I immunoprecipitates were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with anti-TAP or anti-calnexin antibodies, and the bound antibodies were visualized with ^{125}I-protein A (ICN Pharmaceuticals Inc., Costa Mesa, CA) and autoradiography.

Results

Inhibition of Proteasome Activity Results in Prolonged TAP–Class I Association and Impaired Intracellular Transport of Class I Molecules. Before undertaking an analysis of the site(s) on class I molecules that interact with TAP, we wished to examine further the effects of peptides on this association. Previous work has demonstrated that various peptide aldehydes inhibit the supply of peptide ligands for class I molecules, largely through inhibition of cytosolic proteasome activities (35). To assess the effect on the TAP–class I complex of limiting peptide supply, we performed pulse-chase experiments with EL4 cells in the absence or presence of one of the peptide aldehyde inhibitors, LLnL.

As shown in Fig. 1 *A*, TAP–class I complexes could be recovered at the various chase times by immunoprecipitation with anti-TAP2 antiserum. Deglycosylation of immunoprecipitates with endoglycosidase H (endo H) permitted the resolution of TAP-associated K^b and D^b heavy chains along with associated β_2m. In the absence of LLnL, the amounts of TAP–class I complexes increased to a maximum level after 10-min chase for K^b and 30-min chase for D^b and then dissociated such that half of the complexes were lost by 35 min of chase for K^b and 74 min of chase for D^b (Fig. 1 *B*). The different rates of dissociation closely paralleled the different rates at which K^b and D^b molecules are transported from the ER to the Golgi apparatus in EL4 cells, as established previously (9). In contrast, in the presence of LLnL, dissociation of class I molecules from TAP was substantially delayed such that the half time of dissociation was observed at ~62 min of chase for K^b and at ~260 min for D^b. Another effect of LLnL treatment was a dramatic increase in TAP-K^b complexes to a maximum level 4.2-fold greater than that observed in the absence of LLnL. No such increase was observed for TAP-D^b complexes. Presumably, under conditions of normal peptide supply, TAP-K^b complexes do not have the opportunity to accumulate extensively. Consistent with the subcellular localization of TAP in the ER and *cis*-Golgi (5, 6), prolonged association of class I molecules with TAP resulted in accordingly slower intracellular transport of class I molecules as measured by their conversion to forms resistant to endo H digestion. LLnL treatment slowed the half times of ER to Golgi transport from ~34 to ~60 min for K^b and from ~75 to >120 min for D^b (data not shown).

Extracellular Domains of Class I Molecules Are Sufficient for Association with TAP. To identify the site(s) on class I molecules that interact with TAP, we examined various truncated and hybrid class I molecules for their ability to associate with TAP. Fig. 2 *A* depicts the class I molecules used in this study. Full-length D^b, D^b lacking all but three amino acids of its cytoplasmic tail (D^b-cyt), GPI-anchored D^b composed of the extracellular domains of D^b fused to

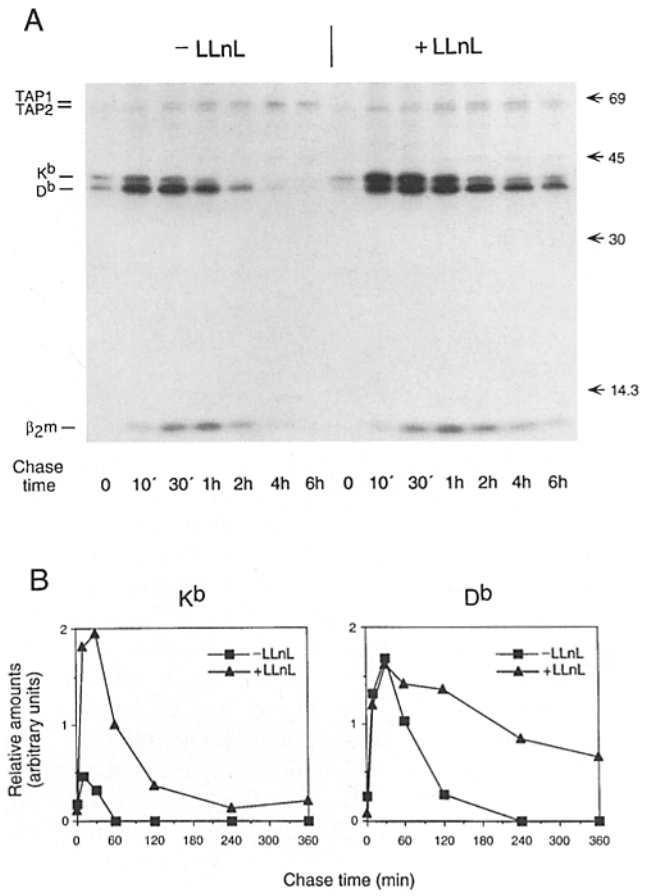

Figure 1. Effects of proteasome inhibition on the TAP–class I interaction. (*A*) Murine EL4 cells were preincubated in the absence or presence of 100 μM LLnL for 40 min, pulse labeled with [^{35}S]Met for 5 min, and then chased for periods of up to 6 h. Cells were lysed in 0.5% digitonin lysis buffer and then immunoprecipitated with anti-TAP2 antiserum to recover TAP–class I complexes. Immunoprecipitates were deglycosylated with endo H and then analyzed by SDS-PAGE. The mobilities of the TAP 1 and TAP 2 subunits, the K^b and D^b H chains, and β_2m are shown. The numbers on the right indicate the mobilities of molecular mass standards in kD. (*B*) Densitometric analysis of the relative amounts of K^b and D^b H chains in *A*.

the COOH-terminal portion of $Q7^b$ in the middle of their connecting peptides (D^bcp/cpQ7^b), and the GPI-anchored class I $Q7^b$ molecule, were all expressed in the murine thymoma cell line, R1.1. The soluble hybrid class I molecule D^d/Q10 was expressed in murine L cells and consisted of the α_1, α_2, and α_3 domains of D^d fused to the COOH-terminal 27 amino acids of the soluble class I molecule, $Q10^b$. Association of the various class I molecules with TAP was

