

The oxidoreductase ERp57 efficiently reduces partially folded in preference to fully folded MHC class I molecules

Antony N. Antoniou, Stuart Ford, Magnus Alphey, Andrew Osborne, Tim Elliott¹ and Simon J. Powis²

Division of Cell Biology and Immunology, School of Life Sciences, University of Dundee, Dundee DD1 5EH and ¹Cancer Sciences Division, University of Southampton School of Medicine, Southampton General Hospital, Southampton SO16 6YD, UK

²Corresponding author
e-mail: s.j.powis@dundee.ac.uk

The oxidoreductase ERp57 is an integral component of the peptide loading complex of major histocompatibility complex (MHC) class I molecules, formed during their chaperone-assisted assembly in the endoplasmic reticulum. Misfolded MHC class I molecules or those denied suitable peptides are retrotranslocated and degraded in the cytosol. The presence of ERp57 during class I assembly suggests it may be involved in the reduction of intrachain disulfides prior to retrotranslocation. We have studied the ability of ERp57 to reduce MHC class I molecules *in vitro*. Recombinant ERp57 specifically reduced partially folded MHC class I molecules, whereas it had little or no effect on folded and peptide-loaded MHC class I molecules. Reductase activity was associated with cysteines at positions 56 and 405 of ERp57, the N-terminal residues of the active CXXC motifs. Our data suggest that the reductase activity of ERp57 may be involved during the unfolding of MHC class I molecules, leading to targeting for degradation.

Keywords: chaperone/endoplasmic reticulum/MHC class I/oxidoreductase ERp57/protein folding

Introduction

Major histocompatibility complex (MHC) class I molecules present short peptides to CD8⁺ T lymphocytes, permitting the detection and elimination of pathogen-infected cells. MHC class I molecules meet and bind peptides during assembly within the endoplasmic reticulum (ER) in an assembly process known to involve a series of interactions with the ER resident chaperones calnexin and calreticulin, the accessory molecule tapasin and the oxidoreductase ERp57 (Lehner and Trowsdale, 1998; Pamer and Cresswell, 1998). An early interaction of the MHC class I heavy chain with calnexin is normally rapidly replaced by the formation of a peptide loading complex containing calreticulin, ERp57, tapasin and the transporter associated with antigen processing (TAP) (Solheim *et al.*, 1997; Diedrich *et al.*, 2001). The MHC class I-specific accessory molecule tapasin is critical for the formation of this complex. Tapasin physically links MHC class I-chaperone complexes to TAP and is also involved in editing the peptide repertoire bound by MHC class I

molecules (Lehner *et al.*, 1998; Grandea and Van Kaer, 2001; Purcell *et al.*, 2001).

ERp57 is a member of the protein disulfide isomerase (PDI) family, whose functions include disulfide bond oxidation, reduction and isomerization (Gilbert, 1997; High *et al.*, 2000; Ellgaard and Helenius, 2001). The active cysteine residues are contained within two CXXC motifs located within N- and C-terminal thioredoxin-like domains (Hirano *et al.*, 1995; Urade *et al.*, 1997). ERp57 forms complexes with the ER chaperones calnexin and calreticulin (Oliver *et al.*, 1997, 1999; High *et al.*, 2000; Molinari and Helenius, 2000). These chaperone complexes possess specificity for monoglucosylated polypeptides, and it has been postulated that through consecutive rounds of deglycosylation and reglycosylation of non-native glycoprotein substrates, cycles of folding occur until a native protein conformation has been adopted (Elliott *et al.*, 1997; Zapun *et al.*, 1998; Oliver *et al.*, 1999).

The association of ERp57 with MHC class I molecules and the TAP complex (Hughes and Cresswell, 1998; Lindquist *et al.*, 1998; Morrice and Powis, 1998) and the detection of ERp57-MHC class I mixed disulfide intermediates (Lindquist *et al.*, 2001) suggests that ERp57 is the main oxidoreductase involved in MHC class I assembly. Recently, ERp57 has also been shown to form a disulfide interaction with tapasin rather than MHC class I molecules when part of the peptide loading complex (Dick *et al.*, 2002).

MHC class I misfolding during normal assembly, or due to deficiency in peptide supply by inhibition of the proteasome or TAP, or by intervention by viral proteins (Wiertz *et al.*, 1996), results in retrotranslocation, deglycosylation of the heavy chain, ubiquitylation and degradation by the proteasome (Hughes *et al.*, 1997; Shamu *et al.*, 1999). Prior to the retrotranslocation step from the ER it is thought that MHC class I molecules must first undergo unfolding and reduction of disulfide bonds (Tortorella *et al.*, 1998; Fagioli *et al.*, 2001). To address whether ERp57 can directly reduce the disulfide bonds present in MHC class I molecules, we have developed a novel assay system to monitor the status of disulfide bonds in folding and folded MHC class I molecules in the presence of recombinant ERp57. Our results indicate that ERp57 can efficiently reduce a partially folded pool of MHC class I heavy chains, whereas folded MHC class I molecules are resistant to reduction. We also define, by site-directed mutagenesis, the cysteine residues that are critical in the reduction process.

