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The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic-reticulum-associated degradation)

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Abstract

Over one-third of all newly synthesized polypeptides in eukaryotes interact with or insert into the membrane or the luminal space of the ER(endoplasmic reticulum), an event that is essential for the subsequent folding, post-translational modification, assembly and targeting of these proteins. Consequently, the ER houses a large number of factors that catalyse protein maturation, but, in the event that maturation is aborted or inefficient, the resulting aberrant proteins may be selected for ERAD (ER-associated degradation). Many of the factors that augment protein biogenesis in the ER and that mediate ERAD substrate selection are molecular chaperones, some of which are heat- and/or stress-inducible and are thus known as Hsps (heat-shock proteins). But, regardless of whether they are constitutively expressed or are inducible, it has been assumed that all molecular chaperones function identically. As presented in this review, this assumption may be false. Instead, a growing body of evidence suggests that a chaperone might be involved in either folding or degrading a given substrate that transits through the ER. A deeper appreciation of this fact is critical because (i) the destruction of some ERAD substrates results in specific diseases, and (ii) altered ERAD efficiency might predispose individuals to metabolic disorders. Moreover, a growing number of chaperone-modulating drugs are being developed to treat maladies that arise from the synthesis of a unique mutant protein; therefore it is critical to understand how altering the activity of a single chaperone will affect the quality control of other nascent proteins that enter the ER.

Keywords

endoplasmic-reticulum-associated degradation (ERAD); folding; heat-shock protein 70 (Hsp70); molecular chaperone; proteasome; ubiquitin

INTRODUCTION

Protein biogenesis is an inherently error-prone process, given the high rate of translation, the synthesis of polypeptides that may be encoded by mutated genes and the fact that cellular stress or nutrient deprivation may result in the incorporation of incorrect amino acids. The resulting defective proteins may be unable to acquire requisite post-translational modifications and assume their native conformations. The cell cannot tolerate and should not risk the resulting threat of aberrant protein accumulation, as defective proteins may then aggregate and/or bind to wild-type functional proteins. These events, in turn, might lead to dominant-negative effects

on essential cellular processes. To offset this threat, multiprotein cellular machines have evolved to help retain aberrant proteins in solution, thereby preventing protein aggregation, and to target defective proteins to proteolytic enzymes. The importance of the proteolytic pathway is highlighted by the facts that perhaps 30% of newly synthesized polypeptides in some cells may be destroyed during or soon after translation [1] and that defects in clearing cytoplasmic aggregates can lead to neurodegeneration [2,3].

Another compartment in eukaryotic cells in which the degradation of aberrant polypeptides is of critical importance is the ER (endoplasmic reticulum). Over one-third of all proteins enter the secretory pathway [4] and the first step in this pathway is the ‘translocation’, or insertion, of a nascent polypeptide into the ER membrane (if the protein contains a transmembrane domain) or into the ER lumen (if the protein lacks membrane-spanning hydrophobic domains and is soluble). In eukaryotes, polypeptides are thought to begin folding co-translationally as they enter the ER [5]. Thus the ER lumen houses a high concentration of enzymes that catalyse the post-translational modification of proteins (e.g. signal sequence cleavage and addition of a core oligosaccharide), that trigger amino acid isomerization and oxidation (peptidylprolyl *cis-trans* isomerase and protein disulfide-isomerase respectively), and that prevent the formation of aberrant off-pathway conformations that would otherwise lower protein-folding efficiency [6–8]. Enzymes in this last class include molecular chaperones, which have been generally defined as factors that attenuate protein aggregation and that, in some cases, catalyse the ATP-coupled folding or refolding of polypeptide substrates. Molecular chaperones reside in every cellular compartment and have been categorized by their apparent molecular masses and by the fact that many were first recognized as Hsps (heat-shock proteins). Constitutively expressed heat-shock cognate proteins are termed Hscs, and, in most cases, but not all (see below), homologous Hsp and Hsc chaperones perform identical functions in the cell.

What happens if aberrant proteins accumulate in the ER? The first answer is that the cell ‘responds’ to this insult by inducing the UPR (unfolded protein response), a transcriptional programme that results in the synthesis of ER chaperones and enzymes required for protein modification, for ER membrane expansion, for protein trafficking from the ER and for the disposal of misfolded proteins [9,10]. The second answer, which may more immediately address the problem at hand, is that defective proteins can be delivered back to the cytoplasm. Here, they are destroyed by the proteasome, a multi-catalytic 26S proteolytic particle that is enriched at the ER membrane [11–15]. The selection, delivery to the cytoplasm (also known as ‘retro-translocation’ or ‘dislocation’) and proteasome-targeting of proteins has been termed ERAD (ER-associated degradation) [16] (see also <http://www.BiochemJ.org/bj/404/0353/bj4040353add.htm> for an animation). In effect, the ERAD pathway clears the secretory pathway of potentially dangerous proteins. As might be expected, this pathway becomes essential if the concentration of misfolded proteins accumulates to very high levels and/or if UPR induction is simultaneously compromised [17, 18]. Accumulating evidence also indicates that the ERAD pathway has been co-opted by some opportunistic pathogens. These pathogens produce toxins that are retro-translocated from the ER or target the destruction of components required for antigen presentation. ERAD is also important for some cellular regulatory systems that destroy specific metabolically controlled enzymes that reside in the ER membrane [19,20].

