An Unstable β2-Microglobulin: Major Histocompatibility Complex Class I Heavy Chain Intermediate Dissociates from Calnexin and then Is Stabilized by Binding Peptide

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Summary

Proper assembly of the class I heavy chain (HC), β_2-microglobulin (β_2m), and peptide must occur in the endoplasmic reticulum (ER) in order for MHC class I molecules to be expressed on the cell surface. Newly synthesized class I HC bind calnexin, an ER resident chaperone. These calnexin-associated class I HC appeared to lack the stable association with β_2m in peptide transporter–deficient T2 cells since β_2m-unassociated class I HC-specific HC10 antibody, but not β_2m-associated class I HC-specific W6/32 antibody, coimmunoprecipitated calnexin. To determine the precursor–product relationship of the pool of HC that bind peptide, class I–restricted peptides were added to lysates of T2 cells in vitro. These peptides stabilized preexisting β_2m-associated HC complexes (β_2m^+:HC:pep$^-$), but had no significant effect on the preexisting pool of calnexin-associated HC that lack β_2m. Release of HC from calnexin appeared to be controlled by the binding of β_2m, since β_2m-deficient FO-1 cells showed a prolonged association of class I HC with calnexin, while β_2m-transfected FO-1 cells displayed a more rapid dissociation of class I HC from calnexin. Consistent with this result, the dissociation of class I HC from calnexin did not appear to be dependent on peptide binding since the dissociation rates were similar in peptide transporter–deficient T2 cells and in wild-type T1 cells. From these observations, we speculate that in the stepwise assembly of class I molecules, calnexin may mediate dimerization of class I HC with β_2m, and that the unstable $\beta2m^+$:HC:pep$^-$ complexes, after dissociation from calnexin, subsequently bind peptide to complete the assembly.

MHC class I molecules present peptide fragments of intracellular antigens to CD8$^+$ cytotoxic T cells (1, 2). Such endogenously synthesized proteins are proteolytically degraded into peptides in the cytoplasm and then are transported into the endoplasmic reticulum (ER[1]) through an ER membrane–associated peptide (pep) transporter encoded by the TAP.1 and TAP.2 genes (3). The assembly of MHC class I heavy chains (HC), β_2-microglobulin (β_2m), and peptides takes place in the ER (4–6). Mutant cell lines that have defects in the assembly of MHC class I molecules, such as β_2m-deficient cell lines (R1E in mouse and Daudi in human), lack cell surface expression of MHC class I molecules (7, 8). Similarly, mice with disruption of the β_2m gene express no MHC class I molecules and display a profound defect in CD8$^+$ T cell development (9, 10). In addition, mutant cell lines that have deleted the peptide transporter (such as RMA-S and .174/T2 cells) appear unable to load peptides onto MHC class I molecules in the ER (11–13) and also have a severe defect in the expression of MHC class I molecules on the cell surface. Thus, the complete assembly of MHC class I molecules in the ER is an essential step for their cell surface expression.

Studies on the assembly of class I molecules have suggested alternative stepwise sequences (14, 15). In one proposed pathway, the HC can form a loose association with β_2m, which is then stabilized by peptide occupying the binding site. Alternatively, a short, high-affinity peptide might stabilize a conformational change of the HC, which can then associate with β_2m (16). In either case, the binding of peptide and β_2m to the HC is cooperative, and peptide binding like the association with β_2m is an inherent component of class I assembly. Although recombinant or purified components of class I HCs, β_2m, and synthesized specific peptides can assembly in in vitro buffer systems, such assembly requires high concentrations of each component and long periods of time to occur (17, 18). Recent evidence has implied that, in vivo, molecular chaperones may facilitate protein folding and assembly (19), and this may be physiologically relevant for class I assembly in the ER. Calnexin (IP90, p88) was identified as an ER resident protein that associates with incompletely assembled TCR/CD3, membrane Ig, and MHC

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; ER, endoplasmic reticulum; HC, MHC class I heavy chain; pep, peptide.

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class I complexes (20–22) or with incompletely folded secretory glycoproteins (23) in the ER. Calnexin associates with free class I HCs soon after their synthesis, but this association is transient and calnexin dissociates from completely assembled class I complexes (24, 25). Thus, calnexin has been hypothesized to play a role in facilitating class I assembly and in mediating retention of incompletely assembled complexes in the ER (26, 27).

In the present study, we characterize a pool of calnexin-associated class I HCs, and delineate a possible stepwise sequence of class I assembly.

Materials and Methods

Cells, Antibodies, and Peptides. T2 and T1 cell lines (28) and T2 cells transfected with HLA-B27 (T2/B27) (13) were provided by Dr. Peter Cresswell (Yale University, New Haven, CT). These cell lines were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 20 mM Hepes, 2 mM glutamine, and 1 mM pyruvate (all from GIBCO BRL) at 37°C in a 5% CO_2 atmosphere. β_2m-deficient FO-1 cells (29) were provided by Dr. Soldano Ferrone (New York Medical College, Valhalla, NY). β_2m-expressing and mock transfectant clones of FO-1 cells were obtained from transfection of either pb2m13 (30) plus pSRα-neo (31) or pSRα-neo alone, respectively, by calcium phosphate precipitation method (32). These transfectant clones were grown in DMEM supplemented with 10% FCS in the presence of 0.5 mg/ml G418 (Geneticin; Sigma Chemical Co., St. Louis, MO). mAbs W6/32 (specific for β_2m^+:HC; IgG2a) (33), L243 (anti-HLA-DR antibody; IgG2a) (34), and P3 (negative control; IgG1) (35) were obtained from the American Type Culture Collection (Rockville, MD). mAb HC-10 (IgG2a) which recognizes β_2m^-:HC, but not β_2m^+:HC (36) was provided by Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge, MA). Anti-calnexin/IP90 antibody, AF8 (IgG1), was generated in our laboratory (20). UPC10 is a negative control antibody (IgG2a) purchased from Sigma Chemical Co. M57-68K62 peptide (14) which binds to HLA-A2.1 was synthesized with a peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA) at the core biopolymer laboratory, Brigham and Women's Hospital. HLA-B27–restricted B27NP peptide (18) was a gift from Dr. William E. Biddison (National Institutes of Health, Bethesda, MD).

