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FORUM REVIEW ARTICLE

Redox-Dependent Protein Quality Control in the Endoplasmic Reticulum: Folding to Degradation

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Abstract

Significance: Nascent polypeptides entering the endoplasmic reticulum (ER) are co- and post-translationally modified by N-glycosylation and the oxidation/isomerization of cysteine residues followed by folding with the aid of molecular chaperones. Only properly folded proteins reach their final destination. The oxidative environment in the ER enables ER-resident oxidoreductases to facilitate disulfide bond formation, which stabilizes protein structures. ER oxidoreductases involve in both the productive folding of newly synthesized proteins and ER-associated degradation (ERAD) of misfolded proteins. ***Recent Advances:*** The ER luminal event of ERAD is composed of three major steps: the recognition and segregation of terminally misfolded proteins from folding intermediates, unfolding of misfolded substrates by oxidoreductases that cleave the disulfide bonds to enable the translocation of the substrates through the retrotranslocation channel, and transport of substrates to be degraded to the dislocon channel. The factors required for these three critical steps have been found to form a supramolecular complex in the ER. ***Critical Issues:*** This complex comprises EDEM1, a lectin-like molecule that recognizes mannose-trimming and segregates the identified substrates from the productive folding pathway into the degradation pathway; ER DnaJ (ERdj)5, a reductase that resides in the ER and reduces disulfides in misfolded proteins; and immunoglobulin heavy chain binding protein (BiP), an heat shock protein (Hsp)70 family molecular chaperone that recruits substrates to the dislocon channel after dissociation from the EDEM1/ERdj5 complex coupled with ATP hydrolysis. ***Future Directions:*** The importance of disulfide bond reduction in misfolded proteins for retrotranslocation through the dislocon channel will be discussed by comparing the function of ERdj5 with that of other oxidoreductases in the ER. *Antioxid. Redox Signal.* 16, 1119–1128.

Introduction

Anfinsen's dogma states that the native structure of proteins is determined by their amino acid sequence; however, this is not applicable to the folding of many cellular proteins, since such proteins and macromolecules interact with each other *in vivo*. Most proteins require the assistance of molecular chaperones to achieve the correct functional structure. Although proteins are most stable when they have attained the lowest free energy conformation, their folding proceeds *via* various folding intermediates, which often expose hydrophobic amino acid clusters on the molecular surface of the protein, and they tend to form aggregates with other unfolded or intermediately folded proteins *via* hydrophobic interactions. Typical molecular chaperones in the cytosol, such as Hsp70 and chaperonin, have an affinity for hydrophobic clusters on protein surfaces, and facilitate correct folding using the energy from ATP hydrolysis. Hsp70 family proteins support the folding of nascent polypeptides transferred from the (nascent polypeptide-associated complex) in eukaryotes or the trigger factor in prokaryotes, both of which bind to ribosomes. On the other hand, chaperonins, such as GroEL/ES in prokaryotes or chaperonin containing t-complex polypeptide 1/TRiC in eukaryotes, assist with the folding of partially folded proteins by capturing them inside a barrel-like structure (24).

The action of folding itself is a complicated process composed of multiple steps, and some portions of newly synthesized proteins do not achieve the correct structure. Indeed, it was reported that ~30% of newly synthesized proteins are degraded before attaining their mature functional conformation (53). Even after proteins attain a mature conformation, cellular stresses such as heat shock can cause proteins to become misfolded, exposing the hydrophobic regions on the molecular surface. Such misfolded proteins are recognized by molecular chaperones or their equivalents and are subsequently polyubiquitinated for degradation by the proteasome (19). This process is known as "quality control of misfolded proteins."

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Membrane and secretory proteins, which make up approximately one-third of all proteins, are synthesized in the endoplasmic reticulum (ER) (18, 31). In eukaryotic cells, membrane or secretory proteins are co-translationally transported into the ER through the Sec61 translocation channel (translocon) and maintained by ER-resident molecular chaperones for proper folding. The predominant molecular chaperones in the ER are BiP, an Hsp70 family protein, and calnexin (CNX) and calreticulin (CRT), lectin-like chaperones that reside in the ER membrane and ER lumen, respectively.

The environment in the ER is different than that of the cytosol. Most secretory or membrane proteins contain numerous cysteine residues, which form disulfide bonds that stabilize both the tertiary and quaternary structure. The ER provides an oxidative environment and facilitates the oxidation of cysteine residues by various ER-resident oxidoreductases, including members of the protein disulfide isomerase (PDI) family (25). In addition, many of the proteins that traverse the secretory pathway are glycoproteins that possess N-linked glycans. The molecular chaperones CNX/CRT assist with protein folding by recognizing those N-glycans and associating with nascent proteins containing monoglucose N-glycans. Thus, oxidative folding and N-glycosylation are critical for the effective quality control of misfolded proteins in the ER quality control, as will be discussed in detail below (see also Fig. 1).

