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Intracellular Signaling by the Unfolded Protein Response

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Key Words

endoplasmic reticulum stress, signal transduction, organelle homeostasis, protein folding, regulated mRNA splicing, translational control

Abstract

The unfolded protein response (UPR) is an intracellular signaling pathway that is activated by the accumulation of unfolded proteins in the endoplasmic reticulum (ER). UPR activation triggers an extensive transcriptional response, which adjusts the ER protein folding capacity according to need. As such, the UPR constitutes a paradigm of an intracellular control mechanism that adjusts organelle abundance in response to environmental or developmental clues. The pathway involves activation of ER unfolded protein sensors that operate in parallel circuitries to transmit information across the ER membrane, activating a set of downstream transcription factors by mechanisms that are unusual yet rudimentarily conserved in all eukaryotes. Recent results shed light on the mechanisms by which unfolded proteins are sensed in the ER and by which the unfolded protein signals are relayed and integrated to reestablish homeostasis in the cell's protein folding capacity or—if this cannot be achieved—commit cells to apoptosis.

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INTRODUCTION AND OVERVIEW

ER: endoplasmic reticulum

ER stress: a condition in which the capacity of the ER to fold proteins becomes saturated. ER stress may be caused by drugs that impair glycosylation or disulfide bond formation or overexpression of or mutations in proteins entering the secretory pathway

All newly synthesized proteins need to fold properly and localize to their appropriate compartments within the cell. In eukaryotic cells, most secreted and plasma membrane proteins first enter the secretory pathway by translocation into the endoplasmic reticulum (ER). Proteins or membrane protein domains enter the ER through the translocon as unfolded polypeptide chains and fold within the lumen of this organelle (Wickner & Schekman 2005). Protein folding in the ER is facilitated by ER-resident chaperones, which prevent the nascent proteins from aggregating and instead steer them down productive folding pathways. Asparagine-linked carbohydrate moieties are added to many proteins entering the ER, and selective processing of the carbohydrate serves as a signal of the protein's folding state (Trombetta & Parodi 2003). Relative to the cytosol, the ER is an oxidizing environment, which facilitates formation of disulfide bonds in maturing proteins, further stabilizing the proteins' structure.

For secreted and membrane proteins to transit through the secretory pathway, they must first complete folding in the ER. The ER therefore constitutes a protein folding factory that imposes exquisite quality control on its products, ensuring that only properly assembled and functional proteins are delivered to their ultimate destinations (Ellgaard & Helenius 2003). Because many cell surface proteins relay important signals that ultimately determine cell fate—i.e., whether a cell is to differentiate, divide, migrate, or die—it is easy to appreciate why the fidelity of assembly of these components is vital for the health of an organism.

The load of proteins deposited into the ER varies between cell types and during the life of a cell. Developmental processes, cell cycle progression, and changes in the surrounding environment all can affect the amount and types of proteins that need to be folded in the ER. Thus, during their life, cells frequently encounter situations that cause the protein folding demand to overwhelm the ER's folding capacity, resulting in ER stress. ER stress can arise transiently as a cell's gene expression program is altered in response to changes in extracellular signals, or can be more permanent in cells bearing mutations that interfere with proper maturation of secretory or membrane proteins.

In some human genetic disorders, mutations in genes encoding important membrane and secretory proteins reduce the levels of these proteins because improper folding in the ER prevents their exit from this compartment. For example, the Z variant of α1 antitrypsin is a folding mutant that is retained in the ER of the hepatocyte, reducing its levels in the lung, where it normally functions (Qu et al. 1996). ER stress can also be caused by environmental perturbations encountered commonly by cells. These include starvation for nutrients; anoxia and ischemia; infection by viruses; and heat, which denatures proteins (Ma & Hendershot 2004, Feldman et al. 2005, Wu & Kaufman 2006). In all these cases, the folding capacity of the organelle is perturbed,

and the entire cell needs to adapt to the new condition.

To cope with and adapt to ER stress, an intracellular ER-to-nucleus signal transduction pathway evolved to match dynamically the ER's protein folding capacity to need. This pathway, termed the unfolded protein response (UPR), increases the amount of ER membrane and its components, including chaperones and protein-modifying enzymes needed to fold proteins. The UPR also decreases translation and loading of proteins into the ER and enhances the targeting of unfolded proteins in the ER for degradation. To this end, unsalvageable unfolded polypeptides are returned to the cytosol to be degraded by the proteasome via ER-associated degradation (ERAD) (Hiller et al. 1996, Wiertz et al. 1996, Meusser et al. 2005, Römisch 2005). If a homeostatic balance is not reestablished after inducing the UPR, i.e., if an acute UPR remains induced for a prolonged time, the cell commits apoptosis. Thus, cells at risk of displaying malfunctioning proteins on their surface are actively eliminated from an organism.

The signaling components that mediate the UPR were first discovered in the yeast *Saccharomyces cerevisiae* more than a decade ago. The two principal components of the pathway are an unfolded protein sensor in the ER membrane, the transmembrane signaling protein Ire1 (Cox et al. 1993, Mori et al. 1993), and a downstream effector, the transcription activator Hac1 (Cox & Walter 1996, Mori et al. 1996). The transcriptional targets of Hac1 ameliorate ER stress by expanding the ER (Sriburi et al. 2004) and with it the protein folding capacity of the cell. The initial understanding of the UPR was that of a simple feedback pathway: increased unfolded proteins → activation of Ire1 → production of Hac1 → activation of UPR target genes → decrease of unfolded proteins. Later, as the salient features of the yeast UPR were confirmed in metazoan cells, it became clear that the UPR in higher eukaryotes contains parallel and cross-wired circuitry, suggesting that the UPR is more accurately described as a signaling network that integrates information transmitted through multiple unfolded protein sensors and their downstream effectors. Recent studies in yeast indicate that the UPR in yeast also possesses the molecular roots for this complexity, upon which mammalian cells have built to adapt and enrich processing of the information flow through the pathway according to their unique requirements (Leber et al. 2004, Patil et al. 2004).

In this review we examine the remarkably conserved ensemble of UPR effectors and their mechanistic interconnections, injecting an evolutionary perspective as we trace the course of the unfolded protein signal between the compartments of the cell. We begin by describing the general circuitry of the different branches of the UPR and the transcriptional programs that they execute. We then follow the signal backward through the cytosol to the ER and close with a description of recent advances in our understanding of how unfolded proteins are recognized in the ER lumen.

UPR: unfolded protein response

ERAD: ER-associated degradation

UPR SIGNALING NETWORK AND TRANSCRIPTIONAL CONTROL

The UPR operates as a homeostatic control circuit that regulates the protein folding and secretion capacity of the cell according to need. At its core, the circuitry features a collection of transcriptional programs, whose targets expand the size and capacity of the entire secretory apparatus of the cell. UPR transcriptional control is exerted by the combinatorial action of a set of transcription factors whose qualitative makeup and concentration regimes are finely controlled by the conditions within the ER.

