

ERp57 is essential for efficient folding of glycoproteins sharing common structural domains

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ERp57 is a member of the protein disulphide isomerase family of oxidoreductases, which are involved in native disulphide bond formation in the endoplasmic reticulum of mammalian cells. This enzyme has been shown to be associated with both calnexin and calreticulin and, therefore, has been proposed to be a glycoprotein-specific oxidoreductase. Here, we identify endogenous substrates for ERp57 by trapping mixed disulphide intermediates between enzyme and substrate. Our results demonstrate that the substrates for this enzyme are mostly heavily glycosylated, disulphide bonded proteins. In addition, we show that the substrate proteins share common structural domains, indicating that substrate specificity may involve specific structural features as well as the presence of an oligosaccharide side chain. We also show that the folding of two of the endogenous substrates for ERp57 is impaired in ERp57 knockout cells and that prevention of an interaction with calnexin or calreticulin perturbs the folding of some, but not all, substrates with multiple disulphide bonds. These results suggest a specific role for ERp57 in the isomerisation of non-native disulphide bonds in specific glycoprotein substrates.

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Introduction

The endoplasmic reticulum (ER) provides an environment that allows the oxidative folding and post-translational modification of proteins entering the secretory pathway (Hwang *et al.*, 1992). The compartmentalisation of the ER away from the cytosol ensures that the correct redox conditions are established to enable a distinct set of folding catalysts to facilitate the formation of native disulphide bonds in proteins. A family of ER oxidoreductases is thought to be responsible for catalysing the formation, isomerisation and reduction of these disulphide bonds (Ellgaard and Ruddock,

2005). In addition to their role in oxidative protein folding, ER oxidoreductases may also be responsible for the redox regulation of ER-localised enzymes and channel proteins (Li and Camacho, 2004; Higo *et al.*, 2005). The oxidoreductases contain active sites homologous to the active site found in the cytosolic reductase thioredoxin, characterised by a pair of cysteine residues (CXXC) that shuttle between the disulphide and dithiol form (Ferrari and Soling, 1999). The reactions that these enzymes catalyse require the individual active sites to be maintained in either the oxidised disulphide form, for disulphide bond formation, or the reduced dithiol form, for isomerisation or reduction of disulphide bonds (Freedman, 1995). Comparative studies of the *in vitro* activities of several of these enzymes demonstrate that they are capable of carrying out similar functions (Alanen *et al.*, 2006); so the question as to why there are so many family members needs to be addressed.

Protein disulphide isomerase (PDI) was the first ER oxidoreductase to be identified and characterised (Goldberger *et al.*, 1964). PDI is itself oxidised by Ero1 allowing it to act as an oxidase and introduce disulphide bonds into substrate proteins (Sevier and Kaiser, 2002). It is clear that PDI is capable of both the formation and isomerisation of disulphide bonds *in vitro* (Lyles and Gilbert, 1991), in yeast (Laboissiere *et al.*, 1995) and in mammalian systems (Bulleid and Freedman, 1988). Hence, it would appear that all the functions of an ER oxidoreductase during the formation of disulphide bonds can be catalysed by just one enzyme. One explanation for the presence of several enzymes capable of fulfilling similar functions is that they all have distinct substrate specificities. To date, we have very little information on the substrates of these enzymes *in vivo*.

However, we do have a general idea of the characteristics of substrates for the ER oxidoreductase ERp57. This enzyme has been shown to form a complex with calnexin or calreticulin, two ER resident proteins that interact specifically with monoglucosylated glycoproteins (Oliver *et al.*, 1997; Oliver *et al.*, 1999). ERp57 has been shown to catalyse the formation of native disulphide bonds in glycoproteins, both *in vitro* (Zapun *et al.*, 1998) and *in vivo* (Oliver *et al.*, 1997; Van der Wal *et al.*, 1998; Antoniou *et al.*, 2002), and to form a stable interaction with the MHC class I loading complex via an interaction with tapasin (Peaper *et al.*, 2005). Hence, ERp57 can be viewed as a glycoprotein-specific oxidoreductase fulfilling this function while in complex with calnexin or calreticulin. Recent evidence using ERp57 knockout cells has highlighted the fact that not all glycoproteins are substrates for ERp57 and that other characteristics of proteins determine whether their disulphide bonds are formed by ERp57 or other enzymes within the mammalian ER (Garbi *et al.*, 2006; Solda *et al.*, 2006). Even though ERp57 is suspected of catalysing the oxidative folding of glycoproteins clearly there may be specificity within this large cohort of secreted proteins.

The large number of proteins present in the mammalian ER oxidoreductase family suggests a complexity that makes

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understanding individual substrate specificities challenging. To begin to tackle such a huge question, we have taken an unbiased proteomics approach to determine the range of substrates that ERp57 interacts with when catalysing reduction or isomerisation reactions. We have taken advantage of the fact that an intermediate of disulphide exchange includes a mixed disulphide between the enzyme and substrate, which is prolonged by mutation of the C-terminal cysteine in the active site. Therefore, we created stable cell lines expressing ERp57 with a V5 tag, where the second cysteine of each of the two active sites was mutated to alanine. Using such an approach, proteins that are substrates for ERp57 were trapped as mixed disulphide intermediates. These were then immunisolated, separated by 2D gel electrophoresis and identified by mass spectrometry. Of the substrates identified, many contained 'small, disulphide-rich' domains, a number of which exhibit an unusually low level of secondary structure, suggesting that ERp57 has a distinct role in the formation of native disulphide bonds in a subset of glycoproteins. Hence, our data provide convincing evidence for a direct role for ERp57 in the isomerisation of non-native disulphide bonds in a distinct set of proteins entering the secretory pathway in mammalian cells and lead us to suggest that each ER oxidoreductase may have specific substrate specificity.

Results

Identification of ERp57 substrates

Our experimental strategy to determine the substrate specificity of ERp57 involved creating HT1080 human fibroblast stable cell lines that express ERp57-V5 (ERp57 with a V5 tag) or ERp57-V5 Cys2,7 (where both CXXC active sites had been mutated to CXXA). The V5-tag replaced the ER retention sequence but was followed by a KDEL sequence to ensure ER localisation. Immunofluorescence staining indicated that ERp57-V5 (Figure 1A) and ERp57-V5 Cys2,7 (results not shown) colocalised with endogenous PDI, demonstrating that they localise to the ER and as such are exposed to the same environment and substrates as endogenous ERp57. To determine whether ERp57 forms mixed disulphides with substrate proteins, both cell lines and wild-type HT1080 cells were treated with NEM to prevent post-lysis thiol exchange. Cell lysates were separated by SDS-PAGE under reducing and non-reducing conditions. Under non-reducing conditions, mixed disulphides containing ERp57-V5 were not visible (Figure 1B, lane 2), suggesting that such species may exist too transiently to detect using this technique. However, mutation of the active sites of ERp57 to CXXA allowed a number of high molecular weight complexes containing ERp57-V5 to be observed (Figure 1B, lane 3). These were disrupted by addition of DTT (Figure 1B, lane 6), demonstrating that they are indeed mixed disulphides. These results confirm those published previously (Dick and Cresswell, 2002) and demonstrate the validity of this approach to trap substrate proteins with ERp57.

To identify the individual substrates forming mixed disulphides with ERp57, we first needed to isolate the mixed disulphides and then separate the substrates from ERp57 and each other. To do this, we first treated cells expressing ERp57-V5 Cys2,7 with NEM to prevent post-lysis disulphide exchange, then subjected cell lysates to affinity isolation with agarose beads conjugated to an anti-V5 antibody. ERp57-V5

and mixed disulphides were eluted from the beads and separated by 2D SDS-PAGE. The first dimension was carried out under non-reducing conditions to separate ERp57 and mixed disulphide complexes. The second dimension was carried out under reducing conditions to resolve the mixed disulphides and separate the substrate proteins according to their individual size. Proteins were then visualised by silver staining (Figure 1C). All of the mixed disulphides isolated contained ERp57-V5, which produced a horizontal line adjacent to ERp57 that had been purified as a monomer. Proteins that had bound non-covalently to ERp57 or the agarose beads migrated in a diagonal line, as these species migrated at the same rate under reducing and non-reducing conditions. The substrate proteins that had formed part of a mixed disulphide resulted in spots or smears that migrated below the diagonal as they migrated further in the second dimension than the first. Control gels generated following lysis of untransfected HT1080 cells contained no proteins migrating faster in the second dimension than the first (results not shown). Resolved spots were excised from the gel and proteins identified by mass spectrometry (Figure 1C and Table I).

