An Essential Function of Tapasin in Quality Control of HLA-G Molecules

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SUMMARY

Tapasin plays an important role in the quality control of MHC class I assembly, but its precise function in this process remains controversial. Whether tapasin participates in the assembly of HLA-G has not been studied. HLA-G, an MHC class Ib molecule that binds a more restricted set of peptides than class Ia molecules, is a particularly interesting molecule because during assembly, it recycles between the ER and the cis-Golgi until it is loaded with a high-affinity peptide. We have taken advantage of this unusual trafficking property of HLA-G and its requirement for high-affinity peptides to demonstrate that a critical function of tapasin is to transform class I molecules into a high-affinity, peptide-receptive form. In the absence of tapasin, HLA-G molecules cannot bind high-affinity peptides, and an abundant supply of peptides cannot overcome the tapasin requirement for high-affinity peptide loading. Addition of tapasin renders HLA-G molecules capable of loading high-affinity peptides and of transporting to the surface, suggesting that tapasin is a prerequisite for the binding of high-affinity ligands. Interestingly, the “tapasin-dependent” HLA-G molecules are not empty in the absence of tapasin but are in fact associated with suboptimal peptides and continue to recycle between the ER and the cis-Golgi. Together with the finding that empty HLA-G heterodimers are strictly retained in the ER and degraded, our data suggest that MHC class I molecules bind any available peptides to avoid ER-mediated degradation and that the peptides are in turn replaced by higher-affinity peptides with the aid of tapasin.
INTRODUCTION

After being targeted to the endoplasmic reticulum (ER), nascent MHC class I heavy chains associate with a multiprotein complex that assists in their assembly with peptides and β2-microglobulin (β2m). This complex includes calnexin, calreticulin, ERp57, transporter associated with antigen processing (TAP), and tapasin (1). MHC class I molecules are divided into two types based on their polymorphism and levels of expression. The highly expressed polymorphic class Ia molecules bind a diverse set of peptides derived from the cytosol, whereas the less abundant, tissue-specific, nonpolymorphic class Ib molecules bind a more restricted set of peptides (2). Tapasin is indispensable for the proper function of the class Ia antigen presentation pathway. In tapasin-mutant mice, the expression and stability of surface class I molecules are strongly reduced. In tapasin double-negative cells, the presentation of cytosolic antigens is markedly impaired (3). Defects in the development of CD8+ T cells and immune responses against some viruses were also noted in tapasin double-negative mice (4). Like class Ia molecules, tapasin plays an important role in the assembly and surface expression of certain class Ib molecules such as HLA-E (5) and H2-M3 (6,7).

Recent studies have implicated that tapasin serves several functions in the assembly of class I-peptide complexes. It functions to bridge the heavy chain complexes to TAP (8). Tapasin expression also enhances the stability of TAP heterodimers, increasing overall peptide transport into the ER (9,10). In insect cells, tapasin retains empty K^b molecules in the ER (11). Likewise, in tapasin-deficient human 721.220 cells, tapasin prevents premature release of K^b molecules from the ER, suggesting that tapasin might participate in retaining class I molecules in the ER until an optimal peptide is
loaded (12). Finally, other recent studies have suggested that tapasin might be involved in the peptide editing of class I molecules (13-15). Although there is little doubt that tapasin is a class I-dedicated chaperone, the actual mechanism for each proposed function of tapasin during its interactions with class I alleles remains to be elucidated. Tapasin dependency could reflect a direct role for tapasin in ER retention of heavy chains, TAP stabilization, peptide editing, or any combination of these functions. Determining which of these interrelated functions are primary rather than secondary manifestations of tapasin’s interactions with class I molecules has proven to be difficult. In addition to the uncertainty regarding the precise functions of tapasin, various class Ia molecules differ in their dependency on tapasin for both efficient surface expression and presentation of antigenic determinants to CTL, as observed in studies of 721.220 transfectants (13,16-18). In 721.220 cells, tapasin is not required for high levels of surface expression of the HLA-B2705 allele or for presentation of viral determinants to CTL (16). In contrast, for the HLA-B4402 allele, functional antigen presentation and surface expression are highly dependent on tapasin; HLA-B0801 falls between the B2705 and B4402 alleles in the spectrum of tapasin dependence (16). These relationships reflect the complexity of tapasin function. In fact, the aforementioned studies regarding the function of tapasin took place before the concept was established that the tapasin dependence of class I molecules is allele-specific. Thus, the conclusions of these studies might be biased, depending on which class I alleles were analyzed.

HLA-G, an MHC class Ib molecule, is expressed primarily in trophoblast cells and has limited polymorphism (2). Due to a scarcity of natural endogenous peptide ligands in most cells, the supply of peptides is the rate-limiting factor for the intracellular transport kinetics of HLA-G (19). Recent evidence shows that HLA-G
protects trophoblast cells from recognition by NK cells (20,21); expression of HLA-G on melanoma cells protects them from lysis by NK cells (22). HLA-G is a particularly interesting molecule in protein trafficking because it recycles between the ER and the cis-Golgi until it is loaded with a high-affinity peptide (19). This feature makes it possible to estimate whether HLA-G molecules are loaded with high-affinity or low-affinity peptides. Whether the process involved in the assembly of MHC class Ia–peptide complexes applies to the assembly of HLA-G-peptide complexes has not been studied. Because HLA-G binds a restricted set of peptides (23,24) and recycles between the ER and cis-Golgi, it is an attractive model for investigating the roles of individual components of the ER peptide-loading complex in the assembly of functional MHC class I molecules.