Results

Monoclonal antibody HC10 recognizes oxidized and reduced pools of MHC class I

Monoclonal antibody (mAb) HC10 recognizes a pool of partially folded, non- β 2-microglobulin (B2m) associated,

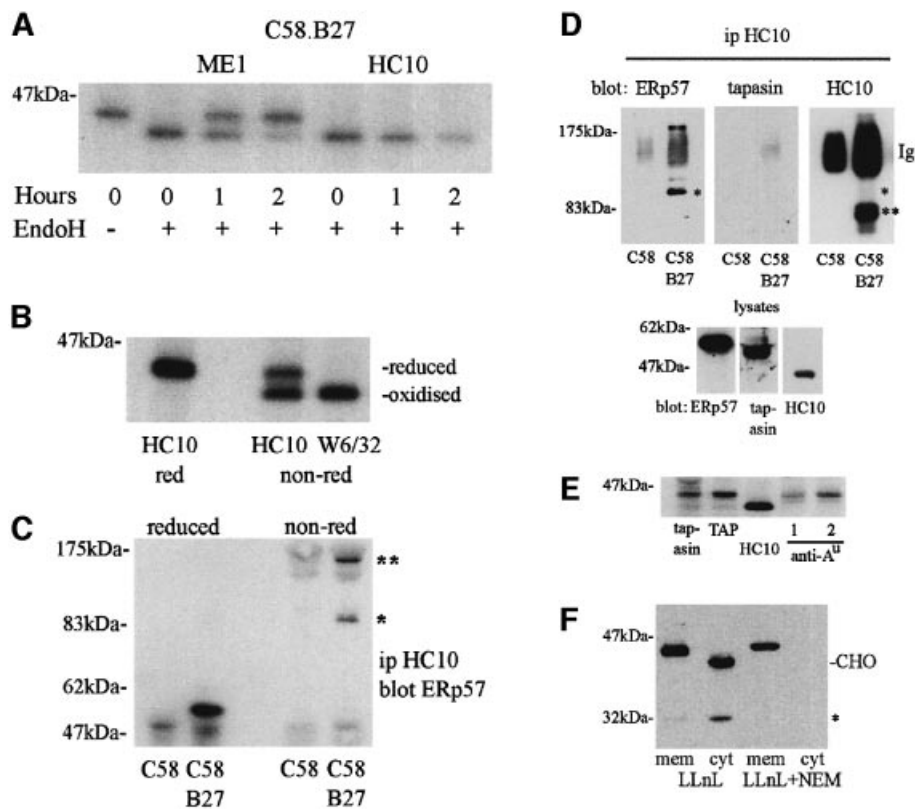


Fig. 1. MHC class I assembly in the ER. (A) C58.B27 cells were pulse radiolabelled, chased for the indicated times and B27 molecules immunoprecipitated with ME1 (folded B27) or HC10 (unfolded), followed by endo H digestion. (B) T3 cells were radiolabelled and MHC class I molecules immunoprecipitated with HC10 (unfolded) or W6/32 (folded). Samples were analysed reduced or non-reduced. (C) C58 or C58.B27 cells were lysed and immunoprecipitated with HC10. Samples were analysed reduced or non-reduced, followed by immunoblotting to reveal ERp57. (D) The experiment shown in (C) was repeated and immunoblotted for ERp57, tapasin and B27. Control blots of whole-cell lysates performed at the same time are also shown. (E) C58.B27 cells were radiolabelled, lysed in digitonin and immunoprecipitated with antibodies to tapasin, TAP, B27 (HC10) and rat RT1.Au (anti-A^u), NR5/10 (lane labelled 1) and GN7/5.B11 (lane labelled 2). (F) C58.B27sv5 cells (C-terminally tagged B27) were incubated with LLnL with or without NEM, membrane and cytosol fractions were prepared and B27 molecules detected by immunoblotting with anti-tag mAb Pk. A cytosolic deglycosylated (CHO) B27 heavy chain product is indicated, as is a novel 32 kDa heavy chain breakdown fragment (*). Similar results were obtained with HC10, except for the absence of the 32 kDa product.

human HLA-B and -C class I molecules (Stam *et al.*, 1986). Rat C58 thymoma cells transfected with HLA-B*2705 (C58.B27) were pulse-chased, immunoprecipitated with HC10 and analysed by SDS-PAGE under reducing conditions. The HC10 reactive class I pool did not acquire endoglycosidase H (endo H) resistance, indicating that the HC10 reactive class I pool is predominantly located within the ER (Figure 1A). The HC10 signal also decreased over 2 h of chase. This loss of HC10 reactive material is indicative of class I molecules achieving fully folded status, but it is also likely to include a pool of molecules that fail to fold and are subsequently degraded (Raposo *et al.*, 1995; Mear *et al.*, 1999). In contrast, fully folded B27 molecules, as detected by mAb ME1, acquired endo H resistance, indicating exit from the ER and transit to the cell surface over the same time period (Figure 1A).

Under non-reducing SDS-PAGE the same pool of HC10 reactive MHC class I molecules shown in Figure 1A resolves as two distinct species with different gel mobilities (Figure 1B). The upper band represents class I molecules with no intrachain disulfides and shares similar mobility to fully reduced HC10 reactive molecules (Tector

et al., 1997). The lower band represents a partially folded oxidized class I species containing disulfide bonds and shares similar mobility to fully folded class I molecules, as detected by conformation-specific antibodies such as mAb W6/32. Similar results were obtained in C58.B27 cells using HC10 and ME1 mAbs and with HC10 in the B2m-deficient cell line Daudi (data not shown). Thus, mAb HC10 can be used to detect pools of partially folding oxidized and reduced class I molecules in the ER.

ERp57 forms a mixed disulfide intermediate with HLA-B27

We have previously shown that ERp57 associates with rat MHC class I molecules in the C58 cell line (Morrice and Powis, 1998). To determine whether ERp57 actively participates in the assembly of B27 molecules in these cells, we looked for the formation of a mixed disulfide intermediate (Lindquist *et al.*, 2001). C58.B27 cells, pretreated with *N*-ethylmaleimide (NEM) to trap transient intermediates and inhibit post-lysis disulfide bond formation, were lysed in NP-40 containing lysis buffer, which disrupts non-disulfide interactions, and immunoprecipitated with HC10. Western blotting with anti-ERp57

antisera revealed a species under non-reducing conditions of mol. wt ~95–100 kDa, the correct weight to be a complex of ERp57 (~56 kDa) and B27 class I heavy chain (~43 kDa), as shown in Figure 1C (single asterisk). An unidentified higher molecular weight species was also detected (Figure 1C, double asterisks), whose weight was difficult to determine due to non-reduced immunoglobulin distorting this region of the gel. Both these high molecular weight complexes are disrupted on reduction to reveal the presence of ERp57.

