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

By Masahiko Sugita and Michael B. Brenner

From the Lymphocyte Biology Section, Department of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115

Summary

Proper assembly of the class I heavy chain (HC), β2-microglobulin (β2m), 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 β2m in peptide transporter–deficient T2 cells since β2m-unassociated class I HC-specific HC10 antibody, but not β2m-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 β2m-associated HC complexes (β2m + :HC:pep−), but had no significant effect on the preexisting pool of calnexin-associated HC that lack β2m. Release of HC from calnexin appeared to be controlled by the binding of β2m, since β2m-deficient FO-1 cells showed a prolonged association of class I HC with calnexin, while β2m-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 β2m, and that the unstable β2m + :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) 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), β2-microglobulin (β2m), 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 β2m-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 β2m 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 β2m, 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 β2m (16). In either case, the binding of peptide and β2m to the HC is cooperative, and peptide binding like the association with β2m is an inherent component of class I assembly. Although recombinant or purified components of class I HCs, β2m, and synthesized specific peptides can assemble 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...
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% CO2 atmosphere. β2m-deficient FO-1 cells (29) were provided by Dr. Soldano Ferrone (New York Medical College, Valhalla, NY). β2m-expressing and mock transfectant clones of FO-1 cells were obtained from transfection of either pb2m13 (30) plus pSRoe-neo (31) or pSRoe-neo alone, respectively, by calcium phosphate precipitation method (32). These transfected 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 β2m :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 β2m :HC, but not β2m - :HC (36) was provided by Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge, MA). Anti-calnexin/IP90 antibody, A8F (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 x 107) 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, 107 cells/ml were labeled for 5 min with 0.2 µCi [35S]methionine (NEG-072 EXPRES®; 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). Assayed cell 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 [35S]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 (1:1000 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 [35S]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-chloro- 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 (cpmCNX) immunoprecipitated with mAb A8F and counts per minute of class I HCs (cpmHC) coimmunoprecipitated with mAb A8F were counted directly by a beta 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 (cpmBG) 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 = (cpmHC - cpmBG)/(cpmCNX - cpmBG).

Results

β2m-free Class I HCs (β2m :HC) Are Associated with Calnexin in T2 Cells. We previously demonstrated that β2m :HC, which are recognized by mAb HC10, are associated with calnexin in β2m-deficient Daudi cells (20). To examine whether β2m :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 β2m :HC from iodinated T2 cells (Fig. 1, lane 3), demonstrating the association of calnexin with class I HCs that had not assembled with β2m. Note that in contrast to the HC10 antibody, a conformation-dependent antibody, W6/32, which recognizes β2m :HC, failed to coimmunoprecipitate calnexin from T2 cells (Fig. 1, lane 4). This implied that calnexin bound β2m :HC, but not β2m + :HC. This led us to assume that there may be several distinct intracellular class I HC pools, including β2m-unassociated HCs (β2m + :HC) either bound to calnexin or not, and β2m-associated HCs (β2m :HC) which may contain a bound peptide (β2m :HC:pep+) or not (β2m :HC:pep−) (Fig. 2).

The β2m :HC pool associated with calnexin was not
Figure 1. Calnexin associates with β2m-:HC but not β2m+:HC. Permeabilized T2 cells were iodinated and lysed in 1% digitonin. Immunoprecipitation was performed with mAbs P3 (control), AF8 (anticalnexin), HC10 (anti-β2m-:HC), and W6/32 (anti-β2m+:HC), followed by resolution on a SDS-PAGE gel under reducing conditions. (Right) The positions of calnexin, MHC class I HC (HC), and β2m 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 [35S]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 β2m-:HC-associated calnexin molecules are from a pool of preexisting, unlabeled calnexin molecules, since β2m-: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 β2m-:HC. However, neither technique revealed a detectable association of calnexin with assembled class I molecules recognized by the conformation-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 [35S]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 β2m+:HC:peptide complexes.

