Different Interaction Modes for Protein-disulfide Isomerase (PDI) as an Efficient Regulator and a Specific Substrate of Endoplasmic Reticulum Oxidoreductin-1α (Ero1α)*

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Background: Ero1α and PDI constitute the pivotal oxidative protein folding pathway in mammalian ER.

Results: Both catalytic domains of PDI and PDI homologues rapidly regulate Ero1α activity while Ero1α asymmetrically oxidizes PDI.

Conclusion: The modes for PDI as efficient regulator and specific substrate of Ero1α are different.

Significance: This study reveals how Ero1α-PDI interplay ensures oxidative protein folding homeostatically.

Protein-disulfide isomerase (PDI) and sulfhydryl oxidase endoplasmic reticulum oxidoreductin-1α (Ero1α) constitute the pivotal pathway for oxidative protein folding in the mammalian endoplasmic reticulum (ER). Ero1α oxidizes PDI to introduce disulfides into substrates, and PDI can feedback-regulate Ero1α activity. Here, we show the regulatory disulfide of Ero1α responds to the redox fluctuation in ER very sensitively, relying on the availability of redox active PDI. The regulation of Ero1α is rapidly facilitated by either α or α’ catalytic domain of PDI, independent of the substrate binding domain. On the other hand, activated Ero1α specifically binds to PDI via hydrophobic interactions and preferentially catalyzes the oxidation of domain α’. This asymmetry ensures PDI to function simultaneously as an oxidoreductase and an isomerase. In addition, several PDI family members are also characterized to be potent regulators of Ero1α. The novel modes for PDI as a competent regulator and a specific substrate of Ero1α govern efficient and faithful oxidative protein folding and maintain the ER redox homeostasis.

Disulfide bonds play important roles in the structure and function of many secretory and membrane proteins. The correct formation of disulfides during the folding of nascent peptides to native proteins, namely oxidative protein folding, takes place mainly in the endoplasmic reticulum (ER) in eukaryotic cells (1). Protein-disulfide isomerase (PDI) and sulfhydryl oxidase ER oxidoreductin-1 (Ero1) constitute the pivotal pathway for oxidative protein folding from yeast to mammals. PDI contains four thioredoxin (Trx) domains arranged as a-b-b’-α’, with two -CGHC- active sites respectively located in domain a and α’. PDI can directly catalyze disulfide formation in reduced substrates, as well as the isomerization reaction to convert aberrant disulfides to correct ones (2). Ero1 flavoproteins can catalyze the re-oxidation of reduced PDI for continuous transfer of disulfides to substrate proteins. The -CXXX- outer active site located in an intrinsically flexible loop of Ero1 transfers electrons from the active site of PDI to the buried -CXXX- inner active site, and the electrons are then used to reduce oxygen into hydrogen peroxide via flavin adenine dinucleotide cofactor (3, 4).

There are two isofoms of Ero1 in mammalian cells: Ero1α is widely expressed (5) and Ero1β is abundantly expressed in select secretory tissues such as the pancreas (6). Both Ero1α and Ero1β activities are regulated by regulatory disulfides formed between catalytic and non-catalytic cysteines to avoid futile oxidation cycles with excess hydrogen peroxide production (7–9). For Ero1α particularly, the formation of two regulatory disulfides Cys94-Cys131 and Cys99-Cys104 in the inactive resting state blocks disulfide transferring from the inner active site (Cys394-Cys397) to PDI via the outer active site (Cys94-Cys99). These two regulatory disulfides need to be reduced to liberate the outer active site for activation of Ero1α. A conserved long-range disulfide Cys85-Cys391 was suggested to also participate in the activity regulation of Ero1α (7, 8), which was challenged later (10).

In cells, the poise of active and inactive Ero1α at steady state was demonstrated to be dependent on the level of PDI (7, 11). Thus, PDI seems not only a substrate but also a physiological regulator of its oxidase Ero1α. However, the dynamics of the transition between active and inactive Ero1α during the fluctuation of ER redox environment and the role of PDI in these processes remain largely unknown. For the interplay between Ero1α and PDI, it has been elucidated that the catalytic active Ero1α preferentially oxidizes the C-terminal active site in domain α’ of PDI, rather than the N-terminal active site in
domain a (8, 12), although the reduction potentials of the two active sites are very similar (13). Also we and others have provided evidence that the primary substrate binding domain b of PDI plays a critical role in binding with Ero1α for functional disulfide relay (10, 12, 14). On the other hand, the molecular mechanism of the reduction/oxidation of the regulatory disulfides of Ero1α by PDI is little understood. There are at least twenty PDI family members (PDIs) in mammalian ER (2), but Ero1α as well as its hyperactive isofrom Ero1β poorly catalyzes the oxidation of other PDIs (9, 15). Meanwhile other PDIs at steady state unsuccessfully modulate the redox states of Ero1α (15). Altogether, to reveal the molecular mechanism underlying the interplay between Ero1α and PDIs is central and crucial for understanding how efficient oxidative folding and redox balance in the ER are maintained in mammalian cells.

