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The *ERO1* Gene of Yeast Is Required for Oxidation of Protein Dithiols in the Endoplasmic Reticulum

Alison R. Frand and Chris A. Kaiser*
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

We describe a conserved yeast gene, *ERO1*, that is induced by the unfolded protein response and encodes a novel glycoprotein required for oxidative protein folding in the ER. In a temperature-sensitive *ero1-1* mutant, newly synthesized carboxypeptidase Y is retained in the ER and lacks disulfide bonds, as shown by thiol modification with AMS. *ERO1* apparently determines cellular oxidizing capacity since mutation of *ERO1* causes hypersensitivity to the reductant DTT, whereas overexpression of *ERO1* confers resistance to DTT. Moreover, the oxidant diamide can restore growth and secretion in *ero1* mutants. Genetic tests distinguish the essential function of *ERO1* from that of *PDI1*. We show that glutathione is not required for CPY folding and conclude that Ero1p functions in a novel mechanism that sustains the ER oxidizing potential, supporting net formation of protein disulfide bonds.

Introduction

The formation of native inter- and intramolecular disulfide bonds is a crucial step in the folding of many secretory proteins. Disulfide bonds can stabilize folded protein domains and cross-link associated subunits of oligomeric protein complexes. In eukaryotic cells, disulfide bonds form as newly translocated polypeptide chains enter the oxidizing milieu found in the lumen of the endoplasmic reticulum (ER) (Braakman et al., 1991). Experiments on oxidative protein folding carried out both in vitro and in vivo have demonstrated that the efficient formation of native disulfide bonds requires both an oxidizing agent and a catalyst for the rearrangement of nonnative disulfide bonds.

In vitro assays for oxidative protein folding typically employ a small molecule, such as oxidized glutathione (GSSG), as an electron acceptor for the oxidation of protein dithiols to disulfide bonds. GSSG can contribute disulfide bonds to the substrate or the protein catalyst through dithiol-disulfide exchange reactions (Saxena and Wetlaufer, 1970). The rate of oxidative protein refolding, regardless of the presence of a catalyst, depends on the redox potential of the assay buffer. For example, the optimum rate of PDI-catalyzed refolding of reduced ribonuclease A occurs at a [GSH]/[GSSG] ratio of 5:1 in the presence of 1 mM total glutathione (Lyles and Gilbert, 1991). Measurements of the redox conditions within the ER lumen show that the most abundant redox buffer present is glutathione and that the ratio of [GSH]/[GSSG] is about 2:1. In contrast, the ratio of [GSH]/[GSSG] in the cytosol is from 30:1 to 100:1 (Hwang et al., 1992). These findings implicate a mechanism that concentrates GSSG in the ER lumen as a potential source of oxidizing equivalents for the formation of protein disulfide bonds. However, a small molecule such as GSSG need not necessarily serve as an intermediate in protein oxidation in vivo, since disulfide bond formation in the periplasm of *Escherichia coli* occurs through a series of dithiol-disulfide exchange reactions mediated entirely by periplasmic proteins (Guilhot et al., 1995; Kishigami et al., 1995).

The most extensively studied catalyst of native disulfide bond formation in the ER is protein disulfide isomerase (PDI), an abundant, 55 kDa ER protein with two domains related to the active site of the dithiol-disulfide oxidoreductase thioredoxin. These domains each contain a pair of cysteine residues in the motif CXXC that can participate in dithiol-disulfide exchange reactions. In vitro, PDI can catalyze either the formation, reshuffling, or reduction of disulfide bonds depending on the substrate and the redox conditions of the assay (reviewed by Freedman et al., 1994). The *PDI1* gene of *Saccharomyces cerevisiae* is essential for the formation of native disulfide bonds in carboxypeptidase Y (CPY) and for cell viability (LaMantia and Lennarz, 1993). Mutational studies on the active-site cysteines in PDI indicate that the essential function of PDI in *S. cerevisiae* is to reshuffle nonnative protein disulfide bonds (Laboissière et al., 1995).

A useful method to selectively perturb the formation of disulfide bonds in living cells is to add reduced dithiothreitol (DTT) to the growth medium. DTT can penetrate cell membranes and rapidly and reversibly inhibit protein oxidation in the ER without disrupting protein synthesis, protein translocation into the ER, or intracellular transport of proteins that do not contain disulfide bonds. For example, addition of DTT to *S. cerevisiae* cells blocks the folding and transport from the ER of CPY, a vacuolar protein whose native form has five disulfide bonds but does not interfere with secretion of invertase, a protein that lacks disulfide bonds (Jämsä et al., 1994; Simons et al., 1995).

Despite the importance of the redox state of the ER for the net formation of protein disulfide bonds, little is known of how cells establish oxidizing conditions within the ER lumen. We have begun to investigate the mechanism of protein oxidation in the ER using a genetic approach. We describe a new *S. cerevisiae* gene, *ERO1* (ER oxidation), mutation of which affects yeast cells in much the same way as treatment with DTT. A conditional *ero1-1* mutant is defective in the oxidation of protein dithiols in the ER, and a membrane-permeable oxidant can bypass the essential function of *ERO1. ERO1* thus appears to define a critical component of the mechanism that sustains the oxidized redox state of the ER.

Results

Isolation of the *ERO1* Gene

In a screen of 1200 temperature-sensitive *S. cerevisiae* mutants (Hartwell et al., 1973) for conditional defects in

*To whom correspondence should be addressed.

export of secretory proteins from the ER, we identified a conditional allele of a novel gene that we designate *ERO1*. A backcrossed *ero1-1* strain (CKY559) failed to grow and exhibited a complete block in maturation of the ER form of CPY at temperatures above 36°C. A plasmid clone that rescued both the growth and secretion defect of *ero1-1* was isolated and the open reading frame YML130c identified as the complementing gene (Cherry et al., 1997). We verified that *ERO1* corresponds to YML130c by showing complete linkage between a *URA3* marker integrated at the YML130 locus and *ero1-1*. A chromosomal deletion of *ERO1* was constructed in a diploid strain by replacing the sequence encoding amino acids 124–500 of Ero1p with the *LEU2* marker. Sporulation of this diploid at 24°C gave only tetrads with two viable *leu2*$^-$ spores. Thus, *ERO1* is essential for yeast viability.

ERO1 Encodes a Conserved ER Glycoprotein

ERO1 specifies a protein with a predicted molecular weight of 65 kDa; an amino terminus that appears sufficiently hydrophobic to function as a signal sequence; and 8 AsnXaaSer/Thr carbohydrate acceptor sites. Searches of GenBank failed to identify any protein of known function with sequence similarity to Ero1p. However, sequence homologs of Ero1p were found in other eukaryotic organisms including microorganisms (*Schizosaccharomyces pombe* and *Trypanosoma brucei*), invertebrates (*Brugia malayi* and *Drosophila melanogaster*), plants (*Arabidopsis thaliana*), and mammals (*Mus musculus* and *Homo sapiens*). In the C-terminal conserved region of Ero1p, 65% of residues are identical between *S. cerevisiae* and humans (Figure 1A).

