Cell

Redox Regulation Facilitates Optimal Peptide Selection by MHC Class I during Antigen Processing

Boyoun Park,[1] Sungwook Lee,[1] Eunkyung Kim,[1] Kwangmin Cho,[1,2] Stanley R. Riddell,[3] Sunglim Cho,[1,4] and Kwangseog Ahn[1,*]
[1] National Creative Research Center for Antigen Presentation, Department of Biological Sciences, Seoul National University, Seoul 151-747, South Korea
[2] College of Life Sciences, Korea University, Seoul 136-701, South Korea
[3] Fred Hutchinson Cancer Research Center, 1174 Columbia Street, Seattle, WA 98104, USA
[4] Research Center for Functional Cellulomics, Seoul 151-747, South Korea
*Contact: ksahn@snu.ac.kr
DOI 10.1016/j.cell.2006.08.041

SUMMARY

Activated CD8$^+$ T cells discriminate infected and tumor cells from normal self by recognizing MHC class I-bound peptides on the surface of antigen-presenting cells. The mechanism by which MHC class I molecules select optimal peptides against a background of prevailing suboptimal peptides and in a considerably proteolytic ER environment remained unknown. Here, we identify protein disulfide isomerase (PDI), an enzyme critical to the formation of correct disulfide bonds in proteins, as a component of the peptide-loading complex. We show that PDI stabilizes a peptide-receptive site by regulating the oxidation state of the disulfide bond in the MHC peptide-binding groove, a function that is essential for selecting optimal peptides. Furthermore, we demonstrate that human cytomegalovirus US3 protein inhibits CD8$^+$ T cell recognition by mediating PDI degradation, verifying the functional relevance of PDI-catalyzed peptide editing in controlling intracellular pathogens. These results establish a link between thiol-based redox regulation and antigen processing.

INTRODUCTION

MHC class I molecules capture peptides derived from both self and foreign intracellular proteins in the endoplasmic reticulum (ER) and present them to the cell surface for screening by CD8$^+$ T cells (Pamer and Cresswell, 1998). Peptides are generated primarily in the cytosol by proteasomes and are translocated into the ER by transporters associated with antigen processing (TAPs) for binding to newly synthesized MHC class I heavy chain (HC)/β_2-microglobulin (β_2m) heterodimers (Momburg et al., 1994b; Rock et al., 1994). TAPs exhibit relatively broad substrate specificity, "preselecting" peptides with regard to their lengths and sequences (Momburg and Hammerling, 1998). Because the peptide binding of individual class I molecules is selectively controlled by allele-specific peptide-binding motifs (Rammensee et al., 1993), most TAP-translocated peptides are suboptimal for binding MHC class I molecules. Quantitative estimations predict that of the peptides translocated into the ER, only 0.001% are optimal peptides with correct binding motifs (Lehner, 2003; Montoya and Del Val, 1999; Princiotta et al., 2003). Furthermore, TAP-translocated peptides survive a peptidase-containing ER environment in which free peptides have a short half-life (Heemels and Ploegh, 1994; Roelse et al., 1994). These situations might reflect the existence of specialized mechanisms for protecting peptides from degradation and transferring peptides to MHC class I molecules. Chaperones may bind some peptides and thereby protect them against degradation (Momburg et al., 1994a; Reits et al., 2003). The direct involvement of these chaperones in optimal peptide loading, however, has not yet been established.

As some viruses can replicate and release viral progeny within 4 hr (Grubman and Baxt, 2004), cytotoxic T lymphocytes (CTLs) have a limited time interval in which they must recognize a cell as infected and control the further spread of infection. Moreover, viruses have evolved multiple strategies to interfere with the MHC class I antigen presentation pathway and prevent CTL recognition (Lehner, 2003). Particularly at early times after infection, antigenic peptides might be present at very low frequency. Under these constraints, speed and efficiency in selection of optimal antigenic peptides may play a critical role in promoting recognition of the infected cell.

These reasons support the existence of a mechanism whereby MHC class I molecules select peptides against a background of prevailing suboptimal peptides and

considerable proteolytic activity in the ER. Current evidence suggests that MHC class I molecules first bind peptides indiscriminately to acquire initial cargo that is subsequently edited (Lewis and Elliott, 1998; Sijts and Pamer, 1997). Peptide editing presumably occurs through a peptide-exchange mechanism. In support of the peptide-exchange mechanism, MHC class I molecules retained in the ER are capable of binding peptides up to 3 hr after their biosynthesis (Sijts and Pamer, 1997). Furthermore, the T134K A2 mutant, which otherwise exits the ER rapidly with binding of suboptimal peptides, efficiently binds optimal peptides when it is retained in the ER of brefeldin A-treated cells (Lewis and Elliott, 1998). The process of peptide exchange may be facilitated by the cofactors present in the ER in a manner analogous to the activity of HLA-DM on MHC class II molecules in endosomal compartments (Kropshofer et al., 1996; Weber et al., 1996). Recent work proposes that the multimolecular peptide-loading complex, which consists of MHC class I, β_2m, TAP, calreticulin, ERp57, and tapasin (Elliott and Williams, 2005), controls peptide editing. This process favors the most stable MHC class I-peptide complexes and thus increases the chance of their recognition by T cells. Despite the potential importance of peptide editing in regulating the specificity of immunity and tolerance, little is known about how the MHC class I peptide repertoire is edited.

Here we demonstrate that protein disulfide isomerase (PDI), as a component of the antigen-processing machinery, plays a critical role in stabilizing a peptide-receptive site by regulating the oxidation of the α2 disulfide bond in the MHC class I peptide-binding groove and that this function is required for selecting optimal peptides. Our findings also suggest that PDI serves as a peptide chaperone that enhances the speed and efficiency of optimal peptide loading. We further demonstrate that human cytomegalovirus (HCMV) targets PDI for immune evasion, verifying a critical function of PDI for epitope selection with relevance to viral infections. Collectively, our studies present a paradigm for how the editing of the MHC class I peptide repertoire operates at the molecular level.

RESULTS

Identification of PDI as a Component of the Peptide-Loading Complex

To identify a component of the peptide-loading complex that could provide insight into the precise molecular mechanism underlying optimal peptide loading, we purified TAP complexes from digitonin lysates of HeLa cells. Both silver staining and subsequent analysis by tandem mass spectrometry (MS/MS) revealed that several proteins, including calreticulin, tapasin, MHC class I heavy chain, β_2m, and Ig γ H chain, specifically associated with TAP (Figure 1A). By MS/MS analysis of the 58 kDa protein band, we found that this band was a mixture of ERp57 and PDI. Given the association of PDI with TAP, we suspected that PDI, whose functions include disulfide-bond oxidation, reduction, and isomerization (Noiva, 1999), might be a component of the peptide-loading complex. We confirmed the identity of the TAP-associated components by reprecipitating anti-TAP1 immunoprecipitates from digitonin lysates of radiolabeled HeLa cells with the indicated antibodies (Figure 1B). TAP1, TAP2, tapasin, class I heavy chain, calreticulin, ERp57, and PDI, but not calnexin, were positively identified by reprecipitation (Figure 1B).

To determine whether identification of PDI as part of the peptide-loading complex was cell-type specific or due to contamination during the isolation of TAP-associated complexes, we used human lymphoid T1 and monocytic U937 cells to repeat the experiments and included ERp72, a member of the PDI family, and BiP, an ER chaperone, as controls. Reprecipitation of the TAP1 immunoprecipitates with the indicated antibodies revealed that, in both cell lines, the TAP immunoprecipitates contained TAP1, ERp57, PDI, tapasin, and class I heavy chain but did not contain ERp72 or BiP (see Figure S1B in the Supplemental Data available with this article online). Also, in the TAP immunoprecipitates from HeLa cells, ERp72 and BiP were not detected, despite their abundance in the ER (Figure S1A). These results demonstrate that PDI is a bona fide component of the peptide-loading complex irrespective of cell type.

Specificity of PDI Activity in MHC Class I Antigen Presentation

To investigate the function of PDI in MHC class I assembly, we used RNA interference for knockdown of endogenous PDI. Because prolonged expression (>3 weeks) of PDI small interfering RNA (siRNA) resulted in cell death, we were unable to establish stable siRNA transfectants. To overcome this limitation, we cotransfected HeLa cells with siRNA and a plasmid conferring puromycin resistance. After 72 hr of puromycin enrichment, the transfectants were used for experiments. As detected with the conformation-specific mAb W6/32, surface expression of fully folded class I molecules was reduced by 30 to 40%, whereas the surface level of unfolded free class I heavy chains as quantitated by mAb HC10 staining was increased by 20% to 30% in PDI knockdown cells compared to cells expressing endogenous PDI (Figure 2A, first panel). Since the mAb HC10 recognizes free class I heavy chains and unfolded free class I heavy chains are retained in the ER (Antoniou et al., 2002), free class I heavy chains at the cell surface probably represent a population loaded with low-affinity peptides that might dissociate during the course of the assay. Unpredictably, depletion of PDI did not affect the surface expression of several disulfide-bonded glycoproteins (CD47, CD58, CD71, CD95, CD59, and CD49f; Figure 2A), suggesting that in protein oxidative folding, although PDI is known as a ubiquitous catalyst, it is not essential for the maturation of these glycoproteins in these cells. Alternatively, this result might indicate that the oxidation of MHC class I molecules is more sensitive to PDI levels than the oxidation of other PDI substrates is. Furthermore, we did not observe increased expression

