ERp57 is a thiol oxidoreductase of the endoplasmic reticulum that appears to be recruited to substrates indirectly through its association with the molecular chaperones calnexin and calreticulin. However, its functions in living cells have been difficult to demonstrate. During the biogenesis of class I histocompatibility molecules, ERp57 has been detected in association with free class I heavy chains and, at a later stage, with a large complex termed the peptide loading complex. This implicates ERp57 in heavy chain disulfide formation, isomerization, or reduction as well as in the loading of peptides onto class I molecules. In this study, we show that ERp57 does indeed participate in oxidative folding of the heavy chain. Depletion of ERp57 by RNA interference delayed heavy chain disulfide bond formation, slowed folding of the heavy chain α domain, and caused slight delays in the transport of class I molecules from the endoplasmic reticulum to the Golgi apparatus. In contrast, heavy chain-β2m-microglobulin association kinetics were normal, suggesting that the interaction between heavy chain and β2m-microglobulin does not depend on an oxidized α domain. Likewise, the peptide loading complex assembled properly, and peptide loading appeared normal upon depletion of ERp57. These studies demonstrate that ERp57 is involved in disulfide formation in vivo but does not support a role for ERp57 in peptide loading of class I molecules. Interestingly, depletion of another thiol oxidoreductase, ERp72, had no detectable effect on class I biogenesis, consistent with a specialized role for ERp57 in this process.

Protein folding within the endoplasmic reticulum (ER) is assisted by a diverse array of folding catalysts and molecular chaperones. Folding catalysts include as many as 17 members of the protein-disulfide isomerase (PDI) family of proteins as well as peptidylprolyl cis-trans isomerases. ERp57 (1–3) is a member of the PDI family and contains four thioredoxin domains with two CXXC active sites located within the first and fourth domains (4, 5). Like PDI (6, 7), ERp57 has been shown to catalyze disulfide oxidation, isomerization, and reduction in vitro (8–10). However, it is distinct from other PDI family members in that it associates noncovalently with the molecular chaperones calnexin (CNX) and calreticulin (CRT) (11). These chaperones bind preferentially to nonnative glycoproteins bearing monoglucosylated Glc1Man5–9GlcNAc2 oligosaccharides (12, 13). It is thought that the ternary complex of ERp57, CNX/CRT, and unfolded glycoprotein enhances the rate of disulfide formation/isomerization by keeping the enzyme in proximity to the substrate. Consistent with this view, the activity of ERp57 toward monoglucosylated glycoprotein substrates is dramatically enhanced in vitro when CNX or CRT is present (10). Furthermore, ERp57 forms mixed disulfide intermediates with model viral glycoproteins in vitro, and these complexes are abrogated when the formation of monoglucosylated oligosaccharides is blocked (14). Despite these suggestive findings, there is little evidence that ERp57 actually promotes oxidative folding of any protein in living cells.

To address the functions of ERp57 in cells, we have been studying the biogenesis of class I molecules of the major histocompatibility complex (MHC), a system in which interactions with ERp57 are well established. Class I molecules bind to cytosolically derived peptide antigens and subsequently display them at the cell surface, where they are screened by cytotoxic T cells. They consist of a glycosylated transmembrane heavy chain (H chain) with three extracellular domains termed α1, α2, and α3, a soluble subunit termed β2m-microglobulin (β2m), and a peptide ligand of 8–10 residues. Assembly of class I molecules begins within the ER, where the nascent H chain binds to the membrane-bound chaperone CNX and, indirectly, to ERp57 (15, 16). At this early stage, H chain disulfide bond formation takes place, although the order of formation of the two disulfides within the α3 and α1 domains remains unclear (17). The efficiency of complete disulfide formation is substantially increased upon H chain association with β2m (17, 18). Correct disulfide bond formation is crucial, since mutagenesis of cysteines comprising either disulfide results in reduced cell surface expression and defects in peptide loading (19, 20). Once H chain associates with β2m, a peptide loading complex assembles that consists of the class I heterodimer, CNX, or its soluble paralog CRT, ERp57, Bap31, the TAP peptide transporter, and another protein, tapasin. The peptides that bind to class I are primarily generated in the cytosol by the proteasome and are transported into the ER by the TAP transporter. Tapasin serves as a bridge between class I and TAP (21, 22), thereby placing class I molecules close to the peptide source. In addition, tapasin stabilizes the peptide loading complexes and enhances the loading of high affinity peptides into the class I binding groove (23, 24). Upon peptide binding to class I, the loading complex dissociates, thereby permitting fully assembled class I molecules to be exported from the ER to the cell surface, a process that is facilitated in the ER by Bap31 (25).

