ELSEVIER

Protein folding and quality control in the endoplasmic reticulum

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The endoplasmic reticulum (ER) is a highly versatile protein factory that is equipped with chaperones and folding enzymes essential for protein folding. ER quality control guided by these chaperones is essential for life. Whereas correctly folded proteins are exported from the ER, misfolded proteins are retained and selectively degraded. At least two main chaperone classes, BiP and calnexin/calreticulin, are active in ER quality control. Folding factors usually are found in complexes. Recent work emphasises more than ever that chaperones act in concert with co-factors and with each other.

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Abbreviations

CNX	calnexin
CRT	calreticulin
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FRET	fluorescence resonance energy transfer
HA	hemagglutinin
LDL-R	LDL receptor
PDI	protein disulfide isomerase
QC	quality control
UGGT	UDP-glucose:glycoprotein glucosyltransferase

Introduction

The endoplasmic reticulum (ER) has many functions, including lipid donation to other organelles (reviewed by van Meer and Sprong in this issue), Ca^{2+} homeostasis [1], biogenesis of organelles [2], protein folding, quality control (QC) [3,4] and protein degradation. Although the native conformation of a protein lies encoded in its primary amino acid sequence, the ER greatly enhances protein folding efficiency [5]. The ER is highly specialised for folding: approximately one-third of all proteins in a eukaryotic cell are translocated into the ER [6]; the ER has unique oxidizing potential that supports disulphide bond formation during protein folding [7•]; and the ER lumen is very crowded, with a protein concentration of >100 mg/ml. In this gel-like protein matrix, chaperones and folding enzymes are abundant, greatly outnumbering the newly synthesised substrates [8]. These folding factors in general prevent aggregation and thereby allow more efficient folding of a large variety of proteins. In this review, we highlight the latest advances in understanding how these chaperones and folding enzymes cooperate in assisting protein folding and mediating quality control.

Co-translational and post-translational folding

Mammalian secretory and membrane proteins are synthesised and translocated into the ER by the ribosome/sec61 translation/translocation machinery, of which various enlightening X-ray structures have recently been determined [9,10•]. During translation/translocation newly synthesised proteins immediately start to fold. Combining these processes allows sequential folding which may greatly enhance folding efficiency, especially of multi-domain proteins [11]. The immunoglobulin molecule with its heavy and light chains undergoes extensive folding and assembly already during synthesis [12]. Another example is the ribosome-bound nascent chain of the Semliki Forest virus capsid protease domain, which was shown to be folded and autoproteolytically active immediately after translocon exit, indicating that folding occurs co-translationally but after translocation [13••]. Other proteins, on the other hand, need extensive post-translational folding to acquire their proper native conformation. Envelope glycoprotein gp160 of HIV-1, for example, is synthesised within approximately five minutes, but resides for hours in the ER with no apparent degradation [14]. The LDL receptor (LDL-R) also folds after synthesis: it collapses into a compact structure with non-native disulphide bonds and then continues to fold into a less compact structure with native disulphide bonds [15]. Both gp160 and the LDL-R need extensive post-translational disulphide isomerisation to fold into the native structure. Thyroglobulin even folds via a high molecular weight complex, which first involves formation of disulphide-linked aggregates that with time unfold and assemble into dimers [16]. Whereas some soluble proteins fold relatively easily, others have more difficulty folding and require more assistance from chaperones and folding enzymes.

Chaperones in complexes

Previous studies that combined chemical cross-linking with immunoprecipitation suggested that chaperones act on newly synthesised proteins in the context of a complex. During folding of the homodimer thyroglobulin (each subunit of which is 330 kD), the Hsp70 chaperone

Chaperone and folding enzyme complexes containing BiP.

Family	Protein	Reference	Related proteins not found in a BiP complex
Hsp90	Grp94	[17–20,21••,30]	
Hsp70/Hsp110	Grp170	[17,21••]	
Lectin	Calreticulin	[18,20]	Calnexin
Lectin	UGGT	[21••]	Calnexin
Co-chaperone	ERdj3	[21••]	ERdj1, ERdj2, ERdj4, ERdj5
Oxidoreductase	PDI	[17,19,21••]	ERp19, ERp44, ERp46, ERp57, TMX, ERdj5
	ERp72	[17,20,21••]	
	CaBP1 (P5)	[21••]	
	Erp29	[19,21••]	
PPIase	Cyclophilin B	[20,21••]	FKBP65

Several laboratories found multimeric chaperone and folding enzyme assemblies that interacted with proteins that fold in the ER. The redox protein family, which rapidly expands, includes important constituents of the BiP complex.

