Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1

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The toxic effect of cholera toxin (CT) on target cells is caused by its A1 chain. This polypeptide is released from the holotoxin and unfolded in the lumen of the ER by the action of protein disulfide isomerase (PDI), before being retrotranslocated into the cytosol. The polypeptide is initially unfolded by binding to the reduced form of PDI. We show that upon oxidation of the COOH-terminal disulfide bond in PDI by the enzyme Ero1, the A1 chain is released. Both yeast Ero1 and the mammalian Ero1α isoform are active in this reaction. Ero1 has a preference for the PDI–toxin complex. We further show that the complex is transferred to a protein at the lumenal side of the ER membrane, where the unfolded toxin is released from PDI by the action of Ero1. Taken together, our results identify Ero1 as the enzyme mediating the release of unfolded CT from PDI and characterize an additional step in retrotranslocation of the toxin.

Introduction

The bacterium *Vibrio cholerae* produces cholera toxin (CT)* which affects mammalian intestinal epithelial cells by causing salt and water secretion resulting in diarrhea (Sears and Kaper, 1996). CT consists of a ring of five B subunits and a single A subunit. The A subunit is cleaved into the A1 catalytic domain and the A2 domain upon secretion of the toxin by *V. cholerae*. The A1 and A2 fragments are connected by a disulfide bridge and by noncovalent interactions. The holotoxin is endocytosed and traffics in a retrograde manner along the secretory pathway to the lumen of the ER (Lencer et al., 1999). In this compartment, the disulfide bridge of the A subunit is reduced, and the A1 chain is released from the rest of the toxin and translocated to the cytosol. Based on coimmunoprecipitation and ribosome inhibition experiments, it seems that the A1 peptide is translocated through the Sec61p channel (Schmitz et al., 2000), the same channel used to translocate secretory, lumenal, and membrane proteins from the cytosol to the ER lumen (for review see Matlack et al., 1998). Although the signal transduction pathway induced by the A1 chain in the cytosol is well characterized, much less is known about the mechanism by which the A1 peptide is transported from the ER lumen to the cytosol, a process termed retrotranslocation (for review see Tsai et al., 2002).

Unfolding of the A1 peptide likely represents the first step in retrotranslocation of the toxin. Using a biochemical fractionation approach that made no assumptions about the nature of this unfolding activity, we previously identified the ER oxido-reductase protein disulfide isomerase (PDI) as the major activity that disassembles the toxin and unfolds the A1 chain (Tsai et al., 2001). More detailed analysis demonstrated that PDI acts as a redox-dependent chaperone; in its reduced state, PDI binds and unfolds the toxin, whereas in its oxidized state, PDI releases it. Release of the A1 chain from PDI upon oxidation must occur prior to its retrotranslocation across the ER membrane. When oxidation is induced with oxidized glutathione (GSSG), an unphysiologically high concentration was required to induce release (Tsai et al., 2001). Therefore, we hypothesized that this process must normally be catalyzed by an enzyme, i.e., an oxidase of PDI.

Here we identify the enzyme responsible for the release reaction, provide a mechanism for the release, and describe an additional step in retrotranslocation of the toxin. Our data demonstrate that the ER oxidase Ero1 is responsible for inducing release of the toxin from reduced PDI through oxidation of the COOH-terminal disulfide bond in PDI. Furthermore, we show that the complex of PDI and unfolded toxin is targeted to a protein on the luminal side of the ER membrane. Subsequently, the toxin is released from PDI by
the action of Ero1, presumably committing the toxin to retrotranslocation across the ER membrane.

## Results and discussion

### An ER activity induces toxin release from PDI

To identify an activity that caused the unfolding of purified A subunit of CT, we have previously used an ER extract from dog pancreatic microsomes that was obtained by the addition of a low concentration of detergent, and contained luminal and some membrane proteins. The ER extract rendered the A and A1 peptides sensitive to trypsin digestion under reducing conditions (1 mM reduced glutathione, GSH; Fig. 1 A, lane 6; Tsai et al., 2001). BSA did not show this effect (Fig. 1 A, lane 2), indicating that a protein in the ER extract induces unfolding of the toxin. The protein was subsequently identified as PDI; indeed, incubation of purified PDI with toxin under reducing conditions similarly caused the A1 chain to be sensitive to trypsin digestion (Fig. 1 A, compare lane 4 with lane 2; Tsai et al., 2001).