Intracellular Iodination. Intracellular iodination was performed as described (20). Briefly, T2 cells (4×10^7) were permeabilized in 8 μM digitonin (Aldrich Chemical Co., Milwaukee, WI) for 5 min. After lactoperoxidase-mediated iodination of permeabilized T2 cells, they were solubilized in 1% digitonin in lysis buffer (140 mM NaCl, 50 mM Tris (pH 7.6), 7.5 mM iodoacetamide, and 1 mM PMSF). Immunoprecipitations with indicated mAbs were analyzed on a SDS-PAGE gel under reducing conditions and visualized by autoradiography on Kodak XAR film.

In Vitro Peptide-dependent Assembly. After 1 h preincubation in methionine-free RPMI 1640 media, 10^7 cells/ml were labeled for 5 min with 0.2 mCi [^{35}S]methionine (NEG-072 EXPRE^{32}S^{32}S; Du Pont Co., Boston, MA). Cells were lysed in 0.5% Triton X-100 in lysis buffer either in the presence or absence of peptides. After 20 min at 4°C, the nuclei were removed by centrifugation and the lysates were precleared overnight at 4°C with *Staphylococcus aureus* Cowan strain I (Pansorbin; Calbiochem-Novabiochem Corp., La Jolla, CA). Assembled class I molecules were detected with 1 μl of mAb W6/32 ascites, followed by immunoprecipitation with 50 μl of 10% protein A–Sepharose CL-4B (Pharmacia, Piscataway, NJ). Pellets were washed four times with 0.5% Triton X-100 in Tris-buffered saline. The samples were analyzed on a SDS-PAGE gel under reducing conditions or, in some experiments, on IEF/SDS-PAGE two-dimensional gels as described (21). The gels were fluorographed using PPO(2.5-diphenyloxazole)-DMSO (Du Pont Co.), dried, and exposed to Kodak XAR film.

Rapid Immunoprecipitation. T1 and T2 cells were biosynthetically labeled with [^{35}S]methionine for 5 min as described above. Cells were then solubilized in 0.5% Triton X-100 in lysis buffer containing mAb UPC10 (2 μg/ml), mAbs W6/32, or HC10 (1:1,000 dilution of ascites). After 15 min at 4°C, the nuclei were removed by centrifugation and the lysates were incubated with protein A–Sepharose CL-4B (Pharmacia) for 30 min. The beads were washed four times with 0.1% Triton X-100 in Tris-buffered saline and boiled in sample buffer. All the procedures before electrophoresis were completed at 4°C within 2 h.

Pulse Chase Experiments. Pulse chase experiments were performed as described (21) with slight modifications. Cells pulse labeled with [^{35}S]methionine for 5 min were washed in PBS and incubated at 37°C in complete RPMI 1640 media/10X methionine/10X cysteine supplemented with 10% heat-inactivated FCS for indicated periods of time. The cells were harvested and washed in PBS, and lysed in 0.5% Triton X-100 or 0.3% CHAPS 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate in lysis buffer, followed by the same immunoprecipitation procedure described above (see in vitro peptide dependent assembly). Counts per minute of calnexin molecules (cpm_{CNX}) immunoprecipitated with mAb AF8 and counts per minute of class I HCs (cpm_{HC}) coimmunoprecipitated with mAb AF8 were counted directly by a blot analyzer (Betascope 603; Betagen Corp., Waltham, MA) by gating on the regions of 90 kD and 42–43 kD respectively, in the same lane. Background counts per minute (cpm_{BG}) was also counted from a separate site at the same position on each lane of the gels. To evaluate the relative amount of calnexin-associated class I HCs, an association index was calculated as follows: association index = $(cpm_{HC} - cpm_{BG})/(cpm_{CNX} - cpm_{BG})$.

Results

β_2m-free Class I HCs (β_2m^-:HC) Are Associated with Calnexin in T2 Cells. We previously demonstrated that β_2m^-:HC, which are recognized by mAb HC10, are associated with calnexin in β_2m-deficient Daudi cells (20). To examine whether β_2m^-:HC also were bound to calnexin in peptide transporter–deficient T2 cells, we carried out iodination of intracellular proteins using a permeabilized cell iodination technique. As an abundant ER protein, calnexin was visualized prominently by this method (Fig. 1, lane *2*). The HC10 antibody coimmunoprecipitated calnexin with β_2m^-:HC from iodinated T2 cells (Fig. 1, lane *3*), demonstrating the association of calnexin with class I HCs that had not assembled with β_2m. Note that in contrast to the HC10 antibody, a comformation-dependent antibody, W6/32, which recognizes β_2m^+:HC, failed to coimmunoprecipitate calnexin (Fig. 1, lane *4*). This implied that calnexin bound β_2m^-:HC, but not β_2m^+:HC. This led us to assume that there may be several distinct intracellular class I HC pools, including β_2m-unassociated HCs (β_2m^+:HC) either bound to calnexin or not, and β_2m-associated HCs (β_2m^+:HC) which may contain a bound peptide (β_2m^+:HC:pep$^+$) or not (β_2m^+:HC:pep$^-$) (Fig. 2).