In light of the recent observation that ERp57 is disulfide-bonded to tapasin in the peptide-loading complex (Dick *et al.*, 2002), we investigated the possible presence of tapasin as part of our B27–ERp57 complex. The experiment shown in Figure 1C was repeated and the membrane sequentially immunoblotted for the presence of ERp57, tapasin and B27. As shown in Figure 1D, we readily detected the species previously noted with ERp57 antisera, comprising an ~100 kDa species (single asterisk) and a larger species. However, in both this experiment and several others we failed to detect any tapasin-conjugated species. Immunoblotting with HC10 revealed the faint presence of the B27–ERp57 conjugate (single asterisk), but the signal was swamped by the presence of cross-reactivity on the HC10 immunoglobulin used in the original immunoprecipitation (labelled Ig), and also by the presence of HC10-reactive B27 homodimers (double asterisk) with mol. wt ~83 kDa (Allen *et al.*, 1999). Simultaneous immunoblotting of whole-cell lysates confirmed the presence of ERp57, tapasin and B27. Taken together these data indicate that the partially folded pool of B27 molecules isolated by HC10 can undertake disulfide interactions with ERp57, but not with tapasin.

We also determined whether, when expressed in rat C58 cells, B27 exhibited a tapasin-independent phenotype previously noted by expression in the tapasin-deficient 721.220 cell line (Peh *et al.*, 1998). Digitonin lysates of radiolabelled C58.B27 cells were immunoprecipitated with anti-murine tapasin or anti-rat TAP antibodies. As shown in Figure 1E, the level of B27 interaction with tapasin and TAP was low in comparison with the endogenous rat MHC class I allele RT1.A^u expressed by C58 cells, indicating that B27 is tapasin-independent in these cells.

NEM has previously been shown to inhibit the degradation of MHC class I heavy chains in Daudi cells, suggesting that reduction occurs prior to retrotranslocation (Tortorella *et al.*, 1998). We therefore tested whether NEM inhibited the retrotranslocation of B27 molecules in C58.B27 cells. Cells expressing B27 epitope-tagged at the C-terminus were treated with the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal (LLnL) with or without NEM, and membrane and cytosol fractions prepared. In the presence of LLnL, a deglycosylated (Hughes *et al.*, 1997) B27 heavy chain was detected in the cytosolic fraction (Figure 1F). We also detected a previously unreported MHC heavy breakdown product of ~32 kDa (single asterisk). The appearance of the cytosolic heavy chain products was inhibited by the additional presence of NEM, indicating that the redox status of components in the ER is critical to the degradation process of MHC class I molecules.

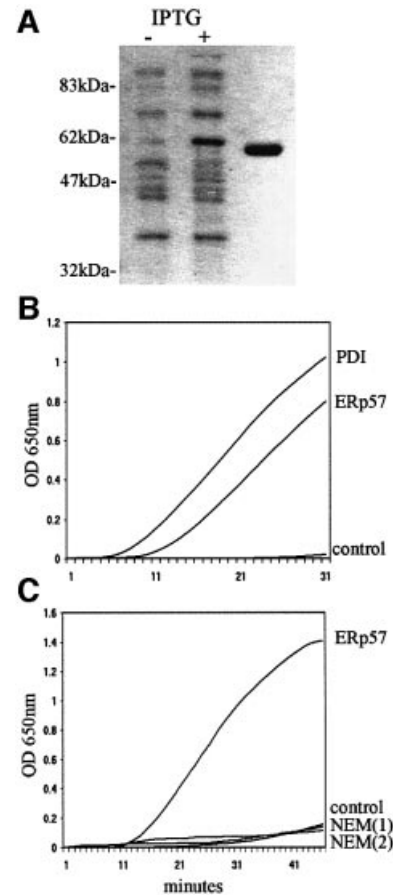


Fig. 2. Purification and activity of recombinant ERp57. (A) *Escherichia coli* expressing histidine-tagged rat ERp57 were cultured in the presence (+) or absence (-) of isopropyl β -D-thiogalactopyranoside (IPTG). Recombinant ERp57 was purified on Ni-agarose, thrombin cleaved to remove the histidine tag, and further purified by anion exchange chromatography (last lane). (B) Insulin reduction. ERp57 and PDI, both at 0.3 μ M, were mixed with 1 mg/ml insulin and DTT (0.32 mM), and the OD₆₅₀ measured at 60-s intervals. Control sample included insulin with DTT alone. (C) NEM inhibition of ERp57 activity. Insulin reduction assays were performed with ERp57 (1 μ M) in the presence of 1 mM NEM [NEM(1)], or by including 1 mM NEM in an ERp57 pre-activation step prior to addition to the insulin [NEM(2)]. Control reaction included insulin and DTT alone.

Recombinant ERp57 reduces MHC class I heavy chains *in vitro*

The ability of HC10 to detect partially folded and oxidized class I molecules, as shown in Figure 1B, and the formation of B27–ERp57 disulfide-bonded intermediates (Figure 1C and D) led us to ask whether we could demonstrate ERp57-mediated reduction of MHC class I molecules *in vitro*. Recombinant rat ERp57 was therefore expressed in bacteria and purified to homogeneity (Figure 2A). To determine activity, the purified product was tested for its ability to reduce and precipitate insulin (Hirano *et al.*, 1995). Recombinant ERp57 displayed reductase activity (Figure 2B), but was less efficient than PDI, an observation previously noted for ERp57 in comparison with both PDI and thioredoxin (Srivastava *et al.*, 1993; Bourdi *et al.*, 1995; Hirano *et al.*, 1995).

