Tissue-specific Expression and Dimerization of the Endoplasmic Reticulum Oxidoreductase Ero1β*

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Endoplasmic reticulum oxidoreductases (Eros) are essential for the formation of disulfide bonds. Understanding disulfide bond catalysis in mammals is important because of the involvement of protein misfolding in conditions such as diabetes, arthritis, cancer, and aging. Mammals express two related Ero proteins, Ero1α and Ero1β. Ero1β is incompletely characterized but is of physiological interest because it is induced by the unfolded protein response. Here, we show that Ero1β can form homodimers and mixed heterodimers with Ero1α, in addition to Ero-PDI dimers. Ero-Ero dimers require the Ero active site, occur in vivo, and can be modeled onto the Ero1p crystal structure. Our data indicate that the Ero1β protein is constitutively strongly expressed in the stomach and the pancreas, but in a cell-specific fashion. In the stomach, selective expression of Ero1β occurs in the enzyme-producing chief cells. In pancreatic islets, Ero1β expression is high, but is inversely correlated with PDI and PDIp levels, demonstrating that cell-specific differences exist in the regulation of oxidative protein folding in vivo.

Disulfide bond formation is an essential component of the protein folding process, and disulfide bonds are required for structural stability, enzymatic function, and regulation of protein activity (7). The catalytic events involving the oxidation, reduction, and isomerization of disulfide bonds take place in the ER. During protein oxidation, PDI introduces native disulfide bonds into substrate proteins, and is reoxidized by the Ero proteins (Ero1p in yeast, Ero1α and Ero1β in humans) (8–11). In yeast, Pdi1p is capable of both oxidizing and isomerizing disulfide bonds, although the relative importance of each function has been debated (12). In humans, PDI also contributes to collagen biosynthesis as a component of the prolyl-4-hydroxylase complex (13) and can act as a component of the ER degradation machinery, particularly with respect to the unfolding and retro-translocation of toxins (14). Numerous PDI homologues exist in yeast (Mpd1p, Mpd2p, Eps1p, and Eug1p) (15) and in humans (e.g. ERp57, ERp72, P5, PD1R, and PDIp) (16). The redundancy of these proteins has made their precise functions difficult to analyze, but ERp57 at least has a specialized b-domain that selectively allows this PDI homologue to interact with the lectin-like ER chaperones calnexin and calreticulin (17).

PDI is the only human protein with an intact WCGHC motif that can be trapped in association with Eros (18, 19), although Ero1α can form complexes with the unconventional PDI family members ERp44 (20) and PDILT (21). In yeast, Mpd1p and Mpd2p both interact with Ero1p (22). The crystal structure of the Ero1p core revealed that the N-terminal CXXXC motif of the protein is likely to transfer electrons to the latter two residues of the C-terminal CXXCXC motif, in close proximity to the isoalloxazine ring of FAD (23). Although co-crystals of Ero1p and Pdi1p are not available, it has been demonstrated biochemically that Pdi1p/PDI binding is disulfide-dependent and requires the N-terminal CXXXC motif of Ero1p/Ero1α (24, 25). A supply of reduced glutathione is also required for appropriate oxidative protein folding in the ER (26, 27).

In humans, Ero1α and Ero1β are up-regulated by hypoxia (28) and the UPR (11), respectively, suggesting that the two proteins have undergone functional specialization in response to the different demands of oxygen tension and high throughput protein folding. However, the relationship between Ero mRNA and protein levels, and the relationship between UPR induction of Ero1β by a chemical versus a physiological (nutritional) stimulus is not known. Cells in culture generally express low levels of Ero proteins. The assumption is that low expression of an (nutritional) stimulus is not known. Cells in culture generally express low levels of Ero proteins. The assumption is that low expression of an

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5 The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; TBS, Tris-buffered saline; MES, 4-morpholineethanesulfonic acid; NEM, N-ε-ethylmaleimide; DTT, dithiothreitol; HA, hemagglutinin; OX, oxidized form; R, reduced form.