To monitor the intracellular distribution of Ero1p, the myc epitope was introduced at the carboxy terminus of the predicted protein coding sequence. The epitope-tagged protein was functional as *ERO1-myc* restored viability to *ero1-*Δ spores. Anti-myc recognized a 96 kDa protein from cells expressing *ERO1*-myc from a centromere plasmid (CKY563). This protein was not detected from cells expressing unmodified *ERO1*, but was abundant in cells expressing *ERO1-myc* from a high copy plasmid (pAF84; Figure 1B). The apparent molecular weight of Ero1p-myc decreased to 81 kDa upon treatment of cell extracts with endoglycosidase H, consistent with removal of 5–6 core N-linked oligosaccharides from Ero1p. Since Ero1p contains N-linked carbohydrate, the protein must enter the ER lumen.

To determine if Ero1p-myc was restricted to the ER or was transported to post-ER compartments, we tested for addition of α1,6-mannose to Ero1p-myc as this modification occurs in *cis*-Golgi. Ero1p-myc could not be reimmunoprecipitated with anti-α1,6-mannose, indicating that the majority of Ero1p-myc resides in the ER. In a control for antibody specificity, the secreted glycoprotein invertase could be reimmunoprecipitated with this antiserum (Figure 1C). Ero1p-myc was included in membrane fractions of cell extracts. The protein could be solubilized by treatment of extracts with 1% Triton X-100, but not by treatment with 0.5 M NaCl, 2.5 M urea, or 0.1 M sodium carbonate (pH 11.5). These treatments did release the luminal ER protein Kar2p from membrane

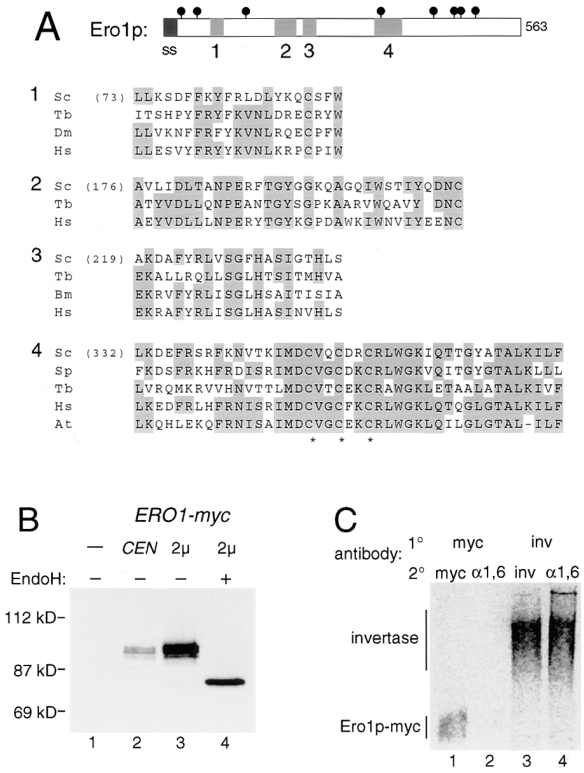

Figure 1. *ERO1* Encodes a Conserved ER Glycoprotein

(A) The predicted amino acid sequence of Ero1p contains a potential signal sequence and eight N-linked carbohydrate acceptor sites. The four highly conserved regions shown here comprise amino acids 73–93 (1), 176–208 (2), 219–239 (3), and 332–375 (4) of Ero1p. The species of origin and accession numbers for these sequences are: Sc, *S. cerevisiae* (gb|z50178); Sp, *S. pombe* (emb|x61926); Tb, *T. brucei* (emb|x60951); Bm, *B. malayi* (gb|AA509062); Dm, *D. melanogaster* (gb|AA202720); At, *A. thaliana* (gb|T45661); Hs, *H. sapiens* (gb|R07093, AA186803, R50884, AA033538).

(B) Ero1p-myc migrates with an apparent molecular mass of 96 kDa, and of 81 kDa after endoH treatment. CKY10 (wild-type) transformants carrying either pAF9 (*CEN ERO1 URA3*; lane 1) or pAF84 (2μ *ERO1-myc URA3*; lanes 3 and 4), and CKY563 (*ero1-*Δ [p*CEN ERO1-myc URA3*]; lane 2) were labeled with [^{35}S] methionine and cysteine for 30 min. Ero1p-myc was immunoprecipitated from cell lysates with monoclonal anti-myc and samples treated with endoH as indicated prior to SDS–PAGE.

(C) Immunoprecipitates of Ero1p-myc were reimmunoprecipitated with either anti-myc (lane 1) or anti-α1,6-mannose (lane 2). As a control, invertase was immunoprecipitated from an extract of CKY10 (wild-type) and then reimmunoprecipitated with either anti-invertase (lane 3) or anti-α1,6-mannose (lane 4). Samples were resolved by SDS–PAGE.

fractions (data not shown). The resistance of Ero1p-myc to extraction by chaotropic agents suggested that the protein was tightly associated with ER membranes. This association may explain how Ero1p is retained in the ER without an obvious KKXX or HDEL motif for ER retention.

ERO1 Is Required for Transport of a Subset of Proteins from the ER

The *ero1-1* mutation inhibited transport from the ER of CPY and the GPI-linked plasma-membrane protein Gas1p, but not of the periplasmic enzyme invertase. In a pulse–chase experiment, an *ero1-1* mutant (CKY559)

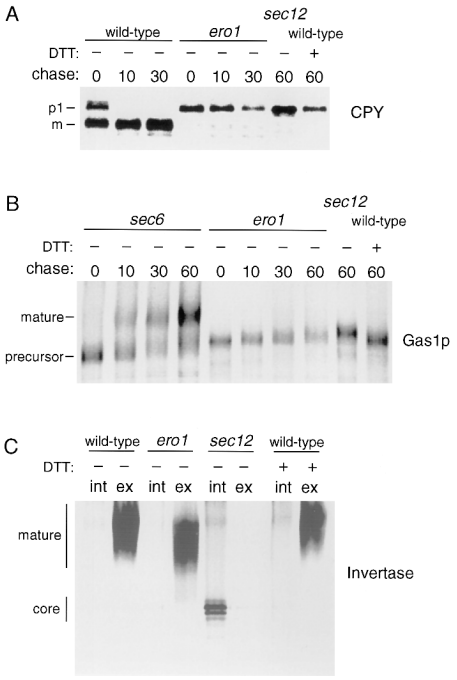

Figure 2. The *ero1-1* Mutant Exhibits a Defect in the Export of a Subset of Proteins from the ER

(A) CKY10 (wild-type), CKY559 (*ero1-1*), and CKY39 (*sec12-4*) were shifted from 24°C to 38°C, pulse-labeled with [^{35}S] methionine and cysteine for 7 min, and chased for 60 min. CPY was immunoprecipitated from cells collected 0, 10, 30, and 60 min after initiation of the chase, and samples were resolved by SDS–PAGE. The effect of DTT on protein transport was assessed by adding 5 mM DTT to cells 10 min before labeling. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

(B) Gas1p was immunoprecipitated from cell lysates as in (A). To follow processing of Gas1p, a temperature-sensitive *sec6-4* mutant (CKY560) was used to prevent degradation of mature Gas1p at the cell surface. The ER (precursor) and Golgi (mature) forms of Gas1p are indicated.