In this study, we report that (i) Cys85-Cys391 disulfide in Ero1α is stable and remains intact during the physiological activation of the enzyme; (ii) Cys94-Cys131 regulatory disulfide responds to the redox fluxuation in ER very sensitively, and its reduction/oxidation can be facilitated by not only PDI but also some other PDIs; (iii) either catalytic domain of PDI is able to facilitate the regulation of Ero1α, and the substrate binding domain b of PDI is not essential for activation/inactivation of Ero1α; (iv) the functional oxidation of PDI catalyzed by Ero1α is asymmetric to make the a’ domain act primarily as an oxidase and the a domain as an isomerase. The above findings shed great light on the mechanism underlying the interplay between Ero1α and PDI proteins, which ensures the efficiency and fidelity of oxidative protein folding and maintains the thiol-disulfide redox homeostasis in the ER.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Protein Preparation**—For protein expression in bacteria, pGEX-6P-1 plasmids encoding Ero1α WT and Ero1α C104/131A were used as previously described (9). pET28a-Ero1p (Phe56-Leu424) was kindly donated by Dr. Yi Yang (East China University of Science and Technology, China). pQE30 plasmids encoding PDI WT, PDI C1, PDI C2, and PDI C1/2 (12), PDI a and PDI a’ c’ (16) were laboratory products. pET23b-PDIp, pET23b-P5, pET23b-EP18, pET23b-EP72, and pET23b-Pdi1p were generous gifts from Dr. Lloyd W. Ruddock (University of Oulu, Finland). pET15b-EP46 and pQE30-EP57 were kindly provided by Dr. Xi Wang (this laboratory). Chimeric PDI-PDIs proteins were constructed by fusing the catalytic domain of PDIp a’ (Leu737-Leu725), EP57 a’ (Lys366-Leu505), EP72 a’ (Lys515-Leu645), P5 a’ (Gly144-Glu280), and EP46 a’ (Asp320-Leu342) to the C terminus of PDI bb’ base (Ala136-Leu355), and inserted into pQE30 vector at XbaI/HindIII sites. For expression in mammalian cells, pcDNA3.1-PDI, pcDNA3.1-Ero1α-myc, and pcDNA3.1-Ero1α-HA were used as described (17). pcDNA3.1-HA-PDI was generated by insertion of an HA tag after the N-terminal ER signal sequence. An aspartic acid residue was inserted before the tag to recreate the signal sequence cleavage site. The cDNA encoding P5 with its signal sequence, a C-terminal myc tag followed by an ER-retrieval motif (KDEL), and a KpnI restriction site on the upstream of the myc epitope, was generated by PCR and inserted into pcDNA3.1 vector at XbaI/HindIII sites. The cDNA for PDIp, ERp18, ERp72, and ERp57 with their own signal sequences and ERp46 with PDI’s signal sequence were amplified by PCR and ligated into XbaI/KpnI digested pcDNA3.1-P5-myc. All the other mutations for Ero1α, PDI, and Pdi1p were created using the Fast Mutagenesis System (TransGen) and verified by DNA sequencing (Invitrogen).

Recombinant Ero1p (18) and Ero1α (12) proteins were expressed and purified as described. PDI proteins and Pdi1p proteins were purified as for PDI (19). For reduced protein preparation, PDI proteins at 100 μM or Ero1α at 10 μM with 100 mM DTT and Pdi1p proteins at 100 μM with 10 mM GSH were incubated in buffer A (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.6) for 1 h at 25 °C. Excess reductants were then removed using a HiTrap desalting column (GE Healthcare) pre-equilibrated with buffer A, and the reduced proteins were kept on ice for use only in the same day. For oxidized protein preparation, PDI proteins at 100 μM or Ero1α at 50 μM was incubated with 50 mM potassium ferricyanide in buffer A for 1 h at 25 °C, and then chromatographed through a Superdex-200 10/300 GL column (GE Healthcare) pre-equilibrated with buffer A. Monomeric protein fraction was collected, concentrated and stored at −80 °C in aliquots.

**Cell Culture, Transfection, and Antibodies**—HeLa cells were cultured in DMEM (Invitrogen) containing 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 5% CO2. Plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h, the transfected cells were harvested, or as needed treated with 50 μM DTT and 50 μM 16F16 (Sigma-Aldrich) dissolved in DMSO for 8 h before harvest.

The following mouse monoclonal antibodies were used: αEro1α (2G4, a gift from Roberto Sitia, Università Vita-Salute San Raffaele, Italy), αEro1α (ab57177, Abcam), αPDI (RL90, Abcam), αGAPDH (GAPDH-71.1, Sigma-Aldrich), αmyc (9E10, Sigma-Aldrich), and αHA (HA-7, Sigma-Aldrich).

**RNA Interference**—The pSUPER-retro-puro vector (Oligoengine) expressing the shRNA targeting PDI sequence 5’-GAGTGTGTCTGACTATGAC-3’ (20) was constructed according to the manufacturer’s instructions. The pSUPER-shEro1α plasmid was used as described (17). pSUPER plasmids were transiently transfected into HeLa cells on day 1. Puromycin was added into the medium to a final concentration of 2 μg/ml on day 2 to kill the negative cells. Then pSUPER plasmids were co-transfected with pcDNA3.1-Ero1α C99/104A on day 3. Cells were harvested on day 5.

