Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex

David R Peaper, Pamela A Wearsch and Peter Cresswell*

Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, USA

We previously showed that the major histocompatibility complex (MHC) class I chaperone tapasin can be detected as a mixed disulfide with the thiol-oxidoreductase ERp57. Here we show that tapasin is a unique and preferred substrate, a substantial majority of which is disulfide-linked to ERp57 within the cell. Tapasin upregulation by interferon- γ induces sequestration of the vast majority of ERp57 into the MHC class I peptide-loading complex. The rate of tapasin–ERp57 conjugate formation is unaffected by the absence of β_2 -microglobulin (β_2 m), and is independent of calnexin or calreticulin interactions with monoglucosylated N-linked glycans. The heterodimer forms spontaneously *in vitro* upon mixing recombinant ERp57 and tapasin. Noncovalent interactions between the native proteins inhibit the reductase activity of the thioredoxin CXXC motif within the N-terminal α domain of ERp57 to maintain its interaction with tapasin. Disruption of these interactions by denaturation allows reduction to proceed. Thus, tapasin association specifically inhibits the escape pathway required for disulfide-bond isomerization within conventional protein substrates, suggesting a specific structural role for ERp57 within the MHC class I peptide-loading complex.

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Introduction

Proper peptide loading onto major histocompatibility complex (MHC) class I molecules requires the coordinated action of multiple endoplasmic reticulum (ER) resident proteins. The chaperone calnexin (CNX) mediates the early folding stages of MHC class I heavy chain prior to its association with β_2 m (Rajagopalan and Brenner, 1994; Vassilakos *et al.*, 1996). Complete heavy-chain oxidation occurs during this interaction, and a role for the thiol oxidoreductase ERp57 in this process has been suggested (Tector *et al.*, 1997; Lindquist

et al., 1998). Once associated and properly oxidized, MHC class I heavy chain/ β_2 m dimers rapidly associate with calreticulin (CRT), ERp57, and the transmembrane glycoprotein tapasin, which is in turn associated with the heterodimeric Transporter associated with Antigen Processing (TAP), forming the MHC class I peptide-loading complex (Cresswell, 2000; Wright *et al.*, 2004). When cells are treated with the sulfhydryl reactive reagent *N*-ethyl maleimide (NEM) or rapidly acidified during extraction, tapasin and ERp57 have been observed to form a mixed disulfide within the loading complex, and this conjugate can form in cells deficient in TAP and to some extent in cells deficient in β_2 m (Dick *et al.*, 2002; Antoniou and Powis, 2003). Prior experiments demonstrated that all TAP-associated ERp57 is conjugated to tapasin, but variable amounts of unconjugated tapasin were seen in these experiments (Dick *et al.*, 2002). A mixture of conjugated and unconjugated tapasin molecules, both associated with TAP, suggested a possible role for conjugate oxidation/reduction reactions in loading complex assembly, peptide loading, and/or complex dissociation (Dick, 2004; Wright *et al.*, 2004).

ERp57 is a member of the thioredoxin (Trx) family involved in glycoprotein folding in the ER. Like protein disulfide isomerase (PDI), ERp57 is comprised of four Trx-like domains with the N- and C-terminal domains containing active Trx CXXC motifs (Sevier and Kaiser, 2002). The N-terminal cysteine within these canonical motifs forms a mixed disulfide with substrates, and the C-terminal cysteine of the motif subsequently attacks the intermolecular disulfide bond, leading to the release of substrate. This is referred to as the escape pathway, and its action leaves the Trx motif in an oxidized state (Walker and Gilbert, 1997). Activation of the escape pathway is rapid and visualization of the transient mixed disulfides formed during protein folding requires cellular treatment with sulfhydryl reactive compounds such as NEM to block the C-terminal Cys of the Trx motif (Braakman *et al.*, 1992). Even when using NEM, however, the only protein that is detectably disulfide linked to ERp57 under normal physiological conditions is tapasin (Dick and Cresswell, 2002). The tapasin/ERp57 disulfide bond involves the N-terminal Trx domain of ERp57 and is formed between Cys57 of ERp57 and Cys95 of tapasin. Mutation of Cys60 of ERp57 to Ala leads to inactivation of the escape pathway and the accumulation of multiple proteins disulfide-linked to ERp57, including the conjugate with tapasin, in the absence of NEM (Dick and Cresswell, 2002; Dick *et al.*, 2002).

Unlike PDI, ERp57 is thought to operate exclusively on N-glycosylated substrates through cooperative interactions with CRT and CNX (Elliott *et al.*, 1997; Oliver *et al.*, 1997; Van der Wal *et al.*, 1998; Molinari and Helenius, 1999). When initially added cotranslationally, the core N-linked glycan contains three terminal glucose residues. The sequential action of glucosidases I and II generates the monoglucosylated glycan recognized by CRT and CNX (Helenius and Aebi, 2004). *In vitro* studies have shown that the ability of ERp57

*Corresponding author. Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, 300 Cedar Street, PO Box 208011, New Haven, CT 06520-8011, USA.
Tel.: +1 203 785 5176; Fax: +1 203 785 4461;
E-mail: peter.cresswell@yale.edu

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to enhance the refolding of glycosylated substrates is greatly enhanced by the presence of CNX and/or CRT (Elliott *et al*, 1997; Zapun *et al*, 1998). Complexes of ERp57, CNX, and/or CRT have been found noncovalently associated with substrates, but these interactions are relatively weak and their detection requires the use of chemical crosslinkers (Elliott *et al*, 1997; Oliver *et al*, 1997; Van der Wal *et al*, 1998; Kang and Cresswell, 2002; Daniels *et al*, 2003). Interactions of CRT and CNX with substrates are blocked by the inclusion of castanospermine (CST) or *N*-butyldeoxynojirimycin, inhibitors of glucosidases I and II (Hammond *et al*, 1994), and mixed disulfides of ERp57 with viral glycoproteins do not form in the presence of CST (Molinari and Helenius, 1999). MHC class I molecules associate with CRT, an interaction that *in vitro* is clearly dependent upon glycosylation and independent of peptide occupancy (Wearsch *et al*, 2004).