All the proteins identified using this approach are proteins that are present in the ER at some stage during their biosynthesis. Most proteins (21 out of 26) are either secreted or cell-surface glycoproteins containing several disulphide bonds, with the remaining five being localised to the ER lumen. One of the proteins that was seen to form a mixed disulphide with ERp57 was tapasin, an interaction that has been characterised previously (Peaper *et al*, 2005). Tapasin is a component of the MHC class I loading complex, where it has been shown to form a stable mixed disulphide with ERp57. Thus, the approach successfully identified a protein that is known to form a mixed disulphide with ERp57, along with several disulphide-bonded glycoproteins that are all potential substrates for this enzyme.

To determine whether the association of substrate proteins with ERp57 was dependent upon glucose trimming of the oligosaccharide side chain, we radiolabelled newly synthesised proteins in the presence and absence of castanospermine. Castanospermine inhibits glucosidases I and II and will maintain oligosaccharide side chains on newly synthesised proteins in a fully glucosylated form, therefore inhibiting their interaction with the calnexin/calreticulin cycle. We immunisolated mixed disulphides with the V5 antibody and then looked for the presence of radiolabelled proteins following SDS-PAGE carried out under reducing conditions. Several radiolabelled proteins were immunisolated with the V5 antibody (Figure 1D, lane 1). These had all formed mixed disulphides with ERp57, as they were absent from cells that had been treated with DTT before cell lysis (Figure 1D, lane 3). As can be seen, the presence of castanospermine blocked interactions with several of the substrates (Figure 1D, lane 2) demonstrating that ERp57 acts on some glycoprotein substrates that enter the calnexin/calreticulin cycle. There were also proteins that were immunisolated even in the presence of castanospermine indicating that ERp57 may also act on substrates even when they are prevented from entering the calnexin/calreticulin cycle.

The ER-localised proteins isolated as mixed disulphides include Ero1L α and ERp72, both of which are oxidoreductases. The ability of Ero1L α to oxidise ERp57 has recently

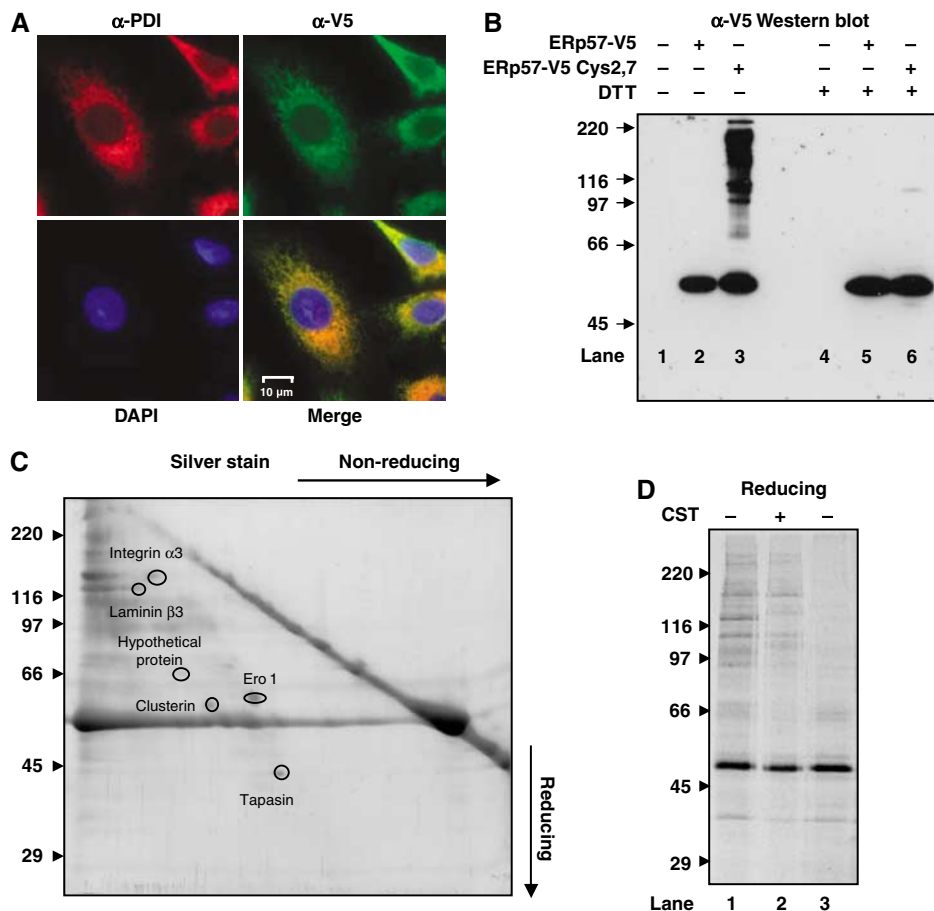


Figure 1 ERp57-V5 Cys2,7 forms mixed disulphides when expressed in HT1080 cells. **(A)** HT1080 human fibroblast cells stably transfected with ERp57-V5 were fixed with methanol. Cells were labelled with a rabbit anti-PDI antibody that was detected with an Alexa Fluor 594 anti-rabbit secondary antibody, a mouse anti-V5 antibody that was detected with an Alexa Fluor 448 secondary antibody and 1,4-diazobicyclo(2,2,2)octane (DAPI). **(B)** Untransfected HT1080 human fibroblasts (lanes 1 and 4), those expressing ERp57-V5 (lanes 2 and 5) or ERp57-V5 where the active sites had been mutated to CXXA (ERp57-V5 Cys2,7; lanes 3 and 6) were treated with 25 mM NEM and lysed. Clarified lysates were separated under non-reducing (lanes 1–3) and reducing conditions (lanes 4–6). Proteins were transferred to a nitrocellulose membrane and probed with an anti-V5 antibody. **(C)** HT1080 cells expressing ERp57-V5 Cys2,7 were treated with NEM and lysed. Clarified lysates were immunoprecipitated with an anti-V5 antibody conjugated to agarose beads. Proteins were eluted by boiling in SDS and separated under non-reducing conditions. Gel lanes were excised and reduced with 50 mM DTT and separated in a second dimension. Proteins were visualised by silver staining. Proteins migrating faster in the second dimension than the first dimension were excised from the gel and identified by mass spectrometry. **(D)** HT1080 cells expressing ERp57-V5 Cys2,7 were untreated (lanes 1 and 3) or treated with 1 mM castanospermine (CST) (lane 2). Cells were radiolabelled for 30 min and chased with cold media for 30 min, where castanospermine was present throughout in treated samples. Samples were then left untreated (lanes 1 and 2) or treated with DTT (15 mM) for 5 min (lane 3). Cells were lysed in the presence of NEM. ERp57 and mixed disulphides were immunoprecipitated with an anti-V5 antibody conjugated to agarose beads and separated by SDS-PAGE under reducing conditions. Radiolabelled proteins were visualised by autoradiograph.

been demonstrated in an *in vitro* assay (Kulp *et al*, 2006), but the fact that ERp57 remains in a reduced state even when Ero1 α is overexpressed has questioned whether ERp57 is a substrate for Ero1 α *in vivo* (Mezghrani *et al*, 2001). To confirm this interaction and to ascertain whether Ero1 α is capable of forming a mixed disulphide with both active sites of ERp57, we created three additional stable cell lines. ERp57-V5 was immunoprecipitated from cell lines expressing ERp57-V5 with either the first (Cys2), second (Cys7) or both active sites (Cys2,7) mutated to CXXA, or with no active site cysteines (Cys1,2,6,7). Immunoprecipitated ERp57 was separated under reducing and non-reducing conditions and Western blotted for Ero1 α (Figure 2A and B). Under reducing conditions an Ero1 α mixed disulphide with ERp57 was detected in cell lines expressing ERp57-V5 containing single point mutations in either or both active sites (Figure 2A, lanes 3–5). A mixed

disulphide was not detected when the active sites of ERp57 remained as CXXC or with the double point mutation of both active sites (Figure 2A, lanes 2 and 6). Ponceau S staining of a blot of the same samples separated under reducing conditions indicated that ERp57 was present in all the immunoprecipitates apart from the untransfected control (Figure 2C). When the same samples were separated under non-reducing conditions, Ero1 α migrated with a higher mass corresponding to that of a mixed disulphide between Ero1 α and ERp57 (Figure 2B, lanes 3–5). The mixed disulphide between Ero1 α and the ERp57 Cys7 is somewhat weaker than to the other mutants but is still present. Thus, it is clear that mutation of the active site(s) of ERp57 to CXXA prolongs an interaction with Ero1 α , and that Ero1 α can form a mixed disulphide with either active site of ERp57. This result confirms the previously published *in vitro* data

