

## Two Conserved Cysteine Triads in Human Ero1 $\alpha$ Cooperate for Efficient Disulfide Bond Formation in the Endoplasmic Reticulum\*

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**Human Ero1 $\alpha$  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 (Cys<sup>85</sup>-Cys<sup>94</sup>-Cys<sup>99</sup> and Cys<sup>391</sup>-Cys<sup>394</sup>-Cys<sup>397</sup>) cooperate in electron transfer, with Cys<sup>94</sup> likely forming mixed disulfides with PDI. Dominant negative phenotypes arise when critical residues within the triads are mutated (Cys<sup>394</sup>, Cys<sup>397</sup>, and to a lesser extent Cys<sup>99</sup>). Replacing the first cysteine in either triad (Cys<sup>85</sup> or Cys<sup>391</sup>) generates mutants with weaker activity. In addition, mutating either Cys<sup>85</sup> or Cys<sup>391</sup>, but not Cys<sup>99</sup>, reverts the dominant negative phenotype of the C394A mutant. These findings suggest that interactions between the two triads, dependent on Cys<sup>85</sup> and Cys<sup>391</sup>, are important for Ero1 $\alpha$  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)<sup>1</sup> 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, Ero1 $\alpha$  and Ero1 $\beta$ , 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 Ero1 $\alpha$  (7, 8, 12, 16). Mutations of these two residues inhibit the oxidation of a reporter protein, confirming that Ero1 $\alpha$  is a pivotal component in mammalian oxidative protein folding (12).

The sequence of human Ero1 $\alpha$  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 Ero1 $\alpha$  (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 Ero1 $\alpha$  deletion or cysteine replacement mutants. We present evidence showing that human Ero1 $\alpha$  contains two essential cysteine triads, suggesting that interactions involving the first 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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<sup>1</sup> The abbreviations used are: ER, endoplasmic reticulum; DTT, dithiothreitol; PDI, protein disulfide isomerase; JcM, Myc-tagged version of J chain.

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), 391–397 (cluster 3), and four scattered cysteines. The numbers refers to human Ero1 $\alpha$ . In the top panel, in 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).

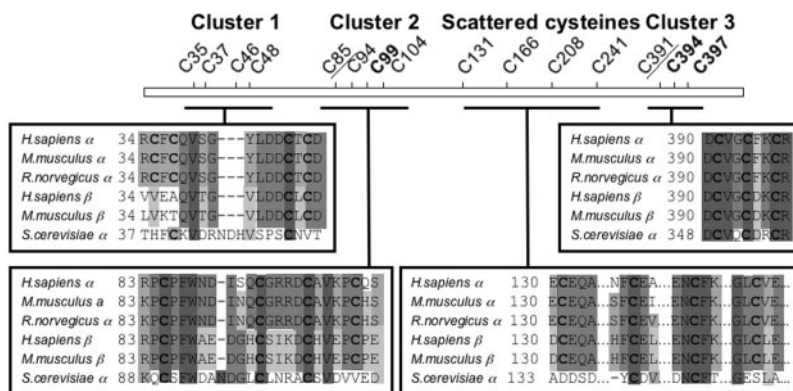


TABLE I  
Functional properties of Ero1 $\alpha$  mutants

The phenotype of each Ero1 $\alpha$  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		<i>ero1-1</i> complementation		PDI binding	Ox1	Ox2
	IP	WB	Spot	Liquid			
wt Ero1 $\alpha$	Active	Active	+	++	+	+	+
Deletion							
$\Delta$ 33–50	ND	Active	+	ND	+	+	+
$\Delta$ 86–95	ND	Dom. neg.	–	ND	+	+	–
Cluster 1							
C35S	ND	Active	+	ND	ND	+	+
C46S, C48S	ND	Active	+	ND	+	+	+
Cluster 2							
C85S	Weakly active	ND	+	+	+ <sup>a</sup>	– <sup>a</sup>	+ <sup>a</sup>
C94S	Weakly active	ND	+	+	±	+	–
C99A	Weak Dom. neg.	Weak Dom. neg.	± (dots)	±	+	+	+
C104S	Active	ND	+	++	+	+	+
Scattered cysteines							
C131A	Active	ND	+	ND	+	+	–
C166A	Active	ND	+	++	±	+	+
C206S	Active	ND	+	ND	±	+	+
C241S	Active	ND	+	ND	±	+	+
Cluster 3							
C391A	Weakly active	Weakly active	+	+	+ <sup>a</sup>	– <sup>a</sup>	+ <sup>a</sup>
C394A	Dom. neg.	Dom. neg.	–	–	+	+	+
C397A	Dom. neg.	Dom. neg.	–	–	+	+	–
Glycosylation sites							
N280A	ND	Active	+	ND	+	+	+
N384A	ND	Active	+	ND	+	+	+
N280A,N384A	ND	Active	+	ND	+	+	+

<sup>a</sup> A pattern that is qualitatively different from wild-type (wt) Ero1 $\alpha$ .