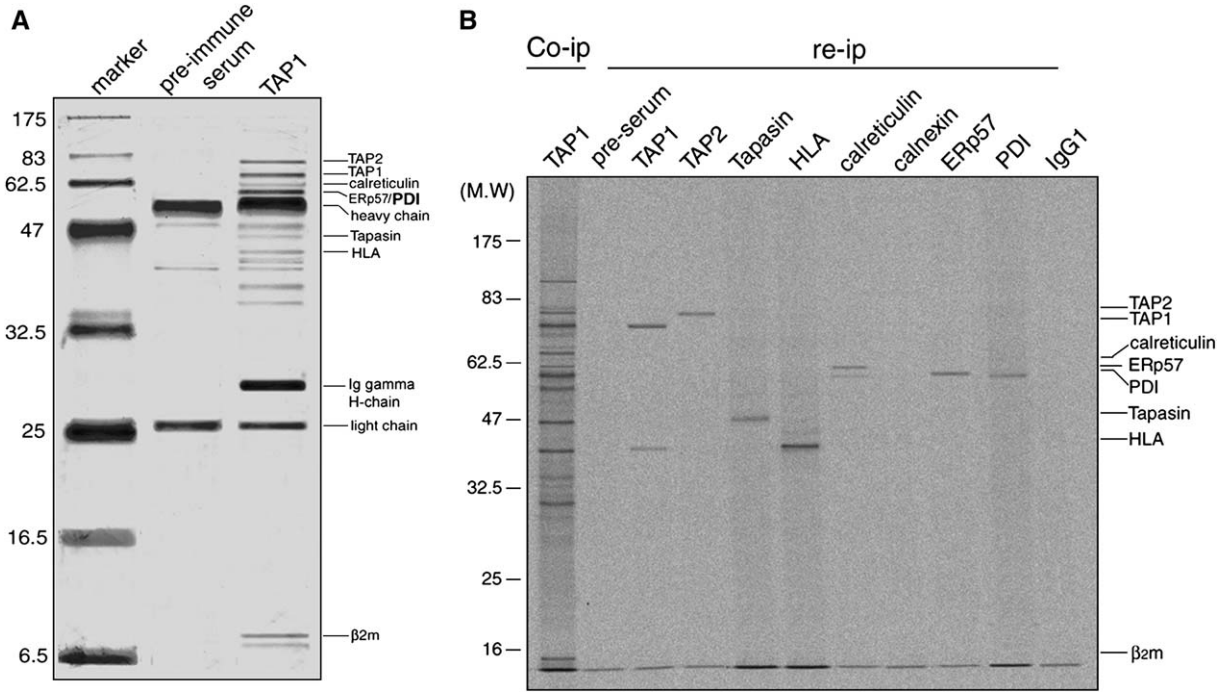


Figure 1. Identification of TAP-Associated Proteins

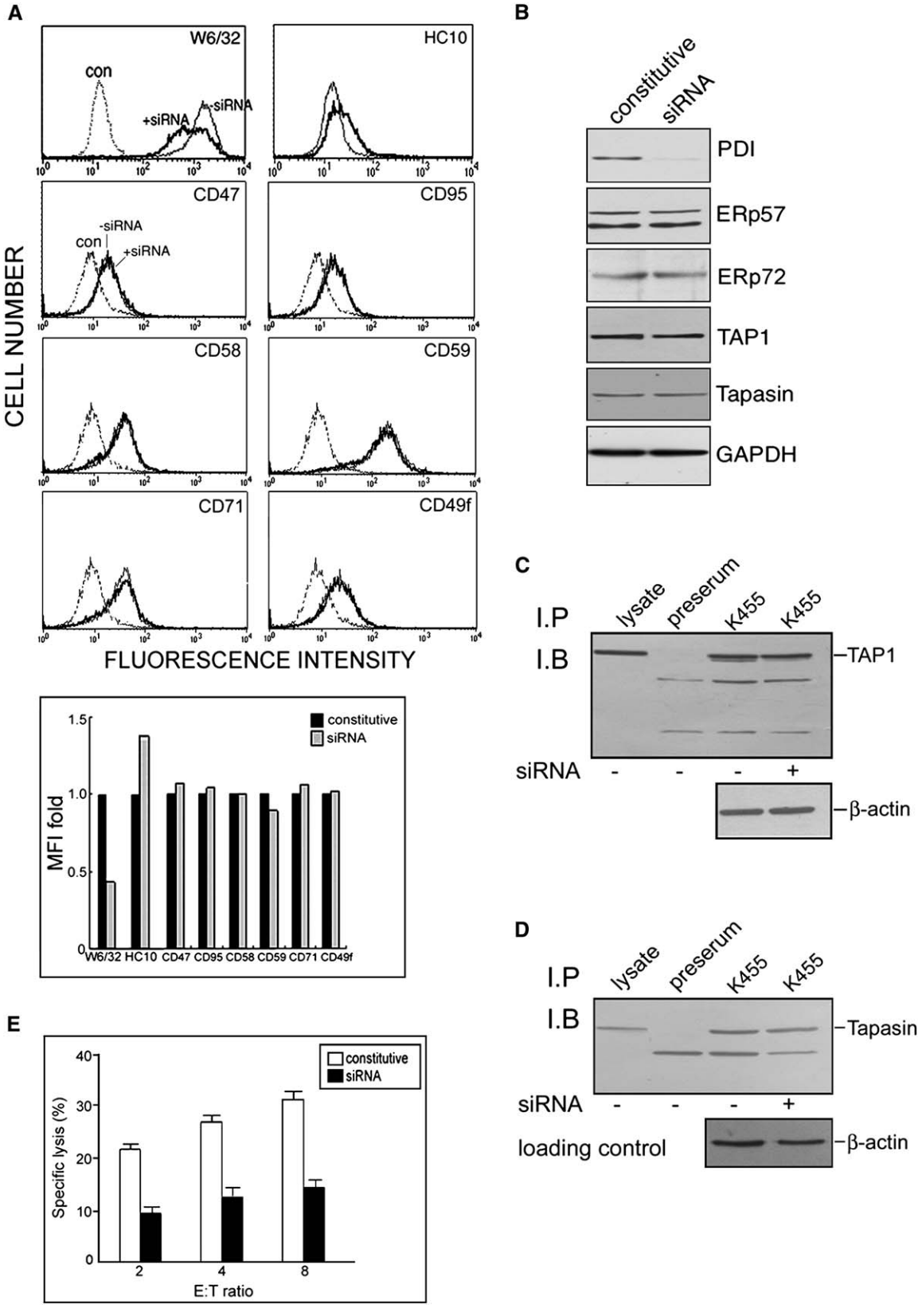
(A) Identification of TAP-associated proteins by MS/MS analysis. HeLa cells were lysed in 1% digitonin with 10 mM *N*-ethylmaleimide (NEM) and immunoprecipitated with TAP1 antiserum. Proteins were resolved by SDS-PAGE, visualized by silver staining, and identified as indicated.

(B) Association of PDI with components of the peptide-loading complex. HeLa cells were radiolabeled for 2 hr, lysed in 1% digitonin with NEM (10 mM), and immunoprecipitated with TAP1 antiserum. Associated proteins were stripped in 1% SDS and reprecipitated with the indicated antibodies.

of other oxidoreductases of the PDI superfamily, such as ERp57 and ERp72 (Figure 2B), which would indicate a compensatory response. PDI depletion did not affect expression of TAP1 and tapasin (Figure 2B) or the association among class I heavy chains, TAP, and tapasin, as shown by immunoprecipitation and reblotting experiments, which revealed normal levels of the class I heavy chains/TAP and the class I heavy chains/tapasin complexes in PDI-depleted cells (Figures 2C and 2D, respectively). To ascertain the functional relevance of PDI, we infected MZ1851 RC cells with a vaccinia virus recombinant expressing cytomegalovirus (CMV) pp65 and analyzed the susceptibility of these cells to lysis by HLA-A2.1-restricted, CMV pp65-specific cytotoxic T cells. PDI depletion inhibited CTL activity (Figure 2E), indicating that PDI plays a physiological role in MHC class I function.

Distinct PDI Domains Control the Maturation and Optimal Peptide Loading of MHC Class I Molecules

PDI consists of four distinct domains arranged in the order a-b-b′-a′ with a C-terminal acidic extension that contains the KDEL ER retention sequence. The a and a′ domains contain thiol oxidoreductase active sites (CXXC) separated by two noncatalytic domains, b and b′ (Edman et al., 1985). The b′ domain provides the principal site for binding small (10 to 15 residues) peptides (Klappa et al., 1998). Mutations within this site greatly reduce the binding affinity for small peptide substrates, with the I272A and F258W mutations having the greatest effect (Pirneskoski et al., 2004). ERp57 does not possess the general peptide-binding site that is found in PDI, suggesting that PDI and ERp57 are dedicated to distinct functions, either oxidation or reduction and isomerization. To identify the discrete role of each domain in MHC class I assembly, we constructed several PDI proteins with deletion or point mutations (Figure 3A). By engineering PDI siRNA oligomers corresponding to the a′ domain, we analyzed the exclusive effect of each mutated protein on class I assembly in a background depleted of endogenous PDI. HeLa cells expressing each mutant were pulse chased, and the lysates were analyzed by an assay that correlates thermostability with the affinity of class I peptide cargo (Williams et al., 2002). In PDI-depleted cells, MHC class I molecules exhibited slow maturation and poor thermostability when compared to cells in constitutive PDI expression (Figure 3B, compare lanes 7–12 and 1–6). In cells with constitutive PDI expression, 95.6% of labeled MHC class I molecules had matured to endoglycosidase H (endo H)-resistant forms 30 min after synthesis, whereas in PDI-depleted cells, only 39.7% of MHC class I molecules were endo H resistant during the same period (Figure 3B, fourth panel, lanes 2 and 8). In cells depleted of PDI by siRNA, MHC class I molecules were less thermostable than cells in constitutive PDI expression, and, in particular,