In this study, we examined the tapasin dependence of HLA-G in terms of tapasin’s critical role in the assembly and intracellular transport of HLA-G. Our findings demonstrate that in the absence of tapasin, HLA-G molecules are not able to bind high-affinity peptides despite an abundant supply of peptides, suggesting that tapasin is a prerequisite for the binding of high-affinity peptides. Interestingly, immediately after synthesis, these tapasin-dependent HLA-G molecules associate constitutively with low-affinity endogenous peptides and exit the ER, thereby avoiding ER-mediated degradation. We propose that a critical function of tapasin is to transform the peptide-binding groove of HLA-G into a high-affinity, peptide-receptive form, which could promote the replacement of low-affinity peptides with high-affinity peptides.
EXPERIMENTAL PROCEDURES

DNA Constructs— All HLA cDNAs and their mutagenized derivatives were inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, Calif.). The cDNA encoding human tapasin was kindly provided by Dr. Cresswell (Yale University, New Haven, Conn.) and was subcloned into the pcDNA3.1/Hygro vector (Invitrogen). The cDNA encoding human β2m was contained in the pcDNA3.1/Neomycin vector (Invitrogen). Point mutations of HLA-G were made by changing the codons by polymerase chain reaction (PCR) with Pfu DNA polymerase (Stratagene, San Diego, Calif.). The sequences for all mutations were confirmed by sequencing.

Stable Cell Lines and Antibodies— NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), penicillin (50 U/ml), and streptomycin (50 µg/ml). NIH3T3 cells were transfected with either human β2m cDNA or human β2m and human tapasin cDNAs. Stable transfectants expressing human β2m (NIH3T3.hβ2m) were selected with 1 mg/ml G418 (Sigma-Aldrich, St. Louis, Mo.). Stable transfectants expressing both human β2m and human tapasin (NIH3T3.hβ2m.hTpn) were selected with 1 mg/ml G418 and 0.35 mg/ml hygromycin (Life Technologies). Tapasin expression was restored in 721.220 cells by transfection with the cDNA encoding human tapasin, and transfectants were selected with 0.35 mg/ml hygromycin, giving rise to the 721.220.Tpn cell line. The 721.220 and 721.220.Tpn cells were cultured in RPMI 1640 medium (Life Technologies) containing
10 % FBS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). The
HLA-G-specific monoclonal antibody (mAb) G233 was a gift from Dr. Loke
(University of Cambridge, UK). The mAb W6/32 recognizes only MHC class I heavy
chains associated with β2m. Polyclonal rabbit K455 antibody reacts with MHC class I
heavy chains and β2m in both assembled and nonassembled forms. The rabbit
polyclonal antibody to human tapasin (R.gp48N) was a gift from Dr. Cresswell. The
rabbit polyclonal antibody to PDI (SPA-890) and the anti-β-COP mAb M3A5 were
purchased from Stressgen (Victoria, British Columbia, Canada) and Sigma-Aldrich,
respectively. The mAb to the cis-Golgi marker GM130 was purchased from BD
Transduction Laboratories (Franklin Lakes, NJ.). Fluorescein isothiocyanate (FITC)-
conjugated goat anti-mouse IgG and Texas-Red-conjugated goat anti-mouse IgG were
purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa.).
Horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Pierce
(Rockford, Ill.).

**Pulse-chase Labeling and Immunoprecipitation**—Cells (5 × 10^6) were
transfected by electroporation, starved for 40 min in medium lacking methionine,
labeled for 20 min with 0.1 mCi/ml [35S]methionine (TranS-label; NEN Life Science,
Boston, Mass.), and chased for the indicated times in normal medium. Cells were lysed
by using 1% NP-40 (Sigma-Aldrich) in phosphate-buffered saline (PBS) with protease
inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C. After preclearing lysates with
protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ.), primary
antibodies and protein G-Sepharose were added to the supernatant and incubated at 4°C
with rotation for 2 h. The beads were washed three times with 0.1% NP-40 in PBS. Proteins were eluted from the beads by boiling in SDS sample buffer and separated by 12% SDS polyacrylamide gel electrophoresis (PAGE). The gels were dried, exposed to BAS film for 14 h, and analyzed with the Phosphor Imaging System BAS-2500 (Fugifilm, Tokyo, Japan). For endoglycosidase-H (endo-H) treatment, immunoprecipitates were digested with 3 mU endo H (Roche, Indianapolis, Ind.) at 37°C overnight in 50 mM sodium acetate (pH 5.6), 0.3% SDS, and 150 mM β-mercaptoethanol (Sigma-Aldrich).