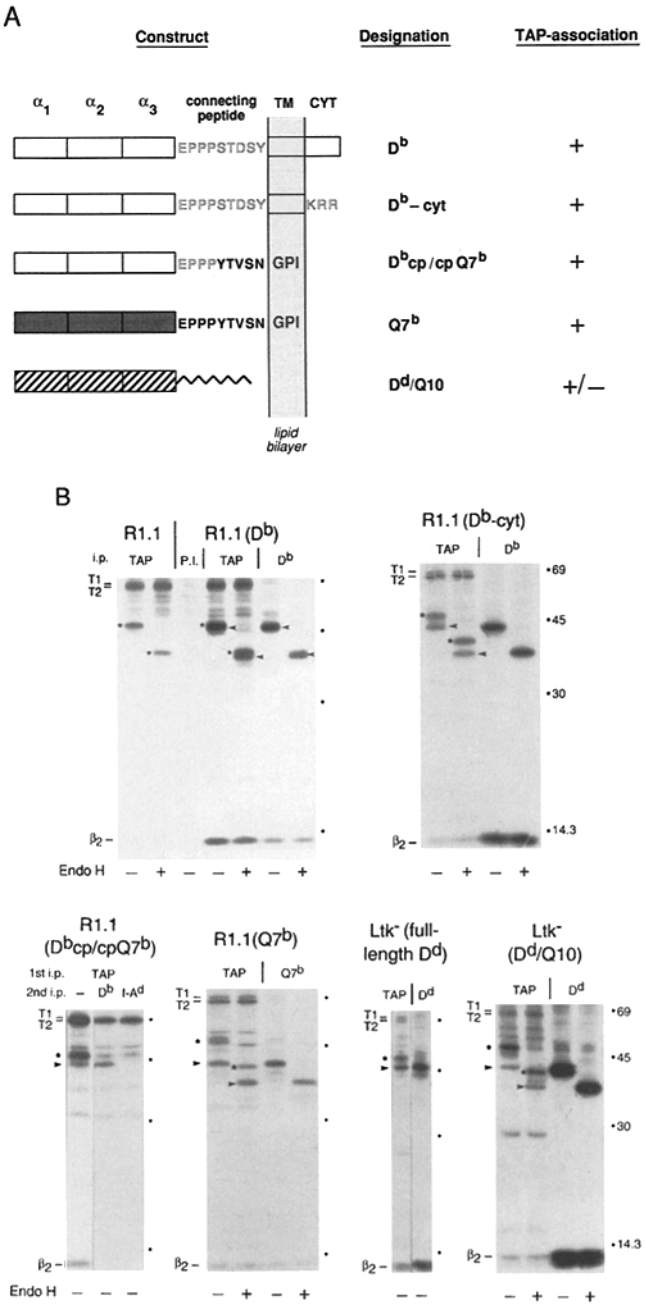

Figure 2. Extracellular domains of class I molecules are sufficient for TAP association. (*A*) Schematic representation of truncated class I molecules used to map sites of interaction with TAP. α_1, α_2, and α_3 represent the three extracellular domains of class I H chains consisting of ~90 amino acids each. TM and CYT represent the H chain transmembrane segment (24 residues for D^b) and cytoplasmic segment (31 residues for D^b), respectively. In the soluble D^d/Q10 hybrid molecule, the hatched boxes correspond to the α_1, α_2, and α_3 domains of D^d and the zigzag line represents the COOH-terminal 27 amino acids of the $Q10^b$ molecule. (*B*) Detection of complexes between TAP and truncated class I molecules. R1.1 or L cell transfectants were radiolabeled for 30 min with [^{35}S]Met, lysed, and then immunoprecipitated with either anti-TAP2 antiserum to isolate TAP–class I complexes or with mAbs β22.249.R1 (for D^b and D^b-cyt), 20-8-4S (for $Q7^b$), and 34-5-8S (for full-length D^d and D^d/Q10) to isolate transfected class I molecules. To distinguish transfected class I molecules from the endogenous K^k and D^k proteins, anti-TAP immunoprecipitates were subjected either to sequential immunoprecipitation (in the case of R1.1(D^bcp/cpQ7^b)) or to deglycosylation by endo H before SDS-PAGE. Mobilities of endogenous H-2^k products are indicated by asterisks and mobilities of transfected class I molecules are indicated by arrowheads. i.p. indicates the specificity of the antibody used for immunoprecipitation and T1, T2, and β_2 denote TAP 1, TAP 2, and β_2m, respectively. For D^bcp/cpQ7^b, the first lane was exposed to film for a half day and the remaining lanes were exposed for 5 days. Note that full-length D^d contains a Glu to Lys mutation at residue 232. This mutant was used instead of wild-type D^d because its synthetic rate was closer to that of D^d/Q10.

assessed by immunoprecipitation of metabolically radiolabeled cell lysates with anti-TAP2 antiserum and identifying co-precipitating class I molecules by SDS-PAGE.

As shown in the first panel of Fig. 2 *B*, anti-TAP antibody coprecipitated the endogenous K^k and D^k molecules from untransfected R1.1 cells (asterisks). These class I molecules were exclusively sensitive to endo H digestion, as expected based on the localization of TAP in the ER and *cis*-Golgi. The K^k and D^k molecules were present in anti-TAP immunoprecipitates of all the R1.1 transfectants but could generally be resolved from the heterologously expressed class I molecule either before or after digestion with endo H. For example, the transfected D^b molecule which was observed to interact with TAP in EL4 cells (Fig. 1), was also found associated with TAP in the R1.1 (D^b) transfectant (Fig. 2 *B*). It could be detected as the lower band (*arrowhead*) of a doublet after endo H digestion of the anti-TAP immunoprecipitate. Examination of the R1.1 transfectant expressing D^b that lacked all but three amino acids of its cytoplasmic tail (Fig. 2 *B*, R1.1 (D^b-cyt)) revealed that the truncated molecule retained the ability to associate with TAP. It was easily resolved from endogenous class I molecules in the anti-TAP precipitate due to its reduced size.

