ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(4): 219–226 **CN** 31-1940/Q

Minireview

Quality Control System of the Endoplasmic Reticulum and Related Diseases

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Abstract The quality control (QC) system of the endoplasmic reticulum (ER) is an important monitoring mechanism in the protein maturation process, which ensures export of properly folded proteins from the ER. Incorrectly or incompletely folded proteins are retained in the ER for refolding or degradation by the ER-residing proteasome. The calnexin/calreticulin cycle and ER-associated degradation are the key elements in QC. These two mechanisms work together to allow incorrectly folded proteins have additional opportunities to achieve their native conformations. The QC dysfunction is involved in many diseases caused by mutant proteins, many of which are causes of neurodegenerative disorders. A better understanding of molecular regulation in the QC system will uncover the molecular pathogenic mechanisms of many diseases caused by protein misfolding and help discover novel strategies for preventing or treating these diseases.

Key words protein folding; quality control; calnexin/calreticulin cycle; endoplasmic reticulum-associated degradation; protein misfolding disease

Introduction

The endoplasmic reticulum (ER) is a highly versatile protein processing factory that is equipped with chaperones and folding enzymes essential for protein folding. ER quality control (QC) guided by these chaperones is essential for life. Correctly folded proteins are exported from the ER, but misfolded proteins are retained and selectively degraded. Although the native conformation of a protein lies in its primary amino acid sequence, the ER greatly enhances the efficiency of protein folding. The compromised function of the ER QC system often leads the organism to suffer the overexpression of misfolded proteins, which may result in various diseases. The ER has unique oxidizing potential that supports disulfide bond formation during protein folding [1]. Chaperones and folding enzymes are abundant, greatly outnumbering the newly synthesized substrates. These folding factors in general prevent aggregation and thereby allow more efficient folding of a large variety of proteins. At least two main chaperone classes, binding protein (BiP) and calnexin/calreticulin, are active in the ER QC. Chaperones and folding enzymes are usually found in complexes. Recent work emphasizes more than ever that chaperones act in concert with co-factors and with each other [2]. Defective protein folding can lead to clinically significant pathologies as seen in cystic fibrosis (CF), Alzheimer's disease (AD), diabetes, familial hypercholesterolemia, and amyotrophic lateral sclerosis [3], as well as Huntington's disease (HD) [4], Creutzfeld-Jacob disease and alpha-1-antitrypsin (α1-AT) deficiency [5]. In this review, we highlight the latest advances in understanding how these chaperones and folding enzymes cooperate in assisting protein folding and mediating QC.

Molecular Chaperones

Mammalian secretory and membrane proteins are synthesized and translocated into the ER by the ribosome and Sec61 translocation machinery. During translocation, newly synthesized proteins immediately start to fold. Some soluble proteins fold relatively easily, whereas others have more difficulty and require more assistance from chaper-

Received: January 7, 2006 Accepted: February 20, 2006

This work was supported by the grants from the National Natural Science Foundation of China (No. 30370506) and the Doctoral Program of Higher Education of China (No. 20050285017)

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DOI: 10.1111/j.1745-7270.2006.00156.x