**Flow Cytometry and Immunofluorescence Microscopy**—The surface expression of HLA-G molecules was determined by flow cytometry (FACScalibur, Becton Dickinson Biosciences, Mountain View, Calif.). Cells (1 × 10⁶) were washed twice with cold PBS containing 1% bovine serum albumin (BSA) and incubated for 1 h at 4°C with a saturating concentration of mAb G233. Normal mouse IgG was used as a negative control for each test. The cells were washed twice with cold PBS containing 1% BSA and then stained with FITC-conjugated goat anti-mouse IgG for 30 min. A total of 10,000 gated events were collected by the FACScalibur cytometer and analyzed with CellQuest software (Becton Dickinson Biosciences). For immunofluorescence staining of permeabilized cells, NIH3T3 cells were fixed in 3.7% formaldehyde, made permeable with 0.1% Triton X-100, and incubated with the appropriate primary antibody for 1 h. MRC-1024 Confocal Microscopy was used for confocal imaging (Bio-Rad, Hercules, Calif.).

**Coimmunoprecipitation and Western Blot Analysis**—Cells were lysed in
1% digitonin in digitonin buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM CaCl$_2$, and 5 mM MgCl$_2$ (pH 7.6) supplemented with protease inhibitors. Lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. For immuno precipitation, samples were incubated with the appropriate antibodies for 2 h at 4°C before protein G-Sepharose beads were added. Beads were washed four times with 0.1% digitonin, and bound proteins were eluted by boiling in SDS sample buffer.

Proteins were separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, blocked with 5% skim milk in PBS with 0.1% Tween-20 for 2 h, and probed with the appropriate antibodies for 4 h. Membranes were washed three times in PBS with 0.1% Tween-20 and incubated with HRP-conjugated streptavidin (Pierce) for 1 h. The immunoblots were visualized with ECL detection reagent (Pierce).

**Microsomes and Peptide Loading Assays**—Microsomes from 721.220 and 721.220.Tpn cells expressing HLA-G or HLA-G/E114H heavy chains were prepared and purified as previously described (25). Biotinylated peptide KIPAQFYIL was conjugated to the photoreactive crosslinker N-5-azido-2-nitrobenzoyloxy succinimide (ANB-NOS; Pierce) as described (9). For the peptide-loading assay, reporter peptides, with or without various concentrations of the unlabeled peptide (KIPAQFYIL), were mixed with 15 µl of microsomes (concentration of 60 A$_{280}$/ml) in a total volume of 50 µl RM buffer (250 mM sucrose, 50 mM triethanolamine-HCl, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM DTT, and 10 mM ATP). The mixture was incubated for 30 min at 26°C in a flat-bottomed 96-well tissue culture plate. The samples were maintained on ice during a 3-min exposure to shortwave (365 nm) ultraviolet irradiation. After centrifugation, the membranes were washed once with cold RM buffer and lysed.
with 1% digitonin, and the cross-linked proteins were immunoprecipitated with mAb G233. The precipitates were separated by 12% SDS-PAGE and transferred to an immobilon-P membrane (Millipore, Bedford, Mass.). The membrane was incubated with HRP-conjugated streptavidin for 1 h, and biotinylated proteins were visualized by using ECL Western blotting reagent (Pierce). Peptide translocation was determined after incubating microsomes with biotin-conjugated reporter peptides in the absence of competitor for 30 min at 26°C with or without 1 mM ATP. Microsomal membranes were recovered by centrifugation at 75,000 × g for 10 min through a 0.5 M sucrose cushion in cold RM buffer. After washing with cold RM buffer twice, the membrane pellet was directly dissolved in sample buffer. The samples were analyzed on Tricine/SDS-PAGE, appropriate for resolution of low mass polypeptides as described (26), and probed with HRP-conjugated streptavidin. The relative densities of the peptide bands were determined by use of an imaging densitometer (GS-700, Bio-Rad, Richmond, CA) and MultiAnalyst densitometer software (Bio-Rad).

RESULTS

HLA-G is highly dependent on tapasin for cell surface expression and intracellular maturation—To determine the function of tapasin for the assembly of HLA-G-peptide complexes, we first examined the tapasin dependence of HLA-G for cell surface expression. The genes encoding HLA-G heavy chains were independently transfected into both 721.220 and 721.220.Tpn cell lines. In the absence of tapasin, low
levels of surface expression were observed (Fig. 1A). Significantly, upon expression of tapasin, surface expression increased more than 5-fold, indicating the dependence of HLA-G on tapasin for its surface expression. In recent efforts to identify the factors that determine the relative tapasin dependence of various class Ia alleles, we have found that the nature of the amino acid residues present at the naturally polymorphic position 114 determines tapasin dependence (unpublished data). To test whether this is also the case for the HLA-G molecules, we constructed substitution mutants and examined their dependence on tapasin for surface expression (Fig. 1A). Surprisingly, a glutamic acid to histidine substitution at position 114 (HLA-G/E114H) allows the otherwise tapasin-dependent HLA-G to have levels of surface expression comparable to the levels seen in the presence of tapasin. The HLA-G/E114Q mutant, in which residue 114 was replaced by the neutrally charged glutamine, fell between HLA-G wild type and HLA-G/E114H in the spectrum of tapasin dependence. These results indicate that like HLA class Ia molecules, the tapasin dependency of HLA-G is also influenced by the nature of the amino acid at position 114. Since it is unlikely that the point mutation causes a gross conformational change in class I molecules, we used the HLA-G/E114H mutant in parallel with wild-type HLA-G as a control for the tapasin-independent phenotype.

