Redox-regulated Export of the Major Histocompatibility Complex Class I-Peptide Complexes from the Endoplasmic Reticulum

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In contrast to the fairly well-characterized mechanism of assembly of MHC class I-peptide complexes, the disassembly mechanism by which peptide-loaded MHC class I molecules are released from the peptide-loading complex and exit the endoplasmic reticulum (ER) is poorly understood. Optimal peptide binding by MHC class I molecules is assumed to be sufficient for triggering exit of peptide-filled MHC class I molecules from the ER. We now show that protein disulfide isomerase (PDI) controls MHC class I disassembly by regulating dissociation of the tapasin-ERp57 disulfide conjugate. PDI acts as a peptide-dependent molecular switch; in the peptide-bound state, it binds to tapasin and ERp57 and induces dissociation of the tapasin-ERp57 conjugate. In the peptide-free state, PDI is incompetent to bind to tapasin or ERp57 and fails to dissociate the tapasin-ERp57 conjugates, resulting in ER retention of MHC class I molecules. Thus, our results indicate that even after optimal peptide loading, MHC class I disassembly does not occur by default but, rather, is a regulated process involving PDI-mediated interactions within the peptide-loading complex.

INTRODUCTION

Assembly of major histocompatibility complex (MHC) class I molecules within the endoplasmic reticulum (ER) is essential for bringing antigenic peptides to CD8⁺ T-cells, which scan and destroy the infected and transformed cells. MHC class I quality control ensures both the ER retention of suboptimally loaded MHC class I molecules and the release of those with a higher affinity to the cell surface. Therefore, the peptide-editing process for optimal peptide loading is critical for increasing the recognition by CD8⁺ T-cells (Van Kaer, 2002; Cresswell, 2005).

MHC class I assembly is a highly regulated process mediated by several ER-resident chaperones and accessory molecules (Cresswell, 2000; Williams *et al.*, 2002a). After initial interaction with the lectin-like chaperone calnexin, MHC class I heavy chain- β_2 -microglobulin (β_2 m) heterodimers are incorporated into the peptide-loading complex (PLC), a multimolecular unit of ER proteins that promotes optimal peptide loading into MHC class I molecules (Hammerling *et al.*, 1998; Pamer and Cresswell, 1998; Elliott and Williams, 2005). The PLC contains calreticulin, tapasin, transporters associated with antigen processing (TAP) and two thiol-dependent oxidoreductases, ERp57 and protein disulfide

isomerase (PDI) (Garbi *et al.*, 2005; Park *et al.*, 2006; Santos *et al.*, 2007).

Several functions have been proposed for tapasin in facilitating optimal peptide loading and optimization of the peptide cargo (Grande and Van Kaer, 2001; Purcell *et al.*, 2001; Momburg and Tan, 2002). Tapasin bridges heavy chain- β_2 m heterodimers to TAP (Sadasivan *et al.*, 1996) and thus provides physical proximity between MHC class I molecules and TAP. Tapasin also retains MHC class I molecules in the ER (Schoenhals *et al.*, 1999; Barnden *et al.*, 2000; Grande *et al.*, 2000), which might enhance peptide loading. In addition, tapasin optimizes the repertoire of bound peptides to achieve maximal affinity of the interaction (Garbi *et al.*, 2000; Myers *et al.*, 2000; Tan *et al.*, 2002; Williams *et al.*, 2002b; Howarth *et al.*, 2004; Elliott and Williams, 2005). Different MHC class I alleles exhibit a distinct dependence on tapasin for surface expression (Grande *et al.*, 1997; Lewis and Elliott, 1998; Peh *et al.*, 1998; Park *et al.*, 2003).

Interestingly, the PLC contains the oxidoreductases ERp57 and PDI, each of which consists of four domains (abb'a') including two thioredoxin-like domains (a and a'). These oxidoreductases catalyze reduction, oxidation and isomerization of disulfide bonds within substrates (Jessop *et al.*, 2004). Emerging evidence suggests that the various transient intra- and intermolecular disulfides are formed among components of the peptide-loading complex (Chambers *et al.*, 2008) and that thiol-dependent redox regulation is vital for the assembly of MHC class I-peptide complexes (Dick, 2004). ERp57 exists in the PLC as a disulfide-linked heterodimer with tapasin (Dick *et al.*, 2002; Peaper *et al.*, 2005). ERp57 forms this disulfide interaction through its N-terminal thioredoxin-like domain with the Cys95 residue of tapasin (Dick *et al.*, 2002). The escape pathway (Walker and Gilbert, 1997) that is activated to release ERp57 from its substrates is inhibited by association with tapasin (Peaper *et al.*, 2005), resulting in a stable tapasin-ERp57 disulfide conjugate. This

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Abbreviations used: BFA, brefeldin A; β_2 m, β_2 -microglobulin; endo H, endoglycosidase H; ER, endoplasmic reticulum; MHC, major histocompatibility complex; NEM, N-ethylmaleimide; PDI, protein disulfide isomerase; PLC, peptide-loading complex; TAP, transporter-associated with antigen presentation.

