Essays in Biochemistry (2016) **60** 227–235
DOI: 10.1042/EBC20160003

Protein quality control at the endoplasmic reticulum

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The ER (endoplasmic reticulum) is the protein folding 'factory' of the secretory pathway. Virtually all proteins destined for the plasma membrane, the extracellular space or other secretory compartments undergo folding and maturation within the ER. The ER hosts a unique PQC (protein quality control) system that allows specialized modifications such as glycosylation and disulfide bond formation essential for the correct folding and function of many secretory proteins. It is also the major checkpoint for misfolded or aggregation-prone proteins that may be toxic to the cell or extracellular environment. A failure of this system, due to aging or other factors, has therefore been implicated in a number of serious human diseases. In this article, we discuss several key features of ER PQC that maintain the health of the cellular secretome.

Introduction

Approximately one-third of the cellular proteome is targeted to the ER (endoplasmic reticulum), the entry point to the secretory pathway. The secretory pathway is a network of membrane-bounded organelles that are topologically equivalent to the extracellular space (Figure 1). This 'luminal' environment is distinct from the cytosol with respect to ion concentration and redox conditions. Proteins destined for the plasma membrane, extracellular space or secretory compartments undergo folding and maturation in the ER before transport via a complex anterograde (and retrograde) vesicle system.

As the protein folding 'factory' of the secretory pathway, the ER hosts a specialized PQC (protein quality control) system of molecular chaperones and folding enzymes. The ER PQC machinery allows unique co- and post-translational modifications that do not occur in the cytosol including asparagine-linked glycosylation and disulfide bond formation. These modifications are essential for the correct folding, assembly and function of the majority of secretory proteins that would misfold if targeted to the cytosol. Interactions with molecular chaperones and enzymes therefore assist protein folding, and their release is a key determinant of exit from the ER.

PQC at the ER is the checkpoint for misfolded proteins entering the secretory pathway. Non-native proteins are retained in the ER and, if terminally misfolded, dislocated across the ER membrane for degradation by the cytosolic ubiquitin–proteasome system, a process called ERAD (ER-associated degradation). Downstream secretory compartments generally lack such efficient systems to capture and dispose of potentially toxic proteins. The ER PQC machinery also regulates the UPR (unfolded protein response), a major cell signalling pathway that halts protein biosynthesis and expands ER folding and degradation capacity in response to protein stress.

In this article, we review ER PQC, which integrates diverse functions from protein folding and degradation to transport and cell signalling.

Protein folding at the endoplasmic reticulum

Targeting and translocation

Proteins targeted to the ER need to be translocated across the ER membrane and, in the case of membrane-spanning proteins, integrated into the lipid bilayer. To accomplish this, many ER-targeted proteins have

Received: 14 January 2016
Revised: 30 April 2016
Accepted: 31 May 2016

Version of Record published: 15 October 2016

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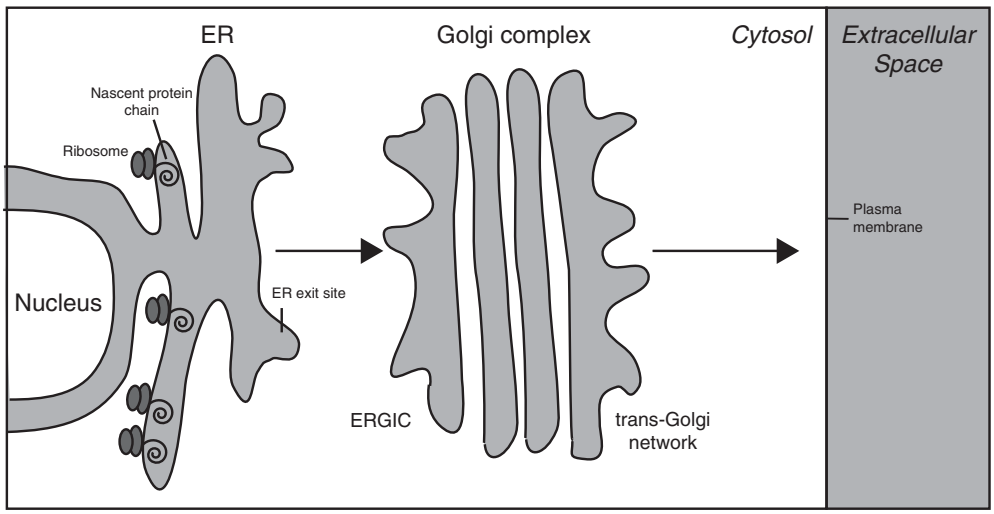

