ERp27, a New Non-catalytic Endoplasmic Reticulum-located Human Protein Disulfide Isomerase Family Member, Interacts with ERp57*

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Protein folding and quality control in the endoplasmic reticulum are critical processes for which our current understanding is far from complete. Here we describe the functional characterization of a new human 27.7-kDa protein (ERp27). We show that ERp27 is a two-domain protein located in the endoplasmic reticulum that is homologous to the non-catalytic b and b’ domains of protein disulfide isomerase. ERp27 was shown to bind Δ-somatostatin, the standard test peptide for protein disulfide isomerase-substrate binding, and this ability was localized to the second domain of ERp27. An alignment of human ERp27 and human protein disulfide isomerase allowed for the putative identification of the peptide binding site of ERp27 indicating conservation of the location of the primary substrate binding site within the protein disulfide isomerase family. NMR studies revealed a significant conformational change in the b’-like domain of ERp27 upon substrate binding, which was not just localized to the substrate binding site. In addition, we report that ERp27 is bound by ERp57 both in vitro and in vivo by a similar mechanism by which ERp57 binds calreticulin.

The formation of native disulfide bonds in the endoplasmic reticulum (ER) is a complex, but essential, process in the biogenesis of many proteins, which pass beyond the carefully regulated environment of the cell. The folding process can occur via multiple parallel pathways within a protein (see for example Ref. 1), and this process can be catalyzed in vivo by different biological molecules. For example, there appears to be at least three parallel pathways for oxidation (disulfide bond formation) in substrate proteins, direct oxidation by Ero1 (2–4) or flavin-dependent sulfhydryl oxidases (5, 6), oxidation catalyzed by proteins belonging to the thioredoxin superfamily (for reviews see Refs. 7–9), and oxidation by low molecular weight compounds such as oxidized glutathione (10, 11). The first class of enzymes reported to be involved in disulfide bond formation was the protein disulfide isomerase (PDI) family (7, 9, 12). Although the founding member of this family, PDI, is sufficient in vitro, when combined with a physiological glutathione redox buffer, to refold reduced protein substrates or to isomerize substrates with incorrect disulfide bonds (13–15), it is clear that in vivo there exists a large family of enzymes. There is evidence that the exact make-up of this family is species-dependent, with, as an extreme example, the yeast Saccharomyces cerevisiae having five reported family members (16), whereas humans have at least seventeen (9), not all of which have been characterized yet. Although the family name suggests that all of the members of the family are able to catalyze isomerization reactions in folding protein substrates, this is not the case. For example, one sub-group of the family, which includes ERp28/29 (17) and Drosophila wind protein (18), lacks both cysteines from the CXXC active site motif that are essential for the catalysis of dithiol-disulfide exchange reactions catalyzed by PDI. Hence they are unable to catalyze any sulfur-based oxidations, reductions, or isomerizations.

To date, all of the non-catalytic PDI family members reported consist of two domains, the first having the thioredoxin fold reported for all PDI domains whose structure has been solved; the second domain is all α-helical (18, 19). Here we report a new human PDI family member, which we call ERp27. ERp27 contains two domains that are homologous to the non-catalytic b and b’ domains of human PDI, and both probably have a thioredoxin fold. ERp27 lacks a CXXC active site and hence is unable to catalyze dithiol-disulfide exchange, however, it is able to bind the small peptide Δ-somatostatin. The binding site is localized to the second domain of ERp27, and mutations in homologous residues to those involved with peptide binding in human PDI (20) and those involved in calreticulin (CRT) binding by human ERp57 (21) also inhibit peptide binding by ERp27, suggesting conservation of the localization of the substrate binding site across the PDI family. ERp27 contains a motif Asp-Glu-Trp-Asp located in a loop in the b’-like domain, which is similar to the Glu-Asp-Trp-Asp motif found at the tip of the P-domains of CRT and calnexin (CNX). This motif in CRT forms part of the ERp57 interaction site (22, 23), and here we report that the homologous motif in ERp27 is bound by ERp57.

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2 The abbreviations used are: ER, endoplasmic reticulum; CNX, calnexin; CRT, calreticulin; DSG, disuccinimidyl glutarate; GFP, green fluorescent protein; PDI, protein disulfide isomerase; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry.
TABLE 1
Plasmids used in this study other than for recombinant Erp27 expression in E. coli
pLWRP64/65/69, pOLR48, pHIA98, and pKEHS69/82/83 were generated previously (21, 24, 25); the rest were generated for this study.