BiP was present in a multimeric complex with Grp94, Grp170 and the redox proteins PDI (protein disulfide isomerase) and ERp72 [17]. The influenza virus hemagglutinin (HA) cross-linked in a 1:1 stoichiometry with the lectin chaperones calnexin (CNX) and calreticulin (CRT), but only with trace amounts of BiP. In the absence of protein synthesis, CRT was found in complex with BiP and Grp94, whereas CNX was not [18]. Several recent studies confirmed that an ensemble of chaperones and folding enzymes act on thryroglobulin [19], on slow folding apolipoprotein B [20] and on unassembled immunoglobulin heavy chain [21••] to assist folding. BiP and Grp94 were always present, whereas additional chaperones and folding enzymes varied (Table 1). The BiP chaperone complex of unassembled immunoglobulin also formed independently of synthesis, suggesting that it is an intrinsic ER chaperone complex [21••].

Large-scale analysis of TAP-tagged proteins in yeast implied that ∼78% of the studied proteins were in multiprotein complexes [22•]. Because chaperones are notoriously 'sticky', they were part of too many of the complexes and were therefore excluded from analysis.

BiP and calnexin: the first to act

Mammalian BiP (Grp78) is one of the most abundant ER chaperones and is closely related to cytosolic Hsp70. Although the recent crystal structure of SecY suggests that the permeability barrier between the cytosol and the ER is a feature of the translocon alone, previous data demonstrated that BiP seals the pore at the ER lumenal side [10•,23]. Because of its location, BiP can immediately interact with the unfolded nascent chain, and hence contribute to the translocation of nascent chains into the ER.

CNX is located near the translocon and can interact with nascent chains of N-glycosylated proteins. A prerequisite for both CNX and CRT binding to newly synthesised glycoproteins is a sequential, initially co-translational, action by α-glucosidases I and II that trim two glucose residues, creating a monoglucosylated glycan. CNX immediately binds to HA when ∼30 residues have entered the ER lumen, suggesting that both glucosidases function in very close proximity to the translocon as well [24].

Immunoglobulin and HA are examples of proteins that exclusively bind BiP or CNX/CRT, respectively, whereas other proteins may need both for folding. When N-glycans were located within the first ∼50 residues of influenza virus HA, CNX interacted with the glycoprotein. When these N-terminal glycans were removed, initial CNX binding was prevented and BiP interacted with the glycoprotein [25]. Which chaperone complex is recruited is determined by the characteristics of the folding protein.

The BiP chaperone complex

How do chaperone complexes assist proteins in folding? Is there a functional physical interaction between chaperones, co-chaperones and folding enzymes? BiP has an ATPase domain and a peptide-binding domain that coordinate repetitive cycles of ATP hydrolysis and ADP exchange, stimulating binding and release of the unfolded protein, respectively [26]. Co-chaperones, as has been shown in detail for cytosolic and bacterial Hsp70s, influence the cycle by modulating ATP hydrolysis (J-proteins) or ADP exchange (e.g. Bag-1). Recently, several J-domain-containing partner proteins for BiP were identified: ERdj 1–5. ERdj3 indeed stimulated ATP hydrolysis of BiP *in vitro* and was found in the BiP complex with unassembled heavy chain *in vivo* [21••,27].

Although a specific nucleotide exchange protein for BiP was identified in the mammalian ER, this protein, BAP, was not found in the BiP complex [28]. Two additional nucleotide exchange factors were found for yeast BiP (Kar2p), not only Sil1p but also Lhs1p, a Grp170 homologue with similarity to Hsp70s and Hsp110. Interestingly, Lhs1p stimulates ADP exchange of Kar2p, whereas Kar2p stimulates ATP hydrolysis of Lhs1p [29••]. This

mutual activation creates an efficient chaperone relay that may initiate sequential action by Kar2p and Lhs1p on the substrate. Mammalian BiP and Grp170 were found in the same complex, which strengthens their cooperativity.

The highly abundant ER chaperone Grp94 (a Hsp90 homologue) binds immunoglobulin during folding after it is released by BiP, participating in another sequential chaperone interaction [30]. Interesting parallels might be found between the elusive ER BiP/Grp94 chaperone complex and the better-defined eukaryotic cytosolic Hsp70/Hsp90 chaperone complex. On the other hand, a recent X-ray structure of the N-terminal Grp94 domain demonstrated conformational differences from Hsp90 [31]. Either the many cofactors that act on Hsp90 have not been identified in the ER yet, or they do not exist. Clearly more research is needed to find out why this protein is so abundant in the ER and what its relation is to ER protein folding in general.

