ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 4, 2003


# Forum Original Research Communication

# Characterization of the ERp57-Tapasin Complex by Rapid Cellular Acidification and Thiol Modification

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## ABSTRACT

**Major histocompatibility complex (MHC) class I molecules bind and present short peptides to cells of the immune system. The oxidoreductase ERp57 is involved in the assembly of MHC class I molecules and is a component of the peptide loading complex, where it is found disulfide-bonded to tapasin. We have studied ERp57 and the ERp57-tapasin conjugate by rapid acidification of the intracellular environment with trichloroacetic acid (TCA), followed by thiol modification with the alkylating agent 4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). By using TCA/AMS treatment, non–tapasin-associated ERp57 is shown to exist almost exclusively in a reduced state, suggesting that both thioredoxin-like CXXC motifs are exposed and reduced. A 110-kDa product is readily detected with this TCA/AMS protocol and is confirmed as an ERp57-tapasin conjugate by its absence from the tapasin-deficient .220 cell line and by immunoblotting with both ERp57- and tapasin-specific antisera. The ERp57-tapasin conjugate can also be modified with the oxidizing agent diamide, indicating that within the pool of ERp57-tapasin complexes the free, non–tapasin-linked CXXC motif exists in both oxidized and reduced states, suggesting availability to undergo redox reactions.** *Antioxid. Redox Signal.* 5, 375–379.

## INTRODUCTION.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) class I molecules assemble within the endoplasmic reticulum (ER), binding short peptides before trafficking to the cell surface for inspection by T lymphocytes (23). The assembly process for MHC class I molecules in the ER involves the formation of a peptide loading complex (PLC), which comprises the MHC class I heavy chain associated with B2-microglobulin in addition to calreticulin, tapasin, and the oxidoreductase ERp57 (12, 15, 20, 22, 25). The PLC further associates with the transporter associated with antigen processing (TAP), which is the source of cytosolic peptides for MHC class I binding (21, 24). The emerging function of the PLC is to ensure quality control in MHC class I assembly and the acquisition of high-affinity peptides.

The presence of ERp57 as part of the PLC originally suggested a role in disulfide bond formation within the MHC class I molecule. However, ERp57-MHC class I disulfide intermediates have so far only been isolated using antibodies capable of detecting relatively unfolded MHC class I molecules (1, 16), whereas within the PLC MHC class I molecules are considered to be almost fully folded. Somewhat unexpectedly, in place of an ERp57-MHC class I conjugate in the PLC, ERp57 has instead been found to disulfide-bond to tapasin (4).

ERp57 is a member of the protein disulfide isomerase (PDI) family of oxidoreductases, and contains two thioredoxin-like CXXC motifs (TR1 and TR2), located in N- and C-terminal domains (11). Mutation of the cysteine residues within these motifs abrogates function, indicating them to be the active sites (1). Some functional cooperativity between the two TR motifs has also been demonstrated in both the tapasin interaction and the ability of ERp57 to form transient intermediates with other substrates (3, 4).

In this study, we set out to detect the presence of ERp57-tapasin conjugates using a rapid acidification protocol that would also enable us to chemically modify cysteines within

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the TR motifs, thus permitting an analysis of the redox state of both free and tapasin-conjugated ERp57. Our data indicate that the available TR motifs in ERp57 can be detected in the reduced state whether tapasin is conjugated or not.

## MATERIALS AND METHODS

### *Cell lines*

C58, T2, and Daudi cell lines were cultured in RPMI 1640 supplemented with 5% bovine calf serum (5-R). T2 cells expressing rat TAP1 and TAP2 (T2-TAP1+2) were cultured in 5-R containing 1 mg/ml G418 (19). The .220 cell line, and transfectants of .220 with human leucocyte antigen (HLA)-B8, and B8 plus human tapasin (14), were gifts from Drs. P. Lehner and E. Hewitt (University of Cambridge, U.K.), and were cultured in 5-R, 5-R plus 1 mg/ml G418, and 5-R plus 1 mg/ml G418 and 500 ng/ml puromycin, respectively. C58 cells expressing rat ERp57 with the C-terminal cysteine residues of both TR motifs mutated to serine (1) were generated by electroporation (160 V, 960 μF) with mutated ERp57 cloned into the pCR3 expression vector. To assist in detection, this construct was engineered to contain also the SV5 epitope tag at the C-terminus (9). The resulting C58.ERp57.(CXXS)2 cells were cultured in 5-R plus 1 mg/ml G418.

