Two Conserved Cysteine Triads in Human Ero1α Cooperate for Efficient Disulfide Bond Formation in the Endoplasmic Reticulum*

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Human Ero1α is an endoplasmic reticulum (ER)-resident protein responsible for protein disulfide isomerase (PDI) oxidation. To clarify the molecular mechanisms underlying its function, we generated a panel of cysteine replacement mutants and analyzed their capability of: 1) complementing a temperature-sensitive yeast Ero1 mutant, 2) favoring oxidative folding in mammalian cells, 3) forming mixed disulfides with PDI and ERp44, and 4) adopting characteristic redox-dependent conformations. Our results reveal that two essential cysteine triads (Cys85-Cys94-Cys99 and Cys391-Cys394-Cys397) cooperate in electron transfer, with Cys94 likely forming mixed disulfides with PDI. Dominant negative phenotypes arise when critical residues within the triads are mutated (Cys394, Cys397, and to a lesser extent Cys99). Replacing the first cysteine in either triad (Cys85 or Cys391) generates mutants with weaker activity. In addition, mutating either Cys85 or Cys391, but not Cys397, reverts the dominant negative phenotype of the C394A mutant. These findings suggest that interactions between the two triads, dependent on Cys85 and Cys391, are important for Ero1α function, possibly stabilizing a platform for efficient PDI oxidation.

A fundamental requirement for secretory and membrane proteins to be efficiently manufactured in the endoplasmic reticulum (ER) is the formation of their correct disulfide bonds. This process relies on a network of protein-protein interactions that precisely regulates the redox equilibrium in the organelle (1–3). Efficient oxidation of substrate proteins is catalyzed by ER resident oxidoreductases such as protein disulfide isomerase (PDI) (4). These proteins transfer oxidative equivalents to their substrates via the formation of mixed disulfide intermediates. In yeast and mammals, the reoxidation of PDI depends on Ero1 (endoplasmic reticulum oxidoreductin 1) proteins (5–9). Whereas yeast exhibits only one Ero1 protein that is induced during ER stress (Ero1p (5, 7)), humans express two isoforms, Ero1α and Ero1β, the latter a UPR (unfolded protein response) substrate (9, 10). In both mammals and yeast, Ero1 proteins form mixed disulfides with PDI and some other ER resident members of the PDI family (6, 11–13), indicating that electrons flow from nascent cargo proteins to Ero1 via PDI (6). Reoxidation of yeast Ero1p seems to involve electron transfer to molecular oxygen via flavin adenine dinucleotide (14, 15).

Considering their role in oxidative folding, Ero1 proteins are expected to have active sites characterized by the presence of conserved cysteine residues. Two cysteine pairs are essential for the function of yeast Ero1p. Cysteines 100/105 seem important for thiol-disulfide exchange with Pdi1p and Mpd2p, whereas cysteines 352/355 are probably in charge of oxidizing the former pair (16). The yeast 352/355 pair is part of a conserved CXXCXXC motif, essential for function (7, 16), and finds its equivalent in residues 394 and 397 of human Ero1α (7, 8, 12, 16). Mutations of these two residues inhibit the oxidation of a reporter protein, confirming that Ero1α is a pivotal component in mammalian oxidative protein folding (12).

The sequence of human Ero1α contains 15 cysteines, many of which are conserved among species and members of the Ero1 family (Fig. 1) and likely play distinct roles. Catalytic cysteines could bind PDI and other upstream or downstream components in the electron transport chain. Structural cysteines may be responsible for the characteristic compaction of Ero1α (17). Another potential function includes homo- or heterodimerization, which has been postulated by complementation assays in yeast (18).

Here we analyzed the functional properties of a wide panel of Ero1α deletion or cysteine replacement mutants. We present evidence showing that human Ero1α contains two essential cysteine triads, suggesting that interactions involving the two cysteines of each triad increase the efficiency of electron transport, possibly stabilizing a platform for efficient PDI oxidation.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Chemicals were purchased from Sigma unless noted otherwise. Polyclonal anti-PDI antibody was a kind gift from A. Benham (Durham, UK) and I. Braakman (Utrecht, Netherlands).

