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Tripartite Management of Unfolded Proteins in the Endoplasmic Reticulum

Minireview

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Unfolding or misfolding of proteins constitutes a fundamental threat to all living cells. In eukaryotes, proteins can be unfolded or misfolded in a variety of subcellular compartments such as cytoplasm, mitochondria, and peroxisomes, but the risk of protein misfolding is particularly acute in the endoplasmic reticulum (ER), in which newly synthesized secretory and transmembrane proteins attain their proper tertiary structure. Efficient quality control systems have evolved to prevent incompletely folded molecules from moving along the secretory pathway. Accumulation of misfolded proteins in the ER would detrimentally affect the function and/or localization of the approximately one-third of all cellular proteins that translocate into the ER after synthesis on membrane-bound ribosomes. Eukaryotic cells have three different mechanisms for dealing with an accumulation of unfolded proteins in the ER: transcriptional induction, translational attenuation, and degradation (Figure 1). This minireview focuses on new pieces of the puzzle that are important for understanding the molecular mechanisms of the induction and attenuation systems, most of which were discovered in the space of only one year after publication of a comprehensive review of this field (Kaufman, 1999), and also discusses coordination and interdependency among the three systems.

Transcriptional Induction in Response to ER Stress

Protein folding in the ER lumen is assisted or facilitated by a number of molecular chaperones, including BiP/GRP78 and GRP94, as well as by folding enzymes such as protein disulfide isomerase and peptidyl-prolyl *cis-trans* isomerase. In response to the accumulation of unfolded proteins in the ER, eukaryotic cells from yeast to man activate an intracellular signaling pathway from the ER to the nucleus known as the unfolded protein response (UPR), resulting in transcriptional upregulation of these ER-resident proteins. As the UPR target genes encode most of the chaperones and enzymes involved in protein folding in the ER, the main purpose of the UPR has seemed to be augmentation of the folding capacity of the ER. Strikingly, however, the number of known UPR target genes has been greatly expanded in yeast using microarray techniques, now identifying a total of 381 regulated genes, and this number might be an underestimate because highly stringent criteria were used for selection (Travers et al., 2000). Based on the finding that UPR targets are not limited to proteins involved in the folding process in the ER but include numerous proteins working at various stages of secretion, the authors proposed that the activated UPR leads to specific remodeling of the secretory pathway to minimize the amount and/or concentration of unfolded proteins in the ER. Characterization of the 173 UPR target genes for which no functional information is currently available will provide a complete catalog of the resources and mechanisms used by cells to remove misfolded proteins and restore the ER to full functionality.

Transcriptional Induction in Yeast

The molecular mechanism of the UPR is well understood in the budding yeast *Saccharomyces cerevisiae*. Ire1p and Hac1p are major players. Ire1p is a type I transmembrane protein whose N-terminal domain is located in the ER lumen; the serine/threonine-specific protein kinase and endonuclease domains of Ire1p are situated on the cytoplasmic or nuclear matrix side of the ER membrane. Since its discovery 7 years ago, Ire1p has been postulated to sense the accumulation of unfolded proteins and thus to transmit signals across the ER membrane. However, the precise mechanism of activation of Ire1p was not known until a very recent paper provided intriguing evidence indicating that chaperone protein BiP/

