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Forum Review

Translational Control and the Unfolded Protein Response

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ABSTRACT

Cellular stresses that disrupt the processing of proteins slated for the secretory pathway induce the unfolded protein response (UPR), a regulatory network involving both translational and transcriptional control mechanisms that is designed to expand the secretory pathway and alleviate cellular injury. PERK (PEK/EIF2AK3) mediates the translational control arm of the UPR by enhancing phosphorylation of eIF2. Phosphorylation of eIF2 reduces global protein synthesis, preventing further overload of the secretory pathway and allowing the cell to direct a new pattern of mRNA synthesis that enhances the processing capacity of the endoplasmic reticulum (ER). PERK also directs preferential translation of stress-related transcripts, including that encoding ATF4, a transcriptional activator that contributes to the UPR. Reduced global translation also leads to reduced levels of key regulatory proteins that are subject to rapid turnover, facilitating activation of transcription factors such as NF-κB during cellular stress. This review highlights the mechanisms by which PERK monitors and is activated by accumulated misfolded protein in the ER, the processes by which PERK regulates both general and gene-specific translation that is central for the UPR, and the role of PERK in the process of cellular adaptation to ER stress and its impact in disease. *Antioxid. Redox Signal.* 9, 2357–2371.

PERK AND TRANSLATIONAL CONTROL ARE CENTRAL TO THE UPR

PROTEINS SLATED FOR THE SECRETORY PATHWAY are transported in an unfolded state into the lumen of endoplasmic reticulum (ER), on which the proteins are folded and processed before being transported to the Golgi apparatus. If the secretory demand exceeds the folding capacity of the ER, the accumulated malfolded proteins elicit the unfolded protein response (UPR), a program of gene expression that collectively enhances the processing, assembly, and transport of secretory proteins. A triad of stress sensors monitors the protein-folding capacity in the ER, detecting the accumulation of malfolded protein, often termed ER stress. One stress sensor, IRE1, is an ER transmembrane protein whose associated RNase activity directs splicing of XBP1 mRNA, encoding a potent basic region/leucine zipper (bZIP) transcriptional activator (15, 73, 84, 97, 113). The second stress sensor, ATF6, is targeted to the membrane of the Golgi apparatus and, on ER stress, is proteolytically cleaved and translocated into the nucleus to direct the transcription of genes subject to the UPR (18, 48, 112). The third UPR sensor is PERK (also designated PEK and EIF2AK3), an ER transmembrane protein kinase that reduces global translation, allowing cells to have sufficient time to correct the impaired protein folding resulting from ER stress before synthesizing additional secreted proteins (42, 45, 101, 102). PERK also directs the translational expression of ATF4, another transcription factor that is central to the UPR transcription program (43, 46, 79, 105).

Although each UPR sensor was originally viewed as acting in a temporally coordinated fashion, recent studies indicate that biologically important differences can be found in the activation of each UPR sensor (33). This review focuses on the third sensor, PERK, and highlights the mechanisms by which PERK recognizes and is activated by accumulated malfolded protein in the ER, the processes by which PERK regulates both gen-

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eral and gene-specific translation that is central for the UPR, and the role of PERK in remediation of stress and prevention of disease processes. This latter topic highlights the discovery that deletion of *PERK* in humans results in Wolcott–Rallison syndrome (WRS), an autosomal recessive disorder that is characterized by developmental or physiologic dysfunctions in specific secretory tissues, including the islet insulin-secreting β cells, leading to neonatal diabetes (25, 98). Mouse model systems disrupted for PERK, or downstream targets, mirror many of WRS-related disorders (42, 118, 119).

PHOSPHORYLATION Of eIF2 REDUCES GLOBAL TRANSLATION IN RESPONSE TO ENDOPLASMIC RETICULUM STRESS

PERK is a member of a family of protein kinases that phosphorylate serine-51 of the α subunit of eIF2. Many different cellular stress conditions have been described that elicit phosphorylation of eIF2α, including those impairing the secretory function of the ER. eIF2 consists of three subunits (designated α to γ) that associate with initiator Met-tRNA$_i^{Met}$ and GTP and participate in the ribosomal selection of the start codon (29, 51). Before the joining of the small and large ribosomal subunits in the translation-initiation process, GTP complexed with eIF2 is hydrolyzed to GDP, releasing the translation factor from the ribosomal machinery (Fig. 1). To facilitate subsequent rounds of translation initiation, the GDP-bound eIF2 is converted to active eIF2-GTP by a reaction catalyzed by the guanine nucleotide exchange factor, eIF2B. The eIF2B is a complex exchange factor, consisting of five different subunits (designated α to ϵ). The ϵ/γ subcomplex of eIF2B carries out the catalytic activity, and the $\delta/\beta/\alpha$ regulatory subcomplex binds to phosphorylated eIF2α, reducing GEF function (29, 51). Phosphorylation of eIF2α by PERK during ER stress converts this initiation factor from a substrate to an inhibitor of the eIF2B (see Fig. 1). The resulting reduction in eIF2-GTP levels reduces general translation, allowing cells to have sufficient time to correct the impaired protein folding resulting from ER stress before synthesizing additional proteins. Concurrently, PERK also induces gene-specific translation, which is important for the expression of stress-remedy genes (43, 46, 79, 105). Emphasizing the importance of this eIF2α kinase stress response, loss of *PERK* in mouse embryonic stem cells exposed to ER stress leads to inappropriately elevated protein synthesis that further exacerbates protein misfolding in this organelle, thus leading to apoptosis (44, 84, 97).

FAMILY OF eIF2α KINASES REGULATES TRANSLATION DURING ENVIRONMENTAL STRESSES

PERK is a transmembrane protein with its regulatory region in the lumen of the ER and eIF2α kinase domain in the cytoplasm (Figs. 2 and 3). Malfolded protein can accumulate in the ER in response to exposure to chemicals that release calcium from the ER organelle, inhibit protein glycosylation, or disrupt the ER oxidizing environment, as well during hypoxic or certain metabolic conditions that enhance the secretory load on the ER. As described further later, ER stress is recognized by the amino-terminal regulatory domain of PERK, leading to activation of the cytoplasmic eIF2α kinase activity. In addition to PERK, three other mammalian eIF2α kinases have been described, and each directly senses distinct stress signals and activates downstream response pathways by regulating translation (see Fig. 1). These eIF2α kinases include PKR (EIF2AK2), which participates in an antiviral defense mechanism that is mediated by interferon (39); HRI (EIF2AK1), regulated by heme-deficiency and oxidative stress in erythroid tissues (17); and GCN2 (EIF2AK4), activated in response to nutritional deprivation, UV irradiation, proteasome inhibition, and certain viral infections (7, 26, 51, 57).

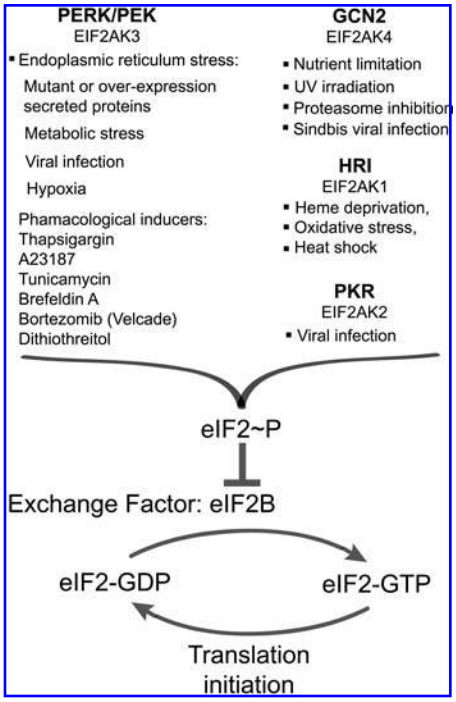

FIG. 1. PERK is a member of a family of protein kinases that regulate eIF2-GTP by phosphorylation of eIF2α. eIF2, combined with GTP and initiator Met-tRNAi^{Met}, associates with the 40S ribosomal subunit, facilitating recognition of mRNA start codons. During this translation-initiation process, eIF2-GTP is hydrolyzed to eIF2-GDP, and a guanine nucleotide exchange factor, eIF2B, is required to recycle eIF2 to the active GTP-bound form. Four different protein kinases, PERK, GCN2, HRI, and PKR, have been identified in mammalian cells that phosphorylate the α subunit of eIF2 at Ser-51 in response to a range of different environmental stresses. Phosphorylation of eIF2α alters this translation factor from a substrate to a competitive inhibitor of eIF2B. Reduced eIF2-GTP levels lessens general translation, allowing cells sufficient time to direct a new gene-expression program that is important for repairing cell damage in response to stress. Each eIF2α kinase is activated by a different set of stress arrangements, with PERK contributing to the UPR in response to perturbations of the ER.