ones and folding enzymes. A chaperone is a kind of protein that will bind transiently to newly synthesized proteins. Calnexin and calreticulin are molecular chaperones involved in protein folding, assembly, and retention/retrieval. Calreticulin binds Ca^{2+} in the lumen of the ER with high capacity and also participates in the folding of newly synthesized proteins and glycoproteins. It is a component of the calreticulin/calnexin pathway [6]. Calreticulin consists of distinct structural and functional domains. The N-domain of calreticulin, together with the central P-domain, is responsible for the protein's chaperone function. Recent studies [7,8] with site-specific mutagenesis show that mutation of a single histidine residue (His153) in calreticulin's N-domain destroys the protein's chaperone function. The P-domain of calreticulin (residues 181–290) contains a proline-rich region, forms an extended-arm structure, and interacts with other chaperones in the lumen of the ER. The extended-arm structure is predicted to curve like that in calnexin, an ER integral membrane chaperone that is similar to calreticulin, forming an opening that is likely to accommodate substrate binding, including the carbohydrate-binding site (**Fig. 1**). As a molecular chaperone, calreticulin binds the monoglucosylated high mannose oligosaccharide (Glc1Man9GlcNAc2) and recognizes the terminal glucose and four internal mannoses in newly synthesized glycoproteins [9]. Both calreticulin and calnexin act as lectins and molecular chaperones, and they bind monoglucosylated proteins and associate with the thiol oxidoreductase ERp57, which is a protein disulfide isomerase (PDI)-like protein resident in the ER and promotes disulfide formation/isomerization in glycoproteins [10]. Together, calnexin, ERp57 and calreticulin comprise the so-called "calreticulin/calnexin cycle", which is responsible for QC and folding in newly synthesized (glyco) proteins. Folding substrates associate transiently with calnexin and calreticulin and enter cycles of de-glucosylation/re-glucosylation, and the process plays an important role in their association with the chaperones. Changes within the ER, such as alterations in the concentration of Ca^{2+}, Zn^{2+} or ATP, may affect the formation of these chaperone complexes and thus the ability of calreticulin to assist in protein folding [11]. The terminal glucose is trimmed by glucosidase (GLS) II and this may be important in the regulation of substrate-calreticulin interactions. The ER also contains a uridine diphosphate (UDP)-glucose, glycoprotein transferase (UGGT), which can re-glucosylate chains that have been glucose-trimmed. Together, UGGT and GLS II establish a cycle of de-glucosylation and re-glucosylation. Importantly, UGGT discriminates between folded and unfolded proteins, adding back a glucose residue to unfolded proteins only. This results in "rebuilding" of the monoglucosylated oligosaccharide on unfolded substrates, enabling them to interact with calreticulin and/or calnexin again. GLS then removes the terminal glucose residue, releasing the bound glycoprotein from its interaction with the chaperone again and again. This de-glucosylation/glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded [12] (**Fig. 2**).

Calreticulin is essential for normal calnexin chaperone function. In the absence of calreticulin, calreticulin substrates are not "picked up" by calnexin but accumulate in