Given the relatively specific nature of the interaction between tapasin and ERp57, we wished to determine the conditions required for the formation and reduction of this mixed disulfide. We have found that tapasin is the preferred substrate for ERp57, and that formation of the tapasin/ERp57 disulfide bond is independent of β_2m expression and glucose trimming. Finally, we present evidence that the disulfide-linked heterodimer is stable, suggesting that the interaction between tapasin and ERp57 inhibits activation of the escape pathway.

Results and discussion

Tapasin expression determines free ERp57 levels

We previously showed that, in NEM-treated B-cell lines, a substantial but unquantitated fraction of cellular ERp57 exists in a mixed disulfide with tapasin (Dick *et al*, 2002). To determine whether free versus tapasin-associated ERp57 levels were dictated by cellular folding requirements or by the levels of tapasin in the ER, we treated HeLa-M cells with interferon (IFN)- γ to increase the expression of tapasin and other components of the MHC class I pathway. We recently found that methyl methanethiosulfonate (MTS) is superior to NEM in preserving the tapasin/ERp57 conjugate (P Cresswell, unpublished observation), and cells were treated with MTS prior to detergent extraction. Based on reducing SDS-PAGE and quantitative Western blotting, we observed that overall ERp57 levels did not change with IFN- γ treatment. However, under nonreducing conditions, the fraction of ERp57 involved in the tapasin/ERp57 conjugate dramatically increased (Figure 1B–E). Less ERp57 was apparently present under nonreducing conditions in the non-IFN- γ -treated cells. We speculate that this is because a fraction of ERp57 exists in mixed disulfides with multiple substrates of differing molecular weights that are individually below the threshold of detection. The amount of ERp57 available for such mixed disulfides is reduced when the proportion associated with tapasin increases upon IFN- γ treatment. Tapasin expression was increased eight-fold by IFN- γ , and conjugate levels increased by the same amount (Figure 1C). In untreated cells, only 15% of the cellular ERp57 was conjugated to tapasin. In contrast, in HeLa-M cells treated with IFN- γ , 80% of the ERp57 was disulfide-linked to tapasin, as confirmed by immunoprecipitating for tapasin and blotting for ERp57 (Figure 1D). Thus, stimulation of tapasin expression by IFN- γ dramatically reduces the level of non-tapasin-

associated ERp57 available to assist glycoprotein folding. Interestingly, in both IFN- γ -treated and untreated cells, free ERp57 was found in at least two different redox states based on its mobility in SDS-PAGE under nonreducing conditions. As shown in Figure 1E, all the detectable tapasin, regardless of IFN- γ addition, is covalently bound to ERp57, indicating that effects of IFN- γ other than the induction of increased tapasin expression do not play a role in conjugate formation. These data argue that tapasin is the preferred *in vivo* substrate for ERp57.

Conjugate formation is independent of β_2m

In a previous study, only a small fraction of total tapasin was detected in a disulfide-linked conjugate with ERp57 in the β_2m -deficient B-LCL Daudi, and we were unable to detect the conjugate in the β_2m -deficient melanoma cell line FO-1. Expression of β_2m in these cells by transfection led to increased steady-state levels of the tapasin/ERp57 conjugate (Dick *et al*, 2002). The level of tapasin in FO-1 cells is relatively low and can be increased by IFN- γ treatment. To determine whether increased tapasin expression in the absence of β_2m could promote greater conjugate formation, or if defective conjugate formation was due to the absence of β_2m , we stimulated FO-1 and FO-1. β_2m , a β_2m -expressing transfectant, with IFN- γ . The cells were then treated with MMTS prior to solubilization in digitonin, which preserves the TAP/tapasin interaction, or Triton X-100, which does not. By a combination of immunoprecipitations, reducing and nonreducing SDS-PAGE and Western blotting, all detectable TAP-associated and total intracellular tapasin was conjugated to ERp57, regardless of β_2m expression (Figure 2). Identical results were obtained with Daudi and its β_2m -expressing derivative Daudi. β_2m (data not shown).

Our previous inability to detect the ERp57–tapasin conjugate in β_2m -deficient FO-1 cells was likely due to a combination of factors, including less efficient conjugate preservation with NEM compared to MMTS and reduced tapasin expression without IFN- γ treatment. It is possible that Cys-60 of ERp57, which is required for activation of the escape pathway and release of tapasin, is less accessible to NEM in the absence of β_2m , while MMTS is able to react equally well in its presence or absence. Tapasin may also exist in slightly different conformations within the loading complex that could affect the accessibility of Cys-60 (see below and unpublished observations). More experiments are needed to resolve this issue, but it is clear from the experiments in Figure 2 that at steady state all the detectable tapasin is disulfide linked to ERp57, and that this is not affected by the presence or absence of β_2m .

