Quality control in the endoplasmic reticulum protein factory

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The endoplasmic reticulum (ER) is a factory where secretory proteins are manufactured, and where stringent quality-control systems ensure that only correctly folded proteins are sent to their final destinations. The changing needs of the ER factory are monitored by integrated signalling pathways that constantly adjust the levels of folding assistants. ER chaperones and signalling molecules are emerging as drug targets in amyloidoses and other protein-conformational diseases.

Aberrant proteins are extremely harmful to cells. Such proteins can originate from mutations, unbalanced subunit synthesis, damaging conditions (for example, oxidation) and also as a side product of normal protein biosynthesis. A certain degree of folding inefficiency can be useful. Misfolded proteins are degraded into fragments (peptides), which are then displayed on the cell's surface. In cells that are not infected, normal cytosolic and nuclear proteins will be displayed; in cells that are infected with a virus, viral peptides are presented and then detected by immune cells. The presence of membrane-bounded compartments in eukaryotic cells further complicates the issue of protein homeostasis, as it implies the existence and coordination of multiple folding and degradation systems.

A proper balance between synthesis, maturation and degradation is crucial for cell survival. At a time when health threats come from viruses, misfolded proteins (which can cause Creutzfeldt–Jakob disease) and inherited diseases, and when protein-based drugs are being introduced, a full understanding of how cells handle proteins is of crucial importance.

In eukaryotic cells, the vast majority of proteins fold either in the cytosol or in the ER. Although the general principles are similar in the both compartments[1,2], fundamental differences exist. Here we summarize the main features of folding and quality control in the ER, highlighting emerging concepts of how the functions of the ER are integrated with other cellular compartments and with transcriptional and translational control to prevent proteotoxicity.

Protein folding in the ER

The ER lumen is similar to the extracellular space: it has high calcium concentrations and is more oxidizing than the cytosol (see refs 1, 2 and references therein), and contains a specialized set of chaperones and enzymes (Table 1). The structural maturation of many proteins synthesized in the ER is slow and inefficient[1], probably because they require several post-translational modifications (for example, signal sequence cleavage, N-linked glycosylation, disulphide-bond formation and reshuffling, addition of glycosylphosphatidylinositol anchors, insertion of membrane proteins in the lipid bilayer). Coordinating these covalent modifications is a challenging task for the folding machinery in the ER. Nevertheless, the ER factory can reach levels of extraordinary efficiency in exocrine glands, plasma cells and other 'professional secretory cells'.

In mammalian cells, proteins are translocated into the ER (Fig. 1), where they start to fold co-translationally. Folding is completed post-translationally, and, generally, individual subunits have folded before assembly and oligomerization take place[1]. Sequential interactions with distinct chaperones are required for each of these steps. For instance, MHC class I molecules exploit calnexin, calreticulin and other devoted ER-resident molecules (for example, tapasin) to negotiate their stepwise assembly into peptide-loaded functional complexes[3]. Although most folding factors in the ER are ubiquitously expressed throughout the body, some are tissue-type specific or cell-type specific, and probably fulfil a particular synthetic task (Table 1). For example, efficient collagen production requires the expression of hsp47, whereas a tissue-specific protein-disulphide-isomerase-like protein, PDIp, is produced in the pancreas and probably permits the massive secretion of digestive enzymes[1,4].

Disulphide-bond formation

Both N-glycosylation and disulphide-bond formation play a crucial role in the folding of secretory and membrane proteins in the ER. The glycans add hydrophobicity to the folding peptide but also act as substrates for the lectin chaperones calnexin and calreticulin, whose role in glycoprotein folding has been extensively reviewed[5,6]. Both calnexin and calreticulin form a complex with ERp57, an ER oxidoreductase, coupling folding assistance to disulphide-bond formation.

The impressive number of oxidoreductases in the ER suggests that catalysis and regulation of disulphide-bond formation is crucial for folding. Energywise, in most cases, the contribution of a disulphide bond is hardly more than that of a single hydrogen bond, yet, without disulphide bonds, native conformations are not obtained. Disulphide bonds cannot force a folding protein into a given conformation: in the sampling of conformations during folding in the ER, native and non-native disulphide cross-links are transiently formed. Continuous activity of oxidoreductases probably ensures that these covalent links remain flexible until folding is completed.

