Functional characterization of 3 thioredoxin homology domains of ERp72

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Abstract Folding of secretory proteins is associated with the formation and isomerization of disulfide bonds. ERp72, a protein disulfide isomerase (PDI) family member, possesses 3 thioredoxin homology domains, but the participation of each domain in disulfide-bond formation and isomerization remains to be determined. We analyzed the function of individual domains in the insulin reduction assay system by site-directed mutagenesis with cysteine-to-serine replacement. All domains contributed to apparent steady-state binding (K_m) and catalysis at saturating substrate concentrations (k_{cat}) but in different manners. A mutant ERp72 with mutations in domains 1 and 2 (ERp72-mut-1+2) exhibited reductions in k_{cat} of 73.9% when compared with wild type, whereas ERp72-mut-1+3 (mutations in domains 1 and 3) and ERp72-mut-2+3 (mutations in domains 2 and 3) exhibited less substantial reductions in k_{cat} . ERp72-mut-1+3 and ERp72-mut-2+3 showed elevations in K_m of 89.9% and 96.2%, respectively, when compared with wild type, whereas ERp72-mut-1+2 exhibited smaller elevations in K_m . These results suggest that domains 1 and 2 make greater contributions to catalyzing efficacy and domain 3 to binding affinity. Domain 2 is involved in binding affinity, in combination with domain 3, in addition to its own contribution to catalyzing efficacy. This assignment of functions to individual domains is similar to that observed in other PDI domains, which is consistent with the high sequence homology between ERp and PDI domains.

INTRODUCTION

The folding of nascent proteins in cellular secretory pathways is associated with the formation and isomerization of disulfide bonds (Pfeffer and Rothman 1987; Rapoport et al 1999; Trombetta and Parodi 2003). Numerous proteins resident in the endoplasmic reticulum (ER) have been identified as chaperones and folding enzymes that assist in protein folding, provide retention, and enable quality control (Gething and Sambrook 1992; Hammond and Helenius 1995). Protein disulfide isomerase (PDI), one of the first described folding enzymes, contains 2 thioredoxin homology domains and catalyzes the formation and isomerization of disulfide bonds during protein folding (Hillson et al 1984; Freedman et al 1995; Gil-

bert 1998). The thioredoxin homology domain contains a Trp-Cys-Gly-His-Cys-Lys (WCGHCK) motif that is identical to the active site consensus sequences of PDI (Lee 1981; Mazzarella et al 1990, 1994; Freedman et al 1995; Gilbert 1998). Additional proteins that possess the thioredoxin homology domains and are localized in the ER, such as ERp61 and protein disulfide isomerase-related protein (PDIR), comprise the PDI family and are regarded as folding enzymes that participate in the formation and isomerization of disulfide bonds in secretory proteins (Mazzarella et al 1990; Martin et al 1991; Srivastava et al 1991; Hayano and Kikuchi 1995; Otsu et al 1995).

ERp72 that has been identified as a luminal ER protein of murine plasmacytoma cells and, together with its rat liver homologue CaBP2, bears 3 thioredoxin homology domains and an Lys-Glu-Glu-Leu (KEEL) sequence at the C-terminus (Mazzarella et al 1990; Lenny and Green 1991; Van et al 1993). ERp72 shares the sequence (WCGHCK) responsible for its redox activity with other

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PDI proteins. However, the functional roles of individual thioredoxin homology domains remain to be elucidated.

In this study, we characterized the functional properties of the individual thioredoxin homology domains of ERp72 using mutant domains in which cysteine in the catalytic center was replaced with serine and by assaying insulin reduction activity.