covalent sequestration of ERp57 by tapasin in the PLC protects the $\alpha 2$ disulfide bond of MHC class I molecules against reduction (Kienast *et al.*, 2007), which might promote optimal peptide loading by stabilizing the peptide-binding groove in a peptide-receptive state. Formation of the tapasin-ERp57 disulfide-linked conjugate was recently shown to be central in recruiting MHC class I molecules and facilitating peptide binding (Garbi *et al.*, 2006). The importance of thiol-based redox regulation during antigen processing was further highlighted by recent studies showing that PDI stabilizes the peptide-receptive groove by oxidizing the $\alpha 2$ disulfide bond in the MHC peptide-binding groove, thereby enhancing optimal peptide selection by MHC class I molecules (Elliott and Neeffes, 2006; Park *et al.*, 2006). It is notable that, unlike the b' domain of ERp57, the b' domain of PDI contains the site for binding small peptides with 10- to 15-amino-acid residues (Klappa *et al.*, 1998).

Considering the physiological relevance of antigen presentation for immune function, the quality control of MHC class I molecules must be tightly regulated. Relative to the mechanism for assembly of MHC class I molecules and their peptide loading, the disassembly mechanism by which MHC class I molecules are released from the PLC after peptide binding and exit the ER is largely unknown. Of particular interest is also the question of whether the structural components of the PLC, containing TAP, calreticulin, and the tapasin-ERp57 disulfide-linked conjugate, fall apart or remain together as the peptide-loaded MHC class I- β_2m dimers are released from the PLC. Whatever the answer to this issue, optimal peptide binding by MHC class I molecules is thought to be sufficient for release of the peptide-loaded MHC class I molecules from the PLC and subsequent exit from the ER (Pamer and Cresswell, 1998). In contrast to the current consensus, we show here that PDI-mediated regulation of the tapasin-ERp57 conjugate dissociation is critical for controlling ER exit of peptide-loaded MHC class I molecules. Our findings suggest that even after optimal peptide loading, MHC class I disassembly undergoes further regulation for quality control of MHC class I molecules.

MATERIALS AND METHODS

Cell Lines and Antibodies

HeLa cells were cultured in minimum essential medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (50 U/ml), and streptomycin (50 μ g/ml). All constructs encoding deletion or point mutations were cotransfected with PDI small interfering RNA (siRNA) into HeLa cells. The cells were partially selected by adding 1 mg/ml puromycin (Sigma-Aldrich, St. Louis, MO) to the growth medium for 3 d before analysis. The human tapasin (Rgp48N) antibody was kindly provided by P. Cresswell (Yale University, New Haven, CT). The mAb ERp57 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal PDI antibody was raised against recombinant PDI purified from *Escherichia coli*.

DNA Constructs

Plasmids carrying PDI and PDI mutants were constructed as described (Park *et al.*, 2006). The cDNAs encoding human PDI and PDI deletion mutants were subcloned into the mammalian cell expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). Isoleucine-to-alanine (I272A) and phenylalanine-to-tryptophan (F258W) replacement mutations within the b' domain of PDI and the cysteine-to-serine (C36, 39S) mutation in the a domain of PDI were made by site-directed mutagenesis with *Pfu* DNA Polymerase (Stratagene, La Jolla, CA). The siRNA construct for human PDI was generated by cloning the cDNA segment encoding residues 1243-1261 (GGACCATGAGAACATCGTC), corresponding to the a' domain of PDI, into the pSilencer2.1-U6 vector carrying a puromycin resistance gene (Ambion, Austin, TX). As a negative control, we generated an siRNA against green fluorescent protein (GFP; GGTATGTACAGGAACGCA).

Metabolic Labeling and Immunoprecipitation

Cells were starved for 60 min in medium lacking methionine and cysteine, labeled with 0.1 mCi/ml [35 S]methionine and cysteine (Trans-label; PerkinElmer, Boston, MA) and chased in normal medium for indicated times. Cells were lysed using either 1% digitonin (Calbiochem, La Jolla, CA) or 1% Nonidet P-40 (NP-40; Sigma-Aldrich) in phosphate-buffered saline (PBS) with a protease inhibitor mixture. After preclearing cell lysates with protein G- or A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), primary Abs and protein G- or A-Sepharose were added to the supernatant and incubated at 4°C with rotation for 2 h. The precipitated proteins were separated by SDS-PAGE and then analyzed by using a phosphorimaging system (BAS-2500; Fuji Film, Tokyo, Japan). For endoglycosidase H (endo H) treatment, immunoprecipitates were digested with 3 mU of endo H (Roche, Indianapolis, IN) 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 Thermostability Assay

Expression of MHC class I glycoproteins on the membrane was determined by flow cytometry (FACScalibur; Becton Dickinson Immunocytometry Systems, Mountain View, CA) and analyzed by CellQuest software after indirect immunofluorescence staining with a saturating amount of monoclonal mouse anti-MHC class I Ab (W6/32) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Thermostability assay was done as described (Williams *et al.*, 2002b). In brief, radiolabeled cells were collected, washed in cold PBS, and lysed in Triton X-100 buffer (1% Triton X-100, 10 mM Tris [pH 7.4] and 150 mM NaCl with PMSF) for 30 min at 4°C. Cell lysates were precleared with protein A-Sepharose beads (Amersham Pharmacia) for 30 min. After preclearing, equal aliquots of cell extracts were incubated at indicated temperature for 60 min and then returned immediately to 4°C. Conformation-dependent W6/32 mAb and protein A-Sepharose beads were then added for 1 h at 4°C with gentle rotation, and immunoprecipitated proteins were subsequently separated by SDS-PAGE and visualized by autoradiography.

