

In and Out of the ER: Protein Folding, Quality Control, Degradation, and Related Human Diseases

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Hebert DN, Molinari M. In and Out of the ER: Protein Folding, Quality Control, Degradation, and Related Human Diseases. *Physiol Rev* 87: 1377–1408, 2007; doi:10.1152/physrev.00050.2006.—A substantial fraction of eukaryotic gene products are synthesized by ribosomes attached at the cytosolic face of the endoplasmic reticulum (ER) membrane. These polypeptides enter cotranslationally in the ER lumen, which contains resident molecular chaperones and folding factors that assist their maturation. Native proteins are released from the ER lumen and are transported through the secretory pathway to their final intra- or extracellular destination. Folding-defective polypeptides are exported across the ER membrane into the cytosol and destroyed. Cellular and organismal homeostasis relies on a balanced activity of the ER folding, quality control, and degradation machineries as shown by the dozens of human diseases related to defective maturation or disposal of individual polypeptides generated in the ER.

I. INTRODUCTION

A. From Genes to Proteins

The flow of genetic information from DNA to RNA to proteins constitutes the basis for cellular life. The replication of the genome is extremely accurate. Correction mechanisms such as editing and repair ensure error rates below 1×10^{-8} in bacteria and 1×10^{-10} in eukaryotes (288). To a certain extent, mutations in the genomic DNA sequence are tolerated and give essential contribution to the evolution process, provided they do not result in replacement of residues essential for the polypeptide's biologic activity, or in mutations, deletions, or premature termination of the polypeptide chain that prevent protein maturation. The information transfer from DNA to mRNA (transcription), from mRNA to linear strings of amino

acid chains (translation), and the conversion of the latter into a correctly shaped and biologically active protein (folding) are error-prone. Sporadic errors in gene transcription (estimated at 1×10^{-4} in bacteria) or translation (see Table 1) as well as a certain level of folding inefficiency rarely have dramatic consequences for cell survival because both messengers and proteins have relatively short half-lives relative to the organisms life span and may rapidly be replaced by new gene products. Moreover, quality control mechanisms are in place to select faulty products to be selectively removed (83, 167).

B. Protein Synthesis

Protein synthesis is operated by ribosomes, macromolecular machines made by ribosomal rRNAs, and several small ribonucleoproteins that act as scaffold. The

TABLE 1. *Error rates in the processes of replication, transcription, translation, and folding*

Process	Error Rate	Reference Nos.
Replication	1×10^{-8} (prokaryotes), 1×10^{-10} (eukaryotes)	288
Transcription	1×10^{-4} per codon (prokaryotes)	328
Translation	$1 \times 10^{-3} - 10^{-4}$	57, 288
Folding	Protein dependent	

large (60S) and the small (40S) ribosomal subunits assemble at the AUG start codon at the 5' -end and disassemble at the UAG/UGA/UAA stop codon at the 3' -end of mRNAs. Transfer tRNAs presenting matching triplet anticodons serve as amino acid donors to elongate nascent polypeptides at a rate of ~ 4 – 5 amino acids/s in mammalian cells (300).

Ribosomes are free in the cytosol, in mitochondria, and in chloroplasts or associated at the cytosolic face of the ER membrane. Cytosolic ribosomes synthesize cytosolic, nuclear, and peroxisomal proteins as well as most proteins of mitochondria and chloroplasts. Membrane-bound ribosomes synthesize polypeptides destined to the secretory and endocytic compartments (ER, Golgi, endosomes, and lysosomes), to the plasma membrane, and for secretion to the extracellular space. Translation has an infidelity rate estimated at 1 amino acid in $10^3 - 10^4$ (57, 288). Errors are caused by misacylation leading to tRNAs loaded with the inappropriate amino acid (221), by selection of an incorrect tRNA during the elongation process (176, 231), by incorrect selection of the start codon, by frame shifts, or by incorrect terminations. During the synthesis of the polypeptide chain (cotranslational phase) and after the release of the newly synthesized polypeptide from the ribosome (posttranslational phase), the folding of the polypeptide chain converts the string of amino acids into a mature, active protein that is eventually displayed at the appropriate intra- or extracellular location.

C. Protein Folding

High fidelity of gene expression is a basic requirement for life of single and multicellular organisms. Rapid and efficient conversion of the information contained in the linear sequence of amino acids in the unique native shape of every one of the individual polypeptides produced by cells is of crucial importance and must fulfill thermodynamic and kinetic requirements. Studies performed in test tubes in the early 1960s (6, 7, 407) revealed that only the information contained in the amino acid sequence is required for proper folding of polypeptides and that the unique native state of a protein in its physiologic milieu is the one in which the Gibbs free energy of

the whole system is lowest (5). The free-surface energy of the "protein-cellular milieu" system is largely increased by exposure of hydrophobic groups. Hence, burial of nonpolar residues or patches in the core of the molecule starts the protein folding process, thus rapidly minimizing free-surface energies. Hydrophilic interactions such as salt bridges and disulfide bonds limit the number of folding states explored by the folding polypeptides, thus allowing termination of the folding process in a biologically acceptable time span.

Whereas few model denatured proteins can refold spontaneously in the absence of cellular factors *in vitro*, protein folding in cells involves other proteins that act as molecular chaperones and as folding enzymes that accelerate rate-limiting reactions in the folding process. They are hosted in all cell compartments and organelles in which protein synthesis or posttranslational protein import occurs. They belong to families that have been conserved during evolution. Small heat shock proteins (Hsp), and proteins of the Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 families act as molecular chaperones and selectively bind nonnative determinants exposed by folding, incompletely assembled polypeptides, or by polypeptides that enter off-pathways eventually leading to irreversible misfolding and destruction (85, 136).

Molecular chaperones are not by definition part of the final polypeptide functional structure and do not convey structural information. They do not normally accelerate the kinetics of the folding process. Folding enzymes or foldases are responsible for accelerating rate-limiting steps for the folding reaction. Protein disulfide isomerases (PDI) catalyze formation of covalent bonds between cysteine residues of a polypeptide. Peptidyl-prolyl *cis-trans* isomerases (PPI) catalyze isomerization of peptidyl-prolyl bonds. These two chemical reactions would be too slow if not assisted enzymatically.

The observation that proteins can be ubiquitinated cotranslationally (333) and might actually be degraded before chain termination if they carry specific degradation signals at their NH₂ terminus (381) show that folding and degradation of a given polypeptide chain may be in kinetics competition (268a). Certainly, folding and degradation are strongly interconnected as it has been established that a functional degradation machinery is required for maintenance of protein folding capacity (86, 114, 268). Despite availability of numerous ER-resident folding assistants, the protein folding process may fail and is substantially affected by errors in the polypeptide sequence. Products derived from faulty genes may be incapable of acquiring functional shapes. These defective products can either be rapidly degraded causing loss-of-function phenotypes or accumulate in or outside cells leading to gain-of-toxic-function phenotypes. Both of these outcomes can cause a number of human diseases, many of which are familial. In this review, we have collected the information

available on mechanisms regulating the efficient transfer of information from DNA to functional proteins. In particular, we focus our attention on the fate of proteins that enter the eukaryotic secretory pathway by cotranslational translocation into the ER. We describe how these proteins fold and are processed with the assistance of resident ER proteins. We explain what is currently understood about how the ER quality control machinery monitors the fidelity of the maturation process and targets aberrant proteins for destruction. Finally, we discuss the pathological consequences of aberrant folding and/or defective degradation of mutated gene products causing several human diseases. Detailed knowledge of the events regulating protein folding, quality control, and degradation may offer therapeutic opportunities to treat these conformational or ER storage diseases.

II. PROTEIN TRANSLOCATION, FOLDING, AND QUALITY CONTROL IN THE ENDOPLASMIC RETICULUM

A. Protein Targeting to the ER

Mammalian ribosomes are only located in the cytosol and in mitochondria. For proteins to be expressed in the ER lumen, two issues must therefore be resolved: 1) how to bring translating ribosomes in close proximity to the ER membrane and 2) how to get the nascent polypeptide chains across the ER membrane. Several groups contributed in the elucidation of these mechanisms. In the early 1950s, Palade (299) showed that in secretory cells a large population of ribosomes is associated with endomembranes. Ten years later, compelling data demonstrated that mRNA for cytosolic proteins is translated on free ribosomes while mRNA for secretory proteins is translated on membrane-bound ribosomes (106, 151, 318) and that membrane-bound ribosomes vectorially discharge nascent polypeptide chains across the ER membrane (319, 320, 331). Finally, it has been established that the *in vitro* translation product of immunoglobulin light chain was larger than the mature protein expressed *in vivo* (123, 238, 259, 335, 338, 364, 374) due to the presence of a string of ~20 hydrophobic residues preceding the NH₂ terminus of the mature secretory protein (336). This led to the hypothesis (26, 259) and later to the demonstration (25) that short signal sequences subsequently removed from mature proteins serve as address tags for protein synthesis at the ER membrane.

A complex of 7S RNA and six polypeptides named signal recognition particle (SRP) binds the signal sequence emerging from the ribosome, thus slowing down chain elongation (397, 398) until the ribosome engages a proteinaceous channel located at the ER membrane and formed by two Sec61 $\alpha\beta\gamma$ heterotrimeric complexes (see

sect. II B7) (120, 261). Only then, protein synthesis is resumed, the short hydrophobic tag is usually removed, and nascent chains are cotranslationally injected into the ER lumen (116, 257, 399). Beyond the function as address tags, signal sequences may have specific posttargeting functions such as regulation of gene expression (245) or of virus assembly (426). Recent data show that the efficiency of polypeptide targeting by signal sequences is variable and may affect the biosynthetic load of the ER during conditions of ER stress (187, 365).

The ER lumen has unique conditions [e.g., is more oxidizing compared with the cytosol (166)], and several proteins expressed in this compartment are subjected to peculiar co- and posttranslational modifications such as the formation of intra- and interchain disulfide bonds between cysteines and addition of preassembled oligosaccharides and lipid anchors. The discrepancy between *in vitro* and *in vivo* folding rates led to the discovery of an enzyme system which catalyzes the rate-limiting step of protein folding in the ER by participating in the formation of native disulfides between the cysteine residues of a maturing protein (118, 390). Nearly half a century of further study clearly showed that complex mechanisms and machineries have evolved to facilitate polypeptide folding, to control the quality of the products, to distinguish native proteins to be transported at their site of activity from aberrant, folding-defective side products of protein biogenesis to be rapidly removed from cells. A basic set of molecular chaperones and folding enzymes has been conserved from eubacteria to higher eukaryotes (137). In addition, in eukaryotes, more sophisticated systems are also in operation.

B. Chaperone-Assisted Protein Folding in the ER

Protein folding in the ER commences cotranslational/translocationally and continues posttranslationally until the native protein structure is reached. The high concentration of calcium ions and oxidizing conditions of the ER create an environment that is topologically equivalent to the extracellular milieu. As an intracellular maturation compartment, the ER prepares secretory proteins to remain stable under challenging extracellular conditions. The ER houses factors that assist proteins in their folding and supports the attachment or formation of protective and stabilizing covalent modifications. Ultimately, properly folded and assembled proteins are packaged into cytosolic coat protein II (COPII)-derived vesicles and transported out of the ER to the Golgi.

1. The classical chaperones

Cellular compartments in which synthesis or translocation of proteins occurs (cytosol, mitochondria/chloroplasts, and ER) contain a high concentration of molec-

ular chaperones that prevent aggregation of unfolded chains, facilitate protein maturation, and retain folding proteins in appropriate micro- or macroenvironments enriched with folding enzymes. Classical chaperones are grouped in several subfamilies, namely, Hsps of 40, 60, 70, 90, and 100 kDa in size (Table 2). The ER lumen does not contain members of the Hsp60 (chaperonins) family; rather, it possesses a member of the Hsp70 family [glucose-regulated protein (GRP)78/BiP (128, 146)]; BiP co-factors classed in the Hsp40 [ERdj1–5 (344)] and GrpE-like families [BAP/Sil1 (56) and GRP170 (358, 405)]; a member of the Hsp90 family [GRP94 (220)]; and a member of the Hsp100 family [TorsinA (32)]. Cytosolic members of the Hsp families are transcriptionally induced upon temperature stress; ER-resident members are not, but their synthesis is strongly enhanced under conditions of ER overload, glucose deprivation, or upon unbalanced calcium or redox conditions homeostasis (220, 347).