MATERIALS AND METHODS

Site-directed mutagenesis

ERp72 complementary deoxyribonucleic acid (cDNA) was kindly provided by Dr M. Green and was amplified by polymerase chain reaction with a primer set (CGGGATCCGAGATGCACAGGAAGATAC and CGGGATCCAGCTCTTCCTTGGTCCTGC), *Pfu* DNA polymerase, and pGEM72-1 as a template (Mazzarella et al 1990). Amplified fragments were digested with *Bam*HI, and the cDNA fragments were subcloned into the *Bam*HI site of p-BluescriptIIKS⁻ (Stratagene, La Jolla, CA, USA) (designated as pBS-ERp72). The *Nco*I fragment was removed from pBS-ERp72, which was then self-ligated (pBS-ERp72Δ*Nco*I). Alternatively, pBS-ERp72 was digested with a combination of *Bam*HI and *Sac*I, and smaller fragments of ERp72 cDNA were subcloned into the *Bam*HI-*Sac*I site of pBluescriptIIKS⁻ (pBS-ERp72BS). Mutations were introduced into the thioredoxin homology domains using the QuikChange[™] site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Primer sets and templates for introducing mutations were as follows: for changing both Cys-60 and Cys-63 to Ser (C60S and C63S), a primer set (CTATGCACCATGGTCTGGACACTCCAAGCAGTTTGCTC and GAGCAAACTGCTTGGAGTGTCCAGACCATGGTGCATAG) and pBS-ERp72BS were used; for changing both Cys-175 and Cys-178 to Ser (C175S and C178S) (mut-2), a primer set (CTATGCCCCGTGGTCTGGACACTCCAAGAAACTTGCC and GGCAAGTTTCTTGGAGTGTCCAGACCACGGGGCATAG) and pBS-ERp72BS were used; and for changing both Cys-524 and Cys-527 to Ser (C524S and C527S) (mut-3), a primer set (CTATGCACCCTGGTCTGGCACTCCAAGCAGCTAGAG and CTCTAGCTGCTTGGAGTGGCCAGACCAGGGTGCATAG) and pBS-ERp72Δ*Nco*I were used. The resultant plasmids were designated pBS-ERp72BS-mut-1, pBS-ERp72BS-mut-2, and pBS-ERp72Δ*Nco*I-mut-3, respectively. Each mutation was confirmed by DNA sequencing.

Expression of recombinant wild-type and mutant ERp72

The *Sac*I fragment from pBS-ERp72 was subcloned into the *Sac*I site of pBS-ERp72BS-mut-1 and pBS-ERp72BS-

mut-2 (designated pBS-ERp72-mut-1 and pBS-ERp72-mut-2, respectively). The *Nco*I fragment of ERp72 was subcloned into the *Nco*I site of pBS-ERp72Δ*Nco*I-mut-3 (pBS-ERp72-mut-3), and the *Nco*I fragment of pBS-ERp72-mut-3 was replaced with the *Nco*I fragments of pBS-ERp72-mut-1 and pBS-ERp72-mut-2 (pBS-ERp72-mut-1+3 and pBS-ERp72-mut-2+3, respectively). The *Nhe*I-*Hind*III fragment of pBS-ERp72-mut-1 was replaced with the *Nhe*I-*Hind*III fragment of pBS-ERp72-mut-2 (pBS-ERp72-mut-1+2). Finally, the *Nco*I fragment of pBS-ERp72-mut-3 was replaced with the *Nco*I fragment of pBS-ERp72-mut-1+2 (pBS-ERp72-mut-1+2+3). All plasmids containing either wild-type or mutant ERp72 were digested with *Bam*HI, and the resultant fragments were separately subcloned into the *Bam*HI-*Bgl*II site of pQE16 (Qiagen, Valencia, CA, USA) (pQE-ERp72, pQE-ERp72-mut-1, pQE-ERp72-mut-2, pQE-ERp72-mut-3, ERp72-mut-1+2, ERp72-mut-2+3, and ERp72-mut-1+2+3, respectively). Expression and purification of the wild-type and mutant sequences were carried out, according to the manufacturer's instructions, using Ni-NTA agarose (Qiagen). Further purification of these proteins was performed using a Resource Q column (Pharmacia Biotech AB, Uppsala, Sweden) after dilution with 20 mM phosphate buffer (pH 7.2) until the proteins were bound.