Figure 1. The endoplasmic reticulum is the entry point to the secretory pathway

The secretory pathway is a network of organelles topologically equivalent to the extracellular space. The luminal environment (grey) is distinct from the cytosol (white) and is connected via a complex system of anterograde and retrograde vesicular transport.

N-terminal hydrophobic signal peptides or transmembrane sequences that are co-translationally recognized by the SRP (signal recognition particle) during exit from the ribosome channel (Figure 2a). SRP engagement stalls translation and protects these hydrophobic sequences from aggregation in the aqueous cytosolic environment during targeting of the ribosome-nascent chain complex to the ER membrane. Upon insertion of these hydrophobic motifs into the Sec 61 translocon pore, translation resumes, and chain elongation at the ribosome drives translocation of the remaining polypeptide across the ER membrane. Proteins with signal peptides are cleaved by the signal peptidase to release the N-terminus of the polypeptide from the membrane.

Signal peptide or transmembrane sequences and their topology can be partially predicted on the basis of the hydrophobicity and polarity of a primary amino acid sequence. However, many transmembrane domains do not necessarily conform to canonical targeting or insertion sequences and may even adopt multiple topologies during folding [1]. This suggests that there are additional determinants of membrane integration beyond SRP recognition. Proteins with signal sequences that are not hydrophobic enough to engage SRP can be post-translationally recruited to the ER via the larger Sec 61 translocon complex with Sec 62/63 and Sec 71/72 [2]. In this case, translocation appears to be driven by ATP-dependent interactions with the ER-resident Hsp70 (heat-shock protein 70) BiP (immunoglobulin heavy-chain-binding protein) on the luminal side of the translocon complex. Proteins with C-terminal hydrophobic transmembrane anchors, called tail-anchored proteins, are also post-translationally targeted to the ER by various mechanisms including the TRC40 (TMS recognition/insertion complex 40)/Get3 pathway [3].

Protein folding begins co-translationally within the ribosome channel and therefore the rate of translation, the kinetics of signal peptide cleavage and the integration of transmembrane domains all influence the folding of secretory proteins. In contrast with protein folding in the cytosol, a major challenge for the investigation of protein folding and quality control at the ER has been to biochemically reconstitute this dynamic membrane environment *in vitro*.

The classical chaperones: BiP and Grp94

As the nascent polypeptide exits the translocon, its conformational freedom is greatly increased and folding is predominantly limited by interactions with molecular chaperones and other folding enzymes within the ER lumen.

The ER hosts members of both Hsp70 and Hsp90 families of molecular chaperones. The ER-resident Hsp70 BiP shares a similar domain organization to its cytosolic homologue and binds to hydrophobic or aggregation-prone regions within nascent polypeptides to assist folding via ATP-dependent cycles of binding and release (Figure 2b). ATP hydrolysis is mediated by interactions with diverse co-chaperones from the ER-resident DNAJ/Hsp40 family, called ERdj (ER-resident DNAJ protein) co-chaperones [4]. Re-fuelling of BiP with ATP also requires two ER-resident