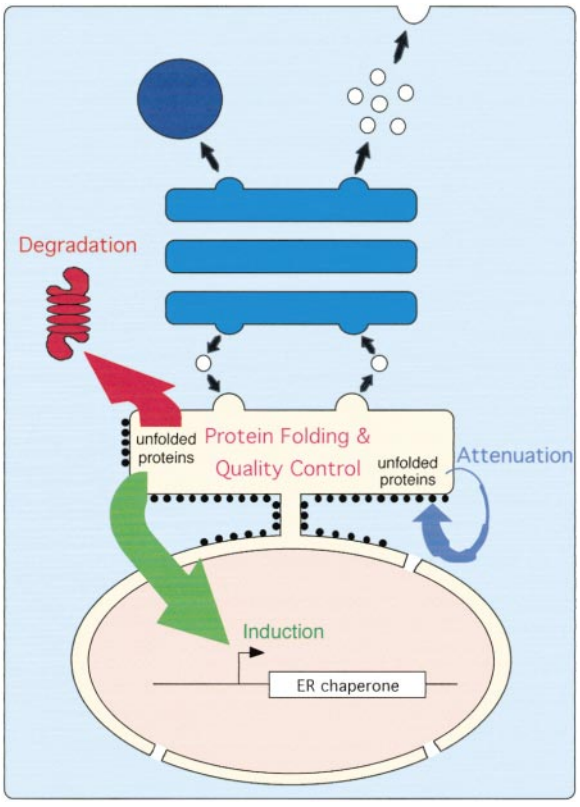

Figure 1. Three Cellular Responses to the Accumulation of Unfolded Proteins in the ER

The ER monitors the folding status of newly synthesized secretory and transmembrane proteins and controls their quality. Three cellular responses are activated to cope with the accumulation of unfolded proteins in the ER: transcriptional induction, translational attenuation, and degradation.

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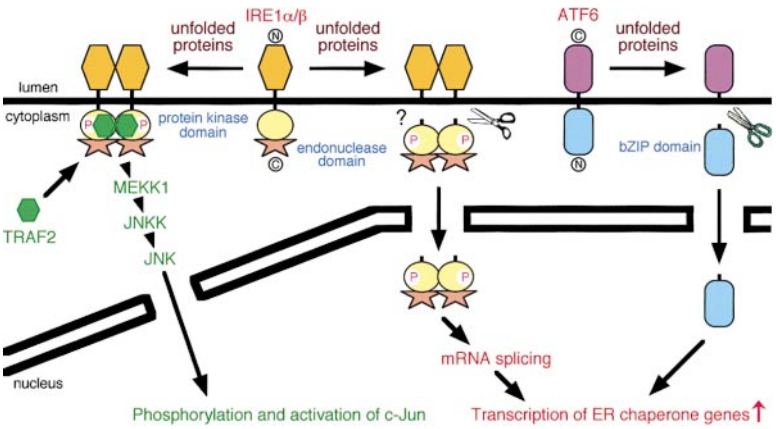

Figure 2. Mechanism for ER Stress–Induced Transcriptional Induction in Mammalian Cells

ER stress–induced oligomerization and autophosphorylation of IRE1α and IRE1β, type I transmembrane proteins in the ER, result in activation of the endonuclease domains that are postulated to initiate splicing of mRNA encoding a putative (unidentified) transcription factor, leading to enhanced transcription of mammalian ER chaperone genes. This series of events may or may not involve proteolysis of IRE1 molecules (thus indicated by the question mark), which allows entering of the C-terminal fragments into the nucleus. In addition, activated IRE1α and IRE1β recruit TRAF2, an adaptor molecule involved in the JNK signaling, culminating in phosphorylation and activation of c-Jun. ER stress also triggers proteolysis of the transcription factor ATF6, which is synthesized as a type II transmembrane protein in the ER. The bZIP-containing N-terminal fragment thus liberated from the ER membrane translocates into the nucleus and activates transcription of mammalian ER chaperone genes.

GRP78 plays a key and direct role in converting Ire1p from a monomeric inactive state to an oligomeric active state in response to ER stress (Bertolotti et al., 2000). ER stress–induced oligomerization of Ire1p is accompanied by its autophosphorylation in *trans*, leading to activation of endonuclease activity present in its C-terminal tail. Ire1p thus activated splices out an intron of 252 nucleotides from the HAC1 precursor mRNA; this encodes the basic leucine zipper (bZIP)-type transcription factor Hac1p, which induces the yeast UPR. The cleaved 5′ and 3′ halves of mature HAC1 mRNA are ligated by the action of the tRNA ligase Rlg1p (reviewed by Sidrauski et al., 1998). This series of events controls the synthesis of active Hac1p at two levels. The intron prevents translation of constitutively synthesized HAC1 precursor mRNA and also separates the DNA-binding domain of Hac1p (encoded by the first exon) from its activation domain (encoded by the second exon). Therefore, ER stress–induced removal of the HAC1 intron allows not only translation of mature HAC1 mRNA but also joining of the two functional domains of Hac1p (Mori et al., 2000). Thus, the HAC1 mRNA splicing system, which represents a novel type of mRNA splicing in eukaryotes, allows yeast cells to synthesize the highly active transcription factor Hac1p only when they need to cope with unfolded proteins accumulated in the ER.

