FEBS Letters 581 (2007) 3641–3651

Minireview

ER chaperones in mammalian development and human diseases

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Received 16 February 2007; revised 13 April 2007; accepted 18 April 2007

Available online 25 April 2007

Edited by Roberto Sitia

Abstract The field of endoplasmic reticulum (ER) stress in mammalian cells has expanded rapidly during the past decade, contributing to understanding of the molecular pathways that allow cells to adapt to perturbations in ER homeostasis. One major mechanism is mediated by molecular ER chaperones which are critical not only for quality control of proteins processed in the ER, but also for regulation of ER signaling in response to ER stress. Here, we summarized the properties and functions of GRP78/BiP, GRP94/gp96, GRP170/ORP150, GRP58/ERp57, PDI, ERp72, calnexin, calreticulin, EDEM, Herp and co-chaperones SIL1 and P58^{IPK} and their role in development and diseases. Many of the new insights are derived from recently constructed mouse models where the genes encoding the chaperones are genetically altered, providing invaluable tools for examining the physiological involvement of the ER chaperones in vivo.

Keywords: Endoplasmic reticulum; Chaperones; Mammalian development; Diseases

1. Introduction

The endoplasmic reticulum (ER) is an essential cellular compartment for protein synthesis and maturation. It also functions as a Ca^{2+} storage organelle and resource of calcium signals. The perturbation of ER functions, such as disruption of Ca^{2+} homeostasis, inhibition of protein glycosylation or disulfide bond formation, hypoxia and virus or bacteria infection, can result in accumulation of unfolded or misfolded proteins and the failure of the ER to cope with the excessive protein load. This leads to ER stress, which is defined as an imbalance between the cellular demand for ER function and ER capacity. To reduce the excessive protein loading, the cells trigger the unfolded protein response (UPR), which signals transient attenuation of protein translation, degradation of malfolded proteins and the induction of molecular chaperones and folding enzymes to augment the ER capacity of protein folding and degradation. However, if the ER stress cannot be relieved, apoptotic pathways are activated in the damaged cells. The ER contains a number of molecular chaperones physiologically involved in the post-translational modification, disulfide bond formation, folding, assembly and quality control of newly synthesized proteins to preserve cellular homeostasis. Upon ER stress, upregulation of ER chaperones is pivotal for cell survival by facilitating the correct folding and assembly of ER proteins and preventing their aggregation. Furthermore, specific chaperones are also involved in stress signaling regulation and protein degradation process to attenuate apoptotic stimuli. While the link between molecular chaperones and human diseases awaits direct proof in most cases, recent construction and characterization of novel mouse models where the gene encoding for the ER chaperone protein is deleted or genetically altered provide new insights on the physiological contribution of these proteins in vivo. This review highlights recent progress in understanding the role of ER chaperones in response to ER stress and their functional roles in mammalian development and human diseases.

2. Chaperoning function of ER proteins

The ER chaperones can be categorized into three groups: (a) chaperones of heat shock protein family including GRP78, GRP94 and the co-chaperones; (b) chaperone lectins like calnexin, calreticulin and EDEM; and (c) substrate-specific chaperones such as Hsp47. Additionally, there are at least two groups of folding catalysts, namely thiol oxidoreductases of the protein disulfide isomerase (PDI) family such as PDI and GRP58/ERp57 and peptidyl prolyl isomerases (PPIs). There are two known chaperone systems in the ER, calnexin/calreticulin and GRP78/GRP94 [1]. The properties and function of ER chaperones, co-chaperones and folding enzymes covered in this review are summarized in Tables 1 and 2.

2.1. Calnexin/calreticulin chaperone system

Calnexin is a 90 kDa type I ER membrane protein and calreticulin is a 60 kDa soluble ER lumen protein with a C-terminal KDEL signal [2,3]. When the newly synthesized polypeptides enter the ER, they are often modified by N-linked glycans ($Glc_3Man_9GlcNAc_2$) and the glucoses are rapidly removed by glucosidases I and II [4]. The nascent protein with monoglucosylated N-linked glycans ($Glc_1Man_9GlcNAc_2$) is recognized by the calnexin/calreticulin system for subsequent

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Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; HHcy, hyperhomocysteinemia; PDI, protein disulfide isomerase; PrP^{sc}, scrapie-associated PrP; STEC, Shiga toxigenic *Escherichia coli*; SubAB, AB5 subtilase cytotoxin; UPR, unfolded protein response

0014-5793/$32.00
doi:10.1016/j.febslet.2007.04.045

Table 1
Summary of function and disease relevance of ER chaperones, co-chaperones and folding enzymes