(C) To follow invertase transport, strains expressing invertase from the *TPI1* promoter were radiolabeled for 10 min and then converted to spheroplasts. Invertase was immunoprecipitated from spheroplast pellet (int) and supernatant (ex) fractions. Core-glycosylated (ER) and mature (Golgi and periplasmic) forms of invertase are indicated.

at restrictive temperature (38°C) exhibited a complete block in conversion of the ER form (p1) of CPY to Golgi (p2) and vacuolar (m) forms of CPY throughout 30 min of chase (Figure 2A). CPY could not be detected after 60 min of chase, suggesting that p1 CPY was eventually degraded in *ero1-1* cells (data not shown). In a wild-type strain (CKY10) under the same conditions, p1 CPY was converted to mature CPY in less than 10 min. Newly synthesized Gas1p is normally processed from an ER form of 120 kDa to a mature, 125 kDa cell-surface form (Schimmöller et al., 1995). Gas1p synthesized in an *ero1-1* mutant at 38°C remained in the ER form and comigrated during SDS–PAGE with Gas1p produced in the ER-to-Golgi vesicle formation mutant *sec12-4* (CKY39; Figure 2B). In contrast, invertase was delivered from the ER to the periplasm in less than 10 min in both wild-type and *ero1-1* strains at 38°C (Figure 2C). The rapid export of invertase from the ER in *ero1-1* cells indicated that the secretory pathway, including the COPII vesicles that transport both invertase and CPY from the ER, functioned in *ero1-1* cells.

An explanation for the selective secretory defect associated with *ero1-1* was based on the effect of the reductant DTT on protein transport in wild-type yeast. Addition of 5 mM DTT to wild-type cells blocks transport from the ER of CPY, but not of invertase (Jämsä et al., 1994; Figures 2A and 2C). Properly folded CPY contains five intramolecular disulfide bonds (Endrizzi et al., 1994). CPY is retained in the ER of DTT-treated cells due to a defect in the formation of these bonds (Jämsä et al., 1994). In contrast, invertase appears to lack structurally important disulfide bonds, as the enzyme has only two cysteines and can fold into an active form when expressed in the relatively reducing conditions of the cytosol (Carlson and Botstein, 1982). The maturation of Gas1p was also blocked by treatment of cells with DTT (Figure 2B), indicating that at least 2 of the 14 cysteine residues in the extracellular domain of Gas1p form a structurally important disulfide bond needed for export of Gas1p from the ER. The selective ER retention of CPY and Gas1p in the *ero1-1* mutant could thus be a consequence of a defect in oxidative protein folding. Consistent with this interpretation, an independently isolated *ero1* allele impaired maturation of an Hsp150-blactamase fusion protein with structurally important disulfide bonds (Pollard et al., 1998 [this issue of *Molecular Cell*]).

ERO1 Is Required for Oxidation of CPY

As the *ero1-1* mutation produced a similar effect on protein transport as treatment with DTT, we postulated that the *ero1-1* mutation interfered with oxidation of protein dithiols in the ER. We therefore used nonreducing SDS–PAGE to examine the redox state of CPY in *ero1-1* cells at restrictive temperature (38°C). Indeed, CPY synthesized in an *ero1-1* mutant (CKY559) at 38°C comigrated on a nonreducing gel with reduced CPY from wild-type cells (CKY10) that had been treated with 5 mM DTT (Figure 3A). CPY produced in a *sec12-4* mutant (CKY39) that had not been treated with DTT served as a standard for oxidized, p1 CPY (Figure 3A, lane 2).

As an independent and explicit test for the presence of free thiols in CPY, we evaluated the reactivity of CPY with the thiol-conjugating reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). The maleimide moiety of AMS reacts with cysteine thiols, thereby increasing the molecular mass of a modified protein by approximately 0.5 kDa per AMS residue added (Joly and Swartz, 1997). When *ero1-1* cells were radiolabeled at 38°C and lysed in the presence of AMS and detergent, the apparent molecular mass of CPY on a nonreducing gel increased by about 15 kDa relative to that of CPY isolated in the absence of AMS (Figure 3B). This mobility shift was consistent with AMS modification of the 10 cysteine residues normally found in intramolecular disulfide bonds in CPY. CPY synthesized in the presence of DTT reacted with AMS to the same extent. In contrast, when *sec12-4* cells were lysed in the presence of AMS, mobility of CPY increased only slightly, consistent with AMS modification of the single

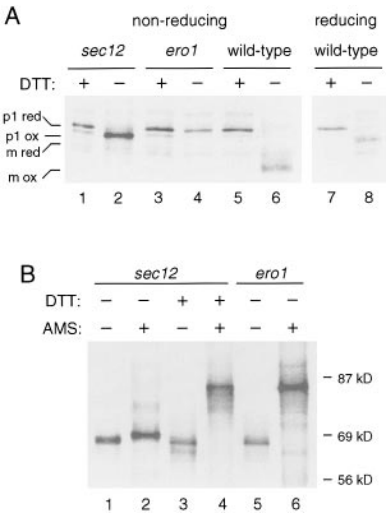

Figure 3. CPY Synthesized in an *ero1-1* Mutant Lacks Disulfide Bonds

(A) CKY10 (wild-type), CKY559 (*ero1-1*), and CKY39 (*sec12-4*) were shifted from 24°C to 38°C and then labeled with [^{35}S] methionine and cysteine for 30 min in the presence of 5 mM DTT (lanes 1, 3, 5, and 7) or in the absence of DTT (lanes 2, 4, 6, and 8). Cells were collected and lysed under nonreducing conditions in the presence of 20 mM NEM to block free thiols. CPY was immunoprecipitated from cell lysates, and samples were resolved by nonreducing (lanes 1–6) SDS–PAGE. Part of each sample was processed under reducing conditions (lanes 7 and 8). Reduced (red) and oxidized (ox) forms of both p1 (ER) and mature (vacuolar) CPY are indicated.

(B) CKY559 (*ero1-1*) and CKY39 (*sec12-4*) were labeled at 38°C as in (A) for 25 min. 5 mM DTT was added to a *sec12-4* sample 10 min before labeling (lanes 3 and 4). Cell pellets were lysed under nonreducing conditions in the presence of 20 mM of the thiol-modifying reagent AMS (lanes 2, 4, and 6), or in the absence of AMS (lanes 1, 3, and 5). CPY was immunoprecipitated from cell lysates and samples resolved by nonreducing SDS–PAGE.

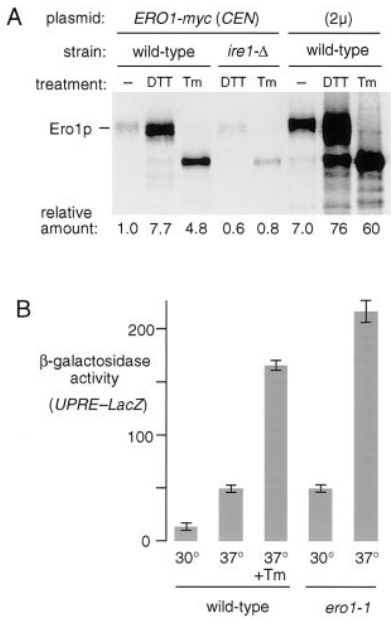

Figure 4. *ERO1* Is Induced by the Unfolded Protein Response, and an *ero1-1* Mutant Activates the UPR

(A) CKY10 (wild-type) and CKY561 (*ire1*-Δ) cells hosting pAF85 (*CEN ERO1-myc LEU2*) as well as wild-type cells carrying pAF84 (2μ *ERO1-myc URA3*) were labeled with [^{35}S] methionine and cysteine for 30 min. 5 mM DTT or 10 μg/ml of tunicamycin (Tm) was added to cells 10 min before labeling as indicated. Ero1p-myc was immunoprecipitated from cell lysates, and samples were resolved by SDS–PAGE. Relative amounts of Ero1p-myc were quantified with a 445si Phosphorimager.