### *Antibodies*

Rabbit anti-ERp57 was a gift from N. Bulleid (University of Manchester, U.K.). Rabbit anti-human tapasin was a gift from Dr. P. Lehner, and rabbit anti-murine tapasin was a gift from Dr. T. Hansen (Washington University, St. Louis, MO, U.S.A.). The monoclonal antibody Pk was used to detect the SV5 epitope tag.

### *Acidification and alkylation of cysteines*

One million cells were resuspended in 0.5 ml of 5-R and incubated for 10 min at 37°C in the presence or absence of 5 m*M* dithiothreitol (DTT) or 0.5 m*M* diamide (Sigma). Acidification was then performed based on the method of Mezghrani *et al.* (18). In brief, cells were resuspended in 100 μl of ice-cold 10% trichloroacetic acid (TCA) in phosphate-buffered saline for 10 min. After a 5-min spin at 20,000 *g*, the pellets were rinsed with 0.5 ml of acetone, and again spun for 2 min at 20,000 *g*. The pellets were then resuspended in 50 μl of nonreducing sample buffer with or without freshly dissolved 20 m*M* 4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes, The Netherlands). Samples were incubated for 30 min at room temperature and for 10 min at 37°C. To facilitate sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, samples were supplemented with a further 50 μl of nonreducing sample buffer and passaged 5 to 10 times gently through a 25-gauge syringe. Samples were resolved by 8% SDS-PAGE, transferred to nitrocellulose (Protran, BA85, Schleicher and Schuell), and immunoblotted with relevant antibodies. Second stage antibodies were horseradish peroxidase-coupled monoclonal anti-rabbit IgG (Sigma) or monoclonal anti-murine Kappa light chain (Caltag Laboratories). Immunoblots were developed using Supersignal West Femto chemiluminescent reagents (Pierce). Immunoblots were stripped of antibody by three washes for 5 min in 0.1% Ponceau S in 5% acetic acid, and then reprobed with relevant antibodies.

## RESULTS

Resuspension of cells in TCA lowers the pH of intracellular compartments, preventing thiol exchange and permitting the detection and analysis of mixed-disulfide intermediates. Subsequent treatment of exposed thiols with the alkylating agent AMS results in an increase in molecular mass that can be detected by SDS-PAGE (18). The initial description of the ERp57-tapasin conjugate relied on preservation of the labile disulfide bond between ERp57 Cys57 and tapasin Cys95 by preincubation of cells in the alkylating agent *N*-ethylmaleimide (4). We therefore asked whether TCA and AMS treatment could also be used to detect the ERp57-tapasin conjugate.

Rat C58 thymoma cells, TAP-deficient human T2, T2-TAP1+2, and human β2m-deficient Daudi cells were preincubated with or without DTT, treated with TCA, and free thiols modified with AMS. Samples were then immunblotted with anti-ERp57 antisera. In each cell line tested, a species of ~110 kDa was detected (Fig. 1A), suggestive of an ERp57-tapasin conjugate. In the absence of AMS modification, no 110-kDa species was detected, suggesting that the disulfide bond can readily be broken when free thiols are not alkylated. Variable amounts of non–tapasin-associated ERp57 with approximate size 57 kDa (non–AMS-treated) or 59 kDa (AMS-treated) could be detected in each cell line. Significantly, AMS-modified ERp57 migrated identically to AMS-modified ERp57 in DTT-pretreated cells. This indicates that the TR motifs of the intracellular pool of non–tapasin-associated ERp57 are almost exclusively in the reduced state. Figure 1 also confirms that formation of the 110-kDa conjugate occurs both in the absence of TAP (T2 cells) and in the absence of MHC class I heavy chain association with β2m (Daudi cells).

