

Species-specific Differences in Chaperone Interaction of Human and Mouse Major Histocompatibility Complex Class I Molecules

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Summary

Previous studies have shown that immature mouse class I molecules transiently associate with a resident endoplasmic reticulum protein of 88 kD that has been proposed to act as a chaperone for class I assembly. Subsequently, this protein was demonstrated to be identical to calnexin and to associate with immature forms of the T cell receptor complex, immunoglobulin, and human class I HLA heavy chains. In this paper we define further the interaction of human class I HLA heavy chains with chaperone proteins and find key differences with the complexes observed in the mouse system. First, calnexin and immunoglobulin binding protein (BiP) both associate with immature HLA class I heavy chains. The two chaperones are not found within the same molecular complex, suggesting that calnexin and BiP do not interact simultaneously with the same HLA class I heavy chain. Second, only free HLA class I heavy chains, and not β_2 -microglobulin (β_2m)-associated heavy chains are found associated with the chaperones. Indeed, addition of free β_2m in vitro induces dissociation of chaperone-class I HLA heavy chain complexes. The kinetics for dissociation of the class I HLA heavy chain-chaperone complexes and for formation of the class I HLA heavy chain- β_2m complex display a reciprocity that suggests the interactions with chaperone and β_2m are mutually exclusive. Mouse class I heavy chains expressed in human cells exhibit the mouse pattern of interaction with human chaperones and human β_2m and not the human pattern, showing the difference in behavior is purely a function of the class I heavy chain sequence.

Functional MHC class I molecules consist of a polymorphic class I heavy chain, β_2 -microglobulin (β_2m)¹ and a bound peptide (1, 2). Class I heavy chains and β_2m are synthesized on membrane bound ribosomes and then translocated into the lumen of the endoplasmic reticulum (ER) (3). Peptides derive from protein degradation in the cytoplasm and are transported into the ER (4–8) where the association of heavy chains, β_2m , and peptides occurs. All three components are required for efficient transport from the ER to the plasma membrane. Cells deficient in either β_2m or peptide supply have greatly reduced levels of class I MHC molecules at the cell surface (9–16).

Degen et al. (17, 18) showed that immature mouse class I molecules associate transiently with a protein of 88 kD, which they proposed acted as a chaperone. This protein was shown to be identical to calnexin (19, 20), a calcium binding protein of the ER (21). Subsequently, calnexin was found

to associate with human class I heavy chain, and also with immature forms of the T cell receptor and immunoglobulin (22–24).

Previous studies have revealed intriguing differences in the interactions of class I MHC heavy chains and β_2m in humans and mice (for reference see 25). HLA class I heavy chains bind β_2m more strongly than do mouse class I heavy chains, and incubation of human cells in the presence of bovine β_2m does not lead to significant β_2m exchange, as is the case for mouse cells (25). In mouse cell lines that lack expression of β_2m , some fraction of class I heavy chains reach the cell surface (26, 27). This is also true for cells of mice rendered β_2m -negative through “knock-out” of the β_2m gene (28, 29), for which incubation with exogenous β_2m and an appropriate binding peptide can reconstitute functional class I molecules at the cell surface (30). In contrast, class I heavy chains of the human cell line Daudi, which is defective in both β_2m alleles, are trapped in the ER and appear actively prevented from reaching the cell surface (12).

Cell lines negative for the peptide transporter genes, TAP, have limited peptide supply resulting in class I molecules largely devoid of peptides. Such “empty” mouse class I molecules expressed in the human TAP-negative cell line T2 are trans-

¹ Abbreviations used in this paper: β_2m , β_2 -microglobulin; BiP, immunoglobulin binding protein; B-LCL, B lymphoblastoid cell line; DSP, dithiobis(succinimidyl-propionate); EndoH, endoglycosidase H; ER, endoplasmic reticulum; TAP, transporter-associated protein.

ported to the cell surface normally whereas their human equivalents are retained intracellularly (31). Furthermore, empty mouse class I molecules, although efficiently transported to the cell surface, are unstable at physiological temperature but can be stabilized by reducing the temperature to 25°C (16). In contrast, empty human class I molecules form stable heterodimers with β_2m , even at elevated temperature (32).

Collectively, these findings provide circumstantial evidence for differences in the biogenesis of class I MHC molecules in human and mouse cells. One potential source of the difference is in the interaction of the class I heavy chain with chaperones, including calnexin. The experiments we report here were undertaken to define further the interaction of HLA class I heavy chains with intracellular chaperones and to compare their interaction with human and mouse class I heavy chains.

Materials and Methods

Cells. Cells lines were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1.4 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (Irvine Scientific, Santa Ana, CA). The C1R cell line, a derivative of the B lymphoblastoid cell line (B-LCL) Hym2 (33) and reduced in class I expression (34), was stably transfected with the B*2705, B*0702, and K^b genomic DNA clones to generate B27C1R, B7C1R, and K^bC1R (provided by Dr. J. Alexander and Dr. P. Cresswell, Yale University, New Haven, CT). EL4, a mouse T cell lymphoma of H-2^b haplotype and MDAY.D2, an H-2^d-expressing anaplastic tumor cell line of lymphoreticular origin, were gifts from Dr. D.B. Williams (University of Toronto, Toronto, Canada). Daudi is a Burkitt's lymphoma cell line defective for β_2m expression (35, 36).

Antibodies. Antibodies with reactivity for different forms of human MHC class I molecules were used: W6/32 and ME1 (37) recognize human MHC class I molecules only when associated with β_2m ; Q1/28 recognizes predominantly free MHC class I heavy chains and to a lesser extent assembled MHC class I molecules (reference in 37); A1.4 (Olympus Co., Lake Success, NY) reacts with free and β_2m -associated class I molecules (38); monoclonal antibodies LA45 (39, 40) and HC10 (41) bind free class I heavy chains. Antibodies L368 (42) and BBM1 (reference 37) were used to detect human β_2m . Reagents specific for mouse MHC class I proteins were the gift of Dr. D. B. Williams (reference in 17): rabbit anti-peptide 8 antiserum reacts with free and β_2m -associated H-2K^b and K^d proteins, and monoclonal antibody 20-8-4s reacts with H-2K^b and K^d when associated with β_2m . AF8 (22), a mouse monoclonal antibody reacting with human calnexin, was a gift from Dr. M. B. Brenner (Harvard Medical School, Boston, MA). The anti-grp78/BiP monoclonal antibody, which also precipitates the grp94/ER99 protein, was purchased from StressGen (Victoria, BC., Canada). Nonimmune rabbit antiserum and monoclonal antibody CVC7 (anti-bovine clathrin light chain, LCa; reference in 37) were used as negative controls.

