TAP Association Influences the Conformation of Nascent MHC Class I Molecules

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TAP Association Influences the Conformation of Nascent MHC Class I Molecules

Barbara A. L. Owen* and Larry R. Pease†

The influence of TAP-MHC class I interactions on peptide binding to the class I heavy chain is assessed during TAP-dependent assembly using Kβ-specific Abs that recognize conformational changes induced by assembly with β2-microglobulin (β2m) and by peptide binding. A significant portion (45%) of Kβ molecules in TAP+, RMA-derived microsomes are associated with the TAP complex as measured by coimmunoisolation of Kβ using anti-TAP1 Abs, while only 20% of the Kβ heavy chain molecules are isolated as Kββ2m complexes with the α-Kβ-specific Abs, Y-3 or K-10-56. The amount of Kβ isolated with Y-3 and K-10-56 increases in proportion to transport and binding of peptide to the Kβ molecules within the RMA microsomes. In contrast, less than 5% of the Kβ within TAP2-RMA-S microsomes associated with the remaining TAP1 subunit. However, greater than 60% of Kβ heavy chain is isolated as K-10-56- and Y-3-reactive Kββ2m complexes. We propose that a TAP-MHC class I interaction serves to stabilize the MHC class I β2m complex in an immature conformation (Y-3 and K-10-56 nonreactive) prior to high affinity peptide binding, preventing the export of class I molecules complexed with low affinity peptide ligands from the ER. The Journal of Immunology, 1999, 162: 4677–4684.

Assembly of the MHC class I complex occurs very shortly after biosynthesis of the MHC class I heavy chain. The nascent MHC class I protein is cotranslationally inserted into the endoplasmic reticulum (ER), associates with the β2-microglobulin (β2m) subunit within minutes of its synthesis, and shortly thereafter is competent to bind peptide. Cofactors in MHC class I assembly have been identified, but the exact pathway is not completely understood. One aspect of MHC class I complex assembly that remains to be fully characterized is how the TAP/tapasin complex mediates peptide binding to the MHC class I heavy chain.

Cell surface expression of MHC class I is barely detectable in β2m-deficient cell lines (2, 3) or β2m knockout mice (4). Likewise, a requirement for the ER membrane protein, TAP, for normal cell surface expression of MHC class I complex has been demonstrated through the use of a variety of TAP-mutant cell lines (5–8) and TAP-knockout mice. (9). Deficiencies in TAP or β2m are associated with aberrantly glycosylated (10) and partially assembled MHC class I molecules, which are retained in the ER/cis-Golgi (11, 12). Therefore, lack of either β2m or peptide results in the inability of the MHC class I heavy chain to traffic efficiently through the secretory pathway, resulting in the severely reduced expression of MHC class I on the cell surface.

MHC class I proteins associate with calnexin (13) and calreticulin (14, 15), two chaperones which reside in the ER and are sensitive to the glycosylation state of their targets. The contributions of these chaperones is not fully understood, but may be overlapping since MHC class I expression is normal in a calnexin-deficient cell line (16). The recently discovered tapasin protein (14, 15, 17, 18), which has been shown to associate with TAP and calreticulin, also modulates the level of MHC class I cell surface expression. The basis of the tapasin requirement has not been determined, but a function similar to that of HLA-DM in MHC class II assembly has been proposed (19). In a tapasin-deficient cell line, 721.220, the levels of MHC class I expression are variable and allele dependent (17).

Although MHC class I heavy chains have been coimmunoprecipitated with anti-TAP Abs, a requirement for a direct TAP-MHC class I interaction for efficient peptide loading remains controversial. Allele-specific differences have been noted with regard to TAP-MHC class I association; for example, some HLA-B heavy chains do not demonstrably associate with TAP, but are able nonetheless to present antigenic peptides (20). Additionally, both membrane-bound HLA-G and soluble HLA-G class I proteins bind a similar peptide repertoire, while only the membrane-bound form has been coimmunoprecipitated with TAP (21). These findings must be interpreted in the context of the fact that failure to demonstrate associations experimentally is not equivalent to the absence of biological associations. Evidence in support of the importance of a TAP-MHC class I interaction is based on the findings that a mutant HLA-A2.1 molecule, T134K, does not associate demonstrably with TAP and is expressed at 20% of the level of the parent molecule (22). However, HLA-A2.1-T134K also apparently does not interact demonstrably with calreticulin, and therefore, the cause of impaired peptide binding may not be solely due to inefficient TAP association with the mutant molecule (23).

Although the phenotype of cell surface MHC class I complexes derived from TAP-deficient cells has been studied extensively, there is less known about the conformation of intracellular MHC class I/β2m complexes in TAP-deficient cells. To better define aspects of the MHC class I assembly process in the endoplasmic reticulum, the role that TAP plays in the formation of early MHC class I-β2m complexes was investigated. We find that the conformation of Kββ2m complexes formed in TAP2-deficient microsomes differs substantially from those formed in TAP-positive microsomes. Our findings demonstrate that in the presence of a

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functional TAP (TAP/tapasin) complex, a significant portion of the nascent MHC class I molecules in sealed microsomes does not assemble into structures recognized by K\(^b\)-specific Abs until appropriate peptides are added. In contrast, in the absence of functional TAP molecules, irrespective of whether K\(^b\)-binding peptides are added to microsomes, the majority of the class I molecules assemble as if they are bound to low affinity ligands within the endoplasmic reticulum. We interpret these findings as evidence that the TAP complex performs regulatory functions beyond peptide transport. We raise the possibility that TAP may be an integral part of an editing process, functioning to assure that most class I molecules exported to the cell surface are bound to high affinity peptides.