**Assay for the in Vivo Redox States of Ero1α, Immunoprecipitation, and Western Blotting**—For Ero1α activation, the harvested cells were re-suspended in DMEM containing 150 μM DTT at 25 °C. Aliquots were taken and immediately blocked by 20 mM N-ethylmaleimide (NEM, Sigma-Aldrich) at different times to trap disulfide bonds. For Ero1α inactivation, after incubation with 10 mM DTT in DMEM at 25 °C for 10 min, cells were quickly washed twice by ice-cold phosphate-buffered saline to remove excess DTT and re-suspended in DMEM at 25 °C. Aliquots were then taken and immediately blocked by 20 mM NEM at different times. Cells were lysed in radio immunoprecipitation assay buffer (Beyotime) containing 1 mM phenylmethylsulfonl fluoride and 20 mM NEM. Post-nuclear super-
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The redox states of Ero1α were analyzed by nonreducing SDS-PAGE to resolve the denatured and reduced BPTI to a final concentration of 30 μM into buffer B containing pre-incubated Ero1 and PDI proteins making a final concentration of 3 μM of each. Aliquots were taken at different time points and quenched by adding 0.1 volumes of 5 M HCl. The samples were then loaded onto a Vydac C18 analytical HPLC column (250 × 4.6 mm, GRACE) and eluted with a flow rate of 1 ml/min using a linear gradient of acetonitrile from 15% to 50% at a rate of 0.7%/min in 0.05% trifluoroacetic acid. The absorbance at 229 nm was monitored. The percentage of each folding intermediate during the refolding was quantified using Chromleach software (Thermo Scientific).

RESULTS

Cys85-Cys391 Remains Intact during Ero1α Activation—Ero1α contains fifteen cysteine residues with the disulfide pattern as shown in Fig. 1A (7, 14, 23). First, we checked the controversial regulatory function of Cys85-Cys391 by taking advantage of retarded protein mobility on nonreducing SDS-PAGE caused by long-range disulfide breakage. Three Ero1α Cys-to-Ala mutants C85/391A, C104/131A, and C85/104/131/391A were prepared to serve as a disulfide ruler, mimicking the reduction of long-range disulfide of Cys85-Cys391, Cys94-Cys131, and the both, respectively (23). As shown in Fig. 1B, purified recombinant Ero1α wild-type (WT) migrated exclusively as oxidized state (Ox2, lane 1), and Ero1α C104/131A migrated slower (Ox1, lane 11). Ero1α C85/C391A migrated a little faster than the Ox1 form (lane 10). Disruption of the two long-range disulfides (Cys94/Cys131 and Cys85-Cys391) made the migration much slower (lane 12) close to the reduced form (red, lane 13). Glutathione (GSH) alone did not reduce Ero1α, while in the presence of PDI, the physiological substrate of Ero1α, the slower migrating species (Ox1) in Ero1α WT gradually emerged with increasing ratios of reduced to oxidized glutathione (GSH/GSSG). This species migrated the same as C104/131A but slightly slower than C85/391A, implying that the long-range disulfide of Cys94-Cys131 was reduced. At 10 mM GSH, the majority of Ero1α shifted to Ox1 state but no band at the mobility of C85/104/131/391A was observed, indicating that Cys85-Cys391 was still intact after the reduction of Cys94-Cys131. Oxygen consumption assays confirmed that the Ox1 species is enzymatically active (Fig. 1C), indicating that the reduction of Cys94-Cys131 results in the activation of Ero1α. Correspondingly, C85/391A (with intact Cys94-Cys131 disulfide) was reduced by PDI and GSH to a slower migrating band, but C104/131A (with intact Cys85-Cys391 disulfide) was not reduced (Fig. 1D), suggesting that Cys85-Cys391 is very stable.

We next examined the influence of PDI on the redox states of Ero1α in HeLa cells. Ero1α C99/104A mutant was used because this mutant retains both long-range disulfides of Cys85-Cys391 and Cys94-Cys131 for redox state examination but lacks intact outer active site (Cys94-Cys99) for catalyzing substrate oxidation, so that the interference from the re-oxidation of Ero1α regulatory disulfides by oxidized substrates can be excluded. As shown in Fig. 1E, Ero1α C99/104A migrated in both Ox1 and Ox2 forms, implying that in cells it exists in both activated and inactivated forms, similar to Ero1α WT. Co-expression of PDI at steady state led to moderate but significant increase of Ox1
form in C99/104A as well as pronounced increase of the disulfide-linked Ero1α-PDI heterodimer, but no fully reduced form was observed, indicating that in cells the increase of PDI level promotes the reduction of Cys94-Cys131 but not Cys85-Cys391. Moreover, mutation of Cys85-Cys391 disulfide dramatically impaired the formation of Ero1α-PDI heterodimer, underlining the importance of this bond for maintaining the Ero1α-PDI functional complex. In summary, during the activation of Ero1α the Cys94-Cys131 regulatory disulfide is reduced resulting in the mobility shift from Ox2 to Ox1, whereas the Cys85-Cys391 disulfide remains intact, which is important for the catalytic activity of Ero1α.

