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Dynamic Retention of Ero1α and Ero1β in the Endoplasmic Reticulum by Interactions with PDI and ERp44

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ABSTRACT

Disulfide bonds are formed in the endoplasmic reticulum (ER) by sequential interchange reactions: Ero1α and Ero1β transfer oxidative equivalents to protein disulfide isomerase (PDI), which in turn oxidizes cargo proteins. Neither Ero1α nor Ero1β contains known ER localization motif(s), raising the question of how they are retained in this organelle. Here the authors show that, unlike endogenous molecules, overexpressed Ero1α and Ero1β are secreted by HeLa transfectants, suggesting saturation of their normal retention mechanism(s). Co-expression of either PDI or ERp44 prevents Ero1 secretion in a KDEL/RDEL dependent way. Covalent interactions between ERp44 and Ero1 are essential for retention. In contrast, a mutant PDI lacking the four cysteines in the two active sites still inhibits secretion, albeit less efficiently. PDI and ERp44 compete for Ero1 binding. PDI also prevents Ero1 aggregation and dimerization, thus chaperoning its own oxidase. This dynamic retention mechanism of Ero1 may be important for fine-tuning the regulation of ER redox homeostasis and quality control. *Antioxid. Redox Signal.* 8, 274–282.

INTRODUCTION

The formation of disulfide bonds is essential for the folding and function of many secretory and membrane proteins, which fold and assemble in the endoplasmic reticulum (ER) (31). Oxidizing equivalents are generated in the ER mainly by members of the Ero1 family (12, 13, 36). Yeast Ero1p binds FAD (16, 37) and is thought to utilize O_2 as the ultimate electron acceptor. In mammalian cells, two members of the family have been identified so far, Ero1α and Ero1β, which differ by tissue distribution and transcriptional regulation patterns (5, 26). Both oxidize protein disulfide isomerase (PDI), which in turn transfers disulfide bonds to cargo proteins (13, 22). PDI (15) is a multifunctional protein, endowed with oxidase, isomerase and reductase (9), as well as a chaperone/anti-chaperone (28, 29) activities. It is composed of four thioredoxin domains (**abb'a'**) followed by an acidic C-terminal domain (**c**). The two redox active sites in the **a** and **a'** domains contain CGHC motifs (19) that are essential for thiol-disulfide exchange (10) but not for chaperone activity (30).

The pathway proceeds via formation of mixed disulfides between donor and acceptor molecules. The specificity of these protein–protein relays is crucial to allow the formation and editing of disulfide bonds in the ER lumen (12). Disulfide isomerization and reduction are essential for protein folding and ER-associated degradation (ERAD) and require PDI to be present in the reduced state. Therefore, excess and/or uncontrolled activity of Ero1 would be deleterious for cell physiology. In both yeast (8) and mammalian cells (23), cytosolic GSH limits Ero1 activity, a step that in view of its importance is likely regulated also by other mechanisms. Amongst the possible regulatory targets are FAD import (35, 37), the folding, the localization and the stability of Ero1α and Ero1β.

Since both human Ero1α and Ero1β rescue yeast Ero1 mutants (26), the pathways of electron transfer are highly conserved between these distant species. In contrast, the mecha-

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nisms of subcellular localization diverge. In yeast Ero1p, a 127-amino acid carboxy-terminal extension is essential for association to the ER membrane and function (27). Human Ero1α and Ero1β lack this tail as well as any known ER retention motifs, raising the question of how they are retained intracellularly. We have shown previously that covalent interactions with ERp44, a protein endowed with a C-terminal RDEL motif, are involved in preventing Ero1 secretion (1). Here we extended our analyses, showing that also the interactions with PDI may contribute to Ero1 localization. Our results suggest that PDI might also regulate folding and activity of Ero1, revealing a possible dynamic pathway for ER redox regulation.

MATERIALS AND METHODS

Cells and reagents

HeLa and NSO cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS), antibiotics, and L-glutamine. FCS, Optimem and culture media were purchased from Gibco BRL (Milan, Italy); Sepharose-conjugated protein G, [^{35}S]methionine/cysteine, and ECL reagents from Amersham-Pharmacia (Uppsala, Sweden). Endo-H and PNGase-F from New England Biolabs (Beverly, MA), and DSP from Pierce (Rockford, IL). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Horseradish peroxidase conjugated goat anti-rabbit Igs and goat anti-mouse Igs were from Southern Biotechnology Associates, Inc. (Birmingham, AL). The anti-myc murine monoclonal antibody 9E10 (7) was used to detect tagged proteins. Rabbit antibodies against PDI and Ero1α were generous gifts of Prof. I. Braakman (Utrecht University, NL) and Dr. A. Benham (Durham, UK). Polyclonal antibodies against anti-ERp44 (B68) were described previously (2) whilst anti-Ero1β (R49) was generated by immunizing a rabbit with three peptides from human Ero1β (KEAFIDWARIC, CQKLPENSPSK, CIRDLQNFKVLLQQSR) coupled to KLH. The specificity of the serum was tested by Western blotting and immunoprecipitation (A. Cabibbo, A. Fassio, and RS, unpublished results).