Table 1 Substrate proteins that interact with ERp57 *in vivo*

Species	DSB	Glyc	Localisation	Domains
Integrin α -2	6	10	Type I cell surface	<i>IntA</i> β -propeller, <i>IntA</i> -thigh, <i>Int A calf</i>
Integrin α -3	9	14	Type I cell surface	<i>IntA</i> β -propeller, <i>IntA</i> -thigh, <i>Int A calf</i>
Integrin α -6	9	9	Type I cell surface	<i>IntA</i> β -propeller, <i>IntA</i> -thigh, <i>Int A calf</i>
Integrin β -1	(42 Cys)	12	Type I cell surface	Int β psi, 4 \times Int β EGF, β TD
Integrin β -5	25	8	Type I cell surface	Int β psi, 4 \times Int β EGF, β TD
Collagen α (VI) chain	4	10	Secreted	Kunitz
Laminin β -1	47	11	Secreted	13 \times Lam-EGF
Laminin β -3	27	3	Secreted	6 \times Lam-EGF
Laminin γ -1	43	14	Secreted	11 \times Lam-EGF
Laminin γ -2	23	4	Secreted	8 \times Lam-EGF
Plexin A1	(53 Cys)	13	Transmembrane	3 \times Plexin
Aggrin	15	4	Secreted	4 \times EGF, 2 \times Lam-EGF, Kazal
Hypothetical fibrillin	(34 Cys)	6	Secreted	<i>IgG domains</i>
EGF-containing fibulin-like protein	15	1	Secreted	6 \times EGF
LDL receptor	30	5	Type I cell surface	3 \times LDL receptor domains
Discoidin	4	6	Type I cell surface	<i>CUB domains</i>
Clusterin	5	6	Secreted	No domains identified
Lysyl oxidase homolog 2	(42 Cys)	3	Secreted	3 \times <i>SRCR</i> (Scavenger protein cysteine rich)
ADAM 17 precursor	7	9	Type I membrane	Disintegrin
ADAM 10 precursor	3	4	Type I membrane	Disintegrin
Melanotransferrin precursor	6	3	GPI-anchored	<i>Transferrin domain</i>
Hypothetical protein	(6 Cys)	3	(ER)	
Lysyl hydroxylase	(13 Cys)	7	Membrane-bound ER	
Tapasin	2	1	ER	
ERp72	Ox-R	0	ER	
Ero1L α	Ox-R	2	ER	

Proteins that formed mixed disulphides with ERp57-V5 Cys2,7 in HT1080 human fibroblast cells were isolated by 2D gel electrophoresis and identified by mass spectrometry. 'DSB' indicates the number of disulphide bonds present in the native structure, or the number of cysteine residues is included in brackets where this information is unavailable. 'Glyc' indicates the number of potential N-linked glycans. Domains that are indicated small and cysteine-rich by SCOP are in normal text, whereas entries in italics refer to other identifiable domains within the protein. Structural domains were assigned to sequences using the FUGUE fold recognition software (Shi *et al*, 2001). Regions of the sequences that were unassigned were submitted iteratively. All abbreviations are as found in SCOP (Murzin *et al*, 1995) or Superfamily (Gough *et al*, 2001).

(Kulp *et al*, 2006) and suggests that Ero1L α is capable of oxidising ERp57 *in vivo*.

To determine whether there was any specificity in the two active sites of ERp57 towards different substrates, we also identified the range of proteins forming mixed disulphides in cell lines expressing ERp57-V5 with a mutation in either the first or second active site. The majority of substrates formed mixed disulphides with both ERp57-V5 Cys2 and ERp57-V5 Cys7. These included β 1 integrin, the laminins β 3, γ 1, γ 2, clusterin, lysyl oxidase homologue 2, the hypothetical protein, lysyl oxidase, discoidin and Ero1L α . Thus, both active sites were able to form mixed disulphides with these substrate proteins. However, some substrates only formed mixed disulphides with ERp57-V5 Cys2. Tapasin was among this group of substrates, which confirms previous results showing that tapasin forms mixed disulphides specifically with the *a* domain active site (Peaper *et al*, 2005). Although not all substrates formed mixed disulphides with both active sites, it is likely that the inability to detect a mixed disulphide could reflect different levels of detection rather than the absence of the product, making it difficult to interpret negative results. However, the data do show that either the active site within the *a* or the *a'* domain of ERp57 can form mixed disulphides with substrate proteins indicating functional equivalence between the active sites.

Substrate analysis

The substrate proteins found to interact with ERp57 were analysed for domain structure against the Structural Classification of Proteins (SCOP) database (Murzin *et al*,

1995). Ero1L α and ERp72 were excluded as they are oxidoreductases and could equally act on ERp57 as opposed to being selected by ERp57. Thirteen out of the remaining 24 proteins contain one or more 'small domains' that are annotated as 'disulphide-rich' domains, equating to 54% of the substrates identified. There are approximately 21 500 known genes in the human genome of which approximately 7500 (35%) enter the secretory pathway (Chen *et al*, 2005). Of the proteins entering the secretory pathway, 3078 can be assigned one or more SCOP domains, and of these domains only 307 or 9.97% are annotated as small and disulphide rich. Assuming that the proportion we can predict domains for is a representative sample of the secreted proteome and using a binomial distribution, this gives a '*P*' value of 3.8×10^{-8} , demonstrating that the population of substrates of ERp57 shows a significant enrichment of 'small, disulphide-rich' domains.

Further examination of the 'small, disulphide-rich' domains revealed that many contain cysteines that are close together in the primary amino-acid sequence, where each cysteine forms disulphide bonds to alternative parts of the molecule (Figure 3). Further analysis of the small disulphide-rich domains reveals that many of them have a surprisingly low level of secondary structure (Figure 3). Such domains might therefore be expected to demonstrate a large degree of flexibility during folding. Fewer restrictions during folding might indicate a higher potential for the formation of incorrect disulphides, as each cysteine would be unconstrained and able to form a non-native disulphide with any of a number of other cysteine residues in temporal proximity.