BiP/GRP78 has been referred to as the master regulator of the ER (146). It maintains the permeability barrier of the ER by sealing the luminal side of inactive translocons (2); facilitates translocation of growing nascent chains in the ER lumen by acting as a molecular ratchet (247); participates in protein folding and oligomerization (128); regulates aggregation of nonnative polypeptides (314); contributes to calcium homeostasis in the ER (227); plays an important role in the preparation of terminally misfolded ER proteins for the dislocation into the cytosol required for their disposal (40, 184, 264, 354); contributes in the regulation of an adaptive signal transduction pathway termed the unfolded protein response (UPR; reviewed in Refs. 242, 340) that has fundamental roles in cells, organs, and tissues differentiation and maintenance; and is required for cell proliferation during embryogenesis (235).

BiP shields immature proteins from aggregation by promiscuously binding to extended hydrophobic domains with relatively low affinity (1–100 mM). Affinity panning of a bacteriophage expressed peptide library demon-

strated that BiP has a preference for alternating aromatic and hydrophobic amino acids (27). These alternating residues can localize to a single surface to support BiP binding. A BiP binding score or algorithm for predicting BiP interacting regions was developed from these results, which can be used to identify potential BiP binding regions. On average, proteins are expected to contain a hydrophobic Hsp70 interacting region once every 36 residues, underscoring the broad range of substrates these proteins are expected to bind during their maturation (330). Consistently, a number of maturing viral and host cell proteins have been found to transiently associate with BiP (128, 133, 312). The binding of BiP to misfolded mutant proteins can be prolonged and, as shown by analysis of transthyretin (TTR) variants, there might be a direct correlation between thermodynamic and kinetic instability of the TTR variants, and BiP capture (354). Moreover, association of BiP with covalent and noncovalent aggregates of misfolded proteins signaled a role of BiP in maintenance of solubility of aberrant proteins to facilitate their eventual disposal (40, 184, 264, 284, 354).

BiP contains two main functional domains: a COOH-terminal peptide binding domain that is controlled by its NH₂-terminal ATPase domain. ADP binding to BiP creates the high-affinity conformation, whereas ATP binding supports peptide release or its low-affinity conformation. J-proteins are thought to deliver substrates to Hsp70 and initiate the hydrolysis of ATP by Hsp70, thereby preparing the high-affinity form of the chaperone. Recent studies have identified several ER J-proteins called ERdj1/Mtj1 (34, 50), ERdj2/hSec63 (349, 382), ERdj3/HEDJ/ERj3/ABBP-2 (24, 217, 432), ERdj4/Mdj1 (313, 344, 345), and ERdj5/JPD1 that has both a J domain and oxidoreductase activity (61, 158). All these proteins bind BiP *in vitro* and stimulate its ATPase activity. In addition, nucleotide exchange factors assist in the swapping of ADP bound to Hsp70s for ATP. These factors help create the low-affinity binding conformation leading to the release of peptide by Hsp70. The bacterial exchange factor GrpE is the most

TABLE 2. *Molecular chaperones in the ER*

Family	Protein	Function	Reference Nos.
Hsp40	ERdj1/Mtj1	Cofactors for Hsp70	34, 50
	ERdj2/hSec63		349, 382
	ERdj3/HEDJ/ERj3/ABBP-2		24, 217, 432
	ERdj4/Mdj1		313, 344, 345
	ERdj5/JPD1		61, 158
Hsp60	None		
Hsp70	GRP78/BiP	Conventional chaperone	128
Hsp90	GRP94/endoplasmic/ERp99	Conventional chaperone	9
Hsp100	Torsin A?	?	32
GrpE-like	BAP/Sil1	Cofactors for Hsp70	56
	GRP170		358, 405
Lectins	Calnexin	Glycoprotein-dedicated chaperones	296
	Calreticulin		305
	EDEM1, EDEM2, EDEM3		161, 290

thoroughly studied. The recently discovered nucleotide exchange factor BAP/Sil1 (56) and GRP170 (358, 405) appear to serve this role in the ER lumen.

BiP levels are tightly controlled and are elevated upon physiological (e.g., cell differentiation increasing the ER load with cargo proteins) or pathological (accumulation of misfolded proteins) stress responses. During embryogenesis, BiP expression remains below detection limit to the morula stage, but becomes abundant at the blastocyst stage in E3.5 embryos (196). It is therefore not surprising that the *BiP*^{-/-} mice cannot survive beyond this stage, and the cells when cultured in vitro cannot proliferate and rapidly degenerate (235). A partial reduction in the level of BiP is tolerated as *BiP*^{+/-} embryos and adult mice are normal. The essential role of BiP in cell, tissue, and organism homeostasis is underscored by the identification of BiP as the cellular target of one of the most potent bacterial toxins ever characterized, the subtilase cytotoxin (SubAB). SubAB is produced by a highly virulent Shiga toxigenic *Escherichia coli* strain responsible for the 1998 outbreak of hemolytic uremic syndrome in Southern Australia. SubAB inactivates BiP through a single-site cleavage that disconnects the substrate binding from the ATPase domain of the molecular chaperone (269, 302). The disruption of BiP activity results in rapid cell death.

GRP94 is the most abundant glycoprotein in the ER. Whereas BiP is evolutionarily conserved from yeast to human, GRP94 is only found in vertebrates (220). GRP94 appears to associate with more advanced folding intermediates than BiP, since it binds some substrates after they have been released from BiP (253). It helps with the maturation of immunoglobulin heavy chains, integrin, and toll-like receptors (253, 317). GRP94 is comprised of three distinct domains, which include 1) an NH₂-terminal regulatory domain, 2) a central substrate-binding domain, and 3) a COOH-terminal dimerization domain. Nucleotide binding to the NH₂-terminal domain appears to control substrate binding; however, the precise mechanism of

regulation is largely unknown (77, 170, 352). GRP94 does not appear to act as an ATPase or have cofactors like its cytosolic paralog, Hsp90. Geldanamycin, which competes for the ATP binding site on the NH₂ terminus of GRP94, inhibits substrate binding and has been used to determine the necessity of GRP94 binding for protein maturation (360). In addition to its role in protein maturation, GRP94 is also known for its ability to induce T-cell immunity (226). Here, it uses its ability to bind small peptides as an antigen delivery system, thereby initiating MHC class I-restricted T-cell response against a variety of pathogenic or cellular antigens. Overall, cells have evolved a diverse set of roles for the fundamental substrate binding properties of the hsp70 and hsp90 chaperones of the ER that extend far beyond their central function in assisting in protein maturation (220).

2. The lectin chaperones

The majority of the proteins that traverse the secretory pathway receive multiple N-linked glycans. These hydrophilic modifications can change the general properties of proteins. They also provide binding sites for carbohydrate-binding chaperones. In contrast to BiP that binds directly to the hydrophobic backbone of the polypeptide, the lectin chaperones bind the glycans or the bulky hydrophilic extensions. However, both chaperone systems appear to serve similar roles in increasing the overall fidelity of the maturation process.

Preassembled glycans composed of three glucose, nine mannose, and two N-acetyl glucosamine residues (Glc₃Man₉GlcNAc₂, Fig. 1) are transferred by the oligosaccharyl transferase (OST) from a lipid pyrophosphate donor in the ER membrane, dolichol-PP, to nascent polypeptide chains (301). Oligosaccharides covalently modify the side chain of asparagines in Asn-X-Ser/Thr consensus sites (Fig. 1).

The functional unit of the OST appears to be a dimer where each dimer is comprised of eight or nine different

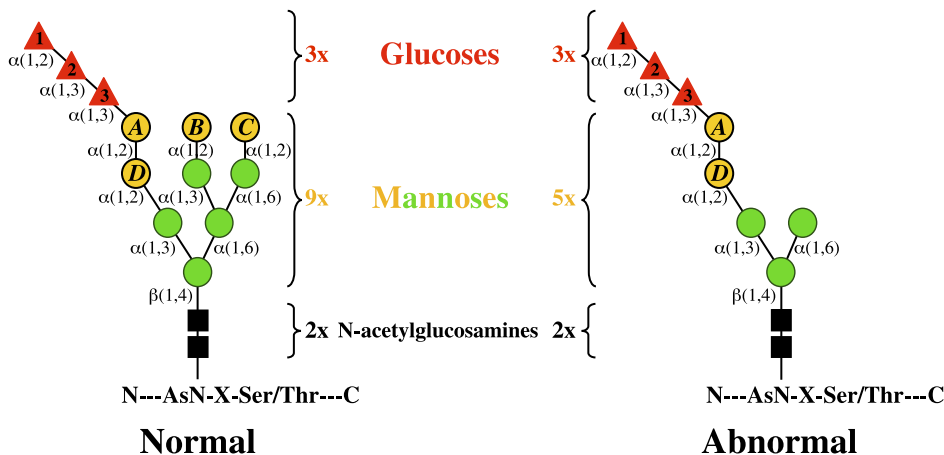


FIG. 1. Structure of N-linked oligosaccharides. The high mannose precursor covalently attached to Asn-X-Ser/Thr sequences of nascent polypeptide chains normally contains 9 mannose residues. Cell lines with defective synthesis of mannosylphosphoryldolichol are characterized by addition of incomplete oligosaccharides with only 5 mannose residues. A, B, C, and D are α1,2-bonded mannose residues that are removed by members of the glycosyl hydrolase family 47 (refer to Fig. 5). Glucose 1 is removed by the glucosidase I; glucose residues 2 and 3 are both removed by the glucosidase II.

subunits (47). A statistical analysis of glycosylation consensus sites indicated that two-thirds of available sites were occupied, and they were often found on exposed areas where secondary structure changes were observed (306). The localization of glycans to flexible regions is at least partially necessitated by the requirement in the transfer reaction for the hydroxyl group of the Ser/Thr residue in the consensus site to loop around and increase the nucleophilic properties of the relatively chemically unreactive Asn residue (17, 171). The transfer generally occurs cotranslationally once the consensus sequence has emerged 12–14 amino acids (30–40 Å) into the ER lumen aligning the Asn with the active site of the OST (282). The glycan is then rapidly trimmed cotranslationally by the sequential actions of glucosidases I and II to generate monoglucosylated side chains ($\text{Glc}_1\text{Man}_6\text{GlcNAc}_2$) that support binding to the lectin chaperones calnexin and calreticulin.

Both calnexin and calreticulin were initially named for their ability to bind calcium (97, 395). Calnexin is a type I membrane protein that contains a single luminal carbohydrate binding domain. The calnexin crystal structure demonstrates that the carbohydrate-binding domain is formed by a β -sandwich structure commonly found in leguminous lectins (339). A long hairpin extends away from the carbohydrate-binding domain forming a second domain termed the P-domain. The P-domain named for its richness in Pro residues creates an arm that recruits an accessory oxidoreductase involved in disulfide bond formation and isomerization called ERp57 (103, 210). Calreticulin is a soluble paralog of calnexin (258). While its crystal structure has yet to be solved, its strong homology to calnexin implies that it will have a similar organization, containing a single carbohydrate-binding domain with a slightly shorter P-domain.

Since calnexin and calreticulin bind monoglucosylated glycans with micromolar affinities (188), their chaperone binding cycles are controlled by the glucosidases and transferase that dictate the carbohydrate composition on maturing glycoproteins in the ER (132, 140, 144, 279, 295, 296, 356). Glucosidases I and II sequentially remove glucose 1 and glucose 2 (Fig. 1), respectively. This generates the monoglucosylated glycans that support the initial binding to the lectin chaperones (Fig. 2, step 1). Glucosidase II is a soluble, heterodimeric glycanase composed of a regulatory (β) and a catalytic (α) subunit (379). The regulatory β -subunit is dispensable for the enzyme activity in vitro (378). However, it has been shown that yeast lacking the glucosidase II β -subunit fails to remove glucose 3 but can still cleave glucose 2 (412). The β -subunit is a sequence homolog of the mannose-6-phosphate receptor. The association of the β -subunit with mannoses in the 6'-tetramannosyl branch of a core *N*-glycan has been recently proposed to cause a change in the conformation and result in the proper positioning of the catalytic

α -subunit for removal of glucose 2 from an additional *N*-glycan unit (74). Consistent with this transactivation model, the presence of more than one *N*-glycan is required for the formation of a complex between calnexin and nascent chains in canine microsomes and semipermeabilized cells (325, 400). Transactivation may not be strictly required for glucosidase II action as the enzyme can efficiently process, at least in vitro, methotrexate conjugates displaying a single oligosaccharide (375).