Plasmids and vectors

Vectors encoding myc-tagged wild-type human Ero1α, Ero1β, and μ-chain have been described previously (1, 5, 26). Plasmid pcDNA-human-PDI was a kind gift of Prof. I. Braakman. Plasmids pcDNA-human-PDIΔKDEL-myc and pGEM-human-PDI-ASAS (mutant human PDI-C36A/C39S/C380A/C383S) were kind gifts of Prof. N. Bulleid (Manchester University, UK). A vector encoding human ERp57 (14) was a kind gift from Dr. Ellgaard (ETH, Zurich). Mutants were generated from Ero1α by PCR amplification with these oligonucleotides. N280A;GAAATGGGGACACGCAATTACAGAATTCCAACAGCGATTTG/CAAATCGCTGTTGGAATTCTGTAATTGCGTGTCCCCATTTC N384A;CTGCATTTTAGAGCTATCTCGAGAATTATGGATTG/CAATCCATAATTCTCGAGATAGCTCTAAAATGCAG. To obtain the double mutant, mutagenesis was performed with the N280A-specific oligos on the N384A encoding vector. After sequencing, mutated DNA was re-inserted into pcDNA3.1 with XbaI/KpnI sites. An EcoRI-SacII fragment from pGEM PDI-ASAS was inserted into pcDNA3.1 to generate pcDNA3.1-PDI-ASAS. To obtain pcDNA3.1-PDI-ASAS-ΔKDELmyc, an EcoRI/Esp3I fragment from PDI-ASAS and an Esp3I/SalI fragment from KDEL-myc from wild-type PDI were inserted into pcDNA3.1 using EcoRI and SalI sites.

To generate HA-tagged ERp57, a KpnI site was first inserted after the HA tag (YPYDVPDYA) of pcDNA3.1-ERp44HA (2) with this pair of oligos: GACTACGCAGAAGGTACCAGTCTTGATAC/GTATCAAGACTGGTACCTTCGCGTAGTC, to obtain pcDNA3.1-ERp44HA-KpnI. The sequence encoding mature ERp57 was amplified from a vector encoding human ERp57 with the following primers, which contain KpnI and HindIII sites, respectively: CCATATCGAAGGTGGTACCTCCGACGTGC/GTTAGCAGCCGGAAGCTTAGAGATCCTCC. The ERp57 PCR products obtained with were digested with KpnI/HindIII and inserted into pcDNA3.1-ERp44HA-KpnI. As a result, the final product, pcDNA3.1-ERp57-HA contains the ERp44 signal sequence, the HA tag, three extra amino acids (EGT) and human ERp57 coding sequence. Its ER localization was confirmed by immunofluorescence; signal cleavage was checked by comparing the electrophoretic mobility of *in vitro* translated and transfected ERp57 (EGR and MO, unpublished results).

The presence of the expected mutations was confirmed by DNA sequencing.

Transfection, [^{35}S]methionine/cysteine labeling, immunoprecipitation and Western blotting

Transient transfection was performed using Lipofectin (Invitrogen) according to the manufacturer's instructions. HeLa cells were (co-)transfected with pcDNA vectors encoding wild type or mutated Ero1α-myc, Ero1β-3myc, PDI, ERp44, or ERp57. Western blotting, [^{35}S]methionine/cysteine labeling and immunoprecipitation were performed as described (23).

Secretion assays

Two days after transfection, cells were cultured for 6–16 h in Optimem (Gibco) and supernatants precipitated with immobilized concanavalin A (Sigma) or anti-myc antibody in the presence of 20 m*M* NEM and protease inhibitors. Cells were lysed in 1% NP-40, 150 m*M* NaCl, 50 m*M* Tris-HCl pH 7.5, 20 m*M* NEM, and protease inhibitors or RIPA buffer (1% NP-40, 0.1% SDS, 150 m*M* NaCl, 50 m*M* Tris-HCl pH 8.0), as indicated.

RESULTS

Overexpression of Ero1α or Ero1β saturates the normal retention mechanism(s)

Consistent with their primary functional role in the ER, endogenous Ero1α and Ero1β are efficiently retained intracellularly (Fig. 1A, lanes 1, 3 and 9,10). In contrast, Ero1α or

