Essential glycan-dependent interactions optimize MHC class I peptide loading

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In this study we sought to better understand the role of the glycoprotein quality control machinery in the assembly of MHC class I molecules with high-affinity peptides. The lectin-like chaperone calreticulin (CRT) and the thiol oxidoreductase ERp57 participate in the final step of this process as part of the peptide-loading complex (PLC). We provide evidence for an MHC class I/CRT intermediate before PLC engagement and examine the nature of that chaperone interaction in detail. To investigate the mechanism of peptide loading and roles of individual components, we reconstituted a PLC subcomplex, excluding the Transporter Associated with Antigen Processing (TAP), from purified, recombinant proteins. ERp57 disulfide linked to the class I-specific chaperone tapasin and CRT were the minimal PLC components required for MHC class I association and peptide loading. Mutations disrupting the interaction of CRT with ERp57 or the class I glycan completely eliminated PLC activity in vitro. By using the purified system, we also provide direct evidence for a role of UDP-glucose:glycoprotein glucosyltransferase 1 in MHC class I assembly. The recombinant Drosophila enzyme reglucosylated MHC class I molecules associated with suboptimal ligands and allowed PLC reengagement and high-affinity epitope exchange. Collectively, the data indicate that CRT in the PLC enhances weak tapasin/class I interactions in a manner that is glycan-dependent and regulated by UDP-glucose:glycoprotein glucosyltransferase 1.

protein folding | peptide editing

The assembly of MHC class I molecules is a critical step in the generation of immune responses against viruses and tumors, and also a highly specialized example of glycoprotein folding in the endoplasmic reticulum (ER). MHC class I molecules display peptides representative of the cellular protein content to CD8+ T cells, and the stable association of the class I heavy chain (HC), β2-microglobulin (β2m), and a high-affinity 8- to 10-aa foreign peptide is essential for T-cell activation. As a result, a specialized adaptation of the glycoprotein folding machinery has evolved to ensure the loading of MHC class I molecules with optimal peptide ligands. Following HC assembly with β2m, the empty heterodimer rapidly and stably associates with the peptide-loading complex (PLC), which facilitates the final peptide-binding step (1). The functions of the MHC class I-specific components of the PLC are well defined. Tapasin interacts with both the HC/β2m dimer as well as Transporter Associated with Antigen Processing (TAP), thereby retaining the empty complexes in proximity to the peptide supply. More importantly, tapasin association stabilizes class I molecules and promotes loading with high-affinity peptides. However, the optimal activity of tapasin requires the presence of calreticulin (CRT) and ERp57, two ER proteins involved in general glycoprotein folding, in the PLC (2–4).

The ER glycoprotein quality control machinery is a complex system that uses the structural state of N-linked glycans to dictate the fate of newly synthesized proteins (1, 5, 6). Initially, a Glc3Man9GlcNAc2 glycan is transferred to polypeptides during translocation and subsequently trimmed by glucosidase I (GlsI) and glucosidase II (GlsII) to a monoglucosylated glycan, which is the substrate for the lectin-like chaperones CRT and calnexin (CNX). Both chaperones have a globular lectin-binding domain and an extended arm known as the P-domain that binds specifically to ERp57, a member of the protein disulfide isomerase (PDI) family. ERp57 has a four-domain architecture of abb′a′ in which the first and last contain a CXXC active site. CRT and CNX work in concert with ERp57 to promote proper folding and disulfide bond formation of newly synthesized glycoproteins. Upon release of the glycoprotein from CRT or CNX, its glycan is deglucosylated by GlsII and is no longer a substrate for the chaperones. If the glycoprotein has not yet acquired its native structure, the enzyme UDP-glucose:glycoprotein glucosyltransferase 1 (UGT1), a folding sensor, reglucosylates it to reinitiate an interaction with CRT/CNX/ERp57. However, a properly folded glycoprotein is not a substrate for UGT1 and can be exported to the Golgi.

