The C-terminal domain of yeast Ero1p mediates membrane localization and is essential for function

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Abstract In eukaryotes, members of the Ero1 family control oxidative protein folding in the endoplasmic reticulum (ER). Yeast Ero1p is tightly associated with the ER membrane, despite cleavage of the leader peptide, the only hydrophobic sequence that could mediate lipid insertion. In contrast, human Ero1-Lα and a yeast mutant (Ero1pΔC) lacking the 127 C-terminal amino acids are soluble when expressed in yeast. Neither Ero1-Lα nor Ero1pΔC complements an *ERO1* disrupted strain. Appending the yeast C-terminal tail to human Ero1-Lα restores membrane association and allows growth of *ERO1* disrupted cells. Therefore, the tail of Ero1p mediates membrane association and is crucial for function. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Disulfide bond; Oxidative folding; Oxidoreductase; Redox; Secretion; Endoplasmic reticulum; Membrane insertion

1. Introduction

In *Saccharomyces cerevisiae*, the essential gene *ERO1* encodes an endoplasmic reticulum (ER) resident oxidoreductin, Ero1p, which plays a key role in the process of disulfide bond formation [1,2]. Ero1p has been shown to bind FAD [3], and transfer oxidizing equivalents to protein disulfide isomerase (PDI) [4]. Its levels of expression correlate with tolerance to exogenous reducing agents [1,2]. We found and characterized two human homologues of *ERO1*, *ERO1-Lα* and *ERO1-Lβ* [5,6]. Also in human cells, PDI forms mixed disulfides with Ero1-Lα [7] and Ero1-Lβ [8]. Altogether, the pathways of disulfide bond formation therefore seem conserved between yeast and human cells, and electrons flow from cargo proteins to Ero1 proteins via PDI. Particularly in professional secretory cells, Ero1 proteins must be continuously reoxidized so as to sustain efficient oxidative folding. However, the redox state of the ER must be precisely controlled, as disulfide bonds are not only formed in this organelle, but also isomerized and reduced before dislocation of misfolded proteins to the cytosol for proteasomal degradation [9,10]. The precise mechanisms that control the redox state of Ero1p are not fully understood.

Both human Ero1-Lα and Ero1-Lβ complement a yeast thermosensitive mutant strain (*ero1-1*) indicating functional conservation amongst the Ero1 family. Indeed, the protein sequences are rather conserved, especially around the CXXCXXC motif, which is crucial for their function [1,5,7]. A striking sequence difference, however, is represented by the last C-terminal 127 residues of the yeast protein, which are absent in the two human proteins. We have already demonstrated that the presence of this C-terminal tail is not essential for rescuing the thermosensitivity of *ero1-1*, as human Ero1-Lα and Ero1-Lβ and a truncated yeast mutant Ero1p-ΔC lacking it allow growth at the restrictive temperature [5].

An intriguing feature of proteins of the Ero1 family is that, despite the absence of hydrophobic sequences that could mediate lipid insertion, they fractionate with the membrane fraction [1,5,6]. In this study, we provide evidence that Ero1p is associated with the ER membrane even after cleavage of the leader sequence. Biochemical and functional analyses exploiting a *ERO1* disrupted strain demonstrate that the C-terminal tail mediates membrane association and is essential for Ero1p function.

2. Materials and methods

2.1. Yeast strains and plasmids

The following *S. cerevisiae* strains were used: SEY6210: *MATα*; *leu2-3, 112; ura3-52; his3-Δ200; trp1-Δ901; lys2-801; suc2-Δ9*; SEY6211: *MAT**a**; leu2-3, 112; ura3-52; his3-Δ200; trp1-Δ901; ade2-101; suc2-Δ9*; MPY201: *MATα/MAT**a**; leu2-3, 112/leu2-3, 112; ura3-52/ura3-52; his3-Δ200/his3-Δ200; trp1-Δ901/trp1-Δ901; suc2-Δ9/suc2-Δ9; lys2-801/LYS2; ade2-101/ADE2; ERO1/ERO1::kan*^{r}; MPY202: *MATα*; *leu2-3, 112; ura3-52; his3-Δ200; trp1-Δ901; lys2-801; suc2-Δ9*; *ERO1::kan*^{r} [pVT-ER01]; CKY559: *MATα*; *leu2-3, 112; ura3-52; ero1-1*.

These strains were grown and manipulated using standard techniques [5,6]. Rich (YEP), minimal (SD) and sporulation media (SP) were supplemented with 2% glucose or galactose (Difco Laboratories, Detroit, MI, USA), as specified. The four genes of interest were cloned into pVT102U, carrying the *URA3* marker gene, and YEP181, carrying *LEU2*, for constitutive and galactose inducible expression, respectively.