The β_2m^-:HC pool associated with calnexin was not

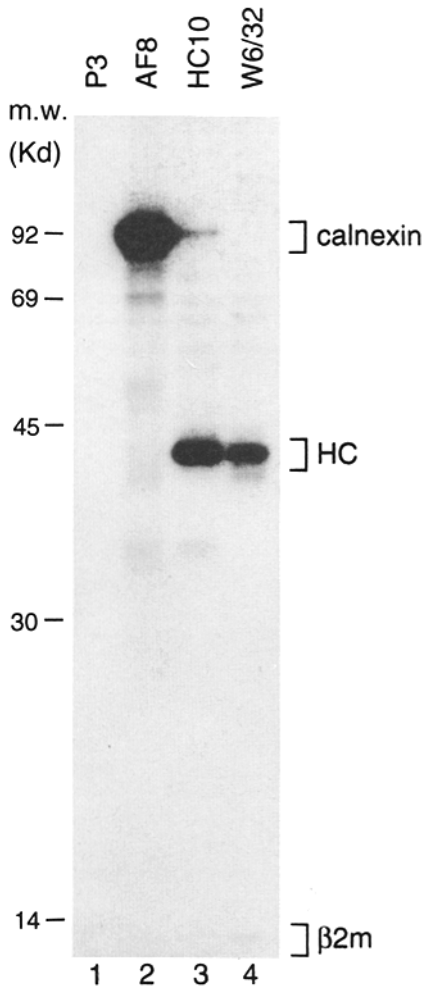


Figure 1. Calnexin associates with β_2m^-:HC but not β_2m^+:HC. Permeabilized T2 cells were iodinated and lysed in 1% digitonin. Immunoprecipitation was performed with mAbs P3 (control), AF8 (anticalnexin), HC10 (anti-β_2m^-:HC), and W6/32 (anti-β_2m^+:HC), followed by resolution on a SDS-PAGE gel under reducing conditions. (*Right*) The positions of calnexin, MHC class I HC (*HC*), and β_2m.

readily visualized by iodination (Fig. 1, lane *2*). This pool was more efficiently identified by coimmunoprecipitation with calnexin when T2 cells were pulse-labeled biosynthetically with [^{35}S]methionine for 5 min. mAb AF8 immunoprecipitated calnexin (Fig. 3 *A*, lane *1*, *arrow*), and also coimmunoprecipitated a number of other proteins. A band was observed in lane *1* (*arrowhead*) that comigrated with class I HCs immunoprecipitated with mAb HC10 (lane *2*). It was confirmed by IEF/SDS-PAGE two-dimensional gel analysis that most of the proteins contained in this band were mAb HC10-reactive class I HCs (Fig. 3 *B*). The efficient visualization of class I HCs associated with calnexin by biosynthetically pulse labeling cells implied that calnexin bound preferentially newly synthesized class I HCs. However, in the reciprocal immunoprecipitation the calnexin band was faintly visualized in the immunoprecipitation with mAb HC10 (Fig. 3 *A*, lane *2*), since in this 5-min biosynthetic labeling only a small fraction of calnexin molecules, which have a half-life of >24 h (data not shown), were labeled. We infer that the major portion of β_2m^-:HC-associated calnexin molecules are from a pool of preexisting, unlabeled calnexin molecules, since β_2m^-:HC-associated calnexin molecules were readily detected after permeabilized iodination which labels all calnexin molecules (Fig. 1, lane *3*). Thus, both biosynthetic and radioiodination techniques revealed the association of calnexin with β_2m^-:HC. However, neither technique revealed a detectable association of calnexin with assembled class I molecules recognized by the comformation-dependent mAb W6/32.

Calnexin-associated Class I HCs do not Appear to be the Immediate Precursors for Peptide-Dependent Class I Assembly. Townsend and co-workers (14–16, 37) have shown that class I assembly can be induced in vitro in lysates of peptide transporter–deficient cells by the addition of specific peptides. In this study we used T2/B27 cells, which synthesize both endogenous HLA-A2 and transfected HLA-B27 HCs. Employing T2/B27 cells made it possible to obtain substantial assembled class I molecules by taking advantage of both HLA-A2 and -B27 alleles for which specific peptides are known. The cells were labeled with [^{35}S]methionine for 5 min, and lysed in 0.5% Triton X-100 in the presence or absence of a mixture of HLA-A2–restricted peptide (M57-68K62) and HLA-B27–restricted peptide (B27NP). mAb W6/32 detected substantial class I complexes after the addition of the mixture of HLA-A2 and -B27 restricted peptides, as compared with that seen in their absence (Fig. 3 *A*, compare lanes *3* and *6*). The faint but significant immunoprecipitation of class I HCs with mAb W6/32 in the absence of peptides (Fig. 3 *A*, lane *3*) may be due either to assembled HLA-A2 molecules which bound peptides derived from signal sequences (38, 39) or to a small quantity of β_2m^+:HC:pep$^-$ complexes.

Despite the abundant appearance of W6/32 reactive class I complexes (Fig. 3 *A*, lane *6*), neither the HC10-reactive β_2m^-:HC pool (lanes *2* and *5*) nor the calnexin-associated β_2m^-:HC pool of class I molecules (lanes *1* and *4*) appeared to diminish after the addition of peptides. The absence of change in these β_2m^-:HC pools by the addition of peptides led us to speculate that in this in vitro peptide-dependent assembly system, β_2m^-:HC, either bound to calnexin or not, were not the direct precursors of the class I complexes being assembled.

Unstable β_2m^+:HC:pep$^-$ Complexes Are Present in T1 and T2 Cells. Several studies have shown that in this in vitro peptide-dependent class I assembly system, unstable β_2m^+:HC:pep$^-$ complexes could be stabilized in detergent by the addition of specific peptide (14, 15, 37, 40). We confirmed the presence of these unstable complexes in mutant T2 cells and wild-type T1 cells by comparing rapid immunoprecipitation with regular immunoprecipitation (see Materials and Methods). With the rapid immunoprecipitation method, a large pool of W6/32-reactive, β_2m^+:HC complexes could be detected even without the addition of peptide in peptide-deficient T2 cells (Fig. 4, lane *8*). On the other hand, when exactly the same procedure was carried out, except that mAb W6/32 was added after overnight incubation of the lysate at 4°C, remarkably fewer β_2m^+:HC complexes were de-