Biosynthetic Radiolabeling, Cell Lysis, and Cross-linking. For pulse/chase experiments, 5×10^6 cells were used for each time point. After starvation in 1 ml of methionine/cysteine-free culture medium/10% FCS for 30 min, cells were pulsed for 10 min by the addition of 100 μ Ci of TranSLabel (ICN Biomedicals Inc., Costa Mesa, CA). Chase was initiated by the addition of 9 vol of culture medium containing 1 mM nonradioactive methionine and cysteine. At various time points, samples were removed, cooled quickly on

ice, washed twice with ice-cold PBS, pH 8.0, and kept on ice until lysis with or without cross-linking. Cell lysis and cross-linking were performed as described (17) with minor modifications: the cross-linking reagent, dithiobis(succinimidyl-propionate) (DSP; Pierce Chemical Co., Rockford, IL), was dissolved freshly in DMSO at a concentration of 10 mg/ml and 3 μ l were added simultaneously with 500 μ l of lysis buffer (0.5% NP-40, 10 mM iodoacetamide, 0.2 mM PMSF in PBS, pH 8.0). Lysis was performed on ice for 30 min. Cross-linking was stopped by adding glycine to a final concentration of 50 mM. Lysates were precleared with 5 μ l of normal rabbit serum and 250 μ l of a 10% solution of Pansorbin cells (Calbiochem-Novabiochem Corp., San Diego, CA) for 4 h at 4°C.

Long-term labeling (15–20 h) was performed in 24-well plates. 6×10^6 cells were resuspended at a cell concentration of 2×10^6 cells/ml in methionine/cysteine-free RPMI 1640/10% FCS and starved for 30 min before radiolabeling with 100 μ Ci/ml TranSLabel. After radiolabeling, cell lysis and cross-linking were performed as described.

Isolation of MHC Class I Molecules. All procedures were performed at 4°C. Precleared cell lysates were immunoprecipitated with 5 μ l of either purified monoclonal antibody or rabbit antiserum or ascites fluid and 50 μ l of a 10% suspension of Pansorbin cells. Isolated immunocomplexes were washed first in 1 ml of 1st washing buffer NTS (0.5% NP-40, 10 mM Tris-HCl [pH 7.4], 0.15 mM NaCl, 1 mM MgCl₂) supplemented with SDS (0.1%) and deoxycholic acid (0.5%) and then in 1 ml of 2nd washing buffer (NTS, diluted 1/10 in dH₂O and supplemented with 0.5 M NaCl). Samples were analyzed by gel electrophoresis. For cleavage of the cross-linker, samples were resuspended in SDS-PAGE sample buffer containing 100 mM dithiothreitol (DTT) and 0.2% 2-mercaptoethanol and boiled for 10 min before SDS-PAGE analysis.

Digestion with endoglycosidase H (EndoH; Boehringer Mannheim Corp., Indianapolis, IN) was performed while immunocomplexes were still bound to Pansorbin cells. Samples were resuspended in 60 μ l of EndoH digestion buffer (0.1 M sodium citrate [pH 5.5], 0.075% SDS, 0.2% 2-mercaptoethanol) and split into two equal aliquots, one of which received 2 mU of EndoH. 2-mercaptoethanol was omitted from DSP-treated samples to preserve cross-linked complexes. Digestion was carried out at 30°C overnight and terminated by the addition of SDS-PAGE sample buffer with or without the reducing agent, DTT.

Analysis of Cross-linked Complexes. Protein complexes isolated from long-term labeled cells were detected by SDS-PAGE under nonreducing conditions. Protein bands were excised from dried gels and eluted twice in 100 μ l of PBS/1% SDS, each time for 24 h at room temperature. Eluted proteins were precipitated with acetone and subjected to EndoH digestion under reducing conditions. Samples were boiled before reanalysis by reducing SDS-PAGE.

One-dimensional Peptide Mapping Using V8 Endoproteinase. Peptide maps for the 84- and 94-kD proteins, calnexin, grp78/BiP, grp94/ER99, and class I heavy chains were created by in-gel digestion using 0.6 μ g of *Staphylococcus aureus* V8 endoproteinase (Boehringer Mannheim Corp.). Radiolabeled 84- and 94-kD proteins were isolated from the 160-kD bands obtained from cell lysates of 200×10^6 B27C1R or B7C1R cells that had been radiolabeled for 20 h and cross-linked as described. Radiolabeled calnexin, BiP, and grp94 were immunoprecipitated from biosynthetically labeled noncross-linked B27C1R or B7C1R cell lysates using monoclonal antibodies AF8 and anti-grp78, respectively. Class I HLA-B*0702 heavy chains migrate as two distinct species on 10% nonreducing SDS-PAGE and both bands were eluted for V8 digestion. Eluted proteins were precipitated with acetone and analyzed by Cleveland mapping as described (43, 44).

Addition of β_2m to Daudi Cell Extracts. Daudi cells were radiolabeled with TranSLabel for 1 h and washed twice in PBS. Pellets of 7×10^6 cells were resuspended in 250 μ l of 0.5% NP-40 lysis buffer containing 0, 0.5, 5, or 50 μ g of either purified β_2m (Calbiochem-Novabiochem Corp.) or chicken lysozyme (Sigma Chemical Co., St. Louis, MO) as a negative control protein. After preclearing the lysates with Pansorbin cells, immunoprecipitation was performed using antibody W6/32 to determine whether HC/ β_2m heterodimer formation occurred under the conditions applied. Precipitated material was analyzed by one-dimensional IEF. For cross-linking experiments, lysis in the presence of exogenous protein was initiated in the absence of cross-linker. After incubation for 15 min on ice, cross-linking was performed by the addition of 250 μ l of NP-40 lysis buffer containing 3 μ l of DSP (10 mg/ml in DMSO) (final concentration of 0.2 mM). Cross-linking and immunoprecipitation of the 160 kD species were performed as described.