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Endoplasmic reticulum oxidoreductases (Eros) are essential for the formation of disulfide bonds. Understanding disulfide bond catalysis in mammals is important because of the involvement of protein misfolding in conditions such as diabetes, arthritis, cancer, and aging. Mammals express two related Ero proteins, Ero1α and Ero1β. Ero1β is incompletely characterized but is of physiological interest because it is induced by the unfolded protein response. Here, we show that Ero1β can form homodimers and mixed heterodimers with Ero1α, in addition to Ero-PDI dimers. Ero-Ero dimers require the Ero active site, occur in vivo, and can be modeled onto the Ero1p crystal structure. Our data indicate that the Ero1β protein is constitutively strongly expressed in the stomach and the pancreas, but in a cell-specific fashion. In the stomach, selective expression of Ero1β occurs in the enzyme-producing chief cells. In pancreatic islets, Ero1β expression is high, but is inversely correlated with PDI and PDIp levels, demonstrating that cell-specific differences exist in the regulation of oxidative protein folding in vivo.
groups of enzyme-producing cells, notably the chief cells in the stomach, and the insulin- and enzyme-producing cells of the pancreas. Constitutively high expression of Ero1β is differentially correlated to PDI expression in professional secretory cells in vivo. At a molecular level, both Ero1α and Ero1β do more than simply engage PDI. Ero1β can form alkylation-independent homodimers, which require Cys<sup>396</sup> of the CXXCXXC active site, and Ero1β complexes are more abundant in the pancreas than the stomach. Our biochemical analysis is supported by the Ero1p crystal structure, which can be modeled as a symmetrical dimer in which interfacial FAD-FAD contacts are prominent. Our results show that the ER oxidation machinery is more complex than anticipated in mammals, demonstrating how disulfide bond formation could be regulated in vivo through differential complex formation.

### Experimental Procedures

**Cell Lines, Antibodies, and Tissues**—Human cervical carcinoma HeLa cells were maintained in minimal essential medium (Invitrogen), and human fibrosarcoma HT1080 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), both supplemented with 8% fetal calf serum (Sigma), 2 mM Glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 5% CO<sub>2</sub>. The polyclonal antiserum against Ero1β was raised against the unique internal Ero1β peptide YTGNAAEADKTTL (Sigma Genosys). Thirteen of the fifteen residues were conserved between mouse and human Ero1β. The polyclonal rabbit anti-sera against PDI, Ero1α (D5), and ERP57 have been described previously (18). The polyclonal PDPl antisera was a gift from M. Lan (29). Polyclonal antiseras against BiP and insulin (H-86) (both Santa Cruz Biotechnology), the mouse monoclonal antibody HA-7 (Sigma), and the anti-myc antibody 9B11 and the anti-myc polyclonal antibody (both Cell Signaling) were commercially available. Normal goat serum was obtained from DAKO, and normal rabbit serum was obtained from a pre-immune rabbit used to generate the PDI antisera. Ero1β antisera was a kind gift from Roberto Sitia.

**Transfections**—Transfections with Lipofectamine 2000 (Invitrogen) were performed according to the manufacturer’s instructions. Sub-confluent cells in 6-cm dishes were washed twice with phosphate-buffered saline (Invitrogen), and transfected with 1 or 2 μg DNA for 6 h in the presence of Opti-Mem serum-free medium (Invitrogen). The medium was replaced after 6 h with complete medium, and the cells were analyzed 24 h post-transfection.

**Construction of Ero1β Mutants**—The wild-type pcDNAEro1β-HA and pcDNAEro1β-myc constructs were a kind gift from Roberto Sita. Site-directed mutagenesis (QuikChange, Stratagene) was used to create CXXCXXC mutants with the following forward primers: C390A, GA-ATATCCTCCGTAATTGACGGTCGCCGATTGACAAATGTGCA; C393A, GTATAGTGGAGGATTGACGGTGGCAAAAATATGCAGGATTATGGGG; and C396A, GACTGTGGTTGGATGGCACAAGCCAGATATGGGGAAATTCAG. Each construct was verified by DNA sequencing. The Ero1α-myc tagged constructs have been previously described (18).

**Gel Filtration**—Gel filtration was performed using a Superdex 200 Precision Column 3.2/30 (Amersham Biosciences/GE Healthcare). Chicken egg albumin (45 kDa) and bovine serum albumin (66 kDa and 132 kDa) (both Sigma) were used as standards. Proteins and mouse tissue lysates injected in a volume of 140 μl were eluted with 50 mM Tris-HCl, 150 mM NaCl, pH 7.0, at a flow rate of 0.5 ml min<sup>-1</sup>, with samples collected every 2 min. Trichloroacetic acid-precipitated fractions were neutralized with Tris and taken up in sample buffer, and equal volumes were analyzed by reducing SDS-PAGE.

**Immunohistochemistry**—Human tissues were sectioned at 4-μm thickness onto Ape's coated slides and placed at 60 °C for 1 h. The slides were cleared through xylene and 100–70% alcohol before blocking endogenous peroxidase activity in methanol peroxidase for 10 min. The slides were washed in cold water, and the optimal digestion technique for each tissue type was determined: for stomach tissue, microwave digestion was in 600 ml of citrate buffer (pH 6.0) for 20 min; for pancreatic tissue, pressure cooker digestion was in 3 liters of citrate buffer (pH 6.0) for 2 min. The slides were washed in tap water and then in TBS (pH 7.4) for 5 min. Tissue sections were blocked in 1% normal goat serum (DAKO) for 10 min before incubating with the optimum dilution of primary antibody in TBS (PDI and PDPl, 1:1000; Ero1β, 1:190; Bp 1:40; and ERP57 and H-86, 1:200) at 4 °C overnight. The sections were washed for 5 min in TBS, and 0.01% biotinylated goat anti-mouse/rabbit antibodies (DAKO) diluted in TBS was added for 30 min. The sections were developed using streptavidin AB complex horseradish peroxidase (DAKO) and 3,3-diaminobenzidine. Counterstaining was achieved with hematoxylin.