Upon substrate release from calnexin (Fig. 2, steps 2 and 2a), glucosidase II also removes the final glucose (glucose 3) creating the unglucosylated substrate, thus inhibiting substrate rebinding to the lectin chaperones (Fig. 2, step 3 for native proteins, step 3a for folding intermediates, and step 3b for folding-incompetent polypeptides). Regeneration of the monoglucosylated state and rebinding to the lectin chaperones is controlled by the UDP-glucose:glycoprotein glucosyltransferase (GT; acronyms such as UGT or UGGT are also commonly used), and it is only possible for nearly native folding intermediates (Fig. 2, step 4a) (43, 44). GT ignores native proteins that are released into the secretory pathway (Fig. 2, step 4) and extensively misfolded polypeptides that are prepared for dislocation into the cytosol and degradation (Fig. 2, step 4b). GT is comprised of two main functional domains: a large NH_2 -terminal domain that binds nonnative structures and a COOH -terminal carbohydrate transferase domain (126). This protein transfers a single glucose onto nonglucosylated side chains of glycoproteins that contain nonnative structures (44, 355, 367). Studies using purified proteins have demonstrated that GT recognizes glycans localized proximal to the misfolded domain (323, 324). However, another study found that GT could modify a glycan that was separated from the protein defect by at least 40 Å (367). GT provides the essential connection that links the nonnative exposed hydrophobic properties of a maturing protein to the composition of its exposed hydrophilic modification responsible for recruiting chaperones.

Calnexin and calreticulin binding exposes maturing substrates to ERp57 (a glycoprotein-dedicated oxidoreductase, Fig. 2) and generally slows the folding reaction helping to increase its overall efficiency (145). Pharmacological inhibition of the lectin chaperone binding with glucosidase inhibitors can lead to faster folding, premature oligomerization, and reduced folding efficiencies. For folding-defective polypeptides, the bypass of the calnexin system normally results in accelerated onset of the degradation program (141; see below). Posttranslational addition of glucosidase inhibitors preserves the *N*-glycans in a monoglucosylated state, which inhibits release from calnexin and calreticulin, and may arrest the global folding and oxidation of glycoproteins (139). Therefore, as with the traditional chaperones discussed above, protein folding appears to take place in the unbound form, with

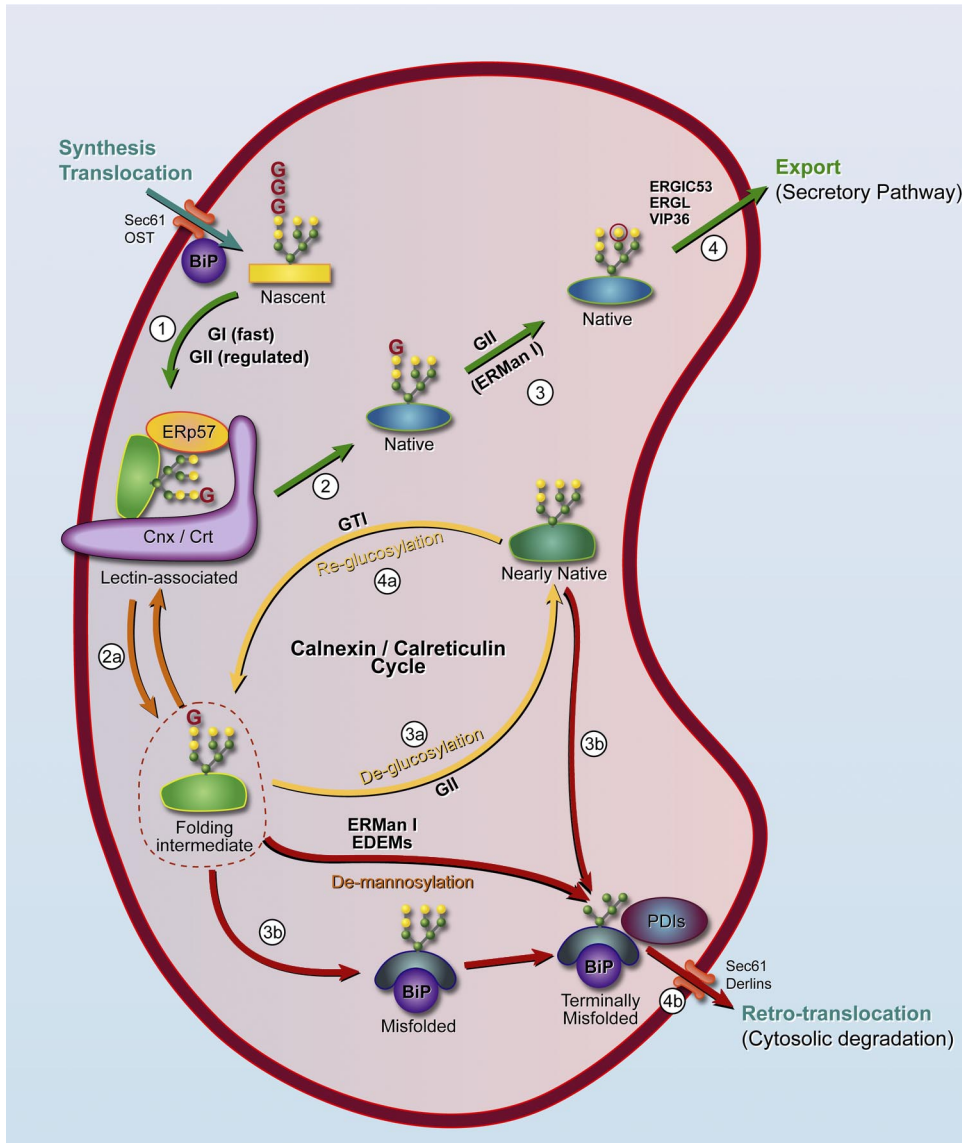


FIG. 2. The fate of newly synthesized glycoproteins in the ER lumen. Nascent chains enter the ER lumen through the Sec61 complex. They are core glycosylated by the oligosaccharyltransferase (OST). The two terminal glucose residues are rapidly trimmed by sequential action of the glucosidase I and II (*step 1*). Mono-glucosylated N-glycans mediate initial association of folding polypeptides with the ER lectin chaperones calnexin and/or calreticulin and exposure to the glycoprotein-dedicated oxidoreductase ERp57. It is likely that most glycopolypeptides are released from calnexin/calreticulin/ERp57 in a native, transport competent state (*step 2*). They are rapidly deglucosylated and partially demannosylated (*step 3*) and eventually sequestered in transport vesicles that leave the ER (*step 4*). For a fraction of newly synthesized glycoproteins, folding is not completed in a single round of association with calnexin/calreticulin/ERp57 in a native, transport competent state (*step 2a*). The folding intermediate released from the lectin chaperones is deglucosylated (*step 3a*), but its forward transport is inhibited by GTI. GTI adds back a glucose residue (*step 4a*) only to glycoproteins with nearly native conformation. These rebound to calnexin/calreticulin and are subjected to additional folding attempts likely to consist in disulfide reshuffling. Glycopolypeptides released from calnexin and displaying major folding defects are ignored by GTI (*step 3b*). Rather, they attract BiP. They are extensively demannosylated and dislocated across the ER membrane for proteasome-mediated degradation (*step 4b*).

chaperone binding helping to control the rate of the folding process and minimizing disruptive interactions that would lead to the formation of terminal aggregates. Glycoproteins may fold properly after a single round of binding to the lectin chaperones (Fig. 2, green arrows). However, cycling by the lectin chaperones as controlled by glucosidase II and GTI (yellow arrows) can lead to proper maturation and transport (green arrows) or ERAD (red arrows).

Having two lectin chaperones with different topologies helps to broaden the scope of substrates that can be assisted. Calnexin binds to glycans found in membrane proximal domains while the soluble calreticulin associates with glycans that emerge deeper into the ER lumen (142). These two lectin chaperones can act to protect a wide range of substrates or work together to assist glycoproteins that possess both membrane proximal and

distal glycans. Expression of calreticulin possessing an added membrane anchor in HepG2 cells demonstrated that substrate selection by membrane-associated calreticulin was similar to calnexin, indicating the importance of membrane topology in chaperone recognition (393).

While carbohydrate binding by calnexin and calreticulin is the central determinant for substrate selection, they also appear to be able to bind directly to the protein backbone in some cases (13, 45, 394; reviewed in Ref. 414). In addition, purified calreticulin and the soluble ectodomain of calnexin can bind and inhibit the in vitro aggregation of nonglycosylated proteins (63, 414). Future studies will be needed to identify where the polypeptide-binding domain is localized on these chaperones and how this binding is regulated.

Deletion of individual members of the calnexin/calreticulin chaperone system is well-tolerated in cultured

TABLE 3. Members of the human PDI family with at least one thioredoxin-like domain

Protein	TLD	Active Site Motif	Postulated Activity
ERdj5	4	CXHC, CXPC, CXPC, CXPC	Reductase
PDIr	3	CXHC, CXHC, CXXC	Inefficient catalyst, lacks essential Glu
ERp72	3	CXHC, CXHC, CXHC	Oxidase/isomerase
ERp46	3	CXHC, CXHC, CXHC	Oxidase
PDI	2	CXHC, CXHC	Oxidase/isomerase
PDIp	2	CXHC, CXHC	Oxidase/isomerase
PDILT	2	SXXS, SXXC	Oxidase/isomerase
ERp57	2	CXHC, CXHC	Oxidase/isomerase
P5	2	CXHC, CXHC	Oxidase
ERp44	1	CXXS	Retention of Erol
ERp18	1	CXHC	Inefficient catalyst, lacks essential Glu
TMX	1	CXXC	?
TMX2	1	SXXC	?
TMX3	1	CXHC	Oxidase
TMX4	1	CXXC	Inefficient catalyst, lacks essential Glu

cells (351 and references therein), but it results in embryonic lethality in mice for GT1 (265), ERp57 (108) and calreticulin (254) deletions. Calnexin deletion causes severe growth and motor disorders, and premature death (73). The lethal outcome of an inherited glucosidase I deficiency (67) confirms that the calnexin/calreticulin chaperone system plays an essential role during protein biogenesis possibly restricted to specific organs or developmental phases. In cultured cells, folding of most polypeptides must progress quite normally because individual chaperone deletions do not result in evident signs of ER stress (108, 263, 265, 351). However, there are at least two relevant exceptions that include influenza virus hemagglutinin (HA) that suffers substantial folding defects when expressed in cells lacking calnexin (263, 307), ERp57 (351), or GT1 (351a) and major histocompatibility complex (MHC) class I molecules that are loaded with suboptimal peptides and show premature release at the surface of calreticulin (107) or ERp57-deficient cells (108).

3. The link between BiP and the lectin chaperone system

Association of newly synthesized glycoproteins with the BiP system often precedes association with calnexin and calreticulin (133, 266, 309, 373, 389, 400). Exceptions are glycoproteins displaying *N*-glycans in the very NH_2 -terminal portion of the molecule (266). As *N*-glycosylation and association with calnexin/calreticulin occur as fast as when ~ 12 residues emerge in the ER lumen (3, 282), this excludes BiP assistance to the nascent chain (266 and references therein). BiP can also intervene after substrate release from the calnexin system to bind extensively mis-

folded polypeptides (18, 40, 68, 76, 98, 113, 162, 184, 203, 239, 243, 263–265, 354, 361, 363).

4. Disulfide bond formation

The formation of disulfide bonds is a critical step in the maturation of the majority of the proteins that traffic through the ER. The conditions of the ER favor the protein-assisted formation of disulfide bonds. Oxidoreductases from the PDI family (Table 3) catalyze these reactions by acting as electron acceptors in the oxidation reaction or electron donors for the converse reduction reaction. These enzymes can also isomerize disulfide bonds, helping a protein to obtain native disulfides by rearranging nonnative linkages (Fig. 3A). PDI family members are defined by containing CXXC motifs in thioredoxin domains. The number and location of these motifs vary depending on the particular enzyme (Table 3) (84).