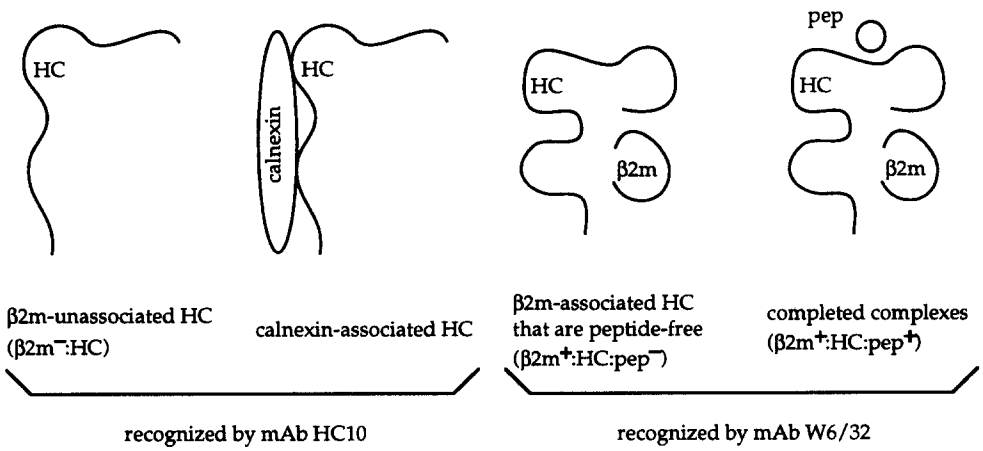
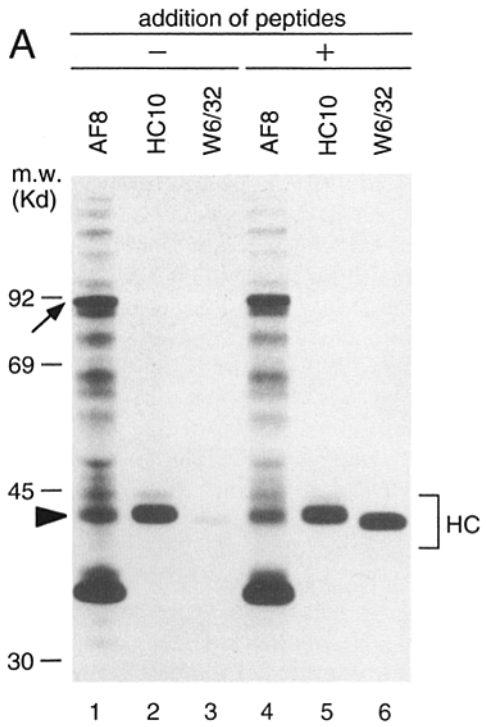


Figure 2. Four distinct pools of class I HC, distinguished by immunoprecipitation with mAbs HC10 and W6/32 which recognize β_2m-free and β_2m-associated class I HCs, respectively.



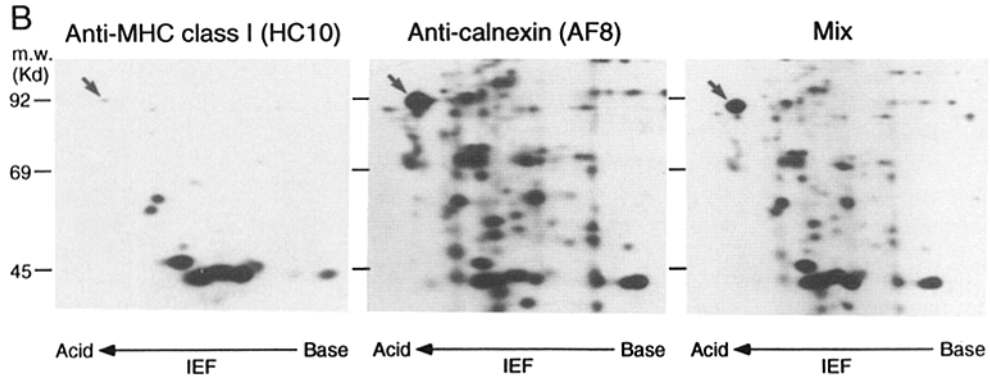


Figure 3. β_2m^-:HC pools are unaffected by peptide-dependent assembly of MHC class I molecules in T2 cell lysates. (*A*) T2/B27 cells were radiolabeled with [^{35}S]methionine for 5 min and lysed in 0.5% Triton X-100 either in the absence (lanes *1–3*) or presence of a mixture of 50 μM M57-68K62 and 5 μM B27NP peptides (lanes *4–6*). Immunoprecipitations were carried out with the antibodies indicated, followed by resolution on a SDS-PAGE gel under reducing conditions. The position of the MHC class I HC is shown to the right. (*B*) T2/B27 cells were metabolically labeled with [^{35}S]methionine for 5 min, solubilized in 0.5% Triton X-100, and immunoprecipitated with mAbs HC10 and AF8. Immunoprecipitates were resolved on two-dimensional gels (IEF followed by SDS-PAGE). Solid arrows identify calnexin.