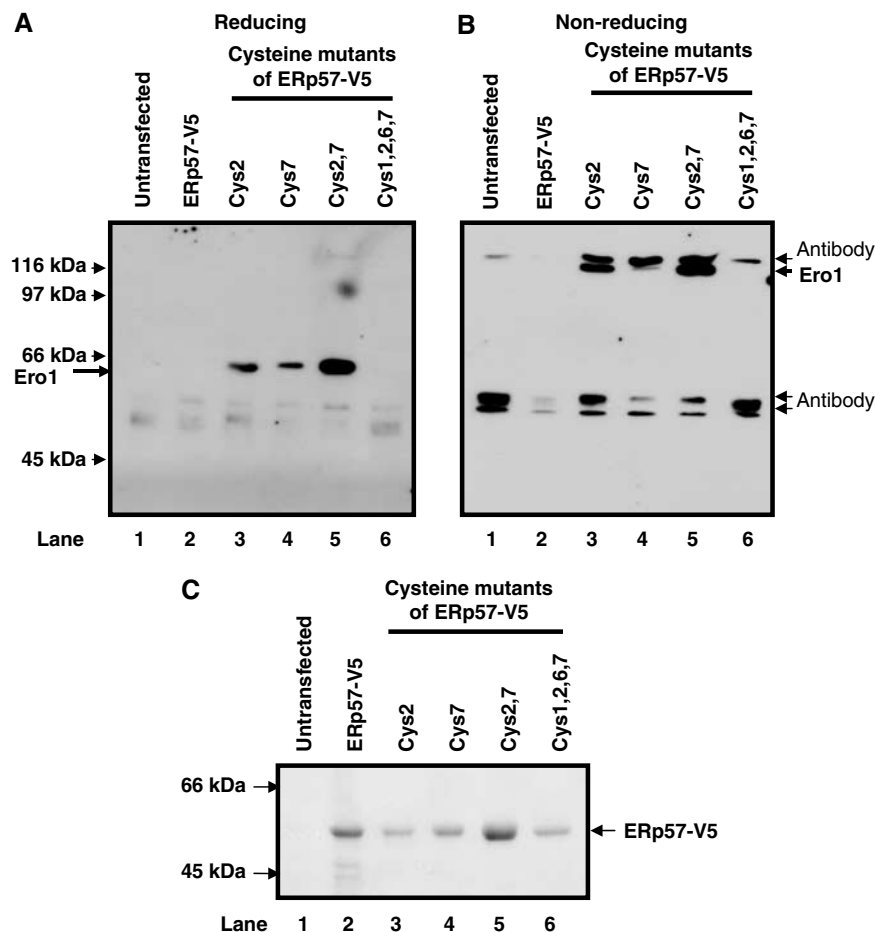


Figure 2 ERp57 forms mixed disulphides with Ero1 α *in vivo*. Untransfected HT1080 cells (lane 1) and stable cell lines expressing ERp57-V5 (lane 2), ERp57-V5 Cys2 (lane 3), ERp57-V5 Cys7 (lane 4), ERp57 Cys2,7 (lane 5) or ERp57-V5 Cys1,2,6,7 (containing no active site cysteines; lane 6) were analysed for mixed disulphides between ERp57 and Ero1 α . Cells were lysed in the presence of NEM to preserve disulphide status and V5-ERp57 was immunisolated with an anti-V5 antibody. The resulting samples were separated under reducing (A) and non-reducing conditions (B, C). Proteins were transferred to a nitrocellulose membrane and probed with an anti-Ero1 α antibody (A, B) or stained with Ponceau S (C).

This, combined with the number of proximal cysteine residues in these domains, suggests that a high level of non-native disulphide bonds could form during folding. Such domains would inevitably require isomerisation by an oxidoreductase such as ERp57.

Interestingly, one of the proteins identified to interact with ERp57 was the LDL receptor (LDL-R). The oxidative folding of LDL-R has previously been well documented (Jansens *et al*, 2002). The peptide chain is thought to quickly become oxidised and tightly packed upon translocation, forming many non-native disulphides. The native conformation, which is much slower to appear, is the result of extensive isomerisation. We note that the LDL-R also contains three 'small disulphide-rich' EGF domains, and that the peripheral EGF-like domains of LDL-R exhibit very little secondary structure, perhaps explaining their propensity to form non-native disulphides during folding.

Requirement of ERp57 for folding endogenous substrates

To investigate whether ERp57 not only interacts with substrate proteins but is also required for their oxidative folding, we examined its role in the folding of two proteins, clusterin

and the β 1 integrin subunit. Clusterin is a small secreted glycoprotein that forms five disulphide bonds, whereas β 1 integrin is a type I membrane protein that potentially forms 21 disulphide bonds and exhibits a number of 'small, disulphide-rich' domains, including four β integrin EGF-like domains.

To study folding and disulphide bond formation, proteins were synthesised in an *in vitro* translation system composed of a rabbit reticulocyte lysate optimised for disulphide bond formation, containing semi-permeabilised cells (SP-cells). Translation reactions were carried out for different times and disulphide status frozen by the addition of NEM to alkylate any free thiol groups. When clusterin was synthesised in the absence of cells, a major translation product was formed with a relative molecular weight of 50 kDa (Figure 4A, lane 1). In the presence of SP-cells derived from mouse fibroblasts (MF), clusterin formed a higher molecular weight species (Figure 4A, lane 2) that was sensitive to digestion with Endo H (Figure 4A, lane 3). A digest with proteinase K, which cannot cross the ER membrane, confirmed that the glycosylated form of clusterin was resident in the ER as it was protected from digestion (Figure 4A, lane 4). Another product of around 52 kDa was also apparent under reducing condi-

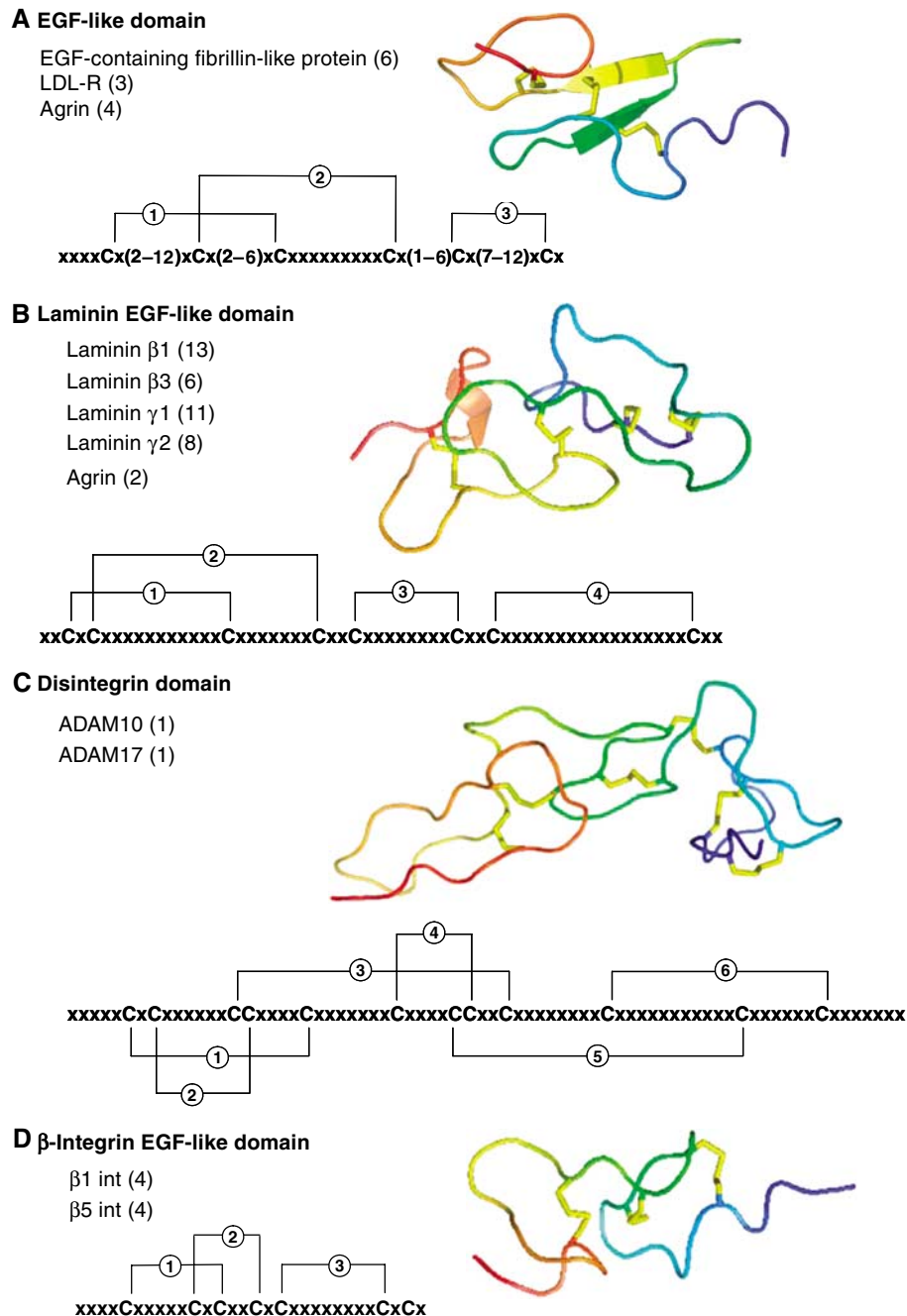


Figure 3 Domain structures of some ERp57 substrates. The domain structures (A–D) common to distinct substrates of ERp57 are illustrated. Proteins listed are substrates of ERp57 that contain domains characterised by little secondary structure and several disulphide bonds, where the number in brackets indicates the number of times the domain occurs in that protein. In the sequences shown, C denotes a cysteine residue, X is any other amino acid and lines represent intradomain disulphide bonds.

tions (Figure 4A, lanes 1–3). However, this species was not translocated into the ER as confirmed by its existence in the absence of cells and its disappearance upon treatment with proteinase K (Figure 4A, lanes 1 and 4). When the translation products were separated under non-reducing conditions, non-translocated clusterin was resolved into two bands, probably representing alternative redox states, as they migrated as a single band under reducing conditions (Figure 4B, lanes 1–3 compared to Figure 4A, lanes 1–3). The glycosylated form of clusterin migrated as a single band, which had a slightly faster mobility than when separated under reducing

conditions (Figure 4B, lane 2 compared with Figure 4A, lane 2). The increased mobility suggests that clusterin formed intrachain disulphide bonds and was fully disulphide bonded after 1 h of translation. A number of higher molecular weight species containing radiolabelled clusterin were also observed in the presence of cells (Figure 4B, lanes 2–4). These were not present under reducing conditions, indicating that they represented mixed disulphides containing clusterin (Figure 4A, lanes 2–4). Their size also decreased following treatment with Endo H, indicating that the mixed disulphides contained glycoproteins (Figure 4B, lane 3). Taken together, these