The picture presented in Fig. 1 suggests that each environmental stress is uniquely recognized by a single eIF2α kinase.

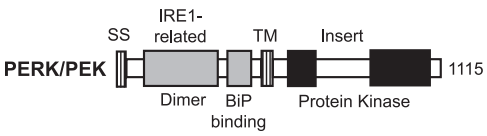

FIG. 2. PERK is a transmembrane eIF2 kinase. PERK is an ER transmembrane protein with an amino-terminal regulatory sequence placed in the ER lumen and a carboxy-terminal protein kinase domain that phosphorylates eIF2α in response to accumulation of misfolded protein in the ER. The PERK polypeptide sequence is depicted as a box. PERK regions important for its regulation and function include the signal sequence (SS), ER transmembrane (TM) region, and ER luminal regions that facilitate PEK dimerization and association with ER chaperones, such as BiP/GRP78. The PERK dimerization region shares sequence homology with IRE1. The protein kinase domain of PERK contains a large insert, a hallmark feature of eIF2α kinases.

This is a simplified view. Analysis of *PERK*+/+ and −/− mouse embryo fibroblast (MEF) cells showed that eIF2α phosphorylation requires PERK early in response to ER stress, but with extended stress conditions (6 h), significant eIF2α kinase activity was found, even in the PERK-depleted cells (58). This suggests that secondary eIF2α kinases can function during ER stress. By combining GCN2 and PERK deletions in MEF cells, both the primary and secondary eIF2α kinase activities were abolished (58). This cooperativity between the primary and secondary eIF2α kinases PERK and GCN2, respectively, was shown to be biologically important for cell-cycle arrest in response to ER stress (40). Phosphorylation of eIF2α facilitates translational repression of cyclin D, which is central for subsequent arrest in the G_1 phase. Although deletion of PERK alone did not restore cyclin D expression in response to ER stress, the combined deletion of PERK and GCN2 in MEF cells fully abolished eIF2α phosphorylation and restored cyclin D translation (40). In certain stress conditions, such as oxidative stress, multiple primary eIF2α kinases can be found, depending on the tissue type, dosage of the environmental agent, and duration of exposure (116). Therefore, eIF2α phosphorylation can contribute not only to the UPR, but also can function in conjunction with other stress response pathways to alleviate cellular injury.

PERK PHOSPHORYLATION OF eIF2α IS INDUCED IN RESPONSE TO ER STRESS

PERK and IRE1 proteins share similar topologic arrangements with amino-terminal regulatory domains in the ER lumen that monitor accumulation of malfolded protein in this organelle and in cytoplasmic protein kinase regions. Because PERK and IRE1 share sequence similarities in a portion of their regulatory regions, it is proposed that these protein kinases have similar mechanisms for recognizing malfolded proteins and inducing protein kinase activity (see Figs. 2 and 3). Supporting this idea, replacement of a portion of the amino-terminal regulatory domain of IRE1 with the related portion of PERK resulted in a functional IRE1 chimera that induced the UPR in response to pharmacologic induction of ER stress (76). Shamu and Walter (99) suggested that IRE1 dimerizes in response to ER stress, leading to intermolecular phosphorylation that enhances IRE1 kinase activity and subsequent mRNA-splicing function. Structural analyses of the luminal portion of yeast and human IRE1 indicate a head-to-head dimerization through an interface that results from a central groove formed by several α-helices situated around an antiparallel β-sheet (21, 120). The deep IRE1 groove shares many features with the peptide-binding domains of major histocompatibility complexes.

Several lines of evidence support the idea that oligomerization is central for activation of PERK in response to ER stress. ER stress has been reported to shift PERK to a high-molecular-weight complex estimated to be ~320 to >600 kDa, as judged by glycerol gradient sedimentation (8, 81, 97). Given that PERK is 125 kDa, this would suggest that PERK may be a dimer or a larger oligomer, and that additional proteins may be in present in the complex. Additional evidence supporting the link between dimerization and activation of PERK is the observation that fusion of a dimerization domain to the PERK kinase domain facilitates constitutive PERK autophosphorylation and eIF2α kinase activity (80). The PERK dimerization domain was localized between amino acid residues 102 and 407 (see Fig. 2), and deletion of this portion of PERK prevents autophosphorylation in response to ER stress (81). Finally, mutations in PERK residues lysine-194 and leucine-196, predicted to participate in the dimerization interface analogous to IRE1, blocked dimerization *in vitro*, further supporting the idea that the PERK shares regulatory features with IRE1 (120). Dimerization of PERK is proposed to trigger intermolecular phosphorylation at a number of different serine and threonine residues. One site, threonine-980, is located in the activation loop of the PERK kinase domain, which is thought to facilitate binding of ATP and to enhance eIF2α phosphorylation (43, 50, 81, 83).

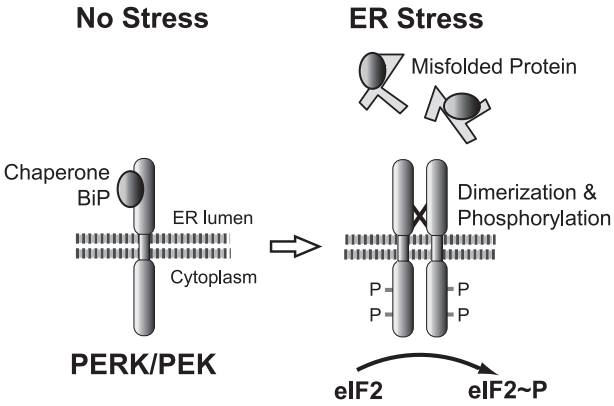

FIG. 3. Model for activation of PERK in response to ER stress. In the nonstressed state, PERK is bound with BiP. In response to accumulation of misfolded proteins during ER stress, BiP is proposed to be released from PERK and assist in the folding of these proteins. Release of BiP from PERK is proposed to facilitate association between PERK polypeptides and intermolecular phosphorylation. Phosphorylation of serine and threonine residues in PERK, such as threonine-980, located in the activation loop of the PERK kinase domain, activates PERK phosphorylation of eIF2α, leading to translational control.

How does accumulation of malfolded protein trigger PERK dimerization and activation? Ron and colleagues (8) reported that PERK is associated with the ER chaperone BiP/GRP78 in the absence of stress. A few minutes after the onset of ER stress, PERK dissociates from BiP, coinciding with PERK oligomerization and autophosphorylation. PERK association with BiP is dynamic, as removal of ER stress agents leads to PERK reassociation with BiP, followed by dephosphorylation of PERK. Reversible association with BiP was also reported for IRE1, further supporting the idea that common mechanisms regulate these two UPR sensors (8). These studies suggest that BiP binds to PERK during nonstressed conditions, maintaining this eIF2α kinase in a repressed state (see Fig. 3). Accumulation of misfolded protein in the ER would compete with PERK for BiP binding. PERK dissociated from BIP would be free to dimerize, leading to intermolecular phosphorylation that favors a PERK protein conformation that directs enhanced eIF2α kinase activity.

The precise mechanism by which release of BiP facilitates dimerization is not well understood. BiP binding to PERK could elicit a protein conformation that does not favor PERK homodimerization, or BiP association with PERK may sterically hinder association between PERK polypeptides. Further supporting this model is the finding that deletion of PERK residues 411 to 481 blocks PERK association with BiP, yet retains the ability to dimerize (81). Consistent with the idea that BiP binding represses PERK, the PERK-Δ411-481 mutant was active, as judged by autophosphorylation independent of ER stress (81). This study supports the idea that separate dimerization and BiP binding sites exist in PERK and serve to enhance or repress eIF2α kinase function, respectively. Features of this model for PERK activation are the subject of some controversy. A scanning mutagenesis analyses found that yeast IRE1 deleted for its BiP-binding region could still be activated by ER stress, suggesting that BiP release may not be the initiating event by which yeast IRE1 senses accumulation of malfolded protein (63). Instead it was proposed that the dimerization region of yeast IRE1 itself recognizes malfolded protein, which would directly facilitate oligomerization during ER stress. In this version of the model, release of BiP would be a secondary consequence of ER stress-induced dimerization of IRE1, and this may serve to restrict IRE1 activation to those stresses that perturb only the ER (63). The differences between these studies may reflect variations between the regulation of PERK and IRE1 in response to ER stress, or may be due to differences in the yeast and mammalian model systems.