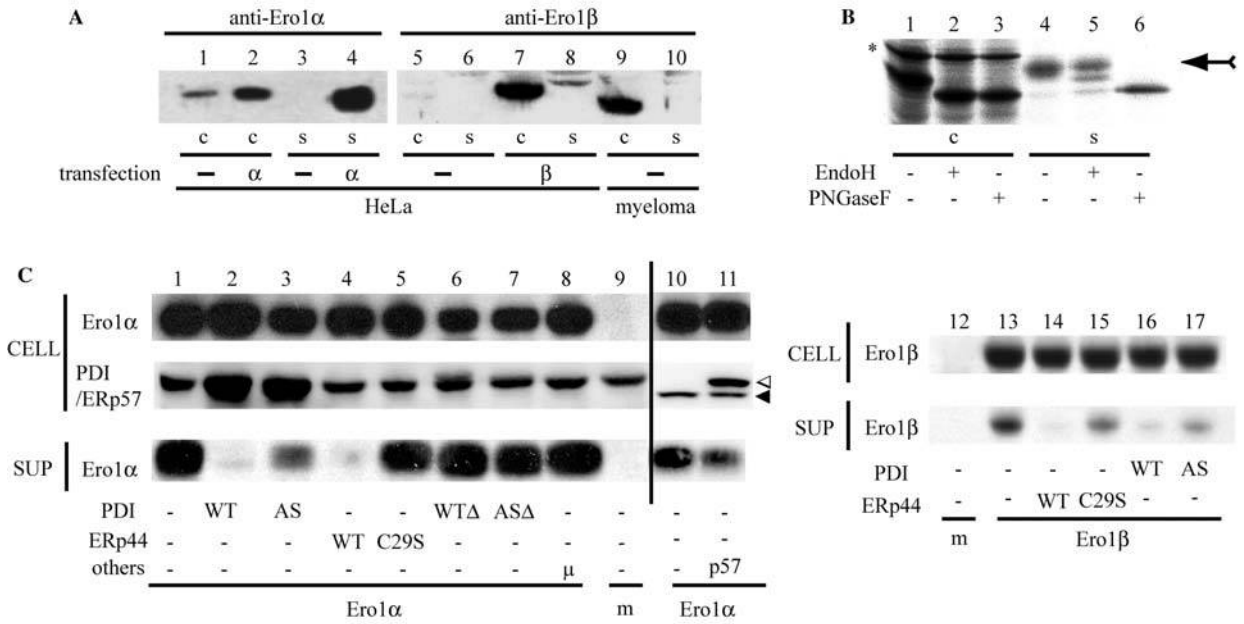

FIG. 1. Secretion of overexpressed Ero1α and Ero1β by HeLa transfectants. (**A**) Unlike overexpressed molecules, endogenous Ero1α and Ero1β are not secreted by HeLa and myeloma cells. HeLa (*lanes 1–8*) or NSO myeloma (*lanes 9–10*) cells, transfected as indicated, were cultured overnight. The supernatants were precipitated with concanavalin A Sepharose, whilst cells were lysed in RIPA buffer. Aliquots were resolved by SDS-PAGE and blots decorated with antibodies specific for Ero1α (*lanes 1–4*) or Ero1β (*lanes 5–10*). Cell numbers are *lane 1*, 2×10^6; *lane 2*, 0.5×10^6; *lane 3*, 5×10^6; *lane 4*, 2.5×10^6; *lanes 5 and 7*, 1×10^6; *lanes 6 and 8*, 3×10^6; *lane 9, 10*; 6×10^6. Even after prolonged exposure of the blots, no specific signal was detected by anti-Ero1α or anti-Ero1β in the supernatants of untransfected cells (*lanes 3 and 10,* data not shown). For endogenous Ero1β, murine myeloma cells were used because Ero1β was not detected with our antibody in HeLa cells (*lanes 9 and 10*). c; cell lysates, s; supernatants, α; Ero1α, β; Ero1β. (**B**) Secreted Ero1α contains endoglycosidase-H resistant N-glycans. Ero1α expressing cells were labeled with [^{35}S]methionine/cysteine for 4 h. Supernatants (*lanes 4–6*) and cell lysates (*lanes 1–3*) were immunoprecipitated with anti-myc and treated with Endo-H and PNGaseF, as indicated. Samples were analyzed in reducing SDS-PAGE and developed to X-ray film. Asterisk points to a background band. The *arrow* points to endoglycosidase-H resistant, secreted Ero1α molecules. (**C**) Co-expression of PDI prevents secretion of Ero1α and Ero1β. HeLa cells transfected with the indicated plasmids were cultured for 6 h. Aliquots from the supernatants (corresponding to 1.2 x 10^6 cells) and cell lysates (corresponding to 0.2×10^6 cells) prepared as described in legend to panel (**A**), were resolved by reducing SDS-PAGE. Blots were immunodecorated with anti-myc (Ero1α), anti-PDI (*CELL, lower panel, lanes 1–9*), or anti-ERp57 (*CELL, lower panel, lanes 10, 11*) as indicated. AS; mutant PDI-ASAS, WTΔ; wild-type PDI without KDEL, ASΔ; mutant PDI-ASAS without KDEL, m; pcDNA empty vector. *Open* and *closed arrowheads* on the right hand margin point to overexpressed HA-ERp57, and endogenous ERp57, respectively (*CELL, lower panel, lanes 10 and 11*). The supernatants from 5 x 10^6 cells and the lysates from 1 x 10^6 cells were analyzed in the case of Ero1β transfectants (*lanes 12–17*), because the levels of expression of this oxidoreductase are generally lower than those of Ero1α.