Secreted proteins enter the ER with reduced cysteines and leave it with oxidized cystines. The requirement for oxidative equivalents is fulfilled by Ero1 proteins, which transfer electrons from protein disulphide isomerase (PDI) to oxygen through a series of specific interchange reactions[7,8]. Additional electron transport pathways exist, and involve proteins such as Erv2p from yeast, although their role under normal conditions remains to be deter-

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mined[7]. The redox gradient between the ER and the cytosol seems to be important for intercompartmental signalling, particularly in the integrated response to oxidative stress, in which adaptive responses emanating from different compartments are coordinated[9]. And redox reactions with opposite electron fluxes must take place in the ER to mediate formation, isomerization and reduction of disulphides[8]. The wealth of redox assistants allows these fluxes to be separate, and channels electron transport through specific protein–protein interactions. The main role of glutathione in redox homeostasis in the ER seems to be that of buffering the oxidative power of Ero1 (refs 7, 8). Because disulphide bonds are so important for folding, we may conclude from the number of ER-resident oxidoreductases that secretory proteins need more help, possibly because they are often larger than cytosolic ones[10]. Perhaps secretory proteins, which are designed to act extracellularly and often must carry their biological messages over long distances, need more protection from denaturing forces outside the cell. This is an issue that glycans are likely to contribute to as well.

Quality control in the ER

Besides providing a unique folding environment, the ER has a crucial quality-control role[1,2,4]. How does it discriminate between native and non-native proteins? The answer to this question depends primarily on ER chaperones. When folding or assembly intermediates expose hydrophobic surfaces, unpaired cysteines or immature glycans, ER-resident chaperones or oxidoreductases interact with them, and as a consequence they are retained in the ER or retrieved from the Golgi complex[1,4]. By forming multimolecular complexes[11], folding factors in the ER may provide matrices that couple retention to folding and assembly. Immature proteins may also form aggregates that are excluded from vesicles exiting from the ER[1,12].

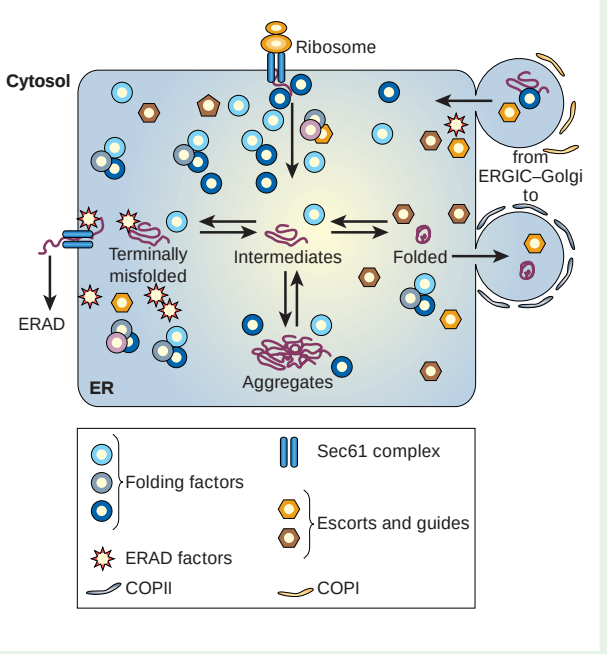

Figure 1 Folding in the crowded environment of the ER. The port of entry for proteins destined for the secretory pathway is the ER. These proteins are synthesized by ER-associated ribosomes and co-translationally translocated across the membrane through the Sec61 complex. The ER is rich in chaperones and folding enzymes (folding factors), in molecules involved in mediating transport to the cytosol for proteasomal degradation (ERAD factors) or to the downstream stations of the secretory pathway (escort and guides[13]). Some ER-resident proteins seem to form multimolecular complexes[1], which can be excluded from transport by size selection, and provide a matrix that couples retention to folding[1,4]. A distinctive feature of the ER is its ability to catalyse opposite reactions: folding and unfolding, oxidation and reduction, protein import and export through Sec61. It is debated whether a 'quality-control compartment'[12,39], involved in the recognition and targeting of terminally misfolded proteins (alluded to in the picture by having ERAD factors concentrated on the left), exists. ERGIC, ER–Golgi intermediate compartment.