A
W6/32
HC10
con
+siRNA
-siRNA
CD47
CD95
CD58
CD59
CD71
CD49f
CELL NUMBER
FLUORESCENCE INTENSITY
constitutive
siRNA
MFI fold
W6/32 HC10 CD47 CD95 CD58 CD59 CD71 CD49f
B
constitutive
siRNA
PDI
ERp57
ERp72
TAP1
Tapasin
GAPDH
C
I.P
I.B
lysate
preserum
K455
K455
TAP1
siRNA
β-actin
D
Tapasin
loading control
E
Specific lysis (%)
E:T ratio

almost no MHC class I complexes could be recovered at 50°C (Figure 3B, lanes 11 and 12). Interestingly, in endogenous PDI-depleted cells, expression of PDI-abb′, but not PDI-ab, restored the maturation rate and thermostability of class I molecules to an extent comparable to the constitutive condition (Figure 3B, lanes 13–18 and 19–24), establishing the importance of the b′ domain in regulating optimal peptide loading and normal maturation of MHC class I molecules. Expression of PDI-F258W/I272A, which serves as a dominant-negative mutant regarding peptide binding, resulted in MHC class I molecules acquiring endo H resistance and thermostability similar to that of cells expressing PDI-ab/siRNA or siRNA alone (Figure 3C, lanes 13–18). These results confirm that the peptide-binding property of the b′ domain is indeed critical not only for optimal peptide loading but also for normal maturation of class I molecules. The PDI-C36,39S point mutation, in which the catalytic site of the a domain is destroyed, also failed to restore thermostability (Figure 3C, lanes 7–12). MHC class I molecules in these transfected cells were rapidly transported out of the ER, as evidenced by endo H resistance. Poor thermostability and faster transport kinetics of MHC class I molecules with the PDI-C36,39S point mutation reflect suboptimal peptide loading and escape from ER quality control. From these experiments, we have established that both the catalytic function of the a domain and the peptide-chaperoning function of the b′ domain of PDI are essential for optimal peptide loading.

Peptide Editing and Thiol-Based Redox Regulation Are Linked

Identification of PDI within the peptide-loading complex suggests that it might regulate the redox state of MHC class I molecules, leading to optimal peptide loading. We examined whether PDI catalyzes intrachain disulfide linkage in class I heavy chains. MHC class I molecules contain two disulfide bonds, one in the α2 domain and the other in the α3 domain, which influence the association of free heavy chain with β_2m (Bjorkman et al., 1987; Smith et al., 1995; Tector et al., 1997). In agreement with a previous study (Warburton et al., 1994), A2.1-C101S, a cysteine-to-serine substitution mutation at position 101 of the A2.1 molecule, failed to form a disulfide bond, as indicated by the reduced form in the nonreducing condition (Figure 4A, lane 9). In cells that express endogenous PDI or PDI-abb′, A2.1 molecules became oxidized, indicative of intradisulfide bond formation (Figure 4A, lanes 8 and 11). In contrast, PDI depletion by siRNA or expression of PDI-C36,39S in cells depleted of endogenous PDI resulted in partial reduction of A2.1 molecules (Figure 4A, lanes 10 and 14, respectively). A2.1 molecules were reduced in the presence of PDI-ab or PDI-F258W/I272A mutants in which the catalytic site of the a domain remains intact (Figure 4A, lanes 12 and 13), demonstrating that not only the oxidoreductase activity of the a domain but also the peptide-binding property of the b′ domain influences the redox state of class I molecules. If the binding of peptides to PDI were vital for PDI-mediated oxidation of class I molecules, depletion of the ER peptide pool should lead to results similar to those observed in cells expressing the mutant proteins but lacking the peptide-binding site, such as PDI-ab or PDI-F258W/I272A. To test this assumption, we blocked the peptide supply into the ER lumen by using the ICP47 or US6 proteins, viral TAP inhibitors (Ahn et al., 1997; Hill et al., 1995). As expected, in the HeLa cells stably expressing US6 or ICP47, the surface expression of MHC class I molecules was downregulated (Figure 4B, left panel). In the same cells, the reduced form of class I heavy chains was increased (Figure 4B, right panel, lanes 7 and 8), indicating that availability of peptides is a crucial factor for determining the redox state of MHC class I molecules.

Next we tested whether, in addition to peptide availability, the nature of peptides influences the redox state of class I molecules. Initially we examined whether PDI preferentially binds high-affinity peptide ligands for MHC class I molecules. Photocrosslinking analysis revealed that both ILMEKIHKV and IDMEKIHK peptides (high- and low-affinity peptides for A2.1, respectively) (Rammensee et al., 1999) can bind TAP, PDI, and PDI-abb′, but not PDI-ab (Figure S2). This result suggests that the b′ domain of PDI binds, although the crosslinking assay is limited in quantitative measurement of affinity differences, a broad spectrum of peptides, not necessarily antigenic ones. Upon exogenous supply of high-affinity peptides, A2.1 molecules became oxidized (Figure 4C, left panel, lane 5). In contrast, in the presence of low-affinity peptides, A2.1 showed predominantly the reduced form (Figure 4C, left panel, lane 6), similar to the redox state of A2.1 molecules in the peptide-depleted condition by ICP47 or US6 (Figure 4B). To confirm the results obtained with synthetic exogenous peptides, we performed a similar analysis by using the minigene expression system. Minigenes

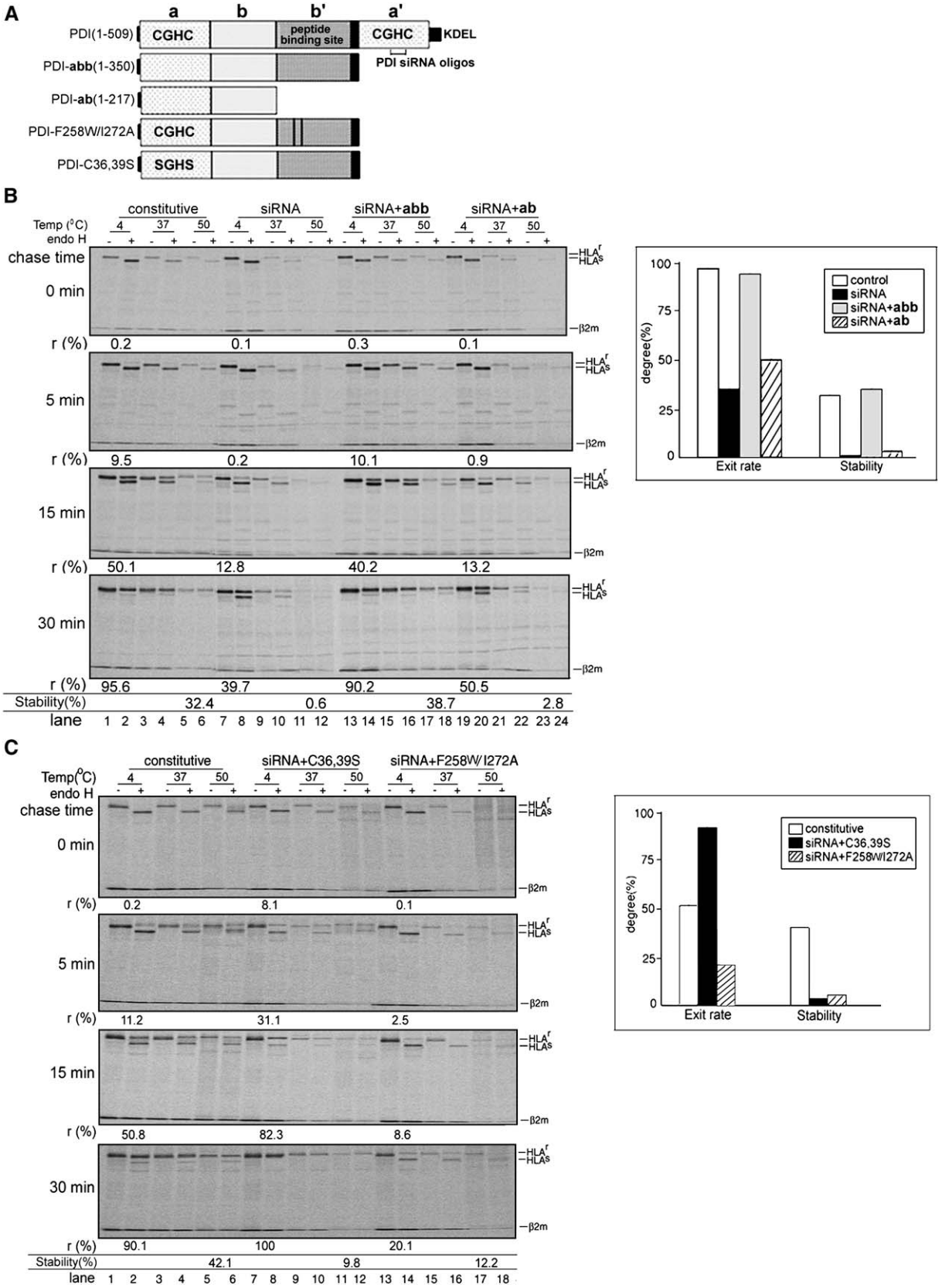
Figure 2. Specificity of PDI in MHC Class I Antigen Presentation

(A) Effect of PDI on the surface expression of class I molecules. Top panels: HeLa cells expressing PDI siRNA (+siRNA) or mock (−siRNA) were assayed for surface levels of MHC class I molecules and disulfide-bonded glycoproteins. −siRNA, thin line; +siRNA, thick line; control antibody staining, dotted line. Bottom panel: The mean fluorescence intensity (MFI) value obtained in PDI siRNA-expressing HeLa cells was set as 1, and the difference in MFI induced by siRNA expression was plotted.