Mutagenesis—Mutants were constructed by PCR using the splicing overlap extension technique (19, 20). Primer sequences are available upon request. The final PCR products were cloned using the pGEM-T-easy cloning kit (Promega, Milan, Italy) and sequenced. The cDNAs were excised with Acc65I and XbaI and inserted in-frame into pcDNA3.1myc/his(−A) (Invitrogen) or into pvT102U (18) for HeLa cell transfection or yeast transformation, respectively. Each mutant is identified by the position number of the mutated residue and the amino acid used to replace it (e.g. C394A).

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**Fig. 1.** Conserved cysteine clusters in Ero1 proteins. Members of the Ero1 family exhibit four conserved cysteine-rich regions, indicated at the top, comprising cysteines 35–48 (cluster 1), 85–104 (cluster 2), 291–307 (cluster 3), and four scattered cysteines. The numbers refer to human Ero1a. In the top panel, bold are shown cysteine residues that when mutated yield a dominant negative phenotype. In the four bottom panels, dark gray indicates identical residues and lighter gray the similarities. The alignment was performed using the program ALIGN (www.expasy.ch).

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**TABLE I**

Functional properties of Ero1a mutants

The phenotype of each Ero1a mutant is described: (i) active, weakly active, or dominant negative (Dom. neg.) in JcM folding assays based on pulse chase and immunoprecipitation (IP) or Western blotting (WB); (ii) capability of complementing ero1-1 cells in spot and liquid assays; (iii) PDI binding; and (iv) presence of the characteristic Ox1 and Ox2 redox isoforms in nonreducing gels. ND, not determined.

| Mutation | Folding assay | ero1-1 complementation |
|----------|---------------|------------------------|
|          | IP            | WB                     | Spot | Liquid | PDI binding | Ox1 | Ox2 |
| wt Ero1a | Active        | Active                 | +    | +      | +           |     |     |
| Deletion |               |                        |      |         |             |     |     |
| Δ53–60   | ND            | Active                 | +    | ND      | +           | +   |     |
| Δ58–65   | ND            | Dom. neg.              | ND   | ND      | +           |     | +   |
| Cluster 1| C35S          | Active                 | +    | ND      | ND           |     | +   |
|          | C46S, C48S    | Active                 | +    | ND      | +           |     | +   |
| Cluster 2| C55S          | Weakly active          | ND   | +       | +           | +   |     |
|          | C94S          | Weakly active          | ND   | +       | +           | ±   | +   |
| C99A     | Weak Dom. neg. | Weak Dom. neg.        | ± (dots) | +     | +           | +   |     |
| C104S    | Active        | Active                 | +    | +       | +           | +   |     |
| Scattered cysteines |               |                        |      |         |             |     |     |
| C131A    | Active        | ND                     | +    | ND      | +           |     | +   |
| C166A    | Active        | ND                     | +    | +       | ±           | +   |     |
| C206S    | Active        | ND                     | +    | ND      | ±           | +   |     |
| C241S    | Active        | ND                     | +    | ND      | ±           | +   |     |
| Cluster 3| C391A         | Weakly active          | +    | +       | +           | +   |     |
|          | C384A         | Dom. neg.              | +    | Dom. neg. | ±           | +   |     |
| C397A    | Dom. neg.     | Dom. neg.              | -    | -       | +           | +   |     |
| Glycosylation sites |               |                        |      |         |             |     |     |
| N280A    | ND            | Active                 | +    | ND      | +           |     | +   |
| N384A    | ND            | Active                 | +    | ND      | +           | +   |     |
| N280A,N384A |             |                        |      |         |             |     |     |

* A pattern that is qualitatively different from wild-type (wt) Ero1a.