Relative to other members of the PDI family, ERp57 appears to enjoy a privileged position in class I biogenesis. ERp57, but not PDI, has been detected in association with both free and β2m-associated H chains and as a component of the peptide loading complex (8, 26, 27). Furthermore, Cresswell and co-workers have demonstrated that between 15 and 80% of the total ERp57 pool can be associated with class I peptide loading complexes and that ERp57 is present exclusively in disulfide linkage to tapasin (28). The introduction of cysteine mutants in tapasin not only abolishes the formation of the ERp57–tapasin complex but also prevents full oxidation of the class I H chain and impedes the loading of high
affinity peptides (29). Whether these phenotypes are due to the lack of ERp57 or a secondary consequence of the tapasin mutations remains to be determined. These studies have led to suggestions that ERp57 may participate in the oxidative folding of the class I H chain, and, together with tapasin, may make class I molecules more receptive to peptide loading through isomerization of the disulfide bond within the peptide binding groove.

In this study, we used RNA interference to reduce the expression of ERp57 as a means to assess its functions in class I biogenesis. We show that in the absence of ERp57, H chain disulfide bond formation and α1 domain folding are substantially delayed, whereas the rate of ER to Golgi transport is slowed only slightly. No effect of ERp57 depletion was observed on the kinetics of H-chain β2m association, assembly of the peptide loading complex, or the loading of peptides onto class I. These findings support a role for ERp57 in H chain disulfide formation but suggest that the ERp57-tapasin disulfide conjugate may not be required for peptide loading. Interestingly, reducing the expression of another ER thiol oxidoreductase, ERp72, was completely without effect on class I biogenesis, consistent with a specialized role for ERp57 in this process and the existence of substrate preferences among different thiol oxidoreductases.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—Mouse L cells stably expressing the class I molecules, H-2Kb or H-2Dd, were maintained in high glucose DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Mouse EL4 cells expressing a truncated version of ovalbumin (residues 253–386; a kind gift of Dr. N. Shastri, University of California Berkeley) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics. The following antibodies (Abs) were used in this study: anti-8, a rabbit polyclonal antiserum, which recognizes the carboxyl terminus of the Kb molecule encoded by exon-8 (30); monoclonal antibody (mAb) 34-2-12S, which recognizes the folded α1 domain of Dd molecules with an intact disulfide bond (17, 31); mAb 34-5-8S, specific for β2m-associated Dd molecules (31); mAbs Y3 and 20-8-4S, which recognize β2m-associated Kb molecules (32, 33); and mAb 25-D1-16, which is specific for Kb molecules complexed with the ovalbumin-derived peptide SINFEKL (34). Rabbit anti-tapasin antiserum directed against the carboxyl-terminal 20 amino acids of murine tapasin and anti-CNX antiserum raised against the ER luminal domain of dog CNX have been described previously (35, 36). Anti-ERp57 antiserum was raised against glutathione S-transferase-fused mouse ERp57. The anti-glutathione S-transferase antibodies were removed by adsorption to glutathione S-transferase-agarose prior to use. Anti-CRT antiserum (SPA-600), anti-ERp72 antiserum (SPA-720), and anti-PDI antiserum (SPA-891) were purchased from StressGen Biotechnologies (Victoria, Canada). Rabbit anti-actin antiserum was purchased from Sigma. Anti-CalBPI antiserum was the generous gift of Dr. D. Ferrari (Max Planck Institute for Biophysical Chemistry, Goettingen, Germany).