To investigate the mechanism by which tapasin affects surface expression of HLA-G, we compared the intracellular maturation and transport of HLA-G in the 721.220 and 721.220.Tpn cells. In the absence of tapasin, 50% of HLA-G heavy chains remained sensitive to endo-H digestion, even after 8 h (Fig. 1B), reflecting retention of these molecules in the ER. Conversely, in the presence of tapasin, most HLA-G molecules had become endo-H resistant by this time (Fig. 1B). These findings suggest that the impaired intracellular transport of HLA-G molecules in the absence of tapasin
accounts for their low levels of surface expression. Pulse-chase experiments from
tapasin-negative and -positive cells revealed comparable acquisition of endo-H
resistance by HLA-G/E114H (Fig. 1C), indicating its tapasin independence for normal
intracellular transport.

We have shown that the availability of high-affinity peptides dictates the
transport kinetics of HLA-G (19). To determine whether the inefficient transport of
HLA-G in the absence of tapasin can be overcome by supplying high-affinity peptides,
we examined the intracellular trafficking of HLA-G and HLA-G/E114H upon
expression of high-affinity peptides by the minigene expression system. The minigene
encoding MIPAQFYIL, a high-affinity peptide ligand for HLA-G (23), was co-
expressed with the gene encoding either HLA-G or HLA-G/E114H in 721.220 and
721.220.Tpn cells. In the presence of tapasin, no discernible difference in the transport
rate between HLA-G and HLA-G/E114H was seen upon expression of high-affinity
peptides (Fig. 1D), but both HLA-G and HLA-G/E114H molecules were transported
with much faster kinetics when compared with the kinetics of molecules without the
supply of high-affinity peptides (Fig. 1B and C, right). In the absence of tapasin, the
supply of high-affinity peptides increased the transport kinetics of HLA-G/E114H
(compare Fig. 1C, left and Fig. 1E, right), whereas this supply did not influence the
transport kinetics of HLA-G (compare Fig. 1B, right and Fig. 1E, left). These results
indicate that for efficient intracellular transport of HLA-G molecules, an abundance of
high-affinity peptides cannot compensate for the lack of tapasin.

Tapasin is a prerequisite for loading high-affinity peptides— In the absence of
tapasin, the impaired intracellular transport and the low surface expression of HLA-G
might be due to its inability to load high-affinity peptides. To test this possibility, we examined peptide loading into HLA-G or HLA-G/E114H molecules as a function of tapasin by using the reporter peptide, KIPAQFYIL, which is known to be high-affinity ligand for HLA-G (23), in the presence of the competitors at different concentrations.

We were surprised at the marked differences in the ability of HLA-G and HLA-G/E114H to load peptides in the absence of tapasin. In the case of no competitor added, the level of binding of the reporter peptide by HLA-G was only 25 % of the binding observed in the HLA-G/E114H substitution mutant (Fig. 2A). Furthermore, the reporter peptides loaded into HLA-G were completely outcompeted by a lower concentration (1.6 µM) of unlabeled peptide, whereas as much as 6.4 µM of unlabeled peptide was not sufficient for completely outcompeting reporter peptide binding to HLA-G/E114H. However, in the presence of tapasin, no discernible difference in the peptide binding ability of HLA-G and HLA-G/E114H was seen (Fig. 2B). These results indicate that HLA-G molecules are highly dependent on tapasin for efficient loading with high-affinity peptides, but the substitution of glutamic acid to histidine at position 114 renders the molecules independent of tapasin for high-affinity peptide loading.

Accordingly, the impaired intracellular transport and reduced surface expression of HLA-G in the absence of tapasin are likely the result of the inability of HLA-G for high-affinity peptide loading.

To test whether the differential peptide loading observed in the absence of tapasin might be due to differences in the luminal availability of the peptide, we quantitated the amount of reporter peptides that were translocated into the ER lumen. Comparable amounts of peptides were recovered between HLA-G and HLA-G/E114H (Fig. 2C). Therefore, we exclude the notion that luminal availability of the peptide plays an
important role in dictating class I loading in tapasin-deficient cells. In control experiments in which ATP was omitted, little peptide was recovered. Since peptide translocation via TAP requires ATP (27), we conclude that the modified reporter peptides entered the ER lumen in a TAP-dependent manner.