The chimeric gene *ERO1-Lα-CY* was obtained by amplifying the C-

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Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase

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terminal domain of the *ERO1* gene using the primers CYfw: GGGGTACCAACGGTTCTGAAAACAGG; CYrev: CGGGATCCCGTTAGCGGCCGCTACT, and cloning it in frame at the end of the *ERO1-Lα* gene, using the restriction sites *Kpn*I and *Bam*HI.

2.2. Construction of the ERO1::kan^r strain

To generate the *ERO1::kan*^{r} strain we used a one step gene replacement of *ERO1* with a *kan*^{r} cassette [11] generated by PCR using the following primers, which contain 40 nucleotides from the flanking sequences of the *ERO1* gene and 19 or 21 nucleotides of the *kan*^{r} gene, respectively: ERO1fw: ACATTTTATTGCATATACCACAGTAACGTGCAGGTAAAACCAGCTGAAGCTTCGTACGC; ERO1rev: CATATAAGATAAACTGTGTTGCTCTTATTGTATATCTAGCGCATAGGCCACTAGTGGATCTGA.

Three primers were designed to verify the *ERO1* disruption: Kan1-rev: CGA TAG ATT GTC GCA CC; ERO1verRev: GCA AGC GCG GTT TAA GC; ERO1verFW: CCA CTT AAA CTG GTT ATA.

Disruption was performed in the diploid strain MPY201. *ERO1/ERO1::kan*^{r} strains were selected on YEP containing 200 μg/ml Geneticin (Gibco BRL, Milan, Italy). This strain was transformed with pVTERO1, pVTERO1ΔC, and pVTERO1-Lα and sporulated. The ascus walls were partially digested with lyticase (Sigma, St. Louis, MO, USA) and the spores individually distributed in a row on a rich medium plate. The viability of the spore clones was then analyzed. One spore clone, *ERO1::kan*^{r} transformed with pVTERO1, was selected (MPY202) and transformed with the four genes cloned in the YEP181 plasmid (Fig. 1B).

Plasmid shuffle was performed on SD supplemented with 2% galactose, 50 mg/l uracil and 5′-fluoroorotic acid (5′-FOA; Sigma) to selectively kill cells containing the pVTERO1, *URA3* plasmid.

2.3. Membrane partitioning

The SEY6210 strain was transformed with pVTERO1myc, pVTERO1ΔC-HA, pVTERO1-Lαmyc and pVTERO1-Lα-CYmyc. The presence of C-terminal myc or HA tags did not alter the capability of the recombinant proteins to rescue growth of the *ero1-1* mutant at the non-permissive temperature ([5], M. Pagani and S. Pilati, unpublished results). Cells growing logarithmically at 30°C were harvested, washed and incubated in Tris–HCl 100 mM, pH 9.4, dithiothreitol (DTT) 10 mM for 10 min at room temperature. Cell were then incubated in 1.2 M sorbitol, 20 mM KPO_4, pH 7.5 with 50 U lyticase for 45 min at room temperature. After two washes with 1.2 M sorbitol, cells were broken by freezing and thawing in 0.6 M sorbitol, 10 mM Tris–HCl pH 7.4, plus a cocktail of protease inhibitors (Sigma). Samples were centrifuged at $10\,000\times g$ for 30 min to remove unbroken cells and debris. The supernatant was further centrifuged at $100\,000\times g$ for 45 min. The supernatant was transferred to a new tube and precipitated with acetone. The pellet was either resuspended in sample buffer, or resuspended in 0.6 M sorbitol, 10 mM Tris–HCl pH 7.4, plus protease inhibitors supplemented with 1% Triton X-100 or 5 M urea and centrifuged again. Samples were resolved by SDS–PAGE and analyzed by Western blotting using monoclonal anti-myc (9E10) or anti-HA (12CA5) antibodies. As controls, carboxypeptidase Y and PDI were identified by Western blotting with antibodies kindly provided by Dr. Karin Romisch (Cambridge, UK).