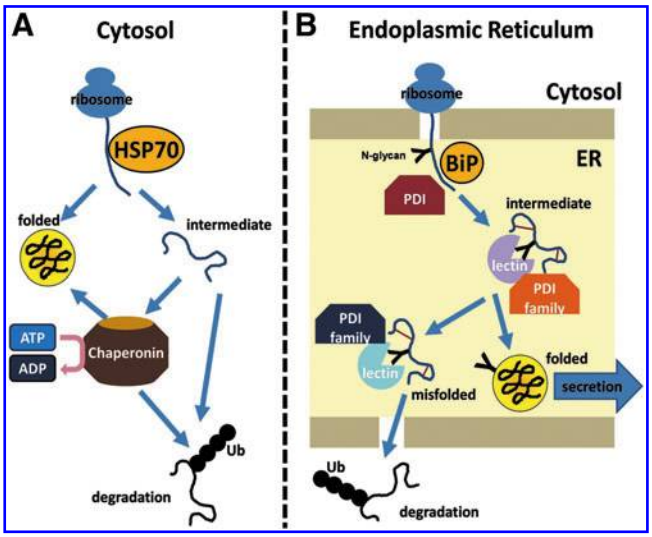

FIG. 1. Chaperone-dependent quality control of newly synthesized polypeptides in the cytosol and in the ER. (A) Newly synthesized polypeptides in the cytosol are recognized by heat shock protein (Hsp) 70 chaperones. If proteins fail to reach their native states, they are transferred to chaperonins for folding. Terminally misfolded proteins are recognized, tagged by ubiquitin (Ub), and degraded by the proteasome. **(B)** In the endoplasmic reticulum (ER), newly synthesized polypeptides are recognized by BiP, which is a HSP70 protein in the ER and protein disulfide isomerase (PDI). If the protein does not achieve the proper conformation, it is transferred to additional PDI family members and lectins for folding. Only correctly folded proteins can be secreted to their final destination. Terminally misfolded proteins are recognized and retained inside the ER by lectins and are finally retrotranslocated into the cytosol, where they are degraded by the Ub-proteasome system. BiP, immunoglobulin heavy chain binding protein. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Redox Environment and Oxidative Folding Cascade in the ER

Productive folding of most membrane or secretory proteins requires the production of disulfide bonds between cysteine residues to stabilize the tertiary structure. Disulfide bond formation and cleavage are accomplished by a reversible thiol (SH)–disulfide (SS) exchange reaction. This reaction is directly catalyzed by PDI (8) and is kinetically and thermodynamically affected by the redox state of the environment. The major redox buffers in the ER lumen are thiol–disulfide pairs and reduced/oxidized pyridine nucleotides (9).

Glutathione (GSH), a tripeptide composed of glutamic acid, cysteine, and glycine (L-γ-glutamyl-L-cysteinyl-glycine) residues, is the most abundant nonprotein thiol in eukaryotic cells. Because of the unique peptide bond between glutamic acid and cysteine (γ-glutamyl bond), GSH is resistant to most proteases and highly stable. GSH is converted to glutathione disulfide (GSSG) through the oxidization of its intrinsic cysteine. In the cytosol, GSSG is reduced by GSH reductase using NADPH, a pyridine nucleotide. The ratio of GSH to GSSG in the cytosol ranges from 30:1 to 100:1, which is a reducing environment that facilitates the formation of disulfide bonds. However, the redox state in the ER is highly oxidative, and the GSH/GSSG ratio ranges from 1:1 to 3:1 (29). As of yet, GSH reductase has not been identified in the ER. The oxidative environment in the ER is suitable for proper protein folding promoted by PDI (Fig. 2). Previously, GSSG was thought to be the primary oxidant of PDI in the ER. However, it has been shown that the ER flavoprotein, ER oxidoreductin 1 (Ero1), catalyzes the oxidation of PDI both *in vivo* and *in vitro* (15, 47). In addition, the deletion of γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step of GSH synthesis, did not affect disulfide bond-dependent protein folding (15). These results suggest that the process of disulfide-bond formation in the ER occurs independently of GSSG. Additionally, recent results indicate that GSH plays a role in maintaining ER oxidoreductases in a reduced state and facilitates reduction or isomerization reactions (42). Why and how the redox balance in the ER is maintained in an oxidative state compared to the cytosol remains to be fully elucidated.

After the discovery of Ero1, oxidative protein folding in the ER was believed to be mediated by a protein electron transfer system composed of PDI, Ero1, and substrate proteins. For the effective introduction of disulfide bonds into substrate proteins, PDI must be maintained in an oxidized state. This is achieved by disulfide exchange with oxidized Ero1. Ero1 captures the flavin adenine dinucleotide (FAD) moiety through which electrons are transferred to molecular oxygen. Thus, the oxidation reaction catalyzed by Ero1 produces hydrogen peroxide, a reactive oxygen species, as a by-product (56).

Although Ero1 is essential for oxidative protein folding in yeast, Ero1-independent disulfide bond formation mediated by PDI is also known to occur in mammalian cells. Mammals possess two genes that encode proteins homologous to yeast Ero1: *Ero1*α (*Ero1-L*α) and *Ero1*β. Both proteins can complement the yeast *ero1-1* mutant *in vivo* and *in vitro*, suggesting