Since early descriptions of the ERAD pathway, a large number of ERAD-requiring factors have been identified, including several classes of molecular chaperones. Previously, seminal work on bacterial chaperones suggested that chaperone-based machines may first attempt to fold a polypeptide substrate, but then target the substrate to one of several proteasome-like complexes if folding is thwarted [21]. It was assumed that eukaryotic chaperones might function similarly during ERAD. Instead, depending on the substrate, a given chaperone may be required for folding or degradation. Furthermore, each ERAD substrate exhibits diverse

chaperone requirements for its degradation. Thus it is difficult to ‘pigeonhole’ a given ERAD substrate, and one cannot state that an ER-associated chaperone is required exclusively for folding or for degradation.

In this review, the general functions of relevant chaperone classes will be presented, and then unique attributes of select ERAD substrates will be discussed. Subsequently, specific examples demonstrating chaperone-specific effects on protein folding in the ER and ERAD will be described. And, finally, models to explain some of these data will be summarized.

CHARACTERISTICS OF DISTINCT MOLECULAR CHAPERONE CLASSES

Perhaps the best-studied class of molecular chaperones is Hsp70. Hsp70s interact preferentially with substrates that expose peptides with overall hydrophobic character. These motifs are most commonly buried in folded proteins, and their exposure in the crowded cellular milieu would give rise to rampant protein aggregation. It is not surprising, then, that the expression of Hsp70 is induced when the cellular concentration of unfolded proteins rises [22], and that Hsp70 binds to polypeptides co-translationally [23]. Importantly, Hsp70s cycle on-and-off substrates in an ATP-dependent cycle. Hsp70s exhibit high substrate affinity when ADP is bound and low substrate affinity when ATP is bound [24], a characteristic dictated by Hsp70 domain organization: the N-terminal ~44 kDa portion of the protein is an ATPase that is followed by a ~15 kDa peptide-binding domain [25–27]. The peptide-binding pocket in this second domain is protected by a more C-terminal, α -helical lid, which opens when ATP is bound, but closes when ADP is bound (Figure 1). The extreme C-terminus of cytoplasmic Hsp70s mediates the interaction between the chaperone and TPR (tetratricopeptide repeat)-domain-containing proteins, some of which couple Hsp70 to the ERAD machinery (see below).

Because ATP hydrolysis is critical to trap a peptide, and, because ADP–ATP exchange is critical for substrate release, Hsp70 co-chaperones have evolved which catalyse individual steps in the Hsp70–ATP hydrolytic cycle. Specifically, Hsp40 chaperones enhance the k_{cat} for ATP hydrolysis and therefore facilitate Hsp70–peptide capture [28]. Hsp40s harbour a ~70 amino acid sequence (known as a ‘J-domain’), which folds into a four- α -helical bundle and most likely interacts with Hsp70’s ATPase domain. Because some Hsp40 homologues also bind hydrophobic peptides [29], these chaperones can target substrates to Hsp70. Then, upon transfer to Hsp70, the Hsp40 enhances Hsp70’s ATPase activity, which in turn locks the substrate on to Hsp70 (Figure 1). In other cases, Hsp40s may simply increase the overall Hsp70 catalytic cycle, thereby facilitating Hsp70 action in the cell. In any event, peptide release requires the exchange of the hydrolysed ADP with ATP, and a growing number of Hsp70 NEFs (nucleotide-exchange factors) have been identified that, like their Hsp70 partners, facilitate protein folding and ERAD [30].

Another abundant chaperone class is Hsp90. Hsp90s possess three distinct domains: an N-terminal ATPase domain (N), a middle (M) region that regulates ATPase activity, and a C-terminal dimerization domain [31–33]. Like Hsp70, residues at the C-terminus of the protein mediate the interaction between Hsp90 and TPR-domain-containing proteins. Also like Hsp70, Hsp90 binds and releases a client protein concomitant with cycles of ATP binding, hydrolysis and release. However, Hsp90 appears to possess more than one binding site for a substrate, at the outer edges of the N domain and M region in an Hsp90 monomer, and ATP hydrolysis, which significantly changes the conformation of the Hsp90 dimer, may subsequently induce a conformational change in the bound substrate. Not surprisingly, then, Hsp70 and Hsp90 can affect protein quality control ‘decisions’ distinctly (see below). Progress on better characterizing the catalytic cycle of Hsp90s has, until recently, been hampered by the difficulty in crystallizing Hsp90, by the ongoing discovery of co-chaperones that modulate various aspects of Hsp90 function and by the complexity brought about by Hsp90’s association with

other chaperone-containing complexes. What is clear, however, is that a large and important group of substrates have been identified as Hsp90 clients, many of which are critical for the maintenance of cellular homeostasis and whose levels are altered in disease [34] (see also see <http://www.picard.ch/research/research.htm>).

Some chaperones that reside in the ER lumen do not bind avidly to hydrophobic peptides, but interact preferentially with distinct sugar residues that are appended on to secreted proteins by the ER-resident oligosaccharyltransferase. As secreted proteins enter the ER, a core Glc₃-Man₉-GlcNAc₂ glycan is added on to asparagine residues within a consensus Asn-Xaa-Ser/Thr sequence, and, soon thereafter, the two terminal glucose residues are cleaved. The monoglucosylated species is a high-affinity substrate for calnexin and calreticulin, which are integral membrane and soluble lectins in the ER respectively. In one model, as the nascent polypeptide cycles on and off the lectins (and perhaps interacts with other chaperones), the final glucose is cleaved and folding may occur [35,36]. If so, the protein can exit the ER by interacting with cargo receptors that associate with the COPII (coatamer protein II) vesicle transport machinery; these vesicles mediate secretory protein transport to the Golgi apparatus [37,38]. If instead the polypeptide is unable to fold, a glucose residue is re-added through the action of UGGT (UDP-glucose:glycoprotein transferase). Because UGGT seems to bind preferentially to partially folded molten-globule-like species [39,40], this enzyme serves as an important sensor of ER folding, and, with calnexin and calreticulin, UGGT helps to retain immature proteins in the ER.