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**RESULTS**

**Construction and Characterization of Ero1a Cysteine Mutants**—The 15 cysteines of human Ero1α can be grouped in three clusters and a central region containing four scattered cysteines. Cluster 3, containing the essential CXXCXXC motif (8, 12), exhibits the highest sequence conservation (Fig. 1). To gain information on the mechanisms of Ero1α function,
we generated a panel of deletion and cysteine replacement mutants and characterized them according to their ability to: i) accelerate oxidative protein folding (12); ii) complement the temperature-sensitive yeast mutant ero1-1; iii) bind its known interactor PDI and ERp44; and finally iv) form characteristic redox isomers (17) in nonreducing gels, giving insight into structural features.

A limitation of this approach is that some mutants could undergo folding defects. To exclude this possibility, we exploited the notion that when over-expressed in HeLa cells, some Ero1α is secreted (13). Some mutants, particularly those in the C-terminal region (e.g. Δ404–420) formed high molecular weight aggregates and were not secreted at all, indicating that they did not pass the ER quality control barriers. A list of partial characterization of the folding mutants, which are not described in this study, can be made available upon request. All of the mutants analyzed herein are detergent-soluble, secreted, and form at least one of the two characteristic molecular weight complexes. The Myc-tagged Ero1α transgenec (Ero-Tg) are also revealed by the anti-Myc antibody. Unlike in immunoprecipitation assays (see Ref. 12 and Fig. 3), JcM-containing covalent complexes are detected in Western blot assay, also immediately after DTT treatment, suggesting that some “old” molecules are trapped in partially DTT-resistant complexes.

FIG. 2. Deletion mutants encompassing clusters 1 and 2 have different functional properties. Cells co-expressing Myc-tagged J chains and the indicated Ero1α mutants were exposed to 5 mM DTT for 5 min, washed at 4 °C, and then cultured at 37 °C for the indicated times without the reducing agent. Aliquots from cell lysates were re-solved by nonreducing gels, and blots were immunodecorated with anti-Myc and SDS-PAGE under nonreducing conditions. Note that few newly synthesized JcM chains are present in immunoprecipitation with anti-Myc and SDS-PAGE under nonreducing conditions. All of the mutants analyzed herein are detergent-soluble, secreted, and form at least one of the two characteristic molecular weight aggregates and were not secreted at all, indicating that they did not pass the ER quality control barriers. A list and partial characterization of the folding mutants, which are not described in this study, can be made available upon request. All of the mutants analyzed herein are detergent-soluble, secreted, and form at least one of the two characteristic molecular weight complexes. The Myc-tagged Ero1α transgenec (Ero-Tg) are also revealed by the anti-Myc antibody. Unlike in immunoprecipitation assays (see Ref. 12 and Fig. 3), JcM-containing covalent complexes are detected in Western blot assay, also immediately after DTT treatment, suggesting that some “old” molecules are trapped in partially DTT-resistant complexes.

FIG. 3. Functional characterization of single-cysteine replacement mutants within cluster 2. HeLa cells co-expressing Myc-tagged J chains and the indicated Ero1α mutants were pulse-labeled with [35S]cysteine and -methionine for 5 min in the presence of 5 mM DTT, washed twice at 4 °C, and chased at 37 °C for the indicated times before immunoprecipitation with anti-Myc and SDS-PAGE under nonreducing conditions. Note that few newly synthesized JcM chains are present in high molecular weight complexes just after the pulse in DTT. The band corresponding to exogenous Ero1α transgene (Ero-Tg) constructs is indicated by an arrow. Jred, reduced J chains; Jox, partially oxidized J chains are also indicated.

FIG. 4. ero1-1 complementation assays. A, spot assays of ero1-1 transformed with Ero1a constructs. The ero1-1 yeast transformants expressing the indicated human Ero1α mutants were plated on rich medium at permissive or nonpermissive temperatures (24 or 37 °C). Note the speckled pattern of growth of the C99A transformant. As reported previously (12), C394A and C397A do not complement ero1-1. U.T., wild type. B, growth rates of ero1-1 transformants. ero1-1 transformants were inoculated at 0.1 A_{600 nm} in rich medium and grown at 37 °C; absorbance was at 600 nm at the indicated times. Note that C85S (−−−), C94S (−−•–•), and C391A (−•−•), each of which scored normal in the spot assay (A), show reduced growth. C99A (−•−−), yielding a speckled pattern in spot assays, supports even slower growth. C394A (−−•−•, upper panel), and C397A (−−•−•, lower panel) do not complement ero1-1, whereas C166A (−−−) and C104S (−•−−) grow at essentially normal rates.