All proteins are subjected to a 'primary' quality control that monitors their architectural design through ubiquitous folding sensors (Table 1). 'Secondary' quality-control mechanisms rely instead on cell-specific factors and facilitate export of individual proteins or classes of proteins (for example, tapasin for class I molecules; see also refs 1, 2, 4 and 13 and references therein for additional examples). Although some proteins can be rerouted to and degraded in the endolysosomal compartment (Fig. 2), the ER is the main test bench where molecules destined for the extracellular space are scrutinized for their potential toxicity. Interestingly, ER quality control seems to monitor primarily local structures within protein domains, allowing transport of proteins originating from exon reshuffling or otherwise 'flexible' molecules[1,4]. A certain degree of freedom from quality control is essential for the evolution of proteins. However, it comes at a price for multicellular organisms. Indeed, many proteins that cause systemic amyloidosis (see the review in this issue by Dobson, page 884) can adopt more than one conformation and can undergo uncontrolled aggregation outside of the cells.

The reasons for having a quality-control system in the ER are easy to understand where protein folding and function are concerned, especially in multicellular organisms where development relies on the fidelity of protein secretion. Quality control can also regulate the transport or the activity of certain proteins during differentiation[14] or in response to stress or metabolic requirements. A clear case in the context of ER physiology is that of the transcription factor ATF6, a transmembrane protein localized in the ER by interactions between its luminal domain and BiP, an abundant ER-resident chaperone of the hsp70 family (Table 1). The unfolded proteins titrate BiP, releasing ATF6 for transport and subsequent cleavage by Golgi proteases[15–17]. In this way, the active cytosolic domain is unleashed and delivered to the nucleus, where it drives transcription of target genes. A similar cleavage mechanism to that for ATF6 underlies the cholesterol-dependent transport of sterol regulatory element binding protein[18]. Other cases of regulated transport are based on the availability of specific ligands (for example, lipids and retinol) or cofactors (calcium ions, vitamin C and so on). Clarifying the mechanisms controlling differential ER to Golgi transport has obvious technological and clinical implications. For example, ligand-induced transport may allow storage of hormones or other proteins in the ER, which can be released when necessary by ligand administration[19].

ER-associated degradation

Mutations or unbalanced subunit synthesis make folding or assembly — and hence exit from the ER — impossible. To maintain homeostasis, terminally misfolded molecules are 'retrotranslocated' or 'dislocated' across the ER membrane to be degraded by cytosolic proteasomes[20,21]. Dislocation is generally believed to occur through Sec61, a protein complex also used by nascent proteins to enter into the ER (Fig. 1), raising the question of how vectorial transport in opposite directions is regulated. Additional mechanisms for delivering molecules across the ER membrane may also exist.

A fascinating problem is how molecules that have not been given the time to fold (and therefore are unfolded)are discriminated from those that have failed to fold after many attempts (misfolded), and must therefore be disposed of. One way of timing glycoprotein quality control involves the sequential processing of N-glycans and in particular mannose trimming in the ER[22]. It remains to be seen how substrates are eventually targeted to the retrotranslocation channels, how these are opened, and to what extent proteins must be unfolded to negotiate dislocation[8,21].

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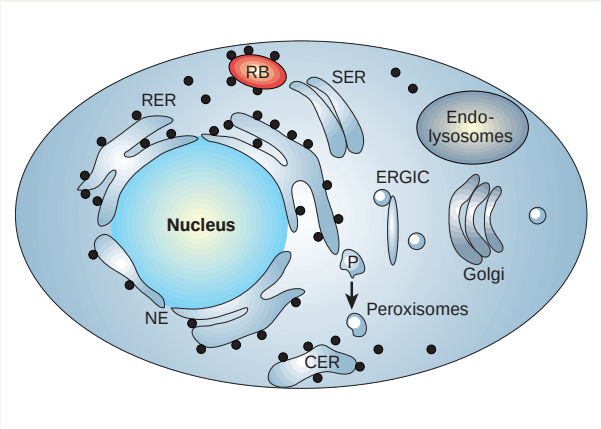