Important roles for CRT and ERp57 in MHC class I assembly and PLC function have been established from studies of KO mice and deficient cell lines (2, 3). In the absence of either component, the cell surface expression and stability of MHC class I molecules are reduced. The cause of the defect has been elucidated for ERp57-deficient cells. Tapasin forms a stable disulfide-linked heterodimer with ERp57, which is required for the structural stability and optimal function of the PLC (4, 7, 8). The disulfide linkage is between Cys95 of tapasin and Cys57 of the ERp57 α domain active site (9), and the dimer is further stabilized by noncovalent interactions between tapasin and the α′ domain active site (10). CRT interacts with both the HC glycan and the β′ domain of ERp57, but the nature and importance of these interactions within the PLC are controversial, particularly in regard to glycan-independent substrate binding (11–15). Finally, the potential roles of GlsII and UGT1 in regulating the CRT/class I interaction in the PLC have yet to be addressed.

By using a variety of biochemical approaches, including reconstitution of a PLC subcomplex entirely from purified components, we have investigated the role of ER quality control components in MHC class I peptide loading. We demonstrate the CRT is recruited to and released from the PLC along with class I molecules. By examination of the CRT/HC stoichiometry and the HC glycosylation state, we found no evidence for glycan-independent interactions within the PLC. Consistent with this, in vitro reconstitution of the PLC required the lectin- and ERp57-binding activities of CRT and could be enhanced by UGT1-mediated glucosylation. Taken together, the data support important roles for CRT and the quality control machinery in regulating class I peptide loading by the PLC.

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Results and Discussion

CRTC Associates with MHC Class I HC/β2m Heterodimers Before Incorporation into the PLC. Work from our laboratory has demonstrated that the tapasin/ERp57 conjugate associates with TAP in cells lacking expression of MHC class I HC or β2m (8, 16), suggesting that this core serves as a scaffold onto which the remaining PLC components assemble. Furthermore, the GisII inhibitor castanospermine (CST) prevents the interaction of MHC class I molecules with the PLC in intact cells (17) and a cell-free assay (4). This suggests that CRTC escorts HC/β2m dimers to tapasin, but this has not been demonstrated experimentally. Initially, we sought to identify PLC-independent complexes of HLA-B8 with CRT by using tapasin-negative .220.B8 cells. We prepared extracts from radiolabeled cells in the absence or presence of DSP, a chemical cross-linker, and performed sequential immunoprecipitations to detect CRT-associated proteins. As shown in Fig. 1A, a CRTC/HC interaction was observed in untreated extracts and stabilized by DSP, β2m, which is not glycosylated, also immuno-precipitated with CRT from cross-linked cell extracts, demonstrating the presence of CRT-associated HC/β2m hetero- dimers (Fig. 1A). To determine whether this complex exists in the presence of the PLC, we performed tapasin immunodepletion from radiolabeled .220.B8.Tpsn cells. As shown in Fig. 1B, a fraction of HLA-B8 molecules were associated with CRT after the complete removal of tapasin. These results are consistent with the hypothesis that CRT/MHC class I complexes are an intermediate in the MHC class I assembly pathway.

To examine whether CRTC binding to MHC class I molecules is required to initiate interactions with the remaining PLC components, we adapted a cell-free assay for PLC activity (4, 10). This system reconstitutes PLC subcomplexes lacking TAP by the addition of recombinant soluble tapasin (S.Tpsn)/ERp57 conjugate to .220.B8 cell extracts, which are a crude source of CRTC and empty class I molecules. We postulated that CRTC/HC/β2m complexes (Fig. 1A and B) are the subset of molecules recruited to the conjugate with this assay. However, a significant portion of these complexes likely dissociate during the extract preparation given the relatively low affinity of CRTC for glycosylated class I HC (Kd of ∼1 μM) (14) and the rapid rate of dissociation determined from kinetic experiments (t1/2 of ∼10 min at 4 °C, Fig. S1). Consistent with our projections, the addition of excess recombinant CRTC to the lysis buffer enhanced HC association with the conjugate (Fig. 1C), presumably because of preservation of CRTC/HC/β2m complexes in the assembly pathway. In agreement, Del Cid et al. (11) have proposed a recruiting role for CRTC in the PLC based on reduced class I association with the PL in CRTC-deficient cells. However, this interpretation is complicated by the analyses being performed at steady state; i.e., the effect of CRTC on the class I /tapasin interaction may be a result of PLC stabilization. Although the two effects cannot be discriminated biochemically, CRTC may serve an important role for both. The discovery that an HC/β2m/CRTC assembly intermediate exists provides the most direct evidence for CRT-mediated recruitment (Fig. 1A and B), whereas the coupling of known interactions between PLC components is sufficient to support CRT-mediated stabilization of existing class I / tapasin interactions (1).