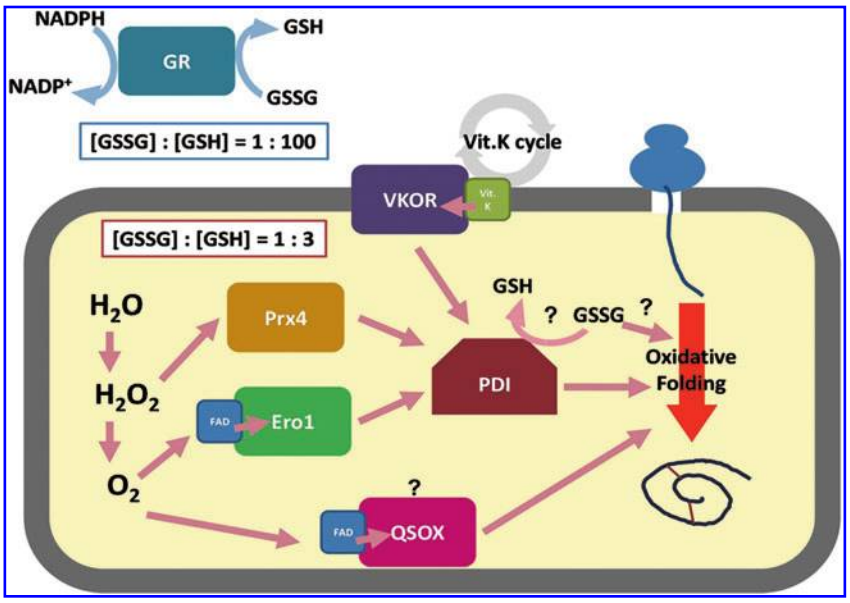

FIG. 2. Oxidation in the ER. The ER redox environment maintained by glutathione is oxidative ([GSH]/ [GSSG] ~ 3) compared to the cytosol ([GSH]/ [GSSG] ~ 100). PDI is oxidized by ER oxidoreductin (Ero) 1, Peroxiredoxin (Prx) 4, and vitamin K epoxide reductase (VKOR), and subsequently facilitates the oxidative folding of client substrates. Quiescin sulfhydryl oxidase (QSOX) probably directly oxidizes substrate proteins. It is unknown whether GSSG and/or PDI directly oxidize substrates. Arrows show the flow of oxidation. Electrons are transferred in the opposite direction of the arrow. There is no evidence of interaction between thiol–disulfide and pyridine nucleotides inside the ER. GSH, glutathione; GSSG, oxidized glutathione. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

that these proteins also function as ER oxidases (5, 38). However, mammalian cells lacking both Ero1α and Ero1β showed only slightly delayed disulfide bond formation (64), which strongly suggests that other oxidation pathways exist in the ER.

Several proteins have been identified as candidates for mediating Ero1-independent protein disulfide bond formation (Fig. 2). The quiescin sulfhydryl oxidase superfamilies are flavin-containing sulfhydryl oxidases that can directly introduce disulfides into unfolded reduced proteins (34). Vitamin K epoxide reductase forms a complex with PDI and is involved in the vitamin K cycle, where it oxidizes PDI (59). The most unique factor to facilitate Ero1-independent oxidization of PDI might be peroxiredoxin IV (PRX4). PRX4 is a 2-cysteine peroxiredoxin localized in the ER that has been reported to reduce H_2O_2 to water by forming an intersubunit disulfide bond (23). PRX4 not only accelerates the H_2O_2-dependent oxidative folding of substrates in the presence of PDI *in vitro* but is also able to rescue a lethal *ero1* mutant in yeast (65). This suggests that PRX4 oxidizes PDI using ER luminal H_2O_2, followed by the oxidative folding of newly synthesized secretory proteins by PDI.

Productive Folding and Quality Control of Glycoproteins in the ER

When nascent polypeptides enter the ER through the Sec61 translocon channel, N-linked glycan composed of three glucoses (Glc), nine mannoses (Mans), and two N-acetylglucosamines (GlcNAc) is covalently attached to the asparagine residue in the consensus motif Asn-Xxx ($\neq$Pro)-Ser/Thr in a target polypeptide by oligosaccharyltransferase (Fig. 3). At the same time, intra- or inter-molecular disulfide bond formation is catalyzed by PDI. In many cases, this bond formation must be edited because the initial disulfide bonds tend to be randomly formed (13). Sequential cleavage of the outermost α1,2-linked glucose residue by glucosidase I and the first α1,3-linked glucose by glucosidase II generates the Glc1-Man9-GlcNAc2 (G1Man9) oligosaccharide chain that allows association with CNX or CRT. ERp57, a member of the PDI family bound to CNX/CRT, facilitates proper disulfide bond formation and subsequent correct folding of newly synthesized glycoproteins through its isomerase activity in conjunction with CNX and CRT. Glycoproteins are then released from CNX and CRT after trimming of the final glucose by GII, which results in the Man9 form of the N-glycan. When the polypeptide portion of the proteins has been properly folded, they are transported downstream in the secretory pathway. However, if folding is incorrect or incomplete, reglucosylation occurs by UDP-glucose:glycoprotein-glucosyltransferase (UGGT), producing the G1Man9 glycoprotein that is again recognized by CNX/CRT. Thus, correct folding can

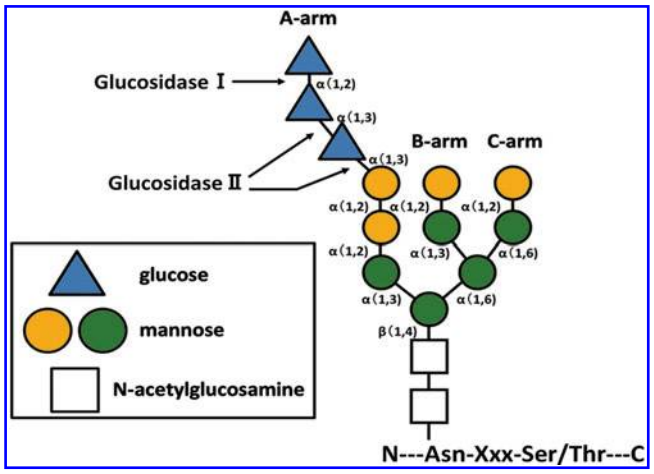

FIG. 3. The composition and structure of N-linked glycans. N-linked glycans composed of three glucose (*blue triangles*), nine mannose (*yellow* and *green circles*), and two N-acetylglucosamine (*white squares*) molecules are added to asparagine residues in consensus Asn-Xxx ($\neq$Pro)-Ser/Thr motifs. α1,2-linked mannose residues are shown in *yellow circles*. The three N-glycan arms are labeled as A-arm, B-arm, and C-arm. The sites of action of glucosidase I (GI) and glucosidase II (GII) are indicated by *arrows*. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

be reattempted. This process is known as the CNX/CRT cycle (6).

In the case that the proteins are terminally misfolded due to missense mutation or cellular stresses, ER-associated degradation (ERAD) occurs to ensure protein homeostasis in the ER (49). In this process, after mannose trimming, misfolded proteins are destined for degradation *via* retrograde transport into the cytosol through a degradation channel (dislocon). In the cytosol, the proteins are polyubiquitinated and finally degraded by the proteasome (1) (Fig. 4). Recent studies revealed that a PDI family oxidoreductase, ER DnaJ (ERdj)5, plays an important role in facilitating the retrotranslocation of misfolded proteins by reductively cleaving inter- or intramolecular disulfide bonds (see below).

