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The Cellular Response to Protein Misfolding in the Endoplasmic Reticulum

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In eukaryotic cells, accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) leads to a stress response. Cells respond to ER stress by upregulating the synthesis of ER resident protein chaperones, thus increasing the folding capacity in this organelle. In addition, this response also activates pathways to induce programmed cell death. The stress-induced chaperone synthesis is regulated at the level of transcription. In *Saccharomyces cerevisiae*, the transmembrane protein, Ire1p, with both serine/threonine kinase and site-specific endoribonuclease activities is implicated as the sensor of unfolded proteins in the ER that transmits the signal from the ER to activate transcription in the nucleus. Activation of the unfolded protein response (UPR) pathway also requires the bZIP transcription factor, Hac1p. Although *HAC1* is transcribed constitutively, the mRNA is poorly translated. Upon accumulation of unfolded proteins, Ire1p generates a new processed form of *HAC1* mRNA that is efficiently translated by removal of a 252 base sequence. Using the yeast-interaction trap system we identified additional components of the UPR. A yeast transcriptional coactivator complex, Gcn5p/Ada, which is composed of Gcn5p, Ada2p, Ada3p, and Ada5p, was identified that interacts with Ire1p and Hac1p. Deletion of *GCN5*, *ADA2*, and/or *ADA3* reduces, and deletion of *ADA5* completely abrogates, the transcriptional induction in response to misfolded protein in the ER. A protein phosphatase, Ptc2p, was also identified as a negative regulator of the UPR that directly interacts with and dephosphorylates activated Ire1p. Recently, two mammalian homologues of Ire1p, *IRE1* and *IRE2*, were identified. hIre1p, is preferentially localized to the nuclear envelope and requires a functional nuclease activity to transmit the UPR. These results indicate that some features of the UPR are conserved from yeast to humans and may be composed of a multicomponent complex that is regulated by phosphorylation status and is associated with the nuclear envelope to regulate processes including transcriptional induction and mRNA processing. We propose that activation of Ire1p induces splicing of *HAC1* mRNA as well as engages and targets the Gcn5/Ada/Hac1 protein complex to genes that are transcriptionally activated in response to unfolded protein in the ER. The transcriptional activation is facilitated by targeting the histone acetylase, Gcn5p in yeast, to promote histone acetylation at chromatin encoding ER stress-responsive genes. In addition, activation of Ire1p leads to increased lipid biosynthesis, thereby allowing ER expansion to accommodate increasing lumenal constituents. Under conditions of more severe stress, cells activate an Ire1p-dependent death pathway that is mediated through induction of GADD153/CHOP.

Unfolded protein response Ire1 Gcn5/Ada complex Ptc2 ER stress

IN eukaryotic cells, *trans*-membrane proteins, as well as proteins that are destined for secretion, transit the secretory pathway consisting of the endoplasmic reticulum (ER) and the Golgi complex. These proteins are synthesized at the ER membrane and are generally co-translationally translocated into the lumen of the ER. The ER is the site for the initial processing events such as signal peptide cleavage, N-linked core-oligosaccharide addition and subsequent trimming of glucose and mannose residues, and disulfide bond formation that are critical for proper folding of nascent polypeptide chains. The ER is also the major site for folding of proteins that are in transit to other cellular compartments or the cell surface. The ER lu-