Protein	Localization	Function	Knockout mouse model	Diseases	Reference
GRP78/BiP	ER lumen ER transmembrane Cell surface Nucleus	Chaperone, Ca^{2+}-binding, ER stress sensor UPR regulator Anti-apoptosis	Embryonic lethality at E3.5 due to failure of embryo peri-implantation	Cancer Alzheimer's disease Parkinson's disease Prion diseases Atherosclerosis	[34,57,65,71,75–78,107]
SIL1	ER lumen	Co-chaperone, nucleotide exchange factor for GRP78	Woozy mouse associated with cerebellar Purkinje cell degeneration and ataxia	Marinesco-Sjögren syndrome	[58–60]
GRP94/gp96	ER lumen Cell surface transmembrane	Chaperone, Ca^{2+}-binding, Anti-apoptosis Tumor immunity	Embryonic lethality	Cancer Prion diseases Autoimmune disease	[65,71,86,87,89]
GRP170/ORP150	ER lumen	Chaperone, potential nucleotide exchange factor for GRP78	Embryonic lethality	Alzheimer's disease	[57,61,129]
GRP58/ERp57	ER lumen Nucleus Cytosol	Thio-oxidoreductase to catalyze disulfide bond formation of glycoprotein	Embryonic lethality (traditional knockout); *Grp58*$^{-/-}$ B cells are defective in antigen presentation (conditional knockout in B cells)	Prion diseases Alzheimer's disease	[35,62,92,130,131]
PDI	ER lumen Cell surface	Thio-oxidoreductase to catalyze disulfide bond formation	ND	Alzheimer's disease Parkinson's disease	[55,92,132]

folding and assembly steps. This process can be co-translational and release of the completely folded glycoprotein from this cycle is usually coupled with transportation from the ER to the Golgi complex. GRP58/ERp57, a member of the PDI family, is also an important component involved in the calnexin/calreticulin system. It contains two thioredoxin motifs and acts as a thiol oxidoreductase to catalyze the disulfide bond formations of the loaded glycoproteins [5]. If the glycoprotein cannot be correctly folded, another ER protein UGGT (UDP-glucose glycoprotein-glucosyltransferase) recognizes the unfolded or misfolded glycoprotein and catalyzes the transfer of a glucose unit from UDP-glucose to a specific mannose residue within the *N*-glycan chain of the glycoprotein [6]. Reglucosylation generates the monoglucosylated *N*-linked glycan that provides a new binding site of calnexin/calreticulin for re-entry of the glycoprotein into the cycle, until the correct folding is achieved [7]. Studies with hydrophobic peptides and glycoproteins have shown that UGGT recognizes surface-exposed hydrophobic regions [8,9]. While it was reported that UGGT can sense a local subtle structural alteration generated by a single point mutation and monoglucosylate glycans distant from misfolded determinants [10], another study using RNaseA/B as the substrate found that UGGT only selectively modifies the *N*-glycans close to or within the unfolded sites [11].

2.2. GRP78/GRP94 chaperone system

GRP78, also known as BiP, is the ER homologue of HSP70 proteins with a conserved ATPase domain and a peptide-binding domain [12,13]. As a chaperone, GRP78 recognizes and binds to the proteins with hydrophobic residues in the unfolded regions [14]. Therefore, some calnexin/calreticulin substrates can bind to GRP78 if the *N*-glycosylation is blocked. GRP78 is in a large multi-protein complex with a set of ER molecular chaperones, GRP94, PDI, ERp72, GRP170/ORP150, UGGT, CaBP1 (calcium binding protein), cyclophilin B and SDF2-L1, which forms an ER chaperoning network processing the unfolded protein substrates [15]. In this complex, GRP94, an ER homologue of HSP90 protein, often functions as a dimer providing a platform for the assembly or oligomerization of loaded protein cargo [16].

3. Regulation of stress signaling by ER chaperones

Cells developed an evolutionarily conserved integrated intracellular signaling cascade, referred to as the UPR, to reduce the unfolded protein load and increase folding capacity. For survival, the UPR signals pathways attenuating protein synthesis, upregulating the transcription of chaperone genes that increase ER capacity of folding and degradation, and retro-translocating misfolded proteins to the cytosol for degradation. There are three major UPR pathways with the ER-resident transmembrane proteins PERK, ATF6 and IRE1 as proximal signal sensors. Molecular chaperones play regulatory roles in UPR signaling pathway. The best characterized is the ER chaperone GRP78 which directly interacts with all three ER stress sensors, PERK, ATF6 and IRE1, and maintains them in inactive forms in non-stressed cells [17]. When accumulation of misfolded proteins occurs, GRP78 is titrated away. Release from GRP78 allows the activation and transduction of the unfolded protein signals across the ER membrane to the cytosol and the nucleus. Further characterization of post-translational modification of the ATF6 reveals

Table 2
Summary of function and disease relevance of ER chaperones, co-chaperones and folding enzymes

Protein	Localization	Function	Knockout mouse model	Diseases	Reference
Calnexin	ER transmembrane Cell surface	Chaperone, glycoprotein folding	Postnatal death and motor disorders	Alzheimer's disease	[46,57,93]
Calreticulin	ER lumen Cytosol Cell surface	Chaperone, glycoprotein folding, Ca^{2+}-binding	Embryonic lethality at E14.5 due to defective embryonic cardiac development	Cardiac hypertrophy Alzheimer's disease Autoimmune diseases	[36,91,131,133,134]
EDEM	ER lumen	Chaperone, recognition and targeting of unfolded glycoprotein for degradation	ND	ND	[30]
ERp72	ER lumen	Thio-oxidoreductase to catalyze disulfide bond formation	ND	ND	[135]
Herp	ER transmembrane	Ubiquitin-like protein involved in ERAD	ND	Atherosclerosis Alzheimer's disease	[109,136,137]
$P58^{IPK}$	Cytosol ER membrane	Co-chaperone, negative regulator of eIF2α kinase PERK and PKR, cotranslocational degradation	Development of diabetes associated with increased β-cell death	Diabetes (mouse)	[22,23,25,118,119]
UGGT	ER lumen	Glucosyltransferase, recognition of misfolded glycoprotein and reglucosylation of *N*-glycan	Embryonic lethality at E13	ND	[37]