**RNA Interference and Transfections**—Two double-stranded small interfering RNAs (siRNAs) corresponding to mouse ERp57 DNA sequences AGCCGACACTTGAGAGATAA (siRNA 1) and AAGAGCCTTGCCCCGATAGT (siRNA 2) were synthesized and annealed by Qiagen (Valencia, CA). siRNA 2 was fluorescein isothiocyanate-labeled to facilitate measurement of transfection efficiency. siRNA targeting mouse ERp72 (AAGCAGTTTGGTCCTACGATATT) and nontargeting control siRNA (AATTCTCCGAACGTGTCACGT) were also purchased from Qiagen. Eighteen hours before transfection, 1 × 10⁶ mouse L cells expressing H-2Kb or H-2Dd were seeded into 6-well dishes. siRNAs were transfected into the cells using oligofectamine (Invitrogen) according to the manufacturer’s protocol with a final siRNA concentra-

**Metabolic Radiolabeling and Immunoisolation—**Pulse-chase radio-labeling experiments with siRNA-transfected L cells expressing either Kb or Dd molecules were performed in 35-mm plates. Cells were starved for 30 min with methionine-free RPMI 1640 and pulse-labeled for 2 min with 0.5 mCi of [³⁵S]Met (>1000 Ci/mmol; Amersham Biosciences). Cells were then washed with Met-free RPMI 1640 and chased for various periods in DMEM containing 1 mM Met and 500 μM cycloheximide. After washing for 3 min in cold PBS containing 20 mM N-ethylmaleimide (NEM), lysis was conducted in 500 μl of PBS, pH 6.8, containing 1% Nonidet P-40, 20 mM NEM, and protease inhibitors (60 μg/ml 2-aminoethylbenzenesulfonylfluoride and 10 μg/ml each leupeptin, antipain, and pepstatin (BioShop, Burlington, Canada)). Following centrifugation at 10,000 × g to remove nuclei and cell debris, the supernatant fraction was incubated on ice for 2 h with either anti-8 or 20-8-4S Ab for Kb and either 34-2-12S or 34-5-8S Ab for Dd. Immune complexes were recovered by shaking for 1 h with 30 μl of packed protein A-agarose beads. Proteins were eluted with SDS-PAGE sample buffer from the beads and analyzed either by nonreducing or reducing SDS-PAGE (10% gel) followed by fluorography. Films were scanned using an EPSON 1680 scanner and were quantified using NIH Image software (National Institutes of Health). In all cases, backgrounds were subtracted by quantifying a blank area of the film corresponding in size to that of the gel band of interest.

In experiments to assess the formation of the peptide loading complex, cells were radiolabeled for 45 min with [³⁵S]Met, washed in cold PBS containing 20 mM NEM, and lysed in digitonin lysis buffer (1% digitonin in PBS, pH 6.8, 20 mM N-ethylmaleimide, and protease inhibitors). The lysate was incubated with anti-tapasin antiserum for 2 h. Immune complexes were recovered with protein A-agarose and then washed twice with 0.2% digitonin in PBS, pH 6.8, before elution and analysis by SDS-PAGE.

For class I ER to Golgi transport assays, Kb and Dd molecules were immunopurified with anti-8 and 34-2-12S Ab, respectively, as described above and then were eluted from protein A beads with 0.1 M citrate buffer, pH 6, containing 0.1% SDS and digested with 2 units of endo-β-N-acetylglucosaminidase H (endo H; New England Biolabs, Beverly, MA) at 37 °C for 2 h prior to analysis by SDS-PAGE.

**Flow Cytometry Analysis**—To assess the cell surface levels of class I molecules, 3–5 × 10⁶ cells were trypsinized from culture dishes and incubated with mouse anti-class I mAbs (1.5 μg of mAb Y3 for Kb or mAb 34-5-8S for Dd) in 100 μl of culture medium for 20 min on ice. After incubation, cells were washed once with fluorescence-activated cell sorting buffer (Hanks’ balanced salt solution, 1% bovine serum albumin, and 0.01% NaN₃) and then incubated with 0.4 μg of phycoerythrin-conjugated goat anti-mouse IgG (H+L chain-specific, Cedarlane, Hornby, Canada) in 100 μl of fluorescence-activated cell sorting buffer for 20 min on ice. Cells were washed twice with fluorescence-activated
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cell sorting buffer and then fixed in 0.5% paraformaldehyde in PBS, pH 7.4. Subsequent flow cytometry was performed using an EPICS Elite flow cytometer (Beckman Coulter, Fullerton, CA).