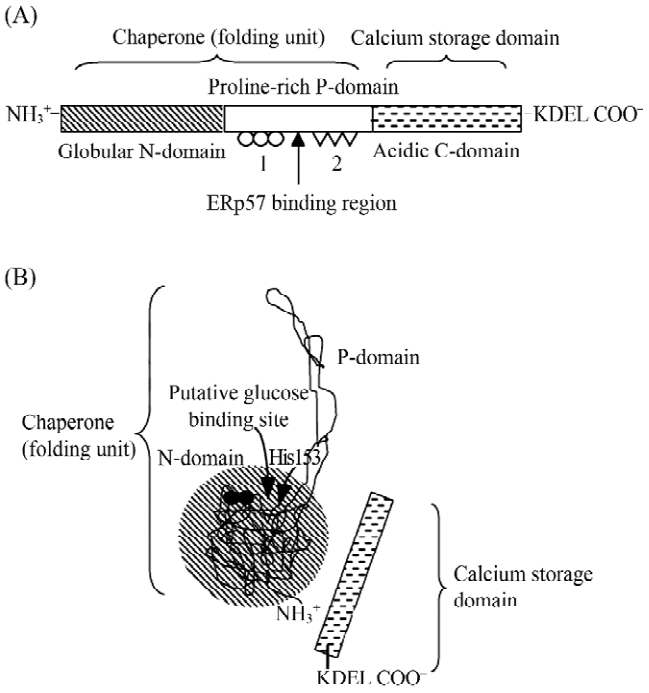

Fig. 1 Model of the 3-D structure of calreticulin

(A) Linear representation of calreticulin domains. The protein contains one N-terminal amino acid signal sequence and a C-terminal KDEL endoplasmic reticulum retrieval signal. Repeat 1 (amino acid sequence PXXIXDPDAXKPEDWDE) and Repeat 2 (amino acid sequence GXWXPPXIXNPXYX) are indicated by circles and triangles, respectively. (B) 3-D model of the N- and P-domain of calreticulin based on the NMR studies of the P-domain of calreticulin [15] and crystallographic studies of calnexin (1JHN) [16]. Calreticulin contains a globular N-domain and central proline-rich P-domain which forms a characteristic loop. The N+P-domain of calreticulin is responsible for a chaperone function of the protein. The C-terminal C-domain contains a large number of negatively charged amino acids and it is involved in high capacity Ca^{2+} storage. Black circles represent the cysteine residue (Cys88–Cys120), which form a C-C bridge in calreticulin. The locations of an essential His153 and a putative glucose-binding pocket are indicated. The N+P-domain is likely to be a chaperone region of calreticulin, whereas the C-domain is responsible for Ca^{2+} storage function in the endoplasmic reticulum lumen.

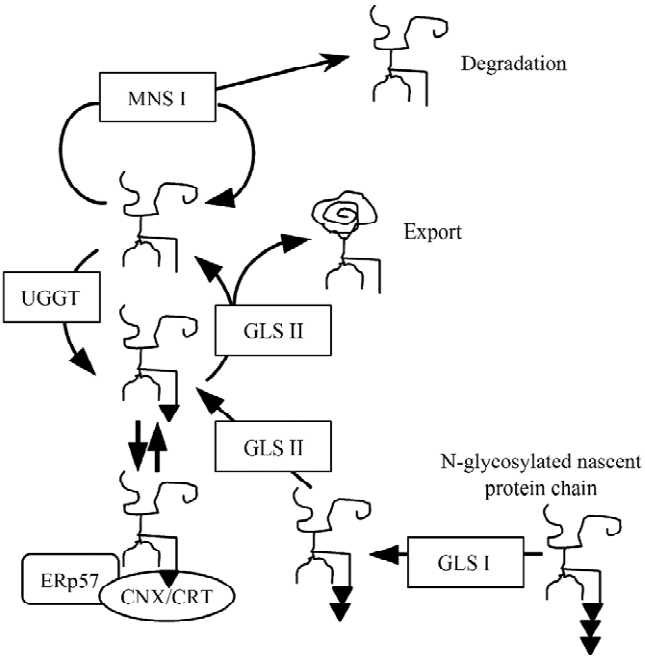

Fig. 2 Model for calnexin/calreticulin (CNX/CRT)-assisted glycoprotein folding in the endoplasmic reticulum (ER)

Nascent protein chains entering the ER lumen are N-glycosylated (black triangles) then immediately trimmed by glucosidase (GLS) I and II. Monoglucosylated glycoproteins bind calnexin or calreticulin and ERp57 complex. GLS II removes the remaining glucose residue if the proteins are correctly folded, preventing lectins interacting with the folding intermediates, and export from Sec61. If proteins are incorrectly folded, they are reglucosylated by UGGT. The deglucosylation and reglucosylation rounds continue until the new correctly folded glycoproteins are no longer reglucosylated and leave the cycle. Mannosidase (MNS) I recognizes the proteins that are always incorrectly folded, removes the oligosaccharides and then delivers them for degradation.

the ER lumen, resulting in the activation of the unfolded protein response (UPR), which is activated to induce transcription of ER-localized molecular chaperones [13]. Pancreatic ER kinase (PERK) and Ire1a, UPR-specific protein kinases, and eIF2a are also activated in the absence of calreticulin [14]. ER molecular chaperones of the calreticulin/calnexin cycle have overlapping and complementary but not redundant functions. The absence of one chaperone can have devastating effects on the function of the others, compromising overall QC of the secretory pathway and activating UPR-dependent pathways.