**Dynamic Regulation of Ero1α Activity in Cells by PDI**—To gain further insights into the dynamic regulation of Ero1α during the fluctuation of the ER redox environment in cells, the activation/inactivation processes of Ero1α were studied by monitoring the interconversion between Ox1 and Ox2 forms of the aforementioned Ero1α C99/104A mutant. Firstly, the activation kinetics of Ero1α was examined by stressing the cells with a low concentration of DTT to mimic the burst of free thiols during protein synthesis. Just in 1 min after DTT challenge, a large portion of Ero1α quickly shifted to Ox1 state, but after 10 min there was still a small fraction of Ox2. When PDI was overexpressed, all Ero1α shifted from Ox2 to Ox1 in 1 min (Fig. 2A). When cells were pre-treated by a small molecule 16F16, which specifically inhibits the thiol-disulfide oxidoreductase activity of PDI (24), the reduction of Ero1α upon DTT addition was almost completely inhibited, strongly suggesting that PDI plays a critical role in mediating the thiol-driven activation of Ero1α (Fig. 2B).

Next, we studied the inactivation process of Ero1α C99/104A by using a DTT pulse-chase assay. In DTT-flooded cells, Ero1α was in Ox1 state with the Cys94-Cys131 regulatory disulfide retained, whereas the Cys85-Cys391 disulfide was explicitly identified to be a structural but not regulatory disulfide in this work. The flexible loop region is represented by an asterisk indicating that in cells the increase of PDI level was examined by stressing the cells with 16F16, which specifically inhibits the thiol-disulfide oxidoreductase activity of PDI (24), the reduction of Ero1α upon DTT addition was almost completely inhibited, strongly suggesting that PDI plays a critical role in mediating the thiol-driven activation of Ero1α (Fig. 2B).

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Ero1α inactivation. As the recovery of ER redox homeostasis (25) and PDI redox balance (26) from a reductive challenge both largely depend on Ero1α oxidase activity, we next looked into whether modulating Ero1α level can affect the inactivation of Ero1α itself. Silencing endogenous Ero1α dramatically inhibited the recovery of Ox2 in Ero1α C99/104A (Fig. 2E). Conversely, co-expression of the hyperactive Ero1α C104/131A markedly accelerated the transition from Ox2 to Ox1 in Ero1α C99/104A and reset the redox poise eventually at 30 min (Fig. 2F). Taken together, in cells Ero1α is very sensitive to the fluctuation of thiol-disulfide redox states in the ER, and the regulatory disulfides of Ero1α can be modulated by the redox active PDI.