Materials and Methods

Cell lines, Abs, and peptides

The murine adenoma cell lines RMA and RMA-S were obtained from P. Cresswell (Yale University, New Haven, CT). The cells were maintained at 37°C, 5% CO\(_2\) in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM l-glutamine, and 100 U penicillin/streptomycin/ml. The mAbs Y-3 (\(\alpha_1\)2-K\(^b\)-specific, \(\beta_m\)-dependent, peptide insensitive) and 28-14-8 (\(\alpha_3\)-L\(^4\)-specific) were purchased from American Type Culture Collection (Rockville, MD) and the mAb K-10-56 (\(\alpha_1\)2-K\(^b\)-specific, \(\beta_m\)-dependent, peptide sensitive) was originally supplied by G. Hammerling (Germany). Peptide was added to microsomes, the majority of the class I molecules assembled as if they were bound to low affinity ligands within the endoplasmic reticulum. We interpret these findings as evidence that the TAP complex performs regulatory functions beyond peptide transport. We raise the possibility that TAP may be an integral part of an editing process, functioning to assure that most class I molecules exported to the cell surface are bound to high affinity peptides.

Cloning (cDNA), RNA transcription, and in vitro translation

A cDNA for the murine class I MHC heavy chain, K\(^b\), obtained from D. Margulies (National Institutes of Health, Bethesda, MD) was excised with EcoRI and subcloned into the PGM7enz (Promega, Madison, WI) vector behind the T7 promoter. Sequence was confirmed by dideoxy sequencing; this particular splice variant of K\(^b\) is missing exon-8, which encodes the last 9 amino acids of the cytoplasmic tail of most K\(^b\) proteins. K\(^b\) cDNA was linearized using HindIII and RNA was transcribed from 4 \(\mu l\) of linearized insert with T7 polymerase (Boehringer Mannheim, Indianapolis, IN). RNA was capped during synthesis with p\(^9\)-5'-\((7\text{-methyl})\)-guanosine-\(\pmb{p}\),\(\pmb{g}\)-guanosine triphosphate, dilithium salt (Calbiochem, San Diego, CA). The RNA was treated with DNase I (Boehringer Mannheim) to remove template DNA, twice extracted with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)-saturated phenol:CH\(_3\)Cl\(_2\) (1:1), and extracted once with isosoyl alcohol:CH\(_3\)Cl\(_2\). The RNA was precipitated overnight at \(-20°C\) with 2.5 vol of 95% ethanol and 0.5 vol of 7.5 M ammonium acetate. Pellet was washed with ethanol, dried in a vacuum centrifuge, and then reconstituted to 1 mg/ml in sterile, HPLC grade water (Burdick and Jackson, Dade International, Muskegon, MI). The K\(^b\) RNA was translated using rabbit reticulocyte lysate prepared in our laboratory by the method of Jackson and Hunt (24). The lysate was stored in liquid \(N_2\) and refrozen no more than once. \(\text{[\(\pmb{5}\text{s}\)]\text{Methionine (SJ 204, Amsler Life}

Microsome isolation, proteinase K protection, and the MHC class I assembly assay

Microsomes were added at a final concentration of 0.1 A\(_{260}\) units (absorbance determined in 0.1% SDS) before the addition of the translation mixture to allow cotranslational insertion of the K\(^b\) protein into the microsomal membrane. Translation was stopped by dilution into 50 mM triethanolammonium acetate, pH 7.5, 150 mM K acetate, and 2.5 mM Mg acetate (microsome isolation buffer) at room temperature. Microsomes were isolated immediately by centrifugation through a sucrose cushion at 90,000 rpm for 20 min in a Beckman Airfuge at room temperature. Microsomes and peptide were washed in 100 \(\mu l\) of microsome isolation buffer and were treated with 50 \(\mu g/ml\) proteinase K (PK) (Boehringer Mannheim) for 45 min in 50 \(\mu l\) 10 mM Tris-HCl, pH 7.5, at 4°C or left untreated. PK treatment has been used extensively to determine both the topology of membrane proteins and the portion of in vitro translated proteins that reside within the interior of the microsome (26). Additionally, PK treatment results in the removal of hydrophobic peptides on the exterior of the microsome (see Results). We have found that inclusion of hydrophobic peptides during translation (cotranslationally (CO-T)) results in an additional 20% loss of the PK-protected microsome fraction. The specificity of PK is determined by bulky aromatics and large hydrophobic side chains of amino acids, residues that are contained frequently in K\(^b\)-restricted peptides. After PK treatment, the microsomes were made 1 mM (final concentration) in PMSF (Sigma, St. Louis, MO) for 10 min at 4°C in order to inactivate the protease prior to solubilization.