Ox1 and Ox2 redox isofoms (Table I), thus excluding gross folding alterations.

To monitor oxidative protein folding in vivo, we used a previously described pulse-chase assay (12) or a simplified version based on Western blotting. Unlike in the previously described assay (12), not all intracellular J chains were reduced in the Western blot assays (Fig. 2), suggesting that some pre-existing molecules are trapped in partially DTT-resistant complexes (12, 21). Nonetheless, the activity of Ero1α mutants can be monitored readily by comparing the shift from reduced to oxidized J chain (Fig. 2).

Using the Western blot protocol, we first examined mutants of cysteine clusters 1 and 2. The deletion mutant Δ33–50, lacking all cysteines of cluster 1, was as active as wild-type Ero1α (Fig. 2). Also single and double cysteine mutants in cluster 1 retained their activity (Table I) confirming that this nonconserved cluster is not essential for function. In contrast, mutant Δ86–95 displayed a weak but reproducible dominant negative activity and also failed to complement the defective yeast mutant ero1-1 (Fig. 2 and Table I), revealing a functional rule for cluster 2.

After this preliminary survey, we refined our tools and ana-
lyzed individual amino acid replacements by immunoprecipitation folding assays. Among individual cysteine-replacement mutants, C85S and C94S were less active than wild-type Ero1α, whereas C99A acted as a weak dominant negative (Fig. 3). Mutation of C104 did not impair the ability to promote JcM folding, further confirming the specificity of the phenotypes observed.

Within cluster 3, as described previously (12, 18), C394A and C397A did not complement erol-1 and behaved as strong dominant negative phenotypes, whereas mutant C391A displayed reduced activity similar to C85S (Table I).

Mutants in the four “scattered” cysteines (C131A, C166A, C208S, and C241S) were almost as active as wild-type molecules, and so were mutants lacking either one or both glycosylation sites (Table I).

In agreement with the functional data obtained in mammalian cells, transformation of erol-1 with the C104S, C131A, C166A, C208S, and C241S mutants allowed vigorous growth at the nonpermissive temperature (Fig. 4; Table I). In contrast, C85S and C94S showed a normal pattern in the spot assay (Fig. 4A) but were clearly deficient in the more sensitive growth curve test (Fig. 4B). The growth of the C99A mutant was severely compromised and was barely detectable in the spot test, where it showed a characteristic speckled pattern (Fig. 4A). As expected, mutants C394A and C397A were not able to complement erol-1 at all. These results confirm a fundamental role for cysteine clusters 2 and 3 in Ero1α activity, as described in yeast Ero1p (16). Interestingly, the Cys85 and Cys391 mutants displayed a similarly reduced capability of supporting yeast growth.

Differential Binding to PDI and ERp44—Next we compared the mutants for their ability to bind PDI and ERp44, two proteins known to form mixed disulfides with Ero1α (11, 17). Four mutants bound considerably less PDI: C94S, C166A, C208S, and C241S (Fig. 5A). Unlike C94S, however, the latter three mutants retained functional activity in both mammalian and yeast cells. This suggests that Ero1α-PDI mixed disulfides can be formed for reasons other than transferring oxidative equivalents to PDI. The four mutants that bound less PDI interacted as well as the wild-type with ERp44, indicating the differing modes of interaction of these two proteins with Ero1α.

An analysis of nonreducing blots revealed additional interesting features (Fig. 5B). The dominant negative mutants C99A, C394A, and C397A bound PDI in more or less normal amounts, the latter exhibiting an additional band of unknown origin (Fig. 5B). The two mutants in the first cysteines of each essential cluster (Cys85 and Cys391) displayed similarities also with respect to PDI binding, accumulating additional high molecular weight bands and fewer 120-kDa heterodimers.

