Endoplasmic Reticulum Oxidoreductin 1-Lβ (ERO1-Lβ), a Human Gene Induced in the Course of the Unfolded Protein Response*

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Oxidative conditions must be generated in the endoplasmic reticulum (ER) to allow disulfide bond formation in secretory proteins. A family of conserved genes, termed ERO for ER oxidoreductins, plays a key role in this process. We have previously described the human gene ERO1-L, which complements several phenotypic traits of the yeast thermo-sensitive mutant ero1-1 (Cabibbo, A., Pagani, M., Fabbri, M., Rocchi, M., Farmery, M. R., Bulleid, N. J., and Siti, R. (2000) J. Biol. Chem. 275, 4827–4833). Here, we report the cloning and characterization of a novel human member of this family, ERO1-Lβ. Immunofluorescence, endoglycosidase sensitivity, and in vitro translation/translocation assays reveal that the products of the ERO1-Lβ gene are primarily localized in the ER of mammalian cells. The ability to allow growth at 37 °C and to alleviate the “unfolded protein response” when expressed in ero1-1 cells indicates that ERO1-Lβ is involved also in generating oxidative conditions in the ER. ERO1-L and ERO1-Lβ display different tissue distributions. Furthermore, only ERO1-Lβ transcripts are induced in the course of the unfolded protein response. Our results suggest a complex regulation of ER redox homeostasis in mammalian cells.

Proteins destined to the extracellular space and to the organelles of the central vacuolar system translocate into the endoplasmic reticulum (ER). In this organelle, they undergo several post-translational modifications, including folding, assembly, glycosylation, and disulfide bond formation, while being monitored by a stringent quality control system that restricts transport to the Golgi of proteins that have successfully completed their folding and assembly pathways. Proteins that do not fulfill these requirements are selectively retained and eventually dislocated across the ER membrane to be degraded by cytosolic proteasomes. These processes are regulated by a vast array of ER-resident chaperones and enzymes (1, 2).

To build an efficient protein folding machinery within the ER, the synthesis of the individual constituents is likely to be precisely and coordinately regulated. That this is indeed the case is evident during the so-called unfolded protein response (UPR). This highly conserved pathway monitors the levels of unfolded proteins present in the ER (3, 4) and induces the synthesis of ER chaperones and enzymes when the ER protein factory is unable to cope with its products. In yeast, the transmembrane protein kinase Ire1p acts as the sensor for unfolded proteins (5, 6). When these proteins accumulate, such as after treatment with dithiothreitol (DTT) or tunicamycin, Ire1p is autophosphorylated (7). Activated Ire1p catalyzes the spliceosome-independent processing of HAC1 transcripts (8) leading to the production of the transcription factor Hac1p. In turn, Hac1p induces the expression of genes containing the UPR elements in the promoter (9). In mammalian cells there are at least two different Ire1 homologs (10, 11). Both Ire1α and Ire1β undergo proteolysis in response to ER stress, releasing fragments that translocate to the nucleus (12), in a mechanism similar to that previously identified for cholesterol homeostasis (13). Similarly, the N-terminal domain of ATF6, a transmembrane type II glycoprotein, is cleaved following ER stress and translocates into the nucleus where it activates UPR target genes (15).

In bacteria, the formation of disulfide bonds takes place in the periplasmic space; DsbA, a soluble protein, directly donates disulfides to newly translocated cargo proteins. DsbA is regenerated into the active oxidized form by DsbB, an integral membrane protein, in which reoxidation is linked to the respiratory chain (16–18). In eukaryotic cells, oxidative protein folding occurs in the ER (2), and work in yeast suggests that a similar chain of events takes place in this organelle. Proteins enter the ER in the reduced state and rapidly form disulfide bonds, often when the nascent chain is still bound to the ribosome (19). A suitable redox environment is necessary for oxidative protein