**Cell Culture and Transfection**—HeLa cells were transfected using Lipofectin (Invitrogen) as recommended by the supplier. All constructs were verified for expression using immunofluorescence as described previously (9).

**JcM Folding Assays**—The radioactive version of this assay was performed by pulse chase and immunoprecipitation as described (12) using a Myc-tagged version of J chain (JcM). For nonradioactive folding assays, transfected cells were trypsinized and incubated for 5 min at 37 °C with 5 mM DTT in Dulbecco's modified Eagle's medium without fetal calf serum. After two washes in phosphate-buffered saline at 4 °C, cells were cultured at 37 °C without DTT for different times and quenched with 10 mM *N*-ethylmaleimide before lysis in radioimmune precipitation buffer supplemented with 10 mM *N*-ethylmaleimide and protease inhibitors. Aliquots from the post-nuclear supernatants were resolved under nonreducing conditions. Western blots were decorated with 9E10 anti-Myc (12) followed by enzyme-conjugated secondary antibodies. Each mutant was tested at least twice.

The nature of these folding assays implies a certain degree of inter-assay variability (compare, for example, Figs. 3 and 7). For reasons that are not entirely clear (cell density, passage number), the JcM folding rate varies also in cells not transfected with Ero1 genes. Extremely reproducible, in contrast, are the promoting effects of wild-type Ero1 $\alpha$  on oxidative folding (in dozens of experiments; see Figs. 3 and 7) and the behavior of each mutant within a given experiment in reference to wild-type and mock-transfected cells. Raw data concerning the many experiments performed are available upon request.

**Complementation of *ero1-1***—The strain CKY559 (MAT $\alpha$ ,*ero1-1*, *ura3-52*, *leu2-3*, 112) was transformed with pT102U-Ero1p wild type or mutated constructs as described previously (18). Transformed yeasts were plated on selective plates (SD–Leu). Two clones for each mutant were selected and grown in liquid culture; one clone for each transformant was plated on SD–Leu and incubated at 37 °C (spot assay).

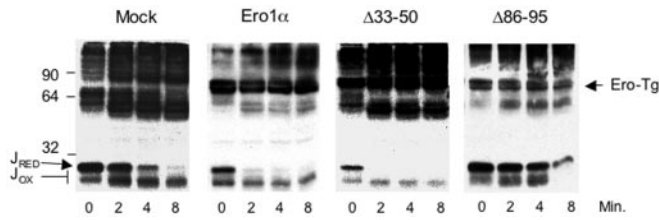
For the liquid growth assay, cells growing logarithmically at 24 °C were resuspended at 0.1 A<sub>600</sub>/ml in rich YEPD medium (yeast extract/peptone/dextrose; Difco Laboratories, Detroit, MI) and incubated at 37 °C. Growth was monitored by measuring absorbance every 2 h at A<sub>600</sub>.

**Analysis of Folding Patterns (Ox1–Ox2)**—Two days after transfection with the different Ero1 $\alpha$  mutants, cells were lysed in radioimmune precipitation buffer plus 10 mM *N*-ethylmaleimide. Aliquots from the post-nuclear supernatants were resolved under nonreducing or reducing conditions and immunoblotted with anti-Myc or polyclonal anti-Ero1 $\alpha$  (D5) antibodies (17).

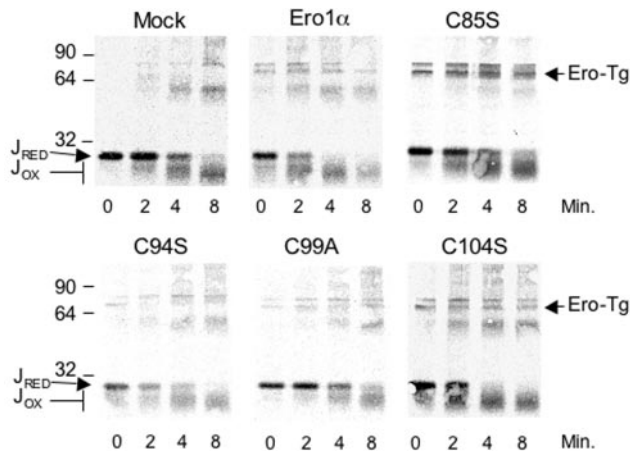
## RESULTS

**Construction and Characterization of Ero1 $\alpha$  Cysteine Mutants**—The 15 cysteines of human Ero1 $\alpha$  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 Ero1 $\alpha$  function,