Immunoblotting

Proteins separated by SDS-PAGE were blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA). These membranes were blocked with PBS containing 5% fat-free milk for at least 1 h. The first antibody, in PBS with 0.01% Tween 20 (PBS-T) and 5% fat-free milk, was added to membranes and incubated for 1 h at room temperature. The membranes were then washed three times with PBS-T and incubated for 1 h with an appropriate secondary antibody conjugated to horseradish peroxidase. Membranes were washed four times with PBS-T. Signals were developed with a Peroxide-Luminol solution (Super Signal West Pico; Pierce, Rockford, IL), and membranes were exposed on Kodak film (Eastman Kodak, Rochester, NY).

RESULTS

PDI-mediated Dissociation of the Tapasin-ERp57 Conjugate Correlates with the Transport Kinetics of MHC Class I Molecules

Formation of the tapasin-ERp57 disulfide conjugate is pivotal in recruiting MHC class I molecules into the PLC and promoting peptide loading (Garbi *et al.*, 2006; Wearsch and Cresswell, 2007). Existence of PDI within the PLC suggests that in addition to the redox state of MHC class I molecules (Park *et al.*, 2006), PDI might also regulate the interaction between tapasin and ERp57. To investigate whether PDI is involved in this interaction, we used RNA interference for knockdown of endogenous PDI as previously described (Park *et al.*, 2006). We observed that the tapasin-ERp57 interaction was substantially increased by depletion of PDI in the reducing SDS-PAGE (Figure 1A, compare lanes 3 and 4). Because transfection of PDI siRNA did not affect expression of tapasin and ERp57 (Figure 1A, right), we exclude the possibility that the observed difference was caused by variable amount of ERp57 and tapasin. Analysis in the nonreducing SDS-PAGE also showed the accumulation of the disulfide-linked tapasin-ERp57 conjugate by depletion of PDI (Figure 1A, compare lanes 5 and 6). These data indicate that PDI affects steady-state level of the ERp57-tapasin disulfide conjugate.

Requirement of the tapasin-ERp57 conjugate for MHC class I assembly (Garbi *et al.*, 2006; Kienast *et al.*, 2007) and

Figure 1. PDI-mediated dissociation of the tapasin-ERp57 conjugate correlates with the maturation kinetics of MHC class I molecules. (A) Enhanced association of ERp57 with tapasin in the absence of PDI. HeLa cells transfected with either control GFP siRNA (–) or PDI siRNA (+). After enrichment of transfectants by puromycin selection (1 μ g/ml) for 3 d, cells were lysed in 1% digitonin and 10 mM NEM. Lysates were immunoprecipitated with the anti-ERp57 antibody, and samples were resolved under reducing and nonreducing conditions and immunoblotted for tapasin. The expression levels of PDI, ERp57, and tapasin were confirmed by immunoblotting aliquots of the lysate with respective antibodies. GAPDH was used as a loading control. (B) Effects of PDI depletion on the transport kinetics of MHC class I molecules. HeLa cells expressing control GFP siRNA (Mock) or PDI siRNA were pulse-labeled for 5 min and chased for 0, 20, and 40 min. 1% NP-40 lysates were immunoprecipitated with conformation-dependent mAb W6/32 before endo H digestion. HLA^r, endo H resistant; HLA^s, endo H sensitive. The intensity of each band at the 40-min chase time point was quantified by densitometry. The ER exit rate (%) was estimated by calculating the ratio of [r] band to total protein ([r] + [s]) for each time point. Data are representative of three independent experiments. (C) Dissociation of the tapasin-ERp57 conjugate is not limited by rates of ER-to-Golgi transport. HeLa cells expressing control GFP siRNA (–) or PDI siRNA (+) were treated with either mock or 5 μ g/ml BFA for 4 h. Lysates in 1% digitonin-NEM were analyzed for formation of the tapasin-ERp57 conjugate in reducing and nonreducing conditions. Aliquots of cell lysate were blotted with the respective antibodies to verify expression levels of proteins.

accumulation of the tapasin-ERp57 conjugate in PDI-depleted cells (Figure 1A) suggest that the export of peptide-loaded MHC class I molecules from the ER may require dissociation of tapasin-ERp57 conjugate. Therefore, we investigated whether the transport kinetics of MHC class I molecules is altered by levels of the tapasin-ERp57 interaction. To this end, we followed pulse-chase experiments with immunoprecipitation of MHC class I molecules by using mAb W6/32 and endo H treatment to analyze their maturation state. In cells with constitutive PDI expression, all labeled MHC class I molecules had matured to endo H-resistant forms 40 min after synthesis (Figure 1B, lane 6), whereas in PDI-depleted cells, only half of the MHC class I molecules became endo H resistant at the same time point (lane 12).