For analysis of the turnover kinetics of cell surface class I molecules, Kb- or Dd-expressing L cells were incubated for 18 h at 26 °C in DMEM containing 10 µg/ml human β2m (Sigma). Human β2m-containing DMEM was then replaced with prewarmed DMEM containing 10 µg/ml brefeldin A (Sigma), and cells were transferred to a 37 °C incubator. Cells (3–5 × 10^6) were removed at the indicated time points and analyzed by flow cytometry as described above.

Kb-SINFEKL complexes on the surface of EL4 TO cells were measured by flow cytometry using mAb 25-D1-16. The SINFEKL peptide loading efficiency was expressed as the Kb-SINFEKL level divided by the surface Kb level (as detected with mAb Y3).

Quantification of Interferon α and β mRNAs—L cells expressing Kb molecules were either untransfected or transfected with control siRNA, siRNA targeting ERp57, and siRNA targeting ERp72. Cells were harvested after 12 h, and total RNA was extracted with the RNeasy Mini Kit (Qiagen). Digestion of contaminating DNA was performed using Fermentas DNaseI according to the manufacturer’s protocol. cDNA was synthesized using 1 µg of RNA in the presence of random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer’s protocol.

For quantitative PCR, reaction components were obtained from the LightCycler® FastStart DNA Master SYBR GreenPLUS I kit (Roche Applied Science). The LightCycler® instrument (Roche Applied Science) and corresponding software was used for all reactions. The following reaction conditions were used: preincubation at 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 10 s, 60 °C for 10 s, and 68 °C for 10 s. The half-time for melting curve analysis at 95 °C for 0 s, 60 °C for 15 s, and a continuous acquisition mode of 95 °C with a temperature transition rate of 0.1 °C/s. The PCR was performed in a final volume of 20 µl containing 1 × Master SYBR GreenPLUS I buffer, 20 pmol of HPRT and ERp72 primers, 10 pmol of ERp57 primers, and 5 µl of template cDNA (concentration 100 ng/µl). The following primer sets were used: for IFN-α2, 5′-AAAGGGGAGCCTCCTCAT-3′ (forward) and 5′-TGTGTTTCTCGTGATGCTGA-3′ (reverse); for IFN-β, 5′-ACACACATTAAACCCATGAAACAACAGGTGGATCTCCAGG-3′ (forward) and 5′-GTTAGGAATTCTCAGTTTTGGAAGTTTCTGGTAAGT-3′ (reverse). Standard curves for IFN-α2, IFN-β, and HPRT were established using serial dilutions of template cDNA in triplicate in the RltQuant software (Roche Applied Science). Levels of IFN-α2, IFN-β, and HPRT mRNA were calculated based on these established standard curves, and the amounts of IFN-α2 and IFN-β mRNA were normalized to the amount of HPRT in each sample. All samples were analyzed in triplicate.

RESULTS

Delayed H Chain Disulfide Bond Formation Is Observed upon Depletion of ERp57 but Not ERp72—To study the function of ERp57 in the process of class I biogenesis, we reduced the expression of ERp57 in L cells expressing the class I H-2Kb molecule by transfecting with 21-bp siRNAs. Following a 4-day transfection with siRNA 1, greater than 90% of ERp57 was depleted (Fig. 1A). No expression differences were observed for several other proteins, including CNX or the thiol oxidoreductases ERp72, PDI, and CaBP1. As a control to assess the effect of reducing the expression of a thiol oxidoreductase not previously implicated in class I biogenesis, we performed a similar experiment using siRNA directed against ERp72. Again, the level of ERp72 was reduced by >90% with no effects on the expression of ERp57 or several other proteins (Fig. 1B).