There are many other typical molecular chaperones in ER, such as BiP, glucose-regulated protein (GRP) 78, GRP94, and PDI [2]. BiP, GRP78 and GRP94 are members of the heat shock protein (HSP) family. They are the most abundant proteins in the ER lumen. The importance of the BiP/GRP94 system in protein folding comes from studies in yeast and mammalian cells. It is the second major ER chaperone system, which is only dependent on the presence of unfolded regions on proteins containing hydrophobic residues. In fact, some calnexin/calreticulin substrates can bind to BiP instead, if N-linked glycosylation is blocked. It has been proposed that during the translocation of a given glycoprotein into the ER a choice is made between chaperone systems (BiP/GRP94 or calnexin/calreticulin) depending on how soon an N-linked glycan occurs on the sequence [17]. PDI can help the formation of disulfide bonds that are the critical structure of protein secondary structure. PDI has two CXXC motifs as the active sites [18]. All of them depend on ATPase activity.

Pharmacological and Chemical Chaperones

Conformational diseases often result from mutations in proteins that are recognized as misfolded by the QC system. Such recognition can lead to two different results: some misfolded proteins can be efficiently ubiquitinated and degraded by the proteasome, leading to a loss of function [19]; whereas others accumulate in cells, forming aggregates that may have toxic consequences and are often referred to as "gain of functions" [20]. In the past decade, efforts to overcome these defects have led to the development of various interventions that successfully rescue proteins from both aggregation and degradation pathways. In particular, treatments with chemical compounds known as either chemical or pharmacological chaperones have been found to stabilize some conformational mutants, promoting their proper transport to their site of action where, in many cases, they can be functional [21–23]. Identifying compounds that can bind to the mutant proteins has been easier for proteins such as channels and receptors for which selective ligands have already been characterized. Because of their involvement in many pathophysiological conditions and the rich pharmacological diversity generated through various drug-screening campaigns, G-protein-coupled receptors have attracted considerable attention for the identification of pharmacological chaperones [24].

Recent studies have demonstrated that pharmacologically selective compounds, termed "pharmacoperones", the short-hand expression for pharmacological chaperones, can stabilize the misfolded receptors, facilitating their export from the ER to the plasma membrane, where they can be active. There are several examples, such as copper [25] and galactose [26]. Such functional rescue suggests that pharmacoperones could represent novel therapeutic

agents for the treatment of protein conformational diseases. Pharmacoperones were first discovered by the work carried out on V2 vasopressin receptor mutants responsible for nephrogenic diabetes insipidus [27]. ER-retained mutant receptors appeared largely as immature core-glycosylated receptor precursors, and they were processed to fully mature receptors harbouring complex carbohydrate arborisation following treatment with the cell permeable V2 vasopressin receptor antagonists. The antagonist treatment also increased the turnover rate of the precursor form of the receptor without affecting the half-life of the mature receptor, which is indicative of an action on the biosynthetic processes. Taken together with the pharmacological specificity of an action, these observations contributed to the emergence of the concept that pharmacoperones assist the folding and ER export of mutant G-protein-coupled receptors. The emerging hypothesis for the action of pharmacoperones suggests that selective lipophilic ligands can penetrate the plasma and ER membranes to bind to the partially folded receptor early during biosynthesis. Ligand binding might alter the thermodynamic equilibrium in favor of the correctly folded protein, increasing the likelihood of the protein escaping the stringent ER QC, and ultimately leading to an increase in the steady-state levels of functional receptors at the cell surface.