(B) CKY10 (wild-type) and CKY559 (*ero1-1*) strains hosting the UPRE-*lacZ* reporter plasmid pCF118 received 0 or 2.5 μg/ml tunicamycin before incubation at either 30°C or 37°C for 2.5 hr. β-galactosidase activity was assayed and normalized to 1 $0D_{600}$ unit of permeabilized cells.

cysteine present in oxidized CPY (Endrizzi et al., 1994). These results show that disulfide bonds do not form in CPY expressed in an *ero1-1* mutant at restrictive temperature.

Ero1p Is Induced with the Unfolded Protein Response

Through the unfolded protein response (UPR), the ER transmembrane kinase Ire1p induces expression of ER chaperones, such as *KAR2* and *PDI1*, when incorrectly folded proteins accumulate in the ER (Cox et al., 1993; Kohno et al., 1993). Ero1p-myc expression increased about 8- or 5-fold upon treatment of wild-type cells with 5 mM DTT or 10 μg/ml of the glycosylation inhibitor tunicamycin (Figure 4A), conditions known to activate the UPR. In cells overproducing Ero1p-myc from a high copy plasmid (pAF84), Ero1p-myc expression increased a further 11- or 8-fold upon treatment with DTT or tunicamycin. Cells with a chromosomal deletion of *IRE1* (CKY561) failed to induce expression of Ero1p-myc in response to these reagents, and a potential unfolded protein response element (UPRE; Mori et al., 1992) was identified 401 bp upstream of the start codon of *ERO1*. The induction of Ero1p by the UPR is consistent with a role for *ERO1* in oxidative protein folding in the ER.

Partial loss of *ERO1* function appeared to produce a stress in the ER that activated the UPR. When incubated at permissive or restrictive temperature, an *ero1-1* mutant induced expression from a *UPRE-lacZ* reporter (pCF118) about 4-fold relative to a wild-type strain (Figure 4B). A similar induction of this reporter was observed when wild-type cells received 2.5 μg/ml of tunicamycin. Growth of the *ero1-1* mutant at semipermissive temperature may require a compensatory increase in *ero1-1* expression by the UPR since *ero1-1 ire1*-Δ double mutants were inviable at 30°C (data not shown).

Ero1p Levels Correlate with the Oxidizing Capacity of the Cell

Otherwise isogenic strains expressing hierarchical levels of Ero1p were tested for their response to membrane-permeable reductants and oxidants. To assay the sensitivity of our strains to exogenous reductant, 10 μl of 3 M DTT was applied on sterile filter disks to lawns of cells incubated at 30°C. The growth of an *ero1-1* mutant (CKY559) was inhibited in a zone 41 ± 0.6 mm in diameter surrounding the DTT source (Figure 5A). The zone of growth inhibition for wild-type cells (CKY10) was 21 ± 0.8 mm in diameter (Figure 5A). Cells incapable of inducing *ERO1* due to deletion of *IRE1* (CKY561) were

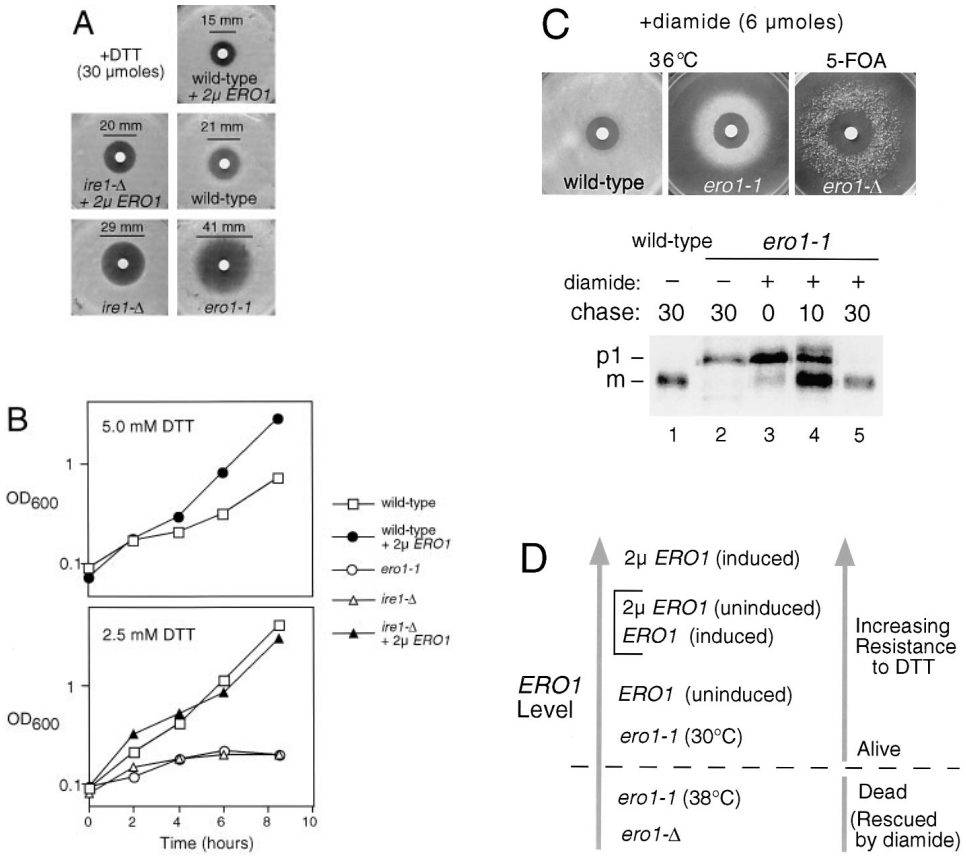

Figure 5. Response of Cells with Either Increased or Decreased *ERO1* Function to Exogenous Reductant (DTT) or Oxidant (Diamide)

(A) CKY10 (wild-type) with or without pAF84 (2μ *ERO1*-myc *URA3*), CKY561 (*ire1*-Δ) with or without pAF89 (2μ *ERO1-myc LEU2*), and CKY559 (*ero1-1*) were each plated as a lawn of 2×10^6 cells on YPD. 10 μl of 3 M DTT was applied to each lawn in a 6 mm filter disk, and the lawns were incubated at 30°C for 1.5 days. The average diameter of the zone of growth inhibition for each strain was determined from three experiments.

(B) Cultures of CKY10 (wild-type), CKY559 (*ero1-1*), and CKY561 (*ire1*-Δ) were grown to exponential phase, resuspended in YPD with 2.5 mM DTT, and incubated at 30°C. Growth of the cultures was measured as the change in optical density (OD600) with time. Growth of CKY10 cells with or without pAF84 (2μ *ERO1-myc URA3*) was also monitored in the presence of 5 mM DTT.