The mammalian enzyme PDI is the most thoroughly studied member of the PDI family. It is a multifunctional protein that can act as both an oxidoreductase as well as a chaperone. PDI contains two catalytically active thioredoxin-like domains (TLD) termed a and a' that are divided by inactive TLD termed b and b'. The recently solved crystal structure of yeast PDI demonstrates that the four TLD form a twisted "U" shape (369). The catalytically active domains are located at the top of the U across from each other, and the two noncatalytic domains are localized to the inside surface of the U in an area enriched in hydrophobic residues. The hydrophobic surface is proposed to play a role in binding misfolded structures, and

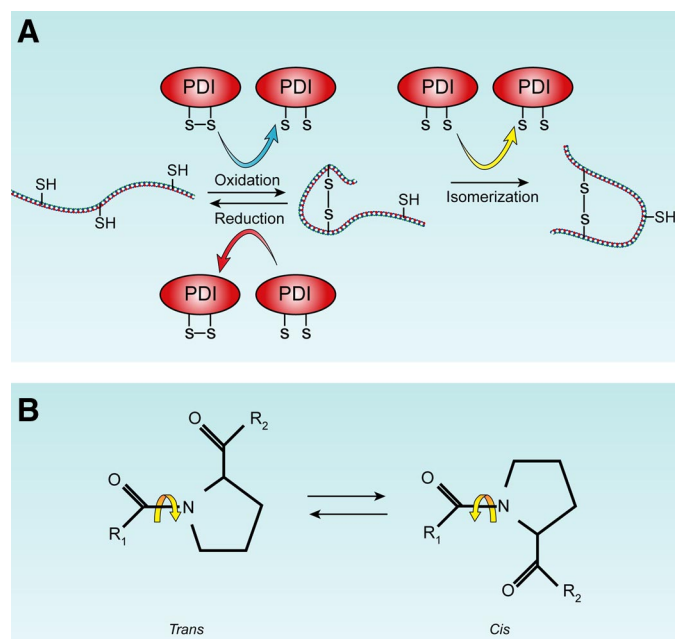


FIG. 3. A: oxidation, reduction, and isomerization of disulfide bonds. B: isomerization of peptidyl-prolyl bonds.

positioning the substrate for the catalytic domains to act upon the substrate.

The cysteines in the catalytically active domains of PDIs can be present in both the oxidized and reduced state. For the catalysis of disulfide bond formation in folding polypeptides, the CXXC motif contains a disulfide bond, acts as an electron acceptor, and leaves the reaction in the reduced state (Fig. 3A). In contrast for reduction of substrate disulfides, the CXXC motif of PDIs intervenes in the reaction in the reduced state (Fig. 3A). Finally, isomerization or disulfide rearrangement both starts and ends with the CXXC in the reduced state (Fig. 3A). A mixed disulfide with a substrate cysteine covalently bonded to a PDI reactive cysteine is a short living intermediate of all redox reactions described above. The oxidation state of the Cys residues found in the catalytic domain determines its function in the maturation process, and their redox state is controlled by its environment and additional proteins, which help to shuttle electrons into and out of the ER such as ERO1p (for review, see Ref. 380). In short, the membrane-associated flavoprotein ERO1 transfers oxidizing equivalents to PDI so that it can act as an oxidizing agent in the oxidation of nascent chains.

Although PDI family proteins are defined by their catalytic CXXC motifs that accelerate the formation and rearrangement of disulfide bonds, an essential function is also their chaperone activity. PDI can inhibit the aggregation of misfolded proteins that do not contain any disulfide bond (42). This activity does not require the active site Cys residues (316). This implies that PDI is not only a foldase that accelerates the folding reaction, but it can also act as a molecular chaperone to increase the fidelity of the folding reaction by inhibiting nonproductive aggregate formation. While the hydrophobic surface formed by the two central noncatalytic domains may play a role in the chaperone activity, how this binding is controlled is currently unknown.

The mammalian PDI family includes well over a dozen different proteins. Some of these proteins contain two TLD (PDI, ERp57, PDIp, PDILT, and P5); however, others have one (ERp18, ERp44, and TMX1–4), three (ERp72, ERP46, and PDIr), or even four of these motifs (ERdj5) (84) (Table 3). The canonical active site motif is the CXHC tetrapeptide characteristic of thiol-disulfide oxidants. It is found once in TMX and in ERdj5; twice in PDI, PDIp, ERp57, and P5; and three times in ERp72 (Table 3). A CXPC motif characteristic for thiol-disulfide reductants is displayed three times in ERdj5. The other PDI family members display a variety of motifs lacking one or both terminal cysteines (Table 3 and Ref. 84 for a review). While it is expected that some of these proteins will serve redundant functions, future studies will be needed to understand the full scope of their roles. Initial studies indicated that a portion of these proteins interact with a

particular subset of substrates while others serve specific roles in protein oxidation, reduction, or isomerization.

ERp57 (also called ER-60 and GRP58) possesses a similar domain organization to PDI but interacts specifically with glycoproteins due to its association with the P-domain of calnexin and calreticulin (237, 293, 294). A conserved positively charged region in the b' domain of ERp57 is responsible for lectin chaperone binding as found by mutagenesis, NMR spectroscopy, and isothermal titration calorimetry (103, 210, 218, 311). This region electrostatically interacts with a negatively charged region at the tip of the P-domains of calnexin and calreticulin (210). The lectin chaperones bind the nascent non-native glycoprotein and position ERp57 to act upon the immature or misfolded glycoproteins possessing monoglucosylated side chains (267, 292, 294). ERp57 exhibits oxidoreductase activity towards a variety of glycosylated proteins (29, 102, 154, 182, 351, 357, 433).

The ERp57 knockout in mice represents the first successful deletion of an oxidoreductase from metazoan cells (108, 351). Influenza HA (351) and class I MHC molecules (108) emerge as the only model glycoproteins among those analyzed thus far to significantly suffer from the deletion of ERp57. Deletion of ERp57 does not affect cotranslational formation of disulfide bonds occurring during entry of HA in the ER lumen. It significantly impairs, however, the posttranslational phases of HA folding. These data were interpreted as an intervention of ERp57 in disulfide bond isomerization during substrate glycoprotein folding (351). Deletion of ERp57 also led to the identification of at least one PDI family member, ERp72, that can act as a surrogate chaperone in catalyzing intra- and intermolecular disulfide bond formation when ERp57 is absent (351). The residues in ERp57 that are involved in association with the P-domains of the lectin chaperones are conserved in ERp72 (77). However, in contrast to ERp57, substrate association with ERp72 remained unaffected by inhibition of substrate binding to calnexin (351). Deletion of ERp57 accelerates the release of MHC class I molecules from the peptide-loading complex, thus resulting in loading with suboptimal peptides and reduced expression and stability at the cell surface (108).

The large number of PDI family members includes enzymes with broad substrate specificity plus additional family members that appear to provide a wide range of specialized functions in the eukaryotic secretory pathway. PDI, ERp57, ERp72, and P5 have been found in functional complexes or transiently associated with folding substrates via mixed disulfides. PDIp is an abundant protein in the ER of pancreatic cells and therefore likely acts upon zymogens, the main pancreatic cargo (75). ERdj5 is an interesting fusion protein with a J-domain (suggesting cooperation with members of the Hsp70 chaperone family) associated with an oxidoreductase

moiety (61, 158). ERp44 has been proposed to form a mixed disulfide with immature proteins and Ero1, thereby retaining them in the ER lumen (4) and to contribute in the regulation of ER calcium homeostasis by associating with the third luminal loop of the channel for calcium export from the ER lumen, the inositol 1,4,5-trisphosphate receptor 1 (152). ERp29 has evolved divergently from other PDI family members but retained the characteristic structural thioredoxin fold in one of its domains. Although the functional characterization of ERp29 is far from completion, all available data point to its important role in the early secretory pathway and allow tentative categorization as a secretion factor/escort protein of a broad profile (16). ERp29 has only a single Cys in its entire sequence, indicating that its catalytic site could only function as an isomerase. It initiates a conformation change in polyomavirus upon entry into the lumen of the ER (240). Therefore, ERp29 is likely used by viruses for their disassembly or uncoating in the lumen, which may involve the rearrangement of disulfide bonds found in their capsid proteins. Certainly, for the nearly 20 members of the PDI family, a number of unanswered questions remain about their structural features, their involvement in protein folding and/or degradation, their redundancy and interchangeability, their intracellular localization, and also about the molecular mechanisms regulating their activity.

5. Peptidyl-prolyl *cis-trans* isomerization

Most peptide bonds in native proteins are connected in *trans* conformation with the exception of Xaa-Pro bonds that can be found in both *cis* and *trans* conformations (Fig. 3B). Refolding experiments demonstrated that *cis/trans* isomerization of peptidyl-prolyl bonds (Fig. 3B) is a rate-limiting step of the polypeptide folding process (194). Prolyl isomerization is catalyzed by dedicated enzymes, the peptidyl-prolyl *cis-trans* isomerases (PPI) (95, 96). Mammalian cells contain three classes of PPI, namely, parvulins, cyclophilins (Cyps) and FK506-binding proteins (FKBPs) (121). The ER contains members of the two latter classes, namely, CypB and FKBP2, FKBP7, and

FKBP10 (Table 4). CypB has been found in complexes containing several other ER chaperones (but not members of the calnexin chaperone system) (255, 435). It has also been reported to form functional complexes with Hsp47, a procollagen-specific chaperone in the ER (350). More recently, it has been reported that ER-resident members of the FKBP family can act as regulators of BiP activity (401, 436) and associate with BiP-bound substrates (65). Although they have been shown to significantly accelerate acquisition of native structure in refolding of denatured proteins *in vitro*, very little information is available on Cyps' and FKFBPs' involvement in protein maturation in the living cell, and data supporting their activity remain, at the best, indirect.

6. Substrate-specific chaperones

While most proteins can fold properly with the assistance of the general chaperone systems, other proteins contain unique structures and/or are present at such a high concentration that specialized assistance is required. Substrate-specific chaperones in the ER include receptor-associated protein (RAP) and Hsp47. RAP facilitates proper folding and prevents aggregation and premature ligand binding by low-density lipoprotein (LDL) receptor (36, 224). It interacts with LDL receptors and helps escort the protein complexes to the Golgi. Hsp47 is a collagen-specific chaperone (278). Collagen is the most abundant mammalian protein, and it possesses an atypical triple-helical structure (185). While it interacts with many of the general chaperones (BiP, calnexin, and calreticulin) and folding factors (e.g., PDI), its proper maturation in the ER also requires Hsp47 (278). Mice with Hsp47 knocked out were deficient in collagen production and died 11.5 days postcoitus (277). The high expression level and unique structure of collagen appear to necessitate its requirement for a specifically tailored chaperone system. A comprehensive list of substrate-dependent chaperones can be found elsewhere (83).

7. Cotranslational folding

The average mammalian protein takes ~2 min to be translated, and protein folding *in vitro* is measured on the millisecond time scale. Therefore, during a 2-min period of translation, extensive protein folding can occur. The Levinthal paradox demonstrates that this time frame is not sufficient to sample all possible conformations available for a given nascent chain to reach its native state (223). Instead, protein folding involves a more direct route to the native structure, assisted in the cell by the temporal and physical constraints placed on the maturing nascent chain, which help to minimize the structures available as folding intermediates. The source of some of these restrictions is that protein folding in the living cell begins while the protein is being translated by the ribo-

TABLE 4. Members of the human PPI family

Protein	Inhibitor	Notes
CyclophilinB/CypB	Cyclosporin A	Part of large multichaperone complexes in the ER (255, 350, 435)
FKBP2/FKBP13	FK506/tacrolimus	ER-stress induced (39)
FKBP7/FKBP23	FK506/tacrolimus	Modulates BiPs ATPase (401, 436)
FKBP10/FKBP65	FK506/tacrolimus	Associates with BiP-bound substrates (65)

Reference numbers are given in parentheses.

some. This process of cotranslational folding can assist the formation of the proper folded structure in several ways. First, it supports a vectorial folding process from the NH₂ to the COOH terminus, which restricts the number of conformations available to a newly synthesized chain. Second, the COOH terminus of the molecule is constrained by the ribosome further limiting the freedom of the nascent chain or the number of available folding intermediates. Third, the bulky ribosome separates the nascent chains of a polysome, preventing nonproductive collisions or aggregation. And fourth, it provides a mechanism for the cell to control and organize the environment of the vulnerable nascent chain. Together, these mechanisms act to optimize the cellular folding process.