Further truncation of D^b was accomplished by replacing its transmembrane and cytoplasmic segments with a GPI anchor (D^bcp/cpQ7^b). Due to low expression levels, unambiguous identification of this hybrid molecule in the anti-TAP precipitate required a sequential immunoisolation procedure. The anti-TAP precipitate was disrupted in SDS and then subjected to a second round of immunoprecipitation with either anti-D^b mAb 28-14-8S or an isotype matched anti-I-A^d mAb (Fig. 2 *B*, R1.1 (D^bcp/cpQ7^b)). The GPI-anchored D^b molecule was selectively recovered in the anti-D^b immunoprecipitate demonstrating that it was indeed associated with TAP. Confirmation of this finding was obtained by assessing the ability of the naturally GPI-anchored $Q7^b$ molecule to associate with TAP (Fig. 2 *B*, R1.1 ($Q7^b$)). The $Q7^b$ molecule was almost quantitatively recovered in the anti-TAP immunoprecipitate indicating that the transmembrane and cytoplasmic segments of class I H chains are not required for interaction with TAP. However, complete abolishment of any membrane anchor dramatically reduced the amount of class I molecule co-precipitating with TAP. Only ~5% of the soluble D^d/Q10 class I molecule was found in association with TAP (Fig. 2 *B*, Ltk^- (D^d/Q10). This was not due to an inherent inability of D^d molecules to bind to TAP since the full-length D^d protein was readily recovered in anti-TAP immunoprecipitates (Fig. 2 *B*, Ltk^- (full-length D^d).

Extracellular Domains of Class I Molecules Are Sufficient for Peptide-regulated Interaction with TAP. To determine whether the extracellular domains of class I molecules contain all of the elements necessary for peptide-regulated interaction with TAP, we tested two aspects of the TAP–class I interaction: dissociation of the TAP–class I complex upon peptide binding to class I and prolonged association of class I with TAP under peptide limiting conditions.

We established previously that upon incubation of digitonin-permeabilized cells with peptide ligands for class I molecules, peptide binding to class I was accompanied by dissociation of TAP–class I complexes (9). Similar experiments were conducted on full-length D^b and GPI-anchored D^bcp/cpQ7^b molecules expressed in R1.1 cells. As shown in Fig. 3 *A*, more than half of TAP-D^b and TAP-D^bcp/cpQ7^b complexes dissociated at 10 nM peptide. Virtually complete dissociation of both complexes was observed at 10 μM peptide.

To test if proteasome inhibition prolongs association of GPI-anchored D^b with TAP, we performed pulse-chase experiments in the absence or presence of LLnL on R1.1 transfectants expressing either full-length D^b or D^bcp/cpQ7^b (Fig. 3 *B*). In the absence of LLnL, D^bcp/cpQ7^b molecules dissociated from TAP more slowly than was observed for full-length D^b ($t_{1/2}$ ~175 min vs ~75 min, Fig. 3 *B*, -LLnL). In the presence of LLnL, the amounts of TAP-associated D^b and D^bcp/cpQ7^b were substantially increased, reaching a maximum by 1–2 h of chase. Furthermore, little dissociation of either complex was observed throughout the re-

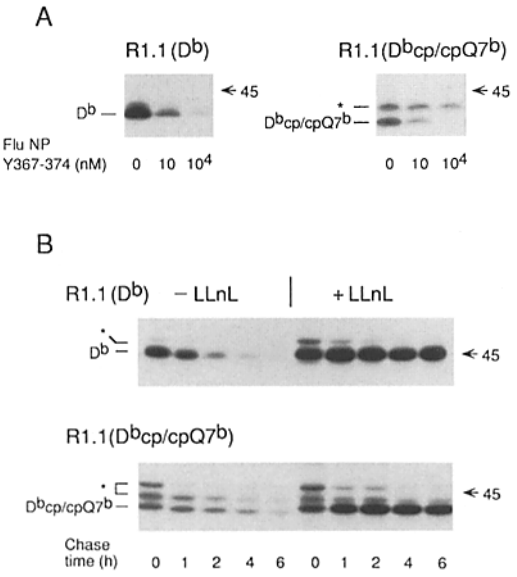

Figure 3. (*A*) Peptide-induced dissociation of D^b and GPI-anchored D^b from TAP. R1.1 transfectants were radiolabeled for 30 min with [^{35}S]Met, permeabilized with 0.003% digitonin, and then incubated in the absence or presence of the indicated concentrations of the D^b-binding peptide, Flu NP Y367-374. TAP-associated D^b molecules were isolated by sequential immunoprecipitation consisting of an initial immunoprecipitation with anti-TAP2 antiserum, solubilization of the immunoprecipitate, and a second immunoprecipitation with the anti-D^b mAb, 28-14-8S. All samples were deglycosylated with endo H before analysis by SDS-PAGE. The protein denoted by the asterisk was also recovered when a control mAb was used in the second immunoprecipitation and thus was judged to be non-specific. (*B*) Both full-length D^b and GPI-anchored D^b remain associated with TAP upon peptide depletion with LLnL. R1.1 transfectants were preincubated for 40 min in Met-free RPMI 1640 medium containing either 0 or 100 μM LLnL, labeled with [^{35}S]Met for 30 min, and then chased for up to 6 h in the continued absence or presence of LLnL. At the indicated times, TAP-associated D^b and D^bcp/cpQ7^b were isolated by sequential immunoprecipitation with anti-TAP2 antiserum followed by mAb 28-14-8S. Asterisks denote proteins recovered non-specifically in the immunoprecipitates.