To probe the interaction between the toxin A chain and PDI, we developed a crosslinking assay (Tsai et al., 2001). Briefly, purified PDI or ER extract were incubated with the A subunit of CT under reducing conditions, followed by addition of a carbodiimide to induce crosslinks between carboxyl and amino groups of the two proteins. This complex is visualized as a higher molecular mass band in SDS-PAGE after immunoblotting with an antibody that recognizes the A subunit of CT. It should be noted that in both the trypsin digestion and crosslinking assays, PDI is in an ~40-fold molar excess over the toxin.

Using this assay, we found that a PDI–A chain complex can be seen with purified PDI, but not with ER extract or BSA (Fig. 1 B, compare lane 3 with lanes 2 and 1; Tsai et al., 2001). Together with the unfolding assay, these results suggest that the toxin must be undergoing cycles of binding and release from PDI when incubated with ER extract, thereby preventing the formation of a stable PDI–toxin complex that can be captured by the crosslinker.

To directly test the possibility that a release activity exists in the ER extract, we first generated the PDI–A chain complex by incubating PDI and purified toxin together under reducing conditions. BSA or ER extract were then added to the preformed complex to induce crosslinking and the dissociation of the complex was probed with a bifunctional carbodiimide crosslinker. Indeed, addition of ER extract, but not BSA, resulted in loss of the crosslinked product (Fig. 1 C, compare lane 2 with lane 1). This result confirms the presence of an activity in the ER extract that induces the release of the A1 chain from PDI. Because addition of high concentrations of the oxidant GSSG can also induce toxin release (Tsai et al., 2001), the release activity is likely to be an oxidase of PDI.

### Ero1 induces the release of unfolded toxin from reduced PDI

Using two different genetic screens in _Saccharomyces cerevisiae_, Ero1 was identified as an enzyme that functions as a PDI oxidase in the ER lumen (Frand and Kaiser, 1998, 1999; Pollard et al., 1998). Ero1 contains the cofactor FAD and may use molecular oxygen to reoxidize itself (Tu et al., 2000). We asked whether the mammalian homologue of...
Ero1 may be responsible for the release activity in the ER extract. In mammals, two different Ero1 isoforms have been identified, Ero1α (Cabibbo et al., 2000) and Ero1β (Pagani et al., 2000). To test whether both isoforms are present in dog microsomes, three different mammalian Ero1 antibodies were used, one that recognizes both mammalian Ero1 isoforms (α/β, antibody 194), one that only recognizes the Ero1α isoform, and one that only recognizes the Ero1β isoform. The antibodies were used to test whether both isoforms are present in dog microsomes.
isoform. The Ero1α/β antibody recognized three distinct bands in the microsomes, with the top band corresponding to Ero1α, the middle band corresponding to Ero1β, and the bottom band corresponding possibly to a novel Ero1 isoform or a degradation product (Fig. 2 A, lane 1). The slower mobility of Ero1α compared to Ero1β is consistent with previous results (Pagani et al., 2000). Treatment of microsomes with endoglycosidase H (Endo H) increased the mobility of all three bands, confirming previous findings that Ero1α and Ero1β are glycoproteins (Fig. 2 A, lane 2; Pagani et al., 2000). The Ero1α- and Ero1β-specific antibodies each recognized only one Endo H–sensitive band in the microsomes (Fig. 2 A, lanes 3 and 5, respectively), which correspond to the positions of the top and middle bands identified using the Ero1α/β antibody, respectively. An additional Endo H–resistant band was observed using the Ero1α-specific antibody (Fig. 2 A, lane 4), which may represent an uncharacterized glycosylated form of Ero1α.

To test the specificity of the antibodies, we expressed and purified mammalian Ero1α from yeast cells (Fig. 2 A, lane 7). Both the Ero1α/β and Ero1α-specific antibodies recognized the purified Ero1α protein, whereas the Ero1β-specific antibody did not (Fig. 2 A, lanes 8–10). We were unable to purify the mammalian Ero1β protein from yeast.

We next used the antibodies to directly test whether the release activity observed in the crude ER extract can be attributed to Ero1. Both isoforms of Ero1 were present in the extract obtained with low detergent concentrations. To deplete Ero1α and Ero1β we used two different antibodies directed against both proteins (antibodies 193 and 194; Fig. 2 B, compare lanes 2 and 3 with lane 1; lanes 5 and 6 with lane 4; and lanes 8 and 9 with lane 7). Although addition of ER extract to the preformed PDI-toxin complex induced loss of the crosslinked product, addition of either of the two Ero1-depleted extracts did not (Fig. 2 B, compare lane 11 with lanes 12 and 13). In fact, with one antibody the crosslinked band was more intense than in the control, suggesting that in the absence of Ero1, the extract is more reducing than 1 mM GSH, perhaps due to the presence of a PDI-reductase. These results demonstrate that Ero1 is required for the release activity. As previously reported, addition of a high concentration of GSSG (30 mM) also resulted in the loss of the crosslinked product (Fig. 2 B, compare lane 14 with lane 10; Tsai et al., 2001).