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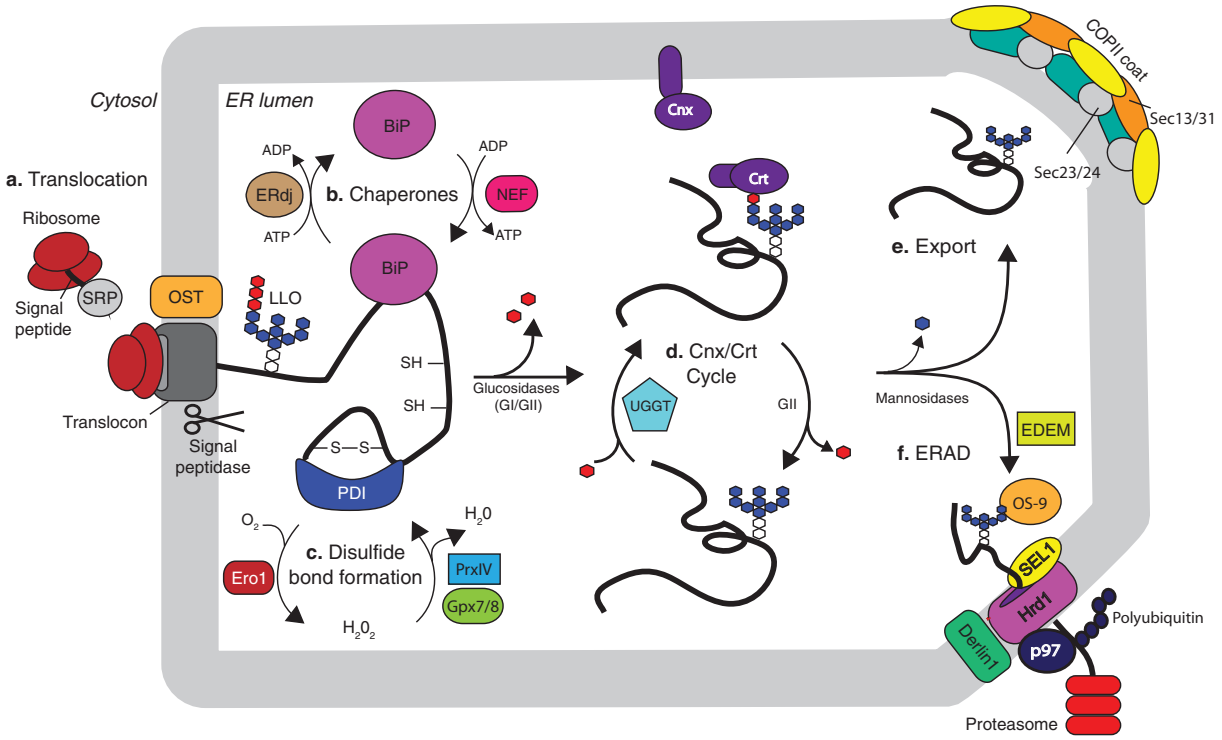

Figure 2. Protein folding and misfolding at the ER

(**a**) Translocation. Targeting and insertion of the signal peptide sequence into the ER translocon is mediated by SRP. OST catalyses the addition of the LLO containing *N*-acetylglucosamine ($GlcNAc_2$; white), mannose (Man_9; blue) and glucose (Glc_3; red). (**b**) Chaperones. BiP binds nascent chains in an ATP-dependent manner assisted ERdjs and NEFs. (**c**) Disulfide-bond formation. PDI oxidizes cysteine residues and is kept oxidized by Ero1 and ER peroxidases PrxIV and Gpx7/8 (glutathione peroxidase 7/8). (**d**) Cnx/Crt cycle. After glucose trimming by GI/GII, the polypeptide is bound by Cnx/Crt lectin chaperones. Trimming of the terminal glucose signals release from the Cnx/Crt cycle. Partially folded proteins are re-glucosylated by UGGT and re-enter the Cnx/Crt cycle. (**e**) Export. Properly folded proteins are released from the ER PQC and packaged into COPII vesicles for anterograde transport to the Golgi. (**f**) ERAD. Terminally misfolded proteins are dislocated across the ER membrane and targeted for degradation by the cytosolic ubiquitin–proteasome system. EDEM, ER degradation-enhancing α-mannosidase-like protein.

NEFs (nucleotide-exchange factors), Sil1 and Grp170 (guanine-nucleotide-releasing protein 170) [3,5]. BiP has been implicated in numerous functions from protein translocation and degradation to regulation of the UPR.