| Plasmid type | Plasmid name | Protein produced |
|--------------|--------------|------------------|
| pET23b (modified; 24) | pLWRP69 | wt PDI a domain (Asp18–Ala269), hexa-His-tagged |
| | pOLR48 | wt PDI b domain (Ala269–Ala349) hexa-His-tagged |
| | pLWRP65 | wt PDI b domain (Lys236–Pro340) hexa-His-tagged |
| | pLWRP64 | wt mature PDI (Asp18–Leu273) hexa-His-tagged |
| | pHIA98 | wt E. coli thioredoxin (Ser1–Ala108), hexa-His-tagged |
| | pKEHS69 | wt mature Erp57, hexa-His-tagged |
| | pKEHS82 | wt mature Erp57 R280A, hexa-His-tagged |
| | pKEHS83 | wt mature Erp57 F299W, hexa-His-tagged |
| | pHIA239 | wt CRT P-domain (Lys206–Ile305), hexa-His-tagged |
| | pHIA199 | wt Erp27 (Met1–Leu273) |
| pcDNA3.1 | pHH37 | ER-targeted EFYP1 (Q69M); CRT P-domain (Lys206–Ile305) |
| | pHH56 | ER-targeted EFYP1 (Q69M) |
| | pHH57 | ER-targeted EFYP2 |
| | pHIA334 | ER-targeted EFYP1 (Q69M); mature Erp27 (Glu26–Leu273) |
| | pHIA338 | ER-targeted EFYP2; mature Erp27 (Glu26–Leu273) |
| | pHIA335 | ER-targeted EFYP1 (Q69M); mature Erp27 (I196W) |
| | pHIA339 | ER-targeted EFYP2; mature Erp27 (I196W) |
| | pHIA336 | ER-targeted EFYP1 (Q69M); mature Erp27 (E231A, W232A, D233G) |
| | pHIA340 | ER-targeted EFYP2; mature Erp27 (E231A, W232A, D233G) |
| pRK7 | pHH34 | ER-targeted EFYP1 (Q69M); mature Erp57 |
| | pHH38 | ER-targeted EFYP2; mature Erp57 |
| | pHH109 | ER-targeted EFYP1 (Q69M); mature Erp57 (R280A) |
| | pHH110 | ER-targeted EFYP2; mature Erp57 (R280A) |
| | pHH36 | ER-targeted EFYP1 (Q69M); mature Erp57 (F299W) |
| | pHH40 | ER-targeted EFYP2; mature Erp57 (F299W) |
| pEGFP-N1 | pHIA203 | GFP-Erp27 (Pro269–Leu273) |
| | pHIA216 | GFP-Erp27 (Pro269–Leu273) |

EXPERIMENTAL PROCEDURES

Generation of Expression Vectors—Plasmids encoding for N-terminal hexa-histidine-tagged human PDI a, b, and b’ domains along with the mature protein, mature human Erp57, and the R280A and F299W mutants therein, and Escherichia coli thioredoxin were generated previously (21, 24, 25).

Plasmids encoding for full-length and mature (Glu26–Leu273) human Erp27 were generated by PCR from IMAGE clone 5207225 using primers that included an in-frame Ndel site 5’ to the first codon of the gene and a BamHI site after a TAA stop codon at the 3’-end. A plasmid encoding for the P-domain of CRT was generated by PCR from a plasmid encoding full-length CRT generously supplied by Prof. S. High (University of Manchester) using primers that included an in-frame NdeI site 5’ to the first codon of the gene and a SalI site after a TAA stop codon at the 3’-end. All inserts were then cloned into pET23b (modified; 24) pLWRP69 wt PDI (Asp18–Leu273) hexa-His-tagged

Bimolecular fluorescence complementation vectors targeted to the ER were made according to the design of Nyfeler and co-workers (26). Oligonucleotides encoding for the signal sequence of calreticulin (MLLSVPLLGLLGLAV) with a Nhel site 5’ and with overhangs complementary to Xbal and Nhel were cloned into the Xbal and Ncol sites of pET23b generating pFH1. The YFP fragments Y1 (1–158) and Y2 (159–239) were generated by PCR using the pLP-EYFP-C1 plasmid (Clontech) as a template with a (Gly-Gly-Gly-Gly-Ser)2-His-terminus. Point mutations in plasmids were performed as recommended by the manufacturer using the QuikChange kit (Stratagene). All plasmids generated were sequenced to ensure there were no errors in the cloned genes.

Protein Expression and Purification—Protein production was carried out in E. coli strain BL21(DE3)pLysS. Strains were grown in LB media at 37 °C, 200 rpm, and induced at an A600 of 0.3 for 4 h with 1 mM isopropyl β-D-thiogalactoside. Cells were pelleted by centrifugation (6,500 rpm for 10 min), and the pellet was resuspended in 1/10th volume of buffer A (20 mM sodium phosphate, pH 7.3) and 1/1000th volume of 10 mg/ml DNase (Roche Applied Science). The cells were lysed by freeze-thawing twice, and the cell debris was removed by centrifugation (12,000 rpm for 20 min). The supernatant was filtered through a 0.45-µm filter before being applied to an immobilized metal affinity chromatography column (chelating Sepharose fast
flow, Amersham Biosciences), pre-charged with Ni$^{2+}$ and equilibrated in buffer A. After loading the column was washed in 20 mM sodium phosphate, 50 mM imidazole, 0.5 M NaCl, pH 7.3 and then in buffer A, before the His-tagged proteins were eluted using 20 mM sodium phosphate, 50 mM EDTA, pH 7.0. The eluant was diluted 5× into buffer A and then applied to a Resource Q anion exchanger. PDI, mature and domain constructs, ERp57, and the P-domain of CRT were eluted from the Resource Q column with a linear gradient (0–100% over nine column volumes) while ERp27 was eluted with a tripartite gradient (0–45% over one column volume, 45–70% over seven column volumes, 60–100% over two column volumes; modified subsequently for 15N-labeled protein to 0–40% over one column volume, 40–60% over eight column volumes, and 60–100% over two column volumes) from buffer A to buffer A containing 0.5M NaCl. Eluted fractions were checked for purity by SDS-PAGE, and fractions containing pure protein were pooled and buffer-exchanged into 20 mM sodium phosphate, pH 7.3, using an Amicon ultra 15 centrifugal filter device (10-kDa molecular weight cut-off membrane filter). The concentration of the protein was determined spectrophotometrically using a calculated absorption coefficients (28) at 280 nm of 18,450 M$^{-1}$ cm$^{-1}$ (ERp27), 6,990 M$^{-1}$ cm$^{-1}$ (ERp27 domain 1), 44,930 M$^{-1}$ cm$^{-1}$ (ERp57), 45,480 M$^{-1}$ cm$^{-1}$ (PDI), and 37,470 M$^{-1}$ cm$^{-1}$ (P-domain CRT). The 15N-labeled ERp27 was produced by growing the expressing strain in M9 media using 15N-labeled NH$_4$Cl (Cambridge Isotopes, Andover, MA) with protein purification as described for unlabeled protein.