To determine whether ERp57 could affect the status of disulfides in partially folded MHC class I molecules, we mixed together HC10 reactive class I molecules with

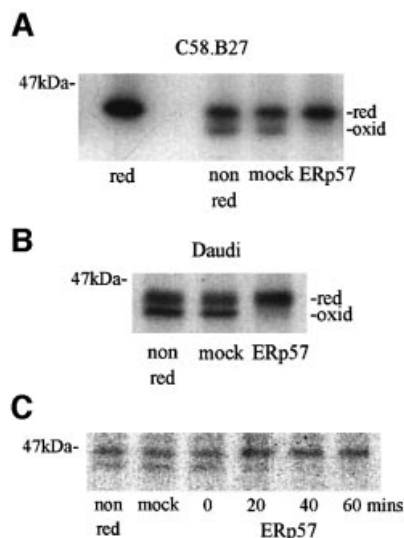


Fig. 3. ERp57 reduction of partially folded MHC class I molecules. (A) HC10 immunoprecipitates from radiolabelled C58.B27 cells were incubated in the presence of ERp57 (1 μ M) or in assay buffer alone (mock). Control samples were analysed reduced (red) or non-reduced (non red) for comparison. (B) HC10 immunoprecipitates from radiolabelled Daudi cells were incubated with 1 μ M ERp57, or mock-treated in assay buffer alone (mock), or analysed non-reduced as indicated. (C) Time course of ERp57-mediated reduction of MHC class I heavy chains. HC10 immunoprecipitates of radiolabelled C58.B27 cells were incubated with 1 μ M ERp57 for the indicated times. Non-reduced and mock-treated controls were as described in (A) and (B).

recombinant ERp57. C58.B27 and Daudi cells were metabolically labelled, lysed in NP-40 buffer and immunoprecipitated with HC10. Pre-activated ERp57 was added for 1 h, followed by non-reducing SDS-PAGE analysis. Control or mock-treated samples retained the lower disulfide-containing band; however, in the presence of ERp57, reduction of the lower band was observed in both C58.B27 and Daudi samples, with an increase in the density of the upper band (Figure 3A and B). No smaller molecular weight species were detected by SDS-PAGE or mass spectrometry, indicating that in this system ERp57 was not acting as a protease. The kinetics of ERp57-dependent reduction of the oxidized MHC class I heavy chain were similar to that of insulin, with much of the reduction being complete within 40 min (Figure 3C). Addition of alkylating agents such as NEM or iodoacetamide during either the assay or during the preactivation of ERp57 inhibited both insulin (Figure 2C) and MHC class I reduction (not shown), indicating that the reductase activity was mediated through cysteine residues.

Folded and peptide-loaded MHC class I molecules are resistant to ERp57 reduction

The above data indicated that partially folded MHC class I molecules were suitable substrates for reduction by ERp57. We next asked whether fully folded MHC class I molecules could also be substrates for ERp57. BB7.2 (anti-HLA-A2 specific) immunoprecipitates from T3 cells (TAP-deficient human T2 cells expressing rat TAP1 and TAP2) and ME1 immunoprecipitates from C58.B27 were incubated with recombinant ERp57 and analysed by non-reducing SDS-PAGE. BB7.2-isolated A2 molecules

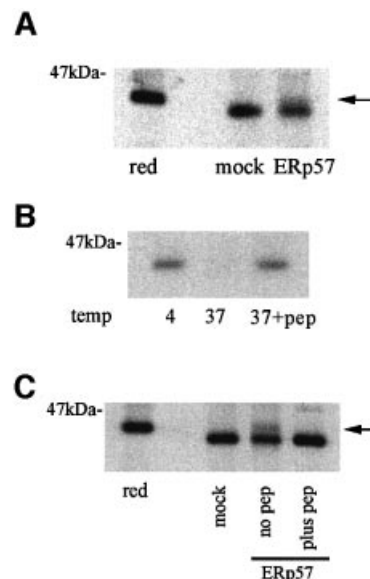


Fig. 4. ERp57 is inefficient at reducing folded and peptide-loaded MHC class I molecules. (A) BB7.2 HLA-A2 immunoprecipitates from radiolabelled T3 cells were incubated with 1 μ M ERp57, or mock-treated with assay buffer alone and analysed non-reduced. A minor population of reduced heavy chains is indicated by an arrow. (B) Peptide stabilization of A2 molecules. Lysates of radiolabelled T3 cells were incubated at 4°C, or at 37°C in the presence or absence of 50 μ M A2 binding peptide LLDVPTAAV, followed by immunoprecipitation with BB7.2. (C) BB7.2 immunoprecipitates from radiolabelled T3 lysates as shown in (A), and a peptide stabilized population (plus pep) as indicated in (B) were incubated with 1 μ M ERp57, or mock-treated with assay buffer alone. The arrow indicates a minor population of reduced A2 heavy chains detected in the non-peptide stabilized sample (no pep).

showed essentially no reduction, apart from a minor population (Figure 4A, arrow). Similarly, ME1-isolated B27 molecules showed no reduction in the presence of ERp57 (data not shown). The minor population of reduced A2 molecules could be derived from two possible sources, either an empty population of folded A2 molecules awaiting peptide loading or a population loaded with weak affinity peptides that may dissociate during the course of the assay, thus permitting ERp57-mediated reduction. To address this issue we utilized the ability of defined synthetic peptide epitopes to stabilize MHC class I molecules when added to detergent lysates treated at 37°C. Under such conditions, peptide-free MHC class I molecules dissociate, allowing the subsequent immunoprecipitation of MHC class I molecules loaded with a defined peptide (Figure 4B). We therefore incubated radiolabelled lysates of T3 cells with the A2 binding peptide LLDVPTAAV and immunoprecipitated peptide-loaded A2 molecules. These were then incubated with recombinant ERp57 and analysed by non-reducing SDS-PAGE. Under these conditions ERp57 did not reduce peptide-loaded A2 molecules, whereas non-peptide-loaded A2 molecules again showed a small population susceptible to reduction (Figure 4C).