To date, three primary branches of the UPR have been characterized; each contributes via unique transcription factors to the execution of the transcriptional response (**Figure 1**). Most centrally, the central logic of transcriptional control by the Ire1 branch is highly conserved. In yeast, the accumulation of unfolded proteins in the ER activates

Ire1, which transmits the information across the ER membrane and excises an intron from *HAC1* mRNA in the cytosol (Cox & Walter 1996, Shamu & Walter 1996, Welihinda & Kaufman 1996, Kawahara et al. 1997, Sidrauski & Walter 1997), which in yeast is Ire1's unique target RNA (Niwa et al. 2005). Fusion of the resulting exons by tRNA ligase (Sidrauski et al. 1996) leads to a spliced mRNA that is efficiently translated to produce the Hac1 transcription factor responsible for activating UPR target genes. Analogously, Ire1-dependent mRNA splicing in higher eukaryotes removes an intron from *XBP1* mRNA, encoding the metazoan Hac1 ortholog (Shen et al. 2001, Yoshida et al. 2001, Calfon et al. 2002). Thus, the key regulatory step in the Ire1-branch of UPR signaling is the nonconventional splicing of the mRNA encoding the transcription activator.

It is likely that the UPR controls a similar basic set of target genes in all eukaryotic cells. A comprehensive study defined the transcriptional scope of the Ire1/Hac1-mediated UPR in yeast to comprise some 400 genes (~5% of the yeast genome), using stringent criteria based on bioinformatics and mutational analyses for inclusion of genes in the set (Travers et al. 2000). Thus, the extent of UPR transcriptional control mediated through the Ire1 branch alone is much larger than anticipated, including genes encoding proteins involved in ER protein folding and modification, phospholipid biosynthesis, ERAD, and

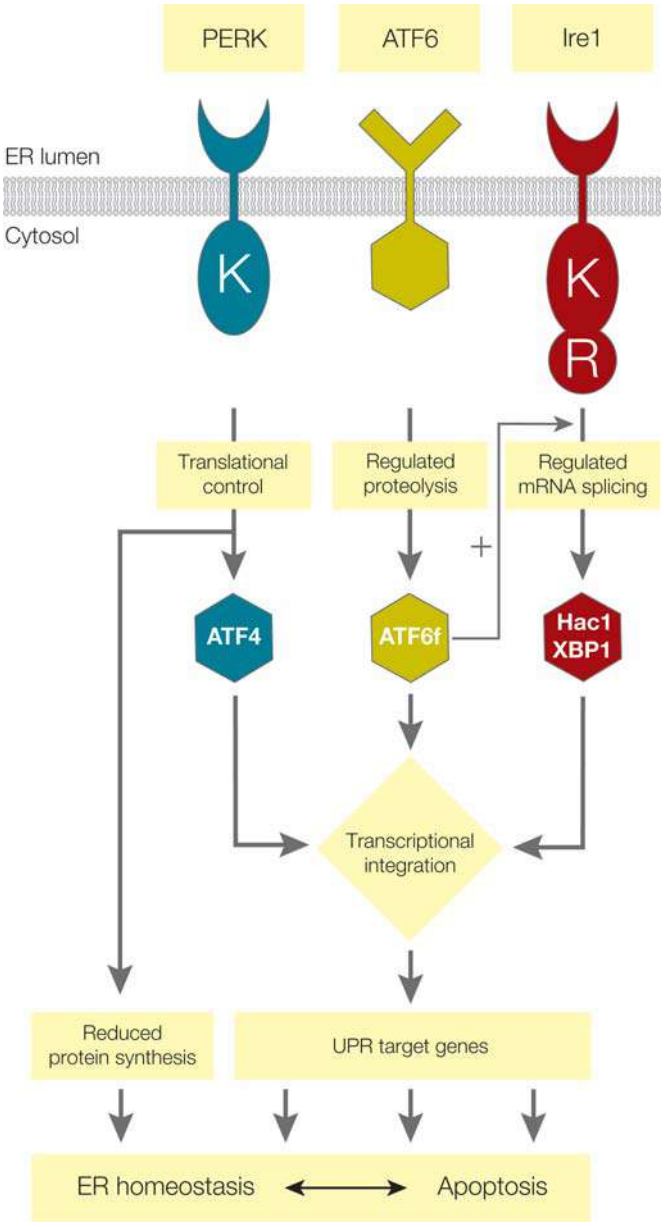


Figure 1

The three branches of the metazoan unfolded protein response (UPR). The three types of endoplasmic reticulum (ER) stress transducers—PERK, ATF6, and Ire1—sense the levels of unfolded protein in the lumen of the ER and communicate this information across the membrane to activate cognate bZip transcription factor via regulation of translational control, regulated proteolysis, and regulated mRNA splicing, respectively. In mammalian cells, ATF6f upregulates expression of *XBP1* mRNA (indicated by *plus sign*). The output of the transcription factors is integrated through their combinatorial action on UPR target genes, whose products increase the protein folding capacity of the cell and hence help the system reestablish homeostasis. PERK also reduces general translation in cells, thereby reducing the protein influx into the ER. If homeostasis in ER protein folding cannot be reached, cells undergo apoptosis. K, kinase domain; R, ribonuclease domain.

vesicular transport in the secretory pathway downstream of the ER. Consequently, the UPR transcriptional program not only increases the capacity of the ER folding machinery but also promotes clearance of proteins from the ER. At present, the inventory of metozoan UPR target genes is still incomplete. Nonetheless, as in yeast, it has been shown that ER folding factors, lipid biosynthetic enzymes, and ERAD components are coregulated during the response (Harding et al. 2003, Lee et al. 2003, Shaffer et al. 2004, Sriburi et al. 2004). Recent gene expression profiling in *Caenorhabditis elegans* classified some 500 UPR target genes according to the UPR branch that controls their activation and their developmental roles (X. Shen et al. 2005).

In yeast, the best-understood upstream activation sequence to which Hac1 binds, the unfolded protein response element 1 (UPRE-1), was identified in the promoter of the UPR target *KAR2* (Mori et al. 1992, Kohno et al. 1993). It came as a surprise that less than a fifth of the yeast UPR target genes contained this sequence element within their promoters. A bioinformatics approach revealed overrepresented motifs in promoters of other UPR target genes that define two additional UPREs (UPRE-2 and UPRE-3), which—although they share no recognizable sequence similarity—also bind Hac1 (Patil et al. 2004). This result suggests that Hac1 binds DNA differently depending on the UPREs present in a given promoter, possibly in combination with other transcription factors. Indeed, a genetic screen identified an additional activator, Gcn4, which, together with Hac1, binds to these two newly identified elements (Patil et al. 2004). Surprisingly, Gcn4 is also required to activate transcription of UPRE-1-driven promoters. Whether utilization of the three types of UPREs affords additional control of the UPR remains unknown. Moreover, the UPREs identified to date still explain the activation of only approximately half the UPR target genes, indicating that, even at the level of yeast target gene promoters, the full extent of regulatory complexity has not yet been revealed.