Figure 3. Release activity is present in Ero1α-containing fractions. (A) The protein in an ER extract was bound to a Q-Sepharose column and eluted with a continuous salt gradient. Fractions were analyzed for its Ero1 content by SDS-PAGE and immunoblotting with antibodies that either recognize both Ero1 isoforms (top), Ero1α only (middle), or Ero1β only (bottom). (B) The fractions were tested for their ability to induce toxin release, as described in Fig. 1 B.
We next asked whether Ero1 is sufficient for stimulating toxin release. Addition of purified Ero1α, either in substoichiometric (1:10) or stoichiometric (1:1) ratio to PDI, to the preformed PDI-toxin complex resulted in the disappearance of the crosslinked product (Fig. 2 C, compare lanes 2 and 3 with lane 1). The same result was obtained upon addition of purified yeast Ero1 (Fig. 2 C, lane 4), either in substoichiometric (1:10) or near-stoichiometric (1:2) ratio to PDI (Fig. 2 C, compare lanes 6 and 7 with lane 5). We conclude that Ero1 is both necessary and sufficient to induce release of the toxin from PDI.

We also separated the ER extract on an ion exchange column (Q-Sepharose) and tested each fraction for its content of Ero1 and its activity to release the toxin from reduced PDI. Two pools of Ero1 were found using the Ero1α/β antibody, one pool corresponding to fractions 5–9 (Fig. 3 A, top, lanes 1–5), identified as Ero1α (Fig. 3 A, middle, lanes 1–5), and another corresponding to fractions 17–21 (Fig. 3 A, top, lanes 13–17), identified as Ero1β (Fig. 3 A, bottom, lanes 13–17). Only fractions 5–9 were active in the release assay (Fig. 3 B, compare lanes 2–6 with lanes 14–18). These data confirm that Ero1α is able to induce toxin release from PDI and raise the possibility that Ero1β may be inactive in this assay. Interestingly, based on the theoretical pl values of the two isoforms, Ero1β is expected to elute before Ero1α, in contrast to the observation. It seems therefore likely that interactions with other proteins alter the properties of the isoforms. A novel ER protein, called Erp44, interacts with both Ero1 isoforms and is therefore unlikely to be responsible for the anomalous behavior of the two proteins (Anelli et al., 2002).

**Ero1 oxidizes the COOH-terminal disulfide bond in PDI and acts preferentially on the PDI–toxin complex**

To demonstrate that the mechanism of Ero1-mediated toxin release is due to oxidation of PDI, we designed an assay that allows us to examine PDI’s oxidation state. Maleimide–PEG5000 is a chemical of ~5,000 daltons that allows the modification of thiol groups in cysteines, and thereby significantly increases the size of the protein. The mobility shift is analyzed by SDS-PAGE followed by immunoblotting with an antibody directed against the modified protein. When PDI was fully reduced by 100 mM DTT and treated with maleimide–PEG5000, a dramatic upward mobility shift of PDI was seen (Fig. 4 A, compare lane 1 with lane 6). Under this condition, all six cysteines in PDI are expected to be in the reduced state and should therefore be modified. When PDI was incubated in the presence of a high concentration of the GSSG (100 mM), a much smaller upward mobility shift was observed (Fig. 4 A, compare lane 5 with lane 6). Under these conditions, only the two noncatalytic cysteines can be modified, whereas the cysteines within the two thioredoxin domains of PDI, each characterized by a CxxC motif, are expected to form disulfide bonds.

Incubation of PDI with GSH (1 mM) followed by maleimide–PEG5000 treatment resulted in an upward mobility shift similar to the mobility shift induced with DTT treatment (Fig. 4 A, compare lane 2 with lane 1), although some degree of smearing was consistently observed. Importantly, addition of stoichiometric amounts of mammalian Ero1α produced an upward mobility shift of PDI that was intermediate to the shifts seen when two or six cysteines in PDI were modified (Fig. 4 A, compare lane 4 with lane 2). Similar results were observed using near-stoichiometric amounts of yeast Ero1 (Fig. 4 B, compare lane 5 with lane 3). The simplest interpretation of this data is that Ero1 oxidizes only one of the two disulfide bonds in PDI, leaving four cysteines available for modification. The ability of Ero1 to oxidize PDI in the presence of 1 mM GSH is consistent with previous findings that yeast Ero1 can oxidize PDI even in 3 mM GSH (Tu et al., 2000).