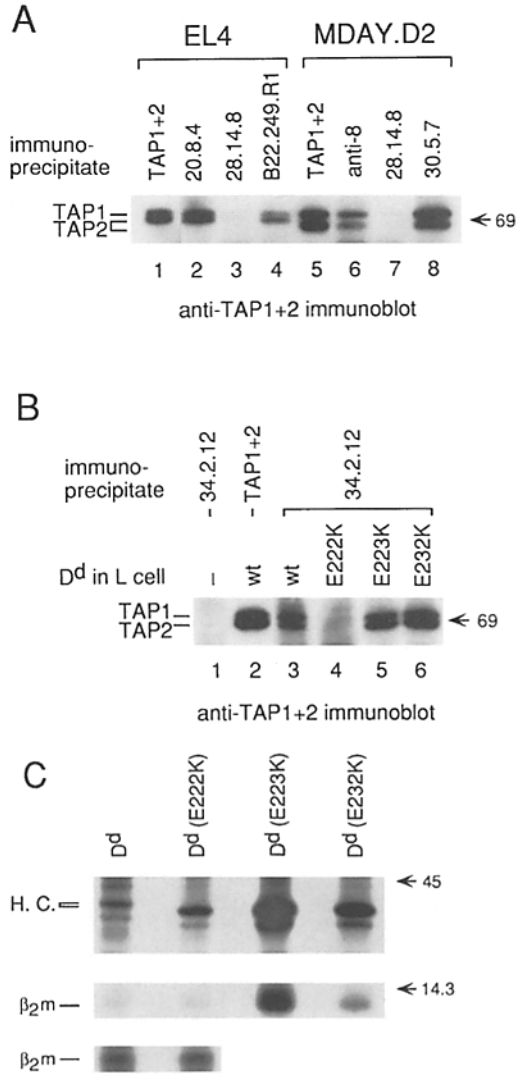

Figure 4. A site of interaction with TAP resides within the α_3 domain of class I H chains. (*A*) Isolation of TAP–class I complexes with various anti-class I Abs. EL4 (H-2^b) or MDAY-D2 (H-2^d) cells were lysed and subjected to immunoprecipitation with antibodies having the following specificities: 20-8-4S (α_1 domain of K^b), 28-14-8S (α_3 domain of D^b and L^d), B22.249.R1 (α_1 domain of D^b and L^d), anti-8 (cytoplasmic tail of K^b and K^d), and 30-5-7S (α_2 domain of L^d). Co-isolated TAP proteins in these immunoprecipitates were detected with a mixture of anti-TAP1 and anti-TAP2 antisera by Western blot analysis. Lanes *1* and *5* depict anti-TAP1 and TAP2 immunoprecipitates from one-twentieth (EL4) or one-quarter (MDAY-D2) the volume of cell lysate used in the other lanes. (*B*) Interaction of mutant D^d molecules with TAP. Untransfected tk^- L cells (lane *1*) or L cells expressing either wild-type D^d (lanes *2* and *3*) or mutant D^d molecules possessing a Glu to Lys mutation at residues 222 (lane *4*), 223 (lane *5*), or 232 (lane *6*) were lysed and then immunoprecipitated with either a mixture of TAP1 and TAP2 antiserum (lane *2*) or with mAb 34-2-12S to isolate D^d molecules. TAP proteins co-immunoprecipitating with D^d were detected by Western blotting with a mixture of TAP1 and TAP2 antisera. Although TAP synthesis was comparable in all transfectants (not shown), wild-type D^d and mutant E222K exhibited low synthetic levels (see *C*). To compensate, these transfectants were treated with 400 U/ml IFN-γ for 24 h before lysis and three times more cells were used than in lanes *1*, *5*, and *6*. The TAP1 and TAP2 signals in lane *2* were obtained from one-sixteenth of the cell lysate used in lanes *1*, *5*, and *6*. (*C*) L cell transfectants (5×10^6 cells) expressing the indicated D^d molecules were radiolabeled with [^{35}S]Met for 30 min, lysed, and then subjected to immunoprecipitation with mAb 34.2.12S. Immunoprecipitates were analyzed by SDS-PAGE after deglycosylation with endo H. (*Top*) H chain region of gel; (*middle*) β_2m region of gel; (*bottom*) a fourfold longer exposure of the first two lanes in the middle panel.

maining chase period up to 6 h. It is noteworthy that in LLnL-treated EL4 cells, significant dissociation of TAP-D^b complexes was observed over a 6-h chase period (Fig. 1). The difference between EL4 and R1.1 (D^b) may be due in part to the longer labeling time (30 min vs 5 min) used for R1.1 cells which was necessitated by the sequential immunoprecipitation technique employed for these cells. Alternatively, EL4 cells may differ from R1.1 cells in the extent to which they produce D^b-binding peptides by a route not inhibitable by LLnL. Based on these data, we conclude that the extracellular domains of class I molecules contain all of the elements required for peptide-regulated interaction with TAP.

The Membrane Proximal Region of the Heavy Chain α_3 Domain May Interact with TAP. Two lines of evidence are consistent with TAP binding to the α_3 domain of class I H chains. First, mAb 28-14-8S, which is directed against the α_3 domain of D^b and L^d molecules, was not able to coprecipitate TAP as revealed by Western blotting of 28-14-8S precipitates from EL4 (H-2^b) or MDAY-D2 (H-2^d) cells (Fig. 4 *A*). In contrast, mAbs 20-8-4S, B22.249.R1, and 30-5-7S that recognize the α_1 or α_2 domains of K^b, D^b, or L^d H chains, respectively, and anti-8 antiserum that binds to the cytoplasmic tail of K^d, were all capable of recovering TAP–class I complexes. This suggests either that the 28-14-8S epitope is rendered inaccessible by TAP binding or that the binding of this mAb disrupts TAP–class I complexes. The latter possibility was excluded because the amount of TAP–class I complexes was not reduced in radiolabeled cell lysates after two rounds of preclearance with mAb 28-14-8S (data not shown).