For secretory cargo, folding starts both cotranslationally and cotranslocationally as the nascent chain emerges in the ER lumen through the Sec61 translocon. These proteins commonly possess NH₂-terminal signal sequences that target the protein to the ER. NH₂-terminal signal sequences of 20–30 amino acids are highly hydrophobic, supporting their integration into the ER membrane in a looplike configuration (143, 245). This places a further constraint on the maturing protein by tethering its NH₂ terminus to the membrane until the signal sequence is cleaved. The timing and efficiency of signal sequence cleavage by the signal sequence peptidase is substrate specific. Signal sequence cleavage of most proteins appears to occur cotranslationally. For the type I membrane proteins influenza HA and tyrosinase, cleavage takes place after ~130 amino acids have been translated; however, the signal sequence for the HIV glycoprotein gp160 is removed posttranslationally (62, 225, 400). Delayed cleavage of gp160 appears to assist the proper folding of this complex viral protein.

Protease protection studies demonstrate that ~40 amino acids can reside within the ~100-Å-long tunnel of the ribosome (15, 248). Its narrow average diameter of ~15 Å permits the folding of some α -helices, as shown by fluorescent resonance energy transfer (FRET) measurements, but precludes the formation of more distal secondary structures (419). FRET studies have also found that a similar level of folding appears to be permitted within the narrow confines of the ER Sec61 translocon (419). In another study, the ribosomal and translocon-arrested Semliki Forest virus capsid protease domain was only able to fold when a linker of 64 amino acids or greater was placed at its COOH terminus, indicating that the protein could not fold to an active state in the translocon (209). Global protein folding is delayed until arrival in the lumen.

The inability for distal folding to take place in the translocon can be explained by recent structural studies, which have shed important light on the translocon architecture. The X-ray structure of the Sec61-related SecYE β from the archaeon *Methanococcus jannaschii* in the ab-

sence of preprotein substrate indicates that the channel possesses an hour-glass shape with its narrowest diameter measuring 3 Å (385). Removal of the proposed plug domain at the center of the membrane would expand the channel to ~17 Å. This value is in sharp contrast to 40–60 Å diameter measured for the functional mammalian Sec61 channel using fluorescence quenching studies (130). Recent cryoelectron microscopic studies of the *E. coli* Sec translocase suggest that it is composed of a dimer of heterotrimers, and a larger functional channel may be created by the joining of the two separate channels (80, 261).

Unless *N*-glycans are displayed at the polypeptide NH₂ terminus (266), BiP is the first luminal chaperone that interacts with nascent proteins upon emergence into the ER lumen (133, 253, 400). BiP is localized in part at the ER translocon entry site where it helps to maintain the permeability barrier also positioning it for early interactions with nascent chains (131). These associations help to drive the directionality of the translocating protein into the ER and protect the nascent chain during its most vulnerable state when it first emerges into the calcium-rich oxidizing environment.

N-linked glycans are generally added cotranslationally once the consensus glycosylation site is ~75 residues away from the peptidyltransferase center (409). The membrane protein calnexin is the first carbohydrate-binding chaperone encountered by monoglucosylated glycoproteins, followed by the soluble calreticulin after the addition of further glycans or chain lengthening (62, 400). Calnexin appears to be recruited to the translocon site through a direct interaction between the ribosome and the cytoplasmic tail of calnexin, which may be regulated by phosphorylation of the calnexin tail (51). The association of lectin chaperones with nascent chains also supports cotranslational interactions with oxidoreductase ERp57 (49, 266, 267).

The translocon environment likely possesses some higher order organization that arranges ER proteins in an assembly line with a general order that allows proteins that act on earlier folding intermediates first (Fig. 4). Folding factors that act on near-native structures would be situated deeper into the lumen, away from the translocon for posttranslational associations. The extent of cotranslational folding and the timing and type of interactions are determined by the nascent chain sequence and structure. The polytopic protein cystic fibrosis transmembrane conductance regulator (CFTR) folds extensively cotranslationally while other proteins fold largely posttranslationally (178, 201).

Currently, it appears that some 20 different polypeptides can interact with ribosome-associated nascent chains including the Sec61 chains (heterotrimeric complex), the OST (8–9 subunits), the signal sequence peptidase (5 subunits), the lectin chaperone system (glucosi-

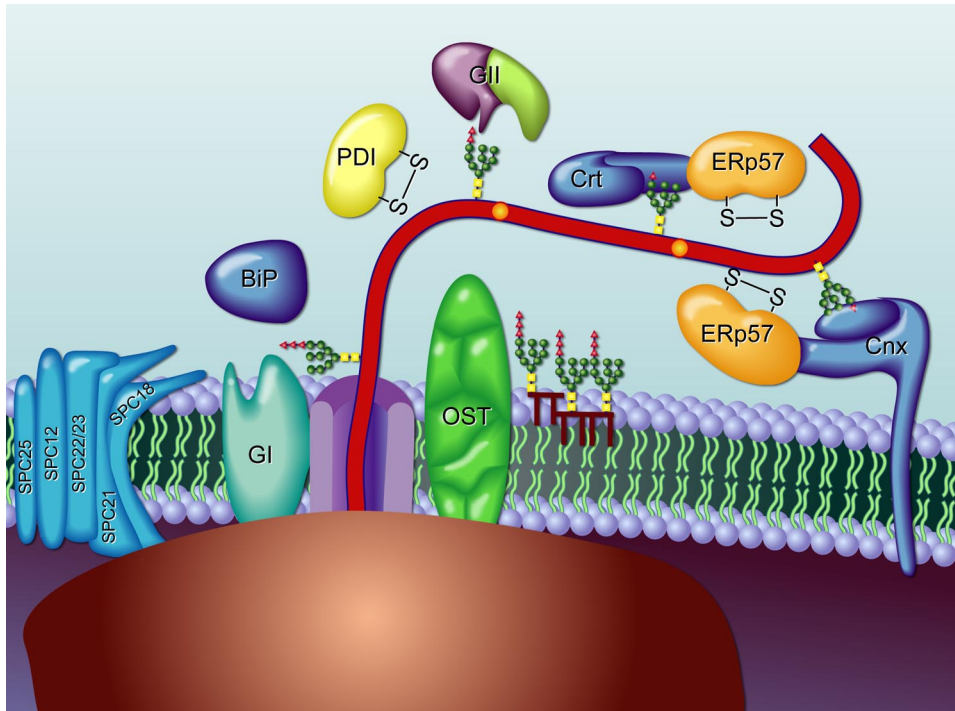


FIG. 4. The translocon-associated environment encountered by the nascent chain. As proteins emerge into the ER cotranslationally and cotranslocational through the Sec61 channel (purple membrane pore), they are subjected to a specialized maturation environment that facilitates their proper maturation. These factors include the signal peptide peptidase (SPC; 5 subunit complex), the oligosaccharyltransferase (OST; comprised of 8 or 9 subunits), glucosylases (GI and II), oxidoreductases (PDI and ERp57), and molecular chaperones [BiP, calnexin (Cnx), and calreticulin (Crt)].

dase I and II, calnexin, and calreticulin), BiP, and oxidoreductases (PDI and ERp57) (Fig. 4). The total mass of these translocon-associated proteins creates a barrier that ensures the vulnerable nascent chains do not come in contact with each other, further helping the maturation process (48, 346).

C. ER-Associated Protein Degradation

1. The efficiency of protein folding

The efficiency of the folding process is strictly protein dependent and cannot be predicted based on the polypeptide sequence. Polypeptides for which acquisition of the correct tertiary and quaternary structures has failed are usually not transported through the secretory line (163, 327), even though exceptions do exist (see sect. II C 4; Ref. 127). Rather, they are dislocated into the cytosol, deglycosylated, ubiquitinated, and fragmented by the 26S proteasome (207). Further processing by the tripeptidylpeptidase II (322) and other peptidases (425) also occurs, until the fragments are reduced to single amino acids that can be recycled for protein biosynthesis. Controversial data have been published on the actual fraction of newly synthesized chains that never attain native structure and are rapidly degraded. These values range from upwards of 30% in one study (341) to substantially less in another (383).

Proteins can be ubiquitinated cotranslationally (333) and might actually be degraded before chain termination

if they carry specific degradation signals at their NH₂ terminus (381). Protein degradation can also be anticipated and become cotranslational when cells are under ER stress and their survival requires a temporary decrease of the biosynthetic burden in the ER (297). Multicellular organisms could profit from a certain degree of folding inefficiency by producing wastes that may be used to monitor the protein set currently in production. To become immunologically relevant, the products of disposal of aberrant proteins must escape complete degradation to amino acids. Short peptides of at least eight amino acids are in fact reimported in the ER lumen through a heterodimeric transporter (TAP1/TAP2) member of the ATP-dependent transporter (ABC) superfamily (124), prepared for loading on class I MHC complexes and displayed at the cell surface for immunosurveillance (376). A high error rate during synthesis of viral proteins would result in surface presentation of viral epitopes, thus warning the immune system that the cell has been infected (337). However, heterologous proteins, such as viral gene products expressed in virus-infected cells, can sometimes show better capacity than the host cell proteins themselves to exploit the cellular folding environment to achieve native structure. Influenza HA folds with near 100% efficiency in infected cells (30). In contrast, biogenesis of the CFTR is a paradigm for cellular proteins with low folding-efficiency, which is further decreased upon gene mutations. It has been estimated that only ~25% of the newly synthesized wild-type CFTR will eventually fulfill ER quality control requirements for transport

to the cell surface, a percentage that drops to pathological levels in the case of mutant CFTR gene products (205, 402).

2. The importance of understanding ER-associated protein degradation

Understanding the mechanisms regulating degradation of folding-defective polypeptides expressed in the ER is one of the central issues in cell biology. Rapid disposal of folding-incompetent polypeptides produced in the ER lumen is instrumental to maintain ER homeostasis (256). The degradation machinery is easily saturated (377). Defective adaptation of the cellular degradation capacity to the ER load may result in accumulation of aberrant polypeptides that eventually impairs the ER capacity to assist maturation of newly synthesized secretory proteins (86).

The mechanisms evolved in metazoans to remove misfolded proteins from the ER lumen are exploited by a number of human pathogens. Bacterial toxins such as cholera and shiga toxin can travel through the secretory line in backflow and invade the host cell cytosol by crossing the ER membrane in a manner similar to ERAD substrates (233). Also, several viral gene products exploit the ERAD machinery to trigger degradation of host cell surface molecules such as viral receptors [e.g., the rapid degradation of the HIV1 receptor CD4 by the HIV1 gene product Vpu (104, 413)] or of molecules involved in immunosurveillance (e.g., the rapid disposal of class I MHC molecules by cytomegalovirus immunoevasins; see sect. II C9).

The degradation machinery also regulates the intracellular level and activity of important cellular factors as exemplified by the case of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol biosynthesis (115) and the inositol 1,4,5-trisphosphate receptor (IP₃) (417). Elevation of the sterol levels increases by twofold the rate of degradation of HMG-CoA, thus controlling by negative feedback the cell production of cholesterol (54). IP₃R can be down-regulated as part of an adaptive response by its polyubiquitination and subsequent degradation, which removes the calcium channel from the ER (417).

Finally, many loss-of-function human genetic diseases are caused by mutations that may not affect protein function, but slow significantly the kinetics of protein folding (refer to sect. III). In such cases, recognition by the ERAD machinery may precede completion of the folding process (268a). It is therefore clear that a detailed understanding of mechanisms regulating disposal of folding-defective and folding-incompetent proteins synthesized in the ER may eventually allow intervention in all the processes described above.

3. Misfolded proteins produced in the ER are degraded in the cytosol

Degradation of misfolded proteins synthesized in the ER requires energy and is unaffected by lysosomotropic agents, lysosomal enzyme inhibitors, and brefeldin A (a macrolide antibiotic that interferes with protein transport between ER and Golgi). These findings led initially to propose that nonnative polypeptides were retained and degraded in the ER lumen by a mechanism that involved ATP consumption (200). The acronym ERAD for ER-associated protein degradation was coined to describe degradation of misfolded proteins progressing in a reconstituted yeast system and requiring unidentified heat-labile cytosolic factors, ATP, and the chaperone calnexin (250). The identification in the mid 1990s of the 26S proteasome as the energy-consuming, heat-labile, and cytosolic factor (23, 153, 180, 315, 353, 403, 406, 410), and the involvement of ubiquitin-conjugating enzymes located in the cytosol (23, 153, 180, 315, 353, 403, 406, 410) led to the surprising conclusion that disposal of misfolded proteins synthesized in the ER lumen requires retro-translocation across the ER membrane into the cytosol.