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men provides an oxidizing environment that favors the formation of disulfide bonds and contain enzymes, such as ERP72 and PDI, that catalyze disulfide bond formation and exchange [see (14,16) for review]. Finally, the ER contains a set of proteins that facilitates protein folding by preventing aberrant folding and aggregation and maintaining proteins in a folding-competent state (14,16). The expression of these protein chaperones that catalyze protein folding can be induced to meet the cellular need. Upon accumulation of unfolded protein in the ER, the cell activates a signal transduction cascade that culminates in the transcriptional induction of genes encoding ER-localized protein folding catalysts and chaperones to correct the protein folding defect. If the defect cannot be corrected, cells activate apoptosis, a programmed cell death. Conditions that overload the ER with unfolded and aggregated protein include the expression of mutant proteins or unassembled protein subunits, overexpression of some wild-type proteins, or by treatment with pharmacological agents that disrupt protein folding in the ER such as tunicamycin to inhibit N-linked glycosylation, β-mercaptoethanol or dithiothreitol to inhibit disulfide bond formation, or ionophores that deplete ER calcium stores. Cells respond to ER stress by increasing the synthesis of ER resident chaperones [(28) and references therein] and thereby increasing the folding capacity in that compartment. This response is conserved in all eukaryotes and is appropriately termed the unfolded protein response (UPR). In this review we summarize the recent advances in elucidating the UPR pathway and other misfolding-related ER stress responses in yeast and in mammalian cells (see Table 1).

THE UPR IS REGULATED AT THE LEVEL OF TRANSCRIPTION INITIATION

The induction of the ER resident protein chaperones in response to the accumulation of unfolded protein is regulated at the level of transcription. In the yeast *Saccharomyces cerevisiae*, genes (*KAR2*, *PDI1*, *EUG1*, *FKB2*, and *LHS1*) that are transcriptionally activated in response to unfolded protein in the ER contain a *cis*-acting 22-base pair promoter element termed the unfolded protein response element (UPRE) that is necessary and sufficient to mediate the transcriptional induction (31,32). The UPREs in these genes contain partially palindromic sequence (CAGCGTG) that has a spacer of one nucleotide that is required for transcriptional induction (31). In contrast, the mammalian *cis*-acting promoter element for the mammalian UPR is more complex. Studies of Chang and coworkers (4) demonstrated that multiple redundant elements are required. Two critical elements identified were CORE and C1. More recently, Yoshida and coworkers (51) identified a common 19-nucleotide sequence motif [CCAAT(N)9CCACG]. This element is termed the ER stress response element (ERSE) and is responsible for the transcriptional induction of GRP78, GRP94, and calreticulin in response to ER stress. The existence of these *cis*-acting elements suggests a mechanism as to how the expression of only a limited set of genes is induced to promote protein folding and relieve ER stress.

TABLE 1
ER STRESS RESPONSES LEADING TO CELL SURVIVAL OR CELL DEATH

ER Stress	Response
Activation of IRE1/2 kinase/endonuclease	
Induction of membrane biosynthesis	survival
Induction of ER protein chaperones	survival
Induction of GADD153/CHOP	death
Activation of eIF-2 kinase	
Inhibition of translation	survival
Induction of cell death	death

Ire1p IS THE SENSOR OF UNFOLDED PROTEIN IN THE ER

The disocvery that the yeast 22-base pair UPRE was sufficient for induction in response to ER stress permitted two groups to isolate mutants in this response by screening defective induction of β-galactosidase under control of a minimal promoter and the 22-base pair element. Both groups simultaneously isolated a second component of the yeast UPR pathway, *IRE1/ERN1*, that encodes a 1115-amino acid transmembrane protein (8,30). The amino-terminal domain (amino acids 1–526), localized to the ER lumen, is followed by a short transmembrane domain (amino acids 527–555) that spans the ER membrane. The carboxy-terminal residues (amino acids 556–1115) are localized to the cytoplasm and/or nucleoplasm. Ire1p is a bifunctional enzyme with both Ser/Thr kinase (38,46) and site-specific endoribonuclease activities (40). The domain structure of Ire1p resembles that of type I growth factor receptors that are activated by ligand-mediated oligomerization and *trans*-autophosphorylation. In fact, Ire1p undergoes oligomerization in response to unfolded proteins in the ER and oligomerization is required for its *trans*-autophosphorylation.