To further investigate a cause-and-effect relationship between regulation of tapasin-ERp57 conjugate dissociation and ER exit rate of MHC class I molecules, we determined the effects of brefeldin A (BFA) treatment on the dynamics of the tapasin-ERp57 conjugate formation and dissociation. The fungal toxin BFA blocks forward transport between the ER and the Golgi apparatus. Either BFA treatment or PDI siRNA transfection did not alter the level of expression of ERp57 and tapasin (Figure 1C, right), but the tapasin-ERp57 conjugate was accumulated by PDI depletion regardless of BFA treatment (Figure 1C, lanes 10 and 11), confirming an essential role for PDI in determining the tapasin-ERp57 conjugate dissociation. Interestingly, BFA treatment also did not alter the steady-state level of the ERp57-tapasin conjugate in

the constitutive PDI expression condition (compare lanes 8 and 9). These results indicate that the steady-state level of tapasin-ERp57 conjugate is continuously regulated by PDI and is not limited by rates of ER-to-Golgi transport.

PDI Interacts with Tapasin and ERp57

Next, to investigate the mechanism by which PDI influences the tapasin-ERp57 interaction, we examined the physical association between PDI and ERp57 or tapasin from digitonin-*N*-ethylmaleimide (NEM) lysates of metabolically labeled HeLa cells. To detect potential disulfide-linked intermediates cleanly, which are normally transient and unstable, we ectopically expressed PDI-myc. To clarify the nature of interactions (disulfide interaction vs. hydrophobic interaction) between PDI and tapasin-ERp57, we performed three rounds of serial immunoprecipitations. Digitonin cell lysates were immunoprecipitated with an anti-myc antibody for PDI-myc (Figure 2, lane 1). Reprecipitation of the PDI-myc immunoprecipitates with anti-ERp57 or anti-tapasin antibodies revealed that in reducing conditions, the PDI-myc immunoprecipitates contained ERp57 and tapasin (Figure 2, lanes 3 and 4). Because coprecipitation of ERp57 and tapasin with PDI could result from being disulfide-bonded to PDI, we examined whether PDI can still interact with ERp57 and tapasin in the presence of DTT with no NEM in the lysate. The cell lysate was immunoprecipitated for PDI-myc with anti-myc antibody and immunoblotted for ERp57 or tapasin. PDI-myc associated with ERp57 and tapasin in reducing conditions (Figure 2B). We could also confirm the

Figure 2. Association of PDI with tapasin and ERp57. (A) HeLa cells expressing PDI-myc were radiolabeled for 5 min, lysed in 1% digitonin-10 mM NEM, and subjected to three rounds of serial immunoprecipitations. In the first round, cell lysates (1% digitonin/no DTT) were immunoprecipitated with anti-myc antibody (lane 1). The anti-myc immunoprecipitates were divided into two aliquots. One aliquot was dissolved in 1% SDS/5 mM DTT (lanes 2–4), and the other aliquot was dissolved in 1% SDS without DTT and boiled (lanes 5–7). Both aliquots were diluted 50-fold with PBS buffer and subjected to a second round of immunoprecipitations. For identification of the disulfide conjugate shown in lane 6, aliquots of the second round immunoprecipitate (1% SDS/no DTT) were supplemented with 5 mM DTT, boiled, diluted 50-fold, and subjected to a third round of immunoprecipitation (lanes 8 and 9). Samples were analyzed under reducing or nonreducing conditions. Data are representative of two experiments. (B) HeLa cells expressing PDI-myc were lysed in 1% digitonin with 0.1 mM DTT without NEM. Digitonin lysates were immunoprecipitated with anti-myc antibody and were subjected to immunoblotting with antibodies specific to ERp57 or tapasin.

physical interactions between endogenous PDI and ERp57 and tapasin in the coimmunoprecipitation experiments using an anti-PDI antibody (Figures 3C and 5B). Hence, we conclude that PDI noncovalently associates with ERp57 and tapasin. In nonreducing conditions, we detected a potential PDI-tapasin conjugate with an apparent molecular weight of 110 kDa (Figure 2, lane 6). To confirm the identity of the conjugate, aliquots of the anti-tapasin reprecipitates were immunoprecipitated again with either an anti-tapasin or anti-myc antibody and analyzed by SDS-PAGE in reducing conditions. Indeed, tapasin and PDI-myc were positively identified (lanes 8 and 9). In contrast, we were unable to detect PDI-ERp57 disulfide-linked conjugates in the same experimental conditions (lane 7). These findings are in agreement with a previous report (Kimura *et al.*, 2005) showing that PDI binds to ERp57 *in vitro* through the noncovalent interaction involving their a and b domains. The interaction of ERp57 with PDI has been also detected by using a membrane yeast two-hybrid system (Pollock *et al.*, 2004). Our results demonstrate that PDI noncovalently binds to both ERp57 and tapasin and that PDI is also disulfide-linked to tapasin.