The ER-resident Hsp90 Grp94 is an enigmatic chaperone. It is one of the most abundant proteins in the ER, yet is not essential for mammalian cell viability and only appears to be required for the folding of a select pool of substrates [6]. Grp94 is structurally similar to its cytosolic homologue and mediates ATP hydrolysis; however, it lacks C-terminal motifs critical for Hsp90 interactions with co-chaperones and Hsp70. Grp94 has been reported to associate within large ER PQC complexes including BiP during the folding of nascent polypeptides, although how it mediates these interactions to confer substrate specificity or ATP hydrolysis is not yet known.

Asparagine-linked glycosylation and the calnexin/calreticulin cycle

Attachment of carbohydrate moieties, called glycans, to asparagine within the Asn-Xaa-Ser/Thr consensus sequence motif is an abundant and highly conserved protein modification in eukaryotes. The addition of hydrophilic glycans enhances the intrinsic solubility of the nascent polypeptide during folding, potentially by masking hydrophobic patches, but also allows critical interactions with the ER lectin chaperones [7].

As the nascent polypeptide exits the translocon, the LLO (lipid-linked oligosaccharide) is added co-translationally by OST (oligosaccharyltransferase) (Figure 2a). Glycoproteins then undergo trimming by GI and GII (glucosidases

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I and II), which sequentially remove two terminal glucose residues. The resulting monoglucosylated species is the substrate for the ER lectin chaperones Cnx (calnexin) and its soluble orthologue Crt (calreticulin) (Figure 2d). During folding, the immature glycoprotein is bound by Cnx/Crt as it attempts to obtain its proper structure. GII then removes the terminal glucose residue to disrupt chaperone binding and release the high-mannose glycoprotein for ER export. UGGT (UDP-glucose:glycoprotein glucosyltransferase) recognizes any solvent-accessible hydrophobic patches within partially folded proteins released by GII and catalyses the readdition of glucose to a nearby glycan to facilitate another round in the Cnx/Crt folding cycle. UGGT therefore represents a major checkpoint in the retention or export of glycoproteins [8].

Disulfide bond formation

In contrast with the reducing environment of the cytosol, many secreted proteins enter the ER with reduced cysteine residues and oxidized cystine. Disulfide bond formation is essential for the folding of many secretory proteins and is catalysed by the PDI (protein disulfide-isomerase) family [9]. There are more than 20 mammalian PDI family members which have diverse structural features and enzymatic activities that allow them to function in many different aspects of ER PQC.

As the nascent polypeptide enters the ER lumen, oxidoreductases catalyse oxidation of adjacent cysteines to form both native and non-native disulfides (Figure 2c). Non-native disulfides may stabilize productive folding intermediates that are critical for the correct folding of proteins. The catalytic sites of PDI contain thioredoxin-like Cys-Xaa-Xaa-Cys motifs that can have either oxidase or reductase activity to facilitate the reshuffling, or isomerization, of non-native disulfides into their native ones as present in the mature protein. To drive the net formation of disulfides, PDI must be also be oxidized by the thiol oxidase Ero1 (ER oxidase 1). Ero1 oxidizes PDI via a series of electron interchange reactions that converts molecular oxygen into hydrogen peroxide (H_2O_2) [10]. PrxIV (peroxiredoxin IV) and other ER-resident peroxidases have been reported to oxidize PDI directly using H_2O_2, which suggests that the consecutive activity of these enzymes may maximize net disulfide biosynthesis in the ER as well as limit oxidative stress due to H_2O_2 production [11].

Diversity and complexity

As nascent polypeptides enter the ER, they are exposed to a crowded environment of molecular chaperones and folding enzymes: BiP binds to hydrophobic side chains, and the lectin chaperones bind hydrophilic glycans and oxidoreductases catalyse disulfide bond formation to support productive folding. These functions, however, are not necessarily exclusive: for example, the lectin chaperones can mediate protein–protein interactions and the position of a glycan can influence BiP binding as well as oxidative folding. ER PQC is therefore a kinetic and energetic process that limits non-productive interactions and maximizes the opportunity to adopt energetically favourable conformations. As the protein folds, for example, it will bury hydrophobic side chains within its tertiary structure, reducing the frequency of chaperone interactions and increasing its likelihood of release. Although further glycan processing and proteolysis occurs within the Golgi complex, protein folding into native tertiary structures and the assembly of quaternary structures takes place within the ER [12].