The second line of evidence comes from analyses of D^d point mutants that were originally generated to map the CD8 binding site on class I molecules. It has been shown that H-2D^d molecules possessing Glu to Lys mutations at residues 222 and 223 lose the ability to bind to CD8 and consequently fail to stimulate CD8-dependent CTL; the same mutation at residue 232 is without effect (26). Interestingly, when wild-type D^d and mutants E222K, E223K, and E232K were immunoprecipitated from cell lysates and the immunoprecipitates blotted with anti-TAP antiserum, the E222K mutant was the only molecule that failed to associate with TAP (Fig. 4 *B*). This was not due to a defect in assembly with β_2m since β_2m coimmunoprecipitated with all of the D^d H chains studied (Fig. 4 *C*). Taken together, these data suggest that at least one site of interaction with TAP resides in the membrane-proximal region of the H chain α_3 domain.

TAP and Calnexin Can Associate with the Same Class I Heterodimer. We previously demonstrated that in addition to associating with TAP, mouse H chain-β_2m heterodimers bind to the molecular chaperone, calnexin (12).

Thus, it was of interest to determine if class I heterodimers interact with TAP and calnexin separately or if the association can occur through a ternary complex consisting of class I heterodimer, TAP1,2 heterodimer, and calnexin.

As shown in Fig. 5 *A*, lane *2*, when a radiolabeled lysate of EL4 cells was treated with the homobifunctional cross-linking agent DSP and then subjected to immunoprecipitation with a mixture of β_2m-dependent anti-K^b and anti-D^b mAbs, cross-linked species corresponding to H chain-β_2m heterodimers (~60 kD) and calnexin-H chain complexes (~160 kD) were detected. These species were absent from a control sample in which DSP was omitted (lane *1*). When anti-TAP2 antiserum was used for immunoprecipitation of the DSP-treated lysate (lane *4*), it coprecipitated class I H chains as expected but also species that comigrated with cross-linked H chain-β_2m heterodimers and calnexin–H chain complexes (compare lane *2* with *4*). To confirm that a calnexin–H chain cross-linked complex was indeed recovered in the TAP immunoprecipitate, samples identical to those in lane *4* were solubilized and subjected to a second round of immunoprecipitation with either anti-K^b, anti-D^b, or anti-calnexin Abs (lanes *5*–*7*). The anti-class I Abs recognized uncross-linked H chains, low levels of a D^b-β_2m cross-linked complex (~60 kD), several species of ~100-kD, and an ~160-kD species. The ~100-kD species have not been identified but the ~160-kD species was also recognized by the anti-calnexin Ab (lane 7), confirming that it represented calnexin cross-linked to either K^b or D^b H chains. The fact that anti-TAP antiserum was capable of recovering cross-linked H chain–calnexin complexes demonstrates the existence of a ternary complex between TAP, class I heterodimers, and calnexin.

We next sought to quantitate the proportion of TAP-associated class I heterodimers that are also bound to calnexin. EL4 cells were radiolabeled for 10 min with [^{35}S]Met and then chased for 10 min to generate maximal levels of TAP–class I complexes. As shown in Fig. 5 *B*, the pulse-chase regimen was effective in producing predominantly β_2m-associated K^b and D^b molecules (lane *1*) with relatively low levels of free H chains (lane *2*). Coimmunoprecipitation and immunodepletion techniques were then used to assess the amounts of H chain-β_2m heterodimers that were associated with TAP, with calnexin, or with both TAP and calnexin. Anti-TAP2 antiserum was used to coimmunoprecipitate complexes of TAP and class I heterodimers (lane *3*). Densitometric analysis revealed that ~40% of K^b heterodimers and ~60% of D^b heterodimers were associated with TAP (compare lane *1* with *3*). Attempts to isolate complexes of calnexin and class I heterodimers by coimmunoprecipitation with anti-calnexin antiserum were complicated by the presence of many other proteins that bound to this chaperone (lane *9*). To circumvent this problem, the amounts of calnexin–class I heterodimer complexes were estimated by clearing cell lysates three times with either preimmune serum or anti-calnexin Ab and then comparing the levels of K^b and D^b heterodimers that could be recovered in a subsequent immunoprecipitation with β_2m-dependent mAbs. As shown in lanes *11* and *12*, the recoveries of both K^b and D^b heterodimers were reduced after preclearance with anti-calnexin antiserum as compared to the recoveries after preclearance with preimmune serum. Densitometric analysis indicated that ~45% of K^b heterodimers and 85% of D^b heterodimers were associated with calnexin. These should be considered minimal estimates given the likelihood that some complex dissociation occurred during the repeated clearances.