Can the cycle of glucose removal and re-addition continue perpetually? For a select number of substrates, it has been shown that mannose trimming (from the Man₉ moiety on the N-glycan) re-directs soluble misfolded secreted proteins from the calnexin cycle to other putative lectins in the ER. These factors may target their substrates for ERAD [41]. Nevertheless, the precise functions of these 'degradative lectins', such as EDEM (ER degradation enhancer, mannosidase α -like) 1, EDEM2, EDEM3 and Yos9, are still being deciphered.

EARLY DEFINITIONS OF THE ERAD PATHWAY THROUGH THE USE OF MODEL SUBSTRATES

One of the earliest hints that the cytoplasmic proteasome degrades an ER protein was provided by Sommer and Jentsch [42], who identified a ubiquitin-conjugating enzyme, Ubc6, that resided in the ER membrane. This observation was notable because most substrates targeted for proteasome-mediated degradation are tagged with multiple copies of a 76-amino acid peptide, ubiquitin. Ubiquitin is appended on to protein substrates through the sequential action of ubiquitin-activating enzymes ('E1s'), ubiquitin-conjugating enzymes (UBCs or 'E2s'), and ubiquitin ligases ('E3s') [43]; a fourth class of enzymes, known as E4s, may be important to elongate the polyubiquitin chain [44]. Sommer and Jentsch [42] also found that disruption of the *UBC6* locus rescued the phenotype associated with a mutant form of the ER translocation channel, Sec61 (see Table 1). These data suggested that the mutant channel was stabilized when the activity of the UPP (ubiquitin-proteasome pathway) was compromised.

Further evidence that the UPP removes aberrant proteins from the ER membrane was provided by investigators who monitored the proteolysis of a mutant form of the CFTR (cystic fibrosis transmembrane conductance regulator). The mutation in the protein (a deletion of a phenylalanine residue at position 508) alters the folding of this chloride channel so that it can no longer traffic to the plasma membrane [45]. As a result, Δ F508-CFTR-expressing homozygotes develop cystic fibrosis, one of the most common lethal inherited diseases in Caucasians [46]. Using recently developed proteasome inhibitors and by co-expressing dominant-negative forms of ubiquitin, it was observed that Δ F508-CFTR was degraded by the UPP [47,48]. In fact, up to ~75% of wild-type CFTR may also be degraded by the UPP,

indicating that the folding of this complex protein in at least some cell types [49] is inefficient. A growing number of other polytopic plasma membrane proteins, particularly multi-subunit ion channels (e.g. the epithelial sodium channel, or 'ENaC'), fold poorly and must be surveyed by the ER quality-control machinery. This ensures that misfolded forms of these channels do not escape the ER. In retrospect, given that: (i) a substantial proportion of proteasomes stud the surface of the ER membrane; (ii) the phenylalanine residue that is deleted in the disease-causing form of CFTR resides in a large cytoplasmic domain; and (iii) mutated cytosolic proteins were known to be proteasome substrates, these discoveries made perfect sense. Since then, a large body of work has been devoted to understanding at the molecular level how the $\Delta F508$ form of CFTR is targeted for degradation by the proteasome, and the examination of CFTR homologues in model systems (e.g. Ste6* in yeast) have helped to elucidate fundamental aspects of ER membrane protein-quality control (see below and Table 1).

Unlike CFTR and other ion channels, many protein passengers through the ER are soluble. Because soluble substrates translocate directly into the ER lumen and do not expose cytosolic domains, it was suggested that defective soluble proteins might be degraded by an ER-resident protease. To investigate this hypothesis, we developed an assay that recapitulated the degradation of a mutant form of a yeast pheromone precursor that had been shown to be proteolysed in yeast [50]. We found that the mutant protein, known as p α F (pro- α -factor), translocated into yeast ER-derived microsomes and was stable, but, if the p α F-containing microsomes were incubated in cytosol and ATP, the protein was rapidly destroyed [16]. To establish the site of degradation, the reaction mixture was centrifuged, and, surprisingly, most of the substrate now resided in the supernatant. This result indicated that the p α F had retro-translocated from the ER into the cytosol. We next determined that the proteasome degraded the retro-translocated p α F because the substrate was stable when wild-type cytosol was treated with proteasome inhibitors or when the cytosol was prepared from a proteasome mutant strain [51]. In parallel, and consistent with these data, Wolf and colleagues observed that the degradation of a mutated form of a vacuole-targeted soluble protein, carboxypeptidase Y, known as CPY*, was slowed in yeast mutated for the proteasome or for an ER-membrane-tethered E2 ubiquitin ligase [52]. CPY* had entered and then exited the ER, and it was noted that CPY* ubiquitination was a prerequisite for degradation. Unlike p α F, CPY* forms disulfide bonds and is glycosylated in the ER before its retro-translocation, indicating that the 'decision' to degrade a soluble protein can take place well after the protein has translocated. To better access the putative retro-translocation channel and the proteasome, these data also suggested that disulfide bonds must be broken before retro-translocation and that N-glycan removal must precede degradation. Consistent with these hypotheses, the breaking of disulfide bonds hastens the degradation of some ERAD substrates [53] and deletion of the cytoplasmic N-glycanase slows the degradation of CPY* [54]. Together, these data indicated that soluble aberrant proteins are somehow selected from the pool of wild-type secreted proteins, and are then exported to the cytoplasm. From an evolutionary perspective, it also made sense that a common quality-control protease, the proteasome, was employed for the disposal of mutated forms of both integral membrane and soluble proteins.