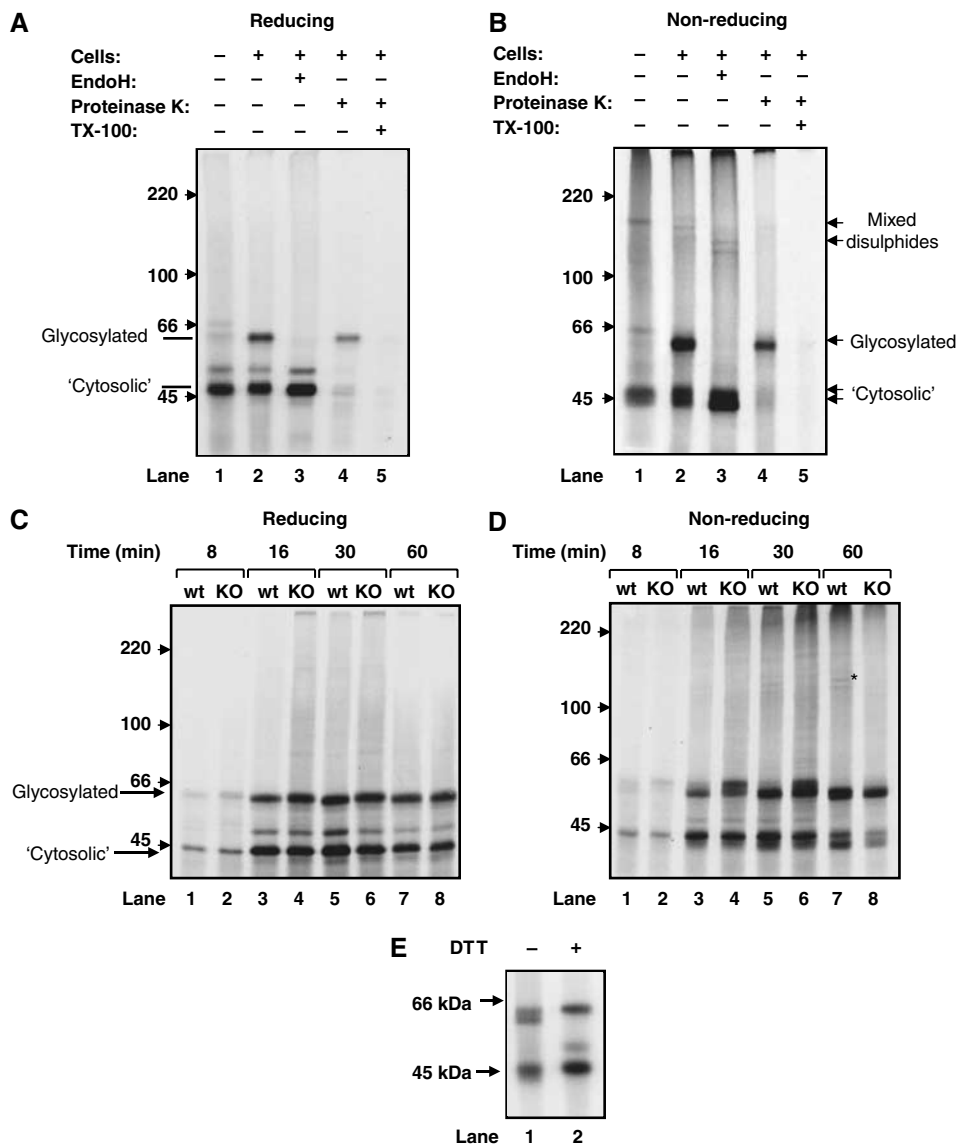


Figure 4 Clusterin requires ERp57 for efficient oxidative folding. (A, B) Clusterin was translated *in vitro* in the presence of reticulocyte lysate and radiolabelled amino acids and in the absence (lane 1) or presence of SP-MF cells (lanes 2–5). Translation was stopped after 1 h by the addition of 25 mM NEM. Following lysis, proteins were treated with Endo H to remove glycans (lane 3). Samples were subjected to digestion with proteinase K before (lane 4) or after solubilising with Triton X-100 (lane 5). Samples were separated by SDS-PAGE under reducing (A) or non-reducing conditions (B) before being exposed to film. (C, D) Clusterin was translated as in panels A and B in the presence of either wt SP-MEF cells (lanes 1, 3, 5 and 7) or *ERp57*^{-/-} SP-MF cells (lanes 2, 4, 6 and 8). Translation was allowed to continue for 5 min before the addition of ATCA to prevent initiation. Reactions were allowed to proceed for the indicated times and were terminated by adding NEM and placing on ice. Cells were isolated and samples separated under reducing (C) or non-reducing conditions (D). * indicates potential mixed disulphides. (E) Clusterin was translated for 16 min in the presence of *ERp57*^{-/-} SP-MF cells. Cells were isolated and samples separated under non-reducing (lane 1) or reducing (lane 2) conditions.

results demonstrate that when translated *in vitro* in the presence of SP-cells, clusterin is translocated, glycosylated and forms intrachain disulphide bonds.

To evaluate the contribution of ERp57 to folding and disulphide bond formation, clusterin was synthesised in the presence of wt SP-cells or *ERp57*^{-/-} MF cells (Garbi *et al*, 2006). Translation was allowed to proceed for 5 min before inhibition of initiation with aurintricarboxylic acid (ATCA), thereby allowing the folding of a small population of clusterin to be followed over time. Under reducing conditions, glycosylated clusterin appeared after 8 min and increased in intensity up to 16 min of translation in both wt or *ERp57*^{-/-} MF cells (Figure 4C). Under non-reducing conditions, clear

differences can be seen in the migration pattern of glycosylated clusterin synthesised in either wt or *ERp57*^{-/-} cells. Glycosylated clusterin quickly became oxidised in wild-type MF cells, becoming fully oxidised by 16 min (Figure 4D, lane 3). However, in *ERp57*-knockout cells, only half of the population of clusterin had become oxidised after 16 min (Figure 4D, lane 4), and was not fully oxidised until 1 h following translation initiation. This difference in mobility was shown to be solely a result of the redox state because glycosylated clusterin migrates as only one band when separated under reducing conditions (Figure 4C). In addition, all forms of clusterin synthesised in the presence of *ERp57*-knockout cells had a different mobility than the reduced