**Western Blotting**—Cells or fresh mouse tissues were lysed in lysis buffer (20 mM MES, 30 mM Tris, 100 mM NaCl, pH 7.4) with 1% Triton X-100, supplemented with 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A and 20 mM NEM where required. Nuclei were removed by centrifugation at 16,100 × g for 10 min at 4 °C. Post-nuclear cell lysates, immunoprecipitates, or comparable amounts of mouse tissue lysates were taken up in sample buffer, boiled, and analyzed by SDS-PAGE. When non-reducing analysis was needed, DTT was left out of the sample buffer. Immunoprecipitations were carried out using 1 μl of anti-HA or anti-myc antibodies immobilized on 50 μl of a 20% suspension of Protein A-Sepharose beads for 2 h, followed by washing twice with lysis buffer. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) at 150 mA for 2 h or 30 V overnight, and the membranes were blocked in 8% milk/phosphate-buffered saline (PBS) for 1 h overnight. The primary antibodies were used at 1:5,000 (α-myc and α-HA), 1:1,000 (αEro1α/D5 and αPDPl), and 1:50 (αEro1β). After washing six times with phosphate-buffered saline, membrane blots were incubated with corresponding secondary antibodies (DAKO) at 1:3,000 for 1 h, washed extensively, and visualized by ECL (Amersham Biosciences) and exposure to film (Eastman Kodak Co.). Protein markers were from Bio-Rad. Determination of protein concentration of the tissue samples prior to gel loading was carried out by using the Bradford assay (Bio-Rad), with samples equalized to 1.8 μg/ml. Each blotting experiment was reproduced at least twice.

### RESULTS

**Ero1β Proteins Are Highly Expressed in Stomach and Pancreas**—Despite much interest in ER oxidoreductases, little is known about the expression of Ero proteins in mammalian tissues. Most work has concentrated on the relationship between Eros and PDI and the mechanism of electron transfer to molecular oxygen. The mammalian cell lines thus far examined have low expression of Eros, making study of endogenous protein behavior in tissue culture difficult. Given that studies at the mRNA level suggest that Ero1β transcripts are high in stomach and pancreas (11), we investigated whether Ero1β proteins could be detected in these mouse tissues. To do this, we raised a polyclonal antibody against an internal peptide unique to Ero1β. To verify that the antisera recognized Ero1β, HeLa cells were mock transfected or transfected with Ero1α-myc, Ero1β-myc, Ero1β-HA, or both Ero1β-HA and Ero1β-myc. The tagged proteins were only detected in
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The relevant individual transfectants (Fig. 1, A and B, lanes 1 and 2) and in the co-transfectant (Fig. 1, A and B, lane 3). The Ero1β serum specifically detected both tagged Ero1β forms (Fig. 1C, lanes 2–4), and no Ero1β could be detected in non-transfected HeLa cells (Fig. 1C, lane 1). Ero1α was not detected by the Ero1β serum, demonstrating that the serum did not cross-react with Ero1α (Fig. 1C, lane 1). As a positive control, Ero1α could be detected by D5 when the membrane from Fig. 1C was reprobed (without stripping the blot) with the specific polyclonal Ero1α serum (Fig. 1D, lane 1). Ero1β-HA migrated faster than Ero1β-myc because of size/charge differences in the C-terminal tag. Tagged Ero1β proteins can restore viability to yeast Ero1p ts mutants (11) and support oxidative protein folding of model substrates (19), demonstrating that tagging does not affect Ero1β function.

Having established the specificity of our Ero1β antibody, we then asked whether Ero1β expression could be detected in mouse tissues. Samples of mouse tissues were lysed and clarified by centrifugation, and equal amounts of protein were analyzed by Western blotting. Pancreas and stomach (Fig. 1E, lanes 1 and 4) expressed high levels of Ero1β, whereas liver, small intestine, kidney, and heart did not (Fig. 1E, lanes 2, 3, 5, and 6). Unlike Ero1β, PDI was expressed in all tissues (Fig. 1F). We conclude that our antibody specifically detected Ero1β and that Ero1β expression was strikingly high in a restricted range of tissues.

Ero1β, PDI, Erp57, and BiP Are Highly and Specifically Expressed in the Chief Cells of the Stomach—We further investigated Ero1β expression in the digestive system by using immunohistochemistry. Human stomach tissue sections (blocked with normal goat serum) were negative when primary antibody was omitted (Fig. 2A) and sections exposed to pre-immune rabbit sera instead of primary antibody gave a faint nonspecific staining (Fig. 2B). However, sections probed with the Ero1β antiserum showed very strong, specific staining in the basal area of the stomach (Fig. 2C). Higher magnification showed that Ero1β had a patchwork distribution, and was present in the enzyme producing chief cells of the stomach (arrow) and absent from the acid producing parietal cells (arrowhead) within the same glands (Fig. 2D).