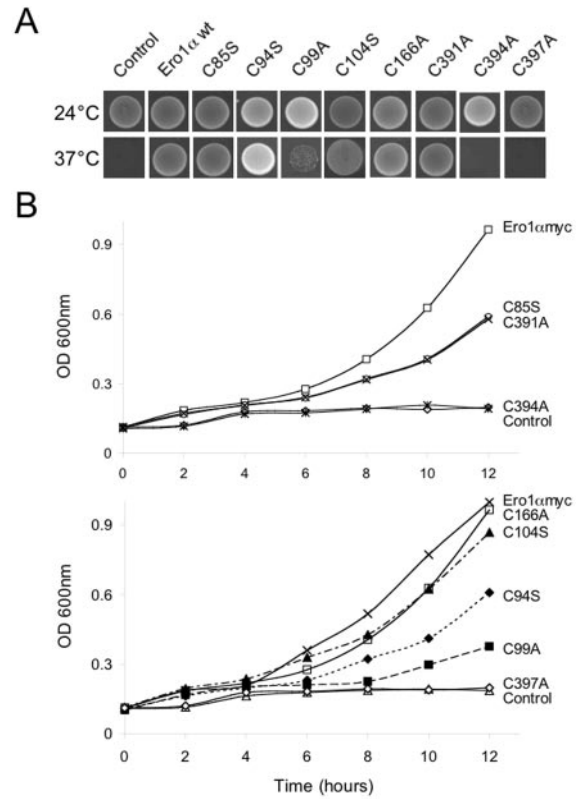
**FIG. 2. Deletion mutants encompassing clusters 1 and 2 have different functional properties.** Cells co-expressing Myc-tagged J chains and the indicated Ero1 $\alpha$  mutants were exposed to 5 mM DTT for 5 min, washed at 4 °C, and then cultured at 37 °C for the indicated times without the reducing agent. Aliquots from cell lysates were resolved by nonreducing gels, and blots were immunodecorated with anti-Myc. Upon formation of intrachain disulfide bonds, monomeric JcM increase their mobility, shifting from the completely reduced state (J<sub>RED</sub>) to partially oxidized J chains (J<sub>OX</sub>). Some JcM form covalent high molecular weight complexes. The Myc-tagged Ero1 $\alpha$  transgenes (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 Ero1 $\alpha$  mutants were pulse-labeled with [<sup>35</sup>S]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 Ero1 $\alpha$  transgene (Ero-Tg) constructs is indicated by an arrow. J<sub>RED</sub>, reduced J chains; J<sub>OX</sub>, partially oxidized J chains are also indicated.

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 interactors PDI and ERp44; and finally iv) form characteristic redox isoforms (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 Ero1 $\alpha$  is secreted (13). Some mutants, particularly those in the C-terminal region (e.g.  $\Delta$ 404–420) formed high molecular weight aggregates and were not secreted at all, indicating that they did not pass the ER quality control barriers. A list 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



**FIG. 4. *ero1-1* complementation assays.** A, spot assays of *ero1-1* transformed with Ero1 $\alpha$  constructs. The *ero1-1* yeast transformants expressing the indicated human Ero1 $\alpha$  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*. w.t., wild type. B, growth rates of *ero1-1* transformants. *ero1-1* transformants were inoculated at 0.1 A<sub>600nm</sub> 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 isoforms (Table I), thus excluding gross folding alterations.

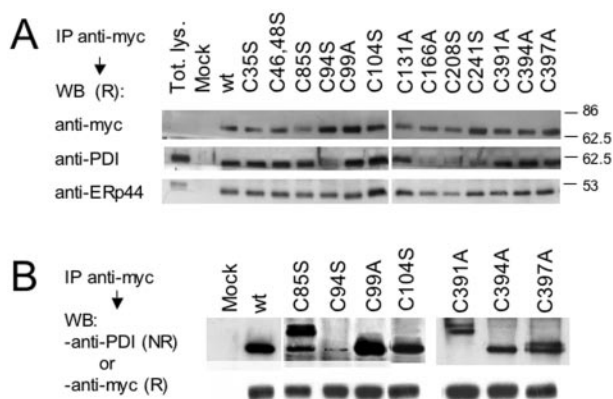
To monitor oxidative protein folding *in vivo*, we used a previously described pulse-chase assay (12) or a simplified version based on Western blotting. Unlike in the previously described assay (12), not all intracellular J chains were reduced in the Western blot assays (Fig. 2), suggesting that some pre-existing molecules are trapped in partially DTT-resistant complexes (12, 21).<sup>2</sup> Nonetheless, the activity of Ero1 $\alpha$  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  $\Delta$ 33–50, lacking all cysteines of cluster 1, was as active as wild-type Ero1 $\alpha$  (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  $\Delta$ 86–95 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 role for cluster 2.