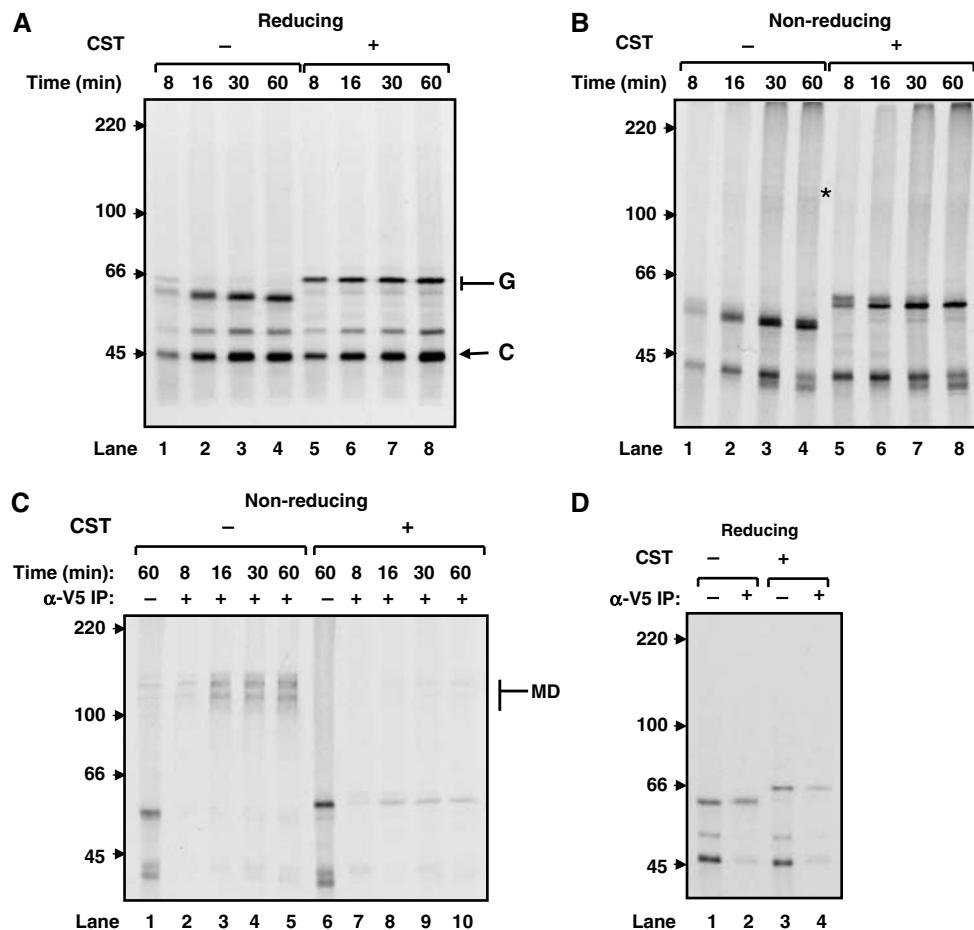


Figure 5 Oxidative folding of clusterin can occur in the absence of an interaction with calnexin or calreticulin. (**A, B**) Clusterin was translated *in vitro* in the presence of reticulocyte lysate, radiolabelled amino acids, SP-MF cells and in the absence (lanes 1–4) or presence of 1 mM castanospermine (lanes 5–8). Translation proceeded for 5 min before initiation was prevented by the addition of ATCA. Following incubation for times as specified, disulphide exchange was prevented by the addition of 25 mM NEM. Proteins were separated by SDS–PAGE under reducing (**A**) and non-reducing conditions (**B**) and visualised by exposure to film. G, glycosylated clusterin; C, cytosolic clusterin; * indicates potential mixed disulphides. (**C**) Clusterin was translated in HT1080 human fibroblast cells expressing ERp57-V5 in the absence or presence of 1 mM castanospermine, as in panels A and B. Following treatment with NEM, samples were analysed directly (lanes 1 and 6) or ERp57 was immunoprecipitated with an anti-V5 antibody (lanes 2–5, 7–10). Samples were separated by SDS–PAGE under non-reducing conditions. MD, mixed disulphides. (**D**) Clusterin was translated for 60 min in HT1080 human fibroblast cells expressing ERp57-V5 in the absence or presence of 1 mM castanospermine, as in panels A and B. Following treatment with NEM, samples were analysed directly (lanes 1 and 3) or ERp57 was immunoprecipitated with an anti-V5 antibody (lanes 2 and 4). Samples were separated by SDS–PAGE under reducing conditions.

form, demonstrating that oxidation still occurred in the absence of ERp57 (Figure 4E). Interestingly, the different redox forms of the untranslocated ‘cytosolic’ clusterin appeared at the same rate in the presence of either source of SP-cells, demonstrating that there was not an intrinsic difference in the redox conditions of the reticulocyte lysate (Figure 4D). Thus, ERp57 is required for the efficient oxidative folding of clusterin.

Role of calnexin/calreticulin in the oxidative folding of clusterin

ERp57 is thought not to bind to substrates directly like PDI but to interact with substrates as a complex with calnexin or calreticulin. To examine the contribution of calnexin/calreticulin binding to the oxidative folding of clusterin, castanospermine was used to inhibit glucose trimming. When castanospermine was included during translation, the glycosylated clusterin synthesised migrated more slowly under reducing conditions owing to the presence of untrimmed

glucose residues on the oligosaccharide side chain (Figure 5A, lanes 1–4 compared to lanes 5–8). However, treatment did not dramatically affect the rate of oxidation of clusterin, as the majority of clusterin still formed a faster migrating band of oxidised protein by 16 min (Figure 5B, lane 6 compared to lane 2). Therefore, either ERp57 can act on clusterin independently of an interaction of the substrate with calnexin or calreticulin, or alternatively releasing a substrate protein from calnexin or calreticulin allows it to interact with other oxidoreductases, which can act in place of ERp57.

To determine if ERp57 can interact with clusterin independently of calnexin or calreticulin, clusterin was translated *in vitro* in the presence of HT1080 cells expressing ERp57-V5, in the absence and presence of castanospermine. NEM was added to prevent disulphide exchange post-lysis and ERp57-V5 was immunoprecipitated. The eluted protein was separated by SDS–PAGE under non-reducing conditions. In the absence of castanospermine, mixed disulphides between ERp57 and clusterin were visible (Figure 5C, lanes 2–5), which appeared

as monomeric clusterin when separated under reducing conditions (Figure 5D, lane 2). However, treatment with castanospermine dramatically reduced the formation of mixed disulphides between clusterin and ERp57 (Figure 5C, lanes 6–10; Figure 5D, lane 4). However, some clusterin was immunoprecipitated with ERp57 as part of a non-covalent complex (Figure 5C, lanes 7–10). Hence, ERp57 may still facilitate disulphide bond formation or isomerisation in clusterin in the absence of an interaction with the calnexin/calreticulin cycle. In ERp57-knockout cells, clusterin may enter the calnexin/calreticulin cycle; however, in the absence of ERp57, the association would not facilitate oxidative folding. In fact, it may only act to sequester clusterin away from other folding factors. Thus, ERp57 is utilised for the efficient folding of clusterin *in vivo* via the calnexin/calreticulin cycle.

Interestingly, a mixed disulphide between endogenous ERp57 and clusterin is consistently seen throughout these experiments when clusterin is translated in wild-type MF cells (Figure 4B, lanes 2–4; Figure 4D, lane 7; Figure 5B, lane 4) but not following treatment with castanospermine (Figure 5B, lane 8) or when ERp57 knockout cells are used (Figure 4D, lane 8). Under such conditions, an alternative mixed disulphide involving clusterin is not visible, even though folding can progress at the same rate. It is possible that either a number of different oxidoreductases then participate in the folding of clusterin, making each mixed disulphide harder to detect, or that the preferred interaction with ERp57 is prolonged owing to the involvement of calnexin or calreticulin.

Role of ERp57 in the folding of the $\beta 1$ integrin subunit

Clusterin is a relatively small substrate with only five disulphide bonds, whereas a number of other substrates that we have shown to interact with ERp57 are much larger, have more disulphide bonds and exhibit 'small cysteine-rich' domains. One such substrate, the $\beta 1$ integrin subunit, was translated *in vitro* in the presence of wt SP-cells or ERp57^{-/-} MF cells. Glycosylated $\beta 1$ integrin that had been translocated into the lumen of the ER was immunoprecipitated and separated by SDS-PAGE under reducing and non-reducing conditions. Under reducing conditions, $\beta 1$ integrin migrated as a slightly diffuse band possibly owing to variations in glycosylation (Figure 6A). Under non-reducing conditions, $\beta 1$ integrin appeared as a faster migrating smear owing to variations in redox state (Figure 6B). When translated in the presence of wt MF cells, $\beta 1$ integrin initially migrated as a diffuse band, which sharpened after 120 min of folding (Figure 6B, lanes 1, 3, 5 and 7). The tighter band migrated faster than the unfolded protein, indicating that it had become more oxidised and had folded to form a compact intrachain disulphide-bonded protein. The mobility of the integrin synthesised in the presence of wt SP-cells comigrates with material synthesised in intact cells, indicating that the protein has attained its native complement of disulphide bonds (Figure 6E). In contrast, in the presence of ERp57^{-/-} cells, $\beta 1$ integrin did not reach a homogeneous redox state even after 120 min (Figure 6B, lane 8), demonstrating that ERp57 is necessary for the formation of the correct disulphide bonds. Interestingly, in the absence of ERp57, $\beta 1$ integrin migrated faster through the gel, indicating that incorrect disulphides had formed between regions distant in the protein leading to a lower hydrodynamic volume.