Walter and colleagues (21) proposed an alternative model for IRE1 activation in response to accumulation of unfolded protein; given the sequence homology between the IRE1 and PERK, this proposed regulatory scheme is pertinent for PERK. In this model, the deep groove identified in the crystal structure of the luminal portion of yeast IRE1 is proposed directly to bind to unfolded protein or partially folded protein, contributing to IRE1 dimerization or interaction between IRE1 dimers that would favor intermolecular phosphorylation and trigger activation of yeast IRE1. This model could also accommodate BiP regulation of IRE1, but the association would provide only coarse control of IRE1 activity when the pool of free BiP is severely depleted (21). This model also is controversial. The crystal structure of the human IRE1 suggests that this groove is too narrow to accommodate polypeptide binding, and its orientation in proximity to the ER membrane would prohibit oligomerization induced by binding to polypeptides (120).

The protein kinase domain of PERK shares specific structural features with the other members of the eIF2α kinase family. Recent structural studies by Dever and Sicheri and colleagues (22, 30) demonstrated that the amino-terminal lobe of the PKR kinase domain facilitates dimerization by a salt bridge in a back-to-back orientation. Alterations in amino acid residues contributing to the predicted salt bridge in PERK block eIF2α activity, suggesting that an additional dimerization interface is present in the kinase domain of PERK (31). Interestingly, residue arginine-587, predicted to participate in the salt bridge in PERK, is mutated to glutamine in a WRS patient, supporting the idea that a defect in PERK dimer formation is the underlying reason for the loss of PERK function.

ATF4 TRANSLATIONAL CONTROL IS INTEGRAL TO THE eIF2α KINASE STRESS RESPONSE

A central regulator of the eIF2α kinase stress response is the transcriptional activator, ATF4 (CREB2). Increased ATF4 expression in response to ER stress occurs predominantly *via* translation control, as illustrated by the observation that the levels of ATF4 protein are sharply increased in response to ER stress, whereas ATF4 mRNA levels remained largely unchanged (43). Furthermore, ATF4 mRNA becomes associated with polysomes in response to eIF2α phosphorylation during ER stress (43). Central to ATF4 translational control is the 5′-leader of the ATF4 mRNA, which is 278 nucleotides in length and contains two upstream open reading frames (uORFs). The first ATF4 uORF encodes a polypeptide only three amino acid residues in length, and the second uORF is 59 amino acid residues in length and overlaps the first 83 nucleotides of the ATF4-coding region (Fig. 4). This uORF configuration is conserved among vertebrates, including humans, mouse, rat, cow, swine, chicken, zebrafish, and frogs (105). The conserved placement of these two uORFs in the 5′-leader of the ATF4 mRNA suggests that this portion of the ATF4 mRNA is important for its translational control. Indeed placement of the 5′-leader of ATF4 upstream of a luciferase coding region conferred ATF4 translational expression on this reporter in response ER stress conditions by a mechanism requiring eIF2α phosphorylation (105).

To determine the roles of uORF1 and uORF2 in the ATF4 translational control mechanisms, site-directed and insertional mutations were constructed in the 5′-leader of the ATF4 mRNA and analyzed for their effects in the luciferase reporter system. These results indicate that uORF1 and uORF2 have opposing functions in the regulation of *ATF4* translation, with uORF1 functioning as a positive-acting element in *ATF4* translational control, enabling ribosomes to overcome the inhibitory affects of uORF2 in response to eIF2α phosphorylation induced during ER stress (79, 105). It is proposed that ATF4 translation begins with the small ribosomal subunit bound to eIF2/GTP/Met-tRNA$_i^{Met}$ processively scanning from the 5′-end of the ATF4 mRNA, initiating translation at the 5′-proximal

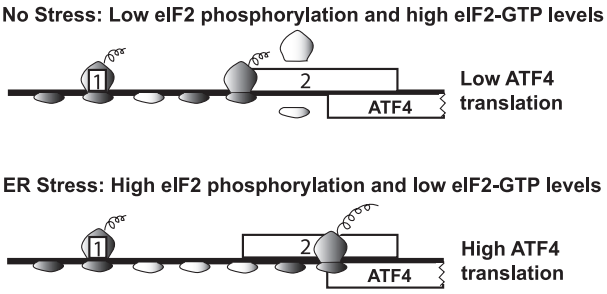

FIG. 4. Phosphorylation of eIF2α enhances ATF4 translation by a mechanism involving ribosomal reinitiation. The *ATF4* mRNA is shown as a line that has uORFs 1 and 2 and the ATF4 coding regions that are illustrated as boxes. The shading of the small ribosomal subunits indicates that it is associated with eIF2/GTP/Met-tRNAi^{Met}. The panels represent translation of the ATF4 mRNA in response to no-stress or ER-stress conditions. Ribosomes translate the 5′-proximal uORF1, a positive-acting uORF that allows ribosomes to reinitiate translation at a downstream ORF. After translation of uORF1, ribosomes resume scanning in a 5′ to 3′ direction along the leader of the ATF4 mRNA. When eIF2/GTP/Met-tRNAi^{Met} is readily available during nonstressed conditions, the small ribosomal subunits rapidly bind eIF2/GTP/Met-tRNAi^{Met} and, after coupling with the 60S ribosome, reinitiate translation at uORF2. The uORF2 is inhibitory and overlaps out of frame with a portion of the ATF4 coding region. Subsequent to translation of the inhibitory uORF2, ribosomes are thought to dissociate from the ATF4 mRNA, reducing expression of the *ATF4* coding region. During ER stress, phosphorylation of eIF2α reduces the eIF2-GTP levels, resulting in an increased time for scanning ribosomes to reacquire this translation-initiation factor. This would result in the scanning ribosomes bypassing the initiation codon of the inhibitory uORF2. In the interval between the initiation codons of uORF2 and the ATF4 coding region, the scanning ribosome can associate with eIF2/GTP/Met-tRNAi^{Met}, allowing enhanced translation of ATF4.

uORF1 (see Fig. 4). After synthesis of the uORF1-encoded polypeptide, ribosomes continue binding to the ATF4 mRNA and reinitiate translation at a downstream coding region. In nonstressed cells when eIF2α phosphorylation is low and abundant levels of eIF2-GTP are present, ribosomes scanning downstream from uORF1 rapidly reinitiate translation at the next ORF, uORF2. The uORF2 overlaps, out of frame, with the ATF4 coding region, and it is proposed that after translation of the uORF2, polypeptide ribosomes dissociate from the ATF4 mRNA, resulting in reduced ATF4 expression.

During ER stress conditions, an elevated phosphorylation of eIF2α reduces the levels of eIF2-GTP. Diminished levels of the eIF2/GTP/Met-tRNA$_i^{Met}$ complex increase the time required for the scanning ribosome to become competent to reinitiate translation. After translation of uORF1, delayed reinitiation would allow a portion of the ribosomes to bypass the uORF2 initiation codon. During the interval between the initiation codons of uORF2 and the *ATF4* coding region, scanning ribosomes would have sufficient time to reacquire eIF2/GTP/Met-tRNA$_i^{Met}$ and initiate ATF4 translation (see Fig. 4). Therefore, ATF4 levels would be rapidly elevated in response to ER stress, allowing this transcriptional activator to direct the UPR. The central regulatory feature in ATF4 translational control—timing of translation reinitiation that depends on the availability of eIF2-GTP—is also a shared characteristic of increased translation of a related bZIP transcriptional regulator GCN4 in yeast *Saccharomyces cerevisiae* (51). In this case, eIF2α phosphorylation serves to direct the bypass of multiple short uORFs in the 5′-leader of the GCN4 mRNA, allowing this master regulator to trigger expression of genes involved in metabolism and salvaging of nutrients.