UPR, unfolded protein response; bp, base pair(s); SRPR, signal recognition particle receptor; MHC, major histocompatibility complex.
folding, a crucial step in the maturation of many secretory and membrane proteins. Protein disulfide isomerase (PDI) is thought to transfer disulfide bonds to newly made molecules. Covalent complexes between PDI and cargo proteins can be detected in the ER (20, 21). To allow efficient protein folding in the ER, PDI must be rapidly re-oxidized, a step that seems to be accomplished by a family of rather conserved ER oxidoreductins (ERO). In yeast, the ERO1 gene product is an essential N-glycoprotein induced in the course of the UPR. Yeast thermosensitive mutants (e.g. ero1–1) show exaggerated DTT sensitivity and a constitutively activated UPR. At the non-permissive temperature, ero1-1 cells are unable to oxidize carboxypeptidase Y or Gas1p (22, 23). All these observations indicate that Ero1p is directly involved in oxidative protein folding. Accordingly, Ero1p interacts with PDI, possibly recycling it from the reduced to the oxidized form (24). Furthermore, the observations that Ero1p also oxidizes ER glutathione (25), and that the chemical oxidant diamide rescues in part the products of the ero1-1 mutant (24), suggest a role for this protein in generating and maintaining oxidative conditions in the ER. The source from which Ero1p derives oxidizing equivalents has not yet been identified.

Recently, we cloned and characterized a human homolog of ERO1, which we called ERO1-L (ERO1-like). We showed that the products of the ERO1-L gene are able to complement several functions of the endogenous ERO1 in yeast, suggesting the existence of similar oxidative protein folding pathways in human and Saccharomyces cerevisiae (26).

The paucity of ERO1-L transcripts in certain human and murine tissues prompted us to search for additional genes encoding proteins with analogous functions in mammalian cells. Here we report the sequence of a novel ERO1-like gene, ERO1-Lb. Like ERO1-L, which will be referred to hereafter as ERO1-La, ERO1-Lb is able to complement several phenotypic traits of the ero1-1 yeast mutant strain. ERO1-Lb displays a distinct tissue expression. In addition, unlike ERO1-La, ERO1-Lb transcripts accumulate in the course of the UPR.

**EXPERIMENTAL PROCEDURES**

Isolation of Human ERO1-Lb cDNA Clones—ERO1-Lb clones were isolated by screening a cdNA library derived from NT2-D1 human embryonal carcinoma cells differentiated by retinoic acid treatment (Stratagene, GmbH, Heidelberg, Germany, catalog no. 937233). Positive plaques were purified and analyzed by standard procedures (27). The longest clone (clone A2) was entirely sequenced (GenBankTM accession no. AF222538).

ERO1-Lb Expression Vectors—The coding sequence of ERO1-Lb was amplified by polymerase chain reaction with the upstream oligo 1 (GC-

GGATCATGGACAGAGGGCTCGG) and reverse oligo 2 (GACAATCG-

GAGTACTCTGTGTAGAAGGAC). The polymerase chain reaction product containing the whole ERO1-Lb sequence was cloned in pBluescript-II-SK (Stratagene), excised with XhoI-Xhol, and transferred into pcDNA3.1 (Invitrogen, San Diego, CA) or excised with BamHI-Xhol and cloned in pVT-102U (28). ERO1-LbGcn was constructed by replacing the stop codon with a NotI site through site-direct mutagenesis and inserting a NotI fragment encoding three copies of the c-Myc epitope EQKILISEEDLN.

Cell Culture and Transfection, Immunofluorescence, Western Blotting, and Yeast Techniques—These techniques were performed as described by Cabibbo et al. (26). To induce UPR stress, cells were cultured for 6 h in the presence of DTT (2 mM), tunicamycin (10 μg/ml), thapsigargin (2 μM), or ERO1-Lb, which will be referred to hereafter as ERO1-La, ERO1-Lb is able to complement several phenotypic traits of the ero1-1 yeast mutant strain. ERO1-Lb displays a distinct tissue expression. In addition, unlike ERO1-La, ERO1-Lb transcripts accumulate in the course of the UPR.

Northern blot analysis was performed using a 35S-labeled ERO1-La or ERO1-Lb resuspended in 15 μl of 0.1 M Tris, pH 8, 1% SDS, 1% mercaptoethanol and boiled for 5 min. In some instances, 4 μl of fresh 2-mercaptoethanol was added to each lane. ERO1-Lb was detected by autoradiography. For proteins, ERO1-Lb digests, cells from a translation reaction were isolated by centrifugation, washed in KHM buffer (110 mM sodium phosphate (pH 7.0), 10 mM KCl, 1 mM MgSO4, 20 mM β-mercaptoethanol), resuspended in 220 μl of lysis buffer (150 mM of Z buffer, 50 μM of CHCl3, and 20 μl of 0.1% SDS), and vortexed for 15 s. β-Galactosidase activity was determined by adding 700 μl of substrate solution (1 mg/ml o-nitrophenyl-β-d-galactoside in Z buffer). The reaction was stopped by adding 500 μl of 1 M Na2CO3, and the absorbance at 420 nm was measured. Units of activity were defined as follows: 1 unit = (A420 x 1.42)/(A00 x π x 0.0045).