that the glycosylation and disulphide bond status of the luminal domain of ATF6 can be utilized as novel sensing mechanisms for the activation of the UPR [18,19]. These studies show that ER stress-induced underglycosylation and reduction favor the transportation of ATF6 from ER to Golgi and the cleavage by S1P and S2P to generate the active nuclear form of ATF6.

For IRE1 signaling, different mechanisms have been reported for its activation. A recent study shows that dimerization of the cLD region (the core region of luminal domain) of the yeast IRE1 retains the full function of the IRE1 luminal domain but is insufficient for the activation, rather the cLD dimer presents an MHC-like groove that is proposed to interact with unfolded proteins and initiate the IRE1 activation [20]. On the other hand, the monomer of the human IRE1α N-terminal luminal domain (NLD) is able to form a dimer that exhibits an MHC-like groove at the interface [21]. Dimerization of the human NLD is able to activate both the kinase and RNase activities of IRE1α and the MHC-like groove is too narrow to bind any peptide, implicating that unfolded protein binding is not required for the activation of IRE1α. Therefore, the study of human IREα supports the conventional view that GRP78 is the primary mechanism for regulating IRE1α activation, whereas the yeast study implies that peptide interaction is the key triggering event. Further studies are required to resolve these apparent differences.

Another ER stress inducible chaperone, $P58^{IPK}$, is proposed to be a negative regulator of PERK activation to inhibit eIF2α phosphorylation and attenuate the UPR [22]. $P58^{IPK}$ belongs to the HSP40 family and contains the tetratricopeptide repeats (TPR) motifs that have been shown to mediate protein-protein interaction [23]. Therefore, it is considered as a co-chaperone. Originally, $P58^{IPK}$ was identified in the cytosol to repress the kinase activity of PKR, the interferon-induced eIF2α kinase, through the direct association with PKR [24]. Instead of the cytosolic localization, $P58^{IPK}$ was recently reported to be a peripheral membrane protein of the rough ER, and in addition to modifying PERK signaling, $P58^{IPK}$ contributes to ER stress induced protein degradation through complex formation with the ER translocon and cytosolic HSP70 chaperone network [25]. However, more recently, it was determined that $P58^{IPK}$ contains a functional ER targeting signal consisting of 26 hydrophobic amino acids at the N-terminus (David Ron, personal communication), implicating that $P58^{IPK}$ may enter the ER. Thus, the precise orientation of the protein complexes and how $P58^{IPK}$ facilitates extraction of the stalled proteins from the ER requires further resolution.

4. ER chaperones facilitate ER-associated protein degradation

The ER employs a mechanism termed ER-associated degradation (ERAD) to clear the aggregated misfolded or unassembled proteins. During ERAD, the target proteins selected by ER quality control system are retrotranslocated to the cytosol and degraded by the ubiquitin–proteasome system. ER chaperones play key roles in ERAD substrate selection and solubilization [26]. The selection process relies on factors that primarily recognize substructures within the misfolded proteins such as hydrophobic patches, unpaired cysteines and partially de-mannosylated *N*-glycans. For example, in the mammalian calnexin/calreticulin system, the glycoproteins undergoing futile folding attempts are eventually subject to terminal mannose trimming (from $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$, or extensive trimming to produce $Man_6GlcNAc_2$ or $Man_5GlcNAc_2$) [27] by ER α 1, 2-mannosidases, leading to

their recognition by EDEM, a class of mannosidase-like proteins, and EDEM then targets misfolded mannose trimmed glycoproteins to degradation [28]. Three EDEM homologues, EDEM1, EDEM2 and EDEM3, have been identified and they are transcriptionally upregulated upon ER stress by the activated IRE1/Xbp-1 branch [29]. EDEM is required for ERAD of glycoproteins and overexpression of EDEM1 accelerates the release of unfolded glycoproteins from calnexin/calreticulin cycle for the onset of degradation [30]. How EDEM delivers substrates to the ER translocon remains to be determined, however, the association of EDEM with putative components of the translocon pore (derlins) has recently been reported [31].

In contrast to EDEM, GRP78 with its co-chaperones selects the ERAD substrates by recognizing the hydrophobic regions of the polypeptides that are usually exposed on the surface of misfolded or unassembled proteins. Simultaneously, they also retain the terminally misfolded proteins in soluble conformations preventing their aggregation in the ER lumen. Apparently all known soluble ERAD substrates require GRP78 and co-chaperones for their degradation [26,32]. After release from GRP78 in an ATP-dependent manner, the targeted misfolded proteins are sorted to the ERAD pathway. PDI is also known as a chaperone that plays a role in ERAD, in addition to its enzymatic activities. PDI has been shown to specifically interact with disulfide-free, misfolded secretory proteins in yeast and target them to the Sec61 translocon for degradation [33].