ATF4 DIRECTS A CASCADE OF GENE EXPRESSION

ATF4 can regulate gene transcription by forming a homodimer or heterodimers with several other bZIP transcription factors, such as the C/EBP isoforms, FOS, JUN, and NRF2. Gene expression microarrays were carried out to delineate the contribution of PERK and ATF4 in the UPR (46). *PERK*$^{-/-}$ and *ATF4*$^{-/-}$ MEF cells, and their wild-type counterparts, were treated for 6 h with tunicamycin, a potent ER stress agent that blocks *N*-glycosylation of proteins in this organelle. Emphasizing the important role of Perk in the UPR, of the 88 genes whose mRNA products were induced by twofold or more, 60% showed a >50% reduction in the absence of PERK (46). The genes regulated by PERK are involved in a diverse array of cellular functions, including folding and processing of secretory proteins, the ERAD pathway for clearance of malfolded secretory proteins, glutathione biosynthesis and the control of the cellular redox status, mitochondrial function, amino acid synthesis and import, regulation of apoptosis, and regulation of signaling and transcription. Of the PERK-controlled genes, only 52% required ATF4 function, with ATF4 having a significant role in the regulation of genes involved in amino acid metabolism, protection from oxidative stress, regulation of apoptosis, and increased expression of additional bZIP transcription factors, ATF3 and CHOP/GADD153 (46) (see Fig. 4). This suggests that PERK can also direct the UPR by mechanisms independent of ATF4, a topic that is revisited later.

The mechanisms by which ATF4 interfaces with the transcriptional machinery to direct transcription is still not fully understood. ATF4 transcriptional control during stress is made more complex by the fact that genes induced by ATF4 can vary depending on the stress conditions that induce eIF2α phosphorylation. For example, the pattern of gene transcription directed by ATF4 is different between ER stress and amino acid deficiency, which activate PERK and GCN2, respectively. This would suggest that other transcription factors, which are induced by each stress condition, independent of eIF2α phosphorylation, function in conjunction with ATF4 to induce transcription. For example, bZIP transcription factors ATF6 and XBP1 function in combination with ATF4 to direct the UPR, whereas during nutrient limitation, ATF4 can work in conjunction with ATF2, whose phosphorylation and activation is induced by mitogen-activated protein kinases (MAPKs) (3, 13).

One of the best-characterized promoters activated by ATF4 is asparagine synthetase (ASNS), which catalyzes the biosynthesis of asparagine and glutamate. The *ASNS* promoter contains two cis-acting elements designated nutrient-sensing re-

sponse element (NSRE)-1 and -2, with ATF4 playing a primary role in increasing ASNS transcription by binding to NSRE-1 in response to ER stress or nutritional deficiency (4, 16, 62). Kilberg and colleagues (16, 62) used reporter assays in combination with transient overexpression of bZIP transcription factors, and *in vitro* and *in vivo* binding experiments, to delineate two phases regulating *ASNS* transcription in response to amino acid limitation. The early phase occurs during the first 4 h of amino acid limitation and involves increased levels of ATF4 binding to the NSRE-1, leading to localized histone acetylation by recruitment of an unidentified histone acetylase (16). The chromatin remodeling directs recruitment of general transcription factors and RNA polymerase II, leading to increased synthesis of ASNS mRNA. In the later phase, which occurs from ~4 to 12 h of amino acid deprivation, ATF3 dimerized with C/EBPβ binds to NSRE-1, coinciding with a diminished rate of *ASNS* transcription (16). Transcriptional expression of *C/EBPβ* is itself induced by amino acid limitation and ER stress, and the elevated levels of C/EBPβ combined with ATF3 may serve as a feedback mechanism to ensure that appropriate transcription levels of the ATF4-targed genes are expressed in response to the stress condition (16, 62).

Given that the same promoter element facilitates increased *ASNS* transcription during the UPR, and the key transcriptional regulators, ATF4, ATF3, and C/EBPβ are temporally expressed in a similar fashion during nutrient and ER stresses, it is thought that some of the basic mechanistic features regulating *ASNS* transcription are conserved among these different stress arrangements (4, 13, 62). Finally, similar studies have been carried out with the *ATF3*, *CHOP*, and *C/EBPβ* promoters and those for stress-responsive genes *VEGF* and *SNAT2* (93). In each case, an early ATF4-binding phase coincides with elevated transcriptional expression, followed by a later phase in which ATF3 binding is thought to direct diminished transcription. Therefore, the central features described for ASNS appear to be broadly applicable to a range of ATF4-targeted promoters induced by amino acid starvation and the UPR.

ATF4, in combination with ATF6 and XBP1, directs the UPR transcription program. IRE1 has two functions in the regulation of mRNA levels during ER stress. In addition to splicing XBP1 mRNA, allowing translation of the activated version of this transcription factor, IRE1 can facilitate selective degradation of mRNAs encoding secreted or membrane proteins (53). This selected mRNA-degradation function has been described in *Drosophila* cells, and would serve to optimize further the transcript levels for encoded proteins that expand the capacity of the ER to fold, process, and transport secretory proteins (53). Therefore, both the synthesis and turnover of mRNAs contribute to directing the UPR.

ATF4 DIRECTS FEEDBACK CONTROL OF eIF2α PHOSPHORYLATION

How are the UPR transcripts translated when a significant reduction in general protein synthesis occurs as a consequence of PERK phosphorylation of eIF2α? Part of the answer lies in the ATF4-targeted expression of GADD34/PPP1R15A. GADD34 was originally identified by subtractive hybridization in a search for mRNA induced by UV irradiation, and was subsequently found to be induced by a broad range of stress conditions that extend beyond DNA damage (38, 52, 90). Like ASNS described earlier, the GADD34 promoter contains an ATF4-binding site that enhances its expression during ER stress or nutritional stress (82). GADD34 serves as a regulatory subunit of the type 1 serine/threonine phosphatase that dephosphorylates eIF2α (14, 20, 89, 90) (Fig. 5). CHOP and ATF3, downstream targets of ATF4, are also suggested to be involved in regulation of GADD34 transcription by this ATF-binding site and an adjacent CHOP-binding element (58, 85). Therefore, once the UPR-directed mRNA program is implemented, ATF4-directed GADD34 expression restores translation, allowing synthesis of those proteins that would maximize the processing capacity of the ER.

It is curious that, like ATF4, many transcripts encoding key UPR regulatory proteins appear to be translated even when global translation is significantly diminished because of reduced eIF2-GTP levels. This is illustrated by the synthesis of CHOP and ATF3, as well as by GADD34 itself, which are synthesized during the phase of UPR when significant eIF2α phosphorylation is present. Although it is still not understood how these regulatory proteins are synthesized during periods of induced eIF2α phosphorylation, it is noteworthy that GADD34 and CHOP each have uORFs (60, 82). Analogous to ATF4, the 5′-leader of the GADD34 mRNA encodes two uORFs that may be important for GADD34 translation by a mechanism of ribosome reinitiation, as described for ATF4 (52, 82). In the case

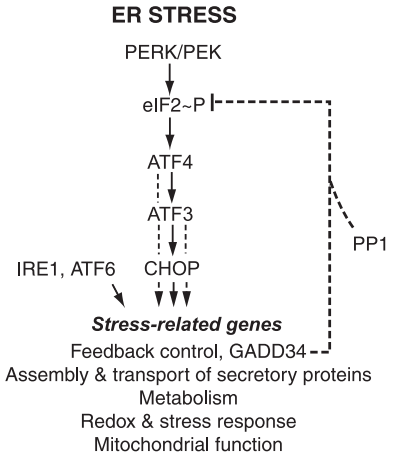

FIG. 5. PERK phosphorylation of eIF2α induces ATF4, ATF3, and CHOP, and together these transcription factors regulate gene expression in response to environmental stresses. ATF4 is a central regulator in the eIF2α kinase pathway that enhances the expression of bZIP transcription factors, CHOP and ATF3, that collectively direct transcription of stress-related genes. ATF4 triggers expression of genes involved in amino acid metabolism and transport, the redox status of cells, and apoptosis. Another target of ATF4 is GADD34, a protein phosphatase regulatory subunit implicated in feedback control of the eIF2α kinase pathway. Additional bZIP transcription factors participating in the UPR are the stress sensors ATF6 and XBP1; XBP1 is activated by IRE1 through splicing of XBP-1 mRNA.

of the human GADD34 mRNA, the two uORFs are separated by only 30 nucleotides, as compared with 87 nucleotides separating uORF1 and uORF2 in the ATF4 mRNA. Curiously, in the mouse GADD34 mRNA, the termination codon of uORF1 overlaps with the initiation codon of uORF2 (82). The close proximity of the uORFs in the GADD34 mRNA suggests that, should GADD34 be subject to translational control, the underlying mechanism is modified from that described for the ATF4 translation reinitiation process. In human GADD34, the shorter distance between uORF1 and uORF2 would suggest that a more modest reduction in eIF2-GTP would be sufficient for bypass of uORF2, or that the RNA structure between the uORFs lends itself to slower ribosomal scanning that provides an adequate temporal window to monitor eIF2-GTP availability. The mouse GADD34 mRNA, in which uORF1 and uORF2 overlap, is even more problematic, requiring ribosomes to back up several nucleotides to implement reinitiation at uORF2.