The Two Catalytic Domains of PDI Can Activate Ero1α Independently—PDI contains two -CGHC- active sites respectively located in domain α and α’. To understand the contribution of the two catalytic domains to the activation of Ero1α, five PDI mutants were prepared (Fig. 3A). We tested the in vitro ability of these PDI proteins at reduced form to reduce the Cys94-Cys131 regulatory disulfide in Ero1α C99/104/166A mutant. The mutation of the unpaired Cys166 to Ala on the background of C99/104A was to avoid aberrant formation of homodimer during the preparation of homogenous oxidized Ero1α monomer (14). Addition of 10-fold reduced PDI WT to oxidized Ero1α resulted in rapid appearance of Ox1 form within 15 s, but up to 5 min there was still Ox2 remaining (Fig. 3B), as the reduction potential of the Cys94-Cys131 regulatory disulfide is much lower than that of PDI as mentioned above. PDI C1 or PDI C2, in which both cysteines were replaced by serines in the active site of domain α and α’, reduced the Cys94-Cys131 regulatory disulfide as efficiently as PDI WT, indicating that either active site of PDI is sufficient to activate Ero1α. PDI C1/2 with both active sites mutated as a negatively control, had little effect on the reduction of Ero1α even though excess GSH was supplied (Fig. 3B and C). The ability of catalytic domains of PDI to activate Ero1α was further assessed by isolated PDI α and α’ domains. Similar to PDI WT both isolated domains reduced ~40% of the Cys94-Cys131 regulatory disulfide in 5 min, although Ox1 form appeared somewhat slower during the initial stage (Fig. 3, B and C). The above results clearly demonstrated that PDI can directly modulate the fast transition of Ero1α from Ox2 to Ox1 state, and the two catalytic domains of PDI can independently perform the task of activating Ero1α.
can occur autonomously. Purified Ero1α protein was firstly treated with excess amounts of DTT for full reduction of the regulatory disulfides. After DTT removal, reduced Ero1α and its mutants were diluted to a final concentration of 3 mM to initiate the self-oxidation. Aliquots were taken out at indicated time points (lanes 3–6) for analyses by nonreducing SDS-9% PAGE and Coomassie staining. As shown in Fig. 4A, WT of Ero1α C394A was incubated with 3 μM GST-Ero1α WT for full reduction of the regulatory disulfides. After DTT was washed out, most of Ero1α protein was firstly rearranged and the autonomous re-oxidation of Ero1α could occur. Purified Ero1α was incubated with excess amounts of DTT for full reduction of the regulatory disulfides. After DTT removal, reduced Ero1α can occur autonomously. Purified Ero1α protein was firstly treated with excess amounts of DTT for full reduction of the regulatory disulfides. After DTT removal, reduced Ero1α and its mutants were diluted to a final concentration of 3 mM to initiate the self-oxidation. Aliquots were taken out at indicated time points (lanes 3–6) for analyses by nonreducing SDS-9% PAGE and Coomassie staining. As shown in Fig. 4A, WT of Ero1α C394A was incubated with 3 μM GST-Ero1α WT for full reduction of the regulatory disulfides. After DTT was washed out, most of Ero1α protein was firstly rearranged and the autonomous re-oxidation of Ero1α could occur. Purified Ero1α was incubated with excess amounts of DTT for full reduction of the regulatory disulfides. After DTT removal, reduced Ero1α and its mutants were diluted to a final concentration of 3 mM to initiate the self-oxidation. Aliquots were taken out at indicated time points (lanes 3–6) for analyses by nonreducing SDS-9% PAGE and Coomassie staining. As shown in Fig. 4A, WT of Ero1α C394A was incubated with 3 μM GST-Ero1α WT for full reduction of the regulatory disulfides. After DTT was washed out, most of Ero1α protein was firstly rearranged and the autonomous re-oxidation of Ero1α could occur. Purified Ero1α was incubated with excess amounts of DTT for full reduction of the regulatory disulfides. After DTT removal, reduced Ero1α and its mutants were diluted to a final concentration of 3 mM to initiate the self-oxidation. Aliquots were taken out at indicated time points (lanes 3–6) for analyses by nonreducing SDS-9% PAGE and Coomassie staining. As shown in Fig. 4A, WT of Ero1α C394A was incubated with 3 μM GST-Ero1α WT for full reduction of the regulatory disulfides. After DTT was washed out, most of Ero1α protein was firstly rearranged and the autonomous re-oxidation of Ero1α could occur.
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FIGURE 5. Asymmetric oxidation of PDI by Ero1α endows PDI with both oxidoreductase and isomerase activities. A and B, loss of reactivity toward mPEG-5k upon disulfides formation in PDI (A) or Pdi1p (B) proteins catalyzed by Ero1α or Ero1p, respectively, was monitored by SDS-10% PAGE and visualized by Coomassie staining. a userdata, αααα, and αααααα indicate the PDI proteins with a domain reduced, a domain oxidized, a′ domain reduced and a′ domain oxidized, respectively. The doublet bands of each PDI species are resulted from one or both cysteines in domain b′ being alkylated by mPEG-5k. Asterisks in B indicate the Pdi1p species with a structural disulfide (Cys90-Cys97) in domain α being reduced and modified by mPEG-5k. C and D, isomerase activities of PDI (C) and Pdi1p (D) proteins for the reactivation of scrambled RNase A were determined after incubation with or without Ero1 oxidases as indicated (mean ± S.D., n = 3). E and F, oxidative folding of denatured and reduced BPTI was catalyzed by Ero1α/PDI (E) or Ero1p/Pdi1p (F) and analyzed by reverse-phase HPLC. The percentage of reduced BPTI, one disulfide containing species, two disulfides containing species and native BPTI at 0, 1, 5, 10, 30, and 60 min were quantified. Values were the mean of two independent experiments with very similar profiles.

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of the kinetically trapped disulfide intermediates (29). The quantitative dissection of BPTI refolding steps showed that in the first 10 min Ero1α/PDI catalyzed the oxidation of reduced BPTI to various intermediates, which were further oxidized to native BPTI at 60 min with the yield over 90% (Fig. 5E). In Ero1p/Pdi1p system, although the oxidation of reduced BPTI in the first 10 min was slightly faster than that in Ero1α/PDI system, the one-disulfide and two-disulfide species remained in later steps and only ~50% BPTI was refolded to native state after 60 min (Fig. 5F). Thus, human Ero1α/PDI system is more efficient than yeast Ero1p/Pdi1p system in proofreading non-native disulfides. In conclusion, the asymmetry in Ero1α catalyzed oxidation of PDI is functionally significant, which makes the a′ domain act primarily as an oxidase and the a domain act as an isomerase so as to ensure efficient oxidative folding of client proteins.