Next, we asked whether other ER chaperones involved in protein oxidation and the unfolded protein response also showed cell-specific expression in the stomach. PDI (Fig. 2, E and F), Erp57 (Fig. 2, G and H), and BiP (Fig. 2, I and J) were all expressed at a high level in the same cell types. In contrast to Ero1β, both PDI and BiP showed some additional expression in the gastric epithelium (Fig. 2, E and I). We conclude that enzyme-producing cells in the stomach express high levels of ER chaperones and the oxidoreductase Ero1β.

Ero1β and PDI Are Both Expressed in the Pancreas but Show Differential Expression in Pancreatic Islets—To determine whether high oxidoreductase expression correlates with high expression of ER chaperones in other tissues, we examined human pancreas. This was of particular interest given the fact that the UPR and protein misfolding contribute to type II diabetes (6, 30). Tissue sections (blocked with normal goat serum) showed faint background staining when TBS (Fig. 3A) or pre-immune rabbit sera (Fig. 3B) were used instead of specific antibody. Pancreas sections stained for the pancreas-specific PDI, PDIP, showed expression of this enzyme in the acinar cells and weak staining of islets, as expected (Fig. 3, C and D) (29). Pancreatic islets were specifi-
FIGURE 3. Immunohistochemistry of the pancreas. Human pancreas sections were probed with TBS (A), normal rabbit serum (NRS) (B), αPDlp (C and D), αinsulin (E and F), αEro1β (G and H), and αPDlp (I and J) and photographed at 4× (C, E, G, and I; bar = 200 μm) and 20× (A, B, D, F, H, and J; bar = 100 μm) magnification. Arrow = pancreatic islets.

Ero1β Expression and Dimerization

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The Ero1βC390A mutant also formed disulfide-dependent complexes (Fig. 4D, lane 2), but hardly interacted with PDI (Fig. 4E, lane 2). Again, Ero1βC390A complexes were preserved in the absence of an alkylating agent, showing that Ero1βC390A could associate with partner protein(s) other than PDI (Fig. 4D, lane 4). Ero1βC393A (Fig. 4D, lanes 2 and 5) and EroβC396A (Fig. 4D, lanes 7 and 8) both interacted with PDI less strongly than wild type in an NEM-dependent manner. Disulfide-linked complexes were dispersed to a single Ero1β band upon reduction with DTT (Fig. 4B and not shown). Note that the 9E11 α-myc monoclonal antibody gave a nonspecific background band at ~130 kDa (Fig. 4D, lanes 9 and 11). The nonspecific nature of this band, and the reproducibility of the results, was confirmed using a polyclonal α-myc antisera, which specifically detected Ero1β-containing complexes only (Fig. 4D, lanes 12 and 13, and not shown). Also note the expected cross-reactive PDI antibody in the myc blot of the immunoprecipitations (Fig. 4E, Ab).

To further confirm the distinct nature of the Ero1β complexes, we performed an experiment in which PDI was depleted from Ero1β-transfected cell lysates by consecutive rounds of immunoprecipitation. All detectable PDI was removed by this procedure, whereas calnexin levels remained unaffected (Fig. 4F, compare lane 1 with lanes 3 and 4). Anal-
forms redox-dependent, NEM independent complexes. A, non-reducing (NR) and B, reducing (R) SDS-PAGE of myc-tagged wtEro1β (lanes 1 and 2), Ero1JC390A (lanes 3 and 4), Ero1JC393A (lanes 5 and 6), Ero1JC396A (lanes 7 and 8), and mock transfectants (lanes 9 and 10) lyed in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of NEM and probed with α-myc. C, non-reducing (NR, lanes 1 and 2) and reducing (R, lanes 3 and 4) SDS-PAGE of myc-tagged Ero1β from HT1080 cells treated with lanes 2 and 4 or without (lanes 1 and 3) 5 mM DTT for 30 min prior to lysis, and subsequently probed with α-myc. D, same as for A, except that lane 10 is loaded with sample buffer only and lane 17 is loaded with mock-transfected cell lysate in the absence of NEM. Lanes 12 and 13 are probed with a polyclonal α-myc antiserum. E, non-reducing SDS-PAGE of myc-tagged wtEro1β (lanes 1 and 2), Ero1JC390A (lanes 3 and 4), Ero1JC393A (lanes 5 and 6), Ero1JC396A (lanes 7 and 8), and mock transfectants (lanes 9 and 10) lyed in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of NEM, immunoprecipitated with α-myc and blotted for PDI. PDI co-immunoprecipitated with wtEro1β, Ero1JC393A, and Ero1JC396A in the presence of NEM. Antibody complexes (Ab) migrate just above the PDI-Ero dimer. F, reducing SDS-PAGE of Ero1β-myct transfected HeLa cell lysates subjected to sequential immunodepletion of PDI prior to detection of calnexin (Cnx) and PDI. All detectable PDI is specifically removed from the lysate. G, non-reducing SDS-PAGE of Ero1β-myc transfected HeLa cell lysates subjected to sequential immunodepletion of PDI, prior to detection of Ero1β-myc with the omc monoclonal antibody. 1st = first immunodepletion, 2nd = second immunodepletion, sup = supernatant, tcl = total cell lysate prior to immunodepletion. The Ero-PDI complex is depleted (arrow), whereas the major Ero complex remains (asterisk).