Based on previous studies suggesting an interaction of ERp57 with class I H chains (8, 27), we anticipated that this enzyme might participate in H chain disulfide formation. Consequently, the effect of ERp57 depletion on H chain disulfide bond formation was monitored in a pulse-chase experiment. After transfecting with ERp57 siRNA 1 or with control siRNA, L cells expressing class I-H-2Kb molecules were radiolabeled for 2 min with [35S]Met and chased in medium containing unlabeled Met and cycloheximide for periods of up to 30 min. Kb molecules were recovered from cell lysates with anti-8, an antiserum that reacts with free or β2m-associated Kb H chains. Isolated Kb H chains were analyzed by SDS-PAGE under nonreducing conditions to allow resolution of species containing zero, one, or two disulfide bonds. The results show that disulfide bond formation was substantially slowed in the absence of ERp57 (Fig. 2A, top; quantified in Fig. 2B, siRNA 1). In control cells, 50% of H chains acquired both disulfide bonds by ~2 min of chase, whereas in ERp57-depleted cells, the rate was 9-fold slower, with 50% of H chains acquiring both disulfide bonds by 18 min of chase. In this experiment, no zero-disulfide bond species was detected, possibly because the first disulfide bond formed co-translationally and therefore was already present following the 2-min pulse. To confirm these results, the same experiment was performed using siRNA 2, which targets a different region of the ERp57 mRNA (Fig. 2A, middle). In this case, the rate of H chain disulfide formation was slowed 11-fold upon depletion of ERp57 (quantified in Fig. 2B, siRNA 2). The half-time for acquisition of both disulfide bonds was ~2 min in control cells versus 22 min in ERp57-depleted cells. In experiments testing the effect of ERp57 siRNA 2 on various control proteins, it was noted that this siRNA caused a ~50% reduction of ERp72 expression in addition to the depletion of ERp57 (data not shown). However, this “off-target” depletion of ERp72 by siRNA 2 did not contribute to the observed slower H chain oxidation, since knockdown of ERp72 expression alone by >90% had no significant effect on H chain oxidation (Fig. 2A, bottom; quantified in Fig. 2B). The lack of effect due to ERp72 depletion, coupled with previous studies showing a lack of PDI interaction during class I biogenesis (26), suggests that ERp57 may be the preferred thiol oxidoreductase promoting oxidative class I H chain folding.

ERp57 Is Required for α3 Domain Folding but Not for H Chain-β2m Association—As an independent means to assess the involvement of ERp57 in H chain disulfide formation, the folding rate of the H chain α3 domain of class I H-2Dd molecules was assessed using mAb 34-2-12S. This antibody only recognizes Dd molecules that have a folded α3 domain with an intact disulfide bond (17). In this experiment, L cells expressing Dd molecules were transfected with ERp57 siRNA 1 or con-

![Image](357x651 to 520x734)

**FIGURE 1.** Silencing effect of RNA interference on ERp57 and ERp72 expression. Kb-transfected L cells (1 × 10^6; well in a 6-well plate) were transfected with 40 ng siRNA targeting ERp57 (A) or ERp72 (B) mRNA or the same concentration of a control siRNA that is known not to affect the expression of mammalian proteins. After 4 days of transfection, cells were lysed, and equal amounts of cell lysate were subjected to SDS-PAGE and immunoblotted with the indicated antibodies.
The Peptide Loading Complex Assembles Properly upon Depletion of ERp57—Cresswell and co-workers (29) have demonstrated that ERp57 is disulfide-linked to tapasin within the peptide loading complex. Consequently, it was of interest to determine whether the peptide loading complex can assemble in the absence of ERp57. To test this, cells transfected with control or ERp57 siRNA were radiolabeled for 45 min and lysed in the mild detergent, digitonin, which preserves the integrity of the peptide loading complex. Anti-tapasin antibody was then used to immunoisolate the complex. As shown in Fig. 5A (left), all components of this protein complex were identified in cells transfected with control siRNA: TAP1, TAP2, CRT, ERp57, tapasin, H chain, and β2m. Under nonreducing conditions (Fig. 5A, right), several components, including CRT, ERp57, and H chain, migrated faster due to the presence of intramolecular disulfide bonds. Also, under nonreducing conditions, the complex containing ERp57 disulfide linked to tapasin could be detected at ∼110 kDa. Upon ERp57 depletion, the ERp57 band (Fig. 5A, left) was absent as expected, whereas the tapasin-ERp57 band (Fig. 5A, right panel) appeared diminished rather than absent. However, a number of faint background bands were present in the tapasin-ERp57 region of the gel, making it difficult to identify this species conclusively following ERp57 knockdown. To address this issue, lysates of control and ERp57-depleted cells were subjected to nonreducing SDS-PAGE and
immunoblotted with anti-tapasin or anti-ERp57 Ab. As shown in Fig. 5B, ERp57 depletion reduced the amount of tapasin-ERp57 complex by 70% as detected with anti-ERp57 Ab and by 90% as detected with anti-tapasin Ab. Despite this extensive loss of the tapasin–ERp57 complex, all other components of the peptide loading complex remained fully associated (Fig. 5A, both panels). Although we cannot exclude the possibility...
that the low level of residual ERp57 might be sufficient to maintain full assembly of the peptide loading complex, it seems more likely that the loading complex is able to assemble normally without ERp57. It is noteworthy that there was no apparent change in the oxidation state of the H chain in the presence or absence of ERp57 (Fig. 5A, compare H chain mobilities in nonreducing lanes). It has been shown previously that when the disulfide cross-link between ERp57 and tapasin is prevented by mutation of tapasin, peptide loading of class I molecules may either be impaired (29) or unaltered (38), depending on the class I molecule examined. To determine if peptide loading is affected upon ERp57 depletion, we first examined the cell surface expression of class I molecules, since loading defects are often accompanied by reduced levels of class I molecules at the cell surface. Surprisingly, the steady state levels of Kb and Dd molecules at the cell surface were about 20% higher when ERp57 was depleted by siRNA treatment compared with control siRNA-treated cells (Fig. 7A).