Significant advances in our understanding of the structure and function of the core ER PQC machinery have revealed a multifunctional and multifaceted system to accommodate the diversity of substrates targeted to the ER. Nuances to ER PQC are mediated (at least in part) by the formation of larger folding complexes: for example, both structural and functional studies of the oxidoreductase ERp57 (ER protein 57) demonstrated a specialized role in glycoprotein folding through its interactions with the lectin chaperones [7]. Additional substrate specificity is provided through interactions with diverse co-chaperones and many other folding factors beyond the scope of this article. The majority of our knowledge however is derived from model protein folding systems and it is difficult to generalize regarding the molecular effects of chaperones on the folding protein within the ER. Therefore, the folding pathways adopted by different substrates cannot yet be predicted from the primary amino acid sequence, structure or topology of a protein. By extension, whether common ER PQC networks are recruited by particular groups of substrates or folding/structural motifs is still unclear.

ER exit

Exit from the ER occurs at specific sites at the ER membrane, termed ER-exit sites, which are segregated spatially from regions where active translocation is taking place (Figure 1). This is where properly folded proteins are packaged into cargo vesicles for forward traffic to the ERGIC (ER/Golgi intermediate compartment) and the Golgi complex. These

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vesicles are called COPII (coatomer protein II) vesicles, which refers to their specific 'coat' complex that drives vesicle assembly and budding (Figure 2e).

As described above, proteins undergoing folding form complexes with the ER PQC machinery and are probably excluded from COPII vesicles simply due to their size. Although there are many open questions regarding how the ER PQC distinguishes properly and partially folded proteins, the release of properly folded proteins from these large complexes may be sufficient to allow their free diffusion to ER-exit sites and incorporation into COPII vesicles. ER cargo can also be selectively packaged into COPII vesicles via Sec 24, a protein that forms part of the COPII coat complex [13]. The ability of Sec 24 to sort diverse substrates has been attributed to the presence of multiple Sec 24 homologues, multiple Sec 24 cargo-binding sites and interactions with different cargo receptors or adaptor proteins. The cargo determinants for uptake by Sec 24 are therefore also quite varied including conformational features and post-translational modifications. ERGIC-53, for example, is a transmembrane lectin receptor that recycles between ER-exit sites and the ERGIC and has a broad specificity for high-mannose N-glycans that helps to recruit soluble cargo into COPII vesicles for forward transport [14].

Protein misfolding at the endoplasmic reticulum

ER-associated degradation (ERAD)

Despite the co-ordinated efforts of the ER PQC machinery, a proportion of translocated proteins may fail to fold correctly. Genetic mutations or environmental stresses that compromise biosynthesis also increase the rate of protein misfolding. With the exception of the proteases required for signal peptide cleavage and degradation, few ER-localized proteases have been identified. Misfolded proteins therefore are dislocated from the ER to be degraded by the cytosolic ubiquitin–proteasome system, a process referred to as ERAD [15] (Figure 2f). ERAD is augmented by ER-derived autophagy, often called ERAD II, to remove aggregates too large to be dislocated across the membrane and targets them for lysosome-mediated degradation. ERAD targeting is best understood for misfolded glycoproteins; however, studies of non-glycosylated proteins suggest that there is significant overlap in the pathways used by both groups of substrates [16].

Misfolded glycoproteins are distinguished from productive folding intermediates via mannose trimming by the ER α-1,2-mannosidase ERManI, and EDEM (ER degradation-enhancing α-mannosidase-like protein) 1, 2 or 3. A major question in the field is how the high-mannose N-glycans of properly folded proteins can be distinguished from misfolded proteins with the same N-glycan structure? Reglucosylation by UGGT is a critical marker of partially folded or misfolded glycoproteins and delays their exit from the ER by returning them to the Cnx/Crt cycle. However, UGGT was recently shown to have no effect on the degradation of misfolded glycoproteins and suggests that the ERAD machinery can efficiently extract misfolded glycoproteins from the Cnx/Crt cycle [17]. Yet this still presents the problem of why slowly folding proteins are not prematurely targeted for ERAD before obtaining a properly folded state. Recent work has described the segregation of ERManI into an ERQC (ER-derived quality control compartment) and that, during the Cnx/Crt cycle, proteins cycle between the ER and the ERQC [18]. Therefore only terminally misfolded substrates that are persistently retained by the lectin chaperones will undergo time- and concentration-dependent mannose trimming by ERManI within the ERQC. Sequential mannose trimming eventually signals recognition by lectins with mannose homology domains OS-9 and XTP3-B, which recruit substrates to the ERAD dislocation complexes at the ER membrane.