(B) Effect of PDI on the steady-state level of components of the peptide-loading complex. Extracts from HeLa cells were analyzed by immunoblotting with the specified antibodies.

(C and D) PDI does not affect the association between class I molecules and TAP or tapasin. HeLa cells were lysed in 1% digitonin with a cocktail of protease inhibitors. Postnuclear lysates were immunoprecipitated (I.P) with class I-specific K455 antibody and immunoblotted (I.B) with anti-TAP (C) or anti-tapasin antibody (D).

(E) PDI knockdown inhibits CTL activity. Error bars represent standard deviations from triplicate wells.


A
a
b
b'
a'
PDI(1-509)
CGHC
peptide binding site
CGHC
KDEL
PDI siRNA oligos
PDI-abb(1-350)
PDI-ab(1-217)
PDI-F258W/I272A
CGHC
PDI-C36,39S
SGHS
B
constitutive
siRNA
siRNA+abb
siRNA+ab
Temp (°C)
endo H
chase time
0 min
5 min
15 min
30 min
r (%)
0.2
0.1
0.3
0.1
9.5
0.2
10.1
0.9
50.1
12.8
40.2
13.2
95.6
39.7
90.2
50.5
Stability(%)
32.4
0.6
38.7
2.8
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
HLAr
HLAs
β2m
degree(%)
control
siRNA
siRNA+abb
siRNA+ab
Exit rate
Stability
C
constitutive
siRNA+C36,39S
siRNA+F258W/I272A
Temp(°C)
endo H
chase time
0 min
5 min
15 min
30 min
r (%)
0.2
8.1
0.1
11.2
31.1
2.5
50.8
82.3
8.6
90.1
100
20.1
Stability(%)
42.1
9.8
12.2
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
HLAr
HLAs
β2m
degree(%)
constitutive
siRNA+C36,39S
siRNA+F258W/I272A
Exit rate
Stability

encoding either MIINFEKL or MIPAQFYIL (low- and high-affinity peptides for A2.1, respectively) were overexpressed under a CMV promoter in A2.1-HeLa cells. A2.1 molecules became oxidized with high-affinity peptides (Figure 4C, right panel, lane 5), whereas A2.1 showed predominantly the reduced form with low-affinity peptides (Figure 4C, right panel, lane 6), essentially the same redox state of A2.1 molecules as observed with exogenous peptides. These results indicate that the nature of peptides also influences the redox state of class I molecules.

Synergistic Action of the α2 Disulfide Bond and Peptide Binding Drives Selection of Optimal Peptides

We investigated separately the redox state of the pool of MHC class I molecules that did or did not associate with the peptide-loading complex. We transfected ICP47-expressing HeLa cells with either mock or PDI siRNA (Figure 5A). After metabolic labeling of cells for 1 hr, we used anti-TAP1 antibody to immunoprecipitate the peptide-loading complex from digitonin lysates containing equal numbers of cells. After two more rounds of immunoprecipitation with anti-TAP1 and anti-β_2m antibodies, the residual TAP-associated proteins and MHC class I-β_2m heterodimers were completely removed from the supernatant (Figure 5B, first panel). Regardless of the availability of peptides, about 10% to 15% of the total pool of cellular PDI was associated with the peptide-loading complex (Figure 5B, second panel, P1 and P3). In the steady state, over 90% of the total pool of cellular MHC class I molecules were associated with the peptide-loading complex (Figure 5B, third panel, compare P1 and S1), and this pool was fully oxidized in mock-transfected cells (Figure 5B, fourth panel, P1). The amount of class I molecules that associated with the peptide-loading complex in either PDI- or peptide-depleted cells was only 40% to 60% of the total pool of class I molecules (Figure 5B, third panel, P2 and P3). Most surprisingly, the class I molecules within the peptide-loading complex were partially reduced (Figure 5B, fourth panel, P2 and P3), suggesting that, in the peptide-loading complex, class I molecules are in dynamic equilibrium between an oxidized and reduced state, with equilibrium predominantly shifted to oxidation at the steady state. In cells that were depleted of both peptides and PDI, only trace amounts of class I molecules were associated with the peptide-loading complex (Figure 5B, third panel, P4), and this population represented predominantly the reduced form (Figure 5B, fourth panel, P4). The pool of class I molecules outside the peptide-loading complex showed exclusively the reduced form (Figure 5B, fourth panel, S1, S2, S3, and S4). The reduced disulfide bond within the peptide-loading complex seems very likely to be the one in the α2 domain (Figure 5E). The disulfide bond in the Ig-like α3 domain forms early after biosynthesis (Tector et al., 1997) and, once formed, is unlikely to be involved in further reduction because it is buried in the hydrophobic core of the Ig-like α3 domain (Dick, 2004).

We hypothesized that if PDI participates in α2 disulfide oxidation and reduction, PDI-class I heavy-chain disulfide intermediates might exist. We dissolved the TAP precipitates in nonreducing buffer containing 1% SDS and 10 mM *N*-ethylmaleimide (NEM), reprecipitated the diluted sample with K455 antibody (which recognizes class I heavy chain and β_2m in both assembled and nonassembled forms), and resolved the eluted material in nonreducing conditions. In mock-transfected conditions (P1), we detected, within the peptide-loading complex, faint but reproducible PDI-class I intermediates with an apparent molecular weight of 105 kDa (Figure 5C, lane 3). We used as many as 5×10^8 cells to detect this intermediate, anticipating that it would be extremely short lived. To confirm the identity of the intermediates, aliquots of the K455 reprecipitates were dissolved in 1% SDS, immunoprecipitated again with either K455 or anti-PDI antibody, and analyzed by SDS-PAGE in reducing conditions. Indeed, class I heavy chains and PDI were positively identified (Figure 5C, lanes 1 and 2). In peptide-depleted cells, the amount of PDI-class I heavy-chain intermediates inside the peptide-loading complex (P3) was significantly increased compared to the amount in mock-transfected cells (Figure 5C, compare lanes 9 and 3). The pool of free class I heavy chains outside the peptide-loading complex (S1 and S3) was also disulfide linked to PDI (Figure 5C, lanes 6 and 12) and was subsequently subjected to proteasome-mediated degradation (Figure 5D, upper panel, compare lanes 2 and 6). HCMV US11 was used as a positive control for proteasome-mediated degradation of class I molecules (Wiertz et al., 1996). In contrast, the class I pool within the peptide-loading complex (P) was stable under the same conditions (Figure 5D, lower panel, lanes 1–4). Thus, the PDI-class I heavy-chain conjugates outside the peptide-loading complex most likely represent the pool released from the peptide-loading complex rather than early folding intermediates during biosynthesis. These results argue strongly that, although PDI functions primarily as an oxidase, PDI can also reduce the α2 disulfide bond

Figure 3. Distinct PDI Domains Control the Maturation and Optimal Peptide Loading of MHC Class I Molecules

(A) Schematic representation of deletion or point mutants of PDI.

(B) Distinct role of each domain in the maturation and peptide loading of class I molecules. HeLa cells expressing PDI siRNA were transfected with the indicated deletion mutants; pulse labeled for 5 min; and chased for 0, 5, 15, or 30 min. After lysis in 1% NP-40, equal aliquots of the lysates were incubated at 4°C, 37°C, and 50°C for 1 hr and then immunoprecipitated with the mAb W6/32. The W6/32 precipitates were digested with endo H and analyzed by SDS-PAGE. For estimating thermostability, the percentage of class I molecules that survived heating to 50°C was plotted in relation to the value at 4°C at the 30 min time point. The ER exit rate was estimated by calculating the ratio of [r] band to the total protein ([r] + [s]) at 4°C for each time point. HLA^r, endo H resistant; HLA^s, endo H sensitive.

(C) Analysis similar to that in (B), except that each point mutant was cotransfected with siRNA.