**Cell Transfections**—COS-7 cells (ATCC, Rockville, MD) were grown on 30-mm diameter Petri dishes with or without glass coverslips in Dulbecco’s modified Eagle’s medium-high glucose medium supplemented with Glutamax (Invitrogen), 10% fetal calf serum, and penicillin-streptomycin. Cells seeded 1 day earlier were transfected with the ERp27-GFP plasmid using 0.5–1 µg/plate and the Fugene6™ transfection reagent (Roche Applied Science) as suggested by the manufacturer. After 24 h, cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% p-formaldehyde for 20 min, and processed for indirect immunofluorescence as described earlier (29). Monoclonal antibodies against protein disulfide isomerase (PDI, Dako A/S, Glostrup, Denmark) and the Golgi matrix protein, Gm130 (BD Biosciences, Lexington, KY) were used as the ER and Golgi markers, respectively, to allow localization of the expressed ERp27-GFP in transfected cells. Fixed and stained cells were examined using an epifluorescence microscope (Olympus BX61) and photographed with a charge-coupled device camera.

For flow cytometric analysis COS-7 cells were grown as above and transfected with 0.5 µg/plate of each plasmid and 5 µl of Fugene6 transfection reagent. After 24 h, cells were rinsed with PBS and then detached from the plate by incubating them with 200 µl of Trypsin-EDTA solution (0.5 mg/ml Trypsin, 0.02% EDTA in PBS) for 7 min at 37 °C. The cells were then collected by centrifugation at 300 × g for 5 min at 4 °C and resuspended in 1.2 ml of PBS containing 2% fetal calf serum. For each sample the yellow fluorescence of 5000 cells was then analyzed using a CyFlow flow cytometer (Partec) with appropriate filter sets. Gating to analyze the average fluorescence intensity of transfected cells was performed using FloMax software (Partec). Transformation with the controls Y1-ERp27 or Y2-ERp57 resulted in transfected cells whose fluorescence intensity overlapped with that of the non-transfected cell population, and therefore the average fluorescence intensity of these controls could not be determined accurately, although it was <1% of that of the Y1-ERp27 plus Y2-ERp57 sample.

**Biophysical Analysis**—Far-UV CD spectra were recorded on a Jasco J600 spectrophotometer. All scans were collected at 25 °C as an average of eight scans, using a cell with a path length of 0.1 cm, scan speed of 50 nm/min, a spectral bandwidth of 1.0 nm, and a time constant of 0.5 s. Protein concentrations used varied between 0.1 and 0.14 mg/ml. The maximal HT voltage was under 750 V in all spectra.

Fluorescence spectra were collected on a PerkinElmer Life Sciences L550 spectrophotometer using a 1-ml cuvette. All scans were collected at 25 °C as an average of four scans, excitation 280 nm, emission 300–400 nm, slit widths 5 nm, and scan speed 200 nm/min. Protein stocks were diluted at least 200-fold into 0.2M phosphate buffer, pH 7.0, containing 0–6 M guanidinium chloride and equilibrated for 5 min at 25 °C before fluorescence spectra were recorded. All spectra were corrected for the blank spectra with no protein added.

The Ellman’s assay (30) was performed by adding 3 µl of 3.4 mg/ml Ellman’s reagent to 200 µl of pre-equilibrated protein solution in either 0.2M phosphate buffer, pH 7.0 (native conditions) or 0.2 M phosphate buffer, 6 M guanidinium chloride, pH 7.0 (denaturing conditions). The rate of change in absorbance at 412 nm was measured in a dual-beam spectrophotometer, and a molar extinction coefficient of 12,830 m$^{-1}$ cm$^{-1}$ (at pH 7.0) was used for the reaction product.