PDI Family Proteins in Productive Folding in the ER

PDI was first identified as an ER oxidoreductase more than 40 years ago (20). Its oxidase and isomerase activities have been demonstrated both *in vitro* (37) and *in vivo* (4). PDI contains four thioredoxin (Trx)-like domains, identified as a, b, b′, and a′. The a and a′ domains contain catalytically active Cys-Xxx-Xxx-Cys (CXXC) motifs, while the b and b′ domains are noncatalytic. Although the structure of full-length mammalian PDI has not been reported, the structures of an isolated partial domain of PDI (33) and the yeast homolog Pdi1p (57) have been solved. Based on these studies, PDI is believed to exist in a U-shape structure with the catalytic a and a′ domains located in the ends of the "U" and the CXXC motifs facing each other. The b′ domain contains a hydrophobic stretch of amino acids within the curved potion of the "U," which mediates binding to the hydrophobic regions of substrates and/or partner proteins.

ERp57, which associates with CNX and CRT, is an oxidoreductase that is also important for productive folding in the ER (40). The crystal structure of ERp57 revealed that it forms a heterodimer complex with tapasin (11). Although the overall structure of ERp57 is similar to that of yeast PDI, a significant difference exists in the b′ domain. While PDI possesses a hydrophobic stretch in the b′ domain, this patch is missing in the b′ domain of ERp57, which instead possesses a positively charged cluster. In addition to a sugar binding domain, CNX and CRT possess a proline-rich P domain, through which the positively charged cluster in ERp57 forms a complex with CNX and CRT (35). When nascent polypeptides display oligosaccharides at their N-terminus, CNX, CRT, and ERp57 recognize them, and ERp57 co-translationally catalyzes intramolecular disulfide bond formation (41). While the *in vitro*

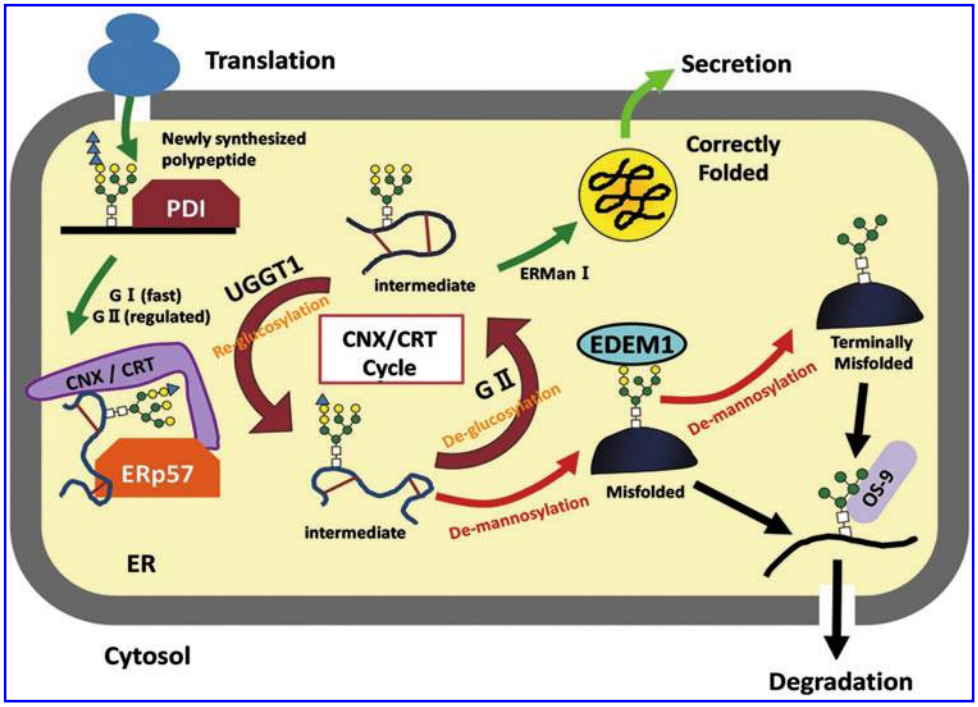

FIG. 4. Quality control of newly synthesized glycoproteins in the ER. Newly synthesized polypeptides enter the ER lumen through the Sec61 translocon channel and N-linked glycans are attached to asparagine residues in the Asn-Xxx-Ser/Thr sequence. The outermost glucose residues are rapidly trimmed by GI and secondary glucose residues are trimmed by GII. Mono-glucosylated N-glycans are recognized by the lectin chaperones calnexin (Cnx) and/or calreticulin (Crt) in complex with the oxidoreductase ERp57. Protein folding proceeds on the CNX/CRT-ERp57 complex until the last glucose residue is removed by GII. Once the protein has obtained its native structure, it is released from the ER and secreted to its final destination. If the protein remains in the intermediate folding stage, it is recognized by UDP-glucose glucosyl transferase (UGGT) and a glucose residue is re-added. It then re-binds to the Cnx/Crt-ERp57 complex and is subjected to an additional folding cycle (CNX/CRT cycle). If the protein is terminally misfolded, de-mannosylation occurs *via* ER mannosidase I (ERMan I) and ER-associated degradation (ERAD) enhancing mannosidase (EDEM) family proteins. Finally, the Man5-7 form of the glycoprotein is recognized by the ERAD complex, and associated lectin osteosarcoma amplified 9 is eventually retrotranslocated into the cytosol for Ub-proteasome degradation. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

oxidative activity of PDI is much higher than that of ERp57, ERp57 exhibits high isomerase activity in the presence of CNX/CRT, suggesting that ERp57 favors glycoprotein substrates captured by CNX/CRT (63). Taken together, these findings indicate that PDI and ERp57 co- and post-translocationally oxidize the cysteines of nascent glycoproteins, and ERp57 then cooperates with CNX/CRT to produce correctly folded proteins. In the case of nonglycoprotein folding, PDI is thought to act as both an oxidase and isomerase during initial folding in the ER.