We also investigated a mutant A2 class I molecule containing a threonine to lysine amino acid change at position 134 (T134K) in the α 2 domain. T134K rapidly transits to the cell surface with a sub-optimal selection of peptides, and whilst it can present synthetic peptides when added exogenously, it is inefficient at presenting endo-

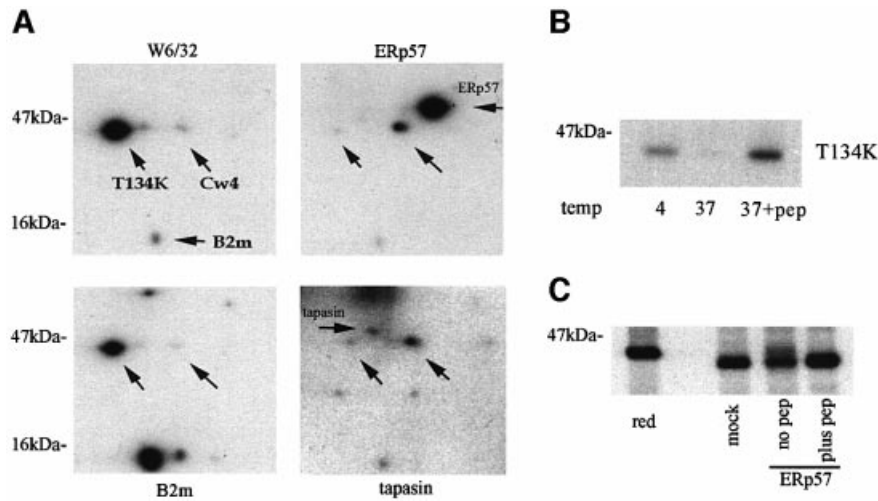


Fig. 5. T134K mutant A2 molecules form weak interactions with ERp57 and tapasin, but display similar resistance to ERp57 reduction. (A) Two-dimensional gel analysis of T134K immunoprecipitations. C1R-T134K cells were radiolabelled and immunoprecipitated with W6/32, anti-human B2m, anti-ERp57 and anti-tapasin. Upward pointing arrows indicated the positions of T134K molecules and the endogenous HLA-Cw4 molecule. Horizontal arrows indicate the position of B2m, ERp57 and tapasin as indicated. T134K is the major assembled MHC class I species in these cells (left panels). A short pre-clearing step results in the detection of T134K/B2m complexes that dissociate under longer incubation times. In comparison with Cw4, T134K forms a weak interaction with ERp57 and tapasin (right panels). Short labelling times result in the detection of the MHC class I molecules and the relevant chaperone being immunoprecipitated, and not the full peptide-loading complex. (B) Peptide stabilization of T134K molecules. Lysates of radiolabelled C1R-T134K cells were incubated at 4°C, or at 37°C in the presence or absence of 50 μ M A2 binding peptide LLDVPTAAV, followed by immunoprecipitation with BB7.2. (C) BB7.2 immunoprecipitates from radiolabelled C1R-T134K lysates, and a peptide stabilized population (plus pep) were incubated with 1 μ M ERp57, or mock-treated with assay buffer alone. The arrow indicates a minor population of reduced T134K heavy chains detected in the non-peptide stabilized sample (no pep).

generously produced epitopes (Lewis *et al.*, 1996). T134K forms only weak or transient interactions with calreticulin (Lewis and Elliott, 1998) and, as demonstrated by immunoprecipitation and two-dimensional gel analysis (Figure 5A), also fails to interact with ERp57 and tapasin, despite being the major assembled product recognized by W6/32 and anti-human B2m antibodies. Thus, T134K appears to circumvent the quality control event(s) in the ER, allowing us to test whether such a mutant would be more susceptible to ERp57-mediated reduction. Immunoprecipitated and peptide-loaded T134K molecules (Figure 5B) as described above were tested for reduction by recombinant ERp57. Non-peptide-loaded molecules were partially reduced, whereas peptide-loaded molecules were completely resistant (Figure 5C). Taken together these results suggest that MHC class I molecules, once having attained a fully folded conformation and bound peptide, are not substrates for reduction by ERp57.

Association of ERp57 reductase activity with Cys56 and Cys405 in CXXC motifs

The CXXC active site motifs are characteristic of oxidoreductases, and ERp57 contains two such motifs (Hirano *et al.*, 1995; Urade *et al.*, 1997). To determine which of the Cys residues were active in the reduction of partially folded MHC class I molecules, we mutated both of the N-terminal Cys residues in each of the motifs (Cys56 and Cys405) and the C-terminal Cys residues (Cys59 and Cys408) to generate (SXXC)₂ and (CXXS)₂ mutants, respectively, as outlined in Figure 6A. Both mutants were purified and tested for their ability to reduce insulin. The (SXXC)₂ mutant retained essentially no reductase activity, whereas the (CXXS)₂ mutant retained a diminished activity in comparison with wild-type ERp57

(Figure 6B). When tested for their ability to reduce partially folded B27 molecules, a similar pattern of results was obtained: (SXXC)₂ showed no activity whereas (CXXS)₂ demonstrated reduced activity (Figure 6C). Mixing the motif mutants with wild-type ERp57 had no inhibitory effect on it (data not shown). These data further confirm that the reductase activity of ERp57 on partially folded MHC class I molecules is mediated by the cysteines in the CXXC motifs. Furthermore, we have identified that retention of the N-terminal pair of cysteine residues alone can maintain limited reductase activity.