Isolation of in vitro-translated K\(^b\) protein from protease-protected microsomes was used to determine the impact of peptide acquired during K\(^b\) synthesis (CO-T) or posttranslationally, concomitant with solubilization (SOL) had on K\(^b\)/\(\beta_m\)-peptide association. K\(^b\) RNA was translated, as described above, in the presence of RMA- or RMA-S-derived microsomes. Peptide was added CO-T and/or SOL at 25 \(\mu l\). In experiments in which microsomes and peptide were both added CO-T, the microsomes were added first, followed by the peptide and then the RNA. We have determined that in TAP-positive, RMA-derived microsomes the yield of conformal K\(^b\), as determined by immunosolubilation (see below), is directly proportional to the amount of high affinity K\(^b\)-binding peptide added.

Immunosolubilation of K\(^b\)/\(\beta_m\) complexes and SDS-PAGE analysis

Immunosolubilation was performed on K\(^b\)/\(\beta_m\) complexes solubilized away from microsomes recovered from in vitro translation reactions as described above. Preadsorption consisted of a 30-min incubation at 4°C with protein A-Sepharose (Sigma). For immunosolubilation using the mAbs Y-3, K-10-56, or 100.3, microsomes were solubilized with 50 \(\mu l\) Nonidet P-40 (NP-40) (Sigma) lysis buffer (1% NP-40, 10 mM Tris-HCl, pH 7.5, and 1 mM PMSF) for 30 min on ice. Ab (5 \(\mu g/ml\), final concentration) was added and incubated overnight at 4°C. Ab-K\(^b\)-complexes were recovered on 60 \(\mu l\) of protein A-Sepharose (1:1 slurry in 1% NP-40 lysis buffer) after an additional 2-h incubation at 4°C. The entire supernatant fluid (135 \(\mu l\)) was saved for analysis by SDS-PAGE as the “unbound” fraction of K\(^b\) and the protein A-Sepharose pellet was collected by centrifugation (14,000 rpm, 10 min, 4°C) for extensive washing. The first wash consisted of 100 \(\mu l\) NP-40 lysis buffer, followed by 100 \(\mu l\) Tris-saline (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl), followed by 100 \(\mu l\) 0.05 Tris, 1.0 M NaCl, and then a final wash with 100 \(\mu l\) Tris-saline. The washes were collected and assayed for [\(\text{\text{[\(\pmb{5}\text{s}\]}\text{Methionine-labeled K}\(^b\)}\text{]}\text{P }\text{counting. It was determined on multiple samples, that the amount of [\(\text{\text{[\(\pmb{5}\text{s}\]}\text{Methionine-labeled K}\(^b\)}\text{]}\text{ contained within the washes represented less than 5% of the total [\(\text{\text{[\(\pmb{5}\text{s}\]}\text{Methionine-labeled K}\(^b\)}\text{]}\text{ contained within the total microsome fraction. Nonspecific binding to K}\(^b\) to either an Ab that recognizes the \(\alpha_3\)-domain of L\(^2\) (28-14-8) or to protein A-Sepharose alone was not detected.}

Immunosolubilation using the anti-TAP Ab was performed as described above, with the following modifications. Microsomes were not treated with PK, as the epitope that the anti-TAP antisem recognizes is present on a cytoplasmic domain of the TAP1 protein. The lysis buffer was ditygonin 5\(\text{[\(\pmb{5}\text{s}\]}\text{Methionine (SJ 204, Amsler Life Sciences, Arlington Heights, IL)]\text{ was added at 20 \(\mu l\) and 25 \(\mu l\) lysis re- sulting in an additional 0.58 mM \(\beta\)-mercaptooctanol; no additional reduc- ing agent was added to the translation mixture. No oxidized glutathione was added during translation; we estimate the final concentration of free SH in the final translation mixture to be about 1.5 mM. This was determined by summing the concentrations of reduced glutathione within the lysate, the added \(\beta\)-mercaptooctanol contained within the [\(\text{\text{[\(\pmb{5}\text{s}\]}\text{Methionine, and the DTT added to the microsomes for storage at \(-70°C\). Translations were performed at 30°C for 90 min, in the absence or presence of potassium/ phosphate-buffered microsomes prepared from cultured RMA or RMA-S cells by the method of Walter and Blobel (25).
After the immunoisolation procedure, the washed beads were solubilized into 30 µl 5% SDS containing 0.1% 2-mercaptoethanol, heated at 100°C for 5 min, and the entire sample, beads included, was analyzed by SDS-PAGE. The total microsomal Kb is comprised of the fraction of Kb in the supernatant fluid, the unbound fraction and the fraction of Kb bound to the Ab and therefore the protein A-Sepharose. SDS-PAGE (12%, reduced) was performed on the bound and unbound fractions, and the gels were fixed, enhanced with (Fluoro-en Hance Research Products International, Boston, MA) for 45 min at room temperature, and dried prior to autoradiography with Bio-MAX (Kodak, Rochester, NY) film. Kb bound and unbound SDS-PAGE bands were measured by densitometry with an Ambis Systems (San Diego, CA) imaging and data analysis system. Identical, size-matched scans of lane backgrounds were subtracted from each protein band to account for film or individual lane variations. Multiple film exposures were used in order to ensure that Kb protein bands were within the linear range of the analytical system.