NMR spectra were collected on a Varian Inova 600-MHz spectrometer from samples of uniformly 15N-labeled ERp27 (0.34 mM) and domain 1 (0.56 mM) in 20 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl and 10% (v/v) D$_2$O. 1H/15N HSQC spectra were collected at 25 °C over 12 and 6 h, respectively, with acquisition times of 125 ms in 1H and 83 ms in 15N and with water suppression using the WATERGATE sequence. Δ-Somatostatin was added to full-length 15N-labeled ERp27 (0.3 mM) at a 5× molar excess (1.5 mM). For NMR analysis of ERp57–ERp27 complex formation 1H/15N HSQC spectra were collected as described above except that the number of transients collected was increased (total acquisition time, 24 h) to compensate for the lower concentration of 15N-labeled ERp27 protein (0.1 mM in both cases). Unlabeled ERp57 was added at a 1.14× molar excess for domain 1 and a 1.5× molar excess for full-length ERp27.

**In Vitro Protein Cross-linking**—Cross-linking was performed using the homobifunctional cross-linking reagent disuccinimidyldimethyl glutarate (DSG, Sigma) and was optimized for different systems by varying protein concentrations, DSG concentration and time of reaction. Proteins were mixed to give a volume of 9 µl in either 0.2 M sodium phosphate, pH 7.2, or phosphate-buffered saline solution (10 mM sodium phosphate, 150 mM NaCl, pH 7.3) and incubated on ice for 10 min. 1 µl of DSG was added and mixed, and the solution was incubated on ice. The cross-linking reaction was quenched by the addition of 2.5 µl of 5× SDS sample buffer.

**Peptide Binding by Cross-linking**—Cell extracts from E. coli BL21(DE3)pLYS S were prepared as described under "Protein
Expression and Purification. Bolton-Hunter \textsuperscript{125}I labeling of \(\Delta\)-somatostatin (AGSKNFWKTFSS) was performed as recommended by the manufacturer (Amersham Biosciences). Cross-linking was performed using DSG as described previously (31).

**Oxidase Assay**—The method of Ruddock and co-workers (32) using a fluorescent decapeptide was used to determine the oxidase activity of each of ERp27 in McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid, pH 7.0) with 0.5 mM oxidized glutathione and 2 mM reduced glutathione.

**RESULTS**

**Identification of a Novel Human PDI Family Member**—A data base search for novel human PDI family members identified a previously uncharacterized open reading frame (Q96DN0, ENSG00000139055, FLJ32115, and c12orf46) encoding a putative 27-kDa ER-resident PDI family member, which we named ERp27. The gene is located at 14,958,241–14,982,750 bp on chromosome 12 and has 7 exons. Serial analysis of gene expression (33) indicates that ERp27 is expressed in humans in a variety of tissue types, including in the spleen, lung, kidney, thymus, and bone marrow and most highly in the pancreas. Unigene (34) cDNA sources include kidney, cervix, breast, placenta, colon, lung, pancreas, thyroid, and marrow. The ERp27 coding region used in these studies was PCR-amplified from IMAGE clone 5207225.

A homologue of human ERp27 can be found in mouse, chimp, dog, cow, rhesus macaque, green puffer fish, and zebrafish; no homologues could be found in other species, including the fully sequenced \(S.\) cerevisiae, Caenorhabditis elegans, or Drosophila melanogaster; although other PDI family members were identified in a large number of organisms using ERp27 as the search. A multiple alignment of the human and mouse ERp27 proteins showed 65.5\% identity, which is considerably lower than the identity observed between other PDI family members between these species, e.g. mature mouse and human PDI show 95.1\% identity.

**Analysis of the Domain Structure of ERp27**—Analysis of the sequence of ERp27 using PSORT (35) and TargetP (36) indicated that the protein would be targeted by a cleavable N-terminal signal sequence to the secretory pathway, with the mature protein starting at A21 (PSORT) or E26 (TargetP). The C-terminal amino acids are KVEL, which probably acts as an ER retrieval motif (usual consensus KDEL).

If the mature protein starts at E26, then it is 248 amino acids long, with a molecular mass of 27.7 kDa and a theoretical pl of 4.6 (ProtParam). The protein contains two cysteine residues but does not contain a CXXC thioredoxin superfamily-active site motif indicating that ERp27 is probably a catalytically redox-inactive member of the PDI family. The protein contains an N-glycosylation site at Asn\textsuperscript{100}.

Multiple alignments of human ERp27 and PDI were performed using a wide range of programs. These alignments all showed a considerable degree of consensus and suggested that ERp27 is a homologue of the \(\text{bb}^\prime\) domains of human PDI, with short N- and C-terminal extensions (Fig. 1). Because other PDI family members also contain a \(\text{bb}^\prime\) domain pair, multiple alignments were done between ERp27 and these proteins. The percentage identity over the \(\text{bb}^\prime\) region was found to be higher between human ERp27 and PDI (33.5\%) than for the other family members (PDIp, 28.1\%; ERp57, 16.5\%; ERp72, 14.7\%; domain boundaries from Ref. 24). No significant homology could be found between ERp27 and the other non-catalytic human PDI family member ERp29.
BLAST searches of mammalian sequences, using full-length ERp27 or either domain, revealed only significant hits with mammalian PDI family members, PDI, PDIp, and PDILT. In all cases the first domain of ERp27 corresponded to the b domain, and the second domain of ERp27 to the b/H11032 domain.