As a control for ERp57 reductase activity, we also included the ER oxidoreductase PDI in several experiments. To our surprise, despite PDI being a more active reductase in the insulin assay (Figure 2B), it was less able to reduce partially folded MHC heavy chains in comparison with ERp57 (Figure 6C). This occurred both when ERp57 and PDI were used at equivalent concentrations and when retested at concentrations demonstrating equivalent insulin reduction (not shown). When ERp57 and PDI were mixed together, efficient reduction was observed, indicating that PDI did not have an inhibitory effect on ERp57 activity (data not shown). This suggests that ERp57 has some as yet undetermined substrate selectivity in addition to that imposed upon it by interaction with its partner polypeptides calnexin and calreticulin.

Discussion

The oxidoreductase ERp57 is a component of the MHC class I molecule assembly complex. In digitonin containing cell lysates, a complex of calreticulin, ERp57, tapasin, MHC class I and TAP can be detected readily (Hughes and Cresswell, 1998; Lindquist *et al.*, 1998; Morrice and

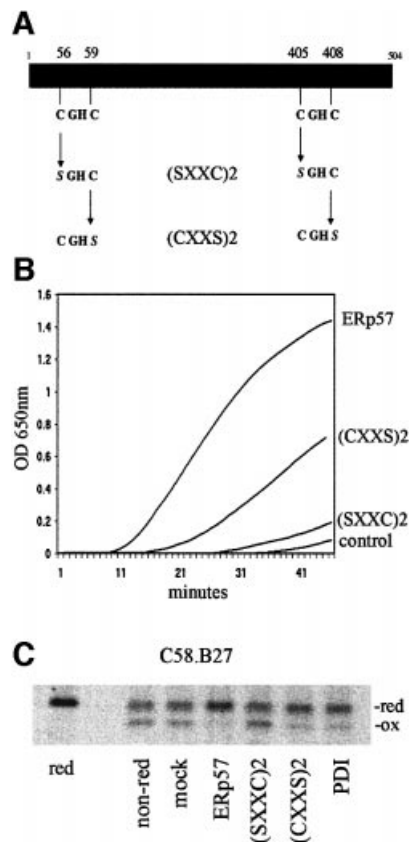


Fig. 6. ERp57 CXXC motif mutations affect reductase activity. (A) Schematic diagram of ERp57 indicating the location of the CXXC motifs and the generation of (SXXC)₂ and (CXXS)₂ mutants. (B) ERp57, (CXXS)₂ and (SXXC)₂ were incubated at 1 μ M with insulin and DTT. The optical density was determined at 60-s intervals. Control sample included insulin with DTT alone. (C) Reductase activity of CXXC motif mutants and PDI on partially folded MHC class I molecules. HC10 immunoprecipitates from radiolabelled C58.B27 cells were incubated with 1 μ M each of ERp57, (SXXC)₂, (CXXS)₂ or PDI. Control samples were treated with assay buffer alone (mock), or analysed reduced (red) or non reduced (non-red).

Powis, 1998), and ERp57–MHC class I interactions have also been observed in an *in vitro* semi-permeabilized cell system (Farmery *et al.*, 2000). ERp57 is thought to form direct interactions with chaperones such as calnexin and calreticulin; therefore, its presence in the class I assembly complex could be maintained purely through these chaperone associations (Oliver *et al.*, 1997, 1999). However, the presence of ERp57 in the TAP complex of cells lacking calreticulin (Gao *et al.*, 2002), and the detection of covalently linked ERp57–tapasin complexes but not ERp57–MHC class I complexes associated with TAP (Dick *et al.*, 2002), suggests that ERp57 is recruited by tapasin at this latter stage of MHC class I assembly. Nevertheless, a disulfide-bonded intermediate of ERp57 with MHC class I has been reported (Lindquist *et al.*, 2001), an observation that we have confirmed here for a human HLA allele (B*2705) assembling in a rat host cell line (Figure 1C). An explanation for these apparently contradictory observations may lie in the use of detection reagents. We and Lindquist *et al.* (2001) have used mAb HC10 to isolate ERp57–MHC class I complexes. Since HC10 only detects non-B2m-associated MHC class I

heavy chains, which are unlikely to be the TAP-associated pool, the ERp57–MHC class I complexes we have detected probably represent both the earliest stage of MHC class I assembly, involving calnexin, and also molecules undergoing reduction prior to retrotranslocation. Thus it is likely that ERp57 plays a dual role in MHC class I assembly, being directly involved in the formation and/or breakage of disulfide bonds in MHC class I heavy chains early during biosynthesis, and then acting in conjunction with tapasin to regulate the peptide loading of B2m-associated MHC class I molecules as part of the TAP complex.

We also detected a higher molecular weight ERp57 disulfide-bonded intermediate by immunoprecipitation with HC10. This higher molecular weight complex may represent a transient complex of class I heavy chains with more than one ERp57 molecule or, alternatively, with other, as yet undefined, components of the early MHC class I assembly pathway. Experiments are ongoing to determine the components of this complex. Our data also indicate that B27 assembles in a tapasin-independent manner in C58 cells (Figure 1E). It may be of interest in the future to compare the nature of HC10 immunopurified complexes of tapasin-dependent and -independent MHC alleles.

During folding and assembly in the ER, non-B2m-associated MHC class I molecules can be detected in at least two different disulfide-bonded isoforms by the mAb HC10 (Figure 1B). We have shown here that one of these isoforms, containing disulfide bonds, can be reduced by recombinant ERp57. The migration of the HC10-reactive ERp57 disulfide-containing product is similar to that of folded MHC class I molecules (Figure 1B), suggesting that it may have both α 2 (Cys101–Cys164) and α 3 (Cys203–Cys259) domain bonds formed. It has been suggested that these bonds are temporally distinct, with the conformation of the α 1/ α 2 domain being dependent on peptide and B2m, while the α 3 domain relies upon the appropriate redox potential (Ribaud and Margulies, 1992; Tector *et al.*, 1997). We are therefore currently investigating whether both disulfide bonds are equally susceptible to ERp57 activity.