Dislocation of aberrant proteins to the cytosol may involve Sec61, a proteinaceous channel that serves as the entry site for nascent polypeptides into the ER (179, 308, 310, 437). Exceptions are known such as substrates that may use the Sec61 homolog Ssh1p in *Saccharomyces cerevisiae* (165) as well as substrates whose retrotranslocation occurs independently of Sec61 (396) or even without involvement of a protein-conducting channel (310a). The derlins and signal peptide peptidase have also been recently implicated as putative components of a retrotranslocon (228, 229, 234, 287, 423, 424). With few exceptions [e.g., the dislocation of class I MHC triggered by the cytomegalovirus (CMV) immunoevasins US2 and US11 (410, 411)] retrotranslocation of ERAD candidates is inhibited upon proteasome inactivation, thus showing that retrotranslocation and degradation are coupled events (53, 222, 241, 249, 260, 264, 396).

4. Protein quality control in the ER: the unfolded versus misfolded conundrum

The ER lumen hosts an estimated 100 mg/ml proteins. Besides resident molecular chaperones and folding factors, thousands of different gene products (most of them displaying *N*-glycans) reside in the ER lumen for the time required to complete the folding program. This time may vary from a few minutes (e.g., ~15 min for HA from the influenza virus) (60) to several hours [for gp160 glycoprotein from the HIV (225) or the blood coagulation protein factors V and VIII (309)]. Proteins that have completed the maturation program are rapidly released from the ER. Therefore, the vast majority of cargo present in the ER lumen is unstructured and/or exposes unfolded or

misfolded determinants that elicit chaperone binding. Complex molecular mechanisms have evolved in eukaryotic cells to distinguish unstructured intermediates of the folding program that have to be preserved from degradation, from terminally misfolded proteins to be removed from the ER lumen. A simplistic view says that one solution selected by evolution to protect unstructured nascent chains synthesized in the ER lumen from unwanted destruction has been the positioning of the multisubunit complex responsible for protein degradation, the 26S proteasome, in the cytoplasm (206). This implies that folding-defective proteins must be actively trapped and extruded from the ER lumen before being exposed to the disposal machinery. It does not answer, however, a central question related to a crucial part of the protein quality control in the ER: how does the quality control machinery distinguish between folding polypeptides to be protected from degradation, native polypeptides to be released from the ER into the secretory line for transport to their final destination, and terminally misfolded proteins to be extracted from the folding machinery and transported into the cytoplasm for proteasome-mediated degradation (83, 256)?

The release of native proteins from the ER is the easiest to envision. Native proteins do not expose motifs (usually hydrophobic patches or unpaired cysteines) that elicit chaperone binding. This is a crucial difference with nonnative polypeptides, for which one mechanism for ER retention relies on association with resident chaperones that carry specific retrieval or retention sequences at their COOH termini that prevent forward transport (luminal KDEL-like sequences for soluble proteins or cytosolic KKXX-like extensions for membrane-bound proteins).

Recent findings show that protein secretion from the mammalian ER is energetically permissive (342) and that ER exit signals may lead to anterograde transport of misfolded polypeptides, at least in yeast (197). In the case of TTR variants (refer to sect. *nBI*), as an example, the ER quality control machinery only prevents forward transport of the most highly destabilized structures but ignores others, disease-associated variants, in spite of compromised folding. This shows that nonnative proteins can induce formation of COPII vesicles at ER exit sites and be transported to the cell surface (127, 342) and may lead to a new definition of the ER quality control that takes into account folding energetics, independent of the acquisition or not of a native state.

Less evident is how the system distinguishes unfolded (defined as a polypeptide on its way to becoming a native molecule) from misfolded (defined as an irreparably unstructured protein to be rapidly removed from the folding compartment) because both conformers expose regions that attract ER-resident molecular chaperones. The regulation of these processes is better known for *N*-glycosylated proteins, and it is widely accepted that the

oligosaccharide appendices and their processing by several ER-resident glucosidases, mannosidases, and one glucosyl-transferase play a crucial regulatory function in this recognition process (141).

5. A timer ticking in the ER lumen

Disposal of folding-defective proteins carrying *N*-glycans is one of the most thoroughly investigated aspects of protein degradation from the mammalian ER. It has been recently shown that protein degradation from the ER can start as soon as the ERAD candidate emerges in the lumen of the compartment when special "degrons" are displayed (381) or under conditions of ER overload (297). These are exceptions, rather than the rule. Normally, newly synthesized polypeptides are afforded a fair time window to explore conformational states that may eventually lead to the native structure. For proteins that will eventually be degraded, this is seen as a lag phase before degradation onset (268a). During the lag phase, *N*-glycosylated ERAD candidates remain trapped in the calnexin/calreticulin chaperone system and undergo formation and/or isomerization and/or reduction of intra- and intermolecular disulfide bonds in hopeless attempts to reach a conformation that fulfills ER quality control standards (264) (Fig. 2, steps 3a and 4a). A long-term retention of polypeptides in futile cycles of folding attempts would eventually interfere with the maturation of the vast array of newly synthesized polypeptides that enter the ER lumen at any given time. Changes in the mannose composition of the polypeptide-bound *N*-glycans are the key that determines the fate of the polypeptide. Removal of terminal mannoses could inhibit binding to calnexin and calreticulin and facilitate the association of folding-defective glycoproteins with ER-resident mannose-binding lectins and with BiP (262, 286) (Fig. 2, step 3b). Consistently, substrate cycling in the calnexin chaperone system and lag phase preceding degradation onset are prolonged by preservation of the Man₉ configuration (69, 264, 420). The finding that inhibition of mannose removal from *N*-glycans protects folding-defective polypeptides from ERAD (359) led to the concept of mannose timer (41, 144), proposing that progressive protein demannosylation terminates the maturation phase and initiates a series of events, still incompletely characterized, that eventually lead to retrotranslocation of the terminally misfolded polypeptide into the cytosol for ERAD (Fig. 2, steps 4b).

6. Protein demannosylation in the ER lumen

Accumulating evidence highlights the crucial role of kifunensine-sensitive α 1,2-mannosidase(s) in timing glycoprotein degradation from the ER (41, 141, 145, 148, 219, 268a, 291a). In *S. cerevisiae*, removal of a single mannose residue from the central branch B of the oligosaccharide displayed by folding-defective polypeptides (Fig. 1) ini-

tiates a series of events eventually leading to retrotranslocation and disposal. The glycanase involved in removal of this specific mannose residue is the MnsI (174). The corresponding mammalian enzyme, the ER α -mannosidase I, shows the same specificity for the mannose residue B (273).

It appears unlikely that the strength of the signal obtained by removal of the terminal B branch mannose residue is the same in the yeast and in the mammalian cell. In higher eukaryotes, in fact, substrate cycling in the calnexin chaperone system offers good protection from disposal, and removal of mannose B is not sufficient to abolish it (141, 219, 274). Irreversible extraction of folding-defective glycopolypeptides from the calnexin system is only obtained upon removal of the mannose on branch A, the sole residue of the protein-bound oligosaccharide that can be reglucosylated by the GT1. Consistently, it has been shown that extensive *N*-glycan processing to Man₅₋₆ configurations precedes or elicits disposal from the mammalian ER (87, 99–101, 159, 198). Moreover, removal of α 1,2-linked mannose is required for degradation of misfolded proteins expressed in mannosyl-phosphoryl-dolichol-deficient cell lines [e.g., B3F7 and MadIA214 (87, 290)] that do not have a cleavable mannose on branch B but display a cleavable terminal mannose on branch A (Fig. 1).

Other advantages of extensive demannosylation are that oligosaccharides lacking branch A cannot bind to ERGIC53/UIP36 cargo receptors (Fig. 2) and that the overall polypeptide volume is reduced, thus facilitating the transit of ERAD candidates across the ER membrane through the proteinaceous channel. The identity of the glycanase(s) that perform the extensive mannose trimming observed during preparation of the folding-defective polypeptides for proteasomal disposal is still a matter of debate and extensive research because data show that the ER α -mannosidase I can proceed with removal of mannoses on branches A and C only at unphysiological condi-

tions (149). At least three possibilities exist: 1) the ERAD substrate and ER α -mannosidase I are segregated to a specialized subregion of the ER (101, 164) where the mannosidase concentration reaches levels similar to those shown *in vitro* to cause extensive mannose removal (149, 420), 2) intervention of Golgi endomannosidase(s) that cleaves A branch mannoses, or 3) intervention of a new class of recently characterized mannosidase-like proteins, EDEM1, EDEM2, and EDEM3 (155, 246, 262, 286, 290, 291, 291a).

7. The EDEM triad as ERAD regulator

The glycosylhydrolase family 47 (GH47; Refs. 147, 274, 291a; Fig. 5) comprises three subfamilies including the ER α 1,2-mannosidase I (ERManI), three Golgi α 1,2-mannosidases (GolgiManIA, IB, and IC), and three EDEM proteins [EDEM1, for ER degradation enhancing α -mannosidase-like protein (160), EDEM2, and EDEM3 (246, 291)]. EDEM proteins are major targets of the ER-stress-induced Ire1/Xbp1 pathway (155, 291, 427). Mammalian cells use this pathway to enhance their capacity for ERAD in response to an increase in cargo load and/or accumulation of misfolded polypeptides (291, 427, 428). RNA interference directed against EDEM proteins (119, 262), and inactivation of the Ire1/Xbp1 pathway that regulates their intraluminal level (427), both reduce ERAD efficiency. Suboptimal ERAD activity eventually inhibits protein folding and reduces secretory capacity, thus revealing important cross-talk between the folding and ERAD pathways (86, 427).

EDEM2 and EDEM3 are soluble ER proteins. The EDEM1 topology remains controversial, as it was initially described as a type II membrane protein anchored at the ER membrane by its uncleaved signal sequence, which was suggested to be required for formation of a functional complex with calnexin (286). More recent reports show that EDEM1 actually forms functional complexes with

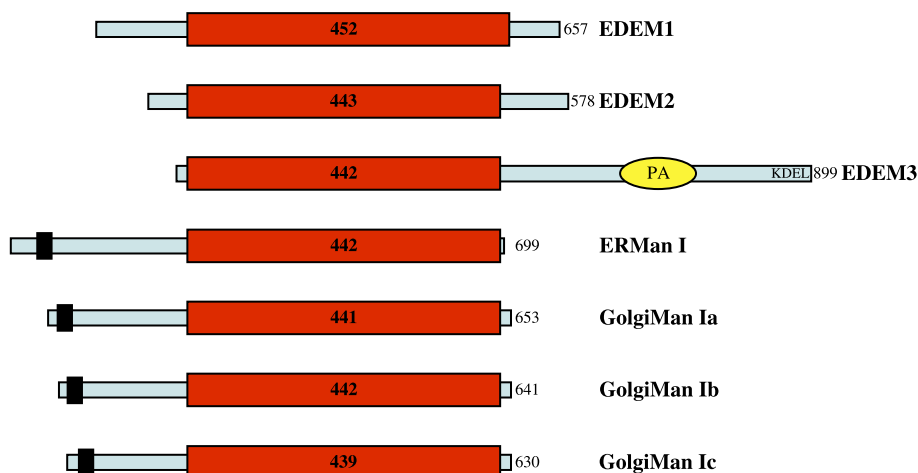


FIG. 5. The members of the glycosyl hydrolase 47 family. Members of this family cleave terminal α 1,2-mannoses (refer to Fig. 1). Numbers show the length of the mannosidase homology domain (red box) and the length of the proteins, respectively. EDEMs are soluble proteins. Only EDEM3 contains a conventional ER-retrieval sequence (KDEL) and a protease-associated (PA) domain. ER and Golgi mannosidases are type II membrane proteins (the membrane anchor is shown as a black box).

derlins (287) and that its signal sequence is efficiently removed, thus releasing it from the ER membrane, as also predicted by computational algorithms (291, 438).