Gcn4 participates in several stress responses, including amino acid starvation, glucose limitation, and UV irradiation. Gcn4 is conditionally translated under such conditions (Yang et al. 2000, Natarajan et al. 2001, Stitzel et al. 2001). To work as Hac1's partner in activating transcription at the UPREs, however, Gcn4 does not require induction of its translation as in the other responses. Rather, its basal expression level is necessary and sufficient. Intriguingly, ATF4, the metazoan ortholog of Gcn4, likewise is a transcription activator of the UPR (Harding et al. 2000, Novoa et al. 2003). By contrast to Gcn4, ATF4 translation is under control of the ER-proximal signal transducer PERK, which defines the second, but metazoan-specific, branch of the UPR (**Figure 1**).

During UPR induction conditions, the level of *HAC1* mRNA does not change. Synthesis of Hac1 is under tight translation control: Only spliced *HAC1* mRNA from which the intron has been removed is translated. When cells suffer from particularly harsh stress conditions, such as ER stress in combination with temperature increase, the transcription of *HAC1* mRNA is upregulated three- to fourfold (Leber et al. 2004). Increasing the cellular *HAC1* mRNA concentration alone has no effect on the production of Hac1 until induction of splicing removes the translational block, leading to higher levels of Hac1. During this enhanced response, termed super-UPR (S-UPR), the transcription of UPR target genes is modified by the higher Hac1 concentrations, eliciting a qualitatively different transcriptional response to adjust to the stress conditions. Under S-UPR conditions, *HAC1* mRNA transcription is upregulated independently of Ire1 and Hac1 activity. Thus, in yeast a second pathway must operate in parallel to the Ire1-dependent branch of the UPR, sensing the conditions inside the ER and affecting a transcriptional response. The molecular components that carry out ER-to-nucleus signaling

UPRE: unfolded protein response element

Kar2: known as BiP in metazoan cells, an ER-resident member of the HSP70 family of molecular chaperones that participates in protein translocation and folding

ERSE: ER stress response element

INO1: encodes inositol 1-phosphate synthase, the enzyme that catalyzes the conversion of glucose 6-phosphate to inositol 1-phosphate

under S-UPR conditions remain to be identified.

A third branch of the metazoan UPR is mediated by ATF6 (**Figure 1**). ATF6 is a bZIP transcription factor, but it is initially synthesized as an ER-resident transmembrane protein. Upon UPR induction, it migrates to the Golgi apparatus, where a cytosolic fragment (ATF6f) bearing the transcription factor function is severed proteolytically from the membrane (Ye et al. 2000). ATF6f activates transcription from promoters containing ER stress response elements (ERSE-I and ERSE-II) (Yoshida et al. 1998, Li et al. 2000, Kokame et al. 2001, Okada et al. 2002). In mammals, a family of ATF6-like proteins includes at least four members, ATF6α, ATF6β, OASIS, and CREBH, that are regulated in a similar fashion during the UPR. Their expression varies among cell types—OASIS and CREBH, for example, have particularly important roles in astrocytes and liver cells, respectively (Omori et al. 2001, Kondo et al. 2005, Zhang et al. 2006). One of the transcriptional targets of ATF6 is *XBP1* mRNA (Yoshida et al. 2000). The concentration of XBP1 is therefore responsive to the conditions in the ER lumen, conceptually parallel to the control of Hac1 concentration afforded by the S-UPR in yeast.

In the yeast UPR signaling network, the Gcn4 and S-UPR branches modulate the basic Ire1/Hac1-dependent ON/OFF switch. The S-UPR acts as a gain control, setting the final Hac1 concentration, and both Gcn4 and the postulated S-UPR-mediating transcription factor combinatorially collaborate with Hac1. All UPR transcription factors identified to date are bZIP proteins, which in principle could form, through their leucine zipper domains, hetero- and/or homodimers, and in doing so they could modulate the response combinatorially. In yeast, for example, Hac1 and Gcn4 bind to the same UPREs, presumably as a heterodimer, to activate these genes (Patil et al. 2004). Thus, it is likely that the promoters of different target genes are tuned to respond to the combination of transcription factors in the cell and that the selective utilization of different UPREs contributes to control. One of the most challenging questions in the field is how varying conditions in the ER are integrated with information about general cell physiology and lead to appropriate stress- and cell-type-specific transcriptional responses.

A still more complex type of transcriptional control is exhibited by a subset of UPR target genes, including the genes encoding phospholipid biosynthesis enzymes, such as *INO1*. These genes are controlled through an upstream activation sequence (UAS_{ino}) element in their promoters (Greenberg et al. 1982, Cox et al. 1997) and in the off state are repressed by Opi1. Upon UPR induction, Hac1 relieves Opi1-mediated repression by an unknown mechanism. Intriguingly, the activation of *INO1* depends on the intranuclear localization of the *INO1* locus: The integral membrane protein Scs2 and recruitment of the *INO1* locus to the nuclear periphery are required for activation (Brickner & Walter 2004).

Depending on the particular state of the cell and what type of ER stress is encountered, these outputs of the UPR can dynamically proliferate the ER, degrade unfolded proteins, or initiate apoptotic programs. Through these outputs, cells increase ER folding capacity and expand the organelle. A remarkable demonstration of the role of the UPR in development is seen during terminal differentiation of B cells into plasma cells as they prepare to convert their secretory system into antibodies factories (Gass et al. 2002). This differentiation process is XBP1 dependent (Reimold et al. 2001, Iwakoshi et al. 2003). The ER proliferates many fold, and nearly all known ER-resident proteins increase accordingly, allowing plasma cells to produce and secrete huge concentrations of immunoglobulins.

If subjected to continuous ER stress such that homeostasis is not regained, cells commit to apoptosis. Apoptotic programs are activated by a combination of signals from each of the three UPR branches as well as Ca^{2+} release from the ER (Scorrano et al. 2003,

Zong et al. 2003). In particular, the PERK and ATF6 branches of the UPR both contribute to transcriptional upregulation of proapoptotic genes, such as CHOP, which is under transcriptional control by ATF4 (Harding et al. 2000) and ATF6f (Yoshida et al. 2000). CHOP downregulates the expression of Bcl-2 (McCullough et al. 2001, Ma et al. 2002), and hence one of its downstream effects is to promote mitochondrial cytochrome *c* release, apoptosome formation, and activation of caspases that lead to cell demise. In parallel, Ire1 activation and binding to TRAF2 are thought to turn on the JNK cascade (Urano et al. 2000) and contribute to proteolytic activation of caspases, including the ER-localized caspase-12 and caspase-4 (Nakagawa et al. 2000, Hitomi et al. 2004). One of the initial proteases believed to trigger the proteolytic cascade is calpain (Yoneda et al. 2001), which responds to Ca^{2+} release from the ER. It is unknown how unfolded protein accumulation leads to Ca^{2+} release, and the molecular details of how cells integrate the various proapoptotic signals to make ultimately a binary life/death decision are not yet understood. The choice to commit to cell death rather than display potentially malformed and improperly functioning protein receptors on the cell surface can be thought of as the ultimate solution to protect the organism from cells that may no longer respond properly to signals from their environment and hence may exhibit uncontrolled growth or differentiation. Thus, cytoprotective and cytotoxic pathways compete to determine whether the cell will survive ER stress.