In sharp contrast to our results obtained with the partially folded pool of MHC class I molecules recognized by HC10, we demonstrated that fully folded MHC class I molecules are resistant to ERp57-mediated reduction. A small population of folded molecules were reduced (Figure 4A), but following loading with a synthetic peptide epitope no reduction was detectable (Figure 4C). The small population that was reduced by ERp57 may arise from the short metabolic labelling times used in these experiments, which may reveal MHC class I molecules that are essentially fully folded but lacking peptides of suitable affinity to survive the assay conditions at 37°C. Our results therefore suggest that even MHC molecules in the latter stages of assembly, once unfolding has been initiated, may still become susceptible to ERp57-mediated reduction. Similar observations obtained with mutant A2 T134K molecules (Figure 5C) strongly suggest that inappropriate peptide loading can also expose MHC class I molecules to ERp57 reductase activity. Furthermore, the poor association of T134K molecules with the peptide-loading complex suggest that the critical disulfide bond formation of MHC class I molecules occurs prior to

the peptide loading and editing functions mediated by tapasin and the TAP complex.

The use of mAbs detecting conformation-dependent epitopes in these experiments raised concerns that steric hinderance in the immunoprecipitates prevented access by ERp57. However, we have also obtained similar results in experiments using folded class I molecules isolated with antibodies recognizing the cytoplasmic tail, thus limiting steric hinderance effects (data not shown). Furthermore, we have also determined that antibodies appear to be very poor substrates for ERp57-mediated reduction under the assay conditions used here (data not shown).

Using site-directed mutagenesis we identified that mutant ERp57 polypeptides containing (CXXS)₂ motifs retained diminished reductase activity, whereas (SXXC)₂ mutants were inactive. The partial reductase activity associated with the (CXXS)₂ mutant again could reflect a population of class I molecules that are undergoing isomerization rather than full reduction. Similarly, (CXXS)₂ PDI yeast mutants exhibited no *in vitro* reductase or oxidase activity, but retained isomerase activity (Laboissiere *et al.*, 1995; Walker *et al.*, 1996) and could rescue inviable cells with a chromosomal deletion of PDI (LaMantia and Lennarz, 1993). No structural data are available as yet for ERp57; however, by comparison with the data available for PDI (Kemink *et al.*, 1997), it can be predicted that the C-terminal cysteine residue of each CXXC motif is inaccessible compared with the N-terminal cysteine, thus allowing the latter to undergo restricted thiol-dependent activities.

Our experimental assay system described here utilizes low concentrations of dithiothreitol (DTT). Thus, cycles of reduction appear to require the presence of a reducing agent or a redox environment that maintains ERp57 in a reduced state. This raises the intriguing question of how the predominantly oxidizing environment of the ER (Hwang *et al.*, 1992) can integrate oxidase, isomerase and reductase activities. In the case of PDI, cycles of oxidative folding activity are regulated by Ero1p (Frand and Kaiser, 1998, 1999). The ERp57 redox state may be maintained in a similar manner to PDI, although no evidence has yet been obtained to suggest that ERp57 utilizes Ero1 polypeptides, and no other candidate regulatory polypeptide(s) has yet been identified. Glutathione in the ER has been shown to serve as a net reductant (Cuozzo and Kaiser, 1999), which may provide the appropriate redox conditions for ERp57 to perform isomerase/reductase activities. Since calnexin and calreticulin have been demonstrated to enhance ERp57-mediated protein folding of RNase B (Zapun *et al.*, 1998), it would be of interest to determine how these chaperones affect the reductase activity that we have described. The apparently relatively stable interaction of tapasin with ERp57 in the TAP complex also raises the intriguing possibility of tapasin regulating ERp57 function (Dick *et al.*, 2002).

Most studies of ERp57 in the MHC class I assembly pathway have focused on the role ERp57 may play in the production of a fully folded, peptide-loaded molecule for export to the cell surface. However, ER quality control mechanisms also target for degradation molecules that do not assemble correctly and fail to load with peptides. The retrotranslocation of MHC class I heavy chains into the cytoplasm is an integral part of this process (Hughes *et al.*,

1997). NEM is capable of inhibiting this process (Tortorella *et al.*, 1998), and whilst the non-specific nature of NEM action is likely to affect several components of the retrotranslocation process, it does indicate that inhibition of ERp57 activity may represent at least part of this process. We have shown in this study that ERp57 can act to reduce partially folded MHC molecules, which potentially would be subjected to quality control in the ER. ERp57 should therefore be detected where the process of MHC class I breakdown is initiated in the ER. Interestingly, in the presence of proteasome inhibitors, immunofluorescent studies have indicated a clustering of MHC class I heavy chains into a subcellular compartment closely associated with the ER. This compartment also contains calnexin and calreticulin, but appears not to contain PDI (Kamhi-Nesher *et al.*, 2001). The presence of calnexin, calreticulin and MHC class I heavy chains in this degradation-associated compartment would strongly suggest that ERp57 may also be present, placing it in the correct environment to perform the reductase activity we have described here.