The degree to which a protein must be misfolded to be targeted for ERAD is still unclear, although distinct degradation pathways appear to be adopted depending on where the folding defect is located; for example, a luminal, cytoplasmic or membrane-associated domain. In each case, a different membrane-associated E3 ubiquitin ligase is used to catalyse the addition of polyubiquitin and target the substrate for degradation at the proteasome [19]. The process of dislocation of misfolded proteins across the ER membrane is also not well understood and the E3 ubiquitin ligase Hrd1, Derlin 1 and the Sec 61 translocon have all been proposed to form the retrotranslocation pore. It has been experimentally difficult to distinguish peptide binding and translocation; however, a recent study successfully reconstituted the yeast Hrd1–SEL1L E3 ligase complex *in vitro* and strongly supports a role for Hrd1 in forming a membrane conduit for misfolded luminal substrates [20]. An ER-resident intramembrane rhomboid protease has also been reported to promote ERAD of membrane-spanning proteins by clipping unstable transmembrane domains

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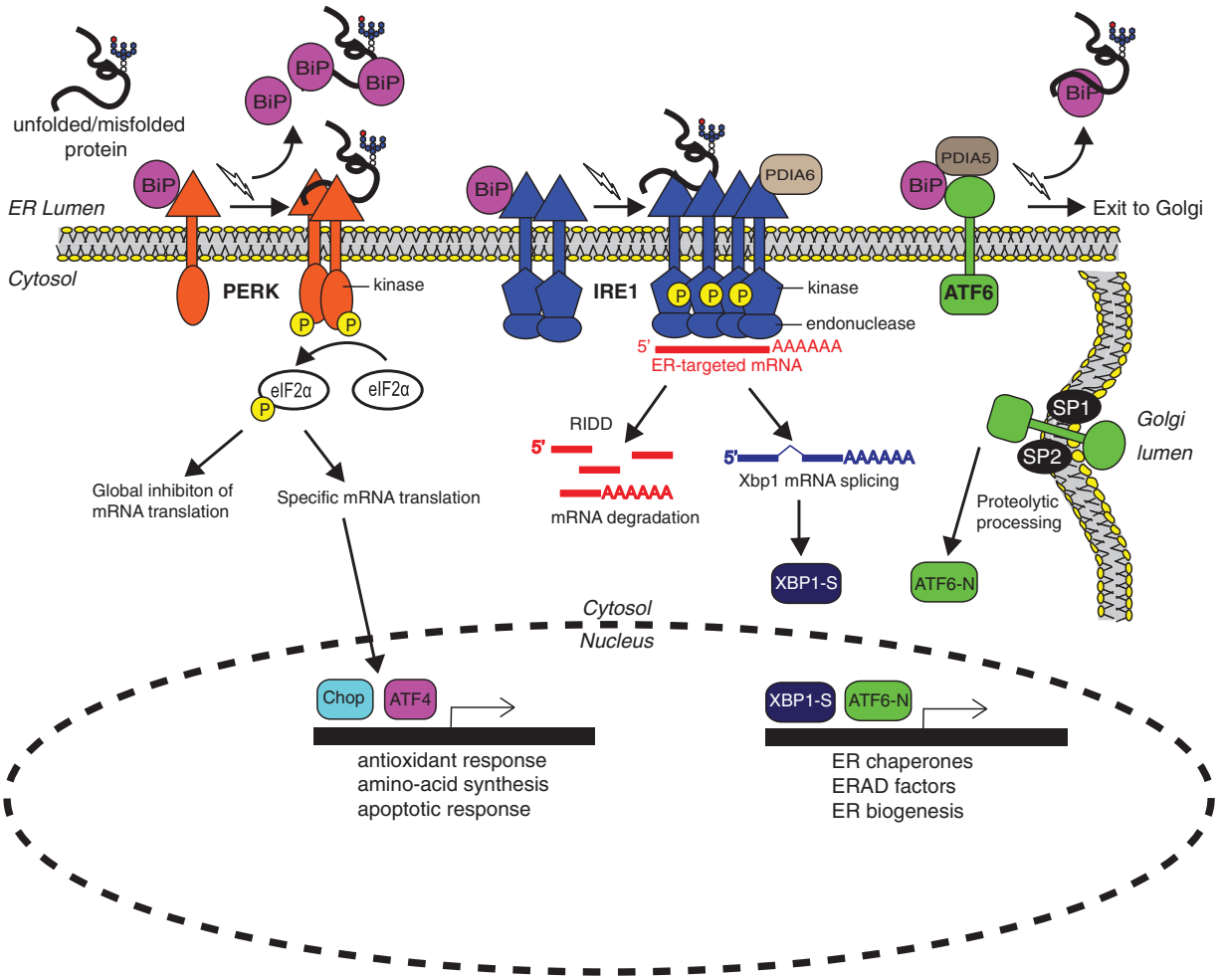