Conjugate formation occurs rapidly after tapasin synthesis and independently of β_2m

We previously showed that tapasin and ERp57 rapidly associate with TAP shortly after synthesis (Diedrich *et al*, 2001). These experiments were conducted without NEM or MMTS treatment, however, so formation of the disulfide-linked conjugate could not be assessed. Additionally, if the kinetics of conjugate formation are related to the incorporation of MHC class I heavy chain or other loading complex components, the rate or order of loading complex assembly may be altered in cells lacking β_2m . To examine these questions, we looked at the kinetics of conjugate formation in IFN- γ -treated

Figure 1 IFN- γ treatment drives tapasin association and decreases the pool of free ERp57. (A) IFN- γ reduces the pool of free ERp57. HeLa-M cells were treated with IFN- γ for 2 days prior to harvesting and MMTS treatment. Three-fold serial dilutions of cell extracts were resolved by SDS-PAGE under reducing or nonreducing conditions and blotted with rabbit anti-ERp57 raised against a C-terminal peptide. After probing for ERp57, membranes were stripped, cut, and re-probed for tapasin with anti-gp48N or for ERp72 as a loading control. The tapasin/ERp57 conjugate and free ERp57 are indicated. (B) IFN- γ reduces the pool of free ERp57. Bands in (A) corresponding to conjugated and reduced ERp57 were quantitated, and the percent of conjugated ERp57 was calculated as Percent Conjugated ERp57 = (Conjugated ERp57/Reduced ERp57)*100. Data shown are the average $\pm$ s.e.m. of three dilutions from two separate experiments. (C) IFN- γ increases conjugate levels to the same extent as tapasin expression. Bands in (A) corresponding to tapasin and conjugated ERp57 with and without IFN- γ treatment were quantitated, and the fold increase in expression was calculated. Data shown are the average $\pm$ s.e.m. of three dilutions from two separate experiments. (D) IFN- γ induction of tapasin reduces the pool of free ERp57. Lysates from (A) were precleared and precipitated with PaSta1, MaP.ERp57, or M.PDI and protein G Sepharose. Samples were resolved under reducing or nonreducing conditions as indicated and blotted with rabbit anti-peptide R.ERp57-C. The upper molecular weight species contains both tapasin and ERp57, and ERp57 exists in at least two oxidation states. The tapasin/ERp57 conjugate and free ERp57 are indicated. (E) All detectable tapasin is conjugated to ERp57 under steady-state conditions. HeLa-M cells were treated with IFN- γ for 2 days prior to harvesting and MMTS treatment. Triton X-100 extracts were resolved by SDS-PAGE under reducing or nonreducing conditions and probed for tapasin and ERp57 as in (A). Membranes were stripped and reblotted for ERp72 as a loading control. Samples were visualized with fluorimetry. The tapasin/ERp57 conjugate, free ERp57, and reduced tapasin are indicated.

FO-1 and FO-1. β_2 m cells using pulse-chase analyses. After a 5-min pulse with [35 S]methionine and cysteine, some TAP-associated tapasin-ERp57 conjugate was already detectable, regardless of β_2 m expression (Figure 3A and B). Conjugate formation then increased with identical kinetics in FO-1 and FO-1. β_2 m cells (Figure 3C). When samples were run under reducing conditions, the signals from both ERp57 and tapasin were unchanged with time, but tapasin was more strongly labeled, arguing that the majority of the dimer signal under

nonreducing conditions arises from newly synthesized tapasin and resident ERp57 (Figure 3D). Identical results were obtained when the primary immunoprecipitations were performed with an mAb specific for ERp57 (data not shown).

We and others detected the presence of the tapasin/ERp57 conjugate in cells deficient in β_2 m (Dick *et al*, 2002; Antoniou and Powis, 2003). This was under steady-state conditions, however, and the low levels of conjugate observed could be explained by its transient formation and rapid reduction

Figure 2 Conjugate formation is independent of β_2m . All TAP-associated and cellular tapasin is conjugated to ERp57 in the presence or absence of β_2m . Post-nuclear supernatants from IFN- γ -treated FO-1 or FO-1. β_2m cells solubilized in the indicated detergents were precipitated with 148.3, MaP.ERp57, PaSta-1, or M.PDI and protein G Sepharose. SDS-PAGE was performed under reducing or nonreducing conditions and blots were probed for tapasin with R.gp48C. The tapasin/ERp57 conjugate and reduced tapasin are indicated.

Figure 3 β_2m expression does not affect the rate of conjugate formation. (A, B) IFN- γ -treated FO-1 or FO-1. β_2m cells pulsed with [35 S]methionine and cysteine for 5 min were chased for the indicated periods prior to solubilization in digitonin. Post-nuclear supernatants were precipitated with the anti-TAP mAb 148.3 or mouse IgG coupled to agarose beads (C = Ctrl). Associated proteins were stripped in 1% SDS, and tapasin and its conjugate with ERp57 were reprecipitated with R.gp48C and protein A Sepharose. The tapasin/ERp57 conjugate, free ERp57, and free tapasin are indicated. (C) Data from panels A and B are presented as a percentage of the maximum ERp57-tapasin conjugate signal throughout the chase period. (D) Newly synthesized tapasin represents most of the radioactivity incorporated into the conjugate. Samples were prepared as in (A) and (B), but gels were run under reducing conditions. Reduced ERp57 and tapasin are indicated.

in the absence of other stabilizing factors within the MHC class I loading complex. In the absence of β_2m , virtually no CRT and very little free MHC class I heavy chain is found associated with TAP. The present data argue that the rate of formation, steady-state level and stability of the conjugate are not affected by β_2m expression, nor by the presence

of other factors recruited into the loading complex by MHC class I heavy-chain/ β_2m dimers.