As an alternative to NP-40 lysis, exogenous proteins were "fed to cells" using sonication. Washed cell pellets were resuspended in 250 μ l of PBS containing purified proteins and sonicated (Branson Sonifer 450; VWR Scientific Instruments, Philadelphia, PA) in pulsed mode at a fixed repetition rate of one pulse/s and a pulse duration of 20% of each second. After sonication, samples were kept on ice for 15 min before cross-linking was performed by addition of 250 μ l of 1% NP-40 lysis buffer containing 3 μ l of DSP (10 mg/ml) to give a final concentration of 0.5% NP-40 and 0.2 mM DSP. Immunoprecipitation was performed as before. No appreciable difference in the amount of HC/ β_2m or 160-kD complexes resulted from the two protocols.

Results

Detection of a 160-kD HLA Class I Heavy Chain/Protein Complex. Using a cleavable cross-linking reagent (DSP)

Degen and Williams (17) showed that transport of murine class I (H-2) major histocompatibility complex molecules from ER to Golgi is controlled by calnexin. We performed similar experiments upon human class I HLA molecules. Cells were biosynthetically labeled and then detergent solubilized in the presence or absence of DSP. Class I HLA molecules were immunoprecipitated and analyzed on nonreducing SDS-PAGE. To ensure the validity of the comparison we repeated the experiments of Degen and Williams, confirming the presence of a 145-kD species in DSP-treated cell extracts after precipitation with anti H-2 antibodies (Fig. 1 a).

Analogous experiments using human C1R cells transfected with either HLA-B*0702 or HLA-B*2705 genes revealed a 160-kD species in DSP-treated cell extracts (Fig. 1 b) that was not seen in immunoprecipitates made from untreated extracts. The cross-linked species from human cells was of slower electrophoretic mobility than that from mouse cells. Comparison of human EBV-transformed B cell lines and C1R cells transfected with class I HLA genes showed that the 160-kD species was more abundant in the C1R transfectants and for that reason our studies focused on them.

Comparison of the immunoprecipitates obtained with monoclonal antibodies directed against different epitopes of class I HLA molecules revealed that detectable amounts of the cross-linked 160-kD species were precipitated only by antibodies reactive with free class I heavy chains (Q1/28, LA45, A1.4, and HC10). Antibodies reactive with the complex of β_2m , heavy chain, and peptide (W6/32, ME1, L368, and BBM1) (Fig. 1 b) gave bands corresponding to the heavy chains (45 kD) and a cross-linked complex of heavy chains and β_2m (55 kD), but no 160-kD species was detected even after long

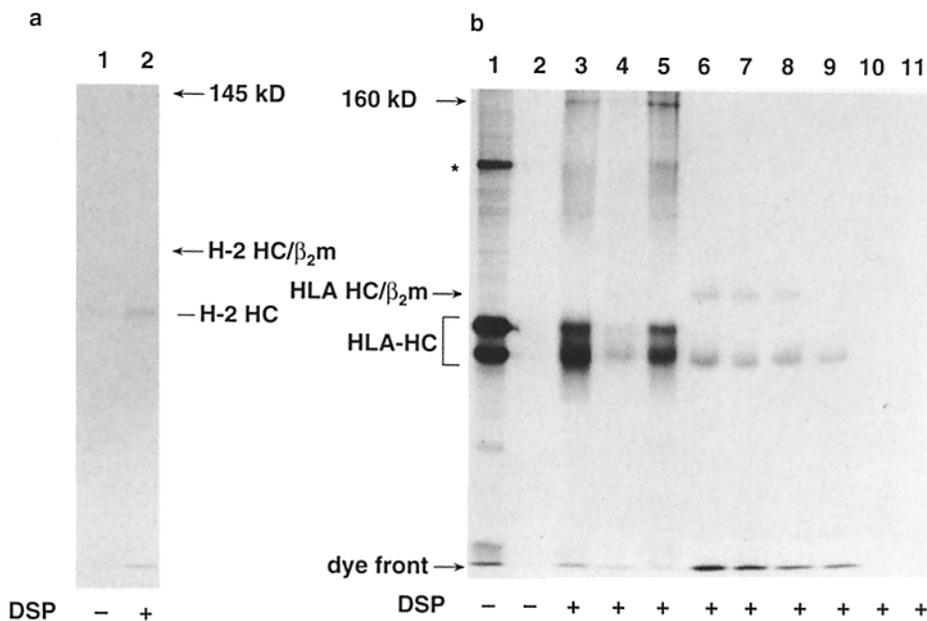


Figure 1. Detection of a 160-kD HLA class I heavy chain/protein complex. 10% nonreducing SDS-PAGE of class I immunoprecipitates from radiolabeled (15 min) mouse (MDAY.D2 [a]) and human (B7C1R [b]) cell lines. Absence (-) or presence (+) of DSP is indicated. Antibody used in (a) is 20-8-4s recognizing free and β_2m -associated H-2 K^b. Antibodies used in (b) are: those specific for human free class I heavy chains (Q1/28: lanes 1 and 5, LA45: lane 3; A1.4: lane 4); antibodies specific for human heavy chain/ β_2m complexes (HLA-HC/ β_2m) (W6/32: lane 8 and ME1: lane 9); those recognizing human β_2m (BBM1: lane 6; and L368: lane 7); anti-bovine clathrin light chain, CVC7 (lane 10) and nonimmune serum (lanes 2 and 11). Arrows indicate mouse H-2 class I/calnexin complex (referred to as 145 kD according to reference 17), human HLA class I/protein complex (apparent molecular mass of 160 kD), mouse (H-2 HC/ β_2m) and human (HLA HC/ β_2m)

class I heavy chain/ β_2m complexes. Asterisk (*) indicates HLA-B*0702 class I heavy chain dimers that are not dissociated under nonreducing conditions. Monomeric HLA class I heavy chains migrate as two discrete bands on nonreducing gels. Both monomeric B*0702 species exhibit identical peptide patterns after digestion with V8 proteinase (Fig. 3).

exposures. This lack of the cross-linked 160-kD band in precipitates using the latter antibodies has been observed consistently in numerous experiments, but has not been seen in mouse cells, for which antibodies against either the H-2 class I complex or β_2m precipitated detectable amounts of the cross-linked product (17). We, therefore, conclude that β_2m is absent in the human 160-kD cross-linked complex or present in amounts undetectable with the antibodies used.