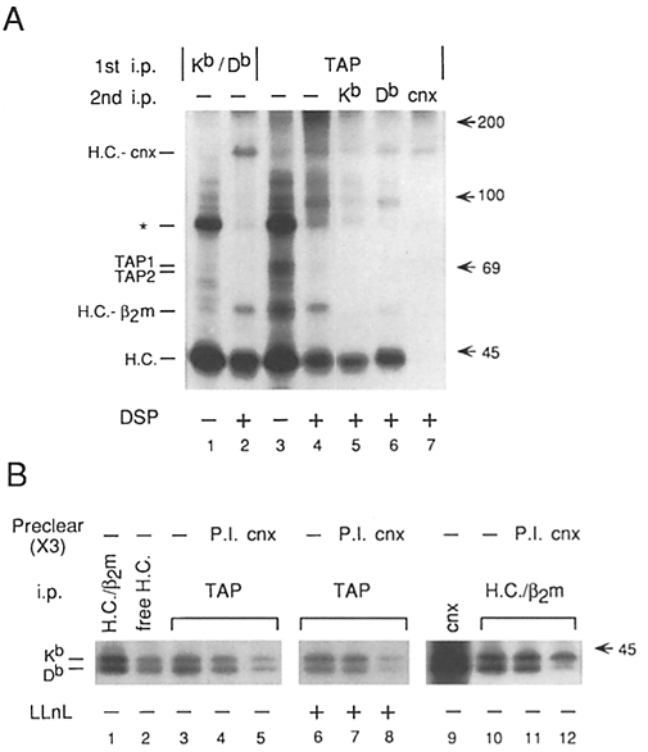

Figure 5. Detection of a complex between TAP, class I heterodimers and calnexin. (*A*) EL4 cells radiolabeled for 15 min with [^{35}S]Met were lysed in digitonin lysis buffer (pH 8) in the absence or presence of DSP. Cell lysates were subjected to immunoprecipitation either with a mixture of mAb Y3 and B22.249.R1 (K^b/D^b) or with TAP2 antiserum. Where indicated, anti-TAP immunoprecipitates were dissociated in SDS and then subjected to a second round of immunoprecipitation with either anti-8 antiserum (K^b), mAb 28-14-8s (D^b), or anti-calnexin antiserum (*cnx*). Samples were analyzed by non-reducing SDS-PAGE (10% gel). The mobilities of cross-linked H chain-β_2m (*H.C.-β_2m*) and H chain-calnexin (*H.C.-cnx*) complexes are indicated. The asterisk denotes an unidentified protein that appears irreproducibly in some immunoprecipitates. (*B*) EL4 cells were radiolabeled for 10 min with [^{35}S]Met and then chased for 10 min. Cells were lysed and equal aliquots of lysate were subjected to immunoprecipitation with either a mixture of mAbs Y3 and B22-249.R1 (to recover β_2m-associated K^b and D^b; *H.C./β_2m*), anti-denatured H chain antiserum (to recover free H chains; *free H.C.*), anti-TAP2 antiserum (*TAP*), or anti-calnexin antiserum (*cnx*). To measure the amount of TAP–class I-calnexin ternary complexes, equal aliquots of lysate were subjected to three rounds of preclearance with either preimmune serum (*P.I.*; lanes *4* and *7*) or anti-calnexin antiserum (lanes *5* and *8*) before recovery of TAP–class I complexes with anti-TAP2 antiserum. To assess the level of calnexin-class I heterodimer complexes, two additional aliquots of lysate were subjected to three rounds of clearance with either preimmune serum or anti-calnexin antiserum before recovery of class I heterodimers with a mixture of mAbs Y3 and B22-249.R1 (lanes *11* and *12*). All immune complexes were digested with endo H before analysis by SDS-PAGE under reducing conditions.

A similar preclearance approach was used to determine the amounts of class I heterodimers that were present in a complex with both TAP and calnexin. Duplicate samples of lysate were subjected to three rounds of clearance with either anti-calnexin Ab to remove all calnexin-associated proteins or with preimmune serum. TAP-associated H chain–β_2m heterodimers remaining after the preclearances were then compared after their recovery with anti-TAP2 antiserum. As shown in lanes *4* and *5*, preclearance with anti-calnexin Ab resulted in a reduction in the recovery of TAP–class I complexes. This confirms that class I heterodimers associate simultaneously with TAP and calnexin. Densitometric analysis revealed that in two separate experiments, 61–71% of TAP-associated K^b-β_2m heterodimers were cleared with the anti-calnexin Ab and thus were bound to calnexin. Likewise, 63–81% of TAP-associated D^b-β_2m heterodimers were present as ternary complexes with calnexin. Again, these should be considered minimal estimates since some dissociation of complexes may have occurred. Indeed, a reduction in TAP–class I complexes was observed when the levels of TAP-associated class I molecules were compared after direct TAP immunoprecipitation (lane *3*) or after three rounds of clearance with preimmune serum (lane *4*). The extent of complex dissociation varied between experiments. For example, when the experiment was repeated using LLnL to limit peptide supply, virtually no losses of TAP–class I complexes were observed when levels were compared after direct TAP immunoprecipitation (lane *6*) or after three clearances with preimmune serum (lane 7). Furthermore, preclearance with anti-calnexin antiserum resulted in a dramatic reduction in the recovery of both TAP-K^b and TAP-D^b complexes (compare lane 7 with *8*); 82% of TAP-associated K^b-β_2m heterodimers and 83% of TAP-associated D^b-β_2m heterodimers were present as ternary complexes with calnexin.

Differential Effects of Peptide Binding to Class I Molecules on the Dissociation of TAP and Calnexin. Although the binding of peptide ligands to class I heterodimers is associated with release of the TAP transporter, it is not clear if peptide binding is sufficient to induce calnexin dissociation.

To assess the effects of peptide binding to class I molecules on the dissociation of TAP and calnexin, EL4 cells were again radiolabeled for 10 min with [^{35}S]Met and then chased for 10 min to generate predominantly β_2m-associated K^b and D^b molecules (Fig. 5 *B*, lanes *1* and *2*). The cells were permeabilized with digitonin and incubated in the absence or presence of K^b- or D^b-specific peptide ligands. After cell lysis, TAP–class I complexes were recovered by coimmunoprecipitation with anti-TAP2 antiserum. Consistent with previous results (reference 9 and Fig. 3 *A*), incubation of permeabilized cells with either the K^b-restricted VSV G 52-59 peptide or the D^b-restricted Flu NP Y367-374 peptide led to the selective dissociation of 80% of TAP-K^b and 70% of TAP-D^b complexes, respectively (Fig. 6, lanes *1–3*; quantitation by densitometry). K^b and D^b molecules bound to calnexin (consisting almost exclusively of H chain-β_2m heterodimers; see previous section) were isolated by sequential immunoprecipitation.