Flow cytometric analysis

Surface expression of Kβ2,m complexes was induced on RMA-S cells by incubating with 10 µM of the indicated peptide at 26°C for 12 h. A total of 5 × 10^6 cells were incubated with either 50 µl of K-10-56 cultured supernatant or 50 µl 100.3 ascites (diluted 1:100) for 25 min at 4°C in FACS buffer (HBSS, Life Technologies) containing 10% BSA, 0.2% sodium azide, and 5 mM sodium bicarbonate, pH 7.4. After incubation, cells were washed three times with FACS buffer and then incubated with fluorescein-conjugated goat anti-mouse IgG and IgM (Biosource International, Camarillo, CA) in FACS buffer for 20 min at 4°C. Cells were then washed three times with FACS buffer at 4°C. Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) instrument and mean channel fluorescence (MCF) values were determined.

Results

Conformational analysis of Kβ2,m complexes within microsomes

Conformational differences of Kb synthesized into microsomes derived from the TAP-positive, murine lymphoma cell line RMA and the variant TAP-negative cell line RMA-S (27) are shown by their differential reactivity with the conformationally sensitive mAb Y-3 (Fig. 1). In peptide-untreated microsomes, about 20% of the intramicrosomial Kb is Y-3 positive, while in RMA-S microsomes, approximately 80% of the Kb molecules can be isolated with the Ab. The specificity of the immunoisolation procedure was confirmed by the absence of Kb recovered with an Ab that does not bind specifically to Kb (L2-specific, 28-14-8, data not shown, see Materials and Methods). The fraction of Kb in any particular conformation can be assessed accurately by analyzing both the Ab bound and unbound fractions after immunoisolation with Kb-specific Abs by SDS-PAGE of [35S]methionine-exposed film. The absence or presence of the OVA8amule (SIINEFKL) peptide (25 µM) is as indicated. Peptide was added either during in vitro translation (CO-T) or coconmitant with SOL. See Material and Methods for additional details.

TAP-Kb complexes formed in microsomes

Due to the unexpected finding that the majority of Kβ2,m complexes formed within RMA-S microsomes in the absence of high affinity peptide are Y-3-reactive, even before adding peptide. Similarly, addition of OVA8 concomitant with solubilization results in no further change in number of molecules bound by the Ab Y-3. Due to the lack of peptide-dependent specificity of Y-3, it is impossible to tell whether peptide-added SOL replaces a possible RMA-S microsome-derived peptide. However, as discussed below, peptide loading of Kb molecules can be assessed using two other Abs that are sensitive to the kind of peptide occupying the class I peptide-binding site. The high affinity Kb-binding peptides OVA8 and SEV9 (FAFGYNPAL) both induce comparable increases in the Y-3 conformation when they are included during translation (CO-T), or concomitant with SOL, in RMA-derived microsomes (Fig. 2A), but neither substantially change the amount of Y-3 reactive Kb in RMA-S-derived microsomes (Fig. 2B).

FIGURE 1. Y-3 complexes formed in TAP-positive and TAP-deficient microsomes. Kβ2,m complexes formed in either RMA or RMA-S microsomes, as indicated, were subjected to immunoisolation with the βm-dependent mAb Y-3. All microsomes were treated with PK after isolation and washing and prior to SOL with NP-40 lysis buffer. This treatment is necessary to determine the intramicrososomal fraction of in vitro-translated Kb, which has been radiolabeled during its synthesis with [35S]methionine. Both Y-3-bound Kβ2,m complexes and unbound Kb were analyzed by SDS-PAGE. The percentage of Y-3-reactive Kb was quantified as the ratio of bound Kb to unbound Kb measured by densitometric image analysis of [35S]methionine-exposed film. The absence or presence of the OVA8amule (SIINEFKL) peptide (25 µM) is as indicated. Peptide was added either during in vitro translation (CO-T) or concomitant with SOL. See Material and Methods for additional details.