(C) CKY559 (*ero1-1*) and CKY10 (wild-type) were plated on YPD, and 6 μmol of diamide was applied to each lawn in a filter disk before incubation at restrictive temperature (36°C) for 2 days. CKY563 (*ero1*-Δ [p*CEN ERO1-myc URA3*]) was plated on SM medium supplemented with 1 mg/ml of 5-FOA to select for segregants that had lost the *URA3* plasmid. 6 μmol of diamide was applied to the lawn in a filter disk before incubation at 30°C for 7 days. To determine the effect of diamide on CPY transport in *ero1-1* cells, CKY559 grown at 24°C was resuspended in media with 0.6 mM diamide and shifted to 38°C. Cells were pulse-labeled with [^{35}S] methionine and cysteine for 7 min and chased for 30 min (lanes 3–5). CKY10 and CKY559 were also labeled in the absence of diamide (lanes 1 and 2). CPY was immunoprecipitated from cell lysates and samples resolved by SDS–PAGE. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

(D) Diagram summarizing how the oxidizing capacity of the cell correlates with *ERO1* expression in a set of isogenic strains.

inhibited in a zone 29 ± 0.7 mm in diameter around the DTT source. The heightened sensitivity of both *ero1-1* and *ire1*-Δ mutants to DTT was also evident in growth assays of liquid cultures: 2.5 mM DTT completely inhibited growth of these mutants at 30°C but only slowed growth of a wild-type strain (Figure 5B). The extreme sensitivity of the *ero1-1* mutant to DTT at a temperature permissive for growth suggested that even a partial loss of Ero1p function greatly diminished the capacity of cells to oxidize proteins in the ER.

Increased Ero1p levels rendered cells more resistant to DTT. In the plate assay described above, the zone of growth inhibition for wild-type cells overexpressing *ERO1-myc* (from pAF84) was only 15 ± 0.7 mm in diameter around the DTT source (Figure 5A). This strain could grow to four times the cell density of a wild-type strain during 8 hr of growth in liquid culture containing 5 mM DTT (Figure 5B). The growth of wild-type cells in the presence of DTT was not improved by overexpression of *PDI1* or *EUG1* from the *GAL1* promoter (data not shown). Furthermore, overexpression of *ERO1-myc* (from pAF89) in an *ire1*-Δ mutant restored to the strain wild-type resistance to DTT as observed both on plates and in liquid medium with 2.5 mM DTT (Figures 5A and 5B). Increased *ERO1* expression can thus account for the natural resistance of cells to DTT afforded by the UPR, and Ero1p appears to be the limiting factor required for cells to overcome toxic levels of DTT.

As the *ero1-1* mutant was hypersensitive to DTT at permissive temperature, we asked whether the growth

and secretion defects of this mutant at restrictive temperature could be suppressed by an exogenous oxidant. The membrane-permeable diazine compound diamide drives formation of disulfide bonds in living cells (Kosower and Kosower, 1995). We applied 6 μmol of diamide to a filter disk on a lawn of *ero1-1* cells and incubated the lawn at restrictive temperature (36°C). The *ero1-1* mutant could grow in a ring surrounding the diamide source, demonstrating that appropriate concentrations of diamide suppressed the temperature sensitivity of *ero1-1* cells (Figure 5C). Wild-type and the *ero1-1* strain displayed equal sensitivity to high diamide concentrations. By a similar plate assay, diamide was also found to rescue the inviability of *ero1*-Δ cells. A strain with a chromosomal deletion of *ERO1* covered by *ERO1-myc* on a *URA3* plasmid (CKY563) was plated on SM medium containing 5-fluoroorotic acid (5-FOA) to select for segregants that had lost the covering plasmid, and 6 μmol of diamide were applied to a filter disk on the lawn. A ring of colonies grew around the filter disk, indicating that appropriate concentrations of diamide could compensate for complete loss of *ERO1* function. These colonies were dependent on diamide for continued growth and were *ura3*$^-$, verifying that they had lost their functional allele of *ERO1*. Diamide could also restore oxidative protein folding in *ero1-1* cells. Newly synthesized CPY was rapidly transported to the vacuole in an *ero1-1* mutant growing in 0.6 mM diamide at restrictive temperature (38°C; Figure 5C). Diamide does not appear to suppress general defects in ER protein folding since the temperature sensitivity of *kar2-159* (CKY222) and *kar2-203* (CKY229) mutants could not be rescued by the oxidant (data not shown).

Figure 5D summarizes the response of cells with different levels of *ERO1* function to exogenous reductant or oxidant. *ERO1* expression correlates with resistance to DTT, and complete loss of *ERO1* function renders cells dependent on diamide. These results support the view that *ERO1* function provides the oxidizing equivalents required for oxidative protein folding in the ER and thereby sets the oxidizing capacity of the cell.

Ero1p and PDI Perform Distinct Essential Functions

As *ERO1* appeared to encode a new type of protein required for disulfide bond formation in the ER, we explored the relationship of *ERO1* to members of the PDI gene family. The finding that exogenous diamide could compensate for loss of *ERO1* provided a useful criteria for defining gene function. We therefore tested the ability of diamide to rescue the inviability of *pdi1*-Δ cells. A strain with a chromosomal deletion of *PDI1* covered by *pGAL1-PDI1* on a *URA3* plasmid (CKY564) was plated on medium containing 5-FOA, and 6 μmol of diamide were applied to the lawn in a filter disk. Colonies were never detected on these plates (data not shown), suggesting that diamide could not provide or bypass the essential function of *PDI1*.

Functional redundancies between members of the PDI family have been revealed by observation that overexpression of either *EUG1* or *MPD1* can restore viability to *pdi1*-Δ cells (Tachibana and Stevens, 1992; Tachikawa et al., 1995). We therefore asked if *ERO1* overexpression could also rescue *pdi1*-Δ cells. A *LEU2* marked plasmid overexpressing *ERO1-myc* (pAF89) was introduced into CKY564 and the transformants plated on medium containing 5-FOA. Colonies never appeared on these plates, indicating that Ero1p overproduction could not provide the essential function of PDI. As a positive control, we confirmed that overexpression of *EUG1* from the *GAL1* promoter (pCT44) allowed CKY564 segregants to grow in the presence of 5-FOA. In addition, overexpression of *PDI1* or *EUG1* from the *GAL1* promoter (pCT37 or pCT44) did not suppress the temperature-sensitive growth defect of the *ero1-1* mutant at 38°C (CKY559; data not shown). Together, these genetic tests indicate that Ero1p and PDI perform distinct, essential functions.

Consistent with the view that Ero1p functions in the same overall pathway for native disulfide bond formation as PDI, we found the growth defect of the *ero1-1* mutant to be exacerbated by a reduction in PDI levels. Removal of the HDEL signal for ER retention from the C terminus of PDI reduces intracellular PDI levels (LaMantia and Lennarz, 1993). In a cross between *ero1-1* (CKY559) and *PDI1*-Δ*HDEL* (CKY395) strains, the double mutant segregants were inviable at 24°C. This interaction appears specific since *ero1-1 KAR2*-Δ*HDEL* double mutants were viable at temperatures below 36°C.

Glutathione Is Not Required for Oxidative Protein Folding in the ER

As oxidized glutathione (GSSG) is an abundant disulfide-bonded species in the ER (Hwang et al., 1992), we assessed the contribution of GSSG to oxidative protein folding. The *GSH1* gene of *S. cerevisiae* encodes γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step in glutathione synthesis. Mutation of *GSH1* produces cells without detectable intracellular glutathione (Ohtake and Yabuuchi, 1991). As previously reported, we found that the presence of DTT substantially restored the ability of a *gsh1*-Δ strain (CKY565) to grow on minimal medium (Ohtake and Yabuuchi, 1991; Grant et al., 1996; Figure 6A). This observation suggested that although GSH has a critical role as a reductant, GSSG could be dispensable as an oxidant in the ER. To test this hypothesis, the *gsh1*-Δ mutant was grown without exogenous glutathione and the processing of CPY monitored by pulse–chase analysis. In the *gsh1*-Δ mutant, p1 CPY matured to the vacuolar form at a normal rate, indicating that oxidative folding of CPY occurred in the absence of glutathione (Figure 6B). Thus, there does not appear to be an absolute requirement for GSSG as an oxidant during disulfide bond formation in the ER.