Putative domain boundaries for the b and b/H11032 domains of ERp27 determined by sequence alignment (allowing for insertions and deletions to fall between the modeled secondary structural elements) were confirmed by making a range of domain constructs of ERp27 (Table 2) with expression in E. coli (data not shown). The full-length mature protein and constructs with deletions at the N terminus to P34 and/or at the C terminus to Leu257 were all partially solubly expressed (>50% soluble expression), suggesting that the N- and C-terminal extensions do exist. Furthermore, a variety of domain 1 constructs based on the hypothetical boundaries were also partially solubly expressed (>50% soluble expression), suggesting that the domain boundaries for this construct were also correct. However, although a variety of domain 2 constructs were made, all formed insoluble inclusion bodies upon expression in E. coli. This is consistent with previous studies showing that the b/H11032 domains of many human PDI family members are difficult to express solubly in E. coli (24). However, these constructs along with the alignments with other PDI family members indicate that the domain boundaries for the two domains of ERp27 are Pro34–Leu141 and Pro142–Leu257. Hence, ERp27 has a 9-amino acid N-terminal extension and a 16-amino acid C-terminal extension. Such extensions are found in all PDI family members, e.g. those in human PDI are of 7 amino acids and 19 amino acids, respectively (24). Interestingly, the inter-domain linker found after the b/H11032 domain of human PDI (20) does not exist after the second domain of ERp27.

Localization of ERp27—ERp27 contains a putative secretory pathway signal sequence and a putative ER-retention signal; hence, it is most probably an ER-resident protein. To investigate the subcellular localization of ERp27, an ERp27-GFP chimera was constructed with amino acids Met1–Pro269 of ERp27 fused in-frame to the N terminus of GFP and Pro269–Leu273 of ERp27 fused in-frame C-terminal to GFP. This chimera retains the putative ER signal sequence at the N terminus and the putative ER-retrieval signal at the C terminus of the chimera and is localized in a fine reticular network and the nuclear envelope (Fig. 2), suggesting that the fusion protein localizes mainly in
Functional Characterization of ERp27

FIGURE 3. Biophysical analysis of ERp27. A, far-UV CD spectra of human ERp27 (solid line) and human ERp27 domain 1 (dotted line). All spectra are the averages of eight scans. The scales on the axes are the same as in Fig. 2B. B, far-UV CD spectra of human PDI a domain (dotted line), b domain (solid line), and b’ domain (dashed line). All spectra are the averages of eight scans. C, fluorescence spectra of native (0.2 M sodium phosphate, pH 7.0; solid line) and denatured (0.2 M sodium phosphate, 6 M guanidinium chloride, pH 7.0; dashed line) human ERp27 at 0.55 μM. D, guanidinium chloride denaturation curve for ERp27 (C) and ERp27 domain 1 (○) showing the change in fluorescence intensity (as a percentage of the intensity at 0 M guanidinium chloride) as a function of guanidinium chloride concentration. Each data point represents an average of at least four scans. Lines of best fit are fitted to the six-component equation for denaturant-dependent changes in ΔG (38).

show similarities with the spectra of the b and b’ domains of human PDI, but also differences. This is consistent with both domains having a thioredoxin fold allowing for previous observations (20, 25) that members of the thioredoxin superfamily appear to have different far-UV CD spectra even though they share the same fold. The CD spectra indicate that, unlike ERp29, ERp27 does not have one all helical domain.

Fluorescence spectra of mature ERp27 under non-denaturing conditions gave a peak with a λ\text{max} of 352.0 nm indicating most probably that both of the two tryptophans of ERp27 are in a hydrophilic environment (Fig. 3C). Upon addition of 6 M guanidinium chloride the fluorescence spectra of ERp27 had a λ\text{max} of 356.5 nm and showed a marked reduction in the signal consistent with denaturation of the protein (Fig. 3C). Fluorescence spectra of the ERp27 domain 1 construct under non-denaturing conditions gave a peak with a λ\text{max} of 355.0 nm indicating that the tryptophan is in a hydrophilic environment. Upon addition of 6 M guanidinium chloride the fluorescence spectra of this construct had a λ\text{max} of 356.5 nm, indicative of a denatured protein (data not shown). Using the six-component equation for denaturant-dependent changes in ΔG (40), ΔG° and midpoints for denaturation were calculated. These values were 36.9 ± 2.0 kJ mol⁻¹ and 2.92 M for mature ERp27 and 35.1 ± 1.5 kJ mol⁻¹ and 2.49 M for the domain 1 construct (Fig. 3D). However, it should be noted that ΔG° values calculated from denaturation curves are prone to error, and hence these values should be treated with some caution.

ERp27 contains one cysteine residue in each domain. To determine the status of these groups an Ellman’s assay for free sulphydryl content was performed under native and denaturating conditions. Mature ERp27 revealed 2.01 free sulphydryl groups under denaturing conditions, with a half time for the reaction of 30.1 s, whereas under non-denaturing conditions no change in absorbance was observed over 30 min. Likewise ERp27 domain 1 revealed 1.02 free sulphydryl groups under denaturing conditions, whereas under non-denaturing conditions no change in absorbance was observed over 30 min. These results indicate that, in the mature protein both cysteine residues exist as free thiols, both are buried and that the cysteine in domain 1 is buried in the isolated domain, i.e. it is not at the domain-domain interface in the mature protein.