Analysis of the MHC Class I/CRTC Interaction in the PLC. To better understand the functions of CRTC in class I assembly, we evaluated the stoichiometry and mode of interaction between CRT and the class I HC in the PLC. The initial assessment of the CRTC/HC ratio (18) was complicated by the subsequent discovery of ERp57 in the PLC, so we used a different approach that involved quantitative immunoblotting. PLCs were isolated by immunoprecipitation from .220.B8.Tpsn cells and analyzed in parallel with purified CRT and class I HC as standards so the relative amounts could be determined. A representative blot is shown in Fig. 2A and the average ratio calculated from five independent experiments was 1.08 ± 0.19 CRTC to 1.00 HC mole- cules. Thus, equimolar amounts of MHC class I HC and CRT are present in the PLC. In further support of this stoichiometry, we performed tapasin immunoprecipitations from CRTC-depleted .220.B8.Tpsn extracts and found that there are essentially no CRTC-deficient PLCs (Fig. 2B).

Monoglycosylated glycans are required for CRTC binding to the class I HC in vitro (14), which presents an apparent discrepancy between the stoichiometry (Fig. 2A) and the mixed glycan structures of PLC-associated HC previously reported (19). We therefore reevaluated the glycosylation state of class I in the PLC with the use of two assays. The first used jack-bean mannosidase (JBM), an exomannosidase that trims nonglucosylated glycans with the use of two assays. The second used quantitative immunoblotting. PLCs were isolated by immunoprecipitation from .220.B8.Tpsn cells followed by JBM or EndoH digestion of PLC-associated tapasin or class I HC. As shown in Fig. 2D, the tapasin glycan contains terminal glucose residues shortly after synthesis (0 min chase), which are subsequently trimmed by the 90-min chase point, consistent with its maturation to the native state. In contrast, the JBM-treated class I HC band did not shift during the chase; thus, its glycan is not trimmed while it is associated with the PLC. To more accurately quantify the fraction of PLC-associated class I HC with monoglycosylated glycans, our second assay used pull-downs with CRT-GST fusion protein immobilized on glutathione beads. As shown in Fig. S2, the binding of class I HC to the CRT-GST beads was completely eliminated by treatment with GisII and is therefore glycan-dependent. Compared with the total amount of PLC-associated class I HC, determined by 3810.7 immunoprecipitations, we found that 97% of the total could be recovered from sequential CRT-GST pull-downs (Fig. 2E). We therefore conclude that virtually all MHC class I molecules in
the PLC are monoglycosylated and bound to CRT via the N-linked glycan.

**CRT and ERp57 Provide Structural Roles in Soluble PLCs Reconstituted from Purified Components.** Conflicting data regarding the mechanism of CRT and ERp57 activity in the PLC have been reported in studies in intact cells (11, 12, 15). We sought to address these discrepancies using an in vitro assay for PLC activity. Since the discovery of tapasin in 1996, two experimental systems have been devised to address its function and both have limitations. Chen and Bouvier used recombinant soluble MHC class I complexes and tapasin purified from *Escherichia coli* (21), but excluding CRT and ERp57 from the system necessitated the addition of leucine zippers to tapasin and class I HC s to induce dimerization. In contrast, we developed a cell-free assay that successfully reconstituted a subcomplex of the PLC (4) but used cell extracts, meaning that a role for unidentified components could not be eliminated. For these reasons, we continued our endeavor to reconstitute a soluble subcomplex of the PLC from purified components (Fig. 3A). This has been a considerable challenge because of the multiple components and specialized interactions within the PLC (1).