The Peptide-binding Property Determines PDI-mediated Dissociation of the Tapasin-ERp57 Conjugates

PDI acts as an oxidoreductase and is also capable of binding small peptides in a chaperone-like manner (Freedman *et al.*, 2002; Winter *et al.*, 2002). The a and a' domains of PDI contain an active site motif (CXXC) that is essential for catalytic activity. The b' domain of PDI provides the princi-

pal peptide-binding site (Klappa *et al.*, 1998). Mutations within this site, in particular, the I272A and F258W point mutations, greatly reduce the binding affinity for small peptide substrates (Pirneskoski *et al.*, 2004). We examined which of these two functions dictates the tapasin-ERp57 interaction. In a previous study, using several PDI proteins with deletion or point mutations and PDI siRNA oligomers corresponding to the a' domain, we were able to analyze the exclusive effect of each domain in a background depleted of endogenous PDI (Park *et al.*, 2006). The digitonin-NEM lysates of HeLa cells expressing either PDI siRNA alone or the PDI siRNA/deletion mutant combination were immunoprecipitated for ERp57, followed by immunoblotting for tapasin. In cells transfected with combinations of PDI siRNA and cDNAs coding for deletion mutants, expression of endogenous PDI was inhibited, whereas PDI mutant proteins were expressed at a level comparable to endogenous PDI in mock-transfected cells (Figure 3A, bottom). Densitometric quantification from three independent experiments confirmed that in cells depleted of endogenous PDI by siRNA, the tapasin-ERp57 interaction significantly increased compared with the interaction in cells with constitutive PDI expression (Figure 3A, compare lanes 3 and 4). The nonspecific band just above the tapasin was observed in Western blots with these anti-ERp57 and anti-tapasin antibodies. This band was present in isotype-matched IgG1 immunoprecipitates but not in direct immunoblot of lysates (lanes 1 and 2). In endogenous PDI-depleted cells, ectopic expression of PDI-abb', but not PDI-ab, decreased the tapasin-ERp57 conjugates to the levels comparable to the constitutive condition (compare lanes 5

Figure 3. Requirement for PDI to be bound to peptides for mediating dissociation of the tapasin-ERp57 conjugate and ER exit of MHC class I molecules. (A) A critical role of the peptide binding (b') domain in dissociation of the tapasin-ERp57. HeLa cells expressing combinations of PDI deletion and PDI siRNA were analyzed essentially the same as described in Figure 1A. The pSilencer vector encoding GFP siRNA was used to compensate for equal DNA amounts. Lysates in 1% digitonin-10 mM NEM were immunoprecipitated with either isotype-matched IgG1 or the anti-ERp57 antibody, and samples were resolved under reducing conditions and immunoblotted for tapasin. One-twentieth of the cell lysates was directly immunoblotted with anti-tapasin antibody to detect tapasin (lane 1). The expression levels of PDI, PDI mutants, ERp57, and tapasin were confirmed by immunoblotting aliquots of the lysate with respective antibodies (bottom panels). NS, nonspecific band. (B) Effect of peptide-binding-site mutation on tapasin-ERp57 conjugate dissociation. The experimental procedures were essentially the same as that described above. (C) Effects of peptide-binding-site mutation on interactions among PDI, ERp57, and tapasin. HeLa cells expressing either GFP siRNA (lanes 1 and 2) alone or combinations of PDI siRNA and PDI-F258W/I272A

I272A (lanes 3 and 4) were metabolically labeled for 5 min. Lysates in 1% digitonin-NEM were immunoprecipitated with the indicated primary antibodies. The resulting precipitates were treated with 1% SDS/5 mM DTT, boiled for 5 min, diluted 50-fold with PBS buffer before reprecipitation with the indicated secondary antibodies, and separated by SDS-PAGE under reducing conditions. GAPDH and PDI were immunoblotted as loading and expression controls, respectively.

and 6), establishing the critical role of the b' domain in regulating the steady-state level of the tapasin-ERp57 conjugates.

To differentiate between the role of peptide-binding property and catalytic activity of PDI in PDI-mediated regulation of the tapasin-ERp57 conjugates, we analyzed the ability of PDI point mutants defective in peptide-binding or catalytic activity in mediating dissociation of the tapasin-ERp57 conjugates. Expression of the PDI-F258W/I272A mutant in endogenous PDI-depleted cells, in which the peptide-binding site is destroyed, failed to dissociate the tapasin-ERp57 conjugates to the level in cells with constitutive PDI expression (Figure 3B, compare lanes 3 and 6). This result confirms that the peptide-binding property of the b' domain is crucial in regulating the tapasin-ERp57 interaction. Unlike PDI-F258W/I272A mutant, ectopic expression of the PDI-C36,39S mutant efficiently led to the dissociation of the tapasin-ERp57 conjugates to the levels comparable to the constitutive condition (Figure 3B, compare lanes 3 and 7), indicating that the cat-

alytic activity of PDI is not required for dissociating the tapasin-ERp57 conjugate.