We recently identified a human homologue (hIre1p) of yeast Ire1p (yIre1p) utilizing degenerate PCR (43). hIre1p is a 110-kDa protein. Like the yeast counterpart, hIre1p is constitutively expressed, but at

a very low level. Autoregulation of hIre1p at the level of transcription and/or transcript stability may account for its low abundance. hIre1p contains high mannose core oligosaccharides (43), which is hallmark of ER resident glycoproteins. Immunolocalization studies revealed that hIre1p colocalizes with the ER resident protein chaperone GRP94, as well as RanGAP1, a component of the nuclear pore complex (43). These results demonstrate that hIre1p is an ER membrane protein preferentially localized to the nuclear envelope. Moreover, like yIre1p, overexpression of wild-type hIre1p constitutively activated the UPR whereas the overexpression of a kinase-defective hIre1p mutant downregulated the UPR (43). Taken together, these data demonstrate that hIre1p is indeed required to activate the UPR pathway in mammalian cells. Recently, we demonstrated that a mutant hIre1p that has intact kinase activity but defective RNAse activity was not able to activate the UPR when overexpressed in mammalian cells. These results provide intriguing evidence that a similar mRNA processing reaction may exist in higher eukaryotes as observed in *S. cerevisiae*. An additional *IRE1*-related gene was recently isolated from murine tissue that displays significant divergence from the human *IRE1* and has been named Ire1βp (45). Murine and human genetic loci for these genes have been designated *ern1* and *ern2* and *ERN1* and *ERN2*, respectively. Overexpression of either full-length Ire1βp, or of the isolated cytoplasmic domain alone, was reported to activate the BiP promoter and induce apoptosis. The remarkable conservation of kinase and the RNase L domains between species suggests that both kinase and RNase activities of Ire1p are critical for transmitting the unfolded protein signal from the ER to the nucleus.

Hac1p IS A *trans*-ACTING FACTOR THAT ACTIVATES THE YEAST UPR

Recently, two groups identified a *trans*-acting factor, Hac1p, that mediates the transcriptional induction of ER stress-responsive genes in yeast (9,29). Hac1p belongs to the basic leucine zipper (bZIP) class of transcription factors. Activation of the UPR pathway is dependent upon the cellular levels of Hac1p. *HAC1* is efficiently transcribed in the presence as well as in the absence of ER stress. When there are no unfolded proteins in the ER, *HAC1* mRNA is exported to the cytoplasm, engaged with ribosomes, but very poorly translated. Kawahara and coworkers (20,21) demonstrated that a 252-nt 3′ untranslated region (3′ UTR) within *HAC1* mRNA attenuates its own translation. Chapman and Walter (5) have shown that the *HAC1* 3′ UTR attenuates not only its own translation but also the translation of GFP mRNA when placed behind the coding region. However, under the conditions of ER stress, *HAC1* mRNA is processed to remove the 3′ UTR in an Ire1p-dependent manner (9,21). Because the new mRNA species lacks the translational attenuator, it is translated efficiently.

The *trans*-acting factors for the mammalian UPR are poorly characterized. The NF-y/CBF transcription factor was reported to bind the CORE region, whereas the YY1 zinc finger-containing protein bound to C1 (25). However, neither of these factors could account for the UPR transcriptional induction because their amounts or modification are not known to be altered upon ER stress. More recently, using a yeast one-hybrid screening approach, Yoshida et al. (50) isolated a bZIP protein, ATF6, that putatively binds the ERSE. Overexpression of ATF6 constitutively induced the BiP promoter in an ERSE-dependent manner (50). In nonstressed cells, ATF6 is constitutively expressed as a 90-kDa protein. However, upon ER stress the 90-kDa protein is posttranslationally processed to a 50-kDa form that is proposed to be the transcriptionally active form of ATF6 (50). Therefore, it appears that activation of the UPR pathway is regulated at different levels in eukaryotes; Hac1p translation attenuation and mRNA processing in yeast and ATF6 posttranslational protein processing in mammalian cells.