Ero1β are easily detected in the supernatant of HeLa transfectants (lanes 2, 4 and 7, 8, respectively), suggesting saturation of the normal retention mechanism(s) upon overexpression.

Most secreted Ero1α molecules contain Endo-H resistant N-glycans (Fig. 1B lane 5) excluding artifactual release by damaged cells. As another indication of the specificity of secretion, the co-expression of wild type ERp44 prevented secretion of Ero1α and Ero1β (Fig. 1C, lanes 4 and 14, see also (1)). In principle, any ER-resident molecule capable of binding the two oxidases with sufficient affinity –either covalently or noncovalently– should inhibit their secretion. Since it is well established that both Ero1α and Ero1β bind PDI, we transfected HeLa cells so as to increase the PDI pool (Fig. 1C, compare lanes 1 and 2). Clearly, the overexpression of wild-type PDI caused the retention of Ero1α and Ero1β (Fig. 1C, *SUP*, lanes 2 and 16, respectively). On the contrary, a PDI mutant devoid of the KDEL sequence, which did not accumulate in large amounts in the ER (*CELL*, compare lanes 2 and 6), did not significantly affect secretion of Ero1α (*SUP*, lane 6). As another important control of the assay, orphan secretory Ig-μ chains, which are retained in the ER and do not directly interact with Ero1α, did not inhibit Ero1α secretion (Fig. 1C, *SUP*, lane 8). The lower secretion of Ero1β probably reflected its lower level of expression, and hence of saturation.

A PDI mutant lacking both active sites interacts noncovalently with Ero1α

PDI forms abundant mixed disulfides with transfected (3, 22) and endogenously expressed Ero1α molecules (Ruffato *et al.*, unpublished observations; Benham *et al.*, submitted for publication). Retention of Ero1α could therefore rely on exploitation of a KDEL motif by covalent interactions with ER

residents. In agreement with this model, an ERp44 mutant lacking the active cysteine (C29S), which does not bind Ero1 (1), failed to inhibit secretion (Fig. 1C, *SUP*, lanes 5 and 15). However, a PDI mutant lacking the four cysteines in its active sites (PDI-ASAS) prevented secretion of Ero1α and Ero1β (Fig. 1C, *SUP,* lanes 3 and 17), albeit less efficiently than wild-type PDI. As expected, the PDI-ASAS mutant did not form covalent heterodimers with Ero1α (Fig. 2A, arrowhead). The interaction between PDI-ASAS and Ero1α, implied by the diminished secretion of the latter in cells co-expressing the two molecules, was confirmed by co-immunoprecipitation studies after cross linking (Fig. 2B), indicating that PDI-ASAS could associate with Ero1α noncovalently. Taken together, these findings suggest that noncovalent interactions precede the formation of covalent linkages between the two molecules.

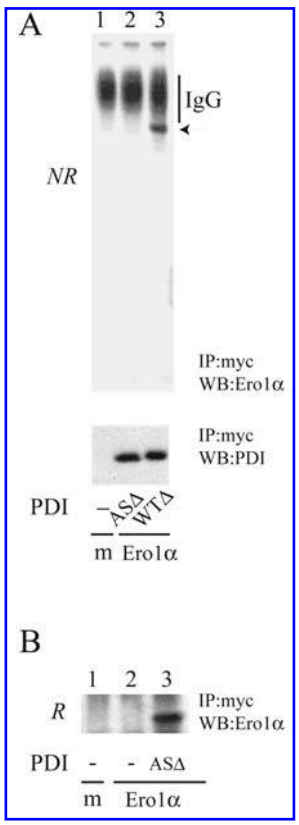

FIG. 2. Interactions between PDI-ASAS and Ero1α. (**A**) PDI-ASAS does not form mixed disulfides with Ero1α. Myc-tagged PDI wild type (WTΔ) or mutant PDI-ASAS (ASΔ) was co-transfected with untagged wild-type Ero1α. Cell lysates were immunoprecipitated with anti-myc antibody and resolved by nonreducing SDS-PAGE. Blots were immunodecorated with anti-Ero1α (*upper panel*) or anti-PDI antibodies (*lower panel*). The high molecular weight band is present in all three lanes consists of IgG molecules detaching from the anti-myc beads used for immunoprecipitation. (**B**) PDI-ASAS interacts noncovalently with Ero1α. HeLa cells expressing untagged wild-type Ero1α alone or co-expressing myc-tagged PDI-ASAS (ASΔ) were treated with 1 m*M* DSP for 30 min at 4°C and quenched with 3 m*M* glycine. Cell lysates were immunoprecipitated with anti-myc and resolved by reducing SDS-PAGE. Blots were decorated with anti- Ero1α to reveal proteins interacting with overexpressed PDI-ASAS.