After this preliminary survey, we refined our tools and ana-

<sup>2</sup> G. Bertoli, T. Simmen, T. Anelli, S. Nerini Molteni, R. Fesce, and R. Sitia, unpublished results.





**FIG. 5. Differential binding of PDI and ERp44 to *Ero1* $\alpha$  mutants.** Lysates from HeLa transfectants expressing the indicated *Ero1* $\alpha$  mutants were immunoprecipitated with immobilized anti-Myc antibodies. Aliquots of the immunoprecipitated material were resolved under nonreducing (NR) or reducing (R) conditions and blotted as indicated on the left of each panel. *A*, filters containing reduced samples were hybridized with anti-Myc (top) to normalize for transgene expression, anti-PDI, or anti-ERp44 (36C9/39; see Ref. 13). The first lane on the left shows the total lysates (Tot. lys.) of HeLa cells expressing a hemagglutinin-tagged ERp44 (migrating more slowly than endogenous molecules because of the tag). Note that C94S, C166A, C208S, and C241S mutants bind less PDI and almost normal amounts of ERp44. *w.t.*, wild type; WB, Western blot; IP, immunoprecipitate. *B*, anti-Myc immunoprecipitates from the indicated transfectants were blotted as indicated on the left and decorated with anti-PDI (NR, upper panel) or anti-Myc (R, lower panel).

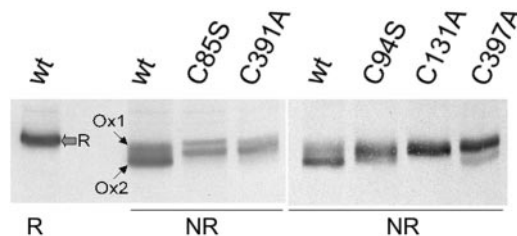
lyzed individual amino acid replacements by immunoprecipitation folding assays. Among individual cysteine-replacement mutants, C85S and C94S were less active than wild-type *Ero1* $\alpha$ , whereas C99A acted as a weak dominant negative (Fig. 3). Mutation of C104 did not impair the ability to promote JcM folding, further confirming the specificity of the phenotypes observed.

Within cluster 3, as described previously (12, 18), C394A and C397A did not complement *ero1-1* and behaved as strong dominant negative phenotypes, whereas mutant C391A displayed reduced activity similar to C85S (Table I).

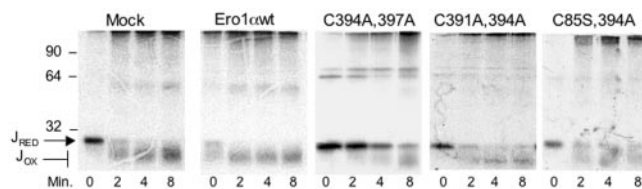
Mutants in the four "scattered" cysteines (C131A, C166A, C208S, and C241S) were almost as active as wild-type molecules, and so were mutants lacking either one or both glycosylation sites (Table I).

In agreement with the functional data obtained in mammalian cells, transformation of *ero1-1* 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 *ero1-1* at all. These results confirm a fundamental role for cysteine clusters 2 and 3 in *Ero1* $\alpha$  activity, as described in yeast *Ero1p* (16). Interestingly, the Cys<sup>85</sup> and Cys<sup>391</sup> 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 *Ero1* $\alpha$  (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 *Ero1* $\alpha$ -PDI mixed disulfides can be formed for reasons other than transferring oxidative



**FIG. 6. Electrophoretic pattern of *Ero1* mutants.** Lysates from HeLa transient transfectants expressing the indicated *Ero1* $\alpha$  mutants or the wild-type molecule were resolved by SDS-PAGE (7.5%) under nonreducing conditions, blotted, and probed with anti-Myc. Fully reduced *Ero1* $\alpha$  wild type (*w.t.*) was also loaded (gray-filled arrow, R). Ox1 and Ox2 are indicated by arrows.