Without tapasin, HLA-G recycles between the ER and Golgi despite an abundant supply of high-affinity peptides—HLA-G molecules that are loaded with peptides of suboptimal affinity are retrieved back to the ER. Loading of HLA-G with high-affinity peptides abrogates this retrieval and allows HLA-G–peptide complexes to transport forward to the cell surface (19). To examine whether tapasin affects the recycling behavior of HLA-G as a function of the availability of high-affinity peptides, we stably expressed minigenes encoding MIPAQFYIL in NIH3T3.hβ2m cells and NIH3T3.hβ2m.hTpn cells and then treated cells with nocodazole. Nocodazole disrupts microtubules, leading to disintegration of the Golgi and interruption of traffic between the Golgi and the ER (28). On the basis of cell size and ease of manipulation, we used NIH3T3 cells for the immunofluorescence and confocal microscopy experiments throughout the study. In the absence of high-affinity peptides, in nocodazole-treated cells, both HLA-G and HLA-G/E114H exhibited the punctate staining pattern around the perinuclear region regardless of tapasin (Fig. 3A and B), an indication of recycling. In the presence of high-affinity peptides and tapasin, the staining pattern for HLA-G and HLA-G/E114H remained unchanged after nocodazole treatment (Fig. 3D), indicating that these molecules were not recycled back to the ER. In the presence of high-affinity peptides but in the absence of tapasin, the distribution of wild-type HLA-G changed to a punctate perinuclear pattern after nocodazole treatment but the staining pattern for
HLA-G/E114H remained unchanged after treatment (Fig. 3C). These results suggest that in the absence of tapasin, HLA-G cannot be transported to the cell surface, despite an abundant supply of high-affinity peptides. Somehow, HLA-G exits the ER but instead of transport to the cell surface, HLA-G is retrieved back to the ER.

To further dissect the fate of HLA-G on the secretory pathway in the absence of tapasin, we examined the intracellular localization of HLA-G and HLA-G/E114H molecules by immunofluorescence staining and confocal microscopy. In the absence of tapasin and high-affinity peptides, both HLA-G and HLA-G/E114H molecules colocalize with GM130 (Fig. 4A), which is a cis-Golgi marker (29). Upon supply of high-affinity peptides, most HLA-G molecules are still found in the cis-Golgi, whereas the predominant distribution of HLA-G/E114H is shown by the pattern of ER and surface staining (Fig. 4B). These results indicate that in the absence of tapasin, HLA-G molecules are not able to transport beyond medial-Golgi despite availability of high-affinity peptides. In the presence of tapasin but absence of high-affinity peptides, the surface staining of HLA-G molecules is clearly observed (Fig. 4C, top panel). Upon expression of high-affinity peptides, the surface staining patterns of HLA-G and HLA-G/E114H are much stronger than the patterns without high-affinity peptides (Fig. 4D), suggesting that in the presence of tapasin, availability of high-affinity peptides can be a limiting factor in determining the surface level of HLA-G molecules. Taken together, these results suggest that in the absence of tapasin, HLA-G molecules are incapable of loading high-affinity peptides, and, subsequently, they are retrieved from the cis-Golgi to the ER. In contrast, HLA-G/E114H molecules can load high-affinity peptides in the absence of tapasin and are directly transported to the cell surface without recycling.

To understand the biochemical basis for the retrieval phenomenon of HLA-G in the
absence of tapasin, we analyzed by coimmunoprecipitation experiments the interaction of HLA-G or HLA-G/E114H with \( \beta \)-COP, a subunit of coatamer, as functions of tapasin and availability of high-affinity peptides. Proteins can be retrieved to the ER by retrograde transport from the Golgi complex by COPI-coated vesicles (30). The \( \beta \)-COP association of HLA-G and HLA-G/E114H with high-affinity peptides was weaker than the \( \beta \)-COP association of these molecules without high-affinity peptides (Fig. 5). In the absence of tapasin and high-affinity peptides, immunoprecipitation of HLA-G coprecipitated a substantial amount of \( \beta \)-COP (Fig. 5A, lane 3), whereas much less \( \beta \)-COP coprecipitated with HLA-G/E114H (Fig. 5A, lane 4). This differential binding between HLA-G or HLA-G/E114H and \( \beta \)-COP was more evident in cells expressing high-affinity peptides but no tapasin (Fig. 5B, lanes 3 and 4). In the presence of both high-affinity peptides and tapasin, a negligible amount of \( \beta \)-COP was coimmunoprecipitated for both HLA-G and HLA-G/E114H (Fig. 5B, lanes 5 and 6). Taken together, these results indicate that tapasin is essential for HLA-G molecules to load high-affinity peptides, escape retrieval, and transport forward to the cell surface.

**Tapasin-dependent HLA-G molecules are not empty in the absence of tapasin**—

In wild-type cells, only peptide-filled and fully conformed classical MHC class I heterodimers transit from the ER to the cell surface (31-33). Given the well-established concept that tapasin plays a critical role in the proper assembly of class I molecules in the ER, we were surprised at the finding that the so-called tapasin-dependent HLA-G molecules were able to exit the ER in the absence of tapasin. It appeared important, therefore, to determine whether the HLA-G molecules that were in transit to the cis-Golgi in the absence of tapasin were actually filled with peptides. Empty MHC class I
molecules are unstable and undergo an irreversible conformational change at 37°C due to dissociation of the heavy chains from β2m. Such unfolded class I molecules cannot be recognized by the conformation-dependent mAb W6/32. Hence, we performed temperature stability assays on cell lysates prepared in pulse-chase experiments. No class I heterodimers for both HLA-G and HLA-G/E114H could be immunoprecipitated from cells expressing ICP47, a herpes simplex virus protein that stops peptide translocation by the TAP heterodimer (34), after the lysates were incubated at 37°C for 1 h at both chase time points (Fig. 6A and B, lanes 9–16). Surprisingly, even in the absence of both ICP47 and tapasin, not only tapasin-independent HLA-G/E114H but also tapasin-dependent HLA-G molecules acquired thermostability even at the 0-min chase point, suggesting that “tapasin-dependent” HLA-G molecules are loaded with endogenous peptides immediately after synthesis.