Amongst the numerous ER oxidoreductases (11), Ero1α preferentially interacts with, and modulates the reoxidation rate of, PDI (22). In agreement with this, the overexpression of ERp57 inhibited the secretion of Ero1α (Fig. 1C, lanes 10 and 11), though much less efficiently than PDI or the PDI-ASAS mutant. Therefore, it seems that ERp57 can weakly bind to Ero1α and mostly in a noncovalent way, since less Ero1α-ERp57 disulfides than Ero1α-PDI disulfides can be detected even when both Ero1α and ERp57 are overexpressed (data not shown).

PDI and ERp44 compete for covalent Ero1 binding

In both normal and transfected HeLa cells, Ero1α accumulates in two distinct monomeric redox isoforms, Ox1 and Ox2 (3), and as mixed disulfides with PDI or ERp44 (Fig. 3A, lane 2). Mutational analyses suggested that PDI and ERp44 can bind to different cysteines in Ero1α (4). However, diagonal gels failed to reveal ternary complexes between the three molecules (22), suggesting that Ero1α binds alternately to PDI and to ERp44, at least in a covalent mode. If that were the case, the overexpression of PDI should inhibit the formation of complexes between Ero1 and ERp44, and *vice versa*. The results shown in Figure 3A indicate that this was indeed the case. Fewer ERp44-Ero1 complexes were detectable following the expression of wild-type PDI (lane 3). A band with a molecular weight and immunoreactivity expected for a PDI-Ero1 complex increased in cells co-expressing the two interacting molecules (see arrow, α+PDI). Interestingly, the expression of wt PDI increased the intensity of monomeric Ero1α, especially the Ox1 form, apparently at the expense of higher molecular weight species. Also PDI-ASAS decreased the Ero1α-ERp44 complexes and increased the accumulation of Ero1 monomers, mainly the Ox1 form (Fig. 3A, lane 5). It seemed that both PDI and PDI-ASAS decreased the intensity of a band consisting of Ero1α homodimers (α_2). As expected, PDI-ASAS did not form covalent heterodimers with Ero1. These results indicate that PDI can bind Ero1α noncovalently, with an affinity that is sufficient to slow down secretion. Formation of mixed disulfides stabilizes the interactions between the two molecules, and favors retention.

The overexpression of ERp44 (Fig. 3A, lane 5) decreased the abundance of PDI-Ero1α mixed disulfides, favoring the accumulation of ERp44-Ero1α complexes with different stoichiometries (1, 2). The C29S mutant had no detectable effects (Fig. 3A, lane 6) confirming that the ERp44 interacts with Ero1α mainly in a covalent manner. Similar results were obtained with Ero1β (Fig. 3B). The overexpression of both PDI and PDI-ASAS reduced the intensity of a band likely consisting of β homodimers (β_2, compare lanes 2–4). Wild-type ERp44, but not C29S mutant, recruited Ero1β into mixed disulfides with a 1:1 or different stoichiometries (lane 5) and also decreased the abundance of β homodimers.

Nonglycosylated Ero1α forms high molecular weight covalent aggregates and is poorly secreted

The overexpression of ERp57 had a minor but reproducible effect on Ero1α secretion. One possible reason for this effect may reside in the well described association of

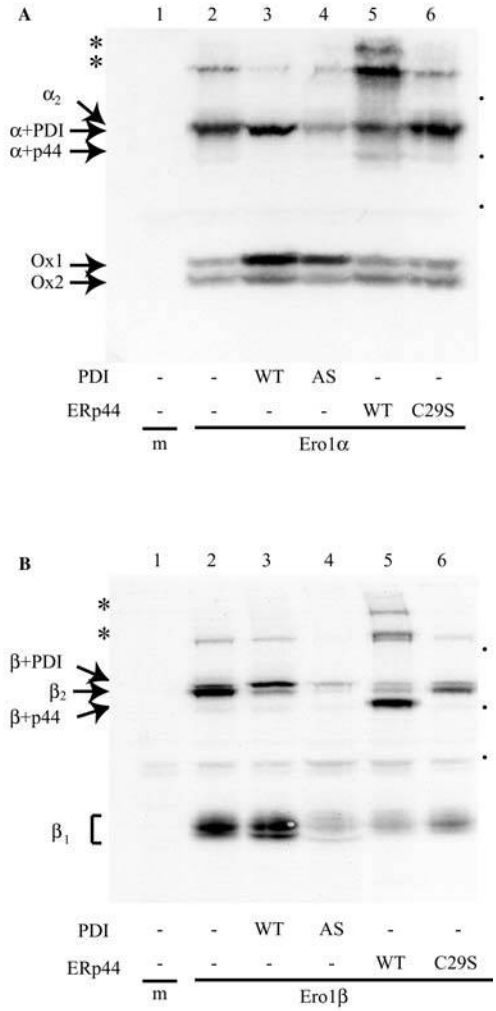

FIG. 3. Competition between PDI and ERp44 for covalent Ero1 binding. Aliquots from HeLa cells transfected as indicated were resolved under nonreducing conditions, and blots immunodecorated with anti-myc. *Arrows* on the left point to monomeric Ero1α (**A**) and β (**B**) and to the different covalent complexes formed with endogenous and/or co-expressed partners. *Asterisks* show the complexes including Ero1α and ERp44 (**A**) or Ero1β and ERp44 (**B**). α_2; Ero1α homodimer, α+PDI; Ero1α and PDI heterodimer, β_1; Ero1β monomer, β_2; Ero1β homodimer, β+PDI; Ero1β and PDI heterodimer. Dots represent 173, 113, 86 kDa markers. Abbreviations as in Fig. 1C.