To confirm the identity of the 110-kDa species, we performed two further experiments. Firstly, tapasin-deficient .220 cells, .220.B8 cells (expressing HLA-B8), and .220.B8.tpn (expressing HLA-B8 and human tapasin) were treated with TCA/AMS. Only in the presence of tapasin could the 110-kDa complex be detected by immunoblotting with both anti-ERp57 (Fig. 1B, upper panels) and anti-tapasin antisera (Fig. 1B, lower panels). Secondly, we confirmed that the 110-kDa species in rat C58 cells was detected by both anti-ERp57 and anti-tapasin antisera (Fig. 1C). The experiments shown in Fig. 1B and C using the .220 series of transfectants and C58 cells also included the analysis of cells pretreated with the oxidizing agent diamide. We included diamide to determine if the labile disulfide bond between ERp57 and tapasin could be broken under oxidizing conditions, possibly by enhancing the oxidoreductase escape pathway involving the C-terminal cysteine residue of the TR motif. In tapasin-deficient .220 cells, diamide increased the mobility of ERp57, indicating complete oxidation of the TR motifs. In .220.B8.tpn and C58 cells, less ERp57-tapasin conjugate was detected in diamide-treated

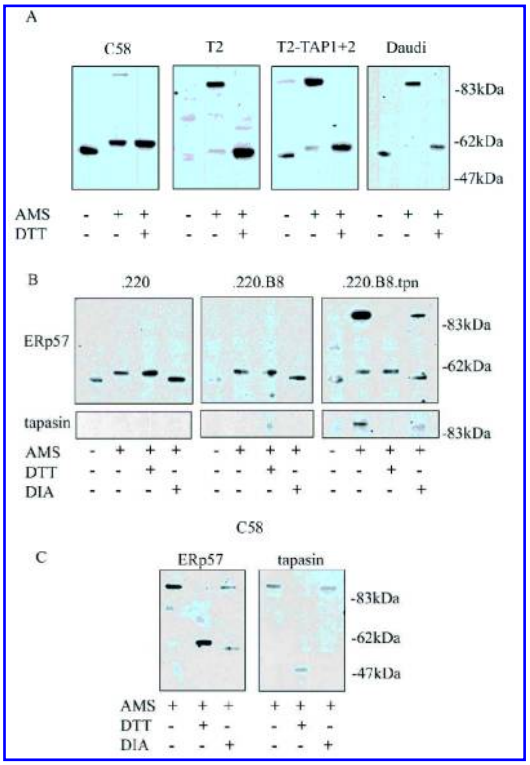


**FIG. 1. ERp57-tapasin conjugates can be detected by rapid intracellular acidification and thiol alkylation.** (**A**) TCA- and AMS-treated C58, T2, T2-TAP1+2, and Daudi cells were immunoblotted for the presence of ERp57. All the cells contain a putative ERp57-tapasin conjugate of size 110 kDa. In addition, nonconjugated ERp57 treated with AMS migrates identically to DTT-pretreated cells, indicating the TR motifs of ERp57 to be in a reduced state. (**B**) Tapasin-deficient .220 cells only display a 110-kDa conjugate when transfected with tapasin. The 110-kDa conjugate in .220.B8.tpn cells is detected by antibodies to both ERp57 and tapasin. (**C**) The 110-kDa conjugate detected in rat C58 cells is confirmed to contain rat ERp57 and rat tapasin. DIA, diamide.

cells; however, there was not a corresponding increase in free ERp57 signal. Diamide-treated samples frequently displayed material that failed to fully enter the resolving gel, thus leading to lower overall levels of protein loading. This may account for reduced signals in these tracks. Nevertheless, diamide treatment failed to cause complete disulfide bond breakage of the ERp57-tapasin conjugate.

In several experiments using rat C58 cells preincubated in diamide, we observed that the ERp57-tapasin conjugate resolved into two bands, whereas in the absence of diamide it resolved as a single species. This was detected using both anti-tapasin (Fig. 2A, with expanded view of ERp57-tapasin region) and anti-ERp57 antisera (data not shown). We considered that this doublet might be the result of diamide oxidizing the C-terminal TR2 motif, which is not thought to interact with tapasin (4). To test this hypothesis, we transfected C58 cells with a mutated form of ERp57 in which the C-terminal cysteine residue of both TR motifs was mutated to serine, thus rendering the TRs insensitive to diamide-induced oxidation. The construct was also epitope-tagged to permit differential detection from endogenous ERp57. The absence of two cysteine residues in this mutant results in a product that does not increase in molecular mass upon AMS treatment as much as wild-type ERp57, as shown by immunoblotting of untransfected C58 and C58.ERp57.(CXXS)2 cells with anti-ERp57 and anti-epitope tag (Pk) antibodies, respectively (Fig. 2B). C58.ERp57.(CXXS)2 cells were then pretreated with or without DTT or diamide and processed with TCA and AMS as before, followed by immunoblotting with anti-epitope tag antibody. The presence of the CXXS "trapping" TR motif resulted in the detection of an ERp57-tapasin conjugate in the absence of AMS alkylation (Fig. 2C), which did not occur with normal TR motifs (see Fig. 1A and B). Furthermore, diamide treatment did not increase the mobility of the non–tapasin-conjugated ERp57, which remained ~59 kDa in