The lectin chaperone complex
With CNX and CRT, the ER has a unique lectin-binding chaperone system that specifically assists in the folding of secretory and membrane glycoproteins [3]. Despite their extensive homology, studies with CRT- and CNX-deficient cell lines showed that their activities and substrate specificities were different [32•]. The distinct phenotypes of mice in which either chaperone was deleted confirmed this difference [33,34]. CRT was found in a complex with BiP and Grp94 whereas CNX was not, although this may reflect the unique membrane association of CNX. On the other hand, we cannot exclude that CNX and CRT function in separate chaperone complexes with distinct functions.

The lectin chaperone ensemble needs accessory proteins including α-glucosidase II, UDP-glucose:glycoprotein glucosyltransferase (UGGT), ERp57 and EDEM (only for CNX) to fold proteins and maintain glycoprotein QC. Some of these were found associated in a protein complex. So far, the accessory factors and activity cycles are indistinguishable for CNX and CRT; they both need assistance of α-glucosidase II as well as UGGT to release and re-bind substrate glycoprotein, respectively [3,35•]. Whereas CNX/CRT merely retains incompletely folded glycoproteins, UGGT functions as the folding sensor. A unique property of UGGT (175 kDa) is that it specifically recognises the innermost GlcNAc moiety on misfolded glycoproteins [36]. Studies on heterodimers consisting of one unfolded RNaseB subunit and one folded RNaseB subunit showed that UGGT specifically 'sensed' and reglucosylated the partially unfolded subunit [37]. With a larger protein containing two Man9-GlcNAc2 glycans, reglucosylation was shown to occur also at a distance from the local disturbance in conformation, although the exact extent of misfolding still needs to be formally determined [38].

Because incompletely folded proteins generally expose hydrophobic stretches, it was no surprise that UGGT, like BiP, has affinity for this type of sequence. UGGT prefers hydrophobic clusters within proteins in a molten globule-like state [39], and reglucosylates hydrophobic glycopeptides of ~20–30 residues [40•]. BiP, however, preferentially interacts with heptameric hydrophobic peptides.

Glycan-independent chaperone function of CNX
Because non-glycosylated proteins frequently bind to CNX and CRT, and because mutations in CNX's lectin site do not disturb this interaction [41], glycan-independent polypeptide binding of the lectin chaperones remains an additional option. Unassembled transmembrane domains of several proteins were found to bind CNX: the CD82 tetraspanin membrane glycoprotein, and an intramembrane point mutant of non-glycosylated proteolipid protein [42,43•]. CNX RNAi knock-down cells displayed slower degradation of the mutant but not the wild-type proteolipid protein [43•]. CNX apparently mediates chaperone function in both the lumen and the membrane of the ER.

Redox proteins
Next to the various chaperones, folding enzymes may be even more important for proper folding. A dominant class are the redox-active proteins, the oxidoreductases, as emphasised by their high number (Table 1). They catalyse thiol redox reactions, leading to disulphide bond formation and isomerization. Next to the well-known abundant oxidoreductases, such as PDI and ERp57, many novel oxidoreductases have been identified over the past few years whose functions and substrates are unknown [19,44]. Database searches, genomics and proteomics screens yielded a wealth of redox enzymes, and we are left to determine the reason for the puzzling number and variety.

Specialised examples are found in the plasma cell, which secretes high quantities of IgM, such that >10^5 disulphide bonds per second are made in these cells (Roberto Sitia, personal communication). During development into a plasma cell, B lymphocytes expand their ER and increase expression of ER chaperones and ER redox enzymes prior to antibody production. These up-regulated ER oxidoreductases, which include ERp44, probably act on IgM J-chain, heavy chains, and light chains, the dominant substrates in plasma cells [45,46••]. Whether these novel redox enzymes are cell-type- and substrate specific, and whether the ordered expansion of ER at times of need applies to other secretory cells, remains to be determined.

Amongst the novel redox proteins recently found, the co-chaperones are especially interesting, as they connect chaperone and folding-enzyme functions. The recently identified ERdj5 protein, which contains a J-domain as

well as four consecutive CXXC thioredoxin-like motifs, might be the 'private' redox protein for BiP just as ERp57 is for CNX and CRT [47].