Constitutively associated peptides confer resistance to ER-mediated degradation of HLA-G molecules—The findings that HLA-G molecules can assemble with low-affinity self-peptides in the absence of tapasin and that the resulting complexes are not able to transport beyond medial-Golgi raises the question as to the physiological function of such assembly. In the absence of peptides, empty class I molecules are retained in the ER and degraded by a quality control mechanism (35-37). To test whether this is also the case for HLA-G, an MHC class Ib molecule, we measured the turnover rate of HLA-G molecules in ICP47-expressing cells. In these ICP47-expressing cells, the resulting empty HLA-G and HLA-G/E114H molecules were being degraded rather rapidly during the longer chases so that we could not detect HLA-G molecules at the 2-h time point (Fig. 7A). In sharp contrast, in the absence of
ICP47, both HLA-G and HLA-G/E114H molecules were relatively stable, even after a 4-h chase. In repeated experiments, we have consistently observed that HLA-G molecules in the presence of tapasin become more stable over time than do HLA-G molecules in its absence (Fig. 7B, compare lanes 11, 12 and lanes 3, 4, respectively). Since the stability of the class I molecules depends on high-affinity association with peptides, this observation suggests that HLA-G molecules are loaded with higher-affinity peptides in the presence of functional tapasin. Overall, our data indicate that even in the absence of tapasin, HLA-G molecules are constitutively associated with peptides, albeit low-affinity peptides, and that the binding of such peptides might stabilize the intracellular HLA-G pool by preventing their degradation. This strategy could confer a longer half-life on HLA-G and provide more chances for HLA-G to exchange the suboptimal peptide ligands for ones with higher affinity.
DISCUSSION

Recent studies have implicated that in the assembly of class I-peptide complexes, tapasin serves several functions, including ER retention of heavy chains, TAP stabilization, and peptide editing. In this study, we addressed the question of whether tapasin is involved in the assembly and trafficking of HLA-G. More importantly, by taking advantage of the unusual trafficking property of HLA-G and the unique feature of peptide ligands of HLA-G, we were able to delineate the precise function of tapasin in the quality control of class I molecules.

Our results demonstrate that tapasin is not required for suboptimal peptide binding but is critical for loading of high-affinity peptides onto HLA-G. Given the tapasin requirement of HLA-E (5) and H2-M3 (6,7), class Ib molecules, for their proper assembly, this suggests that class Ib molecules resemble ‘tapasin-dependent’ class Ia alleles in their dependence for tapasin. Our data rule out retention of class I molecules in the ER as the primary function of tapasin. The rate at which HLA-G/E114H exits the ER is identical with and without tapasin (Fig. 1C). The assembly of HLA-G in 721.220.Tpn cells showed even enhanced transport of HLA-G heavy chains to the cell surface, as compared with the transport of tapasin-deficient 721.220 cells (Fig. 1B). These results indicate that ER retention of HLA-G is relatively unaffected by tapasin. In line with this interpretation, soluble tapasin also fails to retain class I molecules in the ER but still enhances class I surface expression to levels similar to the levels observed for the full-length tapasin construct (10). McCluskey and his colleagues have shown that, based on the endo-H assay, tapasin prevents premature release of K\textsuperscript{b} from the ER, which led them to conclude that tapasin plays a role in the retention of suboptimally loaded
class I molecules in the ER (12). Taking into account the observation that HLA-G associated with suboptimal peptides is not statically retained in the ER but recycles between the ER and the cis-Golgi, together with the fact that the sensitivity of proteins to endo-H digestion cannot distinguish between true retention and retrieval from the cis-Golgi, the apparent steady-state distribution of K\textsuperscript{b} in the ER might be reminiscent of K\textsuperscript{b} retrieval from the cis-Golgi to the ER, rather than K\textsuperscript{b} retention in the ER. In support of this view, in the murine mutant cell line (CMT), class I molecules seem to recycle through the cis-Golgi (37), suggesting that similar quality control by recycling can occur in class I\textsubscript{a} molecules. It appears that tapasin does not affect TAP’s function in translocating peptides into the ER lumen, because peptide transport in microsomes derived from 721.220 cells was similar to peptide transport in microsomes derived from 721.220.Tpn cells (data not shown). Furthermore, as loading of peptides onto HLA-G/114H proceeds normally in the absence of tapasin (Fig. 2A), bridging of class I molecules to TAP, which is one of the proposed functions of tapasin, appears to be unnecessary for enhanced peptide loading. Thus, the most important function of tapasin is exerted by a process that is independent of tapasin’s role in TAP stabilization and in bridging MHC class I molecules with TAP.