Despite the abundant appearance of W6/32 reactive class I complexes (Fig. 3 A, lane 6), neither the HC10-reactive β2m-:HC pool (lanes 2 and 5) nor the calnexin-associated β2m-:HC pool of class I molecules (lanes 1 and 4) appeared to diminish after the addition of peptides. The absence of change in these β2m-:HC pools by the addition of peptides led us to speculate that in this in vitro peptide-dependent assembly system, β2m-:HC, either bound to calnexin or not, were not the direct precursors of the class I complexes being assembled.

Unstable β2m-:HC:peptide Complexes Are Present in T1 and T2 Cells. Several studies have shown that in this in vitro peptide-dependent class I assembly system, unstable β2m-:HC:peptide 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, β2m-: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 β2m-:HC complexes were de-
Figure 2. Four distinct pools of class I HC, distinguished by immunoprecipitation with mAbs HC10 and W6/32 which recognize β2m-free and β2m-associated class I HCs, respectively.

Figure 3. β2m-HC pools are unaffected by peptide-dependent assembly of MHC class I molecules in T2 cell lysates. (A) T2/B27 cells were radiolabeled with [35S]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 [35S]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.
not only in peptide transporter deficient mutant cells but also in normal cells, as suggested by others (14, 15, 40).

**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 β2m 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 β2m 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 β2m-deficient FO-1 mock and β2m-expressing FO-1 β2m cells. (A and B) T1 and T2 cells (A) or FO-1 mock and FO-1 β2m cells (B) were radiolabeled with [35S]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.

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are retained in the ER by calnexin in the absence of β2m (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 β2m 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 cotranslationally) (21, 22). Thus, employing 5 min short pulse labeling with [35S]methionine made it possible to visualize a pool of calnexin-associated class I HCs in immunoprecipitates with an anti-calnexin antibody (Fig. 3A, lane 1). Class I HCs in this pool appeared to be β2m-unassociated since calnexin was coimmunoprecipitated with mAb HC10, which recognizes β2m-:HC, but not with W6/32, which recognizes β2m+ :HC (Fig. 1).

The addition of specific peptides upon lysis of peptide transporter-deficient cell lines has been shown to stabilize β2m+ :HC:peptide+ in detergent (14, 15, 37, 40), which is manifested by an increase in the amount of β2m+ :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 β2m since these peptide addition experiments preferentially detect stabilizing effect of peptide on unstable β2m+ :HC:peptide− complexes.

The presence of unstable β2m− :HC:peptide− complexes which are not bound to calnexin in normal cells and the finding that calnexin binds β2m− :HC led us to speculate that the dissociation of class I HCs from calnexin might be controlled by binding of β2m. Previous work has implied that newly synthesized class I HCs are retained for a prolonged period of time in association with calnexin in β2m-deficient Daudi cells (24, 41), which implied a requirement for β2m for the dissociation of class I HCs from calnexin. We confirmed this by comparing β2m-deficient FO-1 cells, which showed a prolonged association of class I HCs with calnexin, with β2m transfected FO-1 cells, which displayed more rapid dissociation of class I HCs from calnexin (Fig. 5B). In contrast, class I HCs did not show a prolonged association with calnexin in T2 cells in which peptides are not available but β2m is present (Fig. 5A). Class I HCs dissociated from calnexin in T2 cells at a similar rate as in wild-type T1 cells (Fig. 5C), 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 β2m− :HC:peptide− 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 β2m and peptide for the dissociation of class I HCs from calnexin was detected (24) and murine β2m− :HC:peptide− 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 β2m occurs to form a transient trimolecular complex of HC, β2m, and calnexin (shown within parentheses). Calnexin then dissociates from β2m-associated HCs (HC-β2m) 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 β2m-unassociated class I HCs and dimerization of class I HCs with β2m, and the final step of peptide loading onto β2m+ :HC:peptide− 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 β2m+ :HC:peptide− complexes can bind to and dissociate from TAP molecules.

We thank Drs. Peter Cresswell and Soldano Ferrone for the gift of cell lines, Dr. Hidde Ploegh for the
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