Composition of the Cross-linked 160-kD Complex. To analyze its component polypeptide chains the 160-kD cross-linked band was eluted and reanalyzed on SDS-PAGE after reduction of the cross-linker. When cells were biosynthetically labeled for relatively short periods of time (15 min to 2 h) before solubilization and cross-linking only the heavy chains of class I molecules were detected (not shown). However, with overnight labeling bands corresponding to two proteins of 84 and 94 kD apparent molecular mass were detected in addition to the HLA-heavy chain (45 kD) (Fig. 2). As expected from the known specificities of the precipitating antibodies, β_2m was not found in the cross-linked complex.

Treatment of the cross-linked species with EndoH, an enzyme that removes immature N-linked high mannose oligosaccharides from proteins, increased the mobility of all the class I heavy chains (Fig. 2). During glycoprotein maturation EndoH sensitivity is lost due to carbohydrate modification completed by the time glycoproteins arrive at the medial-Golgi (45). Therefore, EndoH sensitivity of the HLA class

I heavy chain in the cross-linked complex is characteristic of class I heavy chains found in the ER or *cis*-Golgi. Neither the mobility of the 84-kD nor the 94-kD protein was affected by EndoH treatment indicating these proteins have no N-linked carbohydrate (Fig. 2).

The failure to detect the cross-linked 160-kD species with β_2m -dependent antibodies and the sensitivity of class I heavy chains to EndoH treatment support a scheme in which the 84- and the 94-kD proteins associate with immature class I heavy chains at a stage in their maturation before the binding of β_2m and egress from the ER.

The Cross-linked Proteins with Apparent Molecular Masses of 94 and 84 kD Correspond to Calnexin and Immunoglobulin Binding Protein (BiP). Calnexin has been shown by Ahluwalia et al. (19) and Hochstenbach et al. (22) to associate with human and mouse class I molecules. BiP, a member of the heat shock protein family (HSP70) and like calnexin localized to the lumen of the ER, was originally described independently as the immunoglobulin heavy chain binding protein (46) and the glucose-regulated protein, grp 78 (47). Its characteristics of binding to immature, unfolded, or misfolded proteins suggested a function as molecular chaperone (48).

To determine if the 84- and 94-kD proteins in the cross-linked species correspond to either calnexin or BiP, proteins precipitated with antibodies against calnexin and BiP were compared with the 94- and 84-kD proteins isolated from the 160-kD cross-linked species precipitated with anti-class I HLA antibodies. Similar electrophoretic mobility on SDS-PAGE indicated that the 94-kD protein corresponds to calnexin and the 84-kD protein to BiP, hypotheses that were substantiated by peptide mapping (Fig. 3).

Calnexin and BiP Form Individual Complexes with Class I Heavy Chains That Coexist within the Cell. Antibodies against calnexin and BiP were used for precipitation from DSP-treated cell extracts and the immunoprecipitates compared to those obtained with anti-class I antibodies. Antibodies against calnexin and class I molecules gave cross-linked products of 160 kD similar in size (Fig. 4 a) further confirming the presence of calnexin in the 160-kD band precipitated with anti-class I antibody. The antibody against BiP, however, did not precipitate the cross-linked species (not shown), suggesting that the epitope recognized by the anti-grp78/BiP antibody has been lost or is inaccessible in the cross-linked complex.

Reduction of the 160-kD bands obtained with either anti-calnexin or anti-class I antibodies gave both the EndoH-sensitive class I heavy chain band and the 94 kD/calnexin band. The BiP band was seen only in the 160-kD cross-linked species immunoprecipitated with antibodies against class I HLA molecules, but not in complexes isolated with the anti-calnexin monoclonal antibody. This suggests that the 160-kD band detected with antibodies against class I HLA molecules represents two individual complexes consisting of either calnexin and class I heavy chains or BiP and class I heavy chains. Ternary complexes consisting of calnexin, BiP, and class I heavy chains with expected molecular mass greater than 160 kD were not detected in our system.

Differences in the Cross-linked Species Formed by Human and Mouse Class I Heavy Chains. Differences in the early matu-

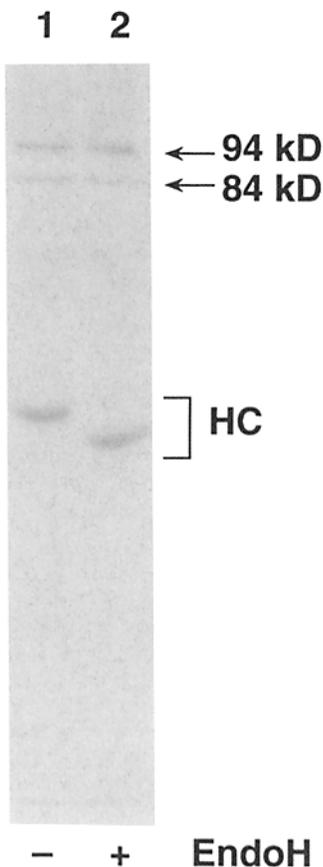


Figure 2. Component proteins of the 160-kD cross-linked species. 10% reducing SDS-PAGE showing the component proteins of the 160-kD cross-linked complex isolated from B7C1R cells. Cells were radiolabeled for 20 h. Digestion with EndoH is indicated (+, -).

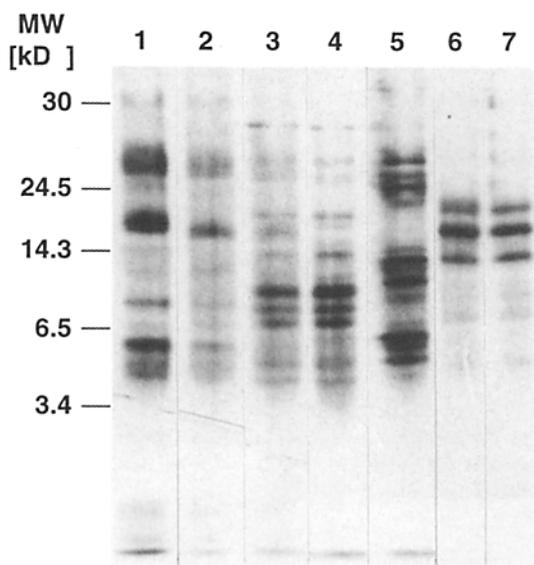


Figure 3. One-dimensional peptide maps obtained by digestion with V8 proteinase. Peptide patterns for the following proteins are shown: calnexin (lane 1), the 94 kD (lane 2), and 84 kD (lane 3) proteins that are the component proteins of the 160-kD complex precipitated with antibody Q1/28, grp78/BiP (lane 4), and grp94 (lane 5). Two species of HLA-B*0702 heavy chains distinguished under nonreducing conditions were analyzed; the band showing slower electrophoretic mobility (lane 6) and the band with faster electrophoretic mobility (lane 7). Peptide patterns for both HLA class I heavy chain species are identical. Unrelated proteins (calnexin, BiP, grp94, HLA class I heavy chains) show distinctively different digestion patterns attesting to the validity of the assay in assessing protein relationships. Enhanced gels were exposed for 5 wk.