CONTROL OF SYNTHESIS OF THE UPR TRANSCRIPTION ACTIVATORS

As expected for homeostatic regulation, the initiation and shutoff of the UPR are tightly controlled, and UPR regulation is exerted at many steps of the pathway. The key regulatory step in the Ire1-dependent branch of the UPR is the removal of an intron from *HAC1* and *XBP1* mRNA in yeast and mammalian cells, respectively. Yeasts and metazoan cells appear to differ in the details of regulation afforded by this splicing event. In metazoan cells, the intron in *XBP1* mRNA is very short (23 or 26 nucleotides, depending on the species) and contained centrally in the open reading frame of the transcription factor. Its removal leads to a frame shift, resulting in production of a spliced mRNA that encodes a qualitatively different protein (the active transcription factor XBP1s) from that encoded on the unspliced mRNA (XBP1u). The role of XBP1u may be to downregulate XBP1s by binding and targeting it into a degradative pathway (Yoshida et al. 2006).

By contrast, the yeast intron in *HAC1* mRNA is 252 nucleotides long, and its presence controls the translation of *HAC1* mRNA (**Figure 2**). Unspliced *HAC1* mRNA is localized to the cytoplasm and engaged with functional polyribosomes, but the ribosomes are stalled on the mRNA owing to the presence of the intron, and no Hac1 is produced (Cox & Walter 1996, Chapman & Walter 1997). The translational attenuation afforded by the intron involves a direct, 16-nucleotide-long base-pairing interaction between the *HAC1* 5′ UTR and the intron (Ruegsegger et al. 2001). The mechanism by which the base-pairing interaction leads to stalling of the ribosomes is unknown, but it conceptually resembles translational control by microRNAs (miRNAs). In *C. elegans*, for example, the small developmentally controlled miRNA *lin-4* binds to *LIN-14* mRNA, inhibiting its translation on polyribosomes (Lee et al. 1993, Wightman et al. 1993, Bartel 2004). Thus, it is intriguing to speculate that the mechanism of translational control mediated by miRNAs in *trans* may be similar to that mediated by the *HAC1* intron in *cis*. By contrast, *XBP1* intron is too short and does not contain sequences that allow pairing to the 5′ UTR. The details of the translational control described for *HAC1* mRNA are therefore yeast-specific. Still, the possibility of translational control of *XBP1* mRNA has been suggested (Calfon et al. 2002) and deserves further investigation.

Caspases: the main effectors of apoptosis, they constitute a family of cysteine proteases that cleave proteins after aspartic acid residues

UTR: untranslated region

miRNA: microRNA

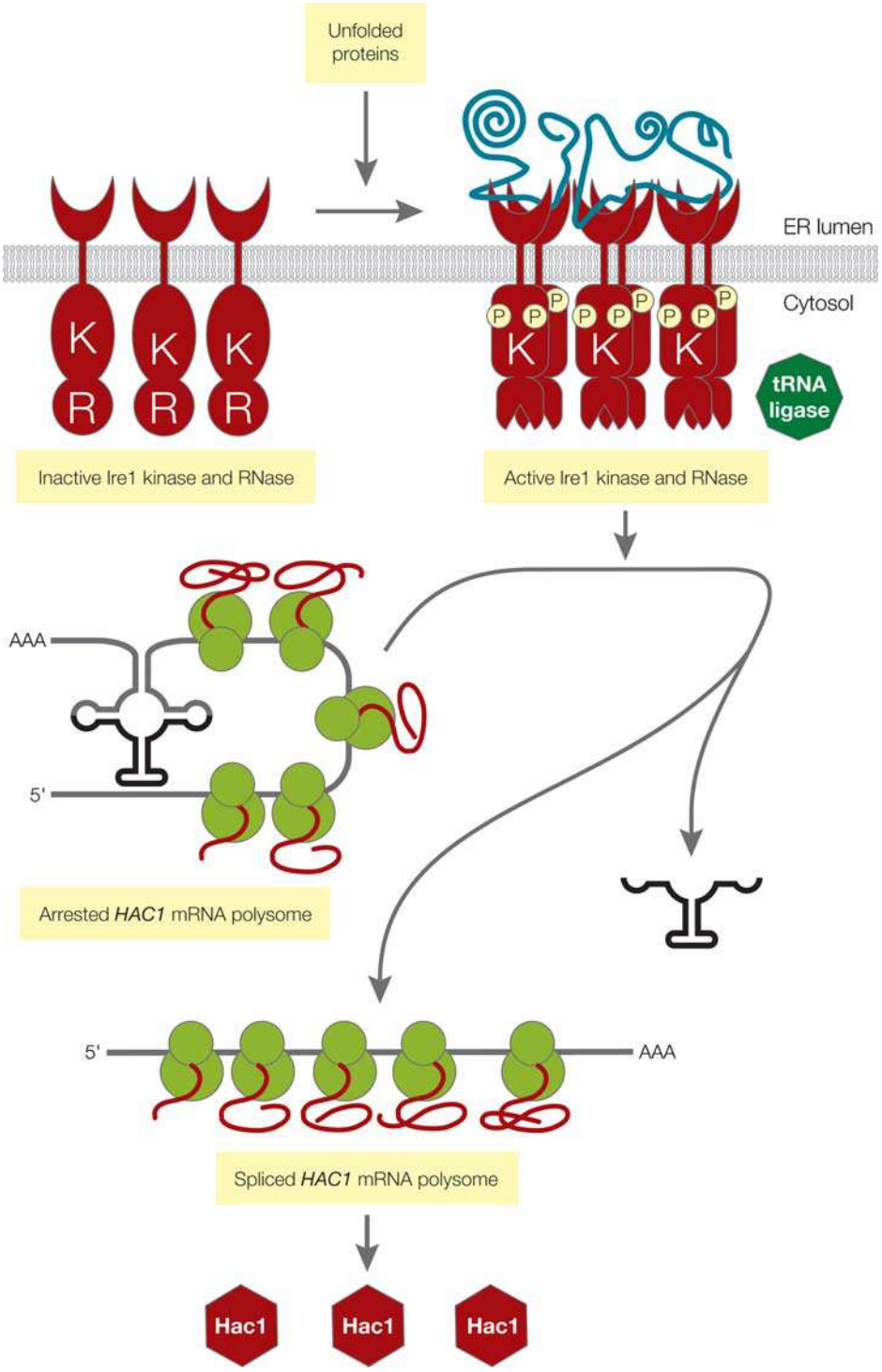


Figure 2

Mechanism of Ire1-mediated mRNA splicing in yeast. Unfolded proteins are recognized by the ER-lumenal domain of Ire1, leading to clustering of this stress sensor in the ER membranes. The Ire1 cytosolic domains become juxtaposed, in turn promoting transautophosphorylation by the kinase domain (K) and concomitant activation of the endoribonuclease domain (R). Base-pairing between the 5′ UTR and the intron of *HAC1* mRNA inhibits its translation; ribosomes are already loaded on the translationally inhibited mRNA. Ire1 excises the *HAC1* mRNA intron, and the resulting exons are ligated by tRNA ligase. Spliced *HAC1* mRNA is efficiently translated, producing the transcription factor Hac1, which travels to the nucleus and activates its target genes.