ysis of the PDI-depleted lysates by non-reducing SDS-PAGE and immunoblotting with α-myc showed that the PDI-Ero1β complex was selectively removed, whereas the lower complex remained (*), along with the Ero1β monomers (Fig. 4G). Thus, in cell lysates, the PDI-Ero population was discrete. We conclude that Ero1β can form non-PDI redox-sensitive complexes that are not lost by disulfide reshuffling in the absence of alkylating agents.

Ero1β Forms Prominent Disulfide-dependent Homodimers—A number of thioredoxin family proteins and redox enzymes can associate as dimers, and this trait may have contributed to the evolution of redox/chaperone protein function (31). To investigate whether Ero1β dimers were a component of the complexes seen in Fig. 4, we took advantage of the fact that Ero1β-myc and Ero1β-HA migrated distinctly on SDS-PAGE (Fig. 1). HeLa cells were either mock transfected (Fig. 5A, lane 4), or transfected with Ero1β-myc (Fig. 5A, lane 1), Ero1β-HA (Fig. 5A, lane 2) and Ero1β-mcy plus Ero1β-HA (Fig. 5A, lane 3). Any mixed disulfide-dependent Ero1β-HA-Ero1β-myc dimer should appear as an intermediate band present only in the double transfectant under non-reducing conditions. The cell lysates were analyzed by Western blotting, first probing the membrane for myc (Fig. 5, lanes 1–4), and then probing the membrane for HA (Fig. 5A, lanes 5–8) without stripping the blot. An intermediate, ~125-kDa Ero1β-myc-Ero1β-HA complex formed and was only present in the double transfectant (Fig. 5A, lane 3 and 7, M+H). Note the extra nonspecific bands of ~130 kDa in both the HA and myc blots (Fig. 5A, lanes 2, 4, and 8). We conclude from this experiment that Ero1β forms SDS-stable, redox-sensitive homodimers at steady state in the endoplasmic reticulum.

Ero1α and Ero1β Form Mixed Disulfide-dependent Heterodimers—We next asked whether the two oxidoreductases, Ero1α and Ero1β, could form mixed complexes at steady state. Thus HeLa cells were transfected with Ero1α-myc, Ero1β-HA, or Ero1α-myc and Ero1β-HA together (Fig. 5, B and C). Cell lysates were analyzed under reducing conditions, and membranes were sequentially probed with the myc antibody (Fig. 5B, lanes 1–4) and the HA-7 antibody (Fig. 5B, lanes 5–8) to confirm the expression of the Ero proteins. Cell lysates were subjected to an HA-7 immunoprecipitation followed by blotting the reducing gels for myc (Fig. 5C). A myc signal could only be observed in the double transfectant (Fig. 5C, lane 3), confirming that Ero1α and β interacted. This experiment therefore demonstrates that the two different oxidoreductases can associate in the endoplasmic reticulum.

Ero1β Complexes Differ in Abundance in Stomach and Pancreas—To see whether Ero1β dimers occurred in vivo, we lysed mouse stomach and mouse pancreas tissues in the absence of NEM, using conditions in which PDIs and Ero1β do not interact (see Fig. 4E, lanes 1 and 2). Our Ero1β antisemur does not immunoprecipitate, or readily detect Ero1β when used for immunoblotting under non-reducing conditions. We therefore subjected tissue lysates to gel filtration and analyzed trichloroacetic acid-precipitated fractions by reducing SDS-PAGE, prior to immunoblotting with the Ero1β serum. Ero1β from stomach was
recovered in fractions 10–13, with a peak in fraction 12 corresponding to the likely size of the glycosylated monomer (Fig. 6A). In contrast, Ero1β from pancreas peaked in fractions 9 and 10, corresponding to the expected size of a glycosylated Ero1β dimer, with a minor amount eluting in fractions 13 and 14 (Fig. 6B). The elution profiles of Ero1β were compared with bovine serum albumin (monomer of 66 kDa and dimer of 132 kDa, Fig. 6C) and chicken egg albumin (monomer of 45 kDa, Fig. 6D). We conclude from this experiment that non-PDI-bound Ero1β complexes exist in vivo and that their relative abundance can be different in the stomach and the pancreas.