Insulin reduction assay

Wild-type ERp72 protein is known to catalyze the reduction of disulfide bonds in proteins such as insulin. The ERp72-catalyzed reduction of insulin by glutathione, reduced form (GSH) (Sigma, St Louis, MO, USA) was monitored by coupling the reduction of the product, oxidized form of glutathione (GSSG), to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation with glutathione reductase, as described elsewhere (Gilbert 1998). In brief, glutathione reductase (16 U, Oriental Yeast Co. Ltd, Tokyo, Japan) was preincubated with GSH (3.7 mM, Sigma) and NADPH (0.12 mM, Oriental Yeast) in reaction buffer (0.2 M phosphate buffer and 5 mM ethylenediamine-tetraacetic acid, pH 7.5) for 30 seconds to remove contaminating GSSG. Insulin (Sigma) was added, and the velocity of nonenzymatic insulin reduction was measured by monitoring the decrease in NADPH absorbance at 340 nm for 2 minutes (velocity of nonenzymatic reaction was 0.0013 to 0.0018 AU/min). The catalytic reaction was then initiated by addition of either recombinant wild-type or mutant ERp72 protein. The enzyme activity in the coupled reaction was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm for 2 minutes using the kinetics assay mode of an Ultraspec 2000 spectrophotometer (Pharmacia Biotech AB) with a peltier-heated cell holder. After

Fig 1. Schematic structures of protein disulfide isomerase (PDI) and ERp72. The PDI and ERp72 possess 2 and 3 thioredoxin homology domains, respectively. (A) Signal sequence and active site of thioredoxin homology domains are represented by hatched and closed bars, respectively. (B) List of mutants with 1, 2, or no active sites in domains 1, 2, and 3.

subtracting the background rate, the specific activity was calculated based on a $\Delta\epsilon_{340}$ of $6.23 \text{ mM}^{-1} \text{ cm}^{-1}$.

Data analysis

Experimental data were analyzed by nonlinear least squares fitting with equation [1]. Individual points were means of multiple replications (duplicate or higher) performed independently on different days.

$$v = V_{\text{max}} \cdot [s] / (K_m + [s]) \tag{1}$$

where K_m represents the Michaelis constant and V_{max} represents the maximum velocity. Calculations of V_{max} and K_m were performed using SPSS software (SPSS Inc, Chicago, IL, USA). Catalysis at saturating substrate concentrations (k_{cat}) represents turnover number per minute.

RESULTS

Purification of recombinant wild-type and mutant ERp72 proteins

We focused on the conserved cysteine residues of thioredoxin homology domains, and each residue was re-

placed with serine using site-directed mutagenesis. Figure 1 shows the active sites of the thioredoxin domains of PDI and ERp72 and lists the mutant proteins generated. Figure 2 shows the Coomassie brilliant blue staining pattern of the purified recombinant wild-type and mutant ERp72 proteins.

Insulin reduction by ERp72 mutants

We performed a pilot insulin reduction study using wild-type ERp72 to determine its K_m . We then measured insulin reduction by wild-type and mutant ERp72 proteins with insulin concentrations being near K_m . The activity traces for wild-type and mutant proteins are shown in Figure 3.

Properties of each thioredoxin homology unit of ERp72

The K_m and k_{cat} values for wild-type and mutant ERp72 proteins are shown in Table 1. The K_m of ERp72-mut-1+3 and ERp72-mut-2+3 were elevated by 89.9% and 96.2%,

	wt	mut-1	mut-2	mut-3	mut-1+2	mut-1+3	mut-2+3	mut-1+2+3
domain 1	x				x	x		x
domain 2			x		x		x	x
domain 3				x		x	x	x

Fig 2. Recombinant wild-type and mutant ERp72 proteins. After purification from *Escherichia coli*, as described in Materials and Methods, wild-type and mutant ERp72 proteins used for insulin reduction assays were applied to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue. Inactive sites of thioredoxin homology domains are depicted by X.