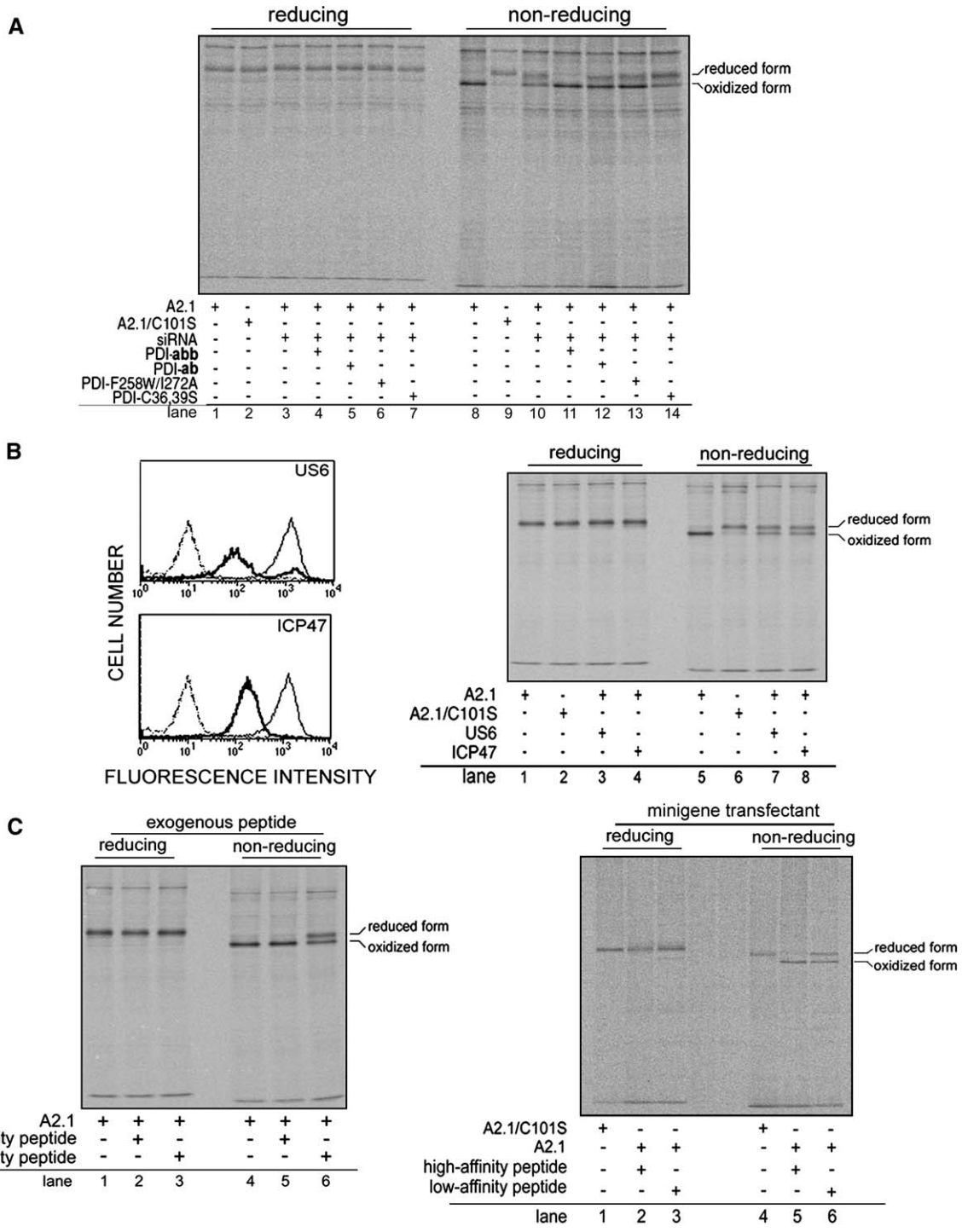


Figure 4. Link between Loading of Optimal Peptides and Thiol-Based Redox Regulation

(A) The catalytic and peptide-binding residues of PDI are critical for regulating the redox state of class I molecules. HeLa cells expressing the indicated combination of constructs were radiolabeled for 10 min. Postnuclear supernatants of 1% NP-40/10 mM NEM lysates were immunoprecipitated with the A2.1-specific BB7.2 antibody, and proteins were separated by SDS-PAGE under reducing and nonreducing conditions.

(B) Peptide starvation increases the level of the reduced class I molecules. The assay was performed as described in (A), but using HeLa cells stably expressing US6 or ICP47.

(C) The nature of peptides influences the redox state of class I molecules. A2.1-expressing HeLa cells were either incubated with exogenous peptides (10 μM) for 48 hr (left panel) or transfected with minigenes encoding either a high-affinity or low-affinity peptide ligand for A2.1 (right panel). The assay was performed as described in (A).

at a rate determined by the availability and nature of peptides.

To further identify the PDI-binding cysteine residue, we constructed a series of A2.1 cysteine mutations and found that they failed to integrate into the peptide-loading complex. In addition, detecting the A2.1 cysteine mutant-PDI conjugates was difficult because they were unstable (B.P. and K.A., unpublished data). We circumvented this problem by overexpressing A2.1 cysteine mutants and myc-tagged PDI simultaneously in HeLa cells and pulsing the cells for only 5 min. We found that cysteine-to-serine mutations in the α2 region (A2.1-C101S and A2.1-C164S), but not the cysteine-to-serine mutation in the α3 region (A2.1-C203S), disrupted the A2.1-PDI conjugate (Figure 5E), indicating that the PDI-binding cysteine is the one in the α2 domain. Together, these results strongly suggest that PDI directly catalyzes the making and breaking of α2 disulfide bonds.

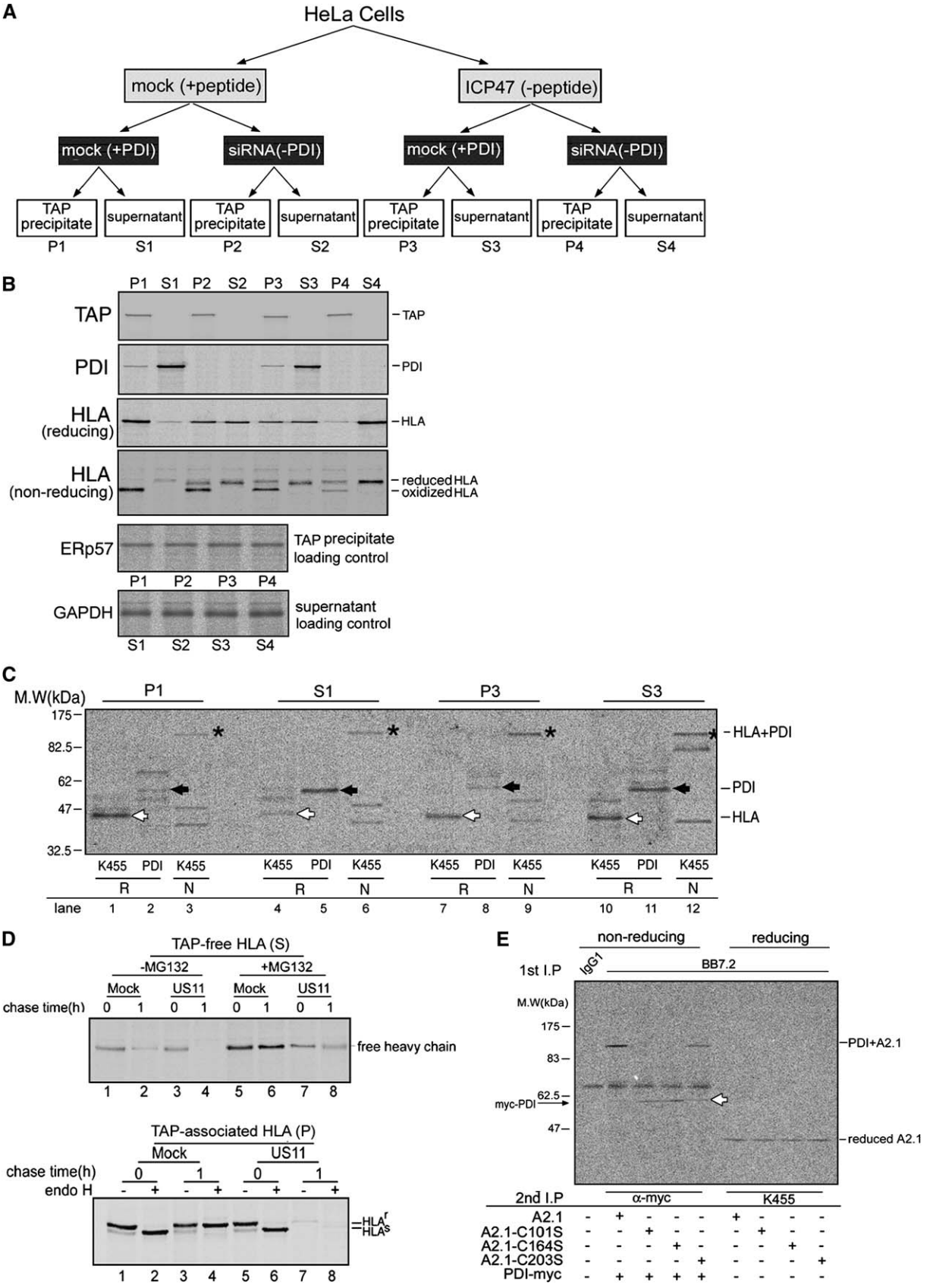
Viral Targeting of PDI-Catalyzed Editing of the Peptide Repertoire for Immune Evasion

We have shown that the human cytomegalovirus US3 protein interferes with the editing of the peptide repertoire of certain class I alleles and that anti-US3 immunoprecipitates obtained from digitonin lysates contain an unknown protein of about 60 kDa, the size of PDI (Park et al., 2004). To explore the possibility that PDI might be an ultimate cellular target for the action of US3, we examined whether US3 and PDI colocalize in the ER. In HeLa cells transiently expressing US3, the staining patterns of both US3 and PDI were analyzed by immunofluorescence staining. Surprisingly, we observed that the staining intensity of PDI was markedly diminished in the cells expressing US3 (Figure 6A), suggesting that US3 might cause PDI degradation. We therefore quantitated PDI in US3-expressing HeLa (HeLa-US3) cells by immunoblotting cell lysates with anti-PDI antibody, which recognizes either the C terminus (residues 499–509) or the b domain (residues 211–370) of PDI. PDI was almost completely absent from the HeLa-US3 cells (Figure 6B, first and second panel). This reduced level of PDI was not due to the masking of antigenic epitopes, as immunoblot analysis of the cell lysates of myc-tagged PDI transfectants with anti-myc antibody (Figure S3) revealed essentially the same result. We hypothesized that US3 must associate with PDI so as to trigger degradation. We examined the physical association of US3 and PDI in digitonin lysates of HeLa-US3 cells in the presence of MG-132. In anti-US3 coimmunoprecipitates, tapasin, class I heavy-chain molecules, and PDI were identified, indicated by reprecipitation with antibodies specific to each protein (Figure 6C). Thus, US3 associates with PDI, although a possible requirement for other proteins in the complex was not excluded. Most importantly, expression of US3 resulted in an increase in the steady-state level of the reduced form of A2.1 molecules in a dose-dependent manner (Figure 6D, lanes 8 and 9), similar to the redox state of A2.1 molecules in the cells depleted of PDI by siRNA (Figure 4A). These results demonstrate that the HCMV US3 protein exploits PDI degradation for $CD8^+$ T cell evasion, reflecting the functional relevance of PDI-mediated peptide editing against viral infections.