To determine whether disrupting the interaction with calnexin or calreticulin could allow correct folding of $\beta 1$ integrin, we monitored folding in wt SP-cells in the presence and absence of castanospermine. To allow a clearer comparison of mobility, samples were treated with Endo H to remove glycans. Under reducing conditions, $\beta 1$ integrin migrated as a single band when translated in the presence or absence of castanospermine (Figure 6C). Under non-reducing conditions in the absence of castanospermine, $\beta 1$ integrin again migrated as a smear following translation, but reached a tightly packed band representing a homogeneous redox state by 60 min (Figure 6D, lanes 1, 3, 5 and 7). However, in the presence of castanospermine, $\beta 1$ integrin still migrated as a smear after 120 min, suggesting that its oxidative folding is impaired in the presence of castanospermine (Figure 6D, lane 8). This demonstrates that $\beta 1$ integrin requires ERp57 to fold efficiently and cannot do so if it is released from the calnexin/calreticulin cycle and allowed to interact with other oxidoreductases. Thus, $\beta 1$ integrin is an obligate substrate of ERp57 in association with calnexin or calreticulin and cannot fold efficiently in its absence or when ERp57 is present but the interaction with calnexin or calreticulin is prevented.

Discussion

The formation of disulphide bonds within the lumen of the ER is known to be catalysed by a family of related oxidoreductases (Ellgaard and Ruddock, 2005), which may catalyse specific reactions or have different substrate specificities. We have now shown that one of these oxidoreductases, ERp57, has clearly defined substrates that share a number of key characteristics; they are all glycoproteins, are heavily disulphide-bonded and are likely to form non-native disulphide bonds. In particular, we have shown that the majority of these identified substrates contain domains, which have an abundance of cysteine residues, are lacking in defined secondary structure, and therefore would be expected to require an isomerase to allow the formation of the correct complement of disulphide bonds. Our results extend previous work carried out with viral proteins, which demonstrated that ERp57 and PDI can form mixed disulphides and exhibit a degree of specificity towards different viral proteins (Molinari and Helenius, 1999), and that some viral proteins are obligate whereas others are facultative substrates for ERp57 (Solda *et al*, 2006). The identification of a number of endogenously expressed proteins that rely upon ERp57 for their correct folding and assembly clearly demonstrates that the ER oxidoreductases have similar activities but defined protein substrates.

Although the approach taken to identify these proteins was successful in highlighting several substrates, this list is likely to be an under-representation of the total number of substrates for this enzyme. This is due to the fact that only proteins forming stable mixed disulphides that are easily resolved by 2D SDS-PAGE and are in sufficient abundance will be detected. The experimental approach is also limited in that we would only expect to identify substrates for ERp57 during its activity as a reductase or isomerase. Alternative substrate trapping experiments that do not mutate the active site residues, but rather increase the stability of the mixed disulphide intermediate by mutation of other amino acids in the sequence, could be considered to identify substrates for

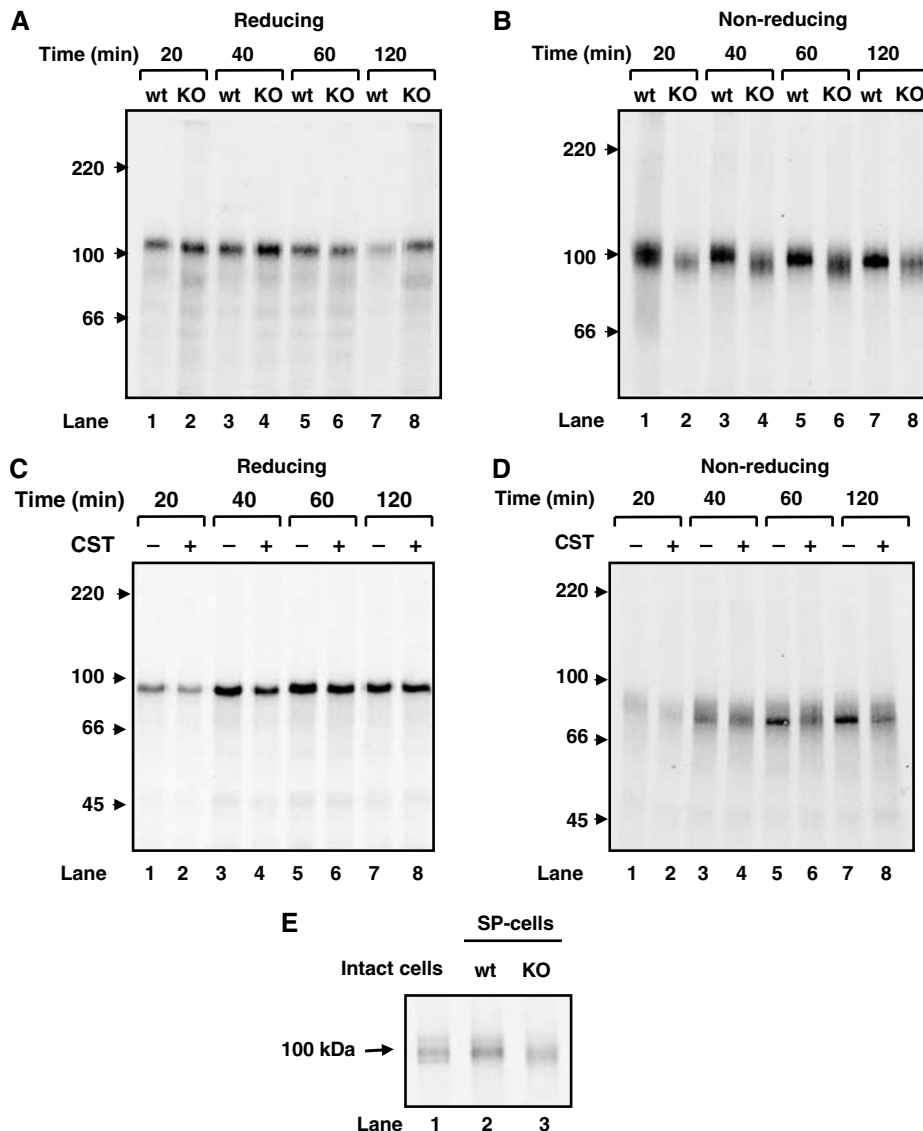


Figure 6 ERp57 is essential for the efficient oxidative folding of $\beta 1$ integrin. (A, B) $\beta 1$ integrin was translated in the presence of reticulocyte lysate, radiolabelled amino acids and either wt SP-MF cells (lanes 1, 3, 5 and 7) or *Erp57*^{-/-} SP-MF cells (lanes 2, 4, 6 and 8). Translation proceeded for 5 min before initiation was prevented by the addition of ATCA. Following incubation for times as specified, disulphide exchange was stopped by the addition of 25 mM NEM. Proteins were separated by SDS-PAGE under reducing (A) and non-reducing conditions (B) and visualised by exposure to film. (C, D) $\beta 1$ integrin was translated as in panels A and B in the presence of SP-MF cells in the absence (lanes 1, 3, 5 and 7) or presence of 1 mM castanospermine (lanes 2, 4, 6 and 8). Samples were separated under reducing (C) or non-reducing conditions (D). (E) Either intact HT1080 cells were radiolabelled for 30 min and chased with cold media for 30 min to allow correct folding and disulphide bond formation (lane 1) or translation of $\beta 1$ integrin was carried out as in panels A and B for 120 min in the presence of either wt MF (lane 2) or *Erp57*^{-/-} (lane 3) SP-cells. Cells were lysed in the presence of NEM. $\beta 1$ integrin was immunoprecipitated from intact cells with an anti- $\beta 1$ integrin antibody (8E3) and separated by SDS-PAGE under non-reducing conditions. Radiolabelled proteins were visualised by autoradiograph.

ERp57 should it act as an oxidase. Such an approach has been used successfully in the past to identify substrates for DsbA (Kadokura *et al*, 2004).

The identification of a mixed disulphide between Ero1L α and ERp57 would suggest that ERp57 can act as an oxidase *in vivo*. Such an activity has been questioned previously owing to the fact that ERp57 is mainly in a reduced form *in vivo* (Mezghrani *et al*, 2001; Jessop and Bulleid, 2004); however it is clear from *in vitro* experiments (Kulp *et al*, 2006) and by its ability to complement DsbA knockouts (Frickel *et al*, 2004) that this enzyme can act as an oxidase. Hence the redox state of the ER oxidoreductase at steady state could simply reflect the relative stability of the active site disulphide. The fact that

ERp57 is predominantly reduced is consistent with the notion that the rate-limiting step in the oxidation reaction is the oxidation of the ER oxidoreductase. An alternative explanation for the formation of a mixed disulphide between Ero1L α and ERp57 would be that ERp57 assists in the oxidative folding of the glycosylated Ero1L α . Interestingly, the formation of a mixed disulphide between two ER oxidoreductases (ERp57 and ERp72) suggests the potential for shuttling electrons between different family members, thereby providing a relay of electrons between substrates and the ultimate electron acceptor or donor.