Fig 3. Rate of insulin reduction by ERp72 as a function of insulin concentration. Reduction was initiated by addition of insulin to assay mixtures containing either wild-type or one of the mutant ERp72 proteins. Solid curves represent the hyperbolic nonlinear least square fits of the data. (A) Circles, wild type; squares, mut-1+2; triangles, mut-1+3; diamonds, mut-2+3. (B) Squares, mut-1; triangles, mut-2; diamonds, mut-3.

Table 1 Kinetic parameters for insulin reduction by either wild-type or mutant ERp72 proteins^a

ERp72	K _m (μM)	% Activity	k _{cat} (min ⁻¹)	% Activity	k _{cat} /K _m (M ⁻¹ min ⁻¹)
Wild type	36.7 ± 10.7	100	46.0 ± 5.3	100	12.5 × 10 ²
mut-1	35.7 ± 5.6	97.4	19.8 ± 1.2	43.0	5.5 × 10 ²
mut-2	57.6 ± 12.9	157.0	26.6 ± 2.9	57.7	4.6 × 10 ²
mut-3	67.7 ± 5.6	184.4	42.8 ± 1.8	92.9	6.3 × 10 ²
mut-1+2	48.7 ± 10.2	132.7	12.0 ± 1.1	26.1	2.5 × 10 ²
mut-1+3	69.7 ± 11.8	189.9	23.4 ± 2.1	50.9	3.4 × 10 ²
mut-2+3	72.0 ± 11.0	196.2	30.7 ± 2.5	66.8	4.3 × 10 ²
mut-1+2+3	ND		ND		

ND, not determined.

^a Experiments were performed at pH 7.5, 25°C, with the ERp72 concentration being 21.6 μM.

respectively, whereas that of ERp72-mut-1+2 was only elevated by 32.7%. In contrast, the k_{cat} of ERp72-mut-1+2 was reduced by 73.9%, whereas those of ERp72-mut-1+3 and ERp72-mut-2+3 were only reduced by 33.2% and 49.1%, respectively. The k_{cat}/K_m ratios for ERp72-mut-1+2, ERp72-mut-1+3, and ERp72-mut-2+3 were 20%, 27.2%, and 34.4%, respectively. The assay for ERp72-mut-1+2+3 was performed with a 3-fold higher amount of mutant protein, but activity was not detectable. Therefore, the K_m and V_{max} for ERp72-mut-1+2+3 were not determined.

Effects of preserving 2 intact thioredoxin homology domains of ERp72

The K_m of ERp72-mut-1 mutant was almost equal to that of wild type, whereas those of ERp72-mut-2 and ERp72-mut-3 were elevated by 57.0% and 84.4%, respectively. In contrast, the k_{cat} values of ERp72-mut-1 and ERp72-mut-2 were reduced by 57% and 42.3%, respectively, whereas that of ERp72-mut-3 was almost equal to that of wild type.

DISCUSSION

ERp72, a member of the PDI family, has been identified as a luminal ER protein and is believed to participate in the formation and isomerization of disulfide bonds in cellular secretory proteins (Mazzarella et al 1990; Van et al 1993; Rupp et al 1994; Feng et al 1996). Although ERp72 shares the sequence (WCGHCK) responsible for redox activity with other PDI family proteins, the functional roles of the 3 thioredoxin homology domains of ERp72 have not been elucidated. In this study, we characterized the functional properties of the 3 individual thioredoxin homology domains of ERp72 by focusing on the reduction of disulfide bonds in insulin. Presumably, this reflected one of the steps involved in the oxidative folding process in which "nonnative" bonds are reduced by ERp72 before rearrangement.

Kinetic analysis of ERp72 mutants with Cys to Ser mutations at 2 cysteine residues in the individual thioredoxin

homology domains revealed that triple mutants with substitutions in domains 1, 2, and 3 had no insulin reduction activity, indicating that at least one of these thioredoxin homology domains of ERp72 is involved in catalyzing insulin reduction.