To further establish that Ero1 can oxidize only one of PDI’s two disulfide bonds, a tenfold excess of yeast Ero1 was added to reduced PDI. Again, only one of PDI’s two disulfide bonds was oxidized (Fig. 4 C, compare lane 2 with lane 1). To determine whether Ero1 oxidizes the disulfide bond in the NH2- or COOH-terminal thioredoxin domain of PDI, we used purified mutants of yeast PDI in which the CxxC motif in either the NH2- or COOH-terminal domain is mutated to AxxA (PDI[NxxC-AxxA] and PDI[CxxC-AxxA], respectively; Fig. 4 D, lanes 1 and 2). Using a tenfold excess of yeast Ero1 over PDI, we found that only the PDI[NxxC-AxxA] mutant was oxidized by Ero1 (Fig. 4 D, compare lane 6 with lane 8). Thus, Ero1 oxidizes only the disulfide bond in the carboxy-terminal thioredoxin domain.

Interestingly, addition of substoichiometric amounts of Ero1α or yeast Ero1 did not alter the mobility shift of PDI (Fig. 4 A, compare lane 3 with lane 2, B, compare lane 4 with lane 3), indicating that this amount of Ero1 is insufficient to oxidize the bulk of PDI. However, under the same conditions, both Ero1α and yeast Ero1 were able to fully induce release of toxin from PDI (Fig. 2 C, compare lane 2 with lane 1; compare lane 6 with lane 5). One explanation is that Ero1 preferentially binds to and oxidizes the PDI–toxin complex. Because the concentration of the toxin is much lower than that of PDI, oxidation of complexed PDI would not be visible in the modification assay. Indeed, when we added a stoichiometric concentration of toxin with respect to PDI, a mobility shift of PDI could be observed even at low concentrations of Ero1 (Fig. 4 E, compare lane 6 with lane 2). The band that runs between the ones corresponding to four and six free thiol groups may perhaps be caused by the formation of a mixed disulfide bond with either the toxin or GSH. Taken together, these data support the idea that Ero1 preferentially acts on the PDI-toxin complex.

**PDI-unfolded toxin is transferred to the ER membrane**

During retrotranslocation of the A1 chain, the unfolded A1 peptide must be targeted to the ER membrane and ultimately engage the translocation channel in order to be transported to the cytosol. We designed an assay to examine transfer of the unfolded A1 chain to the ER membrane, a process that likely mimics an intermediate step in retrotranslocation of the toxin. Purified A subunit of the toxin was initially incubated with ER extract under reducing conditions to stimulate toxin unfolding. Then proteoliposomes were added which were generated by dissolving microsomes in detergent followed by removal of the detergent with hydrophobic beads. These proteoliposomes contain essentially all ER membrane proteins with their orientations randomized. This should allow the PDI–toxin complex to interact with lumenal domains of membrane proteins. To assay for bind-
ing of the PDI–toxin complex, the proteoliposomes were sedimented and the pellet and supernatant fractions analyzed by immunoblotting with toxin antibodies.