Only one uORF is present in the 5′-leader of CHOP mRNA. The CHOP mRNA has a 31-residue uORF that is conserved among humans, mouse, and hamster (60). Mutational analysis of the 5′-leader of the CHOP mRNA fused to a luciferase reporter indicates that this uORF is inhibitory to translation of CHOP mRNA (60). This repression of the CHOP-luciferase reporter was not relieved by pharmacologic induction of ER stress. Therefore, it is currently unclear what role the 5′-leader and encoded uORF play in CHOP expression during the UPR. It is of interest that Her-2/Neu mRNA has a single uORF, and this element is important for regulating Her-2 translational expression (87). Her-2 is a member of the EGF receptor family that direct mitogenic signals in solid-tumor cancers in many different tissues, including breast, ovary, prostate, lung, and pancreas. Like CHOP, the uORF is inhibitory to Her-2 translation, as judged by reporter assays that introduced a cDNA segment encoding the 5′-leader of the Her-2 mRNA between a CMV promoter and the luciferase gene (87). However, inclusion of the 3′-untranslated region (UTR) of the Her-2 mRNA into this plasmid reporter allowed an override of the inhibitory uORF. Embedded in the Her-2 3-UTR is a translational depression element (TDE) that associates with RNA binding proteins, such as HuR, and these bound proteins in combination with polyadenylation binding protein (PABP) are thought to interact with the 5′-leader of the Her-2 mRNA *via* a closed loop between the 5′ and 3′ ends of the Her-2 mRNA (87). It is proposed that ribosomes translating the Her-2 uORF remain stalled at the termination codon, leading to inefficient translation of the downstream Her-2 coding region. The TDE-interacting proteins are suggested to alter termination efficiency of the uORF, allowing ribosomes to be retained on the mRNA and resume scanning and subsequent translation reinitiation at the Her-2 coding region (87). The efficiency of reinitiation could be linked to the availability of eIF2-GTP. Furthermore, this mode of regulation could be adapted to the eIF2α kinase pathway by regulation of the expression or affinities of proteins for the TDE. This model may be applicable to CHOP translation and would suggest that the combined inclusion of segments encoding the 5′ and 3′ UTRs of CHOP mRNA into reporter assays may be important for deciphering possible translational control of CHOP.

Much interest has been expressed in the role of internal ribosomal entry sites (IRESs) in the regulation of translational control in response to eIF2α phosphorylation. The presence of IRESs in the 5′-leaders of mRNA facilitates translation by a mechanism involving direct binding of ribosomes onto mRNAs for targeted recognition of initiation codons in the absence of cap-dependent ribosomal scanning (64). An example of translational control induced by eIF2α phosphorylation that involves an IRES is Cat-1, encoding a high-affinity transporter for arginine and lysine (35, 36, 109). Cat-1 expression is increased in response to either ER or nutritional stress by both transcriptional and translational control mechanisms (35, 47, 78). Similar to the mechanism of ASNS mRNA synthesis described earlier, Cat-1 transcription is induced by ATF4, which binds to the Cat-1 promoter and recruits RNA polymerase II (78). Later in the stress response, Cat-1 transcription is repressed by ATF3 binding to the same promoter element. Translational expression of CAT-1 centers around an IRES positioned within the 5′-leader of the Cat-1 mRNA (35, 109). Translation of an uORF embedded within the Cat-1 IRES facilitates formation of an IRES structure that is conducive to ribosome association to the Cat-1 initiation codon. It is proposed that phosphorylation of eIF2α triggers the synthesis of an IRES-trans-acting factor (ITAF) that associates with the IRES and enhances ribosome recognition and binding to the Cat-1 initiation codon (109). The idea that eIF2α phosphorylation indirectly facilitates translational expression of Cat-1 is consistent with the observation that ER stress or nutrient deprivation activates the IRES only after an extended period, up to 9 h, and Cat-1 translation continues even after eIF2α phosphorylation has returned to basal levels. Recently, it was reported that the 3′-UTR of Cat-1 mRNA can associate with regulatory proteins that modify the ability of micro RNAs to repress Cat-1 translation (9). Therefore, multiple mechanisms may exist for regulation of Cat-1 translation that involves interaction of regulatory factors at either the 5′- or 3′-ends of the Cat-1 transcript. Execution of these different translation mechanisms may depend on the precise stress arrangement and cell types.

PROTEIN KINASE A AND ATF4 TRANSLATIONAL CONTROL

Exendin-4 is a glucagon-like peptide-1 (GLP-1) receptor agonist that stimulates cAMP production by binding to specific receptors on the surface of cells (32). In models of type II diabetes, exendin-4 increases insulin sensitivity peripherally while elevating insulin production in the pancreatic islets. A recent study by Drucker and colleagues (115) addressed how increasing intracellular cAMP improves β-cell function and survival in response to ER stress. PKA signaling elicited by the GLP-1 receptor differentially regulates the PERK portion of the UPR. Specifically, cAMP activation of PKA in ER stressed cells leads to overinduction of ATF4 translation downstream of PERK activation (115). Induced ATF4 protein levels do not involve increased ATF4 mRNA levels or reduced ATF4 protein turnover; this was reproduced by using an ATF4-luciferase reporter, as described earlier. These findings indicate that the target of increased cAMP levels is ATF4 translational control mediated through the 5′-leader of the ATF4 mRNA. The PKA inhibitor H89 blocks the action of forskolin, indicating that

cAMP activation of PKA facilitates ATF4 translation (115). Forskolin by itself does not increase ATF4 levels, suggesting that elevated cAMP functions only in conjunction with stress conditions that induce eIF2α phosphorylation to direct increased ATF4 translation.

It is also important to note that the addition of forskolin does not lead to a further increase in eIF2α phosphorylation induced by ER stress. Diminished phosphorylation of eIF2α occurs with the combination of ER stress and forskolin (115). The rationale for this lowered eIF2α phosphorylation and accompanying increase in protein synthesis is that the overexpressed ATF4 levels increase GADD34, leading to enhanced dephosphorylation of eIF2α. These results suggest that translational induction of ATF4, and its target genes, is subject to PKA signaling that works synergistically with induced eIF2α phosphorylation. For example, PKA may directly or indirectly modify the function of a protein(s) involved in translation initiation, which, in conjunction with eIF2α phosphorylation, facilitates translation of the ATF4 coding region. This may involve key mechanistic features delineated earlier, such as delayed translation reinitiation, or enhanced recognition of the ATF4 initiation codon. It is important to emphasize that PKA is suggested to function only in conjunction with phosphorylated eIF2α; therefore, PKA does not appear simply to trigger a reduction in the function of a translation initiation factor that by itself would delay translation reinitiation and the bypass of the inhibitory uORF2. Such bypass mutants, designated gcd$^-$, have been described in yeast and shown to function independent of eIF2α phosphorylation (51). GLP-1R is widely expressed in the central nervous system, and the prosurvival functions of GLP-1R signaling are suggested to be important for resistance to certain neural injuries. Therefore, the role of GLP-1R and PKA signaling in the regulation of ATF4 translation may broaden beyond that of β-cell survival.

POSTTRANSLATIONAL MECHANISMS REGULATING ATF4

ATF4 is a short-lived protein, with a half-life of less than 30 min. Ubiquitin-mediated degradation of ATF4 *via* the proteasome appears to be central to ATF4 turnover, and addition of proteasome inhibitors, such as MG132, stabilizes ATF4 and enhances its transcriptional activity (71). The F-box protein βTrCP directly binds to ATF4 through an interactive sequence between residues 218 to 224, recruiting ATF4 to the SCF E3 ubiquitin ligase for its polyubiquitination and degradation in the proteasome (71). Mutations in the ATF4 interactive region reduce ATF4 binding with βTrCP and increase the half-life of ATF4. The trigger for ATF4 interaction with βTrCP and ensuing polyubiquitination is not fully understood. It has been suggested that ATF4 phosphorylation at residues not yet defined is a precondition for ATF4 binding to βTrCP binding (71). It is noteworthy that yeast GCN4 is also labile and subject to ubiquitin-mediated degradation by a mechanism involving GCN4 phosphorylation (88). GCN4 is subject to phosphorylation at threonine-165 by a cyclin-dependent protein kinase PHO85, a functional homologue of mammalian CDK5. The cyclin PCL5 is transcriptionally induced by GCN4 during stress conditions, allowing PCL5 to direct PHO85 phosphorylation of GCN4, which mediates GCN4 ubiquitination and degradation (100). This network would thus reduce the level of GCN4 after it had directed gene transcription required for recovery from the underlying stress.