To investigate the effect of peptide-binding-site disruption on interactions among tapasin, ERp57, and PDI, we immunoprecipitated ERp57 or PDI from the 1% digitonin-NEM lysates of radiolabeled HeLa cells, reprecipitated tapasin or ERp57 and resolved the eluted material by reducing SDS-PAGE. In cells treated with PDI siRNA, the level of the tapasin-ERp57 interaction was increased three- to fourfold over control levels (Figure 3C, first panel, compare lanes 2 and 3). Unexpectedly, we found that unlike wild-type PDI, the PDI-F258W/I272A mutant protein interacted poorly with either ERp57 or tapasin (Figure 3C, second and third panels, lane 4). Lack of interaction was not due to the scarcity of available PDI-F258W/I272A protein, evident by the expression of the PDI-F258W/I272A protein comparable to the level of constitutive PDI expression (bottom panels). In the presence of the PDI-F258W/I272A protein, the tapasin-ERp57 interaction increased even further, six- to eightfold

Figure 4. Inverse correlation between dissociation of tapasin-ERp57 conjugate and the ER exit of MHC class I molecules. HeLa cells expressing control GFP siRNA (–) or PDI siRNA (+) were transfected with PDI-abb' or F258W/I272A mutants, pulse-labeled for 5 min, and chased for various periods. Aliquots of 1% NP-40 lysates were incubated at 0, 37, and 50°C for 1 h and then immunoprecipitated with mAb W6/32 before endo H digestion. The ER exit rate (*r*) was estimated by calculating endo H resistance of MHC class I molecules for the 40-min time point. Data are representative of three independent experiments.

over that of the mock-transfected control (Figure 3C, first panel, compare lanes 2 and 4). This observation presumably represents the dominant negative effect of PDI-F258W/I272A against the residual endogenous PDI regarding dissociation of the tapasin-ERp57 conjugates. These results demonstrate that peptide substrate binding of PDI is necessary for its interaction with ERp57 and tapasin and for mediating subsequent dissociation of the tapasin-ERp57 conjugate. Inversely correlated with the intensity of the tapasin-ERp57 interaction, expression of the PDI-F258W/I272A mutant in cells depleted of endogenous PDI delayed the transport kinetics of MHC class I molecules from the ER, as assessed by endo H resistance at 40-min chase point (Figure 4, lanes 19 and 20), whereas the expression of PDI-abb', a parental molecule of PDI-F258W/I272A, restored the transport kinetics of MHC class I molecules to the levels found in cells with constitutive PDI expression (Figure 4, lanes 13 and 14). These results suggest that the peptide-binding function of PDI dictates its interaction with ERp57 and tapasin and the export of MHC class I molecules from the ER.

Peptide Starvation Leads to an Increase in the Level of the Tapasin-ERp57 Conjugate

If the binding of peptides to PDI is vital for PDI-mediated regulation of the tapasin-ERp57 interaction, one would expect that depletion of the peptide pool in the ER would lead to results similar to those observed in cells expressing the PDI-F258W/I272A mutant. To investigate whether depletion of the peptide pool could affect PDI-mediated dissociation of the tapasin-ERp57 conjugate, we depleted the ER luminal peptide pool by blocking the peptide supply into the ER lumen by using the ICP47 or US6 proteins, viral TAP inhibitors (Fruh *et al.*, 1995; Hill *et al.*, 1995; Ahn *et al.*, 1997). As expected, upon expression of US6 or ICP47, the surface level of MHC class I molecules was down-regulated (Figure

5A, left). Neither US6 nor ICP47 affected the levels of expression of ERp57 and tapasin (Figure 5A, right). Immunoprecipitation and Western blot analysis revealed that in the same cells, the levels of the tapasin-ERp57 conjugate were substantially increased (Figure 5A, right panels), similar to the results observed in cells expressing the PDI-F258W/I272A mutant (Figure 3, B and C). This result was confirmed in an experiment by using metabolically labeled HeLa cells. In the presence of either US6 or ICP47, the tapasin-ERp57 interaction was markedly increased (Figure 5B, first panel, lanes 3 and 4). In contrast, depletion of the peptide pool within the ER decreased PDI-ERp57 and PDI-tapasin interactions (Figure 5B, second and third panels, lanes 3 and 4). These results are consistent with the notion that the peptide occupancy of PDI governs the ability of PDI to bind tapasin and ERp57 and to regulate tapasin-ERp57 interaction.

Optimal Peptide Binding by MHC Class I Molecules Is Not Sufficient for the Exit of MHC Class I Molecules from the ER

Optimal peptide loading is believed to be sufficient for triggering MHC class I disassembly and subsequent exit of MHC class I molecules from the ER. The direct scrutiny of the hypothesis has not been possible hitherto due to the rapid transport of MHC class I molecules after optimal peptide loading and the unavailability of MHC class I disassembly mutant phenotypes. An unexpected finding allowed us to uncouple an event of optimal peptide loading from ER exit of MHC class I molecules. Ectopic overexpression of wild-type PDI resulted in about threefold increase in total PDI protein levels (Figure 6B, top panel, compare lanes 1 and 2). In this PDI-overexpressing cells, MHC class I molecules were more thermostable, but their transport out of the ER was unexpectedly slow when compared with cells in constitutive PDI expression (Figure 6A, right panel, compare lanes 1–4 with 5–8). Consistent with the observations