Structural Features of Cysteine Mutants as Revealed by Ox1-Ox2 Formation—The similarities between Cys85 and Cys391 extended to their electrophoretic patterns in nonreducing gels. In addition to forming characteristic mixed disulfides with PDI (12) and ERp44 (11), and perhaps some homodimers, monomeric Ero1α molecules separate into two redox-sensitive isoforms, Ox1 and Ox2 (17). The ratios between the two isoforms can be modified by oxidizing or reducing treatments (11), but their functional significance is still not clear. Both C85S and C391A (see Fig. 6) accumulated partially reduced molecules as well as an additional form migrating between the normal Ox1 and Ox2, rarely seen in wild-type molecules. Pulse-chase experiments (Ref. 17 and footnote 2) suggest that the C85S and C391A mutants share the incapacity to develop a stable Ox1 form. The intermediate band that we detected could likely reflect the formation of what normally generates Ox2 without the previous stabilization of Ox1.

Other mutants, namely C94S, C397A, and C131A (Table I), showed the absence of Ox2. These three mutants are part of different functional groups; C94S is weakly active and shows a weaker binding to PDI, C131A has no distinctive phenotype, and C397A behaves as a dominant negative. As the formation of Ox2 does not directly correlate with functional phenotypes, we did not investigate its significance further.

Double and Triple Cysteine Mutants Reveal Cooperativity between Clusters 2 and 3—To further clarify the functional role of cysteines 85 and 391, we generated double cysteine replacement mutants (Fig. 7). The double mutant C394A,C397A re-
tained strong dominant negative activity. In contrast, when either Cys\textsuperscript{391} or Cys\textsuperscript{394} was replaced concomitantly with Cys\textsuperscript{397}, the dominant negative phenotype was lost. Substitution of Cys\textsuperscript{85} or Cys\textsuperscript{391} reverted the dominant negative phenotype of Cys\textsuperscript{85} as well (not shown). Therefore, the absence of the first cysteine of each triad makes Ero1\textalpha less active, both in promoting and in inhibiting disulfide bond formation. Taken together, these findings indicate that Cys\textsuperscript{391} and Cys\textsuperscript{394} are necessary to coordinate the activity of clusters 2 and 3 in the functional cycle of human Ero1\textalpha.

DISCUSSION

The process of Ero1\textalpha-dependent PDI oxidation likely involves several steps: docking of reduced PDI, formation of PDI-Ero1\textalpha mixed disulfides, detachment of oxidized PDI, and recharging of Ero1\textalpha. By correlating the phenotypes of mutants with predicted functional defects we could assign potential functions to single cysteines. The mutants analyzed were not grossly impaired in their folding, since they formed Ox1 and/or Ox2 and passed the ER quality control to be secreted by HeLa transfectants.

The first conclusion is that, in addition to the conserved cysteine triad located in the C-terminal end of the molecule (cysteines 391, 394, and 397), another cysteine cluster (in the form of the triad Cys\textsuperscript{85}-Cys\textsuperscript{391}-Cys\textsuperscript{99}) is essential for Ero1\textalpha function. Deletion and replacement mutants within these triads are severely impaired in the ability to promote disulfide bond formation and to complement Ero1p-defective yeast mutants. Three dominant negative mutants based on single cysteine substitutions were identified, all mapping within these two triads; these were C99A, C394A, and C397A, the phenotype of the first being less pronounced than that of the latter two. The existence of two active sites has been proposed also in yeast Ero1p (12). The sequence similarities within the crucial regions and the observation that human molecules can complement defective yeast imply that the basic mechanisms of electron transfer are conserved among distant eukaryotes. Besides confirming this notion, our findings reveal that the first two cysteines within each triad, namely Cys\textsuperscript{85} and Cys\textsuperscript{391}, play an important role in the process. Intriguingly, the phenotypes of the two mutants C85S and C391A are similar in several respects. First, both mutants display structural defects, as indicated by the different behaviors of the monomeric proteins under nonreducing conditions and by the paucity of the disulfide-linked complexes with PDI. From a functional point of view, both mutants are less active in promoting JeM oxidation and in complementing ero1-1 yeast cells. Our interpretation of these findings is that interactions between Cys\textsuperscript{85} and Cys\textsuperscript{391} bring together the two active sites, thus facilitating electron transfer from PDI to upstream acceptors. The most obvious way to accomplish this would be the formation of a reversible disulfide bond between the two residues. This bond might lead to the formation of Ox1, absent in both mutants. However, attempts to detect the corresponding disulfide-linked peptides by mass spectrometry failed. Several technical reasons explain these negative findings, including interchange reactions within gel-extracted molecules, reduction of the bond possibly favored by the ionization energy, or differential detectability of certain molecular species. In perfect agreement with our proposal of disulfide linkages between the two Ero1\textalpha triads, analysis of yeast Ero1p crystals revealed the presence of an intra-molecular disulfide between C90 and C349 (24) corresponding to C85 and C391 in human Ero1\textalpha.