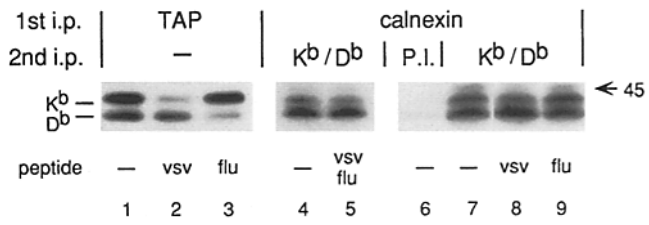

Figure 6. Effects of peptide binding to class I on dissociation from TAP and calnexin. EL4 cells were radiolabeled for 10 min with [^{35}S]Met and chased for 10 min. Cells were permeabilized with 0.003% digitonin and then incubated in the absence or presence of 10 μM D^b-binding peptide, Flu NP Y367-374 (*flu*), or 10 μM K^b-binding peptide, VSV G 52-59 (*vsv*), as indicated. After lysis in digitonin lysis buffer, TAP-associated class I molecules were recovered with anti-TAP2 antiserum (lanes *1–3*). Class I molecules bound to calnexin were isolated by initial precipitation with anti-calnexin antiserum, followed by solubilization of the immunoprecipitate in SDS and subsequent immunoprecipitation of K^b and D^b with a mixture of anti-8 antiserum, mAb 28-14-8s, and anti-denatured H chain antiserum (lanes *4*, *5*, *7–9*). P.I. denotes pre-immune serum. Immune complexes were digested with endo H before analysis by reducing SDS-PAGE.

This involved an initial precipitation with anti-calnexin antiserum to recover all calnexin-associated proteins and, after dissociation of complexes in SDS, immunoprecipitation with a mixture of anti-K^b and anti-D^b antibodies. As shown in Fig. 6, lanes *4* and *5*, incubation of the permeabilized cells simultaneously with the K^b- and D^b-specific peptides resulted in little, if any, loss of calnexin-associated D^b molecules but a significant reduction in calnexin-associated K^b molecules. The experiment was repeated using permeabilized cells incubated with each peptide separately with similar results (Fig. 6, lanes *6–9*). Densitometric analysis revealed that, for the two experiments, peptide binding to class I led to a 15–30% reduction in calnexin-K^b complexes and a 3–9% reduction in calnexin-D^b complexes. Therefore, peptide binding to calnexin-associated H chain-β_2m heterodimers can induce some dissociation from the chaperone, but clearly not to the extent observed for peptide-induced dissociation of TAP–class I complexes.

Discussion

In this report, we demonstrate that class I molecules exhibit prolonged association with TAP under peptide limiting conditions imposed by inhibiting proteolytic activity with the peptide aldehyde, LLnL. This finding supports the notion that class I molecules associate with TAP in order to facilitate acquisition of appropriate peptide ligands. Although both K^b and D^b remained bound with TAP for prolonged periods in the presence of LLnL, they differed in the extent to which LLnL treatment enhanced their association with TAP and in the kinetics of their dissociation. In the case of K^b, LLnL treatment resulted in a roughly fourfold increase in the level of heterodimers bound to TAP whereas little change was observed in the level of TAP-D^b complexes. This observation suggests that under normal conditions, peptides capable of binding to K^b may be more abundant than D^b-specific peptides, and hence TAP-bound

K^b molecules may acquire peptide and dissociate rapidly from TAP with little opportunity to accumulate. Even when peptide supply was inhibited with LLnL, TAP-K^b complexes dissociated more rapidly than TAP-D^b complexes. This may reflect an inherent lower affinity of TAP for K^b or, alternatively, that K^b can acquire peptides more readily than D^b from sources not inhibited by LLnL. The prolonged association of K^b and D^b molecules with TAP in LLnL-treated cells was accompanied by a concomitant slowing of ER to Golgi transport. Although this is suggestive of a role for TAP in retaining peptide-deficient class I molecules in the ER, such a conclusion is complicated by the observation that the TAP–class I complexes also interact with the resident ER chaperone, calnexin (see below).

The finding that class I molecules associate with TAP only after H chain-β_2m assembly (9, 10) and that the interaction is affected either by peptide depletion or by peptide binding to class I (9, 10 and Fig. 1), suggests that regulation of the TAP–class I interaction is achieved by conformational changes associated with β_2m and peptide binding to the H chain. Although there is some evidence that the H chain cytoplasmic tail exhibits structural differences between β_2m-bound and unbound states (36, 37), it is the extracellular domains of the H chain that most clearly reflect conformational changes induced by β_2m and peptide binding (29, 32, 38–42). Thus, it is perhaps not surprising that we found the extracellular (ER luminal) portion of class I molecules to be sufficient for peptide-regulated interaction with TAP.

It is noteworthy that the non-classical, GPI-anchored class I molecule, $Q7^b$, associated efficiently with TAP. Since recent work using a mutant human cell line has suggested that class I association with TAP is necessary for normal acquisition of peptide ligands (43), the existence of a TAP-$Q7^b$ complex is consistent with the finding that $Q7^b$ molecules are occupied by peptides (44). In contrast to $Q7^b$, little of the soluble class I molecule, D^d/Q10, was recovered in association with TAP. This may suggest that a membrane-anchor is required for stable TAP association in vivo or, alternatively, that TAP complexes with soluble class I molecules are more labile during immunoisolation. We favor the latter hypothesis because ~85–95% of these soluble molecules have been shown to be peptide filled (D. Margulies, personal communication) and a soluble form of the $Q7^b$ molecule has also been shown to contain a diverse array of peptides (45). Very recently, a soluble form of the L^d molecule, L^d/Q10 was demonstrated to interact with TAP but no indication of the efficiency of the interaction was provided (20).