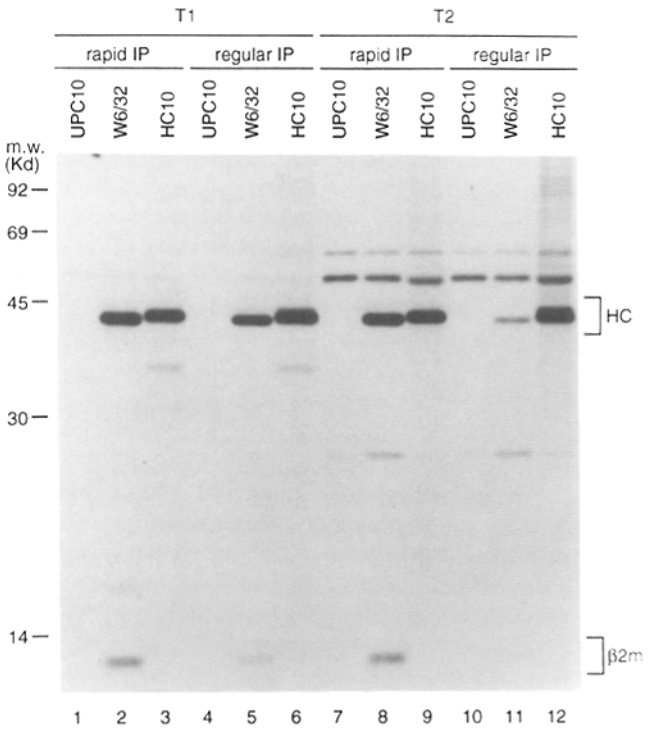


Figure 4. Differences in β_2m^+:HC and β_2m^-:HC pools are detected based on cell type and time after detergent lysis. T1 (lanes *1–6*) and T2 (lanes *7–12*) cells were radiolabeled with [^{35}S]methionine for 5 min. Cells were divided equally into six aliquots and three of them were used for rapid immunoprecipitation (*rapid IP*) (see Materials and Methods, lanes *1–3* and *7–9*). Lysates from the remaining three were incubated overnight at 4°C, and immunoprecipitation was performed on the following day (*regular IP*). UPC10 is an isotype-matched (IgG2a) negative control antibody. The samples were analysed on a SDS-PAGE gel under reducing conditions. (*Right*) The positions of the class I HC and β_2m.

tected by the antibody (Fig. 4, lane *11*). Correspondingly, more β_2m^-:HC was detected with mAb HC10 than in the rapid immunoprecipitation. Since these experiments were performed in the absence of added peptides, we concluded that a substantial pool of β_2m^+:HC:pep$^-$ complexes were generated in T2 cells and were recognized by mAb W6/32, but that these complexes were unstable and presumably disassembled during the overnight incubation at 4°C.

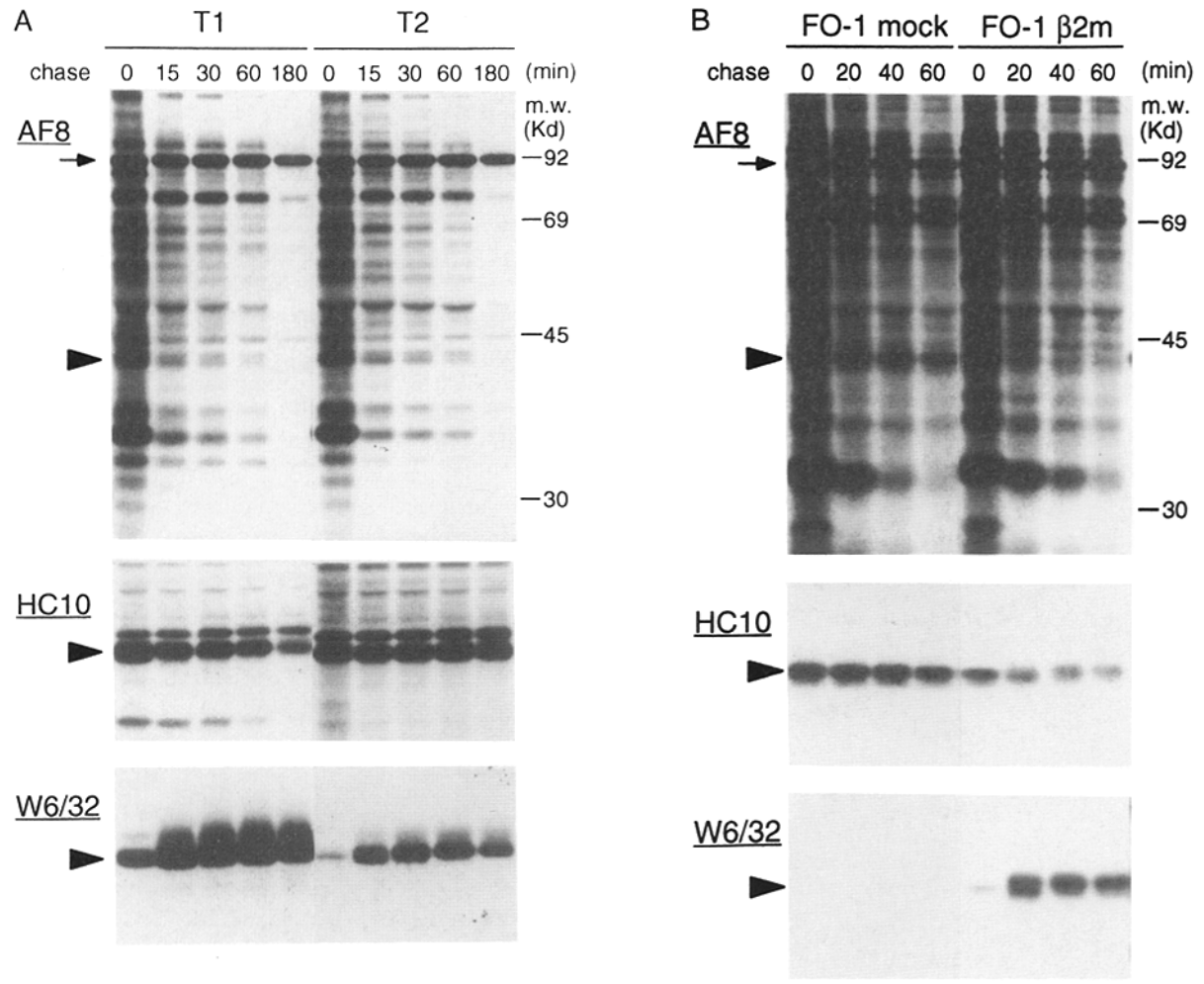
Wild-type T1 cells, which have not deleted the TAP.1 and TAP.2 genes, have apparently normal class I assembly. Unlike T2 cell lysates, T1 cell lysates, which contain a substantial pool of completely assembled class I molecules, still contained substantial quantities of W6/32-reactive class I molecules after overnight incubation (Fig. 4, lane *5*). However, a significant reduction in β_2m^-:HC immunoprecipitated with mAb W6/32 and an increase in β_2m^-:HC immunoprecipitated with mAb HC10 were observed after overnight incubation even in T1 cells (Fig. 4, compare lanes *2* and *5*, and lanes *3* and *6*). Since a greater quantity of W6/32-reactive class I molecules were detected by rapid immunoprecipitation compared to after an overnight incubation, we concluded that relatively unstable β_2m^+:HC complexes were generated not only in peptide transporter deficient mutant cells but also in normal cells, as suggested by others (14, 15, 40).