VARIATIONS ON A THEME: REGULATED ERAD AND ALTERNATIVE PATHWAYS FOR DISPOSAL

In the intervening years, studies of CFTR, p α F and CPY* have provided many details on the mechanisms underlying the ERAD pathway, even as the number of known ERAD substrates has grown significantly. But many other substrates, because of their unique characteristics, have yielded exciting variations on the ERAD theme. For example, Wiertz et al. [55] reported that an HCMV (human cytomegalovirus) gene product, US11, triggers the proteasome-dependent degradation of the MHCI protein during or soon after it translocates into the ER. This may help HCMV evade the immune system. Although MHCI is a single-spanning

membrane protein, the occurrence of rapid U^{PR}-dependent and ubiquitin-dependent degradation suggested that the substrate had not fully integrated into the membrane when it had been selected for ERAD. Indeed, MHC1 dislocation was later shown to require a polytopic integral membrane protein in the ER that may serve as a retro-translocation channel [56,57].

Another ERAD substrate whose degradation is controlled by exogenous factors is apoB (apolipoprotein B). ApoB is the major protein component in LDL (low-density lipoprotein) and VLDL (very-low-density lipoprotein) particles and mediates the interaction between LDL and the LDL receptor. This interaction is essential to deliver cholesterol into endothelial cells via clathrin-dependent endocytosis [58]. Not surprisingly, mutations in the gene encoding apoB can lead to altered levels of circulating cholesterol, and it was shown recently that silencing the *apoB* gene is sufficient to reduce serum cholesterol in non-human primates [59]. As might be expected, apoB secretion is tightly controlled: as the protein is synthesized and inserted into the ER lumen, lipids and cholesterol are acquired because of the presence of an exposed hydrophobic surface. Interestingly, lipidloading is catalysed by a protein complex, MTP (microsomal triacylglycerol-transfer protein) [60], that can be thought of as an apoB-specific chaperone; of note, one subunit of MTP is protein disulfide-isomerase, which is required for the retro-translocation of some ERAD substrates [61,62]. If, however, these lipid ligands are in short supply, the partially translocated protein fails to interact with MTP and is instead destroyed by the UPP [63]. ER-resident lipids also mediate the metabolic control of HMGCoA-R (3-hydroxy-3-methylglutaryl-CoA reductase), which catalyses the rate-limiting step in cholesterol biosynthesis and is the target of the cholesterol-reducing 'statins'. Although the activity of this enzyme is modulated at many levels, in yeast the protein appears to sense an intermediate in the cholesterol-biosynthetic pathway. At a sufficiently high concentration of the intermediate, the conformation of HMGCoA-R is altered so that the enzyme becomes an ERAD substrate [64].

What happens if the ERAD machinery is overwhelmed? As mentioned above, the U^{PR} is induced, which represents an attempt by the cell to circumvent the potentially catastrophic effects of misfolded protein accumulation. The trigger for U^{PR} induction may be through the binding of unfolded polypeptides to an ER membrane U^{PR} sensor, known as Ire1. Alternatively, the U^{PR} may be triggered because the ER luminal Hsp70, BiP (immunoglobulin heavy-chain-binding protein), is recruited from Ire1 by the rise in unfolded proteins [65]. Regardless, data are accumulating that alternative pathways have also evolved to dispose of misfolded ER proteins. One medically relevant substrate for which an alternative pathway for degradation has been demonstrated is AT-Z, the Z variant of AT (α 1-antitrypsin), an abundant protein that is produced in and secreted from the liver. Wild-type AT inhibits elastase in lung connective tissue, but AT-Z is misfolded and can be degraded via ERAD [66]. As a result, AT-Z homozygotes develop emphysema owing to unchecked proteolytic activity. However, AT-Z can also polymerize and form large aggregates that are retained in the ER [67], an event that triggers hepatic cell death and liver failure. By engineering mammalian cells and yeast strains in which AT-Z expression can be controlled, it was determined that AT-Z polymers are delivered to the lysosome and vacuole respectively, where they are degraded [68,69]. In mammalian cells, the lysosome-targeted polymers co-localize with markers of the autophagic pathway, a pathway in which cytoplasmic proteins are encapsulated into lipid vesicles and engulfed by the lysosome. Consistent with autophagic degradation of AT-Z, yeast deleted for autophagy-requiring genes are hypersensitive to high levels of AT-Z; in contrast, low amounts of AT-Z can be tolerated because the ERAD machinery is sufficient to remove this toxic protein [69]. In contrast, another AT mutant, known as AT-NHK (null Hong Kong mutant AT), does not form polymers and in some cells is degraded primarily by the proteasome [66,70]. Together, these data suggest that inherent differences in the expression levels of AT-Z and/or in ERAD efficiency might give rise to the noted variance in liver disease severity among AT-Z homozygotes [71].

Overall, it should be clear from this discussion that ERAD substrates possess widely different characteristics, and thus one might expect that the degradation of one substrate compared with another will exhibit diverse requirements. Although there are general rules that can be applied to ERAD substrates, at least in yeast, on the basis of where a misfolded domain resides [72], this hypothesis has gained credibility as new ERAD substrates are identified and the requirements for their degradation have been delineated.

MOST ERAD SUBSTRATES EXHIBIT DIFFERENT CHAPERONE REQUIREMENTS DURING BIOGENESIS

Even though each ERAD substrate possesses unique attributes, the fact that most chaperones engage their substrates by interacting with misfolded domains suggests that a common chaperone machinery should be responsible for the recognition and turnover of all ERAD substrates. In this section, the impact of individual chaperone classes on distinct ERAD substrates in both yeast and mammals is considered, and, as detailed below, it appears that the search for this common chaperone machinery may be in vain.