How upregulation of EDEM proteins actually facilitates the degradation of folding-defective glycopolypeptides (262, 286) is not fully understood. Recent evidence suggests that EDEM1 (290) and EDEM3 (155) levels determine the rate of ERAD substrate demannosylation (see below). EDEMs may also work as classical chaperones by preventing the formation of disulfide-bonded dimers (161) or covalent aggregates (290) containing terminally misfolded glycoproteins released from calnexin. Inhibition of aggregation seems essential to facilitate disposal of misfolded glycoproteins released from calnexin. Current models claim, in fact, that dislocation across the ER membrane occurs through a narrow proteinaceous channel, even though one report shows that in the US2/US11-modified cells unfolding of the ERAD candidate is not required for retrotranslocation (94, 310a, 370). The chaperone-like activity of EDEM is independent from the capacity to accelerate substrate demannosylation. In fact, overexpression of inactive mutants of EDEM1 still inhibit protein aggregation enhancing substrate degradation (290).

Despite conservation of the $(\alpha\alpha)_7$ barrel catalytic domain of class I mannosidases, EDEM1 was originally described as a putative lectin, rather than an active mannosidase because it lacks a specific disulfide bond conserved in mannosidases (160). However, it was uncovered more recently that the disulfide absent in the EDEM proteins is not conserved among all mannosidases and is, in any case, dispensable for glycanase activity (274). Moreover, despite the relatively low level of sequence identity (35%), EDEM proteins conserve all catalytic residues required for glycolytic activity and for binding of the specific inhibitor of $\alpha 1,2$ mannosidases kifunensine, and structural modeling indicates no difference in their location (160, 189, 190, 368). Interestingly, increase of the intraluminal level of EDEM1 (290) and EDEM3 (155) substantially accelerates demannosylation of folding-defective polypeptides. Although the formal proof of enzymatic activity assessed with purified components *in vitro* is still missing, for both EDEM1 (290) and EDEM3 (155), substitution of a conserved catalytic residue (E220Q and E147Q for EDEM1 and -3, respectively) abolished enhancement of substrate demannosylation upon elevation of the intraluminal level. Acceleration of mannose removal upon upregulation of EDEM1 also occurs in B3F7 cells, which are characterized by the addition of aberrant oligosaccharides to nascent polypeptide chains that lack mannoses on both their B and C branches (Fig. 1) (290). Thus EDEM1 (and possibly EDEM2 and EDEM3) enhances removal of the terminal branch A mannose, the only cleavable terminal mannose present in glycoproteins in this cell line and the only saccharide that can be reglucosylated by GT1 to prolong retention of folding-defective polypeptides in the calnexin cycle (290).

8. Cytosolic lectins prepare ERAD candidates for destruction

N-glycans serve as degradation tags even after release of ERAD candidates into the cytosol. Most misfolded proteins in the cytoplasm including those arriving from the ER are decorated with polyubiquitin chains to promote degradation by the 26S proteasome. Polyubiquitylation occurs by the concerted action of activating E1, conjugating E2, and ligating E3 enzymes located in the cytosol (150). Several E3 variants exist, and one of the best characterized E3 complexes, the SCF (for Skp1, Cull1, Roc1), contains a Fbs1 (F-box sugar recognition) protein in the adult brain and testis or a more ubiquitously expressed Fbs2 protein that confers specificity for glycosylated proteins arriving in the cytosol from the ER (429, 430). Interestingly, Fbs1 can independently act as a monomer or as a Fbs1-Skp1 heterodimer to facilitate degradation of misfolded glycoproteins by acting as a mannose-binding lectin chaperone that suppresses aggregate formation (431). This dual function of the cytosolic Fbs1 (polyubiquitylation + lectin) mirrors the dual function of EDEM family members in the ER lumen. EDEM proteins, in fact, facilitate disposal of glycoproteins from the ER operating in two independent ways, namely, accelerating mannose removal and preventing aggregation of terminally misfolded glycoproteins released from calnexin (161, 290). *N*-glycans are eventually removed from ERAD candidates by cytosolic PNG1 (156, 362). While the removal of glycans facilitates degradation by the proteasome, it is not absolutely required (260).

9. Viral gene products to assess mechanisms of ERAD

A long-lasting coevolution with the hosts led viruses to learn how to make good use of several cellular mechanisms. As described in the previous sections, several model viral proteins have been employed to unravel different aspects of cellular protein biogenesis. An increasing amount of data are now available on how viruses co-opt the ERAD machinery. As an example, the US2 and US11 gene products of the CMV are localized to the ER lumen of infected cells and trigger the rapid retro-translocation into the cytosol and destruction by the proteasome of class I MHC molecules (410, 411). This prevents cell surface expression of viral antigens that could activate immunosurveillance by the host cell (124). Cells expressing US2 and US11 have been used to identify several membrane-bound and cytosolic components involved in mammalian ERAD. Sec61 (410, 411) and/or Derlin1 and Derlin2 (228, 424) have been identified as potential components of proteinaceous channel(s) proposed to be used for retro-translocation of ERAD substrates from the ER lumen into the cytosol. The signal peptide peptidase also appears to be an essential component (with unknown function) of US2 (but not US11) class I MHC

retrotranslocation (234), and SellL (an ortholog of the yeast Hrd3p) is involved in the same process catalyzed by US11 (but not US2) (275). Finally, VIMP, a transmembrane protein that recruits the p97/Cdc48 AAA ATPase and ubiquitin ligases, acts as part of the extraction machinery complex engaged by the heavy chain for degradation (181, 229, 422, 423).

Class I MHC destruction triggered by US2 differs mechanistically from destruction of the same substrate triggered by US11 (105, 138, 228, 234), and both have similarities, as well as striking differences with ERAD of misfolded cellular glycoproteins. As an example, they completely bypass the ER lectins and ER-resident sugar-processing enzymes that play essential roles in substrate selection for protein quality control in noninfected cells. In addition, they apparently do not require unfolding of the ERAD candidate for retrotranslocation (94, 310a, 370). They also are able to uncouple retrotranslocation and degradation, two tightly coupled events for most cellular substrates that normally accumulate in the ER lumen upon proteasome inactivation (53, 222, 241, 249, 260, 264, 396). It still remains unclear to which extent the mechanisms activated by the CMV immunoevasins contribute to the degradation of cellular proteins in noninfected cells, but it is evident that one of the challenges for the future is to understand and characterize all cellular component regulating disposal of folding-defective polypeptides.

10. Alternative degradation pathways

The disposal of misfolded proteins from the ER lumen is normally delayed and not fully prevented upon inhibition of the 26S proteasome. Surrogate degradative systems appear to intervene in the absence of proteasome activity. One example is the giant protease tripeptidyl peptidase II (TP-II), which shows enhanced activity in proteasome-inhibitor adapted cells (111, 117). In addition, recent data suggest that at least upon acute nutrient deprivation for some misfolded proteins produced in the ER lumen [e.g., the mutant α 1-antitrypsin Z protein (186, 211) and fibrinogen (212)], disposal could involve a completely distinct mechanism named autophagocytosis or autophagy (321). This is a major intracellular degradation pathway characterized by bulk sequestration of cytoplasmic constituents within a double-membrane-bound vesicle and subsequent fusion with lysosomes (202). It has been suggested that autophagic degradation may be important for clearance of ubiquitylated protein aggregates (204), thus of aberrant species that are not attacked by proteasomes and may actually cause their inactivation (20). Certainly, autophagy maintains cellular homeostasis, and its inactivation results in intracellular accumulation of protein aggregates leading to neurodegeneration even in the absence of mutant or folding-defective proteins (135).

As a final consideration, in some cases, aberrant substrates may form detergent-insoluble deposits, thus escaping immunoisolation and detection upon proteasome inhibition (363). This can be, and has been, erroneously interpreted as degradation of the substrate performed by alternative ER-resident or cytosolic degradation machineries that cannot be inhibited by conventional protease inhibitor cocktails.

11. ERAD in yeast compared with higher eukaryotes

Many aspects of protein disposal from the yeast ER are conserved in metazoans. In both systems, most folding-incompetent polypeptides must be extracted from the folding machinery and cross the ER membrane to be degraded by cytosolic proteasomes. Due to the facility of manipulating the yeast genome (418), many aspects of ERAD and several factors regulating the processes have been discovered in the yeast *S. cerevisiae*. For some of them, e.g., the recently identified ER lectin Yos9p, which is part of the multimeric Hrd1p complex comprising Kar2p (the yeast BiP) and several membrane-embedded and cytosolic proteins involved in retrotranslocation and ubiquitination of ERAD substrates (reviewed in Refs. 172, 418), functional mammalian homologs have not yet been identified. Also, the specific machineries involved in the detection of structural protein defects [the ERAD-L (or Hrd1p complex) to inspect luminal defects, the ERAD-C (or Doa10p complex) for cytosolic ones, and the ERAD-M for structural defects in the transmembrane regions of newly synthesized polypeptides] have been characterized in yeast (1, 46, 71, 109, 165, 172, 283, 388) but are poorly known in higher eukaryotes. Whereas ERAD-M and ERAD-C substrates are proposed to be targeted directly for retrotranslocation, ERAD-L substrates in yeast first traffic to the Golgi before returning to the ER for subsequent retrotranslocation and proteasomal degradation (388), a trafficking pathway that has no clear counterpart in mammalian ERAD.

Significantly, even homologous proteins may participate in processes that are mechanistically different in yeast and mammalian quality control. In mammalian cells, a "hands off" of folding-defective polypeptides from calnexin to the BiP chaperone system has been shown, and substrate release from the calnexin/calreticulin cycle initiates the degradation process (18, 40, 69, 76, 184, 203, 263–265, 354, 363, 420). Although several components of the lectin chaperone system (chaperones and sugar-processing enzymes) have ortholog proteins in yeast, the differences between the two systems are striking (also refer to sect. 11C6). To summarize some of them, 1) calreticulin is absent from *S. cerevisiae* and calnexin lacks a cytosolic tail; therefore, it is unable to directly interact with cytosolic components. 2) Calnexin (250) and glucose trimming (157, 175) are required for ERAD in lower eu-

karyotes, whilst in mammalian cells, both calnexin and calreticulin are dispensable for ERAD and substrate association with them actually delays disposal. Moreover, it is undisputed that inhibition of glucose removal that results in a bypass of the calnexin cycle substantially accelerates onset of protein degradation in mammalian cells (14, 52, 69, 139, 191, 192, 264, 270). 3) *S. cerevisiae* lacks a functional homolog of GT1 (92) that in mammals prolongs retention of folding-defective polypeptides in the protective calnexin/ERp57 folding cage. Interestingly, *Schizosaccharomyces pombe* possesses GT so these processes can also vary among the various yeast strains (91). 4) The mammalian ER contains at least four members of the glycosyl hydrolase 47 family (ER α -mannosidase I, EDEM1, EDEM2, and EDEM3) and supports extensive demannosylation of folding-defective polypeptides. The yeast ER only contains orthologs of the ER α -mannosidase I and of EDEM1, and removal of a single mannose from branch B seems to be sufficient to elicit polypeptide removal from the ER lumen.

III. CONFORMATIONAL AND ENDOPLASMIC RETICULUM STORAGE DISEASES

A. Human Diseases Caused by Defective Protein Folding or Trafficking: Selected Examples

Secretory and membrane proteins begin their maturation in the ER where they fold, oligomerize, and are

frequently subjected to covalent modifications to become biologically active. To exit the ER and be transported to their site of activity, newly synthesized proteins must pass a tightly controlled quality control test and some proteins must expose cytosolic and luminal signals for forward transport (83). Proteins that fail to do so are rerouted to the cytosol for degradation. It is important to understand the molecular mechanisms that coordinate protein synthesis, folding, transport and degradation because unbalances in these processes are at the basis of many human diseases (Tables 5–7) (thoroughly reviewed in Refs. 10–12, 268a).