The thermal stability of a class I molecule is proportional to the affinity of peptides bound within its binding groove and as such provides a readout of the quality and extent of peptides loaded. We compared the thermal stabilities of cell surface Kb and Dd in the presence or absence of ERp57. This was done by first incubating cells overnight at 26 °C in the presence of exogenous ovalbumin-derived peptide SIINFEKL and presented at the cell surface. EL4 cells expressing a truncated form of ovalbumin were transfected with control siRNA or ERp57 siRNA and assessed for cell surface Kb- and Dd-derived SIINFEKL complexes using mAb 25-D1-16. Following normalization for the differences in Kb expression, the SIINFEKL peptide was presented just as effectively in the absence of ERp57 as in its presence (Fig. 7E). This finding is consistent with the unaltered cell surface stabilities of Kb and Dd molecules and suggests that peptide loading is unaffected by depletion of ERp57.

To address the question of whether the up-regulation of class I molecules on the cell surface was due to the depletion of ERp57 or simply a
Side effect of siRNA treatment, cells were transfected with a variety of siRNAs possessing different target specificities, and then surface class I levels were measured (Fig. 7C). Of the siRNAs tested, those that targeted ERp57, ERp72, and septin 3 were capable of boosting class I cell surface levels, but caveolin and septin 5 siRNAs were not. The ability of septin 3 siRNA to up-regulate class I expression was particularly informative. Septin 3 is a member of the septin subfamily of GTPase domain proteins and, being human neuronal cell-specific, is not expressed in mouse L cells. It is also not expected to be involved in any aspect of class I homeostasis. This suggests that the up-regulation of class I is a side effect of the siRNA transfection. Further support for this view comes from the finding that the up-regulation of cell surface class I is a side effect of the siRNA transfection, not the direct effect of removing a particular protein. Also, it is important to stress that the observed slower oxidative folding of class I molecules (Figs. 2 and 3) is due to the lack of ERp57 and not due to elevated IFN expression. This is based on the fact that ERp72 knockdown dramatically increased IFN-α2 expression as well as up-regulated surface class I (Fig. 7C and D) but had no effect on the rate of oxidative class I folding (Figs. 2 and 3).

