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, 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 for 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 peptide 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), β_2 -microglobulin (β_2m), 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 β_2m , 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/ β_2m 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 GlslI 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 *a* domain active site (9), and the dimer is further stabilized by noncovalent interactions between tapasin and the *a'* domain active site (10). CRT interacts with both the HC glycan and the *b'* 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 GlslI 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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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. (C) Schematic of the JBM assay. (D) .220.B8.Tpsn cells were pulse-labeled for 30 min and chased for as long as 90 min. Primary immunoprecipitations were performed with 148.3 Ab-coupled beads and secondary immunoprecipitations were performed with 3B10.7 or R.gp48C. Digests were then performed with JBM or EndoH and samples were analyzed by SDS/PAGE. Note that, after JBM digestion, the migration of glucosylated species is more similar to that of the undigested band (red asterisk), whereas the migration of deglycosylated species is more similar to that of the EndoH-treated band (blue asterisk). (E) .220.B8.tpsn cells were labeled for 60 min and sequential immunoprecipitation/pull-down assays were performed with 3B10.7 Ab, GST, or CRT-GST immobilized on beads followed by 3B10.7 (Upper). The total amount of HC recovered was calculated from two sequential 3B10.7 immunoprecipitations or from three CRT-GST pull-down steps (Lower).

the PLC are monoglucosylated 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 HCs 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 monoglucosylated *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 assemblies with β_2m 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 β_2m 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 monoglucosylated (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/ β_2m 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/ β_2m /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 *b'* domain (K_d of 0.55 μ M) (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(380-387L) ligand specific for HLA-B8 (4). We incubated HC/ β_2m /RAL complexes with purified PLC components and [125 I]-NP and then determined radiolabeled peptide loading by immunoprecipitations with the mAb w6/32, which binds HC/ β_2m /peptide complexes (Fig. 3C). HC/ β_2m /RAL complexes were capable of a low level of [125 I]-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, β_2m , 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 Δ PDB Erp57 construct (K214A/K274A/K284A) is a combination of the individual point mutations that most profoundly

Fig. 3. In vitro reconstitution of a soluble subcomplex of the PLC. (A) Schematic of the soluble PLC subcomplex. (B) Recombinant proteins (0.5 μ M each) were incubated at RT for 1 h and immunoprecipitations were performed with PaSta1-coupled beads. The tapasin-associated CRT and HC were detected by immunoblotting. (C and D) The indicated recombinant proteins—CRT or Y92A, a CRT glycan-binding mutant; disulfide-linked conjugates of sTpn with C60A, an ERp57 mutant that traps tapasin, with 3X, a redox-inactive ERp57 mutant that traps tapasin, or with Δ PDB, an ERp57 mutant that traps tapasin but does not interact with CRT—were incubated at a final concentration of 0.4 μ M each for 15 min at RT with [125 I]-NP. Peptide loading was measured by w6/32 immunoprecipitation and γ -counting.

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/ Δ PDB 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 Exits 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 β_2m , 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 β_2m -specific siRNA, and samples were harvested for as long a 12 h (Fig. 44). Analysis by coimmunoprecipitation 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 GlsII 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 GlsII 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 GlsII 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 GlsII before or after reimmunoprecipitation of the class I HC, and then JBM digestions were performed as a readout. As shown in Fig. 4C, the glycans of free HCs, but not PLC-associated HCs, were accessible to GlsII (compare lanes 3 and 4 in Fig. 4C). Glycan trimming must therefore occur after release of CRT or MHC class I from the PLC. This finding would appear to disagree with the conclusion implied by the data in Fig. 4B and ref. 27, i.e., that glucose removal is required for dissociation. 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

Fig. 4. Reglucosylation of MHC class I molecules by UGT promotes reengagement with the PLC. (A) Control or β₂m-specific siRNA oligos were introduced into .220.B8.Tpsn cells and samples were harvested at 0, 12, and 24 h after nucleofection. PLC components were detected by immunoprecipitation with 148.3 Ab and quantitative immunoblotting (Left). Amounts of PLC-associated CRT, tapasin, and class I HC were calculated and normalized to TAP (Right). (B) .220.B8.Tpsn cells were labeled with [³⁵S]-methionine for 30 min and chased for 0 to 120 min with or without 2.5 mM CST. Immunoprecipitations were performed with control or 148.3 Abs followed by elution and reimmunoprecipitation with 3B10.7 mAb (Left). Right: Phosphorimager quantification. (C) .220.B8.Tpsn cells were labeled for 60 min and then subjected to immunoprecipitations and enzymatic digestions as shown in the flowchart (Upper). (D) Purified recombinant MHC class I complexes depleted of those with monoglucosylated glycans were incubated with or without UGT and UDP-glucose. Subsequently, [¹²⁵I]-NP loading was measured in the absence or presence of the remaining PLC components (0.4 μM C60A conjugate and CRT).

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 presence of CRT greatly enhances the otherwise weak 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 inter-

action 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 (Novagen). The QuikChange mutagenesis kit (Agilent) was used to generate Y92A CRT in 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 NP(380-387L) 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 cocktail (Roche) and 10 mM methyl methanethiosulfonate (Pierce). Following a 30-min pre-clear, 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 digests 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 Amaxa Nucleofector (Lonza) with program V01 in five 100-μL aliquots. Briefly, 20 × 10⁶ cells were resuspended in 500 μL of