5. ER chaperone function is obligatory for early mammalian development

Gene knockout technology allows definitive tests for the requirement of specific ER chaperone function in vivo. Traditional knockout approach creating homozygous deletion of ER chaperones such as GRP78 [34], GRP94 (Mao and Lee, in preparation), GRP58/ERp57 [35], calreticulin [36] and UGGT [37] results in embryonic lethality. The phenotypes of mouse knockout models of the ER chaperones, co-chaperones and folding enzymes covered in this review are summarized in Tables 1 and 2. These studies provide direct evidence that the function of each of these ER chaperones cannot be compensated during mouse development. Thus, heterozygous mutants and/or tissue-specific knockout mouse models are required to elucidate the critical contribution of these chaperones in mammalian development.

For GRP78, it has recently been shown that complete depletion of GRP78 leads to lethality in 3.5-day-old embryos (E3.5) due to the failure of embryo peri-implantation [34]. The *Grp78*$^{-/-}$ embryos cannot hatch from the zona pellucida in vitro, fail to grow in culture, and exhibit proliferation defects and a massive increase of apoptosis in the inner cell mass, which are precursors of embryonic stem cells. These findings show that GRP78 is essential for embryonic cell growth and pluripotent cell survival. In another study, transgenic mouse lines bearing a *lacZ* reporter gene driven by 3 kb of the rat GRP78 promoter or a mutant promoter with ER stress-response element (ERSE) deletion were used to evaluate the transcriptional regulation of GRP78 during mouse embryonic development. GRP78 is transcriptionally upregulated in both the trophectoderm and inner cell mass of E3.5 embryos and this induction is largely dependent on the ERSE. Since ERSE is an essential *cis*-element of the Grp78 promoter for induction by ER stress, this suggests that physiological ER stress may exist at the peri-implantation stage of early development due to the increased activity of cell proliferation and protein secretion. On the other hand, *Grp78*$^{+/-}$ mice are viable and comparable to wild-type although GRP78 level in the heterozgyotes is about 50% of the wild-type siblings. The GRP94 and PDI levels are mildly elevated in the *Grp78*$^{+/-}$ mice, whereas the levels of the other two ER chaperones, calnexin and calreticulin, are not affected. Thus, during normal mouse development, 50% of wild-type GRP78 level is apparently sufficient to maintain ER homeostasis. This is consistent with the view that compared to normal tissues and organs, GRP78 is more critically needed in cells undergoing physiological or pathological stress.

Embryonic lethality was also observed in the GRP58/ERp57 knockout mice whereas the conditional knockout mice with GRP58/ERp57 deficiency in B cells are viable [35]. The development and proliferation of GRP58/ERp57 deficient B cells are normal, but the MHC class I antigen presentation is impaired. During biosynthesis, MHC class I heavy chain is processed in the calnexin/calreticulin system where GRP58/ERp57 participates in oxidative folding by catalyzing disulfide bond formation and isomerization [38]. GRP58/ERp57 is also detected in the peptide-loading complex consisting of TAP1/TAP2, the MHC class I-specific chaperone tapasin, calreticulin, GRP58/ERp57 and MHC I heterodimers [39], implicating that GRP58/ERp57 plays a role in loading of peptides onto MHC class I molecules. In the GRP58/ERp57-deficient B cells, the amount of cell surface MHC class I-peptide complexes and the peptide loading complex-associated MHC class I are dramatically decreased compared with the wild-type B cells and the dissociation of MHC class I from the complex is much faster. These observations indicate that GRP58/ERp57 recruits MHC class I to the peptide-loading complex and maintains the peptide-MHC I complex in a steady state. In addition, GRP58/ERp57 deficiency in B cells also affects the recruitment of calreticulin into the loading complex [35]. Thus, MHC class I antigen presentation ability is diminished by the deficiency of GRP58/ERp57 in B cells due to the impaired peptide-loading machinery.

Calreticulin deficiency is lethal in mouse embryos at E14.5, resulting from a lesion in cardiac development [36]. The ventricular wall made of cardiomyocytes became thinner in the heart of *calreticulin*$^{-/-}$ mouse embryos. Calreticulin is highly expressed in cardiomyocytes at the early stage of heart development and downregulated after birth in the healthy mature heart. Interestingly, GRP78 and GRP94 are also upregulated during embryonic cardiac development indicating that some ER chaperones may be essential for cardiogenesis [40–43]. In calreticulin-null fibroblast, the Ca^{2+} storage capacity of the ER is reduced [44], whereas overexpression of calreticulin increases the ER Ca^{2+} level [45]. Since Ca^{2+} enhances the client-binding and chaperoning ability of ER chaperones, changes in the ER Ca^{2+} storage capacity or impairment of Ca^{2+} binding to these chaperones affect the quality of ER protein folding and assembly.