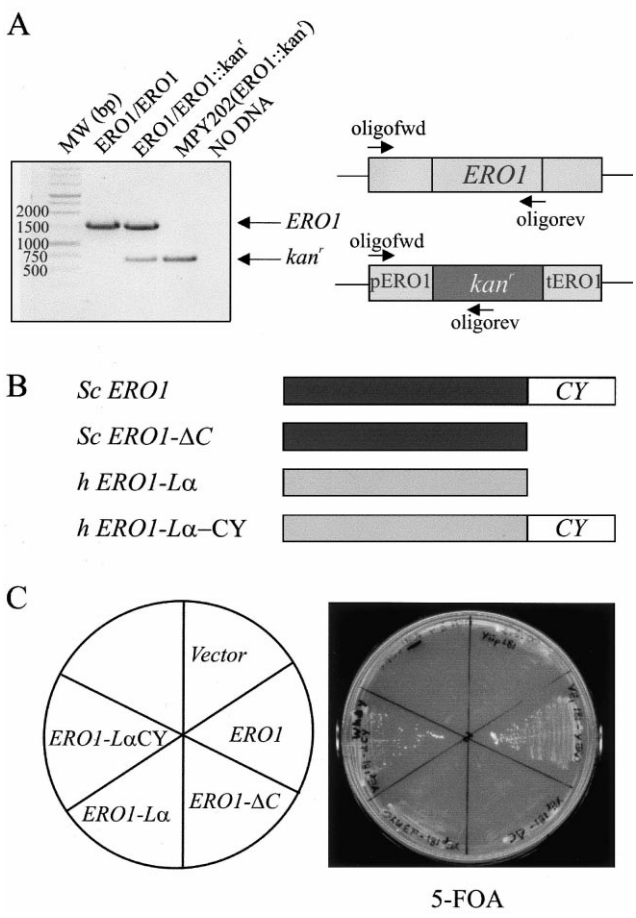

Fig. 1. The presence of the C-terminal tail is essential to rescue an ERO1 deleted strain. A: PCR was employed to confirm the replacement of the *ERO1* gene with the kanamycin resistance cassette in a diploid strain (*ERO1/ERO1::kan*^{r}) and in the haploid strain MPY202 derived after sporulation of the diploid. MPY202 is viable due to an episomal *ERO1* gene carried by an *URA3* plasmid. B: Schematic representation of the four genes introduced in the MPY202 strain: *S. cerevisiae ERO1*, *S. cerevisiae ERO1*ΔC, human *ERO1-Lα* (both lacking the yeast C-terminal domain), and the chimeric human *ERO1-Lα* to which the yeast C-tail was appended. These genes were cloned into a *LEU2* plasmid. C: Growth of different transformants on medium containing 5′-FOA. Since the presence of an *URA3* plasmid does not allow growth in the presence of 5′-FOA, only cells that had lost the episomal *ERO1* gene carried by an *URA3* plasmid proliferate on media containing the drug. The only viable MPY202 transformants are those carrying the *S. cerevisiae ERO1* gene or the chimeric gene *ERO1-Lα-CY*.

3. Results

3.1. The presence of the C-terminal tail is necessary for complementation of ERO1 disrupted S. cerevisiae cells

We have previously shown that human Ero1-Lα and Ero1-Lβ allow the growth of a yeast thermosensitive mutant, *ero1-1* at the restrictive temperature [5,6]. Also a yeast mutant lacking the last 127 amino acids, Ero1pΔC, was effective, confirming that the C-terminal tail is not essential in this assay.

To gain insight into the molecular mechanisms underlying complementation, we constructed an *ERO1* disrupted yeast strain by replacing the whole coding sequence with a *kan*^{r} marker gene [11] in a diploid strain (Fig. 1A). We transformed heterozygous *ERO1/ERO1::kan*^{r} cells with plasmids driving the expression of Ero1p, Ero1pΔC or Ero1-Lα (Fig. 1B). Since *ERO1* is an essential gene in yeast, only half of the spores obtained by tetrad dissection assays are viable unless the gene carried by the plasmid is able to complement. As expected, all the spores derived from the *ERO1* transformant were viable. In contrast, only half of the spores derived from the other two transformants were able to grow, namely those carrying the chromosomal *ERO1* (not shown). These data indicate that mutants lacking the C-terminal tail were unable to complement yeast cells devoid of endogenous Ero1p.