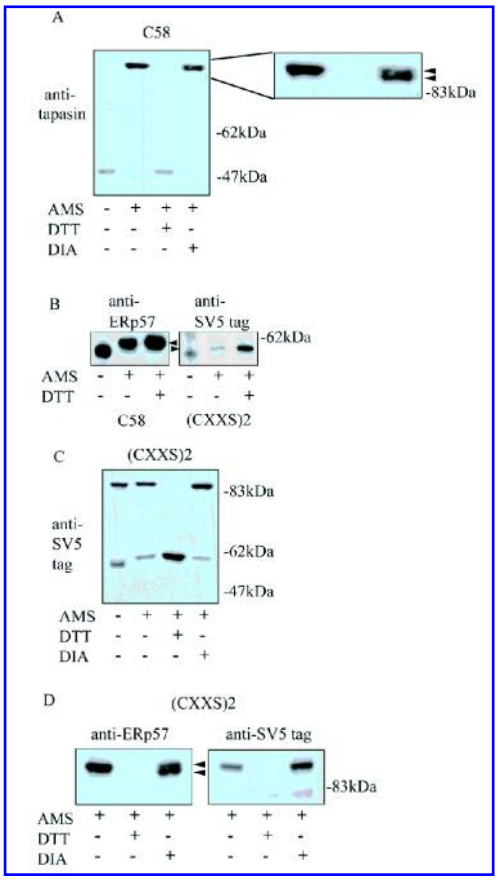


**FIG. 2 Diamide causes oxidation of the free TR motif in the ERp57-tapasin conjugate.** (**A**) Pretreatment of rat C58 cells with diamide (DIA) causes the ERp57-tapasin conjugate, immunoblotted here with tapasin antibodies, to resolve as a doublet (arrowheads). (**B**) A (CXXS)2 motif mutant of ERp57 expressed in C58 cells displays a smaller increase in molecular mass after AMS alkylation in comparison with wild-type ERp57. Arrowheads indicate relative sizes of wild-type and mutant molecules. (**C**) A (CXXS)2 motif mutant of ERp57 expressed in C58 cells traps tapasin without the need for subsequent alkylation. (**D**) Immunoblotting the ERp57-tapasin conjugate in C58.ERp57.(CXXS)2 cells reveals that the TR motif mutant is not oxidized by diamide, whereas endogenous ERp57 is oxidized. Arrowheads indicate the doublet obtained with diamide.

size, indicating a lack of oxidation of the TR motif. Crucially, the ERp57-tapasin conjugate detected by the anti-SV5 tag antibody appeared to migrate as a single species. To test this further, we repeated the experiment and immunoblotted first with antisera to ERp57, followed by anti-SV5 tag antibody after acid stripping of the membrane. The ERp57-tapasin conjugate again resolved as a doublet when revealed by anti-ERp57 (Fig. 2D). However, the ERp57.(CXXS)2-tapasin conjugate resolved as a single species as detected by the anti-SV5 tag antibody. Overlaying the autoradiographs demonstrated that the (CXXS)2 mutant comigrated with the upper band of the ERp57-tapasin doublet. As the CXXS motifs cannot be oxidized, we conclude that the free TR motif in the ERp57-tapasin conjugate is capable of being oxidized by diamide.