β_2m, But Not Peptide, Controls Dissociation of Class I HCs from Calnexin. The in vitro results shown above suggested that a calnexin-unassociated β_2m^+:HC:pep$^-$ complex capable of binding peptide existed and raised the possibility that class I HCs may dissociate from calnexin before peptide binding. If correct, then the dissociation rate of class I HCs from calnexin in vivo would be expected to be similar in T1 and T2 cells. To test this possibility, we carried out a pulse chase experiment of the two cell lines (Fig. 5 *A*). As expected, in T1 cells, the HC10-reactive β_2m^-:HC pool decreased and a large amount of W6/32-reactive class I molecules were detected during a chase period, consistent with normal MHC class I assembly. In contrast, in T2 cells the total amount of β_2m^-:HC pool remained almost unchanged during the chase period and a much smaller amount of assembled class I molecules were observed, consistent with a deficiency in peptides in the ER lumen of these cells. It is important to note that the association of class I HCs with calnexin diminished during the chase period of time in both T1 and T2 cells, and that the rate of dissociation was almost indistinguishable between the two cell lines despite the difference in availability of peptides in the ER of these two cell lines (Fig. 5 *C*, *left*).

To address the possibility that β_2m controls the rates of dissociation of class I HCs from calnexin, we examined β_2m^+ and β_2m^- cells. For comparison with β_2m-deficient FO-1 cells (FO-1 mock), we transfected the β_2m genomic DNA clone and reconstituted β_2m expression in FO1 cells (FO-1 β_2m). In contrast to the observation of peptide-deficient cells above, a significant difference in dissociation of class I HCs from calnexin in vivo was found between β_2m-deficient FO-1 mock and β_2m-expressing FO-1 β_2m cells. In FO-1 β_2m cells, a decrease in the HC10-reactive β_2m^-:HC pool and the appearance of the W6/32-reactive β_2m^+:HC pool was observed during the chase period, whereas in β_2m-deficient FO-1 mock cells, the total amount of β_2m^-:HC pool remained almost unchanged and no appearance of the W6/32-reactive β_2m^+:HC pool was seen (Fig. 5 *B*). More importantly, the association of class I HCs with calnexin diminished during the chase period in FO-1 β_2m cells, whereas class I HCs remained associated with calnexin in the β_2m-deficient FO-1 mock cells (Fig. 5, *B* and *C*, *right*).

Discussion

In order for class I molecules to complete their assembly and exit the ER, at least three events should take place in the ER; namely, binding of β_2m to class I HC, binding of peptide to class I HC, and dissociation of class I HC from calnexin. The former two are critically important for proper expression of class I molecules because lack of either β_2m or peptide has been shown to result in severely impaired class I expression (7–11). Properly regulated dissociation of class I HCs from calnexin seems also essential since class I HCs



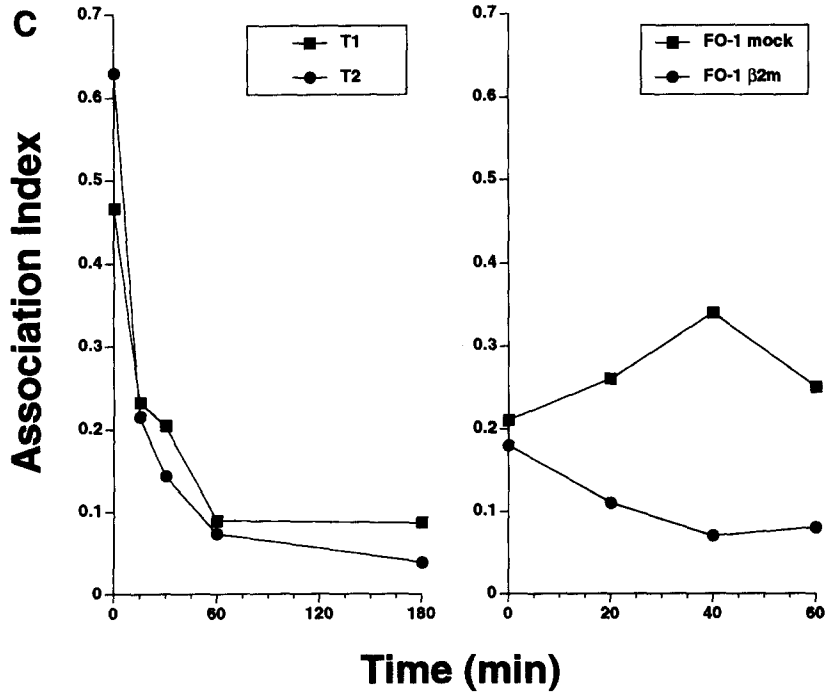


Figure 5. The rates of dissociation of MHC class I HCs from calnexin are similar in peptide transporter–deficient T2 cells and wild-type T1 cells, whereas they differ between β_2m-deficient FO-1 mock and β_2m-expressing FO-1 β_2m cells. (*A* and *B*) T1 and T2 cells (*A*) or FO-1 mock and FO-1 β_2m cells (*B*) were radiolabeled with [^{35}S]methionine for 5 min and chased for indicated periods of time. Cells were harvested and lysed in 0.5% Triton X-100. The lysates were equally aliquoted, and immunoprecipitation was performed with indicated antibodies. The samples were analysed on SDS-PAGE gels under reducing conditions. Immunoprecipitation with mAb P3 (negative control) did not show any appreciable background (data not shown). The positions of calnexin and the class I HC are indicated with an arrow and arrowheads, respectively. (*C*) An association index at each chase period was calculated as described in Materials and Methods and plotted.