ration events of human and mouse class I heavy chains have been found. First, calnexin is found associated with free H-2 heavy chains and with heavy chains bound to β_2m in mouse (17). In contrast, in human cells calnexin (94 kD) is found associated only with free class I HLA heavy chains. Second,

association of BiP with class I was found in human cells but not in mouse cells (17, 18, and Fig. 5 *b*, lanes 1-4). To investigate further these differences we analyzed human C1R cells that had been transfected with the mouse class I heavy chain gene H-2K^b. In these cells, the H-2K^b heavy chain is forced to assemble with human β_2m and interact with human chaperones.

Immunoprecipitation with antibodies specific either for conformational epitopes of the H-2K^b molecule, for free H-2K^b heavy chains, or for human β_2m , all gave a cross-linked species of 160 kD (Fig. 5 *a*). Reduction of the complex revealed bands corresponding to H-2K^b heavy chain and to calnexin, but not to BiP (Fig. 5 *b*). In the environment of the human cell, the H-2K^b heavy chains behave similarly to when they are in mouse cells in that they bind simultaneously to calnexin and β_2m while showing no affinity for BiP (Fig. 5 *b*). This experiment, therefore, suggests that the capacity of β_2m and calnexin to associate simultaneously with class I heavy chains and of BiP to bind class I heavy chains are a function of differences in human and mouse class I heavy chain sequences and not of the species from which either β_2m or the chaperones are derived.

β_2m Binding Can Induce Dissociation of Calnexin and BiP from the Class I Heavy Chains. The binding of calnexin and BiP to class I HLA heavy chains appears to take place before association with β_2m . Indeed, β_2m binding and association with calnexin and BiP seem to represent mutually exclusive interactions. In the context of this hypothesis it was of interest to examine Daudi, a human cell line devoid of β_2m in which HLA class I heavy chains are retained intracellularly (12). Cross-linking experiments showed presence of the 160-kD cross-linked species and results from pulse/chase experiments suggested that in the absence of β_2m the interactions of class I heavy chains with either BiP or calnexin were stable (18).

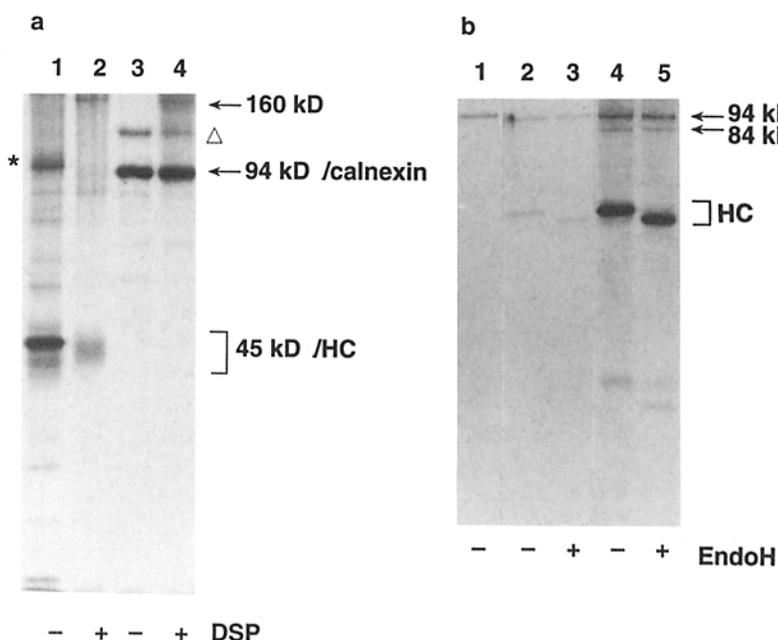


Figure 4. 94 kD/calnexin and 84 kD/BiP are not found in the same cross-linked complex. (a) 10% nonreducing SDS-PAGE showing precipitation from long-term labeled B7C1R using antibody Q1/28 (lanes 1 and 2) specific for free class I heavy chains and antibody AF8 (lanes 3 and 4) recognizing calnexin. Cross-linking was performed as indicated (+/-). Arrows indicate the 160-kD cross-linked complexes and 94 kD/calnexin. HLA-class I heavy chain dimers are indicated by an asterisk (*). The precise nature of the 110 kD band (Δ) is unknown. Upon reanalysis of the excised band only a band of 90 kD corresponding to calnexin was observed (not shown). Therefore, the 110-kD species might represent calnexin dimers or calnexin associated with other, yet unlabeled, proteins. (b) 160-kD cross-linked complexes were eluted from non-reducing gels (shown in *a*, lanes 2 and 4), reduced and component proteins separated on 12.5% SDS-PAGE. (-) and (+) indicate whether EndoH digestion was performed. Lane 1, calnexin precipitated with the anti-calnexin antibody AF8. Lanes 2 and 3, component proteins of the 160-kD cross-linked species precipitated using anti-calnexin antibody; lanes 4 and 5: component proteins of the 160-kD complexes precipitated using antibody Q1/28.