Figure 2 The ER is not always that rough. Distinct ER subcompartments can be clearly identified: the nuclear envelope (NE), and rough and smooth stacks (RER and SER). In plant cells, dilated ER cisternae are used for protein storage. In mammalian cells, they are generally associated with the accumulation of mutated or transport-incompetent proteins produced in excess with respect to ERAD capacity (for example, Russell bodies, RB[30]). Additional ER subdomains were identified recently, including a peroxisome precursor compartment (P) and the cortical ER (CER) in yeast[40,41]. Selected mRNAs seem to be targeted to the storage domains of plant ERs as well as to the yeast CER, thus favouring subcompartmentalization. ER subdomains could exist at an even smaller scale[11]. For instance, different complexes of ER folding factors in the ER may fulfil sequential (and often opposite) tasks during protein maturation in the ER, and membrane sub-domains might exist.

Table 1 **Personnel of the ER protein factory in mammalian cells**

Ubiquitous ER-resident proteins

Family	Main members	Functions
Hsp70	BiP/grp78	Chaperone
Hsp90	Endoplasmin/grp94	Chaperone
Hsp40	ERdj1–ERdj5, Sec63	Co-chaperones regulating BiP?
Lectins	Calnexin and calreticulin	Glycoprotein quality control
	EDEM	Glycoprotein degradation[22]
Glycan-processing enzymes	UGT	Folding sensor. Adds glucoses to misfolded glycoproteins (enters substrates into the calnexin cycle)[5,6]
	ER glucosidases I and II	Remove glucoses from N-glycans (on/off/calnexin cycle)[5,6]
	ER mannosidases I and II	Remove terminal mannoses — ERAD?[22]
Peptidyl–prolyl isomerases	Cyclophilins, FK506-binding proteins	Isomerize *cis-trans* peptidyl-prolyl bonds?
Ero1	Ero1α, Ero1β	Disulphide-bond formation[7,8]
Oxidoreductases	PDI, ERp72, ERp57, p5, and many others	Disulphide-bond formation, isomerization and reduction

Examples of specific folding assistants

Name	Tissue distribution	Function
PDIp	Pancreas	Oxidoreductase
Hsp47 and prolyl-4-hydroxylase	Cells producing collagen	Collagen synthesis and assembly[24]
Invariant chain	Antigen presenting cells	MHC class II assembly and transport
Tapasin	Ubiquitous	Peptide binding to MHC class I[3]
RAP	Ubiquitous	LDL receptor assembly and transport
BOCA/Mesd	?	LDL receptor assembly and transport[42]
Egasyn	Ubiquitous	Quality control of β-glucuronidase
Carboxylesterase	Hepatocytes	Quality control of C reactive proteins
SCAP	Ubiquitous	Retention of SREBP[18]

The unfolded protein response and signalling from the ER

To maintain the efficiency of quality-control mechanisms in diverse physiological conditions, living cells have evolved regulatory circuits that monitor the levels of available chaperones. This is true for both the cytosol and the ER, and compartment-specific responses clearly exist that selectively restore optimal levels of the desired folding factors. The accumulation of aberrant proteins in the cytosol triggers the heat-shock response, resulting in *de novo* synthesis of hsp70 and other cytosolic chaperones[23]. But if aberrant proteins accumulate in the ER, cells activate a different response, the unfolded protein response (UPR), which leads to the coordinated synthesis of ER-resident chaperones and enzymes. The UPR is induced by drugs that block N-linked glycosylation or disulphide-bond formation, or alter calcium ion homeostasis, thus selectively targeting the ER folding machinery[16,17]. Physiologically, ER stress (a condition in which the folding machinery in the ER cannot cope with its protein load) can be caused by synthesis of mutated or orphan proteins, absence of cofactors (an example being scurvy, in which collagen cannot fold because of the lack of vitamin C[24]), or a drastic increase in otherwise normal cargo proteins.

How do the diverse unfolded or misfolded proteins that accumulate in the ER provoke the same pathway? The unifying concept is that BiP and other primary quality-control factors maintain the stress sensors in the ER in the inactive state[16,17], so that chaperone insufficiency triggers UPR whatever the nature of the cargo.