The production of the appropriate PLC substrate (empty MHC class I complexes with monoglycosylated N-linked glycans) has been the most problematic. We previously described the expression of soluble HLA-B8 bearing such a glycan in a *Saccharomyces cerevisiae* glycosylation mutant (14), but the refolding yields without high-affinity peptides were insufficient for extensive structural or functional analyses. Consequently, we tested a different strategy that involved baculovirus expression, as the class I HC assembles with β₂m in insect cells but the complexes are largely devoid of peptides because of the absence of tapasin and TAP (22, 23). Coexpression of the luminal domain of HLA-B8 with human β₂m in Sf21 cells resulted in the formation of heterodimers that were completely retained in the ER as determined by EndoH digestion (Fig. S3A). We therefore postulated that a significant fraction of the HC molecules might possess the correct oligosaccharide structure for CRT binding. Analysis of samples from multiple infections by CRT-GST pull-downs indicated that approximately 20% of the class I molecules produced in Sf21 cells were monoglycosylated (Fig. S3B). HLA-B8 molecules expressed in Sf21 cells were unstable, however, as a result of the absence of bound peptides. To prevent dissociation of HC/β₂m complexes, extraction and purification were carried out in the presence of an intermediate-affinity peptide, the previously described RAL variant of the antigenic peptide EBNA3 (339-447) (4). The RAL ligand binds and stabilizes HLA-B8 molecules (4), but can be displaced by the PLC in vitro (Fig. S4).

Our strategy was to assemble the PLC subcomplex in vitro from purified CRT, sTpsn/ERp57 conjugate, and HC/β₂m/RAL complexes (Fig. S3C). First, we incubated the recombinant proteins in various combinations and assessed PLC reconstitution by the coimmunoprecipitation of CRT and class I HC with tapasin (Fig. 3B). As expected, CRT bound to the conjugate at these concentrations as a result of its specific interaction with the ERp57 β₂m domain (Kₐ of 0.55 μM) (11). MHC class I complexes did not interact with sTpsn/ERp57 alone, but did so efficiently in the presence of CRT, demonstrating that PLC subcomplexes were generated (Fig. 3B). These observations explain the need for leucine zippers to force the dimerization of class I with tapasin in the absence of an HC glycan, CRT, and ERp57 (21). We next tested the reconstituted system for peptide loading activity by using the high-affinity NP(380-387) ligand specific for HLA-B8 (4). We incubated HC/β₂m/RAL complexes with purified PLC components and [³²P]NP and then determined radiolabeled peptide loading by immunoprecipitations with the mAb w6/32, which binds HC/β₂m/peptide complexes (Fig. 3C). HC/β₂m/RAL complexes were capable of a low level of [³²P]-NP exchange that was not affected by the presence of CRT or the sTpsn/ERp57 conjugate alone. However, when both CRT and the conjugate were present, a 10-fold increase in high-affinity peptide binding was observed, which directly demonstrates catalysis of peptide exchange by the PLC. Some laboratories have reported that PDI is a member of the PLC and affects peptide loading (24), but these findings have been contentious (1). Recombinant human PDI was not required for PLC activity, nor did it enhance peptide loading in this assay. These experiments demonstrate successful reconstitution of the PLC from purified proteins and establish MHC class I HC, β₂m, CRT, tapasin, and ERp57 as the only components essential for peptide exchange in vitro.

To directly test the roles of ERp57 and CRT in the PLC, we generated several mutants and tested their ability to support peptide loading. The 3X mutant of ERp57 (C60A/C406A/C409A) allows covalent trapping of tapasin, but is redox-inactive (7). The ΔPDB ERp57 construct (K214A/K274A/K284A) is a combination of the individual point mutations that most profoundly

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**Fig. 2.** Analysis of the CRT/HC interaction in the PLC. (A) TAP immunoprecipitations were performed from the indicated number of 220.B8.Tpsn cells and analyzed by SDS/PAGE along with recombinant class I HC and CRT standards. Quantitative immunoblotting was performed with 3B10.7 or CRT Abs, and the standard curves are shown in Fig. S2. Two sample sets were processed and averaged per experiment, but only one is shown. (B) Extracts from radiolabeled 220.B8.Tpsn cells (60-min pulse) were subjected to four CRT immunodepletion steps. Immunoprecipitations were then performed with control, PaSta1, or CRT Abs and analyzed by SDS/PAGE. Note that, after JBM digestion, the migration of glucosylated species is eliminated. For these reasons, we continued our endeavor to reconstitute a subcomplex of the PLC (4) but used cell extracts, meaning that a role for unidentified components could not be eliminated. For these reasons, we continued our endeavor to reconstitute a soluble subcomplex of the PLC from purified components (Fig. 3A). This has been a considerable challenge because of the multiple components and specialized interactions within the PLC (1).