DISCUSSION

Our findings reveal a mechanism by which thiol-based redox reactions regulate the editing of the MHC class I peptide repertoire. The steady-state level of the reduced class I form was dependent on both the abundance and nature of peptides in the ER. Lack of peptides, or a supply of low-affinity peptides, led to an increase in the reduced class I form (Figure 4). Suboptimal peptides with a faster off rate might render the α2 disulfide bond more vulnerable to PDI reductase activity, whereas a high-affinity peptide, which will firmly fit into the MHC peptide-binding groove and have a longer off rate, makes the bond less susceptible to reduction. This idea is further strengthened by the three-dimensional structures of MHC class I molecules, which predict the burial of the α2 disulfide bond in the presence of peptides (Bjorkman et al., 1987). Our data indicate that, in the peptide-loading complex, most of the class I molecules are oxidized but the reduced form exists (Figures 5B–5D), suggesting equilibrium of a rapid thiol disulfide exchange between PDI and α2 disulfide, with oxidation of α2 disulfide heavily favored in the steady state. Taking into account this notion and the result that oxidation of the α2 disulfide bond is required for optimal peptide loading, we propose that the primary function of PDI in peptide loading is to keep the α2 disulfide bond oxidized. Based on the observation that mutagenesis of the N-terminal cysteines of tapasin disrupts formation of the ERp57-tapasin bond and renders class I molecules partially reduced, it has been proposed that the ERp57-tapasin conjugate is involved in the isomerization of the class I α2 disulfide bond and thus facilitates peptide loading (Dick et al., 2002). Arguing against the direct involvement of the ERp57-tapasin conjugate in isomerization of the class I α2 disulfide bond, analysis of ERp57-deficient mice shows that ERp57 does not influence the redox state of class I molecules but is an essential structural component for stable assembly of the peptide-loading complex (Garbi et al., 2006). Depletion of ERp57 by siRNA also does not affect the redox state of class I molecules and peptide loading (Zhang et al., 2006). Moreover, neither ERp57-class I nor tapasin-class I conjugates, the expected disulfide intermediates if class I heavy chains are reoxidized by ERp57 or tapasin, have been identified. Considering the critical nature of the N-terminal region of tapasin for stabilizing the peptide-receptive class I structure (Bangia et al., 1999), the increase of the reduced class I form with the tapasin mutant likely represents the secondary effect of peptide loading into a loosely folded class I binding groove, which mimics suboptimal peptide binding.

Our work also suggests that PDI is a chaperone for antigenic peptides. Expression of PDI-F258W/I272A, a dominant-negative point mutant regarding peptide binding, results in partial reduction of MHC class I molecules


A
HeLa Cells
mock (+peptide)
ICP47 (-peptide)
mock (+PDI)
siRNA(-PDI)
mock (+PDI)
siRNA(-PDI)
TAP precipitate
supernatant
TAP precipitate
supernatant
TAP precipitate
supernatant
TAP precipitate
supernatant
P1
S1
P2
S2
P3
S3
P4
S4
B
P1 S1 P2 S2 P3 S3 P4 S4
TAP
TAP
PDI
PDI
HLA (reducing)
HLA
HLA (non-reducing)
reduced HLA
oxidized HLA
ERp57
TAP precipitate loading control
P1 P2 P3 P4
GAPDH
supernatant loading control
S1 S2 S3 S4
C
M.W(kDa)
P1
S1
P3
S3
175
82.5
62
47
32.5
HLA+PDI
PDI
HLA
K455 PDI K455
R N
lane 1 2 3 4 5 6 7 8 9 10 11 12
D
TAP-free HLA (S)
-MG132
+MG132
Mock US11 Mock US11
chase time(h) 0 1 0 1 0 1 0 1
free heavy chain
1 2 3 4 5 6 7 8
TAP-associated HLA (P)
Mock US11
chase time(h) 0 1 0 1
endo H - + - + - + - +
HLA^r
HLA^s
1 2 3 4 5 6 7 8
E
non-reducing
reducing
1st I.P
IgG1
BB7.2
M.W(kDa)
175
83
62.5
47
myc-PDI
PDI+A2.1
reduced A2.1
2nd I.P
α-myc
K455
A2.1 - + - - - + - - -
A2.1-C101S - - + - - - + - -
A2.1-C164S - - - + - - - + -
A2.1-C203S - - - - + - - - +
PDI-myc - + + + + - - - -

(Figure 4A) and severely impairs optimal peptide loading (Figure 3C). Although we do not know whether the impaired peptide loading mediated by the PDI-F258W/I272A mutant resulted from the loss of peptide-carrier function or from a side effect that caused loss of catalytic function, this result implicates that the peptide-chaperoning property of the b′ domain might be critical for optimal peptide loading. Of note, in this condition, the MHC class I molecules are not empty but are still loaded with suboptimal peptides, suggesting a role for PDI in facilitating the speed and efficiency of optimal peptide selection. This result reconciles a seemingly contradictory observation by Lammert et al. (1997) in which β-estradiol inhibits peptide binding to PDI but not binding to MHC class I molecules, observed by chemical crosslinking. In the presence of β-estradiol, the peptides crosslinked to MHC class I molecules could be low-affinity peptides. Indeed, we found that, in β-estradiol-treated cells, MHC class I molecules were less thermostable than those in mock-treated cells (Figure S4). Consistent with the proposed role for PDI as a peptide carrier, the b′ domain of PDI, including the hydrophobic binding pocket, recognizes small peptides of 10 to 15 amino acids (Pirneskoski et al., 2004), and PDI is the dominant acceptor for TAP-translocated peptides (Lammert et al., 1997; Spee and Neefjes, 1997). As a peptide chaperone, PDI might facilitate optimal peptide loading either by protecting free peptides from the ER proteases or by delivering peptides into the class I molecules, or optimal loading might involve both mechanisms. Free peptides have a very short half-life in vivo and are most likely protected from degradation by their association with chaperones (Reits et al., 2003).

With the selective pressure of the host immune response, viruses have evolved several strategies to interfere with the antigen presentation pathway (Lilley and Ploegh, 2005). Our previous study showed that the HCMV US3 protein targets the peptide-loading complex and specifically interferes with peptide editing of MHC class I molecules (Park et al., 2004). Interestingly, this study showed that the inhibitory effect of US3 on peptide editing is more profound for tapasin-dependent class I alleles. Since PDI, ERp57, and tapasin are components of the peptide-loading complex and contain free cysteine residues, the possibility exists that PDI controls the redox network of tapasin involving ERp57. Assuming that PDI is indeed an upstream regulator of the tapasin redox network, and given that US3 triggers the degradation of PDI (Figure 6), the inhibition of editing of the MHC class I peptide repertoire observed in the presence of US3 might occur, in fact, via US3-mediated degradation of PDI. Our finding that the HCMV US3 protein targets PDI for immune evasion, regardless of the mechanism, underlines the critical role of PDI-mediated editing of the class I peptide repertoire in normal immune function.

In summary, we propose that editing of the MHC class I peptide repertoire involves two linked equilibriums: a rapid thiol disulfide exchange between PDI and the MHC class I α2 disulfide, and competition between peptides of various affinities for MHC class I binding (Figure 7). After oxidation of the α2 disulfide bond of MHC class I molecules in the peptide-loading complex, PDI transfers a peptide to the class I peptide-binding groove. Then, peptides compete for MHC binding until the MHC-peptide interaction induces a conformational change that is sufficient to trigger the release of the MHC-peptide from the peptide-loading complex, ultimately favoring the presentation of the most stable MHC-peptide complexes. Reduced class I molecules that fail to be rescued by reoxidation are prone to irreversible dissociation from the peptide-loading complex and proteolysis. Thus, the redox status of MHC class I molecules might be a checkpoint for quality control of MHC class I assembly. This regulated process would allow the host to induce rapid and sustained CTL responses against invading pathogens and, at the same time, avoid unwanted immune responses.

EXPERIMENTAL PROCEDURES

DNA Constructs

cDNAs encoding human PDI and PDI deletion mutants, kindly provided by Dr. Y. Nomura (Hokkaido University, Sapporo, Japan), were

Figure 5. PDI Directly Catalyzes Reduction and Oxidation of the α2 Disulfide Bond in the MHC Peptide-Binding Groove

(A) Schematic representation of the experimental protocol. Mock- or ICP47-expressing HeLa cells were transfected without (+PDI) or with (−PDI) PDI siRNA. 1% digitonin/10 mM NEM extracts of radiolabeled (60 min) cells were precipitated by TAP1 antiserum. The TAP immunoprecipitates were designated as P1–P4, and the corresponding supernatants were designated as S1–S4.

(B) Effect of peptides and PDI on the redox state of class I molecules. 1% NP-40/1% SDS/10 mM NEM eluates from P1–P4 were reprecipitated with the class I-specific K455 antibody. S1–S4 were directly immunoprecipitated with K445 and analyzed by reducing or nonreducing SDS-PAGE (third and fourth panels). P1–P4 and S1–S4 were also analyzed for the levels of TAP (first panel) and PDI (second panel).

(C) Existence of the disulfide intermediate between PDI and class I molecules. 1% SDS/10 mM NEM eluates from P1 and P3 were reprecipitated with K455 and analyzed by nonreducing SDS-PAGE (lanes 3 and 9). The identities of the protein bands were confirmed by dissolving K455 reprecipitates in 1% SDS, reprecipitating with either K455 or PDI antisera, and analyzing proteins by reducing SDS-PAGE (lanes 1, 2, 7, and 8). This analysis was repeated for S1 and S3. PDI-class I conjugates (black asterisk), PDI (black arrow), and class I molecules (white arrow) are indicated.