ERp57 with calreticulin and calnexin to form functional complexes that assist glycoprotein folding (25). Therefore, we analyzed mutants lacking either one (N280A or N384A), or both N-glycans (NG-Ero1α). All three mutants were able to complement the yeast *ero1–1* mutant (4), implying that their folding into an active conformation was not totally precluded. However, differences were detected when their secretion was compared (Fig. 4A, *SUP*) the N280A mutant was secreted less efficiently than wild-type molecules (Fig. 4A, *SUP*, compare lanes 2 and 3), while the NG double mutant was not detected in the supernatant after 6 h of incubation (Fig. 4A, *SUP*, lane 5). NG-Ero1α formed abundant high molecular weight covalent complexes (Fig. 4A, lane 10), some of which did not enter the resolving gel (see Fig. 4B, lower panel, lane 2). Ox1 and Ox2 monomers were present in small amounts (Figs. 4A and 4B, lower panels). Also cells transfected with N384A, and to a lesser extent N280A, contained higher proportions of covalent Ero1 complexes.

PDI and PDI-ASAS inhibit aggregation of the NG-Ero1α mutant

The secretion of Endo-H resistant Ero1 implies that these molecules passed the ER quality control scrutiny, and hence they likely adopted a native conformation. Therefore, aggregate formation could explain the reduced secretion of glycosylation mutants. In other words, retention of these mutants could be dependent on ER protein quality control preventing their exit from the ER as for other misfolded molecules (20).

The co-expression of PDI further inhibited secretion of the two single mutants (results not shown), indicating that PDI could interact with Ero1α independently from either sugar. PDI and PDI-ASAS increased the proportion of monomeric NG-Ero1α at the expense of high molecular weight species (Fig. 4B, lanes 3 and 4). On the contrary, ERp57 did not alter the NG-Ero1α oligomerization state (Figure 4B, lane 7).

When cells expressing NG-Ero1α were cultured for longer times (16 h), some NG-Ero1α became detectable in the supernatants (Fig. 4C, lanes 2 and 7). Unexpectedly, the co-expression of PDI or PDI-ASAS promoted the secretion of NG-Ero1α (Fig. 4C, lanes 2–4). ERp44 (Fig. 4C, lane 8) inhibited secretion of this mutant, while ERp57 (Fig. 4C, lane 5) was inactive.

Therefore, the observation that PDI and PDI-ASAS favored the secretion of NG-Ero1α could reflect a role in controlling the oligomerization state of this mutant. ERp44, but not PDI, was present in the high molecular covalent complexes and RNAi of ERp44 promoted some NG-Ero1α secretion (data not shown). These findings suggest that PDI could act as a chaperone for NG-Ero1α, by preventing formation of aggregates and favoring attainment of a secretion competent state, but ERp44 could not. However, as the overexpression of PDI causes retention, the amount of NG-Ero1α secreted was necessarily limited. Indeed, in PDI overexpressing cells, NG-Ero1α was secreted at levels comparable to those of wild-type Ero1α (Fig. 4C, compare lanes 10 and 11).

DISCUSSION

Several mechanisms can contribute to the localization of a protein in the ER. Resident proteins are generally equipped with motifs that mediate retrieval from later compartments (i.e., KDEL or KK) (24, 33). Other proteins are part of supramolecular structures, whose size and slow diffusibility preclude forward transport. Proteins that fail to fold or assemble properly can be retained by the ER quality control machinery. Finally, localization can be achieved by hitchhiking interactions with ER resident proteins.

The studies presented herein suggest that the localization of Ero1α and β in the ER relies primarily on the last mechanism that is parasitizing the localization signals of ER resi-