Chemical chaperones are chemical compounds of low molecular weight, such as dimethylsulphoxide (DMSO), trimethylamine N-oxide (TMAO) and glycerol, which can bind with mutant proteins unspecifically. Many data indicate that many of the mutant proteins adopt a conformation compatible with cell-surface transport when the folding and/or the degradation processes are slowed down by reducing the temperature (kinetic effect). This temperature-dependent recovery of Cl^- channels at the cell surface is mimicked by treating cells with the chemical chaperones. They are believed to function by stabilizing misfolded mutant proteins into conformations that are not targeted for degradation and can escape the ER (conformational effect). The well-studied examples are ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) [28] and α1-AT [29]. For example, glycerol treatment of cells that express ΔF508 CFTR caused an eight-fold increase in cAMP-dependent Cl^- currents. So some nascent ΔF508 molecules can fold correctly, thereby escaping degradation. However, none of the chemical chaperones shown to be active in cell systems could be used in clinical settings. This has led several investigators to search for more specific treatments that could be tolerated in humans.

ER-associated Protein Degradation

In cells, only proteins that have folded or assembled correctly are able to leave the ER, and those that fail to do so are disposed of by proteolytic degradation. This degradation of non-functional proteins was thought to occur within the ER itself, but it has since been recognized that these proteins are exported from the ER and degraded in the cytosol by a process known as ER-associated degradation (ERAD) [30]. This process is dependent on proteasomes and ubiquitin. Most membrane and all soluble proteins are thought to be retrotranslocated to the cytosol by a protein-conducting channel, the Sec61p complex, which also mediates the import of proteins into the ER. Proteasomal degradation of cytosolic proteins is preceded by polyubiquitination which functions as a degradation signal for the proteasome. Polyubiquitination is mediated by the coordinated action of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s), which determine the specificity of the ubiquitin proteasome system. Proteins that fail to fold correctly, however, are retrotranslocated from the ER to the cytosol, and are then recognized by ER-specific E3 ligases that mediate polyubiquitination of the misfolded protein on the cytoplasmic side of the ER, and are subsequently degraded by the proteasome. Several E2s and E3s are located at the ER membrane with their domains active in polyubiquitination facing the cytosolic side [31]. In addition to acting as a degradation signal for the proteasome, polyubiquitination may help to ensure the directionality of the retrotranslocation process by acting as a binding signal for the AAA ATPase p97/Cdc48, which is required for retrotranslocation of several ERAD substrates. Some examples demonstrate that retrotranslocation and proteasomal degradation are not as tightly coupled. Several ERAD substrates are retained in the ER when polyubiquitination is inhibited but are found in the cytosol upon inhibition of the proteasome, for example, major histocompatibility complex class I (MHC I) heavy chain or carboxypeptidase Y. These findings suggest that polyubiquitination and retrotranslocation are coupled processes that are functionally independent of proteasomal activity [32].

QC System and Human Diseases

It is clear that a protein's conformation determines its function, and the folding of a protein after synthesis plays a very important role in the process of the protein's

maturation. QC is an inspector in the ER to ensure the correction of the process. If a protein folds correctly, it will be secreted to the right location and exert its function there. If it is unfolded or incorrectly folded, it will probably lose its function and be degraded through ERAD or aggregates in the ER. However, aggregation of misfolded or incorrectly folded proteins is lethal. When the QC system breaks down, it will probably cause the dysfunction of proteins, and it will lead to pathogenesis of diseases. Research has discovered the relationship between QC and diseases, such as CF, α1-AT, polycystic liver disease (PCLD), Gaucher disease, AD, PD, HD, and so on. Some diseases are caused by dysfunction of mutant proteins, like PCLD. *PRKCSH* and *SEC63* are the two genes that are responsible for PCLD, which encode hepatocystin and sec63 respectively [33]. Both proteins are involved in translocation through the ER membrane and in the oligosaccharide processing of newly synthesized glycoproteins. Mutant hepatocystin results in reduction of the protein levels of normal hepatocystin and GLS II α-subunit, whereas mutant sec63 causes the cytosolic accumulation of full-length protein precursors. Other diseases are caused by the QC excessive degradation of mutant proteins. These proteins can function normally in certain conditions even though they are mutated. But the QC system clears them because of misfolding caused by their mutations, so that the functional proteins are lost. In the case of CF, although the protein CFTR is mutated, it can be corrected when the temperature is reduced to 26 ºC [28].