A different type of translational control is mediated by the phosphorylation of the α subunit of translation initiation factor 2 (eIF2α) via the PERK branch of the mammalian UPR (Shi et al. 1998, Harding et al. 1999) and Gcn2 in yeast (Patil et al. 2004). Like Ire1, PERK is a single-pass ER transmembrane kinase. Upon activation by the accumulation of unfolded proteins in the ER lumen, it phosphorylates eIF2α, which blocks the formation of ribosomal preinitiation complexes and causes general translation attenuation, thereby decreasing the load of proteins translocated into the ER. A direct consequence of this reduction in translation is a rapid decrease in the concentration of cellular cyclin D1 and a concomitant G1 cell cycle arrest (Brewer et al. 1999, Brewer & Diehl 2000, Niwa & Walter 2000). Although translation of most mRNA is attenuated under conditions of limiting eIF2α, a subset of mRNAs that contain small upstream open reading frames (Miller & Hinnebusch 1990, Harding et al. 2000) or internal ribosome entry sites (Fernandez et al. 2002) is preferentially translated under these conditions (Lu et al. 2004). In this way, PERK activation leads to the production of the UPR transcription factor ATF4 (Harding et al. 2000, Scheuner et al. 2001). *XBP1* mRNA as well as some other mRNAs are enriched on the ER surface, where they may be preferentially translated when eIF2α is limiting (Stephens et al. 2005).

ER STRESS SENSORS: TRANSDUCTION OF THE UNFOLDED PROTEIN SIGNAL ACROSS THE MEMBRANE

Each of the three classes of ER stress sensors—Ire1, PERK, and ATF6—independently transduces the unfolded protein signal across the ER membrane. The Ire1-dependent UPR branch is evolutionarily conserved in all eukaryotic cells and is the most ancient, whereas PERK and ATF6 first evolved in metazoans (**Figure 3**). In mammals, the *IRE1* gene became duplicated, giving rise to Ire1α and Ireβ. Whereas Ire1α is expressed in all mammalian cells, Ireβ is expressed primarily in intestinal epithelial cells (Tirasophon et al. 1998, Wang et al. 1998, Bertolotti et al. 2001). It is not known whether Ireα and Ireβ have different activities; the two isoforms appear to have the same in vitro activities, subcellular localizations, and downstream target (*XBP1* mRNA). However, whereas *IRE1α* is essential for mammalian development (Zhang et al. 2005), *IRE1β* deletion does not lead to significant developmental defects (Bertolotti et al. 2001).

PERK evolved from Ire1 by grafting its ER-lumenal unfolded protein–sensing domain and transmembrane region onto an eIF2α kinase domain. This evolutionarily chimeric protein introduces a new function in metazoans: attenuation of translation under ER stress. In mammals this function becomes pivotal, especially for professional secretory cells, as demonstrated by its absence in PERK-deficient homozygous patients with Wolcott-Rallison syndrome (Zhang et al. 2002). Affected individuals have vastly shortened lifespans of their endocrine and exocrine pancreatic cells as well as osteoblasts, all cell types specialized to secrete proteins.

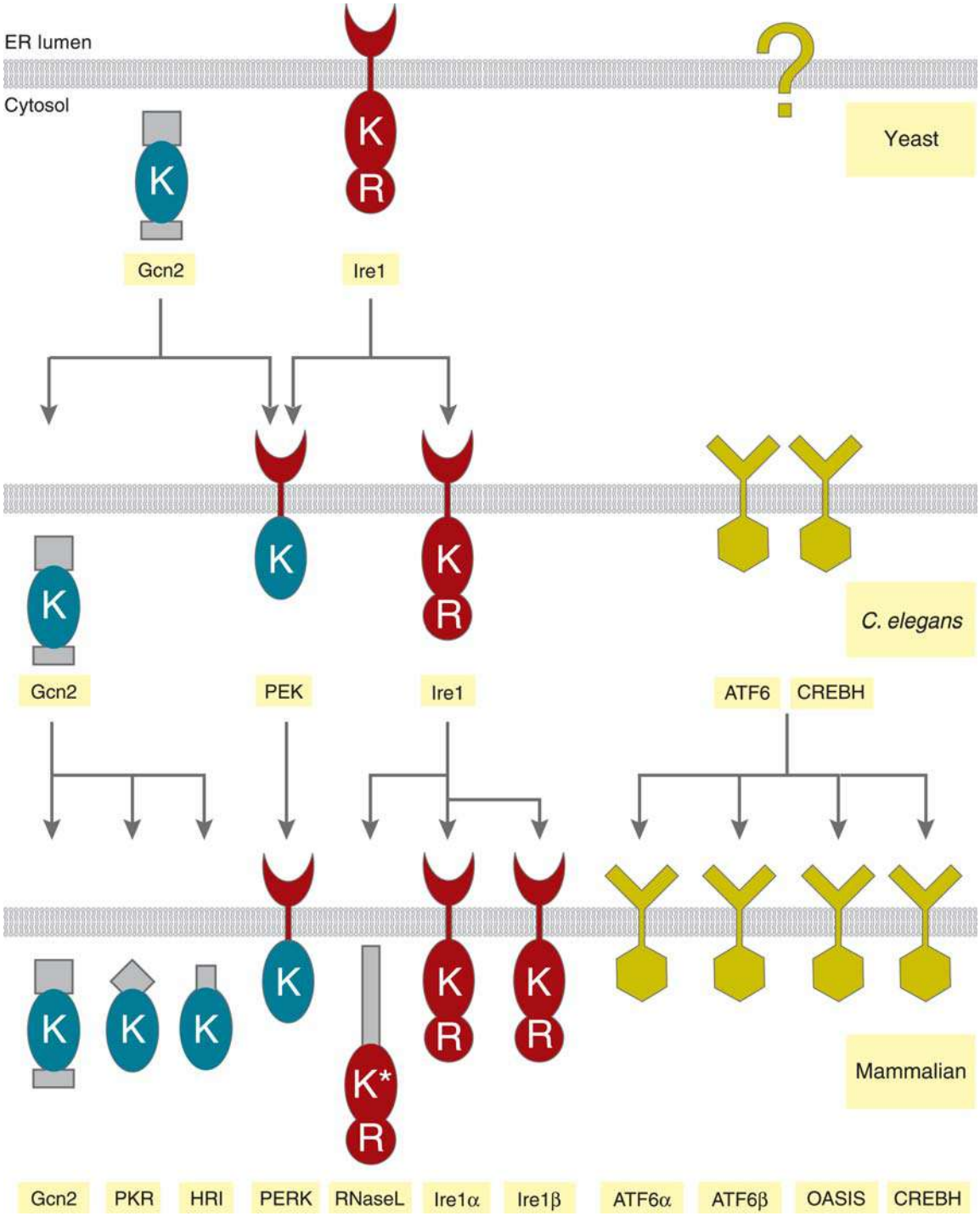
Studies in yeast have shown that the yeast ER-lumenal domain (LD) of Ire1 is functionally interchangeable with the LD of PERK from *C. elegans* (Liu et al. 2000), underscoring their common evolutionary origin and suggesting a similar mode of unfolded protein recognition. In both proteins, oligomerization of the LDs is thought to lead to clustering of the cytosolic kinase domains, which then become activated by transautophosphorylation. In this sense, Ire1 and PERK resemble a plethora of membrane receptor kinases that dimerize/oligomerize in the plasma membrane upon binding of cognate ligands, facilitating their activation.