Transcriptional Induction in Mammals

Key players in the mammalian UPR have started to come to light recently. The mammalian ER contains two Ire1p homologs: IRE1α (Tirasophon et al., 1998), which appears to be universally expressed, and IRE1β (Wang et al., 1998), whose expression is limited to cells of the gut. Overexpression of wild-type IRE1α or IRE1β is sufficient to activate the UPR constitutively, as is the case for yeast Ire1p. Mutant forms containing the luminal and transmembrane domains but lacking the cytosolic effector domains show dominant-negative effects on the UPR, supporting their importance in the mammalian UPR. Because the cytoplasmic regions of both IRE1α and IRE1β contain protein kinase and endonuclease domains capable of cleaving yeast HAC1 precursor mRNA at the same sites that yeast Ire1p does, and because HAC1 precursor mRNA is spliced correctly in mammalian cells in response to ER stress (Niwa et al., 1999), mammalian cells have been postulated to use an mRNA splicing system similar to that in yeast cells to produce Hac1p-like transcription factor(s) that activate the mammalian UPR. However, no such transcription factor has yet been discovered, and HAC1 intron-mediated translational block has not been demonstrated in mammalian cells.

Instead, recent evidence suggests that mammalian IRE1s are subjected to proteolysis in response to ER stress, resulting in the release of soluble C-terminal fragments (Niwa et al., 1999). The IRE1 fragments produced, carrying both kinase and endonuclease domains, enter the nucleus where they are postulated to carry out splicing events, leading to enhanced transcription of ER chaperone genes (Figure 2). Processing of yeast Ire1p induced by ER stress has not been observed, so the above results suggested significant divergence between the UPR in yeast and mammals. However, it is important to note that ER stress–induced proteolysis of IRE1s was observed neither previously (Tirasophon et al., 1998; Wang, 1998) nor recently (Bertolotti et al., 2000) by other researchers. Further studies are necessary to confirm and characterize the proteolytic processing of mammalian IRE1s in response to ER stress.

Recent studies on IRE1α knockout mice revealed quite unexpectedly that IRE1α is dispensable for the mammalian UPR (Urano et al., 2000). Yeast cells cannot transmit signals across the ER membrane without Ire1p whereas the signaling remains intact in IRE1α/IRE1β double-knockout cells (Urano et al., personal communication). When combined with the dominant-negative effects of IRE1 mutants mentioned above, these results may suggest the presence of a third IRE1 molecule sufficient to mediate signaling from the mammalian ER to the nucleus even in the absence of IRE1α and IRE1β. Alternatively, several signaling pathways are involved in transcriptional induction of ER chaperones in mammalian cells, and the absence of one pathway might be fully compensated by others. In support of this idea, the bZIP protein ATF6 was recently identified as a promising candidate transcription factor specific to the mammalian UPR (Yoshida et al., 1998). Its basic region has

significant sequence identity to that of yeast Hac1p, although its overall structure is not similar to that of Hac1p and its expression is not regulated by mRNA splicing. Instead, ATF6 is synthesized as a type II transmembrane protein localized in the ER and activated by ER stress–induced proteolysis (Haze et al., 1999). Upon ER stress, the bZIP-containing N-terminal fragment facing the cytoplasm is liberated from the ER membrane and translocated into the nucleus, resulting in activation of ER chaperone gene transcription (Figure 2). Indeed, overexpression of the cytoplasmic region of ATF6 constitutively activated the UPR (Haze et al., 1999). However, it is not yet known which enzyme cleaves ATF6 or how the proteolysis is regulated.