TAP-Kb complexes formed in microsomes

Due to the unexpected finding that the majority of Kβ2,m complexes formed within RMA-S microsomes in the absence of high affinity peptide are Y-3-reactive, we looked for further detectable differences in the association of RMA-S-derived-Kb molecules with other ER components. It has been previously shown that a significant portion of MHC class I molecules in peptide-untreated TAP-positive cells are associated with TAP. To determine whether a measurable Kb-TAP association occurs within purified microsomes, we compared the amount of Kb cotranslated into either peptide-untreated RMA or RMA-S microsomes by coimmunoisolation with an anti-TAP1 Ab. This Ab, which was raised against the 19 carboxyl-terminal residues of murine TAP1, has been shown to coprecipitate “open” forms of the murine MHC class I protein, Ld (28).
We find that over 40% of the glycosylated-Kb molecules synthesized into peptide-untreated RMA microsomes can be coimmunoisolated with an anti-TAP1 Ab (Fig. 3). Forty percent is a minimal estimate of the TAP-associated molecules, as weak complexes could dissociate during isolation. Our results are comparable with previously described amounts of endogenous Kb co-precipitated from detergent-lysed, Kb-expressing cells using an Ab raised against the terminal one-third of the TAP1 protein (29). The epitope recognized by the anti-TAP1 Ab is contained within the cytoplasmic tail of TAP. PK-treatment most likely destroys this epitope as evidenced by the failure of PK-treated microsomes to yield any Kb in the TAP-bound fraction, as shown in Fig. 3. Therefore, anti-TAP immunosolubilization must be performed on microsomes that have not been treated with protease. It is important to only quantify the glycosylated form of Kb, Kb_gly, which is the highest m.w. species visible in SDS-PAGE of class I molecules coprecipitated with the anti-TAP1 Ab. We have determined that, when microsomes have been treated with PK, only the glycosylated fraction of the total synthesized Kb is protected from digestion. When peptide is added SOL, the amount of Kb associated with TAP drops to 15%. This result is consistent with previously published reports (29, 30), showing that MHC class I molecules dissociate from TAP after binding peptide. In contrast, we find that barely detectable levels of the glycosylated form of Kb cotranslated into RMA-S microsomes is associated with the intact TAP1 subunit, in the presence or absence of high affinity peptide as shown in Fig. 3. A 6× exposure of the bound SDS-PAGE bands to film is required to determine that less than 5% of the Kb_gly fraction can be coimmunoisolated from RMA-S microsomes. Therefore, the association of Kb with TAP1 is much weaker in RMA-S microsomes than with intact TAP1/TAP2 complexes in RMA microsomes. The apparent association of unglycosylated forms of Kb with TAP1 Ab is likely artifactual, as unglycosylated forms of Kb do not reside within either intact RMA-S or RMA microsomes. This is shown clearly by the presence of only a single m.w. species of Kb, which migrates at the expected size for the glycosylated protein after PK treatment (Figs. 1 and 5).

High affinity ligands can bind Kb_β2m complexes formed within RMA microsomes

Abs sensitive to conformation changes induced by bound peptides were used to establish that peptide added CO-T is transported across the microsome membrane and binds to Kb within RMA-derived microsomes. The mAbs K-10-56 and 100.3 bind different epitopes on Kb that are sensitive to peptide occupancy. Both K-10-56 and 100.3 can distinguish the difference between the binding of OVA8 with an amidated or free carboxyl leucine at position 8 (31, 32). As shown in Fig. 4, K-10-56 stains Kb molecules on RMA-S cells cultured at 26°C 150 channels more intensely than cells preincubated with the amidated peptide, OVA8. Binding of Kb to either SEV9 or OVA8 with a free carboxyl terminus results in a 20- to 30-channel increase in the K-10-56 signal. However, the binding of the amidated version of OVA8 to Kb causes a 60-channel decrease in 100.3 binding relative to no peptide, whereas the binding of the OVA8-free COOH results in a drop of over 150 channels of relative staining intensity. The SEV9...
peptide produces a comparable increase in 100.3 binding as it does for K-10-56, approximately 30 channels. These data are summarized in Table I.

The peptide sensitive Abs provide us with the ability to visualize specific peptide occupancy of K\(^{\beta}\). We demonstrate that K\(^{\beta}\) synthesized in vitro in the presence of OVA8 amide loses the K-10-56 epitope in a manner similar to K\(^{\beta}\) molecules loaded with OVA8 amide peptide on the cell surface (31, 32). The amount of K\(^{\beta}\) recognized by K-10-56 in the absence of peptide, 23%, was decreased to 13% when OVA8 amide was added CO-T, and dropped to 7% when OVA8 amide was added with SOL (Fig. 5). These findings demonstrate that peptide added during translation is transported and binds to in vitro-synthesized K\(^{\beta}\) residing within a sealed microsome, directly causing the loss of the \(\alpha_{1}\alpha_{2}\) epitope detected by K-10-56. It is also evident from these results that OVA8 amide displaces a portion of the ligand bound to K\(^{\beta}\) within TAP-positive microsomes, which before peptide addition exhibit the K-10-56-reactive phenotype. We presume that this portion of K\(^{\beta}\) molecules is bound to low affinity ligands, which are readily displaced by the transported high affinity OVA8 amide peptides.