Discussion

We describe a novel gene, *ERO1*, that is required for the net formation of protein disulfide bonds in the ER. Several lines of evidence support this conclusion. (1) Ero1p is an N-linked glycoprotein restricted to the ER lumen. (2) Ero1p expression is induced by the unfolded protein response. This regulation is a hallmark of genes that assist protein folding in the ER. (3) The conditional

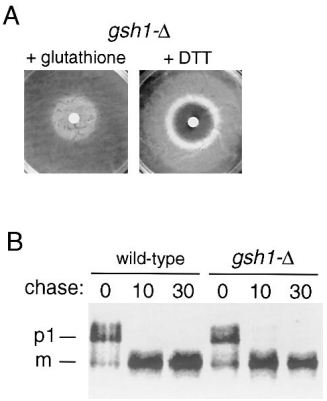

Figure 6. Oxidative Protein Folding in the ER Does Not Require Glutathione

(A) Lawns of CKY565 (*gsh1-Δ::URA3*) were plated on SM medium and either 0.05 μmol of glutathione or 30 μmol of DTT applied to the lawns in filter disks. Plates were incubated at 30°C for 2 or 5 days, respectively.

(B) CKY10 (wild-type) and CKY565 (*gsh1-Δ::URA3*) were grown in SM medium lacking methionine at 30°C, pulse-labeled with [^{35}S] methionine and cysteine for 7 min, and chased. CPY was immunoprecipitated from cell lysates, and samples were resolved by SDS–PAGE. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

mutation *ero1-1* causes a complete block in the ER-to-Golgi transport of CPY, a protein whose native structure contains disulfide bonds, but does not affect the transport of invertase, a secreted protein that lacks disulfide bonds. The *ero1-1* mutation also inhibits maturation of Gas1p, a protein shown to require oxidizing conditions for export from the ER. Moreover, the *ero1-1* mutation prevents the formation of disulfide bonds in CPY, as shown by an increase in the reactivity of CPY with the thiol modifying reagent AMS. (4) A decrease in *ERO1* activity, caused by either the *ero1-1* mutation at permissive temperature or a block in *ERO1* induction imposed by deletion of *IRE1*, renders cells more sensitive to the reductant DTT. Conversely, an increase in *ERO1* activity, caused by overexpression of the gene, renders cells more resistant to DTT. (5) Although *ERO1* is required for both oxidative protein folding and for cell viability, both processes can be restored to an *ero1-1* mutant by addition of the thiol oxidant diamide to the growth medium. These findings indicate that the essential function of Ero1p is to generate oxidizing equivalents in the ER, and further, that the oxidizing capacity of the cell correlates with the level of Ero1p activity.

Examination of the most conserved regions of Ero1p provides clues to the mechanism of *ERO1* function. At least five cysteines in Ero1p are absolutely conserved, and three of these appear in the sequence CXXCXXC (see Figure 1A). This motif is known to specify part of the binding site for an iron-sulfur cluster in proteins related to ferredoxin (Beinert, 1990). This raises the intriguing possibility that the mechanism of *ERO1* function involves an electron transfer reaction at a non–heme iron center, or the CXXCXXC motif could also include an active site of a thioredoxin-like oxidoreductase. Although Ero1p does not exhibit any obvious sequence similarity to proteins of the thioredoxin superfamily, Ero1p may nonetheless contain a thioredoxin-like fold, as primary sequence alone may not be sufficient to identify this domain (Ellis et al., 1992). Cysteine residues not included in the CXXCXXC motif could also form a reactive disulfide bond in Ero1p. We are currently evaluating the potential roles of Ero1p's conserved cysteines in the conjugation of iron and in the formation of reactive disulfide bond(s).

The essential function of Ero1p can be distinguished from that of PDI and related proteins. Whereas overexpression of genes related to *PDI1*, such as *EUG1,* can restore viability to PDI-deficient cells (Tachibana and Stevens, 1992), overexpression of *ERO1* could not. Similarly, the temperature sensitivity of an *ero1-1* mutant could not be suppressed by overexpression of *PDI* or *EUG1*. Moreover, whereas the oxidant diamide can suppress defects associated with complete loss of *ERO1* function, diamide could not restore viability to *pdi1-Δ* cells. One model consistent with these observations is that the essential function of Ero1p in native disulfide bond formation precedes that of PDI.

We have developed an assay for the in vivo redox state of CPY based on modification of free protein thiols with AMS. This assay reveals that Ero1p is necessary for oxidation of protein dithiols in the ER. PDI is necessary for the reshuffling of nonnative disulfide bonds in vivo (Laboissière et al., 1995), but the extent to which PDI may participate in protein oxidation in the ER remains unclear, in part because a direct assay for dithiol oxidation has not been performed on CPY expressed in PDI-deficient cells.

As an abundant disulfide-bonded species in the ER, oxidized glutathione (GSSG) has been suggested to serve as the source of oxidizing equivalents for the formation of protein disulfide bonds in the ER (Hwang et al., 1992). We therefore examined the role of glutathione in oxidative protein folding. A *gsh1-Δ* mutant, which has no detectable intracellular glutathione, carries out oxidative protein folding normally as measured by the rate of CPY maturation. Thus, GSSG does not appear to serve as an obligatory intermediate during disulfide bond formation in the ER. Since the native disulfide bonds in CPY link nonsequential cysteine residues (Endrizzi et al., 1994), glutathione also appears to be dispensable for the rearrangement of disulfide bonds. GSH and GSSG may nevertheless be important for ER function; at their high concentration, GSH and GSSG should act to buffer the redox state of the ER against transient changes.

The pathway for oxidative protein folding in the *E. coli* periplasm provides a useful analogy for thinking about the mechanism of oxidative folding in the ER. Disulfide bond formation in the periplasm proceeds in the absence of a small molecule oxidant such as GSSG. Disulfide bonds are donated to substrates through dithiol-disulfide exchange reactions with DsbA, a periplasmic protein structurally related to thioredoxin that contains a CXXC active site (Bardwell et al., 1991). Oxidized DsbA is regenerated through a dithiol-disulfide exchange reaction with the cytoplasmic membrane protein DsbB (Guilhot et al., 1995; Kishigami et al., 1995). The mechanism for reoxidation of DsbB is not known. The periplasmic oxidoreductase DsbC operates in an analogous