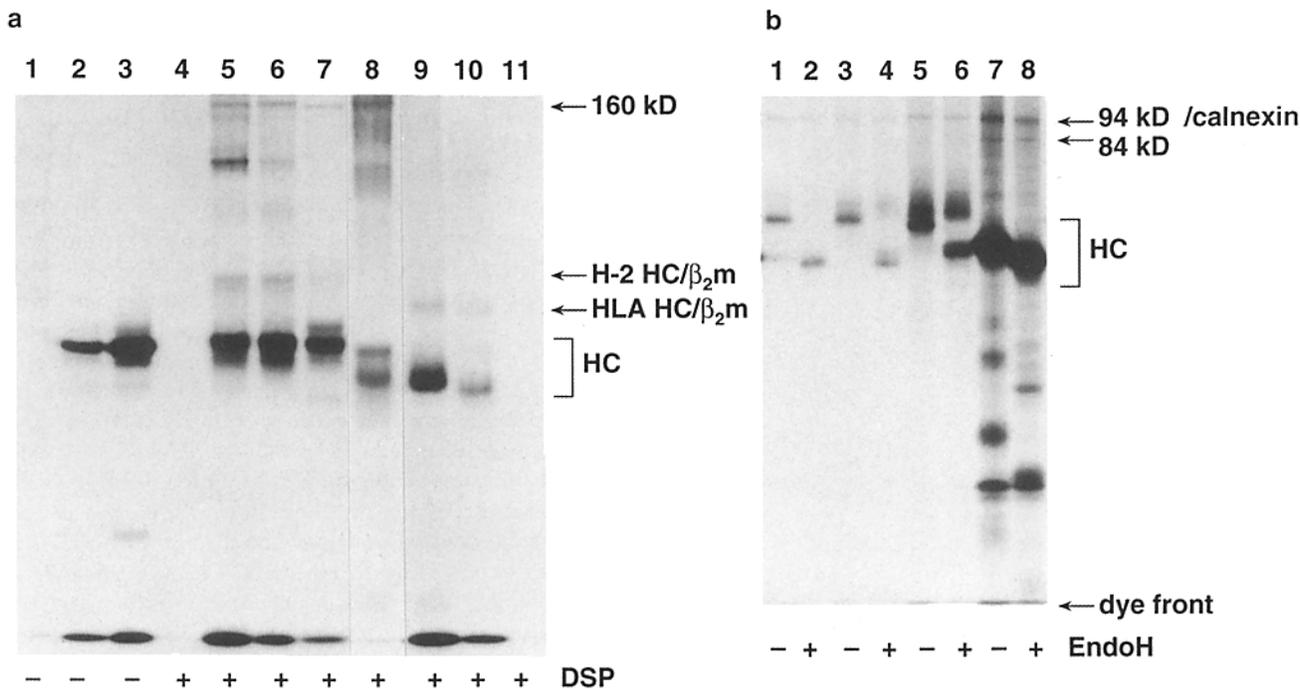


Figure 5. Comparison of the chaperones associated with mouse and human class I MHC proteins. (a) 10% nonreducing SDS-PAGE. Human C1R cells transfected with either mouse H-2K^b (lanes 1–6) or human HLA-B*0702 (lanes 8–11) and mouse cell EL4 (lane 7) were labeled for 15 min and lysed in the absence (–, lanes 1–3) or presence (+, lanes 4–11) of DSP. Exposure time was overnight, except for lane 8 which was exposed for 6 h. Antibodies used for immunoprecipitation were: anti-human β_2m antibody, L368 (lanes 2, 5, and 10); anti-H-2 K^b antibody, 20-8-4s (lanes 3, 6, and 7); anti-human free class I HLA heavy chain antibody, Q1/28 (lane 8); anti-human heavy chain/ β_2m antibody, W6/32 (lane 9); nonimmune serum (lanes 1, 4, and 11). Class I heavy chains (HC), cross-linked class I heavy chain/ β_2m complexes (H-2 HC/ β_2m and HLA HC/ β_2m), and 160-kD cross-linked complexes are indicated. Mouse and human cross-linked complexes have very similar electrophoretic mobilities in the gel systems employed thus they are both referred to as 160-kD complexes. H-2 class I heavy chains and their β_2m complexes exhibit slower electrophoretic mobility than their human counterparts due to more extensive glycosylation. (b) 12.5% reducing SDS-PAGE showing component proteins of different 160-kD complexes. EndoH digestion was performed on half of each samples (+). 160-kD complexes were isolated from: mouse cell lines, MDAY.D2 (lanes 1 and 2) and EL4 (lanes 3 and 4), human cell line C1R transfected with mouse class I allele H-2 K^b (lanes 5 and 6) or human class I allele HLA-B7 (lanes 7 and 8) are shown. Exposure time of enhanced gel was 1 wk. Mouse calnexin has a slightly faster electrophoretic mobility than human calnexin. A band with slightly slower electrophoretic mobility than EndoH resistant K^b heavy chains (lanes 3–6) was not detected consistently in different experiments and most likely is some experimental artifact.

To test the hypothesis that β_2m binding and chaperone association are mutually exclusive interactions, the effect of adding β_2m to Daudi cell extracts before cross-linking was examined. β_2m added in this way was able to associate with class I heavy chains as shown by precipitation with antibodies specific for the heavy chain/ β_2m complex (W6/32; Fig. 6 a). The efficiency to form heterodimers varied among the four allelic products (Fig. 6 a) and overall, it was only a portion of the class I heavy chains that was converted into heterodimers even after addition of the highest concentration of β_2m . The remaining free class I heavy chains were detectable by antibody Q1/28 (not shown). In cross-linked samples, addition of exogenous β_2m decreased the amount of the 160-kD species in a dose-dependent manner (Fig. 6, b and c). The decrease in the amount of the 160-kD species was paralleled by an increase in a 55-kD species consisting of heavy chains and β_2m . This inverse relationship suggests that engagement of β_2m with the heavy chains in vivo leads to dissociation of the 160-kD class I/chaperone complexes. The reactivity of Q1/28 with heterodimers in this experiment that is not observed in situations where heterodimers form natu-

rally, is possibly due to conformational differences between heterodimers forced to form in detergent lysates from free heavy chains and exogenous β_2m and those formed naturally.

Assembly of Class I HLA Molecules Is Slower in C1R Transfectants than in Normal B-LCL. We found that the amount of the 160-kD cross-linked species detected in Daudi cells and C1R cells transfected with HLA-B genes was considerably greater than that in normal B cell lines. In Daudi, this is probably due to lack of β_2m expression. In contrast, C1R cells that express normal level of β_2m have a different defect. C1R was derived from a normal EBV cell line by successive rounds of mutagenesis and selection with anti-HLA-A,B,C antibodies: one HLA-A,B,C haplotype is deleted; for the other haplotype HLA-A is not expressed, HLA-B expression is highly reduced due to a mutation in the initiation codon, and the expression of HLA-C is normal (34). Despite the mutations, transfection of exogenous class I genes into C1R yields levels of surface expression comparable to those found on normal EBV transformed cell lines (not shown). It is possible, however, that the mutation in C1R