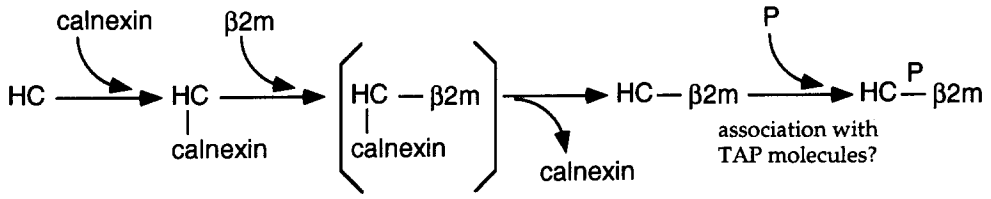


Figure 6. Schematic model of class I assembly. (*HC*) Class I HC; (β_2m) β_2-microglobulin; (*P*) peptide.

are retained in the ER by calnexin in the absence of β_2m (24, 41). In the present study, we describe the stepwise sequence of these events.

One of the important approaches of this study was to discriminate intracellular class I HC pools in terms of association either with β_2m or with calnexin and to assess changes in these pools during peptide-dependent class I assembly (or stabilization) in cell lysates. The interaction of nascent class I HCs with calnexin occurs very soon after their synthesis (possibly cotranslationally or cotranslocationally) (21, 22). Thus, employing 5 min short pulse labeling with [^{35}S]methionine made it possible to visualize a pool of calnexin-associated class I HCs in immunoprecipitates with an anti-calnexin antibody (Fig. 3 *A*, lane *1*). Class I HCs in this pool appeared to be β_2m-unassociated since calnexin was coimmunoprecipitated with mAb HC10, which recognizes β_2m$^-$:HC, but not with W6/32, which recognizes β_2m$^+$:HC (Fig. 1).

The addition of specific peptides upon lysis of peptide transporter–deficient cell lines has been shown to stabilize β_2m$^+$:HC:pep$^-$ in detergent (14, 15, 37, 40), which is manifested by an increase in the amount of β_2m$^+$:HC immunoprecipitated. In our experiments, we used T2/B27 cells expressing both endogenous HLA-A2 and transfected -B27 HCs. As expected, the addition of HLA-A2 and -B27 restricted peptides resulted in a significant increase in W6/32-reactive class I HC complexes (Fig. 3, lane *6*). However, the detectable pool of calnexin-associated class I HCs did not appear to diminish despite the abundant appearance of assembled class I complexes (Fig. 3). This is consistent with the above observation that calnexin-bound class I HCs are unassociated with β_2m since these peptide addition experiments preferentially detect stabilizing effect of peptide on unstable β_2m$^+$:HC:pep$^-$ complexes.

The presence of unstable β_2m$^+$:HC:pep$^-$ complexes which are not bound to calnexin in normal cells and the finding that calnexin binds β_2m$^-$:HC led us to speculate that the dissociation of class I HCs from calnexin might be controlled by binding of β_2m. Previous work has implied that newly synthesized class I HCs are retained for a prolonged period of time in association with calnexin in β_2m-deficient Daudi cells (24, 41), which implied a requirement for β_2m for the dissociation of class I HCs from calnexin. We confirmed this by comparing β_2m-deficient FO-1 cells, which showed a prolonged association of class I HCs with calnexin, with β_2m transfected FO-1 cells, which displayed more rapid dissociation of class I HCs from calnexin (Fig. 5 *B*). In contrast, class I HCs did not show a prolonged association with calnexin in T2 cells in which peptides are not available but β_2m is present (Fig. 5 *A*). Class I HCs dissociated from calnexin in T2 cells at a similar rate as in wild-type T1 cells (Fig. 5 *C*), implying that peptide binding was not required for class I HCs to dissociate from calnexin. These conclusions are strengthened by the recent demonstration that calnexin-free β_2m$^+$:HC:pep$^-$ complexes physically associate with TAP molecules in human (42). These results are in contrast with studies of mouse p88/calnexin, in which a requirement for both β_2m and peptide for the dissociation of class I HCs from calnexin was detected (24) and murine β_2m$^+$:HC:pep$^-$ complexes were efficiently retained by p88/calnexin when expressed in Drosophila cells (26). Although the reason for this apparent disparity is not known, the difference might be explained by variable behavior of subtypes of class I molecules from the different species examined. Species-specific differences in MHC class I assembly and transport have been noted previously, as in the report that mouse class I molecules expressed in RMA-S and T2 cells behave differently from human class I molecules in these cell lines, presumably due to fundamental structural differences of the class I glycoproteins between species (43).

From the observations above, we propose an in vivo pathway for class I assembly (Fig. 6). Newly synthesized class I HCs bind to calnexin which retains them in the ER (HC-calnexin). Subsequently, the association of β_2m occurs to form a transient trimolecular complex of HC, β_2m, and calnexin (shown within parentheses). Calnexin then dissociates from β_2m-associated HCs (HC-β_2m) and finally, these unstable peptide-free class I complexes bind peptide to complete the assembly process and stabilize the MHC class I complexes. Thus, calnexin mediates retention of the β_2m-unassociated class I HCs and dimerization of class I HCs with β_2m, and the final step of peptide loading onto β_2m$^+$:HC:pep$^-$ complexes may be facilitated by physical association of these class I complexes with TAP molecules, as suggested by Cresswell and colleagues (42). It remains to be clarified if TAP molecules exist in close approximation to calnexin and by what mechanisms β_2m$^+$:HC:pep$^-$ complexes can bind to and dissociate from TAP molecules.