In the pulse-chase analysis, some free tapasin was seen at all chase points, in contrast to the results obtained by Western blotting. We have no explanation for these differences, but we have limited our conclusions from each set of

experiments to those appropriate for the technique employed. That is to say, Western blotting most accurately represents the steady-state conditions, and pulse-chase analyses are the only means to assess the kinetics of assembly of the loading complex.

Complete tapasin oxidation can occur after TAP association

Comparing Figure 3B with Figure 3D, the band corresponding to free tapasin became noticeably sharper at later chase points under nonreducing conditions. Under reducing conditions, however, tapasin clearly resolved as a single band. These data suggested that tapasin may initially associate with TAP in a partially oxidized state. To test this hypothesis, we examined the biosynthesis of the ERp57–tapasin conjugate in a pulse-chase analysis by first immunoprecipitating for TAP, releasing associated tapasin under nonreducing conditions with SDS, and resolving free and conjugated tapasin by SDS–PAGE under both reducing and nonreducing conditions. Tapasin initially associates with TAP as a monomer in two oxidation states (Figure 4). Tapasin contains two luminal disulfide bonds, between Cys295 and Cys362 in the membrane-proximal immunoglobulin-like (Ig) domain and

between Cys7 and Cys71 in the N-terminal region. Ig domains fold relatively freely and independently, and a human tapasin Cys295 mutant fails to associate with TAP (Isenman *et al*, 1979; Dick *et al*, 2002; Turnquist *et al*, 2004). It is highly likely, therefore, that upon TAP association tapasin contains an oxidized disulfide bond within the Ig domain, but the Cys7–Cys71 bond is initially reduced (upper band in lane 2). Within 30 min of TAP association, all tapasin resolves as a single band of increased mobility, consistent with full oxidation. The rate of ERp57/tapasin dimer formation is inversely related to the rate of disappearance of the upper band that putatively lacks the Cys7–Cys71 disulfide. FO-1.β₂m cells were used for this experiment for consistency, but identical results were obtained using IFN-γ-induced HeLa-M cells.

ERp57 can form a mixed disulfide with tapasin mutants lacking the Cys7–Cys71 disulfide bond (Dick *et al*, 2002), and it is therefore conceivable that the tapasin/ERp57 conjugate could form during the early folding stages of tapasin and persist after TAP association. Alternatively, the conjugate could form concurrently with or rapidly following complete tapasin oxidation. Our current experiments do not differentiate between these possibilities. Nevertheless, while ERp57 may play a role in tapasin folding after assembly with TAP, in contrast to the behavior expected for a PDI, the association persists after tapasin has acquired the characteristics of a native protein, including full oxidation, functional activity and reactivity with conformation-specific antibodies.

Conjugate formation is independent of monoglucosylated N-linked glycans

ERp57 is thought to assist glycoprotein folding exclusively through glycan-dependent interactions with CRT and CNX. These interactions depend on the generation of mono-glucosylated glycans by the sequential removal of glucose residues by glucosidases I and II from the triply glucosylated core N-linked oligosaccharide (Glc₃Man₉GlcNAc₂). Incubation of cells with CST, a glucosidase inhibitor, blocks the glycan-dependent interactions of glycoproteins with CRT and CNX (Hammond *et al*, 1994).

To ascertain the requirement for the generation of mono-glucosylated N-linked glycans, we examined the effects of CST on the formation of the ERp57–tapasin conjugate. When cells were starved, labeled and chased in the presence of 2 mM CST, the rate of conjugate formation was unaffected (Figure 5A). The CST treatment was effective in that it inhibited the bulk of CRT–substrate interactions (right lane). Additionally, the mobility of monomeric tapasin isolated from CST-treated cells was slightly lower than that from untreated cells. The increased molecular weight likely corresponds to the additional, untrimmed glucose residues resulting from CST treatment. Identical results were obtained using IFN-γ-induced HeLa-M cells.

Both CRT and CNX can interact with nonglycosylated proteins *in vitro* and *in vivo* under conditions of severe protein and cellular stress (Ihara *et al*, 1999). One could therefore imagine that CNX could recruit ERp57 into the loading complex even in the presence of CST. This is not the case, however, as the conjugate formed equally well in CEM.NKR cells deficient in CNX expression, as it did in these cells expressing CNX by transfection (Figure 5B). Human cell lines deficient in CRT expression have not been described. Gao *et al* (2002) reported that, in CRT-deficient

Figure 4 Tapasin completes oxidative folding after TAP association. IFN-γ-stimulated FO-1.β₂m cells pulsed with [³⁵S]methionine and cysteine for 5 min were chased for the indicated times prior to solubilization in digitonin. Post-nuclear supernatants were precipitated with an anti-TAP mAb (148.3) or mouse IgG coupled to agarose beads (C = Ctrl), stripped, and reprecipitated with R.gp48C recognizing tapasin. Samples were incubated with DTT before SDS–PAGE where indicated. The tapasin/ERp57 conjugate, free ERp57, and free and reduced tapasin are indicated. The upper band in non-DTT-treated samples represents partially oxidized monomeric tapasin, and the lower band corresponds to fully oxidized tapasin. The lower panel shows quantitation of the partially oxidized upper band and the conjugate throughout the chase period.