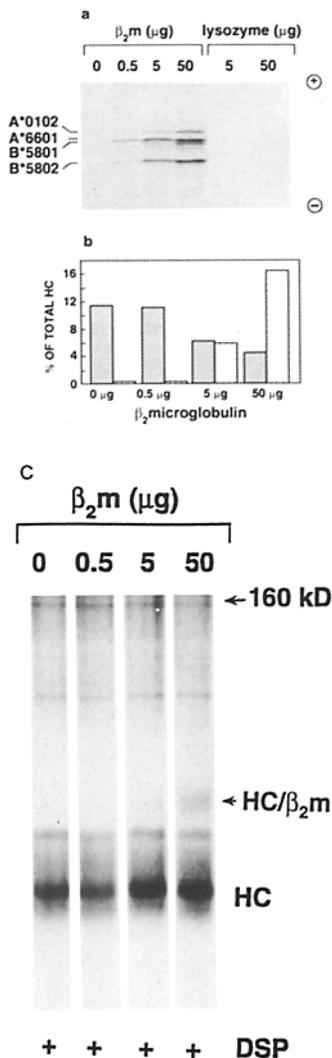


Figure 6. β_2 m binding can induce dissociation of the 160-kD complex in Daudi cells. (a) Radiolabeled Daudi cells were lysed in the presence of exogenously added human β_2 m or chicken egg lysozyme. HLA class I heterodimers were precipitated with antibody W6/32 and analyzed by IEF. Positions for HLA-A*0102, A*6601, B*5801, and B*5802 are indicated. (b) Quantitation of autoradiogram (shown in c). The amount of total class I heavy chains in each lane was calculated as: 45 kD HC plus 55 kD HC/ β_2 m plus 160 kD HC/protein complexes. Radioactivity present in the 160-kD band (dark bars) or 55-kD band (crosshatched bars) is expressed as percentage of the calculated total HC of the same lane. (c) Radiolabeled Daudi cells were lysed and cross-linked in presence of increasing amounts of β_2 m. Proteins were precipitated using antibody Q1/28 and analyzed on 10% nonreducing SDS-PAGE. For quantitation of radioactivity, gels were dried without enhancement and exposed to a PhosphorImage plate (Molecular Dynamics, Sunnyvale, CA). The amount of radioactivity in a protein band was determined and a background value, obtained from an area of the gel not containing a band, was subtracted.

affects the assembly and maturation of class I molecules in a more subtle way that facilitates detection of the cross-linked 160-kD species.

To investigate this possibility, the assembly of HLA-B*0702 and B*2705 molecules in C1R transfectants and normal EBV

transformed cell lines was compared. Significant differences were observed (Fig. 7). The rates of formation of heavy chain/ β_2 m dimers and acquisition of EndoH resistance were more rapid in B cell lines than C1R transfectants. The overall kinetics of class I assembly and transport from the ER through Golgi to the plasma membrane were slower in the C1R transfectants, resulting in a larger pool of free class I heavy chains within the ER. These free heavy chains are probably associated with calnexin and BiP and it is their abundance that probably facilitates detection of the 160-kD complexes.

Discussion

Our experiments identify complexes formed between class I HLA heavy chains and either calnexin or BiP. These complexes have been isolated by immunoprecipitation from detergent lysates and analyzed by SDS-PAGE. Their detection requires chemical cross-linking simultaneous with detergent solubilization, suggesting that the complexes are dissociated by the immunoprecipitation procedure.

The finding of an association between calnexin and class I confirms the results obtained by the groups of Williams (17–19) and Brenner (22, 23). Our analyses, however, reveal differences in the calnexin association of human and mouse class I heavy chains. First, calnexin association is only observed for free HLA class I heavy chains and not for heterodimers of class I heavy chains and β_2 m. Moreover, addition of exogenous β_2 m induces dissociation of the cross-linked class I/protein complexes indicating that simultaneous association of HLA class I heavy chains with calnexin and β_2 m is not a stable situation.

A second difference is that BiP is found associated with human but not with mouse class I heavy chains. These species-specific differences in chaperone association appear to be a function of amino acid differences in the protein sequences of HLA and H-2 heavy chains. Mouse H-2 heavy chains expressed in human cells retain their mouse pattern of behavior, in that they associate with calnexin either as free heavy chains or associated with β_2 m but do not associate with BiP.

A third difference we find is that complexes of class I heavy

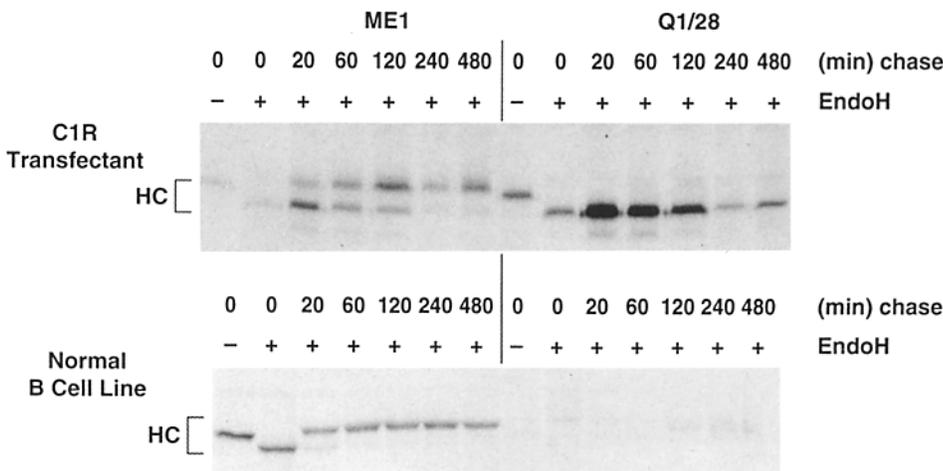


Figure 7. Maturation of HLA-B7 expressed in transfected C1R cells and normal B cell line. C1R cells transfected with B*0702 (top panel) and JY, a normal EBV-infected B cell line that is homozygous for B*0702 (bottom panel) were pulse-labeled for 10 min and chased for the times indicated. For immunoprecipitation antibodies ME1, specific for β_2 m-associated HLA-B7, and Q1/28, reactive with HLA-B7 heavy chains, were used. Samples were treated with EndoH as indicated (+).

chains with calnexin and BiP are more difficult to detect in "normal" human cell lines compared with normal mouse cells. Characterization of the human complexes has been facilitated by the use of two mutant cell lines in which the assembly of class I HLA molecules is impaired. In the Daudi cell line the absence of β_2m prevents assembly and egress of the heavy chains from the ER; in C1R cells the kinetics of assembly is slowed for reasons that are unknown. However, a common feature of the phenotype of Daudi and C1R cells is that class I HLA heavy chains remain in the ER for longer times than in normal cells, a property that probably facilitates detection of the complexes with calnexin and BiP. Supporting this view are the results of our studies on normal EBV-transformed B cell lines. The rate at which class I HLA heavy chains of different alleles are assembled and discharged from the ER varies (49, 50) and the 160-kD cross-linked complexes are more easily detected for allelic products that show slower assembly kinetics, B51 and B27 for example, than for those which assemble more rapidly.