Single mutation in domain 1 (intact domains 2 and 3) reduced k_{cat} compared with that of wild type, but hardly altered K_m values, indicating that the active site of domain 1 chiefly contributed to catalytic activity. Single mutation in domain 2 (intact domains 1 and 3) reduced k_{cat} and increased K_m, indicating that the domain 2 active site contributed to both binding affinity for substrate and catalytic activity. Single mutation in domain 3 (intact domains 1 and 2) doubled K_m without altering k_{cat}, indicating that the principal role of the domain 3 active site was apparently to enhance the recognition and binding of substrate in the steady state.

Preservation of intact domain 2 represented by double mutations in domains 1 plus 3 (mut-1+3) and of intact domain 1 represented by double mutations in domains 2 plus 3 (mut-2+3) had 50–60% activity of wild type but showed about 2-fold elevation in K_m, indicating that the active sites of domains 1 and 2 chiefly contributed to catalytic activity but not binding affinity for substrate. Whereas, preservation of intact domain 3 by double mutations in domains 1 plus 2 (mut-1+2) had similar K_m to that of wild type, indicating that domain 3 made the most significant contribution to binding affinity for substrates among domains 1, 2, and 3. These results of assays using single mutation and double mutations indicated in a consistent manner that the active site of domain 1 contributed to catalytic activity and the active site of domain 3 contributed to binding affinity for substrates. However, the results on domain 2 appeared inconsistent between single mutation study and double mutation study.

We interpret the results on domain 2 as follows. Compared with mut-1+2 (intact domain 3), mut-1 (intact domains 2 and 3) elevated K_m, indicating that domain 2 was involved in binding affinity for substrate. However, compared with mut-2+3 (intact domain 1), mut-3 (intact domains 1 and 2) did not elevate K_m, indicating that domain 2 did not contribute to binding affinity for substrate.

Comparison of N- or C-terminal thioredoxin homology unit of PDI and three thioredoxin homology domains of Erp72 amino acid sequences.

PDI N-terminus (27-117)	N- NVLVLKKSNFEEALAAHKYLLVEFYAPWCGHCKALAPEYAKRAAKLKAEGSEIRLAKVDATEESDLAQYGVRGYPTIKFFKNGDTASPKE -C
Erp72 domain 1 (56-145)	N- GVWVLNDGNFDNFVADKDTVLLLEFYAPWCGHCKQFAPEYEKIASTLKDNDPPIAVAKIDATSASMLASKFDVSGYPTIKILKKQAVDYDG -C
Erp72 domain 2 (171-260)	N- VTLSLTAKDNFDDVNNADIILVEFYAPWCGHCKKLAPEYEKAAKELSKRSPPIPLAKVDATEQTDLAKRFDVSGYPTIKIFRKRPFDYNG -C
Erp72 domain 3 (520-609)	N- VKVVVGKTFDAIVMDPKKDVLIIEFYAPWCGHCKQLEPIYISLGKKYKQKDLVIAKMDATANDITNDQYKVEGFPTIYFAPSGDKKNPIKF -C
PDI C-terminus (371-452)	N- VKVLVGANFEEVAFDEKKNVFVEFYAPWCGHCKQLAPIWDKLGLEYKDHENIIIAKMDSTANEVAVKVSFPTLKFFPASA -C
Erp72 domain 1 (56-135)	N- GVWVLNDGNFDNFVADKDTVLLLEFYAPWCGHCKQFAPEYEKIASTLKDNDPPIAVAKIDATSASMLASKFDVSGYPTIKILK -C
Erp72 domain 2 (171-250)	N- VTLSLTAKDNFDDVNNADIILVEFYAPWCGHCKKLAPEYEKAAKELSKRSPPIPLAKVDATEQTDLAKRFDVSGYPTIKIFR -C
Erp72 domain 3 (520-599)	N- VKVVVGKTFDAIVMDPKKDVLIIEFYAPWCGHCKQLEPIYISLGKKYKQKDLVIAKMDATANDITNDQYKVEGFPTIYFAPS -C

Fig 4. Alignment of homologies between protein disulfide isomerase (PDI) N-terminal thioredoxin homology domain and Erp72 thioredoxin domains 1, 2, and 3 (top), as well as homologies between PDI C-terminus and Erp72 thioredoxin domains 1, 2, and 3 (bottom). Residue numbers are in parentheses. Identical residues are shadowed.