Transcription and Translation in Vitro—Transcription reactions were carried out as described (30). Plasmids pCDNA3.1 ERO1-La and pCDNA3.1ERO1-Lb were linearized with BstEII and transcribed using T7 RNA polymerase (Promega, Southampton, UK). Plasmid pCR.pCR3, encoding for human calreticulin, a kind gift from David Llywellyn (University of Wales, Cardiff, UK) was linearized with NotI and transcribed using T7 RNA polymerase. Reactions (50 μl) were incubated at 37 °C for 10 min followed by chloroform extraction and ethanol precipitation. RNA was resuspended in 50 μl of RNase-free water containing 0.5 mM DTT and 40 units of RNasin (Promega).

mRNAs were translated using a rabbit reticulocyte lysate (FLEXiLy-
sate, Promega) for 60 min at 30 °C. The translation reactions (25 μl each) contained 16.5 μl of reticulocyte lysate, 0.6 μl of 100 mM KCl, 0.5 μl of 1 mM amino acids (minus methionine), 15 μl of 0.1% [35S]methionine (New England Science Products) in 1 μl of in vitro transcribed RNA and, in some instances, 4 μl of semi-permeabilized HT1080 human cells prepared as described previously (30).

Endoglycosidase H and Proteinase K Treatments—To analyze the glycosylation status of the translated products, cell pellets containing [35S]-labeled ERO1-La or ERO1-Lb were resuspended in 15 μl of 0.1 M Tris, pH 8, 1% SDS, 1% mercaptoethanol and boiled for 5 min. In some instances, 4 μl of fresh 2-mercaptoethanol was added to each lane. ERO1-Lb was detected by autoradiography. For proteins, ERO1-Lb digests, cells from a translation reaction were isolated by centrifugation, washed in KHM buffer (110 mM sodium phosphate (pH 7.5) and 150 mM of KAc, 2.5 mM MgAc2 at pH 11 or 13. After a 10 min incubation at room temperature, the samples were loaded on a 15% SDS-PAGE, and visualized using a Fujix Bas 2000 Bioimager.

**RESULTS**

Two Distinct ERO1-like Genes Are Present in Both Human and Mouse—We have recently described the isolation of ERO1-L, a human gene that is likely to be involved in disulfide bond formation in the ER (26). The ERO1-L sequence was used
to perform a survey of the Expressed Sequence Tag division of GenBank™; this led to the identification of a human sequence very similar to, but distinct from, ERO1-L, which was used as a probe to screen a human cDNA library. Among several isolates, a clone was identified that contains an open reading frame encoding a putative protein of 467 residues (Fig. 1).

In consideration of the strong sequence conservation in ERO1-L, the protein was named ERO1-Lb. We will therefore refer to the previously identified ERO1-L (26) as ERO1-La.

The ERO1-Lb gene product shows 50.6 and 74.8% similarity with and 40.2 and 65.4% identity to yeast Ero1p and ERO1-La, respectively. Particularly conserved are the CXXCXXC motif, essential for ERO1-La function in the ero1-1 complementation assay (26) and the "TALK box" located downstream. The greatest differences between the two human sequences are located in the N-terminal region, predicted for both genes to encode for signal sequences for ER translocation. ERO1-Lb contains four N-linked glycosylation sites and, in contrast to ERO1-La, does not contain an EF calcium binding motif.

EROI-Lb Encodes an ER Resident N-Glycoprotein—To determine the intracellular localization of the ERO1-Lb gene products in mammalian cells, the human open reading frame was cloned in the expression vector pCDNA 3.1, and suitable tags were introduced at the C terminus to allow serological identification of the transgene.

Immunofluorescence analyses revealed that ERO1-Lb co-localizes with PDI and calnexin, two ER resident proteins, in both COS-7 (Fig. 2A) and HeLa cells (not shown), indicating that ERO1-Lbmyc accumulates in the ER. To confirm this hypothesis, aliquots from the lysates of transfected cells were digested with endoglycosidase H. A mobility shift of about 6 kDa was observed after treatment, suggesting that ERO1-Lbmyc is a N-glycoprotein and its sugars are not processed by the Golgi enzymes (Fig. 2B). Consistent with a higher number of potential glycosylation sites in ERO1-Lb than in ERO1-La (4 and 2, respectively, Fig. 1), the mobility shift is more pronounced in the former.