Conformational disorders are often familial because mutations in the polypeptide sequences may strongly affect the folding efficiency. They may lead to loss-of-function conditions, in which a membrane or secreted protein is retained and subsequently degraded. Examples are cystic fibrosis, familial hypercholesterolemia, diabetes mellitus, osteogenesis imperfecta, and retinitis pigmentosa (Table 5). However, there are also many examples of gain of toxic functions. If disposal is not efficient, aberrant proteins accumulate in or outside cells and may initiate unfolded protein responses eventually leading to cell death and triggering severe damages to tissues and organs (Table 6). An interesting case is the pathology caused by α 1-antitrypsin mutation. α 1-Antitrypsin is the principal blood-borne inhibitor of the destructive neutrophil elastase in the lungs. Mutated α 1-antitrypsin is not secreted from liver cells and actually accumulates forming intra-

TABLE 5. Diseases caused by protein misfolding resulting in disposal (loss of function)

Protein	Disease	Reference Nos.
α 1-Antitrypsin	Hereditary lung emphysema	230, 315
α -D-Galactosidase	Fabry disease	289
ABCA1 transporter	Tangier disease	280
β -Glucocerebrosidase	Gaucher disease	326, 334
β -Hexosaminidase	Tay-Sachs disease	216
β -Secretase (splice variants)	Alzheimer's disease	262, 264
Capillary morphogenesis factor-2	Infantile systemic hyalinosis	134
CD4	HIV1 infection	413
Class 1 MHC heavy chain	Infantile CMV-linked hepatitis	384, 410
CLD anion transporter	Congenital chloride diarrhea	89
Complement C1 inhibitor	Hereditary angioedema	391
Cystic fibrosis transmembrane regulator	Cystic fibrosis, recurrent nasal polyps, congenital bilateral absence of vas deferens, idiopathic pancreatitis	205, 387
DTDST anion transporter	Diastrophic dysplasia	89
Gonadotropin-releasing hormone receptor	Hypogonadotropic hypogonadism	177
Growth hormone receptor	Laron syndrome	416
HFE	Autosomal recessive hereditary hemochromatosis	19, 125
Insulin receptor	Diabetes mellitus, insulin-resistant syndrome	169
Low-density lipoprotein receptor	Familial hypercholesterolemia	372
Myeloperoxidase	Myeloperoxidase deficiency	70
Pendrin	Pendred syndrome	89
Polycystin-2	Polycystic kidney disease 2	208
Protein C	Venous thromboembolism	371
Tyrosinase	Oculocutaneous albinism, amelanotic melanoma	31, 129
von Willebrand factor	Bleeding disorders	236
Voltage-gated potassium channel	Congenital long QT syndrome	119
21-Hydroxylase	Congenital adrenal hyperplasia	28

TABLE 6. Diseases caused by protein misfolding causing retention/deposition (gain of toxic function and/or loss of function)

Protein	Disease	Reference Nos.
α 1-Antitrypsin	Liver failure, cirrhosis	230, 315
α -Synuclein	Parkinson's disease	59
Aquaporin-2	Autosomal nephrogenic diabetes insipidus	244, 366
Arginine vasopressin	Familial neurohypophysial diabetes insipidus	55
Collagen type I-IV	Osteogenesis imperfecta, Ehlers-Danlos syndrome, idiopathic osteoporosis, Caffey disease	112, 215
Connexin	Charcot-Marie-Tooth syndrome	386
Copper transporter	Menkes disease	195
Fibrillin-1	Marfan syndrome	408
Fibrinogen	Liver failure	33
Granulocyte colony-stimulating factor	Severe congenital neutropenia	81
HERG potassium channel	Hereditary long QT syndrome	193
HMG-CoA reductase	Heart failure	115
Immunoglobulin chains	Heavy chain disease	82, 348, 415
Lipoprotein lipase	Familial chylomicronemia	38
Pael receptor	Autosomal recessive juvenile Parkinsonism	168
Neuroigin-3	X-linked autism, Asperger syndrome	66
Parathyroid hormone-related peptide	Hypercalcemia	252
Peripheral myelin protein 22	Charcot-Marie-Tooth syndrome	276
Prepro-vasopressin	Diabetes insipidus	173
Proteolipid protein	Pelizaeus-Merzbacher leukodystrophy	122
RET protooncogene	Hirschsprung disease, central hypoventilation syndrome	199
Rhodopsin	Autosomal dominant retinitis pigmentosa	332
Sedlin	Spondylo-epiphyseal displasia tarda	110
Several (presenilin, hungtingtin, PrP,...)	Neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's, Creutzfeld-Jakob)	329
TorsinA	Dystonia, myoclonic-dystonia syndrome	214
Thyroglobulin	Congenital hypothyroid goiter	251
Wilson disease protein	Wilson disease	303

cellular deposits. The loss-of-function phenotype observed at the level of patient's lungs (emphysema, Table 5) is therefore accompanied by a gain-of-toxic-function phenotype at the level of the liver (liver cirrhosis, Table 6) (304).

Diseases can also be caused by defects in the cellular machinery that aids in protein biosynthesis or that regulates disposal of folding defective polypeptides (Table 7). ERGIC53 is a carbohydrate-binding sorting receptor that cycles between the ER and ER-Golgi-intermediate compartment (ERGIC). It packages properly folded proteins into COPII vesicles for anterograde trafficking out of the ER by binding to their high-mannose side chains. Mutations in ERGIC53 disrupt the trafficking out of the ER of

the blood coagulation factors V and VIII, thereby causing bleeding disorders (281).

Sil1/BAP is a nucleotide exchange factor that regulates the activity of BiP. Mutations or truncations that affect Sil1 association with BiP cause Marinesco-Sjoegren syndrome, which is characterized by cerebellar atrophy with ataxia, cataracts, mental retardation, and myopathy (8, 343). Loss of Sil1 function dramatically reduces BiP activity. BiP is essential for cell viability, and it is therefore somewhat surprising that only certain organs, and the cerebellum in particular, are affected by Sil1 mutations. It has been postulated that GRP170 might functionally replace the mutate Sil1 protein in most organs (405), as shown for Lsh1p (358), the yeast GRP170 ortholog.

TABLE 7. Diseases caused by mutation/overexpression of ER/cytosolic factors involved in biogenesis/degradation of proteins expressed in the ER

Protein	Disease	Reference Nos.
ERGIC53	Combined factors V/VIII deficiency, bleeding disorder	281
Glucosidase I	Hypotonia and dysmorphism	67
Glucosidase II, β -subunit/hepatocystin	Polycystic liver disease	78
p97/Cdc48/VCP	Inclusion body myopathy, Paget's disease of the bone, and frontotemporal dementia	404
Sec62	Prostate and colorectal cancer	88, 183
Sec63	Polycystic liver disease, small bowel cancer	64
Sil1/BAP	Marinesco-Sjoegren syndrome	8, 343

Inclusion body myopathies are associated with severe weakness caused by muscle cells accumulating cytoplasmic aggregates. In the case of Paget's disease, intracellular accumulation of aggregates is caused by missense mutations of the p97/Cdc48/VCP gene (404) that affect the capacity of cells to degrade misfolded proteins, especially those delivered into the cytosol from the ER lumen. p97 is in fact one coordinator of a complex machinery comprising ubiquitin ligases gp78 and Ufd2, deubiquitinating enzymes VCIP135, and ataxin3 that regulates access of misfolded proteins to the proteasome chambers (404).

The polycystic liver disease is the last selected example presented here. This is an interesting pathological state, in most cases asymptomatic, but that can degenerate in sudden abdominal pain upon cystic rupture. Polycystic liver disease is caused by aberrant cotranslational protein processing associated with mutations in the Sec63 translocon and in the regulatory (β -subunit) of the glucosidase II (79). All pathogenic variants of the glucosidase II β -subunit result in premature termination of translation and loss of the polypeptide COOH-terminal containing the ER retention motif and the sequences required for formation of the functional heterodimeric complex with the glucosidase II α -subunit.

A variety of congenital defects are associated with ER storage diseases caused by protein misfolding in the ER. The source of these mutations can be found in the secretory cargo leading to its degradation or creation of a toxic aberrant by-product that disrupts the strictly regulated ER environment. Alternatively, defects in the secretory machinery can also adversely affect the cargo that is most dependent on their activity for proper maturation. In the end, both types of defects result in the destruction of misfolded proteins supporting the loss of their cellular activity or their accumulation in the ER as a toxic end product.

B. Pharmacological and Chemical Chaperones to Rescue Structurally Defective, Functional Proteins

Several inherited human diseases are caused by mutations in the sequence of specific proteins leading to folding defects that in several cases do not affect the activity of the protein. Rather, they significantly slow the polypeptide folding and result in a disposal of the mutated polypeptide chain which is elicited before acquisition of the native, transport-competent architecture (268a). This premature destruction causes the debilitating loss-of-function syndromes. Examples of this category of diseases include cystic fibrosis, Fabry disease, and nephrogenic diabetes insipidus (Table 5). In these cases, therapeutic approaches based on the use of chemical or

pharmacological chaperones (21, 268a) can be envisioned, that promote and/or accelerate productive folding and inhibit ER retention, thus facilitating the transport of the polypeptide to its site of action.

One indication that the folding defect of the mutant protein may be reverted by the use of chemical chaperones is the increase of the polypeptide folding efficiency upon expression at lower temperature (72, 195). Another is the increase in the export of the select functional polypeptide from the ER upon inhibition of ERAD (392) as the folding and degradation processes are for many proteins in kinetic competition (268a).

Chemical compounds with proven effect in the stabilization of mutant proteins and/or in the facilitation of transport of mutant proteins at the site of activity include 4-phenyl butyrate (PBA), glycerol, trimethylamine *N*-oxide, dimethyl sulfoxide, deuterated water (35), and derivatives of bile acids such as ursodeoxycholic acid (298, 421). Chemical chaperones improve folding capacity by nonspecific mechanisms and require high dosages. Therefore, they often are of limited therapeutic value. However, there is at least one example of a chemical chaperone, PBA, that has been approved by the United States Food and Drug Administration for clinical use. In animal models for type 2 diabetes mellitus (298) or for α 1-antitrypsin deficiency (37), PBA has been shown to alleviate the disease state. The mechanism of action of PBA is unclear, but its capacity to alleviate ER stress and facilitate secretion of the disease-related, mutated proteins may be related to its activity as a chaperone that efficiently inhibits protein aggregation (213). In any case, studies with chemical chaperones offered an important proof of principle and paved the way for characterization of several, substrate-specific pharmacological chaperones that proved efficient in cultured cells or that have already been used in animal and/or clinical trials (22, 58, 232, 271). Here are a few selected examples: V2 receptor antagonists rescued mutant vasopressin receptor from proteasomal degradation (272); gonadotropin-releasing hormone (GnRH) peptidomimetics rescued secretion of several GnRH mutants (177); the chromophore retinal rescued maturation of mutant opsin (285); specific inhibitors facilitated maturation and transport of mutant voltage-gated potassium channels (93), beta-glucocerebrosidase (334), and α -D-galactosidase (90); copper supplementation alleviated maturation defects of the mutant copper transporting ATPase (195); and 4-phenylbutyrate corrected folding defects of several cystic fibrosis channel mutations (434).

IV. CONCLUDING REMARKS

The ER contains a variety of dedicated chaperone systems, protein modifiers, and protein processors that aid in the maturation of the large number of proteins that

traverse the eukaryotic secretory pathway. In recent years, our understanding of the protein maturation process in the ER has become much clearer. Studies have provided evidence for the necessity and the extensiveness of the initial cotranslational and cotranslocational events. During its time of translation, a protein has already been seen and worked on by some 30 polypeptide chains ensuring that the protein gets off to a good start in a tightly regulated translocon-associated environment. This assistance continues posttranslationally until the properly folded protein leaves the ER. A quality control system is also in place that monitors the fidelity of the maturation process, retaining misfolded proteins in the ER and efficiently targeting the terminally misfolded for degradation.

N-linked glycans are employed as both maturation and quality control tags that dictate which proteins should be recruited to interact with the nascent chain. Large advances have been made in translating the glyco-code of the ER or what a given glycan composition means. What does it tell us about the protein's status (folded, unassembled, or terminally misfolded)? What proteins recognize the given glycan composition (chaperones, quality control, or sorting receptors)? What do they do with the glycoprotein once they bind to their specific glycan signal (assist folding or target the protein for destruction)?

Since a source of a large number of human disease states is defects in the protein folding and maturation, a more thorough knowledge of these processes is essential. This will allow us to compare the maturation of normal and aberrant proteins to see where they deviate and possibly provide insight into how they can be rerouted to stay on the normal pathway.

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