By contrast, no sequence similarity is apparent between the LDs of Ire1 and PERK and the LD of ATF6, which is activated through an entirely different process: It is cleaved through regulated intramembrane

eIF2α: α subunit of eukaryotic translation initiation factor 2

Cyclin D1: key regulator of G1-to-S-phase progression of the cell cycle through the formation of active enzyme complexes with Cdk4 and Cdk6

LD: lumenal domain



proteolysis by Site 1 and Site 2 Proteases under conditions of unfolded protein accumulation, resulting in liberation of soluble ATF6f (Ye et al. 2000). Activation of ATF6 resembles activation of SREBP (Sterol Regulatory Element–Binding Protein), a transcription factor involved in cholesterol sensing and biosynthesis. In the presence of sufficient cholesterol, SREBP is retained in the ER by association with an anchor protein (Insig-1) together with its cholesterol-sensing partner protein Scap. When cholesterol levels become limiting, SREBP is released from the anchor and travels to the Golgi apparatus, where it is proteolyzed to release a functional transcription factor (Gong et al. 2006). By contrast to this well-established paradigm, it is not known how the intracellular localization of ATF6 is modulated in response to unfolded protein accumulation. BiP binding may retain ATF6 in the ER (Shen et al. 2002, J. Shen et al. 2005)

Evolution has spawned another Ire1 descendant, RNaseL, which is a component in the innate immune response (Zhou et al. 1993). RNaseL resembles Ire1 in its gross architecture (**Figure 3**) yet yields a radically different function. It is a soluble, cytosolic protein, with a kinase-like domain and an RNase related to Ire1. Like Ire1, RNaseL contains an N-terminal activation domain (in this case comprised of a series of ankyrin repeats) that drives dimerization upon ligand binding (Dong & Silverman 1995, Cole et al. 1996, Nakanishi et al. 2005). The ligands are 2′-5′ oligoadenylates that are produced in response to interferon signaling when viruses infect mammalian cells (Player & Torrence 1998). Dimerization activates the C-terminal RNase domain, which, in contrast to Ire1's site-specific RNase activity, nonspecifically degrades bulk ribosomal and other RNAs, thereby containing viral infection. It is unknown how the respective RNases of Ire1 and RNaseL discriminate their corresponding substrates.

2′-5′ oligoadenylates: molecules formed from ATP of short oligomers of adenosine with 2′ to 5′ phosphodiester linkages; those of three nucleotides in length or greater bind to and activate RNaseL

For Ire1, the kinase is a necessary component of the circuitry that allows transfer of an unfolded protein signal by this sensor. Mutations of catalytically essential kinase active site residues—or residues known to become phosphorylated—demonstrate that Ire1's kinase phosphotransfer function is essential for RNase activation (Shamu & Walter 1996). By contrast, RNaseL has lost phosphotransfer function during the course of evolution, yet its (pseudo)kinase domain is still necessary for activation of its RNase. It is thought that the kinases of Ire1 and RNaseL are dimerization modules and conformational switches that position the attached RNases to control their activation.

Figure 3

Evolutionary relationship of UPR components. The main components of the UPR are conserved through evolution, and many of the protein domains used by the UPR have been duplicated and adapted in higher metazoans, increasing the level of complexity of the response in these organisms. The Gcn2 kinase domain (K) is present in a single gene in yeast; in two genes, *GCN2* and *PEK* (*PERK*), in *C. elegans*; and in four genes—*GCN2*, *PKR*, *HRI*, and *PERK*—in mammalian cells. As such, mammalian cells respond by eIF2 phosphorylation through Gcn2 kinases to four different signals: starvation, double-stranded RNAs, heme, and unfolded proteins in the ER. Yeast *S. cerevisiae* has only Gcn2, but *Schizosaccharomyces pombe* has Gcn2 and two HRIs (Zhan et al. 2004). The PEK/PERK's ER-lumenal domain likely originated from *IRE1*, and both proteins are likely to sense unfolded proteins by similar mechanisms. *IRE1* also gave rise to RNaseL, which inherited the kinase/RNase module (denoted by K and R, respectively). The kinase/endoribonuclease domain of Ire1 can also be found in RNaseL, but the phosphotransfer activity of RNaseL's kinase domain has been lost in evolution. Two ATF6-like unfolded protein sensors in *C. elegans* gave rise to at least four [and possibly more (DenBoer et al. 2005, Stirling & O'Hare 2006)] family members in mammalian cells (ATF6α, ATF6β, OASIS, and CREBH). There is at least one additional way of transducing the unfolded protein signal in yeast (denoted by the *question mark* and defined phenotypically by the S-UPR), but the protein(s) mediating this branch remains to be identified.

cLD: core lumenal domain

Major histocompatibility complex (MHC): a set of membrane proteins displayed on cell surfaces that present small peptide antigens to lymphocytes for immunesurveillance

Adenosine nucleotide binding to the active kinase of Ire1 and to the pseudokinase of RNaseL stimulates the attached RNase activities. Interestingly, the requirement for both the kinase activity and phosphorylation of Ire1 is alleviated if a small ATP mimic, 1NM-PP1, is provided to a mutant Ire1 enzyme that has an expanded active site designed to accommodate 1NM-PP1. Thus, mere binding of a ligand in the active site of Ire1 is sufficient to propagate the unfolded protein signal through the kinase domain, and phosphotransfer can be bypassed (Papa et al. 2003). In response to adenosine nucleotide binding, the kinase domain may switch conformation and/or change its oligomeric state such that the RNase now becomes active. By analogy, the adenosine nucleotide ligand-occupied kinase domain of RNaseL may serve as a module that participates in activation and regulation of the RNase function. The elucidation of the roles of the kinase domains of Ire1 and RNaseL as conformational switches may shed light on the functions of other multidomain proteins containing kinase or enzymatically inactive pseudokinase domains.