Phosphorylation of ATF4 has also been reported to be important for enhanced transcriptional activity. Such regulation of ATF4 by protein phosphorylation has recently been shown to be important in osteoblast differentiation in bone development (111). Coffin-Lowry syndrome (CLS) is a rare genetic disorder that is characterized by skeletal deformities. Underlying CLS is a defect in the *RSK2* gene, encoding a ribosomal serine/threonine protein kinase (111). ATF4 is well expressed in osteoblasts, and phosphorylation of ATF4 at serine-251 by RSK2 enhances ATF4 transcriptional activation of genes involved in osteoblast differentiation, such as osteocalcin (111). Emphasizing the importance of this transcription-regulatory pathway, mice deleted for either RSK2 or ATF4 exhibit skeletal deformities. ATF4 deficiency in osteoblasts leads to decreased type 1 collagen synthesis and secretion. Given that ATF4 is important for amino acid synthesis and uptake, it is proposed that reduced amino acid levels in ATF4-deficient osteoblasts would lead to a reduced translation, thereby reducing the major biosynthetic product, collagen. Consistent with this idea, feeding *ATF4*$-/-$ or *RSK*2$-/-$ mice a high-protein diet helped alleviate the developmental defects and low bone mass (34).

ATF4 transcriptional activity is also controlled by PKA *via* phosphorylation of ATF4 residue serine-254, and this regulatory mechanism is important for bone biology (34). PKA phosphorylation of ATF4 is proposed preferentially to enhance transcription of a different set of promoters than that targeted by ATF4 phosphorylated by RSK2. An important target of PKA-phosphorylated ATF4 is RANKL, and increased osteoblast secretion of RANKL can enhance differentiation of osteoclasts that direct bone resorption (34). PKA activation of ATF4 is germane to the genetic disorder neurofibromatosis type 1 (NF1), which results in neural tumors and bone deformities. The underlying genetic defect in NF1 is in the *neurofibromin (NF1)* gene, encoding a RAS GTPase activating protein, and this genetic lesion has also been shown to enhance cAMP and activate PKA (34, 41). The ensuing activation of ATF4 and increased expression of RANKL in response to NF1 deficiency is suggested to be the underlying reason for increased osteoclasts in bone and resorption in NF1 patients. Consistent with the model that NF1 is an upstream negative regulator of ATF4, mice overexpressing ATF4 in their osteoblast cells display a skeletal phenotype similar to those deficient in NF1 (34).

Another mechanism that appears to be fundamentally important for posttranslational regulation of ATF4 involves protein–protein interactions. As highlighted earlier, ATF4 can form heterodimers with other bZIP transcription factors, and this association can modulate the DNA-binding selection and affinity of ATF4 (97, 106). Numerous other proteins have been reported to bind with ATF4, and a portion of these are suggested to alter ATF4 transcriptional function. For example, histone acetyltransferase p300 binds to an N-terminal region of ATF4, stabilizing ATF4 and enhancing its transcriptional activity, as measured by enhanced CHOP promoter function (70). Although ATF4 can be acetylated by p300, this modification does not appear to be important for p300 stabilization of ATF4. Mutant

p300 devoid of acetyltransferase activity can bind ATF4 and reduce its turnover (70).

ATF4 also can bind to regulatory proteins that repress its transcriptional activity. The protein FIAT contains a leucine zipper that mediates binding to ATF4 (114). Overexpression of FIAT in COS-7 cells blocked ATF4-directed transcription by blocking ATF4 binding to DNA. Interestingly, FIAT expressed in osteoblasts in transgenic mice by using the α1(I) collagen promoter leads to reduced osteoblast activity and bone mass. However, FIAT did not affect osteoblast differentiation or collagen synthesis and secretion, as described for *ATF4*$-/-$ mice (114). Given that differential phosphorylation of ATF4 is suggested to direct changes in ATF4-directed transcription, it is inviting to suggest that ATF4 interaction with FIAT can also lead to repression or activation of selective gene promoters.

REDUCTION IN GLOBAL PROTEIN SYNTHESIS BY eIF2α PHOSPHORYLATION CAN ACTIVATE NF-κB

Another mechanism by which phosphorylation of eIF2α can regulate gene expression derives from its role in reducing global protein synthesis. This would not lead to a static change in the composition of proteins in the cell, but rather preferentially would reduce the levels of key regulatory proteins that are subject to rapid turnover. This is illustrated by recent studies of the activation of NF-κB in response to several different cellular stresses, including ER stress and exposure to UV irradiation (27, 56, 59, 107). NF-κB is a dimer of the Rel family of proteins that regulates the expression of genes involved in immune and inflammatory responses, stress remediation, cell growth, and apoptosis (6, 74). In its inert state, NF-κB is in the cytoplasm bound to proteins known as inhibitors of NF-κB (IκB), the most prominent and well studied being IκBα. Exposure of cells to cytokines, such as TNF-α, leads to the rapid phosphorylation of IκBα at serine-32 by the IκB kinase (IKK), targeting it for ubiquitination and proteasome-mediated degradation. The resulting reduced levels of IκBα release NF-κB from this repressor, facilitating translocation of NF-κB into the nucleus to regulate transcription of its target genes.

Activation of NF-κB in response to eIF2α phosphorylation does not involve IKK phosphorylation of IκBα (27, 56, 59, 107). Phosphorylation of eIF2α, and the accompanying reduction in total protein synthesis, is rapid, within 1 h after exposure to UV-B or UV-C (26, 56). The primary eIF2α kinase activated in MEF cells subjected to UV irradiation is GCN2, with PERK appearing to have a secondary role, as judged by analysis of a collection of MEF cells deleted for each of the eIF2α kinases (26, 56). Accompanying this reduction in global protein synthesis is a significant reduction in the translation of IκBα (56, 107). MAPK p38 is proposed to direct CK2 phosphorylation of IκBα, facilitating IκBα ubiquitination and degradation in response to UV irradiation (61). The combined actions of reduced IκBα translation and rapid turnover would reduce the levels of IκBα and release this inhibitory protein from NF-κB. NF-κB would then be available to direct the transcription of genes involved in remediation of stress damage and the regulation of apoptosis. These central ideas are applicable to an artificial system of induced eIF2α phosphorylation that was engineered by fusing the kinase domain of PERK to a protein module that mediates dimerization on treatment with an inert chemical AP20187 (27). As highlighted earlier, dimerization is central to activation of PERK, and the exposure of MEF cells expressing this PERK fusion protein to AP20187 elicited eIF2α phosphorylation independent of stress. In this case, reduced IκBα synthesis resulted from reduced global protein synthesis that led to activation of NF-κB (27). In addition to the labile repressor proteins, this regulatory scheme could be applicable to transcription activators that are subject to rapid turnover.

PERK phosphorylation of eIF2α also is important for activation of NF-κB in response to ER stress. As judged by EMSA and reporter assays, loss of eIF2α phosphorylation in MEF cells either deleted for *PERK* or containing eIF2α with alanine substituted for serine-51 (A/A) blocked NF-κB in response to different pharmacologic inducers of ER stress (59). The precise molecular details leading to this activation have not yet been fully resolved. The levels of IκBα have been suggested to be partially reduced early after ER stress, with a restoration of IκBα levels after 1 h of this stress arrangement (27, 54). Furthermore, phosphorylation of IκBα at serine-32 was suggested to be modestly elevated in response to the ER stress agent thapsigargin, although other studies have not detected such IKK involvement during ER stress (54, 59). It is noteworthy that treatment with the proteasome inhibitor MG132 suppressed activation of NF-κB in response to UV irradiation but did not impede its induction during ER stress (56, 59). This observation has led to the suggestion that phosphorylation of eIF2α can also signal a release of IκBα from NF-κB, although the mechanistic features remain unresolved.