(D) Fate of PDI-conjugated class I molecules outside the peptide-loading complex. HeLa cells expressing the HCMV US11 glycoprotein were radiolabeled for 10 min and chased for 1 hr with or without 50 μM MG-132. The TAP immunoprecipitates (P) and corresponding supernatants (S) were prepared as described in (A). The supernatants were directly used for immunoprecipitation with K455 and analyzed by reducing SDS-PAGE (upper panel). The TAP immunoprecipitates were stripped in 1% SDS, and the class I molecules were reprecipitated with K455 prior to endo H treatment (lower panel).

(E) Identification of cysteine residues that bind PDI. MG-132-pretreated HeLa cells were cotransfected with PDI-myc and HLA-A2.1 or A2.1 cysteine mutants. Transfected cells were radiolabeled, lysed in 1% digitonin with 10 mM NEM, and immunoprecipitated with mAb BB7.2. One percent SDS eluates from the BB7.2 immunoprecipitates were reprecipitated with anti-myc or K455 antisera and analyzed by reducing or nonreducing SDS-PAGE. PDI is marked with the white arrow.

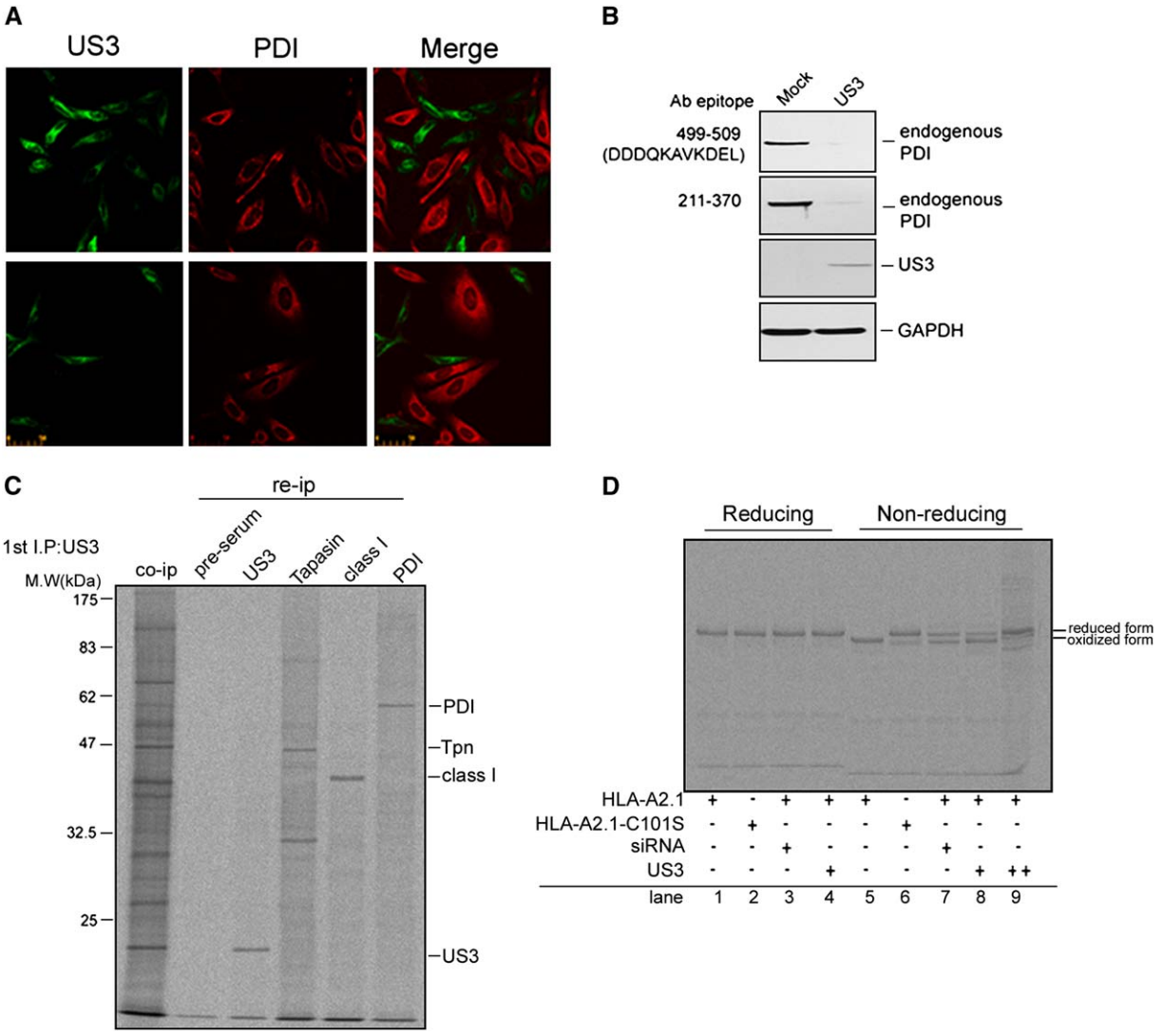


Figure 6. Virus Subversion of PDI for Immune Evasion

(A) Intracellular distribution of PDI in the presence of US3. HeLa cells were transfected with US3-GFP cDNA. Cells were stained with the anti-PDI C-terminal antibody. Bound primary antibodies were labeled with Texas red-conjugated anti-mouse secondary antibodies.

(B) Reduced levels of PDI in cells expressing US3. Lysates from HeLa-US3 cells were immunoblotted for US3 and PDI with the indicated antibodies.

(C) Association of US3 with PDI. HeLa-US3 cells were radiolabeled, lysed in 1% digitonin/NEM (10 mM), and immunoprecipitated with the anti-US3 antibody. Associated proteins were stripped in 1% SDS and reprecipitated with indicated antibodies.

(D) US3 results in an increase in the level of the reduced class I molecules. HeLa cells (1×10^6 cells) were cotransfected with A2.1 (2 μg) and either 2 μg (lane 8) or 4 μg (lane 9) of US3 cDNAs and radiolabeled for 10 min. Cell extracts were immunoprecipitated with mAb BB7.2, and the redox state of A2.1 was analyzed by SDS-PAGE in reducing and nonreducing conditions.

inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA, USA). 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, San Diego, CA, USA). 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 neo vector (Ambion, Austin, TX, USA). This siRNA provides knockdown of only PDI that contains the a′ domain.

Cell Lines and Antibodies

All constructs encoding deletion or point mutations were cotransfected with PDI siRNA into HeLa cells. The cells were partially selected by adding 1 mg/ml of puromycin to the growth medium (Sigma-Aldrich, St. Louis) for 3 days prior to analysis. T1, U937, and HLA-A2.1-expressing renal carcinoma MZ1851 RC cells were grown in RPMI 1640 medium supplemented with 10% FBS. Most antibodies used in this study have been described (Park et al., 2004; Wiertz et al., 1996). The human tapasin (R.gp48N) and human calnexin antibodies were kindly provided by Dr. P. Cresswell (Yale University, New Haven, CT, USA). The mAb ERp57 and anti-β_2m antibody BBM.1 were

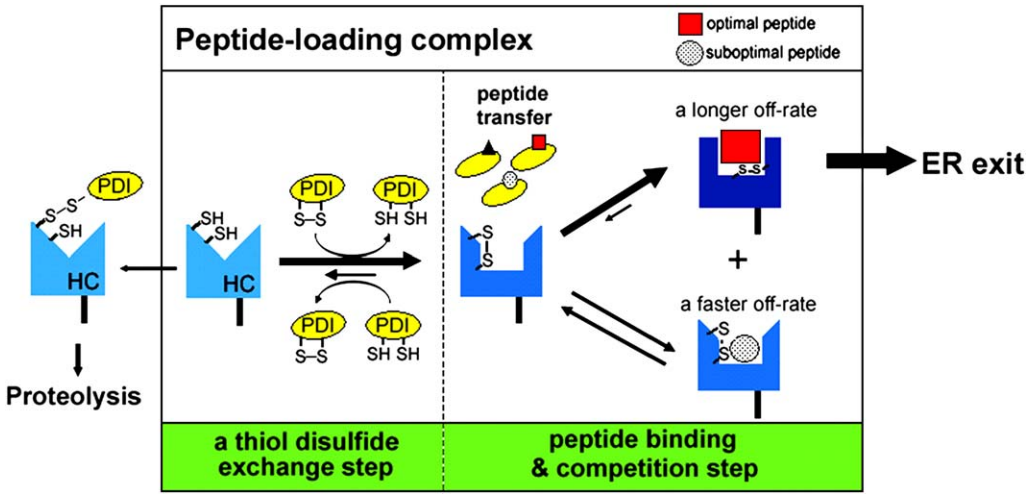


Figure 7. Hypothetical Model for PDI-Mediated Editing of the MHC Class I Peptide Repertoire

In the peptide-loading complex, the α2 disulfide of the MHC class I molecule is in dynamic equilibrium between oxidized and reduced states, with equilibrium predominantly shifted to oxidation at the steady state. Peptides of various affinities that are transferred by PDI compete to bind the oxidized class I molecule, a peptide-receptive form. Only MHC class I molecules with the α2 oxidized and that contain high-affinity peptides are selected for ER exit. Reduced class I molecules are either rapidly rescued by reoxidation for peptide binding or subjected to irreversible loss via dissociation from the peptide-loading complex and proteolysis.

purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-PDI antibodies recognizing the C terminus or b domain were from StressGen Biotechnologies (Victoria, BC, Canada) and BD Biosciences (San Jose, CA, USA), and anti-ERp72 and anti-CD series antibodies were from BD Biosciences.