**FIGURE 3.** K\(^{\beta}\)-TAP complexes isolated from RMA or RMA-S microsomes. K\(^{\beta}\) was in vitro translated into either RMA or RMA-S microsomes as indicated, and TAP-associated K\(^{\beta}\) was immunosolated with an anti-TAP1 antiserum. Microsomes were not treated with PK, as had been done previously for immunosolation of Y-3 complexes, as the epitope recognized by the anti-TAP1 Ab resides within the cytoplasmic tail of TAP1 and is proteolytically removed. This is shown in the third lane of the RMA microsome data; PK treatment results in one m.w. species of K\(^{\beta}\), which is not communiosolated (ND) by the anti-TAP1 antiserum. In contrast, in peptide-untreated RMA microsomes, 45% of the glycosylated K\(^{\beta}\) fraction, designated K\(^{\beta}\)\(_{\text{gly}}\), in the figure, was communiosolated with the anti-TAP Ab. The K\(^{\beta}\)\(_{\text{gly}}\) fraction, which is the highest m.w. species isolated from protease-untreated microsomes, is the only species that resides within microsomes after treatment with protease: see Materials and Methods for details. When peptide is added with SOL, only 15% of the K\(^{\beta}\)\(_{\text{gly}}\) fraction can be communiosolated with the anti-TAP Ab. An increased exposure time (288 h vs 48 h) of only the bound lanes of the SDS-PAGE revealed that less than 5% of the K\(^{\beta}\)\(_{\text{gly}}\) fraction isolated from RMA-S microsomes could be communiosolated with the anti-TAP antiserum. Only the OVA8 amide derivative of the OVA8 peptide was used for this particular experiment.

**FIGURE 4.** The K-10-56 Ab distinguishes peptide occupancy by K\(^{\beta}\). RMA-S cells were cultured overnight at 26°C in RPMI 1640 medium containing 8% bovine calf serum containing 10\(^{-6}\) M peptide as indicated. Cells were stained with the mAb K-10-56 and fluorescein-conjugated goat anti-mouse IgG and then analyzed by flow cytometry. The control was a non-specific isotype-matched mAb.

**Table 1.** The K-10-56 and 100.3 Abs can distinguish peptide occupancy by K\(^{\beta}\).

<table>
<thead>
<tr>
<th>Peptide (concentration)</th>
<th>K-10-56</th>
<th>100.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>543</td>
<td>608</td>
</tr>
<tr>
<td>OVA(_{COOH})</td>
<td>570</td>
<td>445</td>
</tr>
<tr>
<td>OVA8(_{amide})</td>
<td>344</td>
<td>547</td>
</tr>
<tr>
<td>SEV9</td>
<td>561</td>
<td>633</td>
</tr>
<tr>
<td>Control</td>
<td>129</td>
<td>138</td>
</tr>
</tbody>
</table>

* RMA-S cells were cultured overnight at 26°C either without peptide (none) or in the presence of the high affinity K\(^{\beta}\)-binding peptides, OVA8 or SEV9 (10 \(\mu\)M) added to the culture medium. OVA\(_{COOH}\), OVA8 (SIINFEKL) peptide with a free carboxyl terminus; OVA8\(_{amide}\), OVA8 (SIINFEKL) peptide with an amidated carboxyl terminus; and SEV9, SEV9 (FAPGYNPAL) peptide with a free carboxyl terminus. Flow cytometric analysis was performed by incubating the cells with either the K-10-56 or 100.3 mAb (as indicated in Fig. 4) and stained with fluorescein-conjugated goat anti-mouse IgG. The control was a non-specific isotype-matched mAb. Staining intensity is represented as the MCF for the population.
The fact that K-10-56 recognizes molecules bearing a mature folding pattern is demonstrated using a second peptide, SEV9, which does not mask the epitopes bound by the Abs K-10-56 or 100.3. When Kb is translated in the presence of the SEV9 peptide, the amount of K-10-56-positive molecules was nearly doubled to 39%, and increased to 54% when SEV9 was added SOL (see Fig. 6A). Comparable results were obtained with the Ab 100.3, as summarized in Table II.

The K-10-56 Ab was used to probe conformation changes occurring in Kbβ2m complexes within RMA-S-derived microsomes. In contrast to our findings that only 20% of the Kb in RMA microsomes were K-10-56-positive, over 50% of the Kb in RMA-S-derived microsomes was already in a K-10-56-reactive conformation (see Fig. 5). This finding is similar to that seen with Y-3 Ab (Fig. 1) and again demonstrates that, in the absence of the TAP complex, Kb molecules fold into a mature conformation as defined by Ab reactivity. The addition of the OVA8 amide peptide CO-T did not result in a decrease in the K-10-56 signal (expected if OVA8amide is loaded into the Kb molecule), nor did the addition of the SEV9 peptide result in a substantial increase of configured Kb molecules (Fig. 6B). These results are consistent with the loss of peptide transport previously noted for TAP-deficient microsomes.

The fact that the K-10-56-reactive Kb fraction does not decrease when the OVA8amide peptide is added CO-T to RMA-S microsomes demonstrates the effectiveness of the PK treatment in removing hydrophobic peptides from the surface of microsomes.

### Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% K-10-56</th>
<th>% 100.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23 ± 0.8</td>
<td>29 ± 1.5</td>
</tr>
<tr>
<td>OVA8 (CO-T)</td>
<td>13 ± 1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>SEV9 (CO-T)</td>
<td>39 ± 1.8</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>OVA8 (SOL)</td>
<td>7 ± 2</td>
<td>11 ± 2.5</td>
</tr>
<tr>
<td>SEV9 (SOL)</td>
<td>54 ± 3</td>
<td>66 ± 1</td>
</tr>
</tbody>
</table>

*The percentages of K-10-56 or 100.3 values were derived from a minimum of six separate experiments with SDs as indicated. The percentage of Ab binding was determined as explained in the legend of Fig. 6. The OVA8 peptide derivative used in these experiments was the amidated form (OVA8amide).*

**FIGURE 5.** K-10-56-positive Kbβ2m-ligand complexes isolated from RMA and RMA-S microsomes. Kbβ2m complexes formed in either RMA or RMA-S microsomes, as indicated, were subjected to immunosoliation with the peptide-sensitive, β2m-dependent mAb K-10-56. Microsome isolation, PK-treatment and SOL, peptide addition, SDS-PAGE, and data analysis were exactly as described in Fig. 1. Only the OVA8amide derivative of the OVA8 peptide was used for this particular experiment.

**FIGURE 6.** K-10-56-positive Kbβ2m complexes formed in RMA and RMA-S microsomes. A, Kbβ2m complexes formed in RMA microsomes were analyzed by SDS-PAGE and quantified as described in Figs. 1 and 2. B, Kbβ2m complexes formed in RMA-S microsomes were analyzed exactly as described for A. Microsome isolation, PK treatment and SOL, peptide addition, SDS-PAGE, and data analysis were exactly as described in Figs. 1 and 2. The results are representative of six independent experiments, with error bars depicting SD. Only the OVA8amide derivative of the OVA8 peptide was used for this particular experiment.
had any OVA8amide peptide remained associated with the RMA-S microsomes after the PK treatment, it would be expected to bind to preexisting K\(^\beta\)m complexes during SOL and result in the loss of the K-10-56 signal. Addition of OVA8amide, concomitant with SOL, does result in the loss of the K-10-56 epitope, demonstrating that the peptide-binding site of microsomal Kb, which has an antigenic dominance signal, is accessible to peptide.

The SEV9 peptide also is believed to replace endogenous ligands when added with SOL. As expected, however, no significant change in the K-10-56 signal was discernible (Fig. 6B). By manipulating the amount and nature of peptides added during translocation and SOL, we were able to demonstrate that high affinity peptides in the class I-binding site are not readily displaced, even by other high affinity peptides. When K\(^\beta\)m complexes formed with the high affinity peptide, SEV9, were solubilized away from TAP\(^+\) RMA microsomes and subsequently incubated with 25 \(\mu\)m OVA8amide peptide added with SOL, replacement of SEV9 was minimal (30% binding to K-10-56 following SOL incubation with OVA8amide vs 33% without OVA8amide addition) (data not shown). This provides the basis of our conclusion that K\(^\beta\)m complexes formed in the absence of added high affinity peptide are occupied by what are functionally defined as low affinity ligands. Very few (7%) of Kb molecules synthesized in the absence of high affinity peptide are peptide unreactive (Fig. 6A).

**Discussion**

The possibility that a direct TAP-MHC class I interaction is important for peptide acquisition is intriguing, but strong evidence supporting this hypothesis has been elusive (13, 33, 34). Although TAP has been shown to associate only transiently with MHC class I molecules, this association may serve to increase interactions with other accessory proteins within the ER that are required for the folding or peptide binding of MHC class I molecules, such as tapasin and/or calreticulin, calnexin, or as yet unidentified ER-resident proteins. For example, the HLA-A 0201 mutant (T134K), defined previously by the absence of demonstrable association with TAP, improper folding, and an inability to present Ag at the cell surface, also does not interact demonstrably with calreticulin (22). Additionally, in cells lacking TAP, a redistribution of MHC class I associations with other ER-resident proteins has been demonstrated. Specifically, MHC class I molecules within TAP-negative cells lose associations with calreticulin and tapasin and concomitantly increase their associations with calnexin and BiP (35). Finally, changes in trafficking and maturation of carbohydrate moieties on class I molecules have been shown in the TAP-deficient RMA-S cells and tapasin-deficient 721.220 cells (17, 33).

In order to determine whether a measurable difference in an MHC class I-TAP interaction might influence early MHC class I complex assembly, we assayed specific conformational changes in ER-resident K\(^\beta\)m complexes within microsomes derived from TAP-positive or TAP-negative cells. High affinity peptide added CO-T to K\(^\delta\) synthesized in the presence of TAP-positive microsomes induced folding with endogenous \(\beta_m\) as measured by the gain of the \(\beta_m\)-dependent and peptide-inducible Y-3 epitope within the K\(^\delta\) heavy chain. The K-10-56 and 100.3 antigenic profiles of K\(^\delta\) synthesized in the presence of OVA8amide peptide indicate specific peptide transport and binding within TAP-positive, protease-resistant microsomes. When peptide is added concomitantly with SOL of the microsomes, preexisting-K\(^\beta\)m complexes generated within intact microsomes also bind peptide. TAP, a protein that should span the membrane multiple times, should contain several extracytoplasmic loops that most likely are destroyed by the PK treatment step that precedes SOL. In support of this hypothesis, Fig. 3 shows the loss of the communiosolated K\(^\delta\) fraction after PK treatment, suggesting the loss of the epitope recognized by the anti-TAP antiserum. K\(^\beta\)m-peptide complexes that bind peptide added with SOL therefore are most likely doing so in a TAP-independent manner.