Role of Hydrophobic Interaction in Ero1α–PDI Interplay—We further explored the mechanisms underlying the intriguing phenomenon that both catalytic domains of PDI can react with the regulatory disulfides of Ero1α without discrimination but only the a′ domain can mediate efficient electron transfer to the outer active site of Ero1α. We and others previously found that the principle substrate binding domain b′ in PDI is critical for the Ero1α/PDI disulfide relay (12, 14), therefore we studied whether the substrate binding ability of PDI is also required for the activation/inactivation of Ero1α. Here we took the advantage of the mutation of two residues Phe-275 and Ile-289 in the b′ domain of PDI, which dramatically eliminates binding with peptide or Ero1α (10, 30). Surprisingly, PDI binding mutant F275W/I289A at reduced form was able to reduce the Cys94-Cys131 regulatory disulfide in Ero1α as efficiently as PDI WT (Fig. 6A), and the oxidized binding mutant also facilitated the re-oxidation of Ero1α (Fig. 6B). Thus, the peptide binding ability of PDI b′ domain is not required in the reduction/oxidation of the regulatory disulfides in Ero1α.

To explore which form of Ero1α binds with PDI, HA-tagged PDI WT was further used to co-immunoprecipitate Ero1α in HeLa cells. Only Ox1 but not Ox2 species of Ero1α was detected with PDI under nonreducing conditions (Fig. 6C), indicating that PDI specifically recognizes the active form of Ero1α through non-covalent interaction. A surfactant Triton X-100 and a hydrophobic probe 1-anilinonaphthalene-8-sulfonate (ANS) both markedly inhibited the oxygen consumption by active Ero1α during the oxidation of PDI (Fig. 6, D and E), emphasizing the functional role of hydrophobic binding in Ero1α and PDI disulfide relay. Interestingly, Triton X-100 and ANS only slightly affected Ero1p/Pdi1p activity (Fig. 6, D and E), implying that the hydrophobic interaction between Ero1α and PDI is a critical requirement for mammals. Gel filtration chromatography of mixed PDI and Ero1α showed a new peak fraction with the molecular weight larger than that of the separated proteins (Fig. 6F), confirming the presence of a stable complex between PDI and Ero1α. The majority of this complex was formed via non-covalent interaction and the minority was linked by intermolecular disulfide bridges (Fig. 6F). In line with activity assays, negligible complex of Ero1p and Pdi1p was
detected on gel filtration (Fig. 6G). Collectively, all the above data support our model that modulation of the regulatory disulfides in Ero1α by both PDI active sites is independent from the $b'$ domain, while the hydrophobic interaction is necessary for the catalytic oxidation of PDI $a'$ domain by active Ero1α. In contrast, there is no stable hydrophobic binding between yeast Ero1p and Pdi1p, and both active sites of Pdi1p can freely react with the regulatory disulfides (31) as well as the catalytic disulfide (28) and Fig. 5B) in Ero1p due to less conformational restriction.

Interplay between Ero1α and Other PDIs—It is known that Ero1α and its hyperactive homologue Ero1β poorly catalyze the oxidation of other PDI family members (9, 15), but transient mixed-disulfides between Ero1α and several PDIs were trapped in cells (32, 33). We speculated these intermediates might be formed during the reduction/oxidation of the regulatory disulfides in Ero1α by PDIs. Therefore PDIp, P5, ERp46, ERp57, and ERp72 were overexpressed in HeLa cells to check whether they were able to function as regulators of Ero1α. Overexpression of these PDIs to a similar level had only moderate effects on the Ox1/Ox2 ratio of Ero1 (Fig. 7A, first lane in upper panel). However, when the cells were treated with a low concentration of DTT, these oxidoreductases except ERp72 rapidly promoted the reduction of Ero1α (Fig. 7A), underscoring their roles in facilitating Ero1α activation against reductive challenge in the ER. Thus, the inefficient functional oxidation of other PDIs by Ero1α may not be attributed to poor Ero1α activation. As the substrate binding domain $b'$ in PDI plays a critical role in enzymatic disulfide relay between PDI and Ero1α, we realized that lack of the unique $b'$ domain of PDI in other PDIs could be a reason for their inefficient oxidation by Ero1α. To test this possibility, chimeric PDI-PDIs proteins were constructed by fusing one catalytic domain from each of the five PDIs to the C terminus of the rigid $bb'$ base of PDI. The rates of Ero1α catalyzed oxygen consumption were dramatically increased in the presence of the chimeras PDI-P5, PDI-ERp57 and PDI-ERp72 (Fig. 7B). PDI-ERp46 only modestly increased the reaction rate and PDI-P5 was not effective (Fig. 7C). As the catalytic domains from PDIp, ERp57, and ERp72 used to generate the chimeras are $a'$-type while those from ERp46 and P5 are $a$-type, these results explained why other PDIs are poor substrates of Ero1α, and strengthened the molecular mechanism we proposed that both the $b'$ and $a'$ domains are necessary for the functional disulfide relay between Ero1α and PDI (12). As expected, all the PDIs and PDI-PDIs chimeras efficiently promoted the transition of Ero1α from Ox2 to Ox1 state during Ero1α catalyzed reactions (Fig. 7D), indicating that all PDIs tested are capable to activate Ero1α in vitro.