## DISCUSSION

Oxidoreductases usually undergo only transient disulfide interactions with substrate polypeptides, assisting in the oxidation, reduction, or isomerization of bonds (6, 13). The ERp57-tapasin conjugate appears not to follow this general rule. Our data suggest that the majority of ERp57 and tapasin, in the cell lines tested here, are complexed together. The absolute amount of ERp57-tapasin conjugate is subject to some variation between experiments, for example, compare the images obtained for ERp57 in C58 cells in Fig. 1A and C. Figure 1A represents an atypically low level of ERp57-tapasin conjugate, and was included primarily to illustrate the identical SDS-PAGE migration of reduced ERp57 in DTT-treated cells and free ERp57 after AMS alkylation. Nevertheless, some variation was detected in ratios of ERp57-tapasin conjugate levels in most of the cell lines used in this study, for reasons that we do not currently understand.

The disulfide bond between ERp57 and tapasin is certainly very labile, and its detection absolutely requires either alkylation of the adjacent cysteine in the TR motif or its removal by mutagenesis (Fig. 2C). The fragile nature of this ERp57-tapasin disulfide bond raises questions as to how ERp57 is maintained as part of the PLC. Calreticulin is probably bound through a combination of associations with the conserved Asn-linked monoglucosylated glycan at position 86 of the MHC class I heavy chain (10), and other hydrophobic interactions with the heavy chain that remain to be fully defined (17). The extended P-domain of calreticulin directly interacts with ERp57 (8), thus providing one possible explanation for the residency of ERp57 within the PLC. However, the affinity of the calreticulin–ERp57 interaction is low, a feature that is thought to benefit the transient associations that calreticulin and ERp57 have with most folding polypeptides in the ER. Therefore, to prolong the presence of ERp57 within the PLC, tapasin may have evolved the ability to use the N-terminal TR motif to generate the ERp57-tapasin conjugate. Because, as previously mentioned, this disulfide bond is very labile, it may be that ERp57 is caught in a sandwich of transient associations between calreticulin and tapasin.

ERp57 is homologous to PDI, which is considered to be the major oxidoreductase activity in the ER. However, there are less data at present showing a role for PDI in the assembly of MHC class I molecules in comparison with ERp57, either before or after the formation of the PLC (5, 26). There is evidence for PDI interacting with calreticulin (2), but an interaction with tapasin has not been reported.

A major observation of our data is that non–tapasin-associated ERp57 is present almost totally in a reduced state. In contrast, data for PDI suggest it is present normally in an oxidized (7) or only partially reduced state (18). This raises the question if, and how, the redox state of ERp57 is regulated. PDI is oxidized by Ero1, but Ero1 does not affect ERp57 (18). Could tapasin regulate the redox status of ERp57? This intriguing possibility arises because frequently the majority of ERp57 and tapasin are complexed together. Some evidence for cross-talk between the two TR motifs in the ERp57-tapasin conjugate does exist (3, 4), and our data indicate that the free TR motif is only partially affected by diamide. The tapasin-ERp57 conjugate has been characterized so far in lymphoid cell lines, or epithelial cells stimulated with interferon-γ, all likely to express tapasin. It would be interesting to determine the status of ERp57 in other nonlymphoid cells. With relevance to this point, in tapasin-deficient .220 cells ERp57 does not form a predominant conjugate with any other polypeptide, and again appears fully reduced. Thus, in contrast to PDI, perhaps ERp57 does not require an oxidizing partner polypeptide.

The detection of ERp57 disulfide-bonded to tapasin is likely to be of fundamental importance to our understanding of the assembly of MHC class I molecules. As yet, we do not fully understand what each component of the PLC contributes to the final outcome of peptide-loaded MHC class I molecules. However, in studying this complex, we are likely to learn much about the quality control of polypeptide folding within the ER.

## ACKNOWLEDGMENTS

We thank Adam Benham for advice and discussions during the conceptualization of this work, and those listed in Materials and Methods for continuing generosity with reagents. S.J.P. is a U.K. Medical Research Council Senior Research Fellow.

## ABBREVIATIONS

AMS, 4′-maleimidylstilbene-2,2′–disulfonic acid; DTT, dithiothreitol; ER, endoplasmic reticulum; HLA, human leucocyte antigen; MHC, major histocompatibility complex; PDI, protein disulfide isomerase; PLC, peptide loading complex; 5-R, RPMI 1640 supplemented with 5% bovine calf serum; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAP, transporter associated with antigen presentation; TCA, trichloroacetic acid; TR, thioredoxin.

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Received for publication August 5, 2002; accepted May 9, 2003.

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