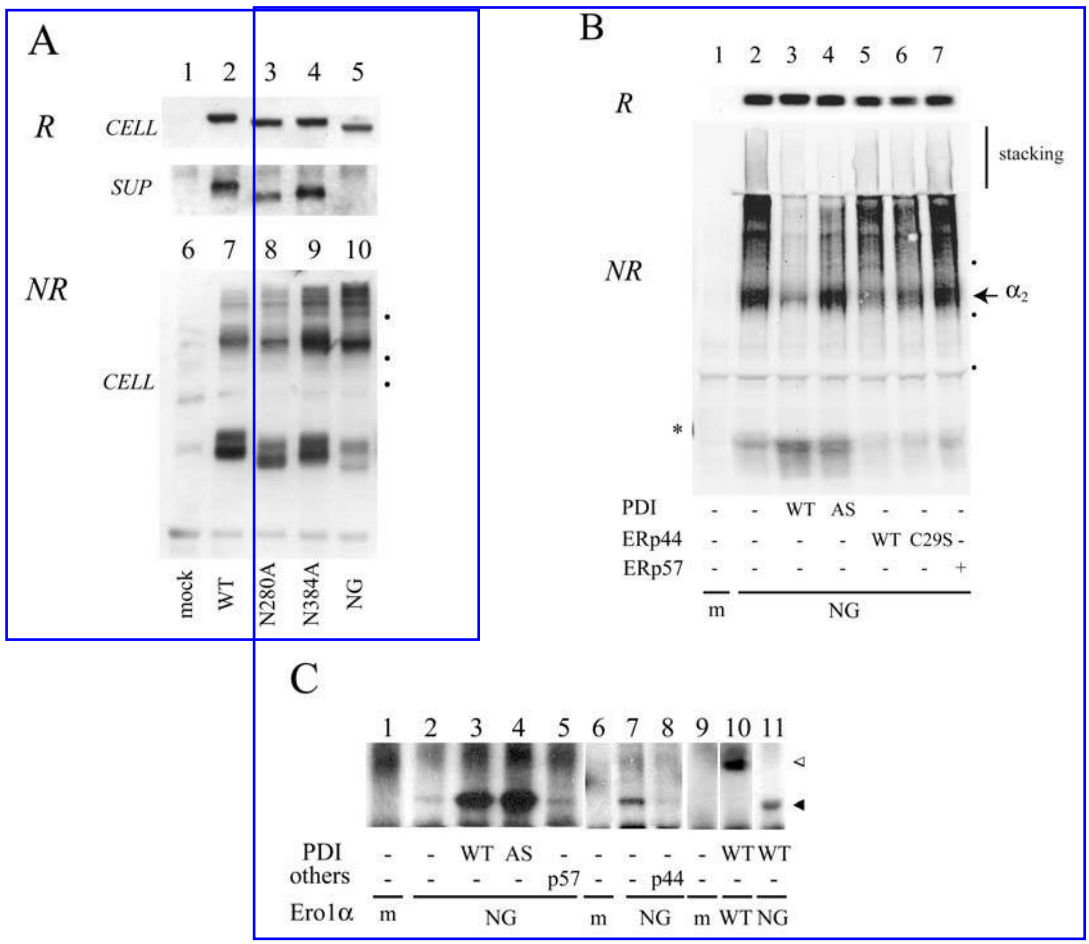

FIG. 4. N-linked glycans favor Ero1α folding and secretion. (**A**) Nonglycosylated Ero1α forms covalent aggregates and is poorly secreted. HeLa cells expressing the indicated Ero1α mutants were cultured for 6 h. The supernatants (*SUP*) were immunoprecipitated with immobilized anti-myc and resolved under reducing conditions. Aliquots from cell lysates were resolved under reducing (*top panel*) or nonreducing (*bottom panel*) conditions. Abundant high molecular weight complexes accumulate intracellularly in the double mutant (*lane 10*, NG), which is not detected in the supernatants (*lane 5*). R; reducing SDS-PAGE, NR; nonreducing SDS-PAGE. WT; wild-type Ero1α, NG; Ero1α mutant N280A/N384A. Dots represent 173, 113, 86 kDa markers. (**B**) The co-expression of PDI and PDI-ASAS inhibits aggregation of NG-Ero1α. Aliquots from the lysates of HeLa cells transfected with the indicated plasmids were resolved under reducing (*upper panel*: R) or nonreducing (*lower panel*: NR) conditions, and blots decorated with anti-myc. Note that monomers (*arrow*) are more abundant in cells co-expressing PDI or PDI-ASAS. The former decreases the intensity of the band with the features of Ero1α homodimers (α_2, see *arrow*). The *asterisk* points to a position of *in vitro* reduced NG-Ero1α monomers. Dots represent 173, 113, 86 kDa markers. Abbreviations as in Fig. 1C. (**C**) The co-expression of PDI and PDI-ASAS favors NG-Ero1α secretion. HeLa cells transfected with wild-type (WT) or nonglycosylated (NG) Ero1α as indicated were cultured for 16 h (*lanes 1–8*) or 6 h (*lanes 9–11*). The supernatants were immunoprecipitated with immobilized anti-myc and resolved under reducing conditions, and blotted decorated with anti-myc antibodies. Note that both PDI and PDI-ASAS increase NG secretion. Owing to the fact that PDI causes the retention of transport competent Ero1 molecules, small amounts of NG and WT molecules were secreted by PDI overexpressing cells (*lanes 10–11*): to reveal them, therefore, longer exposure times were utilized compared to Figure 1C. *Open* and *closed arrowheads* on the right hand margin point to wild-type Ero1α and Ero1α mutant N280A/N384A, respectively. Abbreviations as in Fig. 1C.

dent proteins by virtue of specific interactions. The main interactors are PDI and ERp44, two proteins known to form mixed disulfides with both α and β. In the case of ERp44, the formation of mixed disulfides seems to be the main, if not the only way to establish specific interactions with Ero1 molecules. In contrast, noncovalent interactions play an important role in favoring the binding between Ero1 and PDI.