**FIG. 7. Cooperativity between clusters 2 and 3.** Oxidative folding of JcM chains was compared in HeLa cells co-expressing Myc-tagged JcM chains and the indicated *Ero1* $\alpha$  double-mutants, as described in the legend for Fig. 3. The mobility of reduced and oxidized JcM monomers is indicated.

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 *Ero1* $\alpha$ .

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 (Cys<sup>85</sup> and Cys<sup>391</sup>) 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 Cys<sup>85</sup> and Cys<sup>391</sup> 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 *Ero1* $\alpha$  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<sup>391</sup> or Cys<sup>85</sup> was replaced concomitantly with Cys<sup>394</sup>, the dominant negative phenotype was lost. Substitution of Cys<sup>85</sup> or Cys<sup>391</sup> reverted the dominant negative phenotype of Cys<sup>99</sup> as well (not shown). Therefore, the absence of the first cysteine of each triad makes Ero1 $\alpha$  less active, both in promoting and in inhibiting disulfide bond formation. Taken together, these findings indicate that Cys<sup>85</sup> and Cys<sup>391</sup> are necessary to coordinate the activity of clusters 2 and 3 in the functional cycle of human Ero1 $\alpha$ .

#### DISCUSSION

The process of Ero1 $\alpha$ -dependent PDI oxidation likely involves several steps: docking of reduced PDI, formation of PDI-Ero1 $\alpha$  mixed disulfides, detachment of oxidized PDI, and recharging of Ero1 $\alpha$ . 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<sup>85</sup>-Cys<sup>94</sup>-Cys<sup>99</sup>) is essential for Ero1 $\alpha$  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<sup>85</sup> and Cys<sup>391</sup>, 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 JcM oxidation and in complementing *ero1-1* yeast cells. Our interpretation of these findings is that interactions between Cys<sup>85</sup> and Cys<sup>391</sup> 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 $\alpha$  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 $\alpha$ .

Ero1 $\alpha$  can fold into Ox1- and Ox2-like isoforms also, when translated *in vitro* and hence in the absence of PDI and ERp44. In contrast, Ero1 $\alpha$ -PDI and Ero1-ERp44 mixed disulfides are seen only when ER membranes are added (12, 17).<sup>2</sup> If indeed

Ox1 is caused by an intramolecular bond linking Cys<sup>85</sup> and Cys<sup>391</sup>, 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),<sup>3</sup> is in line with the pivotal role of Ero1 $\alpha$  in promoting disulfide bond formation (12).

Further evidence for a common functional role of Cys<sup>85</sup> and Cys<sup>391</sup> stems from the analysis of double mutants. Clearly, replacing either residue in the Cys<sup>99</sup> or Cys<sup>394</sup> 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 1 have any functional significance as isomerases, as proposed by Woycechowsky and Raines (22), remains to be tested. Also the two glycosylation sites are apparently dispensable for Ero1 $\alpha$  function, since mutating either one or both did not affect activity in either JcM folding or yeast complementation assays.

Mutants lacking Cys<sup>94</sup>, Cys<sup>166</sup>, Cys<sup>208</sup>, or Cys<sup>241</sup> bound less PDI at steady state. Similarly, Frand and Kaiser (16) report defective PDI binding for the Cys<sup>100</sup> and Cys<sup>208</sup> yeast Ero1p mutants. However, only in the case of C94S (and its counterpart in yeast Ero1p, Cys<sup>100</sup>) 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 $\alpha$  in its capacity of quality controller (23). ERp44 bound similarly well to all Ero1 $\alpha$  cysteine replacement mutants, including the four showing reduced interactions with PDI. We have recently proposed that the interaction with ERp44 is important in retaining Ero1 $\alpha$  intracellularly (13). Mass spectrometry analyses of over-expressed Ero1 $\alpha$  revealed that Cys<sup>166</sup>, Cys<sup>208</sup>, and Cys<sup>241</sup> are mainly in thiol form,<sup>4</sup> which could make Ero1 $\alpha$  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 Ero1 $\alpha$  and to the upstream acceptors. Although not essential for function, the stabilizing role of Cys<sup>85</sup> and Cys<sup>391</sup> clearly enhances the activity of the ER oxidizing machine, likely by aligning cysteines 94, 99, 394, and 397.

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<sup>4</sup> S. Camerini, G. Bertoli, T. Anelli, R. Sitia, and A. Bachi, unpublished results.

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## Two Conserved Cysteine Triads in Human Ero1 $\alpha$ Cooperate for Efficient Disulfide Bond Formation in the Endoplasmic Reticulum

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