Other Induction Systems

Mammalian cells respond to ER stress by activating a variety of transcriptional programs. One such example is the ER overload response; the transcription factor NF-κB is activated when large amounts of membrane proteins accumulate in the ER, culminating in induction of proinflammatory proteins and cytokines such as interferons and interleukins. This response is likely to be involved in cellular defense against viral infection. However, no sensor molecule(s) for this response has yet been identified (see review by Kaufman, 1999).

It is also known that certain cytoplasmic protein kinases such as c-Jun amino-terminal kinases (JNK) or stress-activated protein kinases (SAPK) are stimulated under conditions of ER stress, leading to phosphorylation and activation of the transcription factor c-Jun. Surprisingly, analysis of IRE1$\alpha^{-/-}$ cells revealed that ER stress–induced activation of JNK/SAPK is mediated by IRE1 (Figure 2); the cytoplasmic kinase domains of IRE1s interact with TRAF2, an adaptor protein involved in JNK/SAPK signaling, only when IRE1s are activated by ER stress (Urano et al., 2000). Thus, mammalian IRE1s perform an additional function that yeast Ire1p apparently lacks. In this regard, it is noteworthy that Ire1p is the only transmembrane protein kinase in yeast whereas many transmembrane protein kinases are expressed at the surface of mammalian cells and respond to extracellular stimuli by binding to their ligands. As eukaryotic cells acquired transmembrane signaling systems across the plasma membrane during evolution, including the tumor necrosis factor receptor-TRAF2-JNK/SAPK pathway, the events downstream of IRE1 might have diversified to include both initiation of mRNA splicing and activation of the kinase cascade.

Translational Attenuation in Response to ER Stress

Continuous delivery of newly synthesized proteins is a burden to the ER when proper folding is prevented under ER stress conditions. Cells might be able to restore normal function more efficiently if they could suppress protein synthesis, and indeed translation is attenuated in response to the presence of unfolded proteins in the ER (reviewed by Brostrom and Brostrom, 1998). Understanding the molecular mechanism of ER stress–induced translational attenuation has recently made great progress through the discovery of PEK/PERK, a type I transmembrane protein kinase localized in the ER (Shi et al., 1998; Harding et al., 1999). The luminal domains of PERK, IRE1α, and IRE1β show significant sequence homology (~20% identity), suggesting that all three proteins may use a similar sensing mechanism,

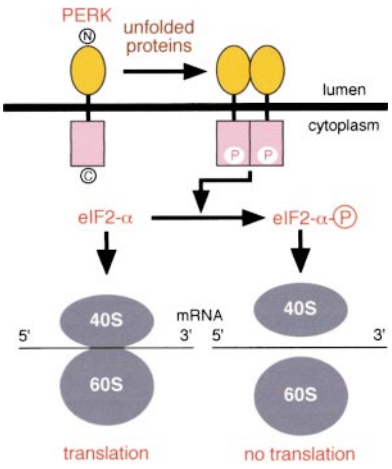

Figure 3. Mechanism for ER Stress–Induced Translational Attenuation

ER stress–induced oligomerization and autophosphorylation of PERK, a type I transmembrane protein kinase in the ER, result in phosphorylation of eIF2-α at serine 51, leading to inhibition of translation initiation.

allowing coordination of the induction and attenuation systems. Recent studies showed that the luminal domains of PERK and IRE1 are indeed functionally interchangeable and that BiP/GRP78 is directly involved in activation of PERK as well as IRE1 by ER stress (Bertolotti et al., 2000).

The cytoplasmic domain of PERK is highly homologous (~40% identity) to PKR, a cytoplasmic protein kinase known to phosphorylate the α subunit of eukaryotic translation initiation factor 2 (eIF2-α) on serine 51. This phosphorylation of eIF2-α leads to inhibition of translation initiation by preventing the association of mRNA with ribosomal 60S and 40S subunits through interference with the formation of a 43S initiation complex. When activated by ER stress, PERK also phosphorylates serine 51 of eIF2-α and can therefore function as both a sensor and an effector by itself (Figure 3). It is interesting to note that only metazoans have gained the ability to attenuate translation in response to ER stress; genome sequencing projects revealed that no PERK-like transmembrane eIF2-α kinases are present in yeast whereas *Caenorhabditis elegans* and *Drosophila melanogaster* both have counterparts of mammalian PERK.