The ability of ERO1-Lb to be inserted into ER membranes was also assessed in vitro in a semi-permeabilized (SP) cell system (30). ERO1-La and ERO1-Lb were translated in the absence (Fig. 2C, lanes 1 and 8) or presence (lanes 2–7 and 9–14) of SP-HT1080 cells. Upon the addition of cells, a molecular weight (Mw) shift of the translated bands is apparent, which can be reversed by endoglycosidase treatment (lanes 7 and 14). The glycosylated isosform is resistant to proteinase K unless detergent is added (lanes 3–4 and 10–11). Altogether, these data indicate that, when translated in the presence of SP cells, both ERO1-La and ERO1-Lb are inserted into ER membranes and become glycosylated. Also, in this assay, the mobility shift induced by deglycosylation is greater for ERO1-Lb than for ERO1-La. As previously observed for ERO1-La translated in the presence of canine microsomal membranes (26), the electrophoretic mobility of the translocated and deglycosylated ERO1-La is undistinguishable from that of the protein synthesized in the absence of membranes. This seems to be the case also for ERO1-Lb, suggesting that the leader peptides of the two human ERO1-L genes might not undergo cleavage.

Both ERO1-La and -b Are Soluble ER Proteins—We had previously shown (26) that upon translation in the presence of canine pancreatic microsomes and extraction of isolated microsomes with a carbonate buffer, ERO1-La could not be extracted from the membranes, apparently behaving as an integral membrane protein. To have further insights on the association between ERO1 proteins and the ER membranes, an alternative
FIG. 2. Like ERO1-Lα, ERO1-Lβ is a N-glycoprotein localized to the ER. A, ERO1-Lβ is localized in the ER. 48 h after transfection with pCDNA3.1-ERO1-Lβ, COS-7 cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and co-stained with mouse anti-myc and rabbit anti-calnexin or rabbit anti-PDI, as indicated, followed by fluoresceinated goat anti-mouse Ig and rhodaminated goat anti-rabbit Ig. B, ERO1-Lβ carries endoglycosidase H-sensitive glycans. 48 h after transfection with pCDNA3.1-ERO1-Lα, pCDNA3.1-ERO1-Lβ, or an empty vector (mock), COS-7 or HeLa cells were lysed in Nonidet P-40. Aliquots corresponding to 20 μg of total protein were treated with or without endoglycosidase H (Endo-H), resolved by SDS-PAGE under reducing conditions, and transferred to nitrocellulose filters. Blots were decorated with anti-myc antibodies and processed for ECL. C, like ERO1-Lα, ERO1-Lβ is glycosylated and protected from proteinase digestion when translated in the presence of semi-permeable cells. In vitro translated RNAs encoding ERO1-Lα (lanes 1–7) or ERO1-Lβ (lanes 8–14) were translated for 1 h at 30 °C in the presence (lanes 2–4, 6, 7, 9–11, 13, and 14) or absence (lanes 1, 5, 8, and 12) of semi-permeable HT1080 cells (SP-cells). Samples were treated with proteinase K in the presence (lanes 3, 5, and 7) or absence (lanes 4 and 11) or of Triton X-100 (lanes 3 and 10) or with Endo H (lanes 7 and 14) and resolved by SDS-PAGE. Gels were dried and processed for fluorography. Closed arrows indicate the mobility of glycosylated, protease-protected translation products, and open arrows point to non-translated products.

approach was used. ERO1-Lα and ERO1-Lβ were translated in vitro in the presence of human SP cells. After translation, cells were isolated, washed, and resuspended in 30 mM Hepes, 150 mM KAc, 2.5 mM MgAc2 buffer at alkaline pH. Each sample was then fractionated on a 25 μm sucrose gradient that reflected the composition and pH of the buffer used during extraction. As controls, we utilized calreticulin, a soluble ER protein, and MHC class I, an integral membrane protein.

Miller et al. (31) demonstrated that the signal recognition particle receptor α (SRPα) subunit is anchored to membranes by a strong protein–protein interaction with the SRPβ subunit. As a result, SRPα is only partially extracted from membranes at pH 11, whereas a complete extraction is achieved at pH 13. Conversely, pH 13 is unable to extract transmembrane proteins such as SRPβ. To further clarify the issue of ERO1-Lα and -Lβ membrane association, extraction experiments were performed at both pH 11 and 13.