Calnexin and calreticulin: folding facilitators, murderous lectins or unique players?

The impact of calnexin and calreticulin on a process is usually examined by the application of small molecules {i.e. CST (castanospermine) and DNJ (deoxymannojirimycin) [73]} that block the trimming of the terminal glucose residues on N-linked oligosaccharides. As a result of their application, nascent glycoproteins in the ER are unable to interact with the lectins [74]. Mannose trimming, which may be essential to redirect some nascent proteins to the degradative lectins ([75–77] and see above), is inhibited by other small molecules {i.e. KIF (kifunensine) and deoxymannojirimycin [78]}. In theory, then, one can ascertain whether calnexin/calreticulin are involved in folding a protein and protect the substrate from ERAD (CST and DNJ should accelerate ERAD) (Figure 2A), or whether they are intermediates in the pathway by which proteins are targeted for ERAD (CST and DNJ should inhibit ERAD) (Figure 2B).

When these experiments are performed in mammalian cells expressing various substrates, disparate results have been reported. For example, it was found that the degradation of the polymer-forming AT-Z protein was slowed in the presence of CST and that some of the protein was even secreted [79]. In contrast, the degradation of the soluble mutant, AT-NHK, was accelerated in the presence of CST [70]. Evidence suggesting that calnexin/calreticulin are intermediates in the ERAD pathway and are thus 'pro-degradative' has been obtained for AT-NHK in a cell type other than that used above [80] and for wild-type CFTR (also see below; [81]). Minimal effects of CST addition on other ERAD substrates have also been noted, even under conditions in which disrupted calnexin–substrate association was confirmed [82–84].

The role of these quality-control lectins on CFTR protein biogenesis has also been examined. Both immature wild-type CFTR and the $\Delta F508$ variant interact with calnexin [85], but the importance of this interaction has remained something of a mystery. Degradation is unaffected in CFTR-expressing yeast deleted for calnexin [86], and, in mammalian cells, variable results have been obtained when the impact of calnexin on CFTR and $\Delta F508$ -CFTR biogenesis was examined. To some extent, the disparity in experimental outcomes derives from the unique attacks that have been used to explore calnexin function. For example, the degradation of $\Delta F508$ -CFTR was unaffected when cells were treated with CST, DNJ or KIF, but the degradation of wild-type CFTR required mannose trimming [87]. However, calnexin overexpression had no effect on wild-type CFTR biogenesis, but slightly accelerated the degradation of $\Delta F508$ -CFTR. In contrast, Kai and co-workers reported that calnexin overexpression slowed the ERAD of $\Delta F508$ and wild-type CFTR [88], and that the

overexpression of a calnexin fragment even led to the trafficking of a small amount of $\Delta F508$ -CFTR to the plasma membrane [89].

Calnexin, and possibly calreticulin, may function as pre-protein ‘stabilizers’ for only the most aggregation-prone substrates [90], and, on the basis of their similar lectin-like properties, one might suspect that calreticulin and calnexin affect ER protein biogenesis identically. Accumulating data indicate that this may not be the case. Although simultaneous binding of both lectins to a substrate can be observed (see, e.g., [91,92]), calreticulin and calnexin can interact with different groups of substrates. In fact, converting calnexin into a soluble protein and calreticulin into a membrane-anchored protein appears to swap their substrate specificities [93]. Some substrates are even known to bind to only one lectin or to the other (see, e.g., [94]), and the secretion and folding efficiencies of different proteins are uniquely altered when calnexin or calreticulin are knocked-down in mammalian cells [95].

Hsp70 and Hsc70: does the ‘p’ make a difference?

In any given species, Hsp70 and Hsc70 are 80–90% identical at the amino acid level. On the basis of this fact, and on the fact that the ATPase and peptide-binding activities of Hsp70 and Hsc70 are similar, it is generally assumed that the only difference between the homologues is that Hsp70s are stress-inducible [22]. However, data that ‘not all Hsp70s are created equal’ began to emerge from studies in yeast, in which different Hsp70s in a given cellular compartment possess unique activities (see, e.g., [96,97]), and more recently in cancer cells, indicating non-overlapping functions for cytoplasmic Hsc70 and Hsp70 [98]. Also, recently, experiments using *Xenopus* oocytes suggested that Hsc70 and Hsp70 have different effects on the biogenesis of CFTR and ENaC: the functional expression of these proteins was significantly reduced when oocytes were micro-injected with cRNA corresponding to Hsc70, but their functional expression increased when Hsp70 cRNA was instead introduced ([99], and R. C. Rubenstein, personal communication). These experiments were born of the observation that Hsp70/Hsc70 bind to CFTR [100–102], and that 4-phenylbutyrate enhances Hsp70 synthesis, decreases the levels of Hsc70, and improves the trafficking of $\Delta F508$ -CFTR to the plasma membrane [103]. In addition, purified Hsc70 retains in solution and prevents the aggregation of CFTR’s first nucleotide-binding domain (NBD1), the domain in which the disease-causing $\Delta F508$ mutation resides, and facilitates its folding [104]. Consistent with antagonistic functions between Hsc70 and Hsp70, overexpression of Hsc70 counteracted the protective effects of Hsp70 overexpression on ENaC levels in oocytes [99]. Nevertheless, it is important to note that Hsp70 overexpression on its own was reported to have no effect on wild-type or $\Delta F508$ -CFTR biogenesis, although the overexpression of both Hsp70 and an Hsp40 co-chaperone, Hdj1, stabilized immature (i.e. ER-resident) forms of the channel [105]. One model to explain these observations is that various cell types express different endogenous levels of the Hsp40s, which can pair with overexpressed partners to exert unique effects on the biogenesis of a secreted protein. In any event, the data suggest that Hsp70 and Hsc70 can perform unique functions in the cell, and, in the future, it will be exciting to discover whether the Hsp70/Hsc70 division of labour is mediated by distinct co-chaperone interactions, subcellular localizations or activities.