Table 1. Yeast Strains

Strain	Genotype	Source
CKY8	*MAT*α *ura3-52 leu2-3, 112*	Kaiser Lab Collection
CKY10	*MAT***a** *ura3-52 leu2-3, 112*	”
CKY39	*MAT*α *sec12-4 ura3-52 his4-619*	”
CKY406	*MAT*α *suc2-Δ9 ura3-52 leu2-3, 112*	”
CKY560	*MAT*α *sec6-4 ura3-52 leu2-3, 112*	This study
CKY558	*MAT***a** *ero1-1 ura3-52 leu2-3, 112 ade2*	”
CKY559	*MAT*α *ero1-1 ura3-52 leu2-3, 112*	”
CKY561	*MAT***a** *ire1-Δ::URA3 ura3-52 leu2-3, 112*	”
CKY562	*MAT***a**/α *ero1-Δ::LEU2/ERO1 leu2-3, 112/leu2-3, 112 ura3-52/ura3-52*	”
CKY563	*MAT*α *ero1-Δ::LEU2 ura3-52 leu2-3, 112* [pAF82]	”
CKY222	*MAT***a** *kar2-159 ura3-52 leu2-3, 112*	Mark Rose (MS174)
CKY229	*MAT***a** *kar2-203 ura3-52 leu2-3, 112 ade2-101*	Mark Rose (MS1032)
CKY190	*MAT*α *KAR2-ΔHDEL suc2-Δ9 ura3-52 leu2-3, 112 his4-619*	Mark Rose
CKY395	*MAT*α *pdi1-Δ::TRP1-PDI1-ΔHDEL leu2-3, 112::LEU2-UPRE-lacZ ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100*	Caroline Shamu (CS297)
CKY564	*MAT*α *pdi1-Δ::HIS3 ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 can 1-100* [pCT37]	Tom Stevens
CKY565	*MAT***a**/α *gsh1-Δ1::URA3/gsh1-Δ1::URA3 leu2-Δ1/LEU2 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/LYS2 trp1-Δ1/TRP1 trp5/TRP5*	Martin Grey (M65312)

pathway for the isomerization of nonnative disulfide bonds and is maintained in its dithiol form by DsbD, another dithiol-disulfide oxidoreductase found in the cytoplasmic membrane (Missiakas et al., 1995).

The properties of *E. coli dsbB* are strikingly similar to those of yeast *ERO1*. Overexpression of *dsbB* increases the resistance of *E. coli* cells to DTT, whereas mutation of *dsbB* renders cells more sensitive to DTT (Missiakas et al., 1993). In addition, defects in disulfide bond formation associated with null alleles of *dsbB* can be suppressed by providing cells with an exogenous source of oxidizing equivalents such as GSSG or cystine (Bardwell et al., 1993). We propose that Ero1p performs a function in the ER membrane similar to that performed by DsbB in the cytoplasmic membrane. A disulfide bond in Ero1p could ultimately be donated to a substrate protein either through a direct dithiol-disulfide exchange reaction, or through a series of such reactions mediated by the oxidoreductases related to PDI that reside in the ER lumen. In addition to *PDI1* and *MPD1*, *S. cerevisiae* has two ORFs, YOL088c and YIL005w, which also specify proteins that contain a thioredoxin-like motif and would be predicted to reside in the ER (Cherry et al., 1997). As a group, the five yeast proteins related to PDI could catalyze disulfide bond formation and rearrangement in the ER in much the same way as DsbA and DsbC jointly accomplish these activities in *E. coli*. In this respect, it is significant that mammalian PDI can partially substitute for DsbA in the periplasm of *E. coli* (Humphreys et al., 1995).

The mechanism by which Ero1p could be oxidized is unclear. Presumably, an oxidation-reduction process in the ER membrane could provide the necessary oxidizing equivalents. One possibility is that oxidation of Ero1p could be coupled to one of the redox reactions of fatty acid or sterol modification that take place in the ER membrane.

With the means to isolate Ero1p from cells and examine its interactions with other proteins, it should be possible to place Ero1p in a pathway for disulfide bond formation in the ER. The apparent conservation of Ero1p suggests that the mechanism for ER oxidation in yeast is likely to be conserved in all eukaryotes.

Experimental Procedures

Strains and Plasmids

S. cerevisiae strains were grown and genetically manipulated using standard techniques (Kaiser et al., 1994). YPD is rich medium with 2% glucose, YEP is rich medium with a specified carbon source, and SD and SMM are minimal media (Difco Laboratories) with either 2% glucose or a specified carbon source. SMM is supplemented with 16 amino acids, not including cysteine.

Genotypes of strains used in this study appear in Table 1. The *ero1-1* mutant was isolated from a collection of temperature-sensitive (Ts^-) strains in the genetic background A364A (Hartwell et al., 1973) and backcrossed to our wild-type genetic background S288C to produce CKY558 and CKY559. In final backcrosses, temperature sensitivity and a CPY transport defect cosegregated 2:2 in all tetrads examined. pAF9 is a YCp50 library (Rose et al., 1987) plasmid that rescued growth of CKY558 at 38°C and contains a 6.5 kb genomic insert including YML130c. pAF23 carries a 2.6 kb SalI–XbaI fragment of pAF9 in pRS306 (Sikorski and Hieter, 1989). To show linkage between YML130 and *ERO1*, pAF23 linearized with EcoRI was introduced into the chromosome of CKY10 by homologous recombination. When integrants were crossed to CKY559, temperature sensitivity and uracil auxotrophy cosegregated 2:2 in all tetrads examined. *ERO1-myc* was constructed by introducing a NotI site after the last codon of *ERO1* through site-directed mutagenesis and inserting a NotI fragment encoding three copies of the c-myc epitope: EQKLISEEDLN. pAF82 contains *ERO1-myc* as well as genomic sequence 1156 bp 5′ of the *ERO1* ATG and 394 bp 3′ of the stop codon in pRS316 (*CEN URA3*). The pAF82 insert was cloned into pRS315 (*CEN LEU2*), pRS306-2μ (2μ *URA3*), and pRS305-2μ (2μ *LEU2*; Sikorski and Hieter, 1989) to create pAF85, pAF84, and pAF89, respectively.

LEU2 (from pJJ252; Jones and Prakash, 1990) was cloned into pAF23 digested with BglII and HindIII to yield pAF25. To generate CKY562, the XhoI–NotI *ero1-Δ::LEU2* fragment from pAF25 was introduced at the *ERO1* locus of a *ura3-52 leu2-3,112* diploid by one-step gene replacement. Sporulation of CKY562 transformed with pAF9 or pAF82 gave viable Ura^+ Leu^+ spore clones dependent on episomal *ERO1* for viability since Ura^- segregants could not be isolated from them on 5-fluoroorotic acid plates (Toronto Research Labs). CKY563 is one such clone carrying pAF82.

To construct CKY561, the *ire1-Δ::URA3* fragment from pCS109A (Cox et al., 1993) was introduced at the *IRE1* locus of CKY10 by one-step gene replacement. The *ire1-Δ* phenotype was verified by the inability of CKY561 to induce *lacZ* expression from pCF118, a *CEN LEU2* plasmid containing the 5′ region of *KAR2* fused to *lacZ* (Sidrauski et al., 1996). The phenotype of the *gsh1-Δ::URA3* strain (CKY565; Schmidt et al., 1996; courtesy of Martin Brendel) was verified by plating 2×10^6 cells on either SD with adenine or SMM, applying either 0.05 μmol of glutathione or 30 μmol of DTT to the

lawns in 6 mm sterile filter disks (Fisher Scientific), and incubating the lawns at 30°C for 2–5 days. On SD, CKY565 grew only in response to glutathione or DTT.

pNV31 carries *pTPI1-SUC2* in a *CEN URA3* vector (Mike Lewis). pCT37 carries *pGAL1-PDI1* in a *CEN URA3* vector; pCT44 carries *pGAL1-EUG1* in YEp351 (2μ *LEU2*; Tachibana and Stevens, 1992). pAF92 contains *pGAL1-PDI1* in pCD43 (*CEN URA3*) and was shown to overproduce PDI by immunoblotting with anti-PDI (courtesy of Peter Walter).