Ero1\textalpha can fold into Ox1- and Ox2-like isoforms also, when translated \textit{in vitro} and hence in the absence of PDI and ERP44. In contrast, Ero1\textalpha-PDI and Ero1-ERP44 mixed disulfides are seen only when ER membranes are added (12, 17).\textsuperscript{3} If indeed Ox1 is caused by an intramolecular bond linking Cys\textsuperscript{85} and Cys\textsuperscript{391}, then the presence of PDI and ERP44 is not essential for its formation. The capability of forming disulfide bonds in a reducing environment, as found in DTT-treated cells (12), is in line with the pivotal role of Ero1\textalpha in promoting disulfide bond formation (12).

Further evidence for a common functional role of Cys\textsuperscript{85} and Cys\textsuperscript{391} stems from the analysis of double mutants. Clearly, replacing either residue in the Cys\textsuperscript{99} or Cys\textsuperscript{394} mutants abolished the dominant negative phenotype. Therefore, the promoting function of the 85–391 couple is evident not only in productive reactions but also in the limiting of disulfide bond formation.

With the screening methods used, no function could be assigned to the N-terminal cysteine-rich cluster. Whether the two CXC motifs found in cluster I have any functional significance as isomerases, as proposed by Woyczechowsky and Raines (22), remains to be tested. Also the two glycosylation sites are apparently dispensable for Ero1\textalpha function, since mutating either one or both did not affect activity in either JcM folding or yeast complementation assays.

Mutants lacking Cys\textsuperscript{94}, Cys\textsuperscript{166}, Cys\textsuperscript{394}, or Cys\textsuperscript{394} bound less PDI at steady state. Similarly, Frand and Kaiser (16) report defective PDI binding for the Cys\textsuperscript{100} and Cys\textsuperscript{208} yeast Ero1p mutants. However, only in the case of C94S (and its counterpart in yeast Ero1p, Cys\textsuperscript{100}) was functional activity compromised, suggesting that this residue, located in essential cluster 2, is responsible for forming intermediate mixed disulfides with PDI in electron transfer reactions. These results also imply that mixed disulfides between Ero1 and PDI can be formed for reasons other than transferring oxidative equivalents to PDI. Perhaps PDI can bind excess or mutated Ero1\textalpha in its capacity of quality controller (23). ERP44 bound similarly well to all Ero1p cysteine replacement mutants, including the four showing reduced interactions with PDI. We have recently proposed that the interaction with ERP44 is important in retaining Ero1p intracellularly (13). Mass spectrometry analyses of over-expressed Ero1\textalpha revealed that Cys\textsuperscript{166}, Cys\textsuperscript{208}, and Cys\textsuperscript{394} are mainly in thiol form,\textsuperscript{4} which could make Ero1p a multivalent substrate for ERP44.

In conclusion, clusters 2 and 3 are part of distinct domains that might come in close contact for efficient electron transfer from PDI to Ero1p and to the upstream acceptors. Although not essential for function, the stabilizing role of Cys\textsuperscript{85} and Cys\textsuperscript{391} clearly enhances the activity of the ER oxidizing machine, likely by aligning cysteines 94, 99, 394, and 397.

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