Calnexin is another major component of the calnexin/calreticulin chaperone system. Interestingly, calnexin deficient mice are viable, but 50% of the *calnexin*$^{-/-}$ mice died within 2 days of birth and the surviving mice are smaller than the littermates and exhibit obvious motor disorders [46]. The *calnexin*$^{-/-}$ mice are further characterized by a dramatic loss of large to medium

myelinated nerve fibers, thereby decreasing the size of the sciatic nerve, implying that calnexin plays a tissue-specific role in mammalian physiology. Furthermore, deletions of calnexin, calreticulin and GRP58/ERp57 have very specific consequences on glycoprotein maturation. For example, defective maturation of MHC class I in cells lacking calreticulin [47], of influenza virus hemagglutinin in cells lacking calnexin [48] or ERp57 [49] and of a series of heavily glycosylated proteins sharing common structural domains in cells lacking ERp57 [50] have been reported.

6. ER chaperones ameliorate protein misfolding in neurodegenerative diseases

Neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease are pathologically characterized by the intracellular or extracellular accumulation of misfolded proteins or mutated gene products [51]. Thus, occurrences of ER stress and UPR activation have been observed in the affected neuronal cells [52]. Mounting evidence suggests that ER chaperones are highly induced to ameliorate the accumulation of misfolded proteins and protect neuronal cells against neurotoxicity [53,54]. Impairment of the functions of the involved chaperones leads to the failure of attenuating ER stress and eventual apoptosis of neuronal cells. This is supported by the development of neurological disorders in knockout mouse models of ER chaperones and co-chaperones as summarized in Tables 1 and 2.

S-nitrosylated PDI was found in the brain samples of Parkinson's or Alzheimer's patients and exposure of the cultured neurons to NMDA that induced Ca^{2+} influx and nitric oxide production also resulted in *S*-nitrosylation of PDI [55]. PDI catalyzes thio-disulfide exchange facilitating the disulfide bond formation and rearrangement reaction. In response to ER stress, PDI is usually upregulated and protects neuronal cells against ischemic injury [56]. *S*-nitrosylation of PDI inhibits its enzymatic activity and leads to the accumulation of polyubiquitinated proteins and activation of UPR. On the other hand, overexpression of wild-type PDI attenuates UPR and protects cells against apoptosis induced by ER stress inducers such as tunicamycin which inhibits *N*-linked protein glycosylation and thapsigargin which is an inhibitor of ER Ca^{2+}-ATPase [55].

Overexpression of GRP78, calnexin and GRP170/ORP150 suppressed the production of β-amyloid peptides (Aβ), a major component of extracellular senile plaques in Alzheimer's disease [57]. Further, co-immunoprecipitation identified the association of amyloid precursor protein (APP) with GRP78, suggesting that GRP78 overexpression inhibits APP maturation through retention of APP in the ER and thus reduces the Aβ production by proteolysis of APP. The protective role of GRP78 in neurodegeneration is supported by the studies of woozy mutant mice and Marinesco-Sjögren syndrome (MS) patients. Human MS syndrome is a rare disease associated with cerebellar ataxia, progressive myopathy and cataracts. Recently, mutations of SIL1, an adenine nucleotide exchange factor of GRP78, have been identified to cause the MS syndrome [58,59]. Further, in vivo study with the SIL1 mutant woozy mouse model revealed that loss of functional SIL1 results in cerebellar Purkinje cell degeneration and ataxia [60]. SIL1 stimulates the release of ADP from GRP78, activating the ATPase cycle to promote the binding and folding of the substrate proteins. It was observed that ubiquitinated proteins aggregate in the ER and cytosol and the UPR pathway is activated to upregulate GRP78, GRP170/ORP150 and CHOP in response to the ER stress induced in cerebellar Purkinje cells. It is noted that the cerebellum is particularly sensitive to loss of SIL1 function, and within the cerebellum only some lobules have degenerated Purkinje cells. One possible explanation for this observation is that other co-chaperone(s) can compensate for SIL1 function in the unaffected cerebellar lobules. GRP170/ORP150 has recently been implicated to substitute for SIL1 as an alternative nucleotide exchange factor for GRP78 in an in vitro experimental system [61]. Thus, in different tissues or in different regions of specific tissues, the distribution and activity of various co-chaperones could vary, giving rise to distinct phenotypes.

GRP58/ERp57 has been implicated in prion diseases, because it is consistently upregulated in the prion replicating brain areas of the scrapie prion-infected mice and its overexpression protects neuronal cells against PrP^{sc} toxicity and ER stress-induced apoptosis [62,63]. Prion diseases, also known as transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders characterized by accumulation of the misfolded form of the cellular prion protein (PrP), denoted PrP^{sc} (scrapie-associated PrP), and consequent neuronal dysfunction and death [64]. The conformational changes of PrP^{sc} lead to the generation of the protease-resistant and insoluble form of the prion protein, which is believed to be neurotoxic. ER stress resulting from PrP^{sc} aggregation induces the apoptosis of neuron cells [65]. The upregulation of ER stress chaperones, such as GRP78, GRP94 and GRP58/ERp57, was also observed in PrP^{sc}-infected neuroblastoma cells [65] and the cortex samples of TSEs patients [64]. Furthermore, GRP58/ERp57 was found to interact with PrP^{sc} and this interaction was enhanced when cells were infected with scrapie prion or treated with proteasome inhibitor, suggesting that GRP58/ERp57 selectively binds to PrP^{sc} [62]. Since GRP58/ERp57 is a thiol oxidoreductase to catalyze disulfide bond formation, it is possible that the interaction of GRP58/ERp57 enhances correct folding of PrP and thus reduces the PrP^{sc} neurotoxicity. Furthermore, a recent study reveals that ER stress facilitates the generation of a misfolded PrP isoform that is more prone to be efficiently converted into PrP^{sc} and that ER damaged cells might be more susceptible to prion replication, contributing to rapid progression of prion disease [66].