The biological role of ligand occupancy is unknown. For Ire1, the in vitro adenosine nucleotide stimulatory effect is most pronounced when ADP is used. If ADP is the natural stimulatory ligand of Ire1's kinase domain in vivo, it may be providing some information about the cell's nutritional state. For instance, ADP levels rise temporarily in proportion to nutritional stress in many professional secretory cells, such as the β-cells of the endocrine pancreas. ATP levels also fluctuate but not as much as ADP levels (because ADP is normally maintained at low concentrations). Thus, ADP is poised to serve as a cofactor—or second messenger—that could signal a starvation state. ADP-mediated conformational changes may increase the dwell time of activated Ire1, serving as complementary input for activation of Ire1 (the other input is unfolded proteins). Protein folding becomes inefficient as the nutritional status of cells declines, triggering the UPR. Through this mechanism, information about nutritional stress may be relayed to the UPR in the face of energy depletion. As such, Ire1 may have evolved this regulatory mechanism to monitor the energy balance of the cell and to couple this information to activation of the UPR. Indeed, one proposed role of the UPR is that of a nutrition-sensing device, matching protein synthetic activity to energy supply (Kaufman et al. 2002).

THE MECHANISM OF SENSING UNFOLDED PROTEINS IN THE ER

The recent crystal structure of yeast Ire1 LD and structure-guided functional analyses of this domain provide a first glimpse at the mechanism by which unfolded proteins may be recognized in the ER lumen (Credle et al. 2005) (**Figure 4**). The structure revealed an ordered conserved core region (cLD), flanked on either side by disordered and functionally dispensable sequences. Whereas the cLD is a monomer in solution, two cLD monomers associate in an almost perfectly twofold symmetric head-to-head arrangement in the crystal lattice, burying a large interface. The most remarkable feature of the cLD dimer is a deep central groove formed by a β-sheet floor and walls composed of α-helices. In its architecture and dimensions, the groove resembles that of the peptide-binding pocket of major histocompatibility complexes (MHCs) (Bjorkman et al. 1987), suggesting that unfolded polypeptide chains bind there directly.

Mutational analyses suggest that cLD dimers form higher-order oligomers necessary for UPR activation across both head-to-head and tail-to-tail interfaces seen in the crystal lattice (Credle et al. 2005). Experimental dimerization of Ire1 mutants with engineered leucine zippers yielded partial activation of the RNase (Liu et al. 2000), perhaps indicating that the activation state of Ire1 is regulated in a continuum depending on the extent of oligomerization. According

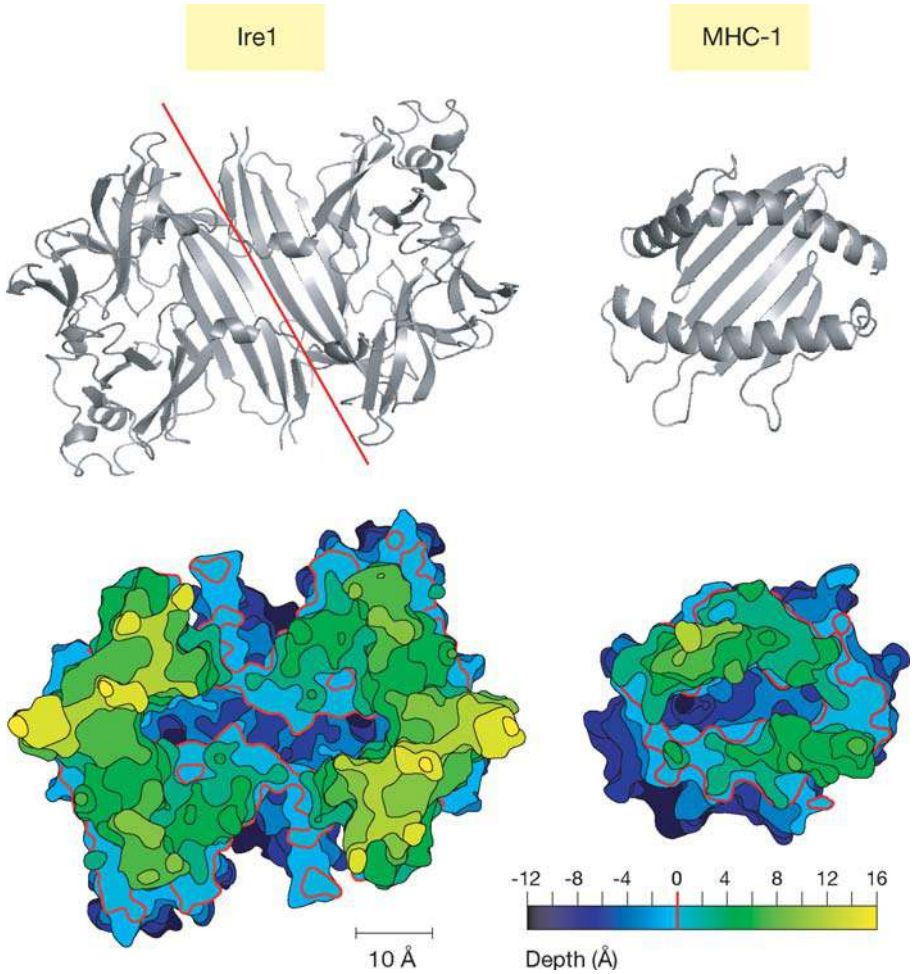


Figure 4

Structure of the Ire1 unfolded protein–sensing domain. (*Top row*) Ribbon diagrams of the cLD dimer (*left*) and MHC-1 (*right*) shown in the same scale for comparison. These two proteins have convergently evolved toward similar architectures, each containing a β-sheet floor on which two α-helices form a deep central groove. Ire1 cLD is a homodimer; the red line demarcates the division between two cLD monomers. (*Bottom row*) A topographic map of cLD and MHC-1 seen from the top. The map displays the grooves as deep canyons of roughly equivalent depths and widths in the two structures. The vertical spacing of the contour lines connecting points of equal depths is 2 Å, and different elevations are colored according to the scale provided. The red index line at depth 0 is set in both structures at the point where the rim becomes discontinuous. Relative to this contour, the grooves in both structures are 11-Å deep at their lowest point. The canyon of Ire1 is lined with conserved alternating hydrophobic and polar residues that may recognize unfolded proteins, which are proposed to bind there (modified from Credle et al. 2005).

to this notion, unfolded proteins may tether cLD dimers into higher-order oligomers. In turn, such an event may change the quaternary association of Ire1 in the plane of the ER membrane to position the kinase domains in the cytoplasm optimally for autophosphorylation and RNase activation. Indeed, Ire1 aggregates into higher-order structures (with a stoichiometry greater than dimeric) upon UPR activation (Shamu & Walter 1996),

resembling the activation mechanism of other membrane-localized sensing proteins (e.g., aspartate chemoreceptors of eubacteria).