Figure 5. Peptide starvation inhibits PDI-directed dissociation of the tapasin-ERp57 conjugate. (A) Enhanced association of ERp57 with tapasin in peptide-depleted cells. HeLa cells expressing viral proteins US6 (HeLa-US6) or ICP47 (HeLa-ICP47) were analyzed for MHC class I surface expression by flow cytometry after preincubation with mAb W6/32. Bold lines indicate down-regulation of MHC class I surface expression in the HeLa-US6 and HeLa-ICP47 cells. Aliquots of these HeLa-US6 and HeLa-ICP47 cells were lysed in 1% digitonin-10 mM NEM. Lysates were immunoprecipitated for ERp57. Eluted proteins were separated by SDS-PAGE under reducing conditions, followed by Western blotting for tapasin. Five percent of the cell lysates was directly immunoblotted with anti-tapasin antibody to detect tapasin (first lanes). NS, nonspecific band. (B) Reduced interactions of PDI with ERp57 and tapasin concomitantly with enhanced tapasin-ERp57 interaction. The same experiments were done as described in Figure 3C by using metabolically labeled HeLa-US6 and HeLa-ICP47 cells. Data are representative of three independent experiments.

that PDI-abb' is able to complement PDI depletion (Figures 3A and 4), overexpression of PDI-abb' also showed almost

the same phenotype in regard to the transport kinetics and thermostability of MHC class I molecules observed for over-

Figure 6. Optimal peptide binding by MHC class I is not sufficient for ER exit of MHC class I molecules. (A) HeLa cells were transfected with mock (constitutive), wild-type PDI, or PDI-abb', pulse-labeled for 5 min, chased for the indicated time period and lysed in 1% NP-40. For an assay that correlates thermostability with the affinity of MHC class I peptide cargo, lysates were preheated for 1 h at 0 and 42°C and then immunoprecipitated with the mAb W6/32, which recognizes the MHC class I heavy chain- β 2m heterodimer only. Eluted proteins were digested with endo H and resolved by 10% SDS-PAGE. The ER exit rate was estimated as described in Figure 4. (B) The expression levels of PDI, PDI-abb', ERp57, and tapasin in each transfectant were confirmed by immunoblotting aliquots of the lysates with the respective antibodies. GAPDH served as a loading control. Data are representative of three independent experiments.

expression of wild-type PDI (compare lanes 5–8 with 9–12). Noteworthy, overexpression of wild-type PDI increased the tapasin-ERp57 interaction three- to fivefold compared with that of cells in constitutive PDI expression, comparable to the level in the PDI-depleted condition (Figure 3B, lanes 3–5). These results are of particular interest because accumulation of the tapasin-ERp57 conjugate appears to inversely correlate with the ER exit of MHC class I molecules. Collectively, our data suggest that PDI-mediated dissociation of ERp57 from tapasin after optimal peptide loading might be a key regulatory event for disassembly of the peptide-loading complex and subsequent ER exit of peptide-loaded MHC class I molecules.

DISCUSSION

The current model of MHC class I assembly does not explain how peptide-loaded MHC class I molecules are released from the PLC and exit the ER. In general, optimal peptide binding by MHC class I molecules is thought to be sufficient for export of peptide-filled MHC class I molecules from the ER. Here we show that in contrast to the current consensus, the release of MHC class I molecules from the PLC and their subsequent exit from the ER is a regulated process. Our data establish that the PDI-regulated dynamics of tapasin-ERp57 interaction could play a critical role in regulating MHC class I disassembly.

PDI facilitates optimal peptide loading into MHC class I molecules by catalyzing the oxidation of the $\alpha 2$ disulfide bond in the MHC peptide-binding groove (Park *et al.*, 2006). In PDI-depleted cells, MHC class I molecules exhibit delayed transport kinetics and poor thermostability, indicative of suboptimal peptide loading (Park *et al.*, 2006). Our current study suggests another role for PDI in regulating the tapasin-ERp57 disulfide conjugate, which appears to control the exit of MHC class I from the ER. Therefore, it was difficult to clarify the primary cause of delayed transport of MHC class I molecules in PDI-depleted cells. We made the interesting observation that the optimal peptide loading and ER exit of MHC class I molecules could be separated. In PDI-overexpressing cells, MHC class I molecules are loaded with optimal peptides but are retained in the ER (Figure 6), providing a conceptual basis that even after optimal peptide loading, MHC class I disassembly undergo further regulation before the ER exit of MHC class I molecules. Several lines of evidence indicate that PDI-mediated redox regulation of the tapasin-ERp57 interaction plays a critical role in controlling exit of MHC class I molecules from the ER. First, in cells overexpressing PDI, the ER retention of MHC class I molecules was accompanied by accumulation of the tapasin-ERp57 conjugate. Second, consistent with a central role for the tapasin-ERp57 conjugate in retention of MHC class I molecules within the ER until they become loaded with optimal peptides, ER exit of MHC class I molecules is accelerated in ERp57-deficient cells (Garbi *et al.*, 2006). MHC class I molecules associating with poorly with TAP and tapasin are exported more rapidly from the ER than are the MHC class I molecules efficiently associate with TAP and tapasin (Neeffjes and Ploegh, 1988; Neisig *et al.*, 1996). In addition, the function of tapasin has been proposed to retain empty MHC class I molecules in the ER (Schoenhals *et al.*, 1999; Grandea *et al.*, 2000). These individual observations can be explained by a function of the ERp57-tapasin conjugate in retaining MHC class I molecules in the ER and argue that impaired function of the tapasin-ERp57 conjugate results in a loss of stringency in quality control of MHC class I molecules. Finally, the importance of the dissociation of the ta-