Finally, it has been reported that IRE1 can recruit the scaffolding protein TRAF2 to the ER membrane, and this interaction can facilitate IRE1 interaction with IKK, leading to activation of NF-κB. This model is supported by the observation that like *PERK*-deficient MEF cells, deletion of *IRE1*α can block NF-κB activation in response to ER stress (54).

PERK DEFICIENCY IS THE UNDERLYING CAUSE OF WOLCOTT-RALLISON SYNDROME

WRS is a rare autosomal recessive disorder that is characterized by neonatal or early-infancy insulin-dependent diabetes, exocrine pancreas atrophy, severe osteopenia, skeletal dysplasias, and growth retardation. Frequently, afflicted patients also have multisystemic pathologies including hepatic and renal complications, cardiovascular disease, and mental retardation. Genetic studies of inbred families by Julier and colleagues (25) identified mutations in *PERK* (*EIF2AK3*) as the underlying cause of WRS. Analysis of 12 families with WRS indicates that with the exception of one case, all patients carried *PERK* mutations resulting in truncated or missense versions of the protein (98). The activities of missense versions of *PERK* were characterized *in vivo* and *in vitro* and found to have a complete lack of activity, with the exception of one mutant (N655K) that

displayed residual kinase activity. Remarkably, the onset of diabetes was relatively late (30 months) in the patient expressing the partially defective *PERK* mutant compared with other patients (<6 months) (98).

Mice deleted for *PERK* have the hallmark diseases described in WRS patients. Harding and Ron and colleagues (42) proposed that cells highly specialized for secretion encounter fluctuating ER stress under normal physiologic conditions, and that the UPR is required to adapt to normal physiologic stresses, as well as to overt stress caused by disease or environmental perturbations. Thus, it was predicted that genetic ablation of the major components of the ER stress pathway could result in chronic physiologic dysfunctions. In particular, blocking one arm of the UPR might exacerbate ER stress, resulting in the increased response of the other two arms of the pathway. This hypothesis was proposed as the explanation for the multitude of defects that are observed both in the human WRS and in mice deficient for *PERK* (42). These anomalies were initially interpreted as a dysfunction in regulating the ER stress-response pathway. In particular, higher rates of proinsulin synthesis were observed in the insulin-secreting β cells, as well as abnormal retention of proinsulin in the ER of a subpopulation of these cells (42). In addition, procollagen-1 was found to accumulate abnormally in the ER of osteoblasts and interpreted as the cause of the severe osteopenia and skeletal dysplasias (118). However, these studies did not report an increase in ER stress as predicted or directly show that the abnormalities were caused by dysfunctions in regulating the UPR. The *in vivo* analysis of PERK in β cells and osteoblasts is in contrast to cell-culture studies of $PERK^{-/-}$ embryonic stem cells that continue to enhance translation aberrantly in response to ER stress and trigger apoptosis (44). The difference between these *in vivo* and *in vitro* studies on PERK function may arise either from differences in the cellular environment and stress arrangements or from differences in the specific tissue types.

Recently, a more detailed study of the cause of neonatal diabetes in PERK-deficient mice found no evidence that dysfunctions in regulating ER stress were the cause of the early onset of diabetes, and instead showed that PERK is critically required to regulate the proliferation and differentiation of insulin-secreting β cells during fetal development (119). Moreover, mice that have had a loss of PERK in the β cells after the initial proliferation and differentiation do not develop diabetes, demonstrating that PERK is not required in adults to maintain normal β-cell functions. In addition, mutations of downstream targets of PERK in the ER stress pathway, including ATF4 and CHOP, do not exhibit defects similar to those in *PERK*-deficient mice (49, 86, 92). *ATF4*-deficient mice do display developmental anomalies, including growth retardation, severe microphthalmia due abnormal lens development (49, 86), and hypotrophy of the exocrine pancreas (Iida and Cavener, unpublished data). The developmental defects in the exocrine pancreas are not associated with an increase in the levels of other components of the ER stress pathway, suggesting that the ATF4-dependent regulation of pancreatic development may involve other pathways. These studies strongly support the idea that an important function of PERK is to regulate fetal and neonatal development through pathways largely independent of the UPR.

Although the knockout mutations in *PERK* and *ATF4* underscore the important functions of these genes in regulating specific aspects of development that appear not to be related to dysfunctions in the ER stress-response pathways, these studies do not preclude a significant role of PERK and ATF4 in the UPR in whole organisms. As highlighted in this review, considerable evidence supports important physiologic roles for these genes in the regulation of ER stress responses in postnatal adult tissues. Rather, analysis of *PERK*-deficient mice argues that some genes involved in the UPR can have multiple biologic functions. In addition to the ER stress response, PERK has a role in development, and this activity may be facilitated through pathways that operate independently or in combination with a subset of the defined UPR players.

PHOSPHORYLATION OF eIF2α REPRESSES PROTEIN SYNTHESIS DURING ISCHEMIA

Ischemia, the acute loss of blood flow to tissues and organs, results in a rapid induction of the UPR (23). Protein synthesis is sharply reduced in the hippocampus and cerebral cortex after an episode of cerebral ischemia and reperfusion, and this repression is accompanied by phosphorylation of eIF2α (24). Deletion of *PERK*, but not other eIF2α kinases, blocks cerebral ischemia–induced eIF2α phosphorylation and significantly alleviates translation repression (68, 91). Ischemia-induced repression of protein synthesis in the brain is thought to be a key regulator of cell death. It is proposed that eIF2α phosphorylation contributes to an acute early phase that can be followed by formation of stress granules containing mRNAs associated with components of the translation apparatus, including 40S ribosomal subunits, many different translation initiation factors, and selected RNA-binding proteins (23). Induction of eIF2α phosphorylation, and the resulting stalled 48S preinitiation complexes, is thought to be essential for configuration of stress granules in response to different environmental stresses, including ischemia (23). Stress granules are thought to serve as sorting centers, directing mRNAs for resumption of translation, transcript decay, or storage (1, 2). During ischemia, stress granules are proposed to be sequestered into larger protein aggregates that include both ribosomal subunits and unfolded proteins that signal irreversible inhibition of protein synthesis and death of the affected neural tissue (23, 117). Another contributor to neural cell death elicited by ischemia may be PERK-dependent activation of ATF4 translation and subsequent transcriptional activation of the proapoptotic gene *CHOP* (85).

The induction of eIF2α phosphorylation during ischemia raises an unresolved question: Is activation of PERK during acute ischemia adaptive or maladaptive? In cell-culture models severe and sustained ER stress results in cell death, whereas less severe, transient ER stress results in adaptation and enhanced survival (96). A single episode of transient ER stress can enhance tolerance to a second insult of ER stress, and this adaptation is dependent on PERK and ATF4 (10, 46, 80). In some situations, cell death may be an advantageous response of multicellular organisms to remove dysfunctional cells and thereby alleviate the negative physiologic impact of these cells on the organism.

PERK AND CANCER

Growth of solid tumors requires adaptation to transient fluctuations in the supply of oxygen and nutrients. In response to acute hypoxia, two distinct phases contribute to reduced protein synthesis (65, 67). In the initial phase of hypoxia, PERK phosphorylates eIF2α, leading to a sharp reduction in global protein synthesis, which would reduce the protein load on the secretory pathway and reduce energy expenditure (65–67). Currently, the mechanisms by which hypoxia activates PERK, and the possible role of BiP in this regulatory process, are not well understood. Accompanying this reduction in global translation is elevated expression of ATF4 and its target genes that are involved in amino acid sufficiency and the redox status of cells (12). After prolonged hypoxic conditions, translation can be reduced by lessening the ability of the mRNA cap-binding protein, eIF4F, to function in translation initiation (65, 67). Reduced eIF4F activity during prolonged hypoxia occurs by decreased TOR-directed phosphorylation of 4E-BP, resulting in enhanced binding of this repressor protein to the eIF4E subunit of the cap-binding protein (19, 65, 77). Inhibition of TOR by hypoxia involves energy depletion and activation of AMP-activated protein kinase (AMPK), which can also inhibit eEF2 and ribosome elongation (77). Additionally, prolonged hypoxia increases eIF4E association with the shuttling protein 4E-T, which reduces cap-dependent translation by directing transport of the cap-binding subunit to the nucleus (65).