The mammalian ER sensors, Ire1, PERK and ATF6, guarantee a tripartite response with synergic strategies[16,17]. By phosphorylating eIF2α, PERK transiently attenuates translation, limiting protein load. ATF6 drives the transcriptional upregulation of many ER-resident proteins and folding assistants. Ire1 activates XBP-1, which in turn induces transcription of factors that facilitate ER-associated degradation (ERAD). The two-step activation of XBP-1 (transcriptionally induced by ATF6 and post-transcriptionally regulated by Ire1) guarantees the proper timing of the UPR; attempts to fold proteins precede the decision to degrade them[25]. If the response fails to clear the ER, apoptosis is induced through several pathways[16,17,26]. This link has important consequences for the pathogenesis of degenerative disorders (see the progress in this issue by Goldberg, page 895).

The UPR is multi-faceted and regulates proteins involved in quality control, ERAD and many aspects of the secretory pathway[27]. It is emerging as a key controller of normal development as well. Particularly compelling in this respect is the observation that plasma-cell differentiation requires XBP-1 (ref. 28). However, attenuating translation through PERK would be counterproductive for plasma cells, which must release antibodies in large quantities[26]. It remains to be seen whether cells can selectively activate individual branches of the UPR. One possibility is that the different ER-resident proteins containing a DnaJ motif (ERdj)[29] might preferentially release BiP from one of the three UPR sensors, similar to the way in which E3 ligases confer specificity on E1 and E2 proteins.

ER quality control and disease

Quality control must be a balance between retaining and degrading potentially harmful products and not preventing export of biologically active proteins. CFTR mutants in cystic fibrosis illustrate an overzealous quality control, where biologically active mutants cannot leave the ER. In this case, relaxing the quality control could cure the patient. But disease can also originate from defective degradation. If the rate of synthesis of a protein exceeds the combined rates of folding and degradation, a fraction of it will accumulate intracellularly. At least two bottlenecks are encountered by ERAD substrates: dislocation across the membrane and actual degradation by the proteasome. Inefficient proteolysis often results in the formation of deposits of ubiquitylated proteins in the pericentriolar region, called aggresomes[30]. Studies with the prion protein suggest that mislocalization to the cytosol is sufficient to cause conversion into the PrP^{sc} form[31,32]. By contrast, inefficient dislocation results in substrates accumulating in the ER. Many ER storage diseases are characterized by the presence of dilated ER cisternae containing mutated pro-

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teins[30]. It is not clear whether these aberrant structures are toxic *per se* or whether they represent a defence mechanism that segregates dangerous proteins into specialized ER subcompartments (Fig. 2). The tendency of the mutated protein to sequester chaperones, and hence to induce a prolonged UPR leading to apoptosis, is probably important in determining cell damage.

Proteasome inhibitors are powerful inducers of the UPR, probably because dislocation of many substrates is coupled to degradation. Receiving substrates from both ER and cytosol, proteasomes are crossroads of the two main protein quality-control systems. Conditions that hamper proteasome function, such as the presence of proteins with polyglutamine repeats[33,34] or a lack of E3 ligases[35], therefore can cause accumulation of ERAD substrates in the ER and activate UPR-dependent apoptotic pathways. In this way, proteotoxicity could be transmitted across compartments, with important implications for degenerative diseases (reviewed in refs 35, 36).

Perspectives

Over the past few years, much has been learned about how proteins are handled by the ER folding and quality-control machineries, and some of this knowledge has begun to be translated to industry and to the clinic. Yet, many questions remain: how does aggregation relate to degradation? Are aggregation and degradation sometimes mutually exclusive, with aggregation preventing dislocation? In what circumstances does this happen? How does aggregation relate to amyloid formation, and how can aggregates be eliminated? How do cells secrete large molecular ensembles, such as procollagen and viruses? Even before complete answers are provided, new strategies for intervention are being considered to prevent viral replication, protein aggregation or amyloid formation. The most important question is whether the folding of a protein can be influenced specifically, with the goals either of curing a disease or of preventing virus assembly. Chemical chaperones and ligand-induced transport opens options for designing specific drugs to control protein (mis)folding or transport[37,38]. Likewise, tissue-specific chaperones might be therapeutic targets and might provide important tools in biotechnology. Will we be able to reduce protein misfolding in degenerative disorders, or induce it in some tumours so as to cause apoptosis? Last but not least, the UPR signalling cascades clearly offer targets for pharmacological intervention. Drugs that modulate the UPR may alleviate a variety of age-related diseases resulting from protein misfolding and aggregation. □

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