A plasmid shuffle experiment was performed to determine whether overexpression of Ero1-Lα or Ero1pΔC could suppress lethality of *ERO1* disruption. For this purpose, we employed a strain in which the lethal chromosomal *ERO1* deletion is complemented by the yeast *ERO1* gene on an episomal *URA3* marked plasmid. This strain does not grow on plates

containing 5-FOA because this compound is converted to a toxic product by the *URA3* gene product [12]. The ability of high copy *ERO1-Lα* or *ERO1*ΔC plasmids, *LEU2* based, to replace the *URA3* marked plasmid bearing *ERO1* was assayed by growth on 5-FOA. As a positive control, a *ERO1*/*LEU2* plasmid was tested. Unlike wild type *ERO1*, neither *ERO1-Lα* nor *ERO1*ΔC rescued *ERO1::kan*^{r} cells, confirming that the C-terminal tail is essential for function. In agreement with this, the chimera *ERO1-Lα*-CY, in which the C-terminal tail of the yeast gene is appended to human Ero1-Lα, restored viability of the *ERO1* disrupted strain (Fig. 1C).

3.2. The leader sequence is cleaved from both yeast Ero1p and human Ero1-Lα

Apart from the N-terminal leader peptide, the sequence of Ero1p does not contain hydrophobic stretches that could be predicted to mediate transmembrane insertion. Nonetheless, membrane partition experiments (Fig. 2A) confirmed that, as previously reported [1], Ero1p was tightly associated with membranes. A possible explanation for this would be that Ero1p is a type II transmembrane protein, with an uncleaved leader peptide. To ascertain whether this was the case, the endogenous N-terminal hydrophobic sequence was replaced by the invertase leader peptide, which is efficiently recognized by leader peptidase [13]. The resulting chimeric protein (SUC-Ero1p) was also associated with membranes. Both Ero1p and SUC-Ero1p could be partially extracted from the membrane pellets by urea, confirming that they are not integral membrane proteins. Mass spectrometry and N-terminal sequencing confirmed that, as suggested by our previous fractionation experiments [6], the leader sequence of human Ero1-Lα is efficiently cleaved between residues G23 and E24.

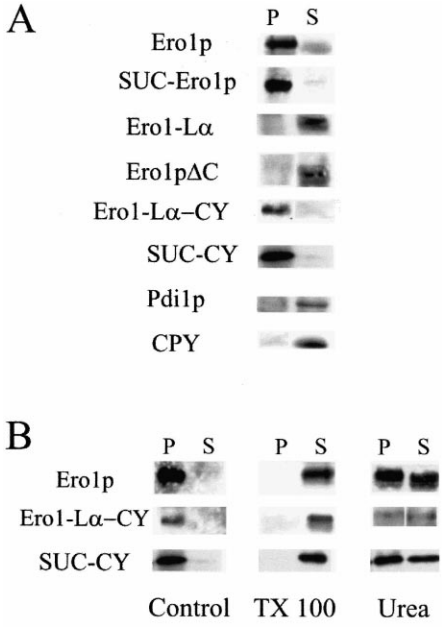

Fig. 2. The C-terminal tail mediates the association of Ero1 proteins with the ER membrane. A: After homogenization in the absence of detergents, membrane associated (P) and soluble (S) proteins were separated by centrifugation at 100 000×*g* and resolved by SDS gel electrophoresis under reducing conditions. Western blots were decorated with 9E10 anti-myc antibodies. Pdi1p and CPY, two soluble proteins localized in the ER lumen and in the vacuole respectively, were used as controls and detected using specific polyclonal antibodies. B: Ero1 proteins which segregated with the membrane fraction were treated with 5 M urea or 1% Triton X-100 for 30 min on ice, centrifuged again at 100 000×*g* and analyzed as in A.

3.3. The presence of the C-terminal tail is necessary to anchor the protein to the membrane

Since the leader peptide is probably cleaved also from yeast Ero1p, membrane association [1,5,6] could not reflect insertion in the lipid bilayers as type II proteins. We therefore proceeded to further investigate the topological distribution of the different mutants (Fig. 2A). Whilst the vast majority of yeast Ero1p and SUC-Ero1p partitioned into the membrane containing pellet, both Ero1-Lα and Ero1pΔC accumulated in the soluble fraction, suggesting that the presence of the tail was important for membrane association. In agreement with this, the Ero1-Lα-CY chimera accumulated in the pellet, similarly to Ero1p. To further confirm its topological role, we fused a myc-tagged version of the yeast C-tail to the invertase leader peptide. Like Ero1p, the resulting chimeric protein (SUC-CY) accumulated in the pellet and was in part solubilized by urea. Ero1p, Ero1-Lα-CY and SUC-CY were solubilized by mild detergents, excluding the possibility that their accumulation in the pellets was due to aggregation. Taken together, these results indicated that Ero1p is a peripheral membrane protein and its C-terminal domain plays an essential role in membrane association.