Therefore, domain 2 appeared to be involved in binding affinity in a somewhat indirect manner, that is, only in combination with domain 3. Altogether, all 3 domains contributed to catalyzing insulin reduction, and individual domains acted synergistically rather than having simply additive effects.

The PDI has 5 μM of K_m for insulin, and loss of C-terminal cysteines of PDI affects the apparent steady-state binding of substrate as evidenced by a 4-fold increase in K_m (Gilbert 1998). Compared with this, elevation in K_m caused by double mutations in domains 1 plus 3 or domains 2 plus 3 of Erp72 was relatively small (approximately 2-fold), which could be because of the innate low binding affinity for insulin (36.7 μM for wild-type Erp72). Therefore, it is natural to raise a question whether the differences in function among domains of Erp72 have biological significance. We claim that they do, on the basis of the following discussion. First, a decrease in k_{cat} caused by double mutations in domains 1 plus 2 of Erp72 is comparable with that caused by loss of N-terminal cysteines of PDI (30–40% residual activity for N-terminal mutated PDI). Second, there is no information regarding the accurate concentration of ribonuclease (Rnase) or insulin in the ER, whereas the major folding enzymes and chaperones, PDI, BiP/GRP78, GRP94, and Erp72, are known to be present in millimolar concentrations in the ER (Hillson et al 1984; Lyles and Gilbert 1991). Therefore, we consider that if functional differences between thioredoxin domains of PDI have biological significance in disulfide bond formation and isomerization of RNase or insulin *in vivo*, functional differences among individual domains of Erp72 must also be biologically significant *in vivo*. We propose in this study that there may be a transfer of substrate to a low affinity site to allow catalysis and

that binding of substrate to the higher affinity site may cause a decrease in the folding rate.

The manner of action of the thioredoxin homology domains of Erp72 was similar to that of the 2 thioredoxin homology domains of PDI (Lyles and Gilbert 1994). Both thioredoxin homology domains of PDI have been reported to contribute to some extent to K_m and k_{cat} . However, the method of contribution differs in that the C-terminal domain contributes more to K_m and the N-terminal domain contributes more to k_{cat} (Lyles and Gilbert 1994). Figure 4 shows the alignment of homologies between the PDI N- and C-terminal thioredoxin homology domains and the Erp72 thioredoxin homology domains 1, 2, and 3. There is complete conservation of the 11 amino acid-sequence EFYAPWCGHCK in the PDI and Erp72 thioredoxin homology domains, but the homologies of the amino acid sequence preceding or following the 11 amino acid-sequence are very different among the thioredoxin homology domains. Erp72 domains 1 and 2 are more homologous with the PDI N-terminal domain (48% and 51% identical, respectively) when compared with the homology between the Erp72 domain 3 and the PDI N-terminal domain (24% identical). In contrast, Erp72 domain 3 is more homologous with the PDI C-terminal domain (46% identical) when compared with the homologies of Erp72 domains 1 and 2 with the PDI N-terminal domain (26% and 23% identical, respectively). Domains 1 and 2 contributed more to catalyzing efficacy, and domain 3 contributed more to binding affinity. This assignment of function to individual domains is similar to that observed in PDI domains, which is consistent with the high homology between the Erp domains 1 and 2 and the PDI N-terminal domain, and that between the Erp72 domain 3 and the PDI N-terminal domain.

Given the general principle that essentially important sequences that exert fundamental biological functions are strictly conserved through evolution, the amino acid sequences flanking the active site cysteines, which are well conserved between ERp72 and PDI regardless of the very different sequences preceding and following this site, must represent essentially important roles, such as enzymatic catalysis of disulfide bond formation or isomerization and formation of secondary or tertiary domain structures. Further characterization of the thioredoxin homology domains of ERp72 requires future studies on oxidative folding and thiol-dependent isomerization of substrates such as reduced or scrambled RNase.