pasin-ERp57 conjugate in regulating the release of MHC class I molecules is further emphasized by studies indicating that human cytomegalovirus has evolved to specifically target the tapasin-ERp57 conjugate for immune evasion. The human cytomegalovirus US3 glycoprotein inhibits the transport of MHC class I molecules from the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996). Recently, the US3 protein has been shown to target the degradation of PDI for CD8⁺ T-cell evasion (Park *et al.*, 2006). We found that US3-mediated degradation of PDI results in inhibiting dissociation of tapasin-ERp57 disulfide bonds, thereby retaining MHC class I molecules in the ER (B. Park and K. Ahn, unpublished data). PDI strongly binds to ERp57 in vitro via their a and b domains (Kimura *et al.*, 2005). In agreement with this finding, PDI binds to both tapasin and ERp57 in vivo and induces dissociation of the tapasin-ERp57 disulfide conjugate, which correlates with ER exit rate of MHC class I molecules.

Noncovalent interactions between tapasin and ERp57 prevent escape pathway activation by eliminating the ability of Cys60 of ERp57 to attack the mixed disulfide bond between tapasin and ERp57, which leads to maintaining the tapasin-ERp57 conjugate in the PLC (Peaper *et al.*, 2005). Our data demonstrate that PDI noncovalently binds to both ERp57 and tapasin. We also detected the PDI-tapasin but not the PDI-ERp57 disulfide conjugate (Figure 2). Therefore, it seems likely that upon the binding of PDI to either tapasin or ERp57, the noncovalent interactions between tapasin and ERp57 are disrupted, allowing escape pathway activation and conjugate dissociation. The transient disulfide bonds are formed both between class I heavy-chain PDI (Park *et al.*, 2006) and class I heavy-chain tapasin (Chambers *et al.*, 2008). Taking into account these observations and the formation of PDI-tapasin disulfide and the involvement of PDI in dissociation of the tapasin-ERp57 disulfide conjugate, we suggest that PDI might play a central role in the regulation of class I heavy-chain tapasin-ERp57 ternary redox network of the peptide-loading complex during MHC class I assembly and disassembly.

Our work indicates that the ability of PDI to regulate the interactions between tapasin and ERp57 is dependent on its property to bind peptide substrates. In the b' domain, PDI has a hydrophobic binding pocket that recognizes small peptides (Pirneskoski *et al.*, 2004) and the mutation of b' domain inhibits peptide binding by causing a conformational change in PDI (Nguyen *et al.*, 2008). Moreover, the interaction between the peptides and PDI is specific and can be saturated, reversed, and abolished by the denaturation of PDI (Klappa *et al.*, 1997). We found that the PDI-F258W/I272A point mutant, in which the peptide binding is disrupted, exhibited significantly lower affinity for tapasin and ERp57 than wild-type PDI (Figure 3C), preventing dissociation of the tapasin-ERp57 conjugate. Depletion of the peptide pool in the ER decreased the interactions of PDI with tapasin and ERp57, which resulted in inhibition of the tapasin-ERp57 conjugate reduction (Figure 5). These observations suggest that in the peptide-bound state, PDI binds with higher affinity to tapasin and ERp57 and executes its function in activating an escape pathway and thereby dissociating the tapasin-ERp57 conjugate. Thus peptides might be cofactors for this activity of PDI and PDI might be a redox regulator that is transiently recruited into the PLC in a peptide-dependent manner. Consistent with this idea, overexpression of PDI led to inactivation of the escape pathway and ER retention of MHC class I molecules. Either depletion of the peptide pool or overexpression of PDI would increase "empty" PDI pool that could not bind to tapasin and ERp57 and thus could not activate an escape pathway. This notion

could also explain the paradoxical observations that both knockdown of PDI and overexpression of PDI apparently have the same effect regarding regulation of the tapasin-ERp57 conjugate levels.

By what mechanism does binding of peptides influence PDI to regulate the interaction between ERp57 and tapasin? The recently determined U-shaped structure of yeast PDI (Tian *et al.*, 2006) reveals that the peptide-binding site in the b' domain is in close proximity to the other domains of PDI. This structural feature may provide an explanation as to why the binding of peptides to PDI influences the catalytic activity of PDI (Vuori *et al.*, 1992; Klappa *et al.*, 1998; Freedman *et al.*, 2002; Winter *et al.*, 2002). The chaperone activity of heat shock proteins to assist in protein folding also relies on their ability to bind peptides (Mayer *et al.*, 2000), suggesting that peptide binding to chaperones, in general, accompanies a conformational change in the proteins. Conceivably, peptide binding to PDI could induce a structural change in PDI, thereby altering its interactions with either tapasin or ERp57 and regulating ER exit of the peptide-loaded MHC class I molecules. Our data support the view that the structural components of the PLC, in particular, the tapasin-ERp57 disulfide-linked conjugate, do not remain together but rather fall apart as the peptide-loaded MHC class I- β_2m dimers are released from the PLC. Given that TAP-translocated peptides specifically bind to PDI (Lammert *et al.*, 1997; Spee and Neefjes, 1997) and that the peptide-binding activity of PDI is unique among human PDI family members characterized to date, PDI may have evolved to sense peptide availability in the ER, functioning as a gatekeeper for quality control of MHC class I molecules.

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