Next, the abilities of PDIs to inactivate Ero1α were studied by using the reconstituted system. PDIp, P5, ERp46 and ERp57 re-oxidized reduced Ero1α as efficient as PDI, and ERp72

FIGURE 6. Role of hydrophobic interaction in Ero1α/PDI interplay. A and B, activation (A) and inactivation (B) of 1 μM Ero1α C99/104/166A by 10 μM PDI F275W/I289A were monitored as in Figs. 3B and 4C, respectively. The percentage of Ox1 species in A or Ox2 species in B was quantified by densitometry, compared with the PDI WT plots in Figs. 3C or 4D, respectively (mean ± S.D., n = 3). C, lysates from HeLa transfectants expressing Ero1α-myc without or with HA-PDI as indicated were analyzed directly by nonreducing SDS-8% PAGE and WB (input) or after immunoprecipitation (IP) with αHA. D and E, oxidation of 20 μM PDI or Pdi1p, respectively, by 2 μM hyperactive Ero1α C104/131A or Ero1p C150/295A was determined by monitoring the rate of oxygen consumption in the presence of 10 mM GSH and various concentrations of Triton X-100 (D) or ANS (E) as indicated. The oxygen consumption rate was calculated from the slope of the linear phase of oxygen decrease, and the rates obtained in the absence of Triton X-100 or ANS were taken as 100% (mean ± S.D., n = 3). F and G, human (f) or yeast (g) PDI and Ero1 proteins, as well as their mixtures after a 30 min pre-incubation at a molar ratio of 1:1.3 were analyzed by a Superdex 200 10/300 GL column under room temperature at a flow rate of 0.5 ml/min. Apparent molecular weight of each peak as shown was calculated using protein standards: blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa). P3 fraction in F was collected, quenched by NEM, and then analyzed by nonreducing SDS-10% PAGE and visualized by Coomassie staining.
showed somewhat weaker effects in terms of the disappearance of fully reduced Ero1α/H9251 (Fig. 7 E). We were then interested in whether the -CGHC- active site containing motif, which is conserved among these PDIs, is sufficient to reduce/oxidize the regulatory disulfides in Ero1α/H9251. A synthesized octapeptide (PWCGHCKA) derived from PDI in its reduced form indeed promoted the activation of Ero1α/H9251 in a dose dependent manner, and the oxidized octapeptide facilitated the inactivation of Ero1α/H9251 in a similar way (Fig. 7 F), suggesting that the -CGHC- active site is the minimal element for regulation of Ero1α/H9251. The high-dose of octapeptide used here implied that the intact catalytic Trx domain is optimized for efficient reduction/oxidation of Ero1α. Altogether, the above data strongly suggested that PDIs with catalytic domain containing the -CGHC- active sites, if not all, are potent regulators of Ero1α. However, active Ero1α can only efficiently catalyze disulfide production via PDI due to the specific recognition of PDI b'-a' domains.

DISCUSSION

Ero1α activity is tightly regulated in mammalian ER by a feedback mechanism. At resting state of cells, formation of the two regulatory disulfides (Cys94-Cys131 and Cys99-Cys104) in Ero1α restricts the availability of the Cys94-Cys99 outer active site (7, 23). The inactive Ero1α must be promptly activated once robust protein oxidative folding capacity is required, and the activated Ero1α must be adequately inactivated to prevent over-oxidation within the ER. Here, we demonstrate that both the activation and inactivation of Ero1α by PDI in vitro occur very fast (<15 s). Similarly, when cells suffer or recover from a reductive challenge the reduction/oxidation of the regulatory disulfides in Ero1α happens rapidly within 1 min. Therefore, the interconversion between inactivated and activated Ero1α responding to the ER redox environment is prompt. Interestingly, we also find that after reductive challenge substantial oxi-
dase activity of Ero1α is required to re-establish the thiol-disulfide balance in the ER on a relative longer time-scale. Previous studies have shown that after reductive challenge the resetting of the steady-state ratio of GSSG to total glutathione is very fast on a time-scale of seconds (25), but the re-oxidation of disulfides in protein substrates takes longer time to be completed (34, 35). Thus, we propose when cells encounter a reductive challenge Ero1α is quickly activated by reduced PDI to catalyze the oxidation of GSH by cooperating with PDI, which results in fast reset of GSH/GSSG balance within 1 min, and Ero1α is then partly inactivated. The remaining active Ero1α drives the oxidation of protein substrates until the thiol-disulfide status in the ER reaches rebalance. After that, Ero1α is inactivated by oxidized PDI to prevent futile consumption of GSH and excessive peroxide production.