Analysis of mouse embryonic stem cells deficient in PERK clearly showed that PERK$^{-/-}$ cells had lost the ability to phosphorylate eIF2-α and attenuate translation even with the accumulation of unfolded proteins (Harding et al., 2000). Furthermore, PERK$^{-/-}$ cells are more sensitive to ER stress than PERK$^{+/+}$ cells, presumably due to enhanced accumulation of misfolded proteins. These results indicated that PERK plays a major role in the translational attenuation induced by ER stress that is required for cell survival. However, because the effects of PERK-mediated phosphorylation of eIF2-α appeared not to be limited to ER membrane–bound ribosomes under the conditions of ER stress tested, the basis for the compartment-specific phenomenon is unclear. In addition, it remains to be determined how ER

chaperones escape from PERK-mediated general inhibition of translation initiation to be ultimately induced to cope with unfolded proteins.

Cooperative Interaction between Induction and Degradation Systems

Recent advances in understanding the mechanism of the protein degradation system have established that misfolded proteins are transported out of the ER through the translocon to the cytoplasm, where they are ubiquitinated and degraded by the action of the 26S proteasome. This series of processing is often called ER-associated degradation (ERAD) (reviewed by Bonifacino and Weissman, 1999). Three recent reports (Casagrande et al., 2000; Friedlander et al., 2000; Travers et al., 2000) provided convincing evidence indicating that efficient degradation of unfolded proteins accumulated in the ER requires the UPR to be functional, at least in yeast. Mouse major histocompatibility complex class I heavy chain (H-2K^b) expressed as a model for misfolded transmembrane proteins, and CPY*, a well-known soluble misfolded protein substrate, were degraded in wild-type yeast cells with half-lives of $\sim$15 min and $\sim$25 min, respectively, in a manner dependent on proteasome activity. In marked contrast, both proteins were stable (half-life $>$ 1 hr) in a strain lacking Ire1p, the proximal sensor for yeast UPR. Importantly, the defect in degradation in *ire1*Δ cells could be overcome by introduction of a constitutively active form of the transcription factor Hac1p into these cells.

Why is the induction system required for the degradation system? An important clue comes from the finding that a number of the implicated proteins are upregulated by the UPR (Casagrande et al., 2000; Friedlander et al., 2000; Travers et al., 2000). It seems that the capacity of the ER to cope with misfolded proteins is so rapidly saturated that UPR-mediated induction of chaperones and other components required for degradation is necessary to sustain high rates of degradation of misfolded ER proteins. It is also possible that degradation rates are accelerated by the action of an oligosaccharide-processing enzyme induced by the UPR that catalyzes the putative rate-limiting step in clearing the ER. Although strains defective in only one of the two systems grow normally, strains defective in both induction and degradation systems grew slowly at 25°C and failed to grow at 37°C (Friedlander et al., 2000; Travers et al., 2000), indicating that unfolding or misfolding of newly synthesized proteins occurs even under normal growth conditions, and the cell copes with this by activating the induction and/or degradation systems.

Perspective

The cellular responses to the accumulation of unfolded proteins in the ER are much more extensive than previously recognized. Three disparate defense systems respond to signals emanating from the ER. These systems work in a coordinated fashion to improve the efficiency of folding, processing, and export of secretory proteins, to remove the fraction of polypeptides that fail to fold, and to reduce the flow of proteins into the ER compartment. Interestingly, two groups recently reported that the UPR is weakened in cells carrying a mutation or deletion of presenilin-1 involved in Alzheimer's disease (Katayama et al., 1999; Niwa et al., 1999). Consistent with this finding, compared to age-matched controls, the brains of sporadic and familial Alzheimer's disease patients contained significantly less BiP/GRP78 and GRP94 (Katayama et al., 1999). Under normal circumstances, these chaperones may play a central role in suppressing the formation of amyloidogenic peptides in the ER (see review by Gething, 2000). Thus, further understanding of the three cellular responses will provide new insights into not only fundamental principles in cell biology but also the pathogenesis of diseases that result from problems in protein folding in the ER.

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