Both CNX and CRT were found in heterodimeric complexes with the PDI-like redox protein ERp57 [48]. Recent NMR interaction studies showed that ERp57 interacted with the extreme tip of the CNX/CRT P-domain [49] with its *b* and *b'* domains [50,51]. ERp57 mediates oxidative folding of glycoproteins when complexed with CNX or CRT, and its redox capabilities are only slightly different from those of PDI [52]. Although present in different chaperone environments, ERp57 and PDI show strong analogies in function.

Important questions to answer are why we need this plethora of redox proteins in the cell and what will be their individual contribution to disulphide making and breaking reactions in the ER. These redox proteins all accept electrons during formation of disulphide bonds in substrate proteins and donate electrons upon reduction of disulphide bonds. PDI donates its electrons to Ero1, which in turn donates them to molecular oxygen through FAD [7•]. How does the electron flow of all the other redox proteins work? Do these other redox proteins have their own, yet unidentified electron acceptor like Ero1 or can Ero1 also facilitate electron relay of other redox proteins besides PDI?

ER-associated degradation

If, after release from chaperones, a secretory protein is folded correctly, it will exit the ER, possibly via the lectin ERGIC-53 if it is a glycoprotein [35•]. If, on the other hand, a protein cannot fold, even after a prolonged time, it needs to be removed from the folding pathway and targeted for ER-associated degradation (ERAD). The ERAD pathway retrotranslocates (dislocates) misfolded proteins back into the cytosol where proteasomal degradation takes place.

Work first done in yeast demonstrated that both BiP and PDI are involved in ERAD. BiP might keep misfolded proteins in a reversibly aggregated state whereas PDI directly targets misfolded proteins for retrotranslocation

Figure 1
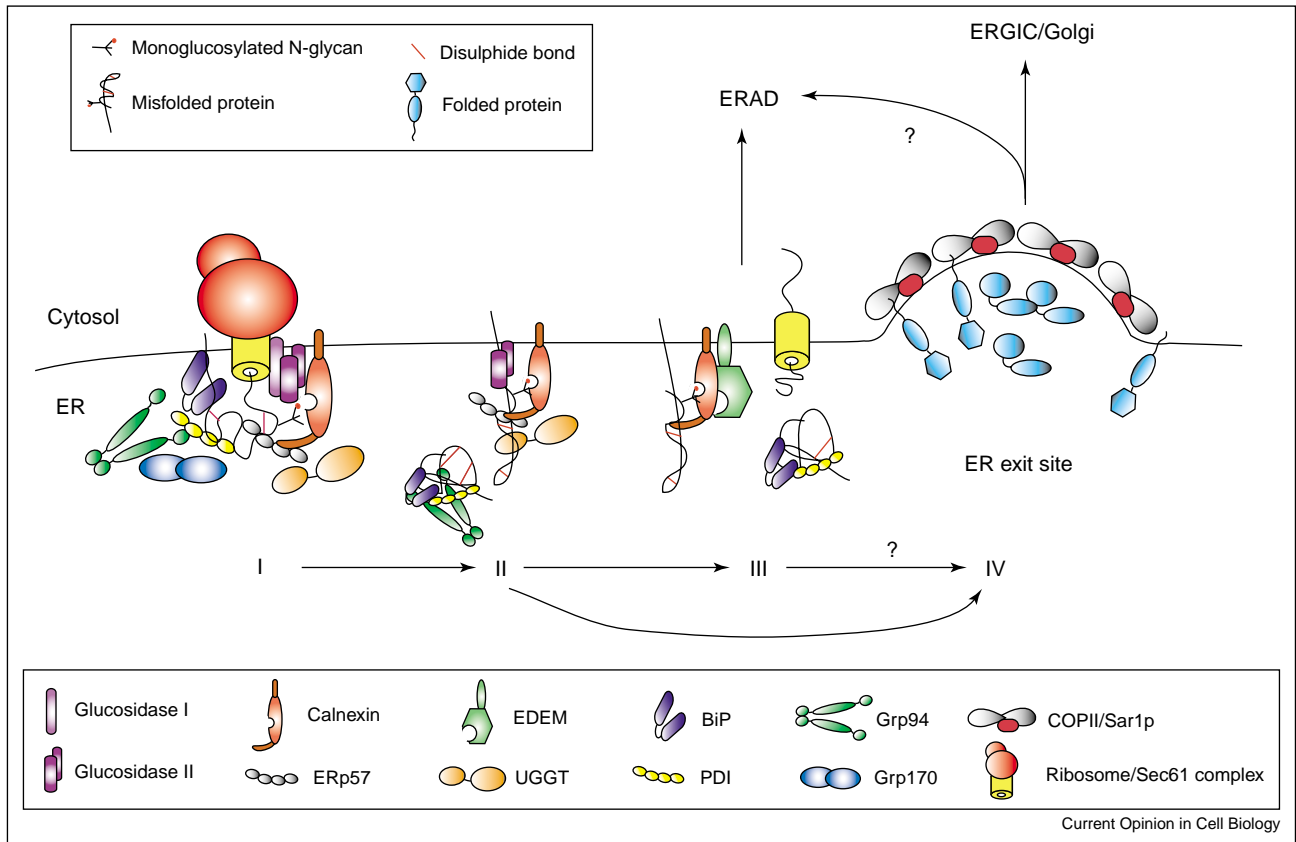
QC by BiP- and CNX-containing complexes follows successive steps. BiP and CNX with their accessory proteins associate with nascent chains as soon as they enter the ER lumen (I), and continue to assist folding after translation (II). When the protein has acquired its correctly folded structure, it is ready for exit from the ER (IV). If the protein fails to reach its native state the chaperones and folding enzymes target and guide the misfolded protein for ERAD (III). For at least some proteins, ER to Golgi traffic is necessary before ERAD, probably via COPII exit sites (IV).