Radiolabeling and Immunoprecipitations

Strains were grown in SMM lacking methionine or SD with auxotrophic supplements to about 1×10^7 cells/ml and resuspended at 1×10^8 cells/ml. Cell proteins were labeled with 40 μCi of [^{35}S] methionine and cysteine (NEN-Dupont) per OD_{600} unit of cells for 7 min and then chased with excess methionine and cysteine. Samples of 4×10^7 cells were collected in 20 mM NaN_3 and lysed by resuspension in 30 μl of 80 mM Tris–HCl (pH 6.8), 2% β-mercaptoethanol, 2% SDS, and 1 mM PMSF; boiling for 2 min; and agitation with glass beads. Extracts were suspended in 1 ml of IP buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, I mM PMSF) and preadsorbed with fixed Staphylococcus A cells (Sigma) before incubation with primary antibody for 2 hr at 24°C. 1 μl of anti-CPY, 1 μl of anti-invertase, or 4 μl of anti-Gas1p (courtesy of H. Reizman) was used per OD_{600} unit of lysate. Immune complexes were collected with protein A–Sepharose (Pharmacia), washed in IP buffer, and solubilized in 40 μl of sample buffer (80 mM Tris–HCl [pH 6.8], 100 mM DTT, 2% SDS, 1 mM PMSF, 10% glycerol, 0.1% bromphenol blue). Samples were resolved by SDS–PAGE and analyzed with a 445si Phosphorimager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Ts$^-$ mutants were grown at 24°C and shifted to 38°C for 25 min before labeling. As indicated, cultures received 0.6 mM diamide upon shift to 38°C or 5 mM DTT 10 min prior to labeling. For detection of invertase, cells carrying pNV31 were labeled for 10 min and then converted to spheroplasts as described (Hong et al., 1996). Spheroplast pellet and supernatant fractions received CKY406 extract along with anti-invertase. Gas1p processing was monitored in a temperature-sensitive *sec6-4* mutant (CKY560) that blocks fusion of post-Golgi secretory vesicles with the plasma membrane and stabilizes mature Gas1p from proteolytic degradation.

To monitor Ero1p-myc expression, strains grown at 30°C were labeled with [^{35}S] methionine and cysteine for 30 min. When indicated, cells received 5 mM DTT or 10 μg/ml of tunicamycin (Sigma) 10 min prior to labeling. Ero1p-myc was immunoprecipitated with 1.5 μl of 9E10 (monoclonal anti-myc) per OD_{600} unit of lysate and digested with endoH as described (Hong et al., 1996). 9E10 immune complexes were solubilized by boiling in sample buffer and divided prior to reimmunoprecipitation with either 9E10 or anti-α1,6-mannose. Cell fractionation was performed on CKY10 hosting pAF84 as described except that spheroplasts were lysed gently with a dounce homogenizer (Espenshade et al., 1995).

Assays of Disulfide Bond Formation in CPY

To examine the mobility of CPY during nonreducing SDS–PAGE, strains were shifted from 24°C to 38°C for 20 min and radiolabeled as described for 30 min. Strains received 0 or 5 mM DTT 10 min before labeling. Cell samples were processed in the presence of 20 mM N-ethylmaleimide (NEM, Sigma) and lysed in the absence of β-mercaptoethanol. CPY immune complexes were solubilized in sample buffer without DTT and resolved by SDS–PAGE. Half of each cell pellet was processed under reducing conditions (as above).

To assay the reactivity of CPY with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Molecular Probes), cell samples derived from strains radiolabeled at 38°C for 25 min were divided prior to lysis in 50 μl of either 80 mM Tris–HCl (pH 6.8), 1% SDS, 1 mM PMSF, and 20 mM AMS or an identical buffer without AMS. Extracts were further incubated with 100 μl of lysis buffer for 1 hr at 24°C prior to immunoprecipitation of CPY. Samples were resolved by nonreducing SDS–PAGE.

Induction of the Unfolded Protein Response

Strains hosting pCF118 (Sidrauski et al., 1996) were grown selectively at 24°C and resuspended at 1×10^7 cells/ml in YPD. Cultures were given 0 or 2.5 μg/ml of tunicamycin and incubated at 30°C or 37°C for 2.5 hr. Cells were permeabilized and β-galactosidase activity assayed as described (Guarente, 1983). Two transformants were assayed per strain and the experiment repeated twice.

Assays for Growth in the Presence of DTT or Diamide

Strains were grown selectively to exponential phase at 24°C prior to assays for DTT sensitivity. Triplicate lawns of 2×10^6 cells were plated on YPD and 10 μl of 3 M DTT applied to the lawns in 6 mm filter disks. Diameters of the zone of inhibition were measured after incubation for 1.5 days at 30°C. Growth was also assayed by the change in OD_{600} over time of duplicate cultures given 0, 2.5, or 5.0 mM DTT and incubated at 30°C in YPD. To test for diamide rescue of Ts$^-$ strains, lawns of 3×10^6 cells were plated on YPD and given 6 μmol of diamide in a filter disk before incubation at 36°C (for CKY559) or 33°C (for CKY222 and CKY229) for 2 days. CKY563 and CKY564 (*pdi1*-Δ pCT37; courtesy of Tom Stevens) were grown overnight in 0.4 mM diamide in YPD or YEP 2% raffinose, 2% galactose. Lawns of 3×10^6 cells were then plated on SMM supplemented with 1 mg/ml 5-FOA to select for cells that had lost the plasmid. The lawns received 6 μmol of diamide on a filter disk and were incubated for 7 days at 30°C. Colonies derived from CKY563 that grew in a ring around the filter were isolated and plated surrounding a disk with 6 μmol of diamide on SMM with or without uracil.

Tests for Genetic Interactions between *ERO1* and *PDI1*, *KAR2*, and *IRE1*

CKY559 transformants carrying pAF82, pCT37, pCT44, or pRS316 as well as CKY564 and CKY564 transformants carrying pCT44 or pAF89 were grown selectively in SMM 2% raffinose, 2% galactose and then resuspended at 2×10^7 cells/ml. CKY559 transformants were spotted on YEP 2% raffinose, 2% galactose plates and incubated at 38°C for 3 days. CKY564 transformants were spotted on SMM with either 2% raffinose, 2% galactose, or 2% glucose as well as 1 mg/ml 5-FOA and incubated at 30°C for 4–5 days.

CKY558 or CKY559 (*ero1-1*) was crossed with CKY395 (*PDI1*-Δ*HDEL*), CKY190 (*KAR2*-Δ*HDEL*), or CKY561 (*ire1*-Δ*::URA3*). Tetrads were dissected on YPD at 24°C, and double mutant progeny were identified after viable spore clones were tested for growth between 30°C and 38°C in addition to either a dominant Kar2p secretion phenotype (Elrod-Erickson and Kaiser, 1996) or uracil prototrophy. Crossing CKY558 to CKY395 gave tetrads with 4, 3, or 2 viable spores where segregation of Ts$^-$ growth was consistent with the assignment of inviable spores as *ero1-1 PDI1*-Δ*HDEL* mutants.

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