From their studies of mouse class I molecules, Degen and Williams found that association of calnexin correlates with EndoH sensitivity and thus transport from the ER to the Golgi (17). In contrast we find that for human class I molecules the dissociation of calnexin and BiP appears more strongly correlated with β_2m association than with the site of intracellular localization.

Since its first definition as an immunoglobulin binding protein (46), BiP association has been described for other proteins and its function as a molecular chaperone established (48). More recent studies (17, 51, 52) provide evidence that another ER protein, calnexin, might also function as a molecular chaperone. We and others found that calnexin and BiP bind to newly synthesized molecules including class I heavy chains. These chaperones may act positively by facilitating correct folding and β_2m association of class I heavy chains or negatively by ensuring ER retention and intracellular degradation of misfolded class I heavy chains. That BiP and calnexin bind independent sets of class I HLA heavy chains suggests they have complementary roles in class I biogenesis, a hypothesis consistent with what is known of calnexin and BiP function in other systems: calnexin preferentially associates with immature, but not aggregated glycoproteins (24, 53),

whereas BiP binds to incompletely folded polypeptides that may be aggregated (24). One possible scheme is that the complexes of class I HLA heavy chains with BiP and calnexin represent two distinct pathways of folding, for example calnexin association may mark the pathway leading to functional class I molecules, whereas BiP association may mark the pathway to intracellular degradation. An alternative scheme is that complexes with BiP and calnexin represent successive intermediates in the same pathway of class I HLA folding. In an attempt to distinguish the two schemes we performed pulse/chase experiments. However, under pulse labeling conditions neither BiP nor calnexin incorporated detectable radioactivity although the 160-kD cross-linked band was detected on account of radioactivity incorporated into the class I HLA heavy chains.

The order in which β_2m and peptide bind to class I heavy chains in vivo is uncertain. Most evidence favors an initial association with β_2m (54) but experiments supporting the binding of peptide first have also been reported (55, 56). The issue is complicated by differences in species, alleles, in vitro systems and the possibility that both pathways may be operative. Of relevance to this debate is the finding of a physical association between class I HLA molecules and the TAP/peptide transporter which resulted in peptide loading (57). Only β_2m -associated heavy chains were found to be associated with the TAP proteins showing that association of heavy chains with β_2m precedes association with peptide.

These findings suggest a scheme for class I HLA biosynthesis (Fig. 8) in which newly synthesized heavy chains associate with either calnexin or BiP. Both chaperone proteins may function similarly to retain class I heavy chains in a conformation compatible for β_2m binding. However, we favor a model in which calnexin and BiP have distinctive roles. Compatible with the features described for BiP association in other systems (24, 48, 58, 59) it seems most likely that misfolded class I heavy chains will form complexes with BiP resulting in their retention and eventual destruction. In contrast, calnexin association may be targeted towards immature class I heavy chains which have conformations compatible with β_2m binding. Association with calnexin may help maintain such conformations until association with β_2m occurs. Binding of β_2m will release calnexin and facilitate associa-

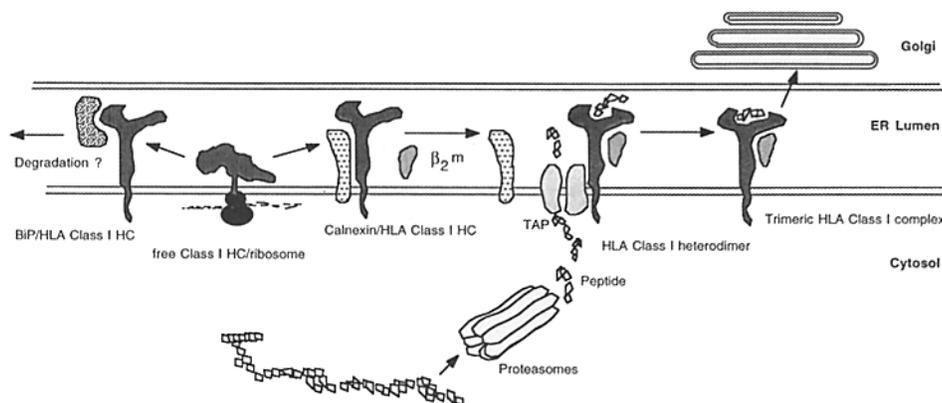


Figure 8. Hypothetical model for the events controlling HLA class I assembly in the ER.

tion with the peptide transporter proteins. After peptide loading class I molecules are released from the ER for transit through the Golgi to the cell surface. In such a model the chaperone proteins, BiP and calnexin, and proteins for the peptide transporters provide "quality control" functions on the assembly of human class I MHC proteins.

Association with β_2m profoundly influences the conformation of class I MHC heavy chains. This stems from the extensive interaction of β_2m with all three extracellular domains (α_1 , α_2 , and α_3) of the class I heavy chain (60, 61). For some time it has been appreciated that the association between class I heavy chains and β_2m in mice is weaker than in humans and in contrast to the human situation association with β_2m is not a necessary requirement for mouse class I heavy chains to reach the cell surface (26, 28). A similar lassitude seems to extend to the association of putative chaperones with the class I heavy chains of the two species.

Whereas calnexin binds to both free and β_2m -associated H-2 heavy chains, its interaction in human cells is restricted to free class I heavy chains. Further adding to the control of human class I MHC assembly is a second putative chaperone, BiP, not detected in the mouse system which is also specifically targeted to free class I heavy chains. For class I MHC molecules, association with β_2m represents a critical point in the biosynthesis and assembly of functional antigen presenting molecules. The impression emerging from this analysis is that intracellular HLA class I heavy chains are either associated with β_2m or with one of two chaperone molecules, BiP and calnexin. Association with BiP and calnexin appears to serve the dual purpose of ensuring that class I heavy chains cannot reach the cell surface in the absence of β_2m and to facilitate that association. All in all, the controls upon class I assembly appear more stringent in humans than in mice.

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