Although addition of ER extract or proteoliposomes alone did not induce transfer of the A1 peptide to the pellet fraction, addition of ER extract followed by proteoliposomes...
caused a fraction of the A1 peptide (30%) to be transferred to the pellet (Fig. 5 A, compare lanes 1 and 3 with lane 5). Neither liposomes lacking proteins nor intact microsomes (which do not have the luminal domains of membrane proteins exposed) stimulated toxin transfer (Fig. 5 A, compare lanes 7 and 9 with lane 11), indicating that the unfolded A1 chain does not bind to lipids or irrelevant proteins. This is supported by the fact that proteoliposomes generated from a proteinase K-treated detergent extract (lanes 15 and 16) failed to transfer toxin. The results indicate that the unfolded A1 chain binds to the ER membrane, likely by virtue of its luminal domains.
proteinase K-treated detergent extract did not show binding activity (Fig. 5 A, compare lanes 15 and 13). Thus, it appears that the unfolded A1 peptide specifically binds to a protein at the luminal side of the ER membrane. An unfolded state of the A1 peptide is insufficient to achieve binding to the ER membrane as demonstrated with A chains that were chemically unfolded with urea. The urea-treated A and A1 peptides were as sensitive to trypsin treatment as the A1 peptide unfolded with an ER extract (Fig. 5 B, compare lanes 6 and 4 with lane 2), but they did not bind to proteoliposomes (Fig. 5 B, compare lane 7 with lane 9). Thus, it appears that the formation of a PDI-toxin complex is necessary for transfer to the ER membrane, perhaps because PDI specifically interacts with a membrane protein. To further test this point, we asked whether PDI is required for the transfer reaction by using ER extract immunodepleted of PDI (Fig. 5 C, compare lane 3 with lane 2). Indeed, the PDI-depleted extract did not support toxin transfer to the membrane (Fig. 5 C, compare lane 6 with lane 4). Furthermore, purified PDI was competent in inducing transfer of the A1 chain to proteoliposomes (Fig. 5 C, compare lane 10 with lane 8). Therefore, PDI is both necessary and sufficient for the transfer of unfolded A1 peptide to the ER membrane. These experiments also show that Ero1 in the ER lumen is not required. About 10% of total Ero1 is found in the proteoliposomes, but this population also does not seem to be required for the transfer of the toxin to the ER membrane, because proteoliposomes generated from microsomes of the yeast ero1-1 mutant bound the PDI–toxin complex with unreduced efficiency (unpublished data). However, the mutant proteoliposomes were inactive in releasing the toxin from PDI, in contrast to wild-type vesicles (Fig. 5 D, compare lane 4 with lanes 2 and 3). Thus, these results indicate that the complex of toxin and PDI is first transferred to the ER membrane and then dissociated by the action of Ero1.

Role of PDI-Ero1 unfolding in retrotranslocation of CT

Based on our findings, we propose the following model for retrotranslocation of CT. Upon reaching the ER lumen, the A1 chain is released from the rest of the toxin and unfolded by PDI. The reduced form of PDI binds to the A1 chain and unfolds it. We assume that the PDI-A1 complex is then targeted to the ER membrane because the A1 chain alone, even when unfolded, does not have a high affinity for the ER membrane. Because PDI, but not Ero1, is required for membrane targeting of the toxin, it appears that the PDI–toxin complex binds to a protein at the luminal side of the ER membrane via PDI. Our results suggest that the next step is oxidation of PDI by Ero1 and release of the toxin from PDI at the membrane. Because the A1 chain refolds when it is released from PDI into buffer, we assume that the unfolded toxin is transferred directly into the translocation channel. This would be possible if either the PDI-interacting protein at the ER membrane or Ero1 were associated with the channel. In this connection it will be interesting to identify the proteins that appear to be associated with the two isoforms of Ero1 in the ER extract.

Although we have seen release of the toxin from PDI with both Ero1 in the ER detergent extract and with Ero1 present in reconstituted proteoliposomes, in vivo the active species is likely to be membrane-bound Ero1. Because Ero1 lacks a transmembrane domain and a K/HDEL sequence at its COOH terminus, it may be kept in the ER by an association with a membrane protein. Its appearance in the ER extract may be caused by disruption of this interaction by even low concentrations of detergent. Our results show that the α-isoform of Ero1 is capable of releasing the A1 chain from PDI, whereas fractions containing Ero1β were inactive. This raises the possibility that Ero1β may serve a different function in the cell than Ero1α, perhaps in the refolding of misfolded ER proteins, but we cannot exclude the possibility that Ero1β was inactivated during sample preparation.

Our data show that Ero1 only oxidizes the COOH-terminal disulfide bond in PDI to cause toxin release. This finding is consistent with previous results showing that the COOH-terminal thioredoxin domain plays a more critical role than its NH2-terminal counterpart during the in vitro refolding of carboxypeptidase Y (CPY), and of bovine pancreatic trypsin inhibitor (Westphal et al., 1999). In addition, the NH2-terminal thioredoxin domain does not appear to play a role in the PDI-mediated retrotranslocation of misfolded prepro-α factor (Gillece et al., 1999). However, in vivo the NH2-terminal thioredoxin domain was found to play a more important role than the COOH-terminal domain in the folding of CPY (Holst et al., 1997). The reasons for the differences between the in vitro and in vivo results remain to be clarified. Our results also indicate that Ero1 is more efficient in oxidizing toxin-associated PDI compared to free PDI. Although the molecular mechanism for this preference is unclear, it could prevent futile redox cycles of PDI in the absence of substrate.