HAC1 mRNA IS PROCESSED BY AN UNCONVENTIONAL MECHANISM

Sidruski and Walter (40) demonstrated that the yeast Ire1p is an endoribonuclease that can cleave *HAC1* mRNA at two specific sites within the 3′ end of the mRNA in vitro. This is consistent with the observations that in vivo *HAC1* mRNA processing requires Ire1p but not the components of the spliceosome (9). Remarkably, cleaved *HAC1* exons are ligated by the tRNA ligase, Rlg1p (41), underscoring that the *HAC1* mRNA processing is unique and is different from spliceosome-mediated conventional pre-mRNA splicing. Like the yeast counterpart, human Ire1p is also a site-specific endoribonuclease (43). However, hIre1p cleaves *HAC1* mRNA at the 5′ exon junction, but not at the 3′ exon junction, suggesting that the cleavage specificity of hIre1 for the 3′ splice site may have diverged from the yeast enzyme. The kinase activity of hIre1p is required for the in vitro *HAC1* mRNA cleavage, indicating that oligomerization and *trans*-autophosphorylation precedes the RNase activation.

The subcellular localization of hIre1p reveals not

only the potential site of *HAC1* mRNA processing but also a possible signaling route from unfolded protein signal in the ER to transcriptional activation in the nucleus. Localization of hIre1p to the nuclear envelope and yeast Rgl1p close to the nuclear pore (6) suggests that *HAC1* mRNA processing occurs at the nuclear pore as it is being exported to the cytoplasm for translation. Hac1p is synthesized in the cytoplasm and is translocated to the nucleus where it activates transcription of the target genes. Perhaps utilization of an RNA processing machinery different from the spliceosome allows quick and efficient processing that ensures a rapid response required for cell survival under the conditions of ER stress. It seems rather wasteful that eukaryotes have invented this unique RNA processing machinery to process only *HAC1* mRNA. Considering the surprising features of the UPR pathway, it is possible that activation of Ire1p directly mediates the processing of yet unknown mRNAs required for the UPR and also indirectly activates ATF6 protein processing.

THE Gcn5/Ada COMPLEX IS REQUIRED FOR THE UPR

To elucidate how Ire1p transmits the unfolded protein signal from the ER to the nucleus, we searched for signaling molecules downstream from Ire1p in *S. cerevisiae*. The transcriptional co-activator, Gcn5p, was isolated as a specific interactor of Ire1p in a yeast two-hybrid screen. In eukaryotes, activation of transcription requires functional interaction between basal factors that occupy the TATA box and the activators that bind the upstream activating sequences. It is thought that this functional interaction is mediated by the transcriptional co-activators. Gcn5p (Ada4p) is a component of a multimeric transcription co-activator complex (13,27) that is composed of Ada1p (19), Ada2p (13,27), Ada3p (18), and Ada5p (26). Gcn5p is an acetyltransferase (2) and is believed to facilitate transcription by acetylating histones and opening up chromatin structures. Yeast strains lacking Gcn5p, Ada2p, or Ada3p were partially defecive in both activating transcription from a UPRE-driven lacZ reporter or of the endogenous *KAR2* gene (47). These results implicate that the Gcn5/Ada complex plays a role in the UPR. In contrast, strains carrying deletions in Ada5p were completely defective for the UPR, indicating a strict requirement for Ada5p in this response (47). However, heat-mediated induction of *KAR2*, from a heat shock element downstream of the UPRE, was intact (47). Therefore, the Gcn5/Ada complex is specifically required for the UPR but not for the heat shock response. We have recently demonstrated that Gcn5p directly interacts with Hac1p (Welihinda et al., manuscript in preparation). Because Ada2p and Ada5p are known to interact with basal factors, it is conceivable that Gcn5/Ada complex plays a co-activator role to maximally activate Hac1p-mediated transcription of genes encoding the ER resident chaperones.

Interestingly, ada5 and ire1 null mutant strains shared common phenotypes. Both were defective in the UPR and both require inositol for growth. Interestingly, Ire1p directly interacts with Ada5p in vitro (Welihinda et al., manuscript in preparation). Indeed, the original interaction detected between Gcn5p and Ire1p in the yeast two-hybrid system was probably indirect and mediated by endogenous Ada5p. The direct interaction between Ire1p and Ada5p led us to test the intriguing possibility that these co-activators are involved in *HAC1* mRNA processing. To our surprise, ada5 knockout cells were defective in *HAC1* mRNA processing in response to unfolded proteins in the ER, suggesting a novel role for this transcriptional co-activator in HAC1 mRNA processing.