Hsp40s and ER protein biogenesis: the proverbial tip of the iceberg

As noted above, Hsp70/Hsc70 function requires Hsp40 interaction, and, in some cases, J-domain-containing co-chaperones can interact with polypeptides and prevent substrate aggregation [29,106–108]. Defining the action of these chaperones is hindered by the fact that each cellular compartment contains multiple Hsp40 subtypes, and the rules that govern whether a given Hsp40 interacts with all or a subset of Hsp70s in the same compartment are not clear. Regardless, there are notable examples in which Hsp40s can exert different effects during ERAD.

First, Cyr and colleagues reported that two cytoplasmic Hsp40s, Hdj1 and Hdj2, interact with CFTR, and, by synthesizing different CFTR fragments in coupled translation/translocation assays, these investigators found that Hdj2 binds to the channel only when NBD1 emerges from the ribosome [109]. In contrast, Hdj1 binds to CFTR throughout its synthesis [109]; consistent with the localization of Phe⁵⁰⁸ in NBD1 and that the deletion of this residue results in the complete degradation of the protein, ~2-fold more Hdj2 binds to Δ F508-CFTR than to wild-type CFTR. Secondly, Frizzell and co-workers found that overexpression of the Csp (cysteine string protein), a cytoplasmic Hsp40 previously implicated in vesicle trafficking [110], prevented the maturation of wild-type CFTR beyond the ER [111]. In accordance with a gatekeeping holdase-like function for this protein, Csp knockdown resulted in a significant increase in plasma-membrane-resident CFTR [112]. Interestingly, co-expression of Csp and Hdj2 led to an additive increase in ER-localized CFTR that was independent of Hsp70 association, and Csp on its own retained NBD1 in solution and prevented its aggregation [112]. These data suggested that Csp protects and retains CFTR in the ER during its synthesis. Thirdly, the ERAD of CFTR and Ste6p* [an aberrant form of a yeast ABC (ATP-binding cassette) transporter; see Table 1] requires two ER-associated Hsp40 chaperones in yeast [113,114], indicating that Hsp40s can also play pro-degradative roles. One of these Hsp40s has also been shown to facilitate the degradation of a cytosolically disposed ER membrane-tethered form of CPY* [115] and of a soluble cytosolic form of CPY* [116]. In line with a pro-degradative role of Hsp40s, CPY* is degraded poorly in yeast containing mutations in two luminal Hsp40s [117]. Overall, because the mammalian genome potentially encodes for ~40 Hsp40 homologues, it is likely that additional members of this chaperone family, working either with or in the absence of Hsp70/Hsc70, will be involved in ERAD and folding.

Unique effects of Hsp90 on protein folding and ERAD: it all depends on the substrate and how you make it

The Hsp90 chaperone exhibits a more restricted substrate specificity than Hsp70s, but, on the basis of its role in regulating cancer cell growth and its unusual ATP-binding pocket, specific inhibitors for the chaperone have been developed [34,118]. Using two different inhibitors, it was shown that Hsp90 helps to fold an enzyme reporter in mammalian cells, but that aborted folding results in proteasome-mediated degradation [119]. These data suggested that Hsp90 can switch between being a pro-folding and pro-degradative chaperone, and thus may decide a nascent polypeptide's ultimate fate. Consistent with this early study, a select number of secreted proteins has been shown either to require Hsp90 for folding (e.g. Ste6p* [114]) or degradation (e.g. a mutant form of the insulin receptor and apoB [120,121]). What is surprising, however, is that the administration of an Hsp90 inhibitor affects either the folding or degradation of CFTR, depending on how the experiment was performed. In one case, the Hsp90 inhibitor, GA (geldanamycin), freed Hsp90 from full-length CFTR and prevented its degradation after the wild-type channel had been translated in rabbit reticulocyte lysate and translocated into mammalian microsomes [122]. This result hinted that Hsp90 is important in facilitating the ERAD of CFTR. In contrast, GA enhanced the degradation of CFTR in mammalian cells [123], suggesting instead that chaperone release destabilizes CFTR and leads to its subsequent degradation. In support of these data, CFTR levels were examined in yeast containing a fast-acting thermosensitive allele in the gene encoding Hsp90: at the non-permissive temperature, CFTR was degraded faster than in wild-type cells, and purified Hsp90 maintained NBD1's solubility [113]. It is, of course, possible that Hsp90 is involved in both folding and degrading CFTR. If so, the observed role of the chaperone is dictated by the way in which the balance is tipped: a more folded conformation may allow Hsp90 to protect the protein until the final conformation is achieved, whereas a less mature form may be selected by Hsp90 for proteasome-targeting. Pertinent to this discussion is the recent report that Hsp90 co-chaperones can play either pro-folding or pro-degradative roles during Δ F508-CFTR

maturation in mammalian cells [124]. Overall, the construction of new tools that can better probe the conformations of ERAD substrates *in situ* will help address this hypothesis.