7. ER chaperones promote cancer progression and tumor immunity

Due to hypoxic conditions and glucose deprivation caused by poor vascularization, the microenvironment of tumors represents physiological ER stress and the UPR is activated for the survival of tumor cells [67,68]. ER chaperones serve as a novel class of pro-survival components protecting the host against death induced by ER stress when expressed at high levels. In cell culture systems, it has been established that GRP78 [69,70], GRP94 [71], calreticulin [72] and the homocysteine-induced endoplasmic reticulum protein, Herp [73] protect cancer cells against ER stress-induced apoptosis. Among them, GRP78 is best-characterized with respect to its role in cancer progression, drug resistance and possibly metastasis [74, Table 1].

Fibrosarcoma cells where GRP78 expression was suppressed by antisense were either unable to form tumors or quickly regressed [69], implying that GRP78 is required for tumor growth. Recent investigations using mouse knockout models reaffirmed the important role of GRP78 in tumor development and progression (Dong and Lee, in preparation). GRP78 expression is highly upregulated in varieties of cancer cell lines and human cancer specimens, including breast cancer, lung cancer, liver cancer and prostate cancer, correlating with malignancy, metastasis and drug resistance [75]. In a variety of human cancers, GRP78 upregulation protects tumor cells from chemotherapeutic agents and knockdown of GRP78 with siRNA or antisense sensitizes these cells to those agents [75,76]. GRP78 protects cancer cells through multiple mechanisms. Under glucose starvation and severe hypoxic conditions in solid tumors, GRP78 is induced by activated UPR signaling to ameliorate misfolded protein aggregation in the ER. Through its ability to bind Ca^{2+}, GRP78 serves as a buffering agent in the ER, preventing Ca^{2+} efflux from the ER to the cytosol and alleviating the ER stress-induced apoptotic stimuli. Furthermore, the cytoprotective role of GRP78 also attributes to its inhibition of the activation of pro-apoptotic components, such as BIK and BAX, as well as suppressing the cleavage of procaspase-7 and procaspase-12 through complex formation [77–79]. Since a subfraction of GRP78 exists as an ER transmembrane protein with the amino portion in the cytosol, it can potentially directly interact with pro-apoptotic components such as BIK, BAX, caspase-12 and caspase-7 which are known to be localized to the ER [75,78,79]. It is also possible that GRP78 in the lumen of the ER complexes with ER-transmembrane proteins that interact with the cytosolic pro-apoptotic components. Since BIK, BAX and caspase-7 are activated by a variety of chemotherapeutic regimens, inhibition of their activation by GRP78 may confer general chemo-resistance to cancer cells [75]. Additionally, BIK is required for estrogen-starvation induced apoptosis in estrogen receptor positive human breast cancer cells and its apoptotic activity is blocked by GRP78 in human breast cancer cells [79,80]. BIK is a BH3-only protein of the BCL-2 family and plays a pro-apoptotic role through regulating the oligomerization of BAX and BAK in the mitochondrial outer membrane and release of mitochondrial cytochrome c. BIK is known to inhibit Bcl-2 activity by direct association. Therefore, inhibition of BIK by GRP78 in human breast cancer could lead to resistance to chemotherapy as well as anti-estrogen therapy. In retrospective studies, GRP78 level in patient tumor specimens has been shown to associate with poor survival, resistance to adriamcyin therapy in breast cancer patients [81,82] and recurrence in prostate cancer patients [83,84]. Thus, GRP78 is a potential novel biomarker for tumor behavior and development of resistance to therapy in cancer [74].

GRP94, the ER homologue of HSP90 also referred to as gp96, shares common transcriptional regulatory elements with the GRP78 promoter and is coordinately regulated with GRP78 [85]. Induction of GRP94 is commonly associated with upregulation of GRP78 in the tumor samples and cell lines. Overexpression of GRP94 is correlated with cellular transformation, tumorigenicity and decreased sensitivity to X-rays in cancer cell lines, whereas decrease of GRP94 level by antisense results in the enhanced sensitivity of tumor cells to etoposide treatment [71]. Interestingly, etoposide-induced cell death triggers proteolytic cleavage of GRP94 by calpain, which also cleaves Bcl-xL during apoptosis, therefore converting a pro-survival protein into a pro-apoptotic molecule [86]. In addition, the role of GRP94 in cancer has been widely recognized due to its ability to induce a tumor-specific protective immunity in various experimental tumor models (Table 1). GRP94 is found to associate with diverse peptides including tumor-specific antigens in the ER and the complexes are captured by antigen-presenting cells, which in turn present the peptides to MHC class I complex leading to the cell-mediated immune response [87]. Based on this mechanism, GRP94 vaccines have been extensively developed in cancer therapy. The isolated GRP94-peptide complexes from specific cancer tissues or modified cancer cell lines are currently used as vaccines to immunize animal tumor models or patients in clinical trials. The studies observed that the immunization elicits the specific immune response and tumor regression and the efficiency is improved by low dose of cyclophosphamide, an inhibitor of the induction of suppressive regulatory T cells [88]. Cell surface GRP94 has also been implicated in systemic autoimmune diseases [89].