DISCUSSION

Despite observations that ERp57 catalyzes disulfide bond formation in vitro (10) and associates with a variety of newly synthesized glycoproteins, including MHC class I molecules (14, 26, 27, 43, 44), direct demonstrations of its in vivo functions have remained elusive. To address this issue, we examined disulfide formation in the presence and absence of ERp57. In the presence of ERp57, the first of the two H chain disulfides formed very rapidly within the 2-min pulse labeling period, whereas the second disulfide formed more slowly, requiring more than 6 min for completion in all radiolabeled H

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chains. Using RNA interference to deplete the expression of ERp57 to less than 10% of its normal level, we show that this enzyme does indeed promote disulfide bond formation and domain folding of the class I H chain in vivo. Specifically, little effect of ERp57 depletion was observed upon the rapid formation of the first disulfide bond, but the rate of formation of the second disulfide was slowed by ~10-fold. This was confirmed by demonstrating that the rate of oxidative folding of the α3 domain in the closely related H-2D\textsuperscript{b} molecule, defined by mAb 34-2-125 reactivity, was similarly slowed. Surprisingly, the kinetics of H chain-\beta\_2m association were unaltered upon ERp57 depletion. This indicates that \beta\_2m, which makes contacts with residues in the \alpha\_1, \alpha\_2, and \alpha\_3 domains (45), is capable of associating with H chains before a native \alpha\_3 conformation has been achieved. This is in line with previous reports suggesting that \beta\_2m association may actually enhance the formation of the second H chain disulfide (18).

ERp57 is known to interact directly with both CNX and CRT (11, 46, 47), suggesting that these chaperones may recruit ERp57 to folding glycoproteins. This concept is supported by the finding that prevention of CNX and CRT interactions with glycoproteins, through treatment of cells with oligosaccharide processing inhibitors, often abrogates ERp57 interactions with glycoprotein substrates (14, 44). Furthermore, Zapun et al. (10) have shown that oxidative folding of monoglycosylated ribonuclease B by ERp57 in vitro is more efficient when either CNX or CRT are present. Consequently, we assessed the involvement of CNX and CRT in the ERp57-catalyzed oxidation of class I H chains by measuring the kinetics of H chain disulfide formation in CRT-deficient mouse cells and in L cells transfected with \beta\_2m to deplete CNX. Surprisingly, in the absence of either chaperone, the activity of ERp57 was not compromised (i.e. no delayed disulfide bond formation was observed (data not shown)). These observations indicate that although it is widely thought that CNX and CRT determine ERp57 substrate specificity, it is not the case for MHC class I molecules. This finding is consistent with a recent report demonstrating that CNX and CRT are also not required for the formation of the tapasin-ERp57 disulfide-linked conjugate within the peptide loading complex (28).

If CNX and CRT do not function as carriers to bring ERp57 into the proximity of folding class I molecules, how does ERp57 gain access to H chains? Tapasin is unlikely to recruit ERp57 during early oxidative folding of the H chain, since it associates with class I molecules mainly within the peptide loading complex after disulfide bond formation is largely complete (Fig. 5). Therefore, we propose that ERp57 is capable of recognizing newly synthesized H chains directly, and this is one reason for its preferential involvement in early class I biogenesis over other thiol oxidoreductases, such as PDI (26). Presumably, these or other thiol oxidoreductases can function to some extent during class I folding when ERp57 is absent, since our findings demonstrate that ERp57 depletion slows but does not arrest H chain disulfide formation.

It has been demonstrated that as much as 80% of the cellular pool of ERp57 can exist as a mixed disulfide cross-linked to tapasin within the peptide loading complex (28). What then is the role that ERp57 plays within this complex? Previous studies have provided conflicting results. When formation of the ERp57-tapasin cross-link was prevented by nuclease B by ERp57, Emsley et al. (10) have shown that oxidative folding of monoglycosylated ribonuclease B by ERp57 in vitro is more efficient when either CNX or CRT are present. Consequently, we assessed the involvement of CNX and CRT in the ERp57-catalyzed oxidation of class I H chains by measuring the kinetics of H chain disulfide formation in CRT-deficient mouse cells and in L cells transfected with \beta\_2m to deplete CNX. Surprisingly, in the absence of either chaperone, the activity of ERp57 was not compromised (i.e. no delayed disulfide bond formation was observed (data not shown)). These observations indicate that although it is widely thought that CNX and CRT determine ERp57 substrate specificity, it is not the case for MHC class I molecules. This finding is consistent with a recent report demonstrating that CNX and CRT are also not required for the formation of the tapasin-ERp57 disulfide-linked conjugate within the peptide loading complex (28).