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disrupt CNX/CRT P-domain binding (25). Both ERp57 mutants efficiently formed disulfide-linked heterodimers when coexpressed with sTpsn and were purified. We also produced a Y92A mutant of human CRT based on the analogous mutations of rabbit and mouse CRT that disrupt glycan binding (11, 26). Consistent with the crystal structure of the sTpsn/ERp57 conjugate (10) and the characterization of the 3X mutant in ERp57-deficient cells (7, 15), inactivation of ERp57 redox activity did not impair the function of the PLC (Fig. 4D). However, when either Y92A CRT or the sTpsn/APDB ERp57 conjugate was used, no tapasin-mediated peptide loading was observed. Thus, the CRT/glycan and CRT/ERp57 interactions are required for PLC function in vitro. Contradictory results have been reported on this issue with the use of mutants expressed in CRT- or ERp57-deficient mouse cells (11, 12, 15). With an independent experimental system involving soluble components, our results agree with the findings of Del Cid et al. (11).

**CRT Exit the PLC with MHC Class I, but Release Is Not Induced by GlisII.** The events that govern MHC class I peptide loading and dissociation of the PLC are poorly understood. Based on our finding that empty MHC class I heterodimers and CRT are corecruited to the core complex of tapasin/ERp57/TAP (Fig. 1), we postulated that they are also simultaneously released from the PLC upon peptide binding. To test this hypothesis, we used siRNA to knock down β2m, preventing the association of newly synthesized MHC class I molecules with tapasin, and then followed the fate of the preexisting PLC components. Transient transfections of 220.B8.Tpsn cells were performed with control or β2m-specific siRNA, and samples were harvested for as long as 12 h (Fig. 4A). Analysis of communoprecipitation and blotting showed that the amount of TAP-associated tapasin/ERp57 conjugate remained constant over the 12-h period after transfection, whereas the CRT and class I HC levels decreased in parallel. This suggests that CRT is released from the core PLC components following peptide loading, and agrees with the observation that CRT does not associate with TAP in the absence of MHC class I heterodimers (16).

Because CRT and MHC class I molecules are released simultaneously from TAP (Fig. 4A) in a manner that is apparently affected by GlisII inhibition (27), we considered the possibility that trimming of the HC glycan triggers dissociation of the PLC. To address this question in greater detail, we labeled 220.B8. Tpsn cells and followed the kinetics of HC release from TAP in the absence or presence of CST. We found that the interaction of class I molecules with the PLC was dramatically prolonged when GlisII activity was inhibited during the chase (Fig. 4B). This suggested that glycan trimming is required for release of class I from the PLC. To further investigate this, we compared the ability of purified GlisII to trim the monoglucosylated glycan of PLC-associated and free HC. TAP immunoprecipitations were performed from radiolabeled 220.B8.Tpsn cells, samples were incubated with GlisII before or after reimmunoprecipitation of the class I HC, and then JBM digestions were performed as a readout. As shown in Fig. 4C, the glucosylase of free HCs, but not PLC-associated HCs, were accessible to GlisII (compare lanes 3 and 4 in Fig. 4C). This suggests that glycan trimming must therefore occur after release of CRT or MHC class I from the PLC. To reconcile these findings, we suggest that the prolonged interaction of MHC class I molecules with the PLC is maintained by a dynamic equilibrium that results from multiple rounds of release and reengagement of the class I glycan provided it is monoglucosylated.