Cell surface localization of GRP78, PDI, calnexin and calreticulin have been reported [90–93]. In prostate cancer cells, GRP78 serves as a receptor for activated macroglobulin and is postulated to promote proliferation, survival and cell motility [94,95]. Cell surface expression of GRP78 in tumors but not in normal organs has prompted new directions in targeting cytotoxic agents into cancer cells [71,74,90].

8. ER chaperones alleviate atherosclerosis stress

Atherosclerosis is a chronic and progressive disease physiologically characterized by the hardening and narrowing of the arteries due to the formation of atheromatous plaques on the inside walls of arteries. It is well-established that atherosclerosis is a principle cause of cardiovascular disease, such as atherothrombotic disease, myocardial infarction, stroke, etc. [96,97]. Numerous clinical and epidemiology studies have demonstrated that hyperhomocysteinemia (HHcy) is an independent risk factor for atherosclerosis and thrombotic disease [98,99]. Deficiencies in homocysteine metabolism, such as nutritional deficiencies in B vitamin cofactors or mutations in cystathionine β-synthase (CBS) gene or 5,10-methylenetetrahydrofolate reductase (MTHFR), can lead to hyperhomocysteinemia or even a severe form of HHcy, homocystinuria [100]. Studies on the potential cellular mechanism by which homocysteine promotes atherosclerosis revealed that HHcy induces activation of proinflammatory factors [101–103], oxidative stress [104] and ER stress associated with activation of the UPR [105,106]. As summarized in Tables 1 and 2, increased expression of ER stress response genes including GRP78, GRP94, Herp and RTP (reducing agents and tunicamycin-responsive protein) has been observed as a consequence of high levels of intracellular homocysteine [107–109]. Homocysteine-induced ER stress leads to overexpression of pro-apoptotic factors including GADD153/CHOP and TDAG51 (T cell death-associated gene 51) and activation of caspase-3 [108,110–112], which contributes to HHcy-associated vascular endothelial cell injury that may promote the development of atherothrombotic diseases. In addition, homocysteine-induced ER stress also induces the expression of the sterol regulatory element-binding proteins (SREBPs) in hepatocytes, vascular

endothelial and smooth muscle cells [113]. SREBPs are ER-resident transcription factors responsible for the activation of genes involved in the cholesterol and triglyceride biosynthesis pathways. Increased expression of SREBPs is also associated with intracellular accumulation of cholesterol. Stable overexpression of GRP78 is able to inhibit the activation of SREBPs and the genes under their regulation [113], which implies that GRP78 may be a potential factor to inhibit atherosclerosis. Further, GRP78 was discovered to suppress thrombin generation by inhibiting tissue factor (TF) procoagulant activity [114], whereas homocysteine induces TF procoagulant activity. TF is a transmembrane glycoprotein and plays a role in initiation of the extrinsic coagulation cascade and increased expression of TF has been detected in atherosclerotic plaques [115]. Thus, increased level of ER chaperone GRP78 can potentially suppress the development or progression of hyperhomocysteinemia and atherosclerosis through: (i) alleviating the homocysteine-induced ER stress; (ii) preventing apoptosis of vascular endothelial cells; (iii) inhibiting the activation of genes responsible for cholesterol/triglyceride biosynthesis; and (iv) suppressing the procoagulant potential of cells.

9. Link of diabetes to UPR pathways and ER chaperone function

Diabetes is a prevalent metabolic disease characterized by perturbed glucose metabolism. Usually, this disease is initiated by failure of pancreatic β-cells associated with autoimmunity (type 1) or insulin resistance in peripheral tissues (type 2). Defects in specific UPR pathways have been linked to diabetes in mouse models and humans. One example is the human Wolcott-Rallison syndrome, a rare autosomal-recessive disorder associated with pancreatic β-cell death and infancy-onset diabetes [116]. PERK mutations resulting in truncated or dysfunctional PERK protein are responsible for this disorder. Similar defect was also observed in $Perk^{-/-}$ mice and increased β-cell apoptosis has been detected in the mouse islets [117]. The activation of PERK upon ER stress involves dissociation from GRP78, oligomerization of PERK and autophosphorylation of serine/threonine kinase domain. The activated PERK further phosphorylates eIF2α (α subunit of translational initiation factor), which results in transient inhibition of global translation, thereby preventing further protein accumulation in the ER and suppressing ER stress-induced cell death [17]. Therefore, PERK is a crucial pathway in pancreatic β-cells to attenuate the stress signaling for survival.