Among the oxidoreductases in the ER, ERp72, and P5 have been reported to be involved in the productive folding of nascent polypeptides. ERp72 and mammalian orthologs of yeast PDI are able to compensate for yeast PDI in mutant strains (21). Because P5 binds to BiP, it is believed to be involved in BiP-dependent folding of polypeptides in the ER (30). However, simultaneous disruption of ERp72 and P5 did not affect productive folding and protein secretion (50), which suggests that ERp72 and P5 may act as a back-up system for PDI or ERp57 (Fig. 5).

An interesting property of Ero1 is that it can localize to the ER without an ER-retention signal. This localization is due to the functional involvement of ERp44, which acts as an anchoring protein for ER-resident proteins that lack an ER retention signal. ERp44 contains an active Trx domain with a CRFS motif. Because it only possesses one cysteine, ERp44 is not a fully active oxidoreductase. However, it can produce long-lived mixed disulfides with other proteins. ERp44 interacts with Ero1 through mixed disulfide bond formation and retains it in the ER (2). The crystal structure of ERp44 has been solved and it was revealed to have a cloverleaf structure containing three Trx-like domains, a, b, and b′, and a flexible C-terminal loop region (C-tail). It is thought that the C-tail regulates ERp44 substrate binding and oxidoreductase activity (60).

ERp44 also acts as a negative regulator of the secretion of intermediately oligomerized immunoglobulin M (IgM) and adiponectin (7, 61) by selectively binding to intermediately oligomerized proteins in a thiol-dependent manner to retain them in the ER or ER-golgi intermediate compartment. This process ensures that only mature client substrates are exported to their final destination. At present, no PDI family members have been reported to accelerate the secretion of membrane and secretory proteins (Fig. 6).

Components Necessary for ERAD

EDEM1 as recognition machinery for mannose trimming

Terminally misfolded proteins in the ER are prevented from entering the secretory pathway and are segregated to the degradation pathway. Misfolded substrates are then retrogradely transported from the ER to the cytosol through a channel in the ER membrane called a dislocon channel. This degradation system is called ERAD.

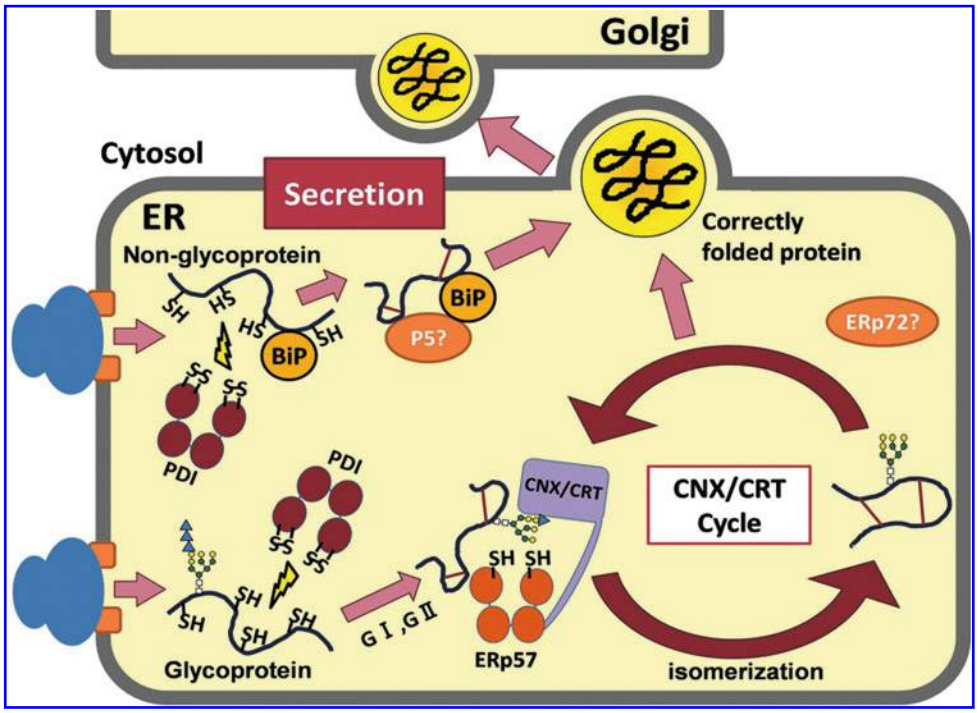

FIG. 5. Productive folding by PDI family proteins and lectins. In the case of nonglycoproteins, the folding of newly synthesized polypeptides is dependent on PDI and BiP. PDI likely acts as both an oxidase and isomerase of nonglycoproteins. P5 also acts as an oxidoreductase of BiP client substrates. In the case of glycoproteins, PDI initially acts as an oxidase that binds to newly synthesized polypeptides through a hydrophobic interaction and forms disulfide bonds. After the two outermost glucoses (Glc) are removed by GI and GII, CNX and/or CRT recognize the mono-glucosylated N-glycan and form a complex with ERp57 through the P-domain. ERp57 mainly acts as an isomerase of folding intermediates to facilitate the formation of the native structure. When the intermediates are correctly folded, the protein is released from the CNX/CRT cycle and secreted to its final destination. ERp72 is suggested to contribute to oxidative folding, but many aspects of its *in vivo* function are still unknown. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