Fig. 3A shows that at pH 11 the treatments used fail to extract MHC class I, which is entirely recovered in the membrane pellet upon sucrose fractionation. Calreticulin, a soluble protein, is preferentially recovered in the supernatant (lane 3), although approximately 30–40% of the material remains in the pellet (lane 2). These membrane-associated calreticulin molecules might be interacting with membrane glycoproteins, although this remains to be established. ERO1-Lα and ERO1-Lβ behave in a comparable way to calreticulin.

Although pH 13 does not affect the partitioning of MHC class I, the membrane association of calreticulin, ERO1-Lα, and ERO1-Lβ is nearly completely abolished under these conditions (Fig. 3A, lane 4). The recovery of MHC class I in the pellet is not because of aggregation, as the presence of Triton X-100 in the extraction buffer completely solubilized MHC molecules (Fig. 3B, lane 5).

These results indicate that both ERO1-Lα and ERO1-Lβ are soluble proteins and suggest that ERO1-Lβ establishes strong interactions with some component of ER membranes. Understanding the details of these interactions will require further experimental work.

ERO1-Lβ Complements the Yeast Mutant ero1-1—To assess whether human ERO1-Lβ is a functional homolog of the S. cerevisiae Ero1p, we expressed this protein in the mutant ero1-1 (CKY559). This strain is unable to grow at 37 °C and...
The expression of ERO1-Lβ allowed the growth of the mutant ero1-1 at 37 °C (Fig. 4A), suggesting that the human protein is able to replace the function of Ero1p in yeast. Next, the effects of the two human genes on the S. cerevisiae UPR pathway were assessed. ERO1-Lα was almost as active as the yeast gene in this assay, ERO1-Lβ also significantly reduced the UPR element-dependent β-galactosidase transcription, albeit less efficiently than ERO1 or ERO1-Lα. These findings indicate that both of the human genes are able to complement partially the ero1-1 defect. The lower efficiency of ERO1-Lβ in the UPR assay correlated with the fact that reduced amounts of the ERO1-Lβ were found to accumulate in the secretory pathway of yeast cells.2

Analysis of ERO1-L Gene Expression in Human Tissues—The above findings indicated that at least two genes exist in humans that share the capability of complementing a yeast mutant strain defective in the generation of oxidative conditions in the ER. It is possible that some functional specialization(s) hallmarks the two genes. We therefore investigated their transcriptional regulation patterns.

To this end, a filter containing different human tissues and cell lines (Multiple Tissue Expression Array, CLONTECH) was sequentially hybridized with probes for ERO1-Lβ and ERO1-Lα. The housekeeping gene ubiquitin was used as a control (Fig. 5). Clearly, ERO1-Lα and ERO1-Lβ transcripts display a different but partially overlapping tissue distribution. The two genes show a curious distribution in the upper digestive tract, ERO1-Lα being abundant in the esophagus but much less so in the stomach and duodenum. Conversely, ERO1-Lβ transcripts are abundant in the stomach and duodenum but barely detectable in the esophagus. Other tissues rich in ERO1-Lβ transcripts are the pancreas, testis, liver, appendix, thyroid, and pituitary gland.

ERO1-Lβ Expression Is Induced by the UPR Pathway in Mammalian Cells—A feature of ERO1 in yeast is its increased expression in the course of the UPR. We therefore sought to determine whether the transcription of ERO1-Lα and ERO1-Lβ is also modulated during the UPR. Three cell lines (U937, COS-7, and 293-T) were treated for 6 h with tunicamycin and DTT, two drugs that induce a robust UPR by preventing N-linked glycosylation and disulfide bond formation, respectively (Figs. 6, A and B). Although ERO1-Lβ transcripts are induced by both tunicamycin and DTT, neither treatment significantly modulates the levels of ERO1-Lα or ERO1-Lβ.

To further dissect the regulation of ERO1-Lβ in different cellular stress conditions, 293-T cells were treated with different UPR-inducing agents (tunicamycin, DTT, thapsigargin, A23187, EGTA, and deoxyglucose) or compounds that causes cellular stress (serum deprivation, heat shock, and UV irradiation) but do not up-regulate UPR genes (Fig. 6C). Although the levels of ERO1-Lβ transcripts increased with all of the UPR-inducing treatments, other cellular stresses did not significantly modulate its expression.