Ero1β CXXCXXC Mutants Can Dimerize with Wild-type Ero1β—Next, we analyzed the molecular requirements for Ero1β dimerization. We were interested in the fact that, although the Ero1αC394A and C397A mutants cannot rescue a temperature-sensitive yeast ero1–1 mutant (10), both Ero1α and Ero1β CXXCXXC and CXXXVXA mutants can form disulfide-dependent dimers with PDI (18) (Fig. 4). However, Ero1αC391A can rescue the ero1–1 yeast mutant despite interacting poorly, if at all, with PDI (10). This observation implies that Ero activity requires more than just PDI-Ero heterodimerization.

First we investigated whether mutant Ero1β proteins could dimerize with wild-type Ero1β. Thus Ero1β-mycC390A (Fig. 7, A and B), Ero1β-mycC393A (Fig. 7, C and D), and Ero1β-mycC396A (Fig. 7, C and D) were all analyzed for their ability to interact with wild-type Ero1β-β-HA. Reducing blots showed that single and double transfectants expressed the expected proteins (Fig. 7, A and C). Cell lysates were subjected to immunoprecipitation with the HA antibody, followed by blotting with the myc antibody (Fig. 7, B and D). No signal could be detected in the mock or single transfectants as expected. Wild-type Ero1β-myc could be co-immunoprecipitated with wild-type Ero1β-HA, verifying this interaction (Fig. 7B, lane 4). Ero1β-mycC390A (Fig. 7B, lane 6), Ero1β-mycC393A (Fig. 7D, lane 4), and Ero1β-mycC396A (Fig. 7D, lane 5) specifically co-immunoprecipitated with wild-type Ero1β-HA. Thus complex formation between wild-type Ero1β and the Ero1βC390A, C393A, and C396A mutants occurred.

C396 Is Required for Ero1β Homodimer Formation—Having shown that mixed Ero1β wild-type/CXXCXXC mutant complexes could form (Fig. 7), we investigated whether mutant-mutant dimers were possible. To do this, we constructed HA-tagged versions of Ero1βC390A, Ero1βC393A, and Ero1βC396A and co-transfected them into HeLa cells with the equivalent myc-tagged mutants. Western blotting was performed with the αEro1β serum to confirm the expression of the proteins (Fig. 8A), or proteins were first immunoprecipitated with α-myc and then probed for the presence of both HA- and myc-tagged Ero1β by blotting with the αEro1β serum (Fig. 8B). The α-myc monoclonal antibody did not bring down any HA-tagged proteins in the single transfectants, as expected (Fig. 8B, lanes 2–5). Wild-type αEro1β-myc and αEro1β-HA co-immunoprecipitated (Fig. 8B, lane 6), as did the...
C390A mutants and the C393A mutants (Fig. 8B, lanes 7 and 8). However, the C396A mutants did not co-immunoprecipitate (Fig. 8B, lane 9).

Our results demonstrate that an Ero1β AXXCXXXC mutant can interact with itself and with a wild-type Ero1β protein. An Ero1β CXXCXXXA mutant can interact with wild-type Ero1β protein but cannot dimerize with the mutant counterpart. These data are consistent with a symmetrical, redox-dependent Ero dimer in which the Cys196 residue is required for dimerization.

**Modeling the Ero1 Dimer**—To identify a molecular basis for the dimer of Ero1β, and to understand the role of the C396A mutant in disrupting it, we analyzed the recently determined crystal structure of Ero1p (23). Although there is only a single monomer in the asymmetric unit of the two different crystal forms of the enzyme reported (PDB codes 1RP4 and 1RQ1), the examination of the crystal contacts revealed a single dimer interaction between monomers that was found in both structures (Fig. 9). This is consistent with the symmetrical dimer suggested by the immunoprecipitation experiments in Figs. 7 and 8. From our model of the dimer, the N termini are brought into close proximity, and therefore the N terminus, which is absent in the construct of the enzyme used for the crystal structure, could also interact and form a part of the dimer interface. The crystal structure was of a truncated *Saccharomyces cerevisiae* Ero1p protein, which shares ~25% sequence identity with full-length human Ero1β investigated here. The human enzyme has several amino acid inserts ranging in size from 5 to 25 residues. Analysis of the crystal structure confirms that they are all found at the surface in positions where additional structure can be accommodated. In addition, none of the inserts would disrupt the presumed dimer interface.