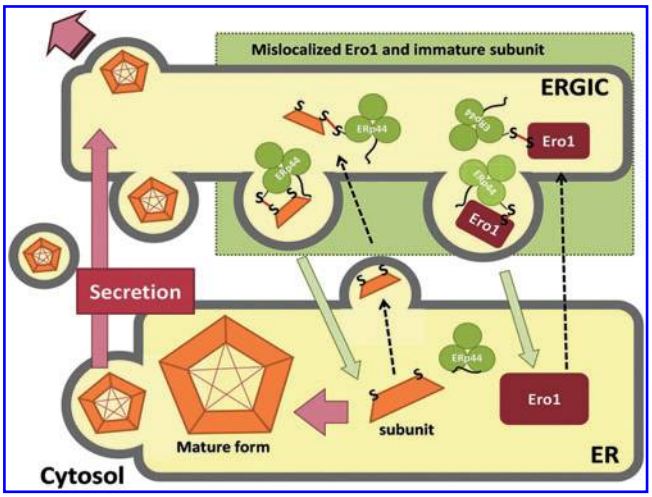

FIG. 6. ERp44 negatively regulates the secretion of ER localized proteins and intermediately oligomerized proteins. Although ERp44 has an ER retention signal, the protein is primarily localized to the ER-Golgi intermediate compartment and the cis-Golgi (3, 61). ERp44 contributes to the retention of Ero1 proteins by forming mixed disulfide bonds through the CRFS motif in an active Trx domain. In the same fashion, ERp44 forms transient disulfides with intermediately oligomerized immunoglobulin M (IgM) and adiponectin. This process ensures that only mature client substrates are exported. The C-tail region of ERp44 (black line) regulates its substrate binding ability. Ero1, ER oxidoreductin 1; Trx, thioredoxin. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

For the selective degradation of misfolded proteins, there must be a cellular mechanism able to discriminate terminally misfolded proteins from intermediate structures in the process of folding. This is accomplished through the trimming of N-linked oligosaccharide chains. As stated previously, the monoglucose form (G1Man9) of oligosaccharide attached to nascent polypeptides is recognized by CNX or CRT, which assist in protein folding. However, in terminally misfolded proteins, the outermost mannoses of the oligosaccharide chain are specifically trimmed from the A and B branches by ER mannosidases such as ER ManI and other glycosyl hydrolase 47 (GH47) family members. The removal of a single mannose residue is not sufficient for the efficient retrograde transport of misfolded glycoproteins from the ER (55), and N-glycans on terminally misfolded proteins are known to be further processed to Man5-6 *in vivo* (16, 28, 32). Once the terminal mannose on the A branch is trimmed shorter than Man7, re-glucosylation by UGGT1 is no longer possible and the substrates are irreversibly segregated from the CNX/CRT cycle into the degradation pathway.

EDEM1 is a member of the GH47 family in the ER. To date, three homologs, *EDEM1*, *EDEM2*, and *EDEM3*, have been cloned and implicated in ERAD (45). EDEM proteins contain all catalytic residues required for glycolytic activity and are assumed to be involved in substrate de-mannosylation. In fact, the overexpression of EDEM3 markedly accelerates mannose removal from misfolded glycoproteins, as does the overexpression of EDEM1 to a lesser degree (26, 44). More importantly, the overexpression of EDEM1 accelerates the release of terminally misfolded glycoproteins from CNX/CRT for transport into the ERAD pathway (39, 43).

Trimming of the outermost α1,2-linked mannose on the C-arm exposes α1,6-linked mannose residues. These are recognized by the mannose-6-phosphate receptor homology (MRH) domain of osteosarcoma amplified 9 (OS-9), which has also been shown to be critical for ensuring the degradation of misfolded glycoproteins. Recently, the crystal structure of the human OS-9 MRH domain was solved and the molecular mechanism of recognition of mannose residues by OS-9 was elucidated (52).

ERdj5 cleaves disulfide bonds on misfolded substrates

ERdj5 was identified as an EDEM1 binding protein (58). ERdj5 has a unique domain structure that contains a J domain at the N-terminus for binding to BiP and four Trx domains, Trx1–Trx4, with redox-active CXXC motifs (Fig. 7A). ERdj5 is the only protein that possesses both a J domain and Trx domains in a single polypeptide (10, 27). ERdj5 binds to BiP through the histidine, proline, and aspartic acid, motif in the J domain, and accelerates the ATPase activity of BiP. Recently, the crystal structure of ERdj5 was solved and it revealed two additional redox-inactive Trx domains, Trxb1 and Trxb2, located between Trx1 and Trx2. The full-length structure can be divided into two clusters: an N-terminal cluster containing the J domain and four Trx domains (Trx1, Trxb1, Trxb2, and Trx2) and a C-terminal cluster containing the Trx3 and Trx4 domains connected by a linker region (Fig. 7B). Systematic mutation analysis of the Trx domains showed that the C-terminal cluster is responsible for binding to both EDEM1 and ERAD substrates and is the primary site of reducing activity as well as ERAD-enhancing activity. ERdj5 forms a complex with EDEM1 and this complex transfers ERAD substrates from CNX to BiP (22).

To act as a reductase in the oxidative environment of the ER, ERdj5 must possess molecular features unique to PDI family oxidoreductases. For example, the common active-site motif in folding-related PDI family members is CXHC, which is assumed to act as an oxidase. By contrast, the Trx domains (Trx3 and Trx4) of ERdj5 in the C-terminal cluster, which has reductase activity, contain a CXPC motif and are assumed to be thiol-disulfide reductants. In fact, the *in vitro* redox equilibrium constants (K_{eq}) for Trx3 and Trx4 are higher than for other PDI family members and the ER redox environment, 83 and 210 mM, respectively. These data suggest that ERdj5 functions as a reductase that exists in an oxidized form in the ER. Once it is reduced by other factors or proteins, ERdj5 returns to a stable oxidized form by reducing the substrates.

The overall molecular structure of ERdj5 is also different from that of other PDI family members. When the Trx1 domain of Pdi1p is superimposed onto that of ERdj5, the remainder of Pdi1p does not align with ERdj5 (Fig. 7C). ERdj5 forms a U-shaped structure composed of Trx1, Trx2, Trx3, and Trx4, the narrow pocket of which does not contain a hydrophobic patch, in contrast to PDI. All of the CXXC motifs in ERdj5 are sterically exposed on the outer surface of the U-shaped structure and lie close to the J domain, which might enhance efficient substrate transfer from EDEM1 to BiP (22). The crystal structure of mammalian ERp57 and Erp44 also show clear differences from that of ERdj5 (11, 60).