DISCUSSION

In all eukaryotic cells, oxidizing conditions must be generated and maintained within the ER to allow disulfide bond formation in proteins destined to the extracellular space. In S. cerevisiae, this function involves Ero1p, an essential ER resident protein (22–25). Ero1p homologs are present in most species (Fig. 1), including Mus musculus, Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, and Schizosaccharomyces pombe (22, 23, 26), defining the existence of a family of genes that are likely to be functionally related.

Although a single ERO1 gene is present in S. cerevisiae, the results presented in this study reveal that at least two members of this family exist in Homo sapiens. The sequences of the two human genes are very similar, suggesting a conserved function between these two proteins. Our data show that in cells transfected with either ERO1-Lαmyc or ERO1-Lβmyc (Fig. 2), the staining patterns obtained with anti-myc largely overlap with the distribution of calnexin and PDI, two ER resident proteins. Preliminary results obtained with polyclonal antibodies specific for ERO1-Lα or ERO1-Lβ suggest also that the endogenous gene products are localized to the ER.

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2 M. Pagani, S. Pilati, and R. Sitia, unpublished observations.
The similarity between ERO1-\(\alpha\) and ERO1-\(\beta\) is evident also from the functional viewpoint. Both genes are able to complement the growth of the yeast mutant \(ero1-1\) at the non-permissive temperature and to alleviate the constitutive UPR that characterizes this strain (Fig. 4). ERO1-\(\beta\) appeared to be less efficient than ERO1-\(\alpha\) in the latter assay as well as in attenuating DTT sensitivity or restoring carboxypeptidase Y oxidative folding in the \(ero1-1\) mutant (data not shown). This finding might be explained in part by the observation that lower levels of glycosylated ERO1-\(\beta\) accumulated in yeast transformants, suggesting an inefficient translocation of this human protein in the yeast ER. Nonetheless, although a quantitative functional comparison of the two genes is not possible at present, it appears that both ERO1-\(\alpha\) and ERO1-\(\beta\) are capable of generating oxidizing conditions in the ER of yeast cells.

Despite their overall similarities in sequence and function, important differences between ERO1-\(\alpha\) and ERO1-\(\beta\) are evident when their transcriptional patterns are compared. First, our studies identify ERO1-\(\beta\) as a novel member of the UPR family in human cells. In this respect, ERO1-\(\beta\) transcripts are induced by tunicamycin, DTT, thapsigargin, and other treatments known to activate the UPR but not by other stresses such as serum deprivation or exposure to UV. In contrast, the abundance of ERO1-\(\alpha\) transcripts is not significantly modulated by any of the above treatments.

Second, the expression of ERO1-\(\alpha\) and ERO1-\(\beta\) shows remarkable variations in different tissues. ERO1-\(\beta\) transcripts are quite abundant in secretory tissues (such as the pancreas and salivary gland) in which the exocytic pathway is well developed to cope with the production and export of proteins destined to the extracellular space. It remains to be seen whether the expression of ERO1-\(\beta\) in these tissues correlates with an ongoing UPR.

How do ERO1-\(\alpha\) and -\(\beta\) exert their function? In yeast, disulfide-linked heterodimers between PDI and Ero1p can be detected, which might represent a transient intermediate of the reaction in which Ero1p oxidizes PDI (24). We have re-
cently obtained evidence that human ERO1-\(\alpha\)\(^3\) and ERO1-\(\beta\)\(^4\) also covalently bind PDI. Hence, at least some players and key molecular events involved in disulfide bond formation appear to be conserved from yeast to humans. In bacteria, disulfide bonds are transferred from DsbB to DsbA to “cargo” proteins in the periplasmic space. DsbB ultimately donates electrons to the respiratory chain, an observation that may explain how the periplasmic space. DsbB ultimately donates electrons to the respiratory chain, an observation that may explain how the periplasmic space.