Purification of TAP-Associated Proteins

HeLa cells were lysed in 1% digitonin with 10 mM NEM, and postnuclear lysates were immunoprecipitated with the anti-TAP1 antibody. Proteins were separated by SDS-PAGE and visualized by silver staining. Proteins in gel slices were digested with trypsin (Shevchenko et al., 1996) and analyzed by MS/MS (Bahk et al., 2004) as described. Proteins were identified by comparing experimental data to the NCBInr database by using the MASCOT search program (Matrix Science).

TAP Depletion Assays

HeLa cells expressing ICP47 or PDI siRNA were radiolabeled for 1 hr and solubilized in 1% digitonin with 10 mM NEM and protease inhibitors. Postnuclear lysates were immunoprecipitated with anti-TAP1 antibodies, and the TAP immunoprecipitates were designated as P (pellet fraction). The pool of pure free heavy chains was obtained by further preclearing of the resulting supernatants by immunoprecipitation with the β_2m-reactive BBM.1 antibody, which removed the class I-β_2m heterodimers, and was designated as S (supernatant fraction). For analysis of the redox state of class I heavy chains in the TAP precipitates or supernatant fractions, TAP-associated proteins were eluted by heating to 95°C in 1% SDS, and the eluted proteins were then diluted into 1% NP-40 in PBS and reprecipitated with K455. The supernatant fractions were used directly for immunoprecipitation with K455 and eluted by reducing or nonreducing buffer. For detecting the PDI-class I heavy-chain intermediates and to confirm the identity of the intermediates, labeled cells were lysed in 1% digitonin in the presence of 10 mM NEM, immunoprecipitated with K455, and analyzed by nonreducing SDS-PAGE. Aliquots of the K455 immunoprecipitates were eluted in 1% SDS, and the eluted proteins were diluted into 1% NP-40 in PBS, reprecipitated again with K455 or PDI antibody, and analyzed by reducing SDS-PAGE.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/2/369/DC1/.

ACKNOWLEDGMENTS

We thank Y. Nomura for PDI cDNA constructs, M. Theobald for MZ1851 RC cell lines, and P. Cresswell for anti-tapasin antibodies. We also thank Drs. L. Ruddock, T. Dick, and P. Cresswell for helpful discussions. This work was supported by National Creative Research Initiative Program of MOST/KOSEF. E.K. was supported by the BK21 fellowship.

Received: August 22, 2005
Revised: June 14, 2006
Accepted: August 11, 2006
Published: October 19, 2006

REFERENCES

Ahn, K., Gruhler, A., Galocha, B., Jones, T.R., Wiertz, E.J., Ploegh, H.L., Peterson, P.A., Yang, Y., and Fruh, K. (1997). The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. Immunity *6*, 613–621.

Antoniou, A.N., Ford, S., Alphey, M., Osborne, A., Elliott, T., and Powis, S.J. (2002). The oxidoreductase ERp57 efficiently reduces partially folded in preference to fully folded MHC class I molecules. EMBO J. *21*, 2655–2663.

Bahk, Y.Y., Kim, S.A., Kim, J.S., Euh, H.J., Bai, G.H., Cho, S.N., and Kim, Y.S. (2004). Antigens secreted from Mycobacterium tuberculosis: identification by proteomics approach and test for diagnostic marker. Proteomics *4*, 3299–3307.

Bangia, N., Lehner, P.J., Hughes, E.A., Surman, M., and Cresswell, P. (1999). The N-terminal region of tapasin is required to stabilize the MHC class I loading complex. Eur. J. Immunol. *29*, 1858–1870.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. Nature *329*, 506–512.

Dick, T.B. (2004). Assembly of MHC class I peptide complexes from the perspective of disulfide bond formation. Cell. Mol. Life Sci. *61*, 547–556.

Dick, T.P., Bangia, N., Peaper, D.R., and Cresswell, P. (2002). Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. Immunity *16*, 87–98.

Edman, J.C., Ellis, L., Blacher, R.W., Roth, R.A., and Rutter, W.J. (1985). Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. Nature *317*, 267–270.

Elliott, T., and Williams, A. (2005). The optimization of peptide cargo bound to MHC class I molecules by the peptide-loading complex. Immunol. Rev. *207*, 89–99.

Garbi, N., Tanaka, S., Momburg, F., and Hammerling, G.J. (2006). Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. Nat. Immunol. *7*, 93–102.

Grubman, M.J., and Baxt, B. (2004). Foot-and-mouth disease. Clin. Microbiol. Rev. *17*, 465–493.

Heemels, M.T., and Ploegh, H.L. (1994). Substrate specificity of allelic variants of the TAP peptide transporter. Immunity *1*, 775–784.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and Johnson, D. (1995). Herpes simplex virus turns off the TAP to evade host immunity. Nature *375*, 411–415.

Klappa, P., Ruddock, L.W., Darby, N.J., and Freedman, R.B. (1998). The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. EMBO J. *17*, 927–935.

Kropshofer, H., Vogt, A.B., Moldenhauer, G., Hammer, J., Blum, J.S., and Hammerling, G.J. (1996). Editing of the HLA-DR-peptide repertoire by HLA-DM. EMBO J. *15*, 6144–6154.

Lammert, E., Stevanovic, S., Brunner, J., Rammensee, H.G., and Schild, H. (1997). Protein disulfide isomerase is the dominant acceptor for peptides translocated into the endoplasmic reticulum. Eur. J. Immunol. *27*, 1685–1690.

Lehner, P.J. (2003). The calculus of immunity: quantitating antigen processing. Immunity *18*, 315–317.

Lewis, J.W., and Elliott, T. (1998). Evidence for successive peptide binding and quality control stages during MHC class I assembly. Curr. Biol. *8*, 717–720.

Lilley, B.N., and Ploegh, H.L. (2005). Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond. Immunol. Rev. *207*, 126–144.

Momburg, F., and Hammerling, G.J. (1998). Generation and TAP-mediated transport of peptides for major histocompatibility complex class I molecules. Adv. Immunol. *68*, 191–256.

Momburg, F., Roelse, J., Hammerling, G.J., and Neefjes, J.J. (1994a). Peptide size selection by the major histocompatibility complex-encoded peptide transporter. J. Exp. Med. *179*, 1613–1623.

Momburg, F., Roelse, J., Howard, J.C., Butcher, G.W., Hammerling, G.J., and Neefjes, J.J. (1994b). Selectivity of MHC-encoded peptide transporters from human, mouse and rat. Nature *367*, 648–651.

Montoya, M., and Del Val, M. (1999). Intracellular rate-limiting steps in MHC class I antigen processing. J. Immunol. *163*, 1914–1922.

Noiva, R. (1999). Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. Semin. Cell Dev. Biol. *10*, 481–493.

Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I–restricted antigen processing. Annu. Rev. Immunol. *16*, 323–358.

Park, B., Kim, Y., Shin, J., Lee, S., Cho, K., Fruh, K., and Ahn, K. (2004). Human cytomegalovirus inhibits tapasin-dependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion. Immunity *20*, 71–85.

Pirneskoski, A., Klappa, P., Lobell, M., Williamson, R.A., Byrne, L., Alanen, H.I., Salo, K.E., Kivirikko, K.I., Freedman, R.B., and Ruddock, L.W. (2004). Molecular characterization of the principal substrate binding site of the ubiquitous folding catalyst protein disulfide isomerase. J. Biol. Chem. *279*, 10374–10381.

Princiotta, M.F., Finzi, D., Qian, S.B., Gibbs, J., Schuchmann, S., Buttgereit, F., Bennink, J.R., and Yewdell, J.W. (2003). Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity *18*, 343–354.

Rammensee, H., Bachmann, J., Emmerich, N.P., Bachor, O.A., and Stevanovic, S. (1999). SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics *50*, 213–219.

Rammensee, H.G., Falk, K., and Rotzschke, O. (1993). Peptides naturally presented by MHC class I molecules. Annu. Rev. Immunol. *11*, 213–244.

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J.W., and Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunity *18*, 97–108.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Roelse, J., Gromme, M., Momburg, F., Hammerling, G., and Neefjes, J. (1994). Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. J. Exp. Med. *180*, 1591–1597.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850–858.

Sijts, A.J., and Pamer, E.G. (1997). Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. J. Exp. Med. *185*, 1403–1411.

Smith, J.D., Solheim, J.C., Carreno, B.M., and Hansen, T.H. (1995). Characterization of class I MHC folding intermediates and their disparate interactions with peptide and beta 2-microglobulin. Mol. Immunol. *32*, 531–540.

Spee, P., and Neefjes, J. (1997). TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. Eur. J. Immunol. *27*, 2441–2449.

Tector, M., Zhang, Q., and Salter, R.D. (1997). Beta 2-microglobulin and calnexin can independently promote folding and disulfide bond formation in class I histocompatibility proteins. Mol. Immunol. *34*, 401–408.

Warburton, R.J., Matsui, M., Rowland-Jones, S.L., Gammon, M.C., Katzenstein, G.E., Wei, T., Edidin, M., Zweerink, H.J., McMichael, A.J., and Frelinger, J.A. (1994). Mutation of the alpha 2 domain disulfide bridge of the class I molecule HLA-A*0201. Effect on maturation and peptide presentation. Hum. Immunol. *39*, 261–271.

Weber, D.A., Evavold, B.D., and Jensen, P.E. (1996). Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. Science *274*, 618–620.

Wiertz, E.J., Jones, T.R., Sun, L., Bogyo, M., Geuze, H.J., and Ploegh, H.L. (1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell *84*, 769–779.

Williams, A.P., Peh, C.A., Purcell, A.W., McCluskey, J., and Elliott, T. (2002). Optimization of the MHC class I peptide cargo is dependent on tapasin. Immunity *16*, 509–520.

Zhang, Y., Baig, E., and Williams, D.B. (2006). Functions of ERp57 in the folding and assembly of major histocompatibility complex class I molecules. J. Biol. Chem. *281*, 14622–14631.