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did not give rise to partially oxidized H chains within the loading complex, and peptide loading appeared normal.

While this manuscript was under review, Garbi et al. (48) reported an examination of class I biogenesis in B cell blasts derived from mice with an ERp57-deficient B cell compartment. In contrast to our experiments, they observed that although class I H-2Kb molecules were recruited into the peptide loading complex, the loading complex was unstable. This had the effect of reducing surface expression of H-2Kb by about 50%, with an impairment in the loading of some, but not all, peptides tested. However, minimal effects of ERp57 depletion were observed on the surface expression or peptide content of H-2Db molecules. They further concluded that the redox state of class I molecules either within or outside the peptide loading complex was unaffected by the lack of ERp57 (48). Whereas this latter finding agrees with the lack of effect we observed of ERp57 depletion on the redox state of class I molecules within the loading complex, it contrasts sharply with our finding that ERp57 promotes early H chain disulfide formation.

The discrepancies between our work and that of Garbi et al. are most likely due to the different experimental approaches used. To assess class I redox state, Garbi et al. examined the entire cellular pool of H chains by immunoblotting, a pool that is dominated by mature H chains at the cell surface (48). In contrast, our pulse-chase approach permitted the analysis of a very small pool of class I H chains immediately after entry into the ER lumen, a technique capable of revealing differences in the folding kinetics of the H chain in the presence and absence of ERp57 (Figs. 2 and 3). It is less clear why our studies differed concerning the effect of ERp57 depletion on the stability of the H-2Kb-containing peptide loading complex and subsequent peptide loading and surface expression. One possibility is that the ~10–30% residual ERp57-tapasin complex remaining after siRNA treatment (Fig. 5D) is sufficient to maintain full assembly of Kc molecules within the peptide loading complex. We also cannot exclude the possibility that increases in IFNα2 following siRNA treatment might influence loading complex function by an unknown mechanism. Further studies using different cell types, different class I molecules, and different approaches will be required to clarify this issue, particularly in light of the variable results already observed with different class I molecules and species (29, 38, 48). Nevertheless, the collective results underscore the involvement of ERp57 both in the early oxidative folding of class I H chains and in maintaining the integrity of the loading complex.

Our findings raise interesting questions concerning the mechanisms governing the substrate specificities of the 17 PDI family members that exist within the mammalian ER (49). ERp57 is clearly the preferred family member engaged in class I biogenesis, and its substrate specificity seems to reside within ERp57 itself rather than through recruitment by either CNX or CRT (see above). Our depletion studies have suggested that the absence of ERp57 may be partially complemented during early stages of H chain folding and possibly fully complemented by other PDI family members at the level of the peptide loading complex. To what extent do these other family members normally participate in class I biogenesis? Of the 17 members identified thus far, only PDI, PDlp, ERp72, and ERp57 share similar characteristics, which include four thioredoxin-like domains (termed a, b, b’, and a’); at least two -CGHC- or closely related active site sequences that permit oxidation, reduction, and isomerization reactions; a conserved arginine residue thought to modulate the pKₐ of the active site; conserved charged pair sequences that are involved in proton transfer reactions; and a b’ domain that contains a high affinity substrate binding site (49). In this study, ERp72 was depleted by siRNA treatment, and no effect was observed on the oxidative folding of class I H chains or on subsequent assembly steps. Furthermore, when both ERp57 and ERp72 were depleted by an unex-pected cross-targeting siRNA, the phenotype was indistinguishable from that of ERp57 depletion alone (Figs. 2 and 3). These observations reinforce the viewpoint that different thiol oxidoreductases are specific for a particular group of substrates (50, 51). It will be of considerable interest to extend our findings by depleting PDI and PDlp either alone or in combination with ERp57 and ERp72 to explore further their specificities and potential functional overlap in the biogenesis of the MHC class I molecule.

During final review of this manuscript, Molinari and co-workers (52) published a report demonstrating that ERp57-deficient fibroblasts exhibit impaired oxidative folding of influenza hemagglutinin but not of Semliki Forest virus glycoproteins, E1, and p62.

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