The proposed Ero1p dimer buries ~1100 Å² of predominantly non-polar solvent-accessible surface area per monomer, and the monomers are arranged so that the active sites are on the same side of the dimer. The dimer brings together the adenosine groups of the two FAD moieties in close approximation; in fact, the two hydroxyls of the ribose sugars of the adenosine moiety of FAD form hydrogen bonds to each other (Fig. 9A). The FADs contribute 20% of the dimer interface and therefore may be essential for dimer formation. Interpreting the mutagenesis data in light of the crystal structure, it is notable that the Ero1β C396A mutant does not dimerize given the buried location of this residue and its contact with the FAD cofactor (Fig. 9B). It is highly unlikely that this residue is involved in any disulfide to form the dimer but, rather, disrupts the FAD binding so that the FAD-FAD dimer interface is lost.

## DISCUSSION

Our experiments show that Ero proteins can homodimerize and heterodimerize, as well as interact with PDI in a redox-dependent manner (Figs. 4–8). Prior to this study, it was known that Ero proteins and PDI were required for disulfide bond catalysis in the ER. However, an Ero1α C391A mutant can rescue a temperature-sensitive yeast ero1D-1 mutant, despite having little affinity for PDI (10, 11, 25). Taken together with this observation, the experiments in this report suggest that both dimerization and binding to PDI are required for appropriate maintenance of disulfide bond formation in the ER. The active C390A Ero1β mutant binds poorly to PDI but can homodimerize, whereas the inactive C396A Ero1β mutant interacts with PDI but cannot homodimerize (Figs. 4 and 8), suggesting that Ero-Ero dimers are important for the functional activity of this protein family.

Our results show that dimerization is partly controlled by the CXXCXXC motif, although other covalent and non-covalent interactions are likely to be important for dimer establishment. Both the Ero-Ero interactions and the Ero-PDI interactions are stable under non-reducing conditions (in the presence of SDS). However, the strength of these interactions is different, given that the PDI-Ero interaction requires trapping by an alkylating agent, whereas the Ero-Ero interaction does not. Dimers are required for disulfide bond formation in vivo and in transfected cells, can occur between various tagged and non-tagged forms of Ero1α and Ero1β, and can be retrieved from the yeast Ero1p crystal structure, suggesting that Ero-Ero dimers are conserved throughout evolution and are biologically...
significant. The fact that mutating Cys^{396} gives rise to no dimers also suggests that dimerization is genuine, because nonspecific dimers resulting from misfolding would be expected to increase, not decrease, upon exposure of the free -SH of C393.

Reconstruction of an Ero-Ero dimer from the Ero1p crystal structure shows that the Ero1p, Ero1/H9251, and Ero1/H9252 glycosylation sites are all located away from the dimer interface, and would not prevent Ero-Ero interactions (Fig. 9). The likely site of PDI docking, via a flexible Ero hinge, is also positioned away from the dimer interface, suggesting that PDI-Ero heterodimers and Ero-Ero dimers are not necessarily mutually exclusive. Our immunodepletion data (Fig. 4G) suggests that Ero-PDI complexes are independent of Ero-Ero dimers in a Triton X-100 lysate. However, it remains possible that tetrameric Ero-PDI complexes could operate under different situations in vivo. Regulation of a dimer/tetramer pool, for example, could allow for rapid mobilization or storage of disulfide donors in response to the fluctuating secretory requirements of a tissue.

The Ero1p dimer that we have modeled is held together by a hydrogen-bonding network at the dimer interface (Fig. 9). Two hydrogen bonds link the two FAD moieties, which are likely to be important for dimer stability, given that the C396A mutation of Ero1β disrupts the dimer. Cys^{396}, in concert with Cys^{393}, probably passes electrons on to FAD for donation to oxygen. Thus the loss of Cys^{396} is likely to lead to loss of dimerization indirectly, by disturbing FAD binding. In the light of our studies, further experiments can now be conducted to determine whether targeting the dimer interface can be used to specifically engineer and manipulate the process of disulfide bond formation. The proximity of the N termini in the model of the truncated Ero1p dimer also suggests that N-terminal cysteines upstream of the CXX/CXC redox active site may be close enough to form an intermolecular disulfide bond. This would explain the appearance of dimers under non-reducing conditions and can now be tested using the relevant cysteine mutants.

Prior to this study, it was known that Ero1β could be induced by the UPR and that mRNA levels were highest in secretory tissues (11). Nothing was known about the expression or behavior of the Ero1β protein in vivo. Our results show that Ero1β, a UPR-responsive protein, is constitutively yet cell specifically expressed in stomach and pancreas (Figs. 1–3). The balance between monomeric and complex forms of Ero1β is

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**FIGURE 9. Structural model of an Ero dimer. A**, two Ero1p monomers form significant interactions (non-bonded and four hydrogen bonds) between the two symmetrically related FAD molecules (space filling). The location of the four likely glycans per monomer, away from the interface, is shown (ball and stick representation). **B**, detail of the dimer interface showing the Cys^{90}-Cys^{346} disulfide bond (Cys^{81}-Cys^{390} in Ero1β), the Cys^{352}-Cys^{355} active site disulfide (Cys^{393}-Cys^{396} in Ero1β), and Cys^{100}-Cys^{105}, which is not labeled for clarity. Cys^{355} (Cys^{396}) is buried within the monomer, in Van der Waals contact with the FAD (stick and space filling representation), and is unlikely to form a dimeric, intermolecular disulfide. Graphics were generated using the PyMOL program (DeLano, W. L., The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA).
also different in the pancreas, where complexes predominate, and in the stomach (Fig. 6). The data imply that Ero1β is subject to tissue- and cell-specific regulation, at both the transcriptional and post-translational level. It will be very interesting to identify these regulatory factors and to determine whether Ero1β expression levels and/or dimerization depend on the nutritional status of an animal.