In this review, we mainly focus on the role of QC in neurodegenerative diseases.

QC System and Neurodegenerative Diseases

Protein misfolding and aggregation occur in most neurodegenerative diseases. This fact has led to the widely accepted view that protein QC plays a critical role in neuronal function and survival. Its importance is underscored by studies showing that manipulating QC pathways alters the pathogenesis of neurodegenerative diseases [34]. Early research implied that aggregations of protein are the biochemical hallmark of several neurodegenerative diseases.

In the last few years, evidence has accumulated that supports the premise that the ubiquitin proteasome system (UPS) [35], which some reports also call it the ubiquitin proteasome pathway [36], plays a role in many neurodegenerative diseases. The UPS is also a major player in cellular protein quality control, and is involved in the degradation of misfolded and other aberrant proteins. Most neurodegenerative diseases are characterized by intracellular deposits of aggregated and misprocessed proteins, many of which are proteasomal components and substrates. Furthermore, several mutations in UPS components have been associated with neurodegenerative diseases, and it is therefore highly conceivable that the UPS is involved in the neuropathogenesis of these diseases.

QC System and AD

AD is a well-researched disease. The aberrant and misprocessed proteins that accumulate in the brains of AD patients constitute the neuropathological hallmarks of the disease. The two most pronounced hallmarks are neurofibrillary tangles, formed by intracellular accumulations of the hyperphosphorylated protein tau, and plaques, which are extracellular deposits of the 40–42 amino acid amyloid peptide, processed from the amyloid precursor protein. The presence of ubiquitin and ubiquitinated proteins in the brain is the initial clue suggesting that the UPS is involved in the pathogenesis of AD. An aberrant form of ubiquitin (UBB^{+1}) also accumulates in the neuropathological hallmarks of AD. This UBB^{+1} is translated from ubiquitin-B mRNA, which contains a dinucleotide deletion near a GAGAG repeat. The two nucleotides are likely to be deleted during or after transcription, as the mutation cannot be detected in the *UbB* gene of AD patients. UBB^{+1} accumulates in the earliest affected brain areas of patients with AD, such as neurons in the transentorhinal hippocampal cortex area. All three proteins mentioned earlier, tau, amyloid peptide and UBB^{+1}, accumulate in the AD brain, and all were reported to affect the proteasomal pathway [37].

QC System and HD

HD is also induced by the aggregation of aberrant proteins. HD is caused by an expansion of the polyglutamine tract in the protein named huntingtin. The bio-hallmark of mutant huntingtin is the formation of intranuclear inclusions and cytoplasmic aggregates in neurons in vulnerable brain areas. Expression of mutant huntingtin in cultured cells also causes the formation of intranuclear inclusions and aggregates in the cytoplasm. The inclusions and aggregates are usually formed by small N-terminal huntingtin fragments and are co-localized with other cellular proteins involved in proteolysis, vesicle trafficking and protein degradation. The formation of huntingtin aggregates and intranuclear inclusions has been proposed to play a role in

HD pathogenesis [38].