Deletion of PERK in MEF cells subjected to prolonged hypoxia results in a partial restoration of protein synthesis and enhanced activation of caspases, which lead to elevated levels of cell death. It is suggested that that the inability to reduce translation further exacerbates the underlying ER stress, as proteins slated for the secretory pathway continue to flood the ER. To address the importance of PERK in the progression of solid tumors, $PERK^{+/+}$ and $PERK^{-/-}$ MEF cells were transformed with oncogenic *Ki-RasV12*, and these transformed cells were injected into the flanks of nude mice. Tumors from the *PERK*-deficient *RasV12* cells grew more rapidly and were larger than the $PERK^{+/+}$ *RasV12* counterparts (10). Loss of PERK in these tumors led to a significant reduction in eIF2α phosphorylation, accompanied by expanded areas of hypoxia and increased apoptosis (10). PERK also was found to promote microvessel formation in tumors (11). Interestingly, microarray analyses of transcripts associated with polysomes suggested that the angiogenetic property of PERK involves translational control mechanisms. For example, PERK phosphorylation of eIF2α phosphorylation in response to hypoxia enhances translation of VEGF and type-1 collagen-inducible protein (VCIP), which facilitates cell adhesion and capillary morphogenesis, and matrix metalloproteinase 13 (MPP13), which is important for cellular adhesion and capillary morphogenesis and is suggested to promote tumor invasion and metastasis (11). Neither VCIP nor MMP13 mRNAs share a 5′-leader configuration analogous to ATF4, which suggests that these transcripts are regulated by distinct mechanisms. In the case of VCIP, it has been suggested that an IRES is critical to its translation, although the mechanistic role of eIF2α phosphorylation in its regulation is presently not understood (11). Analogous xenograft experiments using human colorectal carcinoma cells stably expressing a dominant-negative version of PERK (PERK-ΔC) have also been carried out and indicate that PERK is required for enhanced tolerance to hypoxia and tumor growth (10). Furthermore, immunohistochemical measurements of ATF4 and CHOP in human cervical carcinomas injected with a hypoxia-sensitive dye showed selective expression of these PERK-target genes in hypoxic regions in these tissues (10). Together these studies support the idea that UPR, including the PERK/ATF4 pathway, can provide resistance to hypoxia, thus promoting the progression of solid tumors.

The UPR is also suggested to have antioncogenic functions, contributing to premature senescence that can limit the transformation of human melanocytes. For example, amplified $HRAS^{G12V}$ is associated with small melanocytic neoplasms, which are not known to progress to melanoma (28). Stable expression of $HRAS^{G12V}$ in normal human neonatal melanocytes can significantly enhance senescence by a mechanism involving the PI3-kinase/AKT pathway triggering of the UPR (28). RNA interference against ATF4 or XBP-1 substantially reduces senescence, suggesting a causative role for the UPR pathway. By contrast, activation of the MAPK pathway by sustained expression of $BRAF^{V660E}$, a mutation prominently found in human melanomas, does not lead to PI3-kinase induction of the UPR and the senescence program. The precise reasons that $HRAS^{G12V}$ signals activation of PI3-kinase and the UPR, whereas $BRAF^{V660E}$ does not, is not well understood (28). Together these studies suggest that PERK and the UPR can have important cytoprotective or cytotoxic effects, depending on the type of tumor and the cellular microenvironment.

PERK DIRECTS GENE EXPRESSION CENTRAL FOR THE UPR AND THE PREVENTION OF DISEASE

The expression of a large number of genes is induced by the ER stress response, and several have been shown to be direct targets of ATF6, IRE1, and PERK—the primary effectors of the UPR. Among these, the *Wolfram* gene, which underlies the WFS, has been investigated extensively in humans and mice (5, 37, 55, 94, 95, 103, 108). Wolfram syndrome in humans is characterized by juvenile diabetes, optic atrophy, deafness (5). WFS1 is upregulated by IRE1 and PERK during ER stress and therefore predicted to be part of the adaptation to ER stress (37). Mouse *WFS1* knockout mutants exhibit impaired glucose homeostasis and, in some cases, overt diabetes, but other anomalies associated with human WFS have not been reported in these mice (55). WFS1 is highly expressed in the insulin-secreting β cell (103), and a β-cell specific knockout of the *WFS1* gene also results in impaired glucose homeostasis. WFS1 is associated with the ER membrane, and recent evidence suggests that it modulates ER calcium homeostasis (55). Unlike deficiencies in *PERK*, inactivating mutations of *WFS1* result in the induction of a robust ER stress response in the islets of Langerhans, including elevated expression of BiP, CHOP, HRD1, $p58^{IPK}$, phosphorylated PERK and eIF2α, and the spliced form of XBP1, which collectively represent the three sensors of the UPR (95, 103). Moreover, increased β-cell death, impaired cell-cycle progression, postnatal loss of β-cell mass, and reduced insulin secretion is evident in $WFS1^{-/-}$ mice (55, 95, 108).

The expression of the co-chaperone $P58^{IPK}$ (DNAJ) also is induced by ER stress, and a knockout mutation of $P58^{IPK}$ exhibits postnatal growth retardation, lipodystrophy, and impaired glucose homeostasis in adult male mice (69, 104, 110). Similar to WFS1 deficiency, the loss of $P58^{IPK}$ results in a reduction in the number of insulin-secreting β cells, as caused by cell death and hypoinsulinema. Growth retardation and lipodystrophy in $P58^{IPK-/-}$ mice precedes the onset of diabetes, suggesting that these defects are independent of hypoinsulinema, which develops much later.

An important prediction of the fluctuating ER stress model proposed by Harding and Ron (42) is that elimination of a key component of one of the arms of the UPR impairs adaptation to ER stress, leading to misregulation of the stress-response pathway. This hypothesis, although not fully borne out in PERK deficiency, is supported by the finding of ER stress and cell death associated with mutations in WFS1 and $P58I^{PK}$. Moreover, it was shown that overexpressing the ER chaperone BiP in a WFS1 mutant β-cell line could reverse the ER stress associated with the absence of the WFS1 protein in these cells (108). An alternative explanation for the increased ER stress and cell death in WFS1 and $P58I^{PK}$-deficient mice is that these genes perform essential ER functions in addition to their role in the UPR. Therefore, in their absence, the ER becomes dysfunctional, resulting in ER stress. Consistent with this hypothesis, WFS1 and $P58I^{PK}$ are abundantly expressed in highly secretory cells under normal conditions. WFS1 is important in regulating calcium homeostasis in the ER, whereas $P58I^{PK}$ is a key component of cotranslocational protein degradation, which may participate in normal quality-control functions of the ER (92). Therefore, the absence of WFS1 or $P58I^{PK}$ may seriously compromise normal ER functions and lead to an ER stress response.

As illustrated by genetic deficiencies in *PERK*, *WFS1*, and $P58I^{PK}$, the loss of UPR-related genes particularly affect the development or function of the insulin-secreting β cells. Recently, IRE1α has also been implicated in positively regulating proinsulin synthesis in these cells in response to glucose stimulation (75). Unexpectedly, regulation of proinsulin expression by IRE1α does not involve the catalysis of XBP-1 splicing, the well-known target of IRE1 in the ER stress-response pathway. Genetic ablation of *ATF4* and *XBP-1*, the primary downstream targets of PERK and IRE1, respectively, in the ER stress response does not result in early-onset impairment of β-cell function UPR (49, 72, 86), which also argues that the functions of PERK and IRE1 are not limited to UPR. Curiously, the loss-of-function mouse mutants of XBP1 and ATF4 result in abnormal development of the exocrine pancreas (72) (K. Iida and D. Cavener, unpublished data). Therefore, several of the ER stress genes have important pancreatic functions, but many of these activities appear to be independent of their function in the UPR.

The genetic analysis of the ER stress genes summarized herein shows that the loss of some of these genes results in a vigorous UPR, either as a consequence of a failure to control normal levels of ER stress or a failure to perform normal ER functions. The ER stress genes, however, also have developmental or physiologic functions that are not directly related to the UPR, and therefore, the loss of these genes has revealed primary functions, some related to the function of the ER, and other activities related to the regulation of organogenesis and cellular development.

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ABBREVIATIONS

CLS, Coffin-Lowry syndrome; EIF2AK, eukaryotic initiation factor 2-α kinase; ERAD, endoplasmic reticulum–associated degradation; IRES, internal ribosome entry site; ITAF, IRES-trans-acting factor; PEK, pancreatic eIF2 kinase; PERK, PKR-like ER kinase; uORFs, upstream open reading frames; WFS, Wolfram syndrome; WRS, Wolcott–Rallison syndrome.

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