ERp27 Has a Conserved Substrate Binding Site—Unlike most of the other PDI family members, human ERp27 does not contain a CXXC active site motif, and therefore it is unlikely to be

the ER. This was confirmed by staining of the transfected cells with antibodies against the known ER and Golgi markers (PDI and Gm130). The ERp27 chimeric protein co-localized with PDI but not with Gm130.

Biophysical Analysis of ERp27—Mature ERp27 (Glu²⁶-Leu²⁷³) and the isolated domain 1 construct (Glu²⁶-Leu¹⁴¹) with N-terminal hexa-His tags to aid purification, were expressed in the cytoplasm of E. coli and the constructs purified to apparent homogeneity by a combination of immobilized metal affinity chromatography and anion exchange chromatography (data not shown). Mass spectrometric analysis of the purified protein by MALDI-MS (mass accuracy 0.1%) gave a mass of 28838.9 Da for hexa-His-tagged ERp27 of (calculated mass, 28837.8 Da) and 13795.9 Da for the hexa-His-tagged domain 1 construct (calculated mass, 13798.5 Da). Purified ERp27 was then analyzed by a variety of techniques.

The far-UV CD spectra of purified mature ERp27 and the domain 1 construct (Fig. 3A) indicated that the proteins were very similar and well structured and contained both α-helix and β-sheet. All members of the thioredoxin superfamily, including thioredoxin and the a and b domains of human PDI share the same α/β fold (see Refs. 37–39 as examples). Because ERp27 showed considerable homology with the bb’ region of human PDI, the far-UV CD spectra of purified ERp27 was compared with those of the a, b, and bb’ domains of human PDI (Fig. 3B). The spectra of both mature ERp27 and the domain 1 construct
PDI was localized to a hydrophobic pocket comprising primarily hydrophobic interactions (Fig. 4A). The insignificant shift in line widths (>1 Hz) and the lack of heterogeneity in the NMR spectra upon peptide binding imply a single Δ-somatostatin binding site in ERp27. A comparison of the free and peptide-bound spectra in Fig. 5B with the overlay of domain 1 and full-length protein in Fig. 5A revealed that 32 of the shifted signals originated from the b'-like second domain, one originated from the b-like domain 1, and one was ambiguous. This is consistent with the localization of the Δ-somatostatin substrate binding site in domain 2, as identified by chemical cross-linking. The shift in signal for 32 backbone NH groups in domain 2 upon substrate binding implies that 32 of the 127 residues in domain 2 experience a change in environment. This relatively large number of residues suggests that substrate binding is associated with a conformational change; a finding supported by behavior of the tryptophan side-chain resonances. ERp27 contains two tryptophan residues, one in each domain, and their side chain indole NH resonances can be identified in the 1H/15N HSQC spectra by their charac-
teristic chemical shift positions (Fig. 5). The single tryptophan in domain 2 is located, by alignment with PDI, in the loop between \( \mathrm{H}9252 \) and \( \mathrm{H}9252 \) of the thioredoxin fold. This loop is on the opposite face of thioredoxin from the binding site defined by Leu166, Met168, Tyr182, and Ile196, but the peak for this tryptophan shows a significant shift upon \( \mathrm{H}9004 \)-somatostatin binding. This result indicates that the conformational change induced by substrate binding is not just localized around the substrate binding site but is also transmitted to peripheral regions in the protein.

**ERp27 Interacts with ERp57 in Vitro and in Vivo**

The loop between \( \mathrm{H}9252 \) and \( \mathrm{H}9252 \), which contains the tryptophan, which shifts environment upon ligand binding by ERp27, has the sequence Asp-Glu-Trp-Asp. This motif is similar to the Glu-Asp-Trp-Asp motif at the tip of the P-domain of CRT, which is bound by ERp57 (22, 23), hence we speculated that ERp57 might also bind ERp27. In vitro cross-linking revealed that ERp57 could be cross-linked to either ERp27 or to the P-domain of CRT under a range of conditions (Fig. 6A, lanes 1 and 2; Fig. 6B, lane 1). No ERp27-CRT P-domain interactions were observed (Fig. 6A, lane 3) or interactions between PDI and ERp27 (Fig. 6B, lane 7).

To investigate whether ERp27 and/or ERp57 use the substrate binding sites in their \( \mathrm{b}^\prime \) domains to interact, in vitro cross-linking was performed using the I196W mutant of ERp27, which cannot bind substrates (see above), and the previously characterized F299W and R280A mutants in the \( \mathrm{b}^\prime \) domain of ERp57, which are unable to bind CRT (21). The results (Fig. 6B) revealed that the I196W mutant of ERp27 can be cross-linked to ERp57 (lane 2) but that the cross-links observed between wild-type ERp27 and the F299W or R280A mutants of ERp57 are very much reduced (Fig. 6B, lanes 5 and 6). This implies that ERp57 binds ERp27 through the same site that it binds CRT. The most likely site in ERp27 that is bound by ERp57 is the Asp-Glu-Trp-Asp sequence. This
interaction site was confirmed using the mutations E231K and E231A/W232A/D233G in this loop sequence both of which showed greatly reduced in vitro cross-links with ERp57 (Fig. 6B, lanes 3 and 4). No evidence of light scattering was observed for the ERp27 wild type or the I196W, E231K, and E231A/W232A/D233G mutants at 6 mg/ml, and the CD spectra of these constructs were effectively identical (data not shown), indicating that there was no gross structural change induced by the mutations.