3 A. M. Benham, A. Cabibbo, A. Fassio, N. Bulleid, R. Sitia, and I. Braakman, submitted for publication.

4 A. Fassio, A. Mezghrani, and R. Sitia, unpublished results.

The latter served as a control for sample abundance in individual wells. B, the Northern blots shown in panel A were quantitated by Phosphor-Imager. For each treatment, ERO1-\(\alpha\), ERO1-\(\beta\), and BiP mRNAs were first normalized against the corresponding GAPDH mRNA levels. The levels of ERO1-\(\alpha\), ERO1-\(\beta\), and BiP (black, gray, and white bars, respectively) transcripts are expressed as fold of induction relative to untreated controls (mRNA arbitrary units). Tm, tunicamycin. C, total cellular RNA was extracted from 293-T cells treated for 6 h with the indicated drugs. Blots containing 20 \(\mu\)g of RNA were hybridized with probes specific for ERO1-\(\alpha\), ERO1-\(\beta\), and BiP (black, gray, and white bars, respectively). Transcripts are expressed as fold of induction relative to untreated controls (mRNA arbitrary units). Tm, tunicamycin. C, total cellular RNA was extracted from 293-T cells treated for 6 h with the indicated drugs. Blots containing 20 \(\mu\)g of RNA were hybridized with probes specific for ERO1-\(\alpha\), ERO1-\(\beta\), and BiP (black, gray, and white bars, respectively). Transcripts are expressed as fold of induction relative to untreated controls (mRNA arbitrary units). Tm, tunicamycin.

In conclusion, we report herein the finding that the Ero1p function, essential in S. cerevisiae for the formation of disulfides in secretory proteins, is at least duplicated in humans. The two genes have different tissue distributions and appear to be differently regulated, with only ERO1-\(\beta\) being up-regulated during ER stress. Despite these divergences, their function is at least partially conserved and related to disulfide bond formation, as both gene products are able to reverse phenotypic aspects of Ero1p deficiency in S. cerevisiae. The precise role of ERO1-\(\alpha\) and ERO1-\(\beta\) in the biochemical pathway that leads to disulfide bond formation in the mammalian ER is currently under investigation.

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rather divergent in terms of sequence. It remains to be seen whether members of the ERO1 family can complement DsbB mutants.

If the basic chain of events is similar, an important difference is evident when the topology of these crucial molecules is compared. Thus, although DsbB is an integral membrane protein, the products of ERO1-\(\alpha\) and ERO1-\(\beta\) can be extracted from membranes in SP cells at alkaline pH, a characteristic of soluble or peripheral membrane proteins. Previous experiments performed on canine microsomes (26) suggested a type II topology for ERO1-\(\alpha\). In those experiments, we demonstrated that when microsomes were extracted at pH 11, ERO1-\(\alpha\) stayed in the pellet fraction. In the experiments presented in Fig. 3, both ERO1-\(\alpha\) and ERO1-\(\beta\) codistribute with calreticulin and therefore behave as soluble or peripheral membrane proteins. In these experiments, a fraction of both polypeptides was recovered in the pellet at pH 11, as demonstrated previously in microsomes, perhaps reflecting residual interactions with membrane elements. These interactions are almost completely abrogated at pH 13, a treatment that fails to extract integral membrane molecules such as MHC class I heavy chains. Taken together, these findings suggest that neither ERO1-\(\alpha\) nor ERO1-\(\beta\) are inserted in the ER membrane. In agreement with this conclusion, traces of ERO1-\(\alpha\) are found in the supernatants of HeLa transient transfectants overexpressing the protein. The extracellular form displays slower electrophoretic mobility.\(^{3, 4}\)

Soluble ER resident proteins generally possess a C-terminal KDEL motif (35). As neither ERO1-\(\alpha\) nor ERO1-\(\beta\) displays known ER localization motifs, the question arises as to how they maintain their subcellular localization. Both ERO1-\(\alpha\) and ERO1-\(\beta\) establish intermolecular interactions with ER resident proteins, including PDI.\(^{3, 4}\) These interactions may be important for determining the localization of a functional complex in the ER. Deletion and mutagenesis experiments are in progress in the attempt to verify the mechanisms of ERO1-\(\alpha\) and ERO1-\(\beta\) localization. In conclusion, we report herein the finding that the Ero1p function, essential in S. cerevisiae for the formation of disulfides in secretory proteins, is at least duplicated in humans. The two genes have different tissue distributions and appear to be differently regulated, with only ERO1-\(\beta\) being up-regulated during ER stress. Despite these divergences, their function is at least partially conserved and related to disulfide bond formation, as both gene products are able to reverse phenotypic aspects of Ero1p deficiency in S. cerevisiae. The precise role of ERO1-\(\alpha\) and ERO1-\(\beta\) in the biochemical pathway that leads to disulfide bond formation in the mammalian ER is currently under investigation.

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