In contrast, K\(^\beta\)m complexes synthesized within TAP-deficient microsomes can only bind high affinity peptide when added concomitantly with SOL. As predicted from previous findings, peptide added CO-T to TAP-deficient microsomes is not transported as determined by the retention of the K-10-56-defined conformation of K\(^\beta\)m molecules within sealed RMA-S microsomes. Interestingly, in peptide-untreated RMA-S microsomes, the majority of the microsomal Kb is present as Y-3- or K-10-56-reactive K\(^\beta\)m complexes. This differs from the K\(^\delta\) molecules within RMA microsomes in which only 20% of the total can be isolated as Y-3- or K-10-56-reactive K\(^\beta\)m complexes. Since the RMA-derived microsomes have a functional TAP (TAP/tapasin) complex one would predict that the concentration of available peptide ligands would be substantially higher compared with RMA-S. However, the majority of Kb molecules in RMA microsomes are not only unoccupied by peptide, but do not maintain association with \(\beta_m\) after SOL as determined by low Y-3 and K-10-56 binding (Figs. 1 and 5). This finding implies that the presence of intact TAP molecules plays an important function in preventing the premature assembly of class I molecules before high affinity peptides are available for binding to the peptide-binding site.

When peptide is added CO-T to RMA-derived microsomes, peptide translocation and peptide binding by K\(^\beta\)m occurs readily as measured both by changes in the Y-3 and K-10-56 conformations. Since K-10-56 is able to distinguish whether the OVA8amide or SEV9 peptide is bound to Kb\(^\delta\), it is possible to speculate about the nature of the ligands bound to K\(^\beta\)m in peptide-untreated microsomes. Nearly half of the K\(^\beta\)m complexes that are K-10-56 reactive in RMA microsomes prior to addition of high affinity peptide exchange endogenous ligands for the OVA8amide peptide. Additionally, when OVA8amide is added with SOL, nearly 70% of the endogenous ligands are replaced by the high affinity Kb-binding peptide. However, OVA8amide added upon SOL replaces very little of the high affinity SEV9 peptide added CO-T. Therefore, the low amount of K\(^\beta\)m complexes formed in TAP-positive microsomes are occupied by what functionally resembles low affinity ligands.

In TAP-deficient, RMA-S microsomes, most of the Kb molecules are associated with \(\beta_m\), but appear to be bound to low affinity ligands. A majority of these molecules readily bind the OVA8amide peptide-added SOL. The nature of the low affinity ligands has, as yet, not been determined, but several potential candidates exist. The most obvious candidate is low affinity peptide which, in the case of TAP-deficient microsomes, should be predominantly signal peptides or, perhaps, incompletely synthesized and, therefore, misfolded secretory proteins that normally traffic through the ER. However, one would assume that both RMA and RMA-S microsomes would have equivalent amounts of such peptides. Given this assumption, the importance of the finding that RMA microsomes untreated with high affinity peptide maintain such low levels of MHC class I-\(\beta_m\) complexes is accentuated. We could not detect a significant portion of the K\(^\beta\)m complexes formed in RMA-S microsomes associated with the remaining TAP1 subunit. In TAP\(^+\) RMA microsomes, more Kb heavy chain was found associated with TAP by communiosolation than could be recovered by direct communiosolation with Kb-specific Abs. It is possible that this association is important for the maintenance of peptide-receptive class I molecules prior to peptide loading and could serve a peptide editing function, permitting only higher affinity peptides to induce the mature folding phenotype required for...
class I export from the ER. Such a proposed role for the TAP in this pathway does not minimize the possibility that other ER-resident proteins could also contribute by binding to and stabilizing K\(^{+}\)β2m complexes or precursors.

To date, no other deficiencies in the cofactors involved in MHC class I assembly have been detected within RMA-S cells. Therefore, we conclude from our findings that a functional TAP1/TAP2 complex, either directly or indirectly through its association with tapasin (tapasin/calreticulin), prevents the premature folding of nascent MHC class I proteins in the endoplasmic reticulum. Recent reports indicated that some class I molecules, such as HLA-B8, are able to assemble efficiently without apparent TAP association (36). A similar finding was reported for the HLA-A2 mutant T134K, a molecule that is not detected in complexes with either TAP or calreticulin, but nonetheless assembles and is exported as a peptide-receptive class I molecule to the cell surface (22, 23). While it has long been clear that TAP is not absolutely required for surface expression of class I, none of these early studies addresses the regulatory role TAP expression may have on selection of peptides that bind maturing class I molecules, or on the influence of TAP on the range of peptide concentrations that are required to efficiently package Ag-presenting molecules with appropriate peptides. Our finding that TAP molecules associate with nascent class I heavy chains and prevent them from folding into a mature antigenic configuration is consistent with the view expressed earlier that retention of class I molecules in the ER plays an important role in the process by which class I molecules acquire high affinity peptides for presentation as Ags (23).

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References