Materials and methods

Cell lines and antibodies

The rat T-cell line C58 (Silva *et al.*, 1983), human Daudi, human T3 cells (Momburg *et al.*, 1992; T2 expressing rat TAP1 and TAP2) and C1R-T134K cells (Lewis *et al.*, 1996) were maintained in RPMI 1640 (Gibco-BRL) supplemented with 5% fetal calf serum and 1 mg/ml G418 (PAA Laboratories) as required. C58.B27 was generated by electroporation (160 V, 975 µF) of C58 cells with a cDNA encoding HLA-B*2705 (kindly provided by Dr J.Taurog) cloned into vector pCR3 (Invitrogen). The C58.B27sv5 line expressing B27 epitope tagged at the C-terminus with the SV5 tag sequence SRGKPIPPLLGLDST (Hanke *et al.*, 1992) was generated using PCR primers incorporating the SV5 tag, cloned into pCR3 and electroporated as above.

mAb HC10 (Stam *et al.*, 1986), recognizing unassembled HLA-B and -C molecules, was kindly provided by Drs J.Neeffes and P.Lehner; ME1 (Ellis *et al.*, 1982), recognizing folded HLA-B molecules, was kindly provided by Dr J.Taurog; BB7.2 (Parham and Brodsky, 1981) recognizes folded HLA-A2 molecules; and W6/32 (Parham *et al.*, 1979) recognizes folded HLA-A, -B and -C molecules. Anti-human B2m was obtained from Serotec and rabbit anti-ERp57 was a gift from Dr N.Bulleid. Anti-human tapasin was a gift from Drs P.Lehner and P.Cresswell. Anti-murine tapasin (Harris *et al.*, 2001) was a gift from Dr T.Hansen. mAb anti-RT1.A^u antibodies NR5/10 and GN7/5.B11 were gifts from Dr G.Butcher. mAb Pk, recognizing the SV5 epitope tag, was a gift from Dr R.Randall. Anti-rat TAP antiserum was as described previously (Powis, 1997).

Metabolic labelling and immunoprecipitations

Methionine-starved cells were radiolabelled for between 5 and 10 min with 3.7MBq Trans label (ICN), and resuspended in lysis buffer [150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NEM, 10 mM Tris pH 7.5] containing either 1% NP-40 or 1% digitonin (Sigma) as required. Immunoprecipitations were performed with the indicated antibodies and protein A/G-Sepharose (Sigma) as required (Powis, 1997). HC10 immunisolates were washed with lysis buffer excluding NEM, and then used in ERp57 *in vitro* assays.

For pulse-chase analysis cells were labelled as above, returned to normal medium and samples lysed at the indicated timepoints. After immunoprecipitation the samples were incubated with 5 mU endo H (Roche) for 1 h at 37°C. For peptide stabilization assays, cells were labelled and lysed as above and incubated in the presence or absence of 50 µM A2 binding peptide LLDVPTAAV (Hill *et al.*, 1995) for 1 h at 37°C. Control lysates were incubated on ice. Heat-treated lysates were then returned to ice and immunoprecipitated as above.

For the isolation of B27-ERp57 intermediates, C58 and C58.B27 cells were incubated in 10 mM NEM in phosphate-buffered saline on ice for 10 min prior to lysis in NP-40-containing buffer and immunoprecipitation with mAb HC10. SDS-PAGE under non-reducing and reducing

conditions was performed on Bio-Rad mini-gel apparatus. Two-dimensional electrophoresis of immunoprecipitates was performed as described previously (King *et al.*, 2000). Autoradiography was performed using Kodak Biomax MS1 film (Merck).

Cell fractionation

Cells were treated with or without 100 μ M LLnL (Affiniti Research Products) and 1 mM NEM for 2 h at 37°C, pelleted and freeze-fractured three times on dry ice (Hughes *et al.*, 1997). Samples were resuspended in buffer containing 10 mM Tris pH 7 and 50 μ M LLnL and 1 \times mini-protease inhibitors (Roche) and spun at 2500 g for 3 min to remove nuclei. Supernatants were then spun at 50 000 g for 30 min and the subsequent membrane and supernatant fractions analysed by SDS-PAGE and western blotting. Western blotting was performed on BA85 nitrocellulose (Schleicher and Schuell) using Supersignal Femto chemiluminescent reagents (Pierce and Warriner).

Recombinant ERp57

Rat ERp57 cDNA lacking residues 1–20 of the signal sequence, was subcloned into pET15b vector with an N-terminal His₆ tag (Novagen). ERp57 pET15b was transformed into BL21-competent bacteria. For protein purification, bacterial cultures were grown up and induced overnight at 25°C with 0.4 mM isopropyl β -D-thiogalactopyranoside (IPTG). Bacteria were lysed in BPER reagent (Pierce) according to manufacturer's instructions and the lysate bound to Ni-NTA agarose (Qiagen) for 2 h at 4°C. The histidine tag was removed by sequential digestion of Ni-agarose beads with 2 U bovine/0.5 U human thrombin (Sigma) at room temperature for 1 h. Thrombin was removed by incubation with *p*-aminobenzamide agarose beads (Sigma). Recombinant ERp57 was then dialysed overnight against 50 mM Tris-HCl pH 8 and further purified by high-performance liquid chromatography using anion exchange chromatography. ERp57-containing fractions were pooled and dialysed against 50 mM HEPES, pH 7.5, and stored at –80°C.

Insulin and MHC class I reduction assays

ERp57 reduction of insulin was performed essentially as described previously (Hirano *et al.*, 1995). Briefly, insulin (Sigma) was prepared freshly at 1 mg/ml in assay buffer (100 mM KAc pH 7.5, 2 mM EDTA and 10 μ M DTT). Insulin (50 μ l) was mixed with ERp57 or PDI (Sigma) in a final volume of 10 μ l assay buffer and then 2 μ l of 10 mM DTT was added. The OD₆₅₀ of the assay was read every minute.

For the MHC class I reduction assays, immunoprecipitates were resuspended in 10 μ l assay buffer as above and then supplemented with a further 10 μ l of buffer containing pre-activated ERp57 or PDI, or DTT alone. Pre-activation was performed by incubating ERp57 in assay buffer for 5 min at 37°C in the presence of 100 μ M DTT. ERp57 reduction assays were performed at 37°C for 1 h. Samples were then analysed on non-reducing SDS-PAGE and autoradiography.

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