The physiology of the latter interaction is clear, since PDI is the main Ero1 substrate in the pathway of oxidative folding. In all likelihood, PDI first binds Ero1 noncovalently. The different conformations adopted by PDI depending on its redox state (21, 34) could be exploited to optimize delivery of oxidative equivalents. In normal cells, PDI is in excess with respect to Ero1. How does Ero1 "find" reduced PDI? If indeed reduced PDI has higher affinity for partially unfolded molecules (34), Ero1 could somehow disguise itself as a misfolded protein, so as to preferentially attract reduced PDI.

The available crystal structure of yeast Ero1p (16) suggests an elegant disposition in which two cysteine couples sequentially transfer electrons from PDI to a FAD molecule. However, the structure reveals no channel for O_2. Perhaps the interaction with PDI induces a conformational change in Ero1. This may couple O_2 consumption to PDI oxidation, limiting futile reactions that could yield to ROS accumulation.

It is not clear, at present, whether the binding to ERp44 reflects additional functional role(s) apart from preventing the secretion of Ero1 molecules. Considering that PDI and ERp44 can bind to different cysteines in Ero1α (4), the absence of ternary covalent complexes is surprising and may reflect temporal and/or spatial regulation within the ER. ERp44 retains cargo proteins with exposed thiols, probably acting as a "late check point" in quality control (1). The dynamic retention mode of Ero1 by alternative interactions with PDI and ERp44 could therefore provide multiple sites for disulfide bond formation that may correspond to the execution of sequential steps in the structural maturation of complex molecules. As recent findings showed that ERp44 can associate with IP3 receptor 1 regulating its activity (17), another exciting possibility is that the interaction between ERp44 and Ero1 might couple redox regulation and calcium homeostasis in the ER.

Besides allowing multiple Ero1 locations or functional states, the dynamic retention mechanism we describe may also act as a safety valve. In case too much oxidases are produced, like in our transfected cells, excess molecules can be secreted. It remains to be seen whether secreted Ero1 retains the capability of oxidizing PDI, which is also secreted by many cell types (6, 18, 32, 38), so as to generate extracellular protein oxidizing complexes.

Our secretion assays reveal that Ero1α establishes weak interactions with ERp57. Despite the similarity between PDI and ERp57, however, the two oxidoreductases seem to interact differently with Ero1α. The lack of effect on the nonglycosylated mutant implies that glycans, besides being clearly necessary for efficient Ero1 folding, might be needed also for Ero1–ERp57 interactions. Inhibiting the calnexin–calreticulin pathway by castanospermine treatment, inhibited in part also the effect of ERp57 on Ero1α secretion (data not shown). This suggested that the calnexin–calreticulin-ERp57 pathway is involved in assisting Ero1 folding. An important question that remains to be addressed is what regulates the redox state of ERp57 and other ER oxidoreductases (11, 22).

The overexpression of PDI or PDI-ASAS increased the fraction of monomeric NG-Ero1α at the expense of high molecular weight complexes, suggesting that the substrate (PDI) can act as a chaperone on the enzyme (Ero1) independently from its oxidoreductase activity. Our data do not allow us to establish whether PDI also affects the folding of wild-type Ero1, but the emerging ying–yang model might offer additional possibilities to finely control ER redox. PDI overexpression also prevented the accumulation of a band of 130 kDa. Several lines of evidence suggest this band consists of Ero1α and Ero1β homodimers. Overexpressed Ero1α and Ero1β were detected below the diagonal in reducing/nonreducing gels without corresponding PDI or ERp44 spots ((22) and our unpublished results). Further, functional complementation assays in yeast implied the association between human Ero1α/Ero1β and yeast Ero1p (26). As to the potential functional significance of Ero1 homodimers, the observation that their formation is inhibited by PDI and PDI-ASAS, suggests a regulatory function for this species, perhaps as a way to store Ero1 molecules in a potentially active conformation.

In conclusion, our studies indicate that Ero1 is localized in the ER by interactions with its main substrate, PDI, and with ERp44, a protein that plays important roles in quality control (1) and calcium signaling (17). Cell fractionation studies indicated that Ero1 molecules can interact with membrane proteins (5, 26). The "polygamic nature" of Ero1 may offer interesting regulatory possibilities for integrating redox control, calcium homeostasis and protein transport-degradation within the early secretory compartments. These features may explain why the localization mechanism of yeast Ero1p, based on functions endowed in the C-terminal tail (27) diverged in mammals, whilst the basic pathway remained similar.

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ABBREVIATIONS

DSP, dithiobis-(succinimidylpropionate); Endo-H, endoglycosidase-H; ER, endoplasmic reticulum; IP, immunoprecipitation; NEM, N-ethyl maleimide; PDI, protein disulfide isomerase; PNGaseF, peptide N-glycosidase F.

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