[53]. Recently, Ero1 was found to mediate the release of unfolded cholera toxin bound to PDI before retrotranslocation [54]. In another study mixed disulphide bonds were found between PDI and the membrane glycoprotein BACE457 prior to ERAD [55]. By contrast, PDI was also shown to bind substrate redox-independently [56].

When glycoprotein folding fails, α-mannosidase I will have trimmed one mannose, which will target the protein for degradation through EDEM, another lectin [57]. CNX interacts via its transmembrane domain with EDEM to hand over misfolded α1-antitrypsin and BACE457 [58••,59]. The CNX/EDEM interaction supports an important relay from chaperone to ERAD. Because CRT cannot bind EDEM, more lectins are likely to be found. Moreover, detailed analysis of the oligosaccharide composition of EDEM-bound substrate showed that additional glycoforms predestine a protein for ERAD.

Both BiP and the lectin chaperones are involved in sending misfolded proteins to ERAD. As misfolded BACE457 is substrate for both PDI and EDEM it remains unclear whether both chaperones and their cofactors and redox enzymes work together in this task. As ERp57 closely resembles PDI, this private redox partner of CNX may also function itself in disulphide reduction for retrotranslocation.

Conclusions

The question remains of how the ER decides at the molecular level between the protein folding and ERAD pathways (Figure 1). The kinetics of the folding and degradation pathways may simply differ, as glucosidase activity is suggested to be higher than mannosidase activity. This may favour the folding cycle over ERAD [60]. After multiple rounds of chaperone binding, proteins that inefficiently fold are eventually targeted for degradation. On the other hand, protein folding might be spatially separated from protein degradation by distinct ER subdomains. Two ERAD substrates, asialoglycoprotein receptor H2a and unassembled MHC class I, accumulated before ERAD in ER subcompartments that colocalised with Sec61, CNX and CRT but not with BiP and PDI [61,62]. Genetic evidence in yeast suggests that the Sar1p/COPII machinery is essential in proteasomal sorting [63]. These various observations narrow down the location of the decisions to the ER exit site (reviewed by Watanabe and Riezman in this issue), which then would be involved in the triad decision between protein folding, protein degradation and protein export. Flexible protein complexes are likely to be at the molecular core of this fate-determining process. Exactly how these folding factor complexes work in mammalian cells can now be addressed using siRNA. Folding factors can be knocked-down one by one to study the effect on the complete folding factor network and hence on protein folding and quality control.

Update

Co-translational folding has received increasing appreciation over the last years. Two recent studies by Woolhead *et al.* and Gilbert *et al.* put focus on the ribosome in relation to folding. Fluorescence resonance energy transfer (FRET) showed folding (increasing compactness) of a transmembrane segment inside the ribosomal tunnel [64•]. Comparing density maps of Cryo-EM on translating versus non-translating ribosomes demonstrated occlusion inside the tunnel when ribosome is engaged in protein synthesis of Ig1, Ig2, or GFP [65]. Both laboratories present compelling evidence that newly synthesised proteins may begin to fold inside the ribosome.

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