The topic of the mechanism by which unfolded proteins are recognized in the ER lumen has generated lively debate. Previous models ascribed a negative regulatory role to the ER chaperone BiP (Bertolotti et al. 2000, Okamura et al. 2000). It was proposed that, as BiP binds to the LD of Ire1, it acts as a negative regulator, thus preventing Ire1 activation. This notion derives from the observation that Ire1 activation is temporally linked to reversible dissociation from BiP. In this view, free BiP levels fall as BiP engages unfolded proteins, and Ire1 becomes free to self-associate and activate. However, genetic and structural evidence supporting the idea that BiP dissociation causes, rather than simply being correlated with, Ire1 activation has not been readily forthcoming. Furthermore, this previous model was fraught with inconsistencies. First, BiP is present in the ER lumen at very high concentrations (in the millimolar range). Therefore, the UPR would not become activated unless and until large concentrations of unfolded proteins accumulated to provide a sufficiently large sink for free BiP. However, the UPR seems to respond to small fluctuations in the ER protein folding state, as would seem appropriate for a sensor that adjusts the ER protein folding capacity homeostatically. Second, recent studies identified the BiP-binding site in Ire1 to lie outside the cLD and showed that deletion of this region did not impair Ire1 regulation by the presence or absence of unfolded protein (Kimata et al. 2004, Oikawa et al. 2005).

Structure-guided analyses of LD provoke a new model wherein BiP binding and release in Ire1 activation are irrelevant or possibly only important under extreme activation conditions when the pool of free BiP becomes severely depleted. Such situations may arise under nonphysiological experimental conditions or upon prolonged UPR induction. BiP release under such conditions may serve to enter a different activation state, perhaps signaling that the UPR is not able to reestablish homeostasis in the ER and leading the cell down an apoptotic pathway. Conversely, BiP binding may dampen activation of Ire1 under conditions of mild unfolded protein accumulation (i.e., during conditions that may be dealt with through existing concentrations of ER chaperones). In this view, BiP binding would buffer Ire1 against normal fluctuations of ER unfolded proteins, thereby reducing "noise" in UPR signaling.

The gross resemblance of Ire1 cLD to the peptide binding domain of MHC suggests that unfolded proteins bind in the groove (**Figure 4**). Indeed, the groove is lined with a phylogenetically conserved patchwork of hydrophobic and polar amino acid side chains. Their substitution to alanine reduces UPR signaling (Credle et al. 2005). Thus, unfolded polypeptide chains and/or possibly partially folded proteins with exposed loops on their surface may bind to Ire1 directly in this groove, providing the primary signal mediating its activation.

If the groove in cLD indeed serves to bind portions of unfolded polypeptides, a variety of different—yet not mutually exclusive—mechanisms may provide the means for recognition. Hsp70-type chaperones such as BiP recognize a signature motif on unfolded proteins, which consists of hydrophobic amino acids in every other position (Flynn et al. 1991, Blond-Elguindi et al. 1993). Such a sequence resembles a β-strand, one side of which is destined to pack onto the hydrophobic core of a folded protein but has not yet been properly accommodated in the protein fold. Indeed, the groove in cLD contains a patchwork of conserved hydrophobic and hydrophilic residues. Thus, recognition of specific side chains or classes of side chains in preferred positions may play an important part in unfolded protein recognition by cLD.

Although sequence specificity may influence binding of particular polypeptides to cLD, the simple property of accessibility by itself may allow discrimination between the folded and unfolded states. By analogy,

unfolding of ER proteins exposes interior regions to UDP-Glc glycoprotein glucosyltransferase, a quality-control activity of the ER. The enzyme recognizes innermost sugars in the oligosaccharide moiety and hydrophobic polypeptide cores that become accessible only in misfolded glycoproteins (Trombetta & Parodi 2005). Given the depth of the cLD groove, it is inaccessible to surface residues on compactly folded proteins. In the extreme, interactions in the groove may be limited to backbone contacts only, paying little or no attention to the amino acid sequence of the polypeptide. On the other hand, these mechanisms need not be mutually exclusive, and both accessibility and sequence specificity may be important parameters in recognition of the unfolded protein by cLD. The next challenge in the field is to ascertain whether cLD binds unfolded proteins through these or yet other means.

Ultimately, it will be important to compare and contrast the mechanistic details of unfolded protein recognition by each of the different sensor proteins in the ER. PERK and Ire share a basic molecular architecture of the cLD but may differ in unfolded protein binding strength or kinetics. Similarly, the ATF6-like sensors may recognize unfolded proteins with distinct binding characteristics. Thus, the individual branches of the UPR may be activated differentially (Yoshida et al. 2003), perhaps by fine-tuning the response to a particular signature of the inducing signal or causing a particular temporal sequence to engage the UPR transcriptional effectors. Without question, much of the physiological importance of the UPR circuitry remains to be discovered.

SUMMARY POINTS

1. The unfolded protein response (UPR) is a homeostatic signaling pathway that adjusts ER protein folding capacity according to need.
2. The UPR employs three types of sensors that recognize unfolded proteins in the ER lumen and activate separate branches of the signaling network. Structural modules and mechanistic concepts are phylogenetically conserved; some have been duplicated and rearranged in evolution to generate higher complexity.
3. The UPR employs a variety of mechanisms in signal transduction, including regulated splicing, translational control, and regulated proteolysis.
4. The transcriptional output of the UPR is determined by the combinatorial action of the transcription factors activated through its signaling branches.
5. Structural and mutational analyses of the Ire1 unfolded protein-sensing domain suggest that unfolded proteins are recognized in the ER lumen by binding to Ire1 directly.

FUTURE ISSUES

1. If the UPR cannot reestablish ER homeostasis, cells commit to apoptosis. It is unknown how, mechanistically, this important binary life/death decision is made.
2. The three branches of the UPR use different unfolded protein sensors. It is unknown whether they recognize unfolded proteins differently and thus allow for differentiated responses that are tailored to specific needs. We have only incomplete information regarding the scope of the UPR transcriptional programs and how they relate to cell type or ER-stress-specific needs.

3. Many exciting mechanistic details of the signal transduction devices in the UPR remain to be explored. How are unfolded proteins recognized? How does the Ire1 kinase domain activate the RNase function? How is the ER → Golgi movement of ATF6 regulated? How is translation regulated by the *HAC1* mRNA intron? How does Ire1 recognize the splice site with such high specificity?

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