Because the cross-linking efficiency was relatively poor NMR was used to further demonstrate an interaction between ERp27 and ERp57 and to show that this interaction is mediated by the second β'-like domain. ¹H/¹⁵N HSQC spectra were collected for domain 1 and full-length ¹⁵N-labeled ERp27 protein (0.1 mM) in the presence of a slight molar excess of unlabeled ERp57, and the results are shown as spectra overlays in Fig. 7. The addition of ERp57 to domain 1 of ERp27 had no significant affect on the domain 1 spectrum (Fig. 7A). Essentially no peak shifts were seen, and the relative peaks intensities and peak shapes remained unchanged. The addition of ERp57 to full-length ERp27, however, changed the appearance of the ERp27 spectrum markedly (Fig. 7B). The majority of the ERp27 peaks disappear in the presence of ERp57, with the only peaks remaining being those that are very sharp in the ERp27 spectrum and have ¹H shifts typical of a random coil conformation (8.0–8.5 ppm) or are clearly NH₂ side-chain resonances (paired peaks in the top right-hand corner of the spectrum). These results suggest tight binding of ERp57 to full-length ERp27, the molecular mass of the complex (84 kDa) being sufficiently large to broaden the resonances of the majority of the ERp27 signals to an extent where they are no longer resolved. The sharp peaks remaining are those from the unstructured regions of the ERp27 protein and side chains that continue even in the complex to retain sufficient movement (i.e. molecular tumbling) to appear as distinct peaks in the spectrum. As this effect was not seen with isolated domain 1, the result confirms that the ERp57 binding site resides in domain 2 of the protein.

To investigate if ERp57 interacts with ERp27 in vivo, a bimolecular fluorescence complementation assay was set up. Similar constructs to those used by Nyfeler and co-workers (26) were made with parts of yellow fluorescence protein (YFP) being fused to mature ERp57 and ERp27 with an N-terminal signal sequence to direct the constructs to the ER. Upon expression of the constructs in COS-7 cells, negligible fluorescence was observed by microscopy with the Y1 alone, Y2 alone, and minimal fluorescence with Y1–Y2 co-transfection negative controls (data not shown). However, ER-localized fluorescence was seen when the Y1–ERp57 and Y2–ERp27 or Y2–ERp57 and Y1–ERp27 constructs were co-expressed (Fig. 8A) indicating that ERp57 and ERp27 interact in the ER of COS-7 cells. Flow cytometric analysis of co-transfected COS-7 cells revealed that the Y1–ERp57 and Y2–ERp27 pairing gave the strongest fluorescence signal and that this was ~20-fold higher than the Y1–Y2 negative control (Fig. 8B). To confirm the nature of the interaction, point mutations were made in the Y1–ERp57 and Y2–ERp27 constructs, and a series of co-expressions was performed. In each case the ER localization of bimolecular fluorescence complementation was confirmed by fluorescence microscopy and quantified by flow cytometric analysis (Fig. 8B). The I196W mutant of ERp27 still interacted strongly with ERp57. Because this mutant showed greatly reduced substrate binding, this indicates that the substrate binding site of ERp27

![Figure 7. Interactions between ERp27 and Erp57 by NMR. Interaction of ERp57 with domain 1 (A) and full-length ERp27 (B). Both panels show ¹H/¹⁵N HSQC spectra for ¹⁵N-labeled ERp27 protein (0.1 mM) in the presence (red contour lines) and absence (black contour lines) of a slight molar excess of unlabeled ERp57.](image-url)
is not required for the ERp27-ERp57 interaction. However, the interaction between ERp57 and the E231A/W232A/D233G mutant of ERp27 was significantly weaker than that observed between the two wild-type proteins, as was the interaction between the ERp57 R280A mutant and wild-type ERp27. Although the Erp57 R280A mutant has been shown to significantly reduce ERp57-CRT interactions in microsomes and in in vitro transcription-translation based systems (21), it has not been tested in a cellular system. To confirm that this mutation has a similar effect on ERp57-CRT interactions as it has on ERp57-ERp27 interactions Y1-CRT-P was co-expressed with Y2-ERp27 and Y2-ERp27 was co-expressed with Y2-ERp57 or Y2-ERp57 R280A. Fluorescence microscopic analysis revealed ER-localized fluorescence for both pairings, with the R280A mutation showing a significant reduction in fluorescence (data not shown). Flow cytometry analysis of co-transfected cells revealed a 60% reduction in fluorescence due to the R280A mutation (see Fig. 8C). These in vivo results are fully consistent with the in vitro data showing that ERp57 binds ERp27 by the Asp-Glu-Trp-Asp sequence in domain 2 and that it uses the same site by which it interacts with the tip of the P-domain of CRT.