Figure 5 Monoglucosylated *N*-linked glycans are not required for conjugate formation. **(A)** FO-1.β₂m cells were incubated with or without 2 mM CST throughout starvation, pulse-labeling, and chase. All samples were treated as in Figure 3 and immunoprecipitated tapasin and its conjugate with ERp57 were subjected to SDS-PAGE without reduction. The right panel depicts the percent maximum conjugate signal throughout the chase period. **(B)** CNX is dispensable for conjugate formation. CNX-negative CEM.NKR cells expressing HLA-A2 or HLA-A2 and CNX were solubilized in Triton X-100 and post-nuclear supernatants were precipitated with M.PDI, PaSta1 (anti-tapasin mAb), or MaP.ERp57. SDS-PAGE was performed under reducing or nonreducing conditions, and, following transfer, membranes were probed for tapasin with R.gp48C. The tapasin/ERp57 conjugate and free tapasin are indicated.

mouse embryonic fibroblasts (MEFs), ERp57 was associated with the loading complex, but they did not assess conjugate formation. These experiments are complicated by the fact that mouse MHC class I molecules possess at least one additional *N*-linked glycan, and a greater role for loading complex-associated CNX has been suggested for mouse cells. Nevertheless, in preliminary experiments, albeit complicated by the absence of ideal antibody reagents, we found that regardless of the expression of CRT all tapasin was conjugated to ERp57 and a substantial portion of ERp57 was conjugated to tapasin in IFN-γ-induced MEFs (data not shown).

These data provide new insight into MHC class I peptide-loading complex formation. Previously, TAP with noncovalently associated tapasin was thought to comprise the core functional unit of the loading complex, but our present data indicate that ERp57 conjugated to tapasin is also a core component. Conjugate formation does not correlate with the incorporation of MHC class I-β₂m heterodimers into the loading complex, arguing against a role for MHC class I in recruiting ERp57. We previously suggested that CNX could fulfill this role, but cells deficient in CNX are fully competent for conjugate formation. A role for CRT in recruiting ERp57 to TAP is unlikely because CRT is not found associated with TAP or tapasin in the absence of β₂m (Sadasivan *et al*, 1996; Diedrich *et al*, 2001), a condition we now find compatible with full conjugate formation. Additionally, the kinetics of conjugate formation and ERp57

recruitment to TAP are not affected when the generation of CRT- and CNX-binding sites is blocked by CST. The covalent linkage of tapasin and ERp57 may facilitate the assembly of the peptide-loading complex by stabilizing the much weaker noncovalent interactions between tapasin and MHC class I heavy chain, CRT and ERp57, and the class I heavy-chain glycan and CRT.

While there are implications of our findings specific to loading complex formation, the data are also significant in the general context of glycoprotein folding. The nature of the interaction of ERp57 with tapasin and the loading complex is quite different from that with other substrates. We and others have reported that the interaction between the MHC class I heavy chain and CRT is abolished in the presence of CST, and CST inhibits the interaction of ERp57 with all substrates examined to date (Sadasivan *et al*, 1996; Elliott *et al*, 1997; Oliver *et al*, 1997; Van der Wal *et al*, 1998; Molinari and Helenius, 1999; Kang and Cresswell, 2002). The mixed disulfide formed with tapasin is the only identified exception. Additionally, conjugate formation occurs equally well in the presence or absence of CNX in human cells or, preliminarily, CRT in MEFs. Doubly deficient cells would be needed to fully address the requirement of CNX and CRT for conjugate formation, but the CST experiments clearly indicate that conjugate formation is independent of the presence of a monoglucosylated *N*-linked glycan on tapasin or within the loading complex.

Our ability to detect a disulfide-linked heterodimer of ERp57 and tapasin is surprising. Although trapping mutants of ERp57 can form stable mixed disulfides with a variety of undefined substrates (Dick and Cresswell, 2002), chemical crosslinking reagents are required for the detection of mixed disulfides containing wild-type ERp57, with the exception of viral glycoproteins in virally infected cells (Molinari and Helenius, 1999). In such cells, host protein translation is inhibited and viral proteins are highly expressed. Current models of oxidative protein folding predict the transient existence of mixed disulfides due to the rapid activation of the Trx escape pathway and enzymatic reduction (Sevier and Kaiser, 2002). When particular substrates are abundant, mixed disulfides may exceed the minimal levels needed for detection. This most likely explains the findings in virally infected cells and could potentially explain the ease of detection of the tapasin-ERp57 conjugate. However, especially given the unusual glycan-independent formation of the conjugate, it seems more likely that tapasin has evolved to slow or block the escape pathway normally used by ERp57 to release folding substrates.