The necessity for ERp57 specifically to catalyse the efficient formation of native disulphide bonds under normal

physiological conditions was demonstrated for two of the substrates identified. The results clearly demonstrate that for substrates that potentially require extensive rearrangement of disulphides, there is an obligate requirement for this enzyme. Even for substrates with relatively few disulphide bonds, there is a requirement for Erp57 when the substrate binds to either calnexin or calreticulin. Hence, although other oxidoreductases are able to catalyse oxidative folding, the fact that the substrates are bound to calnexin or calreticulin mean that access to the substrate is limited. So the specificity of the substrates for Erp57 may be determined by a combination of the oligosaccharide interacting with calnexin or calreticulin and the requirement for extensive isomerisation. These results suggest that Erp57 may have evolved to interact with calnexin or calreticulin as a consequence of the sequestration of glycoproteins into the calnexin/calreticulin cycle. In yeast, a calnexin homologue exists, but its role in glycoprotein folding is uncertain (Simons *et al*, 1998) and there is no indication of an association with an ER oxidoreductase. Just as the presence of Erp57 is necessary for efficient disulphide bond formation in glycoproteins associated with calnexin or calreticulin, the interaction of glycoproteins with calnexin or calreticulin may be required for Erp57 function. Evidence to support this idea comes from the inability of Erp57 to catalyse the efficient oxidative folding of β 1 integrin when an interaction between substrate and calnexin or calreticulin is blocked. However, the fact that some substrates still interact with Erp57 even when their association with the calnexin/calreticulin cycle is blocked would suggest that Erp57 can still act on specific substrates that do not enter the calnexin/calreticulin cycle.

In this paper, we have focused on one mammalian ER oxidoreductase, Erp57; however, there are many other ER oxidoreductases for which the function and substrate specificity remain to be elucidated. Within this family, Erp57, Erp72, PDI and PDIP contain similar characteristics in that they each contain similar active site sequences (CGHC) and are composed of both catalytically active and inactive thioredoxin domains (Ellgaard and Ruddock, 2005). The substrate specificity of PDI is thought to be determined primarily by an inactive thioredoxin domain (the b' domain) (Pirneskoski *et al*, 2001) whereas the binding of Erp57 to calnexin or calreticulin is also mediated through its b' domain (Russell *et al*, 2004). Hence, the basis for substrate binding and specificity at least for PDI and Erp57 is likely to be determined by the ability to bind to calnexin or calreticulin. Whether Erp72, PDI and PDIP have distinct substrate specificities remains to be addressed. In addition, whether the other ER oxidases mediate substrate binding through their active thioredoxin domains requires clarification, but identification of the substrates for each oxidoreductase will begin to address the question of their precise function and specificity.

Materials and methods

Antibodies

A mouse monoclonal antibody (8E3) specific for β 1 integrin subunit was a kind gift from Martin Humphries (University of Manchester, UK). A monoclonal anti-Ero1L α antibody was generously donated by Roberto Sitia (DIBIT, Milan). The monoclonal anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA, USA) and the V5-agarose beads from Sigma (St Louis, MO, USA).

Construction of stable cell lines

A PCR product containing the entire coding sequence for Erp57 was amplified from cDNA that was a gift from Stephen High (University of Manchester, UK). The forward primer added an *EcoRI* site at the 5' end of the coding sequence. Two reverse primers were used in sequential rounds of amplification to add a V5 tag followed by a KDEL ER retrieval sequence, stop codon and a *NotI* restriction site in place of the original ER retrieval sequence and stop codon. The final PCR product was ligated into *EcoRI*-*NotI*-cut pcDNA3.1(+ (Invitrogen). Mutagenesis of the C-terminal cysteines in both active sites of Erp57 was performed by PCR.

Plasmids were linearised with *SspI* before transfecting into subconfluent HT1080 human fibroblasts with Fugene8 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Stable cell lines were selected with G418 (Sigma, St Louis, MO, USA) for 14 days before colonies were isolated and screened for expression of Erp57-V5.

Immunofluorescence

Immunofluorescence was performed as described previously (Wilson *et al*, 1995). Cells were permeabilised and fixed in methanol at -20°C for 10 min. A rabbit anti-PDI antibody (John *et al*, 1993) was detected by an Alexa Fluor 594 anti-rabbit antibody, whereas a mouse anti-V5 antibody was detected by an Alexa Fluor 448 anti-mouse antibody. DNA was stained with DAPI. Cells were visualised on an Olympus BX60 upright microscope at $\times 40$ magnification.

2D gel electrophoresis

HT1080 cells expressing Erp57-V5 Cys2,7 were treated with NEM (25 mM) to preserve mixed disulphides. Cells (1×10^8) were lysed in 1% (v/v) Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) (lysis buffer). Clarified lysates were preincubated with protein-A Sepharose for 30 min to limit nonspecific binding, before incubation with anti-V5 antibody conjugated to agarose beads (Sigma, St Louis, MO, USA) for 16 h at 4°C . Beads were washed four times with 100 times bed volume 1% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 50 mM Tris-HCl pH 8.0, 500 mM NaCl and 0.5 mM PMSF (RIPA buffer). Proteins were eluted in non-reducing SDS-PAGE sample buffer (0.25 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue) and separated by SDS-PAGE on a 7.5% acrylamide gel. Gel lanes were excised and incubated in buffer containing 50 mM DTT for 10 min before separation through a second SDS-PAGE gel (7.5%). Proteins were visualised by silver staining. Spots were excised from the gel, digested with trypsin and peptides identified by tandem mass spectrometry (LC/MS/MS) on a quadrupole-time-of-flight (QTOF) instrument. Peptides were matched against predicted peptides in the current version of Mass Spectrometry Database (MSDB) using Mascot software.

Transcription and translation in vitro

DNA was transcribed and proteins translated essentially as described previously (Wilson *et al*, 1995). Human clusterin was transcribed from a pCMVSPORT6 vector, which was linearised with *XhoI*. Human β 1 integrin was transcribed from a pSPUTK vector, linearised with *EcoRV*. SP6 polymerase was used for both transcription reactions. RNA transcripts were translated in a rabbit reticulocyte lysate (Flexilysate, Promega, USA) and where specified in the presence of SP-cells. Initiation of protein synthesis was allowed to proceed for 5 min at 30°C before inhibition with 1 mM ATCA (Sigma, St Louis, MO, USA), followed by incubation at 30°C to allow elongation and post-translational modification. At specified times, 25 mM NEM was added at 4°C to prevent disulphide exchange. SP-cells were isolated and resuspended in SDS-PAGE sample buffer. For β 1 integrin, translation products were immunoprecipitated with antibody specific to β 1 integrin before electrophoresis. Samples were separated by SDS-PAGE under reducing and non-reducing conditions on a 7.5% acrylamide gel, which was dried and exposed to Fuji BioMax MR film (GRI, Essex, UK).

Electrophoresis

Samples for SDS-PAGE were resuspended in SDS-PAGE sample buffer (0.25 mM Tris-HCl pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.004% w/v bromophenol blue). DTT (50 mM) was added to reduce samples where indicated. All samples were boiled for 5 min

before electrophoresis. Proteins resolved through 7.5% acrylamide gels were either transferred onto nitrocellulose for Western blotting or fixed in 10% (v/v) acetic acid and 10% (v/v) methanol and dried. Radiolabelled products were visualized by autoradiography using Kodak Biomax MR film (GRI, Essex, UK).

Radiolabelling

Approximately 10^6 HT1080 cells were deprived of essential amino acids for 45 min in the presence or absence of 1 mM castanospermine (Sigma, St Louis, MO, USA). Radiolabelled methionine/cysteine protein labelling mix (50 μ Ci; NEN, Boston, MA, USA) was added for 30 min. Cells were washed and incubated in medium containing 0.5 mM cycloheximide to prevent further protein synthesis and castanospermine where appropriate. After 30 min, disulphide exchange was inhibited by the addition of 25 mM NEM in PBS. Cells were lysed in lysis buffer (see above) containing 25 mM NEM. Clarified lysates were incubated for 1 h at 4°C with

Sepharose beads to remove proteins that may bind nonspecifically, before being incubated with anti-V5 antibody conjugated to agarose beads overnight at 4°C. Beads were washed three times with lysis buffer and three times with RIPA buffer (see above) before being resuspended in SDS-PAGE sample buffer containing 50 mM DTT and boiling for 2 min. Samples were separated by SDS-PAGE and exposed to autoradiograph.

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