4. Discussion

In bacteria, disulfide bonds are transferred from DsbB to DsbA to cargo proteins in the periplasmic space. DsbB ultimately donates electrons to the respiratory chain [14–16]. PDI has been shown to complement DsbA null mutants [17,18], although the two proteins are rather divergent in terms of sequence. In eukaryotes and prokaryotes, therefore, Ero1p and DsbB carry out similar functions, ensuring that their main downstream partners, PDI and DsbA respectively, are oxidized so as to transfer SS bonds to cargo proteins. Nonetheless, while DsbB is a polytopic integral membrane protein [19], Ero1p can be in part extracted from membranes in *S. cerevisiae* by urea treatment, a characteristic of soluble or peripheral membrane proteins. Moreover, also SUC-Ero1p, which carries a cleaved leader sequence, fractionates in the membrane containing pellet. Therefore, the endogenous Ero1p signal sequence does not carry additional functions apart from targeting to the ER. When expressed in human cells, also Ero1-Lα and Ero1-Lβ associated with the membrane pellet, but could be solubilized following treatment at pH 13 [6]. Cleavage of the leader sequences was confirmed by mass spectrometry and N-terminal sequencing of Ero1-Lα purified from HeLa cells (A. Bachi, F. Talamo, B. Valsasina and R. Sitia, unpublished observations).

Taken together, these findings indicate that neither Ero1p nor Ero1-Lα is inserted in the ER membrane. Their accumulation in the membrane fractions could reflect interactions with integral membrane proteins.

Several lines of evidence point to a topological role for the 127 residue yeast tail. First, a deletion mutant lacking it (Ero1pΔC) no longer accumulates in the membrane pellet. Interestingly, however, Ero1pΔC is neither secreted nor localized in the periplasmic space of yeast cells, implying that ER

residency does not solely rely on membrane association. Second, appending the yeast C-terminal tail to Ero1-Lα is sufficient to cause membrane association of the human protein, otherwise soluble when expressed in yeast cells. Finally, when fused to the invertase leader sequence, a tagged version of the yeast tail displayed a topology similar to Ero1p, indicating that the 127 amino acids contained the information necessary to mediate membrane association.

Membrane association correlates with the capability to complement the growth of a yeast strain in which the endogenous gene has been disrupted (Δ*ERO1*). Like Ero1pΔC, human Ero1-Lα expressed in yeast behaves as a soluble protein and fails to complement Δ*ERO1* cells. This is somewhat unexpected, as the majority of Ero1-Lα accumulated in the membrane fraction of human cells [6]. Therefore, the mechanisms that mediate membrane association of Ero1 proteins have diverged in yeast and mammalian cells. Appending the 127 residue yeast tail to human Ero1-Lα restores membrane association and allows complementation of Δ*ERO1* cells. It will be of interest to identify proteins that specifically interact with the yeast tail, as these molecules may have a role in controlling redox homeostasis within the ER.

Whilst unable to rescue the growth of the *ERO1* disrupted strain, both human Ero1-Lα and yeast Ero1pΔC complement several phenotypic traits of the thermosensitive mutant *ero1-1* [5,6]. Co-expressing SUC-CY and either Ero1pΔC or Ero1-Lα was not sufficient to allow growth of Δ*ERO1* cells (data not shown) indicating that physical continuity with the tail is necessary for function. Taken together, these findings suggest that in *ero1-1* transformants, functional heterodimers are formed in which the enzymatic activity is provided by the tail-less transgenes (Ero1-Lα or Ero1pΔC), while the topological information is contributed by the tail of the endogenous Ero1p. These heterodimers, likely mediated by interactions between the 'heads' of the molecules, do not appear to be stable, since the vast majority of the tail-less proteins are found in the soluble fraction also in *ero1-1* cells. In all likelihood, a few active heterodimers are sufficient to fulfil the growth requirements of this mutant strain. Whilst allowing growth at the non-permissive temperature, human Ero1-Lα and Ero1-Lβ restore other phenotypic traits of *ero1-1*, oxidative folding of carboxypeptidase Y or DTT sensitivity, less efficiently than yeast Ero1p [5,6].

Since FAD is important for Ero1p function [3], mechanisms must exist that allow its import into the ER lumen. The association of Ero1p with the ER membrane may be important for FAD binding. However, Ero1p is active also in anaerobic conditions [20], implying that electron acceptors other than oxygen can be utilized. Electrons produced during the process of disulfide bond formation may be ultimately transported into the cytosol. In view of the importance of tightly controlling the redox in this organelle, the observation that membrane association is essential for function may reflect topological requirements for an efficient and regulated (re)oxidation of Ero1p.

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