**DISCUSSION**

ERp27 is a previously unidentified member of the PDI family. Unlike the majority of PDI family members it lacks a CXXC active site motif, having a two-domain structure homologous to the non-catalytic \( b b' \) domains of PDI. Although ERp27 shares a two-domain structure with the other reported non-catalytic PDI family members, e.g. ERp29, it appears not to follow the same pattern of having one domain with a thioredoxin fold and one all \( \alpha \)-helical domain; rather, ERp27 appears to have two domains that have a thioredoxin fold and therefore may form a distinct subfamily in the PDI family.

Of the fifteen human PDI family members reported to date only a few have a clear physiological function (9). PDI in vitro and in vivo is a general catalyst of native disulfide bond formation, whereas PDJustin appears to play a similar role but with different substrate specificity and a specific localization in the acinar cells in the pancreas (41, 42). ERp57 appears to be a general catalyst of native disulfide bond formation for glycosylated proteins, which it does not bind directly, but rather binds via an interaction with the ER-resident lectins CRT and CNX (43, 44). No clear physiological function has been identified for the other PDI family members, although they appear to have distinct properties, e.g. ERp18 is the only family member to date whose catalysis of diithiol-disulfide exchange is rate-limited by peptide oxidation rather than enzyme reoxidation (25), or distinct interaction partners, e.g. ERdj5 contains a dnaJ motif and interacts with BiP (45), or whose knockdown/knock-out generates a phenotype, e.g. ERp46 whose knock-down makes cells sensitive to hypoxia (46). Likewise, no clear physiological function is discernable yet for ERp27, which appears to show a wide, yet selective, tissue distribution. It is noteworthy that data base searches revealed ERp27 homologs only in a very limited range of organisms, although the high homology with other PDI family members and the unusually low sequence identity between human and mouse ERp27 suggests that other ERp27s may exist but have been missed on the data base screen.

ERp27 does contain a \( b' \)-like domain and, like PDI, binds the 14-amino acid test peptide \( \Delta \)-somatostatin via this domain. ERp27 is one of five PDI family members that contain a \( b' \)-like domain, which has been shown to provide the primary substrate binding site of PDI (20). The data presented here for ERp27 show the conservation of the molecular localization of the substrate binding site across a third PDI family member, and the fact that it can bind \( \Delta \)-somatostatin, as per PDI, suggests that it may use this to interact with a range of unfolded or partially folded polypeptides in the ER. The NMR analysis of \( \Delta \)-somatostatin binding revealed that the \( b' \)-like domain of ERp27 undergoes a significant conformational change and that this is not just limited to the immediate environment of the substrate binding site. However, this conformational change does not appear to be transmitted to the \( b \)-like domain. Similarly, conformational changes occur in the \( b' \) domain of PDI\(^3\) and a conformational change occurs in the \( a \) and \( a' \) domains of PDI, which are linked to modulation of the \( pK_a \) of the active site cysteines and hence are linked to the catalytic cycle (47). ERp27 is also bound by ERp57. This interaction utilizes the CRT P-domain interaction site in the \( b' \) domain of ERp57, with a similar motif in a loop region being bound on ERp27 (Asp-Glu-Trp-Asp) and CRT or CNX (Glu-Asp-Trp-Asp). Because ERp57 uses the same site to bind similar motifs on both ERp27 and CRT/CNX, it cannot bind both simultaneously. Furthermore, because all of the major physiological functions of ERp57 are linked to its ability to inter-
act with the ER-resident lectins CRT and CNX, for N-glycoprotein protein folding and quality control and for major histocompatibility complex-class I protein folding and peptide loading (48–51), the overexpression of ERp27 is likely to have a deleterious effect on all of these processes, even if they are not linked to the physiological function of ERp27. Indeed, ERp27 may have a function in any or all of these processes or may have another function such as bringing non-glycosylated protein substrates to ERp57 to fold, just as CRT/CNX brings N-glycosylated substrates to ERp57, but it is not yet known under what physiological circumstances the ERp57-ERp27 interaction occurs in preference to the ERp57-CRT/CNX interaction. Furthermore, because the tryptophan in the Asp-Glu-Trp-Asp motif in ERp27 significantly changes environment upon substrate binding by ERp27, it is probable that the affinity of ERp57-ERp27 interactions is modulated by ERp27-substrate interactions. However, because the interactions of ERp57 with substrates may also modulate the ERp57-ERp27 interaction until the distinct substrate specificities of ERp57 and ERp27 are known this cannot be tested. This is further complicated by the reported distinct binding sites in the catalytic $a$ and $a'$ domains of PDI (52), which are likely to also be found in the catalytic domains of ERp57, and the lack of available information on the function of the $b$ domains of the PDI family and the interactions they make.

Although detailed characterization has yet to be undertaken on many PDI family members, it appears that only a small subset of PDI family members combine a generalized substrate binding domain and a catalytic domain, a combination that has been shown to be essential for efficient isomerization by PDI (53). Hence the nomenclature to identify this family of ER-resident folding catalysts may be inappropriate, because few, if any, other than PDI are likely to be efficient, generic, protein disulfide isomerases.

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