Detergent	TBS		TBS		Tx		SDS		Tx		
MMTS	-		+		+		+		+		
Boil in 1% SDS											
IP	Tapasin →								Ctrl		
DTT (+/-)	-	+	-	+	-	+	-	+	-	+	
220 kDa											← Conjugate
97											
66											
46											
37											
Lane	1	2	3	4	5	6	7	8	9	10	

Detergent	MMTS	% conjugated tapasin
TBS	-	0
TBS	+	~85
1% Tx	+	~58
0.1% SDS	+	~32

but the elimination of noncovalent interactions by the denaturation of ERp57 and/or tapasin relieves the inhibition and allows reduction to proceed normally, as it would for a typical ERp57 substrate.

The tapasin-ERp57 conjugate exists within the peptide-loading complex, which contains multiple noncovalently interacting proteins (Wright *et al.*, 2004). We performed the experiments in Figure 6 with .221 cells that do not express HLA-A, -B, or -C alleles to limit possibly confounding effects arising from cooperative interactions within the MHC class I loading complex, but identical results were obtained with IFN- γ -treated HeLa-M and FO-1. β 2m cells fully competent for MHC class I loading (data not shown). To fully rule out a role for additional components in the ability of tapasin to inhibit the ERp57 escape pathway, we examined the formation and stability of the conjugate *in vitro* using purified recombinant soluble tapasin, recombinant wild-type ERp57 and the trapping mutants C60A and C409A, affecting the Trx sites of the

Figure 7 Tapasin alone stabilizes a mixed disulfide with ERp57. **(A)** The C60A mutant of ERp57 forms a mixed disulfide with native tapasin *in vitro*. Wild-type, C60A, and C409A mutant ERp57 molecules were mixed with soluble, recombinant tapasin in the presence of the indicated ratios of reduced (GSH) and oxidized (GSSG) glutathione. SDS sample buffer was added to all samples before SDS-PAGE. **(B)** *In vitro* formed conjugates contain both ERp57 and tapasin. Samples were treated as in (A), but after SDS-PAGE the samples were transferred to PVDF membranes and probed for tapasin with R.SinA or for ERp57 with a rabbit antiserum to the intact protein. **(C)** Noncovalent interactions between tapasin and ERp57 maintain the conjugate. Soluble recombinant ERp57 and tapasin were incubated alone or in combination as indicated. Samples were subsequently treated with 10 mM MMTS for 10 min at RT before or after the addition of urea (final concentration 8 M, right panel) or 10 × nonreducing SDS-PAGE sample buffer (left panel). Gels were run under nonreducing conditions and proteins detected by Coomassie Blue staining. The tapasin/ERp57 conjugate, free ERp57, and free tapasin are indicated. The conjugate composed of tapasin and WT ERp57 dissociates upon treatment with urea or SDS, but preincubation with MMTS or deletion of Cys-60 inhibits escape pathway activation and preserves the disulfide linkage.

N- and C-terminal *a* and *a'* domains, respectively. Previously, we showed that only the C60A mutant traps tapasin *in vivo* (Dick *et al*, 2002). Initial experiments were performed

under differing redox conditions (Figure 7A), followed by the addition of nonreducing SDS sample buffer without MMTS treatment. Consistent with the cellular data and the

data presented above, no conjugate was seen in nonreducing SDS-PAGE when tapasin was mixed with wild-type ERp57 or the C409A mutant, while the conjugate was clearly formed and maintained with the C60A mutant. We confirmed the identity of these species by blotting for both tapasin and ERp57 (Figure 7B). When MMTS was added to samples prior to heating in SDS sample buffer, however, similar levels of conjugate formation were seen with wild-type ERp57 and C60A (Figure 7C). Under conditions in which the tapasin-ERp57 conjugate readily formed, we were not able to observe mixed disulfides of ERp57 with other substrates, nor were we able to detect tapasin conjugation to PDI (data not shown). Disruption of the noncovalent interaction between tapasin and wild-type ERp57 in Figure 7C without the addition of MMTS led to escape pathway activation and conjugate reduction, while conjugates containing C60A ERp57 were maintained. Furthermore, exposure to MMTS prior to denaturation with 8 M urea prevented conjugate reduction for wild-type ERp57.

The behavior of conjugates formed *in vitro* using purified ERp57 and tapasin perfectly mimics the behavior of the tapasin-ERp57 heterodimer isolated from cells. Disruption of the noncovalent interactions between ERp57 and tapasin by denaturation relieves the inhibition of ERp57 function, allowing escape pathway activation and conjugate reduction. These data reinforce the concept that ERp57 can form a stable mixed disulfide with a native protein, and clearly demonstrate that the tapasin interaction alone is sufficient to inhibit the ability of Cys60 in the N-terminal Trx-like domain to mediate the escape pathway.

The results depicted in Figures 6 and 7 are surprising, and our current view of this interaction is summarized in Figure 8. Members of the Trx family have evolved to rapidly facilitate protein folding in the cytosol or ER (Sevier and

Kaiser, 2002). Detection of mixed disulfides between folding substrates and Trx family members traditionally requires mutagenesis or dramatic shifts in equilibria driven by viral infection (Walker and Gilbert, 1997; Molinari and Helenius, 1999; Dick and Cresswell, 2002). Detection of the tapasin/ERp57 conjugate is facilitated by the high levels of this mixed disulfide present in cells, but these levels arise from the preferential recruitment of ERp57 into the MHC class I loading complex and inhibition of the escape pathway by tapasin. The ability of tapasin to prevent escape pathway-mediated reduction of the conjugate stabilizes ERp57 within the loading complex by either preventing or dramatically reducing its exchange with the total pool of ERp57 within the ER.