Polyubiquitinated proteins are targeted for degradation by the proteasome, which is a large enzymatic complex found in all eukaryotic cells. The proteolytic core of the proteasome or "20S proteasome" is a 28 subunit multicatalytic particle consisting of four heptagonal rings. The 20S proteasome can also be associated with one or two 11S (PA28) particles, which consist of α- and β-subunits that can be induced by interferon γ (IFN-γ) and result in so-called immunoproteasomes. The association of 11S and 20S particles is thought to also lead to a rearrangement of α-subunit chains, resulting in the widening of the openings to the 20S barrel, thereby facilitating the access of substrates and the exit of peptide fragments at the other end of the proteolytic chamber. In response to IFN-γ, hybrid proteasomes can also assemble into particles consisting of 20S proteasomes with a 19S particle at one end and an 11S particle at the other [39]. As a result of this switch in regulatory particles, there is an increase in ATP-independent degradation of small peptides, but not proteins [40]. In mammals, IFN-γ also induces changes in the 20S proteasome; and the three catalytic subunits in 20S particles are replaced by IFN-γ inducible subunits, namely LMP2 (βi1), LMP10 (βi2) and LMP7 (βi5) [41]. Proteasomes are referred to as immunoproteasomes when they contain the inducible subunits. Oxidative modification might make proteins susceptible to degradation by the immunoproteasome, facilitating peptide generation and antigen presentation [42]. A significant increase in LMP2 and LMP7 subunits was found in HD, indicating an induction of the immunoproteasome. This increase correlates with a rise in 20S proteasome activity assayed with fluorogenic substrates that are processed in a Ub-independent manner. The immunoproteasome induction seems to take place specifically in degenerating neurons in both huntingtin transgenic mice (HD94) and HD patient brain extracts [43]. Cell lines expressing polyglutamine expansion constructs also show an increase in LMP2 subunit expression [44]. These results imply that the immunoproteasome can be involved in neurodegeneration.

QC System and Prion Diseases

Prion (proteinaceous infectious particles) diseases are fatal neurodegenerative disorders that have attracted enormous scientific attention because they exemplify a novel mechanism of biological information transfer based on the transmission of protein conformation rather than on the inheritance of nucleic acid sequence. PrP^{Sc} is the key protein involved in the origin of prion disease. It is a conformationally altered isoform of the cellular protein PrP^{C}, a protein of unknown function that is encoded by a cellular gene [45]. PrP^{Sc} and probably other neurotoxic forms of PrP are structurally distinct from PrP^{C}, and these molecules would be recognized as "abnormal" by the ER QC machinery. They are retained in the ER and degraded by proteasomes. It is possible that PrP^{Sc} damages neurons by activating stress-induced signaling pathways that are engaged by the accumulation of misfolded proteins in the ER, such as UPR. Furthermore, some other PrP molecules carrying disease-related mutations are only partially trapped, and a fraction of them are able to reach the cell surface. Retained PrP might trigger ER stress response pathways, those that reach the surface are known to become aggregated and weakly proteinase K-resistant and might damage cells by altering membrane properties, interacting abnormally with other proteins, or other mechanisms. Interestingly, in Tg (PG14) mice, an animal model of prion, mutant PrP accumulates continuously as the animals age, until a threshold level is reached for the development of neuropathology and clinical symptoms. This phenomenon could reflect a gradual decrement in the efficiency of the proteasomal degradation system during aging, with consequent build-up of mutant PrP in the ER. If ER retention of mutant PrPs does occur in familial prion diseases, then this finding would link these diseases to other human disorders, both inherited and sporadic, that are thought to involve the action of protein QC mechanisms [46]. These include other neurodegenerative diseases such as AD, HD and PD, as well as disorders that affect peripheral organ systems such as CF, congenital hypothyroidism, and α1-AT deficiency. The common theme of all of these diseases is thought to be the misfolding of a polypeptide that is recognized by the cell as abnormal and is then subject to retention in the ER (for secreted or membrane proteins) or sequestration in cytoplasmic inclusions (for soluble proteins), followed by proteasomal attack.

Summary

It remains unclear how the ER decides at the molecular level between the protein folding and ERAD pathways. The kinetics of the folding and degradation pathways may simply differ, as glucosidase activity is suggested to be higher than mannosidase activity. This may favor the folding cycle over ERAD. After multiple rounds of chaperone binding, proteins that inefficiently fold are eventually

targeted for degradation. However, as the molecular mechanisms of a growing number of genetically inherited diseases are uncovered, it is increasingly appreciated that errors in folding and cellular trafficking are more frequent than anticipated. Thus, the development of strategies aimed at promoting proper folding and maturation of mutant proteins could provide new therapies for a wide spectrum of diseases.

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Edited by
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