This hypothesis presents interesting implications for the mechanism of peptide optimization, including a potential role for UGT in promoting repeated peptide loading events until the highest-affinity ligand is acquired. In support of this conclusion, we have found and reported in a companion paper in PNAS that the assembly of MHC class I molecules is impaired in UGT1-deficient murine fibroblasts (28). To further these observations, we adapted our in vitro assay to directly demonstrate that MHC...
class I complexes with intermediate-affinity peptides are substrates for reglucosylation by using the *Drosophila* homologue (i.e., UGT) of human UGT1. Purified HC/β2m/RAL preparations were depleted of monoglucosylated species with CRT-GST beads, incubated with or without recombinant UGT and UDP-glucose, and then subsequently tested for PLC-mediated loading of [125I]-NP. As shown in Fig. 4D, no peptide exchange was observed following the CRT-GST depletion, indicating that all HC-bearing monoglucosylated glycans had been removed. However, peptide exchange was observed when the depleted HC/β2m/RAL complexes were enzymatically reglucosylated by using UGT and UDP-glucose. Thus, as demonstrated directly in the companion paper (28), UGT recognizes MHC class I molecules loaded with suboptimal ligands, promoting peptide optimization by the PLC.

**Summary.** Taken together, the results from the present study support a critical role for the lectin-like chaperone activity of CRT in the PLC. Although it has been speculated that CRT escorts class I molecules to tapasin and TAP (11, 17), we provide experimental evidence for the existence of this intermediate. In addition to a recruiting function, CRT stabilizes class I molecules within the PLC via an equimolar, glycan-dependent interaction. By coupling interactions with the HC glycan and ERp57, the intermediate affinity between MHC class I molecules and the tapasin/ERp57 heterodimer. The glucosylation state of the class I molecule therefore dictates its engagement with the PLC, permitting a role for UGT1 in the optimization of the peptide repertoire as described in another study in PNAS (28).

For more than a decade, the analysis of the PLC and its components has been largely restricted to the biochemical characterization of cell lines. Although such studies have been highly informative, they can provide only an indirect analysis of PLC function. The development of an in vitro assay for PLC function now permits precise analysis of the mechanisms of peptide loading. This objective has been hampered for many years by the inability to produce the appropriate class I substrate (4, 14, 21). By using soluble HLA-B8 expressed with β2m in insect cells, we were able to reconstitute a soluble subcomplex of the PLC with purified CRT and sTpsn/ERp57. In addition to establishing the minimal components required for function, we unambiguously established a role for the CRT/ERp57 interaction and for UGT1 in peptide exchange and optimization. The reconstituted system finally opens the door for the detailed mechanistic analysis of the core PLC components, as well as possible accessory roles of other ER factors such as peptidases in MHC class I peptide loading.

**Materials and Methods**

**Plasmids.** A baculovirus construct was generated for the coexpression of soluble HLA-B*0801* (AA-24–273) with human β2m. Inserts were amplified by using PCR and cloned into pFastBac Dual (Invitrogen). The mature domain of human CRT with a C-terminal 6×His tag was cloned into pET15b and the two ERp57 mutations (ΔPDB and 3X) in pFastBac Dual with sTpsn.

**Cell Lines and Reagents.** The human .220.B8 cell line and its WT tapasin transfectant (.220.B8.Tpsn) have been described previously (18). The antibodies used were 3B10.7 (free class I HC), w6/32 (class I conformation-specific), PaSta1 (tapasin), R.gp48C (tapasin), R.ERp57 (full length ERp57), and CRT (4, 8). The peptides NP380-387L and EBNA3(339-447) RAL were synthesized by GenScript and iodinated as described (4).