The precise function of ERp57 within the MHC class I loading complex has yet to be resolved. The generation and characterization of an ERp57-deficient mouse will surely provide insight into this function. Our previous data suggested that ERp57 plays a role in regulating the oxidation status of MHC class I heavy chains within the loading complex (Dick *et al*, 2002), but a mechanistic understanding of this phenomenon awaits the generation of suitable reagents. Nevertheless, from a cell biological perspective, the interaction of ERp57 with tapasin does not fit current models of the role of the Trx domain in protein folding. Our data indicate that tapasin is the preferred substrate for ERp57 and that the conjugate is extremely stable compared to other Trx family-substrate complexes. Furthermore, the stability of the conjugate arises from the inhibition of the escape pathway by tapasin. These findings suggest that tapasin has evolved to preserve the conjugate with ERp57, and that the heterodimer may play a key structural and/or functional role in the MHC class I peptide-loading complex. Within the complex, the transmembrane domain of tapasin

Figure 8 Noncovalent interactions between tapasin and ERp57 prevent escape pathway activation. Under native conditions, noncovalent interactions between tapasin and ERp57 eliminate the ability of the sulfhydryl of Cys-60 of ERp57 to attack the mixed disulfide between tapasin and ERp57. We hypothesize that tapasin reduces the access of Cys-60 to the mixed disulfide and/or alters the local environment to prevent Cys-60 deprotonation. When denatured, the interactions of tapasin with ERp57 are disrupted, and the sulfhydryl of Cys-60 is now able to attack the mixed disulfide, leading to conjugate reduction. We speculate that the mixed disulfide with tapasin serves a structural rather than enzymatic function, and the altered redox state of MHC class I heavy chain in loading complexes deficient in ERp57 is likely due to the absence of the active site within the *a'* domain of ERp57.

noncovalently interacts with the TAP heterodimer. The covalent association of ERp57 with tapasin, combined with low affinity noncovalent interactions between CRT and ERp57, CRT and the monoglucosylated N-linked glycan of the MHC class I heavy chain, and the MHC class I heavy chain and tapasin would appear to provide the cooperative interactions required to stabilize the peptide-loading complex in a manner that facilitates peptide binding to the class I molecule.

Materials and methods

Cell lines and antibodies

Cell lines used in this study were: HeLa-M (Tiwari *et al*, 1987), FO-1, FO-1.β₂m (Maio *et al*, 1991), CEM.NKR.A2, CEM.NKR.A2.CNX (Sadasivan *et al*, 1995), and the MHC class I-negative B-cell line .221 (Shimizu *et al*, 1988). For experiments involving HeLa-M, FO-1, or FO-1.β₂m, the cells were treated with 200 U/ml human IFN-γ (R&D Systems) for 2 days prior to harvesting. All cells were maintained in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 5% bovine calf serum (Hyclone), and penicillin/streptomycin (Invitrogen). The following mouse monoclonal antibodies were used: 148.3 (anti-TAP1) (Meyer *et al*, 1994), PaSta-1 (anti-tapasin) (Dick *et al*, 2002), MaP.ERp57 (anti-ERp57) (Diedrich *et al*, 2001), and SPA-891 (anti-PDI) (Stressgen). Rabbit antisera to soluble tapasin (R.SinA) (Diedrich *et al*, 2001), to the tapasin C-terminus (R.gp48C) (Bangia *et al*, 1999), to the tapasin N-terminus (R.gp48N) (Lehner *et al*, 1998), to soluble full-length ERp57 (R.ERp57) (Diedrich *et al*, 2001), to the C-terminus of ERp57 (R.ERp57-C) (Hughes and Cresswell, 1998), and to ERp72 (Stressgen SPA-720) were also used. R.gp48N and R.SinA were used for blotting and precipitating whole-cell extracts to minimize the presence of crossreactive bands. R.gp48C was used for blotting immunoprecipitates and for secondary immunoprecipitations, situations in which crossreactive species were not apparent.

Quantitative immunoblots

IFN-γ-induced or uninduced HeLa-M cells were harvested, washed in cold PBS, and incubated with 10 mM MMTS (Pierce) for 5 min on ice. Cell pellets were solubilized in 1% Triton X-100 (Sigma) in 0.15 M NaCl, 0.01 M Tris-Cl, pH 7.4 (TBS) with 2 mM MMTS and phenyl-methylsulfonyl fluoride (PMSF) for 30 min on ice. The protein content of the post-nuclear supernatants was quantitated by Bradford assay (Bio-Rad), and three-fold serial dilutions were made in 1% Triton X-100 in TBS. SDS-PAGE (10 ×) sample buffer with or without 50 mM dithiothreitol (DTT) (5 mM final concentration) (American Bioanalytical) was added to all samples as indicated prior to heating at 95°C for 5 min. All samples were subsequently treated with iodoacetamide (IAA) (15 mM final concentration) for 5 min at room temperature (RT) and resolved by SDS-PAGE. Separated proteins were transferred to PVDF membranes (Millipore) and blocked in 5% skim milk (Carnation) in PBS with 0.2% Tween 20 (Blotto) for 1 h at RT. Membranes were probed with anti-peptide R.ERp57-C (1:1000) in Blotto for 1 h at RT. After washing, they were incubated with alkaline phosphatase-coupled goat anti-rabbit IgG serum (1:5000) (Jackson Labs) in Blotto for 30 min at RT and imaged using a fluorimager with ECF substrate (Amersham). After probing for ERp57, membranes were washed in 1% SDS for 15 min at RT, followed by extensive washing in PBS with 0.2% Tween 20 before cutting into half and reprobing with rabbit anti-tapasin gp48N or rabbit anti-ERp72 as a loading control. The percent conjugated ERp57 was determined by the following equation: Percent Conjugated ERp57 = (Conjugated ERp57/Reduced ERp57) × 100.