In yeast cells, Pdi1p and Htm1p, a yeast homolog of EDEM1, form a complex similar to the ERdj5/EDEM1

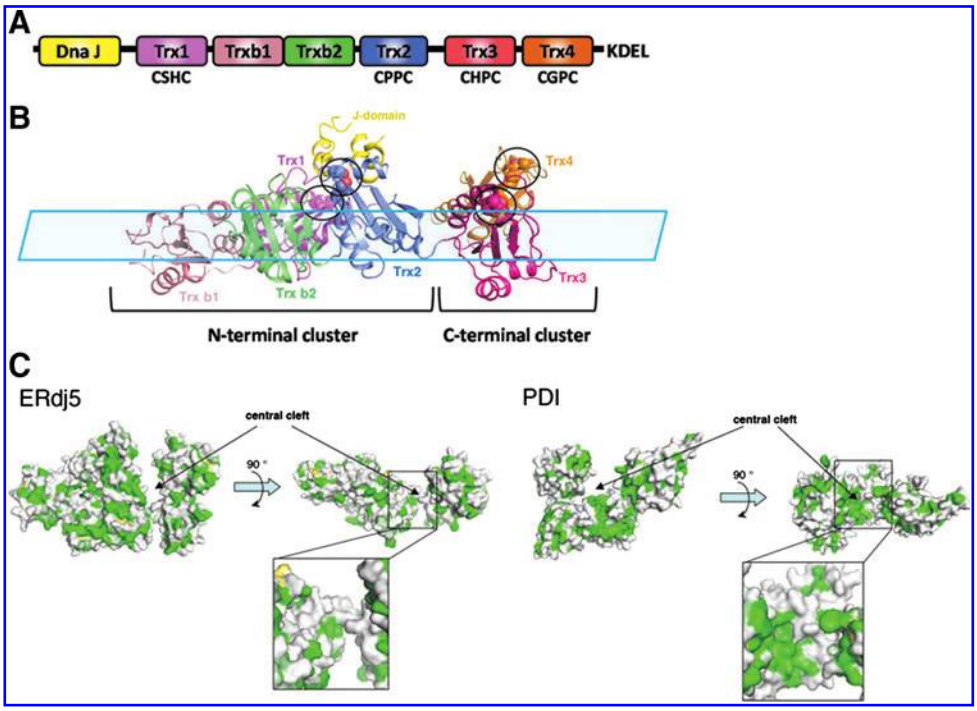

FIG. 7. The overall structure of ERdj5 and the hydrophobic surface features of ERdj5 and Pdi1p. (A) Domain architecture of ERdj5. ERdj5 contains a J domain in the N-terminus, six tandem Trx domains, and a KDEL ER retention sequence in the C-terminus. **(B)** Ribbon diagram of ERdj5. All of the Trx domains are contained in the same plane (flat blue surface). ERdj5 can be divided into two domain clusters: the N-terminal cluster containing the J domain and four Trx domains (Trx1, Trxb1, Trxb2, and Trx2) and the C-terminal cluster containing the Trx3 and Trx4 domains. The redox active sites, which are encircled, are located in the vicinity of the J domain. **(C)** Surface representations of ERdj5 and yeast PDI. Hydrophobic or aromatic residues are light green and charged, and polar residues are gray colored. The central cleft of ERdj5 is narrow compared to that of yeast PDI and has no patch of hydrophobic amino acids. ERdj, ER DnaJ.

complex. The Pdi1p/Htm1p complex has also been reported to participate in glycoprotein ERAD in yeast, although by a different mechanism than in mammalian cells. In yeast, Pdi1p does not act as a reductase but is instead involved in the oxidative folding and stabilization of Htm1p (17, 51). Moreover, substrate recognition is attributed to Pdi1p, and Htm1 does not appear to participate in distinguishing misfolded proteins from folding intermediates (17). Thus, the mechanism and components required for substrate recognition in ERAD are different in mammalian cells and yeast (Fig. 8).

BiP as a molecular chaperone that recruits misfolded substrates to the dislocon channel

After the cleavage of inter and/or intramolecular disulfides by ERdj5, the substrates are transferred to BiP, which is postulated to transport them to the ERAD complex composed of SEL1L, HMG-CoA reductase degradation 1, Derlins, and other component proteins. It is well known that ATP-bound BiP exists in an open form in terms of substrate binding and has a strong binding affinity for ERdj5. The J domain of ERdj5 accelerates the ATPase activity of BiP and converts the ATP-bound form of BiP to the ADP-bound form, which then loses binding affinity for ERdj5 (58). However, the ADP-form of BiP adopts a closed conformation that exhibits a higher affinity for substrates. During dissociation from ERdj5, the ADP-bound form of BiP is postulated to pull the substrates from the ERdj5/EDEM1 complex.

At present, at least eight DnaJ domain-containing proteins have been reported in mammalian cells. ERdj1 and ERdj2 are membrane proteins in the ER. ERdj1 has been reported to regulate translation by binding to ribosomes (36), and ERdj2 is a component of the Sec translocon complex (14). ERdj3 and ERdj4 are soluble luminal proteins in the ER that are induced by ER stress. While ERdj3 has been reported to be involved in the productive folding of proteins (54), ERdj4 was reported to participate in ERAD of misfolded proteins (46) and was also shown to cooperate with ERdj5 in ERAD (12). DnaJB12, a transmembrane protein harboring a cytosolic J domain, was also recently reported to accelerate ERAD of membrane proteins in cooperation with cytosolic Hsp70 (62). Finally, the function of ERdj7 remains to be elucidated.