Ptc2p IS THE "TURN OFF" SWITCH FOR THE UPR PATHWAY

In the search for proteins that interact with Ire1p in *S. cerevisiae*, we also isolated *PTC2* that encodes a serine/threonine phosphatase of type (48). The Ptc2p interaction with Ire1p was specific, direct, dependent on Ire1p phosphorylation, and mediated through a kinase interaction domain within Ptc2p (48). Ptc2p dephosphorylated Ire1p efficiently in a Mg^{2+}-dependent manner in vitro, providing initial clues to its role in the UPR pathway. Like other components of the UPR pathway, *PTC2* was nonessential for growth. Overexpression of wild-type, but not catalytically inactive mutant, Ptc2p inhibited cell growth (48). Similar to ire1 knockout cells, yeast overexpressing the wild-type Ptc2p were more sensitive to Tm, suggesting that the UPR pathway is compromised upon Ptc2p overexpression.

The role of *PTC2* in the UPR pathway was investigated by construction of two yeast strains carrying null alleles of ptc2 (48). These strains contained a UPRE-lacZ reporter plasmid that permitted convenient quantitaion of the UPR. Compared to the wild-type cells, both null mutants showed a three- to four-fold higher induction of the UPR indicating that the UPR pathway was hyperactive in cells devoid of Ptc2p. In agreement with these results, cells overexpressing wild-type Ptc2p showed a sixfold reduction in the UPR, strongly indicating that *PTC2* is a negative regulator of the UPR pathway (48). To investi-

gate whether the catalytic activity of Ptc2p is required for its in vivo regulation of the UPR, the invariant Glu residues within the catalytic pocket were mutagenized. The double mutant (E37A/D38A) destroyed the catalytic activity without affecting Ire1p interaction. Overexpression of Ptc2pE37A/D38A substantially increased both basal and Tm-induced UPR, indicating that the catalytic activity of Ptc2p is required for the negative regulation of the UPR pathway (48). In addition, Ptc2pE37A/D38A appears to compete with the endogenous Ptc2p to permanently activate the UPR pathway in a dominant negative manner (48).

Because activation of the UPR pathway is initiated by Ire1p-dependent *HAC1* mRNA splicing and subsequent synthesis of Hac1p (9,20), the role of *PTC2* in *HAC1* mRNA splicing was studied. In comparison to the wild-type cells, the Tm-dependent *HAC1* mRNA splicing was reduced to 60% in cells overexpressing wild-type Ptc2p. In contrast, either ptc2 null mutants or cells overexpressing catalytically inactive Ptc2p displayed an increased Tm-dependent *HAC1* mRNA splicing (48). Moreover, spliced *HAC1* mRNA was detected in cells devoid of Ptc2p, as well as cells overexpressing catalytically inactive Ptc2p, even in the absence of ER stress, indicating that the *HAC1* mRNA splicing is deregulated in these cells. These results confirm the role of *PTC2* as a negative regulator of the UPR and, more importantly, demonstrate that *PTC2* functions upstream of the *HAC1* mRNA splicing step.