Membrane preparation

.221 cells were harvested, washed in PBS, and incubated for 1 h in medium without cysteine or methionine at 37°C. Cells were labeled with [³⁵S]methionine/cysteine labeling mix (MP Biomedicals) for 30 min, chased in an excess of cold methionine/cysteine for 30 min, washed with ice-cold PBS, pelleted, and frozen. Frozen cell pellets were resuspended in ice-cold TBS with 1 mM CaCl₂ and PMSF and centrifuged at 300 g at 4°C for 10 min. Supernatants were preserved

on ice, and the pelleted material was resuspended in ice-cold 10 mM Tris-Cl, pH 7.4 and centrifuged at 300 g for 10 min. The pooled supernatants were pelleted at 100 000 g for 1 h at 4°C, and the membranes were treated as described in the figure legends.

Immunoprecipitations and immunoblotting

Cells (5 × 10⁶ cells per gel lane) were treated with MMTS as above and extracted in 1% digitonin or 1% Triton X-100 in TBS containing 2 mM MMTS, 1 mM CaCl₂, and PMSF for 30 min on ice. Post-nuclear supernatants were precleared with protein G Sepharose and normal mouse serum, and immunoprecipitated with the indicated antibody and protein G Sepharose. After washing three times with 0.1% detergent in TBS with 1 mM CaCl₂, the beads were heated to 95°C in nonreducing sample buffer. Samples were divided and heated for an additional 5 min at 95°C with or without 5 mM DTT. IAA (15 mM final concentration) was added prior to separation by SDS-PAGE, transfer to PVDF membranes and blocking. Membranes were probed with R.gp48C (1:2000) or anti-peptide R.ERp57 (1:1000), washed, and incubated with goat anti-rabbit IgG serum coupled to horseradish peroxidase (Jackson Labs). Blots were developed with peroxide-luminol solution (Supersignal West Pico, Pierce) and exposed to film.

Pulse-chase analyses

IFN-γ-induced FO-1 or FO-1.β₂m were harvested, washed in PBS, incubated for 1 h in medium without methionine/cysteine as above, pulse-labeled with [³⁵S] labeling mix for 5 min, chased in an excess of unlabeled methionine/cysteine for the indicated times, washed with cold PBS, and incubated with 10 mM MMTS in PBS on ice for 5 min. For experiments examining the glucosylation dependence of conjugate formation, cells were starved, labeled, and chased in the presence of 2 mM CST (Sigma) as indicated. For all pulse-chase studies, cells were solubilized in 1% digitonin in TBS with 2 mM MMTS, 1 mM CaCl₂, and PMSF. Post-nuclear supernatants were precleared with normal rabbit serum and protein A Sepharose (Sigma) prior to specific immunoprecipitation. For reprecipitations, immunoprecipitated proteins were eluted by heating to 95°C in 1% SDS. The eluted material was then diluted into 1% Triton X-100 in TBS and immunoprecipitated with R.gp48C and protein A Sepharose prior to SDS-PAGE. All pulse-chase analyses were repeated at least twice, and representative experiments are shown.

Protein expression and purification

Recombinant soluble tapasin expressed in SF21 cells was generated and purified as described (Chen *et al*, 2002). A construct encoding human ERp57 lacking the signal sequence (amino acids 25–418) was cloned into the pQE60 vector (Qiagen) with a C-terminal 6 × His tag, expressed in *Escherichia coli* and purified according to the manufacturer's instructions. The C60A mutation was introduced into the pQE60 ERp57 construct using the Quick Change Mutagenesis Kit (Stratagene).

In vitro tapasin-ERp57 conjugations

For experiments examining conjugate formation under differing redox conditions, purified tapasin and ERp57 (2–3 μg of each) were incubated together in a total volume of 6 μl TBS with 0.5 mM CaCl₂ for 30 min at RT. The samples were diluted to 60 μl with TBS and 3 mM reduced glutathione and then divided into three. Oxidized glutathione was introduced gradually to the indicated ratios and allowed to incubate for an additional hour at RT. Nonreducing sample buffer was added to the samples, which were analyzed by SDS-PAGE followed either by staining with Coomassie Blue or immunoblotting with R.SinA and rabbit anti-full-length ERp57 antiserum R.ERp57.

To examine denaturation-induced conjugate reduction, purified tapasin and ERp57 (1–2 μg of each) were incubated in a total volume of 3 μl TBS with 0.5 mM CaCl₂ for 30 min at RT. The conjugation reactions were diluted to a final volume of 30 μl with TBS-CaCl₂, incubated at 4°C overnight, and then treated with or without 10 mM MMTS for 10 min at RT. Following the addition of nonreducing sample buffer, the samples were separated by SDS-PAGE (9% acrylamide) and stained with Coomassie Blue. Where indicated, the conjugation reactions were performed as above but urea was added to a final concentration of 8 M instead of TBS-CaCl₂, either before or after the treatment of the samples with MMTS.

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