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REFERENCES

- Feng W, Bedows E, Norton SE, Ruddon RW. 1996. Novel covalent chaperone complexes associated with human chorionic gonadotropin β subunit folding intermediates. *J Biol Chem* 271: 18543–18548.
- Freedman RB, Hawkins HC, McLaughlin SH. 1995. Protein disulfide-isomerase. *Methods Enzymol* 251: 397–406.
- Gething M-J, Sambrook J. 1992. Protein folding in the cell. *Nature* 355: 33–45.
- Gilbert HF. 1998. Protein disulfide isomerase. *Methods Enzymol* 290: 26–50.
- Hammond C, Helenius A. 1995. Quality control in the secretory pathway. *Curr Opin Cell Biol* 7: 523–529.
- Hayano T, Kikuchi M. 1995. Cloning and sequencing of the cDNA encoding human P5. *Gene* 164: 377–378.
- Hillson DA, Lambert N, Freedman RB. 1984. Formation and isomerization of disulfide bonds in proteins: protein disulfide-isomerase. *Methods Enzymol* 107: 281–294.
- Lee AS. 1981. The accumulation of three specific proteins related to glucose-regulated proteins in a temperature-sensitive hamster mutant cell line K12. *J Cell Physiol* 106: 119–125.
- Lenny N, Green M. 1991. Regulation of endoplasmic reticulum stress proteins in COS cells transfected with immunoglobulin mu heavy chain cDNA. *J Biol Chem* 266: 20532–20537.
- Lyles MM, Gilbert HF. 1991. Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. *Biochemistry* 30: 613–619.
- Lyles MM, Gilbert HF. 1994. Mutations in the thioredoxin sites of protein disulfide isomerase reveal functional nonequivalence of the N- and C-terminal domains. *J Biol Chem* 269: 30946–30952.
- Martin J, Pumford N, LaRosa A, Martin B, Gonzaga H, Beaven M, Pohl L. 1991. A metabolite of halothane covalently binds to an endoplasmic reticulum protein that is highly homologous to phosphatidylinositol-specific phospholipase C- α but has no activity. *Biochem Biophys Res Commun* 178: 679–685.
- Mazzarella RA, Marcus N, Haugejorden SM, et al. 1994. Erp61 is GRP58, a stress-inducible luminal endoplasmic reticulum protein, but is devoid of phosphatidylinositol-specific phospholipase C activity. *Arch Biochem Biophys* 308: 454–460.
- Mazzarella RA, Srinivasan M, Haugejorden SM, Green M. 1990. ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase. *J Biol Chem* 265: 1094–1101.
- Otsu M, Urade R, Kito M, Omura F, Kikuchi M. 1995. A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. *J Biol Chem* 270: 14958–14961.
- Pfeffer SR, Rothman JE. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu Rev Biochem* 56: 829–852.
- Rapoport TA, Matlack KE, Plath K, Misselwitz B, Staack O. 1999. Posttranslational protein translocation across the membrane of the endoplasmic reticulum. *Biol Chem* 380: 1143–1150.
- Rupp K, Birmbach U, Lundström J, Van PN, Söling H-D. 1994. Effects of CaBP2, the rat analog of ERp72, and of CaBP1 on the refolding of denatured reduced proteins. *J Biol Chem* 269: 2501–2507.
- Srivastava SP, Chen NQ, Liu YX, Holtzman JL. 1991. Purification and characterization of a new isozyme of thiol: protein-disulfide oxidoreductase from rat hepatic microsomes. Relationship of this isozyme to cytosolic phosphatidylinositol-specific phospholipase C form 1A. *J Biol Chem* 266: 20337–20344.
- Trombetta ES, Parodi AJ. 2003. Quality control and protein folding in the secretory pathway. *Annu Rev Cell Dev Biol* 19: 649–676.
- Van PN, Rupp K, Lampen A, Soling HD. 1993. CaBP2 is a rat homolog of ERp72 with protein disulfide isomerase activity. *Eur J Biochem* 213: 789–795.