Disparate effects of cytoplasmic chaperones during the degradation of apoB

As should be apparent, chaperones may either hinder or facilitate the degradation of a given substrate, or even perform both functions. Furthermore, chaperones that are pro-folding or pro-degradative for one substrate may switch their roles, or have no effect, when another substrate is examined. Support for this notion is provided when one further considers the apoB-degradation pathway: using an *in vitro* assay in which the ERAD of apoB can be recapitulated using either hepatic or yeast lysate, it was found that both Hsp70 and Hsp90 are required for the degradation of this substrate [120]. In contrast, the Hsp110 chaperone, which has no impact on CFTR degradation in yeast or mammals [113] but facilitates the degradation of a soluble human protein in yeast [125], stabilizes apoB (S. L. Hrizo, J. L. Goeckeler, V. Gusarova, D. M. Habel, E. A. Fisher and J. L. Brodsky, unpublished work). Together, these observations indicate that chaperones are not one-trick ponies, but perform a diverse number of functions depending, potentially, on the family to which they belong, on the identity and conformation of the bound substrate and on co-chaperone partners.

CONCLUSIONS AND PERSPECTIVES

One model to explain some of the data presented here is that a given ERAD substrate can display a collection of aberrant conformations, and, consequently, that a distinct subset of chaperones recognizes the substrate. The resulting chaperone complex, which varies from substrate to substrate and is conformation-specific, dictates whether the substrate is ignored, can fold (or can be repaired if previously denatured) or should be targeted for ERAD (Figure 3). Inherent in this model are several assumptions: first, that distinct chaperone complexes bind to different substrates; secondly, that variations in the conformational state of a substrate dictate its fate; and thirdly, that chaperones can 'choose' to fold, ignore or degrade a substrate. Indeed, evidence supporting each of these assumptions exists. For example, even though there may be stable chaperone-containing complexes in the ER [126,127], different chaperone composites may bind to different polypeptide substrates and chaperone-substrate interactions and mobility in the ER appear to be quite dynamic ([128,129], and see above). In support of the second assumption, Kelly, Balch and colleagues analysed a large number of mutations in one aggregation-prone amyloidogenic secreted protein and concluded that the decision to select a protein for ERAD can best be described by a combined kinetic and thermodynamic 'score': mutant proteins outside a range of scores might aggregate or might be ignored by the ER quality-control system [130]. In support of the third assumption, individual chaperones have been identified that can differentially recognize wild-type and mutant forms of a substrate. In one germane example, we found that a member of the small Hsp (sHsp) family enhances the ERAD of $\Delta F508$ -CFTR, but has no effect on wild-type CFTR biogenesis. Furthermore, the sHsp binds preferentially to the mutated form of the channel [131]. These data suggest that sHsps specifically select $\Delta F508$ -CFTR for ERAD.

How do distinct chaperone combinations target a protein for ERAD? For soluble proteins in the ER, a key, yet poorly defined, step during ERAD is the delivery of the substrate back to the presumptive retro-translocation channel. To date, the identity of this channel is contentious, but a growing number of lectins, which includes the EDEM homologues and Yos9, probably act as intermediaries between BiP/calnexin-mediated recognition of a nascent polypeptide [132,133] and polypeptide retro-translocation (see above). Therefore it is possible that simultaneous binding or efficient coupling of BiP and BiP co-chaperones to these lectins may be necessary to trigger polypeptide disposal, as evident by the association between BiP and Yos9 in the yeast ER [134]. Moreover, different chaperone combinations may recruit unique

E3 ubiquitin ligases, which are important mediators of protein degradation via the UPP [43]. In one recent example, the ubiquitination of CFTR during its biogenesis was shown to be catalysed by the Rma1 E3 ubiquitin ligase, which resides in the ER membrane. In contrast, fully synthesized CFTR was ubiquitinated by CHIP (C-terminus of the Hsc70-interacting protein) [135], an E3 ligase that couples with a different E2 enzyme and associates with both Hsc70 and Hsp90 [102,136]. Notably, the interaction between CHIP and the chaperones is mediated by the N-terminal TPR domain [137]. However, the gp78 ubiquitin ligase is required for the disposal of another group of membrane-associated ERAD substrates in mammals [138,139] and binds to the chaperone-like p97 protein (see below; [140]). In the future, it will be interesting to examine the complete chaperone-relay (or parallel pathway) that directs Rma1 and gp78 to their substrates.

Finally, by no means is this review meant to suggest that all chaperones and chaperone-like proteins play multiple roles during ER quality-control decisions. For example, yeast Cdc48 (also known as VCP or p97 in mammals) is a member of the AAA ATPase family of protein 'engines' that forms hexamers and exhibits diverse functions in the cell, depending upon the accessory factor(s) with which it is coupled [141,142]. Purified Cdc48 maintains the solubility of aggregation-prone substrates [143], and is thus a *bona fide* chaperone. However, when Cdc48 is associated with the Ufd1 and Npl4 proteins, it can bind to the cytoplasmic face of the ER membrane and drive the extraction of ERAD substrates concomitant with ATP hydrolysis [144]. To date, no evidence has been presented to suggest that Cdc48 is required for the folding of a nascent protein in the ER. Nevertheless, it is important to keep in mind that new functions for chaperones and chaperone cofactors have emerged as the molecular determinants for the degradation of additional ERAD substrates are delineated. Needless to say, this pursuit will keep many of us busy for some time.

Abbreviations used

ABC, ATP-binding cassette
 apoB, apolipoprotein B
 AT, α 1-antitrypsin
 AT-NHK, null Hong Kong AT mutant
 AT-Z, Z variant of AT
 BiP, immunoglobulin heavy-chain-binding protein
 CFTR, cystic fibrosis transmembrane conductance regulator
 CHIP, C-terminus of the heat-shock cognate protein 70-interacting protein
 CPY*, carboxypeptidase Y mutant
 Csp, cysteine string protein
 CST, castanospermine
 DNJ, deoxynojirimycin
 EDEM, ER degradation enhancer, mannosidase α -like
 ENaC, epithelial sodium channel
 ER, endoplasmic reticulum
 ERAD, ER-associated degradation
 GA, geldanamycin
 HCMV, human cytomegalovirus
 HMGCoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase
 Hsc, heat-shock cognate protein
 Hsp, heat-shock protein
 KIF, kifunensine
 LDL, low-density lipoprotein
 MTP, microsomal triacylglycerol-transfer protein
 NBD1, nucleotide-binding domain 1

pαF, pro-α-factor
 TPR, tetratricopeptide repeat
 UBC, ubiquitin-conjugating enzyme
 UGGT, UDP-glucose:glycoprotein transferase
 UPP, ubiquitin–proteasome pathway
 UPR, unfolded protein response
 VLDL, very-low-density lipoprotein.