Whether the PDI-Ero1–driven unfolding mechanism is used to translocate other substrates, such as misfolded ER proteins, remains to be explored. However, PDI and PDI-related proteins have been implicated in the retrotranslocation of misfolded ER substrates (Gillece et al., 1999; Wang and Chang, 1999), suggesting that the proposed pathway may be general.

Materials and methods

Antibodies

The A subunit of CT was purchased from Calbiochem and mammalian PDI from Takara. Antibodies against different proteins were kind gifts from the following sources: mammalian PDI from H. Ploegh (Harvard Medical School), Ero1α and β from H. Ploegh and C. Kaiser (Massachusetts Institute of Technology, Cambridge, MA), Ero1α from A. Benham (University of Durham, UK), A subunit of CT from W. Lencer (Harvard Medical School), and yeast PDI from T. Stevens (University of Oregon, Eugene, OR). Antibodies against mammalian Ero1β were generated using a peptide containing the sequence SKDCHVEPC with the NH2 terminus acetylated.

Purification of yeast PDI mutants

Plasmids expressing mutant yeast PDIαcut-A543 and PDIαcut-C543 were gifts from J. Weissman (University of California, San Francisco, CA). Purification of the proteins was performed as described in Tu et al. (2000).

Purification of yeast Ero1 and mammalian Ero1α

The yeast strain overexpressing Ero1-Myc-His6 (AFY383) was a gift from C. Kaiser. The yeast strain overexpressing the mammalian Ero1α-Myc-His6 protein was constructed as follows. The gene encoding Ero1α was excised from pC3N3.1-Ero1α-Myc-His6, a gift from R. Sita (University Vità-Salute San Raffaele, Milan, Italy) by digesting with Xbal and Pmel. The fragment was ligated between the Xbal and Smal sites into a yeast overexpression vector (p416-GALL). Yeast cells containing this construct
(p416-Ero1α) were grown overnight and then cultured in YEP Raf/Gal. The cells were harvested and homogenized in a french press, followed by centrifugation at 5000g to remove unbroken cells. The supernatant was centrifuged at 13,000 g for 30 min to collect the membrane fraction which was lysed in a buffer containing 1% Triton X-100, 5% glycerol, 50 mM Tris-HCl, pH 7.4, and protease inhibitors. The soluble material was collected by centrifugation at 100,000 g for 40 min. Affinity purification of the proteins was performed by binding to a Ni-NTA column. After washing with 10 mM imidazole, proteins were eluted with 500 mM imidazole in a buffer containing 0.1% Triton X-100, 5% glycerol, 50 mM Tris-HCl, pH 7.4 and protease inhibitors. The eluted sample was dialyzed and bound to a 1 ml Hi-Trap Q-Sepharose column (Amersham Biosciences). Bound material was eluted with a linear 0–1 M potassium acetate gradient. Fractions of 0.5 ml were collected and Ero1 content analyzed by SDS-PAGE and Coomassie staining.

Preparation of ER extract

An ER extract was prepared by adding 0.2% digitonin to a suspension of 2.2 equivalents/µl canine microsomes followed by centrifugation. The supernatant fraction represents the ER extract.

Immunodepletion of mammalian Ero1 and PDI

100 µl of ER extract derived from dog microsomes were incubated with 10 µl PDI antibodies or 10 µl Ero1β antibodies overnight at 4°C, followed by addition of 20 µl protein A Sepharose beads. Mock depletion was performed without antibodies.

Thiol modification with maleimide-PEG5000

Isolated A subunit (70 nM) was incubated with 3 µM PDI in the presence of DTT (100 mM), GSSG (100 mM), or GSH (1 mM) for 30 min at 30°C. To samples incubated in GSH (1 mM), Ero1 was added and incubated for 20 min at 30°C. 1/10 of the samples was precipitated with trichloroacetic acid, washed with acetone, and resuspended in a buffer containing 2% SDS, 50 mM Tris (pH 7.4), and 5 mM maleimide-PEG5000 (Shearwater). After incubation for 60 min at 30°C, the samples were analyzed by nonreducing SDS-PAGE and immunoblotted with an antibody directed against PDI.

Toxin transfer assay

Isolated A subunit (70 nM) was incubated with either ER extract, PDI-depleted extract, or 3 µM PDI for 20 min at 30°C. 10 equivalents of proteoliposomes were added as described in Gorlich and Rapoport (1993) to prevent degradation of the proteasome by the Sec61p complex. The samples were centrifuged at 13,000 rpm in a tabletop ultracentrifuge using a TLA 100.4 rotor. The supernatant fraction represents the ER extract.

References