The link of molecular chaperones to diabetes is just emerging. A recent study suggests that deficiency of $P58^{IPK}$, an inhibitor of PERK activation, causes type 1 diabetes and late-stage type 2 diabetes in mice associated with hyperglycemia and hypoinsulinemia concomitant with increasing apoptosis of pancreatic islet β-cells [118]. Deficiency of $P58^{IPK}$ has no effect on the insulin secretion by viable pancreatic β-cells and insulin sensitivity of peripheral tissues. However, the adult $P58^{IPK-/-}$ mice with hyperglycemia showed increased β-cell death and upregulation of pro-apoptotic genes, suggesting that loss of functional pancreatic β-cells leads to hypoinsulinemia and subsequent high blood glucose level. Although $P58^{IPK}$ is a known inhibitor of both PERK and the cytosolic eIF2α kinase PKR, its depletion only activates PERK, but not PKR [119]. The diabetic phenotypes of $P58^{IPK-/-}$ mice showed that sustained activation of PERK could lead to prolonged ER stress and induce apoptosis of pancreatic β-cells (Table 2).

As a downstream effector of PERK, the heterozygous loss-of-function mutation of eIF2α in mice fed on high fat diet results in development of diabetes and obesity [120]. This mutation substitutes alanine at Ser^{51}, the phosphorylation site of eIF2α by PERK or PKR for translational inhibition. This diabetes mouse model is characterized by glucose intolerance, abnormal ER distension and inadequate insulin secretion in β-cells. Further, in eIF2α mutant mice fed with high fat diet, increased amount of proinsulin was associated with GRP78 and production of mature insulin was decreased, suggesting that the stable association with GRP78 inhibits the processing of proinsulin into mature insulin. Thus, the S/A mutation of eIF2α abolishes the ability of β-cells to adapt to ER stress through translational attenuation in the high-fat diet fed mice. The increased expression of proinsulin exceeds the folding capacity of the ER and GRP78 retains the proinsulin molecules in the ER for further folding process or degradation through ERAD, which contributes to the reduced insulin level in islets and diabetic symptoms.

10. ER chaperones and bacterial pathogenesis

Bacterial toxins are major determinants of bacterial virulence and even lethality and the main causes of human diseases in bacteria-infected populations. Generally, there are two types of bacterial toxins, lipopolysaccharides, which are associated with the cell walls of gram-negative bacteria, and proteins, which are typically soluble proteins acting as enzymes intracellularly or extracellularly. Bacterial protein toxins usually contain two components: A subunit possesses enzymatic activity and B subunit provides binding to the specific cell surface receptor. In general, toxins bound to the cell surface receptor are endocytosed, and transported retrogradely to the Golgi and the ER as a holotoxin. When released from the AB native toxin, the enzymatic A subunit becomes active. Evidence indicated that PDI mediates the disassembly of cholera toxin (CT) holotoxin [121]. CT has a typical AB5 arrangement of protein toxins, which contains five B subunits and a single A subunit that is cleaved into A1 catalytic chain and A2 chain upon secretion. When CT holotoxin is taken up by intestinal cells and translocated into the ER, the reduced form of ER chaperone PDI binds to the A1 chain, reduces the disulfide bridge between the A1 and A2 chains [122], and releases the A1 chain from the holotoxin when the C-terminal disulfide bond of PDI is oxidized by endoplasmic reticulum oxidoreductin 1 (Ero1) [123]. Eventually, the A1 chain is transferred across the ER membrane to the cytosol where it refolds into an active enzyme that ADP-ribosylates a trimeric G protein and in turn activates adenylyl cyclase to elevate cAMP level for activation of chloride channels at the cellular plasma membrane, which leads to diarrhea. Thus, the unfolding process by PDI facilitates the retrotranslocation of the A1 chain of CT. However, other PDI family members modify toxin retrotranslocation differently. A recent study revealed that ERp72, a PDI-like protein retains the A1 chain of CT in the ER and may potentially reduce the cytosolic activated A1 chain [124].

Another interesting discovery is that a bacterial toxin, named AB5 subtilase cytotoxin (SubAB), causes cell lethality

through specific cleavage of GRP78 at a single amino acid, thus inactivating GRP78 function [125]. SubAB has been detected in a virulent strain of Shiga toxigenic *Escherichia coli* (STEC) and several other STEC serotypes. The STEC strains are known to produce Shiga toxins that have been found to cause the recent US outbreak of *E. coli* from fresh spinach and cause haemolytic uraemic syndrome in humans [126]. The A subunit of Shiga toxin is exported from the ER lumen to the cytosol, where it enzymatically cleaves the 28S rRNA resulting in inhibition of protein synthesis and death of host cells [127]. In contrast, the A subunit of SubAB remains in the ER lumen instead of being retrotranslocated to the cytosol and cleaves GRP78 at the region linking the ATPase domain and the peptide-binding domain, thus destroying the cytoprotective GRP78 and leading to cell death. Therefore, STEC strains bearing SubAB are expected to pose serious threat to human health. On the other hand, SubAB may be a potential tool in medical application regarding to its function of specifically eliminating GRP78, since induction of GRP78 expression is correlated with tumor growth and drug resistance in a variety of human cancers [128].

Acknowledgements: This work was supported in part by the NIH Grants CA27607 and CA111700 (A.S.L). We thank Changhui Mao, Risheng Ye and members of the Lee laboratory for helpful discussions.

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