In the final analysis, our data favor the hypothesis that a critical function of tapasin is to transform class I molecules into the high-affinity peptide-receptive form. In the absence of tapasin, HLA-G molecules cannot bind their natural ligands with high-affinity peptides, despite an abundant supply. Increasing the high-affinity peptide pool cannot overcome the requirement of tapasin for fast transport kinetics and surface expression of HLA-G. These results support the notion that a conformational change of HLA-G by tapasin is a prerequisite for the binding of high-affinity peptides. In contrast
to tapasin-dependent HLA-G, HLA-G/E114H, the tapasin-independent phenotype, has a peptide-binding groove that seemingly forms the open state to fit the appropriate peptides, regardless of tapasin. Accordingly, HLA-G/E114H molecules are capable of binding a broad spectrum of the peptide repertoire without the assistance of tapasin and are subsequently transported to the cell surface. The functional consequence of the failure of HLA class I heavy chains to associate with tapasin is substantial impairment in the loading of endogenously generated peptides. Interestingly, our results show that the tapasin-dependent HLA-G molecules are not empty in tapasin-negative cells but are associated with endogenous self-peptides, ones with low-affinity. These HLA-G complexes recycle between the ER and the cis-Golgi until they bind high-affinity peptides, underscoring the role of not only the ER but also the post-ER compartments in the quality control of HLA-G molecules. Under the otherwise identical experimental conditions, the addition of tapasin renders HLA-G molecules capable of loading peptide ligands with high affinity and allows their transport beyond the medial-Golgi to the cell surface. The phenotype of HLA-G in the absence of tapasin clearly differs from the phenotype of class I molecules expressed in cells either lacking functional TAP or β2m. If β2m is absent, class I heavy chain molecules fail to transport to the cell surface and are degraded (38). MHC class I complexes that are expressed in mutant cell lines lacking TAP function are devoid of peptides and are also degraded in the ER or in the ER-Golgi compartment (39,40). In corroboration of these observations, in ICP47-expressing cells, the resulting peptide-deficient HLA-G is strictly retained and degraded in the ER. Given the fact that the correct assembly of the class I heavy chain with β2m and a peptide is necessary for transport of the complex out of the ER to the cell surface, class I molecules that leave the ER in tapasin-knockout mice (4) could be fully
conformed with peptide, but these peptides would be of generally lower affinity. These results suggest that tapasin, although not required for assembly of MHC class I molecules, provides a gating mechanism for reducing the expression of class I molecules containing suboptimal peptides. However, the suboptimal peptides might play an important role in preventing HLA-G from the otherwise prolonged-ER retention that could increase the risk of recognition by a quality control system in the ER. In this way, HLA-G molecules might have multiple chances to bind high-affinity peptides in a single lifetime.

In summary, the role of tapasin in peptide loading can be viewed as a process with several distinct steps. In order to avoid ER-mediated degradation, newly synthesized HLA-G initially binds a variety of peptides with different affinities, whichever peptides are available in the vicinity. Upon association with tapasin, the binding cleft of heavy chains undergoes the conformational change into a high-affinity, peptide-receptive form, probably by breaking conserved hydrogen bonds between the peptide backbone and MHC class I side chains. In turn, the substitution of suboptimal to optimal peptides occurs by the competition of high-affinity peptides with preloaded low-affinity peptides in the ER. Finally, high-affinity peptides close the peptide-binding groove efficiently and thereby initiate dissociation of peptide-loaded MHC class I molecules from tapasin and subsequent transport to the cell surface. In support of this model, it has been reported that tapasin-associated and peptide-loaded H-2L^d class I molecules display different conformations (41) and that tapasin interacts with regions of the class I heavy chains that are sensitive to the presence of peptide in the antigen binding cleft (42). These events reflect the potential of tapasin to discriminate between the high-affinity and low-affinity peptide-loaded conformation. Independent evidence supporting an
intracellular editing mechanism for class I molecules has been provided by the observation that a specific peptide-K^d complex dissociates more rapidly when retained in the ER than when expressed at the cell surface and that sequential peptide binding can occur in the ER (43). In this regard, tapasin function might be comparable to the function proposed for HLA-DM, which influences peptide loading and peptide selection in the MHC class II pathway. HLA-DM is suggested to keep "empty" MHC class II molecules in a peptide-receptive, and probably more open, conformation (44,45).

The findings presented in this study not only reveal a critical function of tapasin in class I assembly in the ER, but also reveal cooperation of post-ER subcellular compartments for maximizing the quality control of proteins. Similar quality control mechanisms might be imposed upon other class Ib molecules, such as HLA-E or HLA-F, for effective presentation of a restricted set of peptide antigens.