Reducing potential in an oxidative environment

An important issue to be addressed is the reductive source that makes it possible for ERdj5 to reduce other proteins in the ER. Because of its reducing potential, ERdj5 exists in an oxidized form *in vivo*. To act as a reductase, ERdj5 must attain a reduced form, which requires an upstream donor with reducing power. One possible mechanism for this is *via* the direct reduction of ERdj5 by GSH. In fact, ERdj5 is able to successfully catalyze the reduction of insulin *in vitro* using the reducing power of GSH. However, the ratio of GSH/GSSG in the ER might not be high enough to directly reduce ERdj5 and therefore would require the import of GSH from the cytosol through the ER membrane. The mechanism or machineries by which this may occur are unknown. Alternatively, a reduction relay similar to an oxidation cascade may also result in the

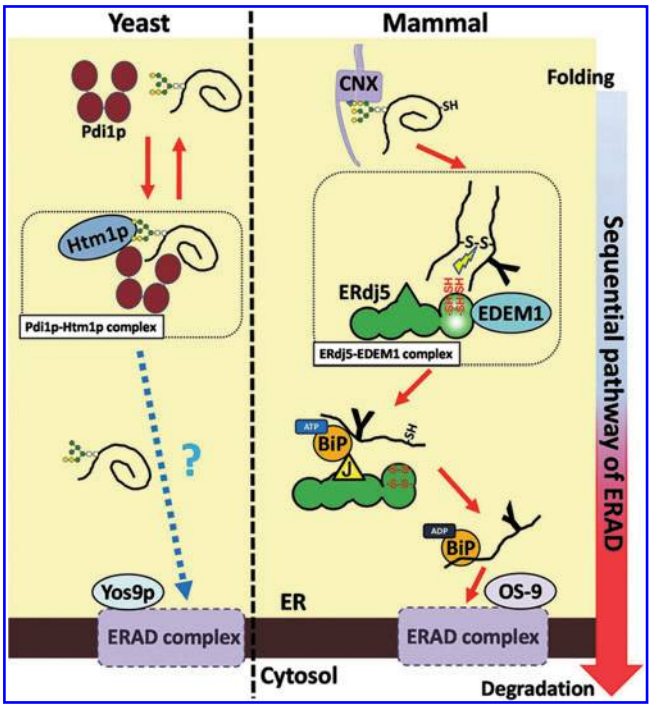

FIG. 8. Differences in yeast and mammalian ER-associated degradation pathways. In yeast, free Pdi1p and Pdi1p-Htm1p bind to unfolded proteins with equal affinities. Unfolded proteins bind to Pdi1p for extended periods to allow mannose residues of the Mannose (Man) 8 oligosaccharide to be trimmed by Htm1p, generating Man7. Man7 glycoproteins are recognized by Yos9p and transferred to the ER membrane-localized ERAD complex. However, which ER-resident proteins transfer Man7 glycoproteins to Yos9 remains unknown. In mammals, terminally misfolded proteins are released from CNX and recognized by ER degradation-enhancing mannosidase-like proteins (EDEM) 1. EDEM1 forms a complex with the C-terminal cluster of ERdj5 and reduces the disulfide bonds of misfolded proteins *via* active Trx domains in the C-terminal cluster. Reduced and extended substrate polypeptides are then transferred to BiP *via* the J domain in an ATP-dependent manner. ADP-BiP captures the extended polypeptides and transfers them to the ERAD complex.

reduction of ERdj5. Recently, the ER flavoprotein (ERFAD), which is associated with ERAD, was shown to interact with ERdj5 (48). ERFAD contains two consensus motifs for binding FAD and NADPH; however, the function of ERFAD in ERAD has not been fully elucidated. Identification of the reductive source or reduction cascade within the ER will be necessary for a full understanding of ERAD mechanisms.

Conclusion and Perspective

Quality control of newly synthesized proteins in the ER is unique compared to that in the cytosol; it depends on a redox environment and the activities of oxidoreductases and lectins. Although its complexity varies among species, the fundamental mechanism itself is likely conserved from yeast to mammal. Mechanistically, at least three indispensible successive steps are necessary. First, a mechanism for recognizing and discriminating substrates to be degraded from the folding intermediates of normal proteins. Second, unfolding and disulfide cleavage of misfolded substrates is necessary for the proteins to pass through the dislocon channel. Third, a mechanism must exist for recruiting the substrates to the ERAD complex on or close to the dislocon channel. As noted above, EDEM1, ERdj5, and BiP, which form a supramolecular functional complex, appear to be the major players in these three steps. Further study of these molecules, including a thorough structural analysis, will enhance our understanding of the molecular basis of ERAD.

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Abbreviations Used

BiP = immunoglobulin heavy chain binding protein
CNX = calnexin
CRT = calreticulin
EDEM = ER degradation-enhancing mannosidase-like proteins
ER = endoplasmic reticulum
ERAD = ER-associated degradation
ERdj = ER DnaJ
ERFAD = ER flavoprotein
ERGIC = ER-Golgi intermediate compartment
Ero = ER oxidoreductin
FAD = flavin adenine dinucleotide
GH47 = glycosyl hydrolase 47
GI = glucosidase I
GII = glucosidase II
G1Man9 = Glc1-Man9-GlcNAc2
Glc = glucose
GlcNAc = N-acetylglucosamines
GSH = glutathione
GSSG = glutathione disulfide
Hsp = heat shock protein
IgM = immunoglobulin M
Man = mannose
MRH = mannose-6-phosphate receptor homology
OS-9 = osteosarcoma amplified 9
PDI = protein disulfide isomerase
PRX = peroxiredoxin
QSOX = quiescin sulfhydryl oxidase
Trx = thioredoxin
Ub = ubiquitin
UGGT = UDP-glucose:glycoprotein-glucosyltransferase
VKOR = vitamin K epoxide reductase

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