ADDITIONAL CELLULAR RESPONSES OCCUR IN RESPONSE TO UNFOLDED PROTEINS IN THE ER

Overexpression of secretory proteins in mammalian cells causes dramatic dilation of the ER as well as induction of ER resident chaperones (10), providing an initial clue that synthesis of lumenal and membrane components is coordinately regulated. In yeast, the ER membrane is the primary site for lipid biosynthesis in the cell. The ER membrane has a high glycerophspholipid content and is particularly rich in phosphatidylinositol. Genes including *INO1*, *CHO1*, and *OPI3* that encode lipid biosynthetic enzymes are responsive to intracellular levels of inositol (1,17,22), a precursor for phosphatidylinositol. Inositol-responsive genes contain a 10-base pair *cis*-acting DNA element known as UAS_{INO} that is necessary and sufficient to mediate the transcriptional induction in response to low intracellular inositol [for review, see (3)]. At high intracellular inositol, a lucine zipper protein, Opi1p, represses these genes (49). Interestingly, mutations in *IRE1* (34), *HAC1* (41), *RGL1* (41), and *ADA5* (36) confer inositol protrophy strongly, suggesting a link between the lipid biosynthetic and the UPR pathways. In fact, Cox et al. (7) demonstrated that is indeed the case. Cells devoid of Ire1p or Hac1p show reduced *INO1* expression in response to inositol starvation. Furthermore, accumulation of unfolded proteins in the ER also induced *INO1* transcription in an Ire1p- and Hac1p-dependent manner. Taken together, these results indicate that the lipid biosynthesis and the UPR are intimately linked. Therefore, the UPR coordinates the synthesis of ER chaperones as well as ER membrane components to increase the folding capacity and the space required to accommodate accumulation of unfolded proteins.

In mammalian cells, the accumulation of unfolded proteins also leads to activation of apoptosis (23,35). Two pathways have been implicated in this response. First, upon ER stress, protein synthesis is inhibited by phosphorylation and inactivation of an essential eukaryotic translation initiation factor, eIF-2 (42). In addition, phosphorylation of eIF-2 and translational inhibition are sufficient to induce an apoptotic response (42). There are several dedicated eIF-2 kinases that have been described (37). Interestingly, a recently identified eIF-2 kinase was described that appears to be a transmembrane protein (39). This localization is suggestive that this kinase may respond to different conditions in the lumen of the ER to feedback and control protein synthesis. Initial inhibition of protein synthesis may limit the accumulation of unfolded proteins under stressful conditions. Upon prolonged stress, activation of the eIF-2 kinases may induce apoptosis through a yet unidentified mechanism.

A second apoptotic pathway appears mediated through GADD153/CHOP. CHOP (CEBP homologous protein) is a bZIP transcription factor that is known to promote apoptosis (12). Although GADD153/CHOP was originally identified as a growth-arrest and DNA damage-induced protein of 153 kDa (11), more recent studies indicate that GDDD153/CHOP is transcriptionally induced under conditions of ER stress to activate programmed cell death (44). Consistent with this, CHOP/GADD153 null mice exhibit threefold reduced apoptosis in response to ER stress (51). It is unclear why cells initiate constructive and self-destructive responses simultaneously under the conditions of ER stress. However, one can envision that the degree and/or the duration of the ER stress determine the appropriate response. For example, if the condition of stress is mild and tolerable, induction of pathways leading to preferential synthesis of chaperone and membrane components occurs. On the other hand, under the

conditions of severe and/or prolonged ER stress cells activate the apoptosis pathway.

POTENTIAL LIGANDS THAT TRIGGER Ire1p ACTIVATION

Although the signal emanating from Ire1p and the cellular responses generated by them are understood, the molecular event(s) occurring in the ER lumen that activates Ire1p remains largely unknown. It is argued that Ire1p detects the exposed hydrophobic regions of unfolded proteins in general. Binding of misfolded proteins would trigger oligomerization and activation of Ire1p, leading to initiation of the UPR. It is also possible that Ire1p senses the level of free BiP in the ER. According to this model, in the absence of unfolded proteins in the ER, BiP is bound to Ire1p and prevents oligomerization and subsequent activation of Ire1p endonuclease activity. When protein misfolding occurs, BiP becomes complexed with unfolded polypeptides, allowing Ire1p to oligomerize. A number of experiments have been performed in an attempt to distinguish between these two possibilities. Both in yeast (15) and mammalian cells (24) reduction of cellular BiP levels is sufficient to induce the UPR. Moreover, overexpression of BiP downregulates the UPR (33). Because BiP levels inversely correlate with misfolded polypeptides, it is still unclear what really is the ligand for Ire1p. As the lumenal domains of yeast and human protein share no apparent homology it is possible that they recognize different ligands.

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