Figure 3. The unfolded protein response (UPR)

The accumulation of misfolded proteins in the ER lumen activates the UPR stress sensors PERK, IRE1 and ATF6. This initiates a signalling cascade of transcriptional and post-transcriptional programmes to resolve stress by enhancing ER folding, secretion and degradation capacity or halting protein biosynthesis. Under severe or prolonged stress, the UPR switches to an apoptotic response. Chop, C/EBP (CCAAT/enhancer-binding protein)-homologous protein; eIF2α, eukaryotic initiation factor 2α; RIDD, regulated IRE1-dependent decay.

[21]. Whichever route dislocation takes, it appears to be an energetic process that, together with ubiquitin-binding proteins, requires the cytosolic ATPase p97 to deliver the polypeptide to the proteasome.

The unfolded protein response (UPR)

The UPR is a signal cascade that is activated upon the accumulation of misfolded proteins in the ER. The UPR is a primarily a protective response that initiates a complex series of transcriptional and post-transcriptional programmes to restore ER PQC and protein homoeostasis [22]. The UPR is mediated by three major stress sensors: PERK [PKR (dsRNA-dependent protein kinase)-like ER kinase], IRE1 (inositol-requiring enzyme 1) and ATF6 (activating transcription factor 6) (Figure 3). Each is a transmembrane protein with an ER luminal 'stress-sensing' domain and a cytosolic functional domain. The UPR performs a particularly important physiological role in specialized secretory cells that demand a rapid increase in ER folding and secretory capacity in response to extracellular stimuli. For example, in response to high glucose, pancreatic β-cells experience a dramatic increase in proinsulin biosynthesis, and the UPR is required for both the quality control of insulin as well as the adaptation of β-cells to ER stress [23].

The UPR sensors are negatively regulated by interactions with BiP which is sequestered to misfolded proteins during ER stress. However, a loss of BiP binding is not sufficient to activate the UPR and the direct binding of

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polypeptides to the IRE1 luminal domain is necessary for oligomerization and downstream signalling [24]. IRE1 binds to peptides primarily composed of basic and hydrophobic residues suggesting that the UPR may discriminate between folded and misfolded polypeptides in the ER lumen. A similar mechanism of activation is predicted for the PERK luminal domain that shares significant homology with the IRE1 luminal domain. The ATF6 luminal domain forms disulfide-mediated oligomers that, upon ER stress, are reduced by the oxidoreductase PDIA5 to facilitate traffic of the activation-competent monomer to the Golgi [25]. In this way, the UPR forms a highly integrated part of the PQC apparatus to communicate changes in protein homoeostasis at the ER.