Previously, it was reported that several other PDIs (including ERP46, ERP57, ERp72, and P5) can hardly modulate the redox states of Ero1α at steady state, although their redox equilibrium constants with glutathione are close to that of PDI (15). By real-time monitoring the redox states of Ero1α, we clearly show that the activation of Ero1α upon reductive challenge is indeed significantly promoted when PDI, PDIp, P5, ERP46, or ERP57 was individually overexpressed in cells, and these PDIs at oxidized form can accelerate the inactivation of Ero1α in vitro (Fig. 7). One exception with unknown reasons is ERp72, which seems less efficient than the other PDIs to regulate Ero1α. Our new finding that many PDIs are potent regulators of Ero1α leads us to propose that the redox state of Ero1α is controlled by the redox balance of PDIs ensemble in the ER (Fig. 8). Since PDIs have substrate specificity in different tissue or cell types, the oxidase activity of Ero1α can thus be precisely regulated in cells with distinct proteomes. Recently, studies have reported the redox regulation of the different unfolded protein response (UPR) sensors was mediated by specific PDIs (36). Therefore, a redox-based feedback regulation loop for controlling the strength of UPR signaling likely exists in the ER, that fine-tuning Ero1α activity by PDIs maintains ER redox homeostasis, which will in turn keep balance of the redox states of PDIs and promote the adaptive UPR as well as attenuate the fatal UPR to avoid cell death.

In this report, we find that the reduction/oxidation of the regulatory disulfides in Ero1α relies on the catalytic activity of PDI but is independent from the peptide binding activity of PDI, and either active site of PDI can facilitate the regulation of Ero1α independently. This novel mode for PDI-mediated regulation of Ero1α is supported by several evidences: 1) single catalytic domain a or a’ of PDI functions well to promote the reduction/oxidation of the regulatory disulfides of Ero1α; 2) an octapeptide containing the -CGHC- active site of PDI alone is capable of regulating Ero1α; 3) the activation and inactivation of Ero1α in cells are dramatically abolished by a PDI inhibitor 16F16, which modifies the active sites of PDI; 4) the substrate binding deficient mutant PDI F275W/I289A regulates Ero1α as efficiently as PDI WT. This mode also well explains why other PDIs containing the -CGHC- active site are also potent regulators of Ero1α, even though they lack the unique b’ domain of PDI. During preparation of this paper we noticed a very recent study claiming that the substrate binding ability of PDI is crucial for the inactivation of Ero1α, according to the observation that a binding mutant PDI I272A/D346A/D348A (residue numbered without the 17-residue signal sequence) was not able to inactivate Ero1α under anaerobic conditions (37). This triple mutant was originally screened out for stabilization of the b’x fragment of PDI in a capped conformation (38), but in full-length PDI the existence of the neighboring a’ domain limits the x-linker to be in a fully capped conformation (39). Thus, the inefficiency of the I272A/D346A/D348A mutant to inactivate Ero1α in their experiments may not be properly attributed to the loss of peptide binding activity.

Once being activated, the reduction of the regulatory disulfides probably increases the conformational flexibility of the regulatory loop between Cys-94 and Cys-131 (14), so that Ero1α can specifically bind to PDI via hydrophobic interactions (Fig. 6), like an unfolded nascent peptide being captured by the substrate binding site of PDI. By recognizing the b’-a’ fragment of PDI, Ero1α specifically catalyzes the oxidation of the active...
site in the α’ domain of PDI. Other PDIs are poor substrates of Ero1α and cannot increase the ratio of activated/inactivated Ero1α at steady state because they cannot bind and stabilize the active form of Ero1α due to the lack of the peptide binding domain b’ in PDI. Intriguingly, if the α’-type domain of PDIP, ERp57 or ERp72 is fused to the bb’ base of PDI, the chimera becomes competent substrate for Ero1α oxidative activity. The two different interaction modes between Ero1α and PDIs described above ensures that the activity of Ero1α can be elegantly regulated by sensing the redox states of the PDIs ensemble, while Ero1α specifically produces disulfides through the α’ domain of PDI, so that the ER is protected from over-oxidation caused by promiscuous oxidation of PDI α domain and other PDIs.

The biological implication of the strong preference on the α’ domain by Ero1α oxidative activity has been revealed in this study. In the Ero1α-driven oxidative folding, PDI α’ domain acts primarily as an oxidase to transfer disulfides into folding substrate, while PDI α domain acts as an efficient isomerase to proofread incorrect disulfides. Under physiological conditions the semioxidized state of PDI is important for the efficiency and fidelity of oxidative protein folding (Fig. 8). Distinct from the mammalian system, there is little stable hydrophobic binding between yeast Ero1p and Pdi1p, which makes less conformational restriction for active Ero1p to transfer disulfides into either catalytic domain of Pdi1p. The strong oxidation of Pdi1p by Ero1p favors fast disulfide generation but compromises the isomerase activity required for catalyzing native disulfide formation (Fig. 5). Our results are in line with the observation that Pdi1p oxidase activity is critical to yeast growth and viability and less than 6% of its isomerase activity is needed (40). Actually, Pdi1p exists predominantly in oxidized state in yeast cells (27), whereas a majority of the active sites of PDI in human cells is in reduced state (41). It is known that only ~1% proteins (78 in 6,623 total proteins) in Saccharomyces cerevisiae are predicted to contain disulfides, much smaller than the percentage of ~16% (3,297 in 20,258 total proteins) in human (www.uniprot.org). Therefore, the less demand of isomerase activity in yeast than in mammals appears reasonable, and the Ero1α/PDI system in mammals has evolved to adapt to the folding of larger and more complicated disulfide proteome.

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