**Immunoprecipitations and Immunoblotting.** Cells (5 × 10⁶ per sample unless indicated otherwise) were lysed in 1% digitonin (EMD Biosciences) in 150 mM NaCl, 25 mM Tris-Cl, pH 7.4, 1 mM CaCl₂ (TBS-C) with complete EDTA-free protease inhibitor tablets (Roche) and 10 mM methyl methanesulfonate (Pierce). Following a 30-min preclear, immunoprecipitations were performed for 1 h at 4 °C with the indicated Abs and protein G Sepharose or directly conjugated beads. Normal rabbit serum was used as a control Ab. After three washes with 0.1% digitonin in TBS-C, proteins were eluted in sample buffer or in 0.5% SDS for subsequent reimmunoprecipitation after dilution into 1% Triton in TBS-C. The following steps were used when indicated. Pulse/chase experiments were performed as described (14). Immunodepletions were executed with four 45-min incubations with Ab-coupled beads. Enzymatic digestions were performed overnight at 37 °C with EndoH (NEB) or JBM (Sigma). The standard blotting procedures and detection with ECL (Pierce) were performed as described (4, 7). For quantitative immunoblots, CRT and soluble HLA-B8 purified from *E. coli* (14) were used as standards, and proteins were detected with alkaline phosphatase-conjugated secondary Ab (Jackson Labs) and ECF substrate (GE Healthcare). PhosphorImager and FluorImager quantifications were performed by using the Storm860 system and ImageQuant software (GE Healthcare).

β2m siRNA. Cells (.220.B8.Tpsn) were transfected with β2m-specific or control siRNA oligos by using the Amazza Nucleofector (Lonza) with program V01 in five 100-μL aliquots. Briefly, 20 × 10⁶ cells were resuspended in 500 μL of
Solution R with 250 pmol control (GCUUCAACAGGGCCACUC) or 125 pmol each of β2m-specific (GAGUAUGCCUGCGUGUGAUU and GCAAGAGCGG UCUUCUCAAU) siRNA oligos (Dharmacon). Samples were harvested at the indicated times and frozen for subsequent analysis. Immunoprecipitations and blotting were performed as described earlier, and the data were normalized to TAP levels before calculating the percentage of control siRNA-treated value.

Protein Purification. All CRT and tStpsn ERp57 recombinant proteins were purified by using the established protocols (4, 14). Unless indicated otherwise, the C60A conjugate (WT sTpsn disulfide-linked to C60A ERp57), which is fully active (4), was used for experiments. Free class I HCs (soluble HLA-B8) included in the lysis, purification, and storage buffers. Pig liver GlisII was purified from all data sets. For reglucosylation experiments, the HC/β2m dimers, 25 to 50 μM RAL peptide was included in the lysis, purification, and storage buffers. Pig liver GlisII was purified as described (6). Drosophila UGT produced in Sf9 cells was a gift of Karin Reinisch (Yale University, New Haven, CT).

Peptide Loading Assay. Purified components (0.4 μM) were incubated in 50 μL TBS-C with 0.8 μM [125I]-NP (380–387L) for 15 min at room temperature (RT). (The residual RAL concentration from the purified MHC was 5 μM.) Next, 750 μL of 0.1% Triton in TBS-C with 50 μM cold NP peptide was added and w6/32 immunoprecipitations were performed. After three washes with 0.1% Triton in TBS-C, the amount of peptide bound on the beads was measured by using a Wallac 1420 counter (Perkin-Elmer). Reactions were performed in duplicate and the baseline values (i.e., no recombinant protein) were subtracted as background from all data sets. For deglycosylation experiments, the HC/β2m/RAL preparations were depleted of monoglycosylated complexes by using CRT-GST beads and then incubated with 1 μM UGT and 100 μM UDP-glucose (Sigma) in 50 mM NaCl, 10 mM CaCl2, 25 mM Tris-Cl, pH 8, for 1 h at 30 °C before the peptide loading assay.

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Supporting Information

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Fig. S1. Dissociation kinetics for the CRT/MHC class I HC interaction. The rate of $^{125}$I-labeled CRT release from the monoglucosylated glyans of denatured class I HC was monitored by using a plate binding assay (1). Briefly, soluble HLA-B8 was purified as described from the S. cerevisiae YG780 mutant under nonnative conditions. The reduced and denatured protein was then immobilized on 96-well plates and incubated with 1.5 μM $^{125}$I-CRT (∼25-fold molar excess) for 1 h at 37 °C in TBS-C. The plates were then chilled and washed four times with ice-cold TBS-C to remove the radiolabeled CRT. Next, 75 μL of TBS-C with 0.1 μM unlabeled CRT was added to the wells and incubated at 4 °C or 25 °C. At the indicated times, sample wells were washed with ice-cold TBS-C and the remaining CRT was eluted with 10 mM EDTA in TBS solution and quantitated by γ-counting.