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References

1. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:213.
2. Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
3. Monaco, J.J. 1992. A molecular model of MHC class-I–restricted antigen processing. *Immunol. Today.* 13:173.
4. Nuchtern, J.G., J.S. Bonifacino, W.E. Biddison, and R.D. Klausner. 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. *Nature (Lond.).* 339:223.
5. Yewdell, J.W., and J.R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science (Wash. DC).* 244:1072.
6. Lapham, C.K., I. Bacík, J.W. Yewdell, K.P. Kane, and J.R. Bennink. 1993. Class I molecules retained in the endoplasmic reticulum bind antigenic peptides. *J. Exp. Med.* 177:1633.
7. Williams, D.B., B.H. Barber, R.A. Flavell, and H. Allen. 1989. Role of β_2-microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules. *J. Immunol.* 142:2796.
8. Seong, R.H., C.A. Clayberger, A.M. Krensky, and J.R. Parnes. 1988. Rescue of Daudi cell HLA expression by transfection of the mouse β_2-microglobulin gene. *J. Exp. Med.* 167:288.
9. Zijlstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, an R. Jaenisch. 1990. β_2-microglobulin deficient mice lack CD4$^-$8$^+$ cytotoxic T cells. *Nature (Lond.).* 344:742.
10. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β_2M, MHC class I proteins, and CD8$^+$ T cells. *Science (Wash. DC).* 248:1227.
11. Townsend, A., C. Ohlén, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Kärre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature (Lond.).* 340:443.
12. Schumacher, T.N.M., N.-T. Heemels, J.J. Neefjes. W.M. Kast, C.J.M. Melief, and H.L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell.* 62:563.
13. Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (Lond.).* 345:449.
14. Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. *Cell.* 62:285.
15. Elliott, T., V. Cerundolo, J. Elvin, and A. Townsend. 1991. Peptide-induced conformational change of the class I heavy chain. *Nature (Lond.).* 351:402.
16. Elliott, T., V. Cerundolo, and A. Townsend. 1992. Short peptides assist the folding of free class I heavy chains in solution. *Eur. J. Immunol.* 22:3121.
17. Kozlowski, S., T. Takeshita, W.-H. Boehncke, H. Takahashi, L.F. Boyd, R.N. Germain, J.A. Berzofsky, and D.H. Margulies. 1991. Excess β_2 microglobulin promoting functional peptide association with purified soluble class I MHC molecules. *Nature (Lond.).* 349:74.
18. Parker, K.C., B.M. Carreno, K. Sestak, U. Utz, W.E. Biddison, and J.E. Coligan. 1992. Peptide binding to HLA-A2 and HLA-B27 isolated from *Escherichia coli.* Reconstitution of HLA-A2 and HLA-B27 heavy chain/β2-microglobulin complexes requires specific peptides. *J. Biol. Chem.* 267:5451.
19. Gething, M.-J., and J. Sambrook. 1992. Protein folding in the cell. *Nature (Lond.).* 355:33.
20. Hochstenbach, F., V. David, S. Watkins, and M.B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA.* 89:4734.
21. David, V., F. Hochstenbach, S. Rajagopalan, and M.B. Brenner. 1993. Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin). *J. Biol. Chem.* 268:9585.
22. Degen, E., and D.B. Williams. 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol.* 112:1099.
23. Ou, W.-J., P.H. Cameron, D.Y. Thomas, and J.J.M. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature (Lond.).* 364:771.
24. Degen, E., M.F. Cohen-Doyle, and D.B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both β_2-microglobulin and peptide. *J. Exp. Med.* 175:1653.
25. Galvin, K., S. Krishna, F. Ponchel, M. Frohlich, D.E. Cummings, R. Carlson, J.R. Wands, K.J. Isselbacher, S. Pillai, and M. Ozturk. 1992. The major histocompatibility complex class I antigen-binding protein p88 is the product of the calnexin gene. *Proc. Natl. Acad. Sci. USA.* 89:8452.
26. Jackson, M.R., M.F. Cohen-Doyle, P.A. Peterson, and D.B. Williams. 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science (Wash. DC).* 263:384.
27. Rajagopalan, S., Y. Xu, and M.B. Brenner. 1994. Retention of unassembled components of integral membrane proteins by calnexin. *Science (Wash. DC).* 263:387.
28. Riberdy, J.M., and P. Cresswell. 1992. The antigen-processing

mutant T2 suggests a role for MHC-linked genes in class II antigen presentation. *J. Immunol.* 148:2586.
29. D'Urso, C.M., Z. Wang, Y. Cao, R. Takake, R.A. Zeff, and S. Ferrone. 1991. Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B_2m gene expression. *J. Clin. Invest.* 87:284.
30. Güssow, D., R. Rein, I. Ginjaar, F. Hochstenbach, G. Seemann, A. Kottman, and H.L. Ploegh. 1987. The human β_2-microglobulin gene. Primary structure and definition of the transcriptional unit. *J. Immunol.* 139:3132.
31. Takabe, Y., M. Seiki, J.-I. Fujisawa, P. Hoy, K. Yokota, K.-I. Arai, M. Yoshida, and N. Arai. 1988. SRα promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* 8:466.
32. Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745.
33. Barnstable, C.J., W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A.F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—New tools for genetic analysis. *Cell.* 14:9.
34. Lampson, L.A., and R. Levy. 1980. Two populations of Ia-like molecules on a human B cell line. *J. Immunol.* 125:293.
35. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511.
36. Stam, N.J., H. Spits, and H.L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299.
37. Elvin, J., C. Potter, T. Elliott, V. Cerundolo, and A. Townsend. 1993. A method to quantify binding of unlabeled peptides to class I MHC molecules and detect their allele specificity. *J. Immunol. Methods.* 158:161.
38. Henderson, R.A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D.F. Hunt, and V.H. Engelhard. 1992. HLA-A2.1–associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science (Wash. DC).* 255:1264.
39. Wei, M.L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature (Lond.).* 356:443.
40. Baas, E.J., H.-M. van Santen, M.J. Kleijmeer, H.J. Geuze, P.J. Peters, and H.L. Ploegh. 1992. Peptide-induced stabilization and intracellular localization of empty HLA class I complexes. *J. Exp. Med.* 176:147.
41. Rajagopalan, S., and M.B. Brenner. 1994. Calnexin retains unassembled major histocompatibility complex class I free heavy chains in the endoplasmic reticulum. *J. Exp. Med.* 180:407.
42. Ortmann, B., M.J. Androlewicz, and P. Cresswell. 1994. MHC class I/β_2-microglobulin complexes associate with TAP transporter before peptide binding. *Nature (Lond.).* 368:864.
43. Anderson, K.S., J. Alexander, M. Wei, and P. Cresswell. 1993. Intracellular transport of class I MHC molecules in antigen processing mutant cell lines. *J. Immunol.* 151:3407.