ACKNOWLEDGEMENTS

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REFERENCES


41. Carreno, B. M., Solheim, J. C., Harris, M., Stroynowski, I., Connolly, J. M., and


FIGURE LEGENDS

FIG. 1. Tapasin dependence of HLA-G for cell surface expression and intracellular maturation. 721.220 and 721.220.Tpn cells were transfected with DNA encoding the indicated heavy chains. (A) Surface expression level of HLA-G and its mutant alleles was measured by FACS analysis. FACS histograms are shown for cells without tapasin (thin lines) and with tapasin (thick lines). Staining of mock-transfected cells is shown by dotted lines (control). To examine the effect of tapasin on intracellular transport of HLA-G (B) and HLA-G/E114H (C), cells were labeled with [35S]methionine for 25 min and chased for the indicated times. To test whether a supply of high-affinity peptide ligands can overcome the lack of tapasin for efficient transport of HLA-G, we cotransfected 721.220.Tpn (D) and 721.220 cells (E) with minigenes encoding high-affinity peptides for HLA-G and DNAs encoding HLA-G or HLA-G/E114H. Cell lysates were immunoprecipitated with mAb G233 and then digested for 16 h with (+) or without (−) endo H. Proteins were separated by 12% SDS-PAGE. Endo-H resistant (r) and endo-H sensitive (s) protein bands are indicated.
FIG. 2. Tapasin dependence of HLA-G for high-affinity peptide loading. Peptide-loading of HLA-G and HLA-G/E114H was measured by using microsomal membranes derived from 721.220 cells (A) and 721.220.Tpn (B). The peptide transport assay was performed using the microsomal membrane derived from tapasin-negative 721.220 cells. The amount of translocated peptides into the microsomal membrane derived from expressing HLA-G/E114H was compared to the value obtained from cells expressing wild-type HLA-G (C).

FIG. 3. Effect of tapasin on retrieval of HLA-G. NIH3T3.hβ2m cells (A, C) and NIH3T3.hβ2m.hTpn cells (B, D) were transfected with the minigene encoding high-affinity peptide ligands for HLA-G (C, D) or with the empty vector (A, B). The cells were incubated in the presence (+Nz) or absence (-Nz) of 20 µM nocodazole for 5 h. Cells were then fixed, permeabilized, and labeled with the G233 antibody. The samples were analyzed for intracellular localization by immunofluorescence microscopy.

FIG. 4. Intracellular fate of HLA-G in the absence of tapasin. NIH3T3.hβ2m (tapasin-negative) (A, B) and NIH3T3.hβ2m.hTpn (tapasin-positive) (C, D) cells were transfected with HLA-G alone (A, C) or HLA-G and the minigene encoding high-affinity peptides (B, D). Cells were fixed, permeabilized, and double-immunostained with the K455 antibody for HLA-G (left column) and with anti-GM130 for endogenous cis-Golgi marker proteins (middle column). Co-localization of HLA-G or HLA-G/E114H with marker proteins was analyzed by confocal laser microscopy. The right column shows the merged images.
FIG. 5. Interaction of HLA-G with β-COP as a function of tapasin and high-affinity peptide ligands. 721.220 and 721.220.Tpn cells were transfected with DNA encoding the indicated heavy chains alone or with DNAs encoding the heavy chains and high-affinity peptide ligands. Cells were lysed by 1% digitonin, and lysates were immunoprecipitated (I.P.) with the indicated antibodies. The immunoprecipitated proteins were separated by SDS-PAGE and blotted (I.B.) with anti-β-COP antibody. As a loading control, the same membranes were re-blotted with the K455 antibody (bottom panels).

FIG. 6. Effect of tapasin on thermostability of HLA-G complexes. 721.220 (A) and 721.220.Tpn cells (B) were transfected with DNA encoding the indicated heavy chains alone or with DNAs encoding the indicated heavy chains and ICP47. Cells were metabolically labeled for 15 min and chased for 30 min. After lysis in 1% NP-40, lysates were divided into two aliquots and either kept on ice or incubated at 37°C for 1 h prior to immunoprecipitation with the mAb W6/32.

FIG. 7. ER-mediated degradation of empty HLA-G molecules. 721.220 and 721.220.Tpn cells were transfected with DNA encoding the indicated heavy chains alone (B) or with DNAs encoding the indicated heavy chains and ICP47 (A). Cells were metabolically labeled for 15 min and chased for the indicated time periods. The cells were lysed in 1% NP-40, and the resulting lysates were immunoprecipitated with the K455 antibody. Immunoprecipitates were analyzed by 12% SDS-PAGE.
figure 1.
continued figure 1.

D  
high-affinity peptide(+), tapasin(+)  

E  
high-affinity peptides(+), tapasin(−)
figure 2.

A

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C

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figure 3.

A

High-affinity peptides(−)  
Human tapasin(−)

-HLA-G

-HLA-G/E114H

B

High-affinity peptides(−)  
Human tapasin(+)  

-HLA-G

-HLA-G/E114H

C

High-affinity peptides(+)  
Human tapasin(−)

-HLA-G

-HLA-G/E114H

D

High-affinity peptides(+)  
Human tapasin(+)

-HLA-G

-HLA-G/E114H
figure 4.

A

- Human tapasin, -high-affinity peptides

![Images showing HLA-G, cis-Golgi, and Merge for both conditions: HLA-G and cis-Golgi with HLA-G/E114H and cis-Golgi with Merge.]

B

- Human tapasin, +high-affinity peptides

![Images showing HLA-G, cis-Golgi, and Merge for both conditions: HLA-G and cis-Golgi with HLA-G/E114H and cis-Golgi with Merge.]

continued figure 4.

C

+Human tapasin, -high-affinity peptides

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D

+Human tapasin, +high-affinity peptides

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figure 6.

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HLA-G

β2m
figure 7.
An essential function of Tapasin in quality control of HLA-G molecules
Boyoun Park and Kwangseog Ahn

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