IRE1 endonuclease activity and the unconventional splicing and re-ligation of *XBP1* (X-box-binding protein 1) mRNA in the cytosol results in the active transcription factor XBP1-S. IRE1 also cleaves other ER-targeted mRNAs and targets them for degradation, a process called RIDD (regulated IRE1-dependent decay). Activation of the PERK cytosolic kinase phosphorylates eIF2α (eukaryotic initiation factor 2α), a key factor in the translation initiation machinery, and blocks global translation to inhibit protein biosynthesis at the ER. Several mRNAs are exempt from translational suppression including the transcription factors ATF4 and Chop [C/EBP (CCAAT/enhancer-binding protein)-homologous protein], required for apoptotic UPR signalling (Figure 3). ATF6 is cleaved by proteases in the Golgi to release its cytosolic transcription factor domain (ATF6-N). ATF6-N up-regulates the transcription of a number of ER chaperones, including BiP, and also plays a key role in ER biogenesis. ATF6-N and XBP1-S can heterodimerize to co-ordinate the transcription of a distinct subset of ER stress responsive genes including ERAD components [26]. Therefore activation of one or multiple arms of the UPR can initiate a highly complex and nuanced response to a loss of proteostasis at the ER.

Discussion

As every protein exhibits its own unique folding pathway, it is remarkable that the ER PQC system ensures the correct folding of such an abundance of diverse substrates. To do this, it has developed a specialized repertoire of chaperones, enzymes and other cofactors. Integral membrane proteins fold across three distinct topological environments – the ER lumen, the cytosol and the lipid bilayer – and are subject to multiple PQC systems. This complex and dynamic folding environment is difficult to reconstitute *in vitro* and the majority of studies to date have been performed in intact cells or using crude ER microsomes which, owing to their inherent heterogeneity, are generally less amenable to biochemical manipulation or structural determination. However, recent advances in cryo-electron microscopy have reported the first high-resolution structures of the actively translating ribosome–translocon complex using ER microsomes and therefore may pioneer other advances in this area [27]. It is not yet possible to predict folding pathways based on the sequence, structure or topological features of a protein, and common chaperone networks or degradation routes cannot be easily inferred. The application of increasingly sophisticated biochemical and proteomic tools to build such interaction maps is therefore a major focus of current research in the field and is already revealing novel functional ER PQC partnerships [28].

Conclusions

The fidelity of the cellular secretome is of critical importance as many of these proteins are responsible for communicating biological information to surrounding tissue and even distal sites within an organism. The secretion of potentially 'toxic' proteins may have serious consequences far beyond the immediate cellular environment and a failure of ER PQC because of aging or other factors has therefore been implicated in a number of serious diseases. A more complete molecular understanding of this system aims to provide new avenues for therapeutics to treat protein-misfolding disorders including pharmacological chaperones to rescue protein folding and inhibitors of apoptotic stress signalling to limit cell death and tissue degeneration in disease.

Summary

- ER PQC ensures the fidelity of folding of proteins destined for the plasma membrane, extracellular space or other secretory compartments.
- The ER has a distinct folding environment to that of the cytosol.

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- Asparagine-linked glycosylation and disulfide-bond formation are essential for the folding and function of secretory proteins.
- ER PQC integrates diverse functions from folding and degradation to membrane transport and cell signalling.

Abbreviations

ATF, activating transcription factor; BiP, immunoglobulin heavy-chain-binding protein; Cnx, calnexin; COPII, coatomer protein II; Crt, calreticulin; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERdj, ER-resident DNAJ protein; ERGIC, ER/Golgi intermediate compartment; Ero1, ER oxidase 1; ERQC, ER-derived quality control compartment; GI/GII, glucosidase I/II; Grp, guanine-nucleotide-releasing protein; Hsp, heat-shock protein; IRE1, inositol-requiring enzyme 1; LLO, lipid-linked oligosaccharide; NEF, nucleotide-exchange factor; OST, oligosaccharyltransferase; PDI, protein disulfide-isomerase; PERK, PKR (dsRNA-dependent protein kinase)-like ER kinase; PQC, protein quality control; PrxIV, peroxiredoxin IV; SRP, signal recognition particle; UGGT, UDP-glucose:glycoprotein glucosyltransferase; UPR, unfolded protein response; XBP1, X-box-binding protein 1.

Funding

Dr Kathleen McCaffrey would like to gratefully acknowledge funding support from the Australian Government Endeavour Fellowship Program [grant number 5380_2016].

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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