In a simple model of Ero-PDI function, a low abundance electron acceptor (Ero) can fuel a large number of catalytic cycles. Our results show that this model is too simple and that cells can accommodate high amounts of Ero1β in vivo. This suggests that Ero1β is either an inefficient catalyst, that it is performing an additional function, or that it can remain in the ER in an inactive state. The difference between Ero1β and PDI expression in pancreatic islets and acinar cells suggests that the expression levels of these two proteins can be independently controlled (Fig. 3). Perhaps PDI is not the only electron donor for Ero1β in the pancreas. The PDI homologue PDiP is expressed specifically in this organ, and although PDiP can use hydroxaryl groups as ligands (32), its dual-catalytic activity may be due to the presence of Ero1β. This suggests that further analysis of the islet-specific physiology of Ero1β electron donors may be worthwhile, because tissue-specific differences in the control of disulfide bond formation may provide an opening for therapeutic intervention in pancreatic protein secretion and type 2 diabetes. In this respect, it is interesting to note that QSOX1 is highly expressed in the islets of Langerhans (33). The QSOX (Quescin-sulphydryl oxidases) family utilize oxygen to generate disulfide bonds with the production of peroxide. However, the true function of QSOX1 in vivo and its relationship to Ero and PDI proteins is not yet known.

Our data raise the unexpected possibility that intracellular electron transfer between two Ero molecules might occur via FAD (Fig. 9). This may seem unlikely given the weight of evidence for a linear Ero-PDI-substrate oxidation chain (34, 35). The Ero-PDI heterodimer can mediate oxidative protein folding in vitro, but it is possible that Ero-PDI dimers contribute to this task in vivo. Many thioredoxin family proteins can dimerize, and mutant bacterial periplasmic DsbC molecules that are unable to do so are converted from isomerases into oxidases (36). Engineered dimerization by domain hybridization also confers novel properties onto redox enzymes (31).

The very high relative expression of the Ero1β oxidoreductase in stomach and pancreas raises the question of how the ER protects itself from free radical damage and oxidative stress in chief cells and islets. Perhaps Ero dimerization is related to the need to protect irrelevant proteins from random oxidative damage by "expensive" electrons, with dimerization preventing inappropriate electron flow from an exposed flavin to a bystander protein. Alternatively, the equilibrium between the dimeric and PDI-bound forms of Ero could be used to regulate oxidoreductase activity through occupation or protection of the active site. In this respect, it will be interesting to determine whether overexpression of Ero1β mutants, which cannot dimerize, generate more oxidative stress in the ER than the C390A mutant (which is active but can dimerize).

In terms of physiology, we note that, although a chemical UPR is necessary to induce Ero1β expression in cell lines, the default state of Ero1β in the chief cells and islets of the stomach and pancreas is "on." Specific cells in some organs are therefore pre-primed for oxidative protein folding. Whether this is due to constitutive or partial maintenance of a UPR state requires further investigation, for example by assessing the status of XBP1, ATF6, PERK, and other regulators of the UPR in chief cells and islets. Evidently, some cells can sustain high levels of Ero1β expression without incurring sufficient oxidative damage to induce cell death, whereas neighboring cells remain refractory to Ero1β activation. Our studies emphasize the importance of relating work in cell lines to the whole animal, and in this respect an Ero1β tissue-specific knock-out mouse may be very informative. Finally, mixed Ero1α/β heterodimers (Fig. 5) add another level to this disulfide bond formation pathway, indicating that Ero1α dimers may be important in the response to combined UPR and hypoxia. This may be relevant to protein misfolding diseases in the CNS or in tumor development, where hypoxic conditions induce the expression of Ero1α (37). It is apparent that the interactions between PDI and Ero proteins are more complex than previously imagined in mammals. Our analysis of the in vivo expression pattern of Ero1β and the molecular requirements for homodimer formation significantly develops our cellular and molecular knowledge of an essential biological process, the control of protein folding in the endoplasmic reticulum.

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Note Added in Proof—A regulatory role for Ero-Ero dimers has also been postulated by the Sitia group (Otsu, M., Bertoli, G., Fagioli, C., Guerini-Rocco, E., Nerini-Molteni, S., Ruffato, E., and Sitia, R. (2005) Antioxid. Redox Signal., in press).

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