TAP associates with class I heterodimers through the TAP1 subunit (9, 46). To localize where a TAP interaction site resides within the extracellular portion of class I molecules we probed different domains of class I molecules with a panel of antibodies and also tested H chain point mutants for their ability to associate with TAP. Both approaches suggest a TAP interaction site within the α_3 domain. The antibody experiments revealed that reagents directed against α_1, α_2, or cytoplasmic domains recognized TAP–class I complexes but the α_3 domain-specific mAb, 28-14-8S, did not. It is noteworthy that another α_3 domain-specific mAb, 34-2-12S, was capable of recognizing TAP–class I complexes. This difference is likely due to the distinct locations of their respective epitopes. The 28-14-8S epitope appears to be within β strand 6 of the α_3 domain of D^b or L^d involving residues 260-262 (47 and G. Waneck, unpublished data) whereas the 34-2-12s epitope involves residue 227 within the loop joining β strands 3 and 4 in the D^d α_3 domain (26). Of three D^d point mutants tested, only a Glu → Lys mutation at position 222 failed to bind to TAP. This residue resides in a conserved acidic loop (amino acids 222-229) in the membrane proximal portion of the α_3 domain that constitutes a major interaction site for CD8 binding (26, 48). Although preliminary, this observation raises the interesting possibility that to associate with a broad range of class I molecules, the TAP transporter uses a portion of the highly conserved CD8 binding site. A TAP-interaction site on the membrane-proximal area of the α_3 domain is also consistent with the predicted small loops of the transporter that penetrate the lumen of the ER (3, 4).

It is of interest that most of the anti-class I mAbs we found to be capable of recognizing TAP–class I complexes were directed against folded determinants in the H chain α_1- and α_2-domains. Recent work by Carreno et al. (20) concluded that only "open" forms of the H-$2L^d$ molecule associate with TAP and that folded L^d molecules detectable with conformation-dependent mAbs such as B22.249.R1 and 30-5-7S do not bind to TAP. Our findings are not consistent with this conclusion. Not only did mAb 30-5-7S recognize folded L^d-TAP complexes, but the conformation-dependent mAbs B22.249.R1 and 20-8-4S were effective in recovering folded D^b-TAP and K^b-TAP complexes, respectively (Fig. 4 *A*). The basis for this discrepancy is unknown but may reflect the different cell lines employed in the two studies. The same group also examined a human cell line lacking classical class I H chains and obtained evidence suggesting that TAP may associate directly with β_2m (20). This raises the possibility that TAP interaction with class I heterodimers could be mediated through β_2m. Such a possibility is not supported by our data. First, we have consistently been unable to observe any newly synthesized free β_2m associating with TAP at early chase times after pulse radiolabeling (e.g., Fig. 1 *A*). Second, the Glu → Lys mutant at position 222 of the D^d H chain retains association with β_2m but does not bind to TAP. Perhaps a low affinity TAP-β_2m interaction can be detected only under conditions of H chain deficiency or, alternatively, the presence of non-classical H chain-β_2m heterodimers could be responsible for the observed association.

Since class I heterodimers interact with calnexin as well as with TAP it is important to determine if these complexes occur separately or if TAP, class I heterodimers, and calnexin associate in a large, muti-component complex. This issue is particularly relevant from the standpoint of quality control in the biogenesis of class I molecules. Peptide-deficient heterodimers are largely retained within the ER (49) and there is some controversy concerning the relative roles of calnexin and TAP as effectors of this retention (10, 20).

We confirmed our previous observations that a substantial portion (40–60% or more) of K^b and D^b heterodimers could be detected in complexes with TAP (9). Furthermore, most heterodimers could be detected in association with calnexin (45% for K^b and 85% for D^b) consistent with our earlier findings (12). Note that the relatively low recovery of calnexin-K^b heterodimer complexes seems to be a consequence of a particularly labile interaction since another K locus product, K^d, exhibits near quantitative binding of H chain-β_2m heterodimers to calnexin (12). Most importantly, we observed that as much as 70–80% of those heterodimers that associated with TAP were also associated with calnexin. Thus, it appears that heterodimers exist mainly as binary complexes with calnexin or as ternary complexes with TAP and calnexin; the pool of heterodimers that bind only to TAP is quite small.

Our findings suggest a model for the major pathway in the biogenesis of mouse class I molecules. In this model, newly synthesized H chains bind quantitatively to calnexin (12, 13) and the interaction is maintained during H chain assembly with β_2m. Most of the calnexin-bound H chain-β_2m heterodimers then associate with TAP to form a large, ternary complex. After their acquisition of peptide ligand, class I molecules dissociate from TAP but remain largely bound to calnexin. This latter point is based on our observation that the introduction of peptide into permeabilized cells results in almost complete dissociation of K^b- and D^b-β_2m heterodimers from TAP whereas only 15–30% of K^b and <10% of D^b heterodimers are released from calnexin. The inability of peptide ligand to dissociate calnexin-L^d complexes in detergent lysates has also been reported recently, although it was not clear if the complexes contained predominantly free L^d H chains or L^d-β_2m heterodimers (20). In the last stage of the model, dissociation from calnexin occurs and the fully assembled class I molecule is exported from the ER for transport to the cell surface. The nature of the event that permits class I release from calnexin remains unknown, but it appears to occur shortly after dissociation from TAP since the kinetics of dissociation from TAP, release from calnexin, and transport to the Golgi are essentially identical within the resolution of our assays (9, 12). In this model, both TAP and calnexin may participate in the ER retention of peptide-deficient heterodimers, but calnexin is the molecule that ultimately determines if a mouse class I molecule is exported to the Golgi apparatus. It is important to consider the possibility that additional components may participate in the formation or maintenance of the TAP–class I–calnexin complex. The discovery of a gene linked to the human MHC that seems to be required for TAP–class I association is consistent with another component playing a role either in delivering class I molecules to TAP or in mediating their interaction (43).

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