Fig. S2. (A) Standard curves from a representative quantitative immunoblotting experiment. The data used to generate the standard curves were from the quantification of four immunoblots including the one shown in Fig. 2A. AU, arbitrary intensity units. (B) CRT-GST binding assay is glycan-dependent. CRT-GST and GST were expressed in E. coli and immobilized on glutathione Sepharose as described (1). The amount of immobilized protein per milliliter of resin was determined by SDS/PAGE and SYPRO Orange staining. To isolate MHC class I molecules for the pull-down assay, 220.B8.Tpsn cells were radiolabeled for 1 h and PLCs were immunoprecipitated by using 148.3 Ab-coupled beads. After three washes, the TAP-associated proteins were released from the beads by denaturation in 0.5% SDS in 50 mM Tris-Cl, pH 7.4, for 10 min at 65 °C. Samples were renatured by a 10-fold dilution into 1% Triton in TBS-C and re-immunoprecipitated with 3B10.7 Ab. The MHC class I HC samples were then incubated overnight in 0.1 M sodium phosphate with no enzyme (i.e., untreated), EndoH, purified GlsII, or GlsII plus 5 mM CST. The class I HCs were stripped again and subjected to a final pull-down assay with 3B10.7-coupled beads, GST beads, or CRT-GST beads and analyzed by SDS/PAGE. The final concentration of GST and CRT-GST in the pull-down step was approximately 2 μM.

Fig. S3. Recombinant expression of PLC components. (A) EndoH digestion of MHC class I HC expressed in insect cells. Extracts were prepared from BV-infected SF21 cells and incubated with or without EndoH (New England Biolabs) overnight according to the manufacturer’s instructions. Samples were analyzed by SDS/PAGE and immunoblotting with 3B10.7 for the class I HC. (B) Glycan analysis of MHC class I molecules expressed in SF21 cells. Extracts were prepared from BV-infected cells and incubated with CST (control, lane 4) or CRT-GST beads (lane 5) for 45 min at 4 °C. The beads were washed four times and bound proteins were eluted for analysis by SDS/PAGE and immunoblotting with the 3B10.7 Ab. A standard curve for the input material (lanes 1–3) was used to calculate the percentage of CRT-associated (i.e., monoglucosylated) MHC class I HC (lane 5). That fraction was calculated to be 18% for this experiment and the average from six independent BV infections and analyses was 20%. (C) Purification summary gel. Approximately 2 μg of the purified recombinant proteins was analyzed by SDS/PAGE and staining with Coomassie blue. The C60A conjugate (WT sTpsn with C60A ERp57) and MHC class I heterodimers (HC with β2m) were purified from insect cells, and CRT was purified from E. coli.

Fig. S4. The tapasin/ERp57 conjugate promotes the displacement of the suboptimal HLA-B8 binding peptide RAL. (A) The tapasin/ERp57 conjugate, but not soluble tapasin (sTpsn), reduces the steady-state binding of RAL peptide to HLA-B8. Extracts from .220.B44 (control, black bars) or .220.B8 cells (blue bars) were incubated with 2.5 μM [125I]-labeled RAL peptide in the absence or presence of the indicated recombinant proteins (0.25 μM) for 45 min at RT. Peptide binding was determined by immunoprecipitation with w6/32 and γ-counting. Samples were processed in duplicate and error bars represent SEM. (B) Dissociation kinetics for HLA-B8/RAL peptide complexes. [125I]-labeled RAL (2 μM) was preloaded onto MHC class I molecules from digitonin extracts of 5 million .220.B8 cells for 45 min at RT. Unlabeled RAL peptide was added to a final concentration of 150 μM, and the sample was divided into two and then further incubated with no recombinant protein (black circles) or 0.3 μM C60A conjugate (blue circles). Aliquots were removed at the indicated times and w6/32 immunoprecipitations were performed for exactly 1 h at 4 °C. After three washes with 0.1% digitonin in TBS-C, the amount of bound peptide was determined by γ-counting.