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Figure 1. The Hsp70 cycle

The ATPase domain of Hsp70/Hsc70 is indicated as being bound to either ATP or ADP. ATP hydrolysis is catalysed by a J-domain-containing Hsp40, which may interact with the Hsp70 on its own or with a bound polypeptide substrate (shown in orange). ATP hydrolysis induces a conformational change in the nucleotide- and peptide-binding sites, which results in lid closure and polypeptide trapping. ADP release, which may be triggered by an NEF (nucleotide-exchange factor), and ATP re-binding are required for polypeptide release.

Figure 2. Models for calnexin function in the ER

Calnexin (Cnx) and calreticulin (not shown) bind most avidly to monoglucosylated oligosaccharyl side chains, which result from trimming of the triply glucosylated species by glucosidases. Calnexin binding either may stabilize the bound substrate (**A**) or the binding may be competed for by other pro-degradative lectins that facilitate ERAD (**B**), such as EDEM or Yos9. The polypeptide is shown as a thin blue line, *N*-acetylglucosamine is shown as a yellow square, mannose is shown as a blue circle, and glucose is shown as an orange triangle.

Figure 3. A model for chaperone-based diversity during ER substrate degradation compared with folding

A wild-type polypeptide substrate (Substrate 1) is shown as a thin blue line. This polypeptide interacts with a unique chaperone composite (depicted here as an orange circle) that facilitates folding. In contrast, mutant (A or B) forms of the polypeptide result in conformers that favour interactions with other chaperone composites, of which only one may be sufficient to trigger ERAD (mutant A). This conformation may be found in other aberrant proteins (Substrate 2), and, assuming that the same chaperone composite binds to this mutated substrate, ERAD also occurs. Consequently, specific conformations, even in different proteins, may have identical chaperone requirements for ERAD. In contrast, a given substrate, and certainly different

substrates, depending on the nature of the mutation, may exhibit different chaperone requirements for ERAD or may even escape ERAD.

Table 1

Select ERAD substrates: properties and uses

Substrate	Topology	Description
pαF	Soluble	Wild-type pαF (pre-pro-α-factor) is a yeast preprotein that is triply glycosylated after its signal sequence (the 'pre' sequence) is removed in the ER; the glycosylation-defective mutant is retrotranslocated and degraded by the proteasome; pαF has been used to dissect the ERAD pathway <i>in vitro</i>
CPY*	Soluble	A point mutation in the vacuole-targeted enzyme, CPY (carboxypeptidase Y), results in a destabilized protein (CPY*) that is a widely used ERAD substrate in yeast; CPY* is glycosylated and disulfide-bonded and may traffic to the Golgi before degradation
AT-Z/NHK	Soluble	Antitrypsin (or the α1-protease inhibitor), Z variant (also known as α-1 antitrypsin, Z); the mutant, Z, allele gives rise to lung and, in some cases, liver disease; although soluble forms of AT-Z are degraded efficiently via ERAD, the protein is aggregation-prone and can be degraded via autophagy; the NHK (null Hong Kong) allele is also an ERAD substrate, and its folding/quality control is mediated by calnexin and EDEM, another ER lectin
ApoB	Soluble, secreted protein	~550 kDa plasma protein that is the major protein component in cholesterol-carrying LDL and VLDL particles; degradation is regulated in the ER membrane by phospholipids, cholesterol and/or oleic acid availability
MHCI	Single transmembrane segment	Normally resides at the plasma membrane and presents antigenic peptides; MHC1 ERAD is triggered by HCMV US2 and US11 gene products
Sec61	Polytopic (ten transmembrane segments)	Major component of the ER protein import (translocation) channel; mutant form is an ERAD substrate
CFTR	Polytopic (12 transmembrane segments)	Epithelial chloride channel that matures poorly in the ER and ~75% is an ERAD substrate; ~100% of the ΔF508 mutant form is an ERAD substrate, and the absence of this protein from lung epithelia results in cystic fibrosis (CF); a member of the ABC family of transporters, the protein contains six membrane-spanning segments, followed by a soluble nucleotide-binding domain (NBD1), a regulatory domain, and then another membrane-spanning domain and nucleotide-binding domain; Phe ⁵⁰⁸ (F508) resides near the end of NBD1
Ste6*	Polytopic (12 transmembrane segments)	Ste6 is a yeast ABC transporter that, like CFTR, resides at the plasma membrane, except that it lacks a regulatory domain; a C-terminally truncated form (Ste6*) becomes an ERAD substrate
HMGCoA-R	Polytopic (eight transmembrane segments)	Catalyses the rate-limiting step in cholesterol biosynthesis and thus its levels are tightly controlled by cholesterol and cholesterol-precursor levels, which, when in abundance, induce HMGCoA-R ERAD
ENaC	Polytopic, three subunits (two transmembrane segments per subunit)	Inefficient assembly of the three homologous subunits of ENaC (α, β and γ) results in ERAD; reduced ENaC activity at the plasma membrane leads to salt wasting and hypotension